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Transformation and synthesis of humic substances during bioremediation of petroleum hydrocarbons

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ABSTRACT

The aim of this paper was to investigate whether changes in the quantity and structure of humic acids (HA) occur during bioremediation of waste mazute (heavy residual fuel oil). The effects of humification on the structure of HA were evaluated by Fourier Transform Infrared Spectroscopy (FT-IR), potentiometric titrations, the ratio of absorbances at 465 and 665 nm (E4/E6 ratio) and the C/H ratio. Also, the quality of humification was monitored by determining parameters which are important for measuring the evolution of humic substances (HS), such as C_{HA}/C_{FA} (the humic acid/fulvic acidratio), the rate of extraction (TE) and the index of polymerization (IP). An increase of the content of HA from 0.23% to 0.70% was observed during the bioremediation process. The FT-IR spectra and C/H ratio of HA extracted at the beginning and the end of the process indicate structural changes during the bioremediation process. The groups containing aromatic and carboxylic carbon increased, resulting in HA structures of higher aromaticity. An increase of the E4/E6 ratio during bioremediation treatment implied the enrichment of HA with functional oxygen groups. Potentiometric titrations of HA solution showed increases in the buffering and the redox capacities of HA during the bioremediation process. The results of parameters of humification showed an increase of the compositional carbon in HA at the end, compared to at the start, of bioremediation, while the carbon that belonged to fulvic acids (FA) reduced by 44%. Transformation into HA is the likely cause of this observed reduction. The TE and the IP demonstrated that humification occured during the bioremediation process. According to the available data, this is the first report on synthesis of HS during bioremediation of petroleum hydrocarbons.

1. Introduction

Petroleum and its derivatives are key sources of energy and raw materials for production, and are used in vast quantities in all domains of life and work. Accidental spillage during exploitation, transport, processing, storage and use of petroleum and its derivatives leads to pollution of soils and waters (Yanto and Tachibana, 2013). The toxic effects of petroleum hydrocarbons are cumulative, while some are carcinogenic, mutagenic or teratogenic, and they therefore can endanger the health of future generations (Singh and Ward, 2004).

One of the technologies that has had remarkable world-wide success in the remediation of oil pollution is bioremediation. Bioremediation is a process in which polluted substances are transformed into non-toxic compounds or are completely degraded to carbon dioxide and water (Gomez and Sartaj, 2014). Specific microorganisms are the most commonly utilized biological agents used in bioremediation, because they have a natural capability to degrade and transform pollutants (bioremediation potential) due to the diversity of their metabolism and their ability to incorporate favourable genetic changes to enhance these processes (Korda et al., 1997; Aleksander, 1999; Alvarez and Illman, 2006; Das and

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Chandran, 2011). The main advantages of bioremediation processes are feasibilty, cost-effectiveness and their environmentally friendly properties (Gomez and Sartaj, 2014; Wu at al., 2016).

It is believed that during biodegaradation of polycyclic aromatic hydrocarbons (PAH), substances similar to humic compounds are created, which is very important, considering that humic substances (HS) are one of the key elements of soil quality (Henner et al., 1997; Ressler et al., 1999).

Fractionation of HS into humic acids (HA), fulvic acids (FA) and humin is based on their solubility in alkaline and acid dilute solutions. The FA compose the fraction soluble in both alkali and acid dilute solutions. The HA are soluble in alkali but not in acid, while the humin fraction is insoluble in both alkaline and acid conditions (Schnitzer, 1991; Stevenson, 1994; Fourti et al., 2010).

Some authors have proposed one indicator of humification based on monitoring the transformation of HA (Castaldi et al., 2005; Xiaoli et al., 2007), whereas others think that the quality of humification can be evaluated by determining C_{HA}/C_{FA} (the humic acid/fulvic acid ratio) (Sugahara and Inoko, 1981; Jiménez and Garcìa, 1992; Lopez et al., 2002; Domeizel et al., 2004), C_{HS}/C (TE), C_{FF}/C_{HS} (IP) (Sugahara and Inoko, 1981; Domeizel et al., 2004), where C_{HA}, C_{HS},C_{FF}, C_{FA} and C are, respectively, the dissolved organic carbon in the HA, in the HS, in the fulvic fraction (FF), in the FA and the total organic carbon in the solid material (Domeizel et al., 2004).

There is extensive information in the literature about humification processes in soil, composts, ground water and sediments (Steelink et al., 1985; Stevenson, 1994; Gaffney et al., 1996; Maccarthy, 2001; Pajaczkowska et al., 2003; Domeizel et al., 2004; Fourti et al., 2010). However, there is a shortage of information on humification processes taking place during bioremediation of petroleum hydrocarbons.

The aims of our study were to investigate the possibility of using a consortium of zymogenous microorganisms (Suja et al., 2014) for simultaneous bioremediation and humification of waste mazut, to determine quantitative and qualitative changes of HA, and to monitor humification parameters during an *ex situ* bioremediation process.

2. Materials and methods

2.1. Experimental design

This pilot bioremediation study was realized in open intermediate bulk containers (IBC) of 1 m^3 in quantities of 0.6 m^3 during 90 days. Control test (CT) was prepared in an IBC in which sawdust, sand and waste mazut (heavy residual fuel oil) were mixed. Softwood sawdust was added to increase the water holding capacity (WHC) and aeration, and as an alternative source of carbon. The CT consisted of 0.105 m^3 of waste mazut from an energy power plant mixed with 0.045 m^3 of softwood sawdust and 0.45 m^3 of ungraded river sand. The dimensions of the CT were 1 × 1 × 0.6 m (length x width x height). Biotic test (BT) was prepared in the same way as the control, but sources of nitrogen, phosphorus and potassium were added for biostimulation. An optimal ratio of C:N:P:K (approx 100:10:1:0.1) (Beškoski et al., 2011), was achieved by spraying a solution of dissolved ammonium nitrate, diammonium phosphate and potassium chloride over the BT. Spraying also achieved the required moisture level in the BT (80–100% WHC). During bioremediation, the BP was watered, turned and mixed every 30 d to maintain the required moisture and aeration levels. The zymogenous consortium of hydrocarbon-degrading microorganisms was prepared as previously described (Beškoski et al., 2011), and was also added to the BT so that it initially contained 3.5 × 10^7 CFU g^{-1} hydrogen-degrading microorganisms. Among isolated strains, the most abundant genera were *Pseudomonas*, *Nocardia, Achromobacter, Acinetobacter, Bacillus, Micrococcus, Rhodococcus, Penicillium, Aspergillus* and *Rhodotorula*. Numbers of microorganisms were determined as previously described (Milić at al., 2009; Gojgic-Cvijovic at al., 2012). The BT was re-inoculated every 30 d during the study. The initial number of naturally-occurring hydrocarbon-degrading microorganisms in CT was 1.2 × 10^4 CFU/g. IBC were placed in a closed hangar which was previously sterilized using UV lamps. The air temperature inside the hangar was, all the time, at a constant temperature of 25 ± 3 °C. At the beginning of the study prior to addition of sawdust, sand and waste mazut, as well as sources of nitrogen, phosphorus and potassium and zymogenous consortium, the IBC were thoroughly sterilized with ethanol and then washed with large amount of sterile water.

Aerial microbial contamination in the hangar during the study was monitored by determining numbers of chemo-organoheterotrophic bacteria (on nutrient agar) and yeasts and moulds (on malt agar). Levels of those microorganisms in the hangar air were determined weekly and never exceeded 17 CFU m^{-3}, so that the impact of these microorganisms was considered negligible. For active air sampling in the hangar, the Cherwell Laboratories' SAS super 100 microbial air sampler with sampling volume 100 L min^{-1} was used. The bioremediation study was conducted from April to June, and in this period the average temperature was 19.1 °C.

Composite samples for analyses were taken at the beginning and the end process from the BT and CT by "zig-zag" sampling with an Eijkelkamp auger soil sampler from 20 random places. The composite samples (approx. 2 kg) were sieved (1 mm grid), collected in stopped glass jars, and stored at 4 °C. Analyses were conducted within 12–24 h after sampling (Beškoski et al., 2011). The results presented are the average of five replicates prepared from each sample (at the beginning and the end process).

2.2. Extraction of HS

The extraction of HS and fractions was carried out using three protocols. All protocols were adapted from procedures described in the literature.

The HA and FA fractions of the HS were extracted following the flow diagram, Protocol 1 (Fig. 1). Additionally, total HS were extracted according to Protocol 2, while HA, necessary for elemental analysis, FT-IR spectra analysis, potentiometric titrations and E4/E6 ratio, were extracted and purificated according to Protocol 3.

2.2.1. Protocol 1 for the extraction of HS

Step 1. Air-dried sample (20 g) was treated with 100 ml of NaOH/$Na_4P_2O_7$ solution (15 g of crystalline sodium pyrophosphate ($Na_4P_2O_7$ × $10H_2O$) and 7 g of sodium hydroxide dissolved in 1000 ml of water), according to (ISO 5073, 1999) in the boiling water bath for 2 h with frequently shaking. The suspension was cooled to room temperature and supernatant S1 was separated from residue R1 by centrifugation at 3000 rpm (residue R1 was discarded). After centrifugation, the supernatant solution was filtered through a 45 μm filter.

Step 2. Supernatant S1 contained dissolved HA and FA. HA was precipitated from S1 by acidifying with 6 M HCl to pH 1. After centrifugation, residual solution (supernatant S2) contained FF (FA and non-humified fraction (NHF)). The HA constituting the residue R2 were redisolved in 0.1 M NaOH.

Step 3. Extract S2, obtained in step 2 and filtered through a 0.45 μm filter, was placed into a column containing 5 ml of DAX 8 (Supelite™ DAX-8 - polymethylmethacrylate resin) resin with a flow corresponding to 15 times the volume of the bed of the column