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Tracking polycyclic aromatic hydrocarbons in lichens: It's all about the algae

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ABSTRACT

Lichens, symbioses of fungi and algae and/or cyanobacteria, have the remarkable ability to uptake and accumulate semivolatile organic compounds (SVOC) from air, including polycyclic aromatic hydrocarbons (PAHs), but the mechanism of accumulation is still unknown. Understanding these mechanisms is critical to standardize the use of lichens as environmental bioindicators and to further integrate them in air monitoring networks. Through a series of experiments we show that gas phase PAHs easily cross lichen's surface and accumulate in the photosynthetic algal layer of lichens. Once accumulated, they remain in the algal layer and not within the fungus hyphae, or adhered to lichen's surface, as it was previously supposed to happen. Additionally, when lichens are washed, gas phase PAHs still remain in the algal layer. Our results reveal that lichens may be utilized as bioindicators of gas phase PAHs, overcoming current limitations of air monitoring.

1. Introduction

Lichens are among the most sensitive organisms in terrestrial ecosystems, responding as early-warning of environmental stresses, including air pollution (Niemi and McDonald, 2004). Unlike plants, lichens do not have roots, depending totally on atmospheric fallout for nutrition. Together with nutrients, they end up accumulating atmospheric pollutants which may cause their own death and affect lichen biodiversity (Contia and Cecchettib, 2001). Due to their outstanding bioaccumulative performance, lichens have been used to monitor a wide range of pollutants all over the planet, ranging from metals and radionuclides to persistent organic pollutants (POPs), including PAHs (Studabaker et al., 2012; Augusto et al., 2004, Augusto, 2012; Schrlau et al., 2011; Shukla and Upreti, 2012; Shukla et al., 2013).

Accumulation mechanisms of lichen symbioses have been described for most pollutants, especially metals (Mikhailova and Sharunova, 2008; Brown, 1987; Nash, 1996); but no information is available regarding accumulation mechanisms of gas phase PAHs or other semivolatile organic compounds (SVOC) in lichens. PAHs are a large group of toxic SVOC composed of two or more fused aromatic rings, which occur naturally in the environment, generated by forest fires and volcanic eruptions (Mastral et al., 2003; Nadal et al., 2004, 2006). However, the largest amount is released by human activities, including incomplete combustion of organic materials during industrial activities, home heating, power generation, incineration, vehicle emissions, petroleum cracking, refining in petrochemical industries, and chemical manufacturing (Mastral et al., 2003). Once released to the atmosphere, gas phase PAHs are capable of traveling long distances before depositing, becoming a concern not only to human populations living in urban areas but also to natural ecosystems (Halsall et al., 1997; Hung et al., 2005; Nadal et al., 2011).

In this study we monitored and tracked one of the most abundant PAHs found in lichens, the 4-ringed compound fluoranthene (FLU); and one of the most toxic PAHs, the 5-ringed compound benzo[a]pyrene (BaP) in the foliose lichen *Xanthoria parietina*, commonly known as golden shield lichen. This lichen species is often found growing on a diverse range of substrates (trees, house roof tiles, walls, rocks) in agricultural, urban and to some extent in industrial areas, where pollutant levels tend to be higher than in

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forestry and natural areas. *X. parietina*, because of its wide distribution, is frequently collected and chemically analyzed to achieve pollutants' concentrations in environments with anthropogenic activities (Augusto et al., 2004, 2013a).

The thallus of *X. parietina* has a flat shape and is composed of four layers: an upper cortex covered by the orange compound parietin which has the role of protecting the algae from sunlight radiation; an algal layer mainly composed by individuals of *Trebouxia* genera (single-celled green algae); a medulla where fungus hyphae form a well-structured net; and a lower cortex with rhizines, with the role of attaching the lichen to the substrate. The upper cortex represents the first barrier pollutants need to cross to enter the lichen.

The main aim of this study was to study the uptake and accumulation of gas phase PAHs (namely FLU and BaP) in the foliose lichen *X. parietina*. Aspects such as influence of washing in the accumulation performance and effect of the lichen growth form were also addressed.

2. Materials and methods

In this study, thalli of the foliose lichen *X. parietina* (Fr.) Th. Fr. were exposed to gas phase fluoranthene (FLU) and benzo[a]pyrene (BaP) over a 16 day contamination period. For comparison with lichens with a different thallus structure, other lichen species with a non-stratified structure (with algae dispersed all over the thallus) were studied as well. A fluorescence microscope (FM) was used to monitor uptake and accumulation fate of each compound within lichen thalli.

The lichen species *X. parietina* was chosen because it has previously been utilized to monitor PAHs in different countries and because it is easy to find growing in almost all types of habitat (Contia and Cecchettib, 2001).

Fluoranthene was selected as one of the target compounds because it is one of the most abundant PAHs in lichens. Being a 4-ringed PAH, it can be found either in gas or particulate phase of air. BaP was selected because it is one of the most toxic PAH studied so far. Having 5 rings in its structure, it exists mainly associated with the particulate phase of air. Nevertheless the partition between gas and particulate phases depends on the physicochemical characteristics and environmental conditions, notably vapor pressure and temperature. Due to their aromatic structure, both target compounds fluoresce when irradiated with UV light and thus are suitable for FM-UV visualization.

Lichens were collected in the mountains of L'Albiol, Tarragona, Spain, from branches and trunks of the available phorophytes, and immediately transported to the lab. After a 24 h hydration period, individual thalli of lichens were exposed to FLU and BaP in separate glass Petri dishes (10 cm diameter, 1.5 cm height, 17.8 cm^3).

Fluorescent grade FLU (99.5%) and BaP (99.6%) were obtained from Dr. Ehrenstorfer GmbH (Germany), and for each Petri dish, approximately 2.5 mg was dissolved in 20 mL acetone and then 250 μl were placed in the middle of the dish using a glass pipet. Lichen thalli (approximately 10 per dish) were placed close to the borders without being in direct contact with the compounds. Uncontaminated control samples (lichens exposed to acetone without PAHs) were used in all experiments.

The Petri dishes were closed and placed in sealed plastic boxes (whitish transparent color), which were placed inside a climatic chamber under constant conditions of temperature (23 °C), relative humidity (23%) and polychromatic radiation (24 W/m^2) throughout the experiment. Three Petri dishes were placed in each plastic box. Lichen thalli were taken for FM analysis after 42 h, 8 and 16 days. Thalli were removed from the contamination Petri dish, placed in glass slides, sectioned using a razor blade, and transferred immediately to the FM for analysis. At least three replicate samples (lichen thalli inside a Petri dish) were analyzed for each compound and at each time period. Uncontaminated control lichens were also analyzed on each occasion.

In order to evaluate the effect of a washing event in the accumulation of PAHs within lichen thalli, after 10 days of exposure to gas phase FLU and BaP, lichen thalli were washed for 3 min with 100 mL of distilled water. After washing, thalli were placed in clean Petri dishes and kept in the same controlled conditions as before. After 5 days thalli were prepared and taken for FM analysis.

The same exposure experiment was repeated for other species of lichens, namely for: *Ramalina canariensis* Steiner, *Ramalina fastigiata* (Pers.) Ach., *Evernia prunastri* (L.) Ach., *Parmotrema hypoleucinum* (Steiner) Hale, *Flavoparmelia caperata* (L.) Hale. Microscope observations of lichens' sections were made after 42 h and 8 days of exposure (results in Supplementary material).

A Nikon Eclipse TE2000-E inverted microscope equipped with bright field, phase contrast and fluorescence capabilities, coupled to a Hamamatsu ORCA digital camera (C8484), was used. The filter set was composed of four filters – red, green, blue and ultraviolet (UV). The UV filter was used to visualize PAHs inside lichens. The remaining filters allowed visualizing all lichen components, including upper cortex with pigment layer, algal layer, medulla with fungus hyphae and the lower cortex. 10× and 20× working distance phase contrast objectives were used.

Images were collected using NIS-Elements BR (3.00, SP1) software. Exposure time in acquisition of images varied depending on the compound, the filter used, and the time period of the experiment, but was kept constant within the same compound and the time period to allow comparisons between exposed and control samples. Hardware gain was set at 10 and binning 1 × 1, plan fluor10×, calibration (μm/px) 0.65, ORCA numerical aperture 0.3, refractive index 1. Composite images were obtained by merging the four color channels using the public software ImageJ 1.47v (Wayne Rasband, National Institutes of Health, USA).

3. Results and discussion

In order to understand the uptake mechanisms of PAHs by lichens and the role of each symbiotic partner in the accumulation process, we performed a laboratory study aiming to simulate exposure of lichens to gas phase FLU and BaP. We exposed *X. parietina* thalli to fluorescent grade FLU and BaP in stable controlled conditions of temperature, humidity and radiation for different time spans, ranging from 42 h to 16 days, and followed their path inside lichen tissues using a fluorescence microscope (FM).

In less than 42 h, FLU migrated across the lichen's surface, crossing the parietin layer and upper cortex, and was observed to be within the algal layer (Fig. 1A). After 8 days, FLU was observed to be mostly within the algal layer and no FLU was observed in other parts of the lichen structure (Fig. 1B). BaP was observed to be within the algal layer only after 8 days of exposure (Fig. 2A and B). Both compounds were observed to remain within the algal layer after 16 days of exposure (Figs. 1C and 2C). Different uptake rates of FLU and BaP may be related to different molecular weights of compounds. FLU is a small molecule (202.26 g/mol) in comparison with BaP (252.31 g/mol). When entering the lichen, both compounds need to cross several resistances imposed by the thallus morphology (Collins and Farrar, 1978). The smaller the molecule, the weaker the resistance. On the other hand, vapor pressure of BaP (7.0×10^{-7} Pa at 25 °C) is lower than of FLU (1.2×10^{-3} Pa at 25 °C), whereas log K_{oa} (octanol-air partition coefficient) is higher (11.48 for BaP and 8.81 for FLU), meaning that it will take longer to volatize (Mackay et al., 1992).