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**Cadmium accumulation in *Scenedesmus vacuolatus*
under freshwater conditions**

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STEFANIE TÖPPERWIEN

Dipl.-Agr. Biol., University of Hohenheim (Germany)

born July 25th, 1974

Citizen of Germany

accepted on the recommendation of

Prof. Laura Sigg, examiner

Prof. Bernhard Wehrli, co-examiner

Prof. Vera Slaveykova, co-examiner

Dr. Renata Behra, co-examiner

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Summary

Metal uptake and accumulation from freshwater systems can lead to toxicity effects in organisms when either essential trace element concentrations (Zn, Mn, Cu, Co) are above nutritional level, or when non-essential metals exhibit detrimental effects at already low concentrations (Cd, Ag, Pb, Hg). In order to assess the toxicity of a given metal it is not sufficient to know the total metal concentration, but also its bioavailability which is controlled by the speciation of the metal in water.

Cadmium is known to compete with other cations for uptake into organisms and is toxic at low concentrations. The goals of this work were to study factors determining Cd uptake and accumulation in *Scenedesmus vacuolatus*, a freshwater algal species, under conditions relevant for metal uptake in freshwaters, and to link the Cd accumulation with chemical speciation measurements of Cd in the water. Therefore, Cd accumulation and speciation was investigated in synthetic solutions and in freshwater samples.

In laboratory experiments zinc and manganese competition on Cd uptake was assessed. Cd accumulated in *S. vacuolatus* between 5×10^{-18} to 6.2×10^{-17} mol/cell in the range of free Cd^{2+} from 1×10^{-11} to 1×10^{-9} M and decreased in the presence of Zn when the $\text{Zn}^{2+}/\text{Cd}^{2+}$ ratio exceeded 14. In presence of increasing Mn, a decrease in Cd accumulation was observed from a $\text{Mn}^{2+}/\text{Cd}^{2+}$ ratio higher than 11000. Based upon these studies it was concluded that for *S. vacuolatus* the competition between Cd and Zn for binding and uptake is dominant, whereas the competition of Cd with Mn is much less evident. Thus, in this algae species Cd is transported mainly through the Zn-transport system.

Freshwaters are characterized by the presence of varying natural organic ligands which influence the metal speciation in the water. A large part of these ligands consist of fulvic or humic acids which are heterogeneous in size and binding characteristics. Therefore, the interaction between a range of standardized fulvic

acid concentrations (5 to 40 mg/L) and cadmium accumulation in *S. vacuolatus* was investigated at pH 7.5. This study shows that fulvic acids control cadmium uptake by binding to the metals in solution, thus decreasing the bioavailable metal fraction in the water. Effects of fulvic acids on cadmium uptake by interactions of the fulvic acid with the cell surface are of minor importance at fulvic acid concentrations up to 40 mg/L. Algae were shown to excrete ligands upon growth in presence of fulvic acids, which contributed to cadmium complexation to a limited extent.

Natural ligands such as fulvic and humic acids, colloids, and variations in pH and temperature influence metal accumulation and metal speciation in freshwaters. Thus, Cd accumulation in *S. vacuolatus* exposed to freshwaters (from two hardwater and a softwater site) was investigated in relation to the actual metal speciation in these waters. Cd accumulation in *S. vacuolatus* was related to speciation measurements of the free Cd^{2+} in the respective waters by competitive ligand exchange-stripping voltammetry (CLE-SV). Other speciation methods which determined total, labile or free Cd fraction were also applied within these fieldworks and Cd accumulation was compared to these results. The Cd accumulation in all investigated freshwaters increased linearly in relation to the free Cd^{2+} concentration, but the Cd accumulation in the softwater was lower than expected compared to Cd accumulation from hardwater and culture media and was not simply dependent on free Cd^{2+} or labile Cd. In this case, other factors such as the composition of the humic acids and the presence of colloidal iron are likely to influence Cd accumulation.

The combination of metal accumulation and speciation studies presented in this thesis helps to better understand the limitations of current metal availability prediction models and is a step forward towards the incorporation of these predictions into water quality criteria.

Zusammenfassung

Die Aufnahme und Akkumulation von Metallen aus Süßwassersystemen ist für Organismen toxisch, wenn die Konzentration von essentiellen Spurenelementen den Nährstoffbedarf deutlich übersteigt (z.B. bei Zn, Mn, Cu, Co) oder wenn ein nicht-essentielles Metall bereits bei geringen Konzentrationen schädliche Einflüsse zeigt (z.B. Cd, Ag, Pb, Hg). Um die Toxizität eines Metalls zu beurteilen, ist die Kenntnis seiner totalen Konzentration nicht ausreichend, da die tatsächliche Bioverfügbarkeit von der Speziation des Metalls im Wasser abhängt.

Kadmium ist bereits in sehr geringen Konzentrationen giftig und konkurriert mit anderen Kationen um die Aufnahme in Organismen. Ziel dieser Arbeit war es, die wesentlichen Faktoren für die Kadmiumakkumulation in die Süßwasseralge *Scenedesmus vacuolatus* unter Bedingungen zu bestimmen, wie sie für die Metallaufnahme aus Gewässern relevant sind. Weiterhin wurde die beobachtete Cd-Akkumulation in Beziehung zur chemischen Speziation von Cd im Wasser gesetzt. Dazu wurden Cd-Akkumulation und -speziation in verschiedenen synthetischen Medien und Gewässerproben untersucht.

Die Konkurrenz von Zink und Mangan mit Cd bei der Aufnahme wurde anhand von Modelluntersuchungen im Labor bestimmt. In Gegenwart einer freien Cd Konzentration von 1×10^{-11} bis 1×10^{-9} M lag die Cd-Akkumulation in *S. vacuolatus* bei 5×10^{-18} bis 6.2×10^{-17} mol/Zelle und nahm in Anwesenheit von Zn ab, wenn ein $\text{Zn}^{2+}/\text{Cd}^{2+}$ -Verhältnis von 14 überschritten wurde. Wurde die Mn Konzentration erhöht, sank die Cd-Aufnahme erst ab einem $\text{Mn}^{2+}/\text{Cd}^{2+}$ Verhältnis > 11000 . Diese Experimente zeigten, dass für die untersuchte Süßwasseralge eine starke Konkurrenz zwischen Zn und Cd um Bindung und Aufnahme vorliegt, während sie im Fall von Mn und Cd nur schwach ausgeprägt ist. In der untersuchten Alge scheint der Zn-Transportmechanismus für die Cd-Aufnahme massgeblich zu sein.

Natürliche Gewässer weisen unterschiedliche Mengen von organischen Liganden auf, die die Speziation von Metallen im Wasser beeinflussen. Ein

grosser Teil dieser Liganden sind Fulvin- und Huminsäuren, die sowohl in ihrer Grösse als auch in ihrer Bindungscharakteristik sehr heterogen sind. In dieser Arbeit wurde untersucht, wie die Gegenwart einer standardisierten Fulvinsäure (von 5 bis 40 mg/L) die Cd-Aufnahme in die Süsswasseralge bei pH 7.5 beeinflusst. Es zeigte sich, dass die Bioverfügbarkeit von Cd durch die Bindung an Fulvinsäure verringert, und damit die Aufnahme von Cd beeinflusst wird. Eine Bindung der Fulvinsäuren an die Algenoberflächen und eine daraus resultierende Veränderung der Cd-Aufnahme ist dagegen bis 40 mg/L Fulvinsäure vernachlässigbar. Unter Wachstumsbedingungen in Gegenwart von Fulvinsäure gaben die Algen Liganden in das Wachstumsmedium ab, diese trugen in geringem Umfang zu einer Komplexierung der Metalle bei.

In natürlichen Gewässern wird die Metallaufnahme und -spezifizierung von Faktoren wie Fulvin- und Huminsäuren, Kolloiden und variierenden pH- und Temperaturwerten beeinflusst. Ein Schwerpunkt dieser Arbeit war ein Vergleich der Cd-Akkumulation in *S. vacuolatus* aus natürlichen Gewässern (zwei harte und ein weiches Gewässer) mit der dort tatsächlich vorliegenden Spezifizierung der Metalle. Die Cd-Akkumulation in die Algen wurde zu Cd^{2+} in Bezug gesetzt, das mit „Competitive ligand exchange-stripping voltammetry (CLE-SV)“ bestimmt wurde und mit den totalen, labilen und freien Metallkonzentrationen verglichen, die durch weitere Untersuchungsmethoden bestimmt wurden. Die Akkumulation von Cd aus natürlichen Gewässern hängt linear von der Konzentration des freien Cd^{2+} ab, allerdings zeigte ein Vergleich zwischen harten und weichen Wässern, dass die Cd Aufnahme nicht nur in Funktion des freien oder labilen Cd erfolgte, sondern im weichen Wasser kleiner ist als im Vergleich mit hartem Wasser. Weitere Faktoren, wie z.B. die Zusammensetzung der Huminsäuren und die Gegenwart von kolloidalem Eisen scheinen die Cd-Akkumulation aus den verschiedenen Gewässern zu beeinflussen. Die in dieser Arbeit vorgestellte Kombination von Studien zur Metallaufnahme und -spezifizierung hilft, die Beschränkungen der aktuellen Modelle zur Vorhersage von Bioverfügbarkeit von Metallen aufzuzeigen, und ist

ein wichtiger Schritt in Richtung der Aufnahme solcher Modelle in die Beurteilungskriterien für die Wasserqualität.

Introduction

1.1 Cd in the environment

Trace metals (e.g. copper, cobalt, iron, manganese, zinc) are essential micronutrients for algae and other organisms (Frausto da Silva and Williams, 2001; Tessier and Turner, 1995). They exist in a variety of redox states and coordination species which influence their biological availability. Most exist in natural waters as metal cations that are complexed to varying degrees by inorganic and organic ligands (Morel and Hering, 1993; Stumm and Morgan, 1996). Essential metals are of vital importance in enzymatic reactions, in co-enzymes, for stabilizing the cell membrane, or for osmotic stability, for the nucleic acid metabolism as well as for electron transport and redox reactions (Borchardt, 1996; Lehninger, 1982; Marschner, 1994). However, at high concentrations they are becoming toxic for algae and other organisms (Allen et al., 1980; Genter, 1996; Walker et al., 2001). In contrast, other metals such as cadmium, lead, silver, and mercury are not essential and are toxic for organisms at already very low concentrations. Cadmium with its similar coordination properties to those of essential metals, particularly Zn, exhibits a strong affinity for sulfhydryl groups, and can easily substitute essential metal ions, block essential functional groups, or modify the conformation of biomolecules (Mason and Jenkins, 1995), and also affects the cellular antioxidant capacity of algae (Pinto et al., 2003).

Cd input sources are mainly of anthropogenic origin (via industrial runoff or discharges, wastewater, fossil fuel combustion, mining activities, and sewage effluents) whereas natural input to aquatic systems occurs via erosion, dissolution of rocks, or volcanic action. Typically, at uncontaminated sites Cd is present at total concentrations of about 0.1 nM with a free metal concentration of about 0.001 nM (Xue and Sigg, 1999) whereas total dissolved background concentrations of Zn range from 10-51 nM with free metal ion concentrations

from 0.3 to 5.6 nM (Borg, 1983; Meylan et al., 2004b; Xue and Sigg, 1994). In Stream Furtbach which was investigated in this work together with other freshwater systems the Cd concentration was found to raise up to 0.19 nM during a rain event (Meylan et al., 2003). Cd concentrations between 0.2 nM (Mylon et al., 2003) up to 7 nM (Croteau et al., 2002) were found at other contaminated sites.

Investigations showed that the elevated total metal concentration does not necessarily lead to an increase of this metal in organisms, but can be matched by and increase in ligand concentration that keeps the free metal concentration at low levels (Behra et al., 2002). To correlate metal concentrations in waters to metal contents in organisms, algae are used as model systems and combined with speciation methods ((CNTC), 2000).

This work aimed at investigating the relation between Cd speciation in waters and accumulation of Cd in *Scenedesmus vacuolatus*, a freshwater alga, under different conditions relevant in freshwater systems.

1.2 Organism - metal interactions

The interaction between algae and metal involves several steps, such as diffusion of the metal from the bulk solution to the biological surface, adsorption of the metal ion on the algal surface and intracellular uptake (Campbell et al., 2002; Simkiss and Taylor, 1995). The metal diffuses through the diffusion layer to the cell and binds on the active sites on the cell surface. The adsorption to the cell surface is considered being very fast followed by slower diffusion across the cell membrane (Bates et al., 1982; Knauer et al., 1997; Sunda and Guillard, 1976). The algal surfaces contain various functional groups with high affinity for metal ions and carry a net negative charge, mainly due to carboxylic, phosphoric and sulfhydryl groups (Crist et al., 1990; 1981;

Kiefer, 1994; Xue et al., 1988). The metals bind to the functional groups and migrate through this matrix to the plasma membrane, which with its lipid bilayer acts as natural barrier of the cell. Within the membrane various types of transport proteins, forming channel proteins or carrier proteins, facilitate the transport of the metal through the membrane. Channels might be gated and their functioning influenced by their chemical and electrical conditions. Free ions and hydrated ions, charged metal complexes, uncharged ionic complexes, or organometallic complexes are able to pass the membrane by facilitated diffusion along an electrochemical gradient or active transport which requires energy (Simkiss and Taylor, 1995). Inside the cell, metals are sequestered rapidly to varying degrees by intracellular ligands, which are conceptually categorized into different groups depending on their physiological actions upon binding (Mason and Jenkins, 1995).

1.3 Free ion activity and biotic ligand model

The bioavailability of metals has been shown to be controlled by the metal speciation (Luoma and Rainbow, 2005; Morel and Hering, 1993; Sunda and Huntsman, 1998a). Laboratory experiments suggest that uptake, growth and toxicity are mainly a function of the free metal ion activity which evolved in the description of the free ionic activity model (FIAM) (Anderson et al., 1978; Campbell, 1995; McBride, 1994; Sunda and Guillard, 1976) or its derivative, the biotic ligand model (BLM) (Campbell et al., 2002; Paquin et al., 2002; Slaveykova and Wilkinson, 2005).

Figure 1.1: Conceptual model of metal uptake (after Campbell, 1995)

This model states that the influence of a metallic compound on organisms is governed by the activity of the free metal ion and not by its total concentration. FIAM has been confirmed for a number of different metals (Cu, Zn, Mn, Fe, Cd), organisms, and biological responses (growth, uptake, nutrients limitation, toxicity (Anderson et al., 1978; Brand et al., 1986; Sunda and Guillard, 1976; Sunda and Huntsman, 1992; 1998a)). The underlying assumptions of the model are comprehensively described in a review of the FIAM model by Campbell (1995). The free metal ion $[Me^{2+}]$ is considered to form a metal-surface ligand complex $[M-X-cell]$ (figure 1.1). The concentration of this complex is proportional to the free metal concentration in the solution in equilibrium with the other metal species. With the assumption that $[M-X-cell]$ is proportional to the biological response, the $[Me^{2+}]$ concentration is influencing the response of the organism.

However, since the development of the FIAM, limitations and exceptions have been and are still reported. Observed exceptions were categorized depending on

the type of ligand involved namely organic ligands, inorganic anions, and low molecular weight ligands (Campbell, 1995; Errécalde and Campbell, 2000; Errécalde et al., 1998; Fortin and Campbell, 2001). Natural organic ligands such as fulvic or humic acid can decrease metal bioavailability by forming labile or non-labile complexes with the metal in solution, thus decreasing the free metal concentration. Labile complexes are characterized by their ability to dissociate within the diffusion layer of the cell membrane, thus contributing to the diffusional flux of metals to the cell membrane. Non-labile complexes have a higher stability constant and slower dissociation rate; therefore they do not dissociate within their residence time in the diffusion layer of the organism, and do not contribute to the metal flux towards the cell surface.

Decreased metal uptake is also observed when cations of the same size as the metal in the solution compete for the binding sites at the algal surface (Campbell et al., 2002; Sunda and Huntsman, 1981; 1983; 1996).

The Biotic ligand model (BLM) introduces the concept of the biotic ligand, which is the site of action where the metal binds at the cell surface prior to internalization (Di Toro et al., 2001). The biotic ligand is in competition with environmental ligands such as organic matter for the metal of interest and other major cations such as Ca^{2+} , Mg^{2+} , or even H^{+} which can also bind to the biotic ligand.

A critical point of the BLM is the case of rate limiting internalization when the uptake rate of the metal of interest is higher compared to the mass transfer of the metal to the biological interface. This has been shown for silver by Fortin and Campbell in *Chlamydomonas reinhardtii*, because this metal has a very high uptake rate compared to the diffusion of the metal to the cell (Fortin and Campbell, 2000). The BLM assumes that there is no diffusion limitation from the bulk solution to the cell wall and the metal concentration at the cell wall is in equilibrium with the biotic ligand. In case of “equilibrium control”, the rates of dissociation and formation of M-X-cell with Me^{2+} are much greater than the

uptake rate. In that case the uptake is controlled by the equilibrium between the bound metal to the biotic ligand and the free metal concentration (Hudson, 1998). The possibility of uptake rate limitation due to kinetic control was studied theoretically (Van Leeuwen, 1999) and has been demonstrated for copper in field studies in a freshwater system (Meylan, 2003). Further need of improvement of the applicability of BLM in predicting metal availability from natural systems has been pointed out by (Slaveykova and Wilkinson, 2005).

1.4 Metal speciation

The particular chemical form in which an element exists in water is called its *speciation* (Stumm and Morgan, 1996). An element can be present as a simple hydrated ion, as a molecule, as a complex with other ions or molecules. In freshwater the speciation of metals is influenced by inorganic anions (e.g. HCO_3^- , CO_3^{2-} , SO_4^{2-} , Cl^-), organic ligands, and binding sites of particle surfaces (Fe-oxides, biological material). Ligands include acetate, oxalate and other small acids, amino acids or larger polymers such as humic and fulvic acids (Campbell, 1995; Stumm and Morgan, 1996), as well as siderophores or phytochelatins excreted by bacteria or algae (Lee et al., 1996; Wei and Ahner, 2005), and also synthetic ligands such as EDTA and NTA (Giger et al., 1991). In most freshwaters, inorganic Cd-, Cu-, or Zn-species occur as hydroxo- or carbonate complexes. Labile complexes are considered playing an important role, depending on their relative kinetics for uptake and dissociation (van Leeuwen, 1999).

Cd has been shown to be strongly complexed in sea and freshwaters as well as by isolated fulvic and humic acids (Bruland, 1992; Muller, 1996; Pinheiro et al., 1994). Fulvic and humic acids are abundant in natural freshwaters and influence metal speciation by reducing the free metal concentration in the bulk solution.

They are heterogeneous with respect to their composition and their metal binding groups which comprise various functional (phenolic-, carboxylic-, sulfhydryl-) groups. They vary in size from 500 - 2000 g/mol⁻¹ for FA to 2000 > 5000 g/mol⁻¹ for HA, which complicates their role in metal speciation (Sigg and Behra, 2005; Tipping, 2002).

A very strong class of ligands was found to govern the Cd speciation at the ambient level in lakes (Xue and Sigg, 1998; 1999; 2002) and the presence of these ligands was linked to occurrence of biological activity in eutrophic lakes (Xue and Sigg, 1999). In lakes with higher metal concentration and low biological productivity the ligands more closely match the fulvic acids characteristics (Xue and Sigg, 1999). In natural freshwaters cadmium was found to be complexed to 85-90 %, copper up to more than 99%, mainly in complexes with organic ligands, and zinc up to 50% (Meylan, 2003; Sigg and Xue, 1994; Xue and Sigg, 1993; 1998). Thus, compared to Cd, copper is more reactive with respect to complex formation, whereas zinc is less reactive. Therefore, zinc is more easily available for the algae than Cd because the free Zn ion concentration is higher than the free Cd ion concentration at similar total metal concentration.

Seasonal variations such as temperature and physico-chemical conditions need also be considered in natural systems as they influence the bioavailable fraction of the metal due to binding of trace metals to suspended particles, algae, or dissolved ligands (Xue and Sigg, 1993; 1994).

The small amounts of trace metals in natural environments require very sensitive and precise methods to determine total, dissolved, or free ion concentration of a metal (Batley et al., 2004). To measure the free metal ion concentration, various methods can be used. A summary of methods determining total, labile and free metal concentrations is given in Chapter 2. From the results obtained by the various methods the free metal concentrations usually has to be derived by means of thermodynamic modelling, which is based on the equilibrium between

metal and ligands (Sigg and Xue, 1994). For this approach, the exact composition metal, ligand, and the stability constants involved need to be known (Martell and Smith, 1976-1989). Modelling of natural waters is complicated by the fact that the ligands themselves are not exactly known and the properties of the ligands are poorly defined. Data processing is generally performed using modelling programmes to estimate ion activities (Gustafsson, 2005; Tipping, 1994).

1.5 Implications for water quality criteria

The relation of total metal concentrations to their actual speciation and availability for organisms should be considered when defining water quality guidelines. However, present regulations involve only dissolved metal concentrations. Within the Swiss Ordinance of Water Quality (WQC), dissolved metal concentrations are expected to be lower than 0.05 µg/l for Cd, 5 µg/l for Zn, and 2 µg/l for copper (Behra et al., 1994). The European Union Water Quality Directive aims at protecting aquatic systems by the definition of suitable environmental quality standards based on appropriate, clear and transparent criteria. In this directive metal speciation methods are considered being a key element in defining relevant criteria (EU-Commission, 2000) and algae are considered as suitable model organisms which are sensitive enough for testing water quality. To date, the introduction of metal speciation in water quality criteria is still a matter of discussion.

1.6 The BIOSPEC project

The work presented in this thesis was performed within the European Union research project BIOSPEC, which aimed at comparison and evaluation of several speciation methods for metals in freshwaters and solutions, and to develop a method for routine prediction of metal biouptake and detection in freshwater systems (Town, 2001).

The main goals of the BIOSPEC project were

- (a) The determination of reliable and predictive quantitative parameters for assessing the biouptake potential of trace heavy metals (Cu, Pb, Cd, Zn, Ni) by development of robust, well-characterized sensors, which can be applied in situ.
- (b) To assess the importance of a dynamic approach to speciation for more reliable prediction of both short term and long term biouptake (related to the acute and chronic toxicity) of heavy metals.
- (c) Rigorous comparison of dynamic speciation techniques in-situ in the field and the laboratory.
- (d) Comparison of methods with metal uptake by organisms under a range of conditions to assess the relevance of speciation measurements for bioavailability and biouptake, and to evaluate the best predictive parameters.

The comparison of analytical techniques has been already covered in publications (van Leeuwen et al., 2005) where the importance of dynamic

speciation as well as factors influencing the kinetics of trace metal binding and uptake were stressed. Speciation of metals in natural samples and comparison of measured speciation parameters to modelling were assessed from field campaigns performed within the project (Sigg et al., 2006; Unsworth et al., 2006).

1.7 Scope of this work

This thesis investigates different factors determining Cd accumulation in *Scenedesmus vacuolatus* under freshwater conditions. Metal uptake experiments were performed to relate bioaccumulation with chemical speciation measurements. Various factors were assessed to evaluate the possibility of relating bioaccumulation to speciation measurements and to other water composition parameters.

The thesis is divided in the following chapters:

- Methods determining metal speciation in freshwaters. This chapter gives an overview about different methods of metal speciation determination. The methods determining total, labile, or free metal concentration in the solution applied within the BIOSPEC project are outlined in this chapter. Problems of speciation determination are evaluated with respect to in-situ field measurements and the significance of the measured parameters for bioavailability is discussed.
- Competition among cadmium, zinc, and manganese uptake in the freshwater algae *Scenedesmus vacuolatus*. This chapter investigates the relations of Zn and Mn competition on Cd uptake in *S. vacuolatus*. Relation to freshwater systems is discussed. This chapter is submitted for publication to *Environmental Toxicology and Chemistry*.

- Fulvic acid interactions on Cd uptake in *S. vacuolatus*. This study assessed the direct and indirect influence of a standard fulvic acid on Cd accumulation. Investigation of ligand excretion by the algae during growth, and the capacity of the ligands to reduce Cd availability in presence of fulvic acids complete this chapter.
- Cadmium accumulation in algae under freshwater conditions. Algae were exposed to real freshwater samples in the laboratory to assess the accumulation of Cd in natural systems. Cd accumulation from several freshwaters is compared to data obtained during a field campaign at the respective field sites, where a suite of speciation measurements were applied in-situ and under controlled conditions in the laboratory. Two hard-water systems from Switzerland and a soft-water system from Great Britain were investigated. Several factors influencing Cd uptake in these natural waters are discussed.

Methods for determining metal speciation

2.1 Introduction

The knowledge about metal speciation in freshwaters is of environmental relevance as it allows the determination of reactivity, mobility, bioavailability, and toxicity of the respective metal (Batley et al., 2004; Campbell, 1995).

The determination of metal speciation relies on measurements of different fractions of metals. No single analytical method can provide a detailed description of all the species involved because every method measures a certain proportion of the total complexes that lie within a given characteristic thermodynamic and kinetic window (van Leeuwen et al., 2005). Complementary speciation data can be obtained by the application of different sensors to measure metal fluxes over a range of well defined time scales (Sigg et al., 2006). The small amounts of trace metals in the natural environment require very sensitive and precise methods for determining total, dissolved, or free ion concentration of the metals.

Many methods determining the labile or free fraction of the metal are of indirect nature, where the metal concentration is measured by a pre-concentration step.

Usually, different types of sensors, either dynamically or equilibrium based are applied. Dynamic sensors are characterized by their response time, which is determined by the thickness of the diffusion layer, and their accumulation time i.e. the signal measured is the result of an integral over the exposure time (van Leeuwen et al., 2005). Equilibrium based methods have to consider the kinetic features to verify whether equilibrium is attained at the given time scale (van Leeuwen and Jansen, 2005; van Leeuwen and Town, 2005).

Normally, the free metal is consumed at the sensor surface but the free metal is in equilibrium with coupled diffusion and kinetics of conversion processes between the free metal and other species. The labile fraction of the metal is given by the amount by which the kinetic flux arising from dissociation of the

complex into the free metal species exceeds the diffusion limited flux. Lability is thus operationally defined and depends on the effective time scale of the analytical technique (van Leeuwen et al., 2005).

Within the BIOSPEC project the labile fractions of Cd, Zn, Pb, Ni, and Cu were determined by Gel-Impregnated Microelectrodes (GIME) (Tercier and Buffle, 1996), Diffusive Gradient in Thin Films (DGT) (Davison and Zhang, 1994), or Permeation Liquid Membrane (PLM) (Parthasarathy et al., 1997). The free fraction was determined by Competitive Ligand Exchange Anodic Stripping Voltammetry (CLE-SV) (Xue and Sigg, 2002), the Donnan Membrane Technique (DMT) (Temminghoff et al., 2000), or the Hollow Fibre Permeation Liquid Membrane (HFPLM) (Tomaszewski et al., 2003). The options of the methods to determine different bioavailable metal fractions are discussed.

2.2 Metal determination methods

Total metal concentrations

To determine total dissolved, or free metal concentrations usually samples are divided in fractions. Filtration is widely used to separate the solids from solution species. In aquatic chemistry, the term “total dissolved” species is operationally defined by filtration using 0.45 μm or 0.2 μm pore size membranes. This is a practical approach to separate solids from solution species, as particles might change the sample properties due to coagulation or flocculation. Filters with small pore size (0.01-0.2 μm) or ultrafiltration can be used to separate small particulate and even colloidal species (Batley et al., 2004; van den Berg and Achterberg, 1994).

The obtained metal fraction is measured by a technique with sensitivity which is suitable for trace metal analysis, usually inductively coupled plasma mass spectrometry (ICP-MS) or optical emission spectrometry (ICP-OES).

Gel-Integrated Microelectrode (GIME)

This method is based on a gel impregnated microelectrode (GIME) consisting of a mercury plated iridium microelectrode array covered by a 300 μm thin layer of agarose gel. (Buffle and Tercier, 2000; Tercier and Buffle, 1996; Tercier et al., 1995; Tercier-Waeber and Buffle, 2000; Tercier-Waeber et al., 2002). The agarose acts as dialysis membrane thus allowing diffusion of small molecules or ions, but excludes colloidal material. It also provides a controlled diffusion medium at the electrode, and eliminates impacts of the bulk solution convections which especially might appear in-situ. In particular it was shown to be effective in river waters with high concentrations of humics or fulvics (up to 30 mg/L) and high loads of suspended matter (50-78 mg/L) when combined in-situ with direct square wave stripping voltammetric measurements. The application involves a pre-concentration step with diffusional flux through the gel layer, followed by complex dissociation and metal accumulation and determination at the electrode by square wave anodic stripping voltammetry (SW-SV). The labile species at the time-scale of the electrode are measured. A great advantage of this method is that it can be applied in-situ in combination with a voltammetric in-situ profiling systems or a voltammetric in-line analyzer (GIME-VIP or GIME-VIA-FIELD (Tercier-Waeber et al., 2003)).

Diffusive Gradient in Thin-films (DGT)

This is a non-equilibrium method developed by Davison and Zhang (Davison and Zhang, 1994; Zhang and Davison, 2000) and can be employed in-situ. The

DGT measures kinetically labile trace metals in sea, freshwaters and soil pore waters. Its principle is diffusion of the metal of interest through a gel layer defined in terms of thickness and size and the subsequent collection on an ion exchanger.

The standard DGT sampler consists of a gel impregnated with a chelating resin (Chelex-100), covered by a diffusive hydrogel (0.4-0.8 mm, polyacrylamide) topped with a 100 µm thick, 0.4 µm pore size cellulose nitrate or polysulfone membrane. It is mounted in a plastic holder with a 2-cm diameter window. Typical deployment times for DGT are 24-72 hours. After exposure the Chelex resin is removed and placed in a known volume of dilute nitric acid and the acid extract is subsequently analyzed for trace metals by ICP-MS. The advantage of the resin embedded in the gel is that it constrains mass transport, making it independent of the hydrodynamics in solution above a level of convection. Thus, it is only controlled by molecular diffusion.

In waters the flux of metal ions through the gel can be related to the bulk solution concentration by the relationship

$$M/At = DC/\Delta g$$

where M is the accumulated mass, A the Area, t the exposure time, C the concentration in the bulk solution, D the diffusion coefficient in the gel, and Δg the gel thickness. The knowledge of the diffusion coefficient of the migrating species in the particular diffusive gel is necessary for calibration of the gel.

DGT can be deployed in-situ or in the laboratory and it can measure several elements at the same time (Zhang and Davison, 1995). By manipulation of the gel composition, a “restricted” gel can be prepared that is able to retard larger metal-humic complexes compared to metal ions. When using the results of two gels having different pore sizes, it is possible to discriminate between labile complexes on the basis of size. Small species are considered being labile

inorganic and larger species being labile organic. By varying the gel layer thickness, labile complexes can be separated by their dissociation rate in the diffusive gel (Sally et al., 2003).

Permeation Liquid Membranes (PLM)

This method is based on diffusive transport of the analyte across a hydrophobic liquid membrane positioned between the sample and a small volume of receiving (or stripping) solution (Parthasarathy et al., 1997; 2003; 2001; Salaun and Buffle, 2004; Tomaszewski et al., 2003).

The membrane consists of a water-insoluble organic solvent containing an organic carrier molecule that is selective for the metal of interest and held in the pores of a hydrophobic chemically inert membrane. The measured signal is the flow of metal through the PLM, which is maintained by a chemical gradient and it is speciation dependent as it is based on the competition between the carrier and the free or labile metal species. Lipophilic species may also cross the membrane and contribute to the total flux. Depending on the specific characteristics of the PLM either the free metal ion or the labile metal species are measured (Tomaszewski et al., 2003).

Hollow Fibre PLM

In this further development of the PLM, a hydrophobic polypropylene hollow fibre membrane is used (Tomaszewski et al., 2003). It is impregnated with the strip solution by slowly running this solution outside the fibre. The strip (acceptor) solution is filled inside the HFPLM and the two ends of the fibre are connected to form a loop which is attached to a Plexiglas frame hung inside the test water body and is largely open to flow. After deployment the strip solution is removed with a syringe and collected for subsequent analysis by ICP-MS.

Donnan Membrane Technique (DMT)

This technique is based on Donnan equilibrium between the free metal ion concentration in the sample and an acceptor solution separated by a hydrophilic negatively charged ion-exchange membrane (Kalis et al., 2006; Temminghoff et al., 2000; Weng et al., 2005). Cations are transported across the membrane driven by the negative electrostatic potential (the Donnan potential) across the membrane, until equilibrium is achieved. Matching of the ionic strength of the donor and receptor solution is necessary. Since the cationic species exchange faster than neutral and anionic species, it is assumed that the measurement is more closely related to the free metal ion.

For in-situ measurements a DMT cell designed for field application is used. The lab DMT cell consists of two chambers, a donor and an acceptor side, the field cell has one chamber which is separated from the donor solution on two sides (i.e. lake, or river water) by the negatively charged membrane. Before deployment the membranes are washed repeatedly with 0.1 M HNO₃, 1M Ca(NO₃)₂, and the background solution of the acceptor side. The acceptor side contains Ca(NO₃)₂ with an ionic strength approximately equal to the ionic strength of the surface water and 30 mg dm⁻³ purified humic acid to accumulate metal ions and to increase the limit of detection for the free metal ions (Kalis et al., 2006). After deployment of the cells for 2 or 4 days samples are taken from the surface water and the acceptor solution of the DMT and metals are subsequently measured by ICP-MS. The calculation of the free metal ion concentrations is calculated based on the Donnan membrane equilibrium or ion transport kinetics.

Competitive Ligand Exchange Stripping Voltammetry (CLE-SV)

The principle of anodic stripping voltammetry (ASV) is based on accumulating the metal on a mercury drop suspended from an electrode for a given time at a given potential. After the deposition step at the electrode surface, the potential is reversed and the current signal is interpreted as concentration of the labile metal. The labile metal complexes are accumulated during the accumulation step.

A competing ligand with known complexation properties (complex stoichiometry and stability constants) and known concentration is added to a water sample. A new equilibrium between the original metal species and the complex with the competing ligand is established. The concentration of the complex with the competing ligand is measured specifically as labile complexes by ASV. By titration of a water sample with a metal, complexation parameters are determined and the free ion concentration is obtained by equilibrium calculations and can be extrapolated for the original sample. Competitive ligand exchange followed by voltammetric measurements can be applied to metal speciation at ambient levels in sea- and freshwaters (Bruland, 1992; Donat and van den Berg, 1992; 1986; Xue and Sigg, 2002).

Cadmium can thus be determined by adding ethylenediamine (EN) as ligand and ASV measurement of the Cd-EN complex (Xue and Sigg, 1998; 2002), and Zn using EDTA as complexing ligand (Xue and Sigg, 1994). Recent work has highlighted that kinetic limitations may affect some of the results obtained by CLE-SV (van Leeuwen and Town, 2005; van Leeuwen et al., 2005).

Stripping Chronopotentiometry (SCP)

Like ASV stripping chronopotentiometry is a two step technique. It was developed originally by Jagner et al. as Potentiometric Stripping Analysis (PSA) (Jagner, 1978; Jagner and Graneli, 1976) and was further developed by Town

and van Leeuwen (Town and van Leeuwen, 2002; 2004a; van Leeuwen and Town, 2003). It relies on measurements of the reoxidation time of the metal deposited by electro-deposition on an electrode (usually coated with mercury, similar to ASV).

The deposition step is identical to stripping voltammetry. Reoxidation of the accumulated metal is achieved by application of a constant oxidizing current, thus the sample, as in ASV, has to be oxygen free (Town, 2001; Town and van Leeuwen, 2001; 2004b). The analytical signal is the time which is required for reoxidation of the metal from the surface of the electrode (Town and van Leeuwen, 2001). The measurements of labile metal species are similar to GIME if the same microelectrode is used.

2.3 Thermodynamic modelling

Thermodynamic calculations are based on the equilibrium between metals and potential ligands if the concentrations and the stability constants of the different species involved are known (Sigg and Xue, 1994, Martell and Smith, 1976-1989). This approach might lead to problems in its application to natural waters, as an important part of the ligands is usually not defined and is present as fulvic and humic acids or other unknown ligands. Several equilibrium calculation programmes now include models for fulvic and humic acids. Thermodynamic calculations are usually performed with modelling software programs such as visual MINTEQ 2.32 (Gustafsson, 2005) which incorporates the Stockholm humic acid model (SHM) (Gustafsson, 2001), the Windermere Humic Acid Model (WHAM) (Tipping, 1994) with the ModelVI (Tipping, 1994; Tipping and Hurley, 1992; Tipping et al., 2002), or the nonideal competitive adsorption model (NICA-Donnan model) incorporated in the ECOSAT code, (Wageningen University, The Netherlands) (Benedetti et al., 1995).

The SHM model is based on a discrete site approach where 8 binding sites of different acid strength are considered. Cations may form monodentate and bidentate complexes. The bulk of humic substances are considered to form gels and the model takes into account the electrostatic effects. Surface interactions are modelled using the Basic Stern Model with the possibility for some humic molecules to have groups outside the gel. Model VI takes into account the complexation of metals by humic and fulvic substances. This model assumes a discrete site distribution with an average and distribution of complexation constants. Electrostatic effects are corrected by terms based on the Debye-Hückel and Gouy-Chapman theories. The NICA-Donnan model is based on the NICA equation, an isotherm for the adsorption of multicomponents to heterogeneous surfaces (Koopal et al., 2005). The binding site heterogeneity is characterized by the use of a distribution equilibrium constants, representing carboxylic (strong) and phenolic (weaker) binding sites. The Donnan approach considers the humic substances as an electrically neutral “gel phase” (Kinniburgh et al., 1996).

2.4 Speciation and uptake

It is generally assumed that it is the free metal ion which is available for uptake up by aquatic organisms (FIAM or BLM model). However, exceptions have been and are reported (Errécalde and Campbell, 2000; Meylan, 2003).

Chemical speciation techniques and speciation models detect and predict the different forms of metals in the aquatic system but up to now they can not give direct quantitative data on adverse biological effects. Bioassays or toxicity tests are generic tests that use living organisms as indicators of contaminants in aquatic systems. Thus, it is of interest to be able (by a suite of analytical techniques) to measure different fractions of the metal in the bulk solution. The

speciation techniques described in this chapter measure either the free or the labile part of metals. In this case the term labile applies to metal complexes which can dissociate within the diffusion layer of the membrane or electrode used. Theoretical considerations stressed the need of investigating conditions where labile species are of importance as diffusion limitation (and therefore depletion of the metal on the sensor surface) can occur and labile species might contribute towards the free metal concentration (Slaveykova and Wilkinson, 2005; van Leeuwen and Jansen, 2005; van Leeuwen and Town, 2005; van Leeuwen et al., 2005). However, labile metal species are not *necessarily* bioavailable. Rather, they are *potentially* bioavailable, depending on the magnitude of the metal flux through the plasma membrane. The larger this flux, the larger will be the fraction of dynamic species consumed. Therefore, the notion of bioavailability is of an organism-specific nature, and meaningless if the organism is not specified (van Leeuwen et al., 2005). If the metal flux is inhibited by some external factor, the speciation methods deployed will *overestimate* the Cd bioavailability. In chapter 5 this is demonstrated for Cd accumulation in softwaters under conditions of high DOC and iron concentrations. Physico-chemical factors influencing metal uptake should therefore also be taken into account in some cases. In summary the combination of bioassays with various metal speciation techniques will provide the best assessment of the bioavailable fraction of metals under the given conditions.

*Competition among Cadmium, Zinc and
Manganese uptake in the freshwater alga
Scenedesmus vacuolatus*

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3.1 Abstract

In this work Zn and Mn competition with Cd uptake was investigated in the freshwater alga *Scenedesmus vacuolatus*. *S. vacuolatus* was exposed to experimental media with Cd and either Zn or Mn in short-term experiments (one hour). Long term experiments were undertaken to investigate the effect of growth on Cd accumulation. Cd is accumulated into *S. vacuolatus* from very low levels of free Cd in relation to the free Cd^{2+} . Cd accumulated in *S. vacuolatus* between 5×10^{-20} to 6×10^{-17} mol/cell in the range of free Cd^{2+} from 1×10^{-14} to 1×10^{-9} M. Zn was found to be an effective competitive inhibitor of Cd uptake with cellular Cd contents decreasing from a $\text{Zn}^{2+}/\text{Cd}^{2+}$ ratio of 14, whereas Mn did not compete with Cd for uptake below a $\text{Mn}^{2+}/\text{Cd}^{2+}$ ratio > 10000 . Binding constants for Cd and Zn affinity to the transport sites were determined. K_{Zn} values of 1.7×10^9 to 6.8×10^9 were higher than values for K_{Cd} (3.1×10^8 to 6.0×10^9) stressing the fact that Zn is essential for the cells. In contrast, Cd seems not to compete with the Mn binding sites for uptake. Determined values for the binding constants for Zn and Cd show that a simple model can be applied to predict Cd uptake at known Zn and Cd concentrations. The environmental implications of these results are discussed with respect to potential Cd toxicity for aquatic organisms.

3.2 Introduction

Metals are of environmental interest both as limiting nutrients and as toxicants. Some metals, like zinc and manganese, are important micronutrients for algae and other organisms, but can have toxic effects at elevated concentrations (Campbell, 1995). Anthropogenic input of metals to natural systems enhances the likeliness of toxic effects of those metals for aquatic organisms. Also, non-essential metals such as cadmium can enter aquatic systems from various sources and cause risks for the algae. Toxic metals interact for example with essential functional groups, by displacing or substituting essential ions, or modifying conformational structures of biomolecules (Mason and Jenkins, 1995). Exceptions from this scheme include Cd showing beneficial effects to the Zn-limited and growth-inhibited marine diatom *Thalassiosira weissflogii*. Here, growth recovery was observed based upon the formation of a specific Cd-carbonic anhydrase, a Zn-dependent enzyme involved in carbon acquisition (Lee and Morel, 1995).

Interaction between algae and metals involve diffusion of the metal from the bulk solution to the biological surface, binding of the metal ion on the algae surface and intracellular uptake (Campbell et al., 2002). Transport across the cell membrane and internalization is usually considered to be the limiting step for metal uptake (Bates et al., 1982; Knauer et al., 1997; Sunda and Guillard, 1976). Experiments with algae in chemically controlled media have shown that uptake, growth and toxicity are most often a function of the free metal ion, according to the biotic ligand model (BLM) which is a derivative of the free metal ion activity model (FIAM) (Anderson et al., 1978; Batley et al., 2004; Brand et al., 1986; Campbell et al., 2002; Morel and Hering, 1993; Paquin et al., 2002; Sunda and Huntsman, 1992). This model states that the influence of a metal on

organisms in aqueous media is governed by the availability of the free metal ion for the organism.

For Cd, uptake has been mostly demonstrated to depend on free Cd^{2+} (Kola and Wilkinson, 2005; Sunda and Huntsman, 2000; Vigneault and Campbell, 2005), albeit some exceptions have been observed (Campbell, 1995; Errécalde and Campbell, 2000; Errécalde et al., 1998; Phinney and Bruland, 1994). Competition of Cd mainly with Zn and Mn for uptake has been observed especially in a number of marine algal species (Campbell, 1995; Sunda and Huntsman, 2000).

Typically, Cd is present at unpolluted natural freshwater sites with a total dissolved concentration of less than 0.1 nM (Xue and Sigg, 1998). Usually, other metals such as Zn, Cu and Mn are present in higher concentrations than Cd (Xue et al., 1996; 1994). It is therefore hypothesized that Cd uptake is suppressed in presence of these ions and Cd accumulation does not reach levels where it interferes with cell metabolism. However, at elevated concentrations, Cd can outcompete other cations and pose a potential risk for the organism due to an enhanced accumulation.

In this paper we investigate Cd accumulation in the freshwater green alga *Scenedesmus vacuolatus* in competition to two essential metals, Zn and Mn. Cd accumulation in *S. vacuolatus* is evaluated under varying Zn or Mn concentrations. Short term (one hour) and long term (several days) experiments are compared to assess the differences in accumulation within different time frames. From short term experiments the uptake of metals in *S. vacuolatus* without changing the speciation of Cd, Zn and Mn in the medium are assessed, and from long term experiments the influence of algal growth on Cd accumulation can be evaluated. These results provide insights into the uptake mechanism of cadmium.

3.3 Materials and methods

3.3.1 Algae cultures

Isolates of the unicellular green alga *Scenedesmus vacuolatus* 211-8b (Chlorophyceae) were obtained from the culture collection of the Institute for Plant Physiology of the University of Göttingen, Germany. Prior to each experiment the algae were acclimatized to the culture medium by successive subculturing. Cultures were prepared by transferring an inoculum of algae in exponential growth phase into fresh culture medium. Algae were cultured in glass erlenmeyers in a HT Infors shaker (Infors, Bottmingen, Switzerland) at 90 rpm and 25°C under continuous light exposure of $276 \mu\text{Em}^{-2}\text{s}^{-1}$ provided by cool white fluorescent lamps. Acclimatization was assumed to be complete once the growth rates of successive cultures were constant. Experimental batch cultures were prepared by transferring an inoculum of algae into the experimental medium (described below) with a starting population density of 8×10^5 cells/ml.

3.3.2 Chemicals

Metal solutions and major nutrient solutions were prepared with salts from Fluka (puriss. grade; Buchs, Switzerland). Ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA), 3-morpholinopropanesulfonic acid (MOPS) were puriss. grade from Fluka, nitric acid (HNO_3 ; 65%), hydrochloric acid (HCl ; 30%) hydrogen peroxide (H_2O_2 ; 30%) were suprapure® chemicals from Merck (Darmstadt, Germany). Sodium hydroxide (NaOH ; 50%) used for medium preparation was from Baker Analysed® (JT Baker; Deventer; Netherlands). Cd

addition to the experimental media was applied from standard Cd ICP-MS stock solutions (J.T.Baker; Deventer, NL).

All glassware and polycarbonate containers were presoaked in 0.01 M HNO₃ and rinsed with deionized nanopure water (18 MΩ Q-H₂O grade, Barnstead Nanopure, Allschwil, Switzerland) before utilization. Erlenmeyers and glassware used for media preparation or experiments were autoclaved before use to avoid microbial contamination.

3.3.3 Preparation of culture and experimental medium

Culture medium was prepared by addition of a concentrated EDTA-trace metal solution (1000-fold) to a solution containing major nutrients and major freshwater cations and anions. The EDTA-trace metal solution was prepared separately by addition of sterile-filtered (0.22 μm) metal stock solutions in 0.01 M HCl (Co, Mo, Cu, Mn, Zn) to an Fe-EDTA solution to ensure complete complexation (Price et al., 1988). The free metal ion concentrations were buffered by EDTA (total final concentration in medium 2×10^{-5} M). The nutrient solution was buffered to pH 7.45 with 1×10^{-2} M of MOPS and sterilized by autoclaving (all but for the carbonate solution which was passed through a 0.22 μm filter). The final EDTA-trace metal solution was added to the major ion nutrient solution resulting in the following total concentrations in the growth medium: 5×10^{-4} M CaCl₂·2H₂O, 1.5×10^{-4} M MgSO₄·7H₂O, 1.2×10^{-3} M NaHCO₃, 5×10^{-5} M K₂HPO₄·3H₂O, and 1×10^{-3} M NaNO₃, 5×10^{-8} M Co(II), 5×10^{-5} M H₃BO₃, 8×10^{-8} M MoO₄²⁻, 1×10^{-7} M Cu(II), 1×10^{-6} M Mn(II), 1.25×10^{-7} M Zn(II), 9×10^{-7} M Fe(III). The free concentrations of Cu, Mn, Zn, and Fe were 3×10^{-14} M, 1.7×10^{-8} M, 9×10^{-12} M, and 1×10^{-20} M, respectively.

In experiments where EDTA was substituted by NTA as the buffering ligand, the free concentrations of Cd, Zn and Cu were kept as in the EDTA culture medium by adjusting their total concentrations.

In experimental short term media, the trace metal solution was replaced by addition of EDTA, Cd, and Zn or Mn only. Total metal concentrations were varied to adjust the free Cd^{2+} , Zn^{2+} , and Mn^{2+} concentration to obtain the required metal concentrations according to the experimental setup.

Media were prepared at least 24h in advance to allow for complete equilibration. The ratios of the free metal ions evaluated were varied from 1 to 80 for the Zn/Cd experiments and from 3 to 50000 for the Mn/Cd experiments, respectively. Where Cd was varied, the free concentration ratios were varied from 0.03 to 100 for the Cd/Zn, and from 0.01 to 225 for the Cd/Mn experiments. In the control medium (no added Cd) the total Cd concentration due to contamination from the chemicals used was 0.1×10^{-9} M corresponding to a pCd ($-\log [\text{Cd}^{2+}] = \text{pCd}$) ≈ 13.7 .

Total metal concentrations were measured in all media by inductively coupled plasma mass spectrometry (ICP-MS; Element2; Thermo Finnigan, Bremen, Germany) before and after the experiments, and free metal concentrations were subsequently computed using the software VMINTEQ (Gustafsson, 2005).

3.3.4 Experimental methods

Competition of Cd uptake by Zn and Mn was examined in short term experiments by exposing aliquots of algae for one hour to experimental media containing varying Cd and Zn or Mn concentrations.

For uptake experiments, exponentially growing algae were separated from the culture medium by centrifugation for 10 minutes at 2300 g at 4°C and resuspended in medium without trace metals. For long term experiments (72

hours) algae were grown in the experimental media with pCd 14 to 9. In a separate experiment, EDTA was substituted by NTA to investigate the influence of a weak ligand on the accumulation of Cd. Free Cd, Cu and Zn were kept at the same level as in the culture medium by varying the corresponding total metal concentrations.

To determine metals in the algae three aliquots from each experimental culture were filtered on acid washed filters (cellulose nitrate; 0.45µm; Sartorius, Göttingen, Germany). To differentiate in long term experiments between adsorbed extracellular metal and intracellular accumulation, algal cells were washed by adding 4 mM EDTA, pH 8, for 10 minutes to the culture medium. The intracellular concentration was defined as the cellular metal determined after the EDTA wash and adsorbed metal as the fraction being removed through the EDTA wash. Adsorbed Cd was found not to exceed 10 % of the total Cd accumulated (data not shown).

The filters were digested in Teflon® flasks in a high performance microwave digestion unit (mls 1200 mega; Microwave Laboratory Systems, Oberwil, Switzerland) by addition of 4 mL HNO₃ and 1mL H₂O₂ for 15 minutes followed by transferring the solutions into 25-mL graduated flasks. Total metal concentrations in digested solutions were determined by ICP-MS using rhodium as an internal standard. The digestion procedure was checked by the analysis of plankton reference material (error < 15 %; CRM 414, Community Bureau of Reference, Commission of the European communities, Brussels, Belgium), ICP-MS measurements were checked using SLRS-4 reference water (River Water Reference Material for Trace Metals; National Research Council Canada, Ottawa, error < 10 %).

A 1 mL sample of the algae suspension was taken to determine cell size and cell numbers (Coulter Multisizer II, 50 µm orifice) and 5 mL were taken for chlorophyll a and b determination. Chlorophyll was determined by the rapid HPLC method according to Murray et al. (1986) after extraction in 90% ethanol.

Additionally, in the long term experiments algal dry weight was determined after the experiment by weighing the filters and normalizing to cell units.

3.3.5 Calculation of metal binding parameters

According to the free ion activity model (FIAM), the Cd concentration in a unicellular organism can be described as

$$\{Cd_{int}\} = k \cdot \{X - Cd\} \quad (3.1)$$

where $\{Cd_{int}\}$ is the accumulated Cd (mol/cell), $\{X - Cd\}$ represents the concentration of transport sites that are occupied by Cd and k is a proportionality constant. The reactions of the transport sites with Cd and other metals can be described as (pseudo)equilibrium constants (Croteau et al., 1998; Hare and Tessier, 1996):

$$Cd^{2+} + X \rightleftharpoons X - Cd \quad K_{Cd} = \frac{\{X - Cd\}}{[Cd^{2+}] \cdot \{X\}} \quad (3.2)$$

$$M^{2+} + X \rightleftharpoons X - M ; \quad K_M = \frac{\{X - M\}}{[M^{2+}] \cdot \{X\}} \quad (3.3)$$

where X and X-M are the concentration of free transport sites and those occupied by the competitive metal respectively and {} indicates mol/cell. The metal competing with Cd is Zn or Mn respectively. The mass balance for the sites is

$$\{X_t\} = \{X\} + \{X - Cd\} + \{X - M\} \text{ (mol/cell)}$$

For competition with Zn, combining equation (3.1)-(3.3) leads to

$$\{Cd_{int}\} = kK_{Cd}\{X_T\} \cdot \frac{[Cd^{2+}]}{1 + K_{Cd}[Cd^{2+}] + K_{Zn}[Zn^{2+}]} \quad (3.4)$$

where K_{Cd} and K_{Zn} represent the affinity of the transport sites for the respective metal, and $\{X_t\}$ the total sites available.

For $M = Zn^{2+} > Cd^{2+}$, the term $K_{Cd}[Cd^{2+}]$ can be neglected in equation (3.4) and Cd_{int} can be expressed as a function of K_{Cd} , K_{Zn} , and the available transport sites X_t .

$$\frac{[Cd^{2+}]}{\{Cd_{int}\}} = \frac{1}{kK_{Cd}\{X_T\}} + \frac{K_{Zn}}{kK_{Cd}\{X_T\}} \cdot [Zn^{2+}] \quad (3.5)$$

$kK_{Cd}\{X_t\}$ and K_{Zn} are calculated from a plot of $[Cd^{2+}]/\{Cd_{int}\}$ versus $[Zn^{2+}]$

From the ratio $\{Cd_{int}\} / \{Zn_{int}\}$ versus Cd^{2+}/Zn^{2+}

$$\frac{\{Cd_{int}\}}{\{Zn_{int}\}} = \frac{kK_{Cd} \cdot [Cd^{2+}]}{kK_{Zn} \cdot [Zn^{2+}]} \quad (3.6)$$

the ratio K_{Cd}/K_{Zn} and K_{Cd} can be derived.

For $M = Cd^{2+} > Zn^{2+}$ the same equations were used and in eq (3.4) the term $K_{Zn}[Zn^{2+}]$ was subsequently neglected.

3.4 Results

In short term experiments the accumulation of metals in *S. vacuolatus* was assessed without depletion of the free metal concentration in the medium, and without changing the speciation in the medium due to potential ligand release of the algae. In long term experiments the accumulation of Cd was investigated in relation to algal growth.

3.4.1 Competition between cadmium and zinc

Short term Cd accumulation in *S. vacuolatus* has been examined by varying pCd from 13.8 to 9 and keeping the Zn concentration at pZn 11.4, resulting in $\text{Cd}^{2+}/\text{Zn}^{2+}$ ratios ranging from 0.003 to 100. Within the investigated low concentration range of Cd (pCd 13.8 to 13), constant Cd content of 5.4×10^{-20} mol/cell was observed (Figure 3.1). From pCd 10.6 on (corresponding to a ratio of free $\text{Cd}^{2+}/\text{Zn}^{2+}$ of 20), Cd accumulation increased 100 times to 5×10^{-18} mol/cell followed by further increase up to 1000 times to 6.2×10^{-17} mol/cell at pCd 9. Constant Zn contents of about 4.45×10^{-18} mol/cell were observed over the range of pCd investigated (Figure 3.1).

Figure 3.1: Cd (open squares) and Zn content (filled squares) in *S. vacuolatus* as a function of the free Cd^{2+} (as pCd) at constant pZn of 11.4 in short term experiments. Error bars represent the standard deviations of 6 replicate cultures (n= 6)

Competitive short term interactions of Zn on Cd uptake were examined by keeping pCd at ~ 10.6 and varying the Zn concentration from pZn 11.4 to 9 (figure 3.2), thus varying the $\text{Zn}^{2+}/\text{Cd}^{2+}$ ratio from 1 to 80. Within the range of pZn 10.6 to 9.7, Cd content was constant and similar to data obtained at pCd 10.6 in figure 1. At pZn higher than 9.7, Cd accumulation decreased by a factor of 6 from 6×10^{-18} to 1×10^{-18} mol/cell (figure 3.2).

Figure 3.2: Zn (full squares) and Cd content (open squares) as a function of pZn and at constant pCd of 10.6 in short term experiments (n=6). The low Cd accumulation values at pZn 11.2 to 11.4 account for the control medium where no Cd was added to the media

The competition of Zn on Cd accumulation is observable from a $\text{Zn}^{2+}/\text{Cd}^{2+}$ ratio of 14 (table 3.1)

At low pZn from 11.4 to 9.7, Zn content in the cells was observed to be constant at 1.7×10^{-17} mol/cell. From pZn 9.7 to 9, Zn accumulation increased by factor 5.8 from 1.7×10^{-17} to 1×10^{-16} mol/cell.

In long term experiments intracellular Cd and Zn accumulation was investigated in two different media with EDTA or NTA as ligand to control the free metal concentrations. Cd accumulation has been assessed in the media by varying pCd from 14 to 9 and keeping the Zn concentration at pZn 11. Cd accumulation in *S. vacuolatus* increased linearly 1400-fold from 2.5×10^{-20} to 3.6×10^{-17} mol/cell (figure 3.3) with increasing pCd both in EDTA and NTA medium, in agreement with FIAM. Concomitantly, Zn contents decreased by factor 4 from 1.2×10^{-17} to 3×10^{-18} mol/cell. The $\text{Cd}^{2+}/\text{Zn}^{2+}$ ratios were varied from 0.002 to 46. In both culture media with either EDTA or NTA increasing Cd accumulation with simultaneously decreasing Zn accumulation was observed.

Table 3.1. Inhibition of Cd accumulation at pCd 10.5 (for Zn) or 10.6 (for Mn) in function of pZn or pMn and the ratio of free metal ion concentrations

pZn	$\text{Zn}^{2+}/\text{Cd}^{2+}$	Cd (mol/cell)	pMn	$\text{Mn}^{2+}/\text{Cd}^{2+}$	Cd (mol/cell)
10.7	1	6.9×10^{-18}	9.9	3.1	5×10^{-18}
10.4	2	4.7×10^{-18}	9.6	7.8	3.6×10^{-18}
10.2	3	4.2×10^{-18}	9.4	12.1	3.9×10^{-18}
9.9	5	4.3×10^{-18}	9.3	16.2	4.7×10^{-18}
9.8	6	6.1×10^{-18}	8.8	50.4	4.6×10^{-18}
9.4	14	5.2×10^{-18}	8.7	62.1	4.5×10^{-18}
			7.8	505	4.8×10^{-18}
average Cd content		5.2×10^{-18}			4.5×10^{-18}
standard deviation		1.9×10^{-18}			4.9×10^{-19}
9.1	40	2.8×10^{-18}	6.7	11370	2.9×10^{-18}
9	81	1×10^{-18}	6.7	11103	2.3×10^{-18}
			5.6	48686	1.8×10^{-18}
			5.5	50687	1.2×10^{-18}

Figure 3.3: Zn (full symbols) and Cd (open symbols) content as a function of varying pCd and constant pZn of 11 in long term experiments with EDTA (squares) and NTA (triangles) as a ligand (n=9).

3.4.2 Competition of cadmium and manganese

Effects of Mn on Cd accumulation were evaluated in short term experiments by varying the Mn concentration from pMn 10 to 5.5 and keeping pCd constant at 10.5, thus varying the ratio of $\text{Mn}^{2+}/\text{Cd}^{2+}$ from 3 to 50000 (figure 3.4).

Figure 3.4: Contents of Mn (filled squares) and Cd (open squares) as a function of varying pMn and at constant pCd 10.5 in short term experiments (n=6).

Within the range of pMn 10 to 7.6, Cd accumulation was observed to be constant at 5×10^{-18} mol/cell. At a pMn higher than 7.6, which corresponds to a $\text{Mn}^{2+}/\text{Cd}^{2+}$ ratio of 10000, Cd accumulation decreased from 5×10^{-18} to 1.2×10^{-18} mol/cell. Over the range of free Mn^{2+} investigated, this corresponds to a 4 fold decrease in Cd accumulation within an increase of the free Mn^{2+} by factor 25000. (table 3.1).

Constant Mn content in the cells was observed from pMn 10 to 8, followed by a gradual increase by factor 17 from 9.4×10^{-18} to 1.6×10^{-16} mol/cell from pMn 8 to 5.5.

Competition of manganese uptake by cadmium was also examined in short term experiments. Cd was varied from pCd 13.6 to 6 and Mn kept constant at pMn 9 corresponding to a $\text{Cd}^{2+}/\text{Mn}^{2+}$ ratio of 4×10^{-5} to 226. Within the range of pCd 13.7 to 12 constant Cd accumulation of 5×10^{-19} mol/cell was observed, from pCd 11.5 Cd starts being accumulated from 2.3×10^{-20} up to 4.3×10^{-17} mol/cell (figure 3.5) at pCd 9. At pCd 9, Cd accumulation reached a plateau of 5.9×10^{-17} and did not increase further.

Figure 3.5: Mn (filled squares) and Cd (open squares) contents as function of varying pCd and at constant pMn of 8.0 in short term experiments (n=6).

Mn accumulation remained constant from pCd 13.7 to 8 at 4×10^{-18} mol/cell, above pCd 8 the Mn accumulation decreased from 4×10^{-18} to 7.3×10^{-19} mol/cell.

In the long term experiments constant Mn accumulation was observed at 1.3×10^{-17} mol/cell in the EDTA and 1.8×10^{-17} mol/cell in the NTA medium over the range of pCd investigated. The difference in Mn accumulation by factor 1.4 between the EDTA and NTA medium can be attributed to the differences in free Mn concentration which varied by a factor of 1.7 (data not shown).

3.4.3 Calculation of metal binding parameters

Binding parameters obtained by short term competition experiments shown in figure 3.2 were evaluated according to equation (3.5) to determine the constants for the transport sites of Cd and Zn, K_{Cd} and K_{Zn} (table 3.2).

Table 3.2: Calculated K_{Zn} , K_{Cd} and kX_t values for Zn/Cd short term competition and long term experiments

Experiment	pCd	pZn	Zn_{tot} (M)	K_{Cd}	K_{Zn}	$k \cdot X_t$ (mol/cell)	K_{Cd}/K_{Zn}
Zn/Cd 1h	10.5	11.5 - 9	$6 \times 10^{-8} - 1 \times 10^{-7}$	3.1×10^8	1.7×10^9	4.8×10^{-16}	0.32
EDTA 3days	14 - 9	11.0	1.2×10^{-7}	6.0×10^9	6.8×10^9	1.5×10^{-16}	0.88
NTA 3 days	14 - 9	10.9	1.15×10^{-7}	1.5×10^9	2.4×10^9	2.1×10^{-16}	0.62

A good relation between increasing free Zn^{2+} in the media and the ratio of Cd^{2+}/Cd_{int} was observed ($r^2 = 0.78$, $p < 0.05$) (figure 3.6), as well as a good correlation of Cd^{2+}/Zn^{2+} to Cd_{int}/Zn_{int} ($r^2 = 0.93$, $p < 0.05$) (figure 3.7).

Figure 3.6: Correlation of Cd^{2+}/Cd_{int} to free Zn^{2+} in short term Zn/Cd experiments, ($r^2 = 0.78$, $p < 0.05$) $n=3$

Generally, values for K_{Zn} were higher than values obtained for K_{Cd} with K_{Zn} ranging from 1.7×10^9 to 6.8×10^9 and K_{Cd} from 3.1×10^8 to 6.0×10^9 . In long term experiments values for K_{Cd} were up to one order of magnitude higher (6×10^9

compared to 3.1×10^8) than in the short term experiments. Values calculated for $k \times X_t$ in table 3.2 represent the maximum of total transport sites available for the metals. The results indicate that it is possible to describe the Cd and Zn competition with a simple 1:1 binding model.

Figure 3.7: Correlation of Cd_{int}/Zn_{int} to Cd^{2+}/Zn^{2+} in short term Cd competition experiments, ($r^2 = 0.93$, $p < 0.05$) $n=3$

As Mn/Cd interaction was less pronounced, relevant uptake parameters for Mn could not be calculated. The Mn/Cd competition cannot be treated according to the simple scheme used for calculation of Zn/Cd binding parameters, probably also due to a small number of data with competition effects.

3.5 Discussion

Cd is known to compete for Zn binding sites at the algal surface due to similar chemical properties (charge, coordination, and ligand preferences) (Maeda et al., 1990; Sunda and Huntsman, 1996). This competition effect is known to lead to an uptake of the non-essential cation Cd (Campbell et al., 2002; Errécalde and

Campbell, 2000). Competition of Cd for Zn or Mn uptake has been demonstrated in a marine diatom (Sunda and Huntsman, 1996; 2000; 2005). In the present study we have examined the competition of Zn or Mn on Cd accumulation under short term and long term conditions in the freshwater alga *S. vacuolatus*. Competition experiments with Zn indicate that Cd enters the cells mainly through the Zn transport system.

3.5.1 Competition between Cd and Zn

Short term experiments with Cd were carried out keeping the concentrations of Zn^{2+} in the experimental medium constant and similar to the Zn^{2+} concentration in the culture medium. Thus the Zn content in the algae was at steady state and is not expected to increase further during the 1 hour exposure in experimental medium, or to decrease upon Cd addition. Accordingly, with increasing pCd in short term competition experiments, cellular Zn content remains constant, and Cd contents increase linearly from pCd 11 to 9.

Zn was found to be very effective in inhibiting Cd uptake when varying the free Zn^{2+} concentration in the experimental media. Competitive effects of Zn on Cd uptake were evident at a $\text{Zn}^{2+}/\text{Cd}^{2+}$ ratio > 14 . Below this ratio the cellular Cd contents are constant, above this ratio Zn competes with Cd which results in decreasing Cd and increasing cellular Zn contents.

In long term competition experiments, Cd did compete with Zn for uptake. When the algae divide, the Zn contents did not increase due to competition with Cd and even decreased since it is biodiluted while Cd is accumulated in the cells during growth (Sunda and Huntsman, 2005). Cd contents are similar to cellular concentrations obtained in short term experiments indicating that the short term experiments represent steady state conditions.

Cd accumulated slightly (about 4×10^{-20} mol/cell) under conditions of Zn excess at very low free Cd^{2+} ($\text{pCd} = 14$) concentrations ($\text{Zn}^{2+}/\text{Cd}^{2+} > 100$). The accumulation indicates that Cd might be also transported through a different transport system than the one for Zn. This hypothesis is supported by modelling Cd contents at low free Cd^{2+} using the parameters obtained for K_{Zn} and K_{Cd} . Calculated Cd contents are lower than the experimentally measured Cd values (data not shown).

Several studies have established competition between Cd and Zn for uptake and enzymatic binding. Kola and Wilkinson (2005) observed a 3-fold reduction in intracellular Cd at 50-fold excess of Zn in *C. reinhardtii*. Ting et al. (1991) showed that, for *C. vulgaris* equimolar concentrations of Cd and Zn the presence of the second metal did not affect the binding of the other. However, they also observed inhibition of Zn uptake in the presence of Cd in long term experiments. Hassler et al. (2004) described decreasing Zn uptake fluxes by factor 4 at a Cd/Zn ratio of 10 and by factor 320 at a Cd/Zn ratio of 200 in *C. reinhardtii* in short term experiments. Zn uptake regulation due to two affinity systems was described by Sunda and Huntsman (1992; 1998b) for some marine algal species. A low affinity system provides Zn supply with little dependence on the actual cell status, whereas the high affinity system is under negative feedback control, and increases at low total Zn concentrations. Under conditions of low Zn concentrations, Cd uptake occurs simultaneously with Zn by the high Zn affinity system. At elevated dissolved Zn concentrations, Cd uptake is suppressed as Zn is present in excess and Zn is taken up by the low affinity system.

The good correlation between the free $\text{Cd}^{2+}/\text{Zn}^{2+}$ ratio and the content ratio $\text{Cd}_{\text{int}}/\text{Zn}_{\text{int}}$ indicates that a simple competition model can be applied. Overall higher values for K_{Zn} compared to K_{Cd} were found, in agreement with Zn as an essential element being more important for the cell and therefore taken up

preferentially. The data for K_{Zn} and K_{Cd} obtained indicate that the interaction of Zn and Cd on the cell surface can be described by the simple binding model used.

The determined K_{Cd} values in this work (3×10^8 - 9×10^9) are several orders of magnitude higher than the values determined for *C. reinhardtii* (10^6 to $10^{6.7}$) by Kola and Wilkinson (2005), however experimental procedures differed, namely with the free Cd^{2+} concentration applied, and pH. The determined K_{Cd} value of 3.1×10^8 derived from short term experiments is in good agreement with the affinity constant of transport sites of 1.5×10^8 found by Vigneault and Campbell (2005) for *P. suscapitata*. Also, determined K_{Zn} values are within the range of K_{Zn} ($10^{7.5}$ and $10^{9.8}$) found for some oceanic species in marine waters (Sunda and Huntsman, 1992; 2000).

3.5.2 Competition between Cd and Mn

In agreement with experiments evaluating the interaction of Cd/Mn competition, it was shown that Cd is not a very effective competitor for Mn.

As in the Cd/Zn competition experiments, Cd is accumulated by *S. vacuolatus* from medium with constant free Cd and varying Mn concentrations. Cd uptake is not inhibited by even a 500-fold excess of Mn and accumulation only decreases at a free Mn^{2+}/Cd^{2+} ratio above 10000. As expected, Mn accumulation occurs at Mn^{2+} concentrations higher than those present in the culture medium.

Cd is not very effective in competing with Mn under conditions of varying Cd and constant Mn. Cd does not interfere with Mn accumulation below a very high Cd^{2+}/Mn^{2+} ratio (> 225). Therefore it is concluded that the Cd uptake is not occurring via the Mn transport system. This conclusion is supported by the fact that Mn accumulation was not affected at all by an increase of pCd in long term experiments in this freshwater alga.

Kola and Wilkinson (2005) also observed no clear interaction of Mn on Cd uptake in *C. reinhardtii* whereas Sunda and Huntsman (1998a; 2000) described a regulation mechanism in diatoms for Cd under Zn or Mn control depending on the total Zn concentration. They found Cd uptake by the Mn transport sites at high free Zn^{2+} and low Mn^{2+} concentrations in some marine species. However, Mn concentration is found to be very low in oceanic waters in general. Thus, marine algae have to increase their Mn transport capacity to prevent Mn limitation, and Cd uptake through the Mn transport system may become enhanced as well.

From these results we conclude that Mn/Cd competition in *S. vacuolatus* cannot be explained by a simple competitive model as assumed in our calculations for Zn and Cd, and that only weak competition from Mn with Cd is observed.

3.5.3 Role of Zn and Mn competition for Cd uptake under freshwater conditions

In summary, competition experiments with Mn or Zn on Cd accumulation show that for *S. vacuolatus* the Zn transport system is the main uptake path for Cd and the Mn transport system is of no important role.

Typically, total dissolved background concentrations of Zn found in natural freshwaters are 10-51 nM with free metal ion concentrations from 0.3 to 5.6 nM (Meylan et al., 2004b; Xue and Sigg, 1994) whereas Cd is present at uncontaminated sites at total concentrations of about 0.1 nM (Odzak et al., 2002; Xue and Sigg, 1998). In the case of a small stream with metal input from contaminated sediments Zn was found to rise to total dissolved concentrations of 95-280 nM during a rain event with Cd increasing up to 0.19 nM (Meylan et al., 2003). Cd concentrations up to 7 nM were found at other contaminated sites (Croteau et al., 1998). From experiments presented in this work it can be

concluded that under conditions of low concentrations of Cd and high Zn concentrations Cd uptake is reduced but might still occur due to Cd transport through other binding sites than for Zn.

Thus, systems with low natural Zn background concentrations might be potentially at risk due to anthropogenic input of Cd, for example streams or lakes in which free Zn^{2+} might be further decreased by complexation. Under these conditions Cd input (e.g. industrial input or through accidents) may lead to a sudden increase of the available Cd concentration which then might be higher than the available Zn.

3.5.4 Acknowledgements

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Fulvic acid interactions on Cd uptake in
Scenedesmus vacuolatus

4.1 Abstract

Direct and indirect interactions of fulvic acids on Cd accumulation by *Scenedesmus vacuolatus*, a freshwater alga, were investigated in short term accumulation experiments.

Cd accumulation was found to be in relation to the free Cd^{2+} available from the experimental media and decreased with increasing amounts of fulvic acid (from 5 to 40 mg/L) added. Electrophoretic measurements revealed that fulvic acids only had a slight influence on the algal surface charge at pH 7.5. No indirect effect of fulvic acid was detected in experiments where the FA concentrations were varied while keeping the free Cd^{2+} constant by adding EDTA at pH 7.5. Potential algal ligand production was evaluated in a 2 day experiment to assess the capacity of the algae to reduce the Cd availability by excreting ligands. Indeed, algal ligand production was detected in presence of fulvic acids, and their stability constants were very similar to constants of fulvic acids. The total amount of ligands produced indicate that the ligand production is not sufficient to decrease Cd concentrations at high levels of Cd, however at low levels they might help to decrease potential metal toxicity. The results are discussed with respect to the role of fulvic acids on metal detoxification.

4.2 Introduction

Natural dissolved ligands such as fulvic or humic acids can interact with metals in water and regulate the concentration of the free metal ions by complexation. Algae are influenced by dissolved ligands which can either change the bioavailability of metals towards the organism by binding to the metal in solution or by binding to the algal surface. In this context it is of interest whether the presence of humic substances in aquatic systems influences metal uptake and accumulation to such extent that it either leads to an enhancement or a decrease of the bioavailability of metals for the algae. In particular the question arises if (and how) effects other than metal complexation influence metal uptake in the presence of fulvic acids.

Contradicting results have been found on the influence of humic substances (HS) on metal binding and its influence on metal uptake for aquatic organisms, describing either an enhanced uptake (Campbell, 1995; Kozuch and Pempkowiak, 1996; Slaveykova et al., 2003), or a decrease of metal availability in the presence of HS (Campbell, 1995; Campbell et al., 2002; Hassler and Wilkinson, 2003; Knauer and Buffle, 2001; Mylon et al., 2003). Availability models such as the FIAM (Campbell, 1995; Morel and Hering, 1993) or BLM (Di Toro et al., 2001; Paquin et al., 2002) describe metal uptake being proportional to the free metal concentration in solution and take into account the binding of ligands to the metal in solution. An additional effect may be the binding of the ligand itself to the cell surface, which subsequently may affect metal uptake. It was found that natural organic matter adsorbs on the cell surfaces (Campbell et al., 1997) and that this sorption is favoured at lower pH (Vigneault et al., 2000). Moreover, fulvic acid (FA) sorption at the algal surface was recently hypothesized to increase the lead uptake by *C. kesslerii* (Slaveykova et al., 2003).

The interactions of Cd and fulvic acids are of particular interest since Cd is toxic at already low levels (Hudson, 1998), it is present in most contaminated waters and it is known to form moderately strong complexes with natural ligands (Vigneault and Campbell, 2005). Fulvic acids on the other hand represent a large fraction of naturally occurring ligands in freshwaters.

The objectives of the present work are to examine the influence of a well characterized standard fulvic acid (Suwannee River Fulvic Acid, SRFA (Averett et al., 1989)) on Cd accumulation in a unicellular freshwater alga (*Scenedesmus vacuolatus*) at relevant natural concentrations of fulvic acid and low concentrations of Cd. In short term experiments, we therefore tested the influence of varying fulvic acid concentrations on Cd accumulation in *S. vacuolatus* and the potential of SRFA to induce changes of Cd accumulation due to indirect effects on the algal surface. For this purpose, the electrophoretic mobility (EPM) of the algal cells exposed to varying fulvic acid concentrations at constant pH was examined. Additionally, we determined the ligand production of *S. vacuolatus* during growth in presence of fulvic acid and compared the properties of these ligands with SRFA.

4.3 Materials and methods

4.3.1 Algae cultures

Isolates of the unicellular green algae *Scenedesmus vacuolatus* 211-8b (*Chlorophyceae*) were obtained from the culture collection of the Institute for Plant Physiology of the University of Göttingen, Germany. Algae treatment was described comprehensively in chapter 3.3.1. Briefly, this involves acclimatisation of the algae to the culture medium by successive culturing followed by transferring them to experimental medium after separating them

from the growth medium by centrifugation. Algae were cultured in glass Erlenmeyers in a HT Infors shaker (Infors, Bottmingen, Switzerland) at 90 rpm and 25°C under continuous light exposure of $276 \mu\text{Em}^{-2}\text{s}^{-1}$ provided by cool white fluorescent lamps. To obtain a sufficient amount of cells, an initial population density of 8×10^5 cells/ml for short term experiments and 4×10^5 cell/ml for 2 day growth experiments was chosen.

4.3.2 Chemicals

Stock solutions of standard Suwannee River Fulvic acid (SRFA) from the International Humic Substances Society (Colorado School of Mines, CO) (Averett et al., 1989) with a concentration of 1 g/L were prepared in Milli-Q water and stored at 4 °C until use. The FA stock solution was prepared at least 24 hours before use to ensure complete equilibration and was prepared freshly when needed. Dissolved Organic Carbon (DOC) content of the stock solution and the experimental media was measured by combustion (Elementar, High TOC II, Gerber Instruments, Switzerland).

Metal solutions and major nutrient solutions were prepared with salts from Fluka (puriss. grade; Buchs, Switzerland). Ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA), 3-morpholinopropanesulfonic acid (MOPS) were puriss. grade from Fluka, nitric acid (HNO_3 ; 65%), hydrochloric acid (HCl ; 30%), hydrogen peroxide (H_2O_2 ; 30%) were suprapure® chemicals from Merck (Darmstadt, Germany). Sodium hydroxide (NaOH ; 50%) used for medium preparation was from Baker Analysed® (JT Baker; Deventer; Netherlands). Cd addition to the experimental media was applied from standard CdCl_2 salts from Fluka.

All glassware and polycarbonate containers were pre-soaked in 0.01 M HNO_3 and rinsed with deionised nanopure water (18 M Ω Q- H_2O grade, Barnstead

Nanopure, Allschwil, Switzerland) before utilization, glassware used for algae experiments or media preparation were autoclaved before use.

4.3.3 Preparation of culture and experimental medium

Culture medium was prepared by addition of a concentrated EDTA-trace metal solution (1000-fold) to a solution containing major nutrients and major freshwater cations and anions (Price et al., 1988). The procedure is explained in detail previously (see chapter 3.3.1). Final concentrations in the medium are: 5×10^{-4} M $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.5×10^{-4} M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.2×10^{-3} M NaHCO_3 , 5×10^{-5} M $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, and 1×10^{-3} M NaNO_3 , 5×10^{-8} M Co(II) , 5×10^{-5} M H_3BO_3 , 8×10^{-8} M MoO_4^{2-} , 1×10^{-7} M Cu(II) , 1×10^{-6} M Mn(II) , 1.25×10^{-7} M Zn(II) , 9×10^{-7} M Fe(III) . The corresponding free concentrations of Cu, Mn, Zn, and Fe are 3×10^{-14} M, 1.7×10^{-8} M, 9×10^{-12} M, and 1×10^{-20} M, respectively.

In short term experiments algae were exposed to a medium containing the major cations and anions plus varying amounts of SRFA, Cd, and EDTA (when needed) based on calculations performed by the modelling software VMINTEQ (Gustafsson, 2005). Total Cd concentrations were varied to adjust the free Cd^{2+} concentration according to the experimental setup.

Growth experiments were performed in adapted culture media with 10 mg FA/L and 5 μM NTA. The free trace metal concentrations were controlled by NTA to avoid depletion in the medium during algal growth, as with FA only the total metal concentrations would not have been sufficient for growth.

All media were prepared at least 24h in advance to allow for complete chemical equilibration. The control medium (no added Cd) showed a residual total Cd concentration of about 0.1×10^{-9} M, corresponding to a pCd ($-\log [\text{Cd}^{2+}] = \text{pCd}$) of about 13.7 due to contamination from the chemicals.

Total metal concentrations were measured in all media by inductively coupled plasma mass spectrometry (ICP-MS; Element2; Thermo Finnigan, Bremen, Germany) before and after the experiments, and free metal concentrations were subsequently computed using the modelling software VMINTEQ (Gustafsson, 2001) or WHAM6 (Tipping, 1994) for calculation with fulvic acids.

In VMINTEQ the SHM model (Gustafsson, 2001) and in WHAM6 the Model VI (Tipping, 1994) describe the interaction of metals with natural organic matter. For both models, total measured metal concentrations, and the concentration of fulvic acids applied were used as input parameters for calculations. Comparison of modelling the free Cd^{2+} concentration by the SHM and WHAM model revealed differences of 5-25 percent between the models depending on total Cd input (data not shown).

4.3.4 Experimental methods

Influence of SRFA on Cd uptake was examined in short term (1 hour) and long term (48 hours) experiments. Exponential growing algae were separated from the culture medium by centrifugation for 10 minutes at 2300 g at 4°C and resuspended in a metal free medium at pH 7.5.

For uptake experiments aliquots of algae were exposed for one hour to experimental media containing major cations and anions, varying fulvic acid concentrations from 0 to 40 mg/L and added Cd concentrations of 0, 7, and 14 nM, respectively. To investigate indirect effects of FA on the algal surface, Cd accumulation was assessed by exposing *S. vacuolatus* to experimental media where the FA concentrations were varied from 0 to 30 mg/L and EDTA was added as ligand to buffer the Cd concentration and to keep the free Cd constant.

For the EPM measurements, a contact time of 30 minutes was chosen. The FA concentration was varied from 0 to 30 mg/L and the pH kept constant at 7.5 by adjusting the pH of the MOPS buffer in the medium.

Algal ligand production and ligand properties were investigated under growth conditions (exposure time 48 hours) in a culture medium containing Cd and 10 mg/L FA and 5×10^{-6} M NTA at pH 7.5. Cd was added to the media to cover the range of pCd of 14 to 9.1. In this case, 5×10^{-6} M NTA instead of EDTA was used as buffering ligand in order to be able to detect additional ligands (which can not be detected in the presence of EDTA). Free Cd, Cu and Zn concentrations were kept at the same level as in the normal culture medium by varying the corresponding total metal concentrations. Growth experiments were performed in a Multitron shaker.

For determining algal metal concentrations, aliquots from the experimental culture were filtered at the end of the experiment on acid washed filters (cellulose nitrate; 0.45 μ m; Sartorius, Göttingen, Germany). The filters were digested in Teflon® flasks in a high performance microwave digestion unit (mls 1200 mega; Microwave Laboratory Systems, Oberwil, Switzerland) by addition of 4 mL HNO₃ and 1 mL H₂O₂ for 15 minutes. Total metal concentrations in digested solutions were determined by ICP-MS using rhodium as internal standard. The digestion procedure was checked by the analysis of plankton reference material (error < 15 %; CRM 414, Community Bureau of Reference, Commission of the European communities, Brussels, Belgium), ICP-MS measurements were checked using SLRS-4 reference water (River Water Reference Material for Trace Metals; National Research Council Canada, Ottawa, error < 10 %).

A 1 mL sample of the algal suspension was taken to determine cell size and cell numbers (Coulter Multisizer II, 50 μ m orifice) and the cell surface area was subsequently calculated assuming spherical shape of the algae. In long term experiments algal dry weight was determined additionally.

Electrophoretic mobility measurements (EPM) were performed by laser Doppler velocimetry with a Malvern Zetasizer II. Calibration of the instrument was performed with ζ -potential latex particle standards (Lawson Labs Inc., Malvern).

4.3.5 Calculation of uptake and diffusion fluxes

For comparison between measured uptake flux and the theoretical diffusion flux of free Cd^{2+} , uptake fluxes were calculated by eq. 4.1

$$J_u = U/s \quad [\text{mol cm}^{-2} \text{min}^{-1}] \quad (4.1)$$

where U is the measured Cd uptake (as mol per minute) and s is the cell surface area (cm^2). Cd uptake was determined after 60 min of exposure where metal uptake is assumed being at steady state and linear increase of Cd during this time is assumed. For all experiments a mean average cell radius, r, of 2.4×10^{-4} cm obtained from the particle counter was used. Results were compared to the theoretical diffusive flux which was calculated according to Slaveykova and Wilkinson (2002) with eq. 4.2

$$J_{\text{diff}} = D_m \times [\text{Cd}^{2+}] \times \left(\frac{1}{\delta} + \frac{1}{r} \right) \quad [\text{mol cm}^{-2} \text{min}^{-1}] \quad (4.2)$$

where D_m is the diffusion coefficient for the free Cd, Cd^{2+} is the free metal concentration in the solution, and δ is the diffusive boundary layer which was assumed to be 5 times the cell radius of 1.2×10^{-3} cm. The diffusion coefficient was taken from Li and Gregory (1974) with $D_{\text{Cd}^{2+}} = 7.17 \times 10^{-6} \text{cm}^2 \text{s}^{-1}$.

4.3.6 Determination of ambient free cadmium and ligands by Ligand Exchange - Stripping Voltammetry

Stripping voltammetry was performed by competitive ligand exchange-anodic stripping voltammetry (CLE-SV) according to Xue and Sigg (1998; 1999; 2002). In this method, a distinction is made between electrochemically labile and inert Cd species.

Within the time scale used, the Cd-NTA complexes (from the medium) are fully labile. Consequently, the current measured by CLE-SV originates from the free ions, inorganic and some weak organic complexes, and the Cd-NTA-complexes. From a titration set of the sample with known Cd addition, Cd^{2+} can be calculated by mass balance of the measured labile Cd concentration and inorganic and NTA complexing coefficients. The corresponding total ambient Cd concentration includes inert complexes other than Cd-NTA (Xue and Sigg, 1999). With the assumption of a one ligand model for 1:1 complexes, the titration data sets of the ratio of Cd-FA to Cd^{2+} can be fitted by Scatchard plots or assuming a one ligand model by the modelling programme FITEQL (Westall, 1982). Thus the conditional stability constants and available ligand concentrations can be obtained. The ambient free Cd^{2+} concentrations can be computed at the total ambient Cd concentration, using these complexing parameters (Xue and Sigg, 2002). Samples for titration were taken before and after algae exposure and stored at 4°C until analysis. They were buffered with 10 mM MOPS adjusted to pH 7.5, kept at constant ionic strength by adding 10 mM KNO_3 and equilibrated at room temperature overnight. For titration, Cd addition was applied in the range from 2 to 140 nM. Labile Cd was measured by differential pulse ASV with a Metrohm 757 voltammeter (Metrohm, Switzerland) by HMDE with an Ag/AgCl/ KNO_3 sat. as reference electrode and a Pt counter electrode, 300 s purging with N_2 , 240 s deposition time, -1.1 V deposition

potential, stripping potential from -0.9 to -0.4 V, voltage step 6 mV, voltage step time 0.4 s and sweep rate 0.015 V/s.

Modelling of the free Cd concentrations was subsequently done with the Stockholm Humic Acid Model using Visual Minteq, version 2.32 (Gustafsson, 2005).

4.4 Results

Effects of fulvic acids on Cd accumulation in *S. vacuolatus* were assessed by short term and long term experiments.

4.4.1 Influence of fulvic acid on Cd accumulation

The effect of fulvic acids on short term Cd accumulation is shown in figure 4.1. Under the experimental conditions, the free Cd^{2+} concentration in the medium is controlled by the concentration of SRFA only. Figure 4.1a shows the Cd contents of *S. vacuolatus* in relation to pCd in the media. With total Cd varying from 4.3×10^{-10} to 1.4×10^{-8} Mol/L, corresponding to pCd 9.5 to 7.8, the Cd content of the cells increases from 8×10^{-20} to 3.8×10^{-18} mol/cell with increasing free Cd^{2+} concentration. No systematic difference between the various FA concentrations can be detected at pH 7.5

Figure 4.1b shows the accumulation of Cd in the same experiment in relation to the FA added to the media. Cd accumulation in *S. vacuolatus* is reduced with increasing fulvic acid concentrations in the media due to the corresponding decrease in free Cd^{2+} . In absence of added Cd, the algal Cd content ranged from 8×10^{-20} mol/cell (no FA added) to 4×10^{-19} mol/cell (40 mg/L FA) due to Cd in the FA stock solution (Cd increased from 4.3×10^{-10} to 1.2×10^{-9} mol/L with

increasing FA concentrations added). In presence of 7 nM total Cd, the cellular Cd concentrations decreased from 1.2×10^{-18} mol/cell (no FA) to 4.2×10^{-19} mol/cell (40 mg/L FA). With 14 nM total Cd, cellular contents decreased from 3.8×10^{-18} mol/cell (no FA) to 4.3×10^{-19} mol/cell (40 mg FA).

Figure 4.1: The upper panel (a) shows Cd accumulation (as mol/cell) in presence of various FA concentrations as a function of pCd at pH 7.5. The legend indicates the FA concentrations applied (in mg/L). The lower panel (b) shows the same experiment (Cd as mol/cell) in function of the FA concentrations applied. The legend indicates the total Cd concentrations (in nM/L) (n=6).

4.4.2 Effect of Fulvic acid on the electrophoretic mobility of *S. vacuolatus*

The electrophoretic mobility (EPM) values of *S. vacuolatus* were assessed in dependence of varying fulvic acid concentrations at pH 7.5 (figure 4.2).

The EPM of the algae shifts to slightly more negative values from -1.5 to -2.1 ($\times 10^8 \text{ m}^2 \text{ V}^{-1} \text{ s}^{-1}$) with increasing concentrations of fulvic acid applied. The experiments were performed at constant pH and ionic strength, thus the EPM values can be directly interpreted as variation of the algal surface charge due to interactions with fulvic acids.

Figure 4.2: Electrophoretic mobility (EPM) of *S. vacuolatus* as a function of fulvic acid concentration at pH 7.5, contact time 30 minutes, n=3

4.4.3 Indirect effects of FA on Cd uptake

To evaluate potential indirect impacts of fulvic acids on Cd accumulation by interactions with the cell membrane, Cd uptake experiments were performed at pH 7.5 by varying the FA concentration and keeping pCd constant (figure 4.3).

The Cd concentration applied was kept constant at pCd 9.9 by adding 1.8×10^{-5} M EDTA and the SRFA concentration was varied from 0 to 30 mg/L.

Indirect effects were not observed, the Cd accumulation was constant (about 1.4×10^{-17} mol Cd/cell) in presence of the varying fulvic acid concentrations. The Cd accumulation is in the same range as accumulation from the culture media (see chapter 3.4).

Figure 4.3: Cd accumulation in *S. vacuolatus* at pH 7.5 in presence of varying SRFA concentrations at constant pCd of 9.9 (total Cd 2.1×10^{-6} M). 1.8×10^{-5} M EDTA was added to control the free Cd²⁺, (n=6).

4.4.4 Algal ligand production during growth

The ligands produced by algae were compared to the properties of fulvic acids. The FA content of 10 mg/L in this experiment corresponds to dissolved organic carbon (DOC) concentrations in an eutrophic lake assessed within the present project (Sigg et al., 2006). NTA controls the free metal concentrations of the trace metals necessary to ensure optimal growth.

The Cd concentration in the algae increased by factor 30 from 4×10^{-20} mol/cell at the lowest Cd concentration measured (pCd 14) to 1.1×10^{-18} mol/cell at pCd 9.1 (data not shown).

Ligand production by the algae after 48 h exposure is observed from a titration set of free Cd^{2+} vs. Cd_{tot} . The shift of the free Cd^{2+} before and after algal exposure indicates algal ligand production (figure 4.4). The offset of the curve after algal exposure (eg. no points at low Cd_{tot}) is due to the fact that the first points at low Cd concentrations could not be measured by CLE-SV as the labile Cd was below the detection limit of the CLE-SV.

The shift of free Cd^{2+} concentration before and after algae exposure is less clear with higher Cd background concentrations in media 4 and 5, probably due to the stronger ligands being already titrated out. This leads to decreasing measured ligand concentrations with increasing total Cd concentrations.

Figure 4.4: Titration curve of growth media with 0.24 nM Cd before (filled points) and after (open points) algal exposure. The shift of the points to the right indicates algal ligand production.

Table 4.1 shows the total Cd concentration before and after algal exposure, the ambient free Cd^{2+} concentration, the determined overall conditional stability constant ($\log K$) before and after algal exposure, and the produced algal ligand concentration determined by CLE-SV. The stability constant for the algal ligand K_{LA} was calculated according to

$$K_{LA} = \frac{K_i L_i - K_{FA} L_{FA}}{[L_{AL}]} \quad (4.3)$$

where $K_i L_i$ represents the overall complexing coefficient after algal exposure, $K_{FA} L_{FA}$ the complexing coefficient before algal exposure and $[L_{AL}]$ the ligand concentration produced by the algae.

As expected, the total and free Cd concentrations decreased in all media after algal exposure compared to Cd concentrations before algal exposure. The ligand concentration measured after algal exposure decreased from 5.7×10^{-8} to 7×10^{-9} mol/L with increasing Cd concentrations applied. The stability constant of the ligands are very close to the stability of the fulvic acid complexes before exposure in media 1 to 3. In medium 4 and 5 the stability constant decreased.

Table 4.1: Total Cd, ambient free Cd²⁺, overall conditional stability constant (K_i), and algal ligands constant (K_{AL}), and ligands produced before and after algal growth (48 h) in media with 10 mgL⁻¹ FA and 5x10⁻⁶ M NTA at pH 7.56 (± 0.01).

Medium	Cd _{tot} (M)	Cd ²⁺ (M)	Log K _i	log K _{AL}	L _{AL} (M)
1 before exp.	4.9x10 ⁻¹¹	1.2x10 ⁻¹⁴	10.9		
after exp.	3.1x10 ⁻¹¹	1.0x10 ⁻¹⁴	10.9	10.91	5.7x10 ⁻⁸
2 before exp.	2.4x10 ⁻¹⁰	9.2x10 ⁻¹³	10.8		
after exp.	5.0x10 ⁻¹²	6.5x10 ⁻¹⁵	10.4	10.31	4.9x10 ⁻⁸
3 before exp.	1.7x10 ⁻⁹	1.0x10 ⁻¹¹	10.3		
after exp.	2.1x10 ⁻¹⁰	4.8x10 ⁻¹³	10.2	10.19	2.9x10 ⁻⁸
4 before exp.	1.4x10 ⁻⁸	3.3x10 ⁻¹¹	9.3		
after exp.	3.4x10 ⁻⁹	1.1x10 ⁻¹¹	10.3	9.19	2.3x10 ⁻⁸
5 before exp.	3.6x10 ⁻⁸	7.6x10 ⁻¹⁰	9.9		
after exp.	1.3x10 ⁻⁸	2.3x10 ⁻¹⁰	9.6	8.17	7.1x10 ⁻⁹

4.4.5 Comparison of fluxes

The transport of free metal ions to the membrane (theoretical diffusion flux) and the internalization of the metal (uptake flux) were compared under various conditions (figure 4.5). Uptake fluxes were calculated according to equation (4.1) assuming steady state and compared to the diffusion flux calculated by equation (4.2). The solid line in figure 5 represents the maximal theoretical diffusion flux for *S. vacuolatus* for the free Cd²⁺ concentrations applied in the experiments. The uptake fluxes for the FA experiments vary from 7x10⁻¹⁵ mol cm⁻² min⁻¹ to 9x10⁻¹⁴ mol cm⁻² min⁻¹ within the range of pCd investigated and are several orders of magnitude below the diffusion limit and below Cd uptake fluxes from culture medium. Uptake fluxes in short term culture and 2 day growth experiments are closer to diffusion limitation for pCd lower than 13.

Figure 4.5: Calculated uptake fluxes for FA experiments in comparison to a reference growth medium with added Cd. The solid line represents the maximal theoretical diffusion flux, the solid points uptake fluxes for culture medium, open points the short term FA experiments, and the triangles the 2 day growth experiments.

4.5 Discussion

The role of humic substances in metal speciation in waters is of interest as the understanding of the mechanism of metal binding in solution or FA binding to the organism helps to improve current models predicting metal-organism interactions and bioavailability. The role of fulvic acids in metal uptake is not clear yet as literature showed contradictory results (Hudson, 2005; Lamelas et al., 2005; Slaveykova et al., 2003; Vigneault and Campbell, 2005).

4.5.1 Role of FA on Cd speciation

In the experiments presented here it is shown that fulvic acids reduce the bioavailability of Cd by reducing the free Cd²⁺ in solution. Indirect effects such

as interaction of FA with the cell surface of *S. vacuolatus* were found to be negligible at pH 7.5. The results lead to the conclusion that Cd accumulation in *S. vacuolatus* depends on the free Cd in solution and that uptake of Cd seems not affected by low to intermediate concentrations of fulvic acids present at neutral pH. These results agree with findings by Vigneault and Campbell (2005) who recently reported that fulvic acids cause no additional effect on Cd uptake besides the reduction of the bioavailability of Cd by complexation in the medium at pH 7 and pH 5 for *C. reinhardtii* and *P. suscapitata*. However, in an earlier work they found that interactions with the algal surface increase at lower pH in case of Cd (Boullemant et al., 2004) or a lipophilic solute (Vigneault et al., 2000). The probability of interactions between the cell surface and FA increases with decreasing pH due to increasing protonation of the cell surface functional groups as well as of FA. Thus, repulsion of negatively charged surfaces decreases at lower pH values and facilitates interaction. At the pH investigated in this work (7.5), sorption effects of the fulvic acid on the cell surface can be excluded from EPM measurements performed.

Contrasting the results from Vigneault and Campbell, Slaveykova et al. (2003) showed that the presence of SRFA decreased lead uptake in *C. kesslerii* at pH 6 with respect to non-complexed Pb compared to a system without SRFA added. However at the same time they observed that uptake fluxes, cellular Pb, and Pb bound to transport sites were higher than expected from the Pb^{2+} concentration and related this effect mainly to chemical changes of the biological membrane due to SRFA adsorption. Lamelas et al. (2005) concluded that a slight shift in the surface charge is not sufficient for this enhanced uptake (compared to the free Pb^{2+}), and that the formation of ternary complexes on the cell surface enhances metal uptake. In this case, the binding of Pb complexed by another ligand on the cell surface precedes Pb uptake. However, under conditions given in experiments presented in this work, this effect seems not evident for Cd uptake in *S. vacuolatus*.

Comparison of uptake fluxes for algae exposed to varying SRFA concentrations showed no diffusion limitation. This implies that the Cd uptake in the presence of fulvic acids is thermodynamically rather than kinetically controlled, suggesting that transport through the cell membrane is the rate limiting step in the uptake process. Similar conclusions were reached by Vigneault and Campbell (2005), showing that for at pH 7 and 5 the presence of fulvic acid seems not to limit the kinetics of Cd transport to the uptake sites compared to the trans membrane transport for two algae species.

4.5.2 Algal ligand production

After a growth period of 2 days, additional ligands were detected in the media in presence of fulvic acids. The ligands produced by the algae during growth seem unspecific for Cd, as they are produced at already very low Cd concentrations. Ligand production by algae as a result of cellular metabolism was also reported by Soldo et al (2005). *Oocystis nephrocytioides* chronically exposed to copper (0.04 and 2×10^{-6} Mol/L) did not increase ligand production with increasing metal concentration but ligand production increased with exposure time from $1.3 \mu\text{M}$ to $3.3 \mu\text{M}$ over 13 days with unchanged stability constant of 10^{13} at pH 7.3.

The conditional stability constants determined by CLE-SV in the media after algal exposure are very close to the constants determined in the same media before algal exposure indicating the algal ligands having similar complexing properties as the fulvic acids. Thus, the stability constants determined after algal exposure very likely represent the constant of the sum of the ligands present in the media and were found to be in good agreement to values determined by Xue and Sigg (1998; 1999) for ligands in lakes with increased biological activity. Biological ligands binding Cd might comprise polysaccharides (Lamelas et al.,

2005) or thiol-containing components such as phytochelatins excreted by the algae, which are known to bind strongly to Cd (Lee et al., 1996).

In conclusion it was shown that *S. vacuolatus* is able to produce extracellular ligands during growth and that these ligands might be able to prevent the cell from enhanced Cd availability at low total Cd concentrations due to their affinity to Cd.

4.5.3 Implications for natural waters

The role of FA in natural waters under the experimental conditions examined here with respect to Cd binding seems dominantly characterized by its ability to bind the free Cd^{2+} , resulting in a decreased bioavailable fraction of Cd. Additional ligand production can enhance this effect to a limited extend.

For predictions of the availability of metals a comprehensive characterization of the natural humic substances present in waters helps to understand its potential binding behaviour and its role in metal speciation. In freshwaters, a large fraction of metal has been found to be complexed by natural organic matter (Achterberg et al., 1997; Meylan et al., 2004a; Mylon et al., 2003). At lower pH values, natural ligands comprising fulvic acids have been found to play an important role for trace metal binding in freshwaters (Mylon et al., 2003; Tipping and Hurley, 1992). However, ligands other than fulvic acid might play a role in trace metal binding. Complexation of Cd by synthetic or biological ligands has also been proposed (Giger et al., 1991; Lamelas et al., 2005; Xue and Sigg, 1999). Compounds comprising sulphydryl groups such as phytochelatins or glutathione have been reported to reduce the bioavailability of Cd in algae (Le Faucheur et al., 2005) whereas only few studies have succeeded demonstrating the release of phytochelatins into the water to reduce the bioavailability of metals (Lee et al., 1996; Wei and Ahner, 2005).

However, any additional ligands produced by algae, even at low concentrations of Cd, maybe effective to lower the available Cd^{2+} concentration and thus to prevent toxic effects of Cd.

*Cadmium accumulation in algae under
freshwater conditions*

5.1 Abstract

Cd accumulation in *Scenedesmus vacuolatus* was investigated from freshwater systems (two hard- and a softwater system) during a campaign aiming at comparison of various analytical methods for trace metal determination. *S. vacuolatus*, a freshwater alga, was exposed to the natural water samples which were spiked with Cd under controlled laboratory conditions. The Cd accumulation is compared to total, labile and free Cd measured by various methods applied in these campaigns. In the hardwater systems Cd accumulation was found to follow the free ionic activity model and accumulation was similar to accumulation from the standard culture medium. In the softwater sample, Cd accumulation increased linearly with increasing free and total Cd concentrations but was lower than expected from the free Cd^{2+} concentrations compared to the other freshwaters and culture media. Modelling of the Cd uptake revealed that beside cation and proton competition, other factors such as dissolved organic carbon composition and the presence of colloids might reduce Cd accumulation in the softwater samples. The data are discussed with respect to freshwater conditions.

5.2 Introduction

The knowledge about metal speciation in freshwaters is of importance as it allows the determination of reactivity, mobility, bioavailability, and toxicity of the respective metal (Batley et al., 2004; Campbell, 1995). Bioavailability of metals to organisms has been shown to be mainly controlled by the activity of the free metal ion, which is described by the free ion activity model (FIAM), or the biotic ligand model (BLM) (Campbell, 1995; Campbell et al., 2002; Hudson, 2005; Paquin et al., 2002). The majority of studies determining the fraction of metal available for uptake have been performed in well defined synthetic systems in the laboratory (for review see (Campbell, 1995) whereas the influence of natural ligands has been assessed in few cases only (Campbell et al., 1997; Kozuch and Pempkowiak, 1996; Lamelas et al., 2005; Meylan, 2003; 2004a; Vigneault and Campbell, 2005). Recent data suggest that the FIAM is a simplified limiting case of the general dynamic situation, where compound fluxes, and complex dissociation rates should be considered as well (Galceran and van Leeuwen, 2004; van Leeuwen, 1999; Wilkinson and Buffle, 2004).

Cd accumulation in freshwater algae is of relevant research interest since Cd is toxic already at low levels (Newman, 1998; Walker et al., 2001) and Cd contamination is mainly of anthropogenic origin. As it is difficult to control the parameters influencing metal speciation in natural freshwaters, research on Cd accumulation has been generally focused on the assessment of Cd toxicity in well defined media under controlled laboratory conditions. Only few studies have assessed uptake and accumulation of metals from freshwaters (Croteau et al., 1998; Hare and Tessier, 1996; Meylan et al., 2003; Mylon et al., 2003; Orvoine et al., 2006).

Thus, the objective of this study is to determine Cd bioavailability in freshwaters by comparing Cd accumulation into a freshwater alga, *Scenedesmus vacuolatus*,

to the measured Cd speciation in these freshwaters and to interpret Cd accumulation based on speciation models available for freshwater conditions. For this purpose, short term Cd accumulation experiments with freshwater samples were performed in which *S. vacuolatus* was exposed to water samples from three freshwater systems (a hardwater stream and lake in Switzerland, and a softwater stream in England). Cd speciation at these field sites was assessed by several methods (Sigg et al., 2006; Unsworth et al., 2006). Accumulation data from *S. vacuolatus* are compared to the measured free Cd^{2+} by Competitive Ligand Exchange Anodic Stripping Voltammetry (CLE-ASV) (Xue and Sigg, 2002). Accumulation data are also compared to the measured Cd parameters by the methods Gel Impregnated Microelectrode (GIME) (Tercier and Buffle, 1996), Diffusive Gradient in Thin Films (DGT) (Zhang and Davison, 2000), Donnan Membrane Technique (DMT) (Temminghoff et al., 2000), and the Hollow Fiber Permeation Liquid Membrane Technique (HFPLM) (Buffle and Tercier, 2000). Other factors influencing Cd accumulation besides Cd speciation are evaluated and discussed.

5.3 Materials and Methods

5.3.1 Field sites

The fieldworks at Lake Greifen and stream Furtbach, Switzerland, were carried out from September 2 to 5, 2003. Lake Greifen is a small eutrophic lake located in Eastern Switzerland near Zurich. Its maximum depth is 32 m, covering a surface area of 8.5 km² and its volume is 150x10⁶ m³. Thermal stratification of Lake Greifen lasts from about May to December, with a thermocline located around 10 m depth. Samples were taken at the deepest point of the lake from the

oxic epilimnion at 2.5 and 5 m depth, in which intensive algal photosynthesis is taking place.

The Furtbach is a small stream located in Eastern Switzerland near Zurich, which flows for approximately 10 km through the Furttal Valley, with an average discharge of $0.4 \text{ m}^3 \text{ s}^{-1}$. Its depth at the sampling site is 0.4 – 0.5 m. The trace metal concentrations in the Furtbach are influenced by the vicinity of metal-handling industries and the presence of metal-contaminated sediments.

The River Wyre was sampled at Garstang in Lancashire, NW England, from April 19 to 22, 2004. The Wyre is an unpolluted river with quite high concentrations of dissolved organic carbon (DOC), due to drainage from a moorland area partly covered with peat. The water level was high and coloured by dissolved organic matter due to rainfall the day before the fieldwork started (6 mm rain recorded by the metrological office field station Hazlerigg DCNN7236 situated a few kilometres north of the fieldwork site).

5.3.2 Water sampling and preparation

Water samples from Lake Greifen were collected with Go-Flo water samplers in 1L polyethylene bottles. Stream Furtbach was sampled by peristaltic pumping using Teflon tubes into 1L polyethylene containers. Bulk water samples from River Wyre were collected by submerging acid cleaned 4L plastic bottles and removing and replacing the cap underwater.

The water samples were filtered in the laboratory under clean-bench conditions within a few hours of collection, using a polysulfone filtration unit (Nalgene) and $0.45\text{-}\mu\text{m}$ filters (cellulose nitrate filters, Sartorius). Subsamples of these filtered samples were prepared for either speciation measurements or bioaccumulation experiments and were left at their original pH. Experiments with Lake Greifen and Stream Furtbach water were performed within 24 hours

of sampling, River Wyre samples were stored at 4°C in the dark before use and processed within 8 days. Samples for measurements of total dissolved metal concentrations were immediately acidified with suprapure HNO₃. A complete description of the parameters surveyed in both field campaigns is given in (Sigg et al., 2006) and (Unsworth et al., 2006).

To avoid contamination, all glassware, samplers, containers, filters and filtration devices were presoaked in 0.01 M HNO₃ and rinsed with deionized nanopure water (18 MΩ Q-H₂O grade, Barnstead Nanopure, Allschwil, Switzerland) before utilization. All devices for field water sampling and trace metal analysis were protected during transport with plastic bags. All handling was performed with plastic gloves.

5.3.3 Test organism and culture conditions

Isolates of the unicellular green algae *Scenedesmus vacuolatus*, 211-8b (Chlorophyceae), were obtained from the culture collection of the Institute for Plant Physiology of the University of Göttingen, Germany. Treatment of algae as well as the preparation of the culture medium is described in detail in chapter 3.3.1. Algae were cultured in glass Erlenmeyers in a HT Infors shaker (Infors, Bottmingen, Switzerland) at 90 rpm and 25°C under continuous light exposure of 276 μEm⁻²s⁻¹ provided by cool white fluorescent lamps.

All experiments were performed in a clean lab and bottles and Erlenmeyers used for algae experiments or media preparations were autoclaved before use to avoid microbial contamination.

5.3.4 Accumulation experiments and metal detection

For the experiment, exponentially growing algae were separated from the culture medium by centrifugation for 10 minutes at 2300 g at 4°C. This washing step was repeated twice with the algae being resuspended in culture medium without trace metals. The total background Cd concentration in the medium was 1×10^{-10} M, corresponding to a pCd ($-\log [\text{Cd}^{2+}] = \text{pCd}$) of about 13.7. Aliquots of washed algae (population density 8×10^5 cells/ml) were exposed for short term experiments (60 min) to field water samples which were previously spiked with Cd to cover a range of free Cd^{2+} from pCd 12.7 to 9.5 for Lake Greifen, from pCd 14 to 12.2 for Furtbach, and pCd 11.5 to 7.5 for River Wyre samples, corresponding to total Cd concentrations from 1.2×10^{-10} to 4.5×10^{-7} mol/L. Previous to the experiment, the spiked water samples were equilibrated overnight at room temperature.

Algal metal concentrations were determined by filtering aliquots of the experimental culture through previously acid washed filters (cellulose nitrate; 0.45 μm ; Sartorius, Göttingen, Germany). The filters were subsequently digested in Teflon® flasks in a high performance microwave digestion unit (mls 1200 mega; Microwave Laboratory Systems, Oberwil, Switzerland) by addition of 4 ml HNO_3 and 1 ml H_2O_2 . The digestion procedure was checked by the analysis of plankton reference material (error < 15 %; CRM 414, Community Bureau of Reference, Commission of the European communities, Brussels, Belgium). Total metal concentrations in the digested algal solutions and water samples were determined by ICP-MS (Elan 5000, Perkin-Elmer, or Element2, Thermo Finnigan) using rhodium as internal standard and were checked by using a reference water (SLRS-4, River Water Reference Material for Trace Metals; National Research Council Canada, Ottawa, error < 10 %).

A 1 ml sample of the algae suspension was taken to determine cell numbers by either Neubauer chamber or cell counter (Coulter Multisizer II, 50 μm orifice),

respectively. Accumulation data were normalized to cell units and related to the measured speciation in the water samples.

5.3.5 Cd uptake flux calculation

To compare the Cd accumulation data from the freshwater samples to accumulation from culture medium (where Cd was added), uptake fluxes were calculated by eq. 5.1:

$$J_u = U/s \quad [\text{mol cm}^{-2}\text{min}^{-1}] \quad (5.1)$$

U represents the measured Cd uptake (expressed as mol/minute) and s the cell surface area (cm^2). Metal uptake was assumed being linear and at steady state after 60 minutes when Cd uptake was determined. A mean average cell radius of $2.5 \pm 0.2 \times 10^{-4}$ cm obtained from the cell counter was used for all experiments. Uptake flux calculations were subsequently compared to the maximal theoretical diffusive flux which was calculated according to Slaveykova and Wilkinson (2002) with

$$J_{\text{diff}} = D_m \times [Cd^{2+}] \times \left(\frac{1}{\delta} + \frac{1}{r} \right) \quad [\text{mol cm}^{-2}\text{min}^{-1}] \quad (5.2)$$

D_m is the diffusion coefficient for the free Cd in water, Cd^{2+} is the metal free concentration in the solution, and the diffusive boundary layer δ was assumed to be 5 times the cell radius. The diffusion coefficient was taken from Li and Gregory (1974) with $D_{Cd^{2+}} = 7.17 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$.

5.3.6 Determination of free Cd^{2+} and of ligands by Competitive Ligand Exchange - Anodic Stripping Voltammetry

Stripping voltammetry was performed by competitive ligand exchange-anodic stripping voltammetry (CLE-SV) according to Xue and Sigg (1998; 1999; 2002). This method distinguishes between electrochemically labile and inert Cd species. Inert Cd represents the non-reducible complexes and includes strong organic ligand complexes and Cd adsorbed on colloids which do not dissociate within the ASV timescale. The labile species include the free aqua ions, inorganic and rapidly dissociating weak organic complexes. Addition of ethylenediamine (EN) increases the fraction of SV-labile Cd, which increases the detection signal and therefore allows a better detection of SV labile Cd. The current measured by SV originates from the free ions, inorganic and some weak organic complexes, and the Cd-EN complexes. The free Cd^{2+} can be calculated by mass balance of the measured labile Cd concentration, inorganic, and EN complexing coefficients. The corresponding total ambient Cd concentration includes inert complexes other than Cd-EN (Xue and Sigg, 1999). With the assumption of a one ligand model for 1:1 complexes, the titration data sets of the ratio of Cd-L to Cd^{2+} can be fitted by Scatchard plots, or with the modelling program FITEQL (Westall, 1982) assuming a one ligand model. The conditional stability constants and the available ligand concentrations can thus be obtained. Using these complexing parameters, the ambient free Cd^{2+} concentrations can be computed at the total ambient Cd concentration.

Samples were buffered with 10 mM MOPS adjusted to the natural pH measured, 1 mM EN and Cd in the range from 2-120 nM were added and equilibrated at room temperature overnight. Labile Cd was measured by SV with a Metrohm 757 or Metrohm 694 voltammeter (Metrohm, Switzerland) by HMDE with an Ag/AgCl/ KNO_3 sat. as reference electrode and a Pt counter electrode, 300 s purging time with N_2 , 240 s deposition time, -1.1 V deposition potential,

stripping potential from -0.9 to -0.4 V, voltage step 6 mV, voltage step time 0.4 s and sweep rate 0.015 V/s.

Modelling of the free Cd concentrations was subsequently performed with the Stockholm Humic Acid (SHM) Model used in Visual Minteq, version 2.32 (Gustafsson, 2005), or the Windermere Humic Acid Model (WHAM) from Tipping (1994) using either the determined ligand stability constant and the ligand concentration determined, or the fulvic acid concentration measured (as DOC) as input parameter. For the River Wyre samples calculations were performed with WHAM estimating the total iron measured as colloidal iron according to Unsworth et al.(2006).

Free Cd^{2+} measured by CLE-SV was compared to the free Cd^{2+} measured by DMT and HFPLM, and to the labile fraction of Cd determined by GIME and DGT, as described in (Sigg et al., 2006). A detailed description of these methods is given in chapter two.

5.4 Results

5.4.1 Water characteristics at the three field sites

Table 5.1 shows measured total metal, pH and DOC concentrations, and the free Cd^{2+} concentration, total ligand concentration $(L)_{\text{tot}}$, and stability constants ($\log K$) determined by CLE-SV in samples from the three freshwater sites. The total metal concentrations are average values and were taken from (Sigg et al., 2006).

Comparison of unfiltered field water samples to water samples filtered in the field and the laboratory filtered water samples revealed that the laboratory filtered samples from River Wyre were probably contaminated with Cd (Sigg et al., 2006). As the laboratory filtered water samples were used for the subsequent experiments, their measured values are reported here.

The measured pH in the freshwaters is very high in Lake Greifen under the late summer conditions (8.7), somewhat lower in Furtbach (8.2 to 8.5), and lower in River Wyre (7.7 to 8.6). River Wyre showed a distinctively higher DOC content (average 15 mg dm^{-3}), which was assumed to consist largely of fulvic acid, than Lake Greifen (4.7 mg dm^{-3}) and Furtbach (1.9 mg dm^{-3}). Very low iron concentrations were found in Lake Greifen ($3 \times 10^{-8} \text{ Mol/L}$) in previous studies (Emmenegger et al., 1998) compared to much higher concentrations of $5.2 \times 10^{-6} \text{ Mol/L}$ in River Wyre.

Ligand concentrations, stability constants and free Cd^{2+} in the original water samples were determined by CLE-SV. In Lake Greifen the determined free Cd^{2+} is $2 \times 10^{-13} \text{ Mol/L}$ and the determined stability constant $10^{10.5}$, with a total ligand concentration of $10.5 \pm 3.1 \text{ nM}$. Equilibrium in Furtbach samples could not be achieved within three days (normal equilibration time is ~ 15 hours), therefore free Cd^{2+} was calculated using the stability constant determined in Lake Greifen,

and a high ligand concentration (500 nM). These estimates lead to a low free Cd^{2+} concentration of 8×10^{-15} Mol/L. In River Wyre the free Cd^{2+} concentration found (3.2×10^{-12} Mol/L) was higher than in the hardwater systems, the determined stability constant lower ($10^{9.3}$), and the total ligand concentrations found to vary from 6 to 18 nM.

Table 5.1: Water parameters from Lake Greifen, Stream Furtbach and River Wyre (from Sigg et al., 2006). Total ligand concentration, ($[\text{L}]_{\text{tot}}$), stability constants ($\log K$), and pCd were determined by CLE-SV (averages over the sampling period).

	Lake Greifen	Furtbach	River Wyre
Cd_{tot} (M)	1.3×10^{-10}	4.0×10^{-11}	1.1×10^{-9}
pCd	12.7	14.2	11.5
$[\text{L}]_{\text{tot}}$ (nM)	10.5	< 500	11.6
Log K	10.5	10.5	9.3
Zn_{tot} (M)	1.2×10^{-8}	6.3×10^{-8}	4.1×10^{-8}
Mn_{tot} (M)	2.0×10^{-8}	2.6×10^{-8}	2.6×10^{-7}
Ca_{tot} (M)	7.5×10^{-4}	2.4×10^{-3}	4.0×10^{-4}
Pb_{tot} (M)	1.5×10^{-10}	4.7×10^{-10}	1.3×10^{-9}
Al_{tot} (M)	nd	nd	3.5×10^{-6}
Fe_{tot} (M)	$3 \times 10^{-8*}$	nd	5.2×10^{-6}
DOC (mg/L)	4.7	1.9	15
pH	8.7	8.2	7.9

(* (Emmenegger et al., 1998), nd = parameter not determined)

5.4.2 Cd accumulation from the freshwater sites and culture medium

Cd accumulation in *S. vacuolatus* in the two hardwater and one softwater field samples and the culture medium is compared as a function of pCd (figure 5.1). In the culture medium accumulation is very low (5×10^{-20} mol/cell) at pCd 13.7 and increases linearly from pCd 12.8 (6×10^{-20} mol/cell) to 2×10^{-17} mol/cell at pCd

9.0. From Lake Greifen water Cd accumulated up to 1×10^{-19} mol/cell at ambient pCd 12.7 and increased to 1×10^{-18} mol/cell at pCd 9.5. From Stream Furtbach water Cd was accumulated with 3×10^{-19} mol/cell at the ambient free Cd^{2+} concentration (pCd 14.2) and increased to 1×10^{-18} at pCd 12.2. Accumulation from both hardwater systems is comparable to Cd accumulation from the culture medium with added Cd.

Figure 5.1: Cd accumulation in *S. vacuolatus* from Lake Greifen, Furtbach, River Wyre, and culture medium as a function of pCd. Standard deviations are given when larger than symbol size (n=9). The legend indicates the different water samples.

Cd accumulation from the River Wyre samples differs significantly from the hardwater systems. Cd accumulation increased linearly with increasing pCd, from 3×10^{-20} mol/cell at pCd 11.5 (ambient Cd) to 3×10^{-18} at pCd 7.4, but the amount of Cd accumulated per cell is two orders of magnitude lower than from the hardwater samples and from the culture medium (figure 5.1). To verify the accumulation data, River Wyre was sampled again in September 2005, where similar Cd accumulation data were obtained (see filled triangles in figure 5.1). Thus, the free Cd^{2+} concentration alone does not explain this lower Cd accumulation.

5.4.3 Cd uptake and diffusion fluxes

Cadmium uptake fluxes in *S. vacuolatus* were calculated for the various experimental conditions and compared to the maximal theoretical diffusion flux (figure 5.2). In Lake Greifen and Stream Furtbach *S. vacuolatus* shows similar uptake fluxes as in the culture medium. At similar pCd, uptake fluxes in River Wyre are about one order of magnitude lower than in the hardwater systems. Uptake flux from Lake Greifen water ranged from 5×10^{-15} to 1×10^{-13} mol cm⁻²min⁻¹ in the range of pCd from 12.7 to 9.7 and in Stream Furtbach uptake fluxes range from 5×10^{-15} to 2×10^{-14} in the range of pCd from 14.2 to 12.2. In contrast uptake flux from River Wyre ranged from 2.5×10^{-16} to 4×10^{-14} mol cm⁻²min⁻¹ in the range of pCd from 11.5 to 7.4. Thus, Cd uptake in the River Wyre is clearly not diffusion limited whereas uptake flux from Furtbach is closer to diffusion limitation.

Figure 5.2: Comparison of uptake fluxes in *S. vacuolatus* from Lake Greifen (open rectangles), Furtbach (open squares), River Wyre (open triangles), and the culture medium (filled squares) in function of pCd. The solid line represents the calculated maximal diffusion flux.

5.4.4 Relation of Cd accumulation to speciation methods

Figure 5.3 compares Cd accumulation in *S. vacuolatus* to measured total, labile, and free Cd concentrations. The labile Cd fraction was obtained by in-situ GIME and DGT measurements, and free Cd was measured by HFPLM and DMT. The data obtained from the hardwater systems are in good agreement with the observed Cd accumulation from culture media. All methods determined very low free (<0.001 nM) and labile (< 0.05 nM) Cd fractions in Lake Greifen and Furtbach. Cd accumulation in *S. vacuolatus* from Lake Greifen and Furtbach samples seems to depend on the labile Cd concentrations. In River Wyre, higher free (0.003 nM) and labile (0.1 to 0.2 nM) Cd fractions were determined, with the exception of the free Cd^{2+} concentration data obtained by HFPLM. In this case, measured free Cd^{2+} concentration correlates positively with the Cd accumulation in *S. vacuolatus*. A possible explanation for the increased measured free Cd^{2+} by HFPLM is the presence of a large fraction of CdCO_3° species in the hardwater samples which might diffuse through the HFPLM as described in (Unsworth et al., 2006) and thus increase the fraction of free Cd measured.

Thus, Cd accumulation from the River Wyre samples is in contrast to the total, labile or free Cd concentrations measured by the various analytical speciation methods, with exception of HFPLM.

Figure 5.3: Cd accumulation from Lake Greifen, Furtbach and River Wyre as function of A) labile GIME-Cd, B) labile DGT-Cd, C) free DMT-Cd, and D) free HFPLM-Cd, E) total dissolved Cd.

5.5 Discussion

Cd accumulation was investigated from three freshwater sites with respect to its speciation. Current models propose that metal uptake follows the FIAM or BLM (Campbell, 1995; Campbell et al., 2002; Paquin et al., 2002), which seems to apply only partially to the freshwaters investigated in this work.

5.5.1 Cd accumulation and Cd speciation

Cd accumulation by *S. vacuolatus* as function of the free Cd^{2+} from the hardwater samples is in reasonable agreement to accumulation from the culture medium. For these freshwaters, Cd accumulation indeed seems to follow the FIAM model. The data obtained from the hardwater samples show that the Cd accumulation follows the free Cd^{2+} concentration. In contrast, the two orders of magnitude lower Cd accumulation for *S. vacuolatus* from River Wyre samples (compared to the culture medium and the hardwater samples) is a function of the free Cd, but the lower accumulation (at higher free Cd concentrations) than in the hardwater samples is not in line with the FIAM or BLM model.

The comparison of the Cd uptake fluxes of all accumulation studies presented here to the maximal theoretical diffusion flux clearly shows that the uptake fluxes in the two hardwater sites and the culture medium were similar and closer to diffusion limitation than calculated uptake fluxes for Cd accumulation from River Wyre. Uptake of Cd from Stream Furtbach is closest to diffusion limitation probably because of the very low free Cd^{2+} concentration. The calculated concentration of free Cd^{2+} in the original water sample is 6×10^{-15} M, which is indeed very low. Thus, it seems possible that in this water diffusion limitation conditions are reached and not enough free Cd is provided by the water body within the diffusion layer of the cell membrane. Previous experiments in Stream Furtbach revealed that in case of Cu the free Cu fraction did not explain Cu uptake (Meylan et al., 2003). In this study, the Cu availability for the cells was explained by the contribution of labile (weak) Cu complexes which could dissociate within the diffusion layer of the cell membrane and thus provide copper available for uptake. For Zn the free Zn concentration was found to be sufficient to explain Zn uptake (Meylan et al., 2003). In previous studies, Cd was found to be complexed by strong organic ligands with a higher

complexing capacity whereas Zn was found to be complexed to a lesser degree (Xue and Sigg, 1998) in some hardwater lakes including Lake Greifen. As lakes are not subject to dramatic changes in terms of ligand stability and metal concentrations, this might explain the low free ambient Cd^{2+} concentrations in the hardwater systems.

Uptake flux calculations of Cd in *S. vacuolatus* from River Wyre samples showed that Cd uptake is clearly not diffusion limited. However, though Cd accumulation followed the free Cd^{2+} concentrations, the accumulated Cd in alga is much lower compared to the predicted accumulation by the FIAM. The discrepancy between the low Cd accumulation in *S. vacuolatus* compared to the high measured free and labile Cd species in River Wyre shows that the chemical speciation methods do not help to relate Cd accumulation from River Wyre to Cd accumulation from the other field sites. Other factors seem to control Cd availability for *S. vacuolatus* from River Wyre. Cd uptake might be influenced by (i) competing cations (Mn, Zn, Pb) and pH, (ii) the presence and composition of organic matter (humic acids), and (iii) the formation of iron colloids in the water.

5.5.2 Influence of competing cations and pH on Cd accumulation

According to findings in chapter 3, measured Cd accumulation was compared with calculated Cd accumulation using equation (4) from chapter 3.3.5 by taking into account competition by metals and H^+ :

$$\{Cd_{int}\} = kK_{Cd}\{X_T\} \cdot \frac{[Cd^{2+}]}{1 + K_{Cd}[Cd^{2+}] + K_{Me}[Me^{2+}] + K_H[H^+]} \quad (5.3)$$

where Me^{2+} represents Zn, Mn, and Pb.

Competition with these cations was assessed by calculating competition for all parameters available (figure 5.4). Measured parameters for pH and total metal concentration were taken from table 5.1, parameters for the binding sites available (X_t) and values for K_{Zn} were taken from previous experiments (see Chapter 3). For K_{Pb} and K_{Mn} values were estimated from the literature (5×10^5 for Pb (Slaveykova et al., 2003) and 1×10^7 for Mn (Sunda and Huntsman, 1996)). For proton binding sites on the cell surface, a constant K_H of 1×10^9 was estimated.

This constant K_H seems high compared to a constant which takes into account the carboxylic groups on the cell surface. A constant of 1×10^9 may take into account sulfhydryl groups on the cell surface, and amino groups. Cd is known to bind preferentially to these groups as they exhibit a strong binding capacity for Cd.

Cation competition was calculated in several steps, first on the basis of competition of Cd with Zn, Mn, Pb, or H alone. In a second step two competing cations were taken into account and finally competition was modelled with all cations involved.

Calculations revealed that protons are the strongest competitor for uptake sites and predicted the lowest Cd accumulation (figure 5.4). The introduction of Zn competition for uptake sites further enhanced the competition whereas competition with Mn or Pb was not relevant. Modelled Cd accumulation was found to decrease most (about one order of magnitude) when all competing cations were taken into account. From these findings it can be concluded that the lower pH and higher free Zn concentrations in River Wyre contributed to decreased Cd uptake.

Figure 5.4: Modelling of Cd uptake in competition with Zn, Mn, Pb, and H. The legend indicates the cations taken into account (Pb not shown). The open squares show measured Cd accumulation from the culture medium, and the filled squares Cd accumulation from River Wyre. Total metal concentrations are indicated in table 5.1.

5.5.3 Effects of dissolved organic carbon and colloidal iron on Cd accumulation

The high organic matter contents in the River Wyre compared to Lake Greifen and Furtbach were presumed to induce changes in the Cd availability for the algae. However, from results presented in chapter 4, the DOC, (which is assumed to consist largely of fulvic acid (Sigg et al., 2006)), was not expected to influence the Cd accumulation other than by controlling the free metal concentration in the water (see section 4.5.1 in chapter 4). The total DOC concentration measured in River Wyre samples were reported to be in the same range as concentrations investigated under laboratory conditions in Chapter 4. Liquid chromatography coupled with organic carbon detection (LC-OCD (Huber and Frimmel, 1992)) of the River Wyre and Lake Greifen water samples revealed, that the composition of the DOC in River Wyre strongly differs from

that of Lake Greifen (figure 5.5). A 5 times higher humic substances fraction and a 10 times higher fraction of low molecular weight acids and humics was found in the River Wyre samples whereas in Lake Greifen a higher amount of polysaccharides was found. The high amounts of humic substances in River Wyre suggest that the composition of the DOC itself might influence metal uptake in organisms by interfering with the metal binding on the cells surface.

The low available free Cd concentration in Lake Greifen could be partially explained by Cd binding to these polysaccharides (Lamelas et al., 2005). The LC-DOC measurements also revealed a high fraction of colloids present in the River Wyre samples, (data not shown). From the high total iron concentrations measured, Unsworth et al. (2006) described the colloids mainly as iron colloids while Mn-colloids and Al-colloids play no substantial role. In Lake Greifen and Stream Furtbach iron is only present in small amounts and is thus not considered of importance in forming colloids.

Thermodynamic calculations by WHAM showed that the formation of Fe-colloids in River Wyre leads to binding of Pb on their surface whereas binding of Cd onto the colloids was found to be of minor importance compared to Cd binding by fulvic acids (Unsworth et al., 2006).

Figure 5.5: LC-DOC measurements from River Wyre and Lake Greifen samples. The different measured fractions are indicated by arrows.

Freshly precipitated iron colloids are reported to be approximately spherical and quite small, with particle sizes ranging from 1 to 10 nanometres (Dzombak and Morel, 1990; Tipping, 1981), which means that they are able to pass the membrane filter of 0.45 μm easily. The colloids might thus be able to interfere with the metal binding at the cell membrane in such way that further metal accumulation is inhibited. Therefore, interactions of the iron colloids with the cell membrane and a subsequent change in uptake behaviour seem possible.

5.5.4 Implications for bioavailability of metals under freshwater conditions

Experiments presented here show that the FIAM is able to accurately predict the accumulation of cadmium for hardwater systems with low DOC contents. However, in softwater systems with high DOC and colloidal iron contents, FIAM failed to predict the accumulation and overestimated the bioavailable fraction of Cd.

From the results presented here, it can be summarized that cation competition in combination with a low pH influences metal uptake which is taken into account in the FIAM or BLM (Campbell, 1995; Slaveykova and Wilkinson, 2005). One example of competition of protons with Cd for uptake sites has been shown for *Chaoborus* (Hare and Tessier, 1996; Orvoine et al., 2006).

The actual composition of DOC seems to interact more strongly with metal accumulation than anticipated by the current models describing metal accumulation in the presence of biotic ligands (Luoma and Rainbow, 2005; Slaveykova and Wilkinson, 2005). In systems with a substantial amount of iron, the formation of colloids should be considered as it cannot be excluded that they might interact with the organisms' cell surface, resulting in a decrease of metal availability for the cells.

Recent theoretical and experimental work indicates that the consideration of metal species fluxes for determining metal bioavailability helps to improve current availability models (Galceran and van Leeuwen, 2004; Meylan et al., 2004a; van Leeuwen, 1999). However, relationships between the parameters measured by the various methods presented here and the available fraction of metals for aquatic organisms have to be further developed as the analytical methods failed to predict the Cd accumulation under conditions encountered in River Wyre. More information on competing cations, DOC composition and on

the presence of colloids is important to evaluate the potential accumulation of Cd.

6

Outlook

This study presents evidence that the Cd speciation controls the Cd bioavailability for *Scenedesmus vacuolatus*. It was shown that competition with other cations and the presence of fulvic acids influences Cd accumulation from culture media and that both can be predicted by a FIAM and BLM approach. In hardwaters, metal prediction by FIAM followed the free Cd concentration whereas in softwaters Cd availability is overestimated by the chemical speciation methods as well as by the uptake models. In freshwaters, especially softwater systems, other parameters such as pH, the occurrence and composition of DOC, and the presence of colloids have to be taken into account to accurately predict Cd availability.

To better understand the influence of these other parameters, further studies covering a range of hard- and softwater conditions would improve the understanding which factors have the strongest impact in each system. Up to now, accumulation experiments were performed in synthetic media designed to match hardwater conditions. Experiments with softwater media would allow the evaluation of factors relevant for metal uptake from softwater systems. Consequently, chemically controlled accumulation experiments with synthetic softwater media are proposed to study the influence of humic acids and/or varying iron concentrations on metal uptake in algae.

The collection of a significant amount of field data similar to those described in chapter 5 is needed. Both hardwater and softwater sites should be more thoroughly investigated to be able to underpin the presented results. Furthermore, metal competition and toxicity models could be validated by investigating other metals. The necessity of the adaptation of water quality criteria to the properties of each water system would be emphasized.

More research on interactions of DOC and colloids with cell surfaces is necessary. As DOC was reported to influence metal speciation and accumulation to varying degrees, a better characterization of the DOC itself and of its interactions with algae cell surfaces would help to understand differences of

metal accumulation behavior from waters of different origin. Specifically, the interaction of the different fractions of DOC or of colloids with the cell surfaces should be more thoroughly assessed.

Finally, the development of a metal accumulation sensor using microorganisms that could be exposed in-situ at the same time with speciation measurement devices is proposed. Such sensors would have the advantage that the algae would be grown under standard conditions and could be exposed in situ for well defined amounts of time. The application of a standard exposure sensor would allow comparison of in-situ speciation to in-situ metal accumulation determinations between different freshwater systems.

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Curriculum Vitae

Stefanie Töpperwien

Born on July 25th, 1974

in Northeim, Germany

1981 - 1985	Primary school in Westerhof, Germany
1985 - 1987	Secondary school in Kalefeld, Germany
1987 - 1994	High school in Northeim, Germany
1995 - 2001	Diploma in Agrarbiologie (Dipl.-Agr. Biol.) at the University of Hohenheim, Germany
1999 - 2000	Diplomarbeit at the University of Hohenheim and Hans Merensky Technical Services, Tzaneen, Republic of South Africa
2001 - 2001	Internship at the Swiss Federal Institute for Forest, Snow, and Landscape (WSL), Birmensdorf, Switzerland
2002 - 2006	PhD thesis at the Swiss Federal Institute for Environmental Sciences and Technology (Eawag) and the Swiss Federal Institute of Technology Zurich (ETHZ)
2002 - 2005	Teaching assistant in aquatic chemistry at the ETHZ

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