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ORIGINAL PAPER

Joint Application of Biological Techniques for the Remediation of Waste Contaminated with Hydrocarbons

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Abstract

Cleaning the oil industry's fuel storage and management facilities generates high levels of hazardous waste. This research aims to assess the use of biological bioremediation treatments, most commonly used for decontaminating soil, by applying them to hydrocarbon-contaminated waste. Turned pile composting using food-derived sludge as a co-substrate and the necessary proportion of bulking agent enabled the bioremediation of the initial mixture via the succession of microbial populations (PLFAs), with a 70% lower TPH concentration obtained 6 months after the start of the process. Subsequent bioassays using the composted material showed survival rates of over 80% with earthworms (*Eisenia andrei*) and a larger decrease in TPH in the joint treatment with earthworms and plants (*Pennisetum clandestinum*). The composting process reduces the concentration of hazardous organic compounds, allowing for the proper development of fauna and flora in the compost by improving the biodegradation rate.

Graphical Abstract

Keywords Phytoremediation · Bioremediation · Composting · Organic pollutants

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Statement of Novelty

This original research article presents a new approach for the integral management of a hydrocarbon-contaminated waste through the combination of biological processes: composting, vermicomposting and phytoremediation. Co-composting with bulking agent and food-derived sludge and intensive turnings during thermophilic phase are necessary to activate an efficient composting process with higher TPH removal rate and quality compost. By stabilizing the organic matter and reducing the pollutant load, the bioremediation process can continue using fast-growing seeds and earthworms. This method may be a practical solution for solid hazardous waste disposal.

Introduction

The accelerated growth of the global economy and the increase in the world's population have led to us consuming more petroleum-derived substances. These derivatives, made up of hydrocarbons, are highly toxic substances for living organisms, constituting a potential health risk. Various in situ and ex situ techniques have been employed to bioremedy soils and other contaminated waste [1, 2], and composting has been one of the most successful methods for improving polluted soils [3]. In order to provide the required conditions for composting contaminated materials, these can be mixed with residues such as sludge, which contribute nutrients and microorganisms, facilitating the bioremediation of waste contaminated by hydrocarbons [4]. Furthermore, this waste can be mixed with a bulking agent that is able to deliver sufficient porosity for the microorganisms present in the mixture to obtain the necessary amount of oxygen [5].

The quality and improvement of treated products can be assessed using plants or animals before and after the bioremediation process. Thus, bioassays with plants and animals are carried out to assess the toxicity on living organisms of a contaminated material due to the presence of different pollutants that can have varying physiological consequences [6, 7]. Earthworms are animals that filter the soil or substrates where they live to feed on the nutrients present. Several studies address toxicity tests on earthworms to evaluate the bioavailability of organic pollutants such as hydrocarbons [8], and various standards, ISO and OECD test protocols, use these organisms as representative of soil fauna for ecotoxicity studies. Other pieces of work use earthworms as a mechanism for the bioremediation of soils or other contaminated materials [9], giving rise to the concept of vermiremediation [10].

Meanwhile, the use of plants as a means of eliminating hydrocarbons is an increasingly common technique due to the inherent sustainability and low cost of the process [11]. Biological degradation in the rhizosphere, volatilisation and phytodegradation are among the mechanisms adopted by the plant that allow pollutants to be treated [12]. There are differing experiences of the effectiveness of phytoremediation in areas contaminated by petroleum derivatives. Günther et al. [13] showed reductions in total hydrocarbon concentration of over 97%, starting from an initial concentration of 4330 mg TPH/kg soil, in soils planted with ryegrass (*Lolium multiflorum*). Phytoremediation is also used to reduce the amount of other pollutants such as heavy metals [14], which can be present in both soils and contaminated waste.

The aim of this study is to determine whether it is possible to apply different biological techniques, usually employed in soil remediation, to waste with a high hydrocarbon content. Treatment by pile co-composting will be evaluated, after improving the structure, composition and microbial load of the source waste, as will the product obtained through tests with fast-growing plants and earthworms as representatives of soil fauna. As a starting hypothesis, it is believed that: the high temperatures reached during the thermophilic stage and the passage through the maturation stage make it possible to stabilise the waste and reduce the concentration of hydrocarbons to values that enable the subsequent treatment of the product obtained using vermicomposting and phytoremediation.

Materials and Methods

Composting

The hydrocarbon-contaminated waste (PW) came from cleaning the sediment from a liquid fuel supply company's petrol and diesel storage tanks. This solid waste had a high hydrocarbon content and a significant sand content that gave it a sediment-like texture. The main physico-chemical characteristics of the materials used in the experiment are summarised in Table 1. As co-substrates for composting PW, food industry sludge and shredded plant material were used in volume ratio 1:2:3 respectively. The sludge was obtained after the treatment of wastewater from the production of ready-made fish products and was used in double proportion to the PW, as indicated by Alves et al. [15]. According to these authors, the double proportion of the sludge and the hydrophobic characteristics of both sludge and PW allow for a homogeneous mixture of materials. The final volume of the compost pile was 6m3. Temperature data and oxygen levels were taken weekly in the pile at depths of 80 cm and 40 cm. In order to homogenise the material and break up the aggregates, the pile was turned using a front-end loader at

Table 1 Physico-chemical characteristics of the waste used in the composting test: PW (polluted waste), sludge from a food-processing plant and bulking agent

	PW	Sewage sludge	Bulking agent
Moisture (%)	33.66 ± 3.01	62.29 ± 2.25	45.07 ± 1.12
Organic matter (% dw)	41.03 ± 4.92	89.43 ± 3.50	92.75 ± 0.40
Total carbon (% dw)	16.82 ± 0.99	51.99 ± 1.14	55.82 ± 0.28
Total nitrogen (% dw)	0.21 ± 0.08	2.32 ± 0.32	1.28 ± 0.02
pH	6.94 ± 0.21	4.66 ± 0.70	6.67 ± 0.01
Electrical conductivity (mS/cm)	0.35 ± 0.1	0.55 ± 0.04	1.51 ± 0.01
Apparent density (kg/m^3)	1701 ± 9.32	854 ± 2.12	424 ± 5.05
TPH (g/kg)	96.03 ± 8.92	ND	ND
Respiratory activity (mg O_2/kg V S * h)	82 ± 11.69	565 ± 51.10	191 ± 15.61
Fats (% dw)	4.91 ± 0.51	208.28 ± 22.33	ND
Germination index (%)*	5–20	20–35	80–95

dw dry weight, *ND* not detected

*Test developed with three plant species: *Lepidium sativum*, *Brassica rapa* and *Lactuca sativa,* the results show the range of the germination index

weeks 2, 4, 7, 10 and 14. As the pile was turned, the material was sampled at random points and the pile was watered to offset the high temperatures produced during the process. Finally, after 180 days the composted material was sifted through a 10 mm sieve (CM).

Bioassays with Earthworms and Plants

In order to assess the effects of composted material on the growth and development of plants and soil decomposers, several tests were carried out. A survival test was carried out with the *Eisenia andrei* earthworm using a 500 mL capacity culture system, which was filled with a layer of sifted and moistened vermiculite as a shelter substrate for the earthworms, the advantage of which is that it is a biologically inert material. A plastic mesh (5 mm mesh size) was placed on the surface of the vermiculite to avoid mixing the materials with the substrate. 150 grammes of each treatment was introduced along with 5 mature *E. andrei* individuals (clitellate) at least 2 months old from the same culture and fed with horse manure. Four treatments were carried out: fresh untreated polluted waste (PW), polluted waste + food sludge + bulking agent (FIM), composted material (CM) and composted material + horse manure (CMHM). The materials used in the test were sifted through a 5 mm mesh and the treatments were replicated five times. The culture systems were kept in darkness under the same conditions for 14 days. The moisture content remained above 70% throughout the process.

Germination tests were performed throughout the pile composting process with three different plant species: cress (*L. sativum*), turnip rape (*B. rapa*) and lettuce (*L. sativa*). The germination index (GI) was calculated using the test proposed by Zucconi and De Bertoldi [16], using fast germinating seeds and root elongation by incubating them in petri dishes. GI is calculated using the percentage of germination and the average length of the seed roots.

$$GI = \frac{G}{G_0} \times \frac{L}{L_0} \times 100$$

where G_0 and L_0 are respectively the germination and length of the seed roots of the control.

The *Pennisetum clandestinum* plant (Whittet variety) was used for the bioremediation test. Its common name is kikuyu and it is a tropical perennial species in the Poaceae family. The earthworm species *E. andrei* was used as a representative of decomposing mesofauna due to its enormous capacity to feed off and live in different organic wastes, as well as its high rate of reproduction. As a replication system, 10 L plastic containers with a perforated base were used to facilitate drainage and to stop the material becoming waterlogged, with a 1 mm mesh to prevent the earthworms from escaping. In total, 20 containers were each filled with 9 L of CM with the following treatments: 5 containers with 10 grammes of pre-clitellate *E. andrei*, 5 containers with 100 *P. clandestinum* seeds, 5 containers with 10 grammes of earthworms and 100 *P. clandestinum* seeds and, lastly, 5 containers were filled with only the composted material, in order to serve as a control treatment. The containers were stored for 3 months in a room with the appropriate light, temperature and humidity conditions to favour the development of the plants without harming the earthworms. The 420 earthworms used in the experiment weighed 280 mg ± 60 on average. All of the containers were watered with the same frequency and under the same conditions of sunlight, temperature and water quality. As with the survival test, the composted material was sifted through a 5 mm mesh.

Physico-chemical Analysis

During the composting pile, 2 kg of material was sampled at each sampling time and its physicochemical characterization was carried out. Electrical conductivity and pH were determined in aqueous extracts (1:10, w/v) using a pHmeter Crison Basic 20 and a conductivimeter Crison CM 35. Moisture and organic matter contents of samples were calculated gravimetrically after drying at 105 °C until constant weight and combustion at 550 °C for 4 h, respectively. Total carbon (TC) and total nitrogen (TN) contents were determined by combustion using a LECO 2000 CN elemental analyser. NH4 + –N and NO_3^-–N were determined by distillation of 2 g of sample in a Selecta Pro-Nitro M and subsequent colorimetric titration with hydrochloric acid using mixed indicator. Respiratory activity at 4 days (AT4) was measured using manometric respirometers by OxiTop® system (WTW GmbH, Weilheim, Germany).

To measure the pollutant load, the term TPH (Total Petroleum Hydrocarbon) was used, which describes a family of several hundred compounds derived from petroleum. Protocol established by standard UNE-EN 14039 [17] was applied for the extraction and determination of TPH. Briefly, about 20 g of homogenised sample was extracted by mechanical agitation with acetone and n-heptane. Subsequently, acetone was removed by means of a separating funnel. An aliquot of the organic phase was transferred to Dual layer SPE Tube Florisil/sodium sulphate (Supelco®) for the polar compounds removal and an aliquot of the purified extract was collected in a chromatography vial. Gas chromatographer with flame ionisation detector (GC-FID, Agilent model) endowed with a capillary column (HP-5 Agilent Technologies, 30 m length, 0.32 mm diameter, 0.25 µm film thickness) was used for TPH determination. The GC was operated with the helium carrier gas. The program of the oven temperature was 80 °C to 325 °C up to 10 °C/min. Injector and detector was set at constant temperature of 300 °C and 350 °C, respectively. Integration area between n-decane and n-tetracontane peaks was used for hydrocarbon content determination and percentage of biodegradation of TPH was calculated between initial and final samples. The percentage of TPH degradation on each sampling day was determined with the following equation:

$$TPH\ removal = \frac{TPH_0 - TPH_i}{TPH_0} \times 100$$

where TPH_0 is the TPH on sampling day 0, and TPH_i is the TPH on each sampling day.

The microbial community composition and biomass were determined by phospholipid fatty acid analysis (PLFAs) following the method described by Gómez-Brandón et al. [18] for organic samples. Briefly, total lipids were extracted by stirring from 200 mg of each freeze-dried sample with 60 mL of chloroform–methanol (2:1, v/v). Phospholipid fraction was obtained after separation on silicic acid columns and was subjected to derivatization with trimethylsulfonium hydroxide (TMSH). Fatty acid methyl esters (FAMEs) obtained were analysed by gas chromatography and mass spectrometry (GC–MS). GC–MS analysis was performed with a CP-Select FAME, 100 m × 0.25 mm. Identification was done by comparison of retention times and mass spectra with known external standards (Larodan Fine Chemicals AB, Malmo, Sweden) and quantification was performed with methyl nonadecanoate fatty acid (C19:0) as internal standard. The sum of bacterial and fungal PLFAs (totPLFAs) was used as an indicator of the viable microbial biomass [19]. The diversity of the fatty acids was calculated with the Shannon-index (H) with the following equation:

$$H = \sum_{i=1}^{n} p_i \times \ln p_i$$

where p_i is the relative abundance of each fatty acid in the total sum and n is the number of detected fatty acids.

Data Analysis

All statistical tests were performed using R software [20]. Differences among treatments in the bioremediation test were determined with ANOVA followed by Tukey's significant difference as a post hoc test. Differences among treatments in the survival test and germination test were determined with repeated measures ANOVA. All statistical tests were evaluated at the 95% confidence level and values are given as the mean ± standard deviations.

Results and Discussion

Composting Pile

The evolution of the temperatures in the pile (Fig. 1) indicated that two days after it had been formed the material reached the thermophilic stage, and it remained in this state for 50 days, during which time it was turned and watered according to the needs of the process. Later, the process entered the cooling and maturation phase until temperatures similar to the ambient temperature were reached by day 180. Several pieces of research on composting substrates with high hydrocarbon content develop primarily mesophilic degradation processes, in which the use of co-substrates does not provide enough easily assimilable compounds to achieve microbial development that raises the temperature to thermophilic conditions [4, 21]. By contrast, Marin et al. [22] observed a short mesophilic stage with maximum

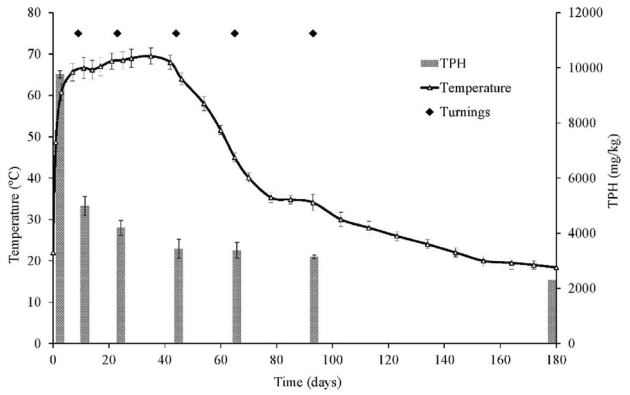

Fig. 1 On the left axis, temperature profile during the composting in pile of polluted waste (PW) (line). On the right axis, total petroleum hydrocarbon (TPH) evolution (bars). Asterisks indicate the day of the turnings on the pile

thermophilic temperatures of 50–55 °C, which were maintained for several days, during composting in a refinery sludge pile with bulking agent and pig manure. Similarly, but to a greater extent, adding co-substrates (bulking agent and food industry treatment sludge) to PW together with the frequency of turning, gave optimal results in terms of self-heating capacity. Managing the pile by turning it succeeded in homogenising the material without interrupting the biodegradation process and, consequently, reaching temperatures above 70 °C and prolonging the thermophilic conditions. In addition, the high level of fats from the sludge prolonged the thermophilic temperatures due to the fact that the lipids present provide a greater amount of energy than other organic compounds [23]. The high temperatures (> 70 °C) reached during the process, together with the long duration and stability of the thermophilic stage, caused a significant degradation of organic matter (Table 2), ensuring adequate sanitisation of the material [24].

With regard to the concentration of TPH, a sharp drop was detected in the first ten days of the process, possibly due to the aeration of the mixture caused by the effect of the bulking agent and the high temperature reached during the activation phase, which facilitated the volatilisation of lower molecular weight alkanes. It is acknowledged that the first stage of composting is characterised by an increase in temperature, which is key to changes in organic pollutants [25]. After the first ten days, the concentration of hydrocarbons continued to fall, but gradually and with less intensity, until it reached 64.8% of TPH eliminated at the end of the thermophilic phase. Similarly, Amir et al. [26] noted a reduction of over 75% in PAH content during the first 30 days of lagooned sewage sludge composting. During the cooling and maturation stage, from 44 days in, over 76% of TPH had been eliminated. A rise in temperature during the composting of hydrocarbon residues may not be an adequate indicator of the TPH degradation process, given that a variety of microorganisms that cause its degradation develop under mesophilic conditions [27]. However, temperature will affect the physico-chemical structure of TPH and influence microbial activity [28] and the succession of different microbial populations with varying degradation capacities.

The total amount of PLFA can be used as an indicator of viable microbial biomass, and specific PLFA assigned to certain microbial groups were useful biomarkers of different composting stages [19, 29, 30]. Microbial biomass, measured as total amount of PLFA, fell throughout the composting process (Table 2). This was an expected decrease as the most assimilable substrates are consumed, reducing the food available for microbial growth. AT4 respiratory activity also showed a similar downward pattern. In the first days of the process, and as a result of the rapid increase in temperature, there was a sharp drop in microbial biomass. Temperature is known to be a key selective factor in microbial communities during composting [31]. High temperatures inhibit the

Table 2 Evolution of the composting parameters in the samples taken during pile turnings

Parameters	Time (days)						
	0	9	23	44	65	93	180
pH	6.61	7.81	8.48	8.87	8.83	8.37	8.18
EC (mS/cm)	1.42	2.18	2.18	1.75	1.58	1.66	0.97
Moisture (%)	44.06	42.01	35.01	34.40	36.96	40.39	42.50
Volatile solids (%)	39.94	36.44	32.87	33.29	32.05	28.36	27.38
AT_4 (mg O_2/g dw)	89.37	37.83	23.06	12.16	6.81	5.50	5.02
NH_4^+ (mg/kg dw)	1217.3	3220.6	2900.3	2007.1	1044.0	65.8	37.7
NO_3^- (mg/kg dw)	52.80	104.19	103.15	108.70	167.61	240.77	172.09
NH_4^+/NO_3^-	21.75	30.98	28.29	17.63	6.25	0.30	0.21
DOC/DON	1.65	2.33	3.19	3.65	2.93	1.55	1.65
C/N	14.96	15.08	14.70	12.94	12.42	12.00	11.38
Microbial biomass (µg/g dw)	688.8	329.0	189.5	54.4	36.3	30.2	26.7

dw dry weight

growth and reproduction of most bacteria, with the exception of thermophilic bacteria. Gram + bacteria, particularly those belonging to the genus *Bacillus*, dominate during the thermophilic phase of composting [31]. It was therefore observed that the Gram + bacteria diversity index increased during the thermophilic phase (Fig. 2). Bacteria have been reported to contribute more to the TPH reduction than fungi [32] so, the drop of TPH in this phase can be consequence of bacterial degradation joint to the volatilization of the lightest hydrocarbons as discussed above The fungal diversity index remained at similar values for the first 65 days, coinciding with the thermophilic conditions and in accordance with the observations of Villar et al. [23] during the composting of fatty food waste, which indicates the presence of thermotolerant or thermophilic fungi, making it possible to extend the high temperatures by managing the material through turning. As a consequence of the easily assimilable organic compounds being consumed, microbial biomass continued to decrease until it stabilised from the fourth turning, corresponding to the cooling and maturation phase. At this stage of composting, the decrease in temperature causes the succession to mesophilic microorganisms, which generally leads to an increase in microbial diversity. Nonetheless, an increase in diversity was not observed in the groups studied. The reduction of organic matter content, which reaches around 30% in the cooling phase and can increase competition for resources, along with the degradation of hydrocarbon compounds, which can release some harmful substances that could inhibit microbiota development, cause selection of microbiota and decreased diversity [33]. Likewise, the reduction of moisture content (below 40% in the cooling phase) can lead to a decrease in microbial diversity.

It is worth noting that, in line with the parameters analysed (Table 2), typical composting process patterns were observed, with the compost reaching values of stability and maturity. Changes in the pH show that the mixture was

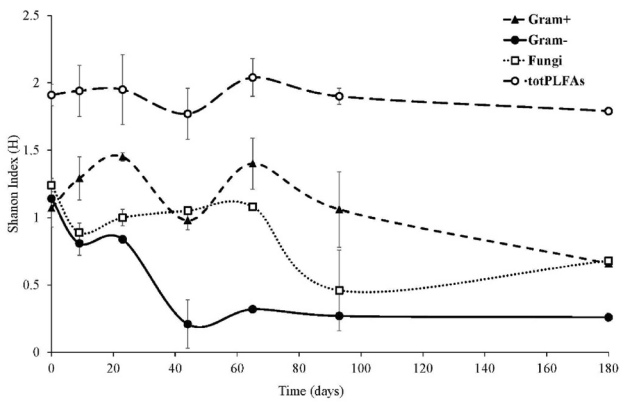

Fig. 2 Changes in Shannon Index for Fungi, Gram-positive bacteria, Gram-negative bacteria and Total PLFAs, estimated by fatty acid analysis of phospholipids (PLFAs) in the composting process

initially slightly acidic in nature, however during the process its value changed to basic. The end pH of around 8 demonstrates the maturity of the compost. With regard to electrical conductivity, an initial upward trend was observed but, as the material matured, it began to decrease, hitting values below the initial recordings and in ranges suitable for plant growth (less than 2 mS/cm). Humidity was below 50% throughout the process because the materials used were very hydrophobic and with low water retention capacity. Consequently, attempts were made to keep the pile at humidity values that would not limit microorganism growth and that, at the same time, would not produce an excess of leachates [15]. The CCQC [34] provides general guidelines for classifying compost using the C/N ratio, and Bernal et al. [35] suggest that numerous parameters can be used to assess the stability and maturity of a compost. Initially the pile contains a low C/N ratio, around 15, due to the presence of inorganic materials in the contaminated material [36]. Later, as the process progresses day by day, there is a decrease in the C/N ratio because of the fall in concentrations of the different forms of carbon and nitrogen, achieving values near maturity [35]. Meanwhile, the concentration of $N–NH_4^+$ increased exponentially during the first 15 days and decreased steadily until the end of the process, coinciding with the highest values detected with the thermophilic phase [37]. By contrast, the concentration of $N–NO_3^-$ increased during the process and, from 50 days, this increase was more marked, coinciding with the cooling and maturation of the material. The NH_4^+/NO_3^- ratio was around 0.21, reaching values very close to maturity according to the maximum ratio of 0.16 suggested by Bernal et al. [38]. Another ratio indicative of the maturity and stability of a compost is the DOC/DON ratio, which represents the forms of carbon and nitrogen that are easily assimilated by microorganisms [39, 40]. This remained low throughout the process. Said-Pullicino et al. [41] showed that the DOC/DON ratio in mature compost is around 5–6.

As for the respiratory activity values, these show that, initially, the pile had high microbial activity but at the end of the process it was very low. In accordance with the recommendations of various standards, the compost obtained has a value below 6 mg O_2/g TS and is considered a completed compost [42, 43]. In view of the results, the composting process was properly developed, generating a stable and mature compost with a significantly lower TPH content than the initial mixture ($p < 0.05$), but that requires bioassays to ensure that there is no ecotoxicity.

Bioassays with Earthworms and Plants

The survival test with the *E. andrei* earthworms showed very high mortality values in treatments with fresh materials (FIM and PW). These substrates had higher concentrations of compounds that are harmful to earthworms, such

as hydrocarbons and ammonium content (Tables 1 and 2), which negatively affected their survival and development. Furthermore, fat content also appears to negatively affect the earthworms, with significantly ($p < 0.05$) higher mortality detected in fresh sludge treatment (FIM) and with high fat content than in PW. Wu et al. [44] proved that the higher the percentage of fat in the food, the less the earthworms would grow because the lipid layer makes it difficult to ingest and because the material has a low oxygen content. The survival of the earthworms in both the CM and the CMHM treatment was very high (Fig. 3), indicating that the organic pollutant content did not cause lethal toxic effects on the earthworms in the short term. The earthworms that managed to adapt to the material in the first 7 days were found to have good mobility at 14 days. Growth was significantly greater ($p < 0.05$) in the CMHM treatment at 14 days than in the CM treatment, with a significant decrease ($p < 0.05$) of biomass in the CM treatment being observed between 7 and 14 days. Gunadi and Edwards [45] proposed that pre-composting could reduce bioavailable nutrients for earthworm development, inhibiting the growth rate and the number of cocoons and hatchlings produced by *E. fetida*. Although no ecotoxicological effect of the CM compost on the survival of the earthworms was noted, the resources available are limiting, with negative effects on growth. It may therefore be necessary to adjust the density of earthworms or add another source of food. Abdollahinejad et al. [46] showed that co-vermicomposting can help earthworms to survive exposure to pollutants and improve the efficiency of soil biodegradation. Thus, the presence of fresh horse manure in the CMHM treatment made it possible for the earthworms to increase in biomass.

The seed germination test is an effective and inexpensive bioassay for evaluating the potential toxicity of compost, but the response is different depending on the plant species [47]. Germination tests performed throughout the composting process highlighted significant differences between plant species ($p < 0.05$), with *B. rapa* showing the greatest differences. While the initial materials—PW and food sludge—showed high toxicities (Table 1), once the substrates had been mixed the germination rate was higher in the three species, with lower initial phytotoxicity in *B. rapa* (Fig. 4). After the first turning, although the germination rate declined in all species, the decline was sharpest in *L. sativum* and *L. sativa*, reaching values below 25%. This toxicity may be due to the increased concentration of ammonia and electrical conductivity (Table 2), as observed by Luo et al. [47]. Although on the following days of the process the index increased steadily, the various plant species presented significantly different evolutions ($p < 0.05$), and while the germination rate with *B. rapa* was maintained at values above 80% throughout the process, with *L. sativa* these values were reached at day 65, and day 91 with *L. sativum*. Cress seeds are one of the most widely used species in phytotoxicity tests, with a compost being considered stable and mature when germination values greater than 80% are reached [16].

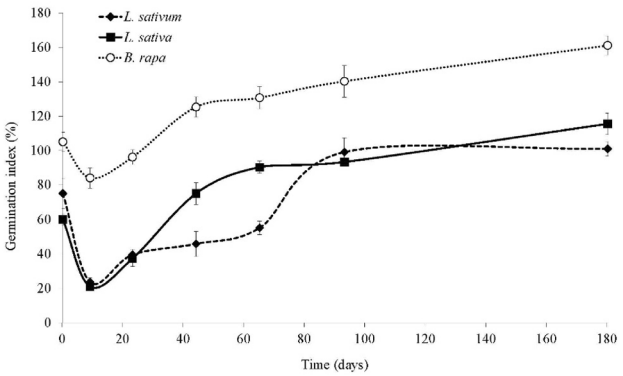

Fig. 4 Changes in the germination index for *L. sativum*, *L. sativa* and *B. rapa* during the composting process

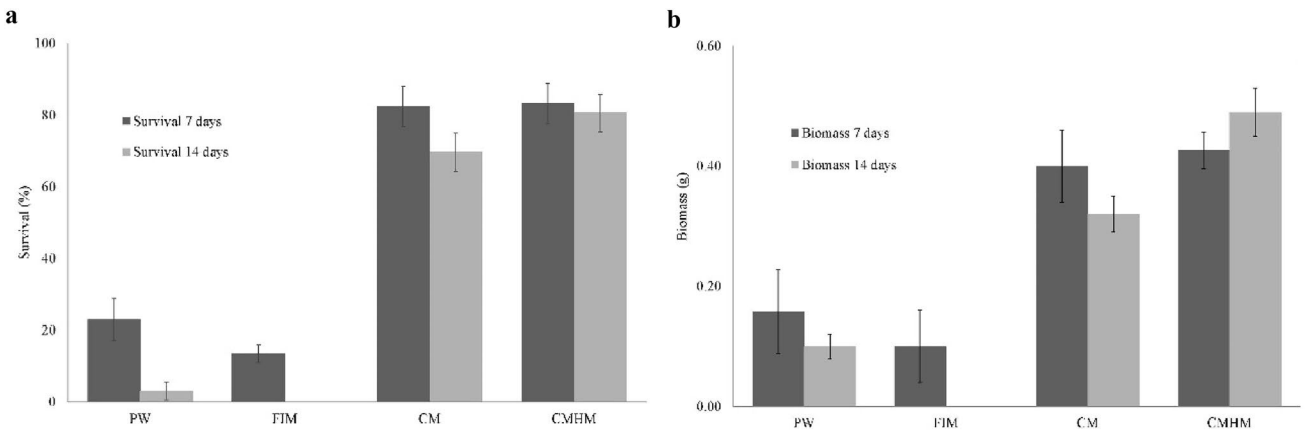

Fig. 3 **a** Changes in survival and **b** biomass of the survival test with *E. andrei* for the treatments; fresh and untreated contaminated waste (PW), contaminated waste + fresh food sludge + bulking agent (FIM), composted material (CM) and composted material + horse manure (CMHM)

As a result, from day 91 of the process the composted material did not have negative effects on plant growth, with the compost being considered mature. However, phytotoxicity is species-dependent, so plant species that are less sensitive to certain compounds in the substrate, such as in this case *B. rapa,* will allow for plant growth in fresher composts. Consequently, for bioremediation using plants, the choice of plant species will depend on the different degree of maturity of the compost, among many other factors.

Table 3 shows the results of the bioassay of bioremediation with plants and earthworms after 3 months of the process. The germination of *P. clandestinum* seeds in compost with and without earthworms was similar ($p > 0.05$) and was maintained at very high values in all containers, such that the concentration of hydrocarbons and the presence of earthworms was not instrumental to the germination of the seeds. The choice of this plant species to carry out the test was determined by the rapid and continuous growth of its root system and by the fact that it has a strong ability to adapt to different environmental conditions. In turn, this species shows moderate tolerance in saline soils and high content in lignocellulosic compounds that can be broken down by microorganisms to produce large quantities of biogas [48]. On the other hand, their rooting and the growth of the stems arise from the same thickening, giving this herb strong anchorage capabilities, meaning that it is often used on land with a high risk of erosion. In the containers, competition between *P. clandestinum* specimens may lead to an increase in above-ground biomass investment to obtain higher solar radiation and this may have an impact on investment in root biomass. After 3 months of growth, the *P. clandestinum* specimens obtained long, robust roots that covered a great deal of the container in the two treatments. The presence of *P. clandestinum* did not affect the growth and development of the earthworms, with similar survival, biomass and cocoon production being observed in the treatments with *E. andrei* ($p > 0.05$). Other studies reported survival and cocoon generation similar to those shown by the treatments of this bioassay [49].

In this bioassay a significant decrease ($p < 0.05$) in the concentration of TPH was detected where the species *P. clandestinum* was present after a 3-month process (9.3% reduction), with the reduction being significantly greater ($p < 0.05$) in the joint treatment of *P. clandestinum* with *E. andrei* (15.2% reduction). The action of the earthworms together with that of the plants' root biomass allowed a decrease in the total concentration of hydrocarbons. This effect may be due to the stimulation or creation of favourable conditions for the growth of the microbial communities responsible for degrading hydrocarbons, or to the transfer of the hydrocarbons in the earthworm's body to the rhizosphere of the plant, where these pollutants are degraded to a greater extent [36]. Hydrocarbons can pass through the earthworm's body without causing any apparent damage. Schaefer et al. [9] researched the effects of the different species of earthworms (*Lumbricus terrestris, Allolobophora chlorotica* and *Eisenia fetida*) on the microbial community of soil contaminated by crude oil (10,000 mg/kg of TPH) in a 28-day process. This experiment found that both the respiration and the concentration of the microbial biomass were considerably higher in earthworm treatments than control treatments, while TPH concentrations were significantly reduced. At the same time, earthworms and plant roots modify substrate conditions by increasing porosity and aeration, and they increase the availability and type of nutrients due to the passage of the material through the earthworms' intestinal tracts [50].

The presence of *P. clandestinum* alone reduced TPH, and together with earthworms this effect was more noteworthy. Numerous articles discuss the role of plants in soil remediation and how these organisms, through various processes, reduce the degree of toxicity. Phillips et al. [51] undertook research into the phytoremediation of hydrocarbons in soils with six different grass species and the combined effect of

Table 3 Evolution of the parameters at the beginning and at the end of the bioassay in pots with earthworms and plants. (Mean ± sd, N = 5)

Parameters	Initial values	Control	*E. andrei*	*P. clandestinum*	*E. andrei* + *P. clandistinum*
Germination (%)	–	–	–	75.55 ± 1.32	74.71 ± 1.03
Stem biomass (g dw)	–	–	–	3.19 ± 0.15	2.92 ± 0.21
Root biomass (g dw)	–	–	–	1.01 ± 0.27	0.87 ± 0.15
Root/stem biomass ratio	–	–	–	0.32	0.30
Stem length (cm)	–	–	–	33.50 ± 5.60	35.20 ± 7.70
Root length (cm)	–	–	–	11.80 ± 1.20	10.30 ± 1.80
Root/stem length ratio	–	–	–	0.35	0.29
Earthworm survival (%)	–	–	91.02 ± 0.78	–	90.31 ± 0.66
Earthworm biomass (g/pot)	10.41 ± 0.1	–	12.08 ± 0.65	–	11.22 ± 0.91
Number of cocoons	–	–	10.61 ± 3.3	–	7.36 ± 3.05
TPH (mg/kg)	2311 ± 11	2298 ± 49	2302 ± 37	2095 ± 42	1960 ± 39

these; after 4.5 months it was found that the largest decreases in the concentration of hydrocarbons (50%) occurred in the individual treatments (*Festuca rubra*). Peng et al. [52] conducted experiments with ornamental species such as *Mirabilis jalapa L.*, measuring the capacity of this species to bioremediate soils contaminated with oil-derived hydrocarbons. After 127 days they obtained reductions of between 40 and 60% in the hydrocarbons. In addition, they concluded that *M. jalapa* has a tolerance to hydrocarbon contamination to values of around 10,000 mg/Kg.

Thus, the application of bioremediation with plants and earthworms after co-composting waste with a high TPH content reduces the presence of these organic pollutants, therefore the association of these techniques allows the conversion of extremely hazardous waste into products with a lower risk to health and the environment that could be used for the regeneration of soils and replanting of degraded areas.

Conclusions

The co-composting of waste with a high hydrocarbon content together with optimal amounts of organic amendment and bulking agent effectively reduced the concentration of TPH by enabling an intensive thermophilic stage and a prolonged maturation stage. Determining the quality and maturity of compost should be accompanied by ecotoxicity studies to establish possible toxic effects on the development of plant species and on the fauna. It is therefore advisable to carry out survival tests with earthworms and germination tests with various plant species.

Sowing plant species along with the addition of *E. andrei* earthworms are techniques that encourage the remediation of compost, as the joint action of the different organisms involved in the process makes it possible to reduce the hydrocarbon content in the compost. However, more research is necessary to know how this root-earthworm interaction allows TPH reduction.

Although the contaminated material is not a soil but a sludge, it can be treated using the same processes used in the bioremediation of contaminated soils. It is necessary to condition the contaminated waste for treatment by composting and then apply bioremediation with plant species and earthworms. This study paves the way to treating waste and soils contaminated with high concentrations of hydrocarbons by applying a set of biological techniques that progressively reduce these pollutants as an alternative to the current management of this contaminated waste, which in many cases consists of disposing of it in hazardous waste landfills.

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