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Article

Unravelling Relationships between In Vivo Effects on Plants and Detected Pesticide Mixtures in Freshwaters of a South-European Agro-Ecosystem

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Abstract: The multiple benefits agriculture provides to society depend on the long-term sustainable management of water resources, including the preservation of a good ecological and good chemical status of the water bodies. Presently, this good chemical status has not been reached in the majority of European river basins. Implemented monitoring strategies are targeted to identify the presence and magnitude of the ecological impacts that come from mixtures of chemicals but fail to give information on the causes of the ecosystem disruptions. This work aims to contribute to assessing the quality of surface waters used for irrigation in the LGVFX agricultural area (Central Portugal) by applying non-conventional in vivo phytotoxicity tests on three primary producers, a monocotyledon (*Sorghum saccharatum*) and two dicotyledons (*Lepidium sativum* and *Sinapsis alba*), complemented by chemical screening and mixture-risk modelling with component-based methods (summation of risk quotients) based on the classic concept of concentration addition (CA). Although inhibition percentages of the phytotoxicity parameter germination and root and shoot growth may be related to the presence of mixtures of pesticides, it was not possible to establish the fingerprinting of the detected compounds with the observed biological effects, mostly due to the large gap of ecotoxicological data on terrestrial plants exposed to contaminated water. In addition, pesticides can interact within the plant, leading to antagonism and synergism phenomena.

Keywords: irrigation waters; risk assessment; pesticide mixtures; bioassays; phytotoxicity

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1. Introduction

Plants, throughout their life cycle, are exposed to a large number of conditions or stressors, which can be grouped into biotic and abiotic. Biotic stress is that caused by the action of living beings, such as small or large animals, other plants, and the so-called pathogens (bacteria, fungi, viruses, and viroids). Abiotic stress, depending on the nature of the causal agent, can be divided into physical and chemical. Among physical–chemical parameters, there are water deficit, salinity (in its osmotic component), extreme temperatures (heat, cold, freezing), excessive or insufficient irradiation, anaerobiosis produced by stagnation or flooding, the mechanical stress produced by the wind or excessive compaction of the soil, and stress induced by wounds or injuries. Chemical stress is caused by salinity (in its ionic or toxic component), by the lack of mineral elements and by environmental contaminants, such as metals and pesticides [1]. With regard to organic compounds, pesticides can enter the aquatic environment from point sources, such as the discharge of wastewater effluents, and diffuse sources, such as runoff of agricultural origin [2]. Given the fact that freshwaters contain species taxonomically related to the target organisms of pesticides, there is a potential for undesirable side effects to occur in aquatic ecosystems. Several studies provided strong evidence that contamination with pesticides has a clear impact on aquatic communities and thus on the ecological status of a water body [3,4]. Despite the recognition that this group of contaminants can pose environmental concerns, there is still a lack of information on how plants react to these compounds. The quality of the irrigation waters is strictly related to physical–chemical and biological parameters that, depending on the culture, may cause different impacts. Several studies [5–8] have shown that emerging pollutants, when present in irrigation waters, cause phytotoxicity, morphological, and physiological alterations in crops. During stress, plants react by slowing down or stopping their basic physiological functions and reducing their vigor.

This study aims to assess the quality of surface waters used for irrigation in the "Lezíria Grande de Vila Franca de Xira" (LGVFX) by conducting toxicity analyses on terrestrial plants in conjunction with chemical analyses to link measurements of biological effects with pesticide compounds or other irrigation water quality parameters when possible. The MicroBioTests phytotoxicity test will be applied for determining the toxic effects on the germination of seedlings and the initial growth of the monocotyledon, sorgho (*Sorghum saccharatum* (L.) Moench), and the dicotyledons, garden cress (*Lepidium sativum* L.) and mustard (*Sinapis alba* L.). This assay is intended to detect not only point source effects but also the cumulative effects of non-point sources of pollution such as agricultural runoff.

2. Materials and Methods

2.1. Characterisation of the Study Area

The LGVFX, constituted by an area of approximately 13,420 ha and surrounded by a peripheral dyke that protects it from the flooding of the Tagus and Sorraia rivers, is located on the left bank of the Tagus river, about 30 km from Lisbon. It is limited to the north and west by the Tagus River, to the southeast by the "Mar da Palha", to the east by the Risco and Sorraia rivers, and to the northeast by the Vau River, according to the demarcation approved by Decreto 33210, of 11 November 1943 [9]. The LGVFX operates almost entirely in the Vila Franca de Xira municipality (north) and in a very small area in the Azambuja municipality. It is divided, roughly in half, by the "reta do cabo", "Estrada Nacional 10", which connects Vila Franca de Xira to Porto Alto, giving rise to "Lezíria Norte" (6620 ha) and "Lezíria Sul" (6800 ha) [10]. In the LGVFX, the Portuguese State built a set of hydraulic infrastructures, which constitute the "Lezíria Grande de Vila Franca de Xira" hydro-agricultural operation (hereinafter AHLGVFX). The "Associação de Beneficiários da Lezíria Grande de Vila Franca de Xira" (hereinafter ABLGVFX) manages and explores the collective use equipment of this hydro-agricultural operation (Campos and Madaleno, 2020) since 2009.

2.2. Crop Occupation in the Study Area

In 2021, the AHLGVFX had a total irrigated area of 9386.42 ha. Taking into account that the irrigated surface of temporary crops, as the main crop (ha) of agricultural holdings, in Portugal was 260,823 ha according to data of the Agricultural Census—2019 of the National Institute of Statistics [11], the total irrigation area of the AHLGVFX corresponds to approximately 3.6% of this total. The areas of the cultivated crops were: 4831.33 ha rice; 3467.8 ha tomato; 498.62 ha maize; 373.2 ha horticultural crops (pepper, pumpkin, potato, pea, melon, broccoli); 101 ha forage crops; 2.83 ha oilseed crops (especially sunflower); and 111.64 ha of sorghum and lucerne [12].

2.3. Selection of the Sampling Surface Sites

The AHLGVFX has several adduction stations on the Tagus, Sorraia, and Risco rivers, although the main water adduction station is at the Conchoso water intake, which feeds blocks I and II. This important water intake (A1, Figure 1) and another one located in the "Vale do Sorraia" (A3, Figure 1) were selected for surface water sampling on three different dates: 27 April, 22 June, and 27 July 2021. This sampling period took into account the timing of pesticide application and irrigation. The importance of carrying out the present study in this agricultural area can be emphasized by the fact that this area: (i) presents an intense agricultural activity; (ii) is occupied by several crops, mainly rice, tomato, and maize, followed by other secondary crops and, therefore, subject to several pesticide application scenarios; (iii) it has intrinsic vulnerability (Tagus Vulnerable Zone); (iv) contains a Special

Protection Area for Birds, which incorporates the Tagus Estuary Natural Reserve, included in the Wetlands of International Importance (Ramsar Sites); (v) is irrigated by surface waters from the Tagus and Sorraia rivers, through stations existing in the 62 km dyke; (vi) was identified as contaminated by various pesticides (including mixtures), as indicated in previous studies carried out by the ISA research team [13] and others included therein; and (vii) is located only a short distance away from the facilities of the Instituto Superior de Agronomia (ISA) in Lisbon.

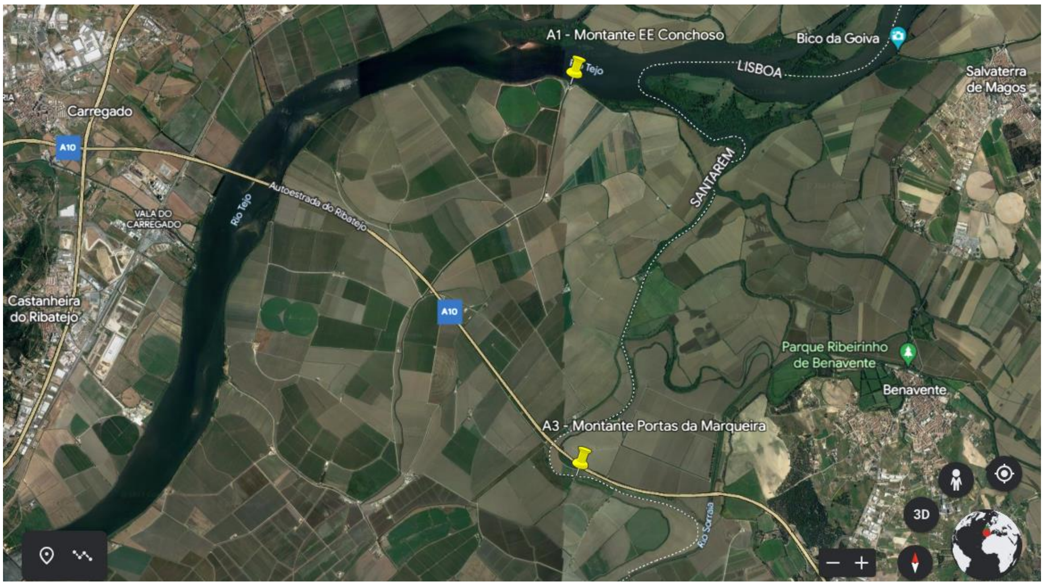

Figure 1. Location of the two sampling points A1 and A3 in the AHLGVFX.

2.4. Surface Water Sampling

Taking into account that the sampled waters were from a moving surface (river), they were taken where the current was normal, avoiding eddies or stagnant water areas. Surface water samples were collected at about 30 cm depth and, when possible, in the center of the current with a Van Dorn bottle. They were stored in five types of glass containers (for analysis of pesticide residues), with different volumes, and 500 mL plastic containers (for analysis of toxic effects), well cleaned and rinsed, at least three times, with the water from which the sample was taken. The containers were well filled, free of air bubbles, and corked. After being identified, the surface water samples were transported in a refrigerated box to the Ecotoxicology Laboratory of the ISA, where they were stored in a refrigerator until analysis at a temperature that did not exceed 5 °C.

2.5. Analytical Methods

2.5.1. Individual Parameters for Water Quality Intended for Irrigation Use

Considering that the sampled surface water aims to satisfy or complement the water needs of agricultural crops, some of the parameters related to the water quality intended for irrigation use established in Annex XVI of the Decree-Law No. 236/98, of 1 August [14] were analyzed.

Metals, such as arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), lead (Pb), nickel (Ni), and zinc (Zn), were determined following the Pharmacopoeia EU 7 Method A (exl. Filtration); chloride and nitrate (as NO_3) by a discrete analyzer according to NEN-ISO 15923-1; sulphate dissolved (SO4) according to ISO 22743; total suspended solids as the dry matter undissolved part (NEN 6484) following NEN 6499/NEN 6484; acidity (pH) following the NEN-EN-ISO 10523; and electric conductivity 25 °C according the NEN ISO 7888. The tests were performed in the laboratory of Eurofins Analytico B.V. under accreditation NEN EN ISO/IEC 17025: 2005, RvA L010.

2.5.2. Pesticides

Pesticides are not included in the list of parameters intended to characterize the quality of irrigation water, but are considered as an additional requirement for water quality and monitoring when there is clear scientific evidence that the risk originates from reclaimed water intended for agricultural irrigation and not from other sources, according to Regulation (EU) 2020/741 of the European Parliament and of the Council of 25 May 2020 on the minimum requirements for water reuse [15].

Around seven hundred and thirty pesticides were searched for analysis in surface waters of the AHLGVFX, including those approved for use in the main crops of the LGVFX. Pesticide compounds were determined through gas chromatography-tandem mass spectrometry (GC-MS/MS) and LC-MS/MS (liquid chromatography-tandem mass spectrometry), following internal methods, DIN 38407-F36, 2014-09, and DIN 38407-F35, 2010-10, mod., namely for phenoxy carboxylic acids and other acidic herbicides in water (screening of about 30 compounds). The tests were performed in the laboratory of Eurofins SOFIA GmbH under accreditation DIN EN ISO/IEC 17025:2018 DAkkS D-PL-19579-02-00. The lowest concentration of the analyzed pesticides that can be measured with certainty (limit of quantification) varies between 0.03 and 5 $\mu g\ L^{-1}$ (for pyrethrins).

2.5.3. Phytotoxkit Liquid Samples

The Phytotoxkit liquid sample microbiotest (MicroBioTests Inc., Gent, Belgium) [16] is a variant of the Phytotoxkit solid samples assay, which measures the decrease (or the absence) in germination and the early growth of plants in contaminated soils, in comparison to the germination and growth in a reference soil.

In the Phytotoxkit liquid sample test, the lower compartment of the test plate is not filled anymore with soil, but with a foam pad and a thick filter paper, which is subsequently spiked with one selected concentration of a chemical compound or an aqueous sample. This alternative test procedure allows the determination of the direct intrinsic effect of a chemical compound (at the selected concentration) or an aqueous sample on the plant.

The Phytotoxkit liquid sample limit test contains tubes with three types of plants seeds that were selected for their rapid germination and growth of the roots and shoots, which allows the completion of the assay after only three days of incubation: the monocotyl Sorgho (*Sorghum saccharatum*) and the dicotyls garden cress (*Lepidium sativum*) and mustard (Sinapis alba). Seeds of the three test plants were positioned at equal distance near the middle ridge of the test plate, on a black filter paper placed on top of the spiked thick white filter paper. The control test plate was spiked with distilled water.

After closing the test plates with their transparent cover, the test plates were placed vertically in a holder and incubated at 25 °C (+/−1 °C) for three days. At the end of the incubation period a "digital" picture was taken of the test plates in which the germinated plants can clearly be seen underneath the transparent cover. The pictures were stored in a computer file for subsequent analyses and length measurements of the roots and the shoots were made with an Image analysis program. This test is intended for phytotoxicity screening of chemicals, leachates of soils or solid wastes, sediment pore waters and elutriates, wastewaters, pesticides and biocides, and other research applications. This cost-effective and user-friendly phytotoxicity assay strictly adheres to ISO Standard 18763 [17].

2.6. Environmental Risk Assessment

Under the European Union (EU), environmental quality standards (EQSs) are used as regulatory values to verify whether the risk of substances regulated under the Water Framework Directive (WFD) is acceptable. These are substance-specific concentrations of individual chemicals in the aquatic environment below which no harmful effects on aquatic organisms are expected. The risk is considered acceptable if the measured environmental concentration (MEC) is lower than the EQS. The derivation of EQSs is laid down in the CIS guidance document 27 [18] by the European Commission. As in other EU guidance documents for assessing the risk of chemical substances for surface waters, e.g., under REACH [19] or the Biocidal Products Regulation (BPR) [20], it is largely based on the Technical Guidance Document on Risk Assessment [21]. Two separate values are determined in each case. The acute EQSs (MAC-EQSs) is intended to provide protection against short-term exposure peaks, while chronic EQSs (AA-EQSs) are intended to provide protection against prolonged exposure. Under REACH and the BPR, the term predicted no-effect concentration (PNEC) is used instead of EQSs.

Risk quotients (*RQs*) were calculated by dividing the *MEC* of a compound *i* by the corresponding regulatory-adopted EQS_i values or (ad hoc) proposals (as lowest $PNEC_i$ values) downloaded from the EQS List Swiss Ecotoxcentre [22] and the NORMAN Ecotoxicology Database [23], respectively.

$$RQi = \frac{MECi}{EQi \text{ or } PNECi} \quad (1)$$

Mixture toxicity was expressed by *RQsum*. Assuming concentration addition (CA), all available $RQ_{EQS,i}$ values and the $RQ_{PNEC,j}$ values were summed up:

$$RQsum = \sum_{n=i}^{n} RQi \quad (2)$$

If *RQsum* is below 1, the risk metric indicates a sufficient safety of the sample.

3. Results

3.1. Individual Water Quality Parameters

The values of parameters determined in the surface water samples from the AHLGVFX, considered in water quality intended for irrigation use, were compared with the maximum recommended (MRV) and maximum admissible values (MAVs), established by the Decree-Law No. 236/98, of 1 August [14].

It can be concluded that not all the six surface water samples are considered in compliance with the respective quality standards, as they did not respect the respective values. This situation occurs, simultaneously, for the parameters of chlorides (MRV 70 mg/L) and total suspended solids (MRV 60 mg/L), exceeding these standards in a surface water sample sampled at the A3 site, on 27 July, by 71% and 83%, respectively, as well as for the total suspended solids, in other from the same site on 27 April, exceeding the MRV by 27%. The A3 sampling site is located in the "Vale do Sorraia", very close to the civil parishes of the Benavente municipality, and may have a direct influence on discharges from wastewater treatment plants originating from domestic and/or industrial activities.

The presence of chlorides in water can cause phytotoxicity when the irrigation water is applied by self-moving sprinklers at low speed, which favors the evaporation of water between two consecutive passes of the sprinkler, concentrating the salts dissolved in the irrigation water on the leaves, which are then absorbed by the leaves, which show burn-like necroses. This problem is aggravated in hot and dry climates and can be mitigated by night watering [24]. The presence of suspended solids, in high concentrations, can cause clogging in soils and silting in irrigation networks, as well as clogging in drip and sprinkler irrigation systems; in addition, in the latter system, water can cause deposits on leaves and fruits [24].

3.2. Detected Pesticide Compounds

Within the set of six surface water samples, 10 pesticides out of 730 analyzed target compounds were detected in at least one sample (Table 1).

Table 1. Pesticides detected in surface water samples collected at the A1 and A3 sites, on three sampling dates (27/04, 22/06, 27/07, in 2021), in the AHLGVFX.

Pesticide	Concentration (μg L^{-1}) 27 April A1	27 April A3	22 June A1	22 June A3	27 July A1	27 July A3
Fungicide						
azoxystrobin	<0.03	<0.03	<0.03	<0.03	<0.03	0.04 (±0.02)
Herbicide						
azimsulfuron	<0.05	<0.05	<0.05	0.2 (±0.1)	<0.05	0.12 (±0.06)
bentazone	<0.05	0.51 (±0.26)	<0.05	3.4 (±1.7)	0.2 (±0.1)	8.0 (±4.0)
clomazone	<0.05	0.1 (±0.1)	<0.05	0.16 (±0.08)	<0.05	<0.05
glyphosate	0.058 (±0.029)	0.13 (±0.07)	0.19 (±0.10)	0.24 (±0.12)	0.061 (±0.031)	0.091 (±0.046)
imazamox	<0.05	<0.05	<0.05	0.092 (±0.046)	<0.05	0.09 (±0.05)
MCPA	<0.05	<0.05	<0.05	<0.05	<0.05	0.066 (±0.033)
oxadiazon	<0.03	0.031 (±0.016)	<0.03	<0.03	<0.03	0.16 (±0.08)
Herbicide metabolite						
AMPA	0.41 (±0.21)	0.36 (±0.18)	0.62 (±0.31)	0.58 (±0.29)	0.62 (±0.31)	0.68 (±0.34)
Insecticide						
flonicamid	<0.03	<0.03	<0.03	<0.03	<0.03	0.079 (±0.04)

Herbicides were the pesticide type with the most active substances detected in the total of surface samples from the A1 and A3 with eight different ones (including an herbicide metabolite), while fungicides and insecticides only showed one active substance each.

The herbicide glyphosate and its metabolite aminomethylphosphonic acid (AMPA) were detected in all sites (A1 and A3) and sampling dates (27/04, 22/06, and 27/07). Comparing by sampling date, the highest concentrations of glyphosate and AMPA were quantified at the A3 and A1 sites, respectively, with the exception of a higher AMPA concentration at the A3 site, on 27 July. These data are within the concentration values found in Europe in various water sources, where growing genetically modified crops is not allowed [25,26].

The herbicides bentazone and oxadiazon were detected in all sampling dates, at the A3 site. The first one was also detected at the A1 site, on 27 July. The herbicides azimsulfuron and imazamox were only quantified on 22 June and 27 July, at the A3 site, with higher concentrations in June, while the herbicide clomazone was only detected on 27 April and June, at the same site. The herbicide MCPA, the fungicide azoxystrobin, and the insecticide flonicamid were only quantified on 27 July, on the last sampling date, at the A3 site. In general, the largest spectrum of active substances was detected at the A3 site on 27 July, with the exception of the herbicide clomazone, not detected in this month.

Based on the Plant Protection Product Authorization Management System, SIFITO [27], we can see that all detected pesticides are authorized for the rice crop, which occupies the largest area in the LGVFX, with the exception of oxadiazon, whose use is no longer approved. In fact, this herbicide was no longer marketed from 31 December 2020, but was allowed to be used until 30 June 2022, according to a search in canceled sales authorizations [27]. In addition, this active substance is very persistent in soil (DT50 = 502 days) and shows slow degradation in the aquatic phase (DT50 = 17.9 days), according to the classification of the Pesticide Properties DataBase [28]. The herbicide glyphosate, one of the most frequently detected in surface water samples collected at the A1 and A3 sites, is approved for two of the three main crops in the LGVFX, rice and maize, as well as for other crops with less agricultural expression, such as potatoes, peas, sunflower, and sorghum. The herbicide bentazone, the second active substance most frequently detected, was quantified with the highest concentration (8 μg L^{-1}), being also registered for crops occupying the LGVFX, namely rice, maize, potatoes, peas, alfalfa, and sorghum crops. Bentazone was also found to be the herbicide with the highest concentration (up to 180 μg/L) and abundant pesticide in the Ebro River Delta, a typical Mediterranean delta ecosystem with 80% of the land devoted to rice cultivation [29].

The greatest diversity of detected active substances occurred in surface water samples collected at the A3 sampling site. This point is located at the end blocks of the "Aproveitamento Hidroagrícola do Vale do Sorraia", whose largest cropped areas are rice and maize [30]. The higher exposure to pesticides, at the A3 site, may be due, most likely, to the use of these compounds in the "Vale de Sorraia" and consequent runoffs, while the A1 site captures in an upstream point on the Tagus River, with greater dilution and with less influence of agricultural crops.

3.3. Environmental Risk Assessment

None of the detected pesticides is a priority substance in the field of water policy [31]. However, the herbicide bentazone is a river basin specific pollutant in Portugal, but it does not exceed the respective quality standard in inland surface waters (80 μg L^{-1}; [32]). The herbicide oxadiazon was included in the first watch list of substances for Union-wide monitoring in the field of water policy pursuant to Directive 2008/105/EC [33]. During 2017, the Commission analyzed the data from the first year of monitoring of substances in the first watch list. On the basis of that analysis, the Commission concluded that sufficient high-quality monitoring data are available for the substance oxadiazon and others, and that, therefore, those substances should be removed from the watch list [34]. Azoxystrobin was identified as a suitable candidate for including in the fourth watch list [35].

Environmental risk assessment of contamination strongly relies on reliable toxicity data. PNEC values from the Ecotox Center of the Federal Office for the Environment in Switzerland [22] and NORMAN Ecotoxicology Database [23], based on experimental endpoints (incl. regulatory-adopted EQS values or (ad hoc) proposals), were available for fungicide azoxystrobin (0.55 μg L^{-1}), herbicides bentazone (470 μg L^{-1}), clomazone (53 μg L^{-1}), glyphosate (360 μg L^{-1}), imazamox (2.1 μg L^{-1}), MCPA (6.4 μg L^{-1}), oxadiazon (0.3 μg L^{-1}), and metabolite AMPA (1500 μg L^{-1}), while PNECs of herbicide azimsulfuron (0.11 μg L^{-1}) and insecticide flonicamid (1000 μg L^{-1}) were predicted by the authors. These were derived as a MAC-QS for the freshwater pelagic community [18], applying an assessment factor of 100 to the lowest L(E)C50 from three short-term tests using species from three trophic levels (fish, invertebrates (preferred Daphnia), and algae) [28].

Taking into account the proposals for quality standards for the detected pesticides that assesses the likelihood of possible damage to the aquatic organisms within the next 24 to 96 h, there is risk to the aquatic ecosystem caused by short-term individual exposure to the herbicide azimsulfuron, when compared with the respective measured concentrations in surface water samples collected at the A3 site on 22 June and 27 July. Only in these two surface water samples, the mixtures of pesticides constitute a risk for the aquatic ecosystem, as azimsulfuron the major risk driver.

The occurrence of these pesticides in the water compartment reflects the intrinsic characteristics of these compounds, such as physico-chemical properties and partition coefficients, reflected in their prediction distribution to the water compartment, the environmental factors, but also their use, as previously discussed, in the main crops of the AHLGVFX, namely the in rice crop, whose production system is strictly linked to water.

3.4. Phytotestkit

3.4.1. Germination

As previously mentioned, the toxic effects on the dicotyledonous *Lepidium sativum* and *Sinapsis alba*, as well as monocotyledon *Sorghum saccharatum* were evaluated, when exposed to the surface water samples collected at the two sites (A1 and A3) on three sampling dates (27/04, 22/06, and 27/07, in 2021), in the AHLGVFX, by calculating the inhibition percentage relative to germination, and to the growth of roots and shoots.

In general, through Figure 2, positive as well as negative percentage inhibition values can be observed. The latter are considered because a stimulation process has taken place, when compared to the control samples. This result was especially observed with *Sinapsis alba*, in the two sampling sites, A1 and A3, with the exception of the surface water sample collected at the A1 site, on 27 July, which had a value equal to zero. However, it was with the only exposed monocotyledonous species, *Sorghum saccharatum*, that the highest negative percentage inhibition value (−12.5%) occurred in a surface water sample collected at the A3 site, on 27 July. All other percentage inhibition values were equal to or higher than zero, reaching a maximum value of 10.34% in the surface water sample exposed to the *Sorghum saccharatum* collected at the A1 site on 22 June.

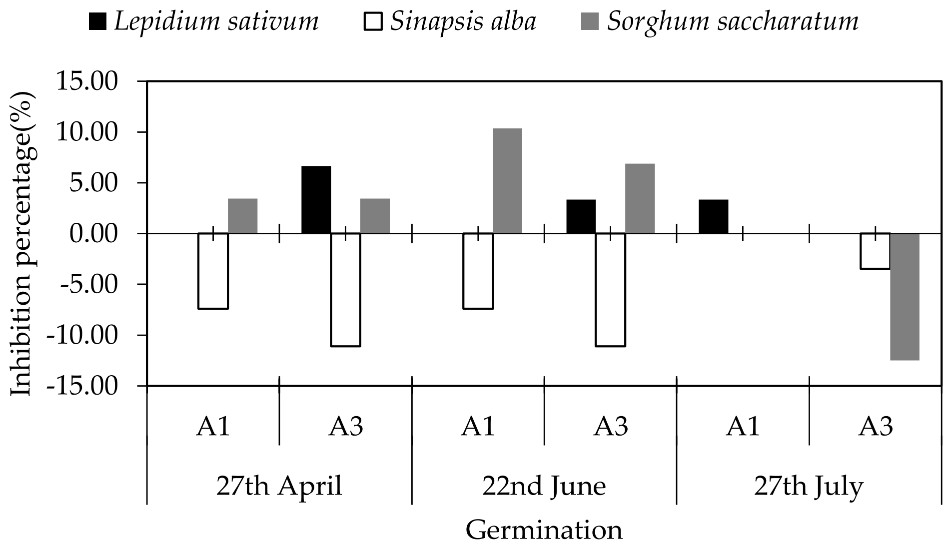

Figure 2. Percentage of inhibition regarding the germination of the dicotyledonous *Lepidium sativum* and *Sinapsis alba*, and the monocotyledonous *Sorghum saccharatum*, exposed to surface water samples collected at the A1 and A3 sites, on three sampling dates (27/04, 22/06, 27/07, in 2021), in the AHLGVFX.

3.4.2. Growth of Roots and Shoot

Through Figure 3, it can be observed that the growth of the roots of the three species of plants benefited from exposure to the sampled surface water samples, namely the monocotyledonous *Sorghum saccharatum*, reaching a negative value of 40.84%, in the surface water sample collected at the A1 site, on 22 June. The dicotyledonous *Lepidium sativum* also grew favorably, in relation to the control, at all sites and sampling dates. It reached the maximum negative value in a surface water sample collected at the A3 site on 22 June. The plant species, *Sinapsis alba* and *Sorghum saccharatum*, ranged between positive and negative values for the surface water samples collected at the two sampling sites, A1 and A3.

The results of Figure 4, relative to the percentage of inhibition in relation to the growth of shoots from the three plant species, showed a pattern similar to the results immediately preceding them. The dicotyledonous *Lepidium sativum* also grew favorably, in relation to the control, in all six surface water samples. It reached the maximum negative value in the surface water sample collected at the A3 site on 22 June, similar to previous results. Once again, the plant species, *Sinapsis alba* and *Sorghum saccharatum*, oscillated between positive and negative values for surface water samples collected at the A1 and A3 sites, reaching the monocotyledonous *Sorghum saccharatum*, the maximum inhibition percentage value in the surface sample collected at the A3 site on 27 April (41.01%).

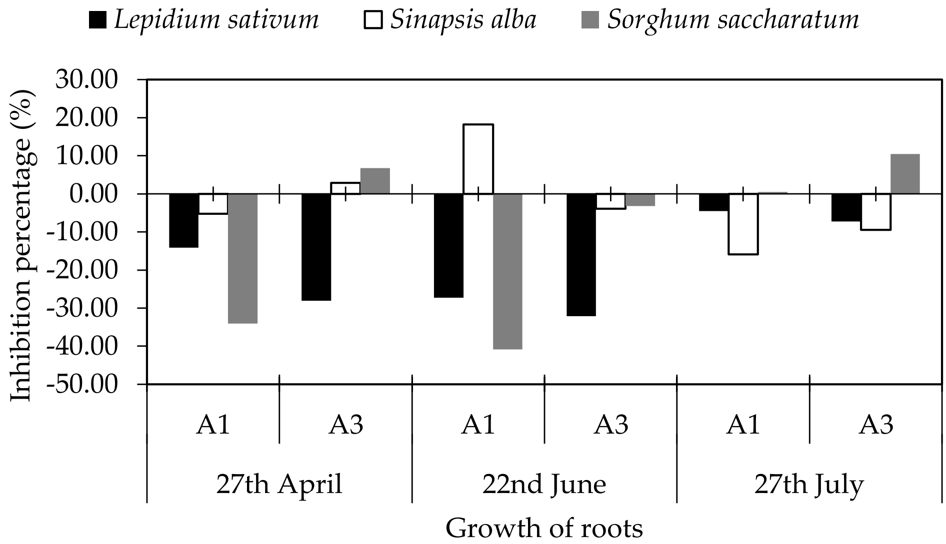

Figure 3. Percentage of inhibition regarding the growth of roots of the dicotyledonous *Lepidium sativum* and *Sinapsis alba*, and the monocotyledonous *Sorghum saccharatum*, exposed to surface water samples collected at the A1 and A3 sites, on three sampling dates (27/04, 22/06, 27/07, in 2021), in the AHLGVFX.

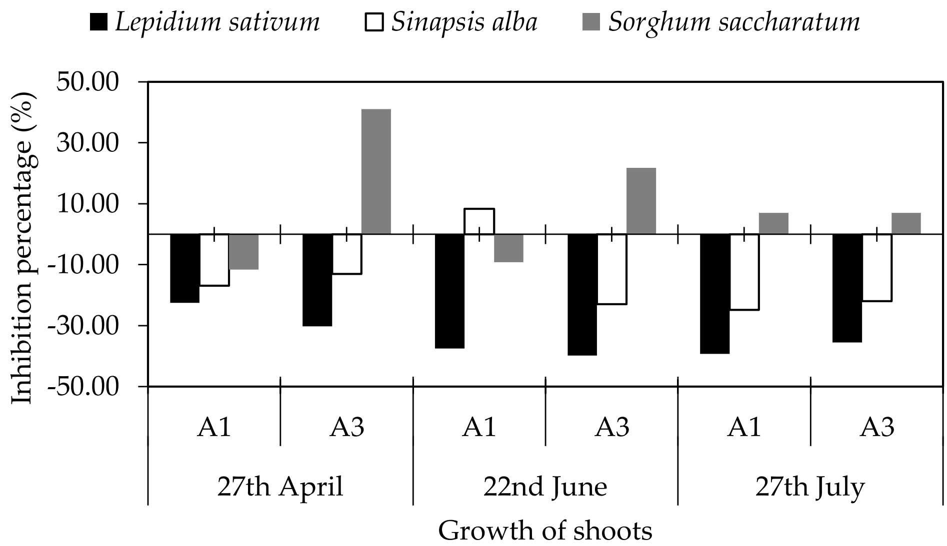

Figure 4. Percentage of inhibition regarding the growth of shoots of the dicotyledonous *Lepidium sativum* and *Sinapsis alba*, and the monocotyledonous *Sorghum saccharatum*, exposed to surface water samples collected at the A1 and A3 sites, on three sampling dates (27/04, 22/06, 27/07, in 2021), in the AHLGVFX.

Analyzing, in general, the results, we can say that the dicotyledonous *Sinapsis alba* was the most frequently positively favored by exposure to surface water samples, considering all evaluated parameters, namely for seed germination.

However, it was with another species of the same group of plants, *Lepidium sativum*, that the results were more consistent. This means that, in all surface water samples, this dicotyledon species benefited positively for the growth of its roots and shoots, with percentage inhibition values ranging from −4.5 to −32.1 (root growth), as well as between −22.56 and −39.82 (shoot growth). However, also, in all these surface water samples, it showed inhibition in the germination of its seeds, with inhibition percentage values equal to or greater than zero, reaching the value of 6.67%.

In contrast, the monocotyledonous *Sorghum saccharatum* was the one that revealed more surface water samples with positive inhibition percentage values, reaching the highest value obtained, among all (41.01%; A3 on 27 April).

4. Discussion

Regarding the study, there was, mainly, a stimulus on seed germination and early growth of plants relative to the control samples, performed by distilled water. These results may seem strange, as at least one pesticide was quantified in all surface water samples, in addition to chloride ions and total suspended solids, exceeding the MRV in two surface water samples, collected at the A3 site, on 27 April and 27 July, from the AHLGVFX. As we know, distilled water consists of chemically pure water, that is, purified by distillation in order to eliminate the salts dissolved in it and other compounds. However, the composition of surface water samples, with the exception of those two surface water samples, allowed to provide the plant with a desirable dissolution of nutritional elements such as the main macronutrient nitrogen and the macronutrient secondary sulfur, absorbed in the form of nitrate (NO_3^-) and sulfate (SO_4^{2-}) ions, respectively, through irrigation water.

The highest inhibition percentage value was recorded for the monocotyledon *Sorghum saccharatum*, when exposed to the surface water sample collected at the A3 site, on 27 April. This sample showed the presence of a cocktail of pesticides, namely the herbicide compounds bentazone, clomazone, glyphosate, oxadiazon, and AMPA. Furthermore, a total suspended solid value of 76 mg/L was also recorded, a value higher than the RMV (60 mg/L).

The plant species *Sorghum saccharatum* was also the one that presented the highest number of samples with positive percentage inhibition values, when exposed to surface water samples, namely for the seed germination parameter. Germination is the process that begins with the absorption of water by the dry seed (imbibition) and ends when one part of the embryo (embryonic stem in dicotyledons or radicle in monocotyledons and gymnosperms) go through the surrounding structures (emergence). In the case of endosperm seeds (such as grass seeds), the resistance that these structures (test and endosperm) oppose to the embryo is so great that, for the production of emergence, it needs the enzymatic degradation of several zones of these structures [1]. Maybe for this reason, this class of angiosperms, in this case the *Sorghum saccharatum* plant species, was more susceptible to the exposed surface water samples.

The phenomenon of hormesis could have been verified in several samples collected at the A1 and A3 sites, as negative inhibition percentage values were observed. In these samples, a large number of pesticides were detected in concentrations between 0.031 $\mu g\ L^{-1}$ (oxadiazon, A3 site on 27 April) and 8.0 $\mu g\ L^{-1}$ (bentazone, A3 site on 27 July). The hormetic effects on plants have been described, through the enumeration of various examples, namely with the herbicide glyphosate [36].

With regard to the list of priority substances in surface waters, which include some pesticide compounds, quality standards have been established that must not be exceeded to achieve a good chemical status. However, these were calculated with toxicity values of aquatic organisms belonging to the primary producers (algae, aquatic plants), first order consumer (aquatic invertebrates), and second order consumer (fish) taxonomic groups. By means of a search in the ECOTOX database [37], toxicity data for the plant species under study were observed, but only related to the soil. There is a big gap in toxicity values for terrestrial plants in the aquatic environment, and this fact may be one of the reasons why the parameters related to the quality of irrigation water have not yet been updated.

This study aimed to determine the direct effects of surface water collected in two supply ports of one of the most important agricultural areas in Portugal, LGVFX, on seed germination and early growth of three plant species, one of which (sorghum) cropped in that area. It was not possible to establish a direct relationship between cause and effect, because a set of various conditions or stressors that can affect the growth and development of plants, may have acted independently or additively, and have contributed to the responses evaluated on plant species. The potential mixture of component interactions as synergism or antagonism can also occur with some specific pesticide combinations or when involving a pesticide and other pollutants such as metals or antifoulants [38,39]. However, as true synergistic interactions between chemicals are rare and often occur at high concentrations, addressing the cumulative rather than synergistic effect of co-occurring chemicals, using standard models as CAs, is therefore regarded as the most important step in the risk assessment of chemical cocktails [38].

The adaptation strategies used by plants for surviving in the changing environment allow them to tolerate stress and are based on the induction of anatomical, structural, and biochemical changes. Some of the adaptations that plants present are specific to a particular type of stress, although most can be considered common to many of them [1].

5. Conclusions

Contrary to what one might think at the beginning of this study, in some surface water samples, a negative percentage of inhibition values were observed for the germination, and the growth of roots and shoots parameters. This event occurred because, probably, the plant species were supplied with several nutritional elements, such as the main macronutrient nitrogen and the secondary macronutrient nitrogen, absorbed in the form of nitrate (NO_3) and sulfate (SO_4^{2-}) ions, respectively, in contrast to the control samples with distilled water, devoid of plant nutrients.

Despite the positive percentage of inhibition values of germination, the growth of roots and shoots parameters may be related to the presence of a cocktail of pesticides. It was not possible to establish the relationship between these compounds and the observed toxic effects, due to a large gap in the ecotoxicological data of terrestrial plants in the water environment and, consequently, of quality standards of these compounds in irrigation water, even if these come to be considered as requirements for the reuse of wastewater. In addition, pesticides can interfere with each other in the plant, which can determine the phenomena of antagonism and synergism.

The results generated in this study are important for knowledge on the quality of irrigation water in the AHLGVFX, in addition to having been applied in approaches currently considered in the European Union, as tools for decision making about those chemical substances that can pose in risk the European surface waters, in relation to ecosystems and human health. This is important in the framework of the River Basin Management Plan, e.g., for the Tagus.

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References

1. Azcón-Bieto, J.; Talón, M. *Fundamentos de Fisiología Vegetal*, 2nd ed.; McGraw-Hill—Interamericana de España, S.L.: Madrid, Spain, 2008.
2. TOPPS—Water Protection. Surface Water Protection. 2018. Available online: www.toppslife.org/ (accessed on 6 July 2023).
3. Pereira, A.S.; Dâmaso-Rodrigues, M.L.; Amorim, A.; Daam, M.A.; Cerejeira, M.J. Aquatic community structure in Mediterranean edge-of-field waterbodies as explained by environmental factors and the presence of pesticide mixtures. *Ecotoxicology* **2018**, *27*, 661–674. [CrossRef] [PubMed]
4. Knillmann, S.; Orlinskiy, P.; Kaske, O.; Foit, O.; Liess, M. Indication of pesticide effects and recolonization in streams. *Sci. Total Environ.* **2018**, *630*, 1619–1627. [CrossRef]

5. Christou, A.; Michael, C.; Fatta-Kassinos, D.; Fotopoulos, V. Can the pharmaceutically active compounds released in agroecosystems be considered as emerging plant stressors? *Environ. Int.* **2018**, *114*, 360–364. [CrossRef]
6. Carvalho, P.N.; Basto, M.C.P.; Almeida, C.M.R.; Brix, H. A review of plant–pharmaceutical interactions: From uptake and effects in crop plants to phytoremediation in constructed wetlands. *Environ. Sci. Pollut. Res.* **2014**, *21*, 11729–11763. [CrossRef] [PubMed]
7. Margenat, A.; Matamoros, V.; Díez, S.; Cañameras, N.; Comas, J.; Bayona, J.M. Occurrence of chemical contaminants in peri-urban agricultural irrigation waters and assessment of their phytotoxicity and crop productivity. *Sci. Total Environ.* **2017**, *599*, 1140–1148. [CrossRef] [PubMed]
8. Marsoni, M.; De Mattia, F.; Labra, M.; Bruno, A.; Bracale, M.; Vannini, C. Uptake and effects of a mixture of widely used therapeutic drugs in *Eruca sativa* L. and *Zea mays* L. Plants. *Ecotoxicol. Environ. Saf.* **2014**, *108*, 52–57. [CrossRef] [PubMed]
9. Decreto 33210, de 11 de Novembro. Diário do Govêrno n.º 245/1943, Série I de 1943-11-11, 743-744. Available online: https://diariodarepublica.pt/dr/detalhe/decreto/33210-1943-257447 (accessed on 6 July 2023).
10. Campos, J.; Madaleno, C. Edições Anteriores da Revista Agrotec. *AGROTEC®* **2020**, *34*, 22–24. Available online: http://www.agrotec.pt/noticias/seccao/edicoes-anteriores/ (accessed on 6 July 2023).
11. Instituto Nacional de Estatística (INE). *Recenseamento Agrícola—Análise Dos Principais Resultados—2019*; Instituto Nacional de Estatística: Lisboa, Portugal, 2021.
12. Narciso, G.A. Contribuição para o Estudo da Relação Entre a Qualidade da água de Superfície da área Agrícola Lezíria Grande de Vila Franca de Xira e os Efeitos Tóxicos Sobre três Espécies de Plantas Terrestres. Master's Thesis, Unviversity of Lisbon, Lisbon, Portugal, 2021.
13. Cerejeira, M.J.; Silva, E. Aquatic Risk Assessment of Pesticides. In *Concepts and Applications in Portuguese River Basins*; ISAPress: Lisboa, Portugal, 2019; 189p.
14. Decreto-lei 236/98, de 1 de Agosto. Diário da República n.º 176/1998, Série I-A de 1998-08-01. Available online: https://diariodarepublica.pt/dr/detalhe/decreto-lei/236-1998-430457 (accessed on 6 July 2023).
15. Regulation (EU) 2020/741 of the European Parliament and of the Council of 25 May 2020 on minimum requirements for water reuse. *Off. J. Eur. Union* **2020**, *177*, 32–55. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32020R0741 (accessed on 6 July 2023).
16. MicroBioTests. Phytotoxicity Test with Phytotoxkit Liquid samples. Available online: https://www.microbiotests.com/toxkit/phytotoxicity-test-with-phytotoxkit-liquid-samples/ (accessed on 6 July 2023).
17. *ISO 18763:2016*; Soil Quality—Determination of the Toxic Effects of Pollutants on Germination and Early Growth of Higher Plants. ISO: Geneva, Switzerland, 2016.
18. European Commission. Technical Guidance for deriving Environmental Quality Standards. In *Guidance Document No. 27—Updated Version 2018, Document Directors at Their Meeting in Sofia on 11–12 June 2018*; European Commission: Sofia, Bulgaria, 2018.
19. European Chemicals Agency (ECHA). *Guidance on the BPR: Volume IV Environment, Assessment & Evaluation (Parts B+C), Version 2.0, Reference: ECHA-17-G-23-EN*; European Chemicals Agency (ECHA): Helsinki, Finland, 2017; ISBN 978-92-9020-151-9.
20. European Chemicals Agency (ECHA). *Guidance on Information Requirements and Chemical Safety Assessment Part E: Risk Characterisation, Version 3.0, Reference: ECHA-2016-G-04-EN*; European Chemicals Agency (ECHA): Helsinki, Finland, 2016; ISBN 978-92-9495-055-0.
21. European Commission. *Technical Guidance Document on Risk Assessment*; Part 2 EUR 20418 EN/2; Joint Research Centre: Brussels Belgium; European Communities: Luxembourg, 2003.
22. Oekotoxzentrum. ad hoc EQS List Swiss Ecotoxcentre. Available online: https://view.officeapps.live.com/op/view.aspx?src=https%3A%2F%2Fwww.ecotoxcentre.ch%2Fmedia%2F195581%2Fquality-criteria-used-by-oz-for-aquatic-risk-assessment.xlsx&wdOrigin=BROWSELINK (accessed on 6 July 2023).
23. Norman. Norman Database System. Available online: https://www.norman-network.com/nds/ (accessed on 6 July 2023).
24. Monte, H.M.D.; Albuquerque, A. *Reutilização de Águas Residuais*; ERSAR: Lisboa, Portugal, 2010.
25. Van Bruggen, A.H.C.; He, M.M.; Shin, K.; Mai, V.; Jeong, K.C.; Finckh, M.R.; Morris, J.G., Jr. Environmental and health effects of the herbicide glyphosate. *Sci. Total Environ.* **2018**, *616–617*, 255–268. [CrossRef]
26. Carlesa, L.; Gardona, H.; Josepha, L.; Sanchísb, J.; Farréb, M.; Artigasa, J. Meta-analysis of glyphosate contamination in surface waters and dissipation by biofilms. *Environ. Int.* **2019**, *124*, 284–293. [CrossRef]
27. Sistema de Gestão das Autorizações de Produtos Fitofarmacêuticos (SIFITO). Available online: www.sifito.dgav.pt (accessed on 6 July 2023).
28. Pesticide Properties DataBase, PPDB. Available online: http://sitem.herts.ac.uk/aeru/ppdb/en (accessed on 6 July 2023).
29. Barbieri, M.V.; Peris, A.; Postigo, C.; Moya-Garcés, A.; Monllor-Alcaraz, L.S.; Rambla-Alegre, M.; Eljarrat, E.; Alda, M.L. Evaluation of the occurrence and fate of pesticides in a typical Mediterranean delta ecosystem (Ebro River Delta) and risk assessment for aquatic organisms☆. *Environ. Pollut.* **2021**, *274*, 115813. [CrossRef] [PubMed]
30. Associação de Regantes e Beneficiários do Vale do Sorraia, ARBVS. Available online: www.arbvs.pt (accessed on 6 July 2023).
31. Directive 2013/39/EU of the European Parliament and of the Council of 12 August 2013 amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy. *Off. J. Eur. Union* **2013**, *226*, 1–17.
32. Agência Portuguesa do Ambiente (APA). Critérios Para a Classificação das Massas de Água—Ficha Técnica. Ficha Técnica, DRH/DEQA. Available online: https://www.apambiente.pt/sites/default/files/_Agua/DRH/ParticipacaoPublica/PGRH/2022-2027/3_Fase/PGRH_3_SistemasClassificacao.pdf (accessed on 6 July 2023).

33. Commission Implementing Decision (EU). 2015/495 of 20 March 2015 establishing a watch list of substances for Union-wide monitoring in the field of water policy pursuant to Directive 2008/105/EC of the European Parliament and of the Council. *Off. J. Eur. Union* **2015**, *78*, 40–42. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32015D0495&rid=4 (accessed on 6 July 2023).
34. Commission Implementing Decision (EU). 2018/840 of 5 June 2018 establishing a watch list of substances for Union-wide monitoring in the field of water policy pursuant to Directive 2008/105/EC of the European Parliament and of the Council and repealing Commission Implementing Decision (EU) 2015/495. *Off. J. Eur. Union* **2018**, *141*, 9–12. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32018D0840 (accessed on 6 July 2023).
35. Commission Implementing Decision (EU). 2022/1307 of 22 July 2022 establishing a watch list of substances for Union-wide monitoring in the field of water policy pursuant to Directive 2008/105/EC of the European Parliament and of the Council. *Off. J. Eur. Union* **2022**, *197*, 117–120. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32022D1307 (accessed on 6 July 2023).
36. Brito, I.P.F.S.; Tropaldi, L.; Carbonari, C.A.; Velini, E.D. Hormetic effects of glyphosate on plants. *Pest Manag. Sci.* **2018**, *74*, 1064–1070. [CrossRef]
37. United States Environmental Protection Agency (US EPA). Ecotox Knowledgebase. Available online: https://cfpub.epa.gov/ecotox/ (accessed on 6 July 2023).
38. Cedergreen, N. Quantifying Synergy: A Systematic Review of Mixture Toxicity Studies within Environmental Toxicology. *PLoS ONE* **2014**, *9*, e96580. [CrossRef] [PubMed]
39. Weisner, O.; Frische, T.; Liebmanna, L.; Reemtsma, T.; Roß-Nickoll, M.; Schäfer, R.B.; Schäffer, A.; Scholz-Starke, B.; Vormeier, P.; Knillmann, S.; et al. Risk from pesticide mixtures—The gap between risk assessment and reality. *Sci. Total Environ.* **2021**, *796*, 149017. [CrossRef]