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Effect of environmental conditions on biouptake of Cu and Pb from natural freshwaters by *Chlamydomonas reinhardtii*: a case study, Litani River, Lebanon

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ABSTRACT

Of the unicellular green algae species, *Chlamydomonas reinhardtii* has only recently gained greater attention for the treatment of heavy metal contamination in aqueous solutions. In the present study, *C. reinhardtii* was used to remove Cu and Pb metal ions from the Litani River in Lebanon. Three samples were collected from three different sites located along the tributaries of Litani. Short-term (40 min) metal biouptake experiments were performed using 0.2 g L^{-1} of freeze-dried and living algal cells. In the studied samples, dead cells showed higher removal efficiency for both metal ions than living cells. The percentage removal of dead algae for Cu (28.66%) is about 1.8 times higher than that of living cells (16%). For Pb, biosorption (23.4%) is about 2.75 times higher than bioaccumulation (8.5%). Water chemistry significantly affects metal speciation, bioavailability, and biouptake by living and dead cells. As predicted by Visual MINTEQ program, only very small fractions of Cu and Pb were present as free ions with the majority being bound to humic substances (HS) or carbonate ligands. $PbCO_3$ and $CuCO_3$ dominated in water samples containing very low amount of organic compounds. As stated by the free ion activity model (FIAM) and the biotic ligand model (BLM), Cu uptake seems to increase with an increase in the free Cu^{2+} concentration in water and it is dependent on ligands complexation and co-cations competition effects. However, equilibrium models cannot explain the bioaccumulation results obtained for Pb. Although further investigations are needed, the obtained results are very promising as a starting point for a potential application of these micro-organisms as an efficient and economic biomaterial for the removal of heavy metals from metal-contaminated freshwaters.

Keywords: Bioaccumulation; Biosorption; *Chlamydomonas reinhardii*; *Copper*; Lead; Natural freshwaters; Litani River; Metal speciation

1. Introduction

Heavy metal pollution has become one of the most serious environmental problems today. Heavy metal ions are of great concern, due to their mobility in natural water ecosystems and due to their toxicity even at relatively low concentrations [1]. The multilayer cell walls and the presence of a large number of diverse functional groups on algal biomass make it highly suitable candidate to be used as a cheap source of adsorbents for heavy metal removal. The role of algae for wastewater treatment and their affinity for heavy metal ions have been demonstrated in several studies [2–8]. The unicellular green algal species, *Chlamydomonas reinhardtii* have only recently gained greater attention for the treatment of heavy metal contamination in aqueous solutions in spite of their presence in freshwater ecosystems and their central role in the fixation and uptake of heavy metals. These species are often used in studies of metal transfers from freshwater phytoplankton to herbivorous organisms [9]. Recent studies revealed that living and dead algal cells of *C. reinhardtii* are suitable for the removal of Cu (II), Pb(II) and Cd (II) ions from monometallic and multi-metal systems [10–16]. It was shown that biouptake processes are much more dynamic in complex multi-metal systems than simple monometallic systems. However, these studies do not pertain directly to real wastewaters composed of a mixture of metals and inorganic and organic ligands. In most aquatic ecosystems, only very small proportions of dissolved metals are present as free hydrated ions with the majority of the metal being complexed by ligands or being adsorbed on the surfaces of particles and colloids [17]. The equilibrium among all these metal species is interchangeable and depends on such environmental factors as temperature, pH, and alkalinity as well as on the biota thriving in the water [18,19]. Over the past few years, equilibrium models, the free ion activity model (FIAM) and the biotic ligand model (BLM) have been increasingly developed in order to quantitatively predict the manner in which water chemistry affects the speciation and biological availability of metals in aquatic systems. Because the BLM, assumes that the metal species in solution and those adsorbed to the biological surface are at thermodynamic equilibrium, the interactive effect of a binary metal mixture is necessarily antagonistic, i.e. in the presence of a second ion, lower (or unchanged) uptake or biological effects is always predicted [20–25]. The FIAM predicts that the bioavailable free metal ion, rather than the total metal concentration, is the determinant of the metal bioaccumulation and toxicity [22,26]. According to these models, complexation and competition processes, could result in a decreased interaction of the metal with the uptake sites on the surface of the organism [20–26]. The study of the relationships between micro-organisms and metals in natural waters are essential for the formulation of relevant water quality criteria. One of the major problems arising in Lebanon is the metal pollution of the Lebanese rivers. One of the rivers suffering the most from this type of pollution is the Litani River and its tributaries. The contamination of Litani by heavy metals was reported and has been attributed to agricultural, industrial, and other anthropogenic activities (sewage network outlets, municipal solid waste dumping, food-processing plants, and animal-raising farms) around the river [27]. In this work, the unicellular green alga *C. reinhardtii* were used to remove Cu (II) and Pb(II) from natural freshwaters ecosystems mainly due to the relative ease by which it is possible to culture and to grow. It is also a useful species due to an existing complete knowledge of its genetic makeup [28]. Water samples were collected from three different sites along the tributaries of the Litani. The physicochemical and biological characteristics of each water sample have been analyzed to assess the effect of environmental conditions and water properties and composition on the removal efficiency of dead and living algal cells. Cu and Pb metal ions selected for this study are important contaminants of estuaries and freshwaters and exhibit different chemical and biological characteristics in the aquatic environment (e.g. speciation, reactivity, essentiality, toxicity). A study of the environmental factors that control metal bioavailability and reactivity is sorely needed as society strives to manage resources, restore ecosystems, and ameliorate the effects of metal pollutants such as lead and copper to the environment.

2. Materials and methods

2.1. Test organisms and growth conditions

Chlamydomonas reinhardtii (wild type 2137 mt$^+$), a unicellular green alga, was used for this study. Algal cells were transferred from a week-old Tris-acetate-phosphate (TAP) agar plate to a (4×) diluted TAP solution ($I = 10^{-2}$ M) [29]. Cells were grown in an incubation chamber at 20°C, under a 12:12 h light: dark regime using fluorescent lighting (80 μmol photons m^{-2} s^{-1}) and rotary shaking (100 rpm) until a density of $(2–3) \times 10^6$ cells mL^{-1} was achieved, generally after 4 d. Cells were then diluted in fresh media to 1.0×10^5 cells mL^{-1} and once again allowed to attain logarithmic growth. Once cells attained mid-exponential growth ($(1–3) \times 10^6$ cells mL^{-1}) generally after 2 more days, they were harvested by centrifugation (3,700 × g, 4 min) into 50 mL of sterile (polypropylene) centrifuge tubes, resuspended in 10^{-2} M MES (2-(*N*-morpholino)ethanesulfonate, sodium salt, Sigma) at pH 6, centrifuged and harvested a second time, then resuspended in exposure media. Cell density was determined using a UV–visible spectrophotometer. The optical density of a cell culture was measured at 665 nm. All manipulations of algal cultures were performed under laminar flow conditions and all bottle borders were flame sterilized.

2.2. Sampling sites

Water samples were collected in May 2014 from three sites S_1 (Qabb Elias), S_2 (Ghzayyel), and S_3 (Chtaura) along the tributaries of the Litani, which is the longest (170 km) river in Lebanon. The river's source is the Al Oleik spring in the Bekaa Plain (west of Baalbeck). Then Litani River flows southward, between Mount Lebanon and the Anti-Mount Lebanon ranges, before turning westward to discharge into the Mediterranean Sea 7 km north of Tyre city. The climate around the river is semiarid characterized by moderately cold winters and the summers that are distinctly dry and hot. In Lebanon, there is usually little or no precipitation throughout the country between June and August, while the heaviest rainfall occurs between November and April. The annual precipitation rate is high (800 mm) in the Litani basin [27]. Geomorphologically, the Litani basin is divided into three sub-basins and the largest is the upper one stretching from the Bekaa Plain to the Qaraaoun Dam (built between 1958 and 1965).

2.3. Water collection

Sampling was conducted in the dry season of the year 2014 when minimal dilution of waterborne pollutants can be assumed. Sampling sites were selected after a preliminary survey. The three sites S_1–S_3 are along the tributaries of Litani River. Water samples were collected at each sampling site at mid depth from the surface using automatic sampling device, and then water transferred to 1-L polyethylene bottles. The collected water samples were immediately filtered through 0.45-μm pore cellulose filter by vacuum suction and divided into two portions, each in 1-L polyethylene bottle. One bottle was stored in the dark for biological and chemical analysis. The other bottle was sterilized prior to biouptake experiments. The method of sampling and collection are in accordance with Standard Methods APHA, AWWA, WPCF (1992) [30].

2.4. Water analysis

Temperature, electrical conductivity (EC_w), total dissolved solids (TDS), and pH were measured *in situ* using (Hach Model 44600 Conductivity/TDS meter, resolution conductivity 0.1 μS cm^{-1}, TDS 0.1 mg L^{-1}). A Turbiquant 3,000 IR turbimeter was used to measure the turbidity. The anions NO_2^-, NO_3^-, Cl^-, SO_4^{2-}, and PO_4^{3-} were analyzed by ion chromatography (Ion Chromatography, "Shimadzu"-Shim-pack IC-A3S). Total hardness was measured by titration with ethylenediaminetetraacetic acid (EDTA). The analysis of total carbon (TC), inorganic carbon (IC), and organic carbon (OC) was carried out with total organic carbon analyzer (Formacs^{HT} TOC Analyser, Autosampler LAS-160 SKALAR). Biological oxygen demand (BOD) and chemical oxygen demand (COD) were measured as an indicator of organic pollutants in water using standard methods [31]. Analysis of metals (Ca, Mg, Na, K, Fe, Cr, Co, Ni, Cd, V, Se, Tl, Ba, Cu, Pb) was determined by ICP–MS (Thermo Scientific XSERIES 2). The speciation of Cu and Pb in water samples was predicted using the software Visual MINTEQ v. 3.0.

2.5. Bioaccumulation measurements

In the bioaccumulation experiments, metabolically active living cells were used in the concentration of 0.2 g L^{-1}. Bioaccumulation was quantified in short-term experiments (40 min) in order to decrease the likelihood of physiological changes in the algae or physicochemical changes in the medium (e.g. decreasing metal concentrations, increasing complexation with extracellular ligands, production of exudates). Metal uptake was stopped by adding 5 mL of 10^{-2} M ethylenediaminetetraacetic acid (EDTA in 10^{-2} M MES at pH 6.0) to natural water samples [20]. Internalized or bioaccumulated metal concentrations were determined following the digestion of the filtered (5.0-μm polyvinylidene fluoride membrane, Millipore) EDTA/MES-washed algae using 0.3 mL of concentrated (65%) ultrapure HNO_3 at 85°C until solutions became colorless. Bioaccumulation capacity q (mg metal ions/g active biomass or living cells) was determined using the following equation:

$$q = \frac{(C_{\text{int}}) \times V}{m} \tag{1}$$

where C_{int} is the internalized metal concentration (mg L^{-1}) after bioaccumulation; V is the volume of the aqueous phase (L), and m is the amount of the active biomass (g). In order to evaluate biological and analytical variability, each experiment was repeated three times using independent cultures of *C. reinhardtii*. The experimental results given are the average values.

2.6. Biosorption measurements

Algae cells were harvested by centrifugation and washed with Milli-Q water three times. The biomass was freeze-dried (lab-made equipment at −100°C, 35 h) and milled to a final particle size of 1 mm. Short-term biosorption tests (40 min) were performed using dead biomass concentration of 0.2 g L^{-1}. Biosorption capacity q (mg metal ions/g dry biomass or dead cells) was obtained using the following equation:

$$q = \frac{(C_0 - C) \times V}{m} \qquad (2)$$

where C_0 and C are the concentrations of the heavy metal in the initial solution (mg L^{-1}) and after biosorption, respectively; V is the volume of the aqueous phase (L), and m is the amount of the dry biomass (g). Each biosorption experiment was repeated three times and the experimental results given are the average values. The percent removal of metal ions by dead or living algal cells was calculated using the following equation:

$$\text{Removal}(\%) = \frac{q}{(C_0 - 0.2)} \times 100$$

where q is the biosorption or bioaccumulation capacity (mg metal ions/g algal cells); C_0 is the concentration of the heavy metal in the initial solution (mg L^{-1}), and 0.2 is the concentration of algal cells in the exposure solution (g L^{-1}).

3. Results and discussion

3.1. Analysis of water samples

3.1.1. Physicochemical and metal analysis

Table 1
Physicochemical parameters and metal analysis of water samples

Parameters	S_1	S_2	S_3
Temperature (℃)	24.9	25.6	26.1
pH	7.38	7.4	7.5
ECw (µS cm^{-1})	756	476	500
Turbidity (NTU)	1.41	0.4	0.2
TDS (mg L^{-1})	483.84	304.64	320
SPM (mg L^{-1})	4.94	2.71	3.7
Total hardness	119.47	78.83	93.75
Ca^{2+} (mg L^{-1})	86.21	60.89	57.55
Mg^{2+} (mg L^{-1})	33.26	17.94	36.2
Na^{+} (mg L^{-1})	7.1	3.7	2.4
K^{+} (mg L^{-1})	2	0.6	0.4
Cl^{-} (mg L^{-1})	15.02	9.01	7.01
NO_3^{-} (mg L^{-1})	33.6	2.12	6.82
SO_4^{2-} (mg L^{-1})	21.25	19.5	9.75
NH_4^{+} (mg L^{-1})	0.047	0.102	0.052
PO_4^{3-} (mg L^{-1})	0.05	0	0.03
Fe^{3+} (mg L^{-1})	0	0.005	0.38
NO_2^{-} (mg L^{-1})	0.019	0.016	0.017
COD (mg L^{-1})	6.2	29.21	0
BO_5D (mg L^{-1})	9	8	8
TC (mg L^{-1})	38.37	53.43	42.3
TOC (mg L^{-1})	1.32	0	0.28
IC (mg L^{-1})	37.05	53.43	42.02
Cr (µg L^{-1})	10.5	10.25	10.25
Co (µg L^{-1})	0.6	0.55	0.55
Ni (µg L^{-1})	8.70	3.1	3.85
Cd (µg L^{-1})	16.15	2.65	3.55
V (µg L^{-1})	0.173	1.731	0.794
Se (µg L^{-1})	1.044	0.829	0.588
Tl (µg L^{-1})	0.014	0.015	0.006
Ba (µg L^{-1})	28.30	13.95	9.01
Cu (µg L^{-1})	52.30	52.70	50.55
Pb (µg L^{-1})	73.95	60.43	62.95

The main physicochemical properties and metal analysis of each water sample studied of the Litani River are listed in Table 1. All water samples have neutral pH (7.4–7.5), optimal temperature values (24–26℃), and low conductivity levels (50–1,500 µS cm^{-1}). Water pH affects significantly metal bioavailability and toxicity in natural environments. Acidification affects metal availability to algae not only by proton inhibition of facilitated metal uptake but also by affecting membrane permeability [32]. Other parameters that can affect metals biovailability are those that can effect metal speciation and mobility such as total hardness, cations, dissolved solids (TDS, SPM), anions, and organic compounds (TOC). The highest levels of these parameters are detected in water sample S_1 (Table 1). Calcium, magnesium, sodium, and other metal ions can interact as competitors, thereby reducing metal bioaccumulation by algae [14,21–23]. Suspended solids can accumulate high amounts of metals which reduces metals mobility and bioavailability. Anions such as Cl^{-} and SO_4^{2-} tend to form inorganic complexes with metal ions, which can significantly influence metals iternalization and biological effects [24,25]. In addition, in real aquatic environments, dissolved organic matter influences metal bioavailability by decreasing the amount of metal ion available for the biotic ligand. Whereas bioavailability of metals depends on the type of organism and route of exposure, dissolved metal speciation and the solubility of particles (both outside and inside the organism) are two properties that influence accessibility to exposed organisms [18,33]. As shown in Table 1, water sample S_1 is also the richest site in nitrate and phosphate. This is mainly due to the fact that mostly agricultural fertilizing activities line the path of S_1 compared to the paths of the other river sampling sites. It can be deduced that the eutrophication phenomenon is most pronounced in S_1. This is indicated by the high levels of biochemical oxygen demand after 5 d (BOD_5) detected in S_1 compared to water samples S_2 and S_3 (Table 1). Eutrophication was obvious in many of the Lebanese rivers [34]. On the other hand, of the metals studied, levels of Pb and Cu were the highest in the three water samples (Table 1). Lead emissions are mainly from the combustion of tetraethyl lead as an additive in fuels. The pollution of copper is mainly due to agriculture activities [27]. Nonetheless, the interpretation of bioaccumulation and toxicity results from metal mixtures is complex because there are chemical interaction with constituents in the media, interactions with physiological processes, and interactions at the site(s) of toxicity [13,14]. These interactions vary depending on the metals, their concentrations, the exposure pathway and duration, and the species and organs studied. The speciation of metal forms in water provides information on the potential availability and toxicity of metals to particular aquatic organism under various environmental conditions.

3.1.2. Metal speciation

The speciation of dissolved metals in each water sample was determined using Visual MINTEQ (v. 3.0). Water physicochemical parameters and metal content (Table 1) were used as input data for model calculations. The properties of organic compounds were modeled according the Stockholm Humic Model (SHM) with the humic substances (HS) defined entirely as fulvic acid (FA). Fig. 1 shows the speciation variation (% total metal concentration) of Cu (Fig. 1(a)) and Pb (Fig. 1(b)) in the studied water samples of Litani River. Visual MINTEQ program with HS input as 100% fulvic acid predicted that 56% of Cu and 29% of Pb were present as metal–humic species in water sample S_1 (Fig. 1). However, only very small fractions of Cu (0.8%) and Pb (0.5%) were bound to FA in water sample S_3 containing lower amount of organic compounds compared to S_1 (Table 1). Measurements of trace metal species *in situ* in natural freshwaters were compared to the metal speciation calculated using two models, WHAM 6 and Visual MINTEQ [35]. According to the modeling speciation results, more than 99% of Cu and Pb were predicted

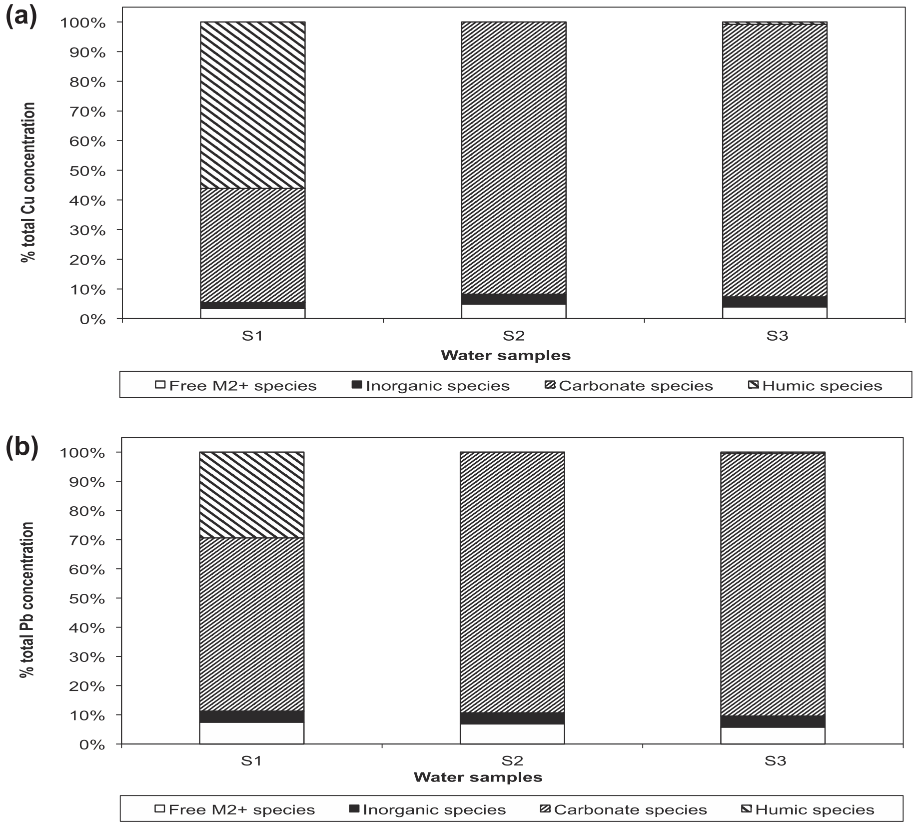

Fig. 1. Speciation of Cu (a) and Pb (b) in the studied water samples modeled by Visual MINTEQ.

Table 2
Distribution of dominant metal species in the studied water samples

	Cu		Pb	
	%	Mol L^{-1}	%	Mol L^{-1}
Sample S_1				
Free ion M^{2+}	3.4	2.792×10^{-8}	7.5	2.673×10^{-8}
MOH^+	1.9	1.574×10^{-8}	3.4	1.198×10^{-8}
MCO_3 (aq)	37.6	3.095×10^{-7}	47.8	1.705×10^{-7}
$MHCO_3{}^+$	0.4	3.265×10^{-9}	11	3.944×10^{-8}
M–HS	56	4.623×10^{-7}	29.3	1.05×10^{-7}
Sample S_2				
Free ion M^{2+}	4.9	4.067×10^{-8}	6.9	2.013×10^{-8}
MOH^+	3.1	2.572×10^{-8}	3.4	1.0016×10^{-8}
MCO_3 (aq)	89.5	7.410×10^{-7}	72.5	2.110×10^{-7}
$MHCO_3{}^+$	0.9	7.373×10^{-9}	15.8	4.6×10^{-8}
M–HS	0	0	0	0
Sample S_3				
Free ion M^{2+}	4	3.873×10^{-8}	5.8	2.155×10^{-8}
MOH^+	3.2	3.119×10^{-8}	3.6	1.354×10^{-8}
MCO_3 (aq)	89	6.977×10^{-7}	75	2.223×10^{-7}
$MHCO_3{}^+$	0.7	5.890×10^{-9}	13	3.988×10^{-8}
M–HS	0.8	9.178×10^{-9}	0.55	2.017×10^{-9}

to be bound to FA in natural freshwaters using the two models with HS input as 100% FA. In addition, it was shown that the free metal ions dominated the inorganic species in softwater, while both free metal ions and carbonate species were important in hard waters [35]. In this study, carbonate species dominated the inorganic species and free metal ions in all water samples (Fig. 1). More than 89% of Cu and Pb were present as carbonate species in water samples S_2 and S_3. Smaller carbonate fractions of Cu (38%) and Pb (59%) were predicted in water sample S_1. The dominant carbonate species were $PbCO_3$ and $CuCO_3$. The concentration of $PbCO_3$ was the highest in all water samples. However, Cu–HS dominated the carbonate species in water sample S_1. As shown in Table 2, a fraction of carbonate species of Pb such as $PbHCO_3{}^+$ was present with 10–16% in all water samples. However, the fraction of $CuHCO_3{}^+$ was very low (<1%). The dominant inorganic species of Cu and Pb were $CuOH^+$ and $PbOH^+$ in all water samples (Table 2).

3.1.3 Biological analysis

Biological quality is evaluated by the presence of micro-organisms in the three samples of the Litani River. The results are shown in Table 3. Significant levels of aerobic germs are found in all water samples of Litani. The highest levels are obtained in S_1, while S_2 has consistently the lowest bacterial levels (Table 3). These germs are witness of fecal contamination of human origin and manure and animal excrements. Most of the microbial pollution is attributed to human fecal matter. This is due to the fact that Lebanon is one of the most densely populated countries. In addition, the presence of *E. coli* is a direct result of the fact that, to date, wastewater is still flowing untreated into rivers and valleys across the Lebanese territories [34]. Metal speciation is largely affected by bacterial activities; whenever bacterial activity increases, environmental conditions can become anoxic and reducing; metals then tend to precipitate as metal sulfides.

3.2. Bioaccumulation of Cu and Pb

Fig. 2 shows the bioaccumulation capacities of metabolically active living cells of *C. reinhardtii* for Cu and Pb metal ions in the three natural water samples. The bioaccumulation capacities of living algal cells for both metal ions are the highest in water sample S_2. However, the lowest potential uptake by algal cells for both metal ions is observed in sample S_1 (Fig. 2). These results can be attributed to the properties and compositions of water samples (Table 1) and their effects on metal speciation (Table 2). The presence of organic compounds in sample S_1 can form metal complexes and so provide alternative binding site for the metal

Table 3
Bacteriological results in Litani water samples in dry season

Amount of micro-organisms (CFU/mL)	S_1	S_2	S_3
Aerobic germs (22˚C–72 h)	100	10	90
Aerobic germs (37˚C–24 h)	90	<10	10
Total coliformes (37˚C–24 h)	0.01	0.01	<0.01
Fecal coliformes (44˚C–24 h)	<0.004	<0.004	<0.004
Escherichia coli (44˚C–24 h)	<0.004	<0.004	<0.004
Streptocoques group D (37˚C–24 h)	<0.004	<0.004	<0.004
Pseudomonas aeruginosa (37˚C–24 h)	<0.004	<0.004	<0.004

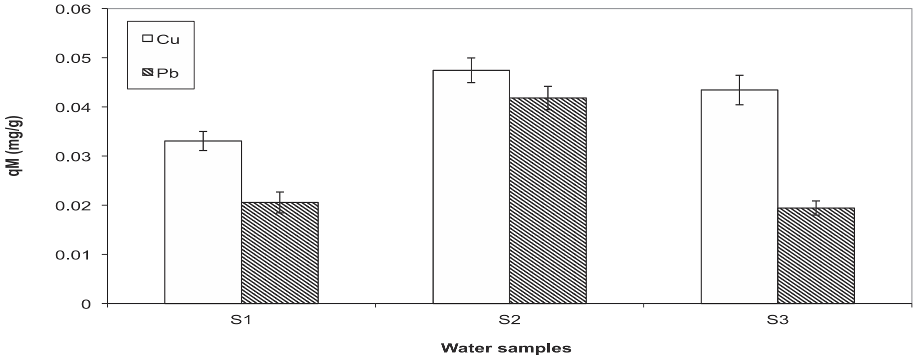

Fig. 2. Bioaccumulation capacities of *C. reinhardtii* for Cu and Pb ions in Litani freshwater samples.

ion. Also, the high levels of dissolved cations (e.g. Na, Ca) in S_1 (Table 1) can lower metals bioavailability due to competition effects [24]. Cu and Pb were present in sample S_1 with 56 and 30% of the metals, respectively, being bound to humic substances (Fig. 1). However, 90% of Cu and Pb were present as carbonate species in water samples S_2 and S_3. Metal bioavailability and biological effects are most often correlated with the concentration of non-complexed metal [36]. Indeed, in many studies, relationships between the free metal ion concentration and metal uptake by aquatic organisms have been reported, even in the presence of complexing ligands [21–23]. The presence of organic matter and humic substances especially reduces the Cu toxicity to aquatic organisms following a reduction of the concentration of free Cu^{2+} ions in the medium [37–39]. Increased pH, hardness, sodium, dissolved organic matter, and suspended solids each caused bioavailability and toxicity to decrease on the basis of total copper concentrations [39,40]. Previous results have shown a reduced Cd (II) uptake in the presence of fulvic and humic substances for the same algae *C. reinhardtii* under well-defined laboratory conditions [29,41]. Recently, it was shown that the increase in intracellular Cd, Cu, and Pb contents in microalga *Chlamydomonas reinhardtii* and that metal bioavailability were directly related to the measured free metal ions concentrations [25]. On the other hand, it can be seen in Fig. 2 that the bioaccumulation potential of algal cells was higher for Cu than for Pb in the three water samples ($q_{Cu} > q_{Pb}$). As shown in Table 2, the concentration of free metal ion Cu^{2+} was higher than the concentration of free Pb^{2+} in all water samples. In addition, the highest concentration of free Cu^{2+} was predicted in sample S_2 ($[Cu^{2+}]_{free} = 4.067 \times 10^{-8}$ mol L^{-1}). Also, the highest value of q_{Cu} was observed in sample S_2 (Fig. 2). Bioaccumulation of Cu in the three water samples can be explained by equilibrium models such as BLM and FIAM. As the concentration of free Cu^{2+} increases, the bioaccumulation capacitiy of Cu increases. However, the variation of bioaccumulation capacities of Pb with an increase in the free Pb^{2+} concentration is not in agreement with equilibrium models. The highest bioaccumulation capacity of Pb (q_{Pb}) was observed in sample S_2 (Fig. 2), where the lowest concentration of free metal ion ($[Pb^{2+}]_{free} = 4.067 \times 10^{-8}$ mol L^{-1}) was predicted (Table 2). Recent studies on the bioaccumulation of Cu and Pb by *C. reinhardtii* have shown that living algal cells have the same bioaccumulation capacity for Cu and Pb in simple monometallic systems.

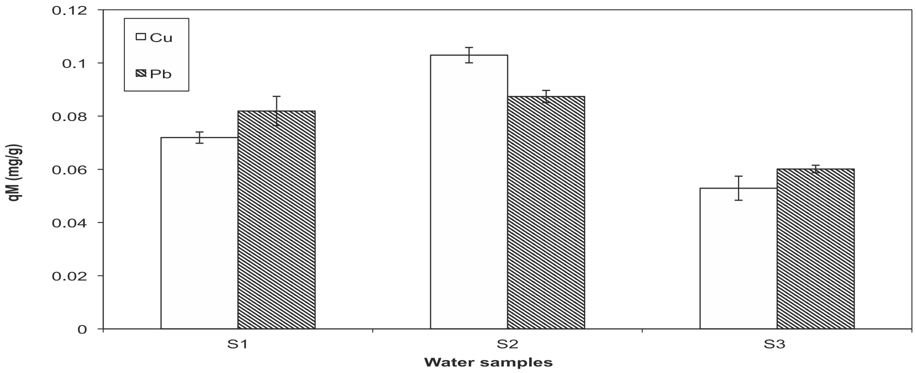

Fig. 3. Biosorption capacities of *C. reinhardtii* for Cu and Pb ions in Litani freshwater samples.

However, synergistic and antagonistic effects between copper and lead were observed in binary metal systems [14–16]. Moreover, no competitive effect of Pb at low concentration on Cu bioaccumulation was observed implying that the ability of algae to uptake copper is greater in the presence of lead [16]. These observations clearly evidence the role of metal speciation on metal bioavailability and internalization by active living algal cells in natural freshwaters.

3.3. Biosorption of Cu and Pb

Fig. 3 shows the biosorption capacities of non-living algal cells of *C. reinhardtii* for Cu and Pb metal ions in each water sample of the Litani River. The presence of functional groups responsible for biosorption of Cu and Pb metal ions on algal biomass surface was identified by FT-IR analysis in a previous study [42]. The highest biosorption capacities of *C. reinhardtii* for both metal ions are observed in sample S_2, whereas the lowest are observed in S_3 (Fig. 3). In addition, dead algal cells seem to have higher affinity to Pb than to Cu ($q_{\mathrm{Pb}} > q_{\mathrm{Cu}}$) in water samples S_1 and S_3 (Fig. 2). Whereas, in water sample S_2 where TOC is 0 mg. L^{-1} (Table 1), dead algal cells have higher affinity to Cu than to Pb (Fig. 3). The presence of organic matter affected metal speciation of Pb and Cu. As predicted by Visual MINTEQ program, organic species such as Cu–Hs and Pb–HS dominated in water sample S_1. In addition, the concentration of Cu–HS was more than four times higher than the concentration of Pb–HS species in water samples S_1 and S_3. However, carbonate species of Cu and Pb dominated in water sample S_2 and S_3. The concentration of $CuCO_3$ was more than 2.5 times higher than the concentration of $PbCO_3$ (Table 2). These results show that environmental conditions and water composition play an important role also on metal uptake by dead algal biomass. Organic compounds may serve as complexing agents for metal ions, thereby affecting metal availability and affinity to algal binding sites. The binding of metal ions to cell wall components of microalgae was well reported in the literature [5,43–47]. Biosorption is a non-metabolic, rapid process dependent on a number of parameters: pH, heavy metal species, type of algae, and biomass concentration [7,8,44–47]. It has been well recognized that pH has significant effects on solubility, speciation, and biosorption capacity of heavy metals [2,44,45]. In fresh natural waters, the affinity of heavy metals to algal surfaces is somewhat reduced because of the presence of Ca^{2+} and Mg^{2+} in large excess [46]. In addition, anions may clearly affect biosorption through the formation of insoluble metal precipitates and the formation of complexes [47]. Other important factors that determine overall biosorption performance include metal concentration, as well as the concentrations of coexisting metal(s), where competitive effects may occur, and the availability of surface ligands [7]. Ligand preferences in metal complex formation are highly relevant for understanding biosorption. Ligand-binding sites on the external or internal surfaces of organisms are often soft base and thus bond strongly with soft and borderline soft acid cations such as Cu^{2+} and Pb^{2+}. Soft metals (acids) bind preferentially with soft S and N ligands, forming weaker bonds with hard base species such as hydroxide and sulfate. The tendency of Cu and Pb metals ions to bind to soft ligands or to organic substrates (which are usually soft), based on their HSAB qualities, follows the order $Pb^{2+} > Cu^{2+}$. The hard/soft scheme predicts that bonds formed between hard acids and hard ligands will be predominantly ionic, whereas soft acid–ligand complexes are more covalent in character, which has significant effect on metal biosorption [7].

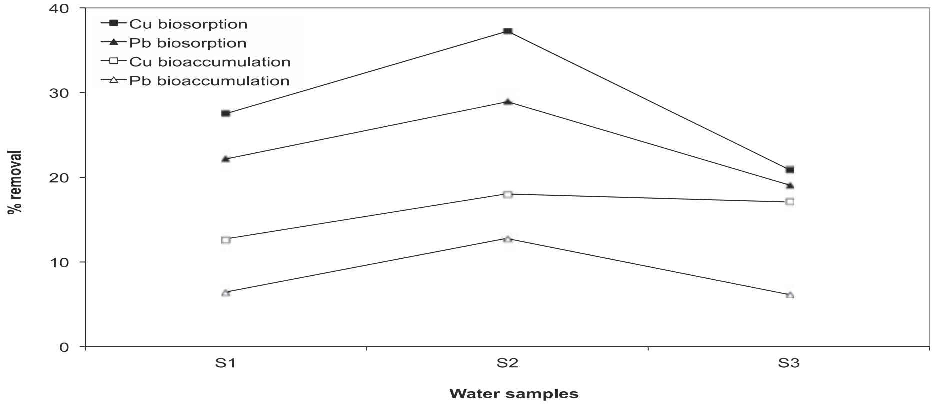

Fig. 4. Percentage removal efficiency of dead and living cells of *C. reinhardtii* for Cu and Pb ions in Litani freshwater samples.

3.4. Comparison between bioaccumulation and biosorption capacities

The efficiency of dead and living algal cells *C. reinhardtii* for the removal of Pb(II) and Cu (II) ions from natural environments are represented in Fig. 4. For both tested metal ions, the percentage removal of metals by living cells is well below the percentage uptake of metals by dead algal biomass. The mean percentage of Cu (II) uptake by algal biomass (28.66%) in the three water samples of Litani River is approximately 1.8 times higher than the average percentage of Cu (II) uptake by living cells (16%). For Pb(II), the average percentage of biosorption removal (23.4%) is approximately 2.75 times higher than the percentage of bioaccumulation (8.5%) (Fig. 4). Dead algal cells are more effective than active living cells of *C. reinhardtii* for the removal of Pb and Cu from natural freshwaters. These results can be compared to previous results obtained in our laboratory for the bioaccumulation and biosorption of Cu (II) and Pb(II) ions by *C. reinhardtii* in single and binary metal systems [16]. This can be related to the metabolically active mechanism of bioaccumulation process, which limits the internalization of the metal through the cell wall [3,20]. However, biosorption is a physicochemical and metabolically independent process based on a variety of mechanisms including absorption, adsorption, ion exchange, surface complexation, and precipitation. On the other hand, the removal efficiencies of dead and living algal cells for Pb and Cu obtained in natural environments are lower than the values obtained previously in our laboratory in simple monometallic systems [16]. As predicted by the equilibrium models FIAM and BLM, in natural waters, the bioavailability of free metal ion is reduced due to competition and complexation effects. According to equilibrium models, the free metal ion concentration [M^{n+}] has been reported to be often a good predictor of ecotoxicity in freshwaters. Accurate determination of [M^{n+}] in freshwaters is an important requirement for studying the interactions of metals with natural organic ligands in freshwaters, and also for the purpose of correlating the free metal ion concentration with ecotoxicity.

4. Conclusion

The results of this work indicated that viable and non-viable algal cells of *C. reinhardtii* are capable of effectively removing lead (II) and copper (II) from natural water environments. Dead algal cells showed higher removal efficiency than living cells and intracellular accumulation of metal ions seems to be limited by living algal cells. Environmental conditions were found to affect considerably the biosorption and bioaccumulation capacities of algal cells. In addition, chemical composition of water plays an important role in metal speciation, biovailability, and biouptake. The decrease in metal uptake was more pronounced in the water sample containing the highest levels of organic and inorganic ligands. The free or aquo ion seems to be more biologically active than complex metal ion. Bioaccumulation results obtained for Cu seem to be qualitatively consistent with FIAM and BLM equilibrium models that state that metal uptake increases with the concentration of free metal ion and decreases due to competition and complexation effects. However, FIAM and BLM models cannot explain the bioaccumulation results obtained for Pb which implies that bioaccumulation process is much more dynamic than assumed in the equilibrium models. On the other hand, the results of this study have revealed that the most advantageous process of metal ions binding is biosorption and the green microalgae *Chlamydomonas reinhardtii* constitutes a promising material for the development of an efficient and economic biosorbent for the removal of heavy metals from metal-contaminated aquatic environments. Improved trace metal speciation predictions, especially at low, environmentally significant metal concentrations, will be important for the prediction of metal bioavailability and toxicity in natural aquatic systems. Treatment, reusability, and tolerance of the algal biomass also need to be evaluated to improve removal efficiency and assess the suitability of the biosorbent *C. reinhardtii* in industrial-scale process.

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References

[1] R.P. Mason, Trace Metals in Aquatic Systems, Wiley-Blackwell, Chichester, 2013.

[2] M. Bilal, J.A. Shah, T. Ashfaq, S.M.H. Gardazi, A.A. Tahir, A. Pervez, H. Haroon, Q. Mahmood, Waste biomass adsorbents for copper removal from industrial wastewater—A review, J. Hazard. Mater. 263 (2013) 322–333.

[3] K. Chojnacka, Biosorption and bioaccumulation—The prospects for practical applications, Environ. Int. 36 (2010) 299–230.

[4] R. De Philippis, G. Colica, P. Mecarozzi, Exopolysaccharide-producing cyanobacteria in heavy metal removal from water: molecular basis and practical applicability of the biosorption process, Appl. Microbiol. Biotechnol. 92 (2011) 697–708.

[5] B. Debelius, J.M. Forja, A. DelValls, L.M. Lubia, Toxicity and bioaccumulation of copper and lead in five marine microalgae, Ecotoxicol. Environ. Saf. 72 (2009) 1503–1513.

[6] S. Dwivedi, Bioremediation of heavy metal by algae: Current and future perspective, J. Adv. Lab. Res. Bio. 3 (3) (2012) 229–233.

[7] M. Fomina, G.M. Gadd, Biosorption: Current perspectives on concept, definition and application, Bioresour. Technol. 160 (2014) 3–14.

[8] J. He, J.P. Chen, A comprehensive review on biosorption of heavy metals by algal biomass: Materials, performances, chemistry, and modeling simulation tools, Bioresour. Technol. 160 (2014) 67–78.

[9] M. Lavoie, J. Bernier, C. Fortin, P.G.C. Campbell, Cell homogenization and subcellular fractionation in two phytoplanktonic algae: Implications for the assessment of metal subcellular distributions, Limnol. Oceanogr. Methods 7 (2009) 277–286.

[10] J. Wang, C. Chen, Biosorbents for heavy metals removal and their future, Biotechnol. Adv. 27 (2009) 95–226.

[11] X.H. Cai, T. Logan, T. Gustafson, S. Traina, R.T. Sayre, Applications of eukariotic algea for the removal of heavy metals from water, Mol. Mar. Biol. Biotechnol. 4 (1995) 338–344.

[12] I. Tuzun, G. Bayramoglu, E. Yalcın, G. Basarana, G. Celik, M.A. Yakup, Equilibrium and kinetic studies on biosorption of Hg(II), Cd(II) and Pb(II) ions onto microalgae *Chlamydomonas reinhardtii*, J. Environ. Manage. 77 (2005) 85–92.

[13] P. Abboud, K.J. Wilkinson, Role of metal mixtures (Ca, Cu and Pb) on Cd bioaccumulation and phytochelatin production by *Chlamydomonas reinhardtii*, Environ. Pollut. 179 (2013) 33–38.

[14] Z. Chen, L. Zhu, K.J. Wilkinson, Validation of the biotic ligand model (BLM) in metal mixtures: Bioaccumulation of lead and copper, Environ. Sci. Techn. 44 (2010) 3580–3586.

[15] R. Flouty, G. Khalaf, Role of Cu and Pb in the bioaccumulation of Ni by *Chlamydomonas reinhartii*: Validation of biotic ligand model, Ecotoxicol. Environ. Saf. 113 (2015) 79–86.

[16] R. Flouty, G. Estephan, Biaccumulation and biosorption of copper and lead by a unicellular algae Chlamydomonas reinhardtii in single and binary metal systems: A comparative study, J. Environ. Manage. 111 (2012) 106–114.

[17] J. Buffle, Complexation Reactions in Aquatic Systems: An Analytical Approach, Ellis Horwood, Chichester, UK, 1988.

[18] E. Tipping, Cation Binding by Humic Substances, Cambridge University Press, Cambridge, UK, 2002, p. 434.

[19] I. Komjarova, R. Blust, Multimetal interactions between Cd, Cu, Ni, Pb, and Zn uptake from water in the zebrafish danio rerio, Environ. Sci. Techn. 24 (19) (2009) 7225–7229.

[20] C.S. Hassler, V. Slaveykova, K.J. Wilkinson, Some fundamental (and often overlooked) considerations underlying the free ion activity and biotic models, Environ. Toxicol. Chem. 23 (2004) 283–291.

[21] I.A.M. Worms, K.J. Wilkinson, Ni uptake by a green alga. 1. Validation of equilibrium models for complexation effects, Environ. Sci. Technol. 41 (2007) 4258–4263.

[22] V.I. Slaveykova, K.J. Wilkinson, Predicting the bioavailability of metals and metal complexes: Critical review of the bioticligand model, Environ. Chem. 2(1) (2005) 9–24.

[23] G. Yang, Q.G. Tan, L. Zhu, K.J. Wilkinson, The role of complexation and competition in the biouptake of europium by a unicellular alga, Environ. Toxicol. Chem. 33 (2010) 2609–2615.

[24] I.A.M. Worms, K.J. Wilkinson, Ni uptake by a green alga. 2. Validation of equilibrium models for competition effects, Environ. Sci. Technol. 41 (2007) 4264–4270.

[25] I.A.M. Worms, D. Adenmatten, P. Miéville, J. Traber, V.I. Slaveykova, Photo transformation of pedogenic humic acid and consequences for Cd(II), Cu(II) and Pb(II) speciation and bioavailability to green microalga, Chemosphere 138 (2015) 908–915.

[26] F.M.M. Morel, Principles of Aquatic Chemistry, John Wiley & Sons Inc, New York, NY, 1983.

[27] S.I. Korfali, M.S. Jurdi, Speciation of metals in bed sediments and water of Qaraaoun Reservoir, Lebanon, Environ. Monit. Assess. 178 (2011) 563–579.

[28] E.H. Harris, The Chlamydomonas sourcebook: A comprehensive guide to biology and laboratory use, Academic Press, San Diego, CA, 1989.

[29] H. Kola, K.J. Wilkinson, Cadmium uptake by a green alga can be predicted by equilibrium modelling, Environ. Sci. Technol. 39(9) (2005) 3040–3047.

[30] APHA, AWWA, WPCF, Standard Methods for the Examination of Water and Wastewater, 18th ed., American Public Health Association, Washington DC, 1992.

[31] L.S. Clesceri, A.D. Eaton, E.W. Rice, Standard Methods for Examination of Water and Wastewater Method 5210B, American Public Health Association, American Water Works Association, and the Water Environment Association, Washington, DC, 2005. http://www.standardmethods.org.

[32] M. Lavoie, S. Le Faucheur, A. Boullemant, C. Fortin, P.G.C. Campbell, The influence of pH on algal cell membrane permeability and its implications for the uptake of lipophilic metal complexes, J. Phycol. 48(2) (2012) 293–302.

[33] G.P. Aiken, H. Hsu-Kim, J.N. Ryan, Influence of dissolved organic matter on the environmental fate of metals, nanoparticles, and colloids, Environ. Sci. Technol. 45 (2011) 3196–3201.

[34] A. Houri, S.W. Jeblawi, Water quality assessment of Lebanese coastal rivers during dry season and pollution load into the Mediterranean Sea, J. Water Health 5(4) (2007) 615–623.

[35] E.R. Unsworth, K. Warnken, H. Zhang, W. Davison, F. Black, J. Buffle, J. Cao, R. Cleven, J. Glaceran, P. Gunkel, E. Kalis, D. Kistler, H. Vanleeuwen, M. Martin, S. Noel, Y. Nur, N. Odzak, J. Puy, W.V. Riemsdijk, L. Sigg, E. Temminghoff, M.L. Waeber, S. Toepperwien, R. Town, L. Weng, H. Xue, Model predictions of metal speciation

in freshwaters compared to measurements by in situ techniques, Environ. Sci. Technol. 40 (2006) 1942–1949.

[36] P. Sánchez-Marín, J. Santos-Echeandía, M. Nieto-Cid, X.A. Álvarez-Salgado, R. Beiras, Effect of dissolved organic matter (DOM) of contrasting origins on Cu and Pb speciation and toxicity to *Paracentrotus lividus* larvae, Aquat. Toxicol. 96 (2010) 90–102.

[37] M. Gardner, J. Ravenscroft, The behaviour of copper complexation in rivers and estuaries: Two studies in north east England, Chemosphere 23(6) (1991) 695–713.

[38] D. Langmuir, Aqueous Environmental Geochemistry, Englewood cliffs, NJ, Prentice Hall, 1997.

[39] C. Lamelas, J.P. Pinheiro, V.I. Slaveykova, Effect of humic acid on Cd(II), Cu(II), and Pb(II) uptake by freshwater algae: Kinetic and cell wall speciation considerations, Environ. Sci. Technol. 43 (2009) 730–735.

[40] R.J. Erickson, D.A. Benoit, V.R. Mattson, E.N. Leonard, H.P. Nelson Jr., The effects of water chemistry on the toxicity of copper to *fathead minnows*, Environ. Toxicol. Chem. 15(2) (1996) 181–193.

[41] B. Vigneault, P.G.C. Campbell, Uptake of cadmium by freshwater green algae: Effects of ph and aquatic humic substances, J. Phycol. 41 (2005) 55–61.

[42] R. Flouty, Removal of Pb(II) and Cu (II) from aqueous solutions by *Chlamydomonas reinhardtii*: Infrared and thermal analysis, Environ. Eng. Manage. J. 14(4) (2015) 871–877.

[43] G. Naja, B. Volesky, The mechanism of metal cation and anion biosorption. in: P. Kotrba, M. Mackova, T. Macek (Eds.), Microbial Biosorption of Metals, Springer, Dordrecht, 2011, pp. 19–58.

[44] Y.C. Lee, S.P. Chang, The biosorption of heavy metals from aqueous solution by *Spirogyra* and *Cladophora* filamentous macroalgae, Bioresour. Technol. 102(9) (2011) 5297–5304.

[45] I. Michalak, K. Chojnacka, A. Witek-Krowiak, State of the art for the biosorption process—A review, Appl. Biochem. Biotechnol. 170 (2013) 1389–1416.

[46] H.B. Xue, W. Stumm, L. Sigg, The binding of heavy metals to algal surfaces, Water Res. 22(7) (1988) 917–926.

[47] G.M. Gadd, Biosorption: Critical review of scientific rationale, environmental importance and significance for pollution treatment, J. Chem. Technol. Biotechnol. 84 (2009) 13–28.