



# Biomass burning particles in the Brazilian Amazon region: Mutagenic effects of nitro and oxy-PAHs and assessment of health risks<sup>☆</sup>



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## ABSTRACT

Emissions from burning of biomass in the Amazon region have adverse effects on the environment and human health. Herein, particulate matter (PM) emitted from biomass burning in the Amazon region during two different periods, namely intense and moderate, was investigated. This study focused on: i) organic characterization of nitro- and oxy-polycyclic aromatic hydrocarbons (PAHs); ii) assessment of the excess lifetime cancer risk (LCR); and iii) assessment of the *in vitro* mutagenic effects of extractable organic matter (EOM). Further, we compared the sensitivity of two mutagenicity tests: *Salmonella*/microsome test and cytokinesis-block micronucleus (CBMN) with human lung cells. Among the nitro-PAHs, 2-nitrofluoranthene, 7-nitrobenz[a]anthracene, 1-nitropyrene, and 3-nitrofluoranthene showed the highest concentrations, while among oxy-PAHs, 2-methylanthraquinone, benz[a]anthracene-7,12-dione, and 9,10-anthraquinone were the most abundant. The LCR calculated for nitro-PAH exposure during intense biomass burning period showed a major contribution of 6-nitrochrysene to human carcinogenic risk. The EOM from intense period was more mutagenic than that from moderate period for both TA98 and YG1041 *Salmonella* strains. The number of revertants for YG1041 was 5–50% higher than that for TA98, and the most intense responses were obtained in the absence of metabolic activation, suggesting that nitroaromatic compounds with direct-acting frameshift mutagenic activity are contributing to the DNA damage. Treatment of cells with non-cytotoxic doses of EOM resulted in an increase in micronuclei frequencies. The minimal effective dose showed that *Salmonella*/microsome test was considerably more sensitive in comparison with CBMN mainly for the intense burning period samples. This was the first study to assess the mutagenicity of EOM associated with PM collected in the Amazon region using *Salmonella*/microsome test. The presence of compounds with mutagenic effects, particularly nitro- and oxy-PAHs, and LCR values in the range of  $10^{-5}$  indicate that the population is potentially exposed to an increased risk of DNA damage, mutation, and cancer.

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**Abbreviations:** B[a]P<sub>eq</sub>, benzo[a]pyrene equivalency concentration; CBMN, cytokinesis-block micronucleus assay; EOM, extractable organic matter; LCR, excess lifetime cancer risk; MED1.5, minimal effective dose to increase the effect 50%; MN, micronuclei; NDI, nuclear division index; PAHs, polycyclic aromatic hydrocarbons; PM, Particulate matter; TEF, toxicity equivalency factor.

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## 1. Introduction

The Amazon represents over half of the remaining tropical rain forests on the planet and contains one of the largest biodiversity in the world. Further, it corresponds to 61% of the area of Brazil (Malhi et al., 2008). Deforestation and land use have changed about 18% of the original tropical rain forest, mostly in the southern and western Amazonia. The land is used mainly for establishing pastures and preparing for crops, consequently causing many alterations in atmospheric dynamics (Artaxo et al., 2013). Interestingly, most of the forest fire hotspots in the Brazilian Amazon are located in an area of approximately 500,000 km<sup>2</sup>, with a population of over 10 million, known as the deforestation arc (de Oliveira Alves et al., 2015).

In October 2013, the International Agency for Research on Cancer (IARC) classified outdoor air pollution as carcinogenic to humans (Group 1) (IARC, 2016). In addition, IARC considers indoor emissions from household combustion of biomass fuel (primarily wood) as probably carcinogenic to humans (Group 2A) (IARC, 2010). Particulate matter (PM) is a heterogeneous mixture of chemicals that changes in space and time according to the emission source, and it is considered one of the main air pollutants. PM, with adsorbed chemical species such as polycyclic aromatic hydrocarbons (PAHs) and other mutagenic compounds, is suspected to increase the risk of human lung cancer and cardiovascular diseases (Brown et al., 2013; Raaschou-Nielsen et al., 2013; IARC, 2013). The PAHs are formed by incomplete combustion or pyrolysis of organic material, together with their nitrated (nitro-PAHs) and oxygenated (oxy-PAHs) derivatives, represent one of the largest contributors to the biological effects of PM (Boström et al., 2002; Kelly and Fussell, 2012; Nemmar et al., 2013).

Many of the biological effects of PAHs, including mutagenesis and carcinogenesis, are believed to be mediated by activation of a series of enzymatically-catalyzed reactions to form their active metabolites. The mutagenic potential of PAHs is probably associated with the structural differences between DNA adducts and the consequent effects of their removal by DNA repair mechanisms (Boström et al., 2002; Jarvis et al., 2014). It is speculated that nitro- and oxy-PAHs are more mutagenic than unsubstituted PAHs because of their ability to act as direct mutagens; however, such quantification of risk is limited by sparse availability of toxicological data (Fu, 1990; Jung et al., 1991; Jariyasopit et al., 2014a, 2014b; Tomaz et al., 2016; Bandowe and Meusel, 2017).

The combination of chemical-analytical methods, along with specific extraction strategies, and short-term bioassays has been successfully used to evaluate effects of a variety of mutagenic compounds in complex environmental samples such as air (Kessler et al., 2012; Jarvis et al., 2013; Palacio et al., 2016). Cell-based bioassays that target relevant biological endpoints complement chemical analysis for environmental quality assessment (Escher et al., 2014). Many mutagenicity assays, such as *Salmonella*/microsome test (Umbuzeiro et al., 2008a; Alves et al., 2016), micronuclei analysis using plants as *Tradescantia pallida* (de Oliveira Alves et al., 2011; de Oliveira Galvão et al., 2014), human lung cell lines (de Oliveira Alves et al., 2014), and exfoliated buccal cells from exposed human populations (Bruschweiler et al., 2014; Wultsch et al., 2015; de Oliveira Galvão et al., 2017), have been used to evaluate the effects of different pollutants emitted by biomass burning. The exposure to fine particulate matter (PM<sub>2.5</sub>) generated in the Amazon region was reported to contribute to an increased micronuclei frequency in exfoliated buccal mucosa cells from schoolchildren (Sisenando et al., 2012).

Recently, de Oliveira Alves et al. (2015) determined the human

health risk associated with exposure to PAHs emitted due to burning of biomass in western Amazon. The estimated cancer risk calculated during the dry seasons ( $17 \times 10^{-5}$ ) exceeded the WHO health-based guideline ( $8.7 \times 10^{-5}$ ). These facts point out the need to understand mutagenic mechanisms associated with the exposure to emissions from Amazon biomass burning.

The *Salmonella*/microsome test has long been used to detect potentially mutagenic environmental compounds. It is a short-term bacterial test that is able to detect mutagens that may directly alter the DNA, using selected mutant strains of *Salmonella enterica* serovar Typhimurium<sup>2</sup> (Mortelmans and Zeiger, 2000). In environmental studies, TA98 and TA100 strains are usually employed for the detection of frameshift and basepair mutagenic activity, respectively. More recently developed strains with different metabolic capacities such as YG1041 and YG1042, with enhanced nitroreductase and acetyl transferase activities have been adopted in air pollution monitoring of specific classes of PAHs present in environmental samples (Claxton et al., 2004; DeMarini et al., 2004; Umbuzeiro et al., 2014). However, the *Salmonella*/microsome assay is able to detect solely point mutations. The inclusion of a test that detects chromosome damage is, therefore, a good strategy to supplement the information on the mutagenic hazards of a substance or complex mixture (Thybaud et al., 2007; Kirkland et al., 2011).

The cytokinesis-block micronucleus assay (CBMN) is a well-established *in vitro* genetic toxicology assay and has become an accepted standard method to assess the genotoxic hazard of chemicals (OECD, 2016). This protocol has been used in environmental studies investigating air quality with several different cell lines, as reported by Bocchi et al. (2016) and de Oliveira Alves et al. (2014) for A549 cells; León-Mejía et al. (2016) for V79 cells; Xin et al. (2014) for HepG2 cells; and Zhai et al. (2012) for HBE cells. Micronuclei (MN) are biomarkers of mutagenicity that may originate from whole chromosomes that are unable to migrate to the poles during the anaphase stage of cell division and/or acentric chromosome fragments, both induced by aneugenic or clastogenic substances, respectively (Kirsch-Volders et al., 2011, 2014). The cytochrome approach of this assay provides information about chromosome breakage and/or loss (e.g., MN), gene amplification (e.g., nuclear buds) and chromosome rearrangement, as dicentric chromosomes (e.g., nucleoplasmic bridges) (Kirsch-Volders et al., 2011, 2014).

The aims of this study were: i) to identify and quantify nitro-PAHs and oxy-PAHs present in the PM emitted from burning of biomass in the Amazon region; ii) to estimate the excess lifetime cancer risk for the exposed population; and iii) to assess the *in vitro* mutagenic effects of extractable organic matter collected during the periods of moderate and intense biomass burning, comparing the sensitivity of a prokaryote (*Salmonella*/microsome test) and a eukaryote (CBMN with human lung cells) mutagenicity assays.

## 2. Materials and methods

### 2.1. Air sampling and solvent extraction

PM<sub>10</sub> fraction was collected from the rural area of Porto Velho, state of Rondônia, in the western part of the Amazon region. The sampling site (8.69° S, 63.87° W) is located in a region with large land use and associated regional biomass burning (de Oliveira Alves

<sup>2</sup> We adopted the taxonomy revision that occurred for the genus *Salmonella*, where *Salmonella typhimurium* was revised as *Salmonella enterica* serovar Typhimurium (Brenner et al., 2000).

et al., 2015). The sampling conditions and instrumentation details were previously described by Brito et al. (2014). Sample collection was conducted during two distinct periods: intense biomass burning – dry season (August–October/2011), and moderate biomass burning – wet season (November/2011–March/2012).

The aerosol samples were collected using a high-volume air sampler with a flow rate of 1.13 m<sup>3</sup>/min. Quartz fiber filters were pre-cleaned by heating in an oven at 800 °C for 8 h. Thereafter, the filters were weighed after equilibrating them in a room with constant temperature and relative humidity (50%) for 24 h. The extractable organic matter (EOM) was obtained with dichloromethane using the Soxhlet apparatus, as previously described by de Oliveira Alves et al. (2015). In addition, the EOM mass concentration contained in the PM<sub>10</sub> was determined according to Valle-Hernández et al. (2010). Previously, each vial was pre-cleaned by heating at 400 °C for 24 h, until constant weight. After, 200 µL of the EOM was added and was completely concentrated with a high purity N<sub>2</sub> gas. Finally, the vials were weighed again using an electronic micro-analytic 1 µg precision Mettler scale. The amount of EOM was assessed taking into consideration the difference of mass between the vial with and without organic extract as well as the air volume sampled.

The filters obtained for each period – moderate and intense biomass burning – were extracted individually for chemical analyses and pooled together to perform biological assays.

## 2.2. Organic chemical analysis

Non-substituted PAHs' results were published in de Oliveira Alves et al. (2015). The detection of their nitro- and oxy-derivatives was performed later, and is described here. Eleven nitro-PAHs (9-nitrophenanthrene, 3-nitrophenanthrene, 2-nitrophenanthrene, 2-nitroanthracene, 3-nitrofluoranthene, 2-nitrofluoranthene, 1-nitropyrene, 4-nitropyrene, 7-nitrobenz[a]anthracene, 6-nitrochrysene, 6-nitrobenzo[a]pyrene) and four oxy-PAHs (9-fluorenone, 9,10-anthraquinone, 2-methylanthraquinone, benz[a]anthracene-7,12-dione) were detected. The quantitative and qualitative analyses of nitro-PAHs (Crimmins and Baker, 2006) and oxy-PAHs (Wei et al., 2012) were performed using gas chromatography with mass spectrometer (GC-MS; Agilent 7820A + 5975 MSD, USA). An inert 5% phenyl - methyl column, VF-5ms (30 m × 0.250 mm, 0.25 µm film thickness) was used for separating the compounds and 1 µL of the sample was injected in splitless mode. For plotting the calibration curve, the nitro-PAHs were procured from NIST (SRM 2265). The oven temperature program was: 40 °C (held 1.7 min) ramped to 150 °C at 20 °C min<sup>-1</sup>, held for 10 min, then to 220 °C at 10 °C min<sup>-1</sup>, held for 10 min and finally ramped to 310 °C and held for 15 min. The oxy-PAHs standard was procured from Sigma-Aldrich and the chromatographic conditions were as follows: injector temperature 290 °C and detector temperature 250 °C. The temperature ramp was: an initial oven temperature of 80 °C maintained for 4 min, and increased to 150 °C at 40 °C min<sup>-1</sup>, maintained for 5 min, then increased at 5 °C min<sup>-1</sup> to a maximum of 300 °C for 10 min. Helium was used as carrier gas (at a flow rate of 1.2 mL min<sup>-1</sup>). The limits of detection (LOD) and quantification (LOQ) for the nitro and oxy-PAH compounds are presented in Table S1.

## 2.3. Cancer risk assessment

To estimate the excess lifetime cancer risk (LCR) for inhalation exposure to nitro-PAHs, the benzo[a]pyrene equivalency concentrations (B[a]P<sub>eq</sub>) were determined using the current relative potency factors (RPF) scales with known toxicity equivalency factor (TEF) values, Table 2. The cancer risk was determined by

multiplying the concentrations of each nitro-PAHs compound with its corresponding TEF and then by the unit risk, as:

$$\text{Cancer risk} = \sum ([\text{nitro-PAHs}] \times \text{TEF}_{\text{nitro-PAHs}}) \times \text{unit risk}$$

The unit risk used was defined by the World Health Organization (WHO) for B[a]P as 8.7 × 10<sup>-5</sup> ng/m<sup>3</sup> (theoretical upper limit of the possibility of contracting cancer when exposed to B[a]P at a concentration of 1 ng/m<sup>3</sup> of air for a 70-year lifetime, based on an epidemiological study of coke oven workers in Pennsylvania) (OEHHA, 1994; 2005, 2011; WHO, 2000; Callén et al., 2013).

## 2.4. Salmonella/microsome microsuspension assay

The *Salmonella*/microsome assay was performed using the microsuspension version of the method, as described by Kado et al. (1983, 1986), where overnight bacteria cultures are centrifuged to obtain a higher cell density compared to the traditional protocol. The exposure of a higher number of bacteria enhances the detection of mutagenic compounds in complex mixtures (Mortelmans and Zeiger, 2000), and it is a good strategy when sample quantity is limited. Due to limited amount of the extracts, only *Salmonella enterica* serovar Typhimurium strains TA98 (*hisD3052*, *rfa*,  $\Delta$ *bio*,  $\Delta$ *uvrB*,  $\Delta$ *chlD*, plasmid pKM101) that preferably detects frameshift mutations and its derivative YG1041 (=TA98 with plasmid pYG233), with high levels of both nitroreductase and acetyltransferase activity, were tested (Maron and Ames, 1983; Hagiwara et al., 1993).

All assays were performed in the absence and presence of *in vitro* metabolic activation, S9 mix (4%, Moltax Inc., NC, USA). Three independent tests were conducted for each extract, with doses varying between 1 and 500 µg of EOM equivalent per plate. Positive controls used were 0.125 µg/plate of 4-nitroquinoline-1-oxide (4NQO - Alfa Aesar), 2.5 µg/plate of 4-nitro-*o*-phenylenediamine (4NOP - Sigma-Aldrich), 0.625 µg/plate and 31.25 ng/plate of 2-aminoanthracene (2AA - Sigma-Aldrich) for TA98-S9, YG1041-S9, TA98 + S9 and YG1041 + S9, respectively.

## 2.5. Cell culture and exposure

Human lung adenocarcinoma epithelial cells, A549 (American Type Culture collection) were cultured in Dulbecco's modified Eagle's medium (DMEM) – high glucose, supplemented with fetal bovine serum (FBS) 10% (v/v), antibiotics (100 U/mL of penicillin and 100 µg/mL of streptomycin), L-glutamine (2 mM) and maintained at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere. The EOM from intense and moderate biomass burning periods were dissolved in dimethyl sulfoxide (DMSO) at the time of use, never exceeding 0.1% of DMSO in the culture. The final concentrations used were 50 µg/mL, 100 µg/mL, 200 µg/mL, and 400 µg/mL for 24 h of treatment period. The untreated cells, vehicle (DMSO 0.1%), and a mix of PAHs (10 ng/mL, Supelco/Sigma SS EPA 610 PAH MIX) were used as control groups, similar to Roubicek et al. (2007) and de Oliveira Alves et al. (2014). The experiments were conducted in triplicate.

## 2.6. Viability assay

Cell viability was determined by the mitochondria-dependent reduction of MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, Sigma) to formazan as described by Mosmann (1983). Briefly, the cells were plated at a density of 4 × 10<sup>3</sup> cells per well in 96-well plates until cell adhesion (24 h). After treatment, the cells were washed with PBS followed by incubation in serum- and phenol-red free medium containing 0.5 mg/mL of MTT solution for 4 h. Thereafter, the medium was removed and 100 µL of

DMSO was added to each well to dissolve the formazan crystals. The plates were then protected from light and kept under agitation for 5 min. After shaking, the absorbance was read at a wavelength of 570 nm in a  $\mu$ Quant-microplate spectrophotometer (Biotek, USA). Data were expressed as percent of control.

### 2.7. Cytokinesis-block micronucleus assay (CBMN)

The CBMN assay was conducted using the standard technique proposed by Fenech (2000, 2007), with some modifications. Briefly, A549 cells were plated in 6-well plates at a density of  $1.5 \times 10^5$  cells per well until cell adhesion (24 h). Subsequently, the cells were exposed to the different concentrations of EOM. After the exposure time (24 h), EOM was removed, and cytochalasin B (final concentration 5  $\mu$ g/mL, Sigma-Aldrich) was added. The cells remained in the incubator during 24 h, followed by trypsinization and centrifugation at 300g for 5 min. Finally, the cells were fixed in three centrifugation cycles (300 g, 5 min) with freshly made cold methanol: acetic acid (9:1) fixative solution, and then homogenized. A drop with 20–30  $\mu$ L of the material was evenly dropped onto a previously washed slide, placed above a hot water bath (40–60 °C) for 10–20 s, and stained using 10% Giemsa in potassium phosphate buffer solution for 8 min (Cornélio et al., 2014). Three thousand binucleated cells were scored per treatment for evaluation of nuclear alterations such as micronuclei, nucleoplasmic bridges, and nuclear buds, as classified by Fenech et al. (2003).

The nuclear division index (NDI) provides important information about cytostatic properties of the test chemical or mixture. NDI was calculated as follows:

$$\text{NDI} = [\text{M1} + 2(\text{M2}) + 3(\text{M3}) + 4(\text{M4})]/\text{N}$$

where M1–M4 indicate the number of cells with 1–4 main nuclei and N is the total number of viable cells scored as recommended by Fenech (2007).

### 2.8. Minimal effective dose – MED 1.5

To be able to compare such different endpoints, as the *Salmonella* test and the micronucleus assay results, the MED1.5 was calculated. The MED1.5 corresponds to the concentration/dose that can cause a 50% increase of the effect observed in comparison to the control values. The MED1.5 can be derived using the linear regression curve for each of the assays. Although a ratio of 2 is considered as a positive criterion for the *Salmonella* test, which would be a 100% increase in the number of revertants above the control values, the MED1.5 was chosen because, as pointed out by Escher et al. (2014), it is employed in several guideline documents, is close to the detection limit of many biological assays, and is an interpolation not an extrapolation of the dose-response curve.

### 2.9. Statistical analysis

The *Salmonella*/microsome microsuspension test results were statistically analyzed by applying the Bernstein model (Bernstein et al., 1982) with the *Salanal* computer program (*Salmonella* Assay Analysis, version 1.0, Integrated Laboratory Systems, RTP, NC, USA). Samples were considered mutagenic when there was a significant difference between doses (ANOVA,  $p < 0.05$ ), positive dose-response curve ( $p < 0.05$ ), and mutagenicity ratio (MR)  $\geq 2$ . The mutagenic potency of a sample is the slope of the regression curve obtained, and is expressed as revertants per unit of mass or volume of the sample tested. In this study, to allow comparisons with other studies alike, the *Salmonella*/microsome results were expressed as the number of revertants per mg of EOM (rev/mg EOM), per ng of

PAHs (rev/ng PAH), per  $\mu$ g of PM<sub>10</sub> (rev/ $\mu$ g PM<sub>10</sub>), and per m<sup>3</sup> (rev/m<sup>3</sup>), as shown in Table 3.

For cytotoxicity and mutagenicity tests with A549 cells, one-way ANOVA followed by Dunnett's test was used to determine the significance level between the treated and control group. Statistical differences were considered significant at  $p < 0.05$ .

## 3. Results

### 3.1. Airborne PM and EOM concentrations

Time series of PM<sub>10</sub>, EOM, and fire spots are shown in Fig. 1. In accordance with the results obtained with the MODIS sensor, larger number of fire spots occurred between July and October/2011 (INPE, 2014). Higher peaks of PM<sub>10</sub> concentrations were observed during intense biomass burning, with concentrations reaching close to 60  $\mu$ g/m<sup>3</sup>.

Table 1 shows the number of filters, total collected air volume, concentration of PM<sub>10</sub>, EOM and PAHs for each of the two samples tested for mutagenicity. The concentration in Table 1 corresponds to the sum of the mass of the all filters by the total air volume and was used to calculate the concentrations in Table 3.

### 3.2. Analysis of organic compound content in air PM extracts

The unsubstituted PAHs profile for the moderate (retene > phenanthrene > anthracene > chrysene > dibenzo[a,h]anthracene) and intense biomass burning period (retene > anthracene > dibenzo[a,h]anthracene > phenanthrene > benzo[e]pyrene) were previously published by our group (de Oliveira Alves et al., 2015). Nitro- and oxy-PAHs are a part of the complex mixture in the organic extract of PM<sub>10</sub> fraction collected. The concentrations of these compounds are shown in Fig. 2. Among the nitro-PAHs identified, 2-nitrofluoranthene > 7-nitrobenz[a]anthracene > 1-nitropyrene > 3-nitrofluoranthene > 6-nitrochrysene were most abundant for the intense biomass burning period. On the other hand, 2-methylanthraquinone > benz[a]anthracene-7,12-dione > 9,10-anthraquinone > 9-fluorenone showed highest concentrations among the oxy-PAHs. The average concentrations of 1-nitropyrene and 6-nitrochrysene during intense biomass burning

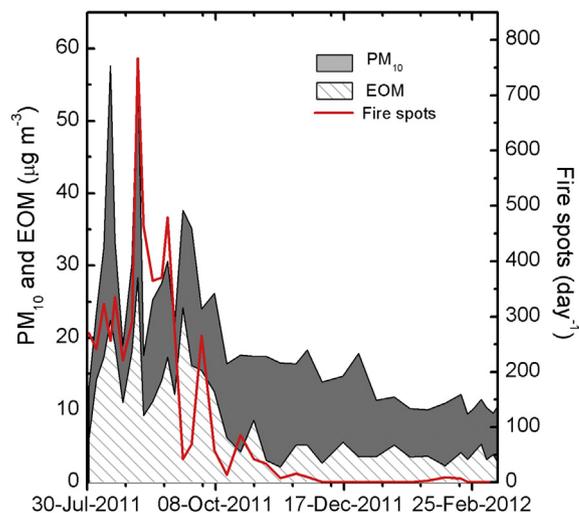


Fig. 1. Concentration time series of PM<sub>10</sub>, extracted organic matter (EOM) and fire spots obtained by MODIS sensor for intense biomass burning (August–October/2011) and moderate biomass burning (November/2011–March/2012) periods in the Brazilian Amazon region.

**Table 1**

Number of filters, total collected air volume, concentration of PM<sub>10</sub>, EOM, and PAHs for each of the two samples tested.

Sample	# filters	Volume (m <sup>3</sup> )	Mass (mg)	PM <sub>10</sub> (μg/m <sup>3</sup> )	EOM (μg/m <sup>3</sup> )	ΣPAHs* (ng/m <sup>3</sup> )
Moderate	23	189,874.1	2889.1	13.7	4.4	1.3
Intense	17	103,813.6	2597.3	27.8	14.9	2.9

EOM: Extractable Organic Matter. \*PAHs - fluorene, phenanthrene, anthracene, fluoranthene, pyrene, retene, benz[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*e*]pyrene, benzo[*a*]pyrene, indene[1,2,3-*c,d*]pyrene, dibenz[*a,h*]anthracene and benzo[*g,h,i*]pyrene.

period were  $125 \pm 28$  pg/m<sup>3</sup> (range 83–206) and  $83 \pm 27$  pg/m<sup>3</sup> (range 53–131), respectively. Whereas, the average concentration of 4-nitropyrene for the same period was  $39 \pm 13$  pg/m<sup>3</sup> (range 30–62), which was approximately twice the concentration found during the moderate biomass burning period. Furthermore, high levels of oxy-PAH, 9,10-anthraquinone, were observed during fire incidences. The average concentration values of all nitro- and oxy-PAHs are shown in Fig. 2.

### 3.3. Cancer risk assessment

The mean, minimum, and maximum concentrations of the nitro-PAHs with known TEF, B[*a*]P<sub>eq</sub> values and LCR for nitro-PAH exposure are shown in Table 2. Although 11 nitro-PAHs were quantified, only 3 of them have defined TEF values. The excess lifetime cancer cases per 100,000 people exposed were 4.2 and 7.3 for moderate and intense biomass burning seasons, respectively (Table 2).

### 3.4. Salmonella mutagenicity assay

The *Salmonella*/microsome results for TA98 and YG1041 are presented in Table 3. The significant difference between doses, positive dose-response curve, and mutagenicity ratio for both periods and stains are presented in Tables S2 and S3. The EOM from the intense burning period was more mutagenic than that from the moderate burning period, for both the *S. Typhimurium* strains. Interestingly, potencies were higher in the absence of metabolic activation, regardless of the strain used, showing a large contribution of direct acting mutagens. The much higher potencies observed with YG1041 demonstrate the significant presence of nitro-compounds (Tables 3 and S3).

### 3.5. Cell viability

The cytotoxic effects of EOM are shown in Fig. 3. Significant

**Table 2**

Estimation of the excess lifetime cancer risk (LCR) for inhalation exposure to nitro-PAHs during intense and moderate Amazon biomass burning periods.

Nitro-PAHs (ng. m <sup>-3</sup> )	TEF <sup>a</sup>	Moderate biomass burning		Intense biomass burning		Excess lifetime cancer cases/100,000 people <sup>c</sup>	
		B[ <i>a</i> ]P <sub>eq</sub> <sup>b</sup>		B[ <i>a</i> ]P <sub>eq</sub> <sup>b</sup>		Moderate	Intense
		Mean (Min - Max)	Mean (Min - Max)	Mean (Min - Max)	Mean (Min - Max)		
1-nitropyrene	0.1 <sup>d</sup>	0.07 (0.044–0.102)	0.007 (0.004–0.01)	0.125 (0.082–0.205)	0.012 (0.008–0.020)	–	–
4-nitropyrene	0.1 <sup>d</sup>	0.02 (0.014–0.032)	0.002 (0.001–0.003)	0.04 (0.030–0.062)	0.004 (0.003–0.006)	–	–
6-nitrochrysene	10 <sup>d</sup>	0.048 (0.028–0.085)	0.48 (0.28–0.85)	0.083 (0.053–0.131)	0.83 (0.53–1.31)	–	–
Σ3Nitro-PAHs	–	0.138	–	0.248	–	–	–
ΣB[ <i>a</i> ]P <sub>eq</sub>	–	–	0.489	–	0.846	–	–
Cancer risk	–	–	–	–	–	4.2	7.3

<sup>a</sup> TEF is toxicity equivalency factor of individual nitro-PAHs relative to benzo[*a*]pyrene.

<sup>b</sup> B[*a*]P<sub>eq</sub> is the B[*a*]P equivalency concentrations.

<sup>c</sup> The unit risk used was defined by the WHO for B[*a*]P set to  $8.7 \times 10^{-5}$  ng/m<sup>3</sup>.

<sup>d</sup> Obtained from OEHHA (1994, 2005, 2011).

**Table 3**

Mutagenicity potencies in *Salmonella* strain TA98 and YG1041 per unit of mass of extracted organic matter (rev/mg EOM), per ng of PAHs (rev/ng PAH), per μg of PM<sub>10</sub> (rev/μg PM<sub>10</sub>), and per m<sup>3</sup> (rev/m<sup>3</sup>), with and without *in vitro* metabolic activation (S9) for moderate and intense Amazon biomass burning periods.

strain	Moderate biomass burning				Intense biomass burning			
	TA98		YG1041		TA98		YG1041	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
rev/mg EOM	64	27	730	99	680	240	3800	1500
rev/ng PAHs <sup>a</sup>	0.4	0.2	4	0.6	30	10	166	65
rev/μg PM <sub>10</sub>	4	2	44	6	280	99	1570	620
rev/m <sup>3</sup>	0.3	0.1	3	0.4	10	4	57	22

<sup>a</sup> PAHs - fluorene, phenanthrene, anthracene, fluoranthene, pyrene, retene, benz[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*e*]pyrene, benzo[*a*]pyrene, indene[1,2,3-*c,d*]pyrene, dibenz[*a,h*]anthracene and benzo[*g,h,i*]pyrene.

differences in cytotoxicity were found neither at any of the EOM concentrations tested, nor between the different organic extracts. The mix of PAHs (10 ng/mL) did not significantly influence the cell viability and, thus, cells could be exposed to all concentrations in the mutagenicity assays.

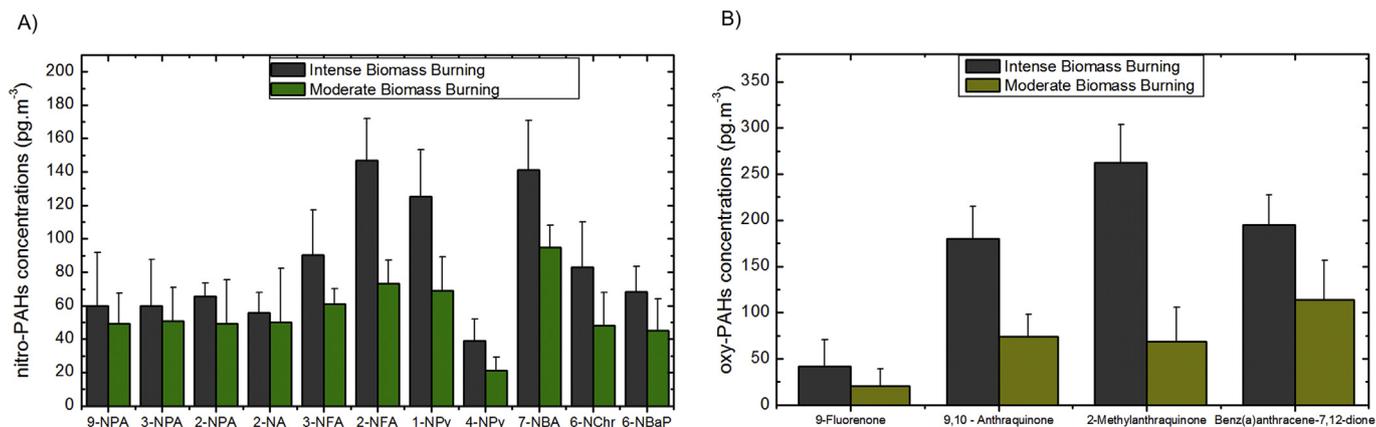
### 3.6. Micronuclei and nuclear abnormalities tests

Micronuclei frequencies and NDI are presented in Fig. 4. A dose-dependent increase in the MN frequency was observed for both moderate period and intense burning period extracts, the differences were not statistically significant. Moreover, no significant increase in the nucleoplasmic bridges and nuclear buds formation compared with negative controls were observed (data not shown), regardless of the extract tested.

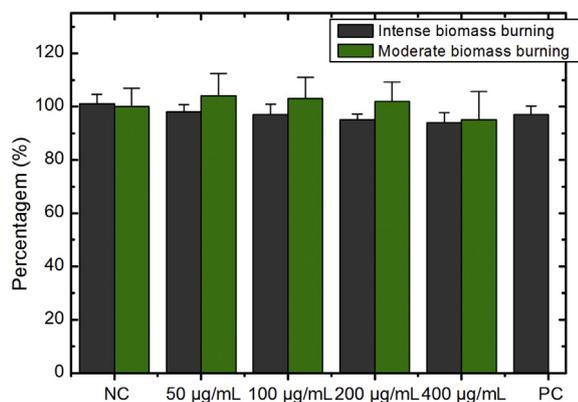
The NDI ranged from 1.60 to 1.78, and 1.65 to 1.70 for moderate and intense burning period, respectively, indicating no significant difference between the samples and the negative control. Thus, it was concluded that the samples did not have any cytotoxic effect in the concentrations used (Fig. 4). These results corroborate those of cytotoxicity data obtained by MTT assay (Fig. 3).

### 3.7. MED1.5

The minimal effective doses that were able to induce a 50% increase in the number of revertants and in the frequency of micronuclei induced by the extracts are shown in Fig. 5. They varied from 11 to 383 μg of EOM depending on the test and condition used (with/without metabolic activation). The *Salmonella*/microsome test was extremely sensitive to the mutagens contained in the intense burning extract, as only 11 μg of the EOM was enough to

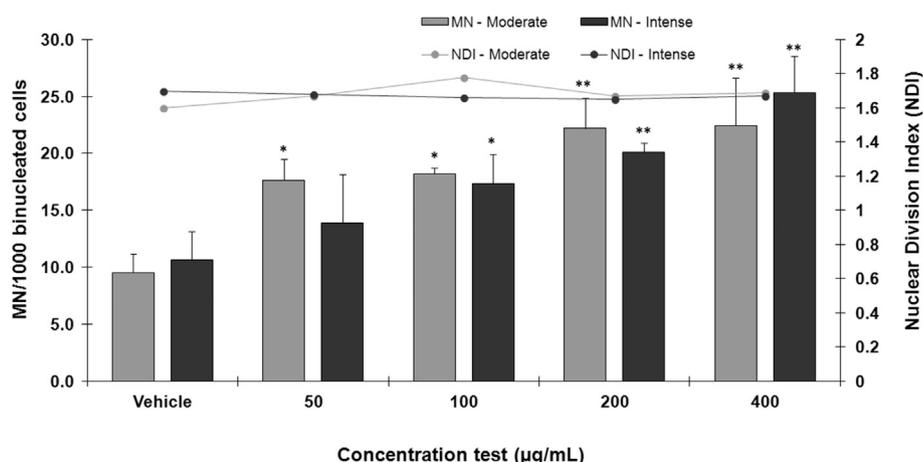


**Fig. 2.** Average concentrations of (A) nitro-PAHs and (B) oxy-PAHs for intense and moderate biomass burning periods. 9-NPA (9-nitrophenanthrene); 3-NPA (3-nitrophenanthrene); 2-NPA (2-nitrophenanthrene); 2-NA (2-nitroanthracene); 3-NFA (3-nitrofluoranthene); 2-NFA (2-nitrofluoranthene); 1-NPy (1-nitropyrene); 4-NPy (4-nitropyrene); 7-NBA (7-nitrobenz[a]anthracene); 6-NChr (6-nitrochrysene); 6-NBaP (6-nitrobenzo[a]pyrene).



**Fig. 3.** A549 cells viability after 24 h of exposure to different concentrations of extractable organic matter (EOM) for moderate and intense biomass burning samples. NC = negative control (DMSO 0.1%); PC = positive control (10 ng/mL of a PAHs mixture).

induce 50% increase in mutations. In comparison, the micronucleus assay required 176 µg of the EOM to induce a 1.5-fold increase in the micronucleus rate. For the moderate burning period extract, the two tests showed only small differences in sensitivity.



**Fig. 4.** Micronuclei frequencies and nuclear division index (NDI) in A549 cells for moderate and intense biomass burning samples, at all testes concentrations of Amazon extractable organic matter. Vehicle: DMSO 0.1%. \* $p < 0.05$  e \*\* $p < 0.01$ , statistically significant compared to negative control according to Dunnett's test.

#### 4. Discussion

In this study, we quantified the nitro-PAHs and oxy-PAHs present in the emitted PM from the Amazon biomass burning and estimate excess lifetime cancer risk as well as the *in vitro* mutagenic effects of the extractable organic matter. We also compared the sensitivity of a prokaryote (*Salmonella*/microsome test) and a eukaryote (CBMN with human lung cells) mutagenicity assays.

PM from burning of biomass can substantially degrade air quality, leading to adverse effects on human health (Jacobson et al., 2014; Reddington et al., 2015). Johnston et al. (2012) demonstrated that landscape fire emissions are an important contributor to global mortality. Around 15% of the Brazilian Amazon was deforested between 1976 and 2010 (Aragão et al., 2014) and the emissions resulting from burning of biomass from the Amazon region significantly enhanced the organic content of the fine aerosol above to 90% (Artaxo et al., 2013). The PM toxicity seems to be strongly influenced by the organic fraction, and is probably associated with organic components in addition to the commonly measured unsubstituted PAHs (Bølling et al., 2012).

During the intense period of biomass burning in Amazon, 7-nitrobenz[a]anthracene, 1-nitropyrene and 3-nitrophenanthrene outdoor concentrations were  $141 \pm 30$  pg/m<sup>3</sup> (range 63–174),  $125 \pm 28$  pg/m<sup>3</sup> (range 83–206), and  $60 \pm 28$  pg/m<sup>3</sup> (range 34–97),

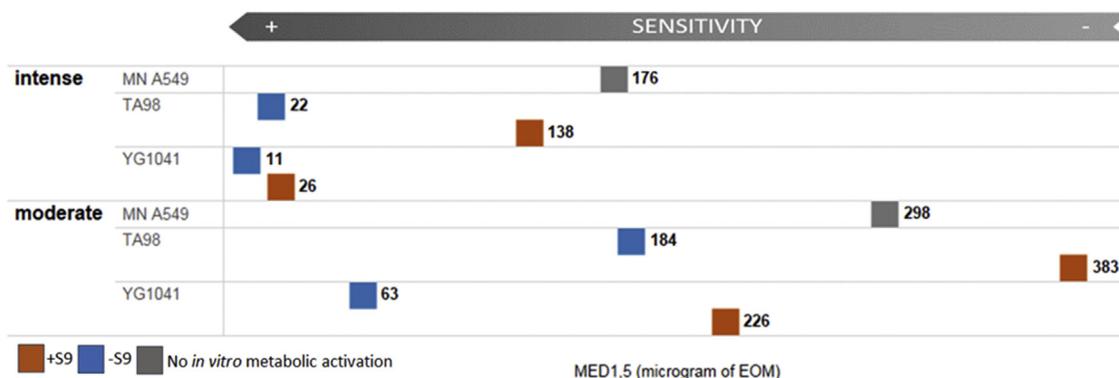


Fig. 5. Minimal effective doses (MED1.5) in µg of extractable organic matter (EOM), able to induce a 50% increase of the observed effect (revertants or micronuclei) in comparison with control values, for the intense and moderate biomass burning extracts.

respectively, in the ambient air (Fig. 2A). Shen et al. (2016) assessed the personal inhalation exposure to nitro-PAHs from residential solid fuel combustion in rural China and found values for 7-nitrobenz[a]anthracene (<30 pg/m<sup>3</sup>), 1-nitropyrene (<30 pg/m<sup>3</sup>), and 3-nitrophenanthrene (<15 pg/m<sup>3</sup>) below those found in the current study.

The LCR for nitro-PAH exposure was of  $4.2 \times 10^{-5}$ , and  $7.3 \times 10^{-5}$ , for moderate and intense burning periods, respectively (Table 2). Although these values did not exceed the WHO health-based guideline value ( $8.7 \times 10^{-5}$ ), they are relevant because LCR might be underestimated as only the three nitro-PAHs (1-nitropyrene, 4-nitropyrene, 6-nitrochrysene) with established TEFs were taken into account (OEHHA, 1994; 2005, 2011). To the best of our knowledge, no other study has demonstrated the inhalation cancer risk estimated for nitro-PAHs from biomass burning. The LCR calculated for the  $\Sigma$ 8PAHs identified in the same samples were  $7 \times 10^{-5}$ , and  $17 \times 10^{-5}$  for moderate and intense seasons, respectively (de Oliveira Alves et al., 2015). While LCR values between  $10^{-4}$ – $10^{-6}$  suggest potential risks, values smaller than  $10^{-6}$  are considered as a negligible risk, and above  $10^{-4}$  represent a high risk (Chen and Liao, 2006).

Interestingly, studies that estimated LCR for exposure to PAHs in urban PM samples in New York, USA ( $4.2 \times 10^{-5}$ ) (Jung et al., 2010); Nanjing, China ( $1.44 \times 10^{-5}$  for male adults) (Wang et al., 2016), and Madrid, Spain (Mirante et al., 2013) estimated a lower carcinogenic risk than the established standards by environmental and health agencies. Higher LCR values were obtained for Kandy, Sri Lanka ( $3.31 \times 10^{-3}$ ) (Wickramasinghe et al., 2011); Zaragoza, Spain ( $1.9$ – $8.2 \times 10^{-5}$ ) (Callén et al., 2014) and Cordoba, Argentina ( $5.13$ – $12.3 \times 10^{-5}$ ) (Amarillo et al., 2014).

Despite the more pronounced toxic effects of several nitro-PAHs than those of PAHs, among the derivatives PAHs identified in this study, only few of them have IARC classification (Bandowe and Meusel, 2017). 1-nitropyrene and 6-nitrochrysene are classified as human probable carcinogens (Group 2A), and 4-nitropyrene and anthraquinone as possibly carcinogen (Group 2B) (IARC, 2014). Further, despite the knowledge about genotoxicity and/or carcinogenicity of PAHs, there are limited/few studies concerning isolated nitro- and oxy-PAH compounds (Table S4).

Amongst the nitro-PAHs found in Amazon biomass burning in the current study, 1-nitropyrene and 6-nitrochrysene were reported as mutagenic for TA98 and TA100 *Salmonella* strains, mostly with a higher response without S9 than with S9 (Tokiwawa et al., 1981; Salmeen et al., 1982; Fu et al., 1988). 1-nitropyrene is the most studied compound which has also showed a positive response in mutagenesis assays (Durant et al., 1996). Bonnefoy et al. (2010) showed increase of mutagenicity for 1-nitropyrene in human

lymphocytes and the majority of micronuclei (63%) were generated without centromeres, suggesting a clastogenic effect of this compound. Recently, Cervena et al. (2016) found higher micronuclei frequencies in human bronchial epithelial cells (BEAS-2B) exposed to 1-nitropyrene.

Although oxy-PAHs are classified as possibly carcinogens by IARC, they are less studied than the nitro-derivatives. In addition, anthraquinone has been shown to lack any mutagenic effect in multiple models such as the *Salmonella* test, mouse lymphoma forward mutation assay, chromosomal alterations, and *in vivo* mouse bone marrow MN assay (Butterworth et al., 2001). Similar negative results were obtained by Durant et al. (1996) in the forward mutagenesis assay using h1A1v2 cells. Further, no significant DNA damage, as measured by the Comet Assay, was found in A549 cells exposed to 9,10-anthraquinone (Shang et al., 2013). 2-methylanthraquinone was identified at high concentrations in our samples, especially during intense biomass burning period (Fig. 2B). Shang et al. (2014) exposed A549 cells to this compound, and did not find statistically significant changes in the level of DNA tail moment (Comet assay) (Shang et al., 2014). This suggests that nitro- and oxy-PAH fractions present in the complex mixtures such as EOM, evaluated in this study, contribute differently to the mutagenic effects observed. Further, the contribution of nitro-PAHs seems to play a major role in the effects of EOM.

There are very few genetic toxicology studies that have assessed nitrated and oxygenated PAHs emitted from biomass burning. The use of different strains in the *Salmonella*/microsome assay is a strategy to detect compounds from different chemical groups that induce mutagenic activity (Mortelmans and Zeiger, 2000; Marvin and Hewitt, 2007; Mutlu et al., 2013). The YG *Salmonella* strains are more sensitive indicators of nitroaromatic mutagens than the classic TA strains (Claxton et al., 2004). In our study, the mutagenic responses were higher with *Salmonella* strain YG1041 compared to TA98 for both moderate and intense burning extracts, indicating the possible contribution of nitroaromatic compounds contained in the EOM from the air sampled in the Amazon region (Hagiwara et al., 1993; Umbuzeiro et al., 2008b). This can be explained by the fact that the elevated levels of nitroreductase and *O*-acetyltransferase in YG1041 could activate low concentration compounds present in the samples that could not be activated by the normal levels of nitroreductase present in TA98 (Einistö et al., 1991; DeMarini et al., 2004).

In addition, nitro-PAHs are known to be direct-acting mutagens (Fu, 1990; Jung et al., 1991; Claxton et al., 2004; Jariyasopit et al., 2014a, 2014b), and the higher mutagenic potencies were obtained for both strains (TA98 and YG1041) and both samples (moderate and intense) in the absence of metabolic activation (Table 3,

Tables S2 and S3).

Umbuzeiro et al. (2008a) used TA98 and YG1041 *Salmonella* strains to investigate the mutagenicity of organic fractions of PM from sugar cane harvesting season. The most polar fractions analyzed (nitro and oxy-PAHs) possessed highest mutagenicity. Consistent with the results obtained in the current study, a strong increase in the mutagenic activity using YG1041 stain and a decrease of response in the presence of *in vitro* metabolic activation were also observed. Similar results were obtained for urban atmospheric PM samples from other Brazilian cities, as São Paulo (Umbuzeiro et al., 2008b), Limeira (Alves et al., 2016), Montenegro and Santo Antônio da Patrulha (Pereira et al., 2013), and Esteio, which is under the influence of atmospheric emissions from an oil refinery (Coronas et al., 2009).

In this study, although the results point out to a strong involvement of nitro-PAHs in the mutagenicity observed, the experiments were done using whole extracts. From the *Salmonella*/microsome data it is not possible to directly correlate the increase of the mutagenic activity with only a specific class of compounds such as nitro-PAHs derived from the Amazon biomass burning. For this purpose, fractionation of the extracts and evaluation of the specific PAH derivatives should be performed.

The CBMN has become one of the standard cytogenetic assays for genetic toxicology testing in cultured human cells because scoring is restricted to binucleated cells, i.e. those that have divided once (Fenech, 2000, 2007). Moreover, the increase in MN formation is associated with early events in carcinogenesis, is predictive of cancer (Bonassi et al., 2011), and is a sensitive and specific predictor especially of lung cancer risk (El-Zein et al., 2006, 2008; Lloyd et al., 2013).

There are few reports assessing CBMN biomarkers in cultured cell lines exposed to PM from environmental air samples. The EOM from both moderate and intense Amazon biomass burning were able to induce an increase in the frequency of MN in A549 cells (Fig. 4). A pioneer study carried out by our group demonstrated genotoxic effects of organic PM collected in Alta Floresta city, also belonging to Brazilian Amazon deforestation arc (de Oliveira Alves et al., 2014). Despite the differences in the PAH concentrations between both regions, the data obtained for Porto Velho in this study are in consonance with those from Alta Floresta. Similar to treatment with samples from Porto Velho, a significant increase in the MN frequencies without an increment in the nucleoplasmic bridges and nuclear buds induction was observed on treatment of A549 cells with samples from Alta Floresta.

According Gutierrez-Castillo et al. (2006) and Roubicek et al. (2007) the water-soluble transition metals and the organic-soluble fraction of PM are both important in inducing DNA damage. However, organic extracts appeared to be more effective for the biological effects induced by the different wood combustion particles than the corresponding washed particles (Bølling et al., 2012). León-Mejía et al. (2016) assessed the genotoxic effects in Chinese hamster lung fibroblast cell line (V79) exposed to coal fly ash particles at concentrations similar to the ones used in this study (37.5–800 µg/mL). They found a dose-dependent increase in micronuclei formation, with statistical significance only at the concentration of 600 µg/mL. Our results show significantly increased MN formation due to exposure to organic extracts at concentrations ranging from 50 to 400 µg/mL (Fig. 4). Similar increase in MN frequencies was observed for the organic fraction from coke oven emissions in HepG2 cells (Xin et al., 2014) and for an urban sample from Italy in A549 cells (Bocchi et al., 2016).

The MED1.5 values showed that the *Salmonella*/microsome test was extremely sensitive to the mutagens contained in the intense burning period extract compared with CBMN in A549 cells (11 µg vs. 176 µg) (Fig. 5). Even bioassays that are indicative of the same

mode of action have slightly different numeric trigger values due to differences in their inherent sensitivity (Escher et al., 2015). As metabolism is the most crucial modifier of the biological effects of organic pollutants, detoxifying many chemicals and activating others, the metabolic capacity of a bioassay needs to be considered when selecting or designing a bioassay (Escher et al., 2014). The A549 cell line was chosen mostly because it maintains many features of normal type II cells from human pulmonary epithelium, expresses wild-type p53 and mRNAs for CYPs 1A1, 1B1, 2B6, 2C8-19, 2E1, 3A5, and 3A7 (Jia et al., 1997; Foster et al., 1998; Hukkanen et al., 2000; Roubicek et al., 2007). MED1.5 data (Fig. 5) reinforce the idea that majority of organic compounds contained in the samples act as direct mutagens, and suggest possible detoxification reactions in A549 cells.

It is important to point out that most of the PM<sub>10</sub> measurements did not exceed the 50 µg/m<sup>3</sup> limit established by the World Health Organization (WHO, 2005). Nevertheless, we observed mutagenic effects that can be associated with PAHs and their derivatives found in the particles from emissions due to biomass burning in the Brazilian Amazon region.

## 5. Conclusions

This study addressed the effects of extractable organic matter of PM emitted from Amazon biomass burning on DNA damage and showed its mutagenic activity using different bioassays. The *Salmonella*/microsome test was considerably more sensitive to the organic matter in comparison with the CBMN using human lung cells mainly for the intense burning period samples. This was the first study to assess the mutagenicity of the samples collected in Amazon region using *Salmonella*/microsome test. The data suggest that nitroaromatic compounds with direct-acting frameshift mutagenic activity contribute significantly to DNA damage. This study revealed that even though majority of PM<sub>10</sub> concentrations were lower than the air quality standards set by the World Health Organization, the strong presence of compounds with mutagenic effects, particularly nitro-PAHs and oxy-PAHs, and LCR values in the range of 10<sup>-5</sup>, indicate that the population is potentially exposed to an increased risk of DNA damage, mutation and cancer.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.envpol.2017.09.068>.

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