Could saponins be used to enhance bioremediation of polycyclic aromatic hydrocarbons in aged-contaminated soils?

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Abstract

Polycyclic aromatic hydrocarbons (PAH) are persistent organic compounds of major concern that tend to accumulate in the environment, threatening ecosystems and health. Brownfields represent an important tank for PAHs and require remediation.

Researches to develop bioremediation and phytoremediation techniques are being conducted as alternatives to environmentally aggressive, expensive and often disruptive soil remediation strategies.

The objectives of the present study were to investigate the potential of saponins (natural surfactants) as extracting agents and as bioremediation enhancers on an aged-contaminated soil. Two experiments were conducted on a brownfield soil containing 15 PAHs. In a first experiment, soil samples were extracted with saponins solutions (0; 1; 2; 4 and 8 $g.L^{-1}$). In a second experiment conducted in microcosms (28°C), soil samples were incubated for 14 or 28 days in presence of saponins (0; 2.5 and 5 $mg.g^{-1}$). CO_2 emissions were monitored throughout the experiment. After the incubation, dehydrogenase activity was measured as an indicator of microbiological activity and residual PAHs were determined. In both experiments PAHs were determined using High-Performance Liquid Chromatography and Fluorimetric Detection.

The 4 $g.L^{-1}$ saponins solution extracted significantly more acenaphtene, fluorene, phenanthrene, anthracene, and pyrene than water. PAHs remediation was not enhanced in presence of saponins compared to control samples after 28 days. However CO_2 emissions and dehydrogenase activities were significantly more important in presence of saponins, suggesting no toxic effect of these surfactants towards soil microbiota.

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Keywords

PAH; saponin; extraction; bioremediation; soil; brownfield

Abbreviations

Ace	Acenaphtene
Anthr	Anthracene
BaA	Benzo(a)anthracene
BaP	Benzo(a)pyrene
BbF	Benzo(b)fluoranthene
BghiP	Benzo(ghi)perylene
BkF	Benzo(k)fluoranthene
Chrys	Chrysene
CMC	Critical Micellar Concentration
DBahA	Dibenzo(ah)anthracene
DMSO	Dimethylsulfoxide
DW	Dry Weight
F	Fluoranthene
Fle	Fluorene
IcdP	Indeno(123-c,d)pyrene
INTF	Iodonitrotetrazolium formazan
N	Naphtalene
PAH	Polycyclic Aromatic Hydrocarbon
Phen	Phenanthrene
Pyr	Pyrene

SDS	Sodium dodecyl sulfate
VI	Intervention value (of the Walloon legislation: pollutant content over which brownfield soils are to be systematically cleaned-up)
VR	Reference value (of the Walloon legislation: natural background of a pollutant, ideal value to reach when there is a soil remediation)

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous organic compounds that are brought in the environment through natural and anthropogenic incomplete combustions that occur during forest fires, industrial manufacturing, fossil fuel use, or waste incineration [Johnsen *et al.*, 2005]. PAHs are composed of two or more condensed aromatic rings, and are characterized by high hydrophobicity and low aqueous solubility [Lakra *et al.*, 2013]. Once emitted in the air or in water, those compounds can accumulate on solid phases, making soil and sediments the main receptor for hydrophobic contaminants in general. Furthermore, PAHs present multiple health-concerning properties such as mutagenicity, carcinogenicity or teratogenicity, explaining why they have been of major concern [Zhang *et al.*, 2006]. They are classified in two main categories: the low molecular weight PAHs, including molecules bearing three rings or less (naphthalene, acenaphtene, fluorene, phenanthrene, and anthracene) and the high molecular weight PAHs, including molecules of four rings or more (fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, dibenzo(ah)anthracene, benzo(ghi)perylene, and indeno(123-c,d)pyrene) [Megharaj *et al.*, 2001; Von Lau *et al.*, 2014].

Many remediation strategies have been applied to contaminated soils but often they turn out to be environmentally aggressive, expensive and disruptive towards soil. Some techniques even tend to postpone the treatment of the pollutants by either confining or translocating them to

another environmental compartment (air or water). Bioremediation is a process relying on microorganisms, plants or their respective enzymes to degrade pollutants [Megharaj *et al.*, 2001].

The bioremediation mechanisms are influenced by pollutants availability to soil microorganisms (and their degrading enzymes) and the microbiota global fit. The pollutants availability greatly depends upon their physico-chemical properties (e.g. aqueous solubility, hydrophobicity, and molecular structure). Environmental factors (like organic matter and clay minerals can chemically or physically segregate the compounds) influence this availability by decreasing the accessibility to degrading agents. Furthermore, interacting factors such as pH, salinity, water content, temperature, redox potential, and water-dissolved oxygen and mineral nutrients will provide conditions more or less favourable to the activity of the degrading agents [Masciandaro *et al.*, 2013].

The bioavailability number has been defined as "the rate of mass transfer of a compound to a microbial cell to the rate of uptake and metabolism i.e. the intrinsic activity of the cell" [Bosma *et al.*, 1997; Johnsen *et al.* 2005]. Therefore, the biodegradation rate is mainly controlled by the mass transfer to the cell or by the cell activity when the ratio is respectively >1 or <1 [Johnsen *et al.*, 2005]

Surfactants are surface-active molecules of amphiphilic nature. When present in an aqueous solution, these compounds can associate into different structures, depending on their nature, their concentration, and abiotic conditions (pH, ionic force, occurrence of solid phases). When present in low concentrations, surfactants remain as monomers and place themselves at the interface between a hydrophobic and a hydrophilic phase (e.g. air and water). Surfactants form micelles (aggregates of monomers) above a defined concentration called critical micellar concentration (CMC) [Lakra *et al.*, 2013]. This surfactant property has been widely investigated over the last decades in order to use surfactants in soil "washing technologies" [Von Lau *et al.*, 2014] or to increase mass transfer of contaminants towards degrading cells [Kobayashi *et al.*, 2012] by

increasing the apparent solubility of PAHs in water. Finally it is noteworthy that when solid phases such as soil are present; surfactants can also aggregate into structures that adsorb onto particles. Two well-known structures are the hemimicelle (a single layer of monomers adsorbed on a solid phase) and the admicelle (similar to the hemimicelle but with a second layer of monomers bond to the first one) [Makkar and Rockne, 2003].

Saponins are a class of natural non-ionic surfactants that are largely distributed in higher plants. They are composed of a sapogenin (hydrophobic) skeleton of either steroidal or triterpenoidal nature coupled to a glycose (hydrophilic) moiety [Oleszek & Bialy, 2006]. Even though saponins are nowadays frequently used in pharmaceutical and cosmetic industries, they originally were employed for their foaming property as natural detergents [Sparg *et al.,* 2004]. Therefore, the potential of saponins to enhance PAHs solubilisation has been investigated in recent studies. Zhou *et al.* (2011) have shown that saponins derived from *Quillaja saponaria* Molina bark are more effective at enhancing apparent solubility of phenanthrene in water than synthetic non-ionic surfactants (Tween 80, Triton X-100 and Brij58) whereas Kobayashi *et al.* (2012) have demonstrated an increase of the apparent hydrosolubility of phenanthrene, pyrene, and benzo(α)pyrene. They also showed that both biodegradation of pyrene and growth of *Sphingomonas* sp were related to the occurrence of saponins. They concluded that saponins had no antimicrobial activity, in spite of some previous experiments reporting that some saponins were capable of inhibiting microbial growth of low-density populations [Killeen *et al.,* 1998]. Finally the same authors reported a removal of freshly-spiked pyrene from soil samples presenting a low organic carbon content (<0.1 %) using aqueous solutions of saponins.

The objective of the study presented herein was to investigate the possibility of using saponins as extracting agent and as bioremediation enhancer on an aged-contaminated soil containing several PAHs. Therefore, two experiments were conducted on a brownfield soil presenting 15 PAHs of interest. The first experiment was conducted to determine whether saponins solutions could

147 extract more PAHs compounds than distilled water. Several concentrations of saponins were
148 tested and extracted concentrations of the 15 PAHs were determined and compared. In the
149 second experiment, contaminated soil was treated with saponins and incubated. Two
150 concentrations of saponins and two incubation periods were tested. Several parameters were
151 examined: (i) the carbon dioxide emission was monitored during the incubation process; (ii) the
152 soil dehydrogenase activity was determined at the end of the incubation period as an indicator of
153 saponins' toxicity towards the microbiota; and (iii) the residual PAHs contents were determined
154 on soil samples after each incubation period.

155 **2. Materials and methods**

156 *2.1.Soil material*

157 The aged-contaminated soil used for this study was sampled on a brownfield in Saint-Ghislain,
158 Belgium in a former coking plant which has been exposed for 70 years to petroleum
159 hydrocarbons, PAHs, cyanides and trace elements. The particle size distribution (81.1 % sand,
160 10.7 % silt, 8.2 % clay) identified the soil as loamy sand. Other characteristics were pH_{H_2O} = 6.7
161 (according to ISO 10390:2005), total organic carbon (according to Springer and Klee, 1954),
162 was 9.44 ± 0.22 % (W/W), and total nitrogen content (according to Bremner, 1982), was
163 0.16 ± 0.02 % (W/W). Soil was sampled, allowed to dry at ambient air, sieved through a 2-mm
164 sieve and stored in sealed boxes until further use. Before the experiments, the contents of 15
165 PAHs were determined to range from 2.9 ± 0.1 mg.kg^{-1}DW to 65.9 ± 7.1 mg.kg^{-1}DW (Table 2).
166 The compounds were naphtalene (N), acenaphtene (Ace), fluorene (Fle), phenanthrene (Phen),
167 anthracene (Anthr), fluoranthene (F), pyrene (Pyr), benzo(a)anthracene (BaA), chrysene (Chrys),
168 benzo(b)fluoranthene (BbF), benzo(k)fluoranthene (BkF), benzo(a)pyrene (BaP),
169 dibenzo(ah)anthracene (DBahA), benzo(ghi)perylene (BghiP), and indeno(123-c,d)pyrene
170 (IcdP). The Belgian Walloon legislation sets the reference value (i.e. the natural background) for

each PAH in soils regardless of their occupation, at 0.01 mg.kg^{-1}DW except N and Phen for which reference values are set at 0.1 mg.kg^{-1}DW. This reference value (VR) is the ideal value to reach when there is a soil remediation. Depending on the soil's occupation (industrial, commercial, residential, agricultural or natural), different intervention values (VI: over which brownfield soils are to be systematically cleaned-up) and threshold values (VS: over which at least a risk assessment and a monitoring must be implemented) have been defined and are available in supplementary data. The experimental soil shows PAHs contents higher than the threshold values for a commercial occupation for the 15 PAHs. All but F are also above the threshold values for an industrial occupation and N, Anthr, BaA, BbF, and BaP are above the intervention values for the industrial occupation [Décret relatif à la gestion des sols, 2009].

2.2.Saponins material and surface-active properties characterization

Crude extracts of saponins (batch number 14L190008) derived from *Quillaja saponaria* bark were purchased from VWR International (Leuven, Belgium) and used without further purification. The total organic carbon and the total nitrogen contents were 42.57 ± 0.22 % and 0.13 ± 0.02 % (W/W) respectively.

The CMC was determined using a Langmuir Kibron film balance composed with a 20 mL teflon tank and a rod used to measure surface pressures. Increasing solutions of raw saponins were prepared in dimethylsulfoxide (DMSO) by dilution of a 100 g.L^{-1} stock solution. 15 µL of solution were injected in ultrapure water (15 mL) in order to reach concentrations from 1 mg.L^{-1} to 100 mg.L^{-1} in the subphase. Changes in surface pressure were recorded until they reached a plateau. The same volume of pure DMSO was injected in the subphase and no change of surface pressure was observed. The measures were taken at a temperature of 25°C. When plotting the evolution of the maximal surface pressure as a function of the saponins concentration, the CMC is the point at which the surface pressure no longer increases with the concentration. This point

was determined as the intersection of two linear regression lines: one fitting the ascending part and one fitting the plateau, as described by Gatard *et al.*, 2013.

2.3.Experimental devices

Extraction experiments

Extraction experiments were conducted in glass flasks. Saponins solutions were prepared in water above the CMC, at respectively 1, 2, 4 and 8 $g.L^{-1}$ and tested as extracting solutions. Distilled water was used as a control. Each extraction was repeated five times. Briefly, 5 g of dry experimental soil were placed at 80 % of water holding capacity and extracted using magnetic stirring with 10 mL of aqueous solution for 24 h, in the dark. The aqueous phase was recovered by filtration. Results related to soil samples extracted by 1, 2, 4 and 8 $g.L^{-1}$ of saponins solutions have been named Sap1, Sap2, Sap4 and Sap8, respectively.

Incubation experiments

Incubation experiments were conducted in microcosms according to the norm AFNOR XP U44-163. Soil humidity conditions were chosen according to Barnier (2009) and Louvel (2010). Briefly, 15 g of dry experimental soil were placed at 80 % of water holding capacity and allowed to pre-incubate for 3 days. Once saponins were added to samples, two vessels were placed next to each sample in a sealed jar. One vessel was filled with distilled water to prevent soil desiccation and one was filled with NaOH solution to control carbon dioxide emission. Jars were incubated in the dark, at 28°C. At the end of the incubation period, soils were sacrificed for dry weight, dehydrogenase activity and PAHs measurements. Saponins were added to the soil samples in order to reach concentrations of 2.5 $mg.g^{-1}$DW or 5 $mg.g^{-1}$DW respectively. Those amendments are a compromise both to the norm AFNOR XP U44-163, limiting the organic carbon amended to a soil to 0.02 ‰ of the soil dry weight, and to soil composting recommendations to observe a C/N ratio between 100 : 5 and 300 : 5 (Colombano *et al.*, 2010).

Untreated soils served as controls and two incubation periods (14 and 28 days) were investigated. All modalities were repeated four times for a total of 24 samples. Results related to soil samples with 2.5 and 5 mg saponins.g^{-1}DW have been named Sap2.5 and Sap5, respectively.

2.4.Chemical analyses

Dry weight determination

Soil samples dry weight determination was based on ISO 11465:1993 cor 1994.

Carbon dioxide emission

Carbon dioxide emission was monitored for each soil sample throughout the whole incubation following a method described in AFNOR XP U44-163. A vessel containing 15 mL of 0.5 M NaOH was placed in each jar as a carbon dioxide trap. Remaining NaOH was measured using automated pH-metric back-titration by acid (1 M). Before titration, barium chloride was added to precipitate carbonates. The equivalence point was set at pH 8.6. CO_2 emissions were measured after 1, 3, 7, 14, 21 and 28 days of incubation. Each time, fresh NaOH solution was replaced in the vessel and a blank was analysed to subtract ambient CO_2 from the measures. CO_2 emissions have been expressed in mg $CO_2.g^{-1}$DW.

Dehydrogenase activity

Dehydrogenase activity was measured for each soil sample after the incubation following a method described by Shaw and Burns (2005). Each sample was split in two sub-samples. Both were analysed the same way but one was previously sterilised by 3 cycles of 20 min at 121°C. One gram of fresh soil sample (sterilised or not) was added with 4 mL of iodonitrotetrazolium chloride 0.2 % (W/V) and incubated 48 h at 25°C in a sealed container. Samples were extracted with 10 mL of a 50:50 (V/V) N,N-dimethylformamide: ethanol mixture, centrifuged and the iodonitrotetrazolium formazan (INTF) produced by the enzymatic reduction was detected spectrophotometrically at 464 nm. INTF quantification was realised using external standard

calibration. The signals measured for the sterilised samples served as blanks and were substracted from the regular sample signals. Dehydrogenase activity is expressed in µg INTF.g^{-1}DW.$48h^{-1}$.

PAHs determination in aqueous samples

PAHs determination in the aqueous samples was based on ISO 17993:2002. The aqueous phase was extracted twice with n-hexane during 1 h, and separated in a funnel. The organic phase was dried on anhydrous Na_2SO_4, eliminated with a rotative evaporation device, and replaced with acetonitrile. The final extract was weighed for volume determination and analysed for PAHs.

PAHs determination in soil samples

PAHs determination in soil samples was based on ISO 13877:1998. Briefly, soils were dried with an equivalent amount of anhydrous Na_2SO_4 and homogenised. The mixture was extracted with dichloromethane on a Soxhlet device for 16 h. The resulting organic phase was filtered on anhydrous Na_2SO_4, eliminated with a rotative evaporation device and replaced with n-hexane. Then the extract was purified on basic aluminium oxide before n-hexane was eliminated with a rotative evaporation device, and replaced by acetonitrile. The final extract was weighed for volume determination and analysed for PAHs.

PAHs analysis

PAHs (20 µL of acetonitrile extract) were injected on an Agilent reverse-phase C18 column (Eclipse PAH 4.6 X 250 mm, 5 µm) with external guard column (Eclipse PAH 4.6 X 12.5 mm, 5 µm) using a mixture of acetonitrile and water as eluents. Both mobile phases were acidified with formic acid (0.1% V/V). The separation was performed at a constant 1.5 mL.min^{-1} flow rate using the following optimized gradient with the acetonitrile/water ratios: 0-15 min, linear increase from 50:50 to 75:25; 15-20 min, linear increase from 75:25 to 100:0; 20-40 min, 100:0. Finally: 40-40.1 min, linear decrease from 100:0 to 50:50 with a final isocratic hold of 5 min.

PAHs were detected fluorimetrically according to ISO 13877:1998 and their quantification has been achieved using external standard calibration.

2.5.Statistics

All statistical analysis was carried out using Minitab 17.0. Data were analysed by general linear model or one-way analysis of variance and mean values were compared by Tukey's test at the 5 % confidence level.

3. Results and discussion

3.1.Saponins CMC

Figure 1 shows the measured surface pressures for raw commercial saponins solutions. The first part of the graph shows a sharp increase of the surface pressure with the saponins concentration before reaching a plateau (second part). The intersection of the two parts is calculated to be 30.2 $mg.L^{-1}$ for a 26.9 $mN.m^{-1}$ surface pressure. As a comparison, Tween 80 (a synthetic nonionic surfactant) has a CMC of about 15 $mg.L^{-1}$ [Tween®80 product information] and the CMC of rhamnolipids (a type of biosurfactant produced by *Pseudomonas aeruginosa*) was reported at 150 $mg.L^{-1}$ [Gabet, 2009]. The saponins solutions, prepared at 1, 2, 4 and 8 $g.L^{-1}$ and used in the extraction experiments thus ranged from 30 to 260 fold the CMC, meaning there were enough molecules to form micelles.

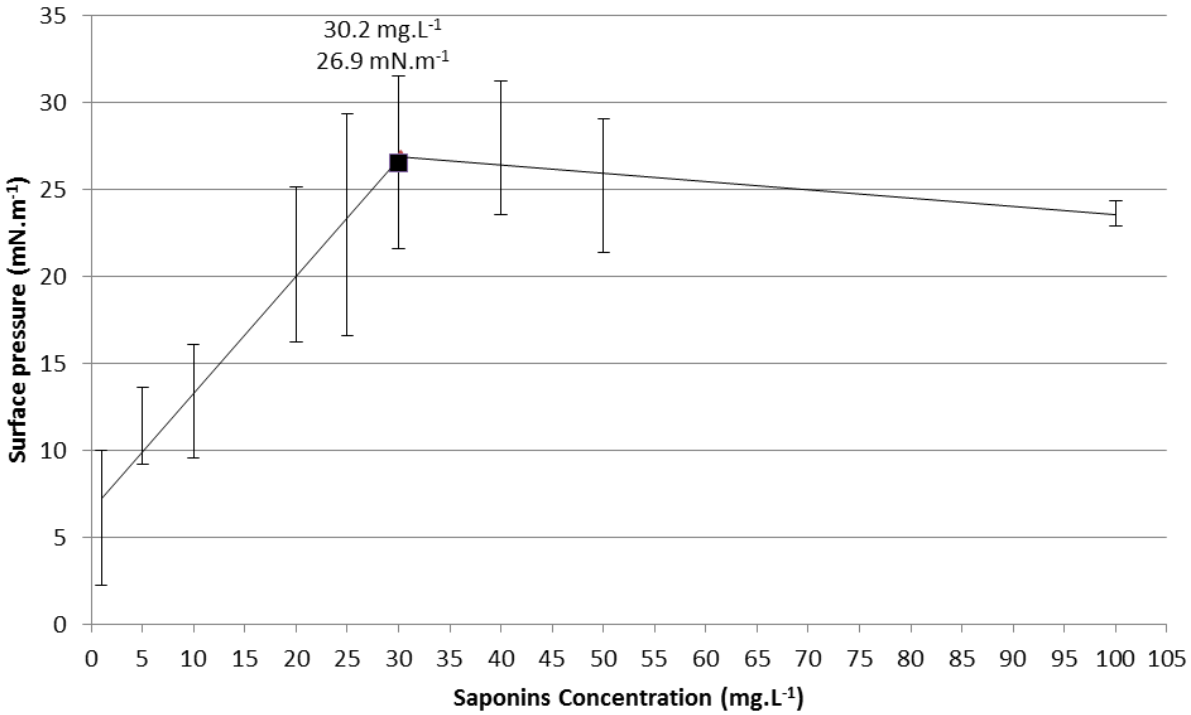


Figure 1. Determination of the critical micellar concentration of commercial *Quillaja saponaria* bark saponins saponins as the intersection of the two linear regression lines fitting the ascending part and the plateau.. Values are means ± confidence interval

3.2.PAHs extractions by saponins

The extractions of soil samples by different saponins solutions (water, Sap1, Sap2, Sap4 and Sap8) allowed extracting PAHs contents ranging from 3 to 864 $ng.g^{-1}$DW (Table 1). Statistical analyses show significant differences between the different extraction solutions for a few compounds.

When comparing each saponins solution to water, it appears that: (i) Sap2 extracted significantly more Ace, Fle, and Anthr than water; (ii) Sap4 extracted significantly more Ace, Fle, Phen, Anthr, and Pyr than water; and (iii) Sap8 extracted significantly more Fle, Phen, and Anthr than water.

When comparing, for one PAH, the saponins solutions that provided a significantly better extraction than water, it appears that: (i) Ace was significantly more extracted by Sap2 and Sap4 solutions, but there was no statistical difference between these two solutions; (ii) Fle and Anthr

were significantly more extracted by Sap2, Sap4 and Sap8 but here again there was no statistical difference between the three solutions; (iii) Phen was significantly more extracted by Sap4 and Sap8, with no statistical difference between the two solutions; and (iv) Sap4 was the only solution that extracted significantly more Pyr than any other.

Given the previous statements, it appears that the Sap4 solution is the best compromise among the different tested solutions as it allowed the extraction of the highest diversity of PAHs (Ace, Fle, Phen, Anthr, and Pyr).

It is interesting to examine the amounts extracted by the Sap8 solution. As it contained twice more surfactants than the Sap4 solution, Sap8 was expected to extract more PAHs than Sap4. However in some cases (Ace, Anthr, and Pyr) the statistical means structuration showed that not only were the extracted amounts not statistically different from Sap4 but also that they were not significantly different from water (Ace and Pyr) and from Sap1 (Anthr), meaning Sap8 provided a less efficient extraction than Sap4 for these compounds. Zhou *et al.* (2011) have determined that in aqueous conditions, the apparent solubilities of naphthalene, acenaphtene (not detected in the present contamined soil), phenanthrene and pyrene increased linearly with the saponins concentration above the CMC. However, their tested saponins concentrations ranged from 1 to 25 fold the CMC (versus 30 to 260 fold the CMC in the present study) and their data does not show whether the PAHs solubilisation enhancements reach a maximum at higher saponins concentrations. Also, their experiments do not involve soil. Kobayashi *et al.* (2012) reported that an aqueous saponins solution with a concentration above the CMC significantly extracted pyrene from low organic carbon soil. However they used freshly pyrene-spiked soil. Haigh (1996) in her review on surfactants/soil/organic contaminants interactions mentions several factors that would prevent non-ionic surfactants to desorb hydrophobic compounds from soil particles. Hydrophobic interactions exist between soil particles and surfactants which could explain the lower extractions for Ace, Anthr, and Pyr by the Sap8 solution: the PAHs could be partitioned

inside micelles, but the saponins constituting the micelles could bind to soil particles. Therefore, the benefit of the PAHs hydrosolubility being raised by the surfactants would be lost because the adsorption of the micelles to solids indirectly binds PAHs back to soil. This explanation could highlight a limitation to techniques that attempt to extract PAHs from soils by washing them with surfactants solutions: in some cases if the surfactant concentration is under or even close to the CMC, no desorption can be expected because monomers bond to soil particles are not capable of forming micelles, but if the surfactant concentration is too high, then micelles could raise the apparent sorption of the organic pollutants onto soil particles.

Table 1. PAHs extractions by different solutions (ng.g^{-1} DW).

PAH	Solution					p-value (α=0.05)
	Water	Sap 1g.L^{-1}	Sap 2g.L^{-1}	Sap 4g.L^{-1}	Sap 8g.L^{-1}	
Naphtalene	132^{a} ± 31	203^{a} ± 50	305^{a} ± 85	294^{a} ± 124	270^{a} ± 128	NS
Acenaphtene	320^{b} ± 85	539^{ab} ± 173	818^{a} ± 303	864^{a} ± 121	706^{ab} ± 254	0.009
Fluorene	106^{b} ± 35	184^{ab} ± 66	338^{a} ± 136	354^{a} ± 78	344^{a} ± 118	0.004
Phenanthrene	129^{c} ± 47	209^{bc} ± 72	385^{abc} ± 160	459^{ab} ± 152	471^{a} ± 151	0.003
Anthracene	41^{c} ± 14	65^{bc} ± 21	113^{ab} ± 35	124^{a} ± 22	119^{ab} ± 33	0.001
Fluoranthene	101^{a} ± 33	141^{a} ± 41	202^{a} ± 79	227^{a} ± 41	225^{a} ± 82	0.027
Pyrene	68^{b} ± 17	103^{ab} ± 43	135^{ab} ± 49	167^{a} ± 29	144^{ab} ± 51	0.024
Benz[*a*]anthracene	26^{a} ± 12	37^{a} ± 9	44^{a} ± 17	58^{a} ± 19	55^{a} ± 36	NS
Chrysene	30^{a} ± 14	46^{a} ± 12	51^{a} ± 20	64^{a} ± 19	63^{a} ± 39	NS
Benzo[*b*]fluoranthene	37^{a} ± 12	48^{a} ± 23	55^{a} ± 26	63^{a} ± 26	47^{a} ± 17	NS
Benzo[*k*]fluoranthene	12^{a} ± 4	19^{a} ± 8	17^{a} ± 8	23^{a} ± 8	20^{a} ± 10	NS
Benzo[*a*]pyrene	20^{a} ± 7	29^{a} ± 14	27^{a} ± 13	36^{a} ± 11	28^{a} ± 14	NS
Dibenzo[*ah*]anthracene	10^{a} ± 5	9^{a} ± 7	9^{a} ± 8	15^{a} ± 12	3^{a} ± 3	NS
Benzo[*ghi*]perylene	14^{a} ± 7	26^{a} ± 14	17^{a} ± 6	40^{a} ± 48	19^{a} ± 10	NS
Indeno[1,2,3-*cd*]pyrene	10^{a} ± 5	19^{a} ± 12	17^{a} ± 6	15^{a} ± 4	18^{a} ± 8	NS

Values are means ± confidence interval (n=5).
p-values (5% confidence level) indicate whether amounts of a PAH extracted by different solutions are significantly different (NS means differences are not significant).
Letters accolated to the values show Tukey's means structuration groups.

3.3.PAHs bioremediation in the presence of saponins

Respiration curves and dehydrogenase activities

Figure 2 presents the CO_2 emissions of (un)treated soil samples during incubation. All samples show a rapid emission during the first two weeks of incubation then slow down towards a plateau. Cumulated emissions at days 14 and 28 are statistically different for the three incubation modalities and increase with the saponins content. One could hypothesize that the increase of the CO_2 emission is simply linked to the degradation of saponins. Nevertheless, assuming that all the saponins added to Sap2.5 and Sap5 samples had been completely degraded during the incubation, the maximal increase of CO_2 emission (calculated according to saponins carbon content) would be of respectively 0.26 and 0.52 mg $CO_2.g^{-1}$DW. However, the differences of CO_2 emitted after only 14 days of incubation between Sap2.5 or Sap5 samples and the control are respectively of 0.80 and 2.92 mg $CO_2.g^{-1}$DW which is about three to five times more. So the presence of saponins increases the global CO_2 emission to a greater extent than their degradation.

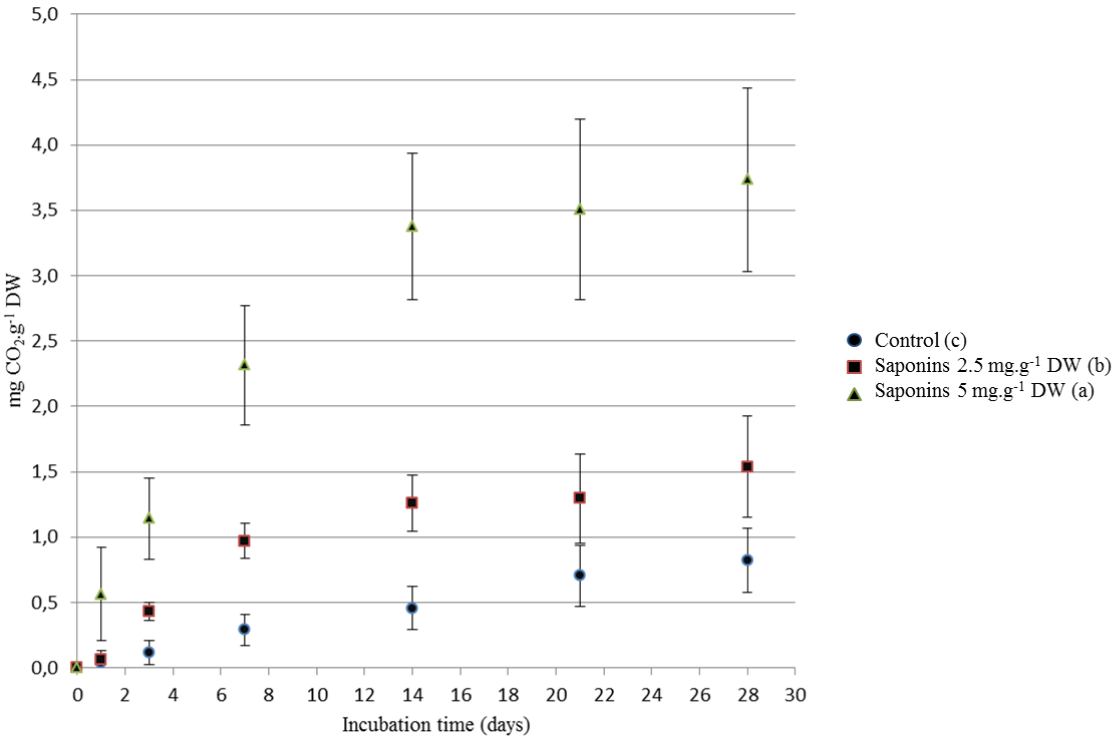
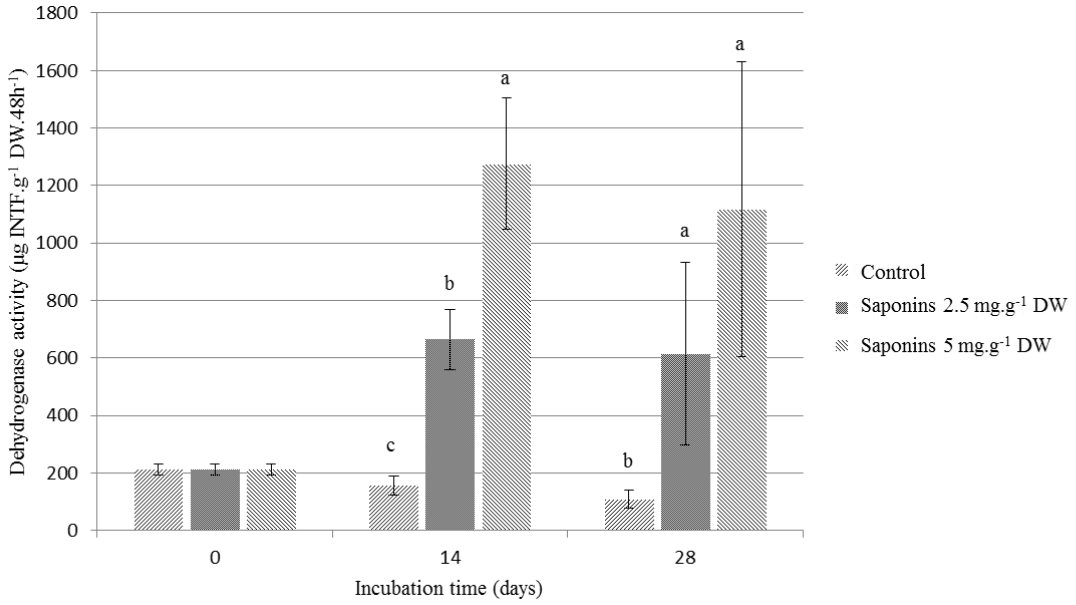


Figure 2. CO_2 emissions during the incubation of soils treated with saponins.
Values are means ± confidence interval.
Treatments followed by the same letter are not significantly different ($p > 0.05$)

Figure 3 shows the dehydrogenase activity in the different (un)treated soil samples after 14 and 28 days of incubation. The activities of the control samples slowly decrease with time. On the other hand soil samples treated with saponins show a sharp increase of their enzymatic activities during the first two weeks then a diminution during the next two weeks of incubation, regardless of the amended concentration. Besides, the dehydrogenase activity of Sap5 samples is about twice the activity of Sap2.5 samples and is statistically different at day 14. Dehydrogenase activity is a common indicator for soil biological activity [Das and Varma, 2011]. Therefore it is reasonable to assume that the diminution of this activity, consistent with the slowing of CO_2 emission (Figure 2), represents the slowing of the global microbial activity in soil samples. Given the higher amounts of CO_2 emitted when saponins are supplied, an explanation is that this carbon source, being rapidly available for microorganisms, is rapidly metabolized and boosts the soil global activity until it starts to lack. At this moment (14 days) the enzymatic activity slows down along with the CO_2 emission. Therefore both CO_2 emission and dehydrogenase activity

363 sets of data suggest that there is no toxic effect of the added saponins towards the soil
364 microbiota.



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366 Figure 3. Dehydrogenase activity of the soils treated with saponins after different incubation periods.
367 Values are means ± confidence interval.
368 Within each time group, sticks with the same letter are not significantly different ($p > 0.05$)

369 *PAHs residual contents*

370 Residual PAHs contents of (un)treated soils after 14 and 28 days of incubation are presented in
371 Table 2. Residual mean values, when compared to the values of the Belgian Walloon legislation
372 norms available in supplementary data, show that even though none of the incubation modalities
373 were able to lower the PAHs down to their respective reference value (0.01 or 0.1 $mg.kg^{-1}DW$),
374 some compounds have been lowered enough to change soil occupation criteria.

375 A few observations can be made from examining each PAH residual mean after each incubation
376 scenario: (i) in all incubation modalities N lowered under 25 $\mu g.g^{-1}DW$ (industrial VI) and Ace
377 under 19 $\mu g.g^{-1}DW$ (residential VI) as soon as after 14 days of incubation; (ii) in control samples
378 and after 14 days, Anthr reached 13.3 $\mu g.g^{-1}DW$ (industrial VI); (iii) in control samples and after
379 28 days, Anthr passed under the industrial VI, Fle passed under 9 $\mu g.g^{-1}DW$ (both residential and
380 commercial VS) which is also under 26 and 16 $\mu g.g^{-1}DW$ (natural and agricultural VIs,

respectively), F passed under 47 µg.g^{-1}DW (industrial VS) and thus under 48 µg.g^{-1}DW (agricultural VI), and Chrys passed under 25 µg.g^{-1}DW (both residential and commercial VIs); and (iv) in Sap5 samples and after 28 days, Anthr passed under the industrial VI, and Fle under both the natural and agricultural VI.

Statistical analyses give complementary information: (i) when comparing the residual PAHs contents after either 14 or 28 days, it appears that the values in samples treated with saponins (both Sap2.5 and Sap5) are not statistically different from the control samples at any incubation time; and (ii) there is a significant effect of the time: N and Ace, on one hand, and Phen, F, and Pyr, on the other hand, are statistically different from the initial content after 14 and 28 days respectively. However in Sap2.5 and Sap5 samples this time-effect on the residual PAHs content is only observed for N and Ace whereas the controls also show such diminution for Phen, F, and Pyr. These observations point towards an inhibition of the PAHs disappearance in the presence of saponins rather than an enhancement.

When the experiment was imagined, it was based on the hypothesis that the addition of surfactants to an aged-contaminated soil would enhance PAHs remediation. Bouchez *et al.* (1995) demonstrated the capacity of PAHs-degrading bacterial strains to degrade some normally recalcitrant PAHs through co-metabolism pathways; Rentz *et al.* (2005) showed that the degradation of BaP by *Sphingomonas yanoikuyae* was enhanced in the presence of a primary, more available source of carbon such as salicylate or plant roots extracts; and finally Kobayashi *et al.* (2012) reported that the biodegration of pyrene by *Sphingomonas* sp. was enhanced in the presence of saponins. Similar events were expected in the present study but the results do not suggest likewise.

Table 2. PAHs residual contents in soils treated with saponins and after different incubation times (mg.kg^{-1} DW).

PAHs	Initial	Control		Saponins 2.5mg.g^{-1} DW		Saponins 5mg.g^{-1} DW	
		14 days	28 days	14 days	28 days	14 days	28 days
Naphtalene	28.9 ± 1.7	17.4 ± 1.0	18.1 ± 0.8	18.5 ± 4.9	21.4 ± 4.2	20.1 ± 0.8	16.7 ± 4.8
Acenaphtene	19.4 ± 1.2	12.2 ± 2.6	10.0 ± 1.4	14.4 ± 2.3	12.1 ± 2.7	13 ± 2.7	10.9 ± 2.8
Fluorene	12.5 ± 1.1	9.4 ± 1.4	8.4 ± 3.1	10.4 ± 0.8	10.6 ± 3.7	10.8 ± 3.0	8.7 ± 3.4
Phenanthrene	46.5 ± 5.5	37.2 ± 6.5	30.5 ± 2.9	38.1 ± 3.8	39.4 ± 11.9	40.6 ± 10.1	39.0 ± 9.4
Anthracene	16.0 ± 1.4	13.3 ± 2.4	11.7 ± 8.7	14.6 ± 1.0	16.1 ± 3.3	19.0 ± 5.9	12.4 ± 7.3
Fluoranthene	65.9 ± 7.1	55.1 ± 11.3	45.6 ± 5.9	53.4 ± 6.5	53.3 ± 8.2	53.7 ± 12.2	52 ± 10.3
Pyrene	45.6 ± 4.8	38.3 ± 1.3	34.4 ± 2.2	38.2 ± 1.0	38.0 ± 6.7	39.3 ± 5.0	38.0 ± 6.7
Benz[*a*]anthracene	28.3 ± 3.6	27.6 ± 3.4	22.8 ± 0.3	26.2 ± 0.4	27.4 ± 2.5	26.2 ± 2.4	27.6 ± 2.4
Chrysene	32.4 ± 4.0	32.9 ± 4.2	23.9 ± 13.9	31.1 ± 1.0	32.9 ± 3.1	31.6 ± 3.9	31.6 ± 6.4
Benzo[*b*]fluoranthene	23.1 ± 3.3	26.1 ± 5.8	19.6 ± 1.6	21 ± 0.8	22.0 ± 2.8	18.7 ± 2.2	22.1 ± 2.2
Benzo[*k*]fluoranthene	11.8 ± 1.6	10.7 ± 0.1	10.1 ± 0.5	10.8 ± 0.2	11.3 ± 1.0	10.7 ± 1.1	11.2 ± 1.0
Benzo[*a*]pyrene	18.3 ± 2.6	18.3 ± 2.4	17.3 ± 1.7	17.7 ± 0.2	19.4 ± 0.3	17.5 ± 1.5	19.2 ± 2.2
Dibenzo[*ah*]anthracene	2.9 ± 0.1	2.5 ± 0.8	2.3 ± 0.4	2.3 ± 0.5	2.4 ± 0.1	2.7 ± 0.3	2.7 ± 0.5
Benzo[*ghi*]perylene	14.1 ± 3.6	13.4 ± 1.6	11.2 ± 1.1	11.5 ± 0.9	12.6 ± 2.0	11.0 ± 1.0	11.4 ± 1.0
Indeno[1,2,3-*cd*]pyrene	15.0 ± 2.6	15.5 ± 2.6	14.4 ± 2.9	14.8 ± 1.0	16.2 ± 2.0	13.4 ± 2.1	13.8 ± 0.5

Values are means ± confidence interval (n=3 or 4).

403

Zhu & Aithken (2010) conducted degradation experiments on aged-contaminated soil in the presence of two non-ionic synthetic surfactants: Brij® 30 (polyoxyethylene (4) lauryl ether: a hydrophobic surfactant) and $C_{12}E_8$ (octaethylene glycol mono *n*-dodecyl: a hydrophilic surfactant) and suggested the following conclusions: (i) the hydrophilic surfactant did not enhance PAHs degradation, at any concentration; and (ii) in the presence of the hydrophobic surfactant, the degradation of 3-rings PAHs (such as Phen) rose with the surfactant concentration but the degradation of 4-rings PAHs (F and Pyr) was less enhanced at a surfactant concentration above the CMC. However no inhibition of the degradation process was mentioned. Also Tiehm (1994), in an attempt to enhance phenanthrene availability to *Mycobacterium* sp., in the presence of Phen and SDS (sodium dodecyl sulfate: a hydrophilic non-ionic synthetic surfactant) observed that the microorganisms metabolized SDS as a primary nutrient source instead of Phen. These observations are in line with the results of the present study which has given strong evidence that saponins are used as a carbon source instead of PAHs and that co-metabolism did not take place during the incubations. Indeed, even though the total organic carbon is increased by less than 0.02 %, the added carbon source (saponins) is much more available for biotransformation than PAHs.

The lower diminution of PAHs contents in the presence of saponins could also be related to the extraction results mentioned previously: if PAHs were secluded by saponins micelles or hemimicelles, either in the soil solution or adsorbed on soil particles, the pollutants would be less available for biodegradation.

Finally, it is important to bear in mind that given the higher surface tensions of N and Ace compared to the other compounds (10.5 Pa and 0.356 Pa at 25°C, respectively), their diminution with time in Sap2.5 and Sap5 samples might simply be a loss by volatilization. Such hypothesis would have to be verified by monitoring the gas emissions in the jar by solid phase micro-extraction sampling. Such case scenario would mean that only Phen, F, and Pyr are significantly

429 degraded in the control samples and that the diminution of N and Ace in all samples (control,
430 Sap2.5 and Sap5) is not significant.

431 **4. Conclusions and perspectives**

432 It is of major interest to extend the general research on PAHs bioremediation enhancement. One
433 could imagine experiments similar to the ones previously describes (involving weathered soil
434 and several PAHs) being carried out with other types of biosurfactants or plant-based
435 amendments such as plant-root exudates, rhamnolipids, surfactin, humic and fulvic acids …
436 However the purpose of the exposed extraction and incubation experiments was to evaluate the
437 potential of saponins from *Quillaja saponaria* bark as a PAHs bioremediation enhancer by
438 confronting this non-ionic surfactant to an aged-contaminated soil.

439 The extraction experiment has proven to be limited in efficiency as it has allowed the significant
440 extraction of only a few compounds (Ace, Fle, Phen, Anthr, and Pyr). Besides, it seems that
441 extraction decreases over a surfactant concentration threshold given the fact that a solution of
442 $8g.L^{-1}$ of saponins could statistically not extract higher amounts of PAHs than water (Ace and
443 Pyr) or than a $1g.L^{-1}$ solution of saponins (Anthr).

444 However this opens the debate towards the application of saponins in stabilization technologies.
445 One could imagine that the present surfactant (saponins from *Quillaja saponaria* bark) could be
446 used as a secluding agent that would help slowing down the migration of a fresh plume of
447 pollution involving PAHs towards a sensitive compartment (such as groundwater) through the
448 binding of PAHs to soil particles. Given the overall biodegradability of biosurfactants, such an
449 application would be temporary and have to be associated to a more permanent treatment.
450 Besides, complementary studies would have to be conducted because as reviewed by Haigh
451 (1996), the interactions of surfactants strongly depend on the soil mineralogy and organic matter.

The incubation experiment results strongly suggest that the presence of saponins in the experimental soil has no enhancement effect on the PAHs bioremediation and even slows down this process. Therefore, there would be no advantage in treating a polluted soil with saponins from *Quillaja saponaria* bark during a bioremediation treatment.

On the other hand, the increase in the dehydrogenase activities and the higher emissions of carbon dioxide when soil was treated show that the saponins do not have a toxic effect on soil microbiota and even seem to increase its activity. Therefore it would be interesting to start over a similar experiment and conduct it for a longer time to assess whether the regular input of saponins could allow the soil microbial activity to last longer by regularly boosting the microbiota. Maybe such action would allow the PAHs remediation to be conducted on a longer period but in a more thorough way.

When crossing incubation and extraction results, two main hypotheses stand out that would explain the greater diminution of PAHs contents in the absence of saponins: (i) the surfactant is preferably degraded over the pollutants; and (ii) the surfactants partitioned the available PAHs into micelles, making them less bioavailable to biodegradation. The first hypothesis would have to be verified by implementing a cell culture similar to the one realised by Tiehm (1994) to assess whether PAHs-degraders could use saponins from *Quillaja saponaria* bark as primary nutrients over PAHs and the second by evaluating the bioavailability of PAHs in the presence of saponins through the use of Tenax® beads for example [Cornelissen *et al.*, 2001].

The conclusion that stands out from the results and interpretations exposed in the present article is that saponins from *Quillaja saponaria* bark, if they were added to an aged-contaminated soil in the tested concentrations, would not enhance PAHs bioremediation in the short run (28 days).

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561 **6. Appendix**

PAHs norms in brownfield soils in the Wallon region (in Décret relatif à la gestion des sols, 2009).

Occupation		Soil (mg/kg_{DW})				
		natural	**agricultural**	**residential**	**recreational or commercial**	**industrial**
Naphtalene (N)	VR	0,1	0,1	0,1	0,1	0,1
	VS	1,1	0,7	1,7	1,7	2,5
	VI	4	2,5	9	9	25
Acenaphtylene (A)	VR	0,01	0,01	0,01	0,01	0,01
	VS	0,3	0,3	0,8	8	43
	VI	3	3	8	78	410
Acenaphtene (Ace)	VR	0,01	0,01	0,01	0,01	0,01
	VS	2,6	1,6	3,9	3,9	6
	VI	9	6	19	19	56
Fluorene (Fle)	VR	0,01	0,01	0,01	0,01	0,01
	VS	4	2	9	9	16
	VI	26	16	46	46	163
Phenanthrene (Phen)	VR	0,1	0,1	0,1	0,1	0,1
	VS	9	6	12	12	16
	VI	27	16	60	60	164
Anthracene (Anthr)	VR	0,01	0,01	0,01	0,01	0,01
	VS	0,3	0,2	0,7	0,7	1,3
	VI	2,2	1,3	3,7	3,7	13,3
Fluoranthene (F)	VR	0,01	0,01	0,01	0,01	0,01
	VS	8	5	23	23	47
	VI	77	48	126	126	475
Pyrene (Pyr)	VR	0,01	0,01	0,01	0,01	0,01
	VS	1,4	0,9	3,6	3,6	6,4
	VI	10	6	18	18	64
Benzo(a)anthracene (BaA)	VR	0,01	0,01	0,01	0,01	0,01
	VS	0,8	0,5	1	1	1,5
	VI	2,5	1,5	5	5	15
Chrysene (Chrys)	VR	0,01	0,01	0,01	0,01	0,01
	VS	5	3	5	5	6
	VI	10	6	25	25	60
Benzo(b)fluoranthene (BbF)	VR	0,01	0,01	0,01	0,01	0,01
	VS	0,7	0,4	0,3	0,9	1,3
	VI	2	1,5	4	4	13
Benzo(k)fluoranthene (BkF)	VR	0,01	0,01	0,01	0,01	0,01
	VS	2,5	1,6	1,3	3,1	4,7
	VI	7,6	4,7	12,8	15,5	47
Benzo(a)pyrene (BaP)	VR	0,01	0,01	0,01	0,01	0,01
	VS	0,2	0,2	0,5	0,9	1,3
	VI	2,2	1,3	4,5	4,5	13
Dibenzo(ah)anthracene (DBahA)	VR	0,01	0,01	0,01	0,01	0,01
	VS	0,8	0,1	0,6	1	1,4
	VI	2,3	0,7	5	5	14

Benzo(g,h,i)perylene (BghiP)	VR	0,01	0,01	0,01	0,01	0,01
	VS	2,5	1,5	3	3	5
	VI	7	5	15	15	46
Indeno(1,2,3-c,d)pyrene (IcdP)	VR	0,01	0,01	0,01	0,01	0,01
	VS	1	0,6	0,2	1,2	1,5
	VI	2,5	1,5	2,5	6	15

VR (Reference Value): ideal value to reach when there is a soil

VS (Threshold value): over which at least a risk assessment and a monitoring must be implemented

VI (Intervention value): over which brownfield soils are to be systematically cleaned-up

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