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Physiological changes of the lichen *Parmotrema tinctorum* as result of carbon nanotubes exposition ☆

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ABSTRACT

Carbon nanotubes (CNT) is one of the more abundant nanomaterial produced in the world. Therefore, it is desirable to access its effects in all environment compartments, in order to mitigate environmental distress. This study aims to verify the potential use of lichens – classical atmospheric pollution indicators – as biomonitors of carbon nanotubes aerosols. To examine cause–effect relationships, preserving environmental microclimatic parameters, the lichen *Parmotrema tinctorum* (Nyl.) Hale was transplanted to open top chambers where aerosols of CNT were daily added. Physiological parameters such as cell viability, photosynthetic efficiency, cell permeability as well as nanoparticle internalization were assessed.

Carbon nanotubes exposure led to reduction on the cell viability of *P. tinctorum*. The treatment with 100 μg/mL of MWCNT-COOH resulted in intracellular ion leakage, probably due to changes in membrane permeability. No alterations on photosynthetic efficiency were detected. Carbon nanotubes entrapment and internalization into the lichen thallus were observed. Short term exposition of CNT produced measurable physiological changes in *P. tinctorum* lichen. This suggests the possibility of use of lichens as models to assess the environmental impact (air related) of engineered nanomaterials.

1. Introduction

Due to the innumerable application possibilities, the production and commercialization of nanomaterials is increasing. The responsible use of this technology requires a better understanding of the nanomaterial life cycle and therefore, generates the necessity of the development of assessment protocols for environmental impact (European Commission, 2012). The potential environmental, health and safety (EHS) impacts of the technology are however, not yet well established (Nel et al., 2013; Savolainen et al., 2013). One of the main challenges in nanoecotoxicological research is the choice of bioindicators to be assessed in a realistic scenario (Behra and Krug, 2008; Savolainen et al., 2013). To this date, several reports (Baun et al., 2008; Long et al., 2012; Pereira et al., 2014) have been published focusing on the nanoparticles effects on aquatic organisms. Cytotoxicity due to nanomaterials is also well described, but there is a lack of data describing the effects of nanomaterial air exposition in the biota.

Carbon nanotubes are virtually weightless and able to stay for a long time in the air, generating aerosols with high potential of dispersion and therefore, increasing the chance of exposition (Savolainen et al., 2013). Atmospheric exposure experiments (essential for inhalation studies, for instance) is particularly challenging due to technical difficulties and cost involved in simulating real-life exposures (Nel et al., 2013).

Aiming the development of environmental risk assessments models, we investigated the effects of carbon nanotubes aerosols in lichens, classical biomonitors of atmospheric pollution. Since 1866 (Nylander, 1866), lichen forming fungi were theme of several (Conti and Cecchetti, 2001; Nimis et al., 2002; Fuga et al., 2007; Käffer et al., 2014; Paoli et al., 2015) biomonitoring studies, being recently, subject of an international standard protocol for environmental exposure (British Standard Institution, 2014). Lichens are symbiotic organisms where a photosynthetic partner (green algae or cyanobacteria) and a mycobiont (usually an ascomycete) live together as a unique morphology called thallus. Lichens are the model of choice to assess atmospheric condition, mainly, due

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to their nutritional air dependence and absence of excretory mechanisms. Two approaches are applied when lichens are used as biomonitors: the "active" – through transplant techniques – or through a "passive" survey – using data on the lichen community diversity in a determined area. The lichen transplant technique, selected for this study, is commonly used to assess biological effects of atmospheric contaminants. It is a versatile tool that can be applied in different exposure scenarios with a multitude of contaminants (Pelegrini et al., 2014; Malaspina et al., 2014; Paoli et al., 2015).

Our main objective was to analyze the possible use of this organism as a biomonitor of carbon nanotubes aerosols. To this purpose, an epiphytic foliose lichen was transplanted to open top chambers (OTCs) and treated with CNT aerosols. CNTs effects were measured in terms of lichen cellular viability, membrane damage and photosynthetic efficiency. This report is, as far as we know, the first to describe the physiological effects of carbon nanotubes in lichens.

2. Materials and methods

2.1. Lichen sampling and preservation

The epiphytic foliose lichen *Parmotrema tinctorum* (Nyl.) Hale was selected for this study because of its good availability, well-established use in biomonitoring studies (Käffer et al., 2012) and facile handling, providing for easy harvest and identification. Sampling was carried out at Serra do Cipó National Park (19°15′S and 43°33′W), far from known pollution sources, on October 2013. Whole thalli, measuring 7–10 cm of diameter, were detached from the substratum using a stainless steel knife. The material was put inside paper bags, to avoid mold formation, transported do the laboratory and carefully cleaned from foreign material and contaminants. Each thalli was divided (radial cuts) using a porcelain palette knife into 7–9 sections, with similar size and weight (approx. 40 mg and 5 cm^2). Seven experimental groups containing at least five samples were formed (each group containing a section of each specimen), and kept inside Petri plates for 16 h to dehydrated in the presence of silica gel. The Petri plates were then sealed, placed in plastic bags and the air from the samples removed. Samples were kept at −20 °C until the experiment execution.

2.2. Multi-walled carbon nanotubes

Multi-walled carbon nanotubes (97.5% pure, with outer diameters between 10 and 30 nm, and lengths between 10 and 60 μm) were synthesized by chemical vapor deposition using Fe, Co and MgO as metallic catalysts and ethylene as carbon source, under atmospheric pressure and temperatures between 600 and 1000 °C at the Nanomaterials Laboratory at UFMG. To make the MWCNT soluble in water, –COOH moieties were generated in 100 mg of the pristine nanomaterial by heating it in a microwave oven during 30 min, in 5 min cycles in an 1:1 solution of H_2SO_4/HNO_3 (adapted from Datsyuk et al., 2008). After functionalization, the mixture was cooled at room temperature and washed, through centrifugation and filtration cycles, until a neutral solution was reached. The solid product was dried in an oven at 80 °C for 24 h. Sample characterization was performed through scanning electron microscopy (SEM), transmission electron microscopy (TEM), Raman spectroscopy and thermogravimetric analysis (TGA). SEM images were obtained in a FEI scanning electron microscope fitted with a cannon FEG (Field Emission Gun), model Quanta 200. TEM images in a microscope model Tecnai G2, also manufactured by the FEI. Raman data was acquired using an Andor Technology Sharmrock Sr 303i spectrometer in the backscattering configuration, trough a 60 × immersion oil objective. The light excitation was provided by a 561.4 nm He–Ne laser source. For the thermogravimetric characterization data the equipment model SDT2960 from TA Instruments (USA) was used.

2.3. Preliminary assessment – cell viability

A preliminary exploratory experiment was conducted by transplanting lichen samples, kept in the laboratory at ambient temperature, and treated daily for 3 days with 9 different concentrations, ranging from 0.001 to 100 μg/mL, of MWCNT-COOH water dispersion. Water was used as control treatment. After the exposure period, the samples were rapidly washed with 5 mL of distilled water. The superior cortex and the algal layer (always from the thallus edge), was carefully removed using a stylet until the internal medulla was reached (white thaline area) (Käffer et al., 2012).

Photobiont cells were stained using Neutral Red and live, dead and plasmolyzed cells were counted (Le Blanc, 1971; Zetsche and Meysman, 2012). The IVF (Index of Photobiont Vitality) was calculated as described in Käffer et al. (2012), applying the formula $IVF=[V(Pl/2)/M(Pl/2)]$ where, V is the number of live cells, Pl is the number of plasmolyzed cells and M is the number of dead cells.

2.4. Open top chamber exposures

In order to examine cause–effect relationships in a more realistic environmental approach, the lichen samples were transplanted to open top chambers (OTCs). Two exposure set ups were performed, one using a functionalized carbon nanotube sample (MWCNT-COOH dispersed in distilled water) and another using the pristine nanomaterial (MWCNT as a dry aerosol). For the first set up, lichen thalli were carefully placed inside an open top chamber and daily sprayed with 0.01, 1.0 or 100 μg/mL of MWCNT-COOH water dispersion. Samples were treated during 15 days. As positive control, activated carbon at 100 μg/mL was used, and water treatment was used as negative control.

For the second set up, lichen samples were exposed to an enriched atmosphere of 0.01, 0.1 and 0.5 g of MWCNT per m^3 during 7 days. Another group was continuously treated during 21 days. These samples were exposed in an open top chamber coupled with a suspension unit where CNT were daily added. In a second, nearby chamber, the control samples were kept in the same environmental conditions.

The OTCs were made of a translucid material, and comprises the exposure chamber, the ventilation system and the chamber's top. The exposure chamber is the OTC's main structure, where the lichen samples are placed. A ventilation system directly connected to this chamber continuously provides fresh air. The suspension unit, specially developed to this work, is connected by a tube to the OTC's ventilation system. This unit has a metallic structure with a cylindrical air turbine inside, a tube for the carbon nanotubes entrance and another tube for the formed aerosol exit.

All samples were placed facing south, as they normally grow in the field, and the treatment occurred early in the morning, when a higher relative humidity is observed. Temperature and relative humidity were monitored inside both chambers and double-sided carbon microscopy tapes were placed inside the OTCs (i.e., by the side of the lichen samples) to confirm CNT availability and distribution inside the chamber. Scanning Electron Microscopy images were taken from the thallus surface and shaved cortex after the exposure. After each treatment, cell membrane damage, cellular location of target elements and photosynthetic efficiency were analyzed as follows.

2.5. Cell membrane damage

To verify the integrity of the lichen cells membrane, a piece of the lichen thallus was submerged in 25 mL of deionized water, kept in agitation for 1 h (at 20 °C) and the variation of electrical conductivity was measured (adapted from Munzi et al. (2009).

2.6. Cellular location of target elements

The cellular location and concentration of Ca, Co, Fe, Mg, Mn and Zn were determined using the sequential elution technique adapted from Brown and Brown (1991) and Figueira et al. (1999). The distribution patterns of essential elements is a way to assess lichens physiological condition. To estimate the soluble fraction present on the lichen surface, the water used for the membrane permeability protocol was stored for elemental analyses. Samples were dried at 80° C for 16 h, and the dry weight was determined. Then, the intact thalli were continuously agitated in 5 mL of $NiCl_2$ (20 mM) solution for 30 min, in order to remove the elements bound to the cell wall. To determine the elemental composition of the intracellular fraction, samples were agitated in 5 mL of HNO_3 (1 M) solution for 3–4 h until complete digestion. All the three fractions – water, $NiCl_2$ solution and HNO_3 solution – were analyzed through ICP-AES at the Atomic Spectroscopy's Laboratory at CDTN – Brazil to quantify the target elements. For quality assurance, standards (MERCK, ICP Multielement Standard Solution IV Certipur, Lot.-no. HC133638) were used and samples, when possible, were analyzed in duplicate.

2.7. Photosynthetic efficiency

Chlorophyll "a" fluorescence emission (Chl_aF) of dark-exposed samples was measured by a pulse-amplitude-modulated fluorimeter Mini-PAM; Walz, Effeltrich, Germany. The maximum quantum yield (Fv/Fm), which characterizes the potential quantum efficiency of photosystem II (PS II), was measured on dark adapted thalli kept for 48 h inside Petri plates covered with humid paper upon a microscopy glass.

2.8. Statistical analysis

Statistical analysis was performed using One Way Repeated Measures Variance Analyses of Variance (ANOVA) at Sigma Plot 13 Software, where values of $P \leq 0.05$ were considered significant. Normality was checked using K–S test. Each treatment was replicated six times. Data are presented as mean $\pm$ SD (standard deviation).

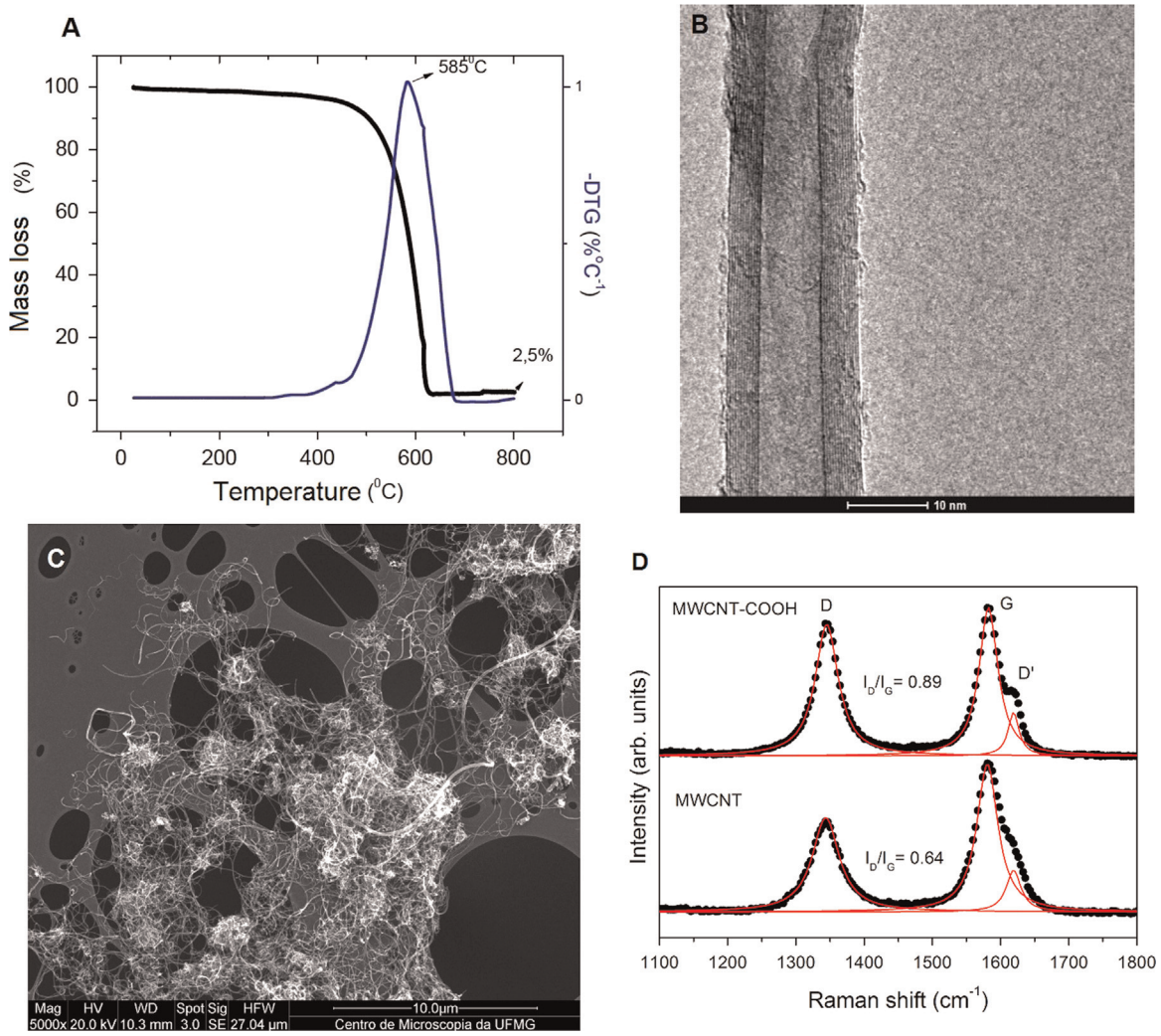

Fig. 1. Sample physical–chemical characterization. (A) Thermogravimetrical analyses of the sample. A single peak at 585 °C was observed with a 2.5% (w/w) residue left after treatment. (B) Transmission electron microscopy analyses showing a single multiwall CNT with approximately 15 nm diameter. (C) CNT are normally agglomerated and impurities are seldom observed. (D) Raman spectra of pristine (MWCNT) and functionalized (MWCNT-COOH) carbon nanotubes.

3. Results and discussion

3.1. Multi-walled carbon nanotubes characterization

Carbon nanotubes physical–chemical characterization is an essential step in any project dealing with the material, ensuring the correct causality analyses of the experiments.

The sample was considered of high purity based on thermogravimetric analyses (Fig. 1A). TG curve shows a single stage of decomposition, between 500 and 600 °C and, the DTG depict the presence of only one peak with a loss of nearly 98% of the initial mass after analyses. The residue of 2.5% indicates the presence of minor impurities, probably remains of synthesis catalysts (MgO, Co and Fe).

Scanning Electron Microscopy images from the pristine and functionalized samples showed high aggregation of carbon nanotubes, which are commonly grouped together forming bundles and clumps (Fig. 1C). Tubes diameter, illustrated in Fig. 1B, varied between 10 and 30 nm with variable lengths. Nanotubes with more than 50 μm in length were observed.

The molecular disorder degree and presence of other carbonaceous material was analyzed by Raman Spectroscopy (Jorio et al., 2004). In Fig. 1D, it is possible to observe the three characteristic spectral regions of CNT: the D (around 1336 cm^{-1}) and D′ (around 1620 cm^{-1}) bands – associated with the sample structural disorder – and the G band (around 1577 cm^{-1}) – relative to the tangential vibration modes of graphite. Furthermore, the CNT disorder degree was estimated by the relative intensities of D and G bands (ID/IG). After functionalization, the ID/IG ratio increased, reflecting the tubes structural change due to the carboxyl group's addition on the tubes surface.

3.2. Investigations on MWCNT and lichen thallus interaction

Raman Confocal images and Scanning Electron micrographs were used to investigate the carbon nanotube deposition and internalization into the lichen thallus. The MWCNT ability to penetrate the lichen thallus, and approach the photobiont cells was analyzed obtaining a confocal Raman image followed by punctual spectra acquisition. It was possible to observe the presence of MWCNT characteristic spectra (in accordance with the performed sample characterization) on the photobiont area. The D (~1295 cm^{-1}), G (~ 1653 cm^{-1}) and G′ (~2933 cm^{-1}) bands were detected on the cell area, and four illustrative data points are shown in Fig. 2.

Control samples did not present similar peaks in this spectral region. In addition, points outside the cell also did not present the nanotubes characteristic Raman spectra. These findings are in agreement with the work of Khodakovskaya et al. (2012) where the presence and relative intensity of the G band was associated with the existence of MWCNT inside plant cells.

The MWCNT aggregation and entrapment on the cell wall region, one of the principal CNT toxic mechanisms (Wei et al., 2010; Schwab et al., 2013) was confirmed by SEM of the photobiont surface (images available as supplementary information).

P. tinctorum's upper cortex was also observed through SEM after MWCNT dry exposure. It was possible to observe carbon nanotubes trapped on the lichen surface, confirming effective aerosol exposition, resulting in dry deposition. Unfortunately, a quantitative estimation of CNT on the thallus is virtually impossible to be achieved. Carbon nanotubes aggregation on the lichen thallus after a wet exposition (with MWCNT-COOH) was also observed. Several studies (Brown and Brown, 1991; Viana et al., 2011; Paoli et al., 2014) suggest that lichens are effective biosystems for entrapment of particulate airborne matter, mainly due the presence of considerable intercellular spaces between the fungus hyphae and the photobiont (Brown and Brown, 1991; Nieboer and Richardson, 1981). Besides these facts, the CNT entrapment on *P. tinctorum* can be also due to the surface chemical composition of this specie, which have – as reported by Oliveira et al. (2009) – high amounts of chelating agents, such as atranorin and lecanoric acid.

Although the specific location of the carbon nanotube cannot be completely assured, these results demonstrate the lichen ability to retain and internalize this nanoparticle in a realistic exposition scenario.

3.3. Effects of MWCNT exposition on cell viability

The lichen sensitivity to MWCNT exposition was first assessed through live, dead and plasmolyzed cell counting followed by the Index of Photobiont Vitality (IFV) calculation. Significant

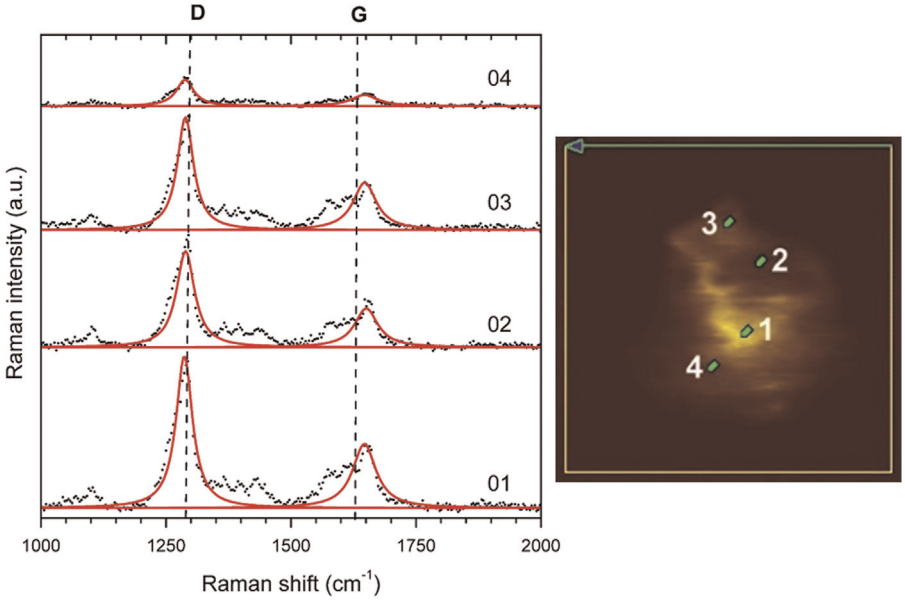

Fig. 2. Raman spectra obtained through Raman Confocal Microscopy in four different points of *Parmotrema tinctorum*'s photobiont cell. The nanotube-specific bands D and G were detected in all four points.

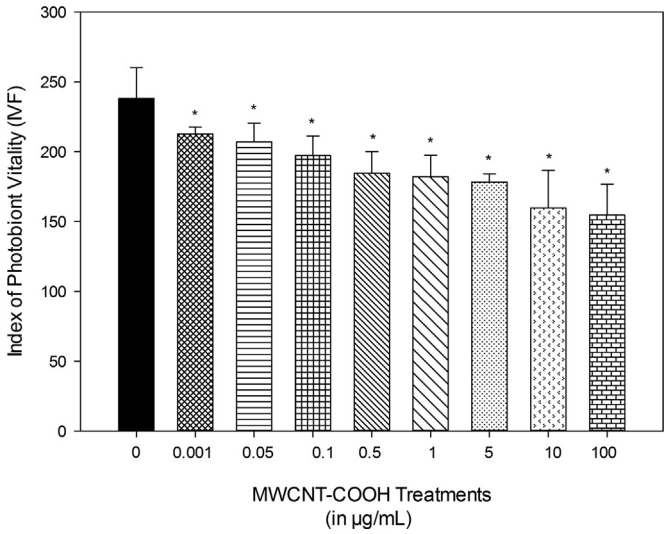

Fig. 3. Effect of MWCNT-COOH exposition on the Index of Photobiont Vitality (mean ± SD, *significantly different from control).

differences ($p \leq 0.02$) on IFV values were estimated comparing control (water) and MWCNT-COOH treatments. Lichen photobiont vitality was reduced due to the carbon nanotubes exposure (Fig. 3). However, dose–response effect was not clearly observed: the comparison between treatments shows significant correlation ($p \leq 0.05$) when the concentration increases in a factor of 100. However, no significant differences in the photobiont vitality were observed by increasing a factor of 10 in the nanotubes concentration, when comparing 100 and 10 µg/mL treatments. These data are in accordance with a similar study considering CNT effects on algae cells vitality (Pereira et al., 2014), where the cell viability was also altered by CNT exposition, but in a non-concentration dependent manner. Considering that our main objective is to evaluate the potential use of lichens for biomonitoring purposes, the organism sensitivity to the exposition is an essential question. *P. tinctorum* is known for its sensitivity to different pollutants (Ohmura et al., 2009), a characteristic related with its foliose nature (Käffer et al., 2012). Our study is the first to report *P. tinctorum* sensitivity to carbon nanotube exposure. However, as a dose response correlation was not observed, the limit of detection of the method could not be determined.

3.4. Cell membrane damage

P. tinctorum membrane does not have its permeability altered after 7 days or 21 days exposure with pristine MWCNT. The multi-exposure (21 days) samples presented the higher conductivity values. This could be due to a short exposure effect related to the transplant technique (as reported by Tretiach et al. (2007). For the second exposure set up (B), there is a statistically significant difference among the control, 100 µg/mL MWCNT-COOH and activated carbon treatments ($p \leq 0.05$). Although the treated groups mean values did not show significant differences between themselves, the ion leakage increases in a direct relation with the concentration of functionalized carbon nanotube.

A direct comparison of the effects of pristine (Fig. 4A) against functionalized CNT (Fig. 4B) cannot be evaluated due to the inherent differences on experimental conditions. However, knowing that lichens are pokilohydric organisms, where the water availability is directly related to their metabolism (Honegger, 1995; Tretiach et al., 2012), it is possible to hypothesize that the treatment with functionalized carbon nanotube (dispersed in water) is more likely to result in metabolic activation. Beckett (1996), studying *P. tinctorum,* showed that approximately 20% of the water associated with the lichen thallus is in the intercellular spaces, and 5% in small cell wall pores (5–10 nm). Considering this water

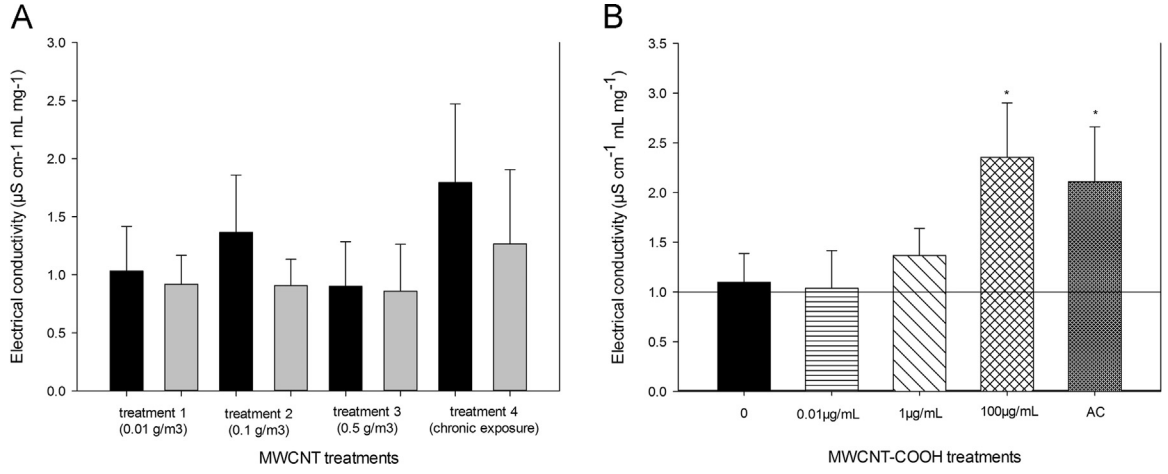

Fig. 4. Cell membrane damage in the lichen *Parmotrema tinctorum*, expressed as mean electrical conductivity (± SD, $N=6$). In (A) after MWCNT dry exposition and, in (B) after MWCNT-COOH wet exposition. (*significantly different from control).

Table 1
Changes in elemental distribution in *Parmotrema tinctorum* after MWCNT-COOH treatments, expressed as average values (in mg/L dry weigh).

Element	0		0.01		1		100		AC	
	Wall-bound	Intracellular	Wall-bound	Intracellular	Wall-bound	Intracellular	Wall-bound	Intracellular	Wall-bound	Intracellular
CA^{2+}	0.15 ± 0.01	3.28 ± 0.3	0.76 ± 0.08	0.34 ± 0.03	0.52 ± 0.05	0.21 ± 0.02	0.73 ± 0.07	0.43 ± 0.04	0.03 ± 0.003	0.21 ± 0.02
CO^{2+}	0.01 ± 0.001	< 0.1	0.02 ± 0.002	< 0.1	0.02 ± 0.002	< 0.1	0.02 ± 0.002	< 0.1	0.02 ± 0.002	< 0.1
MG^{2+}	0.05 ± 0.005	0.21 ± 0.02	0.06 ± 0.006	0.13 ± 0.01	0.07 ± 0.007	0.11 ± 0.01	0.07 ± 0.007	0.11 ± 0.01	0.06 ± 0.006	0.10 ± 0.01
MN^{2+}	0.03 ± 0.003	< 0.1	0.04 ± 0.004	< 0.1	0.03 ± 0.003	< 0.1	0.03 ± 0.003	< 0.1	0.02 ± 0.002	< 0.1
ZN^{2+}	0.13 ± 0.01	0.06 ± 0.006	0.12 ± 0.01	0.03 ± 0.003	0.12 ± 0.01	0.03 ± 0.003	0.15 ± 0.01	0.06 ± 0.006	0.17 ± 0.01	0.03 ± 0.003

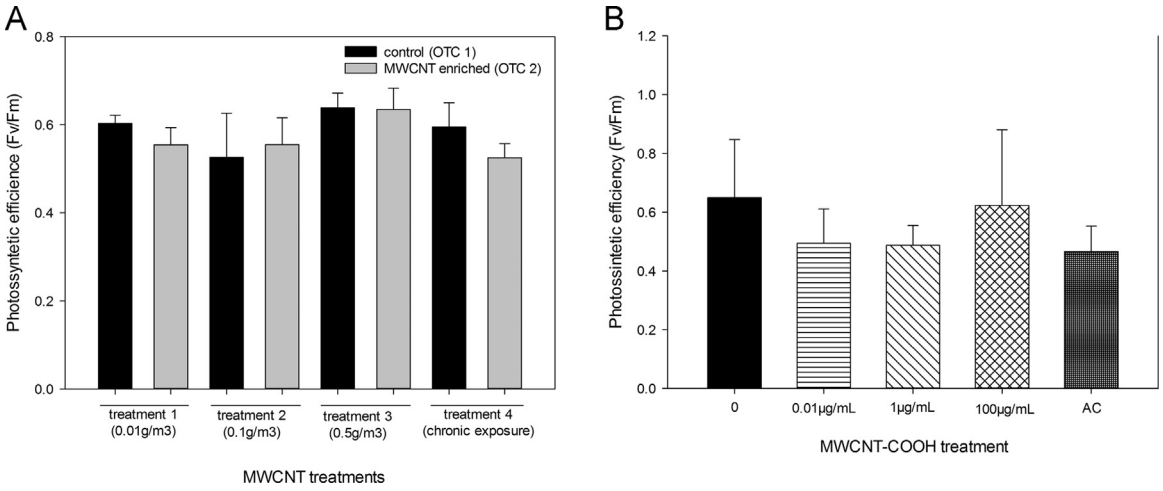

Fig. 5. Effects of carbon nanotubes exposition on photosynthetic efficiency of *P. tinctorum* (mean ± SD, $N=6$). In (A) the results for MWCNT treatment and in (B) for MWCNT-COOH treatment.

distribution pattern, the samples direct watering with a MWCNT dispersion, would eventually allow the MWCNT an easier access to the lichens' intercellular space.

3.5. Cellular location of target elements

The elemental analysis of the different extract fractions showed alterations in the cellular location of some chemical elements after carbon nanotubes exposition, meaning that *P. tinctorum's* mineral cycling dynamic was altered by the treatments. Calcium and magnesium presented a different distribution pattern comparing treated and control samples. As shown in Table 1, their intracellular concentration decreased in the treated samples and increased in the wall-bound fraction.

This corroborate the cell membrane damage results, and permit hypothesizing that the membrane permeability was altered by CNT exposition allowing ionic loss. However, no significative alterations on the location of Co^{+2}, Mn^{+2} e Zn^{+2} were found.

For the intercellular (water) fraction, the elemental concentration was inferior to the method's detection limit, equal to 0.1 mg/L, for all elements. The use of other analytical method for elemental quantification must be considered.

3.6. Photosynthetic efficiency

No differences in photosynthetic efficiency between the treatments and controls were observed. Fv/Fm values varied from 0.525 to 0.638 (Fig. 5).

The chlorophyll “a” fluorescent emission is a common method used in biomonitoring studies (Jensen and Kricker, 2002; Piccotto and Tretiach, 2010; Bertuzzi and Tretiach, 2013) with lichens and plant biology surveys, because of its practicality and accuracy. Through the maximum photochemical quantum yield measurement, expressed by Fv/Fm ratio, the functionality of the photosynthetic process can be assessed. According to several studies with other photosynthetic organisms, carbon nanotubes exposure can influence photosynthetic efficiency, just by its blackening effect caused by the deposition of nanotubes on the surface (Schwab et al., 2011; Pereira et al., 2014). Although the deposition of CNT on *P. tinctorum* surface was observed, in our study this deposition did not alter the photosynthetic efficiency. Probably, as our experimental design aimed to a more dynamic and realistic scenario, the CNT deposition was not sufficient to cause a shadow effect. Also, as observed by Paoli et al. (2015), in our study, the chlorophyll a fluorescence emission remained unchanged even when the membrane permeability was altered. Maybe, the extension of the membrane damage was not sufficient to lead to an impact on the photosynthetic efficiency. In addition, as a first assessment of the carbon nanotubes effects on a lichen physiology, we focused in a short-term exposure, future studies should investigate those parameters in a long-term exposure survey.

4. Conclusions

In this first assessment of CNT effects on a lichen, *P. tinctorum* was exposed to three experimental conditions and commonly used physiological parameters were measured. We could observe that the organism was sensitive to the treatments, but no toxic effect can be alluded. Our preliminary assessment, led to lichen vitality reduction, but no severe damage was reported, and no mortality was observed. Inside the open top chamber, the dry and wet depositions were simulated. In this scenario, only the water treatment with high amounts of MWCNT-COOH altered the lichen membrane permeability. An important parameter to assess the lichen health, the photosynthetic efficiency was not altered by MWCNT exposure, even after 21 days of continuous treatment. The investigations on the CNT–lichen interactions demonstrated evidences on CNT entrapment and internalization on the lichen thallus. Future studies should comprise the carbon nanotube biodistribution evaluation, and a long-term exposure.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2015.05.034.

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