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# Physico-Chemical Characterization and Oxidative Reactivity Evaluation of Aged Brake Wear Particles

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**Brake wear dust is a significant component of traffic emissions and has been linked to adverse health effects. Previous research found a strong oxidative stress response in cells exposed to freshly generated brake wear dust. We characterized aged dust collected from passenger vehicles, using microscopy and elemental analyses. Reactive oxygen species (ROS) generation was measured with acellular and cellular assays using 2′7-dichlorodihydrofluorescein dye. Microscopy analyses revealed samples to be heterogeneous particle mixtures with few nanoparticles detected. Several metals, primarily iron and copper, were identified. High oxygen concentrations suggested that the elements were oxidized. ROS were detected in the cell-free fluorescent test, while exposed cells were not dramatically activated by the concentrations used. The fact that aged brake wear samples have lower oxidative stress potential than fresh ones may relate to the highly oxidized or aged state of these particles, as well as their larger size and smaller reactive surface area.**

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## 1. INTRODUCTION

Road traffic is a dominant source of environmental particulate matter (PM) pollution (Lighty et al. 2000; Riediker et al. 2004; Thorpe and Harrison 2008). Important PM emissions originate from combustion engines, brake and tire wear, and road surface materials. Many toxicological and epidemiological studies have linked traffic pollutants to adverse health effects (Pope et al. 1995; Hoffmann et al. 2007; Maynard et al. 2007), in particular respiratory and cardiovascular illnesses (Lighty et al. 2000; Schwartz et al. 2002, 2005;

Donaldson et al. 2005) and it was recently recognized as a human carcinogen (Loomis et al. 2013).

Brake wear dust was only recently identified as a relevant source of traffic emissions (Riediker et al. 2003; Thorpe and Harrison 2008), and it has not yet been studied in as much detail as diesel exhaust particles (Takano et al. 1997; Xiao et al. 2003; Donaldson et al. 2005). Brake wear dust is a complex and heterogeneous mixture which can be influenced by various factors, including the materials related to the brake design (Garg et al. 2000; Sternbeck et al. 2002; Adachi and Tainosho 2004), driving pattern (Johansson et al. 2009), and road particle resuspension (Lough et al. 2004). The composition of brakes is highly variable between producers, who developed different brake designs with metallic, semimetallic, or nonmetallic brake pads to achieve the desired performance (Nicholson 1995). Braking frequency, stopping distance, traffic density, and other factors can influence the pattern of brake wear generation and release. Weather and road surface condition differences can complicate the dust resuspension process (Lough et al. 2004).

People living near roads but also garage workers or amateurs maintaining brakes can be exposed to brake wear particles. These particles can be freshly emitted when a car slows down or when brakes are being tested in a garage, or they are aged when these particles accumulate in brake drums and can be resuspended by circulating traffic or when brakes are opened during servicing. The recommendation for servicing car brake drums is to wash the drums and to not use pressurized air (OSHA 2006). However, even if the guidelines are followed, dust can be released when opening parts and it is important to assess the potential risk to people working on brakes and the environment.

In order to assess the potential health effects of brake wear particles, a systematic characterization of these complex mixtures is required. Previous studies were using either laboratory simulations (Garg et al. 2000; Sanders et al. 2003; Gasser et al. 2009; Perrenoud et al. 2010) or real-time road measurements (Sternbeck et al. 2002; Adachi and Tainosho 2004; Lough et al. 2004; Hjortenkrans et al. 2007; Johansson et al. 2009) to test the toxicity of “fresh” brake wear particles

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directly after they were released into the environment following their generation in a braking event. Most published studies focused on metal analyses (Garg et al. 2000; Adachi and Tainosho 2004; Hjortenkrans et al. 2007; Hulskotte et al. 2007; Johansson et al. 2009) and particle size distributions (Garg et al. 2000; Sanders et al. 2003; Adachi and Tainosho 2004; Mosleh et al. 2004; Iijima et al. 2007; Perrenoud et al. 2010), which are fundamental ways of characterizing the physico-chemical basis of any toxicity related to brake wear pollutants. We previously assessed the cytotoxicity, oxidative stress, and inflammatory responses in epithelial lung cells exposed to freshly generated brake wear particles (Gasser et al. 2009) and found increased ROS generation in the exposed cells. At that time, we were unable to distinguish if the ROS resulted from a cellular response or from the particles themselves.

In this study, we combined microscopy, metal and carbon analysis, plus acellular and cellular 2',7-dichlorodihydrofluorescein (DCFH) assays to generate a relatively comprehensive characterization of aged brake wear particles. Reactive oxygen species (ROS) generation assessment contributes to a better understanding of their reactivity and the possible mechanisms of how their inhalation affects human health (Brook et al. 2010). The formation of ROS upon contact of PM with aqueous liquids was recognized as an important contributor to PM's potential for provoking oxidative stress (Riediker et al. 2004; Brook et al. 2010). Little is known about the pure oxidative reactivity of brake wear samples measured using an acellular generation assay. This present study focuses on aged brake wear particles, an environmental pollutant where little knowledge exists about their potential biological responses.

## 2. MATERIALS AND METHODS

### 2.1. Sampling Process

The sample collection was done by members of Garage Mirabile SA, Lausanne, Switzerland. Brake wear dusts were collected directly from seven small passenger vehicles with brake drums. Sampling vehicles were chosen to have various ages. Average age of the sampling vehicles was 10 years (max: 17 years; min 4 years); average driving distance was 51,732 km (max: 83,346 km; min: 3762 km). When the garage workers received a car for repair, they carefully opened the brake drums and manually collected the dust using a clean spatula, including both the loose dust and particles attached to all parts of the brake but without scraping. Workers were instructed to pay extra attention during the sampling process to avoid contaminations.

### 2.2. Atomic Absorbance Analysis for Iron (Fe), Copper (Cu), and Manganese (Mn)

Approximately 50 mg of each sample was weighed and transferred into a digi-50 ml tube. To each tube, 2 ml of 48%

hydrogen fluoride was added, followed by 3 ml of aqua regia (a 2:1 mixture ratio of hydrogen chloride and nitric acid). The whole sample suspension was sonicated for 10 min. Milli-Q water (Merck Millipore, MA, USA) was then added to adjust the total volume to 50 ml. The atomic absorbance spectroscopy measurement was subsequently performed using a VARIAN AA240Z Zeeman in our ISO EN ISO/IEC 17025:2005 certified lab based on OSHA-method ID-121 (OSHA, 2002). Blank values were obtained by analyzing pure reagents without brake wear samples.

### 2.3. Carbon Analysis (OC/EC)

EC and OC were determined following the standard NIOSH 5040 procedure (Birch and Cary 1996) using a Sunset OCEC dual optics lab instrument (Sunset laboratory Inc., Hillsborough, NC, USA) with a flame ionization detector (version 6.4), as per the manufacturer's instructions. Briefly, a small amount of the brake wear sample was scattered on a filter quartz punch. The samples went through the processes of thermal desorption and then transformation to methane. The OC concentration was detected in helium gas flow when the temperature reached 850°C. After some cooling time, oxygen was introduced, and the temperature was increased again up to 850°C for EC measurement of the same sample. Clean quartz filters were analyzed as the blank control. Methane was used at the end of each run as the quality control.

### 2.4. Microscopy Analysis

#### 2.4.1. Apparatus

Scanning electron microscope (SEM) and transmission electron microscope (TEM) images were obtained by using an FEI Quanta 650 FEG and an FEI Tecnai G2 F20, respectively. Energy-dispersive X-ray (EDX) measurements were collected using an X-Max SDD with a Silicon Drift Detector of 20 mm<sup>2</sup>. EDX data were processed using the INCA software from Oxford Instrument.

#### 2.4.2. Sample Preparation

For TEM imaging, the dispersed sample suspension was made by adding a small amount of brake wear dust to ethanol, followed by ultrasonication. A droplet of it was then placed on a Cu TEM grid.

For SEM analysis, brake wear dust was sprinkled directly on to carbon tape. Compressed air was applied to blow off excess sample.

### 2.5. Cell-Free ROS Detection

The acellular ROS detection protocol applied in the present study was described previously (Zhao and Riediker 2014). Briefly, a working solution was freshly prepared under a

darkroom lamp emitting outside the excitation range of DCFH shortly before the experiments by first adding a strong base (sodium hydroxide) to the ethanol-based DCFH-DA stock solution, followed by dilution in phosphate buffer with pH 7.2. Horseradish peroxidase (HRP) was added at 0.5 unit/ml to finalize the DCFH-HRP working solution. A defined amount of each brake wear sample was added to the DCFH-HRP working solution to generate concentrations of 2, 5, 10, and 100  $\mu\text{g/ml}$ . The mixture was sonicated for 15 min in a water bath (Branson 5210, 2.8L, 180W) at constant 37°C. At the same time, pure DCFH-HRP solution was sonicated in the same manner to serve as the sonication blank control. In the following, blanks are labelled as “Blank,” while the brake wear samples are named from F4 to F10.  $\text{H}_2\text{O}_2$  was used as the positive control and to generate a calibration curve. The samples and the standards were incubated at 37°C for 15 min. The fluorescent intensity was measured by a 96-well multiple plate reader (Infinite M200, TECAN; DCF excitation/emission @ 485/530 nm). The plate reader was kept at 37°C during the entire measurement. A dynamic reading pattern was used, and sample fluorescence was measured every min up to 1 h. When preparing the standard curve, the fluorescence value of the blank was subtracted from the  $\text{H}_2\text{O}_2$  values.

## 2.6. Cellular ROS Detection

A549 cells (human alveolar adenocarcinoma cells; American Type Culture Collection, ATCC) were cultured at 37°C in a 5%  $\text{CO}_2$  atmosphere using Modified Eagle Medium supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum. Intracellular ROS was detected using the same fluorescent probe used for cell-free ROS detection: DCFH-DA. First, 96-well plates were seeded at a density of 30,000 cells/well or 93,750 cells/cm<sup>2</sup> and incubated for 24 h. Afterward, the culture media were removed, and the cells were incubated with 10  $\mu\text{M}$  DCFH-DA in 1 × Hank’s buffered salt solution (HBSS) for 1 h. The dye solution was then removed, and the cells were exposed for 1 h to either  $\text{H}_2\text{O}_2$  (positive control) or brake wear particles diluted in HBSS at various concentrations. Fluorescence readings were measured using the same plate reader as in the cell-free detection.

## 3. RESULTS AND DISCUSSION

### 3.1. Particulate Matter

TEM showed that the smallest primary particle size for aged brake wear particles is about 10–15 nm (Figure 1). SEM confirmed this finding and also revealed a mixture of particulate matter (PM); diameters ranged from a few to one hundred microns, with many particles showing relatively flat surfaces and sharp edges (Figure 2). We suspect these particles were mechanically shed off from the brake surface. Other particles were very small and round forming chain-like aggregates,

which likely were formed through evaporation from the hot brake surface followed by condensation and aggregation of components. Garg et al. (2000) showed similar results that proposed a variety of generating processes. Particle diameters were estimated across their horizontal axes by analyzing the SEM images with imaging software, and assuming that the particles were pseudospherical. The mean diameter of all the samples was about 2  $\mu\text{m}$ . Approximately 50%–60% of particles were in the range of 1–2.5  $\mu\text{m}$ . Depending on the sample as well as the analyzing area, some images showed about 20% for both below 1  $\mu\text{m}$  and above 2.5  $\mu\text{m}$  (F4, F5, F6). F7 and F10 showed more than 30% of particles with sizes below 1  $\mu\text{m}$ . In contrast, F8 and F9 showed less than 10% of small particles (<1  $\mu\text{m}$ ).

Previous research based on modern brakes reported that they generate relatively large amounts of PM emissions (Garg et al. 2000; Sanders et al. 2003; Mosleh et al. 2004; Iijima et al. 2007; Gasser et al. 2009; Perrenoud et al. 2010). Our estimated size distribution agrees with previous studies (Garg et al. 2000; Sanders et al. 2003; Adachi and Tainosho 2004; Iijima et al. 2007) which reported that up to half of the brake wear dust mass was found to be airborne (Sanders et al. 2003) and with wear emission rate of 5.1–14.1 mg/mile (Garg et al. 2000). However, we did not see a large number of nanosized particles, as were reported in other studies (Gasser et al. 2009; Perrenoud et al. 2010).

Disagreements on particle size distributions may be due to different sampling processes. In this study, we collected aged brake wear dust that collected inside the brake drum while others did experiments using brake pad linings in experimental settings or cars that are either driving or mounted in a garage. Sanders et al. (2003) performed experiments on three popular brake lining materials used in Sweden. They found a mass mean brake wear particle diameter of 6  $\mu\text{m}$ , which was much larger than the number-weighted mean of 1–2  $\mu\text{m}$ . Corroborating these findings, Iijima et al. (2007) measured particle size using an aerodynamic particle sizing instrument and found a peak at 1–2  $\mu\text{m}$ ; mass size data showed the peak range was 3–6  $\mu\text{m}$ . Furthermore, fine particles were estimated to account for 74%–92% of particle numbers and 12%–36% of particle mass. Garg et al. (2000) applied a micro-orifice uniform deposit impactor (MOUDI) to seven popular brake lining designs and reported the average mass median diameter of brake wear particles was in the range of 0.62–2.49  $\mu\text{m}$ , while the overall average of the sampled linings was 1.49  $\mu\text{m}$ . In terms of particle size distribution, 86% were PM10 and 63% were PM2.5, and 33% of particles were found to be smaller than 0.1  $\mu\text{m}$ . Adachi and Tainosho (2004) also reported a similar value of about 1  $\mu\text{m}$ . Mosleh et al. (2004) reported a peak around 0.35  $\mu\text{m}$  in the fine particle fraction, and also showed various peaks in the coarse particle fraction. Thorpe and Harrison (2008) suspected that increasing braking pressure increased the tendency to generate larger particles. In our previous study, we studied the release from brakes on real cars

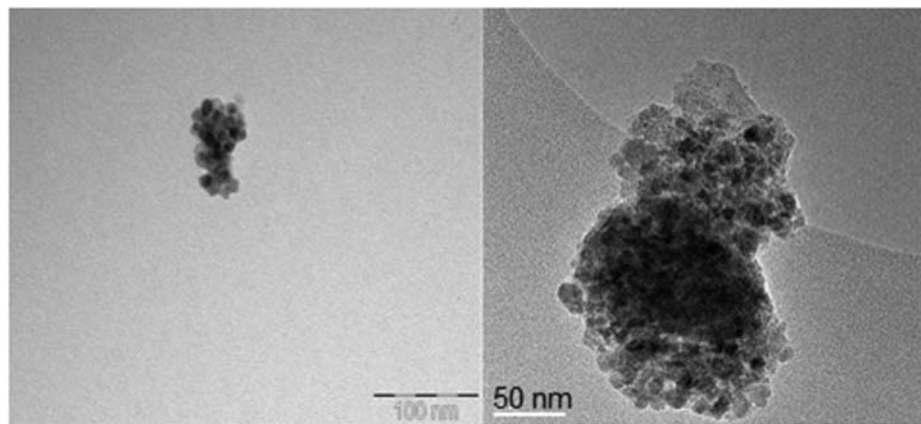


FIG. 1. Representative TEM images of brake wear particles. Mostly clusters of heterogeneous particles were found, the smallest with diameters of 10–15 nm.

mounted in a garage (Perrenoud et al. 2010; Gasser et al. 2009). As expected, full stops produced many more particles than normal decelerations not involving stopping with particle mass distribution finding 87% in the fine particle range, while 12% was in the coarse range (Perrenoud et al. 2010). The particle number distribution showed that 74% of brake dust particles were nanoparticles and only 26% were fine particles. Across all braking patterns, a peak around 60–100 nm was seen for nanoscale particles and a second peak around 300–400 nm for fine scale ones, while only during full stop braking, a third peak for particles smaller than 30 nm appeared (Perrenoud et al. 2010).

### 3.2. Elemental Analysis

#### 3.2.1. Metals

EDX spectroscopy detected 12 metal elements in brake wear samples, including sodium (Na), magnesium (Mg), aluminium (Al), potassium (K), calcium (Ca), titanium (Ti), manganese (Mn), iron (Fe), copper (Cu), zinc (Zn), barium (Ba), and bismuth (Bi) (Figure 2). The composition and concentrations varied from sample to sample. Fe was detected at high concentrations in all seven samples. Ca, Cu, Zn, and Ba were consistently found at more than 1% weight (for a few samples, the amounts were even higher) indicating that they are common components in many brake designs.

Our atomic absorbance analysis (Figure 3) showed that Fe concentrations ranged between 200 and 400  $\mu\text{g}/\text{mg}$  in all seven vehicle samples. About 20  $\mu\text{g}/\text{mg}$  Cu was found in sample F4, which was the highest measured Cu-level. Very small amounts of Mn were detected in all the samples. The metals of interest were chosen based on our previous study and the results agreed with the earlier findings (Gasser et al. 2009).

Previous research suggested that brake wear dust is a significant source of many metals found in traffic emissions, including Cu, Sb, Ba, Zn, Fe, and cadmium (Cd) (Davis et al. 2001; Sternbeck et al. 2002; Riediker et al. 2003; Adachi and

Tainosho 2004; Lough et al. 2004; Hjortenkrans et al. 2007; Hulskotte et al. 2007; Johansson et al. 2009); results vary due to the different vehicle brands and sampling conditions studied. Both Adachi and Tainosho (2004) and Iijima et al. (2007) reported that Fe was one of the primary metals in brake dust. Our data agree and show high amounts of Fe, perhaps explained by the fact that both hematite ( $\text{Fe}_2\text{O}_3$ ) and magnetite ( $\text{Fe}_3\text{O}_4$ ) act as mild abrasives (Nicholson 1995). Several other studies have reported finding Fe (Garg et al. 2000; Sanders et al. 2003; Lough et al. 2004; Iijima et al. 2007). Cu is commonly added to brake pads as a modifier to control heat transport (Nicholson 1995). In agreement with our findings, other studies found Cu in brake dust, although in varying amounts (Garg et al. 2000; Davis et al. 2001; Sternbeck et al. 2002; Sanders et al. 2003; Adachi and Tainosho 2004; Lough et al. 2004; Hjortenkrans et al. 2007; Hulskotte et al. 2007; Iijima et al. 2007; Johansson et al. 2009). Brake wear is one of the leading sources of Cu emissions, contributing between 15% and 90% of Cu emissions in different European countries, with the average estimated to be 50% (Hulskotte et al. 2007). Ba can be added to the fillers and other parts of the brake in the form of  $\text{BaSO}_4$ , thus increasing density and resistance. This element was also found in several other studies (Garg et al. 2000; Sternbeck et al. 2002; Sanders et al. 2003; Adachi and Tainosho 2004; Lough et al. 2004; Iijima et al. 2007). Interestingly, our EDX measurement detected no Sb, although it was found to be a significant component of brake wear dust in other studies (Garg et al. 2000; Sternbeck et al. 2002; Adachi and Tainosho 2004; Lough et al. 2004; Hjortenkrans et al. 2007; Iijima et al. 2007; Johansson et al. 2009). Sb has been proposed as a brake wear emission tracer (Dietl et al. 1997) and Sternbeck et al. (2002) calculated a ratio between Cu and Sb which they suggested could be used to identify brake wear particles in unknown samples. Antimony trisulfide has been suggested as a solid lubricant for brake modifiers, it helps enhance frictional stability (Nicholson 1995; Jang and Kim 2000), and can serve as an oxygen absorber for stability purposes but several of these studies

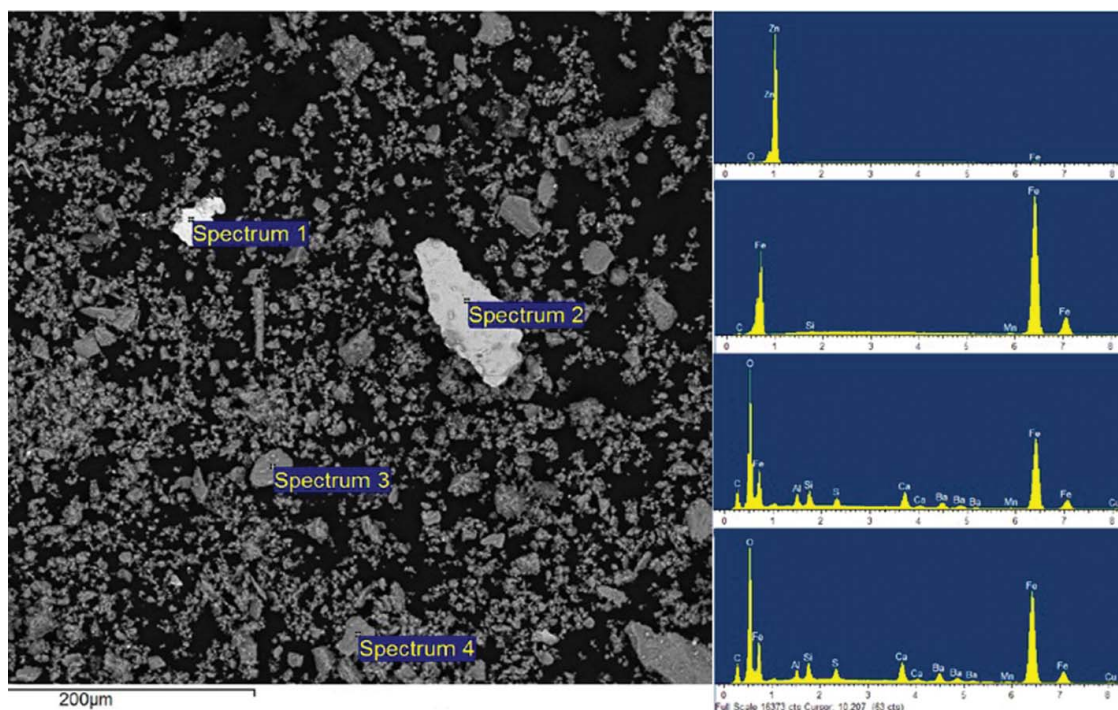


FIG. 2. Representative SEM image with EDX analysis. All samples were mixtures of micron- and nano-scale particles composed of at least 10 different metal types as identified by EDX-spectra. (Y axis of EDX profile represents the intensity.)

warned of its toxicity. We did not find any Sb in our study, possibly because it was discontinued due to the above-mentioned toxicity concerns and seen that is not essential for brakes. In either case, our findings suggest that Sb is not a good tracer for brake wear dust.

### 3.2.2. Carbonaceous Components

The ratios of elemental carbon (EC) to organic carbon (OC), measured in our aged brake wear samples, ranged between 0.086 and 0.28, with an average of  $0.15 \pm 0.085$  (Figure 3). Few studies so far assessed EC and OC specifically on brake wear particles. Most studies investigated the total

mix of traffic emissions. In our previous study, we collected fresh brake wear particles on filters and measured the carbonaceous content. We found an EC/OC ratio ranging from 0.05 to 0.29 (Gasser et al. 2009; Perrenoud et al. 2010). Our samples' EC/OC ratio was smaller than EC/OC ratios of PM<sub>10</sub> found on general traffic emissions. Lough et al. (2004) reported the EC/OC emission ratio as 0.32 for PM<sub>10</sub> and 0.65 for PM<sub>2.5</sub>. Fraser et al. (1998) showed similar results with the EC/OC ratios of  $0.31 \pm 0.04$  and  $0.44 \pm 0.06$  for PM<sub>10</sub> and PM<sub>2.5</sub>, respectively. The similarity between fresh and aged brake wear and the differences to the general traffic particles suggest that EC and OC of our brake samples were mostly influenced by the organometallic brake materials.

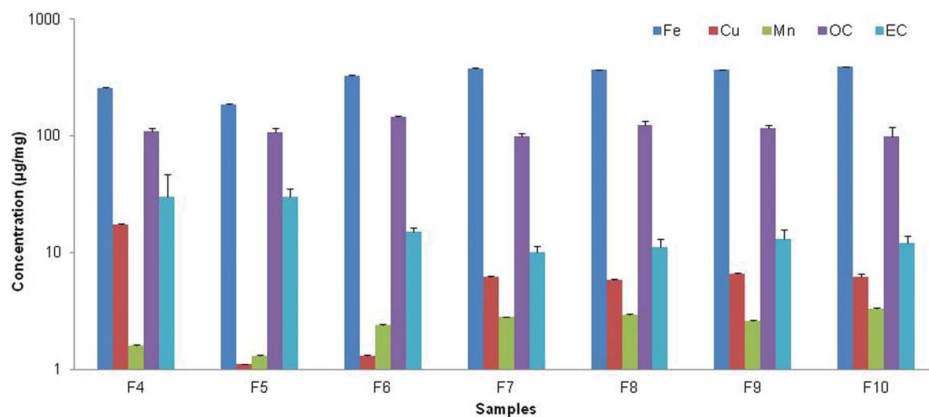


FIG. 3. Brake wear elemental analysis in log scale. (Error bars refer to standard deviation. Results are presented as  $\mu\text{g}$  element per mg particle powder.)

### 3.2.3. Other Detected Elements

Relatively large amounts of carbon and oxygen were found in all seven samples. Carbon was expected because graphite is a cheap material commonly used in modifiers to help increase friction (Nicholson 1995) and because of the abundance of organic resins as binders (Nicholson 1995). We found approximately 20 wt% oxygen in our brake wear samples and interpret these high-oxygen concentrations maybe as a sign for mostly completed oxidation of these aged samples. These particles accumulated on the brake drums over long periods, where they frequently underwent changes in pressure and temperature during braking. High pressure adds strong forces and may cause burning and thus increase temperature. Oxidization is very likely over time, and these conditions would promote it. In fact, some materials added to modifiers are expected to become oxidized in order to control interfacial films (Nicholson 1995). However, it is of note that some elements of the brake pads and additives are in the form of oxides. We also detected silicon (Si) in all seven samples, indicating that quartz may still be added as an abrasive in many designs. Sulfur (S) was detected from six samples and a small amount of chlorine (Cl) was found in four samples.

### 3.3. Acellular ROS Generation

This study investigated the acellular ROS generation as a contribution to oxidative stress using the DCFH assay, which has been demonstrated to measure a range of ROS functional groups, including hydrogen peroxide, organic peroxide, hypochlorite, and alkyl peroxy radicals (Venkatachari and Hopke 2008). It has been verified (Wardman 2007; Venkatachari and Hopke 2008; Sauvain et al. 2012) and widely applied (Hung and Wang 2001; Venkatachari et al. 2005; Zhao and Hopke 2011). An additional advantage of DCFH for this study was that it allowed the use of the same reactant in both the cell-free and the cell-culture-based measurements.

Figure 4a shows the rate of fluorescence activation, in units of fluorescence per unit time. This is a useful way of looking at oxidative reactivity. It focuses more on the ability to generate ROS over time rather than the presence of ROS at a certain time point.

To better illustrate the oxidative potential of aged brake wear samples, we also included data on FW2 as a particle positive control reference. FW2 is a type of carbon black nanoparticle, demonstrated to be highly oxidatively reactive (Sauvain et al. 2012). As shown, FW2 had a much steeper slope than brake wear particles. The fact that our aged samples generated ROS much more slowly over time than a known reactive particle also suggested that our brake wear samples were not very reactive. It is of note that at high sample concentrations FW2 began to have a decreasing slope. We attribute this to a large amount of particles settling at the bottom of the well and blocking the signal, which was confirmed by absorbance

measurements. No optical interferences were observed for any of the tested brake wear samples.

ROS generation was measured at four different concentrations for each sample, at 2, 5, 10, and 100  $\mu\text{g/ml}$  (Figure 4b). A strong and highly significant dose-response relationship between particle concentration and ROS-formation was found for all brake wear samples when calculating the Pearson correlation (correlation coefficients for F4 to F10 were:  $r = 0.9905$ ,  $0.9949$ ,  $0.9990$ ,  $0.7185$ ,  $0.9871$ ,  $0.9650$ , and  $0.9528$ , respectively). The robust Spearman's rank-order correlation test confirmed the results and showed similar and strong monotonic increases among all the seven samples (Spearman- $r$  between  $0.8000$  and  $1.0000$ ). Paired  $t$ -tests with Bonferroni correction showed in addition that samples at 100  $\mu\text{g/ml}$  showed clearly significant ROS generation for F4, F5, F6, F7, F8, and F10 compared to sonicated DCFH-HRP reagent control ( $p < 0.05$ ). We performed statistical tests using the sonicated DCFH-HRP as blank control to take into consideration that the sonication process can generate ROS. It is of note that sonication may produce excess ROS in the presence of particle samples because of the potential particle components involving reactions.

Pearson correlation coefficients were also calculated to determine the possible correlation between acellular ROS and Fe, Cu, Mn, OC, and EC content. Only OC showed a strong correlation ( $r = 0.8989$ , confirmed by Spearman's test) with ROS, agreeing with previous research (Verma et al. 2009; Saf-fari et al. 2014).

Previous studies reported that the ability of particles to generate ROS can change with aging (Hung and Wang 2001; Li et al. 2009; Verma et al. 2009; Rattanavaraha et al. 2011; Chen et al. 2011). Some of them found enhanced ROS generation with aging and attributed this phenomenon to reactions occurring on the particles (Li et al. 2009; Verma et al. 2009; Rattanavaraha et al. 2011). Others observed similar phenomenon as found in the present study (Chen et al. 2011; Hung and Wang 2001). Chen et al. (2011) studied ROS formation using a dynamic chamber that simulated ozonolysis of secondary organic aerosols. They estimated that the initial half-life of ROS was about as short as 6.5 h and suggested rapid ROS analysis. Hung and Wang (2001) also reported different ROS concentrations for aged samples. Far fewer ROS were found from samples aged on filters for 115 h than for 1 h. It seems ROS or the ROS formation potential decayed on the collected samples over time. This might be another explanation for why we saw much less oxidative reactivity in our aged brake wear study than in our previous study in which freshly sampled particles had been tested (Gasser et al. 2009). To conclude, aging is likely to influence particle oxidative reactivity into different directions depending on the specific composition of the particle samples, the storage environment, and possibly other factors such as oxidation state of metals.

Despite measuring less oxidative reactivity with aged versus freshly generated brake wear particles, the  $\text{H}_2\text{O}_2$  equivalence range we calculated from aged brake wear samples



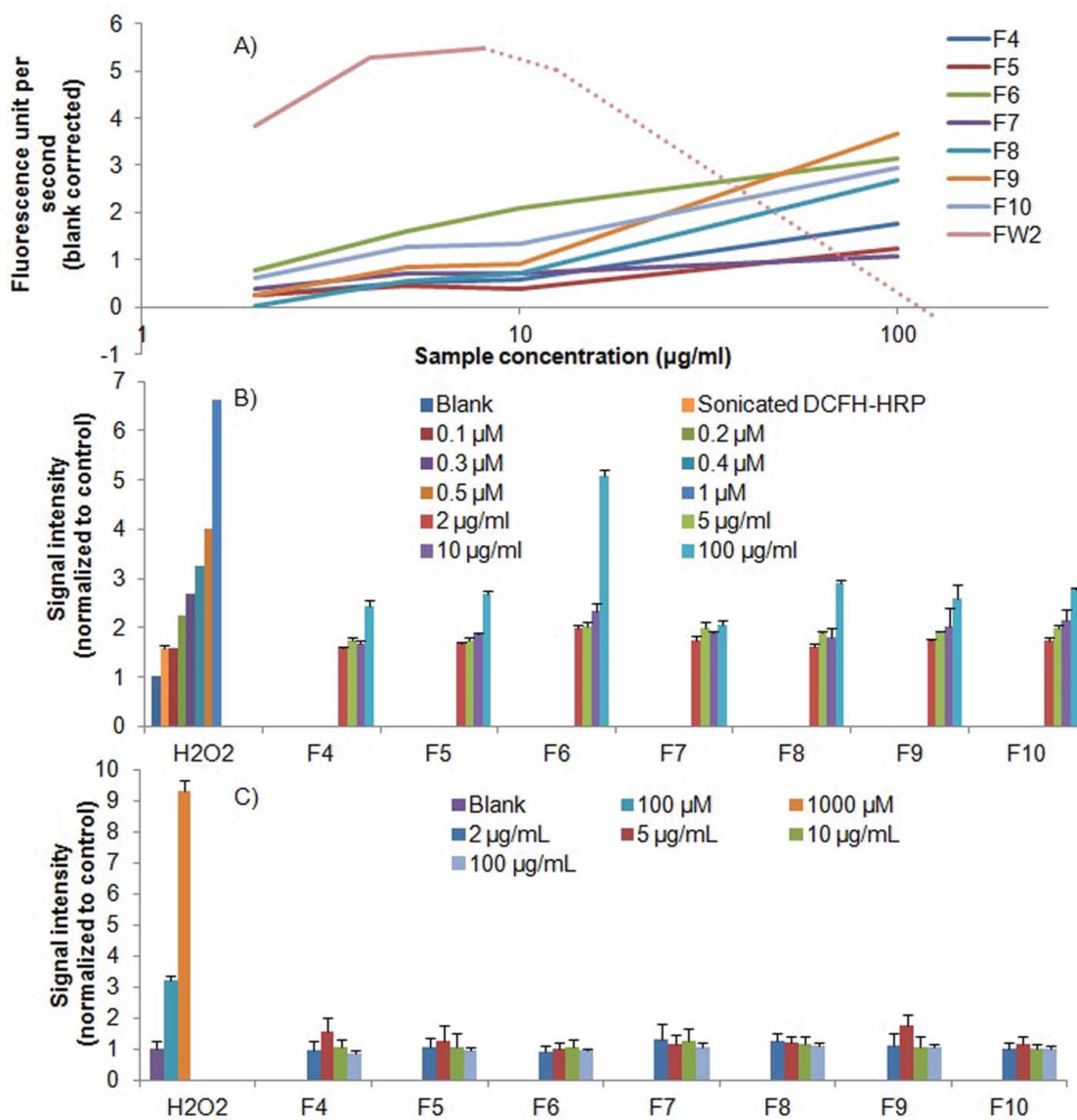


FIG. 4. ROS measurement. (a) Reactivity rates expressed as the slope of the dynamic curve obtained from fluorescence measurements taken every minute over 1 h. FW2 is a reactive carbon black. Above about 8  $\mu\text{g/ml}$ , absorbance interference starts (dotted line). Comparison of DCFH data obtained from different assays at 20 min: (b) acellular test; (c) cellular test.

(Figure 4b) was similar to previously reported measurements on ambient and traffic samples (Hung and Wang 2001; Venkatachari et al. 2005; See et al. 2007; Venkatachari et al. 2007; Wang et al. 2011). However, the ROS content found in our study was significantly lower than in studies on combustion samples (Kao and Wang 2002; Zhao and Hopke 2011). This suggests that our aged brake wear samples may act similarly to most ambient air pollution particles upon deposition in the lungs and lead to ROS-formation.

### 3.4. Cellular ROS Generation

The formation of ROS in cells exposure to brake wear particles was also measured using DCFH-DA. In contrast to the

acellular reactivity results, which indicated a dose-response relationship regarding ROS-formation (Figure 4b), the cellular oxidative stress response to brake wear particles remained low for the same range of tested concentrations (Figure 4c). Using our DCFH-DA cellular protocol, the lowest concentration of  $\text{H}_2\text{O}_2$  that reliably produced elevated intracellular fluorescence values over the unexposed control was 100  $\mu\text{M}$ . This concentration is 100 times greater than the highest  $\text{H}_2\text{O}_2$  standard concentration used in the acellular test. In particle-exposed cells, the sources of intracellular ROS can be generated from the particles themselves but also in response to the presence of and interaction with the particles. And it is also important to consider that unlike in the acellular test, cells can compensate for elevated ROS with an antioxidant response.



Since the particle interaction with the cells could induce intracellular ROS generation, we also measured the change in fluorescence intensity over time. Interestingly, the rate of increase slowed over time in cells exposed to 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$ , and at 60 min these cells yielded a fluorescence intensity similar to unexposed control cells. Since 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$  is below the concentration that induces cell death (i.e., 500–1000  $\mu\text{M}$   $\text{H}_2\text{O}_2$ ), this slowed rate of increase signals a reduction in ROS generation possibly due to an antioxidant response (Davies 1999). As the oxidative stress response of cells is a relatively quick process, we chose to examine the first 10 min for the 10  $\mu\text{g}/\text{ml}$  concentrations when comparing the rate of fluorescence intensity change over time to reduce the influence of cell recovery on the slope. The slopes for all brake wear samples increased similarly to unexposed cells, which was 0.052 $\pm$ 0.01 fluorescence units per second. FW2 was also used as a positive control reference and a much steeper slope of 1.551 $\pm$ 0.71 fluorescence units per second was measured. This value was between the slopes measured for cells exposed to 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$ , 0.513 $\pm$ 0.07 fluorescence units per second, and 1000  $\mu\text{M}$   $\text{H}_2\text{O}_2$ , 2.119 $\pm$ 0.37 fluorescence units per second. Taken together, the brake wear particles have relatively low oxidative reactivity *in vitro* compared to FW2 and  $\text{H}_2\text{O}_2$ , which agrees with the acellular results.

Cell viability was also tested using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. None of the samples showed a decrease in cell viability in cells exposed to 10  $\mu\text{g}/\text{ml}$  of brake wear particles for 24 h. However, reduced viability (75%–80%) was measured for samples F7, F8, and F9 at the highest concentration tested, 100  $\mu\text{g}/\text{ml}$ . It should be noted that exposures to both the oxidative stress and cell viability assays were performed in liquid culture. Not only is liquid culture the standard method for conducting *in vitro* assays, but to correlate acellular particle ROS generation to cellular response, exposure in buffer better matches acellular assay conditions. However, the literature suggests that lung cells respond more sensitively to particle stimuli when exposed in air-interfaced culture rather than submerged culture conditions (Fröhlich et al. 2013). This may, in part, explain why our results differ significantly from those of our previous tests (Gasser et al. 2009), where ROS generation was measured in cells cultured and exposed at the air–liquid interface. Furthermore, since it is proposed that air-interfaced culture conditions are more physiologically accurate, the low oxidative stress measured in this study may be an underestimation of the cellular oxidative stress potential of brake wear particles. However, these differences in exposure route do not seem to fully explain the stark differences observed between freshly generated and aged brake wear particles.

This study applied both acellular and cellular ROS assays to brake wear samples for the purpose of characterizing particle's oxidative stress potential and found this potential to be relatively low, in the range of ambient particles. When comparing these results to our previous study of cellular oxidative stress

after exposure to fresh brake wear particles, during which we found significant activation from cells exposed to dusts generated (Gasser et al. 2009), we can show a clear difference, which we propose to be primarily due to the aging process and to a lesser extent also due to the size of the particles. We explain these differences by the different types of particles and by differences in age. The brake wear dust collected from brakes had less nanoparticles. Metallic nanoparticles are more likely to have a higher level of toxicity because of their relatively large surface areas and smaller sizes (Wilson et al. 2002). Also, the metals in our aged samples were oxidized, probably due to having gone through more corrosion processes. This suggests that the aged brake wear particles collected from brake drums not only have a lower total reactive surface but also otherwise lose a good part of their potential to form ROS.

#### 4. CONCLUSIONS

Taken together, our comprehensive physicochemical analyses of these aged brake wear particles indicate the samples are complex mixtures of a wide size-range of particulate matter with varying elemental compositions.

While our study suggests that aged brake wear particles as present in brake drums are not extremely potent in forming ROS, they still show a potential similar to ambient particles. Research on ambient air pollution suggests that such particles pose a cardiovascular health risk especially to people that are chronically exposed to elevated levels (Brook et al. 2010). Thus, good practices to service brakes should be respected and any excessive release of such dust should be avoided especially in professional settings where these exposures occur repeatedly over many years during a professional life, but also in ambient situations where these particles could contribute significantly to the exposure of nearby populations.

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