ACUTE AND SUB-ACUTE TOXICITY OF CHLOROFORM AND IODOFORM (DISINFECTION BY- PRODUCTS) ON COMMON CARP (*CYPRINUS CARPIO*)

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Islamabad, Pakistan

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A thesis submitted in partial fulfillment of requirements for the degree of

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In

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CERTIFICATE

This dissertation submitted by Ms. Samina Perveen is accepted in its present form, by the Institute of Environmental Sciences and Engineering (IESE), School of Civil and Environmental Engineering (SCEE), National University of Sciences and Technology (NUST), Islamabad, Pakistan as satisfying the partial requirement for the degree of Master of Science in Environmental Science.

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THESIS ACCEPTANCE CERTIFICATE

It is certified that the contents and forms of the thesis entitled **"Acute and sub-acute toxicity of Chloroform and Iodoform (disinfection by-products) on common carp (Cyprinus carpio)"** submitted by Ms. **Samina Perveen**, Registration # 00000172644 has been satisfactory, free of errors and plagiarism for the requirements of the degree of Master of Science in Environmental Science. It is further certified that necessary amendments as pointed out by the GEC members of the scholar have also been incorporated in the said thesis.

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Dedicated to my beloved parents, siblings and friends who have supported me all the way since the beginning of my studies and make me what I am today.

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Samina Perveen

LIST OF ABBREVIATIONS

CBC	Complete Blood Count
$CHCl_3$	Chloroform
CHI_3	Iodoform
DBPs	Disinfection By-products
DMSO	Dimethyl Sulphoxide
THMs	Trihalomethanes
DNA	Deoxyribonucleic Acid
ALT	Alanine Aminotransferase
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium Bromide
GC	Gas Chromatography
HAAs	Haloacetic Acids
LD_{50}	Lethal Dose 50
MCLs	Maximum Contaminant Levels
mg/L	Milligram per Liter
ppb	Parts per billion
ppm	Parts per million
OTM	Olive Tail Movement
PAA	Peracetic Acid
PBS	Phosphate Buffer Saline
SCGE	Single Cell Gel Electrophoresis
OECD	Organization for Economic Cooperation and Development

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ABSTRACT

Disinfection is intended to improve drinking water quality and human health. Though, disinfectants may transform organic matter and form disinfection by-products (DBPs), many of them branded as cyto and genotoxic in nature. Traditionally, research focuses on the effects of DBPs to human health, but cytogenic and genotoxic impacts on aquatic organisms still remained ill defined. Which is why, current study examines the potential toxic effect of chloroform and iodoform (DBPs) on common carp (*Cyprinus carpio)*, selected as a model organism. Healthy fish specimens were exposed to various concentrations of chloroform and iodoform primarily based on LD_{50} values, where acute toxicity was monitored for 96 h. The LD_{50} was determined to be 3 and 90 mg/L for iodoform and chloroform respectively. Headspace SPME analysis through gas chromatography was conducted to assess either applied doses (75.5 – 87.5 and 2.1 -2.9 mg/L for chloroform and iodoform respectively) effects fish blood samples or not. Genotoxicity was monitored using Comet assay. Tail length, tail DNA and olive tail moment values were quantified to be significant ($P<0.05$) as compared to control. Results shows that the mean tail length values for iodoform (11, 17,25,33,41 μm) was significantly higher than chloroform (6, 12, 15, 18.3,22 μm) at all observed concentrations. Cytotoxicity through hematology and biochemistry showed a dose-response relationship. Statistically significant ($p<0.05$) decrease in all blood parameters {(white blood cells (WBC), red blood cells (RBC), platelets (PLT), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and hemoglobin (Hgb)} was observed with increase dose concentration and exposure time. The change was more significant in case of iodoform at all administered doses in comparison to chloroform analogue. The changes in biochemical indices (glucose, total protein and alanine aminotransferase) were also statistically significant ($p<0.05$). But, ALT secretion was significantly increased (93±0.05 and 82.8 ±0.1U/L) at higher concentration compared to control (56±0.1U/L), suggesting liver damage. Thus, results demonstrated that iodoform was statistically more damaging as compared to chloroform.

Keywords: *Biochemical count, chloroform, comet assay, cytotoxicity, disinfectant by-products, genotoxicity, hematology, iodoform.*

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CHAPTER 1

INTRODUCTION

1.1 Background

Water is a prerequisite for life on Earth. The aquatic environment plays a vital role in the functioning of ecosystem. All life forms are highly dependent upon water. Both quality and quantity of water is important to ensure proper survival of life. Water has been used as a symbol of life and purity. Therefore, availability of fresh water is essential for the survival of aquatic life and humans as well. Global water distribution contains only 2.5% of fresh water, the remaining 97.5% of water is too salty to use for human consumption. Among fresh water; 69% is present in icecaps and glaciers while underground contain 30% of total. Less than 1% present is present in form of rivers, lakes and swamps which are consumable forms for humans.

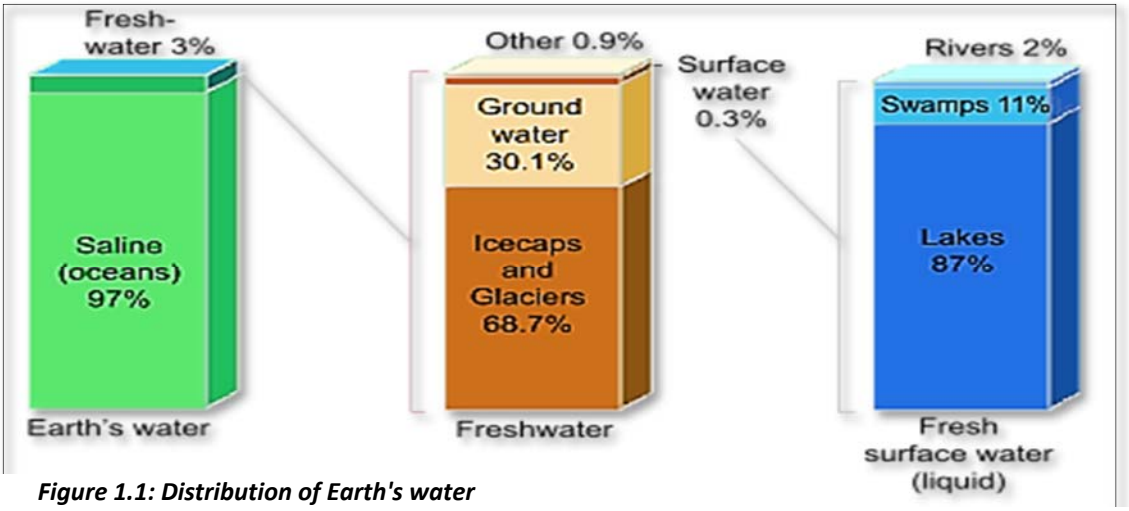


Figure 1.1: Distribution of Earth's water

However, the system is being polluted by natural and anthropogenic ways, releasing toxic chemicals and xenobiotics. These toxic pollutants accumulate and contaminate many parts of water through various water transposition processes. Polluted environment may lead to nutritional, behavioral and reproductive complications within aquatic organisms (Cok *et al*.,

2011). Further, this small portion (less than 1%) of available fresh water is under great stress due to growing needs and developments. The available water resources (lakes, rivers, streams) were contaminated day by day at unprecedented rate. It is said that, today available drinking water is not pure as it is polluted by deadly xenobiotic and chemicals from different sources. It is present dilemma that humans have drinking water which contains chemical cocktail, being not suitable for human consumption. John Archer said in his book entitled **'The Water You Drink, How Safe Is It?"** demonstrate that approximate 60,000 tons of pollutants and chemicals release into drinking water supplying systems and deteriorating its quality.

Water pollution is defined as the addition of something (pollutants, xenobiotic, chemicals etc.) by humans and natural ways within system which deteriorate water quality and alter its physico-chemical composition to extent which harms aquatic life and human health. Modernization, in every phase of development leads to the generation of more pollutants. As with increasing population, the growing needs were increased, so more demand for clean drinking water. Therefore, advancement in every era of development leads to the release of pollutants into aquatic system. These pollutants and xenobiotic were cause physicochemical and biological effects within aquatic system (Goel, 2000).

Water quality is determined by the availability of clean and fresh water. Quality water is indispensable for the proper survival of aquatic life. Therefore, water pollution is being considered as major threats to aquatic life. Dumping of pollutants with increasing pace can deteriorate water quality and disturb ecological balance. Running surface water is under great stress and more vulnerable to carrying pollutants coming from municipal, agricultural and industrial sources. Other than anthropogenic sources; natural influences may also deteriorate water quality.

Water and water resources should be managed to support the human well-being and ecosystem integrity. Provision of clean drinking water for human consumption has become the prime health advancement of 20^{th} century. Sufficient and clean water is made to be available not only for human consumption but also for the survival of aquatic life. Aquatic organism and life forms were survived well under clean water, safe from pollutants and hazardous chemicals.

As for need Disinfection is being considered as ultimate solution for drinking water treatment. The process protects human health via reduction in pathogenic load and generates safe and high quality potable water (Muellner *et al*., 2010). However, disinfectants (chlorine, ozone, UV and chloramines) added during disinfection process have been showed to react with naturally occurring organic matter to produce a wide range of disinfection by-products which showed cytotoxic and carcinogenic effects within aquatic organisms (Gustavino *et al*., 2005). Therefore, disinfection by-products may be considered as an emerging class of water pollutants.

Disinfection can improve the water quality via reduction in pathogenic load. Disinfection by-products (chemicals, organic and inorganic substances) can form as the result of interaction with naturally occurring organic water. Disinfection treatment processes includes chlorine, chloramine, ozone and ultraviolet light disinfectants. Among all disinfectants, chlorine is being as most widespread and commonly used disinfectant around the world, since last century. Chorine is introducing as gas or in form of sodium and calcium hypochlorite in water treatment systems. Major classes of DBPs, especially halogenated disinfection by-products were produces as the interaction of free chlorine or bromine with naturally occurring organic matter. Major constituent of organic matter is humic acid, being produce from microbial decomposition and vegetative decaying processes in surface water (Gustavino *et al.,* 2005). Humic acid may interact with chlorinated disinfectants and produce toxicant by-products. The major classes of halogenated disinfection by-products include Trihalomethanes (chloroform, iodoform, bromoform, bromodichloromethane, and dibromochloromethane), Haloacetonitrile (dichloro-, trichloro-, dibromo- and bromochloroacetonitrile) and Haloacetic acids (trichloro-, dichloro, monochloacetic acid, dibromo- and monobromoroacetic acid) (Nieuwenhuijsen *et al.,* 2017).

Trihalomethanes (THMs) and Haloacetic acids (HAAs) are found to be in highest concentration among all emerging DBPs in water systems. They are considered as mutagenic and carcinogenic to aquatic organism and human health. Epidemiological studies support the evidence of carcinogenic and mutagenic nature of this disinfection by- products. Many invertebrates were used as biomarker to assess pollutants within water systems. Fish being a suitable biomarker, ability to tolerate adverse environments and high sensitivity towards changing conditions (klobucar *et al.,* 2009). Fish are widely distributing aquatic vertebrates and frequently used in water pollution monitoring as they bio-accumulate xenobiotic and toxic pollutants and respond

very rapidly towards ecological disturbance and mutations (Russo *et al.,* 2004). Biomarkers have the ability to forecast change at specie to population and then at ecosystem level. Therefore, they considered to be short term indicator to forecast change that should be arising after long term (whyte *et al.,* 2000).

In the present study the focus was on two most abundant and emerging by-products (chloroform and iodoform), which are genotoxic and cytotoxic in nature. Chloroform is one of the most predominant DBP detected in water (Monarca *et al*. 2004). Iodoform is formed as a result of disinfection when HOI (hypoiodous acid) react with naturally occurring iodide ion (I^-) (Bichsel 2000). Iodinated disinfection by-products (I-DBPs) were classified as emerging and unregulated disinfection by-products (Pan *et al*. 2016). I-DBPs were more cytotoxic and genotoxic than chlorinated and brominated correspondents (Richardson *et al*., 2007). Maximum contamination limit for iodoform is 0.02-5µg/L (Allard *et al*. 2012). The environmental Protection Agency (EPA) has established maximum contamination level (MCL) of 0.08 mg/L for trihalomethanes in drinking water; later on MCL of 0.07 mg/L has been established for most abundant DBP chloroform (USEPA 2006).

1.2 Study objectives

Traditionally, research focuses on the effects of DBPs to human health, but cytogenic impacts of DBPs on aquatic organisms will remained ill defined. Pakistan lacks in conducting research on the genotoxicity of disinfection by-products on human and aquatic life as well. The current study examines the potential toxic effects of chloroform and iodoform (DBPs) by integrating *in vitro* toxicology on Common carp (*Cyprinus Carpio*) using Comet assay, hematological and biochemical analysis.

The objectives are mentioned below:

- Acute and sub-acute toxicity assessment of chloroform and iodoform (DBPs) on Common Carp (*Cyprinus Carpio*).
- Dose-response genotoxicity evaluation using Comet assay.
- Comparative toxicity analysis of chloroform and iodoform through hematological and biochemical analysis.

CHAPTER 2

LITERATURE REVIEW

2.1 Background

Water is basic building block of life. About 71% of the earth surface is covered with water, but among this 97% is salted water that is not fit for human consumption. The only water form; available for drinking, is fresh water account for 2.5% and among this less than 1% is in form that is accessible to humans. This small proportion is under great stress induced by urbanization, rapid development in industrial sectors and unsustainable agricultural practices. The situation is aggravating in developing countries. Available fresh water sources were contaminated at unprcendent rates. The situation is alarming due to excessive use of pesticides, improper disposal of waste and lack of management practices. Water pollution can adversely affect human health and aquatic life. Therefore whole biological community is effected at large scale. Appropriate treatment facilities are required to remove harmful and toxic pollutants from water bodies. Therefore, Water disinfection is a worldwide practice used in the treatment processes for drinking water to lessen the risk of infections associated with pathogens present in drinking water. However, disinfection is going to be one of the main reasons for the water pollution, as a result of disinfection by-products (DBPs).

2.1.1 Disinfectants and Disinfection by-products

Major health advancement of 20th century is "Disinfection", to produce clean drinking water. The process may reduce the outbreak of infectious diseases via reduction in pathogenic load. The commonly used disinfectants include chlorine, chloramine, chlorine dioxide, UV and ozone. Production of clean drinking water, mainly through chlorination, may serve as the eradication and effective reduction of water borne diseases such as dysentery, cholera, typhoid fever and many others (Nieuwenhuijsen *et al.,* 2000). Regardless of ultimate importance of disinfection process, the production of disinfection by-products presents as the matter of interest regarding aquatic and human health.

Carcinogenic and mutagenic nature of DBPs is well identified in many epidemiological studies. Genotoxicity associated with developmental and reproductive problems is also trouble shoot for

present century. Many disinfectants reported as carcinogenic in humans and rodents; and many were posing genotoxicity, mutagenicity and tumors in many fish species as well (Racz *et al.,* 2011).

Disinfection, performing with chlorine and performic acid (PFA) was investigated to evaluate cytotoxicity and genotoxicity in targeted cells (bacterial, plant and mammalian) before and after apoptosis (chlorination) of secondary wastewater effluents coming from municipal waste water treatment plant (WWTP). The Ames test (point mutation), micronucleus and comet tests were performed to detect mutagenicity and chromosomal DNA aberrations. In all above in vitro tests, negative results were obtained for mutagenic and genotoxic effects. Thus, it confirms that disinfection performed with PFA and chlorine applied to secondary effluents; did not contribute towards release of carcinogenic or mutagenic compounds (Ragazzo *et al.,* 2017).

Lin and coworkers (2016) conducted research on newly developed, disinfection by-product dichloroacetonitrile (DCAN), a typical nitrogenous compound to evaluate its toxicity on aquatic organisms. They investigate acute toxicity of dichloroacetonitrile in zebra fish selected as model organism through single cell gel electrophoresis (SCGE) assay. Adult zebra fish individuals were exposed to defined concentration of DCAN for 24 days. The SCGE assay was carried out after every four day to evaluate genotoxicity of DCAN. The results demonstrate; developmental toxicity characterized by the significant decrease in hatchability and neural function disorder. Acute DNA damage was predominant and specifies liver damage.

2.2 Regulations for disinfection by-products

EPA has established regulations covering disinfection by-products and revised regulations on the basis of extensive used of disinfectants. The limit for trihalomethanes must be lowered to 80μg/L. This standard replaces the maximum allowable annual average of 100 ppb. These standards were effective for small surface and all ground level water system as well. EPA has established different allowable limits for different DBPs along their health effects.

- Haloacetic acids (dichloroacetic acid, monobromoacetic acid, dibromoacetic acid, monochloroacetic acid and trichloroacetic acid) are byproducts of chlorination process similar to trihalomethanes. Maximum contamination level (MCL) of (60 ug/L) is allowable in drinking water systems. Excessive levels can leads to liver and nervous system disorder.

- Chlorite was regulated with MCL of (1 mg/L). Excess of chlorite can cause hemolytic anemia.
- Bromate one of newly regulated disinfection by-product, having MCL of (10 μg/L). Whereas, increase concentration impact kidney and gastro intestine.

To reduce the effects of disinfection by-products towards community level, stage 1 and stage 2 rules were set by US Environmental protection agency in 1998 and 2004.

- **Stage 1 rule** (1998) applies to customers including community supply system as well as non-transient non community water supply systems.
- **Stage 2 rule** (2004) strengthen the community health protection by forcing them to compliance with monitoring requirements regarding trihalomethanes (THMs) and Haloacetic acids (HAA) in public drinking water facilities. This rule is built on the basis of former and main focuses on targeted public water systems with greater risk.

These rules set by USEPA ensure safety from disinfectants and disinfection by-products; otherwise, consumption of by-products above permissible limits may leads to adverse health risks.

Over 600 DBPs have been recognized in drinking water facilities. These are somewhat measured and regulated. Among these trihalomethanes and haloacetic acids are the most prevalent ones in chlorinated water (Weinberg & Krasner, 2002).

- Different agencies provided guidelines for disinfection by- products in drinking water;

Table 2.1: Levels of trihalomethanes (THMs) EPA

Total THMs	MCLG	MCL
Chloroform	0.07 mg/L	0.08 mg/L (sum of concentration of total trihalomethanes)
Bromoform	Zero	
Bromodichloromethane (BDCM)	Zero	
Dibromochloromethane (DBCM)	0.06 mg/L	

Table 2.2: US EPA Safe Drinking Water Act (SDWA)

DBPs	MCL (mg/L)
Total THMs	0.08
Haloacetic acid (HAA)	0.06
Bromate	0.01

Table 1.3: World Health Organization (WHO) guidelines

DBPs	Guidelines values
Chloroform	0.2
Bromoform	0.1
Chlorodibromomethane (CDBM)	0.1
Bromodichloromethane (BDCM)	0.06
Dichloroacetaldehyde (DCA)	0.05
Trichloroacetaldehyde (TCA)	0.2
Bromate	0.01

Table 2.4: European Union Standards (EUS)

DBPs	Standard values (mg/L)
Total trihalomethanes	0.1
Bromate	0.01

2.3 Classification of Disinfectants and disinfection by-products

2.3.1 Disinfectants

Most commonly used disinfectants include;

- Chlorine
- Chloramine
- Ozone

- Ultra violet

2.3.1.1 Chlorine

Chlorine was being introduced in urban water supply as disinfectant at the beginning of the 20th century to improve hygienic quality by eliminating waterborne pathogens and the consequent transmission of disease. Chlorine is a reactive agent for chlorination process because of its instant reaction with naturally occurring organic matter present in water systems. This reaction leads to the formation of chlorinated disinfection by-products. Naturally occurring organic matter (NOM) being served as precursor for DBPs formation (Nikolaou & Lekkas, 2001).

When chlorine is added to drinking water system as a disinfectant it reacts to form Hypochlorus acid (HOCl) within seconds. Following reaction will occur

$$Cl_2 + H_2O = HOCl + H^+ + Cl^-$$

The reaction will occur at pH > 4 with chlorine dose up to 100 mg/L (Morris, 1982). The formed HOCl can react with naturally occurring organic matter and form disinfection by-products.

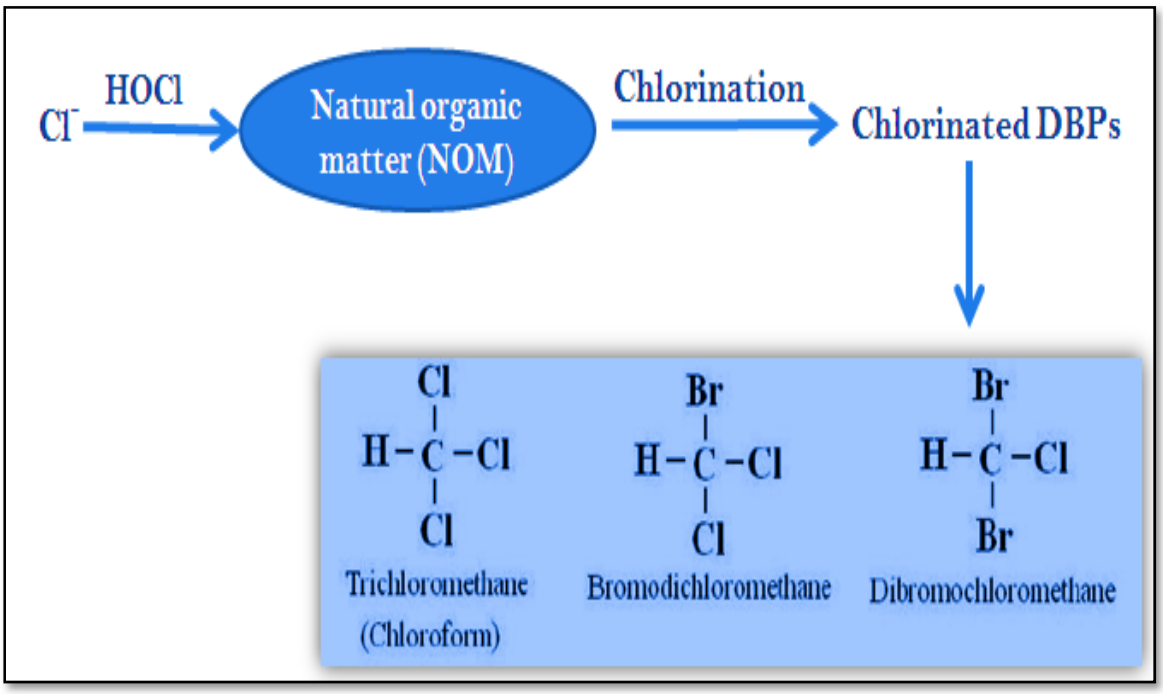


Figure 2.1: Fate of chlorine in drinking water during chlorination process

Chlorine has been used for disinfection process for approximately 100 years. But the concerns were raised due to the formation of toxicant by-products. It is estimated that more than 300 disinfection by-products have been reported to be formed from chlorine treatment (Richardson, 1998). Four of them (chloroform, bromoform, bromodichloromethane, and dibromochloromethane) have been shown to carcinogenic in many epidemiological studies (Bull & Kopfler, 1991).

Advantages

- ✓ Highly effective for most pathogens
- ✓ Protection against biofilm formation
- ✓ Easily controlled, applied and monitored
- ✓ Cost-effective
- ✓ Most reliable in term of operation

Limitations

- ✓ Variety of by-products formation (THMs and HAAs)
- ✓ Form brominated by-products
- ✓ Requires storage and transportation of chemicals
- ✓ Not effective against certain pathogens including Cryptosporidium

2.3.1.2 Chloramine and its by-products

Chloramine is less reactive disinfectant than chlorine. Upon disinfection, chloramine also produce disinfection by-products but at lower concentration. The use of chloramine as disinfectant is being increased in recent year because of limited formation of disinfection by-products (DBPs). On the other hand, monochloramine is about 2000 to 100,000 times less effective than chlorine to inactivate pathogens (E. *coli* and rotaviruses). Therefore, monochloramine is not considered as reliable primary disinfectant. The shift towards monochloramine to control the formation of disinfection by-products may compromise the disinfection process.

Advantages

- ✓ Limited formation of THMs and HAAs
- ✓ Does not oxidize to form brominated by-products
- ✓ Form stable residual than chlorine
- ✓ Less taste and odor than chlorine
- ✓ Excellent in controlling coliform bacteria and biofilm production

Limitations

- ✓ Weak disinfectant
- ✓ Requires controlling of ammonia
- ✓ Toxicity to aquatic life due to ammonia production
- ✓ Cause kidney dialysis

2.3.1.3 Ozone and its by-products

The demand for ozone as disinfectant increased because it readily decomposes and being the most effective disinfectant for all type of microbes especially protozoa which are difficult to inactivate using other disinfectants (chlorine and chloramine). It can provide adequate inactivation of microbes with optimum dose and contact time (Von Gunten, 2003). Different by-products formed as process completion includes aldehydes, formaldehyde, bromate, bromomethanes, carboxylic acids, hydrogen peroxide, brominated acetic acids, brominated acetonitrile's and ketones. However; having all benefits, it is not commonly used disinfectant due to high cost, regrowth of biological entities and lack of disinfectant residual and limited availability of information regarding toxic nature of by-products.

Advantages

- ✓ Easily available and strongest disinfectant
- ✓ Production of non-chlorinated trihalomethanes and haloacetic acids
- ✓ Effective against microbes

Limitations

- ✓ Technical competence in term of operation and maintenance
- ✓ Non protective residual

- ✓ Form non-halogenated and brominated by-products
- ✓ High capital cost compared to chlorine
- ✓ Difficult to control and maintain the overall process

2.3.1.4 Ultraviolet radiation

Ultraviolet radiation is a non-chemical disinfectant; being generated by mercury arc lamp. UV radiation penetrates in the cell wall of the organism and show genetic damage. UV radiations effectively inactivate many microbes while forming limited disinfection by-products.

Advantages

- ✓ Inactivating many pathogens, spores, cysts and viruses
- ✓ Non chemical disinfectant, thus no chemical storage and handling
- ✓ Effective against Cryptosporidium
- ✓ Non effective and limited disinfection by-products

Limitations

- ✓ No residual protection
- ✓ Less feasible for inactivation of some viruses (reoviruses and rotaviruses)
- ✓ Difficult in monitoring
- ✓ Photo-reactivation process occurs as organisms repair and reverse the destructive effects of UV
- ✓ Additional steps required to maintain high clarity drinking water
- ✓ No provision of odor, taste control
- ✓ High cost in term of backup maintenance
- ✓ Environmental toxicity risk due to mercury lamp

2.3.2 Disinfections by-products

Over 600 disinfection by-products have been recognized in drinking water systems, where chlorination is one of the key process (Weinberg & Krasner, 2002). Disinfection by- products have received considerable importance in drinking water facilities, because of their possible

outcomes (genotoxic, carcinogen and cytotoxic) (Hsu *et al.,* 2001). Recently, researchers shifted their interest towards the adverse reproductive effects of disinfection by-products (Cantor, 1994). In 2006, Krasner et al published a nationwide occurrence study. They examined the effect of different treatment conditions and source water on the formation of disinfection by-products and identified many emerging DBPs. They identified choracetaldehyde, dichloroacetaldehyde, bromochloroacetaldehyde and dichloroacetaldehyde as emerging DBPs and categorized them as priority DBPs. Further, DCAL was considered as the most abundant among all DBPs with maximum concentration of (16 µg/L) in simulated distribution water system.

They are categorized as regulated and unregulated disinfection by-products according to US EPA.

2.3.2.1 Regulated DBPs

Regulated DBPs were most commonly practiced in many drinking water distribution systems. They include Trihalomethanes, Haloacetic acids, chlorite and bromate

2.3.2.1.1 Trihalomethanes (THMs)

Among all DBPs, trihalomethanes (THMs) are the most prevalent class and being routinely measured in many developed and developing countries. THMs are volatile group of compounds such as chloroform, chlorodibromomethane (CDBM), bromodichloromethane (BDCM) and bromoform. More than 600 DPBs were recognized and being regulated (Weinberg & Krasner, 2002). Trihalomethanes (THMs) and haloacetic acids (HAAs) are considered as the two main classes of disinfection by-products, being regulated in different countries.

Among these, only 11 have been regulated and mainly originated in drinking water. Chloroform, bromoform, bromodichloromethane and chlorodibromomethane are among the four trihalomethanes regulated by the USEPA in drinking water as a group and are known as total trihalomethanes (USEPA, 2006).

2.3.2.1.2 Chloroform ($CHCl_3$)

Chloroform is usually the most abundant THM often accounting for greater than 90% of the total THM concentration. Typically the most prevalent trihalomethane measured in chlorinated water

and probably the most thoroughly studied DBP. Toxicology studies show carcinogenic and mutagenic nature of chloroform in laboratory animals.

Larson and coworker in 1994 conducted a study by the Centers for Health Research (CIIT) about the toxic effect of chloroform disinfectant by-product. They observed that high dose of chloroform when injected to mice once per day may cause liver damage and eventually cancer. They concluded that high dose of chloroform can overwhelmed the capability of mice liver to detoxify the disinfectant and cause liver damage. Late in 1998, Butterworth *et al* confirm that when chloroform dose is given to mice through drinking water, it will not cause cancer as liver can detoxify the disinfectant. But later, prove that continuous exposure may lead to liver damage and eventually cancer.

USEPA established threshold level of 0.07 mg/L for chloroform and conclude that exposure of chloroform above threshold level cause cell damage and likely to increase the risk of cancer. It is confirmed that chloroform is a carcinogenic at high dose and for meeting drinking water standard set by EPA; chloroform is unlikely to be a health concern for consumers (USEPA, 2006).

The physico-chemical properties of chloroform disinfection by-product are enlisted in Table 2.1

Table 2.5: Physico-chemical properties of chloroform

Characteristics	**Information**
Color	Colorless
State	Volatile, liquid
Odor	Ether-like odor
Water solubility (g/L) at 25 °C	7.2–9.3
Density (g/cm^3) at 25 °C	1.48
Boiling point (°C)	61.3

Log K_{ow}	1.97

2.3.2.2 Unregulated and emerging DBPs

Unregulated and emerging disinfection by-products includes variety of compounds such as haloacetonitriles, halofuranones, halonitromethanes, , haloamides, iodoacetic acids and iodo-THMs (iodo-trihalomethanes).

2.3.2.2.1 Iodo-trihalomethanes (I-THMs)

Iodo-trihalomethanes (I-THMs) may be formed when hypoiodous acid (HOI) is reacting with natural organic matter (NOM). It was estimated that I-THMs were responsible for 25% of the taste and odor in drinking water. Epidemiological studies shows that iodinated disinfection by-products were found to be more mutagenic, genotoxic and cytotoxic than chlorinated and brominated analogues (Richardson *et al.,* 2008). Plewa and coworker (2004) demonstrated that iodoacetic acid (IAA) was found to be more cytotoxic and genotoxic in *salmonella typhimurium* and mammalian cells than chloroacetic and bromoacetic acid. In one study it was observed that cytotoxicity and genotoxicity of iodoacetic acid (IAA) is 100 times higher in mammalian cell than -3-chloro-4- (dichloromethyl) -5-hydroxy-2- (5H)- furanone (MX) which was most mutagenic disinfection by-product (Kronberg & Vartiaine, 1988). The most problematic by-product among I-THMs is iodoform (CHI_3) with threshold level of 0.02-5µg/L (Allard *et al*. 2012).

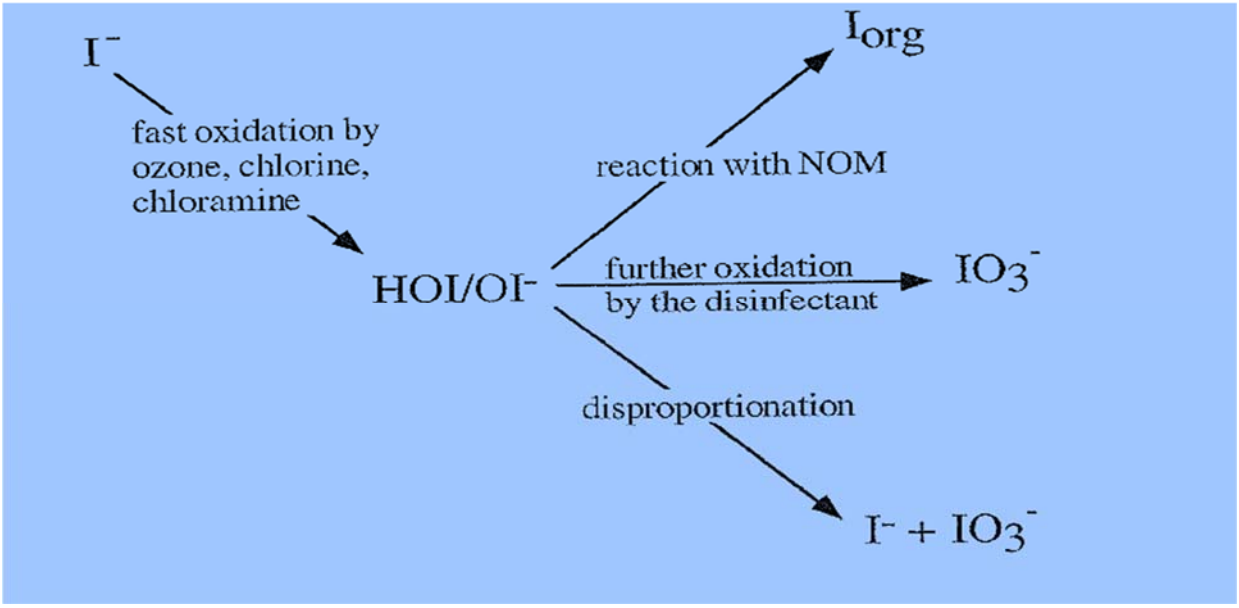


Figure 2.2: Fate of iodide ion during chlorination process

During drinking water treatment, I^- is first oxidized in the presence of ozone, chlorine and chloramine in HOI. While in the second step HOI react with NOM and form organic iodide i.e. iodo-trihalomethanes. While some of OI^- was oxidized to IO_3^- and some were disproportionate again into I^- and IO_3^- specie (Fabian & Gordon, 1997).

Iodoform is one of the most toxic by-product among all I-THMs and regulated disinfection by-products. Therefore, iodoacetic acids and iodoform were recognized and being regulated in drinking water as fate of chlorination due to their toxic nature (Wei *et al.*, 2013).

Some physical and chemical properties of iodoform were listed in table 6.

Table 2.6: Physico-chemical properties of iodoform

Characteristics	**Information**
Color	Yellow powder or crystals
State	Powder or crystal

Odor	Ether-like odor
Water solubility	Very slightly soluble
Density (g/cm3) at 25 °C	13.6
Boiling point (°C)	218
Molecular Wt. (g/mole)	393.73

Carcinogenicity of iodoform was assessed in Osborne-Mendel rats and B6C3F1 mice by the American National Cancer Institute (1978). Test animals were fed orally with define concentrations of iodoform and observed for 78 weeks. The results indicate that high mortality was observed with high dose and with exposure time (Suzuki, 1987).

Mutagenicity of iodoform was observed in different strains of Salmonella typhimurium (TA 98, TA 100, TA 1535 and TA 1537) (Haworth *et al*., 1983). In another study, when iodoform was exposed to Syrian hamster embryo cells an increase in unscheduled DNA synthesis and morphological transformations were observed. The extent of damage was increased with increase dose and exposure duration. Later on, Hikiba *et al*. (2005) observe increase in the level of chromosomal aberrations in Syrian hamster embryo cells exposed to (0-240 μM) iodoform level. Toxicity of iodoform was also assessed in aquatic organisms; rainbow trout and Daphnia magna. It was observed that rainbow trout was more sensitive to iodoform (LC_{50} >0.53 mg/L) where half population was killed. In case of daphnia magna, LC_{50} was found to be > 0.17 mg/L, confirmed that it was less sensitive compared to rainbow trout (Laveroek *et al*., 1995).

2.4 Factors effecting Disinfection by-products formation

Disinfection by-products are formed by the reaction of chemical disinfectants (chlorine, chloramine, ozone and UV) with by- product precursors. Natural organic matter (humic acid and fulvic acid) and inorganic matter (chloride, bromide, iodide etc.) are the most significant disinfection by-product precursors. Formation of disinfection by-products as result of chlorination is quite complex process and driven by different factors;

- Type and concentration of disinfectants

- Concentrations of organic matter and other DBP precursors in water to be disinfected
- Water temperature
- pH
- Contact time
- Length of the distribution network

Many studies were conducted to analyze and understand the significance of relevant factors which influence the formation of disinfection by-products.

Gagnon and coworker (2005) conducted a study to compare the efficacy of chlorine dioxide and chlorite ion for biofilm production control. Annual reactors were dosed with high and low concentrations of chlorine dioxide (0.025 and 0.5 mg/L) and chlorite ion (0.1 and 0.25 mg/L) respectively. Results demonstrate that low level of chlorite ion is not affected in controlling heterotrophic bacteria as compared to chlorine dioxide. This indicates that it would be important to maintain the residual concentration of disinfectant in distribution systems for pathogenic control.

Concentration of trihalomethanes (THMs) is depended upon initial concentration of chlorine dose, water temperature and pH. When the chlorine dose was increased during disinfection process, increased concentration of by-products was formed. Results also demonstrate; at higher concentration of chlorine higher values of trihalomethanes were measured.

The time required for completion of THMs formation varied according to applied chlorine dose and season. It also noted that, formation of trihalomethanes was complete earlier at higher chlorine dose than at lower dose, as concentration of chlorine also important factor affecting THMs formation (Ristoiu *et al*., 2006).

Guo and coworker (2014) conducted research to investigate the effect of different factors such as reaction time, temperature and bromide and iodide concentrations regarding formation of iodinated trihalomethanes (I-THMs) during chlorination process in the presence of chlorine dioxide. Among all I-THMs, iodoform was predominant DBP in all water samples collected from ten water sourcing sites. Longer reaction time leads to the higher level of iodoform formation, as in case of bromide and iodide ions presence. Regarding water temperature, the high level of I-THMs formation was observed at 25°C than at 5°C. Iodoform showed increase trend with increasing chlorine dioxide dose and iodide concentration with molar ration of 1-2 at

pH 8. Thus, results indicate that with increased disinfectant dose, contact time; higher concentration of disinfection by-products was achieved.

2.5 Toxicity of disinfection by-products (DBPs)

Disinfection as the major heath advancement of 20th century seeks attention worldwide due to unintended formation of disinfection by-products. USEPA in 2006 developed stage 2 rules for DBPs to control maximum contamination level (MCL) of certain disinfection by-products, as they are branded as carcinogenic and mutagenic. USEPA established MCL for 11 DBPs including (4 THMs; MCL = 80 µg/L, 5 HAAs; MCL = 60 µg/L, Bromate, BrO_3^-; MCL = 10 µg/L, Chlorite, ClO_2^-; MCL = 1.0 mg/L).

Different in vivo bioassay conducted in National Institute of Cancer (NIC) indicated that THMs are carcinogenic at high dosed level, increasing public health concerns. Many epidemiological studies demonstrate increase cancer risks such as stomach, bladder and colon cancer associated with DBPs in chlorinated water (Lind *et al*., 1989).

In the past decades, Plewa and coworker (2009) has been established first in vitro quantitative and systematic study to evaluate the chronic genotoxicity and cytotoxicity of disinfection by-products in mammalian cells. The toxicity was measured through the reduction in cell density as a function of disinfection by-products for an exposure period of 72 hrs. Comet assay was used to evaluate the genotoxicity as genomic DNA damage in nuclei. This confirms genotoxic nature of DBPs. King *et al*., (2000) conducted population based case study in Canada to evaluate the evidence of DBPs as colon and rectal cancer risks associated with public water supply. In this study 26 males were exposed to chlorinated surface water and results demonstrate that higher cancer risk was observed in males that were exposed to 35-40 years as compared to those exposed >10 year.

2.5.1 Genotoxicity Assessment

To evaluate cytotoxicity and genotoxicity of disinfection by-products within living tissues and to identify sites where these DPBs and other pollutants cause disruption various in vivo and in vitro studies were conducted. Whether used in combination or alone disinfectants produce variety of DBPs that induce toxic and genotoxic activities which is why it has been more than quarter of a

century since the genotoxicity and chemistry of disinfection byproducts has been investigated (Plewa *et al*., 2004).

In vivo is Latin word for "within the living" whereas, in vitro is for "within the glass". In an in vitro experiment, the scientists take parts of a living organism (cells or tissue) and study those using Petri dishes, test tubes, or other lab equipment. While in an in vivo experiments, scientists are conducting their studies in whole living organisms (Seagrave *et al.*, 2003).

Sayes et al (2007) developed successful implementation of different in vitro assays as the predictive screens for the assessment of pollutant toxicity during early development prior to substantive inhalation toxicity. They also suggested that, if these tests were properly validated these early screening would results in fast, simpler and less expensive than in vivo counterparts.

Many advantages were associated with in vitro genotoxicity tests and widely accepted by scientists. However drawback of using in vitro assessment is that, this type of assay dose not integrate DNA damage repair (Fenech, 2013).

In vivo assays integrate the effect of biological assessment and that is why they are more suitable than in in vitro assay for modeling purposes (Brian *et al*., 2007).

2.5.1.1 Genotoxicity assessment tools

Different genotoxicity tests or tools are used for the evaluation of potential toxic effects (alteration in DNA, DNA strand breakage, liver damage and mutations) caused by the pollutants/xenobiotics. About 200 different genotoxic assays are used for the study of genetics, mutations and mutagenesis but all available tests have different genotoxic endpoints (Luttrell *et al*., 2008).

Table 2.7: Common in vivo and in vitro assay for genotoxicity monitoring

Assay names	End points
In vitro assays	
Alkaline single cell gell electrophoresis (SCGE)	DNA strand breakage
Alkaline elusion assay	DNA strand breakage
DNA abduct analysis	DNA adducts
Micronucleus assay in mammalian cells	Clastogenicity, aneugenicity
Sister chromatid exchange in mammalian Cells	DNA repair

CHO Hprt mutation assay	Gene mutation
Chromosomal aberration in mammalian cells	Clastogenicity, aneugenicity
Ames assay	Gene mutation
In vitro assays	
Sister chromatid exchange in rodents	DNA damage (somatic cells)
Rodent micronucleus assay	Clastogenicity; aneugenicity (somatic cells)
Transgenic rodent mutation assay	Gene mutation (somatic and germ cell)
Dominant lethal assay	Clastogenicity (germ cell)
Mouse heritable translocation test	Chromosome mutation (germ cell)
Mouse spot test	Gene mutation (germ cell)
Mouse specific locus test	Gene mutation (germ cell)

2.5.1.2 In vitro assessments

Guzzella and coworker (2004) set a series of short term in vitro assays by using different genetic end-points to evaluate genotoxicity of surface water disinfection by-products with different biocides (NaClO, ClO_2 and PAA). Surface water sample was concentrated on C_{18} silica cartridge before and after disinfection. The concentrates which comprises non-volatile organics were divided into different portion in order to conduct different chemical and biological assay. The following in vitro assays were conducted on water concentrates dissolves in DMSO (dimethyl sulphoxide); the Salmonella mutagenicity assay, SOS Chromotest, Microtox and Mutatox assay, gene conversion, mitochondrial DNA mutability assays and point mutation. The research outcome shows that the SOS Chromotest and Yeast assay are highly sensitive in detecting genotoxicity. Further, water extracts were slightly toxic to different tested organisms, somewhat suppressing their mutagenic nature. The results also demonstrates that NaClO and ClO_2 disinfectants showed increased genotoxicity, whereas PAA somewhat decrease the raw water activity. It was aided that the amount of organic matter in water was varied according to season variability, which increase or decrease raw water activity.

Ragazzo et al (2017) studied the cytotoxicity and genotoxicity effects of wastewater before and after the disinfection using performic acid (PFA), as an emerging disinfectant. The potential genotoxic effects of PFA was evaluated using series of in vitro tests (Ames test, point mutation, micronucleus test, comet test, DNA fragmentation and mitochondrial potential assay) on different targeted cells { plant (allium cepa roots), bacterial (salmonella) and mammalian (human hepatic cells HepG2)}. The sample water was concentrated with silica C_{18} cartridges in order to perform genotoxicity assays. Non-concentrated water samples were also examined for genotoxicity with the help of micronuclei test on Allium cepa. Research outcomes show that in all in vitro tests, before and after disinfection, negative results were obtained for genotoxic and mutagenic effects. In case of Allium cepa tests, a slight increase in the micronucleus frequencies was observed in root cells. Results of salmonella microsome assay expressed that highest doses in strains TA98 and TA100 were toxic to bacteria. Single cell gell electrophoresis test with HepG2 cells demonstrates that PAF application did not induce significant DNA strand breaks and none of the statistically significant increase in the MN test frequencies was observed. In conclusion, it is observed that PAF did not contribute towards the formation of mutagenic and genotoxic compounds after application as disinfectant.

2.5.1.3 In-vivo assessments

Bolognesi et al (2004) conducted in vivo genotoxicity tests (micronucleus and single cell gell electrophoresis) in order to assess the genotoxicity of surface water treated with three different disinfectants (sodium hypochlorite, chlorine dioxide and peracetic acid). Zebra mussel cells were selected as model organism. The toxic effects were detected in different tissues (gills and heamocytes for micronucleus and Comet test respectively). The test organism was exposed to experimental basin supplied with lake water (Lake Trasimeno, Italy) before and after disinfection. The sampling was done according to season variations (October 2000, February 2001 and June 2001). In vivo tests were performed after 3 hrs. 10 and 20 days. The research outcome shows that the peculiar trend in DNA migration was observed according to seasonal variations. The DNA damage was modulated according to seasonal temperature and water quality. The comet data indicates that during worst water quality, i.e. in February 2001, significantly higher DNA migration was observed after 20 days of exposure.

Klobucar et al (2010) conduct a study to evaluate genotoxicity assessment through in vivo assays (micronucleus and comet assay) of fresh water environment using caged Common carp selected as model organism. The selected organism was transferred to two different polluted sites in the eastern Croatia (Belisce and Osijek) and one reference site (Nature Park Kopacki rit). Caged carp were exposed to three weeks and repeated for next three years (2002-2004). Further, in order to evaluate possible difference in the stress responses to polluted water a laboratory exposure in aquaria was also performed during the next year of the study. The results demonstrated that carp exposed to polluted sites showed significant DNA damage in both in vivo tests. Further comet assay was seen to be more sensitive in genotoxicity assessment for both caging and aquaria exposure. Thus, current study suggested that three week caging exposure was successfully applied for genotoxicity biomonitoring of fresh water environment.

Cok *et al*., (2011) studied the genotoxic impact (triggering DNA damage) of pollutants/ xenobiotics in Common carp (*Cyprinus Carpio*) using single cell gel electrophoresis (SCGE). The study was conducted in Ankara (Lake Mogan) which was polluted from different sources (city sewer system and agricultural pesticides). A total of 30 fish samples were collected from different parts of lake. Blood was collected in EDTA vials and transferred to laboratory in controlled conditions. The comet assay was performed and comparison was done with 15 fish samples obtained from "The Research and Applied Fish Farm of University Ankara". Comet results showed that the values of comet parameters (tail length, tail intensity and tail moment) were found to be significant (31.10 ± 10.39, 7.77 ± 4.51 and 1.50 ± 1.48) in comparison to reference site (22.80 ±1.08, 3.47 ± 1.59, 0.40 ± 0.51). Thus, results indicate that the Lake Mogan was polluted with different pollutants having genotoxic impacts on aquatic life and prompted continuous lake monitoring.

2.5.2 Cytotoxicity assessment

Cell viability and cytotoxicity assays are used for evaluation of pollutants effects based on various cell functions such as blood chemistry, enzyme activities, cell adherence, blood parameters viability and nucleotide uptake activities. Cytogenetic damage was detected in Common carp erythrocytes after exposure to chlorinated disinfectants by-products (Sodium Hypochlorite, chlorine dioxide and peracetic acid) with humic acid interaction (Gustavino *et al*.,

2005). Toxicity of Chloroform ($CHCl_3$) along with other THMs was environmentally critical and susceptible to aquatic life (Mattice *et al*., 1981).

2.5.2.1 Hematological and biochemical assessment

Hematology and biochemical parameters are useful and valuable factors for showing physiological disturbance within effected fish and provide useful information regarding the prognosis of infectious diseases. These indices not only assess the physiological and health status of fish but also indirectly assisting the monitoring of aquatic ecosystem at each level of interest (Svetina *et al.,* 2002).

Alterations in hematological count have been extensively used as powerful tool for the determination of health and physiological status of fish (Gabriel *et al.*, 2011). Therefore, allow fast and rapid evaluation of acute and sub-acute toxicities of DBPs and their effect on target organs. Examination of different blood parameters {white blood cells (WBC), red blood cells (RBC), hemoglobin (Hgb) , mean corpuscular haemoglobin concentration (MCHC), hematocrit (Hct), mean corpuscular volume (MCV) , mean corpuscular haemoglobin (MCH) and platelets (PLT)} under the exposure of toxic pollutants provides systematic relationship among certain aquatic species.

The decrease in RBC and hemoglobin also depends upon the metabolism of trihalomethanes. All trihalomethanes were primarily metabolized to carbon dioxide or carbon monoxide. The resulted carbon monoxide reacts with hemoglobin in blood stream and converts it into carboxyhemoglobin (COHb) which unlike oxyhaemoglobin (O_2HB) and prevents hemoglobin from supplying oxygen (O_2) to the body tissues. The cells die because of lack of O_2 and their number begins to decline which consequently lowers the hemoglobin level (Andersen *et al*., 1991).

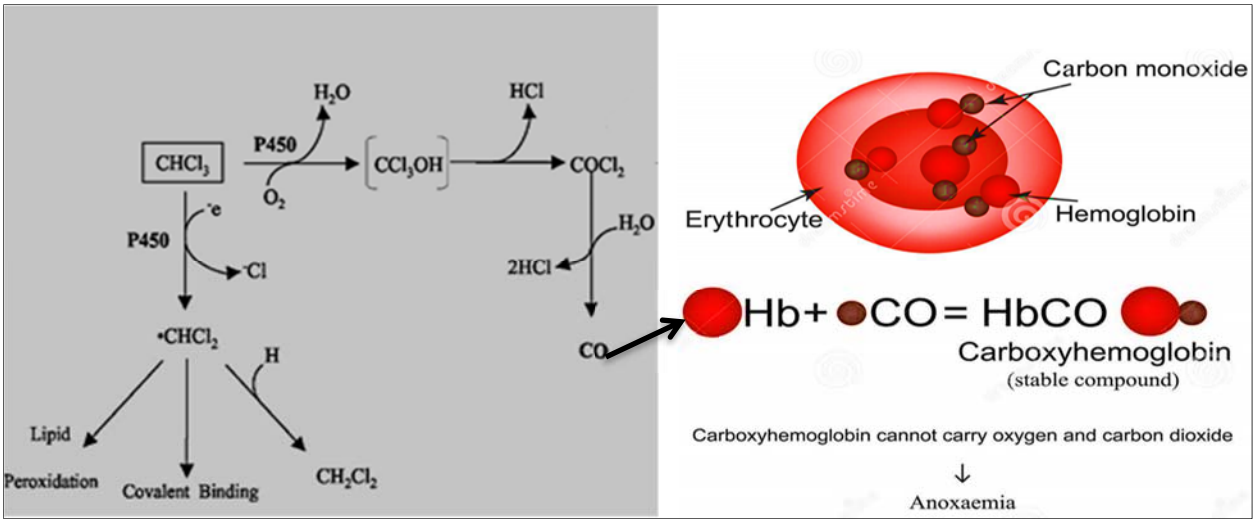


Figure 2.3: Metabolism of trihalomethanes

It is studied that the blood indices exhibits physiological and genetic variation within species. Furthermore, the change is dependent upon fish age, health status and sexual maturity (Baghizadeh *et al.,* 2015).

Fish blood is one of the most important and active component of body, which accompanied to contributes in metabolic process through gas exchange between organisms and with their surrounding (Fazio *et al.,* 2013). For that reason, blood indices were commonly used as the indicator of cyto-toxicological exchange and disturbance in fish.

Alterations in biochemical parameters (Hepatic antioxidant enzymes and total glutathione) were significant after exposure to chlorine compound as compared to peracetic acid disinfectants. Increased enzymatic activity caused potential adverse impacts in carp liver (Elia *et al*. 2008). The decrease in the level of total protein and glucose was observed in cyprinid fish species (Groff & Zinkl, 1999). Physiological (total protein) and immunological (heamocytes) count was investigated in caged Cray fish as a biomarkers of undergone stress (Klobucar *et al*. 2010). Xenobiotic and organochlorine (OC) in Sariyar Dam Lake, Turkey showed decrease in the enzymatic activities (ALT, AST, LDH) in exposed fish liver (*Cyprinus Carpio* and *Capoeta Tinca*) during spring and autumn season (Ozmen *et al*. 2008).

Table 2.8: Hematological and biochemical parameters as cytotoxicity assessment

Hematological parameters	Biochemical parameters

White blood cells (WBC)	Total protein
Red blood cells (RBC)	Glucose
Hematocrit	LDH activity (Lactase dehydrogenase)
Hemoglobin	AST activity (Aspartate aminotransferase)
Mean corpuscular volume (MCV)	ALT activity (Alanine aminotransferase)
Mean corpuscular hemoglobin (MCH)	Triglycerides
Mean corpuscular hemoglobin concentration (MCHC)	Insulin
Platelets	Albumin

Haidar and Ansari (2012) conducted a study to evaluate the cytotoxic effects of Monogenean (flukes) through hematological and biochemical count in Common carp. Study compares the effect of said parasite in healthy and infected fish. Adult healthy and Monogenean infected fishes were collected and kept in separate experimental tanks. Blood samples for hematology and biochemistry were collected in ETDA vials and gel activators. The results of study demonstrates that the lower values of blood parameter (Hb, RBC, WBC) in infected fish as compared to healthy was observed. Whereas, the significant increase in the leucocytes, neutrophils and monocytes in monogenean infected Common carp. In case of blood chemistry, elevated level of serum transamines (Serum Glutamic Oxaloacetate Transaminase and Serum Glutamic Pyruvate Transaminase) indicates liver function disorder in infected fish. Thus, results confirmed that the intensity of monogenean infection is responsible for the alteration in hematology and biochemistry of Common carp.

Rabergh & Lipsky (1997) examine cytotoxicity of two chlorinated disinfection by-products chloroform and carbon tetrachloride. Hepatocyte toxicity in fish was determined by measuring the released concentrations of LDH, GSH and protein as the exposure of chloroform and carbon tetrachloride. At higher concentration of $CHCl_3$, total lysis of cells and released of LDH, GSH was observed.

Villarini et al (2011) evaluate the cytotoxicity of three different disinfectants {sodium hypochlorite (NaClO), chlorine dioxide (ClO_2) and peracetic acid (PAA)} in Common carp for 20 days of exposure. The toxicity was observed through analysis of carp bile. The bile samples

were collected after 0, 10 and 20 days to investigate the effect of exposure time and dose. The aliquot of collected bile samples were adsorbed on C_{18} silica cartridges and toxic potential of whole bile was evaluated. Results indicate that said disinfectants showed significant toxic effects in exposed specimen bile in comparison to control.

CHAPTER 3

MATERIAL AND METHODS

Toxicology study on Common carp (*Cyprinus Carpio*) was carried out in Environmental Toxicology Laboratory under control conditions prescribed for toxicity test (Martinez *et al.,* 2004). Common carp was selected as model organism for current study as it has the ability to tolerate adverse environmental conditions and stresses and high sensitivity towards changing environment. Furthermore, it is a cool to temperate water fish being the main constituent of food chain in many areas of world.

The model fish was exposed to different concentrations of Chloroform and Iodoform (DBPs) to evaluate the geno and cytotoxic impacts on the healthy specimens. Experiments were divided into acute and sub-acute toxicity tests followed by OECD guidelines (OECD 1992). Experimental study was divided into control and experimental groups. Each experimental group contains five fish per batch for both iodoform and chloroform respectively.

3.1. Chemicals and Instruments

Standard Analyte of Chloroform and Iodoform were purchased from Fluka (02487-5ML) and Aldrich (109452-5G) respectively with 99% purity. These standards were stored at -20 °C. Methanol with 99% purity for making dose suspensions was acquired from Merck (Germany). For Comet assay analysis, low melting point agarose (LMPA), normal melting point agarose (NMPA) electrophoresis grade and Tris HCl for molecular biology grade was purchased from Scharlau (Spain). Sodium hydroxide pellets, trizma base, phosphate buffer saline tablets, dimethyl sulfoxide (DMSO), triton X-100 were supplied by Daejung (Korea). EDTA vials with purple and yellow caped for blood collection were purchased from LABOVAC Italiano. For preparation of Iodoform ethanol, iodine crystals and sodium hydroxide pellets were purchased from Fisher Scientific UK Limited and Daejung respectively.

Calibration and optimization of Chloroform and Iodoform was carried out using Gas chromatography (Shimadzu GC-2010, Japan). For Headspace Solid-phase Microextraction (SPME) 75 μm Car-PDMS fiber was obtained from Supelco (USA). Fully automated Sysmex XP-100 was used for hematological analysis and for biochemical analysis AMP Piccos II

Chemistry analyzer was used. Different biochemical indices were analyzed such as glucose, total protein, ALT, LDHL, Amylase, Lipase and Triglycerides. The reagents kits for above indices were purchased from AMP Diagnostic, Austria.

3.2 Cleaning of Glassware

All the required glassware for cyto and genotoxicity (comet assay) had been washed with soapy water and then rinsed with distilled water and finally oven dried at 180 ºC for 12 hours. After this procedure, they were covered with aluminum foil to preserve them from dust. For HS-SPME extraction, SPME glass vials were soaked overnight in concentrated chromic acid solution and rinsed with distilled water and finally oven dried at 180 ºC in order to avoid any contamination experiments which affects the result outcomes.

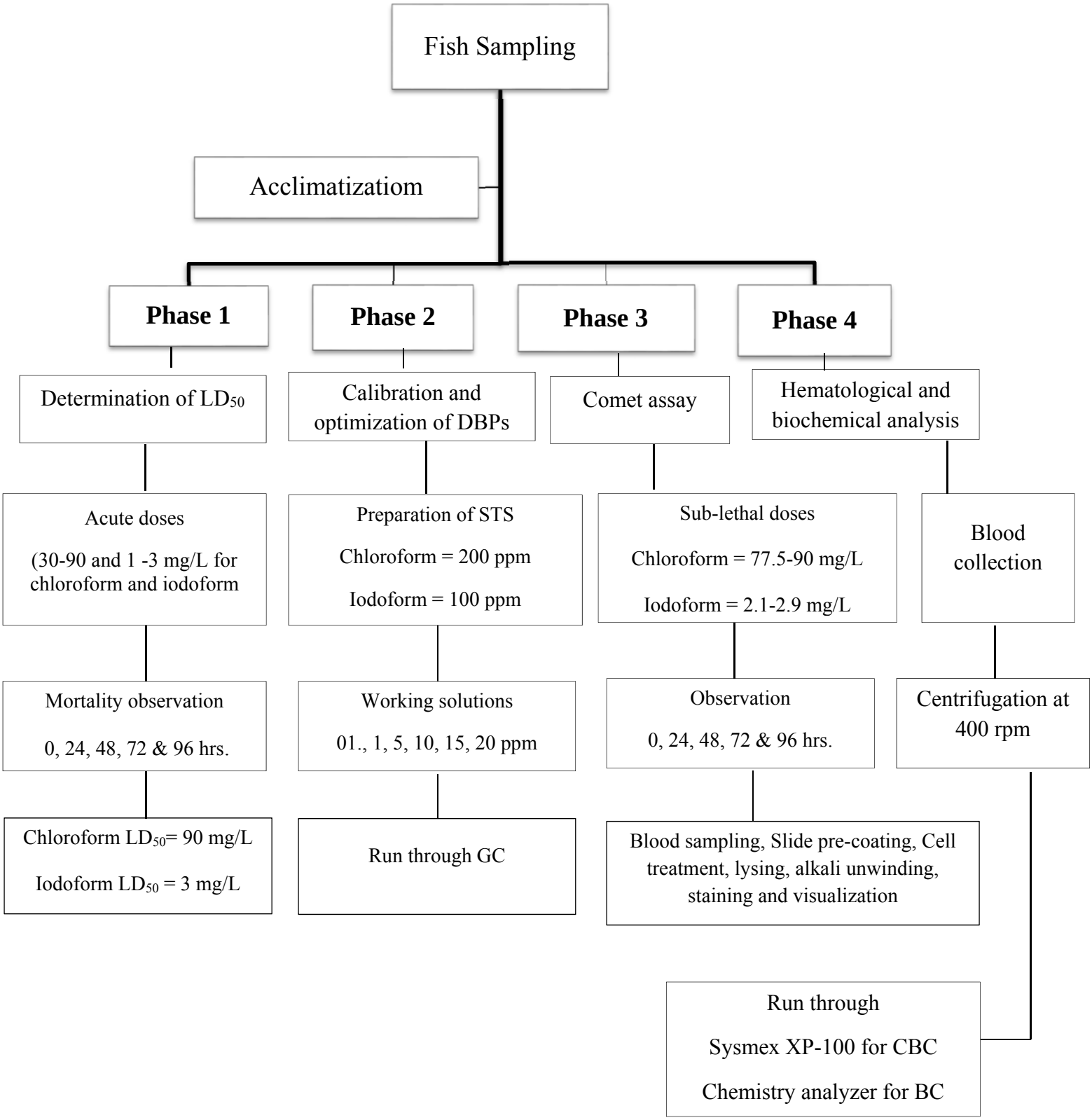


Figure 3.1: Experimental design of research work

3.3 SAMPLING

3.3.1 Site description

Rawal Lake was the study area of present study. The lake is situated in the East of Islamabad and North-East of Rawalpindi, Pakistan (33°42' N, 73°07' E). It provides water to both cities and covers an area of 8.8 km^2. Storage capacity of Rawal dam is 47,500-acre feet and it generates 84,000-acre feet of water on average rainfall. The study site is under great pressure from different human settlements such as Bhara Kahu, Malpur, Bani Gala and Noorpur Shahan etc. Untreated municipal, mainly domestic and agricultural waste is directly dumped into the reservoir which increases the demand of disinfection. Thereby, resulted toxicant by-products showed cyto and genotoxic effects within aquatic organisms. Below map clearly describes the study area.

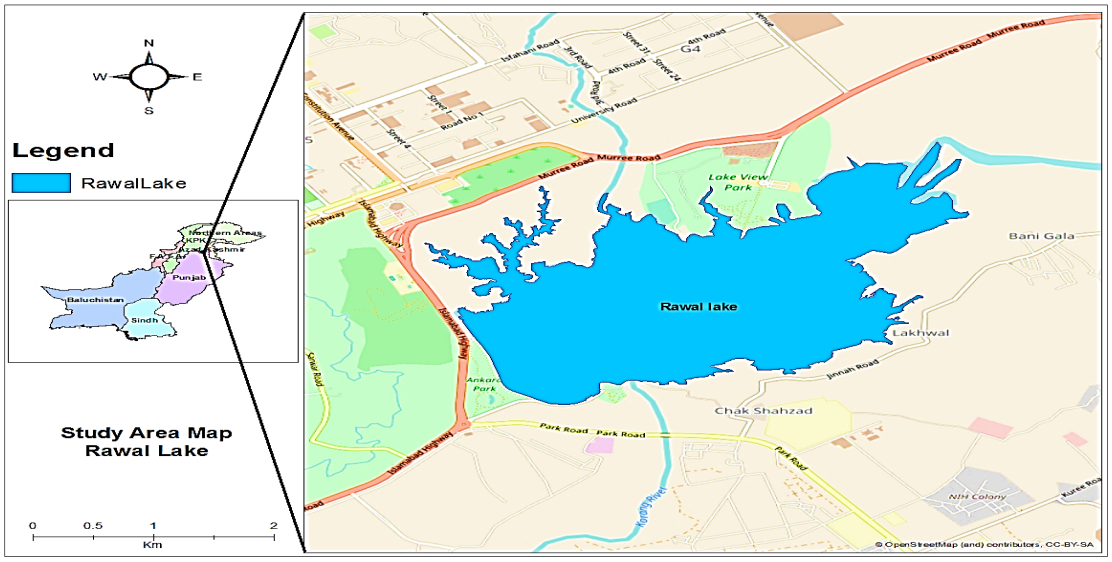


Figure 3. 2: Study area, Rawal Lake

3.3.2 Purchase and Maintenance of Experimental Fish (Common carp)

Healthy Common carp were purchased from Punjab Hatchery Rawal Town (Aquaculture and Fisheries Program and Research Centre), Islamabad. The purchased specimens were transferred to Environmental Toxicology Laboratory of National University of Sciences and Technology

(NUST) in aerated plastic bags. Special care was taken during transportation of samples and then kept in experimental tanks having dimension of 3 X 1.5 X 1.5 ft. Experimental tanks were filled with tap water supplied in laboratory. Fish were acclimatized to laboratory conditions for two weeks and fed with commercially available food pellets.

3.3.3 Acclimatization of fish

Before the start of experiment fish were acclimatized to laboratory conditions for a period of two weeks under control conditions. They were fed with commercial food pellets containing soybean, rapseed, rice, bran, corn, wheat and other agricultural by-products, on daily basis. Experimental tanks were filled with 50 liters of tap water from Environmental Toxicology Laboratory and changed on alternate day, to avoid fouling of tanks dead fish were removed immediately.

Morphometric parameters of experimental fish were determined at the start of experiment.

Table 3.1: Morphometric parameters of experimental fish

Fish specie	Total length (cm)	Total weight (g)	Age (months)
Common carp (*Cyprinus carpio*)	15±0.2	40±0.3	3

3.3.4 Water Parameters of Fish Tanks

The physicochemical characteristic of experimental tank and lake water was assessed using standard OECD (Organization for Economic Cooperation and Development) guideline method, 203, (1992). Water quality was determined at the start of experiment. The water was renewed at every alternative day using tap water from the Environmental Toxicology Laboratory. pH and temperature were measured using Multi parameter analyzer, Consort- C1020. Dissolved oxygen (DO) was measured using Winkler method, whereas titration method was followed to measure total hardness. Fresh water was provided to fish to avoid any damage to tissues or organs.

3.4 PHASE 1

3.4.1 Toxicity Potential of Chloroform and Iodoform

To evaluate toxicity potential of disinfection by-products (chloroform and iodoform), experiments were divided into Control and Experimental group on random selection basis. Five fishes per batch were selected. Fish specimens were kept in experimental tanks and fed with fresh food pellets.

3.4.2 Determination of LD_{50}

LD_{50} was determined by dividing experiment into Control and Experimental group on random selection method. Five batches per experimental test were selected with five fishes per batch and with one control group. Exposure concentrations of chloroform initially introduced were 30. 45, 60, 75 and 90 mg/L for 96 hours of exposure duration and observed after every 24 hours. No mortality was observed below 75 mg/L and none of fishes survived above 90 mg/L for exposure duration. After finding the range, final doses of 77.5, 80, 82.5, 85 and 87.5 mg/L were exposed to experimental fishes and observed for 24, 48, 72 and 96 hours of exposure duration. Iodoform is recently identified disinfection by-product and more toxic than chloroform (Richardson *et al.,* 2007). The initially introduce concentrations for iodoform were 1, 1.5, 2, 2.5 and 3 mg/L for exposure duration. After 96 hours of exposure duration it was observed that below 2 mg/L no mortality was observed and none of fishes survived above 3 mg/L. After finding the range, final doses of 2.1, 2.3,2.5,2.7 and 2.9 mg /L were applied for 24, 48, 72 and 96 hours of exposure duration. Dead fishes were removed immediately during ongoing experiment in order to avoid fouling of experimental tanks.

3.4.3 Acute toxicity Assessment

Acute toxicity was observed according to OECD guidelines 203 (1992). LD_{50} for 96 hours of exposure duration was found to be (90 and 3 mg/L) for chloroform and iodoform respectively. Sub-lethal doses were selected which cause significant health effects to healthy fishes. Again the experiment was divided into Control and Experimental groups. Five batches with one control were selected and toxicity was observed for 24, 48, 72 and 96 hours of exposure duration. Blood samples were collected in EDTA vials after every 24 hours and toxicity was monitored using Comet assay, hematology and biochemical analysis.

3.4.4 Preparation of Iodoform

Iodoform is a pale yellow crystalline solid having characteristic odor. Iodoform is analogue of chloroform being prepared as a result of disinfection process. To make dose suspensions, laboratory analytical grade iodoform was prepared.

3.4.4.1 Apparatus and Reagents

- 25 cm^3 measuring cylinder
- 100 ml beakers
- 100 ml conical flask
- Filter paper
- Spatula
- Iodine
- Ethanol or propane
- Sodium hydroxide

3.4.4.2 Procedure

In the laboratory, iodoform is prepared by reaction of iodine with organic compound containing $CH_3CH(OH)$ group (ethanol, 2-propanol, 2-butanol) or CH_3CO^- group (propanone, 2-butanone) in presence of sodium hydroxide.

Chemical reactions involved are mentioned below;

3.4.4.2.1 Ethanol

$2NaOH + I_2 \rightarrow NaOI + NaI + H_2O$

$CH_3CH_2OH + NaOI \rightarrow CH_3CHO + NaI + H_2O$

$CH_3CHO + 3NaOI \rightarrow I_3CCHO + 3NaOH$

$I_3CCHO + NaOH \rightarrow CHI_3 + HCOONa$

3.4.4.2.2 Propane

$CH_3COCH + 3NaOI \rightarrow CH_3COCI_3 + 3NaOH$

$CH_3COCI_3 + NaOH \rightarrow CHI_3 + CH_3COONa$

Steps involved in the preparation of iodoform are mentioned below;

1. Measure 5 g of iodine using weighing balance.
2. Measure 5 ml of ethanol in 50 ml of beaker using measuring cylinder.
3. Add 5 g of measured iodine in 5 ml of ethanol in a 100 ml of conical flask and dissolve properly.
4. When iodine was properly dissolve, add 5% of sodium hydroxide solution drop by drop, and continuously shaking the flask. Cool the flask from time to time under tap water during reaction.
5. The addition of 5% solution of sodium hydroxide continued, until brown color of the iodine just disappears. Stop the further addition of NaOH solution.
6. Allow the flask to stand for 10 to 15 minutes, until the solution was cleared
7. Then, decant off the clear supernatant liquid.
8. At the end, filter the iodoform in round bottom flask using Whatman filter paper and dry to obtained iodoform crystals.

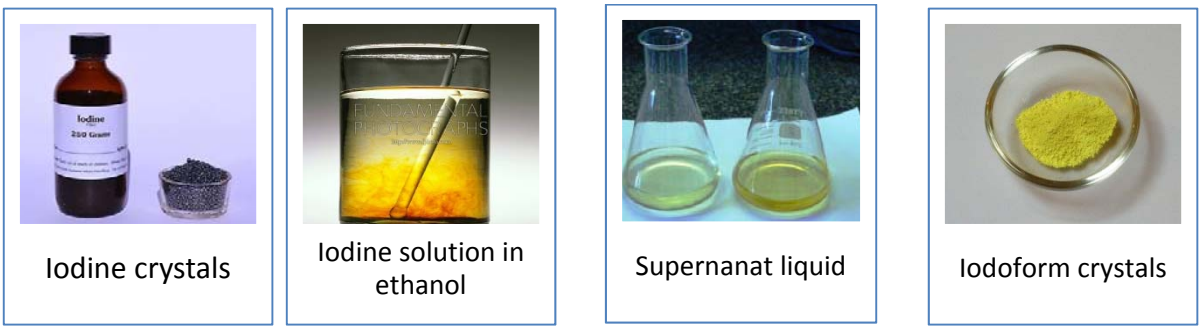


Figure 3.3: Steps involved in iodoform preparation

3.5 PHASE 2

3.5.1 Gas Chromatographic Analysis:

Gas chromatography analysis was developed for calibration and optimization of chloroform and iodoform (DBPs). Standard stock solution of 100 and 200 ppm for iodoform and chloroform were prepared using standards in methanol as a solvent. For the preparation of calibration curves working solutions of five different concentrations (1, 5, 10, 15 and 20 mg/L) were prepared in methanol solvent and run on GC equipped with Electron capture detector.

GC was optimized prior to sample injection. This was done by conditioning process, involving the changing in temperature of injector, column oven and detector. After condition of 20 minutes, retention time and peak area of sample analyte was noted and calibration curves were formulated. Operational conditions for GC analysis were listed in the Table 3.3.

Table3.2: Operational conditions for gas chromatography

Parameters	Values/ Units
1. Injector	
Pressure	48.2 Kpa
Total flow	126.9 mL/min
Temperature	220 °C
Linear Velocity	24.4 cm/sec
2. Column	
Initial temperature	50 °C
Final temperature	200 °C
Temperature ramp	15 C/min
3. Detector	
Temperature	280 °C
Current	0.03 nA
Gas flow	3 mL/min

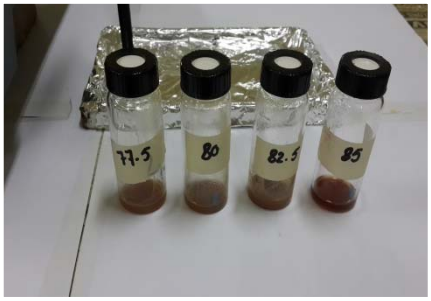
3.5.2 Headspace Solid-phase Microextraction (HS-SPME) technique

Optimization experiment to evaluate the effect of DBPs in fish was determined through Headspace Solid phase micro-extraction technique (HS-SPME) using gas chromatography. Fish

blood samples were fortified with optimized concentrations of chloroform and iodoform in a glass vials having PVC (Polyvinyl Chloride) covers and 1.5 mm thick PTFE (Polytetrafluoroethylene) skiving silicon septum. The analytes were extracted with Divinylbenzene Carboxen-polydimethlysiloxane 75 µm (DVB-CAR-PDMS-75 µm) fiber using hot plate magnetic stirrer. The stirring was done for 30 minutes at 40°C. After the adsorption of analytes, the fiber was retracted back and inserted into GC injection port where they were thermally desorbed under the flux of carrier gas (N_2). Recovery efficiency was calculated using EPA method 555.1 (Munch & Hautman, 1995).

$$R = 100^* (A-B)/C$$

[A = total measured concentration; B = background concentration; C = fortifying concentration]



Treated blood samples

DVB-CAR-PDMS Fiber

Figure 3.4: SPME experimental setup (SPME vials containing treated samples and injection port)

3.6 PHASE 3

3.6.1 Comet Assay Protocol

Comet assay or single cell gel electrophoresis is a flexible, simple and low cost technique for the detection of DNA strand breakage in eukaryotic cells. It may detect DNA damage at very low level and being suitable for minute impairment.

The method protocol is divided into two main steps;

1. Preparation of reagents
2. Preparation of slides for visualization

Table 3.3: Steps involved in comet assay protocol

Preparation of reagents	Preparation of slide for visualization
Phosphate buffer saline	Slide Pre-coating
Agarose (low & normal melting points)	Sample pouring
Lysing solution	Cell lysing
Alkaline solution	Alkali unwinding
Tris base electrophoresis buffer	Electrophoresis
Neutralization solution	Neutralization & staining
Staining solution	Comet visualization

3.6.1.1 Preparation of Reagents

The comet protocol contains different reagents for completion of whole test. All the reagents were prepared using autoclaved distilled water in order to avoid any kind of contamination. Further, glassware was washed and oven dry at 150 ºC to ensure sterilization.

3.6.1.1.1 Phosphate Buffer Saline (PBS)

A tablet of phosphate buffer saline was dissolve in 100 ml of distilled water and autoclaved for 15-20 minutes so that the PBS tablet was completely dissolved and thick consistency transparent solution was formed. Then solution was stored at room temperature (25 ºC).

3.6.1.1.2 Agarose

For the preparation of slides two different percentage (**1 and 2%**) of normal and low melting point agarose solutions were used. The agarose solutions were prepared in phosphate buffer saline solution.

- ✓ **Normal melting point agarose**

For the preparation of 1% normal melting point agarose 500 mg of normal melting point agarose (NMPA) electrophoresis grade was measured and dissolved in 50 ml of phosphate buffer saline (PBS) solution in 100 ml glass beaker. The beaker was placed in water bath at 90 ^{o}C and continuously stirred until the agarose was completely dissolved.

- ✓ **Low melting point agarose**

2% low melting point agarose was prepared by measuring 1000 mg of low melting point agarose (LMPA) electrophoresis grade and dissolving in 50 ml phosphate buffer saline solution in 100 ml glass beaker. The beaker was placed in water bath set at 90 ^{o}C and continuously stirred until the agarose was completely dissolved to avoid formation of lumps.

3.6.1.1.3 Lysing Solution

Lysing solution was prepared in 500 ml of distilled water. The solution comprises of 2.5 N NaCl (73.05 g), 100 mM EDTA (18.6 g) and 10 mM Trizma Base (0.6 g). All the ingredients were dissolved in 350 mL distilled water. Once the ingredient dissolved, 4g of NaOH pellets were added and the solution was mixed vigorously for 20 minutes. After dissolving all ingredients properly, the pH of the solution was adjusted to 10 using HCl and NaOH buffer solutions. 95 mL of distilled water was added to the prepared solution and stored at -4 ^{o}C.

Finally at the time of use 50 mL of 1% Triton X-100 and 5 mL of 10% DMSO was added to make the solution up to 500 ml.

3.6.1.1.4 Alkaline Solution

To prepare 500 mL of alkaline solution, two stock solutions were prepared.

- **10 N NaOH**

It was prepared by dissolving 200 g of NaOH in 500 ml of distilled water.

- **200 mM EDTA**

It was prepared by dissolving 14.8 g of EDTA in 200 ml of distilled water.

Final 500 mL of working solution was prepared by adding 15 mL of NaOH solution and 2.5 mL of EDTA stock solution. The pH of the solution was adjusted to > 13 using HCl and NaOH buffer solution.

3.6.1.1.5 Tris base electrophoresis buffer (TBE)

TBE buffer was prepared by dissolving Tris Base (10.8 g), Boric Acid (5.4 g), and EDTA (1.86 g) in 1000 mL of distilled water. The pH of the solution was adjusted to >13.

3.6.1.1.6 Neutralization Solution

To prepare 500 mL of neutralization solution, 0.4 M Tris HCl was used. The neutralization solution was prepared by dissolving 24.25 g of Tris HCl in 500 mL of distilled water.

3.6.1.1.7 Staining Solution

Ethidium bromide (EtBr) stain was used for comet slide staining.

- **10 X Stock Solution**

The stock solution of 10X was prepared by dissolving 5 mg of EtBr stain in 25 mL of distilled water.

- **1X Working Solution**

Working solution of 1X was prepared by dissolving 1 mL of stock solution in 9 mL of distilled water.

3.6.1.2. Procedure for Comet assay

Comet assay protocol was performed according to method prescribed by Singh and colleagues (1988) with slight modifications. Low humidity environment was preferred during preparation of

slides; otherwise gelling time of agarose was enhanced. The experiment was performed under dimed light so that the slides were protected from UV- induced DNA damage.

3.6.1.2.1 Slide Pre-coating

Before the coating of three layer sandwich slides, they were made grease-free and clean by dipping in 70% ethanol and drying over blue flame.

a. First layer

The pre-coating of slides was done using 1% NMP agarose. The agarose was maintained at 37ºC so that clumps were not formed. Half portion of the slides was dipped in NMPA and immediately lower part is wiped off to make uniform and smooth layer of agarose. The slides were than allowed to solidify at room temperature for 15 to 20 minutes. The pre-coating of slides with NMPA provides enhanced anchorage of second layer of agarose.

b. Second layer with sample pouring

Once the first layer was solidify, LMP agarose with cell suspension was dropped onto dried slides. LMPA-cell suspension was made by mixing 80 µL of LMP agarose and 10 µL of cell sample in an eppendorf tubes. Special care was observed to avoid the formation of bubbles during pouring of sample on agarose layered slides. The slides were allowed to solidify by placing them on ice-pack for 10-15 minutes.

c. Third layer

After solidification of second layer, a third layer of NMP agarose was made to occupy any pores left during second layer, so that complete coverage of sample area was ensured. Immediately after completion of layering, cover slips were carefully placed on the slides and slides were allowed to solidify for 10-15 minutes at -4 ºC.

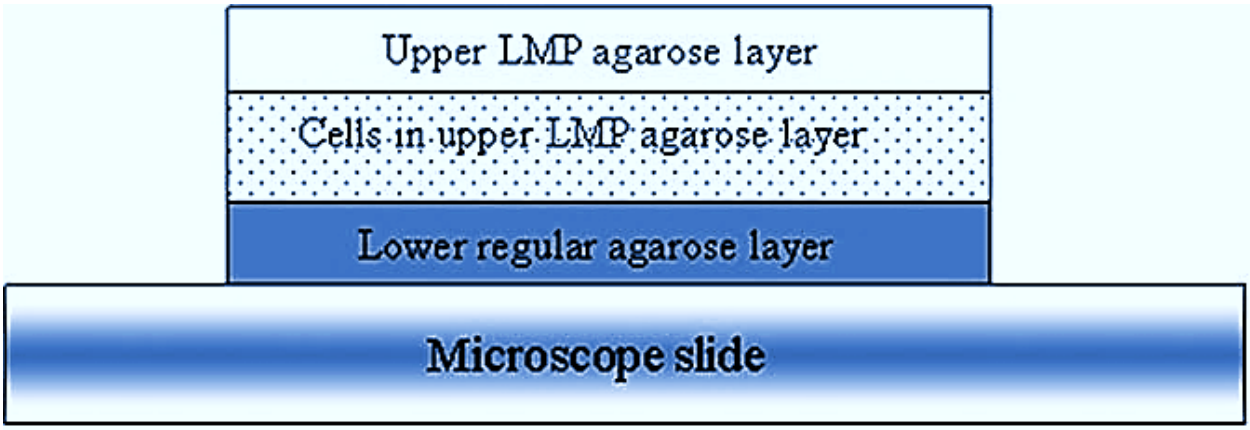


Figure 3.5: Three layered sandwich slide

3.6.1.2.2 Cell Lysing

After solidification of three layered slides, the cover slips were removed carefully and they were dipped in ice-cold lysing solution containing high salts and detergents. The slides were allowed to lyse for 1 to 2 hours at -4 ºC.

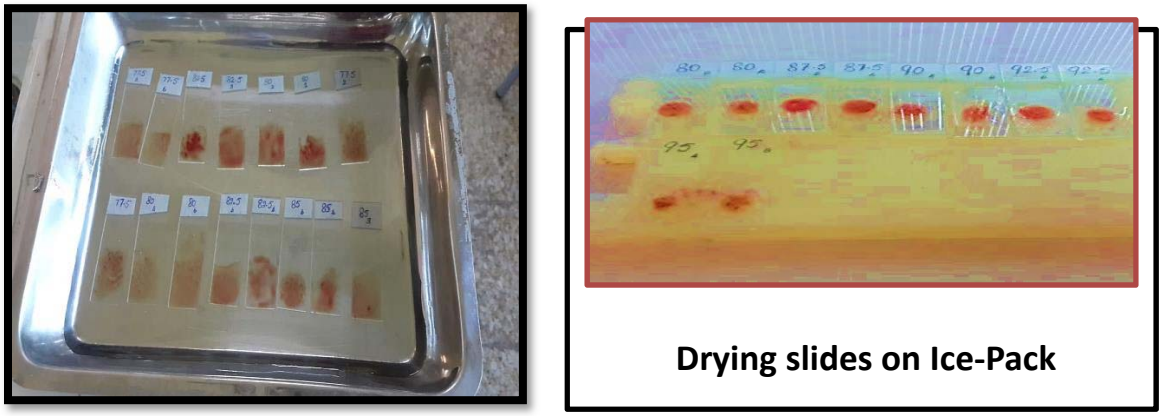


Figure 3.6: Slides dipped in lysing solution

3.6.1.2.3 Alkali Unwinding

After lysing step, slides were submerged in alkaline buffer solution for 20 minutes at room temperature.

3.6.1.2.4 Electrophoresis

After alkali unwinding, the slides were placed in comet electrophoresis tank and filled with TBE buffer solution. The slides were completely submerged in the TBE buffer solution. The tank was covered with lid and allowed to electrophorese for 30 minutes at 24 V and 300 mA.

3.6.1.2.5 Neutralization

After electrophoresing, the slides were gently placed in a flat tray and were neutralized using neutralization solution for 5 to 10 minutes. This step was repeated for 3 to 4 time by dropping neutralization solution using micro syringe. At the end excessive solution was drained out.

3.6.1.2.6 Staining

After few minutes neutralized slides were stained using 80 µL of 1X working solution of ethidium bromide staining solution. This step was also repeated for 3 to 4 times, until yellowish brown color was developed. After staining, excessive solution was drained out.

3.6.1.2.7 Drying

The stained slides were dried at room temperature for 15 minutes before comet visualization.

3.6.1.2.8 Visual Analysis

At the end of comet experiment, the prepared slides were visualized under Trinocular Fluorescent Microscope (Optika- B353FL) using 1000X objective lens. Slides were observed to determine genetic damage after dose exposure through cell suspension. The microscope was equipped with a camera (AIPTEK: AHD-Z600), ocular micrometer of 10µm and white LED/12V 20W illuminator. Images were taken and tail length, tail DNA and olive tail moment (OMT) was measured using CASP Lab software.

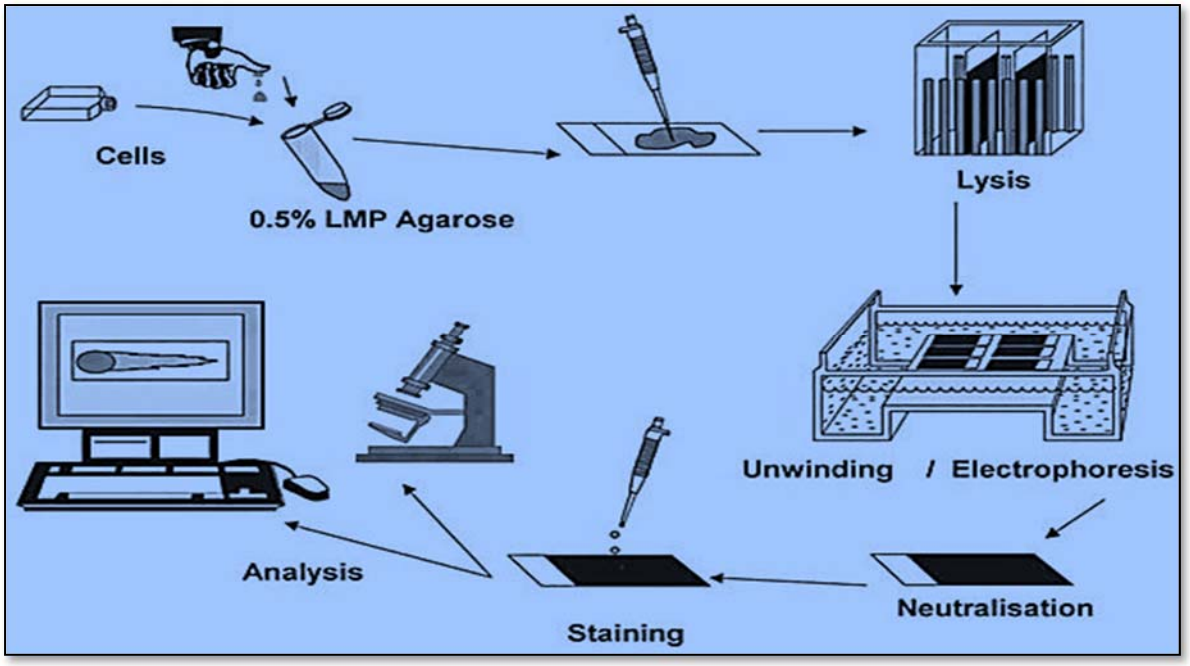


Figure 3.7 Steps involved in Alkaline Comet assay

3.6.1.2.9 Image Analysis

The comet images obtained after visual inspection using fluorescent microscope (Optika-B353FL) were analyzed with the help of CASP Lab software. CASP lab has been developed to interpret colored and gray scale comet images saved in TIF format. It has been optimized for a 600×800 resolution. Unlimited numbers of comet images were loaded successfully into "image view" window. User may adjust various thresholds of sensitivity as a measurement frame is drawn on the screen and size of the image to analyze was also adjusted. The frame was moved on to cell and measurement was started by clicking on "assay the comet". An intensity profile shows up on a "profile" window together with selected result values and the result may be saved. CASP Lab may analyze different parameters such as tail length, tail DNA, tail moment (TM) and olive tail moment (OMT). The obtained results were saved in a spreadsheet in "View Results" window. Where the results may export into text file and interpret.

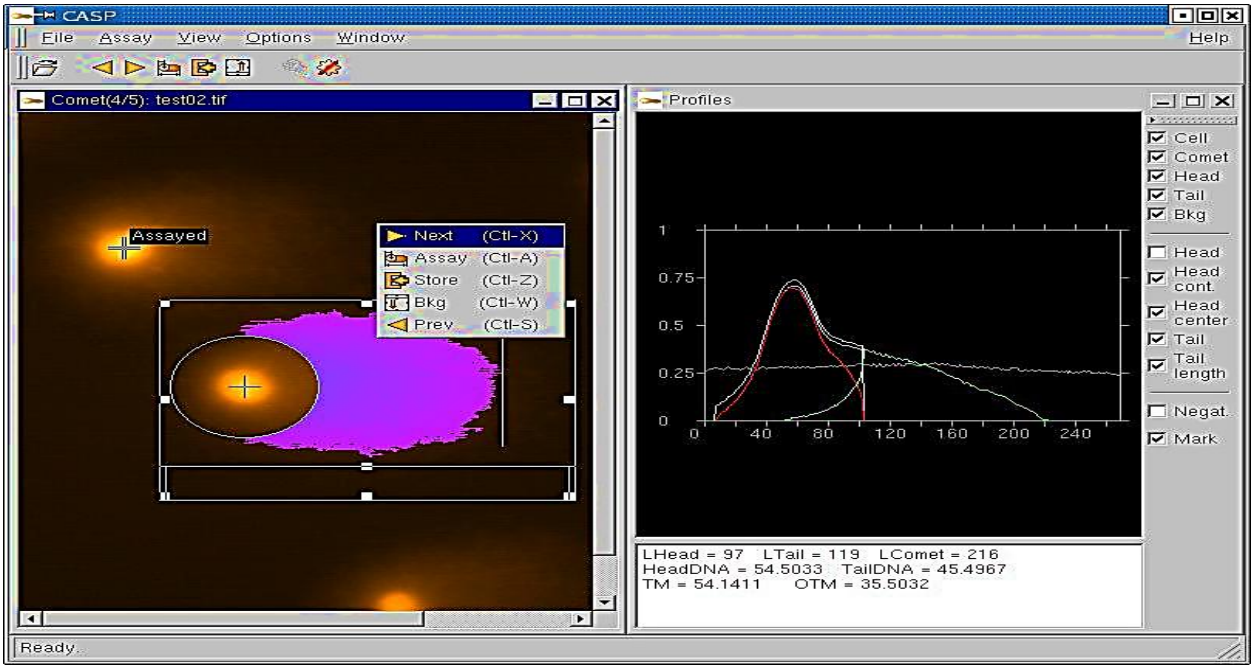


Figure 3.8: CASP Lab software showing comet image analysis

3.7 PHASE 4

3.7.1 Hematological Analysis

After cell treatment, the samples were undergone for complete blood count (CBC). This determines the effect of applied doses on hematological parameters of exposed fish blood samples. Test was carried out for 96 hours of exposure duration and blood sample were collected after every 24 hours. The blood was collected through cardiac puncture from the caudal vein below dorsal fins using 5ml herpinized syringe. The blood was collected in clean purple topped EDTA vials containing anticoagulant. After collecting blood, the vials were slightly shaken by hand to dissolve anticoagulant agent properly. Before the commencement of hematological analysis, the blood samples were centrifuged on Platform shaker LABCON-SPo-MP3 for 10-15 minutes at 300 rpm to avoid formation of any clots. Later, the samples were send to the Pakistan Institute of Medical Sciences (PIMS) for hematological analysis {white blood cells (WBC), red blood cells (RBC), platelets (PLT), hematocrit (Hct), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) , mean corpuscular volume (MCV) and hemoglobin (Hgb)} using Sysmex XP-100 hematology analyzer.

3.7.2 Biochemical Analysis

Biochemistry of fish blood samples was performed to evaluate toxic impact of chloroform and iodoform disinfection by-products. Biochemical indices such as glucose, total protein and ALT (alanine aminotransferase) were used to monitor stress caused by DBPs. Similar procedure was followed for biochemical analysis as for hematology. In this case the blood samples were collected in yellow topped EDTA vials to prepare serum. For serum production the sample were centrifuged for 10 minutes at 400 rpm and then run on chemistry analyzer using commercially available reagent kits.

Sysmex XP-100

AMP Piccos II Chemistry analyzer

Figure 3.9: Blood CBC and chemistry analyzer

3.8 Statistical Analysis

The data were subjected to two way ANOVA to determine significance of difference among control and experimental groups. P-values <0.05 were considered statistically significant. CASP Lab software was used to analyze comet images. GraphPad prism software version (7.01) was used to analyze graphical results.

CHAPTER 4

RESULTS AND DISCUSSION

The present study aimed to assess the genotoxicity potential of disinfection by-products (chloroform and iodoform) on Common carp (*Cyprinus Carpio*). Acute and sub-acute toxicity was determined by healthy exposing fish to different lethal and sub-lethal doses for 96 hours of exposure duration.

This chapter is based upon the results obtained from the series of experiments conducted to determine geno and cytotoxicity of chloroform and iodoform DBPs using comet protocol, hematological and biochemical analysis.

4.1 Acute Toxicity Test

4.1.1 Physicochemical parameters of experimental tank and lake water

Water quality of experimental tanks was determined by investigating different parameters as prescribed by OECD guidelines 203 for toxicity test (OECD 1992). Water quality was analyzed at the start of experiment and compared with physicochemical values of Rawal lake hatchery water from where fish samples were acquired.

Table 4.1: Physiochemical parameters of the experimental tanks and lake water

Parameters Mean values (minimum- maximum)				
	Temperature (ºC)	**pH**	**Dissolved Oxygen (mg/L)**	**Hardness (mg/L)**
Experimental tank	23.41± 3.6 (19.5- 27.5)	7.82± 0.3 (7.5-8.3)	6.9±1.6 (4.5-8.2)	220.25± 68.4 (139-304)
Rawal lake	28.55±2.3 (24-30.3)	7.89±0.4 (7.2-8.4)	6.9±0.9 (7-7.9)	230.75±45.4 (211-298)
OECD guidelines	20-24	6-8.5	80 % of air saturation	10-250

The physicochemical analysis of water parameters is presented in Table 2. The results showed that the mean values of temperature, dissolved oxygen and hardness (28.5 ºC, 6.9 and 230.7 mg/L) increased in lake water in comparison to experimental tanks (23.4 ºC, 5.5 and 220 mg/L). The probable reason for the significant increase may be due to the entry of pollutant load from the nearby areas. Further, increase in pollution load may be due to excessive use of pesticides, improper disposal of poultry and domestic waste coming from the nearby tributaries such as Bhara Kahu, Malpur, Bani Gala and Noorpur Shahan etc. Similar results were presented by Malik and Nadeem in 2011; they found that the quality of lake water deteriorated adjacent to the populated areas, whereas, the quality of water was found to be relatively clean and free of organic waste at the sites which were less impacted from nearby settlements. Ghumman (2011) reported that the lake water received excessive runoff from the adjacent areas during rainy season which increased the dissolve matter. Ayaz and coworker in (2016) conducted a study to evaluate water quality of Rawal Lake and quantified that most of the physicochemical parameters exceed the permissible limits prescribed by World Health Organization (WHO) 2004.

4.1.2 Cumulative Mortality

Cumulative mortality gives the proportion of the individual alive in each group at the start of the experimental test and death over completion of exposure duration. In the current study the experiment was design to determine LD_{50} of both compounds chloroform and iodoform for Common carp (*Cyprinus Carpio*). The results obtained after 96 hours of exposure duration was presented in the Tables 4.1 and 4.2. DBPs were ensured to be present all the time till the end of the toxicity test. Toxicity was observed to increase with increase concentrations of DBPs and exposure time.

Table 4.2: Cumulative mortality of Common carp against iodoform doses

Experimental Groups	**Exposure doses (mg/L)**	**Exposure duration (hrs.)**				**Survived fishes**
		24	**48**	**72**	**96**	
Control	0	5	5	5	5	5

Group 1	1	5	5	5	5	5
Group 2	1.5	5	5	5	5	5
Group 3	2	5	5	4	3	3
Group 4	2.5	5	4	3	2	2
Group 5	3	5	4	2	1	1

Table 4.3: Cumulative mortality of Common carp against chloroform doses

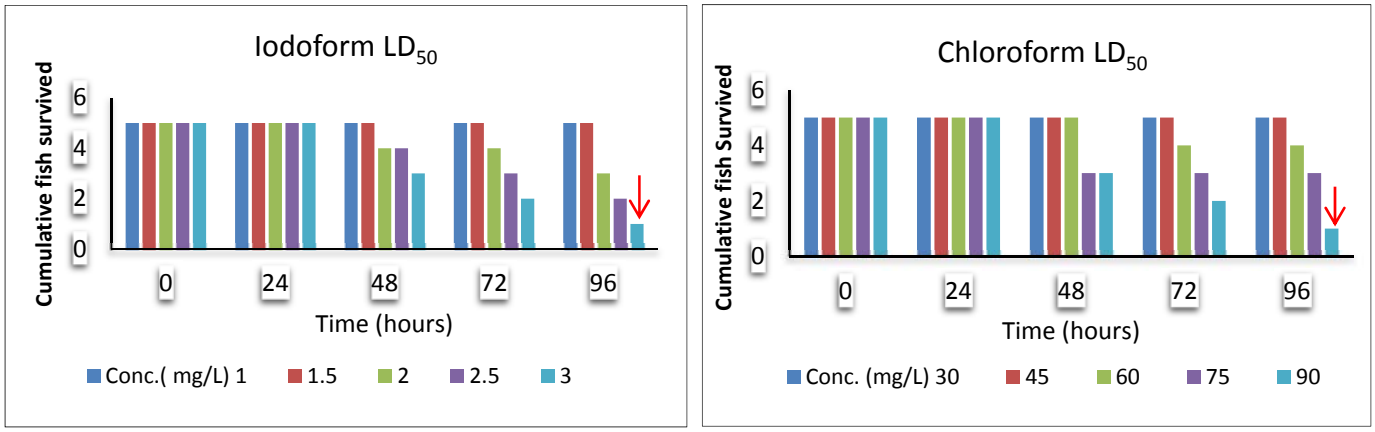
Experimental Groups	**Exposure doses (mg/L)**	**Exposure duration (hrs.)**				**Remained fishes**
		24	**48**	**72**	**96**	
Control	0	5	5	5	5	5
Group 1	30	5	5	5	5	5
Group 2	45	5	5	5	5	5
Group 3	60	5	5	4	4	4
Group 4	75	5	4	3	2	2
Group 5	90	5	4	2	1	1

Rate of survived fish observed after 24, 48, 72 and 96 h of acute exposure depicted that there is a direct relation, as with increase of exposure time and doses, the die off rate was also increased.

At 24 h exposure, no death of single specimen was observed at all exposed concentrations. In case of 48 and 72 h, at exposure concentrations 2.5-3 and 75-90 mg/L half of the population died for iodoform and chloroform respectively. Further, at 96 h it was observed that maximum die off rate was observed. The results showed that with increase dose and exposure time, toxicity of both compounds increased. Thus results in the determination of lethal dose 50 for chloroform

and iodoform respectively. The cumulative mortality observed in the present study suggests that with increasing exposure time and doses, the toxicity also increased. A high dose of iodoform and chloroform results in abnormalities and eventually cause death.

Mortality mainly depends up-on the sensitivity of organism towards toxicant, its concentration and exposure duration. In current study at 96 h, no death was observed in control group but in experimental group half of the population was dead and LD_{50} values were found to be (3 and 90 mg/L) for iodoform and chloroform respectively.



(a) (b)

Figure 4.1: 96 hours LD_{50} of iodoform (a) and chloroform (b)

The results obtained after 96 h of exposure duration for both compounds iodoform (a) and chloroform (b) are depicted in Figure 4.1. Mortality was observed at 48 and 72 hours of exposure duration. But half of the population was killed at 96 h with high concentration. The results are in accordance to the Mattice and coworkers (1981) who determined LD_{50} for chloroform, bromodichloromethane, dibromochloromethane and bromoform (97.2, 67.4, 33.5 and 52.3 mg/L) exposed to Common carp juveniles. LD_{50} of 2.92 mg/L was determined after 96 h for Fathead minnow (Pimephales Promelas) exposed to iodoform DBP.

4.2 Behavioral observations

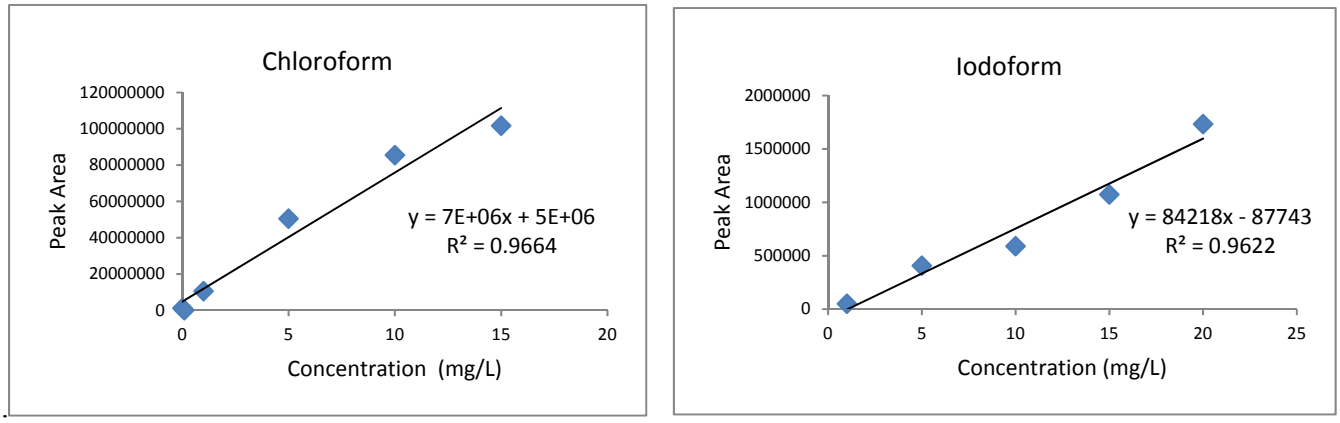
The present study showed that the exposure of DBPs to relatively higher concentrations could negatively affects the health status of exposed specimens (50 {5 fishes/batch}) of common carp. Abnormal behavioral changes were noted in experimental tanks at all observed concentrations for both compounds in comparison to control groups. Prominent behavioral changes were

observed at higher concentrations (2.9 and 87.5 mg/L of iodoform and chloroform respectively) after 24 h of exposure. Mild changes such as restlessness, abnormal swimming and sluggish movement was observed after 48 h at lower concentrations (2.1-2.3 and 77.5-80 mg/L for iodoform and chloroform respectively). Whereas, corrosion of fins, skin injuries and signs of behavior associated with anxiety was observed at medium concentrations for both DBPs. On the other hand, sudden changes such as erratic swimming, lethargy, skin injuries, increase in the frequency of opercular movements and loss of equilibrium was noted at higher concentration (2.5- 2.9 and 82.5-87.5 mg/L for iodoform and chloroform respectively).

Our results are in line with the study conducted by Blahova and co-workers (2014), who reported similar behavioral changes in common carp (Cyprinus carpio). Similar trends of uncoordinated behavior were also reported by Imanpoor & Kabir (2011) in common carp after 96 hours exposure to sublethal concentrations of chloramine T in the range of 1-60 mg/L.

4.3 Gas Chromatography Analysis

Linear calibration curves for both compounds were generated by running different dilutions of both analytes and by plotting against response peak area. Standard calibrations were plotted for all concentrations ranging from 0.1 to 20 ppm for both analytes. The curves obtained show linearity with regression coefficient R^2 of 0.966 and 0.962 for chloroform and iodoform respectively as presented in Figure 1 (a) and (b).



(a) **(b)**

Figure 4.2: Calibration curves of concentration against peak response for chloroform (a) and iodoform (b)

Respective chromatograms for standard and samples of both DBPs (chloroform and iodoform) at dose 20 ppm may be observed in Figure 4.3. The figure clearly depicted that identifiable chromatographic peaks at retention time 10.8 and 2.1 minutes were observed for iodoform and chloroform respectively. The analysis time was 18 minutes.

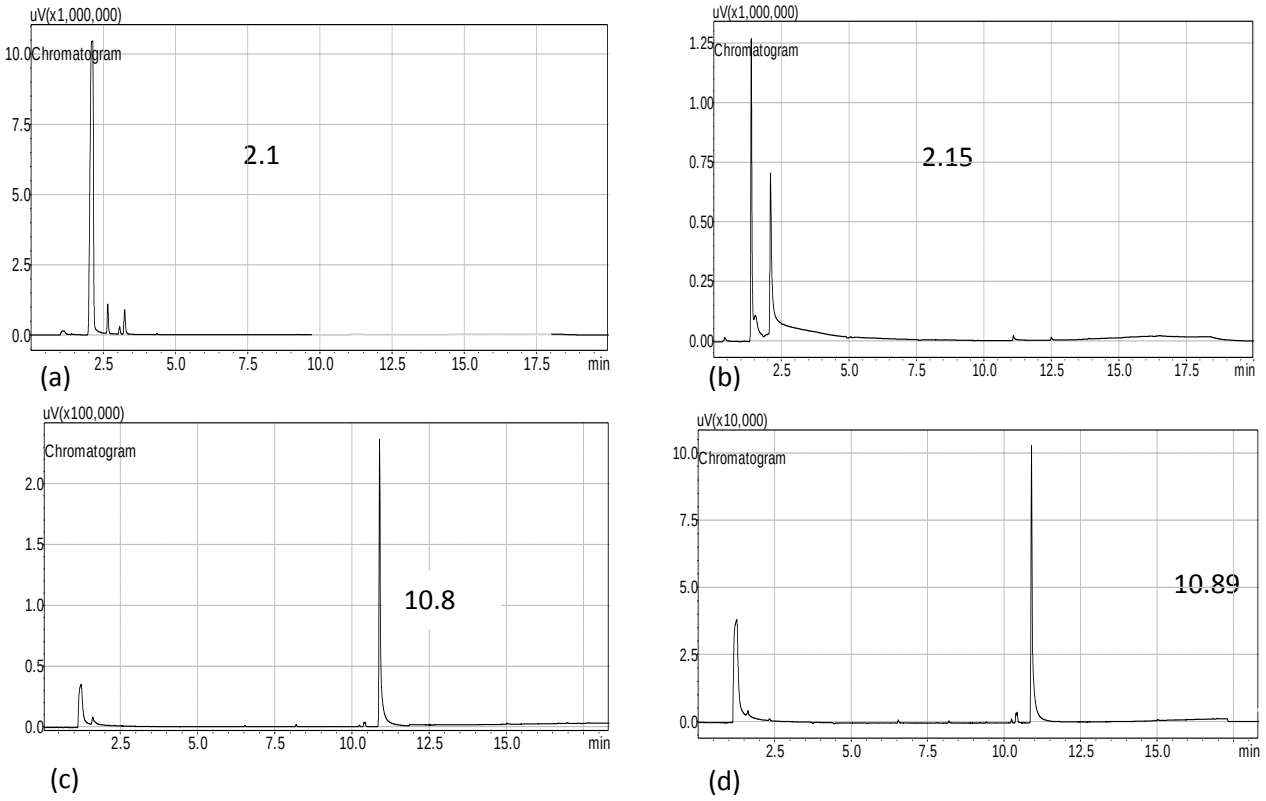


Figure 4.3: Chromatographic peaks for standards and samples of chloroform (a,b) and iodoform (c,d)

Results obtained after chromatographic analysis revealed that the slight change in retention time was observed after cell treatment with iodoform and chloroform disinfection by-products respectively. Thus, indicating that the cell treatment was successful. Moreover, GC operation was manually performed therefore it can be also a cause for these variation in retention time of eluted peaks for both compounds. The change was probably due to volatilization of sample and headspace loss during transferring of sample into SPME vials for extraction (Blount *et al.*, 2006).

4.4 Head space solid phase Microextraction (HS-SPME) analysis

Recovery efficiency for determining DBPs in fish blood samples was calculated through HS-SPME equipped with DVD-CAR-PMDS fiber. The recovery efficiency must fall in the range of 70 to 120 % determined by EPA method 555.1(Munch & Hautman, 1995). The SPME using gas chromatography helped to achieve acceptable recoveries efficiencies for both analytes ranging from (74 to 83 and 68 to 95% for chloroform and iodoform respectively). This verifies that HS-SPME is an accurate, fast and reproducible technique for determining DBPs in fish blood samples. The results for recovery efficiency percentage for both DBPs at each concentration are presented in Table 4.1.

Table 4.4: Percentage recovery efficiency (R) of chloroform and iodoform at each specified concentrations

Compounds	Concentrations (mg/L)	% Recovery efficiency (R)
Iodoform	2.1	95
	2.3	86
	2.5	80
	2.7	74
	2.9	68
Chloroform	77.5	83
	80	81
	82.5	78
	85	76
	87.5	74

The present results were in accordance to Delvaux and coworker (2017) who determined the level of THMs (chloroform, bromodichloromethane, dibromochloromethane and bromoform) in fish blood samples. They quantified that recovery efficiencies for all used THMs were within acceptable ranges (chloroform 78%, bromodichloromethane 120%, dibromochloromethane 102% and bromoform 98% respectively).

Determination of volatile compounds in fish followed by SPME Carboxen-polydimethlysiloxane fiber presents efficient and accurate extraction of analytes at 60°C for 30 minutes of extraction period. Thus, confirms that SPME is useful, fast and sensitive technique in monitoring targeted compounds associated to fish (Iglesias & Medina, 2008).

4.5 Genotoxicity analysis

Genotoxicity of disinfection by-products (chloroform and iodoform) was assessed through comet assay. The results obtained from Comet assay were plotted in a series of bar charts for different comet parameters (tail length, tail DNA % and olive tail moment). Comparison was done for both DBPs at all defined concentrations. For statistical analysis GaphPad Prism version 7.01 was used and two way ANOVA was applied to find out the significance of variance among experimental groups.

4.5.1 Visual analysis

For visual inspection, these are four different classes of DNA damage as prescribed in literature, make it sufficient as quantitative utilization for many purposes. DNA damage was classified in 0 to 4 categories.

This classification was divided as;

- No damage
- Minor damage
- Moderate damage
- Major damage

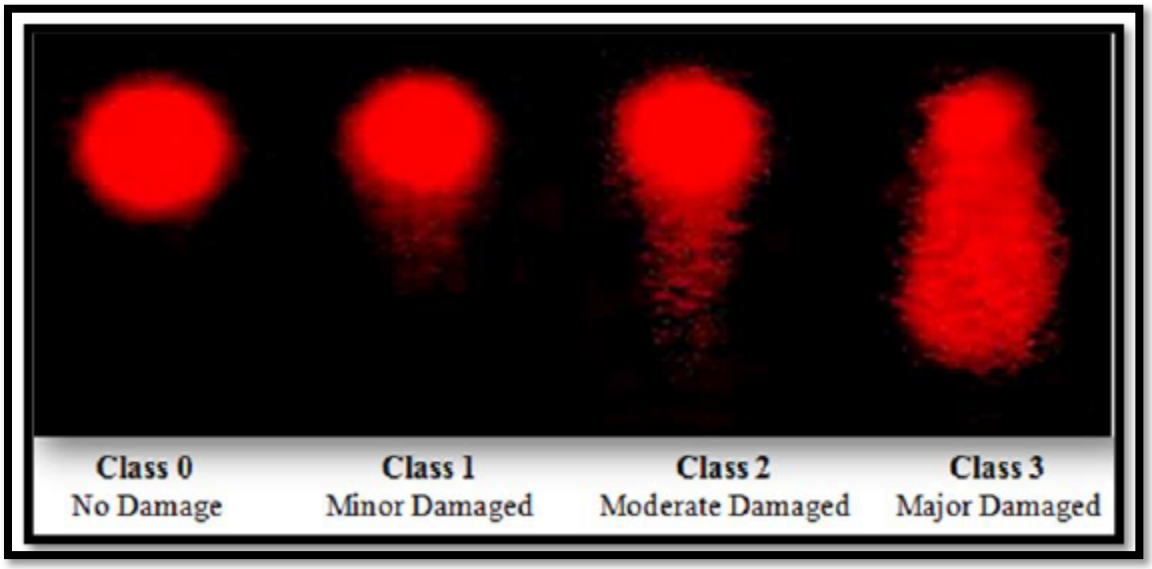


Figure 4.4: Classification of DNA damage

The images of comet parameters for both DPBs (chloroform and iodoform) during the course of current research mostly falls in the classes of 1 to 3, whereas the control groups fall in class 1 i.e. no damage. The images analysis was done using CASP Lab software with focus on three important parameters including tail length, tail DNA % and olive tail moment that are discussed below.

4.5.2 Dose-response Relationship

The dose–response relationship describes the effect of a chemical or stressor on an organism after a certain period of exposure time. This relationship describes the dose-response curves, which refers to the effect of applied chemical against given concentration. Therefore dose-response curves are considered as important tool for determining the applied chemical safe and hazardous level, so that the permissible limits should be created. When evaluating the response, one of the important characteristic is graded relationship, which means that as the amount of chemical or dose increased so is the response towards the chemical also increased.

4.5.2.1 Dose-response curves

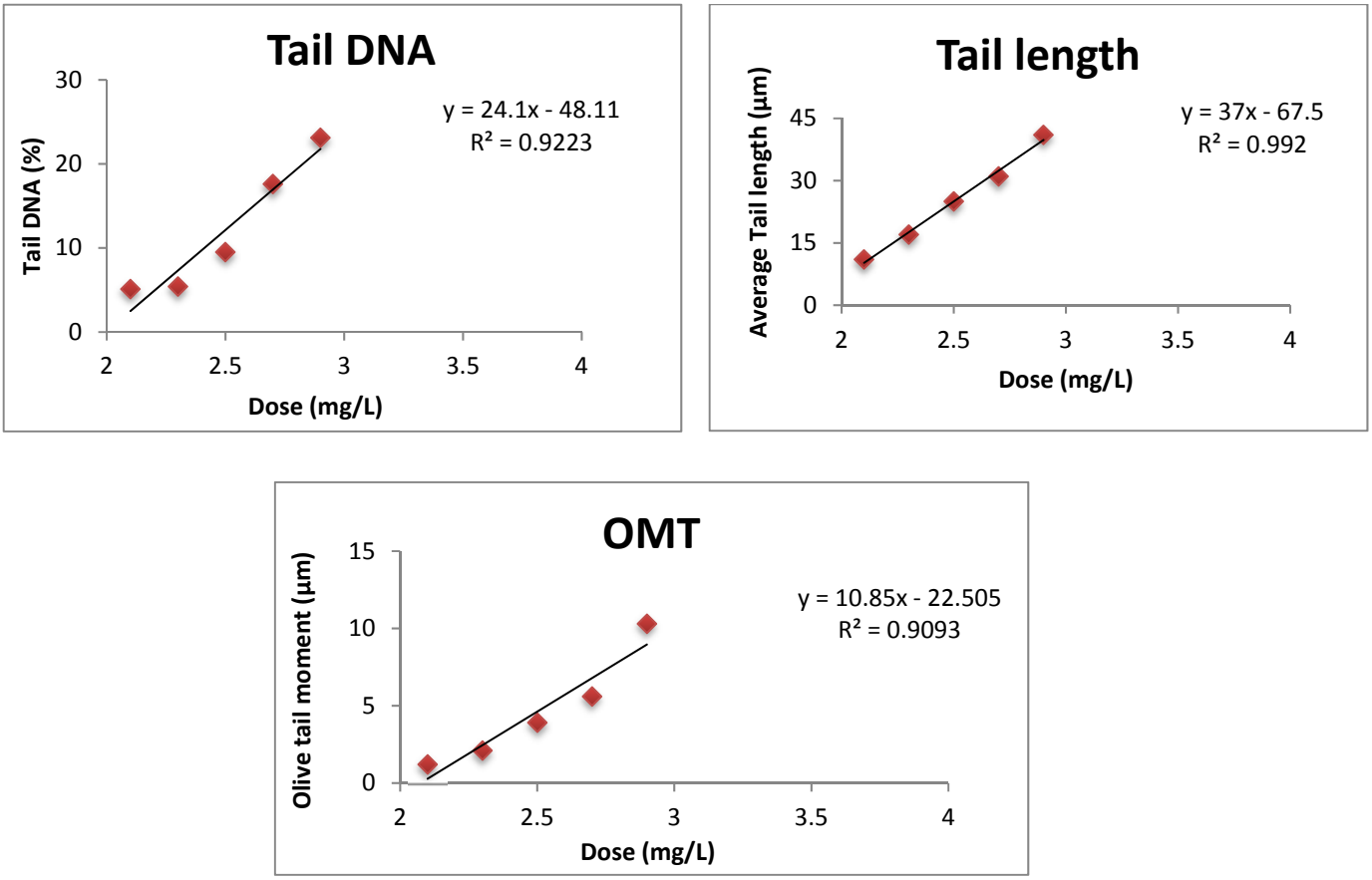


Figure 4.5: Dose response curves of iodoform disinfection by-product

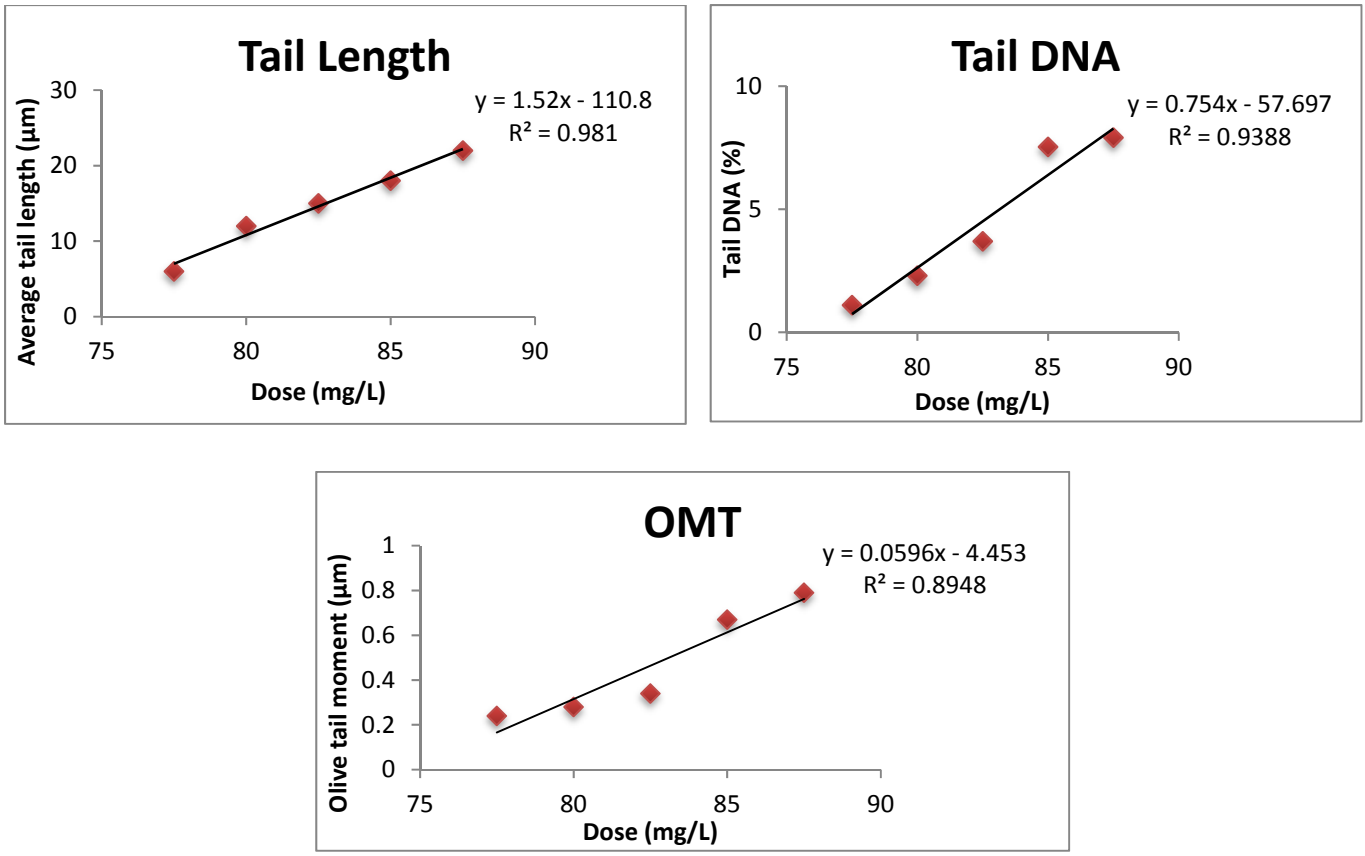


Figure 4.6: Dose-response curves for chloroform disinfection by-product

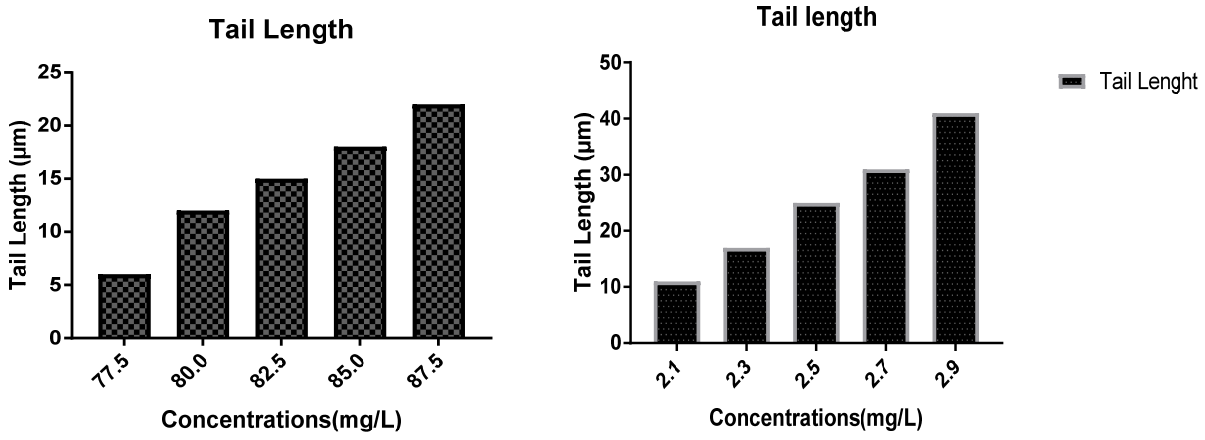
The dose response curves for iodoform and chloroform against tail length, tail DNA and olive tail moment are presented in Figure 4.4 and 4.5 respectively. It was depicted that comet parameters showed linear equation with regression coefficient R^2 of 0.89 and 0.9. A direct relationship against dose concentration and response was observed which means that with the increase in dose concentration the DNA in term of tail length, tail DNA and OMT also increased. Hence it was proved that the fish blood cells were very sensitive against disinfection by-products and comet assay is very useful to detect DNA damage induces by the chloroform and iodoform respectively.

Image analysis was done using CASP Lab software focusing on three important comet parameters that are discussed below.

4.5.3 Tail length

The significant increase in the tail length is determined through the migration of DNA towards tail region which is quantified by fluorescence, measured as tail length (µm). The extent of DNA to travel towards anode after applied current during fluorescence depends upon the level of damage occurred in the DNA. This would further determine the level of DNA damage which classified into low, medium, high and very high level. Tail length is considered to be one of the most important parameters to assess the DNA damage (Kumaravel & Jha, 2006).

The relationship between DNA tail length and varying concentration was observed in Figure 4.5 for chloroform and iodoform respectively, indicating that the model explains the variability of the response data around its mean.



(a) (b)

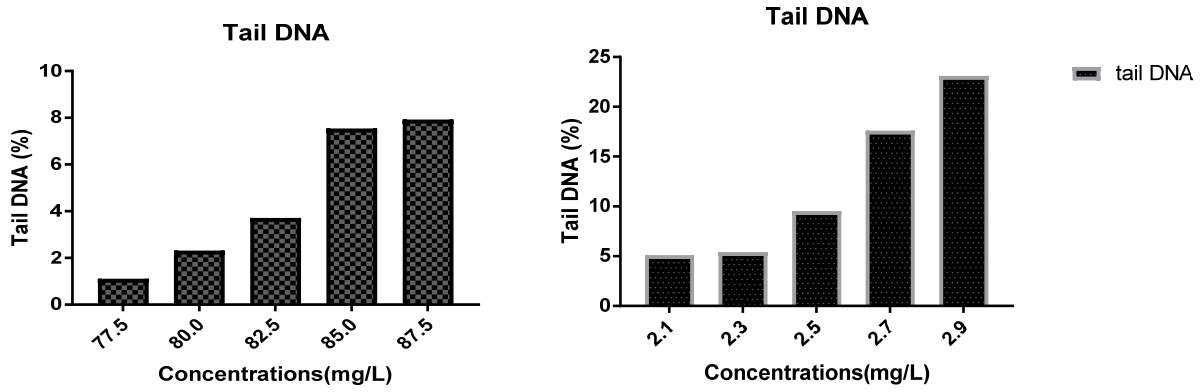
Figure 4.7 Relationship between Tail Length and Concentration of Chloroform (a) and iodoform (b)

The damage associated with tail length is clearly depicts that a direct relation was showed among dose concentration and tail length damage. It was observed from above Figure 4.7 that there is an increasing trend in dose dependent manner for both disinfection by-products (chloroform and iodoform). The mean tail length values for chloroform and iodoform at observed doses were (6, 12, 15, 18.3,22 and 11,17,25,33, 41µm) respectively. These values were quite higher as compared to the control groups (4.4 µm).

When results were compared against both compounds it may be observed that the damage caused by the iodoform is higher than chloroform at every administered dose, which indicated that iodoform toxicity is higher. The comparison of data as DNA migration may determines that at the beginning of exposure concentration (2.1 and 77.5 mg/L) and at the end of exposure concentration (2.9 and 87.5 mg/L) for both compounds (iodoform and chloroform) the damage was statistically significant as compared to control groups.

4.5.4 Tail DNA %

Percentage of DNA in tail is also considered as an important index of evaluating DNA damage. The bar charts below presented in Figure 4.6 depicts the relationship between DNA tail length and varying concentrations of chloroform (a) and iodoform (b) respectively, indicating that the model explains well the variability of the response data around its mean.



(a) (b)

Figure 4.8: Relationship between Tail DNA% and Concentration of Chloroform (a) and iodoform (b)

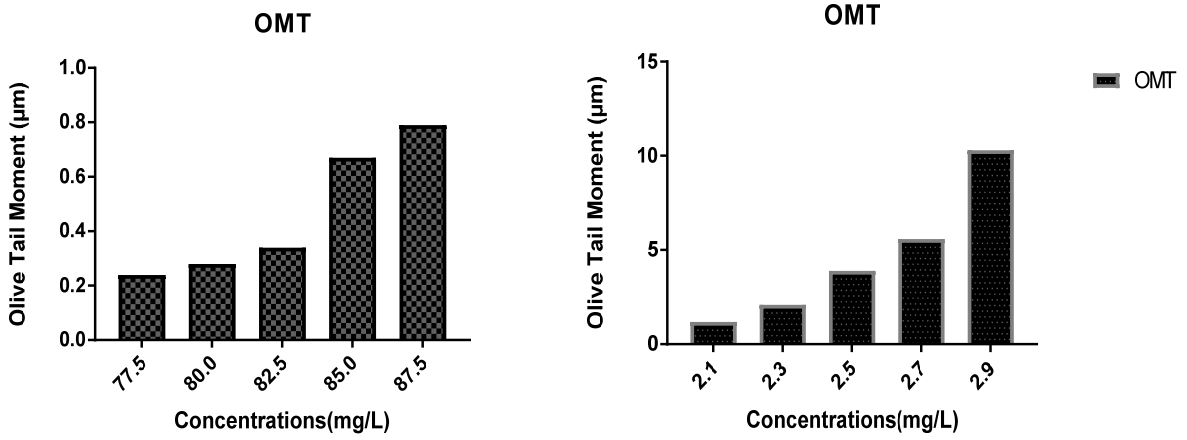
Tail DNA % present in the tail region may quantifies the amount of strands breakage, which increases with increase dose concentration. A greater increased tail DNA % and longer DNA tail length reflects the increase level of DNA damage as response towards disinfection by-products.

The mean tail DNA % values for chloroform and iodoform at the observed doses were 1.1, 2.3, 3.7, 7.53, 7.91 and 5.2, 5.4, 9.5, 17.6, 23.1% respectively. These experimental values were significantly higher than the control groups (0.07 %). The comparison between above charts shows that the percent tail DNA damage was more significant at all administered doses for

iodoform in comparison to chloroform and negative control. Significantly higher damage was observed at dose 2.9 mg/L for iodoform i.e. (23%) as compared to chloroform highest dose (87.5 mg/L) with 7.91% of tail DNA damage.

4.5.5 Olive Tail Moment (OMT)

Another comet parameter which is commonly used for the genotoxicity evaluation as DNA double strands breakage is olive tail moment. Tail moment is defined as tail length product and the total DNA fraction present within tail region. It incorporates a measure of both the smallest detectable size of migrating DNA (reflected in the comet tail length) and the number of relaxed / broken pieces (represented by the intensity of DNA in the tail).



(a) (b)

Figure 4.9: Relationship between olive tail moment and Concentration of Chloroform (a) and iodoform (b)

It may be clearly seen from the above bar charts that the level of genotoxicity in term of olive tail moment is increasing in a dose-dependent manner both for chloroform (a) and iodoform (b) respectively. The OMT values for chloroform and iodoform at all observed doses were 0.24, 0.28, 0.34, 0.67, 0.79 and 1.2, 2.3, 3.9, 5.6, 10.3 µm respectively. These observed values for both compounds were significantly higher in comparison to control group (0.036). Thus results obtained from comet parameters (tail length, tail DNA % and olive tail moment) may prove and strengthen the genotoxicity evaluation of disinfection by-products in fish blood samples using comet assay as promising technique.

The results obtained in the current study shows that the sensitivity of comet assay was somewhat higher at higher concentrations for both compounds, where genotoxic signals increased significantly. Significantly higher DNA migration in zebra mussel cells was observed with increase disinfectants in surface drinking water (Bolognesi *et al*., 2004). Toxicity of chloroform and carbon tetrachloride in rainbow trout hepatocytes revealed that DNA single strand breakage in treated cell was in accordance with higher toxicity (Rabergh & Lipsky, 1997).

Similar results were presented by Klobucar and coworkers in 2012, who detect significant increase in total percentage of tail DNA damage in crayfish juvenile at polluted sites (7.34± 1.25%, Zagreb 8.99± 0.88%, Sisak 14.17 ± 1.70%) compared to reference site (4.46± 0.30%). Notable damage in comet parameters (tail length, tail intensity and tail moment) was observed in Common carp exposed to pollutants in Lake Mogan (Cok *et al*., 2011).

Disinfection by-products indirectly affect human beings, as a bioaccumulation factor in fish. Exposure of trihalomethanes ($CHCl_3$, $CHCl_2Br$, $CHBr_3$ and CH_2Cl_2) to human cell showed decreased in the cell viability, approximate 50 % reduction in cell number for all treatments at higher concentrations. $CHCl_2Br$ and $CHCl_3$ were the most potent genotoxins among treated THMs and caused DNA damage through tail extent moment (Landi *et al*., 2003).

4.6 Hematological analysis

The knowledge about hematological parameters is considered as important tool for effective and sensitive monitoring of morphological and physiological changes within fishes (Kori-Siakpere *et al*., 2005). Literature reviewed showed that the interpretation of blood hematological parameters is quite difficult and challenging. Because of variations in blood due to internal and external factors such as blood sampling, seasonal variations, different laboratory techniques and working conditions and environmental stresses could affect blood hematology.

Hematological parameters of healthy and DBPs infected fish were analyzed using GraphPad prism version 7.01 and represented in bar charts form. The exposure of both compounds (presented in Figures 3 to 6) indicates that the significant discrepancies among all hematological indices were observed at higher concentrations as compared to control. Non-significant changes were observed in case of lowered applied concentrations.

The hemoglobin content of the fish exposed to DBPs for different concentrations was significantly ($p>0.05$) decreased in relation to the control groups. Similarly, hematocrit content for both compounds also decreased throughout the study period. It was observed that maximum percent decrease was noted for both parameters at the end of experiment at 96 h. The hemoglobin and hematocrit content were the indicator of physiological normality and ability of fish to carry oxygen. The significant decrease in said parameters suggests that fish underwent anemic conditions resulted from intensification of disinfection process. Further, it indicates the non-specific immunity and defensive reaction of fish against stress (Narra, 2016). Ali & Ansari (2012) present similar results of significant decrease ($p>0.05$) in the level of hemoglobin and hematocrit in monogenean infected carp as compared to healthy fish.

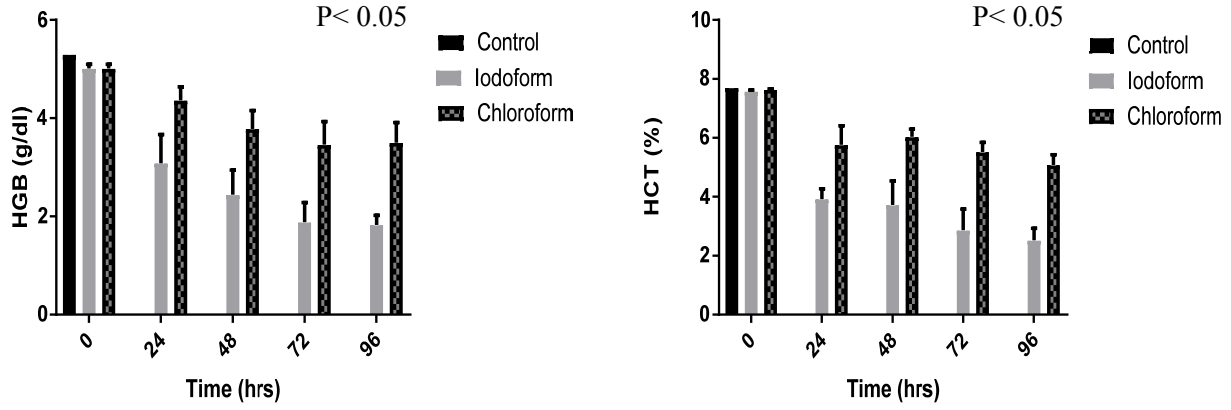


Figure 4.10 Change in Hgb (a) and HCT level (b) in Common carp exposed to iodoform and chloroform for 96 h

Iodoform significantly lowered ($p>0.05$) the platelets level at higher administered doses (2.5 to 2.9) as compared to the control group. Whereas, chloroform had minor effect for most of the study period but significant change was noted at 96 h in comparison to control. In case of MCH values for both compounds (iodoform and chloroform) had minor effect until 72 h of exposure period. Whereas, sudden change was noted for iodoform at 96 h as compared to chloroform and control groups.

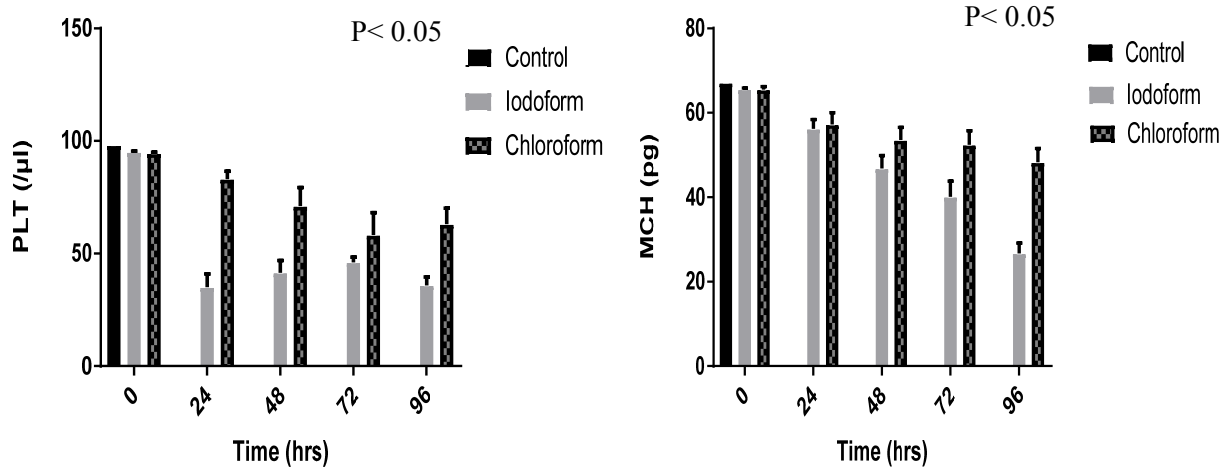


Figure 4.11: Change in PLT (a) and MCH level (b) in Common carp exposed to iodoform and chloroform for 96 h

The significant decrease ($p>0.05$) was observed in case of WBC and RBC content throughout the study period. Normal level of WBC is the indication of immune response and ability to fight against disease, as they are considered as the defensive cells of the body. The reduce level of red blood cell confirms anemia conditions in exposed fish which is due to heamosynthesis and osmoregulatory dysfunction. Further disinfection by-products metabolize to carbon monoxide which react with hemoglobin and convert it into carboxyhemoglobin. Carboxyhemoglobin does not like oxyhaemoglobin compound and prevents hemoglobin to supply oxygen to the body tissues (Andersen *et al*., 1991). As the result of this phenomenon cells decline in numbers and lower the RBC count.

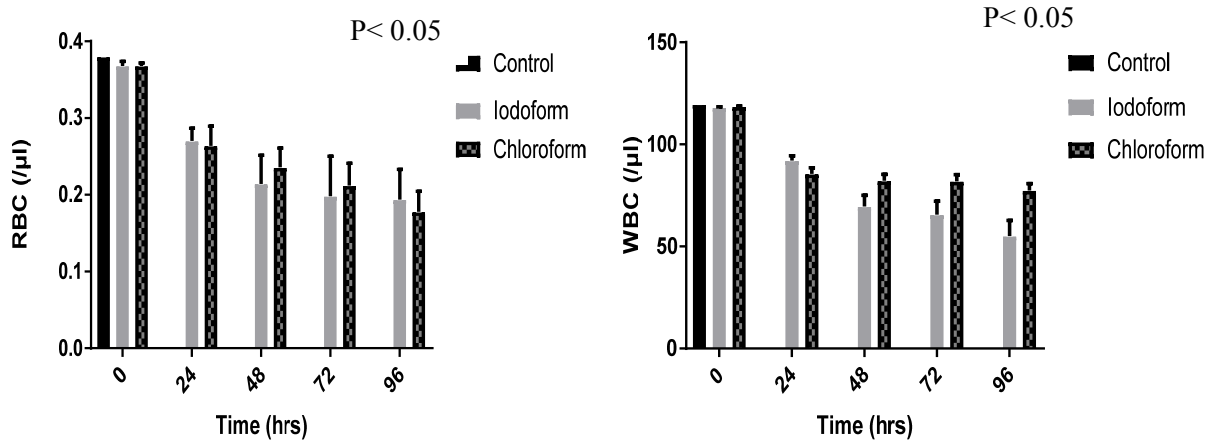


Figure 4.12: Change in RBC (a) and WBC level (b) in Common carp exposed to iodoform and chloroform for 96 h

A similar trend was observed in case of MCV and MCHC values for both compounds. They have minor effects on their values for most of the study period. But in case of MCV values a sudden significant change was noted at 72 and 96 h for iodoform compared to control group. The decrease in the level of MCV and MCHC values at higher concentration and exposure time confirms the toxicity of chloroform and iodoform. The results were in accordance to the Nussey (2000) which showed that the significant decrease in MCV count under the effect of toxicants which cause physiological changes in hematological parameters of fish.

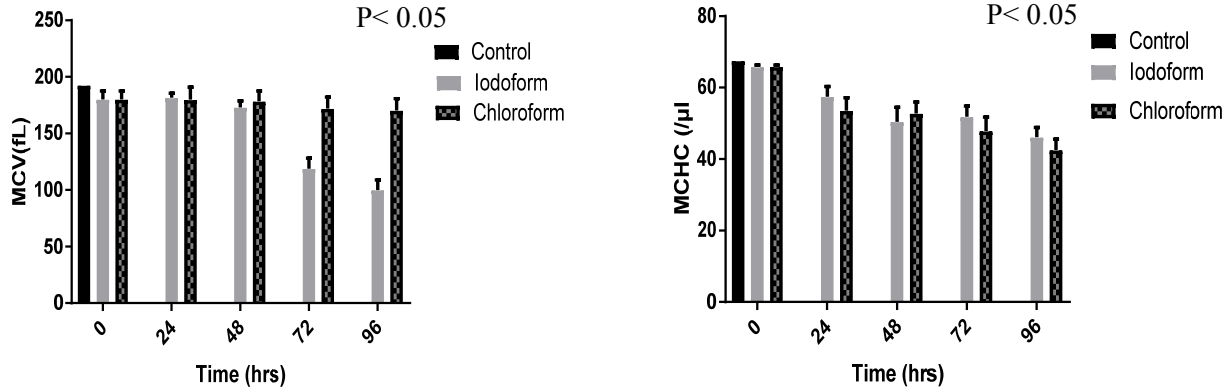


Figure 4.13: Change in MCV (a) and MCHC level (b) in Common carp exposed to iodoform and chloroform for 96 h

Statistical analysis interprets that when hematological parameters were compared for both compounds, significantly higher changes were observed for iodoform as compared to chloroform.

4.7 Biochemical analysis

Biochemical parameters are widely used to monitor pollutants and their impact in aquatic organisms. Moreover, they served as biomarker for pollutant exposure and their toxicity in fish. Among all studied biochemical parameters plasma glucose and total protein level was widely used to assess the stimulated effect caused by different environmental pollutants.

The significant decrease ($p>0.05$) was observed in total protein level with exposure time and spiked dose for both compounds. The decrease in plasma protein level in both treatments might have been caused by the impaired protein synthesis in body as the result of liver disorder. The decrease was also attributed to the increase body energy demand and physiological activities under stress conditions. The current results were in accordance to Ramesh *et al*., 2015 who

studied the impact of furadan pesticide in Common carp and found alterations in hematological and biochemical parameters with decrease level of plasma glucose and protein under high toxicity effect. Ejraei and coworker in (2015) showed that a marked variability was found in hematological and blood plasma indices of grass carp under the effect of age and hormonal treatments.

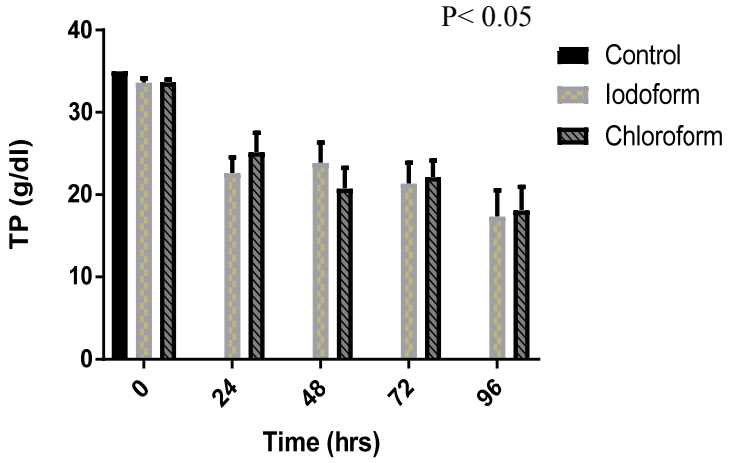


Figure 4.14: Change in TP level in Common carp exposed to iodoform and chloroform for 96 h

The increase or decrease in glucose level in the body is attributed to the feeding habits of organisms. The level of glucose in normal fish or organisms was higher as compared to toxicant exposed. The significant decrease ($p>0.05$) in glucose level for both compounds under acute exposure was observed at 96 h. The decrease level indicates stress response of fish after intensive metabolic activities under stress conditions.

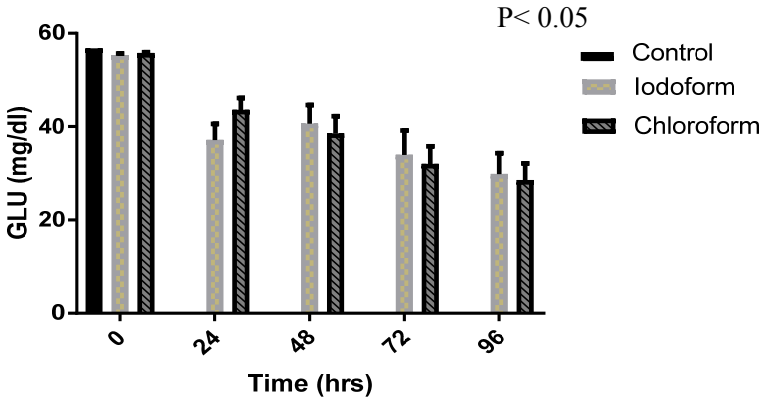


Figure 4.15: Change in GLU level in Common carp exposed to iodoform and chloroform for 96 h

The prolonged environmental stresses in fish species makes them difficult to adapt rapidly changing environments and thereby cause weakness. The weakness seemed to characterize through decrease in liver glycogen and serum cortisol levels. These changes results in the series of alterations in the metabolism process and shorten specie life span (Cicik & Engin, 2005).

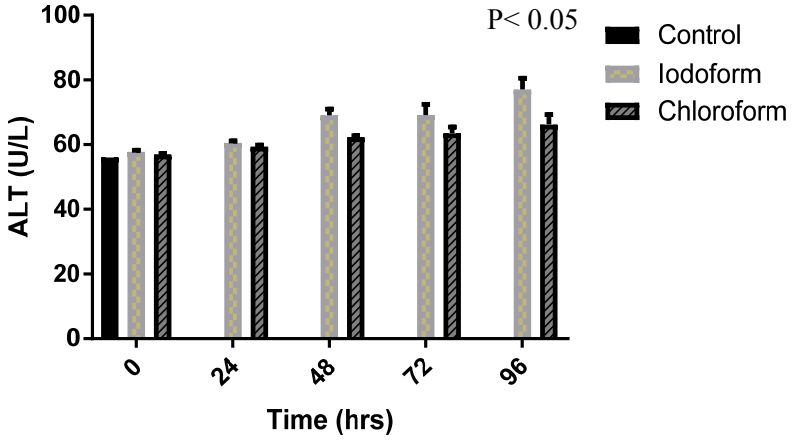


Figure 4.16:Change in ALT secretion in Common carp exposed to iodoform and chloroform for 96 h

The significant elevation in the ALT secretion with increased dose and exposure time for both compounds indicate liver disorder in exposed fishes. The alanine aminotransferase is principally present in the hepatocyte of the body and therefore their increase level reflects liver damage (Mikulikova *et al*., 2013). The sub-lethal exposure of toxic chemical towards fish showed significant decrease ($p>0.05$) in the level of glycogen in liver and muscle tissues as compared to control groups. The decrease in the level of glycogen under higher concentration was measured up to 24 and 29%, respectively (Cicik & Engin, 2005). The effect of disinfection by-products induces toxicity in liver and small intestine leading to cancer, administered in mice exposed drinking water containing by-products for period of one month (Abdelhalim *et al*., 2016). A statistically significant abnormality in liver of carp was reported after chronic exposure of bromodichloromethane (Toussaint *et al*., 2001).

The results of current study regarding blood biochemical investigation indicate that strong alterations in all indices were observed as a stress response of chloroform and iodoform. The significant response was observed for enzymatic activity that is the elevation in ALT secretion as response to DBPs stress. Further decrease in the glucose level reflects an intense metabolic stress exhibited by fish under DBPs exposure.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

The results of current study revealed that the fish habitated in Rawal Lake is contaminated with disinfection by-products. Following conclusions were drawn from the above experimental data;

1. LD_{50} was determined to be 90 and 3 mg/L for chloroform and iodoform respectively.
2. Recovery efficiency was within acceptable range (74-83 and 68-95 %) quantify that HS-SPME is an accurate and efficient technique for determining DBPs within fish blood samples.
3. Comet assay results showed that DNA damages in case of tail length, tail DNA % and olive tail moment were significantly high for both DPBs ($P < 0.05$) in comparison to control.
4. Mean tail length values for iodoform (11, 17, 25, 33, 41 µm) was significantly higher than chloroform (6, 12, 15, 18.3, 22 µm) at all observed doses showing that iodoform has higher genotoxic potential.
5. Effect of iodoform and chloroform on hematological and biochemical indices was statistically significant ($P < 0.05$).
6. For hematology, significant decrease in all blood indices were observed, as more obvious change was observed in case of platelets, hemoglobin and hematocrit.
7. For biochemistry, significant increase was observed in case of ALT secretion from liver of exposed carp, suggesting high degree of liver damage.
8. Statistical analysis represent that iodoform is more toxic and cause significant changes in Common carp in comparison to chloroform, confirming hepatotoxic nature of iodoform.

5.2 RECOMMENDATIONS

1. Histopathological study to find morphological alterations in tissues due to disinfection by-products.
2. Studies to evaluate cyto and genotoxicity of emerging DBPs (Iodotrihalomethanes and nitrogenous disinfection by-products).

3. Study to evaluate cellular defensive mechanism of organisms against oxidative stresses.

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