Original Research

Journal website : www.jeb.co.in ★ E-mail : editor@jeb.co.in

Journal of Environmental Biology

JEB™
p-ISSN: 0254-8704
e-ISSN: 2394-0379
CODEN: JEBIDP

DOI : http://doi.org/10.22438/jeb/42/3/MRN-1524

Evaluation of *Atrichum undulatum* as an effective indicator of lead pollution in soil

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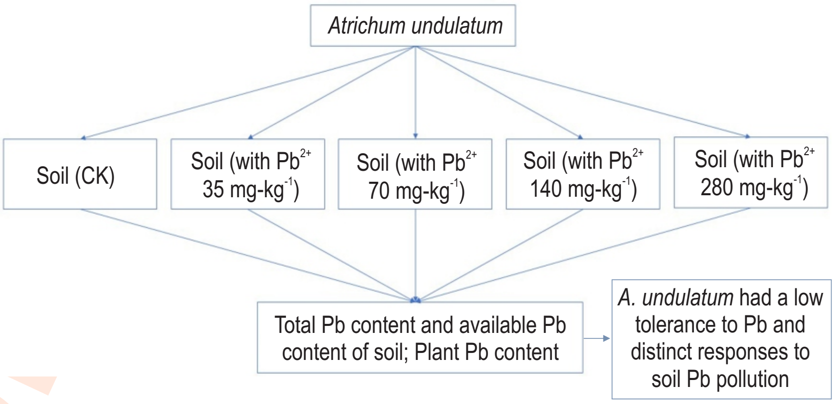
Received: 18.05.2020 *Revised*: 08.10.2020 *Accepted*: 15.12.2020

Abstract

Aim: The aim of this study was to evaluate the sensitivity of *Atrichum undulatum* to soil Pb contamination and check its affectivity as an indicator of Pb pollution indicator.

Methodology: Soil and plant samples were collected and characterized. Plants were grown in different levels of Pb contaminated soil (35, 70,140 and 280 mg kg^{-1}, respectively). After 35 days, the plant samples were cut and ground to determine physiological and biochemical parameters.

Results: Soil available Pb accounted for approximately 15–24% of total Pb, and the ratio of available Pb decreased with increase in treatment concentration. All the above mentioned parameters were closely related to soil Pb stress, especially when soil Pb concentration was higher than 140 mg kg^{-1}. *A. undulatum* had a low tolerance to Pb and distinct responses to soil Pb pollution, with visible symptoms such as damaged sporophytes and gametophytes. When soil Pb concentration was in low concentration (70 and 140 mg kg^{-1}), the leaves turned yellow and brown. When soil Pb concentration increased to 280 mg kg^{-1}, the seta softened and kinked and eventually, the plants withered and died.



Interpretation: Changes in physiological and biochemical parameters of tested plants such as chlorophyll, soluble protein and MDA content were related to soil Pb stress, especially to soil bioavailable Pb content, and corresponded well to changes in soil Pb pollution level. The results suggest that *A. undulatum* can be used as indicators to monitor and evaluate Pb pollution in soil.

Key words: *Atrichum undulatum*, Biomonitoring, Pb-contaminated soil, Stress response

How to cite : Yang, N., M. Dong, Z. Xu, X. Zhou, Z. Xu and W. Ku: Evaluation of *Atrichum undulatum* as an effective indicator of lead pollution in soil. *J. Environ. Biol.*, **42**, 582-588 (2021).

Introduction

Lead is a heavy metal pollutant, considered potentially hazardous, owing to its stabilization in soil; difficult to metabolize and degrade; carcinogenic, teratogenic, and mutagenic to human, livestock and crops; and bioaccumulate in humans and animals via food chain (Shibata *et al.,* 2007; Liu *et al.,* 2008). Currently, as a result of increasing production and use of Pb-containing products, it has been estimated that 4–5 million ha of cultivated land is contaminated by Pb in China, and annually 70–80% of Pb in industrial production waste is discharged into the soil. The increasing use of petroleum fuels with relatively high Pb content has increased Pb pollution in the soil, after atmospheric deposition. In addition, Pb pollution is mostly irreversible once the soil is contaminated, and the effects on crops are long lasting (Liu *et al.,* 2008). Industries mostly use chemical detection methods to evaluate soil Pb pollution; the Pb content of soil is used as a basis to determine the ecological safety of soil Pb. Recently, several researchers (Wang *et al.,* 2014; Zhou *et al.,* 2014) have pointed out that the actual toxicity of heavy metal elements in soil is closely related to soil's biological effectiveness.

Therefore, to test whether a soil has reached safety standards, one can consider the degree of damage caused by heavy metals in the soil to a biological body as a basis for determining toxicity. Thus, if a lower plant with high sensitivity to Pb is used to indicate soil Pb pollution through its growth condition and physiological reaction, it can provide a strong observational basis for determining toxicity levels. If Pb-contaminated soil does not affect plant growth, then soil is considered safe and suitable for planting other crops otherwise, it indicates that the soil has exceeded the safety limit of Pb pollution and there is a risk of Pb contamination, and the soil is not suitable for planting crops. Some plants such as *Juncus effuses* (Sun *et al.,* 2007), *Arabis paniculata* (Tang *et al.,* 2009) can be used as Pb indicators. In contrast to terrestrial vascular plants, the bryophytes have a simple structure and low degree of tissue differentiation. Their surface is not covered with a stratum corneum or waxy layer and therefore, it is sensitive to pollutants in the environment. Heavy metal ions or dust particles, such as Pb, can be directly absorbed by the epidermal parenchyma cells into the plant body, which causes the plant to rapidly display symptoms.

Therefore, it is suitable for real-time dynamic monitoring of environmental pollution. In addition, *in-situ* monitoring of bryophytes causes little disturbance to the environment and is a simple operation with low costs and a short monitoring period, making this method particularly valuable (Zhou *et al.,* 2008; Lodenius, 2013; Abril *et al.,* 2014; Chen *et al.,* 2015; Esposito *et al.,* 2018; Debén *et al.,* 2018) and widely used in air, water and soil pollution monitoring all over the world (Conti and Cecchetti, 2001; Harmens and Norris, 2008; Zhou *et al.,* 2008; Basile *et al.,* 2015; Harguinteguy *et al.,* 2015; Aboal *et al.,* 2017;). Based on the above characteristics, the bryophyte monitoring method has been widely used in monitoring atmospheric and water metal pollution. However, research to determine whether this method can be used to monitor soil heavy metal contamination is limited. Structurally, the roots (pseudo-roots) of bryophytes also exhibit a strong response to the soil environment. Heavy metal ions in the soil can poison plants through pseudo-thin parenchyma cells. The principle of monitoring the atmosphere by aboveground parts of plants can be used theoretically to monitor heavy metal pollution in soil, which is worthy of further exploration. In this study, the bryophyte *Atrichum undulatum*, which is highly sensitive to Pb was used to preliminarily explore its stress response to soil Pb ions. The growth performance was observed and physiological by analyzed after implanted into soils with different concentrations of Pb. The results provide a theoretical basis and technical reference for further development of bio-monitoring technology for soil Pb pollution.

Materials and Methods

Tested plant material: *A. undulatum* plants were collected from farmland soil in Wanzihu, Dongting Lake, Hunan Province (28°51'23.28"N, 112°26'42.71" E). As the base of the plant is generally clustered, the plants, roots, and 2–3 cm of soil were collected in small pieces, using a shovel. The collected source materials were placed in a water tank grid, ensuring that the roots were completely immersed and they were then gently agitated in water for 1–2 hr. During the rinsing process, the roots were protected from physical damage using a special plant root cleaning device developed in-house (CN PAT: CN208146522U). After washing the roots, they were transplanted into plastic pans containing Pb-treated farm soil matrix.

Soil substrate treatment: After air drying and pulverizing, 2 kg of farmland soil was weighed and laid in a flat circular polyethylene shallow pan with a short disc height of 6 cm and diameter of 35 cm. Based on the environmental reference value for Pb pollution in domestic field soil, the corresponding mass of Pb $(No_3)_2$ was calculated; $Pb(NO_3)_2$ was dissolved and fully mixed to ensure that the applied concentrations of Pb^{2+} in the soil matrix were 35.0, 70.0, 140.0, and 280.0 mg kg^{-1}, and soil samples without Pb $(NO_3)_2$ were used as a control. Each treatment was repeated five times. The soil samples were static cultured for 35 days in natural indoor condition to make sure the soil properties were stable. Then, *A. undulatum* plants were transplanted in the shallow pans with treated soils in a net room, under conditions close to their natural environment, *i.e.,* a temperate of 32–35°C and humidity of 86% and were sprayed with 300–500 mL tap water. The morphological characteristics and physiological parameters of the plants were measured once every two days since the second day after transplantation, and repeated five times, and the average test data was taken as the final test result.

Sample collection and analyses: The stems and leaves of plants were cut to determine the physiological and biochemical parameters. The cut samples were ground in a mixture of ethanol and acetone (1:1), extracted, and filtered, and the chlorophyll content was estimated using a 722 spectrophotometer (Shanghai Sunny Hengping Scientific Instrument Co., Ltd, Shanghai, China) (Yang, 2002). The samples were then ground with phosphate buffer (0.1 mmol L^{-1}, pH 7.8, containing 1% PVPK30) and centrifuged for 8 min at 8 000 r min^{-1}. The soluble protein content in the crude extract was estimated by Coomassie Brilliant Blue G-250 colorimetric method, estimated by Malondialdehyde (MDA) content in each sample was estimated by thiobarbituric acid colorimetric method.

Pb content was determined using a Shimadzu AA-6300 graphite furnace atomic absorption spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, MA, USA). For each treatment, 0.3 g of dry soil sample was added to 15 ml of HCl-HNO_3-HF strong acid mixture (3:1:1) to preform microwave-close digestion. The total Pb content in the sample was measured using graphite furnace atomic absorption spectrophotometry (Zhu *et al.,* 1989; Chen, 2008), the soil sample was added to 25 ml of acetic acid at a concentration of 0.1 mol L^{-1}, thoroughly mixed, and shaken for 3 h, and then centrifuged at 10,000 r min^{-1} for 1.5 h, and the process was repeated three times. Finally, the supernatant was collected for evaporation and digestion, and Pb content in the clear liquid sample was determined by graphite furnace atomic absorption spectrophotometry.

Statistical analysis: The data obtained were statistically analyzed using Excel 2013 and SPSS 13.0 software, and presented as mean ± standard deviation.

Results and Discussion

Table 1 shows the total Pb content and available Pb content in the soil culture substrate. As the farmland soil samples used in this study contained a certain amount of Pb, the actual total Pb content measured at each corresponding treatment level was slightly higher than the artificially added level. The data in the table indicates that the available Pb content accounted for approximately 15% of the total Pb content in the blank control sample; the available Pb content accounted for 17–24% of the total Pb content at 35–140 mg kg^{-1} treatment level. In general, the ratio of available Pb decreased with the increase in Pb treatment level. However, when the treatment concentration was 280 mg kg^{-1}, the ratio increased to 28%, which was inconsistent with the above mentioned trend.

This may be due to the addition of excess Pb, short soil aging time, and limited ability of soil colloidal particles to adsorb and hold Pb ions. The concentration of Pb increased with the increase in Pb treatment levels. The enrichment concentration ranged from 2.54 to 89.57 mg kg^{-1}; however, it was still lower than in other plants (María and María, 2014) and had a low enrichment coefficient which was between 31.16% and 45.85% and decreased with increase in Pb concentration. Correlation analysis of soil total Pb content, available Pb content, and plant Pb content (Fig. 1) showed that the plant Pb content was positively correlated with the total Pb content and available Pb content, and its correlation with the available Pb content was higher than that with the soil total Pb content. *A. undulatum* showed an obvious growth response to different levels of Pb stress. In the control plants (raw soil culture without Pb) or those treated with 35 mg kg^{-1} Pb, the growth was normal with fresh green appearance and no obvious symptoms of toxicity.

In plants treated with 70.0–140.0 mg kg^{-1} Pb, on third day, the broad-lanced leaves began to yellow and showed shrinkage and curling. Plants of family Polytrichaceae, such as *A. undulatum*, are sensitive to environmental humidity and the leaves shrink or curl when dry and stretch when wet. However, in this case, the freshness of leaves was not restored even after watering and the yellowing of leaves close to ground became more obvious. With increase in Pb concentration and duration, the degree of damage to leaves gradually increased. Under Pb stress of 280.0 mg kg^{-1}, the degree of damage to leaves of the plant increased further, the sporophyte drooped, and the whole plant appeared dead. Furthermore, the originally sturdy stalk became soft and curved; the sputum gradually browned, wilted, and drooped with time; and the plants began to die. Thus, it can be concluded that *A. undulatum* is sensitive to low levels of Pb contaminated soil. because the

Table 1: Concentrations of total Pb and available Pb in soil and Pb concentration in plants at different treatment

Pb processed concentration (mg kg^{-1})	Total Pb (mg kg^{-1})	Available Pb (mg kg^{-1})	Plant Pb enrichment (mg kg^{-1})
CK	6.24 ± 1.82	0.94 ± 0.18	2.54 ± 1.16
35.0	40.68 ± 3.87	9.76 ± 2.15	18.65 ± 3.67
70.0	74.83 ± 5.43	15.71 ± 3.34	31.89 ± 7.76
140.0	148.49 ± 11.65	25.24 ± 4.86	46.27 ± 9.48
280.0	286.35 ± 22.76	80.18 ± 12.73	89.57 ± 9.89

Table 2: Physiological parameters of *A. undulatum* under different soil Pb stress treatments

Pb concentration (mg kg^{-1})	Chlorophyll content (µg g^{-1} FW)	Soluble protein content (µg g^{-1} FW)	MDA content (nmol $mgprot^{-1}$)
CK	22.45 ± 3.36	351.13 ± 24.77	3.17 ± 0.51
35.0	21.17 ± 3.17	345.76 ± 24.64	3.32 ± 0.68
70.0	18.12 ± 2.95	306.54 ± 22.58	4.79 ± 0.82
140.0	13.76 ± 2.13	271.77 ± 21.65	6.84 ± 1.34
280.0	9.27 ± 1.87	231.68 ± 19.16	9.87 ± 1.85

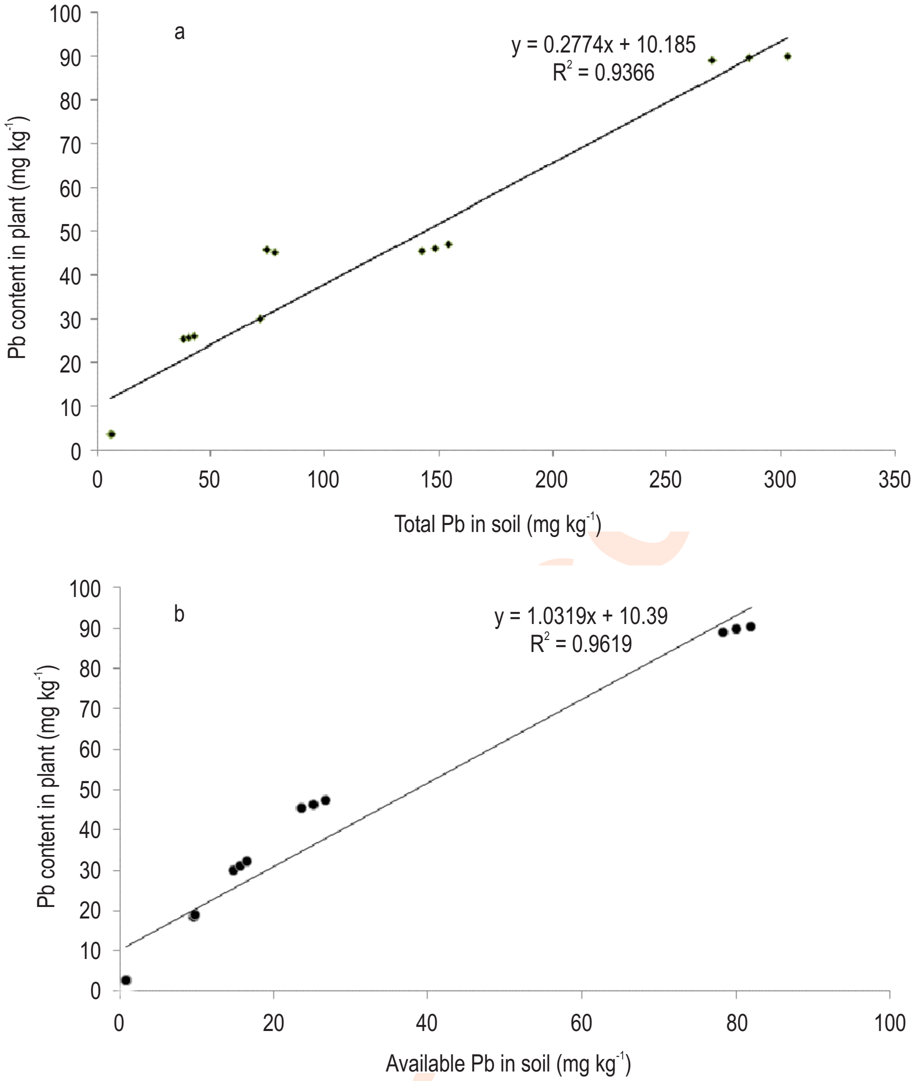


Fig. 1: Correlation between plant Pb concentrations and soil total (a) Pb available and (b) Pb concentrations.

minimum Pb concentration in this experiment was only the Pb background value for primary soil. Therefore, *A. undulatum* is an ideal plant to monitor Pb contamination in soil. Recently, many bryophytes such as peat moss, entodon moss, hypnum moss, and mat moss have been used to monitor atmospheric heavy metal pollution in different regions. Cypress-leaved plait-moss and square pleurochaete moss, widely distributed in the Mediterranean region, were not only effective in monitoring the pollution levels of Pb, Cd, and Hg in the local atmosphere, but also in identifying and locating the source of nitrogen emissions based on isotope labeling of nitrogen in plants (Izquieta-Rojano *et al.,* 2016). Mossy bags containing peat moss have become a standardized atmosphere-monitoring method in countries like Finland (Lodenius, 2013; Boquete *et al.,* 2014). Li *et al.* (2014) conducted heavy metal content analyses using several bryophytes in the Laoshan Area of Qingdao for two consecutive years and obtained satisfactory air pollution monitoring results. However, different kinds of moss are needed to identify different pollutants. In practical application, the morphology of bryophytes is often used to visually reflect environmental pollution.

Therefore, selecting plants of large size and high sensitivity to a specific pollutant can be useful for on-site sampling and pollution indicator. The bryophyte used in this study was sensitive to Pb contamination in soil, and it was also of a suitable size as mature plant height of sporophyte can reach 6–8 cm with a simple body structure, sporophyte parasitic on gametophytes, and uniform structure of monolayers of stems, leaves, and other organs, all of which are suitable for observation of growth indicators and microstructure. The physiological parameters of plants were significantly affected by Pb contamination in soil (Table 2). When the concentration of Pb reached 70 mg kg^{-1}, the chlorophyll and soluble protein content of plants reduced by 19.28% and 12.7%, respectively, as compared to control plants, with a 51.1% increase in the MDA content.

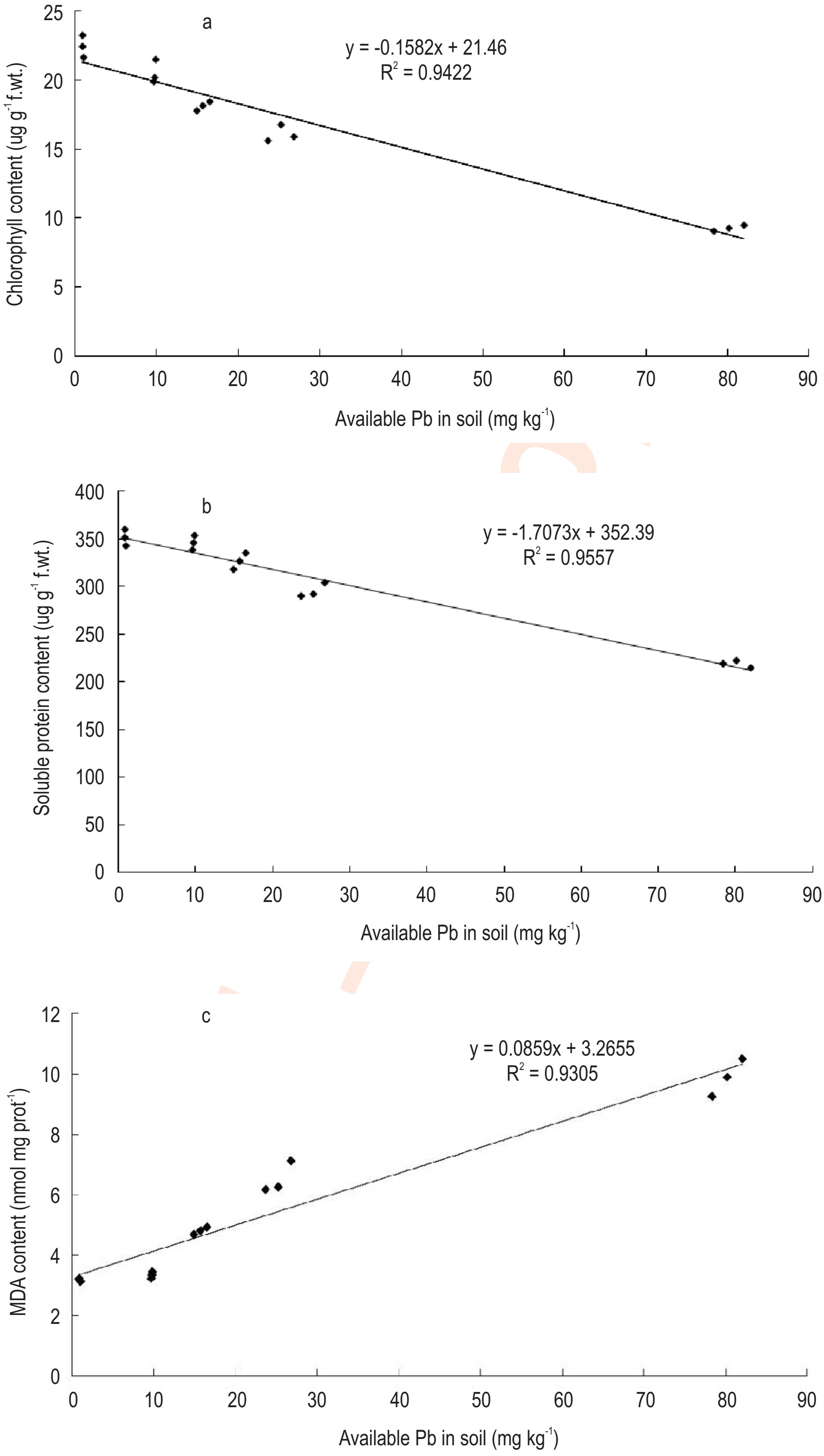


Fig. 2: Correlation between the physiological parameters (a: Chlorophyll; b: Solube protein and c: MDA content) of *A. undulatum* and soil available Pb concentrations.

The physiological parameters of *A. undulatum* changed even more significantly at 140.0 mg kg^{-1} soil treatment, with chlorophyll and soluble protein content decreasing from 38.8% to 58.8% and 22.6% to 34.1%, respectively, as compared to control plants. The increase in MDA content, was 1.16–2.11 times higher than control. As chlorophyll and soluble protein are closely associated with plant growth, stresses that reduce their content in *A. undulatum* could affect plant growth. Malondialdehyde is a substance produced due to peroxidation of membrane lipids when plant cell membrane is attacked by heavy metal ions. An increase in MDA content usually indicates enhanced membrane lipid peroxidation, *i.e.,* the plant exhibits a stress response to adverse conditions (Wang *et al.,* 2009; Li *et al.,* 2012; Li *et al.,* 2013). These three physiological parameters are used as indicator of heavy metal pollution in bryophytes. In *Taxiphyllum taxirameum* and *Eurhynchium eustegium*, soluble protein content, membrane lipid peroxidation, and enzyme activity were affected due to Cd and Pb contamination (Chen *et al.,* 2015). The anthocyanin content and chlorophyll fluorescence values also showed significant changes under stress, which could be used to distinguish the pollution level and toxic effects of heavy metals such as Pb and Cd. In some plants like *Tillandsia usneoides*, chlorophyll content, MDA concentration, and antioxidant enzyme activity can be used to monitor formaldehyde pollution in air through physiological and biochemical parameters (Li *et al.,* 2013).

The growth characteristics of *A. undulatum* under Pb pollution indicated that the bryophyte show strong sensitivity to Pb pollution in soil, and its tolerance to Pb was significantly lower than other plant species (Gjengedal and Steinnes, 1994; Liu *et al.,* 2008; Geffard *et al.,* 2010; Udovic *et al.,* 2013; Salazar and Pignata, 2014). This suggest that bryophyte are suitable for monitoring Pb contamination soil. It should also be noted that this experiment was carried out under a small gradient of Pb stress levels, *i.e.,* four treatment concentration levels were established and the difference between the concentrations were too large to reflect the actual Pb pollution response curve. Therefore, specific "dose-effect relationship" between the two needs to be studied further. To identify the relationship between plant growth status and soil bioavailability, correlation between the changes in physiological parameters of *A. undulatum* and soil available Pb content was analyzed (Fig. 2a-c). The results showed a significant negative correlation between the available Pb content in soil and chlorophyll content and soluble protein content in *A. undulatum* (R^2 = 0.9422 and 0.9557, respectively). A significant positive correlation was observed between available Pb content and MDA content (R^2 = 0.9305). The results demonstrate that the available Pb content in soil was closely related to growth of *A. undulatum* and affected its physiological state. Heavy metals such as Pb persist in soil for a longer time. Due to the influence of internal and external environmental factors, such as oil temperature and humidity, soil acidification process caused by rainfall, soil colloidal particle weathering, mutual antagonism of various metal ions in soil, complexation between chelating agents and lead ions that enter the outside world, causes mutual transformation between different chemical forms (Bolan *et al.*, 2014).

Various dynamic physical and chemical processes such as adsorption-desorption, dissolution-precipitation, and oxidation-reduction; the bioavailability and toxicity also change constantly (Bolan *et al.,* 2014). Previous studies (Remon *et al.,* 2013; Tariq & Ashraf, 2013; Wang *et al.,* 2014) indicate that the absolute value of heavy metals in soil can be used as a basis for measuring the degree of pollution, but its bioavailability is a more scientific parameter for evaluation because heavy metals in soil affect the growth of plants depend only on the proportion in the available state that can be absorbed and utilized by the plants. To a certain extent, the plant monitoring method can evaluate bioavailability of heavy metals. In a study, *Pinus sylvestris* was used to alleviate soil heavy metal pollution, the heavy metal content in pine needles indicated the bioavailability of heavy metals in soil, and also reflected the physico-chemical properties of soil indirectly (Pietrzykowski *et al.,* 2014). After treatment of heavy metal waste liquid with corn seedlings, the biotoxicity and repairing effect of heavy metals in wastewater were evaluated according to the change in the chlorophyll fluorescence value in leaves and the inhibition degree of photosynthetic effect in cyanobacteria (Lucas *et al.,* 2013). Owing to these advantages and application value of bryophytes, *A. undulatum* was selected as a test plants to indicate and evaluate the degree of Pb pollution in soil and its bioavailability.

Therefore, compared with total Pb in soil, available Pb is more suitable for reflecting Pb contamination in soil and ecological safety. *A. undulatum* was susceptible to Pb toxicity in soil, and showed a noticeable growth and physiological response, which revealed its high sensitivity and low tolerance to Pb pollution. Physiological parameters such as chlorophyll, soluble protein, and MDA can be used as indicators to evaluate soil Pb pollution levels and bioavailability. Therefore, the study confirmed that *A. undulatum* has a high application value in monitoring soil Pb pollution.

Acknowledgments

This study was funded by the Chinese National Natural Science Foundation (31700332), Hunan Natural Science Foundation (2016JJ4015; 2019JJ40013), Hunan Science & Technology Innovation Platform and Talent Plan (2017TP2006, 2018TP1042) and Yiyang Science and Technology Innovation Project (2018YR01).

Add-on Information

Author' contribution: N. Yang: Participate the whole process and write the manuscript, **M. Dong:** Guide the experiment and manuscript, **Z. Xu:** Guide the manuscript, **X. Zhou:** Field work and experiment, **Z. Xu:** Field work **and W. Ku:** Experiment.

Research content: The research contents is original and has not been published elsewhere

Ethical approval: Not Applicable

Conflict of interest: The author declares that there is no conflict of interest.

Data from other sources: Not Applicable

Consent to publish: Author agree to publish the paper in *Journal of Environmental Biology*.

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