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The Most Common Laboratory Procedures for the Evaluation of EPB TBMs Excavated Material Ecotoxicity in Italy: A Review

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The rapid development of the mechanized tunneling in current decades has raised serious concerns about the environmental impact of large quantities of the muck. EPB-TBMs require the use of foaming agents for optimizing the soil conditioning. These agents could contain some chemicals (e.g., sodium lauryl ether sulfate – SLES) that are not included in the current legislation at the Italian or EU level. In order to minimize the project costs, it is useful to re-use the excavated soil as a reusable by-product that requires that it does not have any environmental impact on the ecosystems. For this purpose, to draw up a site-specific protocol is a practical and successful tool to evaluate the environmental compatibility of excavated soil during the tunneling. It can rely on one-month experiments at a microcosm or mesocosm scale using soil coming from the excavated site. At fixed times (from 0 to 28 days) the chemical degradation of the chemical together with ecotoxicological tests can be performed on soil or soil-water extracts. Both aquatic and terrestrial organisms are used and the choice of the tests depends on the final destination site. The results of the residual concentration of SLES in soil and in the elutriates, together with those of the ecotoxicological tests, make it possible to evaluate the temporary storage of spoil material and the time necessary for obtaining a safe soil debris to be used as a by-product. These data are usually included in the site-specific protocol to be applied during the excavation phase. This paper describes the main methodological aspects regarding microcosm experiments.

***Keywords**: EPB-TBM, Soil conditioning, Sodium Lauryl Ether Sulphate; Ecotoxicological tests.*

***Review delle procedure di laboratorio più comuni per la valutazione dell'ecotossicità del materiale risultante dallo scavo con TBM EPB in Italia**. Il rapido sviluppo dello scavo meccanizzato negli ultimi decenni ha sollevato serie preoccupazioni per l'impatto dei grandi volumi di smarino sull'ambiente. Le EPB-TBM richiedono l'uso di agenti schiumogeni per ottimizzare il condizionamento del terreno che contengono sostanze chimiche (es. sodio laurileter solfato, SLES) che non sono comprese nella normativa Italiana e comunitaria vigente. Al fine di minimizzare i costi del progetto, è necessario quanto più possibile riutilizzare lo smarino come sottoprodotto. Ciò è possibile se questo non ha alcun impatto ambientale sul suolo o sugli ecosistemi acquatici. A tal fine, redigere un protocollo specifico è pertanto uno strumento efficiente per valutare la compatibilità ambientale dello smarino. Questo può essere realizzato mediante prove della durata di 28 giorni a scala di microcosmo o mesocosmo. Le prove di laboratorio simulano il condizionamento del terreno ed a tempi prestabiliti (da 0 a 28 giorni) è possibile analizzare la degradazione chimica delle sostanze chimiche contenute. A queste prove vanno abbinati test ecotossicologici sui terreni o sugli elutriati valutando l'impatto su specifici organismi acquatici e terrestri. I risultati della concentrazione residua di SLES nel suolo e negli elutriati, insieme a quelli dei test ecotossicologici, consentono di valutare il tempo necessario per la degradazione dello SLES e unitamente ai risultati ecotossicologici, suggeriranno il tempo che deve trascorrere per ottenere un materiale di risulta utilizzabile come sottoprodotto. Questi dati sono inseriti nel protocollo specifico per ogni sito da applicare durante la fase di scavo. Il lavoro descrive i principali aspetti metodologici riguardanti gli esperimenti di microcosmo.*

***Parole chiave**: EPB-TBM, condizionamento dei terreni, tensioattivi anionici; sodio laurileter solfato (SLES), test ecotossicologici.*

1. Introduction

In the past decades, high demand for tunnel construction has resulted in the rapid development of tunneling industries, especially the case of mechanized tunnel boring machines (TBMs). Concerning the use of TBMs in tunneling projects, several million tons of muck are produced every year. For example, the Italian railway Milan-Genoa "Terzo Valico", which belongs to the European railway corridor Genoa – Rotterdam, about 14,000,000 m^3 of excavation material for 36 km of tunnels (Meistro *et al.*, 2020) will be produced. In the case of the frequently used EPB-TBM, muck is generally a combination of soil and rock mixed with a variable amount of chemicals containing foaming agents. The production of this considerable amount of excavated material mixed with chemicals caused growing worldwide concern about the environmental fate of this material (Baderna *et al.*, 2015; Council, 2009; Cserháti *et*

al., 2002; Ivanković and Hrenović, 2010; Jackson *et al.*, 2016). There have been several studies regarding the fate of muck from a waste to a possible resource, by supporting its reuse and recycling as a by-product (Bellopede and Marini, 2011; Grenni *et al.*, 2019; Magnusson *et al.*, 2015; Oggeri *et al.*, 2017; Rahimzadeh *et al.*, 2018). For example, spoil material can supply material to be used for many applications, such as refilling, road construction, and even raw material for industrial production. If the excavated material containing different chemicals is managed as waste, it requires its transportation, treatment, and disposal, with a significant increase in project investments. Furthermore, it is necessary to have sites suitable for the disposal of excavated soil debris, with a significant environmental impact. Consequently, the reuse of excavated material as a by-product is desirable and realized in several EU countries, in line with the circular economy model (D'Aloia Schwartzentruber and Robert, 2019; Murr *et al.*, 2020; Padulosi *et al.*, 2019). Before managing millions of tons of spoil material, ecotoxicological tests on this specific environmental matrix are very useful and effective for ensuring that safe by-products can be used for different purposes. The ecotoxicological tests provide a valuable tool to assess the potential effects of conditioned debris on soil and aquatic biota. Really, the technical and safety data sheets do not report the detailed chemical composition of the foams and information regarding toxicological data at the treatment ratios used in tunneling. The ecotoxicological tests are useful to highlight the presence of all chemicals in a commercial product (including the minor ones and/or metabolites) and eventually, the synergic effects of the mixture. Recent studies on SLES degradation in site-specific studies in real foaming-agent-conditioned soils from construction sites (Barra Caracciolo *et al.*, 2019; Finizio *et al.*, 2020; Patrolecco *et al.*, 2020), show SLES can be biodegraded by natural microbial populations. These works found that several environmental bacteria belonging to the Gamma-Proteobacteria harbor esterase enzymes are involved in SLES degradation (Grenni *et al.*, 2018; Rolando *et al.*, 2020). The authors found that aquatic organisms were the most sensitive to SLES residues in soil water extracts and the SLES concentration is in line with its degradation in spoil material (Finizio *et al.*, 2020). At EU level (European Commission 2012), the use of spoil material as a by-product is theoretically regulated by the EU Directive 98/2008 relating to waste (D'Aloia Schwartzentruber *et al.* 2019). In Italy, excavated soil from tunnels can currently be re-used as a by-product if it is in line with environmental legislative requirements. In particular, the actual legislative framework (Italian Decrees 161/2012, 120/2017 and 152/2006), report threshold values which should not be exceeded for several chemicals, but no limits for foaming agents and anionic surfactants are mentioned. The substances to be analyzed are Hydrocarbons (C > 12), BTEX (Benzene, Toluene, Ethylbenzene, and Xylene), PAHs (Polycyclic Aromatic Hydrocarbons), asbestos and several inorganic elements (As, Cd, Cb, Ni, Pb, Cu, Zn, Hg, total Cr, and Cr VI). However, SLES could be present in residual concentrations in re-used excavated materials and could pose an environmental risk, especially for aquatic ecosystems (Barra Caracciolo *et al.*, 2019; Finizio *et al.*, 2020). The Italian decrees (Decrees 161/2012 and 120/2017) define the appropriate reuse procedures for excavated material. In particular, concerning projects requiring an environmental impact assessment (with excavated material production over 6000 m^3, the so-called large construction sites), a specific "Land Use Plan" report has to be provided by the proposer of the project, in which the final destination of the excavated material has to be identified. The report has to be approved by the Italian Ministry of the Environment. The main requirement is that the spoil material environmental compatibility is in line with the final destination site. In the case of mechanized tunneling with TBM-EPBs, with a precautionary approach, an additional technical document demonstrating that the excavated material is not a risk for the environment (and human health) has to be provided by the Italian authorities (Padulosi *et al.*, 2019). In this paper, the overall procedure of the evaluation of the environmental risk of conditioned material is described, and tests commonly conducted to investigate the effect of foaming agents used for the conditioning process of TBMs on biota are described. It is clear that for a proper study of the conditioning system it is necessary to start from the geotechnical knowledge of the soil and then carry out some conditioning tests on the conditioned materials (Borio and Peila, 2011; Martinelli *et al.*, 2015; Martinelli *et al.*, 2019; Peila *et al.*, 2019; Peila *et al.*, 2016; Todaro, 2016; Salazar *et al.*, 2018; Carigi *et al.*, 2020). When the optimal conditioning is assessed, the ecotoxicology tests are then carried out on specifically prepared samples considering a reasonable increase of the conditioning agents to take into account the possible natural variation of the soil conditioning during the tunnel alignment (Fig. 1). To study the behavior and the biodegradation process of SLES in foaming agent conditioned soils, researches have been recently carried out on microcosm and/or

mesocosm studies. Microcosms are small samples of about a few liters, while mesocosms are large scale samples of about 0.5-1m^3 from which at specific times samples are taken to perform specific ecotoxicological tests.

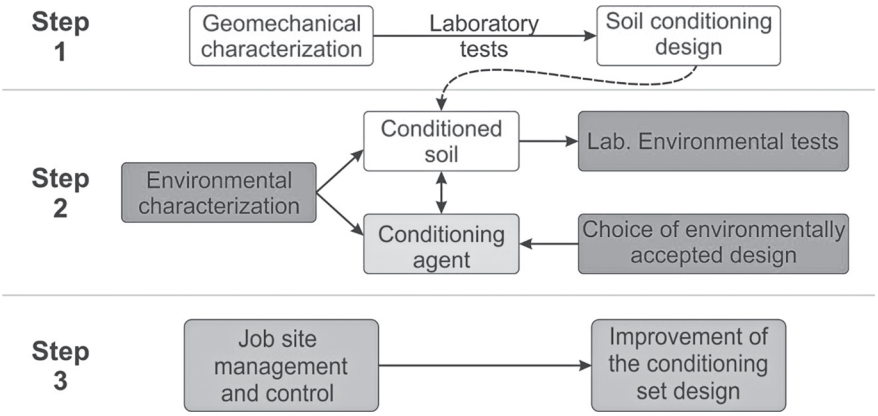

Fig. 1. Different steps in the evaluation of suitable conditioning design.
I differenti passi per lo definizione del condizionamento ottimale.

2. Chemicals in EPB tunneling

In the case of using EPB machines, chemicals are especially essential as the optimized performance depends on the proper implementation of the conditioning process. The main chemicals are foams and polymers (EFNARC, 2005; Gong *et al.*, 2016; Maidl *et al.*, 2013; Milligan, 2000; Peila *et al.*, 2008, 2009; Peila *et al.*, 2007; Peila *et al.*, 2016) which are injected into the tunnel face inside the pressure chamber and along the screw conveyor to modify the excavated material workability and permeability and decrease metal parts wear.

2.1. Foams

By definition, foam is made of bubbles which are produced as a consequence of the reduction in liquid surface tension after using surfactants. Commercial foaming agents contain a chemical mixture, which often includes anionic surfactants. Among anionic surfactants, sodium lauryl ether sulphate (SLES) is one of the main compounds of most commercial products used in tunneling industries. Foam can also contain performance-enhancing soluble polymers to increase its viscosity and improve the thixotropic properties. Various additives, including biocides, to prevent microbial growth and stop biological degradation of the polymers, anti-freeze agents, and of course, dyes for brand recognition can be also used. In some cases, according to project specifications, foam can be enriched with anti-clogging (mainly where tunneling in clay formations) or anti-wear agents. Surfactants are substance that are capable of lowering surface tension, and that is why they are also called surface-active compounds. Properties of surfactants are related to their amphiphilic molecular structure, which consists of a water loving-head and a water-hating tail. Surfactant properties depend on the presence or absence of different hydrophilic and hydrophobic groups (Fig. 2). The hydrophilic group of a surfactant primarily determines its application as well as its chemical-physical properties.

Surfactants are classified in four categories of anionic (mostly SOAP) sulfonates or sulfates ANS, amphoteric/betaines/zwitterionic, (mostly betaines and amino acid derivatives), cationic (mostly quaternary ammoniums) and nonionic (mostly ethoxylated, fatty acid esters) depending on the abi-

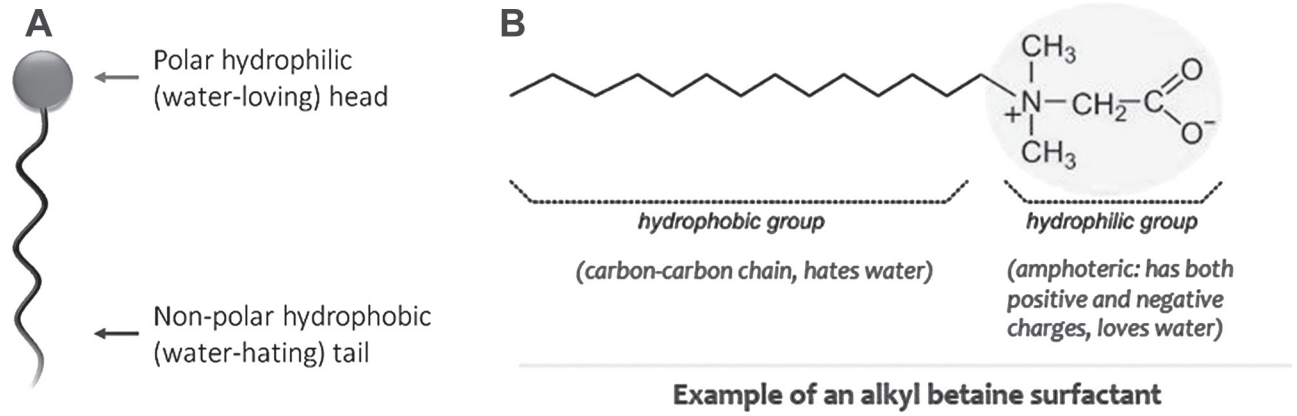

Fig. 2. A: Amphipathic structure of Anionic Surfactant molecules, B: Example of an alkyl betaine surfactant.
A) Struttura di un agente schiumogeno anionico ; B: esempio della struttura di un agente schiumogeno beta alcalino.

lity to produce ions in aqueous solution. In the anionic surfactants, the surface-active feature is related to anions. The molecule is consisting of a water-soluble polar head group (which can be charged or uncharged) and hydrophobic non-polar hydrocarbon tails (Ying, 2006), which is designed to have mainly cleaning or solubilization properties (Swisher, 1986). In cationic surfactants, surface-active features in aqueous solution are related to cations, while in nonionic surfactants, surface-active properties are without ions dissociating and amphoteric surfactants which can react non-ionically, cationically, or anionically, depending on the pH of the aqueous phase. Over the past 50 years, anionic surfactants have been extensively used as detergents and cleaning products (Scott and Jones, 2000). Commercial ANS are industrially available in different forms, such as powders, granules, needles, pastes and solutions (Könnecker *et al.*, 2011) and they are used in many industrial applications (Baderna *et al.*, 2015; Cserháti *et al.*, 2002; Lara-Martín *et al.*, 2008; Van Ginkel, 1996; Ying, 2006). It has been estimated that ANS forms about 60% of worldwide surfactant production (Kronberg and Lindman, 2003). Given that they can be produced easily and cheaply, they also are the main components of most commercial products used for soil conditioning using Eq. 1.

$$CH_3(CH_2)_{10}CH_2(OCH_2CH_2)_nOSO_3Na \quad (1)$$

Among the various types of anionic surfactants, Sodium lauryl ether Sulphate (SLES) and Sodium lauryl Sulphates are the main ingredients in the majority of commercial products. LES is a sodium salt that is obtained through the ethoxylation process of sodium lauryl Sulphate (SLS) (Cserháti *et al.*, 2002) (Fig. 3). There are several synonyms for SLES, e.g., sodium Laureth Sulphate, dodecyl sodium Sulphate Ethoxyethene, polyethylene glycol (1e4) lauryl ether Sulphate, and sodium salt (Robinson *et al.*, 2010). SLES is an important ingredient in cosmetics (e.g., 0.1 to 0.3% in the formulation of mascara), cleaning products (from 0.1% to 50%), and personal care products (e.g., 47% in bath soap and detergents) due to both emulsifying and foaming properties. Extensive use of SLES is also related to inexpensive production and, more importantly, negligible eye and skin irritation properties, which allow the employment of 5% to 25% of SLES concentration in the formulation of baby products (Robinson *et al.*, 2010). SLES is considered to irritate much less than SLS, and consequently, consumer products containing SLES should presumably be milder, while nevertheless maintaining the same characteristics (Rizvi *et al.*, 1996).

2.2. Degradability of SLES

According to the Italian law 120/2017 soil debris can be considered as waste when the chemical thresholds for organic and inorganic contaminants (e.g., heavy metals, hydrocarbons C>12, and other chemical) are exceeded. It is essential to highlight that there are currently neither SLES soil threshold limits in European and Italian legislation nor comprehensive studies on its ecotoxicological effects on soil and water organisms (Barra Caracciolo *et al.*, 2017). Biodegradation, ecotoxicological data on SLES from conditioned soils with foaming agents, and the ecotoxicological effects of SLES on terrestrial organisms are rare and with a limited number of data (Barra Caracciolo *et al.*, 2017; Mariani *et al.*, 2020). The anionic surfactants in foaming agents are generally considered to be biodegradable (Baderna *et al.*, 2015; Barra Caracciolo *et al.*, 2017; Van Ginkel, 1996; Ying, 2006) and non-toxic (Milligan, 2000). Alkyl ethoxy sulphates, are considered among the most rapidly biodegradable anionic surfactants in aerobic conditions (Ying, 2006). According to OECD biodegradability tests (OECD, 2007), both primary and ultimate biodegradation can occur. Primary degradation occurs when the chemical structure loses its surfactant characteristics; ultimate degradation occurs when the molecule is mineralized to CO_2, CH_4, water, mineral salts, and biomass (Scott and Jones, 2000). According to standard tests SLES is basically biodegradable with a rate of 7 h to 30 days, depending on the initial conditions, however data on the biodegradation process of SLES in environmental studies are relatively low. Moreover, most biodegradation tests have been performed on a chemical mixture of alkyl ethoxy sulfate without specification of either the alkyl chain length or the ethoxylate group number. In any case, different bacterial genera isolated from activated sludge, such as *Azotobacter, Bacillus, Pseudomonas, Citrobacter,*

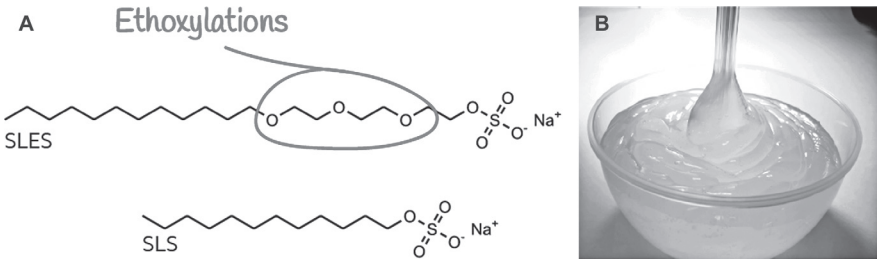

Fig. 3. A: SLES and SLS molecular structures, B: SLES actual form.
A) Struttura molecolare dello SLES e dell'SLS, B) aspetto fisico dello SLES.

Acinetobacter, Klebsiella, and *Serratia* were found to be able to degrade AES in laboratory cultures. Furthermore, there are few data on SLES biodegradation in field studies or environmental risk evaluations examining soil exposure scenarios during excavation with the exception of the researches conducted by (Barra Caracciolo *et al.*, 2017; Rolando *et al.*, 2020). It is of crucial importance to assess if SLES occurrence and persistence in soil debris due to tunneling can pose a risk for the terrestrial and water compartments and human health.

2.3. The aim of ecotoxicological tests

Commercial foaming agents are typically used in EPB soil conditioning between 0.1 and 3 L/m^3 of excavated soil. Because SLES percentage in the commercial foaming agents ranges from 5% to 50%, its expected environmental concentrations are from 40 to 500 mg/kg in treated soils. Degradation studies using a portion of the soil conditioned with the foaming agents in microcosm (Artigas *et al.*, 2012; Barra Caracciolo *et al.*, 2013) or mesocosm experiments and in real environmental conditions (e.g. temperature, light, humidity and so on) were developed in Italy with the goal of assessing the persistence of SLES in soil debris and the soil natural microbial community capability to remove it. A review on ecotoxicological tests on anionic surfactants reports that SLES can potentially have more detrimental effects on aquatic organisms (effective concentrations in the range of few mg/L to dozen of mg/L) than terrestrial ones (Barra Caracciolo *et al.*, 2017). Recent works (Baderna *et al.*, 2015; Grenni *et al.*, 2018) report that SLES ecotoxicity depends on the sensitivity of the organisms tested and from the residual amount of foaming agent in the soil after its disposal in the temporary deposit area at the construction site. That is to say, that it is of crucial importance to assess SLES persistence in soil debris in real conditions to know how to manage spoil material. In particular, bioassays, including the algal growth inhibition test with *Pseudokirchneriella subcapitata*, the acute immobilization test with *Daphnia magna*, the acute toxicity tests with *Danio rerio* and *Vibrio fischeri* and the seed germination test with *Lepidium sativum* were performed as the main tests highlighted by the environmental guidelines (Wieczerzak *et al.*, 2016). In the following paragraphs, the environmental and ecotoxicological tests followed by chemical and physico-chemical methods which can be performed after a geotechnical laboratory assessment are presented and discussed.

3. Sample preparation

Conditioned microcosm soil samples (Fig. 4), are usually prepared with 30% more foaming agent than the optimum amount assessed by laboratory tests because in the actual situation, foam consumption in a job site is larger. Samples are prepared according to the UNI EN 14735:2005 and UNI EN 12457–2:2004 protocols to produce elutriates in a 1:10 (liquid/solid) ratio, using distilled water as the extracting agent (Hubálek *et al.*, 2007; Marguí *et al.*, 2016). 100 g of soil sample is put into a 1 L bottle, and the calculated amount of distilled water (also considering the moisture of the sample) added. The suspension has to be shaken for 24 h at 20 °C in the dark.

After the leaching period, the suspension containers are kept in steady state for 15 min with the aim that the solid particles settle down. The supernatant of the samples is then poured into polyethylene vials and centrifuged for 15 min at 9000 rpm. Required specimens for chemical analyses (elutriate samples) are collected right after centrifugation, while ecotoxicological analyses are performed on filtered elutriate through 0.45 µm cellulose acetate filters. Prepared elutriates for the ecotoxicological and chemical analysis can be stored in polyethylene vials at 4 °C (Fig. 5). However, the pH in each elutriate container needs to be measured.

Soil sub-samples are collected at different days (0, 7, 14, 28 days) in line with SLES degradation over time. Soil elutriates are produced to assess the effects of the foaming agents on target species such as *Pseudokirchneriella subcapitata*, *Daphnia magna*, *Danio rerio*, *Vibrio fischeri*, and *Lepidium sativum*. The

Fig. 4. Microcosm experiments.
Sperimentazione sui microcosmi.

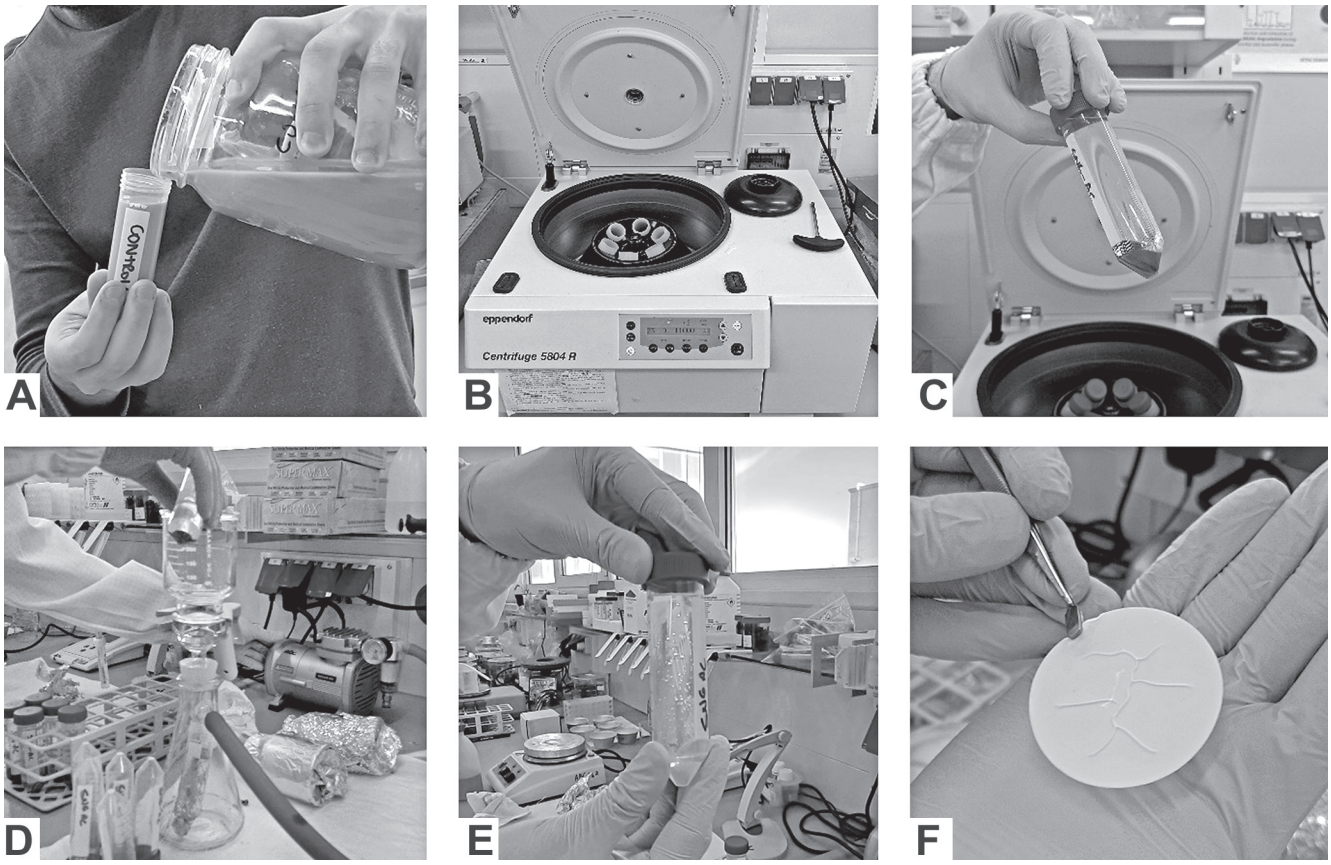

Fig. 5. A: Pouring supernatant of solution into polyethylene vials for centrifuge, B: centrifuge instrument, C: sample after centrifuge, D: extraction of the elutriated solution, E: elutriated solution, F: filter paper.
A: Fase di sversamento della soluzione all'interno della provette per le prove, B: Strumentazione per le prove in centrifuga, C: provino dopo la centrifugazione, D) estrazione della soluzione di elutriato, E: esempio della soluzione di elutriato, F: carta filtrante.

results of the tests are then compared with SLES residual concentrations in the elutriates, determined with the MBAS (methylene blue active substances) spectrophotometric method (Barra Caracciolo *et al.*, 2019). Finally, data can be combined in a battery index, which proved effective at evaluating the overall ecotoxicity in a real-life situation of different excavated soils conditioned with specific products (Grenni *et al.*, 2018).

4. Ecotoxicological tests on foaming agent conditioned soils

Acute toxicity reflects short-term adverse effects resulting typically from aquatic biota exposure to a chemical or formulation and it is generally measured as median effective concentration (EC_{50}), i.e. the concentration of a substance in an environmental medium expected to produce a certain effect in 50% of test organisms in a given population under a defined set of conditions.

4.1. Acute immobilization test

This acute toxicity test is performed in accordance with OECD 202 test (OECD, 2004) using crustacean *Daphnia magna* (Fig. 6).

In order to prepare Daphnia for the test, dormant eggs (ephip-

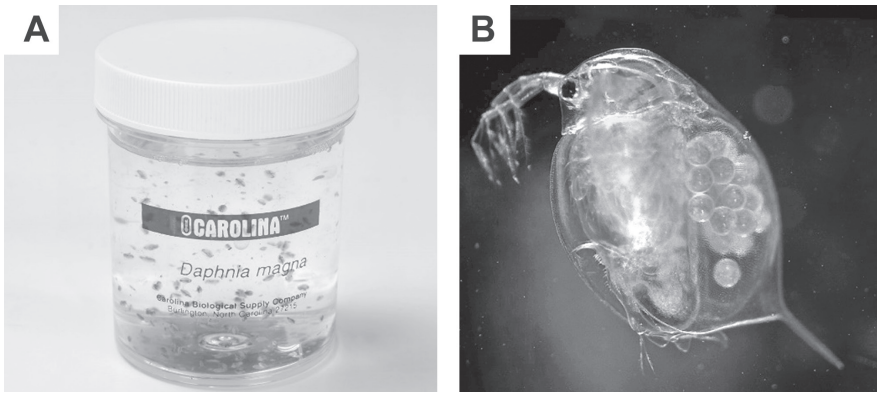

Fig. 6. A: *Daphnia magna* Culture, B: *Daphnia magna* (adult female) (Watanabe, 2011).
A: Coltura della Daphnia magna B: daphnia magna (femmina adulta) (Watanabe, 2011).

pia) are placed in standard ISO freshwater conditions prepared with specific environmental considerations (6000 lx and 20-22 °C). Within 72 h, the daphnids begin to hatch from the ephippia; it should be noted that daphnia should be at least 24 hours old at the beginning of the experiment. The transfer of the daphnids into the test plates is carried out under a microscope at low magnification (10X). For each test 60 daphnia are required that are classified in three separate sets of 20 neonates that are put in four wells (five animals per well); each animal is then treated with 2 mL of elutriate (10 mL in total for each well). The multi-well plates are covered with a parafilm strip and closed with a tight cover in the dark for 48h at 20°C, as specified in the OECD guideline. The test endpoint is immobilization of daphnia in each well by counting the number of immobilized or dead organisms as well as actively swimming ones. The definition of immobilization is related to the neonates' swimming ability, and an observation takes place after a gentle agitation of the liquid for 15 s. It should be considered that daphnia that are only able to move their antennae are considered immobilized. The results are expressed as the effect in percentage (%). Negative and positive controls are carried out using standard ISO freshwater and potassium bichromate, respectively.

4.2. The fish embryo acute toxicity (FET) test

The test is performed according to OECD 2013 based on a 96h exposure of newly fertilized eggs of the (Zebrafish) to a liquid sample and is expected to reflect acute toxicity in fish in general (Fig 7) (Belanger *et al.*, 2012; ECVAM, 2014; Wang *et al.*, 2015).

To provide fish eggs, a breeding stock of *Danio rerio* adults is required which is maintained in a glass aquarium at the optimal living conditions including feeding three/four times a day with a combination of dried food and newly hatched brine *Artemia Salina* shrimps. Fertilized eggs are collected the day before the test using an egg-trap, which is a glass vessel covered with a mesh. The Immersed egg trap in the aquarium is removed at the beginning of the illumination period when zebrafish spawning is done. At least twice the number of embryos required for the test are randomly selected and transferred into petri dishes filled with the test samples/controls. Fish embryos should not be collected later than 1 h post-fertilization. Properly developing fish embryos between the 4- and 32-cell stages and with an intact chorion that are suitable for the test are then selected using an inverted microscope. Dilution water is used as negative and internal controls; dichloroaniline is used in positive controls at a fixed concentration of 4 mg/l. For each test sample 20 embryos are required that each are placed in one well plate filled with 2 mL of elutriate, considering 4 embryos as an internal negative control results in total of 24 embryos for each sample. The incubation is performed for 96 h at 26.0±1.0 °C with a 14:10 h light-dark photocycle on plates that are covered with lids. Up to four apical endpoints are recorded every 24 h, as indicators of lethality in the fish including coagulation of fertilized eggs, lack of somite formation, lack of detachment of the tail-bud from the yolk sac, and lack of heartbeat. At the end of the exposure period, acute toxicity is determined based on a positive outcome in any of the four apical endpoints. The results are expressed as mortality (%) of the fishes.

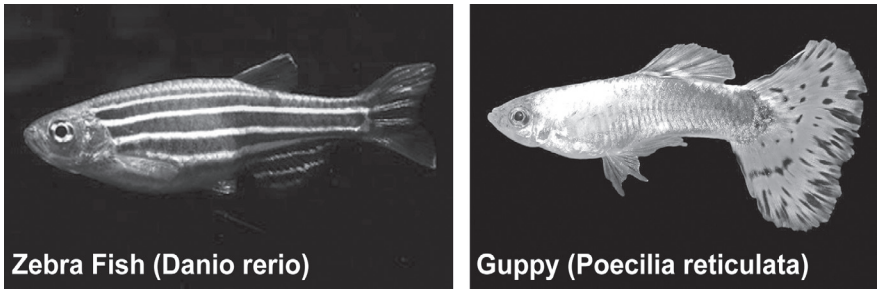

Fig. 7. The two common species used in Fish Embryo Acute Toxicity (FET) testing.
Due comuni specie di pesci utilizzati nelle sperimentazioni per la definizione della tossicità acuta di embrioni di pesci.

4.3. Vibrio Fischeri test

One of the most sensitive tests to SLES occurrence in spoil material is the inhibition of luminescence emitted by the marine bacterium *Vibrio fischeri* (Fig. 8)

This test is based on the inhibition of the naturally emitted luminescence of *Vibrio fischeri* after exposition to a toxic substance. The acute toxicity test with *Vibrio fischeri* is performed in accordance with the UNI EN ISO 11348-3:2019 Protocol. The inhibition percentage is obtained by comparing the light output of the test organism with a toxic-free control after three exposure times (5, 15, and 30 min). The difference in light output of the sample and the control is directly related to the toxicity of a sample. Lyophilized bacteria are rehydrated with the reconstitution solution before using to provide a ready-to-use suspension. Ultrapure water (UPW) is used for the preparation of the diluent solution (2%

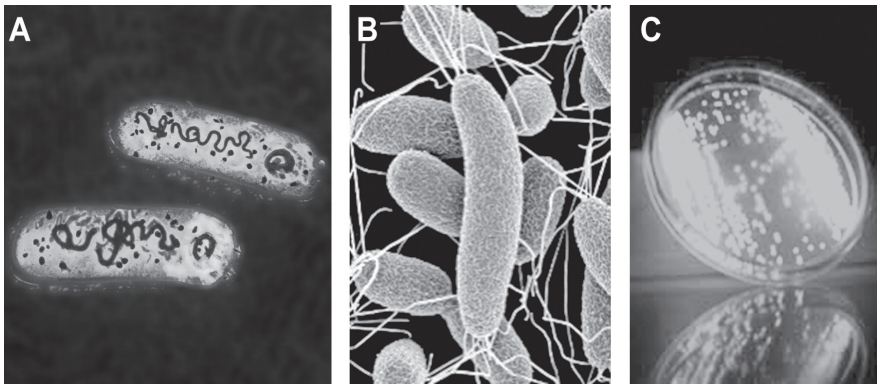

Fig. 8. A: Schematic picture of *Vibrio fischeri*, B: Actual picture, C: Emission of light by *Vibrio fischeri* bacterial population in a petri dish.
A) rappresentazione schematica del Vibrio Fischeri, B) fotografia reale dei Vibrio Fischeri; esempio di emissione di luce da parte di una popolazione di Vibrio Fischeri in un piatto di petri.

W/V NaCl solution) and osmotic adjustment solutions. The osmotic adjustment solution, composed of 22% W/V NaCl solution, is used to bring the salinity of the sample to approximately 2%. According to the UNI EN ISO 11348-3:2019 protocol, the inhibition effect is more than 20% compared to a non-toxic control consisting of a bacterial suspension with distilled water containing 2% NaCl. Subsequently to performing preliminary tests with the negative (solvent control) and positive controls (Dichlorophenol aqueous solution, 3.5mg/L), the acute toxicity of the soils treated with the foaming agent for *Vibrio fischeri* can be evaluated by testing the soil aqueous elutriates obtained from spoil material. According to the ISO protocol, before carrying out the tests, the pH value of each elutriate has to be measured and eventually corrected (range 6.0-8.0) using an HCl 0.1 M solution (Scheerer *et al.*, 2006).

4.4. Algal growth inhibition test

In this test, the inhibition effect of toxic solutions on the growth of an algal population is observed over a period of 72 hours under controlled experimental conditions. The test is performed in accordance with the OECD guideline (OECD, 2011) using green alga *Pseudokirchneriella subcapitata* (Fig. 9).

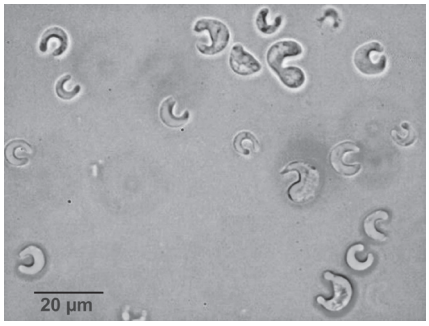

Fig. 9. Picture of Green alga *Pseudokirchneriella subcapitata.*
Fotografia dell'alga Pseudokirchneriella subcapitata.

Test solutions are prepared by adding the whole pool of culture medium nutrients to all the elutriate samples prepared in the aging time of 0 to 28 days, from both treated and untreated soils. Three 50 ml-replicates are set up for each elutriate sample and six 50 ml-replicates for the control, which are prepared by enriching Ultrapure 0.22 µm-filtered water with the standard medium. Moreover, a solution of copper sulphate is used as the positive control toxicant. The test cultures are inoculated with exponentially growing algal cells (10.000 cells/mL) and incubated under controlled conditions (22±1 °C; 5000 lx; 100 rpm). For the test after 72 h exposure to be valid, the acceptability criteria are checked: shift in pH; minimum growth required; maximum variability of both section-by-section growth rates and replicate cultures. The toxicity effects are evaluated using the Yield (Y), which is the 72h cell number minus the 0 h starting number. The algal growth is measured with an electronic particle counter and the cell number (cells/ml) used to calculate the toxicity effect as percent growth inhibition (I%) using Eq. 2.

$$I\% = 1 - \frac{Y_{\text{test culture}}}{Y_{\text{control}}} \cdot 100 \quad (2)$$

Furthermore, the Yield of the elutriate cultures from the untreated soils is used as a control to calculate the toxicity of those from the treated ones and thus to measure exclusively the effect of chemical mixture due to the foaming agent presence.

4.5. Seed germination test

(Baderna *et al.*, 2015) recently evaluated the acute effects of three soil foaming agents containing SLES, in concentrations ranging from 10% to 30%, on three plant species (*Cucumissativus*, *Sorghum saccharatum* and *Lepidium sativum*), in accordance with the guidelines reported by (Martignon,

Fig. 10. Picture of *Lepidium sativum* sprouts.
Fotografia del Lepidium sativum sprouts.

2009). This test is performed in accordance with the US EPA OPPTS 850.4200 guideline (1996) using *Lepidium sativum* seeds (Fig 10). It evaluates the effects of aqueous elutriates on germination and on the lengthening of roots, hypocotyls and epicotyls, expressed as the percent germination index (GI%) (US EPA, 1996; Martignon, 2009).

For each tests a number of 10 *Lepidium sativum* seeds are placed in Petri dishes containing a paper disk with 5 ml of elutriate. To perform the control test the same number of seeds are placed in the petri dish with 5 ml of distilled water instead of elutriate. In the next step petri dishes are placed in a growth chamber at 25 °C in the dark for 72 h. Afterwards, the length of the seedlings are measured, and the germination index (GI) is calculated according to APAT (2004) for each experimental condition, with the formula: GI = N° of germinated seeds x mean seedling length. For each treatment, the percent germination index (GI%) is expressed as the GI percentage of the untreated soil, which is calculated using Eq. 3.

$$GI[\%] = \frac{GI_{treated}}{GI_{untreated}} \cdot 100 \quad (3)$$

Where,

$GI_{treated}$: mean value in the treated soil elutriate;

$GI_{untreated}$: mean value in the untreated soil elutriate.

The inhibition percentage is calculated using Eq. 4.

$$I\% = |100 - GI\%| \quad (4)$$

The untreated soil elutriates used as test controls for the treated soil elutriates to measure the net toxicity of the treatments. The data are reported as the effect percentage net of any possible intrinsic toxicity in the soil, as measured in the untreated soil.

5. Chemical and physico-chemical tests

5.1. Methods of surfactant extraction

Prior to the determination of anionic surfactants and AES in solid matrices (such as soils), a preliminary extraction and pre-concentration phase are required. Soxhlet extraction is one of the most widely used methods for this purpose as it is cheap and easy to perform (Olkowska *et al.*, 2013; Traverso-Soto *et al.*, 2012). However, in recent decades new more efficient extraction methods from solid matrices (in terms of solvent-consumption and required time), have been developed. Among these, microwave-assisted extraction (MAE) is an appropriate technique for the extraction of different anionic surfactants from sediment and sludge in a relatively short time and low required solvent volumes are notable (Traverso-Soto *et al.*, 2012). The other methods that make efficient the extraction in short time from a low solvent volume are: 1) the accelerated solvent extraction (ASE) or pressurized liquid extraction (PLE), which utilizes high temperatures and pressure to maximize the efficiency of extraction in a short time (15-20 min per sample) and with much less consumption of organic solvent than more conventional techniques; 2) supercritical fluid extraction (SFE) which uses the high pressure and supercritical properties of the fluids, generally CO_2 or water (Olkowska *et al.*, 2013; Traverso-Soto *et al.*, 2012).

5.2. Determination of the surfactant in the liquid extracts

For the determination of the anionic surfactant residual concentrations in soil, the analytical determination of the surfactant in the liquid extracts is carried out. Chemical analyses are performed to assess over time SLES concentration in the soil and in the elutriates produced from soils and used for the ecotoxicological tests. HPLC coupled with an ultraviolet (UV) or fluorescence (FL) detector can be applied to the analysis of AES only after a preliminary derivatization step, needed to produce a chromophore group in the surfactant molecule. The use of more sensitive and specific detectors, such as mass spectrometry with different types of ionization interfaces (i.e., electrospray – ESI, atmospheric pressure chemical ionization – APCI) coupled with HPLC (LC-MS) overcomes the complications of this step. Today, LC-MS based on molecular weight and retention time represents the most performant technique for the identification of surfactant homologues and ethoxymers in environmental analysis. Triple quadrupole (MS-MS) (Jahnke *et al.*, 2004) or ion trap MS detectors (Lara-Martin *et al.*, 2006) are the main tools for trace analysis of anionic surfactants in environmental matrices, where interferences due to the matrix are likely. Furthermore, the increasing use of powerful techniques such as time-of-flight (ToF) or quadrupole time-of-flight (Q-ToF) LC-MS systems, allows improved accuracy in the identification and quantification of surfactants and their metabolites. Recently, Gago-Ferrero *et al.* (2015) developed and applied an LC-Q-ToF-MS technique to detected and identify the suspect and unknown contaminants in wastewater, including AES, while (Lara-Martín *et al.*, 2011) applied the LC-Q-ToF-MS to liquid and solid environmental matrices, in order to analyze the most widely used surfactants (linear alkylbenzene sulfonates, LAS, nonylphenol ethoxylates, NPEO, and alcohol ethoxylates, AEO) and their main

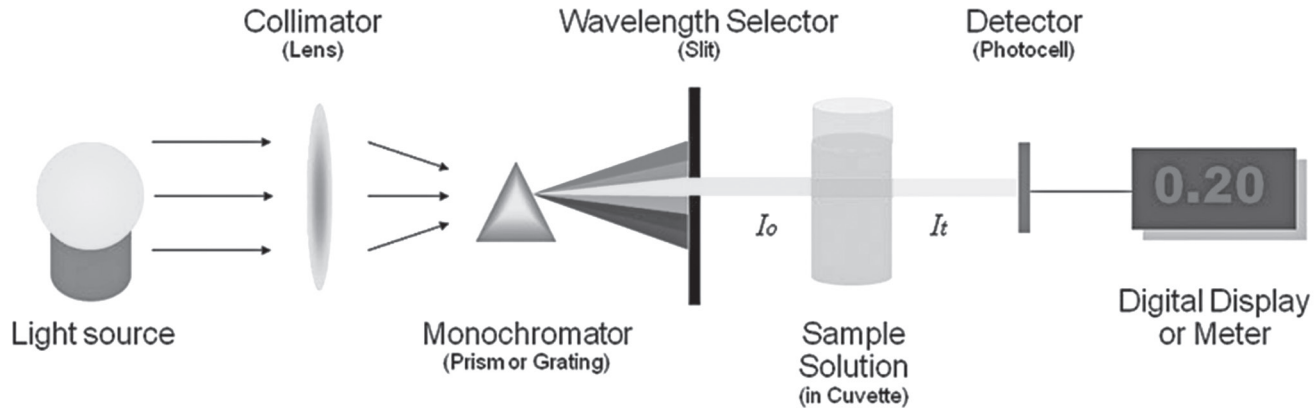

Fig. 11. Basic structure of spectrophotometers (Shim, 2013).
Struttura di base dello spettrofotometro (Shim, 2013).

metabolites. On the other hand, these techniques are not widespread for routine analysis yet, due to their high cost and need of highly skilled lab personnel (González *et al.*, 2008; Lara-Martín *et al.*, 2010; Lara-Martín *et al.*, 2011).

5.2.1. MBAS (Methylene Blue Active Substances) spectrophotometric method

The official methodology for estimating the total concentration of anionic surfactants, including AES, is the methylene blue active substances (MBAS, Standard Methods 5540C, 2012) method. It is based on the ion-pair reaction of these surfactants with methylene blue (cationic ion-pair reagent) and the extraction with solvent chloroform, followed by a spectrophotometric determination of the absorbance at a fixed wavelength (Fig. 11). This technique is relatively simple to use for determining anionic surfactant concentrations and it is suitable for routine analysis, but the major drawback is the production of high volumes of toxic solvents and the lack of sensitivity and selectivity, as it suffers some non-surfactant aqueous-phase interferences (Standard Methods 5540C, 2012).

A similar test can be carried out with an acid methylene blue indicator. The same volumes of the indicator solution and chloroform are added. If the chloroform phase is colorless, the aqueous phase is blue, and the effect is unaltered by the addition of 0.15 mL of 0.2% aqueous solutions of anionic active material such as sodium dodecyl sulphate, then the surfactant is cationic. If the color is transferred to the chloroform phase by the addition, a nonionic surfactant may be present. If the blue color is initially in the chloroform phase and after the addition of 0.15 mL of 0.2 aqueous cetylbenzyldi methyl ammonium chloride (CBC) or Hyamine 1622 the color still remains in the chloroform layer, the surfactant is anionic; if it is transferred to the aqueous phase, a nonionic surfactant may be present (Fig. 12) (Schulz and Bruttel, 1999).

6. Conclusion

The world we are leaving has suffered dramatic changes due to the extensive environmental destruction resulted from industrial advancements in recent decades. Environmental scientists warn that we are close to a no return point in the case of ecological contamination. In this regard, nowadays, much attention is paid to the environment in each aspect of our life to ensure that development is sustainable, by developing regulations and applying an approach that can protect ecosystems from chemical contamination and effects. In this context, ecotoxicological tests using various target species for evaluating the effects of

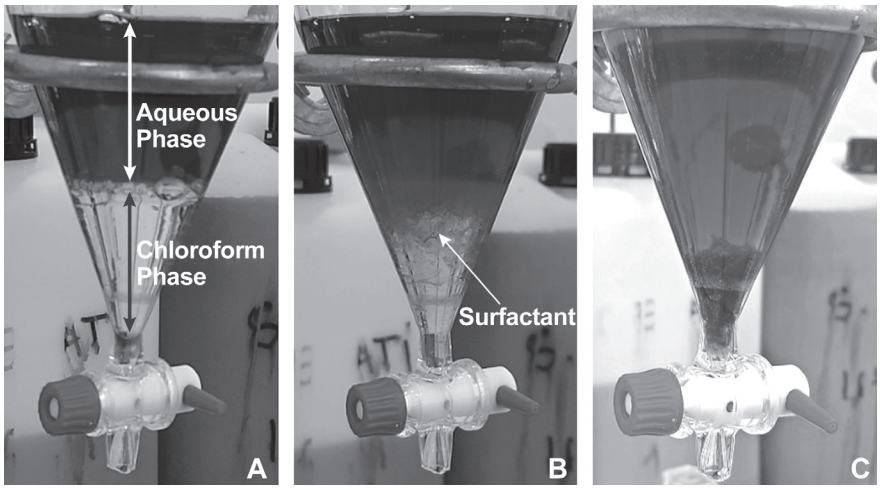

Fig. 12. A: Separation of surfactant after adding chloroform during the first extraction.
A) separazione del contenuto di agente schiumogeno dopo l'aggiunta di cloroformio durante la prima estrazione.

real matrices contaminated by various chemicals are a suitable tool to protect environmental health. In this way, limits are developed for the use of chemicals to preserve the environment. In the case of tunnel construction using EPB TBMs, as huge amounts of chemicals are required for the conditioning of excavated material, the spoils are mixed with these chemicals which might approach the contamination limit. If tunnelling spoils are contaminated they must be considered as waste, which requires decontamination or transportation to a land fil for decomposition. In both cases it will affect the project scheduling and investment. Overall, to prevent unexpected environmental problems the chemicals that are selected for a specific project must be tested to assess their environmental effect in accordance with both the project specifications and environmental conditions and the results should be considered in the selection of chemicals.

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