Influence of zinc and manganese enrichments on growth, biosorption and photosynthetic efficiency of *Chlorella sp*.

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Abstract

Treating biosolids from industrial, urban and agricultural plants produces high amounts of waters. After organic pollutants and non-essential heavy metals have been removed, these wastewaters are still rich in trace elements such as zinc (Zn), copper or manganese (Mn), and have a high conductivity and extremely variable pH. In this study an isolated *Chlorella* sp. strain was grown for 21 days in nutrient solutions enriched with known amounts of Zn or Mn to obtain concentrations three (4.0 mg L^{-1}) and six (1.0 mg L^{-1}) -fold higher than the basal medium levels, respectively, and over the limits permitted in aquatic environments. The green alga exhibited high tolerance to Zn and Mn, with the maximum abatement of Zn (28-30%) and Mn (60-63.5%) after 14 and 7 days of culture, respectively. Mn stimulated the growth rate and biomass production of *Chlorella*, which showed the highest carbon levels just in the first week. In both treatments, the nitrogen and protein content remarkably increased. The photosynthetic pigments increased until the 14th day, with a higher extent in the Zn-enriched solution. An increasing photochemical efficiency was observed after 7 days of treatment, when the microalgae grown in Zn- and Mn-enriched solutions showed a slightly higher maximum photochemical efficiency than control. The autotrophic and controlled growth system adopted was designed to monitor the dynamic balance of Zn and Mn contents in the solutions and in the algal biomass. This system has proved to be useful in identifying the optimal nutritional conditions of the

microalgae, along with the optimal temporal patterns of both metal biosorption capacity for water remediation and element bioaccumulation in the algal biomass.

Keywords bioaccumulation, biofertilisation, chlorophyll fluorescence, photosynthetic pigments, trace elements, Zn and Mn balance

Introduction

Heavy metals are stable and persistent contaminants of environmental and health concerns, and they are released into the environment through waterways due to several anthropogenic activities such as mining, electroplating, the production of electronics, fertilizers, pesticides, and pharmaceutics (Alloway 2013).

Effluents containing heavy metals from industrial, agricultural or urban processes are mainly wastewaters and biosolids, which have historically been underutilized (WHO 1989), but are now becoming beneficial resources both environmentally and economically (WWAP 2012; USDA 2013) after remediation in wastewater treatment plants (WWTPs).

The conventional physico-chemical methods of water remediation are often expensive and inefficient. They lack the specificity required for the treatment of target metals, especially in cases when the metal concentration in the wastewater is relatively low (Fu and Wang 2011). On the other hand, the technologies based on naturally-occurring biological processes have a number of recognized advantages over physico-chemical techniques. In this context, bioremediation, using bacteria, fungi, yeast and algae is an efficient, cost-effective and environmentally friendly strategy for the remediation of sites and recycled matrices and waters contaminated with heavy metals (Fu and Wang 2011; Rawat et al. 2011; Ameen et al. 2020).

In trace amounts ("trace elements") as micronutrients, some heavy metals such as copper (Cu), manganese (Mn), nickel (Ni) and zinc (Zn) are essential to many metabolic processes of living cells. However, they can become noxious or toxic to most prokaryotic and eukaryotic organisms if their concentration exceeds certain thresholds (Prasad 2013). Other heavy metals such as cadmium, lead and mercury are non-essential elements with an unknown biological function and toxic effect in most organisms even at very low concentrations.

The legislative limits of heavy metal inputs in Europe have quite wide ranges, but are subject to common directives (EU Reg. 629/2008), whereas in the USA, Canada and Australasia they have different regulatory limits. In any case, different thresholds have been identified for drinking or domestic waters, agricultural or urban soil systems (Tóth et al. 2016).

Algae are very abundant in the natural environment and adapt well to a wide range of habitats: freshwater and seawater, domestic and industrial effluents, salt marshes, and constructed wetlands. Algae are highly organised forms of life with considerable nutritional value, and they can be an alternative renewable feedstock for third-generation biofuels (Juneja et al. 2013; Safi et al. 2014). Microalgae are unicellular organisms and have an extraordinary capacity to adapt to different environmental conditions and absorb carbon dioxide (CO_2), which they convert into potential biofuel, food, feed and components with high added value (Juneja et al. 2013; Sun et al. 2018).

Microalgae also take up and accumulate heavy metals from their surrounding environment (e. g.: Arunakumara and Zhang 2008; Kumar et al. 2015; Liu et al. 2017). Like bacteria, microalgae respond to heavy metal contamination by preventing the uptake of metals and/or tolerating them inside the cell (Ameen et al. 2020). This process is called the biosorption of metals in microalgae and combines mechanisms of adsorption (rapid and passive sorption of metal ions to cellular surfaces) and absorption (metabolically facilitated internalisation of metal ions) (Das et al. 2010; Kaplan 2013).

The algal cultivation in harvesting/processing systems is both energy- and cost-intensive (Chen et al. 2011; Safi et al. 2014). The beneficial large-scale growing of these organisms produces the highest algae feedstock with minimum nutrients. However, current methods for algal cultivation often use excessive quantities of nutrients which are also very expensive, as their production, unlike light and CO_2, needs natural resources and energy utilization (Blair et al. 2014).

Recycled waters and biosolids from municipal and agricultural activities are rich in trace elements that are also micronutrients. After these effluents have been treated to reduce or eliminate contaminants and non-essential metals they can be used for irrigation, soil fertilization and the management of cultivation systems (Acién Fernández et al. 2018). The metal biosorption capacity of microalgae for the reduction of excess trace elements in waste- and recycled-waters under regulatory limits is a cost-effective strategy for: a) eliminating the costs involved in the production of algal nutrient solutions and the related disposal of the exhausted components along with the considerable environmental benefits; b) remediating municipal, agricultural or industrial wastewaters, which can be re-used for fertigation or other applications after sanitizing treatments; c) producing algal feedstock for high-value compounds and biofuels.

The resulting microalgae biomass enriched in trace elements can be used to produce biofertilizers and feedstocks for use as a food supplement for animal mineral nutrition (Chojnacka 2007; Mahapatra et al. 2018). The trace elements Zn and Mn are at the center of multifaceted acclimation mechanisms that have evolved in algae to ensure that extracellular supply meets intracellular demand. Together with Cu and iron (Fe), Zn and Mn drive the metal homeostasis in algae. This is done by the regulation of the metal ions trafficking across membranes, the intracellular compartmentalization by proteins

and organelles, and metal-sparing/recycling mechanisms aimed at optimizing metal-use efficiency (Blaby-Haas and Merchant 2017).

In this work we investigated the effects of Zn and Mn on the biosorption capacity of a selected population of *Chlorella* sp., the algal biomass and other physiological and biochemical endpoints (photosynthesis, proteins and pigment content) in order to evaluate the microalgae capacity to mitigate excess trace elements in waters while producing high quality biomass.

Our aims were to: i) reduce the excess micronutrient supply in bioreactor algal systems, ii) optimize the micronutrient supply in microalgae cultures, as the quantity of nutrients is one of the most significant economic limiting factors, iii) evaluate the potential of our *Chlorella* strain in the remediation of contaminated waters from excess metals and the production of sustainable biomass.

Materials and methods

Algal pre-culture, culture, experimental set-up and design

The freshwater microalgal *Chlorella* sp. strain was taken from the laboratory collection of the Department of Agriculture, Food and Environment at the University of Pisa (Chiellini et al. 2020). This microalgae strain was cultured and maintained in *in vitro* growth conditions with temperatures of 20 ± 3°C, a photoperiod of 16/8 h day/night, and a photosynthetic photon flux density (PPFD) of 70-100 μmol m^{-2} s^{-1}, 65-70% RH. Freshwater algae were grown in tris-acetate-phosphate medium (TAP) (Gorman and Levine 1965) at pH=7, modified in stock solutions in the nitrogen source: 15 g L^{-1} NH_4Cl was substituted with 11.4 g L^{-1} NH_4NO_3, maintaining the same nitrogen concentration; modified in a trace element solution: 22 g L^{-1} $ZnSO_4$. $7H_2O$ was substituted with 5.94 g L^{-1}, and 5.06 g L^{-1} of $MnCl_2$. $4H_2O$ with 0.61 g L^{-1}. The TAP medium is an algal buffered growth medium containing Tris base and a phosphate buffer system (K_2HPO_4, KH_2PO_4), in which the initial pH (basic) is titrate to pH 7-7.5 with acetic acid. This buffer system reduces the daily pH drift of growth solution due to the consumption of dissolved CO_2 by the photosynthetic activity of algae, and prevents the precipitation of salts.

Purified algal colonies from Petri dishes were selected and transferred to Pyrex glass flasks containing 100 mL of sterile liquid modified TAP medium. Fresh TAP was then added to the algal solution weekly until at least a 6 L volume was obtained for subsequent bioassays. When this pre-culture reached an optical density (OD) of 2.5 at 530 nm, and the chlorophyll pigments were 38.7±0.1 mg L^{-1}, the microalgae were collected by centrifugation (1000 x g, for 10 min.), rinsed twice with sterile distilled water, and re-suspended in fresh TAP until the OD achieved 0.7±0.2.

This algal solution was distributed among the treatments by inoculation in Pyrex flasks containing 500 mL of (i) modified TAP medium with basal Zn and Mn concentrations (20.65 and 3.10 μM, respectively, "Control"); (ii) TAP with 60 μM Zn (Zn 3X, "Zn-enriched"); and (iii) TAP with 20.5 μM Mn (Mn 6X, "Mn-enriched"). The basal Zn and Mn concentrations of control TAP corresponded to 1.35 and 0.17 mg L^{-1} (ppm), respectively, while the "Zn-enriched" corresponded to 4.0 ppm Zn and the "Mn-enriched" to 1.0 ppm Mn. These values are shown in Table 1.

The composition of the basal modified TAP medium used as macronutrients was as follows: NH_4NO_3 (0.285 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.1 g L^{-1}), $CaCl_2 \cdot 2H_2O$ (0.05 g L^{-1}), K_2HPO_4 (0.054 g L^{-1}), KH_2PO_4 (0.054 g L^{-1}), Tris (2.42 g L^{-1}). As micronutrients, 1 mL of the modified Hutner´s trace element solution was used (Hutner et al. 1950) containing Na_2EDTA, $ZnSO_4 \cdot 7H_2O$, H_3BO_3, $MnCl_2 \cdot 4H_2O$, $CoCl_2 \cdot 6H_2O$, $CuSO_4 \cdot 5H_2O$, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ and $FeSO_4 \cdot 7H_2O$ and were added to 1 L of the above solution. The surplus Zn and Mn in the corresponding enriched solutions were added as $Zn(NO_3)_2$ and $Mn(NO_3)_2$, respectively (Table 1). The nitrogen supply (NO_3^-) was equilibrated by reducing the concentration of NH_4NO_3 in the basal solution.

The microalgae exposure to Zn and Mn-enriched media was performed for 21 days, to verify that the exposure was more prolonged than in similar studies. The flasks were capped with air-permeable stoppers and constantly shaken (agitation rate of about 120 rpm) to keep the algae in suspension and to facilitate the transfer of CO_2. The cultures were continuously bubbled with an aeration rate of 0.5 L min^{-1} with air at ambient CO_2 concentration; the assay (21 days) was conducted without renewal of the test solutions. All the microalgae exposure tests were carried out in four replicates (n = 4) with three blanks (the TAP medium solutions, basal and enriched, without algae) for each treatment, with a total of 21 flasks. Figure 1 shows the experimental time-course and design with the control and treatment conditions.

The flask culture apparatus was placed in a chamber with the following growth conditions: 24/22°C, 16/08 h day-night cycle and a PPFD of 100-120 μmol photons m^{-1} s^{-1} from cool-white light lamps (Gavita Lep 330 Plasma fixtures, Gavita Holland Light Emitting Plasma, Netherlands). The microalgae growth was monitored at the beginning of the experimental set up (T_0), after 3 days of acclimation, and weekly for 21 days (on days 7, 14 and 21) as regards the non-destructive parameters (OD, photosynthetic pigments and chlorophyll fluorescence analyses), and at T_0 and weekly for 21 days for the other parameters.

The two treatment doses (4.0 ppm Zn and 1.0 ppm Mn) were selected because they do not lead to symptomatic impairments in the algae but are above the Italian regulatory limits (Italian Environmental Code, Lgs D. 152/2006).

Growth analysis and total protein determination

Microalgae growth was monitored by measuring the optical density (OD) and the algal biomass production of the algal medium at each experimental condition during the 21 day-assay. Every week a small aliquot (1.5-2 mL) of the culture solution was aseptically removed from each flask of the experimental set-up, and the OD was measured at 530 nm, OD_{530} (Vona et al. 2004) using a UV-vis spectrophotometer (UV-1800 Spectrophotometer, Shimadzu). The biomass production was determined by the dry mass of 20-30 mL samples collected from the culture medium, after mechanical agitation for 10 min. The algal biomass is defined as the dry mass (DM) per volume, i.e. mg algae/litre test solution (OECD 201, 2011). The samples were then centrifuged at 1000 x g for 10 min. and the pellets dried at 60°C until constant weight.

The specific growth rate (μ, d^{-1}) was calculated as follows:

$$\mu = \ln (X_f/X_i) / t_f\text{-}t_i,$$

where X_f is the final algal dry biomass concentration (mg L^{-1}) on day t_f, and X_i is the initial biomass concentration on day t_i; t_f-t_i represents the number of days in culture (7, 14, 21).

The total proteins were determined using the combination of a chemical (alkaline) extraction method with a sonication assisted process (Chia et al. 2019). In particular, extracted from *Chlorella* sp. using an alkaline solution: 0.1 M tris-HCl (pH 8) buffer with 0.2% Triton-100, 1 mM EDTA and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). Samples of 2-4 mL of algal solutions were centrifuged at 1000 rpm for 10 min. at 4°C. The supernatant was discharged, and 1-2 mL of extraction buffer (0.1 M tris-HCl pH 8 buffer with 0.2% Triton-100, 1 mM EDTA and 0.5 mM phenylmethylsulfonyl fluoride, PMSF) were added to the pellet. After light stirring for a few minutes to keep the cells in suspension without breaking, the samples were exposed to three sonication (Branson 1210, Bransonic Ultrasonic Cleaner) cycles of 10 min. each (working frequency: 47 kHz ± 6%). At the end of each sonication cycle, the algal samples were transferred onto ice for 5 min. to prevent the microalgae cells from being overheated in the disruption phase. After 30 min. of sonication, each sample was centrifuged at 13,000 rpm for 5 min. at 4°C, and the supernatant was then read at 595 nm with a spectrophotometer (UV-1800 Spectrophotometer, Shimadzu). The total protein concentration in these extracts was determined using Bradford's protein-dye binding method (Bradford, 1976) with bovine serum albumin (BSA) as the standard.

Variations of physico-chemical properties of nutrient and enriched solutions

The pH and electrical conductivity (EC) of the algal growth solutions were monitored weekly throughout the assay period with the Multiparameter AP-700 sensor set (Eijkelkamp Soil & Water, Giesbeek, The Netherlands).

The Zn and Mn concentrations of control and metal-enriched solutions were determined by an inductively coupled plasma-optical emission spectrometer (ICP-OES, Varian AX, Liberty). The culture flasks were kept on a shaker table (80-90 rpm) under a laminar flow hood and, after checking that no cellular aggregates or salt precipitates had formed, they were opened and homogeneous aliquots of 20-30 mL liquid samples from at least three flasks per treatment (maximum total volume 80-100 mL) were collected at the beginning of the experiment and weekly throughout the test period and centrifuged at approximately 1000 x g for 10 min. The supernatant was filtered through a membrane filter (cellulose nitrate filters, Ø 0.2 μm, Sartorius AG, Germany). These samples were read with the ICP-OES instrument by plotting a calibration curve for each element, with a sensitivity of 0.03 ppm. The pellet containing the *Chlorella* sp. cells was used for the mineral analyses of algal biomass. Zinc and manganese concentrations in the solutions were expressed as mg per litre (mg L^{-1}), and contents as total mg per flask volume (mg $flask^{-1}$). At each monitoring time (days 0, 7, 14 and 21), the percentage metal removal was calculated according to the following equation:

$$\%Removal = [(C_0-C_f) / C_0] \times 100,$$

where C_0 is the initial metal content, and C_f is the final concentration of metal in the algal solution.

Contents of carbon, nitrogen, zinc and manganese in microalgae

Samples of *Chlorella* sp. from each test solution at the beginning of the experiment and weekly during the 21 day-assay were collected for the determination of carbon (C), nitrogen (N), zinc (Zn) and manganese (Mn) concentrations. Algal samples were centrifuged at approximately 1000 x g for 10 min., oven dried (60°C) until constant weight and subsequently ground to a fine powder. Using an elemental analyser (Model NA 1500, Carlo Erba, Milan, Italy), approximately 3-4 mg of powder was collected to determine the C and N contents. Carbon and nitrogen amounts in algae were expressed as percentages on a dry mass basis. Approximately 80-200 mg of powder were digested by the microwave (Milestone Ethos 900, Bergamo, Italy) in nitric acid (HNO_3 65%) and hydrogen peroxide (H_2O_2 30%), and analysed for Zn and Mn contents by ICP-OES (Varian AX, Liberty). Zinc and manganese concentrations and contents were expressed on a dry mass basis (mg g^{-1} DM, ppm) or total amount per flask (mg $flask^{-1}$), respectively. At each monitoring time (days 0, 7, 14 and 21), the bioaccumulation factor (BAF) of Zn and Mn in the microalga was calculated as the ratio between the total amount of the metal biosorbed (mg) by algae to the quantity (mg) of the same inorganic pollutant detected in the culture solution (Yan and Pan 2002; Di Baccio et al. 2017).

240
241 **Photosynthetic pigment extraction and quantification**
242 The photosynthetic pigments (chlorophylls and carotenoids) were extracted in methanol as previously
243 described (Chiellini et al. 2020). In particular, samples of algal solution (1 mL) were centrifuged at
244 1500 rpm for 5 min. at 4°C. The supernatant was discharged, the pellet re-suspended in 1 mL of
245 methanol, exposed to 10 min. sonication (Branson 1210, Bransonic Ultrasonic Cleaner), and kept in
246 the dark at 4°C overnight. After centrifugation at 12,000 rpm for 5 min., the absorbance of the
247 supernatant was spectrophotometrically analysed (UV-1800 Spectrophotometer, Shimadzu)
248 compared to a blank containing only methanol at 665.2, 652.4 and 470.0 nm in order to measure the
249 chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and total carotenoid concentration, according to the
250 equations proposed by Lichtenthaler (1987).
251
252 **Chlorophyll fluorescence analysis**
253 Chlorophyll *a* fluorescence was measured using a portable pulse-amplitude-modulated fluorometer
254 (Mini-PAM; Heinz Walz GmbH, Effeltrich, Germany) at different times (days 0, 7, 14 and 21) as
255 previously described (Nestola et al. 2018), using a protocol opportunely modified to determine the
256 fluorescence emission from algal suspensions. Briefly, the fluorescence probe was positioned with a
257 constant distance and angle on the surface of 2 mL algal culture volume for each treatment. The
258 potential efficiency of photosystem II (PSII) photochemistry (F_v/F_m) was determined after adaptation
259 to the dark for at least 30 min. as $F_v/F_m=(F_m-F_0)/F_m$, where F_v represents the variable fluorescence in
260 the dark, F_0 is the minimum fluorescence yield in the dark, and F_m is the maximum fluorescence yield
261 in the dark after application of a saturation flash of light that completely closes all the PSII reaction
262 centres (RCs).
263 The photon yield of PSII photochemistry in the light (Φ_{PSII}) was determined at the algal growth PPFD
264 (100 μmol photons m^{-2} s^{-1}) as $\Phi_{PSII}=(F_m'-F)/F_m'$, where F_m' is the maximum fluorescence yield with
265 all the PSII RCs in the reduced state and F is the fluorescence yield at the actual reduction state of
266 PSII RCs during actinic illumination. F_m' was obtained by superimposing a saturating light flash
267 during exposure to actinic light. Non photochemical quenching (NPQ) was determined according to
268 the Stern-Volmer equation as $NPQ=F_m/F_m'-1$.
269
270 **Statistical analysis**
271 For each time point (days 0, 7, 14 and 21) four Pyrex flasks for each experimental condition (Control,
272 Zn-enriched, Mn-enriched) were prepared. A completely randomized block design was used: the
273 flasks were placed randomly in the culturing apparatus and repositioned daily. Unless otherwise
274 indicated, all samplings were carried out in quadruplicate, corresponding to the single flask (n = 4),

against triplicate blanks (Fig. 1). Consequently, each reported value in the figures and tables represents the mean ± standard error (n = 4). For each time point, a one-way analysis of variance (ANOVA) was carried out to evaluate the effects of the three culture conditions (Control, Zn-enriched and Mn-enriched) on algal performance.

Statistical analysis was conducted using CoSTAT version 6.311, CoHort Software (copyright 1998-2005, Monterey, CA, 93940, USA). Linear regressions between the photosynthetic pigments and the Φ_{PSII} and NPQ parameters were performed by a regression analysis (determination coefficient - R^2 and probability value - P) using STATISTICA 7.0, Stat-Soft, Inc. (Tulsa, OK, USA).

Results and Discussion

Growth and elemental analyses of algae

Our closed culture algae growing system was designed to avoid external pollution, water evaporation and to reduce invading bacteria, and the growth of other algae species. Under these conditions (i.e. 500 mL volume solution and sufficient nutrient supply), the microalgae growth was monitored weekly during the 21 day-assay by measuring the optical density at 530 nm (OD_{530}), the algal total dry mass per volume and the specific growth rate (Fig. 2).

As expected, the OD_{530} in the control solution showed the same trend as the algal biomass, with the highest increase after one week of culture and a plateau pattern until the end of the experiment (Fig. 2A). On the other hand, in the Zn and Mn treated solutions, the OD_{530} reflected the same trend as the algal dry mass concentration (g L^{-1}) up to the first week of growth, then, while the OD_{530} remained unaltered or slightly increased (Mn-enriched), the biomass generally decreased (30-60%) in both treatments during the last two weeks (Fig. 2B).

The OD is a good standard indirect method to monitor the density variations of microalgae culture solutions, as it is correlated to turbidity, the intrinsic properties of the cells and, indirectly, to the algal concentration (Vona et al. 2004; Jia et al. 2015). However, although the OD is usually determined at a wavelength that does not interfere with the photosynthetic pigments which are particularly abundant in this kind of microalgae, the relation between the OD and the quantification of algal biomass concentration may not be very close (Griffiths et al. 2011; Jia et al. 2015). Moreover, the OD not only depends on the concentration of the algal solution, but also on its limpidity (or opacity and granularity), which in turn can be influenced by several factors such as the cell size, aggregation status, interior cellular organelle anatomy, and cell density (Ferrando et al. 2015).

In fact, during the first week of the assay, the algal dry mass increased in all the growth conditions tested. In addition, the total dry mass produced by *Chlorella* sp. during the 21-day assay was generally

higher at all growth conditions compared to the initial level (0.26±0.01 g L^{-1}), and the final algal yield, on average, was higher than 1.0 g L^{-1} (Fig. 2B). This is consistent with large-scale microalgae harvesting systems in which the biomass contents are usually in the range of 0.1–1.0 g L^{-1} (Chen et al. 2011; Acién Fernández et al. 2018). Evidently, after 14 and 21 days of Zn or Mn treatments, the algal solution turbidity was high, but the biomass production started to decrease. This trend was also confirmed by the specific growth rate, representing the degree or velocity of algal growth over time (Fig. 2C). The algal biomass growth rate was maximum on the 7^{th} day of cultivation in each test condition, but decreased in the last two weeks, maintaining the highest levels in the Mn-treated solutions and in the controls.

The high growth rate and biomass productivity of *Chlorella* sp. in the first week of growth was consistent with the nature of such microalgae which are fast growing, and with several *in vitro* studies where the highest performance of *Chlorella* sp. was observed after 7-10 days of batch cultivation (Blair et al. 2014; Safi et al. 2014).

The reduction in algal biomass production and growth rate in the last part of the assay was probably partially due to the closed culture growing system (without renewing nutrients) and partially due to the inhibition effect of excess Zn on the cell multiplication rate. On the other hand, the Mn addition did not negatively affect the biomass or growth rate of *Chlorella* sp., which showed a stimulating effect for almost the entire duration of the assay (Fig. 2B, C).

The C and N contents reflected the high increase in growth after one week of treatment, especially in the algal solutions enriched with Zn or Mn (Fig. 3A-C). The highest levels of C were found in the algal solutions with Mn added, while the highest N enhancement was in the Zn-enriched solution after one week of treatment. However, while the final C balance of the algal growth system remained substantially unaltered from the beginning to the end of the test, the overall N content increased (+32.5%) during the 21-day assay with a general reduction (-27%) in the C/N ratio. This N increase was in agreement with the total protein content in *Chlorella* sp., which increased throughout the three weeks of growth in all the nutrient conditions, from 63.4 mg L^{-1} to an average level of 239.0 mg L^{-1} (Fig. 3D).

Chlorella sp. proteins vary according to the growth stages and conditions, and they play a fundamental role in the maintenance and repair of the cell, as well as acting as cellular motors, chemical messengers, regulators of cellular activities and in defence against foreign invaders (Morris et al. 2009; Safi et al. 2014).

In our experiment, the initial total protein content of *Chlorella* sp. was over 24% DW (data not shown) and remained at this level in the control and Mn-enriched solution, while it increased in the Zn treatment, reaching 50% DW. At days 7 and 21, the highest amount of proteins was measured in the algal solutions enriched with Zn, probably due to the synthesis of structural or enzymatic proteins

involved in the defence or transporter systems of this metal (Blaby-Haas and Merchant 2017; Chen et al. 2016; Zuñiga et al. 2018). The high protein content found in our *Chlorella* sp. strain is in agreement with that found in the same microalga species grown in optimal and supra-optimal growth conditions. This property has attracted the attention of scientists for the use of microalgae as unconventional food sources, and for their antioxidant, immune-modulating, anti-cancer and defence properties (Becker 2007; Morris et al. 2009).

Physical-chemical modifications in the medium

The initial pH value of the algal growth solutions (buffered modified TAP medium) was adjusted to 7.44±0.01 for all the conditions tested (basal and Zn- and Mn-enriched solutions). However, it increased towards the mean value of 9.13±0.03 at the end of the assay (Fig. 4A). Typically, cultivated algae cause marked changes in the pH of culture media, depending on the temperature and CO_2 concentration, by increasing the low pH values and lowering the high values (Grobbelaar 2013; Daliry et al. 2017). In fact, pH is one of the most important factors in algal cultivation because it governs the solubility and availability of CO_2 and essential nutrients, affecting algal metabolism. The uptake in inorganic C by algae can significantly increase the pH of the algal solution (Hansen 2002).

In our experiment, *Chlorella* sp. was cultivated in a closed growth system with controlled light intensity, temperature and CO_2 concentration in order to maintain an autotrophic regime and high biomass production (Safi et al. 2014). Given these growth conditions, the pH increase observed in the monoalgal solutions could be related to the natural ability of *Chlorella* sp. to increase or lower the pH until the stable alkaline value of 9-10. Moreover, in photoautotrophic algae cultures, the replacement of the CO_2 taken up for photosynthesis is relatively slow and results in a decrease in CO_2 partial pressure, thus leading to an increase in pH (Juneja et al. 2013). In natural and artificial growth systems for algae very complex and variable equilibria are formed in the solutions, depending on culture volume, temperature, nutrients availability, CO_2 concentration and light intensity, with continuous drift of pH (Globbelaar 2013; Daliry et al. 2017; de Freitas et al. 2019).

Chlorella sp. can grow in a wide range of pH values (4-10), however the highest biomass productivity is achieved in an alkaline environment (pH 9-10), and in the autotrophic growth regime, the pH of algal solution can increase to as high as 10 (Khalil et al. 2010). Values of pH over 10, 11 and more can induce microalgae cell aggregation and flocculation-sedimentation, which can facilitate the algae biomass harvesting, but reduce their active surface for biosorption (Safi et al. 2014; Chiellini et al. 2020). In our autotrophic closed system, where the optimal alkaline pH (> 9) was reached by the microalgae grown in the control conditions after the second week of culture, while in the Zn- and Mn-enriched solutions, it was reached after three weeks. This effect was probably due to the higher concentrations of Zn and Mn in the treated solutions, where the more reduced chemical status may

trigger an increase in redox potential and a pH reduction (Arunakumara and Zhang 2008; Kumar et al. 2015; Daliry et al. 2017). Under all the conditions tested, any precipitate or cell aggregation was observed and the optimal pH for growth was reached after 21 days, showing that *Chlorella* sp. was able to adapt to the high Zn or Mn concentrations used. Accordingly, in the control and Zn or Mn-enriched solutions, on average the EC decreased from 1643±77 to 776±40 µS cm^{-1} with a progressive increase in pH (Fig. 4B). This was probably due to the general reduction in ion availability in the solution, which was related to the consumption of nutrient ions and trace elements by the microalga population through absorption and adsorption (Kaplan 2013).

Regarding the amounts of Zn and Mn in the growth solutions, in the control conditions, their concentrations remained within the average range of algal Zn and Mn nutrient supplies (Fig. 4 C, D). Micronutrient supplies in algae are very variable, however Mn is usually used in smaller amounts (0.08-0.5 mg L^{-1}) than Zn (from 1.0 to 2.0-2.5 mg L^{-1} or more) (e.g.: Blair et al. 2014; Daliry et al. 2017; Chaudhary et al. 2018). In the algal solution enriched with 4 mg L^{-1} Zn, the Zn concentration decreased by 18.4 and 14.5% after two and three weeks of growth, respectively, reaching 3.2-3.4 mg L^{-1} Zn (Fig. 4C).

In the algal solution enriched with 1 mg L^{-1} Mn, the metal concentration decreased by 50% after the first week of growth and it maintained the same level (0.53-0.54 mg L^{-1}) until the end of the test (Fig. 4D). In both cases, the excess metal was evidently absorbed and/or adsorbed by *Chlorella* sp. In fact, in the Zn-enriched solution the reduction (-58%) in Mn availability was notable, starting from the 7th day of culture (Fig. 4D), when the removal of Zn also occurred (Fig. 4C). This could be explained by the higher biosorption of Mn in the microalgae with a high Zn concentration, as Zn and Mn elements, together with Cu and Fe, show similar balance-storage mechanisms in their homeostasis of algae cells (Blaby-Haas and Merchant 2017).

Biosorption and dynamic balance of Zn and Mn in *Chlorella* sp.

During the three weeks of cultivation, the Zn and Mn concentrations of *Chlorella* sp. increased under all the tested growth nutrient conditions. At the 21th day, the microalga Zn concentration reached a minimum value of 223 mg kg^{-1} (control) and a maximum value of 4837 mg kg^{-1} (Zn-treatment), whereas the Mn concentration ranged from 158 (control) to 589 (Zn-treatment) mg kg^{-1} (Fig. 5). These results, together with the increase in protein content (Fig. 3D), highlighted the efficiency of the algal growth system adopted in Zn and Mn biosorption and accumulation, and showed that most of the microalgae cells were in the exponential growth phase (Daliry et al. 2017; Liu et al. 2017).

The addition of 4 mg L^{-1} of Zn increased both the Zn and Mn biosorption capacity of the algae up to the 21st day of culture, while the treatment with 1 mg L^{-1} Mn stimulated only a higher uptake of Mn (Fig. 5). This is probably due to a synergistic effect between the two metals when neither of them

exceeds the toxicity threshold. Indeed, Zn is less harmful than Mn and other essential transition metals such as Cu and Fe (Maret and Li 2009), as confirmed by the lower permissible limits universally established for Mn than Zn, Cu and Fe. In Italy the permissible limits of Zn and Mn concentrations in groundwater are 3.00 and 0.05 mg L^{-1} (ppm), respectively (Lgs D. 152/2006). In natural surface waters and groundwaters, the Zn concentration ranges from below 10 µg L^{-1} to 40 µg L^{-1}, while in tap water, the Zn concentration is usually higher as a result of leaching from piping and fittings (Elinder 1986). According to the U.S. Environmental Protection Agency (EPA), drinking waters should contain no more than 5 mg L^{-1} of Zn because of the taste, and the recommended dietary allowance (RDA) for this element is 8-11 mg day^{-1}. Manganese concentrations of up to 1.3 mg L^{-1} and 9.6 mg L^{-1} have been reported in neutral and acidic groundwaters, respectively (ATSDR 2000), while the RDA for Mn has been set between 1.8 and 2.3 mg day^{-1} (IOM 2002).

Climate change, extreme weather events and the growth of human activities are increasing the release of trace metals in waters (Acién Fernández et al. 2018). A reduction in excess metals below their threshold values in natural or waste waters by microalgae cultivation would combine the purification of waters through biosorption with the large-scale cultivation of these microorganisms which would be useful for various sustainability applications. The production of biofuels, and mineral-enriched food is of particular interest if the accumulated metals are essential and under the toxicity thresholds. The potential of microalgae in metal remediation has been reported in several studies (e.g.: Das 2010; Rawat et al. 2011; Kumar et al. 2015; Chaudhary et al. 2018). However, the limits of Zn and Mn biosorption by microalgae are not clear, as there are still many uncertainties regarding the optimal doses and / or toxicity thresholds of these metals for microalgae (Grobbelaar 2013; Blair et al. 2014; Daliry et al. 2017).

We monitored the changes in Zn or Mn content in microalgae and in the nutrient solutions in terms of the growth unit used (500 mL volume flasks) in order to evaluate the metal balance and the removal efficiency (Fig. 6A-D). Every week the quantity of Zn or Mn effectively adsorbed and absorbed by the population of *Chlorella* sp. was compared with the corresponding amount of Zn or Mn present in the nutrient solution. In the Zn treatment, the Zn amount in *Chlorella sp.* increased from less than 0.1 mg to about 1 mg (corresponding to 2 mg L^{-1} Zn) after 14 days of growth, while in the solution, it decreased from 2 mg to 1.4-1.5 mg (Fig. 6A, C). In the same conditions, the algal Mn content also increased in each flask, varying from less than 0.10 mg to 0.12-0.18 mg (0.24-0.36 mg L^{-1} Mn), with the maximum level at the 14^{th} day of culture (Fig. 6B, D). In the Mn-enriched solution, the Mn removal by microalgae reached a maximum of 0.13 mg (0.26 mg L^{-1} Mn) per flask after 14 days of growth, with a corresponding decrease in Mn content in the solution from 0.50 to about 0.24 mg (Fig. 6B, D).

These Zn and Mn content dynamics in our algal growth system resulted in a removal efficiency of Zn in the Zn-enriched solution from 4.5 % at the 7th day of growth to 28-30% in the last two weeks, and a Mn removal of over 60% in the Mn-enriched solution from the first week of growth (Fig. 6E, F). These data confirmed the variations in Zn and Mn concentrations in the growth solutions (Fig. 4C, D), and showed that while the maximum Zn accumulation in *Chlorella* sp. occurring at the 14th day also corresponded to the highest reduction in the metal in the treated solution, in the Mn-enriched medium the Mn decrease started one week before (7th day) reaching the maximum Mn content in the microalgae (14th day).

It is thus worth noting that while in the Zn treatment, the maximum metal accumulation reached at the 14th day did not change in the last week of culture, in the Mn treatment the highest algal Mn content reached at the same day started to decrease (-38%) after the 14th day (Fig. 6A, B). This happened when in both treatments the algal concentration of the two metals was increasing (Fig. 5), and despite an effective decrease in biomass being recorded only in the Zn-enriched solution (Fig. 2B). In fact, in algae, as in any other organic matrix, the concentration and content of metals follow different trends depending on the type of metal, the growth conditions and the adsorption/absorption dynamics and mechanisms (Di Baccio et al. 2009; Kumar et al. 2015; de Freitas et al. 2019).

In the Zn-enriched conditions, the 21-day culture of *Chlorella* sp. reduced the Zn concentration by 15% in the solution, from 4 mg L^{-1} to 3.3-3.4 mg L^{-1}, which is very close to the legal limits in Italy for waters (3.0 mg L^{-1}), and the Mn concentration by 94%, from 0.17 mg L^{-1} to 0.01 mg L^{-1}, under the established Mn limit (0.05 mg L^{-1}) (Lgs D. 152/2006). In the Mn-enriched solution, the algal biosorption involved only Mn, which was reduced by half (-50%), from 1 mg L^{-1} to 0.5 mg L^{-1}.

These results confirm the high potential of *Chlorella* sp. in purifying waters that contain dissolved metallic ions (Kumar et al. 2015 and references therein). In fact, the bioconcentration (Fig. 5) and accumulation (Fig. 6A, B) patterns of Zn and Mn in *Chlorella* sp. showed that whereas the excess Zn stimulated the biosorption of Mn, excess Mn did not significantly increase the biosorption of Zn.

In terms of nutrient uptake, microalgae possess molecular mechanisms that enable them to differentiate between non-essential heavy metals for their growth and essential ones (Perales-Vela et al. 2006), such as Zn and Mn. The biosorption of Zn or Mn by *Chlorella* sp. can be represented by their bioaccumulation factor (BAF_{Zn} and BAF_{Mn}, Fig. 7). This factor, together with the bioconcentraction factor (BCF), is currently used in plants to reveal their role in nutrient/metal uptake (Baker and Walker 1990; Di Baccio et al. 2009; Maestri et al. 2010). However, it can also be used for algae and microalgae, showing the algae efficiency in removing nutrients/metals from the medium (Yan and Pan 2002; Abirhire and Kadiri 2011).

The highest metal biosorption performed by *Chlorella sp.* was for Mn (BAF_{Mn}, 3.4-5.1) with excess Zn between 14 and 21 days of growth, followed by the biosorption of Zn (BAF_{Zn}, 0.7-0.8) in the same

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484 growth conditions. A BAF higher than or equal to 1 indicates an accumulator capacity (Baker and
485 Walker 1990; Maestri et al. 2010). In fact, algae are rich in minerals and those found in *Chlorella*
486 include sodium, potassium, calcium, magnesium, iron, manganese, zinc, and copper (Sansawa and
487 Endo 2004). The algal Zn and Mn contents are variable and dependent on the *Chlorella* species,
488 growth phase and conditions (Liu and Hu 2013). However, the average Zn and Mn contents in
489 *Chlorella* sp. strain are about 8 and 2 mg per 100 g DW (Liu and Hu 2013). These quantities are
490 consistent with those measured in our *Chlorella sp.* at the beginning of the experimental set up (time
491 0, T_0): 6.3 mg Zn per 100 g DW and 1.6 mg Mn per 100 g DW (Fig. 5).
492 The increase in Zn microalga content after the first week of growth in the Zn added solution was
493 notable (32 mg Zn 100 g DW^{-1}) and continued until the third week, when the Zn algal level was 484
494 mg 100 DW^{-1}. The Mn content in *Chlorella sp.* grown in the Mn-enriched solution started to increase
495 only after two weeks of growth, but at the end of the assay (three weeks) it had already reached 20.8
496 mg 100 g DW^{-1}, which was 13-fold higher than the initial one. In addition, in the presence of a high
497 Zn concentration, the Mn algal content reached 59 mg 100 g DW^{-1} (Fig. 5). These results highlight
498 that microalgal biomass could be used as dietary and therapeutic supplements in human and animal
499 nutrition (Becker 2007; Safi et al. 2013).
500
501 **Photosynthetic pigments and physiological status**
502 One of the most distinctive characteristics of *Chlorella* sp. is its colour due to the chlorophyll
503 pigments, which can reach 1-2% DW (Safi et al. 2014). *Chlorella* contains chlorophyll *a* (Chl *a*) and
504 *b* (Chl *b*), along with a range of carotenoids such as β-carotene, lutein, zeaxanthin, violaxanthin,
505 neoxanthin and antheraxanthin (Sansawa and Endo 2004). These compounds essentially play the
506 same role as in higher plants, as Chl *a* and Chl *b* belong to the pigment-protein complexes in reaction
507 centres and light-harvesting antennae, and carotenoids are accessory light-harvesting pigments and
508 protective molecules against excess irradiance, chlorophyll triplets, and reactive oxygen species
509 (Masojídek et al. 2013).
510 Due to the relatively high abundance of chlorophylls in *Chlorella* sp., these pigments have been used
511 to measure the algal biomass (Peter et al. 2008). In our experiment, total chlorophylls (Chl *a*+*b*)
512 increased with microalgal growth, with the highest extent in Zn-enriched solution with the 7 day-
513 treatment, and reaching a general plateau between 14 and 21 days (Fig. 8A). The Chl *a*+*b* increase in
514 *Chlorella* sp. grown for 7 days with excess Zn was due to an increase (+66%) in Chl *b* (Table 2), with
515 a decrease (1.3-fold) in the Chl *a* to Chl *b* ratio. This might be due to the increased synthesis of Chl
516 *b*, employed in light harvesting complexes for the dissipation of excess energy in response to high Zn
517 concentrations (Ruban et al. 2004).

Carotenoids also showed a trend similar to Chl *a+b*, with an increase (about 2-fold higher) in the Zn-enriched solution after 7 days (Fig. 7B). This suggests that an enhanced synthesis of photosynthetic pigments by microalgae may improve the efficiency of light energy use with simultaneous protection against the negative effects of the potential stress associated with excess excitation energy in chloroplasts and the over-production of radicals (Masojídek et al. 2013).

After the first week of growth, the chlorophyll content of *Chlorella* sp. grown in the Mn-enriched solution was also impaired, with a significant decrease in Chl *a* (-25%) and consequent reduction in Chl *a*/*b* (1.3-fold, Table 2). Nevertheless, these modifications were not sufficient to alter the total chlorophyll content or the Chl *a+b* to carotenoid ratio. The alterations in photosynthetic pigments of *Chlorella* sp. grown in 4 mg L^{-1} Zn or 1 mg L^{-1} Mn did not result in visible impairment such as chlorosis and reduced biomass or OD. However, the most important variations in these compounds occurred when the rate of the microalgae population growth was at its maximum, that is after the first week of the bioassay (Fig. 2). This was most probably due to the fact that at the time of maximum development and cell proliferation (7 day-culture), *Chlorella* sp. required maximum efficiency and protection in the performance of oxygenic photosynthesis, which is the most important metabolic process carried out by autotrophic eukaryotic microorganisms such as microalgae (Masojídek et al. 2013).

As oxygenic photoautotrophic organisms, microalgae are considered to be very efficient solar energy converters, although they are no more efficient photosynthetically than higher plants (Tredici 2010). In fact, the real advantages of microalgae over plants are due to their unicellular nature, fast multiplication rate, metabolic flexibility, and the sequestration of C in gaseous (CO_2) and nongaseous (bicarbonate, HCO_3^-) forms (Safi et al. 2014, Sun et al. 2018).

Chlorophyll fluorescence has thus become one of the most common and useful techniques for photosynthesis research, also in algae studies, as it is a non-invasive, sensitive and relatively fast methodology to evaluate the performance of photochemical processes in the photosystem II (PSII, Papageorgiou 2007; Hu et al. 2016; Dani et al. 2017). In our study, the potential quantum efficiency of PSII (F_v/F_m) in dark-adapted *Chlorella* sp. reached the maximum levels between the first and the second weeks of growth in the standard medium, with slightly higher values in Zn- and Mn- enriched solutions. These values are in agreement with those detected in *Chlorella* sp. grown under similar experimental conditions (Chen et al. 2016; Jiao et al. 2017; Mykhaylenko and Zolotareva 2017). In particular, the F_v/F_m increased (+4.3-4.7%) in both Zn- and Mn-treatments at the 7^{th} day of algal growth (Fig. 9A), while the actual photon yield of PSII photochemistry in the light (Φ_{PSII}) did not change between the three nutrient algal conditions during the 21 day-culture, but again there was a notable increase after 7 days (Fig. 9B).

The relatively high levels of both F_v/F_m and Φ_{PSII} after 7 days of treatment are consistent with the highest performances shown by the microalgae culture in terms of biomass production, growth rate, and C and N contents (Figs. 2, 3). The relation between Φ_{PSII} and the total chlorophyll content of *Chlorella* sp. (Fig. 9B, inlet) thus supports the key role of these photosynthetic pigments in the use of light by autotrophic organisms (Peter et al. 2008; Ort et al. 2011). This indicates that the chlorophyll concentration of the algal solutions reflects the performance of the photochemical processes with high confidence, providing a good estimation of the *Chlorella* sp. photosynthetic efficiency and biomass production (Ruban et al. 2004; Peter et al. 2008; Ort et al. 2011).

At 7 days of treatment, the NPQ, which estimates the amount of excess excitation energy at PSII dissipated as heat, also increased markedly in the algal solutions enriched with Zn or Mn (Fig. 9C). In these treatments, the NPQ remained high also after 14 and 21 days of growth, when it was also reached by the control. The trend described for NPQ was the same as the variations in total carotenoid content of *Chlorella* sp. algal solutions (Fig. 8B). In fact, the NPQ has two main components, one representing the thermal dissipation of the light through harvest by antennae and the quenching dependent on xanthophylls, and the other that quantifies the energy trapped in the closed reaction centres of PSII (Klughammer and Schreiber 2008). This may explain the positive and highly significant relation between the enhancement of NPQ and the contents of algal carotenoids, as well as the accessory and protective photosynthetic pigments including carotenes and xanthophylls, which are involved in non-radiative light energy dissipation (Fig. 9C, inlet).

Conclusions

Our autotrophic growth culture system for *Chlorella* sp. was able to i) identify the optimal growth and nutrient conditions of these microalgae; and ii) evaluate the microalgae adaptation to supra-natural concentrations of trace elements and their biosorption capacity, through the more convenient temporal pattern of metal bioconcentration and accumulation in the algal biomass. In particular, the highest algal biomass production and growth rate were reached in the Mn-added nutrient solution during the first two weeks of culture. The *Chlorella* sp. strain tested showed a high tolerance to the enrichments of Zn and Mn, with high biosorption and bioremediation capacity of contaminated waters. In the Zn-enriched solution the maximum removal efficiency of Zn (28-30%) was registered between 14 and 21 days, when the Zn concentration in the medium was reduced to values very close to the Italian legal limit for waters (3.0 mg L^{-1} or 46 µM). In the Mn-enriched solution the abatement of Mn was more than 60% from the first week of microalgae culture, with a half reduction (0.5 mg L^{-1} or 9.1 µM) of the initial Mn concentration. A synergistic effect of Zn and Mn uptake by *Chlorella* sp. was observed in the Zn-added solution, where the Mn concentration was reduced from 0.17 mg L^{-1} to 0.01 mg L^{-1}, under the established Mn legal limit (0.05 mg L^{-1} or 0.91 µM).

Commentato [DDB2]: Nel nostro caso non è potenziale perché gli elementi vengono effettivamente determinati nell'alga.

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Commentato [DDB4]:

Commentato [DDB5R4]: Forse qua ci aggiungerei: tutto questo a fronte di un corrispondente incremento di Zn e Mn nelle alghe, sia come accumulo che come concentrazione.

Based on these findings, we believe that our study demonstrates that a suitable distribution of trace elements in the algal nutrient medium and an adequate temporal pattern of growth are cost-effective for culture management. This with the main objectives of i) remediating excess metals in discharge waters, ii) using recycled and sanitized waters rich in trace elements as micronutrients source in algal growth cultivation systems, iii) producing high value biomass enriched or biofortified with essential metals such as Zn and/or Mn.

This pilot culture system was designed to monitor the dynamic balance of moderately high Zn and Mn concentrations in the solutions, combining the optimal growth conditions of *Chlorella* sp. with the two metals bioavailability and biosorption by the microalgae in a relatively long period of culture (21 days). Further studies will be need to adapt this system to specific applications in large-scale plants and field conditions.

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Figure captions

Fig. 1 Schematic description of the experimental set-up with the Zn and Mn enrichments in *Chlorella* sp. culture. Microalgae were grown in 500 mL Pyrex glass flasks and for each treatment (C, control; Zn3X and Mn6X) four replicates ($n = 4$), corresponding to the number of flasks, were used, in addition to three blanks (basal and enriched culture solutions without algae). The assay started (day 0) with the algal inoculation (optical density, OD, 0.7) and was carried out for 21 days, reaching a maximum OD of 2.30. After three days of acclimation, the algal OD, the content of photosynthetic pigments and chlorophyll fluorescence were determined. In addition to these parameters, every week (sampling) the growth and elemental analyses of *Chlorella* sp. and the physico-chemical properties of solutions were performed. C, control, TAP medium with the characteristics indicated in Materials and methods; Zn3X, solutions enriched with a 3-fold higher Zn dose than the control; Mn6X, solutions enriched with a 6-fold higher Mn dose than the control.

Fig. 2 Effects of Zn- and Mn-enriched solutions on optical density (A), biomass production (B), and specific growth rate (C) of *Chlorella* sp. strain during a 21 day-assay. Data (mean values ± standard error, SE) reported in the figures refer to four replications ($n = 4$). The Zn- and Mn-enriched solutions contained 4.0 mg L^{-1} (60 µM) and 1.0 mg L^{-1} (20.5 µM) of Zn and Mn element, respectively. For each time point (7, 14 and 21 days), the results of one-way ANOVA are shown: significant level (P) = * ≤ 0.05; ** ≤ 0.01; where no symbol was shown the P-value resulted not significant. Different letters correspond to significant differences for the post hoc Fisher's LSD-test ($P \leq 0.05$)

Fig. 3 Contents of A- carbon (C), B- nitrogen (N), C- C/N ratio and D- total proteins in *Chlorella* sp. exposed to Zn- and Mn-enriched solutions for 21 days. The Zn- and Mn-enriched solutions contained 4.0 mg L^{-1} (60 µM) and 1.0 mg L^{-1} (20.5 µM) of Zn and Mn element, respectively. Data represent mean values ± SE of four replications ($n = 4$). For each time point (7, 14 and 21 days), the results of one-way ANOVA are shown: significant level (P) = * ≤ 0.05; where no symbol was shown the P-value resulted not significant. Different letters correspond to significant differences for the post hoc Fisher's LSD-test ($P \leq 0.05$)

Fig. 4 Effects of Zn- and Mn-enriched solutions on pH (A), electrical conductivity, EC (B), zinc (C) and manganese (D) concentrations of growth medium of *Chlorella* sp. during a 21 day-assay. The Zn- and Mn-enriched solutions contained 4.0 mg L^{-1} (60 µM) and 1.0 mg L^{-1} (20.5 µM) of Zn and Mn element, respectively. Data represent mean values ± SE of four replications ($n = 4$). The range between the dotted lines represents the algal Zn or Mn nutritional supplies based on a comparison of

several algal nutrient recipes (for references see the text). The dashed line indicates the legal limit in Italy for Zn or Mn (3.0 and 0.05 mg L^{-1}, respectively). For each time point (7, 14 and 21 days), the results of one-way ANOVA are shown: significant level (*P*) = * ≤ 0.05; ** ≤ 0.01; *** ≤ 0.001; where no symbol was shown the *P*-value resulted not significant. Different letters correspond to significant differences for the post hoc Fisher's LSD-test ($P \leq 0.05$)

Fig. 5 Effects of Zn- and Mn-enriched solutions on Zn (A) and Mn (B) concentrations (mg kg^{-1} dry weight) in microalgae *Chlorella* sp. during a 21 day-assay. The Zn- and Mn-enriched solutions contained 4.0 mg L^{-1} (60 µM) and 1.0 mg L^{-1} (20.5 µM) of Zn and Mn element, respectively. Data represent mean values ± SE of four replications ($n = 4$). Statistical analysis as in Fig. 4

Fig. 6 Variations in Zn and Mn contents in the microalga *Chlorella* sp. biomass (A, B), in the growth solutions (C, D) and the percentage metal removal (E, F) after 7, 14 and 21 days of treatment with Zn- and Mn-enriched media. The Zn- and Mn-enriched solutions contained 4.0 mg L^{-1} (60 µM) and 1.0 mg L^{-1} (20.5 µM) of Zn and Mn element, respectively. Data represent mean values ± SE of four replications ($n = 4$). For graphs A, B, C and D statistical analysis as in Fig. 4; for graphs E and F, one-way ANOVA was applied to evaluate differences in Zn and Mn removal by *Chlorella* sp. during the time-course of the experiment. Before statistical analysis, an arc sine or angular transformation to percentage data has been applied.

Fig. 7 Bioaccumulation factor (BAF) of Zn (A) and Mn (B) in the microalga *Chlorella* sp. after 7, 14 and 21 days of treatment with Zn- and Mn-enriched media. The Zn- and Mn-enriched solutions contained 4.0 mg L^{-1} (60 µM) and 1.0 mg L^{-1} (20.5 µM) of Zn and Mn element, respectively. Data represent mean values ± SE of four replications ($n = 4$). For each time point (7, 14 and 21 days), the results of one-way ANOVA are shown: significant level (*P*) = ** ≤ 0.01; *** ≤ 0.001; where no symbol was shown the *P*-value resulted not significant. Different letters correspond to significant differences for the post hoc Fisher's LSD-test ($P \leq 0.05$). BAF, calculated as the ratio between the total amount of the metal biosorbed by *Chlorella* sp. and the metal content in the culture solution at each time point

Fig. 8 Effects of Zn- and Mn-enriched solutions on total chlorophyll (Chl *a*+*b*, A) and carotenoid (B) concentrations in microalgae *Chlorella* sp. during a 21 day-assay. The Zn- and Mn-enriched solutions contained 4.0 mg L^{-1} (60 µM) and 1.0 mg L^{-1} (20.5 µM) of Zn and Mn element, respectively. Data represent mean values ± SE of four replications ($n = 4$). For each time point (7, 14 and 21 days), the results of one-way ANOVA are shown: significant level (*P*) = * ≤ 0.05; where no symbol was shown

the *P*-value resulted not significant. Different letters correspond to significant differences for the post hoc Fisher's LSD-test ($P \leq 0.05$)

Fig. 9 Changes in the maximal photochemical efficiency of PSII in dark-adapted samples (F_v/F_m, A), the effective quantum yield of PSII photochemistry (Φ_{PSII}, B) and non-photochemical quenching (NPQ, C) in *Chlorella* sp. during a 21 day-assay exposed to Zn (4.0 mg L^{-1}, 60 µM) or Mn (1.0 mg L^{-1}, 20.5 µM) enrichments. Data represent mean values ± SE of four replications ($n = 4$). For each time point (7, 14 and 21 days), the results of one-way ANOVA are shown: significant level (P) = * ≤ 0.05; where no symbol was shown the *P*-value resulted not significant. Different letters correspond to significant differences for the post hoc Fisher's LSD-test ($P \leq 0.05$). In graphs B and C, the inlets show the correlations between the photosynthetic pigments and the Φ_{PSII} and the NPQ parameters, respectively. For both correlations, the coefficient (R) and the significance level (***, $P \leq 0.001$) are shown