ELSEVIER

Aquatic Toxicology 58 (2002) 215–227

AQUATIC TOXICOLOGY

www.elsevier.com/locate/aquatox

Bioavailability of waterborne strontium to the common carp, *Cyprinus carpio*, in complexing environments

Mohammed J. Chowdhury [1], Ronny Blust *

Department of Biology, University of Antwerp (RUCA), Groenenborgerlaan 171, B-2020 Antwerp, Belgium

Received 16 January 2001; received in revised form 6 March 2001; accepted 3 August 2001

Abstract

The uptake of strontium (Sr) and calcium (Ca) in the common carp, *Cyprinus carpio*, was studied in chemically defined freshwater in the presence of the complexing ligands, ethylenediaminetetraacetic acid (EDTA), and nitrilotriacetic acid (NTA). The uptake rates were measured in the whole body, gills, and blood of the fish after an exposure period of 3 h. The uptake rates were determined by using the radiotracers ^{85}Sr and ^{45}Ca, and analyzed as a function of the free-ion activity of Sr and Ca in water. Although Sr^{2+} activity decreased, the uptake of Sr showed an increase at relatively low concentrations of EDTA and NTA, and a decrease at relatively high concentrations. This can be explained by the decreased competition between Sr^{2+} and Ca^{2+} at the gill uptake sites due to $\sim$30–140-fold higher affinity of EDTA and NTA for Ca^{2+} than Sr^{2+}. With decreasing Ca^{2+} activity, Ca uptake rates decreased in the presence of EDTA and NTA, but the effect of NTA was less pronounced. A Michaelis–Menten type competitive inhibition model was derived that could predict the whole-body Sr and Ca uptake rates, taking into account the ambient Sr^{2+} and Ca^{2+} activities in the presence of EDTA. In case of NTA, the uptake rates were found to be 1.5–3.2 times higher than what was predicted by the model. When the fish were exposed to complexing environments in the complete absence of Ca, an increased uptake of Sr was still observed in case of NTA, but not EDTA. The increased uptake in the presence of NTA is attributed to the direct uptake of $SrNTA^-$ and $CaNTA^-$ complexes from water. The results reveal that the uptake of Sr and Ca in carp is not merely a function of the free metal-ion activity but that certain complex species may contribute significantly to overall uptake.

Keywords: Strontium; Calcium; Bioavailability; EDTA; NTA; Carp

1. Introduction

Strontium (Sr) is an alkaline earth metal that occurs together with calcium (Ca) in environmental compartments and organisms. The concentrations of Sr are found to vary between about 0.02 and 2.6 μM in freshwater, and between 20 and 92 μM in coastal water and seawater (Bowen, 1966;

* Corresponding author. Tel.: +32-3-21-80347; fax: +32-3-21-80497.

E-mail address: blust@ruca.ua.ac.be (R. Blust).

[1] Present address: Department of Biology, McMaster University, 1280 Main Street West, Hamilton Ont., Canada L8S 4K1.

0166-445X/02/$ - see front matter
PII: S0166-445X(01)00230-2

Crocker and Merritt, 1972; Coughtrey and Thorne, 1983). The environmental importance of Sr lies in its analogous character with Ca in aquatic ecosystems and the toxicity of its radioactive isotopes, particularly ^{90}Sr. This is a high energy β-emitter with a half-life of 28 years that preferentially accumulates in bony tissues of organisms (Eisenbud, 1973). The major sources of radiostrontium in water are wastes from nuclear industries, accidental releases to the environment and nuclear fallout. For example, the Chernobyl disaster in 1986 released an estimated 4×10^{18} Bq radioactivity to the environment. Although less than 3% of the original radioactivity released during the accident remains, there are still substantial inventories of ^{137}Cs and ^{90}Sr in the area due to their long half lives (Savchenko, 1995; Chesser et al., 2000).

Fish take up Sr and its analogue Ca from water, food and sediment. The uptake of these alkaline-earth metals is suggested to occur through Ca transport systems located in the chloride cells of gills and enterocytes of the intestine in fish (Flik et al., 1985; Ishihara and Mugiya, 1987; Flik et al., 1995). As a result, an inverse relationship is observed between the uptake of Sr by fish and the Ca concentration in water (Brungs, 1965; Chowdhury et al., 2000).

Metals in natural waters occur as free hydrated metal ions, complexes with inorganic and organic ligands, and sorbed on or trapped in particles. The bioavailability and toxicity of metal ions to aquatic organisms strongly depends on the chemical form in which the metals occur (Brezonik et al., 1991). In general, it appears that only the free metal ion can cross the exchange surfaces, while most other metal species are not taken up. This observation forms the basis of the free-ion activity model (FIAM), which uses chemical equilibrium principles to relate solution chemistry to metal uptake (Pagenkopf, 1983; Morel, 1983; Campbell, 1995). In this model, it is assumed that the metal (either the free metal ion, *M* or a metal complex, *ML*) interacts with cellular surface sites (*-X-cell*), which are located at the outer surface of the membrane. This interaction can be represented in terms of the formation of a surface complex *M-X-cell*. Since the concentration of free binding sites, *-X-cell*, is considered to be reasonably constant in the presence of low metal concentrations, the FIAM implies that any biological response due to metal exposure or metal transport in a system at equilibrium is proportional to the concentration of the surface complex *M-X-cell*, which in turn depends on the metal ion activity in solution. Complexation of metals with ligands decreases the activity of the free metal ion and hence the uptake of the metal by aquatic organisms.

It has indeed been demonstrated in many studies that differences in metal availability for fish and other aquatic organisms are better explained on the basis of changes in free metal ion activities, rather than total metal concentrations (Campbell, 1995). Most of these studies used organic ligands such as EDTA (ethylenediaminetetraacitic acid), NTA (nitrilotriacetic acid) or citrate to demonstrate the importance of the free metal ion for the uptake or toxicity of metals by aquatic organisms. However, also a number of apparent exceptions have been recorded, in which the uptake of the metal was considerably higher than what was expected on the basis of the free metal ion activity in water (Vercauteren and Blust, 1996; Van Ginneken et al., 1999; Errecalde and Campbell, 2000).

We have recently characterized the uptake kinetics of waterborne Sr in the common carp, *Cyprinus carpio*, at different Ca and pH levels in water (Chowdhury et al., 2000; Chowdhury and Blust, 2001). The results indicate that Ca^{2+} inhibits Sr^{2+} uptake competitively and H^+ inhibits both ions in a partially non-competitive way. Both interactions can be described by a Michaelis–Menten type model for mediated transport (Chowdhury and Blust, 2001).

In the present study, the effect of complexation by two organic ligands, EDTA and NTA, on the uptake of Sr and Ca by carp has been examined. The organic ligands EDTA and NTA form hydrophilic complexes with metals and are often used as metal ion buffers. The main goal of the work was to evaluate the Sr uptake model in complexing environments over a wide range of free metal ion activities, taking into account competition between Sr^{2+} and Ca^{2+} ions for the same uptake sites.

2. Materials and methods

2.1. Experimental fish

Fingerlings (~ 1 g) of the common carp, *C. carpio*, were obtained from the fish hatchery of the Agricultural University of Wageningen, The Netherlands. They were grown for at least three months in the laboratory at 25 ± 1 °C and pH 7.6–8.0 before use in the experiments. They were kept in 150-l glass tanks containing medium-hard freshwater (Ca: 875 μM; Mg: 145 μM), recirculated over a trickling filter and UV system, and fed with commercial food pellets for fish fry (Trouvit, Ghent, Belgium). The levels of NH_4^+, NO_2^- and NO_3^- in the water were maintained below 0.1, 0.1 and 20 mg l^{-1}, respectively, by renewing tank water whenever required.

2.2. Uptake experiments

The Sr and Ca uptake experiments were performed with fish weighing between 2.8 and 6.8 g (4.4 ± 0.9 g). The fish were acclimated at 25 ± 1 °C for a period of 16 days to standard medium-hard freshwater prepared by dissolving the reagent-grade salts ($CaCl_2$: 348 μM; $MgSO_4$: 500 μM; KCl: 54 μM; $NaHCO_3$; 1143 μM; pH: 7.6–8.0) in deionized water (Clesceri et al., 1989). During acclimation 30–40 individuals were held in a plastic tank containing 150-l of standard water that was continuously recirculated over a filter box containing lava stone and synthetic foam. The water quality criteria for NH_4^+, NO_2^- and NO_3^- were maintained as mentioned above. The fish received commercial pellets for fish fry as food (Trouvit, Ghent, Belgium). Fish were not fed 24 h before the start of an experiment.

At the start of an experiment, the fish were individually transferred from their acclimation tank to polypropylene beakers containing 0.5-l standard water of the same composition as used for acclimation. Fish were exposed for 3 h at a water temperature of 25 ± 1 °C and pH of 8.0 ± 0.1. The exposure solutions also contained 10 mM of the non-complexing buffer (H)EPPS (Sigma, St Louise, USA) and approximately 5 mM of NaOH to adjust the pH. The solutions were spiked with the radioactive tracers ^{85}Sr and/or ^{45}Ca (Amersham, Bucks, UK). Stable strontium chloride (Merck, Darmstadt, Germany), was added to the solutions so that the total concentration of strontium was always 0.285 μM. The solutions were prepared and aerated at least 24 h before the start of an experiment to allow equilibration.

Two different experiments were conducted to investigate the uptake of Sr in complexing environments. The first experiment was conducted in the presence of Ca (348 μM). In this experiment, exposure solutions were spiked with 740 kBq l^{-1} ^{85}Sr and 1480 kBq l^{-1} ^{45}Ca as radioactive tracers, and the uptake of both Sr and Ca in the whole body, gills, and blood of the fish was followed. Different concentrations of EDTA (0.5, 0.6, 0.75, 0.8, and 0.9 mM) or NTA (0.5, 0.8, 1.0, and 2.0 mM) were used to cover a broad range of free Sr^{2+} and Ca^{2+} ion activities in the solution. In addition, one control treatment was included which did not contain either of the ligands. In the first experiment, Sr uptake appeared to be affected by a decreased protection from Ca at the gills due to complexation. Therefore, a second similar experiment was performed in a Ca free environment. In this experiment, exposure solutions were spiked with 370 kBq l^{-1} ^{85}Sr as radioactive tracer and only the uptake of Sr in the whole body of the fish was followed. Due to the increased apparent affinity of the ligands for Sr in the absence of Ca, lower concentrations of EDTA (0.2, 0.3, 0.4, 0.46, and 0.56 mM) or NTA (0.3, 0.5, 0.7, 1.0, and 2.0 mM) were used in this experiment. One control treatment was included which did not contain Ca and either of the ligands.

The concentration of the stable Sr and Ca in the exposure solution was measured with a graphite furnace atomic absorption spectrophotometer (SpectraAA. 800Z, Varian, Mulgrave, Australia). The stock solutions of Na_2-EDTA (99.5%, Janssen, Geel, Belgium) and Na_3-NTA (99.5%, Sigma, St Louise, USA) were prepared with ultrapure water, produced by a Milli-Q 185 Plus purification system (Millipore, Bedford, USA). All statistical analyses of the data were performed using the computer software, Statistica (StatSoft, Tulsa, USA).

2.3. Measurement of radioactivity

After exposure the fish were removed from the beakers with a scoop net and rinsed for 10 min in 0.5 l tracer-free water containing 1 mM stable Sr to remove the radioactive tracer adsorbed to the body surface according to the method described by Chowdhury et al. (2000). Three minutes before the end of the rinsing period, 400 μM of MS 222 (ACROS, Geel, Belgium) was added to the rinsing solution to anesthetize the fish. Subsequently, each fish was placed in a 18 ml scintillation vial (Maxi-Vial, Canberra Packard, Meriden, USA) containing 10 ml of the standard water and counted for 1 min in a Minaxi-γ Auto-gamma 5530 counter (Canberra Packard) to determine the whole-body radioactivity of ^{85}Sr. After counting and weighing the fish approximately 100–120 μl of blood was taken with 60 μl Na-heparinized hematocrit tubes by puncturing the heart from the ventral side of the fish. Subsequently, gill filaments were collected and rinsed with 8–10 ml of isotonic solution (0.9% NaCl), and the gill soft tissue was separated from the arches. After weighing, the blood and gill samples were solubilized in 1 ml of Soluene-350 tissue solubilizer (Canberra Packard) and 10 ml of Hionic-Fluor liquid scintillation cocktail (Canberra Packard) was added to the scintillation vials. After stabilization for a period of 24 h, the vials were counted for 15 min for the radioactivity of ^{85}Sr and ^{45}Ca. To measure the whole body ^{45}Ca activity, the fish were transferred to scintillation glass vials, dried at 60 °C for 3 days and ashed at 600 °C in a muffle furnace. The ashes for each fish were dissolved in 1 ml of 20% HNO_3 and 20 ml of Ultima Gold-AB scintillation cocktail (Canberra Packard) was added. After stabilization the ^{45}Ca activity was measured in a Packard Tri-Carb Liquid Scintillation Analyzer (Model 1900 TR) and counts were corrected according to the method described by Van Ginneken and Blust (1995). The counts of the gill samples were corrected for the counts contributed by the blood trapped in the soft gill-tissue, according to the method described by Van Ginneken et al. (1996).

The uptake rate (μmol kg^{-1} h^{-1}) of Sr^{2+} (Sr^{2+}_{uptake}) was calculated from the following equation:

$$Sr^{2+}_{uptake} = \frac{ACT_{Sr}}{60 CE_{Sr} Wt SA_{Sr}} \quad (1)$$

where ACT_{Sr} is the ^{85}Sr activity (cpm) of the whole fish, gills or blood after correction for background radiation and radio-decay, CE_{Sr} is the counting efficiency for ^{85}Sr, W is the wet weight (kg) of the sample, t is the exposure time (h), and SA_{Sr} is the specific activity of ^{85}Sr in the exposure water (Bq ^{85}Sr per μmol total strontium).

The following equation was used to calculate the uptake rate of Ca^{2+} (Ca^{2+}_{uptake}):

$$Ca^{2+}_{uptake} = \frac{(ACT\beta - ACT_{\beta Sr}) D_{Ca}}{60 CE_{Ca} Wt SA_{Ca}} \quad (2)$$

where $ACT\beta$ is the total β-counts (cpm) for ^{85}Sr and ^{45}Ca in the sample, $ACT_{\beta Sr}$ is the β-counts (cpm) due to presence of ^{85}Sr, D_{Ca} is the correction factor for ^{45}Ca decay, CE_{Ca} is the ^{45}Ca counting efficiency in the beta counter, W is the wet weight (kg) of the sample, t is the exposure time (h), and SA_{Ca} is the specific activity of ^{45}Ca in the exposure water (Bq ^{45}Ca per μmol total Ca).

2.4. Kinetic model

In a previous study, we showed that the uptake of waterborne Sr^{2+} and Ca^{2+} in carp is saturable, and can be described by a Michaelis–Menten type model for competitive inhibition of mediated metal transport (Chowdhury et al., 2000). To predict the rates of Sr and Ca uptake in the presence of EDTA and NTA, the same model was used. In this model the free metal-ion activity and rate of metal uptake are related in the following way:

$$j = \frac{J_{max} M^{2+}}{K_m (1 + I^{2+}/K_i + M^{2+})} \quad (3)$$

where j is the rate of metal uptake in the whole body of fish, J_{max} is the maximum rate of uptake, M^{2+} and I^{2+} are the activities of the metal and inhibitor, K_m is the half-saturation constant for the metal in the uninhibited process and K_i is the inhibitor constant.

2.5. Chemical speciation

The equilibrium concentrations of the chemical species considered in the complexing solution were calculated using the computer program SOLUTION (Blust and Van Ginneken, 1998), a modified version of the program COMPLEX (Ginzburg, 1976). This speciation model allows the calculation of the composition of solutions in equilibrium with the atmosphere. The thermodynamic stability constants ($\log K_{ML}$) of the metal ligand complexes for the most important Sr and Ca species used in the speciation model are given in Table 1. The constants are based on the data of Dickson and Whitfield (1981) for the major inorganic interactions and the National Institute of Standards and Technology database for Sr and Ca (Martell and Smith, 1997). For each complex species considered, the stability constants listed for different ionic strengths were fitted to an interpolation function that has the form of an extended Debye–Hückel equation (Turner, 1995). The model's input comprised the total concentrations of the metals and ligands in the solution, the pH, the pE, the temperature, and the atmospheric CO_2 pressure in equilibrium with the solution. The model output comprised the molar concentrations of the free metal ions and complex species. The free ion activities of strontium and calcium were obtained by multiplying the concentrations of the free metal ions with the proper activity coefficients calculated by the Davies equation (Turner, 1995).

Table 1
Thermodynamic stability constants (K_{ML}) for the strontium (Sr) and calcium (Ca) species considered in the chemical speciation model

Sr species	$\log K_{ML}$	Ca species	$\log K_{ML}$
Sr^{2+}	–	Ca^{2+}	–
$SrOH^{+}$	0.82	$CaOH^{+}$	1.15
$SrSO_4^0$	2.30	$CaSO_4^0$	2.31
$SrCO_3^0$	2.81	$CaHSO_4^{+}$	2.28
$SrHCO_3^{+}$	11.54	$CaCO_3^0$	3.15
$SrEDTA^{2-}$	10.33	$CaHCO_3^{+}$	11.43
$SrHEDTA^{-}$	12.98	$CaEDTA^{2-}$	12.48
$SrNTA^{-}$	6.16	$CaHEDTA^{-}$	14.50
$Sr(NTA)_2^{4-}$	7.55	$CaNTA^{-}$	7.61
		$Ca(NTA)_2^{4-}$	8.81

3. Results

3.1. Strontium speciation and uptake in the presence of calcium

The results of the chemical speciation modeling for Sr and Ca in defined freshwater containing 0.285 μM $SrCl_2$ and 348 μM $CaCl_2$ are shown in Fig. 1A. In the absence of organic ligands, Sr and Ca are predominantly present as free metal ions ($\sim 95\%$) and some complexes of sulfate and carbonate ($\sim 5\%$). The presence of the organic ligands EDTA or NTA results in the formation of complexes with Sr^{2+} and Ca^{2+} decreasing the free metal ion concentrations. With increasing ligand concentration, the ionic strength of the medium increased slightly from 8.4 mM in the control to 9.7 or 12.6 mM at the highest concentrations of EDTA (0.9 mM) or NTA (2.0 mM), respectively. As a result, the activity coefficients of Sr^{2+} and Ca^{2+} varied between 0.687 and 0.671 for EDTA and 0.687 and 0.639 for NTA. Due to the greater binding affinity of the ligands for Ca, the free Ca^{2+} ion activity decreased to a much higher extent than the free Sr^{2+} ion activity.

The measured Sr uptake rates in whole body, gills, and blood at the calculated Sr^{2+} activities in the presence of EDTA and NTA are shown in Fig. 2. No mortality or abnormal behavior of fish was observed at any of the concentrations of ligands used. Although complexation with the ligands decreased the free Sr^{2+} ion activity, the uptake rates in whole body, gills, and blood did not follow the same pattern. With increasing concentrations of EDTA or NTA, the uptake of Sr in carp showed an increase at relatively low ligand concentrations and a decrease at relatively high ligand concentrations. A t-test for comparing two independent samples indicates that Sr uptake is significantly higher than in the control condition ($P < 0.001$), except at highest concentration of EDTA and lowest Sr^{2+} activity ($P > 0.05$).

The observed Ca uptake rates in whole fish, gills, and blood decreased from the control with

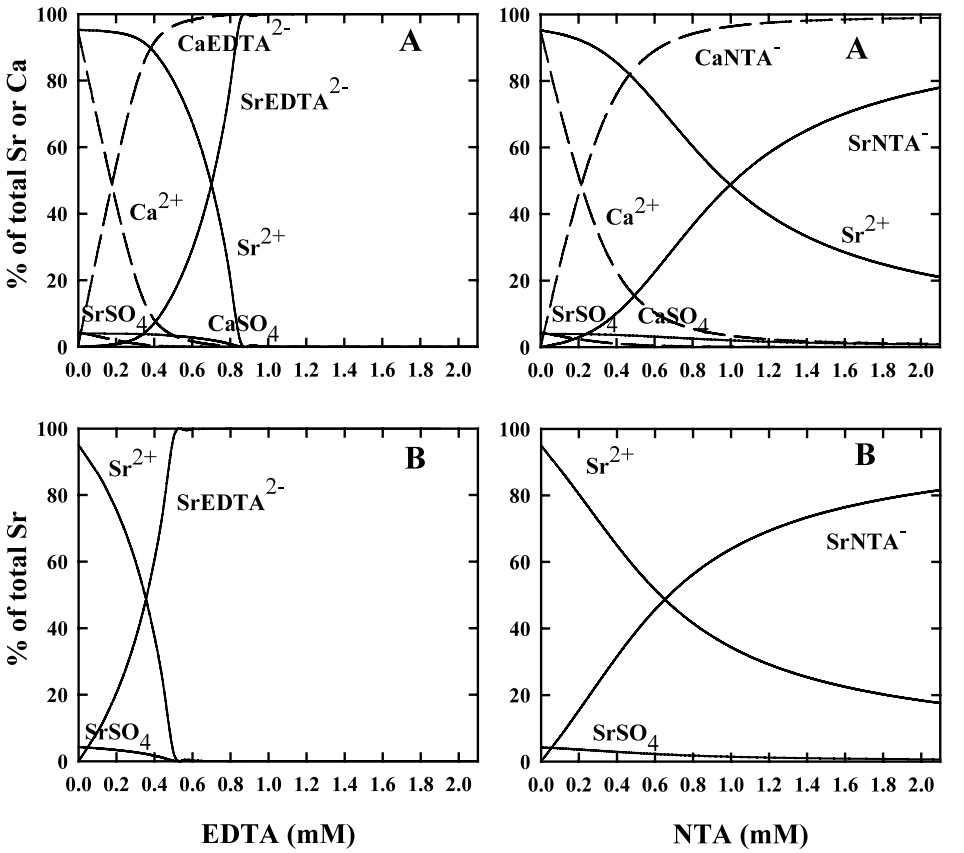

Fig. 1. Effect of complexation by the organic ligands (EDTA and NTA) on the chemical speciation of strontium and calcium in the freshwater used for the experiments (temperature: 25 ± 1 °C; pH: 8.0). (A) Sr and Ca speciation in the water containing 0.285 μM Sr and 348 μM Ca. (B) Sr speciation in the water containing 0.285 μM Sr and 0.0 μM Ca.

decreasing Ca^{2+} activity in the presence of EDTA. However, this effect was not observed for whole fish, gills and blood at lower concentrations of NTA (Fig. 3). The Sr and Ca uptake rates for whole fish, gills and blood in the presence of NTA were higher than those in the presence of EDTA. Calculation of Pearson's correlation coefficient shows that there is a high degree of linear association between the uptake rates of Sr and Ca in the whole fish, gills and blood (Table 2).

A Michaelis–Menten type competitive inhibition model was used to calculate Sr and Ca uptake rates at different metal ion activities in the presence of the organic ligands (Eq. (3)). For this purpose, the parameter values (Table 3) were adopted from previous studies concerning the kinetics of waterborne Sr^{2+} and Ca^{2+} uptake in carp (Chowdhury et al., 2000; Chowdhury and Blust, 2001). The values were estimated over a wide range of waterborne Sr (0.2–10 000 μM) and Ca (10–10 000 μM) concentrations, and a pH range of 5.0–8.5. Since the present study was conducted at a constant pH (8.0), the apparent values of J_{max} for pH 8.0 ($J_{maxSr} = 289.0$ μmol kg^{-1} h^{-1}; $J_{maxCa} = 156.0$ μmol kg^{-1} h^{-1}) were calculated from their true values ($J_{maxSr} = 293.0$ μmol kg^{-1} h^{-1}; $J_{maxCa} = 159.4$ μmol kg^{-1} h^{-1}; pH 5.0–8.5) using the model presented in Chowdhury and Blust (2001). The values of the other parameters (K_{mSr}, K_{mCa}, K_{iCa}, and K_{iSr}) used for the calculation of uptake rates by the competitive inhibition model (Eq. (3)) are shown in Table 3.

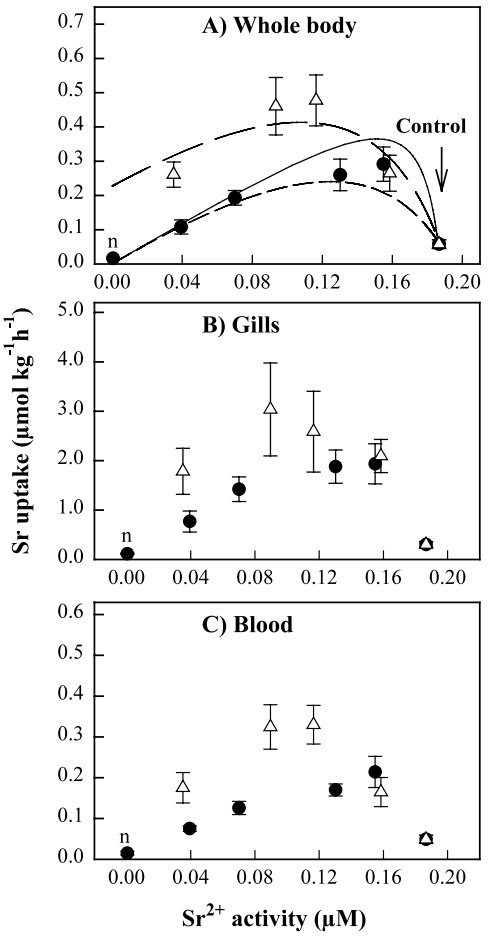

Fig. 2. Effect of complexation by EDTA (circles) and NTA (triangles) on Sr uptake in the whole body, gills, and blood of carp as a function of Sr^{2+} ion activity in the exposure water containing 0.285 µM Sr and 348 µM Ca. Data points represent mean uptake rates with standard deviations (S.D.) ($n = 5–6$; temperature: 25 ± 0.5 °C; pH: 8.0 ± 0.1). The arrow shows the control values for whole body, gills, and blood, obtained in the absence of the ligands. A t-test indicates that the observed uptake rates, except those denoted by 'n' for EDTA, are significantly higher than their control values ($P < 0.001$). The 'solid' and 'short-dashed' lines are calculations of Sr^{2+} uptake by the Michaelis–Menten type competitive inhibition model (Eq. (3)) in the presence of EDTA and NTA respectively, considering that only the free Sr^{2+} ion is available for uptake. The long-dashed line is the calculation by the model (Eq. (4)) in the presence of NTA, considering that both the free Sr^{2+} and $SrNTA^{-}$ complex are available for uptake.

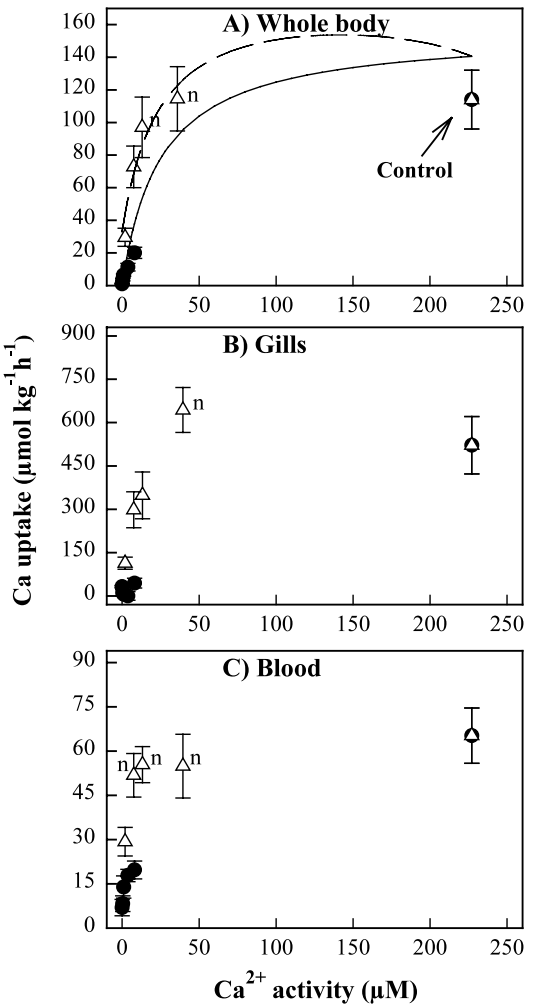

Fig. 3. Effect of complexation by EDTA (circles) and NTA (triangles) on Ca uptake in the whole body, gills, and blood of carp as a function of Ca^{2+} ion activity in the exposure water containing 0.285 µM Sr and 348 µM Ca. Data points represent mean uptake rates with standard deviations ($n = 5–6$; temperature: 25 ± 0.5 °C; pH: 8.0 ± 0.1). The arrow shows the control values for whole body, gills, and blood, obtained in the absence of the ligands. A t-test indicates that the observed uptake rates, except those denoted by 'n', are significantly lower than their control values ($P < 0.01$). The 'solid' line is the calculation of Ca uptake by the Michaelis–Menten type competitive inhibition model (Eq. (3)) in the presence of EDTA and NTA, considering that only the free Ca^{2+} ion is available for uptake. The 'dashed' line is the calculation by the model (Eq. (4)) in the presence of NTA, considering that both the free Ca^{2+} ion and $CaNTA^{-}$ complex are available for uptake.

The observed and calculated uptake rates for whole fish were compared using a t-test in which for each condition the calculated uptake rate is compared with the mean of the corresponding observations (Sokal and Rohlf, 1981). The results show that the model describes the uptake of Sr and Ca in the presence of EDTA reasonably well, but underestimates the uptake of Sr and Ca in the presence of NTA (Figs. 2A and 3A). This indicates that in the presence of EDTA the uptake of Sr and Ca uptake is a function of the free ion activity of Sr^{2+} and Ca^{2+}. However, in the presence of NTA the observed Sr and Ca uptake rates were found to be significantly higher than calculated, implying that Sr and Ca uptake is not only a function of the free ion activities of the metals in the exposure water.

3.2. Effect of complexing in the absence of calcium

In the first experiment, decreased competition with Ca^{2+} at the gills due to complexation of calcium was considered to cause the increased uptake of Sr^{2+} in the presence of the ligands. Therefore, in the second experiment, the effect of complexation on Sr^{2+} uptake was studied in a calcium free environment. In the absence of calcium, the apparent affinity of the ligands for Sr^{2+} increased and as a result, less EDTA or NTA was required to lower the free Sr^{2+} ion activity (Fig. 1B). The ionic strength of the medium changed slightly from 7.4 mM in the control to 8.6 or 12.8 mM at the highest concentrations of EDTA (0.56 mM) or NTA (2.0 mM), respectively. As a result, the activity coefficients for Sr^{2+} varied between 0.702 and 0.685 for EDTA and 0.702 and 0.638 for NTA. The observed Sr uptake in the whole fish as a function of the Sr^{2+} ion activity is shown in Fig. 4. With decreasing Sr^{2+} ion activity, Sr uptake decreased compared with the control level in the presence of EDTA, but again, not in the presence of low concentrations of NTA. However, the Sr uptake rates in this Ca free environment were significantly lower ($P < 0.001$) than what is expected on the basis of the Sr uptake model (dashed line in Fig. 4).

4. Discussion

In this study, the uptake of waterborne Sr and Ca by carp was investigated in the presence of the complexing ligands, EDTA and NTA, to test the validity of the free-ion activity model (FIAM). Generally, complexation of metals with water-soluble ligands decreases the free metal ion activity in the solution and hence decreases the uptake of metals by aquatic organisms. This general finding has been experimentally observed in fish for different essential and non-essential metals like zinc, cobalt, and cadmium in the presence of EDTA and NTA (Pärt and Wikmark, 1984; Playle et al., 1993; Blust et al., 1997; Van Ginneken et al., 1999). Our experimental results show that in com-

Table 2
Pearson correlation coefficient (r) for the degree of linear association between Sr/Ca uptake in the whole body and Sr/Ca uptake in gills and blood of the carp

	EDTA			NTA		
	Whole	Gills	Blood	Whole	Gills	Blood
Strontium						
Whole	1.0 (36)	0.92 (33)	0.97 (33)	1.0 (30)	0.91 (30)	0.98 (30)
Gills	–	1.0 (33)	0.89 (33)	–	1.0 (30)	0.88 (30)
Blood	–	–	1.0 (33)	-	–	1.0 (30)
Calcium						
Whole	1.0 (36)	0.98 (22)	0.97 (30)	1.0 (30)	0.86 (30)	0.83 (30)
Gills	–	1.0 (24)	0.95 (24)	–	1.0 (30)	0.59 (30)
Blood	–	–	1.0 (33)	–	–	1.0 (30)

The values of the coefficient are all significant at $P<0.01$ or 0.001. The values in the parentheses are the number of observations.

Table 3
Kinetic parameters with standard error ($\pm$S.E.) and coefficients of determination (r^2), used for the prediction of the whole body uptake of Sr and Ca in carp in complexing environments (EDTA = 0.5–0.9 mM; NTA = 0.5–2.0 mM; Sr_{total} = 0.285 μM; Ca_{total} = 348 μM; temp = 25 $\pm$ 0.5 °C; pH 8.0 $\pm$ 0.1)

Parameters[a]	Estimates	$\pm$S.E.	r^2	N	P
J_{maxSr}	293.0[b] (289.0)[c]	3.12	0.97	244	<0.001
J_{maxCa}	159.4[b] (156.0)[c]	2.21	0.86	238	<0.001
K_{mSr}	96.3[b]	2.10	0.97	244	<0.001
K_{mCa}	24.9[b]	0.90	0.86	238	<0.001
K_{iCa}	28.5[b]	0.60	0.97	244	<0.001
K_{iSr}	100.9[b]	4.10	0.86	238	<0.001
$J_{max(Sr–NTA)}$	0.28	0.07	0.79	30	<0.001
$J_{max(Ca–NTA)}$	49.0	6.2	0.69	30	<0.001
$K_{m(Sr–NTA)}$	0.06	0.01	0.79	30	<0.001
$K_{m(Ca–NTA)}$	156.2	57.6	0.69	30	0.011

[a] J_{max} in μmol kg^{-1} h^{-1} of free metal ion or NTA complex in fish; K_m and K_i in μM activity of free-metal ion or NTA complex in water.

[b] Values are adopted from Chowdhury et al. (2000) and Chowdhury and Blust (2001) and derived from fitting to the Michaelis–Menten type of inhibition model over a wide range of Sr concentrations (Sr_{total} = 0.2–10 000 μM; $Sr^{2+}_{activity}$ = 0.13–4756 μM), Ca concentrations (Ca_{total} = 10–10 000 μM; $Ca^{2+}_{activity}$: 6.7–4780 μM) and pH (5.0–8.5) in water.

[c] The apparent J_{max} values for pH 8.0, calculated using the model presented in Chowdhury and Blust (2001).

plexing environments Sr uptake does not necessarily increase with the free Sr^{2+} ion activity in the solution (Fig. 2). This observation can be explained by the decreased competition between Sr^{2+} and Ca^{2+} at the gill uptake sites due to the higher affinity of EDTA and NTA for Ca^{2+} then for Sr^{2+}. In the presence of 0.5 mM of EDTA, the free Ca^{2+} ion concentration dropped to 3.5% while the free Sr^{2+} ion concentration only dropped to 80% of the total metal concentrations in the control (Ca_{total} = 348 μM, Ca^{2+}_{free} = 330 μM; Sr_{total} = 0.285 μM, Sr^{2+}_{free} = 0.272 μM; Fig. 1A). In the presence of 0.5 mM of NTA, the free Ca^{2+} and Sr^{2+} ion concentrations dropped to 15 and 80%, respectively.

It is well known that Sr^{2+} and Ca^{2+} compete for the same uptake system and as a result the uptake of one is decreased by the presence of the other (Rosenthal, 1957; Brungs, 1965; Suzuki et al., 1972; Chowdhury et al., 2000). In the present study, this effect was clearly observed for Sr uptake but not for Ca uptake because the total Sr concentration in the water (0.285 μM) was much lower than the total Ca concentration (348 μM) (Fig. 3).

The increase in Sr uptake caused by the decreased protection from Ca^{2+} does not defy the FIAM, because the surface equilibrium assumption of the FIAM is valid in the presence of other competing cations (Campbell, 1995). It is clearly shown that the variation in the whole fish uptake of Sr in the presence of EDTA can be predicted reasonably well by the competitive inhibition model for Sr and Ca uptake in carp (Eq. (3)). In a calcium free environment complexation of Sr^{2+} by EDTA results in a decreased uptake proportional to the decrease in the free Sr^{2+} ion activity (Fig. 4).

In the presence of NTA, however, our results show that the observed Sr and Ca uptake rates are not merely functions of the Sr^{2+} and Ca^{2+} activities (Figs. 2–4). The whole-body Sr uptake rates were 1.6–3.2 times higher than what are calculated by the model, and Ca uptake rates were 1.5–2.8 times higher. Moreover, the competitive effect of Ca is greater with NTA than with EDTA because of the differences in the relative affinities of the two organic ligands for Sr and Ca. Thus, for the same free Sr^{2+} ion activity it is expected that Sr uptake is lower in the presence of NTA than in the presence of EDTA.

The observed increased uptake of strontium may not only be caused by decreased protection from competing ions but also by changes in metal

speciation in the layer lining the gills. The FIAM assumes that diffusion of metal species towards the organism is not rate limiting, taking into account only equilibrium speciation. However, when the delivery of the metal species towards the gill surface is slower than the rate of metal uptake, the equilibrium will be disturbed. In this situation, labile metal complexes might dissociate resulting in a higher metal uptake than expected. Additionally, some metal complexes may be transported across the membrane and directly contribute to the metal uptake (Hudson, 1998; Van Leeuwen, 1999; Van Ginneken et al., 1999; Fortin and Campbell, 2000).

The chemical speciation of metals in the layers lining the solution body interface may be altered because of changes in the pH of the gill environment. In trout, a difference of about one unit in the pH of the expired water (pH 7) compared with the inspired water (pH 8) was observed (Playle and Wood, 1989). However, modeling of the effect of a decrease in pH on the chemical speciation of Sr and Ca in the presence of either

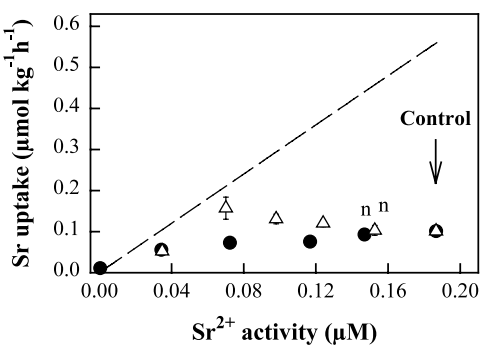

Fig. 4. Effect of complexation by EDTA (circles) and NTA (triangles) on Sr uptake in the whole body of carp as a function of the Sr^{2+} ion activity in the exposure water containing 0.285 μM Sr and 0.0 μM Ca. Data points represent mean uptake rates with standard deviations ($n = 5–6$; temperature: 25 ± 0.5 °C; pH: 8.0 ± 0.1). The arrow shows the control value obtained in the absence of the ligands. A t-test indicates that the observed uptake rates, except those denoted by 'n', are significantly different from the control ($P < 0.01$). The dashed line is the calculation by the Michaelis–Menten type competitive inhibition model (Eq. (3)) for the whole-body uptake of Sr at the calculated Sr^{2+} activities in the absence of Ca. All observed values are significantly lower than their calculated values ($P < 0.01$).

EDTA or NTA showed that the effects on the free Sr^{2+} and Ca^{2+} activities are minimal over this pH range. In addition, the uptake of Sr in carp was found to decrease with the pH of the bulk solution, but the decrease was not significant over the pH range 7.0–8.0 (Chowdhury and Blust, 2001). Thus, the observed deviations from the FIAM can not be explained by a drop in the pH of the solution layer lining the gills.

As stated earlier, the kinetics of metal complexation may influence metal uptake when the transport of the metal ion and/or metal complexes from the bulk solution to the gill surface becomes diffusion-limited and the rate of complex dissociation in the solution is slower than the rate of metal uptake. Generally, the rate of complex formation (k_f) increases with the charge on the ligand, while the rate of complex dissociation (k_d) decreases with an increase in thermodynamic stability ($K_{ML} = k_f/k_d$) of the metal complex (Hering and Morel, 1990). The rate constants for the formation of complexes with Ca^{2+} (3.07×10^8 s^{-1}; 25 °C) and Sr^{2+} (3.58×10^8 s^{-1}; 25 °C) for EDTA and NTA are close (Burgess, 1978). Together with the thermodynamic stability constants listed in Table 1 these values can be used to calculate the dissociation rate constants for the metal–ligand complexes considered. For Sr–EDTA and Sr–NTA, the estimated values are 1.67×10^{-2} and 2.48×10^2 s^{-1}, respectively. For Ca–EDTA and Ca–NTA, the estimated values are 1.02×10^{-4} and 7.54×10^0 s^{-1}, respectively. Although the dissociation rate constants for NTA complexes are orders of magnitude higher than these for EDTA complexes, the Sr and Ca uptake rates in the presence of NTA are only about twice the uptake rates in the presence of EDTA. This indicates that the uptake of Sr^{2+} is not constrained by complexation kinetics.

A number of reports explain the increased uptake or toxicity of metals in fish and other aquatic organisms by the direct uptake of hydrophilic metal–ligand complexes (Pärt and Wikmark, 1984; Daly et al., 1990; Vercauteren and Blust, 1996; Van Ginneken et al., 1999; Errecalde and Campbell, 2000). It has been suggested that the metal complexes are transported by specific systems in the biological membrane. So far, the

multidentate complexes formed by EDTA and NTA with divalent metal ions have not been reported to be bioavailable to aquatic organisms. However, Campbell (1995) cited in his review a report (Laube et al., 1980) that indicates that NTA enhanced the toxicity of Cu, Cd and Pb to a blue green alga. According to Campbell (1995), the increased toxicity might have been caused by the presence of appreciable concentrations of the free-metal ions, due to competition with other metals included in the media (e.g., Fe) or to volumetric errors in dispensing metal and ligand concentrations. Such experimental shortcomings are not likely to occur in the present study and with a similar experimental setup the greater than expected Sr uptake was observed in case of NTA, but not EDTA. In a recent study, Vanbriesen et al. (2000) evaluated the NTA complexes of different metals (Ca, Mg, Co, Fe, Zn, Al, Cu, and Ni) to identify the rate limiting chelate form for biodegradation of NTA by *Chelatobector heintzii*. They concluded that the kinetics of metal complexation reactions had no effect on the degradation. According to them calcium plays the major role and $CaNTA^-$ is the only species that is transported into the cell by an unidentified protein controlling the rate of NTA degradation.

To evaluate the possible contribution of Sr– or Ca–NTA complexes to the uptake of Sr and Ca, Eq. (3) was extended with a term accounting for the transport of the complexes:

$$j = \frac{J_{max} M^{2+}}{K_m(1 + I^{2+}/K_i + M^{2+})} + \frac{J_{max(ml)} ML^-}{K_{m(ml)} + ML} \qquad (4)$$

where $J_{max(ml)}$ is the maximum uptake of metal–NTA complex, ML^- is the activity of the complex in water, and $K_{m(ml)}$ is the half-saturation constant for the complex. The major complexes of Sr and Ca in the presence of NTA are $SrNTA^-$ and $CaNTA^-$ for all ligand concentrations (Fig. 1A). Eq. (4) was fitted to the observed data considering free metal ions and $SrNTA^-$ and $CaNTA^-$ complexes as bioavailable species. The model curves presented in Fig. 2A and Fig. 3A indicate that the calculated uptake is considerably improved for both Sr ($r^2 = 0.79$, $n = 30$) and Ca ($r^2 = 0.69$, $n = 30$). The estimated values for $J_{max(ml)}$ and $K_{m(ml)}$ of $SrNTA^-$ are presented in Table 3. The fitting of this model to the results concerning the uptake of Sr and Ca indicates that the uptake of the organic metal–ligand complexes may contribute to the overall uptake of the metals. However, further studies with radiolabelled NTA are required to demonstrate the availability of metal–NTA complexes.

In a calcium free environment, the uptake of Sr in the presence of the ligands and in the control were lower than what is predicted by the Sr/Ca uptake model (Fig. 4). Exposure to water with very low Ca^{2+} concentration causes regulatory changes and serious disturbances in ion permeability to the epithelia and may result in a net loss of this ion (Hanssen, 1991). In a previous study by Chowdhury et al. (2000) uptake of Sr in carp was found to increase with decreasing Ca concentration in the exposure water down to 10 μM (~ 6 μM Ca^{2+} ion activity). The present study shows that in calcium free conditions uptake of Sr is decreased. Thus, it appears that there is a threshold in ambient Ca concentration below which uptake of Sr decreases strongly, either due to decreased uptake or increased leakage. This effect, however, only occurs at calcium exposure concentrations much below those of natural waters.

From this study, it is concluded that Sr and Ca uptake by carp in the presence of complexing ligands is not always a mere function of the free metal-ion activity, even after taking into account the competitive interaction between Ca and Sr for gill uptake sites.

In the presence of NTA, fish seem to take up $SrNTA^-$ and $CaNTA^-$ complexes directly from water, such that the uptake of Sr and Ca is higher than expected on the basis of the FIAM.

Acknowledgements

This work was financially supported by the Nuclear Fission Safety RTD program (Radiation protection contract # F14P-CT95-0018) of the European Commission. Mohammed J. Chowdhury is thankful to Chittagong University, Bangladesh for granting study leave for doing this research.

References

Blust, R., Van Ginneken, L., 1998. SOLUTION: a Model to Predict the Chemical Spciation of Aqueous Solutions. University of Antwerp, Antwerp.

Blust, R., Van Ginneken, L., Comhaire, S., 1997. Bioavailability of radiocobalt to the common carp, *Cyprinus carpio*, in complexing environments. In: Desmet, G., Blust, R., Comans, R., Fernandez, J.A., Hilton, J., De Bettencourt, A. (Eds.), Freshwater and Estuarine Radioecology. Elsevier, Amsterdam, pp. 299–305.

Bowen, H.J.M., 1966. Trace Elements in Biochemistry. Academic Press, London.

Brezonik, P.L., King, S.O., Mach, C.E., 1991. The influence of water chemistry on trace metal bioavailability and toxicity to aquatic organisms. In: Newman, M.C., McIntosh, A.W. (Eds.), Metal Ecotoxicology: Concepts and Applications. Lewis Publishers, Florida, pp. 1–31.

Brungs, W.A., 1965. Experimental uptake of strontium-85 by freshwater organisms. Health Phys. 11, 41–46.

Burgess, J., 1978. Metal Ions in Solution. Ellis Horwood, Chichester.

Campbell, P.G.C., 1995. Interactions between trace metals and aquatic organisms: a critique of the free-ion activity model. In: Tessier, A., Turner, D.R. (Eds.), Metal Speciation and Bioavailability in Aquatic System. Wiley, Chichester, pp. 45–102.

Chesser, R.K., Sugg, D.W., Lomakin, M.D., Van den Bussche, R.A., DeWoody, J.A., Jagoe, C.H., Dallas, C.E., Whicker, F.D., Smith, M.H., Gaschak, S.P., Chizhevsky, I.V., Lyabik, V.V., Buntova, E.G., Holloman, K., Baker, R.J., 2000. Concentrations and dose rate estimates of ^{134,137}Cesium and ^{90}strontium in small mammals at Chernobyl, Ukraine. Environ. Toxicol. Chem. 19, 305–312.

Chowdhury, M.J., Blust, R., 2001. A mechanistic model for the uptake of waterborne strontium by the common carp (*Cyprinus carpio* L.). Environ. Sci. Technol. 35, 669–675.

Chowdhury, M.J., Van Ginneken, L., Blust, R., 2000. Kinetics of waterborne strontium uptake in the common carp, *Cyprinus carpio*, at different calcium levels. Environ. Toxicol. Chem. 19, 622–630.

Clesceri, L.S., Greenberg, A.E., Trussell, R.R., 1989. Standard Methods for the Examination of Water and Wastewater, 17th ed. American Public Health Association, Washington.

Coughtrey, P.J., Thorne, M.C., 1983. Radionuclide Distribution and Transport in Terrestrial and Aquatic Ecosystems: a Critical Review of Data, vol. 1. A.A. Balkema, Rotterdam.

Crocker, I.H., Merritt, W.F., 1972. Analysis of environmental samples by spark source mass spectrometry. 1. Trace elements in water. Water Res. 6, 285–295.

Daly, H.R., Campbell, I.C., Hart, B.T., 1990. Copper toxicity to *Paratya australiensis*. I. Influence of nitrilotriacetic acid and glycine. Environ. Toxicol. Chem. 9, 997–1006.

Dickson, A.G., Whitfield, M., 1981. An ion-association model for estimating acidity constants (at 25 °C and 1 atm pressure) in the electrolyte mixtures related to seawater (ionic strength 1 mol kg^{-1}). Mar. Chem. 10, 315–333.

Eisenbud, M., 1973. Environmental Radioactivity. Academic Press, New York.

Errecalde, O., Campbell, P.G.C., 2000. Cadmium and zinc bioavailability to *Selenastrum capricornutum* (Chlorophyceae): accidental metal uptake and toxicity in the presence of citrate. J. Phycol. 36, 473–483.

Flik, G., Van Rijs, J.H., Wendelaar Bonga, S.E., 1985. Evidence of high affinity Ca^{2+}-ATPase activity and ATP-driven Ca^{2+}-transport in membrane preparations of the gill epithelium of the cichlid fish *Oreochromis mossambicus*. J. Exp. Biol. 119, 335–347.

Flik, G., Verbost, P.M., Wendelaar Bonga, S.E., 1995. Calcium transport processes in fishes. In: Wood, C.M., Shuttleworth, T.J. (Eds.), Fish Physiology, Cellular and Molecular Approaches to Fish Ionic Regulation, vol. 14. Academic Press, New York, pp. 317–343.

Fortin, C., Campbell, P.G.C., 2000. Silver uptake by the green *alga Chlamydomonas reinhardtii* in relation to chemical speciation: influence of chloride. Environ. Toxicol. Chem. 19, 2769–2778.

Ginzburg, G., 1976. Calculation of all equilibrium concentrations in a system of competing complexation. Talanta 23, 42–149.

Hanssen, R.G.J.M., 1991. Synthesis and Secretion of the Calcium Regulating Hormone Stanniocalcin in Teleost Fish. Ph.D. thesis, Catholic University of Nijmegen, The Netherlands.

Hering, J.G., Morel, F.M.M., 1990. The kinetics of trace metal complexation: Implications for metal reactivity in natural waters. In: Stumn, W. (Ed.), Aquatic Chemical Kinetics. Wiley, New York, pp. 145–171.

Hudson, R.J.M., 1998. Which aqueous species control the rates of trace metal uptake by aquatic biota? Observations and predictions of non-equilibrium effects. Sci. Total Environ. 219, 95–115.

Ishihara, A., Mugiya, Y., 1987. Ultrastructural evidence of calcium uptake by chloride cells in the gills of goldfish, *Carassius auratus*. J. Exp. Zool. 242, 121–129.

Laube, V.M., McKenzie, C.N., Kushner, D.J., 1980. Strategies to response to copper, cadmium and lead by a blue-green and a green alga. Can. J. Microbiol. 26, 1300–1311.

Martell, A.E., Smith, R.M. (Eds.), 1997. Critical Stability Constants of Metal Complexes Database, Ver. 3.0. National Institute of Standards and Technology, Gaithersburg, MD.

Morel, F.M.M., 1983. Principles of Aquatic Chemistry. Wiley-Interscience, New York.

Pagenkopf, G.K., 1983. Gill surface interaction model for trace-metal toxicity to fishes: role of complexation, pH and water hardness. Environ. Sci. Technol. 17, 342–347.

Pärt, P., Wikmark, G., 1984. The influence of some complexing agents (EDTA and citrate) on the uptake of cadmium in perfused rainbow trout gills. Aquat. Toxicol. 5, 277–289.

Playle, R.C., Wood, C.M., 1989. Water chemistry changes in the gill micro-environment of rainbow trout: experimental observations and theory. J. Comp. Physiol. 159B, 527–537.

Playle, R.C., Dixon, D.G., Burnison, K., 1993. Copper and cadmium binding to fish gills: modification by dissolved organic carbon and synthetic ligands. Can. J. Fish. Aquat. Sci. 50, 2667–2677.

Rosenthal, H.L., 1957. The uptake of ^{45}Ca and ^{90}Sr from water by fresh water fishes. Science 126, 669–700.

Savchenko, V.K., 1995. The ecology of the Chernobyl Catastrophe: Scientific Outlines of an International Programme of Collaborative Research. Vol. 16. Man and Biosphere Series, United Nations Education, Science and Culture Organization, Paris.

Sokal, R.R., Rohlf, F.J., 1981. Biometry. W.H. Freeman, New York.

Suzuki, Y., Nakamura, R., Ueda, T., 1972. Accumulation of strontium and calcium in freshwater fishes of Japan. J. Radiat. Res. 13, 199–207.

Turner, D.R., 1995. Problems in trace metal speciation modelling. In: Tessier, A., Turner, D.R. (Eds.), Metal Speciation and Bioavailability in Aquatic Systems. Wiley, Chichester, pp. 149–203.

Van Ginneken, L., Blust, R., 1995. Sequential determination of a combined γ/β and a pure γ-emitter by gamma and liquid scintillation counting: application to the transport of metals across fish gills. Anal. Biochem. 224, 92–99.

Van Ginneken, L., Comhaire, S., Blust, R., 1996. Determination of blood content in teleost gills using a liquid scintillation analyser: procedure for the correction of branchial metal accumulation rates. Comp. Biochem. Physiol. 115C, 33–40.

Van Ginneken, L., Chowdhury, M.J., Blust, R., 1999. Bioavailability of cadmium and Zinc to the common carp, *Cyprinus carpio*, in complexing environments: a test for the validity of the free ion activity model. Environ. Toxicol. Chem. 18, 2295–2304.

Van Leeuwen, H.P., 1999. Metal speciation dynamics and bioavailability: inert and labile complexes. Environ. Sci. Technol. 33, 3743–3748.

Vanbriesen, J.M., Rittmann, B.E., Xun, L., Girvin, D.C., Bolton, H. Jr, 2000. The rate-controlling substrate of nitrilotriacetate for biodegradation by *Chelatobacter heintzii*. Environ. Sci. Technol. 34, 3346–3353.

Vercauteren, K., Blust, R., 1996. Bioavailability of dissolved zinc to the common mussel *Mytilus edulis* in complexing environments. Mar. Ecol. Prog. Ser. 137, 123–132.