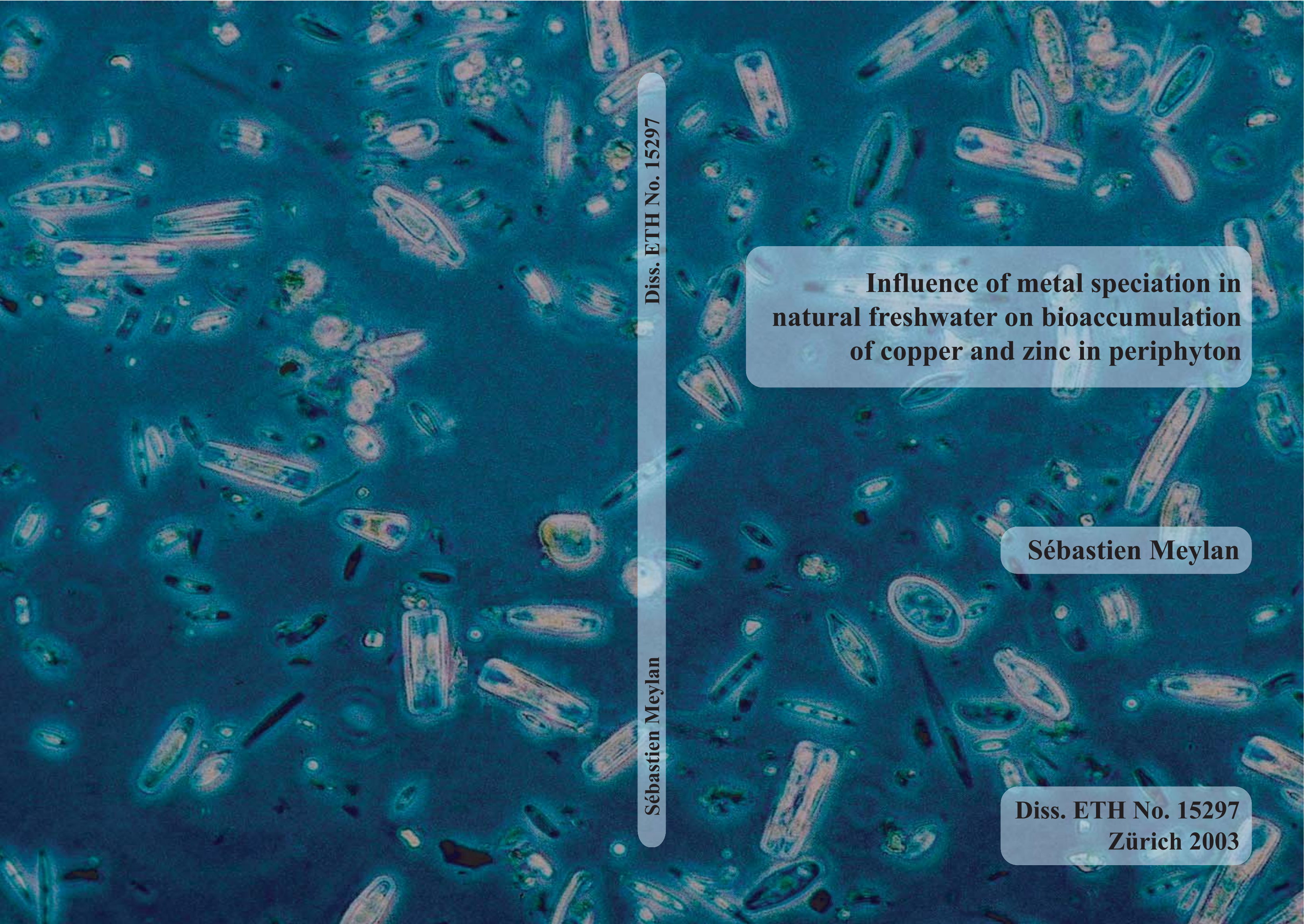

Influence of metal speciation in natural freshwater on bioaccumulation of copper and zinc in periphyton

Sébastien Meylan

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INFLUENCE OF METAL SPECIATION IN NATURAL FRESHWATER ON BIOACCUMULATION OF COPPER AND ZINC IN PERIPHYTON

A dissertation submitted to the
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presented by

Sébastien Meylan

Dipl. Chem. Eng. EPFL
born December 30, 1973
Citizen of Le Lieu (VD), Switzerland

Accepted on the recommendation of
Prof. Laura Sigg, examiner
Prof. Alexander J.B. Zehnder, co-examiner
Prof. Peter G.C. Campbell, co-examiner
Dr. Renata Behra, co-examiner

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Cover: Microscopic photography of periphyton.

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Summary

Copper and zinc are essential trace metals for microorganisms however they are toxic at high concentrations. Metal speciation plays a fundamental role in the bioavailability of copper and zinc, as metals strongly complexed to organic ligands are not bioavailable. Under laboratory conditions, metal bioavailability appears to be generally controlled by the free metal ion concentration, following the free ion activity model (FIAM). Nevertheless, there is a lack of data comparing metal speciation and metal bioaccumulation under natural conditions. Indeed, the presence of large quantities of organic ligands, such as humic and fulvic acids, in natural water drastically complicates the determination of metal speciation. The goals of this work were to study how copper and zinc speciation in natural freshwaters influence the bioaccumulation of these metals in periphyton and to establish if the FIAM is applicable under field conditions. Periphyton, a natural algal biofilm which grows on submerged solid substrates and which plays a preponderant ecological role in natural surface waters, was used as model for an algal community in this study.

In the Furtbach stream (Switzerland), total dissolved copper and zinc concentrations increased drastically during rain events due to the release of metals from contaminated sediments. Metal speciation and bioaccumulation in periphyton were measured over several days during rain events when variations of metal concentration occur. Dissolved metal in the stream increased to maxima of 118 nM Cu and 147 nM Zn. Increases in free copper and zinc ion concentrations and variations in labile metal concentrations in the water were also recorded. Periphyton was collected from glass slides colonized by periphytic communities and fixed to holders directly in the stream. Metal content in periphyton responded rapidly to metal concentration variations in the water. The bioaccumulation of zinc appeared to be controlled by the free metal ion, following the FIAM prediction, whereas a labile fraction of copper (fraction detected by voltammetry after exchanging with catechol) appeared to control the intracellular copper content in periphyton.

These relationships were also investigated in a microcosm study performed with natural river water. This approach allows examining bioaccumulation in identical periphytic communities under controlled conditions but with different copper and zinc concentrations and with different metal speciation. This study supported the observations made in a variable environment, namely that zinc accumulation in periphyton follows the FIAM while labile copper species control copper accumulation in periphyton. This difference is explained by the high extent of copper complexation (>99.99% complexed) which implies that only a small amount of copper is free. The diffusion fluxes of free copper ions from the bulk to the algal cells were estimated and compared to uptake fluxes in periphyton. It showed that the diffusion is largely limiting at environmental copper concentrations. Under these conditions, weakly complexed copper controls the uptake of this essential trace metal by providing metal at the cell membrane.

An important part of this work focused on the evaluation of techniques to determine metal speciation in natural water. An in situ technique, diffusive gradients in thin films (DGT), a laboratory technique, competitive ligand exchange followed by voltammetric measurement, and three speciation programs using models for the complexation by humic and fulvic substances, namely Model VI (WHAM), NICA-Donnan and SHM were compared. The DGT-labile copper and zinc measurements showed significant correlations with labile metal measured by voltammetry. These two techniques measured similar fractions of the total dissolved copper and zinc concentrations. The speciation programs were able to relatively accurately predict zinc speciation whereas the calculated free copper ion concentrations were largely overestimated because these models underestimate the effects of strong copper-binding organic ligands present in natural water.

The results obtained in this study contribute to the comprehension of the concept of metal bioavailability in natural water systems and confirm the importance of metal speciation in estimating the impact of metals on aquatic organisms in natural freshwater. Metal speciation should thus be considered when defining relevant water quality criteria in the future.

Résumé

Le cuivre et le zinc sont des métaux traces essentiels pour les microorganismes, cependant, ils sont toxiques à concentrations élevées. La spéciation des métaux joue un rôle fondamental pour la biodisponibilité du cuivre et du zinc car les métaux fortement complexés à des ligands organiques ne sont pas biodisponibles. Les études effectuées en laboratoire ont montré que la biodisponibilité des métaux est généralement contrôlée par la concentration en ion métallique libre et suit le modèle de l'activité de l'ion libre (free ion activity model - FIAM). Néanmoins, des données comparant la spéciation et la bioaccumulation des métaux sous conditions naturelles manquent. En effet, la présence de grandes quantités de ligands organiques tels que des acides humiques ou fulviques, dans les eaux naturelles complique la détermination de la spéciation des métaux. Les buts de ce travail ont été d'étudier comment la spéciation du cuivre et du zinc en eaux naturelles influence la bioaccumulation de ces métaux dans le périphyton et d'établir si le FIAM est applicable en conditions de terrain. Le périphyton, un biofilm naturel d'algues qui pousse sur les supports solides submergés et qui joue un rôle écologique prépondérant en eaux naturelles de surface, a été utilisé comme modèle pour une communauté d'algues dans cette étude.

Dans le ruisseau Furtbach (Suisse), les concentrations dissoutes totales de cuivre et de zinc augmentent drastiquement lors d'événements de pluie à cause de la libération de métaux provenant de sédiments contaminés. La spéciation et la bioaccumulation dans le périphyton des métaux ont été mesurées pendant plusieurs jours lors de pluies lorsque ces variations de concentrations surviennent. Les métaux dissous dans le ruisseau atteignent des maxima de 118 nM Cu et 147 nM Zn. Les augmentations de concentrations en ion métallique libre et les variations en cuivre et zinc labile dans l'eau ont également été mesurées. Le périphyton a été échantillonné à partir de lamelles de verres colonisées par des communautés périphytiques, les lamelles étant fixées à des supports directement dans le ruisseau. Les métaux contenus dans le périphyton ont montré une rapide réponse aux variations de concentrations dans l'eau. La bioaccumulation du zinc apparaissait être contrôlée par l'ion métallique libre, corroborant les prédictions du FIAM alors que la fraction labile du cuivre (fraction détectée par voltamétrie après échange avec du catéchol) contrôlait le cuivre contenu intracellulairement dans le périphyton.

Ces relations ont également été examinées dans une étude effectuée en microcosme dans de l'eau naturelle de rivière. Cette approche permet l'utilisation de communautés périphytiques identiques sous conditions contrôlées mais ayant différentes concentrations en cuivre et en zinc et différentes spéciations. Cette étude supporte les observations faites dans un environnement variable c'est-à-dire que l'accumulation en zinc dans le périphyton suit le FIAM tandis que les espèces labiles du cuivre contrôlent l'accumulation en cuivre dans le périphyton. Cette différence est expliquée par l'ampleur de la complexation du cuivre (>99.99% complexé) ce qui implique que seul une faible quantité de cuivre est libre. Les flux de diffusion de l'ion libre du cuivre de la solution jusqu'à la cellule d'algue ont été estimés et comparés aux flux d'assimilation dans le periphyton. Cela a montré que la diffusion est largement limitante aux concentrations en cuivre rencontrées dans l'environnement. Sous ces conditions, le cuivre faiblement complexé contrôle l'assimilation de ce métal trace essentiel en fournissant du métal à la membrane de la cellule.

Une partie importante de ce travail s'est concentrée sur l'évaluation de techniques déterminant la spéciation en métal en eaux naturelles. Une technique in situ, gradients diffusifs en films fins (diffusive gradients in thin films - DGT), une technique de laboratoire, l'échange compétitif de ligand suivi d'une mesure voltamétrique et trois programmes de spéciation incorporant des modèles prenant en compte la complexation des métaux par des substances humiques et fulviques, soit le Model VI (WHAM), le NICA-Donnan et le SHM ont été comparés. Les mesures de cuivre et de zinc DGT-labile ont montré des corrélations significatives avec le métal labile mesuré par voltamétrie. Ces deux techniques ont mesuré des fractions similaires par rapport aux concentrations totales dissoutes de cuivre et zinc. Les programmes de spéciation ont été capables de prédire relativement précisément la spéciation du zinc alors que les concentrations en ion libre de cuivre calculées ont été largement surestimées car ces modèles sous-estiment les effets des ligands organiques forts liant le cuivre présent dans les eaux naturelles.

Les résultats obtenus dans cette étude contribuent à la compréhension du concept de biodisponibilité des métaux en eaux naturelles. Ils confirment également l'importance de la spéciation des métaux pour l'estimation de l'impact des métaux sur les organismes aquatiques en eaux naturelles. La spéciation des métaux devrait donc être prise en compte lors de la définition des critères de qualité pour les eaux dans le futur.

1

Introduction

1.1 Overview

Trace metals such as iron, copper, zinc, manganese, cobalt, nickel and molybdenum are essential micronutrients for algal communities, whereas others like lead, cadmium, silver, and mercury are not required. The need for trace metals is due to their function in organisms; they are essential for the catalysis of redox reactions, electron transport, structural functions in nucleic acid metabolism and as cofactor of various enzymes (Lehninger, 1982). Each algal species shows specific requirements in term of trace metal concentration for optimal development. Nevertheless, at high concentrations these trace metals are becoming toxic for algae (Sunda, 1989; Sunda and Huntsman, 1998).

In freshwater, trace metal ions are available under different chemical species. These ions are either under a free metal ion form or complexed by chemical species such as inorganic anions (HCO_3^-, CO_3^{2-}, SO_4^{2-}, Cl^-,…), organic ligands (amino acids, fulvic acids, humic acids,…), and particle surfaces (Fe-oxides, biological material,…). Most of these species are not directly taken up by the aquatic microorganisms and only a small fraction of metal interacts with the organisms. The bioavailability of the metal is thus controlled by the metal speciation (Luoma, 1983; Morel and Hering, 1993; Campbell, 1995; Sunda and Huntsman, 1998).

Laboratory studies suggest that growth and toxicity are mainly a function of the activity of the free metal ion, which inspired the development of the free ion activity model (FIAM) (Morel and Hering, 1993; Campbell, 1995). The FIAM has been confirmed for a number of different metals (Cu, Zn, Mn, Fe, Cd), organisms, and biological responses (growth, uptake, nutrient limitation, toxicity). However, under natural conditions, where natural organic ligands modify considerably the speciation of metals, there is a lack of data on the interactions between metal speciation and effects on algal communities.

The aim of this study is to fill this gap and provide relevant studies on the relationship between metal accumulation in periphyton, a natural algal biofilm, and metal speciation under field conditions.

1.2 Metal speciation in natural freshwater

In natural waters, metals are present under different forms, namely in particulate form, bound to strong or weak organic ligands, bound to inorganic ligands or as free metal ions (Buffle, 1988; Sigg and Xue, 1994). All these metal species are following chemical equilibria that regulate the concentration of the free metal ion as well as all the metal complexes.

In synthetic media with defined chemical composition, chemical equilibrium programs allow the calculation of the different metal species in solution. In natural freshwater, where many components are unknown and where undefined organic ligands are present, such calculations are not possible and free metal ion and other metal species need to be measured. Several methods are available to determine free metal ion concentrations in water. As an example, the ionic selective electrodes (ISE), which measure specifically the activity of the free ion for one metal, is used to investigate relatively high free metal ion concentration (Xue and Sunda, 1997; Rozan et al., 1999; Eriksen et al., 2001). To measure lower free metal ion concentrations, an indirect method, the competitive ligand exchange (CLE) coupled with differential pulse anodic/cathodic stripping voltammetry (DPASV/DPCSV) is generally used (Donat and van den Berg, 1992; Xue and Sigg, 1993; Xue and Sigg, 1994; Xue and Sigg, 2002b). It consists in the addition of a known ligand to the natural water, followed by the measurement of the labile metal concentration, which, with the repetition of this process at different added metal concentrations, allows the calculation of the free metal ion concentration. Whereas the two previous methods are laboratory methods, in situ techniques are as well available but they are usually only measuring the labile fraction of the metal. The diffusive gradients in thin films (DGT) is an in situ technique used to measure over a period of time an integrated value of the dissolved labile metal.

Typical background total dissolved concentrations of copper and zinc found in natural freshwater are 4–24 nM Cu and 10-40 nM Zn in lakes (Borg, 1983; Xue and Sigg, 1994; Xue et al., 1995a; Xue et al., 1996) and 3-31 nM Cu and 11-51 nM Zn in non-contaminated rivers whereas background free metal ion concentrations measured with CLE-DPASV/DPCSV have values, for Cu, ranging from 10^{-16} to $10^{-12.9}$ M (Xue and Sigg, 1993; Xue et al., 1995b; Xue et al., 1996) in river and lakes and, for Zn, ranging from 0.3 to 5.6 nM in freshwater (Xue and Sigg, 1994; Xue et al., 1995a).

1.3 Interactions between metal and aquatic organisms

Interactions of trace metals with organisms are very complex. Globally, the internalization of a metal is divided in two steps. First the metal, complexed or not, present in the water diffuses through the diffusive boundary layer to the cell and binds following chemical equilibrium with the active sites of the cell membrane. Second, uptake of the metal by the cell occurs and the biological utilization of the metal or its accumulation in the cell follows (Tessier and Turner, 1995; Wilkinson et al., 2002).

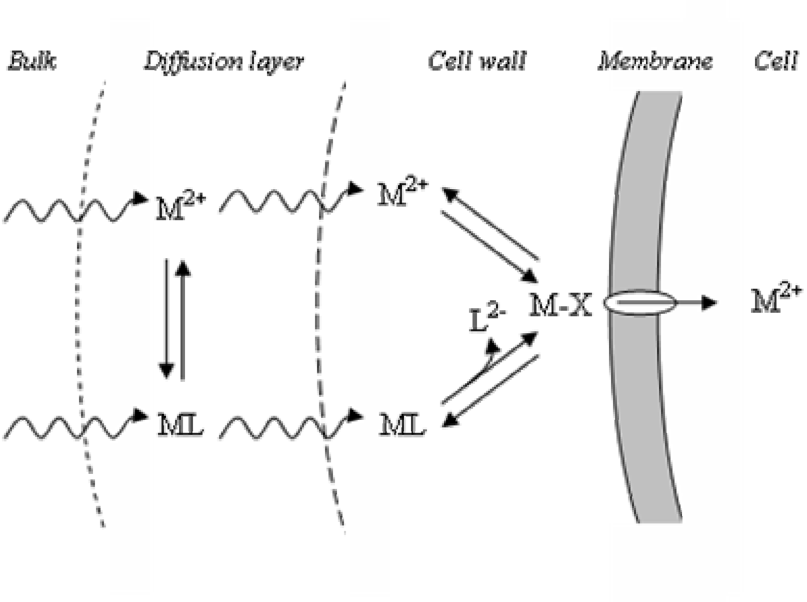


Figure 1: Conceptual model of metal-organism interactions (after Campbell, 1995)

The Free Ion Activity Model (FIAM) was developed based on a convincing body of evidence that the free metal ion concentration is the main factor influencing the bioavailability of the metal and not the total dissolved metal concentration (Morel and Hering, 1993; Campbell, 1995). The applicability of this model has been shown in laboratory experiments with various algal species and various metals (Sunda and Guillard, 1976; Anderson et al., 1978; Petersen, 1982; Sunda and Huntsman, 1992; Knauer et al., 1997b). The goal of this model is to predict the bioavailability of a metal as a function of its metal speciation in water. The formulation of the FIAM and the assumptions underlying it are precisely described by Campbell (1995). Considering the interaction of a free metal ion M^{2+} or a metal-complex ML with the cell membrane forming a M-X-Cell complex (Figure 1), the concentration of the surface complex [M-X-Cell] is proportional to the free metal ion concentration independently to the metal species considered. Assuming that [M-X-Cell] is proportional to the biological response, the M^{2+} concentration is the factor influencing the response of the organism. The metal transport across the cell membrane occurs through physiologically active sites (Simkiss and Taylor, 1995; Hudson, 1998; Bell et al., 2002).

Since the development of the FIAM, limitations and exceptions to the model were rapidly reported. These exceptions were categorized depending on the type of ligand influencing the discrepancy (Campbell, 1995), namely organic ligands (forming lipophilic complexes with the metal), inorganic anions and low molecular weight organic ligands (forming hydrophilic complexes with the metal). These metal-ligand complexes are internalized in the cell using no or other transporters than the conventional metal transporter. Studies focusing on these exceptions reported the possibility of an accidental cadmium- or zinc-citrate complex uptake in presence of citrate where the anionic metal-complex is transported which increase metal uptake (Errécalde et al., 1998; Errécalde and Campbell, 2000). Similar enhanced metal uptake by a green alga was found for silver in presence of thiosulfate (Fortin and Campbell, 2001). In natural systems, these exceptions are not very relevant as they have a very low probability to occur, nevertheless they show the complexity of the interactions between metal and aquatic organisms.

The free ion activity model was developed based on results obtained in laboratory studies and was mostly focusing on the metal speciation. However, the FIAM was not sufficiently focusing on the cell membrane and its interactions with the different components of the water which is essential to have a precise model of the metal bioavailability. In natural systems, the water chemistry is much more complex than in synthetic media. The presence of humic substances offering a large range of binding sites for dissolved metal and influencing considerably the metal speciation is incontestable (Buffle, 1984). Furthermore, major metals interact with the cell and influence the number of sites available for the trace metal to bind to the membrane. Based on the FIAM and on the Gill Surface Interaction Model (GSIM) (Pagenkopf, 1983), the Biotic Ligand Model (BLM) introduces the concept of biotic ligand. This ligand corresponds to the site of action where the metal binds at the cell surface prior to its internalization and thus the occurrence of toxicity (Di Toro et al., 2001). This model combines the competition between the biotic ligand and environmental ligands such as natural organic matter for available metal ions, which include not only the trace metal ion of interest but also major cations such as Ca^{2+}, Mg^{2+} or even H^+ that can bind with the biotic ligands (Figure 2). The formulation and the assumption of the BLM are very similar to the FIAM but consider competing cations and competing ligands (Di Toro et al., 2001; Bell et al., 2002; Campbell et al., 2002).

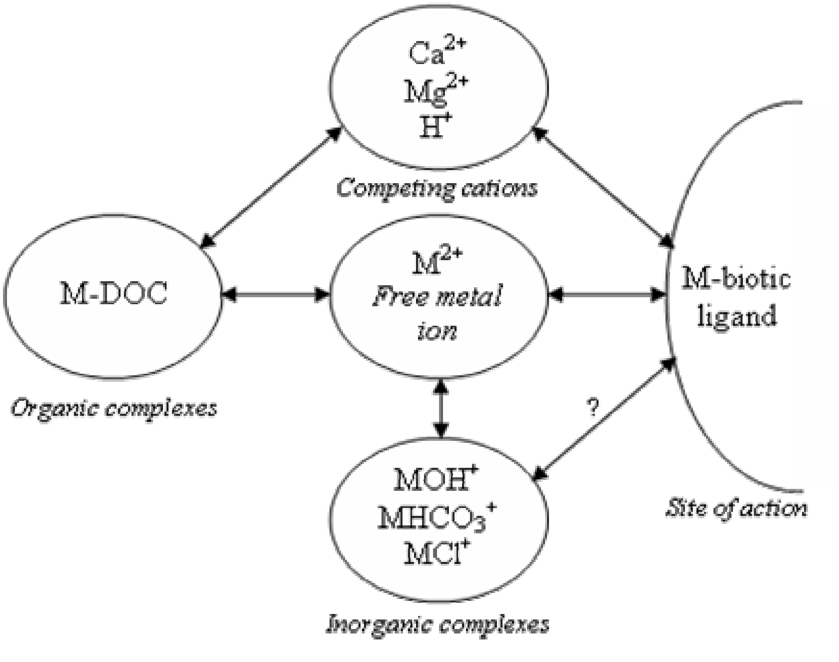


Figure 2: Conceptual diagram of the Biotic Ligand Model (BLM) (after Di Toro et al., 2001)

Assumptions at the basis of the BLM were recently discussed (Campbell et al., 2002) and limitations of the model were emphasized. A critical point for the BLM is the case of the rate-limiting internalization. As an example, total dissolved silver concentration and not free silver ion concentration was found to control silver uptake by *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). Considering that there is no diffusion limitation from the bulk solution to the cell wall (Figure 1), the BLM assumes that the metal concentration at the cell wall is at the equilibrium with the biotic ligand. Indeed, two types of controls (Figure 3) are considered at the cell wall (Hudson, 1998). In the case of an "equilibrium control", rates of formation and dissociation of M-X-Cell with M^{2+} are equal and much greater than the uptake rate. Internalization is then controlled by the equilibrium between metal bound to the biotic ligand and free metal ion concentration. In the case of a "kinetic control", the rates of formation and internalization are nearly equal and much larger than the dissociation rate. This situation would imply that the metal bound to the biotic ligand is immediately internalized and that uptake is faster than supply of free M^{2+} from the solution. The possibility of uptake rate limitation due to kinetic control was raised in a previous study (Hudson, 1998). The limits of these models and the possibility of a diffusion limitation of the metal was treated theoretically in a study comparing the diffusion and uptake fluxes under different conditions (van Leeuwen, 1999). It was shown that, in case of diffusion limitation, the uptake was controlled by a labile form of the metal.

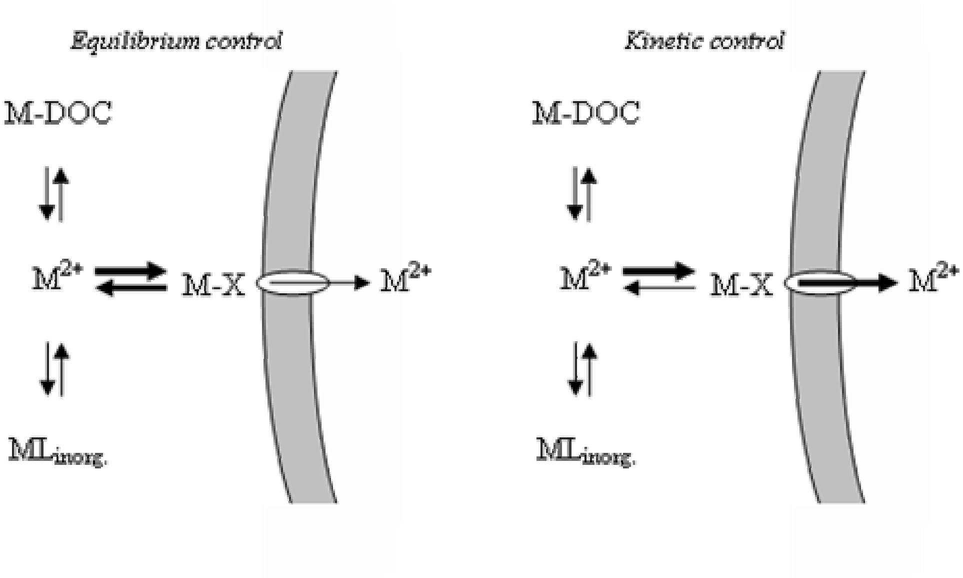


Figure 3: Two limiting cases for control of metal binding to transporters (after Hudson, 1998)

1.4 Significance of periphyton for metal uptake studies

Periphyton, a natural biofilm growing on submerged solid substrates in natural waters, is an ecologically important consortium of microorganisms composed by mainly algae, but also fungi, bacteria and protozoa. All organisms within the community are not necessarily attached but some may live within the community structure of the attached forms (Wetzel, 1983). The periphyton and the plankton are the principal primary producers in the aquatic environment converting nutrients into organic material (Blanck, 1985). In shallow lakes, ponds and rivers, periphyton is even the predominant primary producer (Lewis, 1995; Stevenson et al., 1996). Like all aquatic plants, periphyton is important in oxygen production, nutrient cycling, sediment stabilization, it provides habitat and shelter for aquatic life and food for grazers and herbivores. Periphyton is found in the photic zones of streams and rivers, lakes, wetlands and estuarine and marine systems.

The capacity of periphyton and other algae to take up heavy metal or organic pollutants from the water, producing an internal concentration greater than in their surrounding has been shown in several studies (Newman and McIntosh, 1989; Liehr et al., 1994; Farag et al., 1998; Wright and Mason, 1999; Behra et al., 2002). It has been shown that metal contents in the organisms may be measured even when ambient concentrations are very low (Friant and Koerner, 1981; Smith and Kwan, 1989). Accumulation factors (intracellular concentrations with respect to the metal concentration in water) up to several thousands have been reported (Johnson et al., 1978; Ramelow et al., 1987). Whereas direct chemical analyses of water have been widely carried out, it has frequently been suggested that biotic metal content measurements may give valuable information about metal bioavailability in the surrounding water (Whitton, 1984). Furthermore, it has been suggested that trace metal concentrations in algae are not so subject to erratic fluctuations of metals in water, but integrate the metal concentrations in water over a short period of time. For all these reasons, periphyton has been suggested to be adequate as biomonitor of metal contamination.

Because of differences in sensitivity between the different species in periphyton, the presence of increased metal concentrations in the environment may result in a selection, which favors metal tolerant organisms. Studies using periphytic communities showed that long-term exposure to metals results in the replacement of sensitive algal species by tolerant ones and that these structural changes are accompanied by an increase of the community tolerance to metals (Blanck et al., 1988; Blanck and Dahl, 1996; Knauer et al., 1999; Soldo and Behra, 2000).

The ecological importance of periphyton and its sensitivity to changes in metal concentration in natural water means that field experiments using this consortium of microorganisms as biological material are ecologically significant and particularly relevant. Although influence of metals on single algal species has been considerably studied, periphyton remains mainly to discover and many questions remain open.

1.5 Scope of this work

In order to better understand the ecotoxicological effects of metals on organisms in natural freshwater this work focuses on the influence of the speciation of two metals, copper and zinc, on their bioaccumulation in periphyton in natural freshwater. Whereas these relationships have been studied relatively extensively with culture algae in laboratory (Knauer, 1996), there are still many points concerning cause and effect relationships between metals and aquatic microorganisms that remain unclear in natural systems where the metal speciation is largely influenced by natural organic ligands.

The goal is to find out if the free metal ion concentration controls metal bioaccumulation in natural freshwater and thus if the free ion activity model (FIAM) is applicable under natural conditions.

Copper and zinc have been chosen as metals to be studied because they are both essential for microorganisms but toxic at too high concentrations. Furthermore, they present large difference in their speciation in natural water as copper is mostly complexed to ligands in freshwater and only a small fraction of copper is under the free Cu^{2+} form (<0.01%) (Xue and Sigg, 1993), whereas zinc usually comprises a relatively large proportion of metal under a free form with between 1% to 10% of Zn^{2+} in natural water (Xue and Sigg, 1994).

Such data are essential to better understand the impact of changes in environmental metal concentration on aquatic microorganisms with respect to changes in metal bioavailability. The question is crucial to define relevant water quality criteria for trace metals that take metal speciation into account.

1.6 Outline of the Thesis

Chapter 2 presents a survey of the total dissolved copper and zinc concentrations in rivers and streams from the North-East of Switzerland. This survey was undertaken to find appropriate study sites where to perform experiments in natural freshwater. Criteria that were influencing the choice of the experimental sites are explained and the sites are compared to each other from an environmental perspective.

Chapter 3 attempts to validate the method used to measure the intracellular metal content of periphyton. This method has been previously used in laboratory studies, but its ability to distinguish between intracellular metal and metal adsorbed on the cells and its use in natural systems still remains controversial. Experiments performed to confirm the applicability of the EDTA-wash method to natural algal biofilms are described.

Chapter 4 examines the changes of the metal content in periphyton during dynamic variations of the metal speciation in a stream during rain events. This is the first study that presents simultaneous results of metal speciation and metal bioaccumulation in an algal community and it shows clear indications that the FIAM is applicable in natural water for zinc whereas it is not for copper. Furthermore, these data indicate how environmental variations such as weather conditions or seasonal variations can influence metal content in aquatic microorganisms. This chapter was published in ES&T (Meylan et al., 2003).

Chapter 5 reports on a microcosm study designed to experimentally examine the relationship between copper and zinc speciation and metal bioaccumulation in freshwater. The speciation of the metals was modified by adding metal and/or an organic ligand to the natural water. The results of this study showed clear evidence that diffusion limitation under environmental conditions could lead to the non-applicability of the FIAM, which was the case for copper. This chapter was accepted for publication in ES&T (Meylan et al., 2004a).

Chapter 6 focuses on the techniques used in this study to determine the metal speciation in natural water. The measurements performed with the diffusive gradients in thin films (DGT), a recently developed in situ technique, and with competitive ligand exchange followed by voltammetric measurements, a laboratory technique are compared. Furthermore, the prediction power of three recent speciation software using models for humic substances (WHAM, NICA-Donnan, SHM) was tested in comparison with experimental data. This chapter was published in Analytica Chimica Acta (Meylan et al., 2004b).

2

Selection of study sites in Eastern Switzerland for copper and zinc experiments under field conditions

2.1 Introduction

A survey of the concentrations of copper and zinc observed in small river and streams has been undertaken in the frame of this thesis to identify sites in Switzerland that could be contaminated with these trace metals. Some studies already reported copper and zinc concentrations measured in Swiss water systems. Background concentrations were found in the ranges of 4–24 nM Cu and 10-40 nM Zn in Swiss lakes (Xue and Sigg, 1994; Xue et al., 1995a; Xue et al., 1996) and 3-31 nM Cu and 11-51 nM Zn in non-contaminated rivers whereas in moderately contaminated rivers up to 150 nM Cu and 560 nM Zn are observed (Xue et al., 1996; Sigg et al., 2000; Behra et al., 2002).

The goal of this survey was to find appropriate sites to undertake studies on copper and zinc bioaccumulation in periphyton and on metal speciation in natural freshwaters. Rivers and streams were considered as potential study sites and several criteria had to be met requirements for the site to be selected. The metal concentration had to be relatively high to ensure a detectable response of the metal accumulation in periphyton. The pH had to present values normally found in river system as a too low (<7.0) or too high (>9.0) values would have biased significantly the metal speciation measurements and would thus not be representative of generally encountered natural systems. The dissolved organic carbon (DOC) was also expected not to show too high concentrations indicating an anthropogenic pollution. Furthermore, practical requirements were imposed to the river. As periphyton, a natural algal biofilm growing on submerged substrates, was chosen as target community of organisms for this work, the nature of the river bed and the accessibility of the site were important as this has a direct influence on the experimental setup. Indeed, for in situ colonization of periphyton, racks holding microscopes slides need to be fixed to the river bed. For studying the influence of metal speciation in freshwater on the accumulation in periphyton, the approach includes both field experiments and laboratory analyses, which implies that the river should be located relatively close to the laboratory.

2.2 Materials and methods

2.2.1 Sampling sites

Thirty-seven sites located in Eastern Switzerland were investigated (Figure 1). These sites are roughly divided in three regions (west from the town of Zurich - Table 1, Zurich area - Table 2 and east of Zurich - Table 3). Some sites were sampled several times as they were considered to be particularly appropriate sites for this study following information obtained from the local authorities about their probable elevated metal concentration. The locations of these sites are given on Table 4. The weather conditions during the sampling days were mostly favorable and dry.

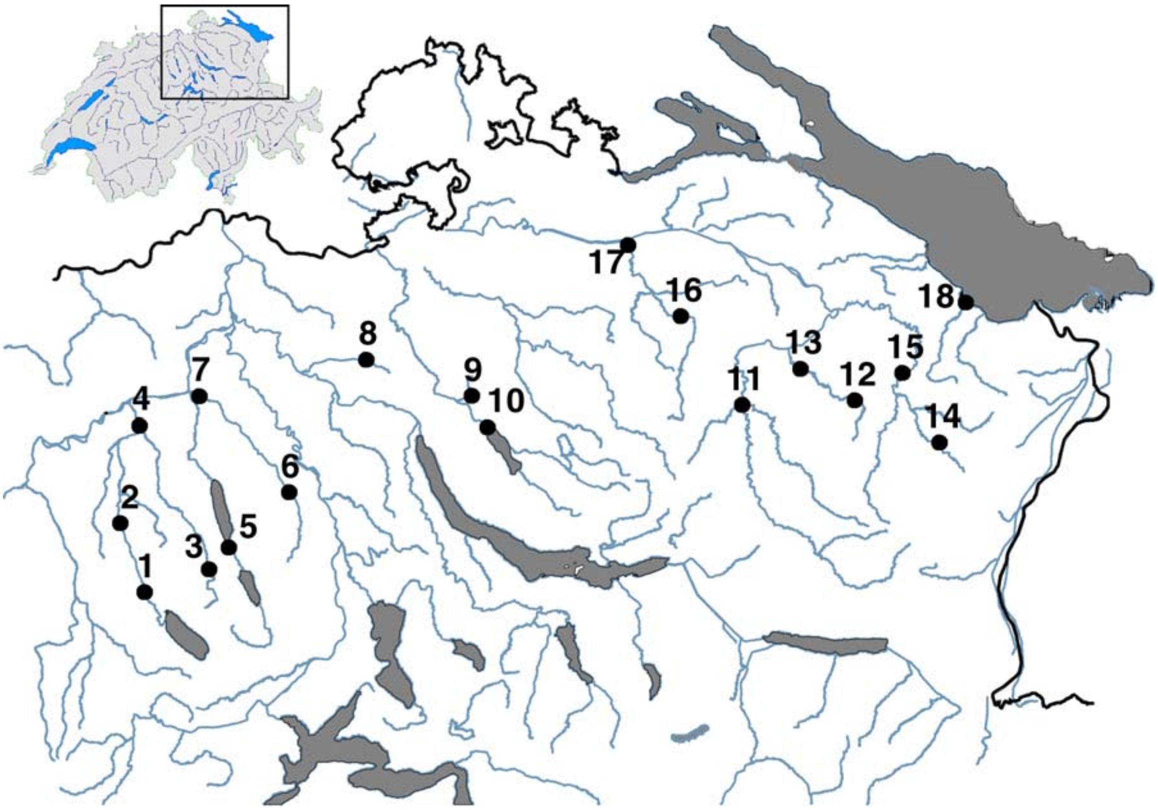


Figure 1: Sampling sites (vector25©2003 swisstopo, vector200©2003 swisstopo, GG25©2003 swisstopo)

In the region situated west of Zurich (Table 1), rather large streams are flowing through agricultural areas and waste water treatment plant (WWTP) connected to these streams do not collect water from adjacent industrial areas. The discharge of these streams varies between 1 to 15 $m^3 s^{-1}$. In the area around Zurich (Table 2) and in the region east of Zurich (Table 3), the sites studied are located in different types of area. The Murg River is flowing in an agricultural region whereas the other rivers are more located in a pre-alpine area in a hilly (Furtbach, Chriesbach, Thur, Glatt #13, Dorfbach, Niederglatt) or even mountainous (Glatt #12 and Sitter) zone. The Altbach, Furtbach, Glatt, Dorfbach and Steinach streams are located in industrial areas. Most of these streams have a discharge between 0.5 and 8 $m^3 s^{-1}$. Only the Thur, Murg, Sitter and Glatt (ZH) Rivers have discharges above 12 $m^3 s^{-1}$, whereas the Trockenloo-Kanal has a discharge under 0.01 $m^3 s^{-1}$.

2.2.2 Water sampling

Water samples were taken from the river shore at every site investigated during this survey. Grab samples (10 ml) for trace metal analysis were taken with a syringe (Once) thoroughly rinsed with river water and filtered on site (0.45 μm, Orange Scientific Gyrodisc). The water was collected in a polypropylene (PP) vials previously acid-washed with 0.01 M with HNO_3 (suprapure) overnight. Samples were then acidified to 0.01 M with HNO_3 (suprapure). All handling was performed with plastic gloves (Semadeni) and samples were kept in plastic bags to avoid contamination.

For dissolved organic carbon (DOC) 100 ml samples were taken in glass bottles and filtered in the laboratory (0.45 μm, Orange Scientific Gyrodisc). Temperature, pH and conductivity were measured directly in the river (Testo 252). Samples were kept at 4°C until analysis.

2.2.3 Sample analysis

Dissolved metal concentrations (Cu, Zn) were measured by ICP/MS (Perkin Elmer Elan 5000) with rhodium used as an internal standard. The accuracy of the ICP/MS measurements was checked using SLRS-4 reference water (National Research Council Canada, error <10%). The detection limits are 1.3 nM for copper and 1.5 nM for zinc. DOC was determined by combustion (Elementar).

2.3 Results and discussion

Background concentrations of copper and zinc measured in this survey were mostly found in streams located in agricultural regions (west of Zurich), in a non-industrial mountainous area (Sitter) or at the outlet of lake Greifen. The background concentration ranges were 8-24 nM Cu and 3-30 nM Zn, which corresponds to values found in previous studies (Xue et al., 1996; Sigg et al., 2000; Behra et al., 2002).

In the region situated west of Zurich (Table 1), rivers are usually containing low levels of metals. The metal concentration is nevertheless expected to be slightly higher during rainy periods due to runoff of fertilizer and pesticide, which contain high amount of copper and zinc. The effect of runoff on metal concentration has already been reported in studies performed in small agricultural catchments or directly in drainage water (Xue et al., 2000; Aldrich et al., 2002). The measurements performed downstream from WWTPs do not show any special metal contamination in this agricultural region. The pH values are relatively high, as expected in a rural area.

Table 1: Rivers in the area west of Zurich, Switzerland. Dissolved copper and zinc concentration, dissolved organic carbon and pH measurements.

#	River/Place (Canton)	Date	Cu (nM)	Zn (nM)	DOC (mg/l)	pH (-)
1	Suhre/Sursee (LU)	22.03.2000	11	11	3.5	8.5
2	Suhre/Staffelbach (AG)*	22.03.2000	14	19	3.6	8.3
3	Wina/Beromunster (LU)	22.03.2000	20	20	2.4	8.5
3	Wyna/Unterkulm (AG)*	22.03.2000	18	26	3.5	8.7
4	Wyna/Aarau (AG)	22.03.2000	19	22	2.9	8.8
4	Suhre/Aarau (AG)	22.03.2000	15	16	3.9	9.0
4	Suhre+Wyna/Aarau (AG)	22.03.2000	18	28	2.9	9.0
5	Aabach/Mosen (LU)*	22.03.2000	12	14	3.2	8.1
6	Bünz/Bünzen (AG)	22.03.2000	24	28	2.4	8.1
7	Bünz/Lenzburg (AG)	22.03.2000	23	20	3.8	8.6
7	Aabach/Lenzburg (AG)	22.03.2000	8	3	3.9	8.9

*: sampling site located downstream of a WWTP.

In the area surrounding the town of Zurich, our survey was focused on two industrial areas (Regensdorf and Dietlikon) and the outlet of lake Greifen (Table 2). The metal concentrations of several samples taken in the two streams flowing through these industrial areas (Furtbach and Altbach) were exceeding the Swiss water quality criteria (WQC), which correspond to 32 nM Cu and 76 nM Zn. The Trockenloo-Kanal is a small affluent of the Furtbach with a discharge <0.01 $m^3 s^{-1}$, which used to serve as outflow for a WWTP. This plant is now out of use but the water running in the channel is probably still in contact with contaminated areas which explains the extremely high concentration of zinc found (960 and 420 nM). These concentrations are nevertheless expected to drop during rain event due to dilution. Samples were taken at different times in the Furtbach and showed relatively large variations between the measured copper and zinc concentration.

The metal concentrations measured at the outlet of Lake Greifen were low and correspond to concentrations expected in lake water. Furthermore, these values are likely to be relatively constant over the year.

Table 2: Rivers in the area of Zurich, Switzerland. Dissolved copper and zinc concentration, dissolved organic carbon and pH measurements.

#	River/Place (Canton)	Date	Cu (nM)	Zn (nM)	DOC (mg/l)	pH (-)
8	Trockenloo-Kanal/Regensdorf (ZH)*	17.04.2000	50	964	6.4	8.0
8	Trockenloo-Kanal/Regensdorf (ZH)*	15.06.2000	42	419	4.2	7.4
8	Furtbach/Quelle (ZH)	15.06.2000	8	15	5.6	7.6
8	Furtbach/Trockenloo-Upstream (ZH)	15.06.2000	38	58	4.6	8.2
8	Furtbach/Regensdorf (ZH)*	17.04.2000	336	71	6.3	8.2
8	Furtbach/Regensdorf (ZH)*	15.06.2000	31	46	4.1	8.1
8	Furtbach/Buchs (ZH)	17.04.2000	22	70	4.9	8.1
8	Furtbach/Buchs (ZH)	15.06.2000	39	65	4.9	8.1
8	Furtbach/Dänikon (ZH)	17.04.2000	39	168	6.6	7.8
8	Furtbach/Würenlos (ZH)	17.04.2000	36	122	4.6	8.1
9	Chriesbach/Wangen (ZH)	19.04.2000	7	27	4.3	8.2
9	Chriesbach/Airport (ZH)	19.04.2000	14	78	3.2	8.1
9	Altbach/Airport (ZH)	19.04.2000	36	92	4.6	8.2
9	Chriesbach/Dübendorf (ZH)	19.04.2000	29	86	3.8	8.0
10	Glatt/Greifensee-Outlet (ZH)	01.02.2000	22	9	3.5	8.0

*: sampling site located downstream of a WWTP, shading: value above the Swiss WQC.

In the region east of Zurich, the metal concentrations in the Glatt, Dorfbach and Steinach Rivers are regularly surpassing the Swiss WQC at the sites located downstream from WWTP collecting wastewater from industrial areas (Table 3). The elevated concentrations are due to the discharge of water by the WWTP, which still contains relatively high concentration of metal into rather small streams. Elevated zinc concentrations were regularly found in the Glatt river in Herisau over several samplings. This site is located just downstream (< 1 km) of a WWTP collecting water from an industrial area.

Table 3: Rivers in the area east of Zurich, Switzerland. Dissolved copper and zinc concentration, dissolved organic carbon and pH measurements.

#	River/Place (Canton)	Date	Cu (nM)	Zn (nM)	DOC (mg/l)	pH (-)
11	Thur/Lütisburg (SG)	15.12.1999	15	24	3.6	8.3
12	Glatt/Johannesbad (AR)	04.02.2000	13	30	1.6	8.6
12	Glatt/Johannesbad (AR)	07.02.2000	15	87	1.3	8.5
12	Glatt/Herisau (AR)*	15.12.1999	44	115	6.5	8.7
12	Glatt/Herisau (AR)*	04.02.2000	24	141	6.1	8.2
12	Glatt/Herisau (AR)*	07.02.2000	25	150	3.1	7.9
12	Glatt/Herisau (AR)*	18.04.2000	77	137	7.3	7.9
13	Glatt/Isenhammer (SG)	07.02.2000	20	75	2.3	8.5
13	Glatt/Isenhammer (SG)	18.04.2000	47	54	4.4	8.7
13	Dorfbach/Gossau (SG)*	15.12.1999	48	161	6.3	8.7
13	Dorfbach/Gossau (SG)*	07.02.2000	26	173	4.7	8.2
13	Glatt/Niederglatt-Upstream (SG)	07.02.2000	31	63	3.3	8.4
13	Niederglatt/Niederglatt (SG)	15.12.1999	68	54	9.0	8.7
13	Niederglatt/Niederglatt (SG)	07.02.2000	26	19	2.3	8.4
13	Glatt/Niederglatt-Downstream (SG)	04.02.2000	30	51	3.8	8.5
13	Glatt/Niederglatt-Downstream (SG)	07.02.2000	29	42	3.0	8.4
13	Glatt/Niederglatt-Downstream (SG)	18.04.2000	73	119	6.8	8.4
13	Glatt/Oberbüren (SG)	07.02.2000	28	47	3.1	8.6
14	Sitter/Appenzell (AI)	15.12.1999	19	30	3.7	8.6
15	Sitter/St-Josephen (SG)	15.12.1999	24	30	4.7	8.6
16	Murg/Rosenthal (TG)	15.12.1999	22	39	3.9	8.3
17	Murg/Frauenfeld (TG)*	15.12.1999	31	42	4.6	8.1
18	Steinach/Steinach (SG)*	15.12.1999	92	184	6.1	8.5

*: sampling site located downstream of a WWTP, shading: value above the Swiss WQC.

In general, the DOC measurements were indicating values in a similar range independently from the region. At the sites contaminated by metals and generally located downstream from WWTPs (Glatt/Herisau, Dorfbach/Gossau, Steinach/Steinach, Furtbach/Regensdorf) higher DOC concentrations are observed than at unpolluted sites. In the area of Zurich, DOC concentrations were as well higher than those found in the agricultural region west of Zurich. This is probably due to the higher density of the population in the region around Zurich compared to the other regions. The low DOC concentration in the agricultural area west from Zurich might be explained by the period of sampling which was performed in spring (March). The sampling site located close to the source of the Glatt in Johannesbad at relatively high altitude had as well a very low concentration of DOC (but relatively high Zn).

2.4 Site selection

No sites were found to be heavily contaminated among those investigated. Some sites were slightly contaminated with copper or zinc concentrations exceeding the Swiss WQC. Although not strongly contaminated, some sites presenting some interest for this study were found during this survey.

The first site chosen for the field studies is the Furtbach in the Zurich area. This site presented relatively high copper and zinc concentrations especially during rain events. Furthermore, DOC and pH were in the appropriate range. The installation of holders to colonize periphyton is easily achievable in this stream.

The second site chosen is the Glatt River in Zurich at the outlet of the Lake Greifen. The metal concentrations at this site are expected to be low and stable over time which is an appropriate requirement for experiments in microcosms such as those planned in this study. DOC and pH measured at the period of sampling were showing adequate values.

Moreover these sites are geographically located at short distance from the laboratory and authorize thus closer links between field sampling and laboratory analysis.

2.5 Sampling locations

The exact locations of the sampling sites are shown on Table 4.

Table 4: Coordinates of the sampling sites

River/Place (Canton)	**Coordinates**	
	east-west	south-north
Aabach/Mosen(LU)	659 700	232 850
Aabach/Lenzburg (AG)	655 250	250 950
Altbach/Airport (ZH)	689 850	251 700
Bünz/Bünzen (AG)	667 200	239 700
Bünz/Lenzburg (AG)	656 075	251 550
Chriesbach/Airport (ZH)	689 850	251 570
Chriesbach/Wangen (ZH)	690 500	251 720
Chriesbach/Dübendorf (ZH)	688 675	251 190
Dorfbach/Gossau (SG)	734 670	252 570
Furtbach/Quelle (ZH)	678 960	254 900
Furtbach/Trockenloo (ZH)	677 480	255 380
Furtbach/Regensdorf (ZH)	677 210	255 800
Furtbach/Buchs (ZH)	676 800	256 080
Furtbach/Dänikon (ZH)	673 770	255 925
Furtbach/Würenlos (ZH)	669 889	255 285
Glatt-ZH/Greifensee-Downstream (ZH)	691 800	247 750
Glatt/Johannesbad (AR)	737 500	248 000
Glatt/Herisau (AR)	737 380	251 000
Glatt/Isenhammer (SG)	734 110	252 600
Glatt/Niederglatt-Upstream (SG)	731 060	254 900
Glatt/Niederglatt-Downstream (SG)	730 650	254 950
Glatt/Oberbüren (SG)	729 120	257 330
Murg/Rosenthal (TG)	715 800	261 450
Murg/Frauenfeld (TG)	709 250	270 300
Niederglatt/Niederglatt (SG)	730 800	254 750
Sitter/Appenzell (AI)	747 900	245 850
Sitter/St-Josephen (SG)	743 350	254 400
Steinach/Steinach (SG)	751 220	263 180
Suhre/Sursee (LU)	649 300	227 300
Suhre/Staffelbach (AG)	646 225	235 825
Suhre/Aarau (AG)	648 675	247 775
Suhre+Wyna/Aarau (AG)	648 650	247 975
Thur/Lütisburg (SG)	723 450	250 450
Trockenloo-Kanal/Regensdorf (ZH)	677 475	255 375
Wina/Beromunster(LU)	657 275	230 150
Wyna/Unterkulm (AG)	651 550	238 750
Wyna/Aarau (AG)	648 800	247 950

3

Determination of intracellularly accumulated metal in a natural algal biofilm by EDTA-wash

3.1 Introduction

The uptake of metal into cells occurs following several steps, starting with the metal adsorption on the cell wall, followed by the transport through the cell membrane and finally the intracellular accumulation. Metals need to be internalized to have an influence on the biochemical mechanisms occurring in the cell and eventually become toxic (Campbell, 1995). It is thus important to be able to differentiate between adsorbed metals on the cell walls and the intracellularly accumulated metal in order to have a relevant measurement of metal bioaccumulation. The consideration of metal accumulation as biological endpoint to determine metal bioavailability is generally accepted by the scientific community (Campbell, 1995; Sunda and Huntsman, 1998; Campbell et al., 2002; Wilkinson et al., 2002). However, no method is generally accepted for the distinction between intracellularly accumulated metal and metal bound to the cell wall.

Methodologically, information on metal accumulation performed by simple acidic digestion of a biofilm followed by the measurement of the metal concentration indicates the sum of intracellular and extracellularly adsorbed metals. The removal of metal adsorbed on the cell walls is commonly performed by washing samples using EDTA (ethylenediaminetetraacetic acid), a strong metal complexing ligand. In laboratory studies, such a procedure is appropriate as the synthetic media usually used contain no other particles. The study of metal accumulation in natural algal communities is not as obvious as metals are also associated to suspended particles in water or included in the natural biofilm matrix under iron or manganese oxide forms. This procedure and its efficiency need thus to be controlled for freshwater algal communities collected under field conditions. Moreover the desorption kinetics of EDTA need to be tested in complex water systems such as natural freshwater.

The experiment described here aims to verify if the EDTA-wash technique is adapted to the conditions applied in the subsequently presented studies, namely the measurement of the bioaccumulation of copper and zinc in periphyton in natural freshwater. Different aspects of the method were tested such as the applied concentration of EDTA, the density of the algal suspension and the duration of the EDTA treatment.

3.2 Materials and methods

3.2.1 Periphyton colonization

Microscope slides were used to colonize a natural periphytic community in a flow-through system. Microscope slides were hold parallel to each other 1 cm under the water surface in polypropylene racks. A pump was continuously providing water from the Glatt River (Canton Zurich, Switzerland). The water was flowing through the colonization tanks before going back to the river. A thin biofilm was rapidly formed on the slides and periphyton was in sufficient quantity for the experiment after 3 weeks.

3.2.2 Exposure of periphyton

Three 50-liters tanks were prepared with natural river water and either copper, zinc or no metal added three days before exposure to allow water to equilibrate (concentration shown on Table 1). Slides with colonized periphyton were then transferred from the colonization tank to the exposure tanks. The periphytic communities were exposed during 2 days.

Table 1: Concentration of metal in the exposure tanks

	Copper (nM)	**Zinc** (nM)
Copper added (Cu)	175	48
Zinc added (Zn)	38	330
No metal added (no)	38	51

3.2.3 EDTA wash experiments

Colonized microscope slides from each of the three experimental tanks were thoroughly rinsed with filtered river water sampled from the tank. Periphytic algae were scratched using a clean microscope slide and a homogenous suspension of periphyton was prepared in river water for every experimental condition. The density of the suspension with 1 microscope slide for 8 ml corresponded roughly to 3-6 mg dw (dry weight) per ml. From these 3 suspensions of periphyton, different assays were performed. First, 6 replicates were taken without any further treatment to measure the total metal content in periphyton which includes intracellular metal content and adsorbed metal on periphyton. Then different EDTA treatments were applied to the periphytic suspensions. Samples (3 replicates) were examined after the application of 4 mM EDTA (stock solution: 0.25 M) during different contact times (0, 5, 10, 20 and 60 min, as well as 2 and 40 min sampling time in the case of copper). Furthermore, different concentrations of EDTA (0.4 mM and 40 mM) were tested with a 10 minutes contact time. In the case of the addition of copper in the exposure tank, the effect of algal density on EDTA-wash was examined. For that a 3-fold dilution of the suspension was treated with 4 mM EDTA during 0, 10 and 60 min. The experiment was carried out at natural river water pH (pH=8.4).

The procedure for each replicate was similar. A 6 ml sample of the homogenous periphyton suspension was taken and filtered on a previously weighed filter membrane. After 15 h drying at 50°C, the periphytic material on the filter was weighed and the dry weight (dw) was deduced. The periphyton was then digested with 4 ml nitric acid concentrated (suprapure) and 1 ml hydrogen peroxide (31%, suprapure) during 13 min in a high performance microwave digestion unit (mls1200 mega). The solution obtained was diluted to 25 ml with nanopure water and measured after settling of the undissolved particles (silicates) for 24 h time with ICP-OES (Spectro Ciros CCD). The digestion procedure was performed as well with plankton reference material (error < 15%, CRM 414, Community Bureau of Reference, Commission of the European Communities), and blank filters to check the quality and the accuracy of the measurements. Blank measurements were under the detection limit (<10.2 nM for Cu, <10.9 nM for Zn).

3.3 Results and discussion

The effect of contact time on the removal of metal from the cell walls of periphytic organisms is shown in Figure 1 for zinc and Figure 2 for copper. In general, the Zn and Cu content decrease rapidly with increasing contact time to EDTA and reach a plateau after already 5 to 10 min, indicating that a large majority of metal is removed after a 10 min treatment. Longer contact times (20-60 min) do not change the Zn and Cu content and small variations are within experimental error. The constant metal content after long contact time confirms that no internalized metal is released from the cell. Furthermore, microscopic observations of samples exposed 60 min indicating that algal cell walls remained intact.

The proportions between intracellular and total metal accumulated differ notably depending on the metal concentration in water (Figure 1 and Figure 2). In natural water containing background concentrations of metals, 89 to 96 % of total cellular Cu and 94 to 96 % of Zn were found to be intracellularly accumulated. At higher concentrations, the proportion of metal adsorbed on the biofilm increased and the proportion of intracellularly accumulated metal obtained was 75 % of Cu and 82 % of Zn.

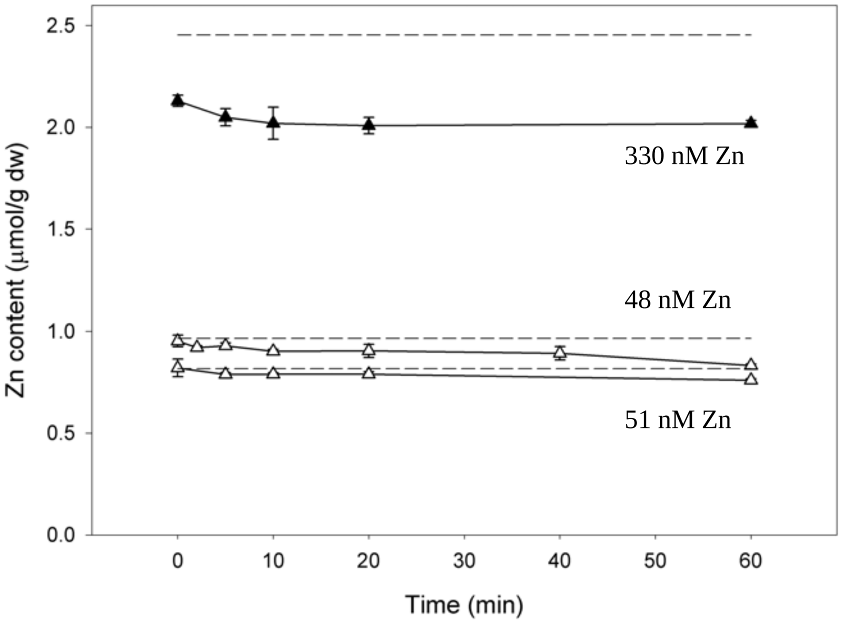


Figure 1: Zinc content in periphyton as a function of contact time with EDTA (4 mM) at background Zn concentration (△) and in water contaminated with Zn (▲). The dash lines (-– –-) represent the total zinc content for each condition.

The treatment of the suspension of periphyton with different concentration of EDTA did not show any significant difference in the metal content (Figure 3). Only small variations were measurable which were within the experimental error. The concentrations of EDTA applied were in a large excess and the complexing capacity of EDTA was sufficient to compete with the ligands in the cell wall. In this range of EDTA concentration, periphyton exposed to background metal concentrations as well as contaminated waters can be efficiently treated.

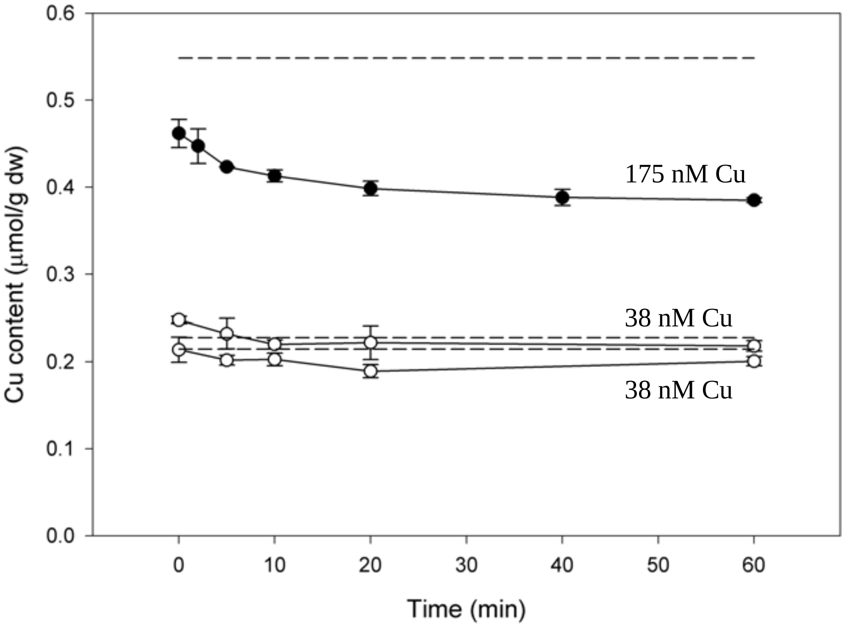


Figure 2: Copper content in periphyton as a function of contact time with EDTA (4mM) at background Cu concentration (○) and in water contaminated with Cu (●). The dash lines (-——-) represent the total copper content for each condition.

The effect of dilution increases the ratio of EDTA concentration to adsorption sites on cell wall surface concentration and might increase thus the efficiency of EDTA. Furthermore, the dilution of the suspension reduces the density of microorganisms which results in more homogenous suspensions and a reduction of the density of inorganic suspended particles embedded in the biofilm. It thus increases the dissolution of trace metals sorbed to particles such as iron or manganese oxides. Nevertheless, no variations between non- and diluted samples were detected (Figure 3). This means that the density of the suspension (roughly 3-6 mg dw per ml) is appropriate as no further trace metal are released by EDTA after dilution of the algal suspension. The vertical position of the microscope slides during the colonization of periphyton and the careful rinsing step with filtered water during the sampling are believed to play a crucial role in reducing the presence of inorganic particles in the biofilm. The eventual contamination due to inorganic particles is thus considered to be within experimental error.

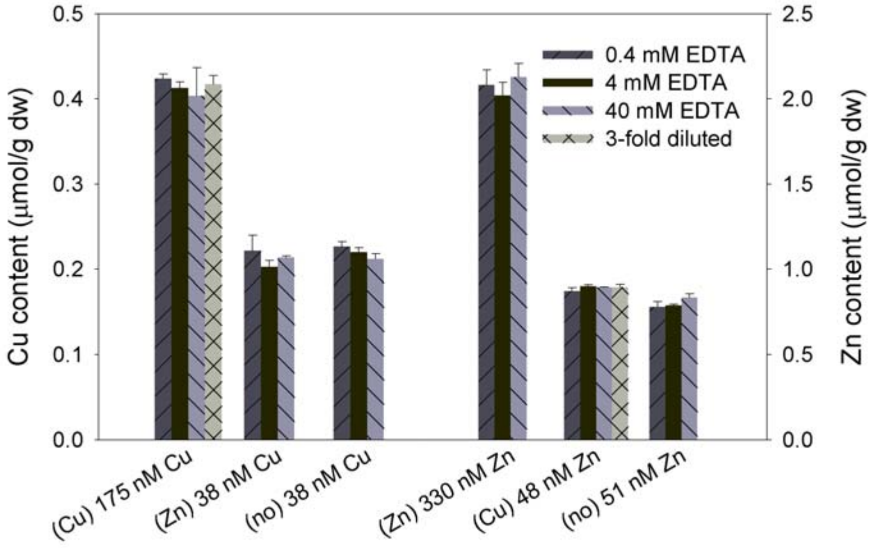


Figure 3: EDTA-washed treatment with different EDTA concentrations and dilution factor. Measurements performed after 10 min contact time.

Several laboratory studies have used EDTA as complexing ligand to determine intracellular copper and zinc content in single species of algae (Bates et al., 1982; Knauer et al., 1997a; Knauer et al., 1997b; Macfie and Welbourn, 2000; Mehta et al., 2002). Only few studies have used a similar technique to examine metal uptake in periphytic communities (Behra et al., 2002; Holding et al., 2003). Copper and zinc in periphyton in natural water was investigated and the ranges of EDTA concentration used were similar to the range found in this study.

3.4 Conclusion

Based on the results obtained in this study, the EDTA-wash technique appears to be appropriate for metal content determination of periphyton samples colonized on glass substrates in natural water. The vertical position of the glass slides reduces the entrapment of inorganic particles in the mat which is held to be minor.

The optimal conditions for the removal of adsorbed copper and zinc to periphyton are a contact time of 10 minutes with an EDTA concentration of 4 mM. These conditions are appropriate to remove the metals without extracting the intracellularly accumulated metals or inducing cell-breakage.

(See pictures related to the experimental setup in Chapter 8)

4

Accumulation of copper and zinc in periphyton in response to dynamic variations of metal speciation in freshwater

Sébastien Meylan, Renata Behra and Laura Sigg, 2003, Environmental Science & Technology, 37(22): 5204-5212.

4.1 Abstract

Although the free ion activity model (FIAM) has been well established in laboratory studies, there remains the need for field data in order to validate the applicability of this model in natural systems. The objective of this study was to investigate the response of copper and zinc accumulation in periphyton to short-term variations in metal concentration and speciation in freshwater. During heavy rain events, dissolved Cu in the Furtbach stream increased from 40 nM to 118 nM, while dissolved Zn increased from 45 nM to 147 nM due to the release of metals from contaminated sediments. Increases in free copper and free zinc ions in the water (from 10^{-14} M to $10^{-11.5}$ M for Cu^{2+}; from 1 nM to 15 nM for Zn^{2+}) were observed during the onset of heavy rain events. Periphytic algae collected from artificial substrates had an intracellular copper content (0.2-2.8 μmol/g dw) which varied as a function of the exchangeable copper in the water (labile form), rather than the free Cu^{2+}. Intracellular zinc content (1.5-8.0 μmol/g dw) was found to follow the same trend as the free zinc ion concentration. Adsorbed Cu and Zn on periphyton showed a very dynamic response to variations in dissolved metal concentration. Different concentrations of dissolved manganese during the two time periods may affect the accumulation of zinc and copper by competition for metal uptake.

4.2 Introduction

Trace metals, such as copper and zinc, are important micronutrients for algae yet are toxic at high concentrations (Sandmann and Boeger, 1980; Sunda, 1989). The bioavailability, and thus the potential toxicity of metals to microorganisms is controlled by the metal speciation in water (Morel and Hering, 1993). In synthetic media, the free ion activity model (FIAM) explains the relationship between metal and bioavailability for aquatic organisms (Campbell, 1995), although some exceptions to this model have been found (Errécalde and Campbell, 2000; Fortin and Campbell, 2001; Campbell et al., 2002). Laboratory studies performed with seawater have pointed up the relationship between the effects to phytoplankton and free copper ion (Sunda and Guillard, 1976; Brand et al., 1986) or free zinc ion concentration (Anderson et al., 1978; Sunda and Huntsman, 1992). Similar laboratory studies performed with freshwater algae have also demonstrated the applicability of the FIAM (Petersen, 1982; Rueter et al., 1987; Knauer et al., 1997b). To predict potential effects of metals in natural systems, results from laboratory studies can be used in conjunction with measurements of the free metal ion concentrations performed in seawater (Bruland et al., 1991) and in freshwater (Xue and Sigg, 1994; Breault et al., 1996; Xue et al., 1996). Nevertheless, it remains unclear how metal speciation influences algae under field conditions due to the lack of data combining both measurements of metal speciation and effects on microorganisms. Relatively few studies relating metal uptake in organisms to speciation under field conditions are available in the literature (Hare and Tessier, 1996; Mylon et al., 2003). Indeed, heterogeneous natural ligands may influence metal uptake in a different manner than synthetic ligands (Campbell et al., 1997; Vigneault et al., 2000). The applicability of the FIAM to natural waters remains unclear and has yet to be directly demonstrated.

Periphyton, a natural algal biofilm, has been relatively well studied in field experiments. Its sensitivity to changes in the surrounding aquatic environment (Wetzel, 1983; Blanck, 1985) and ability to accumulate metals (Newman and McIntosh, 1989) are well documented. Studies with periphyton from freshwater systems have been carried out to assess the effects of metals on algal community tolerance (Soldo and Behra, 2000; Blanck, 2002) and particularly to evaluate the state of aquatic systems with respect to metal pollution due to industrial activities (Johnson et al., 1978; Ramelow et al., 1992; Ivorra et al., 1999). Metal accumulation has frequently been utilized as biological endpoint to measure metal bioavailability as, in most cases, metals need to be internalized to show an effect (Campbell et al., 2002). Studies in rivers (Friant and Koerner, 1981; Behra et al., 2002) have shown significant temporal variability of the metal content in periphyton. This suggests that algae are subject to short-term fluctuations of trace metals in water. Under field conditions, no data are available concerning the response rate of periphyton to variations of metal concentration in water. More data are thus necessary to predict the effect of short-term variations of the environment on periphyton and to fully understand what controls the bioavailability of metals in a natural system.

The objective of this study was to investigate how metal accumulation in periphyton changes in response to large variations in dissolved metal concentration and more specifically to changes in metal speciation over a short period of time. Large increases of copper and zinc concentrations occur during rain events in the Furtbach stream (Zurich, Switzerland). During two periods of several days each (June 2001 and September 2002) simultaneous measurements of dissolved copper and zinc concentrations, exchangeable copper, labile zinc and free metal ion concentrations as well as copper and zinc accumulation in periphyton were performed. Large and rapid variations in the speciation of these metals in a natural environment were examined and related to the bioaccumulation of copper and zinc.

4.3 Materials and Methods

4.3.1 Study site

The Furtbach stream (canton Zurich, Switzerland) originates at the small lake of Katzen, and flows 10 km through the Furttal valley before emptying into the Limmat River. The catchment is primarily agricultural, but also includes an industrial area (Regensdorf). Until the construction of the sewage treatment plant “Wüeri” in 1994, which has its outlet 30 m downstream from the sampling site, the Furtbach stream was periodically contaminated by metal inputs from the surrounding metal handling factories (Kisseleff, 2002). Although the stream has been revitalized, the sediments remain contaminated by metals (AWEL, 2000). The Furtbach stream has an average discharge of about 0.4 m^3s^{-1} which increases to approximately 2.0 m^3s^{-1} during heavy rain events.

Rainfall was measured at a meteorological station located 2 km away from the sampling site with similar measurements being performed at the sampling site for comparison. Two sampling campaigns were carried out, the first at the end of spring 2001 (beginning of June) and the second at the beginning of fall 2002 (end of September).

4.3.2 Periphyton sampling and analysis

Microscope slides fixed to Teflon racks were used to colonize periphyton, a natural algal biofilm. To minimize the collection of suspended particles, the racks were fixed vertically about 10 cm below the water surface. Twelve racks holding 12 microscope slides each (6 per side) were placed next to each other parallel to the water current over a distance of 3 meters. This arrangement insures a homogeneous composition of the periphytic community within each experiment. Periphyton was allowed to grow for 3 weeks prior to sampling. Within a few days of exposure in the water, a thin biofilm was observed on the slides, and the periphyton layer was sufficient for the experiment after about 3 weeks. Microscopic examinations, chlorophyll determinations and fluorometric measurements of some biofilm samples indicated that the majority of particles were algae and that bacterial biomass is negligible (data not shown).

At the time of sampling, 3-4 microscope slides were thoroughly rinsed with filtered river water (0.45 µm, Orange scientific Gyrodisc). The natural algal biofilm was then scratched from the slide with a clean microscope slide and suspended in filtered river water. The suspension was afterwards divided into two fractions. One fraction (20 ml) was treated for 10 minutes with EDTA 4.0 mM (end concentration) to remove the metals adsorbed to the cell wall and most of the inorganic complexes embedded in the biofilm. This process allowed for the measurement of the intracellular metal content in periphyton. The other fraction was used to measure the total amount of metal accumulated in periphyton. The difference between total and intracellular metal content is considered to be adsorbed metal on periphyton. Three aliquots of each fraction were filtered with acid-washed and pre-weighed filters (cellulose nitrate 0.45 µm, Sartorius) to obtain the dry weight (dw) of each sample after 15 h drying at 50°C. Filters were digested with 4 ml concentrated nitric acid (suprapure) and 1 ml hydrogen peroxide (31%, suprapure) for 13 min in a high performance microwave digestion unit (mls1200 mega). After dilution to 25 ml with nanopure water, the metal concentrations of the samples were measured by ICP-OES (Spectro Ciros CCD). The efficiency of the EDTA-wash technique was checked in separate experiments with different EDTA concentrations and exposure times up to 60 minutes. No intracellular metal appeared to be removed and microscopic observations revealed that the cell walls remained intact. The digestion procedure was also performed using plankton reference material (CRM 414, Community Bureau of Reference, Commission of the European Communities), and blank filters to check the quality and the accuracy of the measurements (error <15%). Blank measurements were below the detection limit (<10.2 nM for Cu, <10.9 nM for Zn).

4.3.3 Water sampling for dissolved metal concentration measurements

Water samples were taken automatically at 1-3 hours intervals by an autosampling device (ISCO 6700 Portable samplers), depending upon the rain intensity. One-liter samples were taken in polypropylene (PP) bottles. Within a few hours of sampling, water samples were filtered (10ml) using a plastic syringe and a filter, which both were previously rinsed thoroughly with river water (20 ml syringe, Once; 0.45 μm, Orange Scientific Gyrodisc). Samples were then acidified to 0.01M with HNO_3 (suprapure). All handling was performed with plastic gloves (Semadeni). PP bottles and vials were previously washed with 0.1 M HNO_3 for 24 hours. Dissolved metal concentrations (Cu, Zn, Mn) were measured by ICP/MS (Perkin Elmer Elan 5000) with rhodium used as an internal standard. Field blanks were taken to evaluate the cleanness of the autosampling procedure. The tubing, autosampling device and bottles proved to be negligible sources of metal contamination compared to the metal concentrations found in the river water (<0.15 nM Cu, <1.5 nM Zn and <1.8 nM Mn). The accuracy of the ICP/MS measurements was checked using SLRS-4 reference water (National Research Council Canada, error <10%).

4.3.4 Metal speciation: water sampling and measurement

The water samples used to measure the free metal ion concentration were taken at time intervals varying from 6 hours during heavy rain to 24 hours during dry conditions. Water samples were taken in PP bottles by hand directly from the river. Samples were filtered in the laboratory under a clean bench within 24 hours of sampling with a 0.45 μm membrane filter (Cellulose nitrate, Sartorius) and stored at a temperature of 4°C in PP bottles until analysis. The membrane filters, filtration unit and PP bottles were previously washed with 0.1 M HNO_3 for 24 hours. Free metal ion concentrations were indirectly calculated by competitive ligand exchange (CLE) and electrochemical measurements of the metal or the metal complex depending upon the ligand used (DPCSV/DPASV - Differential pulse cathodic/anodic stripping voltammetry). MOPS (0.02 M, 3-morpholinopropanesulfonic acid) was used to buffer the samples at their natural pH. All the stability constants involved were adapted to the ionic strength of the water (Martell and Smith, 1989).

Measurements of the free zinc ion concentration were performed using EDTA (0, 20 and 30 nM) as complexing ligand. Zinc was measured by DPASV with a voltammeter Metrohm VA Stand 694 coupled to a Metrohm VA Processor 693 (HMDE, Ag/AgCl/KNO_3 sat. as reference electrode, Pt counter electrode, 5 min purging with N_2, 120 s deposition time, -1.2 V deposition potential, 10 mV/s scan rate, 50 mV pulse amplitude, 0.2 s step time) (Xue and Sigg, 1994). The free zinc concentration was measured during the experiment performed in September 2002 only. The labile zinc concentration was obtained by standard addition of zinc (in a labile form) and direct detection of the labile zinc by DPASV.

For the measurement of the free copper ion concentration the complexing ligand used was catechol (1 mM) and the copper-catechol complex was measured by DPCSV (HMDE, Ag/AgCl/KNO_3 sat. as reference electrode, Pt counter electrode, 5 min purging with N_2, 120 s deposition time, -0.05 V deposition potential, 10 mV/s scan rate, -50 mV pulse amplitude, 0.2 s step time) (van den Berg, 1984; Xue and Sigg, 1993). The exchangeable copper was only measured during the September 2002 experiment. $Cu_{exchangeable}$ is defined as the fraction of copper in a free or weakly complexed form which exchanges with the copper-catechol complexes at a catechol concentration of 1 mM. This value is simply obtained by standard addition of copper and detection by DPCSV in the presence of catechol.

4.3.5 Water chemistry

Temperature, pH and conductivity were measured automatically every 30 min by a Testo 252 coupled with a data logger. Water samples taken for the determination of major cation and major anion concentrations, DOC and alkalinity, were collected manually at the same frequency as samples taken for free metal ion concentration measurements. These values are necessary to calculate the water hardness and ionic strength. Major cation concentrations were obtained by ICP/OES, major anions were measured by ion chromatography

(Metrohm), alkalinity measurements were performed by titration (with HCl 0.1 M until pH 4.5) and DOC was determined by combustion (Elementar).

4.4 Results

4.4.1 Influence of rain events on dissolved metal concentration and water chemistry

The Furtbach stream was investigated during heavy rain events over two periods of 8 days, the first in June 2001 and the second in September 2002 (Figure 1). A steep increase of the dissolved metal concentration was observed immediately after the increase of the stream discharge induced by heavy rain event. Similarly, when the rain stopped, the dissolved metal concentration in the stream dropped rapidly.

The period studied in June 2001 presented two distinct rain events separated by a few hours of calm weather. This interruption of rain is clearly reflected in the variation of the metal concentration in the water. During this first study, the dissolved metal concentration increased steeply, from 40 nM to 118 nM for copper and from 45 nM to 147 nM for zinc over a 9 hour period (Figure 1 and Table 1). The rainy period in September 2002 started with some short precipitation that had a clear influence on the dissolved metal concentration. This was followed by a more intense rain event that lasted over a 2 day period and during which the dissolved metal concentrations remained high and reached a maximum at 141 nM for copper and 267 nM for zinc before dropping to background concentration once the rain stopped.

Table 1 shows a summary of the main water parameters measured in the Furtbach in June 2001 and in September 2002. The pH (7.75-8.01), temperature (11.2-17.4°C), DOC (2.6-5.4 mg/l), hardness (2.3-7.2 meq/l) and alkalinity (2.2-6.6 mM) were very similar during these two periods whereas the calculated ionic strength was slightly higher in June 2001 (14.1-19.5 mM) than in September 2002 (3.7-11.4 mM). Manganese dissolved concentrations were clearly higher in September 2002 (26 to 129 nM) than in June 2001 (3.3 to 56 nM). During heavy rain the pH, temperature, alkalinity and ionic strength all decreased. DOC, in contrast, increased during the rain events.

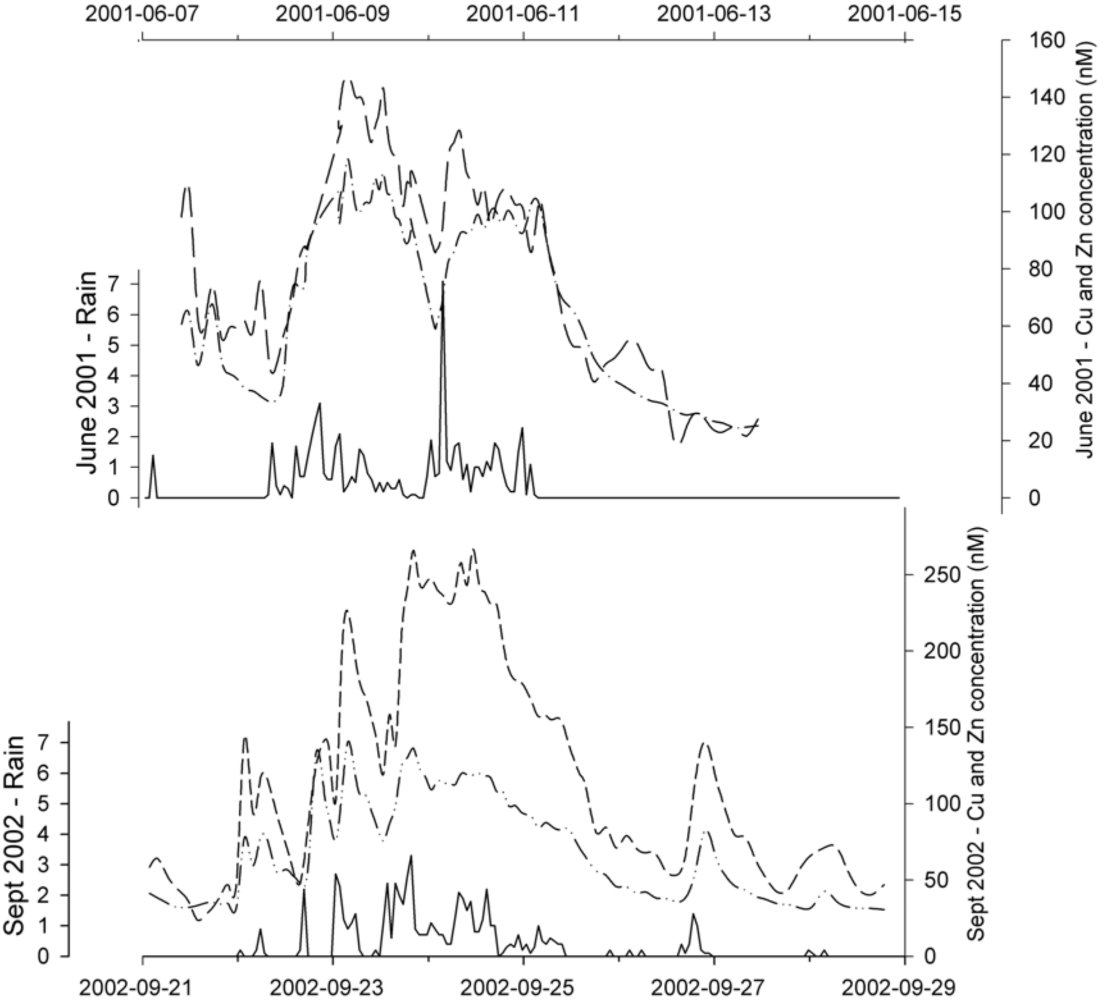


Figure 1: Rain intensity of (mm/h, ——) over two periods of 8 days in June 2001 and in September 2002 and variations of dissolved copper (nM, Jun 01: -·—·-, Sep 02: —··—) and zinc concentration (nM, Jun 01: — —, Sep 02: - — — -) during these rain events.

Table 1: Water parameters in Furtbach in June 2001 and September 2002 under dry and rain conditions and range of measured values over these periods. Cu, Zn and Mn are dissolved concentrations.

Time		Rain	pH (-)	Temp. (°C)	DOC (mg/l)	Hard. (meq/l)	Alk. (mM)	Ionic str. (mM)	Cu (nM)	Zn (nM)	Mn (nM)
June 10 9am		Heavy	7.78	14.1	3.9	4.7	4.3	14.1	93	115	10.4
June 12 4pm		No	7.91	17.0	3.3	5.9	6.1	19.0	29	25	8.7
Sept 24 8am		Heavy	7.79	12.3	3.1	3.1	2.9	5.0	120	258	66
Sept 27 2pm		No	8.01	14.4	2.7	6.9	6.5	11.2	34	42	63
Range											
June 2001	min		7.78	11.8	3.0	4.7	4.3	14.1	25	19	3.3
	max		7.98	17.4	4.1	6.5	6.1	19.5	118	147	56
Sept 2002	min		7.75	11.2	2.6	2.3	2.2	3.7	30	24	26
	max		8.01	16.0	5.4	7.2	6.6	11.4	141	267	129

4.4.2 Concentration of free copper ion and exchangeable copper

The free copper ion concentration measurements performed in June 2001 and September 2002 (Figure 2 and Figure 4) showed large increases of Cu^{2+} at the beginning of the increases of dissolved Cu which correspond to the beginning of the rain events. The background concentrations of free copper ion were found to be around 10^{-14} M, with the highest free copper ion concentration was measured being roughly $10^{-11.5}$ M, which corresponds to a 300-fold increase. More than 99.995% of the total dissolved copper was in a complexed form at any point during the study.

Exchangeable copper was as well showing large increases of concentration during heavy rain events (Figure 4) with a maximum concentration of 103 nM, whereas background concentrations were around 10 nM. Furthermore, at times of high dissolved copper concentrations almost 100% of the dissolved copper was exchangeable whereas at background concentrations of dissolved copper only 30% was exchangeable.

4.4.3 Concentration of free zinc ion and labile zinc

Labile zinc concentrations were measured in June 2001 (Figure 3) and September 2002 (Figure 5) and roughly followed the same trend as the dissolved zinc concentration. The proportion of labile zinc varied from 55% at background concentration of dissolved zinc to 99.5% during heavy rain events. Differences in the dissolved zinc concentrations between June 2001 and September 2002 were also visible in the labile zinc concentrations, which showed a maximum of 113 nM in June 2001 and of 222 nM in September 2002. Background values of labile zinc concentration were 12 nM in June 2001 and 33 nM in September 2002.

Free zinc ion concentrations presented in Figure 5 show that Zn^{2+} varied considerably during the rainy periods. By comparison with the rain data in Figure 1, the Zn^{2+} maximum occurred at the exact time of the rain peak, whereas the increase in labile zinc concentration lagged behind the Zn^{2+} maximum by a few hours. The free zinc ion concentration ranged from 1 nM to 15 nM and represented from 0.2 to 9.5 % of the dissolved zinc concentration.

4.4.4 Bioaccumulation of copper and zinc

The background values measured for intracellular copper content were about 0.8 µmol/g dw in June 2001 (Figure 2) and 0.2 µmol/g dw in September 2002 (Figure 4) whereas the maximum intracellular contents were approximately 3-fold higher (2.8 µmol/g dw in June 2001 and 0.75 µmol/g dw in September 2002) than the background values. For intracellular zinc content, background values were around 1.5 µmol/g dw in June 2001 (Figure 3) and around 1.8 µmol/g dw in September 2002 (Figure 5) while maximum measured values of intracellular zinc content were about 8.0 µmol/g dw in June 2001 and 3.8 µmol/g dw in September 2002.

Background values of total metal content in periphyton were slightly higher than the measurements of intracellular metal content. Background values of total copper content were 1.4 µmol/g dw in June 2001 and 0.3 µmol/g dw in September 2002 and maxima in the total copper content were 9.0 µmol/g dw in June 2001 and 1.4 µmol/g dw in September 2002. Background measurements of total zinc content provided values of 5.5 µmol/g dw in June 2001 and of 2.8 µmol/g dw in September 2002, whereas maximum values observed during rain events were 23.0 µmol/g dw in June 2001 and 10.4 µmol/g dw in September 2002. Curves of total accumulated and intracellular copper were almost overlapping at the beginning of the first rain event (Figure 4) due to the low proportion of adsorbed copper. In the case of copper, periphyton appears to accumulate metal faster than it releases it, which increases gradually copper content during long rain events (September 2002). To underline the relationships between metal speciation and metal accumulation it was necessary to subtract the increasing background of accumulated copper (Figure 4b and c).

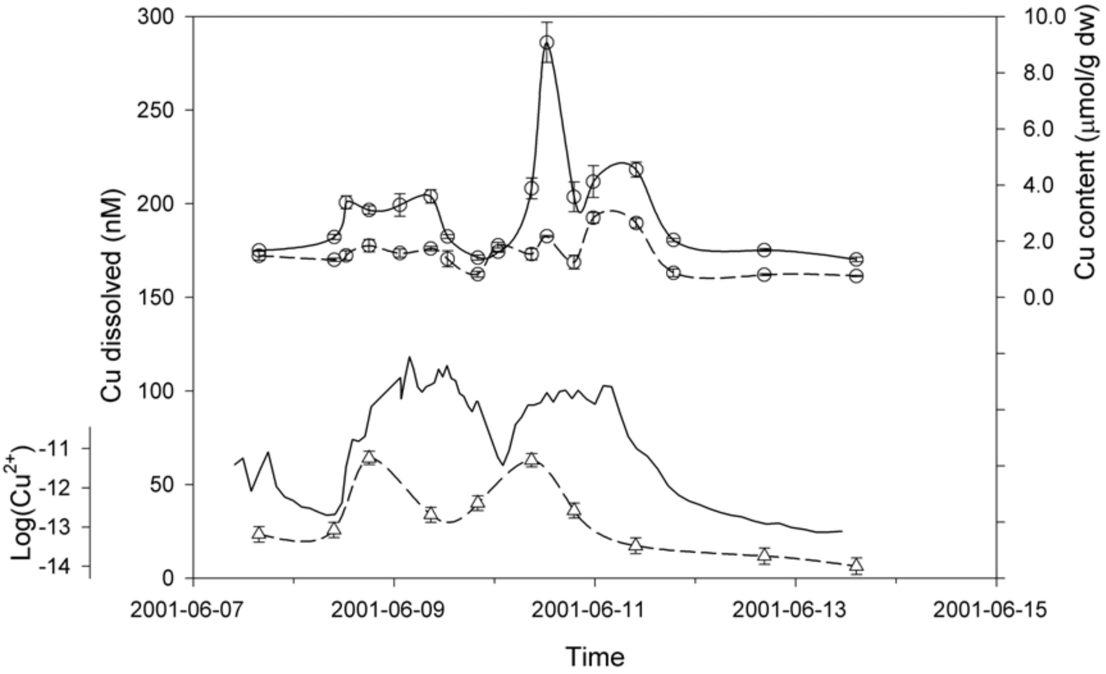


Figure 2: Variations of dissolved copper concentration (nM, ——), free copper ion concentration (M, –△–), total copper content of periphyton (µmol/g dw, —○—) and intracellular copper content of periphyton (µmol/g dw, –○–) in the Furtbach stream, Switzerland, with time over a 8 days rainy period in June 2001.

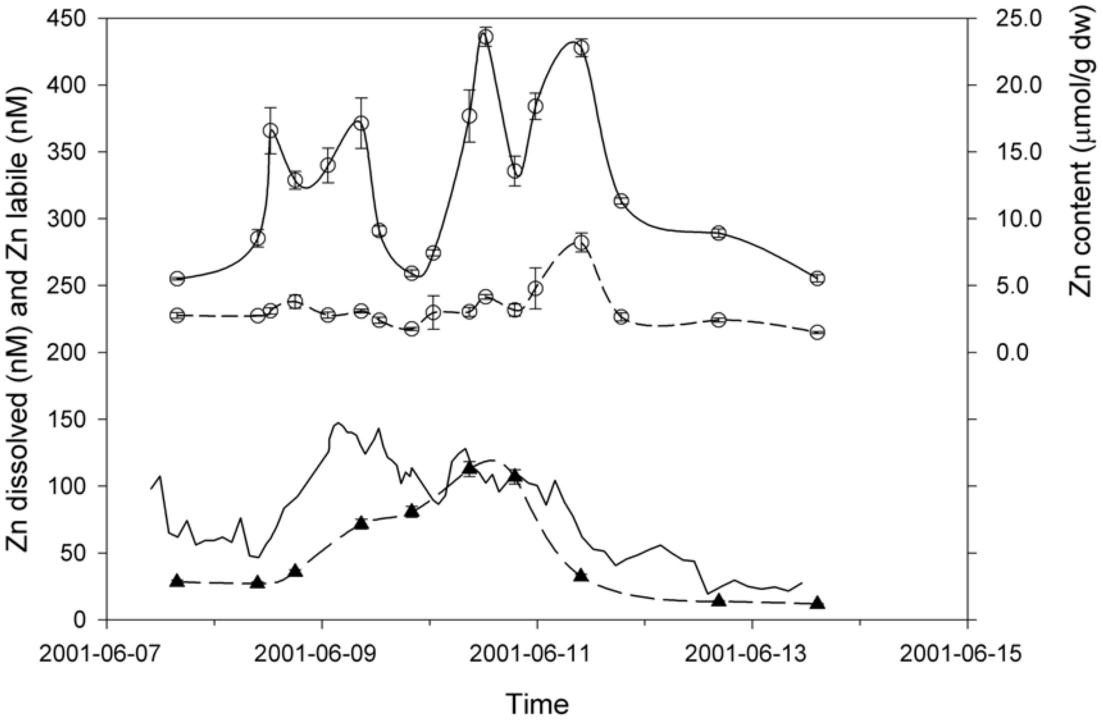


Figure 3: Variations of dissolved zinc concentration (nM, ——), labile zinc concentration (nM, – ▲ —), total zinc content of periphyton (µmol/g dw, —⊖—) and intracellular zinc content of periphyton (µmol/g dw, – ⊖ –) in the Furtbach stream, Switzerland, with time over a 8 days rainy period in June 2001.

Metal adsorbed on the periphyton (Figure 6 and Figure 7) was obtained by calculating the difference between total metal content and intracellular metal content. Adsorbed metal concentrations increased steeply during rain events. Adsorbed Cu increased from below the detection limit to 6.9 µmol/g dw in June 2001 and from undetectable to 0.8 µmol/g dw in September 2002. Adsorbed Zn increased from 2.7 to 19.5 µmol/g dw in June 2001 and from 0.9 to 6.8 µmol/g dw in September 2002. Nevertheless, the proportion of intracellular metal content to the total metal content remained roughly constant during the study for both copper and zinc.

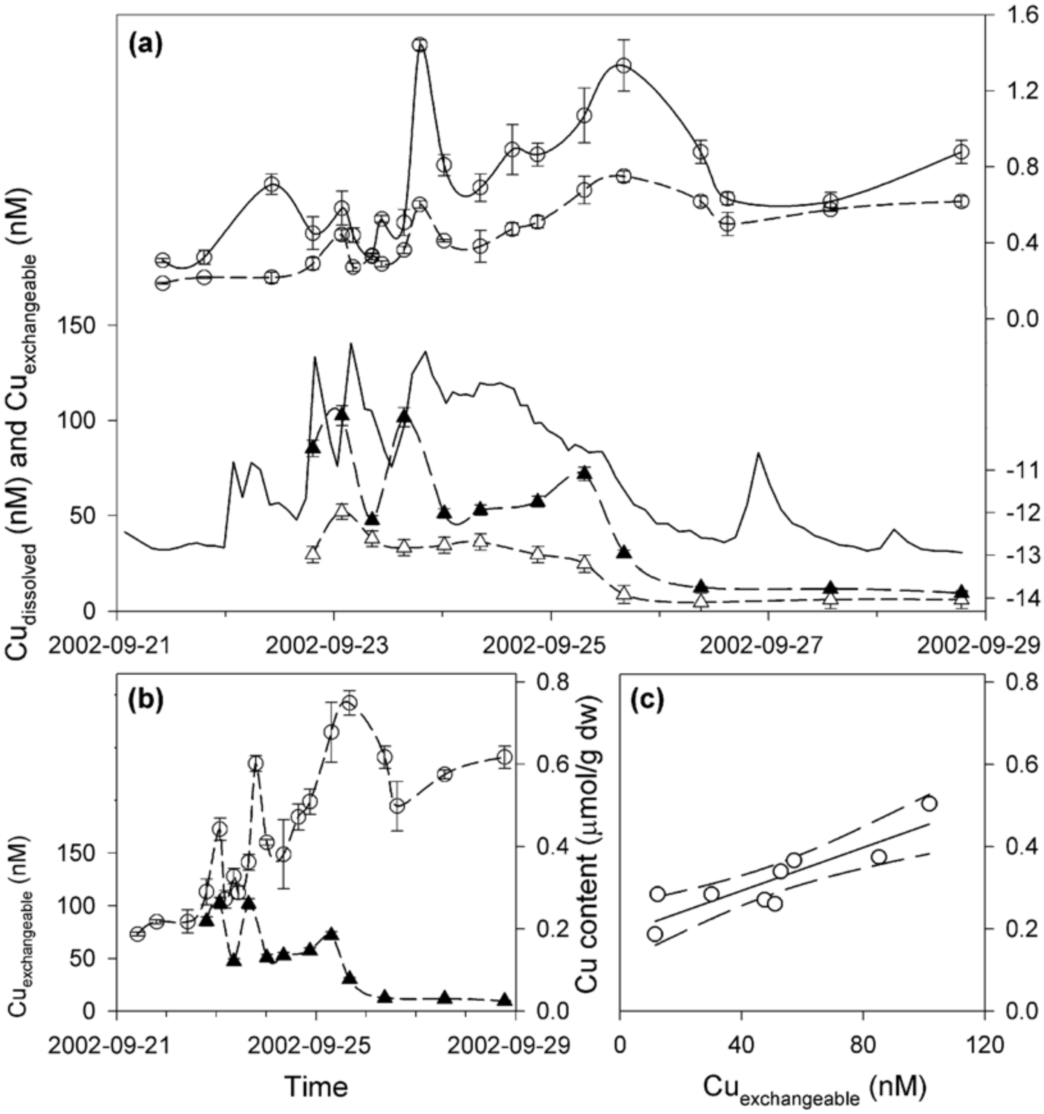


Figure 4: (a) Variations of dissolved copper concentration (nM, ——), free copper ion concentration (M, –△–), exchangeable copper concentration (nM, –▲–), total copper content of periphyton (μmol/g dw, —○—) and intracellular copper content of periphyton (μmol/g dw, –○–) in the Furtbach stream, Switzerland, with time over a 8 days rainy period in September 2002. (b) Enlargement of exchangeable copper concentration (nM, –▲–) and intracellular copper content of periphyton curves (μmol/g dw, –○–) as a function of time. (c) Relation between Cu content with increasing background subtracted (at time t+(6 to 10hours)) with 95% confidence interval and $Cu_{exchangeable}$ (at time t).

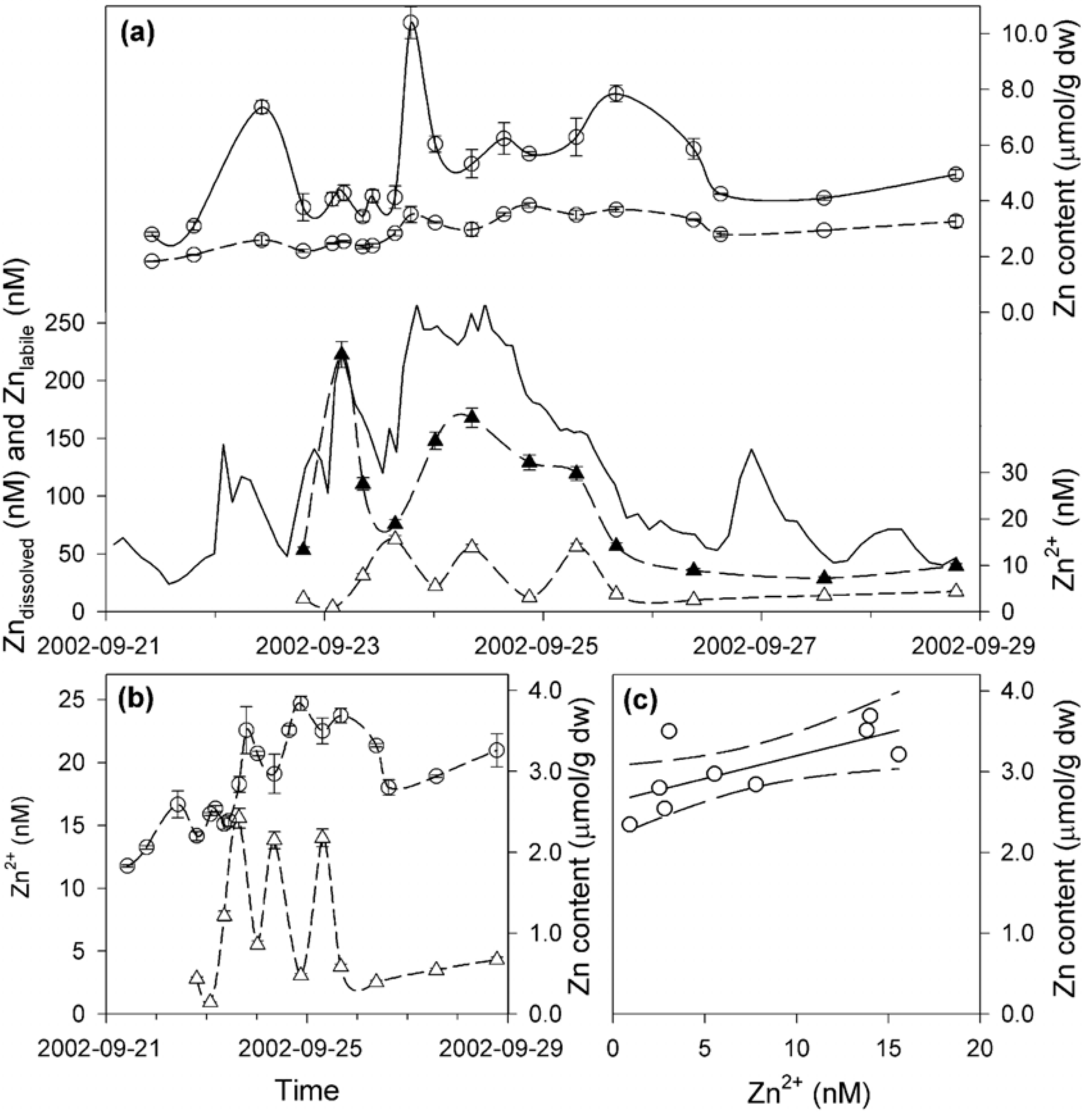


Figure 5: (a) Variations of dissolved zinc concentration (nM, ——), free zinc ion concentration (nM, –△–), labile zinc concentration (nM, – ▲ —), total zinc content of periphyton (µmol/g dw, —○—) and intracellular zinc content of periphyton (µmol/g dw, –○–) in the Furtbach stream, Switzerland, with time over a 8 days rainy period in September 2002. (b) Enlargement of free zinc ion concentration (nM, –△–) and intracellular zinc content of periphyton curves (µmol/g dw, –○–) as a function of time. (c) Relation between Zn content (at time t+(6 to 10hours)) with 95% confidence interval and Zn^{2+} (at time t).

4.5 Discussion

The relationships between rain, metal speciation and metal accumulation in the Furtbach stream during heavy rain events are too complex to be discussed simultaneously. As rain influences the metal speciation, which subsequently influences the metal accumulation, these points will be discussed separately.

4.5.1 Relationship between rain and metal speciation

During rain events, an increase of the dissolved copper and zinc concentration in the Furtbach stream (Figure 1) and dynamic variations of the metal speciation were observed. Two sources of metal pollution that influence the dissolved metal concentration during heavy rain events have been identified in the Furtbach stream. The primary input of metals during high stream discharge induced by heavy rainfall is due to the resuspension of metal contaminated sediments which release metals in the water. The response in the metal dissolved concentration occurs extremely rapidly with the onset of an increase in the rain intensity and every variation of rain is reflected in the dissolved metal concentrations. A decrease in the dissolved metal concentrations follows the slow decrease of the water discharge. A secondary source of metals to the system is runoff from the surrounding industrial region.

Measurements of the speciation of copper and zinc in June 2001 (Figure 2 and Figure 3) and September 2002 (Figure 4 and Figure 5) show that peaks of free or labile species of copper and zinc occur at the beginning of the rain events indicating that the Cu and Zn released from the sediments are in a free or a labile form. The extent of copper complexation appears to be greater under dry conditions than during rain events (Figure 2 and Figure 4). Following a peak in free copper ion concentration at the beginning of the rain events, the decrease in Cu^{2+} whereas $Cu_{dissolved}$ remains high, suggests that ligands present in the water complex Cu^{2+} rapidly. In the case of zinc, labile zinc concentration follows the same trend as dissolved zinc concentration whereas Zn^{2+} is more dynamic and peaks are alternating with rapid decreases. These variations in the free zinc ions may be due to the changes in the dissolved organic carbon content of the water which varies in a manner similar to the labile zinc. The DOC lowers the free zinc ion concentration and simultaneously labile zinc concentration increases.

Differences in metal concentration between non-contaminated and contaminated sites in river and stream systems have been reported in earlier studies (Behra et al., 2002). Similarly, increases in metal concentrations during rain events were observed in a polluted area (Blake et al., 2003). Despite this no data related to the metal speciation during rain events or comparing metal speciation in non-contaminated and contaminated stream waters have been reported until now.

4.5.2 Influence of metal speciation on the intracellular metal content of periphyton

In this study, both total and intracellular metal contents displayed similar patterns with variations in total metal content corresponding to variations in intracellular content. This section is focused on the intracellular metal content of periphyton.

For zinc, a relationship appears between the free zinc ion concentration and the intracellular zinc content of periphyton during the September 2002 campaign (Figure 5b and c). For every increase in Zn^{2+} a response in the intracellularly accumulated zinc is observed after a slight delay. A scatter plot of Zn content (measurement performed 6-10 hours after the speciation measurement) as a function of Zn^{2+} showed a relatively good correlation (r^2=0.5) for such a dynamic environment. The correlation between the Zn^{2+} decreases and the zinc content decreases is also apparent although the response is slower. In both the June 2001 (Figure 3) and September 2002 (Figure 5) data sets, the relationships between the concentration of labile zinc or dissolved zinc and intracellular zinc content are clearly not as straightforward as the relationship between Zn^{2+} and intracellular zinc content.

The variations in the intracellular copper content do not clearly follow the variations of the free copper ion concentration (Figure 2 and Figure 4). Although the large increases of Cu^{2+} are evidently influencing the intracellular copper content, the smaller variations of the copper content cannot be explained. In September 2002, additional measurements of the exchangeable copper concentration (Figure 4) were performed which show a good relationship with the intracellular metal content. Each variation in the exchangeable copper concentration is followed by a modification in the copper accumulation (Figure 4b). By subtracting the increasing background of copper and considering the measurement of the metal content following the measurement of $Cu_{exchangeable}$, a rather good correlation ($r^2=0.76$) was found (Figure 4c), whereas no correlation were found with other fractions of copper. This shows that exchangeable copper is the bioavailable fraction that controls copper uptake.

The fact that the exchangeable copper concentration and the free zinc ion concentration appear to play a crucial role for metal bioaccumulation in this study agrees with the results of a theoretical study (van Leeuwen, 1999)which focuses on the importance of the limiting fluxes of metal involved during metal uptake. For zinc, the Zn^{2+} concentration is rather high and the transfer flux of Zn^{2+} from the bulk solution to the cell membrane is probably much greater than the uptake flux of zinc by algae. Therefore the free zinc ion concentration is predicted to be the determining factor for uptake. For copper, however, where the free metal is present at very low concentration due to its strong complexation, the situation is more complicated. Only a small amount of free copper ions provided by the bulk solution are available at the cell membrane, which may result in a lack of Cu^{2+} in the diffusion layer. The exchangeable copper (labile forms of copper) close to the cell may therefore contribute to maintain a sufficient concentration of free copper ions and because its concentration is much larger than the free copper concentration, exchangeable copper controls the bioaccumulation of copper. This theoretical study explains very elegantly the results obtained in the present study.

Comparisons with field experiments in freshwater show that in Lake Greifen, an eutrophic Swiss lake, Cu^{2+} concentrations typically range between 10^{-14} and 10^{-16} M (Xue and Sigg, 1993), while the copper content in phytoplankton in the same lake (Kiefer, 1994) was found to be between 0.16 to 0.22 µmol Cu/g dw. Background free copper ion concentrations and intracellular copper content in periphyton measured in the Furtbach stream had very similar value in September 2002, while bioaccumulation was slightly higher in June 2001. On the other hand, field data from a polluted river site (Behra et al., 2002) comparing dissolved copper concentration at a polluted site (41-154 nM Cu) and total copper content

(0.9-3.9 µmol/g dw) showed similar results to those obtained in the Furtbach stream in June 2001, and were somewhat higher values than those obtained in September 2002 by this study. In laboratory experiments with the green algae *Scenedesmus subspicatus* (Knauer et al., 1997b) performed with a free copper ion concentration range similar to the natural water concentrations found in our study (10^{-14} to $10^{-11.5}$ M), bioaccumulation of copper was found to be between 0.02 and 1.1 µmol/g dw. These results are similar to those obtained in September 2002, as well as from Lake Greifen.

Field data in rivers (Behra et al., 2002) showed that at a polluted site with dissolved zinc concentrations in the range 95-280 nM, Zn content varied from 12.7 to 16.6 µmol/g dw which are again similar to values obtained in June 2001. Laboratory studies (Knauer et al., 1997b) have shown that, when the free zinc ion concentration was increased from 1.0 to 18 nM, the intracellular zinc content of green algae *Scenedesmus subspicatus* increases from 0.6 to 3.0 µmol/g dw, while our data in September 2002 show an increase from 2.0 to 4.0 µmol/g dw.

Variations in the intracellular metal content in periphyton were found to occur rapidly in response to dynamic variations in either the free zinc ion concentration or the exchangeable copper concentration respectively. Changes in metal concentration were detectable within 4-10 hours depending upon the frequency of sampling. In laboratory studies, increases in intracellular accumulation of copper (Knauer et al., 1997a) or cadmium (Errécalde and Campbell, 2000) in algae were observed within 5 minutes of exposure at higher metal concentrations. These studies also showed that the algae eventually reach a steady-state where they no longer accumulate metal intracellularly relative to their biomass. The time required to reach this steady-state varied from a few hours to days and is highly dependent upon the metal concentration, the presence of competing metals (Sunda and Huntsman, 1996) and the growth rate of algae. Nevertheless, in the present study, because of the large and rapid variations in metal speciation, this steady-state did not appear to be reached. At the opposite, the release of metal when the metal concentration drops, appears to be even slower than metal uptake. Indeed, Errécalde and Campbell (2000) showed that the time for *Selenastrum capricornutum* to reach the steady state in the presence of 600 nM free cadmium ion was about 0.5 hours and more than 4 hours were needed to release the metal to background level. This difference between uptake and release rates explains why in our study increases in metal content were clearly visible and why more time is needed for the intracellular metal content of the periphyton to return to background levels following rain events.

4.5.3 Adsorbed metal on periphyton and intracellular metal content

As a major step in the process of metal uptake is the adsorption of the metal on to the cell wall, the observation of variations in the adsorbed copper and zinc compared with the variations of intracellular metal content is of special interest (Figure 6 and Figure 7).

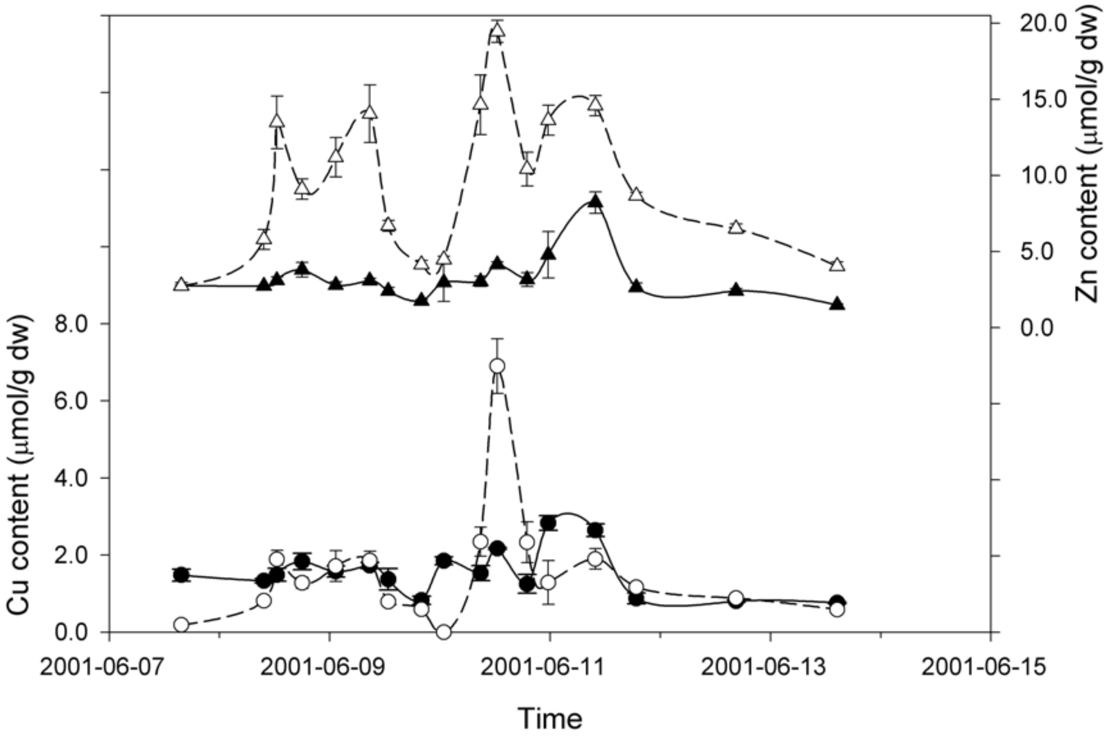


Figure 6: Variations of intracellular zinc content (µmol/g dw, —▲—) and adsorbed zinc on periphyton (µmol/g dw, –△–) and of intracellular copper content (µmol/g dw, —●—) and adsorbed copper on periphyton (µmol/g dw, –○–) in the Furtbach stream, Switzerland, with time over a 8 days rainy period in June 2001.

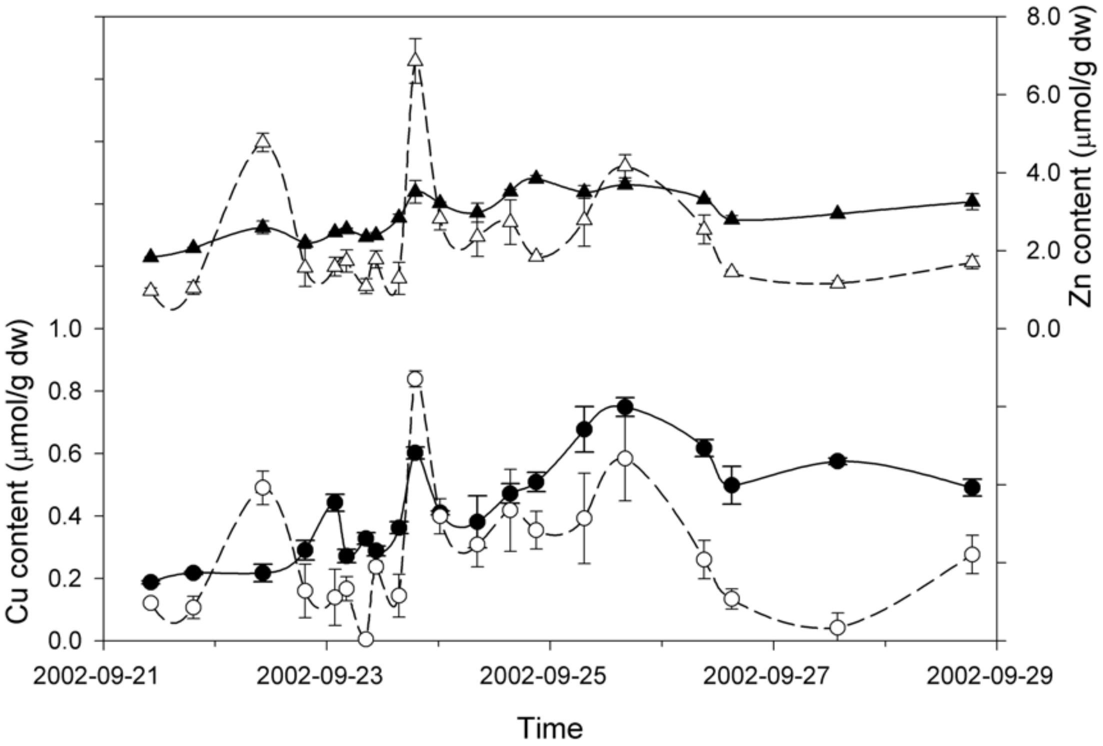


Figure 7: Variations of intracellular zinc content (µmol/g dw, —▲—) and adsorbed zinc on periphyton (µmol/g dw, –△–) and of intracellular copper content (µmol/g dw, —●—) and adsorbed copper on periphyton (µmol/g dw, –○–) in the Furtbach stream, Switzerland, with time over a 8 days rainy period in September 2002.

Peaks in stream copper and zinc concentration were found to immediately influence the adsorbed metal on the periphyton. While all the increases in adsorbed metal are more or less correlated to an increase in the intracellular metal content, it is only during a large and prolonged increase in the labile metal in the water that the uptake occurs and that the intracellular metal content varies. Due to the dynamism of the variations in the metal concentrations, it is not possible to describe a relationship between metal concentrations in the water and adsorbed metals on periphyton. Indeed, the proportion of adsorbed metal to the total metal content in the periphyton varied from 0% to 76% for Cu and from 28% to 83% for Zn with slightly higher ratios in June 2001 than in September 2002. Similar ratios were found in a previous study that compared metal content in periphyton sampled from stones and glass slides (Behra et al., 2002). It was also observed in laboratory studies, that the proportion of adsorbed zinc on algal cells increased from 5% to 80% as Zn^{2+} was varied from 10^{-9} to 10^{-5} M at steady-state (Knauer et al., 1997b).

The fluctuations in the intracellular copper and zinc contents are comparatively small when compared to the variations in the adsorbed metal or the metal concentrations in the water. This is especially true for zinc, where background intracellular zinc contents were relatively high and only small increases were detected, whereas variations of adsorbed metal on the periphyton were very large. The regulation of intracellular metal content by algae at low zinc concentrations has already been observed in laboratory studies in which intracellular zinc content remained nearly constant in function of Zn^{2+} concentrations up to about 10^{-9} to $10^{-8.5}$ M (Sunda and Huntsman, 1992; Knauer et al., 1997b). These concentrations happen to correspond to the range of free zinc ion concentrations occurring in the Furtbach stream.

If we focus more on the values of the metal contents and if we compare the two sampling campaigns performed (June 2001 and September 2002), relatively large differences in the metal contents can be observed. Whereas variations in metal accumulation as a function of the metal speciation had similar relationships between the two periods, higher total copper and zinc contents were measured in June 2001 than in September 2002. Parameters such as pH, temperature, DOC and hardness did not vary significantly between the two periods, nor did copper and zinc speciation differ fundamentally. According to the Biotic Ligand Model (BLM), a model inspired by the FIAM (Di Toro et al., 2001; Campbell et al., 2002), water hardness is a crucial factor that influences the uptake of metals because cations, such as Ca^{2+} and Mg^{2+}, compete with trace metals for binding sites on the cell membrane. Although hardness slightly decreased during the rain events in this study which could increase metal uptake due to a decrease in competition, the effect of hardness alone cannot explain the differences in metal accumulation between June 2001 and September 2002. It is possible that the periphytic communities could differ in species composition from one season to the other, or the thickness of the periphyton layers might vary slightly, but these differences are not expected to play an important role. Differences in the dissolved manganese concentration between the two studies (Table 1) were, in contrast, of more interest. Manganese may influence the uptake of copper and zinc by competing for transport sites (Sunda et al., 1981). Laboratory studies of marine algae lead to the development of a competitive binding model in which Cu^{2+}

would block the uptake or intracellular binding of manganese (Sunda and Huntsman, 1983). Competition between Zn and Mn was also reported in studies where the addition of zinc reduced manganese uptake (Knauer, 1996; Sunda and Huntsman, 1996). Furthermore, a study proposed that manganese (III) hydroxide would adsorb copper on the membrane and prevent its internalization (Stauber and Florence, 1985). In our study, a higher concentration of manganese (Sept 2002) might thus involve a competition between Mn and the trace metals studied, which in turn might have reduced the uptake of copper and zinc. The difference in manganese concentrations observed between studies performed in June 2001 and in September 2002 may thus explain the seasonal differences of metal content in periphyton.

4.5.4 Environmental relevance

The relationship found between the free zinc ion concentration and intracellular zinc content in freshwater periphyton examined in this field study support in the case of zinc the application of the FIAM to freshwaters to prediction of metal bioavailability. The regulation of intracellular zinc content in periphyton at low zinc concentration underlines nevertheless the complexity of natural systems. In contrast, the relationship between exchangeable copper concentration and intracellular copper content in periphyton showed that, in natural water, the very high degree of copper complexation brings the FIAM to its limits. More generally, these results demonstrate the importance of metal speciation in estimating the impact of metals on aquatic organisms under field conditions.

The influence of large increases in copper and zinc concentrations during heavy rain events on periphyton showed that the impact of these environmental changes on microorganisms is clearly measurable and copper and zinc are rapidly intracellularly accumulated. Although no biological endpoints other than the accumulation of copper and zinc were studied, it would be interesting to examine the effects of variations in metal speciation on the growth and the taxonomy of exposed communities. Indeed, previous studies (Soldo and Behra, 2000) showed that prolonged exposure of periphyton to copper levels slightly higher than the Swiss water quality criteria (32 nM for Cu and 76 nM for Zn) and than the Australian and New Zealand guidelines (22 nM for Cu and 122 nM for Zn for

95% protection) induced changes in the species composition of the communities. The ecotoxicological consequences of an extended period of elevated dissolved copper and zinc concentrations (Table 1) exceeding the Swiss water quality criteria are thus reason for concern. These water quality limits are estimations of concentrations that should have no detrimental effect on aquatic organisms, on the basis of ecotoxicological tests. Nevertheless, metal speciation, which was shown to play a fundamental role in the metal bioavailability, should be considered to define future water quality criteria.

(See pictures related to the experimental setup in Chapter 8)

5

Influence of metal speciation in natural freshwater on bioaccumulation of copper and zinc in periphyton: a microcosm study

Sébastien Meylan, Renata Behra and Laura Sigg, 2004, Environmental Science & Technology, in press.

5.1 Abstract

The free ion activity model (FIAM) has already been confirmed under laboratory conditions for many trace metals but has still to be validated under natural conditions where the presence of natural organic ligands influences metal speciation and bioavailability. The goal of this study was to test if the FIAM is followed under natural conditions by measuring copper and zinc speciation as well as metal accumulation in periphyton. Periphyton was exposed in microcosms to natural river water with different added concentrations of copper (25-258 nM) or zinc (18-501 nM) and additions of a synthetic ligand (NTA). Free Cu^{2+} was in the range $10^{-16.5}$-$10^{-14.5}$ M and Zn^{2+} in the range 0.7-8.7 nM, as measured by competitive ligand exchange coupled with cathodic/anodic stripping voltammetry. Other metal complexes were either measured or computed. Bioaccumulation of zinc in periphyton appeared to be controlled by the free zinc ion concentration, confirming the FIAM. In contrast, bioaccumulation of copper was controlled by weakly complexed copper (including Cu^{2+} plus inorganic and weak organic complexes), which is in disagreement with the FIAM, and appears to be caused by limitation of copper diffusion due to very low free Cu^{2+} occurring in natural environments.

5.2 Introduction

Bioavailability as well as toxicity of trace metals to aquatic microorganisms are dependent on metal speciation (Morel and Hering, 1993; Tessier and Turner, 1995). Trace metals, such as copper and zinc, are essential micronutrients (Sunda, 1989). Their bioavailability in synthetic media appears to be controlled by the free metal ion concentration, as predicted by the free ion activity model (FIAM) (Campbell, 1995). Several laboratory studies focused on relating the free metal ion activity to the effect on aquatic microorganisms. Applicability of the model was demonstrated with marine phytoplankton for copper (Sunda and Guillard, 1976; Brand et al., 1986) and for zinc (Anderson et al., 1978; Sunda and Huntsman, 1992) as well as with freshwater algae (Petersen, 1982; Knauer et al., 1997b). Although the FIAM showed relatively good predictability in laboratory studies, some exceptions were found (Campbell, 1995; Errécalde and Campbell, 2000).

Furthermore relationships between free metal ion activity and bioavailability remain to be demonstrated under natural conditions where the presence of natural organic ligands strongly influences the metal speciation (Campbell et al., 1997; Vigneault et al., 2000) and might then influence the metal-organism relationship. Up to now only few studies focused on this relationship in field experiments (Hare and Tessier, 1996; Mylon et al., 2003). The biotic ligand model (BLM), derived from the FIAM, takes into consideration the properties of natural water (DOC, hardness and pH) to account for the influence of competition between cations for the biotic ligand (active sites of microorganisms) and the reduction of metal bioavailability by natural ligands (Di Toro et al., 2001; Campbell et al., 2002). Relationships between microorganisms and metals in natural waters are essential for the formulation of relevant water quality criteria (Allen and Hansen, 1996), thus further experimental data are needed.

Periphyton, a natural algal biofilm growing on solid substrates, is ubiquitous in freshwater systems and plays a prominent role as primary producer in streams. The ecological importance of this consortium of microorganisms and its sensitivity to changes under natural conditions (Wetzel, 1983) and to metal contamination (Newman and McIntosh, 1989; Soldo and Behra, 2000; Blanck et al., 2003) means that field experiments using periphyton are ecologically significant. Metal accumulation is a meaningful endpoint to measure bioavailability, as metal internalization is normally a necessary step for effect (Campbell et al., 2002).

In a previous study, accumulation of copper and zinc was studied as a function of natural variations of their concentrations (Meylan et al., 2003). To evaluate more precisely the relationships between accumulation in periphyton and speciation, experiments in microcosms are carried out. The goal of this research was to study the relationship between metal speciation in natural freshwater and metal accumulation in periphyton and to investigate if the FIAM is appropriate to predict copper and zinc accumulation in a natural system. For these experiments, we focused on two essential metals, copper and zinc. Natural periphytic communities were exposed to Cu and Zn under various conditions in natural freshwater. Experimental conditions were varied by adding metal or/and a ligand NTA (nitrilotriacetic acid) to modify the metal speciation of the natural river water. The copper and zinc dissolved concentrations were in a range from unpolluted to slightly polluted freshwater, which is likely to be found in the environment. Metal speciation was measured and natural ligand concentrations and stabilities were characterized. Accumulation of Cu and Zn in periphyton was measured under the various conditions and was related to the measured and computed Cu and Zn speciation.

5.3 Materials and Methods

5.3.1 Experimental setup

The setup was designed to allow the exposure of identical communities of periphyton to copper and zinc in natural freshwater under stable conditions over a short period of time (2 days). For this purpose, the study was divided into 3 steps: 1) the periphyton colonization of glass slides was achieved in tanks operated as a flow-through system with natural water during a 3 week period. 2) The experimental tanks were prepared and left 3 days to allow the equilibration of the natural water with different concentrations of metal and of synthetic ligand added. 3) For the bioaccumulation experiment, colonized glass slides were exposed in the prepared experimental tanks.

The natural water was taken from the Glatt river (Zurich, Switzerland) at a location situated 1.4 km downstream from Lake Greifen where the periphyton colonization, the bioaccumulation experiments and the sampling were performed.

5.3.2 Periphyton colonization

Microscope slides fixed vertically (short edge) at about 1 cm under the water surface in polypropylene (PP) racks were used to colonize periphyton. Racks holding 10 slides each were placed in colonization tanks during 3 weeks. Low quantities of inorganic particles are expected in the periphytic biofilm due to the vertical position of the microscopic slides on one hand and to the rapid sedimentation of the particles in the tanks where the agitation is much lower than in the river. Already after a few days in the water, a thin biofilm developed on the slides and a growth period of 3 weeks was enough to obtain a homogenously grown periphyton layer sufficient for the experiment. Microscopic examinations of some biofilm samples indicated that the majority of particles were algae (not shown).

5.3.3 Bioaccumulation experiment

Three days before the experiment, 14 experimental tanks of 50 liters were filled with natural water and different added concentration of metal (0-250 nM copper or 0-600 nM zinc) and NTA (0 or 1.8 mM for the copper experiment, 0 or 0.01 mM for the zinc experiment), and allowed to equilibrate (Table 1a and b). Concentrations of NTA were based on a preliminary experiment carried out in January 2001.

At the beginning of the bioaccumulation experiment, microscope slides with a thin biofilm of periphyton were translocated from the colonization tank to the experimental tanks (2x10 slides pro tank). Periphyton samples for the analysis of metal content were taken after 48 hours of exposure in each experimental tank simultaneously with water samples for the determination of the metal speciation. Periphyton samples were as well taken after 0, 4, 8, 24 and 48 hours of exposure in the two experimental tanks containing the highest metal concentration to determine metal uptake rates of the periphytic community. Changes in periphyton biomass during the bioaccumulation experiment were negligible. Water samples for total dissolved metal concentration were taken several times in each tank during the exposure time.

5.3.4 Periphyton sampling and metal content analysis

For each measurement of metal content, a sample (5 microscope slides) was taken for metal analyses. Slides were thoroughly rinsed with filtered river water (0.45 μm, Orange scientific Gyrodisc). The natural algal biofilm was then scratched from the slide with a clean microscope slide and was suspended in filtered river water. The suspension was afterwards divided into two fractions. One fraction (20 ml) was treated during 10 minutes with 4.0 mM EDTA (final concentration) added to the river water to remove the metals adsorbed to the cell wall as well as most of the inorganic complexes stacked in the biofilm (Meylan et al., 2003).

Table 1a: Copper experiment performed in April 2003 with the concentration of copper and NTA added to the different tanks.

	copper added (nM)	copper measured (nM)	NTA added (mM)	DOC measured (mg/l)
1	-	25 ± 1	-	4.1 ± 0.4
2	-	25 ± 1	-	4.3 ± 0.4
3	75	79 ± 3	-	4.5 ± 0.5
4	75	80 ± 3	-	4.2 ± 0.4
5	150	132 ± 5	-	4.5 ± 0.5
6	150	130 ± 5	-	4.2 ± 0.4
7	250	204 ± 8	-	4.5 ± 0.5
8	250	191 ± 8	-	4.4 ± 0.4
9	-	26 ± 1	1.8	137 ± 14
10	-	30 ± 1	1.8	134 ± 13
11	150	162 ± 6	1.8	133 ± 13
12	150	164 ± 6	1.8	135 ± 14
13	250	258 ± 10	1.8	138 ± 14
14	250	252 ± 10	1.8	134 ± 13

Copper measured: n=5, DOC: n=2.

Table 1b: Zinc experiment performed in June 2002 with the concentration of zinc and NTA added to the different tanks.

	zinc added (nM)	zinc measured (nM)	NTA added (mM)	DOC measured (mg/l)
1	-	18 ± 2	-	3.6 ± 0.4
2	-	18 ± 2	-	3.6 ± 0.4
3	100	41 ± 4	-	3.5 ± 0.4
4	100	38 ± 4	-	4.0 ± 0.4
5	300	100 ± 9	-	3.6 ± 0.4
6	300	87 ± 8	-	3.6 ± 0.4
7	600	196 ± 18	-	4.4 ± 0.4
8	600	230 ± 22	-	3.6 ± 0.4
9	-	30 ± 3	0.01	4.7 ± 0.5
10	-	30 ± 3	0.01	4.9 ± 0.5
11	300	266 ± 25	0.01	4.2 ± 0.4
12	300	270 ± 26	0.01	5.4 ± 0.5
13	600	501 ± 48	0.01	4.8 ± 0.5
14	600	456 ± 44	0.01	4.9 ± 0.5

Zinc measured: n=5, DOC: n=2.

This process allowed the measurement of the intracellular metal content of periphyton and the other fraction was used to measure the total amount of metal accumulated in periphyton. The difference between total and intracellular metal content is considered as adsorbed metal. The efficiency of the EDTA wash technique was checked in separate experiments with different EDTA concentrations (0.4 to 40 mM) and treatment times (0 to 60 min). Constant values were obtained after 10 min treatment with 4.0 mM EDTA. These conditions showed no intracellular metal removal and microscopic observations revealed that the cell walls remained intact. Three aliquots of each fraction were filtered on acid-washed and pre-weighed filters (cellulose nitrate 0.45 μm, Sartorius) to obtain the dry weight (dw) of each sample after 15 h drying at 50°C. Filters were digested with 4 ml nitric acid concentrated (suprapure) and 1 ml hydrogen peroxide (31%, suprapure) during 13 min in a high performance microwave digestion unit (mls1200 mega). After dilution to 25 ml with nanopure water, the metal concentrations of the samples were measured by ICP-OES (Spectro Ciros CCD). The digestion procedure was performed as well with plankton reference material (error < 15%, CRM 414, Community Bureau of Reference, Commission of the European Communities), and blank filters to check the quality and the accuracy of the measurements. Blank measurements were under the detection limit (<10.2 nM for Cu, <10.9 nM for Zn).

5.3.5 Trace metal clean procedure

All handling was performed with plastic gloves (Semadeni) to avoid contamination. The 50-liters PP tanks used for the exposure experiment were previously cleaned and thoroughly rinsed with river water. They were protected during the experiment by transparent plastic covers to avoid external contamination. Vials, filtering membranes, filtration unit and PP bottles were previously washed with 0.1 M HNO_3 during minimum 24 hours. Filtration was performed in the laboratory under a clean bench.

5.3.6 Water sampling and dissolved metal concentration measurements

Water samples were taken manually in each tank regularly during the 48 hours exposure of periphyton (5 times). The samples (10 ml) were filtered using a plastic syringe and a filter, both previously thoroughly rinsed with water from the tank (20 ml syringe, Once; 0.45 µm, Orange scientific Gyrodisc), and acidified to 0.01 M with HNO_3 (suprapure). Dissolved metal concentrations (Cu, Zn) were measured by ICP/MS (Perkin Elmer Elan 5000) with rhodium used as an internal standard. Field blanks were taken to control the cleanness of the sampling and showed metal concentrations below the detection limit (<0.15 nM for Cu and <1.5 nM for Zn). The accuracy of the ICP/MS measurements was checked using SLRS-4 reference water (error < 10%, National Research Council Canada).

5.3.7 Metal speciation: water sampling and measurement

In addition to the dissolved metal concentration, two different fractions of the metal were determined, the free metal ion concentration (Cu^{2+} and Zn^{2+}) and the labile metal concentration ($Cu_{\text{weakly complexed}}$ and Zn_{labile}).

At the end of the exposure time, a 2-liter water sample in PP bottles was taken from each tank to measure the free metal ion concentration. Samples were filtered within 6 hours after sampling on a 0.45-µm filter (Cellulose nitrate, Sartorius) and kept at a temperature of 4°C in PP bottles until analysis. Free metal ion concentration was indirectly calculated by competitive ligand exchange (CLE) and the measurements of the samples were performed by electrochemical detection of the metal or the metal complex depending on the ligand used (DPCSV/DPASV - differential pulse cathodic/anodic stripping voltammetry). MOPS (0.02 M, 3-morpholinopropanesulfonic acid) was used to buffer the samples at their natural pH. All the stability constants involved were adapted to the ionic strength of the water (Martell and Smith, 1989).

For the measurement of the free copper ion concentration, the complexing ligand used was catechol (1 mM) and the copper-catechol complex was measured by DPCSV with a voltammeter Metrohm VA Stand 694 coupled to a Metrohm VA Processor 693 (HMDE, Ag/AgCl/KNO_3 sat. as reference electrode, Pt counter electrode, 5 min purging with N_2, 120 s deposition time, -0.05 V deposition potential, 10 mV/s scan rate, -50 mV pulse amplitude, 0.2 s step time) (van den Berg, 1984; Xue and Sigg, 1993). The titrations curves were performed using 15 different concentrations of copper added (up to 550 nM) and including 5-8 points to calibrate the sensitivity of the DPCSV titrations (0.2 to 0.46 A M^{-1}, lower in the presence of NTA). The solutions were let to equilibrate over night in the presence of copper and the catechol was added 5 minutes before the voltammetric measurements. For every water sample, this modus operandi was verified to be appropriate. The titration curves of copper were fitted (software FITEQL 4.0 (Westall, 1982)) to a two-ligand model, for which concentrations and conditional stability constants of a strong copper-binding ligand L_{strong} and a weak copper-binding ligand L_{weak} were obtained. "Weakly complexed copper" is defined as the sum of the free copper, of the inorganic copper-species and of the copper bound to weak organic ligands and is assumed not to include the CuNTA-complexes. Weakly complexed copper concentrations were extrapolated from the voltammetric measurements of an exchangeable form of copper ($Cu_{exchangeable}$), which exchanges with the copper-catechol complexes at a catechol concentration of 1 mM and which was obtained by standard addition of copper and DPCSV measurement in the presence of catechol. With the measurements of the free copper ion concentration, the dissolved copper concentration, the water parameters and the deduced values of strong and weak copper ligand concentrations and constants, the proportion between exchangeable copper (measured) and weakly complexed copper can be calculated (equilibrium software vminteq 2.15 (Gustafsson)). $Cu_{weakly\ complexed}$ only includes free copper ion and complexes weaker than CuNTA, whereas $Cu_{exchangeable}$ includes the CuNTA-complexes. Furthermore, the complexation coefficient of the copper-catechol complex (α_{CuCat}) under these experimental conditions can be calculated and, with the ligand concentration obtained from the titration curve, the stability constant of a copper-exchangeable ligand complex can be extrapolated.

For the measurement of the free zinc ion concentration, the complexing ligand used was EDTA (0, 20 and 30 nM). Zinc was measured by DPASV (HMDE, Ag/AgCl/KNO_3 sat. as reference electrode, Pt counter electrode, 5 min purging with N_2, 120 s deposition time, -1.2 V deposition potential, 10 mV/s scan rate, 50 mV pulse amplitude, 0.2 s step time) (Xue and Sigg, 1994). The sensitivity of the DPASV titration was measured for each water sample using 5 points (0.03 to 0.47 A M^{-1}, lower in the presence of NTA) and 8-10 points were used for the determination of the free zinc ion concentration (up to 80-120 nM Zn added). All samples for the titration were prepared and let overnight to equilibrate. Labile zinc was obtained by standard addition of zinc (under a labile form) and direct measurement of the labile zinc by DPASV.

The errors on the determination of the free metal ion concentrations were estimated from the titration curves where the standard deviation of the sensitivity and the error on the replication of each point were used. The errors on the labile metal fraction were obtained from the standard deviations on the measurements by standard addition (Figure 1 and 3).

5.3.8 Water chemistry

Temperature, pH and conductivity were measured automatically every 30 min in a control tank as well as in each tank twice a day with a pH meter WTW 340i Multi. At the end of the exposure time, water samples to measure concentrations of major cations and major anions, DOC, hardness and alkalinity were taken. These values are necessary to calculate the ionic strength. Major cation concentrations were obtained with ICP/OES, major anions were measured by ion chromatography (Metrohm), alkalinity measurements were performed by titration (with HCl 0.1 M until pH 4.5) and DOC was obtained by combustion (Elementar).

5.4 Results

5.4.1 Copper experiment

The copper experiment was performed in April 2003 and the exposure time itself was 48 hours. The concentration of copper added to the tanks varied from 0 to 250 nM and the measurements of $Cu_{dissolved}$ showed values ranging from 25 to 258 nM, 25 nM corresponding to the background concentration of copper in the river water (Table 1a). The copper speciation results in natural freshwater obtained during this study (Figure 1) showed that the free copper ion concentration varied from $10^{-16.5}$ to $10^{-14.5}$ M. A good correlation ($r^2 = 0.89$) was found between Cu^{2+} and $Cu_{dissolved}$ and this relation was not influenced by the addition of NTA. Weakly complexed copper was in contrast influenced by the addition of NTA in the tanks (Table 2a). The calculated value of $Cu_{weakly\ complexed}$ was higher without addition of NTA (3-65 nM) than in the presence of added NTA (0.2-4 nM). R^2 coefficient for the relationships of $Cu_{dissolved}$ with $Cu_{weakly\ complexed}$ in the presence or absence of

NTA were 0.91 and 0.96 respectively (Figure 1). Water parameters measured during this experiment are shown in Table 3.

Table 2a: Experimental speciation data for copper at background concentration without and with NTA added (results for 2 tanks).

		no NTA		**1.8 mM NTA**	
		(nM)	% of $Cu_{dissolved}$	(nM)	% of $Cu_{dissolved}$
Cu^{2+}	measured	0.04 ± 0.02 (x10^{-6})	*0.1x10^{-6}*	0.05 ± 0.04 (x10^{-6})	*0.2x10^{-6}*
$Cu_{inorganic}$	calculated	12 (x10^{-6})	*50x10^{-6}*	10 (x10^{-6})	*40x10^{-6}*
CuL_{weak}	calculated	2.8	*11*	0.2	*1*
CuNTA	calculated	-	-	7.2	*27*
CuL_{strong}	calculated	22	*89*	19	*72*
$Cu_{exchangeable}$	measured	2.8 ± 0.1	*11*	7.4 ± 1.2	*28*
$Cu_{dissolved}$	measured	25 ± 1	*100*	26 ± 2	*100*

Table 2b: Experimental speciation data for zinc at background concentration without and with NTA added (results for 2 tanks).

		no NTA		**0.01 mM NTA**	
		(nM)	% of $Zn_{dissolved}$	(nM)	% of $Zn_{dissolved}$
Zn^{2+}	measured	1.0 ± 0.1	6	0.7 ± 0.1	2
$Zn_{inorganic}$	calculated	2.6	14	1.6	5
ZnL_{weak}	calculated	3.3	18	4.3	14
ZnNTA	calculated	-	-	11	37
ZnL_{strong}	calculated	11	61	13	43
Zn_{labile}	measured	6.9 ± 1.0	38	17 ± 2	57
$Zn_{dissolved}$	measured	18 ± 2	100	30 ± 3	100

Intracellular copper content of periphyton varied between 0.066 and 0.180 µmol/g dw when no NTA was added to the natural river water (Figure 2). In the presence of 1.8 mM NTA, Cu content was lower ranging from 0.038 to 0.058 µmol/g dw. Comparable data on copper content were obtained in a preliminary study performed 1 year before the main experiment with similar $Cu_{dissolved}$ and Cu^{2+} and using the same experimental setup. The relationship between copper accumulation in periphyton and copper speciation in the natural river water was influenced by the addition of NTA. The difference between the slopes of the curves obtained in the presence or absence of NTA for Cu^{2+} was relatively large, whereas weakly complexed copper showed a linear relationship with the intracellularly accumulated copper independently of the addition of NTA (r^2 = 0.92) (Figure 2). The linear relationship between Cu content and $Cu_{weakly\ complexed}$ including conditions in the presence and absence of NTA indicates that $Cu_{weakly\ complexed}$ controls copper accumulation.

Table 3: Water parameters during the 2 experiments

		Range
Cu experiment (April 2003)		
pH	(-)	8.41±0.06
Temperature	(°C)	10.3±5.1
Alkalinity	(mM)	4.1±0.8
Ionic Strength	(mM)	7.8±1.7
Hardness	(meq/l)	4.3±0.4
Zn experiment (June 2002)		
pH	(-)	8.43±0.06
Temperature	(°C)	18.8±3.7
Alkalinity	(mM)	3.8±0.1
Ionic Strength	(mM)	6.8±0.1
Hardness	(meq/l)	4.0±0.1

Total copper content was slightly higher than intracellular copper content of periphyton and the proportion of intracellular content compared to the total content was between 77 and 94%, the rest being adsorbed on the cell walls. The fraction of copper adsorbed to the cell wall is increasing slightly with increasing concentration of copper weakly complexed. The adsorbed fraction is thus lower at low total dissolved copper concentrations and in the presence of NTA.

5.4.2 Zinc experiment

The zinc experiment was performed in June 2002. The concentration of zinc added to the tanks was in the range 0 to 600 nM and the concentration of dissolved zinc measured in the tanks varied between 18 and 501 nM with a background concentration of 18 nM Zn (Table 1b). A relatively large fraction of the zinc added is adsorbing on the tank internal sides in the absence of NTA whereas zinc stays in solution in the presence of NTA. Figure 3 shows that there is a linear correlation ($r^2 = 0.92$) between labile zinc and dissolved zinc and that the addition of 0.01 mM NTA does not influence this relationship. The range of Zn_{labile} found was 4.2-389 nM. The free zinc ion concentration was on the contrary influenced by the addition of NTA, which reduced the Zn^{2+} concentration. A linear correlation between $Zn_{dissolved}$ and Zn^{2+} was found in both cases, with ($r^2 = 0.99$) or without ($r^2 = 0.92$) NTA added and the range of Zn^{2+} was 0.7-8.7 nM. Water parameters measured during this experiment are shown in Table 3.

Intracellular zinc content of periphyton after 48 hours exposure varied between 0.46 and 2.7 µmol/g dw (Figure 4). A linear relationship was found between the intracellular zinc content and the free zinc ion concentration ($r^2 = 0.81$) including the samples with added NTA, which indicates that the free zinc ion concentration controls the zinc accumulation. The relation between Zn_{labile} or $Zn_{dissolved}$ and zinc content shows the influence of the addition of NTA in the natural river water. NTA lowered the zinc content (Figure 4).

The comparison of the measurements of the total zinc content and the intracellular zinc content of periphyton shows that 90±5% of the metal was intracellularly accumulated and that around 10% was adsorbed to the cell walls. Generally, zinc adsorbed on the cell wall increases with increasing free zinc ion concentration.

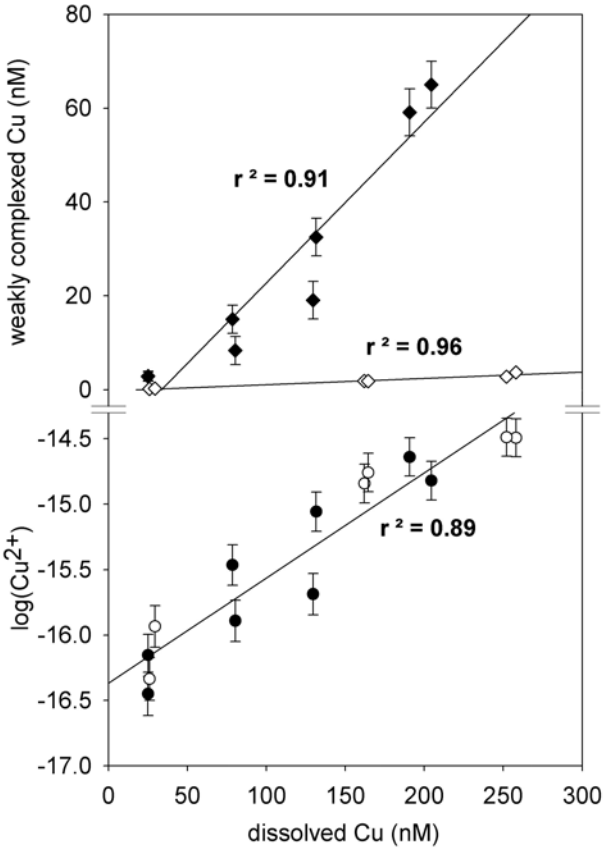


Figure 1: Speciation of copper in natural river water; weakly complexed copper (◆ - no NTA, ◇ - 1.8 mM NTA added) and free copper ion (M) (● - no NTA, ○ - 1.8 mM NTA added) as a function of dissolved copper.

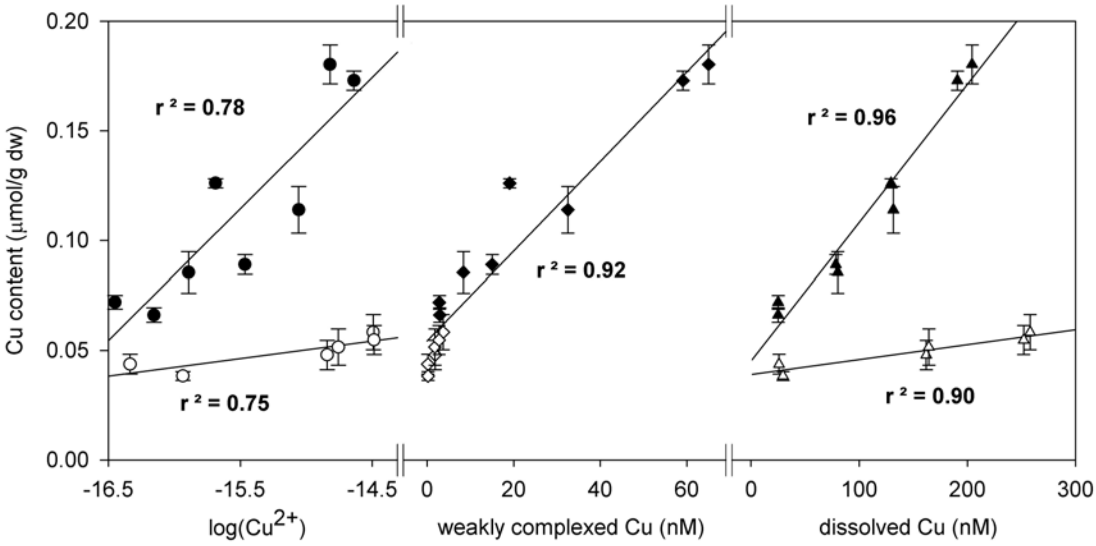


Figure 2: Bioaccumulation of copper; intracellular copper content of periphyton as a function of free copper ion (M) (● - no NTA, ○ - 1.8 mM NTA added), weakly complexed copper (◆ - no NTA, ◇ - 1.8 mM NTA added) and dissolved copper (▲ - no NTA, △ - 1.8 mM NTA added).

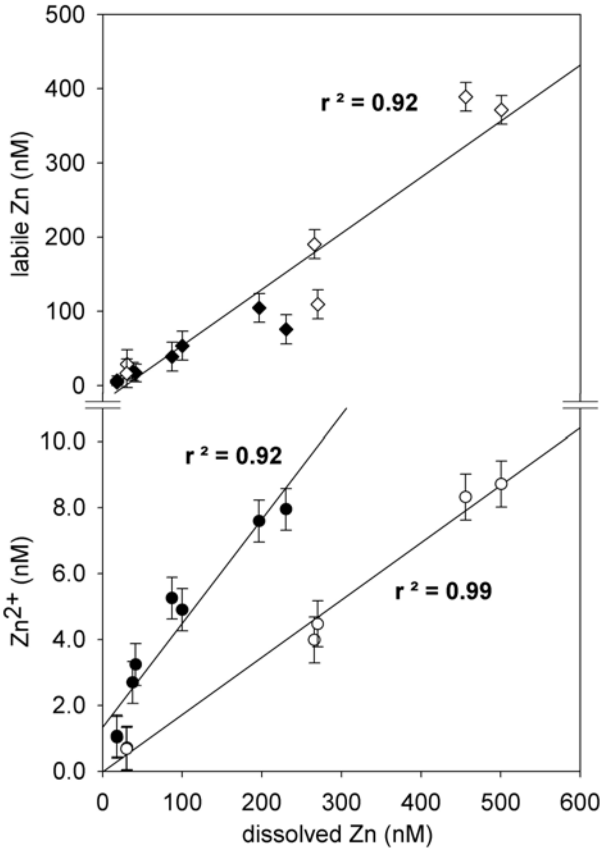


Figure 3: Speciation of zinc in natural river water; labile zinc (◆ - no NTA, ◇ - 0.01 mM NTA added) and free zinc ion (● - no NTA, ○ - 0.01 mM NTA added) as a function of dissolved zinc.

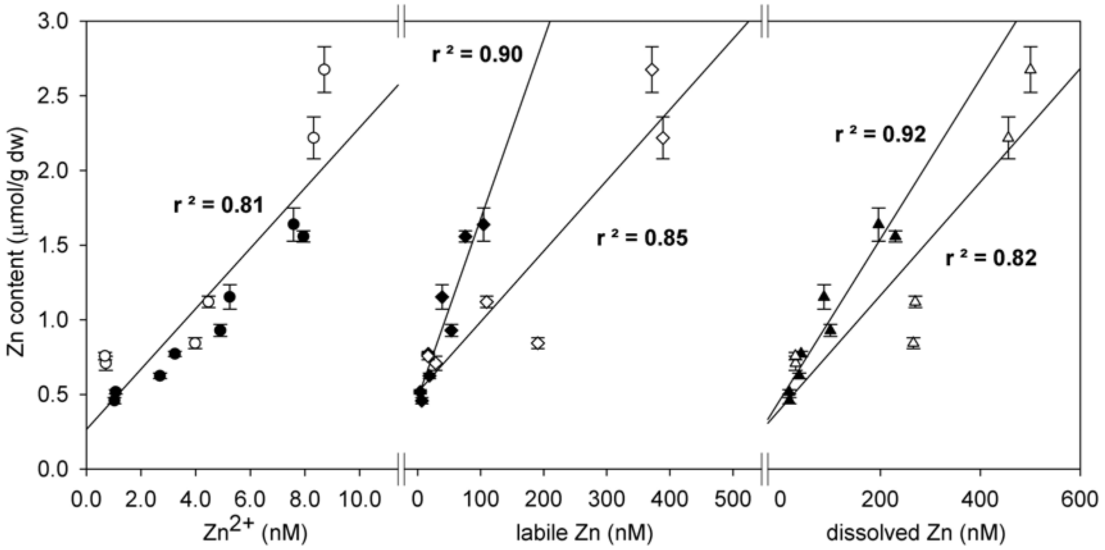


Figure 4: Bioaccumulation of zinc; intracellular zinc content of periphyton as a function of free zinc ion (● - no NTA, ○ - 0.01 mM NTA added), labile zinc (◆ - no NTA, ◇ - 0.01 mM NTA added) and dissolved zinc (▲ - no NTA, △ - 0.01 mM NTA added).

5.4.3 Uptake kinetics and calculations of uptake and diffusion fluxes

The transport of free metal ions to the membrane (diffusion flux) and the internalization of the metal (uptake flux) were compared (Table 4). Copper and zinc uptake fluxes J_u (mol min^{-1} cm^{-2}) and diffusion fluxes J_{dif} (mol min^{-1} cm^{-2}) were evaluated for this study using the following expressions:

$$J_u = u_m \cdot (w/s)$$

$$J_{dif} = c_m \cdot D_m / \delta$$

where u_m (mol min^{-1} g^{-1}dw) is the uptake rate, w/s is a coefficient (gdw cm^{-2}) relating the cell dry weight to the cell surface, c_m (mol cm^{-3}) is the metal concentration in the bulk solution, D_m (cm^2 min^{-1}) is the diffusion coefficient and δ (cm) is the diffusive boundary layer.

Table 4: Diffusion fluxes and uptake fluxes for the copper and zinc experiments considering a "most favorable case" where limitation is minimized and a "least favorable case" where limitation is maximized.

Metal species	**Concentration** **c_m** (M)	**Diffusion flux** **J_{dif}** (mol min^{-1} cm^{-2})		**Uptake flux** **J_u** (mol min^{-1} cm^{-2})
Most favorable case				
Cu^{2+}	1.9×10^{-15}	4×10^{-18}	<<	2×10^{-15}
Cu_{labile}	3.8×10^{-9}	8×10^{-12}	>>	"
Zn^{2+}	7.8×10^{-9}	2×10^{-11}	>>	1×10^{-14}
Zn_{labile}	9.0×10^{-8}	2×10^{-10}	>>	"
Least favorable case				
Cu^{2+}	1.9×10^{-15}	4×10^{-21}	<<	4×10^{-15}
	3.6×10^{-17}	7×10^{-23}	<<	"
Cu_{labile}	3.8×10^{-9}	8×10^{-15}	≈	"
	7.1×10^{-11}	1×10^{-16}	<	"
Zn^{2+}	7.8×10^{-9}	2×10^{-14}	≈	2×10^{-14}
	6.8×10^{-10}	1×10^{-15}	≈	"
Zn_{labile}	9.0×10^{-8}	2×10^{-13}	≈	"
	4.2×10^{-9}	8×10^{-15}	≈	"

The uptake rates u_m were calculated from short-term uptake data obtained with the highest free metal concentration (log[Cu^{2+}]=-14.7±0.1 (M), [Zn^{2+}]=7.8±0.2 nM). The accumulation of metal versus time is shown on Figure 5. Calculated uptake rates of periphyton were 3.1±0.3 nmol hours^{-1} (g dw)$^{-1}$ for Cu and 20±4 nmol hours^{-1} (g dw)$^{-1}$ for Zn. At similar free metal ion concentrations, uptake rates measured in laboratory studies with algae (Sunda and Huntsman, 1992; Sunda and Huntsman, 1995; Knauer et al., 1997a) gave values in a similar order of magnitude. The average size of algal species in periphyton was assumed to be in the range 5 to 10 μm, which is consistent with measurements performed in other studies (McCollum et al., 1998), and the dry weight per cell is about 3x10^{-11}-2x10^{-10} g for algae of this size range. Diffusion coefficients were assumed to be in the range 3.3x10^{-6}-9x10^{-6} cm^{2}·min^{-1} for Cu^{2+} and Zn^{2+} (Li and Gregory, 1974; Mota and Correia dos Santos, 1995) and the diffusive boundary layer of biofilm is estimated to be, in flowing water, 0.1 mm (Kohler-Rink and Kuhl, 2000) and, in still water, 1 mm (Ploug and Jorgensen, 1999), which corresponds to the size of diffusive boundary layers measured for periphyton (Riber and Wetzel, 1987).

A "most favorable case" where diffusion fluxes are optimal (small boundary layer, high diffusion coefficient and highest concentration measured) and uptake fluxes are minimal (smaller periphytic cells) was considered, as well as a "least favorable case" where diffusion is unfavorable and uptake is elevated. J_{dif} and J_u are shown in Table 4.

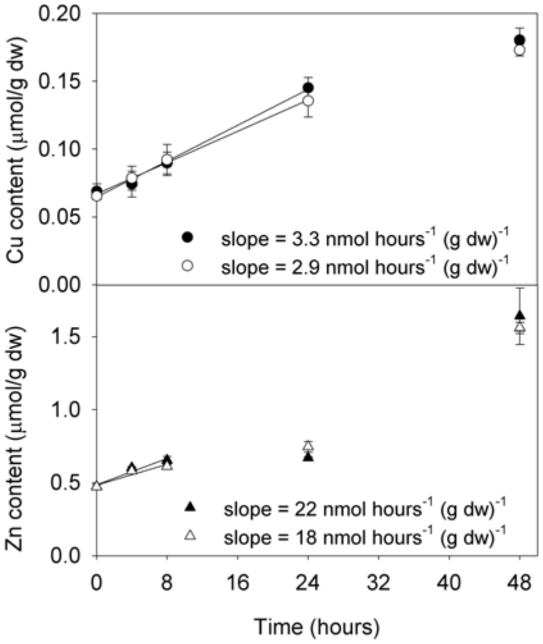


Figure 5: Accumulation of copper (●, ○ - 2 replicates) and zinc (▲, △ - 2 replicates) in periphyton as a function of time, initial linear regression representing the uptake rate (nmol hours^{-1} (g dw)$^{-1}$).

5.5 Discussion

5.5.1 Copper speciation

In this study (Figure 1) the ratio of Cu^{2+} to $Cu_{dissolved}$ is very low (10^{-9}-10^{-8}) and the extent of copper complexation is more than 99.9999%. The fact that the copper experiment were carried out in spring during the period of high biological activity might explain the high proportion of copper bound to organic ligands. These results are in agreement with previous studies of copper speciation using similar methods (Coale and Bruland, 1988; Xue and Sigg, 1993).

Free copper ion concentration (Figure 1) in natural river water is apparently not influenced by the addition of 1.8 mM NTA. The introduction of a relatively strong organic ligand ($\log(K_{CuNTA}) = 13.49$ with $I = 0.01$, complexing coefficient $\alpha_{CuNTA} = 3.8 \pm 0.2 \times 10^{7}$) leads to a competition with weak and to some extent with strong natural organic ligands. The relation of competition between the weak natural organic ligands and NTA influences largely the weakly complexed fraction of copper, whereas it has no detectable effect on Cu^{2+} concentration. As the overall equilibrium is largely influenced by competing reactions and the free metal ion is at very low concentration in the natural water, the difference measured in $Cu_{weakly\ complexed}$ is not reflected in Cu^{2+}. Any difference of free copper ion concentration in the presence of NTA and in the absence of NTA is considered to be within experimental error.

In contrast, the effect of the addition of NTA on the extrapolated value of weakly complexed copper (as well as on the measured value of exchangeable copper) is clearly visible (Table 2a). The measurement of the $Cu_{exchangeable}$ includes CuNTA complexes as these complexes are labile with respect to the exchange with catechol. Indeed, calculation of the complexing coefficient of the CSV-labile copper complexes which corresponds to $\alpha_{CuCatechol}$ ($6.5 \pm 1.2 \cdot 10^7$) is slightly higher than the complexing coefficient of CuNTA ($3.8 \pm 0.2 \cdot 10^7$). The extrapolated values of weakly complexed copper, which considers only the copper complexes with stability constants corresponding to $\log K \leq 12$ (corresponding to the fitted weak ligand L_{weak}), are much lower when 1.8 mM NTA is added to the river water than in the absence of NTA. Indeed, at background concentration, copper bound to weak organic (CuL_{weak}) ligands drops from around 11% of dissolved copper in the absence of NTA to less than 1% of dissolved copper in the presence of NTA (Table 2a).

5.5.2 Copper bioaccumulation

The intracellular copper content of periphyton is largely influenced by the presence of NTA (Figure 2). For similar dissolved concentration, the presence of NTA reduces by 1.5 to 3-fold the intracellularly accumulated copper. Furthermore, in the presence of NTA, the highest Cu content (0.058 μmol/g dw) is lower than the lowest Cu content without added NTA (0.066 μmol/g dw). Only weakly complexed copper, which is as well largely influenced by the introduction of the synthetic ligand, explains the low copper accumulation in periphyton in the presence of NTA, as the good correlation ($r^2 = 0.92$) between weakly complexed copper and intracellular copper content shows (Figure 2). This means that under natural conditions copper accumulation is controlled by the weakly complexed forms of copper. Whereas it was expected that $Cu_{dissolved}$ would not have a direct relationship with the intracellularly accumulated copper, the fact that Cu^{2+} does not explain Cu content of periphyton indicates the limitation of the FIAM under conditions of low copper concentrations. Although the fact that weakly complexed copper is controlling the copper bioaccumulation under natural conditions was already suspected in a previous study (Meylan et al., 2003), this study is the first experimental evidence that at low copper concentrations copper accumulation is controlled by a labile form of the metal.

A theoretical study (van Leeuwen, 1999) predicted that when the free metal ion in solution reaches very low concentration the metal uptake could be controlled by the labile metal concentration. If we consider that, under natural conditions, the large concentration of organic ligands is limiting the availability of Cu^{2+} at the cell diffusion layer, the metal uptake is controlled by the labile copper concentration as these species provide copper for the cell membrane. In our study, where strong organic ligands are present in high concentration, $Cu_{\text{weakly complexed}}$ represents the labile form, which controls the copper uptake.

5.5.3 Zinc speciation

The speciation of zinc in natural freshwater shows a much higher proportion of free metal ion than in the case of copper. In the present study, the measurements obtained for the natural water without any addition of zinc or NTA show a high complexation ratio due to the high pH (8.40) with an average of 5.7% of Zn^{2+}, 38% of Zn_{labile} and about 63% of strong organic complexes (Table 2b). These values are in agreement with previous studies (Xue and Sigg, 1994).

The addition of 0.01 mM of NTA in the natural river water (Figure 3) shows no effect on the fraction of labile zinc because of two reasons. First the complexing coefficient α_{ZnNTA} (106±4) is lower than the complexing coefficient of the zinc-labile ligand complexes $\alpha_{Zn(L\_weak)}$ (177±25) indicating that NTA competes mostly with the weak organic ligands and that the concentration of NTA is not sufficient to compete with the strong organic ligands as it is the case for copper. Second, ZnNTA-complexes are labile to the ASV conditions used. Free zinc ion concentration is in contrast to labile zinc influenced by the addition of NTA, which decreases Zn^{2+} by about 2-fold.

5.5.4 Zinc bioaccumulation

Depending on the presence of NTA, labile zinc and dissolved zinc concentrations show different relationship to the accumulation of zinc in periphyton (Figure 4) indicating that these measured values are not the concentrations controlling zinc accumulation. Intracellular zinc content is however clearly related to the free zinc ion concentration and a good correlation was found independently from the addition of NTA (r^2=0.81). In the case of environmentally relevant zinc concentrations in natural freshwater the FIAM is thus verified.

The curve of intracellular zinc content as a function of free zinc ion concentration shows that, at low Zn^{2+} concentration (<4 nM), the variation of zinc content is very small (Figure 4). This would suggest that algae regulate their intracellular Zn content at background concentration to maintain an optimal level of intracellular concentration needed for growth and metabolism. Zn regulation has been reported with marine and freshwater algae in laboratory studies in a range of free zinc ion concentration up to 3 nM (Sunda and Huntsman, 1992; Knauer et al., 1997b; Sunda and Huntsman, 1998), which corresponds to the range measured in this study.

5.5.5 Uptake and diffusion fluxes

This study covers a range of dissolved concentrations typically found in natural systems up to moderately contaminated sites (Table 1a and b). Indeed, background concentrations of copper and zinc found in natural freshwater are 4–24 nM Cu and 10-40 nM Zn in lakes (Borg, 1983; Xue and Sigg, 1994; Xue et al., 1995a; Xue et al., 1996) and 3-31 nM Cu and 11-51 nM Zn in non-contaminated rivers whereas moderately contaminated rivers have up to 150 nM Cu and 560 nM Zn (Xue et al., 1996; Sigg et al., 2000; Behra et al., 2002). Furthermore, measurements performed in this study showed low background free metal ion concentrations (Table 2a and b) compared to previous measurements performed in freshwater using the same methods. Background free copper ion concentrations range from 10^{-16} to $10^{-12.9}$ M (Xue and Sigg, 1993; Xue et al., 1996) in rivers and lakes, whereas free zinc ion concentrations have been measured between 0.3 and 5.6 nM in freshwater (Xue and Sigg, 1994; Xue et al., 1995a).

At environmentally relevant metal dissolved concentrations, metal accumulation in periphyton is controlled by the metal speciation in a different way for copper and for zinc. Whereas zinc follows the free ion activity model (FIAM), weakly complexed copper controls the intracellular Cu accumulation. The very low free Cu^{2+} concentration is probably not sufficient to sustain copper uptake and this essential metal must be supplied by weak complexes. To confirm this hypothesis, we estimated the transport of free metal ion (as well as of $Cu_{weakly\ complexed}$ and Zn_{labile}) to the membrane (diffusion flux) and the internalization of copper and zinc (uptake flux) (Table 4). The most favorable conditions for the copper uptake were considered to overestimate the chances of Cu^{2+} to provide enough metal to the cell for the uptake. The highest Cu^{2+} concentration measured in this study was taken in consideration ($1.9x10^{-15}$ M) and the highest diffusion flux of Cu^{2+} estimated was compared to lowest uptake flux estimated. The transport of Cu^{2+} to the cell (J_{dif}: $4x10^{-18}$ mol min^{-1} cm^{-2}) appeared to be 3 orders of magnitude smaller than the uptake of copper (J_u: $2x10^{-15}$ mol min^{-1} cm^{-2}). This indicates that, even under the most favorable conditions, the bulk solution does not supply enough free copper ions to the algal cells. The consequence is that labile copper in solution provides the metal to the cell wall. For the range of weakly complexed copper concentrations encountered in this study ($7.1x10^{-11}$-$3.8x10^{-9}$ M), the estimated diffusion flux ranges from $1x10^{-16}$ to $8x10^{-12}$ mol min^{-1} cm^{-2} depending of the conditions of diffusion chosen, which is compatible with the estimated uptake flux of copper ($2x10^{-15}$ mol min^{-1} cm^{-2}). For zinc, the range of diffusion fluxes of Zn^{2+} estimated for the concentrations encountered in this study is $1x10^{-15}$ to $2x10^{-11}$ mol min^{-1} cm^{-2} and the uptake flux of zinc varies between 1-$2x10^{-14}$ mol min^{-1} cm^{-2}, indicating that only under extremely unfavorable diffusion conditions (thick diffusive boundary layer, low temperature, low Zn^{2+} concentration) the transport of Zn^{2+} would be limiting for the cell. Limitation of Zn^{2+} appears thus not to be the case in this study. Conclusions related to the limitation of the diffusion of free copper ion were already reached in laboratory studies for different metals with algae (Hudson, 1998; Wilkinson et al., 2002). Similarly the present results obtained from field experiments fit perfectly the theoretical study of van Leeuwen (1999).

By comparing the uptake fluxes of copper and zinc calculated in this study (Table 4), the uptake flux of zinc appear to be only slightly higher than the uptake rate of copper although copper is under limitation conditions. We can thus consider that these two trace metals have an uptake flux of a similar order of magnitude. If we compare the other parameters involved in the equations of diffusion and uptake fluxes (that is diffusion coefficient, boundary layer, cell size and metal concentration) only the metal concentration differ between copper and zinc in this study as the diffusion coefficients of Cu^{2+} and Zn^{2+} are similar. This means that, to provide enough metal to the cell membrane so that the uptake flux is satisfied, the minimal metal concentration is expected to be in a similar range for copper and for zinc. This makes clear why Zn^{2+} controls the uptake of zinc whereas a labile form of copper ($Cu_{\text{weakly complexed}}$) controls the uptake of copper, as the Cu^{2+} concentration is much too low.

Previous results (Meylan et al., 2003) on relationship between metal speciation and metal accumulation, which were obtained during natural variations of the metal concentration in a river, showed as well that a labile form of copper determines the copper accumulation whereas free zinc ion influences the zinc accumulation. The present study confirms the first observations with more direct and systematic measurements. Furthermore, the previous study was performed in another river (Furtbach, Switzerland) in which the complexing capacity of the natural ligands was slightly lower than in the present study. The same conclusions were thus reached in two different natural systems.

5.5.6 Environmental considerations

These results obtained in a natural system confirm the fact that metal speciation is playing an essential role in the estimation of metal accumulation and similarly in predicting an eventual toxicity for microorganisms, which is crucial to define relevant water quality criteria. This study demonstrates that the applicability of the FIAM under natural conditions is not straightforward as it depends much on the metal considered. At environmentally relevant concentrations, zinc accumulation follows the FIAM whereas copper accumulation is a function of weakly complexed copper. In uncontaminated natural freshwater, copper appears thus to be under diffusion limitation, although at a cellular scale the free copper ion concentration might still be determining. It is thus likely that copper toxicity for algae does not occur before diffusion limitation is reduced. Indeed, laboratory studies showed that copper toxicity for algae appears at relatively high Cu^{2+} concentrations (Sunda and Guillard, 1976; Brand et al., 1986; Knauer et al., 1997b).

(See pictures related to the experimental setup in Chapter 8)

6

Speciation of copper and zinc in natural freshwater: comparison of voltammetric measurements, diffusive gradients in thin films (DGT) and chemical equilibrium models

Sébastien Meylan, Niksa Odzak, Renata Behra, and Laura Sigg, 2004, Analytica Chimica Acta, 510(1): 91-100.

6.1 Abstract

In situ measurements of copper and zinc using diffusive gradients in thin films (DGT) in two distinct natural water systems were compared to metal speciation assessed by competitive ligand exchange (CLE) and voltammetric measurements. In a dynamic river system, where dissolved metal concentrations vary significantly over short time periods, DGT technique provided averaged values of the metal concentrations over time. In microcosms, at different total dissolved concentrations of copper and zinc, DGT technique measured a similar fraction as measurements of labile metal performed by voltammetry. The proportion of DGT and voltammetric-labile zinc to dissolved zinc was 61±4% and respectively 76±9%. DGT technique was measuring 81±8% of exchangeable copper (by exchange with catechol). These two fractions were similarly influenced by the addition of NTA. In the absence of NTA, copper measured by DGT represented 34±4% of dissolved copper whereas in the presence of NTA, this proportion raised to 57±2%. These measurements were compared to calculations performed with speciation programs using several models for the complexation by humic and fulvic substances, namely Model VI (WHAM), NICA-Donnan and SHM. The predicted speciation by these three models was similar. The prediction of free zinc ion and labile zinc concentrations were in agreement with experimental data. Calculated concentrations of free copper ion were overestimated because these models are not considering strong specific copper-binding ligands probably present in natural water.

6.2 Introduction

Metal speciation in aquatic systems has a large influence on the metal bioavailability (Morel and Hering, 1993). Depending on its concentration and degree of complexation, a metal can be growth limiting or toxic for living organisms. Although methods to determine metal speciation in natural freshwater exist, these are generally laboratory methods which imply heavy handling of the samples and might consequently induce inaccuracy and contamination. Furthermore, they are often either time consuming or not accurate at low metal concentration. Competitive ligand exchange followed by voltammetric measurement (CLE-DPASV or CLE-DPCSV) is one of these ex situ methods that showed accurate results in freshwater (Xue and Sigg, 1993; Xue and Sigg, 1994) as well as in seawater (van den Berg, 1984; Donat and van den Berg, 1992). A recent in situ method is the technique of diffusive gradient in thin film (DGT) (Davison and Zhang, 1994; Zhang and Davison, 1995) that may be used to measure metal in natural systems by exposure over a typical time period of a few days. Although studies using DGT devices were already performed in seawater (Dunn et al., 2003; Munksgaard and Parry, 2003) and in freshwater systems (Denney et al., 1999; Gimpel et al., 2003), more data with comparative measurements using other speciation techniques (Odzak et al., 2002; Twiss and Moffett, 2002) under various natural conditions are still needed.

Chemical speciation programs using different models for the metal complexation by humic substances might be used to evaluate the concentration of the different metal species in natural systems. The three currently available models considered in this study are the Model VI (WHAM-Windermere Humic Aqueous Model) (Tipping and Hurley, 1992; Tipping, 1998), the NICA-Donnan model (Non-Ideal Competitive Adsorption) (Benedetti et al., 1995; Benedetti et al., 1996) and the SHM (Stockholm Humic Model) (Gustafsson, 2001). Until now these models have been mainly used to characterize data acquired with various speciation techniques (Kinniburgh et al., 1996; Bryan et al., 2002) or to predict metal speciation when metal speciation data are not available. Although some studies comparing metal speciation and values obtained with models were already performed (Christensen et al., 1999; Christensen and Christensen, 1999; Xue and Sigg, 1999; Gimpel et al., 2003), there is a need for further comparisons (Nordstrom, 1996).

This study aims to compare in situ DGT measurements of the DGT-labile concentrations of copper and zinc with speciation measurements using CLE with either DPASV (for Zn) or DPCSV (for Cu) in natural freshwater systems. Measurements of the voltammetric-labile metal fraction as well as the indirect determination of the free metal ion concentration of copper and zinc were performed. It is of clear interest to compare which fractions of dissolved metal are measured as DGT-labile and as labile by voltammetry, although identical results cannot be expected for these techniques based on different principles. A field study was performed in the Furtbach stream where very dynamic changes in metal concentration occur (Meylan et al., 2003) whereas another experiment was performed in microcosms under stable conditions using river water and considering different metal concentrations. With a rather complete and precise picture of copper and zinc speciation available in the two river water systems studied, the purpose of using the three speciation models is to examine their predictive power, which means to evaluate if the predicted metal speciation corresponds to the metal concentrations and to the fractions of dissolved Cu and Zn obtained with the DGT technique and with CLE-voltammetry.

6.3 Materials and Methods

6.3.1 Experimental setups

Two different field sites have been chosen for this study. First, the Furtbach stream which is subject to large changes of dissolved metal concentration in water over a short period of time has been selected. Second, the Glatt River with rather stable background metal concentrations has been chosen as the water to which additions of metal and of ligand were made in the microcosm study.

In the Furtbach stream (Zurich, Switzerland), large increases of copper and zinc concentrations occur during rain events. During two periods of several days, the first in February 2001 and the second in September 2002, measurements of copper and zinc dissolved concentration and deployments of DGT devices during various time intervals were performed. Short and steep variations of the metal concentration and speciation in this natural freshwater were examined.

The second site was located at 1.5 km from the outlet of Lake Greifen, where natural water from the Glatt River (Zurich, Switzerland) was used to fill several 50-liters tanks. To each tank different concentrations of copper (0, 75, 150, 250 nM) or zinc (0, 100, 300, 600 nM) as well as different concentrations of NTA (0 or 1.8 mM for the copper experiment, 0 or 0.01 mM for the zinc experiment) were added. After 3 days equilibration period, DGT devices were deployed for 2 days. During this period several water samples were taken from each experimental tank for measurements of total dissolved metal concentrations. Water samples for voltammetric measurement were taken at the end of the DGT deployment.

6.3.2 Water sampling and dissolved metal concentration measurements

Water samples were taken either manually in each tank in the Glatt experiment or using an autosampling device (ISCO 6700 Portable samplers) in the Furtbach experiment. The samples (10 ml) were filtered into 15 ml-vials using a plastic syringe and a filter (20 ml syringe, Once; 0.45 µm, Orange scientific Gyrodisc), previously thoroughly rinsed with river water and acidified to 0.01M with HNO_3 (suprapure). Bottles and vials used for the sampling were previously washed with 0.1 M HNO_3 and the experimental setups were previously cleaned and well rinsed with river water. All handling was performed with plastic gloves (Semadeni) to avoid contamination. Dissolved metal concentrations (Cu, Zn) were measured by ICP/MS (Perkin Elmer Elan 5000) with rhodium used as an internal standard. Similar manipulations were performed with nanopure water under field conditions to control the cleanness of the sampling and these field blanks showed metal concentration under the detection limit of the instrument (1.3 nM for Cu and 1.5 nM for Zn). The accuracy of the ICP/MS measurements was checked using SLRS-4 reference water (National Research Council Canada).

6.3.3 Metal speciation: water sampling and measurement

Two-liter water samples were taken in PP bottles to measure free metal ion and labile metal concentrations. Samples were filtered in the laboratory under a clean bench within 6 hours after sampling on a 0.45-µm filter (Cellulose nitrate, Sartorius) and kept at a temperature of 4°C in PP bottles until analysis. Filtering membranes, filtration unit and PP bottles were previously washed with 0.1 M HNO_3.

Free metal ion concentration was indirectly determined by competitive ligand exchange (CLE), and the measurements of the samples were performed by electrochemical detection of the metal or the metal complex depending of the ligand used (DPCSV or DPASV - Differential pulse cathodic or anodic stripping voltammetry). For each sample, several aliquots were prepared with different concentration of metal added in the presence of a competitive ligand. The titration curve obtained allows by chemical equilibrium calculation the determination of the free metal ion concentration (van den Berg, 1984; Donat and van den Berg, 1992; Xue and Sigg, 1993; Xue and Sigg, 1994). MOPS (0.02 M, 3-

morpholinopropanesulphonic acid) was used to buffer the samples at their natural pH. All the stability constants involved were adapted to the ionic strength of the water (Martell and Smith, 1989). A labile fraction of the metal was additionally measured by standard addition of a labile form of the metal and direct voltammetric measurement. The labile fraction of copper measured is referred as “exchangeable copper” and is defined as the fraction of copper that is under a free form or a complexed form weaker than the copper-catechol complexes. Labile zinc by voltammetry is based on the diffusion of Zn species to the electrode and their dissociation within the diffusion layer.

For the determination of the free copper ion concentration, the complexing ligand used was catechol (1 mM), and the copper-catechol complex was measured by DPCSV with a voltammeter Metrohm VA Stand 694 coupled to a Metrohm VA Processor 693 (HMDE, Ag/AgCl/KNO_3 sat. as reference electrode, Pt counter electrode, 5 min purging with N_2, 120 s deposition time, -0.05 V deposition potential, 10 mV/s scan rate, -50 mV pulse amplitude, 0.2 s step time) (van den Berg, 1984; Xue and Sigg, 1993). The titration curve of copper with catechol was fitted to a two-ligand model, for which concentrations and conditional stability constants were obtained by using the software FITEQL 4.0 (Westall, 1982). The measurement of the exchangeable copper that exchanges with catechol at a concentration of 1 mM and forms copper-catechol complexes detectable by DPCSV was performed by standard addition of copper and DPCSV measurement in the presence of catechol.

For the measurement of the free zinc ion concentration, the complexing ligand used was EDTA (0, 20 and 30 nM). EDTA forms a non voltammetric-labile ZnEDTA complex and the addition of this ligand reduces thus the zinc signal which was measured by DPASV (HMDE, Ag/AgCl/KNO_3 sat. as reference electrode, Pt counter electrode, 5 min purging with N_2, 120 s deposition time, -1.2 V deposition potential, 10 mV/s scan rate, 50 mV pulse amplitude, 0.2 s step time) (Xue and Sigg, 1994). The fraction of labile zinc was obtained by standard addition of zinc (under a labile form) and direct measurement of the labile zinc by DPASV. Free Zn^{2+} was calculated from labile Zn after equilibration with EDTA.

6.3.4 DGT measurements

Diffusive gradients in thin film (DGT) devices were deployed either directly in the river and fixed at 10-20 cm from the surface (Furtbach) or submerged in the tanks (Glatt). Three replicates were measured per DGT measurement. The DGT devices were prepared following the slightly modified procedure recommended by Zhang (Zhang, 1997a; Zhang, 1997b) and described in Odzak et al. (2002). The diffusive gel (0.8mm thickness) and the resin hydrogel (0.4mm thickness) were prepared using acrylamide (ultra-pure, Boehringer), agarose cross-linker (2%, DGT Research Ltd, Lancaster, UK) and deionized water. An ion-exchange resin (Chelex-100, 200-400 mesh, sodium form) was used for the resin hydrogel. Chemical polymerization was initiated by ammonium persulphate (10%) and TEMED (N-N-N'-N'-Tetramethylenediamine) (99%). For measurement, the resin gel layer was removed and placed for 24 hours in a vial containing 2 ml of 2 M HNO_3 (Suprapure). After 8-fold dilution, the metals were analyzed by ICP-MS. This procedure was performed under a clean bench. The calculations are as described in another study (Zhang and Davison, 1995) and the diffusion coefficients for free aquo ions are those measured by Hao Zhang, DGT Research Ltd., Lancaster (personal communications).

For each DGT measurement, 1 device consisting of 3 replicates was used. In February 2001, 3 DGT devices were deployed whereas, in September 2002, 9 devices were used. In the microcosm study, 1 device per 50 l tank was deployed.

6.3.5 Water chemistry

For both experimental setups, temperature, pH and conductivity were automatically measured (every 30 min) with a pH meter WTW 340i Multi. Water samples to measure concentrations of major cations, major anions, DOC and alkalinity, were taken at the same time as the samples for metal speciation. These values are necessary to calculate the ionic strength. Major cation concentrations were obtained with ICP/OES, major anions were measured by ion chromatography (Metrohm), alkalinity measurements were performed by titration (with HCl 0.1 M until pH 4.5) and DOC was obtained by combustion (Elementar).

6.3.6 Statistical analysis

Linear correlations between the different concentrations measured were performed and F statistic was used (Microsoft Excel, Office XP) to determine the probability of the correlations.

6.3.7 Speciation models

Three different chemical equilibrium models, namely the NICA-Donnan model (Benedetti et al., 1995; Benedetti et al., 1996), the Model VI (WHAM) (Tipping and Hurley, 1992; Tipping, 1998) and the SHM (Gustafsson, 2001), that all describe the interactions of metals with natural organic matter were used to estimate the speciation of copper and zinc in natural water. A simple two-ligand model was also used to fit the free copper ion concentrations. Simulated concentrations of free metal ion and metal bound to inorganic ligands were obtained. Furthermore, when a synthetic ligand (NTA) was added, labile zinc or exchangeable copper respectively were evaluated by addition of the metal-NTA complexes to the inorganically bound metal. As the voltammetry and the DGT technique measurements of the labile fraction account for the metal bound to NTA, the incorporation of the calculated value of metal-NTA with the speciation programs to the labile fraction makes sense for the comparison of these values. Simulations were performed with the default parameters included in each program for each of the models as the goal is to evaluate the predictive potential of these speciation programs.

The speciation code ECOSAT (Wageningen University, The Netherlands) includes the NICA-Donnan model. This model is based on the NICA equation, an isotherm for the adsorption of multicomponents to heterogeneous surfaces (Koopal et al., 1994). The binding site heterogeneity is characterized by the use of a distribution of equilibrium constants, representing carboxylic (strong) and phenolic (weaker) binding sites. The Donnan approach considers the humic substances as an electrically neutral phase through which there is a uniform electrostatic potential represented as a “gel phase” (Kinniburgh et al., 1996).

Model VI is included in the speciation program WHAM 6 to take into account complexation of metal by humic and fulvic substances (Tipping and Hurley, 1992; Tipping, 1998). This model represents humic compounds by rigid spheres of homogenous size, which carry metal-humic binding sites positioned on the surface with different binding strength, bidentate and tridentate binding sites being permitted. Electrostatic effects are corrected with terms based on the Debye-Hückel and Gouy-Chapman theories.

The SHM (Gustafsson, 2001) is used in the chemical equilibrium program Visual MINTEQ 2.15 (Gustafsson) and it employs a discrete-site approach where 8 sites of different acid strength are considered. Cations may form both monodentate and bidentate complexes. The bulk of humic substances are considered to form gels and the model takes into account the electrostatic effects. The interactions on the surface are modeled using the Basic Stern Model with the possibility for some humic molecules to have groups outside the gel.

Concentrations of the major ions and of the trace metals of interest (Na^+, Mg^{2+}, K^+, Ca^{2+}, Fe(III), Cu(II), Zn(II), Cl^-, NO_3^-, SO_4^{2-}, CO_3^{2-}, H^+ and NTA if present) were introduced in the modeling programs. The concentration of dissolved organic carbon (DOC) measured in the natural freshwater was divided into humic acid for 20% and fulvic acid for 80% (Buffle, 1988; Tipping, 1998). DOC, pH, alkalinity and hardness are presented in Table 1.

Table 1: Parameters used for the simulation

river	Exp.	DOC (mg/l)	pH (-)	Alkalinity (mM)	Hardness (meq/l)	Figure
Furtbach	Sept 2002	3.6	7.87	4.6	4.9	3
Glatt	Cu	4.3	8.43	3.3	4.2	5
Glatt	Zn	3.8	8.44	3.7	4.0	6

A two-ligand model was used to fit experimental data for free copper ions using the computer program FITEQL 4.0 (Westall, 1982). This model considers two copper complexing ligands, one strong (pK=14.5-15.5) and one weaker ligand ($pK \approx 12$). The concentrations of these ligands were determined with this model as well.

6.4 Results

6.4.1 Dynamic concentration variations during rain events

The dissolved metal and DGT-labile concentrations of copper and zinc measured in the Furtbach stream during two rain events are compared in Figure 1 and Figure 2. The rain period in September 2002 was longer than that in February 2001 and the rain was more intense. The measurements of copper and zinc concentration performed by DGT follow the variations of the dissolved metal concentrations integrating these concentrations over a period of time.

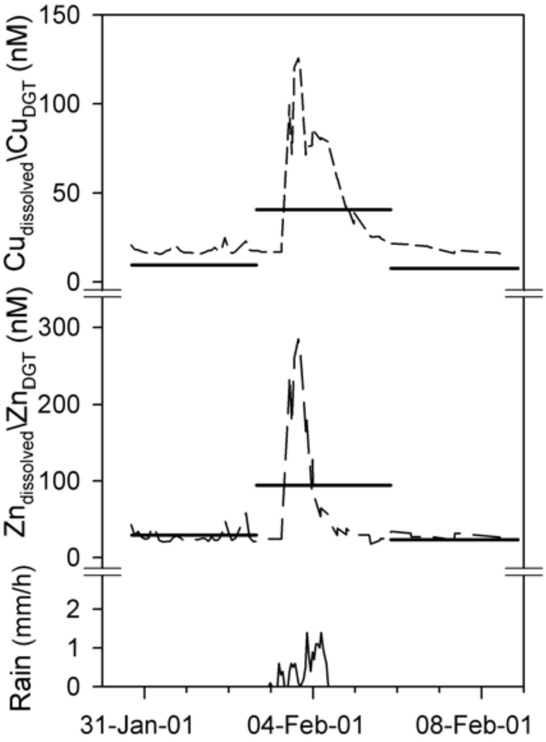


Figure 1: Dissolved copper (- – – -), dissolved zinc (— —) and measurements of copper and zinc with DGT devices (———) over 3 time intervals during a rain event (——) in February 2001 in the Furtbach stream.

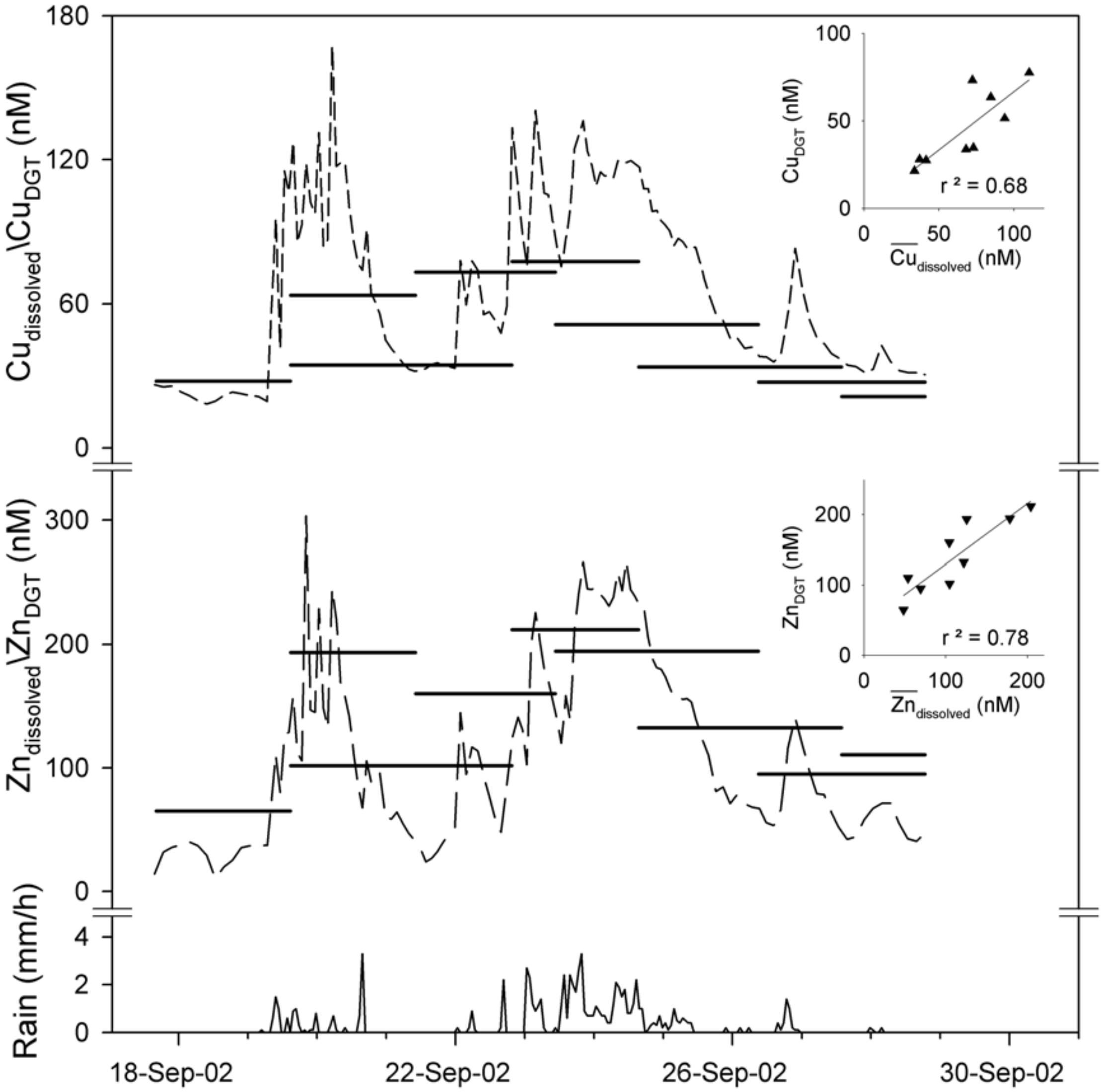


Figure 2: Dissolved copper (- — — -), dissolved zinc (— —) and measurements of copper and zinc with DGT devices (———) over several time intervals during a rain event (——) in September 2002 in the Furtbach stream and relation between Cu (▲) and Zn (▼) concentration measured with DGT device and average dissolved Cu and Zn.

Background concentrations obtained with DGT were higher in September 2002 (21 nM Cu and 65 nM Zn) than in February 2001 (8 nM Cu and 23 nM Zn) although background dissolved metal concentrations were similar for both experiments (16-18 nM Cu and 10-18 nM Zn) because the first September rain event started during the deployment of the first DGT device, which increased the measured background value. During the rain event in February 2001, $Cu_{dissolved}$ reached 130 nM and $Zn_{dissolved}$ 290 nM while DGT measurements showed values of 41 nM Cu and 94 nM Zn. Longer rain events in September 2002 induced higher maximal values for the DGT measurements (78 nM Cu and 212 nM Zn) than in February 2001, whereas $Cu_{dissolved}$ (167 nM) and $Zn_{dissolved}$ (303 nM) were approximately similar during the two rain events. The concentrations of dissolved metal measured during the deployment period of a DGT device were considered together and an averaged value was calculated for each period. These values were compared to the corresponding DGT measurement and a linear relationship was obtained, with r^2 coefficients of 0.68 for Cu and 0.78 for Zn (Figure 2 insert).

Free Cu^{2+} ions in Furtbach had values ranging between 10^{-14} and 10^{-12} M and $Cu_{exchangeable}$ values varied between 9.4 to 100 nM during the September 2002 rain events (Figure 3). Similarly free zinc ion concentration (0.9-16 nM) and labile zinc (29-222 nM) were measured and their relationships with dissolved metal concentration were calculated giving linear correlations (Table 2).

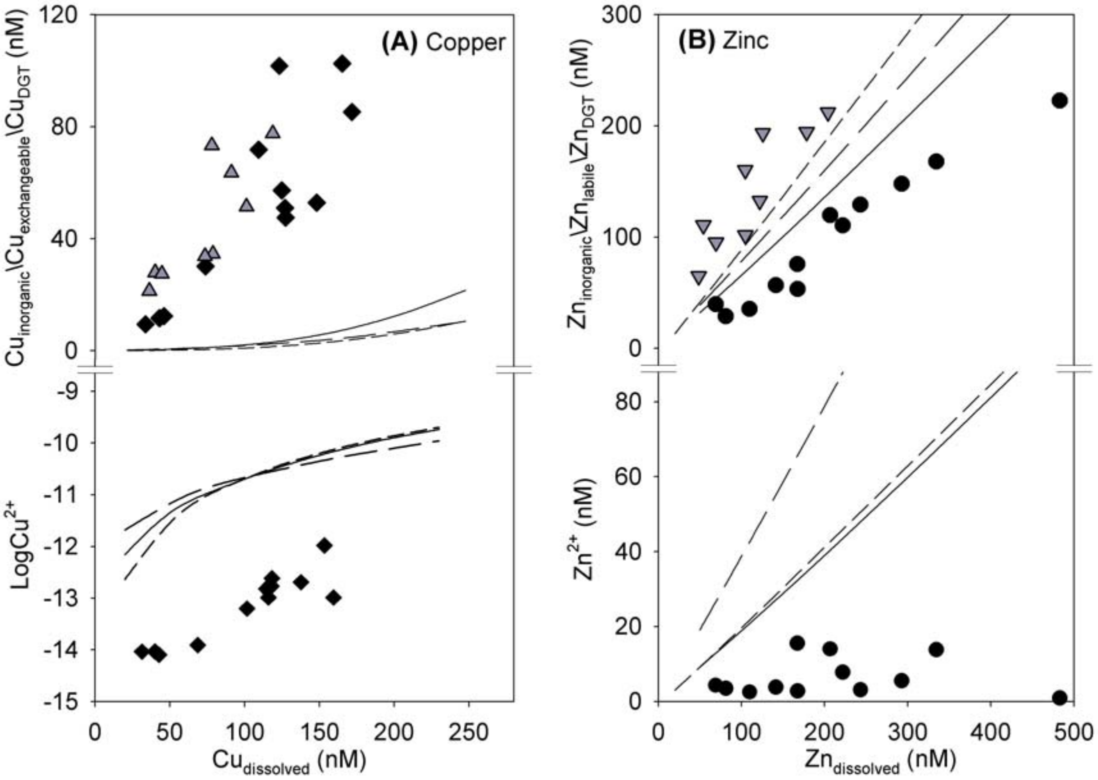


Figure 3: Experimental and simulated data from the Furtbach study; (A) Experimental data (◆) of Cu^{2+} and exchangeable copper and DGT measurements of copper (▲) as a function of dissolved copper; Simulated data of inorganic Cu concentration and Cu^{2+} with WHAM (——), NICA-Donnan (- – – -) and SHM (— —). (B) Experimental data (●) of Zn^{2+} and labile zinc and DGT measurements of zinc (▽) as a function of dissolved zinc; Simulated data of inorganic Zn concentration and Zn^{2+} with WHAM (——), NICA-Donnan (- – – -) and SHM (— —).

Furthermore, computations of free metal ion concentrations (Cu^{2+} and Zn^{2+}) and concentrations of inorganic metal complexes ($Cu_{inorganic}$ and $Zn_{inorganic}$) including free metal ion and metal inorganically bound, with the models WHAM, NICA-Donnan and SHM are shown on Figure 3. The 3 models gave similar results among them, but were not fitting well the experimental data in such a dynamic system.

6.4.2 Microcosm studies

Dissolved metal concentrations were compared to DGT measurements and to labile zinc or exchangeable copper under stable conditions over a period of 2 days in the presence or absence of NTA (Figure 4). Dissolved metal concentrations were in the range 25-258 nM Cu and 18-501 nM Zn whereas $Cu_{exchangeable}$ was depending on the addition of NTA (2.8-65 nM in the absence of NTA and 7.4-190 nM in the presence of NTA), Zn_{labile} was not influenced by NTA (4.2-390 nM). DGT measurements of Cu and Zn showed a similar trend as $Cu_{exchangeable}$ and Zn_{labile}. Cu_{DGT} varied in the range 1.0-61 nM in the absence of NTA and in the range 6.6-139 nM in the presence of NTA, whereas Zn_{DGT} varied between 0.9 and 302 nM. The relations between the various concentrations are linear and the slope and r^2 coefficients are given in Table 2. Moreover, a good correlation was found between $Cu_{exchangeable}$ and Cu_{DGT} with a r^2 coefficient of 0.94.

Free metal ion concentrations were as well measured during these experiments and the range of concentrations measured were $10^{-16.5}$-$10^{-14.5}$ M Cu^{2+} independently of the presence of NTA (Figure 5). For Zn^{2+} values varied between 1.0 and 7.9 nM in the absence of NTA and between 0.7 and 8.7 nM in the presence of 0.01 mM NTA (Figure 6). The different conditions encountered in these experiments were applied to the WHAM, NICA-Donnan and SHM models and the results obtained were compared to experimental data. The results of the three models were in accordance with each other (Figure 5 and 6). Furthermore, the two-ligand model was applied to free copper ion measurements (Figure 5).

The results of the linear regressions from Figure 2-6 and of the statistical analysis are reported in Table 2. The slopes indicate the proportion between the 2 specified metal fractions and percentages of error on slopes were calculated.

Statistical analysis were performed and showed that all the regressions performed are significant ($p<0.005$) and some regressions are significant at the $p<0.001$ level.

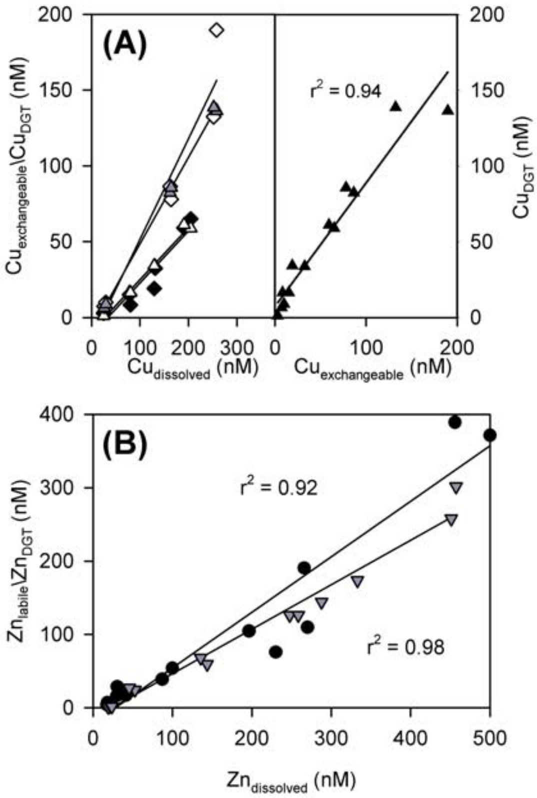


Figure 4: Experimental data from the microcosm study; (A) Concentration of copper measured by DGT (△ - no NTA, ▲ - 1.8 mM NTA added) and exchangeable copper concentration (◆ - no NTA, ◇ - 1.8 mM NTA added) as a function of dissolved copper and DGT measurements (▲) related to exchangeable copper. (B) Concentration of zinc measured by DGT (▽) and labile zinc concentration (●) as a function of dissolved zinc.

6.5 Discussion

6.5.1 DGT measurements of dynamic concentration variations during rain events

The dynamic variations of the metal concentrations in the Furtbach stream appear to have a good correspondence with the DGT measurements that integrate the concentration of DGT-labile Cu and Zn over a certain time period (Figure 1 and 2). Background values of Me_{DGT} and $Me_{dissolved}$ previous to the rain events agree with each other, and as soon as the rain events are starting the increases in metal concentration are detectable. After the rain event the dissolved metal concentration drops rapidly to background levels alike DGT measurements. Although some studies using DGT devices have already been performed in river water (Denney et al., 1999; Sangi et al., 2002), such an experiment combining dissolved metal with DGT measurements during dynamic concentration variations is unique. This study clearly demonstrates the capacity of DGT to provide an integrated picture of the metal concentration over a period of time (Munksgaard and Parry, 2003).

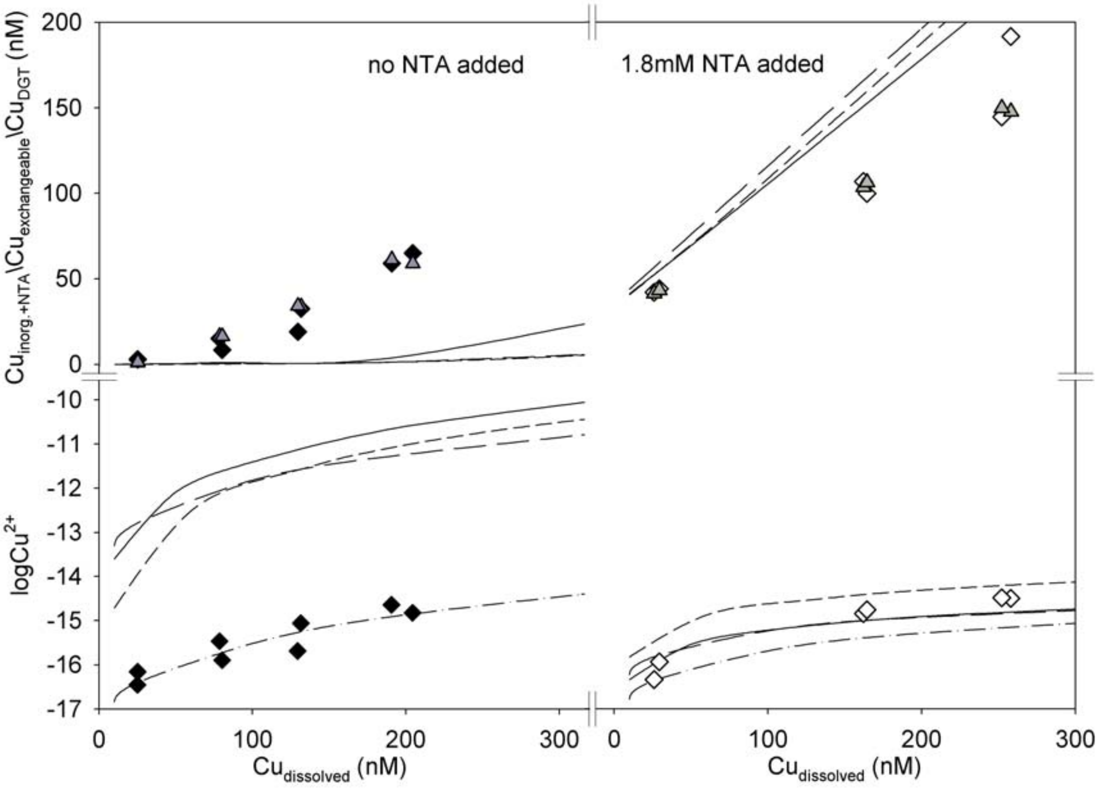


Figure 5: Experimental and simulated data from the microcosm study; Experimental data of Cu^{2+} and exchangeable copper (◆ - no NTA, ◇ - 1.8 mM NTA added) and DGT measurements of copper (▲- no NTA, △- 1.8 mM NTA added) as a function of dissolved copper; Simulated data of Cu^{2+}, of inorganic Cu (in absence of NTA) and of exchangeable Cu (in presence of NTA) with WHAM (——), NICA-Donnan (- – – -), SHM (— —) and a two-ligand model (-· — ·-).

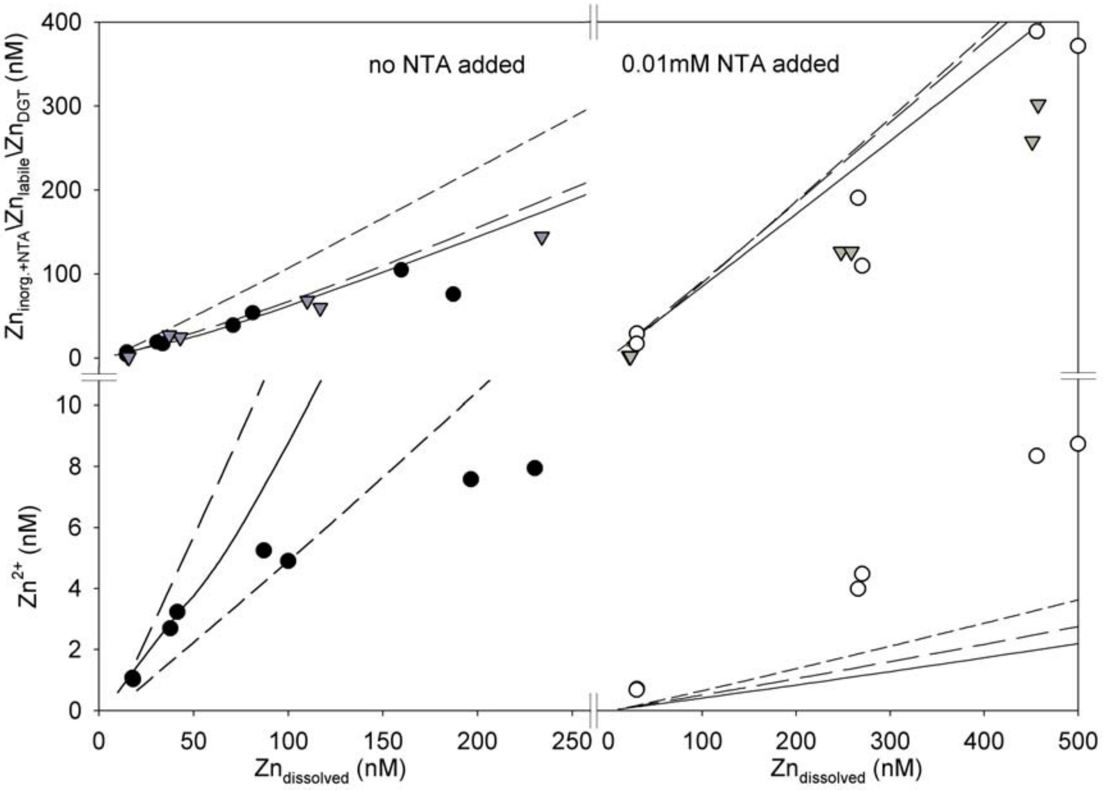


Figure 6: Experimental and simulated data from the microcosm study; Experimental data of Zn^{2+} and labile zinc (● - no NTA, ○ - 0.01 mM NTA added) and DGT measurements of zinc (▽- no NTA, ▽- 0.01 mM NTA added) as a function of dissolved zinc; Simulated data of Zn^{2+}, of inorganic Zn (in absence of NTA) and of labile Zn (in presence of NTA) with WHAM (——), NICA-Donnan (- – – -) and SHM (— —).

The experiment performed in September 2002 shows that the proportions of metal measured by DGT compared to dissolved metal concentration are 66±17% for copper and 86±17% for zinc (see slopes in Table 2). Although the error on this proportion is quite important, the results given by the DGT device are in the same order of magnitude as the values of $Cu_{exchangeable}$ and Zn_{labile} obtained by voltammetric measurements (Cu: 64±12% and Zn: 50±4%, Table 2, Figure 3). Indeed these results show that similar fractions are measured for copper with the two techniques although they are based on different principles. The deployment time of the DGT devices, which was not constant in this experiment, does not appear to have any influence on the results as all the measurements are following the same trend independently from this factor. Although this was expected because the DGT technique is based on the flux of metal through the diffusive gel and is thus not proportional to the deployment time (after normalization to the time unit), this fact was not yet shown in a dynamic natural environment.

Table 2: Measurements of copper and zinc with voltammetry and DGT; comparison of the slopes and intercepts obtained and evaluation of the metal strongly bound to ligands.

	as a function of		slope	error %	r^2	n	p^c	Figure
Dynamic experiment in the Furtbach 2002								
$Cu_{exch.}$	$Cu_{dissolved}$		0.64	*19*	0.73	12	***	3
Cu_{DGT}	$\overline{Cu_{dissolved}}^{a}$		0.66	*26*	0.68	9	**	2+3
Zn_{labile}	$Zn_{dissolved}$		0.50	*7*	0.95	12	***	3
Zn_{DGT}	$\overline{Zn_{dissolved}}^{a}$		0.86	*20*	0.78	9	***	2+3
Microcosm study								
$Cu_{exch.}$	$Cu_{dissolved}$	-	0.34	*13*	0.91	8	***	4+5
Cu_{DGT}	$Cu_{dissolved}$	-	0.34	*4*	0.99	8	***	4+5
$Cu_{exch.}$	$Cu_{dissolved}$	NTA	0.66	*14*	0.93	6	**	4+5
Cu_{DGT}	$Cu_{dissolved}$	NTA	0.57	*2*	1.00	6	***	4+5
Cu_{DGT}	$Cu_{exch.}$	all[b]	0.81	*8*	0.94	14	***	4
Zn_{labile}	$Zn_{dissolved}$	-	0.41	*15*	0.88	8	***	6
Zn_{DGT}	$Zn_{dissolved}$	-	0.53	*4*	0.99	8	***	6
Zn_{labile}	$Zn_{dissolved}$	NTA	0.79	*14*	0.92	6	**	6
Zn_{DGT}	$Zn_{dissolved}$	NTA	0.64	*7*	0.98	6	***	6
Zn_{labile}	$Zn_{dissolved}$	all[b]	0.76	*9*	0.92	14	***	4
Zn_{DGT}	$Zn_{dissolved}$	all[b]	0.61	*4*	0.98	14	***	4

a: mean value over the time interval of the DGT measurement, b: "all" means measurements with and without NTA, c: **: 0.005>p>0.001, ***: p<0.001.

6.5.2 DGT and voltammetry in microcosm experiments

Correlations between DGT measurements and different metal species were evaluated in the presence or absence of 1.8 mM NTA (Figure 4). With increasing $Cu_{dissolved}$, copper measured with DGT devices is increasing but the fraction of metal measured is influenced by the addition of NTA. Indeed, the introduction of a relatively large amount (1.8 mM) of a rather strong synthetic ligand (logK=13.5) in the natural water that has the complexing capacity to compete with the weakest fraction of the strong natural organic ligands increases the proportion of labile copper measured by DGT. The proportion $Cu_{DGT}/Cu_{dissolved}$ increases from 34±1% (no NTA) to 57±1% (with NTA) (Table 2). This explanation is supported by the comparable behavior of $Cu_{exchangeable}$, the proportion of which raises from 34±4% (no NTA) to 66±9% (with NTA) (Table 2). These similar proportions indicate that copper measured with DGT devices is narrowly connected to the exchangeable copper in solution measured with voltammetry. With both techniques the labile fraction measured includes the CuNTA complexes. Indeed CuNTA either diffuses through the diffusive gel (DGT) or exchanges with catechol (voltammetry). Furthermore, the correlation between these two concentrations is significant ($p<0.001$) and the ratio $Cu_{DGT}/Cu_{exchangeable}$ is 81±6% independently of the addition of synthetic ligand. We can thus deduce that CuNTA is measured by the two techniques considered and that it is fully DGT-labile, which is corroborated by a previous study (Scally et al., 2003).

Relationships between DGT-labile zinc and dissolved zinc give significant linear correlations ($p<0.001$, Table 2, Figure 4 and 6) independently of the addition of NTA and the proportion of Zn_{DGT} to $Zn_{dissolved}$ is 61±4%. Similarly Zn_{labile} (ASV) is increasing linearly with increasing $Zn_{dissolved}$ and the proportion calculated is 76±9%. This independence of Zn_{DGT} and Zn_{labile} to the addition of NTA means that ZnNTA is included in the fraction measured by these two techniques. Indeed NTA is not a very strong ligand for Zn ($\log K=11.2$) and competes exclusively with weak labile ligands at this concentration (0.01 mM). The similar values found for Zn_{DGT} and Zn_{labile} indicate that DGT devices measure a concentration corresponding to the voltammetric labile zinc in solution (Xue and Sigg, 1993; Xue et al., 1995a; Xue and Sigg, 2002a).

6.5.3 Use of DGT

It is interesting to notice that for copper and for zinc the ratios DGT-labile to dissolved metal and voltammetric-labile to dissolved metal are in a similar range. This agreement suggests that these two techniques account for a similar fraction of the total dissolved metal concentration although they are based on different principles. The DGT technique takes into account the flux of metal complexes diffusing through a diffusive gel whereas voltammetry measures the metal complexes (either zinc complexes or copper-catechol complexes) diffusing through the diffusive boundary layer to the electrode. Moreover, the labile fractions of metal obtained in this study with the two different techniques are largely depending on variable parameters as the gel pore size and the catechol concentration for the copper voltammetric measurement. The metal complexes formed with the synthetic ligand NTA appear clearly to be measured with the two techniques considered, which reinforce the hypothesis that these techniques measure similar fractions.

Comparisons of results obtained in other studies comparing DGT technique and other speciation techniques are difficult because of differences in the preparation of the gel used for DGT device and of differences in the procedures used to measure the different fractions of copper. Furthermore, differences in the type of water, DOC or range of metal concentration might influence the results. Some studies need nevertheless to be cited (Dunn et al., 2003). Several studies were exclusively comparing DGT measurements with measurements of the total metal concentrations either in seawater (Zhang and Davison, 1995; Dunn et al., 2003) or in freshwater (Alfaro-De la Torre et al., 2000; Sangi et al., 2002; Gimpel et al., 2003). As a matter of fact, only few studies attempted to measure a labile fraction of copper simultaneously with DGT measurement. A set of DGT devices was exposed to stream water containing high concentration of DOC (14.6 mg/l – pH 7.5) and the ratio of Cu_{DGT} to $Cu_{dissolved}$ (33 nM) measured was 91%, whereas estimation of Cu_{labile} with voltammetry (ASV), showed around 50% of $Cu_{dissolved}$ but Cu_{labile} was qualified as poorly reliable by their authors (Zhang and Davison, 2000). A larger study performed in coastal waters and coupling DGT measurements and competitive ligand exchange with voltammetric measurement for the determination of free copper ion and inorganically complexed copper concentrations was the first study that showed convincing comparison of the DGT technique with a speciation technique in marine systems (Twiss and Moffett, 2002). In this study performed at different coastal sites DGT-labile copper represented generally a fraction up to 40% of the dissolved copper fraction whereas $Cu_{inorganic}$ was generally about 10 to 100-fold smaller than the DGT-labile Cu measurement. In lake water, DGT measurements were compared to CLE-DPCSV for Cu and to CLE-DPASV for Zn (Odzak et al., 2002) and showed, compared to the dissolved metal concentration, a proportion of DGT-labile metal of 15-25% for Cu and about 30% for Zn. The proportion of Zn^{2+} to $Zn_{dissolved}$ reached 4% whereas a much lower proportion of Cu^{2+} to $Cu_{dissolved}$ was obtained due to the small concentration of free copper ($10^{-16.5}$-$10^{-13.8}$ M) (Odzak et al., 2002). The order of magnitude of these last results corresponds to the results obtained in this study as the preparation and measurement procedures are very similar.

6.5.4 Copper simulation

The models, WHAM, NICA-Donnan and SHM, predict similar values for free Cu^{2+} and for inorganic Cu (Figure 3 and 5) in the two different rivers studied. Nevertheless, if we consider the free copper ion concentration, the model predictions fit poorly the measured Cu^{2+} concentration and greatly overestimate Cu^{2+}. A difference of about 2 orders of magnitude is found for the experiment performed under dynamic conditions (Figure 3). In the microcosm study where a higher complexing capacity was observed, this difference reaches around 3 orders of magnitude. Large differences in the prediction of Cu^{2+} by speciation models were already observed in a study of copper speciation in different Swiss lakes that compared the results obtained with measurements of synthetic media in the presence of humic substances and the speciation model WHAM (Xue and Sigg, 1999). The strong complexing capacity of the lake Greifen, the Glatt river experimental site being located 1.5 km from the lake outlet, was already measured in previous studies where specific metal binding ligands were measured by CLE-DPASV or CLE-DPCSV (Xue and Sigg, 1993; Xue et al., 1995a; Xue and Sigg, 2002a). The conclusion was drawn that specific strong copper binding ligands of probable biological origin were present in lake water, which provoke a drastic reduction of free copper ion. These observations confirm the results obtained in the present study where CLE-DPCSV measurements showed the presence of strong copper binding ligands ($\log K_1 = 15.3 \pm 0.2$, pH 8.4) at a concentration of 220±80 nM, which is relatively low compared to the concentration of DOC (4.3±0.2 mg/l ≈ 19-29 μM functional groups depending on the model). The conditional stability constants for humic substances used in the three models considered are much lower than the one for this strong copper binding ligand. Binding by the humic substances is far less specific. The effect of this strong copper binding ligand is shown on Figure 5 where, together with a weaker ligand (2 µM, $\log K_2=12$, pH 8.4), Cu^{2+} is plotted using a two ligand model. In these models, the binding strength of humic substances might be underestimated because the model parameters are usually optimized for a free copper ion concentration range higher than the range of Cu^{2+} found in this study, which was performed at environmental relevant level of metal. The assumption that 80% of the DOC consist in fulvic acids, the other 20% being humic acids might influence slightly the difference between the measured and the calculated free copper ion concentration but this difference was evaluated to be less than half an order of magnitude.

The introduction of NTA in the models reduces drastically the predicted Cu^{2+} concentration compared to the value predicted in the absence of NTA. Indeed, in the models, NTA plays the role of a strong specific ligand compared with the humic substances. As these strong ligands are not present in the models, the influence of NTA is disproportionate. On the contrary, the measured Cu^{2+} concentration is practically not influenced by the presence of NTA because even stronger ligands are present in the natural water, as discussed previously.

The fraction of labile metal measured by DGT or voltammetry is depending on the technique used and the labile fraction consists in several metal-complexes of weak strength. In the case of speciation programs, each inorganic species can be estimated but the metal-organic ligand complexes issued from models cannot be directly related to the labile fraction. In the absence of NTA, the determination of a labile form of copper is not possible with the speciation models and only $Cu_{inorganic}$ is calculated which corresponds to the sum of all copper-inorganic ligands complexes and free copper ion. $Cu_{inorganic}$ is a much smaller fraction than the measured $Cu_{exchangeable}$ and Cu_{DGT} (Figure 5). In the presence of NTA, the estimation of a labile fraction of copper which corresponds to the addition of $Cu_{inorganic}$ to CuNTA (calculable with the speciation models) is likely to be more accurate because CuNTA account for a large proportion of labile copper. This fraction is slightly higher than the measured $Cu_{exchangeable}$ and Cu_{DGT}, but of a similar order of magnitude.

In the case of copper the prediction of the speciation of copper at typical concentration in the environment using speciation model such as WHAM, NICA-

Donnan or SHM is thus not straightforward. Information on strong specific ligands in natural water is needed to allow such prediction, as illustrated by the data involving NTA.

Few studies using DGT measurements and comparing these results with simulated data are available. The speciation program WHAM was used to predict the extent of metal complexation in lake water and Cu_{DGT} predicted with WHAM represented 16% of total Cu (measured by dialysis) and the measurement of Cu_{DGT} was found to be around 33% of total Cu (Gimpel et al., 2003). This study showed that Cu_{DGT} predicted using the speciation model WHAM is underestimated compared to the measurement of copper by DGT. Another study attempted to measure the organic plus inorganic fraction (91% of total dissolved) and the inorganic fraction (36% of total dissolved) of copper using DGT devices with different gel thickness and calculation (WHAM) were made to predict these fractions, which gave results between 3 and 80% of total dissolved metal depending on the parameter used for the simulation (Zhang and Davison, 2000).

6.5.5 Zinc simulation

The WHAM, NICA-Donnan and SHM models (Figure 3 and 6) applied to predict free zinc ion and inorganic zinc concentrations under freshwater conditions are showing a better agreement with experimental data and thus a better predictive power than it is the case for copper. The three models give in the case of zinc somewhat different results and their respective accuracy depends on the range of dissolved zinc considered.

In the dynamic river system (Figure 3), calculated values of free zinc ion concentration are clearly overestimated whereas the prediction of Zn^{2+} in the microcosm study (Figure 6) is more accurate. Although the predicted values of Zn^{2+} are relatively good at low $Zn_{dissolved}$ concentration, a discrepancy appears at higher concentration of zinc and Zn^{2+} is overestimated. The Stockholm humic model appears to overestimate Zn^{2+} more than the two other models applied. Like for copper, the introduction of NTA in the simulation decreases drastically the values predicted because of the underestimation of the binding strength of zinc with humic substances compared to the binding strength of ZnNTA.

The calculation of $Zn_{inorganic}$ under the different conditions gives a good approximation of the labile fraction measured either by voltammetry or with DGT. In the study performed in a dynamic environment (Figure 3), the computed data are giving values situated between the values measured with DGT and with voltammetry. In the microcosm study (Figure 6), the WHAM and SHM show good predictive values whereas the NICA-Donnan model overestimates slightly $Zn_{inorganic}$. Nevertheless the labile fraction of zinc includes undoubtedly zinc complexed to weak organic ligands which means that the values calculated with models are overestimated. When NTA is added to the river water, the calculation of $Zn_{inorganic}$ combined with ZnNTA appears to give a good estimation of the labile zinc concentration measured either with DGT technique of voltammetry although the estimated values are slightly overestimated.

The only other study measuring zinc with DGT (74% of total) and using WHAM to predict the proportion of metal inorganically complexed (45%) was performed in lake water and presents a similar trend as what we obtain under dynamic conditions (Gimpel et al., 2003).

6.6 Conclusions

In this study, DGT technique has proved to be an efficient tool to measure in situ metal speciation and appeared to give similar results as voltammetric measurements of labile copper and zinc in natural freshwater. Measurements performed with the DGT technique provide reliable values of the zinc labile fraction and of the copper exchangeable fraction with the advantages of being robust and easy of use. The DGT technique proved to be efficient to measure integrated values of labile metal concentration especially when short-time variations of the concentration are the focus of the measurement. The comparison of these results with computed values obtained with three speciation programs taking humic substances into account, shows relatively good prediction potential for zinc whereas discrepancies in the prediction were found for copper due to its high level of complexation.

Whereas speciation programs need to be used carefully in natural water, DGT devices, providing a labile metal concentration measurement, might be very useful to predict metal speciation as well as metal bioavailability for microorganism in aquatic systems as it was shown that, at copper concentrations found in the environment, copper accumulation in periphyton is proportional to a labile form of copper (Meylan et al., 2003; Meylan et al., 2004a).

(See pictures related to the experimental setup in Chapter 8)

7

Outlook

This study presents clear evidence that the metal speciation controls metal bioavailability to algae in natural freshwater. It was shown that whereas bioaccumulation of zinc in periphyton is determined by the free zinc ion concentration following the FIAM, bioaccumulation of copper is influenced by the fraction of copper weakly complexed by ligands due to diffusion limitation of free copper ion to the cells. These results focusing on the relationship between metal speciation and metal bioavailability are among the first obtained in natural systems. Such results are important to validate the biotic ligand model (BLM) under field conditions and to determine relevant water quality criteria based on metal speciation (Allen and Hansen, 1996; Paquin et al., 2002). The validation of the BLM and the collection of a significant amount of field data on the metal-aquatic organisms interaction is primordial as this model is foreseen to be the basis of an efficient prediction tool of metal toxicity (Di Toro et al., 2001; Batley, 2003). Water quality criteria would then be adapted to the properties of each water system based on total metal concentration, pH, water hardness, and DOC.

Further studies on periphyton in natural freshwater similar to those performed in microcosms and described in chapter 5 would contribute to increase the knowledge concerning the interactions between metals and aquatic microorganisms. In the frame of the BLM, still several points need to be examined, such as the influence of water hardness or of pH on metal accumulation in periphyton. Furthermore, similar experiments performed with other metals would be particularly appropriated. The investigation of the relationship between metal accumulation and the speciation of nickel, an essential metal like copper and zinc, or of cadmium, a non-essential metal would bring significant contribution to the validation of the BLM. Moreover, methods to determine metal speciation of these two metals were already reported (Xue and Sigg, 1998; Xue et al., 2001).

Periphyton was particularly appropriated to represent natural algal communities in this study. The rapid changes in metal content in periphyton were clearly and easily detectable which means that it might be used as an indicator of changes in metal bioavailability. The utilization of periphyton in studies comparing identical communities of organisms is thus particularly suitable. Nevertheless, metal content in periphyton measured in different rivers need to be compared carefully. We saw in this study that the background metal content in this community of organisms differs significantly depending on the time of the year and the river studied which complicates greatly the applicability of such an indicator for comparison between sites. Furthermore, the handling of periphytic communities from the colonization step to the outcome of a metal content value is relatively time consuming meaning that its use for routine analyses is complicated.

The effect of the matrix of periphytic biofilm has probably an influence on the diffusion of metal to the cells and thus on metal bioavailability and would be worth to be studied. This would probably explain partially why background metal contents vary depending on the river sampled as the composition of community and thus the matrix varies.

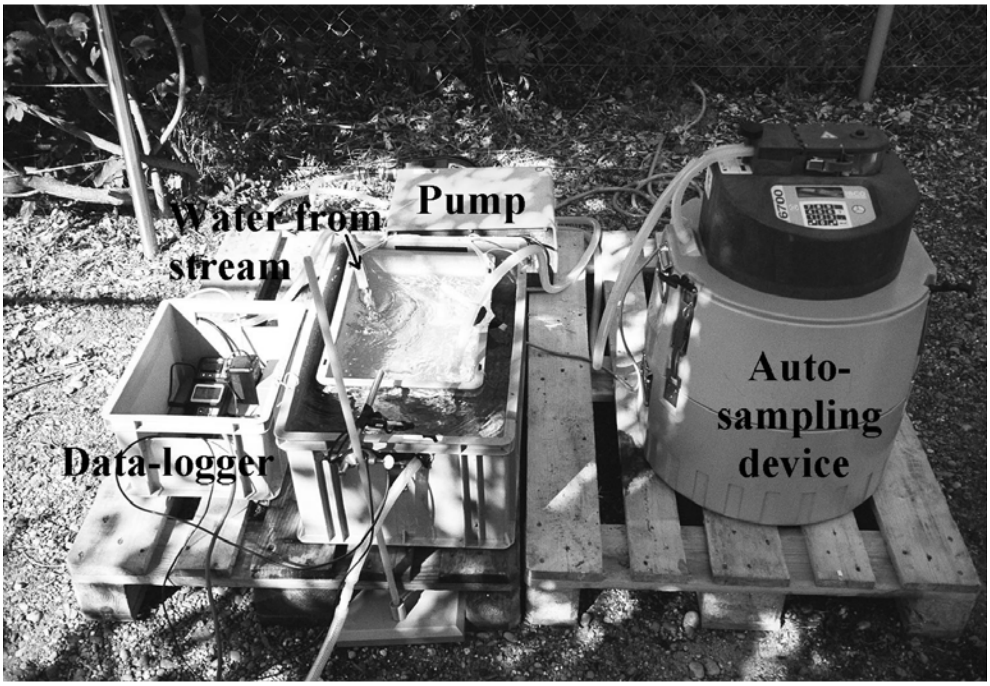
In natural systems, one of the main difficulties while studying the influence of metal speciation on metal bioaccumulation is the determination of the metal speciation. This work was comparing actual laboratory and in situ techniques with chemical equilibrium models and showed that these models were not sufficiently reliable to predict metal speciation at environmental relevant concentrations. Furthermore, the two techniques used were either time consuming or not appropriate to measure free metal ion concentrations. The need for robust and accurate techniques implying low handling for the determination of metal speciation is obvious. The availability of better technique would then allow the direct formulation of water quality criteria in terms of free metal ion concentrations.

8

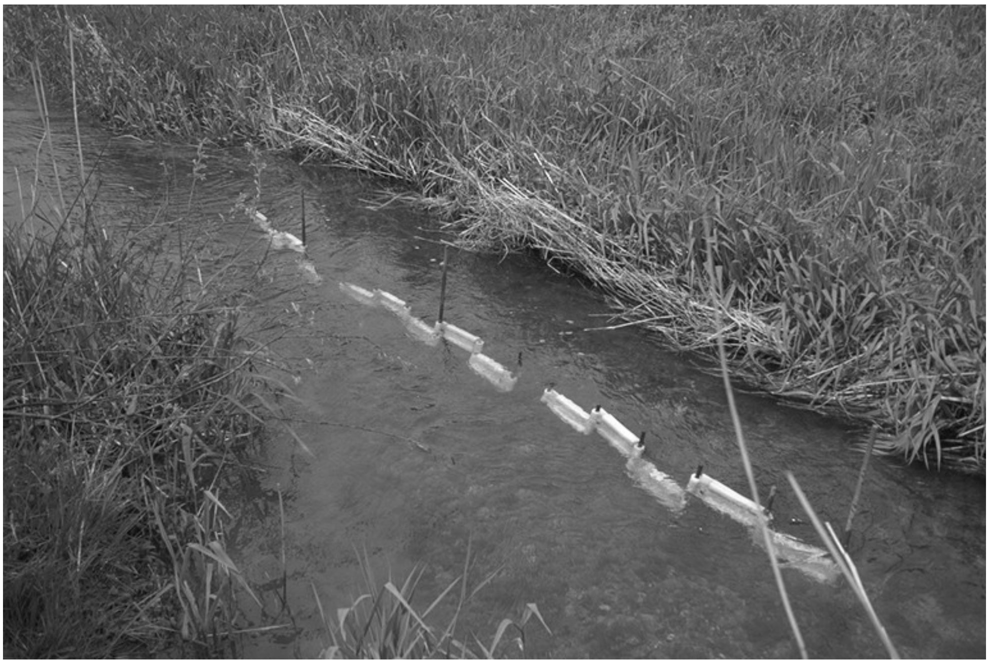
Experimental setup

8.1 Furtbach stream in Regensdorf (Switzerland)

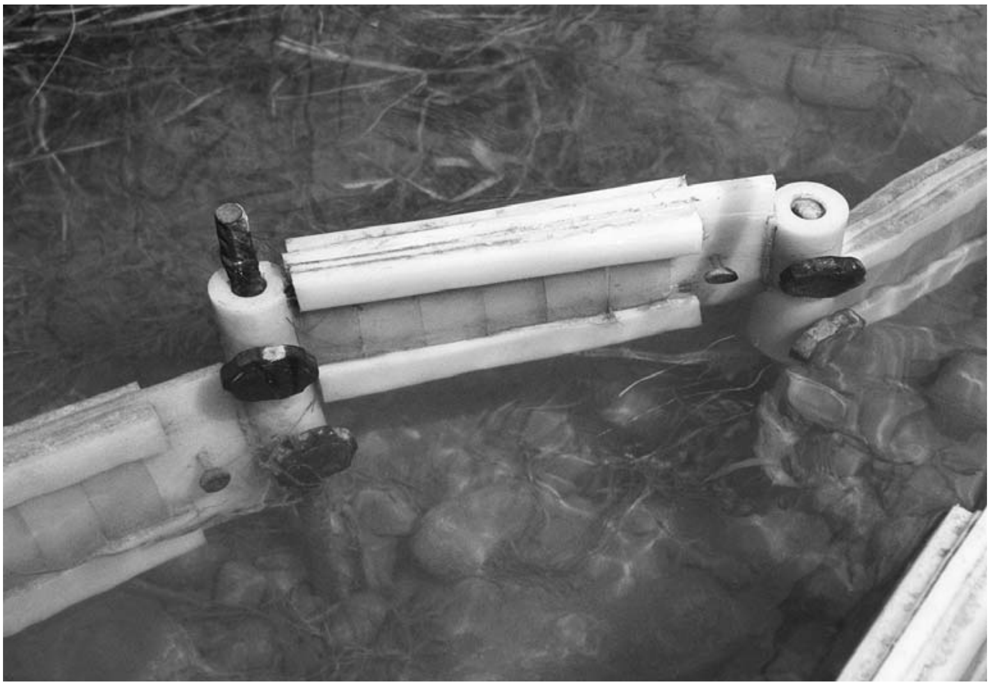
Sampling site at the Furtbach stream, close to WWTP Wüeri



Auto-sampling setup including an auto-sampling device and a data logger



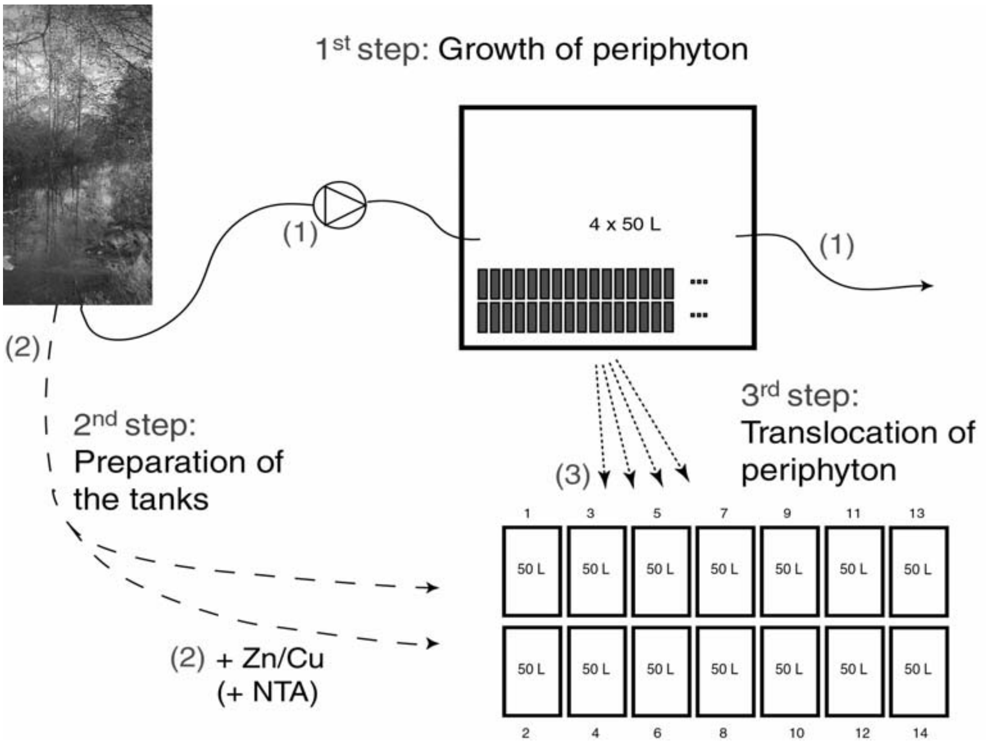
Several Teflon holders fixed in line at the stream bed



Periphytic biofilm growing on microscope slides fixed to Teflon holders

This experimental setup was used for the studies presented in chapters 4 and 6.

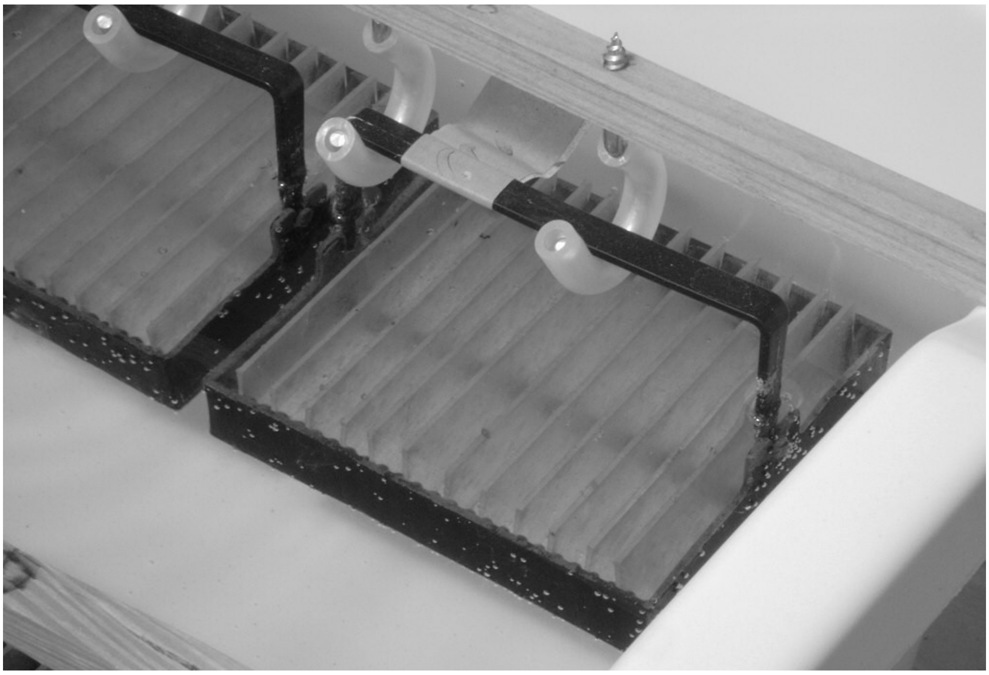
8.2 Glatt river in Fällanden (Switzerland)



“3 steps” design as presented in chapter 5

Tanks operated as a flow-through system and used for periphyton colonization

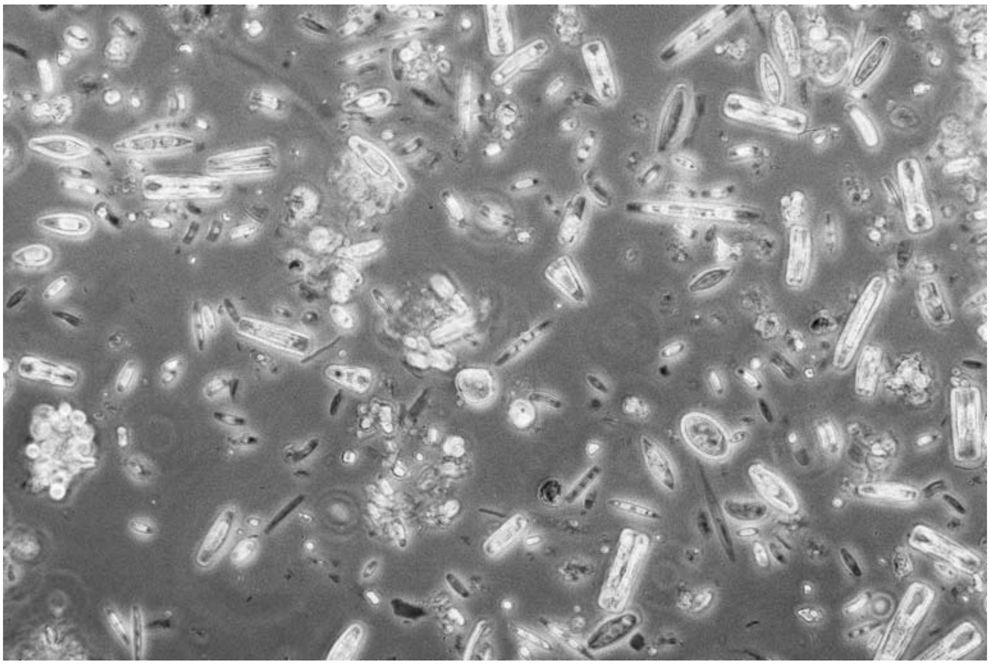
Experimental tanks with periphyton exposed to different metal speciation

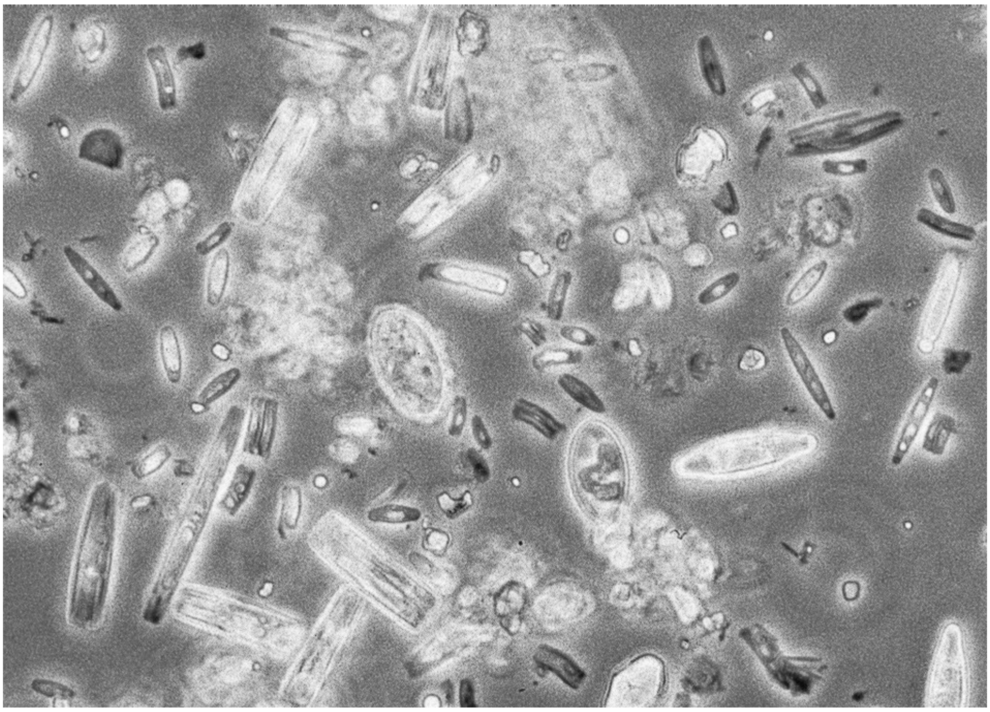


Biofilm of periphyton on microscope slides fixed in a plastic holder

This experimental setup corresponds to the setup used for the studies presented in chapters 5 and 6 and some modifications to this design were performed for the studies related in chapter 3.

8.3 Microscope pictures of periphyton





These two pictures are showing typical microscope shots of periphyton at different magnification (approx. 100x and 300x).

9

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Curriculum vitae

Sébastien Meylan
Born on December 30, 1973
in Lausanne, Switzerland

1979-1986	Primary school in Gland and Grand-Lancy, Switzerland
1986-1989	Secondary school in Onex, Switzerland
1989-1993	High School at Collège de Staël in Carouge, Switzerland
1993-1999	Diploma in Chemical Engineering at the Swiss Federal Institute of Technology of Lausanne (EPFL)
1997	Traineeship at the Chemistry Department of Queen's University, Ontario, Canada
1998-1999	Diploma work at the Chemical Engineering Department of Queen's University, Ontario, Canada
1999-2003	Ph.D. Thesis at the Swiss Federal Institute for Environmental Sciences and Technology (EAWAG) and at the Swiss Federal Institute of Technology of Zurich (ETHZ)
1999-2003	Teaching assistant in aquatic chemistry at the ETHZ