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In vitro and *in vivo* toxicity of coal fly Ash Lechate

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Abstract

Coal Fly ash is a major solid waste from coal-fired power stations. In Brazil, more than 4 million tons per year of fly ash are generated and only 30% is applied as raw material for cement and concrete production. The remaining is disposed in on-site ponds, nearby abandoned or active mine sites and landfills. The inadequate disposal of fly ash may pose a significant risk to the environment due to the possible leaching of hazardous pollutants into the surrounding soil and groundwater. A combination of leaching tests, cytotoxicity and ecotoxicological assays were used in this studyin order toevaluate the possible adverse effects of coal fly ash in non-target organisms. The sample was collected from coal-fired power plant located in Southern Brazil and the coal fly ash was submitted to a leaching procedure using USEPA SW 864 Method 1311. The leachate was prepared in six dilutions: 1.56%, 3.12%, 6.25%, 12.5%, 25% and 50%. Acute toxicity tests were performed on NCTC clone 929 (CCIAL-020) culture cells by neutral red uptake cytotoxicity method; acute ecotoxicity using*Daphnia similis*and *Danio rerio* embryos according to ABNT NBR 12713 and OECD 236, respectively were employed. The cytotoxicity index (CI_{50}) obtained was 33%; the $EC_{50}$of *D. similis* after 48 h of exposure to the leachate was 7.25% and the $LC_{50}$of *D. rerio* after 96 h of exposure was 4.39%. The results of these bioassays indicated toxicity of the coal fly ash leachate toward exposed organisms.

Keywords**:**Ecotoxicity; Coal Combustion Products; eluates; *Daphnia similis*; *Danio rerio*

INTRODUCTION

In Brazil, the main application of coal fly ash is at manufacture of cement and concrete (~30 % of total production). It is also used in small quantities as a road-bed stabilizer and in asphalt mixture. The remaining waste is disposed in on-site ponds, nearby abandoned or active mine sites or landfills (Fungaro *et al.*, 2013).Fly ash production leads to the problem of disposal as well as environmental damage by causing soil and water pollution by leaching of pollutants. The leachability of the potentially toxic pollutants from the based fly ash products is also of particular importance. Thus, fly ash safe disposal and utilization is an important concern to safeguard the cleaner environment (Munawer, 2018).

Previous studies showed that fly ash from Brazilian coal-fired power plants contain high concentrations of important trace elements such as arsenic, boron, chromium, copper, zinc, vanadium, nickel, among many others (Depoi *et al.*, 2008; Levandowski and Kalkreuth, 2009; Fungaro *et al.*, 2013; Kalkreuth *et al.*, 2014). Disposing large amounts of fly ash into landfills can cause leaching of these heavy metals to the water through the soil and may threaten the aquatic life and environment as well as human health (Munawer, 2018).

The properties of coal fly ash are strongly dependent on the geological origin and combustion process of the coal. Therefore, it is important to characterize regional coal ash in detail to evaluatethe environmental impacts of disposal (ash leaching) (Castro, 2013).

Some studies demonstrated the toxicity of coal fly ash leachates toward aquatic or terrestrial microorganisms

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(Tsiridis *et al.,* 2006; 2012). Considering that there is a lack of information about correlation between chemical composition of the eluates from coal fly ash and toxicity effects toward test organisms, it is important to use different species from distinct levels of the food chain for an overall evaluation of the environmental impact of fly ashes disposal.

The aquatic toxicity of chemical substances is evaluated by ecotoxicological assaysusingrepresentative organisms of the water column or sediments. The toxicity knowledge of these substances to different aquatic organisms allows to verify the temporary impact that pollutants can cause to biota in water bodies, besides the determination of trigger valuesfor several chemical substances in order to protect the aquatic biota (Zagatto and Bertoletti, 2006).

The *in vitro* assayby neutral red uptake method can determine the basal cytotoxicity due to the alterations and cellular death caused by a substance (Andrighetti-Fröhner, 2003).Mammalian cell lines are used to monitor landfill leachate because of their high sensitivity to many environmental toxicants when compared to other bioassays (Ghosh, 2015). Considering the lack of information about environmental hazard of coal fly ash, our study aimed to assess both leaching and leachate toxicity of ash samples collected from coal-fired power plant located in the Southern of Brazil.

MATERIALS AND METHODS

Materials

All the reagents used for experimental studies were of analytical grade. The sample of coal fly ash (CM) was collected from bag filter of the Figueira coal-fired power plant, located in Paraná State, Brazil. The sample was characterized about the chemical composition and the leachate obtained on a leaching assay was submitted to *in vitro* cytotoxicity and *in vivo* ecotoxicity assays.

Characterization of material

The semi-quantitative chemical composition of the fly ash was determined by a X-ray fluorescence equipment (Bruker – Model S8 Tiger X-ray Fluorescence Spectrometer). Mineralogical composition was determined by X-ray diffractometry (Rigaku – Multiflex X-ray Diffractometer) using Cu Kα radiation at 40 kV and 20mA. The scan rate was 0.02°/s and ranged between 5 – 90 ° (2θ). Crystalline phases identification was made by using Search-Match computer program and by searching the powder diffraction file database from International Centre for Diffraction Data (ICDD).

Leaching Tests

The leaching of the elements present in the raw fly ash was carried out using the standard Toxicity Characteristic Leaching Procedure, TCLP 1311 (USEPA, 1992). The application of the standard TCLP took place in a rotary agitator using a sample agitation at 30 rpm ± 2 rpm, under a liquid to solid ratio of 20 L/kg at temperature of 23 ± 2 °C. An acetic acid solution at pH 2.88 ± 0.05 was used to prepare the extraction fluid, by diluting 5.7 mL of glacial acetic acid in deionized water to a volume of 1 L. Extraction experiments included the addition of 12.5 g of solid sample in a polyethylene bottle followed by the addition of 250 mL of extraction fluid. The mixture was then agitated for 18h and the eluate was collected by vacuum filtration using a glass fiberfilter. The pH of eluate was adjusted at 7 prior to bioassays testing to avoid pH-induced toxic effects.

Cytotoxicity index (CI_{50})

The determination of cytotoxicity index (CI_{50}) of coal fly ash leachate was carried out according to the ISO 10993-5. This assay was performed by the neutral red uptake method. Diluted solutions of the coal fly ash leachate (50, 25, 12.25, 6.25, 3.13 %) in triplicate were placed in contact to NCTC clone 929 (CCIAL 020) mouse connective tissue cells adhered to the 96 wells of the cell culture microplate. The microplates were supplied by the Nucleus of Cell Culture of the Adolfo Lutz Institute. In this assay, a negative control used was the extract of Al_2O_3 in culture medium (0.5 gmL^{-1}), positive controlwas natural rubber latex (NRL) extract in culture medium (0.1 g 10 mL^{-1})and for cellular control only culture medium was added. Cell viability was verified by the incorporation of neutral red by the living and intact cells. The optical density was read on final test solution,using a spectrophotometer ELISA reader-SUNRISE from TECAN, at 540 nm and 600 nm as a reference filter. The percentage of cell viability was statistically calculated using the standard software installed in this spectrophotometer and the CI_{50} was obtained in the Origin 8.0.

Acute ecotoxicity assay with *Daphnia similis*

*Daphniasimilis*organisms was *maintained at Ecotoxicology Laboratory ofNuclear and Energy Research Institute (IPEN). Organisms were fed with an algal suspension of Pseudokirchneriella subcapitata* at the concentration of 10^5 cells mL^{-1} and a mixture of yeast and fish chow, and maintained in dilution water (MS) under controlled conditions of temperature (20±2ºC) and light-dark cycle (12:12hours) (ABNT, 2016a). The organism sensitivity results were in the stablished variation 2.28 to 2.69mg L^{-1}.

Neonates from 6 to 24 hours wereexposed to coal ash leachate in different concentrations (50.0; 25.0; 12.5; 6.0; 3.0; 1.5 %) with 20 organisms distributed in 4 tubes per concentration, for a period of 48 hours. The control was ofdilution water (MS medium). The endpoint to determine acute toxicity wasthe immobility of each organism, according to ABNT NBR12713. At the end of assay based on the number of immobile organisms as a function of concentration, EC_{50} was calculated using Trimmed Spearman-Karber software (Hamilton, 1977).

Acute ecotoxicity assay with *Danio rerio*(zebrafish)

Danio rerio cultures are maintained in Laboratory of Applied Toxinology (LETA) at Butantan Institute, under controlled temperature conditions (25 ± 2ºC) and light and dark cycle.Adult males and females are kept separate and fed twice daily with fish feed and Artemia spp. Breeders between 6 – 24 months of age (ABNT, 2016c) are placed in small aquaria, suitable for breeding, in the ratio of two males to one female.After spawning the eggs were collected from the aquaria and transported to the Ecotoxicology Laboratory at IPEN.The assay was carried out based on OECD No. 236 Guideline on Fish Embryo Acute Toxicity (FET) Test (OECD, 2013). Eggs collection attended the validation criteria for fertilization rate (≥80%) and development stage up to 4 hours post fertilization (hpf). In 24-well plate, were distributed 1 egg per well at control intern, and 2 eggs per well with 2 mL of test solutions. Statistical analyze was performed using Trimmed Spearman-Karber (Hamilton, 1977) for LC_{50} of coal fly ash leachate to zebrafish embryos. In order to guarantee the quality of the obtained results, the test organism sensitivity was evaluated with zinc chloride ($ZnCl_2$) and the result was according to the limits established.Effects were observed in an inverted microscope Olympus CK40. The lethality of the individuals was identified through: lack of heartbeat, non-detachment of the tail, lack of somite formation and coagulated embryos. The leachate concentrationswere: 3.13%, 6.25%, 12.5%, 25% and 50%, based on the result of CI_{50} concentration.

RESULTS

Chemical Composition

The chemical composition of the ash (% weight) is given in Table 1. The content of material consists of silica (SiO_2), alumina (Al_2O_3), ferrous oxide (Fe_2O_3), potassium oxide (K_2O) and calcium oxide (CaO) as major constituents. Quantities below 2% are also observed.

Table 1 - Chemical composition of fly ash sample.

Oxides	wt%	**Oxides**	wt%
SiO_2	49.2	Cr_2O_3	0.048
Al_2O_3	21.6	PbO	0.049
Fe_2O_3	9.86	As_2O_3	0.16
CaO	2.06	ZrO_2	0.099
K_2O	2.74	Rb_2O	0.014
SO_3	1.65	SrO	0.029
TiO_2	1.20	NiO	0.011
MgO	1.02	CuO	0.022
Na_2O	1.23	Y_2O_3	0.028
ZnO	0.682	V_2O_3	0.082
MnO	0.061	MoO_3	0.03
P_2O_5	0.097	BaO	<0.001

Mineralogical composition

Crystalline phases identified for the fly ash sample are shown in Fig. 1. Ash sample is composed mainly of quartz, mullite and magnetite (which are also confirmed from their chemical compositions - Table 1). These crystalline phases are typically encountered in this type of material and were identified in other studies (Umaña-Peña, 2002; Jha *et al.*, 2008; Lee *et al.*, 2010; Ibanez *et al.*, 2012; Fungaro *et al.*,2013; Widiastuti *et al.*, 2014). The sample presented the amorphous halo between 20 and 30 in the 2θ angular zone which corresponds to the vitreous component of ashes.

Cytotoxicity assay

With the results of cell viability in relation to coal fly ash leachate were placed in the graphic and is shown in the Fig. 2.In this graphic we could calculate the CI_{50}which means the concentration of the sample that provoke injure to 50% of cell population in the assay. The CI_{50} obtained for coal fly ash leachate was 33%.

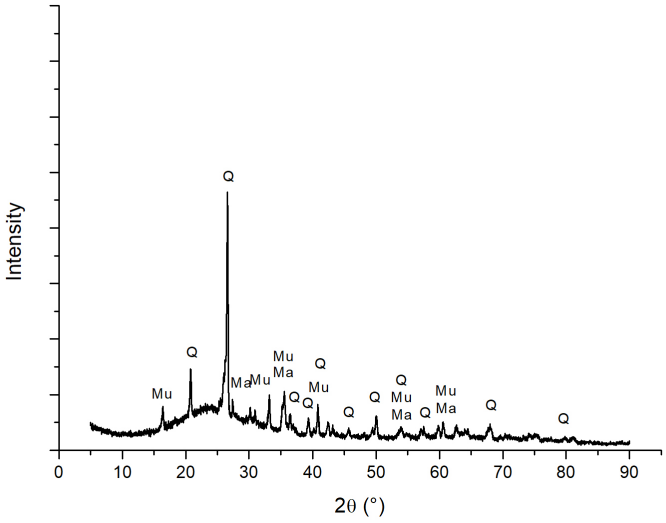

Figure 1 - XRD Patterns of ash sample: Q = Quartz (SiO_2), M = Mullite ($Al_{4.8}Si_{1.2}O_{9.6}$) and Ma = Magnetite (Fe_3O_4).

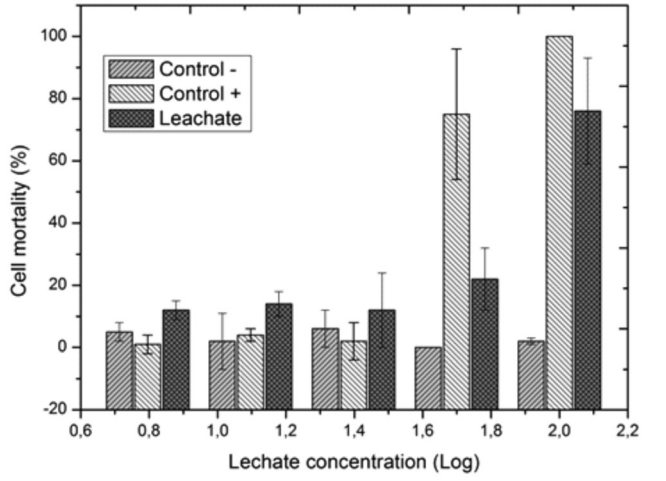

Figure 2 -Mean cell mortality in the cytotoxicity assay by neutral red uptake method.Erros bars are standard deviation.

Acute Ecotoxicity assay with *Daphnia similis*

The obtained results of acute toxicityofcoal fly ash leachate on *Daphnia similis* are shown in Fig.3.

In the Table 2 are presented the EC_{50} with confidence limits of the three performed tests. The coal fly ash leachate EC_{50} average was 7.25%.

Acute Ecotoxicity assay using*Danio rerio*

The coal fly ash leachate acute ecotoxicity results on *Danio rerio* embryos are shown in Fig.4 where it is observed that the mortality increased with the increasing concentration of the leachate.

The LC_{50} with the confidence limits results are shown in Table 3. The coal fly ash leachate LC_{50} average was 4.39%.

DISCUSSION

The fly ash used in this study was predominantly composed of silica and alumina (70%) indicating that this material can be used for the synthesis of zeolite, an alternative adsorbent, for example, and, therefore, minimize environmental impacts. The characteristics and properties of fly ash samples from Brazilian power plants, including Figueira coal-fired were previously discussed (Fungaro *et al*., 2013; Izidoro *et al*., 2012; Izidoro, 2013).

The procedure established by Norm NBR 10005 (ABNT NBR 10005, 2004b) was used for the classification of the ashes (Fungaro *et al*., 2013). The fly ash sample from Figueira coal-fired power plant was considered hazardous and classified as Class I, due to As concentration was above the maximum limit allowed by Annex F of the Norm NBR 10004 (ABNT NBR 10004, 2004a).

Previous studies regarding ash samples of Brazilian coal-fired power plants reported that the concentrations of As, Cd, Mo, Pb, TI, U, Hg, and Zn of Figueira coal-fired power plant samples are higher than others plants (Depoi *et al*., 2008)**.** In other study, the major and trace elements present in coal and ashes of Figueira coal-fired power plant and their mobility were evaluated (Flues *el at*., 2012)**.** The total metal and Asconcentrations in ash samples was As > Zn > Mn > Mo ≥ Pb > Cr > V > Cu ≥ Cd > Ni > Co. Arsenic was considered the most critical element to be leached from ashes due to their high mobility (70%) followed by Mo (>55%) and next by Mn, Zn, Cd, Pb (30–5%). In relation to studies on leachate elements of coal ash, the application of leaching tests and physicochemical analyses of the

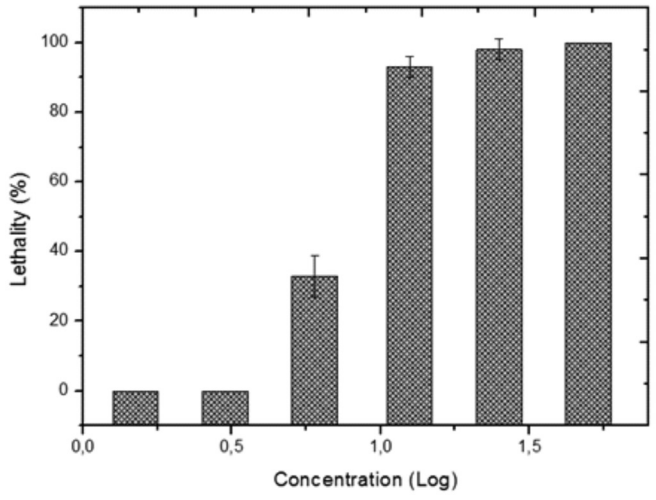

Figure 3 - Mean *Daphniasimilis* immobility results in relation to concentrations of coal fly ash leachate. Error bars are standard deviation.

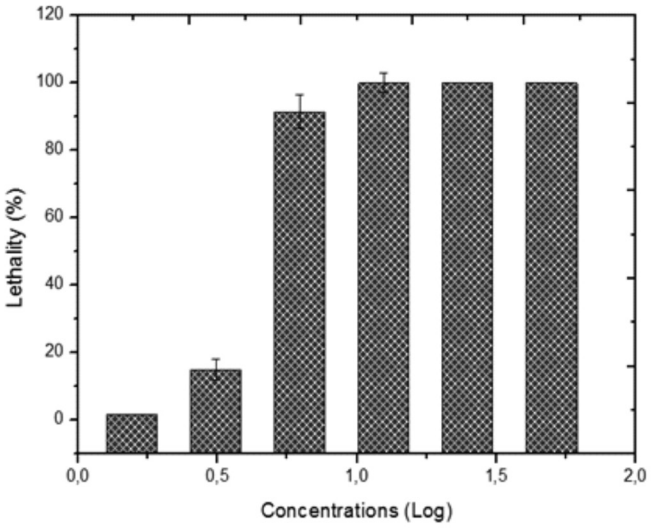

Figure 4 - Mean *Danio rerio* embryos lethality results in relation to concentrations of coal fly ash leachate.Error bars are standard deviation.

Table 2 - Coal fly ashes leachate EC_{50} on *Daphnia similis*.

Assay	EC_{50}(%)	Confidence Limits	
		Lower	Higher
1	7.50	6.29	8.94
2	6.75	5.69	8.01
3	7.51	6.30	8.94
Average	7.25	6.09	8.63

Table 3 - Coal fly ashes leachate LC_{50} on *Danio rerio* embryos

Assaynumber	LC_{50} (%)	Confidence Limits	
		Lower	Higher
1	4.34	4.12	5.27
2	4.54	4.10	5.03
3	4.35	3.48	5.55
Average	4.39	3.9	4.66

resulting leachates is the common practice for assessment of contamination caused by its disposal or utilization. However, is difficult a comparison between various leaching procedures and ecotoxicity tests because there are several mechanisms taking place simultaneously (Tsiridis *et al*., 2006).

A combination of leaching tests (EN 12457-2, EN 12457-3 and NEN 7343), physicochemical and ecotoxicological analyses were used for the evaluation of the environmental hazard of six coal fly ashes collected from different power plants in Europe plants. Physicochemical analyses indicated that the concentration of Cr, Mo, sulfates and total dissolved solids in the leachates of all samples exceeded the leaching limit values for inert wastes. The toxicity of leachates was evaluated using *Vibrio fischeri,* Brachionuscalyciflorus *P. subcapitata* and *Daphnia magna* (*D. magna*). The highest sensitivity was presented by *D. magna* and was described to the presence of Cr, while *Vibrio fischeri* was the least sensitive. The toxicity of the leachates toward *P. subcapitata* was relatively high, but the correlation between the toxicity and heavy metals concentration was poor. The NEN 7341 leaching test was strongly suggested for the implementation of ecotoxicity characterization of these solid wastes (Tsiridis *et al*., 2006, 2012).

Arsenic is considered one of the most significantly toxic elements present in coal ashes. A review article emphasized the concentration of As in fly ash, leaching studies, the behavior as hazardous element on human health, environment quality and the mitigation strategies to accomplish environmental management. The concentrations in fly ash generally range from 2 to 440 mg kg^{-1}, but depending on the concentration in the original coals and the combustion methods, they can be as high as 1000 mg kg^{-1} (Pandey *et al*., 2011).

A broad range of toxicity studies was conducted after the 2008 TVA Kingston ash spill into the Emory and Clinch rivers to evaluate potential effects of fly ash on aquatic biota. The study demonstrated that the risks to resident species were moderate and limited to locations with ash content greater than 40% **(**Sherrard *et al*., 2015).Moreover,the Emory-Clinch system after its remediation presented low risk of excessive element exposure and limited adverse reproductive effects to freshwater turtles **(**Steen *et al*., 2015).

The *in vitro* toxicity assay on mammalian cells showed CI_{50} of 33%. The presence of cytotoxic, compounds as metals can act in the lysosomal membrane stability leading to cell death, verified in the cytotoxicity assay.

In the acute toxicity assay with *Daphnia similis* the obtained $EC_{50}$was 7.25%, probably due to the presence of arsenite (0.16%) and this element showing a high mobility in the leaching process. The obtained result showeda higher acute toxicity when compared with the Castro (2013) results in the percolate of coal fly ash evaluation (EC_{50}: 25% to 84%). The difference between these results can be explained by the different types of elements extraction: in the percolate assay the soil contained in the columns could retain some toxic substances.

The fly ash sample was considered hazardous and can be classified as Class I, due to the concentration of As which was above the maximum limit allowed by Annex F of the Norm NBR 10004 (ABNT NBR 10004, 2004a). Arsenic was considered the most critical element to be leached from ashes due to their high mobility (70%) followed by Mo (>55%) and next by Mn, Zn, Cd, Pb (30–5%).

The LC_{50} results obtained for *Danio rerio* embryos after leachate concentrations exposure was 4.39%, similar to EC_{50} of *D. similis*. Jezierska *et al*. (2009), described that when heavy metals are present in water, they can cause many physiological alterations in fish, the most common mechanism of toxicity is associated with enzymatic alterations in synthesis activity and lead to osmotic disturbs. Early developmental stages are more sensitive to these effects and the exposure during embryonic development can reduce the survival and hatching success. During the egg swelling there is a perivitelline space with a colloidal suspension of proteins secreted by the vitelline membrane that absorb water. The ions can enter due to the high permeability of the egg membrane, changing the chorion structure and even thepermeability (Jezierska *etal*., 2009).

CONCLUSION

The ash sample investigated in the present study contains considerable quantity of arsenic,being classified as hazardous waste. The leachate tested showed a high toxicity to the aquatic organisms as compared to other studies, but considering the lack of information about environmental hazard of coal fly ash, more studies has to be performed in order to assess both leaching and leachate ecotoxicity of ash samples collected from coal-fired power plant. Ecotoxicity results may be different considering the diverse characteristics on the composition of the coal fly ash, because of this, it is very important to perform more studies to characterize and understand in detail the regional coal ash hazard to the environment. More studies using different species and *in vivo* tests should be employed for ecotoxicological evaluations at different conditions to understand the biological response of the fly ash.

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