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Sorption–Desorption of Imazamox and 2,4-DB in Acidic Mediterranean Agricultural Soils and Herbicide Impact on Culturable Bacterial Populations and Functional Diversity

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Abstract: In this study, we investigated the sorption–desorption behavior of imazamox (IMZ) and 2,4-DB (DB) in two typical acidic Mediterranean agricultural soils and the impact of these herbicides on culturable soil bacterial populations, enzyme activities and functional diversity when applied at concentrations higher than recommended doses (10×, 50×, 500×). Herbicide sorption was similar in both soils and IMZ was less retained compared to DB (~0.5 vs. 40 μg g^{-1} soil, respectively). IMZ desorption was remarkable (70–100%) while that of DB was more limited, i.e., ~40%. Three days after spiking (*DAS*), IMZ and DB significantly increased the number of soil-culturable heterotrophic bacteria, actinomycetes and *Pseudomonas* spp., soil respiration and the potential catabolic capacity of soil microbial communities. Soil dehydrogenase activity increased by ~56–70% in IMZ-treated soils while being reduced by ~33–41% in DB-treated ones. β-glucosidase activity showed a soil-dependent behavior, while the pattern of C source utilization suggested a change of soil microbial community structure after herbicide (especially DB) spiking. At 30 *DAS*, the herbicides' impact on soil microorganisms, enzyme activity and functional diversity was still visible. Moreover, a toxic effect of DB (at 50× and 500×), but not IMZ, was recorded vs. *Rhizobium sullae*, the bacterial symbiont of *Hedysarum coronarium*. The obtained results indicated that IMZ and DB are poorly sorbed and highly desorbed by both soils. Moreover, at the tested concentrations, IMZ and DB can have short- and medium-term impacts on the microbial component and the related activity of the investigated soils, likely affecting a range of ecosystem services provided by soil microorganisms.

Keywords: herbicides; soil microbial community; dehydrogenase activity; β-glucosidase activity; Biolog community-level physiological profile; *Rhizobium sullae*

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1. Introduction

Herbicides' extensive application in agricultural systems is a matter of environmental concern due to their potential hazardous impact on non-target organisms, e.g., soil microorganisms, and soil biological processes key to agroecosystem functioning [1]. Although many herbicides are not directly applied to soil, a certain amount can reach its surface during application, thus affecting microbial populations and their activity [2]. Moreover, given the growing use of herbicides, the impact of overuse and of concentration "hot spots" originating from uneven application and/or spill scenarios should be a matter of concern, as they could negatively influence soil biological fertility and health (e.g., organic matter decomposition, nutrient cycling, plant growth stimulation, plant disease suppression, degradation of toxic substances) [3]. This is a well-known possibility, and that is why soil microorganisms were identified as a specific protection goal for pesticides environmental risk assessment by the European Food Safety Authority [4]. As they respond promptly to environmental changes, microorganisms are considered more efficient indicators than

physical and chemical parameters of the impact of agricultural practices on soil quality [5]. For instance, microbial abundance and activity have been used as indicators of soil health, while the analysis of carbon source utilization patterns (functional diversity) effectively detected ecosystem perturbations due to pollutants [6,7]. Additionally, soil enzymes, whose activity is generally affected by pollutants and/or soil management practices, are recognized as valid indicators of soil quality [1,5]. For instance, enzymes such as dehydrogenases, β-glucosidase and urease were revealed as reliable indicators of herbicide impact on soil functioning [1].

2,4-DB (4-(2,4-dichlorophenoxy)butanoic acid; DB) and imazamox (5-(methoxymethyl)-2-(4-methyl-5-oxo-4-propan-2-yl-1H-imidazol-2-yl)pyridine-3-carboxylic acid; IMZ), are very commonly used herbicides in agriculture. The first (DB) belongs to chlorophenoxyalcanoic acid herbicides which act similarly to endogenous auxin [8]. DB is employed in pre- and post-emergence to control annual and perennial broad-leaved weeds of legume species, cereals, grassland, orchards and in forestry [9]. The second (IMZ) belongs to imidazolinones which act by inhibiting the acetolactate synthase (ALS), responsible for the biosynthesis of branched-chain amino acids such as valine, leucine and isoleucine [10]. Additionally, IMZ is used in post-emergence to control grassy and broad-leaved weeds in various forage and crop legumes (e.g., alfalfa, beans, peanuts) and in forestry [9]. As previously highlighted, some phenoxyacid herbicides closely related to DB (such as 2,4-D) can be promptly catabolized by soil microorganisms, producing the growth of selected microbial populations, and short-term changes of soil bacterial populations were reported following 2,4-D application [11]. Despite substantial knowledge being available on the influence of 2,4-D on soil microbial communities and its degradation by selected bacterial strains or consortia, this is not the case for other closely related herbicides such as DB [12]. In particular, essentially no information can be found for this latter herbicide. Likewise, the impact of IMZ on soil microbial communities is poorly studied: for instance, its influence on soil microbial biomass and related C source utilization was investigated by Lupwayi et al. [2], mainly in comparison with other herbicides. However, in this latter study, IMZ was combined with imazethapyr (another imidazolinone herbicide), making it hard to identify the impact of each compound on soil microorganisms. More recently, Sim et al. [13] showed a significant inhibition of soil β-1,4-N-acetylglucosaminidase activity after IMZ application at the recommended dose. In the same study, a low or insignificant IMZ effect was observed at the microbial community level (e.g., on bacterial and fungal abundance), although a significant alteration of nitrifying bacterial community was highlighted.

Moreover, given that DB and IMZ are commonly used for weed management in legumes cultivation, their influence on symbiotic N-fixing bacteria associated with those leguminous species (i.e., rhizobia) should be of outmost importance, while in fact it was poorly investigated. Very few reports are available on the impact of DB and IMZ on rhizobia [14,15], and their effect on several rhizobial species associated with widely grown legumes, e.g., *Rhizobium sullae,* the common bacterial symbiont of Sulla (*Hedysarum coronarium* L.) [16], is essentially unknown.

Overall, the net influence of herbicides on soil microorganisms is influenced by sorption and desorption processes occurring in soil. These latter processes ultimately govern the herbicides' concentration in the soil solution, their mobility and potential bioavailability, as well as their chemical and biological degradability [17–20]. On the other hand, soil physicochemical properties greatly influence sorption and desorption processes [21]. For polar, ionizable chemicals such as imidazolinone herbicides (e.g., IMZ; pK_{a1} = 2.3, pK_{a2} = 3.3, pK_{a3} = 10.8), soil pH is critical for sorption processes: as soil pH decreases, IMZ becomes less ionic, and a greater amount of herbicide is adsorbed. At the same time, soil organic matter can contribute to IMZ retention at low pH values by means of greater hydrophobic interactions [22]. Similarly, phenoxyalkanoic acids retention (e.g., DB, pK_{a1} = 4.95) was also negatively correlated with increasing pH, and positively correlated with soil organic matter content [23]. Although soil pH and organic matter are critical for DB and IMZ mobility, the knowledge of sorption–desorption phenomena occurring in a specific soil is essential

to understand the herbicides' fate, as well as to elucidate their potential impact on soil microbiota and their functioning.

Therefore, this study was aimed to investigate: (i) the sorption–desorption behavior of IMZ and DB in two typical acidic Mediterranean agricultural soils with different physicochemical characteristics; (ii) the short- and medium-term impact of the herbicides (i.e., 3 and 30 days after spiking) on soil-culturable microorganisms, *Rhizobium sullae* growth, soil respiration, dehydrogenase and β-glucosidase activities and (iii) their impact on the structure of soil microbial communities when applied at concentrations higher than the recommended dose.

2. Materials and Methods

2.1. Origin of Soils and Physicochemical Analyses

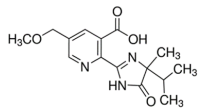
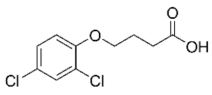
Different soil samples (n = 10 per farm, 1 kg each; 0–25 cm depth) were randomly collected from two pasture-based dairy ewes agricultural farms (farm A: 39°03′53″ N; 8°43′23″ E; farm F: 39°10′02″ N; 8°27′59″ E) located in the Sulcis–Iglesiente district (~2500 km^2; Sardinia, Italy). These two soils were never treated with DB and IMZ and are representative of approx. 3950 million ha of arable land worldwide characterized by soil acidity (soil A and F showed pH values of 5.95 and 6.40, respectively) and limited fertility attributes, e.g., low nitrogen and/or low P availability [24].

Soil samples were pooled in the laboratory according to their origin (i.e., soil A and soil F), and an aliquot was air-dried and sieved to <2 mm before soil physicochemical analyses, while the remaining field-moist soil was used for microcosm set-up. Particle size was determined with the pipette method [25] and allowed us to classify soil A as silt loam (USDA textural classification; 44% sand, 50.7% silt, 5.3% clay) and soil F as sandy loam (64.5% sand, 34.2% silt, 1.3% clay). Soil pH and electric conductivity were measured in 1:2.5 (w/v) solid to water suspension; cation exchange capacity (CEC) and exchangeable K were determined using the $BaCl_2$ and triethanolamine method [26]. Extractable P was determined using the Olsen method [26]. Total organic C and total N were determined using a CHN analyzer (Leco CHN 628) with oatmeal Leco part No. 502-276 as calibration sample.

2.2. Sorption–Desorption Experiments

The herbicides IMZ (99.9% purity) and DB (99.5% purity) were supplied by CPA Chem, Stara Zagora, Bulgaria; selected IMZ and DB physicochemical properties are reported in Table 1. All the solvents of HPLC grade (Carlo Erba Reagenti, Milano, Italy) were used without any additional purification.

Table 1. Selected physicochemical properties of imazamox (IMZ) and 2,4-DB (DB), recommended application rates and mode of action.

	Chemical Structure	pK_a	Water Solubility (g L^{-1})	Log K_{ow}	Recommended Application Rate	Mode of Action
IMZ		2.3;3.3;10.8	4.16	0.73	40 g ha^{-1}	Acetolactate synthase inhibitor [10]
DB		4.95	$46 \cdot 10^{-3}$	3.53	1400 g ha^{-1}	Auxin mimic after conversion to 2,4-D [8]

Herbicide sorption isotherms on both soils were obtained using a batch equilibrium protocol [20,27]. In particular, triplicate samples of 2.5 g of soil (oven-dried at 105 °C) were equilibrated in polyallomer centrifuge tubes, previously checked to verify that they did not adsorb the herbicides, with 5 mL of aqueous herbicide solution. The initial concentration of herbicide solutions ranged between 1 and 5 μM (IMZ), and 42 and 168 μM (DB), respectively. The highest herbicide concentrations corresponded to 3 and 100 μg g^{-1} dry soil for IMZ and

DB, respectively (these concentrations were included in the microcosm study). The tubes were shaken in an end-over-end shaker (70 rpm) at 25 ± 2 °C until equilibrium was reached. Preliminary kinetic batch studies (Supplementary Materials) indicated that the equilibrium was reached, in both soils, within 7 h for IMZ and 24 h for DB and no degradation took place within the aforementioned times. After equilibration, the suspensions were centrifuged at 3500 rpm for 10 min and the supernatant was pipetted off, filtered (0.2 μm, PTFE D. 25 Albet-Jacs) and analyzed immediately (see HPLC analyses). The herbicide amount sorbed was calculated from the difference between the initial and final concentrations of herbicide in solution.

Desorption was performed at the highest initial herbicide concentration used in the sorption isotherms, as previously described by Pinna et al. [27]. Immediately after the sorption process, 2.5 mL of the supernatant solution was withdrawn, and the amount of sorbed herbicide calculated. The remaining slurry was again brought to 5 mL by the addition of 2.5 mL of water, equilibrated for 24 h, and centrifuged as described above. Then, 2.5 mL of the supernatant was withdrawn, and the amount of sorbed herbicide calculated again. These steps (supernatant withdrawing and replacing and re-equilibrating) were repeated four times consecutively.

2.3. HPLC Analyses

The HPLC system was assembled as follows: a Waters 1515 pump equipped with a Waters 2487 UV–Vis programmable detector operating at 254 nm, Breeze chromatography software, a μBondapak C18 analytical column (10 μ, 3.9 × 300 mm) eluting with acetonitrile plus water (30 + 70 by volume for IMZ, and 70 + 30 by volume for DB, respectively) previously brought to pH 2.7 with phosphoric acid at a flow rate of 0.5 mL min^{-1}. The retention times for IMZ and DB under the aforementioned chromatographic conditions were 8.19 and 8.05 min, respectively.

The concentration of IMZ and DB was quantified by HPLC using external standards (CPA chem, Certified Reference Material, IMZ + 99.5% certified purity, DB + 99.9% certified purity). The analytical method was validated with good linearity R^2 = 0.998 (IMZ) and 0.999 (DB). LOD and LOQ were calculated to be 0.10 mg L^{-1} and 0.33 mg L^{-1} (IMZ) and 0.12 mg L^{-1} and 0.40 mg L^{-1} (DB), respectively.

2.4. Processing of Sorption–Desorption Data

Sorption–desorption data were fitted to the logarithmic form of the Freundlich equation (i.e., the most common model used to describe herbicide sorption on heterogeneous surfaces) [28,29]:

$$\log C_s = \log K_{ads} + 1/n_{ads} \log C_e$$

where C_s (μmol kg^{-1}) is the amount of herbicide sorbed by soil, C_e (μM) is the equilibrium concentration in solution, and log K_{ads} and $1/n_{ads}$ are empirical constants representing the intercept and the slope of the isotherm, respectively.

The hysteresis coefficient (H) for the sorption–desorption isotherms was calculated according to the formula:

$$H = (1/n_{des})/(1/_{nads})$$

where $1/n_{ads}$ and $1/n_{des}$ are the Freundlich slopes obtained for the sorption and desorption isotherms, respectively.

2.5. Microcosm Set-Up

Pooled field-moist soil samples (A and F) were used to set up different microcosms (300 g of field-moist soil each) which were arranged in plastic containers with lids loosely fitted to allow aeration. According with Sim et al. [13], a total of forty-eight microcosms were pre-incubated for one week in the dark at 15% of humidity content (*w*/*w*). Soil microcosms were then spiked with the herbicides at 3, 15 and 150 μg g^{-1} (IMZ), and 100, 500 and 5000 μg g^{-1} (DB). These concentrations were higher than respective recommended

doses (e.g., 0.3 and 10 μg g^{-1} soil for IMZ and DB, respectively, under the following assumptions: IMZ and DB mostly localized in the first one cm of soil, soil bulk density 1.3 g mL^{-1}, weight of 1 hectare 1.3×10^5 kg), and were used to simulate potential uneven distribution of herbicides in soil "hot spots" after application [30] (i.e., 3 and 100 μg g^{-1} for IMZ and DB, respectively), as well as very high herbicides concentrations possibly resulting from spills, faulty spray equipment, waste disposal, tank filling and washing [3,31] (i.e., 15–150, and 500–5000 μg g^{-1} for IMZ and DB, respectively). Commercial IMZ- and 2,4-DB-based pure products contain approx. 40 and 400 g L^{-1} of IMZ and 2,4-DB, respectively. They should be diluted up to 600 and 1000 L with water, respectively, before distribution to 1 ha surface (e.g., to alfalfa). This implies that a possible 1 mL spill of the pure products contains approx. 600- and 1000-fold concentrated IMZ and 2,4-DB, respectively.

Three microcosms were spiked for each herbicide dose and soil type, and triplicate untreated microcosms were used as control (2 soil types × 2 herbicides × 4 spiking rates including controls [i.e., 0 μg g^{-1}] × 3 replicates = 48 microcosms). Herbicides were applied as water solution and the humidity content was adjusted to 20% and kept constant during the entire length of the experiment. After spiking, soil within each microcosm was carefully homogenized and incubated in the dark at 22 °C for 30 days. Three and thirty days after spiking (*DAS*), soil samples were collected for microbial and biochemical analyses to investigate possible short- and medium-term effects of IMZ and DB.

2.6. Culturable Microorganisms

Total culturable heterotrophic bacteria, actinomycetes and *Pseudomonas* spp. were enumerated in duplicate soil samples (10 g each) collected from each microcosm at 3 and 30 *DAS* using the serial dilution and spread plate method as previously described [32]. Briefly, soil samples were dispersed in 90 mL of sterile (autoclaved) pyrophosphate solution (2 g L^{-1}) and shaken at 150 rpm for 30 min. Serial (ten-fold) dilutions were then obtained using sterile 0.89% (*w*/*v*) NaCl solution and 150 μL aliquots from each dilution were inoculated and spread on duplicate plates containing selected microbiological media. Solidified 1:10 strength TSA (Tryptic Soy Agar, Microbiol, Cagliari, Italy), AIA (Actinomycete Isolation Agar, Difco, Milan, Italy) and PSA (Pseudomonas Selective Agar, Microbiol, Cagliari, Italy) were used for the respective microbial counts. Colony count, carried out after incubation of the plates at 28 °C for 48 (total culturable bacteria) and 72 h (actinomycetes and *Pseudomonas* spp.), was reported as Log_{10} CFU g^{-1} soil.

2.7. Community-Level Physiological Profile

The community-level physiological profile (CLPP) was determined in each microcosm using Biolog EcoPlatesTM (Biolog Inc., Hayward, CA, USA). Aliquots of 20 mL deriving from the 100-fold dilutions of soil samples (used for enumeration of culturable microorganisms) were centrifuged at 500 rpm for 5 min, filtered with sterile (autoclaved) Whatman 1 filter paper and used to inoculate Biolog EcoPlate wells (120 μL in each well). The Biolog EcoPlate is a 96-well microtiter plate containing a triplicate set of 31 carbon sources (and a control well with no carbon) of soil/rhizosphere relevance [7]. Carbon source utilization by the inoculated microbial community is revealed by purple color formation due to the reduction of tetrazolium violet which is present in each well. After inoculation, Biolog EcoPlates were incubated in the dark for 96 h at 28 °C and carbon source utilization was monitored, at time zero and every 24 h, by recording the absorbance values at 590 nm (OD_{590}) with a Biolog MicroStation™ reader. Biolog EcoPlate data (i.e., raw OD_{590} readings) were processed as reported by Diquattro et al. [7] to obtain: (i) the average well color development (AWCD), i.e., a measure of the potential catabolic capacities of the microbial community; and (ii) the richness value, i.e., the number of substrates catabolized by each microbial community ($OD_{590} > 0.25$) [33]. The use of the different carbon source guilds within the EcoPlate ($n = 5$; i.e., sugar and sugar derivates, sugar phosphates, carboxylic acids, amino acids and polymers) was also obtained and expressed as percentage of total substrate utilization in the plate. Finally, standardized OD_{590} values (i.e., OD_{590}/AWCD) were used

for principal component analysis (PCA). All the Biolog-derived data presented refer to the 72 h incubation time, as this time point allowed the best discrimination among treatments.

2.8. Soil Basal Respiration

Duplicate soil samples (20 g each) from each microcosm were separately placed in plastic containers inside stoppered glass jars together with 4 mL of 1 N NaOH which served to trap evolved CO_2. Each jar was then incubated in the dark at 25 °C for 10 days. A total of five jars without soil were used as control. After incubation, the NaOH was removed from the jar and carbonate precipitated by adding 8 mL of 0.75 N $BaCl_2$. The NaOH excess was finally titrated with 0.1 N HCl until pH 8.8 and the CO_2 evolved was reported as mg CO_2 kg^{-1} soil h^{-1} [34].

2.9. Soil Enzyme Activities

Soil dehydrogenase (DHG) and β-glucosidase (GLU) activities were determined in duplicate soil samples from each microcosm. DHG was determined colorimetrically by quantifying the concentration of triphenylformazan (TPF) produced after incubation of soil samples with triphenyltetrazolium chloride [35]. GLU was estimated by quantifying the p-nitrophenol (p-NP) released following incubation of soil samples with p-nitrophenyl glucoside [35].

2.10. Growth Inhibition of R. sullae

Growth inhibition of several *R. sullae* strains, notably WSM1592 [16] and S1_H1A, S4_11, S6_7 (belonging to the microbial collection of the Dipartimento di Agraria, University of Sassari, Italy), was assessed on yeast mannitol agar (YMA) plates in the presence of IMZ and DB [36]. For each rhizobial strain, different 10 µL aliquots of saturated YM broth culture were inoculated on the surface of YMA plates containing the same DB and IMZ concentrations used in soil microcosms (i.e., 3, 15 and 150 µg g^{-1} for IMZ and 100, 500 and 5000 µg g^{-1} for DB). Control YMA plates with no herbicide were included. Inoculated plates were incubated at 28 °C for 48 h, then colony presence and size were visually examined.

2.11. Statistical Analysis of Data

The statistical analysis was carried out separately within each considered soil (A or F) and within each considered time-period (3 or 30 *DAS*) to assess the impact of herbicide concentrations on the following variables: microbial counts, Biolog-derived indexes (AWCD and richness) and C source consumption, enzyme activities and soil respiration. The variables were analyzed by using the *gls* and *varIdent* functions of the *nlme* R package, while the post hoc comparisons were carried out using the *glht* function of the *mutcomp* R package. In order to be more conservative, the *p*-values of the multiple comparisons between treatments were adjusted using the Bonferroni correction ($p < 0.05$). Pearson correlation between Log_{10}-transformed DB and IMZ concentrations and microbial and biochemical data was also determined. This latter analysis was carried out using the NCSS software (v. 07.1.21). Past3 (v. 3.11) was used for PCA analysis of standardized Biolog EcoPlate OD_{590} values.

Pesticide information of water solubility, pKa and octanol/water partition coefficient (K_{ow}) was obtained from Hazardous Substances Data Bank (HSDB) (information available at https://pubchem.ncbi.nlm.nih.gov/compound/86137 and https://pubchem.ncbi.nlm.nih.gov/compound/1489; accessed on 24 march 2022).

3. Results and Discussion

3.1. Herbicide Sorption–Desorption

Herbicides sorption–desorption was investigated in A and F soils (Table 2) to gain information on their potential bioavailability.

Table 2. Selected chemical properties of A and F soils (mean values ± SE).

Chemical Analyses	Soil A	Soil F
pH	5.95 ± 0.03	6.40 ± 0.01
EC (μS cm^{-1})	480 ± 5	299 ± 1
Cation exchange capacity (CEC, $cmol_{(+)}$ kg^{-1})	16 ± 0.3	19 ± 0.2
Total organic matter (OM, %)	2.54 ± 0.03	2.19 ± 0.05
Total nitrogen (g kg^{-1})	1.35 ± 0.08	0.57 ± 0.05
C/N	11 ± 0.5	22 ± 1.8
Extractable P (mg kg^{-1})	1.6 ± 0.5	3.3 ± 0.6
Exchangeable K (mg kg^{-1})	96.29 ± 1.55	145.53 ± 1.85
USDA textural classification	Silt loam	Sandy loam

The sorption behavior of IMZ and DB on both soils is well described by the empirical Freundlich equation ($R^2 \geq 0.96$; Table 3). In particular, as suggested by the low K_{ads} values, the extent of IMZ sorption was very limited in both soils (approx. 12% when applied at 3 μg IMZ g^{-1} soil). IMZ is an acidic molecule (Table 1), and at pH values of A and F soils (5.95 and 6.40, respectively), more than 99% is present in anionic form, and then weakly bound or repelled by the negatively charged soil constituents [37]. Nevertheless, the slight sorption observed suggested that a lipophilic effect was probably acting in both soils, and/or that a certain interaction with positively charged sites on soil colloids (e.g., surficial -OH_2^+ groups of Fe and/or Al oxyhydroxides, protonated ammino groups R-NH_3^+ of the organic matter) was likely occurring. The $1/n_{ads}$ value < 1 in F soil (typical of L-type sorption isotherms [38]; Table 3 and Figure S1) indicated a relatively higher affinity of IMZ for soil at low concentrations. As the herbicide concentration increased, the extent of sorption progressively decreased, likely because of the difficulty for IMZ to find free sorption sites. On the other hand, the Freundlich parameter $1/n_{ads} > 1$ in A soil (typical of S-type adsorption isotherms; Table 3 and Figure S1) indicated a lower IMZ affinity for soil at low herbicide concentrations and greater sorption as the herbicide concentrations increased. As pointed out by Sposito [39], this was likely because of the cooperative interactions among sorbed organic species that stabilized the sorbate on soil solid surfaces enhancing the IMZ affinity for the latter.

Table 3. Freundlich parameters (mean values ± SE) for imazamox (IMZ) and 2,4-DB (DB) sorption–desorption in A and F soils.

Soil	Herbicide	K_{ads} [a]	$1/n_{ads}$	R^2	K_{des} [a]	$1/n_{des}$	R^2	H [b]
A	**IMZ**	0.18 (±0.04)	1.11 (±0.05)	0.956	0.57 (±0.06)	0.86 (±0.02)	0.996	0.77
F		0.22 (±0.03)	0.79 (±0.01)	0.962	0.22 (±0.05)	1.07 (±0.05)	0.968	1.35
A	**DB**	19.30 (±0.94)	0.54 (±0.02)	0.974	8.49 (±0.16)	0.30 (±0.03)	0.980	0.55
F		7.62 (±0.11)	0.72 (±0.03)	0.962	1.89 (±0.18)	0.40 (±0.04)	0.978	0.56

[a] μmol $^{(1-1/n)}$ L $^{1/n}$ kg^{-1}; [b] hysteresis coefficient $H = (1/n_{des})/(1/n_{ads})$.

Hysteresis occurs when desorption isotherms do not coincide with sorption ones. A hysteresis coefficient (H) value equal to 1 means that desorption proceeds as quickly as sorption does and hysteresis is absent. Conversely, H < 1 indicates that the rate of desorption is lower than the rate of sorption and that hysteresis takes place. In soil A, the calculated H value (i.e., 0.77) suggested a limited hysteresis effect, indicating that the IMZ sorbed is almost totally desorbed (i.e., after three desorption steps, approximately 70% of the initially retained product was desorbed) and, therefore, potentially bioavailable. On the other hand, the $1/n_{des}$ value obtained for IMZ desorption in soil F was higher than the corresponding $1/n_{ads}$ value (Table 3), suggesting a higher rate of IMZ desorption compared to sorption (i.e., a negative hysteresis; H > 1). This also implied an easier release of adsorbed IMZ from soil (i.e., the herbicide was totally desorbed after three desorption steps). This different desorption behavior in the two soils was probably due to a different

interaction strength between IMZ and soil surfaces, which is expected to be reduced in soil F due to a higher pH value. These results agree with Aichele and Penner, who observed a greater percentage of imidazolinones in the soil solution as the pH increased [40].

Like other phenoxyalkanoic acid herbicides, DB was poorly sorbed by A and F soils (even if it was much more sorbed than IMZ; Table 3 and Figure S1), and the extent of sorption was negatively correlated with soil pH (59 and 52% DB was sorbed by soil A and F, respectively, when the herbicide was applied at 100 μg g^{-1} soil). DB is a weak organic acid with a pK_a = 4.95 (Table 1) and, like IMZ, it predominantly occurred in anionic form in both soils which showed pH values of 5.95 (soil A) and 6.40 (soil F). According to Werner et al. [23] and Góngora-Echeverría et al. [29], DB sorption was likely due to partitioning of the anionic herbicide into soil organic matter (i.e., A soil > F soil), which is increasingly important at low soil pH. Moreover, competition phenomena for the same soil sorption sites (e.g., involving phosphate anions which were 2-fold more abundant in soil F; Table 2) could have contributed to higher DB sorption in soil A. L-type isotherms were recorded on both soils ($1/n_{ads} < 1$; Table 3 and Figure S1), indicating high affinity between DB and soils and a progressive saturation of sorbent sites.

As suggested by the hysteresis coefficient values calculated for DB (0.55 and 0.56 in A and F soils, respectively; Table 3), the herbicide sorption was hysteretic on both soils. Moreover, the effectiveness of desorption (i.e., K_{des} and H; Table 3) was positively correlated with soil pH and negatively correlated with organic matter content. After three desorption steps, only 41 and 38% of the sorbed DB was recovered in A and F soils, respectively, in agreement with the reported desorption of phenoxyalkanoic acids from soils containing >1% organic matter [41].

Taken together, sorption/desorption results showed that IMZ (more polar and soluble in water than DB) was the least sorbed and most easily desorbed herbicide in both soils, while DB was relatively more sorbed and retained. From an environmental point of view, this could have worrying consequences for soil microbiota deriving from the relevant presence of both herbicides (particularly IMZ) in the soil solution. This is even more relevant in cases of herbicide overuse and/or spill scenarios, faulty spray equipment, waste disposal, washing spill and others [3,31].

3.2. Influence of DB and IMZ on Selected Soil Culturable Bacterial Populations and Total Bacteria

The number of total culturable heterotrophic bacteria (CHB) significantly increased in both soils after 3 days since IMZ and BD spiking (Figure 1). This was more relevant in soil F where (on average) approx. 20% increase in Log CFU g^{-1} soil were detected in IMZ- and DB-treated microcosms, vs. approx. 5 and 8% increases in soil A, respectively. Positive significant correlations were highlighted in both soils between CHB number and herbicide concentrations (r = 0.61–0.93, p = 0.01–10^{-6}; Table S1). Similar results were observed for actinomycetes and *Pseudomonas* spp., whose numbers significantly increased at 3 days after IMZ and DB addition, especially in soil F (Figures S2 and S3). Additionally, in these cases, positive significant correlations were highlighted in both soils between microbial counts and herbicide concentrations (r = 0.57–0.95, p = 0.02–10^{-6}; Table S1).

These results clearly indicated the presence in both soils of different readily active bacterial populations [42] capable of utilizing IMZ and DB as a carbon and energy source, considerably increasing their number. Apparently, this was more relevant in soil F, likely because of a higher abundance of resident herbicide-degrading bacteria and/or better conditions for bacterial growth (e.g., higher soil pH and greater content of available P; Table 2). Similar increases in total heterotrophic bacteria were reported by Cuadrado et al. [43] at 14 days after DB addition at 500 μg g^{-1} soil, and comparable findings were also noted by Zabaloy et al. [11] and Zhang et al. [44] studying the impact of herbicides closely related to DB on soil microbial community (i.e., 2,4-D and 2,4-D butyl ester).

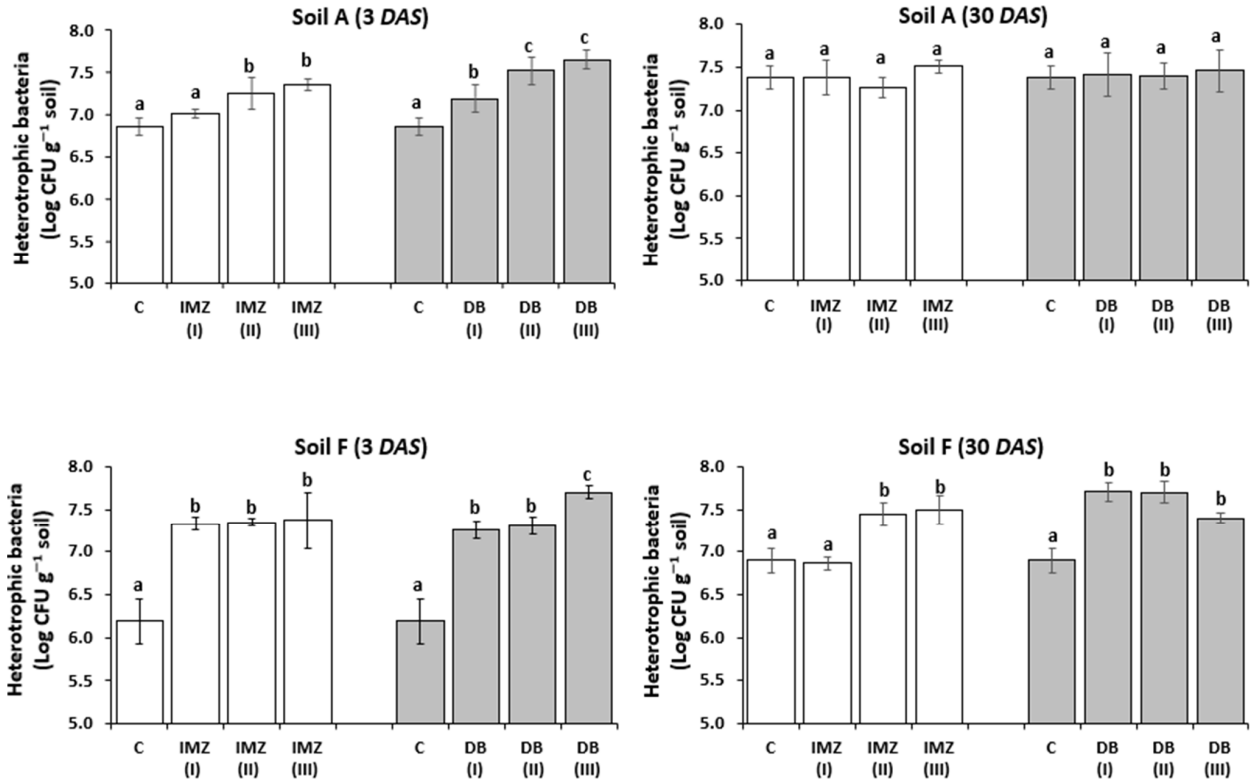


Figure 1. Influence of different concentrations of imazamox (IMZ) and 2,4-DB (DB) on the number of soil total culturable heterotrophic bacteria (mean values ± SE) at 3 and 30 days after herbicide spiking (*DAS*). IMZ(I), (II) and (III) refer to 3, 15, 150 μg IMZ g^{-1} soil, respectively. DB(I), (II) and (III) refer to 100, 500, 5000 μg DB g^{-1} soil, respectively. C, control (untreated) soil. For each soil and herbicide, different letters on top of bars indicate statistically significant differences (Bonferroni corrected p-value < 0.05).

Strains of *Pseudomonas* capable of utilizing DB as a sole source of carbon and energy were previously isolated from different soils [12,45,46]. In this regard, our results seem to support the occurrence of similar strains in soils A and F, whose abundance can be greatly increased by herbicide addition (Figure S3). No information is available to date on DB impact on soil-culturable actinomycetes; however, Zhang et al. [44] showed that 100 μg g^{-1} soil of 2,4-D butyl ester significantly increased their number at 1 and 8 *DAS*, and that a significant decrease occurred for 1000 μg g^{-1} dose. This partly supports our findings (i.e., a stimulating effect of DB on culturable actinomycetes which was noted up to 5000 μg DB g^{-1} soil [(DB(III)]; Figure S2) highlighting possible differences between the two herbicides and/or between the composition of the soil microbial communities investigated in this study and in that of Zhang et al. [44].

Even if poor knowledge exists on IMZ impact on soil microbial community, Vasic et al. [47] reported a significant decrease in soil-culturable bacteria at 7 days after IMZ addition (at the recommended dose, approx. 40 g ha^{-1}) in two forest soils. In the same study, an overall stimulating effect of IMZ on soil actinomycetes was also recorded. This partly supports our results [i.e., differently from Vasic et al. [47], culturable bacteria were significantly increased by IMZ; Figure 1] and strongly suggests specific soil-dependent herbicidal effects on the resident microbial communities [13]. No information is available on possible IMZ effect on soil-culturable *Pseudomonas* spp.; nevertheless, their potential ability to degrade this herbicide was previously reported [48].

Despite the relatively short half-life of 2,4-DB and IMZ (approx. 4 and 10 days, respectively) in the soil environment [12,40], residual herbicidal effects were still apparent for all microbial groups targeted at 30 *DAS* (Figure 1, Figures S2 and S3). At this time point,

the CHB number was significantly higher in soil F treated with IMZ and DB (vs. respective control soils), especially when higher doses were applied (Figure 1). Positive significant correlations between the number of culturable heterotrophic bacteria and the concentration of both herbicides were still recorded in this soil (r = 0.57–0.70, p = 0.02–0.002; Table S1). No differences were recorded in soil A for this microbial population (Figure 1). On the contrary, residual DB and IMZ effects on culturable actinomycetes were present in this latter soil (but not in soil F, except for IMZ at the highest concentration) treated with the higher herbicide doses (Figure S2), while a consistent positive effect of DB on *Pseudomonas* spp. was recorded in both soils (Figure S3). These results suggest a relatively long dissipation time of DB and IMZ in the two soils, as also reported by Cuadrado et al. [43] and Kaur et al. [49], whose effects on soil-culturable microorganisms were still visible after 30 days. As pointed out by sorption/desorption trials, DB and especially IMZ were poorly sorbed by the two soils investigated (Table 3), and IMZ was also completely desorbed in soil F. Together with the high herbicide doses investigated, this could possibly explain the generally increased number of culturable microorganisms recorded at 30 *DAS*.

Taken together, these results indicated that DB and IMZ, when applied at concentrations higher than the recommended doses to acidic Mediterranean soils, can have significant short- to medium-term influence on the increase in soil-culturable microorganisms, which are one of the most active soil components despite their limited occurrence (up to 95% of soil bacteria are unculturable under standard laboratory conditions) [50]. In turn, this could have substantial consequences on different soil processes mainly mediated by promptly active microorganisms, such as oxidation of organic matter, humification, nutrient cycling, plant growth promotion and control of crop fungal pathogens [50]. While these latter could be considered positively, the selective enrichment of herbicide-adapted soil microbial populations could nevertheless reduce the overall community diversity and some associated beneficial effects, e.g., greenhouse gas emission, soil organic carbon stability under climate warming [51,52]. Furthermore, preliminary flow cytometry investigations carried out on soil A, while confirming a limited presence of culturable bacteria (approx. 1% vs. total soil bacteria; Figure S4), indicated a substantial negative impact of DB and IMZ on total bacterial abundance (especially at 3 *DAS*) which deserves further investigation.

3.3. Influence of DB and IMZ on Soil Respiration and Enzyme Activity

In both soils, respiration significantly increased in a dose-dependent mode at 3 days after herbicides spiking (e.g., in average up to ~2.3-fold for both herbicides in soil A; Figure 2), supporting the view that DB- and IMZ-degrading microbial communities were present in both soils and were actively involved in herbicide oxidation/degradation. In this regard, significant correlations between soil respiration and IMZ and DB concentrations were highlighted (r = 0.80–0.91, p = 0.0002–10^{-6}; Table S1). Moreover, soil respiration and Log CFU of culturable heterotrophic bacteria were also significantly correlated (r = 0.55–0.86, p = 0.03–$2{\cdot}10^{-5}$; Table S1) suggesting that such a microbial population, despite its limited size (see data in Figure 1 and Figure S4), played a central role in herbicide catabolism. Overall, differences in soil respiration tended to reduce at 30 *DAS*, even if significantly higher respiration rates (compared to control soils) were still present in soils amended with the highest herbicide doses (Figure 2). Our results agree with Sagliker and Ozsal [53], who reported increased soil respiration after IMZ spiking, and showed that DB can also have short- and long-lasting effects on soil oxidative functions, possibly altering soil biogeochemical cycles. To gain some insight into the above-mentioned aspect, selected soil enzyme activities were assessed at different times (3 and 30 days) after herbicides spiking. In particular, the dehydrogenase activity (DHG), reflecting the ability of a group of intracellular enzymes to oxidize organic compounds, was quantified providing a measure of the total oxidative activity of soil microflora [7]. In both soils (at 3 and 30 *DAS*), DHG followed different herbicide-dependent trends. Dehydrogenase activity increased after IMZ addition (vs. control soil), especially when the herbicide was added at the highest concentration (more than 2-fold in the majority of cases; Figure 3). This was accompanied by a positive correlation

between DHG and IMZ concentration in spiked soils (r = 0.66–0.90, p = 0.005–2 × 10^{-6}; Table S1) and between DHG and soil respiration (r = 0.65–0.71, p = 0.002–0.006; Table S1), confirming DHG and respiration as reliable indicators of soil microbial activity [54]. Such increased DHG activity in IMZ-treated soils is in contrast with the results of Kaur and Kaur [55] who reported a temporary (up to 15 days) toxic effect of imazethapyr (i.e., an herbicide closely related to IMZ) when added at 0.8–1.5 µg g^{-1} soil.

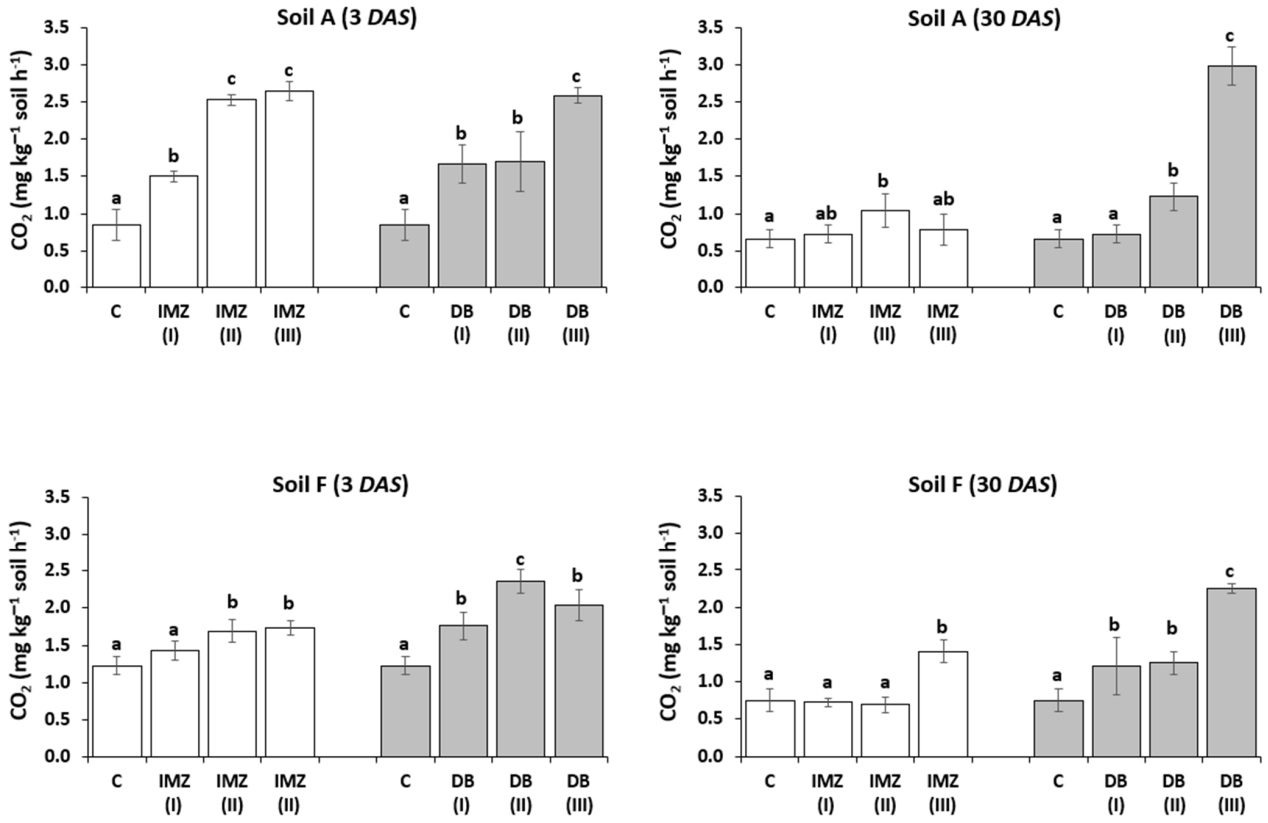


Figure 2. Influence of different concentrations of imazamox (IMZ) and 2,4-DB (DB) on soil respiration (mean values ± SE) at 3 and 30 days after herbicide spiking (*DAS*). IMZ(I), (II) and (III) refer to 3, 15, 150 µg IMZ g^{-1} soil, respectively. DB(I), (II) and (III) refer to 100, 500, 5000 µg DB g^{-1} soil, respectively. C, control (untreated) soil. For each soil and herbicide, different letters on top of bars indicate statistically significant differences (Bonferroni corrected p-value < 0.05).

Differently from IMZ, DB significantly reduced DHG activity at 3 and 30 *DAS* (Figure 3), and significant negative correlations were found between DHG and DB concentrations (up to r = −0.96, p = 10^{-6}; Table S1), and between DHG and respiration (up to r = −0.71, p = 0.002; Table S1). This seems difficult to explain, given the involvement of several dehydrogenases in the respiratory pathway [56]. However, several studies showed some TTC toxicity (TTC is the substrate used for DHG quantification) towards selected bacterial strains or populations, e.g., Gram-positive bacteria [57,58], and it was shown in many instances that 2,4-D or 2,4-D butyl ester (herbicides closely related to DB) were able to select and enrich Gram-positive bacteria [44,59]. Taken together, the above-mentioned studies could indicate that the observed DHG reduction in DB-spiked soils was not the result of a reduced metabolic capability of microbial communities but was rather an indication of an altered (DB-driven) soil microbial composition.

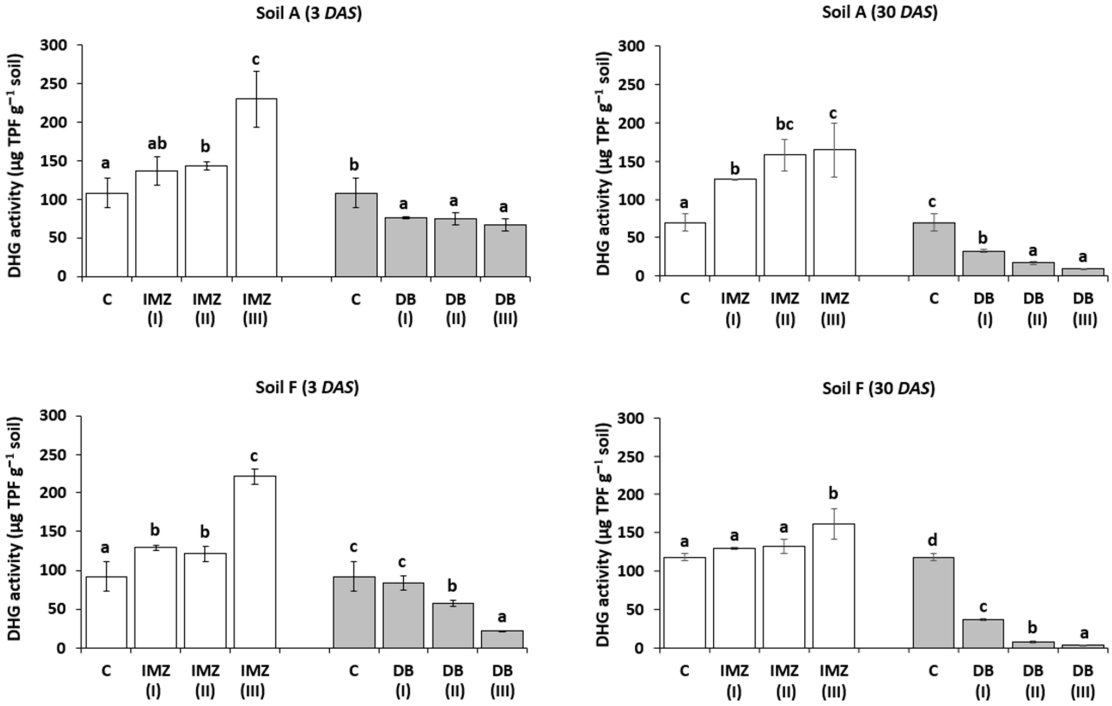


Figure 3. Influence of different concentrations of imazamox (IMZ) and 2,4-DB (DB) on soil dehydrogenase (DHG) activity (mean values ± SE) at 3 and 30 days after herbicide spiking (*DAS*). IMZ(I), (II) and (III) refer to 3, 15, 150 µg IMZ g^{-1} soil, respectively. DB(I), (II) and (III) refer to 100, 500, 5000 µg DB g^{-1} soil, respectively. C, control (untreated) soil. TPF, triphenylformazan. For each soil and herbicide, different letters on top of bars indicate statistically significant differences (Bonferroni corrected p-value < 0.05).

Soil β-glucosidases (GLU) are enzymes involved in C cycle, which catalyze the hydrolysis of cellobiose to form β-glucose units, i.e., an important source of energy and C for soil microorganisms [35]. At 3 days after spiking, IMZ and DB significantly enhanced GLU activity in soil A (approx. 2-fold vs. control), whereas both herbicides showed a limited detrimental effect in soil F, mostly at the highest herbicide concentrations (Figure S5). At 30 days after spiking, consistent effects were recorded in both soils depending on the herbicide: IMZ weakly stimulated GLU while DB significantly reduced it (Figure S5). While contrasting effects (i.e., stimulating or inhibiting) of many herbicides on GLU were previously reported [60,61], to our knowledge, no information is available for IMZ and DB. However, significant β-glucosidase inhibition at 30 days after 2,4-D spiking were previously reported, which is coherent with our findings [62]. Overall, the results presented indicate that DB and IMZ, at the concentrations used in this study, can influence soil biochemical functioning up to 30 days after herbicide spiking, likely perturbing organic matter degradation and selected steps of the C cycle.

3.4. Influence of DB and IMZ on the Biolog Community-Level Physiological Profile

The Biolog AWCD can be used to quantify the potential catabolic capacities of fast-growing soil-culturable bacteria adapted to high-substrate concentrations [63]. In this sense, both herbicides (especially DB) increased the AWCD in both soils at 3 and 30 *DAS* (e.g., up to 2.5- and 3-fold for IMZ in soil A, respectively; Figure 4), suggesting an enhanced catabolic potential in these soils which can, however, be the result of higher microbial counts [64,65]. Moreover, with one exception (IMZ in soil F at 3 *DAS*), AWCD was positively and significantly correlated with DB and IMZ concentrations (r = 0.72–0.95, p = 0.009–2 × 10^{-6}; Table S1). Interestingly, increased AWCD values were accompanied by higher richness, i.e., the number of substrates catabolized by the microbial communities within IMZ- and DB-spiked soils (Figure S6). All this could suggest a likely impact of DB and IMZ on the

structure of soil microbial communities but can be also the result of an increased microbial abundance in herbicide-treated soils [33]. In order to establish if DB and IMZ were merely increasing the size of fast-growing culturable bacteria capable of degrading the herbicides or were changing to some extent the structure of soil microbial communities, standardized C source utilization data were analyzed by PCA [33]. This latter accounted for approx. 70–95% of total variance in the first two components (PC1 and PC2; Figure 5) suggesting a certain influence of the herbicides on the microbial community structure [66]. In particular, at 3 and 30 days after herbicides spiking, C source utilization of microbial communities from DB-spiked soils was clearly different from that of control soil and of IMZ-spiked ones, i.e., they were mostly clustered well apart. On the contrary, C source utilization of microbial communities from IMZ-spiked soils was found to be more similar to that of control soils, and only IMZ (III) soils were consistently distant from control ones (Figure 5). In more detail, three days after DB spiking, the consumption of sugar phosphates significantly reduced in both soils compared to respective controls (e.g., from approx. 9 up to 4% in soil A) while limited, and sometimes inconsistent, changes were recorded after IMZ spiking (Table S2). Thirty days after DB spiking, the consumption of carboxylic acids reduced in both soils (e.g., from approx. 49 up to 28% in soil A), while further differences were highlighted in soil A (e.g., sugar and sugar derivates consumption increased from approx. 15 up to 36%) and soil F (e.g., amino acids consumption increased from approx. 21 up to 28%; Table S2). No significant differences in the consumption of C source guilds were highlighted thirty days after IMZ spiking (Table S2). Taken together, these results indicated that IMZ and DB at the tested doses were able to significantly influence the metabolic activity and composition of soil microbial communities up to one month after herbicide spiking [63–65].

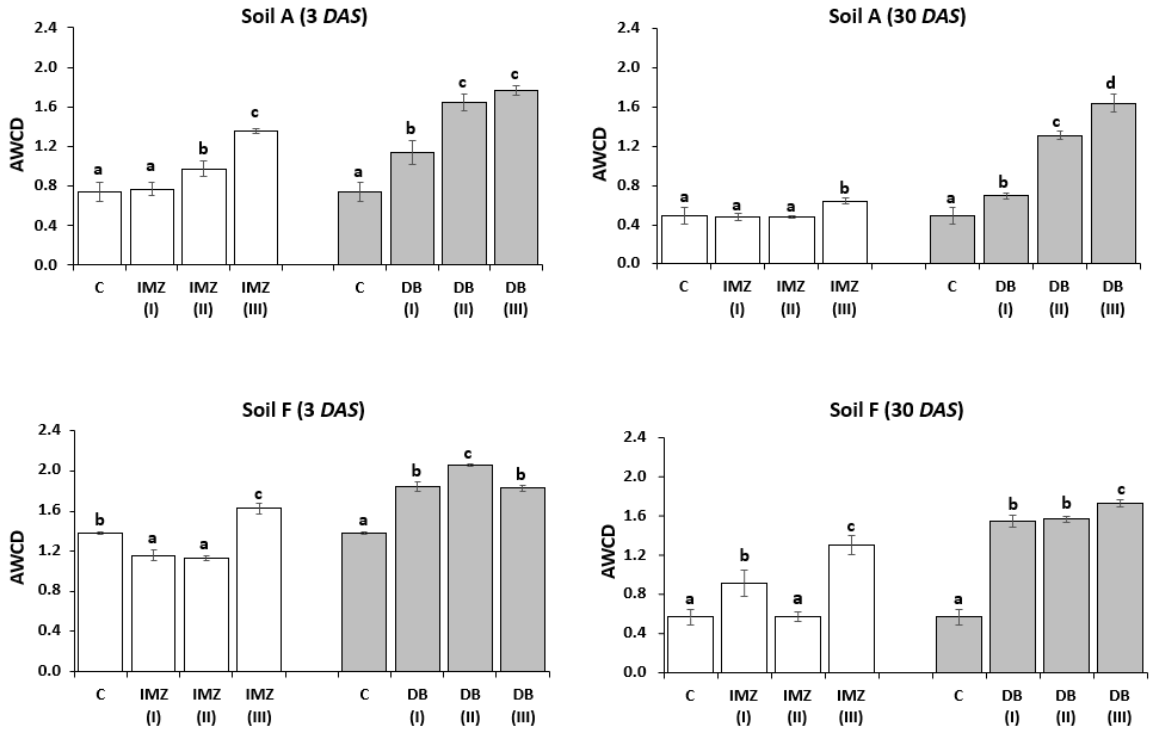


Figure 4. Influence of different concentrations of imazamox (IMZ) and 2,4-DB (DB) on Biolog average well color development (AWCD; mean values ± SE) at 3 and 30 days after herbicide spiking (*DAS*). IMZ(I), (II) and (III) refer to 3, 15, 150 μg IMZ g^{-1} soil, respectively. DB(I), (II) and (III) refer to 100, 500, 5000 μg DB g^{-1} soil, respectively. C, control (untreated) soil. For each soil and herbicide, different letters on top of bars indicate statistically significant differences (Bonferroni corrected p-value < 0.05).

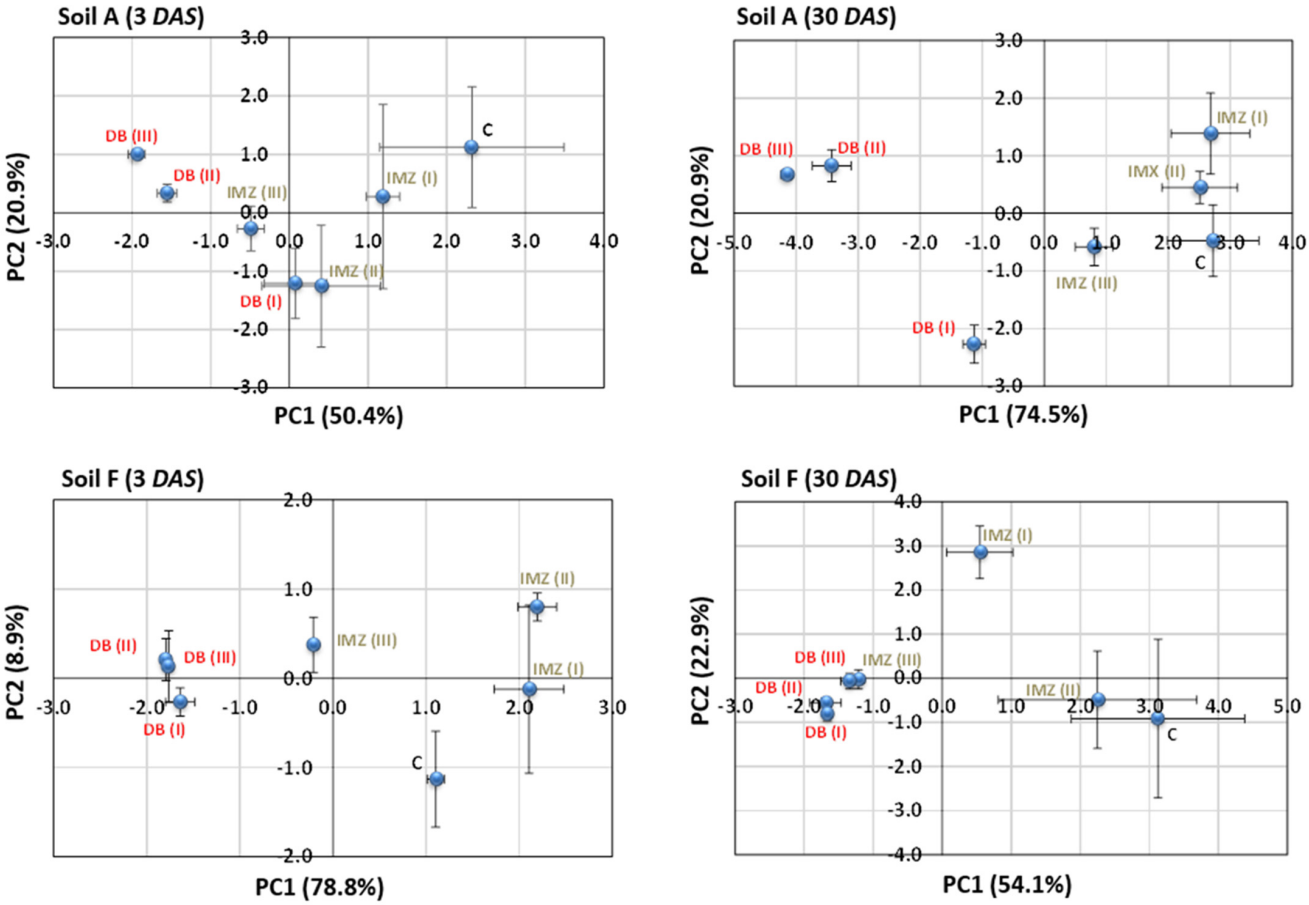


Figure 5. PCA plots of standardized C source utilization data of microbial communities extracted from soils treated with different imazamox (IMZ) and 2,4-DB (DB) concentrations at 3 and 30 days after herbicide spiking (*DAS*). IMZ(I), (II) and (III) refer to 3, 15, 150 μg IMZ g^{-1} soil, respectively. DB(I), (II) and (III) refer to 100, 500, 5000 μg DB g^{-1} soil, respectively. C, control (untreated) soil.

3.5. Influence of DB and IMZ on R. sullae Growth

The possible impact of DB and IMZ on soil rhizobial communities is of great relevance since these herbicides are largely used in legume cultivation [67,68]. To test this hypothesis, the growth of different strains of *R. sullae* on a common microbiological medium, supplemented with IMZ and DB, was assessed. Results revealed a clear toxicity of DB at higher doses, which was, to some extent, strain-dependent. For instance, S4_11 was the only strain able to grow in the presence of 500 μg DB g^{-1} medium while none (including WSM1592, the current Australian commercial inoculant for Sulla) [16] was grown in the presence of 1000 μg DB g^{-1} medium (Figure 6). On the contrary, all the *R. sullae* strains were able to grow in the presence of all IMZ concentrations, suggesting a likely compatibility between the growth of the bacterial microsymbiont and this herbicide. Similar results were showed in the case of *R. tropici* [15]. However, this does not rule out the possibility that IMZ could interfere with the process of nodule formation and/or initiation) [69], as well as N-fixation.

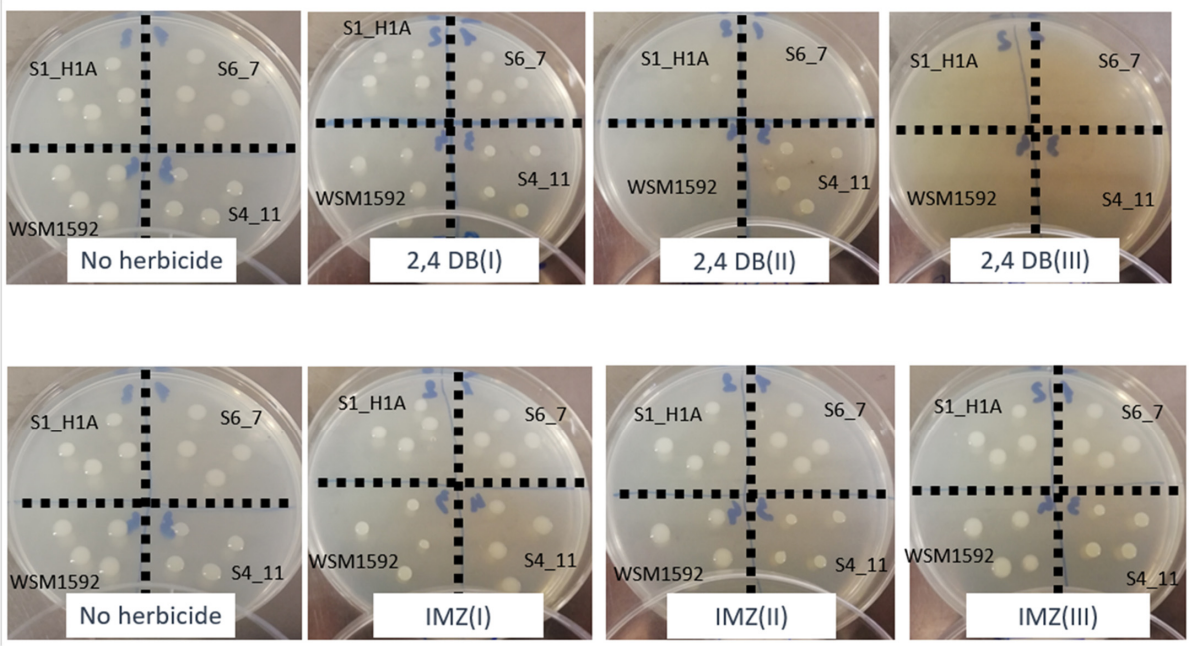


Figure 6. Influence of different concentrations of imazamox (IMZ) and 2,4-DB (DB) on the growth of different strain of *R. sullae* on YMA plates. IMZ(I), (II) and (III) refer to 3, 15, 150 μg IMZ g^{-1} medium, respectively. DB(I), (II) and (III) refer to 100, 500, 5000 μg DB g^{-1} medium, respectively.

4. Conclusions

In this study, we showed that IMZ and DB, some widely used herbicides, are poorly sorbed by typical Mediterranean acidic soils and can have significant short- and medium-term effects (from 3 up to 30 days after spiking) on different culturable soil microbial populations when applied at concentrations higher than recommended ones. Both herbicides mostly increased the abundance of culturable heterotrophic bacteria, actinomycetes and *Pseudomonas* spp., while at the same time increasing soil respiration and the potential catabolic activity of soil microbial communities. Soil dehydrogenase (but also β-glucosidase at 30 days after spiking) increased in both soils after IMZ addition, but significantly decreased after DB spiking. All this pointed to an enrichment of herbicide-degrading microbial populations in soil after herbicide spiking, as well as to a change of the soil microbial community structure, as supported by the Biolog C source utilization data. Clear toxic effects of DB were also noticed against *R. sullae*, emphasizing the potential impact of such herbicides on some microorganisms essential for effective cultivation of *H. coronarium*. Taken together, the results presented provided new evidence on the influence of DB and IMZ on soil-culturable microorganisms and soil functionality, which suggested likely short- and medium-term consequences on different soil processes governed by such promptly active microbial populations. The impact of these consequences in terms of plant growth and other soil ecosystem services, e.g., oxidation of organic matter, humification, nutrient cycling, is mostly unknown and deserves further investigation.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture12111862/s1, and includes Tables S1 and S2; Figures S1–S6; Flow cytometry analysis and preliminary kinetic batch studies [70].

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