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RESEARCH ARTICLE

Mutagenicity assessment of aerosols in emissions from domestic combustion processes

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Abstract Domestic biofuel combustion is one of the major sources of regional and local air pollution, mainly regarding particulate matter and organic compounds, during winter periods. Mutagenic and carcinogenic activity potentials of the ambient particulate matter have been associated with the fraction of polycyclic aromatic hydrocarbons (PAH) and their oxygenated (OPAH) and nitrogenated (NPAH) derivatives. This study aimed at assessing the mutagenicity potential of the fraction of this polycyclic aromatic compound in particles (PM_{10}) from domestic combustion by using the Ames assays with *Salmonella typhimurium* TA98 and TA100. Seven biofuels, including four types of pellets and three agro-fuels (olive pit, almond shell and shell of pine nuts), were tested in an automatic pellet stove, and two types of wood (*Pinus pinaster*, maritime pine, and *Eucalyptus globulus*, eucalypt) were burned in a traditional wood stove. For this latter appliance, two combustion phases—devolatilisation and flaming/smouldering—were characterised separately. A direct-acting mutagenic effect for the devolatilisation phase of pine combustion and for both phases of eucalypt combustion was found. Almond shell revealed a weak direct-acting mutagenic effect, while one type of pellets, made of recycled wastes, and pine (devolatilisation) presented a cytotoxic effect towards strain TA100. Compared to the manually fired appliance, the automatic pellet stove promoted lower polyaromatic mutagenic emissions. For this device, only two of the studied biofuels presented a weak mutagenic or cytotoxic potential.

Keywords Mutagenicity · Polycyclic aromatic hydrocarbon · PM_{10} · Residential wood burning · Ames assay · Domestic combustion

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Introduction

Residential biofuel combustion is widely used for heating in many countries during winter, and it is known to be one of the major sources of regional and local air pollution (Bølling et al. 2009; Gonçalves et al., 2011; Canha et al. 2012), mainly regarding both gaseous and particulate pollutants, polycyclic aromatic hydrocarbons and volatile organic compounds (Alves et al. 2011a, 2014; Singla et al. 2012). Enhanced by the typical weather conditions of the cold winters from European countries (such as stagnation), emissions from residential biomass combustion have a higher impact in the surrounding environment promoting, for instance, levels of particulate matter comparable with areas with high traffic (Glasius et al. 2006). Moreover, these emissions may not have enough time for dilution, chemical oxidation and reaction before the population is exposed to them (Tissari 2008; Vicente

et al. 2015b), which enhances their exposure potential. Even indoor air quality may be affected by outdoor infiltration of wood smoke (Canha et al. 2014) enhancing the human exposure.

Several factors influence the emissions from residential wood smoke, such as the combustion appliances (e.g. open fireplace, wood and pellet stoves, masonry heaters, boilers for wood and wood chips and pellets) and their specific characteristics, where a great variability exists regarding the combustion technology and air supply (Bølling et al. 2009). Moreover, fuel type (e.g. wood logs, wood chips, pellets or agro-fuels) and condition of the fuel (e.g. moisture content, composition and log size) also influence the efficiency of the combustion (Calvo et al. 2014; Claxton et al. 2004; Claxton and Woodall 2007; Vicente et al. 2015a). Therefore, the physicochemical properties of particles emitted from residential biomass combustion differ considerably with combustion conditions and between combustion appliances (Vicente et al. 2015b).

Portugal has an estimated consumption of 2 Mton of wood in residential combustion, whose particulate emissions contribute to around 30 % of the total PM_{10} from all sectors of activity (Gonçalves et al. 2012). The use of new biofuels made of residues from other activities (such as forestry or furniture industry) for house heating has been promoted in the last years by national authorities (Proforbiomed 2012).

The organic chemicals associated with airborne particles, especially polycyclic aromatic hydrocarbons (PAHs), have been linked to the mutagenic and carcinogenic activity potentials (Bølling et al. 2009; Kamal et al. 2015; Kim et al. 2013; Naeher et al. 2007; Zelikoff et al 2002). In 2006, due to the scientific evidence, the International Agency for Research on Cancer (International Agency for Research on Cancer - IARC 2006) classified indoor emissions from household combustion of biomass fuel (mainly wood) as probably carcinogenic to humans (group 2A). Several studies showed that PAH and their derivatives from emissions of domestic cooking and heating are mutagenic, whose potential varies greatly depending on fuels, combustion appliances and extraction methods (Claxton et al. 2004; Claxton and Woodall 2007; Oanh et al. 2002; Vu et al. 2012).

The Ames assay is a plate incorporation test based on genetically engineered microorganisms, which is widely used as a quick screening tool for detecting mutagens (Claxton et al. 2004; Maron and Ames 1983; Mortelmans and Zeiger 2000). Two bacterial strains of *Salmonella typhimurium*—strain TA98 and strain TA100—are used to determine frameshift mutations and base-pair substitution mutations, respectively. Both strains are unable to produce histidine with each one carrying different mutations in various genes in the histidine operon. These mutations act as hot spots for mutagens that cause DNA damage via different mechanisms (Mortelmans and Zeiger 2000). Reversion of the strains to histidine-independent wild type under the application of a test substance is evidence of its mutagenicity, and moreover, its reversion frequency is usually related to the dose of the mutagen (Mukherji et al. 2002). Since these bacterial models lack the activating enzymes that are often responsible for conversion of some chemicals to mutagenic species, activating enzymes prepared from rat liver microsomal fractions (S9) may be added to the assay for identifying these indirect acting mutagens (Mukherji et al. 2002).

The main goal of this study was to evaluate the toxic effect of the fraction of PAHs and their nitrated and oxygenated derivatives (NPAHs and OPAHs) of particles (PM_{10}) from domestic combustion in order to: (i) characterise different biofuels and two typical combustion appliances, usually used in southern European countries, regarding the mutagenicity and, therefore, (ii) identify which fuel poses the least mutagenic risk and, subsequently, should be recommended for domestic use.

Materials and methods

Biomass fuel selection

Typical biofuels used for wood burning to promote household heating in southern European countries were selected. Two types of firewood, *Pinus pinaster* (maritime pine, which is a softwood) and *Eucalyptus globulus* (eucalypt, which is a hardwood), were selected since both represent the most prevalent tree species in Portugal, according to the Portuguese National Forestry Inventory (ICNF 2013) and both also have a large market share in the country. These wood types were used for feeding the manual appliance, which was a traditional cast iron wood stove.

Four types of pellets with different compositions were selected for the experiments in the automatic pellet stove. Pellets type I were composed mostly of pine wood and were EN-Plus labelled. Pellets type II were made of a mixture of lignocellulosic residues (75 %) and of dust from the furniture manufacturing industry (25 %). Pellets type III had the same components as pellets type II but in different proportions: 65 % of lignocellulosic residues and 35 % of dust from the furniture manufacturing industry. Pellets type IV were a blend of 50 % of waste woodchips and 50 % of dust from the furniture manufacturing industry.

Three specific agro-fuels were also selected since they represent common agricultural residues in southern European countries, and their use as energy source has been promoted by the national authorities (Proforbiomed 2012). The chosen agro-fuels were olive pit, almond shell and shell of pine nuts, which were also applied to the automatic pellet stove.

A detailed description of these biofuels can be found in Vicente et al. (2015a, 2015b), where characteristics such as

elemental composition, ash and moisture content and emission factors are described.

Combustion appliances

Two appliances that are common in southern European countries were used to carry out the combustion experiments: a batch-operated wood stove representing the heating device most widely used by households in Portugal (Gonçalves et al. 2012), in which the two types of firewood were burnt, and an automatic pellet stove symbolising the best-selling residential combustion device over the last years in the country, which was used to study the remaining biofuels.

The manually fed wood stove, model Sahara, was supplied by the Portuguese company Solzaima, which has a power output of 9.6–18.2 kW. The top-fed pellet stove, model Alpes, with a nominal output of 9.5 kW, was supplied by the same company.

Both combustion appliances were integrated in a system which was fully monitored regarding combustion parameters. A schematic representation of the experimental installation for both type of appliances can be found in Vicente et al. (2015b) and a fully and detailed description of the combustion facility can be found elsewhere (Calvo et al. 2014; Vicente et al. 2015a,b,c).

Combustion experiments and PM_{10} sampling

The combustion experiments were conducted in order to mimic the user behaviour. Full description of the experimental procedure of the combustion experiments for both combustion appliances can be found in Vicente et al. (2015b), with extensive characterisation of the operating conditions (e.g. rate of fuel consumption, average values of combustion chamber temperatures, among others). Several experiments were conducted for each type of biomass fuel, ranging from four to nine replicate tests per biofuel in the pellet stove (where the rate of fuel consumption ranged from 0.8 to 1.6 kg h^{-1}) and three experiments per firewood in the wood stove (with a rate of fuel consumption ranging from 1.5 to 2.3 kg h^{-1}).

Particulate matter (PM_{10}) was collected in a dilution tunnel (length of 11 m and an internal diameter of 0.20 m) connected to the exhaust stack under isokinetic conditions (Calvo et al. 2014, 2015; Vicente et al. 2015a,b) by a Gent sampler using Teflon membrane filters (47 mm diameter, Pall Corporation) and operating at a flow rate of around 12.4 L min^{-1}. The dilution tunnel enables the simulation of the rapid cooling and mixture that occurs when the exhaust gases are released into the atmosphere. This procedure promotes the decrease of temperature and the lowering of the vapour pressures of the gaseous species, which affects the gas to particle partitioning, increasing, therefore, the condensation of vapours on particle surface (Vicente et al. 2015b).

For the combustion experiment in the wood stove, two filters were collected per each experiment. The first filter was left during around 10 min after loading a batch of fuel until the O_2 content in the flue gas started to drop. This period corresponded to the initial heating and wood drying and the initial steps of the devolatilisation without the existence of a visible flame. The second filter was used for the remaining of the combustion cycle, which includes the flaming and smouldering phases. Each filter was analysed separately in order to evaluate both combustion stages.

For each study of a biofuel in the pellet stove, three levels of power output were evaluated, in order to evaluate the different behaviours by users. In each case, filters were sampled during around 1 hour under steady state conditions, which were evaluated by continuous monitoring of the flue gas composition.

For each type of combustion appliance, one blank sample of the combustion system was collected during the non-operating period.

Analysis of polycyclic aromatic compounds

The extraction of the fraction of polycyclic aromatic compounds (PACs) of the PM_{10} from the different biofuels and respective analysis is fully described elsewhere (Alves et al. 2011b; Vicente et al. 2015b). Table 1 displays the list of compounds that were quantified in samples, which includes 26 parent and alkyl-PAHs, 15 OPAHs (oxygenated-PAHs), 4 AZAs (azaarenes) and 15 NPAHs (nitrated PAHs). The filter samples from the combustion of biofuels in a wood stove were not extracted integrally as the filter samples from the combustion of biofuels in a pellet stove. The fractions of the filter samples that were extracted for these samples were 85 % of filter area of pine (devolatilisation phase) and 90 % of the filter area of the remaining samples (both combustion phases of eucalypt and flaming/smouldering phase of pine). The analytical procedures for the determination of the target compounds were described in detail by Bandowe and Wilcke (2010) and Bandowe et al. (2014). Briefly, the filters were spiked with deutered internal standards and extracted by pressurised liquid extraction with an accelerated solvent extractor. After concentration, the extracts were fractionated in a silica gel column using solvents of increasing polarity. Target compounds in the concentrated extracts were analysed by gas chromatography-mass spectrometry (GC-MS). Afterwards, the dried PAC extracts from each combustion experiment were dissolved in 1.6 mL of DMSO. Dilutions of these extracts were tested in the Ames assays.

Mutagenicity testing method

The total PAH extracts from each combustion experiment were tested for mutagenicity performing the Ames assay with

Table 1 List of the polycyclic aromatic compounds (PACs) measured in the present study and respective abbreviations

PACs	Abbreviation	PACs	Abbreviation
Alkyl + parents-PAHs		OPAHs	
1,2,3,4-Tetrahydronaphthalene	TH-NAP	1-Indanone	1-IND
Naphthalene	NAP	1,4-Naphthoquinone	1,4-NQ
2-Methylnaphthalene	2-MNAP	1-Naphthaldehyde	1-NALD
1-Methylnaphthalene	1-MNAP	2-Biphenylcarboxaldehyde	2-BPCA
Biphenyl	BiPHEN	9-Fluorenone	9-FLU
1,3-Dimethylnaphthalene	1,3-DMNAP	1,2-Acenaphthylenequinone	1,2-ACQ
Acenaphthylene	ACY	9,10-Anthraquinone	9,10-ANQ
Acenaphthene	AC	1,8-Naphthalic anhydride	1,8-NANH
Fluorene	FLO	4H-Cyclopenta[d,e,f]phenanthrenone	CpPHEone
Phenanthrene	PHE	2-Methylanthracene-9,10-dione	2-MANQ
Anthracene	ANT	Benzo[a]fluorenone	BaFLU
Cyclopenta[d,e,f]phenanthrene	CPHEN	7H-Benz[d,e]anthracene-7-one	BANTone
3,6-Dimethylphenanthrene	3,6-DMPHE	Benz[a]anthracene-7,12-dione	BANTdione
Fluoranthene	FLT	Naphthacene-5,12-dione	5,12-NACQ
Pyrene	PYR	6H-benzo[cd]pyrene-6-one	BPYRone
Retene	RET	NPAHs	
Benzo[a]anthracene	BaA	1-Nitronaphthalene	1-NitroNAP
Chrysene + Triphenylene	CHR + TRY	2-Nitrobiphenyl	2-NitroBP
Benzo[b,j,k]fluoranthene	BbjkF	5-Nitroacenaphthene	5-NitroAC
Benzo[e]pyrene	BeP	2-Nitrofluorene	2-NitroFLO
Benzo[a]pyrene	BaP	9-Nitroanthracene	9-NitroANT
Perylene	PER	9-Nitrophenanthrene	9-NitroPHE
Indeno[1,2,3-c,d]pyrene	IcdD	3-Nitrofluoranthene	3-NitroFLUA
Dibenzo[a,h]anthracene	DahA	1-Nitropyrene	1-NitroPYR
Benzo[g,h,i]perylene	BghiP	2,7-Dinitrofluorene	2,7-DNitroFLO
Coronene	COR	6-Nitrochrysene	6-NitroCHR
AZAs		3-Nitrobenzanthrone	3-NitroBANTone
Quinoline	QUI	1,3-Dinitropyrene	1,3-DNitroPYR
Benzo[h]quinoline	BQI	1,6-Dinitropyrene	1,6-DNitroPYR
Acridine	ACR	1,8-Dinitropyrene	1,8-DNitroPYR
Carbazole	CBZ	6-Nitrobenzo[a]pyrene	6-NitroBaP

S. typhimurium TA98 and TA100 strains, with and without metabolic activation by the S9 fraction (Trinova Biochem GmbH, Giessen, Germany) to assess the direct and indirect-acting, frameshift and base-pair substitution mutagens.

Since the sample volume available for analysis was limited (only 1.6 mL), each sample was tested at its maximum concentration and, when results were not conclusive, the sample was tested in series of four to five doses (using geometric progressions) within the range of minimum and maximum concentration, in order to investigate a concentration-dose response. Three replicates were always performed for each analysed dose.

The test was performed in plates containing two distinct layers of agar: 25 mL of the bottom agar (glucose minimal agar) to provide support media and 2 mL of top agar to deliver the extract concentration, the S9 mix (in assays with S9) or buffer (in assays without S9) and the tester strain to the bottom agar. All reagents were prepared according to Maron and Ames (1983) and Mortelmans and Zeiger (2000).

The tester strain was pre-incubated overnight in Oxoid nutrient Broth number 2 (Oxoid, England). The rat liver microsomal fractions for metabolic activation S9 was obtained in lyophilised form, purchased from Trinova Biochem GmbH. In each test, a negative control consisting of sterilised water blank (50 μL/plate) was included to determine the spontaneous revertants. A second negative control using DMSO solvent was included in all the tester strains in order to determine as well the spontaneous revertants.

In addition, a positive control consisting of known mutagens was used to confirm the reversion properties and specificities of each strain, activity of the S9 mix and other components presented in the assay. For experiments without S9, 2-nitrofluorene (10 μg/plate) and sodium azide (10 μg/plate) were used in positive controls for test with *S. typhimurium*

TA98 and TA100, respectively. For assays with the inclusion of S9, 2-aminoanthracene (10 μg/plate) was used in positive control for both bacterial strains, TA98 and TA100. The plates were incubated at 37 °C, for 48 h, and subsequently, the number of revertant colonies formed in each plate was counted.

Statistical analysis

Normality of data was assessed by Shapiro-Wilk test. The homoscedasticity of variances was tested by the Bartlett test. The evaluation of outliers within the dataset was conducted by applying the Grubbs test, which is recommended by ISO 5725-2 (International Standards ISO Guide 5725-2 1994). When suspicions arouse about the potential toxicity/mutagenicity of some extracts, one-way analysis of variance (ANOVA) followed by the multiple comparison Dunnett test were performed to check for a significant difference in the number of revertant colonies on plates in comparison with the negative control. A level of significance of $\alpha = 0.05$ was used to reject the null hypothesis.

Statistical analyses were conducted using software XLSTAT version 2014.5.03. Regression analyses, using the same software, were performed, when an apparent dose-related increase was recorded, to check for significant mutagenic effect.

A mutagenic effect was considered existent (i) when a dose-related significant increase in the number of revertant colonies was observed or (ii) when the average number of revertant colonies in the plates was two times greater than those recorded in the negative control plates (Mortelmans and Zeiger 2000). According to the OECD guidelines (1997), this last criterion is sufficient to assume mutagenic potential, even without a dose-effect relationship.

Results

A full description of the PAC contents is described by Vicente et al. (2015b), where the emission factors per compound, along with the full speciation of the PAC fractions for each studied biofuel, were presented. Figure 1 presents the overall emission factors of each studied PAC fraction, showing a great variability within biofuels and combustion appliances.

The emissions of PACs for the pellet stove ranged from 137 (pellet type III) to 835 (shell of pine nuts) μg kg^{-1} of fuel burned, while the emissions from wood stove ranged from 659 (eucalypt, flaming + smouldering) to 38,813 (pine, devolatilisation) μg kg^{-1} of fuel burned. The phase of devolatilisation showed the highest PAC emissions for both types of woods in the wood stove, although softwood emissions have been around eight times higher than those from hardwood. The combustion of conifer logs is characterised by higher burning rates, which promotes very hot flames and short and local drop of oxygen concentration, resulting in high emissions of PACs (Vicente et al. 2015b), as seen in this work.

Table 2 shows the highest mass of PACs in the extract of each biofuel that was tested by the Ames assay.

The number of the revertant colonies obtained from the mutagenic tests applied to PAC extracts of PM_{10} emitted in the combustion of the different biofuels in the two combustion appliances are presented in Tables S1, S2, S3 and S4 of Supplementary Information.

In some cases, more than one Ames assay was performed for the same sample due to initial high scattered results. Therefore, results were normalised regarding the respective negative controls, and mean ratios (number of revertant colonies of sample/number of revertant colonies of negative control, which is also called mutagenicity ratio (MR)) were calculated for each sample, which are presented in Table 3. When significant difference between the number of revertant colonies of the sample and the number of revertant colonies of the negative control in a specific Ames assay was found, mean ratios of all assays of that sample are shown. The assays with statistical differences are indicated in bold (Table 3).

A first evaluation of the results of the Ames assay testing the maximum concentrations of the PAC extracts regarding the strain TA98 in absence of S9 mixture pointed out four samples with ratios above 1.4, however still below 2 (twofold principle of mutagenicity confirmation), namely almond shell, eucalypt (both phases) and pine (devolatilisation). In these cases and when the standard deviation was high, a repetition of the Ames assay was conducted. When enough sample volume was available, the concentration-dose response for the samples was studied. For the second assay of the eucalypt samples (for devolatilisation and smouldering/flaming phases), ratios above 2 were obtained (both significantly different from the negative control, by an ANOVA followed by a Dunnet test), which confirms the mutagenic effect of these samples towards the strain TA98.

The concentration-dose response for the samples from the combustion of almond shell and the devolatilisation phase of pine was evaluated (Table S5 in Supplementary Information supplies the raw results). Only the sample from the coniferous wood presented a significant dose-related increase in the number of revertant colonies ($R^2 = 0.309$, p value 0.007). Although almond shell did not complied with any requirements to confirm its direct mutagenicity towards TA98 (ratio of revertant colonies of sample/negative control of 2 or significant regression between concentration of PAHs and number of revertant colonies), the ratio of 1.5 ± 0.5 may suggest a mutagenicity effect.

Two samples presented cytotoxic effects towards TA100, namely pellets type IV and pine (devolatilisation) with a significant decrease of 30 and 25 % of the revertant colonies,

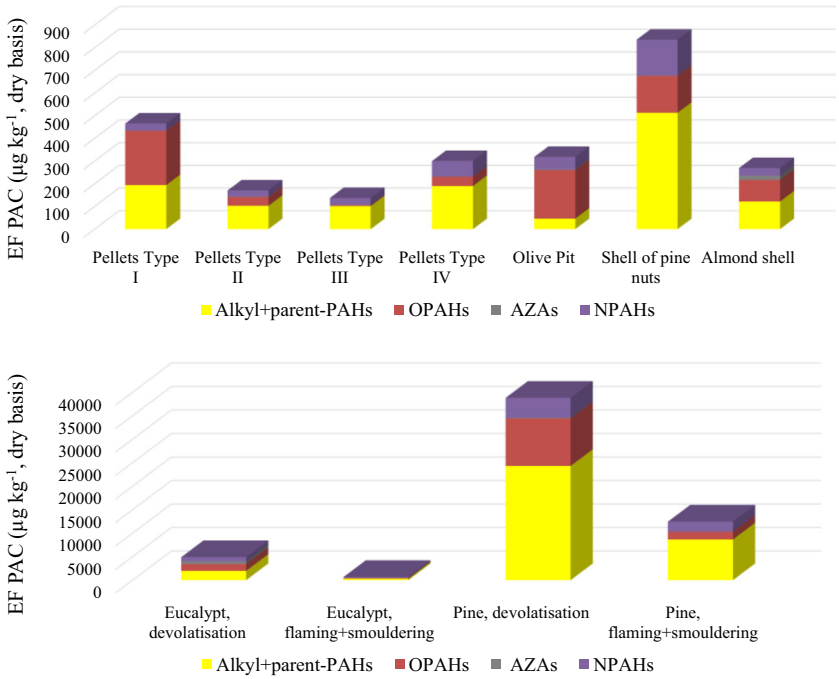

Fig. 1 Emission factors of the studied fractions of polycyclic aromatic compounds (PAC) of the different biofuels, micrograms per kilogram of biofuel burned (dry basis) (Vicente et al. 2015b), burned in an automatic pellet stove (*top*) and in a wood stove (*bottom*)

comparatively to the negative control. The concentration-dose response of pellets type IV was evaluated (Table S6 in Supplementary Information supplies the raw results), but no significant dose-related decrease was found.

Regarding the indirect-acting mutagenic effect towards the strain TA98 (assay with S9 mix), the combustion phase of devolatilisation for both samples of pine and eucalypt presented similar ratios (between 1.3 and 1.4). For the strain TA100, samples of eucalypt (devolatilisation) and pine (smouldering + flaming) also presented ratios around 1.4. However, the existence of mutagenic activity of these samples towards both strains could not be confirmed.

Table 2 Maximum PAC masses (ng) studied by the Ames in each extract solutions

Combustion appliance	Sample	PAC masses (ng)
Automatic pellet stove	Pellets type I	244
	Pellets type II	239
	Pellets type III	222
	Pellets type IV	238
	Olive pit	255
	Shell of pine nuts	278
	Almond shell	254
Wood stove	Eucalypt, devolatisation	699
	Eucalypt, smouldering + flaming	327
	Pine, devolatisation	796
	Pine, smouldering + flaming	755

Discussion

Three of the studied samples had emissions with mutagenic activity in Ames assay with *S. typhimurium* TA98, namely eucalypt (both phases of combustion) and pine during the devolatilisation phase. Almond shell showed a potential mutagenic effect, which was not confirmed latter. Therefore, these results show the direct-acting mutagenicity of the studied extracts, suggesting a shift mutation mechanism in the induction of the mutagenicity (Mortelmans and Zeiger 2000). Moreover, it is important to highlight that the PAC extracts that were evaluated for the existence of a mutagenic potential were diluted, which means that samples presenting a high ratio, although below 2 and not significant (such as samples from almond shell combustion or from the devolatilisation phase of pine in TA98 assays), may present a mutagenic effect for their original concentrations in the extracts.

Pellets type IV and pine (devolatilisation phase) have shown a cytotoxic effect towards strain TA100, since significant lower number of revertants on the plates were found when comparing with the negative control plates. The cytotoxic effect was registered at the highest PAC masses for both samples, namely 238 and 796 ng for pellets type IV and pine extracts, respectively. The toxicity restrained the growth of bacteria, which may mask the mutagenicity of the samples. Vu et al. (2012) also found similar toxic effect of pine wood combustion using a fireplace (cold start) towards the strain TA98 with metabolic activation (PAHs mass of 115 ng) and using a wood stove (hot start) towards the strain TA100, also with metabolic activation (PAHs mass of 474 ng), which

Table 3 Ratios between number of revertants from samples and negative control for all the strains of *S. typhymurium* studied (TA98 and TA100) and different conditions (with and without metabolic activation with S9 mix)

		TA98		TA100		TA98/S9		TA100/S9	
Combustion appliance	Samples	Ratio	*n*	Ratio	*n*	Ratio	*n*	Ratio	*n*
	Positive control	11.3 ± 5.3	11	1.36 ± 0.29	6	30.9 ± 2.3	6	7.22 ± 0.88	3
	DMSO	1.05 ± 0.20	5	0.89 ± 0.20	3	0.97 ± 0.09	3	1.02 ± 0.14	3
Pellet stove	Pellet type I	1.25 ± 0.28	6	0.91 ± 0.18	3	0.88 ± 0.20	3	1.11 ± 0.12	3
	Pellet type II	0.94 ± 0.11	3	0.88 ± 0.16	3	0.70 ± 0.42	3	0.94 ± 0.13	3
	Pellet type III	1.32 ± 0.26	6	1.08 ± 0.12	3	0.92 ± 0.25	3	1.09 ± 0.18	3
	Pellet type IV	0.93 ± 0.10	3	*0.70 ± 0.11/0.81 ± 0.02*	3/3	1.06 ± 0.23	3	1.21 ± 0.23	3
	Olive pit	1.16 ± 0.33	6	0.98 ± 0.07	3	1.03 ± 0.05	3	1.19 ± 0.05	3
	Shell of pine nuts	1.12 ± 0.27	6	0.95 ± 0.06	3	0.82 ± 0.14	3	1.15 ± 0.06	3
	Almond shell	1.49 ± 0.46	6	0.93 ± 0.07	3	1.05 ± 0.08	3	0.99 ± 0.13	3
Wood stove	Eucalypt, devol.	1.62 ± 0.27/*2.08 ± 0.24*	3/2	0.84 ± 0.05	3	1.44 ± 0.09	3	1.41 ± 0.18	3
	Eucalypt, smoul. + flam.	1.43 ± 0.20/*4.12 ± 1.37*	3/3	0.86 ± 0.06	3	0.85 ± 0.23	3	1.20 ± 0.10	3
	Pine, devol.	1.53 ± 0.14	3	*0.75 ± 0.03*	3	1.33 ± 0.33	3	1.22 ± 0.41	3
	Pine, smoul. + flam.	1.09 ± 0.14	3	0.81 ± 0.08	3	1.25 ± 0.17	3	1.39 ± 0.12	3

n number of replicates, *italicized numbers* significant differences by one-way ANOVA followed by a Dunnet test ($p < 0.05$)

indicates that the cytotoxic effect is due to the metabolisation products of the PAC in the studied extracts. However, the present study suggests a direct-acting cytotoxic effect of pine wood towards only the strain TA100.

As seen above, from the studied biofuels in the automatic pellet stove, only pellets type IV (produced with 50 % of residues from furniture industry) showed a direct-acting cytotoxic effect towards the strain TA100. Vu et al. (2012) also found that briquettes, which were made of wastes from forest cleaning activities and/or from local wood processing industries, presented a cytotoxic effect, but only regarding strain TA98 with metabolic activation.

For all the above samples with direct-acting mutagenic or cytotoxic effect, when S9 was introduced into the studied strains, the previous effect disappeared. This indicates, for instance, that the samples containing direct-acting frameshift mutagens lost their mutagenicity after being metabolised by the enzymes from S9 liver fraction. This fact was also observed by Vu et al. (2012), with all studied samples losing their mutagenic effect after addiction of S9 fraction to the strains, as well by Oanh et al. (2002), who reported a decrease of mutagenicity of the organic extract from wood combustion when metabolic activation was added.

The mutagenicity of PAC extracts is greatly associated with moderately and highly polar classes of compounds, which tend to contain nitroaromatic compounds, aromatic amines and aromatic ketones (Claxton et al. 2004). The addition of S9 liver fraction is known to promote a decrease of the mutagenicity of more polar fractions, which is typical for compounds such as nitroarenes (Barale et al. 1994). In our study, the three samples that showed direct-acting mutagenicity regarding the strain TA98 lost their effect when metabolic activation was added. This might indicate that the polar fraction of these samples greatly contributed to their direct-acting mutagenicity. All three mutagenic samples assessed in this study were from wood stove, and all of them had the highest AZAs mass content within the samples studied in this appliance, namely 1695, 205 and 138 ng of AZAs compounds for, respectively, eucalypt (devolatilisation phase), eucalypt (smouldering/flaming phase) and pine (devolatilisation phase). For the pellet stove, almond shell was the only biofuel that showed a weak direct-acting mutagenic effect, being as well the sample with the highest AZA content within the studied biofuels (ranging from 67 ng from olive pit to 188 ng of almond shell) in this combustion appliance. All the four AZA compounds assessed in this study have shown to have mutagenic effect in Ames assay in previous works (Bleeker et al. 2002).

The PAC extracts of emissions from the wood stove were about 4- to 18-fold higher than those from the pellet stove, with the highest aromatic content for both type of woods registered during the start-up combustion phase, which lasts only around 10 min (Vicente et al. 2015b). In the pellet stove, within the biofuels studied, all three agrofuels showed higher PAC masses in the extracts than the four types of pellets.

No correlations between the assessed mutagenicity of the extracts and their PAC concentrations was found, which might indicate that the mixture also contained aromatics that were not determined in this work and may have direct-acting mutagenic properties.

Due to the small volume available per sample and its maximum PAC masses (ranging from 222 ng/plate for pellets type III to 796 ng/plate for samples from the devolatilisation phase of pine), it was not possible to perform further experiments. According to the OECD guidelines (1997), the recommended maximum test concentration in Ames assay is 5 mg/plate, which is higher than the concentrations of this work. Therefore, further evaluation should be conducted to assess the mutagenicity of these types of PAC extracts in higher concentrations to better simulate the real human exposure to biomass burning emissions due to their usual continuous and cumulative exposure in specific periods of the year, such as winter.

Conclusions

The present study evaluated the mutagenicity of PM_{10} emissions from the combustion of different biofuels in two specific combustion appliances commonly used in southern European countries.

PM_{10} emissions from wood stove presented the highest mass fractions of PACs than the pellet stove, for both types of studied wood (eucalypt and pine). The start-up phase of the combustion (devolatilisation) revealed much higher PAC contents than the remaining combustion process. PM_{10} released during the devolatilisation step, for both woods, have shown a direct-acting mutagenic effect through a shift mutation mechanism. A similar mutagenic effect was also registered for PM_{10} emitted during the following flaming and smouldering stages of eucalypt combustion.

The automatic pellet stove promoted lower PAC fractions in PM_{10} emissions, and no strong mutagenic effect was found for any of the studied biofuels. However, pellets made with a 50 % incorporation of industrial residues showed a direct-acting cytotoxic effect towards the strain TA100. A similar behaviour was found for the devolatilisation phase of pine combustion. From the new agro-fuels used in the pellet stove, almond shell presented a weak direct-acting mutagenic effect through a shift mutation mechanism.

This study provides information regarding the mutagenic potential of different biofuels for residential heating available on national southern European markets, where some have shown a strong or weak mutagenic effect, along with some cytotoxic effects. Therefore, these results may be useful for recommendations for policy makers, households and end users when choosing their combustion-based domestic heating systems and which biofuel to select, taking into account its mutagenic risk. Emission requirements for the eco-labelling or certification of small-scale combustion appliances must be mandatory in all countries. The market of firewood sales should be regulated, and all pellets sold at the market must have quality certification.

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References

Alves C, Gonçalves C, Fernandes AP, Tarelho L, Pio C (2011a) Fireplace and woodstove fine particle emissions from combustion of western Mediterranean wood types. Atmos Res 101:692–700

Alves CA, Vicente A, Monteiro C, Gonçalves C, Evtyugina M, Pio C (2011b) Emission of trace gases and organic components in smoke particles from a wildfire in a mixed e evergreen forest in Portugal. Sci Total Environ 409:1466–1475

Alves C, Nunes T, Vicente A, Gonçalves C, Evtyugina M, Marques T, Pio C, Bate-Epey F (2014) Speciation of organic compounds in aerosols from urban background sites in the winter season. Atmos Res 150: 57–68

Bandowe BAM, Wilcke W (2010) Analysis of polycyclic aromatic hydrocarbons and their oxygen-containing derivatives and metabolites in soils. J Environ Qual 39(4):1349–1358

Bandowe BAM, Meusel H, Huang R-J, Ho K, Cao J, Hoffmann T, Wilcke W (2014) $PM_{2.5}$-bound oxygenated PAHs, nitro-PAHs and parent-PAHs from the atmosphere of a Chinese megacity: seasonal variation, sources and cancer risk assessment. Sci Total Environ 473–474:77–87

Barale R, Giromini L, Ry SD, Barnini B, Bulleri M, Barrai I, Valerio F, Pala M, He J (1994) Chemical and mutagenic patterns of airborne particulate matter collected in 17 Italian towns. Environ Health Perspect 102:67–73

Bleeker EAJ, Wiegman S, de Voogt P, Kraak M, Leslie HA, de Haas E, Admiraal W (2002) Toxicity of azaarenes. Rev Environ Contam Toxicol 173:39–83

Bølling AK, Pagels J, Yttri KE, Barregard L, Sallsten G, Schwarze PE, Boman C (2009) Health effects of residential wood smoke particles: the importance of combustion conditions and physicochemical particle properties. Part Fibre Toxicol 6:29

Calvo AI, Tarelho LAC, Alves CA, Duarte M, Nunes T (2014) Characterization of operating conditions of two residential wood combustion appliances. Fuel Process Technol 126:222–232

Calvo AI, Martins V, Nunes T, Duarte M, Hillamo R, Teinilä K, Pont V, Castro A, Fraile R, Tarelho L, Alves C (2015) Residential wood combustion in two domestic devices: relationship of different parameters throughout the combustion cycle. Atmos Environ 116:72–82

Canha N, Freitas MC, Almeida-Silva M, Almeida SM, Dung HM, Dionísio I, Cardoso J, Pio CA, Caseiro A, Verburg TG, Wolterbeek HT (2012) Burn wood influence on outdoor air quality in a small village: Foros de Arrão, Portugal. J Radioanal Nucl Chem 291:83–88

Canha N, Almeida SM, Freitas MC, Wolterbeek HT, Cardoso J, Pio CA, Caseiro A (2014) Impact of wood burning on indoor $PM_{2.5}$ in a primary school in rural Portugal. Atmos Environ 94:663–670

Claxton LD, Woodall GM Jr (2007) A review of the mutagenicity and rodent carcinogenicity of ambient air. Mutat Res 636:36–94

Claxton LD, Matthews PP, Warren SH (2004) The genotoxicity of ambient outdoor air, a review: Salmonella mutagenicity. Mutat Res 567: 347–399

Glasius M, Ketzel M, Wåhlin P, Jensen B, Mønster J, Berkowicz R, Palmgren F (2006) Impact of wood combustion on particle levels in a residential area in Denmark. Atmos Environ 40:7115–7124

Gonçalves C, Alves C, Pio C (2012) Inventory of fine particulate organic compound emissions from residential wood combustion in Portugal. Atmos Environ 50:297–306

Gonçalves C, Alves A, Fernandes AP, Monteiro C, Tarelho L, Evtyugina M, Pio C (2011) Organic compounds in PM2.5 emitted from fireplace and woodstove combustion of typical Portuguese wood species. Atmos Environ 45:4533–4545

ICNF (2013) IFN6 - Áreas dos usos do solo e das espécies florestais de Portugal continental. Resultados preliminares. Instituto da Conservação da Natureza e das Florestas, Lisboa, p. 32. Available at: http://www.icnf.pt/portal/florestas/ifn/resource/ficheiros/ifn/ifn6-res-prelimv1-1

International Agency for Research on Cancer - IARC (2006) Press Release n° 172 - Indoor emissions from household coal combustion carcinogenic: women in low- or medium-resource countries most exposed. Available at http://www.iarc.fr/en/media-centre/pr/2006/pr172.html

International Standards ISO Guide 5725-2 (1994) Accuracy (trueness and precision) of measurement methods and results—part 2: basic method for the determination of repeatability and reproducibility of a standard measurement method.

Kamal A, Cincinelli A, Martellini T, Malik RN (2015) A review of PAH exposure from the combustion of biomass fuel and their less surveyed effect on the blood parameters. Environ Sci Pollut Res 22: 4076–4098

Kim KH, Jahan SA, Kabir E, Brown RJC (2013) A review of airborne polycyclic aromatic hydrocarbons (PAHs) and their human health effects. Environ Int 60:71–80

Maron DM, Ames BN (1983) Revised methods for the Salmonella mutagenicity test. Mutat Res 113:173–215

Mortelmans K, Zeiger E (2000) The Ames Salmonella/microsome mutagenicity assay. Mutat Res 455:29–60

Mukherji S, Swain AK, Venkataraman C (2002) Comparative mutagenicity assessment of aerosols in emissions from biofuel combustion. Atmos Environ 36:5627–5635

Naeher LP, Brauer M, Lipsett M, Zelikoff JT, Simpson CD, Koenig JQ, Smith KR (2007) Woodsmoke health effects: a review. Inhal Toxicol 19:67–106

Oanh NTK, Nghiem LH, Phyu YL (2002) Emission of polycyclic aromatic hydrocarbons, toxicity, and mutagenicity from domestic cooking using sawdust briquettes, wood, and kerosene. Environ Sci Technol 36:833–839

OECD (1997) Test Guideline 471. Bacteria reverse mutation. In: OCED Guidelines for the Testing of Chemicals. Organisation for Economic Co-operation and Development, Paris, France

Proforbiomed (2012) Promotion of residual forestry biomass in the mediterranean basin—situation report on forest biomass use in Mediterranean region. Work package leader: Slovenian Forestry Institute, Ljubljana, Project- MED number (1S-MED 10-009)

Singla V, Pachauri T, Satsangi A, Km K, Lakhani A (2012) Characterization and mutagenicity assessment of PM2.5 and PM10 PAH at Agra, India. Polycycl Aromat Compd 32:199–220

Tissari J (2008) Fine particle emissions from residential wood combustion. PhD Thesis, Kupio University Publications C, Natural and Environmental Sciences, Finland.

Vicente ED, Duarte MA, Calvo AI, Nunes TF, Tarelho L, Alves CA (2015a) Emission of carbon monoxide, total hydrocarbons and particulate matter during wood combustion in a stove operating under distinct conditions. Fuel Process Technol 131:182–192

Vicente ED, Vicente AM, Bandowe BAM, Alves CA (2015b) Particulate phase emission of parent polycyclic aromatic hydrocarbons (PAHs) and their derivatives (alkyl-PAHs, oxygenated-PAHs, azaarenes and nitrated-PAHs) from manually and automatically fired combustion appliances. Air Qual Atmos Health. (in press) Doi: 10.1007/s11869-015-0364-1

Vicente ED, Duarte MA, Tarelho LAC, Nunes TFV, Amato F, Querol X, Colombi C, Gianelli V, Alves CA (2015c) Particulate and gaseous emissions from the combustion of different biofuels in a pellet stove. Atmos Environ 120: 15–27.

Vu B, Alves CA, Gonçalves C, Pio C, Gonçalves F, Pereira R (2012) Mutagenicity assessment of aerosols in emissions from wood combustion in Portugal. Environ Pollut 166:172–181

Zelikoff JT, Chen LC, Cohen MD, Schlesinger RB (2002) The toxicology of inhaled woodsmoke. J Toxicol Environ Health B 5:269–282