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ORIGINAL PAPER

Biodegradation of the trifluralin herbicide by *Pseudomonas fluorescens*

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Abstract

Trifluralin is a xenobiotic pre-emergence and relatively stable herbicide in the soil from the dinitroanilines family. The present study was conducted with the aim of biodegradation and acceleration of its detoxification process in soil by *Pseudomonas fluorescens*. The experiment was based on a completely randomized design that gauged the effect of *P. fluorescens* on the biodegradation of the trifluralin in four media types, including medium with carbon, nitrogen, carbon + nitrogen, and, without carbon + nitrogen. Population growth, chemical oxygen demand, and trifluralin concentration were measured 24, 48, and 72 h after inoculation. Results indicated that 72 h after inoculation, the maximum and minimum values of population growth were 20.28×10^6 and 1.32×10^6 cell mL^{-1} and the highest and lowest percentage of chemical oxygen demand removal was recorded as 42.29 and 19.5% from carbon + nitrogen and without carbon + nitrogen medium respectively. Based on High-Performance Liquid Chromatography results, the highest biodegradation rate was obtained in carbon + nitrogen (63.97%) and carbon medium (45.05%). According to the results, the highest and the lowest percentage of trifluralin biodegradation occurred in the nutrient-rich and nutrient-free medium. It could be concluded *that P. fluorescens* requires an energy source for decomposition, and alone it is not able to break down the structure of trifluralin to use it as a food source, however, with the help of carbon and nitrogen sources as an energy starter; it is decomposed by the possibly co-metabolic method. It seems that fertile soil is needed to activate the degradation capacity of *P. fluorescens*.

Keywords Bacterial population · Chemical oxygen demand · Co-metabolism · Xenobiotic

Introduction

Trifluralin ($C_{13}H_{16}F_3N_3O_4$) herbicide is one of the widely used pre-emergence inhibitors of cell dinitroanilines in the soil, preventing roots and shoots growth of germinating seeds (Fernandes et al. 2013). Dinitroanilines are amongst the least mobile herbicides and could cause environmental contamination (Triantafyllidis et al. 2010). Annual use of herbicides accumulates in the soil and transfers to groundwater, threatening the ecosystem and human health and damaging non-target plants. Trifluralin, as a xenobiotic compound, binds strongly to soil organic matter and, due to its inactivity, damages sensitive plants in rotation (Zeyer and Kearney 1983). Recently the process of herbicide biodegradation has received particular attention because of being cost-effective and environmentally friendly (Fernandes et al. 2013). Microorganisms decompose herbicides into small molecules and metabolize them as nutrients, and unlike physical and chemical degradation, in microbial decomposition, secondary pollution will not produce (Zhang et al. 2017).

Although trifluralin is degraded in the environment by photodecomposition and biological processes (Fernandes et al. 2013), its microbial metabolism is one of the essential degradation procedures in water and soil. Since bacteria and fungi are the most abundant microorganisms in nature, bacteria account for 65% of the total soil microbial biomass; thus, they play a significant role in the biological modification of pesticides (Islam and Wright 2003). So they have attracted significant attention recently (Siddique et al. 2003), and many microorganisms, including bacteria and fungi which can degrade trifluralin, have been isolated from different sources (Singh and Singh 2011; Lee et al. 2006). There are various reports on pesticide biodegradation by different

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microorganisms, like *Arthrobacter* sp. C3 to degrade atrazine (Wang et al. 2016a, b), *Serratia ureilytica* that decompose butachlor (Mohanty and Jena 2019), and *Pseudomonas* biodegrade cypermethrin (Jilani and Khan 2006).

Although considerable research has been done on the degradation of trifluralin in soil, there are few reports of its degradation by microorganisms (Bellinaso et al. 2003). Microbial activity in soil plays a vital role in the degradation of dinitroaniline herbicides (Fernandes et al. 2013). This process was named enzymatic reaction, which included various enzymes destroying herbicides into more minor molecular compounds (Tang et al. 2018). Microorganisms have an extraordinary ability to adapt to the environment, and new compounds, such as herbicides, induce or create new enzyme systems for degradation (Zhang et al. 2015).

Microbial degradation of trifluralin may occur under aerobic or anaerobic conditions, although the results show that further degradation occurred under anaerobic conditions in poorly drained soils (Fernandes et al. 2013). According to Rodrigues and Almeida Guia (2005), trifluralin was degraded by 98% under anaerobic conditions, while the rate of degradation under aerobic conditions was 25%, and trifluralin degrading fungi in this experiment included *Sclerotium tiumrolfsii*, *Fusariums, Aspergillus niger,* and *Trichoderma* sp. In the initial monitoring of the studied soil of Querejeta et al. (2014), 19 strains of bacteria with the capacity to degrade trifluralin were identified, 8 of which belonged to the *Pseudomonas* genus. In another study, *Brevundimonas* isolates degrade trifluralin comparatively and only in media containing carbon, nitrogen, and complex organic matter (yeast extract), evidence of co-metabolism (Hamdi et al. 1969).

There are so many unrequited queries about the environmental fate and metabolism of trifluralin; since it is a xenobiotic compound resistant to biodegradation, some studies proposed co-metabolism as its decomposition route (Zeyer and Kearney 1983; Bellinaso et al. 2003). Co-metabolism was the critical process for degrading herbicides (Ye et al. 2018). Co-metabolic discussed some chemical substances like herbicides, which biodegrade in the natural environment by adding some organic matter as the principal energy source (Zhang et al. 2010). Co-metabolic bioremediation methods have been applied in the field for more than 20 years on some of the most obstinate pollutants, like pesticides, halogenated aliphatic, chlorinated alkenes, PAHs, and aromatic hydrocarbons.

Despite the results of various investigations on trifluralin biodegradation, many questions remain about its metabolism and environmental fate, the influence of abiotic factors on the bio-decomposition rates, and the genes and enzymes accountable for its biodegradation (Coleman et al. 2020). The current study considers other significant knowledge gaps, such as growth-related biodegradation and co-metabolism. This research addresses this question: Is it possible for *P. fluorescens* to intake trifluralin as a source of carbon, nitrogen, and/or carbon + nitrogen to support their growth, or do the microbes degrade trifluralin co-metabolically? Based on cited literature, the biodegradation of trifluralin by different microorganism, including various bacteria and fungi, were investigated; however, the bio-decomposition of trifluralin by *P. fluorescens* under different nutrient sources to find out if the degradation pathway is co-metabolic was not considered.

This study evaluated the biodegradation of trifluralin in different media from nutrient-free to nutrient-rich. So the study was conducted in the central laboratory of the Faculty of Agriculture, Gonbad Kavous University, in 2018 to see if *P. fluorescens* could degrade trifluralin co-metabolically or if it can use the herbicide as a sole energy source. The results of this study may lead to an improved consideration of the fate of trifluralin in nature.

Materials and methods

This experiment was performed in a completely randomized design with three replications in the central laboratory of the Faculty of Agriculture, Gonbad Kavous University, in 2018. The effect of *P. fluorescens* (IBRC-M 10752) on the biodegradation of trifluralin (48% EC-Aria Shimi, Tehran, Iran) herbicide (Table 1) was investigated in modified Mineral Salt Medium (MSM) amended with glucose (10 g L^{-1}) and $(NH_4)SO_4$ (0.608 g L^{-1}) as a carbon and nitrogen source respectively. The Iranian Biological Resource Center (IBRC) collection prepared the bacterial isolate. Modified MS medium (mMSM) including 1.048 g KH_2PO_4, 0.928 g K_2HPO_4, 0.036 g NaCl, 0.036 g $CaCl_2.2H_2O$, 0.124 g $MgSO_4.7H_2O$, and 0.013 g of $FeCl_3.6H_2O$ per liter of distilled water with a pH of 6.9 was prepared based on the method described by Monteiro et al. (2000). All mineral salts were purchased from Merck (Germany). To evaluate the biodegradability of trifluralin and to determine if trifluralin was used as a carbon or nitrogen source, four different media were prepared, including medium with carbon

Table 1 Molecular Formula and physicochemical properties of trifluralin

Molecular formula	Molecular weight	Water solubility (mg L^{-1} at pH = 7)	Vapor pressure (mm Hg 25 °C)	Octanol–water partition coefficient Log (Kow)
$C_{13}H_{16}F_3N_3O_4$	335.28	0.222	4.58×10^{-5}	5.34

National Center for Biotechnology Information (2021)

(C), nitrogen (N), carbon + nitrogen (CN) and without carbon + nitrogen (noCN).

The required concentration of the herbicide was calculated using the minimum inhibitory concentration (Umar et al. 2012). Sterile trifluralin was prepared using a microbiological filter (0.22 µm), and the proper concentration was added to the sterilized MS medium after sterilizing for 15 min at 1.5 psi and wrapped with the cover to avoid photodegradation. Each of the 24 flasks (6 flasks for each medium composition) was taken for the biodegradability experiment.

Preparation of the microbial inoculums

The bacterial isolate was pre-cultured on nutrient agar medium (Merck, Germany) and was re-cultured on complete MSM containing trifluralin (0.2%). Flask was inoculated with bacterial isolate using sterilized loops. Inoculated flasks were kept in a rotary shaker (150 rpm) for 24 h at 25 °C. The bacterial suspension was adjusted equal to 0.5 McFarland and was added to the test flasks as an initial inoculum. No bacterial inoculum was added to control flasks.

Bacterial population growth kinetics

After inoculation with bacterial inoculum, flasks were placed in a shaker incubator at 150 rpm, at 25 °C for 72 h, and the absorption was measured by the spectrophotometer (Model 6300 Jenway-UK) in 600 nm for evaluating growth (Pipkin et al. 1997). To calculate bacterial cell density, the optical density of bacterial suspension was adjusted to 0.1, using a spectrophotometer (600 nm). To calibrate the data given by the spectrophotometer, the standard plate count technique was used to estimate the population density of bacteria in a sample by plating 1 mL of a diluted sample and counting the number of bacteria colonies. Then after, the optical density of each sample was determined severalty, and bacterial cell density was calculated using a calibration curve. In order to choose the best bacterial activity, based on optimum growth in the presence of herbicide, bacterial growth was measured during different hours of incubation.

Chemical oxygen demand measurements

The samples' chemical oxygen demand (COD) was measured 24, 48, and 72 h post-inoculation (*hpi*). The COD measurement was done according to the standard method of Baird et al. (2012). First, the digestion solution containing 1.5 mL of $K_2Cr_2O_7$ was added to the 2.5 mL sample. Then, 3.5 mL sulfuric acid was added to the mixture to create an acid layer under the digestion solution and entirely mixed by inverting it several times. The containers were heated in a block digester at 150 °C and refluxed for 2 h. After cooling to room temperature, the COD of samples was measured using an AL125COD meter.

The equation below calculated the COD removal efficiency (Eq. 1).

$$COD_{rem} = \left(\frac{COD_{int} - COD_{fin}}{COD_{int}}\right) \times 100 \quad (1)$$

COD_{rem} signifies the efficiency of COD removal, COD_{int} represents the initial COD, and COD_{fin} is identified as the final COD.

Trifluralin concentration detection through HPLC

All high-performance liquid chromatography (HPLC)-grade chemicals and trifluralin chromatography analytical standards were attained from Sigma-Aldrich (Germany) and Merck (Germany), respectively. After 72 h, the solution was centrifuged for 10 min twice at 12,000 rpm, and the grown bacterial mass in the shaker incubator was taken from the solution. Then the top solution was filtered through a Millipore filter.

Trifluralin concentrations were quantified by HPLC (KNAUER, Pump 1000, UV 2600) with an injection volume of 60 µL, using a C_{18} column (250 mm × 4.6 mm). The samples were examined by isocratic elution in the HPLC system with the mobile phase of acetonitrile and distilled water (60:40 w/w) and the flow rate of 1.0 mL min^{-1}. The photodiode array detector had a wavelength of 235 nm. The amount of trifluralin concentration was calculated based on the peak reduction of standard trifluralin and observed samples to quantify the area reduction of the peak. The contribution of *P. fluorescens* biodegradation can be calculated by comparing trifluralin concentration in bacterial and non-bacterial samples after 72 h.

Statistical analysis of data

Abnormal data were normalized by logarithmic conversion and then analyzed by SAS software version 9.1, and mean comparison was performed by LSD test at 1% level. The figures were also drawn in Excel software.

Results and discussion

Changes in the population density of P. fluorescens

The effect of trifluralin herbicide on the microbial population of *P. fluorescens* was determined based on the changes in the bacterial population in each medium. During the experiment, bacterial population density showed a significant increase ($P < 0.01$) in all different media up to 72 *hpi*

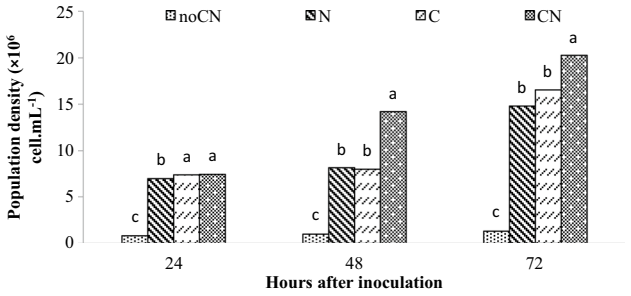

Fig. 1 Population density of *P. fluorescens* in CN (mMSM with glucose and $(NH_4)SO_4$), N (mMSM with $((NH_4)SO_4)$, C (mMSM with glucose) and noCN media contained trifluralin during the period of 24, 48, and 72 *hpi.* LSD values for 24, 48, and 72 h after inoculation were 0.2×10^6, 1.68×10^6, and 3.17×10^6

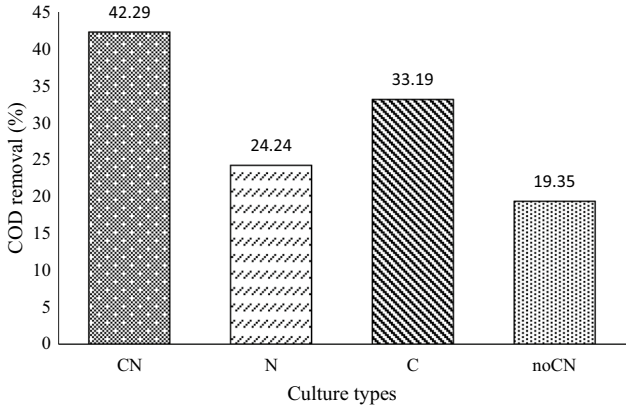

Fig. 2 Chemical oxygen demand removal percent in CN (mMSM with glucose and $(NH_4)SO_4$), N (mMSM with $(NH_4)SO_4$), C (mMSM with glucose) and noCN, media contained trifluralin 72 *hpi* of *P. fluorescens*

(Fig. 1); moreover, its population increased in all media types over time. It seemed that *P. fluorescens* could grow in the presence of trifluralin in all different media. Although the bacterial population in C and CN media was not significantly different at 24 *hpi*, this difference became significant during the next 48 *hpi,* and CN media showed the maximum growth at the end of the experiment. Similarly, there was no significant difference between the bacterial population in the C and N amended medium up to 72 *hpi*. The lowest bacterial population, 24, 48, and 72 *hpi* in noCN medium were 0.8×10^6, 1×10^6 and 1.32×10^6 cells mL^{-1} and the highest amount was obtained from CN medium with 7.44×10^6, 14.19×10^6 and 20.28×10^6 cells mL^{-1} (Fig. 1), respectively. Bacterial population growth of 63.31% in medium containing CN and 37.12% from noCN medium was recorded as highest and lowest at 72 *hpi* compared to 24 *hpi*, respectively. Bacteria need carbon and nitrogen sources to increase their population growth. Through the nature of the media treatments, except for the noCN medium, all or part of the food source needed for the population growth of bacteria was available in all other treatments. *P. fluorescens* could adapt to the herbicide over time; as seen in Fig. 1, the population density increased during the experiment. Jilani (2013) witnessed no difference between *Pseudomonas* growth at low pesticide concentrations and no pesticide sample. She also stated that high pesticide concentrations declined the microbial amount; however, the culture stayed in the lag phase, and no loss happened in the bacterial population. Usually, the growth of microorganisms in a particular element signifies its metabolic ability (Jilani 2013). Sharifi et al. (2015) also explained that bacterial isolates, which showed better activity in media amended with herbicide than other isolates, could decrease the herbicide more.

In the present experiment, the *P. fluorescens* population increased in trifluralin-containing media with rich nutrient sources. The lowest population growth was recorded in the nutrient-free medium (noCN), indicating that *P. fluorescens* is not so effective in using trifluralin as the only energy source. Other nutrient-containing media, primarily carbon and nitrogen, enhanced bacterial population growth, supporting this hypothesis that *P. fluorescens* is not specialized enough to use trifluralin as a sole energy source, so it may be implied as a co-metabolic degrading pathway. Bacterial isolates from trifluralin-containing media as the only carbon and energy source do not necessarily use this compound for active metabolism. This point was emphasized in a study of bacteria isolated from Brazilian soil by enriching the medium in which four distinct strains (one *Klebsiella*, one *Herbaspirillum,* and two *Bacillus* spp). They found that trifluralin could degrade in the medium's presence of succinate and yeast extract (Bellinaso et al. 2003).

Furthermore, the short lag phase, coupled with rapid logarithmic growth of the bacteria in all media except noCN (Fig. 1), showed the effect of external carbon and nitrogen sources for the priming of the degradation mechanism by the bacteria and tended to rapid herbicide degradation. This result is supported by Moneke et al. (2010), who showed that the effective utilization of glyphosate by the selected bacterial isolates, including *P. fluorescens*, coincided with the rapid growth of the bacteria.

Chemical oxygen demand measurement

Chemical oxygen demand is a circuitous valuation of the amount of organic matter, which was significantly different in all four media ($P<0.01$). As shown in Fig. 2, COD removal, the reduction of chemical oxygen demand percentage, was calculated. The highest percentage of COD removal was related to CN (42.30%) and C (33.19%) media. N and noCN medium values were 24.24 and 19.35%, respectively. It should be noted that the highest value of COD removal

represents more herbicide degradation (Jilani and Khan 2006).

Explain that in a noCN medium, the only organic matter present was herbicide, so its initial chemical oxygen demand is lower than in other media. Considering the difference between media nutrients, it seems that part of the difference between COD removal is related to the quantity differences of organic matter used in the medium.

COD is the quantity of oxygen necessary for the oxidation of total organic matter in water, so the more organic matter, the higher COD value (Yao et al. 2014). In the current study, the amount of COD at the end of the experiment was lower than at the beginning in all media, especially in C and CN media 72 *hpi* because trifluralin degraded more in these two media, and it is evident that the COD value depends on the amount of organic matter. Since trifluralin declined over 72 *hpi*, the COD value decreased, and the COD removal increased, following Jilani (2013), who proved a similar correlation between the biodegradation rate and COD removal. The maximum COD removal (42.30%) was recorded from CN culture, and the minimum (19.35%) was measured from noCN culture (Fig. 2). It seemed that the COD-removal efficiency increased as the trifluralin biodegradation increased, consistent with Erguven et al. (2016). They reported that the highest COD removal was obtained from the fungi and bacteria mixtures, leading to the highest trifluralin degradation, up 82%. Another study demonstrated that *B. simplex* had the uppermost COD removal efficiency and elucidated the high capability of chlorsulfuron metabolism in contaminated soil (Erguven and Yildirim (2016).

Trifluralin quantification

Analysis of trifluralin consumption by *P. fluorescens* in four different media containing trifluralin was calculated according to the herbicides reduction on the standard curve. Based on the results of the standard sample of trifluralin, the peak extracted at 17:25 min indicates the concentration of this herbicide (Fig. 3).

Measuring the amount of trifluralin herbicide 72 h later from the no-bacterial sample elucidates that trifluralin degraded about 3.15, 17.81, 25.25, and 1.90 percent compared to the start of the experiment in C, N, noCN and CN medium respectively (Table 2). The herbicide reduction in bacterial-free conditions seems related to chemical degradation. Trifluralin chemical degradation rate in N and noCN media treated with no bacteria was higher than the other two media compared to the beginning of the experiment. The amount of biological degradation can be calculated by comparing herbicide reduction percent between samples "with

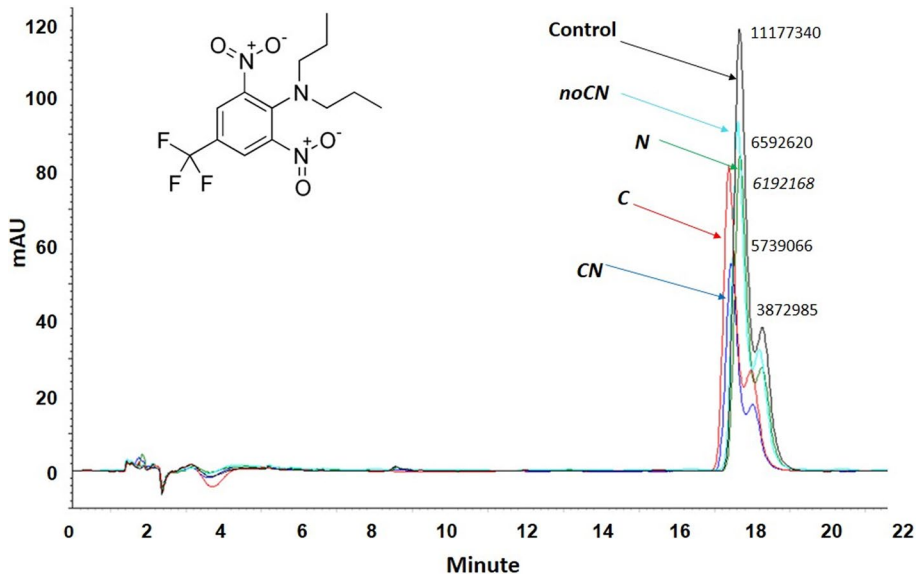

Fig. 3 HPLC chromatogram for trifluralin degradation by *P. fluorescens* in CN (mMSM with glucose and $(NH_4)SO_4$), N (mMSM with $(NH_4)SO_4$), C (mMSM with glucose) and noCN, after 72 h of inoculation. Control is for the standard (trifluralin retention time = 17.25 min)

Table 2 Trifluralin reduction percent in CN (mMSM with glucose and (NH_4) SO_4), N (mMSM with (NH_4) SO_4), C (mMSM with glucose), and noCN, 72 h after the experiment started

	Carbon + nitrogen (%)	Nitrogen (%)	Carbon (%)	Without carbon and nitrogen (%)
Chemical degradation	1.90	17.81	3.15	25.25
Biodegradation	63.97	22.85	45.05	14.76

bacteria" and "without bacteria,". The CN and C obtained the highest biodegradation rate at 63.97 and 45.05%, respectively (Table 2). The amount of biodegradation in N and noCN medium ranged about 14.76–22.85%. According to the HPLC analysis of pendimethalin by Mu'azu et al. (2018), *Bacillus* sp. and *Pseudomonas* sp. revealed a promising ability to reduce this herbicide and confirmed the decontamination potential of these microorganisms. Bio-decomposition of other chemical herbicides, including glyphosate by *Pseudomonas* sp., *Arthrobacter atrocyaneus*, and *Flavobacterium* sp. (Singh and Singh 2016) and pendimethalin degradation by *B. megaterium* (Abdel-Moteleb and Hasan 2013) also reported.

Trifluralin structure is very xenobiotic because of the presence of two nitro groups that are rare in nature (Ju and Parales 2010) and the trifluoromethyl group, which are not found in nature at all (Zanda 2004). Since xenobiotic compounds are unsuitable substrates for microbial enzymes, they are usually resistant to biodegradation (Wang et al. 2016b). However, it should be noted that the reduction of nitro groups and *N*-dealkylation observed in the soil is, at least in some cases, due to microbial function. Theoretically, a significant amount of carbon and nitrogen is present in trifluralin, but its xenobiotic structure and very low aqueous solubility indicate that it is difficult for microbes to access it as food. Generally, the predictions and experimental observations are consistent with the idea that trifluralin's microbial degradation is more co-metabolic than catabolic reactions (Coleman et al. 2020).

In other words, the physicochemical properties of trifluralin, such as low solubility in water (0.22 mg L^{-1} at 20 °C) and high vapor pressure (6.7 MPa at 20 °C) (Coleman et al. 2020), have a significant impact on determining its environmental fate, including rapid disappearance, strong binding to organic matter and little leaching to groundwater. However, considering the xenobiotic structure of trifluralin, its biodegradation still occurs, and pure media containing bacteria and fungi can microbial decompose it by dealkylation and nitro group reduction (Coleman et al. 2020).

One of the first detailed biodegradation studies of trifluralin (Zeyer and Kearney 1983), in which 180 bacteria and fungi were studied, showed that one-third of the isolates released some labeled carbon dioxide from propyl-1-^{14}C trifluralin. However, the overall mineralization rate was meager (1–6%) and required the presence of other food sources, such as sucrose, lactose, and yeast extract, clearly indicating co-metabolism. Their results confirmed that the yeast could not use trifluralin as a carbon or nitrogen source and could not release $CO_2{}^{14}$ from the ring-^{14}C- labeled or $CF_3{}^{14}$-labeled-trifluralin, which characterizes the biodegradation mechanism as a co-metabolite dealkylation. Although a complex mixture of trifluralin metabolites was observed in this experiment, only one of them (3-nitro-5-(trifluoromethyl) benzene-1,2-diamine) was identified, which is related to trifluralin by removing the propyl group and reduction a nitro group to an amine (Zeyer and Kearney 1983). However, the enzymes responsible for the dealkylation or reduction of the nitro group have not been identified. The general conclusion of their study showed that many soil aerobic microbes are capable of trifluralin dealkylation, but the ring structure of the herbicide molecule is not destroyed (Zeyer and Kearney 1983). Zablotowicz et al. (2001) reported the reduction of trifluralin nitro groups by *P. fluorescens* isolates and the production of dealkyl metabolites, but unfortunately, the identification of the metabolite was tentative, and the responsible enzymes were not identified.

There are some conflict reports on dinitroaniline biodegradation. Some believe that bacteria like *B. cirulans* could grow in a medium containing pendimethalin (1 g L^{-1}) as the only carbon source and produce the metabolites 3,4-dimethyl 2,6-dinitroaniline and 6-amino pendimethalin. Based on their results, this bacterium could use the herbicide as a source of carbon and energy for growth by reducing nitro to 6-amino pendimethalin and by oxidative dealkylation to 3,4-dimethyl 2,6-dinitroaniline (Megadi et al. 2010). On the other hand, Avarseji et al. (2021) reported that some bacterial species, such as *Pseudomonas* could decompose pendimethalin effectively in a liquid medium containing a supplementary carbon source.

Conclusion

The maximum population of *P. fluorescence* bacteria was obtained from the C and CN media; the maximum COD removal and trifluralin decomposition were recorded in these two media. Since the lowest population density and herbicide reduction were documented in the noCN media, it could be concluded that *P. fluorescens* cannot consume trifluralin as the sole nutrition. Trifluralin is not an energy source for *P. fluorescens*; its degradation is likely because of a co-metabolic process with glucose and ammonium sulfate as an energy starter. *P. fluorescens* failed to grow at contained trifluralin medium with no glucose and ammonium sulfate compared to others is another reason to suggest a possibly co-metabolic degradation pathway of trifluralin. Biodegradation is a cost-effective, eco-friendly, and efficient procedure for herbicide removal that could be implemented in polluted areas as fast remediation. Microorganisms are the main self-reproductive, abundant weapon in the soil that systematically could degrade the herbicides. So future research is required to understand the biodegradation mechanism and discover the involved enzymatic processes.

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Authors' contributions ZA and FT conceived the ideas. ZA and FT and MF and EGA assembled the data, analyzed the data. EGA helped to interpret the data. ZA and FT wrote the manuscript. FT, ZA and EGA edited the manuscript. All authors improved and approved the manuscript.

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Declarations

Conflict of interest The authors declare no competing interests.

Ethical approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

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