Doctoral Thesis

Interactions of copper and hydrophobic ionogenic organic pollutants in biological membranes and their consequences for bioavailability and toxicity towards algae

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Interactions of copper and hydrophobic ionogenic organic pollutants in biological membranes and their consequences for bioavailability and toxicity towards algae

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Doctor of Sciences

presented by

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Summary

The chemical speciation of copper determines its bioavailability and subsequently its toxicity for aquatic organisms. In the case of algae, the most significant uptake route of copper is the carrier-mediated transport of free copper ions (Cu$^{2+}$). An alternative uptake route for copper was observed in the presence of hydrophobic ionogenic organic compounds like 8-hydroxyquinoline (oxine) and diethyldithiocarbamate, which can form stable complexes with metals.

The scope of this thesis was to evaluate the bioavailability and toxicity of an important group of hydrophobic copper-organic complexes, copper-8-hydroxyquinoline complexes, for the freshwater algae Scenedesmus vacuolatus.

For this study, copper complexes with different substituted 8-hydroxyquinoline ligands were used in order to determine the effect of their different functional groups on bioavailability and toxicity. The test set of ligands consisted of the parent compound 8-hydroxyquinoline as well as of chlorinated and brominated 8-hydroxyquinolines. These ligands exhibit high stability constants with copper and antimicrobial and antifungal properties and are therefore used for the extraction of copper in analytical chemistry and in pesticides. One of their copper complexes, copper-8-hydroxyquinoline, is widely used as a preservative for textile, wood and paint and as a fungicide.

Membrane-water partitioning coefficients of the selected ligands as well as of their copper complexes were determined using liposomes as model membranes. The results showed that the partitioning of copper-8-hydroxyquinoline complexes is dependent on several factors including hydrophobicity, molar volume and polarisation of the complex. The partitioning of the ligands was primarily dependent on their hydrophobicity and charge.

Furthermore, uptake of copper-8-hydroxyquinoline complexes into the unicellular algae Scenedesmus vacuolatus was examined. The goal was to identify physicochemical parameters that influence their bioavailability and to gain mechanistic information about their uptake. In these experiments rapid uptake was observed for all tested copper-8-hydroxyquinoline complexes with exception of the copper-sulfoxine complexes. Uptake rates and therefrom derived bioconcentration factors of copper were found to be not only dependent on the passive diffusion of
these complexes across the cell membrane but also on their fate within the cell. The results suggest that copper-8-hydroxyquinoline complexes undergo ligand exchange reactions with intracellular ligands that exhibit a high binding affinity for copper. Moreover, the comparison of the copper uptake rates via copper-8-hydroxyquinoline complexes with the uptake rate of Cu$^{2+}$ showed that under the chosen experimental conditions passive uptake of copper-8-hydroxyquinoline complexes is less efficient than carrier-mediated uptake of Cu$^{2+}$.

In addition, toxicity tests to determine growth inhibition and inhibition of photosynthesis were evaluated. However, none of the chosen toxicity tests showed significant results. Two reasons could be identified: the low water solubility of the copper-8-hydroxyquinoline complexes and the prerequisite that the experimental algae medium had to be free of metals to avoid competition between them and the added copper. Both issues made it difficult to work with established toxicity test methods.

In conclusion, hydrophobic copper-8-hydroxyquinoline complexes may be a considerable source of copper for algae. They are able to passively diffuse across biological membranes and their uptake into algae is additionally driven by means of ligand-exchange processes in the cytoplasm. For the different tested copper-8-hydroxyquinolines, both processes were influenced by the different functional groups of the ligands and the resulting different molecular properties of the complexes. However, the contribution of copper-8-hydroxyquinoline complexes to the overall copper uptake is dependent on the concentration of Cu$^{2+}$. Hydrophobic copper-8-hydroxyquinoline complexes will play an important role in aquatic environments where the Cu$^{2+}$ concentration is distinctively lower than the complex concentration.
Zusammenfassung


Ziel dieser Doktorarbeit war es, die Bioverfügbarkeit und Toxizität einer wichtigen Gruppe von hydrophoben Kupfer-organischen Komplexen, den Kupfer-8-hydroxyquinolinen, für die Süßwasseralge Scenedesmus vacuolatus zu evaluieren.


Im Anschluß wurde die Aufnahme von Kupfer-8-hydroxyquinolinkomplexen in die einzellige Alge Scenedesmus vacuolatus untersucht. Ziel war es, physikochemische Parameter zu identifizieren, die die Bioverfügbarkeit von Kupfer-8-hydroxyquinolinen beeinflussen. Weiterhin sollten mechanistische Informationen über ihre Aufnahme gewonnen werden. Mit Ausnahme von Kupfer-sulfoxin wurde dabei für alle Kupfer-8-
Zusammenfassung


Zusammenfassend wurde festgestellt, daß hydrophobe Kupfer-8-hydroxyquinolinkomplexe eine bedeutende Kupferquelle für Algen sein können. Sie sind zum einen fähig, passiv durch biologische Membranen zu diffundieren, zum anderen wird ihre Aufnahme in Algen zusätzlich durch Ligandenaustauschprozesse angetrieben. Für die getesteten Kupfer-8-hydroxyquinoline wurden beide Prozesse durch die verschiedenen funktionellen Gruppen ihrer Liganden und die daraus resultierenden unterschiedlichen Eigenschaften der Komplexe beeinflußt.

Der Beitrag der Kupfer-8-hydroxyquinolinkomplexe zur Gesamtkupferaufnahme ist jedoch von der Konzentration an Cu²⁺, die im betrachteten System vorliegt, abhängig. Hydrophober Kupfer-8-hydroxyquinolinkomplex spielt dann eine wichtige Rolle, wenn die Cu²⁺-Konzentration in einem aquatischen System deutlich geringer ist als die Komplexkonzentration.
1.

Introduction
1.1. Copper and its interactions with algae

1.1.1. Copper in the environment

Copper occurs as native copper in mineral form or more frequently in more than 150 minerals, mostly associated with sulphur or carbonate. Examples for frequently occurring copper minerals are chalcopyrite (CuFeS₂), bornite (Cu₉FeS₄), covellite (CuS), chalcocite (Cu₂S), azurite (Cu₃(CO₃)₂(OH)₂), malachite (Cu₂CO₃(OH)₂) and cuprite (Cu₂O). Weathering leads to a slow release of more mobile copper compounds in the form of salts and complexes into the environment. Higher concentrations of copper attain soil and water in the immediate vicinity of copper smelting plants and electrical industry (Samecka-Cymerman & Kempers, 2004). Other sources of copper pollution originate from fertilisers, pesticides and anti-fouling paints (copper oxide and copper thiocyanate) (Stauber & Florence, 1987; Boxall et al., 2000).

As a result of these different pollution sources, total copper concentrations in oxic aquatic systems vary distinctly. Freshwater concentrations range from 0.3 - 3·10⁻⁸ M total copper for non-polluted lakes and rivers (Xue et al., 1995; Xue et al., 1995; Xue et al., 1996) up to 10⁻⁶ M total copper in rivers and lakes in mining areas (Diagomanolin et al., 2004; Samecka-Cymerman & Kempers, 2004). In seawater systems reported concentrations are between 5·10⁻¹⁰ M for non-polluted areas and 6·10⁻⁷ M for largely polluted estuaries (Bruland et al., 1985; Twiss & Moffett, 2002). For non-polluted waters free copper ion concentrations range between 10⁻¹⁶ - 10⁻⁹ M (Xue et al., 1995; Xue et al., 1995; Xue et al., 1996) in freshwater and 10⁻¹³ - 10⁻¹² M in seawater (Twiss & Moffett, 2002).
1.1.2. Speciation of copper and its influence on uptake, bioavailability and toxicity for algae

Common oxidation states of copper include copper(I) (Cu⁺) and copper(II) (Cu²⁺). Due to the standard electrode potential at 298 K for the reduction of Cu²⁺ to Cu⁺ of 0.16 V, copper exists as Cu²⁺ in oxic environments like surface waters.

In surface water, copper exists in different species depending on chemical water properties including pH and the amount of inorganic ions and organic ligands, which form complexes of different character and stability. Thermodynamic stability is vitally important for the copper speciation and is described with the complex stability constant K, which equals the ratio of association and dissociation rate constant (equation 1.1 and 1.2).

\[
\text{Cu}^{2+} + \text{L}^2- \xrightleftharpoons{\ \ k_d \ } \text{CuL} \tag{1.1}
\]

\[
K = \frac{k_a}{k_d} \tag{1.2}
\]

From a kinetic point of view, copper complexes are differentiated in inert and labile ones. Inert complexes are complexes with a small dissociation rate constant, which is dependent on the thermodynamic stability and the factors that influence the association rate constant, the dissociation of water molecules from Cu²⁺ and the formation of the copper-ligand complex. If Cu²⁺ is removed from the system by biological uptake, it is not expected that copper present in inert complexes is released to rapidly reestablish equilibrium. In contrast, labile copper complexes, i.e. copper complexes with a high dissociation rate constant, are able to dissociate rapidly and thus contribute to the supply of Cu²⁺.

Inorganic ligands, which form labile copper complexes, include OH⁻, HCO₃⁻, CO₃²⁻, Cl⁻ and SO₄²⁻. Their charged and uncharged complexes have hydrophilic properties. In the case of copper, hydroxo and carbonate complexes are of main importance in freshwater systems. Organic ligands mainly originate from biological degradation and
conversion processes of natural organic materials, but there are also synthetic ones occurring in natural waters. They cover all types of ligands from inert to labile ones. Natural organic ligands include small carboxylic acids (oxalate, acetate, citrate, etc.), amino acids and phenols. In most cases, their complexes with copper are moderately stable and their importance for speciation is low (Stumm & Morgan, 1996; Escher & Sigg, 2004; Martell & Smith, 2004). A second category of natural organic ligands are humic and fulvic acids, macromolecules with COOH-, OH-, N- and S-groups, which act as strong ligands and buffer the free ion (Cu^{2+}) concentration to low levels (Xue & Sigg, 1993; Xue & Sigg, 1999). Chelating synthetic polycarboxy ligands such as EDTA (ethylenediaminetetraacetic acid) also form stable hydrophilic complexes with copper. Hydrophobic copper complexes may arise from other synthetic compounds like dithiocarbamates and 8-hydroxyquinolines, which are used as pesticides or for the extraction of metals in analytical chemistry (Bruland et al., 1979). The complexation of copper in natural waters is of great environmental relevance, because it determines the bioavailability and subsequently the toxicity of copper for algae and other organisms.

The carrier-mediated uptake of copper into an unicellular alga can be described as a three step process (Campbell, 1995) (see Figure 1.1). First, copper in the aqueous solution diffuses from bulk solution through the diffusion layer around the cell to the cell surface. Second, copper binds at the cell membrane to active sites and third, it is taken up into the cell by carrier-mediated transport (Fraústo da Silva & Williams, 2001). Interaction of copper with algae comprises, in addition to intracellular uptake, adsorption to the algal cell surface.
Chapter 1: Introduction

Figure 1.1. Conceptual model of copper-algae interactions (modified from (Campbell et al., 2002)).

$\text{Cu}^{2+}$: free copper ion; $\text{CuL}$: copper complex in solution; $\text{Cu}-\text{X}-\text{membrane}$: surface copper complex.

The bioavailability of copper for e.g. algae can be described by the Free-Ion Activity Model (FIAM) and the more recent Biotic Ligand Model (BLM). The FIAM assumes metal (M), e.g. copper, uptake via specific transport sites and predicts that the bioavailability of a metal is dependent upon the free metal ion concentration ($M^{2+}$, e.g. $\text{Cu}^{2+}$) (Campbell, 1995). The model also considers surface complexation ($M-\text{X-cell}$) at the cell membrane. It assumes that the concentration of $M-\text{X-cell}$ is proportional to $M^{2+}$ that in turn is determined by the metal speciation in the aqueous phase. Thus, a toxic effect, which is proportional to the concentration of $M-\text{X-cell}$ is also proportional to the concentration of $M^{2+}$. The applicability of the FIAM has been shown in several laboratory experiments with various algal species (e.g. Scenedesmus subspicatus, Chlamydomonas reinhardtii, Scenedesmus vacuolatus (formerly known as Chlorella fusca), Oocystis marsonii, and Oocystis nephrocytioides) (Sunda & Guillard, 1976; Petersen, 1982; Knauer et al., 1997; Knauer et al., 1997).

The BLM represents a refinement of the FIAM. The formulations of both models are relatively similar but the BLM turns the attention to the organism-water interface,
where metal binding sites are located. It also comprises competitive binding between metals and cations like Ca\(^{2+}\), Mg\(^{2+}\) or H\(^+\) (Di Toro et al., 2001; Campbell et al., 2002; De Schamphelaere et al., 2005; Slaveykova & Wilkinson, 2005). The model predicts metal uptake and subsequently toxicity depending on the amount of metal, which is associated with binding sites on the surface of an organism ("biotic ligands"). For algae, it is hypothesised that binding sites on the algal surface are the biotic ligands (De Schamphelaere et al., 2005). Besides the biotic ligands also organic and inorganic ligands compete for the metal (Figure 1.2).

![Diagram of the Biotic Ligand Model](image)

**Figure 1.2.** Conceptual diagram of the Biotic Ligand Model (after (Di Toro et al., 2001)).

Exceptions to these two models were observed in the presence of hydrophobic ionogenic organic compounds like diethyldithiocarbamate, xanthates and 8-hydroxyquinoline, which can form stable complexes with metals (Florence et al., 1983; Ahsanullah & Florence, 1984; Florence & Stauber, 1986; Campbell, 1995). These complexes may diffuse in or across the membrane because of their hydrophobicity and thus open an alternative uptake route for copper into the cell and facilitate its uptake.
1.1.3. Relevance of copper for freshwater green algae

Copper is an essential micronutrient for freshwater algae as it is for other organisms. Both Cu(II) and Cu(I) very effectively bind to organic molecules, particularly to soft Lewis bases, due to their high electron affinity (Fraústo da Silva & Williams, 2001; Atkins et al., 2006). Copper plays a vital role as cofactor of enzymes, in metalloenzymes, or for keeping the redox and electrolyte balance. Thus, it is a constituent of proteins like cytochrome oxidase, catechol oxidase, superoxide dismutase and plastocyanin (Buchanan et al., 2000).

In higher concentrations, however, copper may be harmful to organisms. Therefore copper salts are widely used as fungicides and algicides (Russell, 2005; Borkow & Gabbay, 2005). In general, copper toxicity is believed to be mainly caused by inappropriate nonspecific binding of copper to physiologically important biomolecules. Copper may (i) block essential biological functional groups of biomolecules, (ii) displace essential metals in biomolecules, and (iii) modify the active conformation of biomolecules. Specifically, copper directly inhibits various enzyme systems, e.g. catalase, alkaline phosphatase, Na/K-ATPase, and has also a direct impact on oxidative phosphorylation and on photosynthesis (Baron et al., 1995) (see also chapter 1.1.4).

Other toxic effects of copper include the induction of oxidative stress by generating free radicals. This becomes apparent when observing the major enzymes involved as antioxidants in algae and plants (glutathione reductase, ascorbate peroxidase, superoxide dismutase and catalase), when those organisms are exposed to an increasing amount of copper. The activity of these enzymes first rises to protect the cell from active oxygen species and then decreases again, when the enzyme production cannot meet the need any more. As a result of oxidative copper stress lipid peroxidation and the disruption of plasma membranes occur. Another consequence is also the degradation of chlorophyll a (Nagalakshmi & Prasad, 1998; Mallick, 2004).

For algae it has also been reported that copper leads to an inhibition of cell division and thus to an increase of cell volume with time (Nalewajko & Olaveson, 1995). This effect seems to be related with a reaction of copper with glutathione within the cytoplasm that leads to a decrease of the ratio between reduced and oxidised...
glutathione, which in turn influences mitosis (Florence & Stauber, 1986; Stauber & Florence, 1987). Additionally, in copper-sensitive plants, a change in chloroplast ultrastructure was observed (Droppa & Horvath, 1990).

Copper toxicity or deficiency is dependent on the requirement of a certain algae species and the cellular copper content. For *Scenedesmus vacuolatus* the optimal range for growth was determined in lab experiments as $10^{-13}$ to $10^{-10}$ M free copper ($Cu^{2+}$) (Knauer, 1996). Cellular copper includes metabolic necessary copper that is directly used, e.g. as a cofactor in newly produced enzymes, and additional copper, which is bound to intracellular binding peptides like phytochelatin and which forms a reservoir for copper.

### 1.1.4. The role of copper in photosynthesis

Copper is an essential metal for photosynthesis. It is for example a cofactor of the protein plastocyanin, which serves as an electron carrier between Photosystem I and II (PS I and II), it is also bound to the light harvesting complex in PS II, and involved in the water-splitting side of PS II (Droppa & Horvath, 1990; Baron et al., 1995). However, in higher concentrations, copper has an inhibitory effect on both photosystems with PS II being the more sensitive site (Droppa & Horvath, 1990). The inhibition of PS I by copper occurs via interaction with its donor site, potentially with ferredoxin (Schroder et al., 1994). For PS II it was shown that $Cu^{2+}$ ions inhibit its electron donor as well as its acceptor side (Baron et al., 1995). Among other effects on the donor side, copper reversibly inactivates oxidation of tyrosine and reduces the reduction rate of the oxidised primary electron donor, which in turn irreversibly oxidizes nearby reaction centre components of PS II. Additionally, copper is known to enhance degradation of 17- and 23-kDa proteins, which are components of the oxygen-evolving complex, due to specific interactions with the donor side of PS II (Patsikka et al., 2001). On the acceptor side copper irreversibly interferes in the interaction between the secondary plastoquinone acceptor $Q_A^-$ and $Fe^{2+}$ in the acceptor complex due to structural changes around $Q_A$ (Droppa & Horvath, 1990; Schroder et al., 1994; Baron et al., 1995; Jegerschoeld et al., 1995; Patsikka et al., 2001).
The inhibition of both photosystems by copper also contributes to oxidative stress (Sandmann & Boger, 1980). The inhibition of PS I leads to an increased production of superoxide radicals during the oxygen reduction process, which is subsequently converted into hydrogen peroxide and hydroxyl radicals. PS II inhibition also results in a production of hydroxyl radicals.

### 1.2. Hydrophobic copper-8-hydroxyquinoline complexes

Unsubstituted, chlorinated and brominated 8-hydroxyquinolines represent examples of ionogenic organic compounds (Figure 1.3) and form stable and hydrophobic metal-ligand complexes with copper (Figure 1.4). The formation of these complexes is instantaneous and completely reversible (Nicoletti et al., 1999) (Table 1.1).

**Table 1.1. Acidity constants (pKₐ) of the examined 8-hydroxyquinolines and stability constants of their 1:1 (CuL; β₁) and 1:2 copper complexes (CuL₂; β₂)**

<table>
<thead>
<tr>
<th>compound</th>
<th>abbreviation</th>
<th>pKₐ (SO₃H) b</th>
<th>pKₐ (NH) b</th>
<th>pKₐ (OH) b</th>
<th>log₁₀ β₁ b</th>
<th>log₁₀ β₂ b</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-Hydroxyquinoline-5-sulfonic acid</td>
<td>sufoxine</td>
<td>2.54 ± 0.02 c</td>
<td>3.79 ± 0.01 c</td>
<td>8.41 ± 0.01 c</td>
<td>12.2 ± 0.3 d</td>
<td>22.4 ± 0.6 d</td>
</tr>
<tr>
<td>8-Hydroxyquinoline</td>
<td>oxine</td>
<td>4.89 ± 0.01 c</td>
<td>9.54 ± 0.01 c</td>
<td>12.0 ± 0.1 d</td>
<td>22.9 ± 0.1 d</td>
<td></td>
</tr>
<tr>
<td>5-Chloro-8-hydroxyquinoline</td>
<td>chloroxine</td>
<td>3.71 ± 0.02 c</td>
<td>9.37 ± 0.02 c</td>
<td>15.1 c</td>
<td>25.8 c</td>
<td></td>
</tr>
<tr>
<td>5,7-Dichloro-8-hydroxyquinoline</td>
<td>dichloroxine</td>
<td>2.6 d</td>
<td>7.3 d</td>
<td>10.5 e</td>
<td>17.6 e</td>
<td></td>
</tr>
<tr>
<td>5,7-Dibromo-8-hydroxyquinoline</td>
<td>dibromoxine</td>
<td>2.9 d</td>
<td>7.4 d</td>
<td>10.5 e</td>
<td>18.1 e</td>
<td></td>
</tr>
</tbody>
</table>

a for definitions of pKₐ, β₁ and β₂ see equation 2.2, 2.5 and 2.6.  
 b T = 25 °C, I = 0.1 M.  
 c (Kaiser & Escher, 2006)  
 d Values in aqueous media from (Martell & Smith, 2004).  
 e Derived from stability constants in a dioxan/water medium from (Gupta et al., 1970).
Chapter 1: Introduction

Figure 1.3. Structures of the 8-hydroxyquinoline ligands used in this study.

- oxine
- chloroxine
- dichloroxine
- dibromoxine
- sulfoxine

Figure 1.4. Speciation of the 8-hydroxyquinolines and structures of their copper complexes. Depending on the pH 8-hydroxyquinolines form cationic (H₂L⁺), neutral (HL) and anionic species (L⁻). The anionic species forms 1:1 (CuL) and 1:2 complexes (CuL₂) with copper. (oxine: X = H, Y = H; chloroxine: X = Cl, Y = H; dichloroxine: X = Cl, Y = Cl; dibromoxine: X = Br, Y = Br; sulfoxine: X = SO₃⁻, Y = H). Sulfoxine has an additional negative charge because the sulfonate group is deprotonated with an acidity constant, pKₐ(SO₃H), of 2.54.
Chapter 1: Introduction

Due to their high stability constants these 8-hydroxyquinolines are used for the extraction of metals in analytical chemistry (Bruland et al., 1979) and because of their antimicrobial and antifungal properties (Hollingshead, 1954; Gershon et al., 1999; Gershon et al., 2001) as constituents of antiseptic and disinfectant formulations (Hollingshead, 1954; Hollingshead, 1954; Block, 1991).

Copper-8-hydroxyquinoline (copper-oxine; CAS# 10380-28-6, EC# 233-841-9) is widely used as an excellent preservative for textile, wood and paint, as a fungicide in fruit and vegetable gardening (Yeager, 1991; Kikuchi et al., 1996; Nicoletti et al., 1999; Schultz et al., 2005) and as a weed controlling agent on golf courses (Odanaka et al., 1994). Because of its low human toxicity it is particularly applied in places where this characteristic is desirable, i.e. railroad car interiors, floors and walls of food plants, food freezer rooms, dairies, breweries, hospitals, and apartment houses (Yeager, 1991). It is also featured by its high stability against photodegradation (Liu et al., 1994).

There are several hypotheses why copper-oxine is a highly effective fungicide (Albert, 1951; Yeager, 1991). One hypothesis sees the fungicidal effect in the ligand oxine, which, once entered a cell, chelates metals essential for growth. Another assumes that oxine simply serves as a transporter for copper into the cell, where the latter exerts its toxic effects. A third hypothesis supposes that the complex itself may have toxic effects by catalysing the formation of hydroxyl radicals from molecular oxygen via the Fenton reaction (Florence et al., 1983). The fact that not all metal-8-hydroxyquinoline complexes of toxic metals exhibit antifungal effects (Liu et al., 1994) and that copper-oxine and the likewise fungicidal oxine act synergetically (Nicoletti et al., 1999) support the latter two assumptions. Toxicity studies have already been conducted with fungi (Nicoletti et al., 1999; Nicoletti et al., 1999), bacteria (Liu et al., 1994), marine amphipods (Ahsanullah & Florence, 1984), and algae (Stauber & Florence, 1987; Phinney & Bruland, 1997). They all showed a higher toxicity of copper-oxine in comparison to the single compounds Cu^{2+} and oxine, whereas the toxicity of copper-oxine decreased with an increasing fraction of the charged 1:1 complex presumable due to its smaller bioavailability (Block, 1956). Furthermore, Liu demonstrated that the toxicity of copper-oxine is not affected by the counter-anion of the copper salt, which was used to form the complex (Liu et al., 1994).
In contrast to oxine and the chlorinated and brominated 8-hydroxyquinolines, 8-hydroxyquinoline-5-sulfonic acid (sulfoxine; Figure 1.2) is more hydrophilic. It shows only a very low antifungal activity, which was assumed to be caused by low bioavailability due to its low hydrophobicity (Gershon et al., 2002). Sulfoxine furthermore forms hydrophilic copper complexes, which is the reason for an observed decrease in copper uptake and an alleviated copper toxicity in the presence of sulfoxine (Ahsanullah & Florence, 1984; Phinney & Bruland, 1994).
1.3. Biological membranes and their relevance for the uptake of chemical substances in cells

1.3.1. Structure of biological membranes

Biological membranes are mainly composed of lipids and proteins (Figure 1.5). The amphipathic lipids are arranged in 5-10 nm thick bilayers with their polar, hydrophobic heads towards the surrounding aqueous phase and their non-polar, hydrophobic tails in the core of the bilayer (Buchanan et al., 2000). Both lipids and proteins are held together by cooperative non-covalent forces.

![Figure 1.5. Schematic illustration of a biological membrane.](image)

Phospholipids are the most common type of membrane lipids while galactosylglycerides, glucocerebrosides and sterols are less abundant (Buchanan et al., 2000). The former are composed of a glycerol backbone with fatty acid chains attached to the first two glycerol carbons and a phosphate group on the third (Figure 1.6). The fatty acid tails of phospholipids contain of 14 - 24 carbon atoms and at least one has one or more cis double bonds, which introduce kinks within the chain.
Phosphatidylcholine is one of the most frequent phospholipid in biological membranes and therefore commonly used in biological studies (Figure 1.6).

![Phosphatidylcholine structure](image)

**Figure 1.6.** 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC) as an example of a phospholipid.

Proteins comprise about half of the mass of membranes and are either embedded in the membrane (integral proteins) or attached to the surface (peripheral proteins) (Buchanan et al., 2000) (see Figure 1.5). Integral proteins are usually involved in transporting substances across the membrane, while peripheral proteins maintain the cell's shape, act as enzymes or serve as receptors.

The composition of lipids and proteins, the amount of sterols and the number of kinks in fatty acid chains of phospholipids are amongst other things responsible for the packing properties of biological membranes.

The fluid-mosaic model depicts the structure and properties of biological membranes (Singer & Nicolson, 1972). It describes the membrane as an asymmetric and non-static structure whose constituents can swap places with each others because they are primarily hold together by non-covalent interactions. Therefore lipids are able to move freely and relatively fast within the plane of the membrane. In contrast, the transfer of lipids across the bilayer occurs rarely because the hydrophobic headgroup of a lipid has to cross the hydrophobic core of the membrane. Integral proteins are also able to diffuse laterally within the membrane.
1.3.2. Diffusion and transport across biological membranes

Biological membranes represent highly selective barriers. A molecule can cross this barrier by passive and active processes (Buchanan et al., 2000) (Figure 1.7).

Passive diffusion allows molecules, which are hydrophobic or small, uncharged and hydrophilic (e.g. water), to enter the cell through the lipid bilayer and along a concentration gradient. This process is rendered possible because the membrane constituents are held together by weak, non-covalent forces and these molecules can slip between them and thus cross the membrane. It is assumed that the examined copper-8-hydroxyquinoline complexes enter a cell by passive diffusion.

Some polar or ionic molecules can cross the membrane by means of integral proteins. The so called facilitated diffusion is also a passive process and driven by a concentration gradient. There are two types of integral proteins: channel and carrier proteins. Channel proteins are water-filled pores, which facilitate a controlled flux of ions across the membrane. Carrier proteins have a specific binding site and constantly alternate between two conformations, which allows them to transport bound substances, e.g. free copper ions (Cu^{2+}), across the membrane. In the case of Cu^{2+} it was proposed that its uptake pathway in algae consists of a cell surface reductase, which reduced Cu^{2+} to Cu^{+}, and a Cu^{+} transporter (Merchant et al., 2006).

Active transport is the energy requiring and highly specific pumping of molecules across a membrane by integral proteins (active carriers). It also allows transport against a concentration gradient.
1.3.3. Liposomes - a simple model for biological membranes

The transport of many substances across biological membranes involves proteins, but passive diffusion is the predominant mechanism for hydrophobic compounds. Suitable model systems to study this passive uptake in biological membranes are liposomes (Kraemer, 2001). They are protein-free phospholipid bilayer vesicles of known composition and size, which mimic the anisotropic properties and ordered structure of biological membranes (Stein, 1986) (Figure 1.8). Like in biological membranes, the lipid bilayers of liposomes exhibit regions with hydrophobic, hydrophilic, charged or neutral properties where neutral and charged species of an ionogenic substance may sorb by different sorption mechanisms. Particularly for ionic substances, partitioning into liposomes provides a more realistic impression of their uptake in biological membranes than partitioning into octanol (Escher & Schwarzenbach, 1996), which is a traditionally used model system for biological membranes. Particularly, the extent of partitioning of charged organic species is largely underestimated in the octanol-water system compared to the liposome-water system (Campbell, 1995; Escher et al., 2000a). Liposome-water partitioning also

Figure 1.7. Passive and active movement of substances across membranes.
shows a smaller dependence on ionic strength compared to the partitioning of ions in octanol where formation of ion pairs is an important factor (Escher et al., 2000a).

Within the last 20 years, liposome-water systems have been increasingly applied in environmental sciences to evaluate the partitioning behaviour of organic pollutants between water and biological membranes (Gobas et al., 1988; Escher et al., 2000a).

*Figure 1.8. Cross-section through a liposome.*
1.4. Scope of this work

The scope of this work was to develop parameters and methods to evaluate bioavailability and combined effects of copper and substituted 8-hydroxyquinolines on freshwater green algae (Scenedesmus vacuolatus). Algae are important test species because of their key position as the base of the aquatic food chain.

Figure 1.9 shows a schematic diagram of an unicellular green alga in its aquatic environment. In this environment copper and hydrophobic and hydrophilic ligands, which form complexes with copper, may be found. Hydrophilic complexes influence primarily bioavailability and toxicity by modulating free ion concentration, whereas hydrophobic complexes may directly contribute to the copper uptake into algae.

On the one hand, copper ions (Cu$^{2+}$) are able to enter the cell by carrier-mediated uptake (Williams, 1981). Inside the alga, Cu$^{2+}$ can associate with cellular ligands to form the essential and stored copper pool or cause toxic effects. On the other hand, hydrophobic copper complexes may passively diffuse in or across the membrane due to their high hydrophobicity. So far, there is a lack of systematic studies on this process. Hydrophobic complexes may (i) accumulate in the cell membrane, (ii) diffuse into the cell membrane and dissociate at the membrane-cytoplasm interface and release copper into the cytoplasm by means of ligand exchange with intracellular ligands or (iii) enter the cytoplasm and release copper via a ligand exchange mechanism (Phinney & Bruland, 1994). Toxic effects, which are triggered by hydrophobic copper complexes may be based upon Cu$^{2+}$, when the complex simply serves as a transporter for copper into the cell, or induced by the complex itself (see chapter 1.2).

Phinney and Bruland proposed a model for the uptake of copper-oxine into the marine diatom Thalassiosira weissflogii (Phinney & Bruland, 1994; Phinney & Bruland, 1997) that assumes a dissociation of the complex, a protonation of the ligand in the cytoplasm, and an equilibration between the protonated ligand in the cytoplasm and in the external solution. In their study copper uptake in the presence of copper-oxine and other hydrophobic copper-organic complexes with ligands from other substance groups was determined.

The present thesis extends these findings. It works with five complexes of a single substance group, the copper-8-hydroxyquinolines, and deals particularly with the
influence of the different functional groups of the ligands on the bioavailability of the complexes. The thesis provides quantitative information about the partitioning of copper-8-hydroxyquinolines into biological membranes and about their uptake into the freshwater algae *Scenedesmus vacuolatus*. Additionally, it aims to acquire mechanistic information about the toxicity of these copper-8-hydroxyquinolines for algae.

**Figure 1.9. Processes determining uptake, adsorption and toxicity of copper in algae.**

The cell wall, which surrounds the plasma membrane, is omitted from the diagram for brevity.

- Cu$:^{2+}$: free copper ion; Cu$_{ess}$: essential copper; Cu$_{stored}$: stored copper; P: hydrophilic ligands; L: hydrophobic ligands; CuP: hydrophilic copper complex; CuL$_{2}$: hydrophobic copper complex.
In order to evaluate bioavailability and toxic effects of hydrophobic copper-organic complexes this thesis addresses the following issues:

- Chapter 2 explores the role of combinations of hydrophobic ionogenic organic compounds (8-hydroxyquinolines) with copper for the passive uptake of copper via hydrophobic copper complexes in *in vitro* membrane systems (liposomes). Liposome-water partitioning experiments were conducted (i) to study the partitioning of the neutral and ionic species of selected 8-hydroxyquinolines and their copper complexes in phospholipid membranes and (ii) to investigate dependencies of their liposome-water partitioning on the different functional groups of the ligands.


- Chapter 3 presents the results of a study about the bioavailability and uptake of hydrophobic copper-8-hydroxyquinoline complexes in algae. The objective of this study was to evaluate the uptake of selected copper-8-hydroxyquinolines complexes in the freshwater algae *Scenedemus vacuolatus* under controlled laboratory conditions and to determine physicochemical parameters, which influence the bioavailability of these complexes. To evaluate whether passive diffusion is the dominant process in the copper uptake via copper-8-hydroxyquinoline complexes, the obtained uptake data in algae was also compared with that in liposomes.

  This chapter will be submitted to *Environ. Sci. Technol.* under the title “Sibylle M. Kaiser, Renata Behra, Beate I. Escher, Laura Sigg: Bioavailability and uptake of hydrophobic copper-organic complexes in algae”.

- Chapter 4 summarises various experimental approaches, which were tested to determine toxic effects of hydrophobic copper-8-hydroxyquinoline complexes. The investigation showed that under the experimental conditions none of the approaches was suitable for determining the toxicity of these complexes.
2.

The evaluation of liposome-water partitioning of 8-hydroxyquinolines and their copper complexes

This chapter was published as:

Sibylle M. Kaiser, Beate I. Escher
The evaluation of liposome-water partitioning of 8-hydroxyquinolines and their copper complexes.
2.1. Introduction

It is known that the uptake of low molecular, hydrophobic organic compounds in biological membranes is taking place by passive diffusion (Stein, 1986). In contrast, the uptake of free copper ions usually results from carrier related transport processes. The resulting bioavailability can be described by the Free-Ion Activity Model (FIAM) (Campbell, 1995) and the more recent Biotic Ligand Model (BLM) (Campbell et al., 2002; Slaveykova & Wilkinson, 2005). Exceptions to these models were observed in the presence of hydrophobic ionisable organic compounds like diethyldithiocarbamate, xanthates and oxine, which can form stable complexes with copper and other metals (Florence et al., 1983; Ahsanullah & Florence, 1984; Florence & Stauber, 1986; Campbell, 1995). These complexes may diffuse in or across the membrane because of their hydrophobicity and thus circumvent the carrier-mediated transport systems for copper and facilitate the uptake of copper in cells. There is a lack of systematic studies that examine the interaction of hydrophobic copper complexes with biological membranes. Some of the existing studies state that hydrophobic metal complexes are not retained in the cell membrane but diffuse into the cytosol and rapidly exchange the metal ions with internal cellular ligands (Phinney & Bruland, 1994; Phinney & Bruland, 1997; Croot et al., 1999). In contrast, organic pollutants, even hydrophobic ionic compounds, may intercalate in biological membranes (Gobas et al., 1988; Escher et al., 2000a).

A suitable model system to observe passive uptake of a hydrophobic organic compound in biological membranes is the examination of its liposome-water partitioning (Kraemer, 2001). Liposome-water systems have been increasingly applied in environmental sciences (Gobas et al., 1988; Escher et al., 2000a) to evaluate the partitioning behaviour of organic pollutants between water and biological membranes. Liposomes, which are artificial phospholipid bilayer vesicles of known composition and size, mimick the anisotropic properties and ordered structure of biological membranes (Stein, 1986). Lipid bilayers exhibit regions with hydrophobic, hydrophilic, charged or neutral properties where neutral and charged species of an ionogenic substance may sorb by different sorption mechanisms. Particularly for ionic substances partitioning into liposomes gives a more precise impression of their
uptake in biological membranes than partitioning into octanol (Escher & Schwarzenbach, 1996), which is the most widely used model system for biota. The extent of partitioning of charged organic species is largely underestimated in the octanol-water system compared to the liposome-water system (Campbell, 1995; Escher et al., 2000a). Liposome-water partitioning also shows only a small dependence on ionic strength in contrast to the partitioning of ions in octanol where formation of ion pairs is an important factor (Escher et al., 2000a).

Liposome-water partitioning coefficients include information about partitioning into and binding to membranes but not about membrane permeation of the examined substance (Avdeef, 2003). Partitioning and binding (sorption) are both dependent on van der Waals and hydrogen donor/acceptor interactions between membrane and substance (Schwarzenbach et al., 2002). It has been shown that charged and polar molecules are located in the head group region of a lipid bilayer with their ionic or polar groups pointing outwards while their non-polar groups are orientated towards the hydrophobic core. Uncharged molecules are expected to partition deeper into the membrane (Boulanger et al., 1981; Avdeef, 2003).

8-Hydroxyquinoline (oxine) and chlorinated and brominated 8-hydroxyquinolines (the names and abbreviations are given in Table 2.1 and chemical structures in Figure 1.3) represent examples for hydrophobic ionogenic organic compounds. They form stable and hydrophobic metal-ligand complexes with copper of the type CuL₂ and CuL⁺ (see Figure 1.4). The formation of the complex is instantaneous, requires no energy and is completely reversible, but because of the high stability constants the equilibrium lies at the side of the complex (Nicoletti et al., 1999).

Hydrophobic 8-hydroxyquinolines are used for the extraction of metals in analytical chemistry (Bruland et al., 1979) and as constituents in antiseptic and disinfectant formulations, in preservatives and in agricultural fungicides (Hollingshead, 1954; Hollingshead, 1954; Block, 1991). Their copper complexes show antimicrobial and antifungal properties (Hollingshead, 1954). Copper-oxine is widely used as wood preservative and fungicide (Yeager, 1991; Kikuchi et al., 1996; Nicoletti et al., 1999; Schultz et al., 2005). For copper-oxine passive diffusive uptake in cells has already been shown for the diatom Thalassiosira weissflogii and for several other marine
phytoplankton species (Phinney & Bruland, 1994; Phinney & Bruland, 1997; Croot et al., 1999).

In contrast to the above mentioned hydrophobic 8-hydroxyquinolines, 8-hydroxyquinoline-5-sulfonic acid (sulfoxine) is more hydrophilic (see Table 2.1 and Figure 1.3 and 1.4). It shows only a very low antifungal activity which was assumed to be caused by low bioavailability due to its low hydrophobicity (Gershon et al., 2001). It has also been shown that the assimilation of copper by the diatom *Thalassiosira weissflogii* was about ten times smaller when Cu existed as Cu(sulfoxine)$_2^{2-}$ than as Cu(oxine)$_2$ (Phinney & Bruland, 1994).

The goals of this study were (i) to evaluate liposome-water partitioning of the neutral and ionic species of selected 8-hydroxyquinolines and of their copper complexes, (ii) to investigate dependencies of the liposome-water partitioning of the copper complexes on that of their ligands and on other complex characteristics.
Chapter 2: The evaluation of liposome-water partitioning of 8-hydroxyquinolines and their copper complexes

**Table 2.1. Acidity constants (pKₐ), octanol-water partitioning coefficients of the neutral species \( K_{\text{OW, neutral ligand}} \) and stability constants of the 1:1 and 1:2 Cu-complexes \( \beta_1 \) and \( \beta_2 \) of the examined 8-hydroxyquinolines**

<table>
<thead>
<tr>
<th>compound</th>
<th>abbreviation</th>
<th>( pK_{\text{a}}(\text{SO}_3\text{H}) )</th>
<th>( pK_{\text{a}}(\text{NH}) )</th>
<th>( pK_{\text{a}}(\text{OH}) )</th>
<th>( \log_{10} K_{\text{OW, neutral ligand}} )</th>
<th>( \log_{10} \beta_1 )</th>
<th>( \log_{10} \beta_2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-Hydroxyquinoline-5-sulfonic acid</td>
<td>sulfoxine</td>
<td>2.54 ± 0.02 b</td>
<td>3.79 ± 0.01 b</td>
<td>8.41 ± 0.01 b</td>
<td>-0.03 ± 0.02 b</td>
<td>12.2 ± 0.3 c</td>
<td>22.4 ± 0.6 c</td>
</tr>
<tr>
<td>8-Hydroxyquinoline</td>
<td>oxine</td>
<td>4.89 ± 0.01 b</td>
<td>9.54 ± 0.01 b</td>
<td>1.85 ± 0.01 b</td>
<td>12.0 ± 0.1 c</td>
<td>22.9 ± 0.1 c</td>
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</tr>
<tr>
<td>5-Chloro-8-hydroxyquinoline</td>
<td>chloroxine</td>
<td>3.71 ± 0.02 b</td>
<td>9.37 ± 0.02 b</td>
<td>2.51 ± 0.01 b</td>
<td>15.1 b</td>
<td>25.8 b</td>
<td></td>
</tr>
<tr>
<td>5,7-Dichloro-8-hydroxyquinoline</td>
<td>dichloroxine</td>
<td>2.6 c</td>
<td>7.3 c</td>
<td>2.84 c</td>
<td>10.5 d</td>
<td>17.6 d</td>
<td></td>
</tr>
<tr>
<td>5,7-Dibromo-8-hydroxyquinoline</td>
<td>dibromoxine</td>
<td>2.9 c</td>
<td>7.4 c</td>
<td>3.57 c</td>
<td>10.5 d</td>
<td>18.1 d</td>
<td></td>
</tr>
</tbody>
</table>

\( a \) T = 25 °C, I = 0.1 M \( b \) This study. \( c \) Values in aqueous media from (Martell & Smith, 2004). \( d \) Derived from stability constants in a dioxan/water medium from (Gupta et al., 1970). \( e \) calculated with SPARC (Carreira et al., 1994; Hilal et al., 1995).
2.2. Theoretical considerations

2.2.1. Concentration and speciation in the aqueous phase

8-Hydroxyquinolines dissociates in the aqueous phase according to the reactions

$$H_iL^{(i-1)} \rightleftharpoons H^+ + H_{(i-1)}L^{(i-2)} \quad i = 1...n$$ \hspace{1cm} (2.1)

$H_iL^{(i-1)}$ represents the acidic form, $H_{(i-1)}L^{(i-2)}$ the associated basic form and $H^+$ the aqueous proton. $n$ stands for the maximal number of protonated species of the hydroxyquinolines which is $n=2$ for oxine, chloroxine, dichloroxine and dibromoxine and $n=3$ for sulfoxine. The mass law expressions for these reactions are

$$K_{a,i} = \frac{c_{H_iL}^\circ \cdot a_H^w}{c_{H_iL}} \quad i = 1...n$$ \hspace{1cm} (2.2)

where $K_{a,i}$ is the equilibrium constant, $c_{H_iL}^\circ$ and $c_{H_iL}$ represent the aqueous concentrations of the acid and its associated base and $a_H^w$ the aqueous proton activity ($pH = -\log_{10} a_H^w$). The index $w$ refers to the aqueous phase.

Copper exists in the present experiments in the aqueous phase as free ion Cu$^{2+}$ or bound in complexes with the fully deprotonated species of the 8-hydroxyquinolines (ligands) $L^j$, Cu$L^{(2j)}$ and Cu$L^{(2-2j)}_2$. The complexes are formed according to the reactions

$$Cu^{2+} + L^{-j} \rightleftharpoons CuL^{(2-j)} \quad (2.3)$$

$$Cu^{2+} + 2 \cdot L^{-j} \rightleftharpoons CuL^{(2-2j)}_2 \quad (2.4)$$
Chapter 2: The evaluation of liposome-water partitioning of 8-hydroxyquinolines and their copper complexes

with the mass law expressions

\[
\beta_1 = \frac{a^w_{\text{CuL}}}{a^w_{\text{Cu}} \cdot a^w_{\text{L}}} \tag{2.5}
\]

\[
\beta_2 = \frac{a^w_{\text{CuL}_2}}{a^w_{\text{CuL}} \cdot (a^w_{\text{L}})^j} \tag{2.6}
\]

where \( j \) refers to the charge of the ligand and \( j=1 \) for oxine, chloroxine, dichloroxine and dibromoxine and \( j=2 \) for sulfoxine. \( \beta_1 \) and \( \beta_2 \) are the stability constants of the copper complexes, \( \text{CuL}^{(2j)} \) and \( \text{CuL}_2^{(2j)} \). \( a^w_{\text{Cu}}, a^w_{\text{L}}, a^w_{\text{CuL}}, \) and \( a^w_{\text{CuL}_2} \) are the activities of the free copper ion \( \text{Cu}^{2+} \), the ligand \( L^j \) and the complexes \( \text{CuL}^{(2j)} \) and \( \text{CuL}_2^{(2j)} \).

The total concentration of copper in the aqueous phase \( c^w_{\text{Cu, tot}} \) is defined by

\[
c^w_{\text{Cu, tot}} = c^w_{\text{Cu}} + c^w_{\text{CuL}} + c^w_{\text{CuL}_2} \tag{2.7}
\]

where \( c^w_{\text{Cu}} \) is the concentration of the free copper ion \( \text{Cu}^{2+} \) and \( c^w_{\text{CuL}} \) and \( c^w_{\text{CuL}_2} \) are the concentrations of the two copper organic complexes \( \text{CuL}^{(2j)} \) and \( \text{CuL}_2^{(2j)} \).

The total concentration of the ligand in the aqueous phase \( c^w_{\text{L, tot}} \) is defined by

\[
c^w_{\text{L, tot}} = \sum_{i=0}^{n} c^w_{\text{H}_i \text{L}} + c^w_{\text{CuL}} + 2 \cdot c^w_{\text{CuL}_2} \tag{2.8}
\]

where \( \sum_{i=0}^{n} c^w_{\text{H}_i \text{L}} \) is the sum of the concentration of all species of the 8-hydroxyquinolines and \( c^w_{\text{CuL}} \) and \( c^w_{\text{CuL}_2} \) are the concentrations of the two copper organic complexes \( \text{CuL}^{(2j)} \) and \( \text{CuL}_2^{(2j)} \).
2.2.2. Concentration and speciation in the membrane phase

All species of an 8-hydroxyquinoline and its copper complexes partition between aqueous phase and membrane phase (liposomes) according to a reaction of the type

\[ B^w \rightleftharpoons B^{lip} \]  

(2.9)

where \( B \) is the species that partitions into the lipid bilayer and the superscripts \( w \) and \( lip \) refer to the aqueous phase and the liposome phase. The associated mass law equations is

\[
K_{lipw,B} = \frac{c^{lip}_B}{c^w_B} = \frac{n^{lip}_B \cdot V^w}{V^{lip} \cdot \rho^{lip} \cdot n^w_B} \quad [L \cdot kg^{-1}] 
\]

(2.10)

\( K_{lipw,B} \) is the liposome-water distribution coefficient of \( B \) (L·kg\(^{-1}\)). \( c^w_B \) and \( n^w_B \) are the concentration (mol·L\(^{-1}\)) and the amount (mol) of \( B \) in the aqueous phase, \( c^{lip}_B \) and \( n^{lip}_B \) are the concentration (mole per kg of phospholipid in the membrane) and the amount (mol) of \( B \) in the liposome phase, \( V^{lip} \) and \( V^w \) are the volumes (L) of the lipid and water phase and \( \rho^{lip} \) is the density of the phospholipids (1.015 kg\(_{PL} \)·L\(_{PL}^{-1}\)).

The free copper ion \( Cu^{2+} \) was not expected to partition significantly into the membrane, which had to be proved in the following.

Analogously to the aqueous phase, the total concentrations of an 8-hydroxyquinoline and of copper in the liposome phase are defined by

\[
c^{lip}_{i,tot} = \sum_{i=0}^{n} c^{lip}_{i,L} + c^{lip}_{i,CuL} + 2 \cdot c^{lip}_{i,CuL_2} \quad n = 2,3
\]

(2.11)

and

\[
c^{lip}_{Cu,tot} = c^{lip}_{CuL} + c^{lip}_{CuL_2}
\]

(2.12)
2.2.3. Liposome-water partitioning of 8-hydroxyquinolines

For a given ionic strength, the pH-dependent liposome-water distribution ratio \( D_{\text{lipw},L} \) (L·kg\(^{-1}\)) for all species of an 8-hydroxyquinoline \( L \) in the absence of copper is described by

\[
D_{\text{lipw},L}(pH) = \frac{\sum_{i=0}^{n} c_{H_jL}^{i} \cdot f_i}{\sum_{i=0}^{n} c_{H_jL}^{i}} \quad [\text{L·kg}^{-1}] \quad n = 2,3 \quad (2.13)
\]

which is equal to

\[
D_{\text{lipw},L}(pH) = \sum_{i=0}^{n} f_i \cdot K_{\text{lipw},H_jL} \quad [\text{L·kg}^{-1}] \quad n = 2,3 \quad (2.14)
\]

\( f_i \) are the fractions of the species present at a given pH and \( K_{\text{lipw},H_jL} \) are the related, pH-independent liposome-water distribution coefficients of these species (Kraemer, 2001), defined by equation 2.10.

2.2.4 Liposome-water partitioning of 8-hydroxyquinolines-copper complexes

The partitioning of the 8-hydroxyquinoline-copper complexes is described by analogous equations. Due to limited solubility of the copper complexes, only total copper could be analytically determined. Therefore, the liposome-water distribution coefficients of the complexes, \( K_{\text{lipw},CuL} \) and \( K_{\text{lipw},CuL_2} \), have been obtained by measuring the liposome-water distribution ratio of copper \( D_{\text{lipw},Cu} \).

\[
D_{\text{lipw},Cu}(pH) = \frac{c_{\text{lip}}^{\text{CuL}} + c_{\text{lip}}^{\text{CuL_2}}}{c_{\text{lip}}^{\text{CuL}} + c_{\text{lip}}^{\text{CuL_2}}} = \frac{c_{\text{lip}}^{\text{CuL}}}{c_{\text{lip}}^{\text{CuL_2}}} \quad [\text{L·kg}^{-1}] \quad (2.15)
\]

which is equal to

\[
D_{\text{lipw},Cu}(pH) = f_{\text{CuL}} \cdot K_{\text{lipw},CuL} + f_{\text{CuL_2}} \cdot K_{\text{CuL_2}} \quad [\text{L·kg}^{-1}] \quad (2.16)
\]
2.3. Experimental section

2.3.1. Chemicals

The 8-hydroxyquinolines (names and abbreviations are given in Table 2.1) were purchased from Sigma-Aldrich (Buchs, Switzerland). All chemicals were of purities ≥ 97% and were used as received. 0.5 N KOH and 0.5 N HCl Titrisol® for the potentiometric titrations were from Merck (Dietikon, Switzerland). All pH-buffers were purchased from Fluka (Buchs, Switzerland) with purities > 99%: MES (2-morpholinoethanesulfonic acid, pKₐ = 6.15), MOPS (3-(N-morpholino)propanesulfonic acid, pKₐ = 7.2), CAPS (3-(cyclohexylamino)-propanesulfonic acid, pKₐ = 10.4) and citric acid (pKₐ1 = 3.13, pKₐ2 = 4.76; pKₐ3 = 6.40). Tetrabutylammonium-hydrogensulfate was purchased from Fluka with a purity of > 99%. Methanol used for HPLC was HPLC-grade from Fluka and octanol for octanol-water partitioning was purissimum grade from Fluka. Palladium matrix modifier (Pd(NO₃)₂) and magnesium matrix modifier (Mg(NO₃)₂) for graphite furnace AAS were purchased from Merck. Synthetic 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POGP) in chloroform was supplied by Avanti Polar Lipids (Alabaster, AL, USA).

2.3.2. HPLC method for the detection of 8-hydroxyquinolines

Concentrations of 8-hydroxyquinolines were measured with reversed phase HPLC with UV detection (Summit HPLC System; Dionex, Olten, Switzerland). As stationary phase Nucleodur C8 Gravity 5 μm (Macherey-Nagel, Oensingen, Switzerland) was used. This material was chosen because of its exhaustive endcapping of the residual free silanol groups, which results in a surface with very low silanol activity. This material allows an elution of polar compounds such as 8-hydroxyquinolines with small tailing.

For oxine, chloroxine, dichloroxine, and dibromoxine a mixture of methanol and 10 mM NaH₂PO₄ was used as mobile phase. It was also possible to quantify the copper complexes with the same method. The latter is however disadvantageous for the column because small amounts of adsorbing copper deteriorate the quality of the
stationary material and the column has to be replaced after around 150 injections. Sulfoxine was detected with ion pair chromatography. As a stationary phase Nucleodur C8 Gravity 5 μm was used, the mobile phase consisted of methanol, 10 mM NaH2PO4 and 8 mM tetrabutylammoniumhydrogensulfate. The detailed conditions of elution are summarized in Table 2.2.

Table 2.2. HPLC elution conditions for 8-hydroxyquinolines

<table>
<thead>
<tr>
<th>compound</th>
<th>stationary phase</th>
<th>mobile phase</th>
<th>detection wavelength [nm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>sulfoxine</td>
<td>Nucleodur C8 Gravity, 5 μm</td>
<td>Methanol/10 mM, NaH2PO4 + 8 mM N(C4H9)4(HSO4), pH 7, 30:70 (v/v)</td>
<td>242</td>
</tr>
<tr>
<td>oxine</td>
<td>Nucleodur C8 Gravity, 5 μm</td>
<td>Methanol/10 mM, NaH2PO4, pH 7, 50:50 (v/v)</td>
<td>241</td>
</tr>
<tr>
<td>chloroxine</td>
<td>Nucleodur C8 Gravity, 5 μm</td>
<td>Methanol/10 mM, NaH2PO4, pH 7, 70:30 (v/v)</td>
<td>245</td>
</tr>
<tr>
<td>dichloroxine</td>
<td>Nucleodur C8 Gravity, 5 μm</td>
<td>Methanol/10 mM, NaH2PO4, pH 6, 80:20 (v/v)</td>
<td>249</td>
</tr>
<tr>
<td>dibromoxine</td>
<td>Nucleodur C8 Gravity, 5 μm</td>
<td>Methanol/10 mM, NaH2PO4, pH 6, 80:20 (v/v)</td>
<td>249</td>
</tr>
</tbody>
</table>

2.3.3. AAS method for the determination of total copper

Total concentrations of copper were detected with a graphite-furnace atomic absorption spectrometer (5100 PC AAS equipped with a 5100-ZF Zeeman furnace module; Perkins-Elmer, Norwalk, CT, USA). The instrument parameters of the graphite-furnace AAS were: wavelength 324.8 nm, lamp current 20 A, slit width 0.7 nm, signal type Zeeman AA, read time 4 s.

To determine total copper, 40 μL of sample and 5 μL of matrix modifier were transferred to the graphite tube. The matrix modifier consisted of 0.1 g·L⁻¹ Mg(NO3)2 and 0.1 g·L⁻¹ Pd(NO3)2 in 0.1 M HNO3. The following temperature program was
applied: (a) two-step drying for 20 s at 110 °C and for 30 s at 130 °C, (b) charring with a 10 s ramp from 130 to 1000 °C and holding for 20 s, and (c) atomization for 4 s at 2100 °C and 4 s at 2400°C (during the first atomization step the argon flow was interrupted).

2.3.4. Potentiometric determination of acidity constants and octanol-water partitioning constants of 8-hydroxyquinolines

Determination of acidity constants $K_a$ and octanol-water partitioning constants $K_{ow}$ of 8-hydroxyquinolines was conducted as described in (Avdeef, 1992; Avdeef, 1993) using a PCA 101 automatic titrator (Sirius Analytical Instruments Ltd., Forest Row, East Sussex, UK).

Titrations were performed at 25 °C in 0.5 M KCl solution under argon atmosphere using 0.5 M HCl and KOH. Titrations were conducted from pH 1.8 to 12.2. The mixture was stirred for 90 s after each incremental addition of titrant. Titrations for the determination of $pK_a$ were done five times per substance, those for the determination of $K_{ow}$ with five different volume ratios of octanol and aqueous phase ($V_{oct}/V_{aq} = 0.05$ to 3) and in triplicates. The titration curves were analysed with PKaLOGP software V5.01a (Sirius Analytical Instruments Ltd., Forest Row, East Sussex, U.K.).

2.3.5. Stability constants of the copper-8-hydroxyquinoline complexes

The stability constants of the copper-8-hydroxyquinoline complexes for an ionic strength (I) of 0.1 M and a temperature (T) of 25 °C were taken from literature (Gupta et al., 1970; Martell & Smith, 2004) with the exception of the constants for the copper-chloroxine complexes (Table 2.1). They were determined by potentiometric titration with a copper ion selective electrode (Cu-ISE) according to (Mash et al., 2003) with the following modifications. The titrations was performed in darkness using an Orion 96-29 cupric ion-selective electrode and a 90-02 Ag/AgCl double-junction reference electrode connected with an Orion 720A pH/ISE meter. Experiments were conducted in 50 ml polypropylene beakers which were placed in 150 ml Metrohm titration vessels. The vessels were immersed in a water bath of 25 ±
0.5 °C to keep temperature in the beakers constant. Titrations were performed by manual addition of copper (CuSO₄) solution to a chloroxine solution and pH was measured each second titration step with an Orion 8103 pH electrode. Chloroxine concentration was 1·10⁻⁵ M and total copper ranged from 2.5·10⁻⁶ to 6.5·10⁻⁶ M.

2.3.6. Liposome preparation

For the preparation of liposomes synthetic 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POGP) was used. POGP dissolved in chloroform was dried down to a thin film inside a glass vessel in a rotary evaporator. Subsequently, residual traces of solvent were removed under high vacuum (0.01 Pa) within 12 h. The lipid film was rehydrated by adding aqueous buffer solution of desired pH and shaking the vessel so that the lipid bilayers detach and spontaneously form multilamellar vesicles (MLV). The resulting suspension of MLV was treated with ten freeze-thaw cycles in liquid nitrogen and a 40 °C water bath to complete rehydration and increase vesicle size. Afterwards, MLV were converted into unilamellar vesicles (UV) by membrane extrusion through a polycarbonate membrane of 0.1 μm (Nucleopore, Pleasanton, CA, USA). The MLV suspension was extruded 10 times in a thermobarrel extruder from Lipex Biomembranes, Vancouver, BC, Canada. The diameters of UV were determined with a ZetaSizer from Malvern Instruments, Malvern, Rochester, U.K (courtesy of B. Bommer, EMPA Dübendorf; Switzerland). The average diameter of the UV was 125 ± 35 nm.

These liposomes are of overall neutral charge. They are zwitterionic over the pH-range of all experiments (pH 2-11) because the pKₐ of the phosphate group in phosphatidylcholine is ≤ 1 (March, 1990). Bucher showed that membrane properties of PC-liposomes that play a role for partitioning are not influenced by pH in the pH range of 2-11, because the partitioning of the non-ionising substance progesterone did not vary in that pH range (Bucher, 2006). Additionally, the partitioning of organic acids and bases was also not influenced by pH in pH ranges where the substances are neutral (Escher et al., 2000b).

Furthermore, significant amounts of hydrolysis did not occur during the experiments. It was earlier shown by zeta-potential measurements that phosphatidylcholine (PC)
liposomes, which were incubated at 37 °C for 5 hours, have a zeta-potential of ~0 mV in the pH range from pH 2 to 10.5 and become a gradually negative zeta-potential ≥ pH 11 (Kraemer, 2001). Zeta-potentials of liposomes are in general ~0 mV at pH values where they are net neutral and those of PC liposomes become negative in a pH range where hydrolysis occurs (Kraemer, 2001). PC undergoes slow hydrolysis in aqueous solutions, which it is slowest at neutral pH and accelerates towards higher and lower pH values (Grit et al., 1993). Liposomes produced at pH 6 and 7 were therefore stored at 4 °C and used within three days. Liposomes produced at pH 2 and 11 were directly used to avoid significant amounts of hydrolysis during the liposome-water partitioning experiments.

The phospholipid content of the liposome suspension [lip] was determined by measuring total phosphorus and subtracting its orthophosphate content. The concentration of total phosphorus and orthophosphate was analysed by an ammonium molybdate spectrometric method according to DIN EN ISO 6878:2004 (International Standard Organisation, 2004).

2.3.7. Determination of liposome-water distribution ratios for 8-hydroxyquinoline ligands by equilibrium dialysis

Liposome-water distribution ratios $D_{lipw}$ for the 8-hydroxyquinolines were determined using equilibrium dialysis according to (Escher & Schwarzenbach, 1996).

The experiments were performed in home-built dialysis cells made of two glass chambers with a volume of 1 ml each. The half-cells were separated by a dialysis membrane made of regenerated cellulose with a cutoff of 10000-20000 Dalton (Thomapor, Reichelt Chemie Technik, Heidelberg, Germany).

A single experiment required two dialysis cells, a measurement cell and a reference cell. The first half-cell of each dialysis cell was filled with a solution of the examined 8-hydroxyquinoline in buffer, whereas the second one contained liposomes in buffer (measurement cell) or buffer (reference cell). At a given pH, distribution ratios of the 8-hydroxyquinolines were determined at four to five different concentrations in three replicates each at 25 °C in the dark. Kinetic experiments showed that the time to reach equilibrium between the two half cells was 12 hours. After separation of the
half cells, chemical analysis with HPLC of the content of all half-cells without liposomes allowed derivation of the liposome-water distribution ratio $D_{lw}$ (eq. 2.17)

$$D_{lw} = \frac{C_{ref} - C_w}{C_w \cdot [\text{lip}]} \quad [\text{L} \cdot \text{kg}^{-1}]$$

where $C_{ref}$ is the concentration of 8-hydroxyquinoline in the reference cell (mol·L$^{-1}$), $C_w$ is its concentration in the liposome-free compartment of the measurement cell (mol·L$^{-1}$), and $[\text{lip}]$ is the concentration of liposomes (kg·L$^{-1}$), i.e. the phase ratio.

Liposome-water distribution ratios $D_{lw}$ were determined at three different pH values in the range of pH 2 to 11 for each 8-hydroxyquinoline. The pH values were chosen so that either the cationic, neutral or anionic species was dominant in the aqueous solution. Details of the experiments are given in Table 2.3. Buffers were composed of 10 mM citric acid for pH 2 to 2.6, MES for pH 6 and 6.5, MOPS for pH 7 and 7.5, and CAPS for pH 11. Ionic strength was adjusted to a value of $I = 0.1$ M with NaNO$_3$. Total concentrations of the 8-hydroxyquinolines were in the range of $10^{-4}$ to $10^{-3}$ M. Liposome concentrations $[\text{lip}]$ were between $1.6 \cdot 10^{-3}$ and $2.5 \cdot 10^{-2}$ kg·L$^{-1}$. Volume ratios of water to liposomes ($V_w/V_{lip}$) in the different experiments were chosen dependent on the hydrophobicity of the examined 8-hydroxyquinoline so that a fraction of 50% of 8-hydroxyquinoline was taken up into liposomes (see equation 2.10, $n_{lip}^{B} \cdot n_{lip}^{W} = 1$ and $V_{lip}^{W} = \frac{n_{lip}^{B}}{n_{B} \cdot K_{lipw}^{B} \cdot \rho_{lip}^{op}}$).
Chapter 2: The evaluation of liposome-water partitioning of 8-hydroxyquinolines and their copper complexes

Table 2.3. Experimental details of the equilibrium dialysis experiments for the determination of liposome-water distribution ratios of 8-hydroxyquinolines ($D_{lipw,I}$)

<table>
<thead>
<tr>
<th>Compound</th>
<th>pH</th>
<th>$[lip]$ (kg·L$^{-1}$) $^a$</th>
<th>$f_w^{L^-}$</th>
<th>$f_w^{H^+L^+}$</th>
<th>$f_w^{HL^-}$</th>
<th>$D_{lipw,L}$ (L·kg$^{-1}$) $^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfoxine</td>
<td>3.2</td>
<td>3·10$^{-2}$</td>
<td>0.136</td>
<td>0.671</td>
<td>0.193</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>Oxine</td>
<td>2.2</td>
<td>2·10$^{-2}$</td>
<td>0</td>
<td>0.002</td>
<td>0.998</td>
<td>1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>7.2</td>
<td>2·10$^{-2}$</td>
<td>0.004</td>
<td>0.990</td>
<td>0.006</td>
<td>146 ± 1</td>
</tr>
<tr>
<td></td>
<td>11.1</td>
<td>3·10$^{-2}$</td>
<td>0.967</td>
<td>0.033</td>
<td>0</td>
<td>33 ± 1</td>
</tr>
<tr>
<td>Chloroxine</td>
<td>2.2</td>
<td>5·10$^{-3}$</td>
<td>0</td>
<td>0.004</td>
<td>0.996</td>
<td>41 ± 1</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>2·10$^{-3}$</td>
<td>0.001</td>
<td>0.988</td>
<td>0.011</td>
<td>1913 ± 12</td>
</tr>
<tr>
<td></td>
<td>11.1</td>
<td>2·10$^{-3}$</td>
<td>0.977</td>
<td>0.023</td>
<td>0</td>
<td>123 ± 1</td>
</tr>
<tr>
<td>Dichloroxine</td>
<td>2.0</td>
<td>1·10$^{-3}$</td>
<td>0</td>
<td>0.201</td>
<td>0.799</td>
<td>542 ± 6</td>
</tr>
<tr>
<td></td>
<td>2.7</td>
<td>1·10$^{-3}$</td>
<td>0</td>
<td>0.529</td>
<td>0.471</td>
<td>1247 ± 40</td>
</tr>
<tr>
<td></td>
<td>10.5</td>
<td>1·10$^{-3}$</td>
<td>0.999</td>
<td>0.001</td>
<td>0</td>
<td>298 ± 6</td>
</tr>
<tr>
<td>Dibromoxine</td>
<td>2.0</td>
<td>7·10$^{-4}$</td>
<td>0</td>
<td>0.101</td>
<td>0.899</td>
<td>1042 ± 4</td>
</tr>
<tr>
<td></td>
<td>2.6</td>
<td>6·10$^{-4}$</td>
<td>0</td>
<td>0.334</td>
<td>0.666</td>
<td>3008 ± 16</td>
</tr>
<tr>
<td></td>
<td>10.5</td>
<td>1·10$^{-3}$</td>
<td>0.999</td>
<td>0.001</td>
<td>0</td>
<td>1085 ± 27</td>
</tr>
</tbody>
</table>

$^a$ [lip]: concentration of liposomes, $^b$ $f_w^{L^-}$, $f_w^{H^+L^+}$, $f_w^{HL^-}$: fractions of the anionic, neutral and cationic species in water, $^c$ T = 25°C, I = 0.1 M

The experimental data from the equilibrium dialysis experiments consisted of a set of $D_{lipw}$ for three pH values as well as the information of the speciation of the 8-hydroxyquinolines under those conditions (Table 2.3). The speciation in the aqueous phase was calculated with VMinteq. For each pH value an equation of type 2.14 was set up using the associated $D_{lipw}$ and the fractions $f$ of the species present at the given pH. Subsequently, the $K_{lipw}$ values of the anionic, neutral and cationic species were calculated by solving the resulting linear system of three equations. For the relatively hydrophilic sulfoxine it was only possible to determine the $D_{lipw}$ at pH 3.2. At this pH the amount of sulfoxine that partitions into liposomes is at maximum extent but nevertheless very small ($D_{lipw} = 0.36 \pm 0.01$ L·kg$^{-1}$). At other pH values
partitioning into liposomes was so marginal that it was not feasible to assess $D_{\text{lipw}}$ values. Consequently, only one equation of type 2.14 could be set up for sulfoxine. Because it is not possible to solve an equation with three unknown parameters, the Excel Macro Solver® was used to estimated the approximate $K_{\text{lipw}}$ of the three species of sulfoxine and consequently no standard deviations are reported.

2.3.8. Determination of liposome-water distribution ratios for copper-ligand complexes by ultracentrifugation

Equilibrium dialysis was not suitable for the copper-8-hydroxyquinoline complexes, because copper strongly sorbed to the dialysis membrane. Liposome-water distribution ratios were determined in 13.5 ml ultracentrifugation tubes (Polyallomer, thick wall; Kontron Instruments, Milton Keynes, UK). A single experiment required two tubes, a measurement tube and a reference tube.

For the measurement tube, buffer, liposomes, copper, and 8-hydroxyquinoline solution were pipetted into the tube and equilibrated in darkness at 25 °C for 2 h for the copper complexes of oxine and chloroxine and for 24 h for the copper complexes of dichloroxine, dibromoxine and sulfoxine. Apart from the missing liposomes, the reference tube was composed and treated equally. Afterwards, the aqueous phase was separated from the liposomes by ultracentrifugation (Centrikon T-2000; Kontron Instruments, Milton Keynes, UK) at approximately 250000 g (45000 rpm) for 90 min and total copper was measured in the aqueous solution by AAS. From the total copper concentration the fractions of the different Cu-species were calculated using the substance parameters ($pK_a$, $\beta$) quoted in Table 2.1 and the speciation program VMinteq (Gustafsson, 2003).

Distribution ratios $D_{\text{lipw}}$ of the copper-8-hydroxyquinoline complexes were determined in 10 mM MOPS pH 7.5 and at least four different concentrations in three replicates each. Total concentrations of the complexes were in the range of $10^{-8}$ to $10^{-7}$ M. Working in higher concentration ranges was not possible because an almost complete separation of the complexes from the buffer by ultracentrifugation was observed although no precipitates were visible. It is assumed that this phenomenon occurred because of colloid formation. Liposome concentrations $[\text{lip}]$
were between \(1.6 \times 10^{-3}\) and \(2.5 \times 10^{-2}\) kg\(\text{L}^{-1}\). Volume ratios of water to liposomes \(\left(\frac{V^w}{V^\text{lip}}\right)\) in the different experiments were chosen dependent on the hydrophobicity of the examined 8-hydroxyquinoline so that a fraction of 50% of copper-8-hydroxyquinoline was taken up into liposomes (see equation 2.10, \(\frac{n^\text{lip}}{n^w} = 1\) and

\[
\frac{V^\text{lip}}{V^w} = \frac{n^\text{lip}}{n^w - K_{\text{lipw}} \cdot \rho^\text{lip}}.
\]

The \(D_{\text{lipw}}\) for the copper-8-hydroxyquinoline complexes were determined at different complex concentrations and pH values (Table 2.4) and calculated according equation 2.17. A variation of these parameters led to different speciation conditions. With the determined \(D_{\text{lipw}}\) and the information about the speciation, calculated with VMinteq, \(K_{\text{lipw}}\) of the complexes were calculated. For each concentration an equation of type 2.16 was set up using the associated \(D_{\text{lipw}}\) and the fractions of the species. Subsequently, the \(K_{\text{lipw}}\) values of the neutral and charged copper-8-hydroxyquinoline complexes were calculated by solving the resulting linear system of equations.

Ultracentrifugation was also used for determining the partitioning of the \(\text{Cu}^{2+}\) ion. Total concentrations of \(\text{Cu}^{2+}\) were varied in the range of \(10^{-8}\) to \(10^{-7}\) M and pH was buffered to pH 7.5 by 10 mM MOPS or to pH 2.5 by 10 mM citrate.

### 2.3.9. Comparability of equilibrium dialysis and ultracentrifugation experiments

With equilibrium dialysis and the ultracentrifugation method two different methods for the determination of the \(K_{\text{lipw}}\) of 8-hydroxyquinolines and of their copper complexes were used. In order to show that this was legitimate, both methods were compared for chloroxine at pH 6.0 and \(I = 0.1\) M. The results showed no significant difference between the two experimental approaches. It had also been earlier shown for eight alcohol ethoxylates that the results of both methods are statistically indistinguishable (Müller et al., 1999). Thus, it seemed appropriate to use equilibrium dialysis for the determination of the \(K_{\text{lipw}}\) of the 8-hydroxyquinolines. With this method the separation of the liposomes from the surrounding aqueous phase was less problematic than with the ultracentrifugation method.
The equilibrium dialysis experiments were performed at an ionic strength of 0.1 M, while in the ultracentrifugation experiments, the ionic strength had to be reduced to 0.01 M because the concentrations of the complexes had to be low due to the discussed formation of colloides. Thus, a further dilution of the samples for the analytical determination of total copper by AAS was not possible and an ionic strength higher than 0.1 M caused AAS background signals, which interfered with the copper peak. However, this experimental problem should not cause a problem because ionic strength dependency of the liposome-water partitioning even of charged species is negligible in the linear range of the sorption isotherm (Escher et al., 2000a). Only at very high concentrations when the isotherms go into saturation due to build-up of a negative surface potential, the shielding by the electrolyte starts to play a role. This was certainly not the case in the present experiments where concentrations of the neutral and positively charged complex well below $10^{-7}$ M were used.
**Table 2.4. Details of the ultracentrifugation experiments for the determination of liposome-water distribution ratios of copper-8-hydroxyquinoline complexes \((D_{lipw,c})\)**

<table>
<thead>
<tr>
<th>ligand</th>
<th>pH</th>
<th>([\text{lip}]^a) (kg\cdot L^{-1})</th>
<th>([\text{Cu}]_{tot}^a) (mol\cdot L^{-1})</th>
<th>([L]_{tot}^a) (mol\cdot L^{-1})</th>
<th>(f_{\text{CuL}^2}^w) b</th>
<th>(f_{\text{CuL}^+}^w) b</th>
<th>(f_{\text{Cu}^{2+}}^w) b</th>
<th>(D_{lipw,c}^c) (L\cdot kg^{-1}) c</th>
</tr>
</thead>
<tbody>
<tr>
<td>oxine</td>
<td>7.5</td>
<td>1\cdot 10^{-2}</td>
<td>4.9\cdot 10^{-6}</td>
<td>9.8\cdot 10^{-8}</td>
<td>0.985</td>
<td>0.155</td>
<td>0</td>
<td>38 ± 12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.2\cdot 10^{-6}</td>
<td>1.2\cdot 10^{-7}</td>
<td>8.71</td>
<td>0.129</td>
<td>0</td>
<td>39 ± 12</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.4\cdot 10^{-8}</td>
<td>1.5\cdot 10^{-7}</td>
<td>8.77</td>
<td>0.123</td>
<td>0</td>
<td>41 ± 12</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.6\cdot 10^{-8}</td>
<td>1.7\cdot 10^{-7}</td>
<td>0.880</td>
<td>0.120</td>
<td>0</td>
<td>41 ± 10</td>
<td></td>
</tr>
<tr>
<td>chloroxine</td>
<td>7.5</td>
<td>2\cdot 10^{-3}</td>
<td>4.9\cdot 10^{-6}</td>
<td>9.8\cdot 10^{-8}</td>
<td>0.998</td>
<td>0.002</td>
<td>0</td>
<td>320 ± 64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.1\cdot 10^{-6}</td>
<td>1.2\cdot 10^{-7}</td>
<td>0.999</td>
<td>0.001</td>
<td>0</td>
<td>312 ± 63</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.4\cdot 10^{-8}</td>
<td>1.5\cdot 10^{-7}</td>
<td>0.999</td>
<td>0.001</td>
<td>0</td>
<td>310 ± 75</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>2\cdot 10^{-3}</td>
<td>3.4\cdot 10^{-8}</td>
<td>6.4\cdot 10^{-8}</td>
<td>0.911</td>
<td>0.077</td>
<td>0.012</td>
<td>295 ± 89</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.5\cdot 10^{-8}</td>
<td>8.6\cdot 10^{-8}</td>
<td>0.911</td>
<td>0.077</td>
<td>0.012</td>
<td>288 ± 36</td>
<td></td>
</tr>
<tr>
<td>dichloroxine</td>
<td>7.5</td>
<td>1\cdot 10^{-2}</td>
<td>4.9\cdot 10^{-6}</td>
<td>9.8\cdot 10^{-8}</td>
<td>0.577</td>
<td>0.422</td>
<td>0.001</td>
<td>27 ± 8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.3\cdot 10^{-6}</td>
<td>1.1\cdot 10^{-7}</td>
<td>0.589</td>
<td>0.410</td>
<td>0.001</td>
<td>28 ± 5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.9\cdot 10^{-8}</td>
<td>1.2\cdot 10^{-7}</td>
<td>0.606</td>
<td>0.393</td>
<td>0.001</td>
<td>29 ± 10</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.6\cdot 10^{-8}</td>
<td>1.3\cdot 10^{-7}</td>
<td>0.622</td>
<td>0.377</td>
<td>0.001</td>
<td>31 ± 6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.6\cdot 10^{-8}</td>
<td>1.7\cdot 10^{-7}</td>
<td>0.659</td>
<td>0.340</td>
<td>0.001</td>
<td>29 ± 2</td>
<td></td>
</tr>
<tr>
<td>dibromoxine</td>
<td>7.5</td>
<td>1\cdot 10^{-2}</td>
<td>5.5\cdot 10^{-6}</td>
<td>1.1\cdot 10^{-7}</td>
<td>0.450</td>
<td>0.549</td>
<td>0.001</td>
<td>59 ± 11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.5\cdot 10^{-6}</td>
<td>1.3\cdot 10^{-7}</td>
<td>0.479</td>
<td>0.520</td>
<td>0.001</td>
<td>60 ± 15</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.5\cdot 10^{-8}</td>
<td>1.5\cdot 10^{-7}</td>
<td>0.502</td>
<td>0.497</td>
<td>0.001</td>
<td>62 ± 12</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.5\cdot 10^{-8}</td>
<td>1.7\cdot 10^{-7}</td>
<td>0.523</td>
<td>0.476</td>
<td>0.001</td>
<td>63 ± 6</td>
<td></td>
</tr>
</tbody>
</table>

\(a\) \([\text{lip}]\): concentration of liposomes, \([\text{Cu}]_{tot}\): total concentration of copper in the experiment, \([L]_{tot}\): total concentration of ligand in the experiment; \(b\) \(f_{\text{CuL}^2}^w\), \(f_{\text{CuL}^+}^w\), \(f_{\text{Cu}^{2+}}^w\): fractions of the neutral and charged copper-ligand complex and of Cu\(^{2+}\) in water; \(c\) \(T = 25^\circ C, I = 0.01 \text{ M}\)
Chapter 2: The evaluation of liposome-water partitioning of 8-hydroxyquinolines and their copper complexes

2.4. Results and discussion

2.4.1. pKa and log10 Kow of the examined 8-hydroxyquinolines.

Acidity constants of the 8-hydroxyquinolines, pKa, and the octanol-water partitioning coefficient of the neutral species, Kow,neutral ligand, were determined using potentiometric titration or were calculated with SPARC (Table 2.1). Table 2.1 also lists the stability constants of the two copper-ligand complexes, β1 and β2.

2.4.2. Partitioning of 8-hydroxyquinolines in the liposome-water system.

The Klipw of the 8-hydroxyquinolines are given in Table 2.5.

Table 2.5. Liposome-water partitioning coefficients of the neutral, cationic and anionic species of the examined 8-hydroxyquinolines and their standard deviations

<table>
<thead>
<tr>
<th>compound</th>
<th>log10 Klipw,H (L·kg⁻¹)</th>
<th>log10 Klipw,H⁻ (L·kg⁻¹)</th>
<th>log10 Klipw,L⁻ (L·kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sulfoxine</td>
<td>-0.28</td>
<td>-1.40</td>
<td>-0.28</td>
</tr>
<tr>
<td>oxine</td>
<td>2.17 ± 0.01</td>
<td>0.60 ± 0.02</td>
<td>1.47 ± 0.01</td>
</tr>
<tr>
<td>chloroxine</td>
<td>3.29 ± 0.01</td>
<td>1.51 ± 0.01</td>
<td>1.91 ± 0.01</td>
</tr>
<tr>
<td>dichloroxine</td>
<td>3.35 ± 0.02</td>
<td>2.04 ± 0.09</td>
<td>2.47 ± 0.01</td>
</tr>
<tr>
<td>dibromoxine</td>
<td>3.94 ± 0.01</td>
<td>2.28 ± 0.02</td>
<td>3.03 ± 0.01</td>
</tr>
</tbody>
</table>

a values estimated with the Excel Macro Solver®, b T = 25°C, I = 0.1 M

Figure 2.1 compares the Klipw of the neutral, anionic and cationic species of the 8-hydroxyquinolines with the octanol-water partitioning coefficients, Kow,neutral, of its neutral species. The Kow,neutral was used as a measure of hydrophobicity of the 8-hydroxyquinolines. Figure 2.1 shows generally higher Klipw for the neutral species than for the ionic ones. The uptake of the latter is decreased due to their charge (Smejtek & Wang, 1993; Escher et al., 2000a; Word & Smejtek, 2005). Nevertheless, the uptake of the charged species is only one to two magnitudes lower than that for
the neutral species. From the literature it is known that in octanol-water systems the partitioning of charged hydrophobic compounds is generally several orders of magnitude lower than that of their neutral species and strongly dependent on the ionic strength of the electrolyte. In contrary, in liposome-water systems the characteristics of the electrolyte and the ionic strength are of minor importance and partitioning not only depends on the hydrophobicity of the charged species but also on their specific interactions with the membrane. It is assumed that hydrophobic ions stay at the hydrophobic-hydrophilic interface in the phospholipids where they can undergo optimal van der Waals and hydrogen donor/acceptor interactions (Escher & Schwarzenbach, 1996; Escher et al., 2000a).

![Figure 2.1](image)

**Figure 2.1.** Relationship between the liposome-water partitioning coefficients ($K_{lipw}$) of the neutral (■), anionic (▲) and cationic (♦) species of the examined 8-hydroxyquinoline and the octanol-water partitioning coefficients of the neutral species ($K_{ow, neutral ligand}$). The error bars represent the standard deviations and the lines the linear regressions for the three species. The broken line indicates equal partitioning in the liposome-water and octanol-water system.
Furthermore, the $K_{lipw}$ of the anions are higher than that of the corresponding cations. This can be explained by differences in the free energy of transfer of the ions into the phospholipid membrane ($\Delta G^\circ$). $\Delta G^\circ$ is made up of several free energy terms regarding the change in the dielectric constant between water and membrane, the hydration of an ion, the polarization of water molecules adjacent to the aqueous solution-membrane interface, the interaction of the ion with the intrinsic, positive dipole potential of the membrane and all interactions, which a hypothetical neutral particle (generated by discharging the ion) would have with the aqueous phase and the membrane (Schamberger & Clarke, 2002). The differences in $\Delta G^\circ$ for anions and cations are predominantly caused by the Gibbs free energy of hydration of the ions $\Delta G_{hydr}$ and their influence on the dipole potential of the membrane (Clarke & Lupfert, 1999; Schamberger & Clarke, 2002).

$\Delta G_{hydr}$ increases for an ion with its decreasing stabilization in water and thus leads to a decrease of $\Delta G^\circ$, i.e. to an enhanced partitioning into the membrane (Schamberger & Clarke, 2002). 8-Hydroxy-quinoline anions ought to exhibit weaker hydration energies in comparison with the associated cations. Their negative charge is delocalized over the aromatic ring system while the positive charge of the cation is localized at the nitrogen atom. The delocalisation leads to a more uniform distribution of the charge over the molecule. Interactions with the surrounding water molecules are therefore weaker in comparison to those of the localized charge of the cations.

Furthermore, it was reported that both anions and cations cause a decrease in the dipole potential of the membrane which results in a decrease of $\Delta G^\circ$, i.e. to an enhanced partitioning into the membrane. However, the influence of the anions on the dipole potential is more pronounced (Clarke & Lupfert, 1999).

Both the higher $\Delta G_{hydr}$ and the greater decrease of the dipole potential of the membrane favour the partitioning of anions into phospholipid membranes.

It is also remarkable that the $K_{lipw}$ of all 8-hydroxyquinolines correlate with the $K_{ow, neutral}$ and that there exist good and nearly parallel regressions between $K_{ow, neutral}$ and $K_{lipw}$ for the three species (equations 2.18 to 2.20). The coefficients of determination $r^2$ of the regressions were tested with the F-test using a critical value for the significance threshold value $\alpha = 0.05$ of 9.55. Sulfoxine was omitted from this correlation because Gobas et al. (Gobas et al., 1988) showed that there is no linear
Chapter 2: The evaluation of liposome-water partitioning of 8-hydroxyquinolines and their copper complexes

Correlation between $\log_{10} K_{lipw,\text{neutral}}$ and $\log_{10} K_{ow,\text{neutral ligand}}$ for $K_{ow} < 1$ (i.e. $\log_{10} K_{ow} < 0$).

\[
\log_{10} K_{lipw,\text{neutral}} = 0.991 \pm 0.204 \cdot \log_{10} K_{ow,\text{neutral ligand}} + 0.517 \pm 0.563 \quad (2.18)
\]

$\rightarrow r^2 = 0.92$, $F = 24$, $n = 4$

\[
\log_{10} K_{lipw,\text{anionic}} = 0.937 \pm 0.106 \cdot \log_{10} K_{ow,\text{neutral ligand}} - 0.301 \pm 0.292 \quad (2.19)
\]

$\rightarrow r^2 = 0.97$, $F = 79$, $n = 4$

\[
\log_{10} K_{lipw,\text{cationic}} = 0.991 \pm 0.227 \cdot \log_{10} K_{ow,\text{neutral ligand}} - 1.061 \pm 0.626 \quad (2.20)
\]

$\rightarrow r^2 = 0.90$, $F = 19$, $n = 4$

The slope of the regression line for the neutral species (eq. 2.18) is similar to those for the linear regressions between $\log_{10} K_{lipw,\text{neutral}}$ and $\log_{10} K_{ow,\text{neutral ligand}}$ of 20 phenols (slope = 0.77; $r^2 = 0.92$) (Escher et al., 2000a) and of 21 halogenated and alkylated aromatic hydrocarbons with $\log_{10} K_{ow}$ between 1 and 5.5 (slope = 1.01; $r^2 = 0.96$) (Gobas et al., 1988). The three data sets show congruence as is evidenced by Figure 2.2. It was therefore deduced that the partitioning process of these hydrophobic aromatic compounds is governed by similar molecular interactions.

The regression lines for all 8-hydroxyquinoline species are parallel but the intercept of the anionic species is 0.8 log units smaller than that of the neutral species and the difference between cationic and anionic species is about 0.75 log units. Thus, independent of the hydrophobicity of the differently substituted oxines, there is a constant ratio of $K_{lipw}$ between neutral, anionic and cationic species.

As a result of these good correlations it should be possible for other compounds of this substance class to derive the $K_{lipw}$ of their ionic and neutral species from tabulated $K_{ow}$ of their neutral species.
Figure 2.2. Relationships between $\log_{10} K_{\text{lip},\text{neutral}}$ and $\log_{10} K_{\text{ow},\text{neutral ligand}}$ of 20 chloro- and nitrophenols (○) (Escher et al., 2000) 21 halogenated and alkylated aromatic hydrocarbons (Δ) (Gobas et al., 1988) and the five substituted 8-hydroxyquinolines of this study (▲). The broken line indicates equal partitioning in the liposome-water and octanol-water system.
2.4.3. Partitioning of copper-8-hydroxyquinoline complexes in the liposome-water system

Table 2.6 lists the $K_{lipw}$ of the copper-8-hydroxyquinoline complexes. Passive uptake for the copper-sulfoxine complexes was not detectable, because it is a rather hydrophilic complex. The Cu$^{2+}$ ion also showed no detectable partitioning into liposomes ($\log_{10} K_{lipw,Cu^{2+}} < -1 \text{ L}\cdot\text{kg}^{-1}$).

Table 2.6. Liposome-water partitioning coefficients of the neutral and charged copper-8-hydroxyquinoline complexes with standard deviations

<table>
<thead>
<tr>
<th>ligand</th>
<th>$\log_{10} K_{lipw, CuL_2}$ (L·kg$^{-1}$) $^a$</th>
<th>$\log_{10} K_{lipw, CuL^+}$ (L·kg$^{-1}$) $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>sulfoxine</td>
<td>$&lt;-1$</td>
<td>$&lt;-1$</td>
</tr>
<tr>
<td>oxine</td>
<td>$1.64 \pm 0.01$</td>
<td>$1.05 \pm 0.16$</td>
</tr>
<tr>
<td>chloroxine</td>
<td>$2.49 \pm 0.01$</td>
<td>$1.98 \pm 0.24$</td>
</tr>
<tr>
<td>dichloroxine</td>
<td>$1.61 \pm 0.09$</td>
<td>$1.03 \pm 0.39$</td>
</tr>
<tr>
<td>dibromoxine</td>
<td>$1.97 \pm 0.01$</td>
<td>$1.47 \pm 0.03$</td>
</tr>
</tbody>
</table>

$^a T = 25^\circ\text{C}, I = 0.01 \text{ M}$

Figure 2.3 plots the partitioning coefficients of the neutral (CuL$_2$) and charged copper-8-hydroxyquinoline complexes (CuL$^+$) against the $K_{ow}$ of the neutral species of their ligands. Again the $K_{lipw}$ of the cationic species are lower than those of the neutral species because of their charge. The $K_{lipw}$ of the copper-oxine and copper-chloroxine complexes show an increase of the $K_{lipw}$ of the complexes with increasing hydrophobicity of their ligands. However, the liposome-water partitioning coefficients of the neutral and charged copper complexes of dichloroxine and dibromoxine are smaller than those of copper-chloroxine despite of the higher $K_{ow}$ of their ligands. At the same time, a slight increase of the $K_{lipw}$ of the complexes with the increasing hydrophobicity of their ligands can be recognised within the group of copper-dichloroxine and copper-dibromoxine. The results therefore indicate that besides the
Chapter 2: The evaluation of liposome-water partitioning of 8-hydroxyquinolines and their copper complexes

Hydrophobicity of the ligand additional parameters influence the liposome-water partitioning of copper-8-hydroxyquinoline complexes. Possible reasons lie in the different properties of the ligands due to their different functional groups and hence in resulting properties of the complexes like molar volume and polarisation.

![Diagram showing relationship between liposome-water partitioning coefficients and Kow](image)

**Figure 2.3.** Relationship between the liposome-water partitioning coefficients of the neutral (■) and the charged (▲) copper-8-hydroxyquinoline complexes ($K_{lipw,c}$) and the Kow of the neutral species of their ligands ($K_{ow, neutral ligand}$). The broken line represents the 1:1 line and the error bars the standard deviations.

Effects of molar volume ($V_m$) on bioavailability and liposome-water partitioning have already been reported in literature (Opperhuizen et al., 1985; Gobas et al., 1988). Gobas et al. examined the relationship between $V_m$ and $\log_{10} K_{lipw}$ for several chlorinated and brominated benzenes, biphenyls and dioxins and observed a parabolic relationship (Opperhuizen et al., 1985; Gobas et al., 1988). While the $\log_{10} K_{lipw}$ increased linearly with increasing $V_m$ up to around 180 cm$^3$·mol$^{-1}$, it levelled off beyond and reached a maximum around 300 cm$^3$·mol$^{-1}$. At higher $V_m$ values $\log_{10}$
K\textsubscript{lipw} decreased again. In this study, the molar volumes of the 8-hydroxyquinolines could be estimated using COSMO (Eckert & Klamt, 2005) (courtesy of Ch. Niederer, ETH Zürich; Switzerland). Values of 104 cm\textsuperscript{3}\textper\textsuperscript{mol}\textsuperscript{-1} for oxine, of 117 cm\textsuperscript{3}\textper\textsuperscript{mol}\textsuperscript{-1} for chloroxine, of 130 cm\textsuperscript{3}\textper\textsuperscript{mol}\textsuperscript{-1} for dichloroxine, and of 137 cm\textsuperscript{3}\textper\textsuperscript{mol}\textsuperscript{-1} for dibromoxine were obtained. An estimation of the molar volumes of the copper-8-hydroxyquinoline complexes was not possible with COSMO, but a rough estimation was achieved by assuming that the molar volumes of the complexes are around the volume of the ligand for CuL\textsuperscript{+} and around two times the ligand's volume for CuL\textsubscript{2}. A comparison of the results of Gobas et al. with the estimated V\textsubscript{m} of the CuL\textsubscript{2} complexes shows that their relatively high molar volumes have a hindering effect on their liposome-water partitioning and that this effect increases in the order copper-oxine < copper-chloroxine < copper-dichloroxine < copper-dibromoxine. The molar volumes of the CuL\textsuperscript{+} complexes are smaller and lie below the limit, above which an effect of V\textsubscript{m} on K\textsubscript{lipw} is expected. However, the CuL\textsuperscript{+} complexes of dichloroxine and dibromoxine show a hindrance of their partitioning into liposomes in comparison to those of oxine and chloroxine. Therefore, further factors, which influence the liposome-water partitioning of copper-8-hydroxyquinoline complexes, have to exist.

The polarisation of the complexes is expected to be an important one. In an 8-hydroxyquinoline ligand, electronegative substituents like chlorine (Cl) or bromine (Br) cause on the one hand negative inductive effects (-I effects) and on the other hand positive mesomeric effects (+M effects), i.e. I and M effects work in different directions. In this context, the influence of the -I effect of Cl or Br on other atoms of the 8-hydroxyquinolines is dependent on their relative position towards each other. The -I effect of a Cl or Br in position 5 on the atoms, which participate in the copper complexation (O in position 8 and N in position 1; see Figure 2.4), is small because of the relative long distance between the atoms. This becomes obvious when comparing the acidity constants of the OH-groups (pK\textsubscript{a}(OH); see Table 1.1) of oxine and chloroxine. The OH-group of chloroxine is only slightly more acidic than that of oxine. In contrast, a Cl or Br atom in position 7 causes a stronger -I effect on the O atom in position 8. The resulting polarisation of a Br in position 7 on the O atom in position 8 is not as high as that of a Cl in position 7 because Br (electronegativity according Pauling scale: 2.96; (Pauling, 1932)) is a little less electronegative than Cl.
(electronegativity according Pauling scale: 3.16). This can be likewise shown by a comparison of the pK_a(OH) of chloroxine, dichloroxine and dibromoxine. The OH-groups of the latter ones are much more acidic than that of chloroxine and the OH-group of dibromoxine is slightly less acidic than that of dichloroxine. A polarisation of an 8-hydroxyquinoline ligand caused by a Cl or Br in position 7 renders its copper complexes less stable and makes it more difficult for them to partition into the membrane just like it applies for ions.

A comparison of the K_{lipw} of the copper-8-hydroxyquinoline complexes with those of their ligands reveals further aspects with support the hypothesis that molar volume and polarisation are crucial factors for the partitioning of copper-8-hydroxyquinoline complexes into liposomes.

The results show that the K_{lipw} of the overall neutral CuL_2 complexes (K_{lipw, CuL_2}) of oxine and chloroxine are around one order of magnitude smaller than the K_{lipw} of the corresponding neutral ligand species HL (K_{lipw, HL}), whereas K_{lipw, CuL_2} is around two orders of magnitude smaller than K_{lipw, HL} for dichloroxine and dibromoxine. The differences between K_{lipw, CuL_2} and K_{lipw, HL} can be in general explained by the influence of molar volume, polarisation and possibly also by steric factors. The higher difference for dichloroxine and dibromoxine is most probably due to the higher impact of the first to factors on K_{lipw, CuL_2}.

A comparison of the K_{lipw} of the positive CuL^+ complexes (K_{lipw, CuL^+}) with the K_{lipw} of the corresponding positive ligand species H_2L^+ (K_{lipw, H_2L^+}) shows around 0.5 orders of
magnitude higher $K_{\text{lipw, Cu}^+}$ for oxine and chloroxine and around one order of magnitude smaller $K_{\text{lipw, Cu}^+}$ for dichloroxine and dibromoxine. These differences can be explained by the polarisation of the molecules. For oxine and chloroxine the positive charge of $\text{H}_2\text{L}^+$ is localised, whereas the charges of $\text{O}^-$ and $\text{Cu}^{2+}$ of $\text{CuL}^+$ are distributed over the complex binding. This simplifies the partitioning of $\text{CuL}^+$ into liposomes just like the delocalisation of its charge simplified the liposome-water partitioning of the negative ligand species $\text{L}^-$ in comparison to $\text{H}_2\text{L}^+$. For dichloroxine and dibromoxine $K_{\text{lipw, Cu}^+}$ is smaller than $K_{\text{lipw, H}_2\text{L}^+}$ because for $\text{CuL}^+$ the -1 effect of Cl or Br in position 7 of the ligand seems to negatively affect the distribution of the charges of $\text{O}^-$ and $\text{Cu}^{2+}$ over the complex binding.

In consequence, it can be concluded that the partitioning of copper-8-hydroxyquinoline complexes into liposomes is dependent on their hydrophobicity, molar volume, and polarisation, which are in turn influenced by the functional groups of their 8-hydroxyquinoline ligands.

### 2.5. Environmental significance

Copper is normally taken up into cells by carrier-mediated transport. The relatively high $K_{\text{lipw}}$ of the hydrophobic copper organic complexes, which were considered in this study, indicate the possibility of passive uptake of the complexes and consequently of copper.

So far, it is not clear whether the relatively high accumulation in the membrane, up to a factor of 300, leads to membrane toxicity of the complex itself or whether the additional uptake process simply enhances the internal copper concentration by recomplexation with internal ligands. To examine this in detail more work on in vivo bioavailability, bioconcentration and toxicity is necessary.

However, this study showed that it is important not only to regard hydrophobic organic ligands as modulators of the free copper concentration in the medium but also to consider hydrophobic copper-ligand complexes as potentially bioavailable species and to account for effects caused by them.
3.

Bioavailability and uptake of hydrophobic copper-organic complexes in algae

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Sibylle M. Kaiser, Renata Behra, Beate I. Escher, Laura Sigg
Bioavailability and uptake of hydrophobic copper-organic complexes in algae.
Chapter 3: Bioavailability and uptake of hydrophobic copper-organic complexes in algae

3.1. Introduction

Copper is an important micronutrient for algae but acts as a toxic metal at higher concentrations. The chemical speciation plays a major role for its bioavailability to algae, the copper uptake into cells and subsequently for its potential toxicity. The uptake of free copper ions results from carrier-mediated transport processes (Fraústo da Silva & Williams, 2001) and can be described by the Free-Ion Activity Model (FIAM) (Campbell, 1995) and the more recent Biotic Ligand Model (BLM) (Campbell et al., 2002; Slaveykova & Wilkinson, 2005). These models assume copper uptake via specific transport sites and predict bioavailability of copper in dependency upon the free copper ion concentration or the amount of metal associated with binding sites on the surface of the organism ("biotic ligands"), respectively.

Exceptions to these models were observed in the presence of hydrophobic ionogenic organic compounds like diethyldithiocarbamate, xanthates and 8-hydroxyquinoline, which can form stable complexes with metals (Florence et al., 1983; Ahsanullah & Florence, 1984; Florence & Stauber, 1986; Campbell, 1995). These complexes may passively diffuse in or across the membrane because of their hydrophobicity and thus open a second uptake route for copper into the cell. Phinney & Bruland proposed a mechanism for the diatom Thalassiosira weissflogii and the hydrophobic copper-8-hydroxyquinoline complex that assumes partitioning of the complex into the cell membrane and its diffusion into the cytoplasm, where ligand exchange may take place with copper bound to intracellular binding sites (Phinney & Bruland, 1994). On the other hand, hydrophobic copper-organic complexes may also intercalate in biological membranes as is known for hydrophobic compounds (Gobas et al., 1988; Escher et al., 2000a).

The objective of this study was (i) to examine the uptake of selected hydrophobic copper-organic complexes into the freshwater algae Scenedesmus vacuolatus, (ii) to identify physicochemical parameters that influence their bioavailability and (iii) to gain mechanistic information about the fate of these complexes after their partitioning into the cell membrane. The uptake data in algae was also compared with partitioning into liposomes (see chapter 2), which are artificial phospholipid bilayer vesicles of known composition and size. They mimic the anisotropic properties and ordered structure of biological membranes (Stein, 1986) and are increasingly applied in
environmental sciences (Gobas et al., 1988; Escher et al., 2000a; Escher & Sigg, 2004) to evaluate the partitioning behaviour of organic pollutants between water and biological membranes.

For the present study ligands of the group of 8-hydroxyquinolines were used, among them the unsubstituted 8-hydroxyquinoline (oxine) and various chlorinated and brominated 8-hydroxyquinolines. 8-Hydroxyquinolines are used for the extraction of metals in analytical chemistry (Bruland et al., 1979) and as constituents in antiseptic and disinfectant formulations, in preservatives and in agricultural fungicides (Hollingshead, 1954; Hollingshead, 1954; Block, 1991). They are also able to form stable and hydrophobic copper-ligand complexes of the type CuL₂ and CuL⁺ (see Figure 1.4). These copper complexes show antimicrobial and antifungal properties (Hollingshead, 1954) and copper-oxine is widely used as fungicide (Yeager, 1991; Kikuchi et al., 1996; Nicoletti et al., 1999; Schultz et al., 2005). Passive uptake of copper-oxine in cells has already been shown for the diatom Thalassiosira weissflogii and for several other marine phytoplankton species (Phinney & Bruland, 1994; Phinney & Bruland, 1997; Croot et al., 1999). Additionally to the above mentioned hydrophobic 8-hydroxyquinolines, the more hydrophilic 8-hydroxyquinoline-5-sulfonic acid (sulfoxine) was also investigated.
3.2. Experimental Section

3.2.1. Chemicals

The 8-hydroxyquinolines (names and abbreviations are given in Table 1.1 and chemical structures in Figure 1.3) were purchased from Sigma-Aldrich (Buchs, Switzerland). They were of purities ≥ 97 %. Copper solutions were prepared with copper sulfate pentahydrate from Fluka (puriss. grade, ≥ 99 %; Buchs, Switzerland). Ethylenediaminetetraacetic acid disodium salt dihydrate (Na₂EDTA) and 3-(N-morpholino)propanesulfonic acid (MOPS) were obtained from Fluka (purities ≥ 99 %). Nitric acid (HNO₃; 65 %), hydrochloric acid (HCl; 30 %) and hydrogen peroxide (H₂O₂; 30 %) were suprapure® chemicals from Merck (Darmstadt, Germany). CaCl₂·2H₂O, MgSO₄·7H₂O, NaHCO₃, K₂HPO₄·3H₂O, NaNO₃, CoCl₂·6H₂O, H₃BO₃, Na₂MoO₄·2H₂O, MnCl₂·4H₂O, ZnSO₄·7H₂O, FeCl₃·6H₂O and NaOH used for medium preparation were from Fluka and of purities ≥ 99 %. All solutions were prepared with deionised water (18 MΩ·cm⁻¹, Barnstead Nanopure; Allschwil, Switzerland).

3.2.2. Preparation of culture and experimental medium

Culture medium was prepared by adding solutions of MOPS pH 7.5 (final concentration 10⁻² M), CaCl₂ (5·10⁻⁴ M), MgSO₄ (1.5·10⁻⁴ M), K₂HPO₄·(5·10⁻⁵ M), NaNO₃ (10⁻³ M), NaHCO₃ (1.2·10⁻³ M) and an EDTA-trace metals solution to deionised water. The sterile-filtered solutions (0.22 µm pore size) of NaHCO₃ and the trace metals were added after autoclaving of the rest of the medium components. Finally, the culture medium was pre-equilibrated for 24 h to achieve chemical equilibrium before utilization.

The trace metal solution was composed of 5·10⁻⁸ M CoCl₂, 5·10⁻⁵ M H₃BO₃, 8·10⁻⁸ M Na₂MoO₄, 10⁻⁶ M MnCl₂, 10⁻⁷ M ZnSO₄, 9·10⁻⁷ M FeCl₃ and 1.25·10⁻⁷ M CuSO₄ and prepared by addition of sterile-filtered stock solutions of each metal in 0.01 M HCl to a Na₂EDTA solution (end concentration 2·10⁻⁵ M) whereas the Fe-solution was added first to ensure total complexation.
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The experimental medium was similar to the culture medium. Solely the trace metal solution (trace metals and EDTA) was omitted to avoid competition between the trace metals and copper as well as EDTA with the ligands used in the experiments.

3.2.3. Test organism and culture conditions

As test organism the unicellular green alga *Scenedesmus vacuolatus* 211-8b was used. Algae cultures were obtained from the algal collection of the Institute for Plant Physiology of the University of Goettingen, Germany. The algae were grown in glass Erlenmeyer flasks in a HT Infors shaker (Infors, Bottimingen, Switzerland) at 25°C, 100 rpm and with continuous illumination of 267 µE·m⁻²·s⁻¹ provided by cool white lamps. New batch cultures with an initial cell density of approximately 5·10⁵ cells·ml⁻¹ were prepared by transferring an inoculum of algae in exponential growth phase (after 72 h) into fresh culture medium. Algae were acclimatized to the culture medium by successive batch culturing until growth rates of successive cultures were constant.

3.2.4. Equipment

All used glass and polypropylene vessels were presoaked in 0.1 M HNO₃ for at least 24 h and rinsed with deionised water before utilization. Cellulose nitrate filters for algae filtration (0.45 µm; Sartorius, Goettingen, Germany) were acid-washed with 0.1 M HNO₃ for 12 hours, rinsed with deionised water and dried at 50 °C. Background content of copper after this treatment was determined to be (3.12 ± 0.02)·10⁻¹⁰ mol-filter⁻¹. Prior to experiments with a certain copper-8-hydroxyquinoline complex, filters were saturated with this complex to avoid further adsorption of the complex to the filter during the experiment and dried at 50°C. Copper contents of the presaturated filters were (3.69 ± 0.20)·10⁻¹⁰ mol-filter⁻¹ for Cu-sulfoxine, (6.02 ± 0.06)·10⁻¹⁰ mol-filter⁻¹ for Cu-oxine, (12.19 ± 0.09)·10⁻¹⁰ mol-filter⁻¹ for Cu-chloroxine, (4.17 ± 0.08)·10⁻¹⁰ mol-filter⁻¹ for Cu-dichloroxine and (11.16 ± 0.25)·10⁻¹⁰ mol-filter⁻¹ for Cu-dibromoxine. A presaturation was not necessary for filters used in Cu-EDTA experiments because no significant adsorption of copper to the filters was observed in these experiments.
3.2.5. Analytical methods for the detection of total copper and speciation calculations

Total concentrations of dissolved copper were measured by inductively coupled plasma mass spectrometry (ICP-MS; Perkin-Elmer Elan 5000; Perkin-Elmer GmbH, Ueberlingen, Germany). The accuracy of the measurements was checked using SLRS-4 reference water (River Water Reference Material for Trace Metals; National Research Council Canada, Ottawa, Canada).

Based on the total copper concentration, the copper speciation present in the experiments was calculated using the computer program VMinteq (Gustafsson, 2003).

3.2.6. Experimental procedures

Uptake experiments for copper-8-hydroxyquinoline complexes in *Scenedesmus vacuolatus* were conducted in experimental medium at pH ~ 7.5 over 30 min in four replicates.

Algae were separated from the culture medium after 72 h of growth by centrifugation (Megafuge 1.0 R; Heraeus Instruments GmbH, Hanau, Germany) at 2500 g for 10 min and washed three times with trace metal free experimental medium to remove the residual culture medium and to minimize the internal and adsorbed copper concentration of the algae. After that treatment the background concentration of internal copper ranged between 2.7 and 5.9·10^{-18} mol-cell^{-1} internal copper and adsorbed copper between 0.4 and 1.1·10^{-18} mol-cell^{-1}.

For the uptake experiments copper and 8-hydroxyquinoline were applied in a ratio of 1:2 resulting in a nearly complete complexation (~ 100 %) of copper in copper-8-hydroxyquinoline complexes (for details see Table 3.1). The experimental medium was spiked with the complexes 12 h prior to the experiment to allow equilibration. The speciation, particularly the concentration of free copper ions, was calculated using VMinteq.
Chapter 3: Bioavailability and uptake of hydrophobic copper-organic complexes in algae

### Table 3.1. Experimental conditions for the copper-8-hydroxyquinoline uptake experiments

<table>
<thead>
<tr>
<th>ligand</th>
<th>$c_{Cu,tot}^W$ [mol·L$^{-1}$]</th>
<th>$c_{L,tot}^W$ [mol·L$^{-1}$]</th>
<th>pH</th>
<th>$c_{Cu^{2+}}^W$ [mol·L$^{-1}$]</th>
<th>$c_{complex}^W$ [mol·L$^{-1}$]</th>
<th>$f_{CuL}$</th>
<th>$f_{CuL_2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>sulfoxine</td>
<td>$(7.84 \pm 0.15) \cdot 10^{-8}$</td>
<td>$1.36 \cdot 10^{-7}$</td>
<td>7.50</td>
<td>$(2.78 \pm 0.72) \cdot 10^{-11}$</td>
<td>$(7.82 \pm 0.14) \cdot 10^{-6}$</td>
<td>0.29</td>
<td>0.71</td>
</tr>
<tr>
<td>oxine</td>
<td>$(7.05 \pm 0.06) \cdot 10^{-8}$</td>
<td>$1.37 \cdot 10^{-7}$</td>
<td>7.58</td>
<td>$(9.72 \pm 1.16) \cdot 10^{-11}$</td>
<td>$(6.96 \pm 0.05) \cdot 10^{-6}$</td>
<td>0.14</td>
<td>0.86</td>
</tr>
<tr>
<td>chloroxine</td>
<td>$(7.45 \pm 0.10) \cdot 10^{-8}$</td>
<td>$1.37 \cdot 10^{-7}$</td>
<td>7.50</td>
<td>$(1.55 \pm 0.30) \cdot 10^{-13}$</td>
<td>$(7.44 \pm 0.10) \cdot 10^{-6}$</td>
<td>0.24</td>
<td>0.76</td>
</tr>
<tr>
<td>dichloroxine</td>
<td>$(7.66 \pm 0.24) \cdot 10^{-9}$</td>
<td>$1.37 \cdot 10^{-7}$</td>
<td>7.56</td>
<td>$(4.27 \pm 0.45) \cdot 10^{-11}$</td>
<td>$(7.62 \pm 0.23) \cdot 10^{-6}$</td>
<td>0.73</td>
<td>0.27</td>
</tr>
<tr>
<td>dibromoxine</td>
<td>$(6.05 \pm 0.12) \cdot 10^{-9}$</td>
<td>$1.21 \cdot 10^{-7}$</td>
<td>7.48</td>
<td>$(3.67 \pm 0.27) \cdot 10^{-11}$</td>
<td>$(6.03 \pm 0.12) \cdot 10^{-6}$</td>
<td>0.55</td>
<td>0.45</td>
</tr>
</tbody>
</table>

* $T = 25 \, ^\circ C$, $I = 0.01 \, M$.  

$^a$ $c_{Cu,tot}^W$: total copper concentration; $c_{L,tot}^W$: total ligand concentration; $c_{Cu^{2+}}^W$: free copper ion concentration; $c_{complex}^W$: concentration of copper complexed by the used ligand.  

$^b$ $f_{CuL}$, $f_{CuL_2}$: fractions of the CuL and CuL$_2$ complexes in $c_{complex}^W$.  

Algae were added to the equilibrated medium to start the uptake experiment. The cell density of algae in the experiment was around $3 \cdot 10^6$ cells·ml$^{-1}$ and measured with a Z2 Coulter Particle Count and Size Analyzer (Beckman Coulter, Krefeld, Germany). A high cell density was chosen to provide sufficient algae material for copper analysis.  

For each time step two 15 ml aliquots of algae culture were filtered on cellulose nitrate filters, which were presaturated with the appropriate copper-8-hydroxyquinoline complexes. Filtrates were sampled to determine the copper concentration in the medium and total copper concentrations were measured with ICP-MS. Filters were dried at 50 °C and treated by acidic digestion. Therefore, they were placed in Teflon digestion flasks, 3 ml of 65 % HNO$_3$ and 1 ml 30 % H$_2$O$_2$ were added, and digestion was performed in a high-performance microwave (mls 1200 mega; Microwave Laboratory systems, Oberwil, Switzerland) for 25 min. After
cooling, the solutions were transferred to 25 ml graduated polypropylene flasks and filled up with deionised water. The recovery of the acidic digestion was checked with phytoplankton reference material (CRM 414; Community Bureau of Reference, Commission of the European Communities, Brussels, Belgium).

For the determination of total copper, samples were diluted with 0.1 M HNO₃ and total copper concentrations were measured with ICP-MS. Data were corrected for the copper content of the presaturated cellulose nitrate filters. The cellular copper content was calculated as mol copper per alga cell [mol-cell⁻¹] and mol copper per litre cell volume [mol-L_cv⁻¹] by dividing the former through by the mean cell volume of *Scenedesmus vacuolatus* cells (25-10⁻¹⁵ L_cv·cell⁻¹).

Intracellular copper was defined as copper in the cytoplasm and copper, which was intercalated in the membrane. To differentiate between intracellular uptake and extracellular adsorption of copper, a final concentration of 10 mM Na₂EDTA, pH 7.5 was added to one of the two aliquots of algae prior to filtration for 10 min. Preliminary experiments showed that these conditions were sufficient to detach all desorbable copper and that a longer reaction time or EDTA concentration > 10 mM did not increase the desorption of copper. Total copper uptake was defined as copper content without EDTA wash minus background concentration in the algae, the intracellular copper concentration as copper content after EDTA wash minus background concentration and the adsorbed copper concentration was calculated as difference between total and intracellular copper.

### 3.2.7. Copper-8-hydroxyquinoline uptake kinetics

Under the experimental conditions copper was almost completely complexed by the added 8-hydroxyquinoline ligand (see Table 3.1). The trace concentrations of residual free copper (Cu²⁺) in the different copper-8-hydroxyquinoline complex experiments were between 10⁻¹³ and 10⁻¹⁰ M depending on the complex stability constants of the two copper-8-hydroxyquinoline complexes, CuL(2⁻) and CuL₂(2⁻²⁻), and on the exact pH value (Table 3.1).

Therefore, the experimentally determined uptake rates for intracellular and total copper described the cumulative copper uptake via carrier-mediated transport of free copper ions (Cu²⁺) and via passive diffusion of copper-8-hydroxyquinoline
complexes. Uptake kinetics of copper taken up via copper-8-hydroxyquinoline complexes were obtained by subtraction of the copper fraction that was taken up via Cu$^{2+}$. The fraction of copper, which was taken up via Cu$^{2+}$ in the copper-8-hydroxyquinoline complexes experiments was determined in separate experiments with appropriate Cu$^{2+}$ concentrations adjusted by EDTA (for details see the appendix to chapter 3).
3.3. Theoretical Considerations

3.3.1. Concentration and speciation in the experimental medium

8-Hydroxyquinolines dissociate in the aqueous phase, i.e. in the experimental medium, according to the reactions

\[ K_{a,i} \quad H_iL^{(i-1)} \leftrightarrow H^+ + H_{(i-1)}L^{(i-2)} \quad i = 1...n \]  \hspace{1cm} (3.1)

\( H_iL^{(i-1)} \) represents the acidic form, \( H_{(i-1)}L^{(i-2)} \) the associated basic form and \( H^+ \) the aqueous proton. \( n \) stands for the maximum number of protonated species of the 8-hydroxyquinolines, which is \( n=2 \) for oxine, chloroxine, dichloroxine and dibromoxine and \( n=3 \) for sulfoxine. \( K_{a,i} \) is the equilibrium constant for the mass law expression.

Copper existed in the aqueous phase under the given experimental conditions as free ion \( Cu^{2+} \), bound in hydrophilic complexes with medium components like carbonate and phosphate or bound in hydrophobic complexes with the fully deprotonated species of the 8-hydroxyquinolines (ligands) \( L^j \), \( CuL^{(2-j)} \) and \( CuL_2^{(2-2j)} \). The latter complexes were formed according to the reactions

\[ Cu^{2+} + L^{-j} \leftrightarrow CuL^{(2-j)} \quad \beta_1 \] \hspace{1cm} (3.2)

\[ Cu^{2+} + 2 \cdot L^{-j} \leftrightarrow CuL_2^{(2-2j)} \quad \beta_2 \] \hspace{1cm} (3.3)

where \( \beta_1 \) and \( \beta_2 \) are the stability constants of the copper complexes \( CuL^{(2-j)} \) and \( CuL_2^{(2-2j)} \) (see Table 1.1).

It is known that hydrophilic metal complexes, unlike hydrophobic ones, are usually not bioavailable (Florence et al., 1983). Thus, the total concentration of bioavailable copper \( c_{Cu,\text{bioav}}^w \) in the present experiments was defined by

\[ c_{Cu,\text{bioav}}^w = c_{Cu^{2+}}^w + c_{CuL}^w + c_{CuL_2}^w \] \hspace{1cm} (3.4)
where \( c_{Cu2^+} \) is the concentration of the free copper ion Cu\(^{2+}\) and \( c_{CuL} \) and \( c_{CuL_2} \) are the concentrations of the two copper organic complexes \( CuL^{(2i)} \) and \( CuL_2^{(2-2i)} \).

### 3.3.2. Liposome-water distribution ratios

Liposomes are widely used to mimic biological membranes because they are also characterized by an ordered structure and anisotropic properties (Stein, 1986). In liposome-water partitioning experiments with copper-8-hydroxyquinoline complexes the complex species \( CuL^{(2i)} \) and \( CuL_2^{(2-2i)} \) partition between aqueous phase and liposomes. For a given ionic strength, the pH-dependent liposome-water distribution ratio \( D_{lipw} \) in units of litre water (L\(_w\)) per litre phospholipid (L\(_{PL}\)) for both species \( (i) \) is described by

\[
D_{lipw}(pH) = \frac{\sum_{i=0}^{2} (c_{S_i}^{lip} \cdot \rho_{lip})}{\sum_{i=0}^{2} c_S^{w}} [L_w \cdot L_{PL}^{-1}]
\]

where \( c_S^{w} \) is the concentration of the species \( i \) in the aqueous phase [mol\( \cdot \)L\(_w\)\(^{-1}\)], \( c_S^{lip} \) the concentration of \( i \) in the liposome phase in mole per kg of phospholipid in the membrane [mol\( \cdot \)kg\(_{PL}\)\(^{-1}\)] and \( \rho_{lip} \) the density of the phospholipids (1.015 kg\(_{PL}\)\( \cdot \)L\(_{PL}\)\(^{-1}\)).

The \( D_{lipw} \) can also be expressed as

\[
D_{lipw}(pH) = \sum_{i=0}^{2} f_i \cdot K_{lipw,i} [L_w \cdot L_{PL}^{-1}]
\]

where \( f_i \) are the fractions of the species \( i \) present at a given pH and \( K_{lipw,i} \) are the related, pH-independent liposome-water partitioning coefficients of these species.

The \( K_{lipw,i} \) for the calculation of the \( D_{lipw} \) of the copper-8-hydroxyquinoline complexes under the present experimental conditions (\( D_{lipw,complex} \)) were taken from chapter 2, the fractions of the species \( i \) are given in Table 3.1.
3.3.3. Bioconcentration factors and uptake rates

The bioconcentration factor of copper for algae (BCF; in units of litre water per litre cell volume [L\(_w\)·L\(_{cv}^{-1}\)]) is in general defined as the ratio of the internal copper concentration of the algae (\(c_{Cu,int}^a\); in units of mol copper per litre cell volume [mol·L\(_{cv}^{-1}\)]) and the bioavailable copper concentration in the surrounding aqueous phase (\(c_{Cu,bioav}^w\); in units of mol copper per litre water [mol·L\(_w^{-1}\)]) under steady state conditions. It is dependent on the speciation of copper. The BCF can be calculated by measuring uptake kinetics of copper and determining the uptake (\(k_1\), [min\(^{-1}\)]) and elimination (\(k_2\), [min\(^{-1}\)]) rate constants under the given experimental conditions. It is defined as the quotient of \(k_1\) and \(k_2\) (equation 3.7):

\[
BCF = \frac{c_{Cu,int}^a}{c_{Cu,bioav}^w} = \frac{k_1}{k_2} \quad [L\_w \cdot L\_{cv}^{-1}] 
\]

(3.7)

Both rate constants can be obtained by using the following one-compartment bioconcentration model with first order kinetics (equation 3.8) and fitting the measured uptake kinetics with it.

\[
\frac{\Delta c_{Cu,int}^a}{\Delta t} = k_1 \cdot c_{Cu,bioav}^w(t) - k_2 \cdot c_{Cu,int}^a(t) 
\]

(3.8)

In this model, bioconcentration is the result of two processes, uptake and elimination, and \(c_{Cu,int}^a(t)\) and \(c_{Cu,bioav}^w(t)\) refer to the measured internal copper concentrations in algae and the bioavailable copper concentration in the aqueous phase at a given point of time t [min]. For fitting the copper-8-hydroxyquinoline uptake kinetics, the measured total copper concentration in the medium at a given point of time t during an uptake experiment was used as \(c_{Cu,bioav}^w(t)\) because \(\sim 100\%\) of copper was bound in copper-8-hydroxyquinoline complexes.
3.4. Results and Discussion

3.4.1. Uptake of copper-8-hydroxyquinoline complexes

The uptake experiments revealed that under the experimental conditions (see Table 3.1) hydrophobic copper-8-hydroxyquinoline complexes are bioavailable (Figure 3.1). In contrast, the copper-sulfoxine complex was not significantly taken up (not shown), which is in accordance with its uptake into liposomes (see chapter 2).

In all experiments nearly 100 % of copper was bound in copper-8-hydroxyquinoline complexes but the relative fractions of the 1:1 (CuL⁺) and 1:2 complexes (CuL₂) varied between the different ligands. In the beginning of the experiments, the fraction of CuL₂ was lowest for copper-dichloroxine with 27 % and increased for copper-dibromoxine (45 %), copper-chloroxine (76 %) and copper-oxine (86 %).

Figure 3.1 shows the obtained uptake kinetics for total and intracellular copper in the presence of oxine (Figure 3.1 A), dichloroxine (Figure 3.1 B), chloroxine (Figure 3.1 C) and dibromoxine (Figure 3.1 D) corrected by the copper uptake, which was caused by the residual free copper (Cu²⁺) (see Figure 3.7 in the appendix to this chapter). Hence, the curves describe the copper uptake in Scenedesmus vacuolatus exclusively via copper-8-hydroxyquinoline complexes. On examination of the uptake kinetics, it becomes apparent that the uptake reached a plateau within 30 min with maximum internal copper concentrations of 2.9·10⁻¹⁸ mol-cell⁻¹ for copper-oxine, 4.5·10⁻¹⁸ mol-cell⁻¹ for copper-dichloroxine, 1.3·10⁻¹⁷ mol-cell⁻¹ for copper-chloroxine and 1.5·10⁻¹⁷ mol-cell⁻¹ for copper-dibromoxine. The fast achievement of a plateau can be explained by the fast decrease of the copper-8-hydroxyquinoline concentration in the medium due to algae uptake and the high cell density. It decreased for around 45 % for copper-oxine, 60 % for copper-dichloroxine, 65 % for copper-chloroxine and 70 % for copper-dibromoxine.

The amount of adsorbed copper (c_ads), the difference between total and intracellular copper, was nearly constant for each complex over time and complete saturation of available sorption sites occurred within the first minute. Adsorption constants (K_d = c_ads/c_w, in units of litre water per litre cell volume) varied from averaged 330 ± 680 L_w·L_cv⁻¹ for copper-dibromoxine to 1400 ± 400 L_w·L_cv⁻¹ for copper-oxine, 4010 ± 290 L_w·L_cv⁻¹ for copper-chloroxine and 5310 ± 440 L_w·L_cv⁻¹ for copper-dichloroxine.
The uptake rates ($k_1$) for copper via copper-8-hydroxyquinoline complexes increased in the same order as the maximum internal copper concentrations (Table 3.2), i.e. copper-oxine < copper-dichloroxine < copper-chloroxine < copper-dibromoxine. In contrast to the relatively high and varying uptake rates, the elimination rates ($k_2$) are similar and small with values of 0.1 - 0.2 min$^{-1}$. Consequently, bioconcentration factors (BCF) for copper by means of the uptake of copper-8-hydroxyquinoline complexes, which were calculated from $k_1$ and $k_2$ according to equation 3.7, increased in the same order as $k_1$ (see Table 3.2).

The uptake rate of copper via copper-oxine ($k_1$(copper-oxine)) in *Scenedesmus vacuolatus* (see Table 3.2) was around an order of magnitude higher than those, which were reported for other algae species in the literature, but comparable. Literature values for $k_1$(copper-oxine) were obtained by assuming that no elimination of copper occurs. However, a direct comparison of the published data with $k_1$(copper-oxine) from this study was possible because $k_2$(copper-oxine) was very small in comparison to $k_1$(copper-oxine). Reported $k_1$(copper-oxine) for different phytoplankton species were very variable and lied in the range of $9.4 \times 10^{-12}$ L$_w$·cell$^{-1}$·h$^{-1}$ and $5.7 \times 10^{-7}$ L$_w$·cell$^{-1}$·h$^{-1}$ depending on the algae species (Phinney & Bruland, 1994; Croot et al., 1999).
### Table 3.2. Uptake of Cu²⁺ and copper-8-hydroxyquinolines in Scenedesmus vacuolatus under the experimental conditions \(^a\) and influencing parameters

<table>
<thead>
<tr>
<th>compound</th>
<th>(k_1)</th>
<th>(k_2)</th>
<th>BCF</th>
<th>(\log_{10} \text{BCF})</th>
<th>(f_{\text{CuL}2})</th>
<th>(\log_{10} D_{\text{lipw,complex}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>copper-oxine</td>
<td>460 ± 80</td>
<td>9.0 \times 10^{-6} ± 2.0 \times 10^{-6}</td>
<td>0.2 ± 0.1</td>
<td>2.6 \times 10^3 ± 0.7 \times 10^3</td>
<td>3.42</td>
<td>0.86 ± 0.01</td>
</tr>
<tr>
<td>copper-dichloroxine</td>
<td>550 ± 100</td>
<td>1.1 \times 10^{-5} ± 0.2 \times 10^{-5}</td>
<td>0.1 ± 0.1</td>
<td>4.0 \times 10^3 ± 1.1 \times 10^3</td>
<td>3.60</td>
<td>0.27 ± 0.17</td>
</tr>
<tr>
<td>copper-chloroxine</td>
<td>1790 ± 560</td>
<td>3.6 \times 10^{-5} ± 1.1 \times 10^{-5}</td>
<td>0.1 ± 0.1</td>
<td>2.0 \times 10^4 ± 1.0 \times 10^4</td>
<td>4.31</td>
<td>0.76 ± 0.03</td>
</tr>
<tr>
<td>copper-dibromoxine</td>
<td>3540 ± 930</td>
<td>7.1 \times 10^{-5} ± 1.9 \times 10^{-5}</td>
<td>0.1 ± 0.1</td>
<td>3.0 \times 10^4 ± 1.2 \times 10^4</td>
<td>4.48</td>
<td>0.45 ± 0.01</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>10510 ± 4720</td>
<td>2.1 \times 10^{-4} ± 0.9 \times 10^{-4}</td>
<td>0.1 ± 0.1</td>
<td>2.3 \times 10^5 ± 1.7 \times 10^5</td>
<td>5.37</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) see Table 2. \(^b\) \(k_1, k_2, \text{BCF}\): uptake (\(k_1\)) and elimination rates (\(k_2\)) as well as bioconcentration factors (BCF) for copper via copper-8-hydroxyquinoline complexes. \(^c\) \(f_{\text{CuL}2}\): fraction of the CuL₂ complex. \(^d\) \(D_{\text{lipw,complex}}\): liposome-water distribution ratios of the complexes.
Figure 3.1. Total (■) and intracellular (▲) copper uptake in the green algae Scenedesmus vacuolatus by (A) copper-oxine, (B) copper-dichloroxine, (C) copper-chloroxine, (D) copper-dibromoxine and (E) Cu²⁺ in short-term uptake experiments with the same initial concentrations of bioavailable copper (note that the y-scale of E is different from A-D). The error bars represent standard deviations.
3.4.2. Bioconcentration factors and liposome-water partitioning

Observed uptake kinetics may be affected by several factors: (i) the ability of the complexes to partition into biological membranes, (ii) the relative fractions of $\text{CuL}^+$ and $\text{CuL}_2$, because the partitioning of charged molecules into biological membranes is decreased in comparison with neutral ones (Smejtek & Wang, 1993; Escher et al., 2000a; Word & Smejtek, 2005; Kaiser & Escher, 2006) and the membrane penetration is even lower (Block, 1956; Erickson et al., 2006), and (iii) the fate of the complex after its partitioning into the cell interior.

The relationship between the BCFs of copper (via copper-8-hydroxyquinoline complexes) and the liposome-water partitioning ratios of the complexes ($D_{\text{lipw,complex}}$) under the given experimental conditions was examined (Figure 3.2). The $D_{\text{lipw,complex}}$ include both information about the partitioning behaviour of the $\text{CuL}^+$ and $\text{CuL}_2$ complexes into biological membranes and about their relative fractions and vary by about one order of magnitude for the different copper-8-hydroxyquinolines. The BCF show the same relative variability between the different complexes, but BCF values are two to three orders of magnitude higher than the $D_{\text{lipw,complex}}$ values. For a better comparison, BCFs of the copper-8-hydroxyquinolines in *Scenedesmus vacuolatus* were predicted. The prediction was based on a QSAR (quantitative structure activity relationship) reported by Geyer at al. (Geyer et al., 1991), which was set up using 41 organic chemicals with different structures and physico-chemical properties. The original QSAR was based on $K_{ow}$ and converted to $K_{lipw}$ using the method derived in (Vaes et al., 1997) (equation 3.10).

$$\log_{10} \text{BCF [LW-LCv$^{-1}$]} = 0.65 \cdot \log_{10} K_{\text{lipw [LW-LPL$^{-1}$]}} + 0.37$$ (3.10)

Assuming that copper uptake via copper-8-hydroxyquinoline complexes depends on partitioning of the complexes into biological membranes and that the uptake behaviour of copper-8-hydroxyquinolines is similar to that of the organic chemicals, which were used to establish the QSAR, experimental BCF values similar to the predicted QSAR-BCFs were expected.

However, Figure 3.2 shows distinctively higher experimental BCFs than calculated BCFs. The difference amounts to two to three orders of magnitude. Hence, a higher
amount of copper is taken up via copper-8-hydroxyquinoline complexes than expected from the QSAR and additional factors have to play a role. The BCF model for organic compounds assumes partitioning between external aqueous phase and membrane lipids and between membrane lipids and cytosol. It does not account for any intracellular process. In case of the copper-8-hydroxyquinoline complexes it can be expected that there are ligand exchange reactions occurring in the cytoplasm. Intracellular ligands are expected to be SH-group containing amino acids, peptides, proteins and phytochelatins that exhibit a high binding affinity for copper (Gershon et al., 1975; Phinney & Bruland, 1994). A possible intracellular amino acid ligand may be histidine. Zentmyer et al. (Zentmyer et al., 1960) already showed that copper-oxine exchanges its ligand with histidine ($\beta_2 = 18.3$) and assumed that the ligand exchange primarily takes place with its CuL$^+$ complex, which is formed from CuL$_2$ in the cytoplasm. Ligand exchange reactions would result in an increased passive diffusion of copper-8-hydroxyquinoline complexes into algae along the concentration gradient.
Figure 3.2. Plot of the bioconcentration factors (BCF, \([L_w \cdot L_{cv}^{-1}]\)) of copper via copper-8-hydroxyquinoline complexes versus the liposome-water distribution ratios of the copper-8-hydroxyquinoline complexes \((D_{lipw,complex})\) under the experimental conditions. The straight line shows the QSAR for the BCF of organic compounds (equation 3.10), whose bioaccumulation is only driven by partitioning.

Phinney & Brunland (Phinney & Brunland, 1994) came to the same conclusion for copper-oxine and copper-diethyldithiocarbamate when they observed a much higher uptake into the diatom Thalassiosira weissflogii than expected from equilibrium calculations. Their study additionally showed a difference in the uptake of metal-diethyldithiocarbamate complexes with Cu, Cd and Pb in dependency of their complex stability. Similarly, the present study indicated a role of the complex stability on the uptake of copper-8-hydroxyquinolines in algae. The difference between experimental BCF and QSAR-BCF is slightly increased for copper-dichloroxine and clearly for copper-dibromoxine in comparison to that of copper-chloroxine and copper-oxine. The former complexes exhibit a smaller stability and thus a ligand
exchange reaction is relatively favoured by them in comparison to the other two complexes. However, the difference between BCF and QSAR-BCF increases in the order copper-oxine < copper-chloroxine < copper-dichloroxine < copper-dibromoxine (Table 3.2) while the stability constants for the CuL₂ complex (β₂) decrease in the order copper-chloroxine > copper-oxine > copper-dibromoxine > copper-dichloroxine (Table 1.1), thus showing that the stability of the copper-8-hydroxyquinoline complexes may be an additional but not dominant factor that affects the uptake of copper-8-hydroxyquinoline into algae.

Summarised, several factors related to the penetration of the membrane by copper-8-hydroxyquinolines and their fate within the cytoplasm influence the uptake of copper-8-hydroxyquinolines in *Scenedesmus vacuolatus*.

### 3.4.3 Comparison of uptake rates for copper via Cu²⁺ and via copper-8-hydroxyquinolines

A comparison of the copper uptake and elimination rates (k₁ and k₂) for the same initial concentrations of hydrophobic copper-8-hydroxyquinoline complexes and Cu²⁺ (Table 3.2 and Figure 3.1 E) shows that k₁(Cu²⁺) is 3 to 23 times higher than the k₁ of the complexes and that the k₂ are similar, despite the fact that the bioavailable copper in the medium decreased for >99 % over the whole experiment in the Cu²⁺ experiment while that in copper-8-hydroxyquinoline experiments only decreased for 45-70 %. Obtained values for Cu²⁺ uptake in mol·cell⁻¹·min⁻¹ (k₁(Cu²⁺)·cₖw Cu²⁺ = 2·10⁻¹⁷ mol·cell⁻¹·min⁻¹) were higher but comparable to those, which were reported for other algae species in the literature. Literature values for k₁(Cu²⁺)·cₖw Cu₂⁺ were between 0.5·10⁻²¹ - 6·10⁻¹⁸ mol·cell⁻¹·min⁻¹ for uptake in various algal species (Knauer, 1996; Croot et al., 2003; Quigg et al., 2006).

At first sight, the result that k₁(Cu²⁺) > k₁(Cu-oxine) is in contrast to findings of other researches who reported that the uptake of copper via copper-oxine is higher than that via Cu²⁺ (Phinney & Bruland, 1994; Croot et al., 1999). However, it becomes reasonable when considering that in the present uptake experiments with copper-oxine the copper:oxine ratio was chosen as 1:2, whereas the published data was obtained in experiments with an excess of oxine. An excess of oxine resulted in experimental conditions under which 100 % of copper was bound in CuL₂ complexes,
whereas in the present copper-oxine uptake experiments 86 % of copper was bound in CuL₂ and 14 % in CuL⁺. This difference in speciation led to a decreased copper-oxine uptake in the present study in comparison to the studies in literature.

3.5 Environmental significance

The results of this study showed that copper-8-hydroxyquinoline complexes are bioavailable for Scenedesmus vacuolatus via passive diffusion. In conclusion, this study pointed out that it is important not only to regard organic ligands as modulators of the speciation of copper and thus of the free copper concentration in the medium but also to consider hydrophobic copper-8-hydroxyquinoline complexes as bioavailable species and as a potentially important source of copper for algae. They may especially play a major role in aquatic environments, where the concentration of free copper ions is low. Additionally, their importance for copper uptake in comparison to that of Cu²⁺ depends on the environmental conditions, which became obvious by comparing the results of studies with different copper:ligand ratios.
Appendix:

Calculation of copper-8-hydroxyquinoline uptake kinetics

The copper-8-hydroxyquinoline uptake experiments in *Scenedesmus vacuolatus* were conducted in experimental medium at pH ~ 7.5 (for details on the experimental conditions see Table 3.1). Under these conditions, copper was almost completely complexed by the added 8-hydroxyquinoline ligand (~ $7 \times 10^{-8}$ M). The concentrations of residual free copper (Cu$^{2+}$) in the different copper-8-hydroxyquinoline complex experiments were between $10^{-13}$ and $10^{-10}$ M depending on the complex stability constants of the two copper-8-hydroxyquinoline complexes, CuL$^{(2\cdots)}$ and CuL$_2^{(2\cdots)}$, and on the exact pH value (see Table 1.1 and 3.1). Therefore, the obtained uptake kinetics for intracellular and total copper described the cumulative uptake via carrier-mediated transport of Cu$^{2+}$ and via passive diffusion of copper-8-hydroxyquinoline complexes. Uptake kinetics of copper-8-hydroxyquinoline complexes could be obtained by subtraction of the copper fraction that was taken up via Cu$^{2+}$ from the cumulative uptake.

This procedure is presented in the following and the results are demonstrated by means of the intracellular copper concentrations of the five tested copper-8-hydroxyquinoline complexes.

To determine the fraction of copper, which was taken up via Cu$^{2+}$ in the copper-8-hydroxyquinoline experiments, uptake kinetics over 30 min were determined in several experiments with $7 \times 10^{-8}$ M total copper and between $1.3 \times 10^{-13}$ M and $5.6 \times 10^{-10}$ M Cu$^{2+}$ adjusted by EDTA. EDTA-copper complexes are not directly bioavailable, but maintained a constant aqueous Cu$^{2+}$ concentration in solution.

Uptake kinetics of Cu$^{2+}$ reached a plateau within 30 min (Figure 3.3), which can be explained by the decrease of the Cu$^{2+}$ concentration for around 25 % in the medium due to uptake in algae and high cell densities ($3 \times 10^6$ cells·ml$^{-1}$).
Figure 3.3. Example of typical uptake kinetics of intracellular copper for three Cu$^{2+}$ concentrations in the aqueous medium (▲ 3.97·10$^{-10}$ M Cu$^{2+}$, ■ 4.09·10$^{-12}$ M Cu$^{2+}$, ◆ 1.66·10$^{-13}$ M Cu$^{2+}$).

The tested Cu$^{2+}$ concentrations ($c_{Cu^{2+}}^w$) were not the exact Cu$^{2+}$ concentrations, which were present in the copper-8-hydroxyquinoline experiments, because total copper concentration in solution and pH could be measured but not exactly adjusted. Hence, the uptake kinetics of those Cu$^{2+}$ concentrations that were present in the copper-8-hydroxyquinoline experiments had to be calculated from the measured ones. This was achieved by sorting the obtained data for intracellular ($c_{Cu, int}^w$) and total copper concentrations in algae ($c_{Cu, tot}^w$) of all copper-EDTA experiments after their associated points of time within the kinetic (t). The resulting curves for each point of time could be well described by a sorption isotherm, which reaches saturation (Figure 3.4; equation 3.11). Equation 3.11 can be converted into equation 3.12, which was used to fit the data for each individual point of time (Figure 3.5).
Figure 3.4. Relation between external concentrations of Cu\(^{2+}\) (\(c_{\text{Cu}^{2+}}^w\) ; [mol·L\(^{-1}\)]) and intracellular copper (\(c_{\text{Cu, int}}^a\) ; [mol·L\(^{-1}\)]) for a single point of time (\(t = 1\) min is shown as an example).

\[
c_{\text{Cu, int}}^a = \frac{M \cdot c_{\text{Cu}^{2+}}^w}{K + c_{\text{Cu}^{2+}}^w} \quad \text{M, K = constant} \tag{3.11}
\]

\[
\log(c_{\text{Cu, int}}^a) = \log(M) - \log(K \cdot e^{-2.303\log(c_{\text{Cu}^{2+}}^w)} + 1) \tag{3.12}
\]
Chapter 3: Bioavailability and uptake of hydrophobic copper-organic complexes in algae

Figure 3.5. Fitted curves for individual points of time (t; [min]) of the uptake kinetics of intracellular copper ($c_{Cu, int}^w$; [mol·L$_{-1}$]) via carrier-mediated transport of different concentrations of Cu$^{2+}$ ($c_{Cu2+}^w$; [mol·L$_{-1}$]).

For simplification of the graph only the curves for $t = 1, 5, 9$ and $30$ min are shown.

Using Equation 3.11 and the obtained fitting parameters, M and K, intracellular and total copper concentrations for the considered points of time could be calculated for the Cu$^{2+}$ concentrations, which were initially present in the copper-8-hydroxyquinoline complex experiments (Figure 3.6). For simplification of the graph only the obtained data for $c_{Cu2+}^w = 4.27\cdot10^{-12}$ M and $1.55\cdot10^{-13}$ M is shown, because the data for the other concentrations is close to that for $4.27\cdot10^{-12}$ M. Subsequently, the uptake kinetics for the copper uptake via Cu$^{2+}$ in the copper-8-hydroxyquinoline experiments were fitted with equation 3.8.

The obtained uptake kinetics for Cu$^{2+}$ slightly overestimated the real Cu$^{2+}$ uptake in the copper-8-hydroxyquinoline experiments, because the Cu$^{2+}$ concentration in the
medium decreased for around 30-35 % in the copper-8-hydroxyquinoline experiments, while it decreased for around 25 % in the Cu-EDTA experiments. However, when having a look on Figure 3.5 it becomes apparent that not even a difference of 5-10 % in assumed and real Cu\(^{2+}\) concentration over the whole time of the uptake experiment would change the internal copper concentration significantly.

![Graph showing intracellular copper concentrations](image)

**Figure 3.6.** Calculated data for intracellular copper concentrations \(c_{\text{Cu,int}}\) due to the uptake of \(4.27 \times 10^{-12} \text{ M} \) (■) and \(1.55 \times 10^{-13} \text{ M} \) (▲) \(\text{Cu}^{2+}\) for individual points of time (t) and fitted uptake kinetics (solid lines).

The predicted copper uptake kinetics for \(\text{Cu}^{2+}\) were then subtracted from the cumulative uptake kinetics measured in the copper-8-hydroxyquinoline complex experiments. The subtraction resulted in the uptake kinetics of intracellular copper via passive diffusion of copper-oxine (Figure 3.7 A), copper-dichloroxine (Figure 3.7 B), copper-chloroxine (Figure 3.7 C) and copper-dibromoxine (Figure 3.7 D). Copper-
sulfoxine was omitted because the calculated copper uptake via Cu\(^{2+}\) was equal to the cumulative copper uptake, i.e. the copper-sulfoxine complexes were not taken up.

The data shown in Figure 3.7 (given in mol copper per litre cell volume \([\text{mol Cu-L}_{cv}^{-1}]\)) can be converted to mol copper per alga cell \([\text{mol-cell}^{-1}]\) like it is used in Figure 3.1 by multiplying it with the mean cell volume of the *Scenedesmus vacuolatus* cells \((25 \cdot 10^{-15} \text{L}_{cv}\cdot\text{cell}^{-1})\).
Figure 3.7. Measured cumulative copper uptake (■), predicted copper uptake via Cu\(^{2+}\) (black solid line) and calculated uptake of copper via copper-8-hydroxyquinoline complexes (▲) for copper-oxine (A), copper-dichloroxine (B), copper-chloroxine (C) and copper-dibromoxine (D).
4.

Toxicity of copper-8-hydroxyquinoline complexes
4.1. Introduction

With regard to copper-oxine, previous studies hypothesised that toxic effects are either caused by the fact that oxine serves as a transporter for copper into the cell, where Cu$^{2+}$ exerts its toxic effects (Albert, 1951; Yeager, 1991), or that the complex itself may have toxic effects by catalysing the formation of hydroxyl radicals from molecular oxygen via the Fenton reaction (Florence et al., 1983). A third hypothesis is that the copper-8-hydroxyquinoline complexes intercalate in the membrane, where they disturb the membrane structure or may act through toxic mechanisms in the membrane, e.g. by delivering Cu$^{2+}$ to sites of action in the membrane.

To learn more about the toxic effects of copper-8-hydroxyquinoline complexes, it was planned to complement the in vitro (liposome-water partitioning – chapter 2) and in vivo (uptake in algae – chapter 3) experiments about their bioavailability with in vitro and in vivo toxicity experiments.

Toxicity experiments were planned to focus on more integral endpoints like growth inhibition and reproduction but also on physiological endpoints, which are known to be related to copper because the results of the bioavailability experiments hint that copper-8-hydroxyquinoline complexes serve as transporters for copper into algae (see chapter 3). Therefore, special attention was paid to Photosystem II (PSII) and model systems for PS II, which is an important site of action for copper. The used methods are summarised in Table 4.1.
### Table 4.1. Test methods applied to determine the potential toxic effects of Cu$^{2+}$ and copper-8-hydroxyquinolines

<table>
<thead>
<tr>
<th>Method</th>
<th>tests toxic effects on</th>
<th>by measuring</th>
<th>chapter</th>
</tr>
</thead>
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<td>Growth inhibition test</td>
<td>growth/ reproduction of algae</td>
<td>cell density</td>
<td>4.2</td>
</tr>
<tr>
<td>Photosynthesis rate test</td>
<td>the photosynthesis rate in algae</td>
<td>the amount of incorporated $^{14}$C during photosynthesis</td>
<td>4.3</td>
</tr>
<tr>
<td>Pulse-amplitude-modulation fluorometric method</td>
<td>the efficiency of Photosystem II (PS II) in algae</td>
<td>the quantum yield of PS II</td>
<td>4.3</td>
</tr>
<tr>
<td>Kinspec system</td>
<td>the electron transport chain of a bacterial photosynthetic membrane and the integrity of the membrane (baseline toxicity) using chromatophores</td>
<td>absorbance changes at specific wavelengths to observe components of the electron transport chain</td>
<td>4.4</td>
</tr>
</tbody>
</table>
4.2. Growth inhibition

4.2.1. Introduction

Growth inhibition experiments are normally carried out in algae culture medium, which contains trace metals and EDTA to supply algae with essential metals. However, when working with hydrophobic copper-8-hydroxyquinoline complexes as toxicants trace metals as well as EDTA are problematic. An addition of a copper-8-hydroxyquinoline complex to algae culture medium would lead to a competition between the 8-hydroxyquinoline and EDTA for copper because the complex stability constants of the EDTA copper complexes are similar or higher than that for copper-8-hydroxyquinoline complexes. Consequently, copper would be bound by both ligands or the Cu-complexation would be dominated by Cu-EDTA. Additionally, the growth medium contains other metals than copper, which also form complexes with 8-hydroxyquinolines. Thus, it was necessary to prepare the experimental medium without trace metals and EDTA. However, this means to withdraw several essential algae nutrients from the medium. Thus, the aim of the following experiment was to test whether algae are able to grow unhindered for a certain time span in medium without trace metals. Therefore, algae cultures in culture medium and in trace metal free experimental medium were compared in terms of cell density and fluorescence yield of Photosystem II.

4.2.2. Experimental design

The experiment examined algae in culture medium as it is described in chapter 3.2.2 and algae in that medium without EDTA and trace metals (see experimental medium in chapter 3.2.2). 50 ml batch cultures were prepared by transferring an inoculum of *Scenedesmus vacuolatus* (for details on the algae see chapter 3.2.3) in exponential growth phase into the respective medium. The initial cell density was approximately $5 \times 10^5$ cells ml$^{-1}$ and triplicates were produced for each of the two media. Algae were grown in 100 ml glass Erlenmeyer flasks in a HT Infors shaker at 25°C (for details see chapter 3.2.3). At certain points of time, samples were taken from the algae cultures and cell density and fluorescence yield of Photosystem II ($\Phi_F$) were
determined. The cell density was measured with a Z2 Coulter Particle Count and Size Analyzer (Beckman Coulter, Nyon, Switzerland). The fluorescence yield of Photosystem II was determined with a ToxY-Pam dual-channel yield analyser. This method is based on the fact that the quenching of chlorophyll fluorescence is closely related to the efficiency of energy conversion within that photosystem (Genty et al., 1989). \( \Phi F \) is defined by equation 4.1, where \( F_M \) is the maximal and \( F_0 \) the minimal fluorescence in the light adapted state.

\[
\Phi F = \frac{F_M - F_0}{F_M} \quad (4.1)
\]

4.2.3. Results and discussion

For the first 48 hours both algae cultures showed now no significant difference in cell density (Figure 4.1). However, it was clearly visible that the algae in medium without trace metals were lighter green indicating that the chlorophyll content of the cells must have been decreased. ToxY-Pam measurements showed that the fluorescence yield of Photosystem II was distinctively decreased in comparison with the algae cultures in culture medium (Figure 4.2). This decrease could already be significantly detected after 8 hours of culturing.

In conclusion, it is not possible to conduct growth inhibition tests with hydrophobic copper-8-hydroxyquinoline complexes in trace metal free medium over a longer time span. Thus, 72 h growth inhibition tests in the style of OECD guideline for testing of chemicals 201 (Organisation for Economic Co-operation and Development, 1984) are not feasible. A 24 h growth inhibition test like proposed by Meene (Meene, 2005) may be possible because the decrease of \( \Phi F \) of the algae in trace metal free medium in comparison to those in culture medium was merely around 20 % after 24 h.
Chapter 4: Toxicity of copper-8-hydroxyquinoline complexes

Figure 4.1. Cell density in a culture of Scenedesmus vacuolatus in algae culture medium (♦) and in experimental medium without trace metals (▲) depending on culture time.

Figure 4.2. Fluorescence yield of Photosystem II ($\Phi_F$) in a culture of Scenedesmus vacuolatus in algae culture medium (♦) and in experimental medium without trace metals (▲) depending on culture time.
4.3. Effects on photosynthesis

4.3.1. Introduction

Copper is known to have several inhibitory effects on both, Photosystem I and II (PS I and II; see chapter 1.1.4). Thus, if copper-8-hydroxyquinoline complexes simply act as transporters for copper into cells, they should cause those effects. In the case of an own mode of action of the copper-8-hydroxyquinoline complexes, e.g. by the formation of hydroxyl radicals, the photosystems may also be affected indirectly. To explore potential effects on photosynthesis two test methods were applied.

The photosynthesis rate test (Steemann Nielsen, 1952) was used to examine the effects of copper-8-hydroxyquinoline complexes on the photosynthesis rate of algae. It determines the incorporated of $^{14}$C in algae via assimilation of $^{14}$CO$_3$. The latter is consumed by algae during primary production (equation 4.2).

$$6 \cdot \text{CO}_2 + 6 \cdot \text{H}_2\text{O} \xrightarrow{\text{light energy}} \text{C}_6\text{H}_12\text{O}_6 + 6 \cdot \text{O}_2 \quad (4.2)$$

The pulse-amplitude-modulation fluorometric method was applied to determine the inhibition the quantum yield of Photosystem II by copper-8-hydroxyquinoline complexes by means of a ToxY-PAM dual-channel yield analyser. It uses the saturation pulse method, where chlorophyll fluorescence is measured before and during a saturation pulse of light to derive the quantum yield of PS II (equation 4.1) (Schreiber et al., 2002). The inhibition of $\Phi_F$ is calculated with equation 4.3 where $\Phi_{F_s}$ is the quantum yield of the sample and $\Phi_{F_c}$ that of the control.

$$\text{Inhibition} = \frac{\Phi_{F_c} - \Phi_{F_s}}{\Phi_{F_c}} \quad (4.3)$$

Both bioassays were performed with copper-chloroxine as a first test substance because its bioavailability for Scenedesmus vacuolatus at pH 7.5 is the second highest in comparison with the other copper-8-hydroxyquinoline complexes and its
solubility in water is higher than that of copper-dibromoxine. These characteristics were important because a measurable effect on the photosynthesis was only expected at higher concentrations.

### 4.3.2. Experimental design

For the photosynthesis rate test 2 ml aliquots of the incubation medium ($0.5 \cdot 10^{-3}$ M NaHCO$_3$; $0.09 \cdot 10^{-3}$ M MgCl$_2$; $0.5 \cdot 10^{-3}$ M MOPS, pH 7.5) were pipetted into glass scintillation vials and spiked with three different concentrations of copper-chloroxine. Examined copper-chloroxine concentrations were between $5 \cdot 10^{-8}$ and $9 \cdot 10^{-8}$ M and each concentration was tested in triplicates. Concentrations up to $9 \cdot 10^{-8}$ M are presumed to be soluble, while at higher ones signs of colloid formation or precipitation were observed. Additionally, the same concentrations range was tested for Cu$^{2+}$. Controls just contained incubation medium. An aliquot of *Scenedesmus vacuolatus* (for details on the algae see chapter 3.2.3) in exponential growth phase was added to the incubation medium with copper-chloroxine, so that the initial cell density was approximately $2 \cdot 10^6$ cells·ml$^{-1}$. All vials were incubated in the darkness for 5 h at 25 °C. After incubation, 1 µCi NaH$^{14}$CO$_3$ (GE Healthcare UK Limited, Little Chalfont, UK) were added to each vial and they were incubated a second time for 30 min in a water bath (25°C) at a light intensity of 125 µE·m$^{-2}$·s$^{-1}$. The absorption of $^{14}$C by the algae was stopped by addition of 100 ml of concentrated H$_2$SO$_4$ (98 %), samples were bubbled with air for 30 min to remove excess $^{14}$CO$_2$ and 3 ml Lumagel (Lumac, Basel, Switzerland) were added. The incorporated radioactivity was then measured by liquid scintillation counting (BE-Tamatic I; Kontron Instruments, Milton Keynes, UK) in counts per minute (CPM).

For the pulse-amplitude-modulation fluorometric method 50 ml of culture medium without trace metals (see experimental medium in chapter 3.2.2) were spiked with copper-chloroxine concentrations between $5 \cdot 10^{-8}$ and $9 \cdot 10^{-8}$ M and an aliquot of *Scenedesmus vacuolatus* in exponential growth phase was added, so that the initial cell density was approximately $5 \cdot 10^5$ cells·ml$^{-1}$. For each concentration triplicates were produced and controls consisted of medium and algae only. Algae were grown in 100 ml glass erlenmeyer flasks in a HT Infors shaker (Infors, Bottmingen,
Switzerland) at 25°C, 100 rpm and with continuous illumination of 267 μE·m⁻²·s⁻¹ provided by cool white lamps. After 12 h samples were taken from the algae cultures and the inhibition of the quantum yield of Photosystem II was determined with the ToxY-Pam dual-channel yield analyser (Gademann Instruments GmbH, Würzburg, Germany) in comparison to the controls.

4.3.3. Results and discussion

The results of both experiments showed no significant difference between the controls and algae, which were exposed to similar concentrations of copper-chloroxine or Cu²⁺. Table 4.2 exemplarily shows the results of the photosynthesis rate test.

Thus, copper-chloroxine has no significant effect on photosynthesis within the tested concentration range. Used concentrations of copper-chloroxine were relatively low because the limited solubility of copper-chloroxine and the same concentrations of Cu²⁺ also did not cause effects. Therefore, it can be concluded that water-soluble concentrations of copper-chloroxine do not lead to the uptake of a toxic amount of copper.

Table 4.2. Incorporation of ¹⁴C in algae (in count per minute (CPM)) in the presence of copper-chloroxine and Cu²⁺

<table>
<thead>
<tr>
<th>Incorporation of ¹⁴C (CPM)</th>
<th>copper-chloroxine</th>
<th>Cu²⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>58378 ± 1620</td>
<td>53734 ± 3009</td>
</tr>
<tr>
<td>5·10⁻⁸ M</td>
<td>52087 ± 2128</td>
<td>51676 ± 6086</td>
</tr>
<tr>
<td>7·10⁻⁸ M</td>
<td>57864 ± 6493</td>
<td>53106 ± 6766</td>
</tr>
<tr>
<td>9·10⁻⁸ M</td>
<td>59158 ± 2357</td>
<td>49768 ± 3997</td>
</tr>
</tbody>
</table>
4.4. Toxic effects on the electron transfer chain of a photosynthetic model system isolated from bacteria

4.4.1. Introduction

In vitro membrane toxicity can be measured by time-resolved spectrometry on energy-transducing membranes (Kinspec Test). The Kinspec system is a modularly built spectrophotometer that allows the quantitative distinction and determination of baseline toxicity (Escher et al., 2002), uncoupling (Escher et al., 1997), inhibition of the electron transfer (Escher et al., 1996), and inhibition of the ATP synthesis (Hunziker, 2001). Its design and mode of operation are described in detail in (Escher et al., 1997). Chromatophores of *Rhodobacter sphaeroides* are used as model systems for energy-transducing photosynthetic membranes. They can be prepared from whole bacterial cells (see 4.4.2.2) and are small membrane vesicles containing an intact photosystem, including electron transport chain and ATP-synthase. The cyclic electron transport chain in the chromatophores of *Rhodobacter sphaeroides* is depicted in Figure 4.3 and its constituents are comparable to those of Photosystem II of *Scenedesmus vacuolatus*.

![Figure 4.3. Simplified scheme of the cyclic electron transport chain of Rhodobacter sphaeroides.](image)

Carot.: carotenoids; [BChl]$_2$: primary electron donor - a bacteriochlorophyll dimer; Q$_b$, Q$_i$, Q$_o$: quinone binding sites; cyt c$_1$, cyt c$_2$, cyt b$_{h}$: cytochromes.
The method uses chromatophores, which were incubated in the darkness and exposed to single-turnover flashes of visible light to excite the photosystem. It observes absorbance changes at several wavelengths, which allow evaluation of the redox kinetics of different constituents of the electron transfer chain.

It was planned to determine the effect of copper-8-hydroxyquinoline complexes on the membrane potential by means of measuring the absorbance change ($\Delta A_{503}$) at 503 nm. At this wavelength the electrochromic shift of the carotenoids can be measured which is directly proportional to the membrane potential. It is a relatively general parameter for the status of the electron transport process and additionally, one of the hypothesised modes of action was baseline toxicity caused by the complexes itselfs.

Subsequently, specific inhibition of constituents of the electron transport chain ought to be determined. By measuring the absorbance change of the wavelength pairs 561-569 nm ($\Delta A_{561-569}$) and 551-542 nm ($\Delta A_{551-542}$) cytochrom $b_H$ (cyt $b_H$) and the cytochroms $c_1$ and $c_2$ (cyt $c_{tot} = cyt c_1 + cyt c_2$) could be observe.

Cu$^{2+}$ was planned to serve as a control to see whether detected effects are comparable to those of copper or not and to appraise which of the proposed modes of action may be the most probable. For copper it has already been shown with other test systems that it has specific effects on Photosystem II (see chapter 1.1.4).

4.4.2. Experimental design

4.4.2.1. Redox buffers

For Kinspec measurements with copper-chloroxine changes in the experimental protocol were required. To assure, that almost all copper is available as Cu(II) a redox potential ($E_r$) of > 300 mV is necessary. None of the so far in Kinspec system used redox buffers, which led to potentials $\approx 30$ mV (succinate and fumarate) or $\approx 125$ mV (dithionite and a mixture of the redox mediators duroquinone, 1,2-napthoquinone, 1,4-napthoquinone, 1,4-benzoquinone, 2,3,5,6-tetramethyl-p-phenylenediamine, and N-methylphenazonium methosulfate) were suitable to fulfil this condition. Literature research showed that all redox mediators for a potential around 300 mV contain iron which would compete with copper for chloroxine. Thus,
experiments had to be conducted without a redox buffer system. Consequently, it was necessary to investigate in a first step whether the electron transport chain works properly under redox-unbuffered conditions for several hours.

4.4.2.2. Chromatophores

Chromatophores were produced and characterised according to (Escher et al., 1997) with KOH and KCl replaced by NaOH and NaN03.

4.4.2.3. Method

To test whether it is generally possible to work without redox buffering, a chromatophore suspension was prepared and its redox potential was measured with a voltage-meter (Metrohm, Herisau, Switzerland).

Additionally, the Kinspec was used to observe whether the electron transport chain is still functioning. For an experiment 6 ml of pH-buffer composed of 10 mM MOPS pH 7.5 and 100 mM NaNO3 were mixed in a cuvette. Frozen chromatophores were thawed and added in an end concentration of approximately 1.9-10^-7 M of reaction centre. After an incubation time of 1 and 5 h in the darkness, the suspension was exposed to single-turnover flashes and the absorbance change (ΔA) at 503 nm (membrane potential) was recorded. Furthermore, the wavelength pairs 561-569 nm (cytochrome bH) and 551-542 nm (cyt c_{10t} = cyt c_1 + cyt c_2) were measured after 5 h without and in presence of antimycin, which inhibits the oxidation of cytochrome b_{1} (cyt b_{1}) directly and that of cytochrome c_1 and c_2 (cyt c_1 and c_2) indirectly.

4.4.3. Results and discussion

Without redox buffering the obtained redox potential in the chromatophore suspension was relatively stable and around 570 ± 30 mV, thus confirming that in case of an addition of copper the copper in solution would be present in the appropriate redox state, i.e. as Cu^{2+}.

However, the Kinspec measurement of ΔA_{503} reveal that the maximum membrane potential, which was excited by single-turnover flashes, is distinctively decreased
after 5 hours (Figure 4.4) and hence the activity of the electron transport that generates the membrane potential. Thus, it can be concluded that the external redox potential rapidly increases the membrane's redox potential, which leads to an increasing oxidation of the reduced components of the electron transport chain and a subsequent hindrance of the electron transport.

Figure 4.4. Membrane potential of chromatophores measured as \( \Delta A_{503} \) (in relative absorption units: r.a.u.) after 1 (grey) and 5 h (black) of incubation in an experimental system without redox buffering.

Figure 4.5 and 4.6 shows the absorbance changes of cyt b\(_{H}\) (\( \Delta A_{561-569} \)) and cyt c\(_{tot}\) (\( \Delta A_{551-542} \)) induced by single-turnover flashes after 5 hours without and after addition of antimycin in an unbuffered chromatophore suspension as well as the same curves in a suspension which was buffered to 30 mV. The curves without antimycin overlay
each other. There no changes of $\Delta A_{561-569}$ and $\Delta A_{551-542}$ by the light flashes are visible because the cytochromes are reduced and reoxidised in the range of $\mu$sec.

Figure 4.5 shows that in buffered solution, each flash of light leads to an increasing $\Delta A_{561-569}$ when antimycin causes an inhibition of the oxidation of cytochrome $b_H$ but the electron transfer chain works unhindered in other respects. However, the curve in unbuffered solution and with addition of antimycin reveals only a small hindrance of the reoxidation of cyt $b_H$, which leads to the conclusion that nearly no electrons reach cyt $b_H$.

![Figure 4.5. Inhibition of cytochrome $b_H$ by $10^{-5}$ mol·L$^{-1}$ antimycin in experimental systems with ($E_h = 30$ mV; light grey) and without redox buffering (black) in comparison to a control measurement without addition (dark grey).](image)

Figure 4.6 additionally shows an increasing $\Delta A_{551-542}$ in the case of the buffered system ($E_h = 30$ mV) with antimycin. The cytochromes $c_1$ and $c_2$ are indirectly affected and not able to reoxidise any more. In the unbuffered system the addition of
antimycin does not influence $\Delta A_{551-542}$ at all. This indicates that no electrons reached the cytochromes $c_1$ and $c_2$ before antimycin blocked their reoxidation. It seems that at an earlier stage of the electron transport chain the electron transfer is either blocked or no electron-acceptors are present any more due to the high redox potential in the membrane.

Figure 4.6. Antimycin inhibition of cytochrom $c_{tot}$ in experimental systems with ($E_h = 30$ mV; light grey) and without redox buffering (black) in comparison to a control measurement (dark grey).

Considering that the midpoint potential ($E_m$) of the primary electron donor ([BChl]$_2$; Figure 4.3), the potential when half of the molecules is oxidised and the other half reduced, is 450 mV (Dutton et al., 1975) and that the $E_m$ of the other redox compounds are lower (Schreiber, 2006), these observations can be explained as follows. When exposed to light, the primary electron donor [BChl]$_2$ becomes photooxidised and the released electron is transferred over an intermediate step to
Chapter 4: Toxicity of copper-8-hydroxyquinoline complexes

the quinone binding site \( Q_b \) where a semiquinone is formed. To become fully reduced the latter one needs a second electron which is delivered by \([\text{BChl}]_2\). To make that possible, \([\text{BChl}]_2\) has to be rereduced by cyt \( c_2 \) between the delivery of the first and second electron. However, if the external redox potential also increases the internal redox potential of the chromatophores, cyt \( c_2 \) \( (E_m = 348 \text{ mV}; \) (Meinhardt & Crofts, 1982)) is mainly oxidised and in this redox state not able to rereduce \([\text{BChl}]_2\). Subsequently, the electron transport is hindered and almost no electrons are delivered to cyt \( b_h \), \( c_1 \) and \( c_2 \).

Additionally, the passing down of the external potential leads to a membrane potential where also most of the \([\text{BChl}]_2\) is oxidised. Thus, it is not any more possible to photooxidise already oxidised reaction centre complexes and light has no effect on them any more.

Consequently, a determination of toxic effects on the electron transfer chain of a photosynthetic system with the Kinspec system was not possible for unbuffered conditions \( (E_h = 570 \pm 30 \text{ mV} ) \), when \( \text{Cu}^{2+} \) would not be reduced to \( \text{Cu}^+ \) in comparison to buffered conditions \( (E_h = 30 \text{ or } 125 \text{ mV}) \).
5. Conclusions and Outlook
Chapter 5: Conclusions and Outlook

5.1. Bioavailability of copper-8-hydroxyquinoline complexes and their components

5.1.1. Summary of the results on bioavailability and uptake

Uptake route, influencing parameters and further fate of copper-8-hydroxyquinoline complexes in organisms have been a matter of interest over several years, especially in connection with the fungicidal effect of oxine (Block, 1955; Block, 1956; Liu et al., 1994; Nicoletti et al., 1999) and the fact that copper uptake in presence of copper-oxine represents an exception to FIAM and BLM (Florence et al., 1983; Ahsanullah & Florence, 1984; Florence & Stauber, 1986; Campbell, 1995). Although these and other studies enlightened parts of these processes, a complete and detailed model for the bioavailability of copper-8-hydroxyquinoline complexes is so far not existent. The present study aimed to enlarge the knowledge about their uptake mechanism and led to the following conclusions and assumptions.

The results on the partitioning between liposomes and water showed that hydrophobic copper-8-hydroxyquinoline complexes are able to passively partition into phospholipid membranes. The partitioning of their charged (CuL⁺) and neutral (CuL₂) complexes was dependent on the hydrophobicity of the ligand and on other factors including molar volume and polarisation of the complexes (chapter 2). Depending on the experimental conditions, the relative fractions of CuL⁺ and CuL₂ influenced the amount of a copper 8-hydroxyquinoline, which partitioned into a biological membrane, because the partitioning of charged molecules into biological membranes is decreased in comparison with neutral ones (Smejtek & Wang, 1993; Escher et al., 2000a; Word & Smejtek, 2005; Kaiser & Escher, 2006).

For an expansion of these findings to algae uptake experiments into the unicellular algae Scenedesmus vacuolatus were conducted (chapter 3). Their results showed two to three magnitudes higher copper uptake via copper-8-hydroxyquinoline complexes than it was expected from their partitioning into biological membranes, indicating that more factors than the passive diffusion through the cell membrane influenced the uptake of the complexes in algae. It is assumed that a ligand exchange mechanism within the cytoplasm is responsible for the observed higher uptake in algae. After penetrating the cell membrane, copper bound in copper-8-
Chapter 5: Conclusions and Outlook

Hydroxyquinoline complexes may be exchanged with intracellular ligands, which form complexes of a higher stability than the 8-hydroxyquinolines. Besides the complex stability also the relative concentrations of the intracellular ligands in comparison to the 8-hydroxyquinolines are crucial for the ligand exchange. It is expected that the concentration of intracellular ligands exceeds the concentration of copper-8-hydroxyquinoline complexes distinctively.

Intracellular ligands are expected to be SH-group containing amino acids, peptides, proteins and phytochelatins that exhibit a high binding affinity for copper. Possible intracellular amino acid ligands may be cysteine and histidine (Albert, 1952; Zentmyer et al., 1960; Gershon et al., 1975). Zentmyer already showed that copper-oxine exchanges its ligand with histidine ($\beta_2 = 18.3$) and assumed that the ligand exchange primarily takes place with its CuL$^+$ complex, which is formed from the CuL$_2$ complex that penetrates the cell membrane (Zentmyer et al., 1960). The CuL$^+$ complex exhibits smaller stability constants than CuL$_2$ (see Table 5.1) and the copper-histidine complex and a predominant ligand exchange of histidine with CuL$^+$ seems therefore probable. The same may hold for copper-chloroxine, whose CuL$_2$ complex also has a higher and its CuL$^+$ complex a lower stability constant than copper-histidine. For copper-dichloroxine and copper-dibromoxine also the CuL$_2$ complexes have smaller complex stability constants.

For cysteine it may be assumed that it not only acts as an exchange ligand, but also reduces Cu$^{2+}$ to Cu$^+$ (Pecci et al., 1997).
Table 5.1. Stability constants of the 1:1 and 1:2 Cu-complexes (\( \beta_1 \) and \( \beta_2 \)) of the examined 8-hydroxyquinolines and of histidine

<table>
<thead>
<tr>
<th>ligand</th>
<th>( \log_{10} \beta_1 ) ( ^a )</th>
<th>( \log_{10} \beta_2 ) ( ^e )</th>
</tr>
</thead>
<tbody>
<tr>
<td>oxine</td>
<td>12.0 ± 0.1 ( ^c )</td>
<td>22.9 ± 0.1 ( ^c )</td>
</tr>
<tr>
<td>chloroxine</td>
<td>15.1 ( ^b )</td>
<td>25.8 ( ^b )</td>
</tr>
<tr>
<td>dichloroxine</td>
<td>10.5 ( ^d )</td>
<td>17.6 ( ^d )</td>
</tr>
<tr>
<td>dibromoxine</td>
<td>10.5 ( ^d )</td>
<td>18.1 ( ^d )</td>
</tr>
<tr>
<td>histidine</td>
<td>18.3 ( ^e )</td>
<td></td>
</tr>
</tbody>
</table>

\( ^a \) \( T = 25 \) °C, \( I = 0.1 \) M \( ^b \) (Kaiser & Escher, 2006) \( ^c \) Values in aqueous media from (Martell & Smith, 2004). \( ^d \) Derived from stability constants in a dioxan/water medium from (Gupta et al., 1970). \( ^e \) (Zentmyer et al., 1960)

The assumed dissociation of the copper-8-hydroxyquinoline complexes leads to an ongoing passive diffusion of these complexes into algae along the concentration gradient depending on the external concentration of copper-8-hydroxyquinoline complexes.

The findings of Phinney and Bruland (Phinney & Bruland, 1994) support the hypothesis about a ligand exchange process. They observed a higher uptake of copper-oxine and another hydrophobic copper-organic complex, copper-diethyldithiocarbamate, into the diatom *Thalassiosira weissflogii* than expected from equilibrium calculations under the assumption that uptake is caused by the hydrophobicity of the complexes. Therefore they assumed a ligand exchange process with intracellular compounds. Their study additionally showed a difference in the uptake of metal-diethyldithiocarbamate complexes with Cu, Cd and Pb in dependency on their complex stability. Phinney and Bruland also assumed that the hydrophobic ligand, which is set free by the ligand exchange reaction, becomes protonated within the cytoplasm and may penetrate the cell membrane towards the surrounding medium.

In conclusion of the findings of this study and that of other researchers, the following model for the uptake of hydrophobic copper-8-hydroxyquinoline complexes in unicellular algae is proposed (Figure 5.1). The most important processes that
control the uptake of copper include the copper speciation in the medium, the carrier-mediated transport of Cu\(^{2+}\) and the passive uptake of the copper-8-hydroxyquinoline complexes in the cell, the copper-8-hydroxyquinoline complex speciation in the cytoplasm and the ligand exchange. The copper speciation in solution is predominantly governed by association and dissociation rates of the present copper complexes, among others the copper-8-hydroxyquinolines (\(k_a, k_d\)). The copper transport over the membrane depends on the one hand on the transport rate of the Cu\(^{2+}\) carriers (\(k_t\)) and on the other hand on the partitioning of copper-8-hydroxyquinoline complexes between the aqueous phase and the membrane (\(K_{mw}\)) and on the diffusion rate of them through the membrane from external to internal sorption sites (\(k_{int}\)). In the cytoplasm, the speciation of the copper-8-hydroxyquinoline complexes is again dependent on their association and dissociation rates. The ligand exchange in the cytoplasm is controlled by the dissociation rate of the copper-8-hydroxyquinoline complexes and on the association rate of the new complexes between copper and intracellular ligands (\(k_{ic}\)).
Figure 5.1. Proposed model for the uptake of hydrophobic copper-8-hydroxyquinoline complexes in unicellular algae.

$k_a$, $k_b$ association and dissociation rate constants of the copper-8-hydroxyquinoline complexes, $K_{m_{ew}}$ membrane-water partitioning coefficient, $k_{int}$ diffusion rate constant of the copper-8-hydroxyquinoline complexes through the membrane from external sorption site to internal sorption site, $k_c$ association rate constants of the complexes of copper and internal ligands.
5.1.2. Outlook

For a confirmation of the proposed model about bioavailability of copper-8-hydroxyquinoline complexes for unicellular algae (Figure 5.1) it could be useful to examine the following issue. If a ligand exchange takes place, 8-hydroxyquinoline ligands (L⁻) are set free and may diffuse back to the external medium after protonation (HL). Their ability to partition into biological membranes has been already shown in chapter 2 and the penetration of the neutral species (HL) is most likely. Therefore it should be possible to measure a release of 8-hydroxyquinoline ligands. For that purpose, it is first necessary to develop a measurement method, which can determine 8-hydroxyquinolines in the expected concentration range. The HPLC-detection that was used in the present study (chapter 2) is not suitable, because the detection limit is not low enough. However, the application of an LC-MS/MS may be suitable. An enrichment of the 8-hydroxyquinoline before the measurement may also be considered. Secondly, the release experiment has to proceed in a medium free of 8-hydroxyquinoline and also of Cu²⁺ (and other metal ions) because alternatively the ligand will again form a copper complex. Thus, a copper-8-hydroxyquinoline complex has to be first accumulated in algae. In a second step, algae have to be separated from the accumulation medium, washed and placed in new medium without the 8-hydroxyquinoline and without metals. There, possibly released amounts of 8-hydroxyquinoline can be detected. A problem of this experiment is that the assumed release of HL already takes place during the accumulation of copper-8-hydroxyquinoline complexes and the washing of the algae. Even if a release of HL occurs, it is not sure that there is still HL in the cell, which can be released to the new medium, or that a detectable amount of HL is released.

Furthermore, a separation of different cell components of exposed algae could give information about the localisation of 8-hydroxyquinolines within the cell. For this purpose the development of a separation and extraction method for the cell components of *Scenedesmus vacuolatus* is necessary.

Additionally, for both proposed experiments the application of radioactive labelled 8-hydroxyquinolines could be advantageous because that would ease the observation of their fate and localisation in the cell.
5.2. Toxicity of copper-8-hydroxyquinoline complexes

5.2.1. Concluding remarks

Planned toxicity experiments included the examination of more general toxic effects like growth inhibition and effects, which are known to be caused by copper because the results of the bioavailability experiments indicated that copper-8-hydroxyquinoline complexes serve as transporters for copper into algae. Therefore, special attention was paid to toxic effects on photosynthesis, one of the main processes where copper exerts toxic effects.

For two reasons, the chosen toxicity tests either did not detect toxic effects of copper-8-hydroxyquinoline complexes on the tested biological endpoints or were not feasible. First and most importantly, the water solubility of the copper-8-hydroxyquinoline complexes is very low ($< 10^{-7}$ M). Thus, concentrations used in the toxicity experiments on photosynthesis were low and the same concentrations of Cu$^{2+}$ also did not cause measurable effects, indicating that water-soluble concentrations of copper-8-hydroxyquinoline complexes do not lead to toxic effects on photosynthesis. Secondly, the experimental medium had to be free of metals, so that no competition between them and the added copper took place. In the case of the Kinspec experiments this condition prohibited the application of a necessary redox buffer and thus made the experiment impossible for copper-8-hydroxyquinoline complexes because the used chromatophores could not maintain the redox potential that was necessary for an unhindered electron transfer. For the growth inhibition experiments, the trace metal free medium shortened the possible time over which experiments could be conducted, because the lack of essential trace elements led to a decrease of the chlorophyll content of the algae cells over time. Therefore, a 72 h OECD growth inhibition test (OECD, 1984) was not accomplishable. However, growth inhibition tests over time spans of 24 h or shorter like that proposed by Meene (Meene, 2005) seemed to be possible, because the inhibition of the quantum yield of Photosystem II of the algae at 24 h was merely around 20 % (see chapter 4.2).
5.2.2. Outlook

Further experiments on the toxicity of copper-8-hydroxyquinoline complexes on photosynthesis may be conducted with cosolvents like ethanol or dimethyl sulfoxide (DMSO) to increase available amounts of copper-8-hydroxyquinoline complexes. An important prerequisite would be the knowledge about the solubility of copper-8-hydroxyquinoline complexes under the experimental conditions. Simultaneously, it would be necessary to ensure that the used concentration of cosolvent does not negatively affect the biological endpoint that is tested.

However, it is questionable whether an application of cosolvents is reasonable. The aim of the conducted studies was to examine whether copper-8-hydroxyquinoline concentrations, which may be found in the aquatic environment, have toxic effects on the photosystem of algae. This was already tested in this study and a test system with cosolvents would not represent the aquatic environment. However, in the aquatic environment a delivery of copper-8-hydroxyquinoline complexes from precipitates may occur.

It is yet still interesting to continue the search for mechanisms, which cause toxic effects of copper-8-hydroxyquinoline. Due to the fungicidal and antimicrobial properties of copper-oxine, it is known that toxic effects on organisms exist. A further examination of toxic effects of copper-8-hydroxyquinolines should focus on other processes than photosynthesis, e.g. on the inhibition of enzymes. Florence & Stauber (Florence & Stauber, 1986) reported that they observed a strong inhibition of glutathione reductase by copper-oxine. Nearly the same effect was measured for an equimolar concentration of oxine. An equimolar concentration of Cu²⁺ had a smaller effect on glutathione reductase, but deactivated catalase to a distinctively higher extent than oxine or copper-oxine. However, the results for copper-oxine are to be considered with caution because the tested concentration of $4 \cdot 10^{-5}$ M was more than two orders of magnitude higher than the solubility limit (< $10^{-7}$ M).
5.3. Environmental significance of copper-8-hydroxyquinolines

Due to the high stability constant of copper-8-hydroxyquinoline complexes, they may be formed in water bodies, which are polluted with copper and 8-hydroxyquinolines. Additionally, copper-oxine is widely used as fungicide in agriculture and for preserving goods made of wood or textile (Yeager, 1991; Odanaka et al., 1994; Kikuchi et al., 1996; Nicoletti et al., 1999; Schultz et al., 2005).

While the predominant uptake route for copper into cells is carrier-mediated transport, the present study showed that hydrophobic copper-8-hydroxyquinolines enter cells via passive diffusion along a concentration gradient, which could be proven by their partitioning into liposomes and their uptake into algae. These findings indicate that it is important not only to regard hydrophobic organic ligands as modulators of the speciation of copper and thus of the free copper concentration in aqueous solution, but also to consider hydrophobic copper-8-hydroxyquinoline complexes as bioavailable species. Depending on the conditions in a given aquatic environment and thus on the speciation of copper, passive uptake of copper-8-hydroxyquinoline complexes may be a considerable source of copper to organisms. By reason of their bioavailability it is also necessary to consider possible effects caused by them.

5.4. Transferability of the findings about copper-8-hydroxyquinolines to other hydrophobic metal-organic complexes

The findings about the bioavailability of hydrophobic copper-8-hydroxyquinolines may be partially transferred to other hydrophobic metal-organic complexes. It is however required that the complexes are stable enough to be taken up into a cell. For the penetration of the cell membrane hydrophobicity, polarisation and molar volume are of crucial importance. However, for the passive diffusion of other hydrophobic metal-organic complexes those molecular properties may be of different importance or additional properties may play a role. It is to be assumed that the proposed ligand exchange processes in the cytoplasm also take place for other metal-organic
complexes, because the involved internal ligands may not be specific for copper-8-hydroxyquinolines. The processes are furthermore likely to be dependent on the dissociation rate constants of the considered metal-organic complexes. Therefore, the ligand exchange processes may also be important factors for the bioavailability of other hydrophobic metal-organic complexes.

In conclusion, the obtained results for the copper-8-hydroxyquinolines are transferable to other metal-ligand complexes with similar molecular properties and may serve as a starting point for considerations about those with distinctively other properties.
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