UNIVERSITÉ DU QUÉBEC À MONTRÉAL

DEGRADATION DE L'HERBICIDE DIURON PAR OZONATION ET ANALYSE DE LA TOXICITE SUR *Lemna minor*

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DEGRADATION OF THE HERBICIDE DIURON BY OZONATION AND TOXICITY
ANALYSIS ON *Lemna minor*

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DEDICATION

To my cherished parents,

My beloved husband,

My dear sister and brother.

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LIST OF ABBREVIATIONS, AND ACRONYMS

DCMU: Diuron

DCPMU: 1-(3,4-dichlorophenyl)-3-methylurea

3,4-DCA: 3,4-dichloroaniline

SIS: Swedish Institute for Standards

ROS: Reactive Oxygen Species

Chl a: Chlorophyll a

Chl b: Chlorophyll b

CL 50: Median effective concentration

UHPLC: Ultra-High-Performance Liquid Chromatography

LC-MS/MS: Liquid Chromatography Tandem Mass Spectrometry

LC-MRM: Liquid Chromatography Multiple Reaction Monitoring

UV: Ultraviolet

PH: Potential of hydrogen

PQ: Plastoquinone

PS I: Photosystem I

PS II: Photosystem II

QA: Quinone A

QB: Quinone B

O_2: Oxygen

ATP: Adenosine Triphosphate

OECD: Organisation for Economic Co-operation and Development

LIST OF SYMBOLS AND UNITS

mg/L: Milligrams per litre

µg/L: Microgram per litre

mL: Milliliter

FW: Fresh weight

FN: Frond number

ºC: Celsius Degree

d: Day

RÉSUMÉ

Le diuron (3-(3,4-dichlorophényl)-1,1-diméthylurée), un herbicide largement utilisé dans le monde depuis plus de quatre décennies, a été employé pour le contrôle des mauvaises herbes et de la mousse dans les zones non cultivées et de manière sélective dans diverses cultures. Malgré son efficacité, le diuron est rapporté comme présentant une haute toxicité pour les organismes non ciblés, démontrant un potentiel dommageable au niveau cellulaire et subcellulaire. Son utilisation répandue en agriculture pose un risque pour l'équilibre biologique, conduisant à la contamination des eaux de surface par le drainage agricole. La persistance du diuron dans le sol, avec une demi-vie dépassant les 300 jours, soulève des inquiétudes quant à sa lente pénétration et la contamination des eaux souterraines. Par conséquent, les eaux de ruissellement provenant des terres agricoles utilisant largement le diuron peuvent contribuer à des problèmes environnementaux et de santé. L'objectif de ce projet est d'étudier l'influence de l'ozonation sur la dégradation du diuron et sa toxicité avant et après ozonation en utilisant la plante aquatique *Lemna minor* comme modèle d'étude.

Cette étude fournit des aperçus sur les profils toxicologiques du diuron et de ses métabolites (fénuron ; 1,1-diméthyl-3-phénylurée, DCPMU ; et 3,4-Dichloroaniline, 3,4 DCA) chez *Lemna minor*, mettant en évidence des sensibilités variables à travers des biomarqueurs physiologiques et biochimiques. De plus, cette étude montre la toxicité du diuron après traitement par ozonation sur *Lemna minor* et la tendance à la dégradation du diuron en utilisant des méthodes analytiques.

La recherche révèle que le diuron présente un effet inhibiteur dépendant de la dose sur la plante, avec ses métabolites et montrant des réponses variables : le fénuron induit une réponse hormétique, le DCPMU est hautement toxique, et le 3,4-DCA semble stimuler la croissance, suggérant une adaptation métabolique potentielle ou une détoxification. Cette étude montre également la diminution significative des pigments chlorophylliens et l'augmentation uniforme des niveaux d'Espèces Réactives de l'Oxygène (ROS), soulignant le potentiel nuisible de ces produits chimiques pour la photosynthèse et en induisant un stress oxydatif. En conclusion, cette étude démontre les effets complexes de l'ozonation sur la toxicité du diuron, indiquant des impacts

fluctuants avec des réponses initiales de stress suivies par la récupération et la détoxification au fil du temps.

ABSTRACT

Diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea), an herbicide extensively used globally for over four decades, has been employed for weed and moss control in non-crop areas and selectively in various crops. Despite its effectiveness, diuron is reported to exhibit high toxicity to non-target organisms, demonstrating potential harm at cellular and subcellular levels. Its widespread use in agriculture poses a risk to biological equilibrium, leading to surface water contamination through agricultural drainage. The persistence of diuron in soil, with a half-life exceeding 300 days, raises concerns about slow penetration and groundwater contamination. Consequently, runoff waters from extensively diuron-utilized agricultural lands may contribute to environmental and health issues. The objective of this project is to study the influence of ozonation on diuron degradation and its toxicity before and after ozonation using the aquatic plant *Lemna minor* as a study model.

This study provides insights into the diverse toxicological profiles of diuron and its metabolites (fenuron; 1,1-dimethyl-3-phenylurea), DCPMU, and 3,4-Dichloroaniline, 3,4-DCA) in *Lemna minor*, highlighting varying sensitivities across physiological and biochemical endpoints. Additionally, this study investigates the toxicity of diuron after ozonation treatment on *Lemna minor* and the degradation trend of diuron using analytical methods.

This research reveals that diuron exhibits a dose-dependent inhibitory effect on the plant, with its metabolites showing varying responses: fenuron induces a hormetic response, DCPMU is highly toxic, and 3,4-DCA appears to stimulate growth, suggesting potential metabolic adaptation or detoxification. This study also highlights the significant decrease in chlorophyll pigments and the uniform increase in Reactive Oxygen Species (ROS) levels, underscoring the potential for these chemicals to impair photosynthesis and to induce oxidative stress. Additionally, this study explores the complex effects of ozonation on diuron toxicity, indicating fluctuating impacts with initial stress responses followed by recovery and detoxification over time.

GENERAL INTRODUCTION

Insecticides and herbicides, particularly those in the phenyl urea family, are extensively employed in a variety of sectors, including industry, forestry, gardening, agriculture, and household use (Fatima et al., 2007). While herbicides are predominantly used in agriculture for crop protection, their application is not confined to these areas alone. They are also employed in non-agricultural sectors. Following their use, these chemicals can end up in water bodies through means such as spray drift, rain wash-off, erosion, and surface runoff. A striking fact is that a large portion, about 99.7%, of applied herbicides ends up as residues in aquatic environments, predominantly through runoff and leaching, highlighting a critical environmental issue (Gatidou et al., 2015; Klöppel et al., 1997; Prado et al., 2009). The problem is further compounded by urban-industrial and agricultural wastes, which contribute to the contamination of surface water and sediments (Radić et al., 2011). The increasing use of herbicides in agricultural fields to enhance crop production efficiency has led to the accumulation of substantial herbicide residues in soil and aquatic environments. This raises serious concerns about their impact on water ecosystems and their influence on the diversity and productivity of these ecosystems. Herbicides can have detrimental impacts on non-target organisms within these ecosystems, leading to significant changes in the composition of macrophytes, phytoplankton, and other photosynthetic communities (Kumar & Han, 2010; Relyea, 2005).

Diuron, a globally used substituted urea herbicide, has been a prominent choice in agricultural cultivation since the 1950s. The annual consumption of pesticides, including diuron, is approximately two million tons. Diuron is specifically utilized for weed control, operating by inhibiting photosynthesis in weeds and grasses. Despite its low solubility in water, diuron is bio-recalcitrant and chemically stable, leading to potential contamination risks for both underground and surface water. Its environmental persistence, evidenced by a half-life of about 370 days, adds to its pollutant profile (Dragone et al., 2015; Khongthon et al., 2016; Duc et al., 2022; Bouquet–Somrani et al., 2000). Diuron has been found in various concentrations in wastewater treatment plant effluents, surface water, and groundwater, mainly due to agricultural runoff and distribution systems. This accumulation in aquatic environments poses significant and irreversible threats to

human health, emphasizing the urgency of research on diuron removal from these settings (Xiang et al., 2018). Diuron, while showing slight toxicity to mammals and birds, poses moderate toxicity to aquatic invertebrates. Its primary biodegradation product, 3,4-DCA, is not only persistent but also more toxic in soil, water, and groundwater, thus presenting both direct and indirect toxicity risks. As a result, diuron is identified as a potential environmental contaminant with characteristics of a poisoning pesticide (Giacomazzi & Cochet, 2004).

Effective monitoring and management strategies are essential to preserve the integrity of aquatic ecosystems. Such strategies should be based on accurate quantitative data regarding the detection of herbicides in these ecosystems and their associated risks (Lee et al., 2021). Sophisticated analytical methods like high-performance liquid chromatography (HPLC) and mass spectrometry are commonly employed to measure herbicide residues, but they have drawbacks including complex sample preparation, high costs, and interference from other contaminants (Kumar & Han, 2010). To overcome these limitations and provide ecologically meaningful information on pollution exposure, biological assays, particularly aquatic bioassays, are used. These assays help evaluate the ecological risks of pollutants in water, especially in situations involving mixtures and unknown substances, and offer a more comprehensive approach compared to traditional chemical analysis-based management (Lee et al., 2021). Duckweed (*Lemna minor*) is utilized in water quality studies as a biological indicator, helping to monitor heavy metals and other aquatic pollutants (Radić et al., 2011).

The objective of this study was to assess the toxicological effects of diuron and its by-products on *Lemna minor*. Over a span of seven days, *L. minor* plants were exposed to varying concentrations of diuron and its by-products. The investigation centered on monitoring changes in cellular and biochemical plant markers, such as growth rate, chlorophyll content, and the production of reactive oxygen species. Subsequently, a range-finding test was conducted to establish appropriate test concentrations of diuron for experiments aimed at determining EC50 values (Gatidou et al., 2015).

Following this, the research narrowed its focus to the specific toxic effects of diuron, utilizing a concentration of 100 µg/L after ozonation treatment at different time intervals. The choice of 100 µg/L for the toxicity test was based on its significant impact on *Lemna minor*'s growth rates and biochemical markers observed during preliminary tests, indicating a noteworthy influence on the

organism's physiology. This justified further investigation into the toxic effects of diuron and its by-products. The study primarily emphasized measuring growth inhibition to elucidate the toxicological characteristics of diuron and its transformed compounds post-ozonation.

1. CHAPTER I

LITERATURE REVIEW

1.1 General information about diuron

1.1.1 Background of diuron

Diuron, also known as DCMU, N-(3,4-dichlorophenyl)-N,N-dimethyl-urea, is an herbicide belonging to the phenylamide family and the subclass of phenylurea (Giacomazzi & Cochet, 2004). Diuron is a white crystalline solid and wettable powder, primarily employed as a herbicide. Diuron is extensively utilized for either pre- or post-emergence suppression of a variety of broadleaf and grass weeds in several crops (Tandon & Pant, 2019).

Diuron was first reported in 1951 and has been used worldwide for both agricultural and non-agricultural uses (Briggs, Weddle et al. 2008). It is considered a Priority Hazardous Substance by the European Commission (Malato, Blanco et al. 2002). The chemical structure of the diuron is shown in figure (1.1).
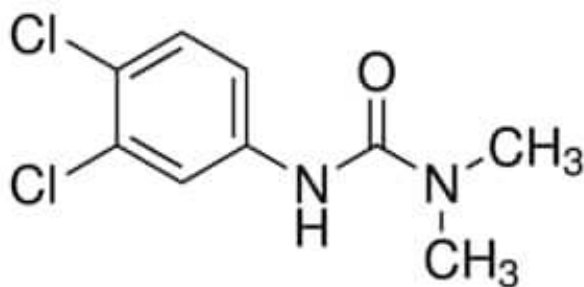
Figure 1. 1 Chemical structure of diuron

In the context of agriculture, this substance is employed for weed management in various fruits, crops, and fallow fields; it is also occasionally applied to irrigation and drainage systems in the absence of water (Giacomazzi & Cochet, 2004). In non-agricultural settings, its primary use is for controlling weeds on solid structures like roads, railway lines, and pathways in industrial areas and

rights-of-way, often in conjunction with other herbicides (Giacomazzi & Cochet, 2004). Diuron is sometimes utilized as an algaecide or biocide in decorative ponds, fountains, and aquariums, though it is not used in natural bodies of water (Moncada, 2004).

Diuron has been identified as highly toxic to certain non-target species, and its potential toxicity at the cellular and subcellular levels has been established (Chauhan et al., 1998; Simon et al., 1998; Teisseire et al., 1999). Its extensive application in agriculture can disrupt biological balance and lead to the contamination of surface waters through agricultural runoff (Polcaro et al., 2004). Additionally, diuron is known to be persistent in soil, especially when used in large amounts, with a half-life exceeding 300 days (Malato et al., 2003). As a result, diuron from agricultural runoff can seep slowly through the soil and potentially pollute groundwater. The runoff from agricultural areas where diuron is widely used may also present environmental and health hazards (Feng et al., 2008).

1.1.2   Basic Overview and Functional Mechanisms

Diuron, a systemic herbicide belonging to the substituted phenyl urea class, is readily absorbed by plants through their roots from the soil solution and quickly moves to the stems and leaves via the transpiration system, mainly through the xylem. Its primary mechanism is to inhibit the Hill reaction in photosynthesis, thereby reducing the synthesis of high-energy molecules like adenosine triphosphate (ATP), essential for various metabolic activities. Diuron acts by binding to the QB-binding site on the D1 protein of the photosystem II complex in the chloroplast's thylakoid membranes, impeding electron transfer from QA to QB. This inhibition hampers CO_2 fixation and the creation of ATP and other high-energy compounds necessary for plant growth. The disruption in QA reoxidation leads to the formation of triplet-state chlorophyll, which generates singlet oxygen upon interaction with ground-state oxygen. Both triplet chlorophyll and singlet oxygen contribute to hydrogen extraction from unsaturated lipids, initiating a chain reaction of lipid peroxidation. This process results in the oxidation and damage of lipids and proteins, causing a loss of chlorophyll and carotenoids, and creating leaky membranes that lead to rapid desiccation and disintegration of cells and cell organelles (Hess & Warren, 2002).

1.1.3 Physicochemical properties of diuron

The summary of the main physicochemical properties of diuron is shown in Table (1.1). Diuron, identified by its CAS registry number 330-54-1, is a non-ionic compound that exhibits a colorless, crystalline structure in its pure state. It has moderate solubility in water, dissolving up to 42

Table 1.1 Physicochemical properties of diuron (Çokay Çatalkaya, 2011)

Properties	
Chemical Name	N-(3,4-dichlophenyl)-N,N-dimethyl urea
CAS Number	[330-54-1]
Water solubility	42 ppm @ 20°C
Melting Point	158 °C - 159 °C
Vapor Pressure	2.97×10^{-3} (mm Hg) @ 50°C
Organic carbon partition coefficient (K_{oc})	2.77
Molecular Formula	$C_9H_{10}Cl_2N_2O$
Molecule Weight	233.1 g/mol
Density	0.619 g/mL @ 20°C

milligrams per litre at a temperature of 20 degrees Celsius. At room temperature, diuron remains in a solid state, with its melting point ranging between 158 and 159 degrees Celsius. Its vapour pressure at 25 degrees Celsius is measured at 0.009 millipascal, and the Henry's law constant for diuron is calculated to be a very low 0.000051 Pascal cubic meters per mole, indicating that diuron has a low tendency to volatilize from aquatic or terrestrial environments (Giacomazzi & Cochet, 2004). Under neutral pH conditions, the hydrolysis rate of diuron is minimal, but it rises significantly in strongly acidic or alkaline environments, resulting in the formation of its primary derivative, 3,4-dichloroaniline (3,4-DCA) (Salvestrini et al., 2002). Diuron demonstrates a stable hydrolysis rate at a neutral pH, but this rate significantly increases under highly acidic or basic conditions, as reported by Spencer in 1982 and Salvestrini et al. in 2002, resulting in the formation of its main by-product, 3,4-DCA. The compound possesses a low to moderate lipophilicity, as reflected by its octanol-water partition coefficient (log K_{ow}) of 2.6. With a soil organic carbon partition coefficient (K_{oc}) of 485, diuron is predicted to have a strong affinity for binding to organic

particles in soil. This elevated K_{oc} value indicates a substantial adsorptive potential, leading to an uneven distribution within the soil matrix (Giacomazzi & Cochet, 2004).

1.1.4   Sources and presence of diuron in the environment

Due to their widespread usage and long-lasting nature, residues of diuron are commonly detected in water, soil, and sediment environments (Tandon & Pant, 2019). In sediment samples from the Brazilian Amazon region, the maximum recorded concentration of diuron reached 55.2 micrograms per kilogram (Viana et al., 2019). In the river basins of Costa Rica, the maximum recorded concentrations of diuron in water and sediment samples reached 22.8 micrograms per litre and 11.75 micrograms per kilogram, respectively (Carazo-Rojas et al., 2018). Reported findings indicate that around 70% of samples taken from European streams had diuron at its highest concentration, which was 864 nanograms per litre (Loos et al., 2009). The European Commission, as of 2019, has included diuron in its list of priority substances. In Europe, the established limits for diuron in surface water are a maximum concentration of 1.8 μg/L and an annual average concentration of 0.2 μg/L (Mori et al., 2018). Besides the parent compound, metabolites resulting from the degradation of diuron have been found in soil and aquatic ecosystems globally (Hussain et al., 2015). Additionally, its considerable solubility and extended half-life in aqueous photolysis (DT50) contribute to its presence in various water bodies, including rivers, streams, lakes, and seawater (Felício et al., 2018). According to the European Food Safety Authority's 2005 report, the half-life (DT50) of diuron in soil under aerobic conditions spans from 14 to 372 days. In research by (Mercurio et al., 2016), it was found that the DT50 of diuron in water reaches 499 days in dark conditions, indicating a notably slow rate of natural degradation. Additionally, various studies, including those by (Camenzuli et al., 2012; Maqbool et al., 2016; Moisset et al., 2015) have verified that diuron makes its way into both surface water and groundwater via irrigation, drainage, percolation, and surface runoff, as depicted in (Figure 1.2).

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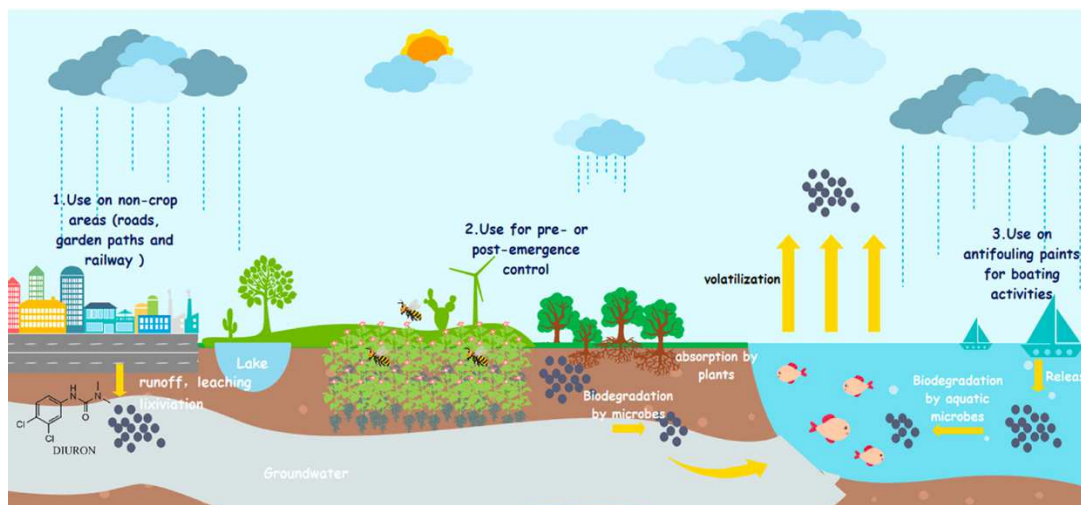
Figure 1.2 The environmental fate and presence of diuron (1) Diuron infiltrates groundwater via leaching. (2) Diuron is introduced into surface water through runoff. (3) Diuron is released into the atmosphere by means of volatilization (Adapted from Li et al. (2021)).

1.1.5 Bioaccumulation of diuron in the aquatic environment

Diuron acts as a pollutant in water environments due to its usage in antifouling paints for boats. A study on Japan's water bodies revealed that 86% of the samples had diuron concentrations of 3.05 µg/L(Okamura et al., 2003). In the coastal and marine areas of the Netherlands, diuron levels exceeded the allowable limit of 430 ng/L (Lamoree et al., 2002). Numerous other investigations have also indicated diuron pollution from antifouling paints in water (Okamura et al., 2003; Okamura et al., 2002). Both diuron and its by-products were found in surface waters and seabed sediments (Thomas et al., 2002), though in-situ biodegradation data is scarce. Laboratory tests showed that diuron did not biodegrade over a 42-day period in seawater at 15°C, while its degradation products were less persistent (Thomas et al., 2002).

Mainly detected in seawater, diuron's sorption to marine sediments increases with the rise in particulate matter, which differs from other biocides (Voulvoulis et al., 2002). This sorption process seems somewhat irreversible (Giacomazzi & Cochet, 2004).

The spread of diuron in agriculture results in the contamination of the aquatic environment through the leaching of soil (Louchart et al., 2000; Thurman et al., 2000). As per the French Environmental Institute (IFEN, 1998), diuron is identified in 28% of the samples taken from rivers within the

national basin system (Giacomazzi & Cochet, 2004). Diuron contributes to aquatic pollution due to its utilization as an antifouling paint biocide (Okamura et al., 2003).

In coastal and marina waters in the Netherlands, concentrations exceeding the allowable maximum of 430 ng/L were identified (Lamoree et al., 2002). Diuron and its degradation products were identified in surface waters and sediment layers (Thomas et al., 2002), but limited information is available regarding in situ biodegradation.

1.1.6 Metabolic pathway of diuron in the environment

This herbicide, when applied to soil, demonstrates considerable longevity, ranging from four to eight months, which varies based on soil moisture and type (Copping & Hewitt, 1998; Rouchaud et al., 2000). However, its presence has been frequently detected in surface water (Garmouma et al., 1998; Rouchaud et al., 2000). Given the increasing concerns about its ability to contaminate both surface and groundwater, extensive research has been conducted on its direct and indirect impacts on both the intended and unintended organisms, with diuron being suspected of having genotoxic properties (Copping & Hewitt, 1998). While most of the toxicity research has been centered on the herbicide itself (Call et al., 1987; Flum & Shannon, 1987; Sobieszczanski et al., 1981; Tan & Chua, 1987), its degradation products are also potential contributors to environmental pollution. Consequently, it's critical to gather information regarding the behavior and fate of this herbicide in soil post-application.

Diuron undergoes both abiotic and biotic degradation in the environment. Abiotic degradation includes hydrolysis and photodegradation, where diuron is transformed into various products, including 3,4-dichloroaniline (3,4-DCA). Biotic degradation primarily occurs through microbial action under both aerobic and anaerobic conditions. Microorganisms in soil and water environments can metabolize diuron, leading to the formation of intermediate compounds. The efficiency and pathways of these degradation processes depend on environmental factors such as soil type, microbial communities, and the presence of other substances. In this study, we used abiotic degradation which we will talk about in the next section. The transformation of diuron in the environment is significant because its degradation products, especially 3,4-DCA, can be more toxic than diuron itself, posing environmental and health risks (Giacomazzi & Cochet, 2004). Once

introduced into aquatic systems, diuron can undergo degradation processes such as single and double demethylation, as well as photodegradation. These processes lead to the creation of several by-products (Figure 1.3) like fenuron (1,1-dimethyl-3-phenylurea), DCPMU (1-(3,4-dichlorophenyl)-3-methylurea), DCPU (1-(3,4-dichlorophenyl) urea), and 3,4-DCA (3,4-dichloroaniline) (Dewez et al., 2002; Jirkovský et al., 1997).
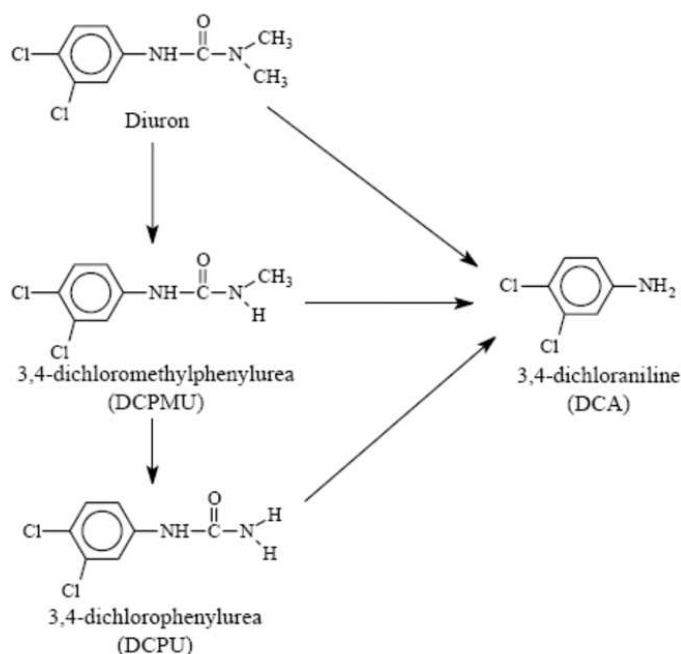
Figure 1.3 Pathways for degradation of diuron (Çokay Çatalkaya, 2011)

1.1.6.1 Abiotic degradation: photolysis and hydrolysis

Diuron demonstrates a notably slow natural hydrolysis rate in neutral solutions at 25 °C, leading exclusively to 3,4-dichloroaniline (3,4-DCA) as its irreversible degradation product in water. This hydrolysis is accelerated by the presence of OH^-, H^+ ions, and a buffer (Salvestrini et al., 2002). This study suggests that in natural environments, both organic and inorganic matter present in the soil, when dissolved in water, can catalyze diuron's chemical degradation. Complementing this, earlier findings by (Frost & Pearson, 1963) indicate that the decomposition of urea, a related

process, results in the formation of isocyanic acids or isocyanates, which are further hydrolyzed into amines and carbon dioxide. This underlines the complex interactions and transformations that diuron undergoes in environmental settings (Figure 1.4).
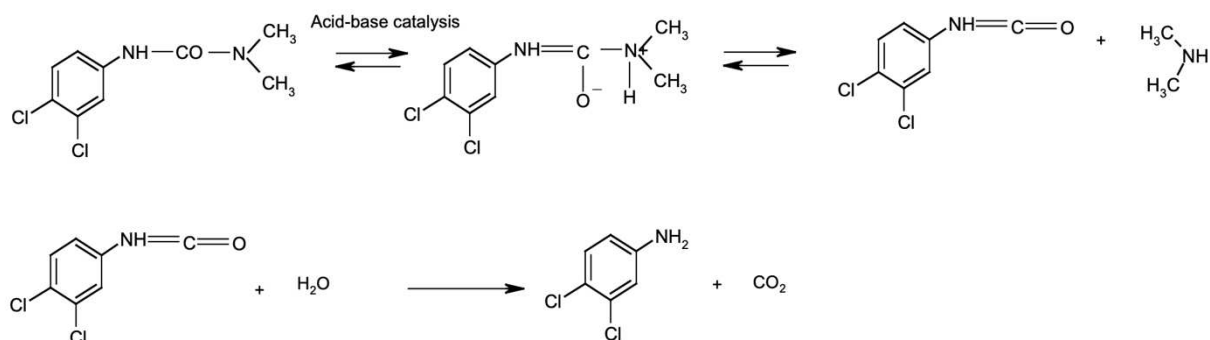
Figure 1.4 Predicted pathway reactions of chemical degradation of diuron (Salvestrini et al., 2002)

Some studies, including (Malato et al., 2002), have explored the reduction of diuron in water through photolysis, specifically using Photo-Fenton or TiO_2 systems that utilize UV light in the 200 to 300 nm range. These methods can achieve up to 90% mineralization (removal of Total Organic Carbon) in about 125 to 159 minutes, though complete mineralization takes over 200 minutes. This shows potential for wastewater treatment. However, under natural UV sunlight in seawater, the photodegradation of diuron is much less effective (Okamura et al., 2002), which could explain its long half-life of one month to a year in such environments.

1.1.6.1.1 Fenuron

Fenuron (1,1-Dimethyl-3-phenylurea), a phenylurea with the formula $C_9H_{12}N_2O$ (Figure 1.5), is commonly used in cereal farming to control weeds (Mazellier et al., 2007). Despite its agricultural utility, Fenuron is environmentally persistent, resistant to microbial degradation, and known for contaminating both surface and ground waters through runoff (Oturan et al., 2010). Its aqueous direct photolysis is relatively inefficient, with a quantum yield of only 0.006 under 254.0 nm irradiation (Mazellier et al., 2007). Fenuron's high solubility (3850.0 mg/L) and environmental longevity mean it can persist as a toxin in both surface and groundwater (Kribéche et al., 2016b;

Blanchoud et al., 2004). Notably, in the Three Gorges reservoir and its tributaries in China, Fenuron was detected in 3 % of surface water samples at concentrations ranging from 0.22 to 0.33 µg/L (Wolf et al., 2013). In rural China's groundwater, it was found in 41.0% of samples from 166 villages, with concentrations up to 58.0 ng/L (Li et al., 2020).
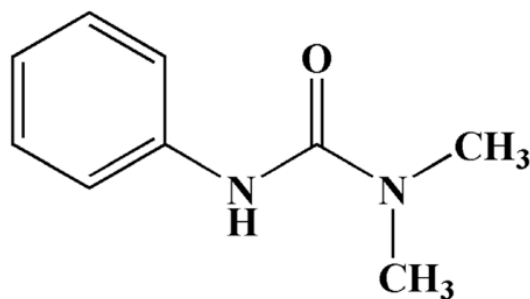
Figure 1.5 The chemical structure of fenuron (1,1-Dimethyl-3-phenylurea) C9H12N2O (Hayat, et al., 2022)

1.1.6.1.2   1-(3,4-Dichlorophenyl)-3-methylurea (DCPMU)

DCPMU is one of the main diuron metabolites (Pesce et al., 2010) herbicide with a chemical structure characterized by a phenyl ring with two chlorine atoms and a dimethylurea group, giving it the formula $C_9H_{10}C_{12}N_2O$ (Figure 1.3).

1.1.6.1.3   3,4-dichloroaniline (DCA)

3,4-Dichloroaniline (3,4-DCA), a common degradation product of diuron, poses significant risks to aquatic environments (Crossland, 1990). Due to its high-water solubility (580 mg L^{-1} at 20 °C) and a relatively long half-life of 18 days without hydrolysis (IHCP 2006), 3,4-DCA is a persistent water contaminant. It endangers the growth, development, and propagation of aquatic organisms, underscoring the need for careful management and monitoring of pesticide use and its environmental impacts (Schafers and Nagel, 1991; Bozena and Danuta, 1998; Palau-Casellas and Hutchinson, 1998; Ramos et al., 2002).

1.2 Aquatic plant growth

1.2.1 Description of *Lemna minor*

Lemna, recognized as duckweed, belongs to the monocotyledonous free-floating aquatic macrophytes within the Lemnaceae family. These plants are typically found in still or slowly moving waters abundant in nutrients across tropical and temperate regions (Mkandawire & Dudel, 2007). *Lemna minor* has a broad distribution, ranging from tropical to temperate regions and inhabiting various environments from freshwater to slightly saline water (Hillman & Culley, 1978). *Lemna minor* exhibits significant potential in phytoremediation, addressing various aquatic pollutants such as organic pollutants, heavy metals, agrochemicals, pharmaceuticals, and more (Ekperusi et al., 2019). Morphologically, *L. minor* is characterized by one or a small number of leaves—termed fronds—and a single root or rootlet. Notably, it lacks a stem. The fronds, which measure approximately 2-4 mm in diameter, tend to cluster and form dense mats on the surface of water bodies, including both freshwater and brackish environments (Figure 1.6) (Correll & Correll, 1972; Rusoff et al., 1980). The frond replication rate for *L. minor* is approximately 1.4 days. When cultivated in a lab setting, Duckweed can grow continuously, given sufficient nutrients, light, and water, thereby yielding an endless supply of Duckweed samples for immediate use (Frick, 1985).
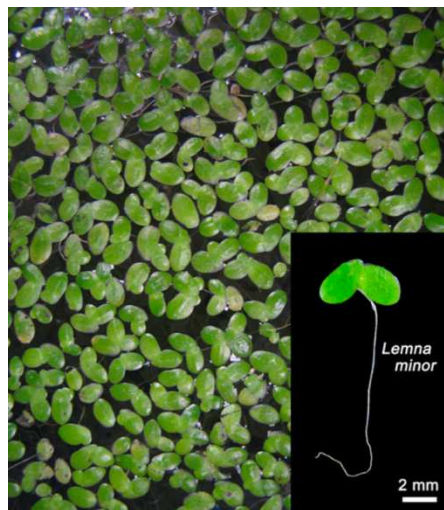
Figure 1.6 The common duckweed *Lemna minor*. Numerous fronds are viewed from above, and one representative plant is shown inside view (Kutschera & Niklas, 2015).

1.2.2 Scientific classification

The duckweed species in this study belong to the Kingdom Plantae, Subkingdom Tracheophytes, Clade Angiosperms, Subclade Monocots, Order Alismatales, Family Araceae, Subfamily Lemnaceae, Genus Lemna, and are specifically identified as *L. minor* (Wang, 1991). The Lemnaceae family encompasses around 40 species globally. This family includes a collection of aquatic monocotyledonous macrophytes, which are distributed across regions such as South America, Central Africa, Europe, South Asia, and southern Australia.

1.2.3 Cultivation of *Lemna minor*

The cultivation of *Lemna minor* necessitates a continuous water supply and nutrient provision from organic manure or fertilizers. Each individual frond can generate up to 10 generations of daughter plants within a span of 10 days to several weeks before eventually perishing. Under optimal conditions of nutrient availability, sunlight, and temperature, the plant's mass can double in less than 2 days, surpassing the growth rate of most other higher plants. To maintain the productivity and well-being of duckweed colonies, regular care and frequent harvesting are necessary throughout the year (Adesina et al., 2005).

In cultivation, nutrient sources for *Lemna minor* encompass animal dung, household food scraps, by-products from food processing facilities, and slaughterhouse waste. When grown under controlled conditions, it's advisable to harvest *Lemna minor* regularly, ideally daily. Approximately 10 to 35 % of the duckweed can be gathered each day, while leaving the rest in the pond to continue growing (Hasan et al., 2009).

For optimal growth, Duckweed necessitates a pH value ranging from 5 to 9, ambient temperatures spanning 6 to 33 degrees Celsius, and a water depth of approximately 0.5 meters (Hasan et al., 2009; Leng et al., 1995). Additionally, it requires approximately 60 mg/L of nitrogen and a minimum of 1 mg/L of phosphorous for its growth. In ideal conditions, a duckweed cultivation area is capable of yielding 10 to 30 tonnes of dried duckweed per hectare annually (Leng et al., 1995). In laboratory settings, *Lemna minor* demands a pH between 6 and 7.5, along with adequate quantities of nitrogen, phosphorus, and potassium, complemented by other vital nutrients such as sulphur and sodium (Ekperusi et al., 2019).

1.2.4 Use of *Lemna minor* in laboratory toxicity studies

Lemna minor has become widely utilized in ecotoxicological studies as exemplary model organisms. The *Lemna* species offer numerous benefits such as their simplistic structural and morphological makeup, compact size facilitating the use of minimal sample volumes, swift growth dynamics, straightforward cultivation and management, considerable uniformity, and a pronounced sensitivity to a diverse array of pollutants (Park et al., 2012).

Lemna minor possesses a notable capacity for accumulating xenobiotic substances from aquatic environments, attributable to its expansive leaf surface area, particularly the abaxial side which maintains constant contact with the water surface (Mohan & Hosetti, 1999; Wang, 1990).

Traditionally, plants in freshwater ecosystems were thought to be less reactive to chemical substances than aquatic animals. Yet, research indicates that *Lemna minor* demonstrates greater sensitivity to a range of toxic metals, effluents, and pesticides compared to fish species (De Carvalho et al., 2007; Demirezen & Aksoy, 2006; Paczkowska et al., 2007; Teisseire et al., 1999).

In 1979, *Lemna minor* was suggested as a macrophyte model to represent aquatic ecosystems for the assessment of chemical product safety in the environment. Consequently, testing methods using duckweed have been endorsed by various national and international bodies, such as the Organization for Economic Co-operation and Development (No, 2006).

1.2.5 Standardized toxicity tests: Measurement parameters

Lemna minor is favored in ecotoxicology and metabolomics research for assessing the toxicity of substances in aquatic environments. The use of this species use has been validated by numerous studies (Aliferis et al., 2009; Kostopoulou et al., 2020; Kummerová et al., 2016; Mkandawire et al., 2014). Recognized globally, both the International Organization for Standardization (ISO) and the Organization for Economic Co-operation and Development (OECD) have established a standard test using *L. minor* to measure growth inhibition.

The primary aim of bioassay testing is to enhance risk assessment and water quality management by establishing representative criteria for exposure conditions. Utilizing multiple endpoints in

testing is more beneficial for comprehensive risk evaluation of toxic substances compared to single endpoint assessments. This multi-faceted approach not only offers deeper insights into the mechanisms of toxicity but also highlights the relative sensitivity of different measured endpoints to the concentration and duration of exposure to toxicants. This method is particularly effective in pinpointing specific endpoints that are most responsive to disturbances caused by particular phytotoxic substances (Nestler et al., 2012).

A variety of endpoints have been utilized in studies involving *Lemna* species. These include measurements of the number of fronds and plants, count of roots, both dry and fresh biomass, dimensions or area of fronds, length of roots, rate of carbon uptake, and chlorophyll concentration, among others (Wang, 1990).

In this study, we explored the impact of diuron on *Lemna minor*, focusing on various endpoints at the physiological level, such as growth and photosynthetic efficiency, and at the biochemical level, including the generation of reactive oxygen species (ROS).

1.2.6 Photosynthesis

Photosynthesis, essential for oxygen production and energy provision on Earth (Bryant & Frigaard, 2006), involves converting light energy into chemical energy by green plants and some other organisms. This process uses protein complexes containing chlorophyll pigments in chloroplasts to transform water into oxygen and light energy. Energy is stored as sugars, later metabolized into complex molecules like amino and fatty acids (Buchanan et al., 2015). During the Calvin cycle, carbon dioxide is assimilated into organic compounds such as ribulose diphosphate (RuBP). Photosynthesis occurs in two phases: firstly, light-independent reactions create energy storage molecules (ATP and NADPH), and secondly, these compounds facilitate CO_2 reduction in biochemical reactions. This process is fundamentally similar across plants, algae, and some bacteria (Ducat & Silver, 2012).

1.2.7 Photosynthetic pigments

Photosynthesis, unique to plants and algae, occurs in chloroplasts, converting light into chemical energy (ATP and NADPH) within thylakoid membranes. These membranes contain two

photosystems, PSI and PSII, each with pigments having an antenna to capture photon energy and a reaction center for energy transfer. The primary photosynthetic pigments, chlorophyll (Chl) *a*, *b*, and *c*, with Chl *a* as the main and Chl *b* and *c* as accessory pigments, alongside carotenoids and xanthophylls, absorb and reflect specific light wavelengths (Lichtenthaler, 1987). Each pigment responds to different spectrum ranges, necessitating multiple types for effective light capture (Perera-Castro & Flexas, 2020). The absorption spectra of these pigments collectively span a wide range of the light spectrum (Figure 1.7). They include the blue-green spectrum (450-475 nm), the red spectrum (630-675 nm), and the range of carotenoids, which extends from 400 to 550 nm (Masojídek et al., 2013).

Chlorophyll's primary function in photosynthesis is to absorb light photons, initiating the process. Carotenoids, in contrast, serve a protective role by safeguarding chlorophyll pigments from photooxidation during intense light exposure (Baroli et al., 2004).
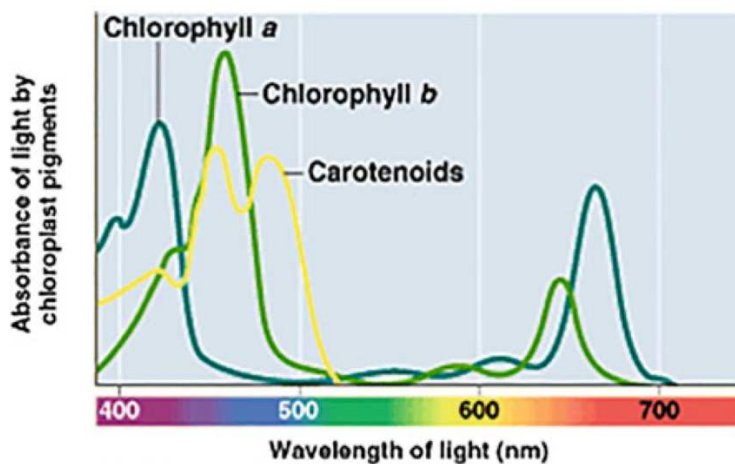
Figure 1.7 Absorption spectra of photosynthetic pigments (Naznin & Lefsrud, 2017)

1.2.8   Oxidative stress caused by contaminants: Reactive oxygen species (ROS)

The environment is an intricate network of interactions between living and nonliving entities, maintained in equilibrium through various natural processes. Every species both influences and is influenced by its environment, indicating a dynamic interplay between all components of the ecosystem (Xie et al., 2019). Environmental factors like salinity, drought, extreme temperatures,

metal pollution, air pollutants, and UV exposure pose significant challenges to the environment. In plants, these conditions, along with the overuse of pesticides and pathogen attacks, can lead to oxidative stress, impacting their growth and physiological health (Choudhury et al., 2013; Foley et al., 2016; Limón Pacheco et al., 2017). Oxidative stress in cells arises from two main sources: direct environmental stressors and the indirect effects of reactive oxygen species (ROS) generation and accumulation. These ROS can cause cellular damage before they are effectively eliminated from the cell (Xie et al., 2019). When the reactive oxygen species (ROS) levels in plant cells exceed their internal defence mechanisms, the cells enter a state of oxidative stress. This stress can significantly impact various aspects of plant growth and development, leading to outcomes such as delayed growth, abscission of flowers and leaves, altered root gravitropism, hindered seed germination, affected polar cell growth, changes in lignin synthesis in cell walls, and accelerated cell senescence (Xie et al., 2019). The increase in reactive oxygen species (ROS) levels is notable for its high reactivity and broad impact on cellular, physiological, and biochemical functions. This increase can lead to significant cellular disruptions, including damage to the plasma membrane through the oxidation of carbohydrates, lipid peroxidation, protein denaturation, and the destruction of vital cellular components like DNA, RNA, enzymes, and pigments (Li et al., 2018; Bose et al., 2013).

The most prevalent reactive oxygen species (ROS) are the superoxide anion ($O_2^{\cdot-}$), molecular oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH^{\cdot}$). Normally, molecular oxygen is inactive due to its electron structure. However, its metabolic imbalance can result in ROS production. This includes free radicals like superoxide radical, hydroxyl radical, perhydroxyl radical, and alkoxy radical, as well as nonradical species such as hydrogen peroxide and singlet oxygen (Hossain et al., 2015; Kalia et al., 2017).

1.3 Advanced oxidation process

It is essential to employ the most effective method to degrade and minimize the toxic effect of herbicides on the environment. Advanced oxidation processes are employed to transform pollutants into end products like CO_2 and H_2O, or into intermediate substances that are more amenable to biodegradation or can be more easily eliminated through adsorption (Sell, 1992). Initially introduced in the 1980s for the treatment of drinking water (Glaze, 1987; Glaze et al.,

1987), Advanced Oxidation Processes (AOPs) use potent hydroxyl or sulphate radicals as primary oxidizing agents. Subsequently, these processes have been widely implemented for treating various types of wastewaters. This is due to the effectiveness of the strong oxidants in readily breaking down stubborn organic pollutants and eliminating certain inorganic contaminants present in wastewater (Deng & Zhao, 2015).

Among many techniques that have been proposed to eliminate herbicides, Advanced oxidation processes (AOPs) are one of the widely used ones – engaging in oxidation reaction with a powerful, non-selective hydroxyl radical (OH•) resulting in oxidation of recalcitrant pollutants that are resistant to conventional treatment approaches and could improve the biodegradability of wastewater (Rekhate & Srivastava, 2020). Their action aims to convert these contaminants into substances that are less harmful or even non-toxic, offering a comprehensive solution for wastewater purification (Huang et al., 1993). (Figure 1.8) represents the mechanism of the advanced oxidation process. The process of pollutant degradation begins with their combination with carbon compounds to form R–HO in an oxygen-rich environment. This reaction leads to the formation of more reactive species that then actively break down the pollutants (Fernandes et al., 2020).
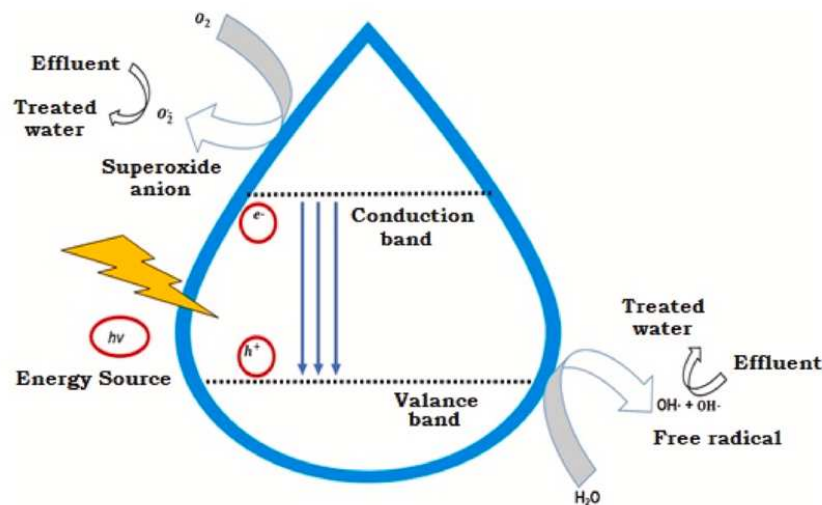
Figure 1.8 Mechanism of advanced oxidation process (Saravanan et al., 2022)

1.3.1 Ozonation process

Among AOPs, ozonation is particularly appropriate for the treatment of eco-friendly water leading to total mineralization of organic pollutants (Shahidi et al., 2014). The effectiveness of ozonation in water treatment is attributed to its oxidative properties, which effectively degrade pollutants. This method is particularly efficient in oxidizing elements like iron, arsenic, and manganese, transforming them into insoluble forms. These oxidized substances are then easily removed from water through subsequent filtration processes (Saravanan et al., 2022).

Ozone is a source of hydroxyl radicals. Ozone, recognized for its specificity and efficiency as an oxidant, possesses a standard oxidation potential of 2.1 volts. The process of oxidizing organic compounds through ozone treatment can occur via two distinct mechanisms. The first mechanism involves direct oxidation by ozone itself, as shown in the following reaction:

$$RCH{=}CHR' + O_3 \rightarrow RCHO + R'CHO + O_2$$

The second mechanism is indirect oxidation, which occurs through the decomposition of ozone and the subsequent formation of hydroxyl radicals. These radicals are generated when ozone reacts with hydroxide ions (OH^-) at neutral or basic pH levels, as indicated in the following reactions:

$$O_3 + OH^- \rightarrow O_2 + HO_2^-$$

$$O_3 + HO_2^- \rightarrow O_2 + O_2^{\bullet -} + {\bullet}OH$$
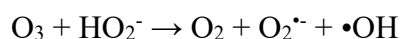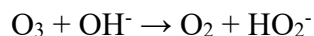
Typically, the indirect oxidation process involving hydroxyl radicals is faster than the direct oxidation method (Singh et al., 1995). The oxidation process is further enhanced in the presence of hydrogen peroxide, leading to a rapid and effective formation of hydroxyl radicals as shown in the reaction below (Sánchez-Lafuente et al., 2002):

$$2\,O_3 + 3\,H_2O_2 \rightarrow 4\,O_2 + 2\,OH{\bullet} + 2\,H_2O$$
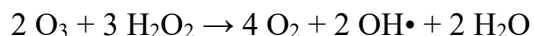
This combined ozone-peroxide oxidation, known as peroxone treatment, is widely utilized for the degradation of microorganic pollutants, such as pesticides, due to its efficiency and simplicity. Once the hydroxyl radicals are formed, they initiate a chain of radical reactions, leading to the

oxidation of contaminants. This follows similar mechanisms to those seen in ozonation at elevated pH levels (Çokay Çatalkaya, 2011).

1.4 Overall issue and goals of the research

Urban and agricultural waste significantly contribute to surface water and sediment contamination, leading to severe water pollution that affects both wildlife and humans. Traditional chemical analysis methods are often inadequate in assessing water quality because these waters contain a vast number of pollutants at concentrations too low for analytical detection. Furthermore, chemical methods cannot predict the toxic or genotoxic effects of these complex mixtures. Therefore, it's crucial to use biological testing systems that employ living cells or organisms. These systems can provide a comprehensive response to the complex mixture of chemicals in water, without needing prior knowledge about the mixture's composition or chemical characteristics. This approach is essential for accurately assessing the health risks posed by water pollution (Radić et al., 2011). Plant assays are highly sensitive to many environmental pollutants, including heavy metals (Fiskesjö, 1985) and have been used for monitoring the potential synergistic effects of mixtures of pollutants (Wang & Freemark, 1995). Duckweed (*Lemna minor*), an aquatic plant that floats on freshwater surfaces, serves as a model organism for evaluating the impact of chemicals in aquatic environments (Zezulka et al., 2013).

Diuron is commonly used in agriculture and as a component of antifouling paints. Despite its widespread application for the selective management of broad-leaved weeds and mosses, the toxicological effects of diuron on species that are not the intended targets remain inadequately explored (Kumar & Han, 2010). Diuron, a harmful herbicide, has led to significant contamination issues in underground and surface waters across numerous nations (Bumroongsakulsawat et al., 2020). Existing research has primarily examined the toxicity of the herbicide, but it's crucial to also consider the environmental impact of its degradation products. To better understand diuron fate after soil application, gathering data is essential. Laboratory-scale studies, alongside field research in contaminated areas, are valuable. To fulfill this goal, this study follows the ensuing aims:

a. Investigate the impact of diuron and its intermediates - fenuron, DCPMU, and 3,4-DCA on the growth and photosynthetic efficiency of plants, as well as on biochemical aspects like pigment synthesis and reactive oxygen species (ROS) levels. The underlying theory is that diuron and its derivatives negatively affect the plant's physiological and biochemical processes, and that the severity of this toxicity is contingent on the concentrations of diuron and its intermediates.

b. Determine the detrimental impacts of diuron at a concentration of 100 µg/L on *Lemna minor* after undergoing ozonation for periods of 5, 10, 20, 30, and 40 minutes, followed by a seven-day exposure. The concentration of 100 µg/L was selected following a range finding test, conducted to establish appropriate test concentrations for experiments aimed at determining EC50 values. Focus on assessing physiological functions such as growth and photosynthetic efficiency. Our hypothesis is that ozonation for varying durations will differentially mitigate the detrimental effects of diuron on *Lemna minor*.

2   CHAPTER II

MATERIAL AND METHODS

2.1   Preparation of vessels

This research employed 250 mL glass Erlenmeyer flasks as containers. All the glass used for culturing *Lemna* underwent rigorous cleaning following three rinses with Milli-Q water sourced from a Barnstead^{TM} Thermo Scientific water purification system (with a conductivity of 18.2 MΩ-cm). Prior to experiments, all materials were autoclaved for sterilization.

2.2   Initial prerequisites and cultivation of the *Lemna minor* plant

2.2.1   Preparation of SIS medium culture

In this study, we used medium culture SIS. This medium culture is a modified version of the Swedish Standardization Institute of *Lemna minor*. It was prepared according to the protocol of the Organization for Cooperation and Development Economic Standards (OECD) for toxicological testing (Guidelines 22 of OECD, 2006) (No, 2006). To prepare this medium, we must prepare seven stock solutions in one litre in advance. The stock solutions are as followed: 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. For sterilising stock solutions, I – VII the membrane filtration (0.2 μm pore size) was used to filter them. All stock solutions are sorted in a cool and dark place (refrigerator). Under these conditions, the shelf life of stock solutions I – V is six months while for stock solutions VI and VII is one month. SIS medium culture is composed of the following: 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 solution VI, 1 mL of solution VII plus 968 mL of Nanopure water. The PH of the medium culture should be at 6.5 ± 0.2 and to adjust the pH the dilute solutions of HCl and NaOH 0.1 M were used. The prepared growth medium culture was transferred to the

autoclaved glass vessel and then autoclaved for 1h. For using this medium culture, it should rest 2 days after preparation.

2.2.2 Culture of *Lemna minor* and conserve it in laboratory conditions

In this research, aquatic cultures of *Lemna minor* were employed for experiments (Figure 2.1). The study followed the OECD's 2006 guidelines for the standard duckweed growth inhibition test to expose the plants to various substances. Young *Lemna minor* plants were grown over seven days in 150 mL containers of SIS medium, maintaining a pH level of 6.5 ± 0.2.

Figure 2.1 Aquatic cultivation of *Lemna minor*.

The stock culture was housed in a controlled environment with regulated conditions, including a temperature of 24 ± 2 °C, relative humidity of $60 \pm 5\%$, and fluorescent lamps (Sylvania Grolux F36W) were used to achieve continuous lighting with a light intensity ranging between 85 and 135 μmol photons $m^{-2}\,s^{-1}$. The containers were covered to prevent evaporation and contamination while allowing essential air exchange for plant growth. Additionally, the culture was renewed every week (7 days) in accordance with OECD (2006) guidelines to ensure the quality of growth. The initial pH of the media was adjusted using HCl and NaOH solutions.

2.3 Preparation and analysis of diuron solutions by UHPLC-UV and UV-Vis

Diuron standard solutions were prepared from pure compound (Diuron $\geq$ 98 %, CAS 330-54-1, Sigma-Aldrich) in seven different concentrations (0.03, 0.3, 0.6, 1.25, 2.5, 5, 10 mg L^{-1}) to develop calibration curves by UV-Vis spectroscopy and HPLC-UV chromatography. To this end, the standard solutions of diuron were prepared by diluting the stock solution of diuron (10 mg L^{-1}) in nano pure water to prepare all standard solutions. By using these standard solutions, UV-Vis spectra were achieved at intrinsic pH and room temperature (Figure S1.a). This allowed the plotting of calibration curves for all four absorption bands (Figre S1.b). The most intense band (249 nm) corresponds to the $\pi \rightarrow \pi^*$ transition. Using another UV-Vis band (254 nm) allowed to HPLC-UV measurements. The calibration curve of HPLC-UV of diuron plotted helps this band which can be used for detecting diuron degradation and most oxidizing intermediates. Diuron peaks were symmetrical and well separated in diuron solutions. The main peak appeared at 4.84 min retention time (Figure S2. a,b). UV-Vis analysis was carried out by means of an Agilent-Cary 60 instrument (1 cm quartz cell). HPLC-UV analysis was performed by Agilent Technologies model 1290 High-Performance Liquid Chromatography coupled to an ultraviolet-visible detector (HPLC-UV). The column used was a C18 column (4.6 x 150 mm, 5 μm particle size). The mobile phase used for HPLC analysis was methanol (Purity >99.8 %, Sigma-Aldrich) and Nanopure water. Methanol used for mobile phase was degassed for 10 minutes in an ultrasonic bath before use. The eluent consisted of 80% methanol and 20% Nanopure water, and the flow rate was 0.5 mL min^{-1}. The injection volume and time were 20 μL and 10 minutes respectively in all samples. Each sample was filtered into HPLC vials. The quantification was carried out at several wavelengths; and finally, 254 nm was chosen.

2.4 Stock solution preparation for diuron and its intermediate

Stock solutions of diuron, fenuron, 1-(3,4-dichlorophenyl)-3-methylurea (DCPMU), 3,4-dichloroaniline (3,4-DCA) can be prepared in medium culture directly, but this process is very slow and time-consuming. In addition, the activation of dissolution of this solution is by ultra-sound followed by filtration. Therefore, all contaminants were dissolved in 1 mL methanol 99.8 % and then added to 1 litre SIS medium culture. They were dissolved in methanol to a final concentration of 0.1%.

Fresh stock solutions of four examined contaminants (100 µg/L) were prepared by dissolving the pure compound (Diuron ≥ 98 %, CAS 330-54-1, Sigma-Aldrich), (Fenuron, CAS 101-42-8, Sigma-Aldrich), (DCPMU, CAS 3567-62-2, Sigma-Aldrich), and (3,4-DCA, 98 %, CAS 95-76-1, Sigma-Aldrich) in the medium of SIS culture at pH 6.5. The prepared solutions were stirred for 2 h or until the solutions no longer contained material not dissolved. Then the stock prepared stock solution was filtered using a 0.2 µm pore size filter. The other concentration of tested solutions was prepared by diluting the 100 µg/L stock solution with SIS medium culture.

2.5 Range finding test for post-ozonation diuron toxicity

In this study, our emphasis was on evaluating the toxicity of the primary pesticide, diuron, after ozonation treatment. Before commencing individual toxicity experiments for diuron following exposure to ozonation, we assessed the effects of the target compounds on duckweed at various concentration levels ranging from 0.03 to 10 mg/L. The objective was to identify appropriate test concentrations for subsequent experiments aimed at determining EC50 values. All preliminary tests were conducted in triplicate (Gatidou et al., 2015). Hence, the concentration of 100 µg/L gave us EC50 values.

2.6 Toxicity test

The toxicity tests for diuron and its three intermediates (fenuron, DCPMU, 3,4-DCA) were conducted in a controlled environment chamber set at $24 \pm 2°C$. This setting included continuous light at an intensity of 100 ± 10 µmol photons m^{-2} s^{-1}, with a photoperiod comprising 16 hours of light and 8 hours of darkness. Each testing vessel held 100 mL of the test solution and was planted with three *Lemna minor* specimens, each having three fronds. For each contaminant concentration, three replicates were performed, as shown in (Figure 2.2). Experimental solutions with various contaminant concentrations (0, 25, 50, 75, 100 µg/L) were prepared by diluting stock solutions of diuron, fenuron, DCPMU, and 3,4-DCA with SIS culture medium, maintaining a pH of 6.5 ± 0.2. The study lasted for a period of 7 days. Upon its conclusion, the *Lemna minor* plants were collected, and their physiological and biochemical parameters were immediately assessed following the OECD's 2006 guideline 221.

Figure 2.2 Testing *Lemna minor* with diuron and its intermediate in a controlled lab environment.

2.7 Assessing the impact of diuron and its intermediates on the growth of biomass

2.7.1 Measuring the number of fronds and fresh weight

Following a 7-day incubation, *Lemna minor* plants were gathered from all containers. The count of individual fronds for each concentration was noted, and the fresh weight of each sample was recorded.

2.7.2 Measurement of the average specific growth rate

The measured response variable is the logarithmic change in both the number of fronds and fresh weight over time, calculated daily for both control and treatment groups. This is done in accordance with the OECD's 2006 formula.

$$\mu\, i - j = \frac{\ln(Nj) - \ln(Ni)}{t}$$

In the formula: $\mu\, i - j$ represents the average specific growth rate between times i and j; Ni is the initial frond count in the test or control container at time i; Nj is the final frond count at time j; and

t is the duration between i and j. The growth rate's average value was computed for each treatment and control group.

2.7.3 Calculation of the percentage of growth inhibition

The percentage of growth rate inhibition (Ir) for each concentration tested was determined using the OECD's 2006 specified formula.

$$\% \, Ir = \frac{\mu C - \mu T}{\mu C} \times 100$$

The formula defines % Ir as the percentage inhibition of the average specific growth rate, where μC is the average specific growth rate in the control group, and μT is the average specific growth rate in the treated group.

2.8 Measurement of photosynthetic pigments

Following a 7-day exposure period, the surviving *Lemna minor* plants were ground in a mortar with 5 mL of 100% methanol at room temperature. This process facilitated the extraction of key biochemical compounds. The resulting extract was then centrifuged at a force of 1968 × g for 5 minutes at 4°C to separate the supernatant (Benghaffour et al., 2023). The absorbance of this supernatant was meticulously measured using a micro-plate reader at specific wavelengths: 665 nm for Chlorophyll *a*, 648 nm for Chlorophyll *b*, and 470 nm for carotenoids (Lee et al., 2021). These readings, conducted at different wavelengths, allowed for a comprehensive analysis of the plant's pigment content and overall health in response to the exposure conditions.

The concentrations of these pigments, expressed in micrograms per millilitre ($\mu g \, mL^{-1}$), were calculated following equations (H. K. Lichtenthaler, 1987; Lichtenthaler & Wellburn, 1983).

$$Chl \, a = 15.65 \times (A_{666}) - 7.34 \times (A_{653})$$

$$Chl \, b = 27.05 \times (A_{653}) - 11.21 \times (A_{666})$$

$$Cx + c = \frac{1000\,(A_{470}) - 2.86\,(Chl\ a) - 129.2\,(Chl\ b)}{245}$$

In these equations, Chl *a*, Chl *b*, and Cx+c represented the quantities of chlorophyll *a*, chlorophyll *b*, and carotenoids, respectively, measured in micrograms per millilitre. The absorbance readings at A666, A653, and A470 correspond to these pigments. For accurate assessment, these chlorophyll concentrations were standardized against the fresh weight of the plants, expressed in milligrams.

2.9 Production of reactive oxygen species (ROS)

To assess ROS levels within the cytoplasm of live cells, the plants underwent a detailed process. They were first incubated in a microplate to 96 wells for 30 minutes at 25°C with CellROX orange (Invitrogen® by Thermo Fisher Scientific) at 5 µM, a fluorogenic probe that has specific absorption and emission peaks (545/565 nm) (Bone et al., 2013). This reagent detects cytoplasmic ROS as described by the manufacturer. Whole plants were incubated in a 96-well microplate with 5 µM CellRox® Orange. After 30 minutes, the plants were washed with phosphate-buffered saline (PBS 1X) at room temperature. Fluorescence was then read at an excitation wavelength of 545 nm and an emission wavelength of 565 nm, using the Infinite M200 fluorescence microplate reader (TECAN®) (Jmii & Dewez, 2021). Results were normalized to fresh weight and were represented as a percentage compared to the control.

2.10 Diuron test and analytical methods after ozonation

The ozonation process, a critical phase in the experimental protocol, was meticulously conducted using a precise ozone generation technique. This involved the introduction of ozone at a calibrated rate of 600 mg per hour, utilizing an A₂Z ozone generator (A₂Z Ozone Inc., USA). The experimental setup comprised plastic centrifuge tubes, each measuring 28 mm by 115 mm, containing 20 mL of a diuron solution at a concentration of 100 µg/L. The ozonation process was executed over a series of time intervals, including 0, 5, 10, 20, 30, and 40 minutes, to assess the efficacy of ozone treatment over time.

Post-treatment, the pH of the samples was meticulously adjusted to mirror that of the SIS medium culture, maintaining a pH level of 6.5 ± 0.2. These experiments were carried out under controlled ambient conditions, specifically at room temperature, within the confines of a laboratory fume hood to ensure safety and consistency.

During the ozonation phase, a continuous stream of the ozone-containing gas mixture was bubbled into the sample tubes to ensure uniform treatment. Subsequently, each ozonated sample was carefully transferred into individual pre-autoclaved Erlenmeyer flasks, each with a capacity of 50 mL, under sterile conditions.

For the toxicity assessment, a standardized approach was adopted. was exposed to three specimens of *Lemna minor*, each with three fronds was added to each ozonated diuron sample.

2.10.1 Identification of diuron solutions after ozonation by UV-Vis and UHPLC-UV

The removal efficiency of diuron after ozonation was calculated by UV-Vis and UHPLC-UV in Nanopure water. The relative peak area (PA/PAo) was calculated as the instant (PA) to initial (PAo) peak area ratio for diuron, the conversion yield being [1-(PA/PAo)]x100 %. These two complementary techniques were respectively used for the qualitative evolution in time of the reaction mixture and quantitative determination of the yields of conversion for both molecules and of production of their main derivatives.

2.10.2 Identification of diuron solutions after ozonation by LC-MRM

To investigate the degradation of diuron and its by-products during ozonation, all samples of SIS medium contaminated with diuron at a concentration of 100 µg/L were analyzed using LC-MRM. This analysis also aimed to identify the by-products, which were previously examined for their toxicity effects on *Lemna minor* following a seven-day exposure period. The LC-MRM method (Liquid chromatography coupled to multiple reaction monitoring) utilizes a Shimadzu Nexera HPLC system and a Sciex QTRAP 5500 Mass Spectrometer. The HPLC conditions include an Agilent Zorbax EclipseC18 column and a binary mobile phase consisting of water with 0.1% formic acid (phase A) and acetonitrile with 0.1% formic acid (phase B), with a flow rate of 0.5 mL/min and an injection volume of 10 µl. The gradient program for phase B starts at 5% and

increases to 95% over 6 minutes. The mass spectrometry is operated in positive Electrospray Ionisation (ESI) -MRM mode, with specific MRM transitions set for diuron and its related compounds, optimized with corresponding declustering potentials (DP) and collision energies (CE).

2.11 Statistical analyzes

In every toxicity assessment, mean values and associated standard deviations were calculated based on triplicate measurements (n = 3). Mean values and standard deviations were calculated for every treatment group. To identify significant differences between treated groups and controls, a one-way ANOVA and subsequent post hoc Tukey multiple comparison tests were employed. Statistical significance was established at $p < 0.05$, using Graph Pad Prism software version 10.1.1.

3 CHAPTER III

RESULTS

3.1 Determination of the effect of diuron and its intermediates on plant growth

In this research, the toxicological impacts of diuron, fenuron, DCPMU and 3,4-DCA in an aquatic environment were examined using the aquatic plant *Lemna minor*. The study demonstrated noticeable alterations in growth rates and sensitive biomarkers, signifying the occurrence of toxicity within the tissues and cells. According to the OECD guideline 221, which pertains to the inhibition of *Lemna* species growth, it is advised to measure plant growth rate by evaluating both the fresh weight and the frond count. The toxicity of diuron, fenuron, DCPMU and 3,4-DCA on the growth of *Lemna minor* at the end of the seventh day was estimated by measuring the fresh weight and the number of plant fronds.

After 7 days of exposure, in the case of diuron (Table 3.1 A), the control shows a rapid growth rate over seven days with an average fresh mass of 179 mg and an average number of fronds of 143. With increasing concentrations of diuron in the medium, a significant decrease in these parameters is observed. At the highest concentration of 100 µg/L, the fresh weight decreases to 8 ± 2.64 mg, and the number of fronds reduces to 10 ± 1.15, indicating a decrease of approximately 95.5% and 93% in comparison to the control, respectively.

After 7 days of exposure to fenuron (Table 3.1 B), the control presents an average fresh mass of 175 mg and an average number of fronds of 135. As the concentration of fenuron increases, we observe different responses in growth parameters. At the highest concentration of 100 µg/L, the fresh weight rises to 216 ± 14.36 mg, and the number of fronds increases to 148 ± 11, suggesting an increase rather than a decrease compared to the control.

When examining DCPMU (Table 3.1 C), the control condition results in an average fresh mass of 190 mg and an average number of fronds of 149. With the addition of DCPMU, there is a considerable decline in both parameters. At the highest concentration of 100 µg/L, the fresh weight

falls to 48 ± 1 mg, and the number of fronds drops to 65 ± 2.51, marking a decrease of about 74.7% and 56.4% in comparison with the control, respectively.

Lastly, in the case of 3,4-DCA (Table 3.1 D), the control has an average fresh mass of 174 mg and an average number of fronds of 146. Contrary to other substances, the presence of 3,4-DCA leads to an increase in both parameters. At the highest concentration of 100 µg/L, the fresh weight reaches 241 ± 11.53 mg, and the number of fronds rises to 154 ± 13.27, indicating an increase of about 38.5% and 5.5% compared to the control.

Table 3. 1 Variation in fresh weight (mg) and number of fronds after 7 days of exposure to diuron (A), fenuron (B), DCPMU (C), and 3,4-DCA (D) with different nominal concentrations in treated Lemna minor.

Label	Control	25 µg/L	50 µg/L	75 µg/L	100 µg/L
Fresh Weight(mg)	179 ± 16.82 a	33 ± 4.93 b	20 ± 1.52 b	12 ± 3.60 b	8 ± 2.64 c
Number of Fronds	143 ± 9.84 a	41 ± 3 b	22 ± 1.73 c	16 ± 2.10 c	10 ± 1.15 c

B

Label	Control	25 µg/L	50 µg/L	75 µg/L	100 µg/L
Fresh Weight(mg)	175 ± 12.48 a	174 ± 11 a	189 ± 12.85 a	180 ± 9.50 a	216 ± 14.36 b
Number of Fronds	135 ± 9.29 a	138 ± 10.40 a	150 ± 6.10 a	136 ± 9.10 a	148 ± 11 a

C

Label	Control	25 µg/L	50 µg/L	75 µg/L	100 µg/L
Fresh Weight(mg)	190 ± 8.73 a	134 ± 8.71 b	70 ± 1.52 c	62 ± 4.58 cd	48 ± 1 d
Number of Fronds	149 ± 8.38 a	119 ± 3.60 b	87 ± 2.51 c	83 ± 3.10 c	65 ± 2.51 d

D

Label	Control	25 µg/L	50 µg/L	75 µg/L	100 µg/L

Fresh Weight(mg)	174 ± 5.13 a	206 ± 8.32 b	230 ± 9.50 b	209 ± 10.81 b	241 ± 11.53 c
Number of Fronds	146 ± 13.27 a	170 ± 9.81a	184 ± 5.29 c	163 ± 11.35 a	154 ± 13.27 a

The results are presented as means ± standard deviation (n=3). When the lowercase letters associated with the values are the same, this indicates that there is no significant difference for $p < 0.05$ between the conditions tested for the same parameter.

3.2 Inhibitory effect of diuron, fenuron, DCPMU and 3,4-DCA on growth of *Lemna minor*

In this research, the percentage of growth inhibition was calculated from two complementary growth parameters, namely the frond number and fresh weight. We evaluated the inhibition of growth caused by the effect of diuron, fenuron, DCPMU, and 3,4-DCA at different concentrations (Figure 3.1). The graph shows a relationship between the concentrations of contaminants in the SIS medium and the inhibition of growth. These results showed an inhibitory effect of diuron on plant growth, which increased significantly depending on the concentration of diuron in the SIS medium culture (Figure 3.1 A). The lowest concentration applied was 0.0 µg/L diuron, showing no growth inhibition. In contrast, the exposure to 25.0 µg/L, 50.0 µg/L, 75.0 µg/L, and 100.0 µg/L of diuron, respectively, caused a corresponding inhibition of growth of 25%, approximately 50%, 75%, and nearly 100%. The percentage growth inhibition increased significantly with each step-up in concentration, particularly noticeable in the number of fronds, illustrating the compound's efficacy at lower concentrations and a possible plateauing effect at higher concentrations for fresh weight.

However, in the case of fenuron, no growth inhibition was observed after 7 days of exposure (Figure 3.1 B). The control group, with 0% growth inhibition, indicates normal growth without the presence of fenuron. As the concentration of fenuron increased from 0 to 100 µg/L, the graph illustrated a fluctuating response in growth inhibition, with both the number of fronds and fresh weight showing variability but no clear trend of increase or decrease. Specifically, at 25.0 µg/L and 50.0 µg/L, there was negligible inhibition or stimulation of growth, as indicated by the data points hovering around the 0% mark. At higher concentrations, 75.0 µg/L and 100.0 µg/L, the

fresh weight showed a slight decrease in growth, but the changes were not significant as they were all marked with the same statistical letter 'a', indicating no significant difference from the control. This suggests that fenuron, within the tested concentration range, has a minimal to no inhibitory effect on the growth of the aquatic plant, contrasting with the more potent herbicide diuron, which exhibited a clear dose-dependent inhibition.

Figure (3.1 C) shows the inhibition effect of DCPMU on plant growth after 7 days of exposure. The graph presents the correlation between the concentrations of DCPMU in the incubation medium and the consequent growth inhibition. As the concentration of DCPMU increases, there is a discernible increment in the inhibition of plant growth, both in terms of frond number and fresh weight. At the initial concentration of 25.0 µg/L, there is a modest but significant increase in growth inhibition for both parameters, shown by the letter 'b'. Progressing to concentrations of 50.0 µg/L and 75.0 µg/L, the growth inhibition continues to amplify, labelled with 'c', and ends in a marked inhibition at 100.0 µg/L for frond number, showing the highest growth inhibition at approximately 40% and labelled with 'd'. The fresh weight at 100.0 µg/L also exhibits significant inhibition but less so than the frond number, suggesting a differential sensitivity of the plant's growth parameters to DCPMU. The trend clearly indicates a dose-dependent inhibitory effect of DCPMU on the aquatic plant's growth.

Like the fenuron case, 3,4-DCA did not show significant inhibition of growth of the plant in SIS medium culture after 7 days of exposure (Figure 3.1 D). Initially, at 25.0 µg/L, there is a slight decrease in growth for frond number, which is then followed by an increase in growth at 50.0 µg/L. The trend in fresh weight somewhat mirrors this pattern but with less pronounced fluctuation. Notably, at higher concentrations of 75.0 µg/L and 100.0 µg/L, the inhibition in the number of fronds steeply declines, indicating a noticeable inhibitory effect. However, the fresh weight response deviates, showing a peak of growth stimulation at 75.0 µg/L before decreasing at 100.0 µg/L. All measured effects are statistically annotated with 'a', signifying no significant difference from the control across the concentration range for both growth parameters. This indicates that 3,4-DCA exhibits a complex interaction with the plants' growth, lacking a clear dose-dependent relationship within the tested concentration spectrum.

(A)

(B)

(C)

36

(D)
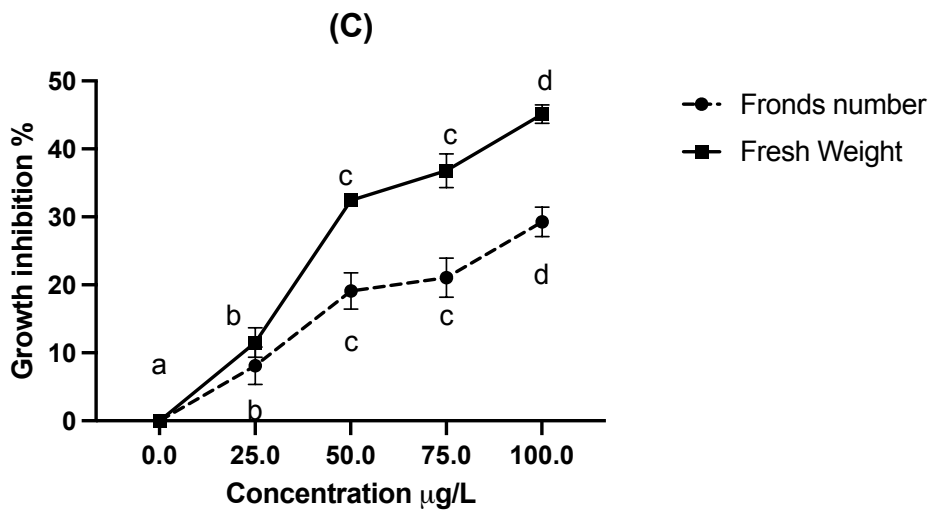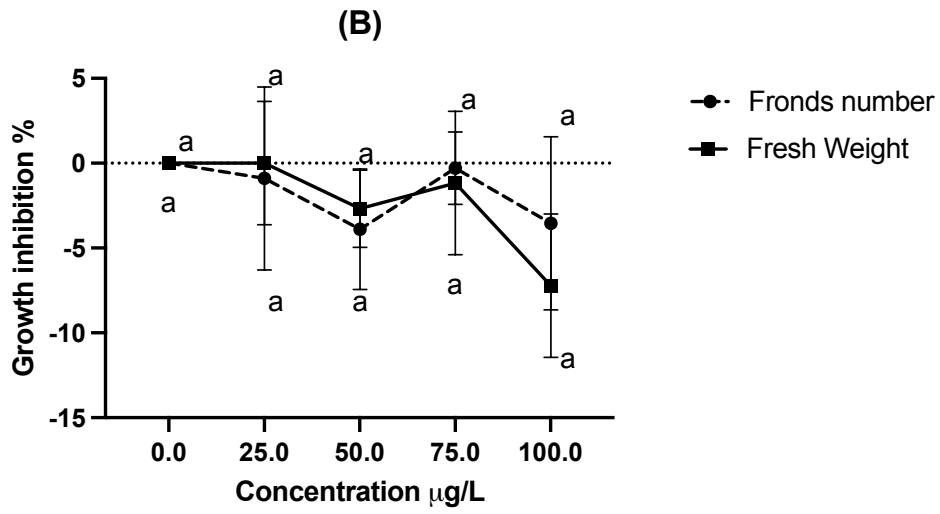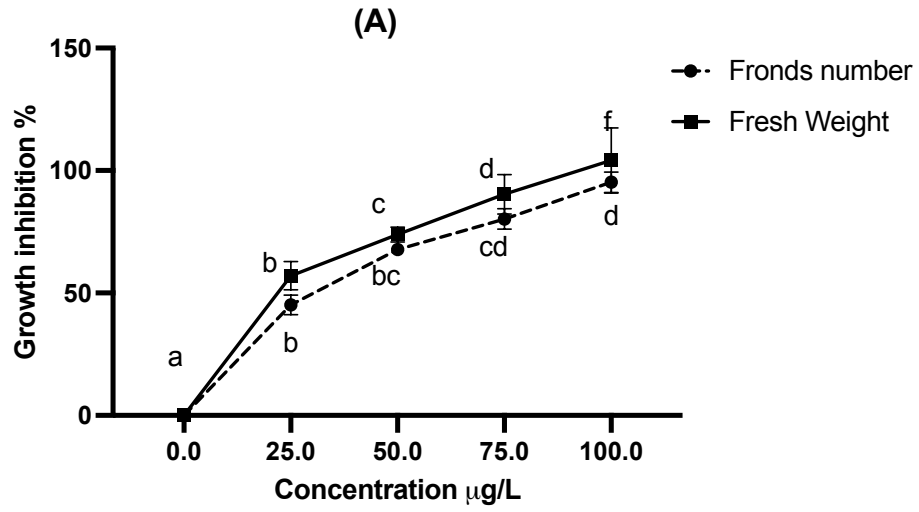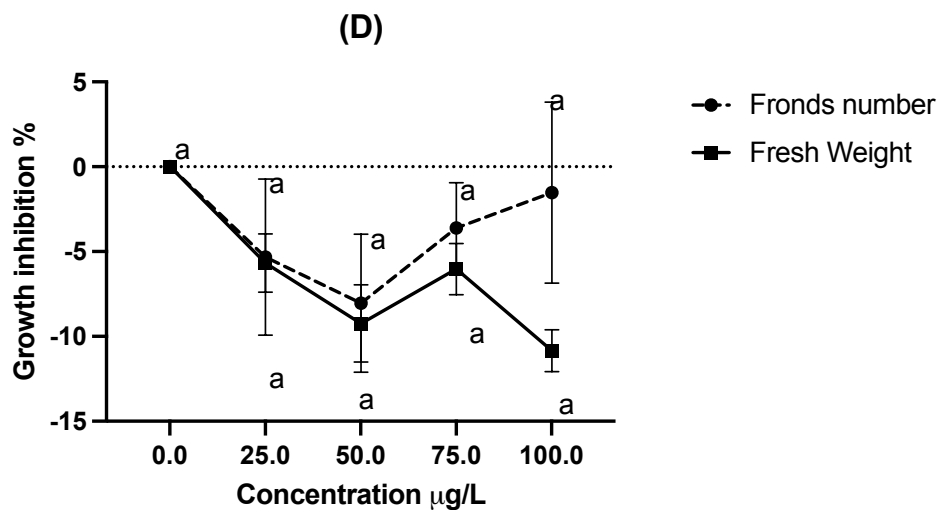
Figure 3.1 Inhibitory effect of diuron (A), fenuron (B), DCPMU (C), and 3,4-DCA (D) on the growth of *Lemna minor* (in percentage %) after 7 days of exposure to different concentrations. When the lowercase letters associated with the values are the same, this indicates that there is no significant difference ($p < 0.05$) between the different conditions tested for the same pH (ANOVA, Tukey HSD test) (n= 3).

3.3   Effect of diuron, fenuron, DCPMU and 3,4-DCA on photosynthetic pigments

Chlorophylls (*a* and *b*) and carotenoids are commonly used as biomarkers to evaluate the toxicity of contaminants. By analyzing the levels of these photosynthetic pigments in *Lemna minor* after a 7-day exposure to contaminants such as diuron, fenuron, DCPMU, and 3,4-DCA (Figure 3.2), a minor alteration in the levels of chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*) was noted.

The results obtained from diuron exposure (Figure 3.2 A) revealed a clear trend of decreasing chlorophyll *a* (Chl *a*) concentration in *Lemna minor* when exposed to increasing concentrations of diuron, ranging from 25 to 100 µg/L. The values are as follows: 4.32, 1.98, 1.25, 0.85, and 0.71 µg/mg FW. This decrease in Chl *a* is significant, as it follows a downward trajectory from the control group, with no overlap of the standard error indicators, suggesting a high level of statistical confidence. In contrast, chlorophyll *b* (Chl *b*) and carotenoids (Car) show a less pronounced decrease. Chl *b* maintains a relatively stable level across the various diuron concentrations, with slight fluctuations that do not indicate a strong dose-response relationship. Carotenoids demonstrate that they are not as significantly affected by diuron exposure as Chl *a*.

Like diuron, a dose-dependent response is observed for fenuron exposure (Figure 3.2 B); thus, the patterns are quite similar for each pigment type. Chlorophyll *a* (Chl *a*) levels peak at the 25 µg/L concentration with a value of 5.63 µg/mg FW and then taper off at 75 µg/L with a value of 4.21 µg/mg FW, indicating a biphasic response where an initial increase in concentration leads to a stimulation of Chl *a* content, followed by a decline at higher concentrations. The same trend is observed for chlorophyll *b* and carotenoids (Car).

The results obtained for DCPMU exposure, as indicated in (Figure 3.2 C), show a sharp drop in chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*) to 25 µg/L with values of 4.45 and 3.02 µg/mg FW, respectively. This is followed by a gradual decrease, hitting a low of 3.18 and 2.39 µg/mg FW for Chl *a* and Chl *b* respectively, and finally reaching the lowest point of content at 100 µg/L for both pigments. Also, in the case of carotenoids (Car) in DCPMU exposure, a slight decline is observed at 25 µg/L, after which the trend remains constant at the subsequent three concentrations.

In the case of 3,4-DCA (Figure 3.2 D), The data, marked by distinct symbols for each pigment, show that the pigment levels remain relatively constant irrespective of the concentration applied, as evidenced by the error bars and the 'a' notation suggesting no statistically significant differences. This indicates that the tested substance has no discernible impact on the pigment content within the concentration range studied.

(B)

(C)
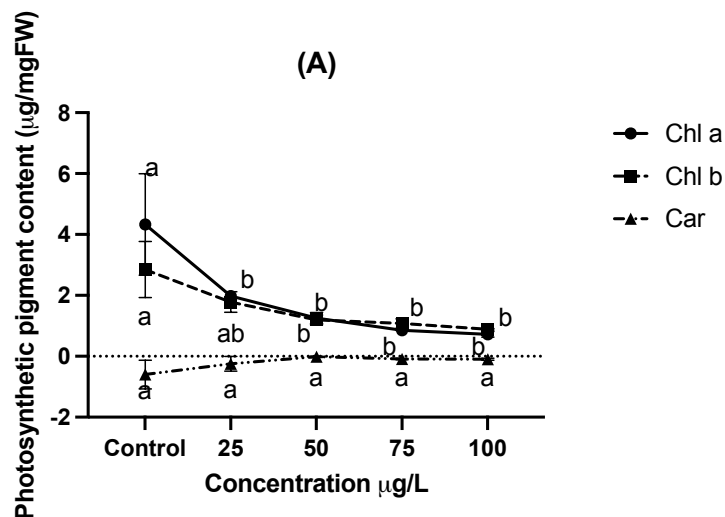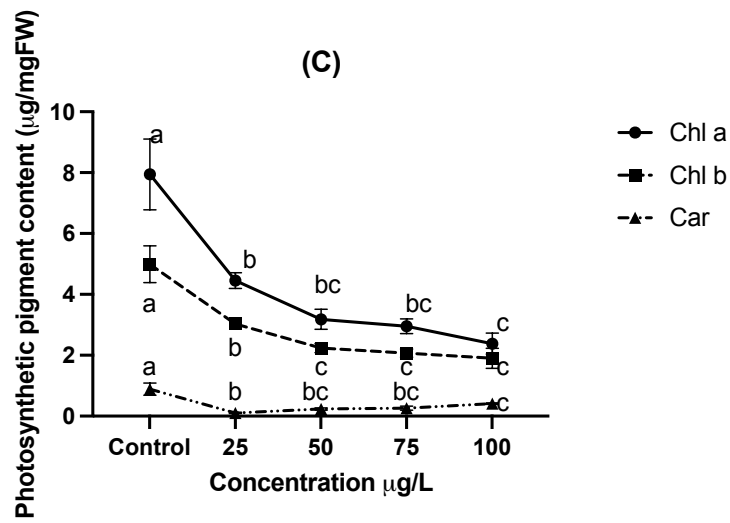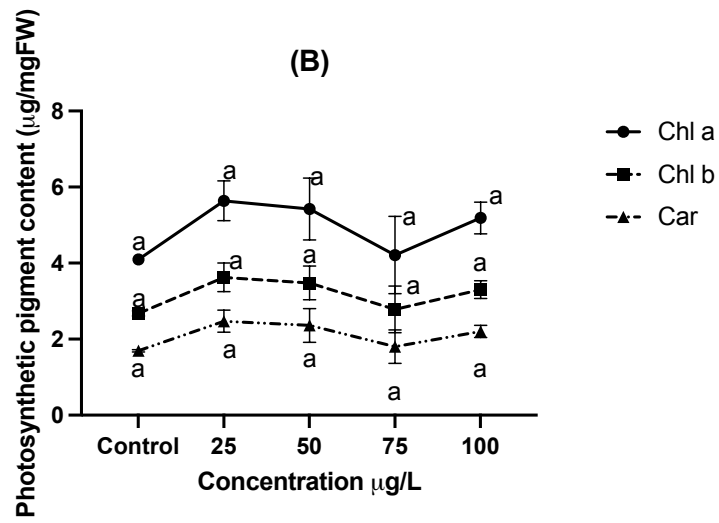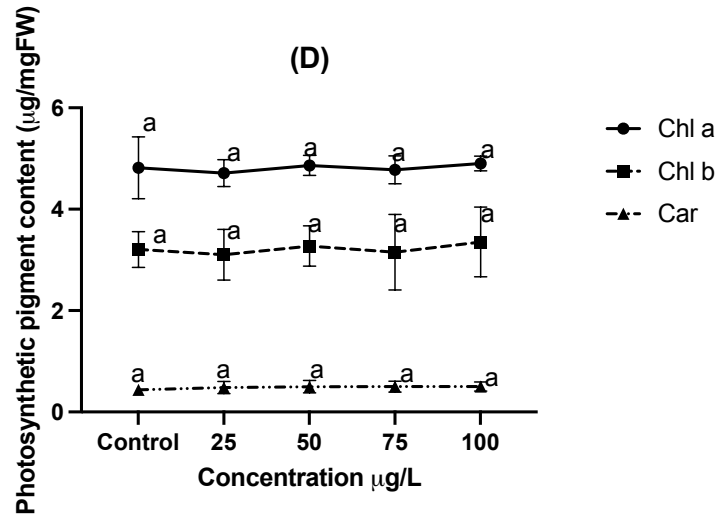
Figure 3.2 Variations in the content of chlorophyll a (Chl a), chlorophyll b (Chl b) and carotenoids (Car) in (µg/mg FW) in *Lemna minor* after 7 days of exposure to different concentrations of diuron (A), fenuron (B), DCPMU (C), and 3,4-DCA (D). When the lowercase letters associated with the values are the same, this indicates that there is no significant difference ($p < 0.05$) between the different conditions tested for the same pH (ANOVA, Tukey HSD test) (n= 3).

3.4 Effect of diuron, fenuron, DCPMU, 3,4-DCA on ROS production

ROS formation is a sensitive biomarker of cellular oxidative stress. The use of CellROX fluorescent probes for the detection of oxidative stress allows us to measure the level of ROS in plants exposed to diuron, fenuron, DCPMU, and 3,4-DCA at different concentrations (Fig. 3.3). After 7 days of exposure, the measurement results of fluorescence show an increase in ROS level in all groups except fenuron in function of the increase in the concentration of contaminants in the SIS medium culture.

In all cases, there is no statistically significant difference between the control and the various concentrations with respect to ROS production, given that all bars share the same marker. A 20% increase is observed at the highest concentration of diuron at 100 µg/L (Figure 3.3 A) and for DCPMU (Figure 3.3 C) and 3,4-DCA (Figure 3.3 D) these increases are 13% and 16% respectively. Figure (3.3 B) shows ROS content for fenuron exposure after 7 days. Despite the other three contaminants, a negative trend was observed in ROS concentration. There is no

significant difference between the control and the various concentrations for fenuron exposure. A slight increase (9%) in ROS content was observed at 75 μg/L.

(A)

(B)

(C)

(D)
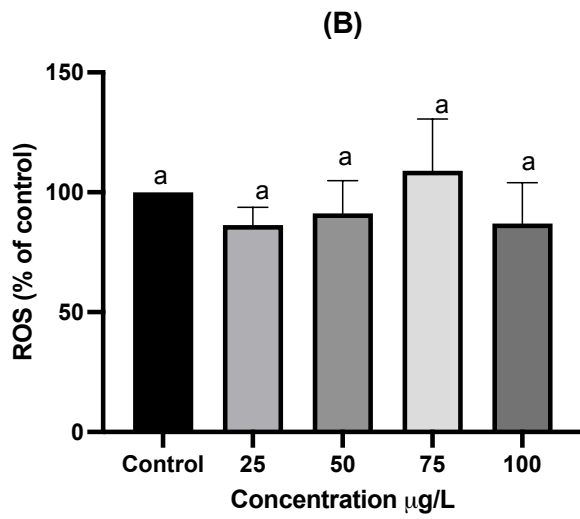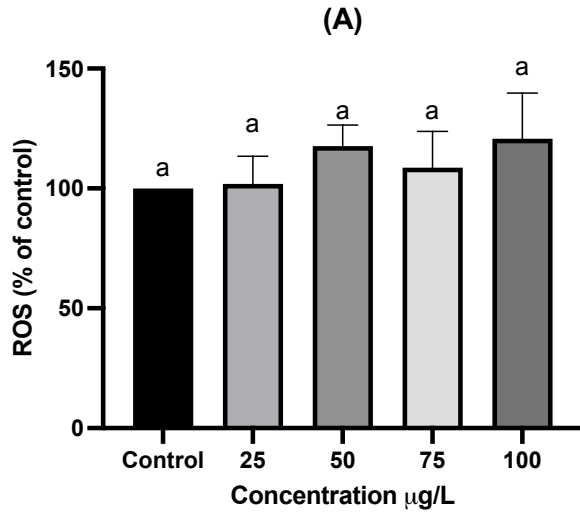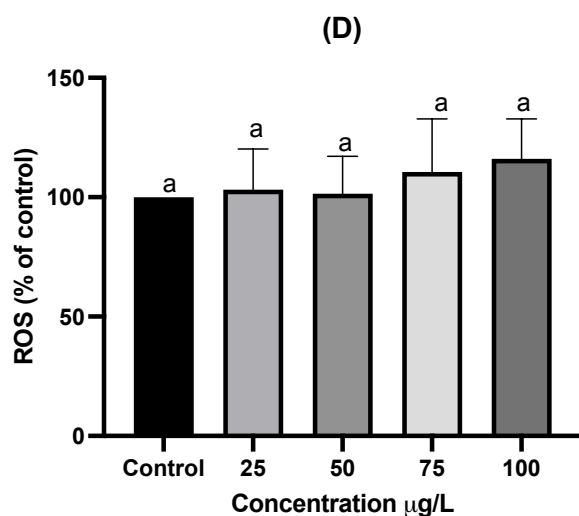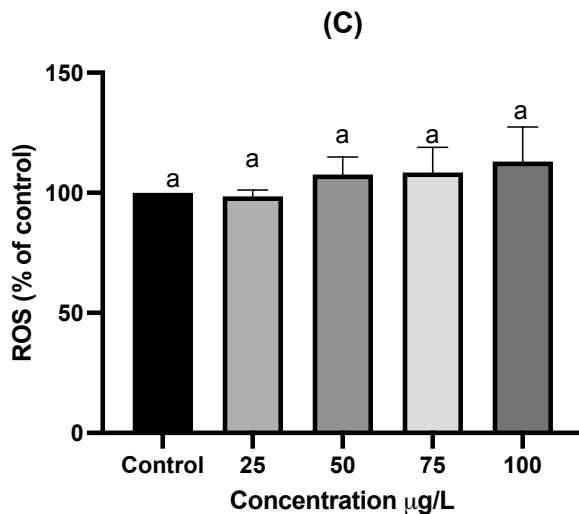
Figure 3.3 The production of intracellular reactive oxygen species (ROS) (in % of control) in *Lemna minor* exposed for 7 days to different concentrations of diuron (A), fenuron (B), DCPMU (C), and 3,4-DCA (D). When the lowercase letters associated with the values are the same, this indicates that there is no significant difference ($p < 0.05$) between the different conditions tested for the same pH (ANOVA, Tukey HSD test) (n= 3).

3.5 Effect of ozonation on the toxicity of diuron in different ozonation time intervals

This section presents the findings of the study investigating the impact of ozonation on the toxicity of diuron over varying time intervals. The experimental approach involved assessing the toxicity at multiple intervals to understand the effectiveness of ozonation as a treatment method. The effect of ozonation was evaluated on the phenotype of *Lemna minor* (fronds number and fresh weight) along with the growth inhibition based on frond number and fresh weight.

Through the results obtained after 7 days of exposure to the post-ozonation contaminated SIS medium culture with diuron, the efficacy of ozonation on mitigating the toxicity of diuron over various time intervals, with implications for plant growth. The summary of the results is shown in (Table 3.2).

Initially, the fresh weight of the control group was measured at 220 ± 9.07 mg, which remained relatively stable at the 40-minute mark with a value of 225 ± 11.93 mg, despite a notable decrease at 20 minutes (70 ± 10 mg). This suggests that plant weight could initially decrease due to ozonation but may recover over time. The second weight parameter, possibly indicating dry weight, significantly dropped from 11.93 mg in the control to 0.57 mg after 5 minutes of ozonation, labelled with a different statistical grouping (b), indicating a pronounced early effect. The number of fronds, a proxy for plant health or growth, decreased sharply from 165 ± 1 in the control to 21 ± 1.73 at 5 minutes, then partially recovered to 172 ± 11.54 at 40 minutes, demonstrating that short-term ozonation may hinder plant growth, but it can rebound or even improve slightly with prolonged exposure. These changes in weight and frond number after ozonation treatments could reflect a detoxifying effect, where initial stress is mitigated over time, potentially contributing to an overall improvement in plant growth post-ozonation. The presence of statistical groupings (a, b, c, d) beside the values suggests that the differences observed at certain intervals are significant and relevant to assessing the impact of ozonation on diuron toxicity and plant health.

Table 3.2 Variation in fresh weight (mg) and number of fronds after 7 days of exposure to ozonated diuron 100 µg/L in different time interval in treated *Lemna minor*

Label	Control	Control 40 min	5 min	10 min	20 min	30 min	40 min

Fresh Weight(mg)	220 ± 9.07 a	225 ± 11.93 a	18 ± 0.57 b	21 ± 2.30 b	70 ± 10 b	184 ± 7.50 a	229 ± 9.0 a
Number of Fronds	165 ± 1 a	160 ± 5 a	21 ± 2.64 b	27 ± 1.73 b	75 ± 11.67 c	169 ± 7.50 d	172 ± 11.54 a

3.6 Efficiency of ozonation on diuron toxicity on the growth of *Lemna minor*

The inhibitory effects of diuron at 100 µg/L on the growth of *Lemna minor* following various ozonation times, measured as a percentage change after 7 days (Figure 3.4). The growth inhibition peaks at 5 and 10 minutes of ozonation (marked with 'b' and 'c'), suggesting that short-term ozonation generates by-products or insufficiently degrades diuron, maintaining or enhancing its toxicity. However, as ozonation extends to 20 minutes and beyond, the inhibitory effect decreases (marked with 'd' and returning to 'a'), indicating that longer ozonation times lead to more complete degradation of diuron or its by-products, reducing its growth-inhibiting impact on *Lemna minor*.

The 'a' label on the controls and the longer ozonation times in both graphs implies a return to non-inhibitory conditions like the unozonated control, revealing a critical threshold where ozonation shifts from being ineffective to effective in mitigating diuron's growth inhibition.

This data suggests a biphasic response where initial ozonation times might increase toxicity, possibly due to the formation of more potent by-products, followed by a reduction in toxicity with longer ozonation, likely due to further breakdown of these by-products or complete mineralization of diuron. The critical observation here is the reversal in growth inhibition at extended ozonation times, underscoring the importance of optimizing ozonation duration for effective water treatment.
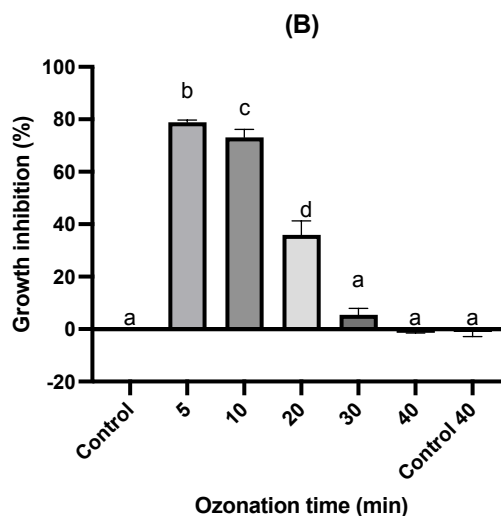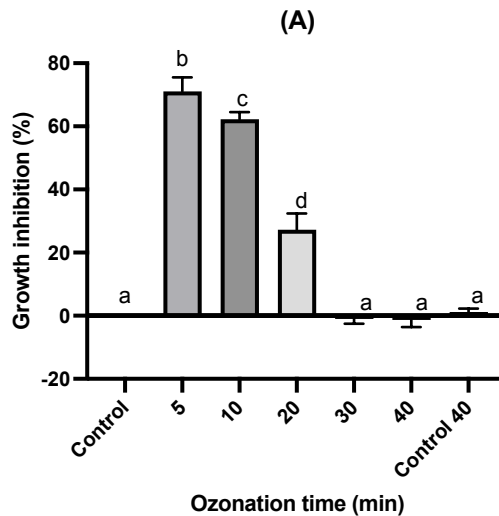
Figure 3.4 Inhibitory effect of diuron 100 μg/L after ozonation in different time intervals on the growth of *Lemna minor* (in percentage %) after 7 days of exposure. When the lowercase letters associated with the values are the same, this indicates that there is no significant difference ($p < 0.05$) between the different conditions tested for the same pH (ANOVA, Tukey HSD test) (n= 3).

3.7   Effect of ozonation on diuron degradation

The UV-Vis spectra of degradation of diuron samples solution during a single ozonation process displayed that the diuron had a major absorption peak from 235 to 265 nm which decreased after

30 minutes of ozonation (Figure 3.5). The efficiency of degradation of diuron in samples increased with increasing the time of ozonation. The absorbance of the diuron at 250 nm (Figure 3.5 B) decreased over the time of ozonation. This decrease gives indications of its consumption.
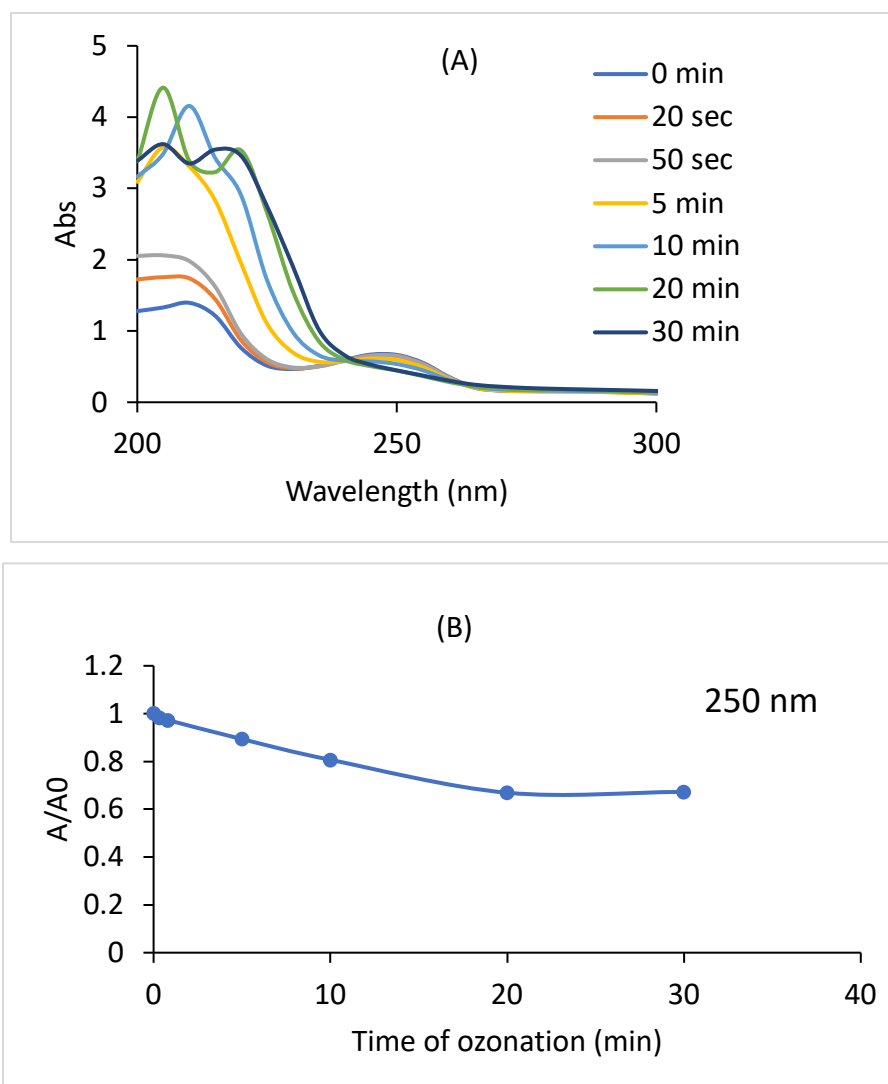
Figure 3. 5 Changes of absorbance in UV-Vis spectra and evolution of the main bands of diuron at 250 nm during ozonation

HPLC-UV analysis of a single ozonation media of diuron showed an overall decrease in the relative area main peak over the time of ozonation in the absence of clay catalysts (Figure 3.6). The retention time for the main peak was 4.8 minutes. The appearance of two intermediate at 6.72 and 7.36 minutes is clear in this analysis. The gradual degradation of diuron over the time of

ozonation of the relative area of the main peak is shown in (Figure 3.7) Between 5-to-30-minute ozonation alone a rapid degradation of diuron and formation of intermediates were clear.

Based on the calibration curves, the conversion rates reach 60 % after completing the time of ozonation (30 minutes).
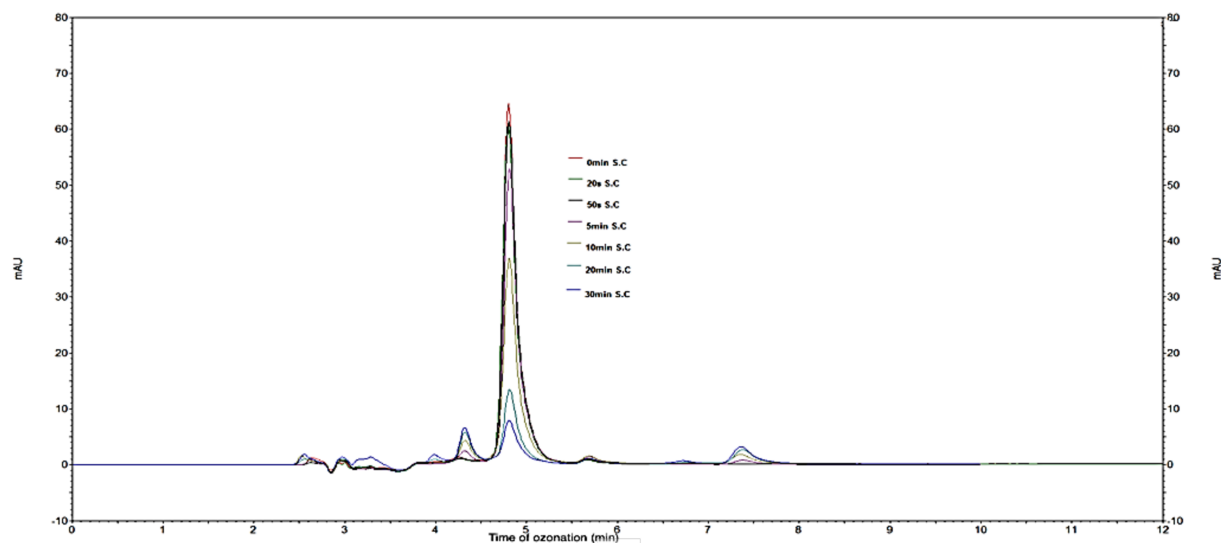
Figure 3.6 Evolution of the relative area of the HPLC-UV peak of diuron solution during the ozonation process. Ozone flow rate: 600 mg/L. Sample volume = 20 mL.
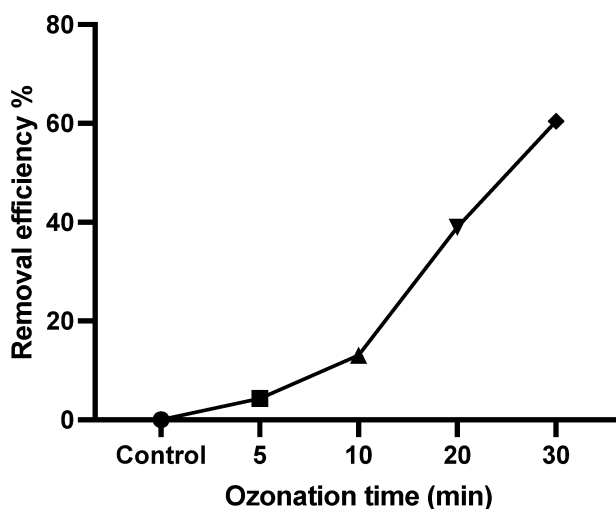
Figure 3.7 Removal efficiency of diuron by ozonation in different time intervals. The relative peak area (A/A0) was calculated as the instant/initial HPLC-UV peak area ratio of diuron. Ozone flow rate: 600 mg/L. Sample volume = 20 mL.

3.8 LC-MRM analysis of diuron after ozonation in SIS medium

The analysis of diuron in the culture medium (SIS) at pH 6.5 using LC-MRM was conducted in duplicate on exposure solutions prior to the cultivation of *Lemna minor* at time zero after subjecting them to varying durations of ozonation (0, 5, 10, 20, 30, 40 minutes). Chromatographic analysis (UHPLC-UV) aimed at quantifying diuron degradation during ozonation in the SIS medium revealed a correlation between the increasing ozonation time and diuron disappearance, with fenuron being the sole by-product detected during the diuron degradation process by ozonation, as shown in (Figures 3.8 and S.3). The degradation efficiency of diuron in the samples rose with the prolongation of ozonation time. The two other diuron intermediates, DCPMU and 3,4-DCA, which were investigated in this study, were not detected in the diuron solutions after various ozonation times using LC-MRM.

Chromatograms depicting diuron degradation, pinpointed at the retention time of 5.3 minutes, were presented as graphical plots of retention time against signal intensity. Graphs also demonstrated the emergence of fenuron during the ozonation of diuron, appearing at a retention time of 3.8 minutes for each replicate.

The aggregate area under the peak corresponding to diuron degradation was directly proportional to the ozonation duration, as illustrated in (Figure 3.9). The degradation efficiency of diuron quickly peaks at approximately 10 minutes of ozonation and then stabilizes, suggesting that the most substantial reduction in diuron occurs within the initial 10 minutes. Concurrently, the peak intensity of diuron, indicative of its concentration, decreases sharply, signalling a notable reduction in diuron concentration during the same timeframe. Statistically significant differences between the data points are indicated by letters (a, b, c, d). The detection of fenuron in the ozonated diuron solutions indicates that the approximate concentration of fenuron in the 10-minute sample was around 40 µg/L, as estimated by comparison with a 100 µg/L fenuron standard, as depicted in (Figures 3.10 and S.4).

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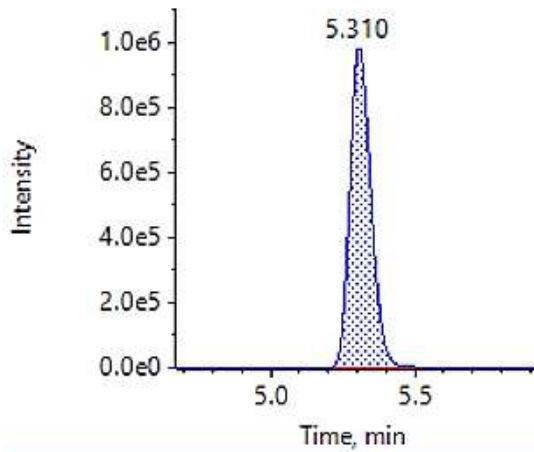
diuron_5min_1 - Diuron_...f), (sample Index: 11)
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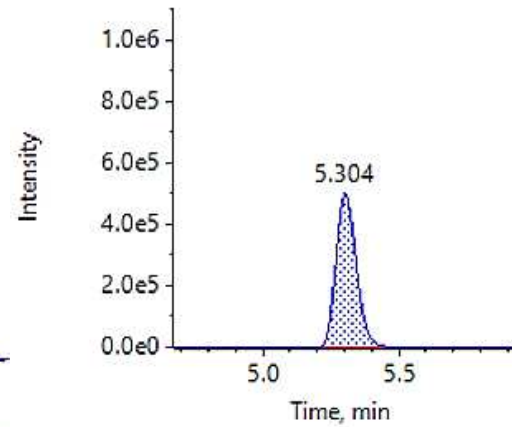
diuron_10min_1 - Diuron...f), (sample Index: 12)
Area: 1.080e6, Height: 2.142e5, RT: 5.31 min
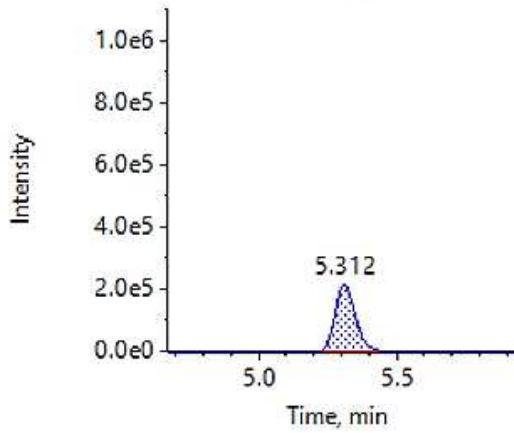
diuron_20min_1 - Diuron...f), (sample Index: 13)
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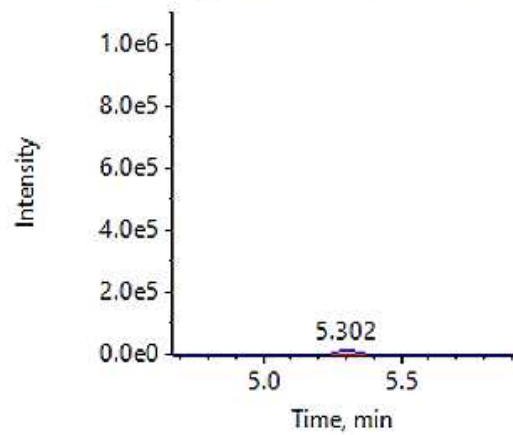
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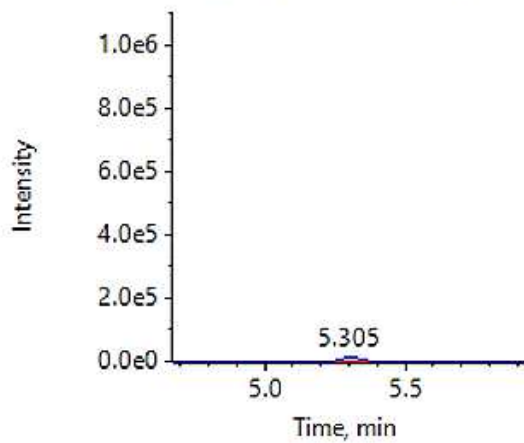
diuron_40min_1 - Diuron...f), (sample Index: 15)
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50
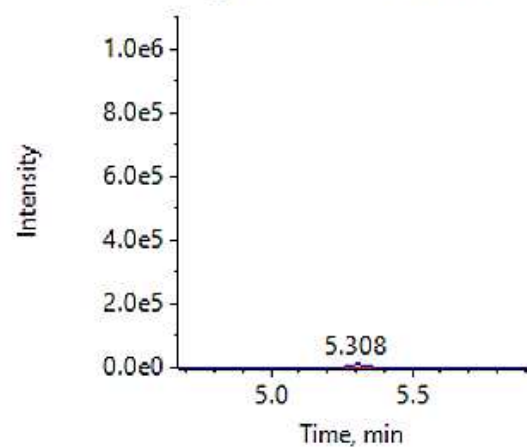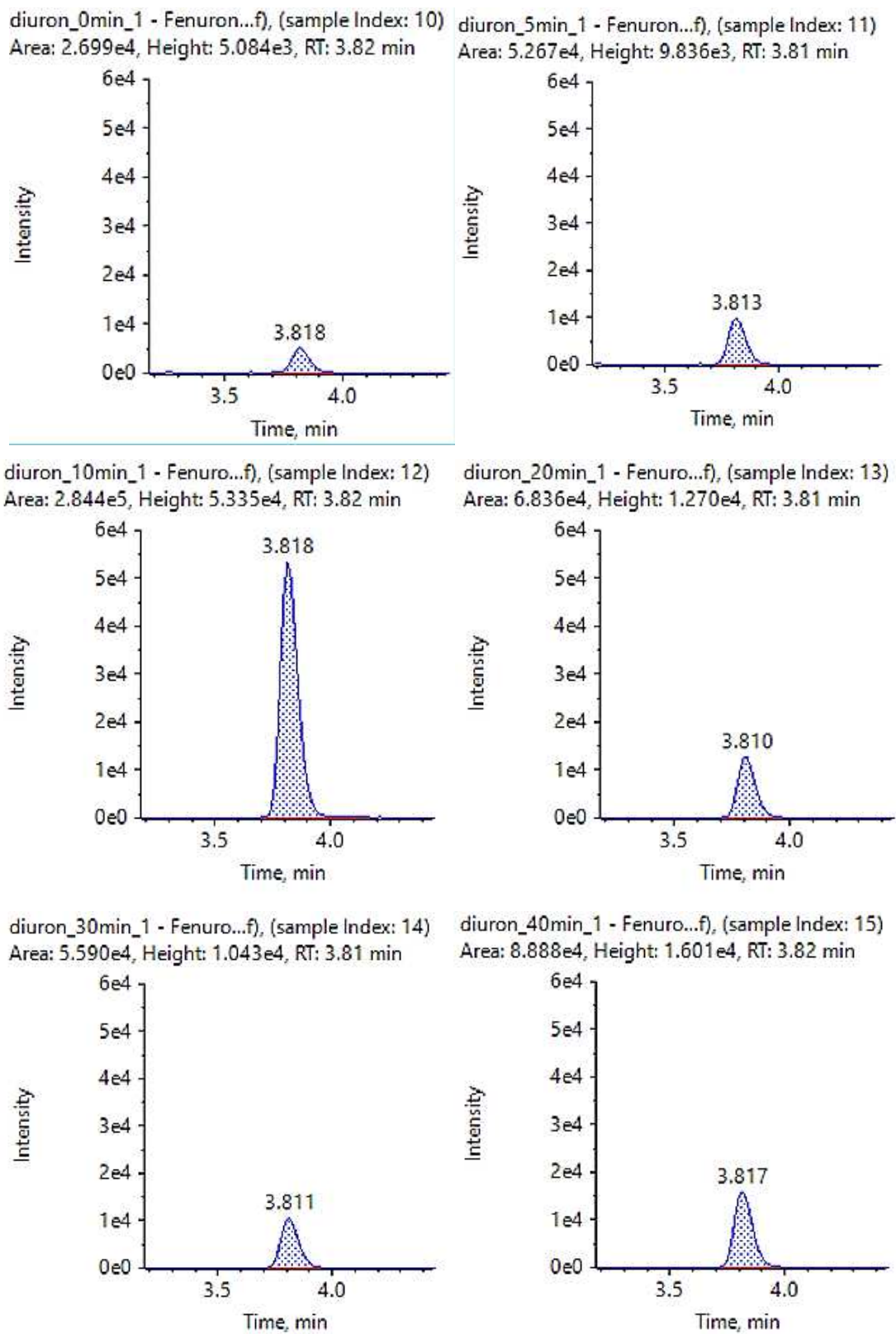
Figure 3.8 Changes in the HPLC-UV peak area of diuron solution over the course of the ozonation process and the emergence of fenuron.
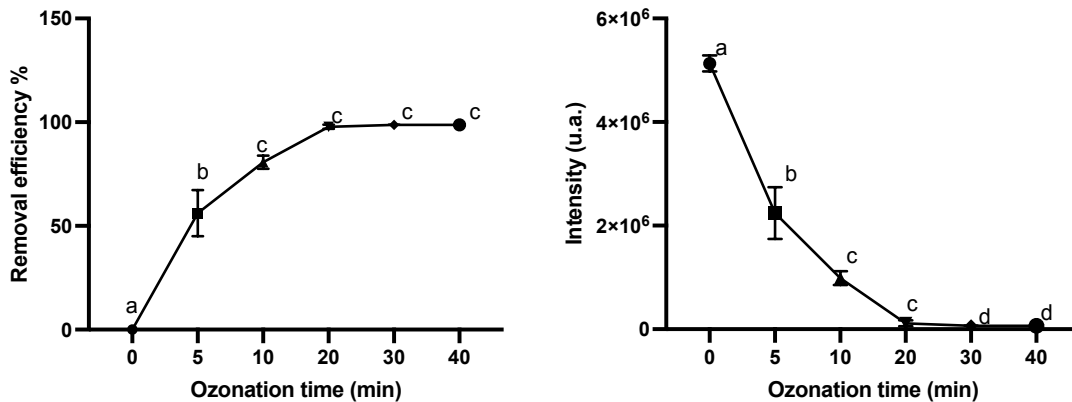
Figure 3.9 Evolution of the relative area and removal efficiency of the HPLC-UV peak of diuron solution during the ozonation process using LC-MRM. Ozone flow rate: 600 mg/l. Sample volume = 20 mL. When the lowercase letters associated with the values are the same, this indicates that there is no significant difference ($p < 0.05$) between the different conditions tested for the same pH (ANOVA, Tukey HSD test) (n= 2).
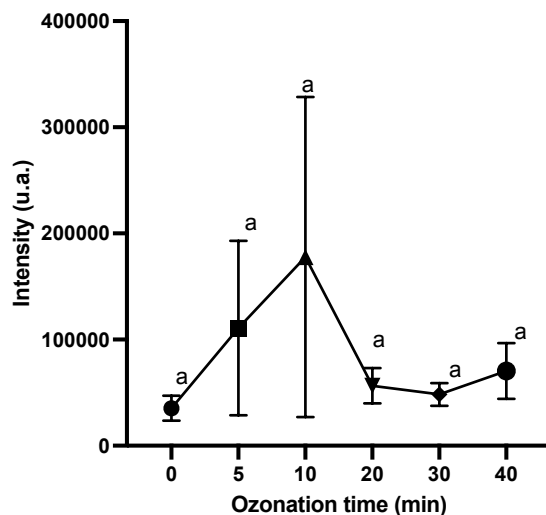
Figure 3.10 The appearance of fenuron during the ozonation process of diuron solution using LC-MRM. Ozone flow rate: 600 mg/l. Sample volume = 20 mL. When the lowercase letters associated with the values are the same, this indicates that there is no significant difference ($p < 0.05$) between the different conditions tested for the same pH (ANOVA, Tukey HSD test) (n= 2).

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4   CHAPTER IV

DISCUSSION

Diuron, a widely used herbicide for the control of weeds in soil and aquatic environments, is synthesized and utilized extensively across various regions (Khongthon et al., 2016). Consequently, its prevalence in aquatic ecosystems has become a focal point of environmental research in recent years, as it has been detected in several environmental matrices (Fatta-Kassinos et al., 2011). Furthermore, the toxicity of diuron has been documented in various aquatic organisms (Burns et al., 2015; Dewez et al., 2002; Gatidou et al., 2015; Lee et al., 2021; Oturan et al., 2008; Mansano et al., 2018; Kao et al., 2019; Shim et al., 2022).

A review of the ecotoxicological literature concerning diuron and its by-products reveals that the majority of research has concentrated on the effects of the herbicide, with minimal attention given to its metabolites such as 3,4-DCA, DCPMU, and fenuron (Bonnet et al., 2007). In this research, we tried to assess the various toxicity endpoints in *Lemna minor* as a measure of fenuron, DCPMU, and 3,4-DCA Stress.

Building upon this foundation, the present study is an extension of the toxicological profile of diuron and its three intermediates in the aquatic milieu, employing the well-established laboratory model for ecotoxicological assays, the aquatic plant *Lemna minor*.

This research examined the detrimental impacts of diuron and its three intermediates fenuron, DCPMU, and 3,4-DCA on *Lemna minor* following a 7-day exposure to these contaminants in the SIS medium culture. Additionally, this study explored the toxicity effects of diuron post-ozonation at different time intervals to assess the degradation efficiency and its subsequent influence on the growth of *Lemna minor*. Statistical analysis of the experimental data was conducted to determine the significance of varying pollutant concentrations on both growth inhibition and photosynthetic processes.

4.1 Effect of diuron, fenuron, DCPMU, and 3,4-DCA on plant growth

4.1.1 Total Fronds Number and Fresh Weight

Frond number and fresh weight are the primary endpoints typically assessed in studies involving *Lemna* species. In this study, we evaluated the fresh weight and fronds number's sensitivity to diuron, fenuron, DCPMU, and 3,4-DCA in comparison with these traditional endpoints. For diuron, the number of total fronds and the fresh weight of *Lemna minor* exhibited a decrease ($p < 0.05$) correlating with the concentration of diuron. Diuron can disrupt plant growth by obstructing the electron transport in photosystem II, which hampers photosynthesis and thus, halts the production of carbohydrates (Kleczkowski, 1994). A similar effect was observed in *Lemna gibba* by (Dewez et al., 2002) and another research using *Lemna minor* by (Lee et al., 2021).

Contrastingly, fenuron demonstrated an unexpected increase in both growth parameters at the highest concentration. This unusual response could suggest a hormetic effect, where low doses of a substance stimulate growth, or it may reflect an adaptive response to stress conditions, which should be explored further in future studies (Calabrese & Baldwin, 2003). In (Ge et al., 2014) study, fenuron showed the weakest toxicity effect on *Selenastrum capricornutum* and *Photobacterium phosphoreum*.

The response to DCPMU showcases a considerable decline in plant health, with a marked decrease in fresh weight and frond number. This is indicative of its toxicological potency, which may be attributed to its persistence in the environment and its potential to interfere with crucial biochemical pathways within the plant. In the research conducted by (Da Rocha et al., 2013) the most potent cytotoxic diuron metabolite was DCPMU, followed by DCPU, 3,4-DCA.

Interestingly, 3,4-DCA induced an increase in both measured growth parameters. The response to 3,4-DCA could be an indication of metabolic adaptation or detoxification processes that some plant species employ to mitigate the effects of certain xenobiotics (Miglani et al., 2022). This hypothesis for 3,4-DCA needs further research and investigation in the laboratory. However, in the study conducted by Dewez et al. (2002), 3,4-DCA showed the weakest toxicity effect on *Lemna gibba*.

The variability in response to different substances underscores the complexity of predicting toxicological effects based on chemical structure alone. It also highlights the importance of considering individual and combined effects of contaminants in risk assessments, as the interactions between various substances can lead to synergistic, additive, or antagonistic outcomes (Trewavas, 2005).

The lack of significant differences between certain concentrations, as indicated by the same lowercase letters, suggests that there may be threshold levels below which the plants can maintain normal growth or even exhibit enhanced growth rates. This observation could be pivotal for environmental management, as it informs the threshold levels of pollutants that aquatic ecosystems can tolerate before adverse effects become apparent (Wilson & Tisdell, 2001).

Considering our obtained results, it is evident that aquatic plants like *Lemna minor* can serve as sensitive indicators of water quality and the presence of pollutants. This study contributes to a growing body of evidence that underscores the need for comprehensive environmental monitoring and stricter regulations on the use of herbicides, to safeguard aquatic ecosystems from the potentially devastating effects of chemical pollutants.

4.1.2   Effect on the growth of *Lemna minor*

(Figure 3.1) indicates the growth inhibitory based on fronds number and fresh weight in percentage for all examined contaminants. Consistent with previous findings (Gatidou et al., 2015; Lee et al., 2021), our results demonstrate a pronounced dose-dependent inhibition of growth with diuron (Figure 3.1 A), where higher concentrations lead to more significant reductions in both fronds number and fresh weight. This is in agreement with the mechanism of action of diuron, which disrupts the electron flow in photosystem II, thereby impeding photosynthetic processes and plant growth (van Wijk & van Hasselt, 1993).

Contrary to diuron, fenuron exhibited no clear pattern of growth inhibition (Figure 3.1 B), echoing the findings of (Ricart et al., 2009) that suggest certain phenyl urea derivatives may have variable effects on different aquatic species. This observation indicates that fenuron's impact may be species-specific or influenced by other environmental factors not captured in this study.

For DCPMU, we observed a significant, yet less aggressive, dose-dependent inhibition compared to diuron (Figure 3.1 C). This resonates with the research by (Dewez et al., 2002), which highlighted the lesser toxicity of certain diuron metabolites. The implication here is that the toxicity of diuron and its derivatives can vary, necessitating careful consideration of their use and the potential for environmental accumulation.

In the case of 3,4-DCA, the absence of a significant inhibitory effect (Figure 3.1 D) suggests a more complex interaction with the plant's physiology, possibly involving compensatory growth mechanisms as described by Suresh and Ravishankar (2004). The lack of a dose-response relationship, indicated by the statistical letter 'a' across all concentrations, aligns with the notion that not all herbicides or their breakdown products equally affect plant growth (Marking & Dawson, 1975).

4.2   Effect on the content of photosynthetic pigments

Chlorophyll plays a pivotal role in the process of photosynthesis, and its levels can serve as a crucial metric for assessing plant damage associated with growth and development caused by metallic and organic pollutants in their surroundings (Li et al., 2008). Additionally, the significance of this measure is underscored by its ability to offer a reliable, albeit indirect, gauge of a plant's nutritional condition. In this study, we measured the contents of chlorophyll pigments (chlorophyll *a*, *b* and carotenoids) in *Lemna minor* after 7 days of exposure to diuron, fenuron, DCPMU, 3,4-DCA at different concentrations. The results obtained (Fig. 3.2) show a significant decrease in the content of chlorophyll *a*, chlorophyll *b* and carotenoid upon exposure to high concentrations of diuron and DCPMU.

Our findings are aligned with (Ridley, 1977; Singh & Singla, 2019) indicating that the chlorophyll content decreases as a result of 7 days exposure of *Lemna minor* to diuron. On the contrary, in the case of fenuron following a slight decrease at concentration of 75 µg/L an increase of chlorophyll *a*, chlorophyll *b* and carotenoid was observed in 100 µg/L concentration. For 3,4-DCA, a steady trend was observed in all examined concentrations after 7 days of exposure. Our finding for chlorophyll content is in contrast with the finding in a study by (Lee et al., 2021). In the study, exposure to diuron resulted in an increase in chlorophyll content in *Lemna minor*. Specifically,

chlorophyll content increased significantly by 38% at a diuron concentration of 50 µg/L after 72 hours of exposure. The increase in chlorophyll *b* was also observed, indicating an overall rise in photosynthetic pigment content under diuron stress. This increase was attributed to the induction of shade-type chloroplast formation by diuron, which may have facilitated more pigment accumulation in the chloroplasts (Williams et al., 2009). The study suggests that this increase in chlorophyll content might be a response of *Lemna minor* to the phytotoxic effects of diuron, though it may lead to more severe oxidative damage.

4.3 Production of intracellular reactive oxygen species

When plants face biological or abiotic stress, they produce reactive oxygen species (ROS), leading to oxidative stress (Gomes & Juneau, 2016). This stress impairs the development of chloroplasts, diminishes root growth and seed survival, and stimulates frond separation and desiccation in *Lemna minor*. Additionally, it causes peroxidation of vital lipids in cell membranes and organelles, weakening the activity of antioxidant enzymes and disrupting the plant's natural defence mechanisms (Kumar & Han, 2010; Philosoph-Hadas et al., 1994).

The study's use of CellROX fluorescent probes to measure ROS levels in plants exposed to diuron, fenuron, DCPMU, and 3,4-DCA reveals insights into oxidative stress responses. The increase in ROS levels with higher concentrations of diuron, DCPMU, and 3,4-DCA (except for fenuron) suggests a stress response, likely due to the contaminants' toxicity. Notably, even with increased ROS at higher concentrations, the differences weren't statistically significant from the control across all contaminants, indicating a somewhat uniform response to varying levels of exposure. The slight increase in ROS for fenuron at 75 µg/L, without significant differences from the control, contrasts with the other contaminants, suggesting a different mechanism of action or lower toxicity. These findings highlight the nuanced nature of plant stress responses to different contaminants and underscore the importance of considering both the type and concentration of contaminants in ecological risk assessments. In study of Lee et al. (2021), a concentration-dependent increase in ROS was observed with diuron exposure, with significant increases at higher concentrations.

4.4 The impact of ozonation on the toxicity of diuron varies with different ozonation time intervals

The efficiency of ozonation in mitigating the toxicity of diuron on the growth of *Lemna minor* is a significant area of investigation, as evidenced by the varying responses observed in the aquatic plant. Ozonation, a powerful oxidizing treatment, has been shown to alter the toxicity profiles of various contaminants, including diuron (Feng et al., 2008; Solís et al., 2016). In the context of *Lemna minor*, a sensitive bioindicator for aquatic ecosystems, understanding the impact of ozonated diuron is crucial. The study indicates that ozonation can modify the effects of diuron, potentially reducing its toxic impact on the plant's growth and development. This reduction in toxicity might be attributed to the degradation of diuron into less harmful by-products through ozone treatment. However, the extent and efficiency of this mitigation vary, likely depending on factors such as the duration of ozonation and the initial concentration of diuron. The findings suggest that while ozonation can be an effective method for decreasing diuron's harmful effects, the process needs to be carefully optimized to maximize its benefits. This understanding is vital for developing effective strategies to protect aquatic plants and ecosystems from the adverse effects of herbicides and other pollutants.

In the latter part of our research, we focused on ozonating diuron-contaminated SIS medium culture in different time intervals ranging from 0 to 40 min. This phase began with a range-finding test, as suggested by (Gatidou et al. 2015), to pinpoint suitable concentration levels for the main experiments. This preliminary step was crucial for establishing the correct dosages needed to accurately assess the EC_{50} values, which are essential for understanding the effective concentration of diuron that causes a 50% reduction in a specified endpoint. This approach ensured that the subsequent experiments were both precise and relevant to our investigation into diuron toxicity and its modulation by ozonation. In this study, the EC50, which represents the effective concentration of diuron to achieve a 50% reduction in a specified biological parameter, was identified at 100 µg/L in the SIS medium culture.

This research was undertaken to address a gap in understanding the impact of diuron toxicity following its degradation by single ozonation, particularly on plants like *Lemna minor*. To explore this area, we investigated how an ozonated SIS medium, contaminated with diuron, affects the

growth rate of *Lemna minor*. Our focus was to shed light on the consequences of ozonation as a treatment method on plant health and development in environments exposed to diuron.

In this study, we explored the effects of ozonation on the toxicity of diuron and its impact on *Lemna minor* growth, significant findings emerged from the analysis of different ozonation time intervals. Initially, there was a noticeable decrease in *Lemna minor*'s fresh weight at 20 minutes post-ozonation, followed by a recovery at 40 minutes, indicating an initial stress response to ozonation that later subsided. Additionally, a significant drop in another weight parameter, possibly indicating dry weight, was observed right after 5 minutes of ozonation, suggesting a pronounced early impact. The number of fronds, a key indicator of plant health, initially plummeted after 5 minutes of ozonation but showed improvement at 40 minutes. This suggests that while ozonation initially induces stress on the plants, it also triggers a detoxifying effect over time. Further, the study revealed a biphasic response in the growth inhibition of *Lemna minor*, where short-term ozonation (5 and 10 minutes) appeared to increase toxicity, possibly due to the formation of more potent by-products. However, as ozonation duration increased beyond 20 minutes, there was a marked decrease in inhibitory effects, implying a more effective degradation of diuron or its by-products. This critical observation underscores the need for optimizing ozonation duration in water treatment processes, as prolonged ozonation leads to significant toxicity reduction, a vital consideration for environmental management and the mitigation of pollutants like diuron.

The UV-Vis spectral analysis demonstrated a notable decrease in Diuron's absorption peak between 235 to 265 nm after 30 minutes of ozonation. This reduction suggests a successful degradation of Diuron, as further supported by the increased efficiency of degradation over time observed in the HPLC-UV analysis. The HPLC-UV results confirmed diuron's decrease, with its main peak retention time at 4.8 minutes and the emergence of intermediates at 6.72 and 7.36 minutes. These changes align with the observed 60% conversion rate after 30 minutes of ozonation, indicating significant breakdown and formation of by-products.

5   CHAPTER V

GENERAL CONCLUSION

The present findings in the first study offer fresh perspectives on the mechanism of diuron toxicity in *Lemna minor*, highlighting the varying degrees of sensitivity across physiological and biochemical. In the second study, we examined the toxicity of diuron after ozonation treatment at different time intervals ranging from 0 – 40 minutes by assessing the physiological endpoint of *Lemna minor*. To better understand the toxicity effect of diuron intermediates after ozonation in our first study along with diuron, we examined the toxicity effect of three diuron intermediates (i.e., Fenuron, DCMU, 3,4-DCA). In our second study, the trend of diuron degradation was analyzed using analytical methods, namely UV-Vis, UHPLC-UV, and LC-MRM.

A key finding is the variable effect of diuron and its metabolites on *Lemna minor* growth. Diuron showed a dose-dependent inhibitory effect, while fenuron demonstrated an unexpected hormetic response, suggesting potential adaptive mechanisms or a lower toxicity profile. DCPMU was notably toxic, consistent with previous studies, and 3,4-DCA induced an increase in growth parameters, indicating possible metabolic adaptation or detoxification processes in the plant. The study also revealed a significant decrease in chlorophyll pigments upon exposure to high concentrations of diuron and DCPMU, underlining the potential of these chemicals to impair photosynthesis. In contrast, fenuron and 3,4-DCA had less pronounced effects, suggesting lesser impact or more complex interactions with the plant's physiology.

The research also focused on the production of Reactive Oxygen Species (ROS) as a stress response to these contaminants. A uniform increase in ROS levels, except for fenuron, indicates a generalized stress response in *Lemna minor*, highlighting the importance of oxidative stress in ecological impact assessments.

Another novel aspect of this research is the exploration of the impact of ozonation on the toxicity of diuron solutions. The ozonolysis was carried out in the absence of *Lemna minor*. The findings indicate a fluctuating impact of ozonation on *Lemna minor*, with initial stress responses leading to

recovery and detoxification over time. This biphasic response underscores the complexity of using ozonation as a treatment strategy and the need for optimization for environmental safety.

The study emphasizes the critical role of aquatic plants like *Lemna minor* as indicators of water quality and pollutant presence, contributing significantly to our understanding of herbicide toxicity in aquatic ecosystems and the efficacy of treatment strategies like ozonation. Future research should aim to understand the molecular mechanisms behind *Lemna minor*'s responses, explore the long-term effects of low contaminant concentrations, and investigate the plant's potential for phytoremediation applications.

In conclusion, this research sheds light on the varying toxicological profiles of diuron and its metabolites and highlights the efficacy and complexity of ozonation as a remediation technique. These findings underscore the need for comprehensive environmental monitoring and stricter herbicide regulations to protect aquatic ecosystems. Additionally, *Lemna minor*'s potential as a bioindicator and phytoremediation agent opens promising avenues for future environmental management and conservation strategies.

6 ANNEX A

ADDITIONAL RESULTS
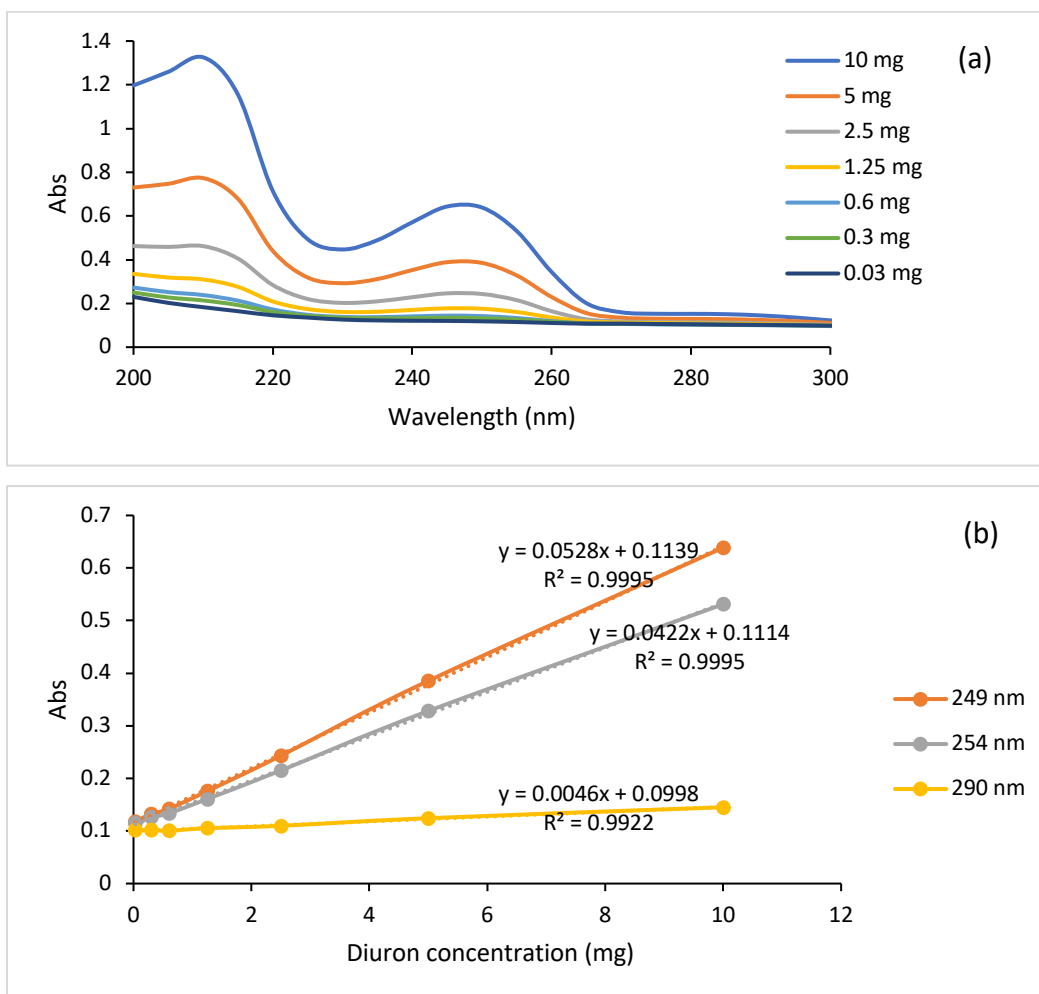
Figure S.1 UV-VIS spectra of diuron (a) and corresponding calibration plot (b) at room temperature. Various diuron solutions (0.03 – 10 mg/L) were prepared by successive dilutions from a stock diuron solution (10 mg/L). Quartz cell: 1 cm; T = 24 ± 2.
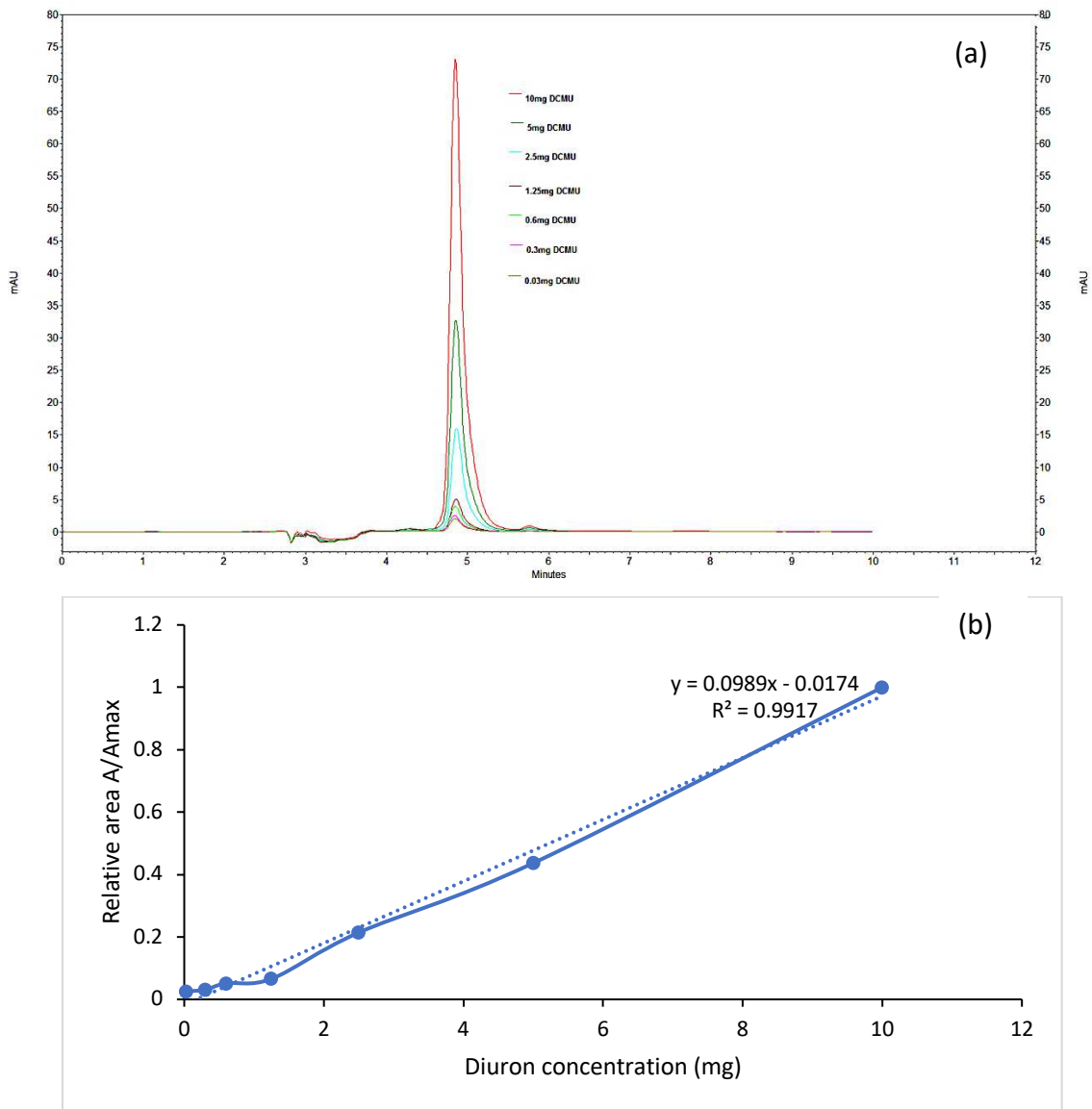
Figure S.2 HPLC-UV diagram of diuron at different concentrations (mg/L) as detected at 254 nm (a) and a calibration curve of diuron peak area (b). Diuron retention time: 4.84 min; C18 column under a 0.5 mL/min; T = 20°C.

Diuron peak areas; retention time 5.3 min

Diuron MRM 233/72 CE22 peak				
Sample Name	Rep1 area	Rep1 %	Rep2 area	Rep2 %
0min	5021000	100.0	5240000	100.0
5min	2594000	51.7	1887000	36.0
10min	1080000	21.5	892000	17.0
20min	75050	1.5	153300	2.9
30min	67390	1.3	64650	1.2
40min	51930	1.0	78280	1.5
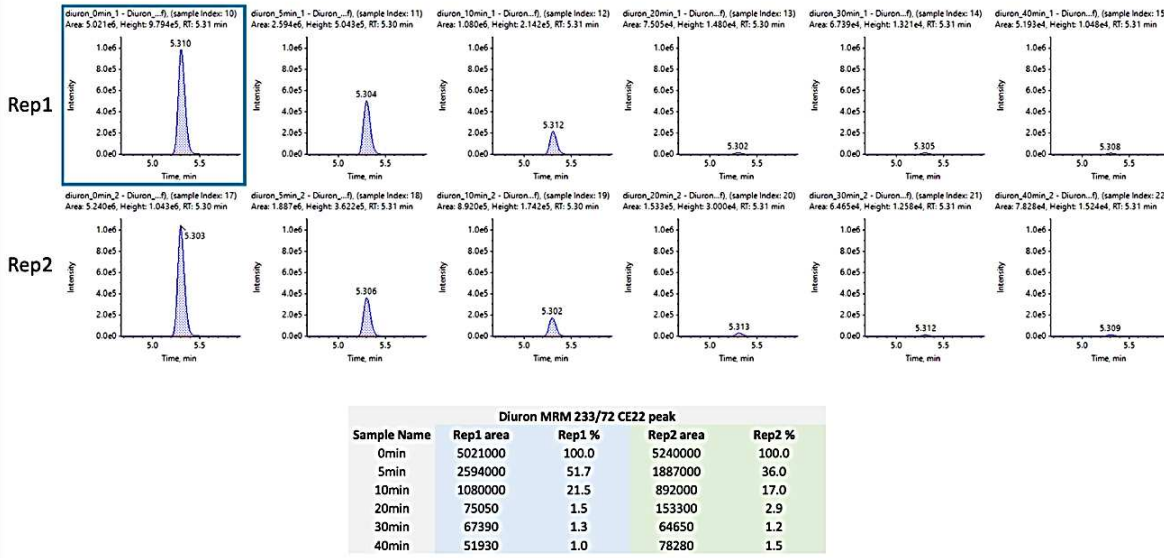
Fenuron peak areas; retention time 3.8 min

Approximate concentration determined on next slide

Fenuron MRM 165/72 CE30		
Sample Name	Rep1 area	Rep2 area
0min	26990	43700
5min	52670	168900
10min	284400	71240
20min	68360	44710
30min	55900	40700
40min	88880	51800
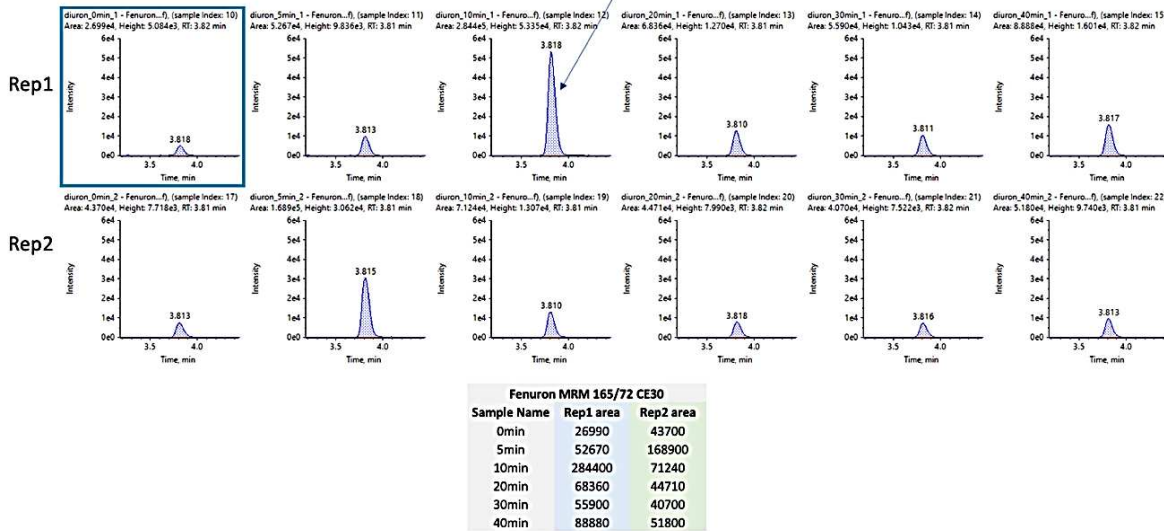
Figure S.3 Chromatogram (XIC) of diuron in SIS medium before cultivation with *Lemna minor* at pH 6.5.

| | diuron_10min_1 - Fenuron_165...e.wiff), (sample Index: 12) Area: 2.844e5, Height: 5.335e4, RT: 3.82 min | Fenuron_1ng/ml_10ul - Fenuro...e.wiff), (sample Index: 26) Area: 7.274e5, Height: 1.360e5, RT: 3.82 min |
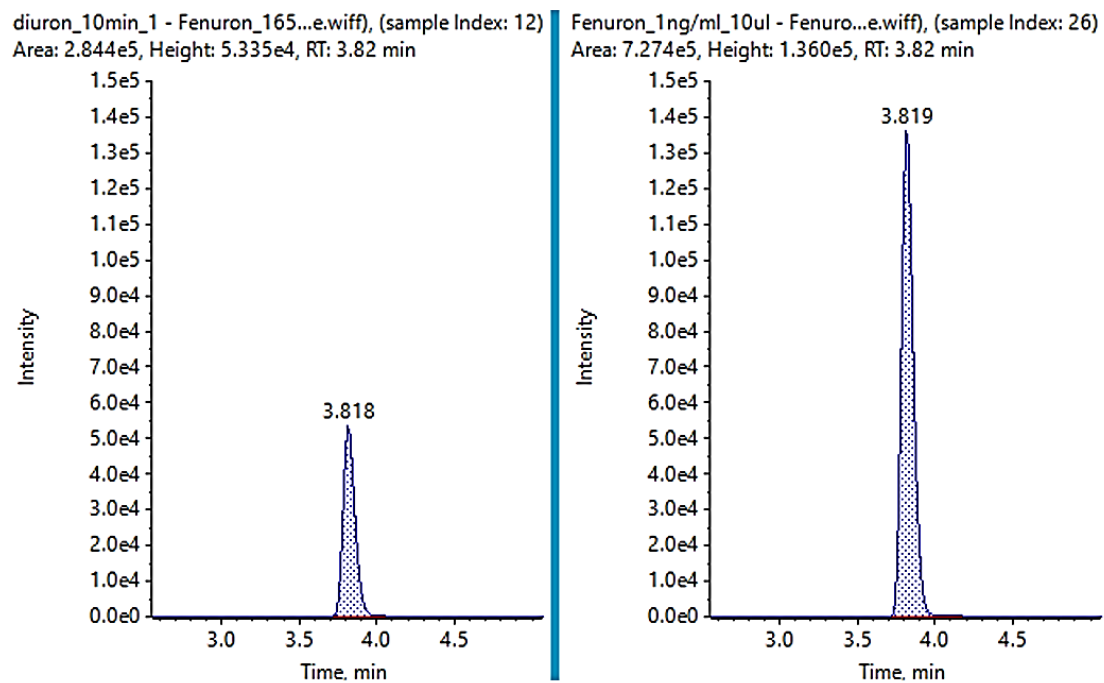
Sample Name	Fenuron peak area 165/72
diuron 10min_1	284400
Fenuron 1ng/ml	727400

Figure S.4 Comparison of Fenuron concentration in a 10-minute sample with 100 µg/L standard.

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