Sains Malaysiana 44(3)(2015): 337–346

Vibrio fischeri Bioassay for Determination of Toxicity in Petroleum Contaminated Soils from Tropical Southeast Mexico

(Bioasai *Vibrio fischeri* untuk Menentukan Ketoksikan dalam Tanah Terlumus Petroleum dari Tenggara Tropika Mexico)

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ABSTRACT

*Using the Microtox bioassay toxicity was determined in 16 areas located in eight sites: In contaminated areas, in areas with natural recovery, after bioremediation and phytoremediation projects and in control areas (uncontaminated). These areas correspond to the following ecosystems: mangrove, fresh water swamp (*Pachira aquatica*), marsh and pasture (in the coastal plain and Pliocene-Pleistocene terraces). A significant toxicity was detected in uncontaminated organic soils (22.2-49.1 toxicity units (TU)) which were comparable to levels found in hydrocarbon contaminated areas (22.3-42.0 TU). Generally, the toxicity in organic soils was much higher than that found in mineral soils (which was from below quantification levels to 9.3 TU). In an area restored by phytoremediation, the simple method used by Petróleos Mexicanos achieved recovery and superficial detoxification in the treated plot, exhibiting a toxicity reduction of 2.4 times with respect to untreated soil and a toxicity even slightly lower than the uncontaminated control in the same ecosystem (22.2 TU). Likewise, a bioremediation project in pasture resulted in a toxicity reduction down to levels comparable to uncontaminated soil (from below quantification levels to 7.9 TU). A tendency to decrease toxicity in organic soils during the rainy season was observed, toxicity drop in 80% of the areas sampled. Based on these findings, recommendations are presented for use of this test method in regional diagnostic studies.*

Keywords: Bioremediation; contamination; ecotoxicology; oil

ABSTRAK

*Penggunaan ketoksikan bioasai Microtox telah ditentukan di 16 kawasan yang terletak di lapan tapak: Dalam kawasan terlumus, di kawasan dengan pemulihan semula jadi, selepas projek bio pemulihan dan fito pemulihan serta kawasan kawalan (tidak terlumus). Kawasan-kawasan ini sesuai dengan ekosistem berikut: Paya bakau, paya air tawar (*Pachira aquatica*), marsh dan Pastura (di dataran pantai dan Teres Pliocene-Pleistocene). Ketoksikan yang ketara telah dikesan di tanah organik tidak terlumus (22.2-49.1 unit ketoksikan (TU)) yang dibanding dengan tahap yang dijumpai di kawasan terlumus hidrokarbon (22.3 - 42.0 TU). Secara amnya, ketoksikan dalam tanah organik adalah lebih tinggi daripada yang diperoleh dalam tanah mineral (daripada tahap pengkuantitian yang rendah kepada 9.3 TU). Dalam kawasan yang telah dipulihkan melalui fito pemulihan, kaedah yang mudah digunakan oleh Petróleos Mexicanos telah mencapai pemulihan dan detoksifikasi superfisial dalam plot yang dirawat, mempamerkan pengurangan ketoksikan 2.4 kali berbanding dengan tanah yang tidak dirawat dan ketoksikan yang sedikit lebih rendah daripada kawalan tidak terlumus dalam ekosistem yang sama (22.2 TU). Begitu juga, projek bio pemulihan di padang ragut mengakibatkan pengurangan ketoksikan ke tahap yang setanding dengan tanah tidak terlumus (daripada tahap ketersediaan kepada 7.9 TU). Kecenderungan untuk pengurangan ketoksikan dalam tanah organik semasa musim hujan juga diperhatikan, dengan ketoksikan menurun 80% dalam kawasan yang disampel. Berdasarkan penemuan ini, cadangan dikemukakan bagi penggunaan kaedah ujian ini dalam kajian diagnostik serantau.*

Kata kunci: Bio pemulihan; ekotoksikologi; minyak; pelumusan

INTRODUCTION

In southeastern Mexico, especially in southern Veracruz State and western Tabasco State, one of the areas most impacted from hydrocarbon contamination in the country can be found (Adams et al. 2011, 1999a, 1999; Beltrán 1993; Palma-López & Obrader 1999; Zavala 1996; Zavala & Adams Schroeder 2006). This area has many petroleum installations that are very old, typically 40 years or more and some dating back to the beginning of de 20th century (for example the Lázaro Cárdenas refinery in Minatitlán, Veracruz which began construction ca. 1902, Petróleos 1988). During the major part of the development of the oil fields and related installations for the transport and processing of petroleum, there was not a national environmental consciousness and practices aimed at avoiding and controlling spills and discharges in place. Consequently, today there are hundreds of hectares contaminated by hydrocarbons in the region, some of the

most important being the Sánchez Magallanes oil field (Benito Juárez, Cárdenas, Tabasco), the marshes and mangroves behind the La Venta Gas Processing Plant (La Venta, Huimanguillo, Tabasco) and the Santa Alejandrina marshlands next to the refinery in Minatitlan (Veracruz).

In the last 30 years, a preoccupation for the environment has started to develop at the national level, leading to policies and programs aimed at preventing or reducing the contamination produced by the petroleum industry. One important component of this process has been the diagnostic study of the affected areas and the remediation of some of the most contaminated sites. With respect to these activities, the Ministry of Environment and Natural Resources (in Spanish, *Secretaría de Medio Ambiente y Recursos Naturales, SEMARNAT*) published the first norm on hydrocarbon contaminated soils in 2002 (NOM-EM-138-ECOL-2002, SEMARNAT 2002), which indicates criteria for the remediation of contaminated sites and maximum permissible levels of hydrocarbons in soil. Later in 2005, the Ministry published an updated norm (NOM-138-SEMARNAT/SS-2003, SEMARNAT 2005). The cleanup criteria in these norms are based almost exclusively on hydrocarbon concentration, but do include permissible levels based on hydrocarbon fraction, this owing to the fact that the low molecular weight hydrocarbons are more toxic (Edwards et al. 1995; Overton et al. 1997). However, toxicity may also vary according to availability in the soil or sediment matrix (Piskonen 2002) and this aspect is not covered in the official norm.

In addition to these norms, a General Law on Ecological Equilibrium and Environmental Protection (*Ley General de Equilibrio Ecológico y Protección al Ambiente*) was published by the Ministry of Environment, Natural Resources and Fishing (*Secretaría de Medio Ambiente, Recursos Naturales y Pesca*, SEMARNAP, 1997). In articles 134 and 152 BIS, it was indicated that contaminated sites must be restored to a level that allows them to be used according to their natural vocation or according to an urban development plan. In article 136, general criteria were also presented for the prevention and control of contamination of soil and subsoil. Among these, fraction II indicates that it was necessary to prevent or avoid contamination of the soil that may result in the noxious alteration of biological processes.

One of the techniques that can be used to determine if the soil meets this criterion is the use of bioassays. The advantage of using assays is that the true toxicity in a sample is measured directly and is not based on a supposition according to the toxicity of some component in the petroleum mixture, or its concentration. This is important, because the toxicity is influenced by the bioavailability of toxic compounds: if they are present but not available in the soil or sediment matrix, there may not be toxicity and a simple chemical determination cannot provide this kind of information.

One of the bioassays considered among the Mexican norms is the Microtox test, which uses *Vibrio fischeri* as a test organism (Mexican norm NMX-AA-112-1995-SCFI, SECOFI 1995). Unfortunately, there are relatively few data on the toxicity of impacted areas and reference areas (uncontaminated) in this region, or even on a national level. For this reason, the toxicity was determined in soils typical of this most contaminated region, as well as in nearby uncontaminated controls of the same soil and ecosystem type.

Materials and Methods

Study Area

The Cinco Presidentes Production Unit (*Activo de Producción Petrolera Cinco Presidentes*) was selected for this study due to its long history of petroleum contamination and the diversity of soil, geoforms and ecosystems including sandy costal soils, various kinds of marshes and swamps, floodplains, as well as hilly areas used for pasture and livestock raising. Furthermore, it is a representative of many impacted areas in tropical southeastern Mexico, and has a very high level of oil pollution as well as potential for natural recovery (Adams et al. 2011, 1999; Zavala 1996, 1993; Zavala & Adams Schroeder 2006).

Samples were taken in various areas with particular characteristics of interest, among them a mangrove swamp, taziste swamp, popay-sedge marsh, cattail-popay-sedge marsh, sandy soil with pasture and Pliocene-Pleistocene terraces with pasture. The samples were taken from uncontaminated reference areas, areas with oil spill conditions and in areas with some natural recovery of after remediation projects (Table 1). A more detailed description of the site-specific characteristics is given below:

La Venta Gas Processing Complex Situated in the extreme western part of Tabasco State, this complex has been a source of chronic contamination for decades due to discharge of wastewater without proper treatment from a crude petroleum dehydration plant. At one point, the contamination was so extensive that approximately 11 ha had an oily crust with concentrations of up to 78% of total petroleum hydrocarbons (Adams et al. 2011, 1999). Later, civil engineering projects contracted by the state run petroleum company, Petróleos Mexicanos (PEMEX), allowed salty water from the Tonalá River to enter the area, which was previously a freshwater marsh with predominantly cattail (*Typha* sp.) vegetation. Today there are still heavily impacted areas, but much of the area has been partially recovered by a young mangrove forests (white mangrove, *Laguncularia racemosa*). The uncontaminated control for this site was in the nearby Cinco Presidentes Oil Field, but from an area with secondary vegetation, predominantly white mangrove, red mangrove (*Rhyzosphora mangle*) and a diverse group of herbaceous and brushy vegetation including mucal (*Dalbergia* sp., Magaña 1995). The soils in both sites have organic surface horizons (epipedons) (Zavala-Cruz

& García-López 2012) composed of vegetable tissues from the same ecosystem, in various degrees of decomposition.

Rodador Oil Field Samples were collected in this oil field in an area corresponding to the geoform of recent coastal plain, in sandy soil, typically used for pasture and/or coconut cultivation (Palma-López at al. 2007; West et al. 1985; Zavala-Cruz & García-López 2012). The control site was a nearby uncontaminated pasture. In addition, soil from an adjacent separation battery that had been contaminated and latter bioremediated was also collected. In this area, the bioremediation project had just been completed, according the property owner.

San Ramón Oil Field This is situated in a semi-saline marsh parallel to the coast between two coastal lagoons, the El Carmen Lagoon and the El Yucateco Lagoon. This ecosystem is dominated by a low growing, spiny palm, the Taziste (*Acoelorrhaphe wrightii*), with an understory of serrated herbaceous marshy vegetation, the navajuela (*Cladium jamaicense* Crantz) (Garcia et al. 2012). The samples of organic soil were collected from an uncontaminated reference site and at a contaminated site near a separation battery. Furthermore, samples were collected from a site that had been contaminated by a petroleum spill and then recovered by phytoremediation, approximately eight years prior to our sampling.

During this period, the social evolution of environmental consciousness in Mexico (and many other tropical countries) was only begging to develop, and the precise processes that resulted in the restoration of contaminated sites was not well understood, even by industry personnel. At this site engineers from the national petroleum company (Petróleos Mexicanos or PEMEX) had observed that in some chronically contaminated areas, sometimes the application of organic peat helped vegetative recovery. For the restoration of this site, they collected the organic peaty layer of soil from a nearby marsh. During routine access road maintenance operations, they cleared out marshy vegetation and organic soil clogging the drainage pipes that let water pass under the road, from one side of the marsh to the other. Most of this material came from the top 50 cm of marsh. It should be noted that although this peat was not from a pristine area, it was not obviously contaminated.

The site recovery method consisted of applying a 20 cm thick blanket of this organic peaty soil on top of the oil stained soil. Later a mixture of cattails and other marshy vegetation, (possibly ferns and some grasses) were collected from nearby marshy areas and were planted on top of the marshy organic peat (personal communication, Juan Perez de la Cruz, previously of the Industrial Safety and Environmental Protection department of the PEMEX subsidiary, PEMEX Exploración y Producción (Exploration and Production, in Agua Dulce, Veracruz). It appeared that the principal species used was cattail (*Typha latifolia*). This was also collected from drainage ditch clearing. The top leaves and the lowest part of the root were cut off – leaving only a bulb with a root crown and cut-off leaves. These were planted into the peaty soil.

At the time of sampling, the area had a complete vegetative cover consisting of the planted species as well as other secondary herbaceous species and a few individuals of the bush *Myrica serifera*. Upon taking samples, the presence of a definite surface horizon of new organic material from the recovery was clearly observable, essentially not contaminated and lying over a deeper horizon that was stained with oil. These two horizons were separated and analysed separately for toxicity.

Ogarrio Oil Field This area is a mosaic of different marshy vegetation types consisting in part of swamp predominated by the Apompo tree (*Pachira aquatica*) and on the other hand, a marsh composed of giant reed (*Cyperus gigantus*) and popay (*Thalia geniculata*). It suffers from chronic problems of leaks and spills due to corroded pipelines, and in the contaminated samples, a large spill had occurred approximately 1-1½ years previously, reaching concentrations of up to 20-40% of total petroleum hydrocarbons (TPH). During the second sampling period, a small leak in the pipeline had also occurred in the control area also (area no. 11) as well. The soil is composed of organic material in the surface horizon (epipedon) (Zavala-Cruz & García-López 2012), in the Apompo swamp as well as in the marshland.

Sánchez Magallanes Oil Field This site is located in a marshy area with organic superficial soil and a floristic composition of secondary vegetation predominated by popay, giant reed and cattails (Zavala 1996, 1993; Zavala-Cruz & García-López 2012). This area also suffers from serious problems of pipeline corrosion which results in a chronic problem of leaks and spills. In the contaminated site, a series of spills had occurred resulting in a stained area of roughly 10-15 ha, with TPH concentrations of 45-72% in the most impacted area (Adams et al. 1999). The reference site was located in the periphery of the oil field in an uncontaminated area.

Agua Dulce Hills These samples were taken from a hilly area just to the south of the town of the same name, in Agua Dulce, Vercruz, near the border with Tabasco State. This area is composed of Pliocene-Pleistocene terraces (SGM 2004; West et al. 1985) with weathered surface soil (red earth soil, a Cambiasol or Luvisol in the World Reference Base-FAO classification system, Palma-López et al. 2007). The samples were taken in an uncontaminated pasture for future reference.

Bacal Oil Field This area is located in the extreme south of the Petroleum Production Unit in very weathered terraces from the Pliocene (SGM 2005). The soil is similar to that from the Agua Dulce Hills but even more weathered and correspond to an Acrisol in the World Reference Base-

TABLE 1. Location and Characteristic of Sampling Sites

Area	Soil Type (WRB-FAO)*	Ecosystem	Location and Coordinates	Observations
1	Histosol	Mangrove/ Secondary Vegetation	Cinco Presidentes Oil Field 18º 12.319 'N, 94º 0.358 'W	Uncontaminated mangrove (reference area)
2	Solonchak/ Histosol	Mangrove	La Venta Gas Processing Complex 18º 5.434 'N, 94º 3.196 'W	Old oil spill/discharge, weathered oil
3	Solonchak/ Histosol	Mangrove	La Venta Gas Processing Complex 18º 5.439 'N, 94º 3.160 'W	Natural attenuation from old oil spill/discharge
4	Arenosol	Pasture (coastal)	Rodador Oil Field 18º 13.639 'N, 93º 59.068 'W	Uncontaminated pasture (reference area)
5	Arenosol	Pasture (coastal)	Rodador Oil Field 18º 13.640 'N, 93º 59.011 'W	Recently treated by bioremediation, near separation battery
6	Histosol	Taziste/Navajuela swamp	San Ramon Oil Field 18º 13.958 'N, 93º 55.004'W	Uncontaminated swamp (reference area)
7	Histosol	Taziste/Navajuela swamp	San Ramón Oil Field 18º 14.102 'N, 93º 54.808'W	Chronic leaks in swamp
8	Histosol	Taziste/Navajuela swamp & Secondary Vegetation	San Ramón Oil Field 18º 14.129 'N, 93º 54.679'W	Area of phytoremediation surface horizon
9	Histosol	Taziste/Navajuela swamp & Secondary Vegetation	San Ramón Oil Field 18º 14.129 'N, 93º 54.679'W	Area of phytoremediation underlying contaminated horizon
10	Histosol/ Gleysol	Apompo swamp	Ogarrio Oil Field 18º 1.479 'N, 93º 56.990'W	Uncontaminated swamp (reference area)
11	Histosol	Popay/giant reed marsh	Ogarrio Oil Field 18º 1.509 'N, 93º 56.963'W	Uncontaminated marsh (reference area)
12	Histosol	Popay/giant reed marsh	Ogarrio Oil Field 18º 1.465 'N, 93º 56.942'W	Recently contaminated marsh
13	Histosol	Popay/giant reed/ cattail marsh	Sánchez Magallanes Oil Field 18º 9.538 'N, 93º 52.279'W	Uncontaminated marsh (reference area)
14	Histosol/ Gleysol	Popay/giant reed/ cattail marsh	Sánchez Magallanes Oil Field 18º 8.882 'N, 93º 52.982'W	Chronic leaks in marsh
15	Cambiasol/ Luvisol	Pasture (Pliocene-Pleistocene terrace)	Agua Dulce Hills 18º 3.449 'N, 94º 8.382'W	Uncontaminated pasture (reference area)
16	Acrisol	Pasture (Pliocene terrace)	Bacal Oil Field 17º 47.968 'N, 93º 56.864'W	Uncontaminated pasture (reference area)

* Zavala-Cruz & García-López 2012. Soil classification is according to the Food and Agriculture Organization of the United Nations (FAO) as published in the World Reference Base for Soil Resources (WRB) 2006

FAO classification system (Palma-López et al. 2007). During our site visit, no contaminated area was found and samples were taken in an uncontaminated pasture for future reference.

SAMPLING METHOD

In each site, three samples were taken using a post-hole digger to a depth of approximately 25 cm. These were placed in black polyethylene bags and transported to the laboratory at ambient temperature. In the laboratory, the samples were conserved in a freezer until analysis. Prior to analysis, the samples were thawed, completely mixed and the large roots, stones and sticks were removed.

BIOASSAY TESTS METHOD

Of the bioassays in official Mexican norms, one with great potential is that using *Vibrio fischeri* (previously classified as *Photobacterium phosphoreum*), which was used in this study. The samples were analysed according to Mexican norm NMX-AA-112-1995-SCFI (SECOFI 1995) using the Microtox analyser and reagents, modifying the method to consider the recommendations of the manufacturer (Mayo-López et al. 2010; SDI 2005). The test uses naturally bioluminescent marine bacteria, which in healthy conditions produce small quantities of light (Isenberg 1993). Under toxic conditions, the bioluminescence is reduced and can be measured by a luminometer (similar

to a spectrophotometer but the bacteria, rather than a lamp, produce the light being measured).

Dilutions (1:10) of the soil samples were prepared in de-ionized water in 100 mL graduated cylinders and let to settle. Subsequently, the clear, non-turbid supernatant was decanted. This was used to prepare a dilution series to which the bacterial suspension was added as indicated by the manufacturer. After 10 min, the bioluminescence in the dilutions was measured with the Microtox analyzer. Using the software provided by the manufacturer, dose-response functions were graphed to determine the amount of sample in the dilution corresponding to a reduction in bioluminescence of 50% with respect to a blank, the effective concentration 50 (EC50). A 10 g sub-sample of the original soil that was used to prepare the dilution was dried in an oven at 60ºC for 18-24 h, to determine the humidity percentage and this value was then used to calculate the EC50 on a dry weight basis.

Like other toxicity parameters, such as LD50 (lethal dose 50), the lower the value, the more toxic it will be. This may be confusing for interpretation by non-specialist. To convert this into a positive indicator, where a higher value indicates greater toxicity, the EC50 values were converted to toxicity units (TU) as per Mexican norm NMX-AA-112-1995-SCFI. This was done using the following equation: TU=1/EC50, where the EC50 is expressed as a proportion (for example if EC50=100000 mg/kg, TU=1/(0.1)=10 TU). This allows a more positive value to indicate higher toxicity.

Results and Discussion

The toxicity results from the different ecosystems are presented in Table 2. The EC50 data had a natural distribution into four groups: Averages of 13400-27900 mg/kg with standard deviations of 1400-9400 mg/kg (sites 1, 2, 9, 12 and 13); averages of 44800-56100 mg/kg with standard deviations of 31500-47800 mg/kg (sites 3, 6, 7 and 8); averages of 80200-132100 mg/kg with standard deviations of 24200-161800 (sites 5, 10, 14 and 16); and sites with basically no tendency to toxicity or levels so low that it could not be accurately calculated (sites 4, 11 and 15).

In general, there are more dispersion in the EC50 data from areas with low toxicity (high EC50 values). According to Isenberg (1993), the bioluminescence data were used to calculate the alfa (α) value according to the formula $\alpha = (B_o/B_i) - 1$, where B_o is the bioluminescence of the blank without sample and B_i is the bioluminescence of a known dilution (concentration) of the sample. When the log(α) vs. log(conc.) function is graphed, a straight line is produced in normal dose-response functions. The EC50 value equals the concentration of sample (in the dilution in the bioassay) which gives a B_i value 50% that of the blank (B_o). In our data evaluation, only those log(α) vs. log(conc.) functions with R>0.90 were accepted for calculating the EC50. In some samples, a tendency was found, but with R<0.90. This was observed to occur especially in those samples with (theoretically) calculated EC50 values >100000 mg/kg, (high EC50 values, corresponding to low toxicity samples). It was considered that this was the approximate limit for this test for reliable measurements of toxicity, and that those samples with very low toxicity (with EC50 >100000 mg/kg), the toxicity could not usually be measured accurately with this bioassay. For this reason, those samples with correlations of R<0.90, or in which no tendency for toxicity was observed, were assigned a numerical value for EC50 of 100000 mg/kg, solely for comparison purposes.

The corresponding values calculated as toxicity units, are presented in Figure 1. In this figure, the data are divided according to site or ecosystem type. In the samples from mangroves (sites 1, 2 and 3), the measured toxicity in the contaminated site is less than in the reference site. This is counter-intuitive, but there is another example of this phenomenon in the cattail/popay/giant reed marsh (sites 13 and 14) in where the reference (uncontaminated) area is much more toxic than the contaminated area. At first, this was difficult for the authors of this study to accept and a measurement error was suspected. For this reason, we returned to the marshy sites to collect more data. Instead of only analyzing the data with n=3 for each site, we finally analyzed a total of eight samples from the reference area and 15 from the contaminated site (n= 8 and 15, respectively). This additional data confirmed this phenomenon. Uncontaminated and contaminated mangrove sites (1 and 2) were significantly different at a 95% confidence interval (p<0.05) and the uncontaminated and contaminated marsh (sites 13 and 14) were significantly different at a 90% confidence interval (p<0.10).

It appears that there is some natural substance in some marshy soils (from mangrove in the first case and the cattail/popay/giant reed marsh in the second) that imparts a high toxicity (around 74-75 TU) to the samples and which is more toxic to the test organism than the petroleum in the soil. In other marshy areas with petroleum spills, for example in the Ogarrio Oil Field (sites 10, 11 and 12; Apompo swamp and popay/giant reed marsh ecosystems) the contaminated samples were indeed more toxic than the uncontaminated reference samples. The Apompo swamp was significantly less toxic than the nearby contaminated marsh at a confidence interval of 90% (p<0.10). However, in the Taziste swamp (sites 6 & 7) the toxicity is similar in the contaminated site and the uncontaminated reference site (no significant difference at 95% confidence interval). In studies carried out in other marshy areas, for example in southern Louisiana (USA), researchers have found that the toxicity does not depend on the total petroleum hydrocarbon concentration, but rather on the concentration of the most toxic and lightest (low molecular weight) hydrocarbons (Overton et al. 1997). These observations are congruent with the data; the spills in the mangrove, Taziste swamp and cattail/popay/giant reed marsh ecosystems are from relatively old spills (approx. 10-40 years old) and many of the lighter, more toxic hydrocarbons in the petroleum have had the opportunity to be volatilized or be biodegraded in this tropical monsoon climate. Conversely, in the site were we observed a more recent spill (less than 1 year, in the

TABLE 2. Soil Toxicity in Different Ecosystems in Southeast Mexico

Area	Characteristics	Effective Concentration 50 (in mg/kg)				Toxicity Units (TU)		
		n	$\bar{x}$	range	σ	$\bar{x}$	range	σ
1	Mangrove/ secondary vegetation (uncontaminated reference)	3	13,500	10,100 – 17,800	3,900	74.1	56.2 - 99.0	21.4
2	Mangrove with spill	3	23,800	19,400 – 26,200	3,700	42.0	38.2 – 51.5	6.5
3	Mangrove in recovery	3	49,200*	19,200 – BQL*	44,200*	20.3	10 -52.1	18.2
4	Pasture (coastal) (uncontaminated reference)	3	100,000*	all BQL or NT*	NA	10	NA	NA
5	Pasture (coastal), bioremediation	3	127,000*	115,000 – BQL*	34,700*	7.9	8.7 - 10	2.1
6	Taziste swamp (uncontaminated reference)	3	45,100*	14,300 – NT*	47,700*	22.2	10 – 69.9	23.5
7	Taziste swamp with spill	3	44,800*	17,200 – NT*	47,800*	22.3	10 – 58.1	23.8
8	Taziste/secondary vegetation, phytoremediation (superficial horizon)	3	56,100	19,800 – 76,300	31,500	17.8	13.1 – 50.5	10.0
9	Taziste/secondary vegetation, phytoremediation (underlying contaminated horizon)	3	23,000	21,800 – 24,600	1,400	43.5	40.7 – 45.9	5.1
10	Apompo swamp (uncontaminated reference)	3	80,200*	40,700 – BNC*	34,200	12.5	10 – 24.6	5.3
11	Popay/giant reed (uncontaminated reference)	3	100,000*	all NT*	NA	10	NA	NA
12	Popay/giant reed with spill	3	27,900	17,100 – 33,800	9,400	35.8	29.6 – 58.5	12.1
13	Cattail/popay/ giant reed (uncontaminated reference)	8	13,400	7,100 – 29,900	8,000	74.6	33.4 –140.8	44.5
14	Cattail/popay/giant reed with spill	15	132,100	3,800 – 530,900	161,800	7.6	1.9 – 263.2	9.3
15	Pasture (Pliocene-Pleistocene terrace) (uncontaminated reference)	3	100,000*	all BQL*	NA	10	NA	NA
16	Pasture (Pliocene terrace) (uncontaminated reference)	3	107,200*	87,400 – BQL*	24,200*	9.3	10 – 11.4	2.1

*non-toxic samples (NT) or samples with toxicity below a quantifiable limit (BQL) were assigned EC50 values of 100,000 mg/kg to calculate averages, range and standard deviation; see text for details.
NA = not applicable

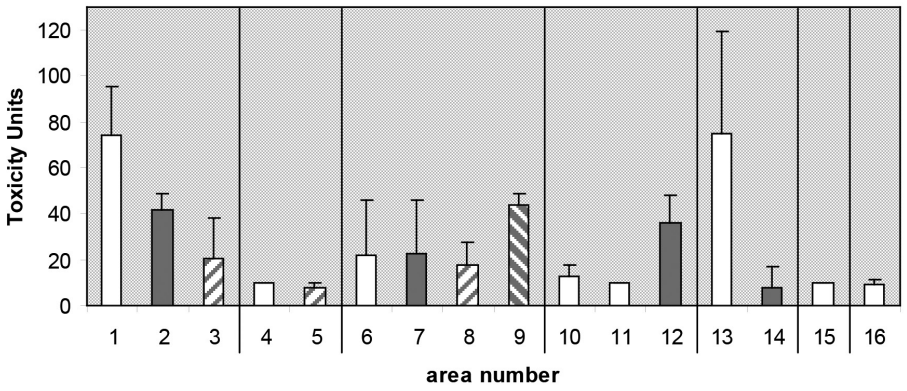

FIGURE 1. Toxicity in soil and sediment at contaminated, remediated and reference areas

Values are averages of three repetitions, with the exception of site 13 (n = 8) and site 14 (n = 15). Some samples did not show toxicity or was too low to quantify with certainty (TU ≤ 10). With these samples, a value of 10 TU was assigned for calculation of averages and standard deviation (error bars). White bars represent uncontaminated reference sites; grey bars represent contaminated sites; hashed bars are used for sites with natural attenuation, bioremediation or phytoremediation. The areas are grouped according to sites or ecosystem:

1 – Mangrove/secondary vegetation (reference)
2 – Mangrove with spill
3 – Mangrove with natural attenuation
4 – Pasture, coastal (reference)
5 – Pasture, coastal with spill
6 – Tazistal swamp (reference)
7 – Tazistal swamp with spill
8 – Tazistal swamp, phytoremediation (superficial)
9 – Tazistal swamp, phytoremediation (deep layer)
10 – Apompo swamp (reference)
11 – Popay/giant reed marsh (reference)
12 – Popay/giant reed marsh with spill
13 – Cattail/popay/giant reed marsh (reference)
14 – Cattail/popay/giant reed marsh with spill
15 – Pasture -hilly area, coastal (reference)
16 – Pasture -hilly area, inland (reference)

Ogarrio Oil Field, sites 10, 11 and 12) the contaminated samples were more toxic than the uncontaminated samples and significantly so (p<0.10). This is probably because some of the more toxic components have not yet had enough time to volatilize and biodegrade.

With respect to natural attenuation, bioremediation and phytoremediation, all of these methods resulted in a reduction in toxicity to below background levels. In the mangrove samples (sites 1, 2 and 3), the toxicity was reduced by roughly one half and was not significantly different from the uncontaminated control (p<0.05). Other authors have also found that natural attenuation and weathering to be effective, as long term means of reducing toxicity (Overton et al. 1997; Steliga et al. 2012) and have used this bioassay as an indicator. For the sandy coastal soil with pasture (sites 4 and 5), the bioremediation also resulted in a toxicity in the same range as the uncontaminated reference site, which is also congruent with the findings of Adams et al. (1996) and Hamzah et al. (2010).

The simple technique used for phytoremediation (or phytorestoration) by petroleum engineers also appeared to have worked well in this site. Toxicity was reduced almost 2½ times to below background levels. Although the underlying layer of soil was still oil stained and had toxicity, almost double that of the uncontaminated reference area, this contamination appeared to be sequestered effectively by the cap of organic soil placed on top. In the field, the difference in the two soil horizons was clearly evident, as well as the fact that the petroleum in the underlying layer seemed trapped in place. The surface layer did not have evidences of petroleum contamination such as stains, odour or sticky consistency and a complete vegetative cover had been established. Furthermore, there was no significant difference in toxicity between the surface layer at the phytoremediated (phytorestored) site and the uncontaminated reference site at a confidence interval of 95%. In this case, the phytoremediation technique used did not (apparently), reduce the hydrocarbon concentration, but did effectively limit the dispersion of toxic hydrocarbons into the surface layer and allow for the establishment of a new vegetative cover.

Finally, in the upland areas in the Agua Dulce hills and the Bacal Oil Field, basically no natural toxicity was detected with this bioassay. This was also the case with the other mineral soil from the sandy coastal area. This is important because it establishes background levels for the soils in this region using this bioassay. Notably, mineral based soils did not present background toxicity, whereas many of the organic based soils did, ranging from 12.5-74.6 TU. Among these the samples from the Apompo swamp and nearby marsh had very low toxicity values (10-12.5 TU), the Taziste swamp had higher values (22.2 TU) and the mangrove swamp and marsh with cattails had the highest values (74.1-74.6 TU). It is possible that the organic material from which the marshy soils are derived plays a role in the toxicity as measured in this bioassay.

To study this in greater depth, toxicity was measured in these organic soils latter in the year, during the rainy, heavily flooded period, in November, in addition to the sampling realized previously, during the dry season (May, Figure 2). In eight of the ten sites studied, the toxicity

dropped during the rainy season. The exceptions (sites 7 and 11) correspond to areas with recent petroleum spills. In site 7, there were contaminated conditions in both sampling periods, and in site 11, we observed a light but very recent spill just prior to our sampling in November.

The drop in toxicity during the rainy season was especially noticeable in the mangrove ecosystem. There are few possible reasons for this and to understand them, it is necessary to consider what may be causing the toxicity measured by this bioassay. As noted, the mineral based soils in this study did not present toxicity. This suggests that either there are toxic compounds that are derived from the organic matter in itself, or due to the transformation of the organic matter. During the first sampling period, it was observed that the most toxic samples were found in ecosystems with mangrove or cattails. However, during the second sampling period, we observed that the most toxic samples came from the Taziste swamp and the popay/giant reed marsh. Thus, although many plants do produce toxic compounds, to reduce the activities of herbivores (especially insects, Vanenesland et al. 1981), it is difficult to confirm that the toxicity comes directly from the plants themselves. Another possible explanation could be the presence of toxic metals that have been found to occur naturally in the Tonalá river watershed (García et al. 2006; UNAM 2002). It is possible that these toxic compounds are diluted and partially washed out of these marshy ecosystems during the wet season, thus diluting the toxicity.

An alternative explanation also exists with respect to the transformation of the organic substances in the soil by heterotrophic microorganisms. It is well known that actinomycete bacteria and fungi produce antibiotic agents during the decomposition of organic compounds (Kilham & Prosser 2007; Thorn & Lynch 2007). The ecological strategy employed by these microbes is to control or kill off other bacterial populations in the soil to reduce competition for organic substrates. It is possible that such a mechanism is also functioning in these marshy, organic rich soils. During the dry season, the water level drops in these marshes and swamps and the surface layers of soil have some aerobic zones appropriate for the growth of actinomycetes and fungi. The production of antibiotics in these soils may be especially important for this particular bioassay, which uses a bacterium as the test organism. During the rainy season these areas flood, reducing the oxygen content in the soil and thus the growth of aerobic microbes (such as antibiotic-producing actinomycetes and fungi), with the subsequent reduction in toxicity as measured by this bioassay.

Whichever mechanism is actually at work, or even if a different mechanism is causing this phenomenon, it is apparent that to use this bioassay effectively it is necessary to take samples from the contaminated areas and the uncontaminated reference areas during the same period of the year.

Conclusion

In this study, we found that there are many factors that may intervene in the evaluation of toxicity in petroleum impacted soil, and that it is not always the most 'contaminated' sites that are the most toxic. The bioassay presented here is a valuable tool for determining the toxicological impact of a combination of environmental factors, among which the type of hydrocarbon and it degree of weathering, its bioavailability in the soil or sediment matrix, as well as the overall hydrocarbon concentrations

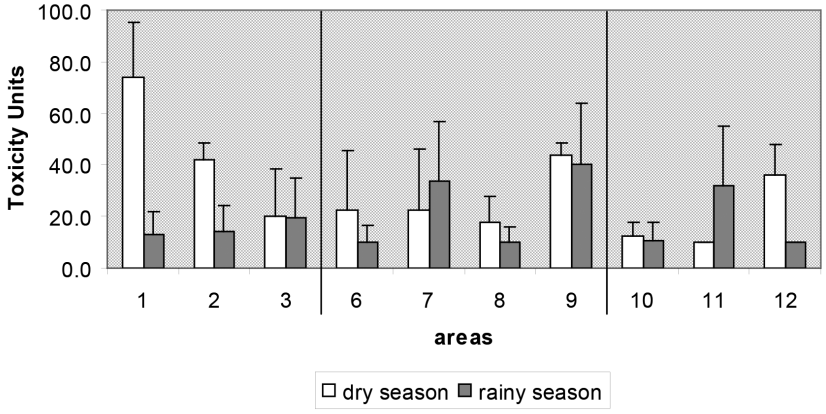

FIGURE 2. Comparison of toxicity in organic soils in dry and rainy season

Data are averages of at least three samples, presented with error bars representing the standard deviation. Areas:

1 – Mangrove/secondary vegetation (reference)
2 – Mangrove with spill
3 – Mangrove with natural attenuation
6 – Tazistal swamp (reference)
7 – Tazistal swamp with spill
8 – Tazistal swamp, phytoremediation (superficial))
9 – Tazistal swamp, phytoremediation (deep layer)
10 – Apompo swamp (reference)
11 – Popay/giant reed marsh (reference)
12 – Popay/giant reed marsh with spill

may be important, as has been found by other authors (Khan et al. 2013; Matejczyk et al. 2011). For these reasons, it is recommended as a site evaluation test to complement the traditional evaluation of hydrocarbon concentration. Furthermore, it may provide evidence to determine if a site not only meets norms, but environmental law as well, such as the clean-up criteria specified in the General Law on Ecological Equilibrium and Environmental Protection, in particular that there not be noxious affects to biological processes (art. 135, section II). As shown in this study, it is very important to take samples in uncontaminated reference areas from the same ecosystem and soil type near the contaminated site, as well as at the same time of the year to avoid false positives. This was especially necessary in organic based soils that tend to present some natural background toxicity with this bioassay. In recent years, this test has been approved by the Mexican federal environmental authority as a cleanup standard in risk based cleanup programs (SEMARNAT 2007), when referenced to background toxicity levels and may be useful in similar tropical areas with petroleum production.

ACKNOWLEDGEMENTS

We would like to thank Mr. Juan Pérez de la Cruz, previously from the office of Industrial Safety and Environmental Protection of Pemex Exploración y Producción, in Agua Dulce, Veracruz who shared valuable information in respect to the site recovery methods used near the San Ramon separation battery (phytoremediation). Thanks also to the Comisión Nacional para el Conocimiento y Uso de la Biodiversidad (CONABIO) and The John T. and Catherine D. MacArthur Foundation for the financial support.

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Received: 7 December 2013
Accepted: 22 September 2014