Free Radical Biology and Medicine 196 (2023) 93–107

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

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

Original article

Responses of individual and combined polystyrene and polymethyl methacrylate nanoplastics on hormonal content, fluorescence/photochemistry of chlorophylls and ROS scavenging capacity in *Lemna minor* under arsenic-induced oxidative stress

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ARTICLE INFO

Keywords:
Antioxidant
Arsenic
Chlorophyll *a* fluorescence transient
Nanoplastic
Polymethyl methacrylate
Polystrene

ABSTRACT

Nanoplastics alter the adverse impacts of hazardous contaminants such as heavy metals by changing their adsorption and accumulation. Few findings are available on the interaction between nanoplastic and heavy metals in plants. However, there is no report on the mechanisms for removing metal stress-mediated oxidative damage by the combination treatments of nanoplastics. To address this lack of information, polystyrene nanoplastic (PS, 100 mg L^{-1}) and polymethyl methacrylate (PMMA, 100 mg L^{-1}) were hydroponically applied to *Lemna minor* exposed to arsenate (As, 100 μM) for 7 days. PS or PMMA caused a reduction in the contents of N, P, K, Ca, Mg and Mn, but the improved contents were detected in the presence of PS or PMMA plus As stress. The hormone contents (auxin, gibberellic acid, cytokinin, salicylic acid and jasmonic acid) reduced by stress were re-arranged through PS or PMMA applications. Based on chlorophyll efficiency, fluorescence kinetics and performance of PSII, the impaired photosynthesis by As stress was improved via PS or PMMA applications. This alleviation did not continue under the combined form of PS and PMMA in As-applied plants. All analyzed antioxidant activity (superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX), glutathione reductase (GR), glutathione S-transferase (GST), glutathione peroxidase (GPX), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR)) decreased or unchanged under As, PS or PMMA. Due to the inactivation of the defense system, *L. minor* had high levels of hydrogen peroxide (H_2O_2) and thiobarbituric acid reactive substances (TBARS), showing lipid peroxidation. After As toxicity, induvial applications of PS or PMMA indicated the activated enzyme capacity (SOD, POX, GST and GPX) and upregulated AsA/DHA, GSH/GSSG and redox state of GSH, which facilitated the removal of radical accumulation. The efficiency of the antioxidant system in As + PS + PMMA-applied *L. minor* was not enough to remove damage induced by As stress; hereby, TBARS and H_2O_2 contents were similar to the As-treated group. Our findings from alone or combined application of PS and PMMA provide new information to advance the tolerance mechanism against As exposure in *L. minor*.

1. Introduction

Plastic pollution has several risks for the ecosystem and negatively affects the life quality of the organisms inducing genetic damage, immune toxicity and reproductive toxicity [1]. The accumulation of plastic reached 368 million tons in 2019 and has continued to rise [2].

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https://doi.org/10.1016/j.freeradbiomed.2023.01.015
Received 14 September 2022; Received in revised form 12 December 2022; Accepted 12 January 2023
Available online 16 January 2023

Polystyrene (PS) and polymers such as polymethyl methacrylate (PMMA) are defined as plastic products [3]. The diameter of nanoplastics is smaller than 1000 nm or 100 nm [4]. PS and PMMA are transferred from roots to leaves, flowers and stems via the vascular system in wheat and lettuce [5]. These two environmental pollutants classified as nanoplastics can have an important alteration on the metabolisms of plants. For example, *Allium cepa* exposed to PS treatments (10-100 mg L^{-1}) exhibited high genotoxic and oxidative damage [6]. In the presence of PS (50 mg L^{-1}), the lipid peroxidation and proline content increased in *Cucumis sativus* [7]. One possible reason for the adverse effects of PS nanoplastics may be benzene production as a result of its degradation [5]. Although the impacts of nanoplastics on the inhibition and disruption of photosynthesis have been studied [8,9], the detailed knowledge regarding the pollution of plastics on photosynthetic performance index, electron transport and capacity of reaction center or antenna complex of photosystems (PSI–PSII) is not available. While Wang et al. [10] reported a decrease in the growth and chlorophyll content in maize exposed to PS nanoplastics, Lian et al. [11] detected an increase in root elongation and photosynthesis in wheat. *Oryza sativa* subjected to PS nanoplastic treatments (50 and 100 mg L^{-1}) had improved growth and development and increased antioxidant enzyme capacity [12]. Therefore, the interaction of nanoplastics varied depending on different plant species as well as exposure dose, surface charge, growth stage of plants. PMMA-applied *Brassica campestris* had reduced seed vigor, germination and biomass [13]. The same study reported that the co-exposure of PMMA and arsenic decreased the root length and biomass. There is a limited toxicity finding of nanosized PMMA in plants. On the other hand, the combination of PS and PMMA nanoplastics could make the joint impact more complex in plants. However, there is no report about the phytotoxicity of the combination form of these pollutants.

Nanoplastics can adsorb chemical substances such as heavy metals (lead, copper, arsenic (As) and cadmium), which may intensify the negative effects of nanoplastics on plants [13]. This property of nanoplastics is dependent on increased polarity, surface area and amorphous structure [14]. For example, Cu^{2+} adsorption capacities of PMMA were 41.03 ± 1.78 μg/g [15]. The surface charge status of nanoplastics alters in the presence of heavy metal. When electronegative As(V) is adsorbed to PS nanoplastic, the negative charge of PS increased and PS easily enter to roots and cells of plant [16]. The adsorption capacity of nanoplastics may change based on their concentrations [17]. Dong et al. [18] noted that the high levels of PS and polytetrafluoroethylene caused a decline in the endogenous As(III) content of rice seedlings. When the metal ions adsorbed by nanoplastics are released into plants, their contents of heavy metal increase. The high accumulation of poisonous metalloids such as arsenic (As, 5–20 mg kg^{-1}) hampers growth, carbon assimilation and redox balance in plants. As exposure in two forms called arsenite (As(III)) and arsenate (As(V)) altered photosynthesis negatively by non-stomatal factors such as disrupted chlorophyll structure, reduced rubisco activity and impairment of thylakoid membrane [19]. The plants exposed to As treatments exhibit oxidative damage, induced thiobarbituric acid reactive substances (TBARS) and destruction of essential molecules such as enzyme, lipid, protein and DNA [13]. The negative effects induced by stress are controlled through the activated antioxidant system, including enzymatic (catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD) and glutathione reductase (GR)) and non-enzymatic compounds (ascorbate (AsA) glutathione (GSH) and α-tocopherol) [18]. Besides, one of the As toxicity is on energy and phosphorus metabolism by decreasing phosphorylation and ATP synthesis [20]. There is a similarity between the atomic structure of As and phosphorus. Therefore, the uptake of As(V) to plants is via phosphate transporters [21].

Only a few findings reported the effects of microplastics in heavy metal-applied plants. The interactions between heavy metal and nanoplastics detect in two different ways. For example, Lian et al. [11] reported that PS nanoplastics decreased cadmium uptake and removed the damage induced by cadmium in wheat. When polytetrafluoroethylene or PS were applied to *Oryza sativa*, As uptake was reduced due to the decreased root activity triggered by PS nanoplastic treatment [18]. Yang et al. [22] mentioned that microplastic treatments decreased zinc translocation from roots to the other parts in maize. On the other hand, copper toxicity was alleviated by polyvinyl chloride plastics in cucumber roots [23]. Higher levels of chlorophyll content, stomatal conductance and intracellular CO_2 concentrations were detected in wheat seedlings under the co-exposure of PS and cadmium stress than in the treatments with a single metal [24]. However, there is no report how co-exposures of different nanoplastics such as PMMA and PS influence on the photosynthetic capacity and defense system against As toxicity in plants.

Lemna minor, a small floating monocotyledon, is the best candidate due to the bio-accumulation of heavy metals, organic pollutants and nanomaterials for the phytoremediation process [25]. Also, *L. minor* has some advantages in the applications of genetic engineering and the production of alcohol and butanol [26]. However, there are no researches on the interaction between alone and combined treatments of PS, PMMA and arsenic stress in *Lemna minor*. Given the above findings, the co-presence of PS and PMMA can produce different results than when applied alone. The aim of the current research is the evaluation of the single or combined effects of PS and/or PMMA on chlorophyll *a* fluorescence transient, hormone contents (auxin (IAA), gibberellic acid (GA), cytokinin (CK), abscisic acid (ABA), salicylic acid (SA) and jasmonic acid (JA)) and antioxidant system in As stressed-*Lemna minor*.

2. Material and methods

2.1. Preparation and synthesis procedures of polystyrene nanoplastics and methyl methacrylate

Styrene, methyl methacrylate (MMA, 99%), ammonium persulfate (APS, 98%) and acetone (99.5%) were purchased from Merck. The ultrapure water used in this study was purified in a laboratory and had a resistivity of 18.25 MΩ cm at 25°C (Millipore, USA). Polystyrene nanoparticles (PS) were synthesized using an emulsion polymerization approach, according to Nuruzatulifah et al. [27]. As for the synthesis procedure, the free radical polymerization of MMA was used to create Poly (methyl methacrylate) nanoparticles (PMMA) to be applied as nanoplastics. 1 g of MMA and 25 mg of APS were dissolved in acetone–water mixture (4/6 mL) in 20 mL vial. The bottle was tightly closed to prevent the acetone from boiling. The mixture was stirred in a 70°C water bath for 3 h. Within 30 min, the reaction becomes milky. The resulting suspension was precipitated by centrifugation and washed twice with ultra-pure water.

2.2. Characterization

The surface morphology of the samples was observed by scanning electron microscopy (SEM, Zeiss EVO LS 10) with X-ray energy dispersive spectroscopy (EDX). Fourier Transform Infrared Spectroscopy (FTIR) measurements were obtained using a FTIR model Vertex 70 Bruker spectrophotometer (Bruker, Germany) equipped with an ATR (Attenuated Total Reflectance). All spectra were recorded at a spectral range between 400 and 4000 cm^{-1} at a scan rate of 180 scans and a spectral resolution of 4 cm^{-1}. The FTIR spectrum was employed in the transmittance mode. FTIR analyses were performed to verify possible interactions among PS, PMMA and As.

2.3. Plant material and experimental design

Duckweed (*Lemna minor* L.) cultures were grown in hydroponic Hoagland solution under controlled conditions (16/8 h light/dark regime at 24°C, 70% relative humidity and 350 μmol m^{-2} s^{-1} photosynthetic photon flux density) and the solutions were refreshed every three days. Based on the previous studies [12,28,29] about the genotoxicity of nanoplastics, the concentrations of polymethyl methacrylate (PMMA, 100 mg L^{-1}) and polystyrene (PS, 100 mg L^{-1}) were chosen as treatment groups, and 100 μM sodium arsenate ($Na_2HAsO_4 \cdot 7H_2O$) was used as the As(V) source. The preparation of PS and PMMA was completed according to the procedure presented by Lian et al. [11]. The plants were harvested after 7 days of treatment.

2.4. The contents of macro- and micronutrients, arsenic/arsenate and PS/PMMA

The contents of N, P^{3-}, K^{+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, As(III) and As(V) and the endogenous contents of PS and PMMA were analyzed by ICP-AES (Varian-Vista) and the measurements were completed for leaves (0.1 g dry weight) [30]. A GC-MS system that connected an Agilent 7890B GC (Agilent Technologies, Santa Clara, CA, USA) to an Agilent 5977A mass selective detector (MSD) and equipped with an Agilent 7693A autosampler (Agilent Technologies, Santa Clara, CA, USA) was used to analyze PS and PMMA contents according to the procedure given by Dong et al. [31].

2.5. The contents of endogenous hormone

Extraction and purification processes for hormone contents were performed according to the protocol detailed by Turan et al. [32] using 1 g of fresh leaves collected from each group.

2.6. Chlorophyll fluorescence

The changes on the photochemistry of PSII were identified through Handy PEA+ (Hansatech).

2.7. OJIP analysis

The function of photosystems was detected by Plant Efficiency Analyser and the definition of the calculated parameters was given in Supplementary Table S1.

2.8. Determination of H_2O_2 content, lipid peroxidation and proline content

The contents of H_2O_2 and lipid peroxidation were calculated [33,34]. Pro content was measured according to Bates et al. [35].

2.9. Identification of isozyme and total activity related to antioxidant system and AsA-GSH cycle

For protein and enzyme extractions, 0.5 g of each leaf sample was homogenized in 50 mM Tris-HCl (pH 7.8) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.2% Triton X-100, 1 mM phenylmethylsulfonyl fluoride and 2 mM dithiothreitol (DTT). The total soluble protein content of the enzyme extracts was determined [36]. Superoxide dismutase (SOD) isozyme/enzyme activity was defined [37,38]. The activity of catalase (CAT) isozyme/enzyme was determined using the procedure suggested by Woodbury et al. [39] and Bergmeyer [40]. The isozymes/enzyme capacity of peroxidase (POX) was measured according to the method suggested by Seevers et al. [41] and Herzog and Fahimi [42]. The enzyme/isozyme activities of glutathione S-transferase (GST) and glutathione peroxidase (GPX) were determined [43,44]. The isoforms and total NADPH oxidase (NOX) activity were calculated [45, 46].

Ascorbate peroxidase (APX) and glutathione reductase (GR) were spectrophotometrically and electrophoretically carried out [47,48]. The contents of ascorbate (AsA) and oxidized ascorbate (DHA) were estimated [49]. The procedure for monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) was performed [49]. Glutathione (GSH) was assayed according to Paradiso et al. [50], utilizing aliquots of supernatant neutralized with 0.5 M K–P buffer. Based on enzymatic recycling, glutathione is oxidized by DTNB and reduced by NADPH in the presence of GR, and glutathione content is evaluated by the rate of absorption changes at 412 nm. Oxidized glutathione (GSSG) was determined after removal of GSH by 2-vinylpyridine derivatization. Standard curves with known concentrations of GSH and GSSG were used for the quantification. GSH redox status was obtained [51].

The Gel Doc XR + System was used to photograph stained gels and subsequently evaluated using Image Lab software v4.0.1 (Bio-Rad, California, USA). Enzyme standards are used in gels for normalization.

2.10. Statistical analysis

The information on statical analysis was given in Supplementary Table S1.

3. Results

3.1. SEM-EDX and FTIR analysis of PS and/or PMMA-applied Lemna minor under As stress

The SEM micrographs of dried samples of control, PS + PMMA, As and As + PS + PMMA plants were represented in Fig. 1A–D. As a result of the penetration of PS, PMMA and As to the plant tissues, a dramatic morphological variation has occurred, as indicated in Fig. 1B–D, compared to the images of the control plant sample (Fig. 1A). SEM mapping and EDX spectra have been used to investigate the distribution of all elements, their compositions, and their existence in addition to dopant element. SEM mapping images shown in Fig. 2A and 2B for the plants exposed to As stress indicated that C, O and As were well distributed throughout the samples. As seen in Fig. 2B, O map was in green, C in red and As in orange, and their combination shown in Fig. 2A confirmed the prepared sample's homogeneity. When the EDX spectrum in Fig. 2C was examined, the presence of arsenic in the plant was seen. Fig. 2D depicted the FT-IR spectra of the pure plant sample and interpretation of the adhesion of the produced PS, PMMA and As to the plant samples. From Fig. 2D, it could be seen that there was an absorption band between 1150 cm^{-1} and 1250 cm^{-1} attributable to the C–O–C stretching vibration. The bands at 1034 cm^{-1} and 828 cm^{-1}, was the characteristic absorption vibration of PMMA. The absorption peak at the wave numbers of 765 cm^{-1} corresponds to C–H out-of-plane bending vibration absorption and indicates only one substituent in the benzene ring (which belongs to PS). The band at 1380 cm^{-1} indicated the C–H bending vibration of the $–CH_3$ group. The bands at 2869 cm^{-1} and 2925 cm^{-1} belong to the C–H bond stretching vibrations of the $–CH_3$ and $–CH_2$- groups, respectively. Also, weak absorption bands at 3297 cm^{-1} could be attributed to the –OH group stretching and bending vibrations. The peak at 763 cm^{-1} was observed in the As alone-treated plant sample. This peak shifted to 759 cm^{-1} in the plant sample exposed to As with PS and PMMA.

3.2. Macro- and micronutrient contents, PS and PMMA accumulation in PS and/or PMMA-applied Lemna minor under As stress

The changes in nutrient content of *L. minor* exposed to PS and/or PMMA treatments were shown in Fig. 3. PS and/or PMMA caused a decrease in the endogenous contents of N, P^{3-}, K^{+}, Ca^{2+}, Mg^{2+} and Mn^{2+} in plants. The same decline in the accumulation of these ions was exhibited under As exposure. As+PS treatment increased the contents of N, K^{+}, $Ca^{2+}$and Mn^{2+}. As+PMMA caused an increase in N, P^{3-}, K^{+}, Ca^{2+}, $Mg^{2+}$and Mn^{2+}. Interestingly, all analyzed ion contents decreased in As + PS + PMMA-applied plants as compared to the As alone. There was a significant increase in endogenous contents of As(III) and As(V) when PS or PMMA alone was applied to *L. minor* (Fig. 3). Both endogenous As(III)

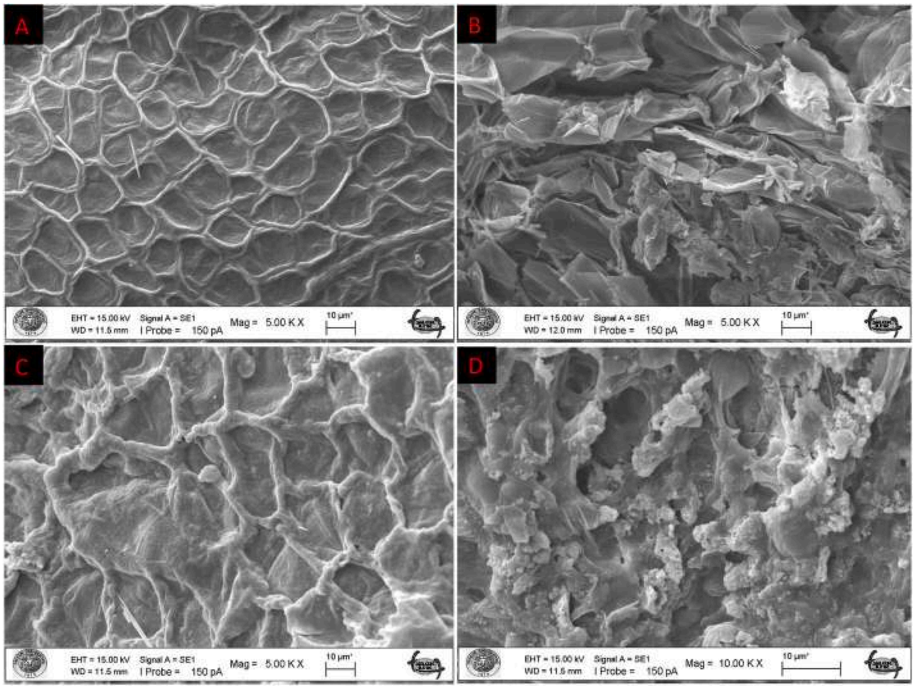

Fig. 1. SEM images of control plant sample (A), the plants exposed to polystyrene (PS, 100 mg L^{-1}) and polymethyl methacrylate (PMMA, 100 mg L^{-1}) (B), the plants exposed to arsenic stress (As, 100 μM) (C) and the plants exposed to PS and PMMA with As stress (D).

and As(V) contents of As-treated plants reached high levels as compared to the control ones. When compared to the As alone, a low As(III) and As (V) contents of leaves measured under PS and/or PMMA in the presence of As toxicity (As + PS and As + PMMA).

As expected, the endogenous PS content in PS-applied plants was 26 times higher than the control (Fig. 3). The combination of PS and PMMA also increased PS accumulation. While As stress had no impact on the PS content, PS and PS + PMMA treatments induced the PS accumulation under As stress. Similarly, addition of PMMA to *L. minor* significantly increased PMMA accumulation under both the control and stress conditions (Fig. 3).

3.3. Hormone accumulation in PS and/or PMMA-applied Lemna minor under As stress

After PS or PMMA treatments, *L. minor* showed low levels of IAA, GA, SA and JA (Fig. 4). In contrast to this, ABA content caused an increase under PS and/or PMMA treatments. Compared with under non-stress conditions, As toxicity decreased the analyzed hormone contents, except for ABA accumulation. In the presence of As stress, the addition of PS and/or PMMA to duckweed plants increased the contents of IAA, GA, SA, CK and JA, reaching the highest levels under As + PMMA. Fig. 4 displayed that the enhancement in ABA and GA contents was recorded under As + PS + PMMA. However, IAA, CK and JA contents in plants exposed to As + PS + PMMA were significantly lower than at the As alone.

3.4. Chlorophyll fluorescence in PS and/or PMMA-applied Lemna minor under As stress

Fig. 5A-5B revealed that PS and/or PMMA-applied *L. minor* exhibited a decrement in the maximum photosynthetic quantum yield (F_v/F_m) and size and number of active photosynthetic reaction centers (F_v/F_o). The lowest level in F_v/F_m was observed by 15% after the combined form of PS and PMMA exposure (PS + PMMA). On the other hand, PMMA alone caused the lowest levels in F_v/F_o of *Lemna* plants by 1.6-fold decrease. Similarly, a decline in F_v/F_m and F_v/F_o was detected in the leaves of As-treated plants. There were more significant inductions in F_v/F_m and F_v/F_o under PS or PMMA together with As stress (As + PS or As + PMMA). Interestingly, after As exposure, PS + PMMA treatment did not change in F_v/F_m, F_v/F_o and F_o/F_m as compared to the stress alone. F_o/F_m related to PSII photochemistry increased under PS and/or PMMA toxicity as compared to the control group. Also, the high level in F_o/F_m detected under As stress (Fig. 5C). *L. minor* leaves grown under As + PS or As + PMMA had a low F_o/F_m level. This trend in F_o/F_m did not maintain under As + PS + PMMA, which it was similar to the plants treated with As alone.

3.5. Fluorescence kinetics and performance of PSII in PS and/or PMMA-applied Lemna minor under As stress

When duckweed plants were exposed to PS, As and PMMA toxicity, an increase in antenna size of active reactive center in PSII (ABS/RC), electron transport flux per reaction center (ET_o/RC), trapping rate of absorbed photons in reaction center (TR_o/RC), dissipation energy flux (DI_o/RC) and the rate of the closure of reaction centers (dV/dt_o) was observed (Fig. 5D). $\Phi P_o/(1\text{-}\Phi P_o)$, $\Psi E_o/(1\text{-}\Psi E_o)$ and $\gamma RC/(1\text{-}\gamma RC)$ associated with the trapped electron fluxes, the efficiency of light reaction and fraction of PSII decreased in the presence of As, PS or PMMA. Similarly, the efficiency for electron transport (ΨE_o) and φRo was decreased by exogenously application of As, PS and PMMA. An enhancement in V_I and V_J showed high accumulation of Q_A^- and closed reaction center under As, PS or PMMA. The performance indexes (PI_{ABS} and PI_{total}) of As, PS or PMMA-treated plants were lower in *L. minor* than that of the control group. Besides, these effects on chlorophyll *a* transient were much more obvious under the combination of PS and PMMA (PS + PMMA) (Fig. 5E). PS or PMMA addition to As-applied plants alleviated the negative effects of stress on PSII photochemistry. However, this effect did not sustain with PS + PMMA application.

3.6. ROS accumulation and lipid peroxidation in PS and/or PMMA-applied Lemna minor under As stress

As observed in Fig. 6A, the excessive exposure of As increased Pro

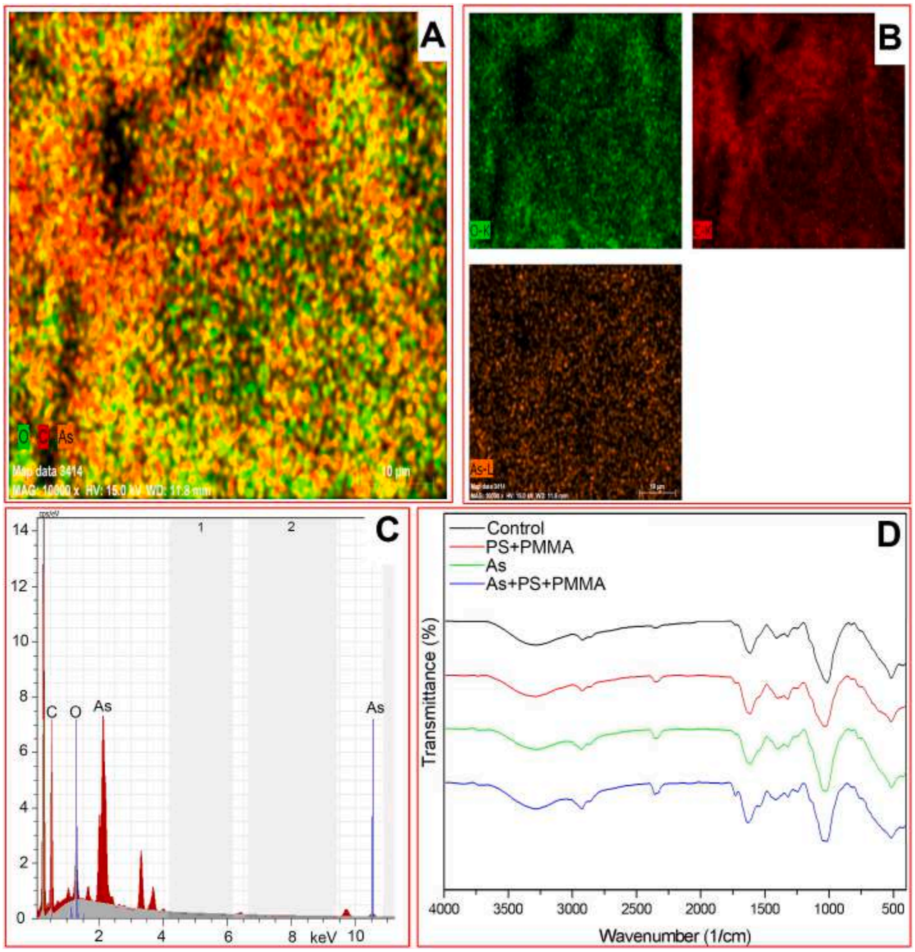

Fig. 2. SEM–EDX elemental mapping images of *Lemna minor* under polystyrene (PS, 100 mg L^{-1}) and polymethyl methacrylate (PMMA, 100 mg L^{-1}) with arsenic stress (As, 100 μM) (A, B), SEM–EDX spectrum of the plants exposed to PS and PMMA with As stress (C), FT-IR spectra of *Lemna minor* under PS and PMMA with or without As stress.

content by 1.5-fold. This increase in Pro content did not observe under PS, PMMA and PS + PMMA. When As was combined with PS and/or PMMA, there was a decrement in Pro accumulation. In comparison with the control group, the high level of H_2O_2 content found under As toxicity (Fig. 6B). Besides, PS, PMMA and PS + PMMA under the control conditions increased H_2O_2 accumulation and the maximum induction was calculated under PS treatment by a 1.8-fold increase in *L. minor*. Except for As + PS + PMMA, H_2O_2 content in combination with PS or PMMA was lower than that of As treatment alone. However, no alteration in H_2O_2 content observed in the presence of PS + PMMA under As stress. *L. minor* exposed to As stress suffered from an induction in TBARS content (Fig. 6C). There was an enhancement in TBARS of As, PS or PMMA-subjected duckweed leaves. PS treatment presented the maximum level in TBARS when compared to the control group. TBARS content was markedly decreased under As + PS and As + PMMA. TBARS content did not change in the duckweed plants subjected to the combination of PS and PMMA under As stress.

3.7. Antioxidant enzyme/isoenzyme capacity related to AsA-GSH cycle in PS and/or PMMA-applied Lemna minor under As stress

As illustrated in Fig. 7A, five bands for SOD isozymes (two Mn-SOD1-2, one Fe-SOD and two Cu/Zn-SOD1-2) were detected in duckweed leaves. A similar activity of SOD was observed between the control groups and As stress (Fig. 7B). The alone application and co-existence of PS and PMMA decreased the total SOD activity, suggested by Mn-SOD1-2 and Cu/Zn-SOD2. Mn-SOD1, Fe-SOD and Cu/Zn-SOD1 were responsible for increasing SOD activity under As + PS and As + PMMA. As + PS + PMMA exposure caused no change in SOD activity as compared to the As alone. Only one CAT isoform was detected throughout the experimental period. As evident by CAT isozyme (Fig. 7C), the total CAT activity decreased or unchanged in *L. minor* exposed to As, PS, PMMA or PS + PMMA (Fig. 7D).

As given in Fig. 8A, four bands (POX1-4) for POX isoforms were shown in *L. minor* leaves. The presence of As, PS or PMMA resulted in decreases in total POX activity (Fig. 8B). All POX isoforms proved this effect, especially POX4 supported it more strongly. The induced activity on POX was detected under As + PS and As + PMMA in plants. The maximum induction in total POX activity was followed by As + PMMA (1.9-fold), as suggested by POX1-4 isozymes. Six APX isozymes (APX1-6) were visualized in duckweed plants (Fig. 8C). As compatible with the all APX isoforms, the decreased or unchanged APX activity was detected in the leaves (Fig. 8D). After As exposure, PS and/or PMMA resulted in a significant increment in APX, which was parallel to the increased intensities of APX2-4-5-6.

All application groups had eight GR isoforms in leaves (Fig. 9A). Except for PS alone, GR activity decreased under As or PMMA toxicity (Fig. 9B). The addition of PS or PMMA to As-applied plants caused a significant increment in total GR activity, but not under As + PS + PMMA. This trend was clearly supported by all GR isoforms. Fig. 9C revealed that five bands for NOX isozymes were given in *L. minor*. As toxicity caused a reduction in the total NOX activity (Fig. 9D), which showed the low intensities of all NOX isozymes in leaves. However, the increased activity in NOX observed under PS alone and PS + PMMA.

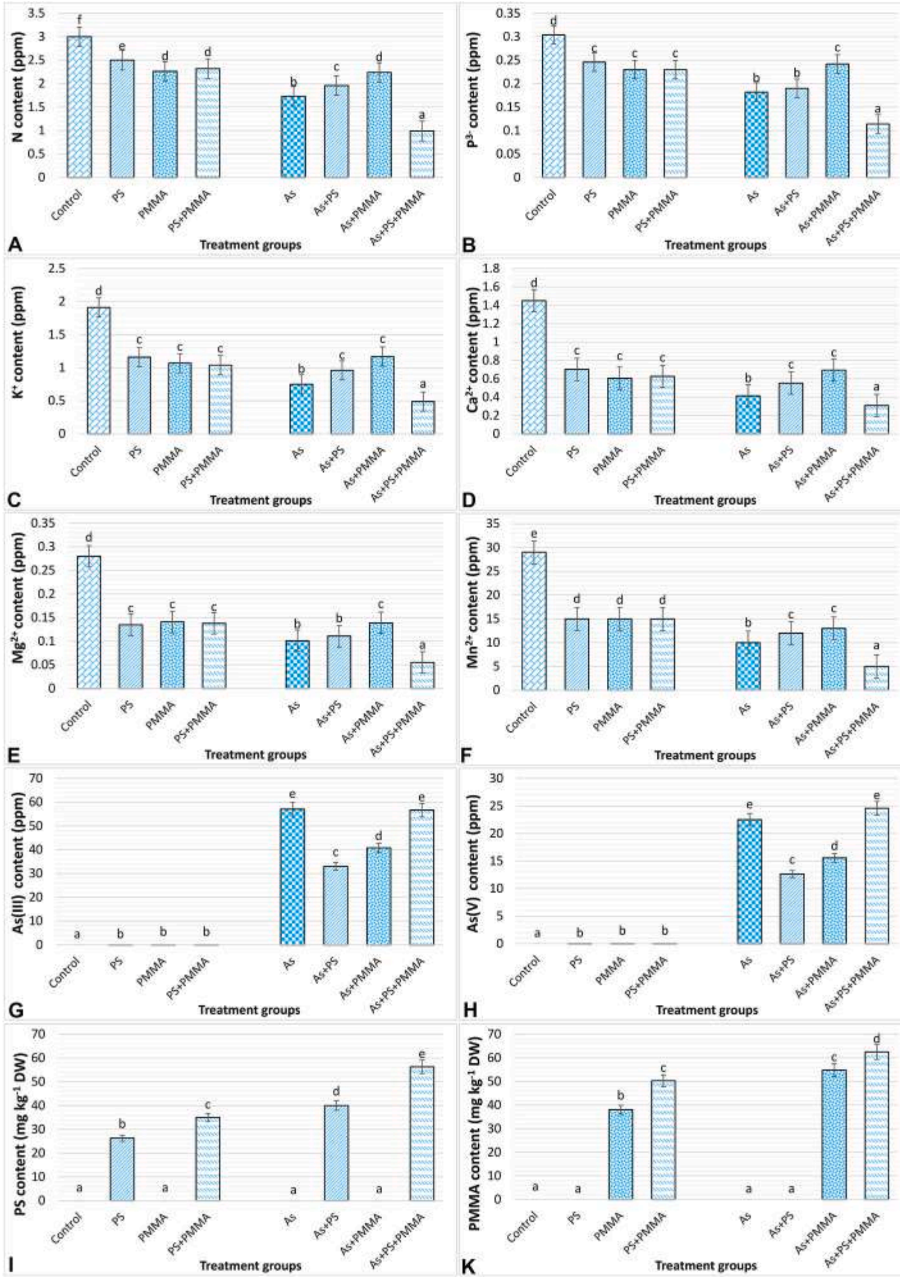

Fig. 3. The endogenous contents of macro- and micronutrients (N, P^{3-}, K^{+}, Ca^{2+}, Mg^{2+} and Mn^{2+}) and arsenic (As(III) and As(V)) and the endogenous contents of PS and PMMA in *Lemna minor* under polystyrene (PS, 100 mg L^{-1}) and polymethyl methacrylate (PMMA, 100 mg L^{-1}) with or without arsenic stress (As, 100 μM).

In leaves, five bands for GST isoforms were identified during the experimental period (Fig. 10A). The low levels of GST activity were measured in As-treated duckweed plants, as observed all GST isoforms (Fig. 10B). There was no change in GST under PS or PMMA alone. However, when PS and/or PMMA were applied under As pollution, the high activity of GST was supplied in *L. minor*. The maximum increment was observed under As + PS by a 2.3-fold increase. This change was parallel to the intensities of all GST isoforms, especially GST5 isozyme. The changes of GPX isoforms consisting of the four bands were shown in Fig. 10C. There was a noticeable drop in GPX under As stress in *L. minor* (Fig. 10D). GPX3-4 isozymes were detected as the major bands for all treatment groups. While PS or PMMA did not affect under the control conditions, the combination forms of PS or PMMA together with As stress caused an increase in *L. minor*. However, this variation did not maintain under As + PS + PMMA.

While, MDHAR decreased in *L. minor* after As exposure, stress did not change DHAR activity (Fig. 11A-11B). PS or PMMA treatments under the control conditions resulted in a decrease or non-change activity of MDHAR and DHAR. However, PS and/or PMMA in the presence of As stress promoted both activities of MDHAR and DHAR compared to the stress alone. In response to As, PS and PMMA treatments, the contents of tAsA and DHA decreased in duckweed plants (Fig. 11C-11D). The leaves treated with As + PS and As + PMMA had the high content of tAsA. In contrast to this, the addition of PS or PMMA to stress-applied plants decreased DHA content. A similar content of tAsA and DHA found under As + PS + PMMA of *L. minor*. As given in Fig. 11E-11F, the opposite results in GSH and GSSG content were calculated throughout the experimental period. GSH content decreased in plants treated with As, PS or PMMA, but GSSG content did not. The induced level of GSH was observed under As + PS and As + PMMA compared to the stress alone. As + PS and As + PMMA application in response to stress resulted in the reduced contents of GSSG, except for As + PS + PMMA. Based on the

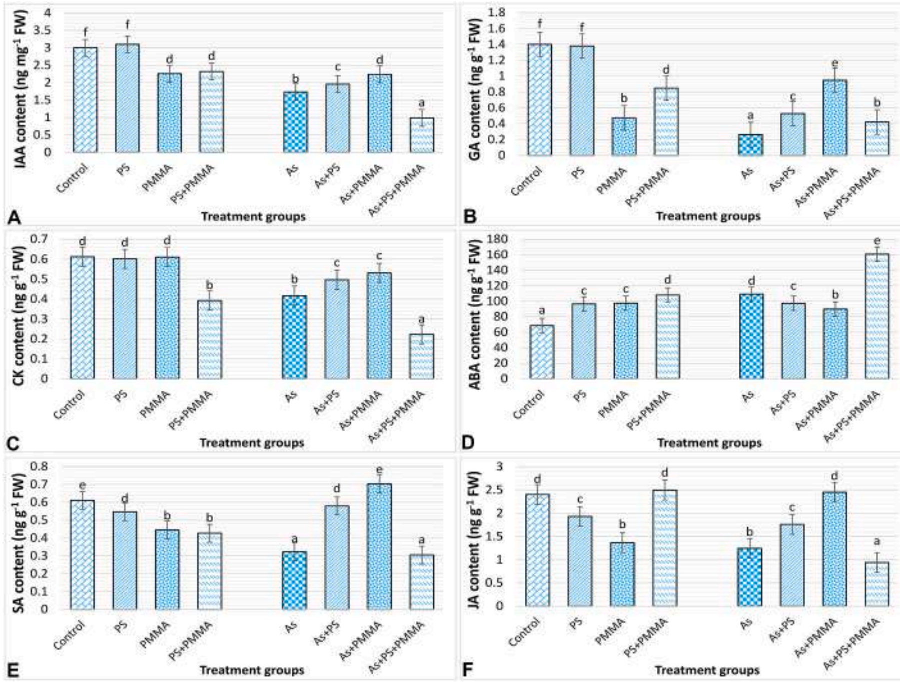

Fig. 4. The endogenous contents of phytohormones (auxin (IAA), gibberellic acid (GA), cytokinin (CK), abscisic acid (ABA), salicylic acid (SA) and jasmonic acid (JA)) in *Lemna minor* under polystyrene (PS, 100 mg L^{-1}) and polymethyl methacrylate (PMMA, 100 mg L^{-1}) with or without arsenic stress (As, 100 μM).

results of tAsA and DHA contents, tAsA/DHA and GSH/GSSG decreased under As, PS or PMMA alone applications, but the combined treatment of tAsA and DHA increased this ratio, except for As + PS + PMMA (Fig. 11G). As given in Fig. 11H, the GSH redox state was impaired by As, PS and PMMA in duckweed plants. On the other hand, the improved redox status of GSH detected after As plus PS or As + PMMA exposure. As well as GSH/GSSG, the induced GSH redox rate did not continue by As + PS + PMMA application.

4. Discussion

4.1. Accumulation of macro/micro-nutrients and PS-PMMA contents

The reported consequences of As contamination are inhibition of nutrient supply, functional groups of enzymes, chlorophyll synthesis and protein content [52]. Farnese et al. [53] reported a decline in magnesium (Mg), calcium (Ca), manganese (Mn), potassium (K) and phosphorus (P) under As stress, which is in competition with them as it shares their transporters. In the present study, the reduction in N, P, K, Ca, Mg and Mn induced by As toxicity might be correlated with disturbances in oxygen evolving complex, growth and biosynthesis, and structure of chlorophyll. These elements take place in these metabolic processes. The observation in As-applied *L. minor* was in congruence with the previous report of Farouk and Al-Amri [54], who found that As pollution impeded nutrient uptake in rosemary plants. The same trend was also detected in *L. minor* treated with PS or PMMA. The results were in line with the data of Lian et al. [55], who reported adverse effects of 0.01–10 mg L^{-1} PS in wheat by reduction of micronutrients. As expected, PS or PMMA exposure increased their endogenous contents. However, the endogenous contents of PS or PMMA under As stress (As + PS or As + PMMA) resulted in a lower accumulation of PS or PMMA compared to As + PS + PMMA. This may explain the positive effects of PS or PMMA on nutrient balance and antioxidant system against As stress in duckweed. Depending on As, PS and PMMA treatments, the induced endogenous As was caused the deficiency of nutrient uptake. As can easily enter through inorganic phosphate (Pi) transporter. Therefore, there is a competition between As and Pi [56], which proved the decline in P content in *L. minor* exposed to As treatment. As-imposed disruptions could be improvement by PS or PMMA treatments. It has been demonstrated that PS can modulate the gene expression related to root development and nutrient transport by transcriptomic analysis in rice [12]. Kitchin et al. [57] identified that the interaction between electronegative As(V) and PS may increase the negative charge on the PS surface. In the present study, this coaction will allow more PS to enter in *L. minor*. In a supportive way, the greater contents of PS were observed in As + PS-treated plants than PS alone. However, no improvement in the uptake of nutrients was observed in the groups where PS and PMMA were applied together (As + PS + PMMA). When PS and PMMA co-exist, it may result in the formation of different surface interactions and less association with As compared to its application alone. Dong et al. [18] reported that PS can reduce As(III) by adsorption of As in rice.

4.2. Hormonal regulation

Phytohormone controls important biological processes such as germination, growth, development and photosynthesis under normal or stress conditions [58]. Except for ABA content, As toxicity inhibited the contents of IAA, GA, SA, CK and JA in *L. minor*. This effect may be through disruptions in transporters of these hormones. After excessive metal exposure, signaling pathways are modulated the regulatory roles of GA and ABA [59]. Under PS or PMMA contamination caused a decline in IAA, GA, SA and JA. Similarly, Sun et al. [60] and Zhou et al. [12] found that PS caused a reduction in JA and ABA after PS exposure. On the other hand, the mechanisms of how nanoplastics regulate phytohormone accumulation are not well determined [61]. The accumulation of plant growth regulators plays role in combating stress conditions by inducing gene expression of anti-stress genes [62]. The increment in CK content contributed to the increased chlorophyll biosynthesis [63]. Under stress, the induction in SA levels is connected with the optimized allocation of nitrogen and improved cellular permeability [64]. Therefore, the alone application of PS or PMMA might play a role in removing As toxicity by providing regulation in the hormonal signaling pathway. However, the combined application of PS and PMMA under As stress did not maintain the same effect on hormones.

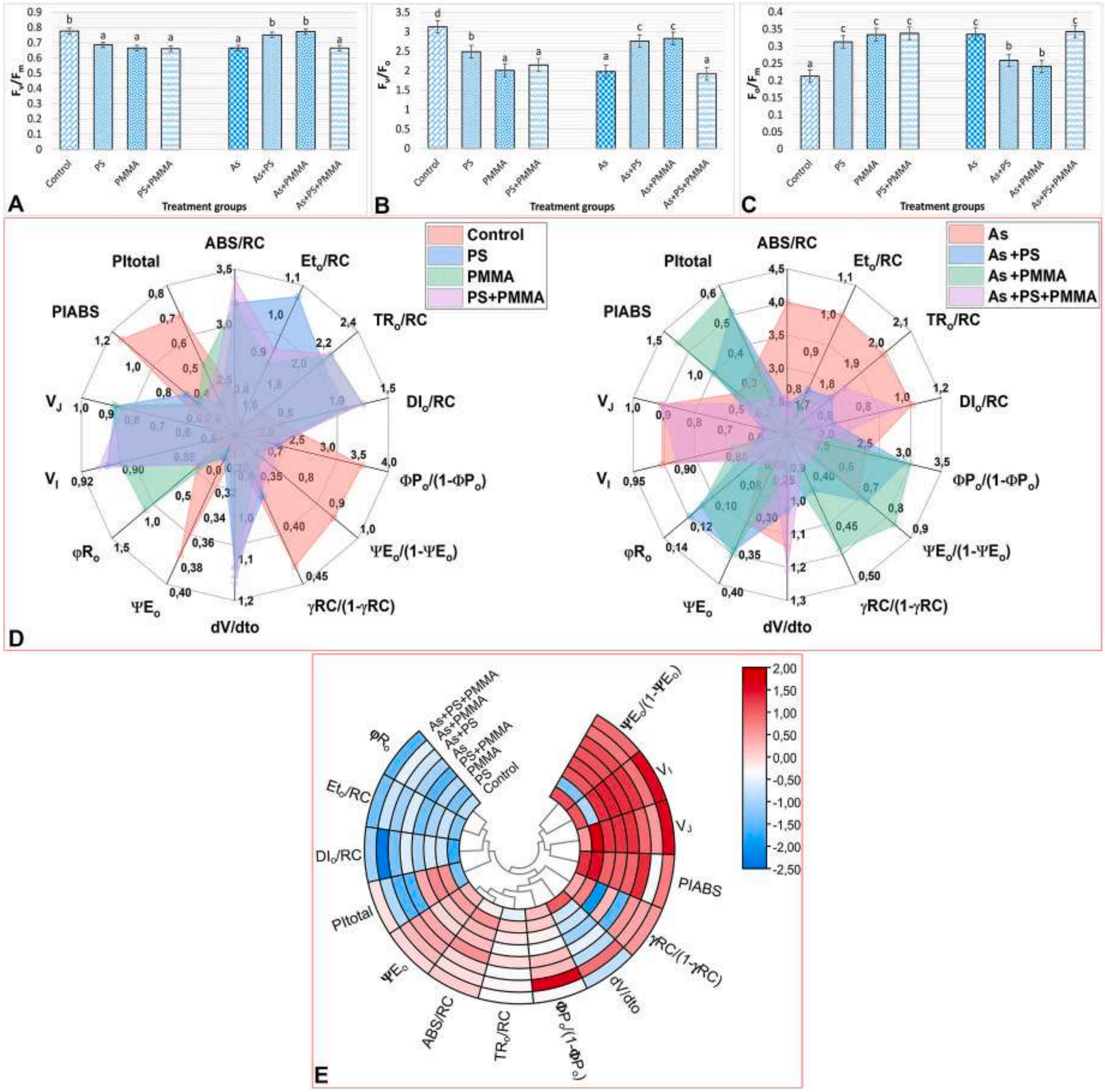

Fig. 5. The maximal quantum yield of PSII photochemistry (F_v/F_m, A), potential photochemical efficiency (F_v/F_o, B) and physiological state of the photosynthetic apparatus (F_o/F_m, C), and radar plots (D) and heat map (E) of OJIP-test parameters in *Lemna minor* under polystyrene (PS, 100 mg L^{-1}) and polymethyl methacrylate (PMMA, 100 mg L^{-1}) with or without arsenic stress (As, 100 μM).

4.3. Chlorophyll fluorescence

As toxicity impairs photosynthetic pigments, thylakoid membrane structure and enzyme activity related to Calvin cycle, signifying that stress disrupts the photosynthetic performance of plants [65]. Nanoplastic or microplastic exposure disrupts the absorption efficiency and photochemistry of PSII [66]. The damage to the oxygen evolving complex of PSII and reduced plastoquinone (PQ) indicated that plants are affected by stress. In the present study, the decreased F_v/F_m in plants treated with As stress showed that stress caused the instability of the photosynthetic apparatus, which was in line with previous study on rice [67]. Also, the donor side of PSII was negatively affected by As, PS or PMMA treatments, which reduced the efficiency of the Hill reaction and declined in the size and active reaction center, as showed low F_v/F_o and high F_o/F_m (dissipation indicators). Thus, the re-oxidation to quinone A proteins (Q_A) from Q_A^- might be reduced under stress conditions, which causes poor electron transfer from PSII to PSI. In our study, when PS or PMMA concentration was applied, the reason of inhibition in the chlorophyll fluorescence might be disruption of thylakoid metabolic functions in *L. minor* as suggested by Du et al. [68]. Furthermore, PS or PMMA exposure was noticed a change in the rate of electron transport system and PSII photochemistry under As stress, as deduced from the high/low values of F_v/F_o and F_o/F_m. After nanoplastic treatments, the accelerated energy metabolism and improved photosynthesis processes were observed in wheat [55]. Also, the restoration in fluorescence parameters under As + PS or As + PMMA was related to increased endogenous content of Mn, an important ion on extrinsic proteins oxygen evolving complex. The alone application of PS or PMMA may be increased electron transfer from this complex to D1 protein associated with core reaction center of PSII. This is in agreement with the findings of Shen et al. [69], who reported that organic compounds can be adsorbed by nanoplastics. Since both PS and PMMA have this property, the accumulation of organic contaminants may be greater in the co-exposure of PS and PMMA in *L. minor*. Therefore, PS + PMMA could not abolish As toxicity in terms of photosynthesis. Wang et al. [70] found that the dissolution rate of phenanthrene in rice differs between phenanthrene alone and phenanthrene plus PS, which is higher in plant culture containing phenanthrene alone. According to the researchers,

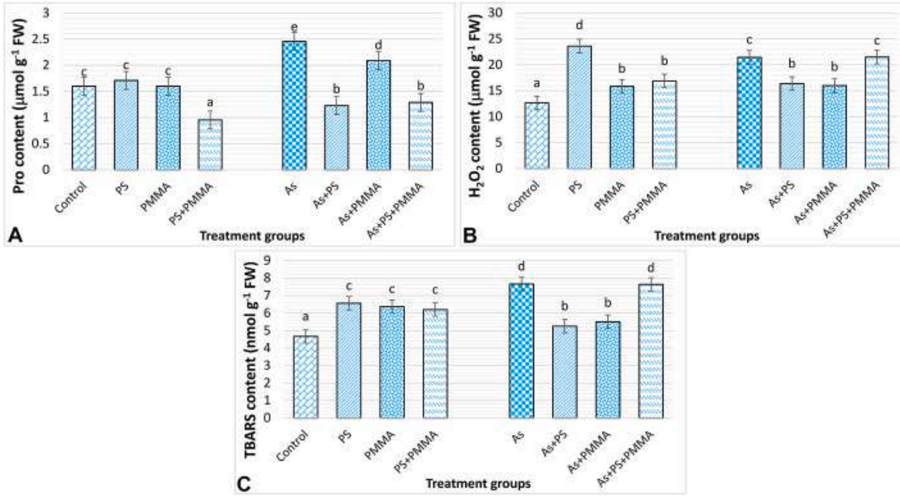

Fig. 6. Proline content (Pro, A), hydrogen peroxide content (H_2O_2, B) and lipid peroxidation (TBARS, C) in *Lemna minor* under polystyrene (PS, 100 mg L^{-1}) and polymethyl methacrylate (PMMA, 100 mg L^{-1}) with or without arsenic stress (As, 100 μM).

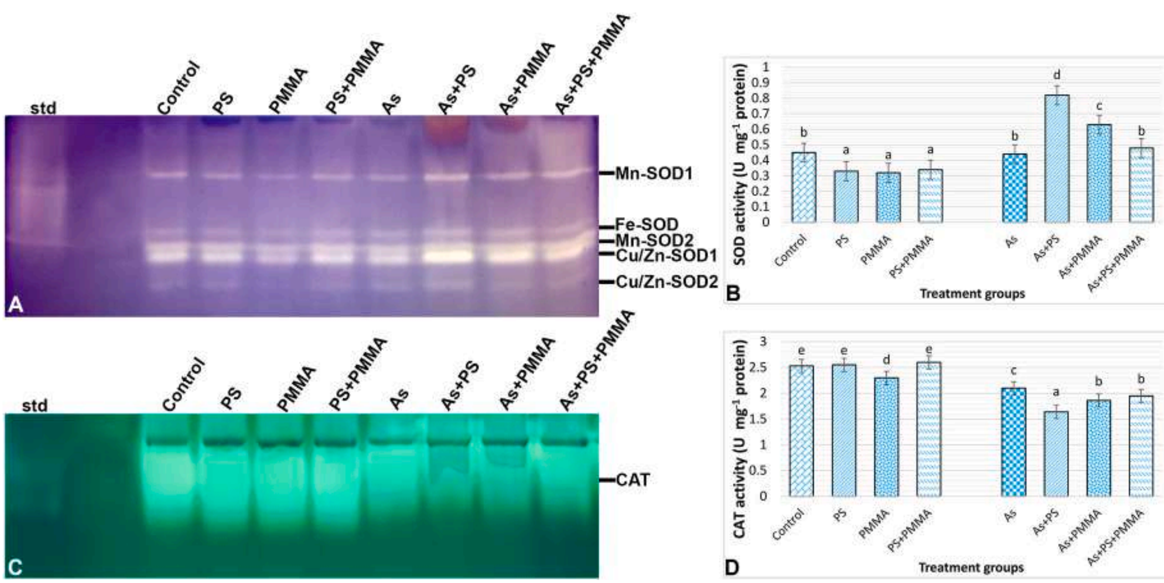

Fig. 7. The relative band intensity of different types of superoxide dismutase isoenzymes (SOD, A) and total SOD activity (B), relative band intensity of different types of catalase isoenzymes (CAT, C) and total CAT activity (D) in *Lemna minor* under polystyrene (PS, 100 mg L^{-1}) and polymethyl methacrylate (PMMA, 100 mg L^{-1}) with or without arsenic stress (As, 100 μM).

this case might be connected with the adsorption of phenanthrene on the PS. In the current study, the co-existence of PS and PMMMA may have reduced the binding capacity with As and the endogenous As(V) content of the plants increased. In As + PS + PMMA-applied *Lemna* plants, the increased As(V) accumulation may cause chlorophyll fluorescence to be more affected by stress.

4.4. PSII photochemistry

As-treated plants exhibit a reduction in ribulose 1,5–bisphosphate carboxylase/oxygenase activity, PSII photochemistry and chlorophyll biosynthesis [71]. Experimental evidence indicates that polystyrene nanoplastics impair the photochemical efficiency of plants by disrupting function of the antenna complex and reaction centers of PSI-II [72,73]. One way of revealing the functional and structural capacity of PSII, reduction and oxidation rate of Q_B, Q_A, and plastoquinone pool is to evaluate measurements followed by the OJIP test. In the current study, As-applied *L. minor* had an enhancement in ABS/RC by increasing absorbed photon flux per RC for protecting photosynthetic machinery. The increased levels of ABS/RC, ET_o/RC, TR_o/RC and DI_o/RC proved the inactivation of PSII and reduction of the subsequent electron carriers under As, PS or PMMA treatments. However, this situation did not provide protection on PSII photochemistry and led to more heat loss (increased DI_o/RC) in *L. minor* subjected to As or nanoplastic toxicity. As or PS-PMMA alone treatment decreased electron flux in PSII as proved the reduced ΨE_o and φR_o involved quantum efficiency. This result showed the adverse impacts of PS and PMMA alone applications as well as As toxicity. Interestingly, this role of PS or PMMA on photosynthetic efficiency of *L. minor* against As stress changed in a positive way. Upon PS or PMMA addition to As-applied duckweed plants, a decrease in ABS/RC, ET_o/RC, TR_o/RC and DI_o/RC protected the stabilization of energy transport for trapping and energy conversion efficiency of PSII. As proved low TR_o/RC indicating transmission rate of electrons of reaction centers, PS and PMMA ensured more electron transfer from reaction center in stress-treated *L. minor*. The high values of $\Phi P_o/(1-\Phi P_o)$, $\Psi E_o/(1-\Psi E_o)$ and $\gamma RC/(1-\gamma RC)$ triggered by PS or PMMA under As

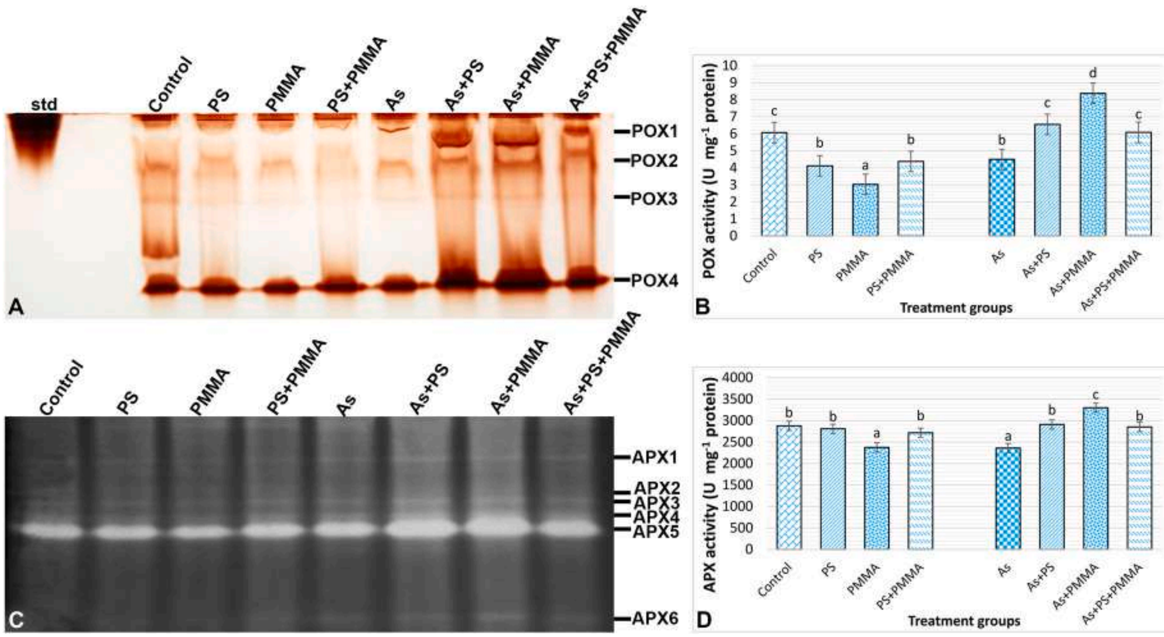

Fig. 8. The relative band intensity of different types of peroxidase isoenzymes (POX, A) and total POX activity (B), relative band intensity of different types of ascorbate peroxidase isoenzymes (APX, C) and total APX activity (D) in *Lemna minor* under polystyrene (PS, 100 mg L^{-1}) and polymethyl methacrylate (PMMA, 100 mg L^{-1}) with or without arsenic stress (As, 100 μM).

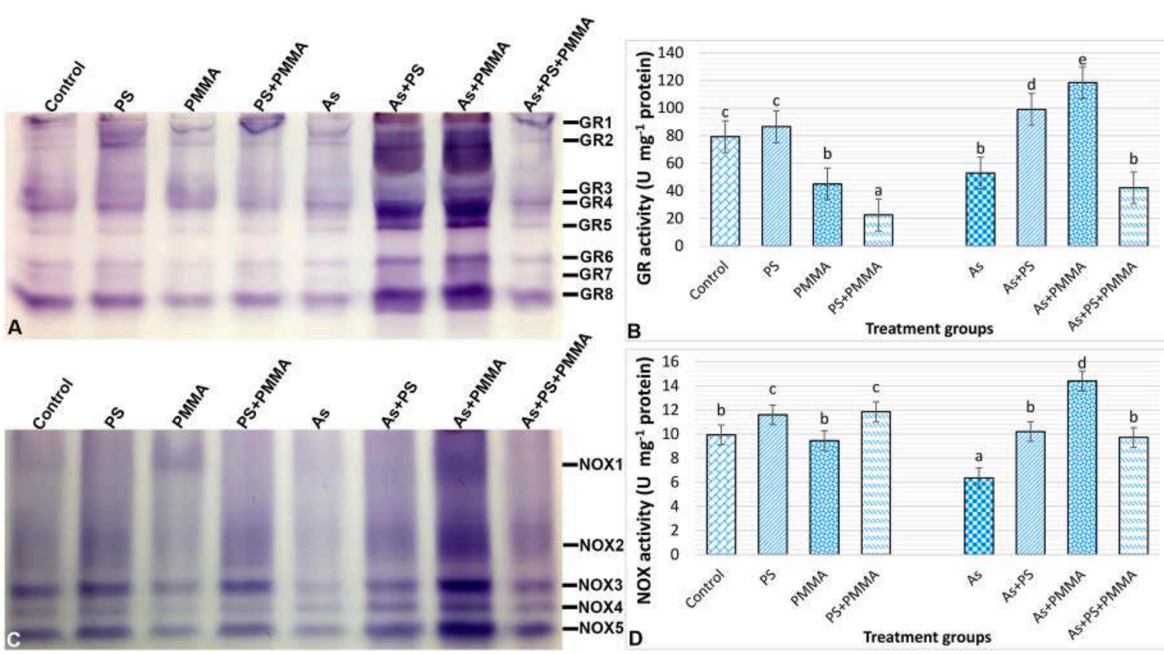

Fig. 9. The relative band intensity of different types of glutathione reductase isoenzymes (GR, A) and total GR activity (B), relative band intensity of different types of NADPH oxidase isoenzymes (NOX, C) and total NOX activity (D) in *Lemna minor* under polystyrene (PS, 100 mg L^{-1}) and polymethyl methacrylate (PMMA, 100 mg L^{-1}) with or without arsenic stress (As, 100 μM).

toxicity showed that PS or PMMA positively affected the trapped energy by active reaction center and transport to the end electron acceptors at PSI against toxicity. In the presence of As stress, PS or PMMA-mediated reduction in DI_o/RC reflected a drop in energy dissipation of un-trapped excitation energy in photosystems of *L. minor*. The declines in ΨE_o and φR_o were reversed by PS or PMMA and the improved efficiency of electron transport from Q_A^- (primer quinone acceptor) to Q_B (seconder quinone acceptor). The reoxidation of Q_A^- was also related to the decrement in V_J and V_I levels observed under As + PS and As + PMMA. Exogenous applications of PS or PMMA to stress-applied plants protected the stabilization of PQ redox state. Similar to this, the low levels of dV_G/dt_o showed that PS or PMMA against to As stress provided no damage on the oxygen evolving complex. Therefore, after As toxicity, exogenously applied PS or PMMA alone was effective in the conversion of light energy to chemical energy in *L. minor*, but not under As + PS + PMMA. This interpretation was supported by our results of photosynthetic indexes, including PI_{ABS} and PI_{total}. The measurements on PI_{ABS} give information about the capacity to transport light energy into NADPH. In this study, in response to As stress, PS or PMMA alone may activate the carbon fixation pathways and conversion of solar energy

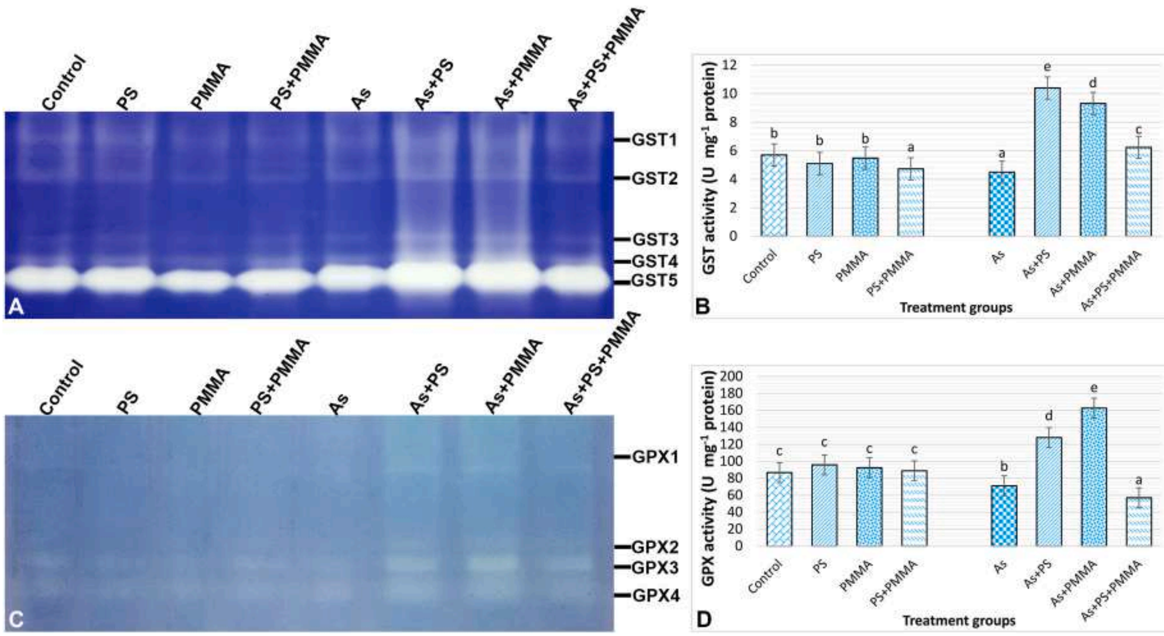

Fig. 10. The relative band intensity of different types of glutathione S-transferase isoenzymes (GST, A) and total GST activity (B), relative band intensity of different types of glutathione peroxidase isoenzymes (GPX, C) and total GPX activity (D) in *Lemna minor* under polystyrene (PS, 100 mg L^{-1}) and polymethyl methacrylate (PMMA, 100 mg L^{-1}) with or without arsenic stress (As, 100 μM).

into ATP and NADPH. These changes induced by PS or PMMA may be effective in providing the energy requirement for plant tolerance under stress. Lian et al. [55] reported the acceleration in energy metabolism in the presence of nanoplastics. On the other hand, the combined exposure of PS or PMMA differed from that of their individual applications the impacts on photochemical efficiency of the photosynthetic system of *L. minor*. PS combination with PMMA had antagonistic effects on photochemical reaction of As-applied *L. minor*. When PS and PMMA co-exposure to plants grown under As stress, it may have created different negative effects on photosynthesis than those of the application alone because of their aggregation between them. Tamayo-Belda et al. [74] reported that the heteroaggregates of PS and poly(amidoamine) dendrimer increased the damages on cell membranes. Parenti et al. [75] confirmed the high sorption capacity of PS for hydrophobic contaminants.

4.5. Enzymatic/non-enzymatic antioxidant capacity and redox balance

As toxicity provokes excessive production of reactive oxygen species (ROS) leading the imbalance in the redox state in plants [76]. The various antioxidant enzymes/non-enzymes (SOD, CAT, POX, APX, GR, MDHAR, DHAR, ascorbate and glutathione) remove the toxic ROS levels in plants treated with As stress [77]. Similarly, nanoplastic exposure upregulated genes involved in antioxidant enzymes [60]. In the present study, the addition of As, PS or PMMA to *L. minor* could not supply the efficient antioxidant capacity. All antioxidant activity (SOD, CAT, POX, APX, GR, GST, GPX, MDHAR and DHAR) decreased or unchanged under As, PS or PMMA, implying that the toxicity inflicted on *L. minor* by As or nanoplastic pollution exceeded the self-regulation capability of the antioxidant system. Due to the inactivation of defense system, *L. minor* had the high levels of H_2O_2 and TBARS showing lipid peroxidation. Highly similar findings were reported by Song et al. [78] and Wang et al. [10] in As or nanoplastic-exposed plants. The disruption in electron flux in photosystems and electron transport system triggered by nanoplastics resulted in the excessive accumulation of ROS such as H_2O_2 producing oxidative damage to plant cells [12]. There are differences in H_2O_2 levels between PS and PMMA alone due to their surface potential and density. PS with positive zeta potential (zeta potential: 8.75 mV) is more toxic than PMMA (zeta potential: -8.24 mV) [79]. In the present study, this information was supported with the higher H_2O_2 levels in PS-applied plants that than of PMMA-treated ones. NOXinduced ROS production might be responsible for the high H_2O_2 levels in PS-treated *L. minor*. After As stress exposure, induvial applications of PS or PMMA indicated the positive effects on the enzyme activities such as SOD and POX, which facilitated the removal of ROS. It was in line with a report suggested by Wang et al. [10], who eliminated the phytotoxicity of cadmium in 100 mg L^{-1} PS-applied *Zea mays*, which was also related to a reduction in the cadmium uptake. Microplastic exposure can potentially impact the toxicity of heavy metals [80]. This trend might be related to the increased endogenous of beneficial nutrients (N, K, Ca, Mg and Mn) contents in *Lemna* plants under As + PS or As + PMMA. In this treatment group, these nutrients might be changed the interaction between PS/PMMA and As. Microplastics can adsorb the amounts of heavy metal on the surface [81]. Similarly, in our study, a decrement in endogenous contents of As(III) and As(V) might result in less toxic effects in *L. minor* under As + PS and As + PMMA compared to the As alone.

The toxic accumulation of H_2O_2 is prevented through the enzyme activity associated with Asada-Halliwell pathway [82]. In response to As stress, the increased APX activity mediated by induvial applications of PS or PMMA eliminated H_2O_2 accumulation by using AsA as a substrate. This expression was consistent with the increased activities of APX, MDHAR and total contents of AsA observed in our study. MDHA and DHA formed as a result of this reaction re-formed AsA by the catalysis of MDHAR and DHAR enzymes [65]. These findings indicated that PS or PMMA, when they were treated alone, provided AsA regeneration against As stress. In the presence of stress, the elevated levels of AsA play an important role as a potential antioxidant and regulator of signaling pathways [83]. GSH renewal, which is a tripeptide antioxidant with low molecular weight, also plays a role in eliminating the negative effects of stress [84]. PS or PMMA applications increased the activities/contents of GSSG, GR and DHAR in order to maintain cellular GSH redox balance when applied with As stress. When these findings were evaluated together with the induced activities of APX, GR, MDHAR and DHAR, *Lemna* plants exposed to PS/PMMA and As stress exhibited the promoted levels of AsA/DHA and, GSH/GSSG and the improved redox state of

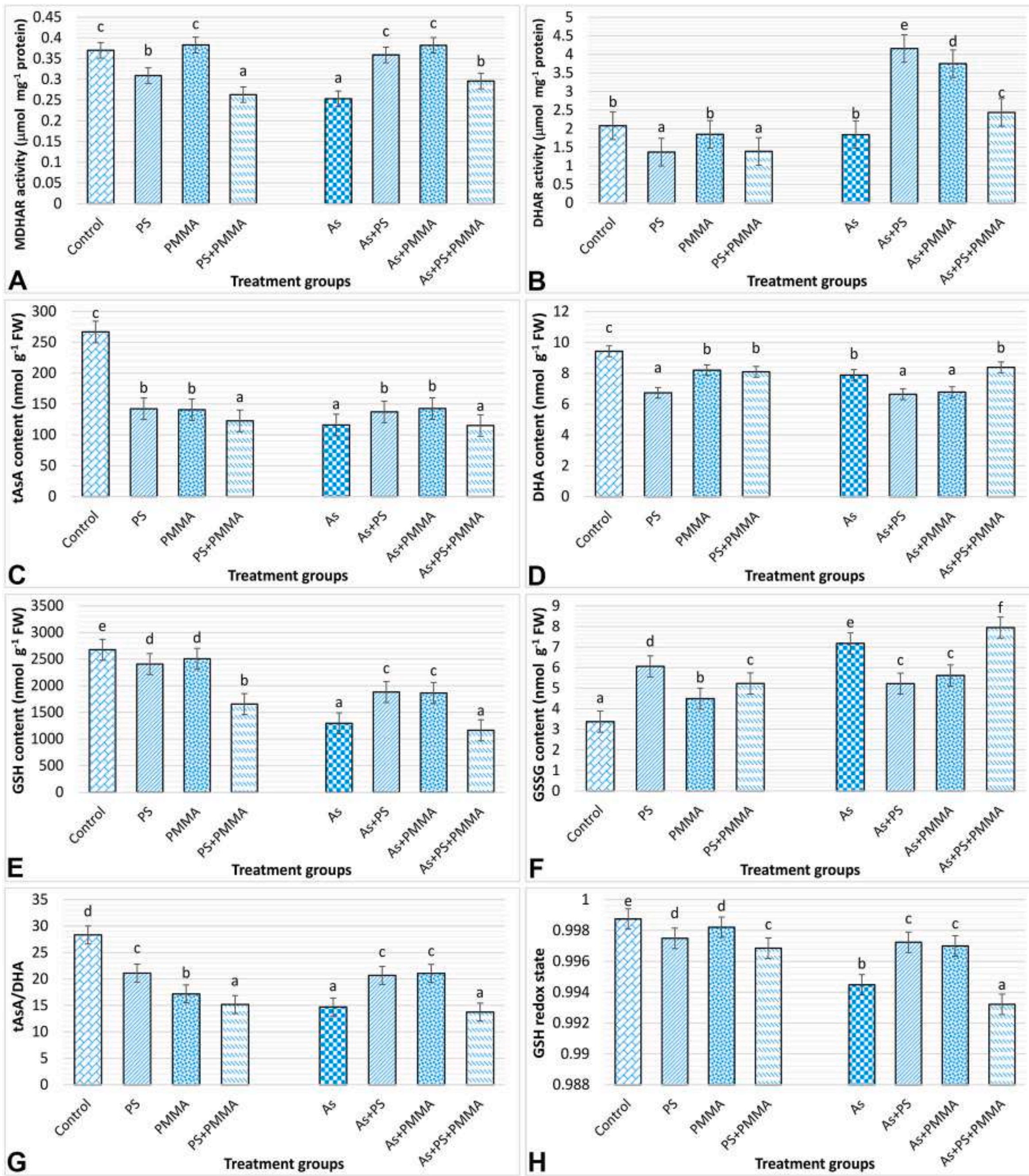

Fig. 11. The monodehydroascorbate reductase activity (MDHAR, A), dehydroascorbate reductase activity (DHAR, B), total ascorbate content (tAsA, C), dehydroascorbate content (DHA, D), glutathione content (GSH, E), oxidized glutathione content (GSSG, F), AsA/DHA (G) and GSH redox state (H) in *Lemna minor* under polystyrene (PS, 100 mg L^{-1}) and polymethyl methacrylate (PMMA, 100 mg L^{-1}) with or without arsenic stress (As, 100 μM).

GSH. The elevated GSH/GSSG and GSH pool are regulated through GR activity by using an electron from NADPH [85]. In the current study, due to the protection of GSH pool, PS or PMMA alone in stress-applied *Lemna* plants successfully removed H_2O_2 content by activating GST and GPX enzymes, which were eliminated H_2O_2 and organic hydroperoxides, as suggested by Zulfiqar and Ashraf [76]. Under As + PS and As + PMMA, the scavenging of ROS production induced by As stress was achieved via activating antioxidant system compared to As alone and subsequently, TBARS content decreased. As well as an antioxidant, Pro accumulation has a vital role in reducing oxidative damage induced by As stress and stabilizing electrolyte leakage [86]. Interestingly, in our study, Pro did not accumulate under PS or PMMA plus As stress. The protection in this group (As + PS and As + PMMA) might be the other osmoprotectants such as glycine betaine, choline, myo-inositol, polyols, sucrose and trehalose as showed by Anzano et al. [87].

After As exposure, both PS and PMMA applications caused an inactivation in SOD, CAT and regeneration of AsA and, GSH of *L. minor*. A combination of PS and PMMA showed increased phytotoxicity compared to either substance alone under As stress. PS and PMMA addition to *L. minor* under stress did not aid in recovering the lipid peroxidation and ROS accumulation. Wang et al. [70] reported that PS and phenanthrene were more toxic for *Oryza sativa*. When the pollution concentrations are high or the different pollutants are present, they will exceed the regulation ability of antioxidant enzyme system, as observed by Sun et al. [88]. In the present study, co-exposure of PS and PMMA in the presence of As reached the endogenous contents of As(III) and As(V) to the levels of stress-applied plants, which may be increased ROS accumulation and lipid peroxidation as a result. Interestingly, in this treatment group, significant declines in IAA, SA, CK and JA might be interacted with decreases in antioxidant enzyme activities. The components of hormone signaling regulates the function of stress-responsive transcription factors which plays roles in tolerance to stress conditions [89]. Li et al. [90] reported that antioxidant enzyme system was controlled by the phytohormone regulatory network in barley treated with nanoplastic toxicity.

5. Conclusion

The single or combined exposure of PS and PMMA decreased the accumulation of micro- or macronutrients such as N, P, K, Ca, Mg and Mn. In presence of As stress, PS or PMMA applications improved the uptake of N, K, Ca and Mn, but their combination did not increase any of the analyzed elements. The chlorophyll efficiency (F_v/F_m, F_v/F_o and F_o/F_m) and photochemistry of photosystems reduced by As stress were alleviated by PS or PMMA alone. This trend was not observed under As + PS + PMMA in *L. minor*. PS or PMMA together with stress exposures might be contributed to the hormone signaling such as IAA, GA, SA, CK and JA compared to the As stress alone, but not under As + PS + PMMA. The combined presence of PS and PMMA accumulated more the endogenous contents of As(III) and As(V) in *L. minor*. The antioxidant capacity impaired by As stress was improved through PS or PMMA treatments by activated SOD, POX, GST, GPX, up-regulated AsA/DHA, GSH/GSSG and redox state of GSH. The efficiency of the antioxidant system in As + PS + PMMA-applied *L. minor* was not enough to remove damage induced by As stress; hereby TBARS and H_2O_2 contents were similar to the As-treated group. All findings indicated that the alone application of PS or PMMA decreased lipid peroxidation and ROS accumulation by controlling beneficial nutrients, hormone contents and electron flux on PSI and PSII, as well as modulated antioxidant system.

Credit authorship contribution statement:

E.Y. and C.O.K. designed experiments; B.A. and F.N.A. carried out data analysis; M.T., H.C. and H.S. the PMMA characterization, C.O.K. interpreted the results and wrote up the first draft of the manuscript; C.O.K. and E.Y. critically edited the whole manuscript. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare no competing financial interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2023.01.015.

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