Impact of the Herbicide Oxyfluorfen on the Activities of Some Enzymes Found in Soil and on the Populations of Soil Microorganisms

Marioara Nicoleta Filimon 1,2, Diana Larisa Roman 1,2, Despina Maria Bordean 3 and Adriana Isvoran 1,2,*

1 Department of Biology-Chemistry, Faculty of Chemistry, Biology, Geography, West University of Timisoara, Pestalozi 16, 300115 Timisoara, Romania; marioara.filimon@e-uvt.ro (M.N.F); diana.roman@e-uvt.ro (D.L.R.)
2 Advanced Environmental Research Laboratories (AERL), Oituz 4, 300225 Timisoara, Romania
3 Faculty of Food Engineering, Banat’s University of Agriculture and Veterinary Medicine King Michael I of Romania, Calea Aradului 119, 300645 Timisoara, Romania; despina.bordean@gmail.com

* Correspondence: adriana.isvoran@e-uvt.ro

Abstract: This study assesses the effects of the herbicide oxyfluorfen on the activities of enzymes and on the populations of soil microorganisms by considering experiments on soil samples maintained in both laboratory and in field conditions. Furthermore, the molecular docking approach was used to evaluate the interactions of oxyfluorfen with enzymes found in soil. There was a dose dependent inhibitory effect of oxyfluorfen against the activities of dehydrogenase, phosphatase, protease and urease. The enzymes activities obtained for the soil samples maintained under field conditions usually reflected a different trend than those obtained under laboratory conditions, emphasizing the influence of the soil physicochemical properties. For soil samples maintained in field conditions and for the normal dose of oxyfluorfen, dehydrogenase activity recovered after 14 days and a minimum of 21 days was necessary for the recovery of phosphatase, urease and protease activity, respectively. The most important parameters of soil influencing the activities of enzymes and the populations of microorganisms were the pH, N-NO₃ and N-NH₄ contents. A dose dependent behavior of populations of microorganisms found in soil treated with oxyfluorfen has been shown. There was a slight grows of the colonies of microorganisms when oxyfluorfen was applied, but this growth decreased with increasing the oxyfluorfen concentration.

Keywords: oxyfluorfen; soil; enzymatic activity; microorganism’s populations; molecular docking

1. Introduction

Soil contains free enzymes and immobilized extracellular enzymes, but also intracellular enzymes. The presence of enzymes in soil is an indicator of biological equilibrium, soil fertility and quality, as well as an indicator of the soil’s biological condition from the pollution point of view [1]. Enzyme activities can reflect the deviations in soil quality since they underpin nutrient cycling and function as indicators of the changed microbial community caused by environmental impact [2]. Furthermore, any change in soil microflora may be one of the possible causes of decrease of agricultural productivity. Soil microorganisms (bacteria, molds, algae, protozoa, actinomycetes and some nematodes) play a vital role in maintaining the soil productivity, microbial biomass being considered as an active and continuous source of nutrients needed for plant growth and development [3]. Application of herbicides can affect the development of bacterial, actinomycete, fungal and protozoal species. The decrease in the populations of microorganisms may be due to the competitive-ness for food, the toxic effect of the applied herbicides or the persistence of herbicides in the terrestrial ecosystem [4]. Consequently, the balance between the pathogens and beneficial microorganisms is affected and it enables the development of opportunistic organisms (which are able to cause disease) [5–10]. Microbial biomass, the diversity of microbial.
colonies and metabolic activities are the parameters used as biological indicators of the impact of application of pesticides on disturbance of soil quality.

Application of herbicides in agricultural crops can have adverse effects such as qualitative and quantitative changes on soil microbial populations and alteration of the activity of enzymes found in soil. These effects are strongly correlated with the concentration of applied herbicide, its persistence, its physicochemical and behavioral characteristics and with the soil physicochemical parameters, respectively [11–14]. Literature data reveal the stimulation or inhibition of the enzymes’ activities in the presence of herbicides, depending on the type of the herbicide, its dose, the incubation temperature, application interval, inorganic and organic soil content, soil type, soil maintenance works, heavy metal content and other environmental factors [15–20]. Soil temperature and humidity are two factors that reduce the herbicides toxicity and their persistence in soil [19,21,22].

In order to highlight the soil availability for nutrients, it is necessary to study the effect of various groups of herbicides on soil microbial transformations of certain nutrients in the soil, especially compounds containing nitrogen and phosphorus [23]. There also are situations in which application of herbicides have the effect of stimulating the development of communities of soil microorganisms due to the ability of microorganisms to degrade herbicides and to use them as a source of biogenic elements for their own physiological processes. However, prior to degradation, herbicides have toxic effects on communities of microorganisms, leading to declining species abundance, diminishing their biochemical activity, and perceiving the diversity of the initial communities. Toxic effects of herbicides occur more severely immediately after application, when their concentration in soil is high [24]. Subsequently, some microorganisms trigger herbicides degradation, the herbicides concentrations and their toxic effects gradually decrease and disappear. After this phase, the herbicides may represent the carbon sources for soil micropopulation, leading to an increase in soil microflora [25]. Sometimes, the soil micropopulation increases over its value at the application of the herbicides [26]. The relationships of commensalism and cooperation between groups of microorganisms can also influence the populations of microorganisms [27]. Furthermore, data from the literature reveal that microorganisms are very sensitive to changes in the environment, are well suited for an early warning indicators and predictors in monitoring the soil health. The activities of enzymes found in soil and the soil microbial community structure have been widely used as indicators to determine the influences of different pollutants on soil quality and fertility status [1].

Computational chemistry is a useful tool allowing to understand the mechanisms by which the enzymes act and to describe how these mechanisms occur at the atomic level, molecular docking being one of the most used methods. This method proved to be useful for characterizing the interactions between pesticides and enzymes found in soil, such as contributing to the understanding of the factors determining the biological activity of pesticides. Molecular docking has been used to assess the interactions of the herbicides chlorsulfuron and nicosulfuron, and the fungicides difenoconazole and drazoxolon with bacterial chitinases [28,29], Bacillus pasteurii urease [30] and Proteus mirabilis catalase [31], respectively, of the herbicide S-metolaclor with enzymes found in soil (dehydrogenase, protease, phosphatase and urease) [32]. These studies illustrated the inhibitory effects of investigated pesticides on the enzymes activities and that herbicides had a higher inhibitory potential than fungicides. Another molecular docking study revealed that three urease inhibitors, hydroquinone, N-(n-butyl) phosphorothiocitriamide and phenyl phosphorodiamate had also negatively affect the hydroquinone glucosyltransferase [33]. Moreover, the inhibitory effect of the biofumigant 2,3-dimethylmaleic anhydride against acetylcholinesterase of P. americana has been revealed and characterized using the molecular docking approach [34].

The aim of this study is assessing the effects of the herbicide oxyfluorfen on populations of microorganisms and on activities of enzymes found in soil. Oxyfluorfen is a diphenyl ether herbicide used in pre- and post-emergence crops due to its broad spectrum of weed control in agricultural crops, orchards and wine-growing areas, as well as
in non-agricultural ornamental regions (forestry, access routes and embankments). The
toxicity studies based on the rate of application revealed that oxyfluorfen was more toxic
in pre-emergent application on soil than by direct spraying [35]. Computational studies
have established that oxyfluorfen is a lipophilic compound with partition coefficient of
4.3 and it exhibits a limited mobility at the soil level due to strong absorption on the soil’s
organic compounds [36]. Other studies also highlighted that oxyfluorfen is persistent and
immobile in soil [37]. The oxyfluorfen half-life at soil level was calculated as 30–103 days
at different doses and varying amounts of precipitations [38]. Taking into account its mod-
erate to high soil persistence, there is an increased risk of environmental contamination
with oxyfluorfen [36,39].

Although there are studies on the effects of oxyfluorfen on soil microbiota, the in-
formation provided is insufficient to reach clear conclusions. In order to complete the
existing information, within this paper the results of both computational and experimental
studies are presented. From the experimental point of view, the effects of oxyfluorfen on
populations of microorganisms (bacteria, molds and yeasts) and on the activity of enzymes
found in soil are assessed. Taking into account that responses of the enzymes’ activities
and of the populations of microorganisms found in soil to the applications of pesticides
are dependent on a variability of physicochemical factors, both experiments conducted
in controlled laboratory conditions such as to exclude other effects than those due to the
herbicide application and experiments conducted in field conditions were considered.
From the computational point of view, the molecular docking approach has been used
to evaluate and to obtain a deeper view of the possible interactions of oxyfluorfen with
specific enzymes found in soil (dehydrogenase, urease, phosphatase and protease).

There are two aspects of the novelty of this study. One aspect of novelty consists of
evaluating the effects of oxyfluorfen on microorganisms and enzymes found in a chernozem
(loamy-clayey) soil that is characteristic for the western region of Romania. Another aspect
of novelty consists of the combination of experimental and computational approaches that
is meant to give an overall idea of the effects of oxyfluorfen on the enzymes found in soil.

2. Materials and Methods
2.1. Material

Oxyfluorfen, with the IUPAC name 2-chloro-1-(3-ethoxy-4-nitrophenoxy)-4-(trifluoromethyl)
benzene, is a herbicide belonging to the nitrodiphenyl ether class. It is used to combat
weeds due to its ability to disturb the membrane permeability, to provoke biochemical
changes and finally to kill the plant cells. Experiments were performed using a product
sold on a local market under the trade name “Goal4F” (a pre- and post-emergence selective
herbicide) with the content of 480 g oxyfluorfen in 1 L of solution.

2.2. Soil Sampling and Treatment

The soil samples were collected from an experimental field located nearby Timisoara
city (45°45′14.54″ N, 21°18′16.66″ E), in an area where insecticides, fungicides, herbicides
or chemical fertilizers were never used. Chernozem soil samples were collected from the
top layer of soil (0–20 cm) from five different spots in quantities varying between 20 and
25 kg. The material was ground, sieved (2 mm) and spooned by random sampling, giving
sub-samples of 10 kg of soil. The samples were preserved in a refrigerator and processed as
soon as possible during the following 30 days. Biochemical and microbiological analyses
were achieved on soil samples grouped in 2 experimental lots (Lot I; Lot II), each with
4 experimental variants, as presented in Table 1.
Table 1. Variants of the experimental lots of treated soil: B—untreated control soil, \( \frac{1}{2} \) D—a half of normal dose (1 g oxyfluorfen/kg of soil), D—normal dose (2 g oxyfluorfen/kg of soil) and \( 2 \times \) D—double dose (4 g oxyfluorfen/kg of soil).

| Oxyfluorfen Dose | Dose Abbreviation | Lot I—Soil Preserved in Laboratory Conditions with Controlled Values of Physiochemical Parameters | Lot II—Soil Preserved in Field Condition with Variable Physiochemical Parameters |
|------------------|-------------------|-----------------------------------------------------------------------------------------------|-----------------------------------------------------------------------|
| untreated soil   | B                 | al measurements are made after 7 days of incubation                                          | al measurements are made after 7 days of incubation                   |
| 1 g/kg of soil   | 1/2 D             | bl measurements are made after 14 days of incubation                                          | bl measurements are made after 14 days of incubation                 |
| 2 g/kg of soil   | D                 | cl measurements are made after 21 days of incubation                                          | cl measurements are made after 21 days of incubation                 |
| 4 g/kg of soil   | \( 2 \times \) D | an measurements are made after 7 days of incubation                                          | an measurements are made after 14 days of incubation                 |
|                  |                   | bN measurements are made after 14 days of incubation                                          | bN measurements are made after 21 days of incubation                 |
|                  |                   | cN measurements are made after 21 days of incubation                                          | cN measurements are made after 21 days of incubation                 |

Oxyfluorfen has been applied in the following doses: 0 g/kg of soil (untreated soil, B), the half of the recommended dose (1 g/kg of soil, \( \frac{1}{2} \) D), the recommended dose (2 g/kg of soil, D) and the double of the recommended dose (4 g/kg of soil, \( 2 \times \) D) Table 1). For every considered dose, the herbicide has been applied only once at the begging of experiment by spraying on the soil surface. The evaluation of activities of enzymes and of the total number of bacteria/molds/yeast CFU (colony forming units)/g of soil have been assessed at 7, 14 and 21 days, respectively, for both samples maintained in laboratory conditions and in the field conditions.

2.3. Monitoring of the Physicochemical Properties of the Soil

In the case of soil, we have monitored the following physicochemical parameters: temperature \( t \) (°C), pH, electrical conductivity (EC), the organic matter content (OM), the contents of water, \( \text{NH}_4 \)-N, \( \text{NO}_3 \)-N and phosphate [40]. Some physicochemical parameters of soil (temperature, pH, EC) were measured by using a handheld multimeter Multi 340i/SET WTW (Weilheim, Germany) fitted with a specific sensor for each parameter. The soil pH plays an important role in availability of nutrients essential for plant growth. The most favorable accessibility of nutrients occurs at a pH between 6.0 and 7.0; pH lower than 6.0 produces lower rates of N mineralization, which is a process reliant on active, viable microbial populations in the soil [41]. In order to measure the pH of the soil, soil samples were extracted with double distilled water (1:1 water to soil suspension). For measuring the soil electrical conductivity, a suspension of 5:1 double distilled water to soil was used.

In order to determine the water content in the soil, thermogravimetric analysis of the samples was carried out using the Sartorius thermobalance which permits the dehydration monitoring of the evaluated products by a constant weighing during the dehydration process. An amount of 5 g of each investigated sample was oven dried at 105 °C to constant weight using thermo-gravimetric method [42].

Determination of the total organic matter content (humus) in the soil is achieved by calcination method. It is based on the principle of elimination by calcination of an organic substance in the sample as a result of the oxidation of carbon with atmospheric oxygen. The weight loss by combustion is determined by weighing. The soil samples were calcined in a muffle furnace, where the temperature was gradually increased up to 550 °C and held constant for 4 h. The amount of organic matter for each soil sample was calculated as

\[
H\% = \frac{m_1 - m_2}{m_1} \times 100\% (1)
\]

where \( m_1 \) is the weight of the soil sample at room temperature and \( m_2 \) is the weight of the ash sample [43].

The determination of ammonium (\( \text{NH}_4 \)-N) content in each of the soil sample solutions was performed by measuring the absorbance using a spectrophotometer T90 UV/Vis (PG Instruments, Alma Park, UK) at a wavelength of 630 nm as described by Baethgen and Alley [44]. The same method has been used for the determination of the contents of phosphate using a wavelength of 882 nm by Amponsah et al. [43] and of the nitrate (\( \text{NO}_3 \)-N) using a wavelength of 543 nm by Uwah et al. [45] in each of the soil sample solutions.
2.4. Biochemical Analyses

The enzymatic activities chosen for assay were: dehydrogenase (EC 1.1.1.1), urease (EC 3.5.1.5), phosphatase (EC 3.1.3.2) and protease (EC 3.4.21.19). All measurements of enzymatic activities were performed by using a T90 UV/Vis spectrophotometer (PG Instruments, UK). For each soil sampling, the analytic protocol was carried out in triplicate, in a controlled laboratory environment, by the same researcher in the same day.

Dehydrogenase activity (DA) was measured using 2,3,5-triphenyltetrazolium chloride (TTC) as substrate to monitor the reaction product (triphenylformazane, TPF) at 485 nm. The reaction mixture containing 3 g soil sample, 0.5 mL of 3% solution of TTC and 1.2 mL Tris buffer (0.1 M, pH 7.6) was kept at 37 °C for 48 h. TPF was extracted with 20 mL acetone and the absorbance of the supernatant was measured at 485 nm. The DA is expressed as mg TPF g\(^{-1}\) soil during 48 h [46].

Urease activity (UA) assesses the rate of urea decomposition in ammonia (NH\(_3\)) and carbon dioxide (CO\(_2\)). For each sample, 5g of soil were put in a sterile polyethylene tube containing 2 mL toluene, 5 mL phosphate buffer and 5 mL of 5% urea solution (CH\(_4\)ON\(_2\)). The mixture was incubated at 37 °C for 24 h. In the collected supernatant, the quantity of produced NH\(_4^+\) was determined using Nessler’s reagent. The absorbance was measured at 445 nm and the UA is expressed as mg NH\(_3\)-N g\(^{-1}\) h\(^{-1}\) soil during 24 h [47].

Phosphatase activity (FA) was estimated based on hydrolytic separation of phenyl phosphate by phosphomonoesterases; the final products are disodic phosphate and phenol. The latter compound reacts with Gibbs reactive (2,6-dibromchinon-chloramide) resulting in a blue precipitate. For each sample, about 2.5 g soil was put into a test tube containing 10 mL of 0.5% disodic phosphate solution. The mixture was incubated at 37 °C for 48 h. Next, 50 mL of ammonium aluminum sulphate, i.e., NH\(_4\)Al(SO\(_4\))\(_2\) × 12H\(_2\)O, were added to each test tube and the mixture was then filtered through ash-free filter paper. From each test tube, 1 mL filtrate was transferred to an empty test tube, together with 5 mL Borax solution (Na\(_2\)B\(_4\)O\(_7\) × 10H\(_2\)O, pH = 9.4). The mixture was brought to a volume of 25 mL with bi-distilled water. FA was determined at 597 nm. The calibration curve was constructed by using a 50 µg/mL phenol (C\(_6\)H\(_5\)OH) solution. FA was defined as mg phenol/g soil during 48 h [48,49].

The protease activity (PA) was estimated by reaction of ninhydrin with the amino acids resulting from the hydrolysis of gelatin used as substrate. For each sample, about 3 g soil was mixed with 7 mL of 2% gelatin and 0.5 mL toluene. The mixture was homogenized (2 min on vortex) and incubated at 37 °C for 24 h. Next, 25 mL of distilled water was added and the mixture was filtered through ash-free filter paper. From each test tube, 2 mL of filtrate was transferred to an empty test tube together with 5 mL of 0.2% ninhydrin solution and the absorbance was measured at 578 nm. The PA was defined as mg amino-N g\(^{-1}\) h\(^{-1}\) soil during 24 h [50,51].

2.5. Microbiological Analyses

In order to evaluate the total number of bacteria, molds and yeast colony forming units (CFU)/g of soil, dilutions of 10\(^{-3}\) to 10\(^{-6}\) starting to 1 g of soil were prepared from soil samples treated with herbicides and that were incubated in laboratory and field conditions for 7, 14 and 21 days, respectively. Equal volumes of 1 mL of soil dilution of 10\(^{-6}\) for bacteria and 10\(^{-3}\) for molds and yeast were inoculated on selective nutrient culture medium. In the case of bacteria, the culture medium was Plate-Count-Agars (Carl Roth GmbH, Karlsruhe, Germany), incubation was carried out at 37 °C for 48 h and the total number of aerobic mesophilic bacteria (TNG) were counted. The culture medium used for growing and identifying molds and yeasts was Potato-Glucose-Agar (PGA) (Carl Roth GmbH, Germany). Incubation of the plates after inoculation was done at 28 °C for 72 h, and the total number of mold and yeast colonies were determined [10,50].
2.6. Statistical Analysis

In order to analyze if the differences in the enzyme activities and populations of microorganisms registered after application of oxyfluorfen in both laboratory and field conditions, the ANOVA test implemented under Origin 6.0 data analysis software has been used. Statistical evaluation of the correlations between the activities of enzymes found in the soil and physicochemical properties of the soil, between the number of the soil microorganism colonies and the dose of applied herbicide and between the soil microorganism’s colonies and the physicochemical properties of the soil have been made using PAST 2.14 [52]. PAST is a free software used for scientific data analysis, with functions for univariate and multivariate statistics as well as ecological analysis. The experimental results were investigated using the Pearson’s correlation coefficient, $r$, cluster analysis and generalized linear models. The correlation coefficient $r$ measures the strength and direction of a linear relationship between two variables on a scatter plot. The value of $r$ is always between $+1$ and $-1$: $r = -1$ means a perfect negative linear relationship, $r = -0.70$ reflects a strong negative linear relationship, $r = -0.50$ reveals a moderate negative relationship, $r = -0.30$ means a weak negative linear relationship, $r = 0$ is for no linear relationship, $r = 0.30$ corresponds to a weak positive linear relationship, $r = 0.50$ reflects a moderate positive relationship, $r = 0.70$ corresponds to a strong positive linear relationship and $r = 1$ reflects a perfect positive linear relationship [53].

2.7. Molecular Docking Study

In order to better understand the effects of oxyfluorfen on the activities of enzymes found in soil, a molecular docking study has been performed. Molecular docking is a method commonly used to predict how a ligand binds to the target protein and the three-dimensional pose of the ligand into the protein receptor is explored. Usually, numerous potential binding modes are sampled and a scoring function is used to order the sampled modes. However, the identification of the correct binding mode is an important issue and can be solved by similarity to the crystallographic structure of the protein in complex with reference ligands. The interactions of oxyfluorfen with some enzymes secreted by microorganisms found in soil were assessed. The structural files of investigated enzymes were extracted from Protein Data Bank (PDB) [54]: alcohol dehydrogenases from the mesophilic bacterium *Clostridium beijerinckii* (PDB ID 1KEV); urease from *Bacillus pasteurii* (PDB ID 3UBP); phosphatase F from *Bacillus subtilis* (PDB ID 4I9C); proteases from *Serratia* sp. E-15 (PDB ID 1SRP) and *Serratia marcescens* (PDB ID 1SMP); and proteinase from *Streptomyces griseus* (PDB ID 3SGA). These structural files have been prepared for molecular docking using Chimera software such as to consider only the A chain for every enzyme, the ligands that are present in the crystallographic structures (where it was the case) have been removed (with the exception of ions), the hydrogens and charges have been added [55]. The structural file of oxyfluorfen has been extracted from ZINC database [56]. Molecular docking study has been performed using the SwissDock web server [57] that is based on EADock algorithm [58]. A blind, accurate and rigid docking has been considered. Analysis of the docking results has been also done using Chimera software.

3. Results

3.1. Assessment of the Activities of Some Enzymes Found in Soil

The activities of dehydrogenase (DA), urease (UA), phosphatase (FA) and protease (PA) have been determined in all soil samples and the results are illustrated in Figure 1.
Figure 1. Enzymatic activities in soil samples maintained in both laboratory (aL, bL, cL) and field (aN, bN, cN) conditions and treated with various doses of oxyfluorfen (B—samples untreated with oxyfluorfen, $\frac{1}{2}$D—a half of the normal dose, D—normal dose, $2 \times$ D—double dose) in relation to the exposure periods: DA (a); UA (b); FA (c); PA (d).

For similar doses, in laboratory conditions, DA decreases in time arriving at 48% of the activity in the 21 days for the normal dose (D) by comparison to DA in the untreated soil ($p < 0.05$). In field conditions and for the normal dose of oxyfluorfen, DA is decreased with 17% after 7 days ($p < 0.01$) and further increases in time and is higher than in the untreated soil with 17% after 14 days and with 33% after 21 days, respectively ($p < 0.05$). It illustrates that the DA is recovered in approximatively 14 days in the soil treated with the normal dose of oxyfluorfen in field conditions. The lowest values are recorded at the double dose of oxyfluorfen ($2 \times$ D) regardless of the conditions of the experiment, thus revealing the increase in the toxic effect of oxyfluorfen against dehydrogenase when increasing the dose. Furthermore, the increased in DA after 14 days is lower for soil treated with 2D of oxyfluorfen by comparison with soil treated with the normal dose of oxyfluorfen underlying the inhibitory effect of oxyfluorfen against dehydrogenase. The different behavior of the DA in the field conditions by comparison to laboratory conditions illustrates the important effect of the soil state and physicochemical properties.

For both laboratory and field conditions, UA is decreased in the presence of oxyfluorfen in a dose dependent manner, a higher dose conducting to a deepest decrease. The lowest values of UA are recorded after 7 days ($p < 0.01$). For longer periods, UA slowly increases in time regardless of the applied dose of oxyfluorfen, but after 21 days the UA in the soil treated with normal dose of oxyfluorfen is only about 54% by comparison to UA in the untreated soil ($p < 0.05$). It emphasizes that a period longer than 21 days is necessary to recover the UA in the soil treated with the normal dose of oxyfluorfen.

Regardless of the incubation conditions, FA shows a decrease over the 21 days of the experiment with the increase of the oxyfluorfen dose ($p < 0.05$). For the application of the normal dose of oxyfluorfen, after 21 days, FA is about 90% from its activity in the untreated soil ($p < 0.05$). These data illustrate that more than 21 days are also necessary for the recovery of the FA in soil treated with the normal dose of oxyfluorfen.
In the case of the laboratory incubated soil samples, PA decreases for all doses of oxyfluorfen (\( p < 0.05 \)), with the observation that the lowest values were recorded in the 2D case. In the case of soil samples maintained under field conditions, in the presence of oxyfluorfen the values recorded for PA after 14 days are increased by comparison with the PA values in the untreated soil (\( p < 0.05 \)). A higher dose of oxyfluorfen conducts to an upper increase in PA after 14 days in soil maintained in field conditions. After this period, PA registers a slight decrease, being probably strongly influenced by the variations in the physicochemical parameters of soil. We must underline that the PA also decreases in time in the soil samples untreated with oxyfluorfen and maintained in field conditions. The PA in the soil sample treated with the normal dose of oxyfluorfen is almost similar after 21 days with that of the untreated soil for samples incubated in experimental and field conditions, respectively.

All these results reveal that DA, UA, FA and PA are affected by the application of oxyfluorfen and they decrease with increasing the oxyfluorfen dose. In the field conditions and for the normal dose of oxyfluorfen, DA recovers after 14 days and minimum 21 days are necessary for the recovery of FA, UA and PA, respectively.

The results concerning the activities of enzymes found in soil and obtained for the soil samples maintained under field conditions usually register a different trend than those obtained under laboratory conditions. It emphasizes the importance of the soil properties and state and the influence of weather conditions. Furthermore, it must be underlined that experimental studies concerning the impact of pesticides on the activities of enzymes found in soil should be done under field conditions, not only in laboratory-controlled conditions.

3.2. Correlation between the Activities of Enzymes Found in Soil for all the Experimental Variants

Values obtained for the enzymatic activities of interest (DA, UA, FA, PA) in all experimental variants were subjected to cluster analysis performed using Paired Group Algorithm based on Euclidian distances to identify the pattern of variations, and to assign observations to groups, respectively (Figure 2a). The Neighbor Joining Cluster is used for bootstrapping analysis, which allows estimation of the sampling distribution using random sampling methods and presents the association of the closest neighbors, highlights the correctness of the obtained results and possible small errors (Figure 2b). It is based on correlation similarity measure and outgroup root and “Boot N” value set to 100 (that is, repeating the clustering 100 times, based on random selections of columns) [59].

![Figure 2. Cluster analysis based on Euclidian distances (a) and the Neighbor Joining Cluster (b).](image-url)
The dendrogram (Figure 2a) has a distance scale, ranging from 0 to 17. Pairs of sites, or groups of sites, are arranged in ‘clusters’ according to the similarity of their enzymatic activities. The inspection of the dendrogram suggests that there are two main groups of sites: PA cluster and a mixed cluster composed by DA, FA and UA. The larger cluster is subdivided into smaller clusters: UA (all experimental variants with similar distance); DA(D) and DA(2×D); FA(D) and FA (2×D); and FA(B) and FA(¼D). DA (B) and DA(¼D) are completely different compared to DA(D) and DA(2×D). Based on the observed clusters, PA cluster presents different behavior compared to UA, FA and DA, as it was also reflected by Figure 1.

3.3. Correlation between the Activities of Enzymes Found in Soil and Physicochemical Properties of the Soil

Enzymatic activity in soil samples treated with xenobiotics is influenced by physicochemical parameters of soil, as well as by incubation conditions with or without variations in environmental factors [60]. Consequently, the influence of the physicochemical parameters of soil samples treated with oxyfluorfen on the enzymatic activities has been analyzed using the linear Correlation Model based on Normal Distribution and Identity Link Function [52]. pH and organic matter (OM) content are considered to be the most important parameters that influence the development of microorganisms and their enzymatic (metabolic) activity. The mathematical models built using the linear correlation of the activity of the four enzymes (DA, UA, FA, PA) to the pH, EC and OM, water, N-NO₃, N-NH₄ and, respectively, the phosphor content are presented. These mathematical models have been used to establish equations reflecting the linear correlations between the activities of the four enzymes and the physicochemical parameters of the soil, the obtained equations being presented in Table S1. Based on the obtained equations, it is possible to determine the value of the enzymatic activity according to the values recorded for different physicochemical parameters and it represent an element of originality of the study. The obtained equations reveal the following: (i) DA is mainly influenced by water, N-NO₃ and N-NH₄ contents, it increases with N-NH₄ content and decreases with N-NO₃ and water contents; (ii) UA decreases with increasing pH, water and N-NH₄ contents and increases with increasing N-NO₃ content; (iii) FA increases with increasing pH value and decreases with N-NO₃ content; (iv) PA is dependent on all the investigated parameters of soil, increases with increasing pH value (the strongest increase), EC, water, OM, N-NO₃ and phosphor contents and decreases with increasing N-NH₄ content. The most important parameters of soil that influence the activities of investigated enzymes are the pH as well as the N-NO₃ and N-NH₄ contents.

3.4. Microbiological Analyses

Analysis of the evolution of the populations of bacteria, molds and yeast in the soil samples treated with oxyfluorfen, in all the experimental variants conducted to different response depending on the dose of applied herbicide (Figure 3).

Figure 3a reveals that in the soil samples treated with oxyfluorfen, the population of bacteria increases in the period of 14 days ($p < 0.05$) and after this period it slightly decreases; the decrease is more accentuated for a higher dose of oxyfluorfen. The values of the bacteria community recorded at the level of the samples incubated in field conditions are smaller than those obtained in laboratory conditions. Oxyfluorfen usually causes an increase in CFU of molds in soil samples maintained both under laboratory and field conditions (Figure 3b). At the level of soil samples incubated under field conditions, the highest increase in CFU/molds/g soil is recorded after 14 days ($p < 0.05$) followed by a slight diminution for the next 7 days. The response of the population of molds in soil was inconsistent in relation with the application dose of oxyfluorfen, especially in the soil samples maintained in field conditions.
Figure 3. Variation of soil population of the bacteria (a), molds (b) and yeasts (c) for the various doses of oxyfluorfen (B—samples untreated with oxyfluorfen, ½ D—a half of the normal dose, D—normal dose, 2× D—double dose) and in relation to the exposure periods.

There is a distinct response of the yeast community to the applied dose of oxyfluorfen and the incubation conditions (Figure 3c). In laboratory conditions, application of oxyfluorfen conducts to an increase of the yeast population in the first 7 days followed by a strong decrease in the next 14 days ($p < 0.05$), for the double dose of oxyfluorfen the yeast community disappears after 7 days. In field conditions, the population of the yeast usually decreases in the presence of oxyfluorfen, the lowest CFU/g soil values are recorded in the treated soil samples with the double dose of oxyfluorfen for all the experimental variants and regardless of the incubation time ($p < 0.05$).

The similarity between bacteria, molds and yeasts data using Neighbor Joining Cluster algorithm was performed to identify the fingerprint of development of the microorganism community (Supplementary Materials Figure S1). There is a similar behavior for the
three groups of microorganisms (bacteria, molds, yeast) in the field conditions and in laboratory experiments.

3.5. Correlation between the Population of the Soil Microorganism and Applied Doses of Oxyfluorfen

The Generalized Linear Model generated using the normal distribution and identity function describes the dependence of the number of soil microorganism colonies on the CFU/g soil of bacteria, molds and yeast in untreated soil samples, respectively. Consequently, a mathematical model describing the dependence of the number of colonies of microorganisms found in soil on the dose of herbicide has been obtained (Table S2). There is a slight increase of the colonies of microorganisms when the herbicide oxyfluorfen is applied by comparison with the control soil samples, the increase being reduced when a higher dose of oxyfluorfen is applied.

3.6. Correlation between the Population of the Soil Microorganism and Physicochemical Properties of the Soil

It has been demonstrated that the population of the soil microorganisms is also influenced by physicochemical parameters of soil, as well as by incubation conditions [61–64]. Consequently, the influence of the physicochemical parameters of soil treated with oxyfluorfen on the populations of soil microorganisms has been analyzed using the linear Correlation Model based on Normal Distribution and Identity Link Function [52]. Equations corresponding to linear correlations between the microorganism populations found in soil and the physicochemical parameters of the soil are presented in Table S3 and illustrate that population of bacteria is the most affected by the physicochemical parameters of the soil treated with oxyfluorfen. This population decreases with increasing the pH, water and nitrate content, the higher decrease being registered under the influence of pH. The increase of the ammonium content conducts to the increase of the population of bacteria. Phosphor content and EC also affect positively the population of bacteria, but their effects are lower. Molds are negatively affected by the OM and N-NH$_4$ content, there is a decrease in molds population with the increase of OM and N-NH$_4$ content. The other parameters of the soil conduct to the increase in molds population, the highest increase being registered under the influence of pH. Water, OM and N-NO$_3$ contents negatively affect the population of yeasts, the increase of the nitrate content producing a strong decrease in this population. The increase of the pH and ammonium content produce the proliferation of the population of yeast, the pH having the strongest influence. Besides the physicochemical parameters of the soil treated with oxyfluorfen, the pH strongly influences the populations of microorganisms: it produces the proliferation of the populations of molds and yeast and the diminution of the population of bacteria.

3.7. Molecular Docking Study

The SwissDock web server has been used for assessing the interactions of oxyfluorfen with a few enzymes secreted by microorganisms found in soil: alcohol dehydrogenase from Clostridium beijerinckii, urease from Bacillus pasteurii, phosphatase F from Bacillus subtilis, protease from Serratia sp. E-15 and Serratia marcescens and proteinase from Streptomyces griseus. The Protein Data bank contains three-dimensional structures of complexes made by these enzymes with substrate and/or inhibitors and it allowed the identification of the best binding modes of the herbicide to the enzymes by comparing the coverage of protein interactions by the reference molecules. Figure 4 illustrates the binding mode of oxyfluorfen (red sticks) to phosphatase from Bacillus subtilis (blue ribbon) corresponding to the highest affinity. Figure 5 illustrates all the binding modes of the oxyfluorfen (red sticks) to urease from Bacillus pasteurii (blue ribbon), none of them corresponding to the active site of enzyme.
Figure 4. Molecular docking result illustrating the binding mode with the highest affinity of oxyfluorfen (red sticks) to phosphatase from *Bacillus subtilis* (blue ribbon). Oxyfluorfen binds in the same region as the inhibitory peptide (yellow sticks) that is present in the crystallographic structure of the complex.

Figure 5. Molecular docking result illustrating the binding modes of oxyfluorfen (red sticks) to urease from *Bacillus pasteurii* (blue ribbon). Oxyfluorfen does not bind in active cavity of the enzyme, this cavity containing the Ni ions (green spheres).

Figure 4 reveals that oxyfluorfen is able to bind to the same place as the inhibitory peptide (yellow sticks) that is present in the crystallographic structure of the complex and it underlines the inhibitory potential of oxyfluorfen against phosphatase from *Bacillus subtilis*. Figure 5 emphasizes that oxyfluorfen is not able to bind to the active site cavity of urease from *Bacillus pasteurii*. It does not exclude the inhibitory effect of oxyfluorfen against this enzyme, as the binding of oxyfluorfen to other region of the protein may conduct to a conformational change that affect the structure of the active cavity and consequently produces an allosteric inhibition of the enzyme.

The outcomes of the molecular docking study concerning the binding energies of oxyfluorfen to the investigated enzymes are synthetized in Table 2.
Table 2. Interacting energies of oxyfluorfen with enzymes secreted by soil microorganisms.

| Enzyme                                                                 | ΔAG (kcal/mol) |
|-----------------------------------------------------------------------|----------------|
| dehydrogenase from *Clostridium beijerinckii*                         | −7.578         |
| phosphatase from *Bacillus subtilis*                                  | −8.006         |
| urease from *Bacillus pasteurii*                                      | It does not bind to the active site of the enzyme. |
| protease from *Serratia* sp. E-15 and *Serratiamarcescens*            | −7.226         |
| proteinase from *Streptomyces griseus*                               |                |

4. Discussion

The activities of dehydrogenase, urease, phosphatase and protease have been chosen within this study to evaluate the effects of oxyfluorfen on the soil biochemistry due to the spreading and significant roles of these enzymes in the transformation of organic matter.

Dehydrogenase activity is considered to be the most effective method of determining the effect of xenobiotics on the communities of microorganism found in the soil, as it is manifested only in viable cells [65]. For the soil samples maintained in field conditions DA decreases in the first 7 days, it recovers after 14 days and after 21 days it increases by comparison to that obtained for the untreated soil sample with 63% at $\frac{1}{2}$ × D, 34% at D and 17% at 2 × D, respectively. The decrease of DA in the first 7 days and the fact that the increase of the applied dose of oxyfluorfen conducts to a reduced increase of DA after 21 days underlines the inhibitory effect of oxyfluorfen against DA. This result is in good agreement with molecular docking results. The increase in DA after 21 days may be associated with the increase in some of bacterial soil populations capable of using oxyfluorfen as a carbon source. A similar behavior has been noticed when the herbicide fomesafen has been used for the soil treatment [14]. The herbicide S-metolachlor revealed an inhibitory effect against dehydrogenase, the decrease in the activities the enzyme being dose-dependent [32]. Another herbicide, butachlor stimulated activity of dehydrogenase for soil under flooded conditions, but had a detrimental affect against dehydrogenase for un-flooded conditions [19]. The effects of stimulation or inhibition of the dehydrogenase activity as a result of application of various pesticides have been reported in numerous studies [9,66–69].

The hydrolysis of urea in soil is catalyzed by the specific urease enzyme, which is susceptible to the changes in soil induced by herbicides [60]. Urease activity is a useful indicator of soil quality and has been widely used alongside DA to determine the effects of xenobiotics on the metabolic activities in the soil. At the level of soil samples treated with oxyfluorfen, the values obtained for the urease activity recorded a decrease with the increase of the herbicide dose compared to the control soil samples, no matter the incubation conditions. The most accentuated decrease has been recorded after 21 days for the higher dose of oxyfluorfen (2 × D): 19.19% for the soil sample maintained under laboratory conditions and 45.91% for that maintained under field conditions. These results illustrate the inhibitory effect of oxyfluorfen against UA and it is also in good agreement with molecular docking results revealing the possible allosteric inhibition of urease by oxyfluorfen. Quite similar effects have been noticed for the herbicides fomesafen and S-metolachlor. When the herbicides have been applied in high doses on the soil samples, the urease activity registered first a decrease followed by an increase. This behavior indicated that fomesafen and S-metolachlor had initially an inhibitory effect against urease, but this effect gradually disappeared with increasing incubation time [14,32]. Literature data also reveal that urease activity decreased with increasing the concentration of various herbicides applied on the soil samples [9,70,71]. Others studies reported that the herbicides 2,4-dichlorofenoxy acetic acid, butachlor and oxyfluorfen did not affected the urease activity when applied on the soil samples [72]. In contrast, there also are studies showing that urease activity was increased in the presence of some herbicides such as alachlor, propaquizafop, imazethapyr and butachlor for soil under flooded conditions [19,73].

Phosphatase activity is responsible for the transformation of phosphorus-containing organic compounds into inorganic compounds that become available for the microorgan-
isms and for the mineral nutrition of plants. Phosphatase activity is considered a viable indicator that highlights the changes caused by the application of herbicides on the soil [60]. Application of oxyfluorfen to soil samples causes either a stimulation or an inhibition of the phosphatase activity, depending on the incubation conditions. In the soil samples treated with oxyfluorfen and incubated for 21 days under laboratory conditions, phosphatase activity decreases compared to the control sample with 8% at 1/2 D, 39% at D and 51% at 2 × D, respectively, and it illustrates the inhibitory effect of oxyfluorfen against phosphatase. For the soil samples incubated for 21 days under field conditions, the values obtained for the phosphatase activity are increased by comparison to the control soil with: 21% at 1/2 D, 11% to D and 10% to 2 × D, respectively. This result also indicates that a higher dose of oxyfluorfen negatively affects FA and it is in good agreement with molecular docking predictions revealing that oxyfluorfen may act as inhibitor for phosphatase. The increase of FA in field conditions may be due to the increase in populations of some microorganisms that are able of using oxyfluorfen as a carbon source. A similar result indicating an increase in FA during 30-day incubation has been obtained when the herbicide fomesafen have been used [14]. Other studies have shown the inhibition of FA [32,74,75], respectively a stimulation of FA in samples of soil treated with other herbicides [22,60,71,76]. Application of the herbicide butachlor conducted to stimulation in the activity of phosphatase under un-flooded conditions and inhibition under flooded conditions [19].

Proteases play an important role in the nitrogen cycle in the soil by hydrolyzing organic protein compounds, resulting in the formation of simple inorganic compounds, the enzyme being generally associated with inorganic and organic colloids in the soil [77]. In the case of soil samples treated with oxyfluorfen and incubated for 21 days under laboratory conditions, PA decreased compared to the control sample with 5% at 1/2 D, 35% at D and 45% at 2 × D, respectively. The decrease of PA in laboratory-controlled conditions illustrates the inhibitory effect of oxyfluorfen against PA, also in good correlation with predictions obtained using molecular docking. For the soil samples treated with oxyfluorfen and incubated for 21 days under field conditions, the values obtained for the PA are increased compared to the control soil after 14 days and they decrease for the next period until 21 days. The increase of PA in field conditions illustrates the importance of physicochemical properties of soil for both activities of enzymes found in soil and for the dynamics of microorganism populations. In another study, the insecticides acephate and buprofezin stimulated PA when applied in small doses, but PA was adversely affected at higher doses of insecticides [78]. On the contrary, application of the herbicide imazethapyr in a field trial in a clay loam soil at the recommended field rate led to an increase in PA [79]. When the herbicide S-metolachlor has been used, the activity of protease decreased initially and increased after 14 days of incubation [32]. Activity of protease presented an inconsistent response towards butachlor usage under both un-flooded and flooded conditions [19].

Reactions of an enzyme found in soil to the applied pesticide cannot be foreseen with confidence as a complex of interactions characterizes soil subsystem and numerous factors may affect the activity of the enzyme: synthesis, stabilization, regulation, persistence, catalytic comportment of the investigated enzymes and also changes of physical, chemical, and biological soil composition [63]. Data presented in this study illustrate that the activities of investigated enzymes are mostly influenced by the soil pH and its content in nitrate and ammonium and that there are changes in the populations of microorganisms in soil samples treated with oxyfluorfen. Furthermore, the obtained results illustrate that in such studies, laboratory results do not always corroborate the findings under field conditions and it makes difficult to make a sweeping statement concerning the trend of the enzyme activity. Similar findings have been illustrated earlier [19,63]. Globally, the outcomes of this study reveal that oxyfluorfen appeared to significantly influence the activity of enzyme found in soil: dehydrogenase, urease, phosphatase and protease.

The application of herbicides has effects on microorganisms in the soil, determining quantitative and qualitative changes. Literature data reveal that evolution of populations of different groups of microorganisms (bacteria, actinomycetes, molds and yeasts) is
dependent on the concentration of applied herbicide, type of herbicide, the incubation time, the physico-chemical properties of the soil, and the relationship in the groups of present microorganisms [80]. Data obtained in this study expose that in the soil samples treated with the normal dose of oxyfluorfen the populations of bacteria and molds increase in the period of 14 days but over this period, these populations decrease. Quite similar behavior is registered for the yeast community, but the increase in this population is registered after 7 days and it is followed by a slight decrease in the next 14 days. Studies concerning the effects of oxyfluorfen and other herbicides on the populations of microorganisms in soil reveal different results. Some studies by Zhang et al. [14] emphasize the inhibition of the development of fungal communities in the soil, while other studies by El Husaein et al. [81] indicate the increase of the fungal population in the soil treated with oxyfluorfen. Bera and Ghosh revealed that application of the oxyfluorfen to the cultivated soil caused a decrease in the number of microorganisms in the soil in the first 10 days after treatment, but due to the degradation of the herbicide, in the next 50 days there was an increase in the microbial population in the soil [82]. Additionally, Bera and Ghosh [82] revealed that among the investigated microorganisms, molds were least affected by the treatments with oxyfluorfen, this result being in good agreement with data presented in the present study. Another published study revealed that the application of oxyfluorfen differently affected the microbial populations of the soil, depending on the type of soil and on the period of assessment of the effect. For the clay soil, application of a dose of 0.72 kg oxyfluorfen/ha did not influence the microbial biomass [13]. A study made by Das et al. [23] showed that the post-emergence uses of oxyfluorfen applied in a field of rice at a dose of 0.12 kg/ha increased the number of bacteria in the soil. Application of fomesafen, a compound belonging to the same class of herbicides as oxyfluorfen, increased the number of bacteria and actinomycetes in the soil and the increase was proportional to the concentration of administered fomesafen [14]. This observation was explained by the ability of some bacteria and actinomycetes found in soil to use fomesafen as a carbon source [14]. The studies by Liang et al. [83] and Feng et al. [84] have also shown that the microbial populations (especially bacteria) have the ability to ensure efficient degradation of fomesafen and to recover after the initial decreases, sometimes exceeding the initial number existing in the soil [4,26]. Data obtained within this study confirm that oxyfluorfen may conduct to the increase of population of soil microorganisms in 7 to 14 days after its application illustrating that microorganisms present in soil perform the degradation of the herbicide. It also explains the slight decrease of the toxic effects of oxyfluorfen against the population of microorganisms for a longer period of observation. Similar trends have been already noticed in specific literature [25,82]. Similarly, responses of the populations of microorganisms found in soil to the applied herbicide cannot be foreseen with confidence as it has been recognized by many researchers that there is a variability of factors affecting the communities of microorganisms: temperature, pH, water content of soil, availability of food [61–64]. Moreover, data obtained in this study also revealed that the populations of soil microorganisms are strongly influenced by the pH, nitrate and ammonium contents of soil.

5. Conclusions

In this study, the effects of the herbicide oxyfluorfen on the activities of some enzymes and on populations of microorganisms found in soil have been assessed by using two distinct experimental approaches, i.e., soil samples preserved in laboratory conditions with controlled values of physiochemical parameters and soil samples preserved in field conditions with variable physicochemical parameters. The molecular docking technique has been also considered to assess the interactions between oxyfluorfen and enzymes found in soil.

The outcomes of the experimental studies reveal a dose dependent inhibitory effect of oxyfluorfen against the activities of dehydrogenase, protease, phosphatase and urease and are in good correlation with the results illustrated by molecular docking approach. Appli-
cation of the normal dose of oxyfluorfen on soil samples maintained in field conditions revealed that dehydrogenase activity recuperated after 14 days and at least 21 days were necessary for the recovery of activities of the other investigated enzymes (phosphatase, urease and protease). Usually, there was a different trend concerning the activities of enzymes in the soil samples maintained under field and laboratory conditions, respectively. It emphasizes the important influence of the soil physicochemical properties on enzymes activities.

The results that are obtained also reveal a dose dependent behavior of populations of bacteria and molds in soil treated with oxyfluorfen. There was a slight increase of the colonies of microorganisms when the herbicide oxyfluorfen is applied by comparison with the control soil samples, the increase was reduced when a higher dose of oxyfluorfen was applied. It illustrates that these microorganisms are able to degrade oxyfluorfen and to use it as a carbon source.

The most important parameters of soil that influenced the activities of investigated enzymes and the populations of the soil microorganisms are the pH and the nitrate and ammonium contents.

All the information presented in the present study underline that the activities of enzymes found in soil and the dynamics of the populations of microorganisms are strongly correlated and also dependent on the physicochemical parameters of soil. Furthermore, these outcomes reveal that any study dealing with the effects of pesticides on soil must take into account all the aspects and that such experiments should be done under field conditions, not only in laboratory-controlled conditions.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11091702/s1, Figure S1: Neighbor Joining Cluster using Euclidian similarity measure (Cluster analysis using Paired Group Algorithm Euclidian similarity measure presents a corr. Coph = 0.9291), Table S1: Equations reflecting the linear correlations between the activities of enzymes found in soil and the physicochemical parameters of the soil (y denotes the enzymatic activity and x denotes the physicochemical parameter), Table S2: Equations describing the evolution of the colonies of microorganisms with the applied dose of oxyfluorfen based on Generalized Linear Model (x denotes number of colonies of microorganisms found in untreated soil; y denotes number of colonies of microorganisms found in soil when various doses of oxyfluorfen were applied), Table S3: Equations reflecting the linear correlations between the microorganism’s populations found in soil treated with oxyfluorfen and the physicochemical parameters of the soil (y denotes the number of microorganisms and x denotes the physicochemical parameter): EC—electrical conductivity, OM—organic matter content.

Author Contributions: M.N.F., D.L.R., D.M.B. and A.I. they conceived and designed the study; M.N.F. performed biological experiments, collected data and interpreted the results; D.L.R. and A.I. performed the bioinformatics study, collected data and interpreted the results; D.M.B. performed statistical analysis and interpreted the results; M.N.F. and A.I. they wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References
1. Utobo, E.B.; Tewari, L. Soil enzymes as bioindicators of soil ecosystem status. Appl. Ecol. Environ. Res. 2015, 13, 147–169.
2. Cao, P.; Hall, E.; Zhang, E. Soil sampling sensor system on a mobile robot. In Proceedings of the SPIE Intelligent Robots and Computer Vision XXI: Algorithms, Techniques and Active Vision, Providence, RI, USA, 27–31 October 2003; Volume 5267, pp. 304-310.
3. Zaid, A.M.; Mayouf, M.; Farouj, Y.S. The effects of post-emergence herbicides on soil microflora and nitrogen fixing bacteria in Pea field. *Int. J. Chem. Environ. Biol. Sci.* 2014, 2, 40–42.

4. Sapundjieva, K.; Kalinova, S.; Kartalska, Y.; Naydenov, M. Influence of pendimetalin herbicide upon soil microflora. *Pochvoznanie Agrokhim. Ekol.* 2008, 42, 49–55.

5. Gupta, P.K. Pesticide exposure—Indian scene. *Toxicology* 2004, 198, 83–90. [CrossRef]

6. Crouzet, O.; Batissonn, I.; Besse-Hoggan, P.; Bonnemoy, F.; Bardot, C.; Poly, F.; Bohatier, J.; Mallet, C. Response of soil microbial communities to the herbicide mesotrione: A dose–effect microcosm approach. *Soil Biol. Biochem.* 2010, 42, 193–202. [CrossRef]

7. Romero, M.C.; Urrutia, M.I.; Reinoso, H.E.; Kiernan, M.M. Benzo[a]pyrene degradation by soil filamentous fungi. *J. Yeast Fungal Res.* 2010, 1, 25–29.

8. Garbisu, C.; Alkorta, I.; Epelde, L. Assessment of soil quality using microbial properties and attributes of ecological relevance. *Appl. Soil Ecol.* 2011, 49, 1–4. [CrossRef]

9. Filimon, M.N.; Voia, S.O.; Popescu, R.; Bordean, D.M.; Vladoiu, D.L.; Mituletu, M.; Ostafe, V. The effect of chlorsulfuron and MCPB-Na on the enzymatic activity of microorganisms. *J. Serb. Chem. Soc.* 2014, 79, 1075–1084. [CrossRef]

10. Filimon, M.N.; Popescu, R.; Verdes, D.; Dumitrescu, G.; Voia, O.S.; Ahmadi, M.; Dronca, D. The effects of difenoconazole treatment on microorganism from soil. *Rev. Chim.* 2018, 69, 1129–1133. [CrossRef]

11. Saeki, M.; Toyota, K. Effect of bensulfuron-methyl (a sulfonurea herbicide) on the soil bacterial community of a paddy soil microcosm. *Biol. Fertil. Soils* 2004, 40, 110–115. [CrossRef]

12. Sofo, A.; Scopa, A.; Dumontet, S.; Mazzatura, A.; Pasquale, V. Toxic effects of four sulphonylureas herbicides on soil microbial biomass. *J. Environ. Sci. Health Part B* 2012, 47, 653–659. [CrossRef]

13. Da Silva, G.S.; Melo, C.A.D.; Fialho, C.M.T.; Santos, L.D.T.; Costa, M.D.; da Silva, A.A. Impact of sulflurazone, isoxaflutole and oxyfluorfen on the microorganisms of two forest soils, Bragantia. *Campinas* 2014, 73, 292–299.

14. Zhang, Q.; Zhu, L.; Wang, J.; Xie, H.; Wang, J.; Wang, F.; Sun, F. Effects of fomesfen on soil enzyme activity, microbial population, and bacterial community composition. *Environ. Monit. Assess.* 2014, 186, 2801–2812. [CrossRef][PubMed]

15. Yang, Z.; Liu, S.; Zheng, D.; Feng, S. Effects of cadmium, zinc and lead on soil enzyme activities. *J. Environ. Sci.* 2006, 18, 1135–1141. [CrossRef]

16. Guo, H.; Chen, G.F.; Lu, Z.P.; Zhao, H.; Yang, H. Alteration of microbial properties and community structure in soils exposed to napropanoxide. *J. Environ. Sci.* 2009, 21, 494–502. [CrossRef]

17. Rasool, N.; Reshi, Z.A. Effect of the fungicide Mancozeb at different application rates on enzyme activities in a silt loam soil of the Kashmir Himalaya, India. *Trop. Ecol.* 2010, 51, 199–205.

18. Xiong, D.; Gao, Z.; Fu, B.; Sun, H.; Tian, S.; Xiao, Y.; Qin, Z. Effect of pyrimorph on soil enzymatic activities and respiration. *Eur. J. Soil Biol.* 2013, 56, 44–48. [CrossRef]

19. Rasool, N.; Reshi, Z.A.; Shah, M.A. Effect of butachlor (G) on soil enzyme activity. *Eur. J. Soil Biol.* 2014, 61, 94–100. [CrossRef]

20. Nguyen, D.B.; Roseb, M.T.; Rose, T.J.; Zwieten, L. Effect of glyphosate and a commercial formulation on soil functionality assessed by substrate induced respiration and enzyme activity. *Eur. J. Soil Biol.* 2018, 85, 64–72. [CrossRef]

21. Ali斯特, C.A.; Gomez, P.A.; Rojas, S.; Kogan, M. Pendimethalin and oxyfluorfen degradation under two irrigation conditions over four years’ application. *J. Environ. Sci. Health Part B* 2009, 44, 337–343. [CrossRef]

22. Filimon, M.N.; Voia, S.O.; Vladoiu, D.L.; Isvoran, A.; Ostafe, V. Temperature dependent effect of difenoconazole on enzymatic activity from the soil. *Zashchita Karantin Rast.* 2015, 51, 199–205.

23. Das, A.C.; Deb Nath, A.; Mukherjee, D. Effect of the herbicides oxadiazon and oxyfluorfen on phosphates solubilizing microorganisms and their persistence in rice fields. *Chemosphere* 2003, 53, 217–221. [CrossRef]

24. Pose-Juan, E.; Sanchez-Martin, M.J.; Andrades, M.S.; Rodriguez-Cruz, M.S.; Herrero-Hernandez, E. Pesticide residues in vineyard soils from Spain: Spatial and temporal distributions. *Sci. Total Environ.* 2015, 514, 351–358. [CrossRef]

25. Sokolova, T.V.; Gulidova, V.A. Change of the biological activity of soils under the effect of herbicides. *Zashchita Karantin Rast.* 2010, 8, 46–47.

26. Ghosh, R.K.; Jana, P.K.; Nongmaithem, D.; Pal, D.; Bera, S.; Mallick, S.; Barman, S.K.; Kole, R.K. Prospects of botanical herbicides in system of crop intensification in the Gangetic Inceptisols of India. In Proceedings of the 6th International Workshop on Software Clones, Hangzhou, China, 17–22 June 2012; pp. 116–117.

27. Mougi, A. The roles of amensalistic and commensalistic interactions in large ecological network stability. *Sci. Rep.* 2016, 6, 29929. [CrossRef]

28. Vladoiu, D.L.; Filimon, M.N.; Ostafe, V.; Isvoran, A. Effects of herbicides and fungicides on the soil chitinolytic activity. A molecular docking approach. *Ecol. Chem. Eng. S* 2015, 22, 439–450. [CrossRef]

29. Vladoiu, D.L.; Filimon, M.N.; Ostafe, V.; Isvoran, A. Computational analysis of difenoconazole interaction with soil chitinases. *J. Phys. Conf. Ser.* 2015, 574, 012012. [CrossRef]

30. Vladoiu, D.L.; Filimon, M.N.; Ostafe, V.; Isvoran, A. Assessment of pesticides interactions with *Bacillus pasteurii* urease. A computational study. *Rom. J. Phys.* 2015, 60, 583–592.

31. Isvoran, A. Computational study concerning the effect of some pesticides on the *Proteus mirabilis* catalase activity. *AIP Conf. Proc.* 2016, 1722, 130001. [CrossRef]

32. Filimon, M.N.; Roman, D.L.; Caraba, I.V.; Isvoran, A. Assessment of the effect of application of the herbicide S-metolachlor on the activity of some enzymes found in soil. *Agriculture* 2021, 11, 469. [CrossRef]
63. Gianfreda, L.; Rao, M.A. The influence of pesticides on soil enzymes. In Soil Enzymology Soil Biology; Shukla, G., Varma, A., Eds.; Springer: Berlin/Heidelberg, Germany, 2011; Volume 22, pp. 293–312.

64. Bhattarai, C.; Bhattarai, B.; Pandey, S. Variation of soil microbial population in different soil horizons. J. Microbiol. Exp. 2015, 2, 00044. [CrossRef]

65. Nannipieri, P.; Kandeler, E.; Ruggiero, P. Enzyme activities and microbially and biochemical processes in soil. In Enzymes in the Environment; Burns, R.G., Dick, R., Eds.; Marcel Dekker: New York, NY, USA, 2002; pp. 1–33.

66. Araújo, A.S.F.; Monteiro, R.T.R.; Abarkeli, R.B. Effect of glyphosate on the microbial activity of two Brazilian soils. Chemosphere 2003, 52, 799–804. [CrossRef]

67. Zabaloy, M.C.; Gómez, M.A. Microbial respiration in soils of the Argentine Pampas after metsulfuron-methyl, 2,4-D and glyphosate treatments. Commun. Soil Sci. Plant Anal. 2008, 39, 370–385. [CrossRef]

68. Muñoz-Leoz, B.; Ruiz-Romera, E.; Antigüedad, I.; Garbisu, C. Tebuconazole application decreases soil microbial biomass and activity. Soil Biol. Biochem. 2011, 43, 2176–2183. [CrossRef]

69. Das, S.K.; Varma, A. Role of enzymes in maintaining soil health. In Soil Enzymology, Soil Biology; Shukla, G., Varma, A., Eds.; Springer: Berlin/Heidelberg, Germany, 2011; Volume 22, pp. 237–239.

70. Lupwayi, N.Z.; Hanson, K.G.; Clayton, G.W.; Blackshaw, R.E.; O’Donovan, J.T.; Johnson, E.N.; Gan, Y.; Irvine, R.B.; Monreal, M.A. Soil microbial biomass, functional diversity and enzyme activity in glyphosate-resistant wheat–canola rotations under low-disturbance direct seeding and conventional tillage. Soil Biol. Biochem. 2007, 39, 1418–1427. [CrossRef]

71. Kucharski, J.; Wyszkawska, J. Biological properties of soil contaminated with the herbicide Apyros 75 WG. J. Elem. 2008, 13, 357–371.

72. Baruah, M.; Mishra, R.R. Effect of herbicides butachlor, 2,4-d and oxyfluorfen on enzyme activities and CO2 evolution in submerged paddy field soil. Plant. Soil 1986, 96, 287–291. [CrossRef]

73. Ramesh, A.; Joshi, O.P.; Billore, S.D. Effect of herbicides on soil dehydrogenase and urease activity in soybean (Glycine max). Indian J. Agric. Sci. 2000, 70, 218–219.

74. Muñoz-Leoz, B.; Garbisu, C.; Charcosset, J.Y.; Sánchez-Pérez, J.M.; Antigüedad, I.; Romera, E.R. Non-target effects of three formulated pesticides on microbially-mediated processes in a clay-loam soil. Sci. Total Environ. 2013, 449, 345–354. [CrossRef]

75. Bačmaga, M.; Borowik, A.; Kucharski, J.; Tomkiel, M.; Wyszkowska, J. Microbial and enzymatic activity of soil contaminated with a mixture of diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium. Environ. Sci. Pollut. Res. 2015, 22, 643–656. [CrossRef] [PubMed]

76. López, M.; Herrera-Cervera, J.A.; Lluch, C.; Tejera, N.A. Trehalose metabolism in root nodules of the model legume Lotus japonicus in response to salt stress. Physiol. Plant 2006, 128, 701–709. [CrossRef]

77. Nannipieri, P.; Sequi, P.; Fusi, P. Humus and enzyme activity. In Humic Substances in Terrestrial Ecosystems; Piccolo, A., Ed.; Elsevier: Amsterdam, The Netherlands, 2011; pp. 293–328.

78. Maddela, N.R.; Venkateswarlu, K. Impact of acephate and buprofezin on soil proteases. In Insecticides–Soil Microbiota Interactions; Springer: Berlin/Heidelberg, Germany, 2011; Volume 22, pp. 293–312.

79. Perucci, P.; Scarponi, L. Effects of the herbicide imazethapyr on soil microbial biomass and various soil enzyme activities. Biol. Fertil. Soils 1994, 17, 237–240. [CrossRef]

80. Wainwright, M.J. A review of the effect of pesticides on microbial activity in soils. Eur. J. Soil Sci. 2006, 29, 287–298. [CrossRef]

81. El Hussein, A.A.; Mohamed, A.T.; El Siddig, M.A.; Sherif, A.M.; Osman, A.G. Effects of oxyfluorfen herbicide on microorganisms in loam and silt loam soils. Res. J. Environ. Sci. 2012, 6, 134–145. [CrossRef]

82. Bera, S.; Ghosh, R.K. Microflora population and physico-chemical properties of soil of potato as influenced by oxyfluorfen 23.5% EC. Univ. J. Agric. Res. 2014, 2, 135–140. [CrossRef]

83. Liang, B.; Lu, P.; Li, H.; Li, R.; Li, S.; Huang, X. Biodegradation of fomesafen by strain Lysinibacillus sp. ZB-1 isolated from soil. Chemosphere 2009, 77, 1614–1619. [CrossRef] [PubMed]

84. Feng, Z.; Li, Q.; Zhang, J.; Zhang, J.; Huang, X.; Lu, P.; Li, S.-P. Microbial degradation of fomesafen by a newly isolated strain Pseudomonas zeshuii BY-1 and the biochemical degradation pathway. J. Agric. Food Chem. 2012, 60, 7104–7110. [CrossRef] [PubMed]