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Short communication

Exploring the response of *Marchantia polymorpha*: Growth, morphology and chlorophyll content in the presence of anthracene

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) were identified as hazardous contaminants that are ubiquitous and persistent in aquatic environments, where bryophytes *sensu lato* (mosses, liverworts and hornworts) are frequently present. *Marchantia polymorpha* (Class *Hepaticae*; thalloid liverwort) is known to respond fast to changes in the environment; it accumulates toxic substances in its tissues due to the lack of vascular and radicular systems and a reduced or absent cuticle. The objective of the present study was to quantify the effects of increasing concentrations of anthracene (0, 50 100, 280 μM) on the germination of propagules, plant morphology and chlorophyll content index (CCI) in *M. polymorpha* under in vitro cultures. The results show that anthracene had no statistical effect on germination or propagula formation. However, plants exposed to anthracene for 30 days showed significantly lowered the content of chlorophyll (measured as CCI), irregular growth patterns and the induction of thalli asexual reproduction as evidenced by the production of multicellular viable propagules in gemmae cups. Results of epifluorescence microscopy also showed concomitant accumulation of anthracene in the cell walls. All of these distinctive morphological and physiological adaptive responses indicators, clearly suggest that *M. polymorpha* are capable of resisting high (coal tar) anthracene concentrations.

1. Introduction

Persistent organic pollutants (POPs) are recalcitrant chemical compounds. They are known to bioaccumulate, they are highly toxic and can be transported far from the contamination source. Some examples of POPs are polychlorinated byphenyls, pesticides and polycyclic aromatic hydrocarbons (PAHs) (Augusto et al., 2013). The latter are ubiquitous by-products that result from the combustion of organic matter from natural and anthropogenic sources, particularly fossil fuels (Spagnuolo et al., 2016). The PAHs can reach the aquatic ecosystems through different pathways, such as the atmosphere (smog), accidental crude oil and refined fuel spills and untreated industrial and domestic effluents (Neff, 1985). PAHs are extremely toxic to mammals, inducing immunosuppression and genotoxicity, among other effects (Haritash and Kaushik, 2009). In the environment, anthracene concentration can range from 0.002 ppm to 0.07 ppm, on unpolluted and aged-PAH polluted soils, respectively, or 100 ppm on coal tar (Wise et al., 1998; Nadal et al., 2004; García-Sánchez et al., 2018). The concentrations compatible with aquatic life are very low, as determined by guidelines from the United States Environmental Protection Agency (USEPA) (8.3 ppm, Maximum Contaminant Level (MCL) – highest level of a contaminant that is allowed in drinking water). In soils, this level is limited to 17 ppm: limit in ppm for individual PAHs established by the Regional Screening Levels (RSL) for Chemical Contaminants at Superfund Sites (USEPA, 2009).

In plants, PAHs are often toxic if the compound is not appropriately immobilised in the cell wall (Harvey et al., 2002). Uptake of non-polar organic contaminants can be due to penetration of the plasmalemma by simple diffusion due to their lipophilic character (Pilon-Smits, 2005) or it can be mediated by an ABC membrane protein (Cobbett and Meagher, 2002). Once inside plant, PAHs can move across the cell tissues via apoplastic and symplastic pathways (Zhan et al., 2018).

The effect of pollutants, such as anthracene, in vascular plants has been widely studied. For example, anthracene can be solubilised in the thylakoid membranes and can cause conformational changes, resulting in alterations to the electron flux, reductions to the pool of co-enzymes,

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and reductions to the biomass. Moreover, much research has been published about aberrant growth patterns or reactive oxygen species (ROS)-induced responses (Aksmann and Tukaj, 2004; Liu et al., 2009; Tomar and Jajoo, 2013).

On the contrary, the effect of anthracene on non-vascular plants is not extensively documented. Studies were found on the ability of *Brachythecium rutabulum* and *Hylocomium splendens* to accumulate phenanthrene and other hydrocarbons in urban areas (Bustamante et al., 2015; Foan et al., 2015) although research on the topic is scarce.

Phylum *Bryophyta* (*sensu lato*) includes three classes—*Anthocerotae, Hepaticae* and *Musci*—with approximately 15,000 species (Chandra et al., 2017). *Marchantia polymorpha* (*Marchantiopsida*) is a species from the second class. It is considered to be tolerant to adverse environmental conditions, and it frequently colonises contaminated soils and wetlands, thus making it a peri-urban species (Shaw and Goffinet, 2000; Alam and Sharma, 2012). Asexual propagule formation during the gametophytic phase is a widely known characteristic of bryophytes; it is displayed by almost 46% of the described liveworth flora (Longton, 1992; Laaka-Lindberg et al., 2000). In addition, *M. polymorpha* undergoes sexual reproduction, which makes niche colonisation easier and may constitute a first and fast response to unfavourable conditions in the environment (Steere, 1970; Ponce de León and Montesano, 2017).

Moreover, bryophytes react faster to changes in the environment as a consequence of the lack of a radicular and vascular system; water, nutrients and pollutants are in direct contact with cells, which means tolerance and plasticity are required characteristics for acclimation to different stressors (Bates, 1992). Bryophytes are known to accumulate pollutants, for example, heavy metals, dioxins, PAHs and emerging contaminants, such as antibiotics or painkillers (Roy and Hänninen, 1995; Carginale et al., 2004; Delépée et al., 2004; Krommer et al., 2007).

The objective of the present study was to investigate the physiological effects of different concentration of anthracene on *M. polymorpha* by measuring plant survival, growth rate and pigment content. In addition, the capability of *M. polymorpha* to accumulate the contaminant was also investigated. To do that, the system was forced using extremely high concentration that is only found in coal tar, in order to predict the possible use of these plants in phytoremediation and/or phytoextraction.

2. Materials and methods

2.1. Sampling

M. polymorpha specimens for the present work were collected from water channels located in the El Mallín area (San Carlos de Bariloche, Patagonia, Argentina; 41° 08′ 32.0” S, 71° 18′ 36.7″W). This site was altered by anthropogenic activities. Specimens established in wet soil under constant shade were selected, collected and deposited in polyethylene bags; transported at ambient temperature to the laboratory and stored at 4 °C. These samples without any previous treatment were used in the following tests. Morphological characteristics were used for the identification of the collected specimens (Shaw and Goffinet, 2000).

2.2. Sterilisation and planting of propagules

The propagules from gemmae cups of *M. polymorpha* without any previous treatment were collected under a binocular stereo dissecting microscope and placed on a piece of filter paper moistened with water. The soil particles that were adhered to the propagules were removed by moving them onto the filter paper. The method described by Silvani et al. (2012) for sterilisation of hepatic thallus was used. They were placed into glass Petri plates containing minimum medium (Becard and Fortin, 1988) that either was or was not supplemented with the contaminant to reach 100 propagules per treatment. Anthracene was added according to the methodology described by Aranda et al. (2013), preparing a stock solution diluted in acetone (5 mM). PAH-enriched culture medium was prepared adding the stock solution to the culture medium immediately after autoclaving, to reach the final concentrations of 0, 50, 100, 280 and 560 μM. The medium was then stirred for 15 min under sterile conditions in order to evaporate the acetone and avoid the toxic effects of the solvent. The used experimental protocol is well established (Alves et al., 2017; Aranda et al., 2013), although formation of crystals cannot be fully excluded. M medium without PAHs as well as M medium containing only acetone (PAH-free) were used as controls. All the samples were washed before each measurement. Four replicates were used for each treatment concentration. The propagules that presented rhizoids were considered to be germinated. After three days, the percentage of total germinations was quantified.

2.3. Plant growth

Four of the germinated propagules were transferred individually into flask-shaped glass pots (50 mL) with minimal medium (control) and with the different concentrations of anthracene, which were previously described, and the development of the plants was monitored. The bases of the flask-shaped glass pots were covered with aluminum foil to avoid the direct incidence of light and prevent the light UV-oxidation of anthracene. The four replicates per treatment were placed in a chamber under controlled light (16/8 h light/dark) and temperature (25 °C) conditions. Light at 45–60 μM photons m^{-2} s^{-2} was supplied by cool white fluorescent tubes. After 30 days of cultivation, plants were harvested, and quantitative and qualitative measurements (thalo morphology) were made. Plant morphology was analysed under binocular microscope (Leica wild M3Z). Plant biomass was determined after oven-drying the samples at 80 °C until a constant weight was reached.

2.4. Fluorescence microscopy and image analysis

Plants material (0.5 cm^2 in size of actively growing leaf) for visualization was carefully rinsed to remove the remainder growing media and any crystal of the contaminant that could has remained on the surface of the sample. The presence of anthracene was examined by fluorescence microscopy as describe Aranda et al. (2013), with an Olympus BX51 epifluorescence microscope (Olympus Optical Co., Tokyo, Japan). Wavelengths were chosen based on the excitation and emission spectra of anthracene (Byron and Werner, 1991). Upon excitation at 365/10 nm, the presence of anthracene was measured at an emission of 420 nm by using a DAPI based cube filter U-MNU2 from Olympus. The TIFF 12-bit images were captured using a LEICA DFC-425 C CCD camera and were analysed using FIJI software (National Institutes of Health ImageJ platform). RAW images were decomposed in three channels (blue, Green, red) and we use the intensity in the blue channel to compare it with the control and other treatments. We directly compare the average measures of each treatment against the control and against different anthracene concentration scenarios in the blue channel of the TIFF format. We took 20 random circular areas of 100 pixels diameter in which we allocate the cellular wall at the centre of the spot the resulting average histogram in the blue range was compared between conditions. Data were expressed as relative fluorescent intensity.

2.5. Chlorophyll content index (CCI)

Clorofilio device (Cavadevices®, Buenos Aires, Argentina) was used to calculate the relative chlorophyll content. It has a measurement area of 0.6 cm^2, and it is calculated using an index value of the chlorophyll content (CCI), giving a ratio of optical absorbance at 655 nm and 940 nm. One sample consisted of a leaf apex of 0.6 cm^2. Measurements were taken for each replicate of each treatment following the methodology proposed by Ling et al. (2011).

2.6. Statistical analysis

Data were analysed by one-way analysis of variance (ANOVA) using Statistica 7.0 software. Normality and homoscedasticity assumptions were tested, and Tukey's honestly significant difference test (HSD) was used to validate the differences found using ANOVA.

3. Results and discussion

3.1. Propagula germination

The rate of germination of asexual propagules did not depend on the concentration of anthracene, with similar rates at all of the tested concentrations, including the highest concentration (560 μM; Data not shown). In contrast, the increase in the concentration of heavy metals decreases the germination of propagules of *M polymorpha* and turns out to be a good indicator of toxicity (Coombes and Lepp, 1974) although it did not happen with anthracene and *M. polymorpha*. On the other hand, the experiments coincide with those of Carginale et al. (2004) who observed that the germination of propagules of *M. polymorpha* is has a particular resistance towards contaminants, which indicates low resolution of this parameter as a general bioindicator of toxicity.

3.2. M. polymorpha growth in the presence of anthracene

After each propagule was germinated, plant growth was followed until day 30. The contaminant altered the development of the plants, which showed irregular growth patterns. Growth was inhibited for plants exposed to anthracene at 560 μM (data not shown). Anthracene induced "rosette" growth morphology and a decrease in biomass (as dry weight) with respect to the controls (Fig. 1.2). The differences were significant ($p = 0.000062$; $F = 18.02$) for the plants grown at 100 μM and 280 μM (Fig. 1). Although the production of propagula was not observed in 50 and 100 μM treatments, plants exposed to 280 μM produced viable propagules in gemmae cups, which were evident after 30 days.

Although published research about the effects of anthracene in bryophytes is scarce, which made difficult the discussion of some of the results, the toxicity effects of PAHs in vascular plants span a wide range of research. These compounds induce stress responses such as growth reduction of the root and shot via inhibition of cell division and

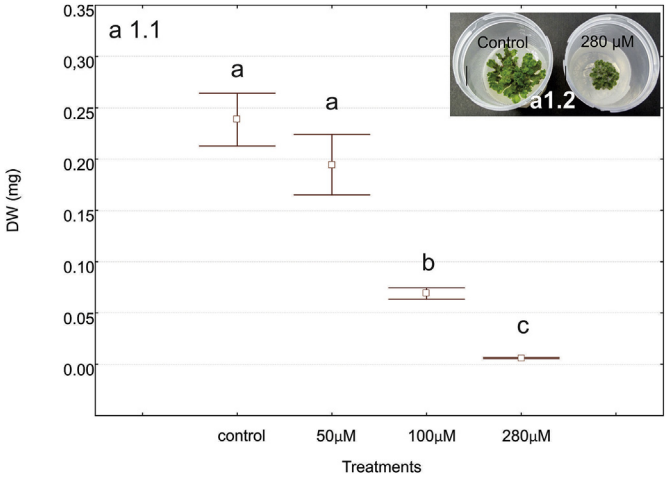

Fig. 1. a) 1.1 Dry weight of *M. polymorpha* (mg) grown during 30 days in the presence of anthracene (0, 50, 100 and 280 μM). Data represent the mean of four replicates per treatment. Error bars denote standard deviation. Values with the same letter are not significantly different between treatments ($p \leq 0.05$), as determined by Tukey's test. b) 1.2 Image of *M. polymorpha* grown in M medium with and without 280 μM anthracene.

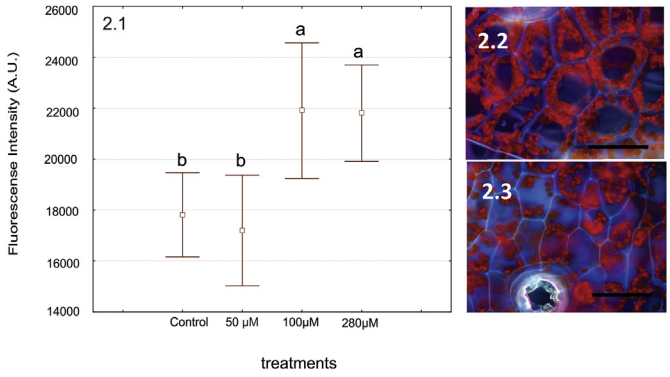

Fig. 2. 2.1 Fluorescence intensity (Absorbance Units A.U.) of *M. polymorpha* grown during 30 days in the presence of anthracene (0, 50, 100 and 280 μM). Data represent the mean of four replicates per treatment. Values with the same letter are not significantly different between treatments ($p \leq 0.05$), as determined by Tukey's HSD test. Vertical bars denote standard deviation. Fluorescence micrograph of a control plant (2.2) and a plant exposed to 280 μM of anthracene (2.3). Scale bars = 10 μm. Differences in the blue light intensity indicate the presence of anthracene. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

elongation; chlorosis; necrosis; and oxidative stress indicators, like production of H_2O_2, programmed cell death and hypersensitive response-like symptoms (Maliszewska-Kordybach and Smreczak, 2000; Alkio et al., 2005). In the present study, plants exposed to anthracene were shown to be less developed than controls and with a "rosette" appearance which indicates that the contaminant could be inducing alterations to the meristem at the base of the heart-shaped slit (apical notch) at the apex of the thallus. In that regard, *M. polymorpha* exhibit altered growth and morphology in the presence of PAH, as a regular stress response-like symptom observed in vascular plant, *Arabidopsis thaliana* (Akio et al., 2005).

3.3. Anthracene uptake by M. polymorpha

The measurements obtained by fluorescence microscopy after 30 days of cultivation showed an increase of fluorescent emission at ~420 μm under UV excitation on the cell walls of plants grown in the presence of 100 μM and 280 μM of anthracene, suggesting the incorporation of the contaminant (Fig. 2.1) ($p = 0$; $F = 51.141$). Although lignin is also fluorescent, as many other molecules with aromatic components under UV irradiation, we considered the increase in the emission as a consequence of the anthracene incorporation, as it has also been described in other studies in vascular plants (Alves et al., 2017; Aranda et al., 2013). Although we cannot confirm that anthracene was transport to plant tissue along apoplastic and symplastic pathways, there is evidence that in bryophytes, the transport route of nutrients and contaminants is apoplastic, following the same route as the circulation of water (Giordano et al., 1989; Carginale et al., 2004). Therefore, nutrients and pollutants are in direct contact with cells and its absorption could occur directly as observed by Harms (1992), who exposed intact cells of plants and cell cultures to another type of PAH (dibenz[a,h]anthracene). It is then possible that at least part of the anthracene is accumulating in the cell walls, as suggested by epifluorescence images of plants exposed to 100 μM and 280 μM of anthracene (2.2 and 2.3). In *M. polymorpha*, the anthracene transported from the substrate to the cell walls could be available for passive and active internalisation. The mechanisms are still unknown, although they could be similar to those described for vascular plants (Zhan et al., 2012).

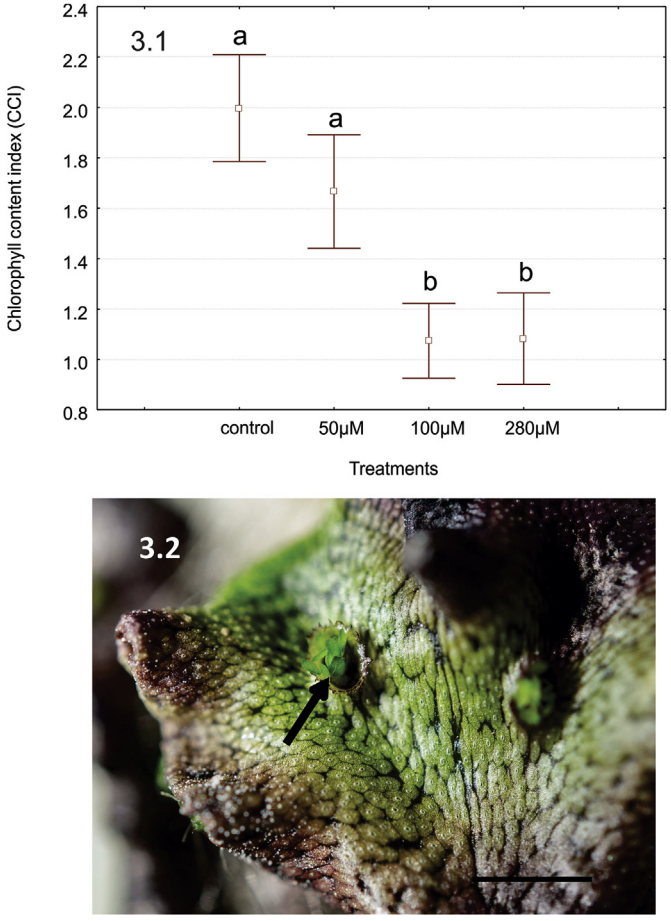

Fig. 3. 1 The chorophyll content index (CCI) of *M. polymorpha* plants treated with 0, 50, 100 and 280 μM of anthracene. Data represent the mean of four replicates per treatment. Values with the same letter are not significantly different between treatments ($p \leq 0.05$), as determined by Tukey's HSD test. Vertical bars denote standard deviation. 3.2 The arrows in the figure indicate the Gemma cup containing viable propagules following exposure to 280 μM of anthracene. Scale bars = 5 mm.

3.4. Effect of anthracene on the chlorophyll content index (CCI)

In order to establish whether the decrease in biomass was caused by an alteration in photosynthetic processes, CCI was measured. The index decreased ($p = 0.000007$; $F = 51$) when the plants were exposed to concentrations higher than 100 μM with respect to the controls (Fig. 3.1). The chlorophyll content is a known and accepted bioindicator of the presence of stressors or contaminants, and it is predictive, to some extent, of plant toxicity (Marwood et al., 2001). The CCI decreased with increasing anthracene concentrations. This experimental result allowed explaining the observed change in the development and growth of the plants. This supports one mechanism of PAH toxicity in which damage to essential cellular components leads to photosynthetic pigments catalysis (Marwood et al., 2001), so energy would be deviated to the maintenance of the photosynthetic apparatus, which would therefore affect biomass production. The difference in growth and photosynthetic pigment content could be a consequence of the energy consumption by the thalli asexual reproduction (Glime, 2017).

The unusual morphology patterns, the differences in biomass production and the decrease in CCI, at least in our experimental conditions, could be related to the ability of *M. polymorpha* to incorporate and deposit appreciable amounts of anthracene into the cell walls. Harvey et al. (2002) proposed that the stabilisation of the contaminant in these structures confers tolerance to the plant, although symptoms of stress were registered. Moreover, in addition to stress, the above mentioned symptoms could be a consequence of the deviation of energy to the production of gemmae cups with propagules, which was observed solely in the specimens exposed to PAH (Fig. 3.2). This is a unique characteristic amongst terrestrial plants (Wyatt, 1994). In this sense, some liverworts are known to reproduce asexually as an adaptive advantage to environmental stress. In many cases it is the environment that determines the reproductive strategy of some species. In this case, the production of fertile gemmae cups of *M. polymorpha* under PAH stress could be interpreted as an effective means for population growth and maintenance. Indeed, the present study supports the hypothesis that asexual reproduction is vital for population survival under stressful environmental conditions (Longton and Schuster, 1983; Kimmerer, 1991; Crow, 1994).

4. Conclusion

Marchantia polymorpha is tolerant to concentrations of anthracene up to 280 μM. The contaminant produces alterations of the chlorophyll content that could be related to the decrease of the growth and the alterations in the growth. However the plant is also able to transport the contaminant from the substrate through the apoplast, to incorporate it into the cell walls. The differences in the monitored morphological and physiological parameters and the production of asexual structures are signals of abiotic stress induced by anthracene; on the other hand, they are also signals of the adaptive advantages of the organism to this type of condition. Further research is needed to understand the mechanism of anthracene toxicity, the tolerance of this species and its potential role in phytorremediation processes.

Author contributions

SN and RN designed and executed the in vitro experiments and identified the plan material. CJ and ST performed the statistical analysis and collaborated in the improvement of the manuscript. AE and SM realized the microscopy comparison, and provided the plant material, critically review the manuscript and improve the text. SM measured the CCI analyzing the statistic differences among concentration and collaborates in the manuscript improvement. SJM wrote the manuscript and critically review the manuscript and designed and executed the in vitro experiments.

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