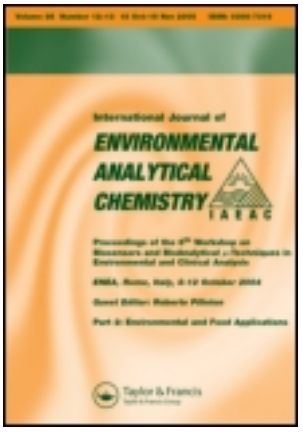

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Bioremediation of hydrocarbons contaminated waters and soils: monitoring by luminescent bacteria test

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Bioremediation has proven successful in numerous applications to petroleum hydrocarbons or chlorinated aromatic hydrocarbons contaminated soils. There is increasing interest in application of biotoxicity tests for ecological assessment and for supporting management decisions for remediation. Luminescent assays, light-emitting bacteria in particular, can be a suitable tool for environmental analysis, and *in vivo* luminescence is a rapid and precise indicator of the toxic effects of xenobiotic on micro-organisms. In this study, three different strains of marine bioluminescent bacteria have been employed to follow the changes in biotoxicity occurring during the laboratory scale bioremediation of water and soil samples contaminated by hydrocarbons and collected at an industrial area. The degradation was made by hydrocarbons degrading bacteria, both of commercial sources and isolated from polluted water and soils. The samples were treated for 45 days. The toxicity of the samples, before and after the bioremediation, was determined directly on water samples or on the extracts of soil samples. The yield of extraction by different solvents (acetone, dioxane, ethanol and dichloromethane) was evaluated by the bioluminescent test. The measurements were carried out using a microplate format both for short time of contact (60 minutes, acute toxicity) and for longer time intervals (24 hours, chronic toxicity). The results have been expressed as percentage of inhibition with respect to the blank emission (100% emission). Original and treated samples have been analysed by gas chromatography to assess the hydrocarbons ($C > 12$ and Poly Chlorinated Biphenyls, PCB) content. The autochthonous bacteria isolated from polluted samples proved less effective, due to the short time for selection in remediation activity with respect to the commercial ones, but their capacity to degrade long chain hydrocarbons was satisfactory. The presented laboratory study can be applied also in case of on-field conditions.

Keywords: hydrocarbons; PCB; soil; bioremediation; bioluminescent bacteria; gas chromatography

1. Introduction

The activities of human beings produce intense and multiple pollution that influence or affect the environment at different levels. Hydrocarbons (HCs) are highly toxic to all

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compartments of the environment [1–3]. Soil and groundwater in the areas of oil terminals or industrial areas are heavily contaminated by these compounds. Remediation treatments in these areas are imposed to decontaminate them and to avoid the spreading of these pollutants in the environment around, limiting the contamination of groundwater [4]. Analytical determinations have to become more and more sensitive, specific and rapid in order to allow the effective realisation of the requested monitoring plans [2–6].

The various remediation procedures are based on thermal, physical, chemical or biological methods [7]. Among them, bioremediation, i.e. the use of micro-organisms to recover solid or liquid matrices, is an alternative, cost-effective, flexible and easy-to-apply technology capable of achieving permanent remediation of contaminated sites [8–10]. When naturally occurring organisms are exposed to organic contaminants they tend to develop, by adaptation, an increased ability to degrade them. Hydrocarbons can be degraded by a large number of micro-organisms belonging to several genera of fungi and bacteria. These bacteria can be isolated from contaminated sites and their cultures enriched using the pollutant as the sole carbon source. At present, many specific bacterial mixtures able to degrade a range of organic pollutants are availabe on the market and ready-to use. The monitoring of remediation procedures can also be based on biological assays [2], behind the well-established chemical analysis, and the presence of any toxic compound can be revealed by measuring the light emitted by luminescent bacteria [11], since its intensity is directly coupled to the energy content of the micro-organisms. The higher the degree of toxicity, the less the amount of light emitted by the bacteria, and several assay kits and dedicated instruments are commercially available to perform these analyses at laboratory or on field conditions [12–14].

We applied an overall microbiological procedure for remediation and toxicity controls of soil and groundwater from an industrial area. The hydrocarbons degrading power of an enriched culture of autochthonous bacteria, previously isolated from polluted samples from the area under treatment, has been tested in a laboratory-scale remediation experiment in comparison with a commercial bacteria mixture selected for its ability to degrade hydrocarbons and PCB. The changes in biotoxicity during these experiments have been evaluated by monitoring the light emission intensity of three strains of *Vibrio* marine bioluminescent bacteria (BLB) [15–16].

2. Experimental

2.1 *Samples*

Soil samples were collected in different areas of the site and at different periods. In the first instance, the samples A1 (depth 0 m), A2 (depth 7–8 m), A3 (depth 11–12 m) and B (depth 10–11 m) were collected and three months later the S1 (depth 1–2 m), S2 (depth 2–7 m) and S.3 (depth 12–13 m) samples were prepared.

Water samples (W1, W2 and W3) have been collected in wells and drilling points in the polluted area. All samples presented an oily film on the water fraction.

2.2 *Gas chromatographic analyses*

The long chain hydrocarbons ($C > 12$) and PCB contents were determined in soil extracts in dichloromethane and in water samples by gas chromatography (GC) before and after

the bioremediation process. Gas chromatographic determinations were made according to the US Environmental Protection Agency (EPA) methods [17].

2.3 *Enrichment culture and bioremediation test*

Two commercially available cultures of hydrocarbons degrading bacteria, both from Micro-Bac International (Round Rock, Texas, USA) were employed:

(A) Micro-Bac® M-1000H* in M-1000PCB, grown at 30°C.
(B) Para-Bac/S in Triphasic 12 grown at 30°C.

Autochthonous bacteria were isolated from polluted water and soil samples and cultured, at 30°C, in two different media: Minimum Medium (MIN) [0.2 g L^{-1} $MgSO_4$, 10 g L^{-1} K_2HPO_4, 1.5 g L^{-1} NH_4Cl, 3.6 g L^{-1} Na_2HPO_4, Yeast Nitrogen Base w/o Amino Acids (Difco) 0.1%, pH 7)] and a Rich Medium (RIC): Yeast Extract 5 g L^{-1} and Bactotriptone 10 g L^{-1} are added to the MIN medium. Four different cultures were prepared by enrichment of the two fractions of water samples:

(C) Bacteria from oil fraction grown in medium MIN.
(D) Bacteria from oil fraction grown in medium RIC.
(E) Bacteria from water fraction grown in medium MIN.
(F) Bacteria from water fraction grown in medium RIC.

When the cultures had grown, they were centrifuged and the pellet was resuspended in sterile physiological solution (9 g L^{-1} NaCl) and washed 3 times. The different cultures were mixed in order to obtain:

Mixture 1 = cultures A + B
Mixture 2 = C + D
Mixture 3 = E + F.

To start the bioremediation treatment the water samples were added to culture media and inoculated (1%, v/v) with the mixtures 1, 2 and 3.

An analogous enrichment procedure was carried out by using a soil sample from each of the two groups, sample B from the first group and sample S1 from the second one. An amount of 10 g of soil were added to 90 mL of RIC medium. The biomasses (Mixture 4 and Mixture 5) obtained from these enrichments have been employed in the bioremediation treatment of the respective group of samples. Aliquots of 50 g of soil were added to 50 mL of culture media and bioremediation started by addition of the various biomasses, as summarised in Table 1. Each sample was incubated in a flask at room temperature in microphilic conditions. Soil sample and water samples were treated for 45 days.

2.4 *Biotoxicity assays*

All samples have been tested by three different bioluminescent bacteria strains:

Vibrio – *Vibrio fischeri (NRRL B-111777)*, from the collection of aerobe bacteria of the Pasteur Institute, Paris, France.
Ucibo – *Vibrio logei*, harvested in the Mediterranean Sea and cultivated at the laboratories of the Department of Metallurgic Science, Electrochemistry and Chemical Techniques, Bologna (SMETEC).

Table 1. Bioremediation mixtures employed in the treatment of the different groups of samples.

Samples	Bioremediation mixtures
A1, A2, A3, B	1 (commercial mixture) 2 (from oil fraction of water samples) 3 (from water fraction) 4 (from sample B)
S1, S2, S3	Mixtures 1, 2, 3 and 5 (from sample S1)
W1, W2, W3	Mixtures 1, 2 and 3

Russi – *Photobacterium phosphoreum* 1883 IBSO, supplied by the Institute of Biophysics (Siberian Branch, Academy of Sciences), Cultures Collection IBSO, Laboratory of Bacterial Bioluminescence, Akademgorodok, Krasnoyarsk, Russia.

The procedure of the bioluminescent toxicity test is based on an ISO Standard method [18], only slightly changed. Acute and chronic toxicity tests were performed [16,19–20].

Bacteria were cultured both on liquid and solid medium and stored lyophilised, as previously reported [6,19]. Lyophilised bacteria are particularly useful since, once reconstituted by 1 mL of distilled water at the moment of use, they produce a constant blank signal [6].

In the acute toxicity test (AT), each well contained 180 μL of sample solution and 20 μL of bacteria suspension in distilled water. In chronic toxicity test (CT), each well was filled with: 100 μL of sample and 100 μL of broth inoculated with reconstituted freeze-dried bacteria. Light emission, expressed as Relative Light Units (RLU), was recorded for 60 min in AT and 24 h in the CT assay.

The results, for both toxicity tests, were expressed as the percentage of inhibition of the blank emission produced by each sample, a relative way that was considered the more correct one. The percentage of inhibition was calculated according to

$$[(B_e - S_e)/B_e]100,$$

where B_e is the emission of the bacteria or the sample not treated and S_e that of the contaminated or treated sample.

The water samples were analysed as they were, while the biotoxicity of soil samples was determined on a solvent extract of the sample. The extraction procedure using ethanol, dioxane and acetone has been optimised in previous research working on soils from oil terminals [6], while the extraction with dichloromethane was performed according to EPA procedures [17]; the same applied for gas chromatographic analysis.

In the toxicity tests on untreated soil samples, the blank was represented by the bacterial solution since a more correct comparison with the extract of a soil not contaminated was not possible because such a sample was not available. In the case of the bioremediated soil samples, the blank was represented by the extract of the corresponding, not treated, samples.

The BLB tests were performed on two similar microplate luminometers: 'Victor 1420' Multilabel Counter (Wallac, Sweden) and 1253 Luminoskan Ascent (Labsystems, Helsinki, Finland); 96-wells black microplates, at room temperature, were used.

Table 2. Hydrocarbons content in water samples before and after the bioremediation treatment, determined by GC.

Water sample	C > 12 content before treatment (mg mL^{-1})	C > 12 content after treatment (mg mL^{-1})
W1	28144	15000
W2	530	270
W3	312	125

Table 3. Light inhibition of three bioluminescent bacteria strains by water samples before and after the bioremediation treatment.

		Biotoxicity light inhibition (%)					
		Ucibo		Vibrio		Russi	
Water sample	Bioremediation treatment (days)	AT[a]	CT[b]	AT	CT	AT	CT
W1	0	86	92	89	94	75	93
	45	99	93	97	97	96	96
W2	0	58	74	59	70	71	62
	45	79	87	74	75	59	75
W3	0	33	45	38	49	24	31
	45	44	54	48	52	49	47

Notes: [a]Acute toxicity.
[b]Chronic toxicity.

3. Results

3.1 *Water*

The PCB content was lower than the detection limit of the GC method in all the three samples under study. The content of C > 12 hydrocarbons was remarkable only in one sample, showing a notable oil fraction; this content resulted reduced after the bioremediation process (Table 2).

Concerning the biotoxicity test (Table 3), the three samples proved toxic to all bacterial strains, both in acute and in chronic tests. In particular, higher toxicity was showed by samples W1 and W2 (inhibition higher than 90%), whereas W3 showed an inhibition of about 40–50%. After the 45 days treatment the toxicity values in both tests showed an increase. This fact has been explained by the increase in the content of short chain molecules, more toxic to the luminescent bacteria and produced by the remediation process [6,16]. As an example, in Figure 1 the graphs obtained for the GC analysis of a sample before and after the bioremediation treatment show clearly an increased presence of shorter hydrocarbons after remediation.

3.2 *Soil*

All the analyses on soil samples required the extraction of the hydrocarbons from the solid matrix and four different solvents, at different dilutions, were tested. Especially in the case

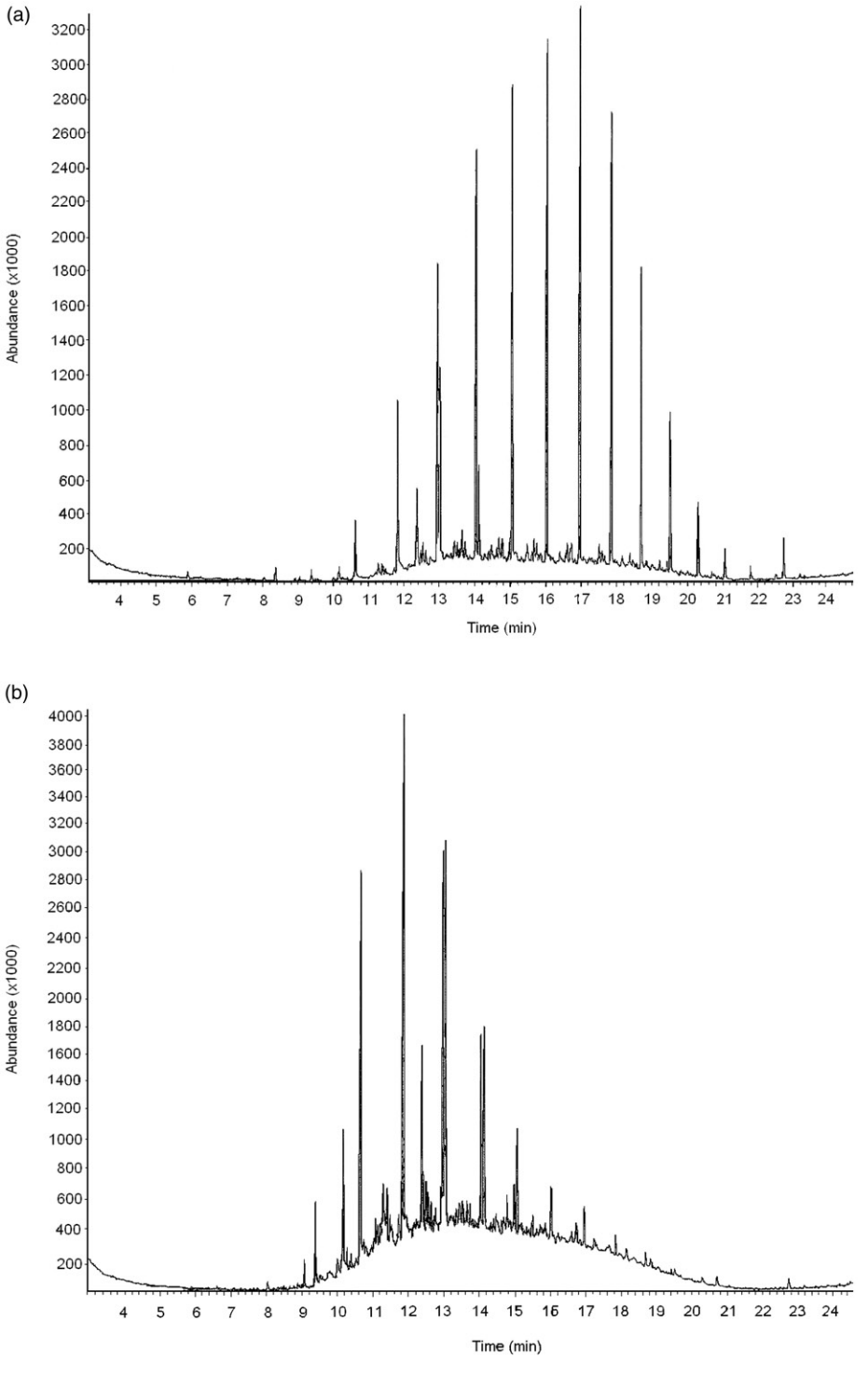

Figure 1. Gas chromatograms of the water sample W2 before (a) and after (b) 45 days of remediation treatment.

of biotoxicity assays, the aim was to highlight potential inhibitory effects of the solvents. The GC data obtained for the different extracts are reported in Table 4.

It is possible to note that the PCB were present, at very low level, only in sample B and that the yield of extraction of hydrocarbons can vary a great deal among the various solvents,

Concerning the biotoxicity of the extracts from untreated samples, only the extract of sample A2 in dichloromethane showed an acute toxicity but, taking into account other parameters of this sample, it is clear this toxicity must be ascribed to components different from hydrocarbons.

On the contrary, the main part of the extracts obtained from the four samples by using the different solvents proved toxic to the bioluminescent bacteria. An example of the results obtained testing the three strains is reported in Figure 2.

Table 4. Gas chromatographic determination of the soil samples hydrocarbons extracted by the various solvents.

		Solvent			
Sample	Analyte (mg kg^{-1})	Acetone	Ethanol	Dioxane	Dichloromethane
A1 (0 m)	C > 12	815	814	810	584
	PCB	<0.01	<0.01	<0.01	<0.01
A2 (7–8 m)	C > 12	906	218	2052	791
	PCB	<0.01	<0.01	<0.01	<0.01
A3 (11–12 m)	C > 12	241	132	781	798
	PCB	<0.01	<0.01	<0.01	<0.01
B (10–11 m)	C > 12	1994	9023	4944	810
	PCB	0.23	1.45	0.81	<0.01

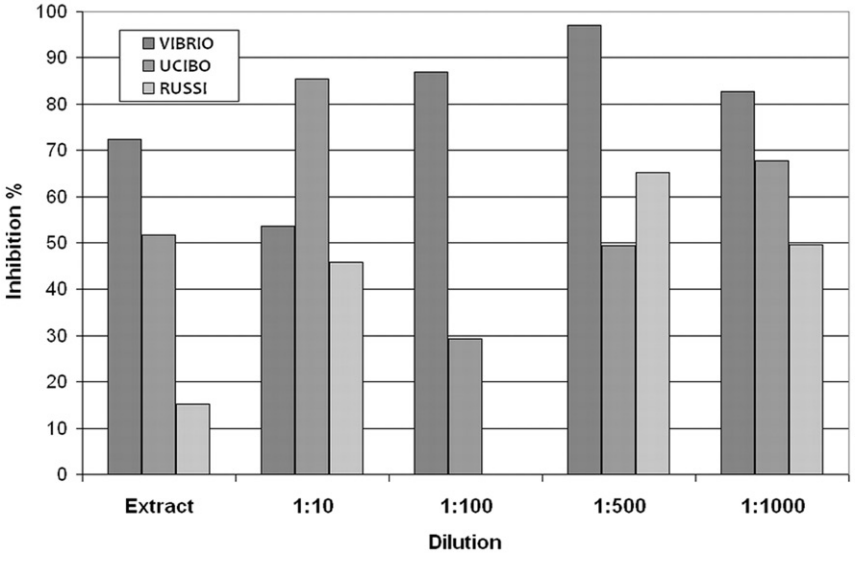

Figure 2. Chronic toxicity of different dilutions of the acetone extract from sample A3. The assay has been performed by using all the three strains of bioluminescent bacteria.

Table 5. CG analyses of S1-S3 samples before and after bioremediation treatment.

Sample	C > 12 before treatment (mg mL^{-1})	C > 12 after treatment (mg mL^{-1})	
		Mixture 1	Mixture 5
S1	1864	924	1230
S2	6186	4330	4832
S3	<0.001	<0.001	<0.001

It is clear that the different strains display individual response to the same sample, as well as different light intensity of the reference blank. It is interesting to note how the light emission was, in various cases, enhanced by the extracts. This phenomenon occurs because the soil is a complex matrix containing compounds which can act as nutrients for bacteria, and then a blank containing the extract of unpolluted soil is necessary to simplify the interpretation of the results. Another surprising behaviour which is possible to observe in Figure 2 concerns the increase of toxicity consequent to increasing dilution of the samples until 1 : 500. This behaviour has been observed in all experiments employing acetone extracts, but never when more lipophilic solvents such as dichloromethane were used: in this case increasing dilutions corresponded to decreased toxicity. The unexpected trend of acetone extracts dilutions can be ascribed to a change in size and equilibrium of the hydrocarbons-bacterial surfactants micelles present in this polar solvent. The concentration of 'free' hydrocarbons would change according to the dilution and to the value of the critical micellar concentration.

Table 5 highlights that the commercial mixture is more efficient in degrading pollutants: it is possible to note a more intense reduction in C > 12 due to long chain hydrocarbons degradation, a mark of the occurring bioremediation. This was an expected result, since the commercial bacteria have been accurately selected to degrade efficiently and specifically the long chain hydrocarbons, while the autochthonous degrading bacteria have been simply, and shortly, isolated and enriched as the organisms able to survive using hydrocarbons as the sole carbon source.

The trend of the experiment described by the chemical assays was confirmed by the biotoxicological analysis made with dichloromethane extracts, both for acute and chronic toxicity (Table 6). After 45 days an increased toxicity was measured, and this has been ascribed to a good degradation activity.

4. Conclusion

The results collected during this research work confirmed that the bioluminescent assay of the total toxicity of a sample offers a reliable and sensitive index of the pollution level in the analysed sample. This kind of assay, easy to perform at low cost, represent an ideal screening tool of environmental and under-processing samples, useful to limit the number of chemical analyses to obtain a quantitative confirmation of the toxicological data.

From the bioremediation procedure point of view, it is interesting to underline the satisfying activity shown by the bacterial mixtures isolated from the same sample to treat.

Table 6. Light inhibition of three bioluminescent bacteria strains by soil samples before and after the bioremediation treatment.

		Biotoxicity light inhibition (%)											
		Ucibo				Vibrio				Russi			
		Mixture 1		Mixture 5		Mixture 1		Mixture 5		Mixture 1		Mixture 5	
Soil sample	Days of treatment	AT[a]	CT[b]	AT	CT	AT	CT	AT	CT	AT	CT	AT	CT
S1	0	67	75	87	83	80	84	89	84	78	83	82	87
	45	79	88	92	94	93	91	94	97	84	89	84	97
S2	0	92	94	97	93	92	89	94	92	89	92	99	97
	45	98	99	99	98	99	99	99	99	98	99	98	99
S3	0	17	12	16	21	22	15	18	13	22	18	16	18
	45	16	14	18	19	13	17	14	16	17	13	19	18

Notes: [a]Acute toxicity.
[b]Chronic toxicity.

Even the commercial Micro-bac mixture, selected for this specific aim, obtained better results. This finding suggest that the laboratory-scale procedure can be applied on field with good possibility of success, since it has been demonstrated that the autochthonous bacteria are enough adapted to perform the remediation of contaminated soil and water, and all this in spite of the short treatment time allowed them in our experiments.

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References

[1] M.T. Del Panno, I.S. Morelli, B. Angeles, and B.L. Corti, FEMS Microb. Ecol. **53**, 305 (2005).
[2] G. Plaza, G. Nalecz-Jawecki, K. Ulfig, and R.L. Brigom, Chemosphere **59**, 289 (2005).
[3] E. Katsivel, E.R.B. Moore, D. Moroukli, C. Strompl, D. Pieper, and N. Kalogerakis, Biodegradation **16**, 169 (2005).
[4] C. Wethasinghe, S.T.S. Yuen, J.J. Kaluarachchi, and R. Hughes, Environ. Int. **32**, 312 (2006).
[5] European Community Directive 2000/60/EC of the European Parliament and of the Council, Off. J. Eur. Commun. (English edition) L 327, 43, 22 (December 2000).
[6] S. Girotti, E. Maiolini, L. Bolelli, E.N. Ferri, A. Pompei, D. Matteuzzi, S. Medvedeva, and P. Fonti, in *Soil, Chemical Pollution, Risk Assessment, Remediation and Security*, edited by L. Simeonov and V. Sargsyan (NATO Science for Peace and Security Series, Subseries: NATO Science for Peace and Security Series – C: Environmental Security, Springer Science, Dordrecht, The Netherlands, 2008), p. 369.
[7] B. Tadesse, J.D. Donaldson, and S.M. Grimes, J. Chem. Technol. Biot. **60**, 227 (1994).
[8] B.K. Gogoi, N.N. Dutta, P. Goswami, and T.R. Krishna Mohan, Adv. Environ. Res. **7**, 767 (2003).

[9] S.K. Brar, M. Verma, R.Y. Surampalli, K. Misra, R.D. Tyagi, N. Meunier, and J.F. Blais, Pract. Period. Hazard. Toxic Radiac. Waste Manag. **10**, 59 (2006).
[10] M.L. McCullough and J.V. Dagdigian, Remed. J. **3**, 265 (1993).
[11] S. Girotti, E.N. Ferri, M.G. Fumo, and E. Maiolini, Anal. Chim. Acta **608**, 2 (2008).
[12] D.J. Dalzell, S. Alte, E. Aspichueta, A. de la Sota, J. Etxebarria, M. Gutierrez, C.C. Hoffmann, D. Sales, U. Obst, and N. Christofi, Chemosphere **47**, 535 (2002).
[13] M.L. Farré, I. Ferrer, A. Ginebreda, M. Figueras, L. Olivella, L. Tirapu, M. Vilanova, and D. Barceló, J. Chromatogr. A **938**, 187 (2001).
[14] A.M. Kuznetsov, E.K. Rodicheva, and S.E. Medvedeva, Field Anal. Chem. Tech. **2**, 267 (1998).
[15] J.A. Marin, J.L. Moreno, T. Hernandez, and C. Garcia, Bioremediat. Biodegrad. **17**, 251 (2006).
[16] S. Girotti, M.G. Fumo, L. Bolelli, E.N. Ferri, A. Pompei, D. Matteuzzi, and P. Fonti, Luminescence **23**, 222 (2008).
[17] EPA 3510C Separatory Funnel Liquid-liquid Extraction (1996) and EPA 8270D Semivolatile Organic Compounds by Gas Chromatography/Mass Spectrometry (GC/MS) (1998). <http://www.epa.gov/>
[18] British Standard – BS EN ISO 11348-3: 1999, Part 3. BSI, Index House, Ascot, SL5 7EU, UK (1999).
[19] S. Girotti, L. Bolelli, F. Fini, M. Monari, G. Andreani, G. Isani, and E. Carpené, Chemosphere **65**, 627 (2006).
[20] Y.S. El-Alawi, B.J. McConkey, D.G. Dixon, and B.M. Greenberg, Ecotox. Environ. Safe. **51**, 12 (2002).