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Uptake and acute toxicity of cerium in the lichen *Xanthoria parietina*

L. Paoli [a], E. Fiorini [a], S. Munzi [b], S. Sorbo [c], A. Basile [c], S. Loppi [a,*]

[a] University of Siena, Siena, Italy
[b] University of Lisbon, Lisbon, Portugal
[c] University of Naples, Naples, Italy

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ABSTRACT

Environmental cerium (Ce) levels are likely to increase in the near future and monitoring of its biological effects will therefore be necessary. The aim of this study was to test if treatment of the lichen *Xanthoria parietina* with Ce-containing solutions (0.1 mM, 1 mM, 10 mM and 100 mM) causes Ce bioaccumulation (both extra- and intra-cellularly) as well as physiological (sample viability, membrane lipids peroxidation, photosynthetic performance, water-soluble proteins content) and ultrastructural alterations. The results showed that treatment with Ce solutions induces Ce bioaccumulation, both extra-cellularly and intra-cellularly, which in turn causes an acute toxicity, evident as decreased sample viability, marked decrease in the photosynthetic performance and important changes in the ultrastructure.

1. Introduction

Cerium (Ce) is a rare earth element (REE) that represents 0.0043 percent of the Earth's crust and occupies the 25th place in the list of the most common elements in the lithosphere. The presence of Ce in the Earth's crust is in the range 20–60 mg/kg, but its occurrence in soils is highly variable, depending on the mineral characteristics of the parent rocks and the human impact, since levels of Ce are higher in anthropic environments. Cerium is the basic constituent of vehicular and industrial catalysts in association with platinoids (Rauch et al., 2002) and is employed in the reduction of pollutant emissions or the maximization of several production processes (combustion, dehydrogenation, hydrogenation, cracking).

The application of Ce in catalytic converters allows platinoids stabilization, increased resistance to high temperature and improvement of catalytic activity (Koltsakis and Stamatelos, 1997). In addition, Ce is used to store oxygen to optimize the efficiency of the air-fuel mixture, and to improve the efficiency of rhodium in the reduction of nitrogen oxides (NO_x). Cerium-containing particles are emitted as a result of the deterioration of catalytic converters, mainly owing to abrasion and thermal stress, and particles of road dust from urban environments show a clear relationship between Ce and platinoids (Rauch et al., 2002).

Cerium is also used as additive in the disposal of particulate matter generated by diesel engines. Diesel engines are provided with a special anti-particulate filter, also known as “trap”, or DPT (Diesel Particulate Trap), which captures and retains carbonaceous particles in exhaust gas. Along with its use, the filter tends to clog and Ce contributes to restore its functionality. These filters can be regenerated after use and the regeneration process involves the addition of Ce to the fuel. Consequently, the wear of catalytic converters and the consumption of additives in diesel engines determine a progressive emission of Ce and accumulation into the environment, especially in urban areas.

Environmental levels of Ce in PM in the air have been reported in the interval 0.19–11 ng/m^3 (HEI, 2001). Cerium dispersion to the environment has been modeled for various types of diesel engines and traffic situations (Samaras, 1994): future ambient levels are estimated to increase in high traffic scenarios up to 1.25 μg/m^3.

Soil samples collected around busy roads are clearly enriched in Ce: e.g. in soil samples collected from urban sites in Japan, Ce ranged 51–160 mg/kg (Sugimae, 1980) and in soils along British highways Ce ranged 47–136 mg/kg (Ward, 1990). The increase of Ce in the soil foreseen for 2050 compared with the actual levels (based on country roads, in the first 10 cm of depth and at a maximum distance of 10 m from the edge of the roadway), has been estimated in the range 5–30 mg/kg (Samaras, 1994). Lichens have been widely used in biomonitoring of air pollution since their dependency on the atmosphere for nutrients and the lack of a waxy cuticle and stomata, allowing many contaminants to be absorbed over the whole thallus surface, make them very sensitive organisms (Ferry et al., 1973). Despite an increasing concern in studying Ce as a pollutant, the biological risk associated with spreading of Ce in the environment is still poorly known. The main routes of exposure to Ce-containing particles are inhalation and

* Corresponding author. Fax: +39 577 232896.
E-mail address: stefano.loppi@unisi.it (S. Loppi).

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ingestion (HEI, 2001). Cerium is released to the environment mainly associated with PM and can be easily oxidized to form a variety of salts (such as chlorides, phosphates, and nitrates). Soluble forms of Ce are likely to reach the circulation and be deposited in several organs (HEI, 2001). Modeling of particle (1 μm in diameter) clearance estimated that about 80 percent of inhaled Ce deposited in the lung would clear through the gastrointestinal tract and about 5–15 percent would translocate to the circulation and the rest to the lymph nodes (HEI, 2001). On these bases, we hypothesized that: (i) lichens not only accumulate Ce at extracellular sites, but this element can enter the cells and exert a physiological effect; (ii) high concentrations of Ce can be deleterious to lichens and effects can be seen at physiological and ultrastructural level. In order to test these hypotheses, we investigated the physiological response of the lichen *Xanthoria parietina* treated with Ce-containing solutions.

2. Materials and methods

2.1. Lichen material

Thalli of the foliose lichen *X. parietina* were collected in a rural area of Tuscany (43°14′07″N, 11°20′26″E, Ville di Corsano, Siena, Italy). This species was chosen being extremely common in Tuscany and already used in biomonitoring studies (Loppi et al., 2006) as well as in laboratory experiments (Munzi et al., 2009; Pisani et al., 2009, 2011). After collection, samples were transferred to the laboratory in plastic bags. Peripheral lobes of the thalli were detached using tweezers, got rid of impurity, rinsed in deionized water and left overnight in a climatic-chamber at 15 ± 2 °C, RH 55 ± 5 percent, 40 μmol/m^2/s photons PAR.

2.2. Cerium treatments

Treatment solutions containing Ce at concentrations of 0.1 mM, 1 mM, 10 mM and 100 mM were prepared using cerium chloride ($CeCl_3 \cdot 7H_2O$). Samples were treated with excess Ce for a short-term to simulate acute Ce pollution and identify physiological parameters sensitive to this element. Lichen samples were incubated and gently shaken for 1 h in 50 mL of solutions, let air-dry on absorbing paper and then stored at light and room temperature for 24 h. The same treatment was repeated three times and data compared with control samples treated in the same way, but incubated in deionized water. All treatments were carried out on five replicates (500 mg of lichen material was prepared for each replicate, to carry out physiological tests and Ce determination).

2.3. Bioaccumulation

Since the biological effect of any element is determined by the amount that penetrates into the cell and not by its total concentration, to distinguish between total amounts and intracellular fractions of Ce, a sequential elution technique (Brown and Brown, 1991) was followed. Samples were divided into two batches (each of 1000 mg): the total Ce content was determined in one batch while the other batch was soaked by shaking for 20 min in 100 mL of 20 mM Na_2EDTA solution and then rinsed in deionized water to remove the extracellular fraction of Ce bound to the cell wall (Branquinho and Brown, 1994). The content of Ce in the samples after this washing cycle corresponds to the intracellular fraction.

Lichen thalli were air-dried to constant weight then pulverized and homogenized in liquid nitrogen with a ceramic mortar and pestle. About 200 mg of lichen powder were mineralized with a mixture of 6 mL of 70 percent HNO_3, 0.2 mL of 60 percent HF and 1 mL of 30 percent H_2O_2. Digestion of samples was carried out in a microwave digestion system (Milestone Ethos 900, Milestone S.r.L, Sorisole, Italy). Cerium levels, expressed on a dry weight basis, were determined by ICP-MS (Perkin-Elmer Sciex 6100, Perkin-Elmer, Waltham, MA, USA). Analytical quality was checked by the Standard Reference Material IAEA-36 'lichen'. Precision of analysis was estimated by the coefficient of variation of five replicates and was found to be within 6 percent.

2.4. Sample viability

Triphenyltetrazolium chloride (TTC) reduction to triphenylformazan (TPF) is a good indicator of dehydrogenase activity (dark respiration) and was used to assess sample viability (Bačkor and Fahselt, 2005). Ca. 15 mg of lichen material was incubated in the dark for 20 h in 2 mL of 0.6 percent TTC and 0.005 percent Triton X 100 solution in 50 mM phosphate buffer. Solutions were then removed and samples rinsed in distilled water until bubbles of Triton X were produced. Water-insoluble formazan was extracted with 6 mL of ethanol at 65 °C for 1 h. Tubes were then centrifuged at 4000*g* for 10 min and absorbance read at 492 nm. Results were expressed as absorbance units/g (dw).

2.5. Membrane lipid peroxidation

Membrane lipid peroxidation was estimated using the thiobarbituric acid reactive substances (TBARS) assay. About 50 mg of lichen material was rinsed in distilled water and then homogenized in a mortar using 2 mL of 0.1 percent (w/v) trichloracetic acid (TCA) with the addition of sand. 1.5 mL of the homogenate were put in eppendorf tubes and centrifuged at 12,000*g* for 20 min. 0.5 mL of the supernatant were collected and added to 1.5 mL of 0.6 percent thiobarbituric acid in 10 percent TCA and put in glass tubes. Tubes were put in the oven at 95 °C for 30 min, cooled in an ice bath and then solutions were centrifuged again at 12,000*g* for 10 min. The absorbance of the supernatant was measured at 532 nm and corrected for non-specific absorption at 600 nm. Concentration of TBARS was calculated using the extinction coefficient for the TBA-MDA complex (155/mM cm) and the results expressed as μmol/g (dw).

2.6. Chlorophyll *a* fluorescence emission

Chlorophyll (Chl) *a* fluorescence emission was analyzed by the classical physiological indicator F_V/F_M, representing the potential quantum yield of primary photochemistry, where $F_v=(F_M-F_0)$ is the variable fluorescence and F_0 and F_M are minimum and maximum Chl *a* fluorescence. In addition, the performance index PI_{ABS}, a global indicator of the photosynthetic performance, was calculated to express the overall vitality of the photobiont in the samples (Strasser et al., 2000). To measure each sample, the lichen material was sprayed with mineral water for a few seconds, once hydrated samples were dark-adapted with a clip (4 mm diameter) for 10 min to allow full dark adaptation of the photosynthetic pigments. Lichens rested on a foam pad whilst in the clip to minimize damage to the structure. Samples were then lightened 1 s with a saturating 3000 μmol/m^2 s light pulse and fluorescence emission was recorded for 1 s. Both parameters were measured with a Plant Efficiency Analyzer (Handy PEA, Hansatech Ltd, Norfolk, UK).

2.7. Water-soluble proteins

The water-soluble protein content was estimated by the dye binding technique (Bradford, 1976). Samples (about 25 mg) were rinsed in deionized water and homogenized in a mortar using 2 mL of 50 mM phosphate buffer (pH 6.8) and sand, and the homogenate was centrifuged at 12,000*g* at 4 °C for 20 min. Then 100 μL of the supernatant were added to 1.5 mL of Bradford solution, the mixture shaken and allowed to react at least for 10 min. Absorbance was read at 595 nm and the concentration (mg/g dw) was determined using bovine serum albumin as a protein standard.

2.8. Transmission electron microscopy (TEM)

Lichen samples were kept for 24 h on filter paper wetted with deionized water to ensure complete hydration of the thalli. TEM preparations (3 replicates for each treatment) were run according to the method reported in Basile et al. (1994). In short, specimens were fixed with glutaraldehyde 3 percent, post-fixed with osmium tetroxide 1 percent, dehydrated with ethanol to propylene oxide and embedded in Spurr's epoxy medium. Ultrathin sections (70 nm thick) were collected on copper grids and stained with uranyl acetate and lead citrate. A FEI EM 208S TEM (FEI, Eindhoven, The Netherlands), with an accelerating voltage of 80 kV, was used for observations. The image analysis of the cellular ultrastructural characters within a median section of algal and fungal cells was performed on electron micrographs by the software program analySIS (FEI, Eindhoven, The Netherlands); cytoplasmic and chloroplast droplets, altered organelles and other ultrastructures were examined. Ten ultrathin sections were examined for each replicate.

2.9. Statistics

Significance of differences was checked by one-way analysis of variance (ANOVA), using the HSD Tukey test for post-hoc comparisons. Differences between total and intracellular concentrations were checked by the *t*-test. Data not matching a normal distribution (Kolmogorov–Smirnov test at the 95 percent confidence interval) were log-transformed prior to analysis. Statistical tests were run using the software *STATISTICA* (StatSoft).