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# Mycoremediation effect of *Trichoderma harzianum* strain T22 combined with ozonation in diesel-contaminated sand

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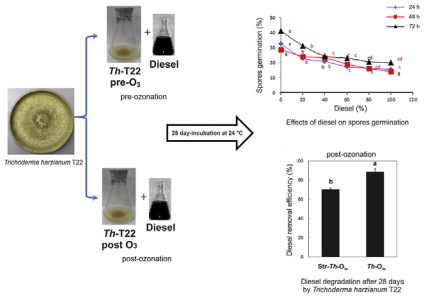
## HIGHLIGHTS

- Bioremediation by *Trichoderma harzianum* in diesel-contaminated sand was studied.
- The effect of ozonation was also examined (pre- and post-treatment).
- Survival, growth and sporulation of *T. harzianum* were monitored.
- *T. harzianum* showed mycoremediation effect in diesel-contaminated sand.
- Pre-ozonation negatively affected the subsequent biodegradation.

## GRAPHICAL ABSTRACT

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## ABSTRACT

This study aimed to determine the ability of the fungus *Trichoderma harzianum* strain T22 (*Th*-T22) to utilize diesel fuel as a carbon source. The potential use of *Th*-T22 for diesel bioremediation in an artificial soil was tested by inoculating a diesel-sand mixture with a fungal mycelial suspension of *Th*-T22. Given the ability of ozone to degrade compounds with low biochemical reactivity, the effect of a pre- and post-ozonation was also evaluated. The survival, growth and sporulation of *Th*-T22 throughout the bioremediation trial were monitored in all the treatments. In the post-ozonation treatments, the biodegradation percentages of diesel removal were 70.16% and 88.35% in *Th*-T22-inoculated sand treated or untreated with the antibacterial streptomycin, respectively. The results showed that ozonation alone caused good removal efficiencies (41.9%) but it was much more effective if combined with *Th*-T22 in a post-ozonation regime, whereas pre-ozonation negatively affected the subsequent biodegradation, likely due to its disinfectant and oxidizing effect on *Th*-T22. The results obtained demonstrated the significant mycoremediation ability of *Th*-T22 in diesel-contaminated sand and its possible use as a bioremediation agent for diesel spills in polluted sites.



## 1. Introduction

Crude oil contains several environmental contaminants, such as polyaromatic hydrocarbons (PAHs), naphthenes, paraffins, organic sulfur/nitrogen compounds and phenols, and its breakdown and

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diffusion into the soil can damage the biological community systems, essential for plants and soil microorganisms (Dariush et al., 2007). In particular, diesel is a complex mixture of total petroleum hydrocarbons (TPHs), that include volatile and extractable petroleum hydrocarbons, that are made by 75% saturated hydrocarbons (primarily alkanes and paraffins, including n-, iso-, and cycloparaffins) and 25% aromatic hydrocarbons (including naphthalenes and alkylbenzenes) (Yu et al., 2005; Lee and Kwon, 2017; Zemo et al., 2017).

Diesel is still widely used as a fuel and it is responsible for soil contamination, mainly caused by leaks from underground storage tanks or pipelines or accidental spills from transporting tanks, vehicles, trains, ships and trucks (Yu et al., 2005). The main impact of diesel spills is due to movement in soil and migration to groundwater and, eventually, to drinking water supplies (Lee and Jang, 2011). In contaminated sites, diesel can exist in different forms: adsorbed on solid particles, dissolved in water and volatile in pores. However, since most compounds have both low solubility and volatility, diesel is generally adsorbed on the grain surface, making the remediation by biodegradation or chemical technologies difficult to adopt (Yu et al., 2005; Lee and Jang, 2011). The persistence of diesel can cause a major change in the physicochemical properties of the soil, and has a direct toxic effect on both aquatic and terrestrial ecosystems, becoming responsible for mutagenic and carcinogenic phenomena in both animals and humans (Argumedo-Delira et al., 2012; Ai-Jawhari, 2014; Andreolli et al., 2016). In particular, the residual oil in the soil is a risk due to its recalcitrant nature, representing a persistent toxicity source for plants and microorganisms, essential for soil functions (Chorom et al., 2010; Wang et al., 2013).

For all these reasons, the degradation of the contaminants from diesel, especially by using innovative and natural bioremediation methods, is of great interest nowadays. The biodegradation process permits the transformation of hazardous substances into non-toxic forms and represents one of the primary mechanisms for removing petroleum and diesel contaminants from the environment (Ganesh and Lin, 2009). TPHs molecules are usually strongly attached to the colloidal surfaces of soil upper layer, the richest in microbial activity, therefore the biological treatment could be useful and give promising results. In this regard, the biostimulation and bioaugmentation solutions are mostly coupled with the biological treatments to improve the mineralization rate, especially in *in situ* chemical oxidation (Ahn et al., 2005; Hamidi et al., 2007; Hong et al., 2008; Russo et al., 2012). The microbial mineralization strategy for TPHs has been examined either using bacteria and fungi, such as *Pseudomonas* (Widdel and Rabus, 2001) and *Phanerochaete* (Moen and Hammel, 1994). Ganesh and Lin (2009) reported the ability of gram-positive bacteria to degrade diesel contaminated soil as much as their biosurfactant production increased. The ability of fungi of degrading crude oil varies along with a wide range, and some of them can efficiently degrade diesel and exhibit high affinity towards crude oil (Maddela et al., 2015; Ghanem et al., 2016).

In the last decade, many researchers have also focused on the use of ozonation as a suitable technique to oxidize organics contained in diesel, otherwise difficult to remove using other technologies (Lee and Jang, 2011). Ozone ($O_3$) is an allotrope form of oxygen considered a strong oxidant, more than oxygen ($O_2$) or hydrogen peroxide ($H_2O_2$), with a redox potential of 2.07 V and about 11 times more soluble than $O_2$ in water solutions (Jung et al., 2005). $O_3$ reacts directly or indirectly via $OH^{\bullet}$ radicals with organic and organometallic functional groups at points of high electron density. Therefore, $O_3$ becomes able to break both double and resonance bonds, so oxidizing many organic pollutants (Liang et al., 2009; Wang et al., 2012; Dong et al., 2015). Particularly, $O_3$ attacks the σ-bonds between C and H atoms utilizing the 1,3-dipolar insertion mechanism and $OH^{\bullet}$ radical reactions, transforming alkanes in short-chain compounds, as well as in their oxidative by-products, such as alcohols, ketones and carboxylic acids (Yu et al., 2005; Liang et al., 2009). The effects of the remediation with intermittent and continuous ozonation, that ensures a higher microbial activity, and consequently promotes mineralization and the oxidation of recalcitrant compounds have been recently applied successfully (Wu et al., 2015; Azubuike et al., 2016).

*Trichoderma* spp. fungi belong to *Ascomycetes* and can adapt in different environmental conditions at all latitudes and in a wide variety of habitats, significantly contributing to soil fertility and plant growth (Sofo et al., 2012; Argumedo-Delira et al., 2012; Vitti et al., 2015). For this reason, *Trichoderma* spp. have been used effectively as potential bioremediation agents through the breakdown and transformation of organic pollutants, such as PAHs, diesel residues, or other micropollutants, such as pharmaceuticals (Zafra et al., 2015, Buchicchio et al., 2016; Palli et al., 2017). They can produce extracellular hydrolytic enzymes able to mineralize a wide range of organic compounds (Vinale et al., 2013). Seventy species of *Trichoderma* are known to degrade high-molecular-weight petroleum hydrocarbons because of their capacity in using common enzymes in alternative ways, such as multicopper laccases, peroxidases and ring-cleavage dioxygenases (Cobas et al., 2013; Maddela et al., 2015; Zafra et al., 2015; Andreolli et al., 2016).

On this basis, this study aimed to test an integrated remediation technology for diesel-contaminated soils, in which the biodegradation using *Trichoderma harzianum* strain T22 (*Th*-T22) was coupled with ozonation treatment before and after an incubation period of 28 days.

## 2. Materials and methods

### 2.1. Sand characterization and spiking

Silica sand, chosen as model soil, was left to rest at laboratory conditions for a week and then characterized to determine its physico-chemical properties, i.e. granulometry, soil organic matter (SOM), water content and pH. To guarantee homogeneous samples, remediation tests were performed on 0.5–2.0 mm sieved samples characterized by the physicochemical properties reported in Table 1. SOM was evaluated by baking 100 g of soil in muffle at 605 °C up to reaching constant weight, subsequently placed in a desiccator at room temperature until complete cooling. Soil water content was measured by drying 100 g of soil in an oven at 105 °C up to reaching constant weight. Subsequently, the 100 g-sample was placed in a desiccator at room temperature until complete cooling. Soil pH was measured in suspension by adding 30 g of soil to 500 g of distilled water in a glass beaker and then stirring the suspension with a magnetic stirrer for 15 min. After covering the beaker with paraffin to avoid water evaporation, the suspension was left to rest at room temperature for 24 h.

Silica sand samples were artificially contaminated with

**Table 1**
Physico-chemical properties of the sieved sand. The values represent means ($n = 5$) of five independent replicates.

| Parameter | Value | Unit |
|---|---|---|
| Texture | coarse sand | – |
| Grain size | 0.5–2.0 | Mm |
| pH | 7.8 | – |
| Water content | 0.11 | % |
| SOM content | 0.07 | % |

SOM: Soil Organic Matter.

commercial diesel-fuel obtained from a gasoline station at a concentration of 6000 mg $kg^{-1}$ (on a dry basis), that is 8 and 120 times higher than Italian threshold limits of 750 mg $kg^{-1}$ and 50 mg $kg^{-1}$ for heavy hydrocarbons (C > 12) in industrial and residential areas, respectively. To simulate homogeneously contaminated soils, before the contamination the sand was firstly autoclaved at 121 °C for 20 min, dried in an electrical oven at 60 °C for 2 h, and then placed in a desiccator at room temperature. A spiking solution was prepared using methylene chloride as a solvent. In detail, 2.43 g of diesel were weighed in a glass beaker, added to 30 mL of methylene chloride, and then stirred for 5 min. Successively, 135 g of sand sample were spiked to 10 mL of the previous emulsion and manually mixed to establish a homogeneously contaminated condition with an initial concentration of 6000 mg $kg^{-1}$. The soil sample was weathered at 20 °C in the hood for 5 d to evaporate solvents and volatile fractions. The effect of diesel contamination on pH value of the soil sample was also observed, proving a negligible alteration not further considered in this work. The preparation and the treatment of the soil samples were carried out at room temperature.

### 2.2. Ozonation treatment

Ozonation apparatus was composed of a laboratory-scale glass column (diameter = 4 cm; length = 10 cm) connected at the bottom to a portable ozone generator for ambient air. A multi-perforated plate and a cotton plug were disposed at the bottom of the column to hold the soil, ensuring a homogenous distribution of the ozone through the soil volume, and avoiding the occlusion of ozone tubing. Then, 135 g of spiked soil were packed into the column, which was exposed to ozone flux in different operational conditions. Ozone was generated from ambient air by a bench ozone generator (Model ZY-H135E; Tommesani, Bologna, Italy), passed through a Teflon tube and then into the contaminated sample from the bottom of the ozonation column. Ozonation tests were performed at 225 L $h^{-1}$ for 30 min in intermittent flux (10 min "on" and 10 min "off"). The ozonation process was tested before (pre-ozonation) or after (post-ozonation) the incubation period of 28 d with *Th*-T22.

### 2.3. Biodegradation by Th-T22

*Tested fungus*. *Th*-T22 was frozen conserved in the mycotheca of the School of Agricultural, Forestry, Food and Environmental Sciences (SAFE), University of Basilicata, Potenza, Italy (collection number 1559) and recultured on Potato Dextrose Agar (PDA) plates.

*Biodegradation assay*. The bioremediation effect of *Th*-T22 in the contaminated soil by diesel was evaluated with the following experimental procedures. The *in vitro* assay was carried out in Potato Dextrose Broth (PDB)-treated/untreated with streptomycin (250 ppm).

#### 2.3.1. Fungal inoculation

Two fresh fungal discs (Ø = 0.5 $cm^2$) were used for inoculating the nutrient substrates into the flasks and, successively, the substraes were divided into six treatments as following:

a) Str-*Th*-$O_{3b}$ = 200 mL PDB + 250 ppm streptomycin + *Th*-T22
b) *Th*−$O_{3b}$ = 200 mL PDB + *Th*-T22
c) Str-*Th*-$O_{3a}$ = 200 mL PDB + 250 ppm streptomycin + *Th*-T22
d) *Th*−$O_{3a}$ = 200 mL PDB + *Th*-T22
e) Str-$O_3$ = 200 mL PDB + 250 ppm streptomycin (control 1)
f) $O_3$ = 200 mL PDB (control 2)

where Str = streptomycin, *Th* = *T. harzianum* strain T22; $O_{3b}$ = ozonation before *Th*-T22 incubation (pre-ozonation); $O_{3a}$ = ozonation after *Th*-T22 incubation (post-ozonation).

All the treatments were incubated at 24 °C in a rotary shaker for 28 d. One sample of about 1 g was taken every week and suspended in 5 mL distilled water, mixed vigorously, and 20 μL-aliquots were cultivated on PDA plate and incubated for 4–5 d at 24 °C. Then, the mycelium diameter was measured in order to evaluate the growth of *Th*-T22 and ensure the absence of any microbial contamination.

#### 2.3.2. Analytical method for evaluating diesel degradation

A 10 g aliquot of the treated sand from each treatment was suspended in 10 g of anhydrous sodium sulfate, vortexed for 2 min, and then added in a Soxlet apparatus with 250 mL dichloromethane for 6 h. The extracted solution was poured in a rotary evaporator for 5 h at 40 °C to remove the organic solvent. The amount of residual diesel was weighed after evaporating the solvent under reduced pressure. The degradation capacity (% R) was evaluated by calculating the differences between the initial ($C_{in}$) and the final weight ($C_{out}$) using the following equation:

$$\%\,R = [(C_{in} - C_{out}) \,/\, C_{in}] \times 100 \qquad \text{Equation 1}$$

where % R = the degradation capacity; $C_{in}$ = initial weight of the sample; $C_{out}$ = final weight of the sample.

As suggested by Rivas et al. (2009), the results were calculated on the basis of the amount of diesel extracted with the Soxhlet procedure. Absolute values were calculated by considering that approximately 50% of diesel was extracted in control analysis. Residual diesel was also detected by gas chromatographic analysis to evaluate the variation of the chromatogram profile and verify the qualitative variation of the composition of extractable residues. In detail, the solution of Soxhlet extraction was filtered on 1 g of sodium sulfate anhydrous and 1 g of Florisil (60–100 mesh). The analysis was carried out using Shimadzu 2010 Plus Tracera gas-chromatograph (Kyoto, Japan) equipped with a Restek Rtx-624 fused silica capillary column (6% cyanopropyl-phenyl − 94% dimethylpolysiloxane; 20 m × 0.18 mm I.D.; film thickness = 1.00 μm) and a barrier ionization discharge (BID) detector. The injector port was set to 200 °C and the oven temperature program was adjusted at 30 °C for 6 min, increasing to 200 °C with a 3 °C $min^{-1}$ rate and holding for 60 min. All the injections (100 μL) were made in a split mode (split ratio = −1.0). Helium 6.0 (SIAD corporation, Bergamo, Italy) was used as carrier gas at a flow rate of 0.49 mL $min^{-1}$. The BID detector operated at 270 °C via a 50 mL $min^{-1}$ discharge gas flow.

### 2.4. Behavior of Th-T22 to diesel-contaminated soil

#### 2.4.1. Fungal mycelium growth

Fungal mycelium growth was measured following the microdilution method using a *96-well* microplate technique (Nunc MaxiSorp®, Denmark), as reported by Elshafie et al. (2019). Briefly, 4 mL of liquid suspension from fresh fungal cultures (96 h) were prepared at $10^8$ colony forming units (CFU) $mL^{-1}$. Diesel samples were incorporated into PDB at a concentration of 6 g $kg^{-1}$ (the same concentration used for the biodegradation assay). Successively, serial dilutions were carried out to obtain the following concentrations: 4.8, 3.6, 2.4 and 1.2 g $kg^{-1}$. Then, 200 μL from each prepared mixture and 100 μL of the prepared fungal suspension were added to each microplate well and then incubated at 24 ± 2 °C for 72 h. The absorbance was measured at λ = 540 using Microplate reader instrument (DAS s.r.l.; Rome, Italy) at the following times (*t*): 0, 24, 48 and 72 h from the beginning of the incubation. Measuring the absorbance of the fungal growth achieved in different diesel concentrations and the incubation times indicated the tolerance of

*Th*-T22 to diesel.

#### 2.4.2. Spore production and germination

The effect of diesel on the spore production capacity and conidial germination of *Th*-T22 was determined using the hemocytometer slide. The possible impact of diesel was examined by preparing the following concentrations: 6.0, 4.8, 3.6, 2.4 and 1.2 g $kg^{-1}$ (Table 2), following the method of Han et al. (2019) and Della Pepa et al. (2019) with minor modifications. In particular, 1000 μL of *Th*-T22 suspension ($10^6$ CFU $mL^{-1}$) were inoculated in 25 L of PDB supplemented with the concentrations described above and vortexed for 30 s. All the prepared suspensions were incubated at 24 ± 2 °C for 96 h. Approximately, 500 μL of each prepared suspension was dropped onto a microscope hemocytometer slide, blended, and incubated at 28 °C and 80% relative humidity. The total spore production (equation (2)), spore germination percentage (equation (3)) and the inhibition percentage (equation (4)) were recorded after 24, 48 and 72 h using a light microscope.

$$\text{Spores / mL} = n \times 10^4 \qquad \text{Equation 2}$$

where $n$ = the average cell count per square of the four corners counted by hemocytometer slide method.

$$\text{SG (\%)} = (n\ \text{spores}_t\ /\ n\ \text{spores}_c) \times 100 \qquad \text{Equation 3}$$

where SG (%) = spore germination percentage; $n$ $\text{spores}_c$ = total number of spores in the control; and $n$ $\text{spores}_t$ = total number of spores in the treatments.

$$\text{SGI (\%)} = \text{SG}_c\ (\%) - \text{SG}_t\ (\%) \qquad \text{Equation 4}$$

where SGI (%) = inhibition percentage of spore germination; $SG_c$ (%) = spore germination percentage of control; and $SG_t$ (%) = spore germination percentage of the treatment.

### 2.5. Statistical analysis

The obtained results were statistically processed and subjected to one-way analysis of variance ANOVA, followed by *Tukey* B Post Hoc multiple comparison test with a probability of $p < 0.05$ using SPSS statistical software package (version 13.0; IBM, Armonk, NY, USA).

## 3. Results and discussion

### 3.1. Diesel biodegradation by Th-T22

Silica sand, in which diesel has been freshly added (6 g diesel $kg^{-1}$ sand), was chosen because of its low content of organic matter, which could interfere and compete with the contaminants during the biodegradation processes, making the evaluation of diesel removal difficult. Furthermore, sandy soils (e.g., costs, beaches, marine environments) are among the most vulnerable to diesel pollution, and the sand itself is the main inorganic component of a wide range of soil types. In all the treatments inoculated with *Th*-T22 (i.e., Str-*Th*-$O_{3b}$, *Th*−$O_{3b}$, Str-*Th*-$O_{3a}$, and *Th*−$O_{3a}$), the mycelium growth was optimal throughout the incubation time of 28 d and no other fungal species have been found. The results highlightedthat the level of diesel decreased during the incubation period due to the ability of *Th*-T22 to consume it as a carbon source, compared to the lower diesel removal obtained in the case of control treatment without *Th*-T22 (Str-$O_3$ and $O_3$) (Fig. 1).

The breakage of the organic compounds with the consequent increase of free active molecules on fungal outer-surface, where they become available for mineralization (Ai-Jawhari, 2014), could have been one of the causes of the observed diesel biodegradation by *Th*-T22. Indeed, Greensmith (2005) studied the ability of *Th*-T22 and found that this fungus produces surfactants able to increase the solubility of hydrocarbons and to make them bioavailable to the bacteria. This hypothesis has been also confirmed by Almansoory et al. (2015), who studied the application of biosurfactant for phytoremediation strategy of gasoline-contaminated soils, discovering that the removal of total petroleum hydrocarbons was raised up to 93%. A similar level of diesel removal (88.35%) was obtained in contaminated soils after a 28 d incubation with *T. longibrachiatum* (Cobas et al., 2013). In the same species, Andreolli et al. (2016) observed the high biodegradation capacity of this fungus after 12 d incubation in sand samples contaminated with phenanthrene (95.2%), anthracene (96.8%), fluoranthene (88.1%) and pyrene (89.7%).

In the *Th*−$O_{3b}$ treatment, the absence of streptomycin likely favored bacteria that contributed to obtain a further diesel removal, acting in a synergic action with *Th*-T22. These bacteria probably came from the diesel samples itself and/or from the surrounding. Indeed, diesel is not a sterile matrix and can contain microorganisms both inside the oil droplets and on their surfaces (Chorom et al., 2010; Wang et al., 2013). The *Th*−$O_{3b}$ treatment allowed to achieve a cumulative diesel removal of 65.92%, compared to 60.19% of Str-*Th*-$O_{3b}$ (Fig. 1b), while in the Str-*Th*-$O_{3a}$ and *Th*−$O_{3a}$ treatments, removal of diesel was 70.16 and 88.35%, respectively (Fig. 1c). The additional degrading effect of streptomycin was not observed in the controls without *Th*-T22 (Str-$O_3$ and $O_3$; both with a diesel removal = 41.9%) (Fig. 1a), so confirming the bacterial biodegrading action only in the presence of the fungus. Recent studies have confirmed the inhibitory effect of some antibiotics against diesel-degrading bacteria (Grenni et al., 2018; Chaudhary et al., 2019).

### 3.2. Effects of ozonation on diesel mycoremediation

Gaseous ozone supplied in contaminated soils decomposes into oxygen through the exothermic decomposition $O_3 \rightarrow 3/2\ O_2$

**Table 2**
Preparation of the treatments at different diesel concentrations.

| Treatment concentration | | Working solution | |
|---|---|---|---|
| Diesel (g $kg^{-1}$ DW) | Diesel (μL $mL^{-1}$) | Diesel (μL) | PDB (mL) |
| 0 | 0 | 0 | 20 |
| 1.2 | 1.8 | 44 | 20 |
| 2.4 | 3.5 | 88 | 20 |
| 3.6 | 5.0 | 125 | 20 |
| 4.8 | 7.5 | 188 | 20 |
| 6.0 | 10 | 250 | 20 |

PDB: Potato Dextrose Broth.

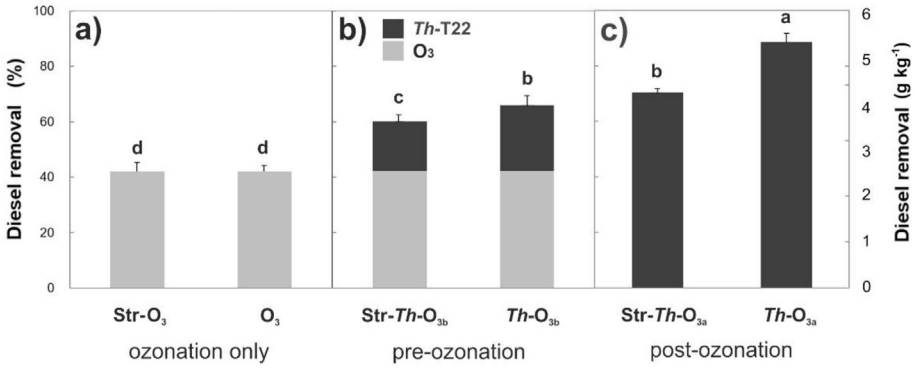


**Fig. 1.** Diesel removal, expressed as percentage or absolute value, by *Trichoderma harzianum* strain T22 (black columns) coupled with pre- or post-ozonation treatments (grey columns). The values represent means ($n = 5$) ± SD of five independent replicates. Bars with different letters are significantly different at $P < 0.05$. Treatments: **(a)** Str-$O_3$ = 200 mL PDB + 250 ppm streptomycin (control 1); $O_3$ = 200 mL PDB (control 2); **(b)** Str-*Th*-$O_{3b}$ = 200 mL PDB + 250 ppm streptomycin + *Th*-T22; *Th*−$O_{3b}$ = 200 mL PDB + *Th*-T22; **(c)** Str-*Th*-$O_{3a}$ = 200 mL PDB + 250 ppm streptomycin + *Th*-T22; *Th*−$O_{3a}$ = 200 mL PDB + *Th*-T22.

(Oyama, 2000), which favors the bioremediation of residuals, contributing to microbial cell growth and reducing the accumulation of toxic fractions in the soil (Liang et al., 2009). According to the data of Fig. 1, the Str-*Th*-$O_{3b}$ and *Th*−$O_{3b}$ treatments (pre-ozonation), the biodegradation by *Th*-T22 ensured a further diesel degradation of 17.95 and 23.68%, respectively, in addition to the oxidation by $O_3$. Contrary to the pre-ozonation treatments (Str-*Th*-$O_{3b}$ and *Th*−$O_{3b}$), in both Str-*Th*-$O_{3a}$ and *Th*−$O_{3a}$ a negligible further extraction was observed after the post-ozonation application (Fig. 1c), probably because the higher water content and the presence of the nutrient solution could have limited the contact between $O_3$ and diesel. Comparing the pre-ozonation (Str-*Th*-$O_{3b}$ and *Th*−$O_{3b}$) and post-ozonation treatments (Str-*Th*-$O_{3a}$ and *Th*−$O_{3a}$), it can be noticed that post-ozonation was able to achieve higher removal efficiencies, whereas pre-ozonation negatively affected the subsequent biodegradation due to the disinfectant effect of $O_3$ on *Th*-T22 (Fig. 1b). This is in accordance with studies reporting the inhibitory effect of $O_3$ against fungi (Cataldo, 2006; Hudson and Sharma, 2009).

Jung et al. (2005) observed that the initial number of heterotrophic bacteria ($10^8$ CFU $g^{-1}$) and alkane-degrading bacteria ($10^7$ CFU $g^{-1}$) was decreased by two, three and four orders of magnitude after 60, 300 and 900 min of ozonation, respectively. Besides, Ahn et al. (2005) reported that 900 min of ozonation followed by biodegradation did not lead to any further significant removal due to the high mortality of bacteria during the chemical treatment. The effects of $O_3$ on biodegradation were also investigated by Stehr et al. (2001), who observed that the capacity of *Sphingomonas yanoikuyae* in degrading phenanthrene decreased by 3−5 times with increasing $O_3$ dose. Therefore, the post-ozonation could be an alternative solution to improve biodegradation and overcome the limitations linked to the bactericidal effect of $O_3$ (Rivas et al., 2009; Wang et al., 2013). However, the results of ozonation strongly depend on the experimental conditions, i.e. the non-homogeneous distribution of $O_3$ through the soil column, total decomposition of $O_3$ molecules before contact with microbes, and consumption of total $O_3$ during chemical reactions with soil contaminants.

The chromatographic fingerprints of the two ozonation treatments (*Th*−$O_{3b}$ and *Th*−$O_{3a}$), recorded at the end of the 28-day incubation period, are reported in Fig. 2, in which the samples treated with *Th*-T22 (black fingerprint) are compared to the untreated one (pink fingerprint) with ozonation only. For the pre-ozonated system (*Th*−$O_{3b}$), the heavier diesel components having longer carbon chains were still present after the combined treatment in the whole chromatographic profile and a decrease in the number of peaks occurred (particularly at 47−85 min retention times) (Fig. 2a). Regarding the post-ozonated system (*Th*−$O_{3a}$), diesel components with shorter retention times (particularly those at 45−52 min and 68−88 min) were partially or removed after the combined treatment with *Th*-T22 and $O_3$ (Fig. 2b). Based on these results, it can be assumed that the biodegradation alone could be an alternative solution to overcome the limitations linked to the bactericidal effect of O3, as pointed out by Rivas et al. (2009) and Chorom et al. (2010).

### 3.3. Fungal growth in diesel-contaminated sand

The fungal mycelium growth showed no significant reduction up to diesel concentrations of 3.6 g $kg^{-1}$ (Table 3). There was a significant decrease in the fungal growth only after 72 h at diesel concentrations of 4.8 and 6.0 g $kg^{-1}$ (Table 3). These results indicated that diesel did not inhibit *Th*-T22 growth at low concentrations but a negative effect on fungal growth occurred at higher diesel levels. This factor is of key importance besides the choice of the microorganism to be used for biodegradation purposes, the efficiency of microbial growth and contaminant removal are related to its levels in soil (Cobas et al., 2013; Lee et al., 2015; Ghanem et al., 2016). Indeed, Machín-Ramírez et al. (2010) found that the removal of benzopyrene by *T. harzianum* after 5 d incubation decreased from 77 to 24% with increasing benzopyrene concentration from 25 to 75 mg $L^{-1}$. The tolerance of *T. harzianum* to diesel was also demonstrated in other studies (Delira et al., 2012; Ai-Jawhari, 2014; Kota et al., 2014). In the same genus *Trichoderma*, Kota et al. (2014) reported that *T. virens* seems to adapt faster than other fungal species in soils contaminated with crude oil, growing and penetrating in the soil grains after 3 d incubation and, consequently, reaching the maximum growth rate after 7 d. Similar results of *Th*-T22 growth (Table 3) have been found by Husaini et al. (2008), who observed that the diameter of the high radial extension of the *T. asperellum* mycelium (1.23 cm $d^{-1}$) also in the presence of motor oil.

Regarding the fungal growth over time, there was generally a constant and significant increase from 0 to 72 h of incubation, that was less marked only at diesel concentrations of 4.8 and 6.0 g $kg^{-1}$ (Table 3), suggesting that the tested fungi was not growing normally and there was a negative impact of the diesel and/or its by-products. From the observed trends of fungal growth, it is hypothesized that any increase in diesel concentration over 6 g $kg^{-1}$ or an incubation time longer than 72 h could inhibit the fungal

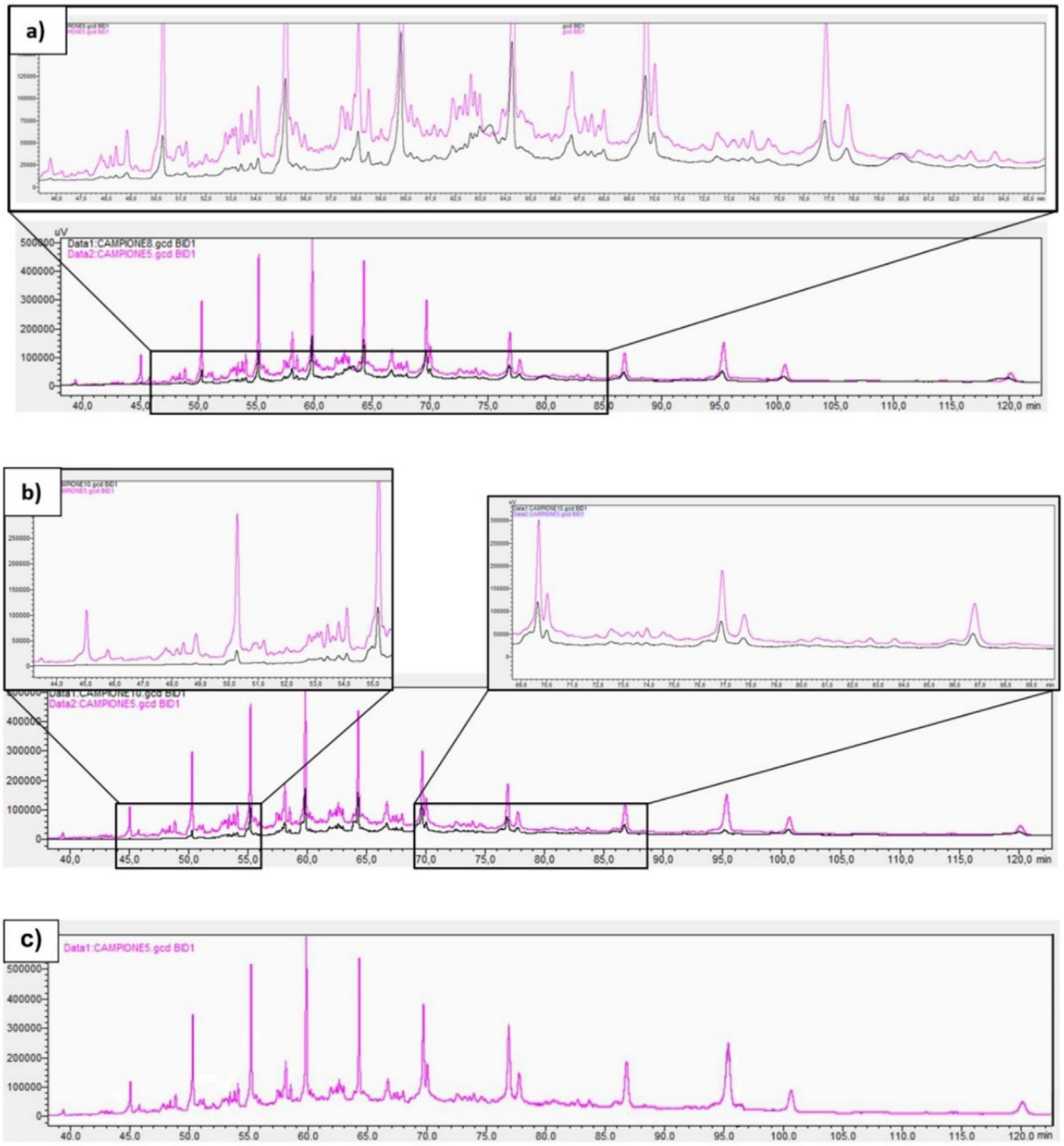


**Fig. 2.** GC-BID fingerprints of **(a)** pre-ozonated treatment (*Th*−$O_{3b}$) and **(b)** post-ozonated treatment (*Th*−$O_{3a}$). The black fingerprints correspond to the samples treated with *Th*-T22 whereas the pink fingerprints are the control treatment with ozonation only (treatment $O_3$). **(c)** GC-BID fingerprint of diesel before any treatment with *Th*-T22 and $O_3$. Treatments: $O_3$ = 200 mL PDB (control); *Th*−$O_{3b}$ = 200 mL PDB + *Th*-T22; *Th*−$O_{3a}$ = 200 mL PDB + *Th*-T22. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

**Table 3**
Fungal mycelium growth, total number of spores, spore germination (SG) and spore germination inhibition (SGI) recorded at different incubation times and diesel concentrations. The values of represent means ($n$ = 5) ± SD of five independent replicates.

| Diesel (g $kg^{-1}$ DW) | Fungal mycelium growth (Abs. λ = 540) | | | | Spore production and germination at 72 h | | |
|---|---|---|---|---|---|---|---|
| | 0 h | 24 h | 48 h | 72 h | Total n. spores | SG (%) | SGI (%) |
| 0 | 0.00 ± 0.00 Aa | 0.29 ± 0.01 Ab | 0.76 ± 0.04 Ac | 1.29 ± 0.04 Ad | 554 A | 41.16 A | 0.00 A |
| 1.2 | 0.00 ± 0.00 Aa | 0.21 ± 0.02 Ab | 0.63 ± 0.06 Ac | 1.21 ± 0.02 Ad | 511 A | 31.31 AB | 9.85 B |
| 2.4 | 0.00 ± 0.00 Aa | 0.20 ± 0.02 Ab | 0.61 ± 0.03 Ac | 1.35 ± 0.02 Ad | 491 A | 24.44 B | 16.72 BC |
| 3.6 | 0.00 ± 0.00 Aa | 0.19 ± 0.02 Ab | 0.59 ± 0.03 Ac | 1.18 ± 0.03 Ad | 464 AB | 23.07 B | 18.09 BC |
| 4.8 | 0.00 ± 0.00 Aa | 0.19 ± 0.01 Ab | 0.55 ± 0.02 Bbc | 0.62 ± 0.04 Bc | 410 AB | 20.61 B | 20.55 BC |
| 6.0 | 0.00 ± 0.00 Aa | 0.17 ± 0.01 Ab | 0.45 ± 0.01 Bbc | 0.50 ± 0.04 Bc | 391 B | 19.95 B | 21.21C |

Values of fungal mycelium growth followed by different uppercase letters within columns (diesel concentration) and by different lowercase letters within rows (incubation period) are significantly different at $P < 0.05$; values of spore production and germination followed by different uppercase letters within columns (diesel concentration) are significantly different at $P < 0.05$.

growth, and hence the biodegradation effect would not be adequately efficient. Finally, although diesel after 72 h of incubation did not significantly inhibit the total number of produced spores up to a concentration of 4.8 g $kg^{-1}$, spore germination was negatively affected starting from a diesel level of 2.4 g $kg^{-1}$ (24.44%), while spore inhibition was inhibited starting from a diesel concentration of 1.2 g $kg^{-1}$ (9.85%) (Table 3).

## 4. Conclusions

Although most of the fungal species are sensitive to crude oil (Maddela et al., 2015; Ghanem et al., 2016), in this study *Th*-T22 showed positive effects in biodegrading diesel, especially with a post-ozonation treatment (Fig. 1). On the other hand, $O_3$ was more efficient in degrading diesel in combination with *Th*-T22 rather than alone (Fig. 1). While the chemical degradation by using $O_3$ are known, the high and unexpected ability of *Th*-T22 in biodegrading diesel contaminants could be due to its capacity to different factors, such as its filamentous nature, heterotrophic nutriment, extracellular enzyme production, nutrient absorption through the cell wall, and hyphae apical growth. This fungus was particularly able to grow well in a diesel-contaminated media even after 28 d from the inoculation, showing high percentages of biodegradation (Table 3 and Fig. 2). Also, the spore germination percentage was still high (19.95%) at a diesel concentration of 6.0 g $kg^{-1}$ (Table 3). However, the results showed a slow increase of fungal growth, especially at diesel doses higher than 4.8 g $kg^{-1}$ (Table 3), indicating that *Th*-T22 needs more exposure time to adapt to the contaminated matrix.

Based on the results obtained, the inoculation of contaminated soils with *Th*-T22 with or without $O_3$ application could be a feasible approach to enhance the natural degradation of hydrocarbons. This could reduce the use of synthetic compounds and products normally used for degradation purposes that could have collateral effects on the environment and human health.

## Declaration of competing interest

The authors declare no conflicts of interest.

## CRediT authorship contribution statement

**H.S. Elshafie:** Methodology, Formal analysis, Investigation, Data curation, Writing - review & editing. **I. Camele:** Data curation, Writing - review & editing. **A. Sofo:** Data curation, Writing - review & editing. **G. Mazzone:** Methodology, Formal analysis, Investigation. **M. Caivano:** Methodology, Formal analysis, Investigation. **S. Masi:** Resources. **D. Caniani:** Conceptualization, Supervision.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2020.126597.

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