**Polycyclic aromatic hydrocarbons contaminated soils: bioaugmentation of autochthonous bacteria and toxicological assessment of the bioremediation process by means of *Vicia faba* L.**

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**Abstract**

Two bacterial strains [*Achromobacter* sp. (ACH01) and *Sphingomonas* sp. (SPH01)] were isolated from a heavily PAH contaminated soil (5431.3 ± 102.3 ppm) for their capacity to use a mixture of anthracene, pyrene, phenantrhene, and fluorene as sole carbon sources for growth and for the capacity to produce biosurfactants. The two strains were exploited for bioaugmentation in a biopile pilot plant to increase the bioavailability and the degradation of the residual PAH contamination (99.5 ± 7.1 ppm) reached after nine months of treatment. The DGGE profile of the microbial ecology of the soil during the experimentation showed that the bioaugmentation approach was successful in terms of permanence of the strains in the soil in treatment. The bioaugmentation of the two bacterial isolates positively correlated with the PAH depletion that reached 7.9 ± 2 ppm value. The PAH depletion has been assessed by the loss of the phyto-genotoxicity of soil elutriates on the model plant *Vicia faba* L. that was found to be a good system for monitoring matrix toxicity, offering several valuable endpoints. The middle phases of the bioremediation process resulted to be the most critical, inducing genotoxic effects and selective DNA fragmentation in the root region corresponding to the stem cell niche. This has been interpreted as a stringent mechanism to eliminate the risk for accumulating mutant stem cells that can also compromise their offspring.

## 1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a cluster of several hundred individual organic substances, constituted by carbon and hydrogen atoms, grouped into a configuration of at least two condensed benzene rings. PAHs are released into the environment as a result of incomplete combustion of fossil fuels, organic matter combustions, volcanic eruptions, but with the majority due to anthropogenic emissions such as automobile exhausts, processing production, and accidental spillage of petroleum (CDC 2009; Eisler 1987; Zhang and Tao 2009). Although PAHs can be present in over 100 different assemblage, the US Environmental Protection Agency has listed 16 PAHs as priority pollutants for remediation (USEPA, 1982). In addition PAHs are included in the inventory of the most recalcitrant pollutants to biodegradation and, due to widespread sources, long range transport and persistent characteristics, PAHs are present almost everywhere in sediments, soil, air, aquifers and biota (Byeong-Kyu Lee 2010). Once released into the environment, the persistence of PAHs is mainly related to their high hydrophobicity and low bioavailability, positively correlating with the increase of the number of fused benzene rings in the molecule.

PAHs affects a wide variety of organisms, including terrestrial vegetation, fish and other aquatic life forms, amphibians, birds, and mammals and they exert their peculiar toxicity depending on molecular weight and structure, on their concentration, on the route and the extent of exposure, causing a variety of hazardous effects *in vivo* and *in vitro*, including genotoxicity, developmental toxicity, immune system alterations and carcinogenesis (Guo et al. 2011).

Depending on the chemical structure, PAHs may be subjected to different type of transformations. Actually microbial biodegradation is the principal process affecting the fate of PAHs in the environment (Cerniglia 1993; Kim et al. 2013) and, among the different driven-strategies that can be employed in the abatement of these pollutants, microbial biodegradation has been recognized as an environmentally friendly remediation approach, worthy of serious consideration in the context of the sustainability of the intervention of decontamination of environmental matrices (Mueller et al.

1997). However, the low bioavailability of the PAHs may be the bottleneck in the process of decontamination, resulting in its failure or, at the best, in the persistence of residual levels of contamination, corresponding only to the downgrading of the dangerousness of the treated matrices, in the frame of the protection of the public health. Most of the time an effective decontamination of the environmental matrices is not reached. In this context, it is worth mentioning the difficulties to define the trend of degradation pathways of different classes of pollutants, especially PAHs, given that the intermediates of degradation are often not known or difficult to identify. It is also noteworthy that these intermediates may be even more toxic than the parental pollutants (Riser-Roberts 1998), they can persists at the end of a bioremediation process and eventually they can be produced during the process of biodegradation and partial oxidation of the above mentioned parental pollutants. For these reasons the sole chemical characterization, actually focusing on the principal contaminants, results to be insufficient to foresee or measure the toxicity of the matrices during and at the end of a bioremediation process. Therefore, it is essential to evaluate the effects that a decontamination process and eventually a decontaminated soil can have on biological systems through eco-toxicological tests, which provide important information on the bioavailability and possible toxicity of either residual primary pollutants or their intermediates of degradation.

One of the purpose of this work was the evaluation of the toxicity of the elutriates of an aged and heavily PAH contaminated soil ($5431.3 \pm 102.3$ ppm) at the end of a process of biological treatment in a dynamic biopile pilot plant, treating 7 tons of the contaminated soil. The description of the pilot plant and the related treatment process is out of the scope of the present work, however it must be stated that the decontamination process determined the depletion of PAHs from $5431.3 \pm 102.3$ ppm up to $99.5 \pm 7.1$ ppm. With reference to the Italian legislation, the obtained levels of residual PAH contamination ([PAH] < 100 ppm) are compatible with the re-introduction of the soil in the context of origin, an industrial site, with any further restriction. The toxicity assessment of the elutriates was performed on the model system of *Vicia faba* L. In facts, plants are widely used in toxicological tests (Giorgetti et al. 2011; Ruffini Castiglione et al. 2014; Sadowska et al. 2001) and

they have a particular value when used for the determination of the toxicity of soil elutriates because of the evidence that these latter are the vehicle for plants for coming in contact with any contaminants. The employment of *Vicia faba* allowed us to evaluate different toxicological endpoints such as seed germination and root elongation. In addition, the possible genotoxic effect at the level of the root apical meristems was determined by cytogenetic and molecular cytogenetic analysis by light and fluorescence microscopy.

The other scope of the present experimentation was the enhancement of the bioremediation process to deplete the final residual PAH contamination of the soil ([PAHs] = 99.5 ± 7.1 ppm) to reach values compatible with the re-introduction of the decontaminated soil not only in industrial but also in public sites ([PAH] < 10 ppm). The residual PAH contamination in soils normally corresponds to the not bioavailable fraction, that actually is matter of restriction for the re-introduction of the decontaminated soils in public contexts. Biosurfactant-enhanced bioremediation technology is still at its early phase, however, biosurfactants can effectively increase the solubility of PAHs and their desorption from soil (Hickey et al. 2007), putatively increasing their rate of biodegradation. At the same time, the bioaugmentation of bacteria with metabolic traits of interest, such as the capacity to utilise a fraction of PAHs as sole carbon source, can increase their biodegradation rate. To this scope two bacterial strains (*Achromobacter* and *Sphingomonas* sps.) were isolated from the original contaminated soil (5431.3 ± 102.3 ppm of PAHs) for their capacity to use a mixture of PAHs such as anthracene, pyrene, phenantrhene, and fluorene as sole carbon sources for growth. The two bacterial strains were selected also for their capacity to produce biosurfactants and they were massively bioaugmented in the biopile pilot plant to eventually increase the bioavailability and the biodegradation of the residual level of PAH contamination. The bioaugmentation effects were evaluated either in terms of PAHs depletion and in terms of depletion of the toxicity of the soil elutriates.

## 2. Materials and Methods

### 2.1 Chemicals and Soil

Chemicals used throughout the experiments were of analytical grade and purchased from Sigma-Aldrich (Milan, Italy). Chemical standards of the 16 PAHs including naphthalene (NA), acenaphthylene (ACY), acenaphthene (ACE), fluorene (FL), phenanthrene (PH), anthracene (AN), fluoranthene (FLU), pyrene (PY), benzo[a]anthracene (BaA), chrysene(CH), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), benzo[a]pyrene (BaP), indeno[1,2,3-cd]pyrene (IP), dibenzo[a,h]anthracene (DA), and benzo[g,h,i]perylene (BP), the deuterated PAH internal standard solutions (naphthalene-d8, acenaphthene-d10, phenanthrene-d10, chrysene-d12, and perylene-d12), and surrogate standard solutions (2-fluorobiphenyl and 4-terphenyl-d14) were obtained from AccuStandard Chem. Co. (USA). Internal and surrogate standards were used for sample quantification and process recovery. The PAH contaminated soil was provided by Teseco SpA (Pisa, Italy). The texture of the soil was sandy (35% silt, 50% sand, and 15% clay) with total phosphorous of 1.5% and total nitrogen of 1.3%.

### 2.2 Bacterial enrichment cultures

Enrichment cultures of potential PAH transforming bacteria were set up in the enrichment medium consisting in Minimal basal medium (MBM) [containing (g/L) $Na_2HPO_4$ 2.2 ; $KH_2PO_4$ 0.8 ; $NH_4NO_3$ 3.0 ; EDTA 0.5, $MgSO_4$ $7H_2O$ 3.0, $MnSO_4$ $H_2O$ 0.5, $CaCl_2$ $2H_2O$ 0.1, $ZnSO_4$ $7H2O$ 0.1, $FeSO_4$ $7H_2O$ 0.1, $CuSO_4$ $5H_2O$ 0.01, $NaMoO_4$ $2H_2O$ 0.01, $NaScO_4$ 0.01, $NaWoO_4$ $2H2O$ 0.01, and $NiCl_2$ $6H_2O$ 0.02] amended with a mixture of DMSO dissolved anthracene, pyrene, phenantrhene, and fluorene at the final concentration of $500 \pm 0.1$ mg/L each. A total of 10 g of  the PAH contaminated soil ($5431.3 \pm 102.3$ ppm)  were incubated in 100 ml of the enrichment medium in Erlenmeyer bottles and  incubated at $28 \pm 1$ °C on an orbital shaker (250 rpm) in the dark. After 30 days of incubation, 1 ml of the suspension was incubated in flasks with 100 ml of fresh enrichment

medium, amended with the mixture of DMSO dissolved anthracene, pyrene, phenantrhene, and fluorene, for 30 days in the same conditions. The passage was repeated five times. Afterward, serial dilutions of the culture medium were plated on agarized MBM (Noble agar 1,5% w/vol) with crystals of anthracene, pyrene, phenantrhene, and fluorene on the lid of the plates. The plates were incubated at $28 \pm 1$ °C for 7 days in the dark. Two diverse bacterial phenotypes were collected and clustered in different OTUs by amplified ribosomal DNA restriction (ARDRA) analysis (Weisburg et al. 1991). The primer used were the 27F and 1492R universal primers (Muyzer et al. 1993). The ARDRA was performed digesting the PCR 16S rDNA amplification products with Sau3A, AluI and HaeIII. All the analyses were performed twice for each isolate. Two different OTUs were recovered. For the corresponding isolates, genomic DNA was extracted. The 16S rRNA gene fragment was amplified and sequenced on both strands and aligned to the database sequences using BLASTN (Altschul et al. 1997). Substrate utilization by the bacterial isolates associated to the different OTU was verified on MBM agarized plates with PAHs crystal on the lid. The evaluation of bacterial growth on agarized plates was verified at least twice for each isolate. To investigate the capacity of the isolates to utilise anthracene, pyrene, phenantrhene, and fluorene as a sole carbon sources, flasks containing 100 ml of MBM amended with $100 \pm 0.1$ mg/L of the different hydrocarbons were inoculated with ten colonies collected by MBM agarized plates for each isolate. The flasks were incubated at 24°C and 120 rpm for 2 weeks. The growth of the isolates was estimated by serial dilutions of the bacterial suspension grown performed on LB agarized plates to calculate the colony forming units (CFU). In order to determine the extent of the PAHs biodegradation, triplicate flasks were collected and acidified at pH 2.0. The analysis of the PAHs were performed by extracting the acidified culture three times with an equal amount of ethyl acetate. The ethyl acetate extracts were combined, dried with anhydrous $Na_2SO_4$ and evaporated under gentle nitrogen flow. The residues were dissolved in methanol and analysed by reversed-phase HPLC (Shimadzu LC-2010HT, Kyoto, Japan) equipped with a UV detector and a 250 mm XDB-C18 column (Agilent, Palo Alto, CA, USA). A methanol-water mixture (80/20 v/v) was used

as the mobile phase at a flow rate of 1 ml/min. The UV absorbance spectra were obtained on-line at 254 nm. The biodegradation percentages of PAHs were calculated as the difference in residual PAH concentrations between the inoculated and the not inoculated flasks.

**2.3 Evaluation of the capacity of the bacterial isolates to produce biosurfactants**

The capacity of the bacterial isolates to produce biosurfactants was evaluated by oil-spreading essay (Youssef et al. 2004). The isolates were cultivated for 6 days at 28 °C and 250 rpm agitation in 125 mL Erlenmeyer bottles containing 50 mL of MBM amended with glucose (20.0 g/L). After incubation, bacterial cells were precipitated by centrifugation and the supernatant was analysed by the oil-spreading test (Morikawa et al. 2000). Briefly, a thin layer of crude oil was added to a petri dish containing distilled water. Then, supernatant was gently placed on the center of the oil layer. When biosurfactant is present, the oil is displaced forming a clearing zone. The adopted criterion for the selection of good biosurfactant producers was the formation of a halo larger than 2.5 cm in diameter, which corresponds to a surfactin concentration of 44.4 mg/L, adopted as the standard for comparison.

The capacity of the bacterial isolates to produce biosurfactants was confirmed by the emulsification assay (Cooper and Goldenberg 1987). The supernatants were used to determine the emulsification of kerosene and hexadecane. In 2-ml tubes the supernatants were mixed with 500 mL of distilled water, 1 ml of kerosene or hexadecane in triplicate. The mixtures were vigorously vortexed for 2 min. The resulting emulsion was allowed to stand for 24 h. A negative control was performed in absence of supernatant amendment. After the period of 24 h, the height of the emulsion layer was measured. The emulsion index was calculated based on the ratio of the height of emulsion layer and the total height of the liquid [EI % = (emulsion/ total h) x 100]. The stability of the emulsification ability of the biosurfactant was confirmed after 15 days.

**2.4 Preparation of microcosms**

A total of 9 experimental replicates (glass pots), each containing 100g of soil were prepared and maintained in a temperature-controlled (24 ± 1°C) dark chamber at the 60% of the soil maximum water holding capacity. Three microcosm out of nine were inoculated with both the isolated OTUs at a density of $10^3$ CFU g/soil each, three microcosms were inoculated with the same bacterial inoculum but after the bacterial inoculum sterilisation at 121°C and 1 atm for 20 minutes. Three microcosms were not inoculated with bacteria. Inocula for bioaugmentation were prepared by massive cultivations of the two bacterial strains in LB. In order to reach the expected bacterial inoculum ($10^3$ CFU/g soil each OTU), appropriate volumes (ml) of the massive OTU's growing cultures have been collected and gently centrifuged. The bacterial pellets have been washed twice with a saline solution (NaCl 0.9 % wt/vol) and inoculated in the pots by manually homogenising the soil. All pots were routinely manually mixed every three days of incubation and checked for water content that was maintained at the 60% of the maximum water holding capacity. At the end of two months of incubation the pots were sacrificed and analysed for the PAH content.

**2.5 Preparation and inoculation of the bacterial consortium**

The two selected bacterial strains were grown in a bench-top bioreactor (Labfor 5, Infors HT) in LB medium up to the microbial density necessary to inoculate the biopile pilot plants (7 tons of soil) with $10^3$ CFU/gr of soil. The bacterial inoculum was centrifuged and pelletted, resuspended in 10 L of saline solution (NaCl 9% w/vol in water) and homogenously disposed on the top of the biopile that was mechanically mixed after the bacterial inoculum. The biopile pilot plant was monitored and maintained by the industrial partner TesecoSpA by controlling the temperature of the pile that was maintained around the 24 ± 2°C and the water content that was maintained at the 60% of the soil maximum water holding capacity. The biopile was mechanically mixed every week.

**2.6 PAH Extraction and Analytical Procedures**

Soil samples collected and analyzed for PAH content, derived from: a) the independently destructive dissection of 3 pots per time-point of analysis; the soil in each pot were roughly mixed and a total of 10 different samples of 0.5 g of sediments were randomly collected. The different 10 soil samples per pot were mixed together and the resulting 5 g of soil were divided into two technical replicates and separately extracted and analyzed; b) the collection of columns of soil from the biopile pilot plant every one square meter grid, operated with a stainless steel probe. The collected columns of soil were roughly mixed and divided in technical replicates and separately extracted and analyzed. Soil samples from biopile was collected initially after 5 weeks (5W), successively after three weeks for 5 times (8W, 11W, 14W, 17W, 20W).

Soil samples, that were collected from the biopile, were dried in a vented oven at 25°C for 36 hrs. All samples were extracted using a soxhlet apparatus with 1 : 1 (v/v) acetone/n-hexane. A total of 10 µL of surrogate standard mixture (2-fluorobiphenyl and 4-terphenyl-d14) solutions was added. The concentration of the surrogates was 250 ng/mL each. Method blanks were prepared following the same procedure without adding sediment sample. The verification of the calibration for quality control was prepared by adding the standard solution to 1:1 (v/v) acetone/*n*-hexane at a concentration equal to the 75% of the last calibration point. After subsequent drying over anhydrous sodium sulphate of the organic fraction and concentration to 1.0mL using a gentle stream of nitrogen, an internal standard mixture (naphthalene-d8, acenaphthened 10, phenanthrene-d10, chrysene-d12, and perylene-d12) solution at a final concentration of 50 ng/mL each was added to the extract to be analyzed using gas chromatography with mass selective detection (GC-MS). The internal standard mixture has been selected to cover all the fragmentation range of the 16 analyzed PAHs. The quantitative analysis of PAH was performed with an Agilent 6890 GC-5975B Series MS system in the selective ion monitoring mode (SIM). Injection of 1 µL of samples was conducted in the splitless mode with a sampling time of 1.0 min. Separation of PAH congeners was carried out with a 30m (long) × (0.25)mm I.D. (inner diameter) HP-5MS capillary column (Hewlett-Packard,

Palo Alto, CA, USA) coated with 5% phenyl-methylsiloxane (film thickness 0.25 µm). The injection temperature was 300°C. The transfer line and ion source temperatures were 280°C and 200°C, respectively. The column temperature was initially held at 40°C for 1min and raised to 120°C at the rate of 25°C/min, then to 160°C at the rate of 10°C/min, and finally to 300°C at the rate of 5°C/min, held at final temperature for 15min. Detector temperature was kept at 280°C. Helium was used as a carrier gas at a constant flow rate of 1 mL/min. Identity of the PAHs in the samples was confirmed by retention time and the relative abundance of selected monitoring ions of the standard PAHs. The 16 priority PAHs were quantified using the response factors related to the respective internal standards based on five-point calibration curve for each individual compound ranging from 10 ng/mL to 1000 ng/mL. Each PAH concentration was corrected using the recovery rate of the surrogate standard and expressed on a dry-weight basis.

## 2.7 Molecular techniques

Standard procedures were used for nucleic acid manipulation and agarose gel electrophoresis. The DNA from soil was extracted by using the UltraClean™ Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA). DNA was manipulated using enzymes purchased from Sigma-Aldrich (Milan, Italy) and sequenced using a PRISM Ready Reaction DNA terminator cycle sequencing Kit (Perkin–Elmer, Milan, Italy) running on an ABI 377 instrument. Nucleotide sequence data were assembled using the ABI Fractura and Assembler computer packages and analyzed using ClustalW and Omega (version 1.1) (Oxford Molecular Group, UK). The V-3 region (position 341–534, *E. coli* numbering) of bacterial 16S rcDNA was amplified by PCR using the primers p3/p2 (Muyzer et al. 1993). The PCR products were separated on polyacrylamide gels [8 % (wt/vol), 37.5:1 acrylamide–bisacrylamide] with a 30–60 % linear gradient of urea. Denaturing gels were run using the Dcode Universal Mutation Detection System (Bio-Rad, USA). The gel images were acquired using the ChemDoc (Bio-Rad) gel documentation system. The denaturing gel gradient electrophoresis (DGGE) profiles, concerning the presence and intensity of the bands, were analyzed

using Quantity One (Bio-Rad) to calculate the Shannon's diversity (Shannon and Weaver, 1963) and the Eveness indexes (Pielou 1975).

## 2.8 Phytotoxicity, Cytotoxicity and Genotoxicity tests

Seeds of *V. faba* L., following the procedure previously described in Giorgetti et al. (2011), were germinated at 24 ± 1 C° for 72 h in soil elutriates. Soil elutriates were prepared as described in Plumb (1981) starting from soil samples collected from the biopile as previously described. In each experiment, the negative control was achieved using distilled water. After 72 h of germination in the different matrices (control = water; T0 = elutriate from the initial contaminated soil; 5W-20W = elutriates from soils treated in biopile for 20 weeks) different endpoints have been evaluated.

Phytotoxicity test was carried out scoring both root length (cm) and seed germination rate (% germinated seeds) in four replicates of ten seeds for each sample. The germination index percentage (GI%) was determined according to the equation:

GI% = (GsLs)/(GcLc) x 100

where Gs and Ls are the seed germination and root elongation (mm) for the sample; Gc and Lc the corresponding values for the control.

Ten roots for each treatment were fixed in ethanol : glacial acetic acid (3:1 v/v). Root tips were squashes and stained following Feulgen technique as described in Venora et al. 2002. At least 1000 nuclei, randomly selected for each slide, were analyzed by means of light microscope for the estimation of the mitotic activity, mitotic aberrations and the micronuclei frequency.

Mitotic activity, expressed as mitotic index MI (number of mitosis per 100 nuclei), indicated the levels of cytotoxicity of the matrices.

Micronucleus frequency assay (MNC test, number of micronuclei per 1000 nuclei), and mitotic aberrations (aberration index AI = number of aberrations per 100 nuclei) were determined for the evaluation of the genotoxicity of the matrices.

The categories of the scored aberrations included chromosomal bridges and fragments, lagging chromosomes, aberrant metaphases and disturbed anaphases in dividing cells, micronuclei in interphase cells (Ruffini Castiglione et al. 2011).

As additional endpoint of genotoxicity at cell and tissue level (genotoxicity *in situ*), DNA fragmentation has been detected by the TUNEL assay, which foresee the incorporation of fluorescein-labelled dUMP at 3'-hydroxyl termini (fragmentation sites) using terminal deoxynucleotidyl transferase. FAA fixed roots were paraffin embedded, sectioned (10 μm) with a microtome and mounted on poly-L-lysine coated slides for TUNEL assay (Ruffini Castiglione et al. 2014). Briefly, after incubation in proteinase K (Sigma-Aldrich, USA) for 15 min and two washes in 1× PBS, we followed the manufacturer's instructions of the Apoptosis Detection System Kit (Promega, WI). Co-staining with DAPI (Sigma-Aldrich, USA) was used to visualise all nuclei. Slides were examined with Axio Observer.Z1, Zeiss Microscopy, Jena, (Germany) equipped with Axiocam MRc5 (Zeiss MicroImaging, Göttingen, Germany). The experiment was conducted twice, including the positive control (treated with 2 μg/ml DNase I) and the negative control (processed without terminal deoxynucleotidyl transferase).

**2.9 Statistical analysis**

Data were elaborated with the aid of the ANOVA, and means were separated by the Bonferroni multiple-comparison test using the specific software Statgraphics 5.1 (Statistical Graphics Corp., USA).

## 3. Results

### 3.1 Isolation and characterisation of bacterial strains for bioaugmentation

A total of two isolates capable of growing in the presence of anthracene, pyrene, phenantrhene, and fluorene as a sole carbon sources were recovered and analyzed by ARDRA. They showed two different profile and they were grouped in two OTUs. The sequencing of the corresponding 16S rRNA gene indicated that the isolates belonged to the *Achromobacter* sp. (ACH01) [98 % homology to *Achromobacter* sp. 15DKVB, accession number HQ448950] and to *Sphingomonas* sp. (SPH01) [98 % homology to *Sphingomonas* sp. IMER-A1-28, accession number FJ434127.2 ]. Both ACH01 and SPH01 were capable to deplete anthracene, pyrene, phenantrhene and fluorene amended as single carbon sources in mineral medium. Moreover the two bacterial strains resulted to be capable to produce surfactants by the oil-spreading and emulsification assays. The metabolic capacities of interest of the two bacterial isolates are reported in Table 1.

### 3.2 Depletion of the PAHs after bacterial bioaugmentation in the microcosms and the pilot plant

The depletion of PAHs in the microcosm test, after two months of incubation, has been observed only in the pots inoculated with $10^3$ CFU/g of soil. Results are shown in Table 2, column B. The 10.8-17.8% of depletion of the different PAHs has been interpreted as an indication of the PAH depletion, that positively correlated with the bioaugmentation of the two strains. The depletion of PAHs in the pilot plant is also shown in Table 2, columns C-H. A decrease from the initial total PAH concentration of 99.2 ± 13.8 ppm value to 7.9 ± 2 ppm has been observed.

### 3.3 Molecular analysis

The 16S rDNA DGGE profiles of the bacterial populations characterising the metagenome of the soil during the pilot plant experimentation is shown in Figure 1. The PCR amplified V3 regions of

the 16S rDNA of ACH01 and SPH01 were exploited as molecular markers to monitor the presence of bands corresponding to the microbial inocula in the different profiles. After 5 weeks of incubation, the amplification products of interest were above the detection limits of the DGGE analysis. The putative bands indicating the persistence of ACH01 and SPH01 as strains present in the soil at the different time of analysis were gel excised and sequenced in order to verify their identity, resulting to match with ACH01 and SPH01. In relation to the microbial ecology of the mesocosms during the experimentation, the increase of the Evenness index from 0.67 up to  0.79 and the increase of the Shannon's diversity index from 1.8 up to 2.2 has been observed.


### 3.4 Plant bioassays

The results of *V. faba* germination and root elongation test, combined in the germination index percentage (GI%) were reported in Figure 2. The recorded values of the GI%  indicated significant changes and unsteadiness in the composition of the matrices with an ability to induce basically two type of response: a) hormetic effects, indication of weak phytoxicity, as in the case of  the soil at the beginning of the experimentation (T0)  and of soils treated in biopile for  8, 14 and 17 weeks (8W, 14W, 17W); b) a strong disturbance  of the germination process after 5 and 11 weeks (5W, 11W). Only the matrix treated for 20 weeks (20W) within the biopile resulted no more phytotoxic, showing values of the GI% in a range comparable to the control.

The mitotic index can be exploited as a clue of the cytotoxicity of the soil elutriate during the bioremediation process: a decrease in the mitotic index value is an indication of the increased toxicity of the matrix in treatment. As shown in Figure 3,  the soil at T0 was toxic with reference to the control. After bioaugmentation of ACH01and SPH01, more precisely after 5 weeks (5W) of treatment in the biopile, the cytotoxicity of the soil strongly increased. An increased cytotoxicity of the matrix with reference to the control has been observed up to 17 weeks of treatment (17W). Successively after 20 weeks of treatment (20W), the toxicity of the matrix decreased up to values statistically not different to the control one.

The genotoxicity of the matrices along the bioremediation process were estimated by analysing the frequency of anomalies and/or aberrations in dividing cells and the frequency of micronuclei (MCN) in interphase of the root meristematic cell population. Some representative examples are reported in Figure 4. The two histograms which describe the trend of the genotoxicity (Figure 5, panel a and b) are basically super-imposable, firstly demonstrating that T0 exerted genotoxic effects on *V. faba*, detectable as clastogenic and aneugenic damages. In addition the values of the aberration index and of the micronucleus test sharply increased following the bioaugmentation, showing a maximum peak of genotoxicity after 5 weeks (5W) of treatment. Successively the genotoxicity decreased with a not linear trend and it was no more detectable after 20 weeks of treatment.

DNA damage induced by the matrices along the bioremediation process has been also evaluated by the TUNEL technique that allows to detect *in situ* single and double DNA strand breaks as fluorescent green signal. TUNEL-positive signals were observed, including in the control, in some xylem vessels and in root cap cells in all the analysed samples belonging to each treatment, with an increase of the signal intensity along the bioremediation process, mainly in cortical cells and at meristem level (Figure 6). In addition the matrix after 8 weeks (8W) of treatment induced a selective DNA fragmentation in a specific region of the root apical meristem corresponding to the niche of root apical stem cells. Positive controls treated with DNAse I showed a general fluorescence involving all the nuclei, while in negative controls, the omission of terminal deoxynucleotidyl transferase led to a lack of signal (data not shown).

## 4. Discussion

Strong adsorption of PAHs to soil organic matter, up to the partitioning of the latter in the hydrophobic portion of the soil organic substance (Cui et al. 2011), is the bottleneck to the processes of biodegradation of PAHs and the reason of their residual permanence in environmental matrices. In fact, it is reasonable to assume that, the residual PAH contamination in the soil used in the present experimentation is associated to its low bioavailability, more than to a putative paucity of microorganisms that have access to it. The presence of bacteria producing bio-surfactants in addition to their capacity to degrade hydrophobic contaminants could play a key role in the complete depletion of such residual contamination. Results obtained confirm the hypothesis, showing that the bioaugmentation of the isolated bacterial specimen ACH01 and SPH01, belonging respectively to *Achromobacter* and *Sphingomonas* sps., is positively correlated with the abatement of the residual PAH contamination in the soil of interest. More precisely, the enrichment of autochthonous bacterial specimen capable to utilise PAHs as sole carbon source and concomitantly to produce biosurfactants, determined the depletion of the residual PAH contamination, from levels corresponding to the possibility to reallocate the soil in industrial areas, to level corresponding to the possibility to reallocate the soil in public areas. The DGGE profile of the microbial ecology during the process showed that the bioaugmentation approach was successful in terms of permanence of the bioaugmented strains during the experimental period of treatment. At all the time of analysis the amplification products of interest were above the detection limits of the DGGE analysis, indicating the persistence of the bioaugmented ACH01 and SPH01 in the system, also when the nearly complete depletion of the PAHs was observed. Moreover, the increase in ecological indexes of biodiversity in the soil in treatment, as the Shannon and eveness indexes, indicated a change in soil properties over time. In fact the depletion of PAHs positively correlated with the improvement in the biodiversity of the bacterial community of the soil. This assumption is based on the increase in the Shannon diversity index that quantifies the abundance in terms of taxonomic units in a bacterial community. Moreover, as indicated by the increase im the eveness

index, the increment in the abundance of taxonomic units during the decontamination process co-occurred with the redistribution of bacterial specimen in the soil among them. This result point out the disappearance of dominant bacterial populations during the process of decontamination, eventually specialised to cope with a specific stress factor. In fact, the depletion of the contamination can be reasonably assumed as the disappearance of a stress factor, and the improvement of the biodiversity of the bacterial community of the soil can be reasonably interpreted as the recovery of the resilience of the matrix that, at the end of the process of decontamination, results to be characterized by the equal distribution of the microbial specimen among a significant number of different bacterial genera. *Achromobacter* and *Sphingommonas* sps were already described as capable to produce biosurfactants (Manickam et al. 2012; Wongwongsee et al. 2013) and involved in PAH degradation (Dave et al. 2015; Farjadfard et al. 2012; Janbandhu and Fulekar 2011, Xu et al. 2015). Both species have been exploited for the bioaugmentation approach in relation to the enhancement of PAH depletion in contaminated soils with encouraging results (Colla et al. 2014). However, as far as we know, both metabolic traits, the capacity to produce biosurfactants and to use PAHs as a sole carbon source, combined with the capacity to persist in the inoculated matrices even in absence of the primary pollutants, were not previously described. In spite of the success obtained in the depletion of the residual portion of PAHs that positively correlated with the bioaugmentation of ACH01 and SPH01, the bio-based process resulted to be associated to the increase of the toxicity of the elutriates deriving from the matrix in treatment. In fact, results here obtained showed the necessity to adopt a toxicological assessment of the process of bioremediation of soils contaminated with toxic chemicals in order to safely decide to reallocate the matrices, in accordance to the legislation. In fact toxic contaminants can be transformed in intermediates potentially even more toxic of the parental chemical structures for the biota. The here observed decontamination of the PAHs in the biopile pilot plants, leading to the depletion of PAHs up to values compatible with a reallocation of the soil in industrial sites, ends up with a high toxicity of the matrix. In this case, the chemical assessment of the depletion of the

PAHs, results to be insufficient in assessing the process of detoxification of the soil. On the other hand, bioassays provide important information for the estimation of pollutant-effects of chemicals on environmental matrices and, actually, complement chemical analyses. In contrast to chemical analyses, bioassays provide direct measurements of impacts of pollutants on the environment and on living organisms (Eisentraeger et al. 2005).

Over the last decades ecotoxicological tests have been used as supplementary tools to monitor bioremediation of hydrocarbons, both in laboratory and field studies (Płaza et al., 2005). In light of this, *Vicia faba* was chosen as biological system providing a large array of endpoints such as phytotoxicity, cytotoxicity and genotoxicity. Recently the micronucleous assay with *V. faba* was standardized and inserted within guidelines for evaluation of mutagens in freshwaters and sediments from the Italian "Istituto Superiore di Sanità" (Gustavino et al. 2013) and the international protocol ISO 29200 (Cotelle et al., 2014).

In our experimentation all the examined elutriates resulted to be toxic. A decrease in toxicity to values similar to the one of the control was detected only after 20 weeks of treatment. Phytotoxicity monitored along the bioremediation process showed a heterogeneous trend of the results, ranging from the induction of biostimulating to inhibitory effects. This is not surprising considering that, during the soil remediation, significant changes in the composition of the matrices can occur, leading to the formation of both strong phytotoxic compounds, with inhibitory effects, and weak phytotoxic compounds that can induce hormetic effects. It is in fact known as the weak activity of a toxicant may lead to an increase in metabolic level and biomass of living organisms (Calabrese and Baldwin 2002). The recorded increase in GI% in some steps of the bioremediation process can be considered a sort of defense mechanism for the plant organism that dilute in a greater biomass the weak toxicant to better cope with it. These considerations are in line with the conclusion of Migliore et al. (2010) concerning the hormetic effects recorded in *Lythrum salicaria* following treatments with antibiotic Sulfadimethoxine. Moreover toxic effects associated to the ongoing of the biodegradation process were evident on further endpoints, especially during the intermediated

phases of the soil treatments. In fact, after 5 weeks of treatment, cytotoxicity and genotoxicity of the elutriates were evident, revealing a toxicity comparable to or even higher than the one deriving from the polluted soil at the beginning of the experimentation. It is noteworthy that the elutriates produced after 5 weeks of treatment were particularly genotoxic, exerting clastogenic and aneugenic effects to a greater extent than all the other tested samples.

The TUNEL analysis, detecting cytohistochemically single and double DNA strand breaks in root apices, showed TUNEL-positive nuclei in the tissues and structures of the root that, as a rule, undergo developmental PCD processes, namely differentiating tracheary elements and root cap cells (Kuriyama and Fukuda 2002). In addition, we have observed in the same root tissues a general intensification of the TUNEL signal during the intermediate phases of bioremediation process. DNA fragmentation was detectable in meristem cells, confirming our cytological evidences on aneugenic and clastogenic effect of the toxic compound present/originated along the bioremediation process. After 8 weeks of treatment the bio-based process of decontamination became selectively hazardous for a small area of cell population corresponding to the site of root apical stem cells. This selective DNA fragmentation can be related to a selective induction of cell death. The process can be reasonably interpreted as a way to eliminate damaged stem cells. In fact it could represent a stringent mechanism to get rid of the risk of accumulating dangerous DNA mutations that can compromise the stem cell niche and its offspring. Similar results have been shown in root and shoot of *Arabidopsis thaliana* in which stem cells and their early descendants were selectively killed by non apoptotic programmed cell death following treatment with radiomimetic drugs, X-rays, that disrupt DNA repair by nonhomologous end-joining (Fulcher and Sablowski 2009).

## 5. Conclusions

The isolation, enrichment and massive re-inoculation of selected autochthonous microbial strains might request extra-resources if applied on a real scale; however, they might also determine a step forward in the designing of sustainable biotechnological approaches to accelerate the process of contaminant depletion in soils and sediments. Results here obtained showed a positive correlation of the bioaugmentation of two autochthonous microbial strains, *Achromobacter* sp. ACH01 and *Sphingomonas* sp. SPH01, capable to use anthracene, pyrene, phenathrene and fluorene as carbon source and to produce biosurfactants, with the depletion of the residual PAH contamination in an aged contaminated soil that impeding its reallocation in public area. The two strains' massive inoculation in the soil constituted the sole condition associated to the depletion of the residual portion of PAH contamination. This evidence suggests that the two strains are good candidates for bioaugmentation approaches and their exploitation as allochthonous *inoculum* in soils contaminated by PAHs will be verified in the near future. A further result here obtained is the evidence of the toxicity of a bio-based processes of transformation of PAHs. The identification of putative mutagens eventually deriving from the biological transformation of the primary toxic contaminants or even the monitoring of their increased bioavailability is not sustainable in terms of costs on a real and laboratory scale. Toxicological assessment must co-occur with the chemical analysis and *V. faba* toxicity assay represents an excellent approach for monitoring the biodegradation processes in relation to the phytotoxicity and the genotoxicity of elutriates of the environmental matrices.

Moreover, toxicological assessments of the process of decontamination can be adopted to determine the length of the decontamination process itself because of the capability to assess both the depletion of the contamination and the detoxification of the matrices. In the case in study, the process of decontamination was performed for 20 weeks, despite the chemical analysis indicated an almost total depletion of PAHs already after the first weeks of treatment. The intermediate phases of the bioremediation process became the most critical in terms of toxicity, inducing genotoxic effects

and selective DNA fragmentation in the root region corresponding to the stem cell niche, interpreted as a plant stringent mechanism to eliminate the risk for accumulating mutant stem cells and compromising their offspring.

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**Figure 1.** PCR-amplified V3 regions of: First lane: the ACH01 and SPH01 in axenic culture; T0, soil at the beginning of the experimentation; 5W-20W, soil after 5 weeks up to 20 weeks of treatment in the pilot plant.

**Figure 2.** Variation in *V. faba* germination index percentage (GI%) during bioremediation process in the biopile pilot plant. Values over 100% (light grey area, hormetic effect) and under 50% (grey, inhibition effect) indicate phytotoxicity of the matrices. (Control = water;  T0 = initial contaminated matrix; 5W-20W = matrices from bioremediation process treated in biopile for 5 - 20 weeks).

 **Figure 3.** Mitotic index variation in *V. faba* root meristems during bioremediation process in the biopile pilot plant. (Control = water;  T0 = initial contaminated matrix; 5W-20W = matrices from bioremediation process treated in biopile for 5 - 20 weeks). Values marked with ∗ indicate statistical significance in respect to the control (P< 0.05).

**Figure 4.** Genotoxicity tests in *V. faba*: a) aberration index; b) micronucleous test, monitored during the bioremediation process in the biopile pilot plant. . (Control = water;  T0 = initial contaminated matrix; 5W-20W = matrices from bioremediation process treated in biopile for 5 - 20 weeks). Values marked with ∗ indicate statistical significance in respect to the control (P< 0.05).

**Figure 5.** Representative anomalies observed during cytological analysis of the root meristems injured by genotoxic effects of the different matrices during bioremediation process. a) chromosome bridges in anaphases; b) chromosome bridge in anaphases with a laggard chromosome; c) laggard chromosomes in anaphase; d) and h) micronuclei; e) disturbed anaphase with a laggard chromosome; f) C metaphase; g) reduction grouping.

**Figure 6.** TUNEL assay on longitudinal sections of *V. faba* root tips from seedlings grown on contaminated matrices during some steps of bioremediation process. a–a'; d-d') DNA fragmentation related to physiological programmed cell death in root cap cells (a') and in tracheary elements differentiation (d') in control samples and at the end of the bioremediation process respectively.

b-b';c-c') representative images  showing the  increase in DNA fragmentation during the intermediate phases of bioremediation process at 8 weeks (8W) and 14 weeks (14W). It is worth noting in b) (white circle) and in (b') the selective DNA fragmentation in the region corresponding to the niche of root apical stem cells.
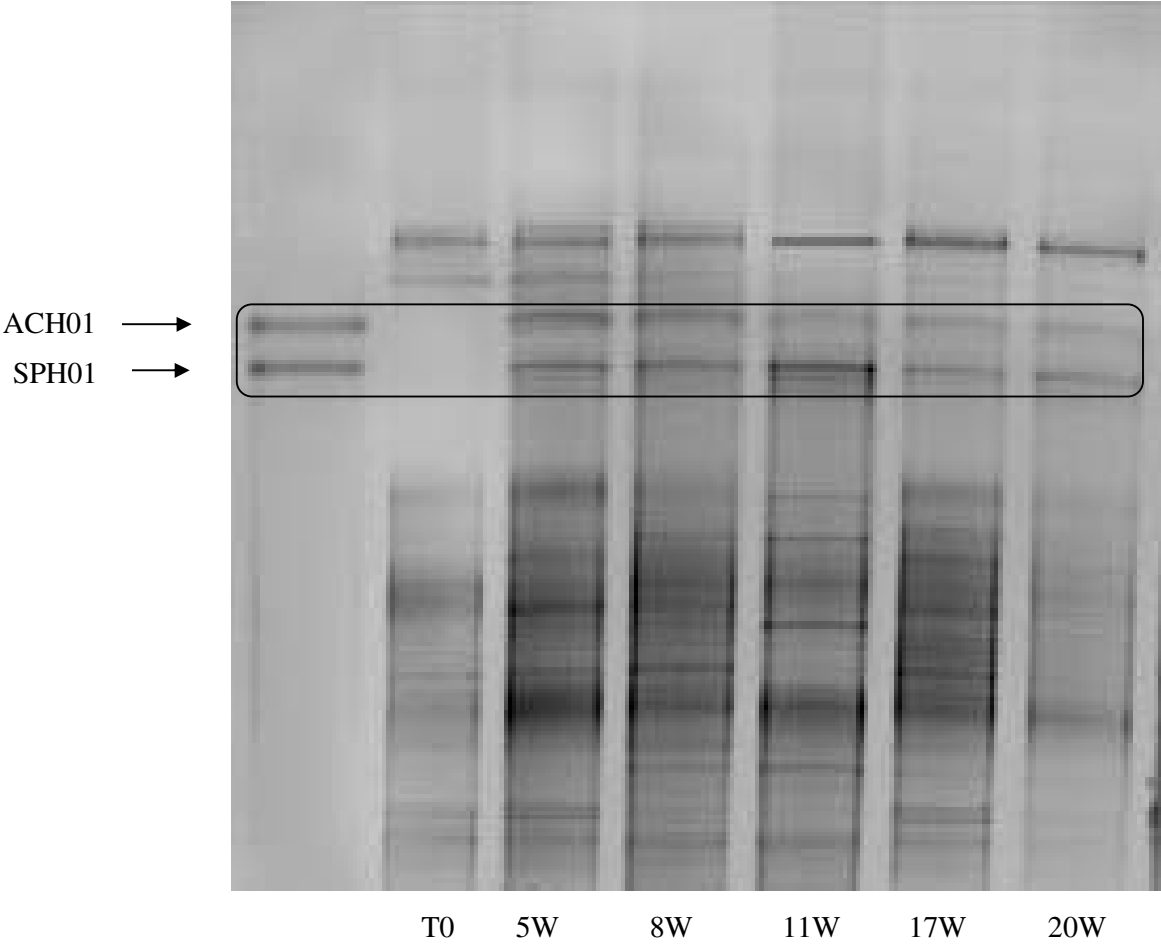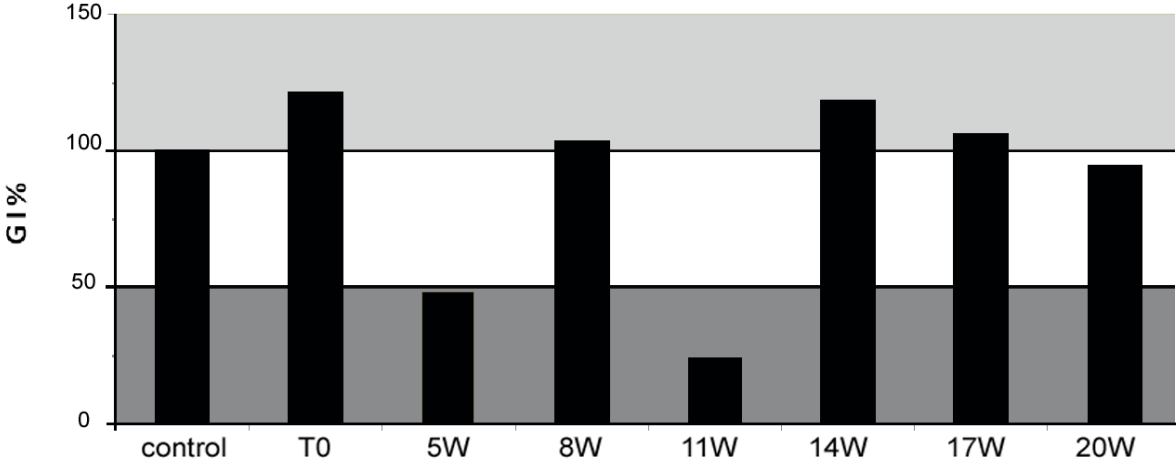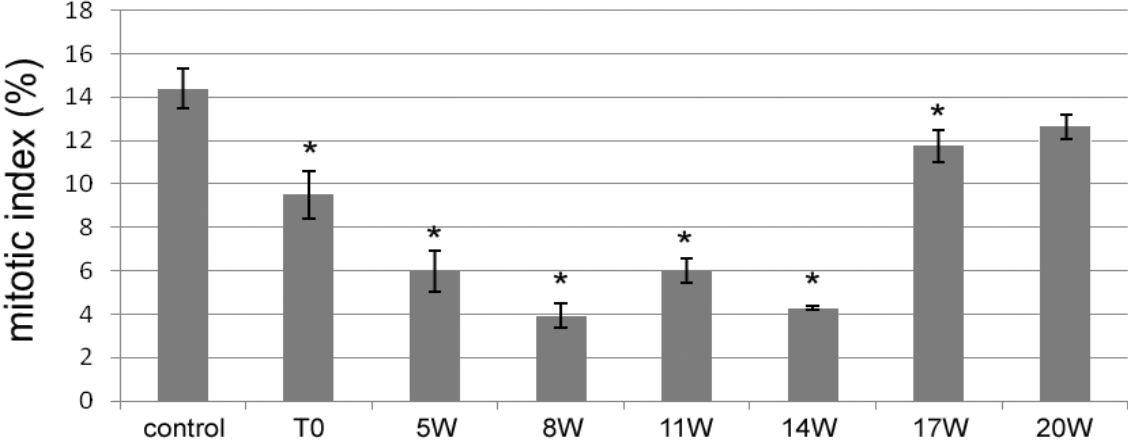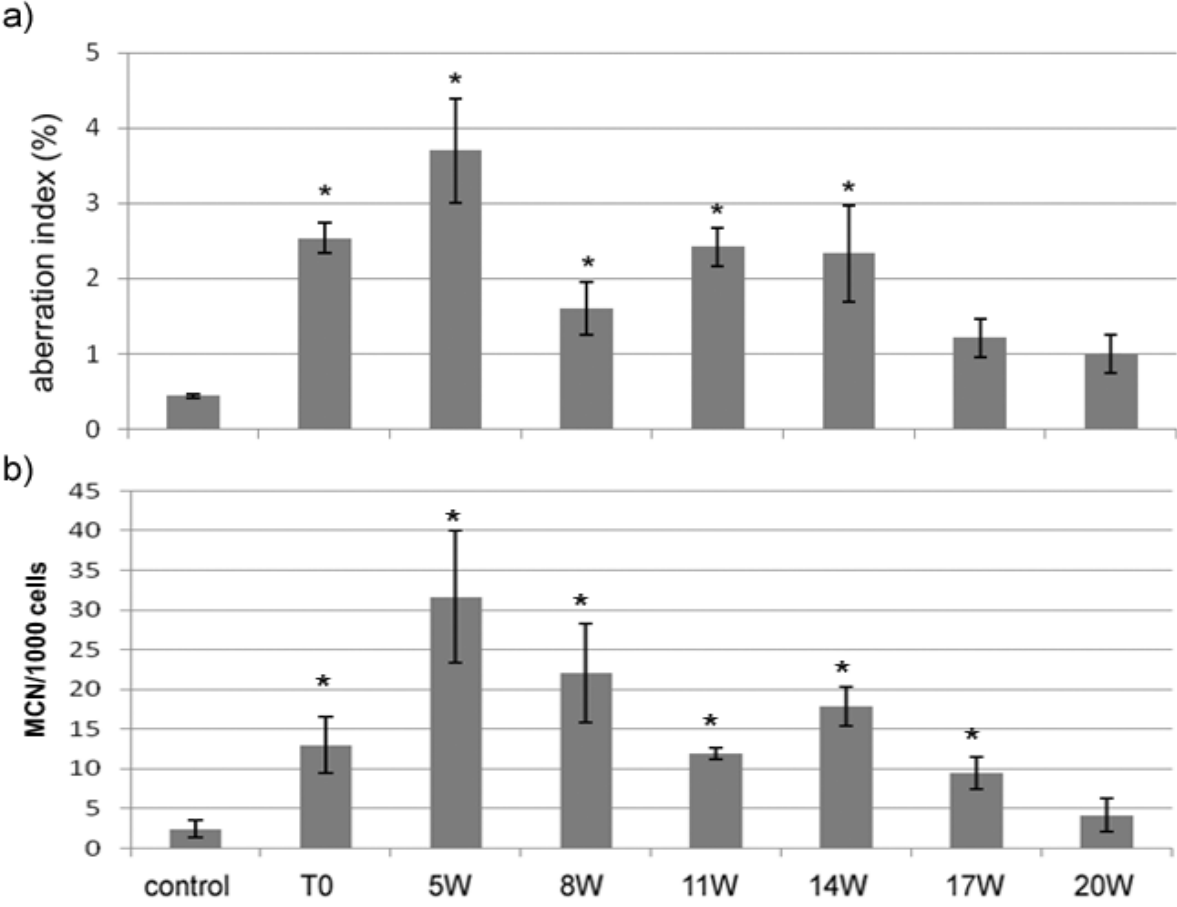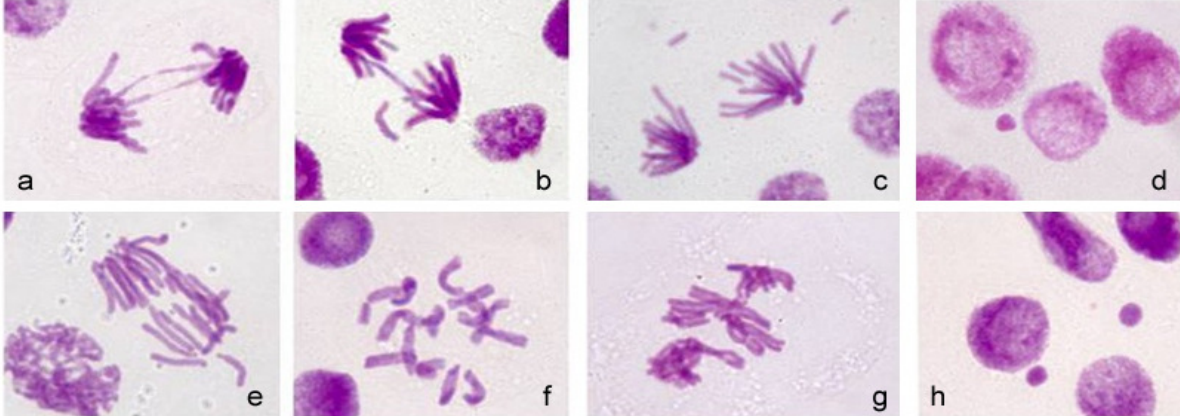
Figure 1

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