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# Biological indices of toxicity in tropical legumes grown in oil-contaminated soil

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ABSTRACT

This study evaluated the toxic effects of total petroleum hydrocarbons (TPH) on growth of the legumes *Crotalaria incana* L. and *Leucaena leucocephala* Lam., and on the development of nitrogen-fixing soil microorganisms, using biological toxicity indices and the toxicity potential index ($TPI_c$), which enable comparison of effects of exposure time and concentration. Growth and biomass accumulation in both plant species decreased with high pollutant concentrations. The $EC_{50}$ and the NOEC were not identified for either species. The Phytotoxicity Relative Index showed that root length was most strongly affected by the oil, and the Impact Index on Nitrogen Fixer Microorganisms indicated that, despite damage to the root system, *L. leucocephala* rhizosphere bacteria doubled at 10,000 mg $kg^{-1}$ TPH after of 240 days of exposure. Finally, the $TPI_c$ revealed that *C. incana* was more sensitive than *L. leucocephala* to chronic TPH toxicity and might strongly depend on beneficial soil bacteria.



## 1. Introduction

The oil industry has altered the natural resources of southeastern Mexico through oil spills and chronic discharge of drilling muds (García-Cuellar et al., 2004). The tropical legumes *Crotalaria incana* and *Leucaena leucocephala* can grow in oil-contaminated soil (Rivera-Cruz and Trujillo-Narcia, 2004; Vázquez-Luna, 2014) and have been reported to improve agricultural soil and to serve as a fodder tree with high protein content in non-contaminated soil (Daimon, 2006; Franzel et al., 2014).

Quantitative indices of soil quality have been developed to assess soil ecological health (Dawson et al., 2007). While these indicators are useful for ecological risk assessment, the tests can be costly because they assess soil enzyme activity (Wang et al., 2010), which requires detailed chemical analysis (Mao et al., 2009). In addition, the protocols (Guideline 208) of the Organization of Economic and Cooperative Development (OECD) only evaluate acute toxic effects on seedlings, and do not consider chronic effects (OECD, 1984).

Płaza et al. (2005) suggest that chemical data are insufficient to evaluate biological effects because of the large numbers of compounds and synergistic effects that contribute to toxicity; in contrast, effects-based assessments provide realistic approaches for determining soil quality. Soil quality can be assessed by analysing physical, chemical, and microbiological characteristics (Giacometti et al., 2013) and their ecotoxicological response on organisms such as worms and bacteria (Tang et al., 2011); however, these tests do not necessarily reveal the toxic potential of chronic oil pollution (Mikkonen et al., 2012) or the potential of beneficial microorganisms to ensure stable agricultural production (Singh et al., 2011). Nitrogen-fixing bacteria can facilitate aggregation and help to protect the soil ecosystem against exposure to biocides; thus, their survival in nodules of legumes and in rhizosphere soil under adverse environmental conditions should be examined (Gupta and Roper, 2010).

Based on these insights, biological activity is an important parameter to consider in evaluating soil quality (Schloter et al., 2003) and enables determination of risk factors associated with exposure of plants to contaminants; thus, it is a measure of the potential effects of contaminants on ecosystems (Fernández et al., 2005).

*Abbreviations:* $PRI_y$, phytotoxicity relative index for the following variables: height ($PRI_H$), root length ($PRI_{RL}$), above-ground biomass ($PRI_A$), root biomass ($PRI_R$), leaf biomass ($PRI_l$), stem biomass ($PRI_s$), and numbers of nodules ($PRI_n$); PI, phytotoxicity index; $MII_x$, microbial impact index for variable (fl, free living;r, *Rhizobium* spp.); $IIM_F$, impact index on nitrogen fixing microorganisms; BTI, biological toxicity index; $TP_x$, toxic potential to concentration ($x$) with respect to the control treatment; WTP, weighted toxic potential with respect to the time of exposure; $TPI_c$, toxicity potential index by a chronic effect; TPH, total petroleum hydrocarbons.

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In Mexico, current environmental regulations establish the maximum permissible limits of hydrocarbons in soil and specify characterization and remediation of hydrocarbon contamination (SEMARNAT-SS, 2003); however, these standards do not include criteria for assessing chronic effects of contamination on soil microorganisms and plants, or the use of bioindicators in assessments. Therefore, the aim of this study was to generate user-friendly indicators of soil contamination to measure the toxic effects of total petroleum hydrocarbons (TPH) on growth of the legumes *C. incana* L. and *L. leucocephala* Lam., and on the development of nitrogen-fixing soil microorganisms (rhizobial and free-living). The indices developed were the biological toxicity indices (BTI) and the toxicity potential index ($TPI_c$) in higher plants. Such studies can help tropical zones that lack well-defined biological indicators of soil quality to develop and implement standards.

## 2. Materials and methods

Uncontaminated soil (as defined by SEMARNAT-SS, 2003) was obtained from Santa Teresa Arroyo Hondo, Tabasco, Mexico. The following characteristics were analysed according to SEMARNAT (2000): organic matter content (Walkley and Black); pH (potentiometry); exchangeable phosphorus (P) and potassium (K) (extraction with 1 N ammonium acetate [pH 7] and quantification by atomic absorption and emission spectrometry); cation exchange capacity (CEC, extraction with 1 N ammonium acetate [pH 7] and quantification by distillation and titration); and texture (Bouyoucos). TPH content was determined using the United States Environmental Protection Agency (EPA) method 418.1 (EPA, 1986).The soil was a Eutric Gleysol (organic matter, 10.2%; pH, 6.3; P, 23.1 mg $kg^{-1}$; K, 3.35 Cmol(+) $kg^{-1}$; CEC, 45.2 Cmol(+) $kg^{-1}$; clay, 18%; silt, 21%; sand, 61%).

### 2.1. Bioassays

Bioassays were established under a completely randomised design with three replications for each plant species. These species were selected because *C. incana* has been recorded from areas with close to 80,000 mg $kg^{-1}$ weathered TPH (Vázquez-Luna, 2014), and *L. leucocephala* has been found in soils containing approximately 2800 mg $kg^{-1}$ weathered TPH (Rivera-Cruz and Trujillo-Narcia, 2004). OECD protocol 208 was used in previous studies of these legumes; both species showed a 5-d delay in seedling emergence when exposed to high concentrations (>20,000 mg $kg^{-1}$ TPH), in addition to reduced height and biomass (Vázquez-Luna et al., 2010). The pretest was modified according to Rivera-Cruz et al. (2005) as follows: established seedlings were grown for 30 d in uncontaminated soil, and plants of the same vigour and height (10 cm for *C. incana* and 7.5 cm for *L. leucocephala*) were selected. The roots were carefully washed, and bare-root plants were transplanted into containers with 1800 g oil-contaminated soil. All plants survived, and plants were thinned to prevent competition. This work followed the recommendations of ISO standard 22,030:2005 for evaluating chronic toxicity in higher plants, which provided a basis for using just two plant species (Tarazona et al., 2013).

Treatments were prepared 72 h before the tests using non-contaminated soil (1800 g dry weight) mixed with different quantities of light crude oil (American Petroleum Institute [API] gravity = 33.6°; sulphur content = 1.3%) (PEMEX, 2004). Water was then added and the mixtures were homogenised. All concentrations were calculated on a dry-weight basis (w/w). *L. leucocephala* was tested with concentrations of 150 (control), 10,000, 20,000, 40,000, 60,000, and 80,000 mg $kg^{-1}$ TPH for 150 d exposure. *C. incana* was evaluated with concentrations of 150 (control), 1700, 3500, 7000, 12,000, 25,000, and 32,000 mg $kg^{-1}$ TPH for 240 d exposure. Distilled water was added throughout the experiment to maintain the soils at field capacity (28–30% moisture); to prevent interference with growth, no nutrients were provided.

### 2.2. Variables

Plant growth (height) was measured every 15 d. Total exposure time was differentiated for statistical purposes according to (1) the life cycle of the species under the experimental conditions, and (2) the time at which one of the treatments reached the median lethal concentration ($LC_{50}$). Thus, *C. incana* was evaluated for 150 d, whereas the $LC_{50}$ for *L. leucocephala* occurred at day 240. At the end of evaluation, the plants were extracted from the containers by removing excess soil and separating aerial parts (leaves and stems) from roots, and the numbers of nodules were counted for each experimental unit. The nodules were washed with sterile distilled water and preserved at 4 °C until microbiological testing. Above-ground and root biomass were measured after oven drying at 75 °C for 48 h.

Microbiological variables were determined in rhizosphere soil and plant nodules using combined-carbon medium for free-living nitrogen-fixing bacteria (Rennie, 1981); yeast mannitol agar medium was used for *Rhizobium* spp. extracted from nodules (CIAT, 1988). Bacterial populations were estimated using a serial dilution viable-count method (Madigan et al., 2003). Serial 10-fold dilutions were prepared with 10 g of rhizosphere soil in 90 ml of sterile water to $1/10^5$ for free-living nitrogen-fixing bacteria, and with 1 g rhizosphere soil in 99 ml of sterile water for nodule rhizobia.

### 2.3. Indices of toxicity

The phytotoxicity relative index ($PRI_y$) was calculated for variable height ($PRI_H$), root length ($PRI_{RL}$), above-ground biomass ($PRI_A$), root biomass ($PRI_R$), leaf biomass ($PRI_l$), stem biomass ($PRI_s$), seeds biomass ($PRI_{se}$) and nodules ($PRI_n$). The $PRI_y$ of each variable was compared with that of the corresponding control (Eq. (1)). The phytotoxicity index (PI) represents the response of the whole plant to the pollutant and was obtained from the sum of all $PRI_y$ values divided by the total number of studied variables (Eq. (2)) (Vázquez-Luna et al., 2010). The microbial impact index (MII) was determined using the formulas reported by Vázquez-Luna et al. (2011) (Eqs. (3) and (4)). The biological toxicity index (BTI) was measured as the sum of PI and $IIM_F$ (Impact Index on nitrogen-fixing microorganisms) (Eq. (5)).

$$PRI_y = 1 - \left[\frac{ot}{\overline{X}ct}\right]_i \quad (1)$$

$$PI = \frac{\sum_{i=1}^{n} PRI_y}{n} \quad (2)$$

$$MII_y = 1 - \left[\frac{ot}{\overline{X}ct}\right]_i \quad (3)$$

$$IIM_F = \frac{\sum_{i=1}^{n} MII_x}{N} \quad (4)$$

$$BTI = \sum_{i=1}^{n} [PI + IIM_F]_i \qquad i = 1, 2, \ldots, r \quad (5)$$

where *ot* symbolizes petroleum treatment in relation to variable (*y*); *i* represents the *i*th variable; $\overline{X}ct$ denotes the average value of each variable for the control treatment; *n* = the number of variables measured in plants; *N* = the number of nitrogen-fixing bacterial species examined; and *r* represents treatment replicate number.

Toxic potential ($TP_x$) was determined by dividing the evaluated oil concentration by the concentration of the control treatment (Eq. (6)). The weighted toxicity potential (WTP) explains the $TP_x$ of each