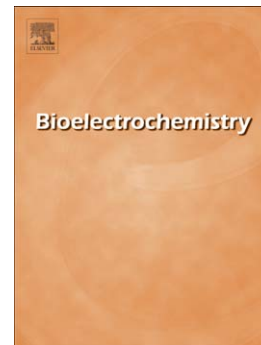# Accepted Manuscript

Interactions between heavy metals and photosynthetic materials studied by optical techniques

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INTERACTIONS BETWEEN HEAVY METALS AND PHOTOSYNTHETIC MATERIALS STUDIED BY OPTICAL TECHNIQUES

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Abstract

In this work studies on rapid inhibitory interactions between heavy metals and photosynthetic materials at different organization levels were carried out by optical assay techniques, investigating the possibility of applications in the heavy metal detection field. Spinach chloroplasts, thylakoids and Photosystem II proteins were employed as biotools in combination with colorimetric assays based on dichlorophenol indophenole (DCIP) photoreduction and on fluorescence emission techniques. It was found that copper and mercury demonstrated a strong and rapid photosynthetic activity inhibition, that varied from proteins to membranes, while other metals like nickel, cobalt and manganese produced only slight inhibition effects on all tested photosynthetic materials. By emission measurements, only copper was found to rapidly influence the photosynthetic material signals. These findings give interesting information about the rapid effects of heavy metals on isolated photosynthetic samples, and are in addition to the literature data concerning the effects of growth in heavy metal enriched media.

Keywords Photosynthetic materials, chloroplasts, heavy metals, Hill Reaction, fluorescence emission

Introduction

Photosynthesis is one of the most studied biological processes. Such interest could be explained by a fascination with this complex process and also by the possibility of applying the inner photosynthetic mechanisms in the most diversified fields. This last topic is really interesting, and examples of applications of photosynthetic materials can be found in the environmental or in the energy transfer fields [1-5].

Photosynthetic materials, such as the chloroplasts, thylakoid membranes, Photosystem II (PSII) from higher plants and the bacterial reaction centers (RC) could provide a great opportunity to built photo-optical electronic devices based on chloroplast proteins. Such applications of the photosynthetic materials are studied by a sub-branch of molecular electronics, called "RC-biotechnology". The main idea of such application is that the photosynthetic proteins behave exactly like molecular machines in which a light signal (which constitutes also the energy to be employed) is used to start a reduction-oxidation (redox) reaction [6, 7, 8].

Photosynthetic materials, and in particular RC proteins specifically interact with some pollutants such as pesticides, herbicides and heavy metals and therefore can be used for their detection. These pollutants are present in the industrial waste effluents and agricultural run-off and pose a risk of contamination of agricultural soils, surface and ground waters and a consequent accumulation in plant and animal tissues. Environmental monitoring is of fundamental importance for preservation of the planet and of its natural resources, and this is why the techniques and the procedures for detecting ecosystem pollutants are always in demand [1].

An interesting way for exploiting photosynthetic samples in the environmental field consists in performing bioassays in solutions: the observation of changes in the chemical-physical properties of the materials under test, in the presence of pollutant substances, can give useful information about mechanisms of interaction between photosynthetic systems and toxic compounds.

For example, one of the most interesting and simple assays for the evaluation of in vitro photosynthetic material activity (considered as electronic transfer) is the Hill Reaction [9-11], as reported in the "materials and methods" section.

Heavy metals such as copper, cadmium, mercury, lead are toxic even at low concentrations, they are not biodegradable, and have the tendency to accumulate in the living organisms [2]. It is known that heavy metals could seriously affect the photosynthetic apparatus by irreversibly binding the components of photosynthetic electron transport chain. At the same time it is also true that at very low concentrations many heavy metals are important for photosynthetic organisms. For example, copper (Cu) is a prosthetic group present in the protein plastocyanin and it is fundamental for photosynthesis: if Cu concentration in the nutrient medium is less than 10^{-14}-10^{-16} M (ranging from organism to organism) Cu deficiency can occur, and plants grown in such conditions were found with changes in the expression of several genes, with reductions in PSI and PSII activities and with morphological changes in the leaf or in the root structures [12, 13]. However, high Cu concentration (similar to other heavy metals) can strongly and negatively affect the photosynthetic electron chain, in particular at PSII level. It has been reported that PSII (rather than PSI) is the main target for Cu [14], although the binding site is still a matter of debate. Some researchers are sure that the site should be on the donor side, probably close to the Oxygen Evolving Complex (OEC) through its binding to the residue of thyrosine in PSII [15-17], but some different scientific suggestions do exist [4]. It is also widely reported that in the PSII-LHCII complexes (where LHCII represents the Light Harvesting Complex II) Cu interacts with chlorophylls (chls) by substituting the central Mg(II) atom. In particular, chls embedded inside the LHCII are considered as the main target pigments for Cu ions in the dark conditions [18-22]. Other heavy metal-substituted chls (hms-chls) have been observed and reported in the literature [18-22], as in the cases of Zn-chl or Ni-chl, and every hms-chl showed reduced fluorescence emission quantum yield and modified excited state levels [23, 24]; moreover, the formation of hms-chls is usually considered to play a key role in the photosynthesis inhibition by heavy metals [18, 22, 25-33].

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Although a great level of information can be found in the literature, about the effects of heavy metal enriched media on different photosynthetic organism growth , less abundant data have dealt with the effects of almost instant heavy metal additions to these bio-material.

In this work, investigations on the influences of heavy metals at different concentrations on photosynthetic materials isolated from spinach leaves were performed using optical methods. In particular, in the first part of the present work the effects of Cu(II), Hg(II), Co(II), Mn(II) and Ni(II) ions on the PSII complex, membrane fraction enriched in PSII (BBYs), thylakoid and chloroplast activities were analyzed by means of Hill Reaction based assays, showing the rapid inhibition in the electron transfer efficiencies especially in the presence of Cu(II) and Hg(II) for each biological sample. In the second part of the work, fluorescence emission measurements were carried out to consider influences of the same metal ions on the pigments present in the photosynthetic materials. Rapid and remarkable responses were obtained by each bio-material used only in the presence of copper, this showing a specificity of the emission test for this heavy metal.

Experimental

Chemicals

Triton X-100, n-dodecyl-β-D-maltoside (DM), n-octyl-β-D-glucopyranoside (OG), [2-N-morpholine]ethane-sulphonic acid (MES), NaCl, CaCl$_2$, CuCl$_2$, CoCl$_2$, NiCl$_2$ and NaHCO$_3$ were purchased from Sigma; HgCl$_2$ and MnCl$_2$ were purchased from Fluka; acetone (99.8%) and sulphuric acid (96%) were purchased from C. Erba.

Isolation of chloroplasts, thylakoids, membrane fractions enriched in PSII, PSII core complexes

Chloroplasts, thylakoids, membrane fractions enriched in PSII (BBYs) and PSII complexes were isolated from market spinach leaves according to Hankamer's procedure [34–37]. Chloroplasts were obtained from spinach leaves using differential centrifugation and thylakoids from chloroplasts by their rupture in a hypotonic solution. Thylakoids were solubilized with Triton X-100 in order to extract PSII enriched membranes. These last membranes were solubilized with OG to detach the LHCII proteins from the PSII core (OG-core complexes). OG-core complexes were then diluted with an aqueous buffer (MNCB) containing MES (25 mM), NaCl (10 mM), CaCl$_2$ (5 mM) and NaHCO$_3$ (10 mM), and DM was added to the solution, in order to obtain a chl concentration of 0.5 mg/ml and a 25 mM DM concentration.

Sucrose gradients, supplemented with DM (0.03 %, w/v), were used to obtain oxygen-evolving PSII core monomers which lack the 23- and 17-kDa extrinsic proteins and the CP29, CP26, CP24 chl binding proteins. The concentration of PSII complex samples was quantified accordingly to chl concentration, in mg mL^{-1} [38].

The photosynthetic materials (chloroplasts, thylakoids, membrane fractions enriched in PSII, PSII core complexes) were stored at -80°C after freezing in liquid nitrogen: these storage conditions prevents the biosamples from ageing effects [7, 39].

Photochemical assays

The oxido-reductive reaction known as the "Hill reaction" was originally observed on chloroplasts by using a suitable electron acceptor "A", as reported in the following:

$$H_2O + A \xrightarrow[\text{chloroplasts}]{h\nu} AH_2 + 1/2O_2$$
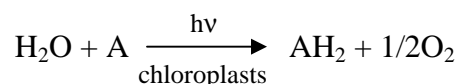
As electron acceptors, several compounds can be used, as in the case of 2,6-dichlorophenol indophenol (DCIP), that is capable to capture the electrons flowing from PSII to PSI during the Z-scheme performed under illumination inside chloroplasts, changing from blue to colourless [11]. In order to observe the photosynthetic activity of photosynthetic materials under the influence of added heavy metals, the Hill reaction was extended to all the bio-samples here studied; the electron transfer was correlated with DCIP photoreduction by an electron donor (water for BBYs, thylakoids and chloroplasts) catalyzed by the biomaterials under study. Absorbance measurements were carried out at 600 nm, and an extinction coefficient of 21 mM^{-1} cm^{-1} was used to calculate the concentration of reduced DCIP. A set of four 13 W fluorescent tubes (Osram, Munich, Germany), each one producing basic cool white light (830 lm), were used to induce the DCIP reduction. In order to compare the different bio-assays in their specific optimal conditions, the time of illumination was optimized and selected individually for each different photosynthetic material employed, as reported in the result section; chl concentration in each tested preparation was 0.005 mg mL^{-1} and DCIP was about 60 µM; the media used for each assay was SMNCB (pH 6.5), composed by 25 mM Mes, 0.3 M Sucrose, 10 mM NaCl, 5 mM $CaCl_2$, 10 mM $NaHCO_3$. The heavy metal ions tested in this work were Cu(II), Hg(II), Co(II), Mn(II) and Ni(II). Visible absorption spectra were recorded using a Varian CARY/5 spectrophotometer (Varian, Inc., Palo Alto, CA, USA) using 1 cm path length quartz cells.

Steady-state fluorescence experiments

Interactions between photosynthetic materials, particularly pigments, and heavy metals were studied by fluorescence emission tests. The experiments were performed by exciting the photosynthetic material suspensions (PSII, BBY and thylakoid membranes) in the absence or in the presence of metals at excitation wavelength 436 nm, and by observing emission in the range 600-800 nm. Every emission experiment was performed on bio-samples, diluted at final chl concentration of 0.015 mg mL^{-1} in SMNCB buffer. The heavy metal ions tested in this work were Cu(II), Hg(II), Co(II), Mn(II) and Ni(II). Fluorescence measurements were carried out using a Varian Cary Eclipse spectrofluorimeter (Varian, Inc., Palo Alto, CA, USA).

Statistical analysis

GraphPad InStat software (Sigma, St. Louis, MO) was used to process the data by analysis of variance (ANOVA) to indicate statistically extremely significant differences between means (one-way ANOVA with post-hoc Tukey test, $p < 0.0001$). All reported data represent mean values $\pm$ standard deviations.

Results and discussion

Hill Reaction assays

In the present study chloroplasts, BBYs, thylakoid membranes and PSII complexes were extracted from spinach leaves according to the section "Materials and Methods". Such variety of photosynthetic materials with different organization levels was used in order to compare the differences in the responses of these biosystems, to the presence of heavy metals, by using optical bio-assays.

The effects of copper, mercury, cobalt, nickel and manganese ions were analyzed by means of the Hill Reaction-like optical assay, since the photosynthetic activity can be determined as proportional to the absorbance decrement at 600 nm of the DCIP signal. This parameter is strongly connected to the electron transfer efficiency in the tested photosynthetic materials giving useful information on the interactions between heavy metals and samples.

Since this method requires that the sample is capable to catalyze the reduction of DCIP and the oxidation of water by exploiting white light illumination as energy, it is necessary that the starting photosynthetic material is highly efficient in the electronic transfer. This prerequisite is easily satisfied by BBYs, thylakoid membranes and chloroplasts which contain all the complexes involved in the electron transfer, but not sufficiently by PSII complexes which are extracted from their natural membranes and can not efficiently conduct the above-mentioned redox reaction. For this reason for PSII complexes some modification of the Hill Reaction would be required. It includes the addition of the 1,5-diphenylcarbazide (DPC) which is employed as a more appropriate electron donor. Nevertheless, in this work the interactions between PSII complexes and heavy metals were not investigated by this specific assay, since tested heavy metals were reacting with DPC, thus influencing the measurements (data not shown). As a result the Hill-like assays were performed with chloroplasts, thylakoid and BBY membranes.

The bio-samples were previously characterized by absorbance measurements and preliminary Hill Reaction-based tests were performed with the purpose to evaluate the optimal conditions for the subsequent analyses.

The absorbance spectra of the assay solution containing photosynthetic material and DCIP, were then recorded at different times under constant white light illumination. The spectra resulted from the overlapping of the DCIP absorption band visible in the range 550-650 nm and the typical thylakoid spectrum (Fig. 1A), having the main peaks at about 675 and 436 nm due to chlorophyll a and shoulders in the range 450–550 nm attributable to carotenoids [40–43].

Although a slight photobleaching effect is produced by light stress on the inner pigments, observable as decrements in the chlorophyll 675 and 436 nm peaks, this does not influence the range 550-650 nm, where DCIP absorbs light radiation; therefore, for these systems, in the range 550-650 nm, the most important light effect is the DCIP reduction by the photosynthetic samples, visible as decrement of its absorption band intensity.

As it can be seen on the Fig. 1B, the photoreduction of DCIP by all studied bio-samples reached its plateau within 5-6 minutes. It is clear that the chloroplast preparations were more efficient than thylakoids and BBY membranes, as demonstrated by the higher value of the photoreduction of DCIP. Nevertheless, all the tested photosynthetic materials were able to reduce DCIP and reach high plateau values.

After the optimization, optical assays, based on the Hill Reaction, were performed in order to verify the influences of the tested heavy metals on photo-induced electron transfer in the photosynthetic materials.

The results obtained for chloroplast solutions are reported in Fig. 2A: as it is shown, the electron transfer of chloroplasts was influenced in different ways by different metals at a fixed concentration of 10^{-4} M; the effects of heavy metals under test were calculated according to the subsequent formula and then plotted as bars:

Electron transfer inhibition % = $100 - (\Delta Abs_M / \Delta Abs_0) \cdot 100$

where ΔAbs_M represents the absorbance change after the Hill-like reaction (within a specified illumination time) for a photosynthetic material in the presence of one of the metals at the specified concentration; ΔAbs_0 represents the absorbance change after the Hill-like reaction for the photosynthetic material in the absence of metals.

The graph shows that when a metal has strong negative effect on chloroplasts, an inefficient DCIP photoreduction is performed, a remarkable metal effect has occurred and a high electron transfer inhibition bar can be observed. The Hill-reaction reached its maximum in 5-6 minutes for chloroplasts, and that is why it was decided to perform the analyses in 5 minutes under constant illumination, in order to test the effects of copper, mercury, manganese, cobalt and nickel ions. In the chloroplast preparations, the effects produced by 10^{-4} M bivalent manganese, cobalt and nickel ions on the DCIP reduction (i.e. on the electron transfer efficiency) were not found to be important. At contrary, Cu(II) and Hg(II) resulted to strongly affect the chloroplast electronic transfer, in a way which remarkably depended on the heavy metal concentrations, as reported in the next paragraphs. In order to evaluate any difference between tested photosynthetic samples, the same experiments reported in Fig. 2A were carried out for BBY and thylakoid membranes, and similar results were obtained from a qualitative point of view. Very slight influences were observed by adding 10^{-4} M of Co(II), Mn(II) or Ni(II) on the electron transfer efficiency, while 10^{-4} M of Cu(II) and Hg(II) showed remarkable effects.

In the Fig. 2B, the percentage effects of only copper and mercury ions on all the bio-samples used were showed. It can be observed that in each case, Cu(II) showed the strongest effects on DCIP photoreduction, with similar results for every bio-sample employed. Slightly less remarkable influences were recorded for mercury, and some differences were observed by changing the photosynthetic material. In particular BBY were found to be more stable with respect to Hg(II) addition than thylakoid and chloroplast preparations.

The dependences of the DCIP photoreduction efficiency on the copper and mercury ion concentrations were analyzed for all the bio-samples and the results are visible in the Fig. 3A and B, respectively.

As it is shown in the Fig. 3A, although the three different bio-samples could respond to copper even at 10^{-7} M concentrations, chloroplasts and thylakoids were found to be more resistant to Cu(II) than BBYs. It was, in fact, observed that in order to inactivate completely the chloroplasts and thylakoids, higher metal concentrations (10^{-5} M) were required, in comparison with BBYs which would be inactivated with 10^{-6} M of Cu(II).

Cu(II) is known to interact preferentially at the level of the LHCII chls in dark conditions, while under strong illumination it seems to directly damage PSII at level of the pheophytin or of the chl inside the reaction center [22]. Moreover, under strong illumination Cu(II) effects are enforced, probably due to rapid formation of OH radicals via a Cu(II)-catalyzed mechanism [44]. Obviously, these mechanisms of damaging produce secondary inhibiting effects on the electron transport chain of the photosynthetic systems. All these reasons could explain the greater sensitivity and more rapid responses of the tested photosynthetic systems to Cu(II) [22], as well as the easier inactivation of BBYs, that are thylakoid membranes enriched in LHCII-PSII complexes.

In the Fig. 3B the effects of Hg(II) concentration are reported: it is evident that Hg(II) concentration can influence the electron transfer in all performed bio-assays, even if all the bio-samples were found to be more resistant to Hg(II) than to Cu(II): in fact, Hg(II) ions inhibited the bio-sample activities at higher concentrations than Cu(II).

The mechanism of photosynthesis inhibition by Hg is less documented in the scientific literature [22]: it is indicated that Hg ions inhibit the electron flow in different photosynthetic organisms at multiple sites [45]: at the oxidizing side of PSII [46, 47], at the RC of PSI [48] or at level of the plastocyanin [46, 49]. The differences between Hg and Cu inactivation mechanisms could justify the greater resistance of the photosynthetic materials to Hg(II).

Emission measurements

 In Fig. 4A steady-state emission spectra of PSII complexes are reported, as recorded during a period of time of 20 minutes at room temperature, after excitation at 436 nm: the emission spectra present a typical band at 683 nm and a shoulder at 740 nm, which is probably due to a vibrational sublevel, as reported in the relevant literature [38, 50–52]. Fluorescence red re-absorption can be considered negligible because of the low PSII concentrations used (chl content was 0.015 mg/ml) [53]. As it can be easily observed, the PSII emission intensity undergoes a rapid decrease: this behaviour has to be ascribed to a partial aggregation involving the photosynthetic proteins in the buffer used, as previously reported [54]. Actually, in aqueous buffers even if stabilizing detergents are employed, PSII complexes (i.e. membrane proteins) can be involved in aggregative processes, that cause the quenching of the emission signal of their chls as long as the proteins aggregate; the emission intensity results to decrease during time, until a stationary intensity value is reached.
In Fig. 4B, the fluorescence emission intensity at peak value ($\lambda = 683$ nm) is measured as a function of time for PSII complexes, BBY, thylakoid membranes and chloroplasts at room temperature. The values are reported as percentage ratios between the absolute intensity sampled at a certain time and the corresponding initial intensity value. In this way the points in the graph indicate the residual emission percentages for the tested samples. Each reported emission measurement was carried using samples with chl concentration equal to 0.015 mg mL^{-1}. It was observed that PSII complexes underwent an evident emission quenching, while in average BBY and thylakoid membrane emissions were found to be more stable than PSII with time; also chloroplasts produced rather stable emission signals during 20 minutes of observation. All the above mentioned bio-samples were chosen for the subsequent investigations on the effects of heavy metals on photosynthetic material emission.
In the Fig. 5A the percentages of BBY emission loss after 10 minutes of incubation with 10^{-4} M heavy metals (copper, mercury, manganese, cobalt and nickel). These values were obtained according to the following equation:

Emission loss % $= 100 - (I_M/I_0)\cdot100$

where I_M represents the emission intensity after 10 minutes of incubation for a photosynthetic material in the presence of one of the metals at the specified concentration; I_0 represents the emission intensity for the photosynthetic material before metal addition. The values reported in the graphs were also corrected by subtracting a blank value, that is the value observed for the same photosynthetic material in the absence of metals after 10 minutes of incubation; this correction was mandatory, since biomaterials showed loss of fluorescence with time (Fig.4), and it was necessary to exclude that this phenomenon could overlap with effects of added heavy metal cations.
First of all, it is evident that the intensity of BBY emission is reduced in the presence of heavy metals, but most of all in the presence of copper ions. From a qualitative point of view, similar results were obtained for other photosynthetic materials: copper dramatically decreased the emission intensities also in PSII complex, thylakoid and chloroplast preparations.
In general, it can be asserted that the presence of each tested heavy metal in PSII, chloroplast and thylakoid solutions slightly affected the emission intensity, but in the case of copper, interesting effects have to be highlighted. The strong and rapid interaction between Cu(II) and photosynthetic samples is confirmed in Fig. 5B where the percentages of emission loss for all tested photosynthetic materials in the presence of copper ions are indicated: BBY emission loss was found as considerably greater than the other bio-samples after incubation with this metal; even for BBY no important effect was recorded in the presence of the other tested heavy metals.
The remarkable effects produced by Cu(II) on emission intensities of photosynthetic samples are in accordance with the data and observations here reported in the Hill assay section. In fact the formation of Cu substituted chls is responsible primarily for the loss of emission intensity [19] and it can strongly reduce the light harvesting efficiencies of antenna [18, 19]. In the past, most of the studies were carried out by means of in vivo tests, evaluating the growth of photosynthetic

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organisms in the presence of heavy metals: not only Cu-substituted, but also other heavy metal-substituted chls were observed in a great number of scientific works [21-24]. In our work effects produced by short- time incubation of different photosynthetic samples isolated from spinach with salts of heavy metals, added information to this stimulating debate. According to the literature, all the hms-chls should show a very important loss in the emission intensities if compared with the natural Mg-chl. In this work, the considerable emission decrements, that are visible only for Cu additions, should be ascribed to the short incubation time (20 minutes): therefore, the data seem to indicate that interaction between Cu(II) and chls occurs in a very short time, i.e. mainly in the first 10-15 minutes (data showing the effect of heavy metals during time were also obtained, but not shown); at the same time the effects observed with Hg, Ni, Mn and Co ions are less pronounced, indicating less rapid interactions with the photosynthetic materials.

The responses of the photosynthetic sample emission intensities to different concentrations of Cu(II) ions, after a fixed incubation time of 10 minutes are reported in Fig. 6, where emission intensity percentages were calculated as indicated for Fig. 4B.

As already found during the Hill assays, copper concentration affects all the photosynthetic materials under test, even if the greatest influence was observed on BBY membranes, which resulted to be strongly and sensitively dependant on the metal concentration, due to the fact that these particular biological preparations are enriched in PSII-LHCII super-complexes.

In fact, it is necessary to highlight that the concentration of chl was the same for each tested bio-preparation. Therefore, in chloroplasts and thylakoids chls are not only located inside LHCII or PSII proteins, but also inside PSI and LHCI proteins, which exploit this class of pigments for capture and conversion of the light energy; due to this, PSII-LHCII enriched membranes (i.e. BBYs) are more enriched in "useful chls" than thylakoids or chloroplasts (and than PSII complexes, which are isolated without their LHCII system). Another important explanation for the differences showed by BBY and PSII proteins could reside in the fact that during the experiments aggregation processes occur, that are more important for PSII proteins than for the other bio-samples; therefore, the accessibility of PSII chls could be significantly decreased. All these reasons could explain the greater sensitivity to the metal concentration for BBYs than for the other bio-samples tested.

In order to analyze the possibility of a practical application for the reported optical method, it would be necessary to discuss the concentration limits that have to be observed according to the relevant laws. In Europe limits of the heavy metal concentrations are determined by the individual European states. For example, in Italy, allowed concentration of Cu(II) in surface water is ≤ 0.1 mg L^{-1}, while in public sewerage waste is < 0.4 mg L^{-1} (which corresponds $\leq 1.57 \times 10^{-6}$ M and $\leq 6.30 \times 10^{-6}$ M, and on the logarithmic scale -5.8 and -5.2, respectively). As it is shown in the Fig. 6, the limits for Cu(II) are comprised in a concentration range, where the BBY emission loss changes sensitively; for this reason, the emission assay could provide a potential tool for the monitoring of copper ions in water on the scale required by legislation. At the same time PSII proteins and the other tested bio-samples did not demonstrate the sensitivity towards the copper which would be sufficient for their practical applications.

Conclusions

The data presented here refers to the effects of heavy metals (Cu(II), Hg(II), Co(II), Mn(II), Ni(II)) directly added to isolated photosynthetic materials, differently from most of literature data, that regard the growth of photosynthetic organisms in heavy metal enriched media. Results showed that assays exploiting fluorescence emission and absorbance techniques can give very useful information about short-time toxic interactions occurring between copper or mercury ions and photosynthetic materials extracted from spinach leaves. In particular, the emission measurements showed a specificity for copper, with higher sensitivity when BBY membranes were employed as a biotool; other bio-materials, i.e. PSII, thylakoids and chloroplasts showed less sensitive responses. At the same time, the Hill Reaction-based methods were found to be more sensitive to Cu(II) than to other metals, even if interesting responses were also observed with respect to Hg(II). Since concentration of 10^{-7} M Cu(II) could be measured, that is lower than the European concentration limits for Cu(II) in water, the data concerning copper ion effects using both optical assays could be considered as a basis for developing practical and rapid copper-specific analytical tools, with reduced costs if compared to the official standard methods for metal determination, like Graphite Furnace Atomic Absorption or Inductively Coupled Plasma Atomic Emission Spectroscopy. The co-operation of the two optical assays could also give interesting analytical information, since while positive responses obtained by the Hill method indicate the presence of copper and/or mercury, the emission test will be capable to give information exclusively about copper ions. Moreover, although the Hill Reaction method has been more widely proposed for the detection purposes, it offers only the possibility of assays in solution, whilst the emission measurements here presented, constitute a background study for possible application of the BBY membranes in the biosensor field.

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Figure captions

Fig. 1 The panel "A" shows room temperature absorption spectra of chloroplasts in the presence of DCIP under white light illumination, recorded after different illumination times within 10 minutes. In the panel "B" the photoreduction of DCIP by BBYs, thylakoid membranes and chloroplasts at different illumination times is reported. DCIP concentration is given in nmol per mg of chl calculated from spectrophotometric measurements.

Fig. 2 Effects of different heavy metals on chloroplast preparations, observed as percentage influences on Hill-like reaction efficiencies in the presence of each tested metal (A). Effects of Cu(II) or Hg(II) at 10^{-4} M concentration on BBY, thylakoids (Thyl) and chloroplasts (Chlor) observed as percentage influences on Hill-like reaction efficiencies in the presence of each tested metal (B). Illumination time was 5 minutes for each assay test; reported data represent mean values ± standard deviations obtained from three replicates.
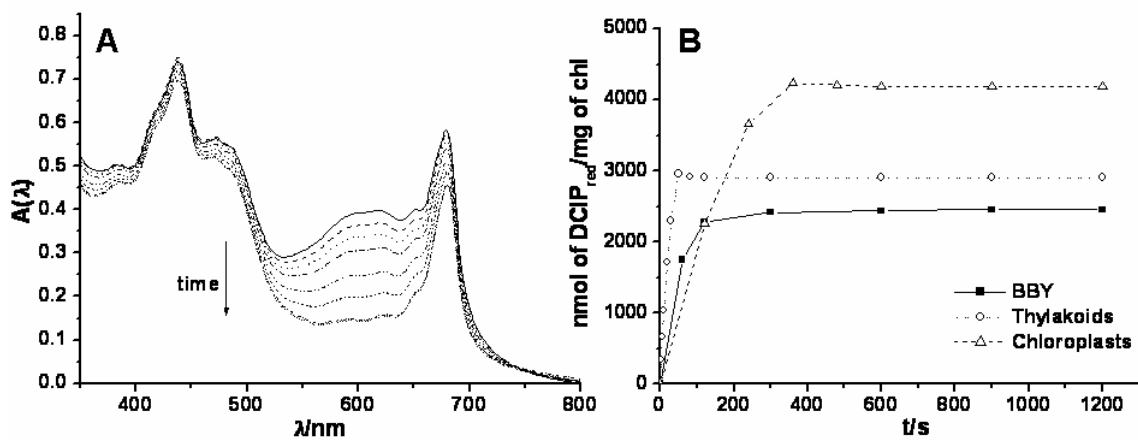
Fig. 3 Effects of different concentrations of Cu(II) (A) or Hg(II) (B) on chloroplasts, BBY and thylakoid membranes. Reported data represent mean values ± standard deviations obtained from three replicates.

Fig. 4 Panel "A": decrease in fluorescence intensity of PSII monomer within 20 minutes at room temperature. Panel "B": fluorescence emission intensity (%) of PSII monomers, BBY and thylakoid membranes at 683 nm as a function of time, at room temperature; the values are normalized with respect to the initial intensities.

Fig. 5 Fluorescence emission loss (%) of BBY membranes at 683 nm after 10 minutes of incubation in the presence of 10^{-4} M heavy metals, at room temperature (A) fluorescence emission loss (%) of PSII, BBYs, thylakoids (Thyl) and chloroplasts (Chlor) at 683 nm after 10 minutes of incubation in the presence of 10^{-4} M copper ions, at room temperature (B). Reported data represent mean values ± standard deviations obtained from three replicates.
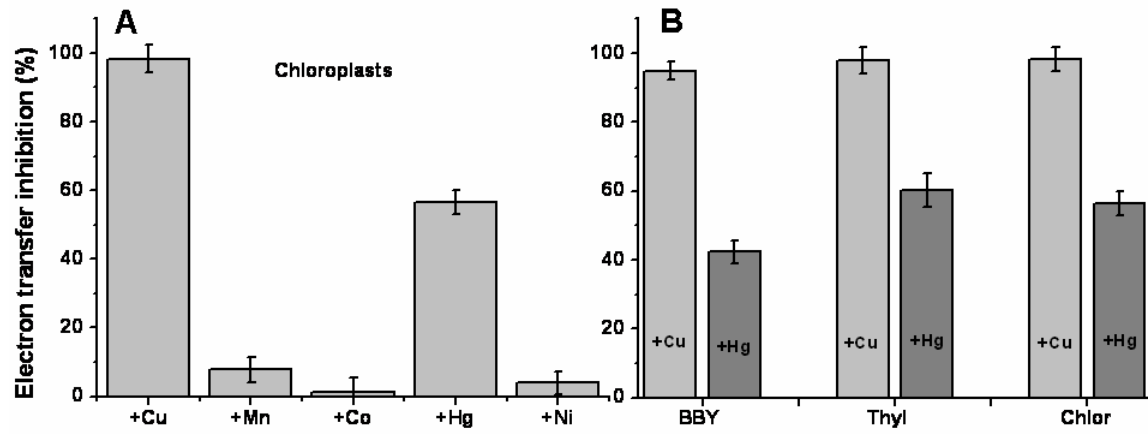
Fig. 6 Fluorescence emission loss (%) of PSII complexes, BBYs, thylakoid membranes and chloroplasts at 683 nm after 10 minutes of incubation in the presence of Cu(II) at different concentrations, at room temperature. Reported data represent mean values ± standard deviations obtained from three replicates.

Figure 1

Figure 2

Figure 3

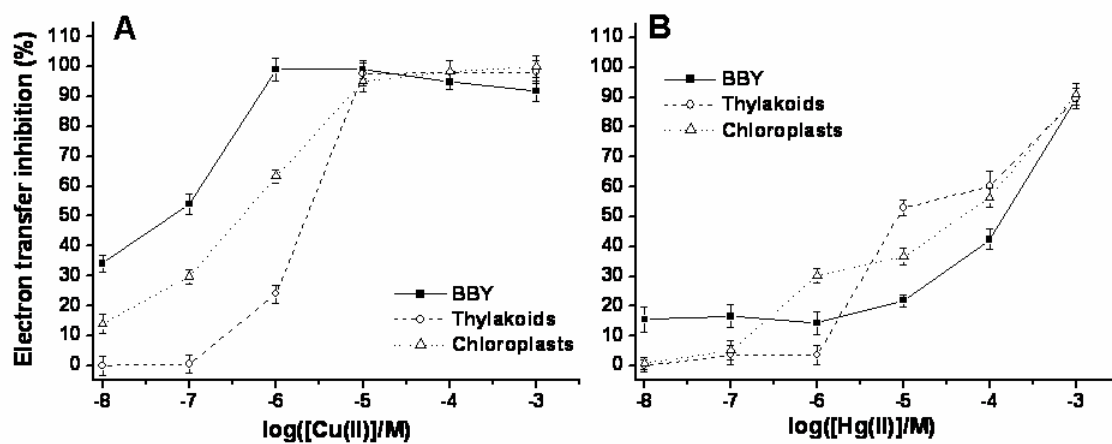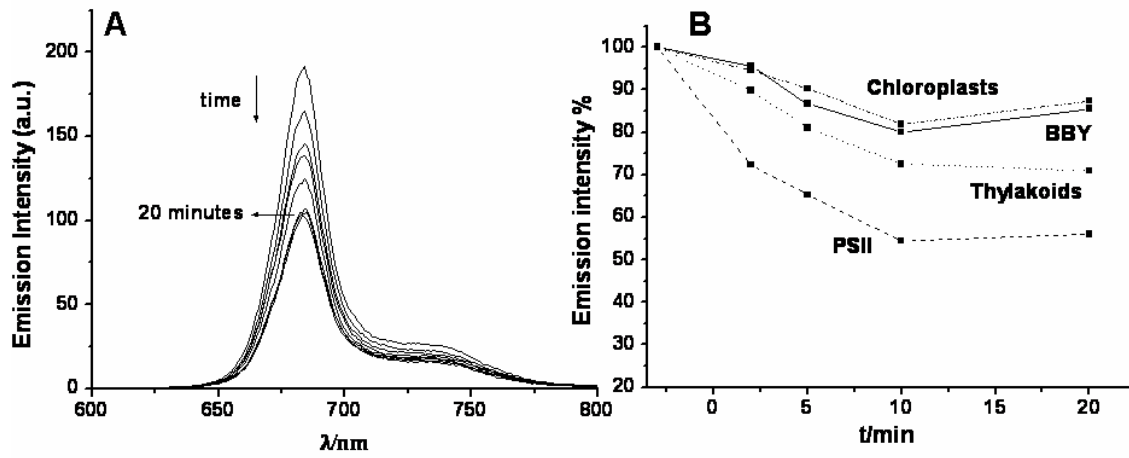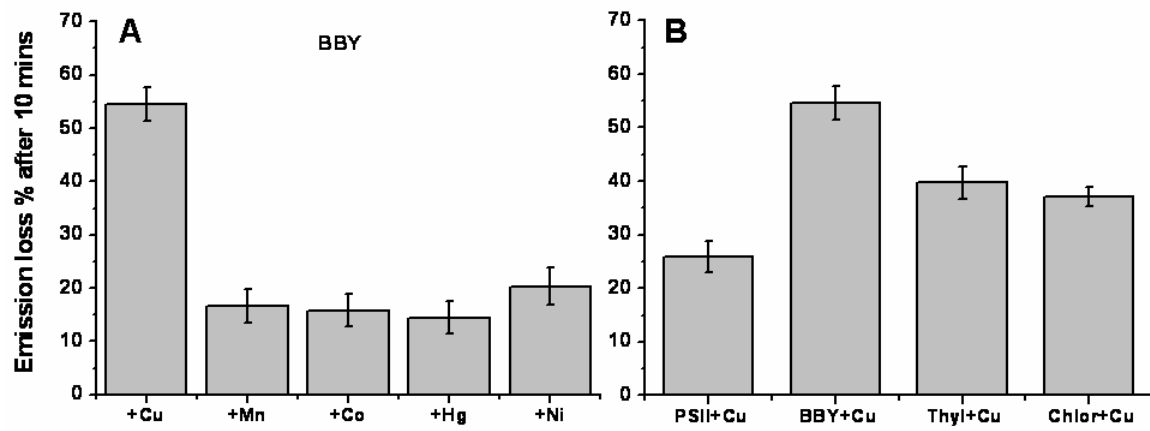**Figure 4**

Figure 5

Figure 6