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Effects of sewage sludge application on unfertile tropical soils evaluated by multiple approaches: A field experiment in a commercial *Eucalyptus* plantation

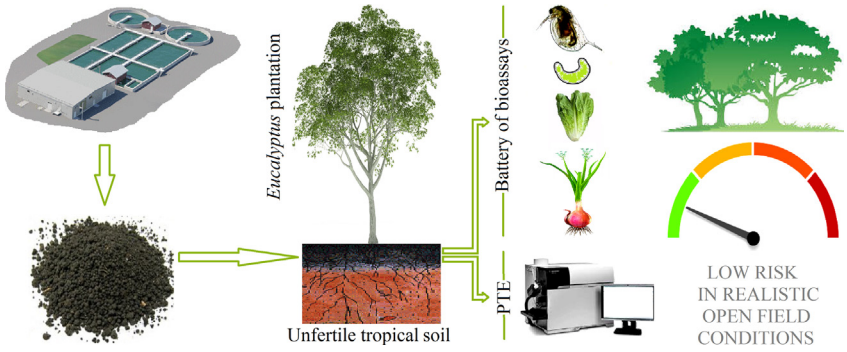
Cassio Hamilton Abreu-Junior [a], Maria Julia de Lima Brossi [b], Regina Teresa Monteiro [a], Paulo Henrique Silveira Cardoso [a], Thays da Silva Mandu [a], Thiago Assis Rodrigues Nogueira [c], Antonio Ganga [d], Peter Filzmoser [e], Fernando Carvalho de Oliveira [f], Lucia Pittol Firme [f], Zhenli He [g], Gian Franco Capra [h,i,*]

[a] University of São Paulo, Center for Nuclear Energy in Agriculture, Piracicaba, SP 13400-970, Brazil
[b] University of Groningen, Gelifes - Microbial Ecology Cluster, Groningen, AG 9747, Netherlands
[c] Department of Plant Protection, Rural Engineering and Soils, School of Engineering, São Paulo State University, Ilha Solteira, SP 15.385-000, Brazil
[d] Dipartimento di Agraria, Università degli Studi di Sassari, Via Colombo n°1, 08100 Nuoro, Italy
[e] CSTAT - Computational Statistics, Institute of Statistics & Mathematical Methods in Economics, Vienna University of Technology, Wiedner Hauptstr. 8-10, 1040 Wien, Austria
[f] Biossolido Agricultura e Ambiente Ltda. e Biossolo Serviços Agronômicos e Ambientais Ltda. Piracicaba, SP, Brazil
[g] Institute of Food and Agricultural Sciences, Indian River Research and Education Center, University of Florida, Fort Pierce, FL 34945-3138, USA
[h] Dipartimento di Architettura, Design e Urbanistica, Università degli Studi di Sassari, Via Colombo n°1, 08100 Nuoro, Italy
[i] Desertification Research Centre, Università degli Studi di Sassari, Viale Italia n° 39, 07100 Sassari, Italy

HIGHLIGHTS

- Sewage sludge (SS) application in forest soils increased considerably in last years.
- Toxicity of SS in unfertile tropical soil under *Eucalyptus* plantation was evaluated.
- A battery of bioassays and PTE availability were assessed by a multiple approach.
- In realistic open field conditions SS risk may be lower than expected.
- Open-field trials should be preferred for evaluating SS toxicity in forestry002E

GRAPHICAL ABSTRACT



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ABSTRACT

Sewage sludge (SS) reuse in forest plantation as soil fertilizer/amendment has tremendously increased in recent years. However, SS may have high concentrations of potentially toxic elements (PTE), representing a potential risk for soil and the whole ecosystem. This paper was aimed to assess the toxicity of PTE in unfertile tropical soils amended with SS in a commercial *Eucalyptus* plantation, with an integrated multiple approaches combining: *i*) the use of a battery of bioassays (*Daphnia magna*, *Pseudokcrichirella subcapitata*, *Lactuca sativa*, and *Allium cepa*); and *ii*) the evaluation of some PTE (Cd, Cr, Cu, Fe, Mn, Ni, Pb, and Zn) and their availability into the pedoenvironment. Differences in total and available PTE between SS doses and time of treatments were evaluated using ANOVA; correlations between PTE and bioassays by a sparse partial robust M-regression (SPRM), while multiple correlations among parameters were performed by principal factor analysis (PFA). Results show that PTE contents in soils tended to increase with SS application doses. However this cannot be assumed

* Corresponding author at: University of São Paulo, Center for Nuclear Energy in Agriculture, Piracicaba, SP 13400-970, Brazil.
E-mail address: pedolnu@uniss.it (G.F. Capra).

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Potentially toxic elements
Multivariate statistics

as a general rule since in all the investigated treatments the PTE concentrations were consistently below both soil natural background concentrations and quality reference values. Bioassays showed a generalized low eco- and genotoxicity of SS with an increase in toxicity at increasing SS doses but with a clear decreasing trend as time went by. *A. cepa* was the most sensitive bioassay followed by *P. subcapitata* > *D. magna* > *L. sativa*. Overall, the results indicate that in realistic open field conditions SS risk may be lower than expected due to dynamic decrease in PTE toxicity with time after application. This study has an important implication that open-field trials should be strongly encouraged for evaluating environmental risk of SS application in forestry.

1. Introduction

Sewage sludge (SS) represents a common by-product of wastewater treatment that should be beneficially used, thus avoiding environmental contamination and providing benefits to the "circular economy" viewpoint (Mosquera-Losada et al., 2017). As reviewed by Cieślik et al. (2015), SS has been used and managed for several purposes such as land reclamation, building manufactures, phosphorus recovery, rare earth metal recovery in industry, pellets, and absorbents. Its application in agriculture is now considered a consolidated practice in several regions worldwide as a cost-effective approach for both waste disposal and improving soil quality by recycling organic matter and nutrients (Franco et al., 2010; Lu et al., 2012). Recently, there has been a surge in the utilization of SS as fertilizer/amendment in forest and degraded areas (Kimberley et al., 2004; Wang et al., 2013; Abreu-Junior et al., 2017; Gutiérrez-Ginés et al., 2017). Its application in forest plantation can address two pivotal questions: *i*) it reduces the risk of contaminants that enter the human food chain (Kimberley et al., 2004), since the generated products are not edible; and *ii*) it increases tree growth and the whole system productivity (Abreu-Junior et al., 2017). Additionally, in the tropical areas, where worldwide market leading countries in wood (and derivate) production are concentrated, SS can significantly improve soil fertility and the general soil physical-chemical conditions (Abreu-Junior et al., 2005); a strategic outcome in areas often characterized by extremely unfertile soils (Guerrini et al., 2017).

On the other hand, depending on its origin and quality, SS can contain high concentrations of potentially toxic elements (PTE), representing a potential risk to soil and, through soil leaching and runoff, contamination to groundwater and the surrounding fluvial systems (Smith, 2009). SS also represents a potential source of organic contaminants (Kim et al., 2017) and pathogens (Smith, 2009; Nascimento et al., 2018). While it is impracticable to analyze all these contaminants in SS and/or amended soils (Selivanovskaya and Latypova, 2003), additional issues can be (co)responsible for an underestimation of the real environmental risk associated with SS application on pedosphere, such as: *i*) contaminants into the soil environment, metabolites and byproducts, cannot be fully detected by chemical analyses; *ii*) soil physico-chemical analysis does not allow an integration of the combined/synergistic effects caused by the presence of multiple chemicals characterizing SS and the corresponding amended soil; *iii*) total PTE concentrations can overestimate the real environmental risk, as aging processes may significantly reduce bioavailability and, subsequently, biotoxicity of the pollutants. Based on these lines of evidence, ecotoxicological test using bioassays are considered to be an excellent integration (albeit, not a substitution) to soil physico-chemical analysis (Huguier et al., 2014). Since both toxic inorganic and organic contaminants are present and bioavailable in water elutriates obtained from soils, bioassays, when used together with chemical analyses, may detect synergistic and antagonistic effects, as well as provide information about the contaminants bioavailability (Ahlf and Förstner, 2001).

The selection of appropriate bioassays should be based on: *i*) high representativeness and sensitiveness to contaminants; *ii*) rapid life cycles and uniformity in terms of reproduction, growth, and phenotypic features; and *iii*) similar routes of exposure. Since the use of a single bioassay cannot provide a satisfactory description of soil quality, it is strongly recommended to use a set of tests, composed of different bioassays, in order to provide an appropriate assessment (Selivanovskaya and Latypova, 2003; Huguier et al., 2014). Monitoring soil quality by biological indices is becoming an essential tool to understand the environmental risks involved in SS application (Melo et al., 2018). To date, minimal information is available on the assessment of SS environmental risk and the quality of amended soils, by the integrated means of PTE speciation and a battery of bioassays. Moreover, most of the previous studies have been focused on short-term and laboratory or semi-field (pots) evaluation (Selivanovskaya and Latypova, 2003; Andrés and Domene, 2005; Carbonell et al., 2009; Cesar et al., 2012; Huguier et al., 2014), there is still lacking of researches on long-term open field observations.

This paper aims to assess, by integrated multiple approaches, combining the use of a battery of bioassays, the availability of PTE in the pedoenvironment, their toxicity in unfertile tropical soils amended with SS using a field experiment in a commercial *Eucalyptus* plantation.

2. Material and methods

2.1. Study area and field procedure

The research was conducted in an experimental area of 3.3 ha, at the Entre Rios farm belonging to the company Suzano Bahia Sul Papel e Celulose S.A., Angatuba municipality, central São Paulo state, southern Brazil (Fig. 1). A detailed pedological survey was carried out before the experiment and the soil was classified as Typic Hapludox (Soil Survey Staff, 2014)/Rhodic Ferralsol (IUSS Working Group WRB, 2015).

Before the experiment, the soil A_p surface horizon (0–40 cm depth) was characterized (Annex 1) according to official procedure (van Raij et al., 2001). PTE concentrations were below the reference values for the soils of the São Paulo State (Nogueira et al., 2018), thus allowing for the sludge applications in this particular area (CETESB, 2016). Soon after, lime (95% relative power of total neutralization, at the rate of 1.8 Mg ha^{-1}) was applied to soil, over the whole experimental area, in order to raise the base saturation to 45%.

The SS was obtained from a domestic sewage sludge treatment plant (Jundiaí municipality, State of São Paulo, south-eastern Brazil). The SS was generated in a biological system composed of a sequence of aerated, mixing, and sedimentation ponds for a period of approximately one year. In order to reduce the presence of pathogenic agents and to obtain material containing up to 25% of solids, the SS was further treated with polymers, centrifuged, and air dried for three months, with periodic mechanical turnover of the piles (Abreu-Junior et al., 2017). At the end of the processing, the SS was characterized according to the official procedure recommended by the Resolution 375 (CONAMA, 2006). The main chemical features and the concentrations of PTE in SS are reported in Annex 2. In brief, the SS contained high concentrations of total OC, N, and P, while PTE contents were below the limits established by CONAMA (2006) and those usually observed in SS (Abreu-Junior et al., 2005; Smith, 2009). These figures allowed the agricultural use of the investigated SS (CONAMA, 2006).

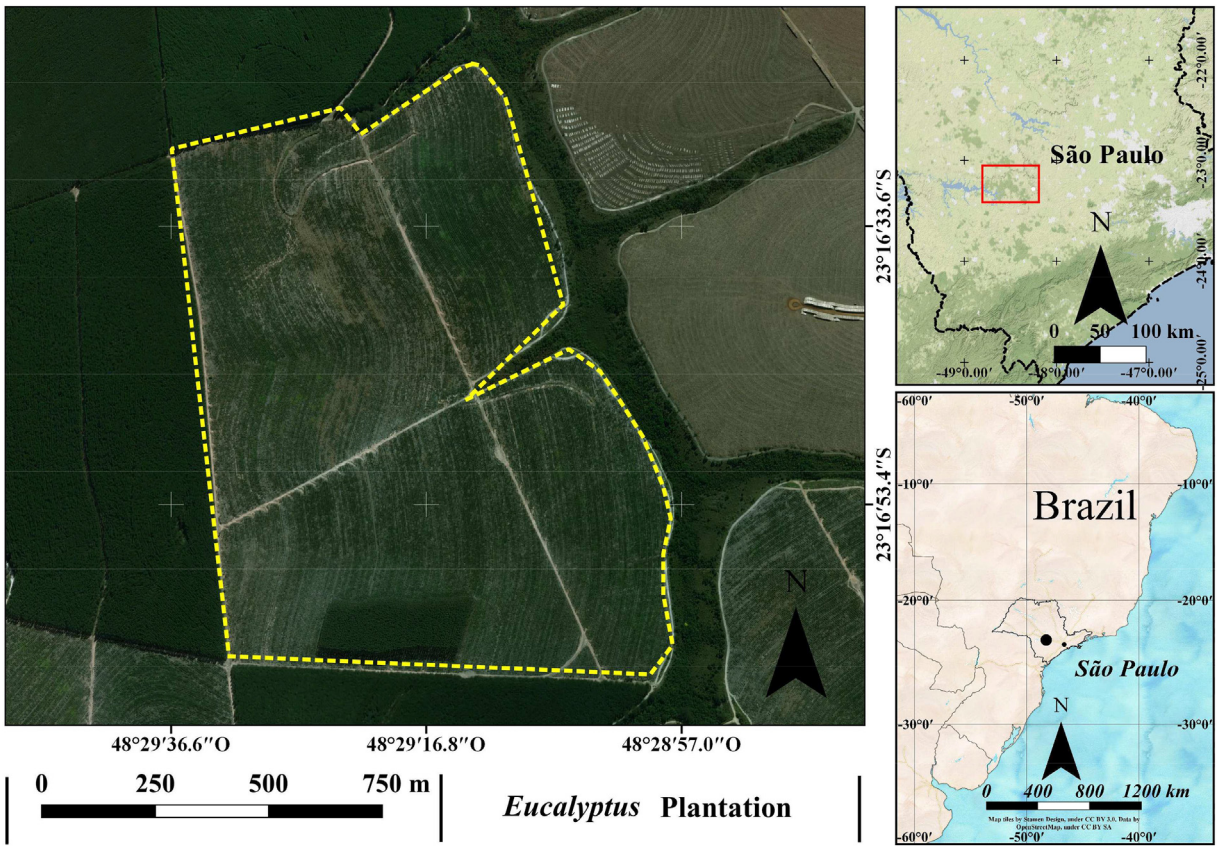

Fig. 1. Study area (Suzano Bahia Sul Papel e Celulose S.A., Municipality of Angatuba, São Paulo state, Brazil; 23°170S and 48°150 W, 650 m asl).

The SS was applied in each row in a continuous band of 60 cm, close to the planting lines, just before *Eucalyptus* planting, at doses of 0, 8, 15, and 23 Mg ha^{-1}, which are equivalent to 0, 50, 100, and 150% of the recommended N supply, based on the N criterion (CONAMA, 2006).

Soils samples were collected in each of the previously reported treatment/doses from A_{p1} horizon (0–20 cm) at 18, 21, 24, 27 and 34 months after SS application. Each soil sample, consisting of six replicates randomly collected around the area of each treatment/doses, was submitted to the following analysis.

2.2. PTE extraction method

The investigated PTE (Cd, Cr, Cu, Fe, Mn, Ni, Pb, and Zn) were selected according to their initial concentration in soil and/or SS (Annexes 1 and 2). Since the initial concentrations of As, Ba, Hg, Mo, and Se were negligible in one or both media they were not further investigated.

Soil samples were air-dried and sieved at 0.5 mm. Concentrations of PTE were determined in terms of: *i*) total recoverable metals, according to USEPA-3051A method (USEPA, 2007) a digestion with HNO_3 + HCl; and *ii*) (bio)available, according to DTPA (van Raij et al., 2001) and Mehlich 1 (Silva, 2009) extractable methods. All analyses were carried out in triplicates and blank samples were analyzed simultaneously. In order to ensure very low limits of detection all acids, reagents and water were ICP-MS compatible grade. Standard reference material (SRM 2709a – San Joaquim) was used to assure the accuracy and precision of the analytical methods. The recovery of metals in SRM was within 89–124%, representing the recommended range for the investigated elements (USEPA, 1996). Concentrations of PTE in the extracts were then analyzed by inductively coupled plasma mass spectrometry (ICP-MS, Model Agilent 7500ce, Agilent Technologies, Tokyo, Japan).

2.3. Ecotoxicity and genotoxicity assays

The toxicity of the SS-amended soils (after 18, 21, 24, 27 and 34 months) were determined by both ecotoxicity and genotoxicity tests using water flea *Daphnia magna* Straus, algae *Pseudokirchneriella subcapitata* (Korshikov) Hindak, and higher plants *Lactuca sativa* L. and *Allium cepa* L. The test procedures were performed according to the following methods.

In all the following methods soils samples were previously air-dried and sieved (Ø < 0.2 mm). Soil sample elutriates (SSE) were prepared by agitating (24 h) soil samples in a soil/solution mixture of 1:4 H_2O ultrapure water. All tests were conducted with fresh decanted (5 min) elutriates.

Acute assays with *D. magna* were conducted using the protocol proposed by Dutka (1997) consisting of a 48-hour static bioassay method. In particular, 5 newborns of *D. magna* were placed in 28 mL of SSE. Six concentrations (100, 95, 90, 85, 80, and 75%) were tested with three replicates for each concentration + a control. After 48 h, the organisms alive were counted. The LC_{50} (48 h) value indicates the elutriate dilution (in percentage) of the tested soil that is lethal to 50% of *D. magna* and was calculated according to the Trimmed Spearman-Karber method (Hamilton et al., 1977); *i.e.*, the higher the LC_{50} value, the lower the soil toxicity.

Assays with *P. subcapitata* were conducted by a cost-effective 72-h exposure of the algae. When exposed to samples containing bioavailable toxicants, growth inhibition of algae will result in relation to unexposed control (Blaise et al., 2000). Overall, six different concentrations (100, 75, 50, 54, 12.6 and 6.3%) + a control with three replicates were performed. The IC_{50} (72 h) value provides the SSE dilution of the tested soil that causes growth inhibition of 50% on *P. subcapitata* and was calculated according to the Trimmed Spearman-Karber method (Hamilton et al., 1977).

The plant germination and growth tests (72 h) with seeds of *L. sativa* were tested according to Dutka (1997). Three replicates for each concentration + a control (20 seeds per replicates) were performed in Petri dishes containing 2 mL of SSE. For each germinated seed root growth was measured in centimeter.

Cito-, geno-, and mutagenic assays were performed in onion seeds (*A. cepa*, Baia Piriforme variety) by a modified version of Grant's (1982) protocol. Onion seeds were germinated in Petri dishes (two replicates per sample) lined with paper filters moistened with SSE. The negative control consisted of seeds allowed to germinate in ultrapure water. All bioassays were conducted in duplicates. When they reached about 2 cm in length, the roots were collected and fixed in Carnoy's solution (ethyl alcohol and acetic acid, 3:1) for 6 to 18 h at room temperature. After fixation, the roots were transferred to a fresh Carnoy's solution and stored in a refrigerator until examined. The first 1 cm of root tips was abandoned, and the next 1 cm (F1-cell, nonmeristematic region; Leme and Marin-Morales, 2008) was placed in 1 mol L^{-1} HCl at 60 °C for 10 min and stained with Schiff's reagent for 2 h. The roots were then washed in distilled water until the dye was completely removed. Slides were prepared using 2% of acetic carmine with root meristems gently pressed between the slides and coverslips. The latter was removed with liquid nitrogen and slides mounted with synthetic resin (Permount) for examination. The microscopic investigation included the analysis of three different variables: *i*) the chromosomal aberrations (CA), by evaluating several types of aberration (such as fragments, bridges and losses, laggard or vagrant chromosomes), within different cell division stages (prophase, metaphase, anaphase and telophase) and classifying them into just one category in order to evaluate it as a single endpoint; *ii*) the micronucleus (MN) induction in the meristematic cells of *A. cepa*; and *iii*) the mitotic index (MI), *i.e.*, the number of cells in division. The analysis was done by scoring 5000 cells per treatment, 1000 cells per slide, comprising a total of 5 slides.

2.4. Statistical analysis

Univariate and multivariate statistical analyses were performed using R (R Development Core Team, 2008) and SAS (SAS Institute Inc., 2011).

Data were compared using ANOVA. Significant differences between means were determined by a Tukey's post-hoc honest significant difference test with $p < 0.05$.

A sparse partial robust M-regression (SPRM) for a partial least squares (PLS) regression was performed in order to understand how and in which extent PTE concentration influenced bioassays by taking into account both SS doses and time. Such a regression was selected, rather than a standard one, because it reduces dataset dimensionality (Hoffmann et al., 2015). Additionally, this kind of analysis is preferable in case of potential outliers (robust), and because it allows setting coefficients with no explanatory power equal to zero (sparse). In particular, the bioassay variables have been considered as response in a regression setting, while PTE concentration data as explanatory variables. In this kind of analysis bioassay variables were treated, for reasons of interpretation, separately by fitting individual models. The explanatory variables were three blocks of compositions: DTPA, Mehlich, and Total recoverable metals. Each block has been separately clr (centered log-ratio) transformed, and the clr variables have been used in the regression.

A factor analysis (FA) was carried out to explain the variation in a multivariate data set with as few 'factors' as possible and to detect hidden multivariate data structures and correlations between investigated parameters. According to Reimann et al. (2002) we (i) Box–Cox transformed the raw data set to approach normality; (ii) performed a correlation matrix (CM) on the Box–Cox transformed data; and finally (iii) carried out the FA based on the CM. Varimax rotation – an orthogonal rotation that minimizes the number of variables that have high loading on each factor – was used to simplify the transformed data matrix, and facilitate results interpretation (Reimann et al., 2002).

Values in the text indicate the mean ± standard error.

3. Results and discussion

3.1. Potentially toxic elements (PTE)

Table 1 shows the PTE concentration, estimated with the different extraction methods, and according to SS doses and period (months after SS application). PTE contents tended to increase with SS application doses, though with some exceptions to extraction methods or individual PTE. For Cd, Cu, and Zn, both the available (DTPA and Mehlich-1) and total recoverable (HNO_3 + HCl) fractions statistically increased ($p < 0.05$) with SS doses. For the other investigated PTE this was true only for available fractions while there were not statistical differences in terms of total recoverable fractions.

The change of PTE content with experimental time varied with PTE element and form. Cadmium and Mn did not show a significant increase in both total recoverable and available fractions during the three years of field experiment. Total recoverable Cr, Fe, Ni, Pb, and Zn increased along the experimental time, while there was no significant difference in their available fractions. Finally, Cu showed an increase in available fraction but not in the total recoverable amount. As reviewed by previous authors (Silveira et al., 2003; Smith, 2009; Silva and Camillotti, 2014), the accumulation of heavy metals in soil is one of the important considerations that cause concerns regarding the reuse of SS in the pedoenvironment. However, in the present field experiment when the total recoverable concentrations of PTE are compared with soil natural background concentrations (NBC; Nogueira et al., 2018) or quality reference values (QRV; CETESB, 2014) for the State of São Paulo, it is clear that they were below those standard values (Table 1) and similar to those usually observed for natural soils worldwide (WS; Kabata-Pendias and Mukherjee, 2007). Even in terms of PTE availability, values of pivotal importance but not ruled by international legislations, the observed values were usually lows and under those reported by previous authors for both worldwide (Kabata-Pendias and Mukherjee, 2007) and São Paulo State (Gabos et al., 2014; Oliveira et al., 2014) soils.

3.2. Ecotoxicity

Values of LC_{50} (48 h), obtained from the toxicological test with *D. magna*, were generally high, indicating low ecotoxicity, ranging from 78 ± 2 to 99 ± 1% (Table 2) with a mean of 88 ± 1%. There were significant differences in LC_{50} values among the different SS doses only after the first two observations (18 and 21 months); during the remaining thirteen months (from 21 to 34) there was no significant trend of increasing ecotoxicity with increasing SS doses. It appears that just after a relative short period of stabilization, SS ecotoxicity for *D. magna* decreased with time; as a matter of fact, LC_{50} values statistically increased, meaning ecotoxicity decreased, over time.

Values of IC_{50} (72 h), obtained from the toxicological test with *P. subcapitata*, were more variable, ranging from values showing both medium-high (34 ± 0) and absence of ecotoxicity (98 ± 1%), with a mean of 54 ± 4% (Table 2). Additionally, IC_{50} (72 h) values significantly decreased with SS dose. During the experimental period, ecotoxicity generally increased, even if during the last observation (34 months) a generalized significant decrease was observed.

Observed values in root growth in *L. sativa* (Table 2) are evidences of a positive interaction of SS doses. Indeed, root growth increased with increasing SS dose. This could be related to N or P in the sludge, an economic benefit. Mean values significantly increased with SS doses: 1.5 (control), 1.7 (8 Mg ha^{-1}), 1.8 (15 Mg ha^{-1}), 1.9 (15 Mg ha^{-1}), and 2.1 (23 Mg ha^{-1}) cm. Such a positive effect was also observed with time, with values statistically increasing as 1.3 (18 months), 1.8 (21 ms), 1.9 (24 ms), and 2.0 (27 and 34 ms) cm. Consequently mean

Table 1
PTE concentrations in SS-amended soils (mean ± SE).

Extraction	Sewage sludge doses (Mg ha^{-1})				Time after SS application (months)				
method	0	8	15	23	18	21	24	27	34
	Cd (mg kg^{-1})								
	WS[a] = 0.01–2; NBC[b] = 0.1; QRV[c] = <0.05								
DTPA	0.01±0.00a	0.03±0.01a	0.04±0.01ab	0.06±0.01b	0.01±0.00a	0.03±0.02a	0.05±0.02a	0.04±0.02a	0.04±0.01a
Mehlich-1	0.01±0.00a	0.02±0.01a	0.04±0.01ab	0.06±0.01b	0.01±0.01a	0.03±0.01a	0.05±0.02a	0.03±0.01a	0.03±0.01a
HNO_3+HCl	0.02±0.01a	0.05±0.01a	0.10±0.02ab	0.12±0.02b	0.02±0.01a	0.08±0.03a	0.10±0.03a	0.09±0.03a	0.08±0.01a
	Cu (mg kg^{-1})								
	WS[a] = 8–80; NBC[b] = NI; QRV[c] = 35								
DTPA	0.5±0.1a	0.5±0.1a	0.5±0.1a	0.5±0.1a	0.5±0.1a	0.5±0.1a	0.5±0.1a	0.5±0.1a	0.5±0.1a
Mehlich-1	0.4±0.1a	0.4±0.1a	0.4±0.1a	0.4±0.1a	0.4±0.1a	0.4±0.1a	0.4±0.1a	0.4±0.1a	0.4±0.1a
HNO_3+HCl	1.5±0.3a	1.5±0.3a	1.5±0.3a	1.5±0.3a	1.5±0.3a	1.5±0.3a	1.5±0.3a	1.5±0.3a	1.5±0.3a
	Fe (mg kg^{-1})								
	WS[a] = 1000; NBC[b] = NI; QRV[c] = NR								
DTPA	41±2a	41±2a	41±2a	41±2a	41±2a	41±2a	41±2a	41±2a	41±2a
Mehlich-1	49±2a	49±2a	49±2a	49±2a	49±2a	49±2a	49±2a	49±2a	49±2a
HNO_3+HCl	6618±697a	6618±697a	6618±697a	6618±697a	6618±697a	6618±697a	6618±697a	6618±697a	6618±697a
	Mn (mg kg^{-1})								
	WS[a] = 7–100; NBC[b] = NI; QRV[c] = NR								
DTPA	1.1±0.2a	1.1±0.2a	1.1±0.2a	1.1±0.2a	1.1±0.2a	1.1±0.2a	1.1±0.2a	1.1±0.2a	1.1±0.2a
Mehlich-1	2.8±0.1a	2.8±0.1a	2.8±0.1a	2.8±0.1a	2.8±0.1a	2.8±0.1a	2.8±0.1a	2.8±0.1a	2.8±0.1a
HNO_3+HCl	33.2±3.1a	33.2±3.1a	33.2±3.1a	33.2±3.1a	33.2±3.1a	33.2±3.1a	33.2±3.1a	33.2±3.1a	33.2±3.1a
	Zn (mg kg^{-1})								
	WS[a] = 1–900; NBC[b] = 22; QRV[c] = 60								
DTPA	1.1±0.1a	1.1±0.1a	1.1±0.1a	1.1±0.1a	1.1±0.1a	1.1±0.1a	1.1±0.1a	1.1±0.1a	1.1±0.1a
Mehlich-1	1.1±0.1a	1.1±0.1a	1.1±0.1a	1.1±0.1a	1.1±0.1a	1.1±0.1a	1.1±0.1a	1.1±0.1a	1.1±0.1a
HNO_3+HCl	8.2±1.6a	8.2±1.6a	8.2±1.6a	8.2±1.6a	8.2±1.6a	8.2±1.6a	8.2±1.6a	8.2±1.6a	8.2±1.6a
	Cr (mg kg^{-1})								
	WS[a] = 5–1500; NBC[b] = 36; QRV[c] = 40								
DTPA	0.01±0.00a	0.02±0.00ab	0.02±0.00ab	0.03±0.00b	0.02±0.00a	0.02±0.00a	0.02±0.00a	0.02±0.00a	0.02±0.00a
Mehlich-1	0.09±0.00a	0.09±0.01a	0.11±0.01ab	0.14±0.01b	0.11±0.01a	0.12±0.02a	0.13±0.01a	0.10±0.01a	0.09±0.01a
HNO_3+HCl	9.90±1.12a	11.14±0.99a	12.27±1.26a	12.68±1.30a	7.19±0.55a	11.35±0.42b	13.81±0.81c	12.81±1.02cd	12.33±0.43db
	Ni (mg kg^{-1})								
	WS[a] = 1–750; NBC[b] = 36; QRV[c] = 13								
DTPA	0.04±0.01a	0.10±0.02ac	0.14±0.03bc	0.20±0.03b	0.06±0.01a	0.16±0.04a	0.15±0.04a	0.12±0.04a	0.13±0.05a
Mehlich-1	0.04±0.00a	0.07±0.01ac	0.11±0.01bc	0.16±0.03b	0.08±0.01a	0.12±0.05a	0.09±0.03a	0.11±0.02a	0.08±0.02a
HNO_3+HCl	1.08±0.12a	1.31±0.17a	1.39±0.15a	1.55±0.19a	0.82±0.06a	1.63±0.13b	1.62±0.13bc	1.46±0.13bd	1.13±0.07ad
	Pb (mg kg^{-1})								
	WS[a] = 2–750; NBC[b] = 10; QRV[c] = 17								
DTPA	0.7±0.0a	0.8±0.1ab	1.0±0.1b	1.3±0.0c	1.0±0.1a	1.1±0.1a	0.9±0.1a	0.9±0.1a	0.9±0.2a
Mehlich-1	0.7±0.0a	0.9±0.1ab	1.0±0.1ab	1.2±0.1b	0.6±0.0ac	1.1±0.2b	1.0±0.1bc	1.0±0.1bc	1.0±0.1bc
HNO_3+HCl	4.2±0.5a	5.0±0.5a	5.3±0.6a	6.4±0.6a	3.1±0.3a	5.4±0.4b	6.4±0.6b	5.8±0.6b	5.4±0.4b

Significant differences ($p < 0.05$) are indicated by different lowercase letters after means within the same row.
NI = not investigated; NR = not ruled.
[a]World soils (Kabata-Pendias and Mukherjee, 2007).
[b]Natural background concentrations (Nogueira et al., 2018).
[c]Quality reference values (CETESB, 2014) in the State of São Paulo.

Table 2
Values of LC_{50} (48 h), IC_{50} (72 h), and root growth (72 h) for the ecotoxicological tests with *D. magna*, *P. subcapitata*, and *L. sativa*, respectively (mean ± SE).

Time after SS application (months)	LC_{50} (48 h) SS doses (Mg ha^{-1})				IC_{50} (72 h) SS doses (Mg ha^{-1})				Root growth (72 h) SS doses (Mg ha^{-1})			
	0	8	15	23	0	8	15	23	0	8	15	23
	%				%				cm			
18	91±1c	88±2bc	81±1d	81±0d	98±1a	70±0a	61±2a	63±0a	1.3±0.0a	1.3±0.0a	1.4±0.0b	1.4±0.0b
21	89±1d	78±2d	79±1e	79±0e	71±0b	43±0d	30±1d	30±0e	1.6±0.0b	1.8±0.0c	2.1±0.0d	2.2±0.0e
24	92±1c	87±2c	83±1c	83±0c	60±1d	48±0c	38±1c	36±0c	1.7±0.0b	1.8±0.0c	2.0±0.0d	2.1±0.0e
27	94±0b	89±2b	89±1b	89±0b	97±2a	48±0c	40±1c	34±0d	1.6±0.0b	1.7±0.0c	1.8±0.0c	1.9±0.0ce
34	99±1a	97±3a	94±1a	92±0a	63±0c	62±0b	47±2b	44±0b	1.2±0.0c	1.6±0.0d	1.9±0.0e	2.0±0.0e

Significant differences ($p < 0.05$) are indicated by different lowercase letters.

values of root growth for all the tested samples ranged between 1.5 and 2.8 cm, a length considered to be appropriate for the first 72 h (Fjllborg and Dave, 2004). Banks et al. (2006) also reported an increase in lettuce growth with SS dose. This could be attributed to the fact that SS is a source of nutrients for lettuce while total and available heavy metals were lows. Indeed, in soils characterized by high heavy metal concentrations lettuce growth was tremendously inhibited by PTE such as As, Cd, Pb, and Zn (Corrêa et al., 2006; Valerio et al., 2007).

Reported results for ecotoxicity showed that: *i*) ecotoxicity generally decreases with time; and *ii*) test with *P. subcapitata* is more sensitive than with *D. magna* and *L. sativa*, at least when performed in long-term field experiments. Indeed, comparisons with previous studies cannot be completely satisfactory because they were realized in laboratory or semi-field (pots) conditions (Selivanovskaya and Latypova, 2003; Andrés and Domene, 2005; Carbonell et al., 2009; Cesar et al., 2012; Huguier et al., 2014). However, in a recent research aiming at assessing the potential ecotoxicity of nine organic wastes, 5 of which belonging to the SS-type, Huguier et al. (2014) observed that *P. subcapitata* were the most sensitive bioassays in a battery of 13 different bioassays with *D. magna* being the less sensitive ones. Our study suggests that one of the reasons for such higher sensitivity could be related to the intrinsic nature of the experiment. Indeed, and unexpectedly, a sort of time-dependent detrimental effect, in terms of ecotoxicity increase, was observed for (and only) *P. subcapitata* in the control, *i.e.*, in soils with no SS. This seems to suggest that the turbidity of the sample, which harms the photosynthetic process in algae, could be at least partially affected the experiment. Other differences could be related to differences in the characteristics of SS such as physical-chemical properties, heavy metal content, availability and speciation. For instance, both Blaise and Férard (2005) and Fernandez et al. (2005) confirmed that *D. magna* were less sensitive than *P. subcapitata*, but Fjllborg et al. (2005) demonstrated that sensitivity of *D. magna* to heavy metals toxicity in SS leachate varied during the year, depending on one (Zn) or more (Cu) metal presence.

3.3. Genotoxicity

Obtained results with *A. cepa* (Table 3) were used as a clue for genotoxic (chromosomal aberrations), mutagenic (micronucleous), and cytotoxic (mitotic index) effects.

Table 3
Genotoxic (chromosomal aberrations), mutagenic (micronucleous), and cytotoxic (mitotic index) effects to *A. cepa* (mean ± SE).

Time after SS application (months)	Chromosomal aberrations SS doses (Mg ha^{-1})					Micronucleous SS doses (Mg ha^{-1})					Miotic index SS doses (Mg ha^{-1})				
	NC	0	8	15	23	NC	0	8	15	23	NC	0	8	15	23
	%					%					%				
18	0.30±0.01a	0.71±0.01b	0.81±0.01b	0.82±0.01b	0.88±0.01b	0a	0.01±0.00a	0.02±0.01ab	0.02±0.02b	0.06±0.01c	20.2a	23.1±0.5a	21.9±0.5a	21.3±0.4a	23.2±1.0a
21		0.47±0.03ab	0.66±0.01ab	0.71±0.01bc	0.96±0.01c		0.04±0.01ab	0.09±0.01c	0.06±0.01bc	0.08±0.01c		24.2±0.5a	25.9±0.1a	25.9±1.0a	32.2±1.0a
24		0.40±0.01ab	0.65±0.03bc	0.75±0.03cd	0.98±0.01d		0.01±0.00a	0.06±0.01ab	0.13±0.03b	0.10±0.01b		35.1±0.0ab	41.8±1.0ab	41.5±0.5b	35.5±4.3a
27		0.52±0.01ab	0.59±0.01b	0.88±0.01c	0.87±0.03c		0.01±0.00a	0.10±0.01b	0.11±0.01b	0.14±0.02c		35.6±0.5b	40.2±0.1c	37.4±4.0b	34.5±5.0ab
34		0.27±0.01b	0.51±0.01ab	0.55±0.01ab	0.87±0.07c		0.01±0.00ab	0.01±0.00ab	0.10±0.04c	0.15±0.05d		36.9±0.2bc	44.3±1.1d	35.3±0.3b	41.0±1.7db

Significant differences ($p < 0.05$) are indicated by different lowercase letters. NC = negative control.

Chromosomal aberrations (CA) in *A. cepa* statistically increased with SS doses, however in all the application doses a time-dependent decrease was observed (Table 3). According to Natarajan (2002) chromosome aberrations can be caused by genotoxic agents, to which many organisms, man included, may be exposed. Thus, the evaluation of chromosomal aberrations as *end-points* of chemical agents has been widely used in environmental monitoring. However, observed values for CA in the SS amended soils can be considered low, ranging from 0.27 to 0.98% with a mean of 0.69%. The research by Caritá and Marin-Morales (2008) showed the values of CA up to 2.63% while Mazzeo et al. (2015) and Martins et al. (2016) reported higher CA values of 9–10% with different SS types and soils. It should be taken into account that the total frequency of CA may indicate the genotoxicity of the compounds present in the tested samples but not the mechanisms of action of these under the genetic material inside the cell. An analysis differentiating each type of chromosomal alteration can better inform about the effects of the compounds on DNA. For instance, chromosomes breaks can be derived from microtubule problems (mitotic spindle structure), which are responsible for the correct segregation of the chromosomes into the daughter cells. However, this type of aberration was rarely found in the present study, indicating that substances present in soil treated with SS did not directly interfere with mitotic spindle. Another fact that can also confirm that the SS tested in this study does not contain substances that are able to interfere with the microtubules, is the observed low frequencies of polyploid and binucleated cells. In the present study, the most frequently observed alteration was chromosome bridges, present in both the anaphase and telophase (Fig. 2). Anaphasic bridges can be formed as a consequence of chromosome adhesion as already observed by Caritá and Marin-Morales (2008) in samples coming from SS amended soils.

Even mutagenic effects were observed in very small amounts, with micronuclei ranging from 0.01–0.20% with a mean of 0.06% (Table 3). At increasing doses of SS micronuclei frequency statistically increased, with an effect that appears particularly enhanced at the highest SS doses. However, even in these cases, values were generally low. Caritá and Marin-Morales (2008) reported values of MN up to 1.31% while Mazzeo et al. (2015) and Martins et al. (2016) reported CA values up to 5% with different SS types and SS amended soils. In our study, the observed micronuclei may have been a consequence of chromosomal fragments resulting from chromosomal bridges. As a matter of fact, data showed a significant correlation between the frequencies of CA and MN, a relationship that was confirmed by MN analysis in F1 cells (non-meristematic region), which showed that meristematic cells carrying CA progressed to micronucleated cells.

The cytotoxic effects, evaluated by the frequency of mitotic index, showed that there were no significant differences between samples treated with different SS doses until 21 months (Table 3). After this period, the MI increased showing a statistically higher value if compared to the negative control (NC) but without statistically significant differences when compared to soils without SS addition. Additionally, in all the investigated samples, the so-called cytotoxic limit value (50%), *i.e.*, the value responsible for lethal effects (Panda and Sahu, 1985) was not exceeded. For all such reasons, the higher mitotic index observed in all the samples when compared to NC may have been related to an increase in organic matter and nutrients rather than to toxic effects. As the growth of the plant occurs by cellular divisions, this may explain the increase in the mitotic level and related index. Overall, the results showed that the SS doses did not cause cytotoxic effects while promoting an increase in the amount of mitosis. This indicated that under the experimental conditions, this test was not very effective in assessing cytotoxic effects as a function of SS doses. The results were more influenced by other factors, such as OM and nutrient contents, rather than PTE.

On the whole, the tests with *A. cepa* provided the following information: *i*) generally speaking, very low values for all three investigated effects even if, at increasing SS doses, some genotoxic and mutagenic effects have been observed, with the firsts statistically characterized by a time-dependent decrease; *ii*) the absence of cytotoxic effects; and *iii*) a generally higher sensibility to SS when compared to *D. magna*, *P. subcapitata*, and *L. sativa*.

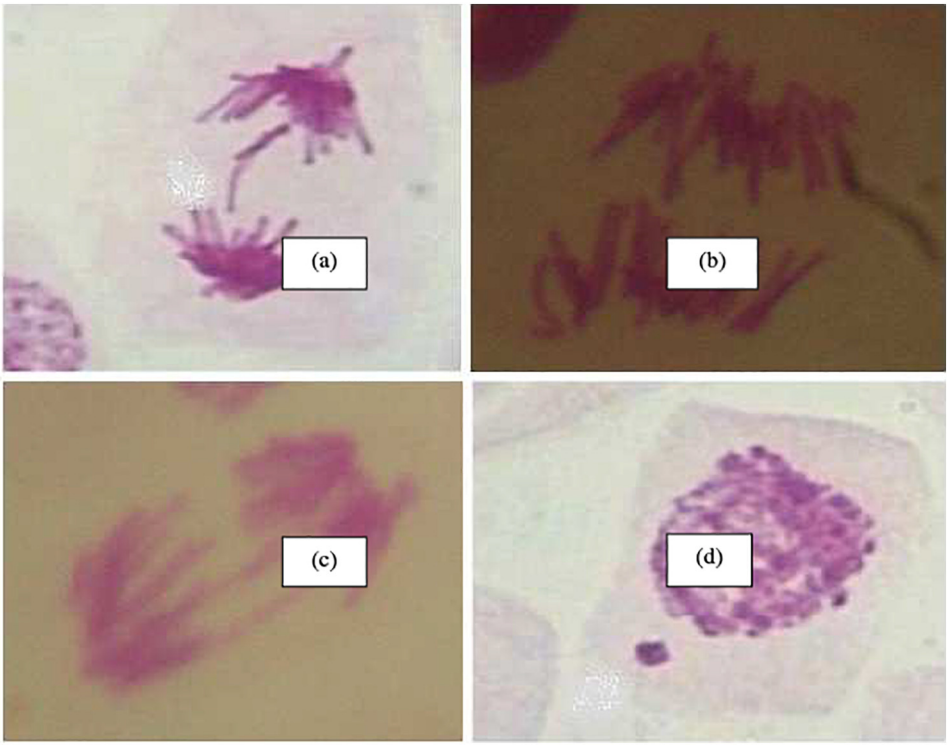

Fig. 2. Genotoxic and mutagenic effects in *A. cepa* cells. (a) and (b) Chromosomal breaks; (c) chromosomal bridge and (d) micronucleus.

4. Multivariate statistics

4.1. Sparse partial robust M-regression (SPRM)

For each of the six bioassay indexes, three different graphs have been elaborated (Figs. 3–4): *i*) the standardized regression coefficients, in order to understand which variables were significant; *ii*) the biplot of the first two PLS components, in which the correlations between bioassay and PTE are shown. In this representation bioassay variables are projected into the plots as rays (in red), which point at the direction of the largest variation of the variables. PTE are shown with their abbreviations (in red), and the numbers (blue) represents the collected samples (see the legend). The orthogonal projection of the scores on the rays informs about the magnitude and sign of the contributions; and *iii*) the measured response *versus* its prediction, explaining what about the statistical reliability of the investigated model.

Fig. 3 shows the three graphs for bioassays used to investigate ecotoxicity. For LC_{50} *D. magna*, regression coefficients show that relatively few variables among the PTE (Fe-DTPA and -total recoverable, Zn-DTPA and -M, Pb-DTPA and -M, Ni-M and -total recoverable) and collected samples (8 among 40) were significant. In the biplot, by considering correlations among significant variables only (PTE *vs* collected samples), the strongest positive correlations were: Fe-DTPA and -total recoverable, Pb-DTPA, Ni-total recoverable *vs* treatments with 0 and 8 Mg ha^{-1} SS and collected after 21 and 24 months (samples number 4, 6, and 16). For IC_{50} *D. magna*, regression coefficients showed a higher number of significant variables for both PTE (Mn-DTPA and -total recoverable, Fe-DTPA and -total recoverable, Ni-DTPA -M and -total recoverable, Zn-DTPA and -M, Cd-DTPA and -total recoverable, Pb-M and -total recoverable) and collected samples (13 on 40). The biplot showed that strongest correlations were between: Mn-DTPA and -total recoverable, Fe-DTPA and -total recoverable *vs* samples with no SS and collected after 18 and 21 months (samples 2, 3, and 4); Ni-DTPA and -total recoverable *vs* samples with 0, 8, and 15 Mg ha^{-1} SS and collected after 15, 24, and 27 months (number 13). For the root growth of *L. sativa*, regression coefficients showed that significant variables among PTE were the Fe Ni, and Mn forms (DTPA, -M, and -total recoverable), Cu-DTPA, Cd-DTPA and -total recoverable, Cr-total recoverable while among collected samples 13 over 40 were significant. The biplot showed the following positive correlations: all the Fe forms *vs* samples without SS and collected after 18, 21, and 34 months (samples 2, 3, 4, 9, 10, and 11); all the Mn forms *vs* samples with 0, 8, and 15 Mg ha^{-1} SS and collected after 18, 24, and 34 months (samples 6, 16, 20, and 22). In all the three investigated cases, predicted responses seem to well fit measured one, showing a good statistical reliability of the investigated model. Summarizing, all the three investigated bioassay seems to be mainly influenced by Ni and Fe with a decreasing exotoxicity with increasing concentration in SS. Other PTE such as Cd, Mn, Pb, and Zn seemed to play a less important role, while Cr and Cu a negligible one at least in terms of ecotoxicity.

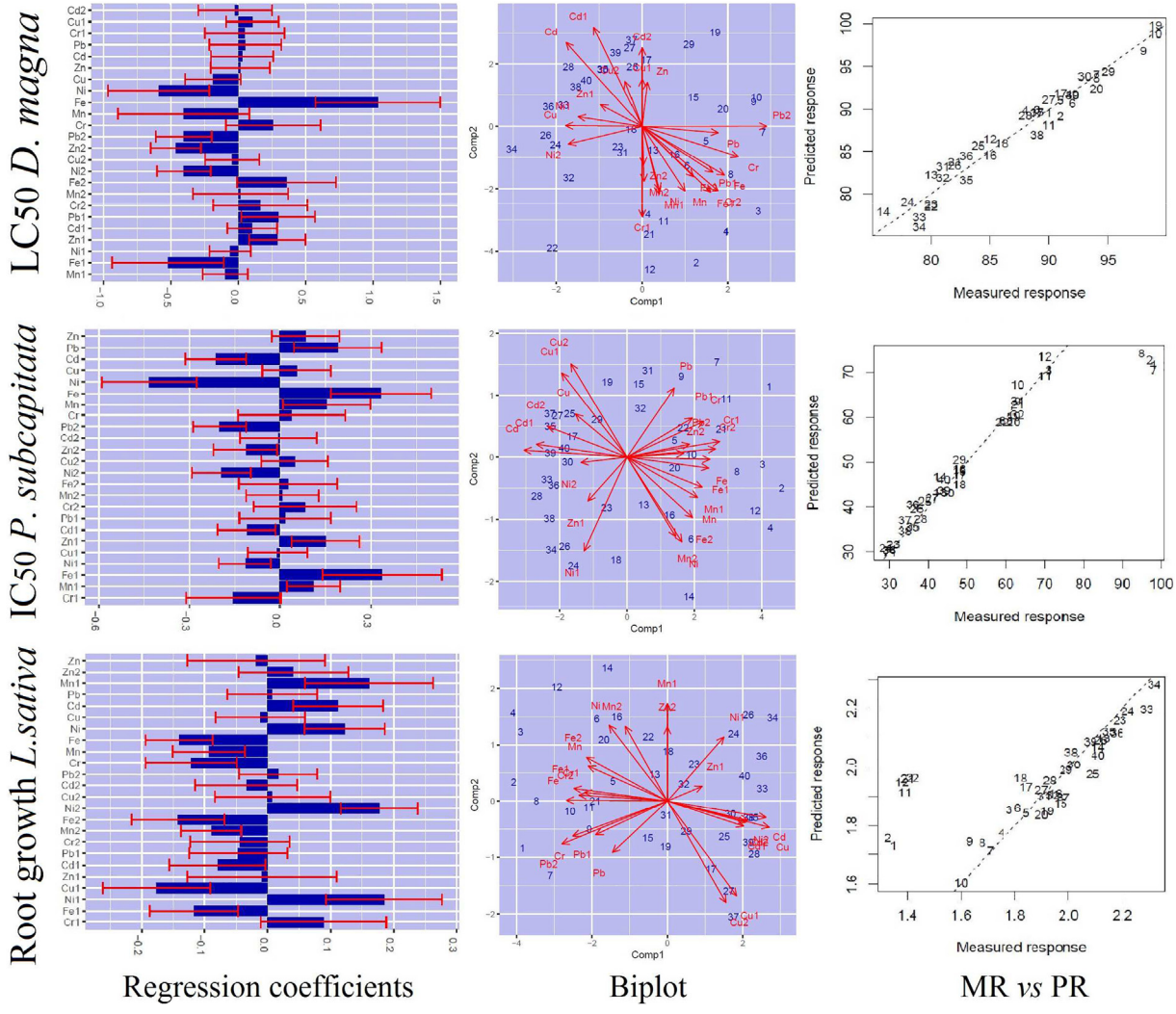

Fig. 3. Sparse partial robust M-regression (SPRM) coefficients, biplots, and predicted *vs* measured responses for ecotoxicity.

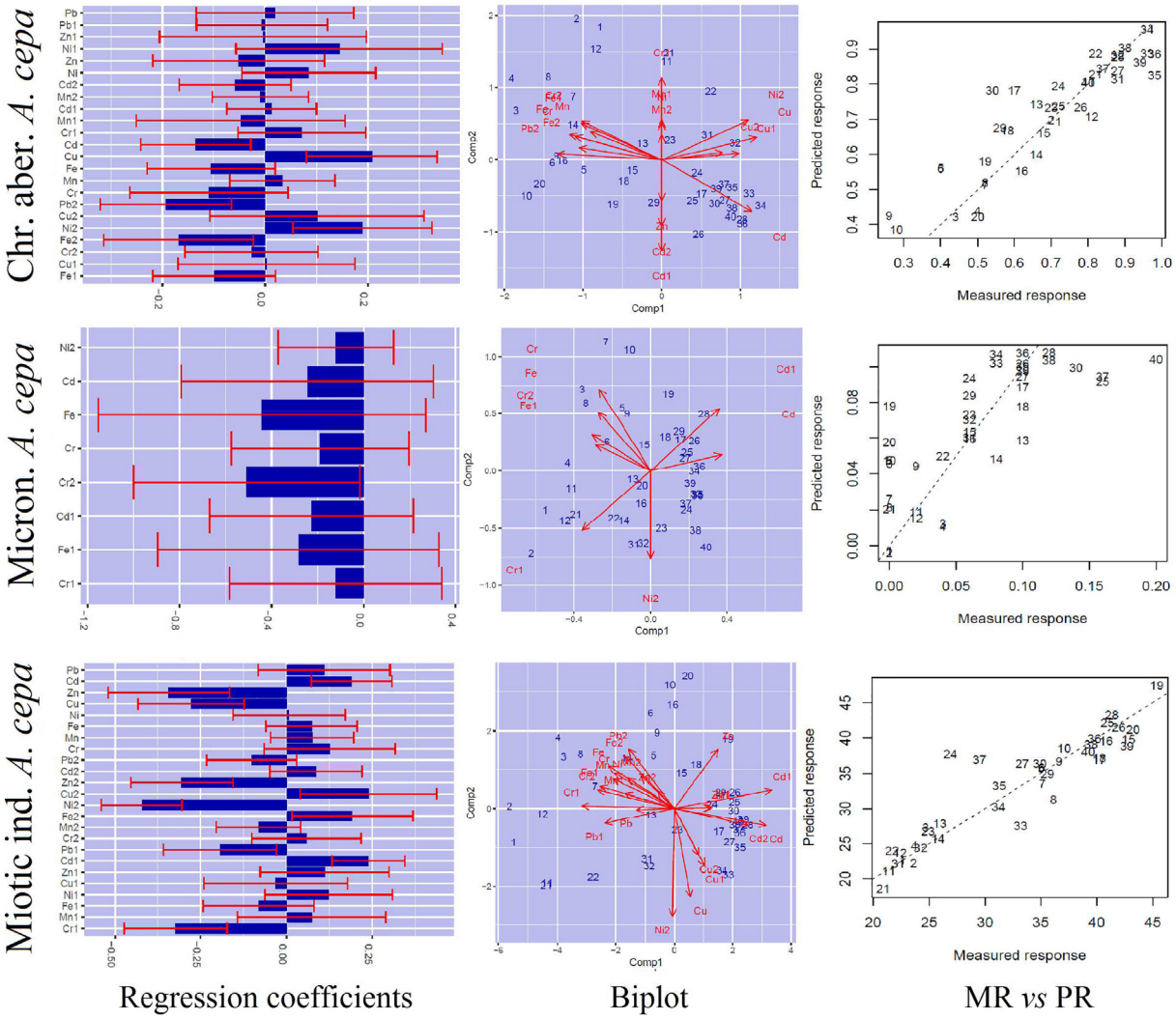

Fig. 4. Sparse partial robust M-regression (SPRM) coefficients, biplots, and predicted *vs* measured responses for genotoxicity.

Fig. 4 showed the graphs for indexes from *A. cepa* as clues of genotoxicity. For chromosomal aberrations (CA), regression coefficients showed which were significant variables among the PTE (Fe-M, Ni-M, Pb-M, Cu- and Cd-total recoverable) and collected samples (5 among 40). The biplot showed both positive (meaning that CA increases) and negative (CA decreases) correlations, with the strongest respectively as: *i*) Pb- and Fe-M *vs* samples with no SS and collected after 21, 24, and 27 months; and *ii*) negative correlations were Cd-total recoverable *vs* samples with no SS and collected after 21–27 months, Ni-M and Cu-total recoverable *vs* samples with no SS and collected after 24 months. For micronucleous, regression coefficients showed that only one variable for PTE (Cr-M) and two for collected samples (2 and 4) were significant. The biplot showed that there was only one significant correlation (positive): Cr-M *vs* samples with no SS and collected after 18–21 months. For miotic index, several variables for both investigated PTE (Cr-DTPA and -M, all the Ni forms, Pb-M and -total recoverable, Cu-M, Cd-total recoverable, Mn-DTPA) and collected samples (10 over 40) were significant. Accordingly, the biplot shows several positive and negative correlations, respectively: *i*) Pb-total recoverable and -M, Cr- and Mn-DTPA, Ni-total recoverable *vs* samples with 0, 8, and 15 Mg ha^{-1} SS and collected after 18, 21, and 27 months; and *ii*) negative correlations were Cd-total recoverable, Ni-DTPA *vs* the same samples of the previous reported correlation (*i*); Ni- and Cu-M *vs* samples with 8 Mg ha^{-1} SS and collected after 27 and 34 months. Predicted responses fitted well the measured one, with the exception of micronucleous with the graph showing a more dispersed distribution. On the whole, as for ecotoxicity, even genotoxicity appeared to be mainly influenced by Ni while, only secondary, by Pb. However, while for ecotoxicity an inverse correlation was observed (at increasing Ni concentration, ecotoxicity decreased) with genotoxicity the opposite was true (at increasing Ni concentration, genotoxicity increased).

On the whole, SPRM confirmed a general low eco- and genotoxicity with just few PTE playing a negative role (only in term of genotoxicity), which was however connected at some extremely specific phase along the investigated 3-years cycle. Additionally, such statistical elaboration confirmed a generalized higher sensibility of *A. cepa* to PTE if compared to all the bioassays tested for ecotoxicity (*D. magna*, *P. subcapitata*, and *L. sativa*).

4.2. Principal factor analysis (PFA)

The eigenvalues of the four extracted factors (Table 4) after the matrix rotation were >1, and the factors could be grouped into a four-component model accounting for 86.4% of all data variation. F1, representing alone an impressive 55.3% of the total variance, extracted several PTE in both their available (Ni, Zn, Cd, Fe) and total recoverable forms (Ni, Zn, Cd, Fe, and Mn), root growth (Root), miotic index (MI), and micronucleous (MN) as all positively concordant, whilst they

Table 4
Factor loadings of a factor analysis ($n = 40$); Extraction Method: principal factor analysis (PFA); Rotation Method: Varimax; bold loadings >0.5.

	F1	F2	F3	F4
Cr-D	−0.036	**0.562**	**0.548**	**0.511**
Mn-D	0.149	−0.048	**0.523**	0.483
Fe-D	0.188	0.468	**0.599**	**0.529**
Ni-D	**0.732**	0.133	0.199	0.469
Cu-D	0.361	0.387	−0.088	**0.788**
Zn-D	**0.656**	0.139	0.086	**0.597**
Cd-D	**0.728**	0.295	−0.169	**0.569**
Pb-D	0.008	**0.748**	0.221	0.490
Cr-M	0.092	**0.582**	0.343	**0.595**
Mn-M	0.350	**−0.855**	0.240	0.164
Fe-M	**0.697**	−0.013	0.129	**0.626**
Ni-M	0.361	0.253	0.160	**0.779**
Cu-M	0.257	0.435	−0.135	**0.810**
Zn-M	0.272	0.247	0.334	**0.756**
Cd-M	**0.581**	0.394	−0.066	**0.651**
Pb-M	0.336	**0.877**	0.099	0.233
Cr-H	0.429	**0.841**	−0.158	0.189
Mn-H	**0.857**	0.236	0.018	0.192
Fe-H	**0.526**	**0.784**	−0.199	0.163
Ni-H	**0.904**	0.058	0.291	−0.083
Cu-H	0.422	0.393	0.045	**0.760**
Zn-H	**0.700**	0.474	−0.098	0.334
Cd-H	**0.764**	0.369	−0.061	0.454
Pb-H	0.354	**0.875**	−0.066	0.237
LC50	−0.160	0.278	**−0.893**	−0.061
IC50	**−0.848**	−0.097	−0.285	−0.302
Root	**0.782**	0.154	0.425	0.231
MI	**0.626**	0.084	**−0.568**	0.093
CA	0.195	−0.172	0.306	**0.798**
MN	**0.717**	0.013	−0.246	0.361
Proportional variance	55.3	13.7	11.7	5.7
Cumulative variance	55.3	69.0	80.8	86.4
Eigenvalues	16.593	4.119	3.514	1.706

D = extraction with DTPA; M = extraction with Mehlich-1; H = extraction with HNO_3 + HCl.

were inversely correlated with IC50. It seems that such bioassays were mainly influenced by Ni, Zn, Cd, and Fe with particular reference to their total recoverable forms (higher loading values). As a matter of fact, this factor showed that at increasing concentrations there was an increase in cytotoxic (miotic index, MI) and mutagenic (micronucleous, MN) effects with, however, a decrease in terms of the whole ecotoxicity (root growth increased, IC50 decreased). While this could look as a contradiction, this represent a confirmation of what previously observed with SPRM elaborations, *i.e.*, ecotoxicological tests with *D. magna*, *P. subcapitata*, and *L. sativa* are less sensitive to treatments with SS respect to those with *A. cepa*. Thus, F1 can be interpreted as the "relative sensitiveness of bioassays to PTE".

When compared to F1, all the other three extracted factors are, in terms of variance and statistical significance (eigenvalues strongly decreased), of marginal importance. F2 (13.7% of the variance) showed that at increasing total recoverable Pb and Cr their available forms increased accordingly, while there was a decrease in available Mn. This factor simply showed some already known geochemical affinity between these elements. Consequently, this factor can be interpreted as "geochemical affinity among the investigated PTE".

F3 and F4 were quite similar, at least in terms of interpretation, to F1. Such factors help us to complete the interpretation previously reported in F1; indeed, while we previously stated that ecotoxicological tests with *A. cepa* were particularly sensitive to the total recoverable forms of PTE, and more sensitive to those conducted with other bioassay, in these last two factors we can additionally underline their less sensitiveness to PTE available forms.

Summarizing, the PFA demonstrated that: *i*) bioassay with *A. cepa* are much more sensitive to those with *D. magna*, *P. subcapitata*, and *L. sativa*; and *ii*) this is particularly true when related to PTE total recoverable forms.

5. Conclusions

This study represents the first research conducted to investigate the toxicity of SS on the pedoenvironment, by the integrated means of PTE speciation and a battery of bioassays, under the realistic open field conditions.

As expected, PTE contents in SS amended soils seemed to increase with SS application doses; however: *i*) this cannot be assumed as a general rule in terms of both investigated extraction methods or detected PTE; *ii*) in all the investigated treatments, PTE concentrations were always consistently below soil natural background concentrations, as well as quality reference values those of natural soils worldwide.

In terms of absolute values, all bioassays showed a generalized low eco- and genotoxicity of SS. In terms of applied doses and time: *i*) toxicity increased with increasing SS doses; *ii*) such an adverse effect diminished over time. In terms of bioassays sensitiveness both SPRM and PFA confirmed that *A. cepa* was the more sensitive one followed by *P. subcapitata* > *D. magna* > *L. sativa*.

As a general conclusion, these results indicate that under realistic open field conditions environmental risks of SS are lower than previously reported from laboratory or greenhouse experiments, due to a time-dependent decrease in metal toxicity. It is suggested that studies in open field conditions should be strongly encouraged in the future to further assess environmental risks of SS application in forestry or agriculture. Indeed, even if a key factor will be always represented by the quality and preparation of the applied SS, these studies are critical in order to determine the toxicity of SS in fields.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.11.334.

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