UNIVERSITÉ DU QUÉBEC À MONTRÉAL

TOXICOLOGICAL PROPERTIES COMPARISON BETWEEN HYPERVALENT IODINE REAGENTS AND METALS: EFFECTS ON GROWTH AND PHOTOSYNTHESIS OF DUCKWEED *LEMNA MINOR*

THESIS

PRESENTED

AS PARTIAL REQUIREMENT OF

MASTER OF CHEMISTRY

BY

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DECEMBER 2020

UNIVERSITÉ DU QUÉBEC À MONTRÉAL

COMPARAISON DES PROPRIÉTÉS TOXICOLOGIQUES ENTRE DES RÉACTIFS À BASE D'IODE HYPERVALENTS ET DES MÉTAUX : EFFETS SUR LA CROISSANCE ET LA PHOTOSYNTHÈSE DE LA PLANTE AQUATIQUE *LEMNA MINOR*

THÈSE

PRÉSENTÉE

COMME EXIGENCE PARTIELLE

MAÎTRISE EN CHIMIE

PAR

BANEENAH AMHARIB

DÉCEMBRE 2020

ACKNOWLEDGMENTS

I would like to sincerely thank my departed father, Abdullah, who I wish he was here today to be proud of me. And my mother, Souad, for all the efforts they made in order to give me confidence and constant encouragement to reach what I am now. I thank them for their patience and valuable advice and for their confidence in my success.

I would like to thank my lab colleagues, Mr. Suliman, and Mrs. Samira for their help, and my support. I would also like to thank Mr. Philippe Juneau's laboratory, as it provided me with assistance and ease in using the equipment I need in this research.

Lastly, I would like to thank Mr. Sylvain Canesi for his guidance. My special and deep thanks to my dear supervisor Mr. David Dewez for the trust he placed in me and for his endless support as well as giving me independence at work as well as his understanding of some of the circumstances that I went through.

DEDICATION

To the soul of my dear father

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS, AND ACRONYMS

Cr: Chromium

IBX: 2-iodoxybenzoic acid

Hg: Mercury

Chl *a*: Chlorophyll *a*

Chl *b*: Chlorophyll *b*

RC: Reaction Centre

EC_{50}: Median effective concentration

ROS: Reactive Oxygen Species

F: Variable fluorescence induced by continuous actinic illumination

F_M: Maximum fluorescence induced by a saturated flash, after an adaptation to darkness

F_O: Basic fluorescence measured by analytical light modulated, after adaptation to darkness

PQ: Plastoquinone

PS I: Photosystem I

F_V: Variable Fluorescence Intensity

PS II: Photosystem II

Q_A: Quinone A

Q_B: Quinone B

H_2DCFDA: 2',7' dichlorodihydrofluorescein diacetate

HMs: Heavy metals

O_2: Oxygen

Cl^-: Chloride

PEA: Plant Efficiency Analysis

LHCI: Light-Harvesting Complexes of Photosystems I

LHCII: Light-Harvesting Complexes of Photosystems II

CAT: Catalase

PQH2: Reduced Plastoquinone

SOD: Superoxide Dismutase

POD: Peroxidase activity

ATP: Adenosine Triphosphate

MDA: Malonyldialdehyde

LIST OF SYMBOLS AND UNITS

mM: Millimolar

µM: Micromolar

mg/L: Milligrams per liter

µg: Microgram

ml: Milliliter

µE.m^{-2}. s^{-1}: Microeinsteins per square meter per second

lux: Illuminance unit

ms: Millisecond

µs: Microsecond

mM: Millimolar

µM: Micromolar

FW: Fresh weight

ºC: Celsius Degree

d: Day

m^2: Meter Square

s: Second

%: Percentage

h: Hour

g: Gram

A: Absorbance

M: Molar

pH: Hydrogen Number

L: Liter

RÉSUMÉ

L'environnement est continuellement contaminé par des métaux traces provenant des activités humaines qui peuvent affecter la santé des écosystèmes. Les métaux traces sont également connus pour être des composés naturels en raison du rejet de la croûte terrestre. Cependant, les activités humaines ont contribué à son augmentation en grande quantité dans plusieurs domaines. Cela conduit à son tour à un affaiblissement des écosystèmes environnementaux, en affectant la santé des organismes vivants. Cela se produit en raison de la bioaccumulation des métaux dans la chaîne alimentaire. De plus, le traitement des métaux traces nécessite beaucoup d'efforts, d'attention, de technologies et de ressources financières consommatrices et non respectueuses de l'environnement. Par conséquent, l'approche de la chimie verte peut réduire les rejets de polluants et les remplacer par des matériaux respectueux de l'environnement. Les réactifs à l'iode hypervalent ont été utilisés comme substitut aux agents oxydants traditionnels dans les réactions de synthèse organique en raison de leur stabilité et de leur efficacité, ainsi qu'ils sont moins chimiquement toxiques que les métaux traces tels que le chrome (Cr) et le mercure (Hg). Pour évaluer leurs propriétés de toxicité, la plante aquatique *Lemna minor* est un modèle approprié pour étudier la toxicité des polluants comme étant un bio-indicateur de la qualité de l'environnement pour le milieu d'eau douce. Le principal objectif de cette étude était de déterminer les biomarqueurs sensibles chez *L. minor* des effets toxiques de l'acide 2-iodoxybenzoïque (V) et du (diacétoxyiodo) benzene (III). Par conséquent, nous avons analysé l'évolution des biomarqueurs capables de décrire la cytotoxicité de ces composés. Les traitements au Cr (VI) et au Hg (II) ont fait diminué la croissance de la biomasse, la teneur en pigments de la photosynthèse, la fluorescence de la chlorophylle et la formation de ROS. Cependant, la croissance de *L. minor* a été complètement inhibée de 81 % en présence de 0,25 mM de CrO_3 et de 83 % en présence de 7 µM de $HgCl_2$. Le niveau de la teneur en pigments chlorophylliens était significativement diminué dans les plantes traitées au Cr et au Hg par rapport au témoin. En outre, la production de ROS a été significativement augmentée de 619 % pour le Cr (VI) et de 1034 % pour le Hg(II) par rapport à 100 % du témoin. Contrairement aux réactifs iodés hypervalents, en presence

d'IBX (V) et de DIB (III), nous avons observé que la croissance de la biomasse de *Lemna* était légèrement inhibée (11 %) par l'IBX et (10 %) par le DIB par rapport au témoin. Le niveau de la teneur en pigments chlorophylliens a été augmenté en présence d'IBX, mais il n'y a pas eu de changement avec DIB. Comme dans la formation de ROS, nous avons remarqué une augmentation significative de 270 % et 289 % à 0,20 mM et 0,25 mM d'IBX respectivement, alors qu'il n'y avait pas de changement en présence de DIB même aux plus fortes concentrations. Ainsi, il a été conclu que les réactifs à l'iode hypervalent étaient moins toxiques que les métaux traces sur la lentille d'eau *L. minor*.

ABSTRACT

The environment is continuously contaminated with trace metals from human activities, which can affect ecosystem health. Trace metals are also known to be naturally occurring compounds due to the discharge of the earth's crust. However, human activities contributed to its increase in large quantities in several fields. This in turn leads to a weakening of the environmental ecosystems, by affecting the health of living organisms. This take place due to the bioaccumulation of metals in the food chain. In addition, trace metals processing requires a lot of effort, attention, technologies and financial resources that are consuming and not environmentally friendly. Hence, the green chemistry approach can reduce the release of pollutants and replace them with environmentally friendly materials. Hypervalent iodine reagents have been used as a substitute for traditional oxidizing agents in organic synthesis reactions because of their stability and effectiveness as well as less chemically toxic in comparison to trace metals such as Chromium (Cr) and Mercury (Hg). To evaluate their toxicity properties, the aquatic plant *Lemna minor* is a suitable model for studying pollutants toxicity as being a bioindicator of environmental quality for freshwater environment. The major goal of this study was to determine sensitive biomarkers in *L. minor* of the toxicity effects of 2-iodoxybenzoic (V) acid and (diacetoxyiodo)benzene (III). Therefore, we analyzed the change of biomarkers able to describe the cytotoxicity of these compounds. Cr (VI) and Hg (II) treatments decreased the growth of biomass, photosynthesis pigments content, chlorophyll *a* fluorescence, and formation of ROS. However, an inhibition of growth in *L. minor* was completely inhibited by 81 % in the presence of 0.25 mM of CrO_3, and by 83 % in the presence of 7 µM of $HgCl_2$. The level of chlorophyll pigment contents was significantly decreased in Cr-treated plants as well as Hg compared to the control. Furthermore, the ROS production was significantly increased by 619 % for Cr (VI) and by 1034 % for Hg (II) compared to 100% for the control. Unlike hypervalent iodine reagents, in presence of IBX (V) and DIB (III), we observed that growth of biomass of *Lemna* was a slightly inhibited by 11 % for IBX and by 10 % for DIB compared to control. The level of chlorophyll pigment contents was increased in the presence of IBX, but there was no change with DIB. As for the formation of ROS, we noticed a significantly increased by 270 % and 289 % at 0.20 mM and 0.25 mM of IBX,

respectively, while there was no change in presence of DIB even at higher concentrations. Thus, it was concluded that the hypervalent iodine reagents were less toxic than trace metals on duckweed *L. minor*.

GENERAL INTRODUCTION

With the industrial revolution continuing in many countries of the world, and with the proliferation of factories and refineries, the exposure of the environment to the danger of pollutants has become very large. Over the past three decades, there has been growing concern about environmental pollution and its impact on human health (Fereidoun *et al.*, 2007). In the modern era, pollution has become one of the problems that has the greatest danger to humans, animals, soil, and water. With rapid industry and random urbanization (Kumar *et al.*, 2013). Pollution has been defined as unfavourable change in the physical, chemical, and biological properties of air, water, and land. Environmental pollution has been attributed to human activities and natural ecosystems (Lal, 2002). Whereas agriculture, transportation, and mining and leather tanning factories are the main sources of these pollutants (Evans *et al.*, 2019; Morera *et al.*, 2011). In addition to the loss of many microbial, plant and animal species due to increased pollution and climate change (Häder et Erzinger, 2018). Trace metals are one of the most important and common pollutants of the environment that still continuing through the progress of the industrial revolution (Dong, W. Q. Y. *et al.*, 2001); Trace metals such as lead (Pb), cadmium (Cd), mercury (Hg), chromium (Cr), and zinc (Zn)

are among the minerals with density greater than 5 g/cm^3, and this type of pollutants not only deteriorates the quality of the atmosphere and water but also threatens the health of living organisms through nutrition (Dong, J. *et al.*, 2011; Li, Z. *et al.*, 2014; Nabulo *et al.*, 2010). In addition, the water is the most important resource for life. Water pollution got worse in many cities due to the high pollution threats that have been identified in many parts of India, China, Europe, America and parts of Africa (Yıldız, 2017). The presence of water is necessary for agriculture as it guarantees the increase and quality of crops (Lu *et al.*, 2015), and a lot of research papers have indicated the relationship between increasing crops and water resources (Deng *et al.*, 2006; Fan *et al.*, 2011). People have become concerned about the complexity of nature and balance within the global ecosystem. Therefore, scientists seek through their studies to understand these interactions so that the surrounding environmental condition can be assessed, and measures taken to prevent the degradation of the ecosystem in the future (Singh et Singh, 2017). Trace metals are produced from a range of natural and human sources. Nevertheless, mineral contamination can occur in environments affected by sedimentation in the atmosphere through the disposal of agricultural or industrial wastes (Ali *et al.*, 2016; Dawson et Macklin, 1998).

The aquatic photosynthetic organisms contribute to the production of biomass in ecosystems and are directly exposed to many pollutants. As considered as a valuable food source, these organisms break down nutrients and chemicals that can pollute

higher organisms in the food chain. These submerged root plants are a natural part of every ecosystem as they stabilize the bottom sediments in ponds and lakes (Yarsan et Yipel, 2013). These organisms absorb pollutants and can transfer them to herbivores, as this is known as bioaccumulation and biomagnification (Szynkowska *et al.*, 2018). Indeed, the duckweed *Lemna minor* represent a good bioindicator to analyze the toxicity effects of hazardous compounds, which is useful for environmental assessment.

Determining the toxicity properties of chemicals is very important for their proper use and management. Therefore, it will be useful to test the toxicity of iodine reagents because of their use in organic synthesis of natural products instead of trace metals that were previously preferred for the same purpose. In fact, the toxicity property of iodine reagents is poorly known. The main objective of this research project was to investigate the toxicity effect of hypervalent iodine reagents in comparison with trace metals by using the duckweed *L. minor*. One of the important reasons that led to study the hypervalent iodine reagents is to bring the scientific society's awareness to the advantages of utilization such compounds as an alternative to trace metals. As they have the feature of environmental sustainability, moderate reaction conditions, and recyclability. This opens wide horizons for discovering new transformations of these compounds, which contribute to understanding of strategies and concepts of improved organic synthesis in green chemistry.

Therefore, this research project is presented in this thesis as two specific studies:

In the first study, the specific objective was to determine the toxicity effect of CrO_3 and IBX on the duckweed *L. minor*. The plants were exposed during 7 days to different concentrations of CrO_3 and IBX (0.05, 0.10, 0.15, 0.20, and 0.25 mM). Indeed, the tolerance level for CrO_3 and IBX of *L. minor* has been poorly studied. Under these experimental conditions, changes in cellular and biochemical parameters indicating the growth rate, chlorophyll pigments, the Chl *a* fluorescence kinetic, reactive oxygen species production and the PSII activity performance were used as indicators of the toxicity effects. The results are presented and discussed in Chapter III.

In the second study, the specific objective was to investigate the toxicity effects of DIB and $HgCl_2$ on *L. minor* plants after exposed during 7 days to different concentrations of DIB and $HgCl_2$ (1, 2.5, 3, 5, 7 μM). Under these conditions the growth inhibition, the PSII activity performance, the content of chlorophylls, the Chl *a* fluorescence kinetic, and the reactive oxygen species were determined to understand the toxicological properties of hypervalent iodine reagents.

CHAPTER I

1.1 Organic synthesis and green chemistry

Many chemists see chemistry as a central science that locate among physics and biology, and its ability to analyze and synthesize molecules from atoms or other molecules. The synthesis is of the utmost importance in our life where through this process we create new chemical entities or molecules to derive from them the important elements we need. One of the branches of synthesis is organic synthesis, the art and science of structuring substances, natural or synthetic, whose primary component is carbon. The pioneer of organic synthesis is plenary synthesis and strive to synthesize living natural molecules in laboratories. The ability of human to iterate the molecules of living organism and create other molecules are prominent in the development of human history. The origin of organic synthesis dates to 1828, when German chemist Friedrich Wöhler synthesized urea as an example of natural substance in every living body (Nicolaou, K. C., 2014). Wohler's discovery followed the synthesis of urea through the complete synthesis of acetic acid, which is a natural product containing two carbon atoms (unlike the urea atom), by German Chemist Hermann Kolbe in 1845 (Nicolaou, Kyriacos C. et Montagnon, 2008). After urea, the most exciting synthesis was (+)-glucose by E. Fischer (Lichtenthaler, 1992). Organic synthesis in general has

a set of benefits for the environment as well as organism, including useful products that start from medical preparations, dyes, and agricultural materials to diagnostics and materials used in technology such as smart devices (Tsonis, 2012). Nowadays the chemistry of natural products is attracting a lot of vital interest. Where new simple or complex materials, whether useful or not, are being investigated and discovered. To determine the structure of molecule there are many effective tools using the physical chemistry. When exploring a complex substance, the first problem that a research faces is synthesis, which is the preparation of substance in various chemical methods for ascertaining the correctness of the composition found, or to increase knowledge of the chemical reactions of the molecule. Antibiotics of medicinal importance are often isolated from microorganisms such as molds or germs. These molecules are the best from medical point of view as they can be considered as a weapon for existence where they can be collected and modified in their structure to discover alternative solutions that are more abundant and low cost. The chemistry of natural products originated in the eighteenth century through the work of pharmacists at that time, among them the Swedish Carl Wilhelm Scheele as he was credited with identifying oxygen, in addition to discovering many naturally occurring organic acids, including citric, malic, oxalic, and lactic acids (West, 2014).

Unsurprisingly an increased pressure is being exerted on chemists to develop sustainable processes and alternatives to dangerous chemicals, as chemical industries are the main actor in human development. From this perspective, the common general

definition of green chemistry is the design, invention, and application of chemical products and processes to reduce or eliminate the use of generation hazardous materials (Black, 2008). Whereas this concept of green chemistry was adopted in the earlier 1990s nearly 20 years ago. Green chemistry is also defined as the process of building chemical products that bring down the usage and production of harmful substances (Li, C.-J. et Trost, 2008). Likewise organic and inorganic solvents are the primary source of waste for any synthetic process (Constable *et al.*, 2002). Thus, from the perspective of the principle of green chemistry, chemical reactions must be under clean conditions without using solvents (Reichardt et Welton, 2011), and water is well known to be a solvent where several biochemical organic and inorganic reactions can take place. One of the advantages of using water as a solvent is that it is inexpensive, more economical, and safe compared to organic solvents that can cause cancer (Andrade et Alves, 2005; Lindström, 2002).

Therefore, green chemistry endeavour to obtain new methods that are neat at the same time economically emulator. The 12 principles of green chemistry are design rules developed by Paul Anastas and John Warner, and this aim to reduce the use of toxic solvents in chemical analyzes as well as not generating wastes resulting from these processes (Figure 1.1). Also, it helps chemists and researchers achieve sustainability through molecular design in order to reduce harmful effects (Anastas et Warner, 1999; Jarvis, 2019).

Fig 1.1 The 12 principles of green chemistry proposed by Anastas and Warner (Anastas et Warner, 1999).

1.2 Hypervalent iodine reagents as alternative oxidative agents

Since 1940s, despite the utilization of the following compounds such as potassium dichromate, mercury chloride, lead chloride, and organothallium compounds as strong oxidants in the synthesis reactions. However, the toxicity of these compounds was negatively affected on ecosystem. For this reason, the choice of hypervalent iodine reagents as alternative selective oxidants and stable catalytic systems of a benign character was a revolution in the field of green chemistry. The compounds of

polyvalent iodine are preferred as metal-free oxidizing agents, also have found practical application in modern organic chemistry due to mild conditions, their diverse reactivity combined with environmental character and commercial availability (Yoshimura et Zhdankin, 2016). This is because of the low toxicity, clean conversion, and reactivity. In addition to transitional metals, simulation processes, the useful ones are the derivatives of 3 and 5 organic iodine's commonly known as hypervalent iodine reagents (Zhdankin, 2009). Iodine (III) and iodine (V) derivatives are now routinely used in organic synthesis as reagents for various selective oxidative transformations of complex organic molecules. Whereas, the reactivity pattern of hypervalent iodine reagents in several aspects is similar as the reactivity of transition metals. However, we can find iodine compound with different oxidative states $^{+3}$, $^{+5}$, and $^{+7}$. Hypervalent bonds are highly valued bonds, they have a high polarity ability longer and weaker than covalent bonds which gives them specific reaction and limited structural properties. The use of these substances has recently been very prosperous as they are very effective oxidizers, non-dangerous, and easy to handle with (Lee *et al.*, 2019).

1.2.1 Classification of hypervalent iodine reagents

The classification of polyvalent iodine compounds depends on the number of carbon bonds associated with the central iodine atom. The chemical properties and applications of these categories differ greatly. Large classes of hypervalent iodine reagents are shown in (Table 1.1) (Loscher et Morselli, 1985). Commonalty categories

of iodine (III) reagents are: (i) iodosylarenes ArIO and their non-cyclic derivatives ArIX2, (ii) pentagonal iodine heterocycles, benziodoxols and benziodazoles, (iii) iodonium salts R2I + X-, and (iv) iodonium halides ArI= CL2 (Table 1.2). Although pentavalent iodine reagents are less sophisticated comparison with trivalent iodine reagents, the chemistry of iodine (v) reagents recently has attracted fundamental attention. The reagent containing iodine in oxidative state +5 are of two types (Table 1.3).

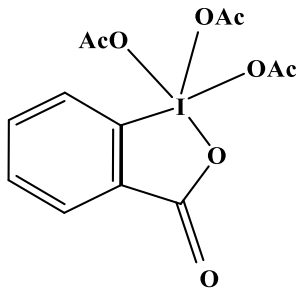
Table 1.1: Classification of hypervalent iodine reagents

Iodine (III) reagents		**Iodine(V) reagents**		
RIL_2 (one carbon ligand, two heteroatoms ligands)	R_2IL (two carbon ligands, one heteroatom ligand)	R_3I (three carbon ligands)	RIO_2 (one carbon bond)	$R_2IO^+ X^-$ (two carbon bonds)
Iodosyl benzene	Bis (Heteroaryl)iodoniu -m salts	Triphenyl iodine	Iodylarenes	Iodyl salts
Iodoaryl halides	Alkenyl iodonium salts	5-Aryl 5*H* dibenziodoles	*o*-Iodoxybenzoic acid	
[bis (Acyloxy)iodo] arenes	Alkynyl iodonium salts	(Dicyano)iodobenz -ene	Dess-Martin periodinane	
[Hydroxy(tosyloxy)i o do benzene] and µ-oxo-bridged iodanes	Alkyl iodonium salts			

Table 1.2: Types of iodine (III) reagents

N-X-L Type	Example	Common name
10-I-3	$PhICl_2$	(Dichloroiodo)benzene
	$PhI\ (OAc)_2$	(Diacetoxyiodo)benzene
8-I-2	Ph_2I^+	Diphenyl iodonium
	$PhI^+\ CH{=}CH_2$	Alkenyl(phenyl)iodonium
	$PhI^+\ C{\equiv}CH$	Alkynyl(phenyl)iodonium
10-I-2	PhIO	Iodosyl benzene
	$PhI^+\ HC{=}CL_2$	Phenyl iodonium methyl ides

Table 1.3: Types of iodine (V) reagents

N-X-L Type	Example	Common name
12-I-3	$PhIO_2$	Iodylbenzene
10-I-5		Dess-Martin periodinane

The named of hypervalent iodine types is according to N-X-L nomenclature (Martin-Arduengo) (Klasen, 2015). Whereas N is the number of electrons that are officially assigned to central atom, X is the symbol of iodine atom, and L is the number of ligands

bonded to iodine atom. In this study, we studied two types of hypervalent iodine compounds and their impact on aquatic ecosystems.

1.2.2 IBX (2-Iodoxybenzoic Acid)

IBX was discovered by Hartmann and Meyer in 1893 (Zhdankin et Stang, 2008). IBX is a penta-iodine, which is mostly used to oxidize alcohols and aldehydes to the corresponding carbonyl compounds (see figure 1.2). Its physical properties are a compound with limited solubility, which it is insoluble in many common organic solvents as well as in water, limiting its application in organic synthesis. To overcome these obstacles, a water- soluble IBX derivative has been synthesized by introducing a carboxyl group onto the phenyl ring of IBX (Wirth, 2003). It was thought that IBX was shock sensitive, however, later it was confirmed that the sensitivity of the compound was due to residual potassium bromate remaining from its preparation. The commercial IBX was then stabilized with carboxylic acids such as benzoic acid and isophthalic acid (Frigerio *et al.*, 1999). By structurally modifying it or by developing polymer-supported analogues, where it can be prepared in the laboratory with a purity of 98% by interacting 2- iodoxybenzoic acid and Potassium bromate. However, potassium bromate was replaced by commercial Oxone to prevent explosion during the reaction

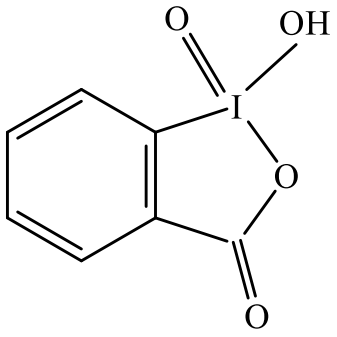


Figure 1.2 Chemical structure of 2- Iodoxybenzoic acid IBX

1.2.3 DIB (Diacetoxyiodo) benzene

DIB is a tri-iodine compound that is used as a nucleophile and oxidizer (Figure 1.3), to perform an acetoxylating reaction with 3-oxo-N-substituted butanamides (Liu *et al.*, 2013), oxidative cleavage, and in oxidation of a transition metal in catalytic cycle. As it is prepared by reacting iodobenzene with a mixture of acetic acid and peracetic acid (Moriarty *et al.*, 2006). Recently, DIB has emerged as a potential catalyst in heterocycles structure, and it resembles IBX in its insolubility in most organic solvents, in addition to its stability and effectiveness as powerful oxidizing agents in synthesis reactions. Besides simple oxidation, hypervalent iodine reagents have applied in many oxidative rearrangement reactions, for their electrophilic nature and premium capacity to leave the group (Jacquemot et Canesi, 2012). Furthermore, it acts to activate double bonds in alkenes, which leads to significantly oxidative rearrangement through ring

expanding (Silva *et al.*, 2008), ring retraction (Silva *et al.*, 2007), or Ariel peregrination (Liu *et al.*, 2013).

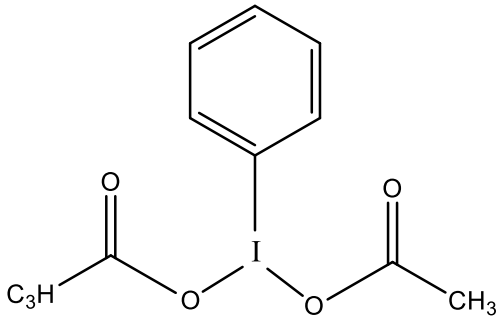


Figure 1.3 Chemical structure of (Diacetoxyiodo) benzene DIB

1.3 Biological and toxic effects

1.3.1 Hypervalent iodine reagents

Along with the diversity and multicity of their chemical behavior, hypervalent iodine reagents exhibit biocidal properties towards a wide range of microorganisms such as bacteria, fungi, and yeast. Furthermore, these substances are environmentally safe, and therefore tend to replace other reagents with similar chemical properties (Yadav *et al.*, 2005). There are a few chemicals available to combat plant bacterial diseases, including copper compounds which fundamentally was used to this intent (Lindow, 2012; Ninot *et al.*, 2002)

1.3.2 Trace metals

1.3.2.1 Chromium

In recent years, pollution of the ambient environment with chromium, especially hexavalent chromium has become a worrying cause. Chrome is the 17th most abundant element in the Earth's crust and widely used in many different industries, including production of paints, dyes, alloying, tanning, refractory bricks, chemicals, and metallurgical. Also, the waste from these industries is used as filling materials in many reclamation sites (Avudainayagam *et al.*, 2003; Salunkhe *et al.*, 1998). Chromium can influence living organism because of its strong toxicity, which has made it a prominent concern. In aquatic ecosystems that are affected by human activities, the concentration of chromium can reach mmol per liter (El-Shafei, 2016; Perreault *et al.*, 2009; Velma et Tchounwou, 2010). Its oxidation states are between 0 and +6 and change easily according to the oxidation conditions of the environment. In addition, under normal circumstances, chromium and hexavalent chromium are more stable (Hörcsik, Z. T. *et al.*, 2007). Since Cr (III) and Cr (VI) have different toxic and epidemiological properties, the Environmental Protection Agency EPA differently regulates them. Cr (VI) is a strong irritant and it is considered a human carcinogen (Kimbrough *et al.*, 1999). It is also toxic to many plants (Shanker, A. *et al.*, 2005), aquatic animals (Velma *et al.*, 2009), and microorganisms (Viti *et al.*, 2014). Unlike Cr (III), it is considered as micronutrient that is necessary for the metabolism of sugar and fats in the human body

(Zafra-Stone *et al.*, 2007). Other than that, the recommended limits for chromium concentration in water are 8 µg ml^{-1} for Cr (III) and 1 µg ml^{-1} for Cr (VI), while in liquid residues resulting from industrial processes, chromium levels are high as 2-5 g L^{-1} (Chandra *et al.*, 1997).

1.3.2.2 Mercury

Among the most important pollutants that have a highly toxic effect on humans, animals, and plants is mercury. Mercury is the second most toxic metal in the world as well as is known to be the cause of several diseases such as Minamata (organic mercury poisoning) (Matsuo, 2003). The natural source of mercury is due to the discharge of the earth's crust through volcanoes eruptions, burning fossil fuels and the melting process of some element's ores such as lead, copper and zinc. Contamination by mercury has received special attention due to its high toxicity and widespread occurrence unlike most minerals that act as nutrients (Dirilgen, 2011; Regier *et al.*, 2013). According to the world health organization, most exposure to mercury is caused by ingestion contaminated fish or mercury gases from dental amalgam or during work in industrial places (Bates, 2006; Mieiro *et al.*, 2009; Park et Zheng, 2012). Indeed, anatomy studies have shown that dental amalgam is the main source of mercury in human tissues. As amalgam, holders have approximately about 2-12-fold more mercury in tissues and brain, than people who do not have amalgam (Björkman *et al.*, 2007; Guzzi *et al.*, 2006; Mutter, 2011). The free ionic form of mercury is Hg^{+2}, which is highly water soluble and can be accumulated in higher plants (Elbaz *et al.*, 2010).

Correspondingly, it can interact with the sulfhydryl group of enzymes and proteins in plant cells (Maggio et Joly, 1995; Zhou *et al.*, 2007).

1.4 The use of biomarkers in toxicological assessment

Toxicology in general is a science that specializes in studying the health of living organisms, whether human, plant or animal. Huge numbers of pollutants enter daily into the environment and exert various pressures on living organisms and ecosystems. It is taken into consideration that assessing the risk of these pollutants is not easy due to the diversity in chemical nature and toxicity range of pollutants, as well as the difference in sensitivity of organisms to these types of pollutants. Even minimal concentrations of pollutants often cause damage to organisms, which are difficult to notice, because only measurable effects resulting from prolonged exposure to pollutants are expressed. Biomarkers in toxicology are molecular, biological, and physiological changes that occur to an organism because of its exposure to pollutants. Therefore, it is important to develop and use early warning indicators or biomarkers that reflect negative biological responses towards anthropogenic toxic compounds, even in precise concentrations (Depledge *et al.*, 1995; Van der Oost *et al.*, 2003). The latest development in molecular biology and biotechnology has permitted to study novel and more sensitive biomarkers to analyze the exposure to water and terrestrial pollutants in laboratory and field studies. Furthermore, methodological use of multiple biomarkers is the most useful and efficient way to assess a pollutant impact (Tsangaris

et al., 2010), preventing pollution and environmental catastrophes (Biswas et Agarwal, 2013; Sanni *et al.*, 2017).

1.5 Plant growth

1.5.1 Biological material: *Lemna minor*

Duckweed *L. minor* is a floating freshwater plant easy to cultivate because of its rapid reproduction and it is a vital indicator of toxic chemicals for aquatic ecosystems (OECD, 2006; Wang, 1990). They are considered as food for waterfowl and invertebrates (Hillman et Culley, 1978). The plants have flat, small oval leaves, and a single root emerges from the bottom of frond (Figure 1.4). The length of individual plant is about 3-5 mm and each one has a root that reaches approximately 5 cm in normal growth conditions. The plants can float all the time, forming a carpet on the surface of water in ponds and freshwater bodies. In addition, it can duplicate its mass in 6 days (Kuznetsova *et al.*, 2019). Due to its accumulation property for metals, plants of *L. minor* have the potential for the removal of trace metals such as Cd, Cu, Cr, Ni, Pb, Hg, and Se. The accumulation capacity of *L. minor* ranges depending on the metal exposed to, for example, in a case of Cd, Cu, and Se, the plant is good accumulator, moderate for Cr, low for Pb and Ni (Shukla et Srivastava, 2019; Zayed *et al.*, 1998), and mercury as well (Varga *et al.*, 2013).

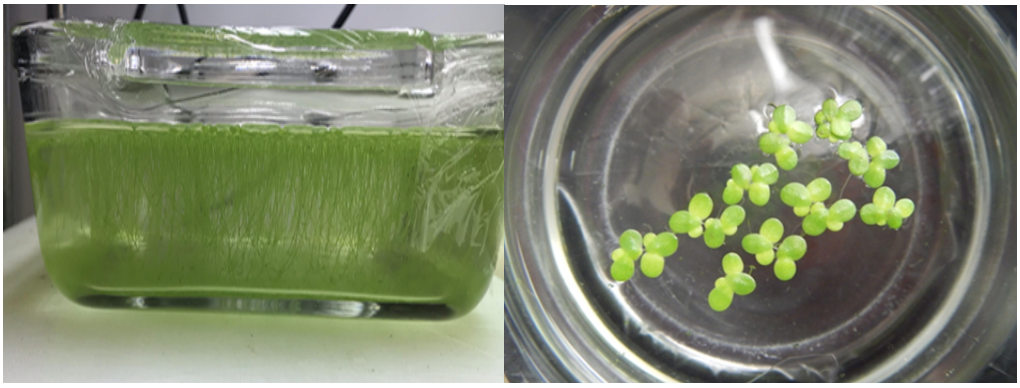


Figure 1.4 Pictures of fronds and roots length of *L. minor* cultures in our laboratory.

1.5.2 Scientific classification

This duckweed is part of Kingdom: Plantae, Sub Kingdom: Tracheophytes, Clade: Angiosperms, Sub Clade: Monocots, Order: Alismatales, Family: Araceae, Sub Family: Lemnaceae, Genus: *Lemna*, and Species: *L. minor* (Wang, 1991). The Lemnaceae family has approximately 40 species around the world. It comprises a group of aquatic monocotyledons macrophytes that are found in South America, Central Africa, Europe, South Asia, and the south of Australia.

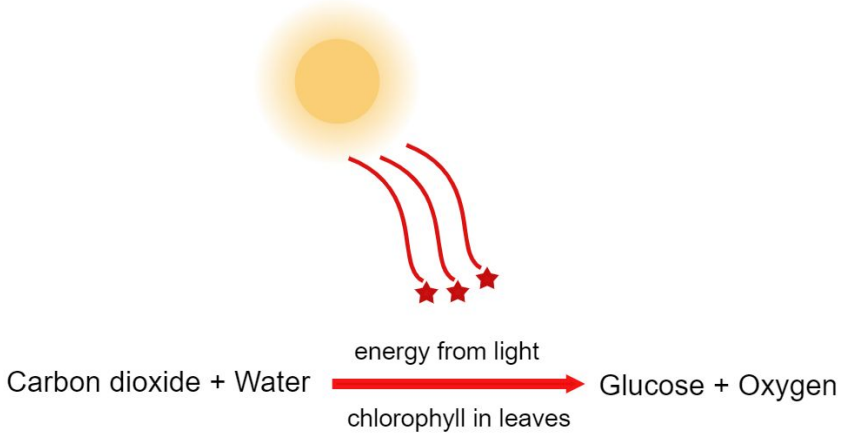
1.5.3 Development phases of *Lemna minor*

There are two phases in the life of *L. minor*, a childhood phase with two tiny fronds, and after two days of growth, it reaches an adult format with four fronds. Then, the four-frond *Lemna* is divided into two new individuals with two fronds on the 3rd day

of growth. Plants are more likely to be “contact pollinated” by colliding with floating roots together. However, sexual reproduction in the life of duckweed is an exception, often they reproduce asexually by forming chains of new stems from plant buds (Lemon *et al.*, 2001). Thus, the result is the production of an individual new daughter (Fertig, 1998).

1.6 Photosynthesis

Photosynthesis is primarily responsible for producing oxygen in the atmosphere and providing the energy needed for life on Earth (Bryant et Frigaard, 2006). Photosynthesis is defined simply as the process that converts light energy into chemical energy by green plants and some other organisms. Some protein complexes containing chlorophyll pigments are used to convert water into oxygen by capturing light energy (Figure 1.5). These proteins-pigments complexes are inside organelles known as chloroplasts found in the cells of plant leaves. In plants, energy is stored in form of sugars which in turn are spread through conductive vessels of phloem and can be metabolized into complex molecules such as amino and fatty acids (Buchanan, Bob B. *et al.*, 2015).



$$6CO_2 + 6H_2O \longrightarrow C_6H_{12}O_6 + 6O_2$$

Figure 1.5 The process of photosynthesis in plants (Bryant et Frigaard, 2006).

The carbon dioxide is combined into organic carbon compounds such as ribulose diphosphate (RuBP) during the Calvin cycle (Ducat et Silver, 2012). Although there are few differences in the process of oxygenic photosynthesis in plant, algae, and some bacteria, all processes are generally quite similar in theses organisms. Photosynthesis in plant occurs in two phases; in the first phase, the light energy from light-independent reactions is used to make energy storage molecules as adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH). In the second phase, biochemical reactions use these compounds to reduce CO_2.

1.6.1 Chloroplast and thylakoids

Chloroplasts are organelles where chlorophylls absorb the energy from sunlight, then store it in energy-storage molecules following the process of releasing O_2 from H_2O (Figure 1.6). Two membranes and a high content of chlorophylls characterize this plastid. There are circular disks having between 3 to 10 µm long and 1 to 3 µm thick (Milo et Phillips, 2015). These plastids consist of a number of membrane ultrastructure called thylakoids submerged as granum in the chloroplast stroma (Buchanan, B. B. *et al.*, 2000; Wise et Hoober, 2007). Thylakoids consist of membrane encircling thylakoid lumen and all photosynthesis processes, especially photosystem (PS) I and II, plastoquinone (PQ), cytochrome b_6f complex (Cyt. b_6f), plastocyanin, ferredoxin, and ATP_{ase} (Whitmarsh, 2001).

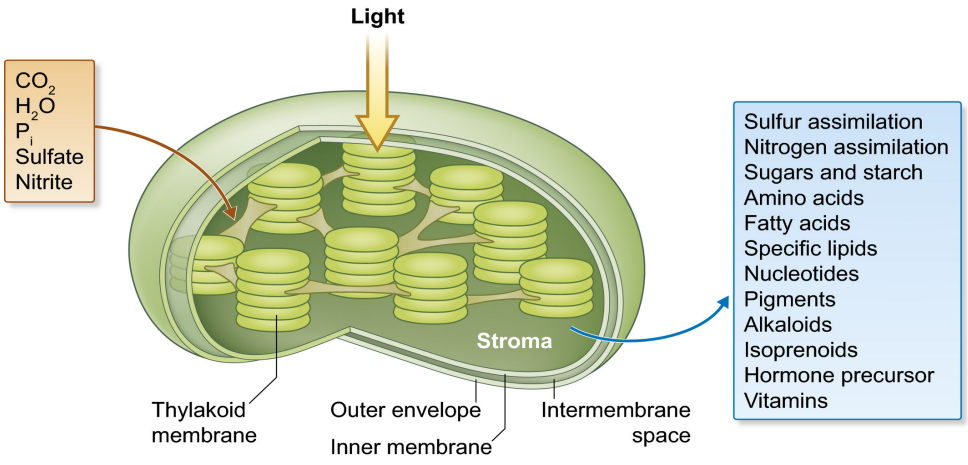


Figure 1.6 Diagram showing the chloroplast ultrastructure in plants (Kirchhoff, 2019).

1.6.2 Photosynthetic pigments

Photosynthetic pigments, including antenna pigment and plastid pigment are molecules reflecting certain wavelengths, while absorbing only specific wavelengths of visible light. This makes them colorful as well as responsible for absorbing electromagnetic radiation by transferring the energy of absorbed photon to the reaction center. Since each pigment interacts with a limited range of spectrum, there is a need to produce several types of pigments with different colors (Figure 1.7). The main classes of pigments are chlorophylls and carotenoids which are fat soluble molecules extracted from thylakoid membranes (Yokthongwattana *et al.*, 2005), while phycobilins and pyridine are water soluble extracted from tissues (El-Khatib *et al.*, 2016).

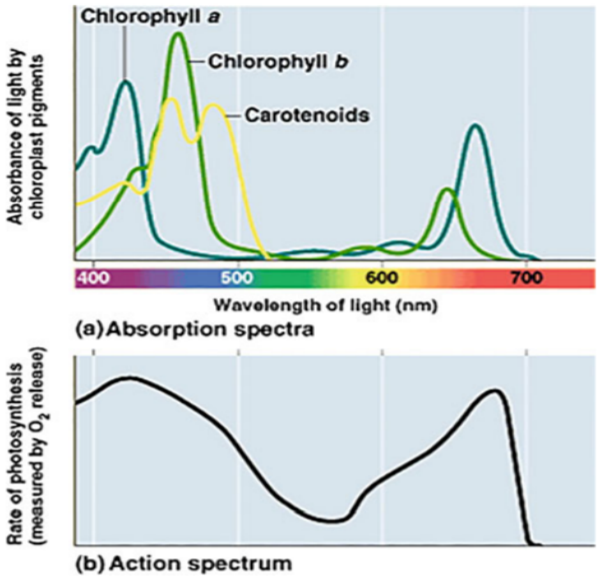


Figure 1.7 Absorption spectra and action spectrum of chlorophylls and carotenoids (Naznin et Lefsrud, 2017).

The light-reactions that occur during the photosynthesis process, whether in plants, bacteria, or algae are stimulated by multi-proteins complexes merged into thylakoid membrane containing photosystem (PS) I and photosystem (PS) II (Freeman, 2006; Golbeck, 2007). Chlorophyll pigments are gathered in truss within the antenna, consisting of a porphyrin ring connected to a magnesium ion (Mg^{+2}), attached to a phytol chain (hydrophobic side) which makes molecule nonpolar. It is worth noting that the biosynthesis of chlorophylls is synthesized of one from the other, where chlorophyll *b* differs from chlorophyll *a* by the presence of the formyl group at the same position of methyl group in the porphyrin ring (Figure 1.8) (Buchanan, Bob B. *et al.*, 2015).

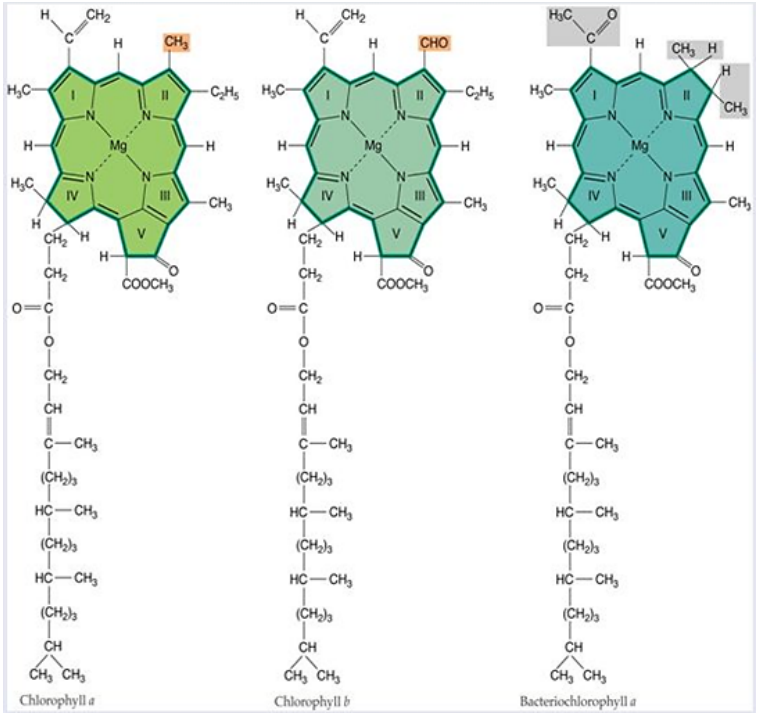


Figure 1.8 Chemical structure of Chl *a* and *b* (Buchanan, Bob B. *et al.*, 2015).

As for carotenoids, they play two important roles for chlorophyll pigments in sweeping harmful reactive oxygen species formed inside the plastid, also in expanding the range of light absorption during photosynthesis (Demmig-Adams, 1990).

1.6.3 Photosystems

Photosystems in green algae and plants are divided into two, the photosystem I (PSI, plastocyanin-ferredoxin oxidoreductase) and the Photosystem II (PSII, water-plastoquinone oxidoreductase). They are multiunit membrane complexes, the first system is found in the stroma of thylakoid lamella while other is in the grana (Dekker et Boekema, 2005). Each system is formed of a central complex (each core complex contains a reaction centre), a light harvesting antenna complex (LHCI) for PSI and for PSII (LHCII), which consisting of hundreds of pigment molecules, carotenoids, polypeptides, ferric ion, plastoquinone (PQ), polypeptides, and manganese pack (Figure 1.9). These compounds have roles divided according to where they are found in the photosystems (Yahia *et al.*, 2019).

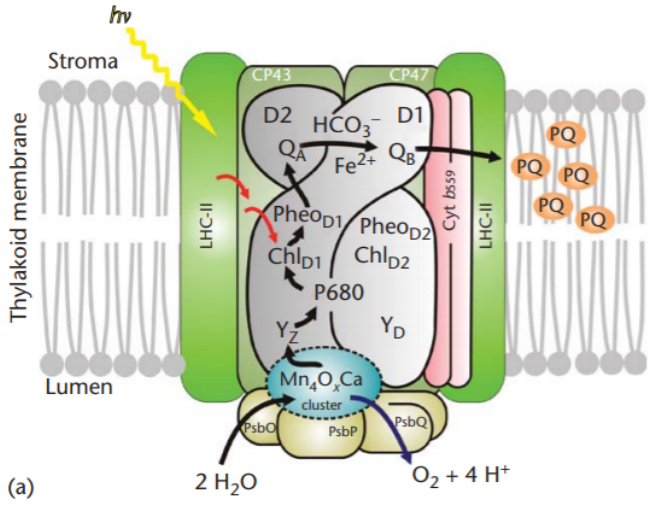


Figure 1.9 Structure of photosystem II in the thylakoid membrane (Govindjee, K. J. F. *et al.*, 2010).

- The photon collecting antenna

These antennae are mainly responsible for absorbing light energy through pigments and transferring it to the reaction center, where an initial charge separation occurs. As there are two kind of antennas. The first is internal containing carotenoids, Chl *a*, and other pigments, and the second is external containing Chl *b* (Sakuraba *et al.*, 2010). The chlorophyll molecule is excited during the absorption of photon's energy, and resulting in the transfer of excited state between antenna pigments to reaction center (Kirst *et al.*, 2017; Pham et Messinger, 2016).

- The reaction center

The reaction center (P680) is known to contain a special pair of chlorophyll *a*, a manganese bundle as well as two quinones (Q_A and Q_B), and two pheophytins (Pheo). The P680 is associated with another protein complex in chloroplast lumen called the oxygen-evolving complex (OEC), which is the one that performs the photolysis process of H_2O by removing the electrons from water molecule and transferring them to the chlorophyll pair for the release of oxygen gas. The Ca^{+2} and Cl^- locate in the second complex considered as stabiliser factors. In fact, the manganese bundle contains positive charges that aid in the reduction processes to separate water molecules (Govindjee, J. F. et Kern, 2010; Xiong *et al.*, 1998).

- The S states

The process of developing oxygen takes place in five separate states S_0, S_1, S_2, S_3, and S_4, which can be achieved through four activations of PSII (Figure 1.10). Each state contains a different number of oxidation equivalents, which stored at S_4 state then the oxygen evolving complex returns to its basic state S_0. The procedure of oxidizing water to molecular oxygen requires the extraction of four electrons and four protons from two water molecules. The S_1 state is often stable because it consists of manganese ions with multi-oxidants, where the OEC allows the electron to pass from manganese to the excited $P680^+$ (Barber, 2017; Joliot *et al.*, 1969; Kuntzleman et Yocum, 2005).

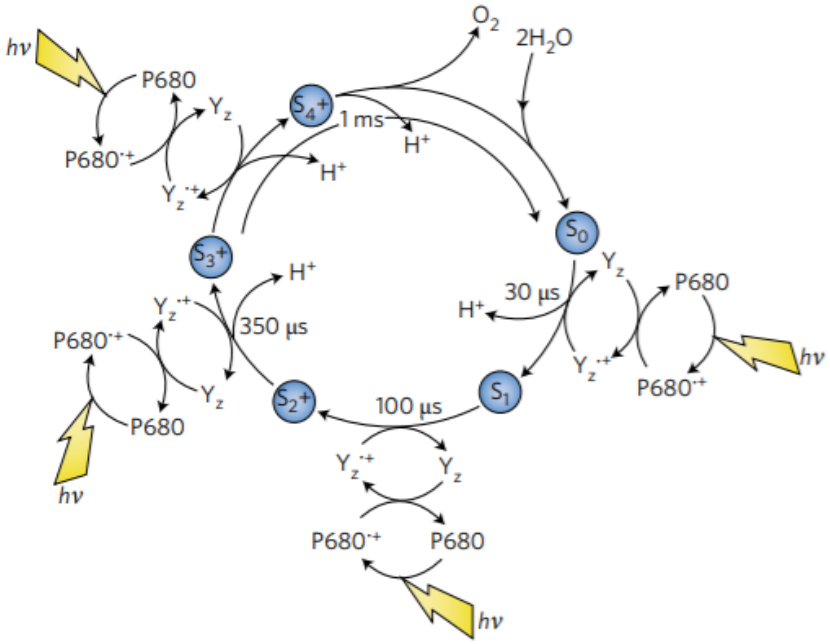


Figure 1.10 The diagram of S-state cycle (Barber, 2017).

1.6.4 Rapid and polyphasic kinetics of Chl *a* fluorescence

To measure plant photosynthetic efficiency, a Plant Efficiency Analyzer HANDY-PEA (HANSATECH®) is used. It is a device that measures the rapid phase motion of chlorophyll fluorescence from green plants, which shows the process of the transfer of electrons within photosystem II, resulting from the photolysis of water (Figure 1.11). The fluorescence of chlorophyll is induced by exposing the molecule to light for a second. Fluorescence exhibit a constant value indicating F_O at 20 µs of exposure to illumination (Strasser *et al.*, 2010). Accurate information can be obtained by

fluorescence about the structure and function of PSII. At the transitional state O, the fluorescence output is almost equal, and after this phase, the fluorescence output is variable reflecting the state of the electron carriers in PSII (PQ, Q_A, and Q_B). Fluorescence induction measurements are made using dark-adapted intact leaves, or any other samples such as micro and macroalgae. The plants exhibit the rapid polyphasic rise called O-J-I-P fluorescence (Mathur, Sonal *et al.*, 2014), where the level O indicates the basal fluorescence level (F_O), the fluorescence intensity at 2 ms (F_J), the fluorescence intensity at 30 ms (F_I). The I level reflects the heterogeneity of plastoquinone PQ during the transfer of Q_A to Q_B, and P is the maximum fluorescence value (F_M). In this research, these following equations are utilized according to (Strasser *et al.*, 2010):

- ABS/RC allows an assessment of the absorption of collecting antennas by active reaction center of PSII:

$$\frac{\boldsymbol{ABS}}{\boldsymbol{RC}} = \frac{\boldsymbol{M_O}}{\boldsymbol{V_J}} = \phi_{\boldsymbol{PO}}$$

- M_0 is the primal rate of induction of variable fluorescence:

$$\boldsymbol{M_O} = \frac{\left(\boldsymbol{F_{300_{\mu s}}} - \boldsymbol{F_{20_{\mu s}}}\right)}{\left(\boldsymbol{F_P} - \boldsymbol{F_{20_{\mu s}}}\right) \times \boldsymbol{0,25}\right)}$$

$F_{300\mu s}$ represents fluorescence level at 300 μs.

- V_J represents the variable fluorescence relative to the reduction of Q_A:

$$\boldsymbol{V_J = \frac{F_{2ms} - F_{20\mu s}}{F_P - F_{20\mu s}}}$$

F_{2ms} represents the fluorescence level at 2 ms for the transition J.

- The yield of the initial photochemical reaction of PSII is almost 0.8 in higher plants:

$$\boldsymbol{\phi_{PO} = \frac{F_P - F_{20\mu s}}{F_P} = \frac{F_V}{F_P}}$$

$F_{20\mu s}$ represents fluorescence level at O transition, and F_M is the maximum fluorescence level at P transition.

- PI_{ABS} is the indicator of the performance of the photochemical activity of PSII.

$$PI_{ABS} = \frac{1 - (\frac{F_O}{F_M})}{\frac{M_O}{V_J}} \times \frac{(F_M - F_O)}{F_O} \times \frac{(1 - V_J)}{V_J}$$

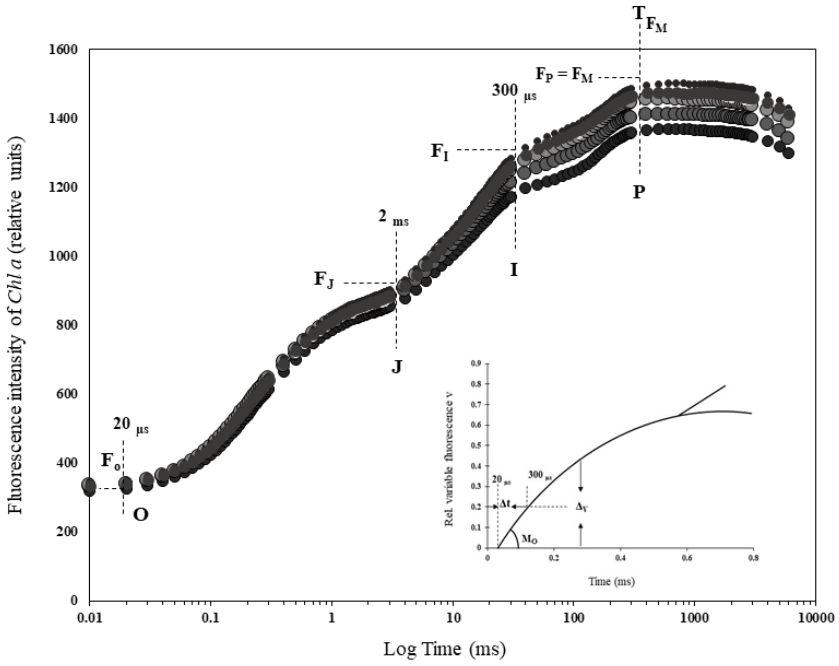


Figure 1.11 A Chl *a* polyphasic fluorescence rise curve (Strasser *et al.*, 2004; Tsimilli-Michael et Strasser, 2008). F_o the value of F at 20 µs of illumination. The O-J phase coincide to the reduction of Q_A (Q_A^-). F_I coincide to the first reduction of Q_B ($Q_A^-Q_B^-$). The I-P phase corresponds to the accumulation of Q_B reduced twice ($Q_A^- Q_B^{2-}$). F_P coincide to maximum reduction of PSII when PQs are reduced PQH_2. Later, F_P fluorescence decreases and coincide to transferring of electrons from PS I to PS II.

1.7 The oxidative stress

Plants and green algae can grow in changing environmental conditions and are often subject to various pressures, causing these organisms to develop different mechanisms including the development of pathways to overcome the stress caused by unsafe environmental factors (Shao *et al.*, 2008). Oxidative stress arises when large cellular molecules such as DNA, lipids, and proteins are damaged. Reactive oxygen species (ROS) as by-products are considered to be toxic to cellular metabolic processes (Bhattacharjee, 2005). They play a primary role in plants by controlling certain processes such as the growth and response to biological and non-biological environmental stimuli. When exposed to metals, these organisms enhance the formation of ROS which is controlled by an antioxidant system that prevents the increasing concentration of these molecules within the cells (Lobo *et al.*, 2010).

1.7.1 The main forms of reactive oxygen species

Reactive oxygen species (ROS) are formed from oxidative reactions catalyzed by metals. Atomic oxygen contains two unorganized electrons in its outer shell, making oxygen susceptible to radical formation. In addition, the serial reduction of oxygen results to the formation of several types of ROS, including singlet oxygen $^1O_2$, superoxide ion O_2^-, hydrogen peroxide H_2O_2, hydroxyl radical $OH^\bullet$, and perhydroxyl radical $^\bullet O_2H$ (Engwa, 2018).

CHAPTER II

MATERIAL AND METHODS

2.1 Preliminary requirements and incubation of *Lemna minor*

2.1.1 Preparation of SIS culture medium

The chosen medium culture is SIS, which is the modified version of medium created by the Swedish Standardization Institute of *Lemna minor* as shown in Annex 4 of the OECD report 221. Seven stock solutions were prepared in glass bottles of one liter capacity, as Solution I: $NaNO_3$ at 8.5 g/L; KH_2PO_4 at 1.34 g/L; Solution II: $MgSO_4.7H_2O$ at 15 g/L; Solution III: $CaCl_2.2H_2O$ at 7.2 g/L; Solution IV: Na_2CO_3 at 4 g/L; Solution V: H_3BO_3 at 1 g/L; $MnCl_2.4H_2O$ at 200 mg/L; $Na_2MoO_4.2H_2O$ at 10 mg/L; $ZnSO_4.7H_2O$ at 50 mg/L; $CuSO_4.5H_2O$ at 5 mg/L; Co $(NO_3).6H_2O$ at 10 mg/L; Solution VI: $FeCl_3.6H_2O$ at 170 mg/L; Na_2.EDTA.$2H_2O$ at 280 g/L; Solution VII: MOPS buffer at 490 g/L. Solutions from I to VI were filtered by porous membrane 20 µm in diameter. Solutions I to V are valid for six months while solutions VI and VII are only valid for one month. All stocks were kept in cool and dark place. Where 1 liter of SIS medium culture consists of 10 mL of solution I, 5 mL of solution II, 5 mL of

solution III, 5 mL of solution IV, 1 mL of solution V, 5 mL of the solution VI, 1 mL of solution VII beside 968 mL of Nanopure water. The medium is prepared two days before use due to the need to stabilize the pH at (6.5 ± 0.2) after adding MOPS buffer. In case the pH is unstable, it will be adjusted using dilute solutions of acid and base such as 0.1 M HCl and NaOH.

2.1.2 Mother culture of *Lemna minor* and how to preserve it

The plant was provided by Professor Philippe Juneau laboratory from the Biological Sciences Department at UQAM sciences faculty. The growth medium was placed into glass vessel, and then *Lemna minor* were added to newly prepared medium. The plants were grown in a controlled climate chamber under a photoperiod of 16-h light and 8-h dark at room temperature 23 ± 1°C. It is essential that the selected *Lemna minor* are young, lesions free or no apparent parts of chlorosis. White fluorescence lamps (Sylvania Grolux F36W) were used to obtain a continuous illumination with the light intensity between 85 and 135 µmol of photons m^{-2} s^{-1}. The container was covered by plastic film to avoid evaporation and contamination, with the necessity of leaving a small part of the container exposed to allow the air to enter and exchange needful gases for plant growth. Lastly, medium culture was changed once a week to preserve the growth quality (Baciak *et al.*, 2016; OECD, 2006).

2.1.3 Stock solutions preparation

2.1.3.1 DIB and IBX

Because most of the hypervalent iodine reagents are insoluble in water, both DIB and IBX stocks were prepared by dissolving them in medium culture of *Lemna*. Many of chemicals used are obtained from commercial specialized places such as Sigma Aldrich® which often are not of high purity or somewhat expensive. Thus, DIB and IBX have also been prepared in Professor Sylvain Canesi's laboratory at UQAM to obtain materials with a purity of 98 %. According to DIB, the stock solution was prepared with a concentration of 1000 µM by weight 0.16 g of di(acetoxyiodo) benzene (DIB), put it in a volumetric flask with a capacity of 500 ml then complete with medium culture. Whilst IBX stock solution was prepared with a concentration of 5 mM by weight 0.700 g of 2-iodoxybenzoic acid (IBX), put it in volumetric flask with a capacity of 500 ml then complete it with medium culture.

2.1.3.2 CrO_3 and $HgCl_2$

Chromium (III) Oxide CrO_3, ≥ 98.0% (NT) was purchased in Sigma Aldrich® Company as well as Mercury chloride $HgCl_2$ ≥ 98.0% (NT). Stock solution of CrO_3 at concentration of 100 mM was produced by weight 0.99 g of CrO_3 salt, put it in volumetric flask with a capacity of 100 ml then the volume is completed with Nanopure purified water (Termo Scientific Barnsted Nanopure ultrapure water purification

system). As for stock solution of $HgCl_2$ at concentration of 1000 µM was produced by weight 0.027 g of $HgCl_2$ salt, then transfer it to a volumetric flask with an ampleness of 100 ml and the volume is supplemented with Nanopure water.

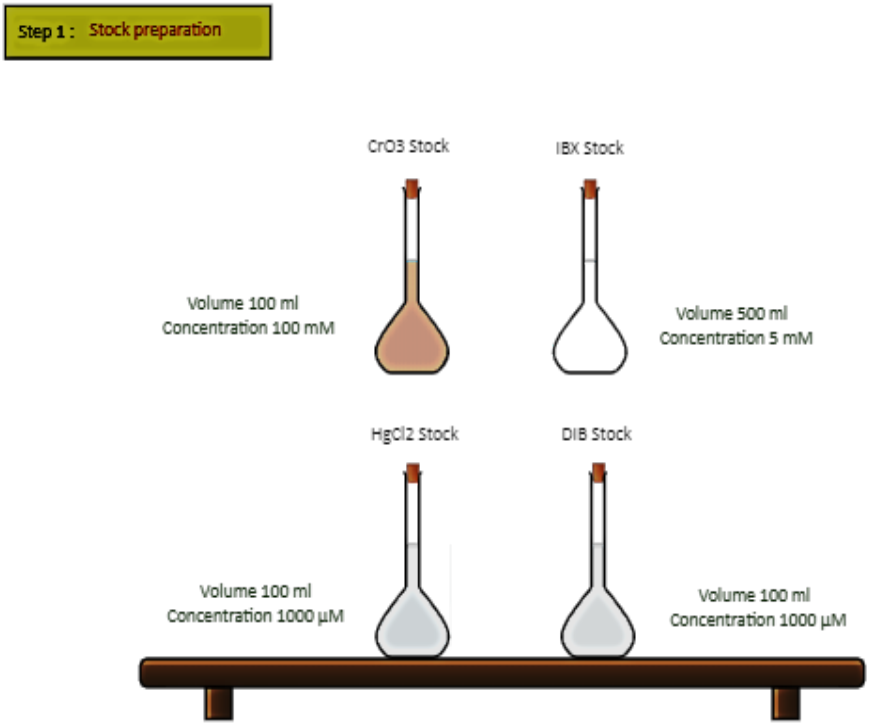
2.1.4 Preparation of contaminated medium culture

Six glass containers with capacity of 500 ml were previously washed with distilled water and diluted nitric acid 5 % to ensure they are free from any contamination with metals. This step followed by contamination of SIS medium by metals stock. Six nominal concentrations of IBX and CrO_3 were prepared separately in medium culture 0; 0.05; 0.10; 0.15; 0.20; and 0.25 mM respectively in final volume of 500 ml for 3 replicates. Each incubation vessel has total volume of 150 ml. The same thing in the case of $HgCl_2$ and DIB each one separately, six concentrations in medium culture were produced: 0; 1; 2.5; 3; 5; and 7 µM in final volume of 500 ml made for 3 replicates. The average of replicates used for most experiments are from 5 to 6 for each test material.

2.1.5 Incubation and test conditions

Plants consisting of 2 to 3 fronds are taken from the stock culture and randomly distributed in the flasks of cell culture (T-75 SARSTEDT®) to 150 ml that are filled with culture medium contaminated with different concentrations of CrO_3, IBX, $HgCl_2$, and DIB. All experiments set-up with zero metal concentration served as control

(Reale *et al.*, 2016), the young plants should be incubated with a minimum of 150 ml of the contaminated medium. Ten individuals *Lemna* with 2 fronds are incubated in each flask for 7 days (Figure 1.12). The test cultures are kept at 24°C ± 2°C. The pH of culture medium for both control and contaminated samples are adjusted at 6.5. All test vessels are closed by plug that allows air to pass through the flask and simultaneously reduce evaporation of medium.



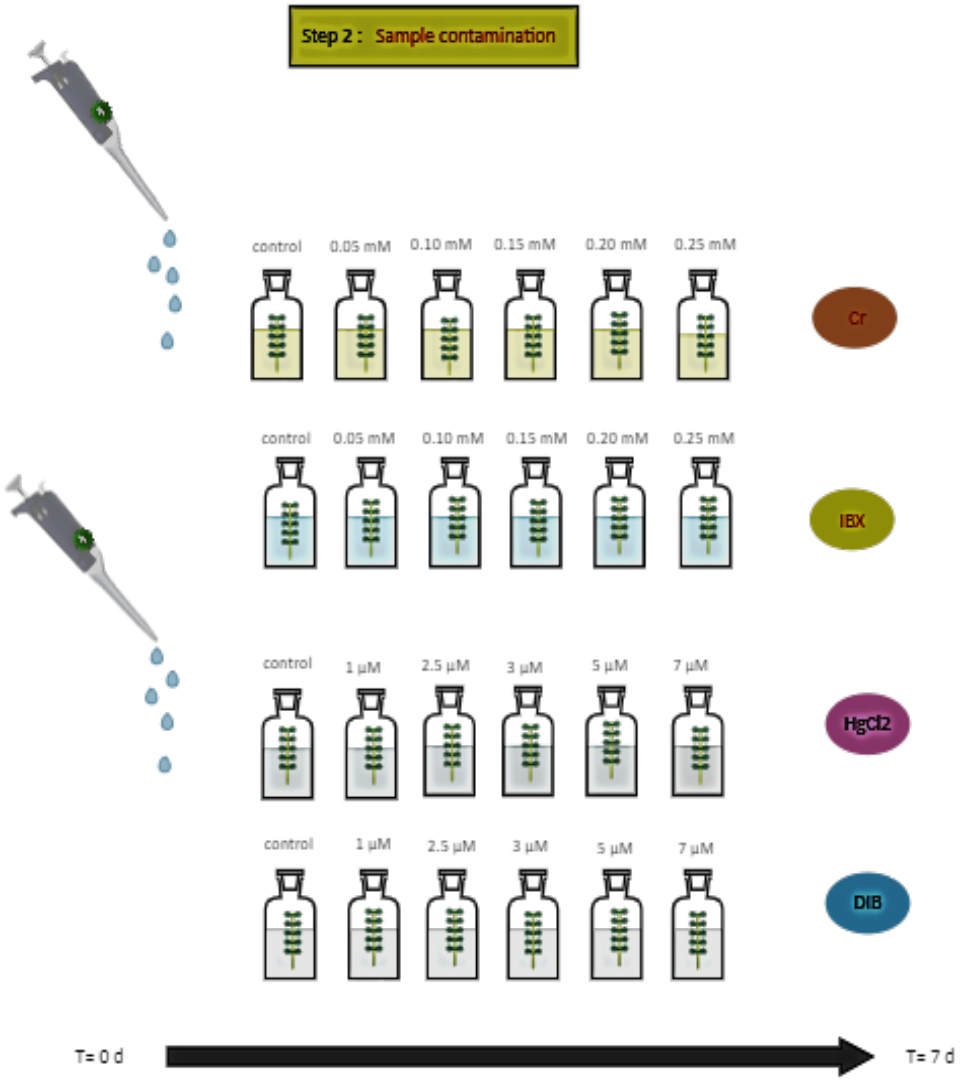


Figure 1.12 Experimental scheme for incubation in a contaminated environment.

Then all containers are placed in the incubator randomly , taking into account the same criteria as those in the mother medium (OECD, 2006). Some experiments are carried out four times (n=4) and some five times (n=5).

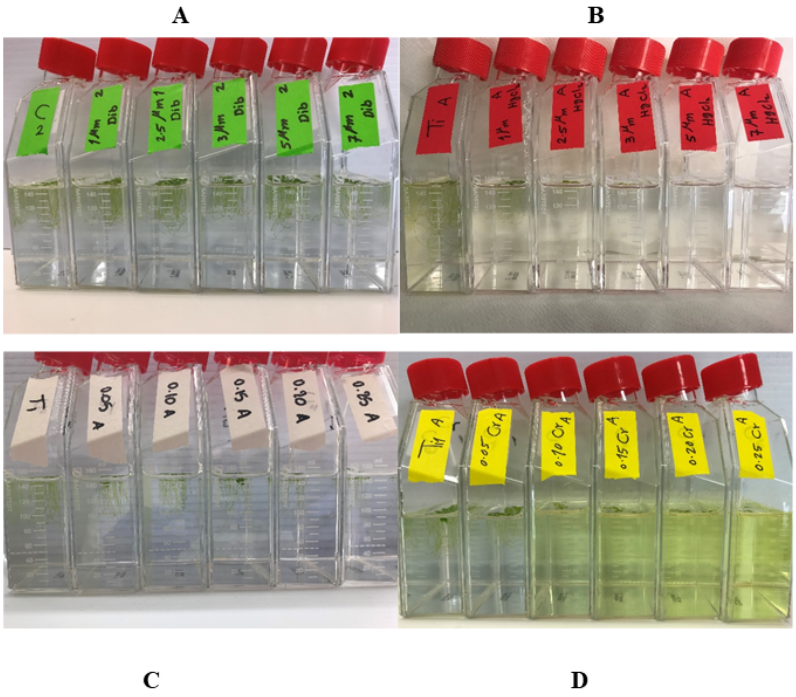


Figure 1.13 A *L. minor* treated with DIB after 7 days of incubation, B treated with $HgCl_2$, C treated with IBX, and D treated with CrO_3.

2.2 Experiments

2.2.1 Calculation of growth inhibition

After 7 days of incubation *Lemna minor* are collected in all containers. The number of individual fronds (a) are counted for each concentration and the fresh weight of each sample are measured (b).

Calculation of the specific growth rate

$$\mu \boldsymbol{i - j} = \frac{\boldsymbol{In(Nj) - In(Ni)}}{\boldsymbol{t}}$$

Where Nj is the variable (a or b) at t = 7, Ni is the variable (a or b) at t = 0, and t = time in days.

- Calculation of the percentage of growth inhibition

$$\%\boldsymbol{I} = \frac{\mu \boldsymbol{T} - \mu \boldsymbol{E}}{\mu \boldsymbol{T}} \times \mathbf{100}$$

Where μE is the specific growth rate of treated samples, μT is the specific growth rate of control (OECD, 2006). The average of the two parameters is calculated.

2.2.2 Determination of reactive oxygen species formation

The production of ROS in *L. minor* was estimated according to (Li, T. et Xiong, 2004). After 7 days of IBX, CrO_3, DIB, and $HgCl_2$ exposure, the treated-plants and the control were collected and washed in culture medium three times and placed in 1 ml new medium solution. The ROS was measured by using the cell penetrating indicator 2',7' dichlorodihydrofluorescein diacetate (H_2DCFDA) (Mitov *et al.*, 2016). The probe of

cellular esters is degraded into a non-fluorescent compound 2',7', dichlorodihydrofluorescein (H_2DCF), which is maintained in the cell. When the ROS and cellular peroxidases are present, H_2DCF is transformed to a high fluorescent compound, the 2',7'dichlorofluorescein (DCF) (Haugland *et al.*, 2005). The plants were treated with 5 μM of H_2DCFDA in 10 ml of medium solution for each sample at temperature 25°C for 30 min. The emission of fluorescent at 530 nm was measured by using exciting wavelength at 485 nm. The fluorescence reading was performed by using an Infinite M200 fluorometer (TECAN ®). The values of 16 readings per sample were averaged.

2.2.3 Determination of photosynthetic pigments

The plants were collected and ground in a mortar in the dark by using 10 ml of ethanol at a concentration of 95 % to extract pigments at ambient temperature. After 24 hours, the samples were centrifuge at 5198 *g* for 5 min at 24°C.

The absorbance of Chl *a* was measured at 665 nm, of Chl *b* at 649 nm, and of carotenoids at 470 nm (Mouzaki-Paxinou *et al.*, 2016). The absorbance of samples was read by using a Cary 60 UV-vis spectrophotometer from Agilent Technologies®. The pigment concentrations were calculated by the following equations:

- $\text{Chl}_a = 13.36 \times (A_{665}) - 5.19 \times (A_{649})$
- $\text{Chl}_b = 27.43 \times (A_{649}) - 8.12 \times (A_{665})$
- $C_{x+c} = (1000 \times (A_{470}) - (2.13 \times \text{Chl}_a) - (97.64 \times \text{Chl}_b)) /209$

Where the Chl $_a$, Chl $_b$, and C_{x+c} is respectively the content of chlorophyll *a*, chlorophyll *b*, and carotenoids in (µg/ml), and the $A_{665,}$ $A_{649,}$ and A_{470} are the absorbance values. The chlorophyll contents were normalized per mg of fresh weight.

2.2.4 Measurement of the rapid kinetics of Chl *a* of PSII

In this experiment, plants of *L. minor* were collected after 7 days of contamination, and the rapid kinetics of Chl *a* were measured by using a HANDY-PEA (HANSATECH®) Plant Efficiency Analyzer. Before starting the measurement process, the plants were left in the dark for 15 min, causing the electron transport chain to discharge (oxidize). Then, the plants were exposed to a 1 sec flashlight stimulation.

2.3 Statistical analysis

The (one-way ANOVA) followed by a Tukey's test from the Statistical Package (ORIGNPRO) software was used to determine whether there was a significant difference between the treated samples and control. For ($P < 0.05$) * and ($P < 0.01$) **.

CHAPTER III

RESULTS

In the current study, we investigated the toxic effects of the following compounds IBX, CrO_3, DIB, and $HgCl_2$ in aqueous medium using *Lemna minor*, which indicated a clear change in the rate of growth and sensitive biomarkers as evidence of the presence of toxicity in tissues and cells.

3.1 Effects of IBX and CrO_3 on the phenotype of *Lemna minor*

Through the results obtained in the case of chromium, we find that the control contains a rapid growth rate for seven days with an average fresh mass of 124 mg, and the average number of fronds of 131.2 in case of chromium (III) oxide (Table 1.4A). We noticed that with an increase in the concentrations of pollutant in the medium, there is a significant decrease in these parameters by an estimated percentage of 99 % from 0.05 mM of exposure to CrO_3, the values are as follows : 64.6 fronds and 46.17 mg, that is a decrease of 37.99 % and 47.15 % in comparison with control. In case of IBX the rapid growth rate of control after 7 days with an average fresh mass of 168.65 mg, and the average number of fronds of 141.2 (Table 1.4B). We have noticed that with the

increased concentrations of IBX in medium of incubation, there is a slight decrease in parameters at confidence level of 10 % from 0.05 mM from exposure to IBX, the values are as follows : 141.2 fronds and 168.658 mg, with a decrease of 2.42 % and 9.99 % compared to the control. Moreover, the color of *L. minor* after being contaminated with CrO_3, changes to become yellowish-white, whereas when *L. minor* is contaminated with IBX, it remains green.

Table 1.4: Decrease in fresh weight (mg) and number of fronds after 7 days of exposure to different nominal concentrations of CrO_3 (A) and IBX (B) in treated *Lemna minor* for ($P < 0.05$) * and ($P < 0.01$) ** compared to control (C), (n = 5).

A

Label	**C**	**0.05 mM**	**0.10 mM**	**0.15 mM**	**0.20 mM**	**0.25 mM**
Fresh weight (mg)	**124.74** (44.48)	**46.17**** (11.80)	**32.99**** (10.47)	**24.5**** (8.89)	**23.07**** (10.42)	**24.60**** (10.85)
Number of fronds	**131.2** (12.33)	**64.6**** (7.93)	**51**** (3.847)	**43**** (5.29)	**33.4**** (5.88)	**27.8**** (5.63)

B

Label	**C**	**0.05 mM**	**0.10 mM**	**0.15 mM**	**0.20 mM**	**0.25 mM**
Fresh weight (mg)	**168.65** (31.22)	**133.77** (28.93)	**129.10** (23.07)	**117.01** (18.00)	**109.73*** (14.84)	**120.03** (25.04)
Number of fronds	**141.2** (24.79)	**132** (31.28)	**123.8** (23.70)	**130.2** (17.37)	**120.8** (17.22)	**128.6** (18.86)

The values in ( ) represent the standard deviation.

3.2 Effects of DIB and $HgCl_2$ on the phenotype of *Lemna minor*

After 7 days of incubation, we find that the control contains a rapid growth rate with an average mass of 183.09 mg and the average number of fronds of 131.6, in case of $HgCl_2$ (Table 1.5A). We have observed that with an increase of $HgCl_2$ concentrations in medium, there is a significant decrease in this parameter by an estimated percentage of 93 % from 1 µM exposure to $HgCl_2$, the values are as follows : 87,6 fronds and 89.58 mg, a decrease of 21.95 % and 34.79 % compared to control. While in case of DIB the rapid growth rate of control after 7 days with an average fresh mass of 188.83 mg, and average number of fronds of 137.2 (Table 1.5B). We remarked that with increasing DIB concentration in medium, approximately, there is no significant decrease in these parameters at confidence level of 6 % from 1 µM of exposure to DIB, and the values are as follows : 130.4 fronds and 177.95 mg, at rate of 2.68 % and 4.22 % respectively, in comparison with control. In addition, the white color appears gradually on plants when exposed to mercury chloride, while they remain green when exposed to the same concentrations of organic iodine reagent.

Table 1.5: Decrease in fresh weight (mg) and number of fronds after 7 days of exposure to different nominal concentrations of $HgCl_2$ (A) and DIB (B) in treated *Lemna minor* for ($P < 0.05$) * and ($P < 0.01$) ** compared to control (C), ($n = 5$).

A

Label	C	1 µM	2.5 µM	3 µM	5 µM	7 µM
Fresh weight (mg)	**183.09** (16.69)	**89.58**** (16.59)	**51.17**** (5.62)	**42.06**** (5.37)	**35.75**** (3.25)	**28.41**** (4.40)
Number of fronds	**131.6** (6.11)	**87.6**** (9.47)	**65.8**** (7.49)	**58.4**** (6.62)	**48.6**** (9.47)	**33.4**** (5.20)

B

Label	C	1 µM	2.5 µM	3 µM	5 µM	7 µM
Fresh weight (mg)	**188.83** (14.04)	**177.95** (14.22)	**182.01** (11,49)	**170.24** (14.62)	**190.31** (11.15)	**179.24** (8.70)
Number of fronds	**137.2** (5.26)	**130.4** (6.52)	**127** (7.66)	**119.4** (5.23)	**116.6** (4.45)	**111.4** (9.62)

The values in ( ) represent the standard deviation.

3.3 Effect of CrO_3 and IBX on growth

In this study, we evaluated the inhibition of growth caused by the effect of CrO_3 and IBX on plants (Figure 1.14). The graph shows a relationship between the concentrations of CrO_3 and IBX in incubation medium and the inhibition of growth. The percentage is 0 % for control, because it coincides with perfect growth. With regard to other concentrations after control, we notice a gradual inhibition of plant growth, as the concentration increase.

The lowest concentration applied was Chromium trioxide 0.05 mM that showed a significant increase in growth inhibition compared to the control, where the percentage is 41.75 % comparison with control.

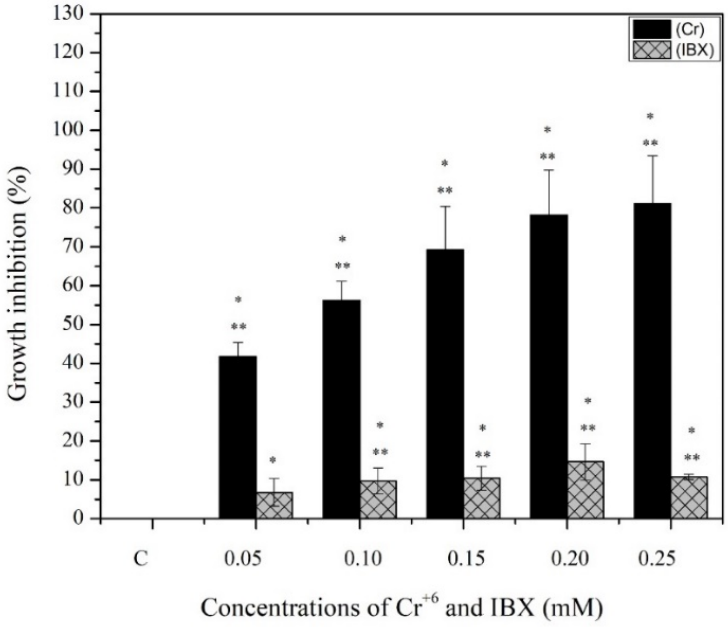


Figure 1.14 Inhibitory effect of CrO_3 and IBX on the growth of *Lemna minor* (%). The inhibition of plant growth of *Lemna minor* was calculated using the number of fronds and the fresh mass after 7 days of exposure to different nominal concentrations of CrO_3 and IBX. Data represents the average of five replicates (n = 5). ($P < 0.05$) * and ($P < 0.01$) **. C, Control.

In addition, exposure to 0.10 mM, 0.15 mM, 0.20 mM, and 0.25 mM of CrO_3 respectively, causes a corresponding inhibition of growth of 56.27 %, 69.19 %, 78.22 %, and 81.14 %. On the other hand, when studying the changes caused by IBX, we find that the percentage is 0 % for control referring to the ideal growth. For the IBX concentration of 0.05 mM, results showed a significant increase of 6.77 % compared to control. Moreover, when exposed to the concentrations of 0.10 mM, 0.15 mM, 0.20

mM, and 0.25 mM consecutively, it leads to a rate of inhibition of 9.711 %, 10.43 %, 14.64 %, and 10.74 %.

3.4 Effect of $HgCl_2$ and DIB on growth

From morphological changes occurring to the plant, we can deduce the effect of $HgCl_2$ and DIB by measuring the number of fronds as well as fresh weight to determine the level of growth inhibition (Figure 1.15). The graph shows a relationship between the concentrations of $HgCl_2$ and DIB in medium and growth inhibition. In case of $HgCl_2$, the percentage is 0 % for control, which refers to optimal growth. Next, we notice a gradual inhibition of biomass growth, which count on the exposure concentration in the first place.

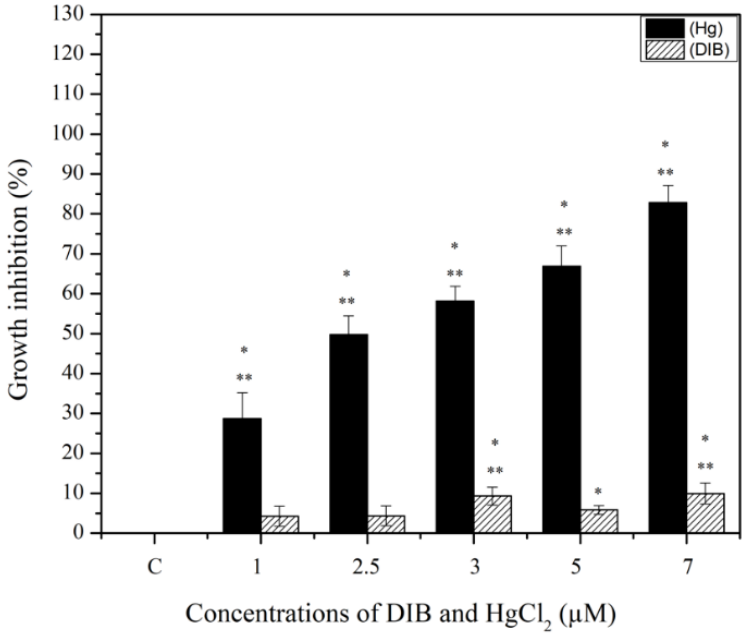


Figure 1.15 Inhibitory effect of $HgCl_2$ and DIB on the growth of *Lemna minor* (%). The inhibition of plant growth of *Lemna minor* was calculated using the number of fronds and the fresh mass after 7 days of exposure to different nominal concentrations of $HgCl_2$ and DIB. Data represents the average of five replicates (n = 5). ($P < 0.05$) * and ($P < 0.01$) **. C, Control.

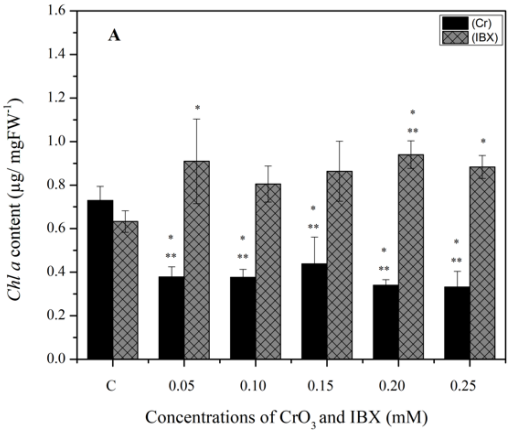
For the lowest applied concentration of $HgCl_2$ of 1 µM, the inhibition level increased significantly compared to control, by 28.66 % with 99 % confidence level. As well as the exposure to concentrations of 2.5 µM, 3 µM, 5 µM, and 7 µM in medium causes inhibition of 49.70 %, 58.15 %, 66.85 %, and 82.82 %, respectively.

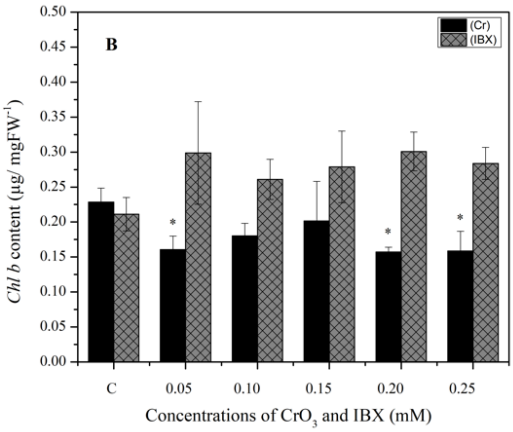
For DIB concentration of less than 3 µM, the inhibition is not significant compared to control, but from 3 µM, we observed a significant increase of 9.29 % of inhibition compared to control with 99 % confidence level. The exposure of 5 µM and 7 µM of DIB in incubation medium caused similar inhibition of 5.84 % and 9.95 %.

3.5 Effect of metals and hypervalent iodine reagents on pigments content

3.5.1 Effect of CrO_3 and IBX

The results obtained from the determination of pigments content (Figure 1.16) exhibit a significant decrease in chlorophyll *a* content compared to control, under the exposure to 0.05, 0.10, 0.15, 0.20, and 0.25 mM of CrO_3 with a confidence level of 99 % and the values are as follows : 0.729, 0.378, 0.376, 0.438, 0.34, and 0.332 µg/mg FW. While chlorophyll *b* content shows an insignificant decrease at 0.10 and 0.15 mM unlike 0.05, 0.20, and 0.25 mM that shows a significant decrease with a confidence level of 95 %. Otherwise, the carotenoid contents take another turn as the results show an insignificant variation with all concentrations in medium. Regarding IBX, the results exhibit a significant decrease at 0.05, 0.25 mM compared to control with 95% confidence level and at 0.20 mM with 99%. On the other hand, there is a non-significant decrease in chlorophyll *b* and carotenoid contents.


A
(Cr)
(IBX)
Chl a content (µg/ mgFW-1)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
C
0.05
0.10
0.15
0.20
0.25
Concentrations of CrO3 and IBX (mM)


B
(Cr)
(IBX)
Chl b content (µg/ mgFW-1)
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
C
0.05
0.10
0.15
0.20
0.25
Concentrations of CrO3 and IBX (mM)

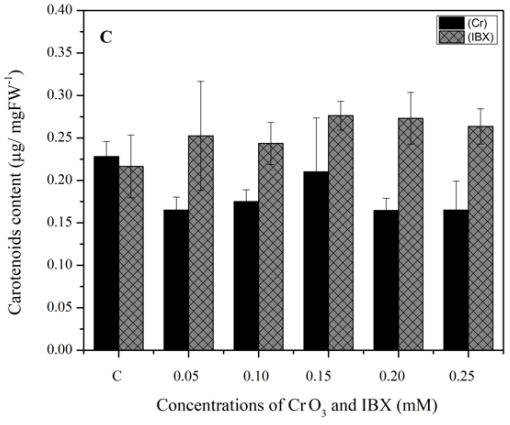
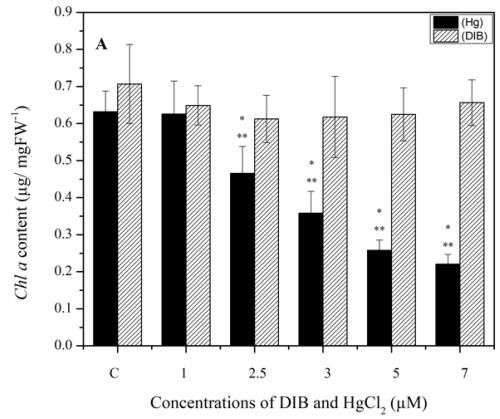
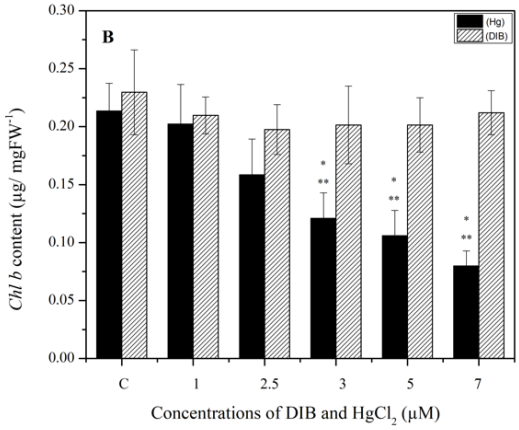

C
(Cr)
(IBX)
Carotenoids content (µg/ mgFW-1)
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
C
0.05
0.10
0.15
0.20
0.25
Concentrations of CrO3 and IBX (mM)

Figure 1.16 Change in chlorophyll *a* (A), *b* (B) and carotenoids (C) (µg/mg FW) content in *Lemna minor* after 7 days of exposure to different nominal concentrations of CrO_3 and IBX. (P <0.05) * and (P <0.01) **, compared to control (C), (n = 5).

3.5.2 Effect of DIB and $HgCl_2$

The measurement of the content of pigments in plant after exposure to several concentrations of DIB and $HgCl_2$ (Figure 1.17) shows at concentrations of 2.5, 3, 5, and 7 µM of $HgCl_2$, a significant decrease in chlorophyll *a* content compared to control by 99 % (P <0.01) **, and the values are 0.465, 0.358, 0.258, and 0.220 µg/mg FW, respectively. Unlike DIB, it does not show any significant change at the same concentrations. As for chlorophyll *b* content, we notice a significant decrease in pigment content at 3, 5, and 7 µM of $HgCl_2$ compared to control by 99 % (P <0.01) **, and the values were as follows : 0.120, 0.105, and 0.079 µg/mg FW. Otherwise, there is no noticeable change caused DIB. For the carotenoid contents, the results show a significant decrease at 5 and 7 µM of $HgCl_2$ compared to control by 99 % (P <0.01), and the results are 0.123 and 0.114 µg/mg FW, while for DIB there is no change worth mentioning.


A
(Hg)
(DIB)
Chl a content (µg/ mgFW-1)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
C
1
2.5
3
5
7
Concentrations of DIB and HgCl2 (µM)


B
(Hg)
(DIB)
Chl b content (µg/ mgFW-1)
0.00
0.05
0.10
0.15
0.20
0.25
0.30
C
1
2.5
3
5
7
Concentrations of DIB and HgCl2 (µM)

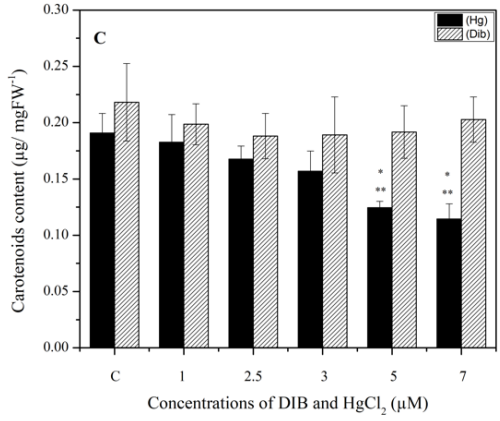

C
(Hg)
(Dib)
Carotenoids content (µg/ mgFW-1)
0.00
0.05
0.10
0.15
0.20
0.25
0.30
C
1
2.5
3
5
7
Concentrations of DIB and HgCl2 (µM)

Figure 1.17 Change in chlorophyll *a* (A), *b* (B) and carotenoids (C) (µg/mg FW) content in *Lemna minor* after 7 days of exposure to different nominal concentrations of DIB and $HgCl_2$. ($P < 0.05$) * and ($P < 0.01$) **, compared to control (C), (n = 5).

3.6 Effect of trace metals and hypervalent iodine reagents on ROS production

3.6.1 Effect of CrO_3 and IBX

The toxicity effect of CrO_3 and IBX was estimated through the intracellular ROS production in *L. minor* plants due to the incitement of oxidative stress (Figure 1.18). we found a high fluorescence emission of reactive oxygen species compared to the control when the plant was exposed to 0.10 mM, 0.15 mM, 0.20 mM, and 0.25 mM of CrO_3, and at these concentrations the significant increase in production of ROS was recorded with a confidence level of 99 %. As the ROS production reached 465.40 %, 586.59 %, 587.85 %, and 618.84 %, respectively. Regarding IBX, the high fluorescence emission was recorded when *L. minor* was exposed to 0.20 mM and 0.25 mM at level of confidence of 95 % where the production of ROS reached 270.46 % and 288.98 %.

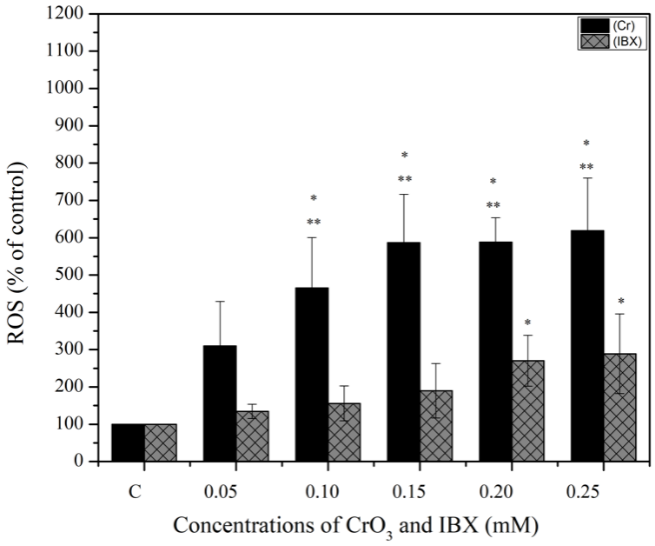


Figure 1.18 The production of intracellular ROS in *L. minor* exposed during 7 days to different concentrations of CrO_3 and IBX. Data represents the average of four replicates (n = 4). The asterisk (*) indicates significant differences for ($P < 0.05$) * and ($P < 0.01$) ** between treatments and the control.

3.6.2 Effect of $HgCl_2$ and DIB

The (H_2DCFDA) probe was taken by living tissue of *L. minor* and oxidized to form a high fluorescence compound, 2',7'dichlorofluorescein (DCF) (Figure 1.19). We observed a high fluorescence emission in comparison with control after exposure to 2.5 µM, 3 µM, 5 µM and 7 µM of $HgCl_2$. For the last two applied concentrations a significant increase in ROS production was recorded, with level of confidence of 99 % and the percentages are as follows: 786.32 % and 1034.06 %. However, we find the opposite when *L. minor* was exposed to the same concentrations of DIB, as no high

fluorescence emission was recorded for ROS production compared to control. The percentages are 114.40 %, 105.49 %, 103.59 %, 94.52 %, and 102.22 %, respectively.

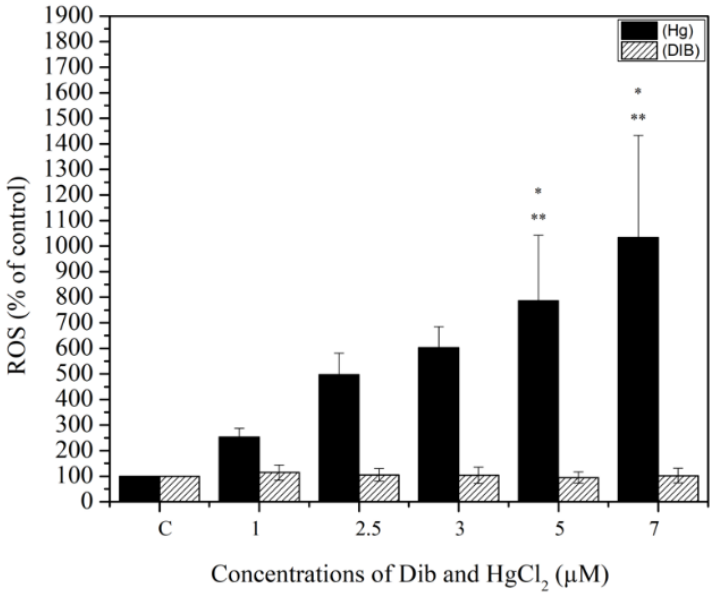


Figure 1.19 The production of intracellular ROS in *L. minor* exposed during 7 days to different concentrations of $HgCl_2$ and DIB. Data represents the average of four replicates (n = 4). Only $HgCl_2$ treatments are significantly different for ($P < 0.05$) * and ($P < 0.01$) ** compared to the control.

3.7 Effect of metals and hypervalent iodine reagents on photosynthetic parameters

3.7.1 Effect of CrO_3 and IBX

Through the parameter of total chlorophyll, we can know the organizational structure of PSII. During exposure to CrO_3, we notice through the results that there is a significant decrease for 0.05, 0.10, 0.15, 0.20, and 0.25 mM of CrO_3 in medium with a

confidence level of 99 % ($P < 0.01$) ** compared to control, with values of 0.269, 0.278, 0.320, 0.248, and 0.245 compared to value of control 0.479. The contrast level of many biomarkers associated with PSII after exposure to CrO_3 is determined by using the Handy-PEA fluorometer. First of all, the results obtained for the rapid fluorescence kinetics of chlorophyll *a* which (F_v/F_o) show us a clear disturbance in the activity of PSII from 0.05 mM of CrO_3, referred to the changing of transitions of JIP, and preventing photoelectron transmission (Figure 1.20A). Then, regarding to ABS/RC, we observed that there is the same behavior with values 3.51, 4.12, 4.59, 4.99, and 5.25 compared to 2.95 of control. In addition, the quantitative efficiency of PSII (F_v/F_m) and performance indicator of PSII (PI_{ABS}) show a significant contrast for all concentrations of CrO_3, at a confidence level of 99 % ($P < 0.01$) ** compared to control. For (F_v/F_m) : 0.647, 0.493, 0.412, 0.330, and 0.327 vs 0.779 of control; and for (PI_{ABS}) : 0.517, 0.202, 0.121, 0.053, and 0.069 against 1.539. Lastly, the relative fluorescence for Q_A reduction increased significantly with 99 % of confidence level from 0.05 to 0.20 mM of CrO_3, with values 0.500, 0.612, 0.663, and 0.699 compared to a base value of 0.464 for the control.

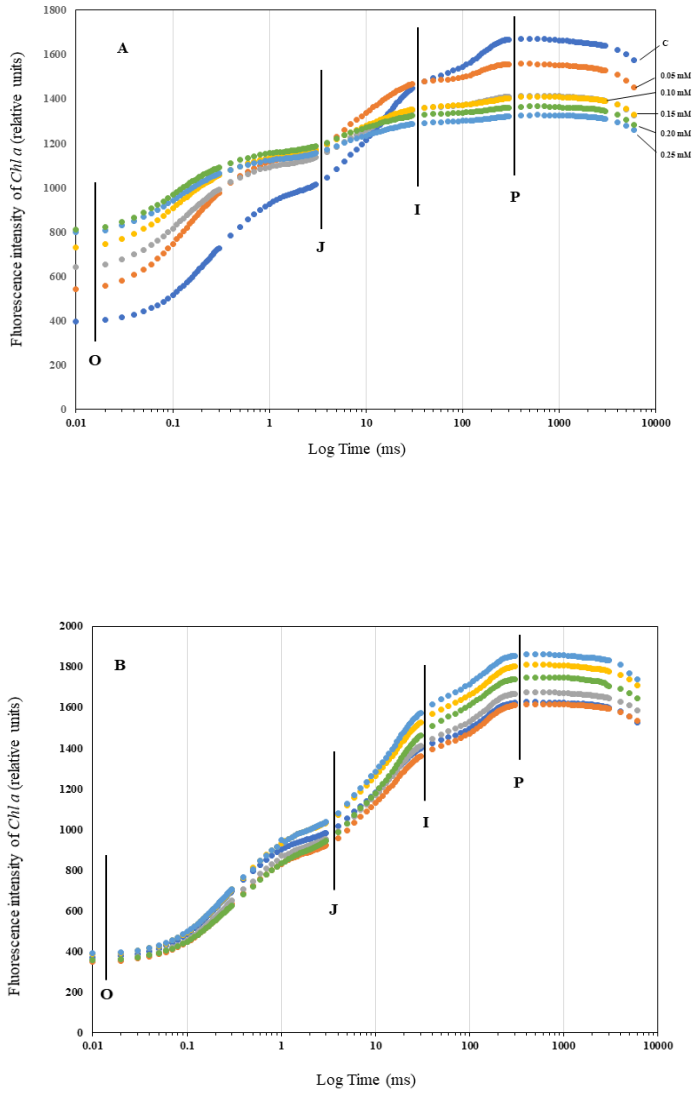


Figure 1.20: A. Change of the rapid and polyphasic rise of *Chl a* fluorescence kinetic for *L. minor* exposed for 7 days to different concentrations of CrO_3. Data represents the average of five replicates (n = 5). B. Change of the rapid and polyphasic rise of *Chl a* fluorescence kinetic for *L. minor* exposed for 7 days to different concentrations of IBX. Data represents the average of five replicates (n = 5).

Concerning IBX, unlike CrO_3, as for measuring the total chlorophyll (Chl *a/* Chl *b*) (Table 1.6), the results show a significant increase at 0.05 and 0.20 mM of IBX in medium with a confidence level of 95 % ($P < 0.05$) ** compared to control, and the values are 0.603 and 0.620 compare to 0.421 value of control.

Table 1.6: Variation of different photosynthetic parameters in *Lemna minor* after 7 days of exposure to different nominal concentrations of CrO_3 (A) and IBX (B). ($P < 0.05$) * and ($P < 0.01$) **, compared to control (C), (n = 5).

A

Label	C	0.05 mM	0.10 mM	0.15 mM	0.20 mM	0.25 mM
Chl a/ Chl b	**0.47** (0.042)	**0.26**** (0.032)	**0.27**** (0.025)	**0.32**** (0.088)	**0.24**** (0.015	**0.24**** (0.049)
Fv/Fm	**0.77** (0.003)	**0.64** (0.021)	**0.49**** (0.108)	**0.41**** (0.117)	**0.33**** (0.084)	**0.32**** (0.117)
ABS/RC	**2.95** (0.23)	**3.51** (0.29)	**4.12** (0.29)	**4.59*** (0.45)	**4.99**** (0.52)	**5.25**** (0.63)
Vj	**0.46** (0.08)	**0.50*** (0.10)	**0.61**** (0.10)	**0.66**** (0.18)	**0.69**** 0.18)	**0.69**** (0.21)
PI_{ABS}	**1.53** (0.55)	**0.51**** (0.22)	**0.20**** (0.25)	**0.12**** (0.14)	**0.05**** (0.05)	**0.06**** (0.09)

B

Label	C	0.05 mM	0.10 mM	0.15 mM	0.20 mM	0.25 mM
Chl a/ Chl b	**0.42** (0.03)	**0.60*** (0.13)	**0.53** (0.05)	**0.57** (0.09)	**0.62*** (0.04)	**0.58** (0.03)
Fv/Fm	**0.78** (0.005)	**0.79*** (0.003)	**0.80**** (0.003)	**0.79**** (0.003)	**0.80**** (0.002)	**0.80**** (0.002)
ABS/RC	**2.90** (0.13)	**2.68** (0.13)	**2.63*** (0.09)	**2.64*** (0.09)	**2.58**** (0.11)	**2.44**** (0.09)
Vj	**0.46** (0.01)	**0.42** (0.02)	**0.43** (0.01)	**0.42** (0.02)	**0.41*** (0.01)	**0.38**** (0.01)
PI_{ABS}	**1.55** (0.21)	**2.05** (0.37)	**2.05** (0.16)	**2.11** (0.32)	**2.37**** (0.25)	**2.78**** (0.19)

The values in ( ) represent the standard deviation.

In addition, the rapid fluorescence kinetics of chlorophyll *a* (F_v/F_o) show a little disturbance in the activity of the PSII as shown in (Figure 1.20B). Then we observed that ABS/RC ratio follows a different trend with values of 2.68, 2.63, 2.64, 2.58, and 2.44 compared to 2.90 for control. Moreover, the (F_v/F_m) and (PI_{ABS}) show a significant variation at a confidence level of 95 % for 0.05 mM, and 99 % 0.10, 0.15, 0.20, and 0.25 mM compared to control. For (F_v/F_m) : 0.79, 0.80, 0.79, 0.80, and 0.80 vs 0.78 of

control; for (PI_{ABS}) at 99 % under 0.20 and 0.25 mM of IBX : 2.37 and 2.78 vs 1.55 of control. At the end, the V_J relative fluorescence of Q_A reduction decreased significantly with 95 % of confidence level for 0.20 mM and with 99 % for 0.25 Mm, compared to basal value of control, and the values are as follows: 0.41 and 0.38 vs 0.46 for control.

3.7.2 Effect of DIB and $HgCl_2$

The Chl *a*/*b* ratio (Table 1.7) show us that there is no change for all concentrations of DIB in medium compared to control. The efficiency of PSII was evaluated on several parameters by comparing the contrast between each parameter after exposure to contamination. The fluorescence kinetic shows no disturbance of the activity of PSII for applied concentrations, this is indicated by JIP transitions (Figure 1.21A).

Table 1.7: Variation of different photosynthetic parameters in *Lemna minor* after 7 days of exposure to different nominal concentrations of DIB (A) and $HgCl_2$ (B). ($P < 0.05$) * and ($P < 0.01$) **, compared to control (C), (n = 5).

A

Label	C	1 µM	2.5 µM	3 µM	5 µM	7 µM
Chl a/ Chl b	**0.46** (0.07)	**0.42** (0.03)	**0.40** (0.04)	**0.40** (0.07)	**0.40** (0.04)	**0.42** (0.04)
Fv/Fm	**0.78** (0.007)	**0.78** (0.006)	**0.79** (0.002)	**0.78** (0.004)	**0.78** (0.005)	**0.79** (0.006)
ABS/RC	**2.99** (0.18)	**3.01** (0.08)	**3.03** (0.10)	**2.97** (0.09)	**2.97** (0.04)	**2.99** (0.11)
Vj	**0.49** (0.07)	**0.47** (0.01)	**0.46** (0.02)	**0.45** (0.01)	**0.44** (0.009)	**0.45** (0.02)
PI_{ABS}	**3.67** (1.35)	**3.67** (0.35)	**4.00** (0.40)	**4.09** (0.32)	**4.22** (0.20)	**4.19** (0.54)

B

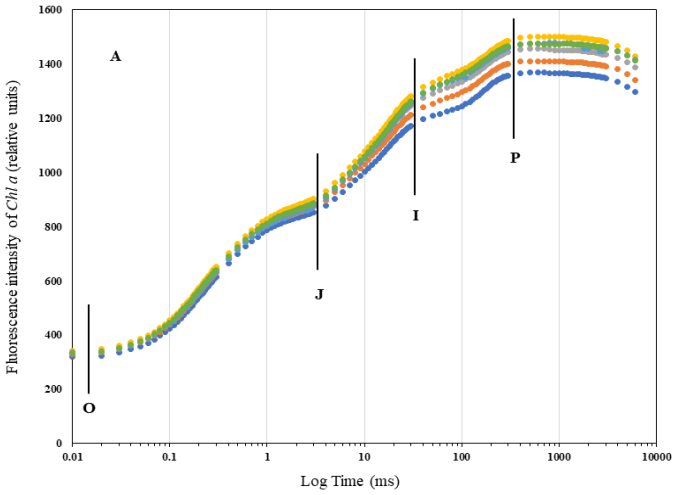
Label	C	1 µM	2.5 µM	3 µM	5 µM	7 µM

Chl a/ Chl b	**0.42** (0.03)	**0.41** (0.06)	**0.31***(0.05)	**0.23****(0.04)	**0.18****(0.02)	**0.15****(0.01)
Fv/Fm	**0.77** (0.005)	**0.80****(0.004)	**0.79** (0.01)	**0.79** (0.003)	**0.75****(0.006)	**0.76** (0.01)
ABS/RC	**3.04** (0.06)	**2.93** (0.09)	**3.35***(0.29)	**3.46****(0.12)	**3.82 ****(0.04)	**3.67****(0.07)
Vj	**0.47** (0.02)	**0.45** (0.01)	**0.51** (0.02)	**0.52***(0.01)	**0.58****(0.006)	**0.54****(0.01)
PI_{ABS}	**1.29** (0.09)	**1.77****(0.09)	**1.18** (0.32)	**1.03** (0.11)	**0.61****(0.02)	**0.77****(0.07)

The values in ( ) represent the standard deviation.

As well (ABS/RC) ratio shows no significant change between the concentrations and control, then we studied the quantitative capacity of PSII (F_v/F_m) and the activity of performance of PSII (PI_{ABS}), both of them show no mentionable change although the values for PI_{ABS} at concentration of 2.5, 3, 5, and 7 µM of DIB show a slight increase compared to control, but this increase is not significant. Finally, the parameter of V_J, shows that there is no change can be mentioned compared to the control. On the other hand if we look at the results related to $HgCl_2$, the Chl *a*/*b* ratio (Table 1.7) decreased significantly for 2.5 µM at 95 % of a confidence level, and for 3, 5, and 7 µM at 99 % compared to the control, and the values are as follows 0.31, 0.23, 0.18, and 0.15 respectively. As for (ABS/RC), the results show a significant increase for 2.5 µM of $HgCl_2$ at 95 % and for 3, 5, and 7 µM at 99 % of a confidence level compared to control, and the values are 3.35, 3.46, 3.82, and 3.67. In addition, we studied (F_v/F_m) parameter, we find that there is a significant increase for 1 µM at a confidence level of 99% compared to control, but at the same time there is a significant decrease for 5 µM compared to the control at 99 %. In addition, the fluorescence kinetic shows a disturbance of the activity of PSII for applied concentrations (Figure 1.21B). As for the performance of PSII (PI_{ABS}) and the V_J, the results show under 1 µM for PI_{ABS} a

significant increase compared to control at 99 % while for 5 and 7 µM the results significantly decreased at 99 % compared to control, the values are as follows 1.77, 0.61, and 0.77 against to 1.29 of control value. Moreover, V_J results significantly increased for 3 µM at 95 % of a confidence level, and for 5 and 7 µM at 99 % compared to control, and the values are 0.52, 0.58, and 0.54 compared of 0.47 of basal value of control.



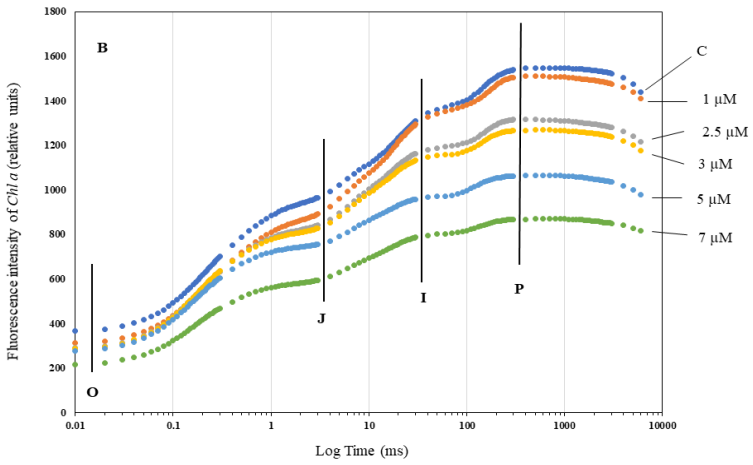


Figure 1.21 : A. Change of the rapid and polyphasic rise of *Chl a* fluorescence kinetic for *L. minor* exposed for 7 days to different concentrations of DIB. Data represents the average of five replicates (n = 5). B. Change of the rapid and polyphasic rise of *Chl a* fluorescence kinetic for *L. minor* exposed for 7 days to different concentrations of $HgCl_2$. Data represents the average of five replicates (n = 5).

CHAPTER IV

DISCUSSION

In this study, the toxicity effects of CrO_3, $HgCl_2$, IBX, and DIB were investigated on *Lemna minor* after incubation in contaminated medium for 7 days. Experimental data was treated statistically to observe the significance of different exposure concentrations on growth inhibition test and on photosynthesis processes.

4.1 Effect on growth

4.1.1 CrO_3 and IBX effect

Chromium trioxide showed to be a potential toxic compound with inhibitory and stimulating effects on plant growth, as well as on some physiological parameters depending on the duration of exposure and concentration. Common duckweed *(L. minor)* is a worldwide species, which is used in ecotoxicological laboratories. Some studies revealed that although CrO_3 has not any role to play in metabolic processes, it is absorbed by plants causing negative effects on the metabolism. In fact, the CrO_3 at low level in liquid medium was not needed for normal growth; but at higher

concentrations, it significantly reduced growth (Huffman et Allaway, 1973; Samantaray *et al.*, 1998; Shanker, A. K. *et al.*, 2005). Exposure of fronds of *L. minor* from 0.05 mM to 0.25 mM of CrO_3 had a significant effect on the growth of plants. At lowest concentration of CrO_3 after 7 days of exposure, it resulted in 50 % reduction in biomass compared to the control. Our results showed the manifestation of morphological and physiological changes as evidence of the toxicity effect of CrO_3 according to (Table 1.4A). *Lemna minor* showed relatively greater tolerance to CrO_3. From 0.05 mM, we observed a significant variation in the inhibition of growth rate of CrO_3 (Figure 1.14). Similar effects had been observed in *Azolla caroliniana* by (Bennicelli *et al.*, 2004). The effective 50 % CrO_3 concentration (EC_{50}) for *L. minor* was found at 0.08 mM, and it indicated that the CrO_3 had a high toxic effect on plant tissues. In addition, there was no previous studies showing the effect of organic iodine reagents on aquatic plants. In this study, the effect of organic hypervalent iodine reagents on *L. minor* was tested. The obtained results showed that the exposure of *Lemna* fronds to the same concentrations of IBX caused a significant decrease in fresh weight at concentration of 0.20 mM ($P < 0.05$) * compared to the control (Table 1.4B).

4.1.2 DIB and $HgCl_2$ effect

Duckweed has been extensively studied for assessment of trace metals in aquatic environment (Basile *et al.*, 2012; Khellaf et Zerdaoui, 2010). In Table 1.5B, the results showed no significant decrease in fresh mass and fronds number after exposed to DIB

concentrations of 1 to 7 µM. Otherwise, at 3 to 7 µM, the results showed a significant decrease in growth inhibition at confidence intervals ($P < 0.05$) * and ($P < 0.01$) ** compared to control (Figure 1.15). Nevertheless, it is not the same for $HgCl_2$, a significant decrease was observed in biomass (Table 1.5A). The growth test showed that the EC_{50} level of $HgCl_2$ was 4 mg L^{-1} (Yang *et al.*, 2018) and 0.2 mg L^{-1} (Dirilgen, 2011). However, they were higher than the value obtained in this study, i.e. 2.5 µM.

4.2 Effect on the content of photosynthetic pigments

4.2.1 Effect of CrO_3 and IBX

From 0.05 mM to 0.25 mM of CrO_3, we observed a significant decrease in the concentration of photosynthetic pigments (Figure 1.16), and a decrease in biomass (Table 1.4A). CrO_3 toxicity has caused severe effects on pigment content in photosynthesis, which can be clearly seen at the lowest concentration tested (0.05 mM). Previous studies have shown that CrO_3 was able to change the ultrastructure of chloroplasts as well as stimulate photosynthetic activity (Appenroth *et al.*, 2001; Reale *et al.*, 2016). Concentrations of Chls significantly decreased in the presence of CrO_3, there was a decrease in Chl *a* content at all tested concentrations after 7 days, while there was no significant difference in Chl *b* content at 0.10 and 0.15 mM concentrations. As for carotenoids, there was also a decrease in the pigments content due to CrO_3, but this decrease was not significant. In similar study, it was indicated that among the

chlorophylls, Chl *a* was the most affected compound to CrO_3 treatments compared to control. However, there were no statistically significant differences observed between CrO_3 concentrations. In contrast, we observed for IBX a marked increase in the photosynthetic pigments content, despite a decrease in the biomass (Table 1.4B). Furthermore, no study has been done on the effect of hypervalent iodine reagents on photosynthetic pigments in plants. These results provide important information for future research on the toxicity effect of these compounds.

4.2.2 Effect of DIB and $HgCl_2$

From the results obtained (Figure 1.17), we remarked that DIB had no effect on chlorophylls content (Chl *a*, Chl *b*), even the carotenoids content did not show any influence with DIB. Given the fresh mass and fronds number for all concentrations (Table 1.5B), results showed no significant change. Otherwise, many studies have been reported that trace metals stress has affected the photosynthetic pigments content in many plants (Hou *et al.*, 2007; Perreault *et al.*, 2014; Sree *et al.*, 2015). In this study, the pigment content of *L. minor* showed a small decrease at minimum concentration of $HgCl_2$, followed by a sharp decrease in the pigments content at higher concentrations, which is significant as the $HgCl_2$ concentration increase (Figure 1.17). This is attributed to insufficient effect of photoprotection and antioxidant production (Lalau *et al.*, 2015), or to the lack of uptake of essential elements such as K and Mn (Boening, 2000). The

large decrease in biomass illustrated the toxic effects of mercury on the environment (Table 1.5A).

4.3 Production of intracellular reactive oxygen species

4.3.1 CrO_3 and IBX effect

Types of reactive oxygen in cells are produced as a mediator during reduction of O_2 to H_2O. Trace metals are starting to produce ROS as a signal of the stress response (Schützendübel et Polle, 2002). It is known that CrO_3 is a toxic and trace element that can generate some types of ROS such as H_2O_2, OH^-, O_2^-, OH_2, etc., which cause oxidative damage to the plants (Choudhury et Panda, 2004; Gupta *et al.*, 2015; Sytar *et al.*, 2013). We observed a considerable increase in the production of ROS in plant cells at 0.10, 0.15, 0.20, and 0.25 mM of CrO_3 (Figure 1.18), with a confidence level 95 % ($P < 0.05$) * and 99 % ($P < 0.01$) **. In the case that there is an excess of ROS formed inside the cells, it is expected that many metabolic processes will be impaired, leading to cell death (Desikan *et al.*, 1998; Epple *et al.*, 2003). Metals had showed the ability to disrupt cellular biochemical processes, thus it is also assumed that CrO_3 is able to disrupt the visible phase of photosynthesis process by preventing the transfer of electrons in the organelle, which increases the formation of ROS within the cells. Plants can suffer from oxidative stress due to exposure to high concentrations of CrO_3, which leads to the initiation of lipid peroxidation process in plant by generating free radicals,

indicating damages to membrane function. Also, the increasing amount of malonyldialdehyde (MDA) in some algae has been reported (Choudhury et Panda, 2005), in wheat leaves as well (Panda et Patra, 2000). The decrease in total Chl (Chl *a*/*b*) content (Table 1.6A), was attributed to the extent of CrO_3 toxicity on cells. In contrast, organic IBX caused a significant increase in ROS production at high concentrations 0.20 and 0.25 mM, with 95 % of confidence level ($P < 0.05$) *. It indicated that the organic iodine reagent was much less toxic on cells than the tested metals and tend to replace other reagents with similar chemical properties. Although these reagents have been used as biocidal against a group of microorganisms such as fungi and bacteria, their effect on plants and on biomarkers such as photosynthetic pigments content has not been studied (Menkissoglu-Spiroudi *et al.*, 2001).

4.3.2 DIB and $HgCl_2$ effect

It was previously showed that the induction of oxidative stress caused a decrease in the growth, pigments content, and damages in cellular membrane such as lipids (Mittler, 2002). After *L. minor* was exposed to different $HgCl_2$ concentrations, the obtained results showed a clear increase in the formation of ROS, despite the increase was high compared to the control, but this increase was not significant at concentrations of 1, 2.5, and 3 µM of $HgCl_2$. At 5 and 7 µM of $HgCl_2$ concentrations, it was significant in comparison to the control at a confidence level of 95 % and 99 %, respectively. Moreover, statistical differences in the results were found between treatments and the

control, and treatments themselves. The excess production of reactive radicals, such as H_2O_2 and O_2^-, is due to an imbalance among the pro-oxidants and antioxidants in the cells (Flora *et al.*, 2008). Otherwise, earlier studies indicated that metal induced protective enzymatic activities (SOD, POD, and CAT) in plants, when oxygen free radicals are generated (Shiyab *et al.*, 2009). The decreased Chl (Chl *a*/*b*) content (Table 1.7B) caused by this metal was attributed to the activation of degradable chlorophyll enzymes and to the inhibition of involved enzymes in biosynthesis (Parmar *et al.*, 2013). For DIB, the results showed no formation of ROS within cells compared to the control (Figure 1.19), and no reduction in total chlorophyll content compared to the control (Table 1.7A). The non-toxicity of hypervalent iodine reagents on plant cells can be explained by the fact that iodine reagents may play a role in biological chemistry. Recently, it has been studied the possibility of using hypervalent iodine reagents as bioconjugates capable of labeling biomolecules such as proteins and peptides to install the structure of iodine (Tessier *et al.*, 2019).

4.4 The change of fluorescence kinetics and parameters related to PSII activity

4.4.1 After exposed to CrO_3 and IBX

From 0.05 mM concentration of CrO_3, we noticed a deactivation in the biosynthesis of chlorophyll pigments (Figure 1.16A and B), since they are the aim of many metals that always show a negative impact on the metabolic processes in plant

(Dewez *et al.*, 2018; Hegazy *et al.*, 2017; Tarrahi *et al.*, 2018). We remarked a gradual decrease in pigments biosynthesis (Figure 1.16), especially at high concentrations, as the biosynthesis depends on Chl *a*. In addition, the Chl *b* is a worthy component of the process of absorbing and transmitting light energy from photons to Chl *a* pair of reaction center of PSII, as well as it is the component of peripheral antennas of PSII (Kirst *et al.*, 2017; Zhu *et al.*, 2010). By looking at (Table 1.6A), we found that the Chl *a*/*b* proportion shows a reduction in the antenna structure of PSII. Chl *b* is the main component for antenna and is responsible to transfer the needed energy to excite the pair of Chl *a*, which has an impact on the efficiency of photosynthesis. This may explain the decrease in the performance index of PSII activity (PI_{ABS}) (Table 1.6A), and it is believed that the reason for this decline is due to the destruction of photosynthetic pigments (Hörcsik, Z., 2006). The ABS/RC parameter was used to determine the affinity of this metal on the reaction center of PSII, showing an increase in parameter value. Moreover, the CrO_3 did block the activity of the reaction center of PSII, which resulted in the replacement of Ca^{+2} as an important factor to stimulate the photolysis of water (Dewez *et al.*, 2018). After, F_V/F_M parameter showed a decrease in the quantitative efficiency of PSII, as a parameter with high sensitivity for the determination of toxicity for this metal (Ayyaz *et al.*, 2020). The absorbed energy is sent back to molecular oxygen, which promotes the increasing of ROS formation (Figure 1.18) and the blockage of photosynthetic electrons transport (Pitzschke *et al.*, 2006). In addition, the V_J parameter was affected by CrO_3, and we observed that the

higher concentration, the greater values in relative fluorescence (Table 1.6A). The J, I, and P phase was affected with the increasing of CrO_3 concentrations, which was caused by the electron transfer inhibition at the PSII leading to the accumulation of $P680^+$ (Figure 1.20) and of Q_A reduced pool (Samson *et al.*, 1999). Furthermore, the decrease in carotenoids content clarify the increased formation of ROS and the inability of PSII to be protected (Figure 1.18). On the other hand, if we look at the effect of IBX, the Chl *a*/*b* ratio is significantly increased at 0.05 mM and 0.20 mM, and this increase may be due to a defect in the structure of PSII antenna. The F_V/F_M ratio represents the conversion efficiency and capture of light energy, which is a good measure to evaluate the quantum yield of photochemistry of PSII (Mathur, S. et Jajoo, 2015). We found a gradual increase in F_V/F_M ratio (Table 1.6 B) compared to control indicating that IBX may influences the efficiency of PSII. Also, the ABS/RC ratio represents the density of active PSII reaction centers of Chl molecules and antenna size, and from the decreasing values of ABS/RC, it can be concluded that IBX does not cause any blockage of electron transfer from reaction center to the Quinone pool (Figure 1.20B). Furthermore, the V_J relative fluorescence of Q_A reduction showed a strong decreased especially at higher concentration of IBX compared to control, which could be explained by the fact that IBX does not have a negative effect on the relative fluorescence of Q_A reduction compared to the effect of CrO_3. Finally, PI_{ABS} is a sensitive index for the stress caused by metals and it is used as a global indicator of all photochemical reactions (Chen et Cheng, 2009). The results obtained (Table 1.6 B)

indicated that the primary photochemical reactions were little affected even with the treatment of high IBX content. It is important to affirm that no previous studies have been conducted on the effect of hypervalent iodine reagents on the photosynthesis processes in plants.

4.4.2 After exposed to DIB and $HgCl_2$

Results on Chl *a*/*b*, F_V/F_M, and ABS/RC ratios (Table 1.7 A) showed no changes remarked between treatments and control for DIB treatment. This might be attributed to the non-toxicity of DIB on photosynthesis, in other words, there was no blocking of electron transfer from PSII reaction center to the plastoquinone pool (Figure 1.21A). As well as for V_J and PI_{ABS} parameters, although the latter showed an increase at the two highest concentrations of DIB, but the increase was not significant compared to control. Hence, it may support an explanation that DIB has no toxic or negative effect on PSII activity, and the results on the formation of ROS (Figure 1.19) showed no increase compared to control. Nevertheless, Hg^{+2} caused a significant decrease in Chl *a*/*b* ratio under 2.5, 3.5, and 7 μM of $HgCl_2$ (Table 1.7 B). This reduction was due to the disturbance in the antenna structure of PSII. The significant change of parameters related to the fluorescence kinetic in the early phase has been reported for mercury (Asztalos *et al.*, 2012). For F_V/F_M ratio, we observed first a significant increase at lower concentration of $HgCl_2$, then a significant decrease at 5 μM of $HgCl_2$ when compared to control. This increase was attributed to the quantum efficiency reduction of PSII,

which referred to the toxicity of $HgCl_2$. Also, the content of Chl *a*, Chl *b*, and carotenoids decreased with the increasing mercury concentration (Figure 1.17), which was in agreement with previous studies (BaČKor et ZetikovA, 2003; Murthy *et al.*, 1995). The Chl *a*, Chl *b*, and carotenoids synthesis were influenced clearly under the stress of mercury, where the decrease of carotenoids did reduce the photochemistry and the oxygen evolution of PSII (Dankov *et al.*, 2009). Moreover, the ratio of ABS/RC was significantly increased at 2.5, 3, 5, and 7 µM, which indicated a decrease in the density of active PSII reaction centers. The decrease of F_M (Figure 1.21B) referred that the electron transport of PSII and PSI was inhibited by Hg^{+2}. On the other hand, V_J showed a significant increase at the last three concentrations due to the accumulation of reduced Q_A. For the last parameter PI_{ABS}, the results obtained showed a significant increase at 1 µM of $HgCl_2$ compared to control, and after it showed a significant decrease under the last two concentrations. This can be explained by the photochemical reactions that were affected by the toxicity of Hg^{+2}, which led to the decrease in the intensity of light that the plant could tolerate resulting in photodamage, as previously done on pea leaves (White *et al.*, 2011; Wodala *et al.*, 2012).

CONCLUSION

Our results showed the toxic effects of CrO_3 and $HgCl_2$ on *L. minor*, which occurred at different stages after the incubation in contaminated culture medium for 7 days. The metals were absorbed causing morphological changes such as chlorosis over time when the plants were exposed to low concentrations of 0.05 mM of CrO_3 and 1 μM of $HgCl_2$. The growth rate was inhibited dramatically by 40 % for CrO_3 and 50 % of $HgCl_2$. In addition, both metals Cr and Hg did accumulate in the plant tissues, which caused the production of ROS in cells and specifically in organelles.

It is most likely that the intracellular uptake of chromium and mercury was triggering a cellular oxidative stress, which may be due to the presence of free ionic form of Cr and Hg within cells. At tested concentrations of CrO_3 and $HgCl_2$, the increasing ROS did affect many biological macromolecules including pigments Chl *a*, Chl *b*, and carotenoids. The biosynthesis of these main pigments was disrupted, leading to a change in the composition of the antennae of photosystems (becoming smaller) and causing a decrease in absorbed energy quantity transferred to the Chl *a* pair of reaction center. The reduction in carotenoids content and their photoprotective ability indicated a defect in the light and dark phases of photosynthesis. The exposure to the highest

concentrations of CrO_3 and $HgCl_2$ did inhibit the growth of *L. minor* up to 80 %, explaining the sharp reduction in all parameters like the efficiency of active PSII reaction centers (ABS/RC). When *L. minor* was exposed to the same concentrations of hypervalent iodine reagents (DIB and IBX), the results showed a little inhibition of growth rate by 14 % for IBX and 9 % for DIB. These changes referred to the non-toxicity of organic iodine reagents. However, this does not mean that organic iodine reagents had no effect on the plants, as the photosynthetic pigments showed a considerable increase in content with the increasing IBX concentrations. This explains the drop in F_M transient, indicating the low efficiency of active PSII reaction centers. Moreover, the increase in the formation of ROS inside the cells was increased by 54 % and 72 % for the last higher concentration. This reinforces the hypothesis that IBX induced oxidative stress within plant cells. Whereas, DIB showed no change in the content of pigments, as well as growth rate. Even the results of ROS production and fluorescence parameters associated to PSII activity did not show any inhibition. It was necessary to emphasize that in the environment there is a gradual pollution, where mechanisms are placed according to the time of exposure. The concentrations tested in this work can lead in long term to large differences compared to the control, particularly since the time for studying the effects caused by CrO_3, $HgCl_2$, DIB, and IBX was 7 days.

The nature of plants enables it to put a certain mechanism to fight the stress resulting from exposure to pollutants if this exposure occurred in natural environment. Through this study, we can clearly conclude that the hypervalent iodine reagents showed no toxicity on the plant biological functions, physiological and morphological properties if we compare it to trace metals. There is no doubt that they are environmentally friendly compounds besides having strong oxidizing chemical properties. The *L. minor* plant is a biological indicator that is very sensitive to any change may occur due to pollution in aquatic environments. It has a potential for bioaccumulation that enables it to be used as a model to absorb metals from contaminated water. Many factories, including leather-tanning factories, release high amount of chromium through their effluents. In addition to the mercury released from fuel and raw material factories, they pose a real risk to water, agricultural, and animal resources destined for human or animal consumption.

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ANNEX A

SUPPLEMENTARY DATA

1- Statistical test of growth inhibition parameter for Cr treatment

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
0.05 0	41.7551	5.44611	10.84273	8.73607E-7	0.01	1	21.06207	62.44813
0.1 0	56.27788	5.44611	14.61392	0	0.01	1	35.58485	76.97091
0.1 0.05	14.52278	5.44611	3.77119	0.1196	0.01	0	-6.17025	35.21581
0.15 0	69.19455	5.44611	17.96805	3.27218E-8	0.01	1	48.50152	89.88758
0.15 0.05	27.43945	5.44611	7.12532	4.84589E-4	0.01	1	6.74642	48.13248
0.15 0.1	12.91667	5.44611	3.35413	0.20579	0.01	0	-7.77636	33.6097
0.2 0	78.22364	5.44611	20.31267	2.95711E-8	0.01	1	57.5306	98.91667
0.2 0.05	36.46854	5.44611	9.46994	8.55576E-6	0.01	1	15.7755	57.16157
0.2 0.1	21.94576	5.44611	5.69875	0.00577	0.01	1	1.25273	42.63879
0.2 0.15	9.02909	5.44611	2.34462	0.57067	0.01	0	-11.66395	29.72212
0.25 0	81.14041	5.44611	21.07008	2.8589E-8	0.01	1	60.44738	101.83344
0.25 0.05	39.38531	5.44611	10.22735	2.42021E-6	0.01	1	18.69228	60.07834
0.25 0.1	24.86253	5.44611	6.45616	0.00156	0.01	1	4.1695	45.55557
0.25 0.15	11.94586	5.44611	3.10203	0.27707	0.01	0	-8.74717	32.63889
0.25 0.2	2.91677	5.44611	0.75741	0.99408	0.01	0	-17.77626	23.60981

Sig equals 1 indicates that the means difference is significant at the 0.01 level.
Sig equals 0 indicates that the means difference is not significant at the 0.01 level.

Powers

2- Statistical test of growth inhibition parameter for IBX treatment

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
0.05 0	6.77654	2.14119	4.47577	0.04269	0.05	1	0.15612	13.39696
0.1 0	9.71477	2.14119	6.4164	0.00167	0.05	1	3.09434	16.33519
0.1 0.05	2.93823	2.14119	1.94064	0.74248	0.05	0	-3.6822	9.55865
0.15 0	10.43367	2.14119	6.89122	7.2987E-4	0.05	1	3.81324	17.05409
0.15 0.05	3.65713	2.14119	2.41546	0.53992	0.05	0	-2.9633	10.27755
0.15 0.1	0.7189	2.14119	0.47482	0.99936	0.05	0	-5.90152	7.33932
0.2 0	14.64924	2.14119	9.67552	6.06309E-6	0.05	1	8.02881	21.26966
0.2 0.05	7.8727	2.14119	5.19976	0.01336	0.05	1	1.25227	14.49312
0.2 0.1	4.93447	2.14119	3.25912	0.23088	0.05	0	-1.68595	11.55489
0.2 0.15	4.21557	2.14119	2.7843	0.38798	0.05	0	-2.40485	10.836
0.25 0	10.74361	2.14119	7.09593	5.10155E-4	0.05	1	4.12319	17.36403
0.25 0.05	3.96707	2.14119	2.62017	0.45326	0.05	0	-2.65335	10.58749
0.25 0.1	1.02884	2.14119	0.67953	0.99643	0.05	0	-5.59158	7.64926
0.25 0.15	0.30994	2.14119	0.20471	0.99999	0.05	0	-6.31048	6.93037
0.25 0.2	-3.90563	2.14119	2.57959	0.47006	0.05	0	-10.52605	2.71479

Sig equals 1 indicates that the means difference is significant at the 0.05 level.
Sig equals 0 indicates that the means difference is not significant at the 0.05 level.

3- Statistical test of growth inhibition parameter for Hg treatment

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
1 0	28.66699	3.21443	12.61225	3.26744E-8	0.01	1	16.45342	40.88056
2.5 0	49.70916	3.21443	21.86991	1.13727E-7	0.01	1	37.49559	61.92272
2.5 1	21.04216	3.21443	9.25765	1.22338E-5	0.01	1	8.8286	33.25573
3 0	58.15596	3.21443	25.58614	7.28929E-7	0.01	1	45.94239	70.36953
3 1	29.48897	3.21443	12.97389	8.24472E-9	0.01	1	17.2754	41.70253
3 2.5	8.4468	3.21443	3.71623	0.12889	0.01	0	-3.76677	20.66037
5 0	66.85024	3.21443	29.41125	1.83884E-8	0.01	1	54.63667	79.06381
5 1	38.18325	3.21443	16.799	3.44763E-8	0.01	1	25.96968	50.39682
5 2.5	17.14108	3.21443	7.54135	2.34189E-4	0.01	1	4.92752	29.35465
5 3	8.69428	3.21443	3.82511	0.11104	0.01	0	-3.51928	20.90785
7 0	82.82918	3.21443	36.44131	1.04449E-8	0.01	1	70.61562	95.04275
7 1	54.16219	3.21443	23.82905	1.22963E-7	0.01	1	41.94862	66.37576
7 2.5	33.12003	3.21443	14.5714	0	0.01	1	20.90646	45.3336
7 3	24.67323	3.21443	10.85517	8.55765E-7	0.01	1	12.45966	36.8868
7 5	15.97894	3.21443	7.03005	5.72482E-4	0.01	1	3.76538	28.19251

Sig equals 1 indicates that the means difference is significant at the 0.01 level.
Sig equals 0 indicates that the means difference is not significant at the 0.01 level.

4- Statistical test of growth inhibition parameter for DIB treatment

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
1 0	4.22101	1.59688	3.73816	0.12511	0.05	0	-0.71645	9.15846
2.5 0	4.32079	1.59688	3.82653	0.11082	0.05	0	-0.61667	9.25824
2.5 1	0.09978	1.59688	0.08837	1	0.05	0	-4.83767	5.03723
3 0	9.29682	1.59688	8.23335	7.03896E-5	0.05	1	4.35936	14.23427
3 1	5.07581	1.59688	4.49518	0.04143	0.05	1	0.13836	10.01326
3 2.5	4.97603	1.59688	4.40682	0.04746	0.05	1	0.03858	9.91348
5 0	5.84657	1.59688	5.17778	0.01385	0.05	1	0.90912	10.78403
5 1	1.62557	1.59688	1.43962	0.90733	0.05	0	-3.31188	6.56302
5 2.5	1.52579	1.59688	1.35125	0.92739	0.05	0	-3.41166	6.46324
5 3	-3.45024	1.59688	3.05556	0.29186	0.05	0	-8.38769	1.48721
7 0	9.95173	1.59688	8.81334	2.60044E-5	0.05	1	5.01428	14.88918
7 1	5.73072	1.59688	5.07518	0.0164	0.05	1	0.79327	10.66817
7 2.5	5.63094	1.59688	4.98682	0.01895	0.05	1	0.69349	10.5684
7 3	0.65491	1.59688	0.58	0.99831	0.05	0	-4.28254	5.59237
7 5	4.10515	1.59688	3.63556	0.14359	0.05	0	-0.8323	9.04261

Sig equals 1 indicates that the means difference is significant at the 0.05 level.
Sig equals 0 indicates that the means difference is not significant at the 0.05 level.

5- Statistical test of Chl *a* content for Cr treatment

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
0.05 0	-0.35083	0.04871	10.18614	2.59135E-6	0.01	1	-0.5359	-0.16576
0.1 0	-0.35253	0.04871	10.23545	2.38794E-6	0.01	1	-0.5376	-0.16746
0.1 0.05	-0.0017	0.04871	0.04931	1	0.01	0	-0.18677	0.18337
0.15 0	-0.29071	0.04871	8.44054	4.92494E-5	0.01	1	-0.47578	-0.10564
0.15 0.05	0.06012	0.04871	1.7456	0.81589	0.01	0	-0.12495	0.24519
0.15 0.1	0.06182	0.04871	1.79491	0.79827	0.01	0	-0.12325	0.24689
0.2 0	-0.38928	0.04871	11.30253	4.05671E-7	0.01	1	-0.57435	-0.20421
0.2 0.05	-0.03845	0.04871	1.11639	0.96668	0.01	0	-0.22352	0.14662
0.2 0.1	-0.03675	0.04871	1.06708	0.97251	0.01	0	-0.22182	0.14832
0.2 0.15	-0.09857	0.04871	2.86199	0.35883	0.01	0	-0.28364	0.0865
0.25 0	-0.39735	0.04871	11.53691	2.72631E-7	0.01	1	-0.58242	-0.21228
0.25 0.05	-0.04652	0.04871	1.35077	0.92749	0.01	0	-0.23159	0.13855
0.25 0.1	-0.04482	0.04871	1.30146	0.93739	0.01	0	-0.2299	0.14025
0.25 0.15	-0.10664	0.04871	3.09637	0.27885	0.01	0	-0.29172	0.07843
0.25 0.2	-0.00807	0.04871	0.23438	0.99998	0.01	0	-0.19314	0.177

Sig equals 1 indicates that the means difference is significant at the 0.01 level.
Sig equals 0 indicates that the means difference is not significant at the 0.01 level.

6- Statistical test of Chl *b* content for Cr treatment

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
0.05 0	-0.0679	0.02068	4.64252	0.03291	0.05	1	-0.13185	-0.00395
0.1 0	-0.04824	0.02068	3.29827	0.22028	0.05	0	-0.11219	0.01571
0.1 0.05	0.01966	0.02068	1.34425	0.92885	0.05	0	-0.04429	0.08361
0.15 0	-0.02714	0.02068	1.85557	0.77568	0.05	0	-0.09109	0.03681
0.15 0.05	0.04076	0.02068	2.78695	0.38697	0.05	0	-0.02319	0.10471
0.15 0.1	0.0211	0.02068	1.4427	0.90658	0.05	0	-0.04285	0.08505
0.2 0	-0.07151	0.02068	4.88953	0.02219	0.05	1	-0.13546	-0.00756
0.2 0.05	-0.00361	0.02068	0.247	0.99997	0.05	0	-0.06756	0.06034
0.2 0.1	-0.02327	0.02068	1.59125	0.86611	0.05	0	-0.08722	0.04068
0.2 0.15	-0.04437	0.02068	3.03395	0.29892	0.05	0	-0.10832	0.01958
0.25 0	-0.06998	0.02068	4.7847	0.02627	0.05	1	-0.13392	-0.00603
0.25 0.05	-0.00208	0.02068	0.14218	1	0.05	0	-0.06603	0.06187
0.25 0.1	-0.02174	0.02068	1.48642	0.89551	0.05	0	-0.08569	0.04221
0.25 0.15	-0.04284	0.02068	2.92913	0.33465	0.05	0	-0.10679	0.02111
0.25 0.2	0.00153	0.02068	0.10483	1	0.05	0	-0.06242	0.06548

Sig equals 1 indicates that the means difference is significant at the 0.05 level.
Sig equals 0 indicates that the means difference is not significant at the 0.05 level.

7- Statistical test of carotenoids content for Cr treatment

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
0.05 0	-0.06286	0.02263	3.92821	0.09612	0.05	0	-0.13284	0.00711
0.1 0	-0.05293	0.02263	3.30769	0.21778	0.05	0	-0.12291	0.01704
0.1 0.05	0.00993	0.02263	0.62052	0.99768	0.05	0	-0.06005	0.07991
0.15 0	-0.018	0.02263	1.12473	0.96561	0.05	0	-0.08798	0.05198
0.15 0.05	0.04487	0.02263	2.80348	0.38067	0.05	0	-0.02511	0.11484
0.15 0.1	0.03493	0.02263	2.18296	0.64101	0.05	0	-0.03504	0.10491
0.2 0	-0.06351	0.02263	3.96853	0.09077	0.05	0	-0.13349	0.00647
0.2 0.05	-6.45255E-4	0.02263	0.04032	1	0.05	0	-0.07062	0.06933
0.2 0.1	-0.01058	0.02263	0.66084	0.99687	0.05	0	-0.08055	0.0594
0.2 0.15	-0.04551	0.02263	2.8438	0.36554	0.05	0	-0.11549	0.02447
0.25 0	-0.06291	0.02263	3.93084	0.09576	0.05	0	-0.13288	0.00707
0.25 0.05	-4.20487E-5	0.02263	0.00263	1	0.05	0	-0.07002	0.06994
0.25 0.1	-0.00997	0.02263	0.62315	0.99763	0.05	0	-0.07995	0.06
0.25 0.15	-0.04491	0.02263	2.80611	0.37968	0.05	0	-0.11488	0.02507
0.25 0.2	6.03206E-4	0.02263	0.03769	1	0.05	0	-0.06937	0.07058

Sig equals 1 indicates that the means difference is significant at the 0.05 level.
Sig equals 0 indicates that the means difference is not significant at the 0.05 level.

8- Statistical test of Chl *a* content for IBX treatment

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
0.05 0	0.27649	0.07784	5.0233	0.01786	0.05	1	0.03581	0.51716
0.1 0	0.17201	0.07784	3.12515	0.2699	0.05	0	-0.06866	0.41269
0.1 0.05	-0.10448	0.07784	1.89815	0.75926	0.05	0	-0.34515	0.1362
0.15 0	0.23085	0.07784	4.1941	0.06539	0.05	0	-0.00983	0.47152
0.15 0.05	-0.04564	0.07784	0.8292	0.99102	0.05	0	-0.28631	0.19503
0.15 0.1	0.05884	0.07784	1.06895	0.9723	0.05	0	-0.18184	0.29951
0.2 0	0.30722	0.07784	5.58169	0.00704	0.05	1	0.06655	0.5479
0.2 0.05	0.03073	0.07784	0.55838	0.99859	0.05	0	-0.20994	0.27141
0.2 0.1	0.13521	0.07784	2.45653	0.5222	0.05	0	-0.10546	0.37588
0.2 0.15	0.07637	0.07784	1.38758	0.9195	0.05	0	-0.1643	0.31705
0.25 0	0.2505	0.07784	4.5512	0.03798	0.05	1	0.00983	0.49118
0.25 0.05	-0.02599	0.07784	0.47211	0.99937	0.05	0	-0.26666	0.21469
0.25 0.1	0.07849	0.07784	1.42605	0.9106	0.05	0	-0.16218	0.31917
0.25 0.15	0.01965	0.07784	0.3571	0.99984	0.05	0	-0.22102	0.26033
0.25 0.2	-0.05672	0.07784	1.03049	0.97635	0.05	0	-0.29739	0.18396

Sig equals 1 indicates that the means difference is significant at the 0.05 level.
Sig equals 0 indicates that the means difference is not significant at the 0.05 level.

9- Statistical test of Chl *b* content for IBX treatment

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
0.05 0	0.08752	0.02983	4.14977	0.06981	0.05	0	-0.0047	0.17974
0.1 0	0.04968	0.02983	2.35551	0.56593	0.05	0	-0.04254	0.1419
0.1 0.05	-0.03784	0.02983	1.79426	0.79851	0.05	0	-0.13006	0.05438
0.15 0	0.06762	0.02983	3.2061	0.24581	0.05	0	-0.0246	0.15984
0.15 0.05	-0.0199	0.02983	0.94367	0.98393	0.05	0	-0.11212	0.07232
0.15 0.1	0.01794	0.02983	0.85059	0.98992	0.05	0	-0.07428	0.11016
0.2 0	0.08962	0.02983	4.24927	0.06023	0.05	0	-0.0026	0.18184
0.2 0.05	0.0021	0.02983	0.0995	1	0.05	0	-0.09012	0.09432
0.2 0.1	0.03994	0.02983	1.89376	0.76098	0.05	0	-0.05228	0.13216
0.2 0.15	0.022	0.02983	1.04317	0.97507	0.05	0	-0.07022	0.11422
0.25 0	0.07254	0.02983	3.43935	0.18508	0.05	0	-0.01968	0.16476
0.25 0.05	-0.01498	0.02983	0.71042	0.9956	0.05	0	-0.1072	0.07724
0.25 0.1	0.02286	0.02983	1.08384	0.97061	0.05	0	-0.06936	0.11508
0.25 0.15	0.00492	0.02983	0.23325	0.99998	0.05	0	-0.0873	0.09714
0.25 0.2	-0.01708	0.02983	0.80993	0.99193	0.05	0	-0.1093	0.07514

Sig equals 1 indicates that the means difference is significant at the 0.05 level.
Sig equals 0 indicates that the means difference is not significant at the 0.05 level.

10- Statistical test of carotenoids content for IBX treatment

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
0.05 0	0.03588	0.0254	1.99722	0.71956	0.05	0	-0.04267	0.11442
0.1 0	0.02699	0.0254	1.50263	0.89123	0.05	0	-0.05155	0.10554
0.1 0.05	-0.00888	0.0254	0.49459	0.99922	0.05	0	-0.08743	0.06966
0.15 0	0.05969	0.0254	3.32311	0.21374	0.05	0	-0.01885	0.13824
0.15 0.05	0.02382	0.0254	1.32589	0.9326	0.05	0	-0.05473	0.10236
0.15 0.1	0.0327	0.0254	1.82048	0.78887	0.05	0	-0.04584	0.11125
0.2 0	0.05658	0.0254	3.14987	0.26238	0.05	0	-0.02196	0.13513
0.2 0.05	0.0207	0.0254	1.15264	0.96188	0.05	0	-0.05784	0.09925
0.2 0.1	0.02959	0.0254	1.64723	0.84881	0.05	0	-0.04896	0.10814
0.2 0.15	-0.00311	0.0254	0.17325	1	0.05	0	-0.08166	0.07543
0.25 0	0.04701	0.0254	2.61701	0.45456	0.05	0	-0.03154	0.12556
0.25 0.05	0.01113	0.0254	0.61978	0.99769	0.05	0	-0.06741	0.08968
0.25 0.1	0.02002	0.0254	1.11437	0.96693	0.05	0	-0.05853	0.09856
0.25 0.15	-0.01268	0.0254	0.70611	0.99573	0.05	0	-0.09123	0.06586
0.25 0.2	-0.00957	0.0254	0.53286	0.99888	0.05	0	-0.08812	0.06897

Sig equals 1 indicates that the means difference is significant at the 0.05 level.
Sig equals 0 indicates that the means difference is not significant at the 0.05 level.

11- Statistical test of ROS production for Cr treatment

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
0.05 0	209.51814	90.38433	3.27826	0.23725	0.05	0	-77.72642	496.7627
0.1 0	365.40161	90.38433	5.71732	0.00843	0.05	1	78.15705	652.64617
0.1 0.05	155.88347	90.38433	2.43906	0.53406	0.05	0	-131.36109	443.12803
0.15 0	486.59402	90.38433	7.61357	4.99496E-4	0.05	1	199.34946	773.83858
0.15 0.05	277.07588	90.38433	4.33531	0.06234	0.05	0	-10.16868	564.32044
0.15 0.1	121.19241	90.38433	1.89626	0.75947	0.05	0	-166.05215	408.43697
0.2 0	487.85546	90.38433	7.63331	4.85285E-4	0.05	1	200.6109	775.10002
0.2 0.05	278.33733	90.38433	4.35505	0.06067	0.05	0	-8.90724	565.58189
0.2 0.1	122.45386	90.38433	1.91599	0.75183	0.05	0	-164.7907	409.69842
0.2 0.15	1.26145	90.38433	0.01974	1	0.05	0	-285.98311	288.50601
0.25 0	518.84734	90.38433	8.11823	2.39734E-4	0.05	1	231.60278	806.0919
0.25 0.05	309.3292	90.38433	4.83997	0.03062	0.05	1	22.08464	596.57376
0.25 0.1	153.44573	90.38433	2.40091	0.55012	0.05	0	-133.79883	440.6903
0.25 0.15	32.25333	90.38433	0.50466	0.9991	0.05	0	-254.99124	319.49789
0.25 0.2	30.99188	90.38433	0.48492	0.99926	0.05	0	-256.25268	318.23644

Sig equals 1 indicates that the means difference is significant at the 0.05 level.
Sig equals 0 indicates that the means difference is not significant at the 0.05 level.

12- Statistical test of ROS production for IBX treatment

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
0.05 0	34.71312	51.63198	0.9508	0.98286	0.05	0	-129.37513	198.80137
0.1 0	55.84104	51.63198	1.5295	0.88242	0.05	0	-108.24721	219.92929
0.1 0.05	21.12792	51.63198	0.5787	0.99826	0.05	0	-142.96033	185.21617
0.15 0	89.82241	51.63198	2.46026	0.52518	0.05	0	-74.26585	253.91066
0.15 0.05	55.10929	51.63198	1.50946	0.8879	0.05	0	-108.97897	219.19754
0.15 0.1	33.98137	51.63198	0.93076	0.9844	0.05	0	-130.10689	198.06962
0.2 0	170.46576	51.63198	4.6691	0.03909	0.05	1	6.37751	334.55401
0.2 0.05	135.75264	51.63198	3.7183	0.14048	0.05	0	-28.33561	299.84089
0.2 0.1	114.62472	51.63198	3.1396	0.27643	0.05	0	-49.46353	278.71297
0.2 0.15	80.64335	51.63198	2.20884	0.63169	0.05	0	-83.4449	244.73161
0.25 0	188.98179	51.63198	5.17626	0.01879	0.05	1	24.89353	353.07004
0.25 0.05	154.26866	51.63198	4.22546	0.07242	0.05	0	-9.81959	318.35692
0.25 0.1	133.14075	51.63198	3.64676	0.15354	0.05	0	-30.94751	297.229
0.25 0.15	99.15938	51.63198	2.716	0.42198	0.05	0	-64.92888	263.24763
0.25 0.2	18.51602	51.63198	0.50716	0.99908	0.05	0	-145.57223	182.60428

Sig equals 1 indicates that the means difference is significant at the 0.05 level.
Sig equals 0 indicates that the means difference is not significant at the 0.05 level.

13- Statistical test of Chl *a* content for Hg treatment

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
1 0	-0.00654	0.04195	0.22032	0.99999	0.01	0	-0.16593	0.15285
2.5 0	-0.1663	0.04195	5.60642	0.00675	0.01	1	-0.32569	-0.00691
2.5 1	-0.15977	0.04195	5.3861	0.00979	0.01	1	-0.31916	-3.75245E-4
3 0	-0.27369	0.04195	9.22693	1.28853E-5	0.01	1	-0.43308	-0.1143
3 1	-0.26716	0.04195	9.00661	1.87146E-5	0.01	1	-0.42655	-0.10777
3 2.5	-0.10739	0.04195	3.62051	0.14648	0.01	0	-0.26678	0.052
5 0	-0.37391	0.04195	12.60562	3.32561E-8	0.01	1	-0.53331	-0.21452
5 1	-0.36738	0.04195	12.3853	5.65168E-8	0.01	1	-0.52677	-0.20799
5 2.5	-0.20761	0.04195	6.9992	6.0423E-4	0.01	1	-0.367	-0.04822
5 3	-0.10022	0.04195	3.37869	0.19965	0.01	0	-0.25961	0.05917
7 0	-0.4113	0.04195	13.86581	0	0.01	1	-0.57069	-0.25191
7 1	-0.40476	0.04195	13.64549	0	0.01	1	-0.56415	-0.24537
7 2.5	-0.24499	0.04195	8.25939	6.72936E-5	0.01	1	-0.40438	-0.0856
7 3	-0.1376	0.04195	4.63888	0.0331	0.01	0	-0.29699	0.02179
7 5	-0.03738	0.04195	1.26019	0.94498	0.01	0	-0.19677	0.12201

Sig equals 1 indicates that the means difference is significant at the 0.01 level.
Sig equals 0 indicates that the means difference is not significant at the 0.01 level.

14- Statistical test of Chl *b* content for Hg treatment

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
1 0	-0.01136	0.01784	0.90017	0.98698	0.01	0	-0.07914	0.05643
2.5 0	-0.05511	0.01784	4.36853	0.05031	0.01	0	-0.12289	0.01268
2.5 1	-0.04375	0.01784	3.46836	0.17842	0.01	0	-0.11153	0.02403
3 0	-0.0927	0.01784	7.34901	3.27701E-4	0.01	1	-0.16048	-0.02492
3 1	-0.08135	0.01784	6.44884	0.00158	0.01	1	-0.14913	-0.01357
3 2.5	-0.0376	0.01784	2.98048	0.31683	0.01	0	-0.10538	0.03019
5 0	-0.10765	0.01784	8.53434	4.19185E-5	0.01	1	-0.17544	-0.03987
5 1	-0.0963	0.01784	7.63417	1.99182E-4	0.01	1	-0.16408	-0.02852
5 2.5	-0.05255	0.01784	4.16581	0.06818	0.01	0	-0.12033	0.01523
5 3	-0.01495	0.01784	1.18533	0.95718	0.01	0	-0.08273	0.05283
7 0	-0.13366	0.01784	10.59592	1.31476E-6	0.01	1	-0.20144	-0.06588
7 1	-0.1223	0.01784	9.69575	5.86148E-6	0.01	1	-0.19009	-0.05452
7 2.5	-0.07855	0.01784	6.22739	0.00232	0.01	1	-0.14634	-0.01077
7 3	-0.04096	0.01784	3.24691	0.23425	0.01	0	-0.10874	0.02682
7 5	-0.02601	0.01784	2.06158	0.69283	0.01	0	-0.09379	0.04178

Sig equals 1 indicates that the means difference is significant at the 0.01 level.
Sig equals 0 indicates that the means difference is not significant at the 0.01 level.

15- Statistical test of carotenoids content for Hg treatment

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
1 0	-0.00821	0.01147	1.01205	0.97814	0.05	0	-0.04366	0.02725
2.5 0	-0.02319	0.01147	2.86017	0.3595	0.05	0	-0.05864	0.01226
2.5 1	-0.01498	0.01147	1.84812	0.7785	0.05	0	-0.05044	0.02047
3 0	-0.03399	0.01147	4.19292	0.0655	0.05	0	-0.06945	0.00146
3 1	-0.02579	0.01147	3.18088	0.25315	0.05	0	-0.06124	0.00966
3 2.5	-0.01081	0.01147	1.33275	0.93121	0.05	0	-0.04626	0.02465
5 0	-0.06625	0.01147	8.17191	7.82745E-5	0.05	1	-0.10171	-0.0308
5 1	-0.05805	0.01147	7.15986	4.56171E-4	0.05	1	-0.0935	-0.0226
5 2.5	-0.04307	0.01147	5.31174	0.01109	0.05	1	-0.07852	-0.00761
5 3	-0.03226	0.01147	3.97899	0.08943	0.05	0	-0.06771	0.00319
7 0	-0.07636	0.01147	9.41846	9.32936E-6	0.05	1	-0.11181	-0.04091
7 1	-0.06816	0.01147	8.40642	5.2228E-5	0.05	1	-0.10361	-0.0327
7 2.5	-0.05317	0.01147	6.55829	0.00131	0.05	1	-0.08862	-0.01772
7 3	-0.04237	0.01147	5.22554	0.0128	0.05	1	-0.07782	-0.00691
7 5	-0.01011	0.01147	1.24655	0.94735	0.05	0	-0.04556	0.02535

Sig equals 1 indicates that the means difference is significant at the 0.05 level.
Sig equals 0 indicates that the means difference is not significant at the 0.05 level.

16- Statistical test of Chl *a* content for DIB treatment

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
1 0	-0.05763	0.05727	1.42312	0.9113	0.05	0	-0.23469	0.11944
2.5 0	-0.09436	0.05727	2.33036	0.57689	0.05	0	-0.27143	0.0827
2.5 1	-0.03674	0.05727	0.90724	0.98651	0.05	0	-0.2138	0.14033
3 0	-0.08881	0.05727	2.19309	0.63663	0.05	0	-0.26587	0.08826
3 1	-0.03118	0.05727	0.76997	0.99361	0.05	0	-0.20824	0.14589
3 2.5	0.00556	0.05727	0.13727	1	0.05	0	-0.17151	0.18262
5 0	-0.08191	0.05727	2.02279	0.70902	0.05	0	-0.25897	0.09515
5 1	-0.02428	0.05727	0.59967	0.99802	0.05	0	-0.20135	0.15278
5 2.5	0.01245	0.05727	0.30757	0.99992	0.05	0	-0.16461	0.18952
5 3	0.0069	0.05727	0.1703	1	0.05	0	-0.17017	0.18396
7 0	-0.05025	0.05727	1.24099	0.9483	0.05	0	-0.22732	0.12681
7 1	0.00737	0.05727	0.18213	0.99999	0.05	0	-0.16969	0.18444
7 2.5	0.04411	0.05727	1.08937	0.96997	0.05	0	-0.13295	0.22118
7 3	0.03855	0.05727	0.9521	0.98328	0.05	0	-0.13851	0.21562
7 5	0.03166	0.05727	0.7818	0.99314	0.05	0	-0.14541	0.20872

Sig equals 1 indicates that the means difference is significant at the 0.05 level.
Sig equals 0 indicates that the means difference is not significant at the 0.05 level.

17- Statistical test of Chl b content for DIB treatment

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
1 0	-0.01946	0.01829	1.50494	0.89061	0.05	0	-0.07601	0.03709
2.5 0	-0.02995	0.01829	2.31541	0.5834	0.05	0	-0.0865	0.02661
2.5 1	-0.01048	0.01829	0.81047	0.99191	0.05	0	-0.06703	0.04607
3 0	-0.02901	0.01829	2.2428	0.61505	0.05	0	-0.08556	0.02755
3 1	-0.00954	0.01829	0.73786	0.99475	0.05	0	-0.06609	0.04701
3 2.5	9.39021E-4	0.01829	0.07261	1	0.05	0	-0.05561	0.05749
5 0	-0.02645	0.01829	2.04484	0.69984	0.05	0	-0.083	0.03011
5 1	-0.00698	0.01829	0.5399	0.9988	0.05	0	-0.06353	0.04957
5 2.5	0.0035	0.01829	0.27057	0.99996	0.05	0	-0.05305	0.06005
5 3	0.00256	0.01829	0.19797	0.99999	0.05	0	-0.05399	0.05911
7 0	-0.01533	0.01829	1.18513	0.95721	0.05	0	-0.07188	0.04122
7 1	0.00414	0.01829	0.31981	0.99991	0.05	0	-0.05242	0.06069
7 2.5	0.01462	0.01829	1.13028	0.96489	0.05	0	-0.04193	0.07117
7 3	0.01368	0.01829	1.05767	0.97354	0.05	0	-0.04287	0.07023
7 5	0.01112	0.01829	0.85971	0.98942	0.05	0	-0.04543	0.06767

Sig equals 1 indicates that the means difference is significant at the 0.05 level.
Sig equals 0 indicates that the means difference is not significant at the 0.05 level.

18- Statistical test of carotenoids content for DIB treatment

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
1 0	-0.01996	0.01844	1.53085	0.88354	0.05	0	-0.07697	0.03705
2.5 0	-0.03214	0.01844	2.46545	0.51838	0.05	0	-0.08915	0.02487
2.5 1	-0.01218	0.01844	0.9346	0.9846	0.05	0	-0.06919	0.04482
3 0	-0.02821	0.01844	2.1638	0.64928	0.05	0	-0.08522	0.0288
3 1	-0.00825	0.01844	0.63295	0.99745	0.05	0	-0.06526	0.04876
3 2.5	0.00393	0.01844	0.30165	0.99993	0.05	0	-0.05308	0.06094
5 0	-0.0282	0.01844	2.1629	0.64967	0.05	0	-0.08521	0.02881
5 1	-0.00824	0.01844	0.63205	0.99746	0.05	0	-0.06525	0.04877
5 2.5	0.00394	0.01844	0.30255	0.99993	0.05	0	-0.05306	0.06095
5 3	1.17337E-5	0.01844	8.99999E-4	1	0.05	0	-0.057	0.05702
7 0	-0.01765	0.01844	1.35366	0.92688	0.05	0	-0.07466	0.03936
7 1	0.00231	0.01844	0.17719	0.99999	0.05	0	-0.0547	0.05932
7 2.5	0.01449	0.01844	1.11179	0.96725	0.05	0	-0.04251	0.0715
7 3	0.01056	0.01844	0.81014	0.99192	0.05	0	-0.04645	0.06757
7 5	0.01055	0.01844	0.80924	0.99196	0.05	0	-0.04646	0.06756

Sig equals 1 indicates that the means difference is significant at the 0.05 level.
Sig equals 0 indicates that the means difference is not significant at the 0.05 level.

19- Statistical test of ROS production for Hg treatment

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
1 0	152.43929	163.19294	1.32102	0.93227	0.05	0	-366.19358	671.07215
2.5 0	396.63209	163.19294	3.43717	0.19761	0.05	0	-122.00078	915.26496
2.5 1	244.1928	163.19294	2.11615	0.67075	0.05	0	-274.44007	762.82567
3 0	503.30743	163.19294	4.36161	0.06012	0.05	0	-15.32544	1021.94029
3 1	350.86814	163.19294	3.04059	0.30705	0.05	0	-167.76473	869.50101
3 2.5	106.67534	163.19294	0.92444	0.98487	0.05	0	-411.95753	625.30821
5 0	686.32059	163.19294	5.94759	0.00598	0.05	1	167.68772	1204.95346
5 1	533.8813	163.19294	4.62656	0.04152	0.05	1	15.24844	1052.51417
5 2.5	289.6885	163.19294	2.51041	0.50432	0.05	0	-228.94436	808.32137
5 3	183.01316	163.19294	1.58597	0.86621	0.05	0	-335.6197	701.64603
7 0	934.06425	163.19294	8.09451	2.48085E-4	0.05	1	415.43138	1452.69711
7 1	781.62496	163.19294	6.77348	0.00173	0.05	1	262.99209	1300.25782
7 2.5	537.43216	163.19294	4.65733	0.03975	0.05	1	18.79929	1056.06502
7 3	430.75682	163.19294	3.7329	0.13794	0.05	0	-87.87605	949.38968
7 5	247.74365	163.19294	2.14692	0.65785	0.05	0	-270.88921	766.37652

Sig equals 1 indicates that the means difference is significant at the 0.05 level.
Sig equals 0 indicates that the means difference is not significant at the 0.05 level.

20- Statistical test of ROS production for DIB treatment

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
1 0	14.40199	20.47881	0.99456	0.97913	0.05	0	-50.6804	79.48437
2.5 0	5.49058	20.47881	0.37917	0.99978	0.05	0	-59.59181	70.57297
2.5 1	-8.9114	20.47881	0.6154	0.99768	0.05	0	-73.99379	56.17098
3 0	3.5955	20.47881	0.2483	0.99997	0.05	0	-61.48689	68.67788
3 1	-10.80649	20.47881	0.74627	0.99427	0.05	0	-75.88888	54.2759
3 2.5	-1.89509	20.47881	0.13087	1	0.05	0	-66.97747	63.1873
5 0	-5.47884	20.47881	0.37835	0.99978	0.05	0	-70.56123	59.60355
5 1	-19.88083	20.47881	1.37292	0.92136	0.05	0	-84.96321	45.20156
5 2.5	-10.96942	20.47881	0.75752	0.99386	0.05	0	-76.05181	54.11297
5 3	-9.07434	20.47881	0.62665	0.99747	0.05	0	-74.15672	56.00805
7 0	2.21951	20.47881	0.15327	1	0.05	0	-62.86288	67.3019
7 1	-12.18247	20.47881	0.84129	0.99008	0.05	0	-77.26486	52.89991
7 2.5	-3.27107	20.47881	0.22589	0.99998	0.05	0	-68.35346	61.81132
7 3	-1.37599	20.47881	0.09502	1	0.05	0	-66.45837	63.7064
7 5	7.69835	20.47881	0.53163	0.99884	0.05	0	-57.38404	72.78074

Sig equals 1 indicates that the means difference is significant at the 0.05 level.
Sig equals 0 indicates that the means difference is not significant at the 0.05 level.

21- Statistical test of total Chlorophyll (*Chl a/b*) for Cr treatment

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
5 0	-0.20936	0.03445	8.59458	3.7803E-5	0.01	1	-0.34026	-0.07847
10 0	-0.20038	0.03445	8.22592	7.12988E-5	0.01	1	-0.33128	-0.06949
10 5	0.00898	0.03445	0.36866	0.99981	0.01	0	-0.12192	0.13988
15 0	-0.15892	0.03445	6.52395	0.00139	0.01	1	-0.28982	-0.02803
15 5	0.05044	0.03445	2.07063	0.68902	0.01	0	-0.08046	0.18134
15 10	0.04146	0.03445	1.70196	0.83087	0.01	0	-0.08944	0.17236
20 0	-0.23039	0.03445	9.45795	8.73003E-6	0.01	1	-0.36129	-0.0995
20 5	-0.02103	0.03445	0.86337	0.98921	0.01	0	-0.15193	0.10986
20 10	-0.03001	0.03445	1.23203	0.94981	0.01	0	-0.16091	0.10088
20 15	-0.07147	0.03445	2.93399	0.33294	0.01	0	-0.20237	0.05942
25 0	-0.23366	0.03445	9.59217	6.97088E-6	0.01	1	-0.36456	-0.10277
25 5	-0.0243	0.03445	0.99759	0.97947	0.01	0	-0.1552	0.10659
25 10	-0.03328	0.03445	1.36625	0.92419	0.01	0	-0.16418	0.09761
25 15	-0.07474	0.03445	3.06822	0.28779	0.01	0	-0.20564	0.05615
25 20	-0.00327	0.03445	0.13422	1	0.01	0	-0.13417	0.12763

Sig equals 1 indicates that the means difference is significant at the 0.01 level.
Sig equals 0 indicates that the means difference is not significant at the 0.01 level.

22- Statistical test of total Chlorophyll (*Chl a/b*) for IBX treatment

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
5 0	0.182	0.05366	4.79685	0.02576	0.05	1	0.01609	0.34791
10 0	0.11084	0.05366	2.9214	0.33739	0.05	0	-0.05506	0.27675
10 5	-0.07116	0.05366	1.87545	0.76807	0.05	0	-0.23707	0.09475
15 0	0.14923	0.05366	3.93314	0.09545	0.05	0	-0.01668	0.31514
15 5	-0.03277	0.05366	0.86371	0.98919	0.05	0	-0.19868	0.13314
15 10	0.03839	0.05366	1.01174	0.97817	0.05	0	-0.12752	0.2043
20 0	0.19842	0.05366	5.22951	0.01271	0.05	1	0.03251	0.36433
20 5	0.01642	0.05366	0.43266	0.99959	0.05	0	-0.14949	0.18233
20 10	0.08758	0.05366	2.30811	0.58659	0.05	0	-0.07833	0.25348
20 15	0.04919	0.05366	1.29637	0.93836	0.05	0	-0.11672	0.2151
25 0	0.16152	0.05366	4.25697	0.05954	0.05	0	-0.00439	0.32743
25 5	-0.02048	0.05366	0.53987	0.9988	0.05	0	-0.18639	0.14542
25 10	0.05067	0.05366	1.33557	0.93064	0.05	0	-0.11523	0.21658
25 15	0.01229	0.05366	0.32384	0.9999	0.05	0	-0.15362	0.1782
25 20	-0.0369	0.05366	0.97254	0.98164	0.05	0	-0.20281	0.12901

Sig equals 1 indicates that the means difference is significant at the 0.05 level.
Sig equals 0 indicates that the means difference is not significant at the 0.05 level.

23- Statistical test of total Chlorophyll (*Chl a/b*) for Hg treatment

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
1 0	-0.00895	0.02953	0.42833	0.99961	0.05	0	-0.10026	0.08237
2.5 0	-0.1107	0.02953	5.30086	0.01129	0.05	1	-0.20202	-0.01938
2.5 1	-0.10176	0.02953	4.87253	0.02281	0.05	1	-0.19308	-0.01044
3 0	-0.1832	0.02953	8.77218	2.78971E-5	0.05	1	-0.27452	-0.09188
3 1	-0.17425	0.02953	8.34385	5.81707E-5	0.05	1	-0.26557	-0.08293
3 2.5	-0.0725	0.02953	3.47132	0.17775	0.05	0	-0.16381	0.01882
5 0	-0.24078	0.02953	11.52961	2.76053E-7	0.05	1	-0.3321	-0.14947
5 1	-0.23184	0.02953	11.10129	5.68311E-7	0.05	1	-0.32316	-0.14052
5 2.5	-0.13008	0.02953	6.22875	0.00232	0.05	1	-0.2214	-0.03876
5 3	-0.05759	0.02953	2.75743	0.39834	0.05	0	-0.1489	0.03373
7 0	-0.27248	0.02953	13.04718	4.0756E-9	0.05	1	-0.3638	-0.18116
7 1	-0.26353	0.02953	12.61885	3.21012E-8	0.05	1	-0.35485	-0.17221
7 2.5	-0.16177	0.02953	7.74632	1.63828E-4	0.05	1	-0.25309	-0.07046
7 3	-0.08928	0.02953	4.275	0.05795	0.05	0	-0.1806	0.00204
7 5	-0.03169	0.02953	1.51757	0.8872	0.05	0	-0.12301	0.05963

Sig equals 1 indicates that the means difference is significant at the 0.05 level.
Sig equals 0 indicates that the means difference is not significant at the 0.05 level.

24- Statistical test of total Chlorophyll (*Chl a/b*) for DIB treatment

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
1 0	-0.03855	0.03775	1.44396	0.90627	0.01	0	-0.18198	0.10489
2.5 0	-0.06215	0.03775	2.32841	0.57774	0.01	0	-0.20559	0.08128
2.5 1	-0.02361	0.03775	0.88445	0.98797	0.01	0	-0.16705	0.11983
3 0	-0.05891	0.03775	2.20671	0.63073	0.01	0	-0.20235	0.08453
3 1	-0.02036	0.03775	0.76275	0.99388	0.01	0	-0.1638	0.12308
3 2.5	0.00325	0.03775	0.1217	1	0.01	0	-0.14019	0.14669
5 0	-0.05418	0.03775	2.02958	0.7062	0.01	0	-0.19762	0.08926
5 1	-0.01563	0.03775	0.58562	0.99824	0.01	0	-0.15907	0.12781
5 2.5	0.00798	0.03775	0.29883	0.99993	0.01	0	-0.13546	0.15142
5 3	0.00473	0.03775	0.17713	0.99999	0.01	0	-0.13871	0.14817
7 0	-0.03279	0.03775	1.22835	0.95041	0.01	0	-0.17623	0.11065
7 1	0.00576	0.03775	0.21561	0.99999	0.01	0	-0.13768	0.1492
7 2.5	0.02937	0.03775	1.10006	0.96869	0.01	0	-0.11407	0.1728
7 3	0.02612	0.03775	0.97836	0.98115	0.01	0	-0.11732	0.16956
7 5	0.02139	0.03775	0.80123	0.99232	0.01	0	-0.12205	0.16483

Sig equals 1 indicates that the means difference is significant at the 0.01 level.
Sig equals 0 indicates that the means difference is not significant at the 0.01 level.

25- Statistical test of (ABS/RC) parameter for Cr treatment

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
0.05 0	1.61492	1.41066	1.61899	0.85768	0.05	0	-2.74675	5.97659
0.1 0	3.34234	1.41066	3.35076	0.20664	0.05	0	-1.01933	7.70401
0.1 0.05	1.72742	1.41066	1.73177	0.8207	0.05	0	-2.63425	6.08909
0.15 0	5.12139	1.41066	5.13429	0.01488	0.05	1	0.75972	9.48306
0.15 0.05	3.50647	1.41066	3.5153	0.16802	0.05	0	-0.8552	7.86814
0.15 0.1	1.77905	1.41066	1.78353	0.8024	0.05	0	-2.58262	6.14072
0.2 0	6.57535	1.41066	6.59191	0.00123	0.05	1	2.21368	10.93702
0.2 0.05	4.96043	1.41066	4.97293	0.01939	0.05	1	0.59876	9.3221
0.2 0.1	3.23301	1.41066	3.24116	0.23586	0.05	0	-1.12866	7.59468
0.2 0.15	1.45396	1.41066	1.45762	0.90288	0.05	0	-2.90771	5.81563
0.25 0	7.31367	1.41066	7.33209	3.37541E-4	0.05	1	2.952	11.67533
0.25 0.05	5.69875	1.41066	5.7131	0.00563	0.05	1	1.33708	10.06041
0.25 0.1	3.97133	1.41066	3.98133	0.08913	0.05	0	-0.39034	8.33299
0.25 0.15	2.19227	1.41066	2.19779	0.63459	0.05	0	-2.1694	6.55394
0.25 0.2	0.73831	1.41066	0.74017	0.99468	0.05	0	-3.62336	5.09998

Sig equals 1 indicates that the means difference is significant at the 0.05 level.
Sig equals 0 indicates that the means difference is not significant at the 0.05 level.

26- Statistical test of (ABS/RC) parameter for IBX treatment

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
0.05 0	-0.22466	0.08089	3.928	0.09615	0.05	0	-0.47476	0.02543
0.1 0	-0.27148	0.08089	4.74657	0.02791	0.05	1	-0.52158	-0.02139
0.1 0.05	-0.04682	0.08089	0.81857	0.99153	0.05	0	-0.29691	0.20328
0.15 0	-0.26351	0.08089	4.6072	0.03479	0.05	1	-0.51361	-0.01341
0.15 0.05	-0.03885	0.08089	0.67919	0.99644	0.05	0	-0.28894	0.21125
0.15 0.1	0.00797	0.08089	0.13938	1	0.05	0	-0.24212	0.25807
0.2 0	-0.32012	0.08089	5.59699	0.00686	0.05	1	-0.57022	-0.07003
0.2 0.05	-0.09546	0.08089	1.66899	0.8418	0.05	0	-0.34556	0.15464
0.2 0.1	-0.04864	0.08089	0.85042	0.98992	0.05	0	-0.29874	0.20146
0.2 0.15	-0.05661	0.08089	0.9898	0.98016	0.05	0	-0.30671	0.19348
0.25 0	-0.45988	0.08089	8.04049	9.82729E-5	0.05	1	-0.70998	-0.20978
0.25 0.05	-0.23522	0.08089	4.11248	0.07374	0.05	0	-0.48531	0.01488
0.25 0.1	-0.1884	0.08089	3.29392	0.22144	0.05	0	-0.43849	0.0617
0.25 0.15	-0.19637	0.08089	3.43329	0.1865	0.05	0	-0.44647	0.05373
0.25 0.2	-0.13976	0.08089	2.4435	0.52781	0.05	0	-0.38985	0.11034

Sig equals 1 indicates that the means difference is significant at the 0.05 level.
Sig equals 0 indicates that the means difference is not significant at the 0.05 level.

27- Statistical test of (ABS/RC) parameter for Hg treatment

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
1 0	-0.11312	0.09834	1.62683	0.85524	0.05	0	-0.41717	0.19093
2.5 0	0.35694	0.09834	5.13333	0.01491	0.05	1	0.05289	0.661
2.5 1	0.47007	0.09834	6.76016	9.17857E-4	0.05	1	0.16601	0.77412
3 0	0.47552	0.09834	6.83866	8.00149E-4	0.05	1	0.17147	0.77957
3 1	0.58864	0.09834	8.46549	4.71817E-5	0.05	1	0.28459	0.8927
3 2.5	0.11858	0.09834	1.70533	0.82974	0.05	0	-0.18547	0.42263
5 0	0.88657	0.09834	12.75002	2.18725E-8	0.05	1	0.58252	1.19062
5 1	0.99969	0.09834	14.37685	0	0.05	1	0.69564	1.30374
5 2.5	0.52962	0.09834	7.61669	2.05345E-4	0.05	1	0.22557	0.83367
5 3	0.41104	0.09834	5.91136	0.00401	0.05	1	0.10699	0.71509
7 0	0.71533	0.09834	10.28732	2.19126E-6	0.05	1	0.41127	1.01938
7 1	0.82845	0.09834	11.91415	1.42811E-7	0.05	1	0.5244	1.1325
7 2.5	0.35838	0.09834	5.15399	0.01441	0.05	1	0.05433	0.66243
7 3	0.2398	0.09834	3.44866	0.18292	0.05	0	-0.06425	0.54385
7 5	-0.17124	0.09834	2.4627	0.51956	0.05	0	-0.47529	0.13281

Sig equals 1 indicates that the means difference is significant at the 0.05 level.
Sig equals 0 indicates that the means difference is not significant at the 0.05 level.

28- Statistical test of (ABS/RC) parameter for DIB treatment

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
1 0	0.01311	0.08065	0.22984	0.99998	0.05	0	-0.23625	0.26246
2.5 0	0.03874	0.08065	0.67936	0.99643	0.05	0	-0.21061	0.2881
2.5 1	0.02563	0.08065	0.44952	0.99951	0.05	0	-0.22372	0.27499
3 0	-0.01873	0.08065	0.32849	0.99989	0.05	0	-0.26809	0.23062
3 1	-0.03184	0.08065	0.55833	0.9986	0.05	0	-0.28119	0.21752
3 2.5	-0.05747	0.08065	1.00785	0.97853	0.05	0	-0.30683	0.19188
5 0	-0.02433	0.08065	0.42673	0.99962	0.05	0	-0.27369	0.22502
5 1	-0.03744	0.08065	0.65656	0.99696	0.05	0	-0.2868	0.21191
5 2.5	-0.06308	0.08065	1.10608	0.96796	0.05	0	-0.31243	0.18628
5 3	-0.0056	0.08065	0.09823	1	0.05	0	-0.25496	0.24375
7 0	-0.00515	0.08065	0.09028	1	0.05	0	-0.2545	0.24421
7 1	-0.01825	0.08065	0.32012	0.99991	0.05	0	-0.26761	0.2311
7 2.5	-0.04389	0.08065	0.76964	0.99362	0.05	0	-0.29324	0.20547
7 3	0.01358	0.08065	0.23822	0.99998	0.05	0	-0.23577	0.26294
7 5	0.01919	0.08065	0.33645	0.99988	0.05	0	-0.23017	0.26854

Sig equals 1 indicates that the means difference is significant at the 0.05 level.
Sig equals 0 indicates that the means difference is not significant at the 0.05 level.

29- Statistical test of (F_V/F_M) parameter for Cr treatment

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
0.05 0	-0.13185	0.06245	2.98566	0.31507	0.05	0	-0.32496	0.06125
0.1 0	-0.28602	0.06245	6.47649	0.00151	0.05	1	-0.47912	-0.09291
0.1 0.05	-0.15416	0.06245	3.49083	0.17338	0.05	0	-0.34727	0.03894
0.15 0	-0.36654	0.06245	8.29978	6.27633E-5	0.05	1	-0.55964	-0.17343
0.15 0.05	-0.23468	0.06245	5.31413	0.01104	0.05	1	-0.42779	-0.04158
0.15 0.1	-0.08052	0.06245	1.82329	0.78782	0.05	0	-0.27363	0.11259
0.2 0	-0.44883	0.06245	10.1632	2.69184E-6	0.05	1	-0.64193	-0.25572
0.2 0.05	-0.31698	0.06245	7.17755	4.42276E-4	0.05	1	-0.51008	-0.12387
0.2 0.1	-0.16281	0.06245	3.68671	0.13412	0.05	0	-0.35592	0.03029
0.2 0.15	-0.08229	0.06245	1.86342	0.77268	0.05	0	-0.2754	0.11081
0.25 0	-0.45118	0.06245	10.21642	2.46447E-6	0.05	1	-0.64428	-0.25807
0.25 0.05	-0.31933	0.06245	7.23076	4.02965E-4	0.05	1	-0.51243	-0.12622
0.25 0.1	-0.16516	0.06245	3.73993	0.12481	0.05	0	-0.35827	0.02794
0.25 0.15	-0.08464	0.06245	1.91664	0.75201	0.05	0	-0.27775	0.10846
0.25 0.2	-0.00235	0.06245	0.05322	1	0.05	0	-0.19546	0.19076

Sig equals 1 indicates that the means difference is significant at the 0.05 level.
Sig equals 0 indicates that the means difference is not significant at the 0.05 level.

30- Statistical test of (F_V/F_M) parameter for IBX treatment

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
0.05 0	0.00992	0.00271	5.17852	0.01384	0.05	1	0.00154	0.01829
0.1 0	0.01223	0.00271	6.38858	0.00176	0.05	1	0.00386	0.02061
0.1 0.05	0.00232	0.00271	1.21006	0.95337	0.05	0	-0.00606	0.01069
0.15 0	0.01213	0.00271	6.3333	0.00193	0.05	1	0.00375	0.0205
0.15 0.05	0.00221	0.00271	1.15478	0.96158	0.05	0	-0.00616	0.01058
0.15 0.1	-1.05866E-4	0.00271	0.05529	1	0.05	0	-0.00848	0.00827
0.2 0	0.01824	0.00271	9.52524	7.79689E-6	0.05	1	0.00987	0.02661
0.2 0.05	0.00832	0.00271	4.34672	0.05201	0.05	0	-4.96545E-5	0.0167
0.2 0.1	0.00601	0.00271	3.13666	0.26638	0.05	0	-0.00237	0.01438
0.2 0.15	0.00611	0.00271	3.19195	0.24991	0.05	0	-0.00226	0.01448
0.25 0	0.02099	0.00271	10.9643	7.13929E-7	0.05	1	0.01262	0.02937
0.25 0.05	0.01108	0.00271	5.78578	0.00497	0.05	1	0.00271	0.01945
0.25 0.1	0.00876	0.00271	4.57572	0.03655	0.05	1	3.88837E-4	0.01713
0.25 0.15	0.00887	0.00271	4.63101	0.03352	0.05	1	4.94703E-4	0.01724
0.25 0.2	0.00276	0.00271	1.43906	0.90747	0.05	0	-0.00562	0.01113

Sig equals 1 indicates that the means difference is significant at the 0.05 level.
Sig equals 0 indicates that the means difference is not significant at the 0.05 level.

31- Statistical test of (F_V/F_M) parameter for Hg treatment

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
1 0	0.02918	0.0056	7.37069	3.15515E-4	0.01	1	0.00791	0.05045
2.5 0	0.01696	0.0056	4.28368	0.0572	0.01	0	-0.00431	0.03823
2.5 1	-0.01222	0.0056	3.08702	0.2818	0.01	0	-0.03349	0.00905
3 0	0.01375	0.0056	3.47422	0.17709	0.01	0	-0.00752	0.03503
3 1	-0.01543	0.0056	3.89647	0.10052	0.01	0	-0.0367	0.00585
3 2.5	-0.0032	0.0056	0.80945	0.99196	0.01	0	-0.02448	0.01807
5 0	-0.02398	0.0056	6.05668	0.00312	0.01	1	-0.04525	-0.0027
5 1	-0.05316	0.0056	13.42737	0	0.01	1	-0.07443	-0.03188
5 2.5	-0.04094	0.0056	10.34035	2.00701E-6	0.01	1	-0.06221	-0.01966
5 3	-0.03773	0.0056	9.5309	7.72319E-6	0.01	1	-0.059	-0.01646
7 0	-0.0116	0.0056	2.9307	0.3341	0.01	0	-0.03287	0.00967
7 1	-0.04078	0.0056	10.30139	2.14079E-6	0.01	1	-0.06205	-0.01951
7 2.5	-0.02856	0.0056	7.21437	4.14684E-4	0.01	1	-0.04983	-0.00729
7 3	-0.02536	0.0056	6.40492	0.00171	0.01	1	-0.04663	-0.00408
7 5	0.01238	0.0056	3.12598	0.26965	0.01	0	-0.0089	0.03365

Sig equals 1 indicates that the means difference is significant at the 0.01 level.
Sig equals 0 indicates that the means difference is not significant at the 0.01 level.

32- Statistical test of (F_V/F_M) parameter for DIB treatment

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
1 0	-0.00162	0.00374	0.61149	0.99783	0.05	0	-0.01318	0.00994
2.5 0	0.00474	0.00374	1.79305	0.79895	0.05	0	-0.00682	0.0163
2.5 1	0.00636	0.00374	2.40454	0.54464	0.05	0	-0.0052	0.01792
3 0	0.00444	0.00374	1.67974	0.83828	0.05	0	-0.00712	0.016
3 1	0.00606	0.00374	2.29122	0.59395	0.05	0	-0.0055	0.01762
3 2.5	-2.99606E-4	0.00374	0.11332	1	0.05	0	-0.01186	0.01126
5 0	0.00469	0.00374	1.77501	0.80547	0.05	0	-0.00687	0.01625
5 1	0.00631	0.00374	2.3865	0.55246	0.05	0	-0.00525	0.01787
5 2.5	-4.77027E-5	0.00374	0.01804	1	0.05	0	-0.01161	0.01151
5 3	2.51903E-4	0.00374	0.09527	1	0.05	0	-0.01131	0.01181
7 0	0.00692	0.00374	2.61549	0.45519	0.05	0	-0.00465	0.01848
7 1	0.00853	0.00374	3.22698	0.23985	0.05	0	-0.00303	0.02009
7 2.5	0.00217	0.00374	0.82244	0.99135	0.05	0	-0.00939	0.01374
7 3	0.00247	0.00374	0.93576	0.98452	0.05	0	-0.00909	0.01404
7 5	0.00222	0.00374	0.84048	0.99045	0.05	0	-0.00934	0.01378

Sig equals 1 indicates that the means difference is significant at the 0.05 level.
Sig equals 0 indicates that the means difference is not significant at the 0.05 level.

33- Statistical test of (PI_{ABS}) parameter for Cr treatment

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
0.05 0	-1.37557	0.18895	10.29556	2.16157E-6	0.01	1	-2.09351	-0.65764
0.1 0	-1.58073	0.18895	11.83107	1.65072E-7	0.01	1	-2.29867	-0.86279
0.1 0.05	-0.20516	0.18895	1.53551	0.88224	0.01	0	-0.9231	0.51278
0.15 0	-1.63667	0.18895	12.24975	7.54165E-8	0.01	1	-2.35461	-0.91873
0.15 0.05	-0.2611	0.18895	1.95419	0.73704	0.01	0	-0.97904	0.45684
0.15 0.1	-0.05594	0.18895	0.41868	0.99965	0.01	0	-0.77388	0.662
0.2 0	-1.69421	0.18895	12.68039	2.70365E-8	0.01	1	-2.41215	-0.97627
0.2 0.05	-0.31863	0.18895	2.38483	0.55319	0.01	0	-1.03657	0.3993
0.2 0.1	-0.11348	0.18895	0.84932	0.98998	0.01	0	-0.83141	0.60446
0.2 0.15	-0.05754	0.18895	0.43064	0.9996	0.01	0	-0.77548	0.6604
0.25 0	-1.6923	0.18895	12.66612	2.81673E-8	0.01	1	-2.41024	-0.97436
0.25 0.05	-0.31673	0.18895	2.37056	0.55939	0.01	0	-1.03467	0.40121
0.25 0.1	-0.11157	0.18895	0.83505	0.99073	0.01	0	-0.82951	0.60637
0.25 0.15	-0.05563	0.18895	0.41637	0.99966	0.01	0	-0.77357	0.66231
0.25 0.2	0.00191	0.18895	0.01427	1	0.01	0	-0.71603	0.71985

Sig equals 1 indicates that the means difference is significant at the 0.01 level.
Sig equals 0 indicates that the means difference is not significant at the 0.01 level.

34- Statistical test of (PI_{ABS}) parameter for IBX treatment

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
0.05 0	0.49626	0.18629	3.7673	0.12024	0.05	0	-0.07974	1.07227
0.1 0	0.49718	0.18629	3.77427	0.1191	0.05	0	-0.07882	1.07319
0.1 0.05	9.18768E-4	0.18629	0.00697	1	0.05	0	-0.57509	0.57693
0.15 0	0.55709	0.18629	4.22901	0.06208	0.05	0	-0.01892	1.13309
0.15 0.05	0.06082	0.18629	0.46171	0.99944	0.05	0	-0.51519	0.63683
0.15 0.1	0.0599	0.18629	0.45474	0.99948	0.05	0	-0.51611	0.63591
0.2 0	0.81853	0.18629	6.21369	0.00238	0.05	1	0.24252	1.39454
0.2 0.05	0.32226	0.18629	2.4464	0.52656	0.05	0	-0.25375	0.89827
0.2 0.1	0.32134	0.18629	2.43942	0.52957	0.05	0	-0.25466	0.89735
0.2 0.15	0.26144	0.18629	1.98468	0.72469	0.05	0	-0.31457	0.83745
0.25 0	1.23046	0.18629	9.34082	1.06324E-5	0.05	1	0.65445	1.80647
0.25 0.05	0.7342	0.18629	5.57352	0.00714	0.05	1	0.15819	1.31021
0.25 0.1	0.73328	0.18629	5.56655	0.00722	0.05	1	0.15727	1.30929
0.25 0.15	0.67338	0.18629	5.11181	0.01544	0.05	1	0.09737	1.24939
0.25 0.2	0.41194	0.18629	3.12713	0.2693	0.05	0	-0.16407	0.98794

Sig equals 1 indicates that the means difference is significant at the 0.05 level.
Sig equals 0 indicates that the means difference is not significant at the 0.05 level.

35- Statistical test of (PI_{ABS}) parameter for Hg treatment

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
1 0	0.47326	0.09588	6.98019	6.24675E-4	0.01	1	0.10894	0.83758
2.5 0	-0.11676	0.09588	1.72207	0.82404	0.01	0	-0.48108	0.24757
2.5 1	-0.59002	0.09588	8.70226	3.14378E-5	0.01	1	-0.95434	-0.2257
3 0	-0.26254	0.09588	3.87219	0.104	0.01	0	-0.62686	0.10179
3 1	-0.7358	0.09588	10.85238	8.5973E-7	0.01	1	-1.10012	-0.37148
3 2.5	-0.14578	0.09588	2.15012	0.65516	0.01	0	-0.5101	0.21854
5 0	-0.68823	0.09588	10.15074	2.74809E-6	0.01	1	-1.05255	-0.3239
5 1	-1.16149	0.09588	17.13092	3.39472E-8	0.01	1	-1.52581	-0.79716
5 2.5	-0.57147	0.09588	8.42866	5.02663E-5	0.01	1	-0.93579	-0.20715
5 3	-0.42569	0.09588	6.27854	0.00213	0.01	1	-0.79001	-0.06137
7 0	-0.52007	0.09588	7.6706	1.86927E-4	0.01	1	-0.88439	-0.15575
7 1	-0.99333	0.09588	14.65078	0	0.01	1	-1.35766	-0.62901
7 2.5	-0.40331	0.09588	5.94853	0.00376	0.01	1	-0.76764	-0.03899
7 3	-0.25753	0.09588	3.7984	0.11521	0.01	0	-0.62186	0.10679
7 5	0.16815	0.09588	2.48014	0.51209	0.01	0	-0.19617	0.53248

Sig equals 1 indicates that the means difference is significant at the 0.01 level.
Sig equals 0 indicates that the means difference is not significant at the 0.01 level.

36- Statistical test of (PI_{ABS}) parameter for DIB treatment

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
1 0	-5.9685E-4	0.46162	0.00183	1	0.05	0	-1.4279	1.42671
2.5 0	0.32624	0.46162	0.99945	0.9793	0.05	0	-1.10107	1.75355
2.5 1	0.32684	0.46162	1.00128	0.97913	0.05	0	-1.10047	1.75414
3 0	0.41692	0.46162	1.27727	0.94192	0.05	0	-1.01039	1.84423
3 1	0.41752	0.46162	1.27909	0.94158	0.05	0	-1.00979	1.84483
3 2.5	0.09068	0.46162	0.27781	0.99995	0.05	0	-1.33663	1.51799
5 0	0.54853	0.46162	1.68047	0.83803	0.05	0	-0.87877	1.97584
5 1	0.54913	0.46162	1.6823	0.83743	0.05	0	-0.87818	1.97644
5 2.5	0.2223	0.46162	0.68102	0.99639	0.05	0	-1.20501	1.6496
5 3	0.13161	0.46162	0.4032	0.99971	0.05	0	-1.2957	1.55892
7 0	0.52341	0.46162	1.60349	0.86242	0.05	0	-0.9039	1.95072
7 1	0.524	0.46162	1.60532	0.86187	0.05	0	-0.9033	1.95131
7 2.5	0.19717	0.46162	0.60404	0.99795	0.05	0	-1.23014	1.62448
7 3	0.10649	0.46162	0.32623	0.9999	0.05	0	-1.32082	1.53379
7 5	-0.02513	0.46162	0.07698	1	0.05	0	-1.45243	1.40218

Sig equals 1 indicates that the means difference is significant at the 0.05 level.
Sig equals 0 indicates that the means difference is not significant at the 0.05 level.

37- Statistical test of (V_J) parameter for Cr treatment

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
0.05 0	0.11753	0.0326	5.09884	0.01578	0.05	1	0.01674	0.21833
0.1 0	0.15534	0.0326	6.7391	9.52272E-4	0.05	1	0.05455	0.25614
0.1 0.05	0.03781	0.0326	1.64027	0.85102	0.05	0	-0.06298	0.1386
0.15 0	0.16064	0.0326	6.96904	6.3698E-4	0.05	1	0.05985	0.26144
0.15 0.05	0.04311	0.0326	1.8702	0.77009	0.05	0	-0.05768	0.1439
0.15 0.1	0.0053	0.0326	0.22993	0.99998	0.05	0	-0.09549	0.10609
0.2 0	0.16939	0.0326	7.3484	3.28052E-4	0.05	1	0.06859	0.27018
0.2 0.05	0.05185	0.0326	2.24956	0.6121	0.05	0	-0.04894	0.15265
0.2 0.1	0.01404	0.0326	0.6093	0.99787	0.05	0	-0.08675	0.11484
0.2 0.15	0.00874	0.0326	0.37936	0.99978	0.05	0	-0.09205	0.10954
0.25 0	0.18936	0.0326	8.21467	7.26974E-5	0.05	1	0.08856	0.29015
0.25 0.05	0.07182	0.0326	3.11584	0.27278	0.05	0	-0.02897	0.17262
0.25 0.1	0.03401	0.0326	1.47557	0.89833	0.05	0	-0.06678	0.13481
0.25 0.15	0.02871	0.0326	1.24564	0.94751	0.05	0	-0.07208	0.12951
0.25 0.2	0.01997	0.0326	0.86627	0.98905	0.05	0	-0.08083	0.12076

Sig equals 1 indicates that the means difference is significant at the 0.05 level.
Sig equals 0 indicates that the means difference is not significant at the 0.05 level.

38- Statistical test of (V_J) parameter for IBX treatment

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
0.05 0	-0.03137	0.01428	3.10738	0.2754	0.05	0	-0.07551	0.01277
0.1 0	-0.02723	0.01428	2.6975	0.42193	0.05	0	-0.07137	0.01691
0.1 0.05	0.00414	0.01428	0.40988	0.99969	0.05	0	-0.04	0.04828
0.15 0	-0.03192	0.01428	3.16231	0.25865	0.05	0	-0.07607	0.01222
0.15 0.05	-5.54496E-4	0.01428	0.05493	1	0.05	0	-0.0447	0.04359
0.15 0.1	-0.00469	0.01428	0.46481	0.99942	0.05	0	-0.04883	0.03945
0.2 0	-0.05006	0.01428	4.95912	0.01983	0.05	1	-0.0942	-0.00592
0.2 0.05	-0.01869	0.01428	1.85173	0.77713	0.05	0	-0.06284	0.02545
0.2 0.1	-0.02283	0.01428	2.26161	0.60685	0.05	0	-0.06697	0.02131
0.2 0.15	-0.01814	0.01428	1.79681	0.79758	0.05	0	-0.06228	0.026
0.25 0	-0.07167	0.01428	7.09996	5.06577E-4	0.05	1	-0.11582	-0.02753
0.25 0.05	-0.04031	0.01428	3.99257	0.08771	0.05	0	-0.08445	0.00384
0.25 0.1	-0.04444	0.01428	4.40245	0.04778	0.05	1	-0.08859	-3.00836E-4
0.25 0.15	-0.03975	0.01428	3.93765	0.09485	0.05	0	-0.08389	0.00439
0.25 0.2	-0.02161	0.01428	2.14084	0.65915	0.05	0	-0.06575	0.02253

Sig equals 1 indicates that the means difference is significant at the 0.05 level.
Sig equals 0 indicates that the means difference is not significant at the 0.05 level.

39- Statistical test of (V_J) parameter for Hg treatment

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
1 0	-0.02474	0.01269	2.75701	0.3985	0.05	0	-0.06398	0.0145
2.5 0	0.0351	0.01269	3.91185	0.09837	0.05	0	-0.00414	0.07435
2.5 1	0.05985	0.01269	6.66886	0.00108	0.05	1	0.02061	0.09909
3 0	0.04769	0.01269	5.31407	0.01104	0.05	1	0.00845	0.08693
3 1	0.07243	0.01269	8.07109	9.31979E-5	0.05	1	0.03319	0.11167
3 2.5	0.01258	0.01269	1.40223	0.91618	0.05	0	-0.02666	0.05182
5 0	0.10368	0.01269	11.55347	2.65022E-7	0.05	1	0.06444	0.14292
5 1	0.12842	0.01269	14.31048	0	0.05	1	0.08918	0.16766
5 2.5	0.06858	0.01269	7.64162	1.9661E-4	0.05	1	0.02934	0.10782
5 3	0.05599	0.01269	6.23939	0.00227	0.05	1	0.01675	0.09523
7 0	0.06947	0.01269	7.7408	1.65409E-4	0.05	1	0.03023	0.10871
7 1	0.09421	0.01269	10.49782	1.54651E-6	0.05	1	0.05497	0.13345
7 2.5	0.03436	0.01269	3.82896	0.11045	0.05	0	-0.00488	0.0736
7 3	0.02178	0.01269	2.42673	0.53504	0.05	0	-0.01746	0.06102
7 5	-0.03421	0.01269	3.81267	0.11297	0.05	0	-0.07346	0.00503

Sig equals 1 indicates that the means difference is significant at the 0.05 level.
Sig equals 0 indicates that the means difference is not significant at the 0.05 level.

40- Statistical test of (V_J) parameter for DIB treatment

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
1 0	-0.01652	0.02457	0.95111	0.98336	0.05	0	-0.09249	0.05944
2.5 0	-0.02744	0.02457	1.57926	0.86968	0.05	0	-0.1034	0.04853
2.5 1	-0.01091	0.02457	0.62815	0.99754	0.05	0	-0.08688	0.06505
3 0	-0.03439	0.02457	1.9796	0.72676	0.05	0	-0.11036	0.04157
3 1	-0.01787	0.02457	1.0285	0.97655	0.05	0	-0.09383	0.0581
3 2.5	-0.00696	0.02457	0.40034	0.99972	0.05	0	-0.08292	0.06901
5 0	-0.04245	0.02457	2.4432	0.52794	0.05	0	-0.11841	0.03352
5 1	-0.02592	0.02457	1.4921	0.89403	0.05	0	-0.10189	0.05004
5 2.5	-0.01501	0.02457	0.86394	0.98918	0.05	0	-0.09098	0.06096
5 3	-0.00805	0.02457	0.4636	0.99943	0.05	0	-0.08402	0.06791
7 0	-0.03525	0.02457	2.02926	0.70633	0.05	0	-0.11122	0.04071
7 1	-0.01873	0.02457	1.07815	0.97127	0.05	0	-0.0947	0.05724
7 2.5	-0.00782	0.02457	0.44999	0.9995	0.05	0	-0.08378	0.06815
7 3	-8.62609E-4	0.02457	0.04965	1	0.05	0	-0.07683	0.0751
7 5	0.00719	0.02457	0.41395	0.99967	0.05	0	-0.06877	0.08316

Sig equals 1 indicates that the means difference is significant at the 0.05 level.
Sig equals 0 indicates that the means difference is not significant at the 0.05 level.