RECOVERING OF DREDGED SEDIMENTS CONTAMINATED BY TOTAL PETROLEUM HYDROCARBON TO PRODUCTIVE SOILS: THE MYCOREMEDIATION APPROACH IN THE BIORESNOVA PROJECT

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Abstract

Chemo-physical treatments to remove salinity and metal contamination from dredged sediments were applied in combination to bio-based approaches (mycoremediation). New fungal specimen were isolated from the contaminated sediments, massively grown and re-inoculated in the matrix in treatment to remove the Total Petroleum Hydrocarbon contamination (TPH). Toxicological assays were exploited to estimate the sediment remediation efficiency over time. Indeed, the only chemical characterization of polluted matrices does not allow to predict the residual toxicity of the latter eventually related to the permanence of a residual contamination by the parental pollutants, to their degradation intermediates and/or to the synergic actions of the both. Higher plants (*Vicia faba* L.) were exploited as indicators of the quality of the treated sediments and used both for the continuous monitoring of the remediation processes and for the evaluation of the final product eco-safety. Biological parameters such as the genotoxicity by means of cytological analysis of mitotic behavior of root meristems were evaluated based on the detection of chromosomal aberrations in mitotic cells, and of micronuclei formation, detectable in interphase cells.

The combination of the Chemo-physical and the Bio-based approach was able to remove the organic contamination (TPH) and the excess of sodium salts that constitute a critical point for the eventual re-allocation

of dredged sediments. At the same time the sediments were detoxified and actually gained the biochemical traits of humified productive soils, eventually suitable for their safe re-allocation in the environment.

1. Introduction

Hydrocarbon pollutants represent a class of contaminants, deriving from human activities or spills and from industrial processes related to petroleum refining, present in solid or liquid matrices such as dredged sediment and marine water. Total Petroleum Hydrocarbons (TPH), the main contaminant present in the sediments affected by hydrocarbon contamination, are partially soluble and volatile toxic compounds resulting as one of the main pollutants that cause a serious risk for the public/human health (Andreoni and Gianfreda, 2007).

TPHs include also an “unresolved complex mixture”. The term UCM describes hump-shaped chromatograms that are often observed in gas chromatograms of environmental matrices contaminated by crude oil and petroleum derivates (Gough et al., 1990; Booth et al., 2008). The UCM of petroleum based samples might contain alkanes, alkenes, alkynes, cycloalkanes, monoaromatics, polycyclic aromatic hydrocarbons (PAHs), steranes and also polychlorinated biphenyls (PCBs) (Gough et al., 1990; Booth et al., 2008). The interest in environmental samples containing hydrocarbons is often motivated by the potentially toxic nature of some of UCM contaminants (Golon, 2012). TPHs induce both acute and chronic effects on living organisms; the aromatic substances might be associate to mutagenic, carcinogenic and genotoxic effects (Cogliano et al., 2008; Covino S. et al., 2010).

The biodegradation of organic compounds operated by basidiomycetes has been described as related to the fungal capacity to produce extracellular oxidative enzymes that have low substrate specificity. They are able of spreading to the solid matrix and of oxidizing organic pollutants that have a similar structure to the lignin (Chiu et al., 2009).

In this study, the ability to deplete the contamination associated to UCM was studied in two meso-scale experimentations. In the first meso-scale trial the basidiomycetes *P.ostreatus* was inoculated. A second meso-scale scale experimentation was conducted to evaluate the capacity of an ascomycetes new isolates, autochthonous to the sediment, to deplete the UCM associated to the profile contamination of the polluted sediments. The degradation of hydrocarbons in soil is generally associated to bacteria, but fungi exhibit favourable characteristics to be considered good candidates for bioremediation treatment of soils contaminated by crude oil (Potin et al., 2004).

The goal of this work was: (i) the depletion of the TPHs contamination, (ii) the reduction of the UCM toxicity, (iii) the restoration of the resilience of dredged sediments here consisting in promoting the bacterial biodiversity recovery and the equi-distribution of the bacterial specimen among the different genera. A toxicological assessment of the process has been performed by evaluating phyto and genotoxicity on root tips of *Vicia faba.*

2. Material and methods

2.1 Depletion of diesel oil

The depletion of diesel oil during the two meso-scale experimentations has been monitored by GC-MS analysis. The samples were analyzed according to the protocol of Klein et al. 2012. Each sample was dried overnight at 105°C and extracted. The extract solutions have been acidified (pH=2.8), amended with 6µg each of deuterated alkanes with 16 (C16) and 24 (C24) carbons as surrogate standard and extracted at room temperature with dichloromethane (30% volume ratio). This procedure was repeated three times, and the dichloromethane phase was concentrated by rotary evaporation at room temperature, followed by evaporation under a stream of nitrogen. The concentration of total extracted and resolved hydrocarbons was determined by high-resolution GC-MS analysis by a Saturn 2200 quadrupole ion trap mass spectrometer coupled to a CP-3800 gas chromatograph (Varian Analytical Instruments, Walnut Creek, CA, USA) equipped with a MEGA 1 MS capillary column (30 m; 0.25 mm i.d., 0.25 lm film thickness, MEGA s.n.c., Milan, Italia).

2.2 Mesocosm experimentation set up

The mesocosm scale experimentation was set up according to the following steps: (i) preparation of the lignocellulosic matrix used to facilitate aeration (bulking agent, BA) and used for the inoculation of the basidiomycota *P. ostreatus*; (ii) mixing sediments and lignocellulosic matrix (30% w/w); (iii) adjustment of macronutrients content N, P (ratio 10:1) (added as NH_4NO_3 and K_3PO_4). The first experimentation has provided the preparation of 15 mesocosms, from the total weight of 1250 g each and divided into 3 mesocosms for time of analysis (T0, T15, T30, T45 and T60) in triplicate. The control was prepared without the amending of BA.

The second meso- scale experimentation provided the inoculation of the selected autochthonous microfungi strain, in each trial, pre-treated with the basidiomycota *P. ostreatus*. The fungal biomass was pre-grown in 200 ml of Malt Extract, in 500 ml Erlenmeyer flasks, at 24°C on an orbital shaker at 150 rpm, then inoculated (5% fresh weight) in the corresponding T60 mesocosms. The analyses were performed after the bioaugmentation of the autochthonous microfungi and after 15, 30, and 45 days of incubation (T75, T90, T105).

2.3 Fungal biomass detection

In order to evaluate the vitality of the *P. ostreatus* and/or the vitality of the autochthonous microfungi during the second phase of the mesocosm scale experimentation, the ergosterol was used as molecular marker of the fungal activity (Giubilei et al., 2008).

2.4 Humic and fulvic acids

Humic and fulvic acids were extracted according to Piccolo A., 1996.

2.5 *Vibrio fisheri bioassay: Microtox solid phase test*

The Microtox Solid Phase test (SPT) protocol (Azur Environmental, Carlsbad, CA, USA) was conducted on

sediment samples; SPT values were normalized to dry weight. On elutriates, the Microtox liquid phase test was applied, following the manufacturer's protocol.

Bacteria (*Vibrio fischeri*) were exposed to a serial dilution of a suspension and then a filter column was used to separate the aqueous phase containing the bacteria from the solid phase. Initially, 7 g of each sediment sample had been mixed with 15 ml of Microtox® solid-phase diluent (3.5 % NaCl), stirred for 10 minutes and 1.5 ml of the suspension had been transferred to SPT tubes. Light output was measured with the bacteria recovered in the aqueous phase with the Model 500 Microtox® Analyzer. The toxicity was calculated using the STI formula (ICRAM, 1999).

2.6 *Vicia faba Bioassay: ecotoxicological test*

The test was conducted on sediment sample and elutriates. The elutriate preparation was carried out mixing 3 parts of bi-distilled water and 1 part of sediment, for 3 hours at 24°C on rotary shaker and settled overnight. Seeds of *Vicia faba* L. were germinated in Petri properly prepared with the two matrices, in controlled conditions for 72 h, according to ISTISAN protocol (Gustavino et al., 2013). The root tips were coloured using the Feulgen technique, specific for the DNA. A glass slide for each root tip squash was obtained. For each slide at least 1.000 nuclei, randomly selected, were analysed by means of light microscope.

Parameters such as phytotoxicity, cytotoxicity and genotoxicity were evaluated.

2.7 Statistical Analysis

All the data were elaborated with the aid of one-way ANOVA, and the means were separated by Bonferroni multiple-comparison test ($P \leq 0.05$) using the specific software Statgraphics 6.1 (Statistical Graphics Corp., USA).

3. Results e Discussion

Petroleum refined products as diesel oil, represent the contaminant of interest in the present study. They are composed by different class of compounds related to THPs but with different chemical structures, biodegradability and toxicity. Biodegradation of hydrocarbons by natural microbial populations is the primary mechanism by which oil contaminants are removed from the environment (Yakimov et al., 2007). In this study, we analyse the ability by allochthonous fungus *P. ostreatus* and autochthonous microfungi isolated from dredged sediments to deplete not only *n-alkanes* but also the UCM fraction. The above metabolic traits were evaluated in two different mesocosm scale experimentation sets. Fungi play an important role for the functioning of the ecosystem soil (Doran and Parkin, 1994; Doran and Parkin, 1996; Hawkswortth et al., 1996). Fungi decompose complex macromolecules like lignin or chitin. Fungal isolates can use petroleum hydrocarbons as nutrient and they can survive in polluted areas. In fact, fungal species were described as capable to utilise oil compound as carbon source and crude oil pollution has been described as associated to the increase of fungal growth (Al-Jawhari et al., 2014; Harayama et al., 1999; Mohsenzadeh et al., 2012).

The results obtained in this study indicate that the bioaugmentation of the autochthonous microfungi strain, isolated from the treated sediment, was more effective than *P. ostreatus* inoculation. GC-MS data show that the microfungi determined a complete depletion (concentration below 60 ppm, according to the law D. L. 152/06

2006) after 30 days of incubation in comparison to the 20%±1.12 of UCM in 60 days. These data confirm that the use of autochthonous microorganisms is more effective than allochthonous ones due to the capability of growing in environment where the selective pressure is high.

Ergosterol data show that there are not significant differences in terms of vitality between basidiomycete and ascomycete. Indeed, after 15 days incubation, both fungi show a decrease in terms of fungal biomass. The decrease in biomass load is actually a positive factor for the bioaugmentation on a real scale plant, because the inoculated fungi do not persist in the environment after the treatment.

The interest in environmental samples, containing hydrocarbons, is frequently motivated by the potentially toxic nature of some of their contaminants. The UCM hydrocarbons toxicology has rarely been studied but its potential is high because include compounds related to PAH or PCB.

In this work, the toxicity of the matrices has been recorded only thanks to the *Vicia faba* ecotoxicological bioassay, while Microtox test, unlike widely standardized, was not responsive in our specific case of study.

In *Vicia faba* system, data related to 3 different endpoints as phyto, geno and cytotoxicity were valuable. The results obtained during phytotoxicity tests show that in TPH contaminated sediment, plant model presents alterations in seeds germination and elongation. The solid matrices have higher phytotoxicity than elutriates. The inoculum of the autochthonous microfungi improved the quality of the matrix, contributing to the reduction of the phytotoxicity in *V. faba*. These data are in agreement with the results concerning the chemical degradations. While the autochthonous microfungi significantly deplete the contamination, *P. ostreatus* determined a reduction of 20% of TPH concentration. Analogous results were obtained from the cytotoxicity assay, with a greater level of toxicity for solid matrix.

The MNC test results obtained are shown in figure 1. The genotoxicity of the matrices increases with the increase in the efficiency related to the depletion of the contaminants up to the condition corresponding to the real depletion of the contamination and the corresponding decrease of the toxicity of the treated matrices. Results recorded by ecotoxicological tests can be associated to the presence of intermediates of degradation coming from the aromatic component of the UCM. Accordingly to the detoxification of the treated sediment the latter have been recovered in terms of microbial biodiversity and the level of humification.

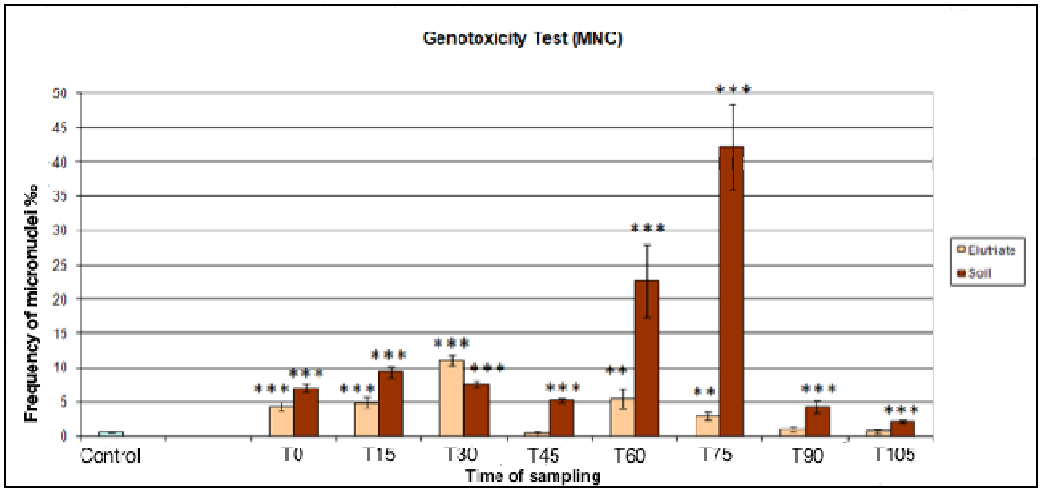

Fig. 1 Genotoxicity trend on *V. faba* evaluating the number of micronuclei/1000 cells (MNC test) from day zero (T0) to 105 days of treatment (T105). The significance of the data compared to the control is indicated by asterisks (p<= 1% **, p<1 ‰ = ***).

4. Conclusions

- The bioaugmentation processes using the autochthonous microfungi, isolated from the dredged sediment, represent an important tool to deplete TPH contamination and the UCM fraction, in a period of time compatible with a real scale pilot system;
- The only chemical analysis are not exhaustive to monitor the decontamination of the matrices that result to be characterised by residual toxicity;
- *Vicia faba* resulted to be exploitable for the monitoring of genotoxicity in both the solid matrices and in their elutriates.

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