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# Environmental relevance of heavy metal-substituted chlorophylls using the example of water plants

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## Abstract

**Following experiments which studied the substitution of the central ion of isolated chlorophylls by heavy metal ions *in vitro*, *in vivo* experiments with submersed water plants were carried out. It was discovered that the substitution of the central atom of chlorophyll, magnesium, by heavy metals (mercury, copper, cadmium, nickel, zinc, lead) *in vivo* is an important damage mechanism in stressed plants. This substitution prevents photosynthetic light-harvesting in the affected chlorophyll molecules, resulting in a breakdown of photosynthesis. The reaction varies with light intensity. In low light irradiance all the central atoms of the chlorophylls are accessible to heavy metals, with heavy metal chlorophylls being formed, some of which are much more stable towards irradiance than Mg-chlorophyll. Consequently, plants remain green even when they are dead. In high light, however, almost all chlorophyll decays, showing that under such conditions most of the chlorophylls are inaccessible to heavy metal ions.**

Key words: Heavy metal chlorophylls, submersed water plants, antenna pigments, copper, zinc.

## Introduction

Several heavy metals such as Fe, Cu, Co, Mn, Mo, and Ni are essential elements to plant metabolism and are often added to mineral fertilizers. In higher concentrations, many heavy metals severely damage plants. This has been extensively studied and reviewed (e.g. Fernandes and Henriques, 1991; Markert, 1993). After continued contamination of a habitat with heavy metals, only species possessing physiological mechanisms to cope with toxic heavy metals (for example, to exclude heavy metals, or to isolate them in vacuoles, or to sequester them by phytochelatins or to integrate infiltrated ions beneficially into their metabolism) will be able to survive (e.g. Kneer and Zenk, 1992).

Many existing assumptions concerning mechanisms of damage by heavy metals argue in terms of inhibition of enzymes (e.g. protochlorophyllide reductase (Stobart *et al.*, 1985)), the oxidizing side of photosystem II (e.g. Clijsters and Van Assche, 1985; Gross *et al.*, 1970), plastocyanin as a component of the photosynthetic electron transport chain (Kimimura and Katoh, 1972), and of heavy metal-induced disturbances of mineral metabolism (Kahle, 1988). Sandmann and Böger (1980) have pointed out the importance of lipid peroxidation by Cu stress. However, experiments of Sheoran *et al.* (1990) and Malik *et al.* (1992) showed that neither inhibition of enzymes of the photosynthetic carbon reduction cycle nor inhibition of stomatal conductance and transpiration can be the mechanism of primary damage. Finally, inhibition of other enzymes, which was found *in vitro*, could not be verified by *in vivo* experiments (Clijsters and Van Assche, 1985; Sheoran *et al.*, 1990).

Heavy metal-substituted chlorophylls (hms-chls) and related porphyrins have been known *in vitro* for a long time. Zn-porphyrins are known from chlorophyll-biosynthesis (Rebeiz and Castelfranco, 1973), as well as from porphyrin decay; $Zn^{2+}$ may complex to the breakdown product biliverdin (Hendry and Jones, 1980). Cu-porphyrins are known from deep-sea sediments, and it was suggested that these substances are recent degradation products of Mg-chlorophyll (Mg-chl), comparable to the much older Ni- and vanadyl-geoporphyrins (Moffett *et al.*, 1990; Palmer and Baker, 1978). The possible role of Zn-porphyrins in the early evolution of photosynthesis has been discussed (Larkum and Barrett, 1983). Several authors (De Filippis, 1979; Gross *et al.*, 1970; Kowalewska and Hoffmann, 1989; Puckett, 1976;

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Samuelsson and Öquist, 1980) have published spectra which show the formation of hms-chls in heavy metal-stressed plants (see Discussion). Some of them (De Filippis, 1979; Gross *et al.*, 1970; Kowalewska and Hoffmann, 1989) suggested substitution of the central ion of chlorophyll by Cu as an interpretation of the spectra.

Watanabe and Kobayashi (1988) noticed *in vitro* that hms-chls have remarkably lower fluorescence quantum yields in comparison with Mg-chl or do not exhibit fluorescence at all (e.g. Cu-chl). Hms-chls also exhibit fluorescence at different wavelengths than Mg-chl. Since the excitation states which are transferred from the antenna pigments to the reaction centres (resonance energy transfer) in the chloroplasts are the same ones which are responsible for fluorescence (Karukstis, 1991), hms-chls are not adapted to light harvesting. In experiments with artificial chl *a* monolayers on an $SnO_2$ electrode, Watanabe *et al.* (1985) also found 'that Mg-chl *a* ... has the highest ability of electron releasing from the singlet excited state ...', compared to other hms-chls. This ability makes Mg-chl only suitable for effective electron transfer in reaction centres. In addition, hms-chls might also be unsuitable for light harvesting for another reason: According to Rebeiz and Belanger(1984) Zn-chl does 'not co-ordinate as readily or as tightly to a second axial ligand' as Mg-chl and may, therefore, be 'difficult to orient in the macromolecular environment of biological membranes'. Similar difficulties might arise also in the case of other metals. So substitution of Mg in the chl molecule by heavy metals (Mg-substitution) *in vivo* makes plants incapable of photosynthesis.

Hms-chls were prepared *in vitro*, examining the reactivity of the different heavy metal ions with pheophytin and Mg-chl. The different properties (absorbance/fluorescence spectra, stability) of the hms-chls formed were also studied. These experiments led to the question whether Mg-substitution *in vivo* contributes to the damage caused by heavy metal stress of plants. To answer this question, *in vivo* experiments were started with water plants. In order to trace the formation of hms-chls in these plants, spectrophotometric and fluorimetric measurements of entire leaves, chloroplasts and extracts from affected and unaffected plants were recorded. These results were compared with measurements of photosynthesis, macroscopic and microscopic observations.

## Materials and methods

### *Preliminary in vitro* experiments

For *in vitro* experiments varying volumes of solutions of $Cu^{2+}$, $Zn^{2+}$, $Cd^{2+}$, $Ni^{2+}$, $Pb^{2+}$, $Ag^{+}$ or $Hg^{2+}$ ($c(hm^{n+})=0.025$ mol $l^{-1}$; Merck p.a.) were added to 96% ethanolic grass extracts (*Agrostis tenuis* Sibth., *Festuca rubra* L., *Holcus lanatus* L.) and pheophytin solutions. Then, after reaction times between 1 min and 2 weeks, Mg-substitution was stopped by dissolving the products in cyclohexane; this lipophilic solvent prevents further substitution of the central ion of chlorophyll. The products were separated by thin-layer chromatography (silicagel sheets; 1:20:200, $H_2O$:isopropanol:isooctane, by vol.), then dissolved in cyclohexane and identified spectroscopically. Pheophytin was prepared following a modified protocol of Fischer and Stern (1940): 25% HCl was added to the grass extracts (1:50, HCl:extract). Then pigments were dissolved in isooctane and separated by thin-layer chromatography (see above).

### *Plant material and growth conditions*

The following species were used: *Elodea canadensis* Michx., *Stratiotes aloides* L., *Myriophyllum spicatum* L., *Ceratophyllum demersum* L., *Callitriche stagnalis* Scop., *Crassula helmsii* (Kirk) Cockayne, *Lemna trisulca* L., and *Lemna minor* L. Plants were collected from uncontaminated ponds and grown in nutrient solution (Gaudet, 1963) for between 2 d and 5 d. Plants were stressed for 2–3 weeks with soluble $CuSO_4$, $ZnSO_4$, $CdSO_4$, $NiCl_2$, $Pb(NO_3)_2$, or $HgCl_2$ (Merck p.a.) which were added to the nutrient solution in initial or constant concentrations. 'Initial' concentration (in our experiments: $c(hm^{2+})=5\times10^{-6}$ to $1\times10^{-3}$ mol $l^{-1}$) means that the heavy metal was added only once at the beginning. The aqueous concentration decreased due to uptake into organic tissue, simulating a single entry of a heavy metal into an aquatic habitat. For 'constant' concentrations, the solution was continuously exchanged, simulating a permanent contamination (in these experiments: $c(hm^{2+})=5\times10^{-8}$ to $5\times10^{-5}$ mol $l^{-1}$). Plants were kept under different light conditions: natural sunlight (up to 120 W $m^{-2}$) or shade (up to 0.8 W $m^{-2}$). The amount of light exposure also influenced the water temperatures: In sunlight they varied between 26 °C (afternoon) and 18 °C (night), in shade between 20 °C and 18 °C.

### *Extraction*

At the end of *in vivo* experiments, plants were dried at 30 °C (<5% loss of chl), ground with sand and extracted (3 d) in cyclohexane ('Uvasol' for spectroscopy, Merck)+0.5 % of isopropanol (Merck p.a.) saturated with $NaHCO_3$ (Merck p.a.). This method of extraction was chosen to prevent complexation of heavy metal ions by chlorophyll during extraction. To check the reliability of this method, controls were carried out: dry heavy metal salts were added (0.1 g $ml^{-1}$) to solutions of Mg-chl and pheophytin in cyclohexane (+ isopropanol, see above) as well as to dried plants which were afterwards extracted in the way described above. In none of these controls were hms-chls formed in detectable amounts. In contrast, experiments with ethanol or acetone as solvents led to the detection of higher Mg-substitution-rates, showing that these solvents were unsuitable for extracting hm-stressed plants. In some cases pigments were separated with thin layer chromatography.

### *Isolation of chloroplasts*

For isolating chloroplasts from stressed and unstressed plants, the procedure described by Walker (1988) was followed.

### *Analytical methods*

Macroscopic and microscopic photographs were taken of all *in vivo* experiments to document changes in colour, growth, tissue- and cell-structure.

All pigment extracts were examined spectroscopically. Spectra of intact leaves and isolated chloroplasts were also measured. Both absorbance and fluorescence spectra of plant extracts were

recorded using a UV/VIS photometer linked to a microcomputer. For quantitative assay of hms-chls in plant extracts the following methods were used.

(a) For Zn-chl, the spectrophotometric method by Jones *et al.* (1977) was used. For this estimation diethylether was used as solvent because previous spectra (recorded in cyclohexane as in the case of Cu-chl) did not have to be considered.
(b) For Cu-chl, the spectrophotometric method by White *et al.* (1977) was applied, but using cyclohexane as solvent, and the absorbtivities of the chlorophylls and pheophytins in cyclohexane for subsequent calculation. This new calibration was made because quantitative estimations of Cu-chl with older spectra, which had all been recorded using cyclohexane, were wanted.
(c) For Ni-chl and Pb-chl, methods similar to those for Cu-chl were used, but since the red absorbance maxima of Pb-chl *a* and Ni-chl *a* are very near to the maximum of pheophytin *a* (Table 1), the results of this calculation were less accurate than those with Zn-chl and Cu-chl.

Cd-chl and Hg-chl could not be quantitatively estimated with methods similar to those used for Zn-chl and Cu-chl because they are too unstable and the absorbance maxima are too close to each other (Table 1). The instability was used for a qualitative detection of these pigments in extracts; the decay of the dissolved pigments under light exposure being measured.

The course of Mg-substitution *in vivo* was examined by the combined measurement of fluorescence and oxygen exchange of intact, unstressed and heavy metal-stressed plants. Fluorescence measurements on intact plants were carried out using a 50 W halogen lamp with an excitation filter (transmission $< 545$ nm). An emission filter (transmission $> 595$nm) separated the fluorescence from the excitation light. The steady fluorescence after 7 min (i.e. after fluorescence induction) was measured. Net photosynthetic oxygen release (subsequently termed 'net photosynthesis') was traced by a Clark-type electrode linked to a digital measuring device. For oxygen measurements the glass vessels containing the plants were placed in front of (30 cm distance, 100 W $m^{-2}$) a 50 W halogen lamp with heat filter for 20 min.

## Results

In the preliminary *in vitro* experiments data were obtained on reactivity, thermodynamic stability and spectroscopic properties of isolated hms-chls. These results (Table 1) were used as a basis for the interpretation of spectra obtained from plant extracts, isolated chloroplasts and leaves.

With the *in vivo* experiments, it was found that the substitution of magnesium in chlorophyll by heavy metals observed *in vitro* also takes place in living plants, making them incapable of photosynthesis.

An almost proportional relationship between toxicity (in constant concentration) and the tendency of an ion to be bound in the centre of the chlorophyll molecule (= complex formation rate) was found (Table 1):

$$Hg^{2+} > Cu^{2+} > Cd^{2+} > Zn^{2+} > Ni^{2+} > Pb^{2+}$$

While *Crassula helmsii* was the most resistant species, *S. aloides* was the most sensitive. Overall, the following sequence of sensitivity was observed: *C. helmsii* < *L. minor* < *C. stagnalis* < *M. spicatum* < *L. trisulca* < *C. demersum* < *E. canadensis* < *S. aloides* (Table 2). A relationship between sensitivity of a species and the extent of Mg-substitution was recorded in shade: to yield the same rate of Mg-substitution, in the case of a more resistant plant, $c(hm^{2+})$ had to be higher than in the case of a less resistant species. Thus, the Mg-substitution rate leading to a decrease of net photosynthesis to zero increased only from 8% (*Stratiotes aloides*) to 14% (*Crassula helmsii*), while the (constant) $Cu^{2+}$ concentration increased from 0.5 to 20 $\mu$mol $l^{-1}$ (Table 2). This shows that the rate of Mg-substitution is more directly correlated to the decrease

**Table 1.** *Properties of heavy metal ions and heavy metal substituted chlorophylls*

The data of reactivity, absorbance maxima and absorbtivities are the results of *in vitro* experiments. They are supported by literature (e.g. Berezin and Dobrysheva, 1970; Boucher and Katz, 1967, Kukhtevich, 1959; White *et al*, 1977; Jones *et al.*, 1977). These authors also found the stability data, which were checked in these *in vitro* experiments using other solvents. The data of toxicity are the results of our *in vivo* experiments.

| Ion | Reactivity[a] | Toxicity[b] | Ranked thermodynamic stability | Absorbance maxima(nm)[c] chl *a*/chl *b* | Absorbtivity[d] chl *a*/chl *b* |
|---|---|---|---|---|---|
| $Mg^{2+}$ | —[e] | —/— | 5 | 662/641 | 89/59 |
| $Cu^{2+}$ | 2 min | 0.8/30 | 1 | 649/626 | 68/49 |
| $Zn^{2+}$ | 3 h | 80/100 | 4 | 657/638 | 90/60 |
| $Cd^{2+}$ | 30 min | 15/500 | 6 | 659/640 | 90/60 |
| $Hg^{2+}$ | 15 s | 0.1/10 | 7 | 671/654 | 90/60 |
| $Ni^{2+}$ | 10.d | 200/1000 | 3 | 652/642 | 70/50 |
| $Pb^{2+}$ | —[e] | 500/1500 | (3–4) | 653/— | 70/— |
| (Pheophytin) | | | | 668/655 | 57/38 |

— Unknown or not existing, d = day, h = hour, min = minute, s = second.
[a] Approximate time for the conversion of 80% of c(pheophytin *a*) to heavy metal chlorophyll at 293 K; (in 85% ethanol; c(pheophytin *a*) ~ 1 $\mu$mol $l^{-1}$; $c(metal^{2+})$ 5 mmol $l^{-1}$.
[b] Concentrations [$\mu$mol $l^{-1}$] needed to lower net photosynthetic $O_2$ release of *Elodea canadensis* to zero within 1 week of heavy metal treatment: $c_{constant}(metal^{2+})$/approximate $c_{initial}(metal^{2+})$.
[c] Red absorbance maximum in cyclohexane (measured by the authors)
[d] Absorbtivities [$\mu$mol$^{-1}$ cm$^{-2}$] at the red absorbance maxima in cyclohexane.
[e] Reaction very slow, a turnover of 80% is not reached even after 3 months. Instead, allomerization leads to a decrease in pigment concentration.

**Table 2.** *Lethal doses of $Cu^{2+}$ and presence of Cu-chlorophyll in the species used in our experiments*

| | *Stratiotes aloides* | *Elodea canadensis* | *Ceratophyllum demersum* | *Lemna trisulca* | *Myriophyllum spicatum* | *Callitriche stagnalis* | *Lemna minor* | *Crassula helmsii* |
|---|---|---|---|---|---|---|---|---|
| $c_{lethal}(Cu^{2+})^a$ ($\mu$mol $l^{-1}$) | 0.5 | 0.8 | 1.0 | 1.5 | 2.0 | 4.0 | 5.0 | 20 |
| Cu-Chl content[b] (% of total chl) | 8.0 | 9.5 | 7.0 | 8.0 | 8.5 | 11.0 | 12.0 | 14.0 |

[a] Constant $Cu^{2+}$ concentration needed for lowering the net assimilation rate down to zero within 1 week in shady conditions.
[b] After 1 week of treatment with $c_{lethal}$ ($Cu^{2+}$) in shade.

of net photosynthesis, than to $c(hm^{2+})$. This relationship was examined with $Cu^{2+}$ because the estimation of Cu-chl was the most exact one. It is very likely that this result is also valid for the other metals because the reaction (Mg-substitution) is the same, as has been shown by the detection of the respective hms-chls in extracts of stressed plants.

Depending on light intensity, there are two types of damage, which lead to different symptoms.

### *The shade reaction*

In shady conditions, heavy metal-stressed plants show symptoms of damage that are directly related to the results of spectroscopic and fluorimetric analyses of cyclohexane extracts. These analyses show the formation of hms-chls (Fig. 1A, C).

Similarly, spectroscopic experiments with isolated chloroplasts and entire leaves from Cu- and Zn-stressed plants yielded the same results: a blue-shift of the red absorbance maximum (Fig. 2) which correlates with the hms-chl formation measured in extracts from the same plant specimens. Treatment of isolated chloroplasts from unstressed plants with phosphoric acid resulted in a shift of the red absorbance maximum from about 682 nm to about 695 nm due to conversion of Mg-chl to pheophytin (this peak at 695 nm (*in vivo*) could be shown to belong to pheophytin by extracting the pigments after the treatment with phosphoric acid and examining the extract again spectroscopically). In the case of Cu- and Zn-stressed plants this shift could not be observed, which once more demonstrates the formation of the more stable Cu- or Zn- chlorophylls (Fig. 2).

As a result of Mg-substitution, chloroplasts of damaged plants exhibit a colour change, corresponding to the colour of the hms-chl formed. This phenomenon has so far been unexplainable (Kahle, 1988).

In shady conditions the chlorophyll content of stressed plants decreases only slightly (Fig. 1B); at least in the case of Cu-stressed plants most of this decrease in absorbance is a matter of the low absorbtivity of Cu-chl (Table 1). The blue-green colour of Cu-stressed plants was extremely stable, as it is typical for Cu-chl (Table 1). So even dead plants appeared vital (especially *E. canadensis*, *C. demersum* and *S. aloides*), but the plants were often fragile and disintegrated when taken out of the vessels at the end of the experiment. This suggested that a breakdown of cellulose took place. Besides other possible reasons, the weakening of tissue may have been caused by the formation of free radicals by $Cu^{2+}$.

In contrast, the more pronounced bleaching of Cd- and Hg-stressed plants in shade is caused by the instability of Cd- and Hg-chl. The significant difference between complex formation rate and thermodynamic stability of hms-chls (Table 1) causes variations of the symptoms described before, specific to single heavy metals.

Since Cu-chl shows no fluorescence, fluorescence measurements were carried out on living plants treated with $Cu^{2+}$. These plants showed a steady decrease in fluorescence being directly related to the decrease in net photosynthesis (Fig. 3). This decrease in fluorescence indicates the formation of (non-fluorescent) Cu-chl (Fig. 1A). However, the fluorescence decrease was much greater than the rate of Mg-substitution (compare Figs 1A and 3).

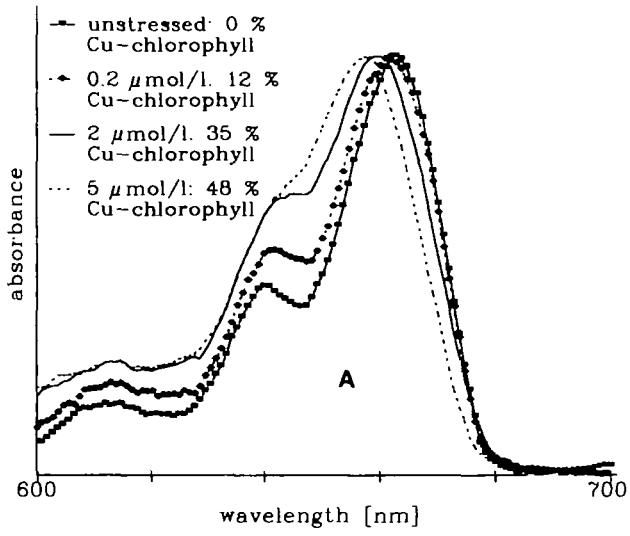
Respiration of stressed plants (except those treated with $Hg^{2+}$) remains nearly constant during the first days of treatment with heavy metals while net photosynthesis decreases greatly. These observations exclude inhibition of the respiration apparatus as a primary damage mechanism.

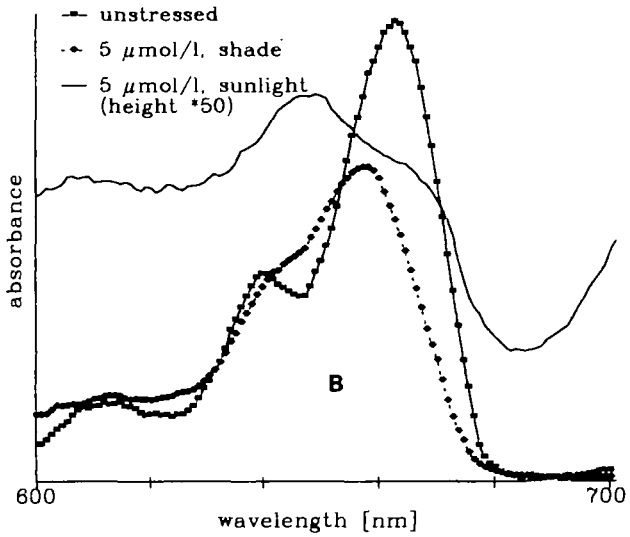
In this laboratory Zn-chl was the first hms-chl which could be traced by means of spectroscopic and fluorimetric analyses in plants taken directly from their natural habitat. It was found in *E. canadensis* specimens taken from a polluted watercourse in the vicinity of Marl (Westphalia, Germany). Although the plants looked quite healthy, they were fragile and did not show any net photosynthesis, as is typical for shade reaction.

### *The sun reaction*

In contrast, in intense light, heavy metal-treated plants bleach almost completely due to chlorophyll decay, even in the case of $Cu^{2+}$. This visual observation could be verified by spectral analyses of cyclohexane extracts (Fig. 1B). In the case of $Cd^{2+}$, plants bleach completely.

After bleaching, the remaining pigment of $Cu^{2+}$-stressed plants contains some Cu-chl, but less than 2% compared to the chlorophyll content of the control (Fig. 1B). Moreover, other pigments are often formed,



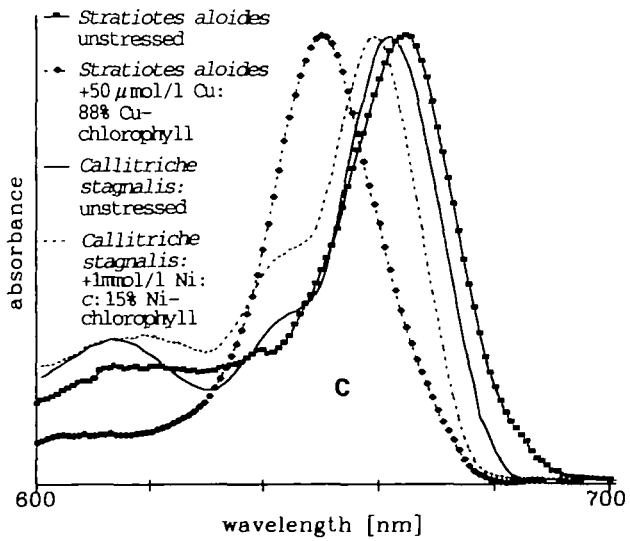




Fig. 1. Some absorbance spectra of extracts from plant experiments. (A) *Elodea canadensis* stressed for 12 d with constant concentrations of $Cu^{2+}$ in shady conditions. These spectra show the formation of Cu-chlorophyll, which at high Cu-chlorophyll percentages leads to noticeable blue-shifts of the red absorbance maximum. For better comparison, the red absorbance maxima are scaled to a common height. (B) *Elodea canadensis* stressed for 12 d with constant concentrations of $Cu^{2+}$ in shade and sunlight: a comparison of the absorbance changes in extracts. The (relatively slight) absorbance decrease in shade is partly due to the low absorbtivity of Cu-chlorophyll. (C) Plants stressed for 20 d with initial concentrations of $Ni^{2+}$ or $Cu^{2+}$ in shade.

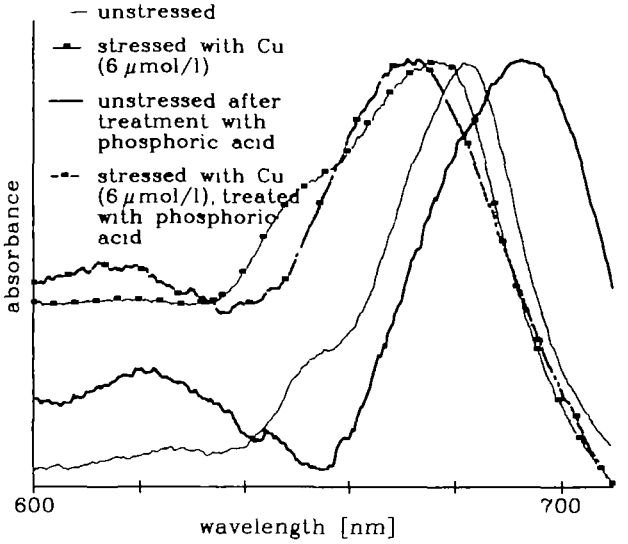



Fig. 2. Absorbance spectra of suspended *E. canadensis* chloroplasts isolated after 12 d of constant copper stress in shade.

which could be allomerization products of Mg-chl. This shows that in sunlight only a small proportion of the chlorophyll molecules (<2 %) is accessible to substitution by heavy metal ions. Otherwise, Mg-substitution by heavy metals would inevitably take place so that stressed plants would remain green, at least in the case of $Cu^{2+}$ and $Ni^{2+}$. Both Cu-chl and Ni-chl are so stable (Table 1) that they do not bleach even after weeks of exposure to direct sunlight, neither in solutions nor in plants which were damaged in shade.

With regard to heavy metal concentration, damage was generally slightly more intense in sunlight than in shade, i.e. lower concentrations were sufficient to make plants die.

## Discussion

This work enabled the discovery and demonstration of a mechanism of high significance with regard to the damage of plants by heavy metals: The incorporation of various divalent heavy metals in the chlorophyll of water plants prevents photosynthetic light-harvesting in the affected antenna pigments and thus inhibits electron transfer in the reaction centres, finally resulting in breakdown of

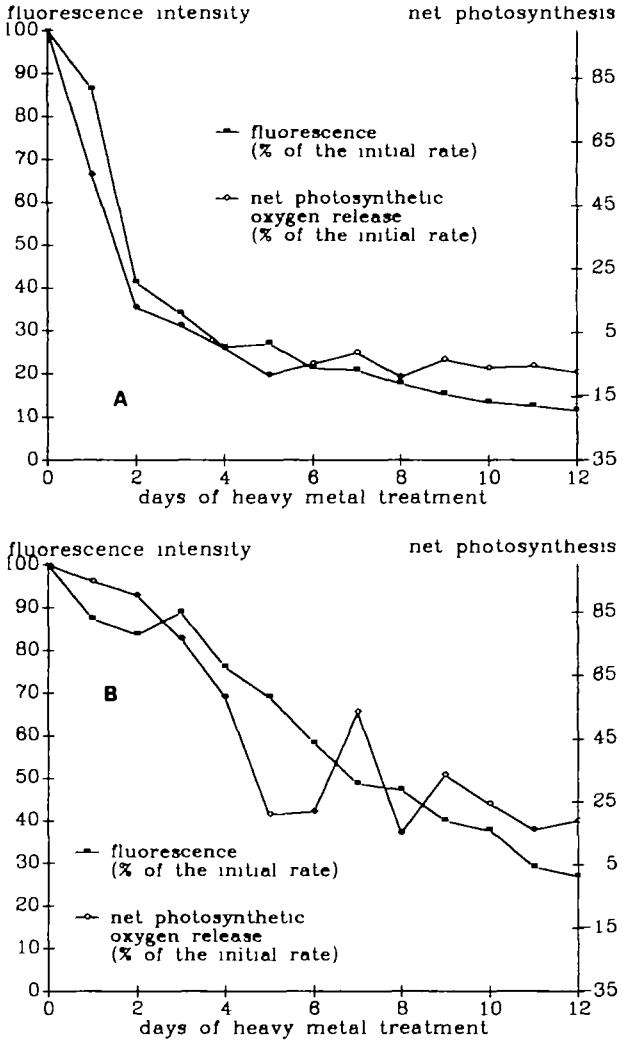



**Fig. 3.** This figure shows a comparison of fluorescence and net photosynthesis of *Elodea canadensis* stressed with Cu in shady conditions. (A) $c(Cu^{2+}) = 2 \times 10^{-6}$ mol $l^{-1}$ (constant). (B) $c(Cu^{2+}) = 2 \times 10^{-7}$ mol $l^{-1}$ (constant).

photosynthesis. This mechanism has a rapid and severe impact on the photosynthetic light reaction, whereas other parts of metabolism (e.g. respiration) are affected much later.

The almost proportional relationship between toxicity on the one side and complex formation rate on the other could be pointed out. The potential of damage to plants by heavy metal ions, with respect to these reactions, does not depend on thermodynamic stability of the complexes formed, but on the tendency to form chlorophyll complexes.

Water plants were used because they are, unlike terrestrial plants, capable of absorbing ions over a much larger part of their surface. This shortened the duration of the experiments. Additionally, growth conditions and heavy metal concentrations available to the plants could be controlled more accurately, compared to terrestrial plants. The major aim of experiments with various species of plants was to prove the general validity of results and to compare differences in resistance to heavy metals. It is worthwhile noticing that the species with the lowest lethal doses of toxic heavy metals are predominantly submersed living plants: *Stratiotes aloides, Elodea canadensis, Ceratophyllum demersum.* In contrast to this, the three species which live amphibiously or at the surface (*Crassula helmsii, Lemna minor, Callitriche stagnalis*) exhibit higher lethal doses of heavy metals. One may argue that this is due to better-evolved capabilities to regulate ion-exchange processes of species living on the borderline of an aquatic habitat. Generally, such plants must be better adapted to cope with changing environmental conditions than completely submersed species.

Many authors who examined the effect of heavy metals on photosynthesis already observed a decrease in fluorescence (Tripathy and Mohanty, 1981; Atal *et al.*, 1991; El-Sheekh, 1993; Wu and Lorenzen, 1984). In general, these results provide a new explanation for this decrease in fluorescence under stress of various heavy metals: At low light intensity it is caused by the formation of low-fluorescent or non-fluorescent hms-chls. Studying chl fluorescence, Wu and Lorenzen (1984) have already suggested this explanation, but only in the case of $Cu^{2+}$ and without any investigations whether Cu-chl is formed or not.

It was reported repeatedly that *in vivo* photosystem I (PSI) is not or only slightly inhibited by heavy metal concentrations that inhibit PSII completely (Clijsters and Van Assche, 1985; Gross *et al.*, 1970; Atal *et al.*, 1991). This fact, combined with the findings about the close relationship between damage caused by heavy metals and Mg-substitution, explains why, in shade, the fluorescence decrease is much greater than the amount of Cu-chl formed. Probably, only the chlorophylls associated with PSII (which are responsible for most of the *in vivo* fluorescence (Krause and Weis, 1984)) are accessible to heavy metal ions at low concentrations. At high concentrations combined with shade, Mg-substitution takes place in all chlorophylls (Fig. 1C).

The decrease in fluorescence in response to $Cu^{2+}$ treatment excludes blocking of the electron transport chain of photosynthesis (on the reducing side of PSII) as the primary damage. Such a blockage (e.g. comparable to that by DCMU at $Q_B$) would have led to an increase in fluorescence due to emission of surplus energy by chl molecules (Karukstis, 1991).

Mushrifah and Peterson (1991) found a large amount of $Cd^{2+}$ in a chlorophyll extract of an *Anabaena flos-aquae* culture which had been treated with $Cd^{2+}$ at low light intensity. With regard to the 'shade reaction', it is likely that $Cd^{2+}$ was complexed in chlorophyll.

Investigating the effect of aqueous sulphur dioxide on lichens, Puckett recorded a blue-shift in the chl spectrum

(Puckett *et al.*, 1973). He interpreted his results as 'allomerization of chlorophyll', possibly because of an unintended formation of Ni-chl: the lichens for this experiment had been collected near a nickel smelter and the aqueous sulphur dioxide might have made Ni-deposits in (or on) the lichens soluble. Thus, this process may have led to Mg-substitution either *in vivo* or during extraction with ethanol (compare with the discussion of extraction methods below). He did not notice important differences, such as, in contrast to Ag-, Cu- and Ni-chlorophyll (Table 1), allomerized chlorophylls are rather unstable. In addition, they exhibit a strong red fluorescence (*c.* 70% compared to Mg-chlorophyll, as Johnston and Watson had already reported in 1956), while Cu- and Ag-chlorophyll do not exhibit any fluorescence and Ni-chlorophyll only very little (Watanabe and Kobayashi, 1988).

Kowalewska *et al.* (Kowalewska and Hoffmann, 1989; Kowalewska *et al.*, 1992) reported to have found Cu-chl in cyanobacteria (*Anabaena variabilis*) stressed with $Cu^{2+}$ at low light intensities as well as in phytoplankton from the Baltic Sea. However, they extracted the algae with acetone, which could have led to Mg-substitution during extraction (see 'extraction'). To avoid this risk of artefacts, cyclohexane was used as solvent in these experiments.

It was already known (Cedeno-Maldonado *et al.*, 1972; Wu and Lorenzen, 1984) that damage is increased with light intensity. Li and Miles (1975), using $Cd^{2+}$ and high light irradiances, found that $Cd^{2+}$ directly inhibits the photoreaction of the PSII reaction centre. This was verified by Atal *et al.* (1991). Li and Miles (1975) suggested that this inhibition was caused by a Cd-induced alteration of the reaction centre chlorophyll. In the sunlight-experiments, especially those with $Cu^{2+}$, it was observed that only a small proportion of the total chlorophyll is accessible to substitution. This may support their interpretation and indicate that the 'alteration' is Mg-substitution.

Using $Cu^{2+}$, Cedeno-Maldonado *et al.* (1972) found that heavy metal ions bind to an unknown substance in the chloroplasts' membranes in shade. These results indicate that the binding of $Cu^{2+}$ is to antenna chlorophylls which are accessible only in shade. The reason of this differential accessibility still requires explanation.

One important reason that the role of *in vivo* Mg-substitution as a mechanism of damage was not discovered earlier might be the high light irradiances used in most studies: Such irradiances led to the sun reaction, which makes the detection of Mg-substitution difficult because the hms-chls formed amount to only a small proportion of the total chlorophyll content.

In most cases, under natural conditions intermediates between sun and shade reaction will take place. For example, in regions like middle and northern Europe, the shade reaction is more likely; in fact, there was difficulty in getting enough stable sunshine periods for carrying out the sunlight-experiments.

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