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Evaluation of the Impacts of Clopyralid and Butisanstar Herbicides on Selected Soil Microbial Indicators

Tohid Rouhi Kelarlou · Ahmad Golchin · Ali-Ashraf Soltani Toolarood

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Abstract Herbicide application is a widely utilized approach for weed management in agriculture. However, it is essential to acknowledge that these chemical compounds can affect non-target soil microorganisms and their functions. To address this concern, a pot experiment was conducted to assess the effects of Clopyralid, Butisanstar, and their combination (But + Clo) at different doses (zero, 0.5x, recommended field dose (x), 2x, and 5x) on microbial indicators, including microbial population, microbial biomass carbon (MBC), basal soil respiration (BSR), substrate-induced respiration (SIR), microbial quotient (q_{micro}), and respiratory quotient (qCO_2) at 10, 30, 60, and 90 days following their application to the soil. The results revealed a significant reduction ($p < 0.01$) in all microbial indicators (except qCO_2, which exhibited an increase) compared to the control soil after herbicide application. Among the treatments, the combination of But + Clo exhibited the greatest decrease, followed by clopyralid and butisanstar. Bacterial populations experienced a more pronounced and persistent inhibitory effect from the herbicides compared to fungi, with a significant decrease (67%) observed from day 10 to day 30, followed by a less substantial decrease (12%) until day 90. The impact of clopyralid and butisanstar on microbial indicators demonstrated a dose-dependent relationship, with the highest and lowest values observed at 0.5 × and 5x doses, respectively. Furthermore, the adverse effects of the herbicides resulted in a substantial reduction of the evaluated indicators, particularly during the initial 30 days following application. However, temporary recovery and an increase in microbial indicators (excluding bacterial populations) were observed on day 60, likely due to the microbial community's adaptation to the applied herbicides. This study emphasizes the negative consequences of herbicide application on the soil microbial community, raising concerns regarding soil health, quality, and fertility in relation to crop production.

Keywords Microbial biomass carbon · Microbial respiration · Microbial quotient · Respiratory quotient

1 Introduction

Weed management is a crucial aspect of agricultural production across various ecosystems, playing a vital role in sustaining productivity and ensuring food security for a rapidly expanding global population (Rana & Rana, 2015). Weeds pose a greater economic threat compared to insects, fungi, and other crop pests (Gharde et al., 2018). Over the past 25 years,

T. R. Kelarlou (✉) · A. Golchin
Department of Soil Science, Faculty of Agriculture, University of Zanjan, Zanjan, Iran
e-mail: tohid.rouhik@gmail.com

A.-A. S. Toolarood
Department of Soil Science, Faculty of Agriculture, University of Mohaghegh Ardabili, Ardabil, Iran

herbicides have become the most widely used agricultural pesticides in advanced cultivation systems, significantly increasing their application (Kniss, 2017; Vats, 2015). Herbicides accounted for more than 50% of all pesticides used globally between 2011 and 2022, encompassing herbicides, insecticides, fungicides, and other categories (Use, 2022). In Iran, where weed infestations contribute to the majority of yield losses (47%) along with pests (28%) and diseases (25%), herbicides have been applied on approximately 7 million hectares of farmland (Anonymous, 2021). However, the escalating use of herbicides as environmental pollutants poses a substantial risk to various components of the ecosystem, including soil, water, and air, and has the potential to harm birds, insects, aquatic organisms, animals (Arunrat et al., 2022; Cech et al., 2022; Mahmood et al., 2016), and human health (Myers et al., 2016; Parlakidis et al., 2022). Given that herbicides are often applied during periods of plant absence or early growth stages, a significant proportion leaches into the soil, impacting non-target organisms within the soil ecosystem (Lupwayi et al., 2004). The presence of herbicides in the soil can adversely affect the biological community, particularly microorganisms, and result in detrimental effects on the diversity and abundance of sensitive species. These anthropogenic agrochemical compounds have the potential to suppress the population and activity of native, non-resistant microorganisms in the soil. Consequently, decomposition and mineralization processes of organic matter (OM) and plant residues may be impeded due to reduced production of soil enzymes such as phosphatase, β-glucosidase, cellulase, and urease (Adetunji et al., 2017; Arunrat et al., 2023). This disruption ultimately hinders the release and cycling of essential inorganic nutrients, including nitrogen, phosphorus, and sulfur. Furthermore, these compounds can impact the breakdown and degradation of diverse pollutants, as well as the production of various metabolites and beneficial soil enzymes within this ecosystem. Consequently, the health and functioning of soil, the principal medium for agricultural production, can become constrained (Adomako & Akyeampong, 2016; Kibblewhite et al., 2008; Lupwayi et al., 2004).

Recent research has indicated that soil's biological properties are more susceptible to disturbance compared to its physical and chemical properties, showing rapid responses to such disturbances. Therefore, these parameters can serve as primary indicators for assessing variations in soil quality (Geisseler & Horwath, 2009). Soil microbial communities exhibit distinct sensitivities and behaviors in the face of stresses caused by chemical compounds and substances (Visser & Parkinson, 1992). Zhang et al. (2016) conducted a study on the impact of heavy metals, including chromium, cadmium, lead, and copper, on soil's microbiological properties. The study found that the influence of these metals on the microbiological properties was more significant than their effect on the physicochemical properties. As pollution levels with these metals increased, microbial biomass decreased, and there were significant changes in the structure of the bacterial community. Another study by Antisari et al. (2013) demonstrated that the utilization of various metal oxide nanoparticles such as CeO_2, Fe_3O_4, and SnO_2 increased qCO_2 and led to alterations in the soil microbial community.

Multiple research evaluations indicate that the introduction of herbicides can impact the abundance, diversity, and activity of soil microorganisms (Adomako & Akyeampong, 2016; Baboo et al., 2013; Baćmaga et al., 2014; Sebiomo et al., 2011). Al-Ani et al. (2019) conducted a study to assess the impact of glyphosate herbicide and the insecticides malathion and alpha-cypermethrin on the abundance and activity of soil microorganisms. The study revealed an inverse relationship between the concentration of pesticides added to the soil and microbial activities, as well as the populations of bacteria, fungi, and actinomycetes. Baćmaga et al. (2014) found that the herbicide metazachlor had an inhibitory effect on the structure and abundance of oligotrophic and organotrophic bacteria, actinomycetes, and fungi in the soil for up to 60 days after application. Lupwayi et al. (2004) reported that the use of clopyralid at the recommended field dose (RFD) increased MBC, while the use of metribuzin, imazamox/imazethapyr, tri-sulfuron, and methyl-metsulfuron resulted in a decrease in MBC. Pertile et al. (2020) conducted a study under laboratory conditions to evaluate the impact of the herbicides imazethapyr and flumioxazin on MBC. The study found that MBC decreased, while both BSR and SIR significantly increased. Nur et al. (2013) demonstrated a significant relationship between the amount and type of herbicides used and their inhibitory effect on microbial populations. The study revealed that paraquat had the highest

inhibitory effect on bacteria and actinomycetes four days after herbicide application, while glyphosate caused the highest inhibition effect on fungi six days after application. The researchers also noted that the herbicide methyl-metsulfuron had the least inhibitory effect on microbial populations. According to various researchers, several factors can influence the differential response of microbial communities to herbicide use. These factors include soil physicochemical properties, weather conditions like temperature and moisture during the season, chemical properties of the herbicide, its concentration, and the types of microorganisms involved (Filimon et al., 2021; Quilchano & Marañón, 2002; Vasic et al., 2022).

In Iran, like many other countries, significant amounts of various herbicides are used to control and eliminate weeds in farms and orchards. Two commonly used herbicides for strategic crop production, particularly canola and sugar beet, are Lontrel (Clopyralid 30% SL) and Butisanstar (Quinmerac + Metazachlor, 41.6% SC). Lontrel as selective herbicides is used for post-emergence at a RFD of 0.8 L per hectare, while Butisanstar is used for pre-emergence at an RFD of 2.5 L per hectare (Shimi et al., 2014; Tomco et al., 2016; Yadaei et al., 2019). Clopyralid belongs to the group of synthetic auxin herbicides. It is a solid compound with low polarity and possesses acidic properties due to the presence of pyridine acids. Additionally, it exhibits a relatively lipophilic nature. It's solubility and washing capacity are particularly high, attributed to its weak absorption, especially in alkaline soils (Roberts et al., 1998). The persistent nature of clopyralid is evident from its relatively low pK value, allowing its residues to persist in the soil for up to 90 days after application at the RFD (Tomco et al., 2016). Furthermore, it has the potential to harm rotational crops for a duration of up to one year (Gillespie et al., 2011). The herbicide Butisanstar is a combination of two herbicides, namely Metazachlor (29.5%, 333 $g.l^{-1}$) from the chloroacetanilide family and Quinmerac (4.7%, 83 $g.l^{-1}$) from the quinoline carboxylic acid family of auxins (Shimi et al., 2014).

The introduction of these compounds into the soil can have irreversible consequences on soil organisms, posing a threat to biodiversity and the proper functioning of soil ecosystems. Recognizing the crucial role of soil biota, particularly microorganisms, in the formation, evolution, stability, and function of the vital production ecosystem, it is imperative to investigate the impact of chemical toxins on soil biota. Given the limited existing research and insufficient information concerning the effects of these two herbicides on soil biological aspects, this study aims to assess the abundance, dynamics, and activity of soil microorganisms, which constitute the most prevalent and influential living organisms, in the presence of clopyralid and butisanstar herbicides.

2 Materials and Methods

2.1 Soil Sampling and Selection

In order to carry out this research, soil samples were collected during late summer from the top layer (0–10 cm) of a wheat farm located in Moghan (Fig. 1). The selected farm had no previous history of clopyralid and butisanstar herbicide use; however, the presence of other pesticides cannot be ruled out. The collected soil samples were air-dried and passed through a 2 mm sieve. Standard methods (Pansu, 2006) were employed to measure certain physicochemical properties of the soil samples. Based on the preliminary results, a soil sample was chosen with the following properties: loam texture (25.4% clay, 33.6% silt, and 41% sand), 42.4% saturation percentage (SP), 32% field capacity in the soil of pots, and the following chemical properties: pH value of 7.52, electrical conductivity (EC) of 3.99 dS/m, calcium carbonate equivalent (CCE) of 20.6%, organic carbon (OC) of 0.46%, 0.05% N, 8.83 ppm P, and 519 ppm K.

2.2 Experimental Setup and Herbicide Treatments

The study was conducted as a factorial experiment based on a completely random design, consisting of 13 treatments with 3 replications, in the growth chamber of the Soil Science and Engineering Department of Mohaghegh Ardabili University. Four different doses (0.5x, RFD = x, 2x, and 5x) of each herbicide were applied. The experimental treatments included various amounts of herbicides: 3.4, 6.9, 13.8, and 34.6 mg a.i./10 kg soil of butisanstar, 0.8, 1.6, 3.2, and 8 mg a.i./10 kg soil of clopyralid, as well as a combination of both herbicides (But + Clo) and untreated soil (control) (Table 1). The selected soil, after air-drying, crushing, and passing through a

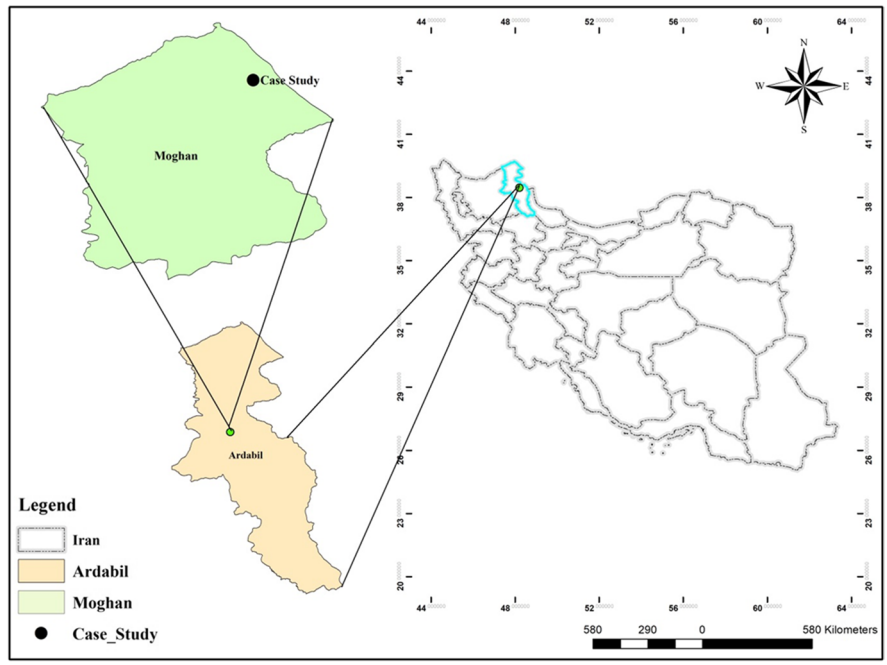

Fig. 1 Location of farm sampling sites

Table 1 Different types and doses of herbicides

Dose* Herbicides	Control** (mg a.i./10 kg soil)	0.5x	x = RFD	2x	5x
Butisanstar	0	3.4	6.9	13.8	34.6
Clopyralid	0	0.8	1.6	3.2	8
But. + Clo	0	3.4 + 0.8	6.9 + 1.6	13.8 + 3.2	34.6 + 8

* The doses were prepared by diluting the herbicide's stock solution (1000 ppm) with 500 ml of distilled water and then sprayed onto the soil. Measurements are made after 10, 30, 60, and 90 days of incubation

** As a control, 500 mL water was sprayed and mixed with soil

4.75 mm sieve, was weighed (10 kg) and then contaminated by spraying with calculated amounts of butisanstar and clopyralid herbicides. The contaminated soils were filled into black plastic pots with diameters and heights of 22 cm and 26 cm, respectively. The pots were transferred to the growth chamber and incubated at a temperature of 25 ± 2 °C with 60% of pot field capacity for 90 days. Soil moisture in the pots was monitored daily by weighing. Soil samples were collected from each pot at 10-day intervals, starting from the 10^{th} day after herbicide application, and continuing at the 30^{th}, 60^{th}, and 90^{th}-day intervals, in order to measure microbial indices such as soil microbial population, MBC, BSR, SIR, q_{micro}, and qCO_2.

2.3 Measurement of Biological Parameters

Standard plate count methods were used to assess the population of bacteria and fungi. Nutrient agar (NA) and potato dextrose agar (PDA) were employed as specific media for bacteria and fungi, respectively. The results were reported as colony-forming units (cfu) (Adomako & Akyeampong, 2016). Soil basal respiration was determined by placing 50 g of soil in glass jars along with 10 mL of 0.05N NaOH in 20 mL glass vials. All samples were incubated for 24 h at 25 ± 1 °C in the dark. The CO_2 evolved during this period was absorbed in NaOH and quantified by titration with 0.1 N HCl after the addition of saturated $BaCl_2$, using

phenolphthalein as the indicator for the titration endpoint (Alef & Nannipieri, 1995). Substrate-induced respiration was determined by measuring the CO_2 production from a 50 g sample of fresh soil. The soil samples, which contained 1% glucose, were placed in a glass jar, and an absorption bottle filled with 25 mL of 0.1N NaOH was carefully placed in the jar, which was then precisely sealed. The sealed jar was incubated at 25 ± 1 °C for 6 h. The evolved CO_2 was trapped by NaOH and determined by titration of the NaOH with 0.1N HCl. MBC (mgC_{micro}-CO_2. g^{-1}soil) was estimated using the chloroform fumigation–extraction method (Jenkinson & Ladd, 2021). q_{micro} ($mgC_{micro}.g^{-1}C_{org}$) was calculated by dividing MBC by soil OC (mgC_{micro}-CO_2/gC_{org}) (Anderson & Domsch, 1986). qCO_2 (mgC_{micro} $g^{-1}.d^{-1}$ / mgC_{resp}) was calculated by dividing the BSR ($mgC\text{-}CO_2.g^{-1}soil.day^{-1}$) by the amount of MBC (Anderson, 1982).

2.4 Statistical Analysis

The statistical analysis included the application of the Analysis of Variance (ANOVA) within the framework of the General Linear Model (GLM). To compare means between treatments, the Duncan test was employed, with the least significant difference values calculated at a significance level of 5%. Pearson correlations were conducted using SPSS (v 26) software. Additionally, figures were generated using Microsoft Excel software.

3 Results

Analysis of variance results demonstrated significant independent and interactive effects of dual and triple treatments involving herbicides, different doses, and duration of incubation on bacterial and fungal population variables, MBC, BSR, SIR, q_{micro}, and qCO_2 at a significance level of $p < 0.01$ (Table 2).

3.1 Response of Bacterial Population to Herbicides over Time

The analysis of variance table indicated that the main effect, as well as the two-way interaction effects of herbicide type × time and time × herbicide doses, on soil bacterial population were all significant at the 0.01 level of significance ($p < 0.01$) (Table 2). The population of soil bacteria decreased significantly with an increase in incubation time. The treatment containing butisanstar herbicide exhibited the highest bacterial population (26.61×10^7 cfu/g soil) on the 10^{th} day, which represented a 15% decrease compared to the control soil (30.1×10^7) (Fig. 2). Between the 10^{th} and 30^{th} days, there was a significant reduction (67%) in bacterial population (from 25.6 cfu/g soil to 8.3 cfu/g soil), followed by a continued decline with lower intensity until the 90^{th} day. The rate of decrease between the 30^{th} and 90^{th} days was 12%. The lowest bacterial count (7×10^7 cfu/g soil) was observed on the 90^{th} day in the soil containing a mixture of butisanstar and clopyralid herbicides. In the control soil, the bacterial population initially increased on the

Table 2 The results of the ANOVA (Mean Squares) show the simple and interaction effects of different types and doses of herbicides and the time elapsed after their use in soil on soil microbial and eco-physiological indicators

Sources of variation	df	Bacterial population	Fungal population	BSR	SIR	MBC	q_{micro}	qCO_2
Type of Herbicides(H)	2	6.8**	1.3**	0.01**	2.67**	231,047**	9.1**	0.00**
Time(T)	3	3539.7**	23.5**	0.10**	0.78**	26,738**	10.6**	0.00**
Dose of Herbicides (DH)	4	3413**	26.9**	1.25**	4.2**	1,108,074**	43.8**	0.00**
H × T	6	2.7**	1.5**	0.01**	0.03**	11,890**	0.5**	0.00**
H × DH	8	0.5ns	1.1**	0.00**	0.17**	27,079**	1.1**	0.00**
T × DH	12	211.7**	1.7**	0.02**	0.06**	23,060**	0.9**	0.00**
H × T × DH	24	0.3ns	0.4**	0.00**	0.02**	5717**	0.2**	0.00**
Error	120	0.27	0.07	0.00	0.00	2113	0.1	0.00
C.V	–	0.99	0.27	0.27	0.38	0.39	0.33	0.39

*, ** indicate significant at a probability level of 0.05 ($p < 0.05$) and 0.01($p < 0.01$), respectively

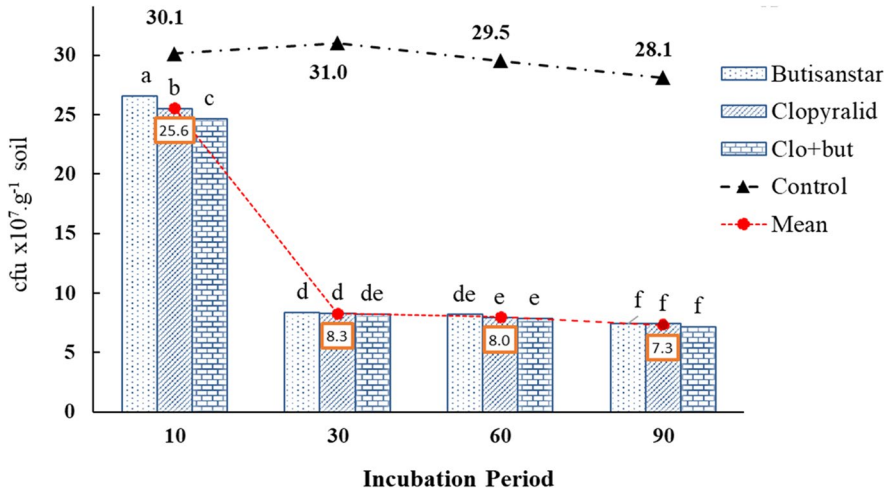

Fig. 2 The impact of types of herbicides and incubation period on the bacterial population. The red dashed lines represent the mean of the three types of herbicides over time. The same letters above the bars indicate non-significant differences ($p > 0.05$) between treatments (Duncan's test)

30th day after incubation but subsequently decreased (Fig. 2).

The mean comparison results indicated the inhibitory effect of herbicide doses on soil bacterial populations over time (Fig. 3). Adding 0.5, 1, 2, and 5 times the RFD of herbicides to the soil caused a significant and severe decrease in bacterial population from the 10th to the 30th day of incubation. There was also a decrease in the bacterial count between the 30th and 90th days. The most substantial decrease (74%) compared to the control treatment was recorded on the 90th day of incubation. Regardless of the incubation period, the highest and lowest cfu were observed in the zero (herbicide-free) and 5 times the RFD treatments, respectively, indicating the negative effects of higher herbicide doses on the bacterial population (Fig. 3).

3.2 Response of Fungal Population to Herbicides over Time

The impact of herbicides, their different doses, and incubation time on the fungal population was significant at the 0.01 level of significance (Table 2). The addition of herbicides during the incubation period resulted in a significant decrease in the fungal population, except on the 60th day compared to the 30th day (Fig. 4A). The inhibitory effects of combining two herbicides on the growth and development of soil fungi were more severe than their

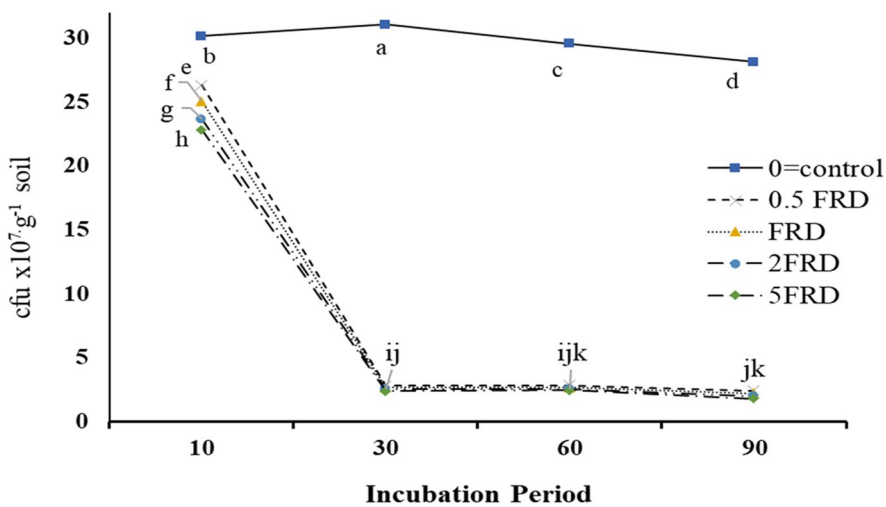

Fig. 3 The effect of different doses of herbicides and incubation period on the bacterial population. The same letters above the lines indicate non-significant differences ($p > 0.05$) between treatments (Duncan's test)

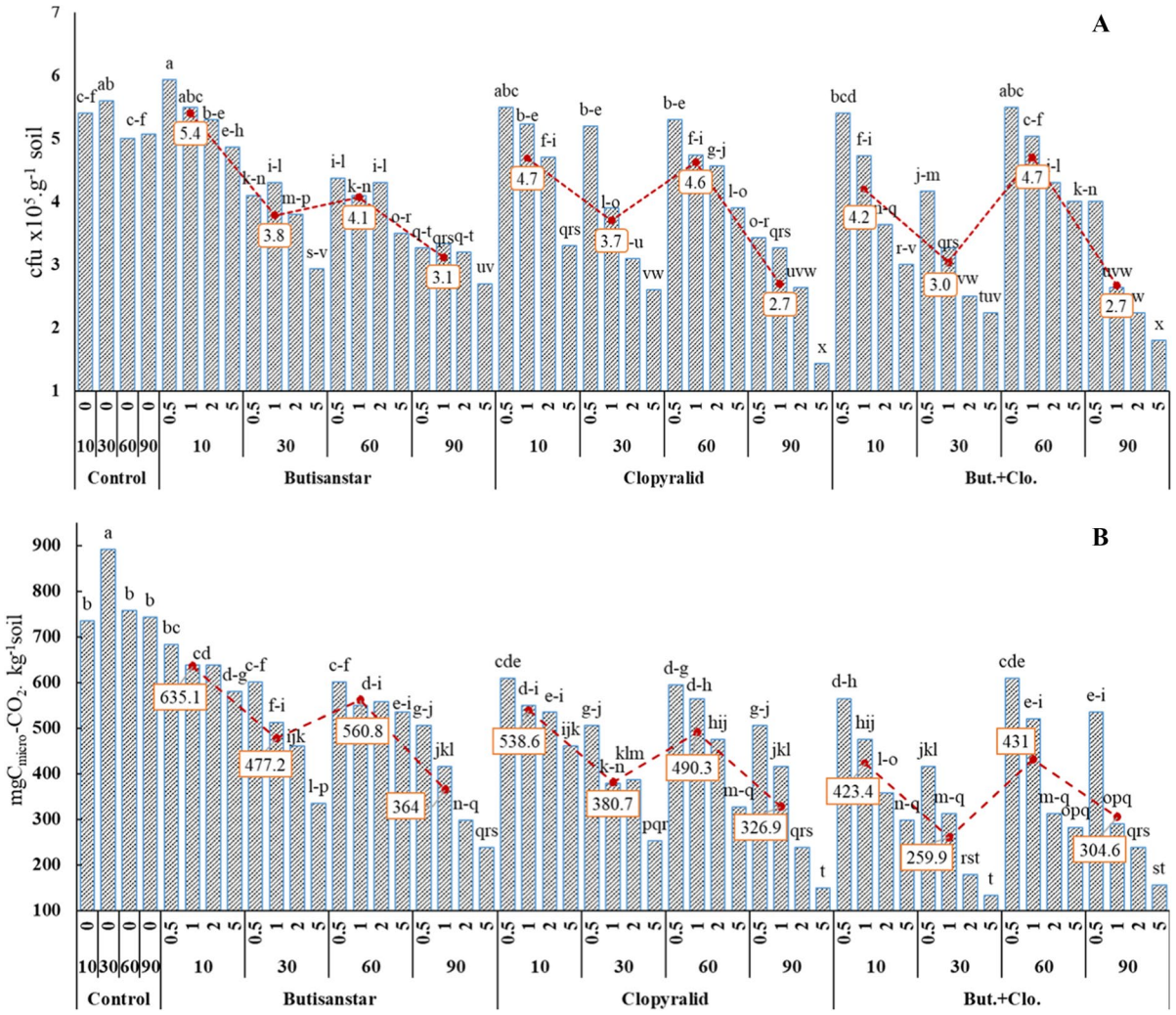

Fig. 4 Fungal population (**A**) and MBC (**B**) were measured in untreated soils (control) and soils treated with different doses (0.5, 1, 2, and 5 times the RFD) of butisanstar, clopyralid, and their mixture (But + Clo) at different incubation times (10, 30, 60, and 90 days after herbicide addition). The values represent means ($n = 3$), and the same letters above the bars indicate non-significant differences ($p > 0.05$) between treatments (Duncan's test). The red dashed lines represent the mean of the four doses of herbicide over time

individual applications. The highest number of fungi was observed on the 10th day for butisanstar (5.4×10^5 cfu/g soil), clopyralid (4.7×10^5 cfu/g soil), and the combination of both (4.2×10^5 cfu/g soil), representing a reduction of 0%, 13%, and 22% compared to the control soil (5.4×10^5), respectively. After 60 days of incubation, there was a temporary increase in the fungal population compared to the 30th day. However, after 90 days of incubation, the population had significantly decreased, and the lowest number of these microorganisms was observed in the combination of two herbicides (2.7×10^5 cfu/g soil), representing a reduction of 46% compared to the control soil (5×10^5 cfu/g soil). In the control soil, there was an initial increase in population up to the 30th day, followed by a significant decrease on the 60th and 90th days. During the incubation period (from 10 to 90 days), the separate and combined application of two herbicides resulted in a reduction in population of 0%, 35%, and 42%, respectively. All herbicides tested showed a significant and substantial reduction in fungal population when 0.5, 1, 2, and 5 times the RFD

were added, compared to the control soil. Among the treatments using equal amounts, the But + Clo treatment showed the highest reduction, followed by clopyralid and butisanstar. The greatest reduction in fungal population during the incubation period was observed with the use of 5 and 2 times the RFD of clopyralid and the combination of clopyralid with butisanstar herbicides, respectively. Additionally, the use of 5 times the RFD of butisanstar also resulted in a significant reduction in the number of these microorganisms. Even with the RFD of herbicides, a significant reduction in fungal population was observed during the incubation period compared to the control soil (Fig. 4A).

3.3 Changes in Microbial Biomass Carbon over Time

The findings of this study indicate that the independent and interactive effects of herbicide type, herbicide doses, and incubation period significantly influenced the MBC index at a significance level of 0.01 (Table 2). Moreover, the addition of all herbicides significantly decreased MBC compared to the control soil on the 10^{th} day. On the 30^{th} day of incubation, a significant and substantial reduction in MBC was observed compared to the 10^{th} day. Specifically, during the 10–30-day incubation period, reductions of 38%, 29%, and 25% were observed for the But + Clo, clopyralid, and butisanstar treatments, respectively. Although there was a slight reduction in MBC on the 60^{th} day compared to the 10^{th} day, a significant increase in this index was observed on the 60^{th} day compared to the 30^{th} day. Specifically, there was no statistically significant difference between the 10- and 60-day intervals at 0.5 and 1 times the RFD. Furthermore, a significant decrease in the mentioned index was observed in all experimental treatments on the 90^{th} day. The highest and lowest values of MBC (635.1 and 259.9 mgC_{micro}-CO_2. kg^{-1}soil) were measured on the 10^{th} and 30^{th} day of incubation, respectively, in the soil containing butisanstar and the combination of two herbicides. Over the 10 to 90-day incubation period, both combined and separate applications of the herbicides clopyralid and butisanstar resulted in reductions of MBC by 28%, 39%, and 42%, respectively. In all of the herbicides under investigation, adding 0.5, 1, 2, and 5 times the RFD led to a decrease in the mentioned index, compared to the control soil. This reduction was more pronounced when using a combination of butisanstar and clopyralid herbicides compared to the individual use of each herbicide. The highest MBC values at 0.5 and 1 times the RFD were observed on the 10^{th} day of incubation with the use of butisanstar herbicide. The lowest values were also observed on the 30^{th} day of incubation, using the combination of two herbicides at 2 and 5 times the RFD (Fig. 4B).

3.4 Changes in Basal Soil Respiration over Time

The main effects and interactions of the studied factors on basal soil respiration (BSR) were found to be statistically significant ($p < 0.01$) over time, as presented in Table 2. When comparing means, it was observed that the addition of all herbicides led to a significant and severe reduction in BSR compared to the control soil. The reduction in BSR was more pronounced when the herbicides butisanstar and clopyralid were used together, as opposed to their individual use. In contrast to the control soil, respiration exhibited a decreasing trend for all the herbicides investigated during the incubation period between the 10^{th} and 30^{th} day. Additionally, in all experimental treatments, despite a temporary increase on the 60^{th} day, a significant reduction in BSR was observed on the 90^{th} day of incubation. The highest and lowest respiration rates on the 10^{th} (0.47 $mgCO_2.g^{-1}.day^{-1}$) and 90^{th} (0.26 $mgCO_2.g^{-1}.day^{-1}$) day, respectively, were obtained with the combined use of two herbicides, representing a 42% and 67% reduction compared to the control soil (0.81 $mgCO_2.g^{-1}.day^{-1}$). Over the incubation period of 10 to 90 days, the combined and separate application of two herbicides resulted in a reduction of microbial respiration by 44% and 29%, respectively. Regardless of the incubation time, the addition of 0.5, 1, 2, and 5 times the RFD led to a decrease in BSR compared to the control soil. The lowest and highest values of the index were measured at levels of 5 and 0.5 times the RFD, respectively, when the combination of two herbicides was added. Even when recommended or lower amounts of herbicides were used, basal respiration showed a decrease compared to treatments without herbicides. Furthermore, BSR exhibited a decreasing trend during the incubation period, with equal levels of herbicides, except for the zero level (Fig. 5A).

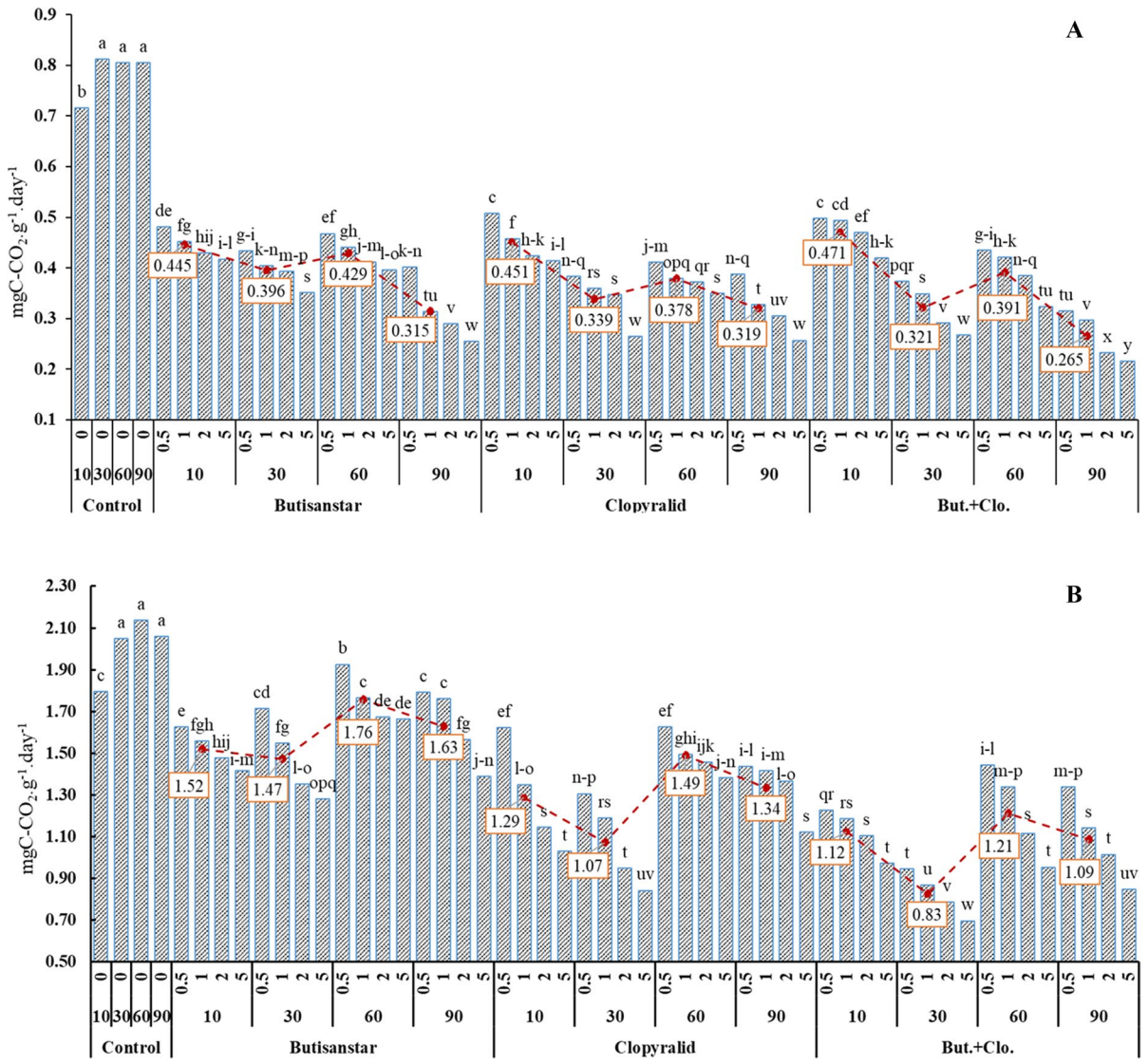

Fig. 5 Illustrates the measurements of BSR (**A**) and SIR (**B**) in untreated soils (control) and soils treated with different doses (0.5, 1, 2, and 5 times the RFD) of butisanstar, clopyralid, and their mixture (But + Clo) at different incubation times (10, 30, 60, and 90 days after herbicide addition). The values represent means ($n = 3$), and non-significant differences ($p > 0.05$) between treatments are indicated by the same letters above the bars (Duncan's test). The red dashed lines represent the mean of the four doses of herbicide over time

3.5 Changes of Substrate-induced Respiration over Time

The main effects of the studied factors and their dual and triple interactions were found to be significant at the 0.01 level (Table 2). The addition of all herbicides resulted in a significant reduction in SIR compared to the control soil on the 10th day. Notably, a significant reduction in this microbial index was observed on the 30th day of incubation compared to the 10th day. During the incubation period of 10 to 30 days, the reduction rates for the combination of two herbicides, clopyralid and butisanstar, were calculated as 26%, 17%, and 3%, respectively. Additionally, in all experimental treatments, despite a temporary increase on the 60th day, a significant reduction in SIR was observed on the 90th day of incubation. The lowest amount of SIR on the 30th day of incubation (0.83

$mgCO_2.g^{-1}.day^{-1}$) was observed with the addition of the combination of two herbicides, while the highest amount (1.76 $mgCO_2.g^{-1}.day^{-1}$) was observed with the use of butisanstar herbicide on the 60th day. In all herbicides under investigation, the addition of 0.5, 1, 2, and 5 times the RFD resulted in a reduction in SIR. The reduction in SIR was more pronounced in all treatments compared to the control soil. Higher amounts were also observed at doses of 0.5 and 1 times the RFD on the 60th and 90th day of incubation with the use of butisanstar herbicide. The lowest amounts were observed at doses of 5 and 2 times the RFD on the 30th day of incubation with the use of the combination of two herbicides (Fig. 5B).

3.6 Changes in Microbial Quotient over Time

This study revealed significant effects of the investigated factors, as well as their dual and triple interactions, on the q_{micro} (mgC_{micro}/gC_{org}.) at a significance level of 0.01 (Table 2). Upon the addition of all herbicides, a notable decrease in q_{micro} was observed on the 10th day compared to the control soil. The reduction in q_{micro} was more pronounced when a combination of two herbicides was used rather than individual herbicides. While there was a decrease in the q_{micro} index up to the 30th day compared to the 10th day for all herbicides under investigation, a temporary increase was observed on the 60th day, with no statistically significant difference found at 0.5 and 1 times the RFD between the 10th and 60th days of incubation. Moreover, a consistent downward trend in the q_{micro} was observed in all experimental treatments in the long term, specifically on the 90th day. The lowest q_{micro} value of 2.76 mgC_{micro}/gC_{org} was recorded on the 30th day when a combination of two herbicides was used, representing a reduction of 68% compared to the control soil. Conversely, the highest q_{micro} value of 3.95 mgC_{micro}/gC_{org} was obtained with the addition of butisanstar on the 10th day, showing a minimal reduction of 4% compared to the control soil. In all herbicides under investigation, the addition of 0.5, 1, 2, and 5 times the RFD led to a significant and noteworthy reduction in q_{micro} compared to the control soil (baseline level). Applying equal amounts of each herbicide resulted in a decrease in q_{micro}, with the most substantial reduction observed in the following order: But + Clo > clopyralid > butisanstar. The most significant decrease in q_{micro} occurred during the incubation period with the use of 5 and 2 times the RFD in the treatments receiving clopyralid herbicide and its combination with butisanstar. Additionally, the application of 5 times the RFD of butisanstar also resulted in a significant decrease in q_{micro} during the 90-day incubation period. Even with the application of the RFD, a significant reduction in q_{micro} was observed during the incubation period compared to the control soil (Fig. 6A).

3.7 Changes in Respiratory Quotient over Time

The findings of this study demonstrated the significant effects of the studied factors and their dual and triple interactions on the qCO_2 at a probability level of one percent ($p < 0.01$) (Table 2). The respiratory quotient exhibited an inverse trend (negative correlation coefficient with other indices) compared to the other investigated indices when herbicides were used. The treatments receiving the herbicides clopyralid and its combination with butisanstar showed the highest qCO_2 values during the incubation period. The rate of increase in qCO_2 was higher in the combined use of the two herbicides, butisanstar and clopyralid, compared to their individual use. The lowest qCO_2 value of 0.03 $mgC_{resp}/mgC_{micro}\ kg^{-1}.d^{-1}$ was obtained with the addition of butisanstar on the 10th day of incubation, representing a 27% increase compared to the control. On the other hand, the highest qCO_2 value of 0.06 $mgC_{resp}/mgC_{micro}\ kg^{-1}.d^{-1}$ was obtained with the combined use of the two herbicides on the 30th day, indicating a 58% increase compared to the control (Fig. 6B). Although there was an increase in the qCO_2 index until the 30th day compared to the 10th day, a temporary reduction was observed on the 60th day of incubation. Furthermore, in the long-term experimental treatments (90th day), a consistent upward trend in the metabolic fraction was observed. When using the same amount of herbicides, the highest increase in qCO_2 was observed for the order: But + Clo > clopyralid > butisanstar. The lowest values were observed at 0.5 and 1 times the RFD on the 10th and 60th days when butisanstar was used. The highest values were also observed at 5 and 2 times the RFD on the 30th and 90th days when the combination of two herbicides was used (Fig. 6B).

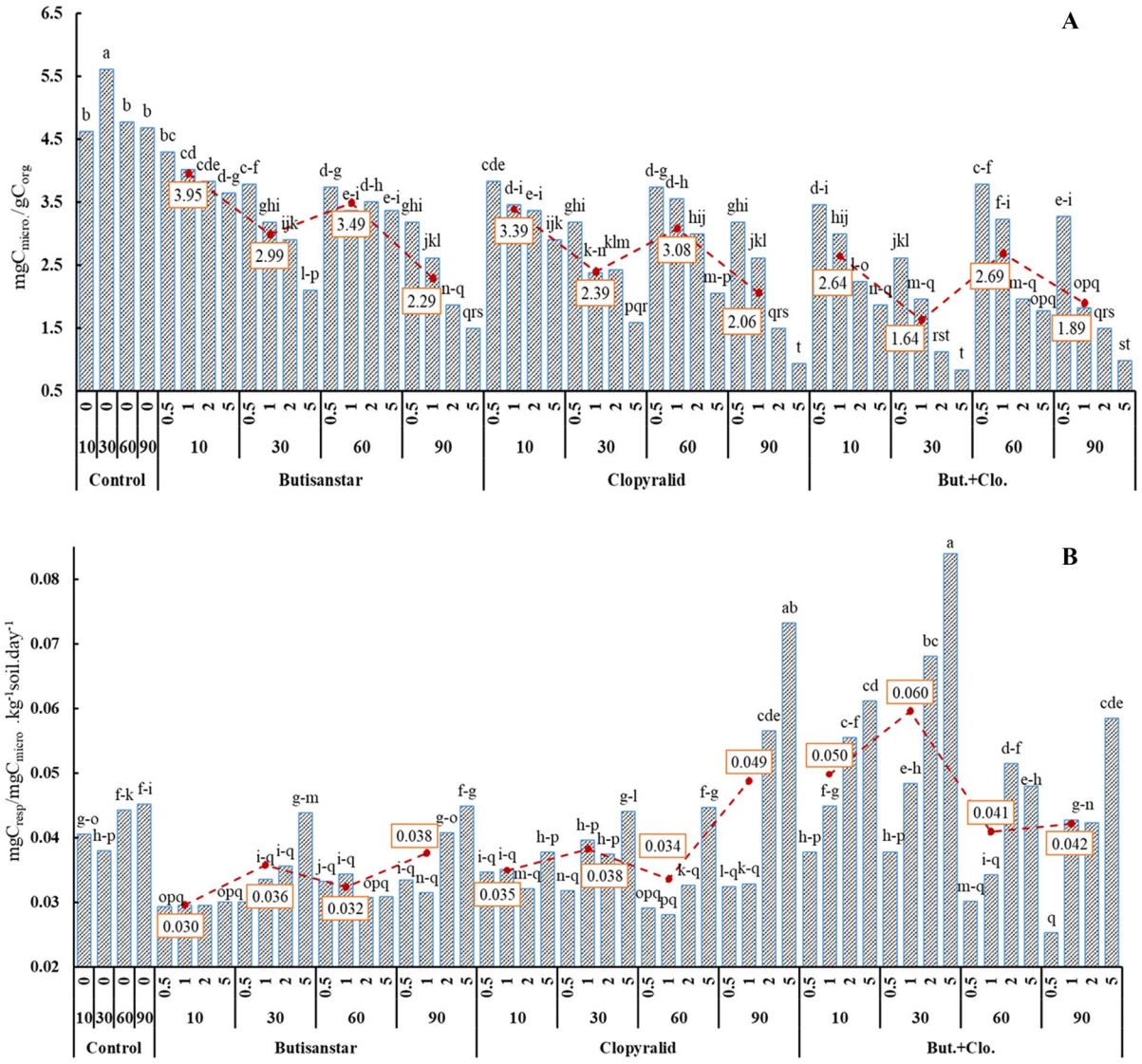

Fig. 6 Illustrates the measurements of the q_{micro} (**A**) and qCO_2 (**B**) in untreated soils (control) as well as soils treated with varying doses (0.5, 1, 2, and 5 times the RFD of butisanstar, clopyralid, and their mixture (But + Clo) at different time points of incubation (10, 30, 60, and 90 days after the addition of herbicide). The presented values represent the means ($n = 3$), and the absence of significant differences ($p > 0.05$) between treatments (Duncan's test) is indicated by identical letters above the bars. The red dashed lines indicate the mean values of the four herbicide doses observed over time

4 Discussion

The soil microbial population is influenced by various agricultural management methods, which alter the physical, chemical, and biological properties of the soil. Herbicides, a prominent group of pesticides used for weed control, also impact non-target organisms. Numerous researchers have reported that the use of different herbicides results in inhibitory effects and reduced soil microbial populations, including bacteria and fungi, compared to herbicide-free soil (control) over varying time intervals (Baćmaga et al., 2014; Sebiomo et al., 2011; Tomkiel et al., 2019). In this study, the application of clopyralid and butisanstar herbicides during the incubation period (10 to 90 days) led to a reduction in the population of

fungi (42–35%) and soil bacteria (71%). The extent of reduction varied depending on the herbicide dosage, with the highest reduction rates observed at 5 times the RFD and the lowest rates at 0.5 times the RFD. Interestingly, when the two herbicides were used in combination, the reduction in microbial populations (especially on day 90 of incubation) was greater than in their separate use. Ayansina and Oso (2006) noted a decrease in microbial populations in soils treated with a combination of two herbicides, atrazine + metolachlor, as well as separately applied atrazine at the RFD and 1.5 times the RFD, compared to the control. Filimon et al. (2021) reported a decline in bacterial population 2 to 4 weeks after adding higher doses of oxyfluorfen to soil samples. Tyagi et al. (2018) also observed an initial decrease in bacterial population up to 30 days after applying acetochlor, atrazine, and 2,4-D ethyl ester herbicides.

Herbicide adsorption onto OM and soil colloids partially leads to inactivation and reduced mobility. Consequently, the herbicide's concentration in the soil solution and its bioavailability decrease, inhibiting its decomposition. Furthermore, OM accelerates the biodegradation processes of herbicides by enhancing the soil's microbiological activity (Bolan & Baskaran, 1996; Takeshita et al., 2019). In this study, the low OC content (0.46%) of the tested soil increased the bioavailability of herbicides, resulting in heightened herbicide toxicity and a decline in microbial populations. Pampulha and Oliveira (2006) also associated the decrease in microbial population at high concentrations of a combination of two herbicides, bromoxynil + prosulfuron, with increased herbicide bioavailability. Certain herbicides, such as sulfonylurea herbicides that inhibit the enzyme acetolactate synthase, affecting weed activity, also impact soil microorganisms and reduce microbial populations (Boldt & Jacobsen, 1998). Low concentrations of clopyralid can stimulate RNA, DNA, and protein synthesis, leading to uncontrolled cell division, irregular growth, and eventual death of living organisms. High concentrations of clopyralid, on the other hand, can inhibit cell division and growth (Tu et al., 2001). Unlike most herbicides, limited research and information are available regarding the stability and mode of action of butisanstar on soil microorganisms.

It appears that due to the morphological and physiological differences between bacteria and fungi, the microbial community structure undergoes changes and alterations due to varying sensitivity of bacteria and fungi to the same herbicides (Baćmaga et al., 2015). Moreover, the discrepancy in resistance between fungal and bacterial species to herbicides enables the survival and proliferation of certain species during the incubation period (Ayansina & Oso, 2006). The significant decrease in bacterial population (67%) compared to fungi (25–38%) one month after herbicide application indicates the high sensitivity and short-term vulnerability of bacterial populations compared to fungal populations when exposed to herbicides such as clopyralid and butisanstar. Consequently, the reduction in bacterial populations persists over the long term (30 to 90 days). Nur et al. (2013) reported a higher inhibition percentage for bacteria compared to fungi when herbicides such as paraquat, glyphosate, glufosinate-ammonium, and methylsulfonyl-methane were applied twice. The negative impact of clopyralid on bacterial populations can be attributed to its high stability against biodegradation, prolonged persistence in soil, and antibacterial properties (Sun et al., 2022; Tomco et al., 2016). Conversely, the sensitivity of certain soil bacteria to the herbicide metazachlor (Baćmaga et al., 2014), the decrease in nitrogen cycle bacteria populations up to 90 days after metazachlor application (Hristeva et al., 2015), and the degradation of metazachlor into stable metabolites (Mamy et al., 2005) as a major component of butisanstar herbicide can explain the long-term reduction of bacterial populations.

In the present study, the fungal population exhibited an increase on the 60th day of incubation, contrary to the decreasing trend observed on other days, as well as the declining trend in bacteria. The reversible effect of the recovery and increase in fungal population with the addition of herbicides can be attributed to the stimulation of herbicide biodegradation by microorganisms as a food source or changes in microbial diversity (Jaiswal et al., 2021), including an increase in resistant fungal species (Pampulha & Oliveira, 2006). Similar findings were reported by Tomkiel et al. (2019), who observed a decrease in fungal population until day 30, followed by an increase until day 60 with the use of herbicides flufenacet + isoxaflutole. Continuing the incubation until day 90, depletion of carbon and energy resources, and the production of secondary metabolites resistant to degradation (Mamy et al., 2005) may explain the decrease in fungal population on day 90.

Furthermore, the decline in fungal populations on days 30 and 90 may be due to disruption and inhibited growth of fungi due to factors such as Hag's disease, inhibition of linear mycelial growth rate, and abnormal growth habits and sporulation patterns (Wilkinson & Lucas, 1969).

Microbial biomass refers to the living OM in the soil, comprising various microorganisms, particularly bacteria and fungi, which account for a small percentage (1–4%) of the organic carbon content (C_{org}). It plays a crucial role in the decomposition of organic residues and acts as a potential reservoir for nutrient release in the soil (Brookes, 2001). In this study, a reduction in MBC and specific microbial activity was observed in all herbicide-treated samples compared to the control soil during the incubation period. The extent of reduction depended on the herbicide type, dosage, and dormancy periods, with the highest reduction observed in the combination of two herbicides, followed by clopyralid, butisanstar, and the control soil. Moreover, higher doses of herbicide application (5 times the reference dose) led to a significant reduction in MBC. Santos et al. (2006) also reported a decrease in MBC and q_{micro} due to the application of fluroxypyr-p-butil and fomesafen herbicides, as well as their commercial mixture. Similarly, Baboo et al. (2013) reported comparable findings regarding the reduction of MBC in soils treated with reference doses of herbicides butachlor, pyrazosulfuron-ethyl, paraquat, and glyphosate. Previous studies on the effects of clopyralid and butisanstar on MBC and q_{micro} are lacking, underscoring the novelty of our results, which demonstrate the negative impact of both herbicides on soil MBC and q_{micro} for up to 30 days of incubation. Despite the overall declining trend of MBC and q_{micro} during the incubation period, a temporary increase in these indices was observed on the 60th day of incubation. Pertile et al. (2020) also documented an initial reduction in MBC up to 15 days after the separate addition of flumioxazin, imazethapyr, and their recommended combination dosage, followed by an increase until the end of the 60-day incubation period. The subsequent increase on the 60th day may be attributed to a rise in fungal population, as indicated by the high correlation coefficient ($r = 0.82^{**}$) (Table 3). This increase could be due to the emergence of resistant species or the activation of resistant forms utilizing lysed microbial biomass resources early in the experiment period. Soil, a significant source of CO_2 emissions, relies on the decomposition of OM by soil microorganisms as a crucial indicator of soil health. Unfortunately, the use of herbicides has a detrimental impact on this process (Sándor et al., 2020). In this study, a notable reduction in microbial respiration was observed in all herbicide-treated soils compared to the control soil, and this reduction became more severe with higher herbicide application rates. Supporting these findings, (Hart, 1995) reported a 13% decrease in CO_2 production in soil treated with 10 and 20 times the RFD of quinmerac herbicide compared to the control soil. Additionally, Sándor et al. (2020) found that 11 out of 14 herbicides, applied at 2 and 3 times the RFD, led to a decrease in microbial respiration. The decline in microbial respiration due to herbicide presence and its severity with increasing concentrations may be attributed to a decrease in microbial populations, particularly bacterial populations. In this study, a positive and significant correlation was observed between BSR and bacterial populations ($r = 0.82^{**}$) compared to fungi ($r = 0.69^{**}$) (Table 3). Furthermore, on the 60th day of incubation, an increase in microbial respiration was observed, potentially attributable to an increase in fungal populations. This explanation is

Table 3 Pearson's correlation coefficients between soil biological indicators

	Bacterial	Fungal	BSR	SIR	MBC	q_{micro}	qCO_2
Bacterial	1						
Fungal	0.65**	1					
BSR	0.82**	0.69**	1				
SIR	0.52**	0.59**	0.79**	1			
MBC	0.71**	0.82**	0.85**	0.82**	1		
q_{micro}	0.71**	0.82**	0.85**	0.82**	1.00**	1	
qCO_2	–0.02	–0.44**	–0.07	–0.33**	–0.53**	–0.53**	1

BSR Basal soil respiration, *SIR* Substrate induced respiration, *MBC* Microbial biomass carbon, q_{micro} Microbial quotient and qCO_2 Respiratory quotient

supported by the relatively strong positive correlation between fungal populations and biomass-specific respiration.

To measure MBC and gain insights into the microbial biomass status, the specific extracellular enzyme-induced respiration (SIR) is a widely used fast and simple physiological method (Visser & Parkinson, 1992). The pattern of SIR (Fig. 4B) also exhibited a relatively strong correlation coefficient ($r=0.79^{**}$) with BSR. Throughout the incubation period, reduced SIR was observed in all herbicide-treated soils compared to the control soil. This finding aligns with a study by Mukherjee et al. (2016) in which florasulam and halauxifen-methyl herbicides caused a decrease in BSR and SIR up to 30 days after incubation at the RFD and 10 times that amount. Similarly, Cycoń et al. (2013) reported a short-term and long-term reduction in SIR upon adding the recommended amount and 10 times that amount of napropamide herbicide due to its negative effect. The addition of herbicides disturbs soil conditions, leading to a decline in active microbial biomass and an increase in resistant forms (such as spores) and dormant states of microorganisms within the ecosystem (Hristeva et al., 2015). The higher SIR levels on days 60 and 90 in the presence of butisanstar herbicide compared to treatments containing clopyralid can be attributed to the lower toxicity of butisanstar herbicide and the presence of resistant species and forms of microbial populations, particularly fungi. The correlation coefficients of SIR with fungal ($r=0.59^{**}$) and bacterial ($r=0.52^{**}$) populations further support the significance of fungi (Table 3).

The qCO_2, a measure of accumulated CO_2 relative to total MBC, is employed to estimate stress and disturbances caused by the presence and consumption of chemicals in the soil (Anderson & Domsch, 1990; Visser & Parkinson, 1992). Various researchers have reported an increase in this biological index as a consequence of herbicide application and presence in the soil (Pertile et al., 2020; Serafini et al., 2022; Sofo et al., 2012). Consistent with these findings, the present study observed an increase in qCO_2 resulting from the application of clopyralid and butisanstar herbicides, particularly at higher concentrations. The elevated concentration of qCO_2 may be attributed to heightened respiration activity in resilient species (characterized by low microbial biomass) during periods of stress, along with the presence of herbicides that possess a significant potential for decomposing carbon resources. In unfavorable and stressful conditions, the respiration rate of microorganisms tends to increase, leading to the allocation of a substantial portion of their carbon and energy budget towards maintaining their survival. Consequently, this allocation results in a reduction in the growth and reproductive capacity of microbial biomass (Anderson & Domsch, 1990). The strong negative correlation coefficient ($r=-0.57^{**}$) observed in this study, as presented in Table 3, provides supporting evidence for this decline. Conversely, a lower qCO_2 value signifies a more efficient and stable utilization of existing resources by the microbial biomass, leading to a decreased release of carbon as CO_2 through respiration (Santos et al., 2006).

5 Conclusion

The findings of this investigation demonstrate that the assessed indicators exhibit high sensitivity to the application of clopyralid in comparison to butisanstar, even when used at recommended dosages. The combined use of these two herbicides resulted in a more pronounced negative impact on biological indicators than when each herbicide was applied individually. The introduction of elevated concentrations of herbicides, known for their potent inhibition of the microbial community, during the short-term experiment led to a significant reduction in microbial population, particularly bacteria, as well as decreased values for the BSR, SIR, MBC, and q_{micro} indicators. The adverse effects and inhibitory influences on the biological indicators persisted throughout the entire incubation period. Consequently, in order to mitigate concerns regarding the adverse effects of the evaluated herbicides on the soil's living organisms, it is recommended to employ herbicides in agricultural settings with minimal quantities and utmost precision to prevent disturbances in the functions of the microbial community. This approach will safeguard the quality and health of the soil, which serves as a paramount medium for agricultural production.

Data Availability Data will be made on available in reasonable request.

Declarations

Conflict of Interest The authors declare that they have no conflict of interest.

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