Short Duration Exposure of 3 µm Polystyrene Microplastics Affected Morphology and Physiology of *Watermilfoil* (sp. Roraima)

Mudalige Don Hiranya Jayasanka Senavirathna (jayasanka@mail.saitama-u.ac.jp)
Saitama Daigaku https://orcid.org/0000-0002-1120-7643
Liu Zhaozhi
Saitama Daigaku
Takeshi Fujino
Saitama Daigaku

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Abstract

Microplastics are one of the most widely discussed environmental issues worldwide. Several studies have shown the effect of microplastic exposure on the marine environment; however, studies on freshwater systems are lacking. This study was conducted to investigate the effect of microplastics on hydroponically growing emergent freshwater macrophytes, *Watermilfoil* (sp. *Roraima*) under controlled environmental conditions. Plants were exposed to 0 mg L^{-1} (control), 0.05 mg L^{-1}, 0.25 mg L^{-1}, 1.25 mg L^{-1}, and 6 mg L^{-1} of 3 μm polystyrene microspheres for seven days. The oxidative stress, antioxidant response, pigmentations, Fv/Fm, and growth parameters in above-water and below-water parts were analyzed separately. Microscopic observations were performed to confirm the tissue absorbance of the microplastics. Exposure to microplastics altered some parameters; however, growth was not affected. The effect of microplastics was not linear with the exposure concentration for most of the parameters and between 1.25 mg L^{-1} and 6 mg L^{-1} concentrations. The response trends mostly followed the second-order polynomial distributions. Under the 1.25 mg L^{-1} exposure, there were significant changes in root length, H_2O_2 content, catalase activity, anthocyanin content, and Fv/Fm. There were differences in parameters between the above-water and below-water parts, and the responses of the microplastics followed different trends. Microscopic observations confirmed the attachment of microplastic particles onto newly formed roots, except for older roots or shoot tissues.

Introduction

The release of plastics into the environment is currently a serious issue worldwide. Although numerous actions have been taken to prevent its environmental risk, there are still significant plastics in the environment from already released plastics and currently releasing plastics owing to the mismanagement of plastic wastes (Thompson et al. 2009; Lebreton and Andrady 2019). Degradation of environmental plastics occurs because of ultraviolet rays (photooxidation), physical force, and biodegradation, which produce small plastic particles. The degraded plastic particles have sizes ranging from 1 μm to 5 mm and are typically called microplastics (MPs); however, there can be different definitions when considering the size of MPs (Hartmann et al. 2019). Furthermore, MPs are purposely added to consumer products such as cosmetics, shower gels, skin scrubbers, and industrial products such as powder coating and synthetic paints to improve their texture and effectiveness (Scudo et al. 2017). Regardless of source, MPs have contaminated most parts of the land and water (rivers, lakes, oceans, and groundwater) worldwide. They have recently been recognized as a significant concern for aquatic systems (Lambert and Wagner 2018). Various studies have been conducted on MPs, focusing mainly on marine and estuarine ecosystems (Shafiq et al. 2019; Bellasi et al. 2020). However, recently, the increased presence of MPs has been reported in freshwater systems worldwide, including in Japan (Wagner et al. 2014; Kataoka et al. 2019). In such environments, MPs can be considered as one of the abiotic factors in modern aquatic ecosystems.

Research on the effects of MPs on marine ecosystems has confirmed various impacts on flora and fauna, and the effects can be physiological and morphological (Rillig et al. 2019; Li et al. 2020; Yu et al. 2020). However, research focused on the effect of plastic particles on aquatic vegetation is lacking, and in freshwater vegetation, the studies are deficient (Mateos-Cárdenas et al. 2019). The effects of MP on aquatic plants have been reported on morphological features such as a change in the root system, reduced root to shoot ratio (*Myriophyllum spicatum* and *Elodia* sp.), and reduced seed germination rate (*Lepidium sativum*) (van Weert et al. 2019). These responses can result from the toxic effect of microplastics on the substrate or absorption of MPs into the plant (Dovidat et al. 2020; Lozano and Rillig 2020). Moreover, evidence revealed that some species may not exhibit morphological changes, although the MPs are attached to the plant surface in the short term, the physiological parameters were not evaluated (Mateos-Cárdenas et al. 2019). The effects of MPs can vary between species and the related plastic type (Horton et al. 2017). In a single MP type, the effect or bioaccumulation can be inversely related to particle size (Lee et al. 2013; Wagner et al. 2014). In such cases, the species sensitive to MPs would perish over time, and the resistant species will remain or new species will occupy MPs-rich ecosystems. The impacts of such occurrences are considerable on species, as it would lead to a shift or collapse of ecosystems (Davies et al. 2012; Downing et al. 2012; Horton and Barnes 2020).

The lack of research on freshwater systems and the uncertainty of impacts due to species and environmental MP variability justifies the need for further research. Therefore, the present study was conducted to determine the effects of microplastics on freshwater macrophytes. The freshwater species watermilfoil (*sp. Roraima*), an emergent macrophyte species, was selected for this study as its morphological responses can be observed in a short duration because of its high growth rate (0.9–1.5 cm day^{-1}). Furthermore, exposure of watermilfoil (*sp. Roraima*) to different concentrations of a polystyrene MP mixture consisting of various particle sizes (20–500 μm) affected the growth parameters (van Weert et al. 2019). Considering these facts, the morphological, physiochemical, pigmentation, and photosystem performance of Watermilfoil (*sp. Roraima*) response to different concentrations of smaller particle size (3 μm) polystyrene MPs was investigated under controlled laboratory conditions.

Methods

Watermilfoil *culture and preparation of cuttings*

Algal and pesticide-free watermilfoil (*sp. Roraima*) cuttings were purchased from a local vendor (Saitama City, Japan) and cultured in glass aquariums (45 × 30 × 25 cm) containing nutrient-washed river sand as the substrate. The nutrient was provided using 5 mg L^{-1} of a commercial nutrient solution (Hyponex concentrated nutrient solution, Hyponex, Osaka, Japan). Cultures were kept in a temperature-controlled room (25 ± 2°C), and light intensity was maintained at 90–100 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR) intensity provided by full spectrum LED straight lights (Model LT-NLD85L-HN; OHM Electric Inc., Japan). The light period was maintained at 12 h light/12 h dark. The culture was maintained

until the well-grown plants emerged. The emerged plants were cut to approximately 10 cm for each experiment.

Plant cuttings of ~ 10 cm in length were planted in 15 cubical (17 × 17 × 17 cm) plastic transparent aquariums (Tetra PL-17KB, Tetra, Japan). In each tank, six plant cuttings were planted inside the tanks by attaching them to holes created on foam rubber cushion strips (rubber cushion; Carboy Inc., Chiba, Japan), which were fixed at the bottom of the tanks (Fig. 1). Nutrients for each tank were provided with 2 L (7 cm water depth) of 10% Hoagland solution. Approximately 3 cm of the cutting emerged from the water once the nutrient was filled. The cuttings were kept for 3 days under 90–100 µmol m^{-2} s^{-1} PAR provided using straight LED lights and 25 ± 2°C for acclimatization and root initiation.

Microplastic treatment

Microplastic concentrations, 0 mg L^{-1} (control), 0.05 mg L^{-1}, 0.25 mg L^{-1}, 1.25 mg L^{-1}, and 6 mg L^{-1}, (supplementary material 1) were introduced to the plants upon completing 3 days of acclimation. Colorless polystyrene microspheres of 3.0 µm diameter (1.05 g mL^{-1} density, 2.6 % in aqueous solution; catalog number 17134-15, Polyscience, PA, USA) were added in required amounts to reach the treatment concentrations. Treatments were performed in triplicate. Microplastic treatment was conducted for 7 days.

Shoot elongations and root lengths

The initial shoot lengths were measured to the nearest millimeter after acclimatization. Shoot lengths were measured from the foam rubber cushion strip to the top of the plant. Shoot elongation after the treatment period was measured using the same procedure. Root length was measured only after treatment. Five randomly selected roots from three randomly selected plants from each treatment were measured to the nearest millimeter.

Sample collection

After the treatments, five plants were harvested, separating the above-water and below-water parts. After the harvest, plants were kept in resealable polybags after removing water from the surface using blotting paper and stored at -80°C until further analysis. The remaining plants were subjected to 30 min of dark adaptation and subjected to chlorophyll fluorescence (ChF) measurement and photosynthetic pigment extraction.

Chlorophyll fluorescence measurement

Chlorophyll fluorescence parameters of the plants were measured using the ChF imaging technique (Handy FluorCam – FC 1000-H, Photon Systems Technology, Brno, Czech Republic). Dark-adapted plants were subjected to ChF measurement. Plants were placed on a thick folded tissue soaked with 10% Hoagland solution and placed under FluorCam to prevent any drying effect on plants during the measurement. The maximum quantum efficiency of photosystem II (Fv/Fm) was quantified using

dedicated chlorophyll fluorescence image analysis software provided with FluorCam (FluorCam 7, Photon Systems Technology).

Plant pigments quantification

The photosynthetic pigment content was estimated by extracting pigments from N, N-dimethylformamide. Pigments of approximately 100 mg of samples collected from the plants were extracted with 5 mL of N, N-dimethylformamide by incubating for 24 h in the dark at room temperature (25–27°C). Pigments were extracted separately from the above-water and below-water parts. The optical absorptions of the extracted pigments were measured at 664, 647, and 480 nm using a spectrophotometer (UV-1280, Shimadzu, Kyoto, Japan). The chlorophyll a (Chl a), chlorophyll b (Chl b), and total carotenoid (Car) content (mg per gram of plant weight) were calculated using the equation provided by Wellburn (1994).

Anthocyanin content was measured according to the method described by Nakata and Ohme-Takagi (2014) with modifications. Approximately 50 mg of plant samples were collected, pulverized in liquid nitrogen, and mixed with 2 mL of extraction buffer containing 45% (v/v) methanol and 5% (v/v) acetic acid in distilled water. The mixture was centrifuged at 2,000g for 15 min at 20°C, and the supernatant was collected. Pigments were extracted separately from the above-water and below-water parts. The optical absorption of the supernatant at 637 and 530 nm was measured spectrophotometrically (UV-1280, Shimadzu, Kyoto, Japan). Anthocyanin content was calculated by taking one anthocyanin unit equivalent to one absorbance unit in 1 mL of extraction solution (Teng et al. 2005).

Hydrogen peroxide and antioxidants quantification

Plant enzymes were extracted using phosphate buffer. Approximately 200 mg of each sample was crushed in liquid nitrogen in the presence of polyvinylpyrrolidone, and the extraction was performed by adding 5 mL of 0.05 M phosphate buffer (pH 6.0) to the crushed samples. The extraction mixture was centrifuged at 2,000g for 10 min at 4°C, and the supernatant was collected for analysis.

The hydrogen peroxide content was measured by mixing 750 µL of the enzyme extract with 2.5 mL of 0.1% (w/v) titanium sulfate in 20% (v/v) H_2SO_4 as per a previously described method (Satterfield and Bonnell 1955). Thereafter, the mixture was incubated at room temperature (25–27°C) for 30 min. The optical absorption of the assay mixture was measured spectrophotometrically at 410 nm (UV-1280). The hydrogen peroxide concentration in the samples was estimated using a standard curve for H_2O_2 and expressed in µmol g^{-1} Fresh weight (FW).

Guaiacol peroxidase (GPX) activity was measured by mixing 100 µL of enzyme extract with 40 µL of 30 mM H_2O_2 and 50 µL of 25 mM guaiacol as per a previously described method (MacAdam et al. 1992). The guaiacol reaction mixture was added to the cuvette, and the reaction was initiated by the addition of enzyme extract. The change in optical absorbance at 420 nm was recorded spectrophotometrically (UV-1280) every 10 s for 3 min. Based on the rate of absorbance increase, GPX activity was expressed as µmol min^{-1} g^{-1} FW using an extinction coefficient of 26.6 mM^{-1} cm^{-1}.

Catalase activity (CAT) was measured by reacting 500 µL of enzyme extract with a reaction mixture containing 100 µL of 10 mM H_2O_2 in 2 mL of pH 7.0, 100 mM potassium phosphate buffer (Aebi 1984). The reaction was initiated by the addition of the enzyme extract to the reaction medium inside the cuvette. The change in optical absorbance at 240 nm was spectrophotometrically recorded (UV-1280) every 10 s for 3 min. CAT activity was expressed as µmol min^{-1} g^{-1} FW, using an extinction coefficient of 40 mM^{-1} cm^{-1}.

Ascorbate peroxidase (APX) activity was measured according to the method described by Nakano and Asada (1981). The reaction mixture contained 100 µL of enzyme extract, 200 µL of 0.5 mM ascorbic acid in pH 7.0, 50 mM potassium phosphate buffer, and 2 mL of 50 mM potassium phosphate buffer. The reaction was initiated by the addition of 60 µL of 1 mM H_2O_2 to the cuvette. The change in optical absorbance at 290 nm was recorded every 10 s for 3 min. Based on the rate of absorbance decrease, APX activity was expressed in µmol min^{-1} g^{-1} FW using an extinction coefficient of 2.8 mM^{-1} cm^{-1}.

Microscopic observations

Plant shoots and roots were microscopically observed for the surface attachment and absorption of MPs. Shoot cross-sections were prepared using a plant microtome (MTH-1, Nippon Medical and Chemical Instruments, Osaka, Japan), and glass slides were prepared. Cross-sections were observed using a digital imaging optical microscope (Zeiss Axiolab 5, Carl Zeiss, ZEISS, Japan). Roots were observed by placing wetted root cuttings directly on glass slides. Roots were cleaned before the observations to remove surface-attached MPs by carefully brushing in a 10% Hoagland solution using a brush containing smooth bristles. Images were captured using specialized software provided with a microscope system (ZEN imaging software 3.0, Carl Zeiss, ZEISS, Japan).

Data analysis and statistical methods

Differences between treatments were tested using one-way analysis of variance (ANOVA) with the post-hoc Duncan's test, with $P < 0.05$, considered significant. Differences between the above- and below-water parts were compared when required using paired sample *t*-tests, considering $P < 0.05$. Statistical analyses were performed using IBM SPSS Statistics version 25 (IBM, Armonk, NY, USA). The antioxidant activities were related to the 0 mg L^{-1} treatment condition by dividing the rest of the treatment condition values by the relevant 0 mg L^{-1} condition values. Descriptive statistics and data visualization were performed using Microsoft Excel 2001 (Microsoft, Washington, USA).

Results

The visual observations did not show any differences in plants (Fig. 1). Microscopic observation of roots confirmed that MPs were absorbed into the roots and the number of MP particles observed inside the roots increased with increasing MP concentration (Fig. 2); however, the stem cross-sections observed did not confirm the existence of MPs in tissues. Shoot lengths showed an increasing trend with the exposure MP concentration; however, the ANOVA test was not grouped (ANOVA $P > 0.05$, F = 2.880; Fig. 3). However,

there were significant differences between the roots. Lengths of the roots were the highest with 1.25 mg L^{-1} MP exposure, whereas 6 mg L^{-1} exposure also showed longer roots than with 0, 0.05, and 0.25 mg L^{-1} MP exposure conditions. The ANOVA testing grouped 0, 0.05, and 0.25 mg L^{-1}; 0, 0.05, and 6 mg L^{-1}; and 1.25 mg L^{-1}MP exposure conditions (ANOVA $P < 0.01$, F = 11.723).

The Fv/Fm was reduced significantly with 1.25 mg L^{-1} MP exposure compared to the rest of the exposure conditions in which the Fv/Fm values were unchanged. The ANOVA test grouped 1.25 mg L^{-1}; and 0, 0.05, 0.25, and 6 mg L^{-1} MP concentrations (ANOVA $P < 0.01$, F = 60.583; Fig. 4).

The Chl-a values in the above- and below-water parts showed different trends with increasing MP concentrations. Chl-a, Chl-b, and Car increased with increasing MP concentration, whereas the Chl a/b ratio decreased. Below-water parts showed inconsistent Chl-a, Chl-b, Car, and Chl a/b ratio trends with increasing MP concentration (Fig. 5). The Chl-a, Chl-b, Car, and Chl a/b ratios of the above-water parts were statistically insignificant between treatment groups (ANOVA $P > 0.05$; F = 0.790, 1.548, 2.328, and 1.657 for Chl-a, Chl-b, Car, and Chl a/b ratios, respectively). The below-water parts showed significant differences, except for Chl-a (Chl-a ANOVA, $P > 0.05$, F = 0.780). The ANOVA test for Chl-b grouped 0, 0.25, and 6 mg L^{-1}; 0.25, 1.25, and 6 mg L^{-1}; and 1.25 and 6 mg L^{-1} MP exposure conditions (ANOVA $P < 0.01$, F = 9.444). The Car was grouped as 0 and 0.25 mg L-1; 0.5 and 1.25 mg L^{-1}; and 0.5 and 6 mg L^{-1} MP exposure conditions (ANOVA $P < 0.01$, F = 11.599). The Chl a/b ratios were grouped 0.5 and 1.25 mg L^{-1}; 0.25 and 6 mg L^{-1}; and 0 and 0.25 mg L^{-1} MP exposure conditions (ANOVA $P < 0.01$, F = 21.959).

The anthocyanin content was significantly higher in below-water parts than in the above-water parts under every treatment condition including 0 mg L^{-1} MP exposure (*t*-test $P < 0.01$ for 0, 0.05, 0.25 and 1.25 mg L^{-1} and $P < 0.05$ for 6 mg L^{-1}). The 1.25 mg L^{-1} MP-exposed above-water parts had a higher anthocyanin content than the rest of the exposure conditions. Below-water parts showed decreasing anthocyanin content till 0.25 mg L^{-1} MP concentration and increased till 6 mg L^{-1} MP concentration (Fig. 6). The ANOVA test grouped 1.25 mg L^{-1} and 0, 0.05, 0.25, and 6 mg L^{-1} of above-water parts (ANOVA $P < 0.01$; F = 8.846). The anthocyanin contents of below-water parts were not different (ANOVA, $P > 0.05$, F = 0.828).

The cellular H_2O_2 content varied under different MP exposure conditions, although it did not follow a trend related to the MP concentration. Under the 1.25 mg L^{-1} MP exposure condition, both above- and below-water parts showed the highest H_2O_2 content (Fig. 7). The below-water parts of 1.25 mg L^{-1} MP-exposed plants had higher H_2O_2 content than the respective above-water portions (*t*-test $P < 0.01$ for 0, 0.05, 0.25 and 6 mg L^{-1} MP; and $P < 0.05$ for 1.25 mg L^{-1} MP). The ANOVA test grouped the below-water parts as 0, 0.05, and 6 mg L^{-1}; and 0, 0.25, 1.25, and 6 mg L^{-1} MP treatments (ANOVA $P < 0.05$, F = 3.482) and above-water parts as 0, 0.05, and 0.25 mg L^{-1}; 0.05, 0.25, and 6 mg L^{-1}; and 1.25 and 6 mg L^{-1} MP treatments (ANOVA $P < 0.05$, F = 5.616).

The GPX activity of both the above- and below-water parts showed the same trend, increasing until the maximum GPX was reached at 0.25 mg L^{-1} MP concentration and recording lower activity at 1.25 and 6 mg L^{-1} MP concentrations (Fig. 8a). The GPX activities of 0.05 and 0.25 mg L^{-1} MP concentrations of the above-water parts were significantly higher than the respective below-water parts (*t*-test $P < 0.01$). The ANOVA test of above-water parts grouped 0 and 1.25 mg L^{-1}; 1.25 and 6 mg L^{-1}; and 0.05, 6, and 0.25 mg L^{-1} (ANOVA $P < 0.01$, F = 18.748), whereas below-water parts did not show differences (ANOVA $P > 0.05$, F = 3.250).

The CAT activity showed an increasing trend with increasing MP concentration, peaking at 1.25 mg L^{-1} in both above- and below-water parts. The CAT activity was then reduced close to the level of that with the 0.05 mg L^{-1} concentration (Fig. 8b). There were no significant differences between the above- and below-water parts for any of the MP concentrations. The ANOVA test for above-water parts grouped 0, 0.05, and 6 mg L^{-1}; 0.25 and 6 mg L^{-1}; and 0.25 and 1.25 mg L^{-1} MP exposure conditions (ANOVA $P < 0.01$, F = 9.020) and below-water parts were grouped as 0, 0.05 and 6 mg L^{-1}; 0.25 mg L^{-1}; and 1.25 mg L^{-1} (ANOVA $P < 0.01$, F = 51.218).

The APX activities of the above and below water parts showed different trends with increasing MP concentrations. The APX activity of 0.05 and 0.25 mg L^{-1} MP exposure conditions showed significantly higher activity in below-water parts than above-water parts (*t*-test $P < 0.01$). The above-water parts did not show a change in APX activity under 0.05 and 0.25 mg L^{-1} MP exposure from that at 0 mg L^{-1} exposure and then increased under 1.25 and 6 mg L^{-1} MP exposure conditions (Fig. 8c). The below-water parts showed a higher APX activity than at the 0 mg L^{-1} MP exposure at every MP concentration. The ANOVA testing grouped 0, 0.05, and 0.25 mg L^{-1}; 1.25 mg L^{-1}; and 6 mg L^{-1} MP exposure conditions of above-water parts (ANOVA $P < 0.01$, F = 27.644) and 0 mg L^{-1}; and 0.05, 0.25, 1.25, and 6 mg L^{-1} MP exposure conditions of below-water parts (ANOVA $P < 0.01$, F = 18.579).

Relationships and Correlations

The regression relationships of CAT, APX, anthocyanin, Chl-a, Chl-b, Chl a/b, Car, and Fv/Fm of the above-water parts with the MP concentrations could be explained by second-order polynomial distributions, whereas the elongation relationship was linear. The R^2 values of the relationships were 0.8213, 0.9591, 0.9644, 0.9161, 0.9868, 0.9768, 0.8905, 0.9692, and 0.8908 for CAT, APX, anthocyanin, Chl-a, Chl-b, Chl a/b, Car, Fv/Fm, and elongation, respectively. The below-water part parameters, H_2O_2, CAT, and root length relationships with MP concentrations could be explained using second-order polynomial distributions. The R^2 values for H_2O_2, CAT, and root lengths were 0.8909, 0.9556, and 0.9291, respectively (Table 1). When the 6 mg L^{-1} MP exposure condition was omitted, the relationships could be explained by linear distributions. The CAT, APX, anthocyanin, Chl-a, Chl-b, Chl a/b, Car, and Fv/Fm linear regression relationship R^2 values were 0.7902, 0.9665, 0.9742, 0.8349, 0.9641, 0.9352, 0.7608, and 0.9808,

respectively. The R^2 values of the linear relationships of H_2O_2, CAT, and root lengths were 0.8806, 0.9321, and 0.9442, respectively (Supplementary data 1).

The linear correlations between H_2O_2 and the remaining parameters were tested. The water CAT, anthocyanin, and Fv/Fm showed a strong correlation with the H_2O_2 content, with R values of 0.7894, 0.7304, and 0.7419, respectively. The APX, Chl-a, Car, and root lengths showed moderate relationships with R values of 0.5682, 0.5159, 0.5464, and 0.6404, respectively. The H_2O_2 content in below-water parts was strongly correlated with CAT, APX, Fv/Fm, and root length, and the R values were 0.8435, 0.7272, 0.8590, and 0.8893, respectively.

Discussion

The MPs were absorbed into the roots of plants under every MP concentration condition, and the number of MPs observed in roots increased with increasing MP mg L^{-1} in water (Fig. 2). Increased availability increases the chances of absorption into the roots. MPs were found only in the root tissues and not in the shoots. The reason could be that only the growing parts absorbed the MP particles. Furthermore, translocation of MPs through the vascular system was not observed in stem cross-sections or surfaces. The size of the particles (3 μm in diameter) in the present study was too large to be absorbed into cells and translocated through the vascular system. Although the roots of the cuttings were already initiated when the treatments were initiated (after 3 days of acclimatization, roots grew throughout the treatment period (Fig. 1). This facilitated the absorption of MPs into the growing root tissues. In addition, the below-water portion of the cuttings had already matured; therefore, MPs were not absorbed. In this study, the tips of the cuttings, which are the growing parts, were kept emerged from the water and were not exposed to MPs; therefore, the absorption of MPs to growing shoots could not be observed.

A slight increase in elongation of plants exposed to 6 mg L^{-1} MP was observed; however, this may be because the short experimental period did not permit a significant change. However, the roots showed a significant difference in length under the 1.25 mg L^{-1} MPs compared to that with other exposure conditions, although the 6 mg L^{-1} MP exposure also showed relatively high root lengths. The 1.25 mg L^{-1} exposure showed a significant difference in other parameters, in which H_2O_2 of below-water parts, anthocyanin of above-water parts, and CAT of below-water parts were significantly increased, and Fv/Fm was significantly decreased. These observations showed that the responses did not linearly follow the MP concentrations. A similar phenomena in which the biomass accumulations did not follow the concentration of MPs for biomass accumulation in the terrestrial plant *Phaseolus vulgaris* (0–2.5% range with 0.5% concentration differences) was reported by Meng et al. (2021). In the present study, the concentration of MPs showed a 5-fold difference between two concentrations, which can be considered different exposure regimes rather than a concentration gradient; therefore, plants may have reached distinct physiological statuses (Senavirathna et al. 2020).

The antioxidant system of plants manages oxidative stress within a non-damaging level, and when the stress level exceeds the threshold, plants will be subjected to oxidative stress (Sachdev et al. 2021). Therefore, antioxidant responses should be appropriately elevated to defend against oxidative stress. The relationship analysis showed that the CAT and APX activities were proportional to the H_2O_2 content and played a primary antioxidant role in the plant. Fv/Fm is an indicator of overall stress on plants, and an Fv/Fm over 0.8 is considered an unstressed status for most plants (Murchie and Lawson 2013; Jägerbrand and Kudo 2016). In this study, an Fv/Fm of below 0.8 was recorded only under the 1.25 mg L^{-1} MP exposure, where H_2O_2 was the highest and CAT and APX activities were also recorded to be the highest. Furthermore, the anthocyanin content, which is elevated under oxidative stress and plays an antioxidant role (Senavirathna et al. 2020), was the highest in the water parts exposed to 1.25 mg L^{-1} MP. These observations suggest that at 1.25 mg L^{-1} MP exposure, although the plant antioxidant capacity was elevated, plants were experiencing some level of stress. The increased root length with the 1.25 mg L^{-1} MP exposure is also a sign of altered physiology of plants. The increased root lengths of *Allium fistulosum* under the influence of MPs was also reported by De Souza Machado et al. (2019).

The measured parameters can be mostly explained using second-order polynomial trends alone with the MP concentrations. However, the responses were mostly distinct at 6 mg L^{-1} compared to those with the rest of the concentrations. This phenomenon is expected because of the broad difference between treatment MP concentrations. This is more convincing when 6 mg L^{-1} is omitted from the relationship analysis, and the relationships become linear. It can be considered that the MPs effect is curving or taking a different trend at a threshold concentration between 1.25 mg L^{-1} and 6 mg L^{-1}. Therefore, the response of watermilfoil with a lower concentration of MPs is different from that at higher concentrations. Under the trifold difference between MP concentrations, *M. spicatum* and *Egeria dense* species exposed to high MP concentrations also showed nonlinear responses with MP concentrations (van Weert et al. 2019). In addition, lettuce exposed to different percentages of PVC particles showed a nonlinear response of pant parameters with particle concentrations (Li et al. 2020).

The above- and below-water parts exhibited different responses to MP exposure. The above-water parts were not directly exposed to MPs, and responses in the above-water portions were triggered by the exposure of the below-water parts to MPs. Different responses in H_2O_2 content, photosynthetic pigments, and anthocyanin content between the above- and below-water parts of plants exposed to different salinity conditions were also observed in another study (unpublished data). Therefore, the above-water-below-water differential responses are a common characteristic of the tested watermilfoil species (sp. *Roraima*). Considering the levels of photosynthetic pigments and anthocyanin, the parameters of the above-water parts were well determined by the MP concentration compared to those of the below-water parts. Such responses are expected because the above-water parts contain growing young tissues, and the below-water parts contain mature tissues. Further, it can be suggested that any effect can be reflected differently in growing tissues compared to that in mature tissues (Cechin et al. 2010; Liu et al. 2019).

Conclusion And Further Directions

This study confirmed the effect of MPs on the growth and physicochemical parameters of watermilfoil. An MP concentration beyond 1.25 mg L^{-1} exhibited significant changes in the parameters; however, the broad MP concentrations selected in this study should be further analyzed to understand the non-linear response between 1.25 mg L^{-1} and 6 mg L^{-1} concentrations. The growing parts of the plants (roots and above-water parts) showed the greatest effect from MP exposure. The solid attachment of microplastics was found only with growing roots and not on mature shoots, indicating that the MPs were absorbed into young growing tissues. However, because the growing shoots of the present experiment were kept above water, further study is required to distinguish whether both growing roots and shoots were affected or only the roots were affected. Furthermore, the present experimental setup did not mix the water, and the water was stagnant; therefore, although the density of MP particles was 1.05 g mL^{-1}, these particles may have slowly reached the bottom of the tanks, allowing only roots to be exposed. This should be focused on in further studies by allowing water column mixing using an air-bubbling-like technique. Evidence shows that the type, size, and shape of MPs affects plants differently (De Souza Machado et al. 2019; Rillig et al. 2019; Li et al. 2020; Meng et al. 2021). This study was conducted with 3 μm diameter, spherical, polystyrene MP particles; therefore, further studies are needed with different sizes, types, and shapes of MPs. Different macrophyte species respond differently to the same stress exposure (Nayek et al. 2010; Senavirathna et al. 2020). Further studies regarding submerged macrophyte species under the same conditions will be conducted to compare species and compare submerged and emerged types.

Declarations

Ethical Approval and consent to participate

Not applicable

Consent for publication

All authors participated and approved the final manuscript to be published.

Availability of data and materials

All data generated or analyzed during this study are included in this published article [and its supplementary information files]

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

MDHJS conceived and designed the experiments; MDHJ and LZ performed experiments and analyzed data; MDHJS acquire the funding; MDHJS and TF contributed to the resources; MDHJS wrote the first draft manuscript; MDHJS, LZ and TF reviewed the manuscript.

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Authors' information (optional)

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Tables

Table 1: The 3µm diameter microplastics mg/L and number of particles per liter

mg/L	Particles/L
0	0
0.05	1.66×10^6
0.25	8.32×10^6
1.25	4.16×10^7
6	2.08×10^8

Figures

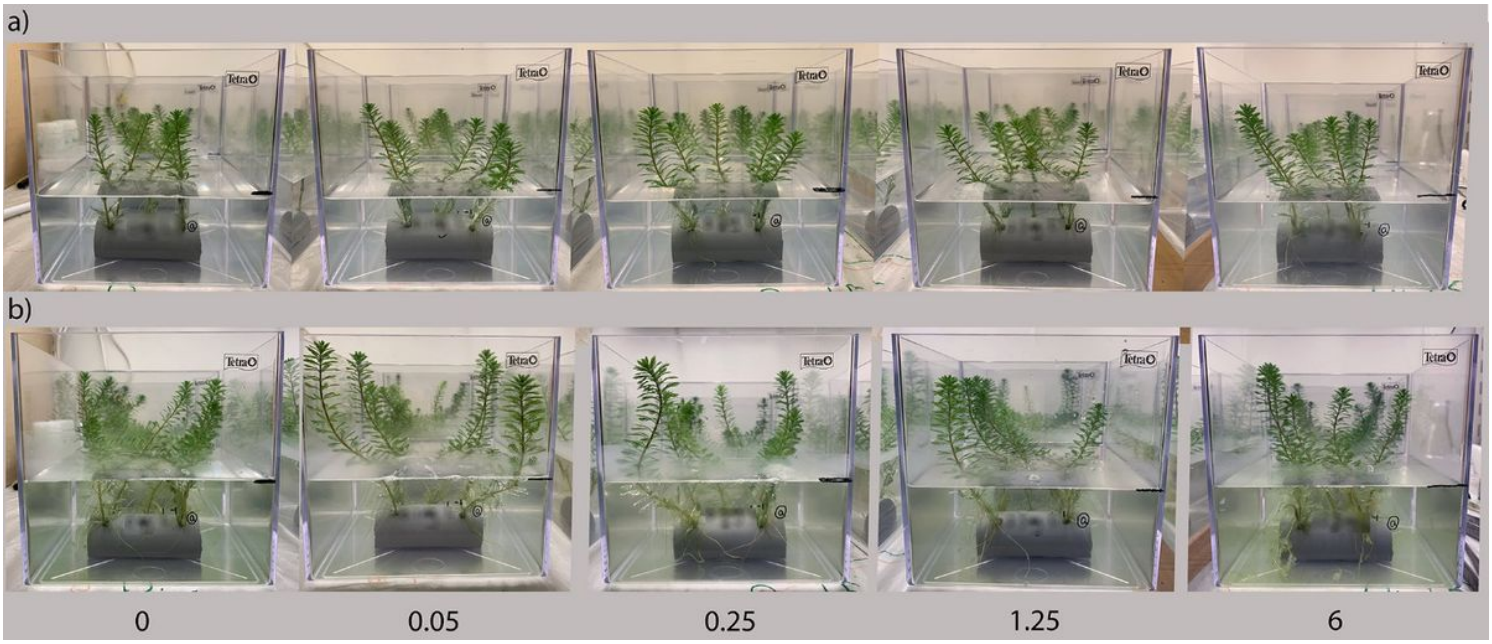

Figure 1

Representative image of Watermilfoil (sp. Roraima) (a) before exposure to microplastics and (b) after 7 days exposure to microplastics. The labels 0, 0.05, 0.25, 1.25, and 6 represent the microplastic content in the substrate in mg L-1

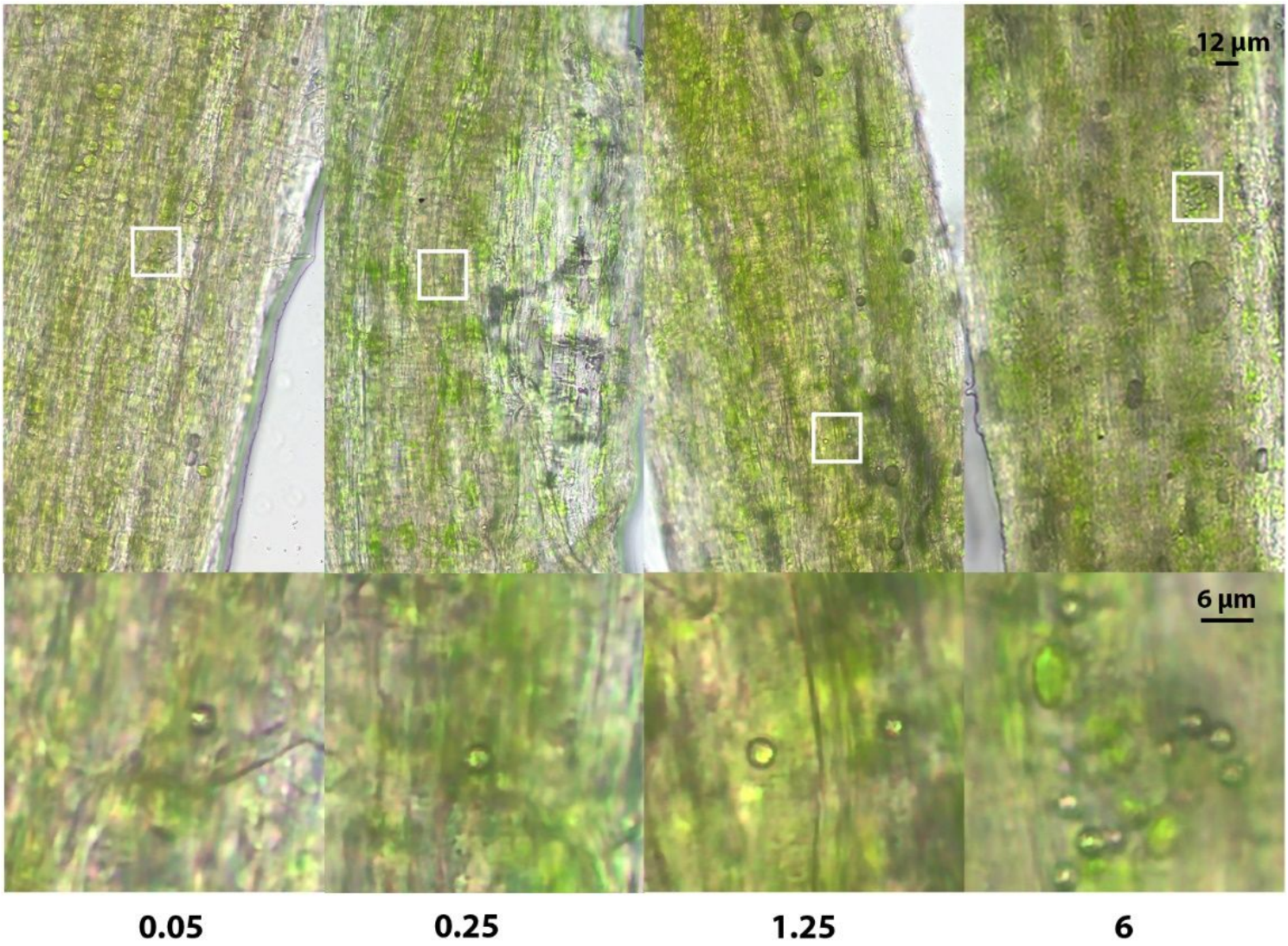

Figure 2

Representative microscopic images of Watermilfoil (sp. Roraima) roots after exposure to different microplastic concentrations. The areas are demarcated by white squares magnified below to the respective image. The labels 0, 0.05, 0.25, 1.25, and 6 represent the microplastic contents in mg L-1

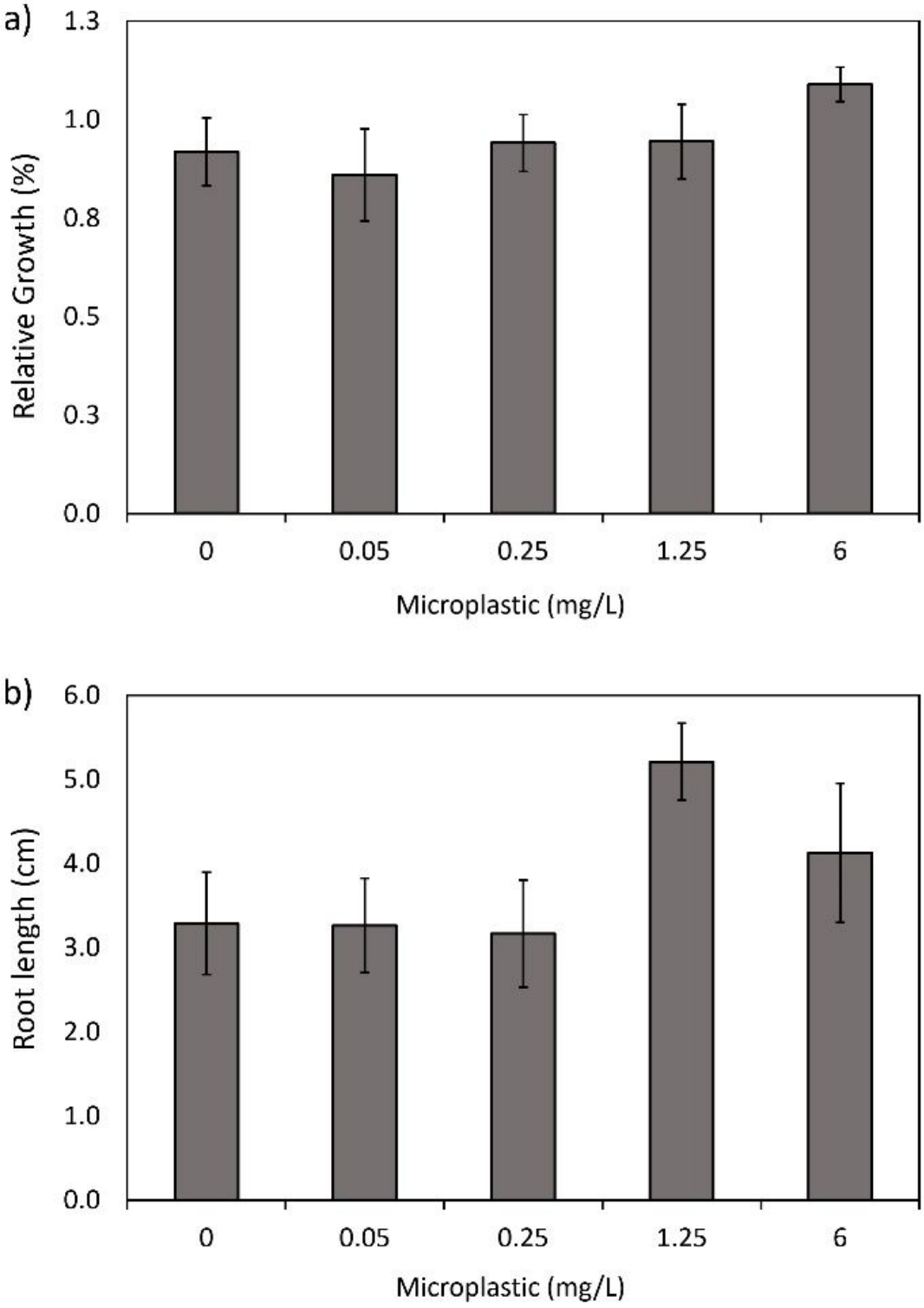

Figure 3

Maximum quantum yield of photosystem II of above water parts of Watermilfoil (sp. Roraima) after exposure to different microplastic concentrations for 7 days. The error bars represent standard deviations

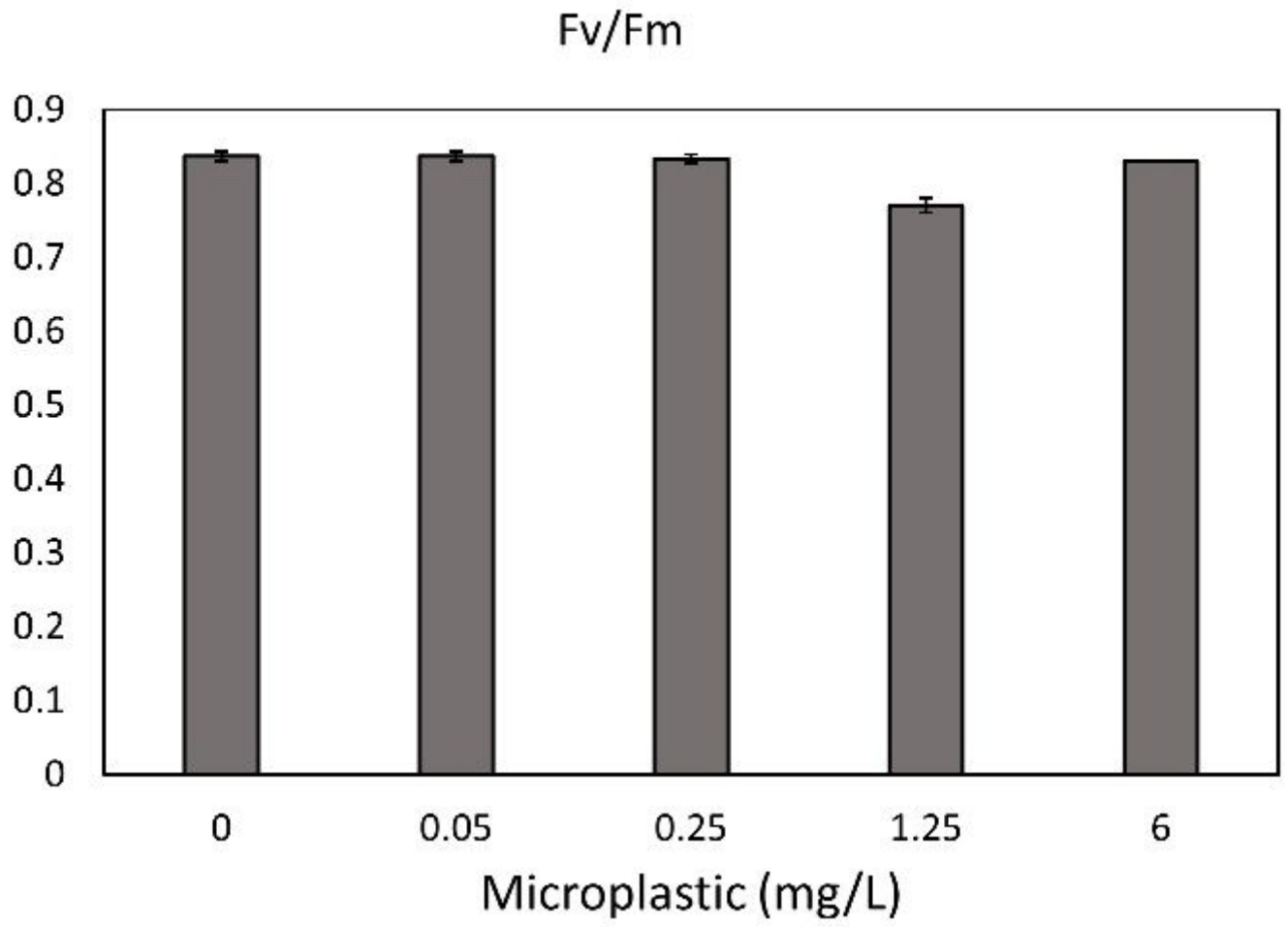

Figure 4

Shoot elongation percentages (a) and root lengths (b) of Watermilfoil (sp. Roraima) after exposure to different microplastic concentrations for 7 days. The labels 0, 0.05, 0.25, 1.25, and 6 represent the microplastic contents in mg L-1. The error bars represent standard deviations

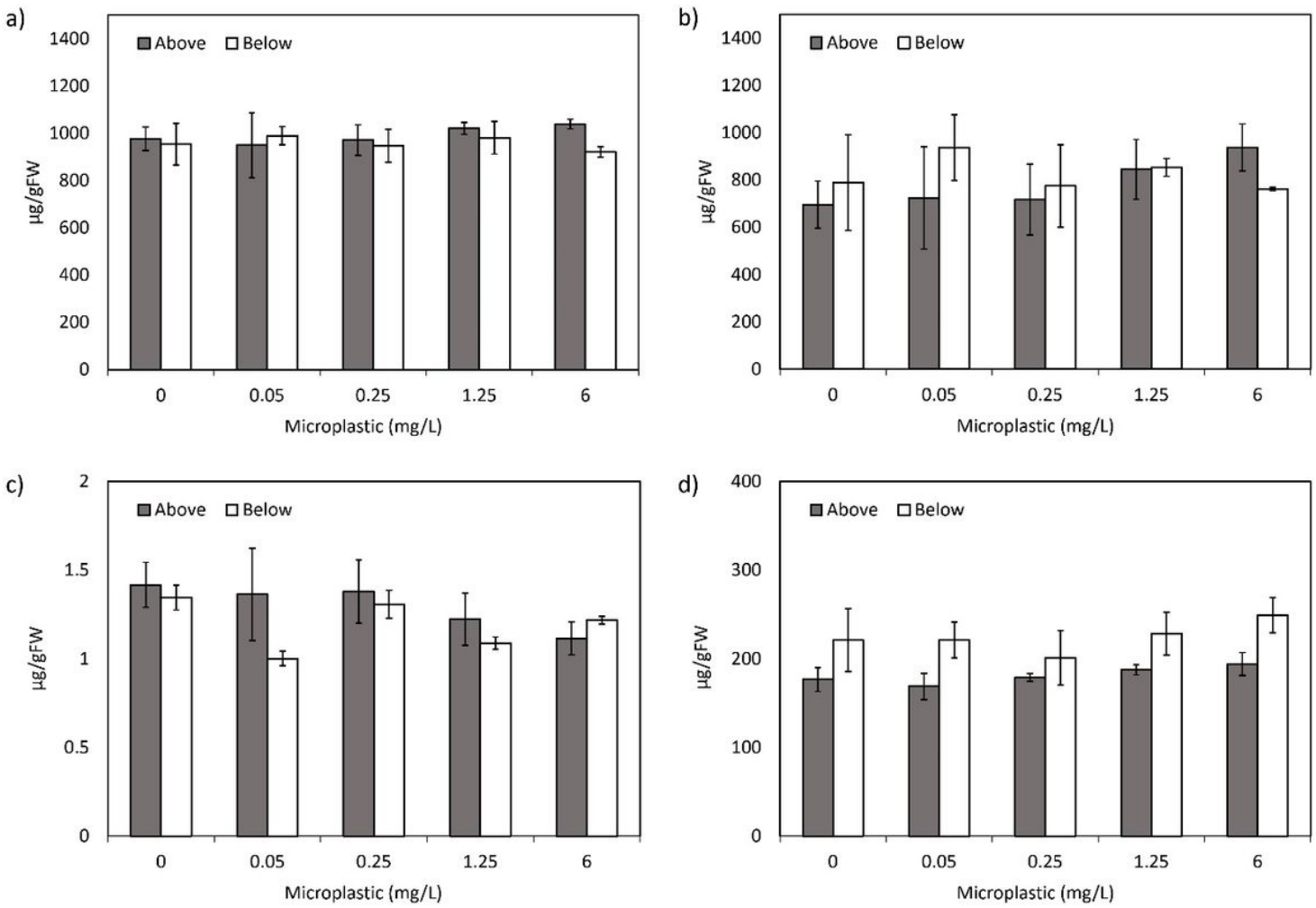

Figure 5

Chlorophyll content (a), chlorophyll b content (b), chlorophyll a/b ratio (g), and carotenoids content (d) of above water and below water parts of Watermilfoil (sp. Roraima) after exposure to different microplastic concentrations for 7 days. The error bars represent standard deviations

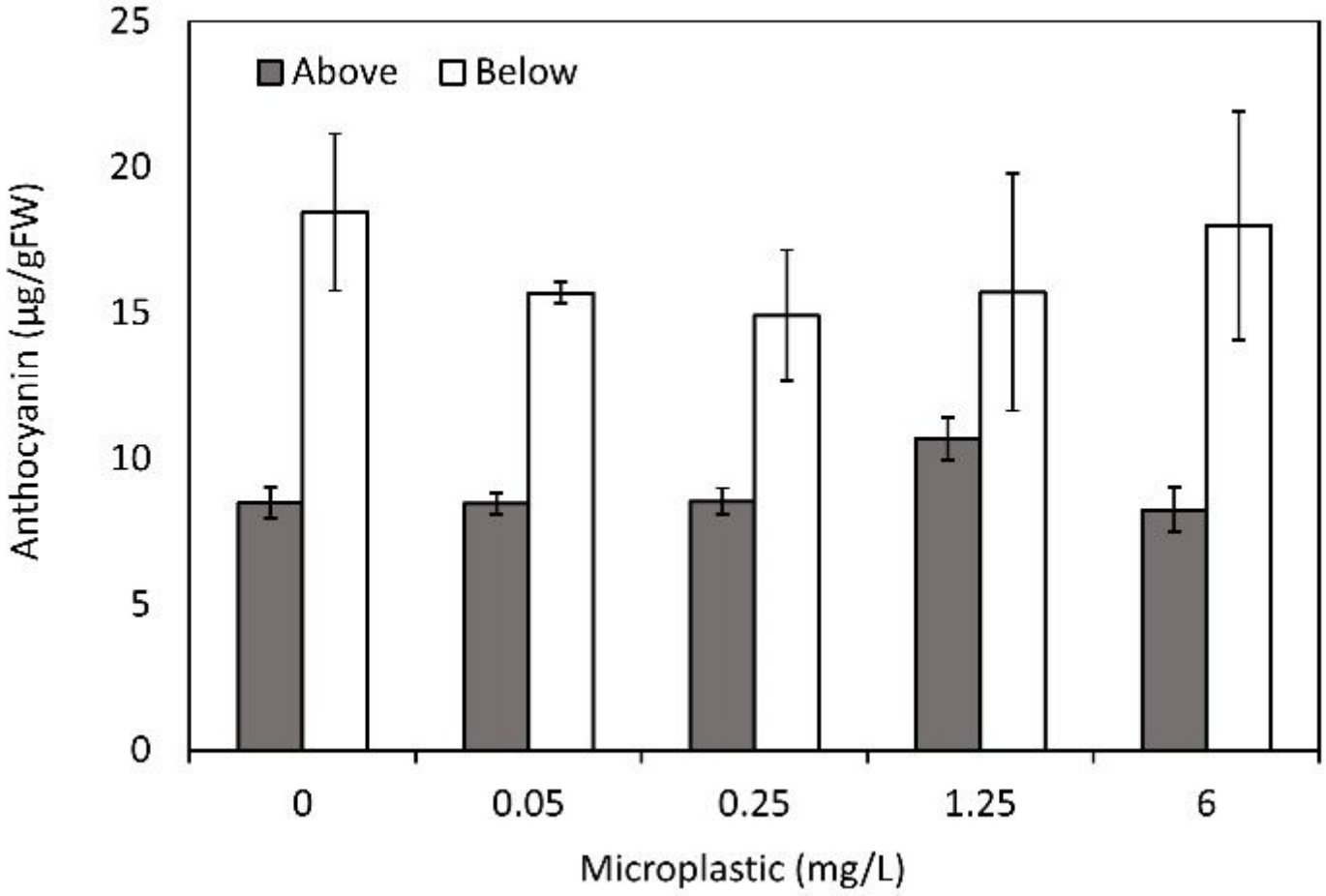

Figure 6

Anthocyanin content of above-water and below-water parts of Watermilfoil (sp. Roraima) after exposure to different microplastic concentrations for 7 days. The error bars represent standard deviations

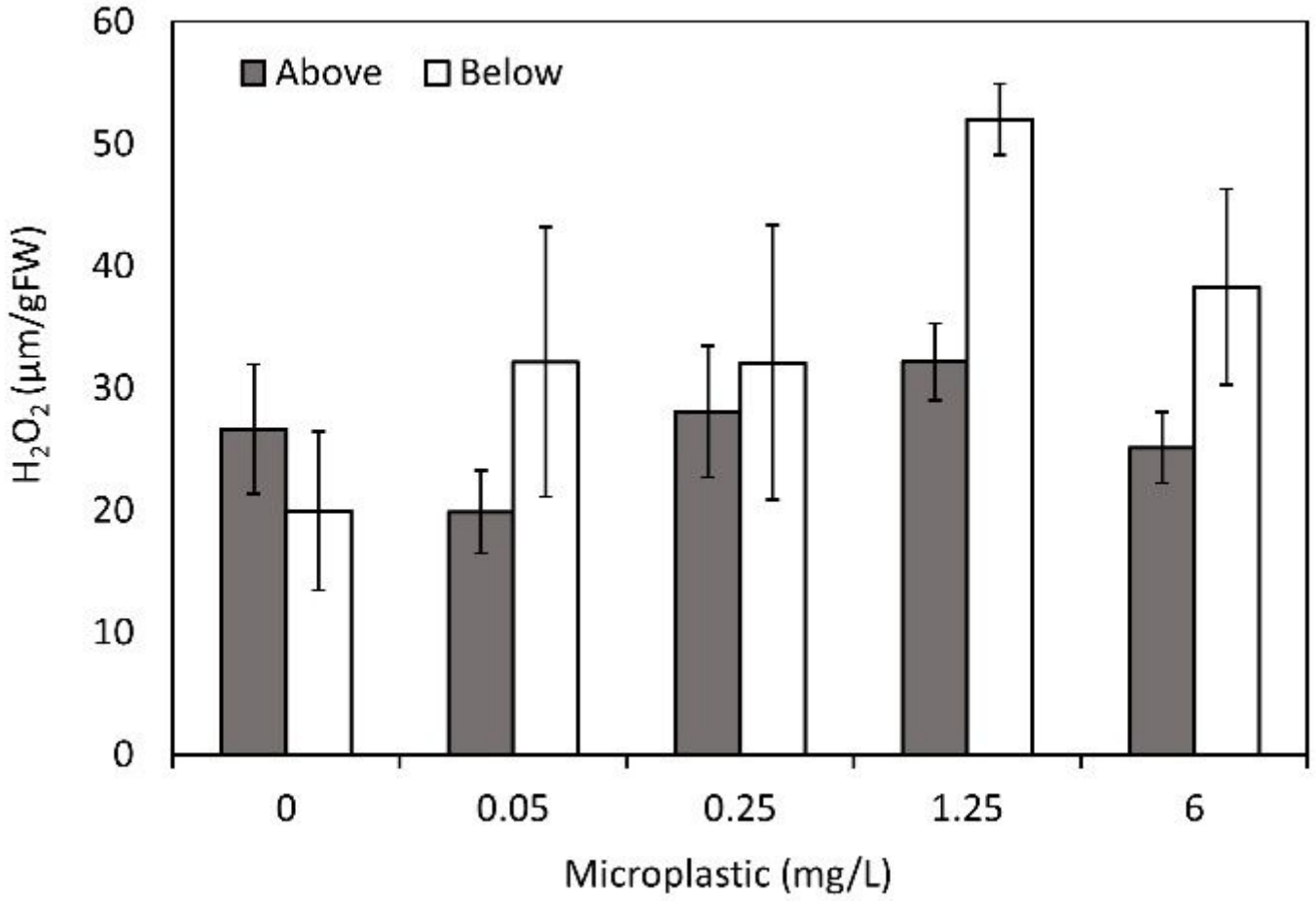

Figure 7

H2O2 content of above-water and below-water parts of Watermilfoil (sp. Roraima) after exposure to different microplastic concentrations for 7 days. The error bars represent standard deviations

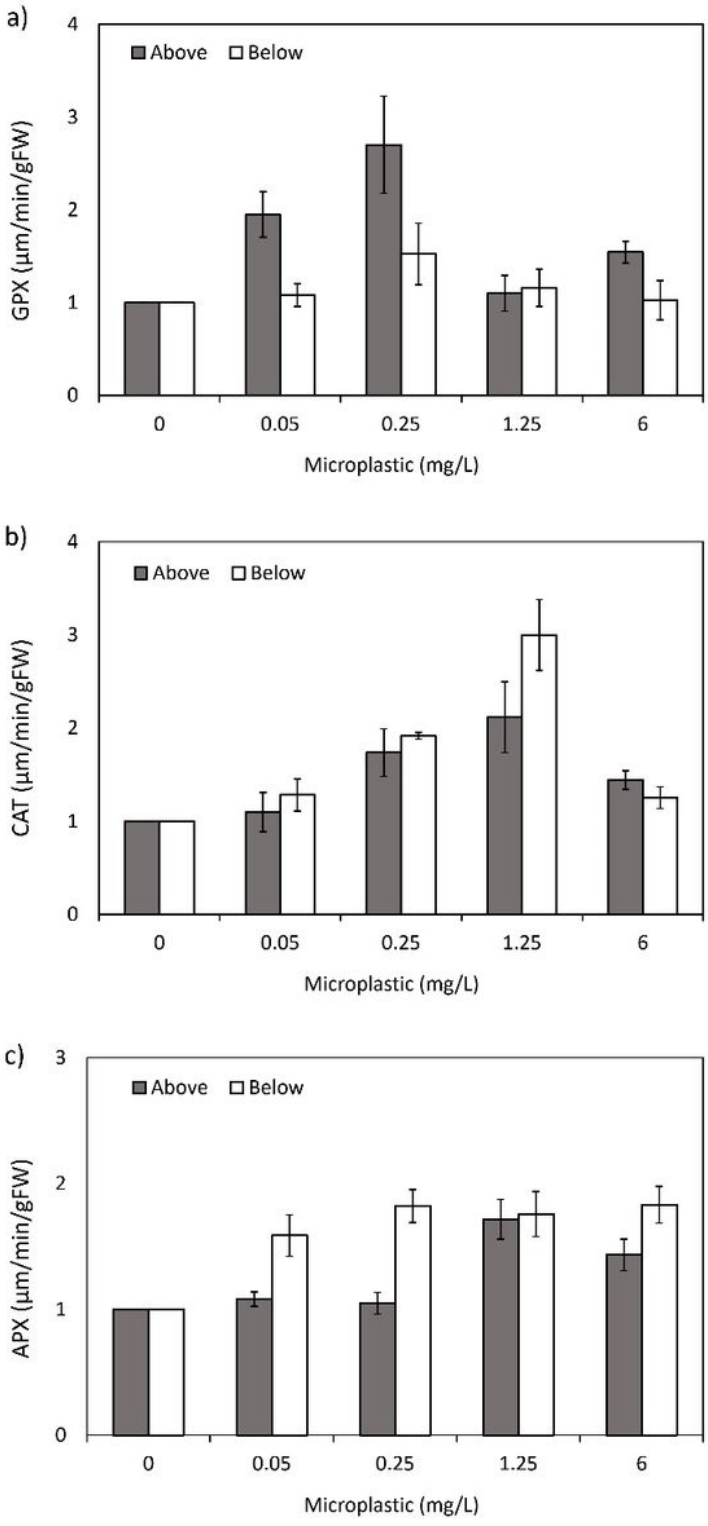

Figure 8

The relative change (compared to control) of antioxidant activities (a) guaiacol peroxidase, (b) catalase, and (c) ascorbic peroxidase of above water and below water parts of Watermilfoil (sp. Roraima) after exposure to different microplastic concentrations for 7 days. The error bars represent standard deviations