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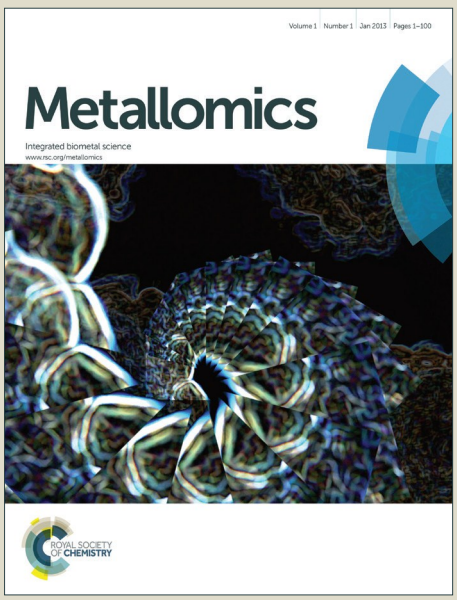

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Critical Review

Mechanisms of metal toxicity in plants

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Metal toxicity in plants is still a global problem for the environment, agriculture and ultimately human health. This review initially addresses the current state of the environmental/agricultural problem, and then discusses in detail the occurrence, mechanisms and relevance of toxicity of selected trace metals (Cd, Cu, Fe, Hg, Ni, Zn). When discussing the mechanisms, special emphasis is laid on a critical review of their environmental/agricultural relevance, because even now many studies in this field of research are performed under highly artificial lab conditions. The typical main problems in published studies are far too high metal concentrations (as they never occur even in highly polluted sites) combined with too short treatment times, as well as environmentally and agriculturally irrelevant growth conditions (e.g. constant light, submerged cultivation of seedlings, etc.). Furthermore, wherever possible an attempt is made to link the mechanisms published so far in terms of discussing which mechanisms are a direct cause of the observed disturbance of plant function, and which are rather a consequence of the primary mechanisms, leading to a complicated toxicity phenotype and ultimately to diminished growth or even death of the plants.

Introduction: Environmental relevance of metal toxicity in plants

Metal toxicity is still a global problem, although public perception is different. After strong efforts towards an improvement of water purification plants has been made the "green" movement started in the 1970s-1990s, in many "western" countries this problem has been regarded as a problem of the past for these countries, and as only still relevant in "developing" and "transition economy" countries. This is, however, a wrong conclusion, because even in the richest countries of the "western" world metal toxicity is still a problem for plants and the environment in general. This is obvious already when looking at recent data e.g. of the European Environment agency on the development of cadmium and mercury discharges into the North Sea, where a rise can be observed since the mid-1990s, or at cadmium emissions.[1] This contamination originates from many sources. A selection of toxic metal concentrations in urban soils, unpolluted and polluted soil environments together with guide values from US and European environmental protection agency is listed in Table 1, respective data for aquatic environments in Table 2. Different soil types and pHs can strongly influence the behaviour of metal in soil and thereby bioavailability of metals[2] and have to be considered.

In many cases, agriculture itself is a major source of both cadmium and copper contamination that can ultimately threat agriculture and human health.[3] As a well-known, but nevertheless still unsolved problem, copper compounds are used as pesticides in vineyards, which can lead to micromolar copper concentrations in agricultural field runoff and small creeks nearby.[4] Such concentrations are lethal to sensitive aquatic plants within a few days, toxicity can start already as low as 20 nM copper.[5] Furthermore, the similarly well-known but unresolved problem of cadmium contamination of phosphate fertilisers leads to cadmium contamination of fields that were used for intensive agriculture for a long time.

In this latter case, strategies for improved phosphor utilization, dramatically reducing the need for phosphate fertilisation, have been developed but are still too rarely used due to a lack of public awareness of the problem. Another strategy to decrease heavy metal toxicity stress and heavy metal accumulation in crop plants is intelligent breeding based on recent insights into mechanisms of metal uptake, translocation, sequestration and storage, as described in the review of Khan *et al.* (2014).[6]

Regarding metals, plants are divided into three groups:

"Excluders" actively remove excess metals from their tissues, resulting in constant concentrations in the shoot over a wide range of metals in the soils. "Indicators" have no avoidance strategy and accumulate increasing amounts of metals proportional to increasing concentrations in the soils. "Hyperaccumulators" actively accumulate metals in their shoots with highest bioaccumulation coefficients at lowest soil concentrations, partially saturating at higher soil concentrations.[7-9]

Toxicity mechanisms are best investigated using indicator plants although the comparison of hyperaccumulating and non-hypperaccumulating plants from the same species can yield valuable insights into the respective toxicity or detoxification strategy.[10,11] Furthermore, plants which are hypertolerant hyperaccumulators for some metals may be just sensitive as indicators to others, which they do not hyperaccumulate. This applies e.g. to the Cd/Zn hyperaccumulator *Noccaea* (formerly *Thlaspi*) *caerulescens*, which tolerates Cd and Zn in very high concentrations,[12] but experiences Cu toxicity like other non-accumulating plants.[13]

Plants are immobile and thus cannot move away from unfavourable conditions, such as toxicity or deficiency of certain elements. In this review, we focus on the biophysical and biochemical mechanisms of metal toxicity in plants and algae for six selected metals (Cd, Cu, Fe, Hg, Ni, Zn). Excellent reviews regarding other toxic metal(loid)s like As[14-16], Al[17-18], Cr[19-20] or Pb[21-22] can be found elsewhere.

In this review, we will critically analyse suggested mechanisms of metal toxicity in view of the fact that many studies were not performed under environmentally relevant conditions. For example, studies using constant light[23] are questionable for the following reasons. In nature, plants and algae always experience changing light intensities due to clouds or to turbid waters; they developed strategies to optimize photosynthesis and minimize photodamage.[24] However, something they never experience under environmentally relevant conditions is constant light. Without a dark phase, plants and algae experience light stress and can be severely injured (reviewed by Velez-Ramirez et al., 2011)[25] and e.g. cannot synchronize cell division to the light cycle.[26] Another problem linked to investigation of toxicity mechanisms is the mode of exposure to the toxic metals. Exposure of leaf discs[27,28], callus cultures[29] or submerged seedlings[30,31] to heavy metals can only give some indications on which impact the metal stress would have on the whole plant. Signalling molecules may be lacking, or toxicity would occur in the roots, which cannot be detected using leaf discs. Further, submerged seedlings may suffer much more from oxygen and carbon deprivation due to lower gas diffusion rates in water than in air. All these artefactual stresses could be stronger than the metal stress that was the subject of investigation (reviewed by Mommer & Visser, 2005 and Voeseneck et al 2006).[32,33] The third, and still most common problem of metal toxicity studies is the use of far too high metal concentrations, which would almost never occur even in the most polluted environments. This often leads to results that are irrelevant for the mechanisms of toxicity in the environment, because at very high concentrations of whichever metal the inhibition becomes unspecific. This is caused by the chemically well known fact that once all high-affinity binding sites are occupied; metal binding will start to occur at low-affinity sites. Important examples of such cases will be discussed in detail in the following sections of this review.

In this review, we generally omitted the topics of metal deficiency unless where needed for understanding toxicity. Resistance mechanisms (e.g. gene regulation, metal detoxification, sequestration, lignification) were included when overlapping with toxicity mechanisms. We summarized the findings presented in the text in a scheme (Fig. 1). Some metals induce toxicity by similar mechanisms and are usually described only for one metal in detail in the following sections.

Toxicity of biologically redox-inert metal ions

Since for many years oxidative stress led the headlines about metal toxicity, it may seem to many readers who don't go into details that this would really be the main explanation for heavy metal toxicity. While redox reactions do play a role in the development of stress symptoms, and there are distinct differences between individual metals as discussed in the following, they are often not the main reason for, but rather a consequence of, metal toxicity. And keeping this in mind it is not so surprising any more that (a) also biologically redox-inert metals ultimately lead to oxidative stress whenever they become toxic, (b) that probably the most toxic metal, mercury, is in plants usually redox-inert.

Zinc - an essential element with low but relevant toxicity

Zinc is, in many parts of the world, more a problem in terms of deficiency than toxicity,[34,35] and Zn^{2+} is far less toxic to most plants compared to e.g. Cd^{2+} regarding concentrations. The New York Brownfield directive (NYS DEC) for soil clean ups allows unrestricted use (incl. agricultural) of soils with 1100 mg kg^{-1} Zn^{2+} (see Table 1). Nevertheless, both naturally Zn^{2+}-rich sites and anthropogenically Zn^{2+}-contaminated sites exist, on which Zn^{2+} toxicity limits plant growth and makes agriculture impossible.

Zn toxicity originates, to a large extent, from a replacement of weakly bound other divalent metal ions from essential sites. One such site is the Mg^{2+} in chlorophyll (Chl). This Mg-substitution, when occurring in an uncontrolled way in a system that evolved for [Mg]-Chl, inhibits photosynthesis for several reasons. First, [Zn]-Chl has, like all other heavy metal-substituted Chls ([hms]-Chls), a less stable singlet excited state[36]. Therefore, electrons from the excited antenna have a reduced likelihood to be transferred to the reaction centre to perform a charge separation, and a higher likelihood to be dissipated as heat instead. Furthermore, like all other [hms]-Chls, also [Zn]-Chl has a diminished tendency to bind axial ligands. Since these are essential for proper binding in Chl proteins, and the tertiary structure of these proteins is only stable with bound Chls,[37] Mg-substitution leads to denaturation of pigment-protein complexes.[38] In environments where cells constantly have to cope with high levels of heavy metals (incl. zinc) and additionally with very acidic conditions, bacteria of the genus *Acidiphilium* have evolved that do photosynthesis with [Zn]-BChl.[39] It seems that in

these organisms the disadvantages of [Zn]-BChl compared to Mg-BChl are over-compensated by the much higher stability of the Zn-complex. This higher stability likely prevents demetallation or uncontrolled transmetallation of their chlorophylls that would most probably occur if these organisms used regular Mg-BChl. The substitution of Mg^{2+} in Chl leads to the degradation of whole photosystems. The loss of Chl and other photosynthetic pigments is the reason for the visible chlorosis, which is a typical visible symptom of Zn^{2+} toxicity besides reduced growth, and leaf necrosis and also reddening of leaves due to anthocyanin production.[40,41] In sugar beet, moderate Zn^{2+} toxicity (50 µM) reduced all photosynthetic pigments and iron (Fe) content, while Chl fluorescence parameters and gas exchange did not change.[42] Comparable results were found in Fe-deficient sugar beet,[43] suggesting Zn-triggered Fe deficiency as the main stressor. Iron is needed in several Fe-S clusters in photosynthesis and respiration, and one would expect a change in Chl fluorescence and gas exchange. Higher Zn^{2+} concentrations (100-300 µM) led to significant inhibition of photosynthesis, which was not related to Fe deficiency. Though using extremely high Zn^{2+} concentrations (1-50 mM, in soil), Bonnet *et al.* noticed a decrease in F_v/F_m in ryegrass, indicating loss of functional photosystems.[44] Chlorophyll a fluorescence kinetics gives information about the photosynthetic performance of a plant and F_v/F_m is the most frequently used parameter. It measures the maximal dark-adapted photochemical quantum yield of photosystem II (review e.g. by Maxwell and Johnson, 2000).[45] However, ryegrass seemingly can endure high Zn^{2+} concentrations before the onset of toxicity symptoms, although not all Zn will be bioavailable for the plants.[2] Unfortunately, the authors did not determine bioavailable (or at least labile) Zn content in the soil.[44] A noticeable decrease of F_v/F_m in plants treated with 1 mM occurred only after 20 days of exposure.[44] No changes in F_v/F_m or Chl content were found in *Myracrodruon urundeuva* plants exposed to Zn^{2+} concentrations up to 200 mg kg^{-1} soil, although phytotoxicity symptoms occurred. Increased carotenoids and antioxidants may have prevented toxicity to the photosystems.[46] Zinc containing (0.5 mM) wastewater from an electroplating unit strongly inhibited PS II mediated electron transport and lowered F_v/F_m accompanied with lower activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo; see also below). In contrast, PS I mediated photoreactions increased (both measured on isolated thylakoids), indicating that enhanced cyclic electron flow dissipated excitons.[47] Inhibited electron transport activity of isolated thylakoid membranes from Zn^{2+}-treated maize plantlets suggested water oxidation complex (possibly by substitution of Mn) and light harvesting complexes of PS II as targets for Zn^{2+} toxicity,[48] conforming earlier results.[49] Decreases in the non-cyclic photophosphorylation are likely due to the inhibition of the electron transport chain rather than direct inhibition of the ATP-synthase, because in this complex ion replacement is not likely.[49] Coherently, no significant effects on the photophosphorylation were observed in *Salvinia* after Zn-rich wastewater exposure.[47] Inhibition of the carboxylase capacity of RuBisCo suggests substitution of Mg^{2+} in the active centre of the enzyme.[50-52]

As Zn^{2+} is an essential ion for normal plant growth, its uptake, storage and use is tightly regulated.[53] Under Zn^{2+} stress conditions (deficiency or toxicity) plants can regulate the expression of the relevant transporters, both at the transcriptional and post-transcriptional level.[54] Zinc (and cadmium) uptake over the plasma membrane is most likely mediated via the ZIP transporter family (ZRT-IRT like protein; Zinc-regulated transporter, Iron-regulated transporter protein). But uptake is possible as well via other transporters with similar affinities for different ions, e.g. iron transporters. A seemingly vicious circle develops: Under iron limitation, plants up-regulate the Fe uptake transporters, which then also translocate more Zn, increasing Zn content in the plant.[53,55] Vice versa, zinc deficient plants often accumulate more iron, proving the competition for uptake.[56] Similarly, reduction of the content of other essential ions (like Mg, Mn, Fe)[57] is recognized and enhances expression of more such transporters. Thereby, the stress of deficiency of other ions enhances the stress of Zn^{2+} toxicity.

The homeostasis of Zn^{2+} is tightly regulated (see above), and it can be remobilized from storages in cases of limitation. Using "control" treatments without added Zn^{2+} will lead to Zn^{2+} deficiency unless chemicals of normal purity (p.a. grade) are used, which may contain enough Zn contamination for the specific species.[58,59]

Although Zn^{2+} is present in the Cu/Zn SOD and thereby involved in the defence against oxidative stress, excess Zn^{2+} leads to the formation of reactive oxygen species. Both Zn^{2+} deficiency and toxicity enhanced hydrogen peroxide concentrations and SOD activity in mulberry leaves.[56] An increased ratio of dehydroascorbate to ascorbate (DHA-to-AsA) indicated disturbed redox-status, shifting towards more oxidized forms in mulberry[56] and rapeseed seedlings.[57] The induction of ROS by the biologically redox-inert zinc is not surprising in view of the many sites in the photosynthetic light reactions where reactive oxygen species can be produced.[60] And the likelihood of such ROS production increases if the normal pathway of excitons and electrons in photosynthesis is inhibited. Even under optimal conditions, ROS arise constantly in metabolism pathways involving oxygen[61] and act as messenger molecules.[55,62] Further, under suboptimal conditions ROS scavenging becomes inhibited. It can result from the substitution of Mg^{2+} in Chl (see above) because [Zn]-Chl has a reduced efficiency for quenching of singlet oxygen.[63] Additionally, the disturbance in other essential nutrients (Cu, Fe, Mg) by excess Zn^{2+} can lead to less functional or reduced activity of Cu/Zn-SOD, Mn-SOD and Fe-SOD.[57] However, Zn^{2+} concentrations in that study were far beyond environmentally relevant conditions, up to 1.12 mM, while hardly any effects were observed at the lowest concentration of 70 µM. This suggests either a too short exposure period to observe effects at environmentally relevant concentrations, or a high chelation capacity in the nutrient solution leading to a far lower bioavailability of zinc. A higher capacity and activity of the antioxidant system in the zinc-tolerant hyperaccumulating ecotypes of *Sedum alfredii* over the non-accumulating ecotype suggests an involvement of the antioxidant system in mediating tolerance.[64] Exogenous ethephon, a precursor of the phytohormone ethylene, reversed negative effects of Zn^{2+} and Ni^{2+} (200 mg/kg soil) on mustard plants through induction of the antioxidant system. Activities of SOD, APX, and GR were increased due to Zn^{2+} or Ni^{2+} treatment compared to the control,

but even higher after ethylene treatment and highest after Zn^{2+} or Ni^{2+} plus ethylene treatment.[65]

Cadmium - only toxic?

For many decades, cadmium (Cd) has been known to be a highly toxic metal, not only to plants but also to animals incl. humans. Concentrations in the soil usually do not exceed 5mg/kg in urban soils and low nM in aquatic environments (Table 2). Even in a heavily contaminated stream in Nigeria, the highest Cd concentration was 1.4 µM, clearly exceeding the limits set by the Federal Environmental Protection Agency,[66] but emphasizing that high µM or even mM concentrations are very unnatural.

Cadmium toxicity mainly originates from non-functional binding to biological ligands that are meant to bind other divalent metals. These are particularly often the amino acids cysteine (Cys) and histidine (His) as most common amino acid residues in various metal centres of enzymes, in particular Zn centres due to the chemical similarity of the metals[67,68] (see below), but also RuBisCo as an enzyme with Mg^{2+} in its catalytic centre. Such Cd-substituted enzymes are usually non-functional. Less known, such a ligand can also be chlorophyll, where Cd^{2+} easily replaces Mg^{2+} as the central ion.[69] In this case, damage to the affected organism originates from degradation of this pigment that bleaches easily, but also from its unsuitability for photosynthesis. As explained already for [Zn]-Chl, also [Cd]-Chl binds axial ligands with much lower affinity than Mg-Chl, leading to protein denaturation. Furthermore, [Cd]-Chl quickly dissipates almost all absorbed excitation energy as heat due to its highly unstable singlet excited state (lifetime still shorter than [Zn]-Chl).[36] Because of the instability of [Cd]-Chl leading to degradation during extraction/separation, as well as the very high similarity of its UV/VIS absorption spectrum with that of Mg-Chl, it is very difficult to actually measure [Cd]-Chl formation in plants.[69] Details of Mg-substitution were reviewed by Küpper et al. (2006).[70] Very recently, Cd incorporation into the major light harvesting complex, LHC II, has been shown to occur already from 5 nM onwards in the aquatic shoot model plant *Ceratophyllum demersum.*[71] In the absence of other potential high-affinity binding sites in this protein, this is most likely due to formation of [Cd]-Chl. Chlorophyll fluorescence data, especially Fv/Fm were less affected by Cd exposure under the tested low light conditions, further suggesting the incorporation into the LHCs.[70] Under high light conditions, the insertion of Cd in the PS II RC (presumably into the pheophytin)[70] is more likely, leading to the loss of the whole photosystem and thereby causing a prominent decrease of F_v/F_m, which was observed under high light, but not under low light conditions.[71]

Analysis of Cd^{2+}-induced chronic inhibition of photosynthesis in *Noccaea* (formerly *Thlaspi*) *caerulescens* indicated that Cd^{2+} inhibits the photosynthetic light reactions more severely than the Calvin-Benson cycle.[72] Further, spectrally resolved analysis of photochemical vs. non-photochemical quenching in the same study showed that Cd inhibits at least two different targets in or around PS II.

Because of their chemical similarity, many transporters for divalent ions like Zn^{2+},[53,73] but also Ca^{+} channels[74] facilitate Cd^{2+} uptake into the roots and further distribution in the plants. The competition for binding sites can reduce the uptake of essential ions (like Cu, Fe, Mg, Mn, Zn) into the roots and cause deficiency, or even dislodge bound Zn^{2+} from binding sites and thereby change the tightly regulated zinc homeostasis in plant cells.[53] There was no effect of toxic Cd^{2+} (200 nM) concentrations on the total accumulation of Zn^{2+} in the tissue of *C. demersum*, but on its distribution: Cd^{2+} apparently inhibited Zn^{2+} export out of the vein, leading to Zn^{2+} deficiency in the mesophyll and Zn^{2+} toxicity in the vein of plants exposed to Cd^{2+}.[75]

A mechanistic uptake study of radiolabelled Cd or Zn in bread wheat (low Cd accumulations) and durum wheat (increased Cd accumulations) root cells revealed mutual uptake inhibition of both ions at the root cell membranes.[76] At the tested concentrations (50 nM-1.5 µM for Cd, 50 nM-50 µM for Zn), both metals yielded non-saturating uptake curves, but with higher K_m values for the non-essential Cd, possibly because high-affinity transporters exist that are specific for Zn.[77]

Another approach identified endogenously produced NO in root cells of *A. thaliana* as important signalling molecule under Cd exposure, mediating Cd stress.[78] Enhanced fluorescence caused by an NO-sensitive dye was detected in roots and shoots (leaves and leaf disks). Using specific mutants, the authors could exclude an involvement of AtNOA1 and NR as catalysts of the NO production. With additional microarray studies, they found a number of NO-dependent genes that were differentially expressed due to Cd exposure. Among the up-regulated genes they found IRT1. This gene encodes an iron transporter in the plasma membrane, which also transports Cd. Apparently a cellular pathway resembling the one of iron deprivation is mediated by NO, giving rise to Cd toxicity.[78]

Although not redox active, Cd^{2+} exposure leads to enhanced production of reactive oxygen species.[79-81] One likely reason is the enhanced mis-transfer of electrons on oxygen instead of their target molecule, e.g. by [Cd]-Chl (see above). Another reason is that Cd^{2+} exposure reduces the capability of scavenging ROS. Several enzymes and non-enzymatic antioxidants are present in plant cells,[82] but Cd^{2+} treatment can alter synthesis or activity, leading to oxidative stress, both in roots and shoots. The replacement of Zn^{2+} in the Cu/Zn-SOD by Cd^{2+} changes the structure of the enzyme [80,83] making it likely functionless and leading to its degradation ($Cu^{+/2+}$ is the redoxactive ion, but Zn^{2+} is believed to have structural purposes). Upon Cd^{2+} exposure, decreased contents and/or activities of the SODs were found.[80,84,85] However, depending on Cd^{2+} concentration, increased activities of enzymes or number of isoenzymes were observed, indicating a protective role of the antioxidant system against moderate Cd^{2+} stress.[86-89] Further information on metal and specifically Cd^{2+}-induced oxidative stress in plants and algae can be found in Sandalio *et al*. (2009)[79] and Pinto *et al.* (2003).[90] If the ROS are not detoxified in time, they can lead to the oxidation of membranes (lipid peroxidation) and produce mutagenic aldehydes.[91] Furthermore, the direct interaction of Cd^{2+} (and other metal ions) with the nucleotides[92,93] or the inhibition of DNA repairing enzymes can induce DNA damage.[94] The *A. thaliana* mutation assay revealed an increasing amount of point mutations from very low Cd^{2+} concentrations (8.8 nM) on, while e.g. Cu^{2+} and Ni^{2+} had much less potential to induce the mutations.[95] Many genotoxicity assays (for plants) are designed

to test contaminated soils or waters and the applied metal concentrations are often beyond natural conditions. The order of metals inducing mutagenetic effects was Hg^{2+}, Cd^{2+} (10^{-7}-10^{-5} M) > Zn^{2+}, Pb^{2+}, Cu^{2+}, Ni^{2+}, Co^{2+}, Al^{3+}, Cr^{3+} (10^{-4}-10^{-3} M) > Mn^{2+}, Mg^{2+} (10^{-2} M) based on the occurrence of micronuclei in onion root tips.[96] Later, other systems were found to be more sensitive, (*Tradescantia* < *Vicia faba* < transgenic *A. thaliana*).[95,97] Therefore, it is not always easy to tell how much genotoxicity adds to phytotoxicity under environmentally relevant conditions.

Plants are immobile and cannot avoid unfavourable heavy metal concentrations in soils. They have developed several ways of detoxification, including chelation, immobilization, exclusion and compartmentalization.[98,99] One major group of ligands are the enzymatically synthesized phytochelatins (PCs)[100,101], which are induced most efficiently by the nonessential metal(loid)s Cd and As, but also by Ag, Pb, Cu, Hg, Zn, Sn, Au.[73,100,101] PC-metal-complexes are most likely transported into the vacuole, where the metals cannot interfere with the photosynthetic and respiratory complexes.[73]

Sequestration into the vacuole is known for many more metals in hyperaccumulating and non-hyperaccumulating plants, although in hyperaccumulators bound to weak ligands, not pyhtochelatins (see Leitenmaier & Küpper, 2013 for a recent review).[9] In some plants special storage cells are located in the epidermis and the metals need to be transported from roots to above ground tissues, requiring many translocation steps against a concentration gradient. Metal concentrations up to hundreds of mM in the vacuole were reported for hyperaccumulating plants.[102,103] The respective transporters have been partially characterized as summarized in Clemens (2006) and [73]Leitenmaier & Küpper (2013).[9] When the rootless macrophyte *Ceratophyllum demersum* was exposed to very low Cd concentrations (2 nM) for 3 weeks, a very homogenous distribution of Cd over the whole cross section of the leaf area was found, and phytochelatins were not yet induced. After prolonged exposure (6 weeks) an increased sequestration of Cd into the epidermis was found. This indicates the onset of the detoxification by sequestration even at very low chronic toxicity.[75]

Expression analyses revealed up- or downregulation of various genes in response to Cd exposure.[104] Though the reason is not always clear and not necessarily directly caused by Cd-toxicity (one should be careful with high Cd concentrations), one can draw conclusions how their regulation may be involved in Cd toxicity. As example, some transcription factors up-regulated in response to Cd in *Arabidopsis thaliana* are constitutively strongly expressed in the Cd-hyperaccumulator plant *Arabidopsis halleri*.[105] Furthermore, miRNAs, small RNAs that usually are involved in negative gene regulation by destroying their respective mRNAs ("silencing"), can act as stress regulators after Cd exposure.[106] The miR393 targets E3 ubiquitin ligase/TIR1 (transport inhibitor response1) lead to less mRNA and thereby down-regulatiion of auxin signalling and possibly less proteolysis of the respective ubiquitin targets.[106]

To avoid patches of unfavourably elevated Cd content in the soil, roots can adapt by enhanced lignification and production of suberin lamellae at the sides facing the Cd contamination.[107] The authors state that these local barriers could restrict the apoplasmic movement of Cd, and thereby also Cd loading into the xylem and its further transport into other root and shoot tissues.

In more recent times, however, at least for some organisms a beneficial role of cadmium could be convincingly shown. This is, as the most prominent case, the expression of an alternative isoform of carbonic anhydrase, which in contrast to the regular isoform works well with Cd^{2+} instead of Zn^{2+} in its active centre. Originally it was found in the marine alga *Thalassiosira weissflogii*,[108] from where it was purified and spectroscopically characterised, and ultimately crystallised.[109-111] It was later found in other algae as well,[111] showing that it likely evolved as a remedy against the widespread extreme zinc limitation in the oceans and that in rare cases Zn^{2+} can functionally be substituted by Cd^{2+}.[111] Finally, it may even occur in Cd-hypertolerant terrestrial plants, but in this case likely for a different reason, more comparable to the occurrence of [Zn]-BChl instead of Mg-BChl in *Acidiphilium* (see above). In *N. caerulescens*, the use of Cd-carboanhydrase could prevent an uncontrolled exchange of Zn^{2+} by Cd^{2+} in normally Zn-containing carboanhydrase. This would explain why in this species Cd^{2+} induced carboanhydrase activity, while in a related non-hyperaccumulator species Cd^{2+} decreased it.[112] The positive effect of Cd^{2+} here can be traced back to the Cd^{2+}-containing enzyme. For many other metals (and other chemicals), positive effects have been found when applied in minute concentrations. This is usually attributed to the hormetic effect, which represents an overcompensation response of the treated organism, thereby triggering favourable effects instead of toxicity.[113]

Mercury – one of the most toxic metal ions

For mercury (Hg), so far no beneficial biological role has been found in any organism, while it is known to be among the most toxic metal ions for all organisms. Highly contaminated zones due to natural Hg sources can be found in Europe (the Almadén district in Spain, the mercury mines in Idrija, Slovenija) and in China (Gouxi in the Guizhou Province) with concentrations up to 76 µg/g in Slovenija[114] and 2 µg/g in China.[115] For comparison, the NYS DEC set a maximum of 0.81 µg/g for agricultural soils (Table 1). Even if concentrations of the bioavailable Hg are lower, the contaminated areas pose a health risk for humans: the surroundings of the Chinese mining districts are used for rice production.[116,117] The methylmercury (MeHg) accumulated in the rice seeds acts as a neurotoxin.[118]

Soluble mercury in the environment and organisms occurs in almost all cases as Hg^{2+}, only some bacteria have an enzyme that is able to reduce it (to Hg^0, metallic mercury), which was used also for making transgenic plants with this property.[119,120] Mercury reduction was observed in many phytoplankton species as well, but how they reduce it is still unknown.[121] Natural mercury concentrations in the oceans rank between 1 and 100 pM.[121, 122] Soils other than the polluted areas range between 20-150 ppb, but fertilizers and manures contaminated with Hg (and other toxic metals) can increase concentrations drastically.[123]

The toxicity of Hg^{2+} is to a large extend caused by its chemical similarity to zinc (Zn), which it can replace in active sites, especially with imidazole N and thiolate S ligands.[67] This

similarity also facilitates the uptake of mercury ions into roots or algal cells via transporters for other essential ions[124,125] and leads to the replacement of other divalent metal ions in their active sites, including Mg^{2+} in Chl.[69]Many studies showed the decrease in Chl content due to Hg^{2+} exposure,[126-128] although initial increases can occur as well. In the early stages of Hg^{2+} exposure (14 days, 100, 200 and 500 mg/kg soil, values of contaminated sites are usually less than 100 mg/kg, Table 1), Chl content was increased in winter wheat compared to untreated control plants.[129] With longer exposure time (28-34 days), Chl was reduced in all samples exposed to Hg^{2+}. However, plant species, experimental conditions and importantly, used Hg concentrations matter. *P. glomerata* plantlets exposed to only 1 µM of Hg^{2+} did not have Chl contents different from the control, but showed an increased activity of the enzyme δ-ALA-D (delta-aminolaevulinic acid dehydratase), which is involved in the biosynthesis of tetrapyrroles, probably balancing the degradation of Chl with enhanced biosynthesis.[130] Earlier works showed that this enzyme can be inhibited by Hg, Pb, Cd and Zn.[131,132] The enzyme NADPH:protochlorophyllide oxidase (POR), which performs photoreduction of protochloropyllide into chloropyllide, gets inhibited by Hg^{2+}.[133] However, leaf homogenates were incubated with very high Hg^{2+} concentrations (there was no effect visible below 10^{-3} M due to very short incubation times of max. 3 h) and the authors doubt that Hg^{2+} ions would react with the enzyme in intact plants.[133] Conclusions about toxicity mechanisms therefore should be taken with care.[134] Similarly, most aquaporins get blocked by Hg^{2+} ions binding to the sulfhydryl group of Cys residues close to the aqueous pore, reducing the hydraulic permeability of root cells (detailed review by Javot and Maurel, 2002)[135]. Binding to nitrogen in the imidazole ring of His was shown as well.[136] However, in how far the blockage of aquaporin contributes to mercury toxicity under environmentally relevant conditions remains unclear, because many experiments were performed as studies to characterize the aquaporins, not as studies of Hg^{2+} toxicity (e.g. expression in *Xenopus* oocytes, or other cell types).[135,137]

Mangroves grown in Hg-amended soil for 12 months showed significantly reduced F_v/F_m values only at the highest Hg^{2+} concentration (160 µg g^{-1}), although Chl content was decreased from 40 µg g^{-1} onwards and hardly any Hg was translocated from the roots into the leaves.[128] However, changes in both F_0 and F_m (F_v=F_m-F_0) can lead to unchanged F_v/F_m for complex reasons.[138] For example, photosystems that became non-fluorescent because of the formation of [Hg]-Chl do not contribute to this parameter although they would be non-functional like other heavy metal substituted chlorophylls.

Photosynthetic oxygen evolution and CO_2 fixation (determined with ^{14}C) declined with increasing mercury concentration (0.5-3 µM) in *Nostoc muscorum*, while respiration increased dramatically compared to control conditions.[139] PS II was more affected than PS I, like shown for many other metals (reviewed e.g. by Küpper and Kroneck, 2005).[8] Tests with exogenous electron donors indicated the inhibition site to be between the oxygen evolving complex (OEC) and PS II. The negative effects were more pronounced under high light conditions,[139] which is in line with previously reported substitution of Mg in reaction centre Chls.[69] A trend of increasing lifetime of the Chl autofluorescence was observed in diatoms exposed to Hg^{2+}, while no change occurred after exposure to MeHg.[140] This emphasizes the different toxicity mechanisms of organic vs. inorganic mercury. Organic mercury cannot substitute other ions in the photosynthetic complexes. But the results were not tested for statistic differences, which unfortunately is a problem in many studies. Spikes in both directions can be easily mis-interpreted as true signals. The consequences of Hg^{2+} insertion in Chl and in proteins are very similar to those triggered by other heavy metals. Mercury-treated plants have higher amounts of reactive oxygen species (ROS) and the antioxidant system resulting from the mis-transfer of electrons on oxygen instead of their electron acceptor.[82] Cultures of *C. reinhardtii* that were exposed to 1-6 µM of $HgCl_2$ for 24 h showed progressively increasing activities of SOD, APX and CAT, while only the highest concentration (8 µM) led to activities lower than that of the control treatment.[127] Not only the activities changed due to Hg-treatment, the expression levels of the respective genes coding for Mn-SOD, APX and CAT were upregulated as well. Comparable results were found for plantlets of *Pfaffia glomerata* shoots, while enzyme activities in roots of *P. glomerata* and rice were rather decreasing with increasing Hg concentrations.[130,141] Depending on the applied Hg concentration and treatment duration, changes in this pattern are possible. Generally, the induction of ROS (hydroxyl anion $OH^{\bullet}$ superoxide anion $O_2^{\bullet-}$ and hydrogen peroxide H_2O_2) followed by lipid peroxidation and decreased membrane integrity seem to occur after Hg treatment.[126,142,143] However, at very high Hg concentrations (and these may be specific for certain plants), an overall inhibition occurs, which is not specific to Hg toxicity anymore. Still, many mercury compounds can induce genotoxicity in plants, including chromosomal aberrations, polyploidy, and the occurrence of micronuclei.[144] But as described for Cd^{2+}, those tests were performed as risk assessment studies, not to unravel mercury toxicity mechanisms.

Non-enzymatic antioxidants, proline, and especially thiol compounds are induced upon Hg stress. Dago *et al*. (2014) extracted glutathione and phytochelatins (PCs) from wild asparagus from the ancient mercury mines in Spain.[145] Higher phytoavailable Hg^{2+} concentrations were correlated to higher concentrations of the PCs.[145] Longer-chain PCs were found in the roots, and shorter ones, especially PC3, in the aerial parts of the plants. Roots generally possessed higher concentrations. As the plant material was ground, however, Hg-PC complexes could have been formed when the vacuole was disrupted. Thus, from such studies it cannot be determined for sure whether the Hg-PC complexes were formed inside the plants, but only that Hg^{2+} induced the synthesis of PCs that could be potential ligands for Hg^{2+}.

Generally, accumulation of mercury seems to be higher in the roots than in the shoots of exposed plants.[123,125,126,146] This could be either a successful translocation stop with the roots acting as a barrier towards the toxic metal, or the blockage of metal transporters, which would lead to more stress as transport of essential metals into the above-ground tissues would be inhibited. To reveal the fate of mercury ions in the roots, X-ray fluorescence (XRF) related techniques allow identification of the tissues or organs in which the mercury binds preferentially.

Carrasco-Gil *et al.* (2013) used μ-XRF to map the distribution and extended X-ray absorption fine structure (EXAFS) to determine the speciation of mercury in roots of *Medicago sativa* and *Marrubium vulgare.*[147] While the first one was hydroponically grown and exposed to Hg^{2+} under controlled conditions, the latter one was collected from a mercury contaminated area in Spain. The distribution of Hg was different in both species. In *M. sativa* Hg was found in the apical regions of primary and secondary roots, in the vascular cylinder and the epidermis. EXAFS spectra revealed that a high proportion of mercury was bound to organic thiols like phytochelatins and GSH.[147] The plant from the contaminated site had a different distribution and speciation of mercury in the tissues. The detected HgS minerals may actually have been from soil microparticles still sticking to the roots.[147] A slightly different set up was used by Debeljak *et al.:* Roots of maize plants grown in Hg-amended soils (50 mg kg^{-1}) were dipped in a special medium that does not penetrate the tissues, rapidly frozen, sectioned with a cryotom and then freeze dried.[148] The sections were analysed with laser-ablation inductively coupled mass spectroscopy (LA-ICPMS). Highest Hg concentrations were found in the outer part of the roots, the epidermis and the endodermis, suggesting that Hg ions cannot cross the endodermal barrier and get transported to the upper part of the plant,[148] which is in consistence with many other studies. Usually more Hg was found in the roots than the shoots and leaves of the plants (see above). However, both studies allow only for the detection of ions on the tissue-, not the cellular level, because samples were freeze-dried. Upon freeze-drying, the vacuole becomes air-filled. And since it does not have an internal solid matrix that could keep them in the middle of the vacuole, solutes that were in the vacuole will stick to the tonoplast membrane surrounding the vacuole. In a dried plant cell, where the cytoplasmic layer is extremely thin, at resolutions achievable with current metal analysis techniques this will be indistinguishable from binding to the cell wall. Possible occurrence of such artefacts should always be checked by measuring an abundant metal with well-known intracellular accumulation (e.g. potassium) as a natural internal reference. True specific cell wall binding of Al was proven for frozen-hydrated tea leaves.[149] Sub-cellular fractionation of tissues also poses the risk of artefacts. Already the step of homogenization of tissues and breaking open of cells brings all cell components into close contact. The results obtained from this technique are rather potential binding sites for heavy metals, not necessarily the occupied binding sites in the intact plant tissues.[125] For example, plant cell walls are composed of compounds which can bind divalent and trivalent ions very effectively. Nevertheless, beyond many cases where binding to cell walls was reported as a result of sample preparation artefacts, they will certainly play a role in binding, uptake, transport and detoxification of trace metals.[150]

Recently, the effect of Hg on plants and algae at the level of microRNAs, genome-wide transcriptomics and signaling molecules (NO, CO and salicylic acid) have been reviewed by Chen and Yang (2012).[134] Briefly, mercury exposure triggered the expression or up-regulation of the general biological defence system, chlorophyll synthesis, cell wall metabolism, biosynthesis of secondary metabolites and Hg tolerance.

Toxicity of biologically redox-active metal ions

Copper - among the most needed, but also among the most toxic metals for plants

Copper is among those "heavy metals" that are known for a long time to be essential micronutrients while easily reaching toxic levels as well. In contrast to animals incl. humans, for plants copper is even more toxic than cadmium, as shown by many studies where both metals were compared.[69,151] Even in the open ocean, where organisms otherwise rarely suffer from toxicity but frequently from deficiency of micronutrients, copper reaches toxic levels - this is known from the Sargasso sea where its natural abundance limits growth of cyanobacteria.[152] In freshwater ecosystems, copper toxicity most often occurs as a result of human activities, which fall into two groups - industry and agriculture. Industrial contamination from various individual sources led to toxic copper levels in major rivers also in Western Europe, e.g. the Rhine with concentrations up to 500 nM in the 1970s. Such concentrations are lethal to sensitive species of cyanobacteria and plants.[5,153]This type of contamination has drastically decreased due to better industrial practices and wastewater treatment. The second source of severe copper contamination, in aquatic ecosystems as well as in soils, however, remains (see Table 1). This is the use of copper-containing pesticides in agriculture, in particular in vineyards. It can lead to very high pollution levels with hundreds of ppm of Cu in the soil.[4,154-156]

Copper toxicity in photosynthetic organisms has been investigated for several decades, leading to a rather detailed understanding as reviewed ten years ago (Küpper and Kroneck, 2005).[8] But more recent research yielded further significant new insights, and there are still important open questions. Like for the other metals discussed in this review, mechanisms of copper toxicity have often been studied using extremely high, environmentally not relevant concentrations. This applies in particular to many older articles, but among those many that are highly cited, such as Gallego *et al*. (1996)[157] and Wecks and Clijsters (1996)[158] that are often cited in relation to oxidative stress caused by copper toxicity. Studies that used much lower, but still toxic copper concentrations came to completely different conclusions concerning the main mechanism of copper toxicity in plants. As a prime target, in many studies with low copper concentrations, the photosynthetic light reactions were found. Inside the photosynthetic system, several targets were identified. Generally, PS II was found to be much more sensitive than PS I. In PS II, in high irradiance (but not related to photoinhibition), the reaction centre was found to be the prime target, while in low irradiance copper caused malfunction of the LHC II by substitution of the Mg^{2+} by Cu^{2+} in its chlorophyll.[69,159,160] As described for Cd^{2+}, Hg^{2+} and Zn^{2+} already, this leads to enhanced thermal dissipation of captured excitons, because like the aforementioned metals [Cu]-Chl has an unstable singlet excited state. However, for [Cu]-Chl it is even less stable, so that absolutely all captured excitons are relaxed thermally.[36] The exact target site of copper toxicity inside the PS II reaction centre has

been a topic of intense research. Inhibition of the primary charge separation in the PS II reaction centre was first suggested by Hsu and Lee (1988),[161] and would make sense in terms of insertion of copper into the pheophytin.[159] A series of detailed mechanistic studies on Cu toxicity to the PS II reaction centre was performed also by Yruela *et al*., already in the first half of the 1990s but still representing the state of knowledge for various individual aspects of this toxicity.[162-165] Using spectroscopic methods, these authors clearly show that under their experimental conditions copper binds in the pheophytin a - Q_a domain, and that copper competes with protons for binding. However, they worked on isolated thylakoids, at relatively high copper concentrations (5-100 µM) and with reduction by dithionite, leading to uncertainty in how far the reaction would be the same in a living plant. Copper is one of the classical redox active trace elements in biological systems, as such being an essential cofactor for many enzymes. Therefore, it was to be expected that copper toxicity enhances ROS production. This has been reported in many studies as reviewed earlier,[8] so that this review will focus on more recent insights. In most of the earlier studies lethal copper concentrations were applied, and it remained unclear whether the ROS production was cause or consequence of the inhibition of activity e.g. of photosynthesis. Recently, copper toxicity was re-investigated using sub-lethal low nanomolar concentrations causing chronic toxicity in the aquatic shoot model plant *Ceratophyllum demersum* under conditions simulating oligotrophic lakes.[5] In this study, copper toxicity first of all affected the PS II reaction centre (see above), while ROS production seemed to be a consequence as it occurred later. In contrast, in an earlier study on copper stress in green algae, 50 and 250 nM copper led to ROS formation, which then led to inhibition of photosynthesis as demonstrated by restoring photosynthesis via an ROS scavenger.[166] In other recent studies, on *Matricaria chamomilla* and *Arabidopsis thaliana* using micromolar concentrations that caused acute toxicity, ROS production occurred within a few hours in roots, i.e. organs without photosynthesis.[151,167] In a study comparing heterotrophic (white) with photosynthetic cells of the same strain of the alga *Euglena gracilis*, ROS production in response to Cu and Cr toxicity was much higher in the photosynthetic cells.[168] In summary, it seems that a basic level of ROS production in response to Cu (and Cr) toxicity is reached directly, without photosynthesis. Damage to photosynthesis strongly aggravates ROS production, while directly copper-induced ROS production in turn also inhibits photosynthesis.

Besides the intensively investigated inhibition of photosynthesis, according to several studies on different species copper toxicity also disturbs nutrient uptake. While differences in these disturbances exist even among ecotypes of the same species,[169] in all cases copper toxicity caused a decrease of iron content in the shoots,[169,170] while acclimation to copper toxicity included a recovery of iron concentrations.[170,171] Already nanomolar chronically toxic Cu^{2+} was furthermore observed to inhibit zinc uptake.[5] Comparison of ecotypes suggests that some of these changes in the shoot are caused by changed uptake in the root.[169] The study of Thomas *et al*. (2013) was performed on the rootless submerged shoot model plant *Ceratophyllum demersum*, clearly showing that nutrient uptake/distribution in the shoot is affected as well.[5] In roots of rice, it was recently shown that copper interacts with vesicle transport.[172] By knockout of genes necessary for this vesicle transport, the authors furthermore found that this vesicle transport is essential for signalling via ROS for activating defences.[172] Further, signalling via nitric oxide (NO) seems to occur during copper toxicity, it was shown to induce proline synthesis at low micromolar copper concentrations inhibiting growth of *Chlamydomonas*.[173] Proline synthesis is known for a long time to be a defence reaction not only against copper toxicity but also other stresses.[174]

In terrestrial plants, roots are the first organs to get in contact with an excess of Cu, and often they accumulate much higher copper concentrations than the shoots, so that they become a primary target for damage.[175,176] This usually results in a decrease in biomass.[177] Changes in the root morphology, numbers of root hairs[178] or cell volume[179] are signs of Cu toxicity. A decreased number of root tips, or organelles (like mitochondria) within root cells[180] indicates stress and will lead to a generally decreased energy production, starch accumulation and finally biomass. However, these findings are consequences of various Cu toxicity mechanisms, and do not *per se* represent mechanisms of Cu toxicity to roots. A study by Pätsikkä et al (2002),[181] however, showed that competition with iron uptake is one of the mechanisms of Cu-induced damage in roots.[181] Some more details on how Cu influences those changes in root morphology (root system architecture) and growth of primary and lateral roots were done in *Arabidopsis thaliana*.[171] Using fusion constructs of specific growth markers with the reporter gene GUS, the authors showed reduced mitotic activity in the respective root tips under Cu stress. The involvement of phytohormone accumulation in inducing (auxin) or inhibiting (cytokinin) lateral root growth at different Cu concentrations was shown. But how Cu induces this (binding sites of Cu, gene transcription, etc.) is not known yet.[171] Defence against copper toxicity on roots involves efflux pumps.[182] Diminishing passive copper inflow by enhanced root lignification, mediated via up-regulation of peroxidase expression, likely plays a role as well.[183]

While all research on copper toxicity mentioned so far had been carried out with dissolved copper, in recent years studies on copper (usually CuO) nanoparticles were added. However, in most cases it remained unclear in how far the nanoparticles dissolved during the experiment, and whether the plants actually took up any nanoparticles or only dissolved copper. Even in the rare cases where dissolution of copper from nanoparticles was measured,[184] it was not done under conditions relevant for soil, nutrient solutions or inside plants, rendering these measurements useless. And typical treatment concentrations of 10,000 to 1,000,000 ppb CuO nanoparticles[184] are very high compared to the roughly 1 ppb where copper toxicity may start in sensitive organisms.[5] Thus, the relevance of significant DNA damage specifically by copper nanoparticles needs to be re-investigated.[184] In another, more recent example of such a study, termed "mechanistic" by the authors, the effects of the CuO nanoparticles matched known effects of dissolved copper such as general growth inhibition, pigment loss, ROS production, and as a defence root lignification.[185] A few years before, however, it had been found that polymer-coated CuO nanoparticles are more toxic to algae than the same particles without coating, because the coated particles could cross membranes more easily.[186] In another

recent study, the authors were able to see some particle-specific effects, and furthermore characterised the aggregation and dissolution of the nano CuO in the tested media.[187] The particle-specific effects happened in the very early parts of the response, within the first five hours. It thus remains to be seen in how far these effects are environmentally relevant, since only at rather high concentrations effects occur within such short treatment times. In any case, this study was very informative concerning the different behaviour of CuO nanoparticles in different plant growth media.

Iron - rarely, but then severely, toxic

Iron toxicity is a topic not often dealt with in plant sciences (incl. algae and phototrophic bacteria), because in the oceans iron is always deficient, and even in terrestrial plants deficiency is more frequent than toxicity. This is due to the redox properties of iron - the abundant redox state in the current atmospheric conditions on Earth is iron (III). This is hardly soluble, and therefore mostly remains biologically inaccessible in minerals. The only chance for iron to become toxic is the reduction of massive amounts of iron (III) to iron (II), which makes it highly soluble. This phenomenon, however, does frequently occur in one very major crop species: rice. The soil of flooded lowland rice fields tends to become anoxic very quickly, and it is often rich in iron. The same occurs in natural freshwater wetlands[188,189] and has recently been found for salt marshes as well, where iron toxicity to the halophyte *Sueda maritima* was described.[190] Once the soil becomes anoxic, the iron is reduced and bioavailable, as described in the review of Becker and Asch (2005).[191] As written in that review, "iron toxicity remains an important constraint to rice production, and together with Zn deficiency, it is the most commonly observed micronutrient disorder in wetland rice". While that review focussed on conditions and management of iron toxicity in rice, we now would like to focus on the current knowledge on mechanisms of iron toxicity in plants.

Iron toxicity was first described as a problem in rice 60 years ago.[192] That publication already accurately described the visible symptoms such as brown spots on the leaves, and could associate it with reducing conditions. The mechanisms behind the symptoms, however, remained unknown. Later it has been shown that strong oxidative stress occurs in plants during iron toxicity.[193] During iron toxicity stress, various reactive oxygen species have been measured since then, such as hydroxyl radical, superoxide radical, singlet oxygen, hydrogen peroxide, alkoxyl and peroxyl radicals, as reviewed already by Becana *et al*. (1998).[194] In the context of human physiology, it was postulated early on that iron toxicity originates from the generation of reactive oxygen species via the Fenton-Reaction or the iron-catalysed Haber-Weiss-Reaction.[195] However, these particular reactions were never really proven to occur *in vivo*, only the rise in reactive oxygen species has been measured. All publications on iron toxicity in plants rely on these postulated reactions ever since.[193,194,196] Therefore, providing clear evidence whether these or other reactions cause oxidative stress during iron toxicity in plants would be an important topic for future research. Further, it still remains to be shown that the occurrence of reactive oxygen species during iron toxicity is actually the cause of the inhibition, and not a consequence of it. These points have to be mentioned in particular since it turned out that in case of other metal(loid)s the toxicity-induced oxidative stress was not directly caused by redox reactions of the metal, but by the malfunction of metal-inhibited photosynthesis (see metals previously discussed in this review). The detailed early study on iron toxicity by Kampfenkel *et al*. (1995)[193] reports a decreased ratio of maximal to minimal chlorophyll fluorescence quantum yields, which at that time was interpreted as photoinhibitory damage to photosystem II. By now it is well known that this ratio (now usually published as $F_v/F_m = ((F_m-F_0)/F_m)$ is not a specific indicator of photoinhibition, but generally shows the dark-adapted maximal quantum yield of PS II photochemistry, i.e. a decline of this ratio generally indicates damage to the PS II reaction centre. Such damage has been reported for many types of metal toxicity (e.g. review by Küpper and Kroneck, 2005)[8]. The first study that showed the involvement of light in the generation of ROS during iron toxicity in plants originates already from 1993.[197] These authors reported that susceptibility to photoinhibition was increased by iron toxicity, and could show that iron toxicity symptoms were absent when the plants were grown in very low light. They postulated that non-heme iron (as present e.g. in the PS II reaction centre) would be responsible for generation of ROS in chloroplasts (mainly via singlet oxygen). Direct evidence for a malfunction of PS II causing the generation of reactive oxygen species under iron toxicity was provided by Suh *et al.* (2002),[198] who could show that iron toxicity causes an increased synthesis of Cyt b_6/f to an extent that it produces singlet oxygen via a photodynamic action, leading to inhibition of PS II.

Iron uptake into the plants under iron toxicity conditions is different from the more known pathway under iron-deficient conditions, where plants developed strategies to enhance it by soil acidification and exsudation of mugeinic acid as a siderophore. The worst toxicity was recently reported for feeding the iron as iron (II) sulphate, although iron (III) citrate was taken up in larger quantities and transported more efficiently from the root to the shoot.[199] This may be due to a so far unknown interaction of ferrous iron with transport proteins for other nutrients and minerals. This thought is supported by earlier results that iron toxicity increases uptake of sodium, and interacts in a more complicated way with the uptake of calcium, magnesium, manganese, molybdenum, phosphorus and zinc.[200] Revealing mechanistic details of these interactions will be an interesting topic for future research.

Defence against iron toxicity is well-known to involve active oxidation in the rhizosphere in order to produce insoluble iron (III) minerals.[201] This has recently been confirmed on the genetic level, where a main quantitative trait locus (QTL) for iron tolerance was modifying root architecture towards conducive air transport into the roots.[202] Furthermore, the precipitated iron was now described as "iron nanoparticles" by comparison with artificial Fe nanoparticles.[203] Once iron toxicity already has started inside the shoots, plants up-regulate enzymes that detoxify reactive oxygen species.[193,194] The pool of weakly bound iron in plants is controlled by the iron-binding protein ferritin, so that its ectopic over-expression leads to enhanced resistance again iron toxicity,[196] and iron ferritin protein levels are up-regulated during iron toxicity stress.[204] Transporters pump iron out of the sensitive cytoplasm into compartments where it does less harm, as shown

by the increased resistance towards iron toxicity upon over-expression of AtNRAMP1.[205] Expression of the iron transporter YSL-1 spreads from the xylem parenchyma to the mesophyll under iron toxicity stress, which was interpreted as re-distributing excess iron to cells with more potential to detoxify it e.g. via vacuolar sequestration.[206] Interestingly, iron toxicity elicits strong ethylene signalling, which in a still unknown way is important for an efficient defence of the plant against the toxicity.[207]

Nickel - an ultra-micronutrient with low toxicity

In plants, nickel is known to be needed for only one enzyme, urease, as reviewed e.g. by Küpper and Kroneck (2007)[208] and Chen *et al.* (2009).[209] For this reason, by most plants it is required only in minute quantities (usually 0.05-10µg/g dw in plants,[210]), so that the study of nickel deficiency involves a lot of effort for lowering the Ni^{2+} uptake by the plants to a critical level.[211,212] Only nickel hyperaccumulator plants, which use its toxicity as a defence against pathogens and herbivores, require much higher levels of nickel for normal growth.[208]

The low requirement for nickel is not paralleled, however, by a low threshold for toxicity. On the contrary, nickel is far (more than 100 times) less toxic to plants than the much needed copper and most other trace elements, as it can easily be seen in a comparison of various potentially toxic metals,[69] and as reviewed previously.[208] For this reason, in many cases when "nickel toxicity" in the environment is reported, it is in reality toxicity of copper that often occurs together with nickel.[213-215] Recently, synergistic effects of toxicity were reported also for the combination of nickel and cadmium. Concentrations of both metals that did not cause toxicity on their own led to severe toxicity when they were combined.[216] The reason for this synergistic action is not clear and cannot be deduced from the current knowledge about the mechanisms of toxicity of the individual metals involved.

Pure nickel toxicity causes several distinct effects, which have been reviewed by Küpper and Kroneck (2007)[208] so that the current review will focus on those that were proven to be important under environmentally relevant low concentrations of nickel. Roots were shown to be sensitive to nickel toxicity, with inhibition measured already at 2.5 µM nickel.[217] The mechanism of this inhibition remained unclear, similar to subtle morphological changes in the rhizodermis, which were observed already at 1 µM Ni^{2+}.[218] Another root-level effect of nickel toxicity that was shown at low micromolar concentrations is the inhibition of the uptake of nutrients.[219-222] This is likely due to interaction of Ni^{2+} with transport proteins. The exact mechanism still remains to be resolved. In shoots of the submerged aquatic macrophyte *Elodea canadensis*, low micromolar concentrations of nickel were found to induce sublethal oxidative stress in terms of lipid peroxidation.[223] It remained unclear, however, whether this oxidative stress was primary, i.e. directly caused by the Ni^{2+}, or a secondary consequence e.g. of malfunctioning photosynthesis, which was severely inhibited under the same conditions. And the inhibition of photosynthesis by exchange of Mg^{2+} against Ni^{2+} inside the chlorophyll was resolved all the way to the molecular level some years earlier. It was first reported *in vivo* by Küpper *et al*. (1996),[69] long after [Ni]-Chl had been shown to dissipate all absorbed photons thermally due to a very unstable excited state like in the case of [Cu]-Chl (see above). This physical property of [Ni]-Chl (like [Cu]-Chl) makes the affected light harvesting systems act as "black holes" for excitons. For Ni^{2+}, it was shown in a very detailed and thorough study on isolated photosystems.[224] In that study, already about three percent exchange of the central Mg^{2+} ions of all chlorophylls in the photosystem against Ni^{2+} were sufficient for complete inhibition of photosynthesis. Besides the thermal relaxation of excitons, also the lack of axial ligands in [Ni]-Chl[225] makes this pigment unusable for photosynthesis, as these axial ligands are required for proper folding of the pigment-protein complexes.[37,226]

Combinations of metals

Most metal-polluted areas have too high concentrations in more than one metal, especially around mining areas. Generally, the interaction possibilities of combined threats are synergistic (total effect is greater than the sum of individual compounds), antagonistic (total effect is lower than the sum of individual compounds) or additive (total effect equals sum of individual compounds). When exposed to binary mixtures of Cd, Cu and Pb from 40 to 640 mg/kg each, *Cucumis sativus* exhibited all three responses (shoots: Cu+Cd and Cu+Pb: antagonistic, Cd+Pb additive; roots: Cu+Cd and Cu+Pb additive, Cd+Pb synergistic). In tertiary mixture however, only antagonistic responses were found.[227] The determination was purely based on root and shoot growth, no physiological parameter was assessed.

Ince et al. (1999)[228] used a statistical approach to predict interactions and found 87% of antagonistic and 13% additive results for duckweed (*Lemna minor*) for various binary mixtures of metals (Co, Cr, Cu, Zn). The microtox assay (bacteria, *Aliivibrio fisheri*) yielded 41% antagonistic, 38% additive and 11% synergistic predictions.[228] Exceeding optimal concentrations, additive or antagonistic effects seem to be the main responses. Again, the determination parameter was based on biomass and rather serves as a criterion for contamination determination, not to unravel toxicity mechanisms.

However, for macrophytes, a synergistic interaction based on various photosynthetic parameters was found for low concentrations of Ni (300 nM) and Cd (3 nM). While Cd only had positive effects and Ni only was slightly inhibitory, Cd&Ni together resulted in increased inhibition.[216] The effective concentrations can differ vastly depending on physical parameters like pH and water hardness. The higher amounts of Ca und Mg in hard water lakes compete with toxic metal ions for binding sites on the organism's surface, usually decreasing their toxicity.[229,230] This does not apply, however, to copper toxicity, because transporters for Cu^{2+} have such a low affinity for Ca^{2+} and Mg^{2+} that the latter metal cannot outcompete Cu^{2+}. Therefore, water hardness does not protect against copper toxicity.[231]

Conclusions

In most cases of toxicity assays, not only high metal

concentrations but also short exposure times were used. It is obvious that at high metal concentrations the toxicity becomes less specific (metal binding to low-affinity sites once the high-affinity sites are occupied). But furthermore, a very recent study on Ni^{2+} toxicity showed on the basis of biotic ligand models that chronic toxicity cannot be predicted by models for acute toxicity,[232] confirming earlier studies on zooplankton with other metals. To unravel the mechanisms of metal toxicity it is important to study the effects under environmentally relevant conditions to ensure a specific effect and not an overall inhibition of the metabolism.

We summarized the mechanisms described in this review in the scheme shown in Fig.1.

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We generally refer to the element name only, when the redox state is unknown or at the beginning of sentences. In biological systems, Cd, Hg, Ni and Zn have the redox state 2+, while Fe and Cu can be $Fe^{2+/3+}$, $Cu^{+/2+}$.

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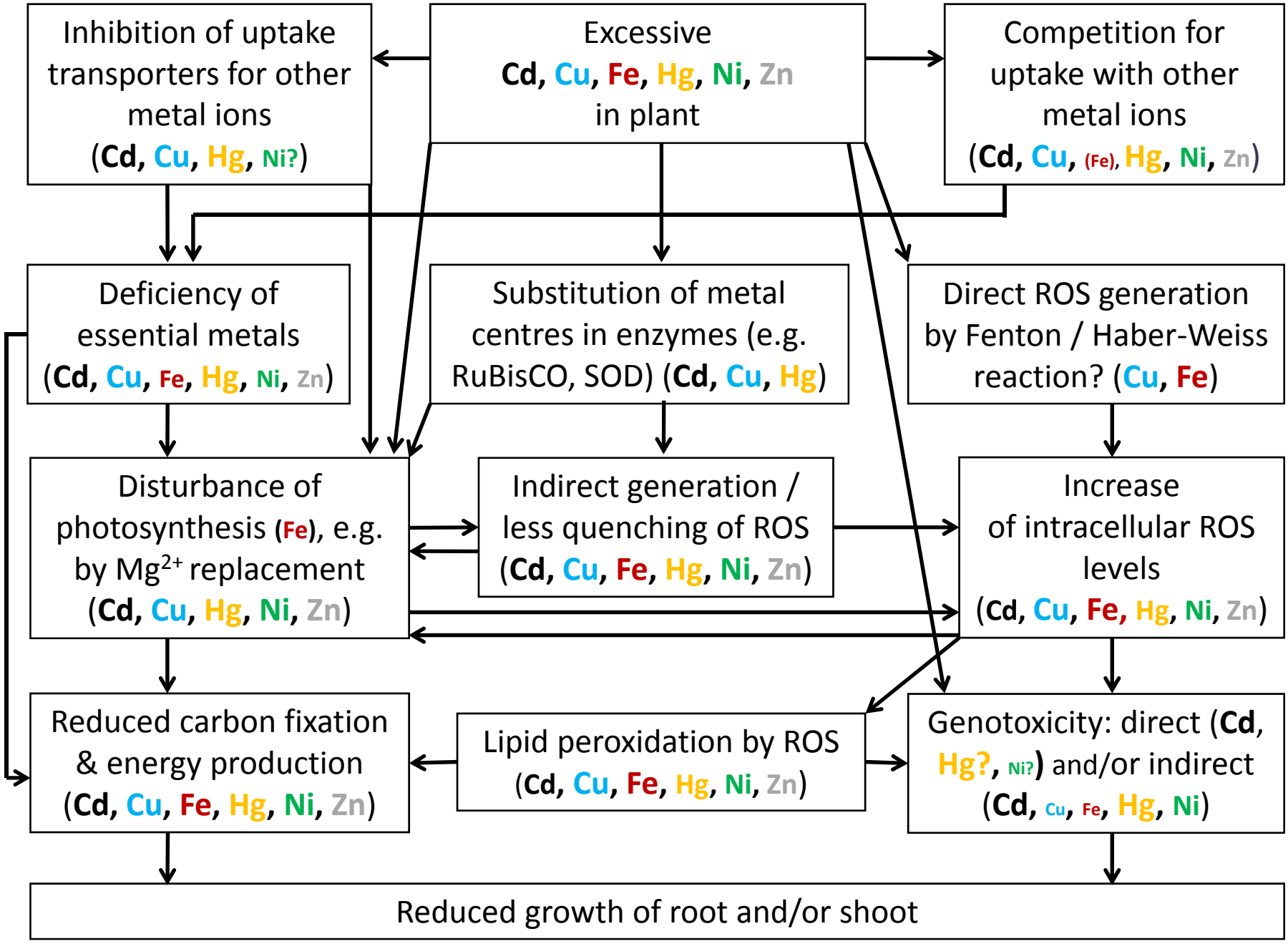

Table 1:
Average metal concentrations in mg/kg in urban soils, contaminated areas and guide values for soil-clean ups.

City/Region	Cd	Cu	Fe	Hg	Ni	Zn	References
Pittsburg, USA	1.2	-	-	0.51	-	-	Carey 1980 [233]
La Coruña, Spain	0.3	60	-	-	28	206	Cal-Prieto 2001 [234]
Madrid, Spain	-	71.7	-	-	141	210	De Miguel 1998 [235]
Aberdeen, Scotland (parkland soils)	-	27	18469	-	14.9	58.4	Paterson 1996 [236]
Aberdeen, Scotland (roadside soils)	-	44.6	18116	-	15.9	113	Paterson 1996 [236]
Hong Kong	2.18	24.8	-	-	-	168	Li 2001 [237]
Palermo, Italy	0.82	75.5	-	1.85	18.8	149	Manta 2002 [238]
Montreal Island, Canada, historic industry area, 3 rail yards	2.3–7.3	160-245	-	-	64 - 98	410 - 547	Ge 2000 [239]
Baltimore, USA, historic industry area	1.06	45	23495	-	27	141	Yesilonis 2008 [240]
Guizhou, China (mining area) at smelter	60.5	202	-	-	24.1	2551	Li 2007 [241]
Guizhou, China (mining area) 15km away	5.1	72.6	-	-	9.9	867	Li 2007 [241]
Sicily, Italy, unpolluted	1.3	34	-	0.066	-	122	Modified from Manta 2001 [238]
New York State Department of Environmental Conservation max. values for unrestricted use (incl. agriculture)	0.43	270	-	0.81	72	1100	NYS DEC [242]
NYS DEC max. values for residental use	0.86	270	-	0.81	140	2200	NYS DEC [242]
Quebec guidelines for soil cleanup: Clean	1.5	50	-	-	50	100	Ge 2000 [239]
Quebec guidelines for soil cleanup: Should be restored	5	100	-	-	500	500	Ge 2000 [239]
Quebec guidelines for soil cleanup: Needs immediate cleaning	20	500	-	-	1000	1500	Ge 2000 [239]

Table 2: Average (or ranges) metal concentrations in nM in freshwater environments.

Region	State of contamination	Cd	Cu	Fe	Hg	Ni	Zn	Reference
Lakes in north UK	unpolluted	0.23	7.5	1251		9.6	56.7	UK EPA, 2008, [243]
Lake Constance, Germany	unpolluted	<0.44	<7.9	134	<0.25	8.5	-	Lake Constance, Zvbwv.de, [244]
Swedish Lakes, Sweden	unpolluted	0.044-0.14	4.8-7.9	-	0.005-0.02	3.4-6.8	13.6-30.3	Swedish EPA, [245]
Swedish streams, Sweden	unpolluted	0.027-0.14	14.3-30.2	-	0.005-0.02	8.52-46	44-86.4	Swedish EPA, [245]
Stream waters Ontario, Canada	polluted	10.4	30.3-59.8	2059-8219	0.2	26.4	181-217	Ontario monitoring, online, [246]
3 lakes in mining area in Ontario, Canada	polluted	-	80-254	-	500-20,000	170-1703	-	Ontario Water quality Report 2012, [247]
Lakes in North UK	polluted	23.9	42.3	-	-	91.4	23897	UK EPA, [243]
Animas River, Colorado after Gold mine spill 2015	polluted	21.4	857	3760	0.4	90	4545	US EPA report, Gold mine response [248]
Water quality criteria for aquatic life - acute	-	17.8	-	-	6.98	8007	1818	US EPA, [249]
Water quality criteria for aquatic life - chronic	-	2.2	-	17907	3.84	886	1818	US EPA, [249]