Toxicity and metal content of organic solvent extracts from airborne particulate matter in Puerto Rico

Alejandro R. Molinelli, Guido E. Santacana, Michael C. Madden, Braulio D. Jiménez

Abstract

The importance of airborne particulate matter (PM) in causing increases in morbidity and mortality in humans has been confirmed by numerous epidemiological and laboratory studies. It has been proposed that PM might deliver transition metals to the airways were they react and generate reactive oxygen species (ROS), thus promoting the expression of inflammatory mediators, and cytotoxicity. In Puerto Rico (PR), the northern Guaynabo area is a US EPA non-attainment zone for PM10 (PM with a mass median aerodynamic diameter ≤10 µm), and a previous study found that organic PM10 extracts from this area were cytotoxic. The purpose of this research project is to compare the toxicity between organic PM extracts from Guaynabo (a coastal urban site) and Fajardo (a coastal rural town) based on their polarity, collection season, and geographical location. We will also evaluate if the metal content of such extracts is associated with their biological activity. PM10 filters from both locations were subjected to a sequential Soxhlet extraction using hexane and acetone. Normal and transformed bronchial epithelial cells were then exposed to the extracts. Using the neutral red assay to measure cell viability we found that coastal urban PM from PR generally exhibits higher cytotoxicity than coastal rural PM. However, this effect is dependent on the polarity of the extracts and the collection season (in winter hexane PM10 is more toxic, whereas during the summer acetone PM10 is more toxic). We also found that non-polar organic constituents in PM from PR are generally more toxic than the polar organic constituents. The main conclusion from this work is that the metal contents of the organic PM extracts from PR could play a minor role in the cytotoxicity observed. This is supported by the findings of elements such as As, V, Ni, and Cu in the most cytotoxic extracts. However, organic compounds probably play the major role. The presence of bioactive fractions of PM underscores the importance of conducting more detailed studies.

Keywords: Particulate matter; Cytotoxicity; Organic extracts; Human cells; Puerto Rico

1. Introduction

During the last decade, the importance of airborne particulate matter (PM) in inducing increases in morbidity and mortality in humans has been confirmed by numerous epidemiological studies showing an association of PM concentration with these two health effects. Human exposure to elevated PM levels can induce airway inflammation leading to airway hyperreactivity (Ghio and Samet, 1999), airway and nasal damage (Gavett et al.,...
1997; Calderón-Garcidueñas et al., 2001), and predisposition to infection (Florey et al., 1979). These responses, observed to occur under controlled exposure conditions in vivo, may also occur in susceptible individuals exposed to ambient PM and could play a role in the associated increases in morbidity and mortality (Ghio and Devlin, 2001; Zanobetti et al., 2000). Laboratory studies have demonstrated that PM can alter the expression of multiple inflammatory mediators, up-regulate the expression of adhesion molecules on inflammatory cells (Salvi et al., 1999), and cause lipid peroxidation (Madden et al., 1999; reviewed in Ghio and Huang, 2004).

It is hypothesized that the toxicity of PM might be due to its organic content (Hannigan et al., 1998), its metal content (Ghio et al., 1996), the presence of endotoxins or other biologicals (Monn and Becker, 1999), the presence of acidic particles (Bascom et al., 1996), or its ultrafine components (Oberdörster et al., 1992). Recent research has focused the attention on PM metal content. It has been proposed that PM might deliver transition metals to the airways with subsequent reaction and generation of reactive oxygen species (ROS), thus promoting the expression of inflammatory mediators, and cytotoxicity (Frampton et al., 1999).

In the island of Puerto Rico (PR) the ambient air of the northern Guaynabo and Cantaño area (Fig. 1) has historically been perceived as polluted by its residents. This stems from the presence of heavy industrial activity and vehicle traffic. Furthermore, for several years this area has been designated by the US EPA as a non-attainment zone for PM$_{10}$ (PM with a mass median aerodynamic diameter \( \leq 10\mu m \)) (Puerto Rico Environmental Quality Board (PREQB), 1994; United States Environmental Protection Agency (US EPA), 2005). Epidemiological data from PR...
examining the relationship between morbidity and mortality and ambient PM concentrations is not available (the Puerto Rico Department of Health catalogs mortality data for multiple diseases, but no studies were found in the literature where it was compared to ambient PM concentrations).

Asthma is a pulmonary disease that affects a considerable amount of the people living in the northern Guaynabo and Cataño areas of PR. An epidemiological study examining the prevalence of asthma in this area shows that approximately 30% of the children between 13 and 14 years old, and approximately 40% of the children 6–7 years old suffer from asthma (Márquez et al., 1990). Another study found parent reported asthma and respiratory condition rates of approximately 40% in schoolchildren (5–7 years old) living in the San Juan metropolitan area (Nazario et al., 2004). These data starkly compare to the US national average for children 0–17 years old, where approximately 7% (at its peak in 1995) suffer from asthma (Akinbami and Schoendorf, 2002). The possible causes for this increased asthma incidence (relative to the US population) are uncertain. Some possibilities include the differential diagnosis of the disease, exposure to indoor air pollutants or allergens, or ambient air components in Guaynabo being inducers of asthma. Because Puerto Rican children living in the US also tend to suffer higher asthma rates (Hunninghake et al., 2006) we cannot discount the effect of genetic susceptibility.

A study by Reyes and collaborators (2000) examined the cytotoxicity, on normal human epidermal keratinocytes (NHEK), of organic extracts of PM from northern Guaynabo and Fajardo during 1996–1997. The Reyes study found that several extracts had biological activity and identified various organic compounds by GC/MS as possible contributors to the observed toxicity.

The findings of biologically active organic fractions in northern Guaynabo PM provide important data in starting to elucidate the nature of the air pollution of the area. However, the association between biological activity and metal content in organic PM extracts has not been evaluated and remains a potential confounding factor in those studies. Given the role metals might play in PM toxicity it is important to evaluate their possible contribution to the cytotoxicity of northern Guaynabo PM. The purpose of this research is to compare the cytotoxicity, in cultured lung epithelial cells, between organic PM extracts from Guaynabo and Fajardo (a coastal reference site) based on their polarity, collection season, and geographical location. We will also evaluate if the metal content of such extracts is associated with their cytotoxicity. Based on previous studies (Reyes et al., 2000) we expect to find that the urban coastal (Guaynabo) PM is more cytotoxic than the rural coastal (Fajardo) PM. We would also expect to find more metals on the polar fraction rather than the non-polar fraction as metals, being ionic compounds, extract better in aqueous conditions (United States Environmental Protection Agency (US EPA), 2004a). Based on this we would expect the polar fractions to be more cytotoxic. In terms of collection season we expect PM collected in the summer to be more cytotoxic than the winter PM as there is a higher amount of PM present in the summer months than in winter months (Fig. 2). Although our findings supported some of our hypotheses the results did differ from what was expected. Finally, it is known that acetylcholine release from parasympathetic nerve terminals is the most important bronchoconstriction pathway in rats and humans (Barnes, 1987). Electrical field stimulation (EFS) of isolated rat tracheas causes the release of endogenous acetylcholine procuring a physiological approach to the study of tracheal responses to a test agent. In this study, we will examine if PM affects tracheal contractility using the intact isolated rat trachea in vitro as a physiological model for airway responsiveness to environmental factors (Gonzalez and Santacana, 2001).

2. Methods

2.1. Description of study sites

The study sites for this project were northern Guaynabo and Fajardo, PR (Fig. 1). The municipality of Guaynabo is located on the north of the island and is part of the San Juan metropolitan area (PM monitoring station at 18.4394°N, 66.1150°W). This is an urban location with heavy industrial activity. The study area that served as reference is located in the municipality of Fajardo, at the northeastern tip of PR (PM monitoring station at 18.3833°N, 65.6194°W). The PM station is located on protected lands managed by the PR Conservation Trust. The area consists mainly of mangrove forest with very light vehicle traffic. Both of our sampling sites are located near the coast and are influenced by the land and sea breeze effect. Prevailing winds in the island blow from the east-northeast (National Oceanic and Atmospheric Administration (NOAA), 1982).

![Fig. 2. Average monthly PM10 and PM2.5 concentrations for the Guaynabo and Fajardo sites during 1999 and 2000 (±SEM). The annual PM10 arithmetic mean during 1999 at Guaynabo was 39.0 µg/m³ (n = 329), and at Fajardo, 27.0 µg/m³ (n = 207, t = 7.742, P < 0.0001). In the year 2000 the annual PM10 arithmetic mean at Guaynabo was 37.0 µg/m³ (n = 314), and at Fajardo, 24.0 µg/m³ (n = 282, t = 11.67, P < 0.0001). The annual PM2.5 arithmetic mean at Guaynabo during 1999 was 10.3 µg/m³ (n = 253), and at Fajardo, 6.8 µg/m³ (n = 143, t = 8.384, P < 0.0001). In the year 2000 the annual PM2.5 arithmetic mean at Guaynabo was 9.4 µg/m³ (n = 282), and at Fajardo, 5.1 µg/m³ (n = 291, t = 16.07, P < 0.0001).](image)
The annual normal temperature for San Juan ranges between 69.6°F and 86.6°F, and for Fajardo ranges between 71.7°F to 86.6°F. Temperature normals for PR between the summer and winter months varies by less than 7°F (National Oceanic and Atmospheric Administration (NOAA), 2004).

2.2. PM sampling and extraction

PM_{10} was collected by the Puerto Rico Environmental Quality Board (PREQB). The PREQB provided \( \frac{1}{2} \) of a quartz PM\(_{10}\) filter (8'' \( \times \) 10'') for each day of the week of August 22–26, 1999 (summer) and February 15–17, 2000 (winter).

All glassware used for extraction and analysis of metals was quantitatively washed using a modified cleanup procedure described in Method 7000A (United States Environmental Protection Agency (US EPA), 1992). PM was extracted from the filters by using a sequential Soxhlet extraction starting with 175 mL of hexane (Fisher: HPLC grade) for 24 h and followed with 175 mL of acetone (Fisher: HPLC grade) for 24 h (based on method 3540C, United States Environmental Protection Agency (US EPA), 1996). To assure consistency between samples the extraction process is adjusted to achieve \( \approx 6 \) cycles/h. Based on the amount of starting material present on the filters and the extract weight obtained the amount of mass extracted from the filters ranges from 15% to 40%.

The samples were then concentrated to approximately 10 mL using a rotary evaporator, split in equal volumes, and dried under a stream of nitrogen. The extracts were weighted and resuspended for cell treatments or atomic absorption spectrometry analysis.

2.3. Measurement of metals

Dried and weighted PM\(_{10}\) extracts were resuspended in 15 mL of HNO\(_3\) (2%). As, Cu, Cd, and Pb were quantified using a Perkin-Elmer (Norwalk, CT) atomic absorption spectrophotometer model 1100B using the graphite furnace mode. Ni, V, and Fe were analyzed using a Perkin Elmer AAAnalyst 800 atomic absorption spectrometer equipped with a Transversely Heated Graphite Furnace with longitudinal Zeeman-effect background corrector. All samples were analyzed based on EPA test methods available from publication SW-846 (United States Environmental Protection Agency (US EPA), 2003). The specific method used and general conditions are summarized in Table 1. The results are presented as \( \mu \)g metal per g of PM extract.

Working standards (at least four) were made by serial dilutions of a stock solution of 1000 ppb. Linear external calibration curves were prepared daily from the intermediate standards. Initial Calibration Verification (ICV) and Continuous Calibration Verification (CCV) standards were prepared from commercially available, mixed metal standards (ULTRAcheck Trace Metals Sample QCI-700, Ultra Scientific, North Kingstown, RI). To assure there was no carry over between samples an Initial Blank Verification (IBV) and Continuous Blank Verification (CBV) were also run. To assure the accuracy of the instrument readings we included blank spikes, matrix spikes, and bench spikes in all runs. Additionally, several preparation blanks were employed to determine if there was contamination from any step in the preparation (Table 1).

2.4. Cell treatments and viability measurements

All cell treatments were performed on normal human bronchoepithelial cells (NHBE) and transformed human bronchoepithelial cells (BEAS-2B). NHBE cells were purchased from Clonetics Corporation (San Diego, CA). The cell media used was Clonetics bronchial epithelial cell growth medium (BEGM) supplemented with 13 mg/mL bovine pituitary extract, 0.5 mg/mL hydrocortisone, 0.5 mg/mL human epidermal growth factor, 0.5 mg/mL epinephrine, 10 mg/mL transferrin, 5 mg/mL insulin, 6.5 mg/mL triiodothyronine, 0.5 mL GA-1000, and 0.1 mg/mL retinoic acid.

BEAS-2B cells (S6 subclone; passages 72–95) were obtained courtesy of Drs. Curtis Harris and John Lechner from the National Institutes of Health (Reddel et al., 1988). This is an immortalized line of normal human bronchial epithelium. The cell media used was Clonetics keratinocyte growth medium (KGM), which is essentially MCDB 153 medium supplemented with 5 mg/mL human epidermal growth factor, 5 mg/mL insulin, 0.5 mg/mL hydrocortisone, 4 mL/L bovine pituitary extract, and 0.5 mL of GA-1000. All cells were grown at 37 °C and 5% CO\(_2\) with fresh media being added every other day. When cells reached 95% confluence they were split, counted, and seeded onto 96-well tissue culture microtiter plates at a density of approximately 3000 cells/well. After 2 days (when cells reached 90–95% confluence) the cell cultures were washed twice with phosphate-buffered saline (NHBE) or HEPES buffer (BEAS-2B) and fresh KGM containing the PM extracts resuspended in DMSO was added. For the NHBE cells we used 6–10 different concentrations of the PM extracts and for the BEAS-2B cells we used three different concentrations (10, 100, and 250 μg/mL). The amount of available material limited the number of doses we could use on the BEAS-2B cells. Also included in the treatments were positive controls consisting of Triton X-100. These controls are included on each plate to assure the assay is performing consistently. Fig. 4 shows the dose-response curve for Triton X-100 plotted using pooled data from all plates. Negative controls containing KGM alone and KGM+DMSO were also present. The DMSO concentration in the media for all exposed groups did not exceed 1% (NHBE) or 3.8% (BEAS-2B). A higher dose of DMSO was required for the BEAS-2B cell assays because material limitations required us to concentrate the PM and consequently its vehicle. However, all viability calculations are performed using controls containing the vehicle and DMSO. DMSO at 3.8% does not reduce cell viability below 70%. Each well contained the extracts or controls at a final volume of 200 μL. After 48 h the neutral red bioassay (NR) was done to determine viability. The

<table>
<thead>
<tr>
<th>Method(^a)</th>
<th>Pb</th>
<th>Cd</th>
<th>Cu</th>
<th>Fe</th>
<th>V</th>
<th>Ni</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method(^a)</td>
<td>7060A</td>
<td>7421</td>
<td>7131A</td>
<td>7211</td>
<td>7380</td>
<td>7911</td>
</tr>
<tr>
<td>( \lambda ) (nm)</td>
<td>193.7</td>
<td>283.3</td>
<td>228.8</td>
<td>324.7</td>
<td>248.3</td>
<td>318.4</td>
</tr>
<tr>
<td>Matrix modifier</td>
<td>Pd</td>
<td>(NH(_4))(_2)HPO(_4)</td>
<td>(NH(_4))(_2)HPO(_4)</td>
<td>Pd</td>
<td>Mg(NO(_3))(_2)</td>
<td>None</td>
</tr>
<tr>
<td>Quantification limit (μg/L)</td>
<td>2.0</td>
<td>0.5</td>
<td>0.1</td>
<td>0.0</td>
<td>0.0</td>
<td>19.5</td>
</tr>
<tr>
<td>Prep. Blk. HNO(_3) 2% (μg/L)</td>
<td>0.4</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>15</td>
<td>0.1</td>
</tr>
<tr>
<td>Prep. Blk. acetone (μg/L)</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>18.9</td>
<td>0.0</td>
</tr>
<tr>
<td>Prep. Blk. hexane (μg/L)</td>
<td>1.7</td>
<td>0.1</td>
<td>0.0</td>
<td>0.0</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>Spike concentration (μg/L)</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>(^b)</td>
</tr>
<tr>
<td>Acetone spike recovery</td>
<td>50%</td>
<td>106%</td>
<td>94%</td>
<td>100%</td>
<td>95%</td>
<td>(^b)</td>
</tr>
<tr>
<td>Hexane spike recovery</td>
<td>102%</td>
<td>88%</td>
<td>84%</td>
<td>92%</td>
<td>99%</td>
<td>(^b)</td>
</tr>
</tbody>
</table>

\(^a\)The method row refers to the EPA preparatory method as referenced in the methodology section.

\(^b\)Vanadium spikes were not included because at the time the extraction was performed, it was not planned to assay for this metal.
neutral red bioassay is based on the ability of viable cells to take-up and bind the neutral red dye (2,8-phenazinediame, N8, N8, 3-trimethyl-monohydrochloride) (Borenfreund and Puerner, 1985). The NR bioassay was previously validated for use with PM by using a standard reference material (SRM-1649) (Reyes et al., 2000).

After PM exposures the cells were incubated with NR (37 °C, 5% CO₂) for 3 h. The dye was then removed and the cells fixed with a formaldehyde solution. A solvent solution containing acetic acid and ethanol was then added to the cells to allow the release of the NR dye. Finally, the absorbance at 540 nm for each well was determined spectrophotometrically by using an Ultramark microplate reader (BioRad, Hercules, CA).

To calculate the percent viability of the treatment groups the average absorbance of its negative control (media alone for the Triton X-100 treated solution. A solvent solution containing acetic acid and ethanol was then added to the cells to allow the release of the NR dye. Finally, the absorbance at 540 nm for each well was determined spectrophotometrically by using an Ultramark microplate reader (BioRad, Hercules, CA).

The average monthly PM10 and PM2.5 (PM with a mass median aerodynamic diameter ≤2.5 μM) concentrations for the Guaynabo and Fajardo sites during the years 1999 and 2000 are shown in Fig. 2 (data provided by the PREQB as reported to the US EPA aerometric information retrieval system in 2000). The mean PM concentrations between Guaynabo and Fajardo are significantly different for both 1999 and 2000. Using the PREQB data we calculated that at the Guaynabo site the PM₁₀/PM₅₀ ratio was 27.3% during 1999 and 25.8% during 2000. For the Fajardo site the ratio is 22.1% for 1999 and 22.7% for 2000. The difference in PM₁₀/PM₅₀ ratios between Guaynabo and Fajardo is statistically significant for both 1999 and 2000 (t = 2.618, p = 0.0180 for 1999; t = 2.176, p = 0.0411 for 2000).

3.2. Metal content of PM₁₀ extracts

Most of the PM₁₀ sample extracts analyzed had higher concentrations of metals in the acetone fraction than the hexane fraction. This finding is not surprising since most metals in ambient PM₁₀ are found in water-soluble form, such as sulfates, humates, or citrates (Frampton et al., 1999), thus, having higher affinity for the polar solvent.

In general the acetone PM₁₀ extracts from Guaynabo (urban site) contained higher concentrations of most of the metals assayed when compared to Fajardo (reference site). The acetone PM₁₀ extract from Guaynabo during the winter consistently had higher concentrations for all metals with the exception of arsenic, which was undetectable. The rest of the acetone PM₁₀ extracts analyzed did not exhibit any consistent seasonal or geographical trend.

Some of the hexane PM₁₀ extracts contained appreciable concentrations of metals and showed a strong seasonal association. All hexane extracts collected in winter had higher metal concentrations than those collected in the summer, independent of location. The hexane extract from Fajardo collected during the winter had the highest concentration of all metals with the exception of arsenic. Furthermore, within the same season, the Fajardo hexane extracts always had higher metal concentration.

A summary of the metal findings per extract is shown in Tables 2a and 2b, and Fig. 3. When the metal concentrations are added, the acetone PM₁₀ extracts from Guaynabo have approximately twice as much of the metal as the Fajardo extracts. However, the hexane PM₁₀ extract from Fajardo in the summer has three times as much of the metal as the Guaynabo extract. The hexane PM₁₀ extract from Fajardo during winter had slightly more metal content than the Guaynabo extract. Fig. 3 also illustrates that copper and iron make up the bulk of the metal content for all extracts. It should be noted that for the PM₁₀ hexane extracts iron makes up most of the bulk. Conversely, for the acetone extracts, copper constitutes the most abundant metal. Arsenic was only present in two samples: the acetone extract from Guaynabo during the summer, and the hexane extract from Guaynabo during the winter.
<table>
<thead>
<tr>
<th></th>
<th>NR$_{50}$ (NHBE)</th>
<th>NR$_{50}$ (BEAS-2B)</th>
<th>Lead</th>
<th>Cadmium</th>
<th>Iron</th>
<th>Copper</th>
<th>Vanadium</th>
<th>Nickel</th>
<th>Arsenic</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) Descending order of toxicity (NR$<em>{50}$ = µg/mL) and metal concentration (µg/g) on hexane PM$</em>{10}$ extracts</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>More toxic</td>
<td>Guaynabo Winter (11.0)</td>
<td>Guaynabo Winter (55.0)</td>
<td>a</td>
<td>Fajardo Winter (2.1)</td>
<td>a</td>
<td>Fajardo Winter (83.5)</td>
<td>Fajardo Winter (13.6)</td>
<td>Fajardo Winter (41.8)</td>
<td>Guaynabo Winter (6.2)</td>
<td>More metals</td>
</tr>
<tr>
<td></td>
<td>Fajardo Winter (12.4)</td>
<td>Guaynabo Summer (28.7)</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>Guaynabo Winter (77.0)</td>
<td>Guaynabo Summer (226.7)</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>Least toxic</td>
<td>Fajardo Summer (83.5)</td>
<td>Guaynabo Summer (&gt; 250.0)</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>(b) Descending order of toxicity (NR$<em>{50}$ = µg/mL) and metal concentration (µg/g) on acetone PM$</em>{10}$ extracts</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>More toxic</td>
<td>Guaynabo Summer (15.2)</td>
<td>Guaynabo Summer (81.3)</td>
<td>Guaynabo Winter (142.0)</td>
<td>Guaynabo Winter (9.1)</td>
<td>Guaynabo Winter (463.9)</td>
<td>Guaynabo Winter (1863.4)</td>
<td>Guaynabo Winter (45.6)</td>
<td>Guaynabo Winter (76.0)</td>
<td>Guaynabo Summer (5.1)</td>
<td>More metals</td>
</tr>
<tr>
<td></td>
<td>Fajardo Summer (51.6)</td>
<td>Fajardo Summer (111.5)</td>
<td>Fajardo Winter (41.9)</td>
<td>Guaynabo Summer (8.9)</td>
<td>Fajardo Winter (317.4)</td>
<td>Guaynabo Summer (1684.4)</td>
<td>Guaynabo Summer (8.0)</td>
<td>Fajardo Winter (54.5)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Guaynabo Winter (75.8)</td>
<td>Fajardo Winter (132.4)</td>
<td>Guaynabo Summer (35.1)</td>
<td>Fajardo Summer (2.6)</td>
<td>Guaynabo Summer (295.0)</td>
<td>Fajardo Winter (984.6)</td>
<td>Fajardo Summer (2.6)</td>
<td>Guaynabo Summer (16.3)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Least toxic</td>
<td>b</td>
<td>Guaynabo Winter (210.3)</td>
<td>Fajardo Summer (5.7)</td>
<td>Fajardo Winter (1.0)</td>
<td>Fajardo Summer (67.7)</td>
<td>Fajardo Summer (591.9)</td>
<td>Fajardo Winter (ND)</td>
<td>Fajardo Summer (7.2)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ND = non-detectable.

* Metal concentration on blank filter extracted with hexane higher than in sample (Pb = 11.9, Cd = 1.9, Fe = 797.0). ND = non-detectable.

* Due to material limitations it was not possible to obtain an NR$_{50}$ value for the Fajardo-winter PM$_{10}$ acetone extract using the NHBE cells. However, at 20 µg/mL (the highest dose tested with the available material) viability remained above 75% of controls.
3.3. Biological activity of PM10 extracts

Both cell types (NHBE and BEAS-2B) were incubated with different concentrations of the membrane-damaging agent Triton X-100 for 48 h after which the NR bioassay was performed (Fig. 4). We calculated an NR50 for the NHBE cells of 33 \( \mu \text{g/mL} \), and for the BEAS-2B cells of 31 \( \mu \text{g/mL} \). These values are similar to the range reported for other mammalian cell types (e.g., keratinocytes), which is 3–32 \( \mu \text{g/mL} \) (Reyes et al., 2000).

To gain a better understanding of the potency of the extracts we calculated the NR50 of residual oil fly ash (ROFA), a combustion derived particle rich in metals such as iron, vanadium and nickel (Ghio et al., 2002). The NR50 value we obtained was \( 2410 \mu \text{g/mL} \).

Tables 2a and 2b show the NR50 values obtained after exposing the NHBE and BEAS-2B cells to varying concentrations of the PM10 extracts. Fig. 5 shows the dose–response curves obtained from the NHBE cells. The results indicate that within the same season and solvent used the extracts from Guaynabo were more toxic than those from Fajardo. We also found that the hexane PM10 extracts from the winter season were more toxic than the corresponding summer extracts, and the acetone extracts from the summer were more toxic than those from the winter. Overall, the hexane extracted PM10 samples from the winter season were the most toxic, followed by the summer extracts in acetone. It is important to consider that the amount of airborne PM10 in the summer is twice of that obtained in winter (Fig. 2).

Our results suggest that in terms of viability the NHBE cells are more sensitive to the PM extracts than the BEAS-2B cells, as the NR50 values were lower and exhibited smaller differences between samples. When we compared the combined NR50 values of the NHBE cells (all treatments included) with those from the BEAS-2B cells we found that their means were significantly different (\( t = 4.022, p = 0.0069 \)). However, it is important to note that the outcome of the toxicity tests were similar for both cell types. The potency ranking order for the PM10 hexane extracts (summer and winter) and the summer acetone extracts is similar among cell types; however, it is inverted for the winter acetone extracts. The NR50 values of the NHBE cells correlate with the NR50 values of the BEAS-2B cells, thus both cell types show a similar association of toxicity (\( r^2 = 0.8563; p = 0.0028 \)), although their specific NR50 values are different.

3.4. Role of metals in the biological effects

When we compared the cytotoxicity of the acetone PM10 extracts with their metal contents (Table 2b), there did not appear to be an association between them. However, we noticed that the Guaynabo PM10 summer sample was the only one that contained arsenic, and in many cases ranked second in various metal concentrations.

We observed similar results with the PM10 hexane extracts. The Guaynabo extract from winter was the only...
that contained detectable amounts of arsenic, and exhibited the highest toxicity. The Fajardo hexane extract from the winter contained the highest concentration of most metals except arsenic and ranked second in cytotoxicity. These results suggest that the toxicity of the Guaynabo extract could be partly explained by the presence of arsenic.

The Guaynabo and Fajardo PM$_{10}$ summer hexane extracts were the lowest in toxicity, and had the least metal content. Thus, among the hexane-extracted samples, there appears to be a seasonal association between metal content and cytotoxicity (where the winter extracts are more toxic), but no geographical association. When we evaluate the toxicity of all PM$_{10}$ extracts and their metal contents, we find no statistical association ($r^2$ values between 0.3819 and 0.0001, $p > 0.05$).

3.5. Effect of EFS on rat tracheal contractility

We compared rat tracheal contractility using isolated rat tracheas exposed to the acetone and hexane PM$_{10}$ extracts from Guaynabo and Fajardo during the summer of 1999. Due to material limitations it was not possible to test the winter extracts. For the Guaynabo-hexane PM the control group EV$_{50}$ was 33.8 V and the treatment group EV$_{50}$ was 37.75 V. For the Fajardo-hexane PM the control EV$_{50}$ was 36.13 and the treatment was 37.50 V. For the Guaynabo-acetone PM the control EV$_{50}$ was 31.32 and the treatment was 34.33 V. For the Fajardo-acetone PM the control EV$_{50}$ was 39.71 and the treatment was 42.30 V. Although none of the EV$_{50}$’s from the voltage response curves were significantly different to the untreated controls ($p > 0.05$) the general impression is that PM shifts the curves to the right (evidenced by the higher EV$_{50}$ values on the treated groups). Thus, further experiments are needed to determine if PM decreases the sensitivity (EV$_{50}$) and contractility ($E_{max}$) of the rat tracheas in response to EFS.

4. Discussion

Numerous studies point out that metals might play an important role in the toxicity of airborne PM. In PR Reyes et al. (2000) found that organic fractions of PM exhibited cytotoxicity and suggest that it might be caused by several of its organic constituents. However, the potential for metals (co-extracted with the organic components) to contribute to the cytotoxicity has not been evaluated. The main objective of this research was to compare the cytotoxicity among organic extracts of airborne PM collected from two sites in PR based on their collection season, relative polarity, geographical location, and metal content.
In PR ambient PM concentration peaks around the summer months. Saharan dusts transported across the Atlantic Ocean also peak around the summer months (Griffin et al., 2001) and could contribute to the PM sampled at both sites. It is unclear what contribution these Saharan PM could make to in vitro toxicological responses or to an in vivo system. During the warm (summer) season, there can also be changes in chemical and photochemical oxidation processes (Greenberg et al., 1993) that account for the increase in PM concentration. Another possible reason for the increase is that of altered pollutant emission patterns during the summer months. These could include increased industrial activity, particularly from the power plants, or increased automobile traffic and activity in the Guaynabo area.

The cytotoxicity of the PM extracts is related to season depending on the extract’s polarity (i.e., polar vs. non-polar). Overall, hexane PM$_{10}$ extracts had lower NR$_{50}$’s than acetone PM$_{10}$ extracts. However, the acetone PM$_{10}$ extracts were more cytotoxic during the summer, whereas hexane PM$_{10}$ extracts were more cytotoxic during the winter. These results suggest that the season affects the composition of PM in such a way that during the summer months there are toxic compounds associated with water soluble fractions, while non-polar toxic compounds predominate in airborne PM during winter. This seasonality difference could be partly attributed to meteorological variables (i.e., winds, temperature, and precipitation) which in turn affect the dynamics and movement of anthropogenic air pollutants and ocean-derived aerosols.

When the geographical location is taken into account, we find that most of the Guaynabo extracts were more cytotoxic than the Fajardo extracts within season and extract polarity. Because the Guaynabo area is more industrialized, the data obtained indicates that the differences in biological responses are associated with inputs of anthropogenic air pollutants. A PM characterization study performed by the US EPA concluded that PM near the northern Guaynabo site comes from at least two sources. These sources could include the Atlantic Ocean (sea aerosols), which may include Saharan dust, and the city of San Juan (automotive and other emissions) (Suhe et al., 1995). A study by Mayol-Bracero et al. (2001) points out that natural oceanic emissions could make up a substantial fraction of trade wind aerosols. That study identified fatty acid esters and phosphates, fatty acids, normal and branched hydrocarbons, ketones, and aromatic hydrocarbons at Cabezas de San Juan in Fajardo, PR. It is possible that the toxicity of the Guaynabo and Fajardo extracts is affected by sea aerosols, however, at the Guaynabo site the toxicity is enhanced by the addition of urban emissions.

The fact that the PM$_{2.5}$/PM$_{10}$ ratio in Guaynabo is higher than that of Fajardo partly supports this statement as combustion particles are mostly associated with fine PM (although this association is not exclusive) (US EPA, 2004a). It is also important to mention that meteorological conditions (which affect the distribution and nature of air pollutants) might be different at the two sites and could play an important role in the different toxicities observed between Guaynabo and Fajardo.

The Reyes et al. (2000) study that compared the cytotoxicity of PM$_{100}$ (PM with a mass median aerodynamic diameter $\leq$100 $\mu$m) and PM$_{10}$ extracts from Guaynabo and Fajardo in three organic solvents (hexane, dichloromethane and acetone) found that the most toxic PM$_{10}$ extracts were extracted with hexane. Furthermore, their most toxic extract (between summer and winter only) resulted from Guaynabo in the winter. Our results confirm those obtained by the Reyes study, with the exception that on our study the winter hexane fraction from Fajardo was highly cytotoxic, comparable to the toxicity obtained from Guaynabo. Furthermore, we found that season plays an important role in the cytotoxicity of the PM extracts. Based on the NR$_{50}$ comparisons the cytotoxicity of the PM extracts in terms of solvent polarity from our study and the Reyes study are similar (non-polar extracts of PM$_{10}$ exhibit the highest cytotoxicity), and both underscore the importance of this variable in the toxicity. However, in terms of season and geographical location our results have several differences (i.e., their most cytotoxic extracts were collected during the summer, with the exception of the Guaynabo-winter extract). These differences could be due to the use of different cell lines NHEK vs. NHBE), different extraction procedures (two solvents vs. three solvents), and a different collection period (1996–97 vs. 1999–2000), as PM components can vary over time.

The epithelial cell type (NHBE and BEAS-2B) used to test our extracts does not appear to be an important factor in determining toxicity. The differences we observe in the responses among both cell types could be attributed to different cellular transport mechanisms of organic compounds or metals (Ballatori, 2002) or differences in metabolic activity or capabilities toward some component in the PM extracts. This last statement is supported by the fact that both cell lines have similar sensitivity to Triton X-100, which does not require to be metabolized in order to cause cellular damage. However, PM is a mixture of compounds and components, each causing a number of effects on the cell, and dependent on the cellular conditions. It is important to reinforce the fact that with both cell lines we obtained similar cytotoxicity results, albeit the NHBE cells were more sensitive (see Table 2). Thus, when designing future experiments the advantages of using NHBE cells (retention of in vivo characteristics) have to be weighted against the disadvantages (difficult culture procedures).

We found no clear statistical association between the presence of metals in the PM extracts and their cytotoxicity. However, two of the most cytotoxic extracts: Guaynabo PM$_{10}$ from the winter in hexane (NR$_{50}$ in NHBE cells = 11 $\mu$g/mL) and Guaynabo PM$_{10}$ from the summer in acetone (NR$_{50}$ in NHBE cells = 15.2 $\mu$g/mL) were the only ones with detectable levels of arsenic. In addition, we observed that the Guaynabo extracts...
contained more metals than the Fajardo extracts (especially As, Pb, Cu, Cd, and V). On our study the arsenic concentration the cells received when they were exposed to the PM extracts that contained the element was approximately 1–2 nM (at a PM10 dose of 15 μg/mL). This concentration is much lower than the NR50 (concentration at which 90% of the cells survive) for NaAsO2 (15 μM in 3T3 fibroblasts) reported by Borenfreund and Puerner (1986). However, our calculation assumes it is elemental arsenic and the results by Borenfreund and Puerner (1986) highlight the fact that the chemical form of the metal is important in determining its cytotoxicity. Furthermore, PM extracts contain complex combinations of metals and other compounds that can interact in a synergistic, additive, or antagonistic manner.

The Guaynabo and Fajardo winter hexane samples had similar cytotoxicity and contain appreciable amounts of V, Cu, and Ni. These metals, common on combustion derived PM, may play a role in the cytotoxicity of the extracts (Ghio et al., 2002). However, the Guaynabo winter acetone fraction contains higher amounts of all metals (including V and Ni) and was the least cytotoxic. It can be hypothesized from this finding that the chemical form of the metals in the non-polar fraction is different and more cytotoxic than the chemical form of the metals partitioning in the polar fraction. To clarify this point would require a detailed chemical speciation of the inorganic fraction. In terms of concentration, the Guaynabo-winter extract in hexane contains ~20 nM elemental copper (the second most abundant element) at a PM10 dose of 15 μg/mL. This concentration is much lower than the NR50 for CuCl2 in 3T3 fibroblasts (~350 μM) (Borenfreund and Puerner, 1986). Thus, we can indirectly infer that the Guaynabo and Fajardo winter hexane extracts exhibit high cytotoxicity because of their organic content.

When we compared the NR50 values in BEAS-2B cells of our extracts with that of ROFA (~100 μg/mL) it is apparent that most of the PR PM extracts are more cytotoxic. The NR50 values for SRM 1649 (NIST urban dust standard containing PAHs and metals) in NHEKs are 6 μg/mL for the hexane extracted fractions and 43 μg/mL for the acetone extracted fractions (Reyes et al., 2000). Thus, SRM 1649 has a similar trend of toxicity (non-polar extracts more toxic than polar) as our PM samples. These ROFA and SRM 1649 NR50 values when compared to the PM10 NR50 values lend support to the concept that organic compounds play a major role in the cytotoxicity of the organic fraction of PM from PR. However, metals could still play a minor role in the cytotoxicity. The magnitude of this effect could be better ascertained by analyzing the cytotoxicity and metal contents of aqueous or acid extracted PM and by analyzing more thoroughly the organic compounds that make up the organic fractions. The study by Reyes et al. (2000) identified several organic compounds (e.g., bis (2-ethylhexyl) phthalate, malathion, 4-morpholine propanamine) on similar PM fractions collected during 1996–97. Further support for a role of organic compounds in the cytotoxicity is evidenced by the fact that polycyclic aromatic hydrocarbons are known to induce biological effects on epithelial cells (Pei et al., 2002).

The use of EFS on isolated rat tracheas exposed to a test agent permits the use of a physiological model to assess airway responsiveness to environmental factors (Gonzalez and Santacana, 2001). Our results from rat tracheal EFS stimulation following PM10 extract exposure were inconclusive given that no significant difference between the control and PM exposed tracheas was found. The lack of statistical significance could be a result of using a very low dose of PM (15–29 pg/mL), which decreases the sensitivity of the assay, together with a low sample number. This is a common challenge when performing low dose studies. Nonetheless, this experiment represents a first step in elucidating a possible role for PM in the increased number of asthma cases among the study area population.

As mentioned in the introduction, PM toxicity can also be caused by endotoxins or acidic components. A role for endotoxin on our experiments is unlikely because BEAS-2B cells lack CD14 receptors (Schulz et al., 2002). Moreover, in separate experiments we treated BEAS-2B cells with lipopolysaccharide (a potent endotoxin) and found no cytotoxicity. The role other pollutants of biological origin might play in the cytotoxicity of the extracts is unknown. We did not examine the presence of acidic particles. However, an air pollution study conducted by Sunie et al. (1995) concluded that the levels of criteria pollutants (including acidic particles) present in the Cataño area were not of concern in relation to the health effects observed due to the low concentration at which they were present. If these levels remain constant through the years, a role for acidic particles in the PM toxicity is low. However, the sampling period for that study was only 4 months during the summer, leaving out the winter months.

The lack of epidemiological data analyzing respiratory disease and ambient PM in PR makes it difficult to relate the toxicological findings with the human health effects. In addition, the neutral red assay, albeit useful in screening for relative toxicity, is an in vitro test. Extrapolating our results to human scenarios must be done with care as the responses of cells in culture might be different to those of the lungs which contain multiple, interacting cell types.

Another common concern in toxicological evaluations is dose extrapolation to humans. If we assume a healthy adult human inhales 21.6 m3 of air per day (Ghio and Devlin, 2001), is exposed to 50 μg/m3 of PM10, and assume 42% of particles deposit in the lungs (Ghio and Devlin, 2001), the amount of PM inhaled is approximately 453.6 μg. Evenly spread over 75 m2 (surface area of the lungs) (Klaassen, 2001), the dose of PM received is approximately 0.061 μg/cm². Using the NR50 obtained for the hexane PM10 extract from Guaynabo during the winter (11 μg/mL for the NHBE cells) the dose received by the cells is 6.9 μg/cm² or approximately 100-fold higher than the hypothetical human exposure. Even though this number appears high many factors can modulate PM toxicity in
vivo. PM deposition is important in the development of many respiratory tract lesions. PM might not deposit equally over the lung surface, thus some areas might receive large amounts, whereas others do not receive much. This depends on the size of the particle. For example, PM$_{10}$ deposits primarily on the thoracic region of the bronchi, while PM$_{2.5}$ tends to deposit in the alveolar region (United States Environmental Protection Agency (US EPA), 2004b). Moreover, it has been shown that PM of the same size does not deposit equally along the lung surface. Some areas such as the carinal ridges might receive doses hundreds of times larger than the average dose for the whole lung (Baláshaży et al., 2003). It is also important to note that the respiratory tract is composed of approximately 50 cell types, at least 12 of which are epithelial (Breeze and Wheeldon, 1977). The various cell types might have different sensitivities to environmental insults or could interact to alter the sensitivity to PM. Additionally, cultured epithelial cells might be more resistant to PM-induced effects compared to the same cell types in vivo. Finally, the estimates presented are done assuming a healthy human adult. The elderly, children, pregnant women and individuals with pre-existent disease can be affected by lower PM concentrations (United States Environmental Protection Agency (US EPA), 2004c). For instance, children tend to inhale a large percent of air through mouth breathing, potentially by-passing nasal scrubbing (Bennett and Zeman, 2004). If the susceptibility factors (e.g., age, pre-existing pulmonary disease, exercising outdoors), the episodic events were airborne PM levels are higher than 50 $\mu$g/m$^3$, and the chronic nature of the exposure is considered, the doses we used are reasonable.

In summary, we have found that urban coastal PM from PR generally exhibits higher cytotoxicity than rural coastal PM. However, this effect is dependent on the polarity of the extracts and the collection season (in winter hexane PM$_{10}$ is more toxic, whereas during the summer acetone PM$_{10}$ is more toxic). This is an interesting finding for an island where the normal temperatures between seasons vary only approximately 7°C. Based on the findings of cytotoxic fractions at Fajardo our data can also be interpreted to hypothesize that PM from PR exhibits a basal cytotoxicity (possibly due to sea aerosols) that is enhanced by anthropogenic emissions at the urban location. We also found that non-polar organic constituents in PM from PR are generally more toxic than the polar organic constituents.

The metal contents of the organic PM extracts from PR appear to play a role in the cytotoxicity observed. This is supported by the findings of elements such as As, V, Ni, and Cu in the most cytotoxic extracts. However, because no clear statistical association was found between cytotoxicity and metal contents, and some extracts containing high amounts of metals (e.g., the acetone extracted Guaynabo PM$_{10}$ from winter) had low cytotoxicity, we believe the role of metals is limited. Moreover, the non-polar extracts (containing less metals) were generally more cytotoxic than the polar extracts (containing more metals), thus we can indirectly infer that organic compounds may play the major role in the cytotoxicity. This work provides an insight into the toxicity of PM on an island’s coastal urban and rural environments. It remains to be determined if aqueous PM extracts (which should contain higher levels of metals) are bioactive. Such a case would not be rare as PM is a heterogeneous mixture of components that can potentially exhibit toxicity across multiple fractions of different polarity.

Disclaimer: The research described in this article has been reviewed by the National Health and Environmental Effects Research Laboratory, US Environmental Protection Agency and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

Acknowledgments

The authors thank Ada Morales from the PREQB for providing filter samples and Darwin Reyes, Lourdes Pérez, Lisa Dailey, Jackie Stonehuerner, Leyda Martínez, and Yolanda Rodríguez for technical assistance. This work supported by US EPA-EPSCoR R827780-01 and NIGMS-RISE Program GM 61838-05. At UNC A.M. was supported by EPA/UNC CT827206 and NIEHS T32-07126.

References


Puerto Rico Environmental Quality Board (PREQB), 1994. Informe sobre la calidad del aire en Puerto Rico durante 1993. Puerto Rico Environmental Quality Board, Data Validation and Management Division, San Juan, PR.


