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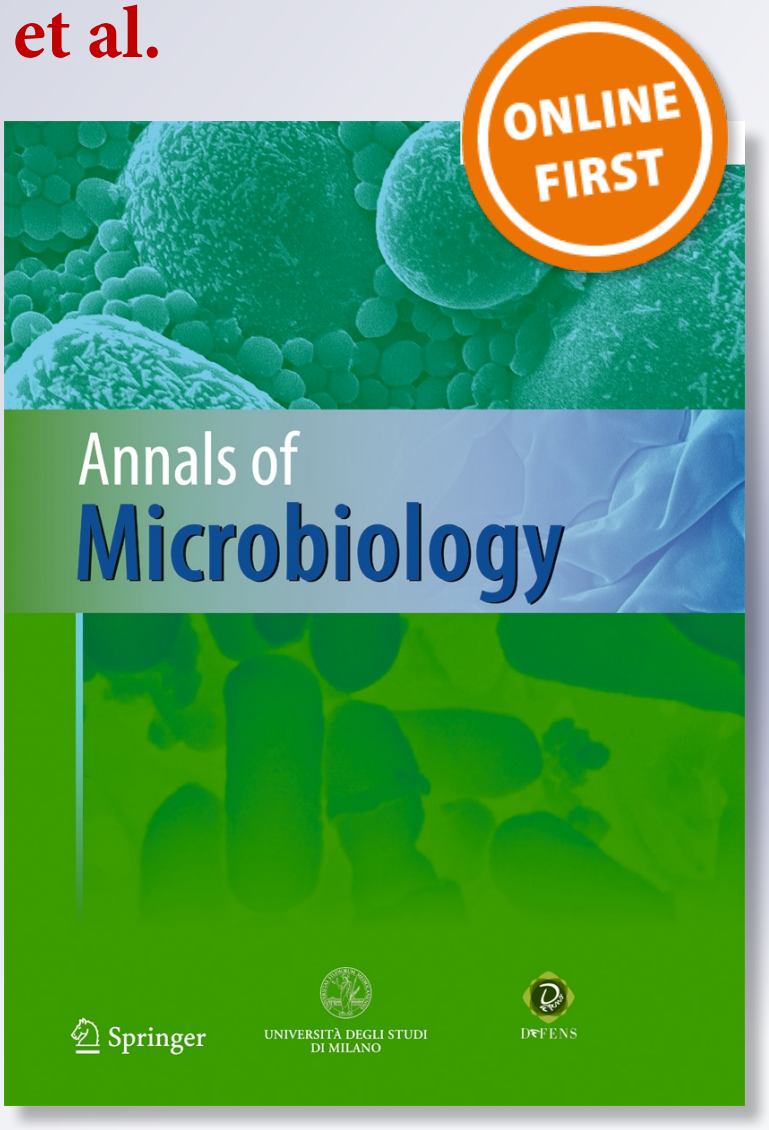

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ORIGINAL ARTICLE

Biodegradation kinetic rates of diesel-contaminated sandy soil samples by two different microbial consortia

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Abstract Mexico is one of the top oil-producing countries in the world and often suffers accidental oil spills, particularly in coastal regions. During the last decade, major spills have occurred in Mexican coastal ecosystems that have resulted in severely heavily oil-polluted shores. Although several remediation processes can be used, those with less servere negative consequences for the environment are preferred. Bioremediation through the use of specific microorganisms has been shown to speed up the decontamination rate process due to the ability of various microorganisms to split recalcitrant pollutants into smaller harmless molecules to be utilized as nutrients. Here, we present the effect of a microbial population succeeding in a bioremediation process carried out under laboratory conditions that may be suitable for the in situ decontamination of diesel in sandy soils. Two bacterial consortia isolated from heavily oil-polluted regions of Tabasco were able to utilize diesel as the sole carbon and energy source, degrading up to 98 % of the original concentration of oil within 30 days. Within 5 days, the bacterial consortia had reduced the levels of diesel in the experimental soil samples to the standard concentrations established by Mexican and U.S. environmental agencies (maximum contaminant level: 1200 mg/kg). Pyrosequencing-based assessment of the bacterial community structure of the two consortia revealed different compositions. Consortium CMic4 had a rich and diverse bacterial community, while CMic10 was dominated by *Enterobacter* sp., which represented 95.9 % of the community. Evaluation of the bioremediation efficiency by biocompatible decontamination analysis with earth worms confirmed the non-hazardous state of the treated soil after the decontamination process.

Keywords Microbial biodegradation · Diesel-contaminated soil · Decontamination analysis · Consortia of microbes for bioremediation · Bioassay

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Introduction

World demand for oil and energy has continues to increase over the years, but oil spill clean-up technologies has not improved at the same rate. Since the late 1990s bioremediation has been considered to be an important waste management process for the in situ decontamination of total petroleum hydrocarbons (TPH; Taylor and Viraraghavan 1999). It has been shown that this process speeds up the rate of decontamination due to the ability of some microorganisms to break recalcitrant pollutants into smaller harmless molecules that they utilize as nutrients (Leahy and Collwell 1990). Therefore, the successful isolation of oil-degrading consortia of microbes and analyses of their properties are important areas of study (Souza et al. 2014). The bioavailability of TPH and its interaction with the soil matrix are also important factors to be considered (Semple et al. 2003, 2004), as demonstrated by Sutton et al. (2013) who compared bioremediation processes on different types of soil and observed that contaminated sand was more

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difficult to bioremediate due to a low bioavailability of TPH. In particular, diesel-polluted soils exhibit irregularly shaped particles (Sielicki et al. 2012) due to the formation of a rich mixture between hydrocarbon compounds of various chains lengths (C10–C28), ashes, sulfates, water, and transition metals, such as arsenic, cadmium, chromium, copper, iron, lead, manganese, nickel, vanadium, and zinc, all bonded to the sand particles encapsulating them (Huffman et al. 2000). Consequently, it can be said that all biodegradation processes are influenced by many different factors, which may include (1) the microbial population present in the bioactive consortia, (2) the hydrocarbons present in the contaminated site, and (3) the environmental compartment in which the process is being carried out (Bamard et al. 2011). Given that Mexico is the tenth largest oil-provider in the world (International Energy Agency 2014) and that its related oil-industry activities, such as exploration, extraction, storage, and distribution, often cause accidental oil spills, the potential for damage to the ecosystems and human health can be considered to be high (Michel and Rutherford 2014). Here we present an evaluation of the effect of microbial diversity on the degradation and kinetics rates of two different microbial consortia exposed to diesel-contaminated sandy soils under specific humidity conditions.

Materials and methods

Isolation of bacterial consortia of TPH degraders

Soil sampling, characterization, and screening for catalase activity Ten soil samples were collected from a 15-year-old spill site in the southern Mexican State of Tabasco (17°50′05″ N, 92°55′09″W) that has been exposed to contamination by different hydrocarbons over the years. Samples were collected manually according to Mexican environmental standards (NOM-138-SEMARNAT/SS-2003). The number of samples was decided upon based on the intention to obtain one sample of each different hydrocarbon present in the soil of the spill site. Samples were transported to the laboratory, and each soil sample was directly tested for the presence of catalase activity in order to validate the presence of biologically active microorganisms. Given that catalase activity is present in almost all living organisms, the detection of the reaction of this enzyme with H_2O_2, to produce oxygen and water is a good biochemical marker to establish the presence of living microorganisms in the sample (Prakash et al. 2009). Only two of the samples tested exhibited strong catalase activity. These samples were selected further consortium development and bacterial isolation. Soil sample humidity was calculated from the difference in weight between the original sample and the same sample after drying to a constant weight (approximately 20 h at 105 °C). To determine the pH of each sample, one part of the soil was mixed with 2.5 mL of $CaCl_2$ 10 mM, and the pH measured with a glass electrode after 2 h of shaking. This process was repeated twice and the mean value reported (Okpokwasili et al. 1986). Total organic carbon (TOC) in the two selected samples was measured by the method of Gaudette et al. (1974). Finally, TPH concentration was determined in a gas chromatography/flame ionization detector (GC/FID) system following standard procedures [Texas Natural Resource Conservation Commission (TNRCC) method 1005] with some modifications, using a Petrocol DH fused silica GC capillary column (100 m × 0.25 mm; inner diameter 0.5 mm; Supelco, Bellefonte, PA).

Isolation of bacterial consortium Benzene, toluene, ethylbenzene, and xylene (BTEX) acclimatization for the development of bacterial communities with good TPH degradation capabilities was carried out on soil samples that exhibited strong catalase activity. Several growth cycles on BTEX-rich medium with minimal nutritional supplements were performed as follows. One gram of soil sample was incubated in mineral medium (MM) (Bordoloi and Konwar 2008) containing: urea (2 g), NH_42SO_4 (2 g), Na_2HPO_4 (3.61 g), KH_2PO_4 (1.75 g), $MgSO_4{\cdot}7H_2O$ (0.2 g), $CaCl_2{\cdot}2H_2O$ (50 mg), $FeSO_47H_2O$ (1 mg), $CuSO_4{\cdot}7H_2O$ (50 μg), H_3BO_3 (10 μg), $MnSO_4{\cdot}5H_2O$ (10 μg), $ZnSO_4{\cdot}7H_2O$ (70 μg), and MoO_3 (10 μg) in 1 L of distilled water. The MM was supplemented with 50 mg/L each of benzene, toluene, ethylbenzene, and mixed xylenes (BTEX) as carbon sources. Incubation was performed in 500-mL serum bottles at 37 °C with shaking at 150 rpm. Supplementary air was passed through the system every 24 h to provide oxygen to the microorganisms. After 5 days, microbial consortia were concentrated by centrifugation (15,000 rpm) and submitted to another two rounds of growth in fresh MM-BTEX, following which the bacterial cells were maintained at −80 °C in fresh MM with 50 % glycerol.

Characterization of bacterial consortia

DNA extraction and tag-pyrosequencing After the BTEX acclimatization process and the validation of hydrocarbon use as carbon and energy source by the bacterial community, DNA from samples 4 and 10 (hereafter referred to as CMic4 and CMic10, respectively) was extracted using a PowerMax DNA Isolation kit (Mo Bio Laboratories Inc., Carlsbad, CA) following the manufacturer's protocol. Following elution, DNA samples were concentrated by ethanol precipitation, resuspended in 10 mM Tris (pH 8.0), and quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Purified DNA samples were submitted to the Research and Testing Laboratory (RTL) (Lubbock, TX, USA) for tag-pyrosequencing. Bacterial tag-encoded amplicon pyrosequencing (bTEFAP) was performed using a

Genome Sequencer FLX System workflow (model 454; Roche Diagnostics, Indianapolis, IN) , with bacteria-specific Gray28F (5′-TTTGATCNTGGCTCAG-3′) and Gray519R (5′-GTNTTACNGCGGCKGCTG-3′) primers, as described previously (Dowd et al. 2008). The HotStarTaqPlus Master Mix kit (Qiagen, Valencia, CA) was used for PCR under the following cycling conditions: 94 °C for 3 min, followed by 32 cycles of 94 °C for 30 s, 60 °C for 40 s, and 72 °C for 1 min, with a final elongation step at 72 °C for 5 min. A secondary PCR was performed using the FLX system for amplicon sequencing under the same conditions, using specially designed fusion primers with different tag sequences (Dowd et al. 2008). Tag-encoded FLX amplicon pyrosequencing analyses were performed with titanium reagents, and titanium procedures were based upon RTL protocols (www.researchandtesting.com).

Data analysis and bacteria identification Following sequencing, all failed sequence reads, low-quality sequence ends and tags, and primers were removed, and sequences collections were depleted of any non-bacterial ribosome sequences and chimeras using B2C2 (Gontcharova et al. 2010), as described previously (Dowd et al. 2008; Ishak et al. 2011). To determine the identity of the bacteria in the remaining sequences, these were denoised, assembled into clusters, and queried using a distributed BLASTn program. The NET algorithm (Dowd et al. 2005) was used against a database of high-quality 16S bacterial sequences derived from the NCBI. Database sequences were characterized as high quality based upon similar criteria utilized by the Ribosomal Database Project (Cole et al. 2009). Using a .NET and C# analysis pipeline, the resulting BLASTn outputs were compiled and validated using taxonomic distance methods, and data reduction analysis was performed as described by Ishak et al. (2011). Based upon the above BLASTn-derived sequence identity (percentage of total length query sequence which aligns with a given database sequence) and validation using taxonomic distance methods, the bacteria were classified at the appropriate taxonomic levels according to the following criteria: sequences with identity scores (relative to known or well-characterized 16S sequences) of >97 % identity (<3 % divergence) were resolved at the species level; those with identity scores of between 95 and 97 % were resolved at the genus level, between 90 and 95 % at the family level, between 85 and 90 % at the order level, between 80 and 85 % at the class level, and between 77 and 80 % at the phylum level. Sequencing reads were aligned and clustered following the RDP-Release pyrosequencing pipeline (http://pyro.cme.msu.edu/). The Shannon, Chao 1, and evenness indices, and rarefaction curves were also obtained using RDP tools.

Accession number All 16S rRNA gene sequences obtained in this study have been deposited in the GeneBank database with accession number SUB306199.

Evaluation of diesel biodegradation capacity

Diesel biodegradation assays were carried out in triplicate in round-shaped 1-L glass containers containing 700 g of a 100 % sandy soil obtained from a local river near the University campus (Monterrey, Mexico) and 150 mL of MM which were then sterilized by autoclaving (20 min at 121 °C and 100 kPa pressure). Each container was then inoculated with 150 μL of one of the isolated consortia (calculated cell count: 10^3 cell/mL by Neubauer chamber). A commercial consortium of *Bacillus* sp. known for its efficient lipid degradation was inoculated into a glass container used as the positive control for diesel biodegradation, and an un-inoculated sterile sample was used as negative control. All experimental sets (glass container + soil sample + MM ± consortium sample) were supplemented with diesel to a final concentration of 3000 mg/kg. Bioassays were conducted under aseptic conditions for 30 days at 30 °C. During the bioassays, aliquots (10 g) were removed from each container at 5-day intervals, and the diesel concentration remaining in each sample was determined by high-performance liquid chromatography according to the Mexican Procedural Standards (issued by the Secretariat of Environment and Natural Resources: Official Mexican Standards NOM-138-SEMARNAT/SS-2003). Additional 1-g aliquots were taken from each flask for pH evaluation.

Diesel biodegradation kinetics and statistical analyses

Diesel half-life ($T_{1/2}$) was calculated according to Dados et al. (2014) using the following equation:

$$T_{1/2} = \ln 2 / K$$

where K represents the biodegradation rate constants using the single first-order kinetic (SFO) model:

$$C = C_0 e^{-kt}$$

The overall and specific diesel biodegradation rate constants for the consortia CMic4 and CMic10 were calculated using the modified Hockey–Stick model (FOCUS 2006). The following equations related to this method were used:

$$C = C_0 \text{ for : } t \leq t_b$$

$$C = C_0 e^{-K(t-tb)} \text{ for : } t > t_b$$

Diesel half-life ($T_{½}$) was calculated according the following equation:

$$T_{1/2} = t_b + \ln 2 / k$$

In both mathematical models, C is the concentration of diesel at a given time (t), C_0 is the initial concentration of diesel in the sandy soil sample, k is the biodegradation rate constant of diesel and t_b is the breakpoint at the time at which rate constant changes and biodegradation starts.

Biocompatibility tests in remediated soil

The effect of diesel and its biodegradation products on the mortality and weight of earth worms (*Eisenia fetida*) was evaluated according to Shin et al. (2005), with some modifications. The earth worms were challenged with increasing concentrations of diesel, ranging from 0 to 1000 mg/kg, to establish the maximum concentration of diesel tolerance. Thereafter, a population sample of ten earth worms was exposed to remediated soils for 14 days. Untreated soil with 3000 mg/kg of diesel was used as the control. Percentages of survivability and changes in average weight were evaluated. All biocompatibility tests were performed in triplicate.

Results

Isolation of bacterial consortia of TPH degraders

Only two of the ten soil samples (samples numbers 4 and 10) exhibited microbial viability based oncatalase activity, with the remaining eight samples testing negative for catalase activity. Total hydrocarbon content, % TOC, pH, and humidity for these two samples are reported in Table 1. Only samples 4 and 10 were analyzed further for BTEX acclimatization. This process was carried out through several rounds of bacterial growth in MM supplied with BTEX as the only carbon source. The bacterial communities obtained from this process were named CMic4 and CMic10. Both consortia were conserved and their community composition characterized by 16S rRNA gene analysis.

Characterization of bacterial consortia

Diversity of bacterial consortia Using the tag-pyrosequencing technique, we generated 13,611 high-quality rRNA sequences with an average read length of 550 bp from the DNA of the CMic4 and CMic10 b. At the phylum and species level, CMic4 showed more than twice the number of operational taxonomic units than CMic10. Richness estimates based on the Chao 1 index and rarefaction curves of both samples (Fig. 1) suggest that most of the estimated diversity contained within these communities was captured by our sequencing efforts. Rarefaction curves showed a leveling off at different genetic distances, indicating that the number of analyzed reads was representative of the communities (Fig. 1). The Shannon diversity index value (H') was higher in CMic4 than in CMic10 at both the phylum and species levels, indicating that CMic4 had not only a higher richness but also a higher bacterial diversity than CMic10. Evenness values indicated that the relative abundances of taxa in CMic10 were more heterogeneous.**Bacterial community structures** All generated reads obtained from the experiment were classified as being bacterial. In both samples, *Proteobacteria* was the dominant phylum, representing 99.34 % of the CMic4 population and 100 % of the CMic10 population. Two additional minor phyla (with abundances of <1 %) were detected in CMic4: *Actinobacteria*, at 0.61 %, and *Firmicutes*, at 0.03 %. Differences in relative abundances between CMic4 and CMic10 at the species level are shown in Fig. 2. While in CMic4 there were ten major species with a relative abundance of >1%, in CMic10 only three major species were identified were a relative abundance of >1 %. In CMic4, the most abundant species were *Pseudomonas* sp. (33.67 %), *Ochrobacterium* sp. (28.23 %), and *Alcaligenes* sp. (16.80 %), while in CMic10, *Enterobacter* sp. dominated by far the community, at 95.93 %, followed by *Klebsiella* sp. at 1.82 %, and *Pantoea agglomerans* at 1.61 %.

Diesel biodegradation efficiency

Bacterial consortia CMic4 and CMic10 were evaluated for their biodegradation potential in a diesel-enriched sandy soil. In experiments where the soil samples were

Table 1 Chemical characteristics of the two soil samples with catalase activity

Soil sample	pH	Humidity (%)	Total organic carbon (%)	Total hydrocarbon content (mg/kg) BS		
				Light fraction (C_5–C_{12})	Medium fraction (C_{12}–C_{28})	Heavy fraction (C_{28}–C_{36})
4 (CMic4)	8.22	72.50	0.89	4220.83	8544.99	69,188.47
10 (CMic10)	4.02	13.44	1.49	N/A	10,209.95	39,699.42

BS, Bharat stage

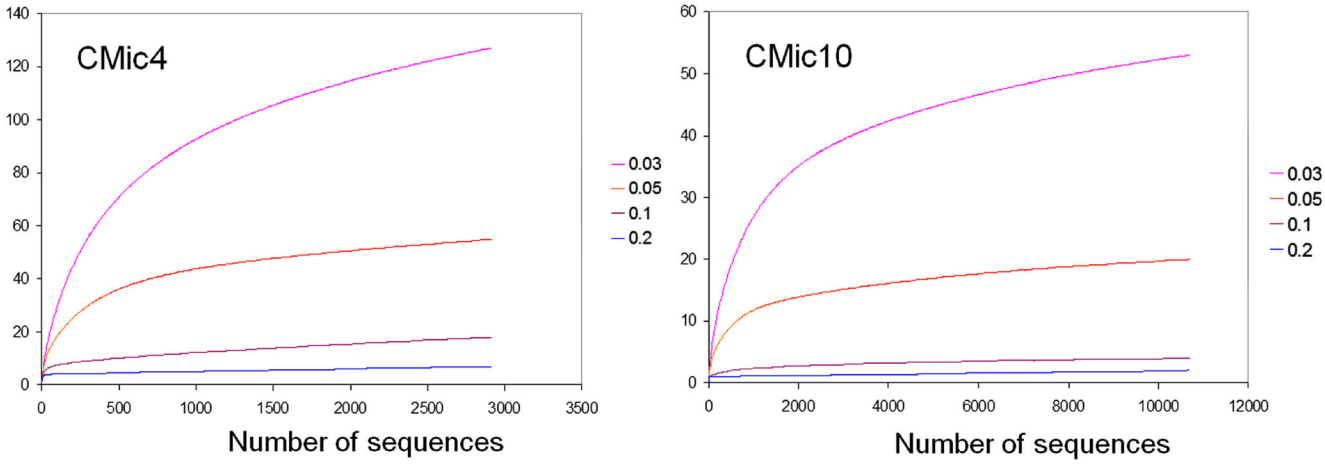

Fig. 1 Refraction curves of CMic4 and CMic10 operational taxonomic units

supplemented with diesel to a final concentration of 3000 mg/kg, both microbiota exhibited high biodegradation potentials, with $T_{1/2}$ values of 9 and 14 days, respectively. The biodegradation pattern exhibited by both consortia consisted of two sequential first-order curves that were characterized by drastic changes in the biodegradation rates after 2.5 days of experimentation (Fig. 3a, b); these could be described by the modified Hockey–Stick kinetic model.

The biodegradation kinetic parameters obtained for CMic4 and CMic10 are presented in Table 2 together with the rate constants determined for both the positive and negative controls. The results suggest that consortium CMic4 biodegraded diesel within a shorter period of time (11.3 days) than CMic10 (16.9 days; Fig. 3a, b). The positive control sample appeared to biodegrade diesel more rapidly than CMic4; however, the biological activity of the control sample ceased completely after 5 days into the experiment (Fig. 3c). The *Bacillus* sp.

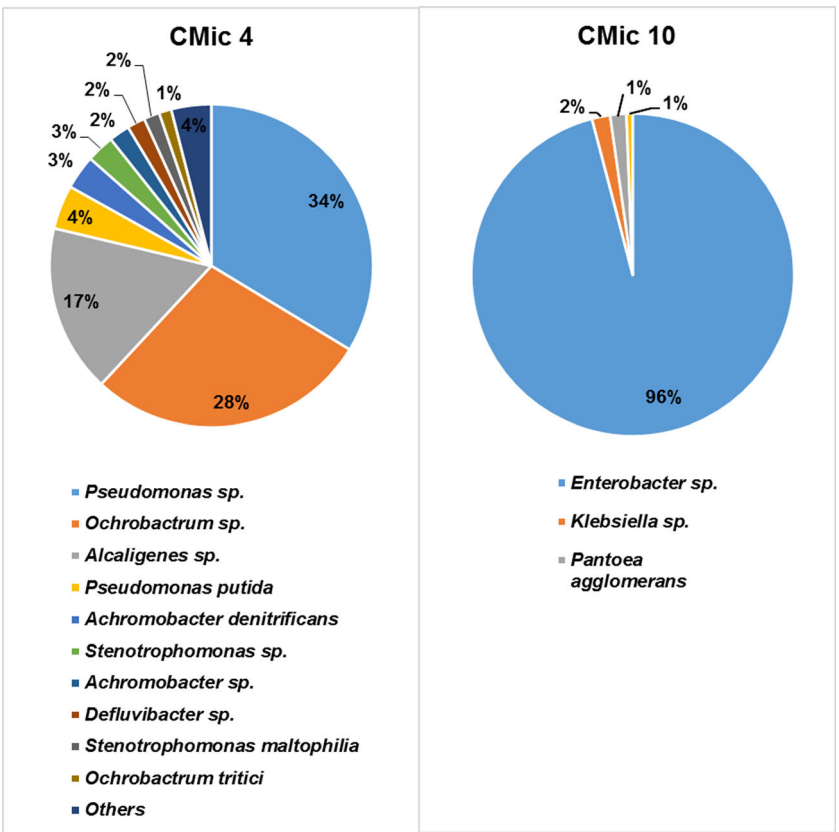

Fig. 2 Relative abundances at 3 % dissimilarity. Composition of the bacterial community of the two consortia (*CMic4*, *CMic10*) isolated from the two oil-contaminated soil samples (no. 4 and 10, respectively)

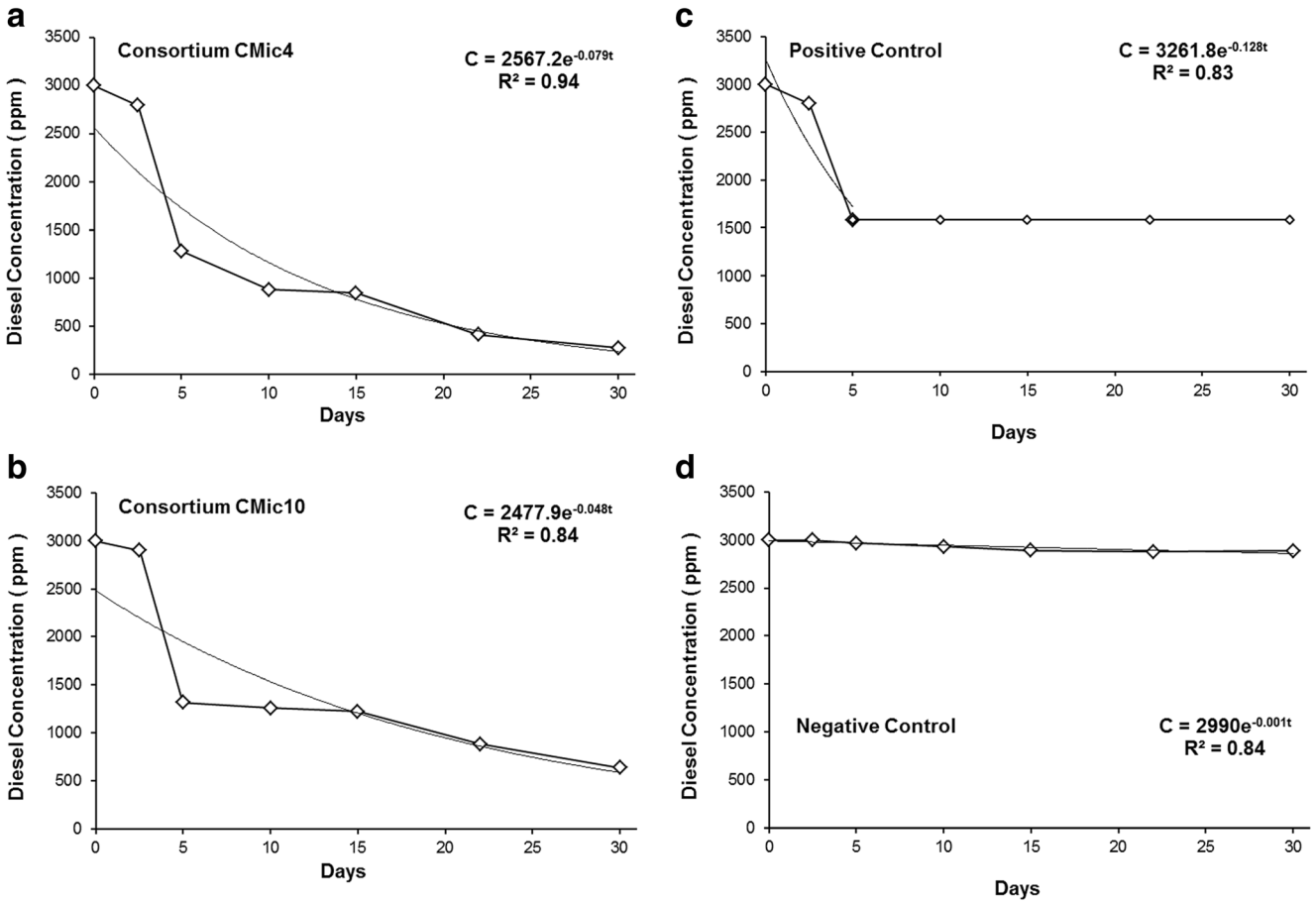

Fig. 3 Biodegradation of diesel by consortia CMic4 (**a**), CMic10 (**b**), *Positive control* (**c**, soil samples supplemented with commercial consortium of *Bacillus* sp. known for its efficient lipid degradation) and *Negative control* (**d**, un-inoculated sterile soil samples). *Lines* Exponential equation estimates of the first-order kinetics model of the sand samples where R^2 is the correlation coefficient. *C* Concentration of diesel at a given time (*t*)

consortium from the positive control was able to biodegrade a considerable fraction of the total diesel composition, reaching nearly 50 % of degradation. In comparison, CMic4 was able to mineralize up to 91 % of the initial 3000 mg/kg of diesel and consortium CMic10 was able to biodegrade up to 79 % of the diesel. Diesel dissipation in the un-inoculated cultures was reported to be 4 %. pH values remained stable in all of the samples and in all of the assays, ranging from 6.5 to 7.5.

Biocompatibility tests in remediated soils

Qualitative analysis Acute toxicity tests were performed using earth worms of the species *E. fetida* in order to evaluate the quality of the sand–soil mixture after the bioremediation assays and its compatibility with sustaining life. When exposed to increasing concentrations of diesel in the range of 0 and 1000 mg/kg, earth worms were observed to survive only

Table 2 Biodegradation kinetic parameters obtained by the Hockey–Stick modified method in sandy soil enriched with diesel

Sample	M_0 (mg/kg)	K (days^{-1})	k (days^{-1}(mg/L)$^{-1}$	R^{2a}	t_b[b] (days)	$T_{1/2}$ (days)
CMic4	3414.2	0.076	1.52×10^{-4}	0.99	2.5	11.3
CMic10	3245.2	0.046	9.2×10^{-5}	0.99	2.5	16.9
Positive control	3000	0.128	2.56×10^{-4}	0.83	–	5.4
Negative control	3000	0.001	–	0.84	–	693.2

M_0, Normalized Initial amount of diesel; t_b, breakpoint at the time that biodegradation starts; K, overall biodegradation rate of the diesel in sandy soil; k, specific biodegradation rate constant determined on 500 mg/L diesel, $T_{1/2}$, diesel half-life

[a] Coefficient of determination of the modified Hockey–Stick model

[b] Constant rate from T= t_b

on concentrations up to 200 mg/kg. When populations of *E. fetida* were incubated on CMic4 and CMic10 treated sand–soil mixtures for 14 days, survivability was reported in 80 % and 50 % of the earth worms, respectively. Earth worms in samples treated with the *Bacillus* mixture used as the positive control exhibited a 50 % worm's survival, while no survivability was observed in the un-treated samples after 7 days (Fig. 4).

Discussion

All biodegradation processes are influenced by a multitude of different factors. Among the most important of these is the species composition of the bacterial community used to biorremediate the contaminated soil. This is primarily due to the metabolic capacities of each bacterial isolate and to its capability to interact with other bacterial isolates in order to access the nutrients more efficiently. This was also the case for the bacterial degraders of TPH isolated in our study. From the ten contaminated soil samples analyzed, only two (samples 4 and 10) were suitable for microorganism acclimatization based on their positive test results for catalase activity. The catalase activity test has been widely applied as a preliminary assay in food production processes, the textile industry, pulp and paper bleaching, manufacturing of antiseptic and disinfecting agents, pharmaceutical research, and clinical, biological, and environmental analyses of microbial activity (Tsiafoulis et al. 2005; Vilian et al. 2014).

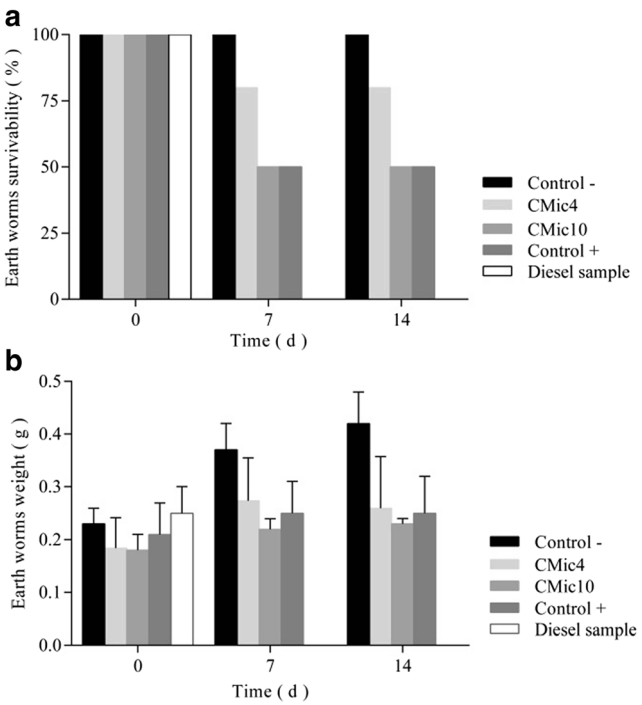

Fig. 4 Acute toxicity tests on earth worms (*Eisenia fetida*), measuring survival (**a**) and weight variations (**b**) at 0, 7 and 14 days

Samples 4 and 10 were further subjected to enrichment and stabilization rounds of acclimatization in MM using BTEX as the sole carbon and energy source, as it has been reported that this mixture of aromatic compounds is the most toxic TPH component for living cells (Peng et al. 2015). After BTEX acclimatization, it was possible to isolate a highly diverse bacterial population (named CMic4) from an acidic soil sample containing 69,188.47 mg/kg of the heavy hydrocarbon fraction. The combination of these two factors, pH and the contamination grade, could have contributed to the elevated diesel-degrading diversity/efficiency of this particular consortium compared with CMic10, which was isolated from an alkaline soil sample with a lower heavy hydrocarbon fraction content (39,699.42 mg/kg). Earlier studies on the effect of pH on soil microbial diversity suggest that acidic soils have less diversity than neutral ones (Bartram et al. 2014). On the other hand, prokaryotic diversity does decrease under alkaline conditions once pH levels rise above 7.0, where an important shift to eukaryotic microorganisms occurs (Liu et al. 2014). Both consortia, CMic4 and CMic10, consisted predominantly of bacteria belonging to the phylum *Proteobaceria* (99.3 and 100 %, respectively). A minor fraction of the CMic4 bacterial community also consisted of *Actinobacteria* (0.61 %) and *Firmicuites* (0.03 %). Despite the abundance of *Proteobacteria*, CMic4 was also composed of three other major bacterial species, accounting for 78.7 % of the population diversity (*Pseudomona* sp., *Ochrobacterium* sp., *Alcaligenes* sp.). In contrast, CMic10 was composed almost entirely of *Enterobacter* sp., which represented nearly 96 % of the bacterial population. All of the bacterial species reported in these consortia belong to genera that have been reported previously to be hydrocarbon degraders (Chikere et al. 2011). For example, *Pseudomonas* is well known as a degrader of gas and crude oil through the considerable activity of its alkane hydroxylases and hydrolases (Arendt et al. 2013). *Alcaligenes* metabolizes gas oil through it dioxygenases (Okpokwasili et al. 1986; Peng et al. 2008). *Ochrabactrum* has been reported to consume diesel using alkane hydroxylases (Watanabe and Hamamura 2003), and *Enterobacter* and *Klebsiella* have also been reported to breakdown alkanes using hydroxylases and dioxygenases (Yousaf et al. 2011; Hanano et al. 2014).

The diesel degradation capacity of both consortia was evaluated by comparing their diesel degradation rates and kinetic constants. We found that CMic4 presented the best efficiency, with a $T_{1/2}$ of 11.3 days, compared with CMic10, which presented a $T_{1/2}$ of 16.9 days. Differences between biodegradation rates may be due to the rich diversity of bacterial species between consortia. It has been demonstrated that different bacterial species have different dissipation potentials depending the type and bioavailability of hydrocarbon (Corseuil et al. 2011; Cyplik et al. 2011). On the other hand, bioavailability of the contaminant is related to the soil type due to its porosity (Semple et al. 2003, 2004). Sandy soils have been found to be

one of the most difficult soil types to remediate (Sutton et al. 2013; Jung et al. 2014; Lisiecki et al. 2014). Consequently, all of these factors contribute t the dynamic process that can be represented mathematically by a kinetic rate constant. The Hockey–Stick model is commonly used to describe dissipation patterns with a lag-phase where the concentration of the pollutant is not constant but declines very slowly up to a point where the biodegradation process starts (FOCUS 2006). In our study, both CMic4 and CMic10 exhibited this pattern at a high correlation (R^2=0.94 and 0.84, respectively; Fig. 3) Our results are similar to those found in the remediation of soil heavily contaminated with hydrocarbons by strains of *Pseudomonas* sp. (Dados et al. 2014) or other microbial consortia (Seklemova et al. 2001; El Fantroussi and Agathos 2005; Delille and Coulon 2008; Nikolopoulou and Kalogerakis 2009; Liu et al. 2010).

The main goal of any bioremediation process is to reduce or mineralize compounds present in the soil that represent an environmental or human health risk. In order to evaluate the biocompatibility of our biodegradation process, we evaluated earth worm survival rates in treated sandy soils. This procedure has been successfully used in earlier studies to demonstrate the non-toxic state of treated soil. Different bioindicators may be used for these biocompatibility tests. Souza et al. (2009, 2011) reported the results of studies with *Allium cepa* and the diplopoda *Rhinocricus padbergi* in soils treated after the concentration of TPHs had been reduced. However, due to their close interaction with soil, some taxonomic groups of invertebrates belonging to the meso- and macro-fauna, such as Isopoda, Collembola, Oligochaeta and Diplopoda, have been proposed as bioindicator organisms (Hopkin et al. 1985). For example, earth worms have features that make them feasible to be used as bioindicators for the toxicity of chemical substances in soil. These organisms are situated in the lowest levels of the terrestrial food webs and serve as food for several animals; consequently, bioassays using earthworms indicate the route of transference and biomagnification of contaminants along these webs (de Andréa 2010). Additionally, assays with *E. fetida* can be considered to be low cost, highly efficiency, and highly reproducible (Fontanetti et al. 2011).

Our results suggest that the earth worms tested in our system were able to tolerate up to 200 mg/kg of pure diesel. However, the relatively high survivability of *E. fetida* in treated soil samples containing the CMic4 and CMic10 consortia, where the residual TPH concentration was 275 and 632 mg/kg, respectively, suggests that diesel toxicity does not necessarily correlate with the concentration level of this contaminant, but that it is more likely to be related to a particular fraction of the hydrocarbon mixture which exhibits a high toxicological activity against *E. fetida*. This notion is supported by the 50 % survivability of *E. fetida* when exposed to a residual TPH concentration of 1582 mg/kg present in treated positive control samples (treated with *Bacillus* sp.). In summary, these results indicate that the fraction of diesel which exhibits toxicological activity against *E. fetida* is quickly metabolized by microorganisms, leaving behind a less hazardous hydrocarbon fraction which the species present in the microbial consortia have difficultly in metabolizing.

Conclusion

The bioremediation process carried out by the bacterial consortia CMic4 and CMic10 was efficient in terms of reducing diesel contamination in a sandy soil model under controlled laboratory conditions. CMic4 exhibited a higher biodegradation efficiency than CMic10 in terms of time and diesel removal. This was apparently due to the richer microbial diversity present in the CMic4 consortium. While both consortia responded to similar kinetic models on their biodegradation rates, CMic4 was faster than CMic10. Assays of biocompatibility of diesel biodegradation products carried out with earth worms confirmed the non-hazardous state of the treated soil after the decontamination process. Additionally, our results suggest that *E. fetida* can be used as a good, low-cost biological marker for TPH toxicity. The biodegradation model presented here represents a good opportunity to carry out further studies which focus on evaluating the mechanism(s) by which each TPH fraction is degraded in accordance with time and on identifying the association(s) between the specific bacterial species/group of species that performs the degradation of specific TPH fractions.

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Conflict of interest The authors declare no conflicts of interest associated with the work presented here.

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