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Lichens “travelling” in smokers' cars are suitable biomonitors of indoor air quality

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ABSTRACT

In this work, two hypotheses have been tested: 1) that lichen transplants “travelling” in smokers' cars accumulate relevant amounts of nicotine and heavy metals from cigarette smoke, and 2) that such exposure affects their vitality. Lichen samples (*Evernia prunastri*) were exposed for two months inside the cabin of 10 volunteer's cars, equally distributed between smokers and non-smokers. Travelling in a smoker's car for two months increased the content of nicotine and heavy metals (Al, As, Cd, Cr, Cu, Ni, Pb and Sb) in the lichen. Exposed to Control (EC) ratios revealed an indoor uptake also for Cu and Sb in non-smoker's cars, caused by traffic pollution. A smoke factor, calculated as the ratio between values of smokers' and non-smokers' cars, indicated a 85.6-fold contribution for nicotine and contributions in the range 1.2 (Pb) to 2 (Ni) for heavy metals; in addition, after travelling in smokers' cars, lichens showed a remarkable (60%) reduction of their vitality, as indicated by the potential quantum yield of primary photochemistry. The study demonstrated that the effects of indoor pollution by cigarette smoke can be detected using lichen transplants.

1. Introduction

The knowledge of the effects of cigarette smoke has increased the awareness on the risks for human health and the depletion of air quality (Cao et al., 2015). Tobacco smoke may contain over 7000 compounds, including 250 known harmful chemicals (among them carbon monoxide, heavy metals, ammonia, PAHS, nitrogen oxides and acetone) and at least 69 that can cause cancer (U.S. Department of Health and Human Services, 2014). Environmental Tobacco Smoke (ETS) is the portion of smoke released by cigarette smoking in the environment and includes mainstream and sidestream smoke (Jaakkola and Jaakkola, 1997; Landsberger and Wu, 1995) as well as pollutants (residual tobacco smoke) that remain on surfaces after smoking, and can react and be re-emitted into the gas phase even after long periods (Matt et al., 2008). Mainstream smoke is that portion of smoke inhaled and then exhaled by smokers; sidestream smoke is the smoke released by the lit up cigarette directly in the surrounding air, between puffs; it has a temperature of 400 °C right after leaving the burning tip, but it cools very quickly to below 100 °C at 10 cm above the tip, causing the condensation of metals present in the gas-phase (Baker and Proctor, 1990; Guerin et al., 1987; Reasor, 1987).

Mainstream and sidestream smoke differ in the quantity of chemical compounds, because during the inhalation the cigarette burns at higher temperature with respect to the burning temperature during the passive smoldering, leading to a more complete combustion of toxic compounds in mainstream respect to sidestream smoke (Jaakkola and Jaakkola, 1997). For the above reasons, ETS has become an important health issue, especially in indoor environments.

Many studies have investigated indoor air quality (IAQ) in relation to cigarette smoke (e.g., Klepeis and Nazaroff, 2006; Maskarinec et al., 2000; Ott et al., 1996; Phillips et al., 1998) and some focused on IAQ in vehicles (e.g., Liu and Zhu, 2010; Müller et al., 2011; Sendzik et al., 2009). Among different indoor environments, vehicle cabins have specific features, such as the small volume that causes a much higher concentration of the pollutants, even after only one cigarette smoked (Sendzik et al., 2009). Moreover, smoking in vehicle cabins is not legally regulated, and in several cases there is a lack of laws that limit the exposure to cigarette smoke in presence of children, pregnant women and other susceptible groups.

It is not easy to assess ETS exposure and IAQ owing to the variability of concentrations, exposure profiles and other factors (Jaakkola and Jaakkola, 1997). Air nicotine and heavy metals are among the most used ETS indicators (e.g., Landsberger and Wu, 1995; U.S. E.P.A., 1992) and their indoor levels have been found to be related to the number of smoked cigarettes (Leaderer and Hammond, 1991).

The interaction of lichen thalli with chemical compounds (e.g.,

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Table 1
Details about the performed experiment elaborated after interviews with drivers; *OW = preference for keeping an open window during driving, CW = closed window during driving, SS = Slow smoker, FS = Fast smoker (> 3 cigarettes per driving hour), EF = driving mainly on suburban high-speed roads, ES = driving mainly on suburban low-speed roads. Samples from non-smoker 5 were removed from the dataset due to a car accident. Relation between mileage and smoked cigarettes (km/cig.), cigarettes per driving hour (cig./h) and number of smoked cigarettes during a typical day (cig./day).

Driver	time (days)	km	cigarettes smoked inside the car	km/cig.	Driving hours	cig./h	cig./day	Notes*
Smoker1	67	4376	130	34	73	1.8	20	OW, SS, EF
Smoker2	69	3190	110	29	55	2.0	15	OW, SS, ES
Smoker3	69	4453	60	74	75	0.8	< 10	OW, SS, ES
Smoker4	62	4800	180	27	80	2.3	15	OW, SS, EF
Smoker5	69	2264	150	15	41	3.7	> 20	OW, FS, ES
Non-smoker1	63	6125	–	–	87	–		OW, ES
Non-smoker2	60	5418	–	–	77	–		CW, EF
Non-smoker3	62	1865	–	–	34	–		CW, ES
Non-smoker4	64	5435	–	–	83	–		OW, ES
Non-smoker5	–	–	–	–	–	–		Car accident

heavy metals) has been extensively and critically investigated (e.g., Paoli et al., 2018). Biomonitoring with lichens allows to detect variations in environmental levels of trace elements and provides useful evidence of the biological effects of atmospheric pollutants (Loppi, 2014). So far, lichens have been widely used as biomonitors of outdoor air quality, but only rarely for the assessment of indoor air quality, such as in the case of schools (Canha et al., 2012, 2014; Protano et al., 2017).

To the best of our knowledge, this is the first lichen-based study of indoor pollution from nicotine and heavy metals in the microenvironment of a cabin car. The present research was carried out to investigate whether the effects of indoor pollution by cigarette smoke can be detected using lichen transplants as a biomonitoring tool. In particular, two hypotheses have been tested: 1) that lichen transplants "travelling" in smokers' cars accumulate relevant amounts of nicotine and heavy metals from cigarette smoke, and 2) that such exposure affects their vitality.

2. Materials and methods

2.1. Experimental design

The lichen *Evernia prunastri* (L.) Ach. has been exposed for about two months inside the cabin of 10 volunteer's cars, equally distributed between smokers and non-smokers. The bioaccumulation of heavy metals and nicotine as well as the vitality (as reflected by chlorophyll *a* fluorescence emission) of lichen transplants have been investigated. The experiment lasted between October and December 2017. Two months have been deemed appropriate for a lichen transplant in indoor environments (e.g., Protano et al., 2017). The experiment took place in southern Tuscany (C Italy), in an area centered in the province of Siena, that is also the native (control) remote area of the lichen material selected for the study (43°11′58″ N, 11°21′32″ E, 310 m a.s.l). Mean temperature during the exposure period was 10.3 °C and relative humidity 71%. To minimize the effects of possible differences due to confounding environmental factors others than tobacco smoke (e.g., heavy metals related to traffic pollution), the selected volunteers (with occasional exceptions) mainly performed their daily routine in and around the urban area of Siena, a small town of ca. 60,000 inhabitants, with limited industrial activity, where vehicular traffic and domestic heating represent the main sources of atmospheric pollution that can affect lichens (Paoli et al., 2013). The cars mainly circulated in peri-urban areas and when not used, they were generally kept outside (only one smoker and one non-smoker regularly parked their car inside a garage). *Evernia prunastri* has been selected since it is easy to collect, transplant and prepare for the analyses, and the thallus has a wide surface/volume ratio, well suited to intercept ambient particles. Furthermore, the material from the mentioned control area has been extensively used in biomonitoring studies, since its chemical content is in line with background values of unpolluted sites (e.g., Loppi and Paoli, 2015; Paoli et al., 2017).

Prior to the exposure, after careful removal of extraneous materials, samples were washed by short sequential immersions (three times) in deionized water. Lichen samples have been exposed using the transplant technique (lichen bags): each lichen transplant is composed by 3–5 thalli (generally 4–5 cm long), gently placed within a plastic net (mesh size 0.8 cm), so that all the material resulted well exposed to the surrounding environment. Two different bags have been placed within the cabin of each car, hanging from the rear-view mirror, or the lateral plastic handles.

All participants were asked to maintain their usual behavior, i.e., ignoring the presence of the monitoring tool. Furthermore, they were asked to report the following information and habits: mileage; driving hours; number of smoked cigarettes during driving and during the day; parking when the car was not used (garages or outdoors); car windows during driving (preferentially open or closed); travelled roads (mainly suburban high-speed roads, or suburban low-speed roads). The participants should have recorded such information on weekly basis. Weekly information was then summarized for the whole period (Table 1). At the end of the experiment, all participants were informed about their own personal results. Due to a car accident occurred to one (non-smoker) volunteer, its samples have been excluded from the dataset.

After retrieval, lichen samples were immediately stored at −18 °C in paper bags to prevent nicotine degradation. To avoid possible cross-contamination, smokers' and non-smokers' samples were kept and handled separately. The marginal parts of the laciniae (up to 2.5 cm from lobe tips), crudely minced with plastic tweezers, were selected for the analysis. Lichens were not washed, since the washing procedure may unpredictably alter their chemical composition (Bettinelli et al., 1996).

2.2. Nicotine content

The lichen material (about 100 mg) was weighed and homogenized in 2 mL of ultrapure H_2O with an electric homogenizer to obtain one sample. The samples were then centrifuged at 12,000 rcf, 4 °C, for 5 min (HERMLE Z 233 MK-2) and the supernatant was recovered and filtered (Minisart Syringe Filter Sartorius, 0.45 μm). From this quantity, 100 μL were taken for the specific HPLC vial to provide a sufficient amount for the analysis. Chromatographic parameters for HPLC (Perkin-Elmer series 200) were set according to Tambwekar et al. (2003), with some modifications: flow rate 0.5 mL min^{-1}, column temperature = room temperature, injection volume = 50 μL; retention time = 9 min; time of analysis = 20 min. The peak of nicotine was read at 280 nm. A calibration curve was set up using a nicotine standard (±)-Nicotine – NO267 (Sigma-Aldrich). The limit of quantification (LOQ) was 5.5 μg/g dw. Three samples were measured for each car.

2.3. Bioaccumulation of heavy metals

Unwashed lichens were pulverized and homogenized with a ceramic mortar and pestle. To get one sample, about 200 mg of lichen material were randomly selected and mineralized with a mixture of 3 mL of 70% HNO_3, 0.2 mL of 60% HF and 0.5 mL of 30% H_2O_2 in a microwave digestion system (Milestone Ethos 900) at 280 °C and 55 bar. The concentrations of selected heavy metals (Al, As, Cd, Cr, Cu, Fe, Ni, Pb, Sb, V and Zn) were determined by ICP-MS (Perkin Elmer Sciex, Elan 6100) and expressed on a dry weight basis (μg/g dw). The investigated elements were selected being of toxicological concern, (e.g., Bernhard et al., 2005; Iskander et al., 1986). A sample of the certified reference material IAEA-336 'lichen' and one procedural blank with the reagents were included in each digestion trial (every 8 samples). Results of accuracy checks were as follows: recoveries in the range 96–107%; imprecision of analysis, expressed by the relative standard deviation of 5 replicates, was below 3% for Al, Cu, Fe, Pb and below 10% for the remaining elements. Three samples were measured for each car.

2.4. Sample vitality

For a rapid screening of the vitality, the potential quantum yield of primary photochemistry F_V/F_M was used as indicator of the photosynthetic performance of the samples, where $F_V = (F_M - F_0)$ is the variable fluorescence and F_0 and F_M are the minimum and maximum Chl *a* fluorescence, respectively. In the laboratory, for each car, 10 randomly selected lichen thalli were reactivated for 24 h by spraying with mineral water and keeping humid at 16 °C in dim light (70 μmol $m^{-2}s^{-1}$). Prior to the measurements, lichen thalli were again sprayed and dark adapted for 10 min. Samples were then lightened for 1 s with a saturating (3000 μmol $m^{-2}s^{-1}$) light pulse, and fluorescence emission was recorded for 1 s with a Plant Efficiency Analyzer (Handy PEA, Hansatech Ltd, Norfolk, UK). Ten samples were measured for each car.

2.5. Statistical analysis

Owing to the limited dataset, non parametric statistics were used. A permutation test was used to check if concentrations of heavy metals and nicotine were higher and vitality lower (one-sided test, $p < 0.05$) in smokers' cars. For the statistical analysis, values < LOQ were replaced by their respective LOQ value. The bioaccumulation of heavy metals and nicotine, as well as the loss in vitality were assessed in terms of the ratio between exposed to control samples (EC ratios). According to Frati et al. (2005), EC ratios > 1.25 and < 0.75 indicate values significantly different from the control. A "smoke" factor (SF) was calculated as the ratio between values of smokers' and non-smokers' cars. Results are presented as means ± bootstrapped 95% confidence intervals. Calculations were run using the free software R (R Core Team, 2018).

3. Results

Smokers reported a consumption of 0.8–3.7 cigarettes per hour (while driving), with partial air circulation in the car (more or less open windows). The number of driving hours was comparable between smokers (65 ± 16) and non-smokers (70 ± 24).

The biological response of the lichen *E. prunastri* travelling in smokers' and non-smokers' cars for eight weeks indicated a significant bioaccumulation of nicotine (min–max 41–997 μg/g dw) and most heavy metals (Al, Cd, Cr, Cu, Ni, Sb, Zn), and a decreased vitality in samples that travelled in smokers' cars (Table 2).

EC ratios calculated for smokers' cars highlighted a significant uptake for nicotine and most heavy metals (Al, As, Cd, Cr, Cu, Ni, Pb, Sb); noteworthy, an uptake emerged also for Cu and Sb in non-smoker's cars (Tab. 3).

Table 2
Concentration of heavy metals and nicotine (μg/g dw) and potential quantum yield of primary photochemistry (F_V/F_M) as indicator of the vitality of the lichen *Evernia prunastri* after travelling for about two months in smokers' and non-smokers' cars (mean ± 95% confidence interval). Values in bold are statistically higher – lower for vitality – ($p < 0.05$) in smokers' cars with respect to non-smokers' cars, values in italics are statistically higher in non-smokers' cars with respect to control samples.

	control (pre-exposure)	non-smokers	smokers
Al	498 ± 106	400 ± 72	**653 ± 27**
As	0.17 ± 0.02	0.21 ± 0.03	0.25 ± 0.06
Cd	0.062 ± 0.005	0.074 ± 0.010	**0.128 ± 0.050**
Cr	1.13 ± 0.21	0.95 ± 0.11	**1.57 ± 0.37**
Cu	4.2 ± 0.4	*7.1 ± 0.8*	**10.0 ± 1.4**
Fe	342 ± 34	336 ± 44	410 ± 46
Ni	1.4 ± 0.1	1.4 ± 0.1	**2.7 ± 0.9**
Pb	1.3 ± 0.1	1.5 ± 0.2	1.8 ± 0.3
Sb	0.047 ± 0.004	*0.064 ± 0.033*	**0.110 ± 0.032**
V	1.10 ± 0.09	0.97 ± 0.1	1.25 ± 0.33
Zn	23.3 ± 2.8	17.5 ± 2.6	**24.7 ± 3.0**
Nicotine	< LOQ	< LOQ	**471 ± 268**
F_V/F_M	0.71 ± 0.06	0.67 ± 0.03	**0.29 ± 0.09**

The smoke factor indicated a 85.6-fold contribution for nicotine and contributions in the range 1.2 (Pb) to 2 (Ni) for heavy metals; in addition, a 60% reduction of vitality, as depicted by the EC of the photosynthetic indicator F_V/F_M, was outlined for lichens that travelled in smokers' cars (Table 3).

4. Discussion

It was assumed that the content of chemical elements in lichen thalli exposed in smokers' and non-smokers' cars reflects the characteristics of the respective indoor environments. During their permanence in smokers' cars, lichen samples accumulated relevant amounts of nicotine and several heavy metals, and showed a decreased vitality, suggesting a worse indoor air quality. This is not surprising, since it is known that smoking cigarettes in the cabin of a car leads to contamination of this indoor environment by residual tobacco smoke pollution (Fortmann et al., 2010). However, the quantification of this phenomenon is a quite hard task, since the level of contamination depends on a wide array of variables such as smoking behavior, air ventilation, car volume, etc. (Ott et al., 2008).

Lichens accumulated relevant amounts of nicotine in smokers' cars, while concentrations in non-smokers' cars were < LOQ. The great accumulation of nicotine in lichens exposed inside smokers' cars can be empirically confirmed also by the characteristic odor of secondhand smoke (Matt et al., 2008), which was strongly maintained also after

Table 3
Exposed to Control (EC) ratios (mean ± 95% confidence interval) and smoke factors (ratios between values of smokers' and non-smokers' cars). Values in bold are significantly different from the control.

	non-smokers	smokers	smoke factor
Al	0.80 ± 0.15	**1.31 ± 0.05**	1.6
As	1.23 ± 0.20	**1.46 ± 0.36**	1.2
Cd	1.19 ± 0.98	**2.07 ± 0.74**	1.7
Cr	0.84 ± 0.09	**1.39 ± 0.27**	1.7
Cu	**1.70 ± 0.19**	**2.38 ± 0.36**	1.4
Fe	0.98 ± 0.13	1.20 ± 0.14	1.2
Ni	0.98 ± 0.01	**1.92 ± 0.71**	2.0
Pb	1.15 ± 0.14	**1.38 ± 0.20**	1.2
Sb	**1.36 ± 0.65**	**2.35 ± 0.61**	1.7
V	0.88 ± 0.08	1.14 ± 0.24	1.3
Zn	0.76 ± 0.13	1.06 ± 0.13	1.4
Nicotine	1	**85.6 ± 49.3**	85.6
F_V/F_M	0.95 ± 0.04	**0.41 ± 0.13**	0.4

storage. We could not find in the literature any report of nicotine concentration in lichens, and the most closely related data are the 0.1–4.5 μg/g dw reported for mushrooms (Cavalieri et al., 2010), values which are consistent with the concentrations < 5.5 μg/g dw found in lichens exposed in non smokers' cars.

It is known that plants fumigated with nicotine may accumulate relevant amounts of this alkaloid: e.g., peppermint plants fumigated for 2 h in a small greenhouse (22 m^3) with the smoke from 11 cigarettes accumulated nicotine up to 60 μg/g dw (Selmar et al., 2015). Nicotine is a suitable indicator of ETS, being specific for tobacco combustion and emitted in large quantities in sidestream smoke (U.S. E.P.A., 1992); it has a high adsorption rate to indoor surfaces, from which it is re-emitted even after weeks in absence of active smoking (Jaakkola and Jaakkola, 1997), therefore it is a suitable indicator also of secondhand smoke (Fortmann et al., 2010; Matt et al., 2004). Nicotine levels have sometimes been measured inside car cabins, to evaluate the amount of residual tobacco smoke pollution in rental cars or in second-hand cars on sale owned by smokers, that could have exposed buyers and non-smoker passengers to possible contamination (Matt et al., 2008; Fortmann et al., 2010). The range of reported average nicotine air concentrations in smokers' cars varied over the wide range of 0.04–0.74 μg/m^3, while for non smokers' cars the values were ca. 0.02 μg/m^3 (Matt et al., 2008; Quintana et al., 2009). These values indicate a smoke factor in the range 2–37, much lower than the 86 found for lichens (min 8 – max 181). However, Matt et al. (2008) reported a smoke factor for surface-based accumulated nicotine of ca. 140, which is consistent with our values in the range 8–181. This suggests that either lichens are very efficient bioaccumulators of atmospheric nicotine or that their accumulation capacity for nicotine is also linked with their high surface/mass ratio. Consistently with the suggestion of Matt et al. (2008), that the number of cigarettes smoked by the owner of the car is more important than the number of cigarettes directly smoked inside the car, our data indicate a correlation (although with $p < 0.10$) between the concentration of nicotine in lichens and the total number of cigarettes smoked by the owner of the car, while no correlations emerged with the number of cigarettes smoked inside the car, the number of cigarettes per km or per hour driven (data not shown). The limited dataset could have influenced such result.

Tobacco smoke is composed of a mixture of gas-phase and particle-phase components, and not only the most volatile compounds such as nicotine may easily adsorb onto materials inside the car, but also particulate constituents can accumulate on surfaces and in dust, and be later resuspended in the air (Fortmann et al., 2010). This is usually the case of most heavy metals, which are widely emitted during cigarette smoking (Benson et al., 2017). Our results indicated that Al, Cd, Cr, Cu, Ni, Sb, Zn are accumulated at higher concentrations in lichens exposed inside smokers' cars compared with non-smokers' cars and that accumulation is significantly different from the control for Al, As, Cd, Cr, Cu, Ni, Pb, Sb. By cross-checking the above data, it emerges that Al, Cd, Cr, Cu, Ni and Sb are of concern for smokers' cars compared with non-smokers' cars. Most of the above elements are reported in tobacco, cigarette paper, filters and particulate matter from cigarette smoke (e.g., Benson et al., 2017; Bernhard et al., 2005; Iskander et al., 1986). Our data highlighted Ni as the main contaminant (smoke factor = 2) among heavy metals. This result is consistent with the fact that cigarette smoking is a significant source of Ni (Stojanovic et al., 2004), which may be emitted not only as particles, but also as gaseous compound (Torjussen et al., 2003). Since Ni is classified as a group I carcinogen by IARC, our results greatly support the hypothesis that people travelling inside smokers' cars are exposed to health risk by second-hand smoke pollution. It is noteworthy that besides accumulating heavy metals in smoker's cars, our lichens significantly taken up Cu (EC = 1.70) and Sb (EC = 1.36) also in non-smokers' cars. This is not surprising, since Cu and Sb are typical traffic-related elements, whose content in lichen thalli is regarded as a good tracer of traffic pollution (Loppi and Paoli, 2015; Paoli et al., 2013). Therefore, the levels of Cu and Sb in smokers' cars represent the contribution of both smoke and traffic pollution.

The vitality of our lichens that travelled inside non-smokers' cars was not statistically different from that of control samples, suggesting that the significantly decreased photosynthetic efficiency measured in lichens that travelled in smokers' cars was caused by smoke and not by the stay inside the car. As laboratory and field studies showed (e.g., Paoli et al., 2014, 2016), that the exposure to heavy metals and other toxic compounds negatively affects the photosynthetic efficiency of lichens, this result is a further confirmation that exposure to second-hand smoke may negatively affect biological systems, not only causing an accumulation of toxic substances, but also with detrimental effects at physiological level.

5. Conclusions

The presence of residual tobacco smoke pollution in air, dust and surfaces of vehicles is largely a dynamic process of adsorption/desorption which determines the accumulation and later re-emission/re-suspension of pollutants associated with smoking, and it is influenced by many variables. Our results, based on the biological response of biomonitors (lichens) that travelled inside the cars for two months, greatly support the hypothesis that car cabins of smokers are contaminated by relevant amounts of nicotine as well as by several chemical elements (Al, As, Cd, Cr, Cu, Ni, Pb, Sb), and that these pollutants negatively affect the vitality of the samples. Thus, the indoor environment of a car is polluted not only during smoking, but for a much longer time, causing additional health risk for the smoking driver and also exposing at risk the health of non-smokers using the vehicle at a later time. Moreover, our results also suggested that the indoor environment of non-smokers' cars is polluted by traffic-related elements such as Cu and Sb.

Declaration of interest

None.

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