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# Bioremediation of marine sediments contaminated by hydrocarbons: Experimental analysis and kinetic modeling

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ABSTRACT

This work deals with bioremediation experiments on harbor sediments contaminated by aliphatic and polycyclic aromatic hydrocarbons (PAHs), investigating the effects of a continuous supply of inorganic nutrients and sand amendments on the kinetics of microbial growth and hydrocarbon degradation. Inorganic nutrients stimulated microbial growth and enhanced the biodegradation of low and high molecular weight hydrocarbons, whereas sand amendment increased only the removal of high molecular weight compounds. The simultaneous addition of inorganic nutrients and sand provided the highest biodegradation (>70% for aliphatic hydrocarbons and 40% for PAHs). A semi-empirical kinetic model was successfully fitted to experimental temporal changes of hydrocarbon residual concentrations and microbial abundances. The estimated values for parameters allowed to calculate a doubling time of 2.9 d and a yield coefficient biomass/hydrocarbons 0.39 g C biomass g-1C hydrocarbons, for the treatment with the highest hydrocarbon biodegradation yield. A comparison between the organic carbon demand and temporal profiles of hydrocarbons residual concentration allowed also to calculate the relative contribution of contaminants to carbon supply, in the range 5–32%. This suggests that C availability in the sediments, influencing prokaryotic metabolism, may have cascade effects on biodegradation rates of hydrocarbons. Even if these findings do not represent a general rule and site-specific studies are needed, the approach used here can be a relevant support tool when designing bioremediation strategies on site.


## 1. Introduction

The contamination of coastal marine sediments by hydrocarbons represents a global concern for the potential consequences on ecosystem and human health [1]. For this reason an increasing attention has been directed toward the research of new strategies and environmental-friendly technologies to be applied for the remediation of sediments contaminated by hydrocarbons. Among these, biotechnological strategies based on the biostimulation of autochthonous microbial communities to speed up biodegradation processes of organic pollutants are of particular relevance.

Numerous studies of sediment bioremediation have been carried out on contaminated beach sediments [2–5], and relatively few studies have been conducted involving hydrocarbon contamination in marine or estuarine sediments [6–8]. These studies have demonstrated that nutrient additions can greatly enhance the biodegradation rate of hydrocarbons. Highest biodegradation rates have been generally reported for saturated hydrocarbons, followed by the low molecular weight aromatics, with high molecular weight aromatics and polar compounds exhibiting extremely low rates of degradation [9]. This pattern is not universal, however, as Cooney et al. [10] reported greater degradation losses of naphthalene than of hexadecane in water-sediment mixtures from a freshwater lake and Jones et al. [11] observed extensive biodegradation of alkyl-aromatics in marine sediments prior to detectable changes in the n-alkane profile of the crude oil tested. Fedorak and Westlake [12] also reported a more rapid attack of aromatic hydrocarbons during the degradation of crude oil. Such discrepancies underpin the need to improve the comprehension of the complex abiotic and biotic interactions occurring in the sediment for formulating objective bio-treatment strategies that will produce a specified outcome in terms of degradation rates and residual contaminant concentrations [9].

The integration of mathematical modeling and experimental testing is a key issue for a better comprehension and prediction of

*Abbreviations:* $C$, Total aliphatic hydrocarbon concentration (μg aliphatic hydrocarbon $g^{-1}$ sediment); HMW, high molecular weight aliphatic hydrocarbons ($C$ > 24–40); $k$, first-order rate constant ($d^{-1}$); $K_0$, semi-empirical model parameter (Eq. (1), $d^{-2}$); LMW, low molecular weight aliphatic hydrocarbons ($C$ > 12–24); PAHs, polycyclic aromatic hydrocarbons; t, time (d); Td, doubling time (d); $X$, prokaryotic abundance ($10^8$ cells $g^{-1}$ sediment); $Y$, semi-empirical model parameter (Eq. (1)) related to yield coefficient ($10^8$ cells $\mu g^{-1}$ aliphatic hydrocarbons), (g C biomass $g^{-1}$ C aliphatic hydrocarbons); $\beta$, semi-empirical model parameter (Eq. (1)) related to inhibiting factor (g sediment $10^{-8}$ cells).

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the efficiency of bio-treatments devoted to hydrocarbon removal in contaminated marine sediments. Kinetic models can be a useful tool for the prediction of residual contaminant concentrations during bioremediation [13–16]. For instance Monod kinetics have been demonstrated for the microbial uptake and oxidation of toluene, a low molecular weight aromatic hydrocarbon of relatively high water solubility, but may not apply to the more insoluble hydrocarbons. Furthermore, the rates of mineralization of the higher molecular-weight aromatic hydrocarbons are related to aqueous solubility rather than total substrate concentrations [17]. Biodegradation rates for many hydrocarbons, therefore, will not display the dependence on concentration which is typically observed with more soluble organic substrates. At the same time most kinetic models have been tested with only one or few substrates, and taking into account single or few bacterial strains growing in culture [18]. Kinetic models of growth of natural bacterial assemblages during bio-treatments of contaminated marine sediments received little attention, but these can be a relevant support for a better understanding of biodegradation rates of hydrocarbons and prediction of residual contaminant concentrations.

In this study we carried out bioremediation experiments on harbor sediments contaminated by aliphatic and polycyclic aromatic hydrocarbons (PAHs), investigating the effects of a continuous supply of inorganic nutrients and sand amendments on the kinetics of microbial growth and hydrocarbon degradation. Inorganic nutrients were added as bio-stimulating agents, whereas the amendment of sand from an unpolluted site was used to potentially increase mass transfer processes of the contaminants. Experimental results were then used to assess the suitability of a rather simple semi-empirical model to predict temporal changes of microbial growth and residual hydrocarbon concentrations during bio-treatments in order to provide a support tool when designing bioremediation strategies on site.

## 2. Materials and methods

### 2.1. Sampling and sample processing

The sediment used for bioremediation experiments was collected from an Italian harbor located in the Adriatic Sea (Mediterranean Sea). Sandy sediment samples used as amendment were collected from an uncontaminated site located ca. 5 km from the harbor. Samples were stored at 4 °C until laboratory analysis and experiment set up. Grain size was determined by wet sieving technique [19]. Sediment water content was calculated as the difference between wet and dry weight and expressed as percentage. For total organic matter (TOM) analyses, sediment samples ($n = 3$) were treated with an excess of 10% HCl to remove carbonates that may interfere with TOM determination. TOM was determined as the difference between dry weight (60 °C, 24 h) of the sediment and weight of the residue after combustion for 2 h at 450 °C [20]. Organic carbon content in the sediment was assumed to represent 50% of the TOM concentrations [21].

### 2.2. Bioremediation experiment

Microcosm experiments were performed in 250 mL flasks containing 20 g wet harbor sediment samples and 100 mL pre-filtered 0.2 μm seawater. Replicate microcosms were amended with: (i) $(NH_4)_2SO_4$ and $K_2HPO_4$ (final concentrations of 0.23 and 0.023 mM of nitrogen and phosphorus, respectively), (ii) 1.3 g wet sandy sediments and (iii) inorganic nutrients and sandy sediments at the same concentrations as reported above. The final concentrations of *N* and *P* were defined on the basis of the organic carbon content in the sediment according to a molar *C*:*N*:*P* ratio of 100:10:1. Microcosms used as controls and those containing sandy sediments were amended daily with 1 mL of pre-filtered 0.2 μm seawater, to compensate for evaporation as verified in preliminary tests, whereas all the other experimental systems were amended with an equal volume of pre-filtered seawater containing inorganic nutrients (0.46 mM nitrogen, as $(NH_4)_2SO_4$ and 0.046 mM phosphorus, as $K_2HPO_4$). All flasks were incubated at a temperature of 30 °C in a shaking incubator at a speed of 180 rpm for 5 wk. Redox potential and dissolved oxygen concentrations were daily measured in the different experimental systems (InoLab multi 720, WTW). Subsamples were collected for prokaryotic counts after 0, 2, 6, 7, 14, 21, 28 and 35 d, whereas other sub-samples were collected for the analyses of aliphatic hydrocarbons and PAHs.

### 2.3. Chemical analysis

Aliphatic hydrocarbons were extracted from the sediment samples according to EPA 3546 method [22] and quantified according to EPA 8015D method [23] by gas chromatography equipped with a flame ionization detector (GC-FID PerkinElmer Clarus 500). Aliphatic hydrocarbons were classified as low (LMW: $C > 12$–$24$) and high (HMW: $C > 24$–$40$) molecular weight. A mixture of hydrocarbons (C10–C35) was used as calibration standard. The limit of detection of the method was equal to 0.1 $\mu g\,g^{-1}$ dry weight.

PAH analysis was performed by high performance liquid chromatography (HPLC PerkinElmer Series 200), according to 3545A EPA method [24]. PAHs were classified as LMW (naphthalene, acenaphthene, fluorene, phenanthrene, anthracene) and HMW PAHs (fluoranthene, pyrene, benzo[a]antrhacene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, dibenzo[a,h]anthracene, benzo[g,h,i]perylene) [25].

### 2.4. Total prokaryotic abundance

Total prokaryotic cells were determined according to standard protocols [26]. Prokaryotes were detached from the sediment by using tetrasodium pyrophosphate and sonication. Sediment subsamples were diluted 100–500 times, stained with acridine orange, filtered on black Nuclepore polycarbonate 0.2-μm-pore size filters, and observed with an epifluorescence microscopy (Zeiss Axioskop 2). Prokaryotic abundance was normalized to dry weight after sediment desiccation.

### 2.5. Data analysis

Analysis of variance (ANOVA) was carried out for testing differences between treatments. When significant differences were observed a post hoc Tukey's test was also performed.

## 3. Results and discussion

### 3.1. Sediment characteristics

The harbor sediments were characterized by the dominance of the silt-clay fraction ($77 \pm 1\%$), high organic matter content ($30 \pm 5\ mg\,g^{-1}$) and high concentrations of total aliphatic hydrocarbons and PAHs ($500 \pm 20$ and $6.1 \pm 0.1\ \mu g\,g^{-1}$ dry weight, respectively). In harbor sediments total aliphatic hydrocarbons were mainly represented by LMW compounds (ca. 60% of the total content), whereas the total PAH pools were almost entirely constituted by HMW compounds (ca. 95% of the total pool). Sandy sediment samples utilized as amendment for bioremediation experiments were characterized by very low silt-clay fraction (ca. 5%), low organic matter load ($5\ mg\,g^{-1}$) and total aliphatic and total PAH concentrations close to analytical detection limits.

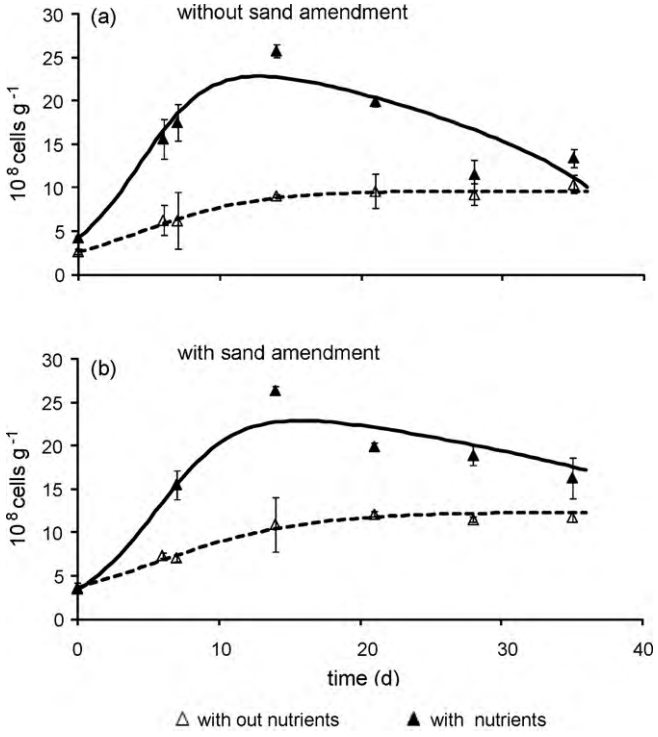


**Fig. 1.** Temporal changes of prokaryotic abundances during bioremediation in the absence (a) and in the presence (b) of sand. Reported are the interpolation lines of experimental data obtained by Eq. (1).

### 3.2. Bioremediation experiments

In this study we assessed the effects of inorganic nutrients and sand amendments on the growth rates of natural prokaryotic assemblages and related biodegradation potential toward hydrocarbons. In the first case, we supplied ammonium sulfate and phosphate di-potassium hydrogen phosphate (as sources of bioavailable nitrogen and phosphorous) to enhance biodegradation rates of hydrocarbons in the sediment ([27] and references therein). In the second case, we used a sand amendment to potentially increase available surface area and consequently help remediation.

Time-course experiments revealed a fast response of the benthic prokaryotic assemblages induced by the addition of inorganic nutrients (Fig. 1). After only 5 d, prokaryotic abundances in these treated sediments were significantly higher than in the microcosms used as control (i.e. without nutrient addition). Conversely, the addition of sandy sediments did not enhance prokaryotic growth since no significant differences were observed between samples with and without sand amendment (Fig. 1). Although the inorganic nutrient supply promoted an exponential prokaryotic growth in the early experimental phase, after 2 wk of incubation microbial abundance progressively decreased. Such a decrease of microbial growth and metabolism in contaminated sediments treated with inorganic *N* and *P* has been related to the progressive inorganic nutrient limitation occurring over time [3,28,29]. However, this was not the case of the present study since we continuously replenished the experimental systems with bioavailable *N* and *P*. Therefore other abiotic factors such as the reduction of the bioavailable organic C and/or biotic factors such an increase of predatory pressure during the time can be invoked to explain the microbial patterns observed in the present study.

Biodegradation extent of aliphatic hydrocarbons and PAHs (calculated as the difference between initial and final concentrations, referred to initial concentration) is reported in Fig. 2. For the aliphatic hydrocarbons, biodegradation was calculated for both the LMW and the HMW compounds, while for the aromatics only the total biodegradation was considered, since aromatics were almost entirely represented by HMW compounds.

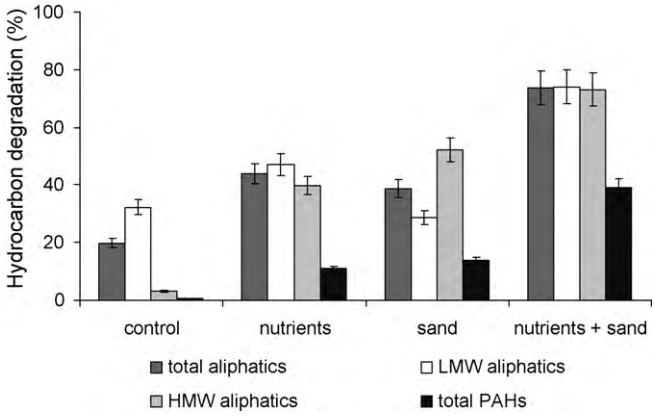


**Fig. 2.** Degradation of total, LMW and HMW aliphatic hydrocarbons and total PAHs in different microcosms at the end of incubations (35 d).

Hydrocarbon removal due to natural attenuation processes was very low since only <5% of the HMW aliphatic and aromatic compounds were removed after 5 wk of incubations. The addition of inorganic nutrients increased significantly hydrocarbon removal, confirming previous laboratory and field experiments which stated that nutrient availability is a key limiting factor for the efficient removal of hydrocarbons by microbes in contaminated sediments [3,30,31]. Also sand amendment increased biodegradation of HMW compounds at the same extent than that achieved by nutrient addition. These results suggest that sand addition to harbor sediments, increasing the solid/liquid interface and enhancing the oxygen diffusion and material transfer rates, may favor the biodegradation of the more recalcitrant HMW hydrocarbons [32,33]. The simultaneous addition of inorganic nutrients and sand provided the highest hydrocarbon biodegradation (>70% for the aliphatic hydrocarbons and 40% for PAHs), indicating a synergistic effect on the whole biodegradation performance. These results suggest that the development of efficient bio-treatments for the remediation of contaminated marine sediments should be oriented not only to increase biomass and activity of hydrocarbon-degrading bacteria, but also to enhance available surface area for the microbial attack.

### 3.3. Kinetic modeling

There is a definite need for robust kinetic models which can contribute to explain the factors influencing biodegradation rates of contaminants in the environment and may ultimately be used to predict timescales and effects of remediation interventions [34–36]. A deterministic approach would request the analyses of temporal changes not only of contaminant concentrations and microbial abundances but also of nutrient concentrations (at least the limiting ones) required to sustain over time the biodegradation processes [37]. Based on the data set acquired in the present study, a semi-empirical approach was applied using hydrocarbons and microbial abundances as dependent variables. Therefore, the results obtained here cannot be generalized and parameter estimation would be requested for each specific site under investigation. The following semi-empirical equations have been fitted to experimental data describing temporal changes of prokaryotic abundance

**Table 1**
Parameters ($k$, $K_0$, $\beta$ and $Y$) estimated by non linear regression analyses in the different experimental systems. Regression coefficients ($R^2$) are also reported.

| Experimental systems | $k$ ($d^{-1}$) | $K_0$ ($d^{-2}$) | $\beta$ ($g\,10^{-8}$ cells) | $Y$ ($10^8$ cells $\mu g^{-1}$ hydrocarbons) | $Y$ (g C biomass $g^{-1}$ C hydrocarbons) | $R^2$ |
|---|---|---|---|---|---|---|
| Control | 0.24 | 0 | 0.10 | 0.07 | 0.16 | 0.97 |
| Nutrients | 0.39 | −0.006 | 0.04 | 0.31 | 0.73 | 0.85 |
| Sand | 0.18 | 0 | 0.08 | 0.04 | 0.09 | 0.90 |
| Nutrients + sand | 0.35 | −0.004 | 0.04 | 0.14 | 0.32 | 0.90 |

($X$) and total aliphatic hydrocarbons (C):

$$\begin{cases} \dfrac{dX}{dt} = kX(1-\beta X) + K_0 \left| \displaystyle\int_0^t X(t)dt \right| \\ \dfrac{dC}{dt} = \dfrac{1}{Y} kX(1-\beta X) \end{cases} \quad X(0) = X_0; C(0) = C_0 \qquad (1)$$

A modified version of the empirical logistic equation [38] was used to mathematically describe the microbial growth rate $dX/dt$, taking into account also the potential decrease of microbial abundance after the stationary phase (due to either a bottom-up control – nutrient limitation or top down control due to predation, i.e. $K_0$ in Eq. (1); [37]. In Eq. (1) the contaminant degradation rate $dC/dt$ was supposed to be associated only to the logistic term of microbial growth through a yield factor, $Y$, hypothesizing that the $K_0$ term was not relevant for contaminant biodegradation.

Eq. (1) has been fitted to experimental data (Fig. 1) and the four adjustable parameters $k$, $K_0$, $\beta$ and $Y$ have been estimated through a nonlinear regression technique [39], minimizing the following objective function, during resolution of Eq. (1) by Runge–Kutta algorithms:

$$\varphi = \sum_j \left( \frac{X_{j,\mathrm{calc}} - X_{j,\mathrm{exp}}}{X_{j,\mathrm{exp}}} \right)^2 + \sum_i \left( \frac{C_{i,\mathrm{calc}} - C_{i,\mathrm{exp}}}{C_{i,\mathrm{exp}}} \right)^2 \qquad (2)$$

where indexes $i$ and $j$ refer to microbial abundances and hydrocarbon concentrations, respectively. The estimated values for the different parameters and the regression coefficients are reported in Table 1. The relatively high values of the regression coefficients confirm the suitability of Eq. (1) to mathematically describe the temporal changes of microbial abundance and contaminant concentrations in the different experimental conditions investigated in the present study.

We found that the estimated values for the kinetic constant ($k$) increased in experimental systems supplied by nutrients (Table 1). Such estimates allowed us to calculate prokaryotic doubling times between 1.8 and 3.9 d, which are typically observed in highly eutrophic marine sediments [40]. Table 1 also shows that the estimated value for $\beta$ (the inhibition/limiting factor of microbial growth and contaminant biodegradation) increased without nutrient inputs [values in the range 0.04–0.10 $g\,10^{-8}$ cells). As expected, $K_0$ was equal to zero in the absence of nutrients (where the prokaryotic abundance reaches a stationary phase), and negative with a continuous supply of inorganic nutrients (−0.004 and −0.006 $d^{-2}$ with and without sand amendment, respectively). The estimated values for parameter $Y$ ranged from 0.04 to 0.31 $10^8$ cells $\mu g^{-1}$ hydrocarbon, corresponding to 0.09–0.73 g C biomass $g^{-1}$ C hydrocarbons (assuming a cellular C content of 20 fg C $cell^{-1}$ [41], and considering a C20 aliphatic compound as model). Such values were higher in the presence of nutrients suggesting that inorganic nutrients can enhance the efficiency of assimilation of degraded organic compounds into prokaryotic biomass [42]. The $Y$ values reported here should be viewed with caution since they were estimated on the basis of the growth rates of the total prokaryotic biomass, which includes both petroleum-degrading prokaryotes and prokaryotes that utilize other organic substrates as primary energy and C source for their metabolism [43,44]. Therefore $Y$ values for the petroleum-degrading microorganisms can be much greater than the values of $Y$ estimated for the entire prokaryotic assemblages. Despite this, the analysis of petroleum-degrading microorganisms based on cultivation techniques would underestimate the actual prokaryotic biomass able to degrade hydrocarbons both directly and/or co-metabolically (i.e. degrading synoptically hydrocarbons and other organic compounds used as primary energy source for their metabolism [9]).

Prokaryotic abundances predicted by Eq. (1) were used to calculate the organic carbon consumption necessary to support the observed microbial growth, assuming a carbon conversion efficiency of 30% [45]. A comparison between the microbial organic carbon demand and temporal profiles of residual concentration of hydrocarbons predicted by Eq. (1) allowed us to calculate that contaminants can supply 5–32% of the total metabolic C requirement. These findings suggest that C availability influencing the prokaryotic metabolism in the sediments may have cascade effects on biodegradation rates of hydrocarbons (being hydrocarbons degraded both directly and co-metabolically) and that this aspect should be taken into account for the prediction of the residual contaminant concentrations.

Eq. (1) also describes the temporal changes of contaminants during all the investigated bioremediation treatments. The profiles show that, without a continuous supply of nutrients, hydrocarbon biodegradation proceeded only for the first 15 d of treatment, reaching an asymptotic value of 300–400 $\mu g\,g^{-1}$ (20–40% biodegradation, Fig. 3a). By contrast, with a continuous inorganic nutrient

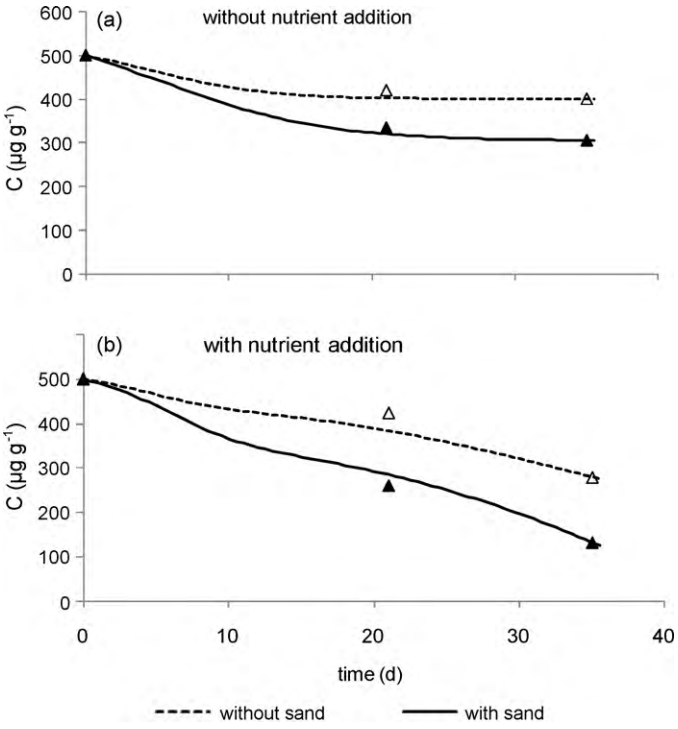


**Fig. 3.** Temporal changes of total aliphatic hydrocarbon concentrations (converted in carbon equivalents) predicted by Eq. (1) in the absence (a) and in the presence (b) of a continuous nutrient supply.

supply (Fig. 3b) the model showed that hydrocarbon biodegradation progressively decreased during the whole treatment.

Even if these findings do not represent a general rule and site-specific studies are needed, the methodologic approach used here can be a relevant support tool when designing bioremediation strategies on site.

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