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ECOTOXICITY AND GENOTOXICITY OF STEEL SLAGS: PRELIMINARY RESULTS

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ABSTRACT

The European iron and steel industry produces a considerable amount of waste and by-products. Also in Italy the steel slag production is very high. Steel slags may be reused in recycled materials, such as materials for the construction industry, road base and asphalt mixtures, allowing to reduce the final disposal in a landfill. The reuse of this recycled material may generate potential release of toxic compounds for the environment and humans. The aim of this study was to assess the ecotoxicity and genotoxicity of steel slags by using an integrated chemical-biological approach. Chemical analysis of leachates obtained by using short-term leaching tests (UNI EN 12457-2) were performed, to evaluate the waste potential reuse according to the Italian legislation (Ministerial Decree 186/2006). Moreover, solutions obtained from leaching tests were assayed by using ecotoxicity tests on plant and animal organisms and genotoxicity tests on bacteria, plant and human cells. Chemical analyses of the eluates were within the limits of the Italian legislation. The preliminary results of the ecotoxicity and the genotoxicity tests demonstrated that this material has a low toxicity and therefore its potential use as a recycled material.

1. INTRODUCTION

The production chain of cast iron and steel generates scrap called steel slag (SS). Two types of slags are mainly generated from the iron and steelmaking industries: basic-oxygen furnace (BOF) steel slags and electric-arc furnace (EAF) steel slag. BOF-SS are residues from the basic oxygen converter, where steel is generated by the pig iron by injecting pure oxygen. EAF-SS are generated in high-power electric arcs where high quality steel is produced by melting recycled steel scrap (Chaurand et al., 2007; Yildirim & Prezzi, 2011). Thus, the production of SS is a result of the steel composition and of the steel production process (Yüksel, 2017).

In Europe, every year, more than 45 million tons of slag from both integral cycle furnace slabs and from electric furnaces are produced (European Slag Association and European Steel Association, 2012). The Italian production is about 6.5 million tons (Federacciai, 2012). According to the European regulations on wastes, this huge amount of material must be reduced in order to save primary resources and enhancedwaste disposal minimization. As a consequence, steel slag recovery is becoming a debated problem (Piatak, Parsons, & Seal, 2015).

Due to the mechanical and physical properties of steel, various applications can be implemented: in the construction industry, as in concrete (Rondi et al., 2016), or in road construction such as road base and asphalt mixtures (Sorlini, Sanzeni, & Rondi, 2012). Moreover, SS can be assimilated to natural hard rocks due to the composition similar to natural aggregates. In fact, SS are characterized mainly by oxides of calcium, iron, silicon, aluminum, magnesium, and manganese (Yüksel, 2017).

The classification of slag as a by-product or waste is variable within the European member states (Euroslag, 2018). The US Environmental Protection Agency classifies iron and steelmaking slags as non-hazardous considering their ignitability, corrosivity, reactivity and toxicity (National Slag Association, 1980). Despite that, according to steel production process characteristics (i.e. cooling conditions, blast furnace charges and temperatures), SS can also contain potentially toxic elements, such as chromium,

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molybdenum, vanadium (Komonweeraket, Cetin, Aydilek, Benson, & Edil, 2015; Primavera, Pontoni, Mombelli, Barella, & Mapelli, 2016; Tossavainen et al., 2007). Moreover, the SS industry remains for a long time in the environment and therefore has a prolonged contact with environmental mixtures such as deep water and soil. It may promote the release (for example through chemical weathering) of substances potentially dangerous both for the environment and for human health (Primavera et al., 2016).

Many mutagenic/genotoxic substances can be present and accumulate in the aquatic and soil environments, and have adverse effects on biocenosis and can affect humans through drinking water (Ceretti et al., 2016; Guan et al., 2017), surface water (Ohe, Watanabe, & Wakabayashi, 2004) and the food chain (Hamilton, Young, Bailey, & Watts, 2018). Consequently, the diffusion of these substances in water and soil could became a public health problem (Drzeżdżon, Jacewicz, & Chmurzyński, 2018; Rashtian, Chavkin, & Merhi, 2019).

The increasing presence of mutagenic/genotoxic pollutants in the environment has caused concern regarding the potentially harmful effects of xenobiotics on human health. Mutagenic compounds are very dangerous pollutants, since their effects may induce damage on an individual level as well as its progeny, throughout generations. Moreover, mutagenicity is related to carcinogenicity: the evidence of mutagenic activity suggests that a substance might be carcinogenic (Bajpayee, Pandey, Parmar, & Dhawan, 2005; Eastmond et al., 2009).

To date, Italian law requires the chemical analysis of these materials, by using short-term leaching tests in the case of re-use as supplementary cementitious materials or aggregates in concrete (Ministerial Decree n. 186, 2006). However, short-term biological tests allow an overall assessment of the analysed samples, that detect synergistic effects of complex mixture components and also predict the risks for the environment and for human health (Eastmond et al., 2009; Kirkland, Aardema, Henderson, & Müller, 2005). In particular, ecotoxicity, mutagenicity and genotoxicity tests are useful tools to predict and prevent risks due to the presence of toxic substances in the environment. The application of these assays in different cells/organisms allows for the assessment of mutagenic hazards for the environment and for humans (Escher & Fenner, 2011; Kirkland et al., 2005).

The aim of this study was to assess the ecotoxicity and genotoxicity of steel slags using an integrated chemical-biological approach, schematically summarized in Figure 1.

2. MATERIALS AND METHODS

2.1 Materials

Four samples of steel electric arc furnace (EAF-C) slags collected from different northern Italian steel-making plants were employed in this study. All the samples were stored from the factories in open areas and each sample collected was considered representative of the whole material storage.

Particle size ranged from 1 to 5 cm, whereas the density ranged between 2.5 and 3.3 g/cm^3.

2.2 Methods

Chemical analysis and biological assays were performed for all the samples.

Leaching tests according to the legislation (Standard CEN EN 12457-2, 2004) were performed on grinded steel slag samples.

Leachate solutions were assayed by using ecotoxicity tests on plant and animal organisms and genotoxicity tests were done on bacteria, plant and human cells.

In particular, the toxicity was tested through the investigation on the crustacean *Daphnia magna* (Standard UNI EN ISO 6341, 2013) and the common onion *Allium cepa* (G Fiskesjö, 1995; Ma et al., 1995). Mutagenicity in bacteria was evaluated by Ames test (Maron & Ames, 1983) and genotoxicity in plant cells was assessed by using micronuclei test in *Allium cepa* (Ma et al., 1995). Moreover, the micronuclei frequency in human leukocytes was studied (Fenech, 2000).

2.2.1 Leaching tests

Leaching tests were performed on naturally dried slag samples according to the CEN regulations (Standard CEN EN 12457-2, 2004), in order to quantify the leachable fraction of the wastes in water. Tests were performed by mixing the homogenized sample with demineralized water at a liquid to solid ratio of 10 l/kg. The mixture was placed on a tightly closed rotary shaker and agitated for 24 h, rotating at 10 ± 2 rpm. All the steel slags were previously subjected to a crushing treatment and a sieving process in order to obtain a particle diameter below 4 mm and the temperature was in compliance with the Directive (about 20°C). The solutions were filtered through 0.45 µm filters.

Chemical analyses of the leachate solutions were performed according to the Italian legislation for the recovery of non-hazardous waste (Ministerial Decree n. 186, 2006). Nitrates, fluorides, sulphates, and chlorides were analyzed through ion chromatography (ICS 1000, Dionex). Metals were measured by means of an optical plasma spectrometer (Optima 2000 DV, PerkinElmer) and cyanides were detected by using the colorimetric method DIN 38 (Nanocolor 400D).

2.2.2 Daphnia magna ecotoxicity test

A preliminary short-term acute test was performed on eluates obtained from the leaching tests using Daphtoxkits F (Ecotox LDS). The assays were conduced according to the European Directive (Standard UNI EN ISO 6341, 2013) and by following the manufacturer's instructions.

As a first step, in order to assess the toxicity of each sample, eluates were tested without any dilution. Totally, 20 neonates of *D. magna* (<24-h-old) were used for each slag leachate solution. Effects on crustacean movements or death were observed after 24 and 48 h of contact.

Eluates that resulted more toxic, were diluted (100%, 50%, 25%, 12.5%, 6.25%) in order to assess the lowest toxic concentration. For each dilution, 20 neonates were observed after 24 and 48 h of incubation.

Standard freshwater was used to dilute eluates and as well as a negative control in every test.

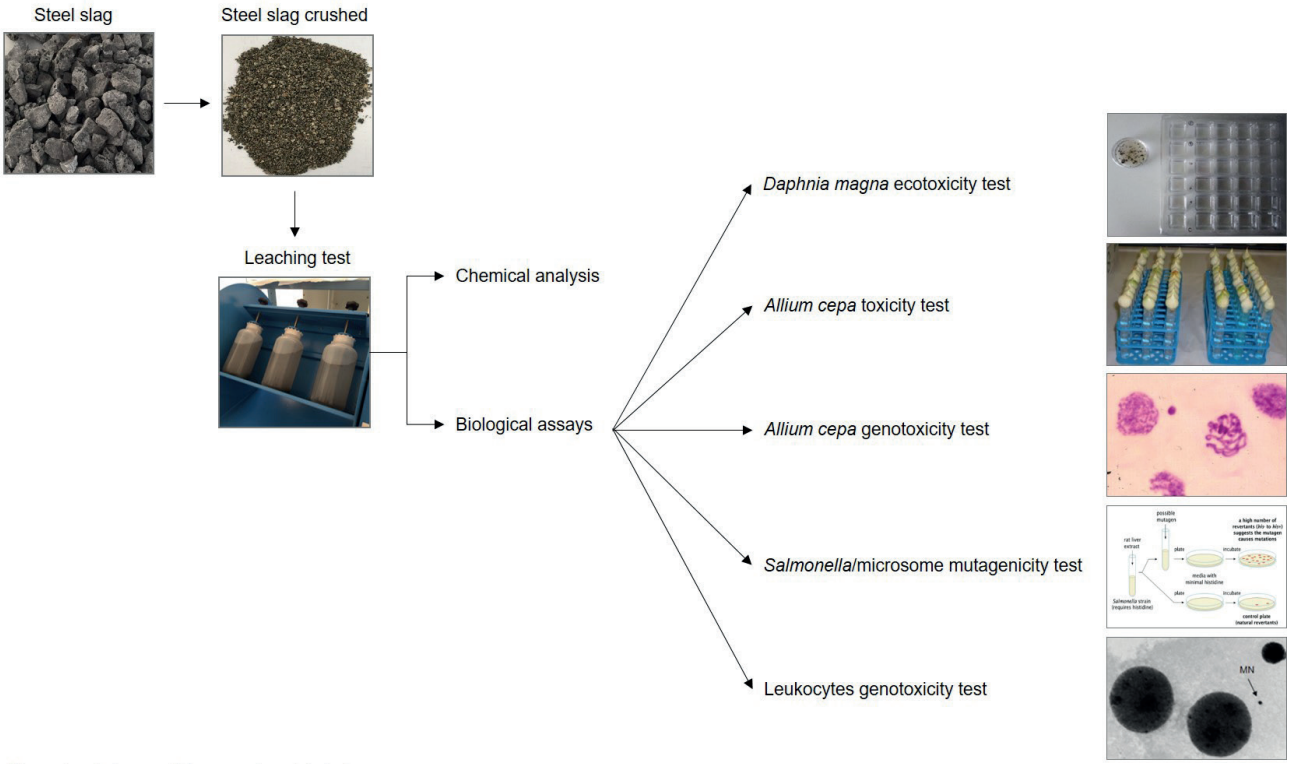

FIGURE 1: Scheme of the experimental design.

2.2.3 Allium cepa toxicity test

In a toxicity assay, 12 equal-sized commercial onion bulbs of A. cepa were cleaned and washed without destroying the primordial roots. They were exposed for 72 h in the dark to different dilutions of leachates (undiluted, 1:2, 1:10, 1:20, 1:100, 1:200 and 1:1000). Distilled water was used to dilute the samples and as a negative control. The roots mean length was used to calculate the EC50 value (Fiskesjö, 1985; Fiskesjö, 1995). Turgescence, consistency, colour change and root tip shape were also used as toxicity indexes.

2.2.4 Allium cepa genotoxicity test

The micronuclei (MN) *Allium cepa* test was performed by using six equal-sized young onion bulbs (2–2.5 cm in diameter) per sample. After 48-h pre-germination in a saline solution, the bulbs were exposed for 24 h to undiluted samples based on the results obtained in the toxicity test. They were then replaced in the saline solution (Rank's solution) for 44 h of recovery time. The roots were cut, fixed in 1:3 acetic acid-ethanol for 24 h and finally stored in 70% ethanol. Staining with acetic orcein was carried out on roots. Rank's solution was used as a negative control. A positive control was performed using maleic hydrazide (10^{-2} M, 6-h exposure) to ensure the effectiveness of the assay. Five roots of each sample were considered for the microscopic analysis (1,000X magnification): 5,000 cells (1,000 cells/slide) were scored for the mitotic index (MI), as a measure of the cellular division and therefore of sample toxicity; 10,000 cells (2,000 cells/slide) were scored for the MN frequency. All the *Allium cepa* experiments were performed in duplicate.

2.2.5 Salmonella/microsome mutagenicity (Ames) test

The leachates from steel slags were tested at increasing doses (2.5, 25, 125 and 250 µl/plate) using *Salmonella typhimurium* TA98 and TA100 strains, with and without *in vitro* metabolic activation (±S9) to detect direct and indirect mutagenic compounds. The experimental procedure was the standard plate incorporation method (Maron & Ames, 1983). The Salmonella TA98 strain detects frame-shift mutagens and the TA100 strain responds to base-pair substitution. Positive controls were 2-nitrofluorene (10 µg/ plate; more than 1,000 revertents/plate) and sodium azide (10 µg/plate; more than 1,000 revertents/plate) for the TA98 without S9 and the TA100 without S9, respectively, and 2-aminofluorene (20 µg/plate; more than 1,000 revertents/plate) for both strains with S9. The distilled water was a negative control. The results were espressed as a mutagenicity ratio (MR) obtained by dividing the revertants of each sample (computed by means of three replicates) by the spontaneous mutation rate (negative control). The test results were considered positive if two consecutive dose levels or the highest non-toxic dose level produced a response at least twice that of the negative control and at least two consecutive doses showed a dose-response relationship (APHA, 2012; Mortelmans & Zeiger, 2000).

2.2.6 Leukocytes genotoxicity test

MN frequency is a biomarker of early genetic effects which is often used in human biomonitoring studies. The MN frequency analysis was obtained with the cytokinesis-block micronucleus (CBMN) assay. The CBMN assay was performed according to the standard protocol de-

scribed by Fenech (Fenech, 2000). Briefly, whole blood was collected from a healthy donor and maintained at 37°C in darkness. Cultures were set up by adding 0.2 ml of whole blood to 2 ml of RPMI-1640 medium (Gibco), supplemented with 10% fetal bovine serum (FBS), 1% phytohaemagglutinin (PHA), 1% glutamine and antibiotics (penicillin: 10,000 U/ml; streptomycin: 10,000 g/ml (Biochrom)). After 24 h of PHA stimulation, leaching solutions were added and after 24 h of exposure, cytochalasin B (Sigma, Steinheim, Germany) was added at a final concentration of 4.5 µg/ml. Cells were harvested at the 72th hour after hypotonic treatment (0.5% KCl, 7 min) and fixed with the Carnoy's fixative 3:1 (methanol:acetic acid) with multiple changes till a clear pellet was obtained. Slides were prepared by the air-dry method and stained with 5% Giemsa for 20 min. With the CBMN assay, the frequency of MN in binucleated cells was measured. In this preliminary study, only the results obtained from the single dose of 2.5 µl of leachates are reported.

3. RESULTS AND DISCUSSION

The steel slag composition is reported in Table 1, in terms of leachable fraction derived from chemical analysis. All the four steel slag samples were characterized by similar concentration of the analyzed compounds. pH values indicated high alkalinity, as seen in the literature (Astrup, Mosbaek, & Christensen, 2006; Gomes et al., 2017). Sample A evidenced higher contents of nitrate, chloride, barium and vanadium with respect to other samples whereas sulphate concentrations were higher in sample B. Vanadium is a common critical element in steel slag, because it is a transition metal typically used in the metal alloy industries and can generate carcinogenic effects in humans (Gomes et al., 2017; IARC, 2006). Sulphate concentrations may affect the possible reuse of this waste in the building field from a mechanical point of view. Indeed, cracks and expansion problems can appear in concrete under sulphate attack (Ali et al., 2011). Another potentially toxic element commonly present in the steel slag leachate solution is chromium (Sas, Głuchowski, Radziemska, Dzięcioł, & Szymański, 2015). Sample C presents the highest concentration of this element (29 µg/l), although it is significantly below the limit value of 50 µg/l. Overall, the chemical analysis of all eluates obtained in our study were within the reference values required by the Italian decree for the recovery of waste (Ministerial Decree n. 186, 2006).

In this study, solutions obtained from leaching tests of four steel slags were assayed by using ecotoxicity tests on plant and animal organisms and genotoxicity tests on bacteria, plant and human cells.

Toxicity tests on steel slag leaching solutions through *D. magna* and *A. cepa* were performed.

Data obtained from the *Daphnia magna* toxicity test are summarized in Table 2. The validity criteria of the assay was respected, where a percentage of immobilization of the controls below 10%.

Preliminary results on crustacean immobilization using undiluted SS leaching solutions revealed differences among the samples: neither after 24 hours nor after 48 hours of observation, did samples A and B show toxic effects. Sample C resulted toxic with a mortality rate of 100% after 24 hours. Sample D showed an increase of immobilization from 30% (after 24 hours) to 75% (after 48 hours). According to the European Standard (UNI EN ISO 6341, 2013), a more detailed test was carried out on samples C and D using different dilutions. Sample C caused a high level of immobilization (90% after 48 hours) even at the lowest concentration (6.25%), while sample D showed a clear dose-response relationship between the sample concentration and Daphnia neonate immobilization. Immobilization by samples C and D could have been due to high pH values. As indicated by the European Standard UNI EN ISO 6341, tests should be performed without pH modification because "an adjustment of pH can alter the nature of the sample" (UNI EN ISO 6341, 2013). However, if toxic effects are observed and the pH of the tested solution is outside the range of the organism survival, experiments can be performed by modifying the pH with no more than 5% of chemicals (UNI EN ISO 6341, 2013). Further investigations are planned to clarify this point.

The *Allium cepa* toxicity test revealed the absence of toxicity in *Allium cepa*. In fact, all samples (both undiluted and diluted) did not influence the length of the roots. Moreover, no other sign of toxicity, as turgescence, consistency, color change and root tip shape, was observed in macroscopic parameters. Due to the absence of toxicity in roots, the undiluted samples were assayed in the *Allium cepa* genotoxicity test.

The results of *Allium cepa* genotoxicity test are reported in Table 3. The mitotic index (MI) showed that the eluates did not negatively influence cell division: MI values of samples are very similar to the negative control value and this allowed to consider the data of the micronuclei. No sample induced a statistically significant increase in the frequency of MN, thus highlighting the absence of genotoxicity in these cells.

Results of the Ames test by using *Salmonella typhimurium* TA98 and TA100 strains expressed as a mutagenicity ratio (MR) are reported in Table 4. No mutagenic activity was found for the SS eluates sample at all doses tested.

Table 5 reports the results of the preliminary micronucleus (MN) test performed at the dose of 2.5 µl of SS leachates. Data are expressed as the frequency of mononucleated (MONO), binucleated (BN), multinucleated (POLY) and the number of MN in each binucleated cell (MN/BN). Since the MN/BN was below 3, which is the cut-off number for the genotoxicity identification, no genotoxicity effects were detected at the tested dose.

The limited number of samples did not allow the authors to perform a detailed statistical analysis.

To date little is known about the toxicity of substances potentially released by steel slags (Suh et al., 2014; Radić et al., 2013), moreover there are no studies about genotoxicity of these materials.

For these reasons, we focused on the integration of chemical analysis, requested and codified by the legislation (Ministerial Decree n. 186, 2006), with biological assays to describe in detail the effects of steel slags on the environment and in humans. This combined procedure rep-

TABLE 1: Chemical characterization of leachates from steel slag. n.d: not detected.

		Sample				
Parameters	**M.U.**	**A**	**B**	**C**	**D**	**Limit value DM 186/2006**
Concentration						
Nitrate	mg/l	5.0	1.1	1.7	1.9	50
Fluoride	mg/l	0.21	0.26	0.31	0.64	1.5
Sulphate	mg/l	1.0	26.2	7.0	9.7	250
Chloride	mg/l	9.2	5.7	4.8	5.8	100
Cyanide	mg/l	< 5	< 5	< 5	< 5	50
Barium	mg/l	0.27	0.16	0.17	0.23	1
Copper	mg/l	< 0.01	< 0.01	< 0.01	< 0.01	0.05
Zinc	mg/l	< 0.01	< 0.01	< 0.01	< 0.01	3
Beryllium	µg/l	< 10	< 10	< 10	< 10	10
Cobalt	µg/l	< 10	< 10	< 10	< 10	250
Nichel	µg/l	< 10	< 10	< 10	< 10	10
Vanadium	µg/l	228	157	68	189	250
Arsenic	µg/l	< 5	< 5	< 5	< 5	50
Cadmium	µg/l	< 4	< 4	< 4	< 4	5
Total Chromium	µg/l	< 10	< 10	29	12	50
Lead	µg/l	< 10	< 10	< 10	< 10	50
Selenium	µg/l	< 10	< 10	< 10	< 10	10
Mercury	µg/l	< 1	< 1	< 1	< 1	1
Asbestos	mg/l	n.d.	n.d.	n.d.	n.d.	30
COD	mg/l	< 15	< 15	< 15	< 15	30
pH	-	10.2	10.5	11.3	10.9	5.5-12

TABLE 2: Results of *Daphnia magna* toxicity test on undiluted solution (samples A and B) and on undiluted and diluted samples (samples C and D).

		Immobilization %	
Sample	**Concentration %**	**24h**	**48h**
A	100	0	5
	control	0	0
B	100	0	0
	control	0	0
C	100	95	100
	50	100	100
	25	100	100
	12.5	90	100
	6.25	10	90
	control	0	0
D	100	30	75
	50	5	20
	25	10	10
	12.5	0	5
	6.25	0	0
	control	0	0

Control solution: standard freshwater

TABLE 3: Results of *Allium cepa* genotoxicity test: mitotic index (MI) and frequency of micronuclei (MN) in *Allium cepa* roots.

Sample	**MI** (%)	**MN** (mean ± SD)
A	12.7	1.0 ± 2.2
B	12.7	1.4 ± 0.9
C	11.8	2.8 ± 3.7
D	13.0	2.0 ± 1.7
Negative control	11.4	0.6 ± 1.3
Positive control	8.1	19.3 ± 14.2

resents an innovative methodological approach that could be improved in the standard procedure for the analysis of SS slag destined for re-use.

In conclusion, chemical composition of all the leaching solutions assayed respects the limits of the Italian legislation on waste recovery. The preliminary results of the *Daphnia magna* test on the leaching solutions of these wastes indicate in general a low inhibition of mobility in the majority of the samples. However, since one sample resulted very toxic for these crustacea, more detailed experiments are in course regarding the effect of different pH and dilution factors of the solutions.

All samples showed the absence of toxicity (length of roots) and genotoxicity (MN frequency) in the root cells in

TABLE 4: Results of Ames test using *Salmonella typhimurium* TA98 and TA100 strains, expressed as mutagenicity ratio (RM).

Sample	Dose (µl/plate)	TA98 - S9 RM	TA98 + S9 RM	TA100 - S9 RM	TA100 + S9 RM
A	250	0.94	1.22	0.75	0.65
	125	1.34	1.16	0.61	0.76
	25	0.90	1.22	0.52	0.69
	2.5	1.16	1.30	0.66	0.82
B	250	0.71	0.84	0.88	1.07
	125	1.19	0.90	1.02	1.07
	25	1.21	1.13	0.90	0.90
	2.5	0.81	1.18	0.83	1.00
C	250	0.77	1.09	0.88	0.98
	125	1.13	1.43	0.94	0.77
	25	0.84	1.48	0.98	0.86
	2.5	1.06	1.24	0.90	0.85
D	250	0.69	1.36	0.69	0.91
	125	0.88	1.39	0.87	0.97
	25	1.17	1.09	0.86	0.93
	2.5	0.73	1.16	0.97	0.96

Negative controls are expressed as revertants/plate: 26.0±8.9 (TA98 – S9), 36.8±6.2 (TA98 + S9), 109.8±11.2 (TA100 – S9), 116.8±11.3 (TA100 +S9).

TABLE 5: Micronucleus test in human cells: frequency of mononucleated (MONO), binucleated (BN), multinucleated (POLY) and the number of binucleated cells with micronuclei (MN/BN).

Sample	Dose (µl)	MONO	BN	POLY	Total cells	MN/BN
Control	2.5	725	165	110	1000	0
Ctrl+ H_2O	2.5	747	157	96	1000	1
A	2.5	776	114	110	1000	3
B	2.5	779	124	107	1000	2
C	2.5	770	128	102	1000	3
D	2.5	769	121	110	1000	1

the *Allium cepa* test. In line with our results, a pot experiment on growth of maize demonstrated the usefulness of EAF steel slag as a non-phytotoxic nutrient supplier (Radic et al., 2013). Moreover, no mutagenic activity was observed in bacteria with the Ames test.

Regarding the biological assay on human cells, the presence of MN in binucleated cells within the limits (normal range 0-3; > 3 genotoxicity index), demonstrated that the cultured cells did not undergo genotoxic damage from the tested leachates. Further concentrations will be evaluated. Also Suh and coworkers characterized the potential toxicity of EAF slag using an *in vitro* human dermal model, discovering that EAF slags were not a dermal sensitizer (Suh et al., 2014).

4. CONCLUSIONS

Our investigation is still preliminary, but results of genotoxicity tests indicate a low toxicity of steel slags. Naturally, it is necessary to further investigate the genotoxicity in other types of cells, especially mammalian cells. Due to the very low number of steel slag samples, a whole battery of biological tests will be repeated on a larger number of samples.

The reuse of steel slag wastes will undoubtedly have an environmental and economic advantages. Knowledge about the potential toxic element release and especially the effect it has on the human and environmental health, is therefore fundamental in finding new applications for steel slags.

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