Biomonitoring of Soil Contaminated with Herbicides

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Abstract: The state of environmental pollution is of random character, and it depends on climatic conditions, landforms, development and industrialization. It is estimated that in the last decade as many pollutants have been released into the environment as in the previous 70 years, and the pollution rate still increases. Many scientific reports indicate that, in addition to metals, pesticides are the most commonly detected compounds in the environment. This situation is mainly due to the irrational use of these chemicals by humans. Mostly, soil environment changes caused by the influence of pesticides can be determined by various chemical analyses, which require the use of sophisticated and expensive equipment. However, biological methods, such as those using microbiological activity and an abundance of microorganisms, e.g., organisms responsible for the cycle of organic matter and nutrients, tend to be neglected. For this reason, the aim of the present study is not only to assess the validity of other research studies that were performed based on the available literature but to compile methods and compare them, which allows for an in depth understanding of the complexity of soil processes following herbicide application by conducting comprehensive soil biomonitoring.

Keywords: herbicides; soil; biotests; biomarkers; microorganisms

1. Introduction

The level of environmental pollution is related to climatic conditions, landforms, development and industrialization. The development of an industrial area and the consequent demand for higher standards of living have entailed the degradation and disintegration of natural environments worldwide. The consequences of excessive human interference in the environment may be irreversible and may constitute a serious threat to biological life, including human existence, in the future [1]. For several years now, the World Health Organization (WHO) [2] has reported that approximately 80% of all human diseases are conditioned by poor environmental conditions, contributing to diseases such as malignant tumors, impaired liver function, bronchial asthma and impairment of the immune system and reproduction (miscarriages, infertility and malformations) [3].

Soil is an integral part of natural environment that includes the interactions between organisms, air, water, nutrients and numerous other parts of the ecosystem. Due to its complex matrix, it is a storehouse of both the nutrients necessary for plants and microorganisms and toxic substances that interfere with biochemical processes [4]. Contaminants introduced into soils in the form of different types of xenobiotics, for instance, persistent organic pollutants (POPs) or heavy metals, can directly or indirectly affect all living organisms present in soil and water (Figure 1). Moreover, they also have a significant impact on human health [5,6]. According to the WHO, in the last 10 years, as many pollutants have entered the environment as in the previous 70 years, and their amount tends to be increasing [2].
Pesticides are among the most common toxic substances detected in soil besides heavy metals [7]. For plant, animal and human health safety, The US Environmental Protection Agency (EPA) and the European Food Safety Authority (EFSA) are responsible for overseeing substances entering the market and deciding when and for how long a pesticide may remain in use [6]. Among all the pesticides, herbicides are the dominant group of compounds and are widely used in various sectors of agriculture, i.e., crops, horticulture, floristry and forestry production. The prevalence of herbicides is related to their ability to destroy or reduce the growth and development of weeds that threaten crop productivity and safety [7,8]. The widespread use of herbicides is of increasing concern to researchers because of the risks they can cause, either through direct or indirect effects on changes that occur in various ecosystems [9]. Calderon et al. [10] reported that, apart from the undoubted benefits of herbicide application, their use carried many environmental risks. Whereas Granados-Galván et al. [5] observed that an improperly applied herbicide dosage or the incorrect disposal of an unused substance can contribute to the formation of their residues in soil and their bioaccumulation, which, as a result, negatively influences the environment. Furthermore, herbicides can affect the population of a community differently, depending on the ecosystem [6]. According to Liess et al. [11], the same concentration of an herbicide in a forest ecosystem will have a more toxic effect on the population living there compared to agricultural lands that are continuously treated with different active pesticide ingredients.

For many years, research conducted in the field of environmental ecotoxicology has focused on the toxic effects caused by synthetic or natural substances on biota. Such studies allow for learning about the levels and types of pollution affecting a single individual or an entire population of organisms occurring in a given environment [12]. According to Vischetti et al. [13], pesticide ecotoxicology is a new area of toxicology that focuses on the
adverse effects of pesticides towards non-target organisms, including a variety of species inhabiting the ecosystems. There are many reports on the toxic effect of a single compound on different groups of organisms, which enable determining the influence of a substance on ecosystem changes [4,14,15]. However, the correct selection of the test organism also seems to be a key issue due to the fact that the same herbicide often yields different toxicity analysis results when investigated on different models organisms. Therefore, the obtained results should be confirmed by tests performed on another model organism, as was suggested by Brink et al. [16].

Unfortunately, in the real conditions of the soil environment, there is never just one active herbicide compound. In fact, mixtures of different active substances of herbicides in soil are present, and their observed effects are as follows: additivity, antagonism or synergism [17,18]. Therefore, the negative changes in soil caused by the influence of herbicide should be determined by various complex chemical analyses that require new methods to be developed and validated and often use sophisticated and expensive equipment [19].

In turn, insufficient attention is paid to biological indicators, e.g., those focusing mainly on microbiological activity and the abundance of microorganisms, including the ones responsible for the cycle of organic matter and nutrients [14].

Biological methods are defined as processes in which living organisms are used. One of the elements of such methods, along with bioremediation and phytoremediation, is biomonitoring. According to Wieczerzak et al. [20], biomonitoring makes it possible to assess the condition of an environment (such as air, water and soil) using biological indicators (bioindicators and biotests) represented by living organisms, i.e., plants, lichens, algae, fungi microorganisms, invertebrates and vertebrates. It can be conducted in two ways: as a qualitative analysis by observing the changes occurring in organisms or as a quantitative analysis by measuring the accumulation of a given substance in the tissues of an organism.

According to the available data, soil ecotoxicological characterization by determining chemical parameters has been intensively studied in the literature, and some methodological guidelines have already been established and published by the Organisation for Economic Co-operation and Development (OECD) [21] and the International Organization for Standardization (ISO) [22]. However, many scientists indicate that the analysis of chemical properties is insufficient and needs to be complemented by biomonitoring and the use of biological techniques, including the molecular ones. A chemical analysis can only partially determine the toxicological effect of tested contaminants by quantifying the level of contamination in the tested material (soil, plant or water). It does not allow, however, for determining the impact of the level of a pollutant on the processes occurring in a living organism, including those at the cell level, and only this provides a complete view of the threat associated with the presence of a given contaminant in the environment [13,22].

For this reason, the aim of the present study is to summarize the biomonitoring of soil using selected biological indicators, allowing for the understanding of the complexity of soil processes following herbicide application. Due to the large amount of available literature on the chemical and physico-chemical methods of analyzing the toxicity of herbicides in soil, this paper presents biotests, bioindicators and biomarkers as integral elements of biomonitoring. In addition, it provides examples of the use of molecular techniques as a tool for herbicide toxicity assessment. This approach allows for the development of the broadest possible view on ecotoxicity of herbicides in soil, which will facilitate conducting comprehensive soil biomonitoring. On the basis of inclusion and exclusion, all study data were analyzed for monitoring the herbicides extracted from PubMed, HRAC, EFSA and OECD. The following key words were used in order to collect the research material: “Herbicides in soil”, “Methods in biological control”, “Biomonitoring of soil”, “Soil microorganisms”, “Biotests and bioindicators soil”, “Biomarkers to assess environment”, “Molecular techniques”, etc. In this manuscript, 90 publications from all available literature data were used.
2. Herbicide Classifications

Herbicides are substances that easily penetrate plants through roots and/or leaves. It is also possible for herbicides to enter through shoots, and they have even been observed to be taken up by swelling seeds. The mechanism of action of herbicides is to interfere with biophysical or biochemical reactions in the plant related to metabolism and various life processes in the plant cells [23].

The classification system for herbicides was established by the Global Herbicide Resistance Action Committee (HRAC) group, and it has proven to be the most comprehensive herbicide classification system in the world. It is based on the targeted inhibited protein (site of action) and similarity of induced symptoms, such as the inhibition of microtubule assembly [24]. Table 1 presents the division of herbicide active substances based on their mechanisms of action and their impact on the environment. According to the adopted classification, group B is the most numerous. It includes compounds that have properties of acetolactate synthase (ALS) enzyme inhibitors. In turn, the commonly used and detected in soil glyphosate is included in the EPSP synthase enzyme inhibitors, i.e., group G [17]. In addition, herbicides can be used in two ways, i.e., foliar or soil application.

According to the OECD [25], when herbicides are applied to plants, they can interfere with the enzymes that catalyze metabolic reactions in the plant, causing deformation through a change in morphology and, ultimately, death. Plants can develop various mechanisms to counteract the effects of the substances by producing an enzyme that inactivates the active herbicide substance or by producing altered target enzymes that are affected by the herbicide. Moreover, plants have the ability to generate barriers that prevent herbicide uptake by plant tissues [25]. However, regarding plants, there are still a few studies determining their condition after herbicide application based on the analysis of physiological parameters, especially changes in structures at the cell and tissue levels [4].

Table 1. Classification of selected herbicides based on active substances, chemical family, mechanism of action and environmental impact [24,26].

<table>
<thead>
<tr>
<th>HRAC Numerical Code</th>
<th>Mechanism of Action</th>
<th>Active Substances</th>
<th>Chemical Family</th>
<th>Environmental Fate [26]</th>
<th>Ecotoxicity</th>
<th>Persistence of Herbicides in Soil (DT50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>Inhibition of acetyl-CoA carboxylase (ACCase) (Graminicides prevent the production of essential fatty acids in weeds from the grass family.)</td>
<td>Butroxydim, quizalofop-ethyl, quizalofop-P-teturyl, cycloxydim, fenthionoprop, clothodim, pinoxaden, propraziquafop, tepraloxynim, tralkoxydim</td>
<td>Aryloxyphenoxy-propiocanes, cyclohexane-diones, phenylpyrazoline</td>
<td>Drainflow: Slightly or moderately mobile; Potential for particle-bound transport: Medium</td>
<td>Moderate alert for birds, daphnia, earthworms and fish [26]</td>
<td>from 0.2 to 45 days</td>
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<td>Group 2</td>
<td>Inhibition of acetolactate synthase (ALS), also named acetohydroxyacid synthase (AHAS) (prevent the production of some amino acids necessary for protein synthesis)</td>
<td>amidosulfuron, chlorosulfuron, ethamsulfuron-methyl, flazasulfuron, florasan, foramsulfuron, imazamox, isodosulfuron-methyl-sodium, mesotulam, penoxsulam, mesosulfuron-methyl, nicosulfuron, pyroxsulam, propoxycarbazulfuron methyl, flucarbazone-Na, thifensulfuron-methyl, thien-carbazone-methyl, tritosulfuron</td>
<td>Sulfonylurea, imidazolinones, triazolopyrimidine, pyrimidinyl benzoates, sulfonanilides, triazolinones</td>
<td>High leachability and mobile</td>
<td>Moderate alert for birds, earthworms and fish [26]</td>
<td>from 1.85 to 200.2 days</td>
</tr>
<tr>
<td>HRAC Numerical Code</td>
<td>Mechanism of Action</td>
<td>Active Substances</td>
<td>Chemical Family</td>
<td>Environmental Fate [28]</td>
<td>Ecotoxicity</td>
<td>Persistence of Herbicides in Soil (DT₅₀) *</td>
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<td>Group 3</td>
<td>Inhibition of microtubule assembly</td>
<td>pendimethalin, benfluralin, butralin, ethalfluralin, pendimethalin, DCPA, trifluralin, propyzamide, diniramine, oryzalin, isopropalin, butamifos, thiazopyr</td>
<td>Dinitroaniline, phosphoromiates, pyridines, benzamides, benzoic acid</td>
<td>Potential for particle-bound transport: High Drainflow: Slightly mobile and moderately persistent; Potential for particle-bound transport: Medium</td>
<td>High alert for fish, chronic ecotoxicity [16,26]; Moderate alert for birds, earthworms, daphnia and fish [16,26]; from 22 to 182.3 days</td>
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<td>Group 4</td>
<td>Synthetic auxins (action like indole acetic acid)</td>
<td>2,4-D, clomeprop, aminopyralid, quinclorac, quinmerac, clopyralid, chlorfenprop, dichlorprop-P, dicamba, fluoroxypr, MCPA, MCPB, mecoprop, picloram, tri chlorpyr</td>
<td>Phenoxy-carboxylates, pyridine-carboxylates, phenyl carboxylates, quinolone-carboxylates, benzoates</td>
<td>High leachability; Drainflow: Mobile</td>
<td>Moderate alert for birds, earthworms, daphnia and fish [26,27]; from 4.4 to 450 days</td>
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<td>Group 5</td>
<td>Inhibition of photosynthesis at PS II (prevent the production of sugar and energy through photosynthesis)</td>
<td>chloridazon, bromacil, brompyrazon, atrazine, desmedipham, khorl, metamitron, metribuzin, trietazine</td>
<td>Triazine, triazines, uracil, pyridazinones, phenylcarbamate</td>
<td>Drainflow: Mobile or moderately mobile; Potential for particle-bound transport: High or medium</td>
<td>Moderate alert for birds, daphnia, earthworms, bees and fish [16,26]; from 31 to 75 days</td>
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<tr>
<td>Group 6</td>
<td>Inhibition of phytoene desaturase (PDS)</td>
<td>bentazon, bromofenoxim, bromoxynil-octanoate, pyridate</td>
<td>Nitriles, benzothiadiazinones, phenylpyridazine</td>
<td>Drainflow: Mobile or non-mobile; Potential for particle-bound transport: Low or medium</td>
<td>Moderate alert for birds and fish; High alert: High fish and daphnia, chronic ecotoxicity [26]; from 2.2 to 53 days</td>
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<tr>
<td>Group 9</td>
<td>Inhibition of 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS) (prevent the biosynthesis of some amino acids)</td>
<td>glyphosate</td>
<td>Glycine</td>
<td>Drainflow: Slightly mobile; Potential for particle-bound transport: Medium</td>
<td>Moderate alert for birds, earthworms, bees, daphnia and fish [26]; from 1.5 to 53.5 days [26]</td>
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<tr>
<td>Group 10</td>
<td>Inhibition of glutamine synthetase</td>
<td>glufosinate-ammonium, bialaphos/bilanalos</td>
<td>Phosphinic acids</td>
<td>No records</td>
<td>Low alert for bees, acute contact and oral ecotoxicity [26]; −7.4 days</td>
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<tr>
<td>Group 12</td>
<td>Inhibition of phytoene desaturase (PDS)</td>
<td>beflubutamide, fluorochloridone, fluridone, diflufenican, picolinat</td>
<td>N-Phenyl heterocycles, diphenyl heterocycles, phenyl ethers</td>
<td>Slightly mobile and moderately persistent; Potential for particle-bound transport: Medium and High</td>
<td>Moderate alert for daphnia and earthworms Moderate and high alert for fish [26]; from 6.1 to 94.5 days</td>
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<tr>
<td>Group 13</td>
<td>Inhibition of 1-deoxy-D-xyllose-5-phosphate synthase (DOXP synthase)</td>
<td>clomazone, bixloxone</td>
<td>Isoxazolidinone</td>
<td>Drainflow: Moderately mobile</td>
<td>Moderate alert for birds, earthworms, bees, daphnia and fish [12]; from 22.6 to 153 days</td>
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<tr>
<td>HRAC Numerical Code</td>
<td>Mechanism of Action</td>
<td>Active Substances</td>
<td>Chemical Family</td>
<td>Environmental Fate [26]</td>
<td>Ecotoxicity</td>
<td>Persistence of Herbicides in Soil (DT50) *</td>
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<td>Group 14</td>
<td>Inhibition of protoporphyrinogen (PPO)</td>
<td>Acifluorfen oxyfluorfen, nitrofen, flumipropyn bifenox, tiafenacil, oxadiazon carfentrazine-ethyl, oxadiargyl, pyraflufen-ethyl</td>
<td>Diphenyl ether, N-phenyl-oxadiazolones, N-phenyl-imides, N-phenyl-triazolines, phenylpyrazoles</td>
<td>Drainflow: Slightly mobile and moderately persistent; Potential for particle-bound transport: Medium</td>
<td>High alert: High fish and daphnia, chronic ecotoxicity [26]; Moderate alert for birds, daphnia, earthworms and fish [26]</td>
<td>from 0.32 to 502 days</td>
</tr>
<tr>
<td>Group 15</td>
<td>Inhibition of very-long-chain fatty acid synthesis (VLCFAs), inhibition of cell division</td>
<td>acetochlor, dimetachlor, dimethenamid-P, benfuresate, flufenacet, metazachlor, anilofos, metolachlor-S, butylate, indanoan, napropamide, pethoxamid, cafenstrole</td>
<td>Azolyl-carboxamides, benzofurans, isoxazolines, oxiranes, thiocarbamates, α-chloroacetamides, α-oxyacetamides, α-thioacetamides</td>
<td>Drainflow: Mobile or moderately mobile and moderately persistent; Potential for particle-bound transport: High</td>
<td>High alert for birds, chronic ecotoxicity; Moderate alert for birds, earthworms, daphnia and fish [26].</td>
<td>from 2.0 to 90 days</td>
</tr>
<tr>
<td>Group 18</td>
<td>Inhibition of dihydropteroate (DHP) synthase</td>
<td>asulam</td>
<td>Carbamate</td>
<td>Drainflow: Mobile</td>
<td>Moderate alert for birds, earthworms, daphnia and fish [26].</td>
<td>−3.2 days</td>
</tr>
<tr>
<td>Group 22</td>
<td>Photosystem I-electron diversion (prevent the production of sugar and energy through photosynthesis; they destroy cell membranes)</td>
<td>diquat, paraquat, morfamquat, cyperquat</td>
<td>Bipyridylium</td>
<td>Very persistent; Potential for particle-bound transport: High</td>
<td>High alert; acute and chronic ecotoxicity for birds [16,26]</td>
<td>from 0 to 3000 days</td>
</tr>
<tr>
<td>Group 23</td>
<td>Inhibition of mitosis/microtubule organization</td>
<td>chlorpropham, propham, carbetamide, barban</td>
<td>Carbamate</td>
<td>Drainflow: Moderately mobile</td>
<td>Moderate alert for birds, earthworms, daphnia and fish [26].</td>
<td>from 5.0 to 13.1 days</td>
</tr>
<tr>
<td>Group 27</td>
<td>Inhibition of 4-hydroxyphenyl-pyruvate-dioxygenase (4-HPPD)</td>
<td>isoxaflutole, topramezone, pyrasulfotole sulcotrione, tembotrione, mesotrione, tefuryltrione</td>
<td>Triketones, isoxazoles, pyrazoles</td>
<td>Drainflow: Mobile and moderately mobile</td>
<td>High alert for fish, chronic ecotoxicity; Moderate alert for birds, earthworms, bees, daphnia and fish [26].</td>
<td>from 0.9 to 218 days</td>
</tr>
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<td>Group 29</td>
<td>Inhibition of cell wall (cellulose) synthesis</td>
<td>dichlobenil, isoxaben, hydroxadiamyl, flupoxam, indaziflam, triaziflam</td>
<td>Nitriles, benzamides, triazolocarboxamides, alkylazines</td>
<td>Drainflow: Moderately mobile; Potential for particle-bound transport: Medium</td>
<td>Moderate alert for birds, earthworms, daphnia and fish [26].</td>
<td>from 25 to 150 days</td>
</tr>
<tr>
<td>Group 34</td>
<td>Inhibition of lycopene cyclase</td>
<td>amitrole</td>
<td>Triazole</td>
<td>Drainflow: Moderately mobile</td>
<td>Moderate alert for birds, earthworms, daphnia and fish [26].</td>
<td>−7.4 days</td>
</tr>
<tr>
<td>Group Ø</td>
<td>The mode of action of herbicides in group Z is unknown.</td>
<td>quinoclamine, bensulide, diphenamid, perfluordione, flamprop-m</td>
<td>Acetamides, phosphorodithioate, inorganic compound, trifluoromethane sulfonamides, arylationampropionic acid</td>
<td>Drainflow: Slightly mobile or very mobile</td>
<td>High alert for bees, daphnia and fish [26]</td>
<td>from 22 to 1000 days</td>
</tr>
</tbody>
</table>

* DT50—half-life.
Herbicides differ in the period of their action and persistence in soil. The half-life in soil, according to the PPDB (Pesticide Properties Database), can vary from 2 days for tralkoxydim to 3000 days for paraquat. On this basis, herbicides can be divided into three groups: persistent (75–100% degradable within 2–3 years), moderately persistent (1–18 months degradable) and non-persistent (up to 12 weeks degradable) [26]. Meena et al. [29] observed that shortly after the application to soil, active herbicide substances are physically and biochemically transformed into various secondary metabolites that can adversely affect the microbial community and may increase phytotoxic effects in plants. As it was also observed by Ahanger et al. [23], the persistence of herbicides and their diversification is difficult to control, and the resulting environmental transformation or migration can pose a major risk. According to Wolejko et al. [4], the changes in the abundance of microorganisms after herbicide application consist of changes in the enzymatic activity of microorganisms, their cellular membranes and cell wall composition. In turn, in plants, pesticides caused changes in physiological mechanisms involving the activation of enzymes responsible for herbicide detoxification and enzymes associated with the alleviation of oxidative stress [29].

3. Herbicide Fate in Soil and Biological Tools Used in Their Biomonitoring

Soil quality can be defined as the specific capacity of soil to function as a life-giving system in natural or managed ecosystems, maintaining plant and animal health and productivity as well as environmental air and water quality and human health [30]. The quality of soil depends on its fertility and yield which, in turn, are related to its physico-chemical properties and biological activity [31].

Herbicides in soil can penetrate other links in the food chain, including plants, animals and humans (Figures 2 and 3). In the environment, herbicides are able to affect living organisms through interactions between the contaminant and the organism, which can lead to an inhibition of growth and physiological functions, changes on the cellular level, mainly in DNA and proteins, and eventually death [32]. The changes that occur after contact with the herbicide are related to the uptake of the contaminant. According to Wolejko et al. [4], the metabolism and degradation of herbicides by microorganisms depend on their ability to use contaminants as a source of carbon and bacterial adaptation to inhabit a contaminated environment. In contrast, the behavior of herbicides in soil may depend on various factors, such as climatic factors, soil physico-chemical properties and characteristics of herbicides influencing persistence in the environment (solubility, degradation time, reactivity, form and dose application) (Figure 3). Living organisms, both animals and human, may not be able to transform the compound or may produce toxic end-products as a result of metabolizing this substance. Obviously, the process depends on the organism species and the bioavailability of the compound [33].

There are still relatively few reports that describe the adverse effects of herbicides on soil environments and the organisms found there. Some groups of herbicides are generally known to be characterized by high toxicity, while others that are considered to be of low toxicity produce metabolites exhibiting acute toxicity and lethal properties through their decomposition [34]. Carles et al. [33] and Golombieski et al. [35] observed that the toxicity of the metabolites of selected herbicides (such as glyphosate, metazachlor or diclofop) is significantly higher than that of the parent compound. However, the majority of these substances are present in trace amounts. Thus, they do not increase the mortality rate of the organisms present. Yet, this does not mean that they are not toxic. The accumulation of these substances in soil may lead to the formation of sub-chronic, chronic or sub-acute toxicity [5,35].
In the past, the effect of herbicides on soil biological properties has mainly been studied by determining soil microbial biomass and/or respiratory and enzymatic activity. However, these are relative indicators, and, in some cases, they may give a false picture of the ecotoxic effects of herbicides. This is because in some cases the application of herbicides as a carbon
source may stimulate the activity of soil microorganisms. Therefore, it was concluded that a new approach and, at the same time, a biomarker may facilitate the characterization of the structure of the population of microorganisms, including the determination of the abundance of functional genes based on the extraction of nucleic acids (DNA/RNA) [13].

3.1. Biotests and Bioindicators Used in Soil Monitoring after Herbicide Application

The biomonitoring of soil environments is a complex and difficult issue that has been neglected in research studies so far. Little scholarly attention has been paid to the various physico-chemical and biological phenomena occurring in soil [30]. According to Wieczerzak et al. [20], the bioavailability of xenobiotics in soil depends on its pH, organic matter content, humidity, granulometric composition and microbial activity. What seems to be dangerous is the occurrence of mixtures of contaminants in soil and their relationships with each other, consisting of synergism and antagonism. One of the measures of the degree of soil contamination and ecosystem disturbance is the carbon cycle data. This is related to the processes of accumulation and decomposition of organic matter in soil contaminated with pesticides or metals. It appears to be a more sensitive and reliable indicator than microbial population change [31,36].

According to Ros et al. [37], some physico-chemical parameters used in soil quality assessment change very slowly, and the effect of treatments or the influence of pollution is observed only in long-term studies. In contrast, soil biological and biochemical properties are extremely sensitive, and even the smallest changes in the soil environment influence their formation. For this reason, biological and biochemical indicators are most often proposed for assessing soil fertility and quality [31,38]. According to Siwik-Ziomek et al. [39], soil biological activity and quality tend to be expressed by parameters such as the microbial biomass or soil respiration, metabolic quotient, microbial population structure, number and composition of functional groups of microorganisms in soil, and enzymatic activity [40].

According to Tatúsko-Krygier and Jakubus [41], to act as a biotest, an organism must meet a number of conditions, such as: (1) it must be characterized by relatively easy breeding under laboratory conditions, allowing for the acquisition of large numbers of individuals; (2) the genetic structures of the organism and its sensitivity to toxic substances should be well-understood; (3) the organisms should be characterized by a high ease of isolation from the environment; (4) the selected susceptible organism should represent the species or cluster to which it belongs and, in addition, it must be a native species that is characteristic of the habitat under study; (5) the test organisms should respond similarly to a dose or concentration of the toxin in different locations and at a given level of exposure to contaminants [42]. Furthermore, according to Brink et al. [16], the test organism used as a bioassay should show a similar sensitivity under field conditions to that observed under laboratory conditions. The scholars noted that the same bioassay cannot be applied to groups of herbicides when assessing their ecotoxicity. On the basis of their own studies, they found that green algae (Selenastrum capricornutum) were more sensitive to metamitron and diuron, duckweed (Lemma minor) was more sensitive to diquat and linuron, while algae (Chlorella vulgaris) were more sensitive to metribuzin. In turn, L. minor was not very sensitive to metamitron [16].

A soil biomonitoring analysis can be conducted by using different biotests (Table 2). According to Sikorski and Adomas [43], three bioassays are commonly employed in order to determine environmental contamination. The first two are intended for laboratory conditions, while the third is performed on populations of organisms living in natural conditions. In the case of laboratory testing, the analysis consists of the incorporation of the contaminant into a medium (e.g., soil) on which toxicity testing is performed on an appropriate indicator organism. In addition, tests can also be performed with appropriate samples collected from contaminated areas, e.g., the polluted ones [36]. The main purpose of such a biotest is a calibration procedure, which will then be used for the estimation of the toxicity of the specific samples [44]. On the other hand, the third group
of in situ biotests is conducted at the site of populations of sensitive organisms in their natural environment [45].

In a field and laboratory study on the biomonitoring of soil after herbicide application conducted by Iwai and Noller [46], the effects of contamination were evaluated by means of biological indicators and parallel ecotoxicological tests in which the woodlouse (Porcellio laevis) and springtail (or collembolan, Cyphoderus sp.) were used. Analyzing the biological indicators, they observed that the applied herbicide atrazine had a significant influence on the density of soil organisms, reducing it by 60–75% compared to the control plots. Furthermore, they noted that the applied atrazine had no significant effect on plant cover diversity [46]. As noted by Łozowicka et al. [6], this comprehensive approach intended for monitoring allows for more accurate tracking and observing changes in, among others, biodiversity and abundance critical to the distribution of a toxicant that occurs in soil, water and air environments and in food.

Table 2. Biomonitoring methods used to assess the quality of soil contaminated with herbicides.

<table>
<thead>
<tr>
<th>Method</th>
<th>Assays/indicators</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biological activity measurement</td>
<td>Soil enzyme activity determination (dehydrogenases, catalase, β-glucosidase, etc.)</td>
<td>effective tool to characterize the microbial status of soil, easy and fast tests</td>
<td>Sensitive to environmental changes</td>
<td>[4]</td>
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<td></td>
<td>Soil respiration analysis</td>
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<td></td>
<td>Soil microbial biomass/number analysis</td>
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<tr>
<td>Biological diversity, community structure and functional gene abundance determination</td>
<td>Fingerprinting methods (DGGE, T-RFLP, etc.)</td>
<td>Easy and fast methods (fingerprint methods), precise methods, independent culture method, highly sensitive</td>
<td>PCR-based methods require DNA sample with high quality and purity, PCR reaction optimization; specialist equipment required (HTS), requires the use of bioinformatics and statistical tools for data analysis</td>
<td>[13,47]</td>
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<tr>
<td></td>
<td>Real-time PCR</td>
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<td></td>
<td>High-throughput sequencing (NGS, TGS)</td>
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<tr>
<td>Cell viability</td>
<td>MTT assay</td>
<td>A large range of applications (bacteria, yeast, animal/human cells), rapid and simple test procedure; highly sensitive (ATP assay)</td>
<td>low sensitivity, possible chemical interferences (MTT assay); specialist equipment required (ATP assay)</td>
<td>[20,48]</td>
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<td></td>
<td>ATP bioluminescence assay</td>
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<tr>
<td>Genotoxicity/mutagenicity analysis</td>
<td>Comet assay</td>
<td>Fast, easy, requiring a small amount of test substance, small amount of sample may be used, high sensitivity, can be applied in different samples (plant, animal/human)</td>
<td>may be too sensitive, reaching saturation at a relatively low level of damage, requires careful calibration, specialist equipment required</td>
<td>[13,20]</td>
</tr>
<tr>
<td>Oxidative stress parameter measurement</td>
<td>ROS analysis</td>
<td>Applied in many different biological models (plant, bacteria, yeast, animal/human cells), easy to conduct, inexpensive, simple, replicable</td>
<td>low sensitivity; the need to maintain a low temperature at each stage of the analysis, high consumption of reagents, limitations related to spectroscopic methods</td>
<td>[49]</td>
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<td></td>
<td>Activity of enzymes (catalases, superoxide dismutase, glutathione peroxidase)</td>
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<td></td>
<td>Non-enzymatic oxidant production (glutathione)</td>
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</tbody>
</table>

Furthermore, to assess the contamination of a soil environment, organisms such as Eisenia fetida (compost pink earthworm), Helix aspersa aspersa (European brown snail) and Folsomia candida (jumpingtail) are used [43,50].

Another approach in the biomonitoring area is to test soil by enriching it with bacteria that are not present in the habitat but could be introduced to support the native population. However, this is a rather difficult task, and there is still a need for many studies to be
conducted over a longer period of time, not only in the laboratory but also in the natural environment, to obtain reproducible results.

3.2. Bacteria as a Bioindicators of Soil Contaminated with Herbicides

Scientific progress in molecular biology has contributed to the development of new techniques that can accurately determine the type of DNA damage caused by a particular active herbicide ingredient. The use of different types/genus/species of bacteria in tests is an appropriate solution for studying mutagenic and genotoxic properties [51]. This is due to their simple structure and free DNA in the cytoplasm, which is easily accessible after entering the cell [42].

Through genetic engineering, strains in which a mutation has been induced in specific genes can be used in tests. When it comes to bacterial tests, they are classified into three groups, which allows for the detection of retroviral mutations, progressive mutations and DNA damage repair [52]. Regarding environmental contaminants, in particular pesticides, tests based on the observation of retroviral or primary mutations are most commonly used, for instance, *Salmonella typhimurium* TA98 (for detecting contaminants causing phase change mutations) and *S. typhimurium* TA100 (for detecting contaminants causing base-pair substitution mutations) [53]. Recently, *Escherichia coli* has also been increasingly included in such tests [54]. The strains used in the tests introduced many mutations affecting their impaired biochemical functions, such as the ability to synthesize one of twenty protein amino acids. Due to the presence of many various DNA defects caused by contamination, each of the strains used in such tests had a different mutation in the same gene, which was responsible for the ability to synthesize different amino acids [55]. In addition, the bacterial strains were sensitized by introducing DNA repair-interfering plasmids into their structures [51].

In addition, the marine bacterium *Alivibrio fischeri*, which exhibits natural bioluminescence, is also used in the bio-testing of pesticide environmental contamination. The toxicity level of a compound is assessed by measuring bioluminescence, which is performed before and after the incubation of the bacterial suspension with the test compound [56]. The bioluminescence intensity of the bacteria is closely related to the population density and metabolic state. Luminescent bacteria emit light when they are in their optimal environment, whereas in the presence of toxic compounds, due to the disruption of physiological processes, the intensity of luminescence decreases. Due to its rapidity and high sensitivity, this assay is well-suited for the analysis of complex environmental matrices [57]. In addition, there are other biotests that present different sensitivities to environmental contaminants so that the effects of contaminants can be observed at different levels of cellular organization, which include Charatox (*Nitellopsis obtusa*), Algaltokit F (*Selenastrum capricornutum*), etc. [42].

Interestingly, more and more frequently, other types of bacteria can be used in bioassays, i.e., *Pseudomonas putida*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Enterococcus faecalis*, *Lactobacillus lactis*, *Lactobacillus fermentum*, *Lactobacillus plantarum* or *Candida albicans*. A study on such bacteria conducted by Jabłońska-Trypuć et al. [58] showed that after the application of dichlobenil and bifenox, cytotoxic effects, especially for *E. coli* and *C. albicans*, were observed. On the other hand, for *P. aeruginosa*, a significant increase in cell viability was noticed, regardless of the applied dose. A similar study was also conducted by Harishankar et al. [59] who reported on the application of herbicide on five models of bacteria, such as *L. lactis*, *L. fermentum*, *L. plantarum*, *E. coli* and *E. faecalis*. The pesticide had significant participation in the metabolism and ability of these strains to utilize 3,5,6-trichloro-2-pyridinol as a carbon and energy source for their growth and development. A study by Medo et al. [60] indicates that dimethachlor and linuron application contributed to changes in soil microbial community and activity. They noted that microbial community *Nitrospirae* and *Proteobacteria* decreased after soil had been treated with linuron, while *Saccharibacteria* and *Gemmatimonadetes* increased. On the other hand,
Since the amount of pesticides, especially herbicides, present in environmental samples does not fully inform about the effects that occur in living organisms, additional biomarkers should be used. They are defined as sub-individual, biochemical, molecular and physiological reactions that provide a valuable tool for understanding the exposure magnitude from all pathways and sources. Biomarkers are primarily implemented in the study of health risks associated with occupational or environmental exposure of the human population to harmful agents or carcinogens [61]. In this context, the biomonitoring of humans, which is carefully developed, uses biomarkers that include either the parent compounds or their metabolites, which are identified in a variety of human specimens, such as urine, hair, blood or/and deciduous teeth [62]. Obviously, when selecting a suitable biomarker, some important aspects need to be taken into account, and on the basis of their analysis, the choice should be made. Figure 4 shows a schematic overview of biomarker selection [61,63].

Figure 4. Factors for selecting the most appropriate biological markers according to Lavezzari and Womack [63].

The situation is different if biomarkers are being used as an early warning for organisms occurring in the natural environment, for example, in soil, and exposed to herbicides or other contaminants. Then, the question arises of which of the available biomarkers is the most adequate for the determination of the toxic effect of active herbicide ingredients. For animals, depending on the measured parameter, biomarkers of exposure, effect and...
sensitivity can be distinguished. Exposure biomarkers provide information on the relationship between the external dose and the absorbed amount of a toxic substance. These assays measure the concentration of a compound in tissues, body fluids, their metabolites or reaction products. Biomarkers of effects can be simple or complex. The former include body weight monitoring and population changes, while the latter determine specific isoenzymes by immunochemical techniques [64].

All biomarkers can be further divided into non-invasive, invasive, and those that indicate pathological damage and detect biochemical changes or responses. The sensitivity of biomarkers, on the other hand, allows for identifying in a population those organisms that have acquired or genetically determined a different sensitivity and establish an individual response, i.e., the activity of enzymes involved in the biotransformation of chemicals related to the polymorphism of the genes encoding them [64,65]. While discussing the biological markers used in the assessment of the risk of ecosystems after pesticide application, it is noteworthy that a decrease in eggshell thickness is an easy marker to measure, because eggshell abrasion of various degrees occurs when DDT, DDE or Dicofol are present in environment. Acetylcholinesterase organophosphate inhibitors are also biomarkers that are present in organisms after the application of carbamates, whose detection is easier and more reliable than a chemical analysis [42].

In the last years, there has been a growth in the interest in the use of biological markers in field surveys of contaminated environments. It arises from the fact that this methodological tool can be useful for ecosystem and habitat protection [4]. It must be recognized that among the biomarkers present each has strengths as well as weaknesses influencing the final result [66]. As noted by Traven et al. [67], not all environmental biomarkers can provide a good early warning of the occurrence of pollution in an environment. This is dictated by the fact that biomarkers can be perturbed by two groups of factors: first, biological (genetic population, age, species, sex, reproductive phase and feeding status) and second, environmental (pH, temperature, salinity and oxygen concentration) [66]. Among the early-warning biomarkers that give the most reliable results, special attention should be paid to those related to reproduction and responding to endocrine disruptors, e.g., vitellogenin in fish, congenital malformations in birds, aromatase, spiggin in three-spined stickleback, intersex in fish and gastropods and skeletal deformities as well as hatching success and brood size for birds. Weichert et al. [68] suggested that while monitoring environment researchers should use several markers with different properties, which allows for a more accurate assessment of environmental contamination and risk for populations.

Plant biomarkers are designed to provide an early warning, and they are applicable to a large number of environmental contaminants; they may act on only one contaminant or a group of different substances [69]. The application of herbicides changes the biological and biochemical properties of the soil environment and, thus, may also affect plants. For example, phenoxy herbicides, to which MCPA belongs, can lead to damage to chloroplasts, membranes and the integrity of the vascular system, premature aging of plants and, as a consequence, their death. It should be remembered that, in addition to the active substance, herbicides include auxiliary substances, emulsifiers and stabilizers that may also affect the soil environment. The literature data indicate that one of the parameters that is sensitive to unfavorable environmental properties is plant enzymes that change their activity simultaneously with the occurrence of a stress factor [54,55]. The most commonly used plant biomarkers include fluorescence of chlorophyll and the accumulation of shikimate, ammonium and 2-aminobutyric acid in plants after the application of herbicides [70]. According to Petersen et al. [69], in response to the application of glyphosate, in plants the accumulation of shikimate increases because glyphosate may inhibit the shikimate pathway. Moreover, Li and Wang [8] indicate that 2-aminobutyric acid is one of the dependable plant biomarkers because it responds in a short time to sublethal levels of herbicide, giving reliable results. In particular, this biomarker may be applicable to a group of herbicides responsible for inhibition of ALS (e.g., triazolopyrimidine sulfonanilides, imidazolines, pyrimidylxy salicylic and sulfonylureas).
4. Molecular Techniques as a Tool for Herbicide Toxicity Assessment

There are many reports describing the utility of various molecular methods for detecting adverse effects of herbicide application on soil microorganisms and non-target organisms, e.g., plants. These are culture-independent approaches involving DNA extraction and sequencing. They are primarily used to characterize microbial populations and assess DNA damage [13]. Table 2 shows the relationship between molecular and biomonitoring methods and their main advantages and disadvantages.

Among the molecular methods for studying the biodiversity of soil microbial communities, PCR-based molecular fingerprinting techniques are represented by DGGE (denaturing gradient gel electrophoresis), TGGE (temperature gradient gel electrophoresis) [71], T-RFLP (terminal restriction fragment length polymorphism), SSCP (single-strand conformational polymorphism), RISA (ribosomal internal spacer analysis) and LH-PCR (length heterogeneity-PCR). Nowadays, they are preliminary techniques for rapidly highlighting the major differences in microbial community composition caused by changes in soil properties [13]. The main limitations of fingerprint-PCR-based methods are the extraction of an adequate quantity and purity of DNA, the selection of primers and restriction enzymes, and PCR reaction optimization. Despite the limitations, these methods can be a useful tool for studying genetic biodiversity and comparing the relationships between different samples collected from soils contaminated with different compounds/substances, including herbicides [47,72]. Often, functional gene abundance, i.e., amoA, is determined using qPCR in addition to the determination of genetic biodiversity [73,74].

The effect of mesotrione on the genetic microbial biodiversity of soils using the t-RFLP technique was presented by Du et al. [73]. Moreover, the authors investigated the abundance of the amoA gene, which mainly exists in ammonia-oxidizing microorganisms (bacteria and archea), by the qPCR technique. In turn, Wydro et al. [14] used the t-RFLP method for evaluating the influence of glyphosate on the genetic community structure of bacteria and fungi [14,74]. Moreover, Mijangos et al. [75] used a DGGE approach for examining the influence of glyphosate on soil microbial communities. The effect of atrazine application on microbial community structure by DGGE assay was studied by Tortella et al. [76].

Currently, high-throughput techniques, such as NGS (next-generation sequencing) and TGS (third-generation sequencing), are being used in order to demonstrate more detailed differences in genetic soil microbiota. These are state-of-the-art tools that provide an accurate picture of soil microbiology, allowing for the discovery of previously unexplored soil microbiome compositions, both cultured and not previously cultured in the laboratory [77]. NGS techniques are based on phylogenetic marker genes, such as the 16S for prokaryotes, internal transcribed spacer (ITS) for fungi, and 18S for the majority of eukaryotes (metabarcoding), or on an analysis of the entire metagenome. Sequencing, apart from the appropriate equipment, requires the use of bioinformatics and statistical tools to analyze the data. Additional limitations of the NGS approach are the quantity and purity of the extracted DNA, optimization of the PCR reaction (metabarcoding) and no live, dead or active microorganism discrimination [78]. However, despite these limitations, this method is the most promising and widely used in environmental studies. The analysis of soil microbial communities after the application of different herbicides (metsulfuron-methyl, bentazon salt, saflufenacil, penoxsulam and pyrazosulfuron-ethyl) performed by 16S and 18S rRNA amplicon next-generation sequencing was described by Serbet et al. [79]. In turn, high-density DNA microarray (PhyloChip) and 16S rDNA amplicon sequencing (NGS) was used to determine the effect of isoproturon [80]. Barcoding sequencing was also applied in order to examine the rhizosphere bacterial community composition after glyphosate treatment [81].

Another technique that deserves attention is the comet assay used to evaluate the genotoxicity of agrochemical substances as well as industrial, pharmaceutical and biocide pollutants. This test can be used for the detection of DNA breaks because fragments of nucleic acid subjected to electrophoresis move outside the nucleus, forming a comet-like tail. The range of migration of DNA fragments is an indicator of its damage. It is a test that can
be used on any cell type [82]. The advantage of this assay, besides being fast, easy to perform and requiring a small amount of test substance, is that it can be used on rapidly dividing cells and may be targeted to only one tissue [83]. Møller et al. [82] indicated differences between laboratories in comet determination procedures and basic descriptors (% DNA in tail). Such significant variability makes it difficult to correctly perform interpretations between laboratories and, thus, also to attempt to standardize methods and promote the use of reference standards [84]. Furthermore, it should be noted here that this test does not directly measure the number of specific DNA lesions but rather focuses on the migration of DNA in gels as a result of the relaxation induced by strand breaks [82]. The comet assay approach was conducted to determine DNA damage in earthworms (*Eisenia fetida*) living in soil contaminated with mesotrione [85]. In turn, Prado et al. [86] have used the comet assay to assess the genotoxic effects of paraquat on microalgae. Furthermore, Cenkci et al. [87] studied 2,4-D and Dicamba genotoxicity in plants using comet and RAPD (randomly amplified polymorphic DNA) assays. The researchers found similar results when using both methods. Additionally, Liman et al. [88] used the comet assay to determine the genotoxic effects of imazethapyr herbicide in *Allium cepa* root cells.

5. Conclusions

Due to the yearly increase in the amount of organic contaminants in soil environments, it is necessary to conduct biomonitoring studies that allow the drawing of reliable conclusions concerning the fate of contaminants present in soils. Moreover, such studies should cover a long period of time, which allows the determination of trends in changes in the level of soil contamination and the effectiveness of herbicide penetration into surface waters. A long-term observation period is necessary for assessing changes in the technology used in the protection and cultivation of plants, especially in the field of active herbicide substances. The above-mentioned aspects are important because they may influence the presence of residues of plant protection products in the soil [89,90].

Moreover, in the course of biomonitoring, other biotests should also be used to enable conducting a comprehensive toxicological assessment of environmental contamination. Therefore, there is still a need for the improvement of soil biomonitoring methods, in particular those treated with pesticides. It is a challenge for scientists because it requires the development of both universal and sensitive methods that can be applied to soils in various climatic zones with different contaminants. In addition to these advantages, molecular biomarkers are important tools for environmental biomonitoring and pollution assessment. As reported in the literature, morphological markers serve their purpose and provide much information on the damage caused by some pollutants [64]. However, the biochemical changes produced after the contact with a toxic substance constitute an early signal of the negative effect of exposure before visible damage [65].

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