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Mesocosm Experiments at a Tunnelling Construction Site for Assessing Re-Use of Spoil Material as a By-Product

Anna Barra Caracciolo [1], Paola Grenni [1,*], Livia Mariani [1], Jasmin Rauseo [2], Martina Di Lenola [1], Valerio Giorgio Muzzini [3], Enrica Donati [4], Ines Lacchetti [5], Paola Margherita Bianca Gucci [5], Antonio Finizio [1,6], Eleonora Beccaloni [5] and Luisa Patrolecco [2]

[1] Water Research Institute, National Research Council (CNR-IRSA), 00010 Rome, Italy; barracaracciolo@irsa.cnr.it (A.B.C.); l.mariani@irsa.cnr.it (L.M.); dilenola@irsa.cnr.it (M.D.L.); antonio.finizio@unimib.it (A.F.)
[2] Institute of Polar Sciences, National Research Council (CNR-ISP), 00010 Rome, Italy; jasmin.rauseo@cnr.it (J.R.); luisa.patrolecco@cnr.it (L.P.)
[3] Institute of Research on Terrestrial Ecosystems, National Research Council (IRET-CNR), 00010 Rome, Italy; valeriogiorgio.muzzini@cnr.it
[4] Institute for Biological Systems, National Research Council (ISB-CNR), 00010 Rome, Italy; enrica.donati@cnr.it
[5] Environmental and Health Department, Italian Institute of Health (ISS), 00161 Rome, Italy; ines.lacchetti@iss.it (I.L.); paola.gucci@iss.it (P.M.B.G.); eleonora.beccaloni@iss.it (E.B.)
[6] Earth and Environmental Sciences Department, University of Milano Bicocca, 20126 Milano, Italy
* Correspondence: grenni@irsa.cnr.it

Abstract: Mechanized excavation of tunnels with Earth Pressure Balance-Tunnel Boring Machines requires the use of foaming agents. The latter contain the anionic surfactant sodium lauryl ether sulphate (SLES) as the main compound. The re-use as a by-product of excavated soil containing foaming agents (spoil material) can pose a risk for soil and particularly for aquatic ecosystems if they are close to the spoil material final destination site. This work reports the chemical results (SLES residual concentrations) and ecotoxicological effects (battery of five tests) of 28 day-mesocosm studies performed at a tunnelling construction site. The soil mesocosms were set up with two different lithologies, which contained four different foaming agent products at the highest amounts used for excavation. The decrease in SLES concentrations and the ecotoxicological tests were performed in soil and its water extract (elutriate) at different times (0, 7, 14, 28 d). Elutriates were prepared in order to simulate a possible SLES leaching from soil to water. The results showed a decrease in SLES over time and different ecotoxicological responses depending not only on the initial amount of each product, but also on the soil lithology and organism tested (aquatic or terrestrial). This study showed how only site-specific ecotoxicological evaluations can ensure a safe management of the spoil material, making possible the re-use of soil and avoiding production of waste.

Keywords: SLES; environmental compatibility; site-specific protocol; *Vibrio fischeri*; *Danio rerio*; *Eisenia fetida*; *Lepidium sativum*; toxicity test battery integrated index

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1. Introduction

The use of millions of tons of excavated soil (spoil material) as a by-product can be a unique opportunity for recycling a useful resource for various purposes, in line with the circular economy [1–3]. It is handled in different ways worldwide. At the EU level, specific guidelines for excavated material management are contained in the Waste Framework Directive 2008/98/EC [4,5]. The latter describes the technical characteristics required for spoil material re-use/recycling, considering both environmental protection and the costs of treatment or use as a virgin raw material.

Some recent publications have posed the question of the re-use of spoil material from mechanized tunnelling [6–12], since high amounts are produced at each excavation site. They highlighted that the final destination of this material needs to be considered with care,

in particular to protect the water compartment. This aspect is not always considered in the framework of spoil material handling at a European level [4,5]. Worksite management of spoil material has to be performed in major construction projects [13] in accordance with the national legislations (e.g., Italian Decrees 161/2012 and 120/2017 [14,15]). For example, the addition of lime (1–6% in weight) can be a practical procedure for the chemical stabilization of excavated material (usually for clayey and silty soils) [7]. It produces short-term modifications (flocculation and agglomeration of clay minerals, reducing plasticity and moisture content) and a long-term reaction resulting in stabilization of the material, cementing and increasing its strength [7]. Moreover, the storage of spoil material in temporary deposit areas for several days before its final use can also be useful for ensuring soil drying and degradation of any chemicals (e.g., those contained in foaming agents) used for soil conditioning during tunnelling [16,17].

The use of commercial products (foaming agents) is necessary in mechanized tunnelling with Earth Pressure Balance-Tunnel Boring Machines (EPB-TBMs) [18–22]. The spoil material coming out of the machine can be maintained at temporary deposits. If, at this point, a set of analyses demonstrates its environmental compatibility, it can be transferred to the final destination site and used as a by-product [23,24]. Currently, there are thresholds for organic and inorganic contaminants (e.g., heavy metals and hydrocarbons with more than 12 carbon atoms) in Italian regulations (Decrees No. 152/2006 and No. 120/2017), but there is no threshold limit for SLES in excavated material in either European or Italian legislation.

Several foaming agents contain anionic surfactants (e.g., alkyl ether sulphates and sodium lauryl ether sulphate-SLES) as their main components, ranging from 5 to 50% [23,25]. Recent studies found SLES to be a biodegradable compound in spoil material [17,23,25], and a bacterial consortium able to degrade SLES was recently selected [26], although it can be toxic for aquatic organisms if residual concentrations persist [16,27–32]. In order to exclude any possible environmental ecotoxicological effects from excavated soil produced during tunnel construction, the Italian Ministry of Environment requires a site-specific technical report, which has to demonstrate that the spoil material at the final destination site does not pose any risk for soil and aquatic ecosystems [16,29,30]. In this respect, this work reports a site-specific experimental study performed at a construction site using mesocosms containing soil conditioned with four different foaming agents at the maximum treatment ratios (TR, litres of foam per m^3 of soil) suggested for this type of tunnelling. Analytical determination of SLES in soil and their water extracts (elutriates), and five ecotoxicological tests were performed from the start of the mesocosm experiment and over 28 days. An initial pre-screening of the four products using *Vibrio fischeri* as the test organism was carried out (effective concentrations producing a 20% or 50% change in the endpoint response, EC_{20} and EC_{50}). In some mesocosms the presence/absence of lime was also tested. At the same time, residual concentrations of SLES were analysed in soil samples and in water extracts. The mesocosm experiment was performed by simulating the spoil material storage at the construction site in a scenario as close as possible to the real situation where natural SLES biodegradation can occur. On the basis of the overall chemical and ecotoxicological results, a site-specific protocol to be applied during the excavation of this tunnel was drawn up. The various steps in the mesocosm experiment, the variation of SLES concentration in the soil and elutriates over time and the corresponding ecotoxicological response were evaluated, discussed and summarized in a toxicity test battery integrated index.

2. Materials and Methods

2.1. Soils and Foaming Agents

The construction site was in the Apennines of Central Italy and involved a tunnel of about 7.5 km for a three-lane highway. The biggest EPB-TBM ever used so far in Europe, with a diameter of 15.87 m, was employed. The overall excavated material to be managed as a safe by-product was estimated at about 1.5 million m^3. Two soil types (MON: gravel

in a sandy-silty soil; density: 1930 kg/m^3; SIL: gravel in a sandy-silty-clay soil; density: 1750 kg/m^3) were identified as representative of the extreme lithologies faced during the tunnel excavation. The soils to set up the mesocosm study were collected at around 50 m below ground. Aliquots of SIL and MON soils were analysed to determine the microbial abundance and organic carbon (OC) content, following methods described in previous works [23].

Four foaming agent products (P1, P2, P3 and P4, equally suitable from a geotechnical point of view for this tunnel excavation) to be used for the two lithologies were suggested by the geotechnical laboratory feasibility studies with slump cone tests. These tests evaluated the workability and consistency of the two soils with the various foaming agents, providing the correct foaming agent treatment ratio (TR) to be applied for each soil [19–21,33]. The tests were conducted at Turin Polytechnic (Environment, Land and Infrastructure Engineering Dept., Tunnelling and Underground Space Lab), using a foam generation system that reproduces the conditioning characteristics used by an EPB-TBM [19,34]. Table 1 reports the density of each foaming agent and the highest amount used (soil Treatment Ratio, TR, L/m^3) [19], together with the percentage of SLES. For the SIL lithology, in one case P3 was used together with P4 to improve the soil workability [18].

Table 1. Foaming agent products (P) and their relative amount used for the soil conditioning. The product density, main components, CAS#, EC# and component % are those reported in the chemical safety data sheets of the commercial products. P4 was used together with P3. SIL: sandy-silty-clay soil; MON: gravel in a sandy-silty soil. The foaming agent amount to be applied in each soil (TR: soil Treatment Ratio) was assessed by slump conic tests.

Product	Density (g/cm^3)	Main Components, CAS# and EC#	Component %	TR (L/m^3)	
				SIL	MON
P1	~1	Sodium lauryl ether sulphate (SLES) 9004-82-4; 618-398-5	10–30	0.53	1.46
P2	1.35–1.45	Alcohols, C12-14, ethoxylated, sulphates, sodium salts (SLES) 68891-38-3; 500-234-8	10–50	0.35	1.2
P3	1.04	(a) Alcohols, C12-14, ethoxylated, sulphates, sodium salts (SLES) 68891-38-3; 500-234-8	10–20	0.59	1.46
		(b) 1,2-benzisotiazol-3(2H)-one 2634-33-5; 220-120-9	0.005–0.01	0.44 *	
P4 **	1.04	Alcohols, C12-14, ethoxylated, sulphates, sodium salts (SLES) 68891-38-3; 500-234-8	25–30	0.165	-

* If P3 is used with P4; in this case P3 is used with a less TR value, ** P4 in this study is used only in addition to P3 foaming agent.

2.2. Soil Mesocosm Set-Up at the Construction Site

Each mesocosm (1 m^3, performed in duplicate) consisted of a concrete storage tank containing the soil (SIL or MON) treated with the various foaming agents, as reported in Table 2. Lime was added at 20 kg/m^3 in some SIL mesocosms. Lime can be added to chemically stabilize fine texture soils and in this case was used only for SIL. A total of eleven experimental conditions were set up. Each mesocosm was maintained at the tunnel construction site (Central Italy, 270 m altitude, April–May 2016) under real conditions (e.g., temperature, humidity) for 28 days in a shed with open sides to protect the soil from any rain, simulating temporarily depositing the spoil material. The air temperature was monitored daily.

Table 2. Summary of the 11 experimental conditions (soil mesocosms) at the construction site.

Soil Lithology	Foaming Agent	Lime	Mesocosm Acronym
SIL	P1	-	SIL + P1
	P2	-	SIL + P2
	P3	-	SIL + P3
	P3 and P4	-	SIL + P3 + P4
	P3	Yes	SIL + P3 + L
	-	-	SIL
	-	Yes	SIL + L
MON	P1	-	MON + P1
	P2	-	MON + P2
	P3	-	MON + P3
	-	-	MON

At fixed times (0, 7, 14, 28 days), soil samples (about 20 kg from each mesocosm) were collected with a soil probe equipped with an auger and a small shovel (deep: 20–100 cm), mixed, transported to the lab in refrigerated bags and then used for the different analyses or ecotoxicological tests.

At each sampling and for each condition, SLES residual concentration and ecotoxicological tests were performed on the elutriates. Moreover, the soil water holding capacity (WHC) was measured at the start of the experiment and soil temperature, moisture and pH were monitored over time. WHC was determined following the ISO method 11274:2019 (Soil quality determination of water retention characteristics—laboratory methods). The pH was measured using a portable pH meter (HI 9124, Hanna Instruments) in a 1:2.5 soil-water suspension.

Elutriates were prepared in a solid/liquid ratio of 1:10 with distilled water (taking the soil moisture of the sample into account), following standardized procedures for waste characterization (UNI EN 12457-2:2004 [35]) with some modifications. In brief, each soil sample (3 replicates, 100 g each) was put into a bottle (1 L) with distilled water and shaken (130 rpm for 24 h at 20 °C, in the dark), simulating the soil leaching process. Further details are reported in Grenni et al. [17] and Mariani et al. [16]. After the solid particles fell to the bottom (15 min), the supernatant was centrifuged (15 min at 9000 rpm). The elutriates obtained were directly used for SLES determination or filtered (0.45 µm, cellulose acetate Whatman) for ecotoxicological analyses as reported in UNI EN 14735:2005 (Characterization of waste-preparation of waste samples for ecotoxicity tests).

2.3. Chemicals and SLES Analyses

All solvents utilised for chemical determinations were of HPLC grade and were obtained from VWR (Radnor, PA, USA). SLES of technical grade purity was purchased from BOC Sciences (New York, NY, USA) and used as the reference compound for the anionic surfactant analytical determinations. SLES was extracted from soil and its water extracts following the method reported in Grenni et al. [17]. Briefly, the target compound was extracted from fresh soil samples (about 2 g) using Pressurized Liquid Extraction (PLE, Dionex ASE 150, Thermo Fisher Scientific Inc., Waltham, MA, USA) followed by the liquid-liquid MBAS extraction method (Methylene Blue Active Substances [36]).

As regards the elutriates SLES was directly measured using the MBAS method. The absorbance of the blue SLES-MBAS complex, obtained both from soil (PLE extracts) and elutriate samples, was then measured by spectrophotometry at 650 nm wavelength (Perkin–Elmer Lambda 25 UV–VIS spectrophotometer). Finally, the analytical SLES concentration was calculated using the equations resulting from the standard calibration curves (0.05–4 mg/L SLES), previously determined, as detailed in Barra Caracciolo et al. [23]. The limit of detection (LOD), calculated in accordance with the IUPAC method [37], was 0.013 mg/L and the PLE extraction recovery was 96.5 ± 1.6%.

2.4. Ecotoxicological Tests

A pre-screening of the foaming agent products (P1, P2, P3, P4 and the combination of P3 and P4, see Table 1) was initially performed to evaluate their intrinsic ecotoxicity. For this purpose, the toxic effective concentration (EC_{20} and EC_{50} values) of the products was evaluated with the bacterium *Vibrio fischeri*.

In the mesocosm experiment the ecotoxicity of all soil samples or soil water extracts was evaluated at different times (0, 7, 14 and 28 d) from the conditioning using the *Vibrio fischeri* toxicity test, *Lepidium sativum* seed germination and seedling growth tests, *Eisenia fetida* tests and the *Danio rerio* test [38]. All tests were conducted in at least three replicates. Positive and negative controls were also considered in accordance with the specific guidelines. All data are reported as the effect percentage (%) in the soil (SIL or MON) (net of any possible intrinsic toxicity measured in the untreated soils) or in the corresponding water extracts.

2.4.1. *Vibrio fischeri* Acute Toxicity Test

The *Vibrio fischeri* acute toxicity test (UNI EN ISO 11348-3:2019 [39]), was performed using a Microtox® analyser (Model 500, Modern Water, London, UK), in accordance with both the ISO and the manufacturer instructions. The test endpoint is the luminescence inhibition of the *Vibrio fischeri* bacterium in contact with a chemical substance or environmental samples at three exposure times (5, 15 and 30 min). The measurements are compared with a negative control (a bacterial suspension in a solution containing 2% NaCl in distilled water). Freeze-dried and lyophilized *V. fischeri* (strain NRRL B-11177) and Reconstitution Solution (used for rehydrating the bacteria) were purchased from Ecotox LDS s.r.l. (Milan, Italy). The saline solution (2% *w*/*v* NaCl) and the osmotic adjustment solution (22% *w*/*v* NaCl, used to obtain about 2% of salinity in the samples) were prepared with Milli-Q water. The toxicant reference 3,5-dichlorophenol was used as the positive control.

The *V. fischeri* test (81.9% Basic Test [39]) was first used to evaluate the intrinsic toxicity of the four foaming agents (P1, P2, P3, P4 and P3 + P4; see Table 1) in terms of concentrations capable of causing luminescence inhibitions of 20% (EC_{20}) and 50% (EC_{50}), respectively. Stock solutions of the four foaming agent products (258.5; 261.0; 255.3; 261.8 mg product/L for P1, P2, P3 and P4, respectively) were prepared with distilled water to produce 7 dilutions (2.6; 5.2; 13.1; 26.2; 52.4; 104.7; 212.1 mg product/L) to be tested for each commercial product. Three tests were performed for each foaming agent and the EC values were calculated using the Microtox software (MicrotoxOmni Software, version 4.2; Modern Water, London, UK).

The *V. fischeri* test (81.9% Screening Test [39]) was then used to evaluate the acute toxicity of soil water extracts (elutriates) over the 28-day mesocosm experiment in all conditions. Before the tests, the salinity (adjusted to 2% with the osmotic solution) and pH (corrected up to the 6.0–8.0 range) of each elutriate were adjusted in line with the ISO protocol. The results are reported as the inhibition percentage (%), calculated using the Microtox software, after 30 min exposure of bacteria to the samples. According to the UNI EN ISO 11348-3:2019 protocol, luminescent inhibition $\geq$20% is considered a toxic effect, compared to the negative control [40,41] and as a validity criterion the coefficient of variation (CV%) between replicate results has to be <20%.

2.4.2. *Lepidium sativum* Seed Germination and Seedling Growth Tests

The phytotoxicity was tested with the seed germination, seedling emergence and early growth tests performed with *Lepidium sativum* seeds (Fratelli Ingegnoli, Milano). For the germination test, seeds (10 seeds, in triplicate) were exposed to the elutriates in accordance with the US EPA Guidelines [42]. The test evaluates if the water extracts have any effect, expressed as the Germination Index (GI%), on germination and lengthening of the roots, hypocotyls and epicotyls of plants [42]. The test was performed in Petri plates (diameter 90 mm) with a paper disk soaked with 5 mL of elutriate or distilled water (negative control). The seeds were put in contact with the elutriates for 72 h in a growth chamber at 25 °C

in the dark. The germinated seeds were then counted and the lengths of the different parts of each young seedling were measured to calculate the GI% [43], as reported in Grenni et al. [17]. A test is considered toxic if the GI < 80% [44].

The seedling growth test [42,43] was performed using pots containing soil samples (about 3 kg, three replicates) of the various conditioned soils, where 20 seeds of *L. sativum* were sown. Water was added to reach 70% of soil water capacity. The soil moisture was kept constant by daily weighing the pots, which were kept in a greenhouse (25 °C ± 10 °C; photoperiod: 16 h light/8 h dark: air humidity: 70% ± 25%; light intensity: 350 ± 50 μE/m^2/s). An OECD soil (sandy-clay soil; total C: 1.46%, total N: 448 mg/kg, P: 110 mg/kg, K: 157 mg/kg of and Mg: 95 mg/kg) was also used as the negative control. After a 21 d exposure period, the plants were counted and their aerial parts used to measure the dry biomass (105 °C for 48 h). The biomass values were compared with those of plants grown in the negative control. The results are expressed as the Growth Index (GrI, %) [45] compared to the growth of the plants in the soils without foaming treatment.

2.4.3. *Eisenia fetida* Acute and Chronic Tests

The earthworm acute toxicity test was conducted in line with the filter paper contact test (OECD guideline 207 [44]), using elutriates (10 replicates) obtained from the soils at each experimental time. Specimens of *E. fetida* were purchased from a commercial earthworm breeding farm (Con.It.A.Lo., Turin, Italy). Before the tests, the organisms were maintained for 3 h in the dark on a wet filter paper to evacuate the earthworms′ gut content, washed with tap water and then dried carefully with an absorbent paper. The test was performed at 20 °C ± 2 °C, in the dark. The ecotoxicological endpoint (earthworm mortality) was assessed after 48 h. The test involved the elutriates (1 mL) and one mature earthworm for each glass vial (8 × 3 cm diameter, flat-bottomed), on the walls and bottom of which a filter paper (80–85 g/m^2, 0.2 mm thickness) was placed. All vials were closed with a stopper with small ventilation holes. The pH of the elutriates to be tested was measured in all cases. Distilled water (1 mL) was added to the negative controls. The results are expressed as mortality (%).

The chronic earthworm toxicity test (28 days of organism exposure) was performed according to OECD Guideline N. 222 [46], using soil samples obtained from each experimental condition (conditioned soils with foaming agents or non-conditioned soils) taken at 0 and 7 days. In addition, an artificial soil prepared according to OECD Guideline No. 207 [47] was used as the negative control to fulfil the validity criteria (mortality <10%). Before the tests, the organisms were acclimated for 7 days, washed with tap water and put on a filter paper to remove any excess of water. For each test (three replicates), 10 *E. fetida* adults with a clitellum (2 months–1 year-old; weight: 260–600 mg) were put in closed glass boxes filled with about 700 g of soil samples and incubated at 20 °C (photoperiod: 16 h light/8 h dark; 400–800 lux). The boxes were opened once a week. The three endpoints tested were mortality and growth (at 28 days) and reproduction (at 56 days). In particular, after the 28 days of organism exposure required by the OECD test, the live worms were counted and any behavioural or morphological changes recorded, whereas after 56 days reproduction was assessed by counting the juvenile earthworms, hatched and unhatched cocoons, and any damage to the organisms was recorded.

2.4.4. *Danio rerio* Acute Toxicity Test

The Fish Embryo Acute Toxicity (FET) test was performed in accordance with OECD Guideline No. 236 [48]. This test uses newly fertilised zebrafish (*Danio rerio*) [49,50] eggs exposed for 96 h to a liquid sample and is intended to reflect acute toxicity in fish in general [17,51,52]. Fertilised eggs were obtained from a breeding stock of zebrafish adults maintained in aquariums and fed 3–4 times a day with a combination of dried food and newly hatched *Artemia salina* shrimps, in line with the OECD guideline recommendations. The eggs were collected with an egg-trap, made of a glass vessel covered with a mesh submerged in the aquarium. Randomly selected embryos (twice the number needed for

each test) were transferred into Petri dishes and observed with an inverted microscope to select only those developing between the 4- and 32-cell stages and with an intact chorion. A 3,4-dichloroaniline (4 mg/L) solution was used as the positive control.

Each test was performed with 24-well plates containing 20 embryos per sample (2 mL of elutriate) and 4 embryos as internal negative controls, which were incubated at 26.0 ± 1.0 °C for 96 h (photocycle: 14:10 h light/dark). Four endpoints were used as fish lethality indicators: fertilised egg coagulation, lack of somite formation, non-detachment of the tail and lack of heartbeat [53]. They were measured every 24 h, up to the end of the exposure period (96 h). The acute toxicity effect was evaluated as a positive result in each of the four endpoints [54]. The results are expressed as mortality (%). The ecotoxic threshold limit is mortality >20%.

2.5. Toxicity Test Battery Integrated Index

The results obtained from each individual test can sometimes give different responses to a specific toxicant or contaminated environmental matrix due to the different sensitivity of each organism tested with the specific chemical.

The use of a toxicity integrated index can overcome this issue by combining the overall results, as suggested by the Guidelines of the Italian National Institute for Environmental Protection and Research (ISPRA) [55] and verified in other studies [17]. An integrated index can be applied to any ecotoxicological test series, no matter what the endpoint number and type. It calculates the overall toxicity and the possible risks in a sample, on a toxicity scale from 0 to 100% [17,56,57]. In this study, the ecotoxicological results obtained from each individual test at 0 and 7 days of the mesocosm experiment were combined in the battery index. This choice was made because the various toxic responses were essentially unvaried 7 days after the start of the experiment. The algorithm, described in detail in Grenni et al. [17], required at least three endpoints and the results reported here are expressed as a toxicity integrated index (T%). Severity, variability and response are specific for each bioassay, whereas the number of endpoints measured and consistency were the same for all sampling times. Outputs of the Integrated Battery Index can vary from 0 to 100% and the corresponding hazard classes are classified from negligible to very high.

2.6. Statistical Analysis

Differences in SLES concentrations between the various conditions were analysed using the Kruskal-Wallis One Way ANOVA (analysis of variance) on Ranks. The post-hoc test used for finding differences between groups was the Tukey test (Sigma Stat 3.5-DUNDAS Software LTD). Correlations between variables (e.g., SLES residual concentrations versus ecotoxicological data) were calculated using MS Excel 2013.

3. Results

3.1. Pre-Screening of the Four Foaming Agents with V. fischeri

The dose-response relationships between the various concentrations tested (mg/L) of each foaming agent and the effect (expressed as % bioluminescence inhibition) on the bacterium *V. fischeri* were calculated with the Microtox software. The data made it possible to calculate the effective concentrations of the foaming agent products (P1, P2, P4 and P3 + P4) causing 20% (EC_{20}) or 50% (EC_{50}) of bacterial luminescence inhibition (Table 3).

The EC_{20} and EC_{50} values for the P3 product refer to those reported in a recent paper [16]. The highest EC_{20} (5.75 mg/L) and EC_{50} (17.86 g/L) values were found for P3, demonstrating it was less toxic than the others. Since P1 and P2 showed similar toxicity and were lower than P4, they were also selected for the subsequent mesocosm experiments.

Table 3. Toxicity of the four foaming agent products (P1, P2, P3, P4 and P3 + P4) evaluated by the *V. fischeri* test; results are expressed as EC_{20} and EC_{50} ± standard errors (s.e.) at 30 min of exposure.

Product	EC_{20} (mg/L)	s.e.	EC_{50} (mg/L)	s.e.
P1	2.21	0.29	6.97	0.87
P2	2.23	0.27	6.88	0.89
P3	5.75	0.45	17.86	1.48
P4	1.96	0.28	6.10	0.89
P3 + P4	4.11	0.00	12.76	0.00

3.2. Soil Mesocosm Experiment at the Construction Site

3.2.1. Organic Carbon and Microbial Abundance, WHC, Soil Moisture, pH, Temperature

The organic carbon content (OC) was 0.5% in SIL and 0.3% in MON and, in line with these values, the microbial abundance was higher (2×10^6 cells/g soil) in SIL than MON (5×10^4 cells/g soil).

As regards the maximum water holding capacity (WHC_{max}) of each soil (SIL or MON) alone or in the presence of lime and the initial moisture of the soils at the mesocosm set-up, the results are reported in Supplementary Materials Table S1. SIL showed a WHC_{max} (33.0% ± 0.8) three times higher than MON (11.4% ± 1.0) owing to its fine lithology. Moreover, adding lime further increased the SIL WHC_{max}. The soil moisture did not significantly decrease during the experimental period (only a slight decrease of 1–4% was observed).

The soil pH and the corresponding water extracts, (Supplementary Materials, Table S2) were close to neutral, except for the soils treated with lime, which had, as expected, a basic pH (about 11).

During the one-month experiment, air temperature changed in line with the season and ranged from 7–12 °C (daily minimum) to 15–26 °C (daily maximum). However, the temperature of the soil mesocosms was essentially constant with an average value of 16.8 ± 0.21 °C at the sampling depth (20–100 cm).

3.2.2. Analytical Determination of SLES in Soils and Elutriates

SLES residual concentrations measured in soils and elutriates from the mesocosms over time (0, 7, 14 and 28 days) are shown in Figure 1A,B, respectively.

At the start of the experiment (day 0), SLES concentrations found in soils ranged from 77 to 160 mg/kg for SIL (SIL + P1; SIL + P2; SIL + P3; SIL + P3 + P4; SIL + P3 + L) and from 276 to 648 mg/kg for MON (MON + P1; MON + P2; MON + P3). These values were in line with the treatment ratios (TRs) applied for the soil conditioning, since the foaming agent amounts were much higher for MON than SIL (Table 1).

SLES concentrations decreased over time in the SIL mesocosms and at 28 days a reduction ranging from 30% (SIL + P3) to 56% (SIL + P3 + P4 and SIL + P1) was observed. The SIL + P3-treated soil showed average SLES concentrations significantly lower (ANOVA, $p < 0.05$) than the other conditions.

The initial (0 d) SLES in the elutriates obtained from the SIL mesocosms (Figure 1B) was from a minimum of 1.2 mg/L for P3 to a maximum of 4.9 mg/L for P3 + P4. These values were in line with the corresponding concentrations in soil. In fact, SLES values decreased over time and starting from day 7 all were lower than 1 mg/L.

On the contrary, no significant variation in SLES was found in the MON mesocosms, either in soils (Figure 1A), or in elutriates (Figure 1B). SLES was significantly (ANOVA, $p < 0.01$) higher in MON than SIL elutriates, with values from 9.6 to 23 mg/L.

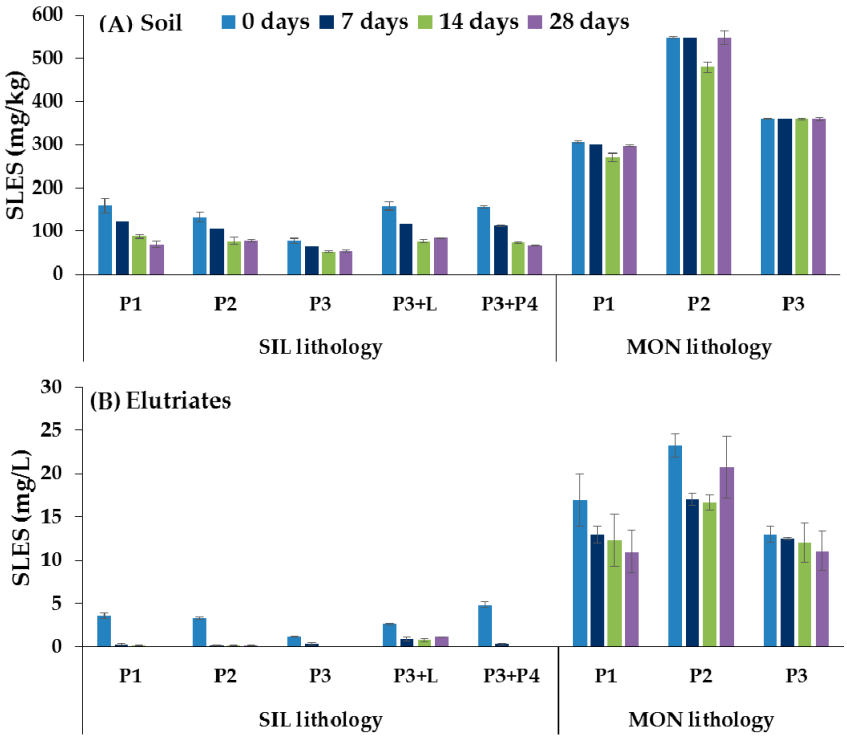

Figure 1. Average values of SLES concentration in the various SIL and MON mesocosm conditions (soils conditioned with P1,2,3,4 and P3 + P4) at the different sampling times (0, 7, 14 and 28 days). (**A**) in soil (mg/kg); (**B**) in elutriates (mg/L). The vertical bars represent standard errors. SIL: sandy-silty-clay soil; MON: gravel in a sandy-silty soil. P1, P2, P3, P4: foaming agent products.

3.3. Ecotoxicological Tests

All the ecotoxicological tests (*V. fischeri* bioluminescence inhibition, *L. sativum* germination and growth, *D. rerio* mortality, *E. fetida* mortality, growth and reproduction) met the validity criteria [39,46,51,58].

The percentages of bioluminescence inhibition (%) for the bacterium *Vibrio fischeri* at 30 min of exposure and at the various experimental times (0, 7, 14 and 28 days) are reported in Figure 2.

An initial bioluminescence inhibition (%) was observed in all P-treated SIL mesocosms except for SIL + P3 where the effect was lower than the toxicity test threshold of 20% (Figure 2A).

The highest inhibition (66%) was recorded in SIL + P3 + P4 due to the SLES concentration of P4. In any case, the effect was transient and never observed 7 days from the soil conditioning (all values were <20% in all samplings). Moreover, a positive correlation ($r = 0.9$, $p < 0.01$) was found between the SLES concentrations in elutriates and their corresponding bioluminescence inhibition values.

The non-treated soils (SIL and MON) did not have any effect on the bioluminescence of the bacterium.

In the case of samples treated with lime (SIL + L and SIL + P3 + L), the pH of the elutriates tested was about 11 and was corrected to neutral, as suggested by the standard protocol, and for this reason no toxicological effect was recorded.

The elutriates produced from the MON P-treated mesocosms (Figure 2B) were toxic over the experimental time, with values ranging from 88 to 95%.

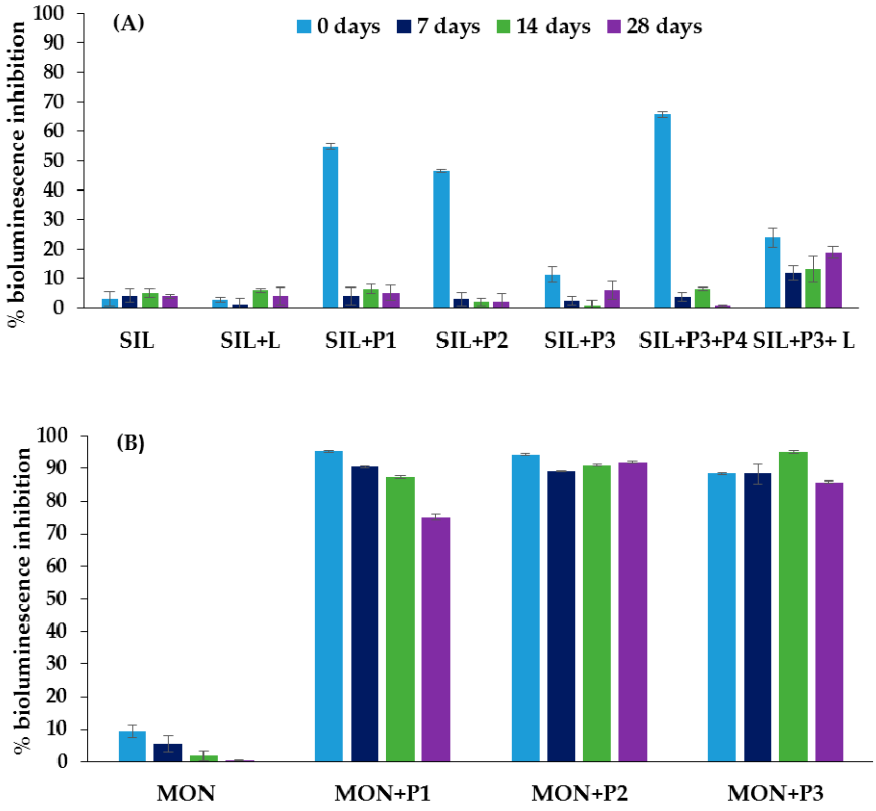

Figure 2. Bioluminescence inhibition (%) of *Vibrio fischeri* at 30 min. of exposure to elutriates from (**A**) SIL and (**B**) MON mesocosms (conditioned with P1,2,3,4 and P3 + P4) at the various experimental times (0, 7, 14 and 28 days). The vertical bars are the standard errors. SIL: a sandy-silty-clay soil; MON: gravel in a sandy-silty soil. P1, P2, P3, P4: foaming agent products; L: lime.

The results of the *L. sativum* germination tests, expressed as the Germination index (GI%), are reported in Table 4. In all conditions, except for SIL + P1 at 0 days, no significant effect on the GI values was observed (values > 80%) and in several cases, a positive effect was even recorded.

Table 4. *Lepidium sativum* Germination index (GI%) ± standard errors (s.e.) obtained from elutriates of the various P-conditioned or non-conditioned (SIL or MON) soil mesocosms at different experimental times (0, 7, 14 and 28 days). SIL: sandy-silty-clay soil; MON: gravel in a sandy-silty soil. P1, P2, P3, P4: foaming agent products; L: lime.

	0 days		**7 days**		**14 days**		**28 days**	
	GI (%)	**s.e.**	**GI (%)**	**s.e.**	**GI (%)**	**s.e.**	**GI (%)**	**s.e.**
SIL	131.4	13.1	96.2	24.4	130.0	8.9	181.6	10.1
SIL + L	97.3	7.6	93.7	15.8	126.8	3.2	140.8	19.6
SIL + P1	55.4	5.3	89.8	11.6	105.4	11.5	146.5	35.0
SIL + P2	122.2	10.9	113.0	10.1	101.1	20.3	151	22.1
SIL + P3	117.1	32.2	136.7	4.6	120.4	3.2	168.9	8.5
SIL + P3 + P4	118.6	5.0	116.5	24.3	135.6	22.2	165.8	13.9
SIL + P3 + L	96.3	9.9	112.5	7.8	88.2	12.6	147.0	3.4
MON	83.3	14.2	92.5	23.4	117.6	3.0	155.6	12.3
MON + P1	108.8	31.5	137.7	8.9	109.9	29.1	157.4	10.7
MON + P2	109.3	14.2	106.9	17.0	106.1	16.2	155.2	11.4
MON + P3	125.7	4.3	132.6	13.7	118.5	2.5	174.2	6.7

The results of the 21 d seedling growth test, expressed as the Growth index (GrI%), from the soil samples collected from P-conditioned and un-treated mesocosms are reported in Table S3. The GrI% was evaluated at the four sampling times (0, 7, 14 and 28 days).

The GrI% values of SIL and MON non-treated soils were very low (SIL: 2.09%; MON: 1.71%), showing both soils were unsuitable for plant growth. These results were ascribable to the very low organic carbon and nutrient content of the soil, which was collected at 50 m depth. However, adding the products to SIL soil not only caused no significant toxic effect, but in the cases of SIL + P2 and SIL + P3 + P4 the plant growth was initially slightly stimulated. In MON soil, adding the product resulted in a worsened stunted seedling growth.

The acute test (organism exposure period: 48 h) with the earthworm *E. fetida* on elutriates showed that all samples had 0% mortality except for the soils treated with lime (SIL + L and SIL + L + P3), in which the mortality was more than 70% (data not shown). These results were due to the high pH value (>11) for the lime treatment which caused a toxic effect on this organism.

The chronic test with the same earthworm (28 days of organism exposure to soil) was performed using soil samples collected at days 0 and 7 (Figure 3). At both sampling times, SIL showed a negligible mortality; in fact all values were lower than the threshold value of 20% [59]. For this reason, the test was not performed at the subsequent sampling times. Moreover, no significant differences with the values for the un-treated SIL and OECD soils were found.

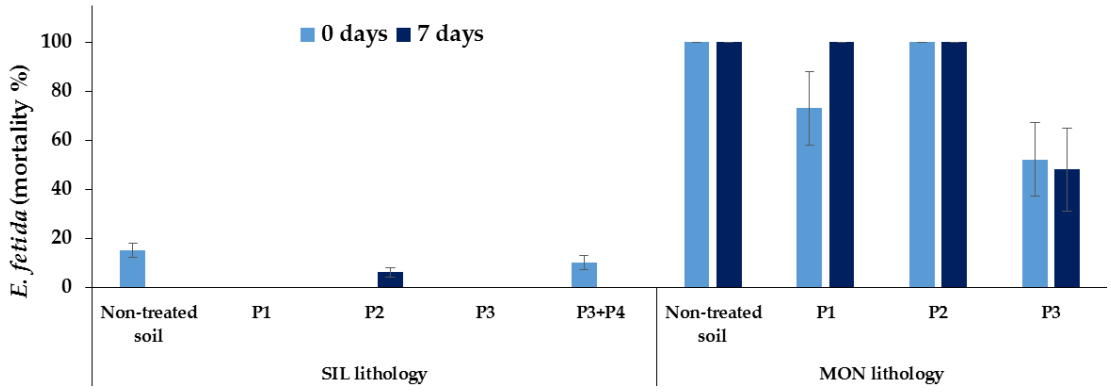

Figure 3. Chronic test (organism exposure period of 28 days) with the earthworm *Eisenia fetida* performed with soil samples at 0 and 7 days of the experiment. The results are expressed as mortality (%) SIL: sandy-silty-clay soil; MON: gravel in a sandy-silty soil. P1, P2, P3, P4: foaming agent products. The vertical bars represent the standard errors.

As in the acute test results, lime was deleterious for worm survival; in fact, the soil treated with it (SIL + L and SIL + P3 + L) was highly toxic, causing 100% mortality for the organisms tested.

The chronic test of 28 days of exposure of the earthworm *E. fetida* to MON-conditioned (MON + P1, MON + P2, MON + P3) and un-treated soils showed a mortality higher than 20%, with values between 50–100%; the high mortality values were presumably due to the lithology (mainly composed of gravel) and a very low organic carbon content in its fine fraction, which caused worm death through starvation. Interestingly, a reduced mortality was found (50% ± 20%) in the MON + P3 condition.

Lastly, the results of the FET test (96 h exposure) performed with the embryo fish *Danio rerio* on elutriates from all soil mesocosms at the different experimental times (0, 7, 14 and 28 days) are reported in Figure 4A for SIL and 4B for MON, respectively. The mortality percentages for SIL (Figure 4A) were negligible; in fact, all values were lower than the 20% threshold and not significantly different from the untreated SIL soil. On the contrary, the MON soil conditioned with the products (Figure 4B) was toxic for *Danio rerio* (mortality

100% over the experimental time), whereas the un-treated MON soil showed mortality values lower than 20%.

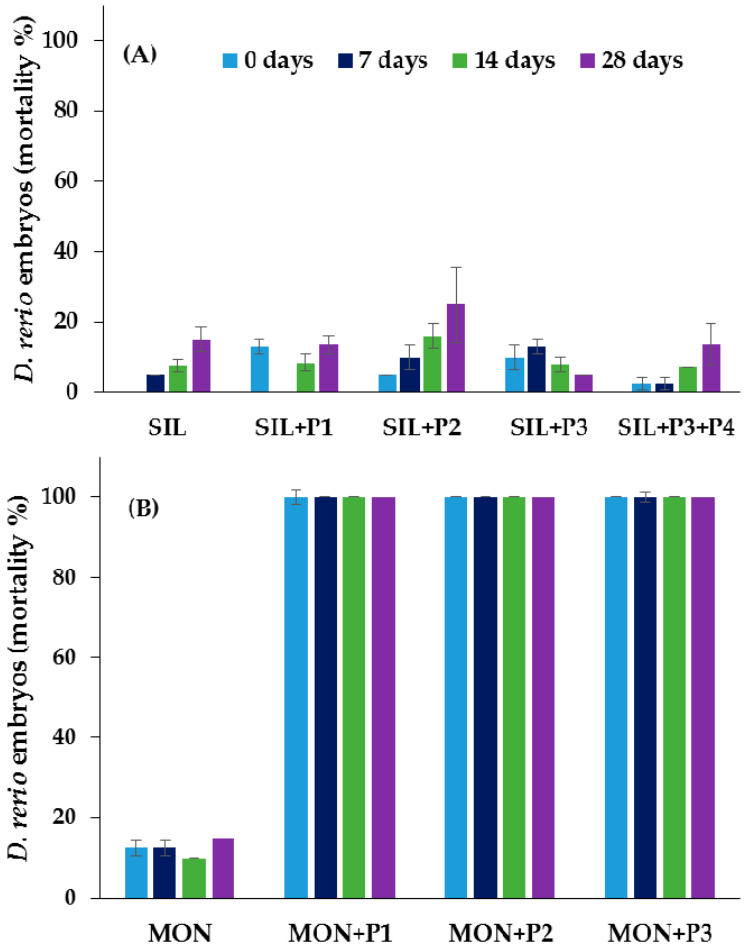

Figure 4. Fish Embryo Toxicity (FET) test at 96h of exposure of *Danio rerio* embryos to soil water extracts from (**A**) SIL and (**B**) and MON mesocosms. SIL: sandy-silty-clay soil; MON: gravel in a sandy-silty soil. P1, P2, P3, P4: foaming agent products.

In the case of the lime treatment, the pH of the elutriate was too high for embryo survival, so that it was not possible to perform the test because it does not foresee any pH correction in the OECD Guideline [48].

The results of the overall ecotoxicological tests of the foaming agent conditioned soils were integrated in a battery index. The results are reported in Figure 5A,B for SIL and MON, respectively.

At day 0 the P3 soil mesocosms (SIL + P3 and SIL + P3 + Lime) showed the lowest toxicity index value (<10%). Subsequently, the toxicity was lower for all SIL-conditioned soils, showing negligible risk battery index values.

As regards the MON-conditioned mesocosms, in all cases (MON + P1, MON + P2 and MON + P3) the toxicity index values were higher than 30% (high toxicity). At 7 days the overall toxicity decreased only slightly, remaining quite high, except for P3 which was only just above 10%.

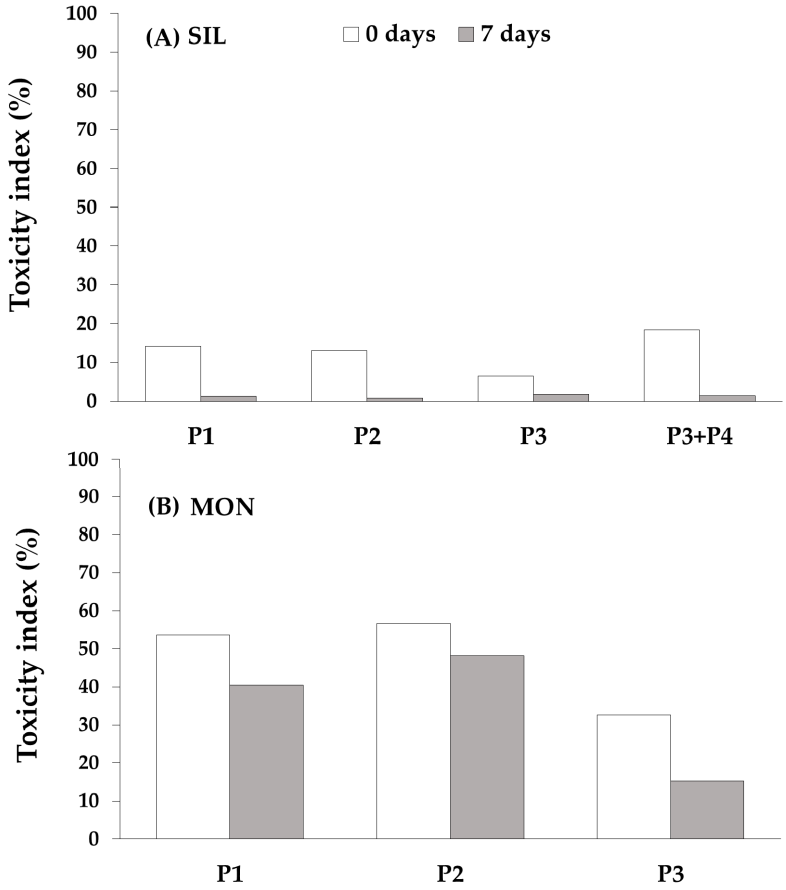

Figure 5. Integrated Toxicity index values at 0 and 7 days obtained from SIL (**A**) and MON (**B**) soil mesocosms treated with the various foaming agents. SIL: sandy-silty-clay soil; MON: gravel in a sandy-silty soil. P1, P2, P3, P4: foaming agent products.

4. Discussion

The current legislation at the national level for the re-use of spoil material as a by-product (in industrial or green areas) establishes chemical thresholds for specific organic and inorganic contaminants (e.g., heavy metals, hydrocarbons with more than 12 carbon atoms; Italian Decrees No. 152/2006 and No. 120/2017) but there is no threshold at either the national or EU levels for anionic surfactants. In this study, the spoil material was analysed for all the contaminants regulated and it always met the legal requirements. However, for precautionary reasons the Italian Ministry of Environment required a site-specific report demonstrating that the spoil material at the final destination site did not pose any risk for terrestrial or aquatic ecosystems. In this context, SLES occurrence in spoil material at concentrations affecting target soil and water organisms could be a risk for ecosystems.

The test results on terrestrial organisms (i.e., *L. sativum* and *E. fetida*) did not show SLES to be toxic, except for P1 at day 0 for plant germination (Table 4), where the highest SLES concentration was found. In line with these results a recent work [25] reported actual concentrations for the worm *E. fetida* of c.a. 300 mg SLES/kg soil, which were much higher than those found in this work. The results suggested that the foaming agents were not toxic for soil organisms at the concentrations used and are in line with those reported in similar studies [45,60]. Interestingly, adding lime was deleterious for the species tested, whether SLES was present or not. This was caused by a sharp soil pH increase and for this reason using lime at worksites is strongly advised against before the spoil material is classified as a by-product.

Regarding the water compartment, anionic surfactants can have a potential impact on aquatic organisms, which are known to be very sensitive to their residues [17,25,28].

For this reason, in this work we used the soil water extracts (elutriates) to check for a possible leaching of SLES from soil to water, following a standardized procedure (UNI EN 12457-2:2004) simulating this phenomenon.

The overall results suggest that the combination of each specific lithological characteristic of the soil (SIL or MON) and foaming agent applied (P1, P2, P3 and P4) influenced the persistence and partitioning of SLES between the soil and aqueous phase (elutriate) and consequently the toxicity, as found in other recent works [17,25,45,60]. In fact, SLES decreased over time only in the mesocosms containing SIL soil. This last lithology showed an organic carbon content (0.5% OC_{SIL} > 0.3% OC_{MON}), microbial abundance values (SIL: 2×10^6 cells/g soil > MON: 5×10^4 cells/g soil) and a water holding capacity (Table S1) higher than MON. These parameters are recognized as key factors in SLES degradation [23,60]. Moreover, the soil lithologies not only influenced the anionic surfactant degradation, but also its leaching from soil to water. The SLES concentrations in the elutriate produced in the case of MON were found to be double those for SIL, both because the initial product concentrations in the soil were higher and because of the predominance of the gravel fraction in MON, which decreased its adsorption capacity. Finally, each foaming agent (P1, P2, P3 and P4) displayed a different environmental fate and toxicity, owing to its different formulation. Soil conditioned with P3 showed the lowest SLES concentrations both in soils and elutriates at the starting time (day 0). The EC_{20} and EC_{50} values obtained in the pre-screening tests conducted on the commercial products, using *V. fischeri*, showed P3 to be the most environmentally friendly and P4 the most toxic and this can explain why, when combined (P3 + P4), the SIL soil showed the highest overall toxicity (Figures 2 and 5). In fact, P3 was selected for use in the subsequent tunnelling.

Regarding the ecotoxicological tests, all the products, except P3, were initially toxic for the bacterium *V. fischeri*, confirming the direct sensitivity of this organism to SLES concentrations higher than 2 mg/L [16].

In the case of MON, the products were always toxic at the treatment ratios used in this work because, as mentioned above, this matrix showed a low water adsorption capacity and high amounts of SLES (from 9 to 23 mg/L) moved from the solid phase to the aqueous elutriates, proving to be toxic for the aquatic organisms *Vibrio fischeri* and *Danio rerio*.

In order to summarize in a number the overall test results and to rank the foaming agent ecotoxicity in SIL and MON soils, a toxicity test battery integrated index was applied. The use of a set of biotests is a common practice when seeking an overview of toxicity [61–63]. It is interesting to note that the battery index applied was initially designed for the aquatic environment [55] and, for this reason, in this case reflects a highly precautionary scenario that takes into account any possible SLES leaching or run off to water bodies [16,17,27].

The toxicity score obtained from the calculation of the battery index made it possible to highlight the P3 foaming agent as the most environmentally friendly one, in the case of both SIL and MON. The simultaneous application of P3 and P4 was the worst case for its potentially higher toxicity than the others (Figure 5A).

On the basis of the results reported above a site-specific Technical Report was performed and the P3 commercial product was proposed for the tunnel excavation. During the actual tunnelling phase, most of the soil excavated was a mix of SIL and MON lithologies and the TR applied sometimes lower; consequently, intermediate ecotoxicological and chemical results, rather than the extreme ones obtained from the mesocosm studies, were expected. To assess the environmental compatibility of the spoil material at the construction site during the tunnelling, the determination of the anionic surfactant SLES and the test with the bacterium *V. fischeri* in the aqueous phase were proposed as monitoring tools. In particular, a site-specific protocol was drawn up and then approved by the Italian authorities, as in previous works [24,64]. The Technical Report recognised 2 mg/L as a threshold value for SLES concentration in elutriates and a bioluminescence inhibition $\leq$20%. If these conditions were met, the soil excavated could be used as a by-product; otherwise, it had to be kept at the temporary deposit construction site until these requirements were verified. It

was implicit that this protocol also had to be in line with the legal framework for excavated material and the circular economy requirements (recovery of scrap material by producing less waste [65]).

The use of foaming agents in EPB-TBM technology is necessary and several companies have been working on new commercial products in order to increase their effectiveness and at the same time lower their potential environmental impact. This means that the chemical formulas of foaming agent products are continuously changing. The use of suitable ecotoxicological tests for evaluating foaming agents and excavated soils containing them should be foreseen by legislation in order to compensate for the lack of legal thresholds not only in Italy, but also at the European level, for tunnel excavation. In fact, the ecotoxicological tests have the enormous advantage of evaluating any possible toxic effects in a commercial product, whatever its composition.

5. Conclusions

The re-use of spoil material as a by-product from tunnelling with a TBM-EPB needs to be evaluated with ecotoxicological studies. Such studies are very useful for establishing the most environmentally friendly product and the period foam-conditioned soil should be stored in temporary deposit areas before its safe use. In this work, the ecotoxicological approach made it possible to define a site-specific technical report with appropriate operating procedures to be applied for monitoring spoil material over the tunnelling time. The tunnel excavation described here has just been completed using the P3 foaming agent (the intrinsically less ecotoxic product and with less toxicity index values in the mesocosm experiment) and the use of the *Vibrio fischeri* test as an ecotoxicological tool proved suitable for defining the environmental compatibility of the spoil material, thus making up for the lack of a SLES threshold in the current legislation.

Supplementary Materials: The following are available online at https://www.mdpi.com/2073-4441/13/2/161/s1, Table S1: Maximum Water Holding Capacity (WHCmax) and moisture of soils (±e.s.) in the various mesocosms. P1, P2, P3 and P4: foaming agent products. SIL: sandy-silty-clay soil; MON: gravel in a sandy-silty soil. L: lime., Table S2: pH values of soil and water extracts for each condition and sampling time. P1, P2, P3 and P4: foaming agent products. SIL: sandy-silty-clay soil; MON: gravel in a sandy-silty soil. L: lime. Table S3: Lepidium sativum Growth index (GrI %) and standard errors (s.e.) at 21 days of L. sativum plants grown in SIL or MON soils collected from mesocosms at 0, 7, 14 and 28 days. P1, P2, P3 and P4: foaming agent products.SIL: sandy-silty-clay soil; MON: gravel in a sandy-silty soil. L: lime.

Author Contributions: Conceptualization, A.B.C. and P.G.; methodology, formal analysis and validation, A.B.C., P.G., L.M., J.R., M.D.L., V.G.M., E.D., I.L., P.M.B.G., A.F., E.B., L.P.; writing—original draft preparation, P.G., A.B.C., L.P., L.M.; writing—review and editing, P.G., A.B.C., supervision, A.B.C., P.G., L.P.; funding acquisition, A.B.C. All authors have read and agreed to the published version of the manuscript.

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