TOXICOLOGICAL ASSESSMENT OF CARBON NANOMATERIALS ON *LEMNA MINOR* L.: INSIGHTS INTO PHYSIOLOGICAL AND BIOCHEMICAL ALTERATIONS

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Abstract

Synthetic carbon-based nanomaterials, such as multi-walled carbon nanotubes (MWCNTs), carboxyl-functionalized nanotubes (MWCNTs-COOH), and fullerene soot, are increasingly being utilised in practical industrial and agricultural applications. This reality raises concerns about their potential unfavourable ecotoxicological impact on aquatic ecosystems where they may accidentally end up. In this context, the present research aimed to evaluate several physiological and biochemical responses of plants belonging to the species *Lemna minor* L. when interacting with these types of nanomaterials, experimentally added at two concentrations (50 and 200 mg/L) to their culture medium over a 14-day cultivation period.

The results obtained demonstrated the appearance in the test plants of functional effects dependent on the dose and nature of the tested nanomaterial, reflected by significant changes in photosynthetic performance (decreases in the content of photo-assimilatory pigments and the efficiency of photosystem II), as well as by the activation of biochemical markers of oxidative stress (increases in the content of flavonoids and polyphenols, changes in POD and SOD activities). The functionalized nanotubes (MWCNTs-COOH) induced the most pronounced biochemical responses, while fullerene soot had more moderate effects, possibly due to its reduced bioavailability in the cultivation media.

The results highlight the sensitivity of *Lemna minor* to chemical stress generated by synthetic carbon-based nanomaterials present in the cultivation medium, thus confirming its usefulness as a model organism in ecotoxicological studies and emphasising the need for rigorous assessments regarding the potential impact of these nanomaterials on aquatic plants in natural ecosystems, to lay the groundwork for responsible ecological management strategies.

Keywords: *Lemna minor*, carbon nanomaterials, oxidative stress, photosynthesis, aquatic ecotoxicology

Introduction

Nanotechnologies represent an emerging field of modern science with remarkable application potential in agriculture, biomedicine, industry, and environmental protection. Their relevance derives from the possibility of designing materials with specific physicochemical properties at the nanoscale (Fang et al. 2017, Patel et al. 2020, Mathew and Victório 2022). Carbon nanotubes (CNTs) stand out due to their unique tubular structure, small dimensions, superior electrical conductivity, and chemical stability, being widely used in laboratory experiments and innovative applications. In the agricultural sector, CNTs have been tested to promote plant growth by improving water and nutrient absorption, as well as serving as vectors for delivering genes and bioactive substances to plant organs (Tan et al. 2009, Jordan et al. 2020). However,

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the widespread use of these nanomaterials has raised concerns regarding their potential toxicity to biological systems, including plants. The effects of CNTs on plant organisms are often contradictory, ranging from stimulating physiological processes to inducing oxidative stress due to the production of reactive oxygen species (Mittler 2002, Chen et al. 2018, Ren et al. 2021, Samadi et al. 2021). In the context of increasingly intense anthropogenic pollution of terrestrial and aquatic ecosystems, synthetic carbon-based nanomaterials are emerging as a potentially omnipresent category of environmental contaminants. This trend is driven by their rapidly expanding industrial applications (Jackson et al. 2013).

In efforts to assess the impact of these nanomaterials on plants, morphological and physiological studies constitute an essential step in elucidating the response mechanisms of vegetation exposed to these compounds. Although numerous studies have investigated CNT interactions with microorganisms, protozoa, or algae, their effects on higher aquatic plants remain poorly understood. In this context, plants from the Lemnoideae subfamily, particularly specimens of the species *Lemna minor* L., are recognized as model organisms in ecotoxicity tests due to their favourable biological characteristics for practical research activities: rapid vegetative reproduction, pollutant bioaccumulation capacity, high ecological tolerance to pollutants, and an important role in phytoremediation (Nasu and Kugimoto 1981, Bokhari et al. 2016, Lanthemann and van Moorsel 2022).

Although species such as *Lemna minor* are well established in studies on wastewater remediation and in monitoring of classic contaminants (e.g., heavy metals, pesticides, dyes), specialised literature still highlights a lack of data on their interaction with CNT-type nanomaterials. Moreover, in the case of microplastic pollution, published results generally indicate a low morpho-physiological impact on *Lemna* colonies, despite their significant bioaccumulation potential. These findings underscore the need to expand knowledge on the effects of CNMs on aquatic plants, particularly concerning the structure and function of their photosynthetic apparatus, redox homeostasis, and overall metabolic dynamics. Given this context, the present study investigates physiological and biochemical responses of *Lemna minor* exposed to synthetic carbon-based nanomaterials, in experimental conditions simulating possible contamination scenarios of natural aquatic environments. By addressing these aspects, the results aim to clarify the bioindicator potential of *Lemna minor* in relation to emerging pollutants and contribute to a better understanding of the ecotoxicological risks associated with the uncontrolled release of nanomaterials into freshwater ecosystems.

Materials and Methods

Nanomaterials

Multi-walled carbon nanotubes (MWCNTs) with an outer diameter of 8 nm (purity > 96%, product code NG01MW0101) and carboxylated MWCNTs (MWCNTs-COOH) with an outer diameter of 8–18 nm (purity > 96%, product code NG01MW0303) were purchased from Nanografi (Ankara, Turkey), while fullerene soot, consisting of a mixture of C_{60}, C_{70} fullerenes, and carbon black (product code 572497-5G), was obtained from Sigma-Aldrich.

Cultivation of *Lemna minor* L. plants

For the experiments, the cultivation protocol for *Lemna minor* L. species followed the recommendations of OECD guideline No. 221 regarding growth tests with *Lemna* spp. (OECD 2006). The base culture was maintained under controlled laboratory conditions (24 ± 2°C, artificial lighting for 17 h/day, light intensity 115–118 μmol m^{-2} s^{-1}), being periodically transferred to fresh media to avoid nutrient depletion and accumulation of residual metabolites. To obtain experimental cultures with reduced phenotypic variability, secondary stock cultures were created starting from a single individual (monoclonal cultures). From these, the plants used in the exposure tests were selected, each cultivation well with culture medium (10 ml volume) receiving a single individual with three fronds. The wells were placed in trays covered with black paint to limit algae development and were incubated at 25°C, with the same lighting regime as previously described, for a cultivation period of 14 days. To ensure the reproducibility and biological relevance of the test, which requires a uniform dispersion of nanoparticles without their aggregation, the CNMs were ultrasonicated. The culture medium in the experimental variants was supplemented with concentrations of 50 and 200 mg/L of each type of CNM tested, and the control variants contained only the simple culture medium. The concentrations were selected based on values commonly applied in ecotoxicological studies with nanomaterials, ensuring both comparability with existing literature and experimental reproducibility. This range was intended to simulate possible contamination scenarios in aquatic ecosystems while providing insights into potential dose-dependent physiological and biochemical effects in *Lemna minor*. Plant development was monitored every 2–3 days throughout the cultivation period, with physiological and biochemical determinations performed at the end (14 days).

Determination of stomatal density

Stomatal density was evaluated by scanning electron microscopy (SEM) on intact leaves/fronds collected at the end of the experiment, selected from morphologically and ontogenetically equivalent regions of all experimental variants. Samples were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 hours at room temperature, then rinsed and dehydrated in a graded series of acetone (30–100%). After critical point drying with liquid CO_2, fragments were mounted on metal supports with conductive adhesive tape and coated with gold (~10 nm) by sputtering (Pathan et al. 2010). Observations were made with a Tescan Vega II SBH SEM microscope at 30 kV, analysing at least three biological replicates per variant and at least three fields per replicate. Stomatal density (stomata/mm²) was calculated based on SEM micrographs with known magnification (Xu and Zhou 2008).

Determination of chlorophyll fluorescence

The functioning of the photosynthetic apparatus of the test plants was evaluated by determining the quantum efficiency of photosystem II (ΦPSII), using a Hansatech FMS II portable fluorimeter. Measurements were made by bringing the device's optical fibre to about 1 cm from the surface of the leaves/fronds, with five readings taken for each experimental variant.

Obtaining plant extracts for biochemical analyses, the leaves/fronds were homogenised in a mortar with quartz sand and 10 ml of solvent (96% ethanol for quantifying photo-assimilatory pigments and secondary metabolites; TRIS-HCl for determining enzymatic activity - SOD and POD and for quantifying protein content), according to the methods described by Wellburn and Artenie (Wellburn 1994, Artenie et al. 2008). The resulting plant extracts were centrifuged for 15 minutes at 4000 rpm at a temperature of 4°C. Determinations were performed in technical triplicate for each of the three cultivated biological replicates, and the results were expressed as arithmetic means.

Determination of photo-assimilatory pigment content

The content of chlorophyll a, chlorophyll b, and carotenoid pigments was determined spectrophotometrically, with readings at wavelengths of 664, 648, and 470 nm of the plant extracts (Wellburn 1994, Zhao et al. 2017). The calculation of pigment concentrations was performed using standard formulas:

$$\text{Chl a} = 13{,}36 \times A_{664} - 5{,}19 \times A_{648}$$

$$\text{Chl b} = 27{,}43 \times A_{648} - 8{,}12 \times A_{664}$$

$$\text{Cx+c} = (1000 \times A_{470} - 2{,}13 \times \text{Chl a} - 97{,}64 \times \text{Chl b}) / 209$$

Total polyphenol content was quantified using the Folin-Ciocalteu method (Herald et al. 2012). Plant extracts were treated with Folin-Ciocalteu reagent and 7.5% sodium carbonate,

and their absorbance was read spectrophotometrically at λ= 765 nm. The content was expressed in mg gallic acid equivalents (GAE)/g fresh material, using a standard curve.
Total flavonoid content was determined by the aluminium chloride method (Herald et al. 2012), by measuring the absorbance of treated plant extracts at λ = 510 nm. Results were expressed in mg quercetin equivalents (QE)/g fresh material.
Antioxidant activity was evaluated using the DPPH test (Sharma and Bhat 2009, Behrendorff et al. 2013). Plant extracts were incubated with 60 µM DPPH solution, and their absorbance was read after 3 hours of incubation at λ = 517 nm. Antioxidant capacity was expressed as percentage inhibition of DPPH radicals, compared to an ascorbic acid control.
Superoxide dismutase (SOD) activity was determined according to the Nitro Blue Tetrazolium (NBT) method, by inhibiting NBT photoreduction by superoxide radicals generated with riboflavin (Artenie et al. 2008). Absorbance of plant extracts was measured spectrophotometrically at λ = 560 nm, and SOD activity was expressed in active enzyme units/gram fresh material.
Peroxidase (POD) activity was determined according to the Winterbourn method adapted by Artenie et al. (Winterbourn et al. 1975, Artenie et al. 2008), by measuring the oxidation of ortho-dianisidine under the action of peroxidase and H_2O_2. Absorbance of plant extracts was determined spectrophotometrically at λ = 540 nm and expressed in POD units/minute/gram fresh material.
Protein content was assayed using the Bradford method (Artenie et al. 2008), with spectrophotometric readings at λ = 595 nm. Results were reported against a standard curve built with bovine serum albumin (BSA).
Statistical analysis of the experimental data was performed using one-way ANOVA with Tukey's post hoc test ($p < 0.05$).

Results and discussions

Stomatal density
Exposure of *Lemna minor* plants to multi-walled carbon nanotubes (MWCNTs) resulted in a moderate increase in their stomatal density, dependent on the tested dose, alongside a significant reduction of individual stomatal surface area (Figure 1). This phenomenon suggests an adaptive mechanism of stomatal miniaturisation in test plants under the abiotic stress conditions. Similar but more pronounced effects were observed with MWCNTs-COOH treatment, indicating an amplified phytotoxic potential of carboxyl groups (Verneuil et al. 2015). Contrary to these trends, fullerene soot treatment reduced stomatal density but increased their surface area, reflecting an alternative compensatory mechanism to maintain optimal gaseous exchange in duckweed plants exposed to these synthetic carbon-based nanomaterials in the cultivation environment. These stomatal modifications highlight the capacity of *Lemna minor* to register structural changes under nanomaterial-induced stress, reinforcing its utility as a morphological bioindicator for detecting sublethal effects of emerging aquatic pollutants, in line with specialised literature reporting stress-induced stomatal patterns characterised by increased density and reduced dimensions. (Xu and Zhou 2008, de Morais et al. 2019).
Chlorophyll fluorescence (ΦPSII)
The efficiency of photosystem II progressively decreased in the test plant leaves/fronds during the experiment, with the most severe effect observed in the MWCNTs-COOH treatment at a concentration of 200 mg/l (Figure 2A). At lower doses, MWCNTs-COOH treatment induced reduced toxicity in *Lemna minor* plants, likely due to better dispersion and lower particle aggregation in the growth medium. Fullerene soot caused only moderate declines in ΦPSII, suggesting limited physical interaction with chloroplasts in the plant's vegetative structures. The decline in ΦPSII efficiency confirms the sensitivity of *Lemna minor* to photochemical

disruption caused by carbon nanomaterials. Such measurable changes in photosynthetic performance can validate its role as a physiological bioindicator in ecotoxicological studies (Chen et al. 2018, Ozfidan-Konakci et al. 2022).

Photo-assimilatory pigments content

Treatments with MWCNTs and MWCNTs-COOH caused quantitative decreases in chlorophylls and carotenoid pigments in *Lemna minor* leaves/fronds, proportional to the applied dose (Figure 2B), indicating varied phytotoxic effects. Fullerene soot at 200 mg/l induced the most pronounced decline in photo-assimilatory pigments in test plants. This effect likely results from either reduced light penetration caused by surface particle aggregation or intensified oxidative stress impacting pigment-producing cells. Dose-dependent pigment reductions reveal *Lemna minor*'s sensitivity to light stress and oxidative damage, confirming its value as a bioindicator of environmental stress (Lang et al. 2019, Subotić et al. 2022).

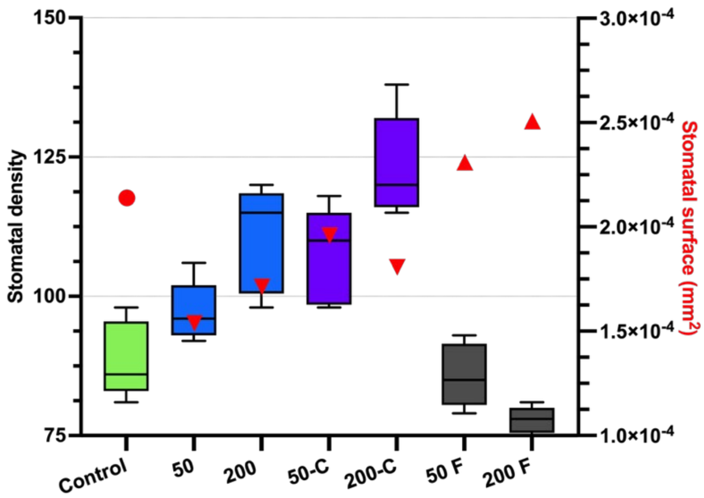

Figure 1. Stomatal density of *Lemna minor* L. individuals cultivated under experimental conditions. Variants: Control, 50 - 50 mg/l MWCNT, 200 – 200 mg/l MWCNT, 50-C – 50 mg/l MWCNT-COOH, 200-C - 200 mg/l MWCNT-COOH, 50 F – 50 mg/l Fullerene soot, 200 F – 200 mg/l Fullerene soot. Statistically significant differences are observed in the case of 50, 200, 50-C, 200-C, and 200F. The stomatal area is marked on the secondary axis (in red) (● - control, ▲ - significant increase in stomatal area, ▼ - significant decrease in stomatal area).

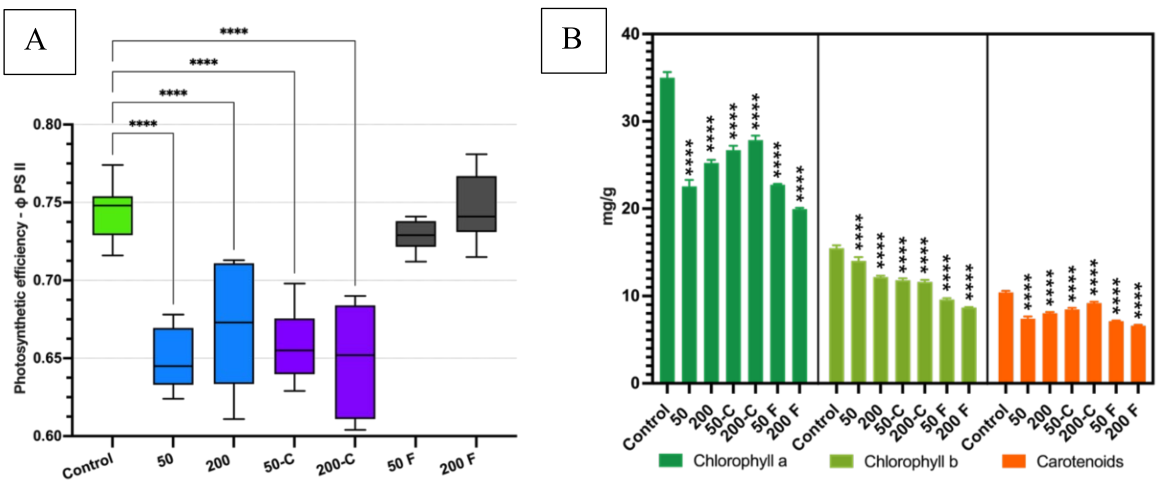

Figure 2. A-Efficiency of the photosynthetic apparatus, B-Assimilatory pigment content of *Lemna minor* L. individuals cultivated under experimental conditions. Variants: Control, 50 - 50 mg/l MWCNT, 200 – 200 mg/l MWCNT, 50-C – 50 mg/l MWCNT-COOH, 200-C - 200

mg/l MWCNT-COOH, 50 F – 50 mg/l Fullerene soot, 200 F – 200 mg/l Fullerene soot. Statistically significant differences are marked with *.

Flavonoid content

MWCNTs-COOH treatments significantly stimulated flavonoid biosynthesis in test plants, particularly at the concentration of 200 mg/l (Figure 3A), indicating an intense response to experimentally induced oxidative stress in the cultivation medium. Unfunctionalized MWCNTs and fullerenes caused a decrease in these compounds, demonstrating a weak effect; the obtained results are consistent with data presented in specialised literature (Chen et al. 2020).

Polyphenol content

All tested nanomaterials experimentally added to the cultivation medium of *Lemna minor* plants induced the biosynthesis of high polyphenol content (Figure 3B), with MWCNTs-COOH treatment at 200 mg/l concentration standing out among experimental variants. The high levels of polyphenol content suggest an important antioxidant role of this group of compounds in ROS detoxification in test plants. Fullerene soot had a weak effect, likely due to its low solubility. (Gohari et al. 2020). Enhanced flavonoid and polyphenol accumulation in *Lemna minor* under nanomaterial stress indicates antioxidant mechanisms activation, highlighting its sensitivity to redox imbalance and utility as a bioindicator of oxidative stress in aquatic systems.

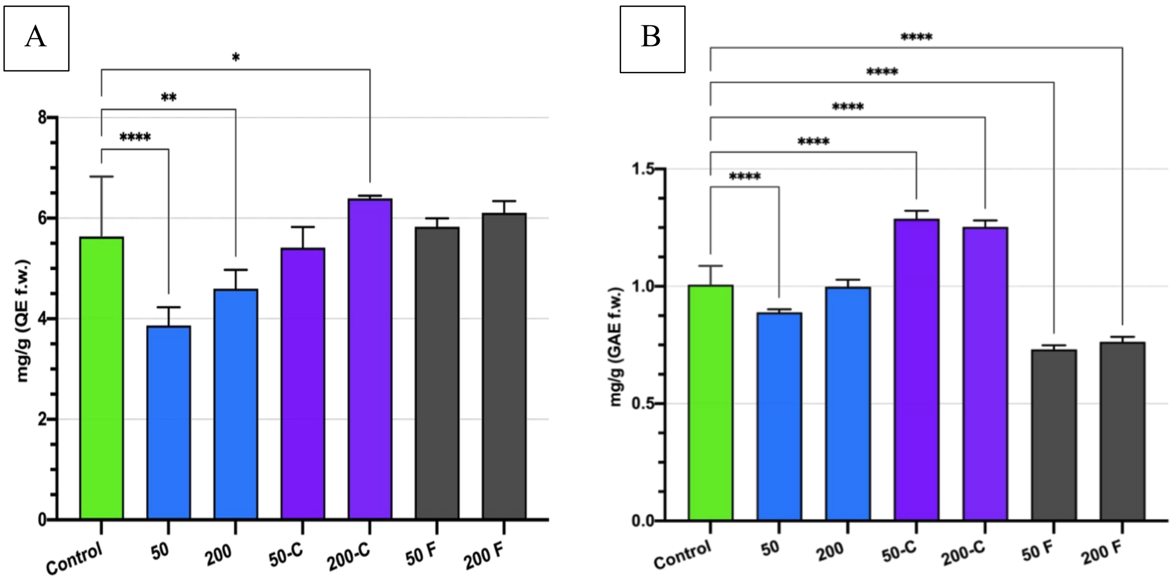

Figure 3. A-Flavonoid content, B-Polyphenol content of *Lemna minor* L. individuals cultivated under experimental conditions. Experimental variants: Control, 50 - 50 mg/l MWCNT, 200 – 200 mg/l MWCNT, 50-C – 50 mg/l MWCNT-COOH, 200-C - 200 mg/l MWCNT-COOH, 50 F – 50 mg/l Fullerene soot, 200 F – 200 mg/l Fullerene soot. Statistically significant differences are marked with *.

Peroxidase activity (POD)

Treatments with MWCNTs and MWCNTs-COOH reduced POD activity, especially in variants with 200 mg/l concentration (Figure 4A), indicating inhibition of the enzymatic antioxidant system in test plants. In the case of fullerene soot testing, POD values remained close to control levels, suggesting moderate stress manifestation in *Lemna minor* plants and conserved enzymatic function (Dietz and Herth 2011).

Superoxide dismutase activity (SOD)

All tested nanomaterials induced decreased SOD activity in test plants (Figure 4B), with the most severe effect observed in treatments with 200 mg/l concentrations. This SOD activity inhibition indicates a possible major redox imbalance in plant structures and an inability to efficiently detoxify the superoxide (Gill and Tuteja 2010).

Soluble protein content

Non-functionalized and functionalized carbon nanotubes caused a decrease in protein content of *Lemna minor* plants (Figure 5), more pronounced in treatment variants with a concentration of 200 mg/l. Fullerene soot induced a hormetic effect: a decrease in protein content at 50 mg/l and a partial recovery at 200 mg/l, suggesting compensatory adaptation mechanisms of test plants to experimentally induced abiotic stress in the cultivation medium (Calabrese and Agathokleous 2021). Reduced POD and SOD activities together with protein depletion reveal impaired antioxidant and metabolic functions in *Lemna minor*, reinforcing its value as a biochemical bioindicator of nanomaterial-induced stress.

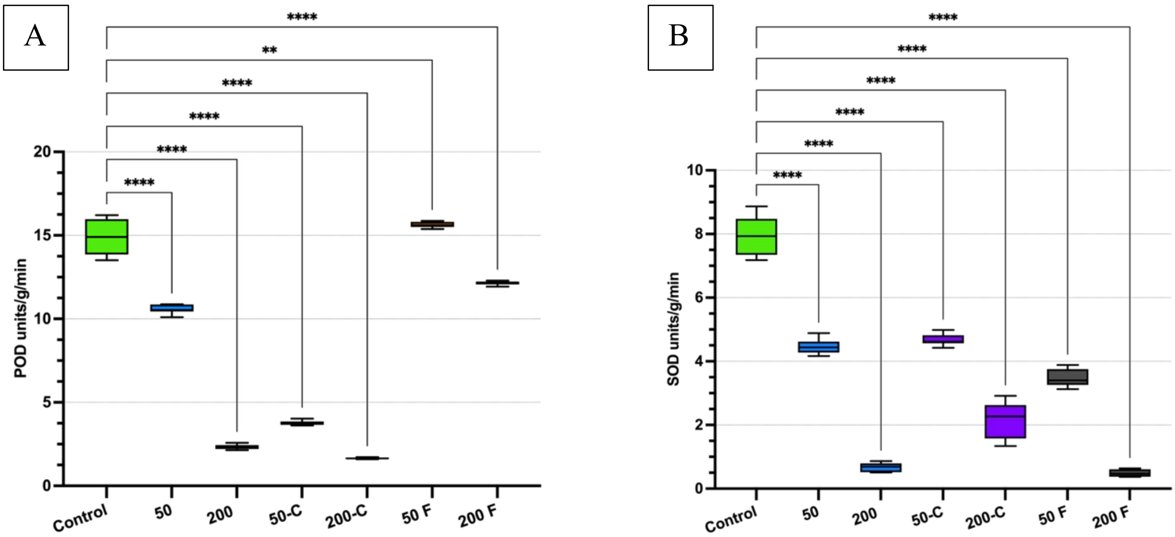

Figure 4. A-POD activity, B-SOD activity of *Lemna minor* L. individuals cultivated under experimental conditions. Experimental variants: Control, 50 - 50 mg/l MWCNT, 200 – 200 mg/l MWCNT, 50-C – 50 mg/l MWCNT-COOH, 200-C - 200 mg/l MWCNT-COOH, 50 F – 50 mg/l Fullerene soot, 200 F – 200 mg/l Fullerene soot. Statistically significant differences are marked with *.

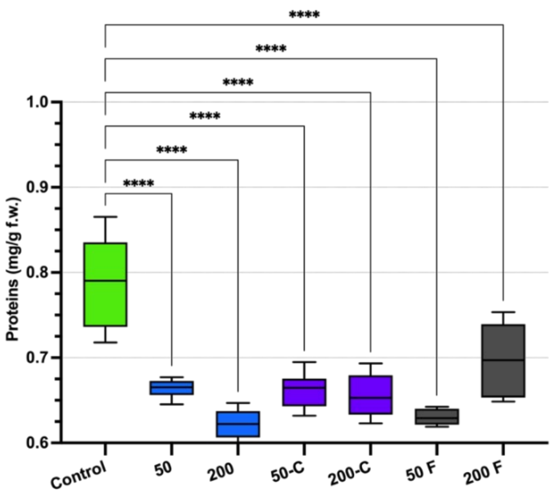

Figure 5. Soluble protein content of *Lemna minor* L. individuals cultivated under experimental conditions. Experimental variants: Control, 50 - 50 mg/l MWCNT, 200 – 200 mg/l MWCNT, 50-C – 50 mg/l MWCNT-COOH, 200-C - 200 mg/l MWCNT-COOH, 50 F – 50 mg/l Fullerene soot, 200 F – 200 mg/l Fullerene soot. Statistically significant differences are marked with *.

Total antioxidant activity

This biochemical parameter increased significantly in the MWCNTs and MWCNTs-COOH treatment variants at a concentration of 200 mg/l, confirming the induction of systemic oxidative stress in plant structures by these nanomaterials (Figure 6). The highest values of total antioxidant activity were observed when applying the MWCNTs-COOH treatment at a dose of 200 mg/l, while fullerene soot generated a nonlinear response, indicating the influence of nanoparticle aggregation on their bioavailability and absorption in test plants. The alteration of antioxidant activity under high-dose treatments demonstrates systemic stress response mechanisms. This parameter provides a robust biochemical signal, confirming *Lemna minor*'s effectiveness in early detection of nanomaterial-induced toxicity. (Zhang et al. 2017).

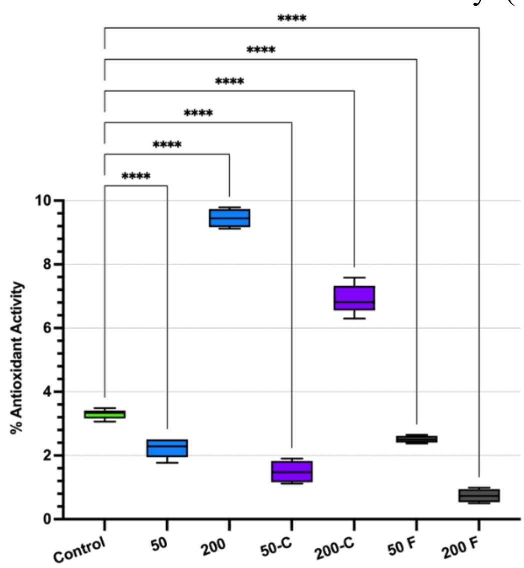

Figure 6. Total antioxidant activity of *Lemna minor* L. individuals cultivated under experimental conditions. Experimental variants: Control, 50 - 50 mg/l MWCNT, 200 – 200 mg/l MWCNT, 50-C – 50 mg/l MWCNT-COOH, 200-C - 200 mg/l MWCNT-COOH, 50 F – 50 mg/l Fullerene soot, 200 F – 200 mg/l Fullerene soot. Statistically significant differences are marked with *.

Conclusions

The results highlight a visible phytotoxicity, manifested across multiple physiological and biochemical levels, of the tested synthetic carbon-based nanomaterials – multi-walled carbon nanotubes (MWCNTs), carboxyl-functionalized nanotubes (MWCNTs-COOH), and fullerene soot – on the aquatic species *Lemna minor* L. The effects produced on the test plants are dependent on the chemical nature of the nanomaterials and the applied dose, confirming that these nanoparticles profoundly influence plant metabolism and the functional integrity of their photosynthetic apparatus.

Stomatal modifications, reduction of assimilatory pigment content, and decreased photosystem II efficiency (ΦPSII) demonstrate severe impairment of photosynthetic performance in duckweed plants exposed to high concentrations of MWCNTs and MWCNTs-COOH, indicating disruptions in electron transport and degradation of chloroplast structures caused by oxidative stress occurring in plant tissues. In the same context, the increased accumulation of phenols and flavonoids in test plants, as an effect of MWCNTs-COOH treatments, suggests a compensatory activation of their antioxidant defences, while fullerene soot generated weaker metabolic responses, possibly due to its limited interaction with plant tissues. These results outline a specific response pattern of test plants to the experimentally induced abiotic stress in the aquatic environment, where effect severity is dictated by dose and nanomaterial surface chemistry, with photochemical and biochemical indicators representing potential reliable markers of these nanoparticles' phytotoxicity in aquatic environments. In the context of increasingly frequent use of functionalized nanomaterials across various fields, the obtained results emphasise the importance of rigorous assessment of these nanoparticles' impact on aquatic ecosystems. The experimental evidence presented in this study consolidates the role of *Lemna minor* as a highly responsive bioindicator for aquatic nanotoxicology. Its ability to exhibit measurable and dose-dependent changes in stomatal patterning, pigment composition, photosynthetic efficiency, antioxidant activity, and enzymatic function underlines its diagnostic value in detecting sublethal stress induced by carbon-based nanomaterials. The present findings underscore the necessity of implementing stringent regulatory frameworks governing the practical use of nanomaterials in order to prevent their accidental or uncontrolled release into the environment.

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