Elsevier Editorial System(tm) for Journal of Hazardous Materials

Manuscript Draft

Manuscript Number: HAZMAT-D-19-04377R2

Title: Use of a biostimulant obtained from okara in the bioremediation of a soil polluted by used motor car oil

Article Type: Research Paper

Keywords: okara biostimulant; used motor-car oil; polycyclic aromatic hydrocarbons; total heavy metals; soil dehydrogenase activity

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Abstract: In this manuscript we studied in the laboratory the bioremediation effects of a biostimulant obtained from okara by enzymatic hydrolysis processes in a soil polluted with used motor-car oil at a rate of 1% (w/w) over an 89-day period. The biostimulant was added to the soil 6 times during the incubation period at a rate of 2%. Dehydrogenase activity and the evolution of polycyclic aromatic hydrocarbons (PAHs) and pseudo total heavy metals in soil were studied. The successive applications of the biostimulant to the polluted soil gradually increased PAHs degradation during the experimental period. Thus, at the end of the experiment, the application of the biostimulant decreased the concentration of naphthalene in soil by 74%, while PAHs with 3, 4, 5 and 6 aromatic rings had been reduced by around 58%, 44%, 30% and 23%, respectively. This degradation is possibly due to the high number of low molecular weight peptides (<300 Da) in the biostimulant which are readily available for PAHs-tolerant soil microorganisms that accelerate the degradation of the said toxins. The concentration of heavy metals in the oil used was not very high and consequently the dehydrogenase activity was not negatively affected.

*Cover Letter

Dear Editor,

Again I remit to you the paper entitled "**Use of a biostimulant obtained from okara in the bioremediation of a soil polluted by used motor car oil**"·corrected and revised according to the reviewer comments for its possible publication in Journal of Hazardous Materials.

In this respect, the new full paper has the following words:

Manuscript: 4419 words

Tables: 1515 words

Yours sincerely

Dr. Manuel Tejada

Novelty Statement (maximum limit:100 words)

Few manuscripts describe the use of organic matter in the bioremediation of soils polluted with used motor-car oil characterised by its high content of polycyclic aromatic hydrocarbons and heavy metals. The aim of this work was to study under controlled laboratory conditions the bioremediation effect of a edaphic biostimulant obtained from okara enzymatic hydrolysis processes applied continuously for a period of 89 days in a soil polluted with used motor-car oil. The authors believe that this work is an advance in the study in the use of different organic compounds in the bioremediation of polluted soils.

Abstract

In this manuscript we studied in the laboratory the bioremediation effects of a biostimulant obtained from okara by enzymatic hydrolysis processes in a soil polluted with used motor-car oil at a rate of 1% (w/w) over an 89-day period. The biostimulant was added to the soil 6 times during the incubation period at a rate of 2%. Dehydrogenase activity and the evolution of polycyclic aromatic hydrocarbons (PAHs) and pseudo total heavy metals in soil were studied. The successive applications of the biostimulant to the polluted soil gradually increased PAHs degradation during the experimental period. Thus, at the end of the experiment, the application of the biostimulant decreased the concentration of naphthalene in soil by 74%, while PAHs with 3, 4, 5 and 6 aromatic rings had been reduced by around 58%, 44%, 30% and 23%, respectively. This degradation is possibly due to the high number of low molecular weight peptides (<300 Da) in the biostimulant which are readily available for PAHs-tolerant soil microorganisms that accelerate the degradation of the said toxins. The concentration of heavy metals in the oil used was not very high and consequently the dehydrogenase activity was not negatively affected.

***Response to Reviewers**

Reviewer #1: I don’t know why I recorded some grammatical errors. Even though the author claimed that the manuscript was edited by a native English speaker, I suggest to author to recheck the language for readability.

The grammar of the manuscript has been reviewed again by a native English speaker. In this sense, the grammar changes made are indicated in the revised manuscript in red. We hope that all grammatical problems indicated by the reviewer will be definitively resolved.

Use of a biostimulant obtained from okara in the bioremediation of a soil polluted by used motor car oil

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Abstract

In this manuscript we studied in the laboratory the bioremediation effects of a biostimulant obtained from okara by enzymatic hydrolysis processes in a soil polluted with used motor-car oil at a rate of 1% (w/w) over an 89-day period. The biostimulant was added to the soil 6 times during the incubation period at a rate of 2%. Dehydrogenase activity and the evolution of polycyclic aromatic hydrocarbons (PAHs) and pseudo total heavy metals in soil were studied. The successive applications of the biostimulant to the polluted soil gradually increased PAHs degradation during the experimental period. Thus, at the end of the experiment, the application of the biostimulant decreased the concentration of naphthalene in soil by 74%, while PAHs with 3, 4, 5 and 6 aromatic rings had been reduced by around 58%, 44%, 30% and 23%, respectively. This degradation is possibly due to the high number of low molecular weight peptides (<300 Da) in the biostimulant which are readily available for PAHs-tolerant soil microorganisms that accelerate the degradation of the said toxins. The concentration of heavy metals in the oil used was not very high and consequently the dehydrogenase activity was not negatively affected.

Keywords: okara biostimulant; used motor-car oil; polycyclic aromatic hydrocarbons; total heavy metals; soil dehydrogenase activity

1. Introduction

At present the use of lubricating oils is widespread because they are necessary for the correct working of motor vehicle engines and other machinery such as pumps, hydraulic motors, compressors and electrical transformers [1]. Due to the physical and chemical reactions that occur during use, used motor oil is characterised by its high content of aromatic and aliphatic hydrocarbons whose chain lengths range from C15 to C50 and heavy metals that

could contribute to the risk of chronic diseases, including mutagenicity and carcinogenicity [1-3].

As a result of their widespread use, terrestrial ecosystems are becoming increasingly contaminated by such lubricants. The bioremediation of soils contaminated with used motor oils is, therefore, an efficient and effective environmental technique for accelerating the cleaning processes [2, 4].

In recent years, applying organic biostimulants obtained from sewage sludge, chicken feathers, wheat condensed distiller's soluble enzymatic hydrolysate, rice bran extract, carob germ enzymatic extract, etc. by enzymatic hydrolysis processes to soils polluted by organic xenobiotics has been a widely-used environmental technique for accelerating the degradation of the said xenobiotics in soil. The low molecular weight proteins, found in large quantities in these biostimulants, are absorbed by the xenobiotics-tolerant microorganisms, increasing both their proliferation and the biochemical activity of the soil, consequently accelerating the degradation of the toxins in the soil [5-8].

Okara is a by-product obtained from the manufacture of soy milk, tofu and their derivatives. It has both a high fibre (56%) and protein (29%) content. Orts et al. [9] obtained a new biostimulant from okara by enzymatic hydrolysis processes using the pH-stat technique in order to remedy a soil polluted with chlorpyrifos. Eighty days after applying the biostimulant to polluted soil, the insecticide had been completely degraded. Consequently, this biostimulant obtained from okara could be of great use for remedying a soil contaminated by motor oil with a high content of polycyclic aromatic hydrocarbons (PAHs).

It should be noted, however, that the effectiveness of these biostimulants obtained by enzymatic hydrolysis processes has been verified after only a single application to polluted soil. It is not known what the behaviour of organic xenobiotics in the polluted soil would be after repeated applications of these biostimulants.

There is an abundant literature indicating that soil biological parameters evolve faster than physical and chemical parameters [6, 10-12]. There is also a lot of information on how PAHs and heavy metals negatively affect soil enzymatic activities, as well as affecting its microbial population [8, 11, 13, 14]. For this reason, a study of these biological properties would be very useful in order to verify the bioremediation capacity of organic matter in polluted soils.

According to Kaczynski et al. [15] and Liu et al. [16], the oxidation-reduction enzymes found in soil play a fundamental role in degrading contaminants, transforming organic matter and in maintaining the metabolism of microorganisms. In this respect, dehydrogenase activity (EC 1.1.1) is an essential intracellular oxidoreductase enzyme in living cells. It participates in redox reactions, it promotes the dehydrogenation of soil organic matter and transmits energy to hydrogen acceptors through the respiratory chain [17]. Due to the above, this enzyme is now used as a soil contamination status bioindicator [18, 19, 20].

The objective of this study, therefore, was to verify the bioremediation efficiency of a biostimulant, obtained from okara by enzymatic hydrolysis processes, in a soil polluted by used motor oil after repeated applications of the said biostimulant to the polluted soil and its influence on the soil dehydrogenase activity.

2. Material and methods

2.1. Soil, biostimulant and motor oil characteristics

The soil used in the study was classified as Calcaric Regosol [21], with 574 ± 31g kg^{-1} sand, 148 ± 35 g kg^{-1} silt, and 278 ± 24 g kg^{-1} clay. Soil pH was 7.9 ± 0.1, 16.4 ± 1.3 gkg^{-1} organic matter, and 0.98 ± 0.05 g kg^{-1} N. The methodology used for determining the soil parameters is described in Tejada et al. [7] and Rodríguez-Morgado et al. [22].

The okara from which the new biostimulant was manufactured was provided by the Soria Natural S.A company. The experimental biostimulant was obtained from the okara by enzymatic hydrolysis using an endoprotease obtained by liquid fermentation of Bacillus licheniformis ATCC 21415. Table 1 shows the chemical characteristics of both the okara and the resulting biostimulant. The characteristics of this process of enzymatic hydrolysis are detailed in Orts et al. [9]. The methodology used for determining organic matter, macro- and micronutrients, and molecular protein mass distribution is described in Rodríguez-Morgado et al. [22].

The motor oil used was a sample of gasoline automobile engine oil taken when the vehicle's oil was changed at 15000 km. Table 2 shows the content of polycyclic aromatic hydrocarbons and total heavy metals in the motor oil.

The PAHs were detected by a GC 7890B Agilent gas chromatograph connected to a MS Triple Q Agilent 7000C mass detector. The analysis conditions were the following:

Column: DB-EUPAH 30X0.25X0.25

Injection: 2 µl

Column pressure: 15.7 psi

Constant flow: 1.2 ml min^{-1}

Injection mode: Splitless 50 psi

Injection temperature: 310 °C

Initial temperature: 80 °C for 1 minute

Temperature ramp: 25 °C $minute^{-1}$ to 200 °C for 8 minutes; 5 °C $minute^{-1}$ to 305 °C for 9 minutes; 50 °C $minute^{-1}$ to 330 °C for 3 minutes; (48.5 total minutes)

115 Table 3 shows the detection limit and quantification limit per each PAH. The internal
116 standards used in GC/MS study were: acenaphthene d8, chrysene d10, naphthalene d8,
117 perylene d12 and phenatrene d10. Recovery analyses were carried out in every treatment,
118 showing values of between 70 and 80%.

119 The concentration of heavy metals in the engine oil was determined following US
120 EPA method 3052. For this, 0.5 g of oil was digested in 9 ml of HNO_3 and 3 ml of HCl using
121 a microwave digester. Digests were filtered through 0.45 mm Millipore filters and diluted
122 appropriately for analysis by a Thermo Elemental ICP-MS X7 Inductively Coupled Plasma
123 Mass Spectrometry system (Thermo Fisher, Cambridge, UK) with quadrupole mass analyser,
124 multichannel detector (Pulse Counting and Analogue Methods), auto sampler ASX-500
125 (CETAC, Omaha, NE, USA). The measurement accuracy (%) of each heavy metal was as
126 follows: Fe: 2.2; Cu: 5.2; Mn: 2.5 Zn: 3.4; Ni: 4.0; Cr: 4.7; As: 1.1; Cd: 3.9; Pb: 5.0; and Al:
127 3.1.

128

129 2.2. Experimental layout

130 Five hundred grams of dried, sieved (<2 mm) soil were preincubated at 25 °C for 7
131 days at 30-40 % of their holding capacity [5]. After this pre-incubation period, soil samples
132 were mixed with gasoline motor oil at a rate of 1% (w/w) in 1-L glass bottles., Motor oil was
133 dissolved in acetone as a carrier solvent at a concentration of 30 mL kg^{-1} according to
134 Ramadass et al. [3]. Acetone was also added to the non-polluted soils (control treatment). The
135 biostimulant was added to the soil 6 times at a rate of 2% once at the beginning of the
136 experiment and then on days 13, 27, 41, 55 and 69 during the incubation period. The
137 biostimulant used was liquid and was solubilised in distilled water (500 l ha^{-1}) before
138 application. Distilled water was added to each soil to bring it to 60% of its water-holding

capacity. An unamended, polluted and an amended, non-polluted soil were used as controls. The incubation treatments are detailed as follows:

1. C, control soil, non-polluted soil and non-organically amended
2. C+O, polluted soil with used motor oil and non-organically amended
3. C+BS, non-polluted soil and amended with the biostimulant
4. C+O+BS, polluted soil with used motor oil and amended with the biostimulant

Triplicate treatments were kept in semi-closed microcosms at 25 ± 1 °C for 50 days, respectively. The moisture content was controlled gravimetrically and moisture loss was replaced as necessary by distilled water.

2.3. Soil analysis

For each experimental treatment, dehydrogenase activity was measured at days 3, 13, 16, 27, 30, 41, 44, 55, 58, 69, 72 and 86 during incubation experiment. The soil samples from days 3, 13, 27, 41, 55 and 69 were selected and analysed before applying the okara biostimulant to the soil.

The method of Trevors et al. [23] as modified by García et al. [24] was used to measure dehydrogenase activity, reducing 2-*p*-iodophenyl-3-*p*-nitro-phenyl-5-phenyltetrazolium chloride (INT) to iodonitrophenyl-formazan (INTF). This was measured in a spectrophotometer at 490 nm.

PAHs were extracted from 30 g of soil. These samples were placed in small glass jars with 10 g of anhydrous sodium sulphate in order to remove moisture from the soil samples. 50 ml of a dichloromethane/pentane 1/1 mixture were then added and the mix was shaken for 30 seconds. The samples were then sonicated for 10 minutes and filtered through anhydrous

sodium sulphate. The total volume extracted was concentrated in a rotary evaporator at a temperature of 50 °C. This process was performed in triplicate. Finally, the volume of the extract was adjusted to 1 mL with isooctane.

Once the PAHs had been extracted, they were measured according to the instruments and conditions previously described.

At days 3 and 86 after the start of the incubation process, the pseudo total heavy metal content in soil was determined, the method of determination being highly similar to that described above. Using a microwave digester, 5 g of soil were digested in 9 mL of HNO_3 and 3 mL of HCl. Digests were filtered through 0.45 mm Millipore filters and appropriately diluted for posterior analysis using the Thermo Elemental ICP-MS X7 Inductively Coupled Plasma Mass Spectrometry system.

2.4. Statistical analysis

With the data obtained from each soil analysis and using Statgraphics Plus 2.1 software package, a two-way analysis of variance (ANOVA) was performed with treatment and sampling time as factors, followed by Tukey's significant difference as a *post hoc* test, considering a significance level of $p < 0.05$ throughout the study. For the ANOVA, triplicate data were used for each treatment and for each day of incubation.

3. Results and discussion

The continuous application of the biostimulant to the non-polluted soil during the experimental period caused an increase in dehydrogenase activity (Table 4). In other experiments carried out by Rodriguez-Morgado et al. [8, 25] and Orts et al. [9], performing a single application to the soil of biostimulants obtained from sewage sludge, chicken feathers and okara by enzymatic hydrolysis processes, it was observed that the dehydrogenase activity

showed maximum stimulation during the first 7 days after application. The dehydrogenase activity then declined progressively until reaching values similar to the non-organically amended soil. According to these authors, the main reason for the behaviour of these biostimulants is a consequence of the protein size distribution obtained in said organic compounds, such behaviour highlighting the influence/high content of the low molecular weight proteins (<300 Da), which are in the majority. The higher content of low molecular weight proteins suggests that N is in a more readily-available form for soil organisms, promoting a greater proliferation of soil microorganisms and, consequently, dehydrogenase activity.

In our experiment, this increase in dehydrogenase activity at the end of the incubation period may be due to the constant accumulation of high molecular weight proteins after the continuous application of the experimental biostimulant, thus requiring more time for the microorganisms to degrade them. Table 1 shows that the protein content of molecular weight > 1000 Da is 26.4%, which may be responsible for stimulating soil microbial activity at the end of the incubation period.

The application of motor oil to the soil showed a significant decrease ($p < 0.05$) in dehydrogenase activity during the whole experimental period (Table 4). At the end of the incubation period and compared to C treatment, dehydrogenase activity decreased significantly ($p < 0.05$) by 62.5% in soils polluted with used motor oil. This inhibition in soil dehydrogenase activity is possibly a consequence of adding different PAHs with 2 to 6 aromatic rings in the used motor oil. These results are in agreement with those obtained by Tejada et al. [6], Rodríguez-Morgado et al. [8] and Lipińska et al. [19], who found that dehydrogenase activity was inhibited in soils polluted by various PAHs due to their toxicity to soil microorganisms. According to these authors, this toxic effect inhibits an abundant population of soil microorganisms and, consequently, reduces soil dehydrogenase activity.

Table 5 shows the evolution of PAHs in soil during the experimental period after polluting it with automobile motor oil. In non-organically amended soils, the concentration of the PAHs remained highly similar throughout the incubation period. Applying the experimental biostimulant to polluted soil increased PAH degradation. This degradation was gradual throughout the experimental period and was greater among those hydrocarbons with the lowest number of aromatic rings. Thus, at day 47 of the incubation period and in comparison with the C+O treatment, the naphthalene concentration decreased significantly ($p < 0.05$) by 68.5% in the C+O+BS treatment, while the hydrocarbons with 3, 4, 5 and 6 aromatic rings decreased by values of around 43%, 33%, 25% and 16%, respectively. At the end of the experimental period, and in comparison with the C+O treatment, naphthalene concentration in the C+O+BS treatment had decreased significantly ($p < 0.05$) by 74%, while concentrations of hydrocarbons with 3, 4, 5 and 6 aromatic rings had decreased by around 58%, 44 %, 30% and 23%, respectively.

There is an abundant bibliography that suggests that PAH degradation in soil is a very slow process carried out by microorganisms conditioned by the number of aromatic rings in the hydrocarbon's structure [8, 26, 27]. According to Edokpayi et al. [28], as the molecular weight of the PAH increases, solubility decreases, increasing in turn environmental persistence and toxicity. For this reason as the number of aromatic rings in its chemical structure increases, PAH degradation decreases.

Applying organic matter to the soil accelerates PAH degradation [6, 8, 27, 29]. According to these authors, soil microorganisms are responsible for PAH degradation. In this sense, applying organic matter to soil contaminated by PAHs stimulates the PAH-tolerant microorganisms, favouring PAH degradation and thus reducing their polluting effect on the soil. In our experiment, successive applications of the experimental biostimulants throughout

238 the incubation period decreased progressively the soil PAHs concentration. Obviously, the
239 degradation was greater in those PAHs with a lower aromatic number.

240 In comparison with our experiment, Tejada et al. [6] and Rodriguez-Morgado et al. [8]
241 found a higher degradation percentage of phenanthrene, pyrene and benzo (a) pyrene after
242 applying to soil different edaphic biostimulants obtained by enzymatic hydrolysis processes
243 from rice bran, wheat condensed distiller's soluble, sewage sludge and chicken feathers. In
244 our experiment the soil was polluted by a varied mixture of PAHs, presenting from 2 to 6
245 aromatic rings. Consequently, although applying the experimental biostimulants accelerated
246 degradation of those PAHs with fewer aromatic rings, the high concentration of PAHs with a
247 greater number of aromatic rings, mainly those with 5 and 6 aromatic rings, possibly
248 continued to exert a very toxic effect on soil microorganisms, as reflected by soil
249 dehydrogenase activity (Table 4).

250 Table 6 shows the evolution of the pseudo total heavy metals content in the
251 experimental treatments during the incubation period. The pseudo total heavy metals contents
252 in treatments C and C+BS were very similar on days 3 and 86 of the experiment, suggesting
253 that the pseudo total heavy metals concentration in the polluted and organically-amended soil
254 did not vary with respect to the non-amended and non-polluted soil. In the same way, and as
255 previously mentioned, the dehydrogenase activity in the C+BS treatment was stimulated when
256 compared to treatment C.

257 A large number of references indicate that a high concentration of heavy metals in the
258 soil solution can inhibit its dehydrogenase activity [11, 30, 31]. Our experiment measured the
259 pseudo total heavy metals content in soil, not merely the content of metals available. Since the
260 dehydrogenase activity was not inhibited in the C+BS treatment, we believe that the heavy
261 metals concentration in the biostimulant applied to the non-polluted did not exert any toxic
262 effect on the above enzymatic activity.

With respect to the C treatment, the concentration of pseudo total heavy metals in the C+O treatment did not vary significantly during the incubation period. This suggests that, possibly, the heavy metal contents in the experimental car motor oil used in our experiment did not have any negative effects on the soil dehydrogenase activity. Therefore PAH content alone would be the cause of soil toxicity. These results do not coincide with those obtained by Dike et al. [1] and Wang et al. [32], who suggest that the toxic effect of used motor oil is mainly a consequence of both the PAHs content and the heavy metals.

According to Ramadass et al. [3], the concentration of heavy metals in a used motor oil is the result of wear and tear in the engine components and the heating and oxidation of the lubricating oil. We believe that the concentration of heavy metals in the used motor oil depends on how the motor has been used. In our experiment the oil was sourced from a 15000-km oil change performed on gasoline car engine. Such a short period of use may not be sufficient for any great amount of heavy metals to be transferred from the engine to the oil.

Applying the biostimulant to the oil-polluted soil showed heavy metal values very similar to those of treatment C, which suggests that applying the experimental biostimulants to the polluted soil did not produce any significant change to its heavy metal content.

Applying the biostimulant to the non-polluted soil did not change the pH value (Table 7). The basic nature of the biostimulant, together with the soil pH value are sufficient reason for a change in the pH of said soil not to occur. On the other hand, neither did applying the biostimulant to the oil-polluted soil change the pH. These results suggest that the concentration of heavy metals, acidic in character, is low in the experimental oil. In addition, it must also be taken into account that the amount of oil applied to the soil was not very high, so the heavy metal concentrations added to the soil was also low. Regarding the PAH concentration in the oil, it should be noted that these chemical compounds do not have an acidic character and, therefore, it also justifies the lack of change in soil pH.

4. Conclusions

It can be concluded that the toxic effect of used gasoline car engine oil is mainly due to its content of polycyclic aromatic hydrocarbons of 2 to 6 aromatic rings. Since the biostimulant has a high content of low molecular weight proteins which are easily assimilated by such microorganisms, the successive applications of the biostimulant to the PAH-polluted soil stimulated PAH-tolerant microorganisms. This accelerated the degradation of PAH in soil, thus reducing its contaminating effect on the soil. The presence of hydrocarbons with a high number of highly toxic aromatic rings did, however, slow down the degradation of those polycyclic aromatic hydrocarbons with fewer aromatic rings.

Notwithstanding the above, and in order to establish with greater precision the bioremedial effect of the said biostimulant, the current results justify further research into using the biostimulant obtained from okara by enzymatic hydrolysis processes at other concentrations and in different types of soils for remedying the effects of used gasoline car engine oil on soil.

Acknowledgments

This work was supported by the Ministerio de Ciencia, Innovación y Universidades (Spain), Plan Nacional I+D+i with reference RTI2018-097425-B-I00.

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Table 1

Chemical characteristics and protein molecular weight distribution (mean ± standard error, n=3) of okaraand biostimulant obtained by enzymatic hydrolysisprocesses

	okara	BS
pH	ND	8.5 ± 0.2
Organic matter (g kg^{-1})	540 ± 19	445 ± 12
N (g kg^{-1})	61.1 ± 2.2	102 ± 13
P (g kg^{-1})	6.6 ± 1.4	5.3 ± 0.3
K (g kg^{-1})	9.4 ± 1.0	9.1 ± 0.5
S (g kg^{-1})	4.0 ± 1.3	24.6 ± 1.1
Ca (g kg^{-1})	1.6 ± 0.3	2.5 ± 0.4
Mg (g kg^{-1})	2.2 ± 0.7	27.8 ± 2.5
Fe (g kg^{-1})	63.6 ± 5.1	64.6 ± 2.7
Cu (mg kg^{-1})	10.7 ± 1.1	13.7 ± 1.3
Mn (mg kg^{-1})	31.0 ± 2.4	26.9 ± 1.4
Zn (mg kg^{-1})	27.8 ± 2.4	24.0 ± 1.4
Protein molecular weight distribution (Da)		
> 10000	90.7 ± 3.4	5,6 ± 1,6
10000 – 5000	1.3 ± 0.4	4.1 ± 1.1
5000 – 1000	1.6 ± 0.3	16.7 ± 3.1
1000 – 300	0.5 ± 0.1	17.5 ± 2.3
< 300	5.9 ± 1.6	56.1 ± 2,5

ND: non determined

Table 2

Polycyclic aromatic hydrocarbons and total heavy metal contents (mean ± standard error, n=3) in motor oil

Polycyclic aromatic hydrocarbons(mg kg^{-1})		Heavy metals(mgkg^{-1})	
Naphthalene	494.0 ± 13.2	Fe	47.4 ± 5.1
Acenaphthylene	11.5 ± 2.4	Cu	6.6 ± 1.3
Acenaphtene	2.1 ± 0.8	Mn	1.2 ± 0.2
Fluorene	122 ± 2.6	Zn	7.6 ± 1.1
Phenantrene	106 ± 3.9	Ni	0.43 ± 0.05
Anthracene	29.4 ± 2.6	Cr	0.19 ± 0.03
Fluoranthrene	108.0 ±2.1	As	0.58 ± 0.1
Pyrene	73.3 ± 5.9	Cd	0.17 ± 0.06
Benzo(a)anthracene	32.6 ± 2.6	Pb	4.6 ± 1.1
Chrysene	50.5 ± 4.4	Al	63.4 ± 2.6
Benzo(b)fluoranthrene	41.4 ± 3.8		
Benzo(k)fluoranthrene	18.6 ± 1.6		
Benzo(a)pyrene	43.8 ± 4.5		
Dibenzo(a, h)anthracene	6.9 ± 1.4		
Indene(1, 2, 3 cd)pyrene	26.8 ± 3.6		
Benzo(g, h, i)perylene	32.3 ± 2.2		

Table 3

Detection limit and quantification limit per PAH

	Detection limit (µg kg)	Quantification limit (µg kg)
Naphthalene	0.0093	< 0.5
Acenaphthylene	0.0144	< 0.1
Acenaphtene	0.0046	< 0.1
Fluorene	0.0249	< 0.1
Phenantrene	0.0282	< 0.1
Anthracene	0.0315	< 0.1
Fluoranthrene	0.0300	< 0.1
Pyrene	0.0267	< 0.1
Benzo(a)anthracene	0.0063	< 0.1
Chrysene	0.0078	< 0.1
Benzo(b)fluoranthrene	0.0295	< 0.1
Benzo(k)fluoranthrene	0.0273	< 0.1
Benzo(a)pyrene	0.0087	< 0.1
Dibenzo(a, h)anthracene	0.0078	< 0.1
Indene(1, 2, 3 cd)pyrene	0.0129	< 0.1
Benzo(g, h, i)perylene	0.0099	< 0.1

Table 4

Evolution of dehydrogenase activity (μg INTF g^{-1} h^{-1}) (mean ± standard error, n=3) in soils amended with the experimental edaphic biostimulants and polluted with car gasoline motor oil during the experimental period

	3	13	16	27	30	41	44	55	58	69	72	86
C	3.4b ± 0.8	3.2b ± 0.5	3.2b ± 0.7	3.5b ± 0.9	3.1b ± 0.5	3.3b ± 0.3	3.5b ± 0.4	3.2b ± 0.8	3.1b ± 0.4	3.5b ± 0.9	3.3b ± 0.2	3.2b ± 0.6
C+O	0.97a ± 0.10	1.3a ± 0.2	1.3a ± 0.3	1.6a ± 0.3	1.6a ± 0.2	1.4a ± 0.3	1.5a ± 0.4	1.5a ± 0.2	1.4a ± 0.2	1.3a ± 0.3	1.3a ± 0.1	1.2a ± 0.2
C+BS	11.4c ± 2.1	3.4b ± 0.4	12.6c ± 1.1	4.0b ± 0.7	20.6d ± 2.9	10.1 c± 1.1	37.0de ± 3.4	11.4c ± 2.0	55.1e ± 4.8	14.5c ± 1.8	64.1e ± 4.1	12.7c ± 2.1
C+O+BS	4.4b ± 1.4	2.1ab ± 0.3	8.5c ± 1.8	2.0a ± 0.2	11.4c ± 2.2	2.8b ± 0.4	17.6d ± 2.4	2.7b ± 0.3	24.9d ± 1.7	2.8b ± 0.2	29.6d ± 3.2	2.8b ± 0.3

Columnsfollowedbythesameletter(s) are notsignificantlydifferent (p> 0.05)

INTF: 2-*p*-iodo-3-nitrophenyl formazan

Table 5

Content of PAHs (mean ± standard error, n=3) in motor oil and evolution of PAHs (mean ± standard error, n=3) in soils amended with the experimental edaphic biostimulants and polluted with car gasoline motor oil during the experimental period

PAH (number of aromatic rings)	3 days		47 days		89 days	
	C+O ($\mu g\ kg^{-1}$)	C+O+BS ($\mu g\ kg^{-1}$)	C+O ($\mu g\ kg^{-1}$)	C+O+BS ($\mu g\ kg^{-1}$)	C+O ($\mu g\ kg^{-1}$)	C+O+BS ($\mu g\ kg^{-1}$)
Naphthalene (2)	4736a ± 3.4	4603a ± 11	4699a ± 17	1482b ± 23	4705a ± 14	1222b ± 26
Acenaphthylene (2)	104a ± 6	91.2a ± 9	102a ± 9	57.9b ± 11	101a ± 8	40.8b ± 14
Acenaphtene (3)	18.9a ± 1.3	16.5a ± 2.4	18.7a ± 1.5	10.4b ± 2.7	18.4a ± 1.3	7.6b ± 2.2
Fluorene (3)	1193a ± 10	1097a ± 16	1176a ± 12	684b ± 18	1164a ± 12	493c ± 17
Phenantrene (3)	986a ± 14	961a ± 18	970a ± 10	554b ± 20	958a ± 16	399c ± 20
Anthracene (3)	279a ± 13	260a ± 15	276a ± 12	152b ± 14	272a ± 10	117b ± 13
Fluoranthrene (4)	994a ± 10	981a ± 13	983a ± 9	654b ± 15	975a ± 8	547b ± 18
Pyrene (4)	720a ± 11	706a ± 19	714a ± 13	487b ± 23	707a ± 12	390b ± 16
Benzo(a)anthracene (4)	314a ± 8	302a ± 12	311a ± 12	203b ± 15	308a ± 10	168b ± 15
Chrysene (4)	489a ± 11	473a ± 18	485a ± 12	325b ± 16	481a ± 7	267c ± 19
Benzo(b)fluoranthrene (5)	400a ± 8	389a ± 14	397a ± 14	296b ± 11	395a ± 11	275b ± 14
Benzo(k)fluoranthrene (5)	175a ± 7	168a ± 10	175a ± 11	131b ± 13	173a ± 10	118b ± 18
Benzo(a)pyrene (5)	423a ± 11	415a ± 16	421a ± 10	313b ± 14	417a ± 9	288b ± 15
Dibenzo(a, h)anthracene (6)	257a ± 9	254a ± 7	256a ± 8	214b ± 10	256a ± 6	199b ± 9
Indene(1, 2, 3 cd)pyrene (6)	58.1a ± 7	55.2a ± 9	57.3a ± 8	48.0b ± 7	57.0a ± 8	43.6b ± 6
Benzo(g, h, i)perylene(6)	310a ± 6	307a ± 7	311a ± 5	259b ± 8	310a ± 6	241b ± 9

Columns followed by the same letter(s) are not significantly different ($p > 0.05$)

Table 6

Soil total heavy metal contents(mean ± standard error, n=3)at the beginning and end of the incubation period for soilsamendedwiththe experimental edaphic biostimulants and pollutedwithcar gasoline motor oilduringthe experimental period

	C		C+O		C+BS		C+O+BS	
	3 days	86 days	3 days	86 days	3 days	86 days	3 days	86 days
Fe (mg kg^{-1})	15879a ± 103	15933a ± 112	15948a ± 155	15996a ± 147	15903a ± 122	15892a ± 102	15939a ± 133	15924a ± 156
Cu (mg kg^{-1})	17.7a ± 1.6	18.2a ±2.1	17.9a ± 1.3	17.9a ± 1.6	18.1a ± 1.4	18.0a ± 1.6	17.7a ± 2.0	18.0a ± 1.1
Mn (mg kg^{-1})	189a ±32	192a ±35	188a ± 26	190a ± 17	190a ±32	189a ±31	191a ± 26	189a ± 20
Zn (mg kg^{-1})	27.8a ±4.1	26.9a ±5.7	28.2a ±2.3	27.5a ± 2.5	27.4a ±3.4	26.0a ±4.1	28.0a ± 2.8	27.7a ± 3.1
Ni (mg kg^{-1})	21.8a ±1.3	21.4a ±1.2	22.0a ± 1.4	21.9a ± 1.8	21.5a ±1.3	21.8a ±1.8	22.0a ± 1.7	21.7a ± 1.8
Cr (mg kg^{-1})	38.4a ± 1.8	39.0a ± 2.2	38.0a ± 1.6	37.6a ± 2.0	38.6a ± 2.0	37.4a ± 1.7	39.3a ± 2.1	37.9a ± 1.7
As (mg kg^{-1})	0.55a± 0.10	0.56a ± 0.12	0.53a ± 0.14	0.52a ± 0.17	0.53a ± 0.11	0.56a ± 0.14	0.55a ± 0.09	0.54a ± 0.11
Cd (mg kg^{-1})	0.33a± 0.18	0.36a ±0.10	0.29a ± 0.12	0.30a ± 0.10	0.30a ± 0.6	0.34a ± 0.9	0.36a ± 1.0	0.38a ± 0.13
Pb (mg kg^{-1})	1.3a± 0.2	1.2a ± 0.3	1.3a ± 0.2	1.3a ± 0.1	1.4a ± 0.4	1.3a ± 0.5	1.4a ± 0.2	1.5a ± 0.3
Al (mg kg^{-1})	2.2a± 0.4	2.5a ± 0.6	2.5a ± 0.3	2.4a ± 0.5	2.0a ± 0.3	2.2a ± 0.5	2.3a ± 0.2	2.4a ± 0.3

Files followed by the same letter(s) are not significantly different ($p > 0.05$)

Table 7

Evolution of pH in soils amended with the experimental edaphic biostimulants and polluted with car gasoline motor oil during the experimental period

	3	13	16	27	30	41	44	55	58	69	72	86
C	7.9a ± 0.1	8.1a ± 0.2	7.9a ± 0.2	8.0a ± 0.1	7.9a ± 0.2	8.1a ± 0.3	8.1a ± 0.1	7.9a ± 0.2	8.2a ± 0.3	8.0a ± 0.2	7.8a ± 0.2	8.0a ± 0.1
C+O	8.1a ± 0.2	8.3a ± 0.2	8.3a ± 0.3	8.0a ± 0.1	8.3a ± 0.2	8.2a ± 0.2	8.2a ± 0.2	8.2a ± 0.2	8.0a ± 0.1	8.3a ± 0.3	8.1a ± 0.3	8.4a ± 0.3
C+BS	8.2a ± 0.1	8.2a ± 0.2	8.4a ± 0.2	8.4a ± 0.3	8.2a ± 0.3	8.4a ± 0.2	7.9a ± 0.2	8.4a ± 0.3	8.3a ± 0.2	8.4a ± 0.2	7.9a ± 0.1	8.1a ± 0.3
C+O+BS	8.2a ± 0.2	8.4a ± 0.3	8.1a ± 0.2	8.4a ± 0.2	8.1a ± 0.1	8.3a ± 0.3	8.0a ± 0.1	8.4a ± 0.1	8.2a ± 0.2	8.0a ± 0.2	8.3a ± 0.3	8.0a ± 0.2

Columns followed by the same letter(s) are not significantly different ($p > 0.05$)

***Highlights (for review)**

- Used motor-car oil caused a negative effect on soil dehydrogenase activity
- Applying the okara biostimulant to polluted soil increased PAH degradation
- Applying the okara biostimulant to polluted soil no affected the heavy metals degradation

Use of a biostimulant obtained from okara in the bioremediation of a soil polluted by used motor car oil

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Abstract

In this manuscript we studied in the laboratory the bioremediation effects of a biostimulant obtained from okara by enzymatic hydrolysis processes in a soil polluted with used motor-car oil at a rate of 1% (w/w) over an 89-day period. The biostimulant was added to the soil 6 times during the incubation period at a rate of 2%. Dehydrogenase activity and the evolution of polycyclic aromatic hydrocarbons (PAHs) and pseudo total heavy metals in soil were studied. The successive applications of the biostimulant to the polluted soil gradually increased PAHs degradation during the experimental period. Thus, at the end of the experiment, the application of the biostimulant decreased the concentration of naphthalene in soil by 74%, while PAHs with 3, 4, 5 and 6 aromatic rings had been reduced by around 58%, 44%, 30% and 23%, respectively. This degradation is possibly due to the high number of low molecular weight peptides (<300 Da) in the biostimulant which are readily available for PAHs-tolerant soil microorganisms that accelerate the degradation of the said toxins. The concentration of heavy metals in the oil used was not very high and consequently the dehydrogenase activity was not negatively affected.

Keywords: okara biostimulant; used motor-car oil; polycyclic aromatic hydrocarbons; total heavy metals; soil dehydrogenase activity

1. Introduction

At present the use of lubricating oils is widespread because they are necessary for the correct working of motor vehicle engines and other machinery such as pumps, hydraulic motors, compressors and electrical transformers [1]. Due to the physical and chemical reactions that occur during use, used motor oil is characterised by its high content of aromatic and aliphatic hydrocarbons whose chain lengths range from C15 to C50 and heavy metals that

could contribute to the risk of chronic diseases, including mutagenicity and carcinogenicity [1-3].

As a result of their widespread use, terrestrial ecosystems are becoming increasingly contaminated by such lubricants. The bioremediation of soils contaminated with used motor oils is, therefore, an efficient and effective environmental technique for accelerating the cleaning processes [2, 4].

In recent years, applying organic biostimulants obtained from sewage sludge, chicken feathers, wheat condensed distiller's soluble enzymatic hydrolysate, rice bran extract, carob germ enzymatic extract, etc. by enzymatic hydrolysis processes to soils polluted by organic xenobiotics has been a widely-used environmental technique for accelerating the degradation of the said xenobiotics in soil. The low molecular weight proteins, found in large quantities in these biostimulants, are absorbed by the xenobiotics-tolerant microorganisms, increasing both their proliferation and the biochemical activity of the soil, consequently accelerating the degradation of the toxins in the soil [5-8].

Okara is a by-product obtained from the manufacture of soy milk, tofu and their derivatives. It has both a high fibre (56%) and protein (29%) content. Orts et al. [9] obtained a new biostimulant from okara by enzymatic hydrolysis processes using the pH-stat technique in order to remedy a soil polluted with chlorpyrifos. Eighty days after applying the biostimulant to polluted soil, the insecticide had been completely degraded. Consequently, this biostimulant obtained from okara could be of great use for remedying a soil contaminated by motor oil with a high content of polycyclic aromatic hydrocarbons (PAHs).

It should be noted, however, that the effectiveness of these biostimulants obtained by enzymatic hydrolysis processes has been verified after only a single application to polluted soil. It is not known what the behaviour of organic xenobiotics in the polluted soil would be after repeated applications of these biostimulants.

There is an abundant literature indicating that soil biological parameters evolve faster than physical and chemical parameters [6, 10-12]. There is also a lot of information on how PAHs and heavy metals negatively affect soil enzymatic activities, as well as affecting its microbial population [8, 11, 13, 14]. For this reason, a study of these biological properties would be very useful in order to verify the bioremediation capacity of organic matter in polluted soils.

According to Kaczynski et al. [15] and Liu et al. [16], the oxidation-reduction enzymes found in soil play a fundamental role in degrading contaminants, transforming organic matter and in maintaining the metabolism of microorganisms. In this respect, dehydrogenase activity (EC 1.1.1) is an essential intracellular oxidoreductase enzyme in living cells. It participates in redox reactions, it promotes the dehydrogenation of soil organic matter and transmits energy to hydrogen acceptors through the respiratory chain [17]. Due to the above, this enzyme is now used as a soil contamination status bioindicator [18, 19, 20].

The objective of this study, therefore, was to verify the bioremediation efficiency of a biostimulant, obtained from okara by enzymatic hydrolysis processes, in a soil polluted by used motor oil after repeated applications of the said biostimulant to the polluted soil and its influence on the soil dehydrogenase activity.

2. Material and methods

2.1. Soil, biostimulant and motor oil characteristics

The soil used in the study was classified as Calcaric Regosol [21], with 574 ± 31g kg^{-1} sand, 148 ± 35 g kg^{-1} silt, and 278 ± 24 g kg^{-1} clay. Soil pH was 7.9 ± 0.1, 16.4 ± 1.3 gkg^{-1} organic matter, and 0.98 ± 0.05 g kg^{-1} N. The methodology used for determining the soil parameters is described in Tejada et al. [7] and Rodríguez-Morgado et al. [22].

The okara from which the new biostimulant was manufactured was provided by the Soria Natural S.A company. The experimental biostimulant was obtained from the okara by enzymatic hydrolysis using an endoprotease obtained by liquid fermentation of Bacillus licheniformis ATCC 21415. Table 1 shows the chemical characteristics of both the okara and the resulting biostimulant. The characteristics of this process of enzymatic hydrolysis are detailed in Orts et al. [9]. The methodology used for determining organic matter, macro- and micronutrients, and molecular protein mass distribution is described in Rodríguez-Morgado et al. [22].

The motor oil used was a sample of gasoline automobile engine oil taken when the vehicle's oil was changed at 15000 km. Table 2 shows the content of polycyclic aromatic hydrocarbons and total heavy metals in the motor oil.

The PAHs were detected by a GC 7890B Agilent gas chromatograph connected to a MS Triple Q Agilent 7000C mass detector. The analysis conditions were the following:

Column: DB-EUPAH 30X0.25X0.25

Injection: 2 µl

Column pressure: 15.7 psi

Constant flow: 1.2 ml min^{-1}

Injection mode: Splitless 50 psi

Injection temperature: 310 °C

Initial temperature: 80 °C for 1 minute

Temperature ramp: 25 °C $minute^{-1}$ to 200 °C for 8 minutes; 5 °C $minute^{-1}$ to 305 °C for 9 minutes; 50 °C $minute^{-1}$ to 330 °C for 3 minutes; (48.5 total minutes)

Table 3 shows the detection limit and quantification limit per each PAH. The internal standards used in GC/MS study were: acenaphthene d8, chrysene d10, naphthalene d8, perylene d12 and phenatrene d10. Recovery analyses were carried out in every treatment, showing values of between 70 and 80%.

The concentration of heavy metals in the engine oil was determined following US EPA method 3052. For this, 0.5 g of oil was digested in 9 ml of HNO_3 and 3 ml of HCl using a microwave digester. Digests were filtered through 0.45 mm Millipore filters and diluted appropriately for analysis by a Thermo Elemental ICP-MS X7 Inductively Coupled Plasma Mass Spectrometry system (Thermo Fisher, Cambridge, UK) with quadrupole mass analyser, multichannel detector (Pulse Counting and Analogue Methods), auto sampler ASX-500 (CETAC, Omaha, NE, USA). The measurement accuracy (%) of each heavy metal was as follows: Fe: 2.2; Cu: 5.2; Mn: 2.5 Zn: 3.4; Ni: 4.0; Cr: 4.7; As: 1.1; Cd: 3.9; Pb: 5.0; and Al: 3.1.

2.2. Experimental layout

Five hundred grams of dried, sieved (<2 mm) soil were preincubated at 25 °C for 7 days at 30-40 % of their holding capacity [5]. After this pre-incubation period, soil samples were mixed with gasoline motor oil at a rate of 1% (w/w) in 1-L glass bottles., Motor oil was dissolved in acetone as a carrier solvent at a concentration of 30 mL kg^{-1} according to Ramadass et al. [3]. Acetone was also added to the non-polluted soils (control treatment). The biostimulant was added to the soil 6 times at a rate of 2% once at the beginning of the experiment and then on days 13, 27, 41, 55 and 69 during the incubation period. The biostimulant used was liquid and was solubilised in distilled water (500 l ha^{-1}) before application. Distilled water was added to each soil to bring it to 60% of its water-holding

capacity. An unamended, polluted and an amended, non-polluted soil were used as controls. The incubation treatments are detailed as follows:

1. C, control soil, non-polluted soil and non-organically amended
2. C+O, polluted soil with used motor oil and non-organically amended
3. C+BS, non-polluted soil and amended with the biostimulant
4. C+O+BS, polluted soil with used motor oil and amended with the biostimulant

Triplicate treatments were kept in semi-closed microcosms at 25 ± 1 °C for 50 days, respectively. The moisture content was controlled gravimetrically and moisture loss was replaced as necessary by distilled water.

2.3. Soil analysis

For each experimental treatment, dehydrogenase activity was measured at days 3, 13, 16, 27, 30, 41, 44, 55, 58, 69, 72 and 86 during incubation experiment. The soil samples from days 3, 13, 27, 41, 55 and 69 were selected and analysed before applying the okara biostimulant to the soil.

The method of Trevors et al. [23] as modified by García et al. [24] was used to measure dehydrogenase activity, reducing 2-*p*-iodophenyl-3-*p*-nitro-phenyl-5-phenyltetrazolium chloride (INT) to iodonitrophenyl-formazan (INTF). This was measured in a spectrophotometer at 490 nm.

PAHs were extracted from 30 g of soil. These samples were placed in small glass jars with 10 g of anhydrous sodium sulphate in order to remove moisture from the soil samples. 50 ml of a dichloromethane/pentane 1/1 mixture were then added and the mix was shaken for 30 seconds. The samples were then sonicated for 10 minutes and filtered through anhydrous

sodium sulphate. The total volume extracted was concentrated in a rotary evaporator at a temperature of 50 °C. This process was performed in triplicate. Finally, the volume of the extract was adjusted to 1 mL with isooctane.

Once the PAHs had been extracted, they were measured according to the instruments and conditions previously described.

At days 3 and 86 after the start of the incubation process, the pseudo total heavy metal content in soil was determined, the method of determination being highly similar to that described above. Using a microwave digester, 5 g of soil were digested in 9 mL of HNO_3 and 3 mL of HCl. Digests were filtered through 0.45 mm Millipore filters and appropriately diluted for posterior analysis using the Thermo Elemental ICP-MS X7 Inductively Coupled Plasma Mass Spectrometry system.

2.4. Statistical analysis

With the data obtained from each soil analysis and using Statgraphics Plus 2.1 software package, a two-way analysis of variance (ANOVA) was performed with treatment and sampling time as factors, followed by Tukey's significant difference as a *post hoc* test, considering a significance level of $p < 0.05$ throughout the study. For the ANOVA, triplicate data were used for each treatment and for each day of incubation.

3. Results and discussion

The continuous application of the biostimulant to the non-polluted soil during the experimental period caused an increase in dehydrogenase activity (Table 4). In other experiments carried out by Rodriguez-Morgado et al. [8, 25] and Orts et al. [9], performing a single application to the soil of biostimulants obtained from sewage sludge, chicken feathers and okara by enzymatic hydrolysis processes, it was observed that the dehydrogenase activity

showed maximum stimulation during the first 7 days after application. The dehydrogenase activity then declined progressively until reaching values similar to the non-organically amended soil. According to these authors, the main reason for the behaviour of these biostimulants is a consequence of the protein size distribution obtained in said organic compounds, such behaviour highlighting the influence/high content of the low molecular weight proteins (<300 Da), which are in the majority. The higher content of low molecular weight proteins suggests that N is in a more readily-available form for soil organisms, promoting a greater proliferation of soil microorganisms and, consequently, dehydrogenase activity.

In our experiment, this increase in dehydrogenase activity at the end of the incubation period may be due to the constant accumulation of high molecular weight proteins after the continuous application of the experimental biostimulant, thus requiring more time for the microorganisms to degrade them. Table 1 shows that the protein content of molecular weight > 1000 Da is 26.4%, which may be responsible for stimulating soil microbial activity at the end of the incubation period.

The application of motor oil to the soil showed a significant decrease ($p < 0.05$) in dehydrogenase activity during the whole experimental period (Table 4). At the end of the incubation period and compared to C treatment, dehydrogenase activity decreased significantly ($p < 0.05$) by 62.5% in soils polluted with used motor oil. This inhibition in soil dehydrogenase activity is possibly a consequence of adding different PAHs with 2 to 6 aromatic rings in the used motor oil. These results are in agreement with those obtained by Tejada et al. [6], Rodríguez-Morgado et al. [8] and Lipińska et al. [19], who found that dehydrogenase activity was inhibited in soils polluted by various PAHs due to their toxicity to soil microorganisms. According to these authors, this toxic effect inhibits an abundant population of soil microorganisms and, consequently, reduces soil dehydrogenase activity.

Table 5 shows the evolution of PAHs in soil during the experimental period after polluting it with automobile motor oil. In non-organically amended soils, the concentration of the PAHs remained highly similar throughout the incubation period. Applying the experimental biostimulant to polluted soil increased PAH degradation. This degradation was gradual throughout the experimental period and was greater among those hydrocarbons with the lowest number of aromatic rings. Thus, at day 47 of the incubation period and in comparison with the C+O treatment, the naphthalene concentration decreased significantly ($p < 0.05$) by 68.5% in the C+O+BS treatment, while the hydrocarbons with 3, 4, 5 and 6 aromatic rings decreased by values of around 43%, 33%, 25% and 16%, respectively. At the end of the experimental period, and in comparison with the C+O treatment, naphthalene concentration in the C+O+BS treatment had decreased significantly ($p < 0.05$) by 74%, while concentrations of hydrocarbons with 3, 4, 5 and 6 aromatic rings had decreased by around 58%, 44 %, 30% and 23%, respectively.

There is an abundant bibliography that suggests that PAH degradation in soil is a very slow process carried out by microorganisms conditioned by the number of aromatic rings in the hydrocarbon's structure [8, 26, 27]. According to Edokpayi et al. [28], as the molecular weight of the PAH increases, solubility decreases, increasing in turn environmental persistence and toxicity. For this reason as the number of aromatic rings in its chemical structure increases, PAH degradation decreases.

Applying organic matter to the soil accelerates PAH degradation [6, 8, 27, 29]. According to these authors, soil microorganisms are responsible for PAH degradation. In this sense, applying organic matter to soil contaminated by PAHs stimulates the PAH-tolerant microorganisms, favouring PAH degradation and thus reducing their polluting effect on the soil. In our experiment, successive applications of the experimental biostimulants throughout

the incubation period decreased progressively the soil PAHs concentration. Obviously, the degradation was greater in those PAHs with a lower aromatic number.

In comparison with our experiment, Tejada et al. [6] and Rodriguez-Morgado et al. [8] found a higher degradation percentage of phenanthrene, pyrene and benzo (a) pyrene after applying to soil different edaphic biostimulants obtained by enzymatic hydrolysis processes from rice bran, wheat condensed distiller's soluble, sewage sludge and chicken feathers. In our experiment the soil was polluted by a varied mixture of PAHs, presenting from 2 to 6 aromatic rings. Consequently, although applying the experimental biostimulants accelerated degradation of those PAHs with fewer aromatic rings, the high concentration of PAHs with a greater number of aromatic rings, mainly those with 5 and 6 aromatic rings, possibly continued to exert a very toxic effect on soil microorganisms, as reflected by soil dehydrogenase activity (Table 4).

Table 6 shows the evolution of the pseudo total heavy metals content in the experimental treatments during the incubation period. The pseudo total heavy metals contents in treatments C and C+BS were very similar on days 3 and 86 of the experiment, suggesting that the pseudo total heavy metals concentration in the polluted and organically-amended soil did not vary with respect to the non-amended and non-polluted soil. In the same way, and as previously mentioned, the dehydrogenase activity in the C+BS treatment was stimulated when compared to treatment C.

A large number of references indicate that a high concentration of heavy metals in the soil solution can inhibit its dehydrogenase activity [11, 30, 31]. Our experiment measured the pseudo total heavy metals content in soil, not merely the content of metals available. Since the dehydrogenase activity was not inhibited in the C+BS treatment, we believe that the heavy metals concentration in the biostimulant applied to the non-polluted did not exert any toxic effect on the above enzymatic activity.

With respect to the C treatment, the concentration of pseudo total heavy metals in the C+O treatment did not vary significantly during the incubation period. This suggests that, possibly, the heavy metal contents in the experimental car motor oil used in our experiment did not have any negative effects on the soil dehydrogenase activity. Therefore PAH content alone would be the cause of soil toxicity. These results do not coincide with those obtained by Dike et al. [1] and Wang et al. [32], who suggest that the toxic effect of used motor oil is mainly a consequence of both the PAHs content and the heavy metals.

According to Ramadass et al. [3], the concentration of heavy metals in a used motor oil is the result of wear and tear in the engine components and the heating and oxidation of the lubricating oil. We believe that the concentration of heavy metals in the used motor oil depends on how the motor has been used. In our experiment the oil was sourced from a 15000-km oil change performed on gasoline car engine. Such a short period of use may not be sufficient for any great amount of heavy metals to be transferred from the engine to the oil.

Applying the biostimulant to the oil-polluted soil showed heavy metal values very similar to those of treatment C, which suggests that applying the experimental biostimulants to the polluted soil did not produce any significant change to its heavy metal content.

Applying the biostimulant to the non-polluted soil did not change the pH value (Table 7). The basic nature of the biostimulant, together with the soil pH value are sufficient reason for a change in the pH of said soil not to occur. On the other hand, neither did applying the biostimulant to the oil-polluted soil change the pH. These results suggest that the concentration of heavy metals, acidic in character, is low in the experimental oil. In addition, it must also be taken into account that the amount of oil applied to the soil was not very high, so the heavy metal concentrations added to the soil was also low. Regarding the PAH concentration in the oil, it should be noted that these chemical compounds do not have an acidic character and, therefore, it also justifies the lack of change in soil pH.

4. Conclusions

It can be concluded that the toxic effect of used gasoline car engine oil is mainly due to its content of polycyclic aromatic hydrocarbons of 2 to 6 aromatic rings. Since the biostimulant has a high content of low molecular weight proteins which are easily assimilated by such microorganisms, the successive applications of the biostimulant to the PAH-polluted soil stimulated PAH-tolerant microorganisms. This accelerated the degradation of PAH in soil, thus reducing its contaminating effect on the soil. The presence of hydrocarbons with a high number of highly toxic aromatic rings did, however, slow down the degradation of those polycyclic aromatic hydrocarbons with fewer aromatic rings.

Notwithstanding the above, and in order to establish with greater precision the bioremedial effect of the said biostimulant, the current results justify further research into using the biostimulant obtained from okara by enzymatic hydrolysis processes at other concentrations and in different types of soils for remedying the effects of used gasoline car engine oil on soil.

Acknowledgments

This work was supported by the Ministerio de Ciencia, Innovación y Universidades (Spain), Plan Nacional I+D+i with reference RTI2018-097425-B-I00.

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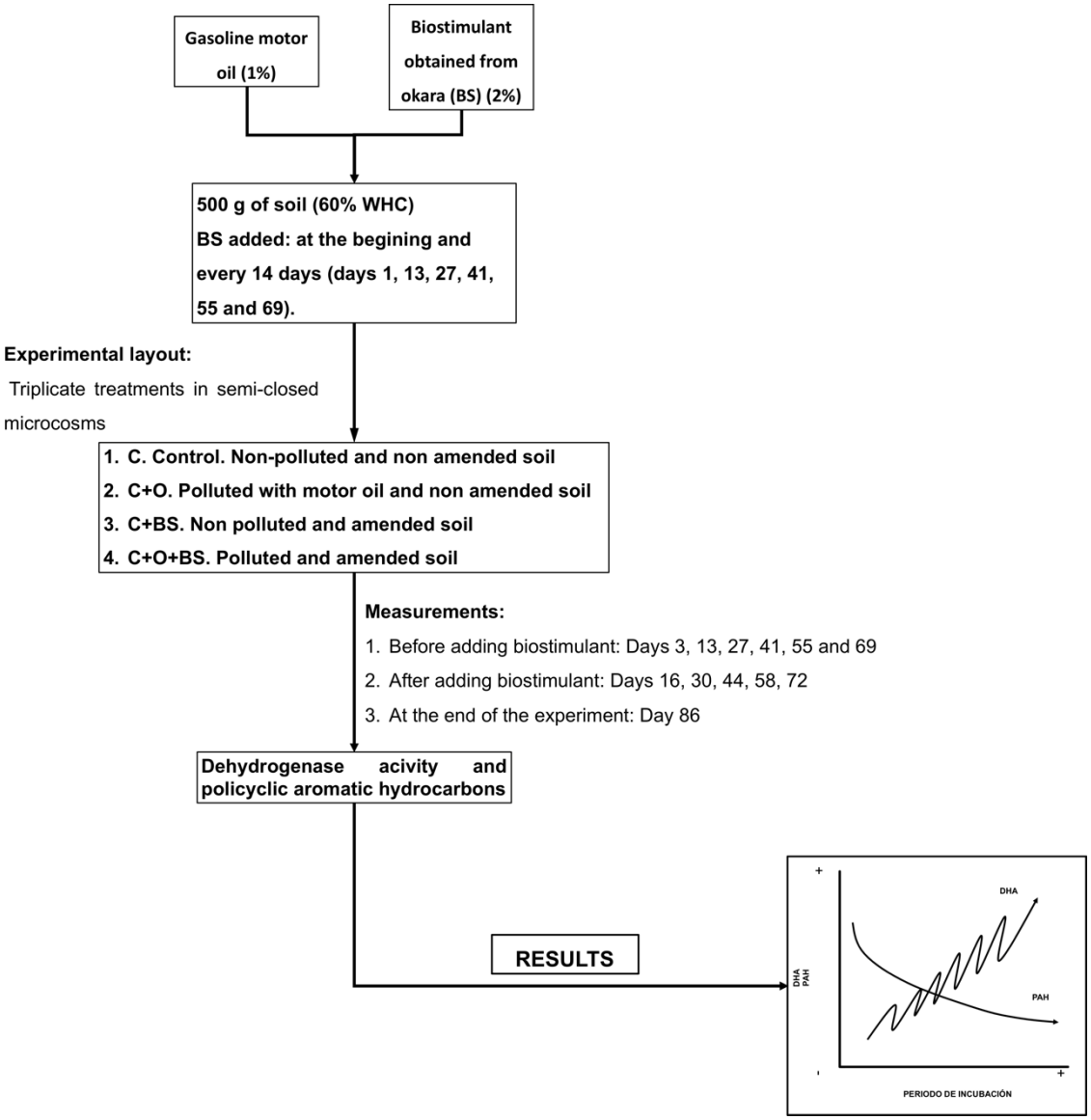
***Graphical Abstract**



***Declaration of Interest Statement**

The manuscript has no conflict of interest

***Author Contributions Section**

All authors have contributed to the manuscript in the same way