Evaluation of soil toxicity using different biotests on *Pisum sativum*: A case study.

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Abstract

In this study we used *Pisum sativum* as model plant, to perform a battery of plant biotests, based on the analysis of biological endpoints, ranging from the macroscopical to the microscopical level, in order to evaluate the toxicity of soils sampled from three different polluted areas (two industrial and one exposed to heavy vehicular traffic). In addition to the conventional germination tests and early root growth analyses, the mitotic index and the percentages of mitotic phases and of aberrations in the root apices were calculated. Moreover, DNA loss and damage were evaluated by flow cytometry and COMET assay, respectively. Root samples from polluted soils showed lower mitotic indices and a higher mitotic aberration percentage and DNA loss in comparison to the controls. Data obtained by COMET tests highlighted the soil genotoxicity, especially in the two industrial areas. All together, our results showed that the three studied sites were characterized by different levels of toxicity: in particular, one of the two industrial sites was the most harmful.

Keywords: *Environmental monitoring*, *genotoxicity*, *mitotic index*, *COMET assay*, Pisum sativum, *soil toxicity*.

Introduction

Soil risk assessment is assuming an increasing importance, in parallel with new technologies for the possible remediation of polluted areas (Ashraf et al. 2014). Chemical analyses are the most typical approach for this purpose, however, the chemical-analytical determinations of the total pollutant content of the soil are not sufficient for the detection of all the potential risks. In addition, though chemical analyses provide extremely punctual information in space and time, they are generally expensive and the content of pollutants in the soil does not always correlates with the toxic effect on living organisms, which are the main target of these studies. In fact, a strong toxicity does not necessarily correspond to a high concentration of pollutants, but depends on the bioavailable fraction (that can interact with living organisms) that is influenced by the physical characteristics and the geochemistry of the substrate (pH, organic matter and clay content, cation exchange capability, electrical conductivity, etc.) (Lasat 2000; Kim et al. 2015). In addition, the interactions among different pollutants can cause extremely diversified effects on living organisms, ranging from attenuation to addition and even synergism, even at low individual pollutant concentrations (Kabata-Pendias 2011; Chibuike & Obiora 2014).

All these limitations have been by-passed by utilizing biotests, based on the use of living organisms (both animals and plants), and their physiological parameters, for the assessment of ecological risk assessment (Dagnino et al. 2008). Biotests are cost-effective and allow the evaluation of short- and long-term soil toxicity (Chatterji 2011).

Plants are sessile organisms, therefore they can be efficient indicators of the presence of stress factors in their habitat. Biotests consist on the analysis of a number of physiological aspects, such as: seed germination and root elongation (Adam & Duncan 2002; Wierzbicka 2015) and cell division (Fiskesjö 1997a; Kwon et al. 2016; Scialabba et al. 2016). Moreover, the genotoxicity COMET test, originally set-up for human cells (Singh et al. 1988), is now widely applied also to plant cells (Ventura et al. 2013; Santos et al. 2015).

This paper is focused on a multi-aspect evaluation of soil toxicity in an area characterized by the presence of different industries combined with many high-traffic roads, located in NW Italy (Fraschetta, Alessandria) using plant biotests employing *Pisum sativum*, that is phylogenetically close to *Vicia faba*, whose use in biotests is well documented (Sang & Li 2004; Lin et al. 2008). *P. sativum*, proposed for chromosome aberration assays (Grant & Owens 2001), has been previously reported for its sensitivity to pollutants (Fusconi et al. 2006; Hattab et al. 2009; Ronchini et al. 2015).

Materials and methods

Soil sampling and processing

Analyses were performed on soils from four sites within the "Fraschetta" area. The choice was based on the prediction of a physical model able to describe pollutant dispersion according to the climatic conditions and the prevalent wind directions (Trivero et al, 2012). The four sites were identified with A, B, C and D (Figure 1) in common agreement among all the participants to the L.I.N.F.A. project: Life Intervention in the Fraschetta Area (http://ec.europa.eu/environment/life/project/Projects/index.cfm?fuseaction=home.showFile&rep=file&fil=LIFE04_ENV_IT_000442_LAYMAN.pdf), whose aim was to evaluate negative effects deriving from the deposition of atmospheric pollutants in soils at a heavily industrialized area (Dagnino et al. 2008). The two soils named A (8.64214°E, 44.8691°N) and B (8.76080°E, 44.89504°N) were collected in sites subjected to the emissions of two factories producing chlorinated and fluorinated reagents and rubber components, respectively, at about 2 km from the sources. The soil identified with D (8.73081°E, 44.82510°N) was collected from a roadside location, at about 50 m from a national straight road constantly exposed to heavy vehicular traffic. Finally, a control soil, named C (8.76397°E, 44.83231°N), was collected in a site not subjected to any specific source of contamination (within 2 Km from the national road and at least 4 km from industrial sites), based on the prevalent wind directions (Trivero et al, 2012).

According to the new Italian soil classification map, the soil in this area is Based Chromic, Haplic, Gleyic, Skeletic e Calcic Skeletic, Luvisol; Haplic Luvisol (Dystric); Eutric Vertic, Dystric, Gleyic, Stagnic e Calcaric Cambisol. Physical/chemical analyses revealed that soil characteristics were comparable among the four sites (sand 38%, coarse silt 9%, fine silt 33%, clay 20%, pH 6.9, organic matter 7%).

In each considered site, the soil was collected at a depth of about 20 cm, in three different points randomly chosen and mixed together, dried at room temperature (RT) for 7 days and sieved at 2 mm.

Soil chemical analyses

The dried processed soils were used for the determination of element concentration (Table I): samples were weighed (1 g) then digested in 10 mL concentrated HNO_3 in a MARS 5 microwave digestor (CEM, Cologno al Serio, BG, Italy). The digested material was filtered through 45-µm filters, and diluted in deionized water to 100 ml final volume. Element concentration was assessed by means of a calibration curve, after measurement by Inductively Coupled Plasma Optic Emission Spectrometry (ICP-OES) using an IRIS Advantage ICAP DUO HR series (Thermo Jarrell Ash, Franklin, MA, USA) spectrometer. Certified standards (BCR 145R, by the Institute for Reference Materials and Measurements, Ratieseweg, Belgium), with known element concentration, were analyzed with the samples in order to confirm the correctness of the procedure.

For dioxins-furans and PCB (Polychlorinated biphenyls) the analyses were performed by gas chromatography-mass spectrometry (GC-MS) according to the 8280a and 8270c EPA (Environmental Protection Agency) methods, respectively.

Seed culture and macroscopic parameters

The experimental system consisted in a Petri dish (15 cm), each filled with 22.5 g of soil saturated with 25 ml of sterile water; the soil was covered with a disk of filter paper according to the method UNICHIM n.1651 (2003). For each soil, five dishes were prepared.

Pea (*Pisum sativum* L. cv. Mezzalama Espresso Generoso) seeds were washed under tap water for 3 hours, put in the dishes described above (25 seeds/dish) and then stored in a growth chamber (24°C), in the dark, for 3 days.

The germination rate (number of germinated seeds/total number of seeds x 100) was calculated for each dish and the length of the main root was recorded for each germinated seed.

Mitotic index, mitotic aberrations and micronuclei evaluation

Root apices were cut from roots of comparable length (about 3 cm). Mitotic activity was evaluated on squashed root apices stained by Feulgen reaction, according to Hooker et al. (1998), using a hydrolysis of 9 min in 1 N hydrochloric acid (Merck, CAS no. 7647-01-0) at 60°C. Ten tips per treatment were evaluated and at least 1000 cells per tip were scored. The mitotic index (MI%) and the mitotic phases distribution were calculated on the same slides. The MI% was calculated as the percentage of nuclei in mitosis over the total number of nuclei observed. The percentage of normal and aberrant mitoses (metaphase sticky chromosomes and breaks; anaphase chromosome bridges and breaks, see Figure 2) as well as the presence of micronuclei were calculated in each slide.

Feulgen-stained nuclei were observed using an Axioscope II optical microscope, connected to an AxioCam camera (Zeiss; Oberkochen, Germany), and digital images were acquired by using an AxioVision II 0.5 software.

Cell nucleus extraction

In order to achieve more comparable samples, roots with similar size ranges were used for the different treatments. Roots atypically small or long were discarded.

The harvested roots were deprived of root tips. Nuclei extraction and sample preparation were performed following Berta et al. (2000). Roots were chopped with a razor blade, in a few drops of extraction solution (0.1 M citric acid, 0.5% Tween 20). Nuclei were extracted after 30 min incubation in this solution by filtering through a 20 μm nylon mesh. All steps were carried out on ice.

For the COMET test, unfixed nuclei were analysed and all the previously described steps were performed in the dark. For the flow cytometry, nuclei were fixed, by adding two volumes of ice-cold ethanol-acetic acid (3:1) to one volume of nuclei suspension, and stored at –20°C.

COMET test

The COMET Assay Kit cod. 0905-050-K by IKZUS (Genova, Italy) was used. All the operations were performed in the dark.

A suspension of 5000 nuclei for each sample was diluted in 10 μl of sodium citrate-buffer, then in 60 μl of Low Melting Point Agarose (LMPA). The resuspended nuclei were uniformly distributed on a microscope slide using a covering slide, and incubated for 5 minutes at 4°C. The covering slide was removed and 100 μl of LMPA were added and distributed as above. After another 5 minutes-incubation at 4°C, the covering slide was removed. The samples were incubated in Lysis solution, additioned of 10% Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich, Milano, Italy), overnight at 4°C. Before electrophoretic run, another incubation was performed in 300 mM NaOH (Sigma-Aldrich, Milano, Italy) pH>13 with EDTA (IKZUS, Genova, Italy) for 15 minutes at 4°C.

The electrophoretic run was performed for 40 minutes, in 300 mM NaOH pH>13 with EDTA (IKZUS, Genova, Italy), at 300 mA. The migration was blocked by incubating in 500 μl of Neutralization Solution, 5 minutes at RT. The nuclei were then fixed with 70% (v/v) ethanol stored at -20°C, 5 minutes at RT, and allowed to dry. 300 μl of IKZUS fluorescent probe

(dilution 1/10 of the stock solution) were added, and samples were incubated for 5 minutes at RT. The probe was uniformely distributed with a covering slide.

The nuclei were analysed by fluorescent microscopy, using an Axiovert 100M inverted microscope connected to an AxioCam camera (Zeiss, Oberkochen, Germany). The comet length, head diameter and area were evaluated with the COMET Score™ software (USA, © 2006 Tritek Corp.). At least 40 nuclei for each treatment were measured.

Flow cytometry

DAPI (4',6-diamidino-2-phenylindole, Sigma- Aldrich, CAS no. 28718-90-3) stained nuclei were analysed with the FloMax software package, associated to the Partec PAS flow cytometer (Partec GmbH, Münster, Germany). Fluorescent rainbow-trout erythrocytes (Partec GmbH, Münster, Germany) were used as an external standard. The Partec PAS instrument was equipped with a mercury arc lamp. UV excitation employed KG1, DUG11 filters and a TK420 dichroic mirror; DAPI fluorescence was detected using an EM455 barrier filter. The following parameters were analysed: relative fluorescence index (calculated as the ratio between the average fluorescence intensity of each sample peak and that of the external standard peak), and coefficient of variation (CV%).

Statistical analysis

For all the considered parameters, the differences between the samples and the control soil were evaluated by one-way ANOVA ($p \leq 0.05$ = significant; $p \leq 0.01$ = highly significant), except for the aberrant mitoses and the percentage of micronuclei; in this cases, being the data not normally distributed, the non-parametric Mann Whitney test was used. The analyses were performed by means of Statview v.4.5 (Abacus Concepts; Berkeley, USA).

206

207 **Results**

208

209 *Soil chemical analyses*

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211 Generally element, dioxin-furan and PCB concentrations in C soil were lower than in the others
212 (D, A, B) (Table I). B soil showed the highest values for all the analysed chemicals, especially
213 for Cr, As, Sn, Sb, Pb, dioxins-furans and PCB, while A and D soils had an intermediate level of
214 pollution.

215

216 *Macroscopic parameters*

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218 The germination percentage of pea seeds was significantly lower in B than in C soil
219 (Table II). A significant decrease (13.6%) of early root length was observed in pea seedlings
220 grown on A if compared to those grown on C, while plants tested on B and D showed similar
221 values to the controls (Table II).

222

223 *Mitotic activity, aberrations and micronuclei evaluation*

224

225 The MI% was significantly lower in A and B samples, while the value of this parameter
226 for D was comparable to C (Table III). Considering the phase index, a significant increase of
227 anaphases was observed for the B sample, while in the A sample no telophases were observed
228 (Table III). The plants grown on B soil showed also a significantly higher number of aberrant
229 mitoses, with a higher number of aberrant prophases (7.44%, Table III and Figure 2), as well as a
230 significantly higher number of micronuclei-forming nuclei (Table III), during both mitosis and
231 interphase (Figure 2b,f,n). The aberrant mitoses observed were: sticky chromosomes either in

prophase (Figure 2 a) or in metaphase (Figure 2c,d,e), broken chromosomes in metaphase (Figure 2g,h) and chromosome bridges in anaphase (Figure 2i,l,m).

In some cases, as micronuclei or aberrant mitoses were detected only in one of the biological samples analysed, the mean value was equal to the standard error.

COMET test

The COMET length (μm) (Figure 3a,b,c,d) was significantly higher (about two-times) in all the three polluted samples (D, A, B) in comparison to the controls (Figure 3e). Comet head diameter (μm) (Figure 3f) and comet area (μm^2) (Figure 3g) showed the same trend.

Flow cytometry

In all the analysed samples, the flow cytometry confirmed the existence of three fluorescence peaks, corresponding to 2C, 4C and 8C nuclear ploidy populations, with the 4C peak as the most represented (Figure 4a).

Fluorescence intensity, expressed as index, was significantly lower in the A sample, for all the three ploidy populations, while in the case of D and B the values were comparable to the control ones (Figure 4b). Data related to CV% of nuclei extracted from pea seedlings grown in B soil were the highest in all the ploidy populations, and showed significant differences with those grown in C soil (Figure 4c).

Discussion and conclusions

Despite the physiological and phylogenetical differences occurring between plants and animals, many studies showed a positive correlation between these two different systems, in particular when exposed to toxic elements. This suggested the potential use of plant-based biotests, at least for first-tier analyses (Panda & Panda 2002). We have adopted this kind of approach to assess the possible risk for environmental and human health, due to the exposure to potentially harmful soils.

In this work we analysed different parameters ranging from macroscopical to cellular level. At our knowledge, this is the first time in which this kind of approach is applied to the soil in a industrial polluted area.

The results of the germination test showed a strong reduction only in one case, i.e. the B sample, while the D and A samples didn't show any difference in comparison to the control (C), in spite of the seed pre-soaking step in water carried out in agreement with Tian et al. (2014). The presence of the tegument offers protection to the seed, since the latter remains intact, but upon germination it becomes permeable and then more susceptible to pollutants. According to our results, many studies reported a concentration-dependent reduction in germination rate (Kranner & Colville 2011). Not only heavy metals, but also highly lipo-soluble organics such as diesel fuel, are likely to exert a significant effect on this parameter, essentially due to the formation of hydrophobic barriers on the seed envelope, preventing the contact with water and oxygen. In agreement with this statement, only the B soil, located in the proximity and likely contaminated by pollutants coming from a rubber industry, was positive to the germination test: this soil was polluted by basic polymers and ingredients containing highly lipo-soluble organics, like dioxins-furans and PCB (Jagadale 2015).

As second step, we used the early root growth assay. Developing rootlets are much more permeable to chemicals than seeds, and therefore their elongation is a more reliable marker of

chemical stress, in presence of both organic and inorganic pollutants (Palmieri 2014). In our case, we observed a significantly lower value of the root length for A sample, compared to the control soil (C). The causes and mechanisms of this phenomenon have been already investigated in a number of species, including *P. sativum*, treated with herbicides: several morphological and physiological changes (i.e. decrease in root length, increase of the wall thickness in the cells of the root cap and of proline concentration) were evidenced in treated compared to non treated plants (Fayez & Kristen 1996; Kristen 1997).

The MI% significantly decreased in A and B samples relative to C. This result confirmed the data obtained from macroscopic parameters. Indeed, MI% is a marker of the meristematic activity: a lower value corresponds to an inhibition of cell division and results in a reduced root growth, and it can be considered a stress symptom (Fiskesjö 1997b; Fusconi et al. 2006). Moreover, the mitotic phase distribution revealed the disappearance of the telophases in A sample and a significant increase of the anaphases in B sample, suggesting a block in the mitotic process. An increase in the percentage of anaphases was already reported by Berta et al. (1990a) for contaminated soils analysed by standardised protocols, such as the *Allium* test, and may be considered as an index of a lower cell division rate.

Aberrant mitoses, including chromosome bridges in anaphases and sticky chromosomes in metaphases were also observed. A number of mechanisms are responsible for mitotic aberrations: some pollutants can interact with spindle proteins, resulting in aneugenesis; others are able to bind chromosomal proteins (e.g. topoisomerase II) causing clastogenesis (Panda and Panda 2002; Khalil et al. 2017); some others can induce defective functioning of specific non-histone proteins involved in chromosome organization causing chromosome stickiness (Türkoğlu 2012). Sticky chromosomes are considered a highly toxic, irreversible effect (Fiskesjö 1993; Abdel-Rahman et al. 2015). Pollutants can also override the physiological control points of mitosis thus preventing possible damages in DNA to be repaired before cell division (Osman 2014).

We observed a significant increase of aberrant mitoses in the B sample, suggesting a genotoxic effect of this soil on cell division. This result is unusual: most of the published works refer to aberrant metaphases and ana-telophases, rather than to prophases, as stress biomarkers (Mišìk & Mičieta 2002; Yekeen & Adeboye 2013). Nevertheless, this phenomenon has been already observed in *P. sativum* treated with cadmium (Fusconi et al. 2006).

The number of micronuclei-forming nuclei was again significantly higher in B sample. This could be explained by the different kinds of pollutants in the three soils. Micronuclei are documented as the product of mitoclastic or chromatoclastic effects, i.e. the results either of spindle malfunction or of chromosome breaks during mitosis (Reddy et al. 1995; Khalil et al. 2017). Micronuclei have been observed in presence of different damaging agents, for example x-rays, alchilating and mitoclastic agents, phenylboronic acids and others (Degrassi & Rizzoni 1982; Khalil et al. 2017).

The presence and intensity of DNA damage evaluated by the COMET test was significantly higher ($p \leq 0.01$) in all the samples grown on the polluted soils, if compared to the controls. Our results confirmed other results reported for this biotest (Panda & Panda 2002; Ventura et al. 2013; Santos et al. 2015) and strongly indicated its usefulness in a set of bioassays, in order to detect even low levels of DNA damage. Although only few papers report pea as a test plant for the COMET assay (Grant & Owens 2001; Ferrara et al. 2004), our results suggested that this species can be successfully exploited.

Concerning flow cytometry analyses, the presence in all the considered samples of three fluorescence peaks, corresponding to the 2C, 4C and 8C nuclear ploidy populations (with the 4C peak as the most represented) was in accordance to previous data described for *P. sativum* (Fusconi et al. 2006). Only the A sample showed a significantly lower value of fluorescence intensity, in comparison to the control. An increase of this parameter was previously considered as an indication of a higher nucleus DNA content (Berta et al. 1990b; 2000). Therefore these results may indicate, by contrast, DNA loss. On the other hand, we observed a significantly

higher CV%, for all the ploidy populations, in B sample. This phenomenon suggests a higher degree of heterogeneity in the nuclear populations and has already been observed by our group in plants subjected to biotic stress (Lingua et al. 1996). Moreover, Rayburn & Wetzel (2002) evidenced the correlation between this parameter and DNA damage due to an unequal distribution of the chromatin during mitosis.

Summarising the results achieved in this work, the B sample, corresponding to a site heavily exposed to the fall-out of rubber industrial emissions polluted by highly lipo-soluble organics, showed the highest occurrence of negative effects on the development and physiology of the tested plantlets. The D sample, corresponding to a site subjected to heavy vehicular traffic, showed a lower number of positive results, suggesting a lower toxicity.

In conclusion, in this study we adopted a number of biotests ranging from low-sensitive (seed germination and root elongation) to high-sensitive (the number of micronuclei-forming nuclei and the COMET test), in order to explore soil toxicity from the cellular to the organism level. In addition, we used also flow cytometry, a very fast way to detect DNA loss and damage, generally not used to evaluate soil toxicity. This kind of analysis can be performed easily and rapidly, using a small amount of plant material and provides a first important indication of soil toxicity. It also allows gaining further information when results of chemical analyses alone are not sufficient. In fact, improving the information obtained with chemical analyses, that alone are not enough: in fact, in our study, biological tests highlighted a real risk while the single chemical element concentration would not suggest a risk for living organisms.

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Table I. Element, dioxin-furan and PCB concentrations (mg kg^{-1}) in the four tested soils (C, D, A, B). For each row different letters mean significant differences between soils ($p< 0.05$) (n=3). In the last column, law limits for public and private green or residential use, according to the Italian legislation (D.Lgs 152/2006) are showed.

	C	D	A	B	Law limits
Be	0.90±0.01 a	0.85±0.13 a	1.27±0.03 a	1.11±0.15 a	2
Va	56.67±0.88 a	73.00±5.57 a	81.00±6.66 a	86.00±10.4 a	90
Cr	105.00±1.53 b	232.67±95.19 b	320.67±92.47 ab	489.33±58.18 a	150
Ni	489.33±58.18 a	172.00±84.58 a	209.67±73.54 a	321.00±78.81 a	120
Cu	24.33±0.33 a	38.33±5.84 a	50.67±19.38 a	62.67±6.33 a	120
Zn	105.33±0.33 a	174.67±22.58 a	152.00±36.83 a	210.67±67.22 a	150
As	11.67±0.33 c	15.00±1.00 bc	19.67±1.45 ab	21.33±3.18 a	20
Co	12.33±0.67 a	20.67±6.23 a	24.00±5.51 a	28.67±5.61 a	20
Cd	0.48±0.01 a	0.57±0.06 a	0.48±0.01 a	0.54±0.03 a	2
Sn*	3.60±0.10 b	4.13±0.20 b	5.23±0.64 ab	6.10±0.86 a	1
Sb	0.27±0.00 b	0.57±0.09 ab	0.62±0.17 ab	0.95±0.15 a	10
Hg	0.12±0.01 a	0.24±0.13 a	0.44±0.15 a	0.25±0.07 a	1
Tl	0.28±0.01 a	0.38±0.05 a	0.42±0.06 a	0.41±0.01 a	1
Pb	20.00±0.58 b	34.67±3.18 b	41.67±13.97 b	75.67±13.22 a	100
Dioxins-Furans	0.56±0.01 b	1.78±0.40 b	1.45±0.84 b	3.92±0.59 a	$1x10^{-5}$
PCB	2.50±0.10 b	4.17±0.81 b	2.63±1.33 b	14.47±2.42 a	0.06

* Sn stands for "organostannic compounds" according to the modifications applied by the art. 13, paragraph 3 bis, Law 11.08.2014 n. 116.

Table II. Germination percentage (n=5 petri dishes, 25 seeds each) and root length of pea seedlings grown on the four different soils (C, D, A, B). Mean values and standard errors are shown. Asterisks indicate significant differences between each soil sample and C soil (* = p< 0.05).

Sample	Germination (%)	Root length (cm)
C	67.20 ± 3.67	1.69 ± 0.10
D	70.40 ± 3.92	1.66 ± 0.10
A	72.00 ± 5.66	1.28 ± 0.09 *
B	39.20 ± 7.74 *	1.46 ± 0.10

Table III. Mitotic index (MI%), distribution of the normal (above) and aberrant (below) mitotic phases and percentage of micronuclei-forming nuclei in pea seedlings grown on the four tested soils (C, D, A, B). no = not observed. Asterisks indicate samples significantly different from the value of C (* = p< 0.05; ** = p< 0.01). n=10 tips, 10,000 counted nuclei.

Normal Phases					
Sample	**MI%**	**Prophase %**	**Metaphase %**	**Anaphase %**	**Telophase %**
C	7.012 ± 0.587	64.485 ± 4.795	21.945 ± 2.515	11.037 ± 1.932	2.533 ± 0.920
D	7.200 ± 0.722	57.181 ± 1.076	23.906 ± 1.887	17.035 ± 2.090	2.398 ± 1.152
A	4.423 ± 0.393 **	59.131 ± 3.588	23.166 ± 3.412	17.703 ± 2.907	no*
B	4.891 ± 0.269 *	55.533 ± 5.495	20.556 ± 3.237	21.961 ± 4.831 *	1.949 ± 1.140
Aberrant phases					
Sample	**%**	**Prophase %**	**Metaphase %**	**Anaphase %**	**Telophase %**
C	0.325 ± 0.325	no	no	0.325 ± 0.325	no
D	1.151 ± 0.431	0.260 ± 0.260	no	0.891 ± 0.302	no
A	2.436 ± 1.410	1.795 ± 1.118	no	0.641 ± 0.641	no
B	7.441 ± 0.718 *	6.026 ± 2.049	0.472 ± 0.472	0.943 ± 0.943	no

Sample	**Percentage of micronuclei-forming nuclei (%)**
C	0.023 ± 0.023
D	0.078 ± 0.078
A	0.023 ± 0.023
B	0.443 ± 0.141 *

553 **Figure legends**

554

555 Figure 1. Map of the sampling sites. A) (8.64214°E, 44.8691°N) and B) (8.76080°E,
556 44.89504°N) were collected in sites subjected to the emissions of two factories producing
557 chlorinated and fluorinated reagents and rubber components, respectively. C) (8.76397°E,
558 44.83231°N), was collected in a site not subjected to any specific source of contamination. D)
559 (8.73081°E, 44.82510°N) was collected from a roadside location.

560

561 Figure 2. Aberrant mitoses observed in our samples after Feulgen staining. Arrows and asterisks
562 indicate micronuclei and broken chromosomes respectively. Irregular sticky prophases without
563 (a) and with (b) micronucleus; c, d, e) metaphases (sticky chromosomes); f) metaphase with a
564 micronucleus; g, h) metaphases with broken chromosomes; i, l, m) anaphases (chromosome
565 bridges); n) interphase with a micronucleus. Bars correspond to 5 μm.

566

567 Figure 3. Fluorescence images of the COMET test of nuclei extracted from pea seedlings: a)
568 nucleus from a control sample; b), c), d) nuclei from plants grown on the D, A and B soils,
569 respectively, showing a "comet" due to DNA fragmentation.
570 Comet length (μm) (e), head diameter (μm) (f) and area (μm^2) (g) values resulting from the
571 COMET test of nuclei extracted from pea seedlings grown on the four tested soils (C, D, A, B).
572 Mean values and standard errors are shown in the graphs (for each soil n = 40). Asterisks
573 indicate highly significant differences between each soil sample and C soil (** = $p < 0.01$).

574

575 Figure 4. Flow cytometry graph (a), with different nuclear populations (2C, 4C, 8C) obtained by
576 nuclei extracted from *Pisum sativum* cv. mezzarama generoso grown on C soil; relative
577 fluorescence in the blue channel (FL4 – arbitrary units) is reported in the x-axis while the
578 number of nuclei in the y-axis. Relative fluorescence index (b) and coefficient of variation

579 (CV%; c) of the different ploidy populations (2C, 4C, 8C) in nuclei extracted from pea seedlings
580 grown on the four tested soils: C, D, A, B (bars from left to right). Mean values and standard
581 errors are shown in the graphs. Asterisks indicate significant differences between each soil
582 sample and C soil (* = $p < 0.05$; ** = $p < 0.01$). n=5.