Removal of MCPA and 2,4D from water and mitigation of phytotoxicity using Vacuum UV treatment.

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Abstract

Freshwater is crucial for food production and human health, but it is unevenly distributed globally and not fully available for use. Freshwater for food production and drinking water is available as groundwater and as surface as water in rivers and lakes. However, the water quality is decreasing due to pollution from human activities. The chemical compounds emitted to the environment due to runoff and leaching threaten the use of water, for human consumption and for irrigation in agriculture, potentially affecting health and crop yield respectively. MCPA and 2,4D are the most common selective herbicides used worldwide, and have been detected in ground and surface waters around the world. These chemicals can have adverse effects on non target aquatic organisms such as algae, and terrestrial organisms such as soil bacteria and commercial crops commercial crops but the concentration effect-range is still being investigated. Thus, there is increasing focus on removing these chemicals from the water cycle to reduce risks associated with use of water for irrigation and human consumption.

This project aims to determine the degradation of MCPA and 2,4D during an Advanced Oxidation Processes (AOP) with Vacuum UV (VUV) irradiation, and to determine the acute ecotoxicity before and after treatment to *Lactuca sativa* (Lettuce), *Lepidium sativum* (Cress), *Raphidocelis subcapitata* (a green microalgae) and *Bacillus subtilis* (a soil bacterium). Toxicity was determined as the EC50 based on growth, after exposing the test organisms to different concentrations of MCPA or 2,4D for a period of 18 to 120 hours, depending on the species. VUV was run for MCPA and 2,4D at an initial concentration of 10 mg/L dissolevd in 4 L of tap water, and samples were taken at 0, 1, 2, 4, 8, 16 and 32 minutes. Remaining concentrations after VUV at each time point, were measured by HPLC using separation in a C18 column with a mobile phase of acetonitrile and a buffer solution at PH 4. The wavelength for UV detection of MCPA and 2,4D was set 230 nm. The initial EC50 values was 0.029mg/L for MCPA and 0.041mg/L for 2,4D using *Lepidium sativum* as test organism; 0.017mg/L for MCPA and 0.068 for 2,4D using *Lactuca sativa*; 50mg/L for MCPA and 100mg/L for 2,4D using *Raphidocelis subcapitata*, and >100mg/L for MCPA and 2,4 D using *Bacillus subtilis*.

Vacuum UV treatment completely removed the initial concentration (10 mg/L) of MCPA and 2,4D after 8 and 32 minutes, respectively. The decay rate coefficient resulted in 0.87(min-1) for MCPA and 0.16 (min-1) for 2,4D. The VUV degradation of 2,4D and MCPA was reflected in increasing apparent EC50 values. For *Lactuca sativa* and *Lepidium sativum*, the toxicity decreased 4-1.5 fold and 7-8 fold after VUV treatment of compound MCPA for 2 and 4 minutes, respectively. After VUV treatment of 2,4D, toxicity for*Lactuca sativa* and *Lepidium sativum* decreased 2-0.57 fold and 4-1.16 fold a times 2 and 4,respectively.

Table of Contents

1 Introduction

Freshwater is an essential resource for both societal activities and natural ecosystems, such as food production and human health. [Gleick, 1993]. However, it is not entirely accessible, and is dispersed inequitably. [Gleick, 1993; Petersen et al., 2019].

Earth's freshwater is contained in surface water (0.3%), ground water (30%); and ice caps, glaciers and permanent snow (69.7%). Only 2.120 km3, equal to 1% of the total amount of water on Earth, is thought to be readily usable and is stored in aquifers, rivers, and lakes. Furthermore, the distribution of surface and groundwater is not uniform around the earth. Water availability and storage varies greatly between regions, and there is no connection between these variations and population density. There are areas with rich water supply where population is low and others in which water is scarce and population is high [du Plessis, 2017].

Due to water extraction and deterioration, economic and human activities decrease the amount of water available [du Plessis, 2017]. According to estimates done by du Plessis [2017] and Kibona D. [2009], the majority of water use worldwide is allocated to agriculture (69%) followed by industry (22%), households (8%) and recreational activities (1%), including drinking water. The practices involved in these activities endanger not only the freshwater availability, but the quality of freshwater used after extraction. When these processes are carried out, synthetic chemicals are required for food production, drinking water desinfection, medical treatment, and manufacturing industries. If these chemicals are not properly disposed or treated of after use, they might end up in the environment, particularly in water. [Hutchinson et al., 2013].

Some of those artificial compounds belongs to the Chemicals of Emerging Concern (CEC), which over the past years, have increased special awareness due to their presence in water bodies [Sengupta et al., 2014; Whitacre, 2010]. These pollutants comprise the chemicals used by society such as pesticides, detergents, and pharmaceuticals, among others [Whitacre, 2010]. Due to their potential impact on ecosystems and human health, particularly due to their propensity for bioaccumulation and biomagnification, Chemicals of Emerging Concern are a topic of public attention [Sengupta et al., 2014; Whitacre, 2010]. The problem with the CEC's is that they are not widely regulated nor regularly monitored, and the hazardous effects are still being investigated [Sengupta et al., 2014]

Agricultural practices and land irrigation are examples of human activities that promote the Chemicals of Emerging Concern in the environment. The use of fertilizers and pesticides increases the surface and ground water pollution due to their loss, through surface runoff and leaching [Petersen et al., 2019]. The extensively use of pesticides for agricultural and non agricultural uses, plus their mobility through water, threaten the

environment [Székács et al.]. Within pesticides, there is a large group of chemicals called herbicides, use to control or remove undesired plants, that may endanger the growth and yield of desired crops [Caudle, 2015]. Chlorophenoxy Herbicides (CPHs) are used in agriculture to avoid the weed growth. They act as an auxin-like growth regulator causing uncontrolled growth which generally ends in the plant death [González et al., 2007]. This group is classified as "possible carcinogenic to humans" by the International Agency for Research on Cancer [IARC, 2022].

Within the chlorophenoxy herbicides, 2,4-dichlorophenoxyacetic acid (2,4-D) and 2-methyl-4-chlorophenoxyacetic acid (MCPA) are the most common selective herbicides used worldwide, to control broad leaved weeds [Yanfeng et al., 2020; Tu M., 2001]. They were developed in 1942 in the United States (US) and United Kingdom respectively. Indeed, 2,4 D was a component of the Agent Orange in the Vietnam War used by US to defoliate vegetation [Tu M., 2001]. Both compounds have been introduced extensively after the World War II due to their selectivity, low cost and easy application. They have been adopted for agricultural and residential purposes due to their ability to kill broad leaved plants without affecting the crops [Freisthler et al., 2022; Zimdahl, 2007]. Nowadays more than 1500 herbicide products contain these compounds as an active chemical [Yanfeng et al., 2020; Tu M., 2001]; and it is expected that as population will increase, and so it will the demand of agricultural products and the agrochemical applications [Cejudo et al., 2021].

As a result of contiguous application, MCPA and 2,4 D have been detected in surface and ground water around the world; including Europe, Australia, Canada, United States, and Latin America [Muszyński et al., 2020; Melgarejo L. et al., 2020]. A technical report made by the European Environmental Agency states that for the period 2007-2017 in the recorded monitoring stations, herbicides exceeded the 0.1 μg/L threshold by 5-15% in surface waters and 7% in ground water [Mohaupt et al., 2020]. For surface waters, over the 13,870 monitoring sites, MCPA was exceed in 1.6%; whereas 2,4D was exceeded in 1.1% in 9,330 sites included in 13 countries [Mohaupt et al., 2020]. Moreover, in ground water over 11 years MCPA and 2, 4 D were detected in 14 countries in 8,871 and 7,883 monitoring sites, respectively [Mohaupt et al., 2020].

The presence of these compounds in surface and groundwater affect the quality of the water for drinking and irrigation uses [Swiatlowski and Čajka, 2018; FAO, 2021, 2008]. Drinking water is safe when it satisfies the required quality standards and when does not represent any significant risk to health over a lifetime of consumption [Dinka, 2018].

The drinking water guideline values established by the World Health Organization (WHO) are 2μg/L for MCPA and 30 μg/L for 2,4D in drinking water [Morton et al., 2020]. However, European Commission Drinking Water Directive established in 1998 (98/83/EC) and its revised version adopted in 2020 by the European Parliament (EU/2020/2184); determines that the maximum concentration of any individual pesticide in drinking water is 0.1 μg/L and maximum concentration of the total sum of all pesticides present is 0.5 μg/L [DWD; Morton et al., 2020].

On the other hand, it is known that the quality of irrigation water influences crop quantities and yield [Zaman et al., 2018]. Irrigation is a relevant component in agricultural water uses (22%) and it is crucial to comply with the food production around the world [du Plessis, 2017; Jägermeyr et al., 2015]. Although physical guideline values exist for irrigation, there is few related to CEC´s, indeed they are still being investigated [Arshad and Shakoor, 2017]. Present parameters for good water quality are based on conductivity, residual sodium carbonate, total dissolved solid and sodium adsorption ratio. [Arshad and Shakoor, 2017; Zaman et al., 2018].

The presence of clorophenoxy herbicides in the water sources due to losses (surface runoff and leaching), may have a negative effect in the environment affecting non target organisms such as commercial crops, soil bacteria and algae community in freshwater [Ma et al., 2006; Nalcaci O.O. and B., 2006; Newton et al., 2020; Sagasta, 2010]. Many commercial crops are broadleaf dicotiledons and are susceptible to growth regulator herbicides such as 2,4D and MCPA [Xinzheng, 2021]. Indeed, the non-target herbicide injury is an arising problem for commercial crops such as *Lactuca sativa* (Lettuce) and *Lepidium sativum* (Cress) [Beate, 2012; Nalcaci O.O. and B., 2006; Xinzheng, 2021]. Despite these compounds are used due to their selectivity to dicotyledons, research studies proved that some monocotyledons species from the Poaceae family, such as *Triticum aestivum L.* (wheat) and *Zea maiz* (maiz) were affected by MCPA and 2,4D [Galon et al., 2021; Kumar and Kumar Singh, 2010]. Furthermore, concern is shared and effects of MCPA and 2,4D are still being investigated also for soil bacterium ans algae community such as *Bacillus subtilis* and *Raphidocelis subcapitata*, respectively [Ma et al., 2006; Newton et al., 2020].

Due to the uses and importance of water quality for food production and drinking water, and possible effects in the environment, it is crucial to treat and control potentially harmful compounds in water. The conventional water treatment processes for herbicides includes filtration, coagulation-flocculation, adsorption and sedimentation [Derylo-Marczewska et al., 2010]. These methods have a relatively high operational cost and may have secondary effects such as sludge formation for example [Syafrudin et al., 2021]. On the other hand, Advanced Oxidation Processes (AOPs) are recognized as clean technologies for water treatment, including VUV Vacuum and Ozone [Syafrudin et al., 2021; Derylo-Marczewska et al., 2010]. Ozone might be attractive, but in some cases the ozone degradation products can be equally harmful and requires chemical addition, whereas VUV does not need chemical addition. Thus, VUV process becomes the adequate technique to remove, as clean as possible, any compounds present [Derylo-Marczewska et al., 2010; Imoberdorf and Mohseni, 2011].

1.1 Problem statement

MCPA and 2,4D are the most common selective herbicides used worldwide to control broad leaved weeds. They have been used since 1492 for food production, residential and recreational purposes. If these compounds are not taken up by the target plants after application or degraded, they can leach from the site of application into different environmental compartments. Their high solubility in water, makes them mobile through the surface and soil profile, and as a result end up in the surface and groundwaters. These natural water sources are used for irrigation and drinking water, and certain concentrations of the mentioned herbicides may affect commercial crop yield and human health. The increasing awareness of the effects of MCPA and 2,4D makes them interesting to study and to provide a solution to remove them from water.

This overall project aim is to determine the degradation of MCPA and 2,4D during an Advanced Oxidation Processes (AOP) with Vacuum UV (VUV) irradiation, and to determine the acute ecotoxicity before and after treatment to Lactuca sativa (Lettuce), Lepidium sativum (Cress), Raphidocelis subcapitata (a green microalga) and Bacillus subtilis (a soil bacterium). The following problem statement has been set:

Does 2,4 D and MCPA affect the growth of *Lactuca sativa* (Lettuce) *Lepidium sativum* (Cress), *Bacillus subtilis* (soil bacteria) and *Raphidocelis subcapitata* (micro algae)? Can these herbicides be removed from drinking water by combined Vacuum UV and UV-C (VUV-UVC) treatment effectively and can VUV-UVC treatment change the ecotoxicity of the herbicides?,

To answer the problem statement the following research questions have been made:

- Can *Lactuca sativa*, *Lepidium sativum*, and *Raphidocelis subcapitata* be used as model organisms to test the phytotoxicity of the herbicides 2,4D and MCPA?
- Can *Bacillus subtilis* be used as model organism to test the toxicity of the herbicides 2,4D and MCPA to soil bacteria?
- Can VUV-UVC treatment degrade 2,4D and MCPA in water, and are there differences in degradation rates for the two herbicides?
- Can VUV-UVC treatment of 2,4D and MCPA in water lower ecotoxicity (EC50) and what is the effect of irradiation time?

2 Theory

2.1 Herbicides

Herbicides are a group of pesticides used to control or remove undesired plants, that may endanger the growth and yield of desired crops [Caudle, 2015]. They are available as formulations rather than the pure chemical, depending on the desired use and application method.

A formulation is made when the active principle of the herbicide is combined with solvents or sufractants, depending on the chemical solubility in water, oil and organic solvents. They come as a powder, solution and pellets, resulting in a wide variety of formulations for the same herbicide [Miller and Westra, 1996].

2.1.1 Fate in the environment

After application, herbicides are expected to be taken by the target plants. If this doesn't occur, they spread within the environment reaching the air and the water, by volatilization and leaching processes, respectively [Zimdahl, 2007]. The path and fate of the herbicides depends on the water and soil characteristics, the climatic conditions and the compound properties, that will promote or inhibit their degradation [Garrido et al., 2015]. When herbicides present in the water and air are not degraded, they may affect non target organisms due to their presence in the water cycle and water uses (Figure1)

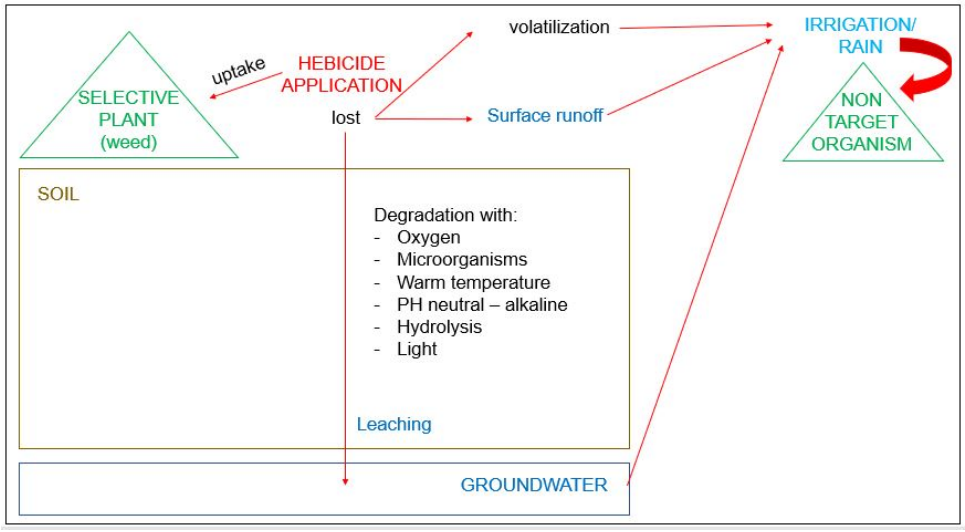

Figure 1. Environmental spread and fate of herbicides. Design elaborated by the author based on literature [Abate Jote, 2019; Garrido et al., 2015; Cabral et al., 2022; Vlaiculescu, 2020; Zimdahl, 2007]

Spread in the environment

Chemicals spread in the air depending on their volatility. Volatilization depends on the vapor pressure which determines the capacity of the chemical to change to the gaseous state [Voutsas, 2007]. The higher the vapor pressure, the more volatile the compound will be [Abate Jote, 2019].

The movement of the chemicals in water is called leaching. By this process, herbicides may reach the groundwater or surface water, as a result of the leaching through the soil profile and surface runoff respectively[Zimdahl, 2007]. Herbicides are transported downwards, dissolved in the mass of water by a process called convection, which is determined by the water velocity and the effective porosity (pore number and connectivity). The time for a chemical to reach the ground water is also influenced by the soil characteristics which define the chemical dispersion and sorption [Loll and Moldrup, 2000] (Figure 2).

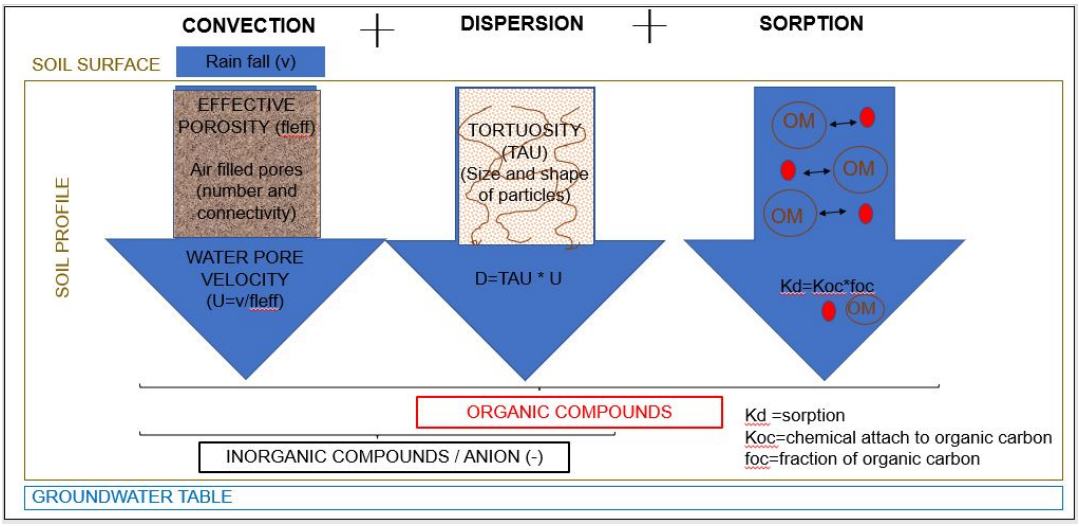

Figure 2. Transport of chemicals in soil. Adapted from Loll and Moldrup [2000]

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The dispersion of the chemical in the soil depends on the tortuosity, which is given by the soil structure. The pores shape, size and orientation, affects the velocity rate at what chemicals travel and the path they take. In narrow pores, velocity will be lower than in wide pores, as well as it will be lower close to the wall pore than in the center [Loll and Moldrup, 2000].

Additionally, soil texture and components determine the sorption capacity, where the solute molecules move from the soil solution onto the solid phase. Sorption is related to the retardation of the chemical to raise the groundwater, because at a higher sorption it will take more time for the pollutant to reach the underground aquifer [Loll and Moldrup, 2000].

Sorption of inorganic chemicals depend on the compound electronic charge and the cation exchange capacity (CEC) of the soil. The attach of the pollutants is done with the smallest particles of the soil (clay). Clay is negatively charged so it attracts pollutants that are positively charged, while repels the negatively charged [Loll and Moldrup, 2000].

Moreover, organic pollutants are retained by the soil depending on their polarity. Polar compounds follow similar sorption mechanisms as the inorganic pollutants like the ion exchange. A polar pollutant is ionizable meaning that it will divide into cations (ion positively charged) and anions (ion negatively charged), which will be retained by the soil or repelled by it, respectively. The soil properties that prevails in this exchange are CEC, clay content and metal oxides [Loll and Moldrup, 2000].

On the other hand, non -polar compounds are sorbed to the organic material present in the soil and the process is driven by hydrophobic interactions, where the pollutant attaches the organic matter rather than water because of the polar similarity. Water is more polar than the organic material so the non-polar pollutant will interact with the element of similar polarity [Loll and Moldrup, 2000].

2.1.2 Degradation in the environment

The presence of herbicides in the environment also depends on the photo, chemical and biological degradation [Zimdahl, 2007]. Persistent compounds have the tendency to conserve their molecular structure after they have been released. Complex compounds are strongly bounded so their degradation is more difficult [Garrido et al., 2015].

Herbicides may be photo decomposed in contact with light [Vlaiculescu, 2020]. The degradation rate is influenced by the amount and intensity of light received and the molecular structure of the chemical [Cabral et al., 2022]. Chemical degradation is given by hydrolysis, oxidation and reduction reactions which are affected by the soil pH [Vlaiculescu, 2020]. Biodegradation is performed by the microorganisms present in the soil [Vlaiculescu, 2020]. This process is affected by the temperature, oxygen concentration, water, nutrient supply and pH [Cabral et al., 2022]. Under anaerobic, cold, dry and acid conditions decomposition will be low [Vlaiculescu, 2020].

As herbicides has the potential to be degraded by natural processes, the artificial removal or remediation of this compounds from the environment, consists on enhancing this natural mechanisms. The elimination process will concentrate on biological, chemical or photo degradation, depending on the type of compound ans the environmental compartment the herbicide may be in [Pileggi et al., 2020; Saleh et al., 2020].

2.1.3 Herbicide removal from water

There are several treatments to remove pesticides from water. Biological processes includes activated sludge, membrane bioreactors, and membrane technologies. Chemical treatments consists on coagulation, adsorption and advanced oxidation process (AOPs) based on chemical supply or on photo decomposition. Each technique has advantages and disadvantages regarding operational costs, efficiency, operability, reliability, environmental impact, pre-treatment requirements, and the sludge production of sludge and toxic transformation products [Derylo-Marczewska et al., 2010; Saleh et al., 2020; Syafrudin et al., 2021].

The advantages of advanced oxidation process (AOPs) is that it have fast degradation

rates, thus less retention time, but the maintenance and operational costs are high. Two of the most common AOPs used to remove pesticides are the ozone-based treatment and ultraviolet (UV)-based treatment. The ozone- based process consists in adding ozone (O3) in the water, leading to a direct removal (reaction of ozone with the contaminant) or indirect by the interaction of the free radicals (oxidative potential), product of the O3 hydrolysis, with the compound. On the other hand, the photochemical decomposition, is based on UV radiation [Saleh et al., 2020]. UV- treatment can be used in combination with catalyst agents such as titanium dioxide (TiO2) and ferric oxide (Fe2O3); or different UV radiation. Due to the lack of chemical supply and residual sludge, vacuum UV-UVC appears to be a promising approach [Derylo-Marczewska et al., 2010].

Vacuum UV- UVC

Ultraviolet radiation is defined as the form in which the energy is emitted by electromagnetic waves. The energy emitted is inversely proportional to the wavelength. This radiation is present in the sun and in the high vapor lamps, and, includes the range between UV-A (380–315 nm), UV-B (315–280 nm), UV-C (280–200 nm), vacuum-UV (VUV) (200–100 nm), and extreme UV (100–1 nm) [Zoschke et al., 2014]. All the radiation emitted within the ultraviolet spectrum, is able to degrade compounds by direct photolysis, defined as the chemical reaction induced by photons [Speight, 2018]. The breakdown depends on the molecule capacity to absorb the UV rays, defined by the Molar absorption coefficient of the molecule, which changes depending on the wavelength. This process is also called direct photo decomposition and it can be represented as Equation 2.1.3 [Benitez et al., 2004]:

$$\text{Molecule} \xrightarrow{hv} \text{M} \cdot \text{intermediate} \tag{2.1}$$

Where "hv" is the photon energy, given by the Plank constant (h) and the frequency of radiation (v) and, "M. intermediate" is the product of the molecule breakdown.

Sometimes molecules can be also photo decomposed in an indirect way. This occurs after photolysis, when the intermediate molecule is a reactive specie that can react with another molecule, oxidizing it, thus decomposing it. In VUV, the most common intermediate reactive species capable of oxidize another molecule is the hydroxyl radical (•OH or HO•) [Kelly and Arnold, 2012].

Water highly absorbs radiation at low wavelength, at 185 the absorption coefficient is 1.8cm-1, whereas at 245 nm is almost negligible [Breinholt, 2020; G. and M., 2012]. High energy UV radiation photodecomposes the water molecules in two ways: photolysis (obtaining hydroxyl radicals (HO•), hydrogen radicals (H)) and photochemical ionization (obtaining hydroxyl radicals (HO•), hydrogen radicals (H+), and free electrons (e-)), Equations 2.1.3 and 2.1.3, respectively [Breinholt, 2020; Imoberdorf and Mohseni, 2011]:

$$\text{H}_2\text{O} \xrightarrow{hv(185nm)} (\cdot\text{OH}) + \text{H} \tag{2.2}$$

$$H_2O \xrightarrow{hv(185nm)} (\cdot OH) + (H^+) + (e-) \tag{2.3}$$

HO• is a very reactive species, which can oxidize and mineralize organic compounds, promoting the removal of contaminants in water [Imoberdorf and Mohseni, 2011; WHO, 2017]. Pollutants are mostly degraded by HO• radical, but may also be degraded by other radicals Equation 2.1.3 [G. and M., 2012] .

$$(\cdot OH) + \text{Pollutant} \longrightarrow \text{TransformationProduct} \tag{2.4}$$

Vacuum ultraviolet (VUV) is one of the known Advanced Oxidation Processes (AOPs). The spectral domain includes the wavelengths between 140 and 200 nanometers with energy sufficient to photo decompose the water [Breinholt, 2020; WHO, 2017]. When the water is hydrolyzed and ionized, the radicals obtained by the reaction oxidize the organic pollutants. This strong oxidation capacity (Equation 2.1.3) and the photolysis breakdown of the chemical (Equation 2.1.3) are the reason why VUV is one of the most effective remediation methods [Chu].

Comparing with the commonly used UV-A, UV-B, and UV-C lamps, VUV lamp can lead to more HO• generation and better removal efficiency of contaminants at the same level of energy consumption [Yangtao et al., 2019] Thus, the advantage of VUV process is that it does not require the addition of any chemicals [Imoberdorf and Mohseni, 2011].

Furthermore, the response of the pollutants to this treatment depends on temperature, pH, flux radiation of the UV light, chemical properties and the compound reactivity. At a higher temperature, the solubility and dissociation of the salts, acids and bases increases [Benitez et al., 2004; G. and M., 2012]. The pH of the solution affects the dissociation, depending on the pKa of the compound. At a low pKa the molecule will have a strong tendency to dissociate in water [Benitez et al., 2004; G. and M., 2012]. On the other hand, the radiation flux of the UV light is related to the wavelength, the lower the wavelength, the higher the photon energy which increases the molecule breakdown [Benitez et al., 2004; G. and M., 2012].

2.2 Chlorophenoxy herbicides

The chemicals 2,4D and MCPA are synthetic chlorophenoxy herbicides used for the post-emergence control of broad-leaved weeds [Derylo-Marczewska et al., 2010]. Both compounds have been used worldwide since 1940, due to their high selective ability to kill weeds, without harming cereals and grass crops [Zimdahl, 2007]. In Europe, United States and England MCPA is most commonly applied and 2,4D in rest of America [Grabińska-Sota and Kalka, 2003; Zimdahl, 2007].

The uses and performances of both compounds are similar [Zimdahl, 2007]. Thus, in the molecular structure, they differ from each other by the presence of a methyl group (CH3) or chlorine (Cl) in the 2nd position of the benzene ring, Figure 3.

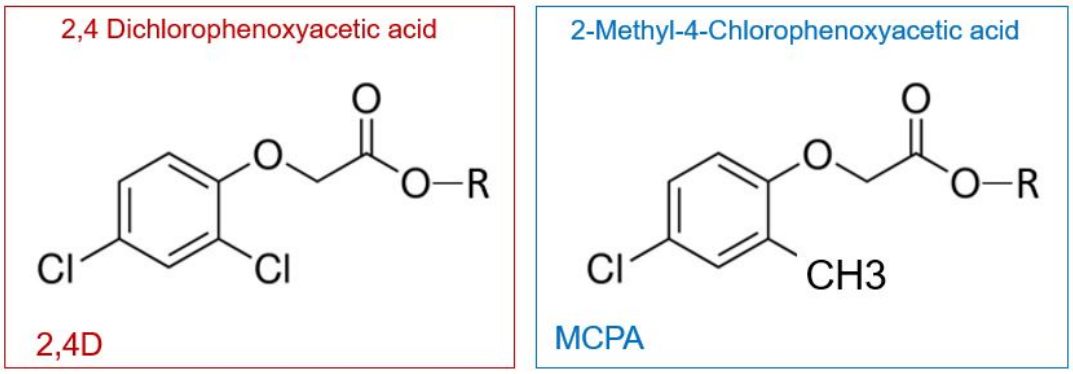

Figure 3. Molecular structure of 2,4D (left) and MCPA (right). Adapted from Trigo et al. [2014] and MERK

Both herbicides are formulated as salts, amines and esters. They are produced by substitution of the "R" in Figure 3 with the mentioned groups [Zimdahl, 2007; Tu M., 2001]. Ester formulations are more readily to be absorbed by cuticle and cell membranes, thus more toxic [Zimdahl, 2007]. The formulations of these compounds include liquids, water-soluble powders, dusts, granules, or pellets alone, or in mixtures with other herbicides [Tu M., 2001].

2.2.1 Mode of action

MCPA and 2,4D are synthetic auxin herbicides, which kill the target weed by mimicking the plant growth hormone auxin (indole-3-acetic acid), causing uncontrolled cell division [Palma-Bautista et al., 2020; Grabińska-Sota and Kalka, 2003; Tu M., 2001]. As a consequence the vascular transport system gets blocked and leads to the plant death [Zimdahl, 2007]. There are three ways in which this herbicides act causing the plant death, by: a) altering the plasticity of the cell walls; b) influencing protein production; and c) increasing ethylene production; [Palma-Bautista et al., 2020]. Although it is known that the sites of action in the plant involve protein families in auxin perception, signaling transport and metabolism; they are still not defined [Palma-Bautista et al., 2020].

Their effectiveness depends on the uptake and translocation to the plant meristems [Palma-Bautista et al., 2020]. These compounds are absorbed by roots, shoots and leaves; and translocated into the plant [Palma-Bautista et al., 2020; Zimdahl, 2007]. The absorbance point depends on the mode of application. The most common method is foliar application by spray drifts, which makes the uptake mainly by leaves [Palma-Bautista et al., 2020].

Also effectiveness, is provided by the high concentrations maintained in the tissues, which plants can not control or regulate [Zimdahl, 2007]. Low concentrations may stimulate the rate of root or shoot growth, seed germination and photosynthesis, but high concentrations can inhibit these processes [Grabińska-Sota and Kalka, 2003].

Despite penetration, translocation and site of action, toxicity depends not only on the retention of the chemical in the plant, but in the soil [Palma-Bautista et al., 2020; Woodford, 1952]. Indeed, to compare effectiveness and toxicity between chemicals is recommended to perform laboratory tests rather than field experiments [Woodford, 1952]. Field experiments may mislead the results due to the dinamics of the chemicals in the soil and, their variance in purity and formulations [Woodford, 1952]. Thus, for assessing auxin and growth activity laboratory experiments are more relevant [Woodford, 1952].

These compounds are classified as selective herbicides, reason why they are so used and well known. Their target is to only affect dicotyledon plants such as weeds present in croplands, without affecting monocotyledon plants [Song, 2014]. It is yet unknown how auxinic herbicides kill dicotyledons while sparing monocotyledons at the molecular level because both plants share the same auxin production, transport, and signal transduction pathways [Palma-Bautista et al., 2020; Zimdahl, 2007]. Recent studies suggested that the underlying reason could be either due to limited translocation or fast degradation of external auxin, changed vascular morphology, or altered auxin perception [Song, 2014]. As plant vascular systems affect auxin transport, the selectiveness of auxinic herbicides may be influenced by the distinction between the vascular tissue structures of dicotyledons and monocotyledons. Phloem and xylem in monocotyledons stems are dispersed in bundles and do not have a vascular cambium; in dicotyledons stems, the vascular tissues are produced in rings [Grabińska-Sota and Kalka, 2003; Song, 2014].

2.2.2 Persistence in the environment

Water

Chlorophenoxy herbicides, threaten the water quality due to its extensive application in agriculture, high mobility and persistence in the aqueous media [Derylo-Marczewska et al., 2010]. Both organic compounds have anionic forms which determines their mobility within the environment [Derylo-Marczewska et al., 2010]. In water, ester and salt formulations are rapidly converted to the anion form by hydrolysis and dissociation, respectively [Abate Jote, 2019]. The rate of hydrolysis depends on the pH, and is higher when pH increases [Abate Jote, 2019]. Due to the negatively charged structure and high solubility, these compounds are repelled by the soil, and stay dissolved in the aqueous soil fraction [Derylo-Marczewska et al., 2010]. As they are not retained by the soil, these chemicals can rapidly move through the soil profile to the groundwater, or over the soil to the surface water [Trigo et al., 2014].

In water, these compounds degrade rapidly under aerobic conditions by microorganisms [Canada, 2010]. The aerobic aquatic half life, is 14 days for MCPA and 13 days for 2,4 D. However under anaerobic conditions the degradation is negligible [Abate Jote, 2019; Morton et al., 2020] (Table 1).

Table 1. Physicochemical properties MCPA and 2, 4 D

PARAMETER	MCPA	2,4 D	References
Molecular weight (g/mol)	200.62	221	[Vergili and Barlas, 2009]
Water solubility (in mg/L, at 25ºC)	640	890	[Vergili and Barlas, 2009]
Hydrolisis half-life in days (at 25ºC, pH=7)	40	39	[Abate Jote, 2019]
Aqueous photolysis half-life in days(at 25ºC)	14	13	[Abate Jote, 2019; Morton et al., 2020; Canada, 2010]
Anaerobic aquatic half-life in days	negligible	312	[Abate Jote, 2019; Canada, 2010]
Aerobic aquatic half-life in days	13	15	[Abate Jote, 2019; Canada, 2010]
Aerobic half-life in days	60	66	[Abate Jote, 2019; Canada, 2010]
Soil photolysis half-life in days	67	393	[Abate Jote, 2019; Morton et al., 2020]
Field dissipation in days	60	59.5	[Abate Jote, 2019; Morton et al., 2020]
Vapor pressure (in Pa at 25ºC)	$4.0\times10^{-}4$ - $1.1\times10^{-}2$	$1.9\text{x}10^{-}5$ - $3.1\times10^{-}3$	[Paszko et al., 2016]
Log Kow (Octogonal -water coefficient)	2.73	2.83	[Vergili and Barlas, 2009]
Log Koc (Soil adsorption coefficient)	1.73 - 2.07	1.3 . 2.03	[Agency, 1995; Morton et al., 2020]

Hydrolysis and photodegradation in natural conditions are not relevant routes in these compounds and is highly dependant on pH, temperature and light intensity. At pH 8, 25ºC under sunlight the aqueous photolitic half life is 15 days for both compounds. On the other hand, hydrolisis half life is around 40 days at pH above 7; at lower pH there is no degradation [Abate Jote, 2019; Canada, 2010; Morton et al., 2020] (Table 1).

Air

Dissipation through air of these chlorophenoxy herbicides is minor due to their low vapor pressure [Abate Jote, 2019] (Table 1) . Ester formulations are more volatile than salts and acid formulations. Anyway it is estimated that 0.3% of the herbicide is lost in the air after a spray drift application, which is the only source that makes volatilizing possible [Morton et al., 2020]. Once in the air, if not degraded, the compounds may return to the earth's surface through wet deposition (dissolved in rain) and dry deposition (as gases or aerosols or sorbed to particles) [Canada, 2010].

Soil

MCPA and 2,4D are not persistence in soil, the field dissipation is around 60 days (Table 1). Indeed the dissipation depends on the soil type, pH and moisture [Canada, 2010]. These factors influence the mobility through the soil profile as well as the degradation [Canada, 2010].

Biological degradation is the most relevant factor in these herbicides breakdown. The most common bacteria are the *Pseudomonas sp.*, *Bacillus sp.* and *Flavobacterium sp.*, *Arthrobacter*, *Xanthobacte*, *Alcaligenes* [Grabińska-Sota and Kalka, 2003; Vlaiculescu, 2020]. Under aerobic, alkaline and warm conditions the degradation rate is higher [Abate Jote, 2019].

2.3 Herbicides effect in non target organisms

Since herbicides are chemicals in nature, frequent and excessive usage could result in residual problems: phytotoxicity to crop plants, negative effects on non-target organisms, and ultimately health risks for people and animals [Sondhia, 2014]. Drawbacks to using herbicides, includes production of toxic byproducts from herbicide partial degradation, modifications to the biogeochemical cycles and microbial communities in soil, changes to

plant nutrition, and enduring environmental contamination. Some herbicides harm non-target bacteria through processes that cause oxidative stress and directed interference with host metabolism [Pileggi et al., 2020].

Because of their presence in the environment and accumulation across the food chain, herbicides can result in health issues. Humans are mostly exposed to these toxins through food, and fish and shellfish have been identified as important vectors for contamination transmission to humans. In aquatic systems 2,4D affects Daphnia *Daphnia magna*, rainbow trout *Salmo gairdneri* and bluegills *Lepomis macrochirus* [de Castro Marcato et al., 2017]. Several metabolic changes and tissue necrosis can be brought on by 2,4-D in non-target organisms, including food chain members. After oral exposure, 2,4-D is readily absorbed in the digestive system; depending on the dose and chemical type of 2,4-D, the agrochemical residues can be seen in plasma levels from 10 minutes to 24 hours after exposure [de Castro Marcato et al., 2017].

Both MCPA and 2,4D while dissolved in water, can travel considerable distances and may affect non targeted ecosystems and organisms [Boutin et al., 2004]. In fact, injuries in plants have been detected as a result of these diffusive point sources where low concentrations of the herbicides were present [Boutin et al., 2004; Xinzheng, 2021]. Although these herbicides are not directly applied to commercial dicotyledon crops, they are still susceptible to their effects. Examples of sensitive dicotyledons are legumes (soybean), ornamental plants and vegetable plants (lettuce) [Song, 2014]. Despite is expected that this compounds should not harm monocotyledons crops such as wheat, sorghum, corn, rice, sugarcane and pastures, recent studies suggested that they me be affected [de Castro Marcato et al., 2017].

2.3.1 Test organisms

Lactuca sativa

Lactuca sativa is an herbaceous plant usually grown for food (Figure 4). It is cultivated in all the continents, and is one of the most consumed green leafy vegetables in the raw form for its taste and high nutritive value [Noumedem et al., 2017; Pink and Keane, 1993]. Some studies reported correlation between Lettuce consumption and prevention of chronic disease. Several minerals such as calcium, magnesium, potassium, zinc and iron are contained in the leaves and are important for human health [Kim et al., 2016].

Figure 4. *Lactuca sativa* (Iceberg). Source: Gug Anlæg og Planteskole

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Its rapid germination and high sensitivity makes it a common model for different studies [Tigre et al., 2012]. It has been investigated that off target herbicides like MCPA and 2,4D, affect commercial crops such as *Lactuca sativa* [Xinzheng, 2021]. Many commercial crops are broad leaved dicotyledons which are particularly susceptible to the auxin regulator herbicides [Xinzheng, 2021]. And although it is known that broad leaved plants are susceptible to this type of herbicides, few is known about their effects at low concentrations [Roesler et al., 2020; Xinzheng, 2021].

Lepidium sativum

Lepidium sativum is an annual, dicotyledonous fast growing edible herb [Grabińska-Sota and Kalka, 2003; Ramadan and Oraby, 2020] (Figure 5) . It is commercially grown in several countries and consumed as food due to its medicinal properties [Juma and Colin, 2011; Ramadan and Oraby, 2020]. Besides shoots and leaves which are consumed as salads, seeds have enriched nutrients stored in the endosperm which makes them more promising [Juma and Colin, 2011]. During imbibition, a mucilage layer is formed around the seed which serves as food storage for the germination [Juma and Colin, 2011].

Figure 5. *Lepidium sativum.* Source: Gug Anlæg og Planteskole

.

Lepidium sativum is easily affected by clorophenoxy herbicides at different concentrations which make this plant, ideal for research purposes [Nalcaci O.O. and B., 2006].

Raphidocelis subcapitata

Raphidocelis subcapitata is a curved unicellular green microalgae, present in freshwater (Figure 6). As part of the aquatic ecosystem, produces oxygen and serve as food for other organisms [Ma et al., 2006]. This algae is highly sensitive to chemicals, reason why is worldwide used as a bioindicator of pollutants and for toxicological bioassays [Suzuki et al., 2018].

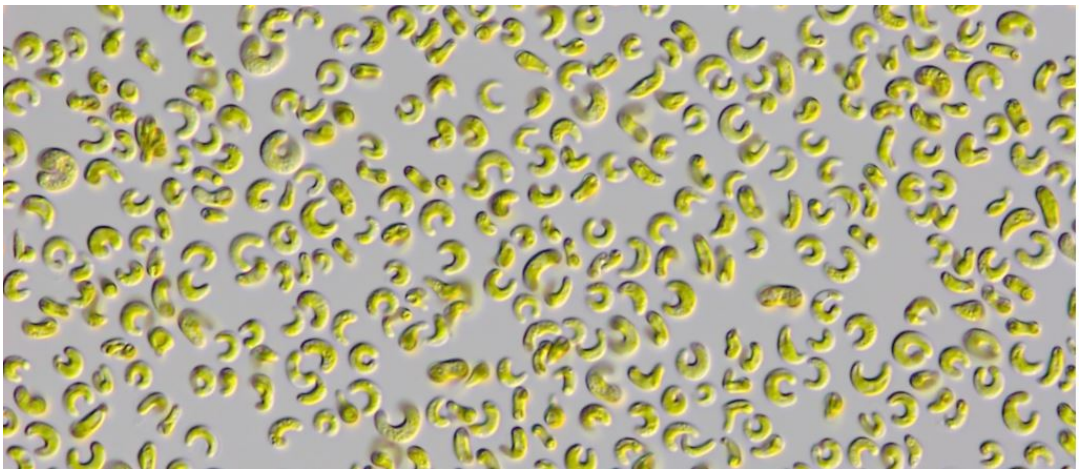

Figure 6. *Raphidocelis subcapitata.* Source: [Göttingen]

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Herbicides may affect the aquatic ecosystem through altering the composition of an algal community [Ma et al., 2006]. Depending on the herbicide mode of action, the effect on the algae will differ [Ma et al., 2006]. This includes affections in photosynthetic process, cell division, synthesis in lipids, hormones and proteins, between others [Ma et al., 2006].

Bacillus subtilis

Bacillus subtilis is a gram positive rod shaped bacterium that form heat resistant spores [Piggot, 2009] (Figure 7). The ability to produce endospores helps the bacteria to survive severe environmental conditions [Hashem et al., 2019]. It also secrete antibiotics and hydrolytic enzymes and modify its environment in a self beneficial manner [Hashem et al., 2019]. This bacteria can form biofilms in inert surfaces which consist of a multicellular bacterial community covered in a self-secreted matrix, that serves as a physical barrier with the surrounding [Hashem et al., 2019; Newton et al., 2020]. The biofilm may be built up in 24 hours in presence of polysaccharides and malic acid [Hashem et al., 2019].

Bacillus subtilis is commonly present in the soil. Indeed, is an important component of the plant rhizosphere, soil region that surrounds the root. It acts as a plant growth-promoting bacteria, with the capacity to form symbiotic relationships with plants and enhance their growth by different mechanisms such as nitrogen fixation [Hashem et al., 2019; Newton et al., 2020]. Colonization of roots by *Bacillus subtilis* is benefitial to both, the bacteria and the plant. While bacteria provides nutrients and compounds that promotes the plant growth and stress protection, the root exudates stilmulate the biofilm production [Hashem et al., 2019].

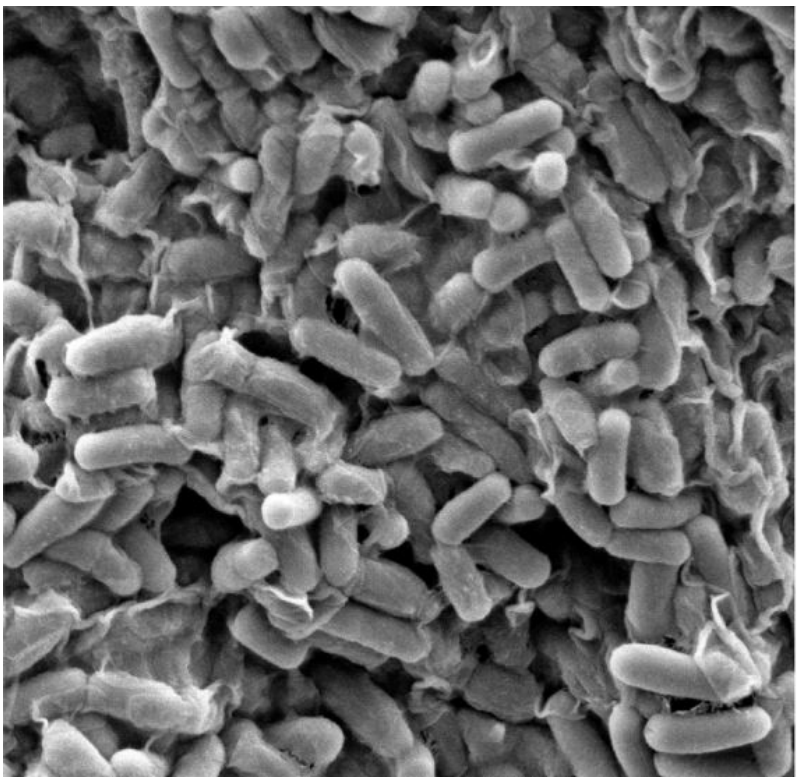

Figure 7. *Bacillus subtilis.* Source: [Kovács, 2019]

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Bacillus subtilis may be affected by the application of the commercial pesticides, inhibiting the biofilm formation [Newton et al., 2020]. On the other hand, some studies suggest that the presence of this bacteria in the soil may enhance the degradation of some herbicides such as glyphosate [Yu et al., 2015]. However, *Bacillus sp.*, may be sensitive to clorophenoxy herbicides, and its growth could be inhibited or promoted depending on the environmental conditions and the concentrations at where they are exposed to [Kizilkaya, 2000].

3 Materials and methods

The present chapter explains the steps that were performed in order to comply with the objectives. It is divided in two main sections:

- Establishing of the dose - response curve to obtain a 0-100% effect range and EC50 in *Lactuca sativa*, *Lepidium sativum*, *Raphidocelis subcapitata* and *Bacillus subtilis*, after exposing them to different concentrations of MCPA and 2,4 D; and test possible endpoints for the seeds
- Degradation of MCPA and 2,4D in drinking water, during Advanced Oxidation Processes with Vacuum UV irradiation and determination of EC50 after treatment in *Lactuca sativa*, *Lepidium sativum* and *Bacillus subtilis*.

3.1 Key chemicals

The main compounds used for all the tests were the two chlorophenoxy herbicides: 2-methyl-4-chlorophenoxyacetic acid (CAS 94-74-6, >95% purity), and 2,4-Dichlorophenoxy acetic acid (CAS 94-75-7, >95% purity), both obtained in Sigma Aldrich.

Stock solutions of MCPA and 2,4D were prepared with distilled deionized water for determination of the dose- response curves and, with tap water from Aalborg University, Denmark for the degradation of the herbicides with VUV. They were stored in a cold room at 5^{o}C in complete darkness.

3.2 Test organisms

Seeds of *Lactuca sativa* (Lettuce: "Calmar" - Iceberg salad) and *Lepidium sativum* (Cress) were obtained from Gug Anlæg og Planteskole, Aalborg and stored in darkness at room temperature. The brand and item code for the seeds were Nelson Garden - D:91273 and Wibulls - 7621, respectively. Growth medium for both cases was pure distilled water.

Raphidocelis subcapitata was grown in the algal medium following the ISO 8692: 2012 preparation. Algal growth medium was prepared in 1L deionized distilled water, adding 10ml of stock solution N^{o}1 and 1ml of stock solutions N^{o}2, 3 and 4. Description of the stock solutions N^{o}1, 2, 3 and 4 are found in the Appendix 7. The exponential growth phase was obtained after 2-5 days of incubating the algae culture at 20^{o}C on a shaker with continuous illumination (6000-10000 lux).

Bacterial strains of *Bacillus subtilis* (DSM10) were obtained from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Germany and stored in complete

darkness at -20^{o}C in the fridge. *Bacillus subtilis* was grown in Davis Minimal Broth (obtained in Merck, Denmark; 93753) on a shaker at 80 rpm and 30^{o}C during 18 hours.

3.3 Methods and experiments

3.3.1 VUV-UVC irradation

The degradation of MCPA and 2,4D in drinking water was investigated using a VUV photoreactor from ULTRAAQUA A/S (Aalborg, Denmark). A total volume of 4 liters of stock solution (10 mg/L) was made for MCPA and 2,4D. The compounds were dissolved in tap water after 1,5 hours under stirring mixing. After being completely dissolved, 4 liters of the stock solution were transferred to the reservoir of the VUV-UVC photoreactor. The reservoir was equipped with a magnetic stir bar to promote mixing, and a stainless-steel cooling spiral to maintain the temperature at 10^{o}C (Figure 8).

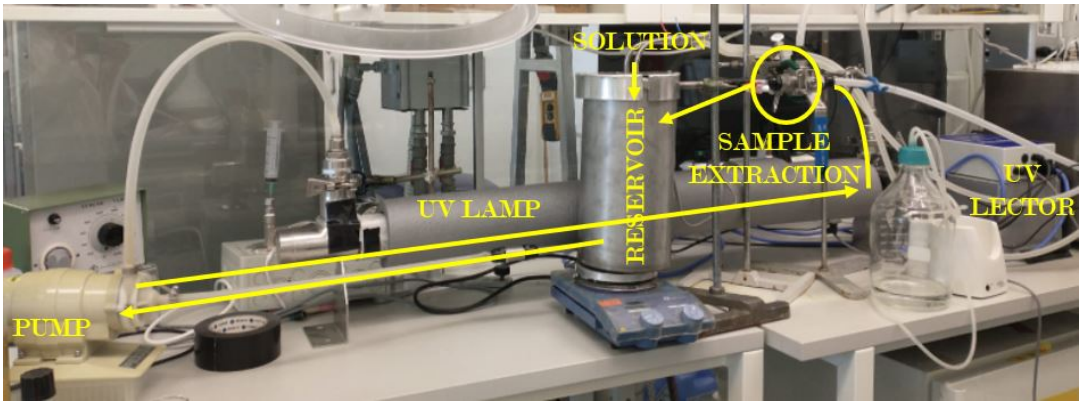

Figure 8. Vacuum UV system

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Each solution run into the system for 34 minutes, passing from the reservoir through the reactor which consisted of a low-pressure VUV Hg transparent lamp with a diameter of 19 mm, covered by a stainless-steel tube with an inner diameter of 53 mm, a length of 1270 mm, and volume capacity of 1.7 L. The UV lamp emitted radiation at a wavelength of 185nm (VUV) and or 254nm (UVC) at 1:4 ration with a radiation of 14 W and 56 W, respectively. The recirculation was made by a diaphragm pump operated at 2 L/min. The reactor was equipped with an UVC detector and the maximum irradiance was detected at 340 W m 2.

Before turning on the lamp, each solution was recirculated for two minutes to stabilize the temperature. Samples were taken with a syringe, extracting 50 mL at each time point. The sample at time 0 was collected before turning on the lamp, and samples at times 1, 2, 4, 8, 16 and 32 were extracted with the lamp turned on (Figure 9).

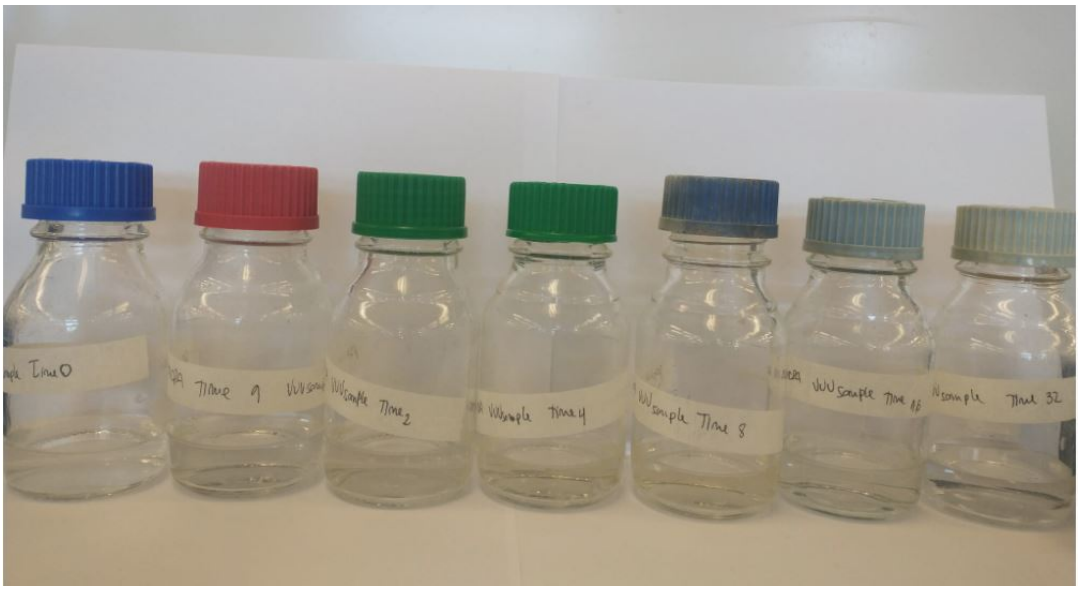

Figure 9. Collected samples for MCPA after 0, 1, 2, 4, 8, 16 and 32 minutes; while performing the Vacuum UV treatment

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After VUV, extracted samples at all times were run through a High-Performance Liquid Chromatography (HPLC) to estimate the degradation rate of both compounds. The separation method was performed in a C18 column with a mobile phase of Acetonitrile (CAS 75-05-8) and a buffer solution of sodium acetate (0.01347 M) and acetic acid (0.006532 M). The gradient elution was determined at a flow rate of 1.0 mL/min, and the wavelength for UV detection was set at 230 nm. Calibration curves ware also made by injecting 50 uL of both compounds at different concentrations (1, 10 and 100 mg/L).

3.3.2 Toxicity of MCPA and 2,4D in *Lactuca sativa*

Dose response curve and alternative endpoint

The experiment set up to determine the phytotoxicity of MCPA and 2,4D in *Lactuca sativa* was performed in 90 mm Petri Dishes. 15 seeds were separately placed over one filter paper of 85 mm. Filter paper was obtained from Frisenette ApA, Knebel. Each Petri Dish was filled with 10 mL of solution: deionized distilled water (control) and deionized distilled water + chemical (treatment). During 48 hours the Petri Dishes with the seeds were incubated at 20^{o}C in darkness and after, transferred to an incubator with a diurnal cycle (16 hours light, 8 hours dark) with white fluorescent light at 4300Lux for 72 hours. The test lasted for 120 hours and was replicated 3 times. The dose-response curve was obtained after testing the seeds at different concentrations of MCPA and 2,4D (0.00076, 0.003, 0.012, 0.048, 0.195, 0.781 and 3.125 mg/L). The endpoint was the elongation of root and shoot (together), and was measured with a ruler.

As an alternative endpoint for *Lactuca sativa*, emergence seedlings were also tested, based on the ISO 17126:2005(E) protocol. Petri Dishes (90mm) were filled with 40 grams of fine sand used as the growth medium. 20 seeds of *Lactuca sativa* were placed over the sand

layer with a distance of 1 centimeter and covered with 30 grams of coarse sand. Each Petri Dish represented one treatment and no replicate was performed. Sand was spiked with 10 mL of 2,4D and MCPA at different concentrations (0.00076mg/L, 0.012 mg/L, 0.19 mg/L, 0.78 mg/L and 3.125 mg/L) except for the two control. The same incubation conditions were applied for all the samples as described above, and the endpoint was the number of emerged seedlings that could be seen with the eye. At the same time, a back up experiment with filter paper was performed in order to compare the differences between the endpoints, considering in this case, elongation. (Figure 41.

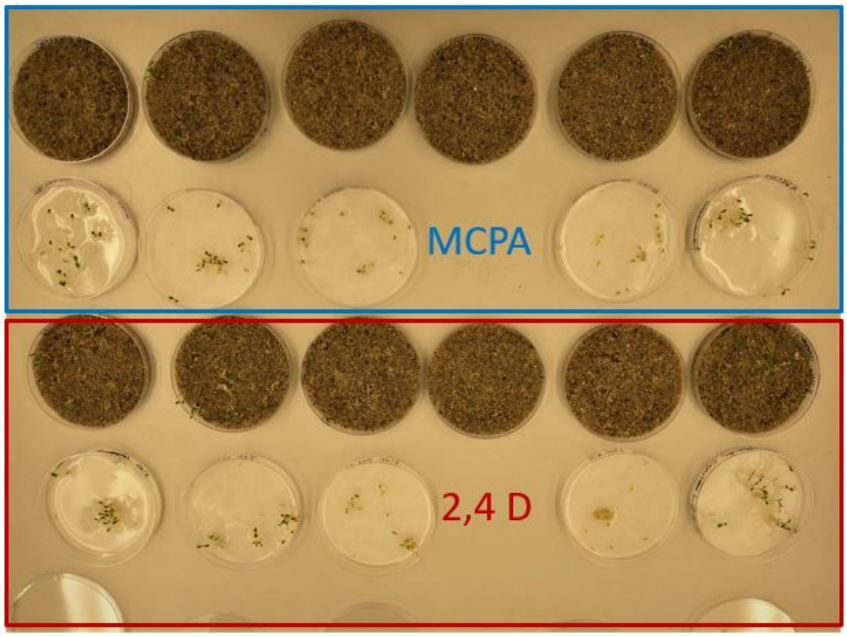

Figure 10. Experiment set up to compare toxicity of MCPA and 2,4D in *Lactuca sativa* with seedling emergence (sand) and elongation (filter paper) as an alternative endpoint.

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3.3.3 Toxicity of MCPA and 2,4D in *Lepidium sativum*

Dose response curve and alternative endpoint

Toxicity of MCPA and 2,4D in *Lepidium sativum* was tested using different experiment set up: using 6 well plates (6WP) and 90 mm Petri Dishes (PD) (Figure 11). This was made to observe the differences in the results between the experiments, while performing the dose-response curve.

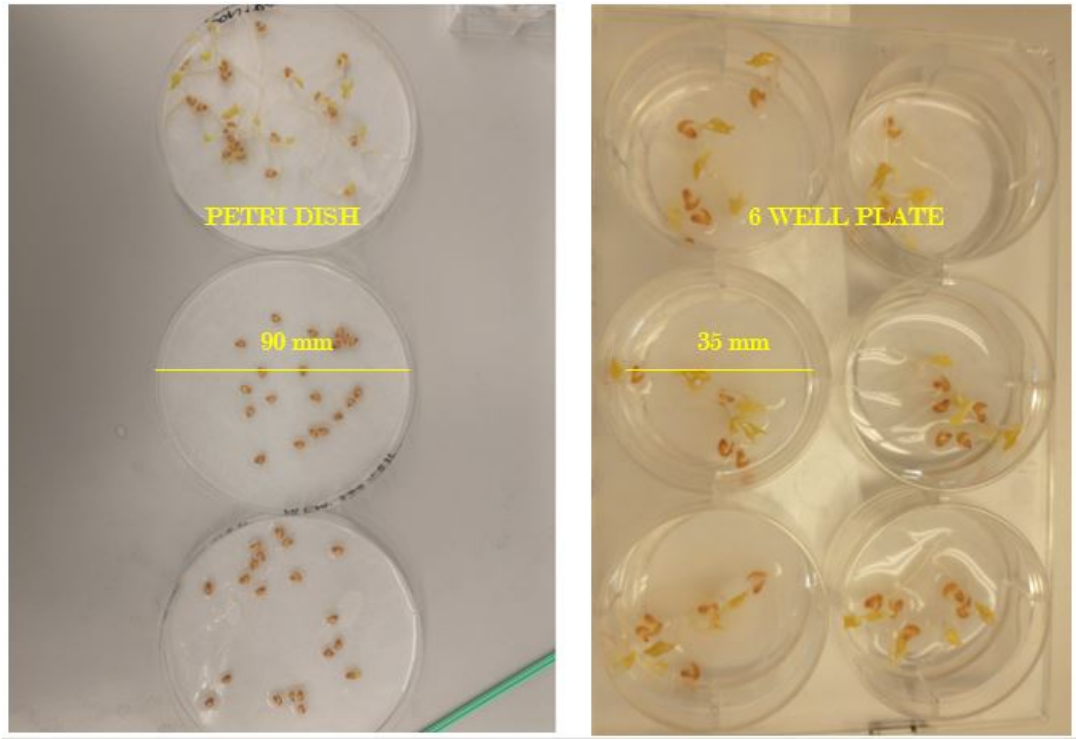

Figure 11. Petri Dish and 6 well plate experiment set up

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Both set up consisted in placing *Lepidium sativum* seeds over one filter paper obtained from Frisenette ApA, Knebel; 30 mm in 6WP and 85 mm in PD. In each well the corresponding solution was added: 1.5mL in 6 well and 10 mL in 90 mm PD. For the control pure distilled water was used, and plates were covered with a lid and incubated for 72 hours in darkness at 20^{o}C.

Tests were performed by changing the amounts of seeds per well: 5 or 10 seeds per well in 6WP and 15 seeds in 90 mm PD. The comparison between the two set up were based on the elongation of the control (0 mg/L) of all the tests performed. The tests were grouped based on the amount of seeds per well in each different test (Table 2).

Table 2. Group of experiment set up per amount of seeds per well for the elongation comparison for the test controls; where (PD) is Petri Dish and (6WP) is 6 well plate.

Experiment Set Up	Number of seeds per well	Volume per well (mL)
6WP	5	1.5
6WP	10	1.5
PD	15	1.5

The concentrations used to determine the dose response curve were for both compounds: 0.00019, 0.00076, 0.003, 0.012, 0.048, 0.195, 0.781, 3.125, 12.5, 50 and 200 mg/L. The endpoint was the elongation of root and shoot (together), and was measured with a ruler.

As an alternative endpoint to determine the toxicity of MCPA and 2,4D in *Lepidium*

sativum, dry weight was measured twice:

- a) while defining the standard curve at different concentrations (0.003, 0.012, 0.048, 0.19 and 0.78 mg/L)
- b) after Vacuum UV treatment at times 4 and 8 for MCPA and 2,4D respectively.

In both cases the time period in the oven was for 24 hs, and each treatment had 30 seeds to weight. Although the seeds were distributed in the 6 well plate during the 72 hours incubation (5 seeds per well), the 30 seeds of the same treatment were measured together in the same metal tray Figure 12.

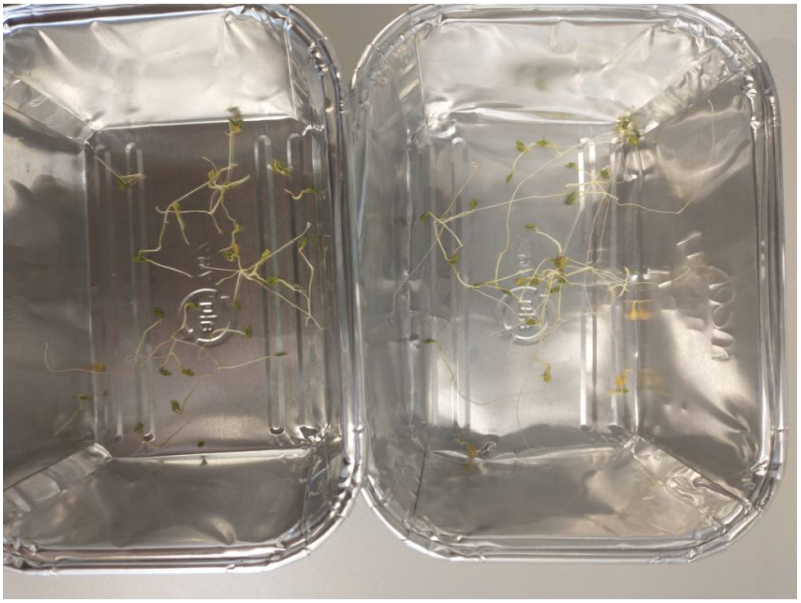

Figure 12. Dry Cress after 24 hs in a 65^{o}C oven, in metal dry

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For test a) seeds with the sprouts were dried in the oven at 60^{o}C and 105^{o}C,for MCPA and 2,4 D respectively. The testing concentrations in mg/L were 0, 0.003, 0.012, 0.048, 0.19 and 0.78. While for test b) only the sprouts (roots and shoots) were dried in the oven at 65^{o}C. The testing concentrations were dilutions D0, D4, D16, D64, D32 at time 8 for 2,4D and time 4 for MCPA (see Section **??** for better understanding).

The dry weight (DW) was obtained by subtracting weight of the empty metal tray (MT), to the weight obtained after the samples were dried in the oven (WSO):

$$\mathrm{DW(g)} = \mathrm{WSO(g)} - \mathrm{MT(g)} \tag{3.1}$$

3.3.4 Toxicity in *Lactuca sativa* and *Lepidium sativum* after VUV-UVC

Samples extracted from VUV were diluted by a factor 10 to obtain the respective Dilution zero (D0). This was made to have a parameter to fit with the standard curve where the

effect range concentrations was lower (1mg/L). For samples extracted at Times 0, 2, 4, 8, 16 and 32; D0 was diluted with pure distilled water by a factor 4, obtaining D4, D16, D64 and D256. Same set up as described before was performed: for *Lepidium sativum* 6 well plate with 5 seeds and 1.5mL solution per well, and for *Lactuca sativa* 90 mm PD with 15 seeds and 10mL solution per well, both using filter paper as the growth medium (Figure 13). Incubation period was the same as described above for the dose-response curves.

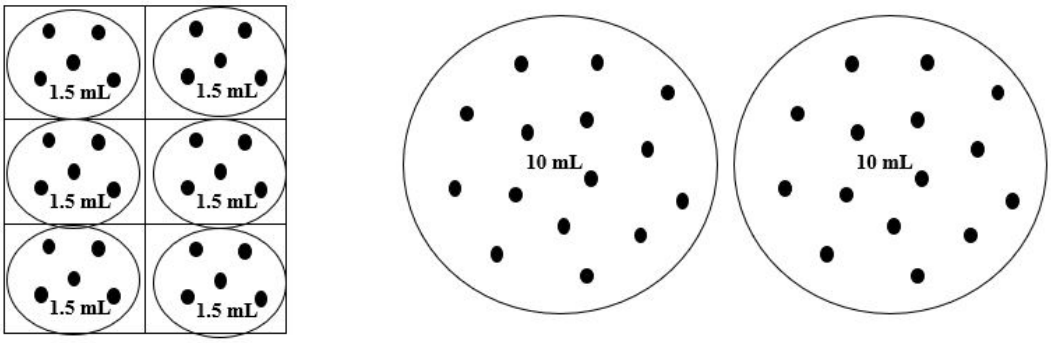

Figure 13. Experiment setup for *Lepidium sativum* (left) and *Lactuca sativa* (right), in 6 well plate and 90 mm Petri Dish respectively.

Lactuca sativa and *Lepidium sativum* were tested with all the respective dilutions for Time 0, 2, 4, 8 and 16 for MCPA, and Time 0, 2, 4, 8, 16 and 32 for 2,4D. The design is shown in Figure14. For each serial dilution at the respective time the standard curve and the EC50 was determined, for both compounds.

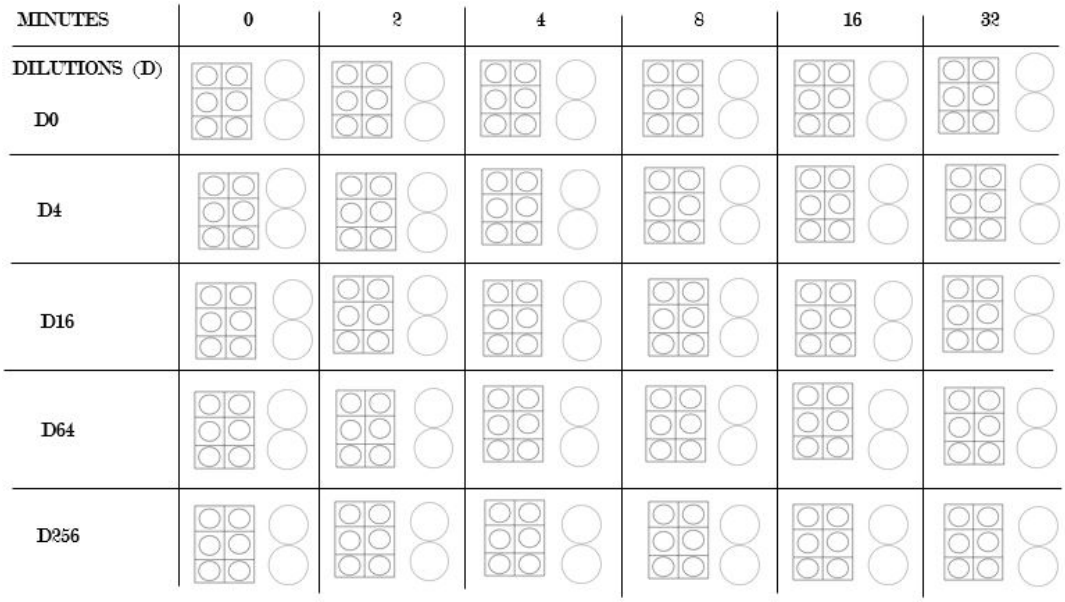

Figure 14. Experiment design to test ecotoxicity of MCPA and 2,4D in *Lactuca sativa* and *Lepidium sativum* after VUV-UVC

.

3.3.5 Toxicity of MCPA and 2,4D in *Raphidocelis subcapitata*

Toxicity in *Raphidocelis subcapitata* was determined by the growth inhibition of the algae, after exposure at different concentrations of MCPA and 2,4D in the exponential growth phase. In order to obtain that, a 96 well plate with 12 columns and 8 rows (A-H) was used to perform each test, which was repeated three times. Columns 1 and 12 were left as the control (algae medium and culture), and as the blank (algae medium), respectively (Table 3). The test medium solution is detailed in Appendix 7

Table 3. Design of the 96 well plate to test toxicity in *Raphidocelis subcapitata* after exposure to different concentrations of the herbicides. M: algae medium, A: algae inoculation, C: compound (herbicide). Source: Roslev 2021

Nº Column	1	2	3	4	5	6	7	8	9	10	11	12
Treatment	M+A	M+A+C	M+A+C	M+A+C	M+A+C	M+A+C	M+A+C	M+A+C	M+A+C	M+A+C	C	M
Concentration (mg/L)	0.00	0.20	0.39	O.78	1.56	3.13	6.25	12.50	25	50	100	Blank
A	Control											Blank
B	Control											Blank
C	Control											Blank
D	Control											Blank
E	Control											Blank
F	Control											Blank
G	Control											Blank
H	Control											Blank

150 μL of algal test medium was transferred with a pipette, from columns 1 to 10 and to column 12 (300 μL). Column 11 was filled with 300 μL of the chemical stock solution (200 mg/L of MCPA and 2,4D). Serial dilution was made by transferring 150 μL from column 11 to column 10, and successively until column 2 using a 8-channel pipette. By this method the stock solution was diluted 10 times by a factor 2. Separately algal culture with *Raphidocelis subcapitata* was diluted in the test medium (1:50). 150 μL of the diluted algal culture was inoculated in columns 1 to 11 using a 8-channel pipette.

The plates were covered with a lid and taken to a room at 20ºC. They were settled on a shaker at 60 rpm under constant light (6000-10000 LUX) for 72 hours. After that period of time, growth was quantified by the absorbance in a Thermo Multiskan Plate Reader at 450 nm (Figure 15).

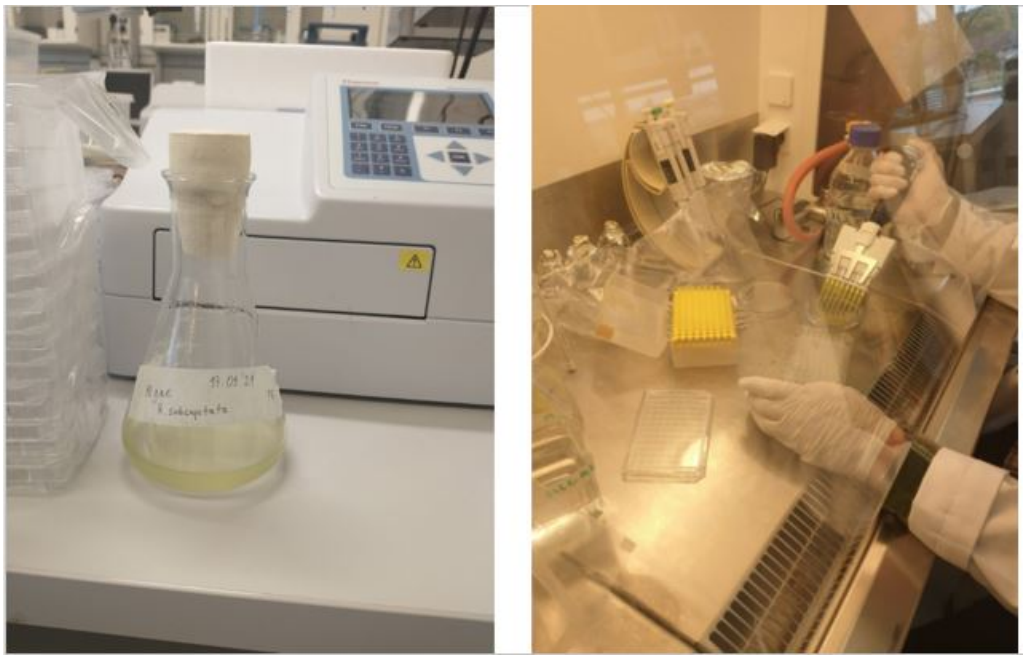

Figure 15. Left: algae culture in an Erlenmeier flask and Thermo Multiskan Plate Reader behind. Right: performance of the serial dilution with an 8 channel pipette in the 96 well plate

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3.3.6 Toxicity of MCPA and 2,4D in *Bacillus subtilis*

Dose response effect

Experiment set up to determine the toxicity of MCPA and 2,4D in *Bacillus subtilis* was similar to the Subsection described above. Both herbicides were tested on the bacteria in the exponential growth at concentrations of 10 and 100 mg/L. A 96 well plate with 12 columns and 8 rows (A-H) was used to perform the test. In the same plate two different samples were tested at the same time by dividing the plate in rows: A-D and E-H. Columns 1 and 12 were left as the control (bacteria medium and culture), and as the blank (bacteria medium), respectively (Table 4).

Table 4. Design of the 96 well plate for the toxicity test in *Bacillus subtilis* with detail treatment. M: bacteria medium, B: bacteria inoculation, C: compound.

Nº Column	1	2	3	4	5	6	7	8	9	10	11	12
Treatment	M+B	M+B+C	M+B+C	M+B+C	M+B+C	M+B+C	M+B+C	M+B+C	M+B+C	M+B+C	C	M
Dilution from stock concentration (C) in mg/L	0.0	C1024	C518	C256	C128	C64	C32	C16	C8	C4	C2	Blank
A-D Sample 1 - Initial CC 10mg/L	Control											Blank
E-H Sample 2- Initial CC 100mg/L	Control											Blank

100 μL of autoclaved distilled water was transferred with a 8 channel pipette, from columns 1 to 10 and to column 12 (150 μL). Column 11 was filled with 200 μL of the chemical stock solution, which changed depending on the sample. Serial dilution was made by transferring 100 μL from column 11 to column 10, and successively until column 2 using a 8-channel pipette. By this method the stock solution was diluted 10 times by a factor 2. 50 μL of concentrated bacterial medium (4x strength Davis Minimal Broth) was transferred to all the columns using a 8 channel pipette. Separately, bacterial culture with *Bacillus subtilis* was diluted in autoclaved saline solution (0.9% NaCl in distilled water) by 1:1000. 50 μL of

the diluted bacterial culture was inoculated in columns 1 to 11 using a 8-channel pipette. The test medium solution is detailed in Appendix 7.

The plates were covered with parafilm and taken to a a Gulv incubator shaker at 30^{o}C for 18 hours. The growth, was quantified by the absorbance read in a Thermo Multiskan Plate Reader at 620 nm.

Toxicity in *Bacillus subtilis* after VUV-UVC

Bacillus subtilis was tested after VUV treatment at times 0, 2, 4, 8, 16 and 32. Same procedure was performed as as described above. The stock concentrations were the extracted samples from VUV-UVC at those different times, as it is shown in Tables 5, 6 and 7, below.

Table 5. Design of the 96 well plate test for the bacteria test with detail treatment after VUV. M: bacteria medium, B: bacteria inoculation, C: compound.

Nº Column	1	2	3	4	5	6	7	8	9	10	11	12
Treatment	M+B	M+B+C	M+B+C	M+B+C	M+B+C	M+B+C	M+B+C	M+B+C	M+B+C	M+B+C	C	M
Dilution from stock concentration (C) in mg/L	0.0	C1024	C518	C256	C128	C64	C32	C16	C8	C4	C2	Blank
A-D Sample 1 - Time 0	Control											Blank
E-H Sample 2- Time 2	Control											Blank

Table 6. Design of the 96 well plate test for the bacteria test with detail treatment after VUV. M: bacteria medium, B: bacteria inoculation, C: compound.

Nº Column	1	2	3	4	5	6	7	8	9	10	11	12
Treatment	M+B	M+B+C	M+B+C	M+B+C	M+B+C	M+B+C	M+B+C	M+B+C	M+B+C	M+B+C	C	M
Dilution from stock concentration (C) in mg/L	0.0	C1024	C518	C256	C128	C64	C32	C16	C8	C4	C2	Blank
A-D Sample 1 - Time 4	Control											Blank
E-H Sample 2- Time 8	Control											Blank

Table 7. Design of the 96 well plate test for the bacteria test with detail treatment after VUV. M: bacteria medium, B: bacteria inoculation, C: compound.

Nº Column	1	2	3	4	5	6	7	8	9	10	11	12
Treatment	M+B	M+B+C	M+B+C	M+B+C	M+B+C	M+B+C	M+B+C	M+B+C	M+B+C	M+B+C	C	M
Dilution from stock concentration (C) in mg/L	0.0	C1024	C518	C256	C128	C64	C32	C16	C8	C4	C2	Blank
A-D Sample 1 - Time 16	Control											Blank
E-H Sample 2- Time 32	Control											Blank

3.4 Data analysis

3.4.1 Toxicity calculation

The endpoints for the four organisms was growth, in terms of: elongation (including root and shoot) for *Lactuca sativa* and *Lepidium sativum* measured with a ruler, and absorbance for *Raphidocelis subcapitata* and *Bacillus subtilis* measured with a spectrophotometer. After measuring the absorbance, the blank values (column 12) were subtracted from the rest of the data (all columns columns and rows). The effect of the herbicides in growth was calculated as the inhibition related to the control ([Xinzheng, 2021]):

$$\mathrm{Y}=\frac{Gc-Gx}{Gc}\cdot 100 \tag{3.2}$$

Where: "Y" is the inhibition in %, "Gc" is the growth in the control and "Gx" is the growth at a certain concentration. The units of "Gc" and "Gx", depended on the endpoint: when absorbance was used the units where "Au" (absorbance units), and when elongation was used the units were "cm" (centimeters).

The inhibition values were used to fit the data in the logistic model following the equation:

$$Y = \frac{1}{1 + \exp(-(a + b \cdot \log(x))} \tag{3.3}$$

Where "Y" is the inhibition in %, "a" and "b" are regression parameters, and "x" is the concentration in mg/L.

After fitting the data, using Excel Solver as the software tool, the regression parameters were obtained, and EC50 was estimated as:

$$EC_{50}(mg/L) = 10 \exp \frac{a}{-b} \tag{3.4}$$

The data fit the model well when R2 was higher than 0.8, which was the model's acceptance criteria, in this project. If this was not the case, the data was fit into a linear model which was obtained by plotting the logarithm of the inhibition (Log(Y)) against the logarithm of the concentration (Log(CC)):

$$Log(Y) = m \cdot Log(CC) + b \tag{3.5}$$

Where "Y" is the inhibition in %, "CC" is the concentration in mg/L; "m" is the slope and "b" is the minimum inhibition. The parameters "m" and b" were given by the displayed equation in Excel after fitting the data in a linear trendline. With those values, EC50 was calculated as:

$$EC_{50}(mg/L) = 10 \exp\left(\frac{Log(50) - b}{m}\right) \tag{3.6}$$

3.4.2 Vacuum UV-UVC degradation of MCPA and 2,4D

The calibration curve was made by plotting the Area results (mAU · minutes) from HPLC against the concentrations (1,10 and 100 mg/L). By fitting the data in a linear trend, the regression relationship was obtained with a R2 around 1 for both compounds (Figure 16).

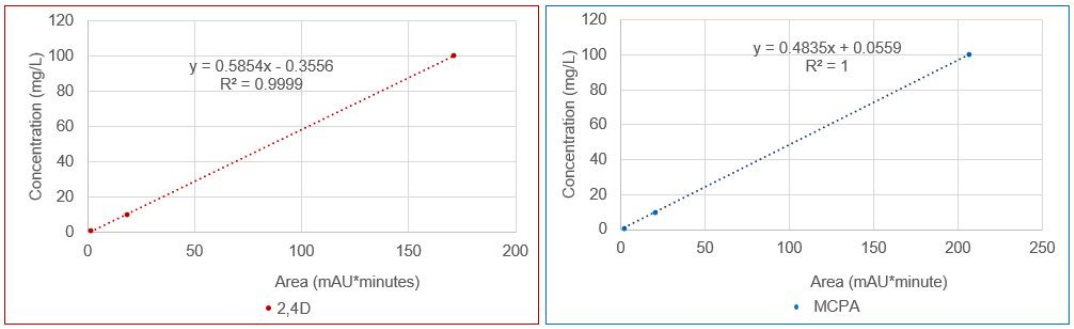

Figure 16. HPLC calibration curves for 2,4D (left) and MCPA (right). Area units are mili Absorbance Units (mAU)

.

Equations obtained by fitting the data, were used to calculate the remaining concentrations of 2,4D and MCPA at each time point after VUV-UVC:

$$\mathrm{Y(2{,}4\,D)} = 0.5854 \cdot \mathrm{x} - 0.3556 \tag{3.7}$$

$$\mathrm{Y(MCPA)} = 0.4835 \cdot \mathrm{x} + 0.0559 \tag{3.8}$$

Where "Y" is the concentration in mg/L and "x" is the Area values obtained after performing the HPLC in mAu (mili Absorbance units).

After estimating the concentrations at all time points, a first order kinetic model was used to calculate the degradation rate for both compounds ([Amin et al., 2016]):

$$\mathrm{ln(C/Co)} = -\mathrm{k(min{-}1)} \cdot \mathrm{t(min)} \tag{3.9}$$

Where "C" is the concentration in mg/L at time "t" (minutes) and and "Co" is the initial concentration at time zero (10 mg/L). "k" is the kinetic value (1/min) which determines the rate at which the compound is degraded. The linear equation, thus "k", were obtained after plotting the natural logarithm of C/Co "ln (C/Co)" over time, in Excel.

4 Results and discussion

4.1 Toxicity of MCPA and 2,4D in test organisms

Toxicity of MCPA and 2,4D was tested in *Lactuca sativa* (Lettuce), *Lepidium sativum* (Cress), *Raphidocelis subcapitata* (green micro algae) and *Bacillus subtilis* (soil bacteria). After exposing the the test organisms to different concentrations of the herbicides, the data was fitted either to the logistic or linear model to obtain the dose- response curves and calculate the EC50. For *Lactuca sativa* and *Lepidium sativum* alternative endpoints were also tested. The results of the experiments are divided per test organism and shown below:

4.1.1 *Lactuca sativa*

Dose response curves

Dose response curves for *Lactuca sativa L.* after being exposed to MCPA and 2,4D, were plot as the elongation inhibition (%) over concentration (mg/L) (Figure17). The data was well fitted in the logistic model with a R-squared of 0.84 and 0.9 for MCPA and 2,4D respectively. By observing both curves, it can be seen that the inhibitory effect is higher for MCPA than 2,4D at all the tested concentrations. Thus, the effective concentration at which the 50% of the growth was inhibited (EC50), resulted in 0.017 mg/L for MCPA and 0.068 mg/L for 2,4D. Despite the differences in EC50, both compounds provoke a 0-100% growth inhibition in *Lactuca sativa* within the concentrations 0.00076 mg/L and 3.125 mg/L.

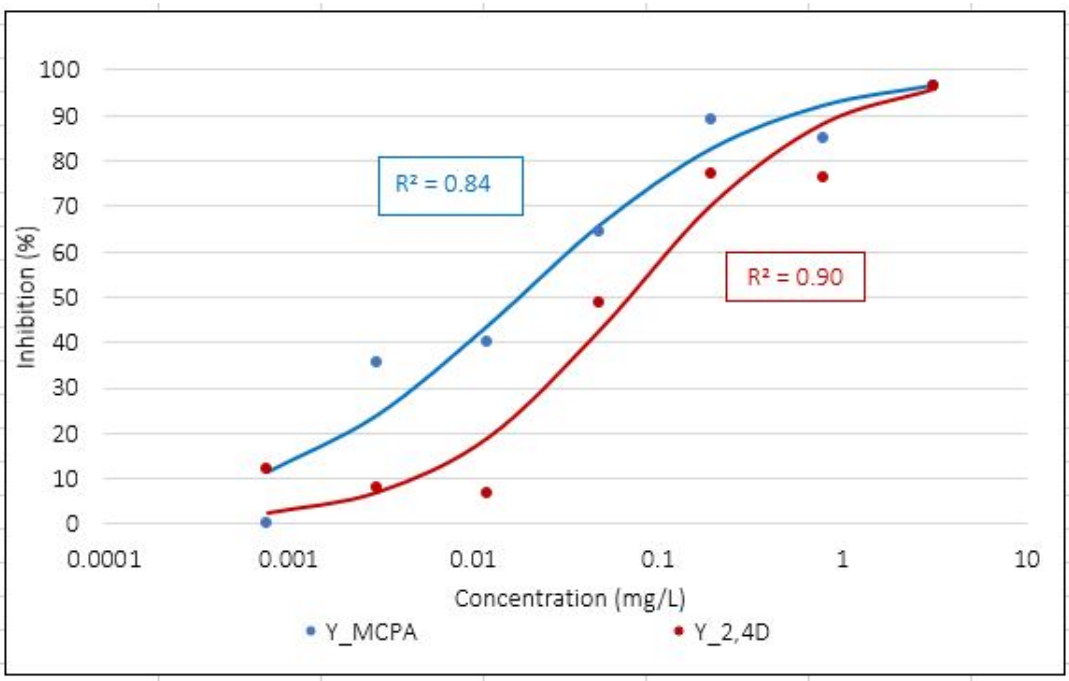

Figure 17. Data fitted in the logistic model for the inhibition in elongation of *Lactuca sativa* at different concentrations of MCPA and 2,4D

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Seedling emergence

After performing the ISO 17-126:2005 test, the number of seedling emerged were counted. The values obtained, for both compounds did not show a correlated tendency between the number of seedling emerged and the tested concentrations. Seedling emergence was expected to increase while reducing the concentrations. On the other hand the emerged seeds at the two control was not similar, in one control the 20 seeds emerged whereas in the other only 1 (Table 8).

Table 8. Number of emerged seedlings of *Lactuca sativa L.* after being exposed to different concentrations of MCPA and 2,4D.

CONCENTRATION (mg/L)	0.00	0.00076	0.012	0.19	0.78	3.125
MCPA	1	4	9	7	5	8
2,4D	20	10	17	13	3	7

ISO 17-126:2005 protocol affirm that the test is valid if the mean emergence seedling of the control is at least 80 %. In this case the mean was 53% and the test was not valid. On the other hand, the EC50 value should correspond to the 50% of the mean seedling emergence in the control, which in this case it was not possible to determinate. Assuming that 10 emerged seeds represents the 50%, it could be said that all the tested concentrations of MCPA are higher than EC50. Following the same logic it could be said that the lowest tested concentrations (0.00076, 0.012, 0.19 mg/L) are equal or below the EC50. Anyway there is not a clear tendency between the concentration and emerged seeds.

In order to define if seedling emergence could be considered as an alternative endpoint for *Lactuca sativa L.* after this particular test performed, the data was fitted into the logistic and linear model. However, the R2 for both models and both compounds, was very low (R2$<$ 0.2), so not relationship between variables could be defined for the seedling emergence test, thus as an alternative endpoint. The dose - response relationship between the % inhibition of the emergence seedling and the applied concentrations are shown in the Appendix 7 (Figure 41A: data fitted in the logistic model and Figure 41B: data fitted in the linear model).

To prove this right, and to compare the results of the endpoints (seedling emergence and elongation), the results of the back up test (filter paper and same concentrations) were collected and analysed. The data obtained from the back up test was fitted into the logistic model with a R2$>$0.9 for both compounds, giving a correlated dose response curve of % inhibition in terms of elongation over concentration (mg/L) (Figure 18).

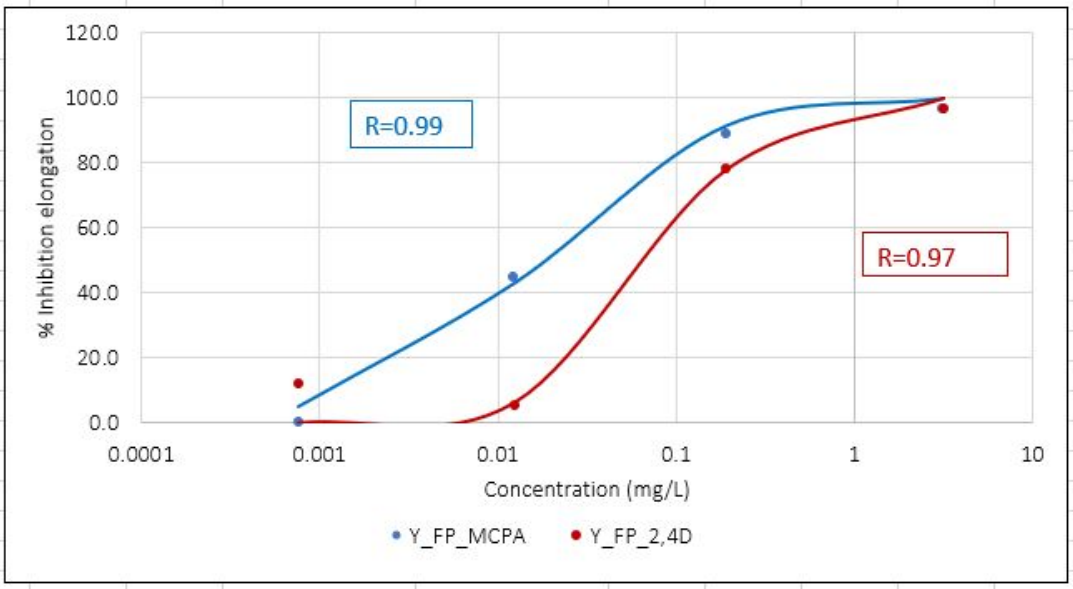


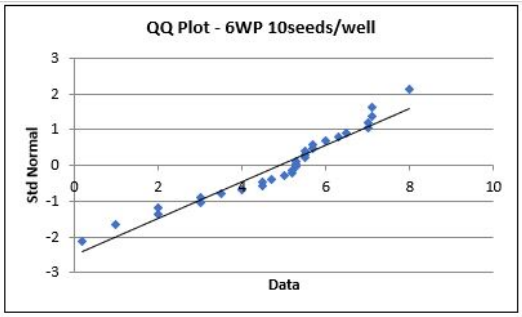
Figure 18. *Lactuca sativa L.* dose response curves of % inhibition in terms of elongation for MCPA and 2,4D, after filter paper test. Data fitted in the logistic model

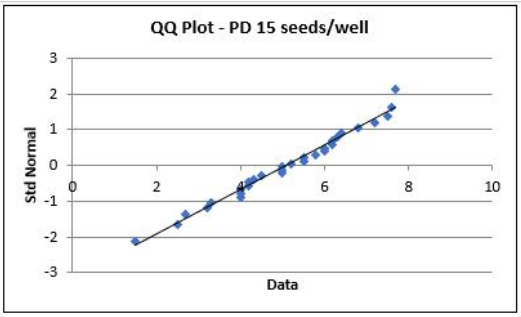
.

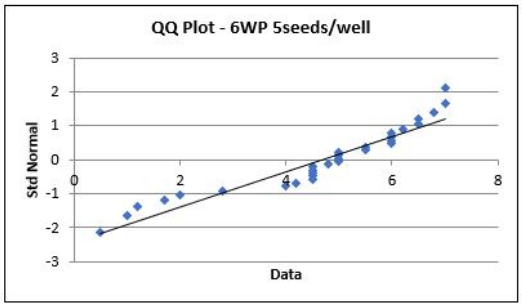
4.1.2 *Lepidium sativum*

Experiment set up

The experiment set up for *Lepidium sativum* was analyzed by using ANOVA - single factor. The comparison was made based on the elongation (cm) of the sprouts in each control at all the set ups: 6WP 5 seeds, 6WP 10 seeds and 90mmPD 15 seeds. Before comparing between the experiments, and to ensure that an ANOVA test could be carried out, a normality test was performed for each of the data set. QQ-plot together with D´Agostino test showed that the data was normally distributed (Figure 19).





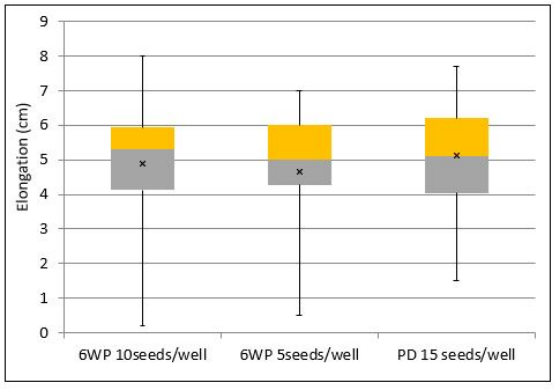


d'Agostino-Pearson

DA-stat	4.125254	4.473468	0.602335
p-value	0.12712	0.106807	0.739954
alpha	0.05	0.05	0.05
normal	yes	yes	yes

Figure 19. Normality test carried out for the the data set of each experiment of *Lepidium sativum*: 6WP (6 well plate) with 5 and 10 seeds per well and PD (Petri Dish) with 15 seeds.

The ANOVA results are reflected in Figure 20, below. The box plot demonstrates how the elongation measurements (cm) is spread, while ordering the data set from the lowest to the highest, in each experiment. The middle value of the ordered data set is also called the median, which is the center line inside the box plot. The average of the data set is given shown as a cross, inside the box. For experiments 6WP-5 seeds and 90PD 15 seeds median and the average mach, whereas for 6WP-10seeds does not. Inversely, 6WP-10seeds has the 50% of the data more concentrated than 90PD-15 seeds and than 6WP-5 seeds, in increasing order. However, the variance in 6WP-10seeds is the highest (3.5cm) and in 90PD 15-seeds the lowest (2.5cm).



SUMMARY

Groups	*Count*	*Sum*	*Average/ariance*	
6WP 10seeds/well	30	146.6	4.887	3.494
6WP 5seeds/well	28	113.8	4.064	3.171
PD 15 seeds/well	30	153.3	5.11	2.506

ANOVA Single Factor

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	17.4	2	8.702	2.849	0.063	3.104
Within Groups	259.6	85	3.054			
Total	277	87				

Figure 20. Elongation results for the different experiment set up (6WP and 90PD) of *Lepidium sativum* given by boxplot (left) and ANOVA test (right)

.

The average for the three set up resulted in 4.1 cm (6WP-5 seeds), 4.8 cm (6WP-10seeds) and 5.1 cm (90PD 15-seeds). Although the average is not the same, it can be said with a 95% of confidence that this dissimilarity between the three set up is not significantly

different. F value (2.8) is below the F critic (3.1), so the null hypothesis can not be rejected, and should be accepted. Null hypothesis states that the elongation between the experiments set up (6WP and 90PD) does not differ hence there is no significant difference.

As no statistical difference was shown between the experiments, the chosen experiment set up, to study the effects of MCPA and 2,4D on *Lepidium sativum* after VUV (Section 4.2.4) was the 6WP- 5 seeds. Another reason was that 6WP was easy to handle, less plastic was used and the amount of seeds made it more comparable with the set up *Lactuca sativa*.

Dose response curves

The set up experiment was also compared by the dose response curves, considering the data obtained by using 6WP, 90PD and the average between both set up. The three curves representing the data fitted in the logistic model while using MCPA is shown in Figure 21. The data fitted best in the model while using the average (Av.) values; the determination coefficient (R2) resulted in 0.97, followed by the 6WP set up (R2=0.95) and by 90PD (R2=0.78).

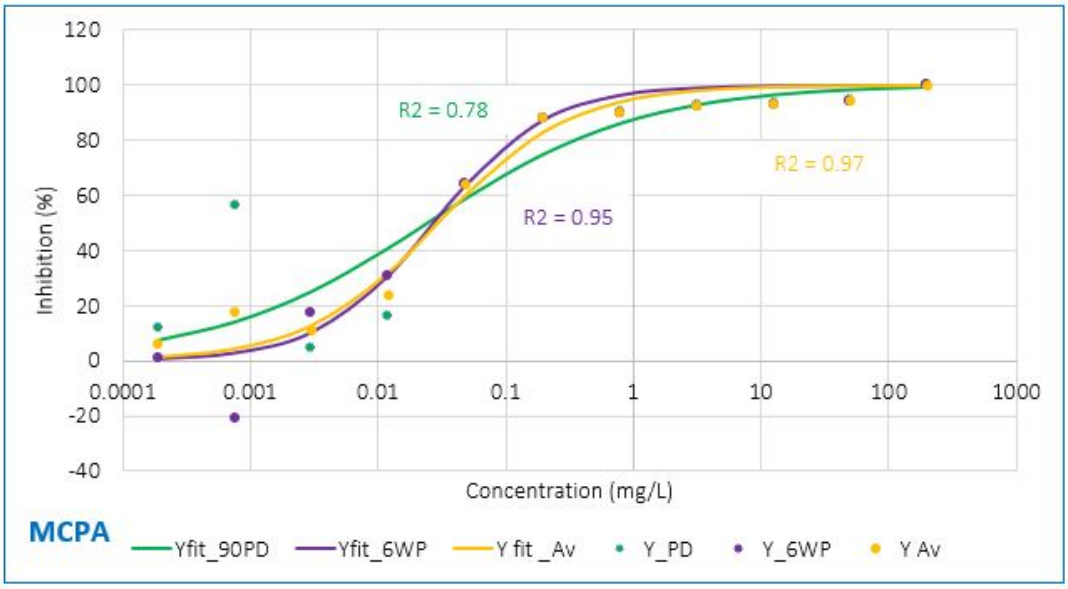


Figure 21. Data fitted to the logistic model for *Lactuca sativa*, in terms of elongation inhibition over concentration of MCPA, while using the different experiment set up: "6WP" (6 well plate), "90PD" (90mm Petri Dish), and "Av." the average between those.

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While using 2,4D, the fitting trendline was similar to MCPA. In decreasing order, the best fit of the data was for the average, with a R2= 0.98, followed by the 6WP set up (R2=0.95) and by 90PD (R2=0.82) (Figure 22).

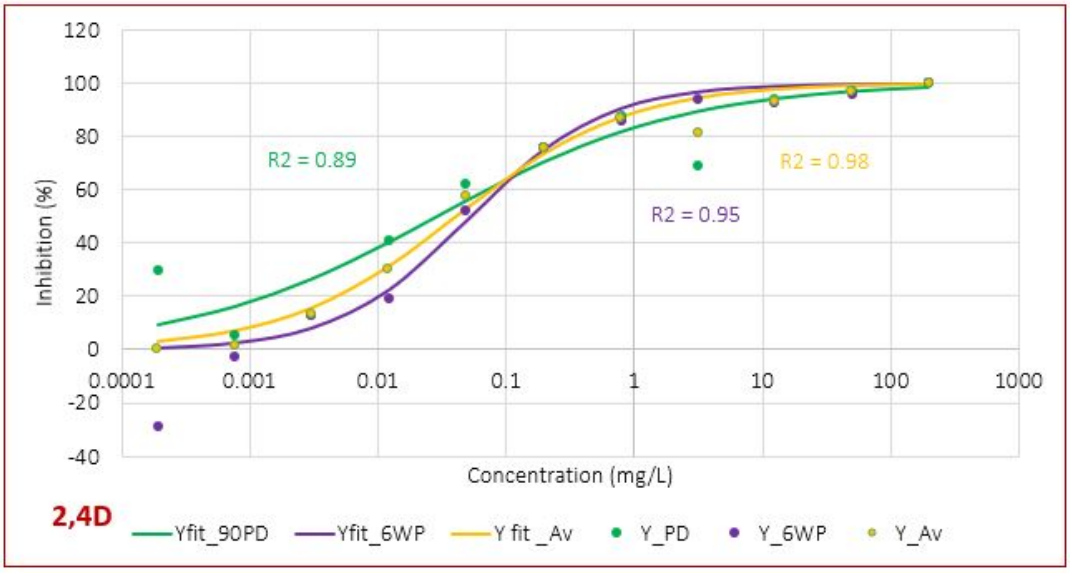

Figure 22. Data fitted to the logistic model for *Lactuca sativa*, in terms of elongation inhibition over concentration of 2,4D, while using the different experiment set up: "6WP" (6 well plate), "90PD" (90mm Petri Dish), and "Av." the average between those.

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By observing the three curves for both compounds in the figures above, is seen that the "S" shape differ. In the two cases "6WP" and "Av." curves have a similar shape between each other compared to "90PD". In increasing order, "6WP" seems to have steeper shape, than "Av." followed by "90PD". The shape is related to the amount of data measured "n", and the "S" profile becomes more pronounced while increasing the number of observations, and in this case less data was obtained for "90PD". The shape also responds to the slope, that relates how much does the % of elongation inhibition changes while increasing the compound concentration. The steeper the slope and shape, the higher the effect; given small changes in concentration, there will be higher changes in the inhibition.

This fact should be also seen while comparing the concentrations in the different curves, that produce the same inhibition. If the shape is steeper that will mean that to reach the same inhibition there will be needed less amount of concentration. The MCPA and 2,4D concentrations that provoke the 10% and 50% inhibition in the three curves, together with the regression parameters used to fit the data in the logistic model are shown in the following Table 9.

Table 9. Regression parameters ("a" and "b") and effective concentrations (EC) of MCPA and 2,4D in *Lactuca sativa*; for different set up experiments 6WP, 90PD and AV (average between both set up)

Set up experiment	a	b	EC10 (μg/L)	EC50 (mg/L)
90PD MCPA	1.93	1.21	0.375	0.025
6WP MCPA	3.53	2.26	2.93	0.027
Av MCPA	2.94	1.93	2.16	0.030
90PD 2,4D	1.59	1.04	0.22	0.029
6WP 2,4D	2.46	1.93	3.82	0.052
Av 2,4D	2.07	1.49	1.39	0.041

Focusing on the effective concentrations that provoke the 50 % (EC50), it can be seen that for the three curves, MCPA values are lower than 2,4D meaning that MCPA has a higher effect. By comparing within the same compound, the EC50 values for "90PD" set up are lower (2,4D: 0.025 mg/L - MCPA: 0.029 mg/L) than the "6WP" (2,4D: 0.052 mg/L - MCPA: 0.027 mg/L) and "Av" (2,4D: 0.041 mg/L - MCPA: 0.030 mg/L). This does not match with the curves shown in the figures. In the curves, if a straight line in drown were the "y" axis is 50 %, the concentration that provoke that effect seems to be higher for 90PD, in both cases, thus should be less toxic, and EC50 should be higher. The reason that could explain this fact is that the R2 is both cases is lower, so the data is not perfectly represented by the curve, and that the amount of observations for 90PD were lower. These low differences could be also explained by biological variation and human error.

Dry weight as alternative endpoint

The dry weight of the seeds together with the sprouts of *Lepidium sativum* was obtained after being dried in the oven. And to evaluate if dry weight could be considered as an alternative endpoint, the inhibition of dry weight was calculated to make it comparable with the elongation as the other endpoint. The following table shows the % inhibition of using as an endpoint elongation (%IE), and using as an endpoint dry weight (%IDW), for both compounds in the same test Table 10.

Table 10. Comparison between the different endpoints for the same testing concentrations. Values are given as % of inhibition for elongation (%IE) and dry weight (%IDW)

Concentration (mg/L)	0.003	0.012	0.048	0.19	0.78
MCPA %IE	-9	27	55	89	89
MCPA %IDW	95	95	95	95	95
2,4D %IE	14	3	39	75	92
2,4D %IDW	95	95	95	95	95

The values in the table show that there is no change in the dry weight inhibition while

increasing the concentrations of MCPA and 2,4D. In all the cases inhibition is the same (95%). On the contrary, there are changes in the elongation inhibition at all the concentrations were MCPA and 2,4D were used. Despite MCPA %IE has a more clear increasing trendline than 2,4D %IE, in both cases is it possible to see the effect at those given concentrations. And, in this case it seems that elongation responds more sensitively than dry weight.

To reevaluate the relationship between inhibition and concentration, the fitting curves for both endpoints were also estimated and plotted to be compared. Figure 23A represent elongation as and endpoint and Figure 23B the dry weight.

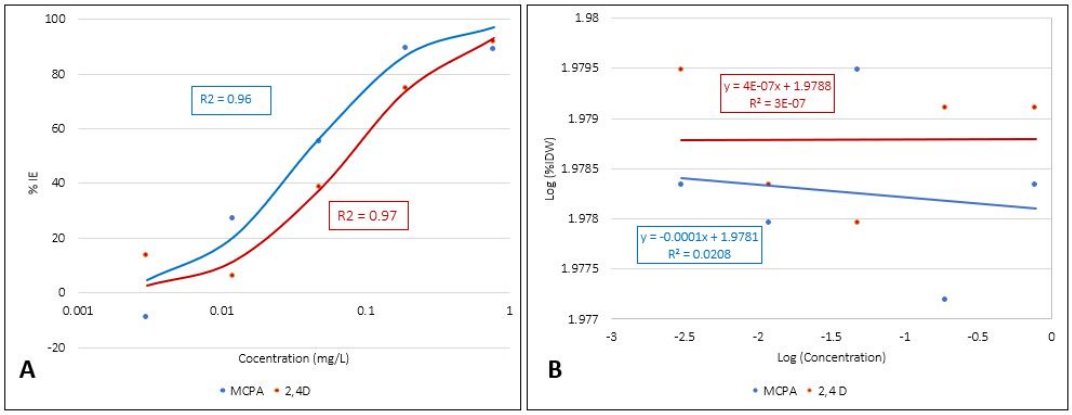

Figure 23. Fitting curves for elongation (%IE) and dry weight (%IDW) for both compounds at the same concentrations. A (left): logistic model fit for elongation; B (right) linear model fit for dry weight

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The data set using elongation as the endpoint, was able to fit in the logistic model (Equation 3.4.1) with a R2 = 0.96 for MCPA and R2=0.97 for 2,4D. On the opposite, while using dry weight as an endpoint the data could not fit the logistic model nor the linear model (Equation 3.4.1). For both compounds the R2 was below 0.02.

Additionally, dry weight was also calculated after VUV at times 4 (T4) for MCPA and 8 (T8) for 2,4D. As each sample (T4 and T8) was diluted by a factor 4, 4 times, the concentrations given are approximations. Following the same procedure as above, inhibition for both endpoints were calculated, values are shown in Table 11.

Table 11. Comparison between the the different endpoints at the same time samples. Values are given as % of inhibition for elongation (%IE) and dry weight (%IDW)

Dilution (D)	D254	D64	D16	D4	D0
Approx. Concentration (mg/L)	0.004	0.016	0.062	0.25	1.00
T4 MCPA %IE	-14	-9	-1	11	52
T4 MCPA %IDW	0	60	60	0	20
T8 2,4D %IE	3	9	31	58	84
T8 2,4D %IDW	-20	20	0	20	0

In this case, there are differences within the inhibition values of dry weight (%IDW) for both compounds. However, within the same time points (T4 MCPA %IDW and T8 2,4D %IDW) there is not a clear tendency nor relation between the inhibition and the concentrations or dilutions. On the other hand, while observing the elongation %IE there is a clear tendency that the inhibition increases with increasing the concentrations, for MCPA (T4 MCPA %IE) and 2,4D (T8 2,4D %IE). The negative values of inhibition indicate that there is growth promotion instead of reduction.

Dose response curves were also plotted to be compared (Figure 24). The data obtained while using elongation as an endpoint (%IE) was able to fit in the logistic model, with a R2 = 0.98 for MCPA and R2 = 0.99 for 2,4D (Figure 24A). On the contrary, the data obtained while using dry weight as an endpoint (%IDW) was not able to fit in the logistic model nor the linear model. The R2 while using the linear model for dry weight, resulted below 0.02 for both compounds (Figure 24B).

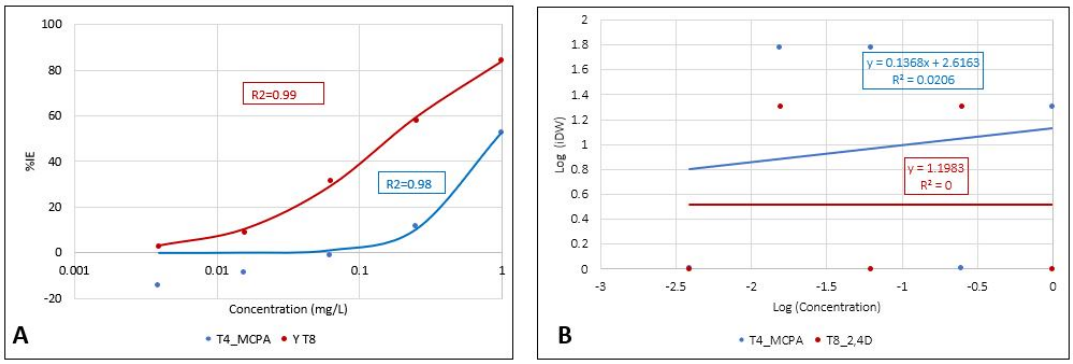

Figure 24. Fitting curves for elongation (%IE) and dry weight (%IDW) for both compounds at times 4 and 8 for MCPA and 2,4D , respectively. A (left): logistic model fit for elongation; B (right) linear model fit for dry weight

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In summary, there was not significant difference between the experiment set up (6WP and PD). This was shown with the ANOVA test, the dose- response curves and the estimated EC50. Regarding the alternative endpoint, dry weight was neither responsive or sensitive to the endpoint, whereas elongation was.

4.1.3 *Raphidocelis subcapitata*

The dose response of *Raphidocelis subcapitata* after being exposed to different concentrations of MCPA and 2,4D is shown in Figure 25. The curve is plotted as the growth inhibition (%), over the concentrations from the serial dilution (0, 0.20, 0.39, 0.78, 1.56, 3.13, 6.25, 12.5, 25, 50, 100 mg/L). Comparing the trend it can been seen that at all concentrations MCPA had a higher inhibitory effect than 2,4D. Indeed, until 12.5mg/L 2,4 D had growth promotion effect, seen as the negative values for inhibition. Furthermore, by observing the is could be induced that the 50% growth inhibition of *Raphidocelis subcapitata* is given approximately at 50mg/L for MCPA and 100mg/L for 2,4D.

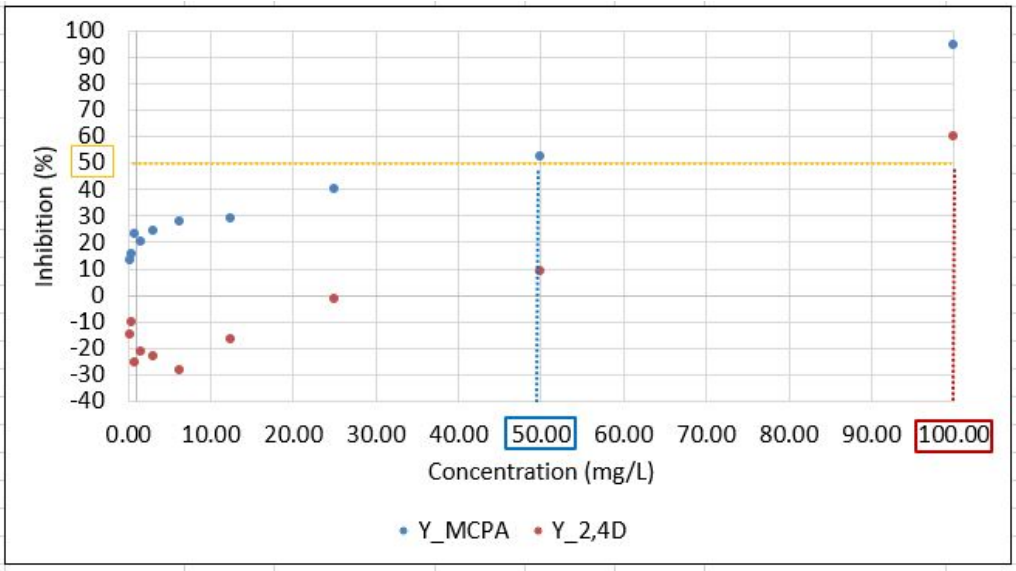

Figure 25. Dose response trend of *Raphidocelis subcapitata* after being exposed to a serial dilution of MCPA and 2,4D

.

The data did not respond to the logistic model so the linear model was performed (Figure 26A). For both compounds the fit was acceptable giving a R2=0.9, in the case of 2,4D the data was fitted only for the positive values. When calculating the EC50, values obtained were 36 mg/L for MCPA and 92 mg/L for 2,4D. To test the closeness of the linear model with the measured value, another linear fit was made by plotting the inhibition against the concentration (Figure 26B). After clearing the equations, EC50 resulted in 43 mg/L for MCPA and 91 mg/L for 2,4D. EC50 values of MCPA and 2,4D estimated with the two linear fit were close to the measured values: MCPA-EC50: 50 mg/L and 2,4D-EC50: 90 mg/L. The reason why Figure 26B shows a curve instead of a line is because the "x" axis (Concentration) is based on a logarithmic scale.

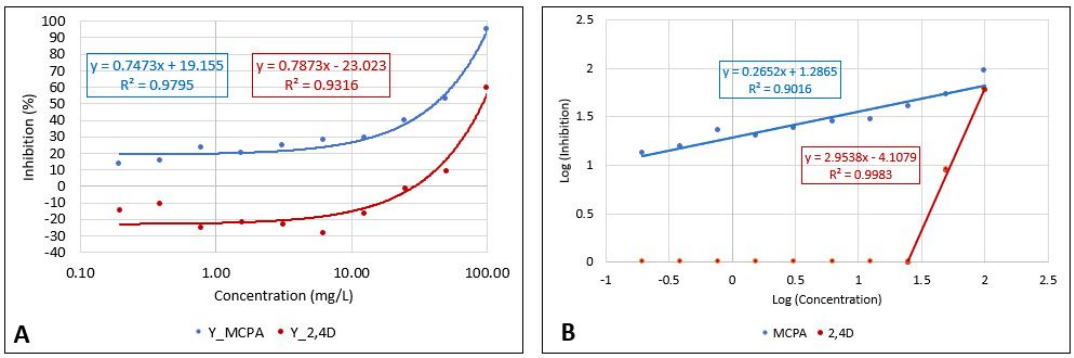

Figure 26. Linear fit of the data obtained after exposing *Raphidocelis subcapitata* to a serial dilution of MCPA and 2,4 D. A: linear fit model, B: linear fit of the data obtained.

4.1.4 *Bacillus subtilis*

Growth inhibition (%), of *Bacillus subtilis* over serial dilutions, using as initial stock concentrations 10 and 100 mg/L are shown in Figure 27A and B, respectively. In both

plots, 2,4D has a highest inhibitory effect than MCPA at almost all the concentrations. Furthermore, while using 10 mg/L as the initial concentration, 2,4D had a positive inhibitory effect (positive values in the Y axis), whereas MCPA had negative values in the y axis, meaning that instead of being inhibited, growth was promoted. Although there is not much visual difference between plots A and B, it can be said that for both compounds at all the dilutions, the inhibition is higher while using 100 mg/L as the initial concentration. However for both initial concentrations there is no inhibitory effect higher than 20 %, so no EC50 can be estimated nor calculated.

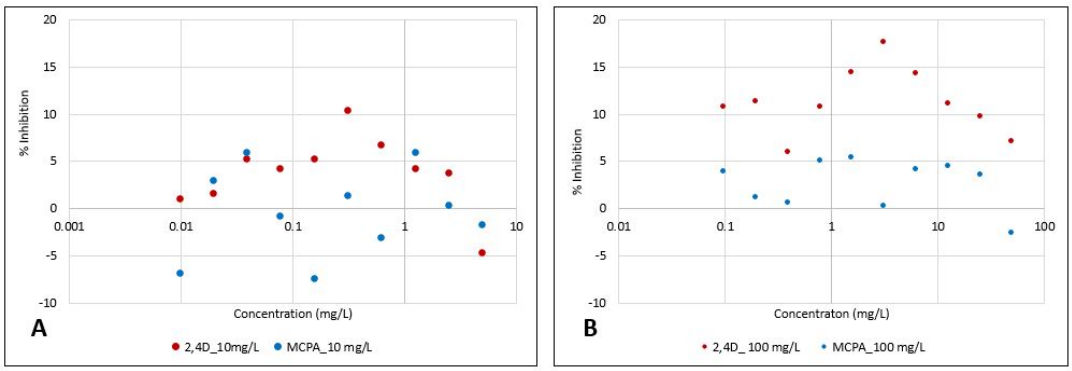

Figure 27. Dose response of *Bacillus subtilis* after serial dilution of MCPA and 2,4D. A (left) serial dilution with a stock concentration of 10 mg/L; B (right) serial dilution with a stock concentration of 100 mg/L

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No clear trend was observed after plotting the response of *Bacillus subtilis* against the exposed doses of both herbicides. Thus, the data was not able to fit in the logistic model nor the linear model. The coefficient of determination (R2) for the four curves was below 0.09, so no relationship could be proved between variables. The data fitted in the linear model is in Appendix 7 (Figure 38A: 10 mg/L, Figure 38B: 100 mg/L).

4.1.5 Comparison between test organisms

A research organism is chosen when it meets accessibility, tractability and resourcing [Dietrich et al., 2020]. These criteria refer to the supply, responsiveness, viability; and to the knowledge, based on previous researches and methodologies performed with that organism [Dietrich et al., 2020]. Except for responsiveness, the tested organisms met these characteristics. Due to their viability and short term growth, it was possible to perform acute tests. Although, they are known to be sensible to CEC, specially pesticides, and as they are used in ecotoxicity tests, different methodologies are standardized, *Bacillus subtilis* did not show defined sensitivity [Ma et al., 2006; Nalcaci O.O. and B., 2006; Newton et al., 2020; Xinzheng, 2021].

Responsiveness of *Lactuca sativa*, *Lepidium sativum*, *Bacillus subtilis* and *Raphidocelis subcapitata* is shown in Figure 28. The plot in the left side shows the responses at different concentrations of MCPA, and the right plot shows the responses while using 2,4D. For both compounds is seen that while using the same concentration, the inhibition changed depending the organism. In general terms, *Lactuca sativa* and *Lepidium sativum*

had similar responses while using the same concentrations and, were more sensitive to both compounds at lower concentrations. On the other hand,*Bacillus subtilis* and *Raphidocelis subcapitata* showed lower inhibition than the plants, while applying the same concentrations of both compounds and, were less sensitive at almost all the concentrations. Indeed, there was not apparent response in *Bacillus subtilis*.

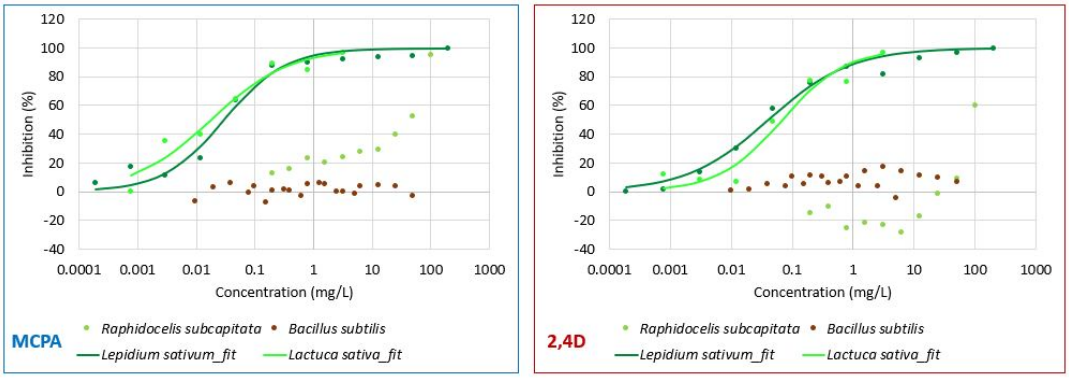

Figure 28. Dose response relation of *Lactuca sativa*, *Lepidium sativum*, *Bacillus subtilis* and *Raphidocelis subcapitata* at different concentrations of MCPA (left) and 2,4D (right)

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Sensitivity of the organisms is also proven with by strength of the dose- response relationship, given by the coefficient of determination (R2). Data obtained from *Lactuca sativa* and *Lepidium sativum* was able to fit in the logistic model with R=9, whereas for *Bacillus subtilis* and *Raphidocelis subcapitata* was not. Indeed, after fitting the data in the linear model only response from *Raphidocelis subcapitata* while applying MCPA had a good correlation.

Raphidocelis subcapitata responded differently to the compounds. MCPA had a higher inhibitory effect than 2,4D along all the tested concentrations, and there was perceived growth promotion with 2,4D until 12,5 mg/L. EC50 could not be precisely calculated but the visible values in the inhibition plot were 100 mg/L for MCPA and 50mg/L for 2,4D. A 96 microplate assay showed that although *Raphidocelis subcapitata* is a standard algae used for ecotoxicity test, is not the most sensitive algae to test herbicides, thus the variation in algal species sensitivity depends, on the chemical mode of action [Nagai, 2019]. The assay also reported that the EC50 values were estimated as higher than 10mg/L for MCPA and higher than 100mg/L for 2,4D [Nagai, 2019]. *Chlorella fusca* could be a potential algae to test because its reproduction may be affected by 2,4D [DeLorenzo et al., 2001].

Responses of *Bacillus subtilis* also differed between compounds, in this case 2,4 had higher inhibitory effects than MCPA. Anyway, the relation between concentration and inhibition is not clear: along the concentrations (range of 0.01mg/L and 50 mg/L) the inhibition with MCPA oscillates between -7% and 6%, and with 2,4D between -4% and 17%. Evangelista et al. [2010] reported that clorophenoxy herbicides, included MCPA, could be partially degraded by *Bacillus subtilis*. The assay concluded that when an easy to use carbon source was present, like glucose, the bacteria could degrade 100mg/L of the compound [Evangelista et al., 2010]. In the present report, although the degradation was not tested,

it can be stated that the herbicides at the given concentrations , did not have a toxic effect to the soil bacteria.

Lactuca sativa was more sensitive with MCPA than with 2,4D. At lower concentrations the inhibition was higher. Thus, by comparing the effective concentrations at where the growth was inhibited by 10%, 50% and 90%, it can be seen that difference in the inhibitory effect between MCPA and 2,4D decreased while increasing the concentration . In terms of the relative differences in concentrations between both compounds, to reach an inhibition of 10% with 2,4D, it was necessary to increase 8 times the concentration applied with MCPA. This difference decreased while higher concentrations were used: to reach 50% of inhibition with 2,4D it was necessary to increase by 4 times the concentration used with MCPA, and to reach 90%, increase it 2 times (Table 12).

Table 12. Effective concentrations of MCPA and 2,4D for *Lactuca sativa* and *Lepidium sativum* .

Organism	Compound	EC10 (μg/L)	EC50 (mg/L)	EC90 (mg/L)
Lactuca sativa	MCPA	0.604	0.0175	0.507
Lactuca sativa	2,4D	4.850	0.068	0.960
Lactuca sativa	Relative difference (2,4D/MCPA) x-fold	8	4	2
Lepidium sativum	MCPA	2.159	0.0298	0.412
Lepidium sativum	2,4D	1.385	0.041	1.218
Lepidium sativum	Relative difference (2,4D/MCPA) x-fold	0.64	1.4	3

Lepidium sativum was also more sensitive to MCPA. While applying the same concentrations, the inhibition was generally higher with MCPA than 2,4D. Nevertheless, this difference was lower than *Lactuca sativa*, and the trendline was the other way round. The sensitivity increased at the higher concentrations. To reach the 90% inhibition with 2,4D, it was needed to increase by 3 times the concentration of MCPA, whereas to reach the 50% 1.4 times. On the other hand, to reach to reach 10% inhibition, it was necessary to add half the concentration used with MCPA (Table 12).

Between *Lepidium sativum* and *Lactuca sativa*, whereas MCPA was more toxic in *Lactuca sativa*, 2,4D resulted more toxic in *Lepidium sativum*. These differences can be seen in the EC10 and EC50 values of Table 12. MCPA had a 50% inhibitory effect with a concentration of 0.0175 mg/L in *Lactuca sativa* and 0.0298 mg/L in *Lepidium sativum*. And, 2,4D had a 50% inhibitory effect with a concentration of 0.041 mg/L in *Lepidium sativum* and 0.068 mg/L in *Lactuca sativa*.

4.2 Vacuum UV- UVC treatment

Advanced Oxidation Processes with Vacuum UV- UVC irradiation was perform to estimate the degradation over time of MCPA and 2,4D in water. The remaining concentrations at each time point (T0, T2, T4, T8, T16 and T32) were estimated using HPLC. At the same time, those concentrations were tested in *Bacillus subtilis*, *Lactuca sativa* and *Lepidium sativum* to determine the toxicity after the treatment.

4.2.1 Degradation of MCPA and 2,4D

Degradation of MCPA and 2,4D over time, during VUV treatment is shown in Figure 29 A. The exponential decrease of the concentration over time is more pronounced for MCPA than for 2,4D. Indeed, the initial concentration (10 mg/L) of MCPA was removed in a 83% and 97%, 2,4D was removed in a 21% and 46%, after 2 and 4 minutes, respectively. Half life time of MCPA and 2,4D resulted in 1 minute and 5 minutes respectively. Furthermore, after applying the first order removal kinetic model, the rate coefficient for MCPA and 2,4D was -0.869 min-1 and -0.1649 min-1 (Figure 29B). This fact demonstrates that the degradation of MCPA was faster than 2,4D.

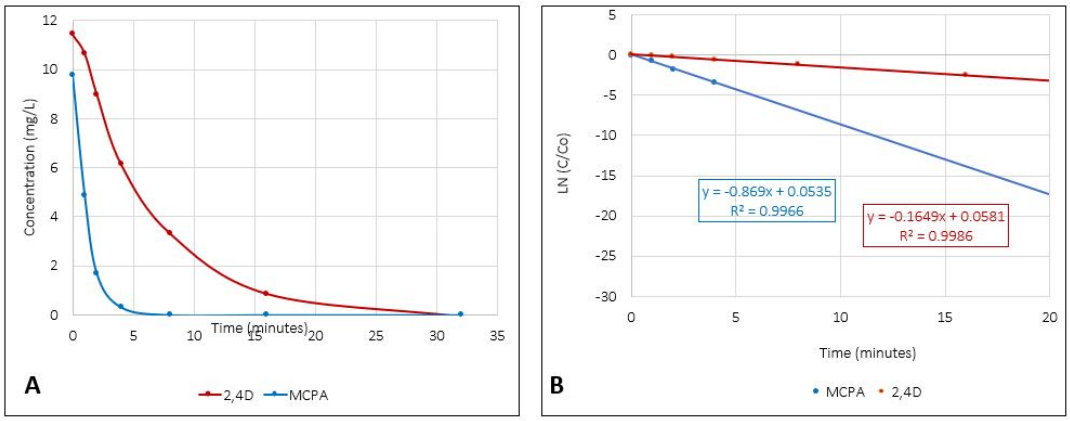

Figure 29. Degradation of MCPA and 2,4D over time during VUV treatment (A) and apparent initial first order removal kinetics (B)

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Both initial concentrations at time 0 should have been equal (10mg/L) because the solution for both compounds was prepared that way. But they seem to differ a bit in the figure: the start concentration of 2,4D is 11.4 mg/L whereas for MCPA is 9.8mg/L. This difference could be explained because the concentrations of Figure 29 A, were estimated by the equation given by the calibration curves while testing the compounds in the HPLC (Section 3.4.2), so the results may not be precise. Another reason could be that the stock solutions were not prepared as precise as they should be. However, the slope in Figure 29 A of MCPA is more steep than 2,4D, so despite the differences in the starting concentrations, MCPA is degraded faster. This fact can be seen by drawing a straight line at a certain concentration ("y" axis): it is needed more time to degrade 2,4D in order to reach the same concentration as MCPA.

Some of the differences between the degradation of both compounds could be explained by the pKa and Molar absortion values, which are higher for MCPA 13.

Table 13. pKa and Molar absortion of MCPA and 2,4D. Source [Benitez et al., 2004].

	MCPA	2,4D
pKa	3.7	2.3
Molar absorption	351.8 M-1.cm-1	172.7 M-1.cm-1

pKa indicates the capacity of the compound to dissociate in water, and the higher the value, the lower the dissociation. In this case 2,4D should have dissociated faster, but as the initial concentration tested (10 mg/L) was made from tap water, the pH was the same. And, at neutral pH, although MCPA has a higher pKa, can still dissociate at the same rate. The rate only increases at low pH, around 3 [Benitez et al., 2004]. So, the differences between the response of both compounds may be due to the Molar absorption. MCPA duplicates 2,4D values which means that MCPA absorbes the radiation two times more, compared to 2,4. This inequality leads to a incresed photochemical degradation for MCPA[Benitez et al., 2004; G. and M., 2012].

4.2.2 Toxicity in *Bacillus subtilis* after VUV-UVC

Toxicity in *Bacillus subtilis* after VUV-UVC was tested and, as expected was not sufficiently responsive to the concentrations of MCPA and 2,4 present in the extracted samples. The inhibition of *Bacillus subtilis* over the concentrations among the different times are plotted in Figure 30 and does not show a clear tendency. It is also seen that while using MCPA and 2,4D there is no inhibition further than 20 %. Times 0 and 16 for both compounds, showed highest peaks for inhibition and growth promotion. Indeed, in both cases the highest growth promotion is seen in the first dilution, estimated to be 5mg/L.

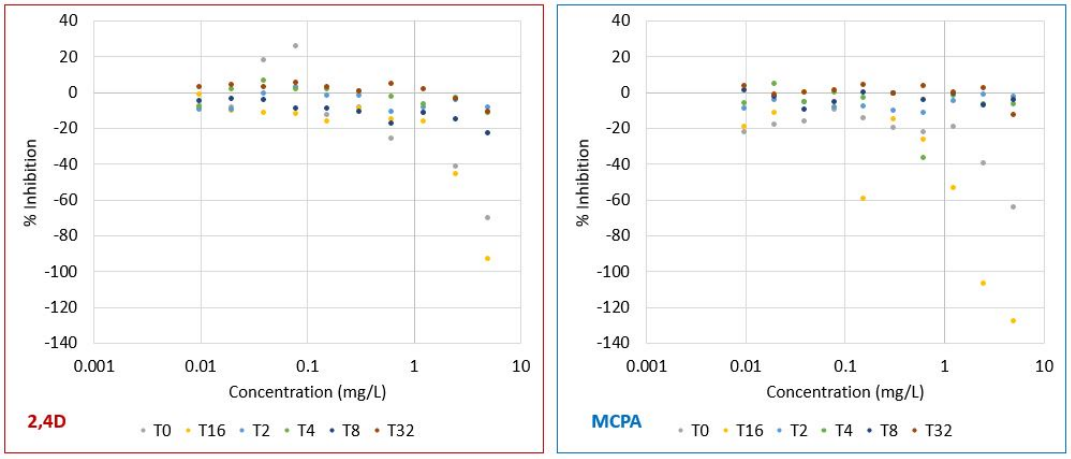

Figure 30. Inhibition of *Bacillus subtilis* with MCPA and 2,4D after VUV-UVC at all the extracted time points.

Furthermore, the obtained data could not be fitted neither in the logistic nor linear model for any of the compounds at all the time points. In the linear model, correlation between inhibition and concentration proved weak due to a low R2 for all the trendlines at the different time samples (Appendix 7 Figures 39 and 40). Although the relation between variables is not strong, it can be seen that for time points the slope, is negative. This means that while concentration decreases the inhibition increases. The cases are times 0, 2, 4, 32 for 2,4D, and time 4 MCPA (Appendix Tables 18 and 19).

4.2.3 Toxicity in *Lactuca sativa* after VUV-UVC

Inhibition of *Lactuca sativa* elongation was estimated after the VUV treatment at all the time points (extracted samples) at each different dilutions (D0, D4, D16, D64 and D256), using both herbicides. An example of the visual effect is provided in Figure 31, where the Petri Dishes show the seeds root and shoot elongation at times 0 (T0) and 16 (T16) after dilution 4 (D4). Between T0 and T16 the visual difference in elongation is clear for both herbicides. Simultaneously, after calculating the inhibition at T0 and T16 for both compounds, it seems that before the VUV-UVC treatment (T0) 2,4 D has a bit stronger inhibitory (77%) effect than MCPA (74%). Furthermore, after 16 minutes of VUV-UVC treatment (T16) MCPA promotes the growth (-20%) and 2,4D still inhibit (24%). This values indicate that after 16 minutes of VUV-UVC treatment, the inhibitory effect is reduced in 0.01-fold for MCPA and 0.33-fold for 2,4D. Furthermore, visual effects for the same time points at dilution 16 (D16) are found in Appendix 7: Figure 42 (MCPA) and Figure 43 (2,4D). As expected is seen that within the same time point, the inhibitory effect from both compounds is reduced while increasing the dilutions.

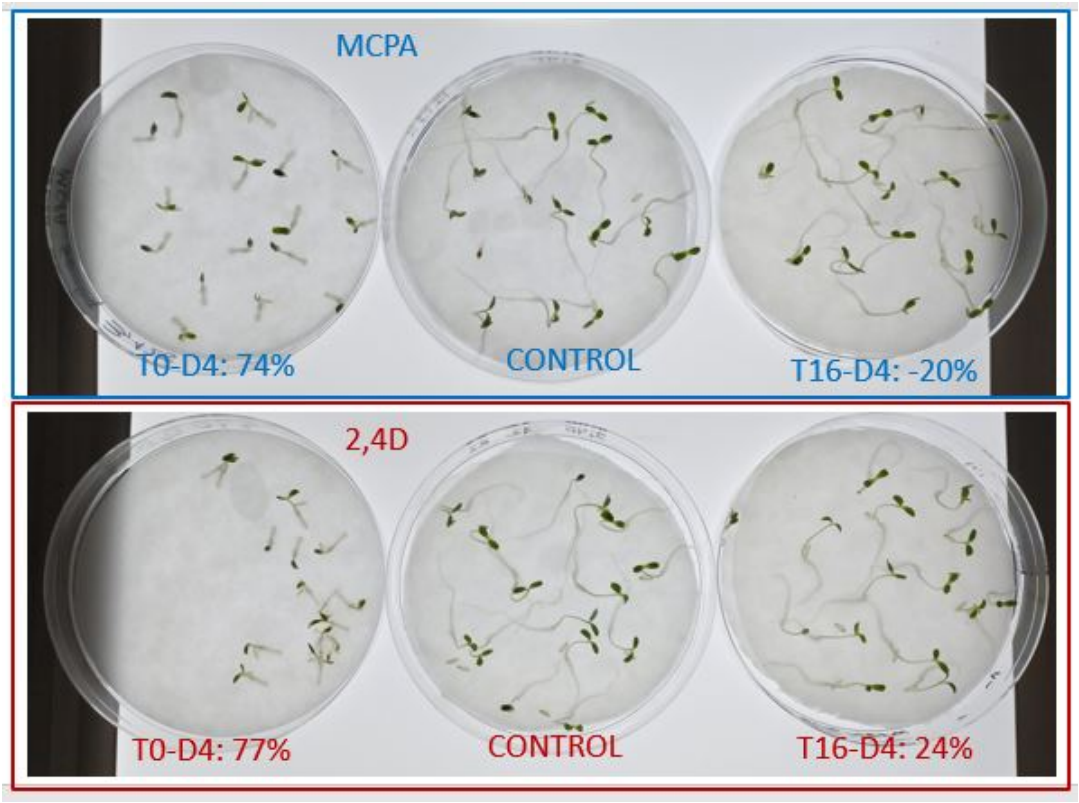

Figure 31. Visual comparison of the inhibition (%) in *Lactuca sativa* after VUV-UVC at times 0 (T0) and 16 (T16) at dilution 4 (D4) with MCPA and 2,4D.

The data obtained while exposing *Lactuca sativa* to 2,4D after VUV-UVC was fitted to the logistic model R-Squared higher than 0.95. Exception was made at T32 where at some dilutions the growth was promoted. On the other hand,while using MCPA, the fit was not that robust. At times T0 and T4 the logistic model was used instead of the linear because the fit was better, R2: 0.65 and 0.72, respectively. At T2, the logistic model was

strong R2:0.91; and at T8 and T16 the linear model was applied due to the negative values obtained (Table 14).

Table 14. Detail of model used to fit the data for *Lactuca sativa* after VUV-UVC treatment of MCPA and 2,4D, and respective R2. NM: Not measured

	MCPA	MCPA	2,4D	2,4D
TIME (minutes)	Fit model	R-Squared	Fit model	R-Squared
T0	Logistic	0.65	Logistic	0.95
T2	Logistic	0.91	Logistic	0.96
T4	Logistic	0.72	Logistic	0.98
T8	Linear	0.81	Logistic	0.98
T16	Linear	0.41	Logistic	0.95
T32	NM	NM	Linear	0.35

The fitted data for MCPA and 2,4D at all time points is shown in Figure 32. A more detailed version of each curve and each compound can be find in Appendix 7, Figures 44 (MCPA) and 45 (2,4D). By observing the fitting curves for *Lactuca sativa* in Figure 32, is seen that for both compounds there is a general decrease of inhibition over time. The decrease for MCPA is higher: in the first dilution (D0), where the curve hits the "y" axis, is seen that at T0 the inhibition is 80%, after 2 minutes 60%, after 4 minutes 50%, after 8 minutes there is almost no inhibition and at time 16 there is growth promotion (negative values of the "y" axis). For the same dilution, the decrease in inhibition due to 2,4D is slower: at T0 inhibition reaches 90%, at T2 and T4 80%, at T8 70%, at T16 55% and after 32 minutes here is no inhibition.

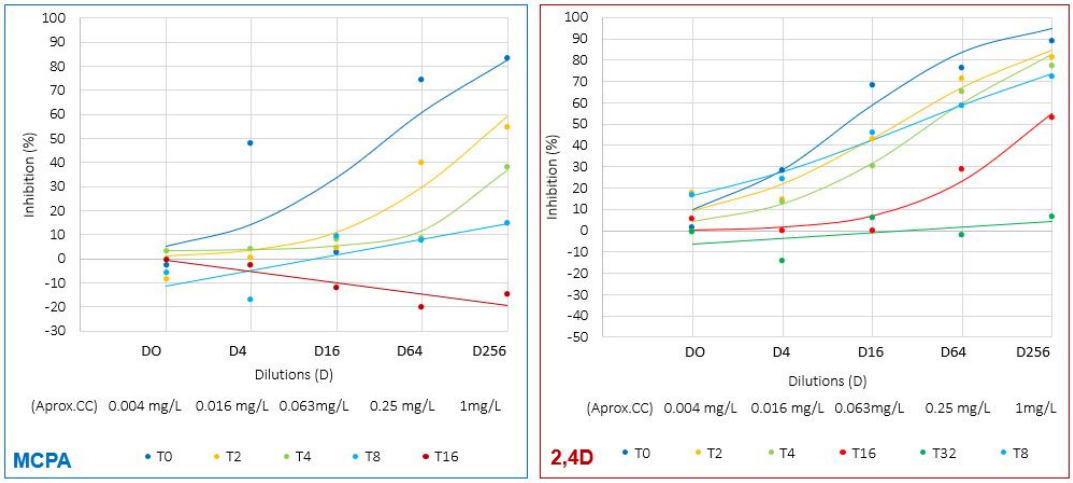

Figure 32. Fit curves of *Lactuca sativa* inhibition (%) over the 5 different dilutions of MCPA and 2,4D at each time point, after VUV treatment

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As expected, while increasing the dilutions within the same curve, for all the cases, the inhibition decreased. The slope of the curves of MCPA are steeper than the ones in 2,4D.

Thus, the responses of *Lactuca sativa* to MCPA are clearly different and the curves do not overlap.

Effective concentrations of MCPA and 2,4D at each time point, for *Lactuca sativa*, are plotted in Figure 33 and detailed in Table 15. For both compounds is seen that EC50 increases over time. MCPA reaches the highest measured concentration (1mg/L) after 4 minutes, whereas 2,4D in the period of 16 and 32 minutes. At time 0 MCPA has a higher EC50 (0.114 mg/L) than 2,4D (0.042mg/L). After 2 minutes, there is a deep increase in EC50 for MCPA around 40% compared to T0, whereas for 2,4D increases a 5% . After 8 minutes seems to be a reduction in the EC50 of 4% for 2,4D in relation to the previous time (T4).

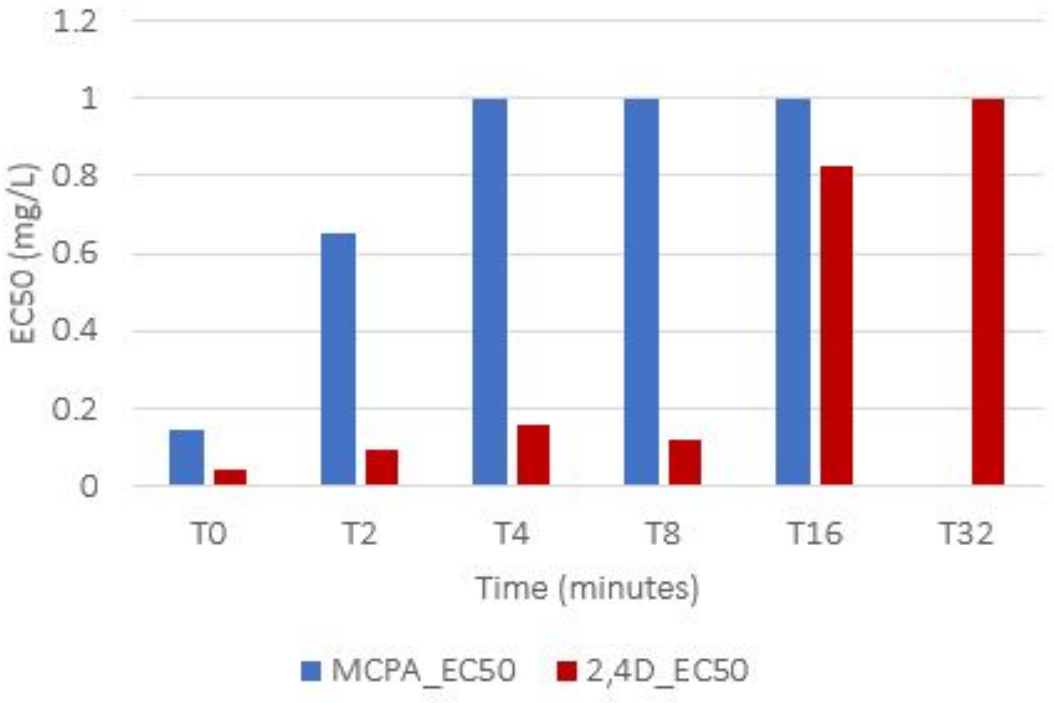

Figure 33. EC50 histogram for *Lactuca sativa* at different time points after VUV treatment

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Table 15. Effective concentrations (EC) in mg/L of MCPA and 2,4D in *Lactuca sativa*. ND: value no determinable due to a low R-Squared of the data fit; NM: not measured

	MCPA	MCPA	2,4D	2,4D
TIME (minutes)	EC10 (mg/L)	EC50 (mg/L)	EC10 (mg/L)	EC50 (mg/L)
T0	0.009	0.144	0.004	0.042
T2	0.056	0.649	0.004	0.092
T4	0.234	>1	0.012	0.156
T8	0.280	>1	0.001	0.119
T16	ND	>1	0.091	0.825
T32	NM	NM	ND	>1

In summary, toxicity of MCPA and 2,4D in *Lactuca sativa* after VUV-UVC irradiation

decreased. This fact was proven in the model fit curves (Figure 32), where the inhibition decreased over time and, in Figure 33 where the EC50 values at T4 (MCPA) and T32 (2,4D) where equal or higher than the initial concentration.

4.2.4 Toxicity in *Lepidium sativum* after VUV-UVC

Inhibition of *Lepidium sativum* elongation was estimated after the VUV treatment at all the time points (extracted samples) at each different dilutions (D0, D4, D16, D64 and D256), using both herbicides. An example of the visual effect is provided in Figure 34, where the Petri Dishes show the seeds root and shoot elongation at times 0 (T0) and 16 (T16) after dilution 4 (D4). Between T0 and T16 the visual difference in elongation is clear for both herbicides. Simultaneously, after calculating the inhibition at T0 and T16 for both compounds, it seems that before the VUV-UVC treatment (T0) MCPA has a bit stronger inhibitory (89%) effect than 2,4D (83%). However, after 16 minutes after VUV-UVC treatment (T16) MCPA promotes the growth (-1%) and 2,4D still inhibit (24%). This values indicate that after 16 minutes of VUV-UVC treatment, the inhibitory effect is reduced in 0.11-fold for MCPa and 0.29-fold for 2,4D. Furthermore, visual effects for the same time points at dilution 16 (D16) are found in Appendix 7: Figure 46 (MCPA) and Figure 47 (2,4D). As expected is seen that within the same time point, the inhibitory effect from both compounds is reduced while increasing the dilutions.

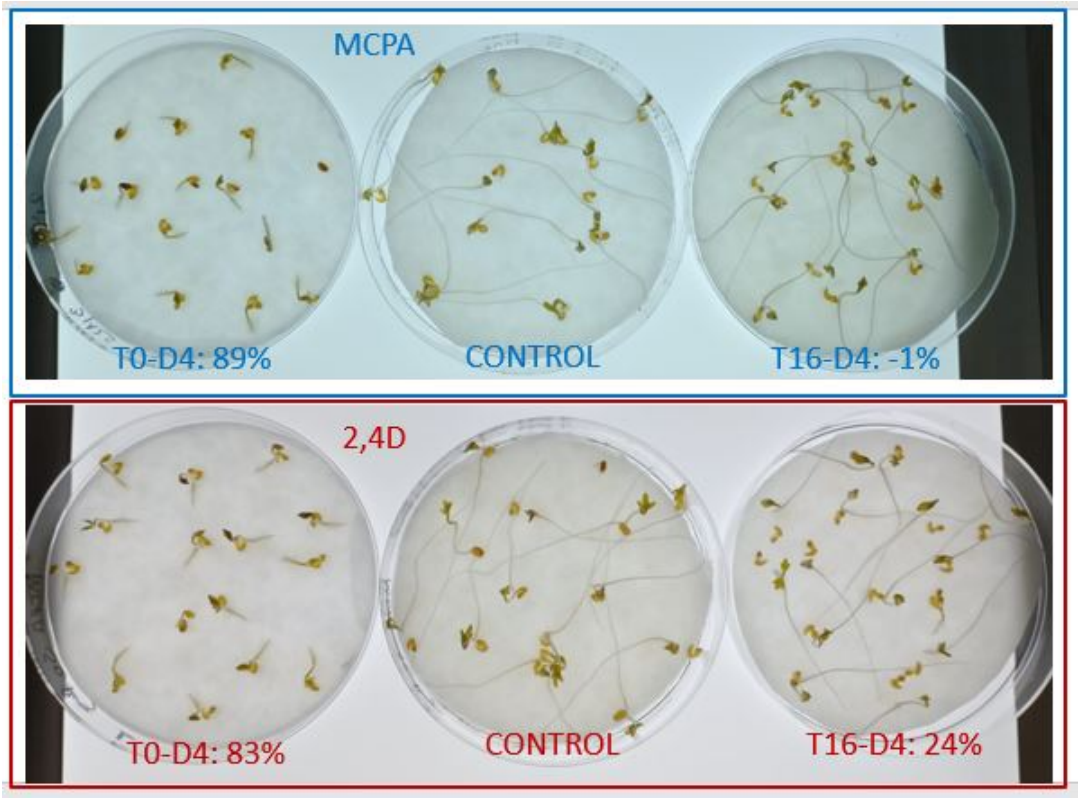

Figure 34. Visual comparison of the inhibition (%) in *Lepidium sativum* after VUV-UVC at times 0 (T0) and 16 (T16) at dilution 4 (D4) with MCPA and 2,4D.

The data obtained for both compounds at almost all the time points was fitted in the

logistic model with a R-Squared higher than 0.83 (Table 16. Exemptions were made in some cases and the data was fitted in the linear model. Linear fit was made while using MCPA at T0, T8 and T16, and while using 2,4D at T32. At all the mentioned time points, excluding T0 of MCPA, the data obtained did not have a clear trend, the values indicated growth promotion instead of inhibition and the R-Sqared proved weak.

Table 16. Detail of model used to fit the data for *Lepidium sativum* after VUV-UVC treatment of MCPA and 2,4D, and respective R2

	MCPA	MCPA	2,4D	2,4D
TIME (minutes)	Fit model	R-Squared	Fit model	R-Squared
T0	Linear	0.57	Logistic	0.83
T2	Logistic	0.90	Logistic	0.99
T4	Logistic	0.89	Logistic	0.99
T8	Linear	0.20	Logistic	0.99
T16	Linear	0.76	Logistic	0.82
T32			Linear	0.14

The fitted data for both compounds at all the time points is shown in Figure 35. Single curves for each time point and each compound are available in Appendix 7, Figure 48 (MCPA) and Figure 49 (2,4D). By observing the curves in Figure 35, is seen that for both compounds there is a general decrease of the inhibition of *Lepidium sativum* over time. The decrease for MCPA is higher: at times 0 and 2, inhibition reaches 70% and at time 4, 50% in the first dilution (D0), whereas at times 8 and 16 there is almost no inhibition. The decrease for 2,4D is gradual, for D0,at times 0, 2, 4 and 8, inhibition reach between 80% and 90%, and at time 16, 50%, whereas at time 32 there is no inhibition. Also, within the same time while increasing the dilutions the inhibitory effect is lower.

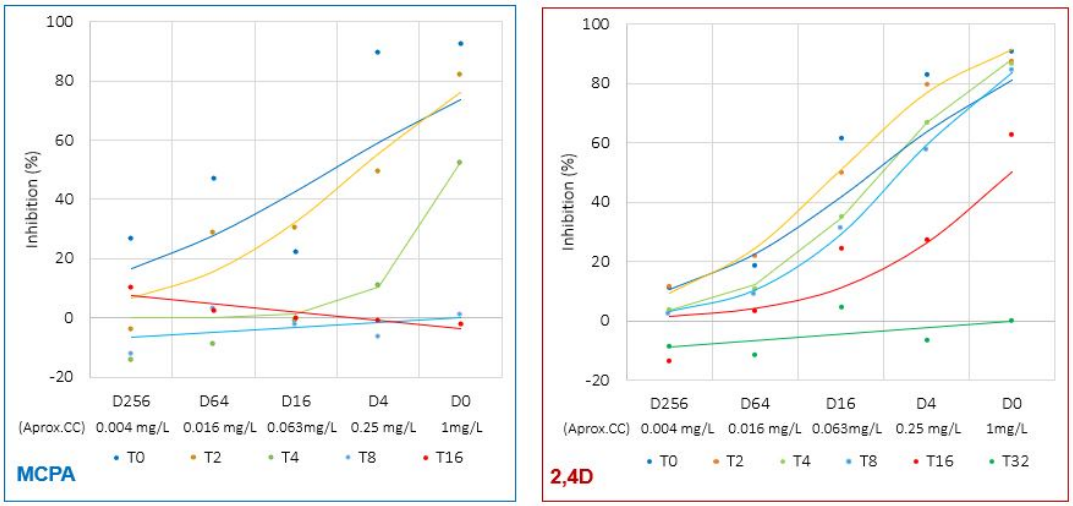

Figure 35. Fit curves of *Lepidium sativum* inhibition (%) over the 5 different dilutions of MCPA and 2,4D at each time point, after VUV treatment

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After the models were performed, the effective concentrations of MCPA and 2,4D in *Lepidium sativum* at each time point, were calculated. Results are plotted in Figure 36 and detailed in Table 17. For both compounds is seen that EC50 increases over time. MCPA reaches the highest measured concentration (1mg/L) after 4 minutes, whereas 2,4D after 16 minutes. At time 0 both compounds have almost the same effect, although EC50 for MCPA is lightly higher (0.114 mg/L) than 2,4D (0.106mg/L). After 2 minutes, compared to T0, EC50 increases for MCPA (0.181mg/L) while for 2,4D decreases (0.061mg/L). This is the only time where 2,4D seem to have more toxic effect related to the no treated time point (T0).

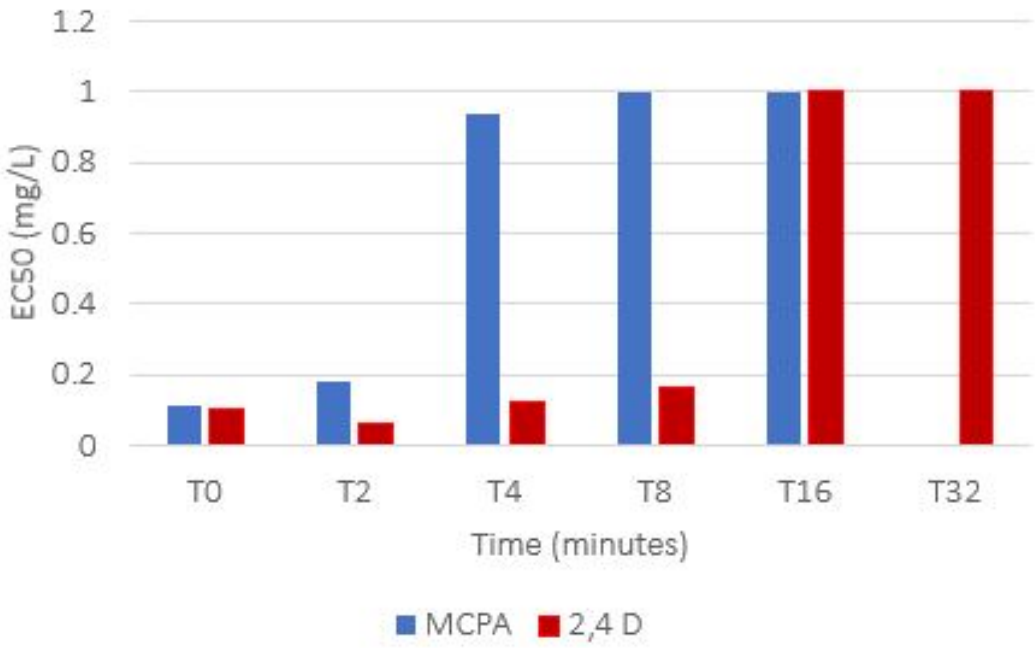

Figure 36. EC50 histogram for *Lepidium sativum* at different time points after VUV treatment

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Table 17. Effective concentrations (EC) in mg/L of MCPA and 2,4D in *Lepidium sativum*.ND:value no determinable due to a low R-Squared of the data fit; NM:not measured.

	MCPA	MCPA	2,4D	2,4D
TIME (minutes)	EC10 (mg/L)	EC50 (mg/L)	EC10 (mg/L)	EC50 (mg/L)
T0	0.001	0.114	0.003	0.106
T2	0.007	0.181	0.004	0.061
T4	0.246	0.941	0.012	0.122
T8	ND	>1	0.015	0.165
T16	ND	>1	0.054	1
T32	NM	NM	ND	>1

In summary, toxicity of MCPA and 2,4D in *Lepidium sativum* after VUV-UVC irradiation decreased. This fact was proven in the model fit curves (Figure 35), where the inhibition decreased over time and, in Figure 36 where the EC50 values at T4 (MCPA) and T16 (2,4D) where equal or higher than the initial concentration.

4.2.5 Comparison between in *Lactuca sativa* and *Lepidium sativum*

The toxicity effect *Lactuca sativa* and *Lepidium sativum* of the compounds before and after the VUV-UVC treatment are summarized in the Figure 37, below. The faster degradation of MCPA compared to 2,4D is reflected in the EC50 values for both organisms. After 4 minutes of irradiation to MCPA, toxicity decreases to 7-fold for *Lactuca sativa* and *Lepidium sativum*; and EC50 reaches the same or higher value than the initial tested concentration 1mg/L. On the other hand, after 4 minutes of irradiation to 2,4D, toxicity decreases to 4-fold for *Lactuca sativa* and to 1.2-fold in *Lepidium sativum*. With 2,4D, EC50 reaches the same or higher value than the initial tested concentration 1mg/L approximately after 16 minutes for both organisms.

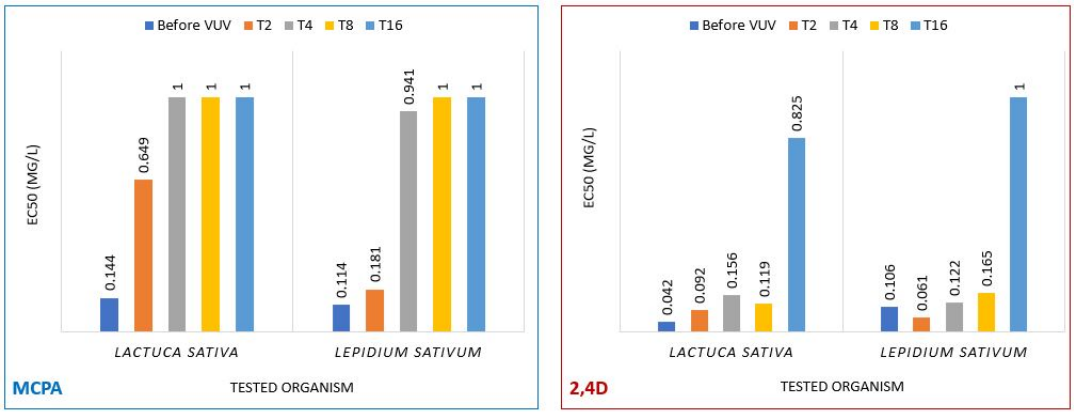

Figure 37. EC50 values of *Lactuca sativa* and *Lepidium sativum*

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It can also be seen that after exposing the herbicides to VUV-UVC irradiation, the toxic effect is reduced in both organisms. At the same time it may be inferred that 2,4D could have a bit more toxic effect than MCPA, this is comparing the EC50 values before VUV.

5 Conclusion

The herbicides 2,4D and MCPA had effects on the growth of some of the tested organisms and, the responses differed when same doses were applied. Growth for *Raphidocelis subcapitata* was inhibited by 50% when 100 mg/L of 2,4D and 50mg/L of MCPA were applied. However, at concentrations below 12.5 mg/L of 2,4D the growth was promoted. Furthermore, *Bacillus subtilis* showed less than 20% and the high tested concentration 50mg/L for both compounds. And, at lower concentrations the growth was promoted. On the other hand, *Lactuca sativa* and *Lepidium sativum* were more sensitive to these compounds. The estimated EC50 values in *Lactuca sativa* were 0.017mg/L and 0.068 mg/L for MCPA and 2,D, respectively. For *Lepidium sativum*, the EC50 resulted in 0.029mg/L for MCPa and 0.041mg/L for 2,4D. Thus, *Lactuca sativa* and *Lepidium sativum* proved to be a model organism to test herbicides, specially at low concentrations, due to the high correlative relation in the dose- response curve.

Additionally, Vacuum UV treatment completely removed the initial concentration (10 mg/L) of MCPA and 2,4D after 8 and 32 minutes, respectively. The degradation was faster for MCPA than for 2,4D. Indeed the half life time of MCPA and 2,4D resulted in 1 minute and 5 minutes respectively. The decay rate coefficient resulted in 0.87(min-1) for MCPA and 0.16 (min-1) for 2,4D. The VUV degradation of 2,4D and MCPA was reflected in increasing apparent EC50 values. For *Lactuca sativa* and *Lepidium sativum*, the toxicity decreased 4-1.5 fold and 7-8 fold after VUV treatment of compound MCPA for 2 and 4 minutes, respectively. After VUV treatment of 2,4D, toxicity for*Lactuca sativa* and *Lepidium sativum* decreased 2-0.57 fold and 4-1.16 fold a times 2 and 4,respectively.

6 Project perspective

The present project has shown that VUV-UVC irradiation is a potential chemical free and non- invasive tool for mitigation of herbicides in water. Due to the extended use of this compounds in food production, is interesting to consider this treatment as an alternative. However more studies are needed to observe the differences in the removal efficiency given lower and higher concentrations. Indeed, lower concentrations should be tested if this treatment is intended to be used as a mitigation tool of herbicides in drinking water. Thus, to reach the standard acceptable values for a safe drinking water.

Additionally, the transformation products after the degradation of the mother compound should be also detected and tested for toxicity. Toxicity of both, mother compound and transformation products could be tested in other sensitive species and in the field, in order to evaluate the chronic and acute effects in a dynamic environment.

7 Appendix

7.1 Materials and methods

7.1.1 Growth medium

Raphidocelis subcapitata

Stock 1: Ammonium chloride 15 mg/L, Magnesium chloride hexahydrate 12 mg/L, Calcium chloride dehydrate 18 mg/L, Magnesium sulfate heptahydrate 15 mg/L, Potassium dihydrogen phosphate 1.6 mg/L.

Stock 2: Ferric chloride (III) hexahydrate 0.064 mg/L, Disodium ethylene diamine tetraacetiate dihydrate 0.1 mg/L.

Stock 3: Boric acid 0.185 mg/L, Manganese chloride tetrahydrate 0.415 mg/L, Zinc chloride 0.003 mg/L, Coblat chloride hexahydrate 0.0015 mg/L, Copper chloride dihydrate 0.00001 mg/L, Disodium molybdate dihydrate 0.007 mg/L.

Stock 4: sodium hydrogen carbonate 50g/L.

Bacillus subtilis

Davis Minimal Medium: Distilled deionized water, 11.6 g/L of Davis Minimal Broth (Sgma Aldrich- code: 93753), 1g/L glucose. Autoclave and add: FeSO4 and Trace Medium [TM] (D1000: 0.5 ml/1000 mL).

4 x strength Davis Minimal Medium: Distilled deionized water, 46 g/L of Davis Minimal Broth, 4 g/L glucose. Autoclave and add: FeSO4 and Trace Medium [TM] (D4: 2 ml/1000 mL).

7.2 Results

7.2.1 *Bacillus subtilis*

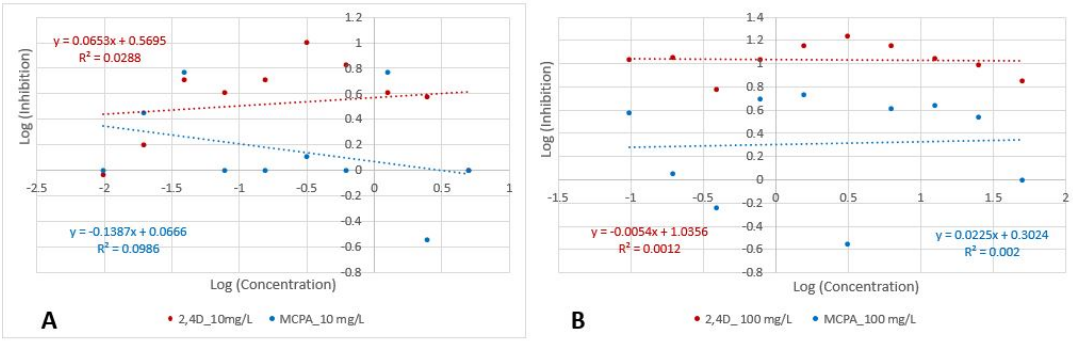

Figure 38. Linear fit for inhibition of *Bacillus subtilis* after being spiked with serial dilution of MCPA and 2,4D. A (left) serial dilution with a stock concentration of 10 mg/L; B (right) serial dilution with a stock concentration of 100 mg/L

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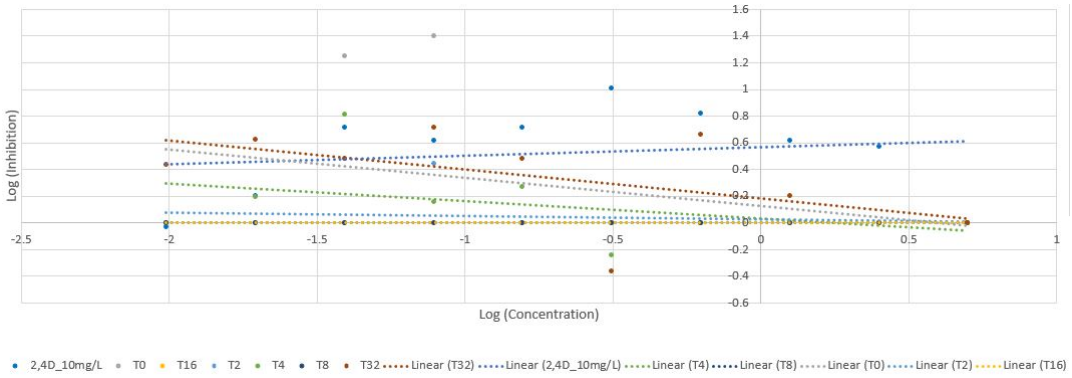

Figure 39. Linear fit for *Bacillus subtilis* after serial dilution of 2,4D at different times points for VUV treatment

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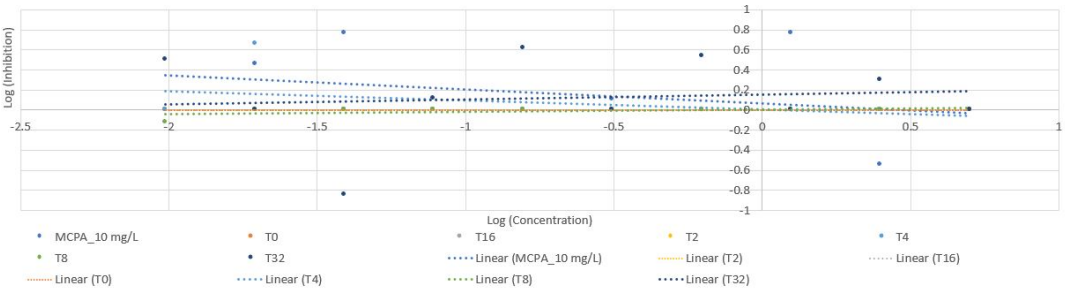

Figure 40. Linear fit for *Bacillus subtilis* after serial dilution of MCPA at different times points for VUV treatment

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Table 18. Parameters from linear fit of *Bacillus subtilis* after serial dilution using 2,4D VUV samples at times 0, 2, 4, 8, 16 and 32

Time	Linear equation (Y = m.x + b)	R2
T0	y = -0.2112x + 0.1276	R^2 = 0.1173
T2	y = -0.0268x + 0.0268	R^2 = 0.0303
T4	y = -0.13x + 0.0355	R^2 = 0.1775
T8	y = 0	R^2 = N/A
T16	y = 0	R^2 = N/A
T32	y = -0.216x + 0.1839	R^2 = 0.3111

Table 19. Parameters from linear fit of *Bacillus subtilis* after serial dilution using MCPA VUV samples at times 0, 2, 4, 8, 16 and 32

Time	Linear equation (Y = m.x + b)	R2
T0	y = 0	R^2 = N/A
T2	y = 0	R^2 = N/A
T4	y = -0.0935x + 0.005	R^2 = 0.165
T8	y = 0.0221x + 0.0023	R^2 = 0.2727
T16	y = 0	R^2 = N/A
T32	y = 0.0497x + 0.1578	R^2 = 0.0116

7.2.2 *Lactuca sativa*

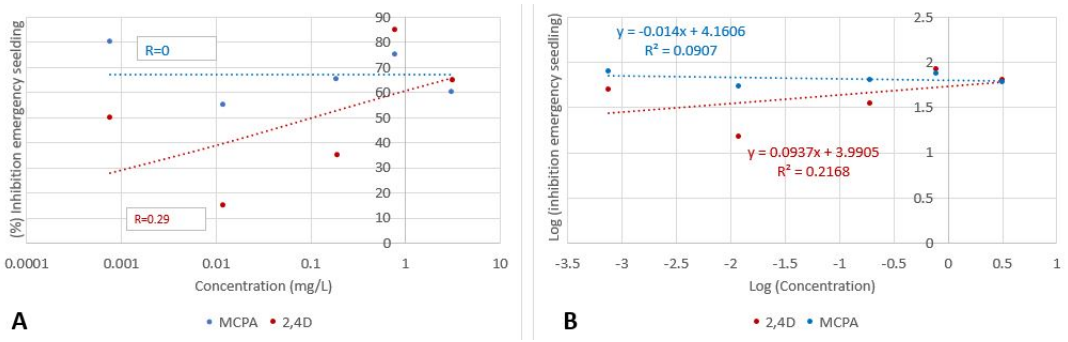

Figure 41. Dose response curves for *Lactuca sativa* using seedling emergence as an alternative endpoint. A (left) data fitted in the logistic model, B (right) data fitted in the linear model

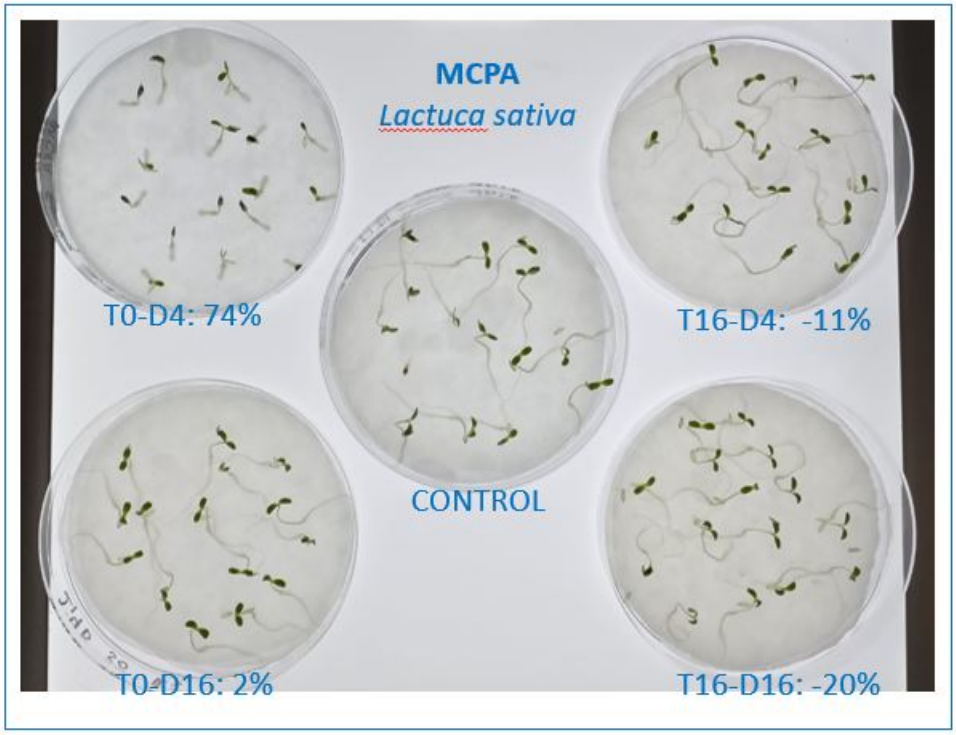

Figure 42. Visual effect after VUV-UVC treatment of MCPA in *Lactuca sativa* at times 0 (T0) and 16 (T16) at dilutions 4 (D4) and 16 (D16)

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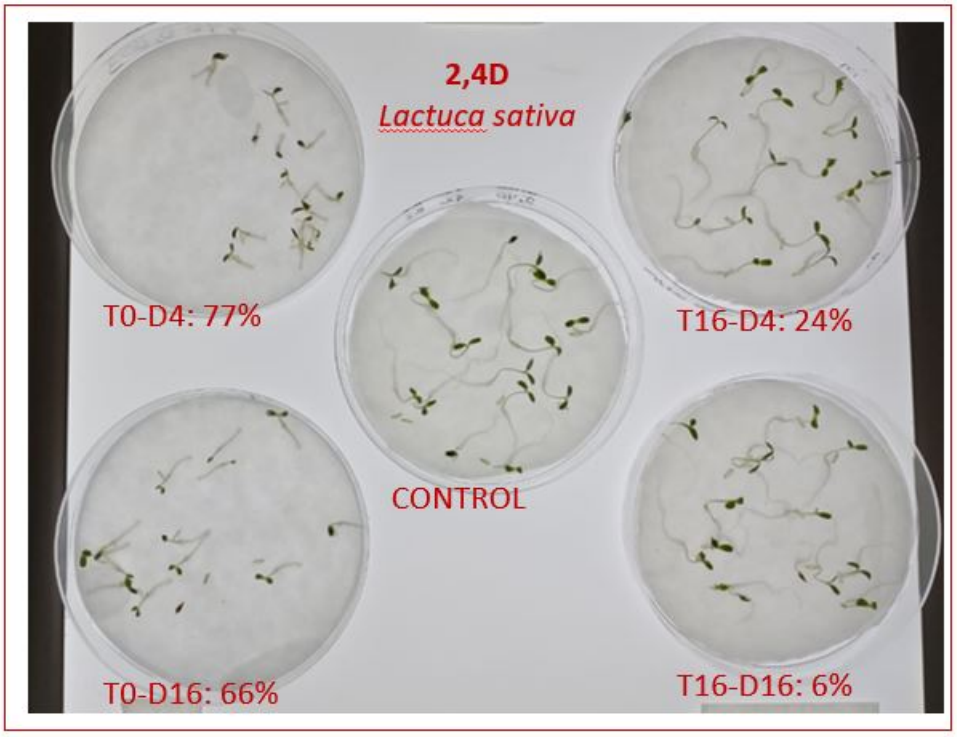

Figure 43. Visual effect after VUV-UVC treatment of 2,4D in *Lactuca sativa* at times 0 (T0) and 16 (T16) at dilutions 4 (D4) and 16 (D16)

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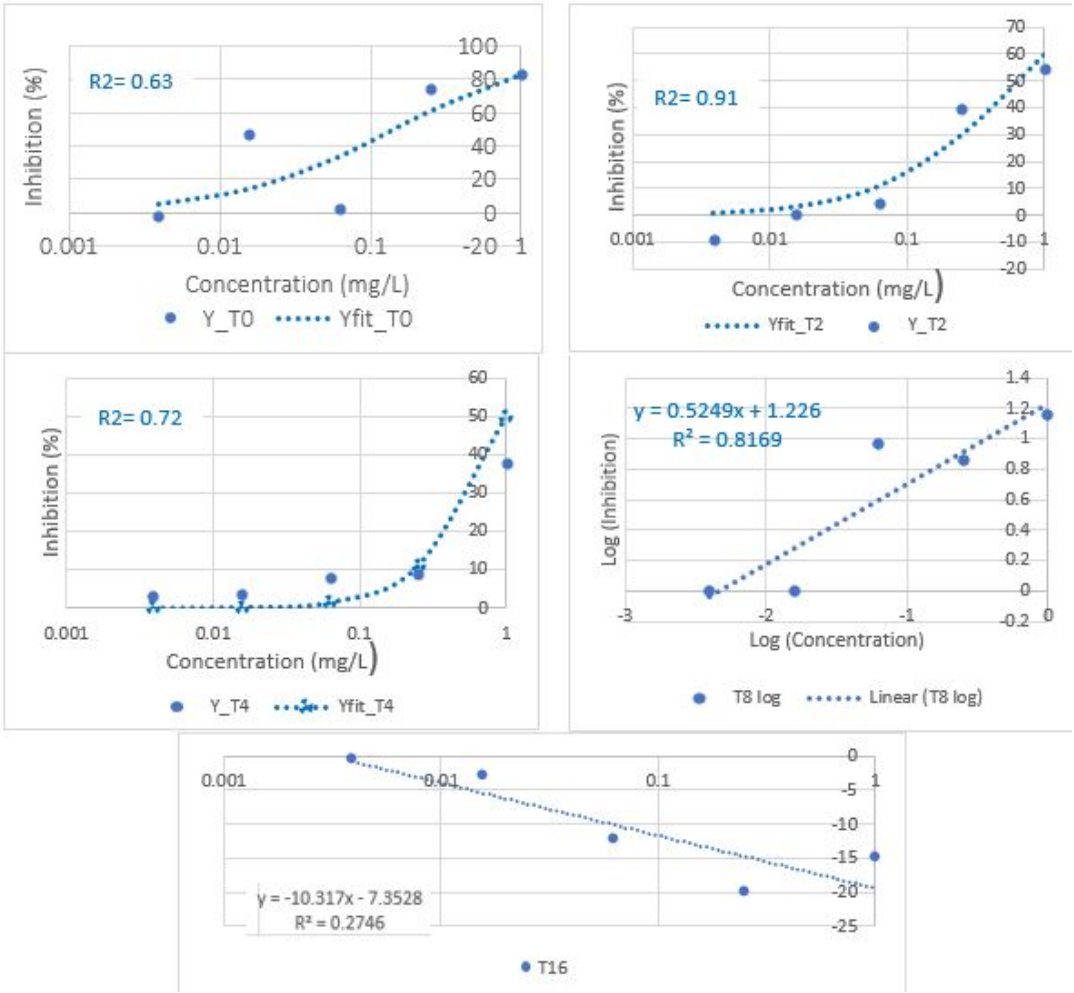

Figure 44. Fit curves for *Lactuca sativa* at time point after VUV-UVC treatment of MCPA

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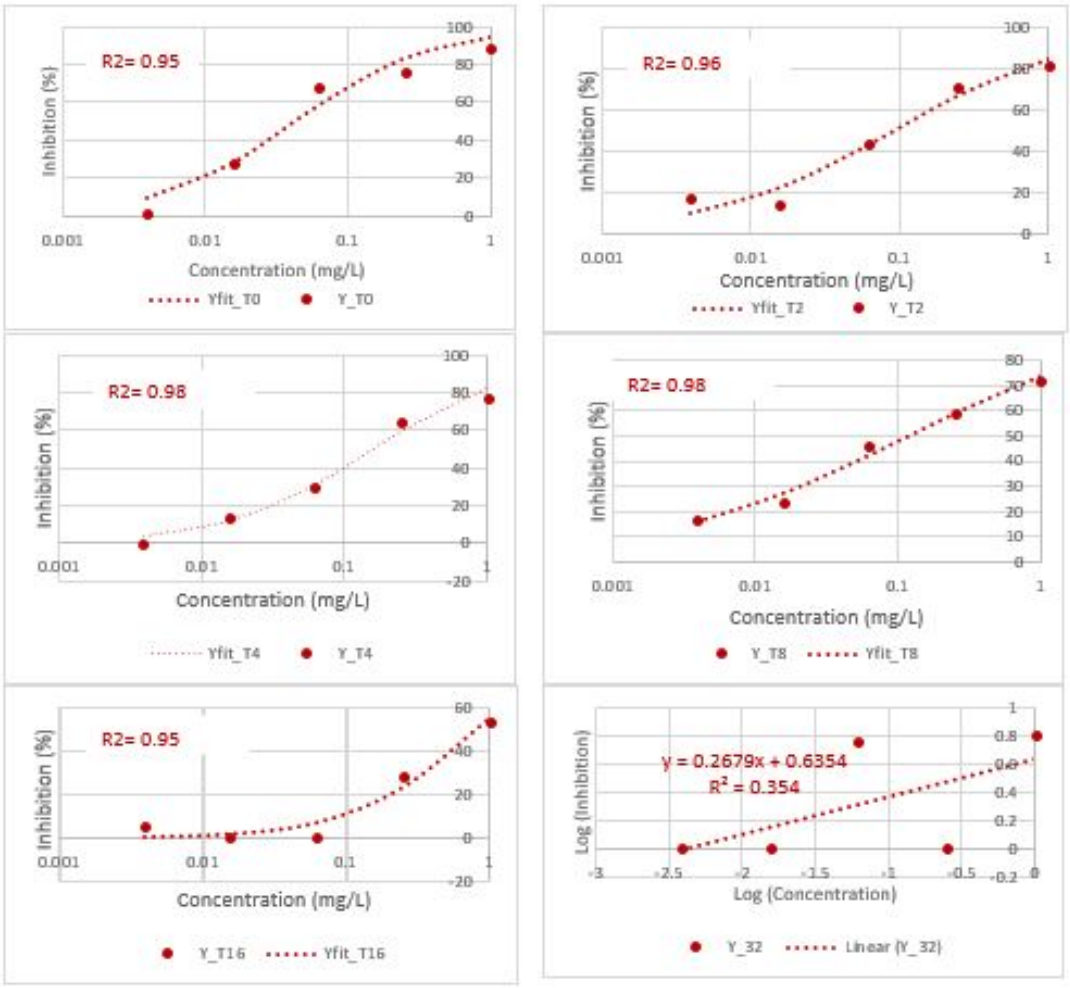

Figure 45. Fit curves for *Lactuca sativa* at time point after VUV-UVC treatment of 2,4D

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7.2.3 *Lepidium sativum*

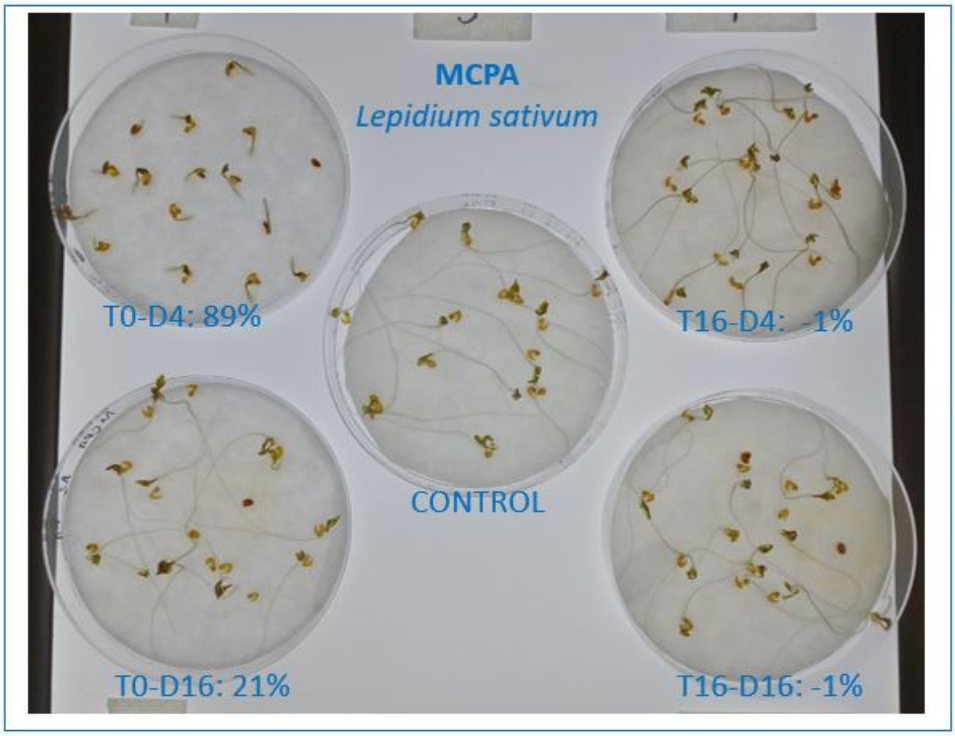

Figure 46. Visual effect after VUV-UVC treatment of MCPA in *Lepidium sativum* at times 0 (T0) and 16 (T16) at dilutions 4 (D4) and 16 (D16)

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Figure 47. Visual effect after VUV-UVC treatment of 2,4D in *Lepidium sativum* at times 0 (T0) and 16 (T16) at dilutions 4 (D4) and 16 (D16)

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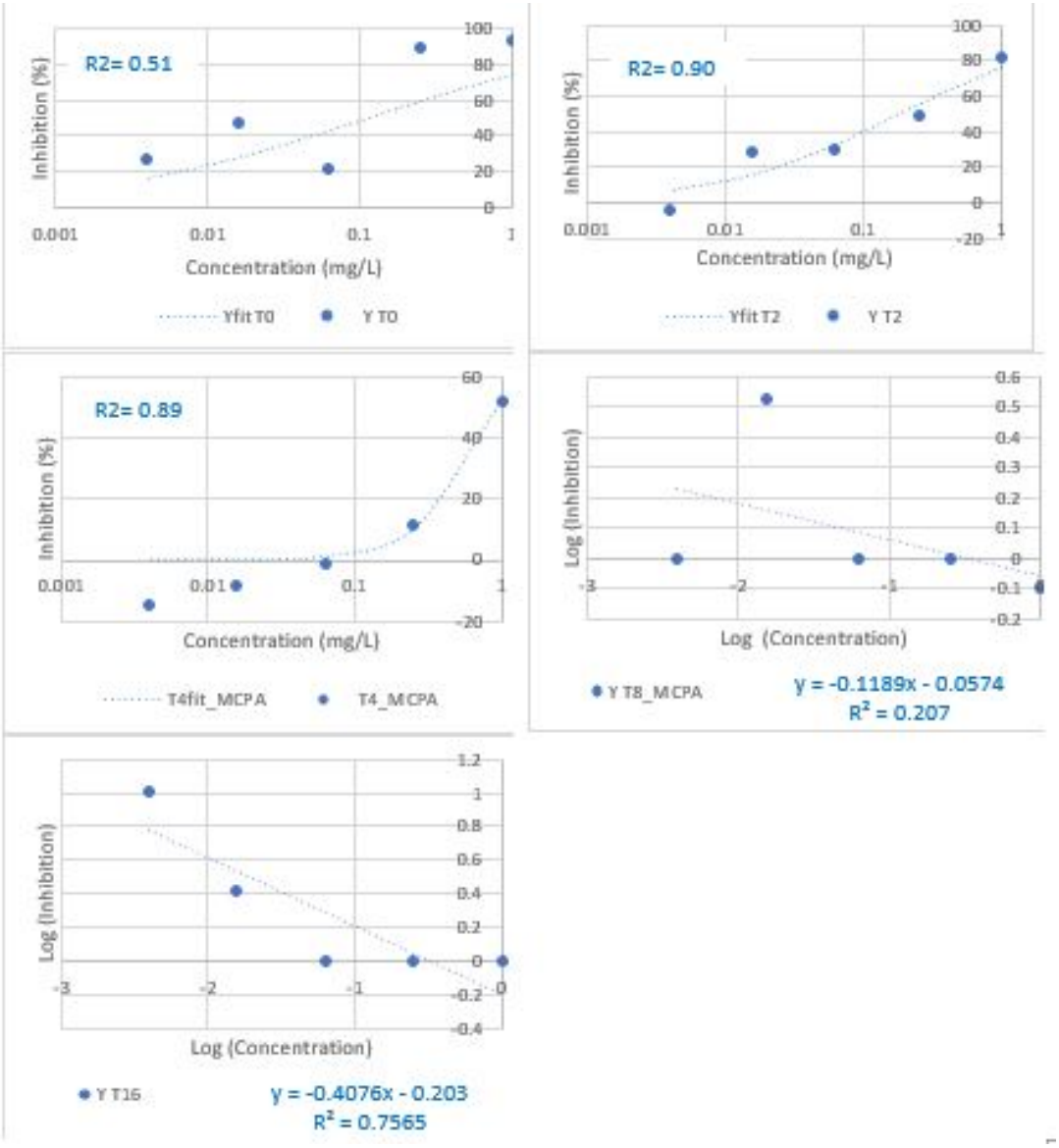

Figure 48. Fit curves for *Lepidium sativum* at time point after VUV-UVC treatment of MCPA.

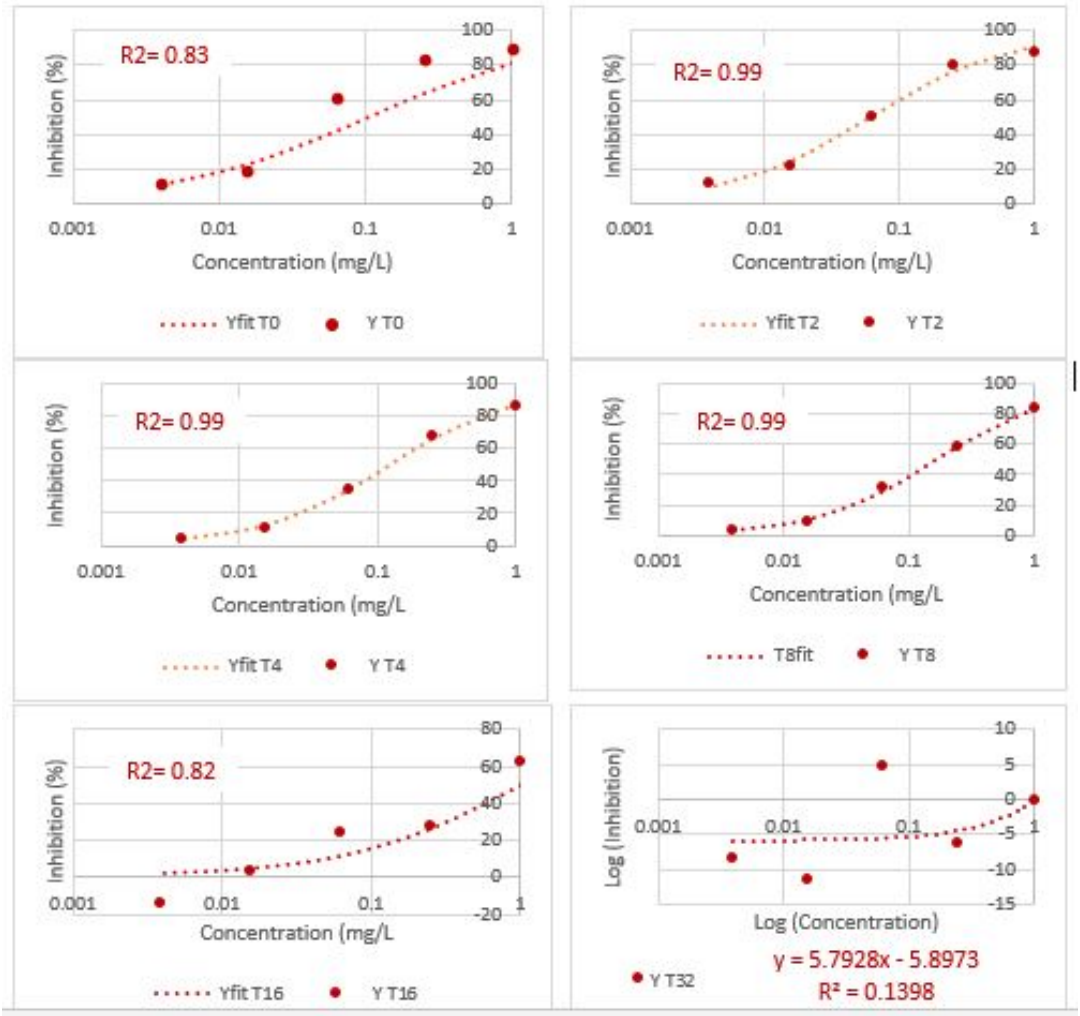

Figure 49. Fit curves for *Lepidium sativum* at time point after VUV-UVC treatment of 2,4D

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Bibliography

Abate Jote, C., 2019. *A review of 2,4-D environmental fate, persistence and toxicity effects on living organisms.* Org. Med. Chem. Int. J., 9(1). URL `https://juniperpublishers.com/omcij/OMCIJ.MS.ID.555755.php`.

Agency, Environmental Protection, 1995. *National Primary Drinking Water Regulations: 2,4-D Technical Version.*

Amin, M. M., Teimouri, F., Sadani, M. and Karami, M. A., 2016. *Application of enhanced nZnO photocatalytic process with ultrasonic wave in formaldehyde degradation from aqueous solution.* Desalination and Water Treatment, 57(20), 9455–9464.

Arshad, M. and Shakoor, A., 2017. *Irrigation Water Quality.*

Beate, S., 2012. *Effects of herbicides on non-target plants: How do effects in standard plant test relate to effects in natural habitats?* Environmental Protection Agency.

Benitez, F.J., Acero, J.L. and Real, S., F.J .and Roman, 2004. *Oxidation of MCPA and 2, 4-D by UV radiation, ozone, and the combinations UV/H2O2 and O3/H2O2.* Journal of Environmental Science and Health, Part B, 39(3), 393–409.

Boutin, C, Elmegaard, N and Kjaer, C, 2004. *Toxicity testing of fifteen non-crop plant species with six herbicides in a greenhouse experiment: implications for risk assessment.* Ecotoxicology, 13(4), 349–369.

Breinholt, L., 2020. *Kinetic modeling and rate constant determination of Vacuum-UV oxidation of BAM in Danish groundwater.* Chemical Engineering.

Cabral, M.K, Mendes, K.F., de Sousa, R.N. and de Paula Medeiros, B.A. Degradation Process of Herbicides in Biochar-Amended Soils: Impact on Persistence and Remediation. In *Biodegradation Technology of Organic and Inorganic Pollutants.* IntechOpen, 2022.

Canada, Health, 2010. *Guidelines for Canadian Drinking Water Quality: Guideline Technical Document 2-Methyl-4-chlorophenoxyacetic Acid (MCPA).*

Caudle, W.M. Chapter 14 - Occupational exposures and parkinsonism. In Lotti, Marcello and Bleecker, Margit L., editor, *Occupational Neurology*, volume 131 of *Handbook of Clinical Neurology*, 225–239. Elsevier, 2015. doi: https://doi.org/10.1016/B978-0-444-62627-1.00013-5.

Cejudo, E., Leal-Bautista, R., Smith, D. and Grimaldo-Hernández, C., 05 2021. *Detection of 2, 4-D in groundwater in northeast of the Yucatan Peninsula Detección de 2, 4-D en agua subterránea en el noreste de la Península de Yucatán Scientific note.* Ecosistemas y Recursos Agropecuarios, 8. doi: 10.19136/era.a8n1.2472.

de Castro Marcato, A.C., de Souza, C.P. and Fontanetti, C.S., 2017. *Herbicide 2, 4-D: a review of toxicity on non-target organisms.* Water, Air, & Soil Pollution, 228(3), 1–12.

DeLorenzo, M.E., Scott, G.I. and Ross, P.E., 2001. *Toxicity of pesticides to aquatic microorganisms: a review.* Environmental Toxicology and Chemistry: An International Journal, 20(1), 84–98.

Derylo-Marczewska, A., Blachnio, M., Marczewski, A. W., Swiatkowski, A. and Tarasiuk, B., Aug 2010. *Adsorption of selected herbicides from aqueous solutions on activated carbon.* Journal of Thermal Analysis and Calorimetry, 101(2), 785–794. ISSN 1572-8943. doi: 10.1007/s10973-010-0840-7.

Dietrich, M.R, Ankeny, N., R.A.and Crowe, Green, S and Leonelli, S., 2020. *How to choose your research organism.* Studies in History and Philosophy of Science Part C: Studies in History and Philosophy of Biological and Biomedical Sciences, 80, 101227. ISSN 1369-8486. doi: https://doi.org/10.1016/j.shpsc.2019.101227. URL `https://www.sciencedirect.com/science/article/pii/S1369848619301165`.

Dinka, M.O. Safe Drinking Water: Concepts, Benefits, Principles and Standards. In Glavan, Matjaž, editor, *Water Challenges of an Urbanizing World*, chapter 10. IntechOpen, Rijeka, 2018. doi: 10.5772/intechopen.71352.

du Plessis, A., 2017. *Global Water Availability, Distribution and Use*, 3–11. Springer International Publishing, Cham, 2017. ISBN 978-3-319-49502-6. doi: https://doi.org/10.1007/978-3-319-49502-6_1.

Evangelista, S., Cooper, D.G. and Yargeau, V., 2010. *The effect of structure and a secondary carbon source on the microbial degradation of chlorophenoxy acids.* Chemosphere, 79(11), 1084–1088. ISSN 0045-6535. doi: https://doi.org/10.1016/j.chemosphere.2010.03.018. URL `https://www.sciencedirect.com/science/article/pii/S0045653510003012`.

FAO, sep 2008. *Climate change, water and food security.*

FAO, dec 2021. *The state of the world's land and water resources for food and agriculture systems at breaking point. Synthesis report.*

Freisthler, M. S., Robbins, C. R., Benbrook, C. M., Young, H. A., Haas, D. M., Winchester, P. D. and Perry, M. J., Feb 2022. *Association between increasing*

agricultural use of 2,4-D and population biomarkers of exposure: findings from the National Health and Nutrition Examination Survey, 2001–2014. Environmental Health, 21(1), 23. ISSN 1476-069X. doi: 10.1186/s12940-021-00815-x.

G., Imoberdorf and M., Mohseni, 2012. *Kinetic study and modeling of the vacuum-UV photoinduced degradation of 2,4-D*. Chemical Engineering Journal, 187, 114–122. ISSN 1385-8947. doi: https://doi.org/10.1016/j.cej.2012.01.107. URL `https://www.sciencedirect.com/science/article/pii/S1385894712001490`.

Galon, L., Brunetto, L., Loureiro da Silva, A.M., Soligo, V., Cavaletti, D.C., da Silva, A.F., Gallina, A., Franceschetti, M.B., Forte, C.T. and Perin, G.F., 2021. *Tank mixture of glyphosate and 2, 4-D applied on RR- maize crop*. Journal of Environmental Science and Health, Part B, 56(11), 948–953.

Garrido, I., Vela, N., Fenoll, J., G.G., Navarro, G., Perez L. and Navarro, S., 12 2015. *Testing of leachability and persistence of sixteen pesticides in three agricultural soils of a semiarid Mediterranean region*. Spanish Journal of Agricultural Research, 13, e1104. doi: 10.5424/sjar/2015134-8339.

Gleick, P. H., 1993. *Water and conflict: Fresh water resources and international security*. International security, 18(1), 79–112.

González, N.V., Soloneski, S. and Larramendy, M.L., 2007. *The chlorophenoxy herbicide dicamba and its commercial formulation banvel induce genotoxicity and cytotoxicity in Chinese hamster ovary (CHO) cells*. Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 634(1), 60–68. ISSN 1383-5718. doi: https://doi.org/10.1016/j.mrgentox.2007.06.001.

Grabińska-Sota, E., E.and Wiśniowska and Kalka, J., 2003. *Toxicity of selected synthetic auxines—2,4-D and MCPA derivatives to broad-leaved and cereal plants*. Crop Protection, 22(2), 355–360. ISSN 0261-2194. doi: https://doi.org/10.1016/S0261-2194(02)00178-3. URL `https://www.sciencedirect.com/science/article/pii/S0261219402001783`.

Göttingen, Georg-A.-Universität. *SAG 61.81 Raphidocelis subcapitata*. URL `https://sagdb.uni-goettingen.de/detailedList.php?str_number=61.81`, Accessed: June 11, 2022.

Hashem, A., Tabassum, B. and AbdAllah, E.F., 2019. *Bacillus subtilis: A plant-growth promoting rhizobacterium that also impacts biotic stress*. Saudi Journal of Biological Sciences, 26(6), 1291–1297. ISSN 1319-562X. doi: https://doi.org/10.1016/j.sjbs.2019.05.004. URL `https://www.sciencedirect.com/science/article/pii/S1319562X19300890`.

Hutchinson, T. H., Lyons, B.P, Thain, J.E. and Law, R.J., 2013. *Evaluating legacy contaminants and emerging chemicals in marine environments using adverse outcome pathways and biological effects-directed analysis*. Marine Pollution Bulletin, 74(2), 517–525. ISSN 0025-326X. doi: https://doi.org/10.1016/j.marpolbul.2013.06.012. URL `https://www.sciencedirect.com/science/article/pii/S0025326X13003032`.

IARC, 2022. *IARC monographs on the identification of carcinogenic hazards to humans.* URL https://monographs.iarc.who.int/agents-classified-by-the-iarc/, [Online; accessed 7-February-2022].

Imoberdorf, G. and Mohseni, M., 04 2011. *Experimental study of the degradation of 2,4-D induced by vacuum-UV radiation.* Water science and technology : a journal of the International Association on Water Pollution Research, 63, 1427–33. doi: 10.2166/wst.2011.321.

Juma, A.H.A. and Colin, R. M. Chapter 61 - Garden Cress (Lepidium sativum) Seeds in Fracture-induced Healing. In Preedy, Victor R., Watson, Ronald Ross and Patel, Vinood B., editors, *Nuts and Seeds in Health and Disease Prevention*, 513–520. Academic Press, San Diego, 2011. ISBN 978-0-12-375688-6. doi: https://doi.org/10.1016/B978-0-12-375688-6.10061-1. URL https://www.sciencedirect.com/science/article/pii/B9780123756886100611.

Jägermeyr, J., Gerten, D., Heinke, J., Schaphoff, S., Kummu, M. and Lucht, W, 07 2015. *Water savings potentials of irrigation systems: Global simulation of processes and linkages.* Hydrology and Earth System Sciences, 19, 3073–3091. doi: 10.5194/hess-19-3073-2015.

Kelly, M.M. and Arnold, W.A., 2012. *Direct and indirect photolysis of the phytoestrogens genistein and daidzein.* Environmental science & technology, 46(10), 5396–5403.

Kibona D., Rwabukambara F., Kidulile G., 2009. *Environment, Climate Warming and Water Management.* Transition Studies Review, 16. doi: 10.1007/s11300-009-0084-z. URL https://doi.org/10.1007/s11300-009-0084-z.

Kim, M.J., Moon, Y., Kopsell, D.A., Park, S., Tou, J.C. and Waterland, N.L., 2016. *Nutritional value of crisphead 'Iceberg'and romaine lettuces (Lactuca sativa L.).* J. Agric. Sci, 8, 734.

Kizilkaya, R., 06 2000. *The effects of herbicides 2,4-D on total bacteria and Bacillus cereus var. mycoides GROWTH IN SOIL.* doi: 10.13140/2.1.4706.6888.

Kovács, A.T., 2019. *Bacillus subtilis.* Trends in Microbiology, 27(8), 724–725. ISSN 0966-842X. doi: https://doi.org/10.1016/j.tim.2019.03.008. URL https://www.sciencedirect.com/science/article/pii/S0966842X19300721.

Kumar, S. and Kumar Singh, A., 11 2010. *A review on herbicide 2, 4-D damage reports in wheat (Triticum aestivum L.).* Journal of Chemical and Pharmaceutical Research, 2, 118–124.

Loll, P. and Moldrup, P., 2000. *Soil Characterization and Polluted Soil Assessment.* Aalborg University. URL https://books.google.dk/books?id=Et3NcQAACAAJ.

Ma, J., Wang, S., Wang, P., Ma, L., Chen, X. and Xu, R., 2006. *Toxicity assessment of 40 herbicides to the green alga Raphidocelis subcapitata.* Ecotoxicology and Environmental Safety, 63(3), 456–462. ISSN 0147-6513. doi:

https://doi.org/10.1016/j.ecoenv.2004.12.001. URL https://www.sciencedirect.com/science/article/pii/S0147651304002453.

Melgarejo L., Morales González J.C., V., Amaral Burity and Torres A.P.L., Côrtes Rocha N., 2020. *Pesticides in Latin America: Violations against the right to adequate food and nutrition.*

Miller, P. and Westra, P., 1996. *Herbicide Formulations.* Colorado State University Cooperative Extension. URL https://erams.com/static/wqtool/PDFs/bmps_colorado/00558.pdf.

Mohaupt, V., Völker, J., Altenburger, R., Birk, S., Kirst, I., Kühnel, D., Küster, E., Semerádová, S., Šubelj, G. and Whalley, C., 06 2020. *Pesticides in European rivers, lakes and groundwaters - Data assessment.*

Morton, P. A., Fennell, C., Cassidy, R., Doody, D., Fenton, O., Mellander, P. E. and Jordan, P., 2020. *A review of the pesticide MCPA in the land-water environment and emerging research needs.* WIREs Water, 7(1), e1402. doi: https://doi.org/10.1002/wat2.1402. URL https://wires.onlinelibrary.wiley.com/doi/abs/10.1002/wat2.1402.

Muszyński, P., Brodowska, M.S. and Paszko, T., Jan 2020. *Occurrence and transformation of phenoxy acids in aquatic environment and photochemical methods of their removal: a review.* Environmental Science and Pollution Research, 27(2), 1276–1293. ISSN 1614-7499. doi: 10.1007/s11356-019-06510-2. URL https://doi.org/10.1007/s11356-019-06510-2.

Nagai, T., 2019. *Sensitivity differences among seven algal species to 12 herbicides with various modes of action.* Journal of pesticide science, D19–039.

Nalcaci O.O., Sirin S. and B., Ovez, 2006. *Toxicity determination of various phenoxyalkanoic acid herbicides using cress seed in phosphate contaminated aqueous media.* Water Pollution VIII: Modelling, Monitoring and Management., 1, 301–307.

Newton, R., Amstutz, J. and Patrick, J.E., 2020. *Biofilm formation by Bacillus subtilis is altered in the presence of pesticides.* Access microbiology, 2(12).

Noumedem, J.A.K., Djeussi, D.E., Hritcu, L., Mihasan, M. and Kuete, V. Chapter 20 - Lactuca sativa. In Kuete, Victor, editor, *Medicinal Spices and Vegetables from Africa*, 437–449. Academic Press, 2017. ISBN 978-0-12-809286-6. doi: https://doi.org/10.1016/B978-0-12-809286-6.00020-0. URL https://www.sciencedirect.com/science/article/pii/B9780128092866000200.

Palma-Bautista, C., Rojano-Delgado, A. M., Dellaferrera, I., Rosario, J. M., Vigna, M. R., Torra, J. and de Prado, R., 2020. *Resistance mechanisms to 2, 4-D in six different dicotyledonous weeds around the world.* Agronomy, 10(4), 566.

Paszko, T., Muszyński, P., Materska, M., Bojanowska, M., Kostecka, M. and Jackowska, I., 2016. *Adsorption and degradation of phenoxyalkanoic acid herbicides in soils: A*

review. Environmental Toxicology and Chemistry, 35(2), 271–286. doi: https://doi.org/10.1002/etc.3212. URL https://setac.onlinelibrary.wiley.com/doi/abs/10.1002/etc.3212.

Petersen, L., Heynen, M. and Pellicciotti, F., 2019. *Freshwater Resources: Past, Present, Future*, 1–12. American Cancer Society, 2019. ISBN 9781118786352. doi: https://doi.org/10.1002/9781118786352.wbieg0712.pub2.

Piggot, P.J. Bacillus Subtilis. In Schaechter, Moselio, editor, *Encyclopedia of Microbiology (Third Edition)*, 45–56. Academic Press, Oxford, third edition edition, 2009. ISBN 978-0-12-373944-5. doi: https://doi.org/10.1016/B978-012373944-5.00036-5. URL https://www.sciencedirect.com/science/article/pii/B9780123739445000365.

Pileggi, M., Pileggi, S.A.V. and Sadowsky, M.J., 2020. *Herbicide bioremediation: from strains to bacterial communities*. Heliyon, 6(12). ISSN 2405-8440. doi: https://doi.org/10.1016/j.heliyon.2020.e05767. URL https://www.sciencedirect.com/science/article/pii/S2405844020326104.

Pink, D.A.C. and Keane, E.M. 40 - Lettuce: Lactuca sativa L. In KALLOO, G. and BERGH, B.O., editor, *Genetic Improvement of Vegetable Crops*, 543–571. Pergamon, Amsterdam, 1993. ISBN 978-0-08-040826-2. doi: https://doi.org/10.1016/B978-0-08-040826-2.50044-8. URL https://www.sciencedirect.com/science/article/pii/B9780080408262500448.

Ramadan, M.F. and Oraby, H.F. Chapter 20 - Lepidium sativum Seeds: Therapeutic Significance and Health-Promoting Potential. In Preedy, Victor R. and Watson, Ronald Ross, editor, *Nuts and Seeds in Health and Disease Prevention (Second Edition)*, 273–289. Academic Press, second edition edition, 2020. ISBN 978-0-12-818553-7. doi: https://doi.org/10.1016/B978-0-12-818553-7.00020-6. URL https://www.sciencedirect.com/science/article/pii/B9780128185537000206.

Roesler, G.D., Jonck, L., C., Gomes, Silva, Rafael Pires S.R., Victoria Jeronimo, A., Silva Hirata, A.C. and Monquero, P.A., 2020. *Decontamination methods of tanks to spray 2, 4-D and dicamba and the effects of these herbicides on citrus and vegetable species*. Australian Journal of Crop Science, 14(8), 1302–1309.

Sagasta, J., M.J.and Burke, 2010. *Agriculture and water quality interactions: a global overview*. URL https://www.fao.org/3/bl092e/bl092e.pdf, Online; accessed 15-May-2022.

Saleh, I.A., Zouari, N. and Al-Ghouti, M.A., 2020. *Removal of pesticides from water and wastewater: Chemical, physical and biological treatment approaches*. Environmental Technology Innovation, 19, 101026. ISSN 2352-1864. doi: https://doi.org/10.1016/j.eti.2020.101026. URL https://www.sciencedirect.com/science/article/pii/S2352186420313262.

Sengupta, A., Lyons, J. M., Smith, D. J., Drewes, J. E., Snyder, S. A., Heil, A. and Maruya, K. A., 2014. *The occurrence and fate of chemicals of emerging concern in*

coastal urban rivers receiving discharge of treated municipal wastewater effluent. Environmental Toxicology and Chemistry, 33(2), 350–358. doi: https://doi.org/10.1002/etc.2457. URL https://setac.onlinelibrary.wiley.com/doi/abs/10.1002/etc.2457.

Sondhia, S., 2014. *Herbicides residues in soil, water, plants and non-targeted organisms and human health implications: an Indian perspective.* Indian journal of weed science, 46(1), 66–85.

Song, Y., 2014. *Insight into the mode of action of 2, 4-dichlorophenoxyacetic acid (2, 4-D) as an herbicide.* Journal of integrative plant biology, 56(2), 106–113.

Speight, J.G. Chapter 7 - Redox Transformations. In Speight, James G., editor, *Reaction Mechanisms in Environmental Engineering*, 231–267. Butterworth-Heinemann, 2018. ISBN 978-0-12-804422-3. doi: https://doi.org/10.1016/B978-0-12-804422-3.00007-9. URL https://www.sciencedirect.com/science/article/pii/B9780128044223000079.

Suzuki, S., Yamaguchi, H., Nakajima, N. and Kawachi, M., 05 2018. *Raphidocelis subcapitata (=Pseudokirchneriella subcapitata) provides an insight into genome evolution and environmental adaptations in the Sphaeropleales.* Scientific Reports, 8. doi: 10.1038/s41598-018-26331-6.

Swiatlowski, T. and Čajka, P., 2018. *Current status of drinking water sources in the world.* Acta Scientifica Academiae Ostroviensis, 12(2), 244–274. ISSN 2300-1739.

Syafrudin, M., Kristanti, R. A., Yuniarto, A., Hadibarata, T., Rhee, J., Al-Onazi, W. A., Algarni, T. S., Almarri, A. H. and Al-Mohaimeed, A. M., Jan 2021. *Pesticides in Drinking Water-A Review.* International journal of environmental research and public health, 18(2), 468. ISSN 1660-4601. doi: 10.3390/ijerph18020468. URL https://pubmed.ncbi.nlm.nih.gov/33430077, 33430077[pmid].

Székács, A., Mörtl, M. and Darvas, title=Monitoring Pesticide Residues in Surface and Ground Water in Hungary: Surveys in 1990–2015 journal=Journal of Chemistry year=2015 month=Sep day=03 publisher=Hindawi Publishing Corporation volume=2015 pages=717948 issn=2090-9063 doi=10.1155/2015/717948, B.

Tigre, R.C., Silva, N.H., Santos, M.G., Honda, N.K., Falcão, E.P.S. and Pereira, E.C., 2012. *Allelopathic and bioherbicidal potential of Cladonia verticillaris on the germination and growth of Lactuca sativa.* Ecotoxicology and Environmental Safety, 84, 125–132. ISSN 0147-6513. doi: https://doi.org/10.1016/j.ecoenv.2012.06.026. URL https://www.sciencedirect.com/science/article/pii/S0147651312002217.

Trigo, C., Spokas, K. A., Cox, L. and Koskinen, W. C., 2014. *Influence of Soil Biochar Aging on Sorption of the Herbicides MCPA, Nicosulfuron, Terbuthylazine, Indaziflam, and Fluoroethyldiaminotriazine.* Journal of Agricultural and Food Chemistry, 62(45), 10855–10860. doi: 10.1021/jf5034398.

Tu M., Randall J. The Nature Conservancy, Hurd C., 2001. *Weed Control Methods Handbook: Tools and Techniques for Use in Natural Areas.* All U.S. Government Documents (Utah Regional Depository). URL https://digitalcommons.usu.edu/govdocs/533.

Vergili, I. and Barlas, H., 2009. *Removal of 2,4-D, MCPA and Metalaxyl from water using Lewatit VP OC 1163 as sorbent.* Desalination, 249(3), 1107–1114. ISSN 0011-9164. doi: https://doi.org/10.1016/j.desal.2009.06.043. URL https://www.sciencedirect.com/science/article/pii/S0011916409009345.

Vlaiculescu, A.A., 2020. *A Bioaugmentation Approach to Pesticide Bioremediation: Pesticide Degradation and Crop Protection.*

Voutsas, Epaminondas. Chapter 11 - Estimation of the Volatilization of Organic Chemicals from Soil. In L., Trevor M., editor, *Thermodynamics, Solubility and Environmental Issues*, 205–227. Elsevier, Amsterdam, 2007. ISBN 978-0-444-52707-3. doi: https://doi.org/10.1016/B978-044452707-3/50013-6. URL https://www.sciencedirect.com/science/article/pii/B9780444527073500136.

Whitacre, D. M., 2010. *Reviews of Environmental Contamination and Toxicology Volume 207.* Springer Science+Business Media, LLC, New York, NY, elektronisk udgave. edition. Reviews of Environmental Contamination and Toxicology, Continuation of Residue Reviews, 207.

WHO, 2017. *Guidelines for Drinking Water Quality: First Addendum to the Fourth Edition.* World Health Organization. International series of monographs on physics.

Woodford, E.K., 1952. *The mode of action of 2: 4-dichlorophenoxy-acetic acid and 2-methyl-4-chlorophenoxyacetic acid.* Journal of the Science of Food and Agriculture, 3 (8), 349–353.

Xinzheng, C., 2021. *Effects of Micro-rates of 2, 4-D and Dicamba on Lettuce and Pumpkin in Nebraska.*

Yanfeng, S., Meiling, C., Yanjian, W., Huaiji, W., Junling, L., Feng, P., Wenlei, H. and He, H., H.and Zhenyu, 2020. *Spatial variation of 2,4-D and MCPA in tap water and groundwater from China and their fate in source, treated, and tap water from Wuhan, Central China.* Science of The Total Environment, 727, 138691. ISSN 0048-9697. doi: https://doi.org/10.1016/j.scitotenv.2020.138691. URL https://www.sciencedirect.com/science/article/pii/S0048969720322087.

Yangtao, W., Lin, D., Lingjun, B., Shumin, Z., Zhou, S. and Shiqing, Z, January 2019. *Degradation of diethyl phthalate (DEP) by vacuum ultraviolet process: influencing factors, oxidation products, and toxicity assessment.* Environmental Science and Pollution Research, 26(6), 5435–5444. doi: 10.1007/s11356-018-3914-x. URL https://doi.org/10.1007/s11356-018-3914-x.

Yu, X.M., Yu, T., Yin, G.H., Dong, Q.L., An, M., Wang, H.R. and Ai, C.X., 11 2015. *Glyphosate biodegradation and potential soil bioremediation by Bacillus subtilis strain Bs-15*. Genetics and Molecular Research, 14, 14717–14730. doi: 10.4238/2015.November.18.37.

Zaman, M., Shahid, S. A. and Heng, L., 2018. *Irrigation Water Quality*, 113–131. Springer International Publishing, Cham, 2018. ISBN 978-3-319-96190-3. doi: 10.1007/978-3-319-96190-3_5.

Zimdahl, R. L., 2007. *Fundamentals of weed science*. Elsevier/Academic Press, Amsterdam ;, [3rd ed.]. edition.

Zoschke, K., Börnick, H. and Worch, E., 2014. *Vacuum-UV radiation at 185nm in water treatment – A review*. Water Research, 52, 131–145. ISSN 0043-1354. doi: https://doi.org/10.1016/j.watres.2013.12.034. URL `https://www.sciencedirect.com/science/article/pii/S0043135413010336`.