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Single-walled carbon nanotubes for enhanced photosynthesis and high-value compound production in microalgae and cyanobacteria

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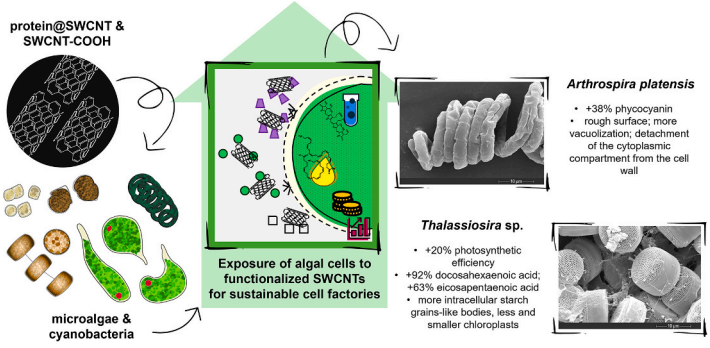
HIGHLIGHTS

- Lysozyme and histone well-disperse single-walled carbon nanotubes (SWCNT) in water.
- SWCNT functionalized with carboxylic acids (COOH-SWCNT) are well-dispersed in water.
- LSZ@SWCNT, HST@SWCNT and COOH-SWCNT are not toxic for phototrophs at 1 mg L^{-1}.
- Photosynthetic efficiency and growth increase after phototroph exposure to LSZ@SWCNT.
- ω-3 fatty acids and phycocyanin increase after phototroph exposure to LSZ@SWCNT.

GRAPHICAL ABSTRACT



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ABSTRACT

Carbon nanomaterials such as single-walled carbon nanotubes (SWCNTs) can improve algal photosynthetic efficiency and increase the production of valuable compounds without decreasing biomass productivity. This work investigates the effects of SWCNTs dispersed by different proteins or functionalized with carboxylic groups (SWCNT-COOH) on several microalgae and a cyanobacterium, aiming to enhance photosynthetic performance and produce valuable compounds. The best SWCNTs' dispersion in water was achieved with lysozyme

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ω-3 fatty acids
Phycobiliproteins

(LSZ@SWCNT), histone (HST@SWCNT) and SWCNT-COOH. In most cases, no toxicity was observed when the phototrophs were exposed for 72 h to these SWCNTs. The cyanobacterium *Arthrospira platensis* and the marine diatom *Thalassiosira* sp. were selected for evaluating the effects of exposure to LSZ@SWCNT for up to 14 days. Increased production of valuable algal compounds (i.e., phycocyanin + 38 % in *A. platensis* after 14 days, docosahexaenoic acid + 92 % and eicosapentaenoic acid + 63 % in *Thalassiosira* sp. after 7 days) was observed without any impairment of the photosynthetic efficiency (+19 % for *Thalassiosira* sp.). The effects observed on both the cell surface and intracellular structures (i.e., cell wall modifications, increased mucus, and vacuolization) suggested that the interaction with LSZ@SWCNT was responsible for the changes in biochemical composition and photosynthetic performance. Therefore, the proposed nanobiotechnological approach, which couples cyanobacteria and microalgae with SWCNTs, may tune the photosynthetic pathways towards the production of high-value compounds exploitable in cosmetics and nutraceuticals, ultimately improving the light-to-chemicals conversion processes without negatively impacting growth.

1. Introduction

Microalgae are a source of biomass that can be employed in a variety of commercial applications, from food and feed to nutraceuticals and cosmetics; microalgae of commercial relevance are considered sunlight-driven cell factories of metabolites with high technological interest and high market value (e.g., antioxidants, pigments, lipids, and vitamins), having additional benefits of growing without the need of (agricultural) land or use of pesticides, the possibility of coupling biomass and biomolecule production with other activities (e.g., wastewater treatment, CO_2 sequestration), and a minor dependence on climatic conditions (Balan et al., 2023). However, most bioprocesses based on natural photosynthesis have low solar energy-to-specific product conversion efficiency, thus resulting in poor process selectivity towards valuable compounds. The production of these molecules can be tuned by changing cultivation conditions during specific growth phases; indeed, it is well-known that microalgae can readily adapt to fluctuations of salinity, temperature, pH value, and illumination by promoting some metabolic pathways rather than others. Therefore, the carbon flux derived from photosynthesis can be directed predominantly towards specific molecules, maximising their concentrations and facilitating their recovery (the hyperaccumulation approach). However, the metabolic imbalance obtainable by diverting the electron energy towards a selected target molecule and away from other components is generally incompatible with multiple-product biosynthesis and high biomass productivity (the so-called "protein *vs* lipid dilemma" (Gifuni et al., 2019)). This issue contributes to even worsening the limited biomass productivity that generally characterized microalgal cultivation ($\leq$ 3 g L^{-1}, while at least a 10-fold higher concentration would be necessary for efficient bioreactor exploitation and downstream operations), with a cascade effect on the downstream processes, including the harvesting phase (i.e., dewatering diluted cultures, use of flocculants that cannot be removed from the collected biomass, centrifugation), and the overall costs. Many efforts have been focused on improving biomass productivity by optimising cultivation conditions, designing advanced cultivation systems, applying genetic engineering, or improving photosynthetic efficiency. In particular, the optimisation of light utilisation represents a critical issue in microalgal biotechnology, as only a small fraction of the wide spectrum of solar radiation is used for photosynthesis by microalgae, but, at the same time, an optimal balance between photosynthesis and photoprotection must be maintained. In this context, a possible solution to increase the selectivity without compromising the growth is the development of hybrid photosynthesis systems, comprising the "living" catalysts (microorganisms or microalgae) and inorganic devices like nanomaterials, which are aimed at optimizing specific phases of the photosynthesis process, such as light harvesting, thus generating surplus energy that could be more efficiently diverted towards the production of valuable chemicals. In particular, the use of low doses of carbon-based nanomaterials (CNMs) has been recently speculated as an innovative approach to improve the cultivation of microalgae and cyanobacteria, by boosting their photosynthetic performance and the synthesis and accumulation of high-value compounds (Agarwal et al., 2022; Aratboni et al., 2023; Lambreva et al., 2015; Lau et al., 2022; Patnaik et al., 2024; Sumathi et al., 2024). CNMs can promote microalgal growth due to hormetic responses, thus improving the production yields and positively impacting the economic efficiency and the sustainability of the whole process. However, it is known that the hormetic responses are only valid when microalgae are exposed to low CNM concentrations since high CNM doses partially or fully inhibit the growth through multiple negative impacts generally due to shading effects (i.e., agglomeration of CNMs-microalgae that reduces the light dose needed for photosynthetic activities), internalization and binding to organelles (with the consequent reduction or inhibition of the metabolic pathways (Wang et al., 2019)), and membrane damage with possible cellular content release, alteration of the membrane permeability, or active site alteration (Moreno-Garrido et al., 2015). CNMs are a family of diverse materials containing carbon and having one critical dimension in the range of 1–100 nm, which include fullerenes, graphene, carbon dots, and carbon nanotubes, i.e., CNTs (Freixa et al., 2018). CNTs consist of one-to-multiple cylindrical hollow-shaped rolled sheets of graphene, and, depending on the number of cylindrical walls, are classified as single (SWCNTs), double (DWCNTs), and multi-walled (MWCNTs) carbon nanotubes (Freixa et al., 2018). Among them, SWCNTs have a diameter of up to 2 nm and can reach lengths of hundreds of nm, thus being one of the most anisotropic materials ever discovered. The chirality of SWCNTs defines their physicochemical properties, which can be tuned accordingly, and depending on their chiral indexes, SWCNTs can be metals or semiconductors, influencing the interaction of these materials with light. One of the main constraints of applying SWCNTs in biological systems (e.g., microalgae) is their insolubility in an aqueous environment. Both covalent and non-covalent derivatisation with amphiphilic dispersants have proven to be a suitable approach to overcome this limitation; the non-covalent approach allows the preservation of the chemical structure of the SWCNTs, without affecting their peculiar optical and electrical properties (Calvaresi and Zerbetto, 2013). The functionalization of SWCNTs with neutral, negatively or positively charged dispersants such as proteins directly affects the potential interaction of such nanotubes with phototrophs (Antonucci et al., 2023, 2022). SWCNTs can absorb radiation along all the visible and infrared regions of the spectrum, and variations in both the absorption and emission spectra can be found depending on their electronic properties (Nepal and Geckeler, 2006). Thanks to their wide absorption spectrum, SWCNTs may act as a supplementary "antenna" for photosynthetic organisms promoting electron transport (Antonucci et al., 2023, 2022; Giraldo et al., 2014; Lambreva et al., 2015), as well as having a protective role against photoinhibition (Antal et al., 2022); hence, SWCNTs may have a potential upcoming role in algal biotechnology, (Patnaik et al., 2024; Sumathi et al., 2024) bioelectricity (Antonucci et al., 2023, 2022), and in the development of living hybrid systems aimed at the production of high-value molecules. Despite these encouraging outcomes, the true mechanisms behind the changes in the photosynthetic machinery of microalgae and cyanobacteria exposed to SWCNTs remain poorly understood (Antal et al., 2022; Lambreva et al., 2015; Lau et al., 2022). Indeed, exposure to SWCNTs can also negatively

influence microalgae, directly or indirectly affecting their growth, cell viability, enzymatic activity, photosynthesis, and membrane integrity (Freixa et al., 2018; Saxena et al., 2020). These negative effects mainly depend on the nanotubes' type, concentration, and exposure time and are likely caused by adsorption, shading, ion release, cell wall disruption, and endocytosis (Lau et al., 2022). Non-toxic and growth/photosynthesis-promoting effects (e.g., improved photosynthetic efficiency, CO_2 uptake, lipid, and polyunsaturated fatty acid accumulation) have been generally reported for concentrations not exceeding 10–15 mg L^{-1} (Aratboni et al., 2023; Freixa et al., 2018; Saxena et al., 2020). Below this threshold, hormesis in phototrophs-CNTs systems is expected, which might cause a sort of "adrenaline rush" in the organism in response to temporary stress, thus stimulating growth and boosting the production of high-value products (Lau et al., 2022).

The present work investigated the potential of using SWCNTs to boost photosynthetic performance and increase both the biomass productivity and synthesis of high-value biomolecules by microalgae and cyanobacteria, to develop efficient cell factories alternative to less productive/non-sustainable sources of bioactive compounds (e.g., fish oil for ω-3 fatty acids). For this purpose, various microalgae or cyanobacteria were exposed to CoMoCAT® SWCNTs dispersed with several proteins (i.e., protein@SWCNT) or carboxylic acid-functionalized SWCNTs (i.e., SWCNT-COOH) to find the best response regarding algal viability, photosynthetic efficiency, biomass productivity, and production of high-value compounds like ω-3 polyunsaturated fatty acids and phycobiliproteins. To our knowledge, this is the first study in which hybrid systems composed of microalgae or cyanobacteria and SWCNTs are investigated, pursuing such multiple objectives.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals, solvents, and analytical standards used in the present work were of analytical grade and were obtained from Sigma-Aldrich or Thermo Fisher. Commercially available SWCNTs have been employed in the present work. CoMoCAT® SWCNTs were purchased from Sigma-Aldrich (diameter/median length: 1–2 nm/1 μm). Carboxylic acid-functionalized (COOH–) SWCNTs were purchased from Cheap Tubes (diameter/median length: 4–5 nm/0.5–1.5 μm). Freeze-dried proteins used for SWCNTs' dispersion were purchased from Sigma-Aldrich, namely lysozyme from chicken egg white (LSZ), bovine serum albumin (BSA), myoglobin from equine heart (MGB), histone (HST), and protamine from salmon (PRT).

2.2. Dispersion of SWCNTs with proteins and stability in water and algal growth media

CoMoCAT® SWCNTs functionalisation was performed by dissolving each protein in 1 mL of Milli-Q water to obtain a 1 mM solution, and then 1.5 mg of SWCNT powder was added to the solution. After sonication for 60 min using a probe tip sonicator (UP200st Ultrasonic Processor, Hielscher Ultrasonics, Berlin, Germany) at 35 % of the maximum amplitude of the sonotrode (S26d7), in an ice bath, the samples were centrifuged for 10 min at 10000 × g at 20 °C. The supernatant was collected and successively used as a stock solution (Calvaresi and Zerbetto, 2013; Di Giosia et al., 2019; Paradisi et al., 2023). SWCNT-COOH were directly suspended in Milli-Q water to reach a concentration of 0.1 mg mL^{-1}, and used as a stock solution, without adding proteins in agreement with the literature (Magnabosco et al., 2020). To assess the stability of protein@SWCNT hybrids, a 100-fold dilution was prepared for each protein@SWCNT stock solution in Milli-Q water and algal or cyanobacterial growth media. Afterwards, all the samples were characterised spectrophotometrically in the range 230–1100 nm at increasing time (i.e., 0, 12, and 24 h), using a Cary60 UV–Vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA).

2.3. Exposure of microalgae and cyanobacteria to SWCNTs

Three exposure tests were conducted to investigate the interaction between SWCNTs and microalgae or cyanobacteria. The experimental setup is summarised below:

- **Selection of the phototrophs**. *Euglena gracilis* (Euglenophyceae, euglenoid, freshwater, strain SAG 1224–5/25), *Scrippsiella* sp. (Dinophyceae, dinoflagellate, seawater), *Thalassiosira* sp. (Mediophyceae, diatom, seawater), *Stephanodiscus hantzschii* (Mediophyceae, diatom, freshwater, strain CCAP 1079/4), and *Arthrospira platensis* (Cyanophyceae, cyanobacterium, alkaline freshwater) were selected as the tested species. Each tested strain was obtained from international culture collections or locally isolated from Italian inland freshwaters and the Northern Adriatic Sea (see the strain characteristics and cultivation in the Supplementary materials). The species were exposed for 72 h to LSZ@SWCNT at the concentrations of 0.1, 1, and 10 mg L^{-1} in 6-well sterile plates (volume of 7 mL) and with an optical density of 0.1. The organisms less affected by the presence of LSZ@SWCNT were selected for SWCNT screening to investigate further the effect of different types of SWCNTs and increasing exposure times.
- **Selection of the SWCNTs**. *Thalassiosira* sp. and *A. platensis* were exposed for 7 days to three different SWCNTs, LSZ@SWCNT, HST@SWCNT, and SWCNT-COOH, tested at a concentration of 1 mg L^{-1} in 6-well sterile plates (volume of 7 mL) and with an optical density of 0.1.
- **Investigation of the effects of LSZ@SWCNT on *Thalassiosira* sp. and *A. platensis***. The cultivation of *Thalassiosira* sp. and *A. platensis* in the presence of LSZ@SWCNT at 1 mg L^{-1} was performed in 0.5 L flasks or bottles with an air supply and followed for 14 days. Three replicates per species and tested conditions were performed, and a control was run in parallel; the optical densities were 0.1 and 0.3, respectively. The growth was monitored every 3 days, and the algal biomass was collected after 7 and 14 days through centrifugation (*Thalassiosira* sp.) or by filtration on nylon filters with a 11 μm porosity (*A. platensis*). pH, conductivity, and the residual inorganic nutrient content ($N\text{-}NO_3$ and $P\text{-}PO_4$) were measured in the filtrates; the contents of proteins, polysaccharides, lipids and total fatty acids, and pigments (i.e., chlorophyll *a*, total carotenoids, and phycocyanin) were determined in the biomass (see Supplementary materials). Data were expressed as a percentage of dry weight (% dw).

The growth of the exposed phototrophs in all the experiments was assessed by monitoring the optical density at 750 nm (OD750) using the JASCO spectrophotometer (UV/VIS, JASCO V-650, Tokyo, Japan), based on a preliminary analysis performed to check the potential influence of SWCNTs on the OD750 measurements to be used instead of cell counting. The appearance of cells after exposure to SWCNTs was visually investigated using light microscopy at 320x magnification (ZEISS Axiovert 100, Carl Zeiss, Germany). Photosynthetic efficiency was used as the endpoint to assess the overall effects on microalgae and cyanobacteria exposed to SWCNTs measuring the maximum quantum yield (Y_{max}) and the effective quantum yield (Y_{eff}) of photosystem II, using a 101-PAM fluorometer (H. Walz, Effeltrich, Germany), equipped with a red or blue high-power LED Lamp Control unit HPL-C for cyanobacteria and eukaryotic algae, respectively. The data have been reported by dividing the measured Y_{max} or Y_{eff} of the SWCNT-exposed cultures by the corresponding values of the controls, expressed as a percentage (%/ctrl, Simonazzi et al., 2021).

The interaction of LSZ@SWCNT with *Thalassiosira* sp. and *A. platensis* was visually investigated in fresh sub-samples collected after 72 h exposure by Micro-Raman spectroscopy, Environmental Scanning Electron microscopy (ESEM), and Transmission Electron Microscopy (TEM) (see Supplementary materials).

2.4. Statistical analysis

The significance of differences observed for the photosynthetic activity (Y_{max} and Y_{eff}) among phototrophs exposed to SWCNTs and controls were assessed by analysis of variance (ANOVA, $p < 0.05$) and post-hoc comparisons (Tukey's test). Before ANOVA tests, data normality and homoskedasticity were examined by the Shapiro-Wilk test and Levene's test, respectively. All analyses were performed on PAST v. 4.17.

3. Results and discussion

3.1. Stability of protein@SWCNT complexes

Single-walled carbon nanotubes have stunning mechanical and electronic properties that make them versatile for application in different fields. However, SWCNTs also have a high surface-to-volume ratio and are composed almost exclusively of carbon atoms. Due to the strong hydrophobic and van der Waals interactions, aggregation phenomena occur in aqueous media, reducing the exposed surface of SWCNTs (≥700 m^2 g^{-1}) to the polar environment. Such water insolubility is an important limitation that hampers their exploitation in the physiological environment.

3.1.1. Stability in water

Five proteins, selected because they i) already demonstrated their efficiency to disperse SWCNTs (Nepal and Geckeler, 2007; Paradisi et al., 2023), and ii) are economically affordable for large-scale industrial applications, were here studied as dispersants for CoMoCAT® nanotubes: lysozyme (LSZ), bovine serum albumin (BSA), histone (HST), myoglobin (MGB), and protamine (PRT). Among them, the dispersion of the SWCNTs with HST, LSZ and BSA was the most efficient. All the protein@SWCNT hybrids showed similar UV–Vis spectra, characterised by a main absorption band centred at 280 nm, which is diagnostic for the aromatic amino acid residues of the proteins (Fig. 1). The bands ranging from 440 to 600 nm are related to the lowest energy van Hove transitions of the metallic SWCNTs, while those from 550 to 800 nm are associated with semiconducting SWCNTs (Di Giosia et al., 2019), in particular the E22 transitions. Well-defined peaks in the visible region due to the van Hove transitions demonstrate that the SWCNTs are well-debundled (O'Connell et al., 2002).

Due to their high content of lysine and arginine, HST and LSZ are basic proteins, thus positively charged at pH 6.9 (Milli-Q water), while BSA has a high content of acidic residues, thus negatively charged at the same pH (see Supplementary materials). It is known that unbundled

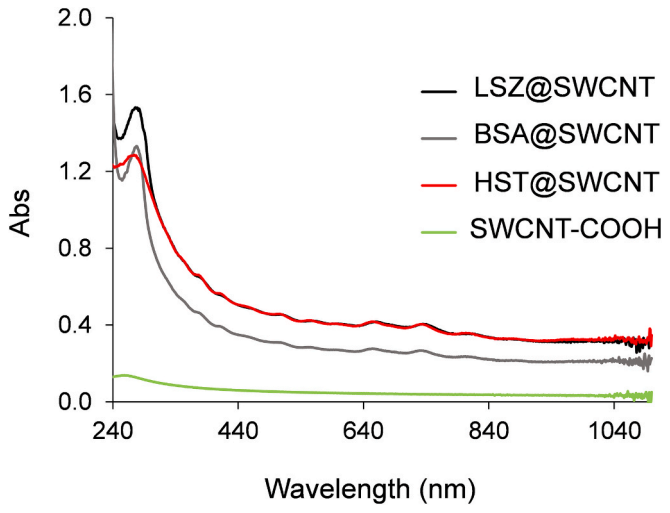

Fig. 1. UV–Vis absorption spectra of LSZ@SWCNT, BSA@SWCNT, HST@SWCNT, and SWCNT-COOH in Milli-Q water.

SWCNTs dispersed by proteins are stable in aqueous media thanks to electrostatic repulsive forces established by their "wrapping", and the factors that tend to neutralize the SWCNTs' superficial coating, like pH values close to the isoelectric point, cause a re-agglomeration of the tubes (Nepal and Geckeler, 2006). PRT has a high isoelectric point (13), but showed a low unbundling capacity, and could not maintain SWCNTs in suspension, probably due to its small dimension (4.3 kDa), which reduces the complementarity with the SWCNTs surface. Indeed, PRT@SWCNT adducts are reported in the literature, but in that case, the interaction is mediated by pyrene molecules, used as non-covalent anchoring groups, to promote the protein adhesion on the surface of the tube (Chen et al., 2001). Shape complementarity is the crucial factor controlling the binding between proteins and SWCNTs; in particular, the interaction between the amino acid residues of the protein and the CNT walls at the atomistic level allows their binding through π-π, cation-π and hydrophobic interactions (Di Giosia et al., 2019). Proteins with a high α-helix content in their secondary structure tend to better unbundle SWCNTs (Matsuura et al., 2006), probably thanks to their surfactant-like behaviour, with the hydrophobic residues exposed on one side of the helix that interact with the CNT surface and the hydrophilic residues interacting with the aqueous environment. This is the case with BSA, which has a high α-helix content (75 %) and a high capacity for unbundling SWCNTs (Matsuura et al., 2006).

Contrary to CoMoCAT® nanotubes, SWCNT-COOH nanotubes do not need to be dispersed by proteins thanks to the presence of the –COOH functional groups. However, a stable suspension of SWCNT-COOH nanotubes was achieved only at 0.1 mg mL^{-1}, 10 times lower than the other protein@SWCNT complexes (stock solutions at 1 mg mL^{-1}). The typical bands of CoMoCAT® nanotubes in the visible region were not present in the SWCNT-COOH spectra, due to the chemical modification of the carbon structure, while the absorption band centred at 280 nm was missing because of the absence of proteins as a supramolecular dispersing agent (Fig. 1).

3.1.2. Stability in algal growth media

Given the low dispersion of SWCNTs in Milli-Q water achieved with MGB and PRT, only the stability of HST@SWCNT, LSZ@SWCNT, BSA@SWCNT, and SWCNT-COOH was tested in the different algae growth media, enriched in salts (e.g., up to 30 ‰ of NaCl in the case of marine growth media) and other components such as vitamins that can affect the stability of the conjugates. As expected by previous studies (Di Giosia et al., 2019), the positively charged proteins (i.e., LSZ and HST, with isoelectric points of 10.7 and 11.7, respectively) were the most efficient dispersing agents for SWCNTs in Milli-Q water, however, their stability in algal growth media was affected by their chemical composition, especially in the case of the Zarrouk's medium, the only one with pH over 9, thus close to the isoelectric point of these proteins. Despite the lower efficiency of the albumin as a dispersing agent, BSA@SWCNT was stable both in Milli-Q water and in all the algal growth media, while the UV–Vis absorption spectrum intensity of SWCNT-COOH was one order of magnitude lower than the protein@SWCNT complexes, also in the tested algal growth media (see Supplementary materials).

3.2. Exposure of microalgae and cyanobacteria to SWCNTs

3.2.1. Selection of the phototrophs

Five phototrophs were tested to investigate their viability after exposure to SWCNTs: *Euglena gracilis*, *Scrippsiella* sp., *Thalassiosira* sp., *Stephanodiscus hantzschii*, and *Arthrospira platensis*. The phototrophs were incubated for 72 h with LSZ@SWCNT, selected because of the promising results achieved in the stability tests in Milli-Q water and algal growth media; LSZ@SWCNT was tested at 0.1 and 1 mg L^{-1}, chosen as the initial concentrations based on the range of concentrations reported in the literature for marine and freshwater microalgae exposed to a variety of carbon-based nanomaterials (Pikula et al., 2018; Saxena et al., 2020). An additional higher concentration of 10 mg L^{-1} was also

tested; however, evident aggregations of nanotubes and algal cells were observed (see Supplementary materials). At this concentration, the nanotubes tended to associate into bundles, which were likely too big to interact with the microalgae cells, resulting in toxicity, as already reported for SWCNTs' absorption by plants' roots (Magnabosco et al., 2020). This toxic effect was also observed here, particularly for the freshwater diatom *S. hantzschii*, for which the photosynthetic efficiency was 40 % of the control.

The maximum photosynthetic activity (Y_{max}, %/ctrl) of the tested organisms exposed for 72 h to the concentration of 0.1 mg L^{-1} was above 85 % of the control (ctrl), with the lowest values observed for the freshwater euglenoid *E. gracilis* (ANOVA, $p < 0.05$) and the highest for the marine dinoflagellate *Scrippsiella* sp. (photosynthetic efficiency of 130 %, above the control, Fig. 2). The optical density of the culture exposed to these conditions, taken as an indication of the algal growth, was similar to the control for the two diatoms *S. hantzschii* and *Thalassiosira* sp. (marine), higher than the control (130 %) in the case of the cyanobacterium *A. platensis*, and around 70 % for the euglenoid *E. gracilis* and the dinoflagellate *Scrippsiella* sp (see Supplementary materials).

The effects of exposure to the concentration of 1 mg L^{-1} were more evident only in the case of *S. hantzschii*, which had a significant decrease of 70 % of the photosynthetic activity (ANOVA, $p < 0.001$, Fig. 2); microscope images also revealed that *S. hantzschii* cells tended to agglomerate more with nanotubes than the other tested organisms (e.g., *Thalassiosira* sp., see Supplementary materials). This effect could be attributed to two factors: i) the smaller dimensions of *S. hantzschii* cells compared to the other tested phototrophs (i.e., diameter of 5 μm *vs* 20–50 μm) and thus a stronger "entrapping" within the SWCNT matrix, and ii) an indirect effect of "shading" induced by SWCNTs, which prevents algae from performing photosynthesis by reducing light transmittance (Schwab et al., 2011). These findings confirm that the effects on algal viability and photosynthetic efficiency caused by the interaction with LSZ@SWCNT can be given by a combination of algal intrinsic characteristics (surface charge, cell dimensions, and cell wall composition) and physicochemical properties of the carbon nanomaterials themselves (Lambreva et al., 2015). However, the general high tolerance towards LSZ@SWCNT here found for the majority of the tested organisms is in line with the results reported in the literature for a variety of algal species and carbon nanomaterials, showing alterations of the photosynthetic performance in a wide range of concentrations over 100 mg L^{-1} (Freixa et al., 2018), e.g., EC_{50} values of 2 and 60 mg L^{-1} for *Porphyridium purpureum* and *Scenedesmus obliquus* exposed to multi-walled carbon nanotubes (Pikula et al., 2023; Sun et al., 2020); no

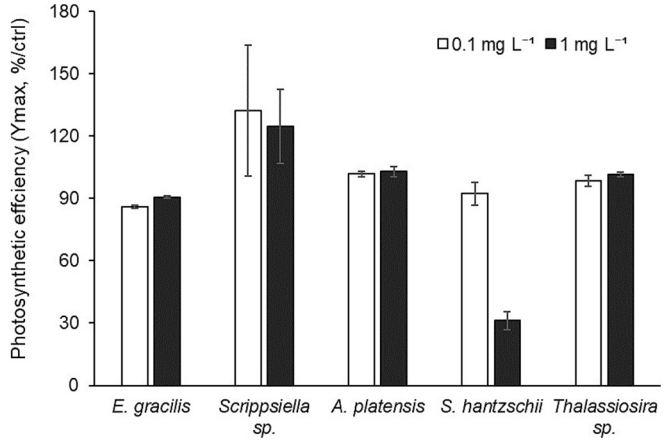

Fig. 2. The photosynthetic activity (maximum quantum yield, Y_{max}, %/ctrl) of *E. gracilis*, *Scrippsiella* sp., *A. platensis*, *S. hantzschii* and *Thalassiosira* sp. measured after 72 h of exposure to LSZ@SWCNT at the concentrations of 0.1 and 1 mg L^{-1}.

effect on the growth of *Skeletonema costatum* and *Heterosigma akashiwo* below concentrations of 5 and 10 mg L^{-1}, respectively (Pikula et al., 2018; Zhang et al., 2018).

3.2.2. Selection of the SWCNTs

The most promising species that were identified in the first screening test, i.e., the marine diatom *Thalassiosira* sp. and the cyanobacterium *A. platensis*, were exposed to LSZ@SWCNT, HST@SWCNT, and SWCNT-COOH, testing the effect of a prolonged exposure time on the cell viability and photosynthetic activity (Fig. 3). It is worth mentioning that the net charge of each protein at the pH of the solution in which SWCNTs are dispersed could not influence just the dispersing capacity of the proteins themselves but also the interaction of the conjugate with microalgae or cyanobacteria that, in turn, have their specific surface charges. All the species selected for the screening tests have a negative surface charge (see Supplementary materials), and the ζ-potential values measured for *A. platensis* and *Thalassiosira* sp. were –19.3 ± 1.0 and –6.0 ± 1.3 mV, respectively; therefore, it is reasonable to assume a stronger interaction with SWCNTs dispersed by positively charged proteins like LSZ and HST than the negatively charged ones like BSA. This was also previously reported for the cyanobacterium *Synechocystis* sp. PCC 6803 exposed to BSA-functionalized SWCNTs (Antonucci et al., 2022). For this reason, although BSA@SWCNT resulted in a stable and dispersed solution (see Section 3.1 "Stability of protein@SWCNT complexes"), this conjugate was no longer tested.

In this second test, the photosynthetic activity of the tested organisms was also measured after exposure to 5 min of actinic light, obtaining the effective quantum yield (Y_{eff}). The photosynthetic activity of *A. platensis* was similar to the control, independently of the type of SWCNTs and the time (Fig. 3). If the photosynthetic activity of *Thalassiosira* sp. exposed to SWCNTs was in line with the control at the beginning of the test, it significantly decreased after 3 days when the microalga was in contact with SWCNT-COOH and, more evidently, with HST@SWCNT (ANOVA, $p < 0.001$, for both). However, Y_{eff} significantly increased after 7 days, reaching a peak of almost 20 % higher in the cultures treated with LSZ@SWCNT than in the control (ANOVA, $p < 0.001$), suggesting a short-term hormetic response to the presence of SWCNTs, as already reported in the literature (Lau et al., 2022).

The optical density measured after exposure to each type of the tested nanotubes was comparable to that of the control after the first day or even higher at the end of the test for both species (see Supplementary materials). If the high tolerance towards SWCNTs of *A. platensis* found in the present work is the first reported evidence of how this cyanobacterium behaves in the presence of carbon nanomaterials, viability tests performed on other marine diatoms (i.e., *Attheya ussuriensis*, and *Chaetoceros muelleri*) showed a high tolerance of these organisms against carbon nanotubes or nanofibers, with no evidence of growth inhibition and EC_{50} values between 360 and 1000 mg L^{-1} after 96 h (Pikula et al., 2020), like what was found here with *Thalassiosira* sp.

It is worth mentioning that substantial mitigation of the toxicity of MWCNTs towards various biological targets has been reported through functionalization with carboxylic and amino groups, suggesting that the length, diameter, shape, purity, and surface area could be the main factors responsible for the toxicity, rather than the surface chemistry (Allegri et al., 2016). Moreover, carboxylated MWCNTs at a dose of 1.5 mg L^{-1} can have a positive effect on the growth and photosynthesis of microalgal-fungal consortia of *Chlorella vulgaris* and *Ganoderma lucidum*. The results found with LSZ@SWCNT, HST@SWCNT, and SWCNT-COOH align with this "mitigation" effect of carboxylic and amino groups reported in the literature for multi-walled carbon nanotubes (Liu et al., 2023).

3.2.3. Investigation of the effects of LSZ@SWCNT on Thalassiosira sp. and A. platensis

To explore the potential biotechnological applications in specific sectors (i.e., cosmetic, nutraceutical and food) where an enhanced

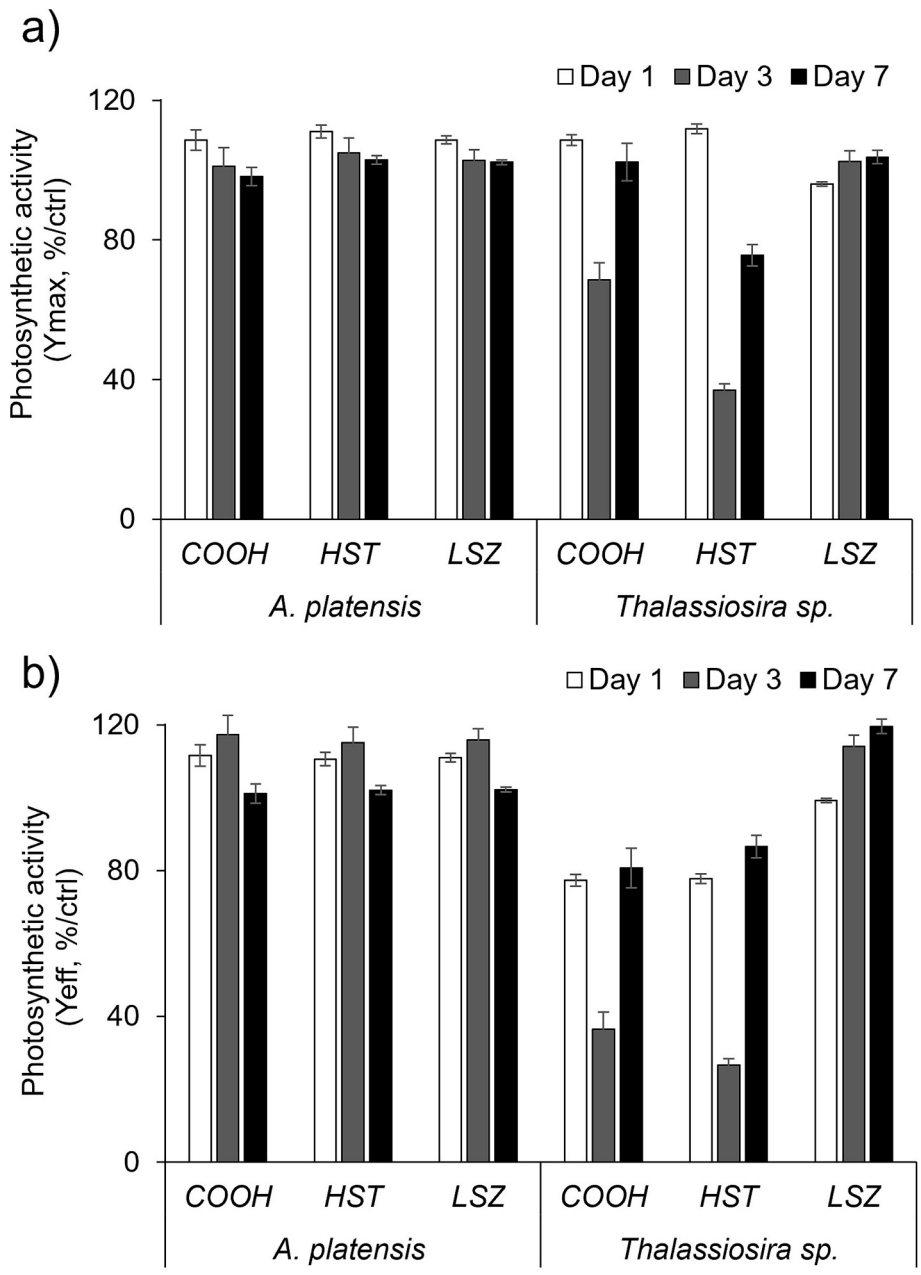

Fig. 3. The photosynthetic activity of *A. platensis* and *Thalassiosira* sp. measured after 7 days of exposure to SWCNT-COOH, HST@SWCNT, and LSZ@SWCNT at the concentration of 1 mg L^{-1}: a) maximum quantum yield, Y_{max}, %/ctrl; and b) effective quantum yield, Y_{eff}, %/ctrl.

production of high-value metabolites from algal biomass is highly desired (Balan et al., 2023), *A. platensis* and *Thalassiosira* sp. were exposed for 14 days to LSZ@SWCNT at the concentration of 1 mg L^{-1}, the conjugate with less effects on the photosynthetic activity of the two organisms at any time tested (see "Selection of the SWCNTs"). The organisms were cultivated in 0.5-L flasks to allow the harvesting of biomass for biochemical characterisation. The physicochemical parameters, in terms of pH and conductivity, of *A. platensis* and *Thalassiosira* sp. cultures exposed to LSZ@SWCNTs, and the residual content of inorganic nitrogen (N-NO_3) and phosphorus (P-PO_4) did not substantially vary during the cultivation period compared to controls; after 14 days, the pH values of *A. platensis* and *Thalassiosira* sp. cultures with or without LSZ@SWCNT were about 10.3 and 8.7, respectively, while the conductivity was about 21 and 50 mS cm^{-1}, respectively. N was still available at the end of the cultivation for both organisms (230 mg L^{-1} for *A. platensis* and 5 mg L^{-1} for *Thalassiosira* sp.), while P was almost halved by *Thalassiosira* sp. (< 1 mg L^{-1}) (see Supplementary materials).

Effects on the photosynthetic activity. The growth of both tested organisms was not negatively affected by the presence of LSZ@SWCNT, and the photosynthetic activity was similar (*A. platensis*) or significantly higher than the control (*Thalassiosira* sp., ANOVA, $p < 0.05$), even after 14 days (see Supplementary materials). Since no LSZ@SWCNT has been added to the cultures after the incubation at day 0, this trend cannot be entirely attributed to SWCNTs' intrinsic shading properties, but it can be tentatively attributed to a response to temporary stress rather than adaptation over time. Growth-promoting effects on photosynthetic microorganisms induced by carbon nanotubes have been previously reported, although in most cases only for short exposure times (< 96 h).

Effects on the production of proteins, polysaccharides, lipids, and lipophilic pigments. After 7 and 14 days of incubation, the protein content of *A. platensis* grown in the presence of LSZ@SWCNT (Fig. 4a) was similar to that of control (i.e., 47 *vs* 44 % on a dry weight basis at day

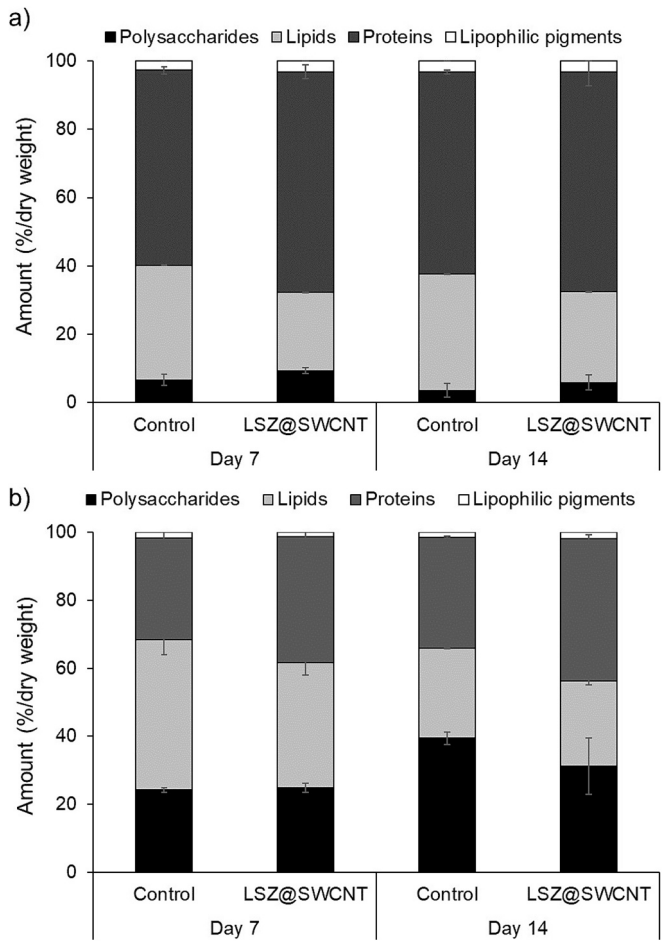

Fig. 4. Amounts of proteins, polysaccharides, lipids, and lipophilic pigments (% dw) of a) *A. platensis* and b) *Thalassiosira* sp. treated for 7 and 14 days with LSZ@SWCNT at the concentration of 1 mg L^{-1} compared to the control.

7, and 46 *vs* 45 % at day 14), but an increase in the phycocyanin content was observed, likely as a response to the shading effect induced by SWCNTs (18 *vs* 13 % on a dry weight basis). Indeed, this pigment-protein complex is used by cyanobacteria to absorb wavelengths in a larger portion of the spectrum and works synergistically with chlorophyll *a* to enhance oxygen production, particularly under low-light environments. Hence, if SWCNTs' agglomerates caused shading of light, *A. platensis* could have responded by increasing the expression of light-harvesting complexes. On the other hand, the lipid content was lower than the control when *A. platensis* was exposed to LSZ@SWCNT (i.e., 25 *vs* 36 % on a dry weight basis at day 7, and 29 *vs* 37 % at day 14), while the fatty acid profile and the ratio between saturated and unsaturated fatty acids did not change over the time and as a consequence of the exposure to nanotubes (i.e., 0.8–1.0).

The protein content of *Thalassiosira* sp. (Fig. 4b) was higher in the cultures grown in the presence of LSZ@SWCNT than in the control (i.e., 37 *vs* 30 % on a dry weight basis at day 7, and 42 *vs* 33 % at day 14). The lipid content of *Thalassiosira* sp. decreased over time in both the control and the treated cultures, while an opposite trend was observed for polysaccharides. The lipid content after 7 days was higher in the control than in algae exposed to LSZ@SWCNT (44 *vs* 37 % on a dry weight basis, respectively) and after 14 days became similar (25–26 %), while polysaccharides were similar after 7 days (24 *vs* 25 % on a dry weight basis, respectively) and after 14 days increased more in the control than in the algae exposed to LSZ@SWCNT (39 *vs* 31 %, respectively). Under various stress conditions (e.g., nutrients, light, temperature), algae and cyanobacteria tend to accumulate reserve compounds such as lipids and polysaccharides, whose cellular ratio can vary according to specific growth. Similarly, the production and secretion of extracellular polymeric substance (EPS) seemed to be promoted in both diatoms (Verneuil et al., 2015) and cyanobacteria (Yuan et al., 2023) exposed to carbon nanotubes as a protective mechanism towards these external agents (Saxena et al., 2020).

The fatty acid profile of *Thalassiosira* sp. was influenced by the exposure to carbon nanotubes, especially for ω-3 long-chain fatty acids like eicosapentaenoic (C20:5) and docosahexaenoic (C22:6) acids: after 7 days of exposure, C20:5 increased from 9 % in the control to 15 % in the samples treated with LSZ@SWCNT (+63 %), while C22:6 increased from 1 % in the control to 1.8 % in the samples treated with LSZ@SWCNT (+92 %) (see Supplementary materials). Consequently, the ratio between saturated and unsaturated fatty acids after both 7 and 14 days was higher in the control than in the treated samples (i.e., 0.9 *vs* 0.6) (see Supplementary materials). It is indeed known that changes in fatty acid profiles are unavoidable when microalgae try to acclimatize under unfavourable conditions; particularly, the production of polyunsaturated fatty acids like eicosapentaenoic and docosahexaenoic acids is enhanced under various stress factors, given the role of polyunsaturated fatty acids in reducing oxidative stress, improving organelle membrane stability and enhancing cell membrane integrity.

The lipophilic pigments (i.e., carotenoids and chlorophylls) of both *A. platensis* and *Thalassiosira* sp. were not markedly influenced by the exposure to LSZ@SWCNT, neither quantitatively nor qualitatively, although the chlorophyll/carotenoids ratio was slightly higher for the organisms exposed for 14 days to LSZ@SWCNT than the controls (4.6 *vs* 4.3 in the case of *A. platensis*, and 1.7 *vs* 1.4 for *Thalassiosira* sp., see Supplementary materials). Particularly, an increase of chlorophyll *a* was observed after 14 days of exposure to LSZ@SWCNT compared to control (2.65 *vs* 2.73 for *A. platensis*, and 0.82 *vs* 1.17 % for *Thalassiosira* sp.) in line with the observed improved photosynthetic performance, suggesting a combined short-term hormetic response to temporary stress conditions caused by the exposure to low doses of CNTs (Lau et al., 2022).

The upregulation/downregulation of specific metabolites as a consequence of exposure to nanomaterials has already been reported for microalgae (Hu et al., 2015); it is known for example that the downregulation of amino acid metabolism in *Chlorella vulgaris* is associated with the enhancement of oxidative stress given by the exposure to nanomaterials, as the decrease in the unsaturated fatty acid content, associated with the deterioration of membrane fluidity and osmotic stress. Other authors reported that the presence of nanomaterials stimulate the production of biomass in *A. platensis* and, while lipids increased upon exposure, proteins, polysaccharides and accessory pigments content did not change (Cepoi et al., 2020). The overproduction of (high-value) target molecules by applying external stimuli can enhance their productivity, facilitate their recovery from the algal biomass in the downstream phase, and, above all, increase the profitability of the entire microalgal biorefining process, given the fact that products like polyunsaturated fatty acids and hydrophobic (i.e., carotenoids) and hydrophilic pigments (i.e., phycobiliproteins) have a small market size but a high market price (10–1000 € kg^{-1}) (Gifuni et al., 2019).

Evaluation of the surface interactions through Raman spectroscopy. The main signals observed in the Raman spectra of the exposed cultures corresponded to the G-band of SWCNTs, found at 1592 cm^{-1} for *A. platensis* and 1595 cm^{-1} for *Thalassiosira* sp. (Fig. 5a). Additionally, Raman peaks at 1520/1527 cm^{-1} for *A. platensis* and *Thalassiosira* sp., respectively, were attributed to carotenoids: the peaks at 1520 and 1527 cm^{-1} are linked to the conjugated C═C stretching vibrations, while the peak around 1156 cm^{-1} corresponds to a combination of C—C stretching and δ(C—H) in-plane bending vibrations of β-carotene within the cells (He et al., 2021). The slight variation in the wavenumbers of β-carotene's vibrational modes within *A. platensis* and *Thalassiosira* sp. cells in the solid state may be due to a loss of electronic delocalisation in the polyene chain of β-carotene. This sensitivity likely stems from the complexity of the biological matrix in which β-carotene is

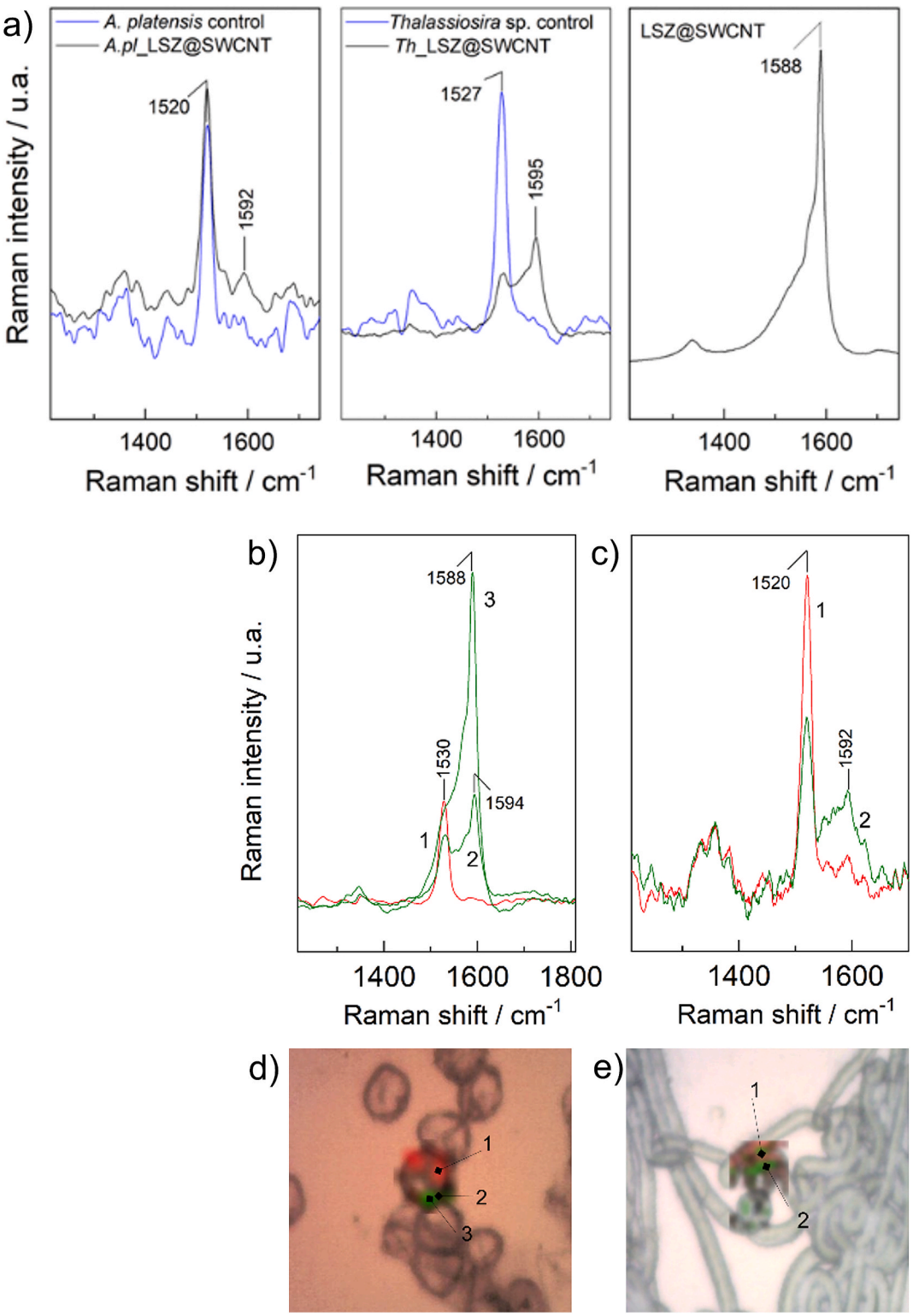

Fig. 5. A) Raman spectra (λ_{exc} = 514 nm) at RT of *A. platensis* and *Thalassiosira* sp. exposed to LSZ@SWCNT at the concentration of 1 mg L^{-1} (black lines) compared to the respective controls (blue lines), and LSZ@SWCNT; Micro-Raman maps at RT and corresponding spectra of *Thalassiosira* sp. (b, d) and *A. platensis* (c, e) treated with LSZ@SWCNT at the concentration of 1 mg L^{-1}, collected from the regions indicated by arrows on the maps. The red areas in the maps and the red lines in the spectra represent *Thalassiosira* sp. and *A. platensis*, while the green areas and green lines correspond to SWCNTs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

embedded (de Oliveira et al., 2010).

Interestingly, a blue shift in the main signal of SWCNTs was observed in the Raman spectra of cultures exposed to nanotubes. The signal shifted from 1588 cm^{-1} for LSZ@SWCNT to 1592 cm^{-1} and 1595 cm^{-1} for *A. platensis* and *Thalassiosira* sp., respectively. This shift may indicate that the SWCNTs were energetically influenced by the presence of phototrophs, suggesting an electrostatic interaction between the two entities. Such interaction could be due to the positively charged

lysozyme and the deprotonated hydroxyl groups of polysaccharides in the cell walls, or possibly H-bonding-like interactions between the amino groups of lysozyme (arginine and lysine residues) and the hydroxyl groups of polysaccharides. Peaks observed in the 1650–1730 cm^{-1} spectral region may correspond to amide groups in lysozyme, likely linked to C═O stretching vibrations coupled with out-of-phase C—N stretching, C—C—N deformation, and N—H in-plane bending vibrations (Kocherbitov et al., 2013).

Raman mapping was also performed on areas of 550 μm^2 and 750 μm^2 to better understand the distribution of LSZ@SWCNT on the surface of *Thalassiosira* sp. (Fig. 5d) and *A. platensis* cells (Fig. 5e). The red colour in the mapping corresponds to the integral of the Raman signals approximately in the 1500–1545 cm^{-1} range, associated with *Thalassiosira* sp. and *A. platensis* cells, while the green colour represents the 1540–1630 cm^{-1} range, corresponding to the SWCNTs. For *Thalassiosira* sp., the signal intensity varied across different areas of the sample, as confirmed by the Raman spectra shown in Fig. 5b, collected from two separate, close points in the green region (SWCNTs). Although the SWCNT signals were intense (peaks 2 and 3), the strong peak at 1588 cm^{-1} (peak 3) was attributed to SWCNT aggregates. The Raman mapping of *A. platensis* (Fig. 5e) showed that the SWCNT signal was more evenly distributed throughout the sample, with no detectable SWCNT aggregates, unlike what was observed in *Thalassiosira* sp. Indeed, the peak corresponding to SWCNTs was less intense in *A. platensis* than in *Thalassiosira* sp. (Fig. 5c *vs* 5b).

Evaluation of the surface interactions through ESEM analysis. The ESEM images of *A. platensis* taken at the magnification of 50000x highlighted some differences observed between control and LSZ@SWCNT-treated cells (Fig. 6a and 6b), where the cellular surface became rough and characterised by irregular wrinkles, lumps, and lateral grooves.

Compared to *A. platensis*, the appearance of *Thalassiosira* sp. cells did not substantially change after LSZ@SWCNT exposure, remaining rigid and geometric (Fig. 6c and 6d), likely due to the nature of its silica cell wall (i.e., frustule). Cells were characterized by chain formation with central mucus filaments, more evident in treated cells. Moreover, some ornamentations of the frustule appeared different in the treated cells compared to the control: particularly, the SWCNT-exposed samples showed enlarged pores of the valve (i.e., areolae) and crown-like spine occlusions (i.e., volae) wide-open or detached from the pore edges.

Similar changes in the morphology of cell walls after SWCNTs' treatment have already been observed, e.g., ruffled cellular surface (Hu et al., 2015; Intrchom et al., 2018). This phenomenon can be attributed to oxidative stress induced by SWCNTs: indeed, SWCNTs' exposure can induce lipid peroxidation through the formation of reactive oxygen species (ROS) that can oxidise the double bonds present in the fatty acid tails of membrane phospholipids (Klaine et al., 2008).

Evaluation of the intracellular effects through TEM analysis. Effects of the SWCNTs' exposure on the cellular ultrastructure of *A. platensis* and *Thalassiosira* sp. were assessed through Transmission Electron Microscope (TEM) imaging acquisition. TEM observations of *A. platensis* showed the typical prokaryotic organisation, multi-stratified cell wall and thylakoids dispersed through the cytoplasm and distinct from the cytoplasmic membrane, having an irregular arrangement. The cells of *A. platensis* exposed to LSZ@SWCNT exhibited a greater rate of vacuolization, together with the detachment of the cytoplasmic compartment from the cell wall (see Supplementary materials); similar evidence was previously reported for *A. platensis* exposed to silver nanoparticles (Cepoi et al., 2020), and it is known that vacuolization could be a defensive mechanism towards CNTs in plant cell models (Lambreva et al., 2015).

Some alterations of cellular ultrastructure were also found in *Thalassiosira* sp., where compromised cytoplasmic compartments with empty spaces were observed inside the cells treated with SWCNTs. Compared to the control, the presence of intracellular starch grain-like bodies seemed to increase, while the chloroplasts appeared altered, less diffused and reduced in number and size (see Supplementary materials). An increase in intracellular starch grains and chloroplast alterations was previously observed in *Chlamydomonas reinhardtii* and *Chlorella vulgaris* exposed to different nanomaterials (Hu et al., 2015;

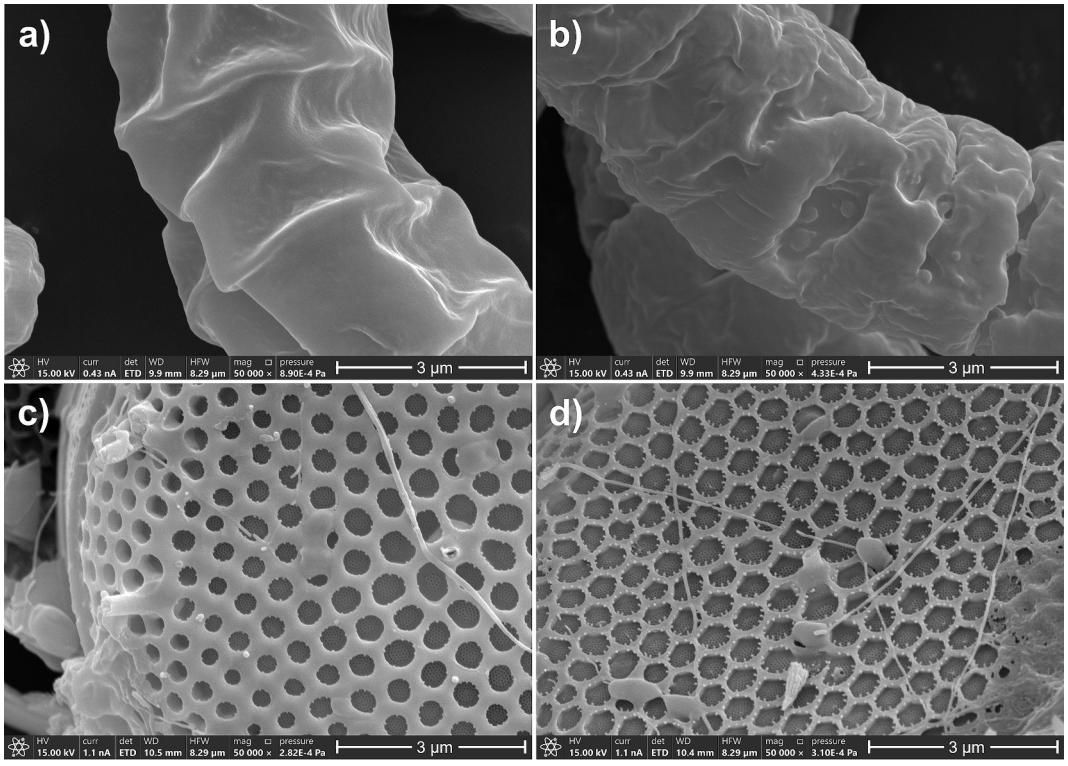

Fig. 6. ESEM images of *A. platensis* and *Thalassiosira* sp. samples after 14 days, untreated (a, c) and treated with LSZ@SWCNT at the concentration of 1 mg L^{-1} (b, d). Images were taken at a magnification of 50000×.

Orlanducci et al., 2020).

Overall, the effects observed in both tested organisms suggest that exposure to LSZ@SWCNT triggered a cellular response, leading to significant changes in the cellular structures, surface, and biochemical profile. These changes originate from the interaction between the microorganisms and carbon nanotubes, as evidenced by Raman spectroscopy characterisation. However, the monodispersion of SWCNTs achieved through protein coating did not allow for the visualisation of the carbon nanomaterial inside the cell via TEM microscopy, contrary to what has been reported in other works (Antonucci et al., 2023). It is worth mentioning that the internalisation of CNMs within microalgae should occur to a lower extent than other types of nanomaterials, resulting in lower adsorption on photosynthetic organelles within the cells, a lower negative impact on photosynthetic efficiency, and an overall lower toxicity (Lau et al., 2022). Moreover, this aspect can have a positive impact in the downstream phase, since the recovery and recycling of SWCNTs from harvested biomass would be easier than for other nanomaterials that are internalised and irreversibly bound to organelles within the microalgal cells.

4. Conclusions

The present study demonstrated that microalgae and cyanobacteria can grow in the presence of SWCNTs, without affecting their growth and photosynthetic performance, while improving the light-to-chemicals processes, boosting the production of specific compounds. Among different SWCNT formulations assessed, LSZ@SWCNT concentrations below 1 mg L^{-1} were not toxic for the tested cyanobacteria and microalgae; moreover, LSZ@SWCNT, HST@SWCNT and SWCNT-COOH were well tolerated by *Thalassiosira* sp. and *A. platensis* during 7 days of exposure. The interaction of LSZ@SWCNT with the cellular surface of *Thalassiosira* sp. and *A. platensis* after 14 days was demonstrated by Raman spectroscopy and ESEM analysis, and this interaction induced variations of the biochemical profiles (i.e., increase of eicosapentaenoic and docosahexaenoic acids, and phycocyanin), increase of the photosynthetic efficiency, and modification of some cellular structures. Overall, this work highlighted the potential of coupling carbon nanotubes with microalgal cultivation towards enhanced photosynthesis and production of high-value compounds.

CRediT authorship contribution statement

Mara Simonazzi: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation. **Sofia Lemaire:** Writing – original draft, Visualization, Investigation. **Alessio Adamiano:** Writing – review & editing, Investigation. **Matteo Calvaresi:** Resources. **Simona Cioffi:** Investigation. **Francesca De Giorgio:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Investigation. **Matteo Di Giosia:** Writing – review & editing, Supervision. **Paola Galletti:** Writing – review & editing, Resources, Funding acquisition. **Marco Malferrari:** Investigation. **Valentina Papa:** Investigation. **Laura Pezzolesi:** Writing – review & editing, Supervision, Resources. **Giampiero Ruani:** Writing – review & editing, Writing – original draft, Resources, Investigation. **Chiara Samorì:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2025.132869.

Data availability

Data will be made available on request.

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