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Research Article

Evaluation of Copper Oxide Nanoparticles Toxicity Using Chlorophyll *a* Fluorescence Imaging in *Lemna gibba*

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Copper oxide nanoparticles (CuO NPs), used in antifouling paints of boats, are released in the environment and can induce toxicity to aquatic organisms. In this report, we used chlorophyll *a* fluorescence imaging to evaluate CuO NPs toxicity in *Lemna gibba*. This approach allowed to evaluate the differential effect of CuO NPs on photosynthesis of whole *L. gibba* plants. Exposure to 0.1 to 0.4 g/L CuO NPs during 48h induced strong inhibition of photosynthetic processes resulting in a decrease of plant growth. By using fluorescence imaging, different photosynthetic parameters were evaluated simultaneously in microplate conditions. Imaging of F_O fluorescence yield showed the decrease of leaf photosynthetic active surface for whole plants exposed to CuO NPs. This method showed that CuO NPs inhibited photosystem II maximal, photosystem II operational quantum yields, and photochemical quenching of fluorescence associated with electron transport. Nonphotochemical fluorescence quenching as an indicator of energy dissipation not used in photosynthesis was shown to be increased by the effect of CuO NPs. Such approach in microplate conditions provides synchronous high repetition measurements for numerous plants. This study may give a reliable methodological approach to evaluate toxicity risk of NPs in aquatic ecosystems.

1. Introduction

Development of nanotechnologies in the recent decades has led to the widespread use of nanomaterials in all kinds of common industrial and medical applications [1]. The use of nanomaterials may represent important toxicological risks because of the difficulty to evaluate the possible toxic effects of such pollutants. The mechanism by which nanomaterials may alter biological systems is dependant to nanomaterial's size, shape, composition, and surface properties [2]. Due to the fast growth of the nanotechnology domain, aquatic ecosystems may be contaminated by nanosized pollutants such as metallic nanoparticles (NPs), which are one of the main types used at large scale for industrial applications [3]. Toxicological properties of metallic NPs are difficult to evaluate because toxicity is dependant on both the nanoparticulate form and the toxic metal ions which can be released by the NPs [4, 5]. In addition, it has been found that the organic compounds used in NPs' synthesis can induce by themselves toxic effects [6]. For these reasons, it has been emphasized that NPs' toxicity is complex and many toxicological aspects still remain to be further investigated [7].

The use of NPs such as copper oxide (CuO) NPs in antifouling paints of boats represents an important source of NPs contamination of aquatic ecosystems. These paints consist of a polymeric film made mostly of acrylic and styrenic monomers covering copper oxides NPs [8]. This kind of paint may release copper species in the environment [9]. Decomposition of these paints may release copper under soluble ionic forms or as NPs, both of which can be toxic to aquatic life [10, 11]. Aquatic photosynthetic

organisms represent the main source of biomass in the aquatic trophic chain and these organisms are very sensitive to the effects of copper species. It has been reported that copper may induce strong inhibition of photosynthetic electron transport mboxprocesses and oxidative stress at cellular level [12, 13]. We reported recently that CuO NPs, having a core of CuO NPs and a shell of polyacrylic acid (PAA), were found to be very toxic to unicellular algae, causing inhibition of PSII electron transport capacity in *Chlamydomonas reinhardtii* cultures [11]. However, toxicity of CuO NPs was not evaluated in other photosynthetic organisms such as higher aquatic plants.

For the evaluation of pollutant's toxicity, the measure of chlorophyll (Chl) *a* fluorescence yield was found to be a sensitive methodological approach [14, 15]. Chl *a* fluorescence induced by light absorption represents a part of the dissipated light energy not used for Photosystem II (PSII) photochemistry and electron transport activity (for a review, see [16]). It is well known that when photosynthetic events related to biochemical or physiological processes are inhibited, the yield and kinetics of dissipated fluorescence are significantly changed [17]. Therefore, Chl *a* fluorescence can serve as a reliable, noninvasive indicator of photosynthetic processes in plants. The pulse-amplitude-modulation (PAM) fluorometric approach can provide useful information concerning photosynthetic electron transport and energy dissipation processes associated with PSII and PSI activity. Using the PAM approach, the yield of variable Chl *a* fluorescence as a measure of the PSII-PSI electron transport was frequently used to determine the toxic effects of metals and herbicides [18–20]. Parameters linked to PSII electron transport capacity (F_V/F_M, F'_V/F'_M), photochemical energy dissipation (qP), and nonphotochemical quenching of fluorescence (qN, NPQ) are sensitive bioindicators of toxicity in plants and algae [14, 21]. Recently, the development of Chl *a* fluorescence imaging system allowed for rapid and simultaneous measurements of a large number of samples. This approach was used for fast screening of plants mutants [22] and to evaluate different pathogen's effect in higher plants [23]. Using microplate bioassays, Chl *a* fluorescence imaging was used for the evaluation of herbicides toxicity in algae and higher plants [24–26]. However, it is not known how CuO NPs may induce toxic effects on PSII photosynthetic electron transport and energy dissipation processes in higher plants.

In this paper, we investigated the use of Chl *a* fluorescence imaging for rapid evaluation of CuO NPs' toxicity in the aquatic higher plant *Lemna gibba* in microplate conditions. In order to differentiate the effect of CuO NPs, we also investigated the possible toxic effects of dissolved ionic copper and polyacrylic acid (PAA), used for NPs' coating. Additionally, Chl *a* fluorescence kinetic was analysed using the PAM fluorometric approach to provide sensitive parameters useful to determine the toxic effect of CuO NPs on photosynthetic processes. Chl *a* fluorescence imaging was used to determine the change of such photosynthetic parameters in whole *L. gibba* plants exposed to CuO NPs' effect. This approach was evaluated concerning its application in ecotoxicological risk assessment.

2. Materials and Methods

2.1. Biological Material. *L. gibba* was obtained from Canadian Phycological Culture Centre (formerly UTCC #310). Plants were grown in an inorganic culture medium as described by [27], in a growing chamber (CONVIRON, Controlled Environments Limited, Winnipeg, Manitoba, Canada), with a 16h/8h light/dark photoperiod. Illumination was of $100\,\mu E\,m^{-2}\,s^{-1}$ provided by cool white fluorescent lamps (Sylvania GRO-LUX F40/GS/WS, Drummondville, Canada). Temperature was kept at 24°C.

2.2. CuO NPs Preparation and Characterization. Detailed synthesis of CuO NPs can be found in [11, 28]. Briefly, 4 g of CuO, provided by MTI Corporation (Richmond, CA, USA), and 110 mg of polyacrylic acid were prepared by RAFT polymerization mediated with trithiobenzyl carbonate and 20 mL of nanopure water. The samples were placed in 50 mL beakers in an ice bath. The mixture was stirred with a magnetic stir bar and sonicated for 3 min with a sonicator (VibraCell 400 W) equipped with a microtip. Under these conditions, an off-white dispersion of CuO in water was obtained. In a 250 mL round-bottom flask, 4.0 mL of styrene, 51.5 mg of sodium dodecyl sulphate, and 20.1 mL of the CuO dispersion were slowly stirred and degassed for 30 min by sparging with argon. The mixture was heated at 80°C. A solution containing three droplets of sodium hydroxide (30%) and 40 mg of 4,4′-azobis-4-cyanovaleric acid in 20.2 mL of nanopure water was added continuously during 4 h (5 cm^3/h) to the CuO dispersion. The use of the sodium hydroxide was to facilitate the dissolution of the initiator (carboxylic acid). The mixture was then kept at 80°C for 1 h in order to bring the reaction to completion. The reaction medium was analyzed by gravimetry (14.8% solids), thermogravimetric analysis (TGA, weight composition: 67% core, 33% shell), and transmission electronic microscopy (TEM). TEM micrographs were obtained with a Tecnai 12 Biotwin microscope equipped with a W source (80 and 120 kV) and a 13 Mpixel Morada camera. The samples were obtained by depositing a dilute sample on a gold grid coated with a formvar film. No contrast agent was used. Using this approach, particle diameter, measured by TEM, was 81 nm (standard deviation 16 nm) and shell thickness was 14 nm (for detailed data, see [11, 28]).

2.3. Pollutant Treatments. For experiments, plants of *L. gibba* developed at the stage of three fronds (during exponential growth) were used. Plants were placed in a 24 well microplate, one plant per well, in 2 mL of culture media. To avoid evaporation of the media during treatment, the microplate was closed with a lid. On the microplate, plants were exposed in duplicates to 0.1, 0.2, and 0.4 g/L of CuO NPs. For all concentrations of CuO NPs, a second treatment was done using only the dissolved ionic copper fraction present in the NPs treatments. To determine the total dissolved copper content of the NPs' treatments, NPs were removed from the treatment media by centrifugation

and analyzed using atomic absorption spectroscopy. Measurements of dissolved copper were done before and after the 48 h of treatments and no differences in the dissolved copper content was found between the beginning and the end of the treatment ($p = 0.33$). These results indicate that NPs' coating effectively prevented the degradation of CuO NPs into ionic copper in the media. From this approach, determined dissolved copper content was 3.29 ± 0.13, 6.60 ± 0.33, and 13.16 ± 0.63 mg/L, for the 0.1, 0.2, and 0.4 g/L CuO NPs' concentrations, respectively. Dissolved ionic copper was added as $CuSO_4$ to the treatment media. On the plate, a third treatment was composed of the PAA organic fraction of the CuO NPs that was used for NPs' coating. PAA was added to the treatment media according to the 67%–33% ratio of organic shell/inorganic core composition of the CuO NPs found in [28]. According to this ratio, PAA treatments were of 0.067, 0.134, and 0.268 g/L for the 0.1, 0.2, and 0.4 g/L CuO NPs concentrations, respectively. The disposition of the treatments on the experimental microplate is presented in Table 1.

2.4. Chl a Fluorescence Measurements. Chl *a* fluorescence kinetics were measured simultaneously in all samples at time 0, 24, and 48 h of treatments using the MAXI-Imaging PAM fluorometer system (Walz, Germany). For these measurements, the microplate was placed in the dark for 30 minutes to obtain equilibrium of PSII oxidoreduction state. Chl *a* fluorescence kinetic was measured according to [29, 30]. The fluorescence F_O, which represents basal fluorescence yield from chlorophylls of light-harvesting complexes, was evaluated by using a modulated light with a low intensity ($1\,\mu E\,m^{-2}\,s^{-1}$) to avoid the reduction of PSII primary electron acceptor, Q_A. The maximal fluorescence yield, F_M, was induced by a short, saturating pulse of white light ($5000\,\mu E\,m^{-2}\,s^{-1}$, 0.8 s duration), causing maximal reduction of PSII. The value of F_S was determined at the steady state of variable fluorescence obtained when algal samples were exposed (10 min) to continuous actinic light ($100\,\mu E\,m^{-2}\,s^{-1}$). The maximal fluorescence yield, F'_M, was determined by application of a saturating light ($5000\,\mu E\,m^{-2}\,s^{-1}$, 0.8 s duration) at steady state of fluorescence when plants were exposed to continuous actinic light. The F'_O fluorescence yield, indicating the fluorescence yield when PSII reaction center was in open state at steady-state conditions, was determined as $F'_O = F_O/(F_V/F_M + F_O/F'_M)$, according to [31]. The maximal PSII quantum yield, indicating efficiency of light energy transfer to primary acceptor Q_A, was determined as the ratio $F_V/F_M = (F_M - F_O)/F_M$ [32]. According to [33], the operational quantum yield was determined as the ratio $F'_V/F'_M = (F'_M - F_S)/F'_M$, and the photochemical quenching value was evaluated as $qP = (F'_M - F_S)/(F'_M - F'_O)$ representing the photochemical energy conversion at PSII reaction centers when the primary acceptor Q_A has been oxidized [29]. The fluorescence quenching which was not related to photochemistry of PSII was measured as the ratio $qN = 1 - (F'_M - F'_O)/(F_M - F_O)$ according to [34]. According to [35], the nonphotochemical quenching parameter was determined as the $NPQ = [F_M - F'_M]/F'_M$.

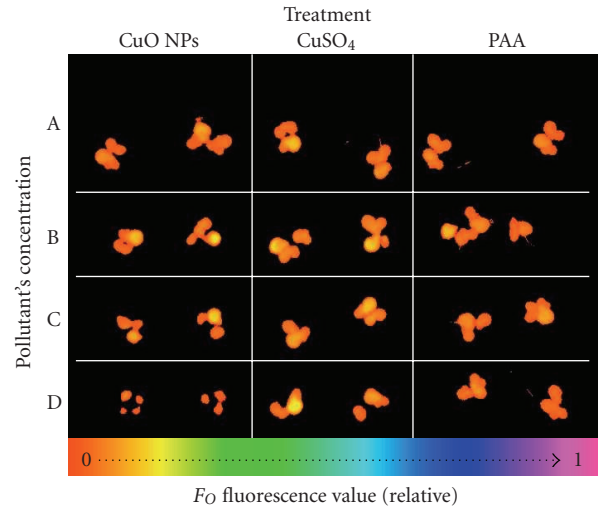

Figure 1: The change of active photosynthetic surface in *L. gibba* whole plants exposed 48 h to different concentrations of CuO NPs, ionic copper ($CuSO_4$), and polyacrylic acid (PAA). The active photosynthetic surface was evaluated by imaging of the F_O fluorescence yield for entire plant distributed in the microplate. Treatments' concentrations in the different rows (A, B, C, D) are distributed in the microplate according to Table 1. The change of leaf area and leaf Chl distribution is indicated on the figure by the change of the size and shape of the fluorescence emission. Color scale on the bottom of the figure indicates the color associated with the relative value of F_O fluorescence yield.

2.5. Data Analysis and Statistics. All treatments were done in duplicates on the microplate, and the experiment was repeated three times. Means and standard deviations were calculated for each treatment. Significant differences between control and exposed plants were determined by analysis of variance (ANOVA) and Tukey Honestly Significant Differences (HSD) test where *P*-value less than .05 was considered to be significant.

3. Results and Discussion

The effects of CuO NPs should be considered as a complex since NPs can go through a degradation process which can release other toxic products. Therefore, such secondary products of NPs' degradation should also be investigated in addition to the investigated NP form to better determine the effect closely associated with the interactions of NPs with cellular system. It has been shown that CuO NPs may release copper ions (Cu^{2+}) [36] which may induce itself toxic effects. It is known for copper ions to inhibit photosynthetic processes in *Lemna minor* [27]. On the other hand, acrylic acid has been indicated to induce toxic effects on algae and aquatic macrophytes [37]. However, in another recent report, polyacrylic acid (PAA) was shown to have no effect on the unicellular algae *Chlamydomonas reinhardtii* [11]. Therefore, by considering those effects, we investigated simultaneously the interactions of CuO NPs, copper ions (added as $CuSO_4$), and PAA with photosynthetic physiological state of *L. gibba*. To determine the change of the active photosynthetic surface

Table 1: Disposition of the different treatments on the microplate. Concentrations of 0.00 indicate control samples.

Treatments	CuO NPs		Ionic copper (CuSO4)		Polyacrylic acid (PAA)	
A	CuO NPs	CuO NPs	$CuSO_4$	$CuSO_4$	PAA	PAA
	0.00 g/L	0.00 g/L	0.00 mg/L	0.00 mg/L	0.00 g/L	0.00 g/L
B	CuO NPs	CuO NPs	$CuSO_4$	$CuSO_4$	PAA	PAA
	0.10 g/L	0.10 g/L	3.29 mg/L	3.29 mg/L	0.07 g/L	0.07 g/L
C	CuO NPs	CuO NPs	$CuSO_4$	$CuSO_4$	PAA	PAA
	0.20 g/L	0.20 g/L	6.60 mg/L	6.60 mg/L	0.13 g/L	0.13 g/L
D	CuO NPs	CuO NPs	$CuSO_4$	$CuSO_4$	PAA	PAA
	0.40 g/L	0.40 g/L	13.16 mg/L	13.16 mg/L	0.27 g/L	0.27 g/L

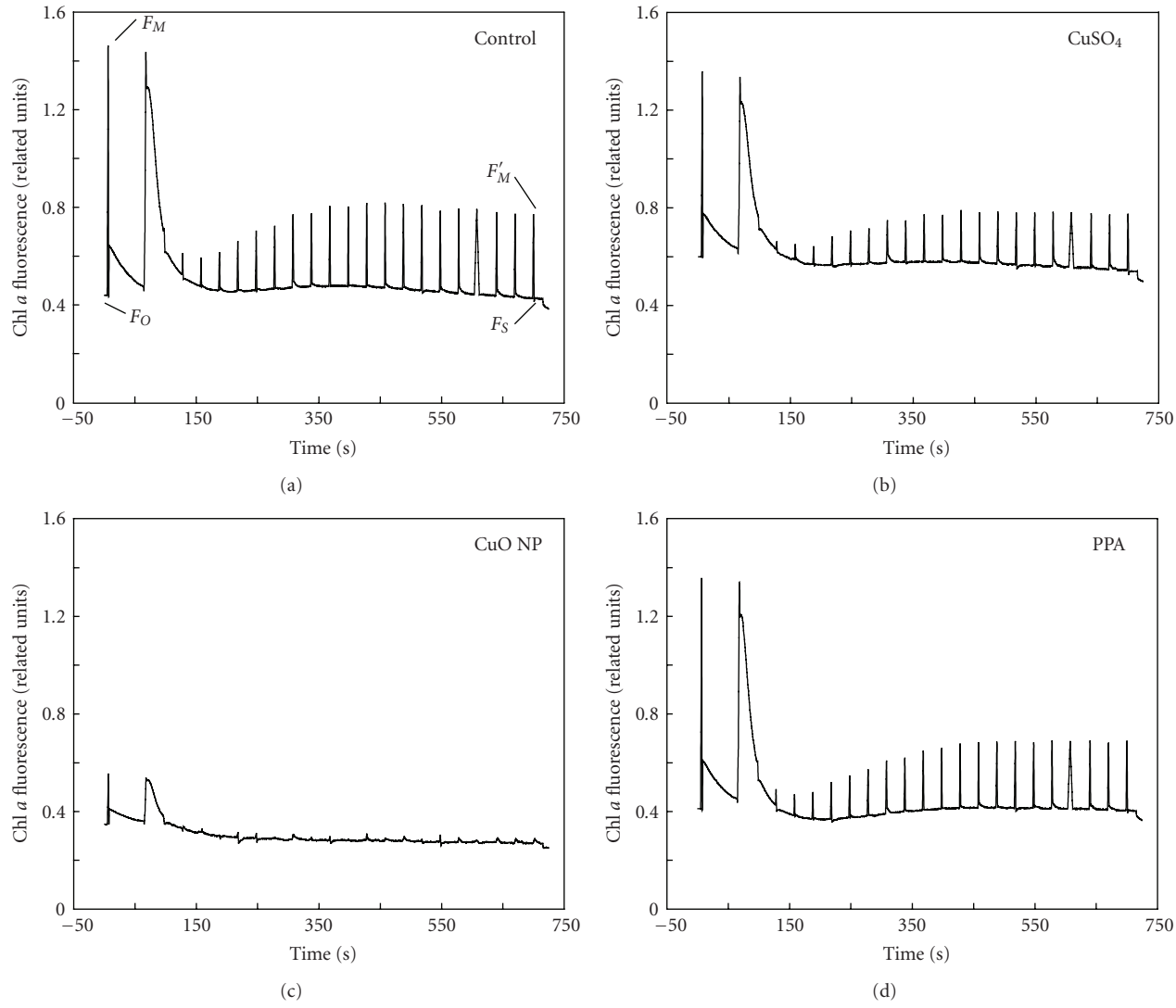

Figure 2: Change in the Chl *a* fluorescence kinetics of *L. gibba* leaves exposed 48 hours to 0.4 g/L of CuO NPs, its ionic copper fraction alone (13.16 mg/L Cu added as $CuSO_4$), and its organic fraction alone (0.27 g/L PAA). For treatments details, see Section 2.

of whole plants, we used Chl *a* fluorescence imaging method permitting to evaluate the relative value of chlorophyll content based on the evaluation of F_O fluorescence value. *L. gibba* plants, exposed separately to treatments in a microplate for 48 h, showed different effect concerning treatments. Here, imaging of F_O fluorescence (indicator of Chl content) showed that CuO NPs induced strong inhibition of plant growth indicated. Some inhibition by copper ions was also found, while no evident effect of PAA was observed (Figure 1). In *L. gibba* exposed to those treatments, we

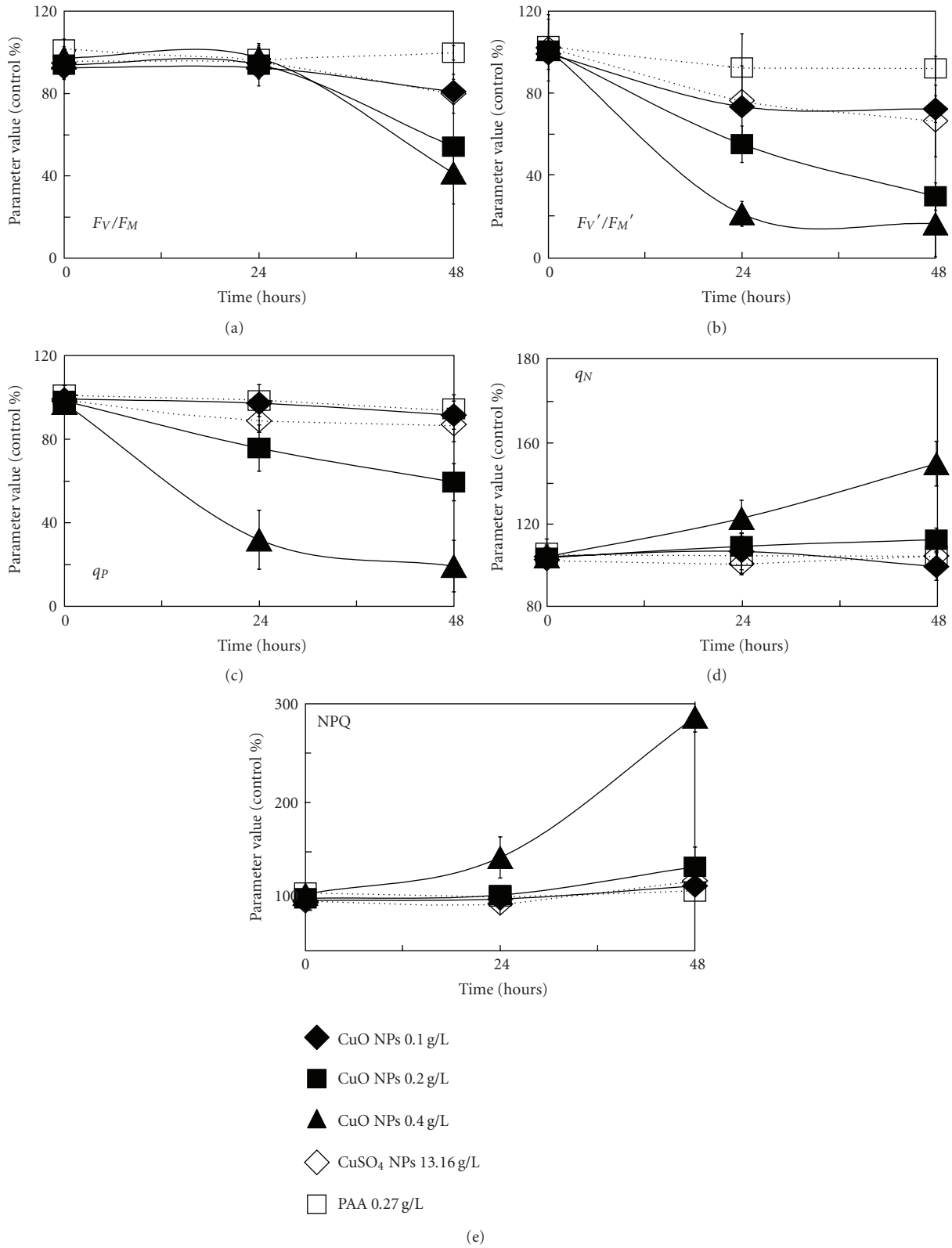

Figure 3: Change of maximal PSII quantum yield (F_V/F_M), operational PSII quantum yield (F_V'/F_M'), photochemical (qP) and nonphotochemical fluorescence quenching coefficient (qN), and the nonphotochemical quenching parameter (NPQ). Different concentrations are indicated by the different symbols on the figure. The error bars indicate the standard deviations calculated on an average of 6 replicates.

investigated the change of functional photosynthetic state by using the PAM-methodological approach. The change of Chl *a* fluorescence induction kinetics induced by 10 min actinic illumination showed high coherence with indications obtained by F_O fluorescence imaging. Indeed, maximal PSII fluorescence yield (F_V/F_M, in dark adapted fronds) and maximum fluorescence yield of PSII at steady-state of fluorescence (light-adapted at steady-state, F'_V/F'_M) were diminished by the effect of CuO NPs and, to a smaller extent, by copper ions (Figure 2). Those effects imposed the interest to evaluate the alteration of PSII functions caused by such treatments. Here, the change of PSII functional parameters during 48 h of treatment indicated the modification of PSII energy transfer associated with electron transport and energy dissipation pathways (Figure 3). After 48 h of treatment, maximal quantum yield of PSII (F_V/F_M) appeared to be strongly inhibited by CuO NPs (41 ± 15% of control sample, $P < .05$). Similar effect was also seen for operational quantum yield (F'_V/F'_M value of 16 ± 12% of control sample for 48 h 0.4 g/L treated plants, $P < .05$), while copper ions had some inhibitory effect which was relatively small (66 ± 17% of control sample, $P < .05$) compared to the effect of CuO NPs. Some effect of ionic copper can be expected since this metal was previously shown to induce inhibition of the PSII water-splitting system in isolated PSII particles [38]. Cu^{2+} was also found to increase the sensitivity of leaves to photoinhibition by light and oxidative stress due to its competition with iron as an essential element [39]. Oxidative stress was also found to have a major role in Cu^{2+} toxicity in photosynthetic organisms [40]. For CuO NPs, a strong production of reactive oxygen species was observed in algal cells exposed under light conditions to CuO NPs concentrations of 0.02 g/L [11]. However, the role of Cu^{2+} ions in CuO NPs toxicity is still a matter of debate. In some studies, it has been suggested for induced toxicity to be due to the dissolved ionic copper fraction [36, 41] while in other reports it has been indicated that ionic copper alone does not explain toxicity induced by CuO NPs [42, 43]. Our results concerning the change of F_V/F_M and F'_V/F'_M in *L. gibba* indicate that CuO NPs' toxicity was mainly caused by the NPs effect. For such change of PSII quantum yields, we may expect also important change within PSII photochemistry and electron transport associated with energy dissipation through regulated and nonregulated nonphotochemical pathways. Indeed PSII photochemical quenching values were strongly decreased by CuO NPs' effect. Such inhibitory effect altered energy dissipation processes via PSII. It appeared that CuO NPs' effect increased the energy dissipation which is not linked to PSII electron transport. The change of qN value (regulated nonphotochemical energy dissipation) at 48 h of treatment showed clearly the ability of CuO NPs to decrease drastically absorbed conversion of absorbed light energy via PSII electron transport. Similar and coherent change was found for CuO NPs' effect on the nonphotochemical quenching parameter based on the Stern-Volmer equation, NPQ (Figure 3).

The use of fluorescence measurements as an indicator of photosynthetic electron transport in whole plants may be associated with some problems since higher plants show great heterogeneity of leaves concerning physiological development and leaf position [44]. In many cases, for whole plants, it is hard to obtain reproducible results and to get some integral indicator concerning photosynthetic processes. We believe that recent fluorescence imaging methods may give some advantages concerning reproducibility and determination of integral value related to the whole plant. Here, we presented the advantages to use fluorescence imaging methods in the study of pollutant's toxic effects on whole plants. Since microplate has optimal conditions to expose *L. gibba* plants to different treatments, this approach may provide the possibility to perform multiple synchronous fluorescence measurements of numerous treatments. Furthermore, fluorescence imaging allows to obtain different photosynthetic parameters concerning the integral state of plants. Such approach to measure the inhibitory effect of CuO NPs on F_V/F_M is presented in Figure 4. In this figure, for visual presentation, different colors were used to determine the value of photosynthetic parameters distributed on the whole plant. The change of color indicated the heterogeneity of plants but this heterogeneity did not prevent to evaluate the integral value on the whole plant body. Here, we were able, for whole plants, to show the decreased value of F_V/F_M when plants were exposed 48 h to CuO NPs. We have to mention that fronds of *L. gibba* showed a different level

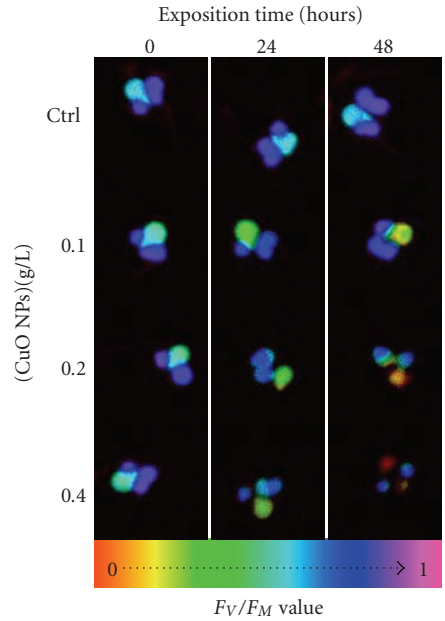

Figure 4: The change of the maximal PSII quantum yield (F_V/F_M) in *L. gibba* whole plants by being exposed to different concentrations of CuO NPs, determined by Chl *a* fluorescence imaging PAM method. CuO NPs' concentrations are indicated on the left side of the figure and exposition time on top of the figure. The change of leaf area and leaf Chl distribution is indicated on the figure by the change of the size and shape of the fluorescence emission. The change of color on the leaf area in the figure indicates the change of the F_V/F_M value on this specific leaf area. Color scale on the bottom of the figure indicates the color associated with the relative value F_V/F_M.

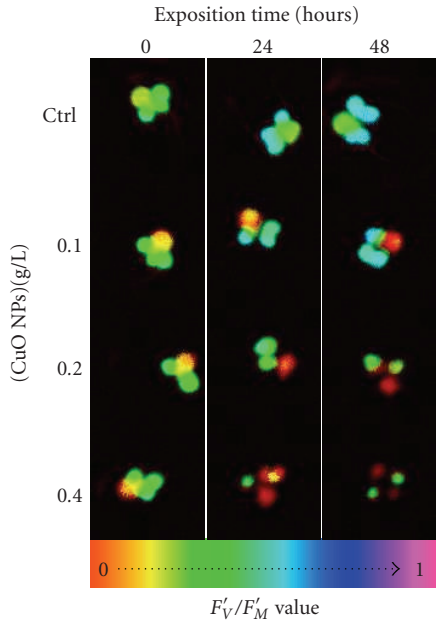

Figure 5: The change of the operational PSII quantum yield (F'_V/F'_M) in *L. gibba* whole plants by being exposed to different concentrations of CuO NPs, determined by Chl *a* fluorescence imaging PAM method. CuO NPs' concentrations are indicated on the left side of the figure and exposition time on top of the figure. The change of leaf area and leaf Chl distribution is indicated on the figure by the change of the size and shape of the fluorescence emission. The change of color on the leaf area in the figure indicates the change of the F'_V/F'_M value on this specific leaf area. Color scale on the bottom of the figure indicates the color associated with the relative value F'_V/F'_M.

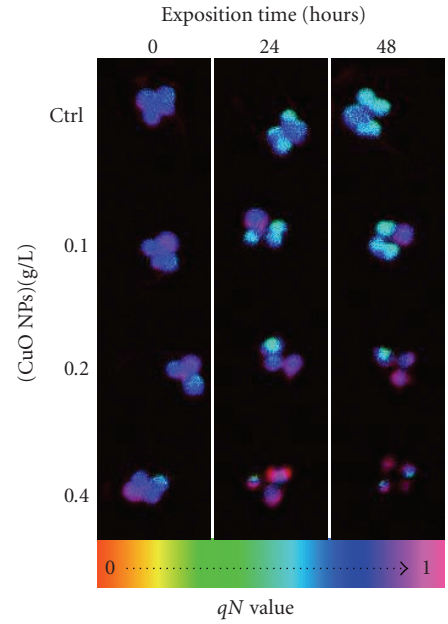

Figure 6: The change of the nonphotochemical quenching of fluorescence (qN) in *L. gibba* whole plants by being exposed to different concentrations of CuO NPs, determined by Chl *a* fluorescence imaging PAM method. CuO NPs' concentrations are indicated on the left side of the figure and exposition time on top of the figure. The change of leaf area and leaf Chl distribution is indicated on the figure by the change of the size and shape of the fluorescence emission. The change of color on the leaf area in the figure indicates the change of the F'_V/F'_M value on this specific leaf area. Color scale on the bottom of the figure indicates the color associated with the relative value of qN.

of inhibition, with some being drastically affected (orange colored) and other at a lesser extent (blue colored) after 48 h of exposure to 0.4 g/L CuO NPs. However, total average value of the whole plants was diminished (see Figure 3). Similar conclusions was found for F'_V/F'_M (Figure 5). Such results were coherent with the change of parameters obtained from PAM fluorescence kinetics. On Figure 6, a strong increase of nonphotochemical energy dissipation can be seen for the whole plant after 48 h of exposure to the highest concentration of CuO NPs. Moreover, the same presentation permitted to indicate a strong decrease of growth for such plants. We believe that no other methods will be able to give such evaluation for numerous samples at the same time. The versatility of this approach and its capacity to provide numerous simultaneous measurements on whole plants indicate that Chl *a* fluorescence imaging may be a valuable approach in CuO NPs' ecotoxicological risk assessment. However, in natural aquatic ecosystems, the degree of contamination by NPs is still hard to evaluate due to the difficulty of quantifying this type of pollutant [45] so we do not know yet the concentrations of nanoparticulate copper that may be encountered in the environment. Using high concentrations on CuO NPs, *L. gibba* photosynthetic electron transport was found to be a reliable biomarker for copper NPs' risk assessment, but the sensitivity of this approach remains to be investigated in naturally occurring conditions.

We can conclude that Chl *a* fluorescence imaging represents a useful tool to evaluate CuO NPs effect on photosynthesis in whole plants. These results showed that in microplate conditions, by using treatments and measuring protocol of *L. gibba* photosynthesis, Chl *a* fluorescence imaging permitted fast and reliable evaluation of CuO NPs toxicity for numerous samples. High-repetition measurements may provide rapid evaluation of the risk of toxicity of pollutants in aquatic ecosystems. Such method will allow for further advance research in environmental toxicological studies and bioassays.

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