Chemosphere 368 (2024) 143718

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Impact of PET micro/nanoplastics on the symbiotic system *Azolla filiculoides-Trichormus azollae*

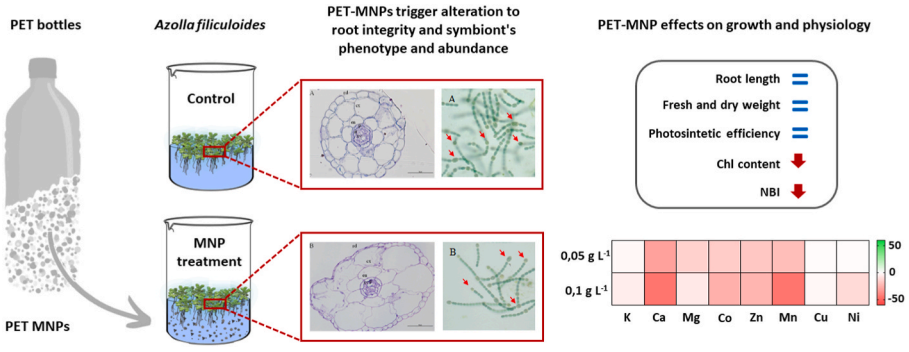
Marco Dainelli [a], Beatrice Chiavacci [a], Ilaria Colzi [a], Andrea Coppi [a], Emilio Corti [a], Matteo Daghio [b], Sara Falsini [a], Sandra Ristori [c], Alessio Papini [a], Elisabetta Toni [b], Carlo Viti [b], Cristina Gonnelli [a,*]

[a] Department of Biology, Università degli Studi di Firenze, via Micheli 1, 50121, Florence, Italy
[b] Department of Agriculture, Food, Environment and Forestry (DAGRI), Piazzale delle Cascine 18, Firenze, 50144, Italy
[c] Department of Chemistry & CSGI, University of Florence, Via della Lastruccia 3, Sesto Fiorentino, 50019, Firenze, Italy

HIGHLIGHTS

- Impact of PET-MNPs was tested for ten days on *Azolla filiculoides-Trichormus azollae*.
- Chlorophyll, ionome and root anatomy, but not biometry, were altered by the treatment.
- PET-MNPs affected morphology and abundance of the cyanobacteria.
- A negative effect of PET-MNPs on nitrogen supply to the fern was proposed.

GRAPHICAL ABSTRACT



ARTICLE INFO

Handling Editor: Paolo Pastorino

Keywords:
Micro-nanoplastics
Polyethylene terephthalate
Symbiosis
Nitrogen fixation
Fern
Cyanobacteria

ABSTRACT

The symbiotic system *Azolla filiculoides-Trichormus azollae* was exposed for ten days to environmentally relevant concentrations (i.e. 0.05 and 0.1 g L^{-1}) of polyethylene terephthalate micro-nanoplastics (PET-MNPs). Plastic particles did not induce any visible toxicity symptoms or growth disorders to the fern, as well as any effects on leaf anatomy and chlorophyll fluorescence parameters. Nonetheless, in treated plants a decrease of chlorophyll content occurred and was coupled to reduction of Nitrogen Balance Index (NBI), an informative parameter of the plant nitrogen status. In the presence of MNPs, plants exhibited a substantial decline in the absorption of essential elements, as evidenced by decreased tissue concentration of Ca, Mg, Co and Mn. The exposure to the pollutants compromised root integrity and possibly its functioning in nutrient accumulation, with evident physical damages not only in the rhizodermis and cortex, but also in the vascular system. In addition, a DNA-based estimation of *T. azollae* revealed a decreasing trend in the relative abundance of the N_2-fixing cyanobacteria for PET-treated samples. This was coupled with an alteration of the symbiont's phenotype highlighted

* Corresponding author.
E-mail addresses: marco.dainelli@unifi.it (M. Dainelli), beatrice.chiavacci@unifi.it (B. Chiavacci), ilaria.colzi@unifi.it (I. Colzi), andra.coppi@unifi.it (A. Coppi), milio.corti93@gmail.com (E. Corti), matteo.daghio@unifi.it (M. Daghio), sara.falsini@unifi.it (S. Falsini), sandra.ristori@unifi.it (S. Ristori), alessio.papini@unifi.it (A. Papini), elisabetta.toni@unifi.it (E. Toni), carlo.viti@unifi.it (C. Viti), cristina.gonnelli@unifi.it (C. Gonnelli).

https://doi.org/10.1016/j.chemosphere.2024.143718
Received 10 June 2024; Received in revised form 6 November 2024; Accepted 7 November 2024

by microscopy analysis, showing a reduction in number of vegetative cells between two consecutive heterocysts and in heterocyst size. This work is the first evidence of MNPs disturbing a strict symbiosis, with possible implications on nitrogen cycling in ecosystems, bio fertilization of agricultural lands and evolutionary pathways.

1. Introduction

Scientific research on micro- and nanoplastic (MNP) pollution has seen marked development in recent years, but the number of publications concerning the impacts of MNPs in freshwater ecosystems is still limited if compared to marine studies (Bhardwaj et al., 2024). Special attention should be devoted to these areas, as freshwater bodies tend to collect and accumulate many MNPs due to slower flow, poor diffusion, and closer proximity to human activities (Y. Wang et al., 2022a). Particularly, polyethylene (PE), polypropylene (PP) and polyethylene terephthalate (PET) are the most frequently detected polymers in rivers and lakes (Pereao et al., 2020). The effect of such contaminants on aquatic vascular plants is of crucial importance, since these organisms not only constitute a habitat for several functional groups, including periphyton, zooplankton, invertebrates, fish and many others, but are also involved in nutrient cycles (Bornette and Puijalon, 2009; Scheffer, 2004).

In the past two years, there has been a surge in research about MNP phytotoxicity on both sediment-rooted and free-floating macrophytes, with a wide range of reported effects dependent on polymer type, particle concentration and size, and time of exposure. Among various studies, for example poly(styrene-co-methyl methacrylate) [P(S-co-MMA)] at two different concentrations (i.e. 0.05 and 0.1 g L^{-1}) has been shown to impair growth and chlorophyll content of *Lemna minuta*, with more evident impacts after a prolonged exposure (i.e. 28 days) compared to short ones (i.e. 7 and 14 days) (Ceschin et al., 2023). Another duckweed (i.e. *Spirodela polyrhiza*), despite not showing a reduced growth rate when exposed for 10 days to the same concentrations of PET particles, was reported to display alterations in nutrient absorption, oxidative status and DNA methylation patterns (Dainelli et al., 2024). Furthermore, the effects of polyvinyl chloride (PVC) MNPs at high concentrations (i.e. 0.1 and 1 g L^{-1}) on the same species in a 7-day-experiment ranged from growth and reproduction inhibition to metabolism adjustments (Y. Wang et al., 2023). Environmentally relevant concentrations of PS-MNPs instead stimulated *Vallisneria natans* root growth after 40 days of exposure, but oxidative stress at the root level was elicited depending on particle size (Fu et al., 2023).

Notwithstanding this increasing body of information, a possible impact of MNPs on macrophytes with microbial symbiotic interactions has not been explored yet, despite these "superorganisms" play a key role in the nutrient cycle of freshwater ecosystems (Rejmankova, 2011). The presence of MNPs and their physicochemical properties (e.g., hydrophobicity, surface roughness and chemical properties) can change the community structure and functions of microorganisms, finally affecting the geochemical cycle of carbon, nitrogen and phosphorus (Bank and Hansson, 2019; X. Wang et al., 2022). In particular, nitrogen cycling has been shown to be widely subjected to the negative impact of MNPs (i.e. W. Wang et al., 2024; Zhang et al., 2024 etc.). This cycle plays a decisive role in biogeochemistry, and largely depends on the microbial driven nitrogen transformation (Shen et al., 2022), Together with nitrification and denitrification, nitrogen fixation is a key process in nitrogen cycling, namely the main way of entrance for the reactive and available forms of this element in most ecosystems (Bellenger et al., 2020). Several macrophytes host microorganisms capable of these processes that help plants to access essential elements otherwise unavailable as N_2 (Cui et al., 2022; Wickstrom and Corkran, 1997). Therefore, an additive detrimental effect of MNPs on these organisms through possible impact on their symbionts cannot be excluded.

In this context, *Azolla filiculoides* is a free-floating aquatic fern and a common species in freshwater ecosystems. The peculiarity of this plant is the stable association with the filamentous nitrogen-fixing cyanobacterium *Trichormus azollae* capable of forming heterocysts, which provide organic nitrogen to the plant (Carrapiço, 2017). The symbiotic relationship between these two organisms is also exploited in the agronomic field. Because of its high potential for biological N fixation carried out by cyanobacteria, *A. filiculoides* is regarded as one of the most promising biofertilizers, especially in rice crops, enhancing the cereal production by 20–30% (Korsa et al., 2024; Verma et al., 2022). This is an important data, since excessive use of chemical fertilizers results in increased environmental pollution while their replacement with biological sources of nitrogen seems to be a good choice to naturally improve crop yields (Maswada et al., 2021).

In this work we investigated for the first time the effects of PET-MNPs at environmentally relevant concentrations on the "superorganism" *Azolla-T. azollae*. Particularly, we aimed at: i) evaluating the impact of PET-MNPs on the water fern's growth, anatomy, photosynthetic efficiency and ionomic profile, ii) assessing if MNP exposure affects *T. azollae* heterocyst frequency and size and the symbiotic cyanobacterium relative abundance in terms of DNA-based estimation. The significance of this study concerns the understanding of not only the MNP-induced alterations in floating macrophytes, but also of the possible consequences on the nitrogen fixation process. This research area has major implications on both ecosystems functioning and yield of agricultural practices.

2. Materials and methods

2.1. Production and characterization of MNPs

PET-MNPs were obtained by breaking down water bottles from an Italian producer, following the standard procedure already used by Dainelli et al. (2024). The size distribution of the plastic particles was determined by Dynamic Light Scattering (DLS) on a Malvern Zetasizer Nano ZS (ZEN 1600 model, Malvern Instruments Southborough, MA), equipped with He–Ne 633.4 mW laser and backscattering detection. In order to ensure quantitative comparison among different samples, the detector attenuation was set to the constant level 10. DLS measurements were performed over 11 runs and in duplicate. The obtained data showed that the mean size of the MNPs was in the range 190–230 nm. Fig. S1 and Table S1 report the results for two independent specimens taken from the same preparation. The polydispersity index (PDI) was 0.5–0.6, revealing a broad size distribution, which was however monomodal and allowed an estimate of the item concentration as 1×10^7 particles/mL from its mean value. This value was calculated from the sample weight, the PET density and by approximating the particle shape to equivalent spheres, as commonly done for DLS data (Caputo et al., 2021).

Zeta Potential (ζ) measurements were performed with a Zetasizer (Zetasizer Pro, Malvern Panalytical Co. Ltd., Malvern, UK) in DTS1070 cells, at 298 K. In these experiments the ζ values were measured by phase analysis light scattering (M3-PALS). Each measurement was carried out in duplicate. Results are reported in Table S1 as mean values with standard deviation over 15 runs. The obtained zeta potential showed that the surface of the MNPs was negatively charged with average ζ values of ≈ −20 mV. This result suggested a moderate, though not negligible, capacity for ions and other small chemicals in solution to be absorbed, and was consistent with prior studies (My Tran et al., 2021; Sitko et al., 2013).

2.2. Plant growth and experimental conditions

The laboratory tests were performed on the floating fern *Azolla filiculoides* following the guidelines of the Organisation for Economic Co-operation and Development (OECD) protocol (Test No. 221, 2006) formulated for *Lemna* sp. for the testing of chemicals, with a few modifications. *Azolla filiculoides* plants were collected from an artificial pond in the Botanical Gardens of the University of Florence as in Bianchi et al. (2020). Plants were cultivated in the climate chamber under controlled conditions: 24/16 °C day/night, light intensity 300 μmol m^{-2} s^{-1}, 16-h (day) photoperiod and relative humidity 60–65%. The beakers used for the cultivation contained 100 mL of H-40 medium growing solution (Table S2, Pereira and Carrapiço, 2009). One beaker was used as inoculum of the culture and 4 to 6 fronds were transferred to each beaker (Fig. 1a–c). *Azolla filiculoides* plants were tested under three different treatment conditions: *i)* control (C), 100 mL H-40 medium prepared in distilled water; *ii)* 100 mL H-40 medium prepared in PET-MNP solution diluted 1:2 (v/v) with distilled water (PET ½, approximative MNP concentration = 0.05 g L^{-1}); *iii)* 100 mL H-40 medium prepared in pure PET-MNP solution (PET, approximative MNP concentration = 0.1 g L^{-1}). For each treatment, 6 replicates were set up, for a total of 18 test beakers. The test lasted 10 days as standard growing time without medium renewal, which would have led to an uncontrolled alteration of the concentration of PET particles. The concentrations of PET-MNPs used to conduct the experiment are fully comparable with those found in polluted freshwater ecosystems (see e.g. Gupta et al., 2023; Li et al., 2020). At the end of the 10 days of treatment (Fig. 1d–f), the plants were sampled from a subset of test beakers. Their fresh and dry weight were determined, while the remaining material was used for the chlorophyll fluorescence, pigment content, and ionome, microscopy and DNA analysis. The root length of each sample was also measured. Photos of the plants (Fig. 1a–f) at the beginning and at the end of the experiment were taken from above using a digital camera (Canon PowerShot SX100 IS) placed at a distance of 10 cm from the top of the beakers. The area (cm^2) occupied by the plants at the two time points was measured with Fiji softwareof ImageJ (Schindelin et al., 2012). According to OECD protocol (Test No. 221, 2006), the following formula was used to calculate the average specific growth rate ($\mu_{0\text{-}10}$): $\mu_{0\text{-}10} = (\ln(N_{10}) - \ln(N_0))/t$; t is the time period of the experiment and N_{10}/N_0 are the measurements of the area occupied by the plants at the beginning or at the end of the test.

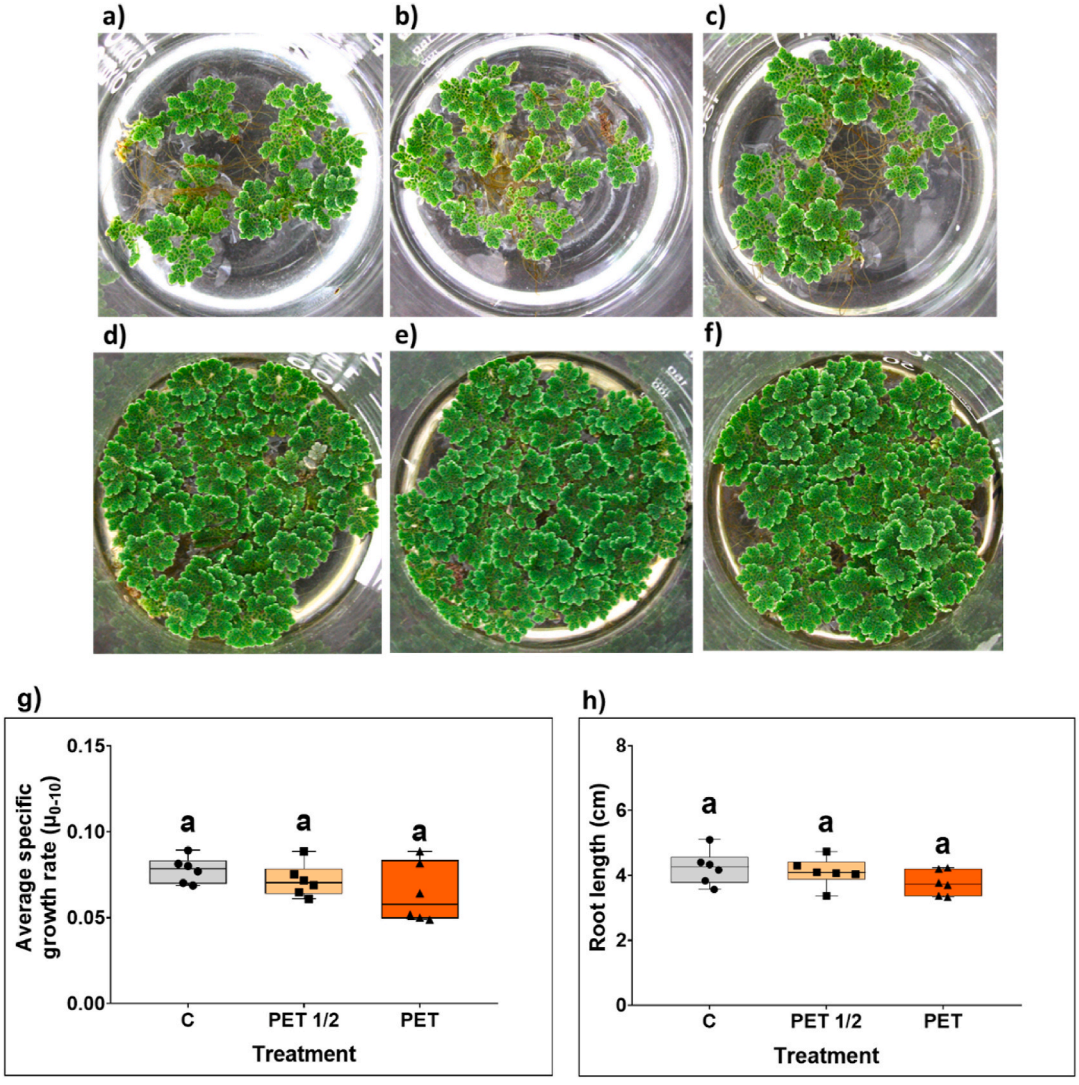


Fig. 1. Photos of *A. filiculoides* plants at the beginning (a–c) and at the end of the experiment after ten days (d–f). One representative beaker for each treatment is shown. a) and d) = C; b) and e) = PET ½; c) and f) = PET. *g)* Average specific growth rate ($\mu_{0\text{-}10}$) and *h)* root length of *A. filiculoides* plants grown in absence (C) or in presence of MNPs at different concentrations (PET ½ and PET) for ten days. Boxplots and whiskers were created using 6 independent replicates. Lower case letters indicate significant differences among the samples (at least $p < 0.05$).

2.3. Chlorophyll fluorescence analysis and pigment content

At the end of the treatment period, a single frond from each beaker was sampled, placed on absorbent paper wetted with distilled water and dark-adapted for 15 min using a leafclip. After that, *Chlorophyll a* fluorescence (i.e. the light re-emitted by the molecules during return from excited to non-excited states) was measured using a portable fluorimeter (HandyPEA, Hansatech Instruments Ltd, Norfolk, UK). The dark adaptation of *A. filiculoides* allows to reach the maximum oxidation of the PSII so that all PSII reaction centres can undertake photochemical reactions. A 1-s long saturating light pulse (3500 μmol m^{-2} s^{-1}, 650 nm) was then applied by the sensor of the fluorimeter and the fluorescence emission recorded, thus obtaining OJIP transients to be analysed. These transients were analysed according to Stirbet and Govindjee (2011) after double normalization between F_0 (step O, minimum fluorescence value at 20 μs) and F_M (plateau P, maximum fluorescence value at plateau). The Biolyzer software (Fluoromatic Software, Geneva, Switzerland) were used to calculate basic parameters (listed and described in Table S3) based on double normalized transients, thus allowing a coherent comparison between the different treatment groups.

A portable Dualex® instrument (Multi-Pigment-Meter MPM-100, Paris, France) was used to measure in a non-destructive way the indices of pigment content (amount of pigment per cm^2 determined through the fluorescence simple ratio), such as chlorophyll content index (CCI), flavonol index (FlvM), and Nitrogen Balance Index (NBI) on a completely expanded leaf. This instrument uses ratio of fluorescence to measure flavonol content (ratios: F660nm/F325nm) and leaf transmission in the near and far infra-red to determine the chlorophyll content (T850/T720). NBI is calculated as the ratio between chlorophyll (T850/T720) and flavonol (F660nm/F325nm) contents.

2.4. Ionome analysis

The concentration of macro- and micro-nutrients (ionome) of *A. filiculoides* plants was analysed following standard methodologies (i.e. Bettarini et al., 2019). The plant material was oven-dried at 50 °C for 48h and then acid-digested using 5 mL of 69% HNO_3 in a microwave digestion system (Mars 6, CEM) with maximum temperature of 200 °C for 20 min. Element concentrations (K, Ca, Mg, Fe, Zn, Mn, Co, Cu and Ni) were measured by atomic absorption spectroscopy (PinAAcle 500, PerkinElmer) checking the reliability (<10% RSD) and accuracy (<5% RSD) of the method with certified reference materials (grade BCR, Fluka Analytical, Sigma-Aldrich). Macro- and micronutrient concentrations were then expressed on a dry weight basis as mg g^{-1} and μg g^{-1} respectively.

2.5. DNA analysis to quantify the relative abundance of Trichormus azollae

Leaf samples were thawed in ice and total DNA was extracted from 300 mg of leaves using the FastDNA™ SPIN Kit for Soil (MP Biomedicals) according to the manufacturer's instructions. The relative abundance of *T. azollae* DNA was calculated by using Ribosomal protein L25 (RPL25) loci as well as the *A. filiculoides* ITS DNA locus in quantitative PCR (qPCR) (Brilli et al., 2022). The qPCR was performed in an ABI StepOne Plus real-time PCR system (Applied Biosystems) in a 10 μL mixture containing 1 X BlasTaq qPCR MasterMix (Applied Biological Materials Inc.) and 400 nM of each primer. The following cycling parameters were used: initial step at 95 °C for 3 min, then 40 cycles each including a step at 95 °C for 15 s and a step at 60 °C for 1 min. Details about the primers used are provided in Table 4. The RPL25 gene was used to estimate the presence of the symbiont *T. azollae* and the *A. filiculoides* ITS was used as a reference gene. The ΔCt between the RPL25 gene and the ITS gene (ΔCt = CtRPL25 – CtITS) was calculated based on the q-PCR results. In this way, the presence of *T. azollae* was normalized to the reference gene. The $2^{(-\Delta Ct)}$ algorithm was used to calculate the relative abundance of *T. azollae* DNA (Schmittgen and Livak, 2008).

2.6. Light microscopy observations

Three plants from each treatment were collected at the end of the experiments and fixed in a solution of Formaldehyde 10%, Ethanol 50%, Acetic Acid 5% (FAA) for 48h at 4 °C. Subsequently, fixed samples were washed in 70% ethanol for 1 h and then dehydrated progressively by treatment with 80% ethanol for 1 h, 95% ethanol for 2 h, and then twice in absolute ethanol for 3 h, cumulatively. Pre-inclusion was performed first with ethanol and historesin in 1:1 ratio for one night, then with 1:2 ratio for 2 h, and in pure historesin for 3 h. Finally, samples were included in a polypropylene capsule with the addition of hardener in ratio 1:15 of basic resin. The included samples were cut in 5 μm thick sections with an ultramicrotome and stained with Toluidine Blue O 0.1% (w/v) in phosphate buffer pH 7.

For the visualization of *T. azollae*, the leaf cavities of fresh samples were gently pressed to ensure the cyanobacteria outflow. Thus, the counting of the cells of the filament between heterocysts and the measurement of the heterocyst size were performed in triplicate for each treatment. We photographed 3 independent samples per treatment (Fig. S2), each from a different beaker, and conducted measurements until reaching a final total of 28 observations. Observations were carried out using a Leitz DM-RB Fluo optical microscope equipped with a Nikon digital camera, and the images analysed by Image J.

2.7. Statistics

The One-way ANOVA was used to check the significance of differences ($P < 0.05$) among means of treatment groups, using GraphPad Prism 7 (GraphPad Software, San Diego, CA). Data normality was verified with the Shapiro-Wilk test, whereas the Bartlett's test was used for checking homogeneity of variances.

3. Results

3.1. Effects of MNPs on plant growth, photosynthesis and pigment content

Azolla filiculoides colonies grown with PET-MNPs medium did not show any significant variation in final fresh and dry weight compared to the untreated plants (Table 1a, Tables S4a–b), nor in the average specific growth rate ($\mu_{0\text{-}10}$) calculated based on the occupied area in the beakers over time (Fig. 1g–Table S4c). Moreover, the length of the roots did not exhibit significant difference with respect to the controls (Fig. 1c–Table S4d). By analysing the parameters of photosynthetic efficiency, most of the considered OJIP-test parameters (Table 1b, F_0, ΦP_o, ΨRE_o, Sm and ABS/RC) did not significantly change upon plant growth in MNP solutions. The only exception was F_0, whose values were significantly reduced in samples treated with the highest PET-MNP concentration (Fig. 2, Table S5) (see Table 3).

The pigment analysis showed a significant decrease of CCI in *A. filiculoides* colonies treated with MNPs at maximum concentration, while the amount of the FlvM remained almost unchanged (Fig. 3, Tables S6a–b). Regarding NBI index, there was a significant reduction of this parameter (Fig. 3, Table S6c).

3.2. Effects of MNPs on element concentration

Concerning macro-elements (Table 2), only Ca and Mg showed a reduction in concentration in MNP-treated plants (Tables S7a–c), while among the micro-elements only Mn and Co concentration was significantly lower in MNP-exposed samples as compared with control plants (Tables S7d–i). Fig. 4 represents the amplitude heatmap of the MNP-treatment-induced decreases in element concentration. Overall, the elemental profile of PET-treated plants was more altered than that of

Table 1
Values of a) fresh and dry weight (FW and DW, expressed in grams), and b) OJIP-test parameters (listed and described in Table S3) of *A. filiculoides* plants grown in absence (C) or in presence of MNPs at different concentrations (PET ½ and PET) for ten days. Values are mean of 6 replicates ± standard deviation. Letters indicate the significant differences among the treatments (at least $p < 0.05$).

a) Fresh and dry weight	Treatments		
	C	PET 1/2	PET
FW (g)	1.892 ± 0.221 **a**	1.983 ± 0.201 **a**	1.869 ± 0.080 **a**
DW (g)	0.173 ± 0.020 **a**	0.178 ± 0.018 **a**	0.171 ± 0.007 **a**
b) OJIP-test parameters	**Treatments**		
	C	**PET 1/2**	**PET**
F_0	441.273 ± 36.968 **a**	421.400 ± 33.711 **ab**	389.600 ± 27.805 **b**
ΦP_o	0.825 ± 0.011 **a**	0.827 ± 0.008 **a**	0.825 ± 0.006 **a**
ΨET_o	0.539 ± 0.029 **a**	0.520 ± 0.018 **a**	0.517 ± 0.025 **a**
ΨRE_o	0.184 ± 0.028 **a**	0.175 ± 0.016 **a**	0.169 ± 0.018 **a**
Sm	19.544 ± 2.280 **a**	18.624 ± 1.178 **a**	18.394 ± 1.518 **a**
ABS/RC	2.586 ± 0.192 **a**	2.651 ± 0.285 **a**	2.671 ± 0.194 **a**
Pi_{abs}	2.171 ± 0.397 **a**	2.030 ± 0.457 **a**	1.923 ± 0.328 **a**

Table 2
Element concentration (mg g^{-1} and µg g^{-1} for K, Ca, Mg and Co, Zn, Mn, Cu, Ni respectively) in *A. filiculoides* plants grown in absence (C) or in presence of MNPs at different concentrations (PET ½ and PET) for ten days. Values are mean of 6 replicates ± standard deviation. Lower case letters indicate significant differences among control and treated samples (at least $p < 0.05$).

Element concentration	Treatment		
	C	PET 1/2	PET
K (mg/g)	70.01 ± 4.38 **a**	68.94 ± 2.22 **a**	67.10 ± 3.74 **a**
Ca (mg/g)	2.30 ± 0.26 **a**	1.75 ± 0.14 **b**	1.52 ± 0.40 **b**
Mg (mg/g)	5.94 ± 0.41 **a**	5.28 ± 0.15 **b**	5.61 ± 0.40 **ab**
Co (µg/g)	81.51 ± 8.59 **a**	70.86 ± 7.53 **b**	66.16 ± 3.34 **b**
Zn (µg/g)	311.54 ± 54.93 **a**	269.25 ± 40.41 **a**	255.80 ± 32.29 **a**
Mn (µg/g)	310.67 ± 69.46 **a**	261.91 ± 56.91 **ab**	204.66 ± 23.26 **b**
Cu (µg/g)	29.71 ± 2.08 **a**	29.34 ± 1.91 **a**	28.99 ± 2.72 **a**
Ni (µg/g)	41.50 ± 9.32 **a**	41.05 ± 6.11 **a**	37.78 ± 3.08 **a**

Table 3
Pigment content indices in *A. filiculoides* plants grown in absence (C) or in presence of MNPs at different concentrations (PET ½ and PET) for ten days. CCI: chlorophyll content index; FlvM: flavonol index; NBI: Nitrogen Balance index. Values are mean of 6 replicates ± standard deviation. Letters indicate the significant differences among the treatments (at least $p < 0.05$).

Pigment content	Treatment		
	C	PET 1/2	PET
CCI	0.48 ± 0.15 **a**	0.42 ± 0.15 **ab**	0.26 ± 0.06 **b**
FlvM	0.07 ± 0.03 **a**	0.06 ± 0.02 **a**	0.06 ± 0.02 **a**
NBI	6.51 ± 2.43 **a**	5.07 ± 1.25 **a**	4.44 ± 1.80 **a**

PET ½ plants, with a notable decrease in Ca, Mg, Mn and Co levels. In general, decreases were more evident for micro-elements with respect to macro-elements.

3.3. DNA analysis to quantify the relative abundance of *Trichormus azollae*

The relative abundance of *T. azollae* DNA was calculated by the $2^{(-\Delta Ct)}$ algorithm. While we observed a decreasing trend in $2^{(-\Delta Ct)}$ mean values of MNPs-treated samples with respect to the control, the differences were not statistically significant (Fig. 5, Table S8).

3.4. Light microscopy observation

Fig. 6 shows light microscopy images of *Trichormus azollae* extracted from leaf cavities of *A. filiculoides* grown in water (Fig. 6a) and in the presence of the two different concentrations of MNPs: PET1/2 and PET (Fig. 6b and c).

Control samples showed a lower number of vegetative cells between two consecutive heterocysts if compared with the filaments collected from plants treated with MNPs, significantly at the higher concentration (Fig. 6d–Table S9a). Moreover, the size of the heterocysts decreased in filaments obtained from plants treated with both concentrations of MNPs in comparison with the cyanobacteria filaments from the control plants (Fig. 6e–Table S9b).

Fig. 7a shows the typical anatomy of *Azolla* root, with regular rhizodermis, a cortex with two layers of parenchyma cells and the internal endodermis with its peculiar hexagonal shape. The internal cylinder was composed of a pericycle that consisted of six parenchyma cells with few plastids, a xylematic vessel, and few sieve tubes. Samples exposed to PET1/2 (Fig. 7b) and PET (data not showed) showed a similar PET-MNP-affected structure as compared to control plants. Their rhizodermis appeared thinned and the cell walls of the cortex severely damaged, with larger intercellular spaces. The vascular elements inside the internal cylinder were much smaller in diameter as compared to control samples.

Regarding frond morphology, *A. filiculoides* leaf was composed of an emerged, dorsal lobes and very thin, ventral lobes which floated on the water surface (Fig. 7c). The dorsal lobe showed a photosynthetic palisade and an ellipsoid cavity (length ≈ 260 µm) that harbours the symbionts. The cavity was open to the outside through a pore (length ≈ 50 µm) covered by protruding teat cells (Fig. 7d). Typical bicellular trichomes were visible as well (Fig. 7d). No differences were evidenced comparing fronds from control plants with those from plants exposed to the two treatments (data not shown).

4. Discussion

The experimental data did not indicate any growth inhibitory effects of PET-MNPs on *A. filiculoides* over the short-term treatment of 10 days. These results differ from those of an earlier investigation on another floating macrophyte, i.e. *Spirodela polyrhiza*, exposed to MNPs of the same polymer at the same concentrations and for the same duration (Dainelli et al., 2024). In fact, although the average specific growth rate remained unchanged between treatments in that study, a plastic particle-induced decrease in fresh and dry weight was observed. The same consideration applied to photosynthetic performance, that was reported to be substantially impacted by PET-MNPs both in duckweeds (Dainelli et al., 2024) and submerged macrophytes (Abduro Ogo et al., 2022; Yu et al., 2022), and in general also in terrestrial plants (Colzi et al., 2022; Dainelli et al., 2023; Pignattelli et al., 2021). In the experimental conditions here adopted, no photosynthetic efficiency indicators (i.e. chlorophyll fluorescence parameters) were altered in *A. filiculoides*. This is probably due to possible adaptive traits of this species, conferring wide tolerance to a variety of environmental stressors, including xenobiotics (Maldonado et al., 2022). Such traits are supposed to promote the invasiveness of this species (Nikkhah et al., 2024; Sood et al., 2012). It is also worth considering that a longer exposure to plastic pollutants could lead to negative consequences on this fern, as demonstrated by Ceschin et al. (2023) on *Lemna minuta* exposed to [P(S-co-MMA)]-MNPs for different times, with more evident

Table 4
Primers used in this study.

Locus	Forward primer (5′-3′)	Reverse primer (5′-3′)	Reference
NARPL25	GTACGGTCACAAAGGCACAG	AGGTGTTCCCTTCGCTGGAT	de Vries et al., 2018
AZITS	GAATTCCGCGAATCATCGAGT	CGGACAACCATCTCCGCT	Brilli et al., 2022

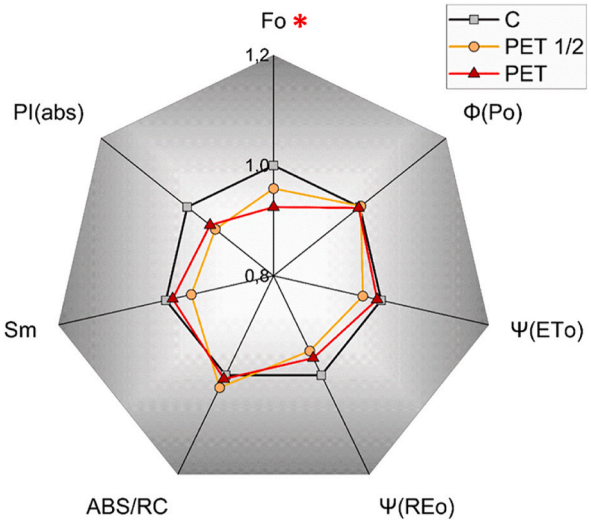


Fig. 2. Radar plot of OJIP-test parameters. Each point represents the average value normalized on the respective average value of control plants. Asterisks indicate significant differences among the samples (at least $p < 0.05$).

effects after 28 days with respect to 7 and 14 days.

The only fluorescence parameter that was impacted by the treatment was F_0, which itself depends partially on the chlorophyll levels of the analysed samples (Bussotti et al., 2012). Consistently with the reduction of F_0, the pigment analysis showed a lower chlorophyll quantity in treated plants, while no changes were reported concerning the flavonol content. These last-mentioned pigments have antioxidant properties and help plants to cope with stressful conditions and pollutants (Daryanavard et al., 2023), including MNPs (see e.g. Z. Yu et al., 2024). In line with the obtained results on growth and photosynthesis, also the stability of flavonol levels suggested lack of PET-MNP-induced stress in *A. filiculoides*, at least in the short term. Therefore, the observed reduction of the NBI parameter (i.e. ratio CCI/FlvM) in treated plants resulted from the decrease in chlorophyll content. NBI is regarded as a reliable indicator of plant nitrogen status (Abdallah and Goffart, 2012; Padilla et al., 2014). Since chlorophyll biosynthesis is expected to be impaired under nitrogen shortage (Mu and Chen, 2021), a probable PET-MNP-imposed deficiency in this element cannot be excluded for the studied system. Considering that the growing medium was nitrogen-free (Table S2), this supposed N-shortage could relate to a possible negative impact of PET-MNPs on N_2-fixing activity of the cyanobacterial symbiont, since the atmosphere was the sole provider of nitrogen for the superorganism. Interestingly, several studies did not observe any decline in chlorophyll content on macrophytes exposed to PET-MNPs in N-rich substrates (e.g. Cui et al., 2023; Dainelli et al., 2024).

As for the analysis of the ionome, a PET-induced nutrient imbalance

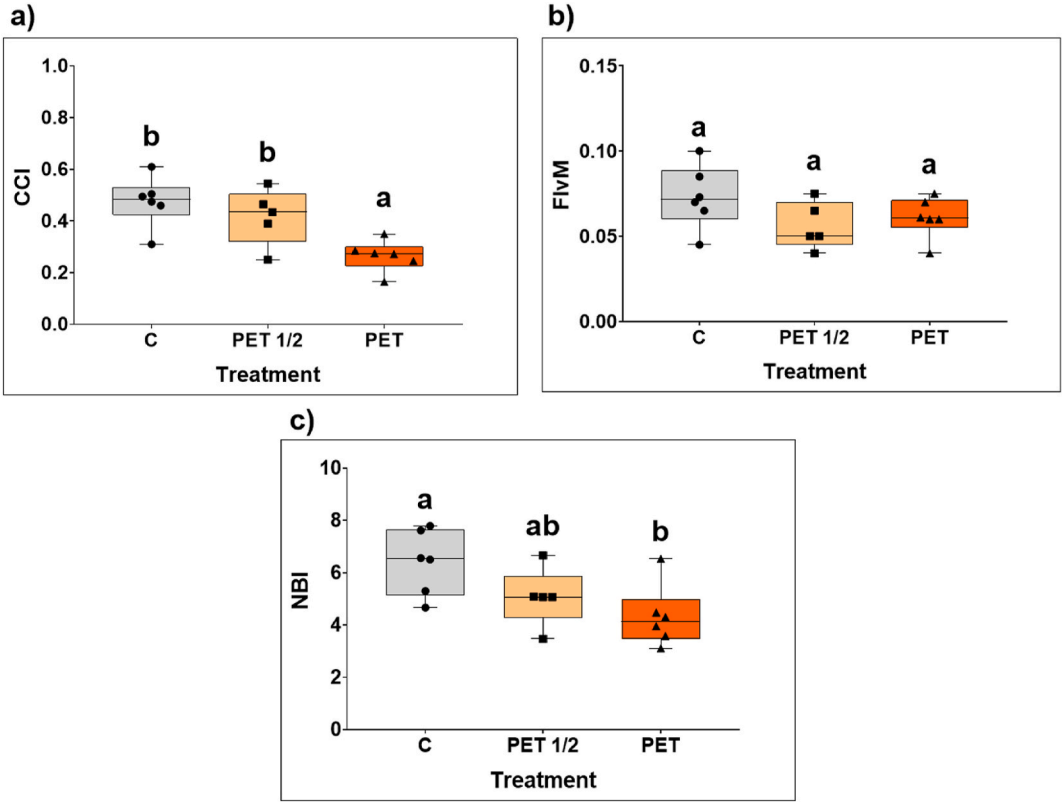


Fig. 3. Pigment content indices in *A. filiculoides* plants grown in absence (C) or in presence of MNPs at different concentrations (PET ½ and PET) for ten days. Boxplots and whiskers were created using 6 independent replicates. Letters indicate the significant differences among the treatments (at least $p < 0.05$).

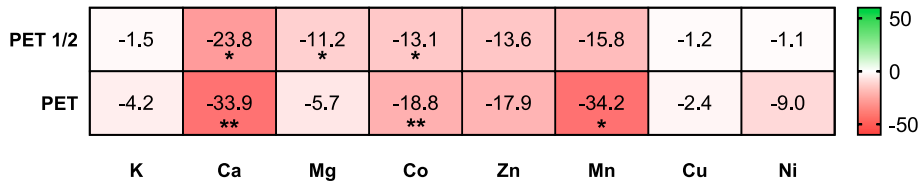


Fig. 4. Heatmap showing ionome variation of *A. filiculoides* plants grown in presence of MNPs at different concentrations (PET ½ and PET) for ten days. Colour scale indicates decreased (red), unchanged (white) or increased (green) element concentration in respect to the control. The percentage of variation is reported, together with asterisks indicating the significant difference between the element concentrations in treated and control plants (at least $p < 0.05$).

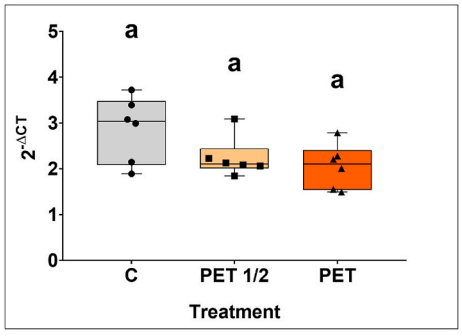


Fig. 5. The relative abundance of *Trichormus azollae* DNA grown in absence (C) or in presence of MNPs at different concentrations (PET ½ and PET) for ten days. Boxplots and whiskers were created using 6 independent replicates. Letters indicate the significant differences among the treatments (at least $p < 0.05$).

emerged with most of the plant elemental concentrations decreased in treated samples. Recent studies suggested several kinds of ionomic changes after MNP exposure (Colzi et al., 2022; Dainelli et al., 2023, 2024; Fu et al., 2022; Tang et al., 2022), but the underlying mechanisms for these variations remain largely unexplained. In our case, negatively charged PET-MNPs could have physically interacted with the roots of *A. filiculoides*. This effect was demonstrated for polystyrene with similar charge on *A. thaliana* roots (Sun et al., 2020), causing a concentration-dependent reduction in the absorption of macro- and micro-nutrients. Physical damages induced by MNPs has been repeatedly reported in the literature, irrespectively of polymer type and plant species (see e.g. Liu et al., 2023; H. H. Wang et al., 2024; J. Wang et al., 2023). In accordance, the root anatomy of the fern was disturbed by the presence of plastic particles, as revealed by microscopy observations. External tissues were particularly damaged, likely due to the direct contact with the pollutants, and the vascular system appeared to be shrunk, thus probably contributing to impaired nutrient absorption and transport within the organism. In addition, negatively charged MNPs surface may have contributed to the amount of cations available for plant metal uptake. In the long term, the PET-induced decrease in element accumulation could lead to significant growth reduction of

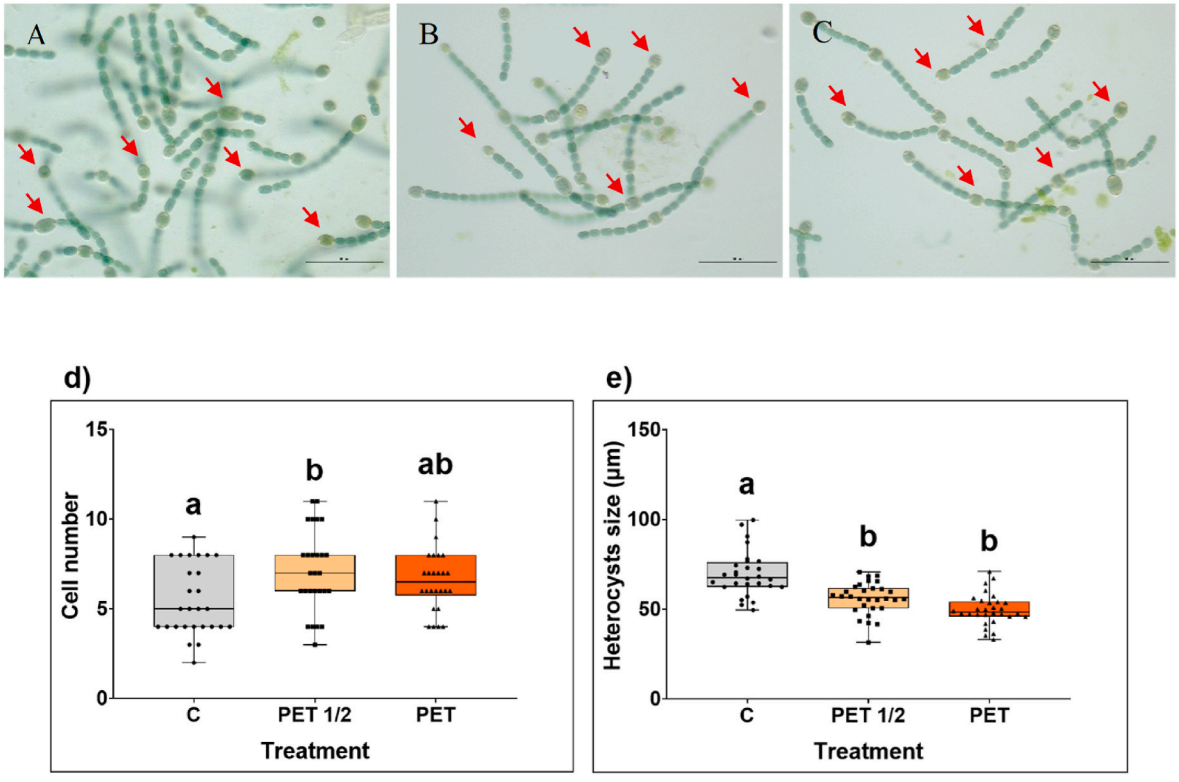


Fig. 6. Images of *Anabaena azollae* flow out from the leaf cavity of *Azolla* grown in the absence *a)* or in the presence of *b)* PET 1/2 and *c)* PET. Scale bars (a, b, c) = 50 µm. d) Number of cells between heterocysts and e) heterocysts area of *Trichormus azollae* collected from *A. filiculoides* cultivated in water or with MNPs at the two different concentrations. Red arrows indicate the heterocysts. Boxplots and whiskers were created using a total of 28 measurements from three independent samples per treatment, each from a different beaker. Letters indicate the significant differences among the treatments (at least $p < 0.05$).

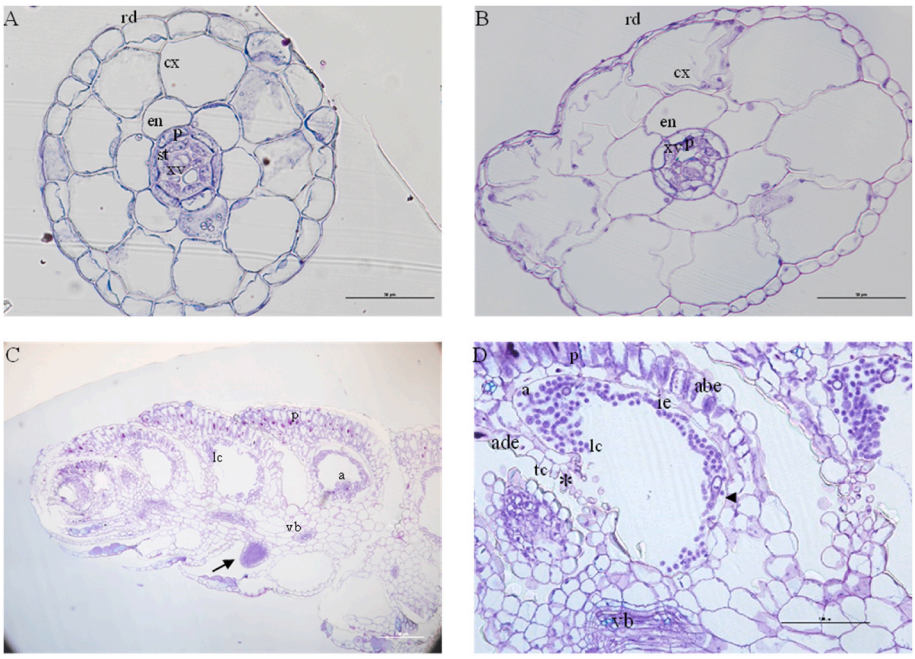


Fig. 7. Cross sections of *A. filiculoides* root grown in culture medium Control (A) and in presence of MNPs treatment (B). Scale bar (A–B): 50 μm. Cross section of a leaf from the control (C). Scale bar (C): 200 μm. Particular of the lobe (D). Scale bar (D): 100 μm. All sections were stained with Toluidine blue. rhizodermis (rd), cortex (cx), endodermis(en), pericycle (p), xylem vessel (xv), sieve tube (st); leaf cavity (lc); palisade (p); *T. azollae* (a); vascular bundles (vb); leaf primordia (arrow), abaxial epidermis (abe); inner epidermis (ie); adaxial epidermis (ade); teat-cell (tc); pore cavity (*); bicellular trichome (arrow head).

A. filiculoides, especially considering the crucial role of Ca in the rapid growth of this fern (Sadeghi et al., 2013) or the key role of Mn in metalloenzymes (Schmidt and Husted, 2019). Interestingly, the PET-decreased concentration of Co deserves further investigation, since this element is required for the growth of the *Azolla-Trichormus* symbiotic system in the absence of combined nitrogen (Johnson et al., 1966). This can be correlated with the indispensability of Co and cobalamin (i.e. vitamin B12) in enzymes for the fixation of atmospheric N_2 into NH_3 and for the survival of *Trichormus azollae* and other nitrogen-fixing bacteria (Hu et al., 2021).

Microscopy analysis emphasized several PET-induced modifications of the symbiont's phenotype (i.e. increased number of vegetative cells between two consecutive heterocysts and reduced heterocyst size). The development of heterocyst patterns is a genetically regulated process, with environmental constraints serving as key regulators (Álvarez et al., 2023; Corrales-Guerrero et al., 2013; Yoon and Golden, 2001). Among these, nitrogen availability is especially significant (Zapomělová et al., 2008) and the reduced heterocyst frequency here reported in *T. azollae* from PET-treated plants is hypothesized to indicate a compromised nutritional status of the holobiont. This hypothesis is further reinforced not only by the plant ionome and NBI data, but also by heterocyst dimensions, as cell size is optimized according to resource availability in nitrogen-fixing symbiosis (Cornejo-Castillo et al., 2024).

Furthermore, DNA analysis showed a mean reduction in the relative abundance of *Trichormus azollae* in the presence of MNPs, although not statistically significant. Plant microbiome alterations induced by plastic particles have been largely reported in the last years (see for example Chai et al., 2024; Ng et al., 2021; Zhang et al., 2023), even if the mechanisms by which this happens remains poorly understood. A pivotal role is undoubtedly played by the plastisphere (i.e. the microbes associated with plastic debris, Amaral-Zettler et al., 2020) that could cause microbial invasion (Li et al., 2021). In any case, chemical byproducts released during plastic degradation and mechanical and physical effects can contribute to the re-shaping of microbial communities as well (Rillig et al., 2024). Multiple factors could have concurred to our observation, including the above-reported Co shortage in treated *A. filiculoides* holobiont. In addition, small particles are dimensionally suited for entering the fern leaf cavity, as the measurements of the pore dimension by microscopy analysis evidenced. They could therefore fill up cyanobacterial vital space and facilitate the transmission of competing microbes or toxic substances (Rillig et al., 2024), ultimately interfering with the performances of the whole symbiotic system, as evidenced by its altered nitrogen status (i.e. reduced NBI parameter). The implications of these observations extend far beyond an ecological point of view. In the long term, MNPs can also pose a significant threat to the global agricultural food production since nitrogen-fixing microorganisms are the mostly used bio-fertilizers (Adhikari et al., 2020).

However, the variability of MNP-exposure consequences on different organisms living in disparate environments emphasizes that many aspects (i.e. type of polymer, particle size, shape, concentration, concerned species etc.; Karalija et al., 2022) contribute to this interaction, making it difficult to predict its outcome. For example, Q. Wang et al. (2023) showed that particles of polylactic acid (i.e. a biodegradable plastic type) boosts N-fixing Rhizobiales bacteria in peanut plants. On the other hand, among the few studies dealing with PET pollution and nitrogen, X. Sun et al. (2022) conducted a microcosm experiment to examine the impact of this polymer in paddy soils. Their findings demonstrated that a low PET-MNP concentration decreased the NH_4^+ level in overlying water, while a higher one had an opposite effect. Anyway, an alteration of the microbial nitrogen fixation process by PET could be hypothesized from these results and ours on the *Azolla--Trichormus* system. In any case, particle concentration seems to exert considerable influence, complicating the prediction of the outcome as stated above.

The present research is an initial step for understanding how plastic particles can disturb *A. filiculoides* and, potentially, its delicate strict symbiosis, a phenomenon previously documented only in the renowned anthozoan-algae symbiotic relationship, namely corals (Lanctôt et al., 2020; Okubo et al., 2018, 2020). In addition, our results could raise concerns of ecological and agricultural importance, as the fern *Azolla* is a multifaceted aquatic resource crucial for ecosystem sustainability (Kollah et al., 2016). This species not only helps to mitigate greenhouse

gas emissions from agriculture and has high bioremediation potential for trace metals, but also serves as major biological source of nitrogen for agriculture and the animal industry (Kollah et al., 2016). Plastic particle pollution is increasingly evident in flooded paddy soils (Ashjar et al., 2023; Y. Wang et al., 2022b) and has been reported to impair rice growth and yield (Chen et al., 2022; Yi et al., 2023). An additional negative effect of microplastic particles (MNPs) on *Azolla*, which is typically dual-cropped with rice to enhance nitrogen supply (Adhikari et al., 2020), could exacerbate the existing threat posed by such contaminants to one of the most important staple foods (Amelba et al., 2022).

5. Conclusion and perspectives

Despite the presence of PET-MNPs did not cause alterations in the growth of *Azolla filiculoides*, ten days of exposure were already sufficient to induced significant decrease in chlorophyll content, in the concentration of Ca, Mn and Co and in altering the root anatomy. These data pointed to a possible PET-MNP-induced shortage of nitrogen to the fern due to a negative impact of the particles on the symbiont *Trichormus azollae*, that in treated samples presented altered morphology and decreased abundance. This work represents the first evidence that MNPs can have a harmful effect on a symbiotic superorganism, like *Azolla-Trichormus*. Long term studies are needed to understand possible outcomes of MNP pollution both on the evolution of eukaryotic organisms with N_2-fixing-prokaryotic partners and on the nitrogen supply in ecosystem functioning and in global agricultural food production.

CRediT authorship contribution statement

Marco Dainelli: Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Beatrice Chiavacci:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Ilaria Colzi:** Writing – review & editing, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Andrea Coppi:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Emilio Corti:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Matteo Daghio:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Sara Falsini:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Sandra Ristori:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Alessio Papini:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Elisabetta Toni:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Carlo Viti:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Cristina Gonnelli:** Writing – review & editing, Writing – original draft, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Marco Dainelli, Ilaria Colzi, Sara Falsini reports financial support was provided by Ministry of Education and Merit. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was carried out within the RETURN Extended Partnership supported under the the Italian PNRR program, Mission 4 Component 2 Investment 1.3 funded from the European Union—NextGenerationEU (project RETURN PE3 – "Multi-risk science for resilient communities under a changing climate", project no. PE00000005). The publication was made with the contribution of the researcher Marco Dainelli with a research contract co-funded by the European Union PON Research and Innovation 2014–2020 in accordance with Article 24, paragraph 3a), of Law No. 240 of December 30, 2010, as amended, and Ministerial Decree No. 1062 of August 10, 2021.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2024.143718.

Data availability

Data will be made available on request.

References

Abdallah, B.F., Goffart, J.P., 2012. Potential indicators based on leaf flavonoids content for the evaluation of potato crop nitrogen status. In: Regency, Hyatt (Ed.), International Conference on Precision Agriculture. Indianapolis, Indiana USA.

Abduro Ogo, H., Tang, N., Li, X., Gao, X., Xing, W., 2022. Combined toxicity of microplastic and lead on submerged macrophytes. Chemosphere 295, 133956. https://doi.org/10.1016/j.chemosphere.2022.133956.

Adhikari, K., Bhandari, S., Acharya, S., 2020. An overview of Azolla in rice production: a review. Reviews in Food and Agriculture 2, 4–8. https://doi.org/10.26480/rfna.01.2021.04.08.

Álvarez, C., Jiménez-Ríos, L., Iniesta-Pallarés, M., Jurado-Flores, A., Molina-Heredia, F. P., Ng, C.K.Y., Mariscal, V., 2023. Symbiosis between cyanobacteria and plants: from molecular studies to agronomic applications. J. Exp. Bot. 74, 6145–6157. https://doi.org/10.1093/jxb/erad261.

Amaral-Zettler, L.A., Zettler, E.R., Mincer, T.J., 2020. Ecology of the plastisphere. Nat. Rev. Microbiol. 18, 139–151. https://doi.org/10.1038/s41579-019-0308-0.

Amelba, A.B.T., Daud, A., Amqam, H., Syafar, M., Ibrahim, E., Syam, A., Harusi, N.M.R., Afdal, M., 2022. Microplastics found in rice Consumed by humans. Journal of Positive School Psychology 6, 7809–7818.

Ashjar, N., Keshavarzi, B., Moore, F., Zarei, M., Busquets, R., Zebarjad, S.M., Mohammadi, Z., 2023. Microplastics (MPs) distribution in surface sediments of the Freidounkenar paddy wetland. Environ. Pollut. 317, 120799. https://doi.org/10.1016/j.envpol.2022.120799.

Bank, M.S., Hansson, S.V., 2019. The plastic cycle: a novel and holistic paradigm for the Anthropocene. Environ. Sci. Technol. 53, 7177–7179. https://doi.org/10.1021/acs.est.9b02942.

Bellenger, J.P., Darnajoux, R., Zhang, X., Kraepiel, A.M.L., 2020. Biological nitrogen fixation by alternative nitrogenases in terrestrial ecosystems: a review. Biogeochemistry 149, 53–73. https://doi.org/10.1007/s10533-020-00666-7.

Bettarini, I., Colzi, I., Coppi, A., Falsini, S., Echevarria, G., Pazzagli, L., Selvi, F., Gonnelli, C., 2019. Unravelling soil and plant metal relationships in Albanian nickel hyperaccumulators in the genus *Odontarrhena* (syn. *Alyssum* sect. *Odontarrhena*, Brassicaceae). Plant Soil 440, 135–149. https://doi.org/10.1007/s11104-019-04077-y.

Bhardwaj, L.K., Rath, P., Yadav, P., Gupta, U., 2024. Microplastic contamination, an emerging threat to the freshwater environment: a systematic review. Environmental Systems Research 13, 8. https://doi.org/10.1186/s40068-024-00338-7.

Bianchi, E., Biancalani, A., Berardi, C., Antal, A., Fibbi, D., Coppi, A., Lastrucci, L., Bussotti, N., Colzi, I., Renai, L., Scordo, C., Del Bubba, M., Gonnelli, C., 2020. Improving the efficiency of wastewater treatment plants: bio-removal of heavy-metals and pharmaceuticals by *Azolla filiculoides* and *Lemna minuta*. Sci. Total Environ. 746, 141219. https://doi.org/10.1016/j.scitotenv.2020.141219.

Bornette, G., Puijalon, S., 2009. Macrophytes: ecology of aquatic plants. In: ELS. Wiley. https://doi.org/10.1002/9780470015902.a0020475.

Brilli, F., Dani, K.G.S., Pasqualini, S., Costarelli, A., Cannavò, S., Paolocci, F., Zittelli, G. C., Mugnai, G., Baraldi, R., Loreto, F., 2022. Exposure to different light intensities affects emission of volatiles and accumulations of both pigments and phenolics in *Azolla filiculoides*. Physiol. Plantarum 174. https://doi.org/10.1111/ppl.13619.

Bussotti, F., Kalaji, M.H., Desotgiu, R., Pollastrini, M., Loboda, T., Bosa, K., 2012. Misurare la vitalità delle piante per mezzo della fluorescenza della clorofilla. Firenze University Press, Firenze. https://doi.org/10.36253/978-88-6655-216-1.

Caputo, F., Vogel, R., Savage, J., Vella, G., Law, A., Della Camera, G., Hannon, G., Peacock, B., Mehn, D., Ponti, J., Geiss, O., Aubert, D., Prina-Mello, A., Calzolai, L., 2021. Measuring particle size distribution and mass concentration of nanoplastics and microplastics: addressing some analytical challenges in the sub-micron size range. J. Colloid Interface Sci. 588, 401–417. https://doi.org/10.1016/j.jcis.2020.12.039.

Carrapiço, F., 2017. The Azolla–Anabaena–Bacteria association: a case of symbiotic Abduction?. In: Algal and Cyanobacteria Symbioses. WORLD SCIENTIFIC, pp. 329–345. https://doi.org/10.1142/9781786340580_0010 (EUROPE).

Ceschin, S., Mariani, F., Di Lernia, D., Venditti, I., Pelella, E., Iannelli, M.A., 2023. Effects of microplastic contamination on the aquatic plant *Lemna minuta* (least duckweed). Plants 12, 207. https://doi.org/10.3390/plants12010207.

Chai, B., Yin, H., Xiao, T., Xiao, E., Dang, Z., Pan, K., 2024. Effects of microplastics on Endophytes in different Niches of Chinese Flowering Cabbage (*Brassica campestris*). J. Agric. Food Chem. 72, 4679–4688. https://doi.org/10.1021/acs.jafc.3c09092.

Chen, S., Feng, Yanfang, Han, L., Li, D., Feng, Yuanyuan, Jeyakumar, P., Sun, H., Shi, W., Wang, H., 2022. Responses of rice (Oryza sativa L.) plant growth, grain yield and quality, and soil properties to the microplastic occurrence in paddy soil. J. Soils Sediments 22, 2174–2183. https://doi.org/10.1007/s11368-022-03232-w.

Colzi, I., Renna, L., Bianchi, E., Castellani, M.B., Coppi, A., Pignattelli, S., Loppi, S., Gonnelli, C., 2022. Impact of microplastics on growth, photosynthesis and essential elements in *Cucurbita pepo* L. J. Hazard Mater. 423, 127238. https://doi.org/10.1016/j.jhazmat.2021.127238.

Cornejo-Castillo, F.M., Inomura, K., Zehr, J.P., Follows, M.J., 2024. Metabolic trade-offs constrain the cell size ratio in a nitrogen-fixing symbiosis. Cell 187, 1762–1768.e9. https://doi.org/10.1016/j.cell.2024.02.016.

Corrales-Guerrero, L., Mariscal, V., Flores, E., Herrero, A., 2013. Functional dissection and evidence for intercellular transfer of the heterocyst-differentiation PatS morphogen. Mol. Microbiol. 88, 1093–1105. https://doi.org/10.1111/mmi.12244.

Cui, J., Zhang, M., Chen, L., Zhang, S., Luo, Y., Cao, W., Zhao, J., Wang, L., Jia, Z., Bao, Z., 2022. Methanotrophs contribute to nitrogen fixation in emergent macrophytes. Front. Microbiol. 13. https://doi.org/10.3389/fmicb.2022.851424.

Cui, R., Kwak, J. Il, An, Y.-J., 2023. Multigenerational effects of microplastic fragments derived from polyethylene terephthalate bottles on duckweed *Lemna minor*: size-dependent effects of microplastics on photosynthesis. Sci. Total Environ. 872, 162159. https://doi.org/10.1016/j.scitotenv.2023.162159.

Dainelli, M., Castellani, M.B., Pignattelli, S., Falsini, S., Ristori, S., Papini, A., Colzi, I., Coppi, A., Gonnelli, C., 2024. Growth, physiological parameters and DNA methylation in *Spirodela polyrhiza* (L.) Schleid exposed to PET micro-nanoplastic contaminated waters. Plant Physiol. Biochem. 207, 108403. https://doi.org/10.1016/j.plaphy.2024.108403.

Dainelli, M., Pignattelli, S., Bazihizina, N., Falsini, S., Papini, A., Baccelli, I., Mancuso, S., Coppi, A., Castellani, M.B., Colzi, I., Gonnelli, C., 2023. Can microplastics threaten plant productivity and fruit quality? Insights from Micro-Tom and Micro-PET/PVC. Sci. Total Environ. 895, 165119. https://doi.org/10.1016/j.scitotenv.2023.165119.

Daryanavard, H., Postiglione, A.E., Mühlemann, J.K., Muday, G.K., 2023. Flavonols modulate plant development, signaling, and stress responses. Curr. Opin. Plant Biol. 72, 102350. https://doi.org/10.1016/j.pbi.2023.102350.

Fu, D., Wu, H., Wang, Z., Huang, S., Zheng, Z., 2023. Effects of microplastics/nanoplastics on *Vallisneria natans* roots and sediment: size effect, enzymology, and microbial communities. Chemosphere 341, 140052. https://doi.org/10.1016/j.chemosphere.2023.140052.

Fu, Q., Lai, J., Ji, X., Luo, Z., Wu, G., Luo, X., 2022. Alterations of the rhizosphere soil microbial community composition and metabolite profiles of *Zea mays* by polyethylene-particles of different molecular weights. J. Hazard Mater. 423, 127062. https://doi.org/10.1016/j.jhazmat.2021.127062.

Gupta, D.K., Choudhary, D., Vishwakarma, A., Mudgal, M., Srivastava, A.K., Singh, A., 2023. Microplastics in freshwater environment: occurrence, analysis, impact, control measures and challenges. Int. J. Environ. Sci. Technol. 20, 6865–6896. https://doi.org/10.1007/s13762-022-04139-2.

Hu, X., Wei, X., Ling, J., Chen, J., 2021. Cobalt: an essential micronutrient for plant growth? Front. Plant Sci. 12. https://doi.org/10.3389/fpls.2021.768523.

Johnson, G.V., Mayeux, P.A., Evans, H.J., 1966. A Cobalt requirement for symbiotic growth of *Azolla filiculoides* in the absence of combined nitrogen. Plant Physiol. 41, 852–855. https://doi.org/10.1104/pp.41.5.852.

Karalija, E., Carbó, M., Coppi, A., Colzi, I., Dainelli, M., Gašparović, M., Grebenc, T., Gonnelli, C., Papadakis, V., Pilić, S., Šibanc, N., Valledor, L., Poma, A., Martinelli, F., 2022. Interplay of plastic pollution with algae and plants: hidden danger or a blessing? J. Hazard Mater. 438, 129450. https://doi.org/10.1016/j.jhazmat.2022.129450.

Kollah, B., Patra, A.K., Mohanty, S.R., 2016. Aquatic microphylla Azolla: a perspective paradigm for sustainable agriculture, environment and global climate change. Environ. Sci. Pollut. Control Ser. 23, 4358–4369. https://doi.org/10.1007/s11356-015-5857-9.

Korsa, G., Alemu, D., Ayele, A., 2024. Azolla plant production and their potential applications. International Journal of Agronomy 1–12. https://doi.org/10.1155/2024/1716440, 2024.

Lanctôt, C.M., Bednarz, V.N., Melvin, S., Jacob, H., Oberhaensli, F., Swarzenski, P.W., Ferrier-Pagès, C., Carroll, A.R., Metian, M., 2020. Physiological stress response of the scleractinian coral *Stylophora pistillata* exposed to polyethylene microplastics. Environ. Pollut. 263, 114559. https://doi.org/10.1016/j.envpol.2020.114559.

Li, C., Busquets, R., Campos, L.C., 2020. Assessment of microplastics in freshwater systems: a review. Sci. Total Environ. 707, 135578. https://doi.org/10.1016/j.scitotenv.2019.135578.

Li, C., Wang, Lifei, Ji, S., Chang, M., Wang, Longfei, Gan, Y., Liu, J., 2021. The ecology of the plastisphere: microbial composition, function, assembly, and network in the freshwater and seawater ecosystems. Water Res. 202, 117428. https://doi.org/10.1016/j.watres.2021.117428.

Liu, Y., Xu, F., Ding, L., Zhang, G., Bai, B., Han, Y., Xiao, L., Song, Y., Li, Y., Wan, S., Li, G., 2023. Microplastics reduce nitrogen uptake in peanut plants by damaging root cells and impairing soil nitrogen cycling. J. Hazard Mater. 443, 130384. https://doi.org/10.1016/j.jhazmat.2022.130384.

Maldonado, I., Moreno Terrazas, E.G., Vilca, F.Z., 2022. Application of duckweed (*Lemna* sp.) and water fern (*Azolla* sp.) in the removal of pharmaceutical residues in water: state of art focus on antibiotics. Sci. Total Environ. 838, 156565. https://doi.org/10.1016/j.scitotenv.2022.156565.

Maswada, H.F., Abd El-Razek, U.A., El-Sheshtawy, A.-N.A., Mazrou, Y.S.A., 2021. Effect of *Azolla filiculoides* on growth, physiological and yield attributes of maize grown under water and nitrogen deficiencies. J. Plant Growth Regul. 40, 558–573. https://doi.org/10.1007/s00344-020-10120-5.

Mu, X., Chen, Y., 2021. The physiological response of photosynthesis to nitrogen deficiency. Plant Physiol. Biochem. 158, 76–82. https://doi.org/10.1016/j.plaphy.2020.11.019.

My Tran, N., Thanh Hoai Ta, Q., Sreedhar, A., Noh, J.-S., 2021. Ti3C2Tx MXene playing as a strong methylene blue adsorbent in wastewater. Appl. Surf. Sci. 537, 148006. https://doi.org/10.1016/j.apsusc.2020.148006.

Ng, E.L., Lin, S.Y., Dungan, A.M., Colwell, J.M., Ede, S., Huerta Lwanga, E., Meng, K., Geissen, V., Blackall, L.L., Chen, D., 2021. Microplastic pollution alters forest soil microbiome. J. Hazard Mater. 409, 124606. https://doi.org/10.1016/j.jhazmat.2020.124606.

Nikkhah, F., Ramazanian, M.R., Rosentrater, K.A., 2024. Prioritizing the valorization strategies of an invasive fern (*Azolla*) in a wetland. Bioresour. Technol. Rep. 26, 101847. https://doi.org/10.1016/j.biteb.2024.101847.

Okubo, N., Takahashi, S., Nakano, Y., 2018. Microplastics disturb the anthozoan-algae symbiotic relationship. Mar. Pollut. Bull. 135, 83–89. https://doi.org/10.1016/j.marpolbul.2018.07.016.

Okubo, N., Tamura-Nakano, M., Watanabe, T., 2020. Experimental observation of microplastics invading the endoderm of anthozoan polyps. Mar. Environ. Res. 162, 105125. https://doi.org/10.1016/j.marenvres.2020.105125.

Padilla, F.M., Teresa Peña-Fleitas, M., Gallardo, M., Thompson, R.B., 2014. Evaluation of optical sensor measurements of canopy reflectance and of leaf flavonols and chlorophyll contents to assess crop nitrogen status of muskmelon. Eur. J. Agron. 58, 39–52. https://doi.org/10.1016/j.eja.2014.04.006.

Pereao, O., Opeolu, B., Fatoki, O., 2020. Microplastics in aquatic environment: characterization, ecotoxicological effect, implications for ecosystems and developments in South Africa. Environ. Sci. Pollut. Control Ser. 27, 22271–22291. https://doi.org/10.1007/s11356-020-08688-2.

Pereira, A.L., Carrapiço, F., 2009. Culture of *Azolla filiculoides* in artificial conditions. Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology 143, 431–434. https://doi.org/10.1080/11263500903172110.

Pignattelli, S., Broccoli, A., Piccardo, M., Terlizzi, A., Renzi, M., 2021. Effects of polyethylene terephthalate (PET) microplastics and acid rain on physiology and growth of *Lepidium sativum*. Environ. Pollut. 282, 116997. https://doi.org/10.1016/j.envpol.2021.116997.

Rejmankova, E., 2011. The role of macrophytes in wetland ecosystems. J Ecol Environ 34, 333–345. https://doi.org/10.5141/JEFB.2011.044.

Rillig, M.C., Kim, S.W., Zhu, Y.-G., 2024. The soil plastisphere. Nat. Rev. Microbiol. 22, 64–74. https://doi.org/10.1038/s41579-023-00967-2.

Sadeghi, R., Zarkami, R., Sabetraftar, K., Van Damme, P., 2013. A review of some ecological factors affecting the growth of *Azolla* spp. Caspian Journal of Environmental Sciences 11, 65–76.

Scheffer, M., 2004. Ecology of Shallow Lakes. Springer, Netherlands, Dordrecht. https://doi.org/10.1007/978-1-4020-3154-0.

Schmidt, Husted, 2019. The biochemical properties of manganese in plants. Plants 8, 381. https://doi.org/10.3390/plants8100381.

Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative CT method. Nat. Protoc. 3, 1101–1108. https://doi.org/10.1038/nprot.2008.73.

Shen, M., Song, B., Zhou, C., Almatrafi, E., Hu, T., Zeng, G., Zhang, Y., 2022. Recent advances in impacts of microplastics on nitrogen cycling in the environment: a review. Sci. Total Environ. 815, 152740. https://doi.org/10.1016/j.scitotenv.2021.152740.

Sitko, R., Turek, E., Zawisza, B., Malicka, E., Talik, E., Heimann, J., Gagor, A., Feist, B., Wrzalik, R., 2013. Adsorption of divalent metal ions from aqueous solutions using graphene oxide. Dalton Trans. 42, 5682. https://doi.org/10.1039/c3dt33097d.

Sood, A., Uniyal, P.L., Prasanna, R., Ahluwalia, A.S., 2012. Phytoremediation potential of aquatic macrophyte, *Azolla*. Ambio 41, 122–137. https://doi.org/10.1007/s13280-011-0159-z.

Stirbet, A., Govindjee, 2011. On the relation between the Kautsky effect (chlorophyll a fluorescence induction) and Photosystem II: basics and applications of the OJIP fluorescence transient. J. Photochem. Photobiol., B 104, 236–257. https://doi.org/10.1016/j.jphotobiol.2010.12.010.

Sun, X., Zhang, X., Xia, Y., Tao, R., Zhang, M., Mei, Y., Qu, M., 2022. Simulation of the effects of microplastics on the microbial community structure and nitrogen cycle of paddy soil. Sci. Total Environ. 818, 151768. https://doi.org/10.1016/j.scitotenv.2021.151768.

Sun, X.-D., Yuan, X.-Z., Jia, Y., Feng, L.-J., Zhu, F.-P., Dong, S.-S., Liu, J., Kong, X., Tian, H., Duan, J.-L., Ding, Z., Wang, S.-G., Xing, B., 2020. Differentially charged nanoplastics demonstrate distinct accumulation in *Arabidopsis thaliana*. Nat. Nanotechnol. 15, 755–760. https://doi.org/10.1038/s41565-020-0707-4.

Tang, M., Huang, Yongchuang, Zhang, W., Fu, T., Zeng, T., Huang, Yongdong, Yang, X., 2022. Effects of microplastics on the mineral elements absorption and accumulation in Hydroponic rice seedlings (*Oryza sativa* L.). Bull. Environ. Contam. Toxicol. 108, 949–955. https://doi.org/10.1007/s00128-021-03453-8.

Verma, G., Prakriti, Kumar, A., Babu, S., Singh, R., Gudade, B., Bhatt, M., Saha, S., Yadav, N.C., 2022. Implications and Future prospects of *Azolla* as a low-cost organic input in agriculture. Agriculation 2.

Vries de, S., Vries, de J., Teschke, H., Dahlen von, J.K., Rose, L.E., Gould, S.B., 2018. Jasmonic and salicylic acid response in the fern *Azolla filiculoides* and its cyanobiont. Plant Cell Environ. 41 (11), 2530–2548. https://doi.org/10.1111/pce.13131.

Wang, H., He, Y., Zheng, Q., Yang, Q., Wang, J., Zhu, J., Zhan, X., 2024a. Toxicity of photoaged polyvinyl chloride microplastics to wheat seedling roots. J. Hazard Mater. 463, 132816. https://doi.org/10.1016/j.jhazmat.2023.132816.

Wang, J., Li, J., Liu, W., Zeb, A., Wang, Q., Zheng, Z., Shi, R., Lian, Y., Liu, L., 2023a. Three typical microplastics affect the germination and growth of amaranth (*Amaranthus mangostanus* L.) seedlings. Plant Physiol. Biochem. 194, 589–599. https://doi.org/10.1016/j.plaphy.2022.12.007.

Wang, Q., Feng, X., Liu, Y., Li, W., Cui, W., Sun, Y., Zhang, S., Wang, F., Xing, B., 2023b. Response of peanut plant and soil N-fixing bacterial communities to conventional and biodegradable microplastics. J. Hazard Mater. 459, 132142. https://doi.org/10.1016/j.jhazmat.2023.132142.

Wang, W., Zhang, Z., Gao, J., Wu, H., 2024b. The impacts of microplastics on the cycling of carbon and nitrogen in terrestrial soil ecosystems: progress and prospects. Sci. Total Environ. 915, 169977. https://doi.org/10.1016/j.scitotenv.2024.169977.

Wang, X., Xing, Y., Lv, M., Zhang, T., Ya, H., Jiang, B., 2022. Recent advances on the effects of microplastics on elements cycling in the environment. Sci. Total Environ. 849, 157884. https://doi.org/10.1016/j.scitotenv.2022.157884.

Wang, Y., Bai, J., Wen, L., Wang, W., Zhang, L., Liu, Z., Liu, H., 2023c. Phytotoxicity of microplastics to the floating plant *Spirodela polyrhiza* (L.): plant functional traits and metabolomics. Environ. Pollut. 322, 121199. https://doi.org/10.1016/j.envpol.2023.121199.

Wang, Y., Zhou, B., Chen, H., Yuan, R., Wang, F., 2022a. Distribution, biological effects and biofilms of microplastics in freshwater systems - a review. Chemosphere 299, 134370. https://doi.org/10.1016/j.chemosphere.2022.134370.

Wang, Y., Cui, W., Duan, Z., Qin, L., Zhang, H., Cheng, H., Wang, L., 2022b. Investigation of microplastic pollution on paddy fields in Xiangtan City, Southern China. Environ. Sci. Pollut. Control Ser. 29, 81300–81307. https://doi.org/10.1007/s11356-022-21415-3.

Wickstrom, C.E., Corkran, J.L., 1997. Nitrogenase activities associated with macrophytes from a lacustrine and a freshwater estuarine habitat. Aquat. Bot. 59, 157–162. https://doi.org/10.1016/S0304-3770(97)00053-3.

Yi, Z., Zhang, Z., Chen, G., Rengel, Z., Sun, H., 2023. Microplastics have rice cultivar-dependent impacts on grain yield and quality, and nitrogenous gas losses from paddy, but not on soil properties. J. Hazard Mater. 446, 130672. https://doi.org/10.1016/j.jhazmat.2022.130672.

Yoon, H.-S., Golden, J.W., 2001. PatS and products of nitrogen fixation control heterocyst pattern. J. Bacteriol. 183, 2605–2613. https://doi.org/10.1128/JB.183.8.2605-2613.2001.

Yu, H., Qi, W., Cao, X., Wang, Y., Li, Y., Xu, Y., Zhang, X., Peng, J., Qu, J., 2022. Impact of microplastics on the foraging, photosynthesis and digestive systems of submerged carnivorous macrophytes under low and high nutrient concentrations. Environ. Pollut. 292, 118220. https://doi.org/10.1016/j.envpol.2021.118220.

Yu, Z., Xu, X., Guo, L., Yuzuak, S., Lu, Y., 2024. Physiological and biochemical effects of polystyrene micro/nano plastics on *Arabidopsis thaliana*. J. Hazard Mater. 469, 133861. https://doi.org/10.1016/j.jhazmat.2024.133861.

Zapomělová, E., Hrouzek, P., Řeháková, K., Šabacká, M., Stibal, M., Caisová, L., Komárková, J., Lukešová, A., 2008. Morphological variability in selected heterocystous cyanobacterial strains as a response to varied temperature, light intensity and medium composition. Folia Microbiol. 53 (4), 333–41.

Zhang, T., Luo, X.-S., Kumar, A., Liu, X., Tong, X., Yao, X., Fan, J., Chen, Z., Chaturvedi, S., 2024. Effects of micro-nano plastics on the environmental biogeochemical cycle of nitrogen: a comprehensive review. Chemosphere 357, 142079. https://doi.org/10.1016/j.chemosphere.2024.142079.

Zhang, Y., Yang, S., Zeng, Y., Chen, Y., Liu, H., Yan, X., Pu, S., 2023. A new quantitative insight: interaction of polyethylene microplastics with soil - microbiome - crop. J. Hazard Mater. 460, 132302. https://doi.org/10.1016/j.jhazmat.2023.132302.