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MOLECULAR DYNAMICS OF METAL UPTAKE, ACCUMULATION AND ELIMINATION IN MARINE ORGANISMS

MOLECULAIRE DYNAMIEK VAN DE OPNAME, ACCUMULATIE EN ELIMINATIE VAN METALEN BIJ MARIENE ORGANISMEN

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Introduction

Biological role of metals

Life has evolved in the presence of metals, available at a wide range of environmental concentrations and chemical characteristics. As a result living organisms have developed many different requirements for metals. Some metals are needed in small amounts, others in larger quantities at millimolar or higher concentrations. Two major biological roles for metals can be defined , maintaining osmotic balance and providing a general cationic environment. As intracellular organic molecules are predominantly anionic, metal cations are important both for osmotic and electrical charge balance. They also serve in the maintenance and reversal of transmembrane electrical potentials and the construction of frames (i.e. shells and bones). Both alkali (i.e. sodium and potassium) and alkaline earth metals (i.e. calcium and magnesium) are involved in these processes and are called the major metals. In addition, specific physiological requirements are known for many metas, including the above mentioned alkali and alkaline earth metals, six generally required mlnor metals (i.e. manganese, iron, cobalt, copper, zinc and molybdenum) and four other metals which are required by at least some organisms (i.e. chromium, vanadium, nickel and tin). The so called minor metals may be defined as metals present in minute amounts, i.e. less than 0.01 percent of the mass of the organism (Ochiai,1977; Hay 1984; Frausto da Silva and Williams,1991).
The six generally required minor metals are all transition metals. One of the characteristics of transition metals is their ability to assume different oxidation states. Therefore they participate readily in redox reactions. Iron and copper serve as the active centres of various metalloenzymes, catalysing electron transfers, oxidation reactions and oxygenation reactions (e.g. cytochrome c, monoamine oxidase, cytochrome P450). They are also the elements that the organisms use as the active sites of oxygen-carrying proteins (e.g. hemoglobin, hemocyanin). With the exception of alkali metal ions, metal ions can generally act as Lewis acids. Some of the transition metals, notably cobalt, manganese and zinc, constitute the active sites of the enzymes that catalyse such reactions as methylation, carboxylation and hydration for a variety of compounds (e.g. methionine synthetase, pyruvate carboxylase, carbonic anhydrase) (Ochiai, 1977; Hay, 1984; Frausto da Silva and Williams, 1991).
The biology of essential metals is largely a chemistry of the light elements which form ionic species with low charges, two or less, and have relatively soluble salts. It appears that the biological evolution has not generated a role for the other elements because any chemical role which they could perform can be performed as well or better by another more readily available element. Cadmium, lead and mercury belong to this second group of metals without any established biological function. All the non-essential metals are of low availability due to their low natural abundance and/or the insolubility of their hydroxides and/or sulphides. However, when made available to an organism they are often extremely toxic at relatively low concentrations. Although some metals are essential for life, all metals are toxic at sufficiently high concentration. For some essential metals, such as copper and zinc, the window between what is essential and what is toxic may be very narrow. Because many organisms exist on the margin of either advantageous or deleterious effect, it is very important, for both essential and non-essential metals, that their physico-chemical distribution in natural environments remains relatively constant (Batley, 1989; Furness and Rainbow, 1989; Foulkes, 1990).

Chemical speciation of metals

Chemical speciation refers to the different chemical forms as which an element exists. Together these different chemical species make up the total concentration of an element in a given system. In the aquatic environment four abiotic reservoirs are distinguished: the suspended matter, the sediments, the surface waters and the pore waters. These four reservoirs strongly interact with each other. Between the suspended matter and the metals in solution, adsorption/desorption and precipitation/dissolution processes take place. The suspended matter and the deposited sediments are interlinked through sedimentation and erosion processes. Processes taking place after deposition and during diagenesis provide the interstitial waters with sometimes high concentrations of metals, which through processes such as diffusion, consolidation and perturbation are able to influence the metal concentrations in the surface waters.
The distribution of a metal between the suspended and dissolved species depends on the concentration and nature of both suspended and dissolved matter. The range of chemical species can include many diverse forms such as: the free hydrated metal ion, inorganic and organic complexes, metals bound to inorganic and organic colloids and solids. Changes in physico-chemical conditions often cause a redistribution of the different metal species among the different phases (Stumm and Morgan, 1981; Salomons and Forstner,1984; Batley, 1989).

Uptake of metals

The mechanisms of metal uptake by aquatic organisms have not yet been fully elucidated although two major routes by which trace metals can be taken up have been identified, i.e. direct absorption of dissolved species from solution and ingestion of food or other particulates. Metal uptake is determined by processes occurring at the interface of solution and organism. This interface is in fact a thin diffusion layer lined by a hydrophobic barrier, the plasma membrane, a lipid bilayer spanned by a host of different proteins. Some of these proteins have a globular structure and they catalyse the transfer of small molecules across the bilayer. Other proteins have only a single hydrophobic helical segment that holds them in the membrane. Some of these are receptors that carry large molecules into the cell. Whereas most of these molecules are free to diffuse laterally around in the liquid bilayer, there are other structures such as the gap junctions and the tight junctions that remain relatively static. In contrast there is also the highly dynamic movement of the membrane during endocytosis, i.e. the uptake of material from the surface of a cell by an infolding of the plasma membrane to form a vesicle. From this it appears that metal species can traverse the interface and enter the body in at least three ways. Mechanisms for carrier facilitated transport, either passive or active, may include, (i) uptake via carriers specific for nutritionally essential cations (e.g. calcium, magnesium), (ii) transport of nutritionally essential metal complexes, (iii) transport of metals complexed with essential nutrients (e.g. amino acids) on carriers specific for the nutrient and (iv) non-specific complexation of metal species with carrier molecules. Alternatively, polar metal species could traverse the bilayer by passive diffusion through water filled pores in the membrane. Hydrophobic or uncharged metal species may be taken up by passive diffusion through the hydrocarbon core of the lipid bilayer. Finally, endocytosis provides a means for

uptake of large complexes, colloids and particulates. The most common kind of endocytosis is pinocytosis. These vesicles are small and contain only solutes. Phagocytic vesicles are much larger and contain solid matter. The endocytic process can be facilitated by the binding of the solute to specific receptors which diffuse across the surface of the cell. Prior to endocytosis they are trapped, forming clusters, at specialised regions of the membrane, called coated pits.
Thus, the biological availability of a metal to aquatic biota is not only determined by the speciation of dissolved and suspended species in solution, but depends also on the instruments available to sequester and ultimately transport the metal across the solution-body interface (Friedman, 1986; Gennis, 1989; Frausto da Silva and Williams, 1991).

Research

The motivation for many trace metal uptake studies is the need to understand the impact of metal pollution on marine communities in order to assess and remediate the effects of anthropogenic activities on environmental health. Therefore it is necessary to understand the relationships between contaminant levels in the environment and the tissues of the exposed organisms.
The physical and chemical conditions in saline water environments such as sea and estuarine areas may be rather different. This does not only influence the speciation of the metals, but also the physiological organisation and condition of the organisms which live in these environments. The most important steps in metal uptake are: (i) the translocation of the metal across the solution-body interface and (ii) the transport from the exchange surfaces to the blood. The intracellular environment of the exchange structures has a high metal binding capacity and can act as a free metal ion buffer. Therefore it is anticipated that the effects of changes in the speciation of the metal with changes in the environmental conditions do not extent beyond the solution-body interface. However, changes in environmental conditions such as temperature, salinity and the hydrogen ion activity are likely to influence the uptake of the metal by altering the organisation of the interface and modulating the activity of the transport systems involved. To obtain a basic appreciation of the mechanisms controlling metal availability, it is necessary to study the separate and combined effects of changes in metal speciation and other environmental factors on the uptake of metals in different types of organisms. This approach must provide the necessary understanding to qualitatively and quantitatively describe the different effects of the environment on the uptake of metals by aquatic organisms.
To contribute to this goal we have studied the uptake of metals by a crustacean and a bivalve which are very different in structural and functional organisation i.e. the brine shrimp, *Artemia franciscana*, and the common mussel *Mytilus edulis*. During these experiments the translocation of the metal across the solution-body interface was followed by measuring metal uptake in animals exposed and/or acclimated to different conditions.

Artemia franciscana

The brine shrimp *Artemia* is a crustacean belonging to the class of the Branchiopoda and the order of the Anostraca. The members of the order are characterised by having stalked eyes, no carapace and an elongated trunk. In most

forms there are eleven pairs of leaf-shaped limbs. The limbs serve for swimming, feeding and respiration. Although most anostracans are freshwater organisms the brine shrimp is distinct, since it occurs in highly saline environments such as salt ponds and lakes. The brine shrimp has a cosmopolitan occurence and is found throughout the tropical, sub-tropical and temperate climatic zones. The brine shrimp is an euryhaline osmoregulator maintaining the osmolarity of the body fluids nearly constant in both low and high salinity waters. This allows the organism to tolerate salinities ranging from 0.5 to 30 %. Reproduction in brine shrimp is either by producing free swimming naupllii (ovoviviparity) or encapsulated embryos (ovoparity). It is only under harsh environmental conditions that these cysts are produced, This is an ecological adaptation mechanism to assure the survival of the population under difficult conditions by formation of dormant stages which are well protected from the environment (Sorgeloos, 1980). The genus *Artemia* is subdivided into a number of bisexual sibling species and a parthenogenetic entity. Sibling species are reproductively isolated in nature but identical or very similar in morphological appearance. For example, the brine shrimps from San Francisco Bay and Great Salt Lake are now designated *Artemia franciscana* (Abreu-Grobois, 1987).

Mytilus edulis

The genus *Mytilus* L. belongs to the family Mytilidae which dates back to the Jurassic and perhaps even Devonian times. Originally containing 22 species, its members have been assigned to several different genera, only 3 being retained in *Mytilus*. The family is recognised by its shell form and sculpture, hinge structure and muscle scars.

Mytilidae belong to the facultative anaerobes i.e. they can live anaerobically but prefer to use oxygen when it is available, as it allows a much more economical use of fuel molecules. They can tolerate also a wide range of oxygen concentrations. *Mytilus* sp, live in inter-tidal and shallow waters which are characterised by large fluctuations in temperature, salinity and oxygen availability (Gosling, 1992).

Mytilus is found in littoral and shallow sublitoral waters. They are living on a variety of substrates, such as rocks, stones, shingle, dead shells and compacted mud or sand. Although the operation of certain local factors such as extreme salinity, or availability of suitable substrate, may limit the local abundance of a species, it is primanly the sea temperature which controls the overall distnbution of mussels. Most species have a wide range of temperature within which they can survive, and a narrower range within which they can reproduce. Those with relatively broad breeding-temperature ranges are cosmopolitan in their distribution, whereas those with more limited ranges are usually more restricted. Salinity is an important factor in determining the distriution of *Mytilus edulis* and *Mytilus californianus* respectively (Bayne et al., 1976)

Molluscs vary in their ability to regulate the ions in their internal environment. Marine and brackish water species do so hardly at all. The composition of the hemolymph of marine molluscs is very close to the surrounding seawater. The small degree of regulation seen in the internal environment of marine molluscs is largely a reflection of the high energy cost of regulation in aquatic animals that have large areas of permeable surfaces.

The temporal changes in environmental conditions (i.e. food availability, temperature, salinity, oxygen concentration) induce associated changes in physiology and biochemistry of the organisms. (Gosling 1992).
Bivalves, such as mussels, are world-wide used as monitoring organisms in environmental programs because:

1. They are dominant members of coastal and estuarine areas and are widely distributed.
2. They are filter feeders that pump large volumes of water over their tissues.
3. They accumulate high concentrations of pollutants, such as heavy metals, in their tissues This makes measurement of trace contaminants in their tissues easier than in seawater.
4. They have a sedentary lifestyle and are therefore more suited than mobile species as integrators of contamination in a given area.
5. Mussel populations are large enough for frequently sampling and they can be readily transplanted to other sites.
6. Mussels are a commercial seafood and measurement of chemical contamination is of interest for public health.

(NRC, 1980; Phillips, 1980; Widdows, 1985; Farrington et al., 1987).

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Modelling the speciation of metals in aquatic environments of low and high ionic strength

INTRODUCTION

The effects and fate of metals in the aquatic environment depends on the chemical speciation of the element. Chemical speciation refers to the actual forms or species in which an ion or molecule is present in solution or sediment. Within this framework interactions of metals with inorganic and organic solutes and particles play a major role in controlling the mobility and distribution of a metal among different compartments (Florence and Batley, 1980; Salomons and Förstner, 1984; Wangersky, 1986).

Three types of metals and three types of metal-ligand interactions need to be considered. In the first group of metals the interactions are mainly ionic. The major metal ions such as sodium, potasssium, calcium and magnesium belong to the first group of elements (class A). The second group consists of metals with a lesser tendency for ionic interactions. Bonding is either ionic, coordinate or covalent. The minor metal ions such as the micronutrients copper and zinc belong to the second group of elements (borderline). In the third group of metals the interactions are mainly coordinate or covalent. The toxicants cadmium and mercury belong to the third group of elements (class B) (Ahrland, 1968; Nieboer and Richardson, 1980; Frausto Da Silva and Williams, 1991).

Several models have been used to estimate the effects of changes in the composition of the solution on the speciation of the elements in the system. These models can be grouped in two major classes: (i) the specific interaction models and (ii) the ion-association models. The specific interaction model have been applied successfully for the prediction of ion activities and mineral solubilities of the major ionic components of solutions over a wide range of pressures, temperatures and ionic strengths. The ion-association model has been applied successfully for the prediction of ion activities and mineral solubilities of the major and minor ionic components of solutions over a limited range of pressures, temperatures and ionic strengths. For many metal-ligand interactions information is not available for calculation of the specific interaction model parameters. The combination of the two approaches however, yields a consistent model that can be used for all components of a solution (Johnson and Pytkowicz, 1979; Whitfield, 1979).

The first step in modelling the speciation of a metal in a particular environment is the characterisation of the different compartments present. If the composition of the system is well defined it is possible to model the speciation of a particular metal using the principle concepts of chemical thermodynamics to describe the equilibrium relations between the different components. The validity of the model depends on the compilation of reactions and stability constants describing the interactions between the different metals and ligands present (Sposito, 1985; Martell et al., 1988; Waite, 1989).

The present work aims to compare some important methods used in the construction of ion-association models and to identify some of the problems encountered during compilation. To evaluate the results of the different methods we have compared estimated and measured conditional stability constants for different proton-ligand and metal-ligand interactions and calculated the impact of errors in the determination of stability constants on the results of the speciation calculations.

RESULTS AND DISCUSSION

Chemical equilibrium

Based upon the principles of chemical thermodynamics a model can be built that describes the most fundamental physical and chemical processes that take place in a well defined environment. Chemical modelling starts with the assumption that the system is in equilibrium. Since the kinetics of reaction processes range over orders of magnitude it is not possible to be absolutely sure about the equilibrium state of the system. A chemical model can therefore only be of use when the chemical reactions of consideration are either very fast or very slow relative to the kinetics of the process studied (e.g. metal uptake in plant or animal). In the former case chemical equilibrium should prevail, wheras in the latter case, the reactants are considered as inert species (Sposito, 1985; Millero, 1990).
The major problem in the construction of a speciation model is the compilation of the reactions and stability constants describing the interactions among the components of the system. It is essential that all prevailing interactions are recognised and described by a set of accurately measured constants. Altough a large number of protonation and metal complex interactions have been characterised and stability constants obtained under various conditions the results show considerable variability. Stability constants for the same equilibrium and reaction conditions can differ over an order of magnitude depending on the source. For many complexes, stability constants have not been measured and estimates must be made by comparison with values obtained for other metal ions with the same ligand, or other similar ligands with the same metal ion (Smith et al., 1985a, 1985b, 1987).

Stability constants

For each equilibrium reaction (i.e. M+L == ML) considered in the ion-association model a thermodynamic stability constant (K(ML)) is given by
K(ML)=a(ML)/a(M)a(L)=m(ML)/m(M)m(L)*f(ML)/f(M)f(L) with a() the activity, m() the concentration and f( ) the activity coefficient of the species. The measured quantity is usually the conditional stability constant (Q(ML)) for the equilibrium in a solution of specified ionic strength (i.e. Q(ML)=(ML)/(M)(L) and K(ML)=Q(ML)xf(ML)/f(M)f(L)= Q(ML)xF(ML)).
Several factors such as ionic strength, temperature and pressure influence the equilibrium position of a reaction. Environmental conditions deviate from standard conditions (i.e. zero ionic strength, temperature of 25 °C and 1 atm total pressure) which makes it necessary to account for changes in the stability constants with variations in the environmental conditions. Variations in the ionic strength of the solution have the most important effect on the stability values. Changes in temperature have a minor effect and the effect of pressure on the stability values is negligible under normal environmental conditions (Byrne et al., 1988; Millero, 1990).
The ionic strength or electrolyte effect results from the electrostatic attractive and repulsive forces that exist between the ions of the electrolyte solution and the ions involved in an equilibrium. These forces cause each ion to be surrounded by a layer that contains a slight excess of electrolyte ions of opposite charge. The consequence of this is usually a reduction in the overall attraction between the ions

involved in an equilibrium. The electrolyte effect is highly dependent upon the charges of the species involved and the ionic strength of the solution.

The ionic strength of a solution is defined as $I=1/2\Sigma(m_iZ_i^2)$ where m_i is the concentration of an ion in moles per liter, Z_i is its charge, and the sum is taken over all the ions in the solution. Stability constants are commonly reported for infinite dilutions or for a specified ionic strength usually either 0.1, 0.5, 1.0, 2.0 or 3.0 M. Neither of these ionic strengths is commonly encountered in environmental solutions and is therefore necessary to correct for the ionic strength effect. To describe the effect of ionic strength on equilibria a concentration parameter called the activity has been introduced. The activity is defined as $a_i=f_i[m_i]$ where a_i is the activity of the ion and m_i is its concentration in moles per liter, and f_i is the activity coefficient. The activity coefficient and the activity vary with ionic strength such that substitution of a_i for m_i in an equilibrium relation frees the numerical value of the constant from dependence on the ionic strength. The activity of an ion can be considered as the effective concentration of the ion. The fraction of the real concentration that determines its behaviour towards other ions with which it may interact.
A theory exists that permits the calculation of activity coefficients from their charge and average size. In this theory, ions are regarded as point charges in a medium with a dielectric constant equal to that of the pure solvent. The validity of the original and of the different extended forms of the mathematical description of the theory is fairly limited. The most frequently used formulae are summarised in Table 1. For solutions with ionic strengths of below 0.1, the electrolyte effect depends largely on the charge of the ions and the ionic strength of the solution. The calculated activity coefficients are increasingly erronous when the ionic strength increases above this point and may amount to several hundred percent. To extend the purely theoretical treatment to higher ionic strenghts and a linear term was added to the original equations. In this way the effect of ionic strength on activity coefficients can be described for solutions upto a few molars.
The linear term has a different value for each ion and has to be determined experimentally. The linear term accounts for short range interactions, i.e. the interactions of the ions and the solvent. The greater the concentration of ions, the greater the portion of the solvent molecules that is oriented thus altering the effect of the solvent on the position of the equilibrium (Beck and Nagypal, 1990).
Since activity coefficients of individual species cannot be measured directly and most sources list stability constants rather than activity coefficients it is more convenient to derive an interpolation function for the effect of ionic strength on the stability constants themselves. The function which also has the form of an extended Debye-Hückel equation (i.e. $\log Q= \log K+1/2z^2I^{1/2}/(1+BI^{1/2})+CI$ with $z^2=\Sigma z^2(\text{products})-\Sigma z^2(\text{reactants})$) and is used to describe the change in the conditional stability constant of an equilibrium with ionic strength. This function can be fitted directly to measured conditional stability constant of an equilibrium within the 0-3.0 M range of ionic strengths(Turner et al., 1981).
The validity of the function to describe the effect of ionic strength on the stability constants of differing proton-ligand and metal-ligand interactions is demonstrated by the results summarised in Fig. 1-7. These results also show that the effect of ionic strength on proton-ligand and metal-ligand interactions is more or less the same for interactions among metal ions and ligands of the same type. This consideration is important for estimating the effect of ionic strength on a proton-ligand or

metal-ligand interaction when stability constants have only been measured at one single ionic strength so that it is not possible to determine the fitting parameters. Analysis of the variation in stability constants with ionic stength has resulted in a compilation for the correction of stability constants measured at one ionic strength (i.e. 0, 0.1, 0.5, 1.0, 2.0 and 3.0) to any other of these ionic strengths. This compilation was originally derived from observed trends in stability constants with ionic strength for interactions between metals and a number of organic ligands for which reliable stability constants were available (Martell et al., 1988). Some examples of the variation of the stability constants with the ionic strength of the solution and the charge of the interacting ions are shown in Table 2. This compilation has been evaluated by comparing measured and estimated stability constants within the 0-3.0 M range of ionic strengths. The results summarised in Fig. 1-7 show that the correctness of the estimates depends on the hydrogen-ligand or metal-ligand interaction studied. In most cases the interactions with inorganic ligands are better described than the interactions with organic ligands.

The effect of ionic strength on the stability constant of a simple metal-ligand (i.e cadmium-chloride) and a proton-ligand interaction (i.e. hydrogen-citrate) is shown in Fig. 1 and 2. In both cases there is very good agreement between the fit of the interpolation function and the measured stability constants. The stability constants were extracted from a critical compilation which is the result of an extensive evaluation of the stability constants listed in various sources (Martell and Smith, 1974-1989). Other compilations provide non-critical listings of stability constants which give a more complete picture of the variation in stability constants for the same interaction (Sillen and Martell, 1964; Hoegfeldt, 1983). Critical and non-critical stability constants for the cadmium-chloride and hydrogen-citrate interaction are compared in Fig 3 and 4.

For many metal-ligand interactions stability constants have only have been measured at one or a few ionic strengths. However, stability constants measured at five ionic strengths are necessary for estimating the two parameters of the interpolation function. If less values are available the first parameter is fixed ($B=1.5$) and only the second parameter is calculated in the regression. The statistics of the regression analysis of the cadmium-chloride and hydrogen-citrate interaction are summarised in Table 3 and 4. In case of the cadmium-chloride and hydrogen-citrate interaction the electrolyte effect is well described over a wide range of ionic strengths (0-3.0). The effect of the number of stability constants measured within a certain range of ionic strenghts can thus be evaluated by fitting the interpolation function to all possible combinations of the six critical stability constants measured (two, three and four values). The results summarised in Fig 5 and 6 show that three or four values are enough to make a reasonable estimate (i.e. error < 0.1) of the stability constant over a wide range of ionic strengths. When only two values are available the error increases considerably but estimates remain reliable for ionic strengths upto unity.

A number of ligands interacts with the alkali metal ions (e.g. Na^+, K^+) that are often introduced in high concentrations with the inorganic salt used to control the ionic strength (i.e. NO_3, ClO_3) of a solution during the determination of a stability constant. In many instances the effect of these interactions on the free ligand concentration has not been recognised so that the reported stability constants is not exactly correct. The effect of this type of error on the stability constant is shown for the hydrogen-citrate interaction in Table 5 and fig 7. The stability constants have been

measured in the either a sodium, potassium or tetraalkylammonium salt solution. Measured stability constants decrease strongly in the presence of high concentrations of sodium or potassium interact with the ligand. When the measuring conditions are well defined and the stability constants for the interfering metal-ligand interaction are known it is possible to correct the stability constant for this effect. Stability constants measured in alkali metal solutions without correction for complexation of the alkali metal ion with the ligand are conditional stability constants of the form, Q'(ML)=m(ML)/m(M)(m(L)+m(NL)) where M is the metal, L the ligand and N the alkali metal. The required stability constants are Q(ML)=m(ML)/m(M)m(L) and Q(NL)=m(NL)/m(N)m(L). Rearrangement of these relations results in an expression for the stability constant Q(ML) which incorporates the conditional stability constant Q'(ML), the alkali metal stability constant Q(NL) and the alkali metal ion concentration. Since, the degree of complexation of the alkali metal ion is always relatively low because of the high alkali metal to ligand concentration, the total concentration of the alkali metal ion will not differ a lot from the concentration of the free alkali metal ion. The results summarised in Table 5 and Fig 7 show that correction of the stability constants for this alkali metal ion effect gives values which are close to the constants measured in tetraalkylammonium salt solutions.

Speciation calculations and propagation of errors

The combination of analytical errors in the determination of stability constants and in the procedures use to estimate stability constants may introduce a considerable amount of uncertainty. The interactions between the major inorganic components of aquatic environments such as Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, Br^-, OH^-, $SO4^{2-}$ are reasoably well characterised and the errors in the stability constants are relatively small (<0.1 log Q). However, for most minor ion components the error is more considerable and varies between 0.1 and 1.0 of the log Q. As such, it is important to know the effect of these errors on the result of a speciation calculation. For this purpose we have developed a model called SOLLUTION, which takes into account the effect of errors in the stability constants on the result of the speciation calculation. The basic algorithm used in the model is based on the mathematical routines used in the model COMPLEX (Ginzburg, 1976). Each stability constant is assigned an error (e.g. 0.1, 0.5, 1.0) and the speciation calculation is repeated until all combinations have been calculated and compared. The final result lists the concentrations of the different species considered using the stability constants without error and the lowest and highest concentrations of the different species when the stability constants are considered with an error. The effect of these errors on the result of a speciation calculation is shown in two examples. The first example, presented in Fig 8, is a calculation of the free copper ion concentration in a seawater environment in function of an increasing total copper concentration. The complexation of copper with natural organic ligands is modelled using two ligands L_1 and L_2 with concentration of 5 nM and 150 nM and conditional stability constants of $LOGK_1=12$ and $LOGK_2=9$. The solution is titrated with copper from 10^{-9} to 10^{-6} M to simulate a complexation capacity determination. In a second example the free cadmium ion concentration is calculated in function of the salinity of the water. The effect of salinity on the free cadmium ion concentration was modelled by the mixing of freshwater and saltwater to simulate a river-seawater interface. The results of the speciation calculations are reasonably accurate when it is assumed that the stability

constants are correct within 0.1 log units of the true log K. However, errors larger than this limit, introduce a considerable amount of uncertainty in the result of a speciation calculation.

CONCLUSIONS

Chemical modelling is a powerfull tool for the study of proton- and metal-ligand interactions in mixtures of electrolytes. The validity of the model is largely depended on the quality of the compilation of reactions and stability constants which describes the system. Selection of the reactions to be included is a first critical step which can only be succesfull when the system is claerly defined. Extrapolation of stability constants obtained for one ionic strength to another ionic strength is the next most critical step in the process of compilation. A minimum of two stability constants obtained for different ionic strengths is required to fit an interpolation function from which stability constants can be obtained for other ionic strengths. If the stability constant has only been obtained for one ionic strength an interpolation function can still be obtained with reasonable confidence from the interaction of the metal with similar ligands or other metals with the same ligand.

The development of a chemical speciation model, that provides estimates of the errors introduced when the value of some of the stability constants are not exactly known, is an important improvement over many earlier models.

Table 1: Equations for the calculation of activity coefficients (Beck and Nagypal, 1990):

Debye and Hückel	$\log f = -Az^2 I^{1/2}$
Debye and Hückel	$\log f = -Az^2 I^{1/2}/(1+BdI^{1/2})$
Güntelberg	$\log f = -Az^2 I^{1/2}/(1+I^{1/2})$
Scatchard	$\log f = -Az^2 I^{1/2}/(1+1.5I^{1/2})$
Debye and Hückel	$\log f = -Az^2 I^{1/2}/(1+BdI^{1/2})+CI$
Guggenhein	$\log f = -Az^2 I^{1/2}/(1+I^{1/2})+CI$
Davies	$\log f = -Az^2 (I^{1/2}/(1+I^{1/2})-0.3I)$
Datta and Grzybowski	$\log f = -Az^2 I^{1/2}/(1+I^{1/2})+CI+DI^{3/2}$

A and B are function of the solvent dielectric constant (E) and the absolute temperature (T): $A=1.823106/(ET^{3/2})$ and $B=50/(ET)^{1/2}$.

C and D are adjustable parameters which are case specific and determined by fitting the equation to experimental data.

Table 2: Variation in stability constants with ionic strength and charge of the interacting ions relative to 0.1 M ionic strength (Martell et al, 1988).

			M^{+}		
IS	0.0	0.5	1.0	2.0	3.0
LOGK L^{-}	+0.2	-0.1	-0.1	0.0	+0.2
LOGK L^{2-}	+0.4	-0.2	-0.2	-0.1	+0.0
LOGK L^{3-}	+0.6	-0.3	-0.3	-0.3	-0.2
			M^{2+}		
IS	0.0	0.5	1.0	2.0	3.0
LOGK L^{-}	+0.4	-0.2	-0.2	-0.1	0.0
LOGK L^{2-}	+0.8	-0.4	-0.4	-0.4	-0.3
LOGK L^{3-}	+1.2	-0.6	-0.6	-0.7	-0.6
			M^{3+}		
IS	0.0	0.5	1.0	2.0	3.0
LOGK L^{-}	+0.6	-0.3	-0.3	-0.3	-0.2
LOGK L^{2-}	+1.2	-0.6	-0.6	-0.7	-0.6
LOGK L^{3-}	+1.8	-0.9	-0.9	-1.1	-1.0

Table 4: Stability constants and concentration products for the first protonation constant of citrate:

IS	0.0	0.1	0.5	1.0	2.0	3.0
Tetraalkylammonium salt background electrolyte						
	6.396	5.80	5.60	5.78	-	-
Sodium salt background electrolyte						
	-	5.67	5.30	5.22	5.10	5.12
	-	5.85	5.66	5.77	5.89	6.14
Potassium salt background electrolyte						
	-	5.70	5.39	5.31	-	-
	-	5.84	5.69	5.78	-	-

Table 5: Comparison of the two and three parameter extended Debye-Hückel equation for describing the effect of ionic strength on the concentration product of the cadmium-chloride equilibrium.

$LOG\ Q = LOG\ K + 1/2x(-4xIS^{1/2}/(1+(1.5xIS^{1/2})) + (CxIS)$

$R^2=0.993$, F=572, n=6

	B	SE	L1	L2
LOG K	1.956***	0.013	1.915	1.997
C	0.186***	0.008	0.161	0.211

$LOG\ Q = LOG\ K + 1/2x(-4xIS^{1/2}/(1+(1.5xIS^{1/2})) + (CxIS)$

$R^2=0.993$, F=218, n=6

	B	SE	L1	L2
LOG K	1.959***	0.021	1.869	2.049
B	1.468**	0.155	0.801	2.135
C	0.191**	0.024	0.088	0.294

Table 6 Comparison of the two and three parameter extended Debye-Hückel equation for describing the effect of ionic strength on the concentration product of the proton-citrate equilibrium.

$LOG\ Q = LOG\ K + 1/2x(-6xIS^{1/2}/(1+(1.5xIS^{1/2})) + (CxIS)$
$R^2=0.936$, F=59, n=6

	B	SE	L1	L2
LOG K	6.444***	0.047	6.294	6.594
C	0.397***	0.031	0.298	0.496

$LOG\ Q = LOG\ K + 1/2x(-6xIS^{1/2}/(1+(1.5xIS^{1/2})) + (CxIS)$
$R^2=0.970$, F=49, n=6

	B	SE	L1	L2
LOG K	6.370***	0.056	6.129	6.611
B	2.075*	0.376	0.457	3.693
C	0.304*	0.053	0.076	0.532

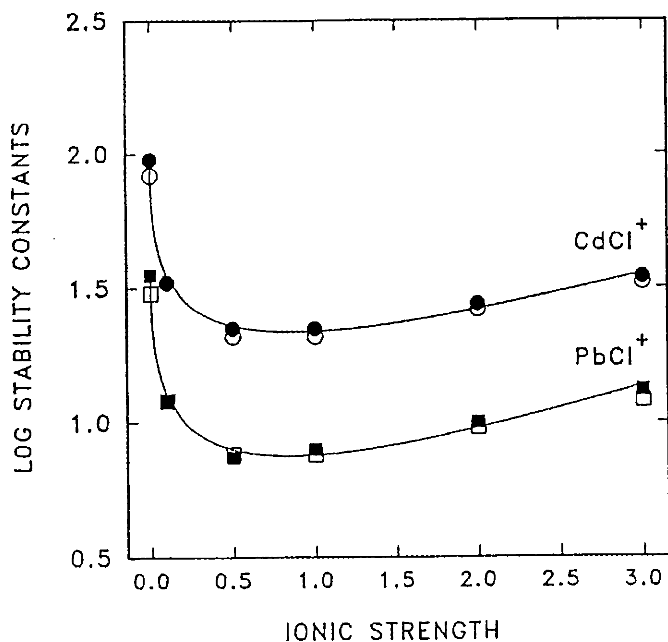

Fig. 1. Effect of ionic strength on the stability constant of the $CdCl^{+}$ and $PbCl^{+}$ complex. Filled circles are critical stability constants, open circles are estimates based on general trends. The line is a fit of the Debye-Hückel interpolation function to the critical stability constants.

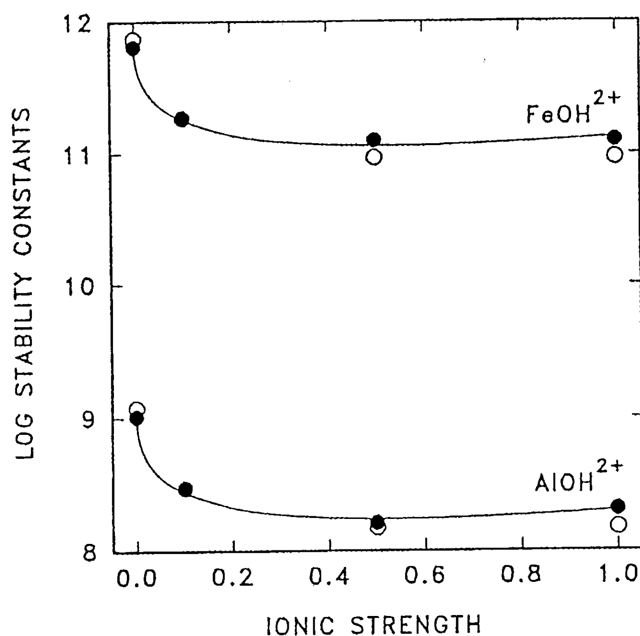

Fig. 2. Effect of ionic strength on the stability constant of the $FeOH^{2+}$ and $AlOH^{2+}$ complex. Filled circles are critical stability constants, open circles are estimates based on general trends. The line is a fit of the Debye-Hückel interpolation function to the critical stability constants.

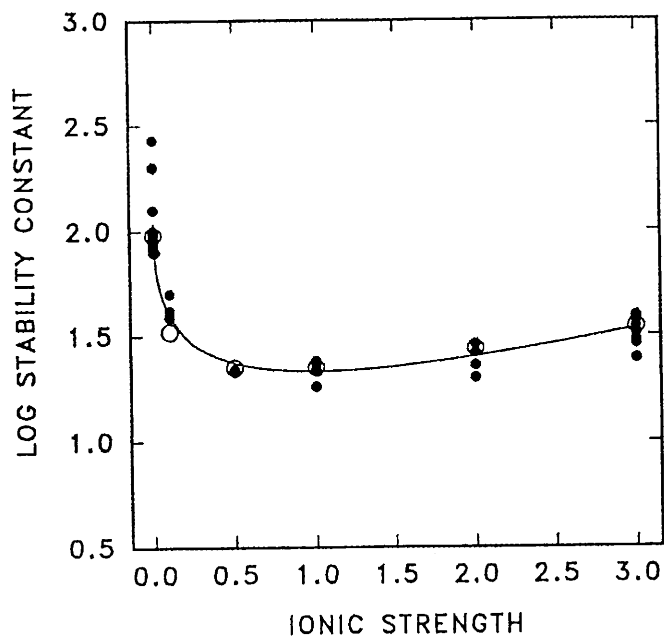

Fig. 3. Comparison of critical (open circles) and non-critical stability constants (filled circles) for the $CdCl^{+}$ complex.The line is a fit of the Debye-Hückel interpolation function to the critical stability constants.

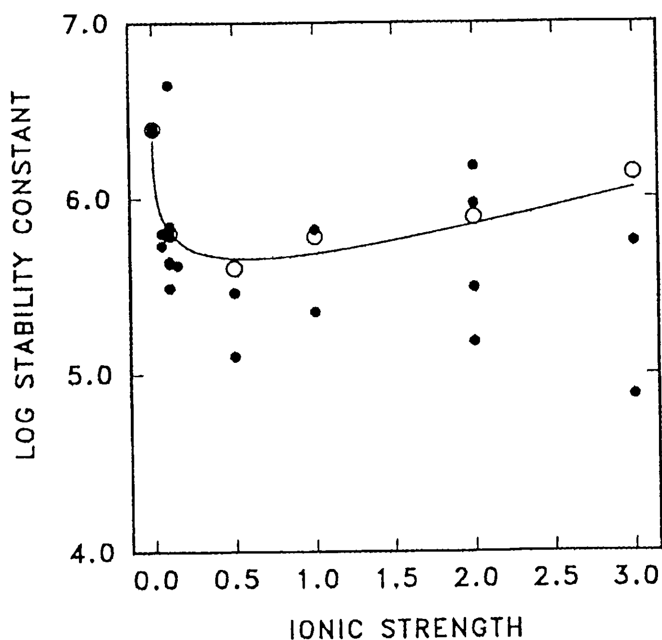

Fig. 4. Comparison of critical (open circles) and non-critical stability constants (filled circles) for the $HCIT^{2-}$ complex. The line is a fit of the Debye-Hückel interpolation function to the critical stability constants.

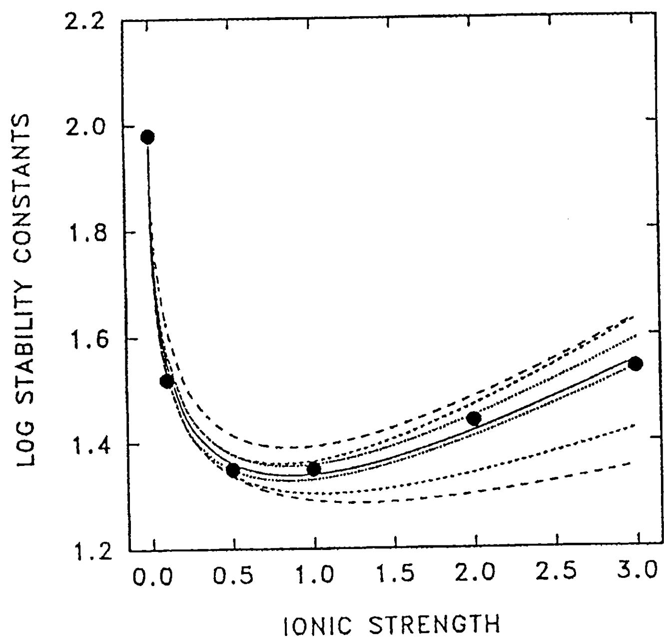

Fig. 5. Effect of fitting the Debye-Hückel interpolation function to two, three or four critical stability constants for the $CdCl^+$ complex.

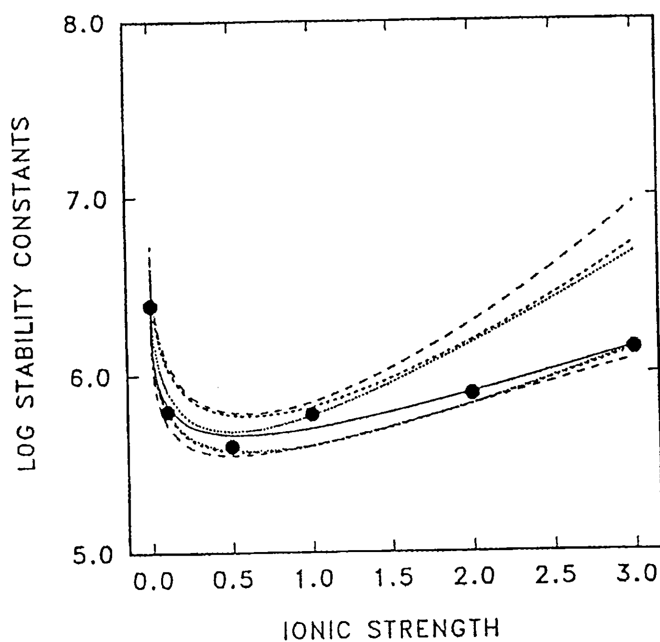

Fig. 6. Effect of fitting the Debye-Hückel interpolation function to two, three or four critical stability constants for the $HCIT^{2-}$ complex.

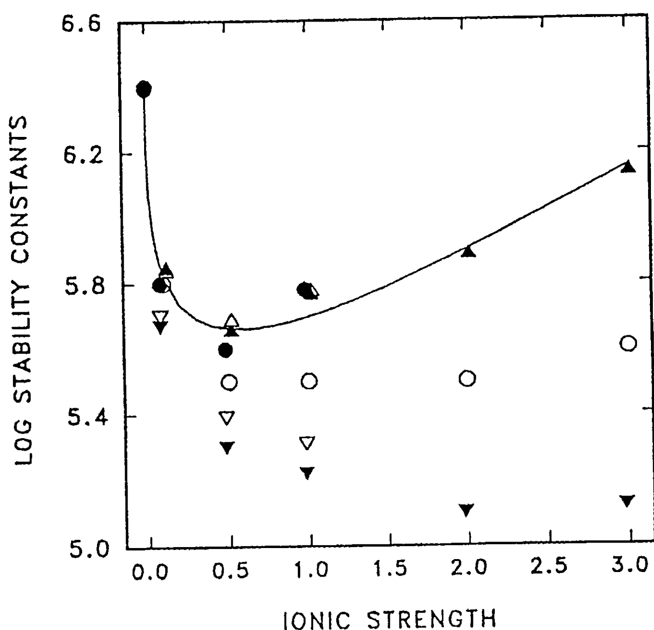

Fig. 7. Effect of the major cation in the solution on the stability constant for the $HCIT^{2-}$ complex. Filled circles are critical stability constants in tetraalkylammonium, open circles are estimates based on general trends, filled triangles down are critical stability constants in sodium salt, open triangles down are critical stability constants in potassium salt. Filled and open triangles up are the respective critical stability constants corrected for the sodium or potassium interaction.

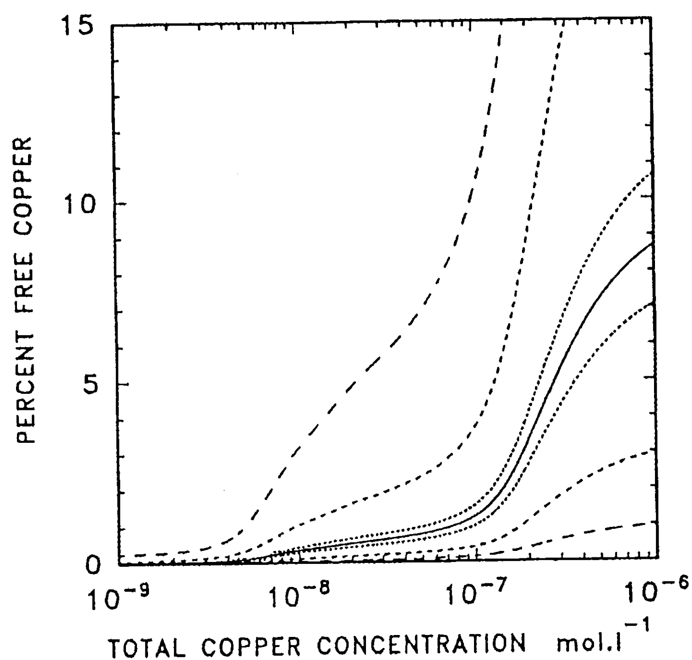

Fig. 8. Propagation of errors for the titration of copper in seawater. Errors are 0.1, 0.5 or 1.0 log units for each copper-complex stability constant considered.

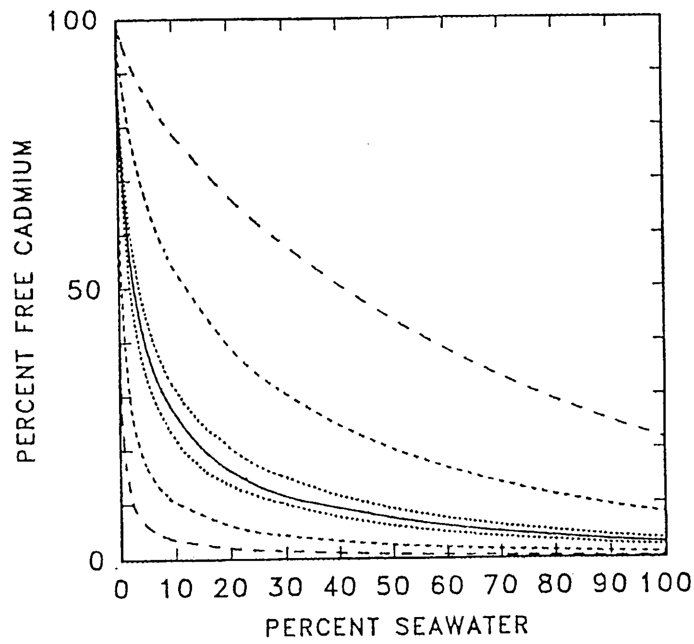

Fig. 9. Propagation of errors for the complexation of cadmium when seawater and freshwater are mixed. Errors are 0.1, 0.5 or 1.0 log units for each cadmium-complex stability constant considered.

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Sequential determination of a combined γ/β and pure β-emitter by gamma and liquid scintillation counting: application to the transport of metals across gills of aquatic organisms

INTRODUCTION

Life has evolved in the presence of metals, available at a wide range of environmental concentrations and chemical characteristics. As a result living organisms have developed many different requirements for metals. Many metals play important roles in the metabolism of many different organisms, maintain osmotic balance, and provide a general cationic environment (Frausta da Silva and Williams, 1991).

Although the biological function of metals is relatively well understood, the mechanisms of metal uptake by aquatic organisms are still not fully known. There are three possible pathways for metal uptake, 1) uptake through the gills, 2) uptake through the skin, and 3) uptake through the gut epithelium. In marine organisms, for instance, metal uptake from the water mainly occurs across the gill epithelium. To obtain a basic appreciation of the mechanisms controlling metal uptake, it is necessary to study the separate and combined effects of changes in metal speciation and other environmental factors on the uptake of metals in different types of organisms (Batley, 1989; Simkiss and Taylor, 1989).

Radioisotope tracer techniques are an expedient tool for the study of metal uptake processes because they provide a very sensitive and accurate technique which makes it possible to follow the transport of several metal ions simultaneously. The use of a second or even a third radioisotope, makes it possible to study interactions between different metal ions or the effects of transport modulators on metal uptake systems in one single experiment. Such experiments are essential to unravel the mechanisms of metal tranport across biological interfaces.

Multiple isotope counting is, however, considerably more difficult than single label counting. The metal isotopes often used are pure β-emitters (^{45}Ca,...) and combined γ/β-emitters (^{57}Co, ^{65}Zn, ^{109}Cd, ^{203}Hg,...). Although simultaneous counting of more than one isotope has been reported (Miyazawa *et al.*, 1991; Bukowski *et al.*, 1992), a detailed systematic description of sample preparation and quench correction procedures in counting a pure β-emitter and a combined γ/β-emitter has not been presented.

In the present paper we describe a combined gamma scintillation/liquid scintillation counting procedure to determine two radioactive metals, in the same biological sample. The method is used in studies on the uptake of metals from water by aquatic organisms of different structural and functional organisation.

The method is demonstrated for calcium and cobalt.

MATERIALS AND METHODS

Gamma Scintillation Counting

Gamma scintillation counting was carried out using a Packard Minaxi Auto-Gamma counting system (model 5530), which was fitted with a three inch thallium-activated, sodium iodide crystal. The crystal contained a 30 mm diameter through-hole in the centre, which can hold scintillation vials up to 28 mm diameter. Radioactive ^{57}Co-chloride, a γ/β-emitter (185 MBq, 1.5 µg Co/ml, carrier free), was purchased from Amersham and was measured in a preset energy window (80-165 keV). Standard stock solutions of ^{57}Co-chloride were prepared as aqueous solutions, and were used to set up a calibration between the pulse height and the γ-energy. Using such a calibration makes it possible to calculate counting efficiency, and therefore to quantify ^{57}Co in a sample.

Liquid Scintillation Counting

Liquid scintillation (LS) counting was carried out using a Packard Tri-Carb 1900 TR liquid scintillation analyzer. Commercially available scintillation cocktail, HIONIC-FLUOR^{TM}, and tissue solubilizer, SOLUENE-350^{TM}, were purchased from Canberra Packard. Polyethylene vials of 20 ml were used with polyethylene screw caps. Radioactive ^{45}Ca-chloride, a pure β-emitter (185 MBq, 0.07 mg Ca/ml), was purchased from Amersham. Standard stock solutions of ^{45}Ca-chloride were prepared as aqueous solutions.

Most substances added to a scintillation cocktail (colors, chemicals, solvents) can reduce the efficiency of the scintillation process. This reduction in counting efficiency is called quench. Quench phenomena can be divided into two major forms : 1) chemical quench and 2) color quench. Chemical quenching occurs when chemical agents (e.g., carbon tetrachloride, water) added to the sample cause energy losses in the transfer from solvent to fluors. Color quenching is due to the attenuation of light photons in the solution, and occurs when colored substances, with absorption spectra that overlap the emission spectra of the fluors, are present in the counting vial. Quenching thus reduces the number of emitted photons from the sample. As a result, the energy spectrum shifts toward lower energies. Consequently, the counting efficiency depends on the degree of quenching and thus on the nature of the sample, the scintillator cocktail and the preparation method used. The relation between efficiency and a quench parameter is the quench curve (Kessler, 1989).

The tissues taken from the fish were highly colored blood samples and moderately colored gill samples. To determine the radioactivity of ^{45}Ca in these samples it is necessary to establish the precise relationship between counting efficiency and quench for each of the two isotopes. For the preparation of the quench curves, a series of 15 standards (three vials per standard) were prepared for each of the nuclides.

^{45}Ca quench series. First, a fixed aliquot of radioactivity was added to the vials, so that each vial contained 18000 dpm. Second, 0.5 ml tissue solubilizer and 10 ml scintillation cocktail were added to each vial and shaken. After an equilibration period of a few hours the samples were counted for 10 minutes in the LS counter using the "efficiency tracing" technique (Ishikawa *et al.*, 1984; Kessler, 1986). This

powerful method, which can be used effectively for almost all pure beta and beta-gamma emitters, requires only a single unquenched ^{14}C standard to calculate dpm results. The radioactivity in each vial was then compared with the mean of the vials, and any vial with a radioactivity that differed more than 1 % of the mean was discarded. Third, varying amounts of a quenching blood solution (100 mg freshly drawn blood/ml SOLUENE) were added and pure SOLUENE was used to make up volumes, so each vial contained 1 ml of SOLUENE.
^{57}Co quench series. First, a fixed aliquot of radioactivity was added to the vials, so that each vial contained 1200 dpm. Second, 0.5 ml tissue solubilizer was added to each vial, shaken and counted in the gamma scintillation counter. The radioactivity in each vial was then compared with the mean of the vials, and any vial with a radioactivity that differed more than 1 % of the mean was discarded. Third, varying amounts of the quenching blood solution were added and pure SOLUENE was used to make up volumes, so each vial contained 1 ml of SOLUENE. Next, 10 ml of scintillation cocktail was added to each vial.
Both series were placed in the LS counter, protected from direct sunlight, to allow chemiluminescence to decrease. After a period of a few hours, they were counted in the liquid scintillation analyzer to obtain the β-spectra. Sample counting was automatically terminated, when a 2 sigma coincidence of 1 % was reached. At the same time, the counting efficiencies were measured, and a quench indicating parameter, tSIE, was determined for each vial by external standardization. The tSIE index, which is the Transformed Spectral Index of the External standard (Kessler, 1989), was related to the counting efficiencies for all the samples by non-linear regression, to obtain the quench curves for each of the nuclides.

Sensitivity Limits

To determine the effect of radioactivity and quench in a sample on the accuracy and sensitivity of the method a number of samples was prepared, using the two nuclides in different combinations. For the ^{57}Co isotope, we used 6 different levels of radioactivity ranging from 0 to 1500 dpm. For the ^{45}Ca isotope, 8 different levels of radioactivity ranging from 0 to 24000 dpm were used. These samples also contained 1 ml of SOLUENE and 10 ml of HIONIC-FLUOR. This resulted in 48 different reference samples. Second, 7 new sets of the same 48 different combinations were prepared and to each set a different amount of a quenching blood solution was added. Thus, a total of 384 different reference samples were prepared.
All these samples were counted in both the gamma and liquid scintillation analyzer to obtain cpm values, which were converted to dpm values as outlined under the calculation methods. Sample counting was automatically terminated, when a 2 sigma coincidence of 1 % was reached.

Statistical Analysis

All sets of data were tested for homoscedasticity by the log-anova test for homogeneity of variances and for normality by the Kolmogorov-Smirnov test for goodness of fit. Linear and non-linear regression methods and analysis of variance were used for analyzing the data. When the data were not normally distributed, the nonparametric Kruskal-Wallis analysis of variance test was performed. Significance

levels of tests are indicated by asterisks according to the following probability ranges (ns=not significant, *=$0.05 \geq P > 0.01$, **=$0.01 \geq P > 0.001$, ***=$P \leq 0.001$). Statistical methods are used as outlined in Biometry (Sokal and Rohlf, 1981).

Cobalt Uptake Experiments

The effect of different external calcium concentrations on the uptake of cobalt through the gills of the common carp, *Cyprinus carpio*, was studied in chemically defined freshwater. This freshwater consisted of a basic composition (1.143 mM $NaHCO_3$, 0.054 mM KCl, and 0.499 mM $MgSO_4$) and a varying amount of calcium ($CaSO_4$). The calcium concentrations of the freshwater exposure solutions were 0.1, 1, 10, 100, 348, 1000, 2500, and 5000 µM.
Prior to the cobalt uptake experiments, fish were acclimated for 16 days to freshwater with a calcium concentration of 1000 µM at 25 °C ± 1 °C. Fish were sampled from the aquaria and individually transferred to beakers containing 0.5 l of a freshwater solution to which ^{45}Ca (1480 $KBq.l^{-1}$) and ^{57}Co (740 $KBq.l^{-1}$) were added 24 hours before the start of the experiments to achieve equilibrium. In addition, 10 mM of the non-complexing buffer (H)EPPS (Sigma) was added to keep the pH at 8.0 ± 0.1 during the cobalt uptake experiments. 1µM of $CoCl_2$ was added to the waters to minimise the effect of variation in the background concentration of cobalt (< 0.02 µM). Uptake of radioisotopes was measured over a three hour period at 25 °C ± 1 °C to determine the metal accumulation rate. Following this incubation period, fish were transferred for 5 minutes to beakers containing 0.5 l freshwater to which 10 mM of the anaestheticum propyl dl-1-(1-phenylethyl)-imidazole-5-carboxylate hydrochloride (Sigma) was added. Subsequently, a ventral incision was made between the pectoral fins. Blood was taken with 60 µl Na-heparinised haematocrit-capillaries by puncturing the heart. After the blood was collected fish were killed by a single blow on the head and gills of both sides of the branchial cavity excised and separated from the gill arches. Both gill filaments and blood were put in separate vials, 1 ml SOLUENE-350 was added, and the vials were placed on a shaker to promote solubilization. 24 hours later, 10 ml HIONIC-FLUOR was added and the vials were counted in both gamma and liquid scintillation analyzer.

RESULTS

β-Spectra and Region Setting

The β-spectra of ^{45}Ca and ^{57}Co, and the effect of blood quench are shown in Fig.1. The pulse height spectrum shifts to lower energy levels when quench increases. From the difference between quenched and unquenched spectra, the settings of the counting regions were determined. Two methods can be used for region setting in dual labelled samples : either two energy regions can be used (one region per isotope) or the two isotopes can be counted in just one single window. In the first method, a compromise between radionuclide separation and counting efficiency must be found, because each energy region inevitably records events from both radio nuclides. We therefore preferred the second method using only one energy window for both isotopes, and set the upper limit to just above the spectral endpoint of the higher energy radio nuclide, i.e. ^{45}Ca. The energy range was therefore set to 0-256 keV.

Quench Curves

The ^{45}Ca and ^{57}Co quench series were counted in the 0-256 keV energy region, and the acquired cpm values (corrected for background and radioactive decay) were divided by the known radioactivity (dpm) of the standards, yielding the counting efficiency of each standard. The tSIE index was related to the counting efficiencies for all the samples by non-linear regression, to obtain the quench curves for each of the nuclides as shown in Fig.2. A rectangular hyperbola, $E=a*tSIE/(b+tSIE)$ (where E is the counting efficiency, $tSIE$ is the quenching index, and a and b are constants) yielded a very good fit to the data. The following equations were obtained :

$$E_{Co} = \frac{1.456 * tSIE}{(399.217 + tSIE)} \qquad R^2 = 0.994^{**} \quad (n = 45) \qquad [1]$$

$$E_{Ca} = \frac{0.994 * tSIE}{(21.790 + tSIE)} \qquad R^2 = 0.930^{***} \quad (n = 43) \qquad [2]$$

where E_{Co} is the ^{57}Co counting efficiency, and E_{Ca} is the ^{45}Ca counting efficiency.

Calculation Methods

First, all 384 reference samples were analyzed for ^{57}Co radioactivity in the gamma counter. The dpm of ^{57}Co, D_{Co}, can be obtained from the following equation :

$$D_{Co} = C_{Co} / 0.88 \qquad [3]$$

in which C_{Co} represents the counts of ^{57}Co in the gamma scintillation analyzer, corrected for background counts. The counting efficiency of ^{57}Co is 88 % in the gamma scintillation counter. The value of D_{Co} is then further corrected for

radioactive decay to obtain D^*_{Co}, the ^{57}Co dpm in the sample at a certain reference date.
Next, all samples were counted in the LS analyzer. The observed counts in the total window, C_T, are the sum of the individual counts of both nuclides. C_T therefore depends on the radioactivity of the two isotopes, D_{Co} and D_{Ca}, their counting efficiencies, E_{Co} and E_{Ca}, and on the background cpm in the LS analyzer, B_β.

C_T equals :

$$ {}_T = E_{Co} D_{Co} + E_{Ca} D_{Ca} + B_\beta \qquad [4] $$

The background cpm in the LS counter, B_β, was obtained by counting ±10 vials in the total window (0-256 keV), containing only cocktail and solubilizer, and averaging the acquired cpm values. When a sample is counted, the obtained tSIE value is used to calculate the counting efficiencies of both ^{57}Co and ^{45}Ca, respectively E_{Co} and E_{Ca}, using quench curve equations [1] and [2]. We now only need the value of ^{57}Co dpm, D_{Co}, to calculate the ^{45}Ca dpm, D_{Ca}. The ^{57}Co dpm value at the time of measurement in the gamma scintillation counter, D_{Co}, can be computed from equation [3]. However, equation [4] is only valid at the time of measurement in the LS counter. The ^{57}Co dpm value needed in equation [4] is therefore the cobalt radioactivity at the time of measurement in the LS counter. The value of D_{Co}, obtained from equation [3], is thus corrected for radioactive decay between measurement in gamma and LS counter to obtain the ^{57}Co dpm in the sample at the time of measurement in the LS analyzer. This value can be substituted in equation [4]. Converting equation [4] yields :

$$ D_{Ca} = \frac{C_T - B_\beta - E_{Co} D_{Co}}{E_{Ca}} \qquad [5] $$

The value of D_{Ca} is then further corrected for decay between measurement in the LS counter and a certain common reference date to obtain D^*_{Ca}, the ^{45}Ca dpm in the sample.

Accuracy and Sensitivity Limits

All reference samples containing the two nuclides in different radioactivities at different quench levels were counted in both gamma and liquid scintillation analyzer. The ^{57}Co and ^{45}Ca dpm values were computed by the method presented above, and were compared with their real dpm values. Percentage deviation from the real dpm values in the reference samples, ε_{Co} and ε_{Ca}, was calculated for both isotopes, using the following equations [6] and [7] :

$$ \varepsilon_{Co} = \left(\frac{D^*_{Co} - D^r_{Co}}{D^r_{Co}} \right) * 100 \qquad [6] $$

$$\varepsilon_{Ca} = \left(\frac{D^{*}_{Ca} - D^{r}_{Ca}}{D^{r}_{Ca}}\right) * 100 \qquad [7]$$

in which D^{r}_{Co} and D^{r}_{Ca} represent the real dpm values of the respective ^{57}Co and ^{45}Ca isotopes.

^{57}Co recovery. Due to the low penetration of β-particles, and because quenching only affects the conversion process of beta particle energy to photoelectrons, it is clear that the presence of ^{45}Ca and blood does not influence the ^{57}Co measurements in the gamma scintillation counter. Therefore only the effect of ^{57}Co radioactivity was considered for calculation of the ^{57}Co recovery. An ANOVA test showed no significant differences among the different ^{57}Co groups. It is shown in Fig.3 that ^{57}Co recovery is very good, i.e. the measurements deviate less than 1 % (with a standard deviation of approximately 2 %) from the real dpm values, even at low ^{57}Co activities.

^{45}Ca recovery. To determine the relative importance of the different factors which influence the variation in ε_{Ca}, we fitted an empirical model through the pooled data (n=336). A non-linear regression model was constructed which considers the effects of ^{57}Co radioactivity, ^{45}Ca radioactivity and quench on the deviation from the real ^{45}Ca dpm values. The mathematical description of the model has the form of a non-linear equation [8] :

$$\varepsilon_{Ca} = C(1) * (D^{r}_{Co})^{C(2)} + C(3) * (D^{r}_{Ca})^{C(4)} + C(5) * (tSIE)^{C(6)} \qquad [8]$$

The first factor in the regression model accounts for the effect of ^{57}Co radioactivity (D^{r}_{Co}). The second factor accounts for the effect of ^{45}Ca radioactivity (D^{r}_{Ca}). The third factor accounts for the effect of quench (tSIE) on the deviation from the real ^{45}Ca dpm values. All the factors are exponential where *C(1)*, *C(2)*, *C(3)*, *C(4)*, *C(5)* and *C(6)* are fitting parameters. The relative importance of the different factors was determined by the forward selection procedure, starting with a single factor and then add factors one at a time and evaluate the effect on the coefficient of determination (R^2) of the regression model. Starting with the factor wich accounts for the effect of ^{57}Co radioactivity explains 13.3 % of the variation. Adding the factor which accounts for the effect of ^{45}Ca radioactivity explains 13.5 % of the variation. Including this effect of ^{45}Ca radioactivity does not improve the descriptive value of the model significantly (F_s=0.996^{ns} / df=1; 333). Adding the factor which accounts for the quench effect explains 14.1 % of the variation, but again this does not improve the descriptive value of the model significantly (F_s=2.084^{ns} / df=1; 332). Thus, approximately 13.5 % of the variation can be accounted for by an effect of ^{57}Co radioactivity. The further addition of other variables does not improve the fit. Thus, almost all variation is caused by random effects, i.e. analytical error.
Fig.4 shows the effect of ^{57}Co radioactivity on the relative deviation from the real ^{45}Ca dpm values. Samples which contained the same amount of ^{57}Co radioactivity were grouped together. This resulted in 6 different groups. Because the first group, which contains no radioactive ^{57}Co, deviates from normality (D_{max}=0.149**), the

nonparametric Kruskal-Wallis ANOVA test and Dunn's Multiple Comparisons test were used to test variation among column medians. The quartile range is calculated as the value of the 75th percentile minus the value of the 25th percentile. This is the width of the range about the median that includes 50 % of the cases. The Kruskal-Wallis test is significant, thus the deviation from real ^{45}Ca dpm values among the different ^{57}Co groups is significant. Still, ^{45}Ca measurements are accurate in all ^{57}Co groups, i.e. deviation from real dpm values is less than 2 % with an approximate quartile range of 2 % in the ^{57}Co and tSIE range tested.
The counting sensitivity of ^{45}Ca was determined at different ^{45}Ca / ^{57}Co dpm ratios. The ^{45}Ca counting deviation increases as the dpm ratio decreases. Fig.5 indicates that at least 5 times more radioactivity is required for ^{45}Ca than for ^{57}Co to obtain reliable accuracy of analysis (deviation from real dpm values smaller than 5 %).

Uptake Experiments

The method presented above was used in metal uptake studies with fish. The cobalt and calcium accumulation rates in gill and blood were determined from measurements of the ^{57}Co and ^{45}Ca radioactivity in the gill and blood samples. The results for gill samples in Fig.6 show clearly that the method presented in this paper can be used to determine cobalt and calcium accumulation rates simultaneously in fish samples. The effect of the calcium concentration in the exposure water on the uptake of cobalt by fish is shown in Fig.6A, representing the cobalt accumulation rate in the gills. There is a clear decrease in the uptake of cobalt with increasing calcium concentrations in the water of exposure. The effect of calcium on cobalt uptake is no longer apparent when the calcium concentration of the water exceeds 2500 µM.
A significant increase was observed in the calcium accumulation rate in the gills when the calcium concentration in the exposure water ranged from 10 to 348 µM. This effect is no longer apparent when the calcium concentration in the exposure water exceeds 348 µM as shown in Fig.6B. An interaction between cobalt and calcium is expected because calcium may compete with transition metals for the same uptake site (Verbost *et al.*, 1989; Spry and Wood, 1989). In addition calcium changes the ion and water permeability of membranes and the transmembrane potential of the gills (Wendelaar Bonga *et al.*, 1983; McWilliams and Potts, 1978).

DISCUSSION

Although radioisotope tracer techniques are an important tool for the study of metal transport processes, the simultaneous determination of two or three radioisotopes in biological samples is not simple nor standard procedure. It is considerably more difficult than single label counting, due to spectrum overlap, more complex energy region setting and effects of quench. Cautious sample preparation, critical selection of counting regions, and preparation of matched quench curves, are necessary before the radioactivities of the individual nuclides can be determined. The method described in this paper is straightforward and independent of the isotopes used.
Counting in a single energy window, has the advantage that the counting efficiency for the high energy isotope remains high, even when the quench level increases dramatically. When using two or more counting windows, efficiency decreases fast with increasing levels of quench. However, in some cases, depending on the degree of spectrum overlap, more than one energy window is still needed for accurate dual or triple label counting.
The method described for the preparation of the quench curves is fast and yields accurate results, but some special considerations must be made. The kind of quenching agent used is important, because there is a fundamental difference between a chemical and a color quencher. The β-spectra for colored samples are spread over a wider energy range than for chemical quenched samples. This effect could alter the shape of the quench curve so that the relation between the quench indicating parameter and the level of quench is no longer constant. In this study a blood solution was used as quencher, because the fish tissues contained blood as the main quenching component. When carbon tetrachloride was used instead of blood as quencher, identical quench curves were obtained with blood and carbon tetrachloride for both ^{57}Co and ^{45}Ca within the tSIE range of interest. This means that carbon tetrachloride can be used as a general quenching agent for the construction of quench curves when different types of samples have to be analysed and it is difficult to obtain quench curves for each of the different tissues. The relation between the quench indicating parameter and the counting efficiency could be described by a rectangular hyperbola. However, this function has only descriptive power and is only used to relate tSIE values to counting efficiency for interpolation purposes.
The amount of quenching agent added to the standards should be kept minimal, so that there is no significant dilution of the fluor molecules. Therefore, it is necessary to use strong quenchers which only need to be added in small amounts to cover the quenching range of the samples. For instance, less carbon tetrachloride is required than aceton to exhibit a same level of quench. The combined gamma scintillation/liquid scintillation counting procedure described in this paper was found very useful in metal uptake studies for the simultaneous determination of ^{57}Co and ^{45}Ca, and also for the simultaneous determination of ^{45}Ca and ^{109}Cd, or ^{45}Ca and ^{65}Zn. It gives reliable and consistent results. ^{57}Co measurements deviate less than 1 % from the real dpm values. A minimum of ±150 cpm (this is 3 times the background count) is required for accurate ^{57}Co determination in the gamma scintillation counter. This means a sample has to contain at least ±170 dpm of ^{57}Co radioactivity. Although ^{45}Ca measurements depend on the ^{57}Co radioactivity in the sample, deviation from real dpm values is less than 2 % within a large ^{57}Co radioactivity range. However, to obtain reliable results the radioactivity of ^{45}Ca in

the sample must be at least 5 times higher than the radioactivity of ^{57}Co. Thus, a minimum of ±850 dpm of ^{45}Ca radioactivity is required.
The recoveries of both isotopes are independent of the quench level in a large tSIE range (190 ≤ tSIE ≤450). In the case where the tSIE value is less than 190, decolorization of the sample can be considered to reduce the level of quench by adding a few drops of hydrogen peroxide (30 %). However, by adding hydrogen peroxide, chemiluminescence is produced. This is an additional production of light as a result of a chemical reaction. When the concentration of hydrogen peroxide added to the sample increases to a certain extend, too much chemiluminescence is produced, which requires nearly one day to decay.
In summary, the method presented makes it possible to quantify a combined γ/β-emitter and a pure β-emitter in different types of tissue of different organisms in the presence of high levels of quench. The method can be applied to different combinations of radiotracers and the procedure can be easily adapted when more than two radiotracers have to be determined.

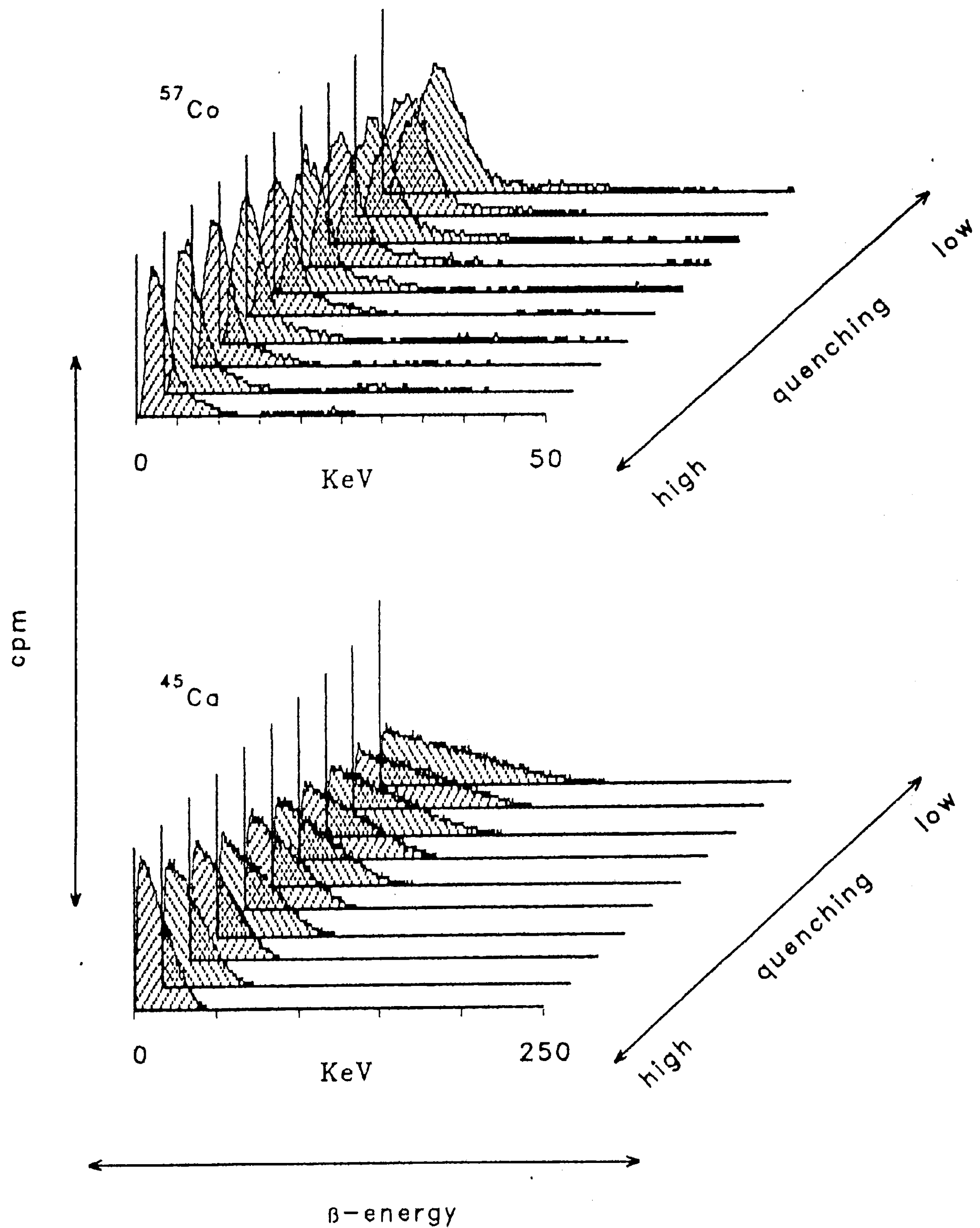

Fig. 1. : Effect of quench on the β-energy spectra of ^{57}Co and ^{45}Ca. Samples are quenched by addition of increased amounts of a blood solution (100 mg freshly drawn blood/ml SOLUENE-350).

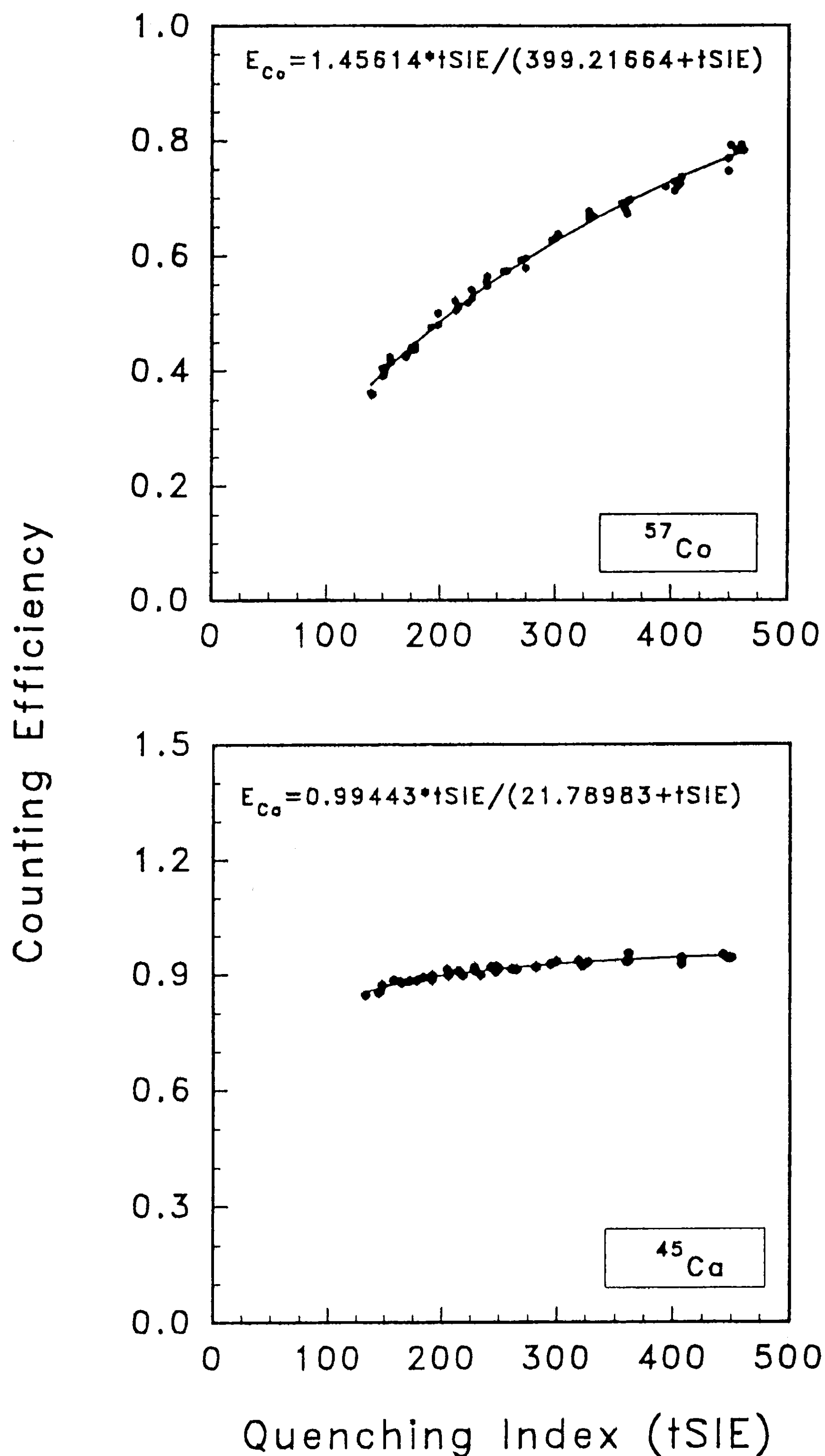

Fig. 2. : Counting efficiency versus quench parameter for ^{57}Co (1200 dpm) and ^{45}Ca (18000 dpm) in the region 0-256 keV. Counting efficiencies were obtained for each of the standard samples by external standardization. These results were related to the tSIE quench index for all standard samples by non-linear regression.

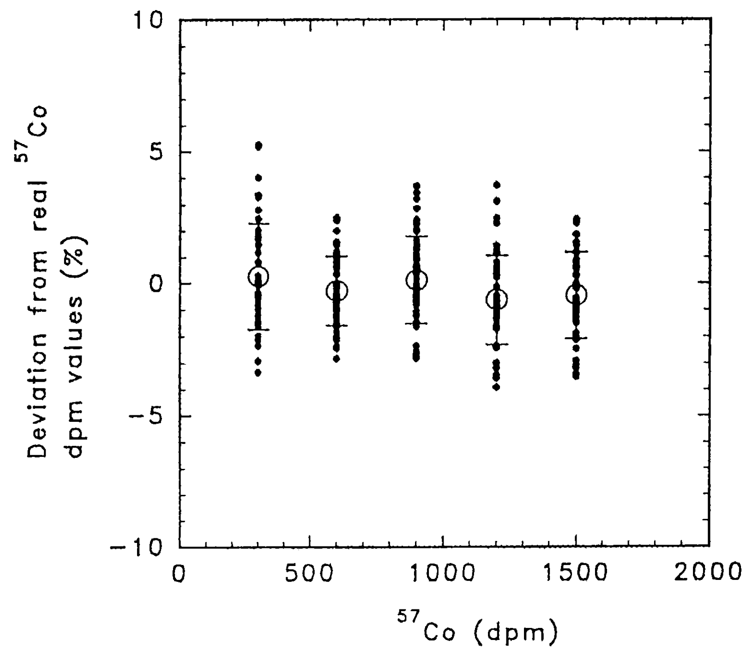

Fig. 3. : Effect of ^{57}Co activity on the percentage deviation from real ^{57}Co dpm values. Deviation from dpm values were calculated by the method presented in the paper. Means (hollow circles) of 48 replicates (full circles) with standard deviations are not significantly different, $p>0.05$.

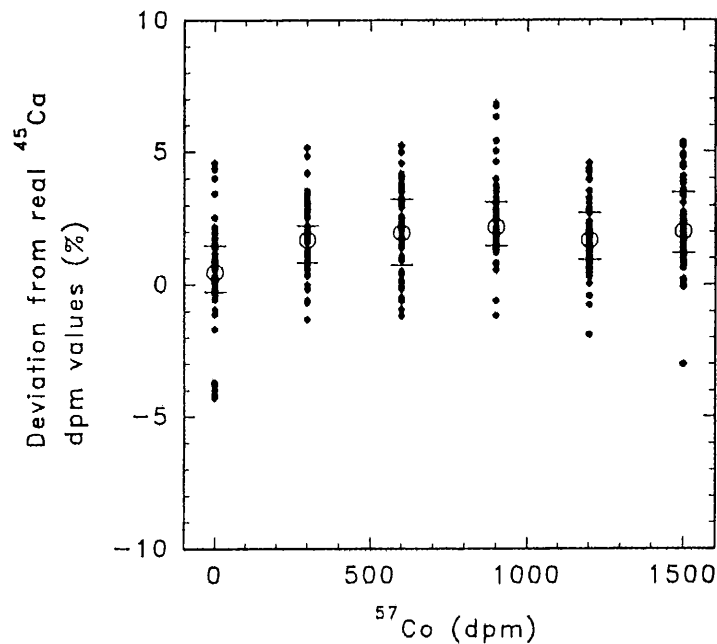

Fig. 4. : Effect of ^{57}Co activity on the percentage deviation from real ^{45}Ca dpm values. Deviation from dpm values were calculated by the method presented in the paper. Medians (hollow circles) of 56 replicates (full circles) and quartile ranges were calculated for each group. Group medians are significantly different from each other, $p<0.001$.

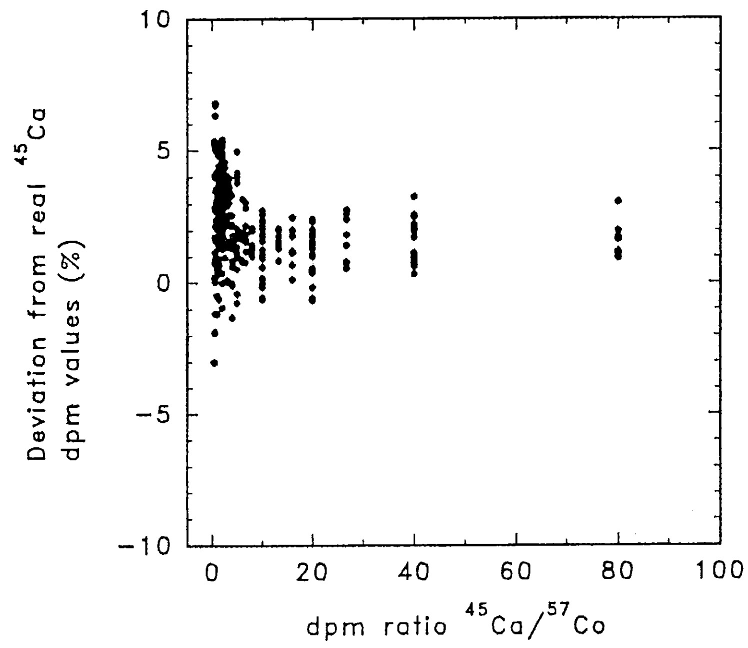

Fig. 5. Counting sensitivity of ^{45}Ca: percentage deviation of ^{45}Ca dpm values determined at different $^{45}Ca/^{57}Co$ dpm ratios.

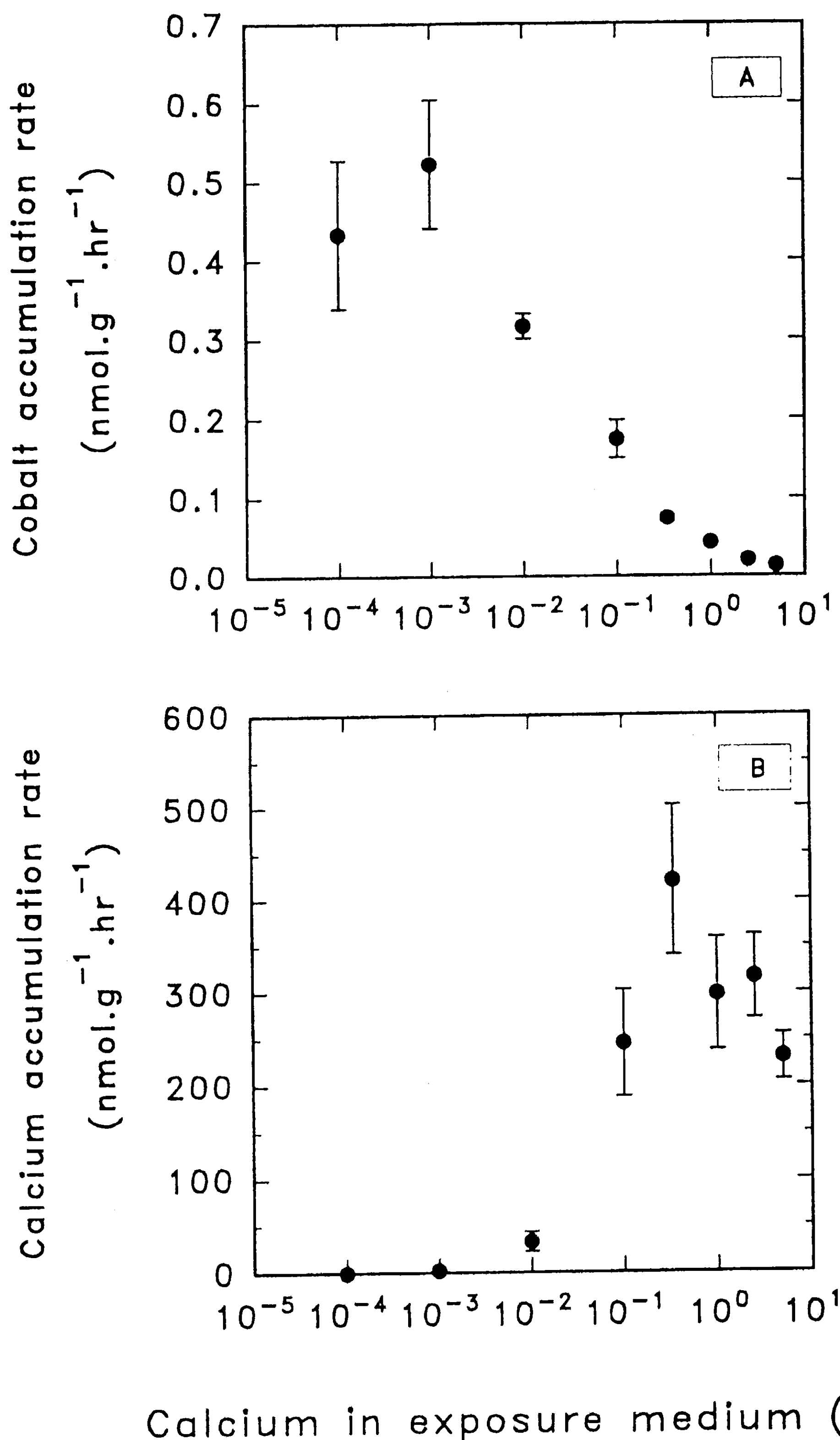

Fig. 6. Effect of calcium concentration in the exposure water on the cobalt accumulation rate (A) and the calcium accumulation rate (B) in gills of the common carp, *Cyprinus carpio.* Fish are acclimated for 16 days to an external calcium concentration of 1000 µM prior to the experiment. Plotted points (full circles) represent data means with standard deviations of 7 replicates (T=25 °C ± 1 °C; pH= 8.0 ± 0.1).

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Multiple-label gamma and liquid scintillation counting in biological samples : a statistical approach

INTRODUCTION

Radioisotope tracer techniques are an expedient tool for the study of metal uptake processes because they provide a very sensitive and accurate technique which makes it possible to follow transport of several metal ions simultaneously. The use of a second or even a third radioisotope makes it possible to study interactions between different metal ions or the effects of transport modulators on metal uptake systems in one single experiment. Such experiments are essential to unravel the mechanisms of metal transport across biological interfaces.

Simultaneous quantitative determination of several radioisotopes in a single sample is, however, considerably more difficult than single label counting (Miyazawa *et al.*, 1991; Bukowski *et al.*, 1992; Van Ginneken and Blust, 1995), and special attention should be paid to the precision of the calculated dpm values of the radioisotopes. Most commercial liquid scintillation counters permit the operator to count in two or more discrete energy regions (channels) simultaneously, which makes it possible to distribute the energy spectra of the radioistopes among these channels. The settings of these channels fix the counting efficiencies of the isotopes. By using these counting efficiencies, the accumulated counts of the radioisotopes, and the accumulated background counts in each different channel, the dpm values of the radioisotopes can be calculated (Kessler, 1989). It is generally recognized that the setting of these channels should depend in some way on the degree of overlapping of the spectra and the relative amounts of the isotopes present. But the nature of this dependence is complex (Bush, 1964), and most operators appear to make these decisions on an intuitive basis, neglecting the statistical nature of the counting proces.

The purpose of the present study is (i) to derive exact expressions to measure the statistical precision of the calculated dpm values, as functions of the counting efficiencies of the isotopes in the different channels, the amount of the isotopes, and the background count rates, (ii) to provide experimental information as to how the precision varies with these quantities, as a basis for a practical approach to the choice of the channels, and (iii) to develop a measuring procedure to determine simultaneously the activities of ^{109}Cd, ^{65}Zn, and ^{45}Ca in one single sample. This method can be used to determine the uptake of cadmium, zinc, and calcium in the tissues of the common mussel, *Mytilus edulis*.

MATERIALS AND METHODS

Determination of the Radioactivity of ^{109}Cd and ^{65}Zn in the Gamma Counter

^{109}Cd and ^{65}Zn are combined γ/β-emitters, which means they emit γ-rays to stabilize their nucleus. These γ-rays generate a signal in the γ-counter. It is important to distribute the energy spectra of ^{109}Cd and ^{65}Zn adequately among two energy channels, A and B, to determine the radioactivities of the two tracers in one single sample. The expressions which are derived to measure statistical precision in the dpm values of the respective isotopes, are suitable for calculations designed to show how the counting errors may be minimized by the best choice of the efficiencies in each channel.
Mathematically, the count contribution of each radionuclide to each region can be calculated from the following expressions in the computation of the dpm values (Kessler, 1989; Burns and Steiner, 1991) :

$$A = E_{CdA} \times D_{Cd} + E_{ZnA} \times D_{Zn} + bkg_A \qquad [1]$$

$$B = E_{CdB} \times D_{Cd} + E_{ZnB} \times D_{Zn} + bkg_B \qquad [2]$$

in which

D_{Cd} = dpm value of ^{109}Cd
D_{Zn} = dpm value of ^{65}Zn
A = total count rate in window A
B = total count rate in window B
bkg_A = background count rate in window A
bkg_B = background count rate in window B
E_{CdA} = counting efficiency of ^{109}Cd in window A
E_{CdB} = counting efficiency of ^{109}Cd in window B
E_{ZnA} = counting efficiency of ^{65}Zn in window A
E_{ZnB} = counting efficiency of ^{65}Zn in window B

These equations can be solved for D_{Cd} en D_{Zn} :

$$D_{Cd} = \frac{A \times E_{ZnB} - B \times E_{ZnA} - bkg_A \times E_{ZnB} + bkg_B \times E_{ZnA}}{E_{CdA} \times E_{ZnB} - E_{CdB} \times E_{ZnA}} \qquad [3]$$

$$D_{Zn} = \frac{B \times E_{CdA} - A \times E_{CdB} - bkg_B \times E_{CdA} + bkg_A \times E_{CdB}}{E_{CdA} \times E_{ZnB} - E_{CdB} \times E_{ZnA}} \qquad [4]$$

We shall assume that efficiencies E_{CdA}, E_{CdB}, E_{ZnA}, and E_{ZnB} are constants whose values may be determined to any desired precision by the counting of standards. The errors of D_{Cd} and D_{Zn} can thus be calculated as a function only of the random nature of A, B, bkg_A, and bkg_B. Uncertainties in the values of the efficiencies, erratic counter performance, etc., are not considered. We also assume that the error in measuring the counting time of the sample, t_s,, is negligible.

By using the above equations, and after application of the rules of propagation of error in the sought quantities D_{Cd} and D_{Zn} (Squires, 1968), the following equations are obtained :

$$t_s \times \varepsilon_{Cd}^2 = F \times \frac{4}{(k \times D_{Zn})^2}\left\{D_{Zn} \times [k \times F_1 + F_2] + F_3 \times (1+a)\right\} \quad [5]$$

with $F_1 = E_{ZnA}^2 \times E_{CdB} + E_{ZnB}^2 \times E_{CdA}$ $\quad F = \frac{1}{(E_{CdA} \times E_{ZnB} - E_{CdB} \times E_{ZnA})^2}$

$F_2 = E_{ZnA}^2 \times E_{ZnB} + E_{ZnB}^2 \times E_{ZnA}$ $\quad k = \frac{D_{Cd}}{D_{Zn}}$

$F_3 = E_{ZnA}^2 \times bkg_B + E_{ZnB}^2 \times bkg_A$ $\quad a = \frac{t_s}{t_b}$

t_b=counting time of a background sample in the gamma-counter.

and

$$t_s \times \varepsilon_{Zn}^2 = F \times \frac{4}{(D_{Zn})^2}\left\{D_{Zn} \times [k \times F_1' + F_2'] + F_3' \times (1+a)\right\} \quad [6]$$

with $F_1' = E_{CdA}^2 \times E_{CdB} + E_{CdB}^2 \times E_{CdA}$ $\quad F_2' = E_{CdA}^2 \times E_{ZnB} + E_{CdB}^2 \times E_{ZnA}$

$F_3' = E_{CdA}^2 \times bkg_B + E_{CdB}^2 \times bkg_A$

In these formulas ε_{Cd} and ε_{Zn} represent the relative 95 % confidence limits (2σ-error) of the calculated D_{Cd} and D_{Zn} values :

$\varepsilon_{Cd} = 2\sigma_{Cd}/D_{Cd}$
$\varepsilon_{Zn} = 2\sigma_{Zn}/D_{Zn}$

It can be seen from Eqns. [5] and [6] that the relative errors of the calculated dpm values are dependent on the counting efficiencies, on the accumulated background counts, the isotope ratio k, the time ratio a, and the counting time t_s of the sample. By using these formulas it is possible to find either the minimum value of the relative 2σ-error, ε, for a chosen counting time, or the minimum counting time which will give an allowed value of ε. The longer the counting time of the sample, the smaller the 2σ-error. When experimental values are substituted in these formulas, it is possible to determine at what efficiencies ε is minimized. Although numerical values of ε so calculated will be smaller than observed values to the extent that counter instability and errors in determining efficiencies contribute to the total experimental error, these calculated values of ε will show how precision varies with channel settings.

Gamma Scintillation Counting

Gamma scintillation counting was carried out using a Packard Minaxi Auto-Gamma counting system (Model 5530), which was fitted with a 3-in. thallium activated, sodium iodide crystal. The crystal contained a 30-mm-diameter through-hole in the center, which can hold scintillation vials up to 28 mm in diameter. Radioactive ^{65}Zn-chloride (185 Mbq, 18 mg Zn/ml) and radioactive ^{109}Cd-chloride (185 Mbq, 1.07 µg Cd/ml), were purchased from Amersham. Standard stock solutions of ^{65}Zn-chloride and ^{109}Cd-chloride were prepared as aqueous solutions and were used to set up a calibration between the pulse height and the γ-energy. In order to determine the counting efficiencies of the two isotopes in the channels A and B at each different window setting, two sets of samples were prepared. To a first set of samples, a fixed aliquot of ^{109}Cd was added to the vials, so that each vial contained 177600 dpm. To a second set of samples, a fixed aliquot of ^{65}Zn was added to the vials, so that each vial contained 444000 dpm. Second, 0.5 ml tissue solubilizer (SOLUENE-350, Canberra Packard) was added to each vial. At the same time, blank samples were prepared, which only consisted of 0.5 ml tissue solubilizer, to determine the accumulated background count rates at each different window setting. These three sets of samples were placed in the gamma counter, and counted for 120 min at each different window setting. The lower limit of channel A was fixed at 15 keV, and the upper limit of channel B was fixed at the spectral endpoint of ^{65}Zn, which equals 1300 keV. The upper limit of channel A and lower limit of channel B were taken the same. Equations [5] and [6] were evaluated at each setting.

Determination of the Radioactivity of ^{45}Ca in a Liquid Scintillation Counter.

The determination of ^{45}Ca radioactivity in a biological sample, which also contains ^{109}Cd or ^{65}Zn, is very complex, because all three isotopes generate a signal in a liquid scintillation analyzer. There is also the additional problem of quench, which is a reduction in the counting efficiency (Kessler, 1989; Burns and Steiner, 1991). The procedure to determine the ^{45}Ca radioactivity of biological quenched samples in the liquid scintillation counter, which also contain ^{109}Cd or ^{65}Zn, has been described previously (Van Ginneken and Blust, 1995). The same procedure can be used to determine the radioactivity of ^{45}Ca in the different combinations $^{109}Cd/^{45}Ca$, $^{65}Zn/^{45}Ca$, and $^{109}Cd/^{65}Zn/^{45}Ca$.

It follows from this procedure that the counting efficiencies do not depend on the window setting, but rather depend on the degree of quenching. It is better to optimize the determination of the ^{45}Ca dpm value as a function of the quenching index and the isotope ratios, because quenching is the principal factor. The principle is demonstrated for the combination $^{65}Zn/^{45}Ca$. It can be demonstrated, that the relative 95 % confidence limit on the calculated D_{Ca} value equals :

$$\varepsilon_{Ca}^2 = \frac{I^2 \times E_{Zn}^2 \times \varepsilon_{Zn}^2}{E_{Ca}^2} + \frac{4 \times I}{E_{Ca}^2 \times D_{Zn} \times t_{s\beta}} \times \left(E_{Ca} + E_{Zn} \times I + \frac{bkg_{\beta} \times I}{D_{Zn}} \times (1 + b) \right) \quad [7]$$

with E_{Zn} = the counting efficiency of ^{65}Zn in the β-counter
E_{Ca} = the counting efficiency of ^{45}Ca in the β-counter
D_{Zn} = dpm value of ^{65}Zn, determined in the γ-counter

ε_{Zn} = 95 % confidence limit of D_{Zn}
I = D_{Zn}/D_{Ca} , the isotope-ratio
bkg_{β} = background count rate in the β-counter
b = $t_{s\beta}/t_{b\beta}$
$t_{s\beta}$ = counting time of the sample in the β-counter
$t_{b\beta}$ = counting time of a blank sample in the β-counter

Formula [7] can be evaluated at different isotope-ratios, I, to determine the optimal ratio of D_{Zn}/D_{Ca}. For other combinations, analogous formulas can be derived.

RESULTS

Determination of the Radioactivity of ^{109}Cd and ^{65}Zn in the Gamma Counter

The γ-spectrum of ^{109}Cd shows one important peak between 10 and 60 keV; the ^{65}Zn spectrum on the other hand shows three important peaks, scattered over the entire energy region, the most important peak being located at 1100 keV. Therefore, the upper limit of region A (and consequently the lower limit of region B) was varied from 60 to 190 keV. At each different window setting, the ^{109}Cd and ^{65}Zn counting efficiencies could be calculated as the ratio of the accumulated count rate (cpm) and the real known activity (dpm). The effect of the window setting on ε_{Cd} and ε_{Zn} at different dpm values for ^{109}Cd and ^{65}Zn is shown in Fig.1, assuming a counting time ratio of 10 and an isotope ratio of 1. From this figure it follows that there is little effect on the relative errors at different window settings, for the three tested radioactivities. There is only a slight increase in the value of ε_{Zn} between 60 and 100 keV, for $D_{Cd}=D_{Zn}=1000$ dpm. As the values of $D_{Cd}=D_{Zn}$ increase, the relative errors decrease. For an identical counting time, ε_{Cd} is always smaller than ε_{Zn}, which can be explained by the higher counting efficiency of ^{109}Cd. It is therefore of interest to choose experimental conditions such that ε_{Zn} is minimal. For other values of $D_{Cd}=D_{Zn}$ and the counting time ratio a, curves were identical to Fig.1. Fig.2. shows the effect of the window setting for different values of the isotope ratio k at $D_{Zn}=100$ dpm and a=10. It seems that, if the amount of radioactive ^{65}Zn is 10 times greater than the amount of radioactive ^{109}Cd (k=0.1), ε_{Cd} increases dramatically if the width of channel A increases, while there is a small decrease in ε_{Zn}. In all other cases there is little effect of the window setting on the relative errors.

Determination of the Radioactivity of ^{45}Ca in the β-counter

Using the same principles as explained above for optimizing D_{Cd} and D_{Zn}, formula [7] was evaluated choosing different experimental conditions in order to find the most optimal conditions to determine the amount of radioactive ^{45}Ca in the presence of ^{65}Zn. For other combinations of tracers, the same approach was used, and comparable results were obtained.

DISCUSSION

Analysis of error sensitivity is a powerful tool to optimize measuring procedures in order to determine one, two, or more radiotracers simultaneously. The formulas derived are straightforward and independent of the isotopes used. It is necessary to know something about the relative activities of the isotopes in the sample, to choose optimum counter settings. It seems reasonable that this ratio will usually be known to within an order of magnitude. If it cannot be guessed from the conditions of the experiment, the order of magnitude can quickly be established from a brief counting of the unknown. When one has obtained this information, error formulas can be analysed and interpreted. Under different experimental conditions (quench, isotope ratios, counting time, etc.), the optimal settings will change, so there is no such thing as a general "optimal condition", but rather will the optimal conditions be dependent on experimental conditions. If the nature of the samples is such that the counting error is much larger for one isotope than for the other, he presumably will choose the channel settings so as to minimize the larger error; if the two errors are approximately equal but cannot be minimized at the same settings, compromize settings will be chosen. In summary, the method presented makes it possible to quantify radiotracers simultaneously in different types of tissue in the presence of high levels of quench.

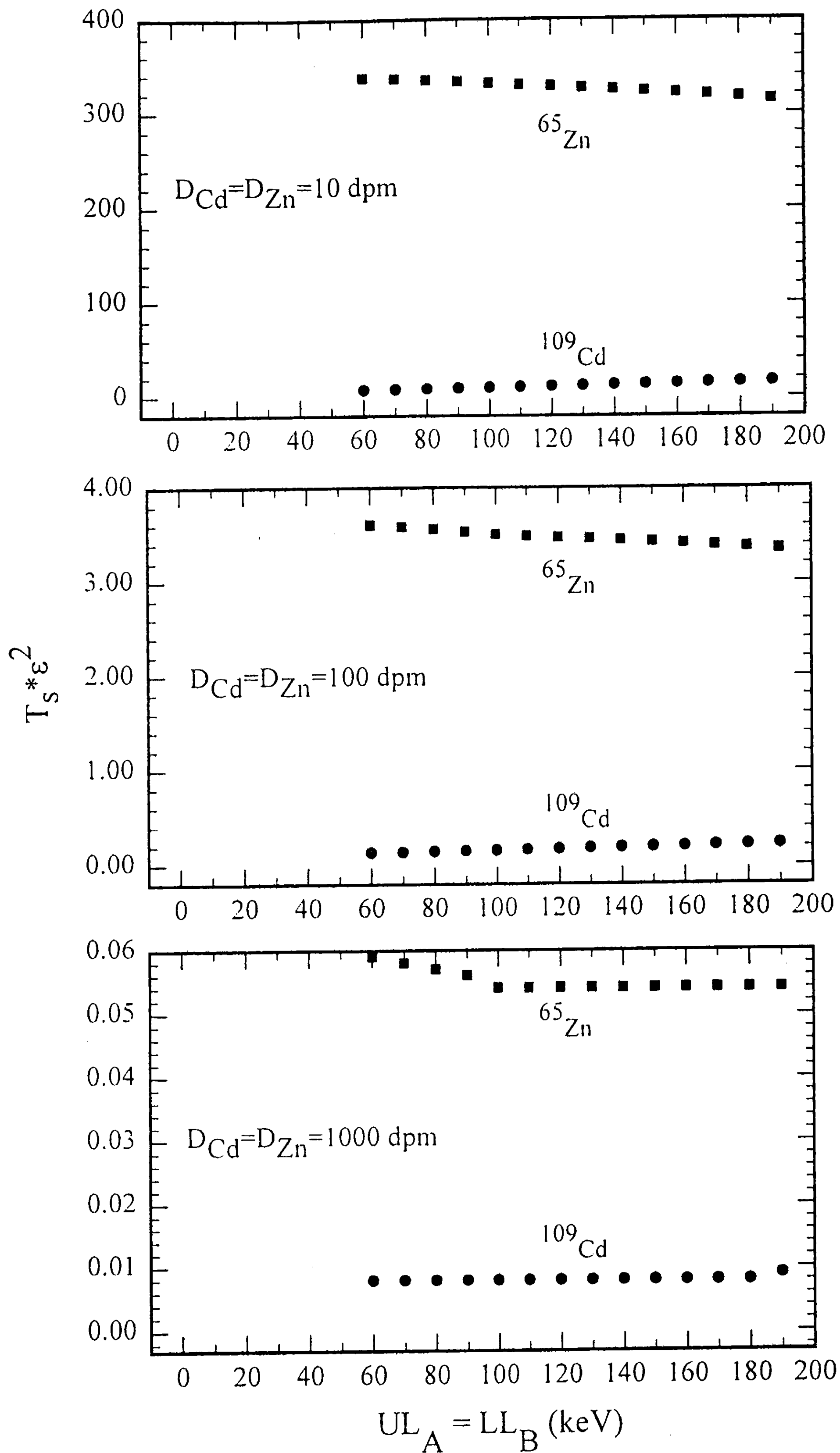

Fig.1. Effect of window setting on the relative errors ε_{Cd} and ε_{Zn} at different dpm values of ^{109}Cd and ^{65}Zn in the gamma-counter. A counting time ratio of 10, and an isotope ratio of 1 was assumed.

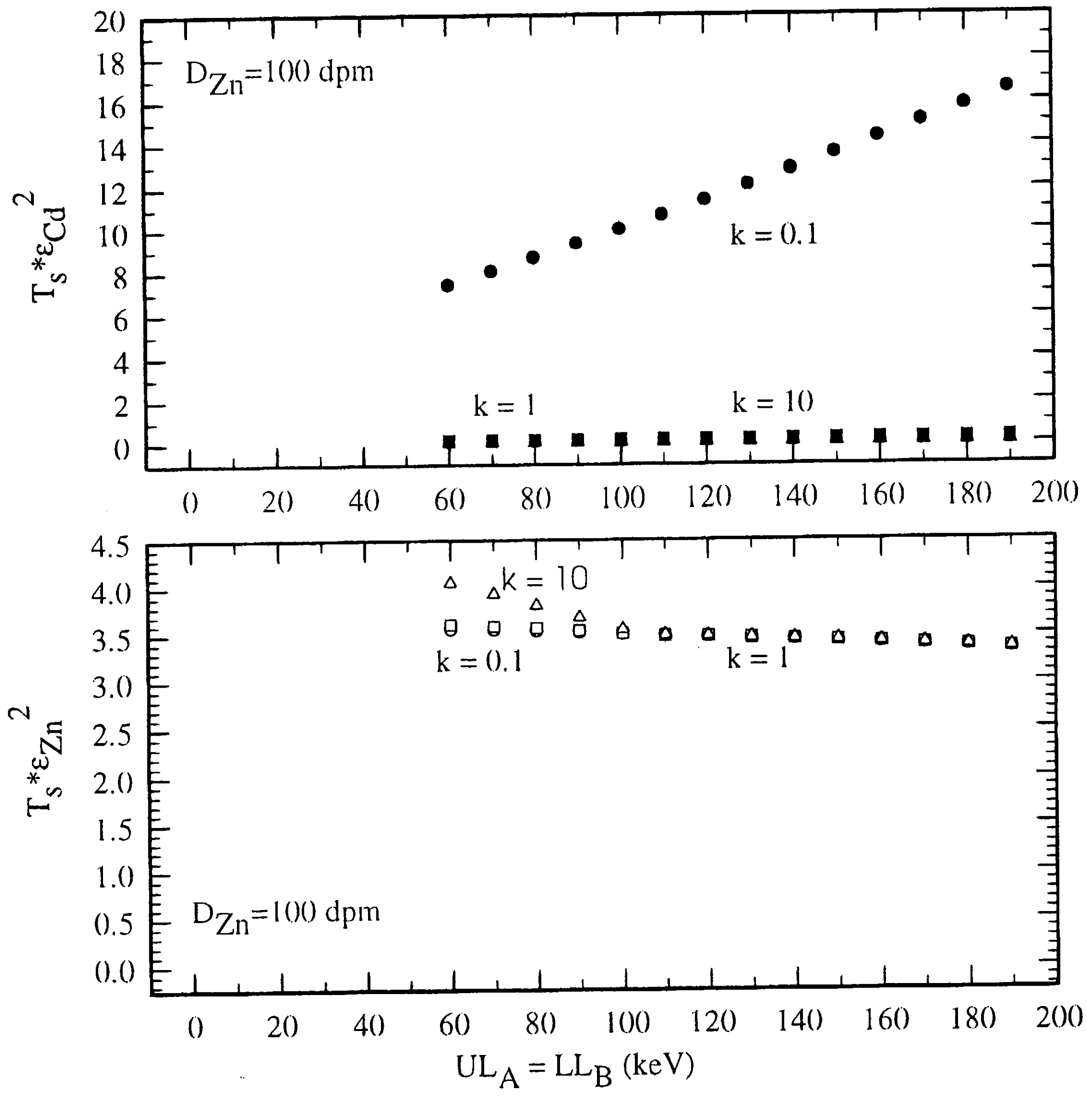

Fig.2. : Effect of window setting on the relative errors ε_{Cd} and ε_{Zn} at different values of the isotope ratio, k, with D_{Zn}=100 dpm and a=10.

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Determination of circulatory fluid content in gills of aquatic organisms using a liquid scintillation analyser: procedure for the correction of branchial metal accumulation rates

INTRODUCTION

Although the environmental fate and the biological function of metals are relatively well understood, the mechanisms of metal uptake by aquatic organisms are still not fully clarified. In most marine vertebrates and invertebrates, metal uptake from the water occurs mainly across the gill epithelium (Olson *et al.*, 1973; Evans, 1987). The gills are, in addition to their role in respiratory gas exchange, acid-base balance and excretory functions (Payan *et al.*, 1981; Wood, 1992), the primary site for uptake of alkaline, alkaline-earth and transition metals from the water (Rankin *et al.*, 1982).

Uptake through the gill epithelium can be determined by *in vivo* experiments. In such experiments, the intact organism is exposed to a solution containing radioisotope tracers of the metal ions of interest. This makes it possible to determine the accumulation rates of the metal ion in the gills and the circulatory fluid, and to unravel metal ion exchange mechanisms in these organisms (Spry and Wood, 1989; Hogstrand *et al.*, 1994). The metal ion accumulation rates can be calculated by using the activity of the accumulated radioactive tracers in the biological tissues of interest (Comhaire *et al.*, 1994). However, in such *in vivo* metal ion exchange experiments, serious problems are encountered with regard to the accuracy of determinations of the branchial metal accumulation rate. Metal ions passing through the apical gill membrane, are transported through the intracellular fluid and the basolateral membrane, and finally appear in the circulatory fluid. Since it is not possible to remove all of the circulatory fluid that is trapped in the gills, it is necessary to correct the measured branchial accumulation rate for the amount of metal ions accumulated in the circulatory fluid to obtain a realistic value for the accumulation in the gill cells. In addition, the weight of the gill tissue has to be corrected for the amount of circulatory fluid carried by it. It is known that gills have a very large extracellular space and circulatory fluid volume (Duff *et al.*, 1987; Gingerich *et al.*, 1987), so that the presence of this extracellular fluid is certainly not negligible in gills. Failing to correct for the contribution of circulatory fluid volume to gill metal accumulation can lead to serious errors in the determination of branchial metal accumulation rates.

In this paper we present a procedure for blood content determination in soft fish gill tissue. This procedure is based on the fact that the tSIE value (i.e., the transformed Spectral Index of the External standard), which is a quench indicating parameter generated when biological samples are analysed for radioactivity in a liquid scintillation counter, can be directly related to the amount of red blood cells (RBC's) present in gill samples. When colored substances are present in a counting vial, they can reduce the efficiency of the scintillation process (quench). The tSIE value makes it possible to correct sample count rate for quench effects by external standardisation when radioactive tracers are determined in biological samples (Van Ginneken and Blust, 1995). With this knowledge, RBC volume (determined by tSIE) can be converted to trapped gill blood volume, using accurate values for whole blood and gill blood hematocrit (Ht). To verify that the tSIE index is directly related to RBC volume in soft gill tissue, it was tested (i) if the tSIE index is a sensitive measure of RBC content in pure blood samples, and (ii) whether or not the gill tissue itself has an effect on the tSIE index. The method is used to determine metal ion accumulation rates in the gill filaments of aquatic organisms of different structural and functional organisation.

The method we propose for the determination of the gill blood volume has a broader application than in metal accumulation studies. It can be used to calculate the intracellular space of trapped RBC's in tissue. Reliable measures of this intracellular space, [DMO] and intracellular ions (Na^+, Cl^-, K^-) contributed by trapped RBC's to the whole tissue values are required, in order to assess the effect of neglecting trapped RBC's on intracellular tissue pH and intracellular ion concentrations. The pH_i can be calculated from the distribution of DMO (5,5-dimethyloxazolidine-2,4-dione) between plasma and tissues, as decribed by Munger *et al.*, 1991.

MATERIALS AND METHODS

Experimental Animals

One-month-old carp, *Cyprinus carpio*, were obtained from the fish hatchery of the Agricultural University of Wageningen, The Netherlands, and were bred in tanks containing 150 liters medium hard fresh water. Fish were held at their optimal temperature of 25 ± 1 °C. The holding tank water was filtered with a trickling filter, treated with UV light (30 W; 820 liter.h^{-1}), and checked every 2 days for NH_4^+, NO_2^-, and NO_3^-. The water was partially renewed if the concentrations of any of these ions exceeded 0.1 mg.$liter^{-1}$, 0.1 mg.$liter^{-1}$ or 20 mg.$liter^{-1}$, respectively. The fish were fed with commercial pellets for under yearling freshwater fish (Joosen-Luickx).
Prior to experiments, the fish (ca. 7-10 g) were kept for a 16-day acclimation period in aquaria containing 150 liters moderately hard fresh water according to Standard Methods (American Public Health Association, 1989) at 25 °C. The same water quality criteria were maintained as described above. Fish were not fed 24 h prior to the start of the experiment.

Experimental Procedures

Fish were sampled from the aquaria and transferred to polypropylene beakers containing 0.5 liter of the moderately hard freshwater solution to which 125 mg.$liter^{-1}$ of the anesthetic ethyl 3-aminobenzoate, methanesulfonic acid salt (MS 222, Aldrich) was added. When the fish were sedated, blood was taken with 60 µl sodium-heparinized Ht capillaries (Hirschmann) by puncturing the heart. After the blood was collected, fish were killed by a single blow on the head and gills of both sides of the branchial cavity were excised and separated from the gill arches. Both blood and gill filaments were put in separate 6 ml pony hang-in vials (Canberra Packard) and fresh and dry weight determined. Measurements of hematological parameters and quench/photometric parameters of these samples were obtained from two different series of experiments, because the fish were too small to do both at the same time.

Series I : Hematology. Blood was collected in sodium-heparinized microcapillary tubes (Hirschmann) and whole blood Ht was determined by centrifugation (Micro-Hematocrit, Heraeus) at 1000 × G for 5 minutes. The Ht value (in %) was read immediately after centrifugation. Whole blood density was determined by weighing out a determined volume of blood, using sodium-heparinized pipette tips.

Series II : Liquid Scintillation Counting. One ml commercially available tissue solubilizer, SOLUENE-350 (Canberra Packard), was added to the samples and the vials were placed on a shaker to promote solubilization. Twenty-four hours later, the solubilized tissues were transferred quantitatively to 20 ml polyethylene vials (Canberra Packard), by rinsing the pony vials with 10 ml HIONIC-FLUOR scintillation cocktail (Canberra Packard). All samples were placed in a Packard Tri-Carb 1900 TR liquid scintillation (LS) analyser, protected from direct sunlight, to allow chemiluminescence to decrease. After a few hours, they were counted to obtain the quench indicating tSIE index.

Series II : Photometric Determination. After the determination of the tSIE values of the samples in the LS analyser, the absorption (OD) of the blood and gill samples, complete with scintillation cocktail, was measured with glass cuvettes (1 ml) from 350 nm up to 650 nm in an SLM/AMINCO DW 2000 spectrophotometer. The peak height at the oxyhemoglobin absorption maximum (413 nm) was used in the results. The tSIE value of the blood and gill samples was related to their absorption at 413 nm. Absorption was measured against a blank, containing 1 ml SOLUENE-350 and 10 ml HIONIC-FLUOR.

Statistical Analysis

All sets of data were tested for homoscedasticity by the log-ANOVA test for homogeneity of variances. Normality of the data was verified by the Kolmogorov-Smirnov test for goodness of fit (Sokal and Rohlf, 1981). Linear and nonlinear regression methods, analysis of variance, and analysis of covariance were used for analyzing the data. Significance levels of tests are indicated by asterisks according to the following probability ranges (ns, not significant; *, $0.05 \geq P > 0.01$; **, $0.01 \geq P > 0.001$; ***, $P \leq 0.001$).

Metal Uptake Experiments

The effect of the correction procedure on branchial metal accumulation rates is illustrated with data from an experiment concerning the availability of calcium and cobalt to fish gills in complexing environments. Accumulation of the metals in the gills and blood of the common carp *C. carpio* was studied in chemically defined fresh water containing one of five organic ligands. This fresh water contained the following components : 1.143 mM $NaHCO_3$, 0.054 mM KCl, 0.499 mM $MgSO_4$, and 0.348 mM $CaSO_4$. To this fresh water 1 µM $CoCl_2$ and different concentrations of either EDTA (ethylenedinitrilotetraacetic acid), NTA (nitrilotriacetic acid), glycine, citrate, or histidine, were added. This created conditions with constant total metal concentrations, but widely varying free metal ion activities. The solutions were spiked with ^{45}Ca and ^{57}Co as tracers. Accumulation of cobalt and calcium was determined over a 3-h period at pH 8.0 ± 0.1 and 25 °C as a measure of the biological availability of cobalt and calcium in the different complexing environments. Experimental procedures, chemical speciation modelling, and calculation of accumulation rates (previous to corrections) were carried out as described previously (Comhaire *et al.*, 1994; Van Ginneken and Blust, 1995).

RESULTS

Hematology Series.

Whole blood Ht was determined to be 23.9 % (± 1.23 %, n=12). The density of whole blood amounted to a value of 1.066 $g.ml^{-1}$ (± 0.0432 $g.ml^{-1}$, n=13). These values were used to calculate gill Ht, and to convert RBC volume to whole blood volume.

Quench/Photometric Series.

In developing the procedure, the initial step was to test whether the tSIE parameter provides a sensitive measure of the RBC content in blood samples. Figure 1 shows the linear relation between RBC concentration ([RBC], in $\mu l.ml^{-1}$) and the absorption (OD) at 413 nm, yielding the following equation :

$$OD = 0.264 \times [RBC] \qquad R^2=0.994^{***}(n=30) \qquad [1]$$

Red blood cell concentration was calculated as follows : fresh weight of the blood was divided by blood density, yielding blood volume. Multiplying this value with whole blood Ht (0.239) yields RBC volume, which was divided by the cocktail volume in the vial.

The tSIE value of the blood samples was related to their absorption at 413 nm. There is a clear relationship (exponential decay) between these two parameters (Fig.2). The following numerical relation [2] was obtained :

$$OD = 1.611 \times EXP(-0.011 \times tSIE) + 1.106 \times EXP(-0.0002 \times tSIE) - 1.010$$

$$R^2=0.999^{***}\ (n=32) \qquad [2]$$

This empirical function is used to relate the tSIE index to absorption. It follows from Fig.2 (solid circles) that the tSIE index can be used to accurately determine the RBC content in a biological blood sample. By measuring the tSIE value of a pure blood sample, one can predict its absorption, and therefore its RBC concentration.

The tSIE value can only be used as an effective measure of RBC content in gill samples if the gill tissue itself has no effect on the tSIE value. Only RBC's, trapped in the gill, must be responsible for the tSIE value. Therefore, the gill samples were also analysed for absorption at 413 nm after analyzing these samples for the quenching index. Without interference from gill tissue on the tSIE value, the data points from these gill samples should follow Eq. [2], which holds for pure blood samples. However, the gill samples slightly deviate from the relation which exists for pure blood samples (Fig.2, open circles). It was tested whether this deviation was the result of an effect of gill tissue on absorption, an effect on the tSIE index, or both. To this end, the concentration of the gill samples was related to absorption at 413 nm. Gill concentration (GC) was calculated as follows : gill fresh weight was divided by gill density, yielding gill volume. This volume was divided by the cocktail volume in the vial, yielding gill concentration. A density of 1.00 $g.ml^{-1}$ was assumed for gills. An additional amount of pure blood (49.5 mg freshly drawn blood) was

added to each vial of a second set of gill samples, and slopes of both lines were compared (Fig.3). The following linear relationships were obtained :

$$OD = 0.0191 \times GC \qquad R^2 = 0.990^{***} \text{ (n=11)} \qquad [3]$$

for pure gill samples, and

$$OD = 0.0190 \times GC + 0.273 \qquad R^2 = 0.966^{***} \text{ (n=9)} \qquad [4]$$

for gill samples with addition of a small amount of blood. Analysis of covariance indicated no effect of gill tissue on the absorption of RBC's at 413 nm (F_S=0.013^{ns}/ df=1;16). It therefore can be concluded that deviation from Eq. [2] is due to a minor effect of gill tissue on the tSIE index. This means that the tSIE index of gill samples underestimates the amount of RBC's present in the samples when Eq. [2] is used. Therefore the tSIE values of the gill samples were corrected in the following manner. The actual absorption of the gill samples was used in Eq. [2] to calculate a corrected tSIE value. The measured tSIE values of the gill samples were correlated to these corrected tSIE values (Fig.4). The following linear relation [5] between measured tSIE index and corrected tSIE index of gill samples ($tSIE_C$) was obtained :

$$tSIE_c = 0.862 \times tSIE + 31.92 \qquad R^2=0.993^{***} \text{ (n=11)} \qquad [5]$$

This relation, which is only valid in the tSIE range between 250 and 400, makes it possible to correct the tSIE values of gill samples for the effect of gill tissue on these values.

Calculation of Trapped Blood Volume in Gill Tissue

With this knowledge, it is possible to determine the whole blood volume, trapped in the gill tissue. There exists a clear relationship (exponential decay) between RBC concentration and tSIE index for pure blood samples (Fig.5). The following numerical relation [6] was obtained:

$$[RBC] = 4.390 \times EXP(-0.0076 \times tSIE) + 728.570 \times EXP(-0.0562 \times tSIE) - 0.119$$

$$R^2=0.998^{***} \text{ (n=30)} \qquad [6]$$

This empirical function can also be used to relate tSIE values of gill samples to RBC concentration by changing tSIE into $tSIE_C$ [7] :

$$[RBC] = 4.390 \times EXP(-0.0076 \times tSIE_c) + 728.570 \times EXP(-0.0562 \times tSIE_c) - 0.119$$

$$R^2=0.998^{***} \text{ (n=30)} \qquad [7]$$

Trapped RBC concentration in the sample (in µl RBC per ml sample) can be obtained by using Eq. [7]. Multiplying this RBC concentration with sample volume yields RBC volume (in µl). The ratio of RBC volume (in ml) and gill volume in the vial (in ml) gives gill Ht (defined as ml RBC per ml of gill homogenate). Dividing gill

Ht by whole blood Ht (defined as ml RBC per ml blood) yields the blood volume, which is trapped in the gill tissue (in ml blood per ml gill tissue).

Correction of Branchial Metal Accumulation Rate

The procedure can be used to correct branchial metal accumulation rates for metal ions accumulated in the blood, which is trapped in the soft gill tissue. Multiplying the trapped blood volume (ml blood per ml gill tissue) with the gill tissue volume (ml) yields the trapped whole blood volume (ml) in the gills (TBV, trapped blood volume). The product of this trapped blood volume and blood density yields the amount of blood (g), trapped in the soft gill tissue (TBA, trapped blood amount). The fresh weight of the soft gill tissue (g) can thus be corrected by subtracting this amount of blood (TBA) from the fresh weight of the soft gill tissue (g). Multiplying the amount of accumulated metal ion in pure blood (nmol/g) with the amount of blood, trapped in the soft gill tissue (TBA), yields the amount of metal ion (nmol), accumulated in the trapped blood (TBMA, trapped blood metal amount). Subtracting this value of TBMA from the total amount of accumulated metal ion in the gill filaments (nmol) yields the corrected amount of accumulated branchial metal ion (nmol). Dividing this corrected amount by the corrected gill fresh weight (g) and the accumulation time (h), yields the branchial metal accumulation rate ($nmol.g^{-1}.h^{-1}$).

Metal Uptake Experiments

The experimentally obtained branchial cobalt accumulation rates for the metal uptake experiments were treated as a single group of results (n=84). First, branchial cobalt uptake rates were calculated without correction for the amount of metal ion accumulated in the trapped blood. These rates were termed not corrected branchial cobalt uptake rates. Secondly, corrected branchial cobalt uptake rates were calculated by the previously presented method. Not corrected as well as corrected branchial cobalt uptake rates were compared (Fig.6A). The same was done for the branchial calcium accumulation rates (n=83) (Fig.6B). The following simple linear relations between corrected and uncorrected cobalt and calcium uptake rates were obtained :

$$C_{Co} = 1.197 \times UC_{Co} \qquad R^2=0.990^{***} \text{ (n=84)} \qquad [8]$$

and

$$C_{Ca} = 1.520 \times UC_{Ca} \qquad R^2=0.955^{***} \text{ (n=83)} \qquad [9]$$

in which C_{co} and C_{ca} represent the corrected, and UC_{Co} and UC_{Ca} represent the uncorrected branchial accumulation rates of respectively cobalt and calcium. This means that the corrected branchial cobalt uptake rates are ± 20 % higher than the uncorrected branchial cobalt accumulation rates. The corrected branchial calcium accumulation rates are ± 52 % higher than the uncorrected values.

DISCUSSION

The method described in this paper, to estimate the gill circulatory fluid volume and to enable subsequent volume corrections, is straightforward and independent of the chemical studied. No additional analyses of the samples, such as determination of the hemoglobin content in the gill homogenate by spectrophotometry, are required. This because one of the quench indicating parameters, provided in a commercial liquid scintillation analyser to correct sample count rate for quench effects, is used to equate the amount of spectral shift in quenched gill tissue to the trapped blood volume. It is shown that the shift and absorbance at 413 nm are closely correlated, and that gill tissue without blood has a smaller contribution to the spectral shift and absorbance. Thus a correction factor can be derived to account for the tissue effect. This approach makes the method applicable to other tissues as well, although the correction factor to account for the tissue effect has to be evaluated for each tissue separately. The correction factor could be influenced by variations in the hemoglobin oxygenation state. However, we did not observe a wavelength shift in our samples : under the chosen conditions, variations in lambda max were smaller than 1.5 nm (very stable), so hemoglobin had a fixed oxygenation state in the SOLUENE/HIONIC FLUOR cocktail combination we used. Variability in lambda max between different samples should be assessed on a case to case basis, when using other combinations of tissue solubilizer/scintillator.

The method uses the tSIE quench indicating parameter of a Packard LS counter. This tSIE parameter, based on external standardisation, relies on the analysis of a Compton spectrum. This Compton spectrum is generated when the external standard (a gamma-emitting isotope) is positioned near the sample vial while it is counted. By analysing the shape or position of the Compton spectrum, the amount of quench in a sample is measured and a quench parameter is generated. The emitted wavelength of light by the external standard can be altered differently in different scintillation cocktails, resulting in different tSIE values. However, since the differences in the spectral properties among commercially available emulsion scintillators is not so great, the impact of the tissue on the tSIE index in these cocktails will be comparable. This means that, although different relations will be obtained for each different scintillator, the approach described in the paper will be adaptable for most commercially available emulsion scintillation cocktails. Other frequently used quench indicating parameters, such as Beckman's H-Number, are also based on external standardisation. This general approach to determine quench indicating parameters by external standardisation implies that trapped blood volume in gills can be correlated to other quench indicating parameters as well.

Most molecules (and most heavy metals) are carried in the plasma, and not in the RBC's. One could thus argue that a correction for plasma volume would be more appropiate. However, it is quite difficult to directly determine plasma volume in the gills. In the previously presented method, therefore, first RBC volume was determined per ml gill tissue and converted to either total blood volume or plasma volume, using the Ht value of trapped blood in the gills. An RBC volume as small as 6.2 µl can be accurately determined in soft gill tissue of average weight (± 0.050 g). The ratio of erythrocyte volume to plasma volume was assumed to be the same in the soft gill tissue as in the peripheral, circulating blood (Duff *et al.*, 1987; Gingerich *et al.*, 1987; Gingerich *et al.*, 1990; Munger *et al.*, 1991).

When considering branchial metal accumulation, one should correct for metal accumulation in the total extracellular fluid volume (ECFV) of the soft gill tissue, instead

of only correcting for metal accumulation in the trapped blood plasma. According to Holmes and Donaldson (1969), this extracellular compartment can be divided into several subcompartments : i) transcellular space, ii) intravascular fluid (or blood plasma), iii) interstitial fluid, and iiii) fluid in the lymphatic system. Correcting for trapped metal in gill ECFV gives additional problems. First, this requires knowledge of the metal accumulation rate per unit ECFV (nmol/ml ECFV). This value cannot be calculated, because the metal-binding protein concentration in the extravascular part of the ECFV is not known. Secondly, Munger *et al.* (1991) reported ECFV estimates in whole body and specific tissues of rainbow trout by using radiolabelled markers, which were infused in vivo. They showed that estimates of ECFV are very marker dependent, probably due to binding or metabolisation of the marker, resulting in an overestimation of the true size of the extracellular compartment in the gill tissues. They reported a gill ECFV estimate of 0.218 ml/ml gill tissue for rainbow trout. Based on their results, we calculated a value of 0.202 ml/ml gill tissue for the plasma volume. This means that for rainbow trout (and probably for other teleosts as well) almost all metal in the ECFV is confined to the plasma volume. Correcting for metal accumulated in trapped blood plasma will therefore provide a good estimate of branchial metal accumulation rates.

Gill filaments contained a relatively large blood volume. Trapped whole blood accounted for about 0.294 ml.g-1 (± 0.0488 ml.g-1; n=84) of the volume of the gill tissue, a value comparable to that reported previously for rainbow trout (Munger *et al.*, 1991). This value was calculated using the presented method and the results of the cobalt uptake experiment. The large vascular space in the gills is consistent with their role in respiratory gas exchange, acid-base balance and excretory functions. Due to this relative large blood volume in gills, there exists a large difference in uncorrected and corrected branchial metal accumulation rate (see Fig.6). The impact of the correction procedure is mainly dependent on the rate of accumulation of the chemical (or the metal) into the circulating blood. If there is no accumulation of the chemical in the blood, one only needs to correct for the weight contribution of the trapped blood in the gill. On the other hand, if the chemical accumulates in the blood, one needs to correct for both the weight and metal contribution of the trapped blood in the gill. This means that the magnitude of the correction factor strongly depends on the uptake characteristics of the chemical under study, and should be assessed for each chemical individually. The method developed for cobalt and calcium shows that a single constant factor is enough to correct for the blood content. This means that there is a linear relationship between the tracer accumulation in the gill and the amount of cobalt and calcium recovered in the blood. However, this relationship does not necessarily remain linear under all circumstances, e.g. accumulation of metal in the gills may result in the inhibition of basolateral ion transporters, so that the rate of metal transfer from the gill epithelium to the blood decreases. Then the correction factor will not be constant. Therefore, linearity of the correction factor should be assessed on a case to case basis.

In summary, our liquid scintillation technique to determine circulatory fluid content (such as blood) in fish gills is acceptable in terms of effort and accuracy, but can no doubt be improved with further modifications for tissues, which generate high background values due to the presence of myoglobin, cytochromes or chromogens.

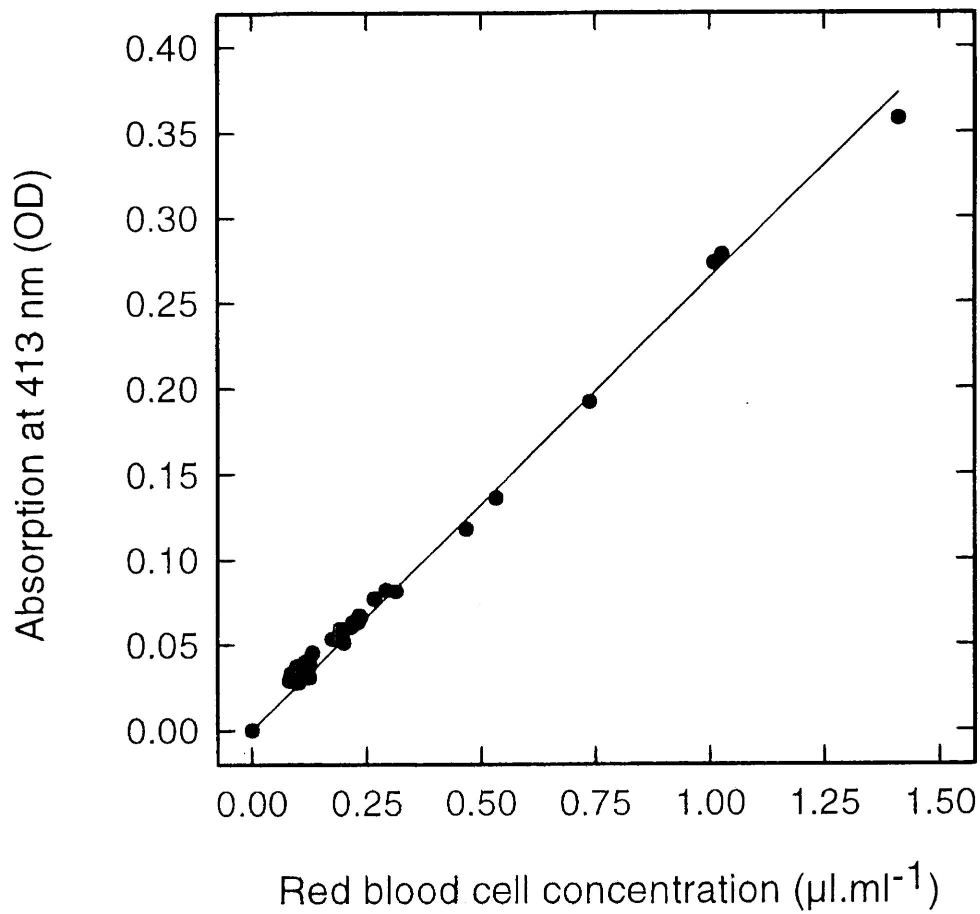

Fig.1. Absorption versus red blood cell concentration of pure blood samples. Absorption (OD) was measured in a spectrophotometer at the oxyhemoglobin absorption maximum (413 nm), and red blood cell concentration ([RBC]) was calculated as explained in the text. Samples contained freshly drawn blood, 1 ml SOLUENE-350 and 10 ml HIONIC-FLUOR. The results were related by linear regression (OD=0.264x[RBC]; n=30; R^2=0.994***).

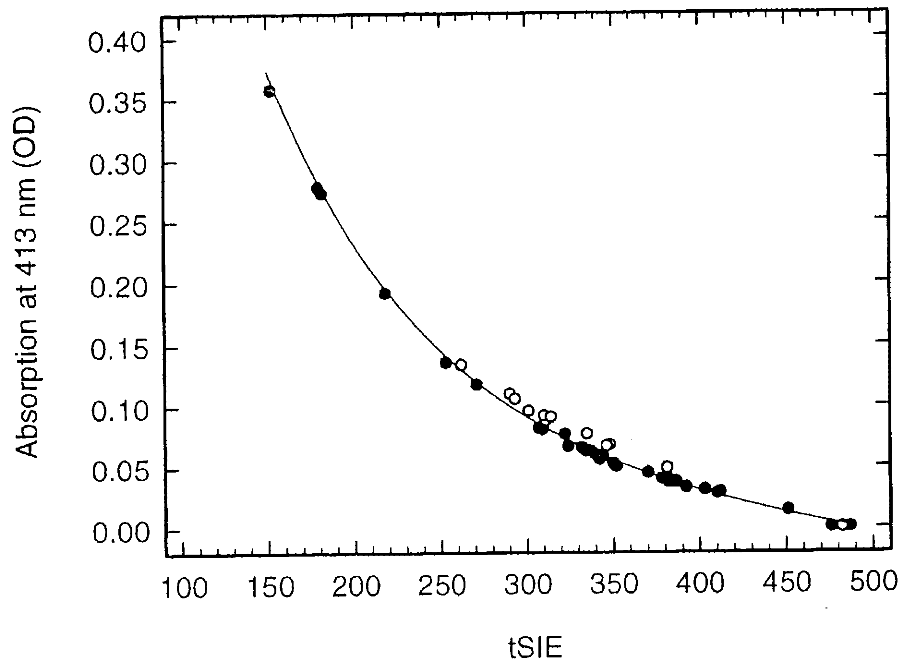

Fig.2. Absorption versus quenching index (tSIE) for pure blood (solid circles) and gill samples (open circles). Absorption was measured in a spectrophotometer at the oxyhemoglobin absorption maximum (413 nm), and tSIE was obtained by external standardisation. Samples contained either freshly drawn blood or freshly dissected gill filaments, 1 ml SOLUENE-350 and 10 ml HIONIC-FLUOR. Gill samples slightly deviate from the relation, which exists for pure blood samples (solid line;

$$OD = 1.611 \times EXP(-0.011 \times tSIE) + 1.106 \times EXP(-0.0002 \times tSIE) - 1.010$$

n=32; R^2=0.999***).

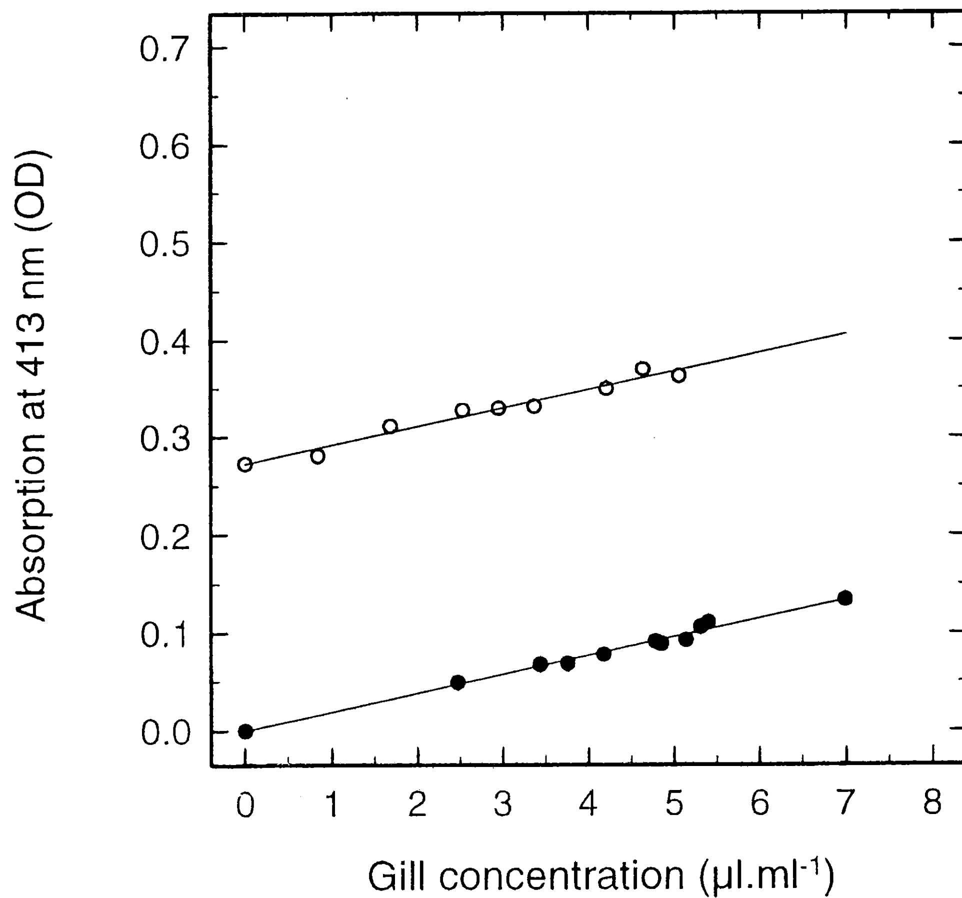

Fig.3. Effect of gill tissue on absorption of blood. Absorption (OD) was measured in a spectrophotometer at the oxyhemoglobin absorption maximum (413 nm), and gill concentration (GC) was calculated as explained in the text. Samples contained either freshly excised gill filaments (solid circles) or gill filaments plus an additional amount of freshly drawn blood (open circles), 1 ml SOLUENE-350 and 10 ml HIONIC-FLUOR. The results were related by linear regression (● OD=0.0191xGC; n=11; R^2=0.990*** and O OD=0.0190xGC+0.273; n=9; R^2=0.966***).

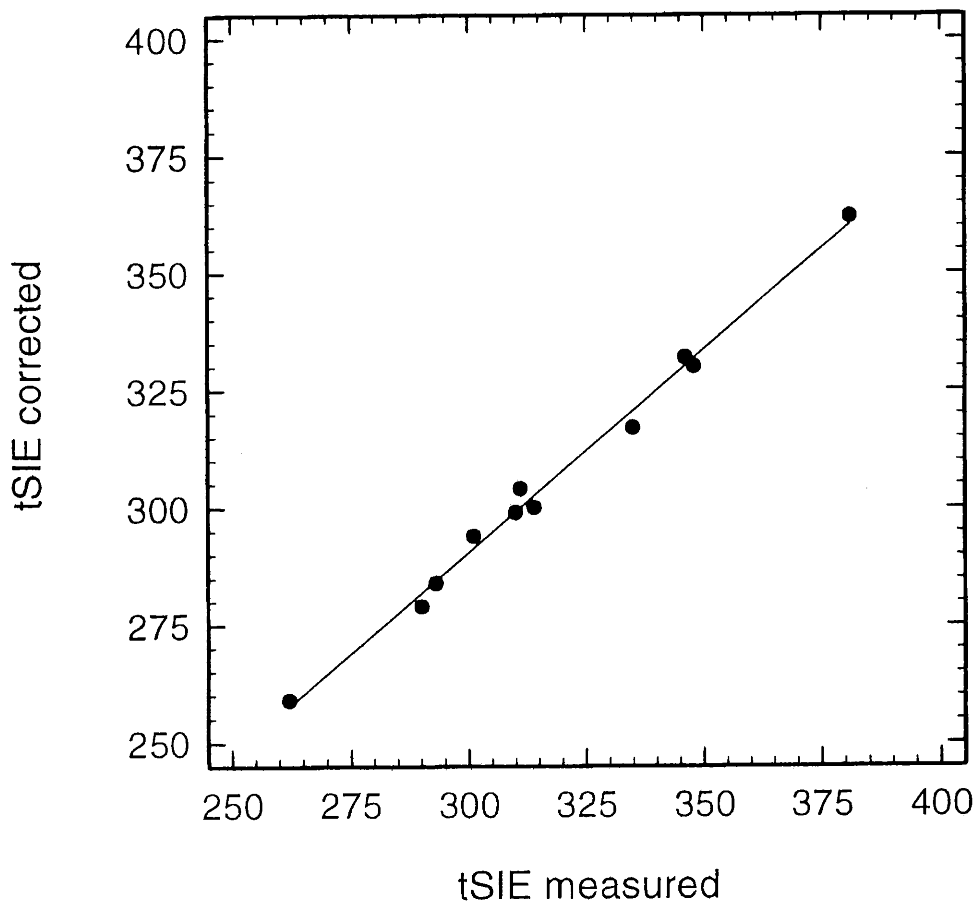

Fig.4. Corrected tSIE values versus measured tSIE values of gill samples. Measured tSIE values were obtained by external standardisation, and corrected tSIE values ($tSIE_C$) were calculated using absorption values of the gill samples and Eq. [2]. A simple linear relation was obtained ($tSIE_C$=0.862xtSIE+31.92; n=11; R^2=0.993***).

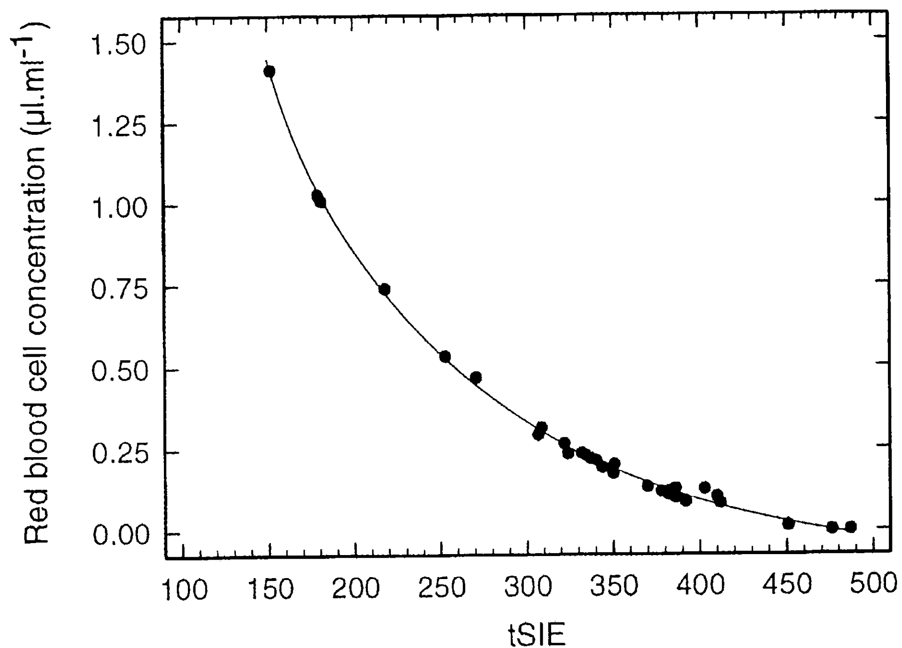

Fig.5. Red blood cell concentration versus quenching index (tSIE) for pure blood samples. Red blood cell concentration was calculated as explained in the text, and tSIE was obtained by external standardization. Samples contained freshly drawn blood, 1 ml SOLUENE-350 and 10 ml HIONIC-FLUOR. This fitting function was used to relate corrected tSIE values of gill samples ($tSIE_C$) with the amount of RBC's, trapped in the soft gill tissue (solid line;

$$[RBC] = 4.390 \times EXP(-0.0076 \times tSIE) + 728.570 \times EXP(-0.0562 \times tSIE) - 0.119$$

n=30; R^2=0.998***).

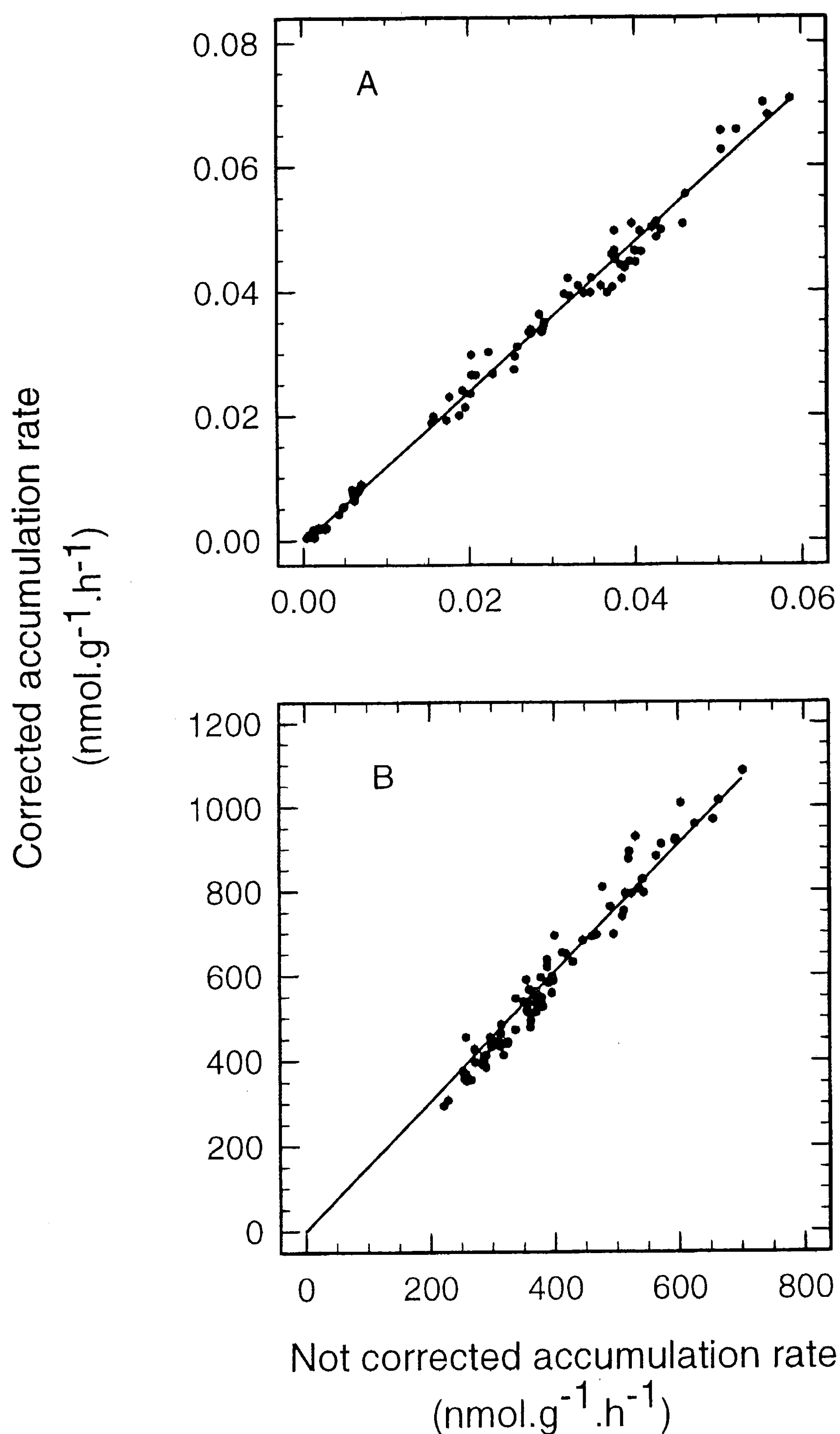

Fig.6. Effect of the correction procedure on branchial cobalt accumulation rates (A) and branchial calcium accumulation rates (B). Plotted points are the result of uptake experiments which were performed in organically complexing fresh water. Simple linear relations were obtained between uncorrected (UC) and corrected (C) values: (A) C_{Co}=1.197xUC_{Co}; n=84; R^2=0.990*** and (B) C_{Ca}=1.520xUC_{Ca}; n=83; R^2=0.955***.

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Effect of salinity on the uptake of cadmium by the brine shrimp, *Artemia franciscana*

INTRODUCTION

The uptake and accumulation of cadmium in aquatic organisms is a long standing environmental problem (Nriagu, 1988, Nriagu and Pacyna, 1988). The chemical speciation of cadmium in saline waters with a composition similar to that of seawater is dominated by the formation of weak complexes with chloride. Only a small fraction of the cadmium exists as the free metal ion while most of the cadmium is found in chloride complexes. In dilute solutions chloride complexation becomes less and less important and the concentration of the free metal ion increases with decreasing salinity (Boyle et al., 1976, Mantoura et al, 1978, Turner et al., 1981).
Cadmium has no known biological function and the internal body concentration is not regulated (Wright, 1977a, Dethlefsen, 1978, Rainbow, 1985, White and Rainbow, 1982, 1986). The availability of cadmium to aquatic organisms is determined by a number of environmental factors of which changes in salinity is one of the most important.
Aquatic organisms control the movement of water and ions across the exchange surfaces by altering the permeability of the body surface and/or by actively regulating the influx and efflux of water and ions. Acclimation of an aquatic organism to salinity involves the reorganisation of the exchange surfaces (e.g. gill and gut epithelium) in order to maintain the composition of the internal environment within certain physiological limits. Generally, the permeability of aquatic organisms is lower in low salinity than in high salinity environments. These physiological alterations in response to changes in salinity have profound effects on the movement of water and ions across the exchange surfaces (Mantel and Farmer, 1983, Gilles and Pequeux, 1983).
Several studies have shown an inverse relationship between the salinity and the uptake or toxicity of cadmium in aquatic organisms. This observation has been explained in different ways including: 1) increased availability of cadmium at low salinity caused by the increased free cadmium ion level (Sunda et al, 1978, Engel and Fowler, 1979, De Lisle and Roberts, Jr., 1988), 2) increased influx of cadmium as a result of the decreased osmolarity of the solution (George et al, 1978), 3) Competition of calcium and magnesium with cadmium for similar uptake sites (Wright, 1977b, Wright and Frain, 1981, Pärt et al., 1985).
The functionally different, but compatible, explanations for the effect of salinity on the uptake of cadmium in aquatic organisms have created considerable controversy concerning the processes controlling the transport of the metal across the solution-body interface. To understand how these chemical and biological processes influence the availability of cadmium to saltwater organisms it is necessary to determine the separate and combined effects of these different salinity components on metal uptake. For this purpose we have studied the effect of changes in salinity and the different salinity components on the uptake of cadmium by the euryhaline brine shrimp *Artemia franciscana*.

MATERIALS AND METHODS

Brine shrimp

Dried *Artemia franciscana* cysts from Great Salt Lake, Utah, USA were purchased from San Francisco Bay Brand, Newark, CA, USA. Cysts were hatched in a funnel-shaped plastic container filled with synthetic seawater (Wiegandt, Krefeld, Germany), and aerated from the bottom. The hatching suspension was illuminated by a fluorescent light tube. Hatching cyst density was 5 g l^{-1}. *Artemia* nauplii were harvested after 36 hours. The larvae were grown from nauplii to adult in 150 l plastic rectangular air-water lift operated race-ways filled with synthetic seawater. Brine shrimp were fed with a suspension of the dried algae *Spirulina*. Animals reached maturity after 3 to 4 weeks and were used before they were 8 weeks old. The methods for intensive culturing of brine shrimp have been described by Sorgeloos et al. (1983).

Experimental procedures

In this study of the uptake of cadmium by the brine shrimp we have used the static test design. The accumulation of cadmium during three hours of exposure was used as a measure for the biological availability of the metal. Experiments were conducted in a thermostated room at 25.0±0.5 °C. Ten days before an experiment was run adult brine shrimp were collected from a batch culture for salinity acclimation. Animals were gradually acclimated to chemically defined solutions of differing salinity over a five day period and kept at the final salinity for the remaining five days (i.e. 0.5, 1.0, 2.0, 3.0, 4.0 %). The last day of the acclimation period the animals were transferred for one hour to a saltwater solution containing 1mM of 8-hydroxyquinoline-5-sulfonic acid. This to remove metal bound to the external surfaces of the animal. For the remaining period the animals were kept in clean saltwater for defaecation. The composition of 1 l of the chemically-defined, saltwater solution with a salinity of 4.0 % was 26.85g NaCl, 4.57g Na_2SO_4, 0.777g KCl, 0.196g $NaHCO_3$, 1.680g $CaCl_2.2H_2O$, 12.32g $MgCl_2.6H_2O$ and 0.026g H_3BO_3. The medium was prepared by dissolving the seven analytical grade products (Merck p.a.) in deionised water. A dispersion of 0.1 mmol l^{-1} manganese dioxide was added to the seawater to remove metals present in the analytical grade reagents. After an equilibration period of 24 hours, the dispersion was filtered through a 0.2 µm membrane filter to remove the manganese dioxide from the solution (Van den Berg and Kramer, 1979). Solutions of lower salinity were prepared by dilution of the 4.0 % solution with deionised water. In some series of experiments the metal chloride salts were totally or partially replaced with metal nitrate salts. In one of these experiments the osmolarity of the solution was controlled by addition of sucrose to the solutions. The osmolarity of the solutions was measured with an osmometer which was calibrated with a stock solution of sodium perchlorate (Advanced Instruments Laboratory Osmometer).
The pH of the solutions was adjusted with HCl or NaOH as required and the media were aerated to promote equilibration of gases with the atmosphere. The dissolved oxygen concentration, the total dissolved carbonate concentration, the hydrogen activity and the redox potential were measured to ensure that equilibrium conditions had been established. Dissolved oxygen was measured with a polarographic oxygen

electrode system (WTW OX191/EO90). Total dissolved carbon dioxide was measured with a gas sensing CO_2 electrode (Ingold 152323000), after acidification of the water sample (pH<4.8) in a sealed measuring vessel. The hydrogen ion concentration was measured with a glass electrode (Ingold 104573002), and pH values expressed on a free hydrogen ion scale (Millero, 1986). Redox potentials were measured with a wire type platinum electrode (Ingold, 105003077). Cadmium nitrate was added to the test solutions from a 0.1 mmol l^{-1} cadmium ion stock. In all series of experiments the total concentration of cadmium in the test solutions was 100 μmol l^{-1}. Experiments were carried out in 0.5 l plastic beakers. Just before an experiment started about 50 animals were collected on a 250 μm screen, rinsed with clean medium and transferred to a beaker. After 180 minutes the beaker was removed and a few ml of the test solution sampled in a plastic vial and stored frozen at -20 °C until analysed for cadmium. The beaker was subsequently emptied over a 250 μm screen. The collected animals were rinsed with deionised water and divided into five plastic vials, dried for 24 hours at 60 °C and stored in a dessication box until analysed for cadmium. All experiments were run at least in duplicate using brine shrimp from different batch cultures. For each treatment group five replicate samples were obtained. The dissolved oxygen concentration, the total dissolved carbonate concentration, the hydrogen activity, the redox potential and the total dissolved cadmium concentration were measured at the beginning and end of an experiment. Generally, all measured values remained within 10 % of the initial values.

Chemical modelling and ion selective potentiometry

The equilibrium concentrations of the chemical species considered were calculated using the computer program SOLUTION (Blust et al., unpubl.) an adaptation of the program COMPLEX (Ginzburg, 1976). This speciation model allows the calculation of the composition of solutions in equilibrium with gas and solid phases. The model uses the ion-association concept which invokes the existence of molecular species like free ions, ion-pairs and complexes. A thermodynamic stability constant data base was built which is based on the data of Dickson and Whitfield (1981) for the major components and the data of Smith and Martell (1976, 1989) and Martell and Smith (1982), for cadmium. For each ion-pair or 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 et al., 1981). Activity coefficients were calculated using the relations given by Millero and Schreiber, (1982). The redox potential of the solutions was calculated from the empirical relation pE=17.6-pH (Baas-Becking et al., 1960). The thermodynamic stability constants and concentration products at the ionic strength of the saltwater solution for all cadmium species included in the model are given in Table 1. Case specific input comprises the total concentrations of the metals and ligands in the solution, the free hydrogen concentration (pH), redox potential (pE), temperature and the gas and solid phases that are maintained in equilibrium with the solution.
The results of the speciation calculations were verified by measuring the free cadmium ion concentrations with a cadmium ion electrode (Orion Model 94-48). The electrode was calibrated for the range 0.1-10,000 μM free cadmium and 0.01-1 M ionic strength. Sodium perchlorate was used as the background electrolyte for these calibration.

Metal analysis

Cadmium was measured by Graphite Furnace Atomic Absorption Spectrophotometry using a Perkin-Elmer 703 Spectrophotometer fitted with a Heated Graphite Atomiser HGA-500 and a deuterium arc background corrector. The method of stabilised temperature platform atomisation was used (Slavin et al., 1983). Biological material was dissolved with concentrated nitric acid in a microwave oven and diluted with deionised water to a 10 % nitric acid solution. Saltwater solutions were diluted ten times with a 10 % nitric acid solution to decrease the salinity. Matrix modificator (200 µg $(NH_4)_2HPO_4$ + 20 µg $Mg(NO_3)_2$ per sample) was added to the solutions and they were analysed against matrix matched calibration standards (Blust et al., 1988).

Statistical analysis

All sets of data were tested for homoscedasticity by the log-anova test for homogeneity of variances and for normality by the Kolmogorov-Smirnov test for goodness of fit. Analysis of variance, single and multiple linear regression and non-linear regression methods were used for analysing the data. The T-method was used to make multiple comparisons among pairs of means with homogeneous variances. The Games and Howell was used to make multiple comparisons among pairs of means with heterogeneous variances. Significance levels of tests are indicated by asterisks according to the following probability ranges (*=$0.05 \geq P > 0.01$, **=$0.01 \geq P > 0.001$, ***=$P \leq 0.001$). Statistical methods are as outlined in Biometry, (Sokal and Rohlf, 1981)) and Primer of Applied Regression and Analysis of Variance, (Glantz and Slinker, 1990).

RESULTS

Chemical speciation of cadmium

The complexation of cadmium in a chemically defined saltwater solution that does not contain organic ligands is controlled by the concentration of chloride. Changes in the hydroxide and carbonate concentration do not alter the speciation of the metal considerably. The calculations and measurements of the free metal ion concentration are in good agreement. The free metal ion is a minor species in waters of high salinity and a major species in waters of low salinity. With increasing salinity the difference between the concentration and activity of the cadmium ion becomes increasingly important. The results of the model calculations and the ion selective electrode measurements concerning the effect of salinity on the speciation of cadmium are summarised in Figs 1 and 2.

Salinity acclimation and uptake of cadmium by brine shrimp

To determine the effect of the salinity of acclimation and salinity of exposure on the uptake of cadmium by the brine shrimp, five groups of organisms were acclimated to five different salinities during a ten day period (i.e. 0.5, 1.0, 2.0, 3.0 and 4.0 %). After the acclimation period organisms from each group were transferred for three hours to solutions of differing salinity (i.e. 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 %) to which 100 μM of cadmium was added. For the five different salinity groups the uptake of cadmium decreased rapidly with increasing salinity but there were marked differences among the acclimation groups. For the same salinity of exposure uptake of cadmium increased with increasing salinity of acclimation. The results of the experiments with the 0.5 and 4.0 % acclimation groups are summarised in Fig 3. These results are representative for the results obtained with the other acclimation groups. The results of the correlation analysis summarised in Table 2 show that there is always a highly significant correlation between the change in salinity (and the covarying factors such as the ionic strength and osmolarity of the solution), changes in the activity or concentration of the free metal ion and the uptake of cadmium by the brine shrimp. The results summarised in Figure 4 show that for the same salinity of exposure and acclimation uptake of cadmium decreased with increasing salinity.

Salinity components and uptake of cadmium by brine shrimp

To determine and separate the effects of the different salinity components (i.e. changes in metal speciation, solution osmolarity and composition) on the uptake of cadmium by brine shrimp, different experiments were performed using animals acclimated to a salinity of either 0.5 or 4.0 %.
To determine the effect of complexation on the uptake of cadmium, animals were exposed to solutions with different chloride concentrations in which the salinity was kept constant by replacing chloride with nitrate. Complexation of cadmium with nitrate is neglible so that the replacement of chloride with nitrate increases the free metal ion activity or concentration while the salinity of the solution remains constant. The results summarised in Figure. 5 show that complex formation decreases cadmium uptake in both the low and high salinity acclimation group. In both cases

the linear association between changes in the concentration or covarying activity of the free metal ion and the uptake of cadmium is highly significant (Sal 0.5%, r^2=0.792 **, n=7 and Sal 4.0%, r^2=0.987***, n=14). Comparison of the results of the effect of changes in salinity (Fig 3) with the results of changes in chloride concentration at constant salinity (Fig 5) show that not all of the variation in metal uptake with salinity is explained by the complexation effect.
To determine the effect of the ionic strength and osmolarity of the solution on the uptake of cadmium, animals were exposed to solutions of differing salinity with very low complexation capacity. To this purpose all chloride was replaced with nitrate. Under these conditions the concentration of the free metal ion is close to the total metal concentration (80-90%) and does not vary appreciably with salinity. However, since the activity of the free metal ion depends on the ionic strength of the solution the activity and concentration of the free metal ion do not covary under these circumstances. In half part of these experiments sucrose was used as an osmolyte to keep the osmolarity the same as the osmolarity of the solutions of acclimation. The results summarised in Figure 6 show that the uptake of cadmium decreases with increasing salinity in both the low and high salinity group and that this effect does not depend on the osmolarity of the solution. In both cases the linear association between changes in the activity of the free metal ion and the uptake of cadmium is always better than the association between changes in the concentration of the free metal ion and the uptake of cadmium (salinity 0.5% nitrate, Cd^{2+}_{act} r^2=0.760***, Cd^{2+}_{conc} r^2=0.089^{ns}, n=13; sucrose r^2=0.927***, r^2=0.727*, n=6 and salinity 4.0% nitrate r^2=0.979***, r^2=0.414*, n=13; sucrose r^2=0.872***, r^2=0.253*, n=13).
To determine the effect of changes in the concentration of sodium, potassium, calcium and magnesium and combinations of either sodium and potassium or calcium and magnesium on the uptake of cadmium, animals were exposed to solutions of differing cation composition and constant salinity. For the preparation of these solutions the concentration of the salts was altered so that the speciation of cadmium and the osmolarity of the solution remained constant. The results summarised in Fig 7. show that changes in the concentration of calcium do not influence the uptake of cadmium in both salinity groups. Similar results were obtained with all other major cations either tested separately or in combination.

Modelling of cadmium uptake

Together these results show that three factors are important in determining the availability of cadmium to the brine shrimp. There are the effects of the salinity of acclimation and exposure on the permeability of the organism and there is the effect of changes in the activity of the free cadmium ion with changes in salinity.
Metal influx does not increase linearly with the activity of the free cadmium ion over the entire range of activities under constant conditions. However, the decrease in metal uptake at the highest free metal ion levels is reasonably well described by a simple nth-order power reaction rate equation (i.e. simple Michaelis behavior). Likewise, the effect of the salinity of acclimation and the effect of the salinity of exposure on metal uptake can be described by the product of two nth-order power equations. Metal influx is then proportional to the product of the terms describing the effect of the salinity of acclimation, the salinity of exposure and the activity of the free metal ion. [i.e. $Sal_{acl}^{k} * Sal_{exp}^{l} * Cd^{2+}_{act}{}^{m}$]. To relate this product to metal

uptake, it is necessary to introduce a coefficient of proportionality (C_f) which relates the activity of the metal ion in the solution to the concentration of the metal in the shrimps. Neglecting the minute amount of metal present in clean organisms equation for the concentration of cadmium in the organisms (Cd_{shrimp}) becomes, $Cd_{shrimp}=C_f*(Sal_{acl}{}^k*Sal_{exp}{}^l*Cd^{2+}{}_{act}{}^m)$. Applying this equation, analysis of the pooled results shows that most of the variation in cadmium uptake is explained when the availability of the metal is determined by the activity of the free metal ion in the solution and that the rate of metal uptake increases with the salinity of acclimation and decreases with the salinity of exposure. The results of the non-linear regression analysis are summarised in Table 3 and Figure 8.

Table 1. Thermodynamic stability constants and concentration products for cadmium species K: thermodynamic stability constant; Q concentration products at salinity 0.5 % and 4.0 %

Species	Log K	Log Q (S=0.5%)	Log Q (S=4.0%)
Cd^{2+}	-	-	-
$CdCl^{+}$	1.97	1.56	1.35
$CdCl_2^{0}$	2.59	1.98	1.70
$CdCl_3^{-}$	2.40	1.76	1.48
$CdCl_4^{2-}$	1.47	1.22	1.38
$CdSO_4^{0}$	2.45	1.62	1.04
$Cd(SO_4)_2^{2-}$	3.44	2.58	1.83
$CdOH^{+}$	3.91	3.49	3.33
$Cd(OH)_2^{0}$	7.64	7.01	6.75
$CdCO_3^{0}$	4.35	3.52	2.94

Table 2. Linear coefficients of determination for uptake in the different salinity groups, between the salinity, the free cadmium ion activity or the free cadmium ion concentration and the cadmium uptake by brine shrimp, *Artemia franciscana*.

Salinity	0.5%	1.0%	2.0%	3.0%	4.0%
Uptake and Salinity	0.540 **	0.480 **	0.549 **	0.596 **	0.502 **
Cd^{2+}_{act}	0.932***	0.966***	0.990***	0.998***	0.968***
Cd^{2+}_{conc}	0.969***	0.935***	0.955***	0.965***	0.900***

Table 3. *Artemia franciscana*: Cadmium uptake, pooled data non-linear regression. B: partial regression coefficients, SE: standard error of partial regression coefficients; L_1, L_2: confidence limits for partial regression coefficients. Activities are in $\mu mol.l^{-1}$ for cadmium in solution and concentrations in $\mu mol.g^{-1}$ for cadmium in brine shrimp, *Artemia franciscana*. ** 0.01>p>0.001; ***p<0.001

(a) $Cd_{shrimp}=C_f(Cd^{2+}{}_{act}{}^{k})$ (R^2=0.578*, n=125)**

Variable	B	SE	L_1	L_2
Coefficient	0.262**	0.087	0.089	0.435
k-exponent	0.766***	0.097	0.573	0.959

(b) $Cd_{shrimp}=C_f(Cd^{2+}{}_{act}{}^{k}Sal_{exp}{}^{l})$ (R^2=0.620*, n=125)**

Variable	B	SE	L_1	L_2
Coefficient	0.419**	0.124	0.173	0.665
k-exponent	0.581***	0.094	0.396	0.766
l-exponent	-0.171***	0.047	-0.263	-0.079

(c) $Cd_{shrimp}=C_f(Cd^{2+}{}_{act}{}^{k}Sal_{acl}{}^{m})$ (R^2=0.863*, n=125)**

Variable	B	SE	L_1	L_2
Coefficient	0.138***	0.027	0.085	0.191
k-exponent	0.866***	0.056	0.754	0.978
m-exponent	0.466***	0.038	0.391	0.541

(d) $Cd_{shrimp}=C_f(Cd^{2+}{}_{act}{}^{k}Sal_{exp}{}^{l}Sal_{acl}{}^{m})$ (R^2=0.937*, n=125)**

Variable	B	SE	L_1	L_2
Coefficient	0.165***	0.025	0.115	0.215
k-exponent	0.571***	0.036	0.500	0.642
l-exponent	-0.338***	0.015	-0.367	-0.309
m-exponent	0.506***	0.027	0.454	0.558

DISCUSSION

Overall, two processes are important in determining the effect of salinity on the uptake of cadmium to the brine shrimp. Acclimation to salinity is important in determining the permeability of the animal. The effect of changes in the activity of the free metal ion are important in determining the availability of the metal to the brine shrimp.

The brine shrimp is an euryhaline hypo-hyperosmotic regulator. Adult animals take up water by drinking and excess ions are removed through the gills. The brine shrimp can regulate its internal environment over a very wide range of external conditions. It maintains its internal osmolarity below that of the medium in environments above 250 mosm, but maintains its internal osmolarity above that of the medium at lower environmental levels. This means that the brine shrimp possesses mechanisms for both hyposmotic and hyperosmotic regulation. In hypo-hyperosmotic animals the energetic demands of osmotic regulation may be reduced by lowering the osmotic gradient between organism and environment and by limiting permeabilities to water and salts in dilute environments (Croghan, 1958a,b c).

Within each acclimation group uptake of cadmium in brine shrimp increases with decreasing salinity of exposure. Among the acclimation groups uptake of cadmium decreases with decreasing salinity of acclimation. Uptake of cadmium in brine shrimp at the salinity of acclimation is maximum at the lowest salinity and minimum at intermediate salinities. The effect of salinity on the uptake of cadmium is therefore the combined result of the effect of the salinity of acclimation on the permeability of the animal and the effect of the salinity of exposure on the availability of the metal. However, it is important to recognise that the magnitude of reduction in permeability following transfer to low salinity may be overestimated if the shift in electrical gradient is not taken into account. The trans-epithelial potential of hypo-osmotic regulators becomes strongly negative after transfer of the animal to low salinity. Thus, the transfer of cations (influx and efflux) is altered independent of an intrinsic change in permeability (Mantel and Farmer, 1983).

The availability of cadmium to the brine shrimp depends on the free cadmium ion level in the solution. Several studies have indicated that the availability of cadmium depends on the free metal ion activity or concentration of the solution (Sunda et al, 1978., Engel and Fowler, 1979, De Lisle and Roberts, Jr., 1988). Most of these studies have not considered the functional difference between these two related factors. The results presented here show that the availability of cadmium depends on the activity rather than on the concentration of the free metal ion. However, since activity and concentration are closely related expressions it is only in the low salinity region where changes in activity and concentration are clearly different that the effect is clear.

It has been shown that several other factors which vary with salinity such as the osmolarity (George et al., 1978) and composition (i.e. calcium and magnesium concentration) of the solution influence the uptake or toxicity of cadmium in aquatic organisms (Wright, 1977a,b, Wright and Frain, 1981, Pärt et al., 1985).

However, the results presented here indicate that changes in the concentration of the major ions or osmolarity of the solution do not appear to alter the availability of cadmium to the brine shrimp. Within each acclimation group changes in the activity of the free cadmium ion with changes in the salinity, chlorinity or osmolarity of the solution explain almost all of the variation in cadmium uptake observed. The fact

that metal availability is related to the activity rather than the concentration of the free metal ion in the solution arises from the fact that the activity coefficient of a species is a measure of the effectiveness with which it influences an equilibrium in which it is a participant. In freshwater solutions, where ionic strength is minimal, the activity coefficient approaches unity. Under such circumstances, the activity and molarity of a species are almost identical. As the ionic strength increases, however, an ion loses some of its effectiveness, and its activity coefficient decreases (Whitfield and Turner, 1979).

The effect of salinity on the availability of cadmium to an aquatic organism should not be directly compared with the effect of salinity on the accumulation or toxicity of cadmium in the organism. The availability of a metal is the fraction of the total concentration in the medium that can be taken up by an organism. The initial step in metal uptake involves the translocation of the metal across the solution-body interface from the external to the internal environment formed by the cells of the exchange surfaces (i.e. gill and gut epithelium). The translocation of the metal across the solution-body interface is considered to be a facilitated process. The transport of the metal from the exchange epithelium to the blood from where it is carried to other parts of the body is considered to be an active process (Verbost et al., 1987, Verbost et al., 1988, Foulkes, 1989).

The apical, basolateral and basal membranes of transporting epithelia are functionally different. Therefore the direct effect of environmental conditions on metal transport across the solution-body interface are not the same as the effects on the other parts of the transport system (Williams, 1983).

To explain the effects of environmental processes on the availability, uptake and accumulation of metals it is important to separate these different steps. It is well established that changes in the chemical speciation of the metal (i.e. changes in the activity of the free metal ion) have a direct effect on the translocation of the metal from the external to the internal environment of the epithelium. Changes in the concentration of the major cations (i.e. calcium and magnesium) do not appear to alter the uptake of cadmium across the solution-body interface. These results seem in contrast with the well established interactions between calcium and cadmium which occur because both are divalent cations with similar ionic radii and are therefore expected to compete for the same binding sites, (Williams, 1981). There are several mechanisms by which calcium may alter the uptake of metals by aquatic organisms. 1) Calcium dependent changes in the permeability of the epithelial structures, causing a decrease in metal uptake as calcium concentration increases. 2) Competition for binding sites on the apical membrane surfaces between calcium and divalent metal ions. 3) Decreased metal transfer from the epithelium to the blood with increasing intracellular levels of calcium (Hull, 1985, Van Os, 1987).

The results concerning the effect of the salinity of acclimation and the composition of the solution do not show a decrease in cadmium uptake with an increase in the concentration of calcium or any of the other major cations. In saltwater organisms the effect of calcium and magnesium on metal uptake does not appear to influence metal uptake directly. This is exemplified by several studies dealing with the effect of calcium and magnesium on the uptake, retention and transfer of cadmium in exchange epithelia of aquatic organisms (Pärt and Svanberg, 1981, Pärt et al., 1985, Verbost et al., 1987, Verbost et al, 1988). These studies have demonstrated that calcium increases the retention of cadmium in the epithelia and decreases the transfer of cadmium to the blood. Hence, it is unlikely to observe an effect of calcium

on total metal uptake in short term studies when metal influx is the most important process. Under these conditions only determination of the retention of the metal in the epithelia or transfer of the metal to the blood can reveal an effect of calcium on cadmium transport. It is however more likely to observe an effect of calcium on cadmium in more long term studies of metal toxicity or accumulation when processes such as metal retention and transfer become more important in determining metal uptake (Pascoe et al., 1986, Wicklund and Runn, 1988, Stephenson and Mackie, 1989).

CONCLUSIONS

To explain the effect of salinity on the uptake of cadmium by the brine shrimp two processes are of paramount importance. The salinity of acclimation determines the overall permeability of the organism for the metal ion. The activity of the metal ion determines the availabiltiy of the metal to the brine shrimp. Much of the variation in metal uptake not explained by the changes in the concentration of the free metal ion is explained by the changes in the activity of the free metal ion.

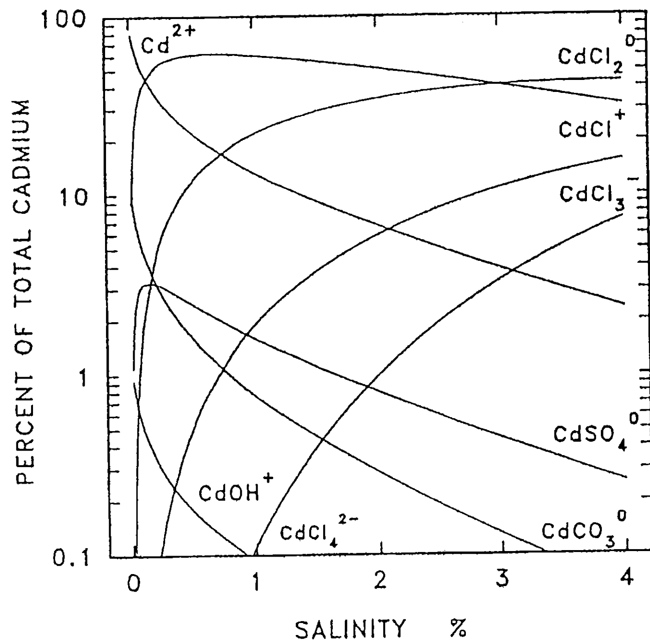

Fig. 1: Speciation model of cadmium as a function of the salinity of the solution (pH=8.1, $pO_2=10^{-0.69}$ atm, $pCO_2=10^{-3.48}$ atm).

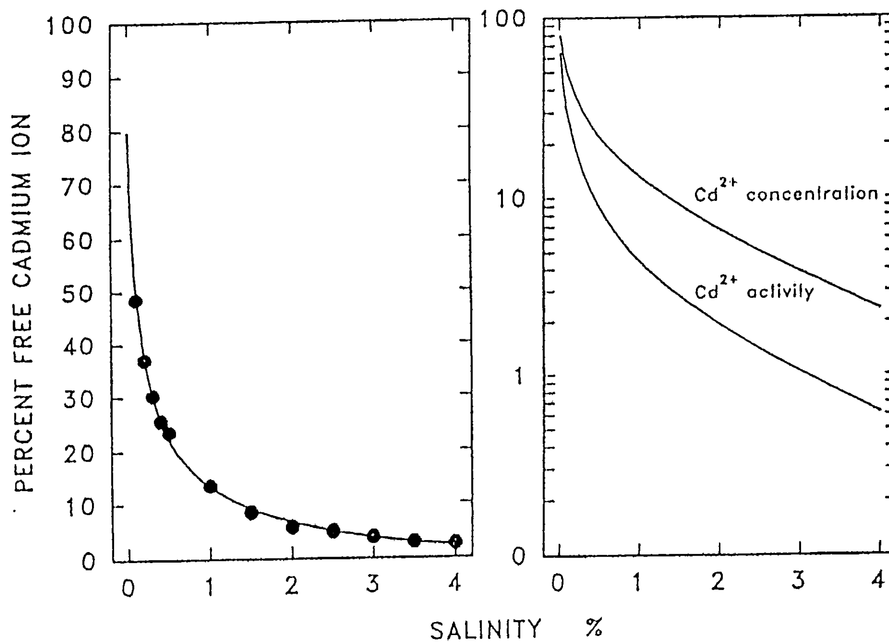

Fig. 2a: Measured (•) and modelled (solid line) free cadmium ion concentration as a function of the salinity of the solution (pH=8.0-8.2, $pO_2=10^{-0.69}$ atm, $pCO_2=10^{-3.48}$ atm).

Fig. 2b: Modelled free cadmium ion activity and concentration as a function of the salinity of the solution (pH=8.1, $pO_2=10^{-0.69}$ atm, $pCO_2=10^{-3.48}$ atm).

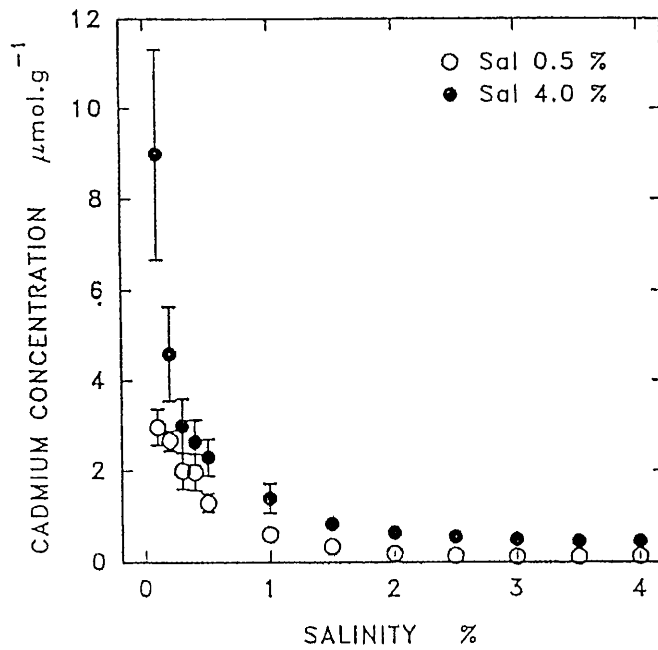

Fig. 3: *Artemia franciscana*. Effect of the salinity of exposure on the uptake of cadmium over a 3 hour period for the 0.5 and 4.0 % acclimation groups (solution contains chloride). Means with standard deviations are significantly different for both the 0.5 and 4.0 % groups, $p<0.01$ (Cd_T=100µM, pH=8.0-8.2, $pO_2=10^{-0.69}$ atm, $pCO_2=10^{-3.48}$ atm).

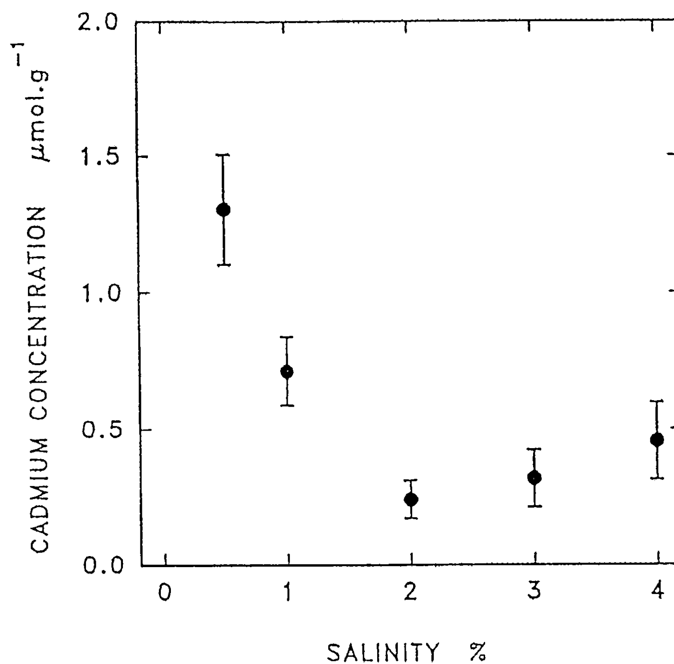

Fig. 4: *Artemia franciscana*. Effect of the salinity of acclimation on the uptake of cadmium over a 3 hour period for the 0.5, 1.0, 2.0, 3.0, 4.0 % acclimation groups. Means with standard deviations are significantly different, $p<0.01$ (Cd_T=100µM, pH=8.0-8.2, $pO_2=10^{-0.69}$ atm, $pCO_2=10^{-3.48}$ atm).

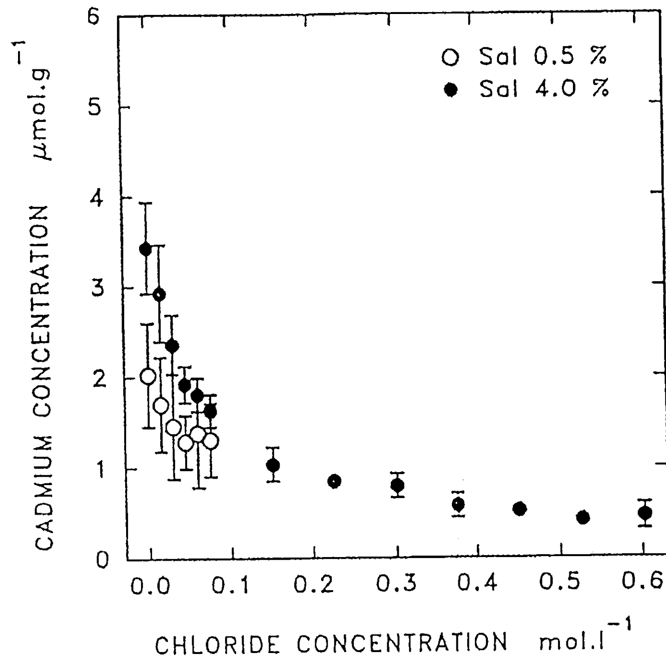

Fig. 5: *Artemia franciscana.* Effect of chlorinity at constant salinity of exposure on the uptake of cadmium over a 3 hour period for the 0.5 and 4.0 % acclimation groups (solution contains chloride and nitrate). Means with standard deviations are not significantly different for the 0.5 % group, $p>0.05$, and significantly different for the 4.0 % group, $p<0.01$ (Cd_T=100µM, pH=8.0-8.2, $pO_2=10^{-0.69}$ atm, $pCO_2=10^{-3.48}$ atm).

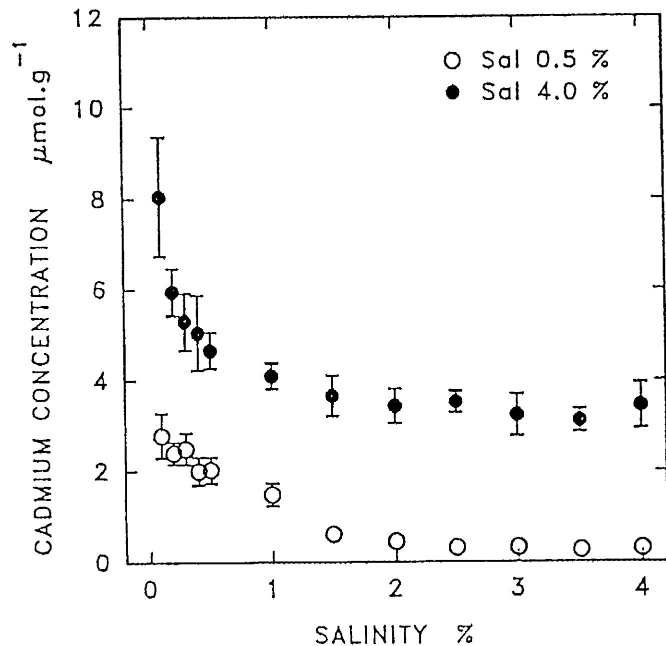

Fig. 6: *Artemia franciscana.* Effect of the salinity of exposure on the uptake of cadmium over a 3 hour period for the 0.5 and 4.0 % acclimation groups (solution contains nitrate). Means with standard deviations are significantly different for both the 0.5 and 4.0 % groups, $p<0.01$ (Cd_T=100µM, pH=8.0-8.2, $pO_2=10^{-0.69}$ atm, $pCO_2=10^{-3.48}$ atm).

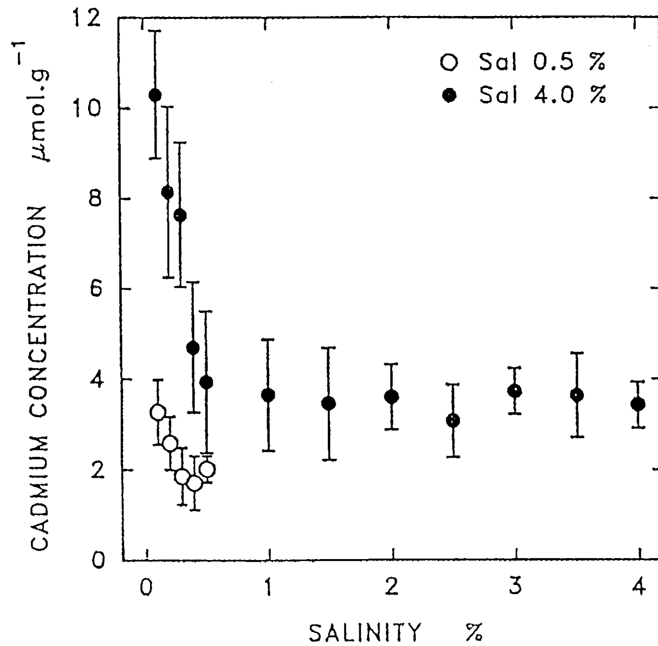

Fig. 7: *Artemia franciscana*. Effect of the salinity of exposure at constant osmolarity on the uptake of cadmium over a 3 hour period for the 0.5 and 4.0 % acclimation groups (solution contains nitrate and sucrose). Means with standard deviations are not significantly different, $p>0.05$ for the 0.5 % acclimation group and significantly different for the 4.0 % group, $p<0.01$ for the low salinity acclimation group (Cd_T=100µM, pH=8.0-8.2, $pO_2=10^{-0.69}$ atm, $pCO_2=10^{-3.48}$ atm).

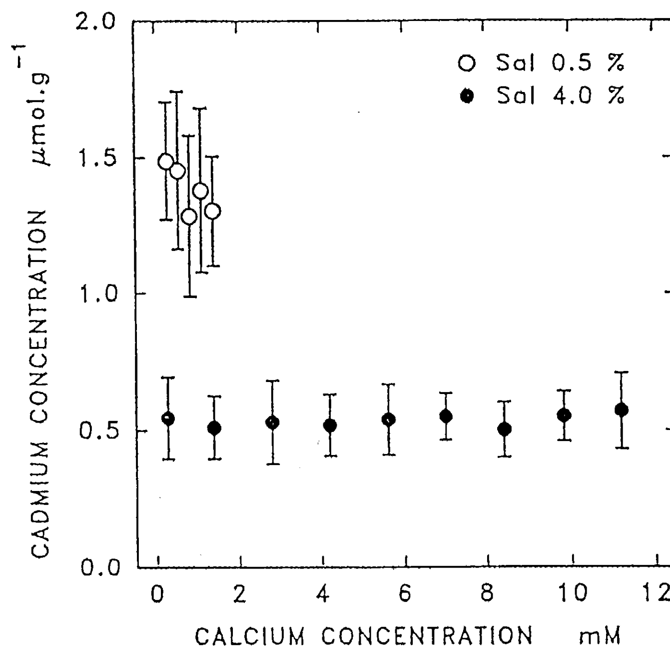

Fig. 8: *Artemia franciscana*. Effect of the calcium concentration at constant salinity of exposure on the uptake of cadmium over a 3 hour period for the 0.5 and 4.0 % acclimation groups (solution contains chloride). Means with standard deviations are not significantly different, $p>0.05$ (Cd_T=100µM, pH=8.0-8.2, $pO_2=10^{-0.69}$ atm, $pCO_2=10^{-3.48}$ atm).

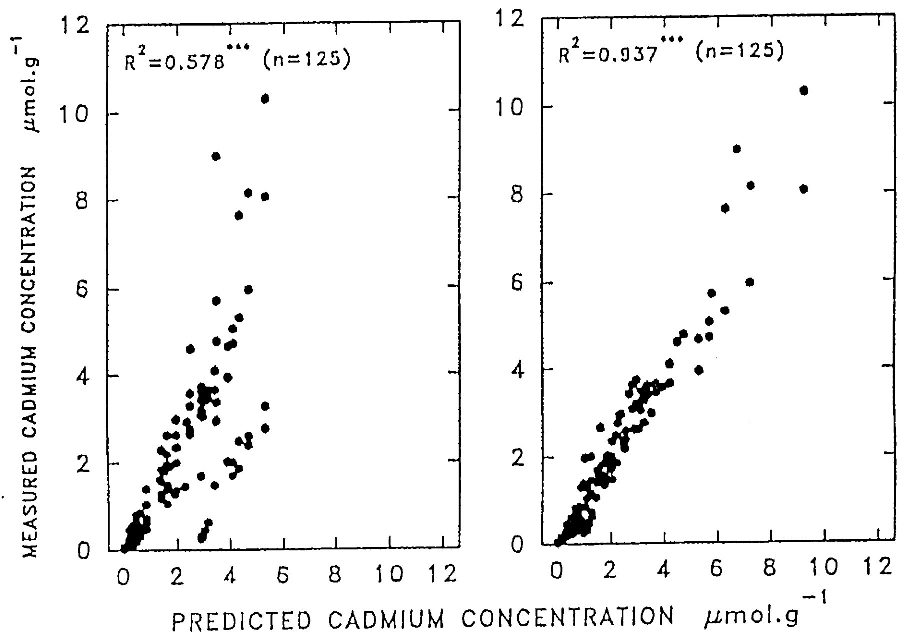

Fig. 9: *Artemia franciscana*. Predicted versus measured concentrations of cadmium in shrimp for two uptake models a) $Cd_{shrimp}=C_f*(Cd^{2+}{}_{act}{}^{m})$, R^2=0.578***, n=125 and b) $Cd_{shrimp}=C_f*(Sal_{acl}{}^{k}*Sal_{exp}{}^{l}*Cd^{2+}{}_{act}{}^{m})$, R^2=0.937***, n=125.

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Effect of hydrogen ions and inorganic complexing on the uptake of copper by the brine shrimp, *Artemia franciscana*

INTRODUCTION

The uptake and toxicity of copper in aquatic organisms is largely determined by the physical and chemical speciation of the metal in the environment and is related to the concentration of the free cupric ion in the solution (Borgmann, 1983, Campbell and Stokes, 1985, Wangersky, 1986, Luoma, 1989). In a saltwater environment most of the copper is complexed by organic and inorganic ligands. Only a small fraction of the total amount of copper exists as the free cupric ion (Waite and Morel, 1983, Van den Berg, 1984, Sunda and Hanson, 1987, Moffett and Zika, 1987, Turner and Whitfield, 1987, Coale and Bruland, 1988).
Experimental work carried out in saltwater solutions, where changes in the hydrogen ion concentration are minimal, have shown that complexation of copper with organic ligands decreases the uptake and toxicity of copper (Sunda and Guillard, 1976, Knezovich et al., 1981, Zamuda and Sunda, 1982, Ahsanullah and Florence, 1984, Zamuda et al., 1985, Blust et al., 1986, Florence and Stauber, 1986, Sunda et al., 1987). Studies carried out in freshwater solutions where changes in the hydrogen ion concentration are more pronounced, have also shown that the complexation of copper with organic ligands decreases the uptake and toxicity of copper (Dodge and Theis, 1979, Guy and Kean, 1980, Borgmann and Ralph, 1983, Buckley, 1983, Starodub et al., 1987). However, it has also been shown that the uptake and toxicity of copper increases with decreasing hydrogen ion concentration and decreasing cupric ion concentration (Andrew et al., 1977, Howarth and Sprague, 1978, Chakoumakos et al., 1979, Miller and Mackay, 1980, Cusimano et al., 1986, Laurén and McDonald, 1986, Starodub et al, 1987). Under these circumstances the direct relation between the concentration of the cupric ion in solution and the uptake and toxicity of the metal in the aquatic organism is lost.
The first step in the uptake of copper from solution by an aquatic organism probably involves interaction with a membrane embedded transport system to form a temporary association which carries the metal across the interface. Most of these systems are sensitive to the binding of other positively charged species, most notably the hydrogen ion. Although the pH of saltwaters remains relatively constant within space and time, the pH at the solution-body interface can vary considerably due to the activity of the specialised tissues which line the interface and create an intermediate environment which differs from the external environment. Hydrogen ions may alter the availability of copper in three distinct ways, i) changing the speciation of the metal in the solution, ii) modulate the activity of the metal transport system and iii) affect physiological processes which either directly or indirectly influence the uptake process (e.g. alter membrane potentials, ionic and osmotic regulation, energy metabolism) (Williams, 1981, Simkiss and Taylor, 1989, Viarengo, 1989).
To understand how these chemical and biological processes influence the availability of copper to organisms it is necessary to determine the combined effect of changes in the hydrogen ion concentration and inorganic speciation of copper on the uptake of the metal in multicellular organisms that live in saltwater environments. For that purpose we have studied the combined effect of hydrogen ions and inorganic complexing on the uptake of copper in the brine shrimp, *Artemia franciscana*.

MATERIALS AND METHODS

Brine shrimp

Dried *Artemia franciscana* cysts from Great Salt Lake, Utah, USA were purchased from San Francisco Bay Brand, Newark, CA, USA. Cysts were hatched in a funnel-shaped plastic container filled with synthetic seawater (Wiegandt, Krefeld, Germany), and aerated from the bottom. The hatching suspension was illuminated by a fluorescent light tube. Hatching cyst density was 5 g l^{-1}. *Artemia* nauplii were harvested after 36 hours. The larvae were grown from nauplii to adult in 150 l plastic rectangular air-water lift operated race-ways filled with synthetic seawater. Brine shrimp were fed with a suspension of Yellow-Mix (Artemia Systems, Ghent, Belgium). Animals reached maturity after 3 to 4 weeks and were used before they were 8 weeks old. The methods for intensive culturing of brine shrimp have been described by Sorgeloos et al. (1983).

Experimental procedures

In this study of the biological availability of copper to the brine shrimp we have used the static test design. The accumulation of copper during three hours of exposure was used as a measure for the biological availability of the metal. Experiments were conducted in a thermostated room at 25.0$\pm$0.5 °C. One day before an experiment was run adult brine shrimp were collected from a batch culture and placed into clean medium to clear their gut contents. The composition of 1 l of the chemically-defined, saltwater solution was 23.50g NaCl, 4.00g Na_2SO_4, 0.680g KCl, 0.196g $NaHCO_3$, 1.470g $CaCl_2.2H_2O$, 10.78g $MgCl_2.6H_2O$ and 0.026g H_3BO_3. The medium was prepared by dissolving the seven analytical grade products (Merck p.a.) in deionised water. A dispersion of 0.1 mmol l^{-1} manganese dioxide was added to the seawater to remove metals present in the analytical grade reagents. After an equilibration period of 24 hours, the dispersion was filtered through a 0.2 mm membrane filter to remove the manganese dioxide from the solution (Van den Berg and Kramer, 1979). The pH of the solutions was controlled by incorporation of the zwitterionic buffers 2-(N-morpholino)ethane-sulphonic acid (MES) and N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) to control the pH between 5.3 and 6.8 and 6.8 and 8.3, respectively. These buffers form only weak complexes with divalent metal ions and complexation of copper is negligible at the concentration of 10 mmol l^{-1} MES or HEPES used to buffer the saltwater solutions (Good et al. 1966). The pH of the test solutions was adjusted with HCl or NaOH as required and the media were aerated to promote equilibration of gases with the atmosphere. At equilibrium the total dissolved carbon dioxide concentration is determined by the pH of the solution and the partial pressure of CO_2 in the atmosphere. To control the total dissolved carbon dioxide concentration at fixed pH the solutions were aerated with synthetic air of known CO_2 concentration (i.e. 0-5.0 %). The dissolved oxygen concentration, the total dissolved carbonate concentration, the hydrogen activity and the redox potential were measured to ensure that equilibrium conditions had been established. Dissolved oxygen was measured with a polarographic oxygen electrode system (WTW OX191/EO90). Total dissolved carbon dioxide was measured with a gas sensing CO_2 electrode (Ingold 152323000), after acidification of the water sample ($pH<4.8$) in a sealed measuring vessel. The hydrogen ion concentration was measured with a glass electrode (Ingold 104573002), and pH values expressed on a free hydrogen ion scale (Millero, 1986). Redox potentials were measured with a wire type

platinum electrode (Ingold, 105003077). Copper was added to the test solutions from a 0.1 mmol l^{-1} cupric ion stock, (i.e. $Cu(NO3)_2$). In a first series of experiments the total concentration of copper in the test solutions ranged between 1 and 100 mmol l^{-1}. Higher concentrations of copper could not be used due to the limited solubility of the metal. In a second series of experiments the total concentration of copper in the test solutions was 5 mmol l^{-1}. Experiments were carried out in 0.5 l plastic beakers. The test solutions were aerated during the experiments to maintain equilibrium. Just before an experiment started about 50 animals were collected on a 250 mm screen, rinsed with clean medium and transferred to a beaker. After 180 minutes the beaker was removed and a few ml of the test solution sampled in a plastic vial and stored frozen at -20 °C until analysed for copper. The beaker was subsequently emptied over a 250 mm screen. The collected animals were rinsed with deionised water and divided into five plastic vials, dried for 24 hours at 60 °C and stored in a dessication box until analysed for copper. All experiments were run at least in duplicate using brine shrimp from different batch cultures. For each treatment group five replicate samples were obtained. The dissolved oxygen concentration, the total dissolved carbonate concentration, the hydrogen activity, the redox potential and the total dissolved copper concentration were measured at the beginning and end of an experiment. Generally, all measured values remained within 10 % of the initial values.

Chemical modelling

The equilibrium concentrations of the chemical species considered were calculated using the computer program SOLUTION (Blust et al., unpubl.) an adaptation of the program COMPLEX (Ginzburg, 1976). This speciation model allows the calculation of the composition of solutions in equilibrium with gas and solid phases. The model uses the ion-association concept which invokes the existence of molecular species like free ions, ion-pairs and complexes. A thermodynamic stability constant data base was built which is based on the model of Dickson and Whitfield (1981) for the major components and the models of Turner and Whitfield (1987) and Sharma and Millero (1988) for copper. For each ion-pair or 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 et al., 1981). The redox potential of the solutions was calculated from the empirical relation pE=17.6-pH (Baas-Becking et al., 1960). The thermodynamic stability constants and concentration products at the ionic strength of the saltwater solution for all copper species included in the model are given in Table 1. Case specific input includes the total concentrations of the metals and ligands in the solution, the free hydrogen concentration (pH), redox potential (pE), temperature and the gas and solid phases that are maintained in equilibrium with the solution.

Metal analysis

Copper was measured by Graphite Furnace Atomic Absorption Spectrophotometry using a Perkin-Elmer 703 Spectrophotometer fitted with a Heated Graphite Atomiser HGA-500 and a deuterium arc background corrector. The method of stabilised temperature platform atomisation was used (Slavin et al., 1983). Saltwater solutions were diluted five times with water. To this solution a one tenth volume of concentrated nitric acid was added. The solutions were analysed directly against matrix matched

calibration standards. Biological material was dissolved with concentrated nitric acid in a microwave oven. The resulting digest was diluted with water and analysed against 10 % nitric acid calibration standards (Blust et al., 1988).

Statistical analysis

All sets of data were tested for homoscedasticity by the log-anova test for homogeneity of variances and for normality by the Kolmogorov-Smirnov test for goodness of fit. Analysis of variance, single and multiple linear regression and non-linear regression methods were used for analysing the data. The T-method was used to make multiple comparisons among pairs of means. Significance levels of tests are indicated by asterisks according to the following probability ranges (*=$0.05 \geq P > 0.01$, **=$0.01 \geq P > 0.001$, ***=$P \leq 0.001$). Statistical methods are as outlined in Statistical Methods, (Snedecor and Cochran, 1980) and Primer of Applied Regression and Analysis of Variance, (Glantz and Slinker, 1990).

RESULTS

The chemical speciation of copper

The complexation of copper in a chemically defined saltwater solution that does not contain organic ligands is controlled by the concentration of hydroxide and carbonate. The concentration of hydroxide is determined by the pH of the solution. The concentration of carbonate is determined by the partial pressure of CO_2 in the atmosphere and the pH of the solution. Changing the partial pressure of CO_2 in the atmosphere makes it possible to alter the carbonate concentration without changing the pH. Measurement of the pH and total dissolved carbon dioxide concentrations in the solutions suffices for the calculation of the carbonate speciation in a chemically defined solution. The results of the measurements concerning the effects of pH and CO_2 on the total dissolved carbon dioxide concentration in the solutions and the effects on the chemical speciation of copper are summarised in Fig. 1 - 4. In a basic environment copper is complexed with hydroxide and carbonate but towards acidic conditions the cupric ion and some covarying inorganic species (i.e. $CuCl^+$ and $CuSO_4^0$), become increasingly more important. For the same total dissolved carbon dioxide concentration, carbonate complexing is important in a basic, but not in an acidic environment.

The uptake of copper in brine shrimp

The results summarised in Fig 5 show that the uptake of copper in the brine shrimp increases linearly with the total concentration of the metal in the solution and that it is much higher in basic and neutral than in acid environments. Apparently copper uptake does not saturate over the copper concentration ranges used (pH=6, Cu_T=10-100 mmol l^{-1}, pH=7 and 8, Cu_T=1-10 mmol l^{-1}). Over the total copper and pH range tested, copper uptake in brine shrimp is positively correlated with the concentrations of Cu^{2+} ($r=0.66^{***}$, n=31), $CuOH^+$ ($r=0.93^{***}$), and $CuHCO3^+$ ($r=0.76^{***}$).
To determine the effect of complexation of copper with hydroxide and carbonate on the availability of copper at different pH values, subsequent experiments were conducted at one total copper concentration of 5 mmol l^{-1}, three different pH values, 6.0, 7.0 and 8.0, and different carbonate concentrations. The results summarised in Fig 6 show that the uptake of copper increases with pH. Over the pH range tested, copper uptake in brine shrimp is positively correlated with the concentrations of $CuOH^+$, ($r=0.79^{*}$, n=8), $Cu(OH)_{20}$, ($r=0.88^{**}$), $CuCO_3^+$, ($r=0.88^{**}$) and $CuHCO_3^+$, ($r=0.82^{*}$). The results summarised in Fig. 7 show that the uptake of copper in the brine shrimp decreases with the complexation of copper with carbonate. This effect is most pronounced at pH 8.0 and 7.0. At pH 6.0 complexation of copper with carbonate is negligible, though a moderate decrease in copper uptake with increasing total carbon dioxide is observed. Over the pH range tested, copper uptake in brine shrimp is positively correlated with the concentrations of $CuOH^+$, ($r=0.98^{***}$, n=21) and $Cu(OH)_2^0$, ($r=0.98^{***}$).
Analysis of the pooled results shows that overall, only the concentrations of the free cupric ion and the two cupric hydroxide species are positively correlated with the uptake of copper in brine shrimp. The results of the correlation analysis are summarised in Table 2.

Linear and non-linear modelling of copper uptake

Multiple linear regression analysis of the pooled results has been used to explain the variation in copper uptake as the result of changes in the speciation of the metal on the uptake of a limited number of metal species. The linear equation for metal uptake assumes the form of a sum of terms. Each term is the product of a regression coefficient (RC) and the concentration of a metal species (MS). Taking into account the amount of copper already present in clean organisms (M_0), the equation for the total amount of copper in the organism (M_T), becomes, $M_T=M_0+RC_1*MS_1+RC_2*MS_2+....$ Using this equation the analysis of the pooled results shows that most of the variation in copper uptake is explained when the free cupric ion and the two cupric hydroxide species are considered to be the metal species that are taken up. The results of the multiple linear regression analysis are summarised in Table 3a and Fig 8a. It has to be noted that the two copper hydroxide species considered show a high degree of collinearity which implies that their separate contribution cannot be assessed in an unambiguous way.

Multiple non-linear regression analysis of the pooled results has been used to explain the variation in copper uptake as the combined effect of changes in the speciation of the metal and an effect of changes in the hydrogen ion concentration on the uptake of a limited number of metal species. The effect of the hydrogen ions on metal uptake is expressed as an effect on the ionisation of the metal transport system. The non-linear equation for metal uptake assumes the form of an equilibrium relation between the concentrations of the hydrogen ion, the copper species taken up and the ionised carrier. The fraction of ionised carrier is determined by the acid dissociation constant (pK) of the ligand and the pH of the solution (i.e. $1/1+10^{(pK-pH)}$). Metal uptake is then proportional to the ratio of the fraction of available copper and the fraction of ionised carrier. To relate this ratio to metal uptake, it is necessary to introduce a coefficient (CF) which relates the sum of the concentrations of the metal species (SMS) in the solution to the concentration of copper that is taken up in the shrimps. Taking into account the amount of copper already present in clean organisms (M_0), the equation for the total amount of copper in the organism (M_T), becomes, $M_T=M_0+CF*SMS*1/(1+10^{(pK-pH)})$. Using this equation the analysis of the pooled results shows that most of the variation in copper uptake is explained when the acid dissociation constant of the transport system is a value of 8 and the cupric ion and the two cupric hydroxide species are considered to be the metal species that are taken up. The results of the multiple non-linear regression analysis are summarised in Table 3b and Fig 8b.

Table 1: Thermodynamic stability constants and concentration products for copper species. K: thermodynamic stability constant; Q: concentration product at ionic strength I=0.614.

Species	log K	log Q
Cu^{2+}	-	-
$CuCl^{+}$	0.40	-0.02
$CuSO_4^0$	2.36	1.32
$CuOH^{+}$	6.01	5.61
$Cu(OH)_2^0$	11.75	10.83
$CuCO_3^0$	6.74	5.73
$CuHCO_3^{+}$	12.25	10.70
$Cu(CO_3)_2^{2}$	10.57	9.27
Cu^{+}	2.61	2.12
$CuCl^0$	5.72	4.85
$CuCl_2^-$	8.03	7.21
$CuCl_3^{2-}$	7.36	6.96

Table 2: Copper speciation and uptake in brine shrimp, pooled data correlation matrix (n=58). ^{ns}Not significant; *:$0.05 \geq p > 0.01$; **:$0.01 \geq p > 0.001$; ***:$p \leq 0.001$.

	$Cu^{2+}{}_1$	$CuOH^+$	$Cu(OH)_2^0$	$CuCO_3^0$	$CuHCO_3^+$	$Cu(CO_3)_2^{2-}$
Uptake	0.283^{*}	0.958^{***}	0.810^{***}	0.257^{ns}	-0.061^{ns}	0.131^{ns}
$Cu^{2+}{}_1$	-	0.171^{ns}	-0.242^{ns}	-0.309^{*}	0.549^{***}	-0.241^{ns}
$CuOH^+$	-	-	0.823^{***}	0.285^{*}	-0.094^{ns}	0.080^{ns}
$Cu(OH)_2^0$	-	-	-	0.520^{***}	-0.279^{*}	0.328^{*}
$CuCO_3^0$	-	-	-	-	-0.102^{ns}	0.886^{***}
$CuHCO_3^+$	-	-	-	-	-	-0.182^{ns}

[1] Cu^{2+}, $CuCl^+$, $CuSO_4^0$ vary together

Table 3: Copper uptake in brine shrimp, pooled data linear regression statistics ($R=0.979^{***}$, n=58)

Variable	B[1]	SE[2]	L_1[3]	L_2
Intercept	1.276***	0.091	1.094	1.458
Cu^{2+}	0.041***	0.006	0.030	0.053
$CuOH^+$	6.032***	0.678	4.673	7.391
$Cu(OH)_2^0$	12.895***	2.152	8.580	17.210

[1]partial regression coefficient
[2]standard error of partial regression coefficient
[3]95 % confidence for partial regression coefficients

Table 4: Copper uptake in brine shrimp, pooled data non-linear regression statistics ($R=0.961^{***}$, n=58).

Variable	B[1]	SE[2]	L_1[3]	L_2
Intercept	1.175***	0.123	0.930	1.421
Factor	7.856***	0.712	6.429	9.283
pK	8.036***	0.063	7.911	8.161

[1]partial regression coefficient
[2]standard error of partial regression coefficient
[3]95 % confidence for partial regression coefficients

DISCUSSION

The results concerning the effect of different total copper concentrations on copper availability show that metal uptake is linear over the pH and concentration range used. This means that copper uptake is either, i) a simple diffusion process with the net rate of copper transport directly proportional to the concentration difference of the copper species across the solution-body interface or, ii) a facilitated diffusion process, with the rate of transport limited by the number of transport sites and the maximum velocity at which they can function. In the latter case, uptake will appear linear until a critical concentration of copper is reached where the transport system becomes saturated. Within the range of total copper concentrations used, saturation of metal uptake in brine shrimp is not observed. However, since the total concentration of copper which can be dissolved is constrained by the limited solubility of the cupric hydroxide and carbonate species, it cannot be concluded from these data whether or not the transport of copper across the solution-body interface is a simple or facilitated diffusion process.

The results concerning the effect of hydrogen ions and inorganic complexing show that the uptake of copper in brine shrimp is not a simply related to the concentration of the cupric ion in the solution. Indeed, over the experimental pH range, the concentration of the cupric ion decreases sharply and yet a pronounced increase in the biological availability of copper is observed. This either means that, i) one or more of the inorganic complexes which prevail in basic conditions are available to the brine shrimp and/or, ii) changes in the pH of the medium alter the metal uptake process, e.g. protonation of binding sites involved in the sequestration and transportation of copper. That is, the biological availability of the free cupric ion and/or the other cupric ion species which are taken up increases with pH. Since changes in the concentration of the cupric ion and the concentrations of the cupric hydroxide species are pH dependent, it is not experimentally possible to separate the effect of pH on the speciation of the metal from the effect of pH on the uptake process in an unambiguous way. Therefore, the observation that most of the variation in the availability of copper is explained by either a multiple linear or non-linear regression model that includes the concentrations of the cupric ion and the cupric hydroxide species as independent variables, does not necessarily mean that there is also a true functional relationship between the concentration of all of these species and the availability of copper to the brine shrimp. It is however clear, that changes in the hydrogen ion concentration have an important effect on the uptake of copper in the brine shrimp and that the observed variation in the uptake of copper is not explained by the changes in the concentration of the free cupric ion alone.

The binding sites in a transport system are often weak acids and the proportion of uncharged conjugate acid and charged conjugate base determines the number of sites available for metal binding. The proportion of conjugate base and conjugate acid is determined by the acid dissociation constant of the site. In this case, the amount of copper that is taken up, is proportional to the product of the proportion of conjugate base of the site and the sum of the concentrations of the metal species taken up. Considering the cupric ion and the cupric hydroxide species as the most important biologically available species, non-linear regression analysis of the data results in a calculated pK value for the empirical binding site of 8.0.

Considering both the chemical and biological aspects of the metal uptake process, a conceptual model that relates metal speciation to metal availability can be built, (Simkiss and Taylor, 1989, Viarengo, 1989). Changes in the speciation of a metal can have

important effects on the movement of the metal in the vincinity and across the solution-body interface. Ions and other polar species in solution are not bare because of interactions with the water molecules. The strength of these interactions with the water molecules increases with the charge density of the bare species, i.e. the ratio of the charge and the size of the species. The organisation of water molecules around the species results in the formation of a solvation sphere. This is a sphere of water molecules which are closely associated to the species and move around with it as one single moving entity. The bound water molecules contribute to the size of the species and it is the number of layers of solvent molecules which determines the effective size of the solvated species. The effective size of the solvated species determines the mobility of the species in the solvent and hence its diffusion rate, (Burgess 1988).
Basically, the solution-body interface has the structure of a negatively charged membrane which is bounded by an electrical double layer. The membrane is a fluid mosaic of proteins in a double layer of phospholipids. Some of these proteins function as carriers that bind solute molecules and carry them across the membrane. Other proteins build channels that perforate the membrane, creating diffusion paths whose resistance is less than that of the membrane itself. The electrical double layer is a sheet of solvated anions and cations with an excess of positive charges to balance the negative surface charge of the membrane. In the bulk phase fluid convection carries the solute species to the proximity of the interface. The solute crosses the electrical double layer by passive diffusion. The flux of a non-electrolyte is determined by the concentration gradient or chemical potential of the solute across the interface. Charged species are subject to electrical forces and the driving force for electrolyte transport is determined by the electrochemical potential gradient rather than by the chemical potential alone (Blank, 1987, Cevc, 1990)
Since the solution-body interface is basically a hydrophobic structure charged and neutral polar species can not permeate the interface by dissolving in the phospholipid bilayer membrane and diffusing across to any extent. There is an enormous interfacial resistance to the transport of polar solutes through a highly apolar phase such as the hydrocarbon core of a biological membrane. It is therefore excluded that metal uptake is a simple diffusion process. This invokes the presence of transport systems that facilitate the translocation of the metal species across the interface (Eisenman and Horn, 1983, Wills and Zweifach, 1987). The strength of the interaction between a metal species and a carrier or channel binding site depends on the nature of the metal species and the binding site and is greater when the interacting sites can approach more closely. The results concerning the effect of inorganic complexing on the uptake of copper presented here together with other studies concerning the effect of inorganic and organic complexing have shown that the effect of complexation on the uptake and toxicity of copper in aquatic organisms does not depend on the stability constant or concentration product of the complexes formed (Sunda and Guillard, 1976, Andrew et al., 1977, Dodge and Theis, 1979, Guy and Kean, 1980, Knezovich et al., 1981, Zamuda and Sunda, 1982, Borgmann and Ralph, 1983, Buckley, 1983, Ahsanullah and Florence, 1984, Zamuda et al., 1985, Blust et al., 1986, Florence and Stauber, 1986, Starodub et al., 1987). This indicates that the translocation of the metal across the solution-body interface only involves weak interactions. Hence, the effect of changes in the speciation of the metal on the uptake process appears the result of i) changes in the concentration of the copper species for which the transport system is selective by charge and/or size and ii) changes in the selectivity and/or activity of the transport system by changes in the hydrogen ion concentration.

CONCLUSIONS

The results presented have shown that changes in the chemical speciation of copper on the uptake of copper in brine shrimp are not directly related to the concentration of the cupric ion alone when changes in the concentration of the hydrogen ion are considered. The observed variations in the uptake of copper are, however, reasonably well described by either a linear or non-linear model that considers the cupric ion and the cupric hydroxide complexes as the most important biologically available species. Since the transport of the metal species across the solution-body interface requires the presence of a facilitating system, the non-linear model for copper uptake appears to be the more feasible integration of the effects of hydrogen ions and inorganic complexation on the availablity of the metal to the brine shrimp.

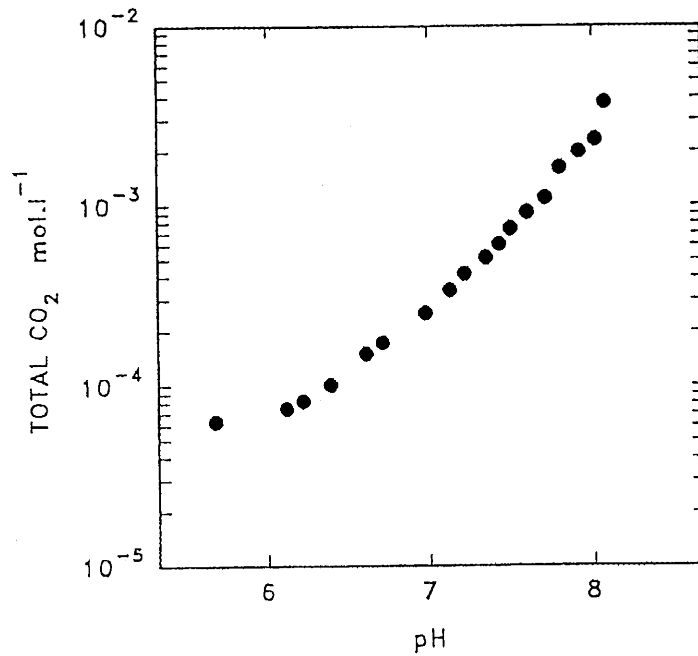

Fig. 1: Measured total dissolved carbon dioxide concentration as a function of pH in a saltwater solution in equilibrium with air (sal=35^0/$_{00}$, $pO_2=10^{-0.69}$ atm, $pCO_2=10^{-3.48}$ atm).

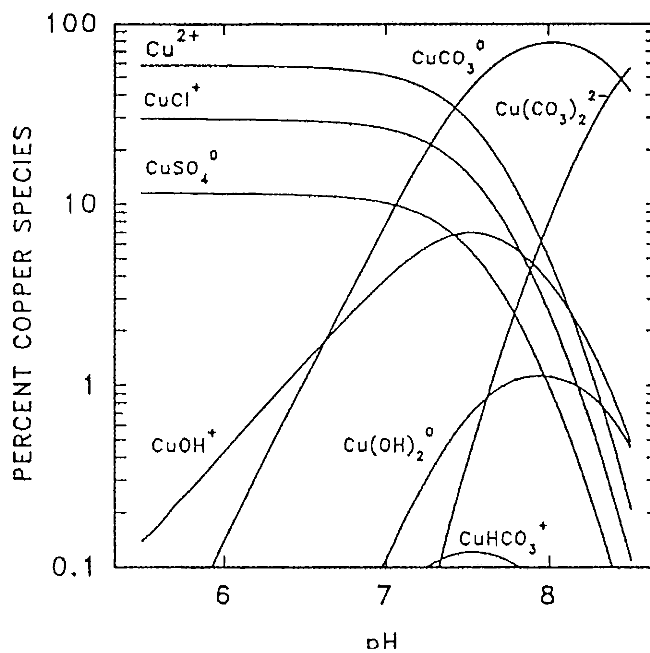

Fig. 2: Speciation model of copper as a function of pH in a saltwater solution in equilibrium with air (sal=35^0/$_{00}$, $pO_2=10^{-0.69}$ atm, $pCO_2=10^{-3.48}$ atm).

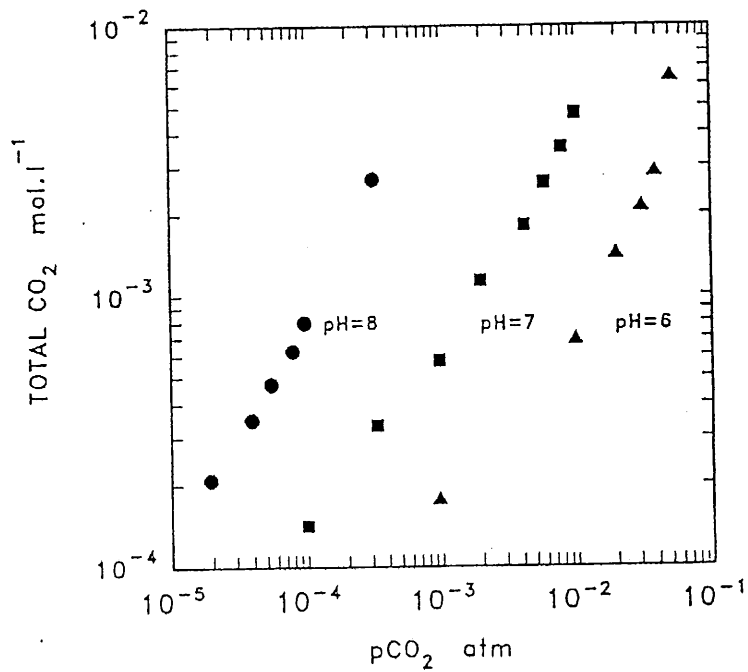

Fig. 3: Measured total dissolved carbon dioxide concentration as a function of pH and pCO_2 in saltwater solution (sal=$35^0/_{00}$, $pO_2=10^{-0.69}$ atm).

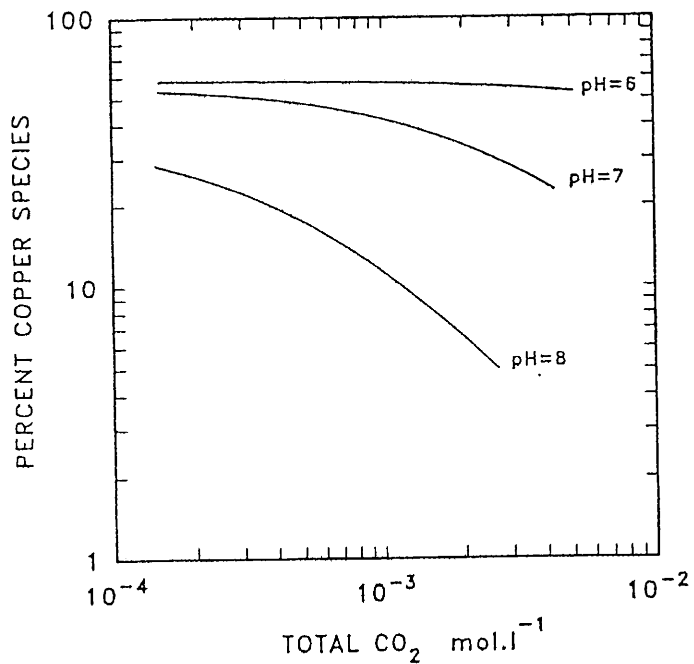

Fig. 4: Speciation model of copper as a function of pH and pCO_2 in saltwater solution (sal=$35^0/_{00}$, $pO_2=10^{-0.69}$ atm). For clarity only the concentration of the cupric ion is shown.

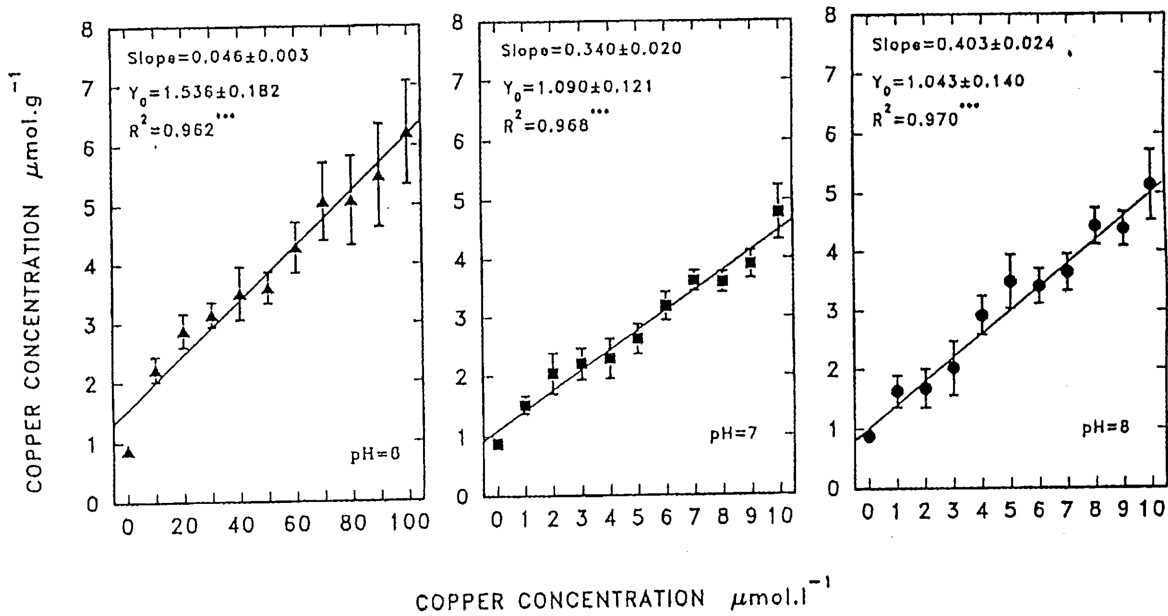

Fig. 5: Uptake of copper by *Artemia franciscana* at pH=6.0, 7.0 and 8.0 as a function of the total copper concentration over a 3 hour period (sal=35^0/$_{00}$, $pO_2=10^{-0.69}$ atm, $pCO_2=10^{-3.48}$ atm). Means of 5 replicates with standard deviation are significantly different, $p < 0.01$.

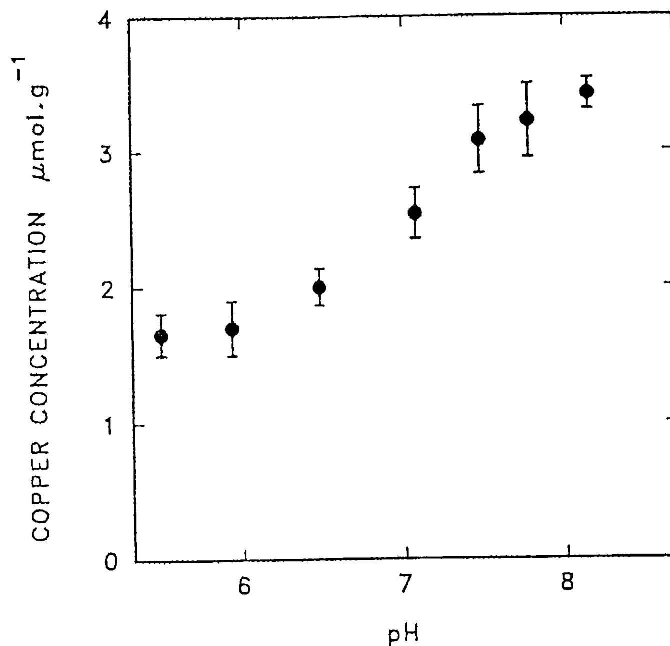

Fig. 6: Uptake of copper by *Artemia franciscana* as a function of pH over a 3 hour period, exposed to a copper concentration of 5.10^{-6} mol l^{-1} (sal=35^0/$_{00}$, $pO_2=10^{-0.69}$ atm, $pCO_2=10^{-3.48}$ atm). Means of 5 replicates with standard deviation are significantly different, $p<0.01$.

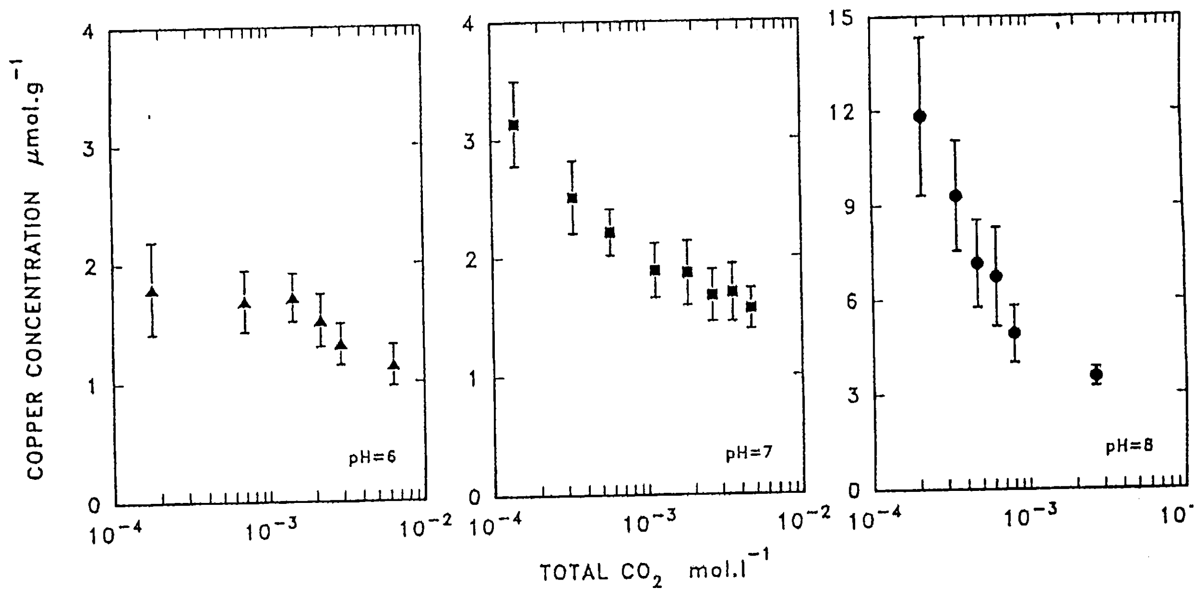

Fig. 7: Uptake of copper by *Artemia franciscana* at pH=6.0, 7.0 and 8.0 as a function of the total dissolved carbon dioxide concentration over a 3 hour period, exposed to a copper concentration of 5.10^{-6} mol l^{-1} (sal=35^0/$_{00}$, $pO_2=10^{-0.69}$ atm, $pCO_2=10^{-3.48}$ atm). Means of 5 replicates with standard deviation are significantly different, $p<0.01$.

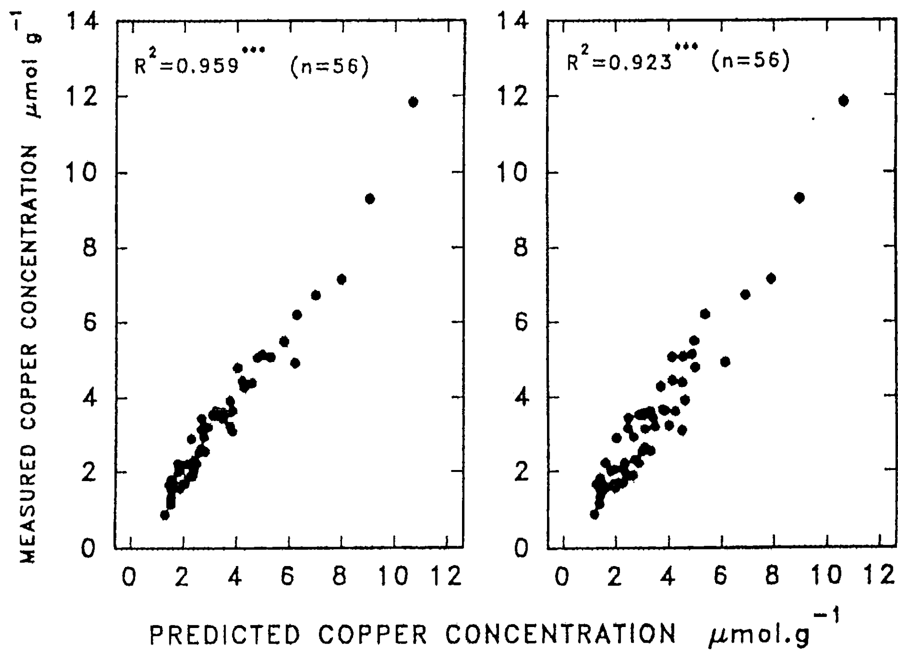

Fig. 8: Predicted versus measured concentrations of copper in *Artemia franciscana* for (a) the linear and (b) non-linear model.

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Effect of total cadmium and organic complexing on the uptake of cadmium by the brine shrimp, *Artemia franciscana*

INTRODUCTION

The uptake of cadmium by aquatic organisms is strongly dependent on the chemical speciation of the metal in the environment. The chemical speciation of an element is the distribution of the element among various chemical forms which together make up the total concentration of the element in the system. Metal complexation involves the formation of several kinds of species which differ in size, charge and stability. In the aquatic environment an organism is not exposed to a metal as one single entity, but to a variety of metal species. The differences in the availability of all these metal species to biological systems make it difficult to determine the availability of metals to aquatic organisms (Batley, 1989; Furness and Rainbow, 1990; Sörensen, 1991).
Metal uptake by aquatic organisms is generally described as involving an initial interaction of the free metal ion with a transport system, either a channel or a carrier, that takes the metal across the external membranes of the exchange surfaces. Transport through alternative transporting systems can occur whenever the metal-ligand complex resembles the original substrate molecule, (Williams, 1981; Cousins, 1985; Simkiss and Taylor, 1989).
Complexation with water soluble ligands generally decreases the uptake of cadmium by aquatic organisms by reducing the concentration of the free metal ion in the solution. Consequently, variations in cadmium uptake are usually better explained on the basis of changes in free metal ion, rather than total metal concentrations (Sunda et al., 1978; Rainbow et al., 1980; Pärt and Wikmark, 1984; Jenkins and Sanders, 1986; Blust et al., 1992). Even after taken into consideration the effect of complexation on the free metal ion concentration, uptake of cadmium may still show considerable variability. Some of this variation may be explained by processes such as the binding of metal-ligand complexes to external body surfaces, the direct uptake of the metal-ligand complex or the role of metal-ligand coordination kinetics. (George and Coombs, 1977; Poldoski, 1979, Muramoto, 1981; Dressing et al., 1982; Winner and Gauss, 1986; Hering and Morel, 1990).
Metal complexation may thus influence metal uptake in different ways depending on the prevailing mode of transport and the ligands present in the solution. In the present work the effect of complexation by five different organic ligands on the uptake of cadmium by the brine shrimp, *Artemia franciscana* has been studied. These complexes were selected on the basis of the thermodynamic stabilities and formal charges of the cadmium complexes formed. Cadmium uptake was studied in solutions of differing complexation capacity to reveal the role of the free metal ion and other species in determining metal availability in relation to the concentration and type of ligand present in the solution.

MATERIALS AND METHODS

Brine shrimp

Dried *Artemia franciscana* cysts from Great Salt Lake, Utah, USA were purchased from San Francisco Bay Brand, Newark, CA, USA. Cysts were hatched in a funnel-shaped plastic container filled with synthetic seawater (Wiegandt, Krefeld, Germany), and aerated from the bottom. Hatching cyst density was 5 g l^{-1}. The hatching suspension was illuminated by a fluorescent light tube. Artemia nauplii were harvested after 36 hours. The larvae were grown from nauplii to adult in 150 l plastic rectangular air-water lift operated race-ways filled with synthetic seawater. Brine shrimp were fed with a suspension of the dried algae Spirulina. Animals reached maturity after 3 to 4 weeks and were used before they were 8 weeks old. The methods for intensive culturing of brine shrimp have been described by Sorgeloos et al. (1983).

Experimental procedures

In this study of the uptake of cadmium by the brine shrimp we have used the static test design. The accumulation of cadmium during three hours of exposure was used as a measure for the biological availability of the metal. Experiments were conducted in a thermostated room at 25.0$\pm$0.5 °C. One day before an experiment was run adult brine shrimp were collected from a batch culture and transferred to a defined saltwater solution to empty their gut. During this day the animals were transferred for one hour to a saltwater solution containing 1mM of 8-hydroxyquinoline-5-sulfonic acid, to remove metal bound to the external surfaces of the animal. The composition of 1 l of the chemically-defined, saltwater solution with a salinity of 35 °/°° was 23.50g NaCl, 4.00g Na_2SO_4, 0.68g KCl, 0.196g $NaHCO_3$, 1.47g $CaCl_2.2H_2O$, 10.78g, $MgCl_2.6H_2O$ and 0.026g H_3BO_3. The medium was prepared by dissolving the seven analytical grade products (Merck p.a.) in deionised water. A dispersion of 0.1 mmol l^{-1} manganese dioxide was added to the seawater to remove metals present in the analytical grade reagents. After an equilibration period of 24 hours, the dispersion was filtered through a 0.2 µm membrane filter to remove the manganese dioxide from the solution (Van den Berg and Kramer, 1979).
The pH of the solutions was adjusted with HCl or NaOH as required and the media were aerated to promote equilibration of gases with the atmosphere. The dissolved oxygen concentration, the total dissolved carbonate concentration, the hydrogen ion concentration and the redox potential were measured to ensure that equilibrium conditions had been established. Dissolved oxygen was measured with an oxygen electrode (WTW OX191/EO90). Total dissolved carbon dioxide was measured with a gas sensing CO_2 electrode (Ingold 152323000), after acidification of the water sample (pH<4.8) in a sealed measuring vessel. The hydrogen ion concentration was measured with a glass electrode (Ingold 104573002), and pH values expressed on a free hydrogen ion scale (Millero, 1986). Redox potentials were measured with a wire type platinum electrode (Ingold, 105003077). Cadmium nitrate was added to the test solutions from a 0.1 mol l^{-1} cadmium ion stock. In a first experiment the uptake of cadmium was followed over a six hour period in solutions containing from 100 to 500 $\mu mol.l^{-1}$ of cadmium. The amount of cadmium present in the digestive tract or

reversibly bound to the body surface was measured in organisms that had been exposed to 100 $\mu mol.l^{-1}$ of cadmium over a three hour period. Organisms were transferred to a clean saltwater solution at the end of the uptake experiments to allow the elimination of the metal. Depuration of cadmium was followed over a six hour period. In half of these experiments, 1 $mmol.l^{-1}$ of the strong metal-ligand 8-hydroxyquinoline-5-sulfonic acid was added to the saltwater solution to promote removal of cadmium bound to the body surfaces. In a second experiment, uptake of cadmium was followed over a three hour period in solutions containing from 10 to 100 $\mu mol.l^{-1}$ of cadmium. In a third experiment uptake of cadmium from a solution containing 100 $\mu mol.l^{-1}$ cadmium was followed over a three hour period as a function of the concentration of one of five organic ligands selected on the basis of their affinity for cadmium and the formal charges of the complexes formed. The five organic ligands were EDTA (ethylenedinitrilotetraacetic acid), NTA (nitrilotriacetic acid), histidine, glycine and citrate. The ligands were added to the solutions from 1-100 $mmol.l^{-1}$ stocks prepared in defined saltwater. In all these uptake experiments the amount of cadmium that could be removed was measured by transferring the organisms for one hour to a clean saltwater solution containing 1 $mmol.l^{-1}$ of 8-hydroxyquinoline-5-sulfonic acid.
Test solutions were prepared one day before an experiment was run to allow metal-ligand speciation equilibration in the solution. The experiments were carried out in 0.5 l plastic beakers. Just before an experiment started about 50 animals were collected on a 250 µm screen, rinsed with clean medium and transferred to a beaker. After 180 minutes the beaker was removed and a few ml of the test solution sampled in a plastic vial and stored frozen at -20 °C until analysed for cadmium. The beaker was subsequently emptied over a 250 µm screen. The collected animals were rinsed with deionised water and divided into five plastic vials, dried for 24 hours at 60 °C and stored in a desiccation box until analised for cadmium. This procedure was repeated five times using animals from separate cultures and newly prepared media and reagents. The dissolved oxygen concentration, the total dissolved carbonate concentration, the hydrogen ion concentration, the redox potential and the total dissolved cadmium concentration were measured at the beginning and end of an experiment. Generally, all measured values remained within 10 % of the initial values.

Chemical modelling and ion selective potentiometry

The equilibrium concentrations of the chemical species considered were calculated using the computer program SOLUTION (Blust et al., unpublished) an adaptation of the program COMPLEX (Ginzburg, 1976). This speciation model allows the calculation of the composition of solutions in equilibrium with gas and solid phases. A thermodynamic stability constant data base was built which is based on the data of Dickson and Whitfield (1981) for the major components and the data of Smith and Martell (1976, 1989) and Martell and Smith (1974, 1977, 1982) for cadmium. For each ion-pair or 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 et al., 1981). The redox potential of the solutions was calculated from the empirical relation pE=17.6-pH (Baas-Becking et al., 1960). Case specific input comprises the total concentrations of the metals and ligands in the solution, the free hydrogen concentration (pH), redox potential (pE),

temperature and the gas and solid phases that are maintained in equilibrium with the solution. The results of the speciation calculations were verified by measuring the free cadmium ion activities with a cadmium ion electrode (Orion Model 94-48). The electrode was calibrated for the range 0.1-10,000 µM free cadmium and 0.5-1.0 M ionic strength. Sodium perchlorate was used as the background electrolyte for these calibrations. Since the cadmium ion electrode has a detection limit of 0.1 $\mu mol.l^{-1}$ free cadmium ion, the total cadmium concentration was 1 $mmol.l^{-1}$ in these test. Measured and calculated free cadmium ion concentrations did not deviate by more than 10 % for any of the organic ligands tested.

Metal analysis

Cadmium was measured by Graphite Furnace Atomic Absorption Spectrophotometry using a Perkin-Elmer 703 Spectrophotometer fitted with a Heated Graphite Atomiser HGA-500 and a deuterium arc background corrector. The method of stabilised temperature platform atomisation was used (Slavin et al., 1983). Biological material was dissolved with concentrated nitric acid in a microwave oven and diluted with deionised water to a 10 % nitric acid solution. Saltwater solutions were diluted ten times with a 10 % nitric acid solution to decrease the salinity. Matrix modificator (200 µg $(NH_4)_2HPO_4$ + 20 µg $Mg(NO_3)_2$ per sample) was added to the solutions and they were analised against matrix matched calibration standards (Blust et al., 1988).

Statistical analysis

All sets of data were tested for homoscedasticity by the log-anova test for homogeneity of variances and for normality by the Kolmogorov-Smirnov test for goodness of fit. Analysis of variance, correlation and regression were used for analysing the data. The T-method was used to make multiple comparisons among pairs of means with homogeneous variances. The Games and Howell method was used to make multiple comparisons among pairs of means with heterogeneous variances. Significance levels of tests are indicated by asterisks according to the following probability ranges (*=$0.05 \geq P > 0.01$, **=$0.01 \geq P > 0.001$, ***=$P \leq 0.001$). Statistical methods are used as outlined by Sokal and Rohlf (1981).

RESULTS

Effect of cadmium exposure and elimination time on cadmium uptake

In a first experiment organisms were exposed to different cadmium concentrations ranging from 10 to 100 $\mu mol.l^{-1}$ and uptake was followed over a 6 hours period. The amount of cadmium that was reversibly bound to the body surfaces or contained in the gut lumen was measured by transferring cadmium exposed organisms to either a clean saltwater solution or a clean saltwater solution to which 1 $mmol.l^{-1}$ of the strong metal ligand 8-hydroxyquinoline-5-sulfonic acid was added and elimination of cadmium followed over a 6 hours period. The results of the uptake experiments conducted in a solution containing 100 $\mu mol.l^{-1}$ cadmium are shown in Fig. 1. The concentration of cadmium in the organisms increased linearly in function of the concentration and time of exposure. Transfer of the organisms to a clean solution showed that elimination of directly exchangeable cadmium was complete within the first ten minutes after transfer. Elimination of cadmium was proportional to the concentration of cadmium in the solution. There were no significant differences in the body concentrations of cadmium among groups taken between 10 and 360 minutes after transfer. Addition of the strong metal ligand 8-hydroxyquinoline-5-sulfonic acid had no significant effect on the removal of cadmium from the body. In subsequent experiments the rate of cadmium uptake was determined by following accumulation for six hours after which the organisms were transferred for one hour to a clean solution to remove directly exchangeable cadmium from the body.

Effect of cadmium exposure concentration on cadmium uptake

In a second experiment the effect of a much broader range of cadmium concentrations, ranging from 0 to 1000 $\mu mol.l^{-1}$, on the uptake of cadmium by the brine shrimp was determined. The results of these experiments are shown in Fig 2. Over the wide range of cadmium concentrations the uptake of cadmium did not remain linear in function of time and concentration. In the higher cadmium concentration exposure groups the accumulation of cadmium by the organisms leveled off within the 6 hour exposure period. At these very high levels of cadmium in the solution the cadmium concentration factor, i.e. the ratio of the cadmium concentration in the organism and the cadmium concentration in the water, was close to unity. This indicates that under these extreme conditions all internal binding sites are saturated and the cadmium in the shrimp is in exchange equilibrium with the cadmium in the water.

Effect of cadmium complexation on cadmium uptake

In a third experiment the effect of different organic ligands on the uptake of cadmium by the organisms was followed over a six hour period in solutions containing 100 $\mu mol.l^{-1}$ of cadmium and different concentrations of organic ligand.
Five different ligands were used, bracketing a wide range of metal-ligand stability constants (EDTA > NTA > histidine > glycine > citrate). The inorganic and organic species considered in modelling the complexation chemistry of the metal, together with their thermodynamic and conditional stability constants are given in Table 1. Chemical equilibrium calculations and measurements of free cadmium ion activities

showed that less than three percent of the total amount of cadmium present exists as the free metal ion in the saltwater solution. Addition of an organic ligand to the solution results in a proportional decrease of all inorganic species and the formation of several organic complexes. The extent of cadmium complexation has been modelled for one fixed cadmium concentration of 100 $\mu mol.l^{-1}$ and different organic ligand concentrations ranging from 1 to 100000 $\mu mol.l^{-1}$. The results presented in Fig 3 show that for the strongest ligand, EDTA, complexation is nearly complete at a 1:1 molar ratio of metal and ligand. NTA, histidine and glycine have intermediate affinities for the cadmium ion under the experimental conditions. For the weakest ligand, citrate, complexation is not even complete at a metal to ligand ratio of 1:1000.

The results of the speciation calculations were used to establish the experimental conditions for determining the effect of organic complexation on cadmium uptake by brine shrimp. Each of the five ligands considered were tested for one fixed cadmium concentration of 100 µmol.l-1 and twenty different ligand concentrations, bracketing a concentration scale of two orders of magnitude. The results of the experiments concerning the effect of organic complexing on the uptake of cadmium by the brine shrimp are given in Fig 4. In all cases cadmium uptake is decreased upon complexation of the free metal ion by the ligand. A direct relation between the change in the free cadmium ion concentration and the change in cadmium uptake is not always apparent over the entire range of free metal ion and ligand concentrations. In case of histidine and citrate uptake of cadmium decreases progressively with the decrease in the free cadmium ion concentration. In case of EDTA, NTA and glycine, uptake of cadmium appears independent of the free cadmium ion activity until a certain level is reached below which uptake of cadmium decreases progressively with the decrease in the free metal ion activity. The calculations of the correlations between the free cadmium ion activity and the rate of cadmium uptake presented in Table 2 show that there is a high degree of linear association between the two variables for all five ligands. Depending on the ligand considered, between 70 % and 90 % of the variation in cadmium uptake is explained by a simple linear relation, that includes the free cadmium ion activity as the single independent variable. The results indicate that for the same free cadmium ion activity, the rate of cadmium uptake from solution is higher in the presence of organic ligands when compared to a pure inorganic solution. The effect is significant for all five ligands tested but most pronounced in case of the strongest ligand. The results of the correlation analysis also show that there is never a high degree of linear association between the concentration of the metal-ligand complexes formed and cadmium uptake for all five ligands. The construction of more complex models for the uptake of cadmium which consider the free cadmium ion and/or one or more of the ligand complexes do not decrease the residual amount of varation in cadmium uptake significantly.

Table 1: Thermodynamic and conditional stability constants for the cadmium species considered in the chemical speciation model. K: thermodynamic stability constant; Q: conditional stability constant in the saltwater solution.

Species	Log K	Log Q
Cd^{2+}	--	--
$CdOH^{+}$	3.91	3.32
$Cd(OH)_2^{0}$	7.64	6.74
$CdCl^{+}$	1.97	1.36
$CdCl_2^{0}$	2.59	1.70
$CdCl_3^{-}$	2.40	1.48
$CdCl_4^{2-}$	1.47	1.34
$CdSO_4^{0}$	2.45	1.07
$Cd(SO_4)_2^{2-}$	3.44	1.89
$CdCO_3^{0}$	4.35	2.97
$CdCIT^{-}$	5.05	3.57
$CdHCIT^{0}$	9.25	7.74
$CdOHCIT^{2-}$	11.16	9.92
$Cd(CIT)_2^{4-}$	6.25	5.51
$CdGLY^{-}$	4.68	4.12
$Cd(GLY)_2^{0}$	8.36	7.53
$CdHIS^{+}$	6.13	5.67
$CdHHIS^{2+}$	11.33	11.33
$Cd(HIS)_2^{0}$	10.55	9.86
$CdNTA^{-}$	11.25	9.36
$CdOHNTA^{2-}$	13.56	12.32
$Cd(NTA)_2^{4-}$	15.46	14.39
$CdEDTA^{2-}$	18.27	16.50
$CdHEDTA^{-}$	21.59	19.20
$CdOHEDTA^{3-}$	18.57	17.21

Table 2: Coefficients of determination (r^2) for linear association between cadmium species activities in solution and rate of cadmium uptake by brine shrimp (n=95-100). The sign in front of the determination coefficient designates whether the correlation is positive or negative. Significance levels are indicated by asterisks according to the following probability ranges: * $0.05 \geq P > 0.01$, ** $0.01 \geq P > 0.001$, *** $P \leq 0.001$.

Cd^{2+}	$CdCIT^{-}$	$CdHCIT^{0}$	$CdOHCIT^{2-}$	$Cd(CIT)_2^{4-}$
+ 0.772***	- 0.830***	- 0.836***	- 0.826***	- 0.704***
Cd^{2+}	$CdGLY^{+}$	$Cd(GLY)_2^{0}$		
+ 0.868***	- 0.518***	- 0.908***		
Cd^{2+}	$CdHIS^{+}$	$CdHHIS^{2+}$	$Cd(HIS)_2^{0}$	
+ 0.897***	+ 0.067*	+ 0.067*	- 0.948***	
Cd2+	$CdNTA^{-}$	$CdOHNTA^{2-}$	$Cd(NTA)_2^{4-}$	
+ 0.704***	- 0.748***	- 0.748***	- 0.875***	
Cd^{2+}	$CdEDTA^{2-}$	$CdHEDTA^{-}$	$CdOHEDTA^{3-}$	
+ 0.718***	- 0.789***	- 0.789***	- 0.788***	

DISCUSSION

The uptake of cadmium by the organism increases with the total concentration of cadmium in the solution when the chemical speciation remains constant. The relation between the concentration of cadmium in the solution and the rate of cadmium uptake by the organism is sigmoidal. This indicates that cadmium uptake is a facilitated process that can be saturated. Cadmium concentrations encountered in natural systems are, however, much lower than the concentrations required to saturate the uptake system (Nriagu, 1988; Furness and Rainbow, 1990; Sündermann, 1994). Thus, under most, except extreme high levels of cadmium exposure, the rate of cadmium uptake increases linearly with the concentration of cadmium in the environment.
Part of the cadmium taken up by the organism, is rapidly lost by the organism after transfer of the animals to a clean solution. Within the linear range of cadmium uptake the amount of cadmium eliminated is proportional to the total cadmium concentration in the solution. The fraction of cadmium that can be removed represents metal present in the lumen of the digestive tract and extracellular spaces of the epithelial structures that constitute the solution-body interface.
The increase in the rate of cadmium uptake with increasing cadmium concentration in the solution may be the result of interactions between the metal and membrane binding sites which could give rise to cooperative binding which is reflected in departures from linearity. Alternatively, increased levels of cadmium cause impairment of membrane functioning and disruption of membrane integrity. The free cadmium ion strongly interacts with the phospholipids of the bilayer displacing membrane stabilising cations such as calcium and magnesium. In addition the cadmium ion alters the transport and electrical properties of cellular membranes by interaction with the functional groups of membrane associated catalysing systems (Webb, 1979; Viarengo, 1989; Foulkes, 1990).
The complexation of cadmium with the organic ligands, EDTA, NTA, histidine, glycine and citrate decreases the uptake of cadmium by the brine shrimp. This observation is in agreement with several other studies showing that complexation reduces the availability of metals to aquatic organisms by reducing the concentration of the free metal ion in the solution (Sunda et al., 1978; Poldoski, 1979; Engel and Fowler, 1979; Rainbow et al., 1980; Muramoto, 1981; Hung, 1982; McLeese and Ray, 1984; Pärt and Wikmark, 1984; Jenkins and Sanders, 1986; Holwerda et al., 1988; Mason et al., 1988; Blust et al, 1992). The rate of cadmium uptake is not, however, a mere function of the free cadmium ion activity in the solution, i.e. cadmium uptake rates may differ by an order of magnitude for the same free cadmium ion activity depending on the complexation conditions. The five ligands used for the assessment of the effect of complexation on cadmium uptake by brine shrimp form a variety of complexes which differ in thermodynamic stability, coordination kinetics, size and charge. All organic ligands tested form hydrophilic complexes with metal ions which do not permeate the solution-body interface of aquatic organisms easily and degradate relatively slowly so that the transport of these metal species across the solution-body interface is of little or no importance.
Metal uptake is believed to involve an initial interaction of the free metal ion with a transporter, either channel or carrier. Transport through channels involves mainly weak electrostatic interactions. In this case metal uptake mainly depends on the size and charge of the metal species taken up. Transport through carrier systems

involves more strong covalent interactions. In this case metal uptake mainly depends on the stability of the metal-ligand complex in the solution and stability of the metal-carrier complex in the membrane, i.e. competition. The thermodynamic stability of the metal-ligand complexes formed covers over ten orders of magnitude but the rate of uptake does not increase with decreasing thermodynamic stability of the complexes formed. This indicates that species selectivity of cadmium uptake sites does not depend on strong covalent interactions but is merely controlled by size and/or charge effects.

Strong organic ligands behave as metal ion buffers when present in sufficiently high concentration. Metal ion buffers provide a controlled source of free metal ion in the presence of interfering complexing agents or competing metal ions. The extent of metal complexation depends, among others, on the hydrogen ion activity and the presence of competing metals. Both protonation and metal competition decrease the effective stability constants of the metal-ligand complexes. It is only when the ligand is present in excess, and almost all of the metal is bound in a complex, that the concentration of the free metal ion is stabilised (Perrin and Dempsey 1974; Da Silva and Williams, 1976; Martell, 1978). Thus, the rate of metal uptake may vary considerably between different environments when the metal ion buffer capacity of the solution is not controlled. Even, an increased uptake of cadmium upon complexation with organic ligands has been observed in a number of cases where the free metal ion concentration was not buffered (George and Coombs, 1977; Dressing et al., 1982; Ramamoorthy and Blumhagen, 1984; Winner, 1984; Winner and Gauss, 1986).

The uptake of a metal is not only controlled by the activity of the free metal ion in the solution but also by the rate of reaction of the metal with the transport systems present in the exchange structures. Slow coordination kinetics may limit the rate of metal uptake across the solution-body interface when the rate of complex diffusion and dissociation in the solution is less than the rate of metal ion transport across the exchange surface. Under these conditions the dynamic equilibrium between the concentration of the free metal ion and the metal-ligand complex is no longer maintained and the free metal ion concentration decreases. Coordination kinetics may become an important factor if the metal exists predominantly in complexed form and the complex itself is not taken up. The rate of complex formation generally increases with the charge on the ligand while the rate of complex dissociation decreases with the thermodynamic stability of the complex formed (Margerum et al., 1978; Hering and Morel, 1990). The results of this study rather show that the rate of cadmium uptake by the brine shrimp increases with the thermodynamic stability of the metal-ligand complex formed. This indicates that coordination kinetics do not constrain the rate of cadmium uptake by the brine shrimp in these experiments.

Consequently, the most likely explanation for the observed differences in the rate of cadmium uptake, which are not explained by the free cadmium ion activity effect, is competitive complex formation in the water layer lining the exchange structures. The presence of relative high concentrations of other metal ions in the boundary layer which compete for the same ligands may alter the effective stability constants of the metal-ligand complexes and hence the speciation of the metals present in the boundary layer. This effect will be more important when the ligand has a high affinity for metals but is present in relative low concentrations so that the metal complexation and buffer capacity of the water is limited. This results in a local

increase of the free cadmium ion activity so that the rate of cadmium uptake increases accordingly.

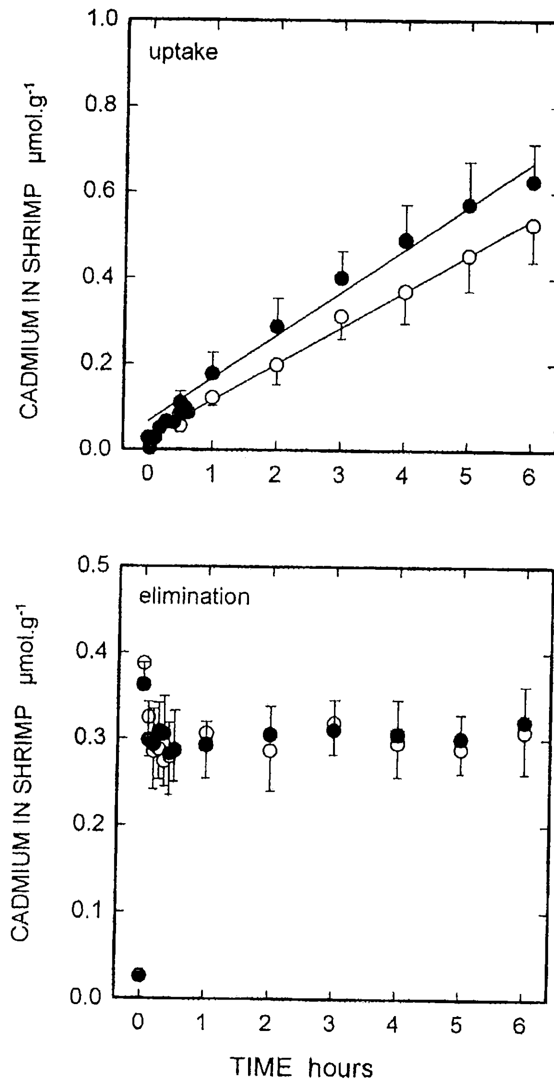

Fig. 1a. *Artemia franciscana*. Uptake of cadmium by brine shrimp as a function of time when exposed to a total cadmium concentration of 100 µmol.l^{-1}. Datapoints represent means with standard deviations for five replicate experiments. Filled data points represent cadmium in shrimp that have not been transferred to a clean solution. Open data points represent cadmium in shrimp that have been transferred for one hour to a clean solution containing 1 mmol.l^{-1} of 8-hydroxyquinoline-5-sulfonic acid. Means are significantly different within, $p<0.01$ and between treatment groups, $p<0.05$.

Fig. 1b. *Artemia francisana*. Elimination of cadmium by brine shrimp as a function of time after 3 hours of exposure to a total cadmium concentration of 100 µmol.l^{-1}. Datapoints represent means with standard deviations for five replicate experiments. Filled data points represent cadmium in shrimp after elimination in a clean solution. Open data points represent cadmium in shrimp after elimination in a clean solution containing 1 mmol.l^{-1} 8-hydroxyquinoline-5-sulfonic acid. Means are only significantly different during the first 10 minutes of exposure, $p<0.05$. There are no significant differences in elimination between the treatment groups, $p>0.05$.

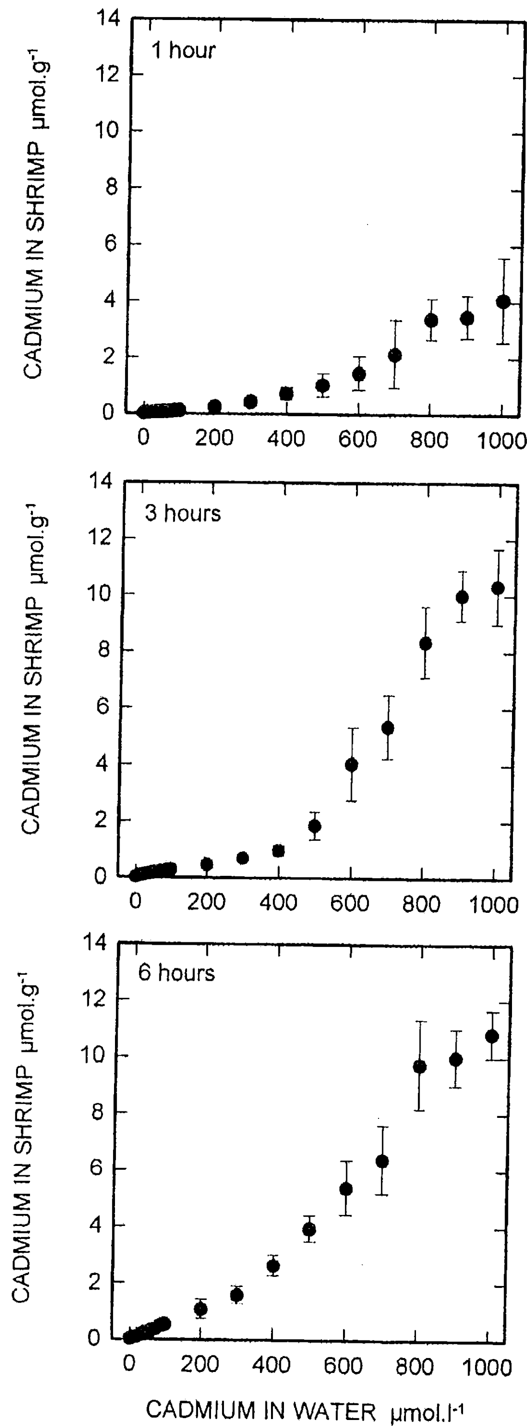

Fig. 2. *Artemia franciscana*. Concentration of cadmium in brine shrimp as a function of the concentration of cadmium in the solution after 1, 3 and 6 hours of epxosure. Datapoints represent means with standard deviations for five replicate experiments. Means are significantly different between the treatment groups, $p<0.01$.

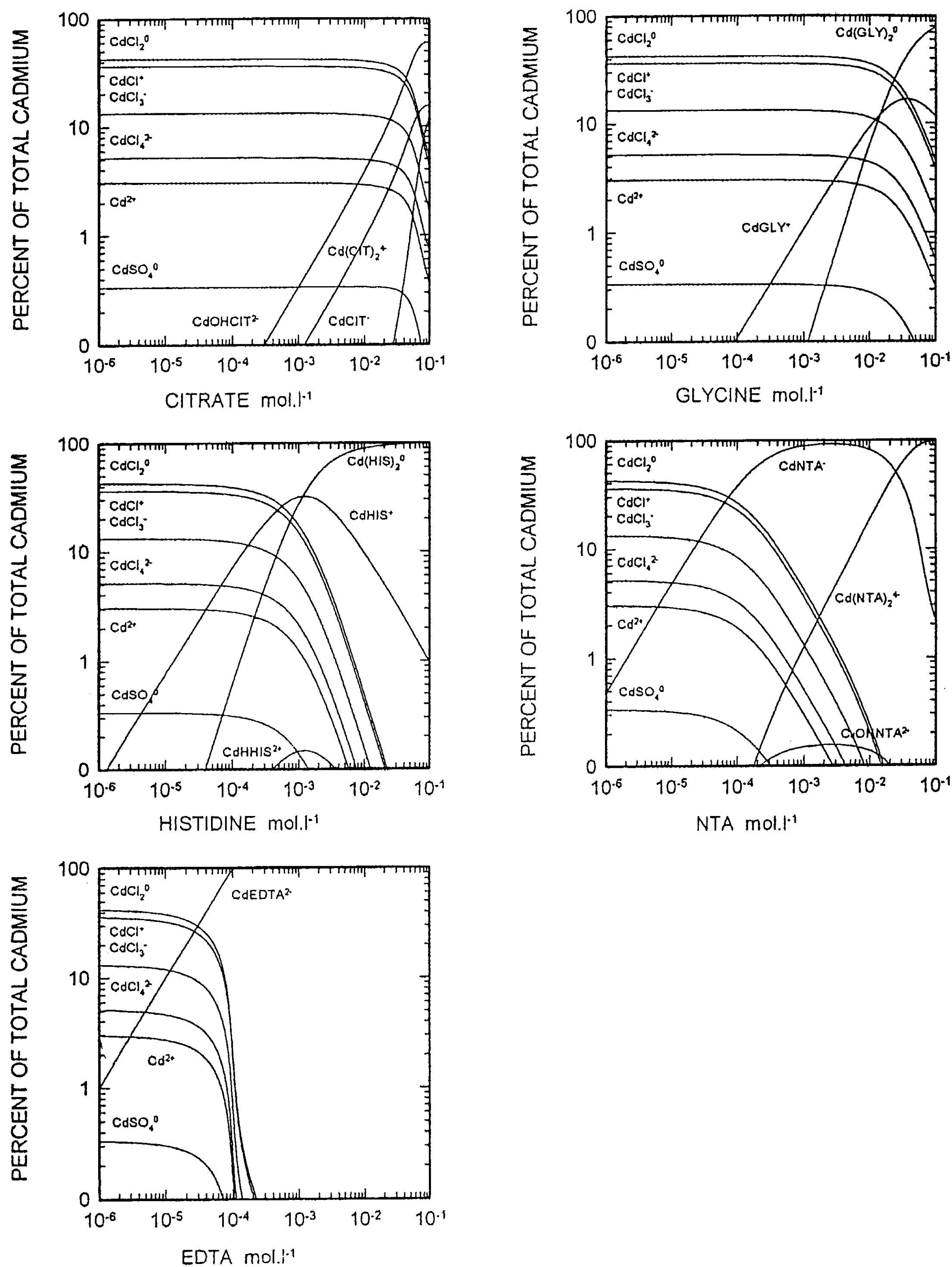

Fig. 3. Effect of complexation by different organic ligands, (i.e. citrate, glycine, histidine, NTA and EDTA) on the chemical speciation of cadmium in a saltwater solution containing 100 μmol.l^{-1} of cadmium (Sal=35 °/oo, pH=8.00, T=25°C).

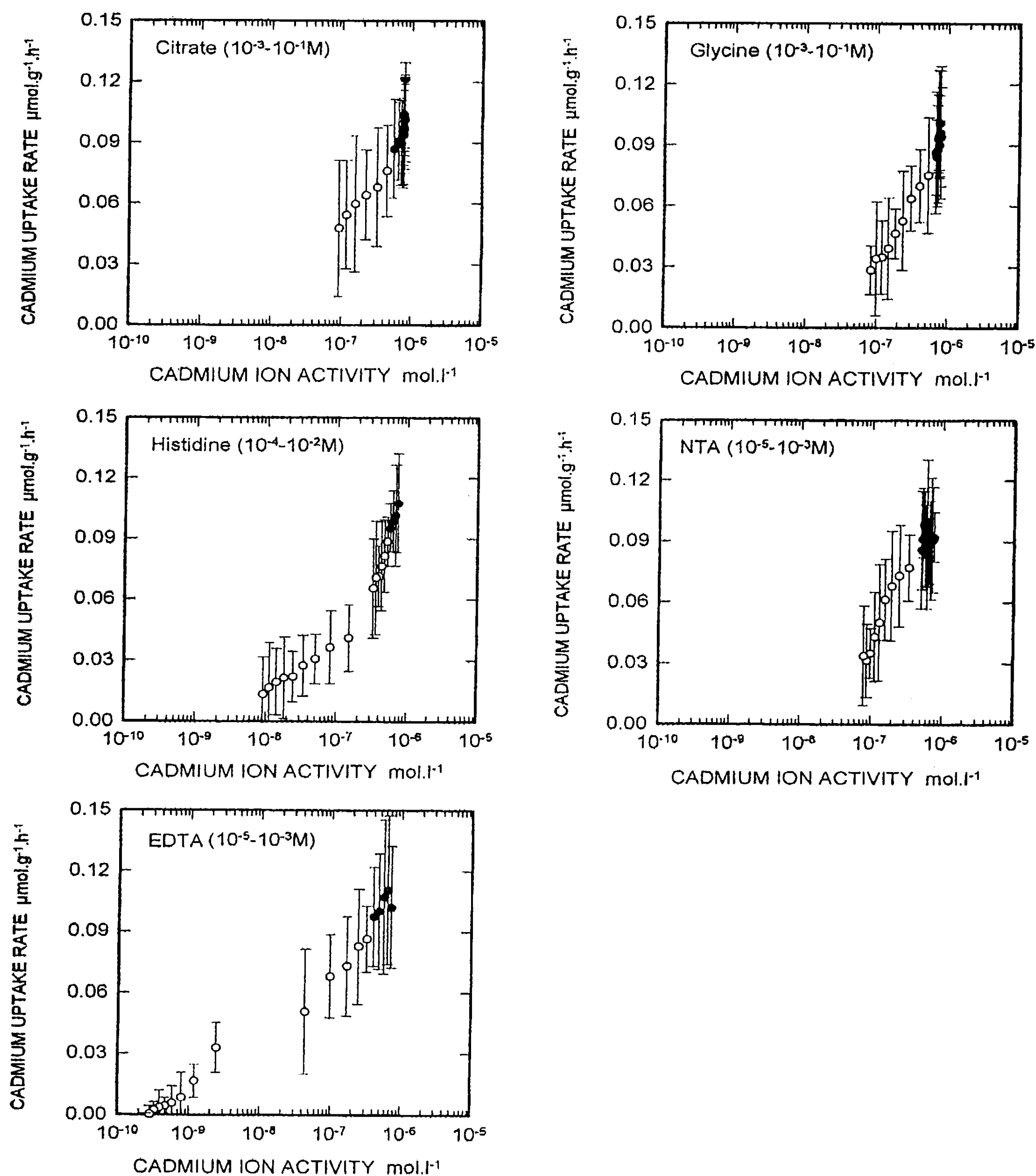

Fig. 4. *Artemia franciscana*. Effect of complexation by different organic ligands, (i.e. citrate, glycine, histidine, NTA and EDTA) on the rate of cadmium uptake by brine shrimp in function of the cadmium ion activity in a solution containing 100 µmol.l^{-1} of cadmium. Datapoints represent means with standard deviations for five replicate experiments. Filled datapoints are not significantly different and open data points are significantly different from uptake rates in solutions without ligand, $p<0.01$.

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Effects of temperature on the uptake of copper by the brine shrimp, *Artemia franciscana*

INTRODUCTION

The uptake and toxicity of metals in aquatic organisms strongly depends on the environmental conditions. Temperature is an important factor influencing both the chemistry of the environment and the physiology of aquatic organisms (Davies and Tribe, 1969; Stumm and Morgan, 1981; Cossins and Bowler, 1987). Most studies indicate that the uptake and toxicity of metals increases with temperature, but the relative importance of chemical and physiological processes are poorly understood (Cairns et al., 1975; Phillips, 1976; Cotter et al., 1982; McLusky et al., 1986; Nugegoda and Rainbow, 1987).

Changes in temperature have an important effect on the extent of metal complexation by altering the equilibrium position of reactions and the solubility of gases. The most important effects are changes in the hydrogen ion activity and total carbonate concentration. These alterations influence the extent of metal hydroxide and carbonate complexation which are controlling factors in copper speciation. (Baes and Mesmer, 1981; Turner and Whitfield, 1987; Byrne et al., 1988).

Most aquatic organisms are poikilothermic, that is, they are unable to control their body temperature so that their physiology is highly dependent on ambient temperature. Variations in temperature influence many different physiological processes, most notably the many catalytic systems involved in transport and metabolism. Within limits, a temperature increase accelerates most of these processes. However, given ample time many aquatic organisms can compensate for temperature changes by appropriate changes in their physiological organisation. (Hazel and Prosser, 1974; Somero, 1978; Burton, 1986).

The most important process in the uptake of metals by aquatic organisms is the transport of the metal across the external membranes of the exchange surfaces. The organisation and functioning of these interfaces are strongly influenced by the temperature of the environment. Thus, temperature may alter metal uptake in different ways depending on the predominant effect. To determine the relative importance of physical, chemical and biological effects of temperature on the uptake of copper by a saltwater organism we have studied the effect of different temperature acclimation and exposure regimes on the rate of copper uptake by the brine shrimp, *Artemia franciscana*.

MATERIAL AND METHODS

Brine shrimp

Dried *Artemia franciscana* cysts from Great Salt Lake, Utah, USA were purchased from San Francisco Bay Brand, Newark, CA, USA. Cysts were hatched in a funnel-shaped plastic container filled with synthetic seawater (Wiegandt, Krefeld, Germany), and aerated from the bottom. The hatching suspension was illuminated by a fluorescent light tube. Hatching cyst density was 5 g l^{-1}. *Artemia* nauplii were harvested after 36 hours. The larvae were grown from nauplii to adult in 150 l plastic rectangular air-water lift operated race-ways filled with synthetic seawater. Brine shrimp were fed with a suspension of the dried algae *Spirulina*. Animals reached maturity after 3 to 4 weeks and were used before they were 8 weeks old. The methods for intensive culturing of brine shrimp have been described by Sorgeloos et al. (1983).

Experimental procedures

The uptake of copper by brine shrimp was followed during three hours of exposure to the metal in a static test system. Copper uptake is linear with time during the short exposure period in the static system, displaying first order uptake kinetics (Blust et al., 1988). Experiments were conducted in thermostated rooms at six different temperatures, ranging from 10±0.5 to 35±0.5 °C. Fifteen days before an experiment was run adult brine shrimp were collected from a batch culture for temperature acclimation. Animals were gradually acclimated to solutions of differing temperatures over a five day period and kept at the final temperature for ten days (i.e. 10, 15, 20, 25, 30 and 35 °C). On the last day of the acclimation period the animals were transferred for one hour to a saltwater solution containing 1mM 8-hydroxyquinoline-5-sulfonic acid. This strong metal ligand, which is not acutely toxic to brine shrimp, was used to remove metal bound to the external surfaces of the shrimp. For the remaining period the animals were kept in clean saltwater for defecation. Copper uptake by brine shrimp was measured for all 36 temperature of acclimation and exposure combinations possible. This experimental design allows the separation of short term exposure and long term acclimation effects of temperature on metal uptake.
The composition of 1 l of the chemically defined saltwater solution with a salinity of 3.5 % was 23.50g NaCl, 4.00g Na_2SO_4, 0.680g KCl, 0.196g $NaHCO_3$, 1.470g $CaCl_2.2H_2O$, 10.78g $MgCl_2.6H_2O$ and 0.026g H_3BO_3. The medium was prepared by dissolving the seven analytical grade products (Merck p.a.) in deionised water. A dispersion of 0.1 mmol.l^{-1} manganese dioxide was added to the seawater to remove metals present in the analytical grade reagents. After an equilibration period of 24 hours, the dispersion was filtered through a 0.2 mm membrane filter to remove the manganese dioxide from the solution (Van den Berg and Kramer, 1979). The pH of the solutions was adjusted with HCl or NaOH as required and the media were aerated to promote equilibration of gases with the atmosphere. The dissolved oxygen concentration, the total dissolved carbonate concentration, the hydrogen ion activity and the redox potential were measured to ensure that equilibrium conditions had been established. Dissolved oxygen was measured with a membrane covered amperometric electrode system (WTW OX191/EO90). Total dissolved carbon dioxide was measured with a gas sensing CO_2 electrode (Ingold 152323000), after acidification of the water sample ($pH<4.8$) in a

sealed measuring vessel. The hydrogen ion activity was measured with a glass electrode (Ingold 104573002). Redox potentials were measured with a wire type platinum electrode (Ingold, 105003077).
Cupric nitrate was added to the test solutions from a 100 mmol l^{-1} cupric ion stock. In all series of experiments the total concentration of copper in test solutions was 5 mmol l^{-1}. Experiments were carried out in 0.5 l plastic beakers. Just before an experiment started about 50 animals were collected on a 250 mm screen, rinsed with clean medium and transferred to a beaker. After 180 minutes the beaker was emptied over a 250 mm screen and the collected animals transferred to a beaker with a saltwater solution containing 1mM 8-hydroxyquinoline-5-sulfonic acid. Ten minutes later the beaker was emptied over a 250 mm screen and the collected animals were rinsed with deionised water and divided into five plastic vials, dried for 24 hours at 60 °C and stored in a dessication box until analysed for copper. This procedure was repeated five times using animals from separate cultures and newly prepared media and reagents. The dissolved oxygen concentration, the total dissolved carbonate concentration, the hydrogen activity, the redox potential and the total dissolved copper concentration were measured at the beginning and end of an experiment. Generally, all measured values remained within 10 % of the initial values.

Chemical modelling

The equilibrium activities of the chemical species considered were calculated using the computer program SOLUTION (Blust et al., unpubl.) an adaptation of the program COMPLEX (Ginzburg, 1976). This speciation model allows the calculation of the composition of solutions in equilibrium with gas and solid phases. A thermodynamic stability constant data base was built which is based on the data of Dickson and Whitfield (1981) for the major components, and the data of Smith and Martell (1976, 1989) and Martell and Smith (1982) for copper. For each ion-pair or complex considered, the stability constants listed for different ionic strengths were fitted to an interpolation function which has the form of an extended Debye-Hückel equation (Turner et al., 1981). Enthalpies to describe the effect of temperature on the equilibrium position of the reactions were taken from Byrne et al. (1988) and Smith and Martell (1989). The thermodynamics of the carbon dioxide system were described using the model of Whitfield and Turner (1986). The thermodynamic stability constants and the effect of temperature and ionic strength on the conditional stability constants of all copper species included in the model are given in Table I. Case specific input comprises the total concentrations of the metals and ligands in the solution, the free hydrogen ion activity (pH), redox intensity (pe), temperature and the gas phases that are maintained in equilibrium with the solution.

Metal analysis

Copper was measured by Graphite Furnace Atomic Absorption Spectrophotometry using a Perkin-Elmer 703 Spectrophotometer fitted with a Heated Graphite Atomiser HGA-500 and a deuterium arc background corrector. The method of stabilised temperature platform atomisation was used (Slavin et al., 1983). Biological material was dissolved with concentrated nitric acid in a microwave oven and diluted with deionised water to a 10 % nitric acid solution. Saltwater solutions were diluted ten times with a 10

% nitric acid solution to decrease the salinity and analysed against matrix matched calibration standards (Blust et al., 1988).

Statistical analysis

All sets of data were tested for homoscedasticity by the log-anova test for homogeneity of variances and for normality by the Kolmogorov-Smirnov test for goodness of fit. Analysis of variance, linear and non-linear regression methods were used for analysing the data. Significance levels of tests are indicated by asterisks according to the following probability ranges (*=$0.05 \geq P > 0.01$, **=$0.01 \geq P > 0.001$, ***=$P \leq 0.001$). Statistical methods are as outlined in Sokal and Rohlf, (1981) and Glantz and Slinker, (1990).

RESULTS

Chemical speciation of copper in saltwater

The speciation of copper in an inorganic chemically defined saltwater solution changes with the hydrogen ion activity in the solution and is controlled by the concentration of hydroxide and carbonate. The ion product of water decreases strongly with temperature so that the hydrogen and hydroxide ion activity in the solution change with temperature. The solubility of carbon dioxide and the conditional stability constants for the two carbonic acid equilibria (i.e. HCO_3^- and H_2CO_3) decrease with temperature. In an open system these different processes result in a decrease in the total carbonate concentration and an increase in hydrogen ion activity. As such, a temperature change alters the speciation of copper by simultaneously shifting the equilibrium position of the different metal complexation reactions and the concentrations of the most important inorganic ligands. The effect of these different processes on the free cupric ion activity were calculated using the chemical speciation model SOLUTION and the results are summarised in Fig 1a. Depending on the degree of phase equilibration the pH ranges from 7.8 to 8.2 in the temperature range 10 to 35 °C. Over this range of temperatures and hydrogen ion activities, the free cupric ion activity varies between 0.9 and 4.7 % of the total copper concentration.

Diffusion of copper in saltwater

Changes in temperature have an important effect on the free diffusion of solutes. The Stokes-Einstein relation (D=RT/6pnrN) provides an approximate expression for the diffusion coefficient (D) of a spherical solute if the hydrated radius (r) of the solute and the viscosity (n) of the solution are known (Robinson and Stokes, 1970). The equation predicts that the diffusion coefficient is inversely proportional to the solute radius and the viscosity of the solution. The hydrated radius can be obtained from the effective size of the solvated ion given by the molar hydrated volume of the solute sphere ($V=Nk(4p/3)r^3$) with N Avogadro's number, k the solvent packing coefficient and r the hydrated radius. The molar volume of the cupric ion with an hydration number of 10.3 is 147.8 $cm^3.mol^{-1}$ for an infinite dilute aqueous solution of 25 °C. This results in an estimate for the radius of the hydrated ion of 404 pm for a water packing coefficient of k=0.888 (Marcus, 1985). Given the effect of temperatire on the viscosity of the saltwater solution and the radius of the solute, the diffusion coefficient of the cupric ion can be calculated in function of the temperature. The results of these calculations are summarised in Fig. 1b. Over the temperature range 10 to 35 °C the diffusion coefficient increases from 3.699 to 7.232 10^{-6} $cm^2.s^{-1}$.

Flux of copper in saltwater

Flux is the amount of solute which crosses a plane of unit area perpendicular to the direction of flow in unit time. Fick's first law (J=Da) provides an expression for calculating the flux in one dimension when the diffusion coefficient of the solute (D) is known and the chemical potential difference (a) does not change with time. Hence, the effect of temperature on the flux of the cupric ion in one dimension is given by the product of the molar activity and the diffusion coefficient of the free cupric ion divided by the unit

distance travelled (i.e. Cu^{2+}_{act}*Cu^{2+}_{dif}/distance). The results of these calculations are summarised in Fig. 1c. Over the temperature range 10 to 35 °C the flux of the cupric ion in the solution increases from 0.161 to 1.475 $pmol.cm^{-2}.s^{-1}$. Thus, temperature has an important effect on the chemical speciation and the diffusion rate of copper and consequently on the flux of the cupric ion. These results are further used to determine the effect of changes in the chemical speciation and diffusion of copper with temperature on the rate of copper uptake by brine shrimp.

Uptake of copper by brine shrimp

The effect of temperature on the uptake of copper by brine shrimp has been studied for six different temperature acclimation groups and six different temperature exposure groups. This experimental design makes it possible to separate the effect of temperature acclimation from the effect of temperature exposure on the uptake of copper by the organism. To minimise the effect of temperature on the speciation of copper, the hydrogen ion activity was kept constant in all treatment groups (pH=8.0±0.1). The results summarised in Fig. 2 a-f and 3 a-f show that within each temperature acclimation group, copper uptake increases with increasing temperature of exposure. The effect of the temperature of exposure on copper uptake decreases with increasing temperature of acclimation. Thus, among the exposure groups, copper uptake decreases with increasing temperature of acclimation. The results of the analysis of variance for the effect of exposure and acclimation to the different temperatures on copper uptake are given in Table II. Both the effects of the temperature of exposure and the temperature of acclimation on copper uptake by brine shrimp are highly significant.
To determine the relative importance of the different factors that contribute to the variation in copper uptake by the brine shrimp with temperature a non-linear regression model was constructed which considers the effects of physical, chemical and physiological processes on the rate of copper uptake. The mathematical description of the model has the form of a non-linear equation:

$$Cu_{uptake}=Cf*Cu^{2+}_{act}*Cu^{2+}_{dif}*T_{exp}{}^{a}*T_{acl}{}^{b}$$

The first factor in the regression model accounts for the effect of temperature on the activity of the free cupric ion (Cu^{2+}_{act}). The second factor accounts for the effect of temperature on the diffusion rate of the free cupric ion (Cu^{2+}_{dif}). The third factor accounts for the effect of the temperature of acclimation on the rate of copper uptake ($T_{acl}{}^{a}$). The fourth factor accounts for the effect of the temperature of exposure on the rate of copper uptake ($T_{exp}{}^{b}$). The two last factors are exponential where a and b are temperature coefficient which are negative when the rate of copper uptake decreases with temperature and positive when the rate of copper uptake increases with temperature. To relate the product of these four factors to copper uptake, it is necessary to introduce a coefficient of proportionality (Cf), which relates the activity of the cupric ion in the solution to the rate of copper uptake by the brine shrimp. The relative importance of the different factors was determined by the forward selection procedure, starting with a single factor and then add factors one at a time and evaluate the effect on the coefficient of determination of the regression model. The results of the non-linear regression analysis are summarised in Table III. Starting with the factor which accounts for the effect of the temperature of exposure on the activity of the free cupric ion in the solution

explains 42.1 % of the variation in copper uptake. Adding the factor which accounts for the effect of the temperature of exposure on the diffusion rate of the cupric ion in the solution explains 76.9 % of the variation in copper uptake. Adding the factor which accounts for the effect of the temperature of exposure on the rate of copper uptake by brine shrimp explains 84.0 % of the variation in copper uptake. The coefficient for the effect of the temperature of exposure on the rate of copper uptake is positive, which indicates that the rate of copper uptake increases the temperature of exposure. Adding the factor which accounts for the effect of the temperature of acclimation on the rate of copper uptake by brine shrimp explains 88.9 % of the variation in copper uptake. The coefficient for the effect of the temperature of acclimation on the rate of copper uptake is negative, which indicates that the rate of copper uptake increases with the temperature of exposure. Overall, the temperature acclimation effect is such that, except for the lowest temperature of acclimation, exposure to the temperature of acclimation has no effect on copper uptake. Thus, although copper uptake increases with the temperature of exposure this effect is largely set-off by the effect of the temperature of acclimation.

Table 1: Thermodynamic stability constants and concentration products for copper species. K: thermodynamic stability constant; Q: concentration quotient at ionic strength 0.614 $mol.l^{-1}$.

Species	Log K	Log Q (10°C)	Log Q (20 °C)	Log Q (30°C)
Cu^{2+}	-	-	-	-
$CuCl^{+}$	0.40	-0.10	-0.04	0.01
$CuSO_4^{0}$	2.35	1.24	1.29	1.35
$CuOH^{+}$	6.01	5.67	5.63	5.59
$Cu(OH)_2^{0}$	11.75	10.63	10.77	10.89
$CuCO_3^{0}$	6.74	5.61	5.69	5.76
$CuHCO_3^{+}$	12.25	10.70	10.70	10.70
$Cu(CO_3)_2^{2-}$	10.75	9.27	9.27	9.27

Table 2: Two way analysis of variance for the effect of the temperature of exposure and the temperature of acclimation on copper uptake by the brine shrimp (36 treatments groups with 5 replications).

Source of Variation	Degrees of Freedom	Sum of Squares	Mean of Squares	F_s
Exposure	5	7.00	1.40	195.50^{***}
Acclimation	5	0.66	0.13	18.38^{***}
Interaction	25	0.58	0.023	3.25^{***}
Error	144	1.03	0.0072	
Total	179	9.27		

Table 3: Copper uptake rate in brine shrimp, non-linear regression analysis of the pooled data. B: partial regression coefficients; SE: standard error of partial regression coefficients; L_1, L_2: confidence limits of partial regression coefficients. Free cupric ion activities in the solution are in $mmol.l^{-1}$ and copper uptake rates in brine shrimp are in $mmol.g^{-1}.h^{-1}$.

1) $Cu_{uptake}=Cf*Cu^{2+}_{act}$ **(**$R^2=0.421^{***}$**, n=210)**

Variable	B	SE	L_1	L_2
Cf	4.478^{***}	0.137	4.209	4.747

2) $Cu_{uptake}=Cf*Cu^{2+}_{act}*Cu^{2+}_{dif}$ **(**$R^2=0.769^{***}$**, n=210)**

Variable	B	SE	L_1	L_2
Cf	0.863^{***}	0.016	0.832	0.894

3) $Cu_{uptake}=Cf*Cu^{2+}_{act}*Cu^{2+}_{dif}*T_{exp}^{k}$ **(**$R^2=0.840^{***}$**, n=210)**

Variable	B	SE	L_1	L_2
Cf	0.213^{***}	0.036	0.142	0.284
Exponent (k)	0.434^{***}	0.050	0.336	0.532

4) $Cu_{uptake}=Cf*Cu^{2+}_{act}*Cu^{2+}_{dif}*T_{exp}^{k}*T_{acl}^{l}$ **(**$R^2=0.883^{***}$**, n=210)**

Variable	B	SE	L1	L2
Cf	0.455^{***}	0.076	0.306	0.604
Exponent (k)	0.437^{***}	0.043	0.353	0.521
Exponent (l)	-0.255^{***}	0.029	0.198	0.312

Table 4: Apparent activation energy (E_a) for the effect of the temperature of exposure on the uptake of copper by brine shrimp.

Acclimation	E_a kJ.mol^{-1}	SE	R^2	N
10 °C	25.27***	2.91	0.766***	25
15 °C	17.46***	2.61	0.661***	25
20 °C	22.59***	2.59	0.768***	25
25 °C	33.45***	3.69	0.821***	20
30 °C	39.50***	4.21	0.759***	30
35 °C	29.71***	4.14	0.648***	30

DISCUSSION

The effect of temperature on biological processes is primarily caused by changes in reaction rates and/or a changes in the position of equilibria. The temperature dependence of a reaction can be described by the Arrhenius equation, $k=Ae^{-Ea/RT}$ in which k is the velocity constant of the reaction, A is a constant related to the collision frequency of molecules, E_a is the activation energy, R is the gas constant, T the absolute temperature and e is the base of the natural logarithm. A plot of the natural logarithm of the rate against reciprocal temperature yields a straight line with a slope of E_a/R (Alexandrov, 1977, Bowler and Fuller, 1987, Cossins and Bowler, 1987). The relation between reciprocal temperature of exposure and the natural logarithm of the rate of copper uptake is linear over the temperature range 15-35 °C, in all but one case as shown in Fig. 4. The apparent activation energy for copper uptake depends on the temperature of acclimation but a general trend is not apparent as shown in Table IV. A number of plots show a break, with the activation energy larger at the lowest than at the higher temperatures. These breaks can be interpreted as transitions from one rate-limiting step to another, each with different activation energies, so that at low temperatures a reaction with a high activation energy is rate-limiting, whilst at higher temperatures a reaction with a lower activation energy is rate-limiting. The breaks in the curves disappear with increasing temperature of acclimation, indicating that the acclimation conditions alter the temperature sensitivity of the transport system.
The diffusion rate of the free cupric ion in solution, the rate of copper uptake by the brine shrimp and the metabolic activity of the brine shrimp all increase with temperature. The diffusion rate of the free cupric ion in the solution has a temperature dependence characterised by a low activation energy of 19 $kJ.K^{-1}.mol^{-1}$ A typical value for physical processes which are not very temperature sensitive. The activation energy for copper uptake by brine shrimp varies with the temperature of acclimation and ranges from 17.5 to 39.5 kJ.K-1.mol-1. The metabolic activity of brine shrimp has a temperature dependence characterised by a high activation energy of 49 $kJ.K^{-1}.mol^{-1}$ at 25 °C (Decleir et al, 1980). Thus, the apparent activation energy for the uptake of copper by brine shrimp is equal or higher than the activation energy for diffusion of the free cupric ion in the solution but considerably less than the activation energy for metabolic activity. Activation energies for the diffusion of chemical species in water are between 17 and 21 $kJ.mol^{-1}$. Activation energies for passive and facilitated diffusion of chemical species across membranes are between 18-38 $kJ.mol^{-1}$. These activation energies are generally larger than for diffusion in water which is due to the fact that the activation energy for movement across membranes includes the activation energy of both the membrane binding and membrane tranport process (Stein; 1986, 1990; Hille, 1991).
In each temperature exposure group copper uptake decreases with increasing temperature of acclimation. As a result there is no effect of temperature on the rate of copper uptake when the brine shrimp are acclimated to the temperature of exposure. Only for brine shrimp exposed to the lowest temperature of acclimation does the rate of copper uptake remain low. This shows that temperature acclimation influences copper uptake so that similar transport rates are maintained at different temperatures. Acclimation is not complete at the lowest temperature, which reflects the physiological inability to fully compensate for the effect of the temperature change.
The decreased rate of copper uptake at the lowest temperature may be the result of the effect of low temperatures on membrane transport processes. Membrane phospholipids

are packed in a regular array, head-to-head, shoulder to shoulder, and tail-to-tail. Such arrays of phospholipids are crystalline but they can be ordered or disordered. The ordered bilayer melts as the temperature is raised through a critical temperature range over which the phase transition occurs. A protein (e.g. transport system) present in such a bilayer may well display quite different kinetic properties when it is surrounded by phospholipids in an ordered, in contrast to a fluid, state. As the temperature of a membrane is lowered, the membrane phospholipids undergo a thermal transition from a highly fluid state to a much more rigidly ordered state. (Overath and Thilo, 1978; Stein; 1986, 1990). This process often alters the functionality and temperature sensitivity of membrane embedded systems and may explain the break in the Arrhenius plots of the effect of temperature on copper uptake by brine shrimp.

In summary, it has been shown that the uptake of copper by brine shrimp increases with the temperature of exposure and decreases with the temperature of acclimation. The temperature of exposure effect can be explained by the effect of temperature the activity and diffusion rate of the cupric ion in the solution. The magnitude of the apparent activation energy for copper uptake indicates that it is a facilitated diffusion process. Temperature acclimation appears to involve a number of physiological alterations which within certain limits compensate for the effect of an increasing temperature of exposure on the transport of copper across the exchange surfaces.

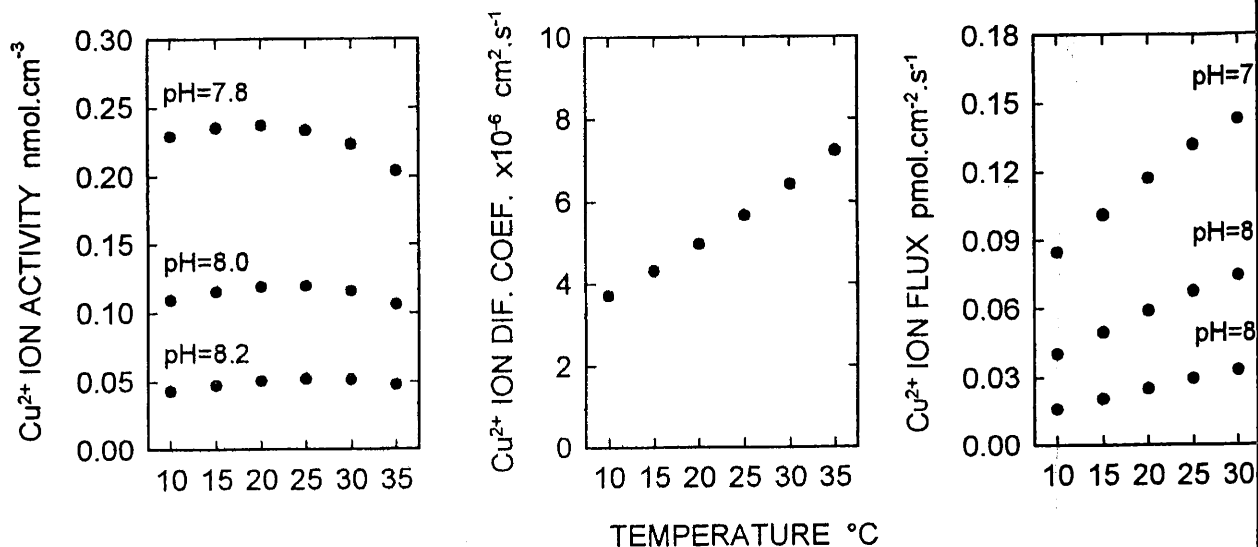

Fig. 1. a) Effect of temperature on the activity of the free cupric ion in the saltwater solution; b) Effect of temperature on the diffusion rate of the free cupric ion in the saltwater solution; c) Effect of temperature on the flux of the free cupric ion in the saltwater solution (Cu_{Total}=5mmol.l^{-1}, sal=3.5%).

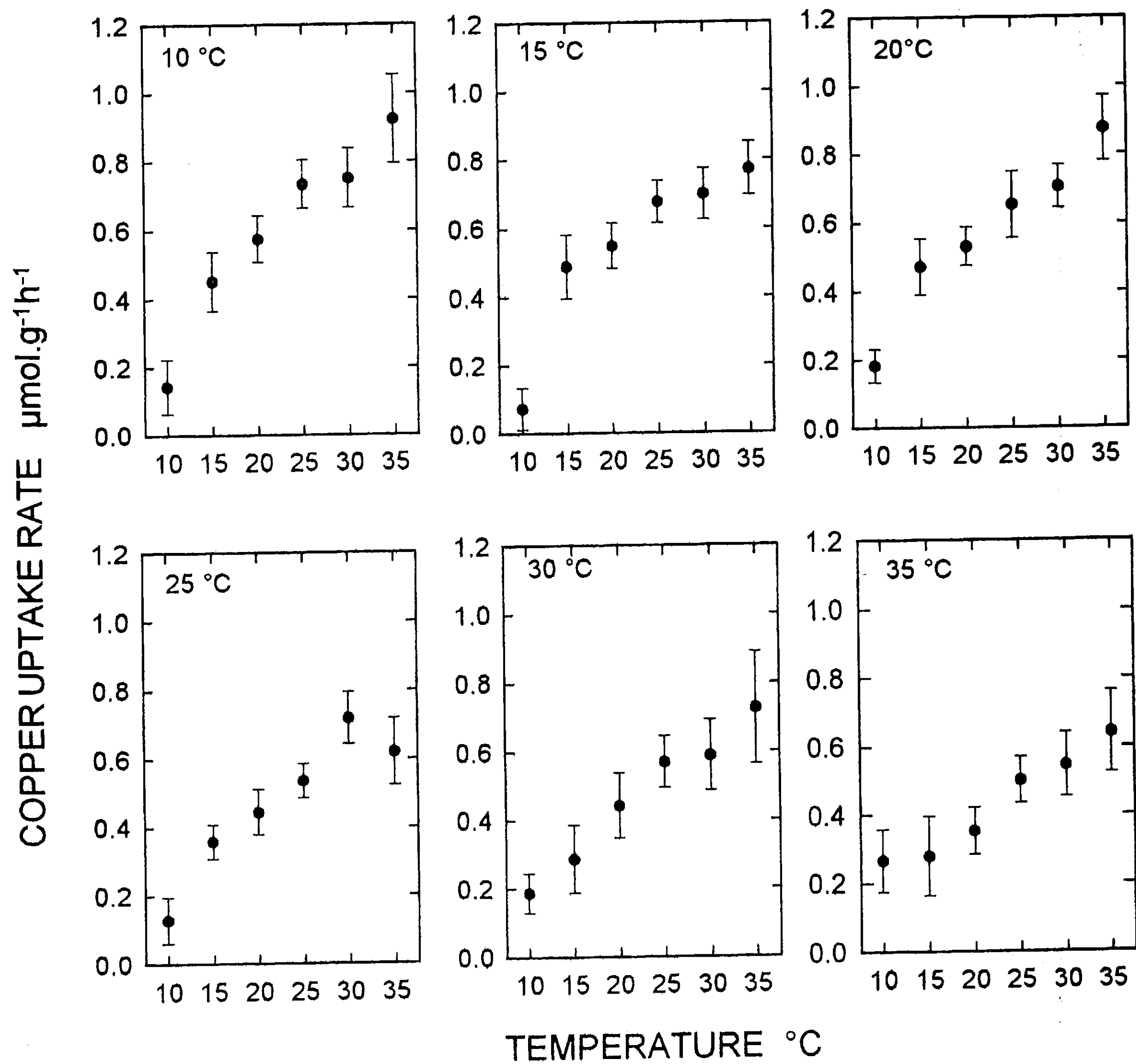

Fig. 2. Effect of the temperature of exposure on the rate of copper uptake by brine shrimp for the different temperature acclimation groups (Cu_{Total}=5mmol.l^{-1}, sal=3.5%, pH= 8.0$\pm$0.1). Means with standard deviations for 5 replicates are significantly different within groups (ANOVA: $p<0.001$).

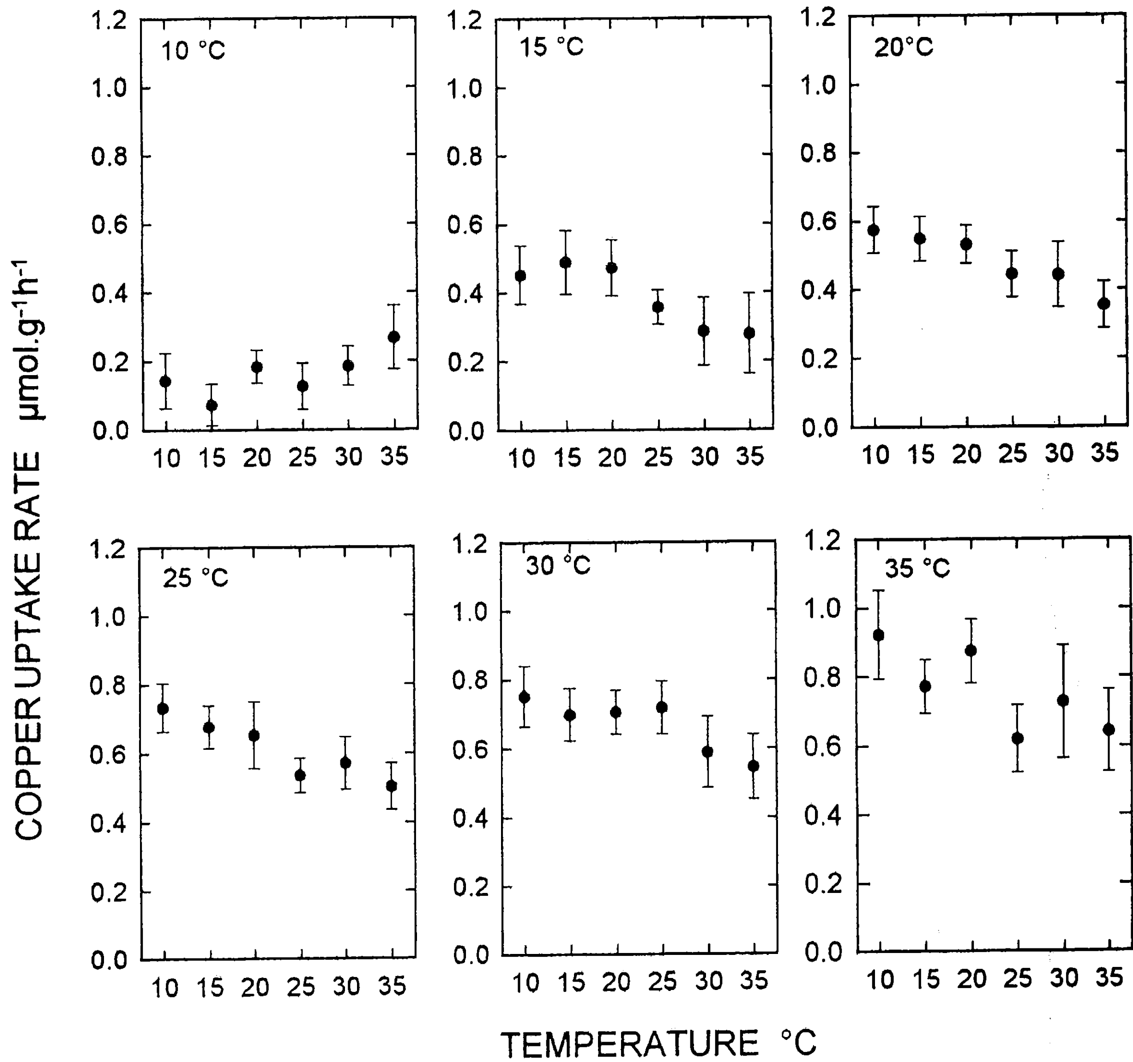

Fig. 3. Effect of the temperature of acclimation on the rate of copper uptake by brine shrimp for the different temperature exposure groups (Cu_{Total}=5mmol.l^{-1}, sal=3.5%, pH= 8.0±0.1). Means with standard deviations for 5 replicates are significantly different within groups (ANOVA: $p<0.01$).

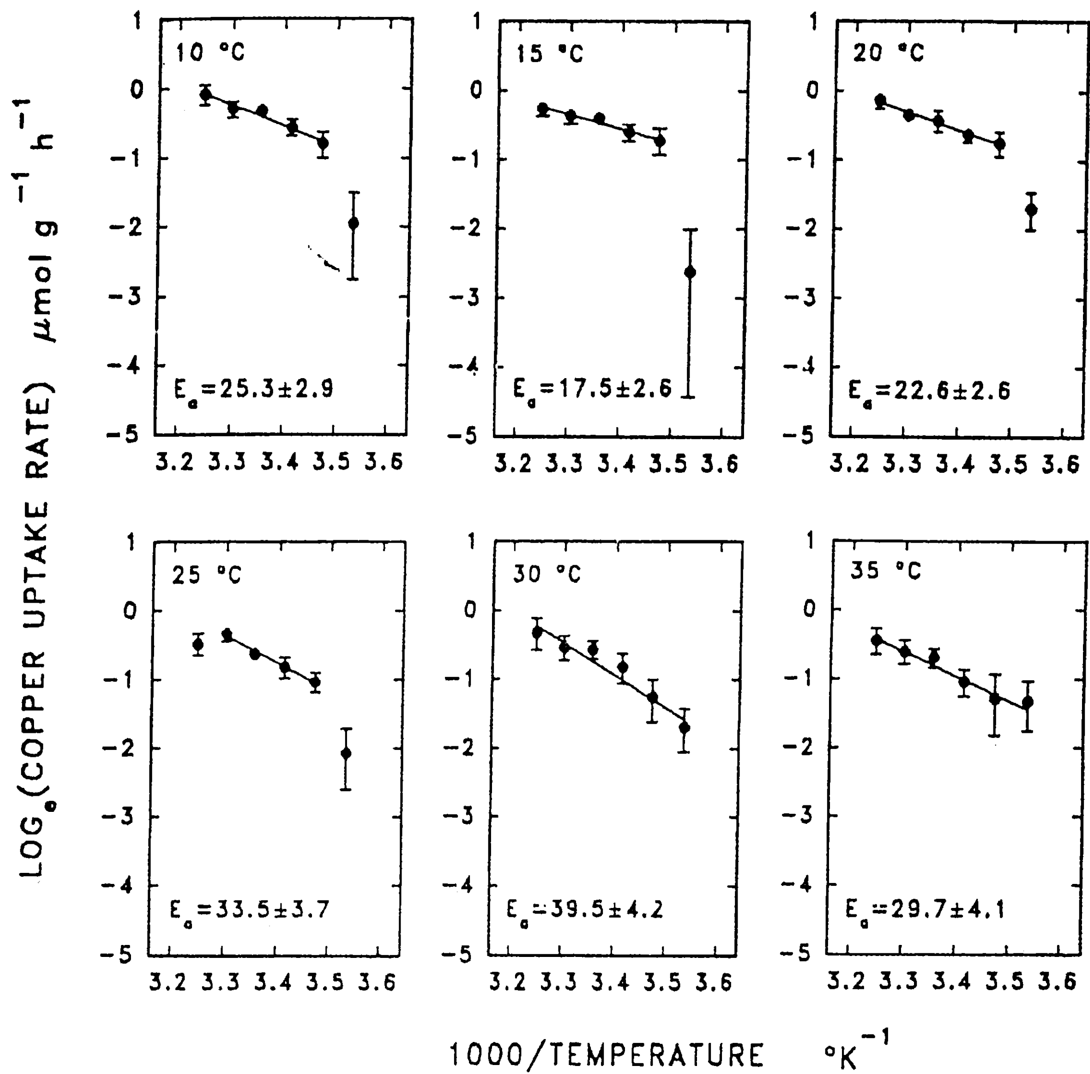

Fig 4. Arrhenius plots for the effect of temperature of exposure on the rate of copper uptake by brine shrimp for the different temperature acclimation groups (Cu_{Total}=5mmol.l^{-1}, sal=3.5%, pH= 8.0±0.1). The fit of the regression line to the data is significant for all groups ($p<0.001$), there are no significant deviations from regression ($p>0.05$) and the slopes of the regression lines are significantly different ($p<0.001$).

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Bioavailability of dissolved zinc to the common mussel, *Mytilus edulis*, in complexing environments

INTRODUCTION

In seawater dissolved trace metals occur as a variety of complexes with inorganic and organic ligands (Turner et al 1981, Motekaitis & Martell 1987, Bruland 1989). Turner et al. (1981) modelled the inorganic speciation of trace metals in seawater and showed that about half of the total zinc concentration exists as the free zinc ion, with the remaining zinc distributed among a series of inorganic species. Bruland (1989) demonstrated the existence of organic ligands in low nanomolar concentrations which form zinc complexes that strongly decrease the concentration of the free zinc ion.

The major factor controlling the uptake of metals by marine organisms is the bioavailability of the metal. The biologically available fraction of a metal is that part of the total concentration in a form that can be taken up by an organism (Turner 1984). The bioavailability of metals to aquatic organisms strongly depends on the chemical speciation and it is generally accepted that the free metal ion is the most biologically available species (Zamuda & Sunda 1982, Blust et al. 1992, Rainbow et al. 1993).

A widely accepted mechanism for metal uptake is the binding of the free metal ion to a membrane embedded channel type protein which mediates the transport of the metal ion across the membrane. The selectivity of these transporters depends on the size and charge of the channel which could impede the transport of certain metal species (Simkiss & Taylor 1989).

In the present work the effect of complexation by different water soluble ligands on the uptake of zinc by the common mussel, *Mytilus edulis* has been studied in chemically defined seawater. The speciation of zinc was controlled by varying the concentration of five organic ligands. The ligands studied were citrate, glycine, histidine, NTA (nitrilotriacetic acid) and EDTA (ethylenedinitrilo-tetraacetic acid). These ligands were selected on the basis of differences in the thermodynamic stability and charge of the formed zinc complexes which are representative for the wide variety of ligands in natural waters. Zinc uptake was studied in solutions of differing complexation capacity to reveal the role of the free metal ion and other species in determining the uptake of zinc in relation to the type and concentrations of the ligand present in the solution.

MATERIALS AND METHODS

Mytilus edulis

Mussels of approximately 6-8 cm in length, were collected at low water sites at Westkapelle (The Netherlands) and were kept in a seawater aquarium containing 350 litres of synthetic seawater at a salinity of 35 ‰ (pH 8.0, 15°C ± 1°C). The aquarium water was filtered over a trickling filter and weekly tested for NH_4^+ (< 0.25 $mg.l^{-1}$), NO_2^- (< 0.1 $mg.l^{-1}$) and NO_3^- (< 10 $mg.l^{-1}$l) (Hawkins 1981). The mussels were fed with a mixture of dried *Spirulina platensis* and *Saccharomyces cerevisiae* every 2 days (8 mg dry weight per animal). Under these conditions the animals could be maintained for several months. Zinc uptake rates for the mussels did not change during this period.

Experimental procedures

Experiments were performed in static exposure systems containing 0.5 l chemically defined seawater (table 1). Zinc uptake by individual mussels was measured over a 24 hours period. Chemically defined seawater was obtained by dissolving the seven analytical grade reagents (Merck, Darmstadt, Germany) in deionised water. A dispersion of 1.10^{-4} $mol.l^{-1}$ manganese dioxide was added to the seawater to remove the metal impurities present in the analytical grade reagents. After an equilibration period of 24 hours, the dispersion was filtered through a 0.2 µm membrane filter to remove the manganese dioxide from the solution (Van den Berg & Kramer 1979).
Although mussels were exposed to chemically defined seawater the complexation properties of the seawater changed during the experimental period. This is due to the fact that the mussels excrete organic compounds (e.g. amino acids) in the water which increases the complexation capacity of the water. At very low zinc concentrations (e.g. 0.01-0.1 $\mu mol.l^{-1}$) this causes a significant change in the chemical speciation and biological availability of the metal. To minimise this effect, the mussels were exposed to a higher zinc concentration (5 $\mu mol.l^{-1}$) so that only a small fraction of the zinc is complexed by the organics which accumulate in the water during the experiment.
Seawater zinc concentrations reported in literature typically range from 1.5 to 150 $nmol.l^{-1}$, but much higher concentrations may be reached in highly polluted areas (Brewer, 1975, Martin & Whitfield, 1981, Langston, 1990). Exposing mussels to elevated zinc concentrations may cause toxic effects. Toxicity varies strongly with exposure conditions and the developmental stage of the organisms. For adult mussels 96-hours LC_{50} values of 38 $\mu mol.l^{-1}$ (Ahsanullah, 1976) and 119 $\mu mol.l^{-1}$ (Abel, 1976) have been reported. Exposure to zinc also has acute effects at much lower concentrations. Filtration rates decrease when mussels are exposed to concentrations of 7.65 $\mu mol.l^{-1}$ or higher (Abel, 1976). Pumping rates decrease when mussels are exposed to concentrations of 4.74 $\mu mol.l^{-1}$ or higher. (Redpath & Davenport, 1988). Hence, exposing mussels to 5 $\mu mol.l^{-1}$ zinc could result in responses which may affect metal uptake rates. To test the effect of 5 $\mu mol.l^{-1}$ zinc on the mussels, filtration rates of control and zinc exposed mussels were measured over a period of 24 hours using the procedure described by Abel (1976). The results showed that filtration rates did not decrease significantly in the zinc exposed

mussels. This, together with the fact that zinc uptake remained linear over the 24 hours exposure period, indicates exposing the mussels to 5 $\mu mol.l^{-1}$ of zinc does not affect uptake rates.
Before each experiment, the mussels were scrubbed to remove epibionts attached to the shells. Twenty four hours prior to the start of the experiment the exposure medium was spiked with 296 $kBq.l^{-1}$ ^{65}zinc (Amersham Bucks, UK). The 1 ml ^{65}zinc stock solution contained 37 MBq for 1.530 mg zinc. Diluted stock solutions of ^{65}zinc were prepared in 1 $mmol.l^{-1}$ HCl. The mussels were exposed to this medium for 24 hours at 15 ± 1°C. pH was checked before and after the experiment and varied less than 0.1 units.
After exposure, each mussel was transferred for 20 minutes to 0.5 litre chemically defined seawater to replace the radioactive water contained in the mantle cavity. This seawater contained 5 $\mu mol.l^{-1}$ $ZnCl_2$ and 1 $mmol.l^{-1}$ of the ligand 8-hydroxyquinoline-5-sulfonic acid but no radioactive tracer. This ligand was added to remove metal bound to the body surfaces. The whole mussel was placed in a preweighed 18 ml Maxi Vial (Canberra Packard, Meriden, USA) and counted in a Packard Minaxi Auto-Gamma counter (Model 5530) for 1 minute to determine ^{65}zinc activity.
Using a file with a triangular cross section, an insertion was made in the shell at the height of the posterior adductor muscle. The shell valves were slightly pried open with an oyster knife, allowing water to drain off the mantle cavity. Approximately 0.5 ml hemolymph was obtained by direct puncturing the sinus of the posterior adductor muscle using a syringe (0.45 x 12 mm, Terumo Europe, Leuven, Belgium) (Zurburg & Kluytmans 1980). The shell valves were opened by cutting the posterior adductor muscle. The mussels were dissected for gills and digestive system. The shells were removed and placed in a preweighed Maxi Vial. Hemolymph and the different soft parts were each placed in a 6 ml preweighed Pony Scintillation Vial (Canberra Packard, Meriden, USA). Fresh weight of the samples was determined and 1 ml of tissue solubiliser (Soluene-350, Canberra Packard, Meriden, USA) was added to hemolymph, gill and digestive system to obtain a homogeneous sample and improve energy detection in the gamma counter. The hemolymph samples were weighed and the total amount of hemolymph was calculated based on the data of Martin et al. (1958). The vials were shaken for 24 hours to promote solubilisation of the tissues. After solubilisation the samples were placed in the gamma counter for 10 minutes to determine ^{65}zinc activity.

Chemical speciation model

To calculate the concentrations and activities of the metal species, a chemical speciation model was constructed. The model calculates the equilibrium speciation from a compilation of the interactions among the components present in the solution (sal 35 ‰, pH 8.00, 15°C). Stability constants used in the calculations are based on the data of Dickson & Whitfield (1981) for the major components, and on the data of Smith & Martell (1976, 1989) and Martell & Smith (1974, 1977, 1982) for the zinc species. For each species the stability constants at different ionic strengths were fitted to an interpolation function that has the form of an extended Debye-Hückel equation (Turner et al. 1981). These fitted equations were used in the computer program SOLUTION (Blust et al. unpubl.), an adaptation of the program COMPLEX (Ginzburg 1976). Enthalpies to describe the effect of temperature on the equilibrium

position of the reactions were taken from Byrne et al. (1988) and Smith & Martell (1989).
Results of the chemical speciation calculations are expressed on the molar concentration scale and multiplied by the appropriate activity coefficients to obtain species activities. The activity coefficients were based on the data of Millero (1982) for the free zinc ion, and on the data of Sposito & Traina (1987) for the other zinc species. The thermodynamic stability constants and the stability constants at the ionic strength of seawater are given in table 2.

Determination of zinc uptake rates

Measurements of ^{65}zinc activity were used to calculate zinc uptake rates. Radioactive zinc was measured in a preset energy window (165-1300 keV). Stock solutions were used for the construction of a calibration curve, so that the counting efficiency could be determined. The counts per minute (CPM) were corrected for background and radioactive decay. ^{65}Zinc can be determined with a 17.8% counting efficiency in a Minaxi Auto-Gamma counting system fitted with a 3 inch thallium activated sodium iodide crystal. The counting efficiency gives the relation between the counts per minute (CPM) and the disintegrations per minute (DPM) of ^{65}zinc. $Zinc^{2+}$ uptake ($nmol.g^{-1}.h^{-1}$) was calculated according the formula:

$$Zinc^{2+}{}_{uptake} = \frac{DPM^{65_{Zn}}}{WxtxSAx60}$$

in which W is the fresh weight (g), t is the exposure time (hours), SA is the specific activity ($Bq.nmol^{-1}$) and 60 is a constant factor to transform dpm to Bq (1 Bq = 1 disintegration per second and 1 Bq = 60 dpm).

Uptake of zinc as function of time and concentration

To test the linearity of uptake in function of time and concentration two preliminary experiments were conducted. In a first experiment accumulation of zinc by mussels exposed to 5 $\mu mol.l^{-1}$ $ZnCl_2$ was followed for 24 hours. After 1, 3, 6, 12, 24 hours the mussels were transferred to a rinsing solution for 20 minutes and processed. Preliminary experiments showed that this period was sufficient to remove the rapidly exchangeable pool of zinc adsorbed on the external surfaces of the animal. In a second experiment accumulation of zinc was followed in solutions containing 1 to 20 $\mu mol.l^{-1}$ $ZnCl_2$ over a 24-hour period. The same experimental procedures were followed as described previously.

Uptake of zinc in complexing environments

To determine the effect of complexation on the uptake of zinc, five organic ligands: EDTA (ethylenedinitrilotetraacetic acid), NTA (nitrilotriacetic acid), histidine, citrate and glycine, were used. The differences in stability constants between the zinc-ligand complexes generated a wide range of free zinc ion activities. The ligands were added to the solutions from concentrated stocks prepared in deionised water.

The experiments were performed in either the absence or presence of a metal ion buffer. In the first case, 5 $\mu mol.l^{-1}$ $ZnCl_2$ and a fixed concentration of one of the ligands was added. Each of the five ligands were tested for 9 different concentrations. In the second case, an excess of $ZnCl_2$ (1×10^{-4} $mol.l^{-1}$) and EDTA (9.55×10^{-5} $mol.l^{-1}$) and a fixed concentration of one of the ligands was added, so that the same free zinc ion activity in both, buffered and non buffered, environments was obtained. The same experimental procedures were followed as described previously.

Statistical analysis

Regressions, correlations and analysis of variance were performed with STATISTICA (StatSoft Inc.,Tulsa). Significance levels of tests are indicated by asterisks according to the following probability ranges (ns: $p>0.05$, *: $0.05 \geq p > 0.01$, **: $0.01 \geq p > 0.001$, ***: $p \leq 0.001$).

RESULTS

Uptake of zinc as function of time and concentration

In a first experiment uptake of zinc was followed in mussels exposed to 5 $\mu mol.l^{-1}$ zinc over 24 hours (figure 1). The uptake of zinc by the soft tissues increases linearly with time of exposure during the first 24 hours. The slope of the absorption curve is the zinc uptake rate (v_{gill} = 0.188 $nmol.g^{-1}.h^{-1}$, $v_{hemolymph}$ = 0.017 $nmol.g^{-1}.h^{-1}$). Adsorption of zinc by the shell does not remain fully linear, indicating saturation of the shell binding sites.
In a second experiment uptake of zinc was determined after 24 hours of exposure to different zinc concentrations ranging from 1-20 $\mu mol.l^{-1}$. As shown in figure 2 a rectangular hyperbola yielded a good fit to the data indicating that zinc uptake displays saturable uptake kinetics. Uptake in gills remained linear up to a zinc concentration of 16 $\mu mol.l^{-1}$ and uptake in hemolymph remained linear up to a zinc concentration of 5 $\mu mol.l^{-1}$. These two experiments show that zinc uptake by the tissues does not saturate over a 24 hours period for mussels exposed to 5 $\mu mol.l^{-1}$ zinc. Therefore the following experiments were conducted over this time interval and at this zinc concentration. Table 3 shows the relative and absolute distribution of zinc in the different organs after 24 hours of exposure to 5 $\mu mol.l^{-1}$ zinc.

Chemical speciation model

The results of the chemical speciation calculations are shown in figure 3. In chemically defined seawater of pH 8.2, salinity 35 ‰ and 15°C, zinc is distributed over the following inorganic complexes: 52% as the free Zn^{2+} ion, 33.5% as chloro-complexes, 6% as carbonate complexes, 4.5% as sulphate complexes and 4 % as hydroxide complexes. The addition of an organic ligand decreases the free zinc concentration by the formation of several organic complexes. For the weakest ligands, like glycine, an excess of ligand must be added before the metal is completely complexed. For stronger ligands, like EDTA, the metal is already completely complexed at an equimolar concentration of ligand and metal.

Uptake of zinc in non-metal buffered complexing environments

Zinc uptake experiments were performed in chemically defined seawater with a fixed total zinc concentration of 5 $\mu mol.l^{-1}$ and 9 different concentrations of ligand. The 45 combinations generate a wide range of free zinc ion activities ranging from 1.5 x 10^{-11} to 5.5 x 10^{-7} $mol.l^{-1}$. In figure 4 the zinc uptake rates are plotted against the zinc ion activity in the seawater for digestive system, gills and hemolymph. In the three organs the uptake increases with increasing free zinc ion activity.
The correlations between the free zinc ion activity and the zinc uptake rates presented in table 4 show that there is a high degree of linear association between the two variables. The correlations between the activities of the zinc complexes and the zinc uptake rates are negative, implying that complexation decreases uptake. However, in the case of histidine the correlations between the activity of $ZnHis^{+}$ and zinc uptake rates are significantly positive indicating that $ZnHis^{+}$ promotes zinc uptake.

The results of a multiple linear regression analysis concerning the effect of histidine on zinc uptake are given in table 5. Considering the free zinc ion as the only species that is taken up by the mussel explains between 54 and 71% of the variation observed depending on the organ considered. Including the $ZnHis^+$ complex in the model explains between 84 and 87 % of the variation. Also including the $Zn(His)_2^0$ complex in the model does not decrease the amount of variation explained by the model.
To test whether there are differences among the five groups in zinc uptake, a linear regression model through the origin was fitted to the data for each of the five ligands. The model was fitted through the origin since there is no zinc uptake if there is no zinc in the medium. A convenient method for systematically comparing all pairs of regression slopes is to compute upper and lower confidence limits for each regression slope such that two slopes are significantly different when their limits do not overlap (Gabriel 1978). Regression slopes which confidence intervals overlap are not significantly different at the experimentwise error rate of $\alpha = 0.05$. Those which intervals do not overlap are significantly different. The regression slopes and their intervals for the five different ligands for digestive system, gills and hemolymph are shown in figure 5.

Uptake of zinc in metal buffered complexing environments

Metal ion buffers provide a controlled source of free metal ions. If free metal ions are removed from the solution, by adsorption or absorption, they are replenished by the reversible dissociation from the reservoir of metal in the buffer complex. Figure 6 gives the results of the uptake in non-metal buffered and metal buffered environments for gills, digestive system and hemolymph. The different groups were tested by one way analysis of variance (ANOVA) followed by Tukey Kramer multiple comparisons test. For gills the uptake of zinc is comparable for both environments. In contrast uptake of zinc by the digestive system and hemolymph is much higher in metal buffered environments. As before, histidine appears to enhance the uptake of zinc, since uptake in the presence of histidine is far above what is expected from the free zinc ion activity in the solution.

DISCUSSION

During the first 24 hours zinc uptake increases linearly with the time of exposure. Hence, a constant concentration gradient is maintained across the exchange surfaces during at least the first 24 hours of metal uptake. Pentreath (1973) and Renfro et al. (1975) followed zinc accumulation and showed that uptake remained linear over the first 2 to 3 days of exposure and that a steady state situation was only reached after 3 to 4 weeks.

As shown in table 3 the gills and the digestive system are the most important organs for zinc uptake. The gills and the digestive system of bivalve molluscs are known to be critical organs for the uptake and accumulation of metal ions from seawater (Pentreath 1973, Janssens & Sholz 1979, Amiard et al. 1986). Considering the absolute amounts of zinc, hemolymph becomes the most important compartment, since it is the largest soft body compartment. The blood volume of *Mytilus californianus* is about 51% of the wet weight of the soft tissues (Martin et al. 1958).

The relation between the total zinc concentration in the exposure medium and zinc uptake rate followed a hyperbolic curve. This means that zinc uptake is a saturable process indicating that a membrane transport system, i.e. a channel or carrier, takes the metal across the lipid bilayer. Such a system can be described by Michaelis-Menten kinetics which characterises the system by two parameters: V_{max} and K_m. V_{max} is the maximum velocity and K_m, in physical terms, can be thought of as a measure of the affinity of the transporter for the metal. The lower the K_m value the higher the affinity (Palmer 1991). The values for V_{max} and K_m in gills and hemolymph are respectively 65.9 and 1.7 $nmol.g^{-1}.h^{-1}$ and 44.03 and 4.1 $\mu mol.l^{-1}$. This means that zinc uptake is much faster in gills than in hemolymph. The affinity of the transporter which takes the metal from the epithelia to the hemolymph is much higher than the affinity of the transporter which takes the metal from the solution across the apical gill membrane.

Transport of metal ions across apical membranes is generally considered to be passive, involving the passage of the metal through size and charge selective channels. The interactions of the metal ion with the channel are rather weak involving mainly electrostatic forces. The transport of metal ions across the basolateral membranes is generally considered to be an active process involving stronger covalent interactions between the metal and the carrier. In general rates of channel transport are also higher than carrier mediated transport (Friedman 1986).

A number of previous studies have demonstrated the importance of the free metal ion activity in the bioavailability of trace metals to aquatic organisms (Zamuda & Sunda 1982, Jenkins & Sanders 1986, Blust et al. 1992). Specifically for mussels, some studies demonstrated an increase in metal uptake in the presence of organic ligands (George & Coombs 1977, Pempkowiak et al. 1989). However, the current study shows that organic ligands decrease metal uptake by reducing the bioavailable form of the metal, i.e. the free metal ion. In all cases, except histidine, there is a negative correlation between the activities of the zinc-ligand complexes and the uptake rates. This indicates that the metal complexes are poorly or not available to the animal. However, a direct relation between the free zinc ion activity and zinc uptake rate is not always apparent over the entire range of complexation conditions.

The observed differences can be caused by changes in the chemical speciation of the metals in the layers lining the exchange surfaces where conditions may diviate

considerably from seawater. This is certainly true for the digestive tract which secretes large amounts of mucus and digestive enzymes (Morton 1992).
The kinetics of metal complexation can also influence metal uptake when the rate of complex dissociation is slower than the rate of metal uptake. In this case the metal transporting system may deplete the surrounding solution of free zinc so that the rate of metal uptake decreases. Generally the rate of complex dissociation decreases with the thermodynamic stability constants of the metal ligand complex. Thus, for the same free metal ion activity, the rate of metal uptake should decrease with the thermodynamic stability of the metal complexes. Such a dependence is not observed indicating that zinc uptake is not constraint by complexation kinetics (Hering & Morel 1990).
In the case of histidine most of the variation in zinc uptake can be explained when the free zinc ion and a zinc-histidine complex are considered to be the metal species that are taken up. Zinc forms two different complexes with histidine (i.e. $ZnHis^+$ and $Zn(His)_2^0$). At low histidine concentrations $ZnHis^+$ is the prevalent complex while at higher histidine concentrations the $Zn(His)_2^0$ complex becomes more important. Formation of the $Zn(His)_2^0$ complex results in a decrease of zinc uptake indicating that only the $ZnHis^+$ complex facilitates the uptake of zinc. Previous studies have shown that histidine, increased the rate of copper uptake by facilitating the delivery of the metal to the copper transporting molecule on the membrane surface (Ettinger et al. 1986). Other authors proposed the direct cellular uptake of zinc-histidine complexes, via an amino acid transport system (Gachot et al. 1991, Aiken et al. 1992). Whether the mechanism of facilitation involves the direct tranport of the $ZnHis^+$ complex across the membrane cannot be deduced from the present study.
Zinc uptake rates in gills are not significantly different for the five ligands used. The slopes of the regression lines of zinc uptake by digestive system and hemolymph are not significantly different for citrate, glycine, NTA and EDTA. However the slope of the regression line for histidine is significantly different from the slopes of the other lines. This is the result of the contribution of $ZnHIS^+$ to the zinc uptake rate. As a consequence the fit is rather poor which is reflected in the broad confidence limit.
Comparing zinc uptake in non-buffered and buffered environments shows that for gills, uptake is comparable in both environments and proportional to the free zinc ion activity in the water for all ligands except histidine. This confirms the observation that histidine facilitates the uptake of zinc. In the digestive system zinc uptake is higher in the buffered environment, indicating that zinc is released from the ligands and the free zinc ion activity increases accordingly. The magnitude of the effect varies strongly with the ligand considered so that the relation between the free zinc ion activity and zinc uptake is totally lost under these conditions. Since hemolymph receives metal from both uptake sites, uptake of zinc in this compartment is also higher in a buffered than in a non-buffered environment.
This study has shown that uptake of zinc strongly depends on the chemical speciation of zinc in the solution. Complexation of zinc with organic ligands may increase or decrease zinc uptake depending on the ligand involved. Most ligands decrease zinc availability by reducing the activity of the free zinc ion in the solution but there is a marked exception. Histidine appears to promote the uptake of zinc even in the presence of a strong chelator. For the same free zinc ion activity the availability of zinc is higher in the digestive system than in the gills, indicating that zinc is released from its complexes when it enters the gut.

Table 1: Composition of 1 litre chemically defined seawater of salinity 35 $^{o}/_{oo}$. The medium was prepared by dissolving the seven analytical grade products in deionised water.

$NaCl$	23.50 g
Na_2SO_4	4.00 g
KCl	0.68 g
$NaHCO_3$	0.196 g
$CaCl_2.2H_2O$	1.47 g
$MgCl_2.6H_2O$	10.78 g
H_3BO_3	0.026 g

Table 2:Thermodynamic and conditional stability constants for the zinc species considered in the chemical speciation model. K: thermodynamic stability constants; Q: conditional stability constants (sal: 35 $^{o}/_{oo}$; pH 8.00; T: 15° C).

Species	log K	log Q
Zn^{2+}	-	-
$ZnOH^{+}$	4.99	4.58
$Zn(OH)_2$	10.20	9.00
$Zn(OH)_3^-$	13.90	13.06
$Zn(OH)_4^{2-}$	15.50	15.49
$ZnCl^{+}$	0.53	-0.17
$ZnCl_2$	0.69	-0.19
$ZnCl_3^-$	0.70	-0.29
$ZnCl_4^{2-}$	0.32	-0.37
$ZnSO_4$	2.32	0.94
$Zn(SO_4)_2^{2-}$	3.26	1.66
$Zn(SO_4)_3^{4-}$	2.03	1.84
$ZnCO_3$	5.10	3.83
$ZnHCO_3$	11.03	9.72
$ZnCIT^-$	6.07	5.09
ZnHCIT	10.00	8.12
$Zn(CIT)_2^{4-}$	7.22	6.51
$ZnGLY^{+}$	5.38	4.85
$Zn(GLY)_2$	9.81	9.02
$Zn(GLY)_3^-$	12.30	11.48
$ZnHIS^{+}$	6.88	6.38
$ZnHHIS^{2+}$	11.60	11.60
$Zn(HIS)_2$	12.67	11.88
$ZnNTA^-$	12.04	10.12
$Zn(NTA)_2^{4-}$	15.02	13.95
$ZnEDTA^{2-}$	17.94	16.37
$ZnHEDTA^-$	20.09	19.53
$ZnOHEDTA^{3-}$	19.75	18.38
ZnH_2EDTA	22.79	20.07

Table 3: Mytilus edulis. Relative importance of different organs for zinc uptake (N = 35; total zinc concentration : 5 $\mu mol.l^{-1}$).

A. Zinc uptake per gram fresh weight ($nmol.g^{-1}.h^{-1}$) ± standard deviations

Organ	Zinc uptake ($nmol.g^{-1}.h^{-1}$)
digestive system	10.3 ± 3.5
gills	5.3 ± 1.4
mantle	4.6 ± 2.7
foot	2.9 ± 0.9
muscle	2.2 ± 0.8
hemolymph	1.3 ± 0.5

B. Absolute zinc uptake in different tissues ($nmol.h^{-1}$) ± standard deviations

Organ	Zinc uptake ($nmol.h^{-1}$)
hemolymph	0.2 ± 0.1
muscle	0.11 ± 0.04
digestive system	0.09 ± 0.03
gills	0.05 ± 0.01
mantle	0.02 ± 0.01
foot	0.004 ± 0.001

Table 4: Mytilus edulis. Coefficients of determination (r^2) for linear correlations between the activities of the different zinc species in solution and the zinc uptake rate for hemolymph, gills and digestive system. The different complexes are represented by ZnL_n where L indicates the ligand used (EDTA, NTA, histidine, citrate, glycine) and n the number of ligands in the complex. Sign in front of determination coefficient designates whether the correlation is positive or negative. Significance levels indicated by asteriks according to the following probability ranges: ns = $p>0.05$; * = $0.05 \geq P > 0.01$, ** = $0.01 > P > 0.001$, *** = $P \leq 0.001$.

HEMOLYMPH

	Zn^{2+}	ZnL	ZnL_2	N
EDTA	0.75 ***	- 0.75 ***		57
NTA	0.70 ***	- 0.70 ***	- 0.14*	59
Histidine	0.45 ***	0.02 ns	- 0.61 ***	53
Citrate	0.66 ***	- 0.66 ***	- 0.34 ***	58
Glycine	0.83 ***	-0.13 *	- 0.71 ***	60

GILLS

	Zn^{2+}	ZnL	ZnL_2	N
EDTA	0.77 ***	- 0.77 ***		62
NTA	0.56 ***	- 0.56 ***	- 0.25 ***	60
Histidine	0.10 *	0.16 **	- 0.33 ***	60
Citrate	0.36 ***	- 0.36 ***	- 0.29 ***	61
Glycine	0.52 ***	- 0.00 ns	- 0.70 ***	62

DIGESTIVE SYSTEM

	Zn^{2+}	ZnL	ZnL_2	N
EDTA	0.79 ***	- 0.79 ***		62
NTA	0.72 ***	- 0.73 ***	- 0.22 ***	60
Histidine	0.36 ***	0.08 *	- 0.64 ***	59
Citrate	0.42 ***	- 0.42 ***	- 0.42 ***	60
Glycine	0.64 ***	- 0.04 ns	- 0.73 ***	61

Table 5: Mytilus edulis. Fitting parameters (C_1, C_2) and coefficients of determination (r^2) for multiple linear regression between the activities of the free zinc ion, the different zinc-histidine species and the zinc uptake rate for hemolymph, gills and digestive system. Concentrations of zinc species are in $nmol.l^{-1}$ and uptake rates in $nmol.kg^{-1}.h^{-1}$. Significance levels indicated by asteriks according to the following probability ranges: ns = $p > 0.05$; * = $0.05 \geq P > 0.01$, ** = $0.01 > P > 0.001$, *** = $P \leq 0.001$.

	C_1	C_2	C_3	N	r^2
$Zn_{uptake} = C_1 \times Zn^{2+}$					
Hemolymph	3.390***			53	0.71***
Gills	11.475***			60	0.54***
Digestive System	23.310***			59	0.67***
$Zn_{uptake} = C_1 \times Zn^{2+} + C_2 \times ZnHIS^{+}$					
Hemolymph	2.789***	0.345***		53	0.84***
Gills	7.620***	2.234***		60	0.85***
Digestive System	17.910***	3.295***		59	0.87***
$Zn_{uptake} = C_1 \times Zn^{2+} + C_2 \times ZnHIS^{+} + C_3 \times ZnHIS_2^{0}$					
Hemolymph	2.770***	0.369***	-0.033^{ns}	53	0.83***
Gills	7.794***	2.034***	0.273^{ns}	60	0.86***
Digestive System	17.729***	3.511***	-0.288^{ns}	59	0.83***

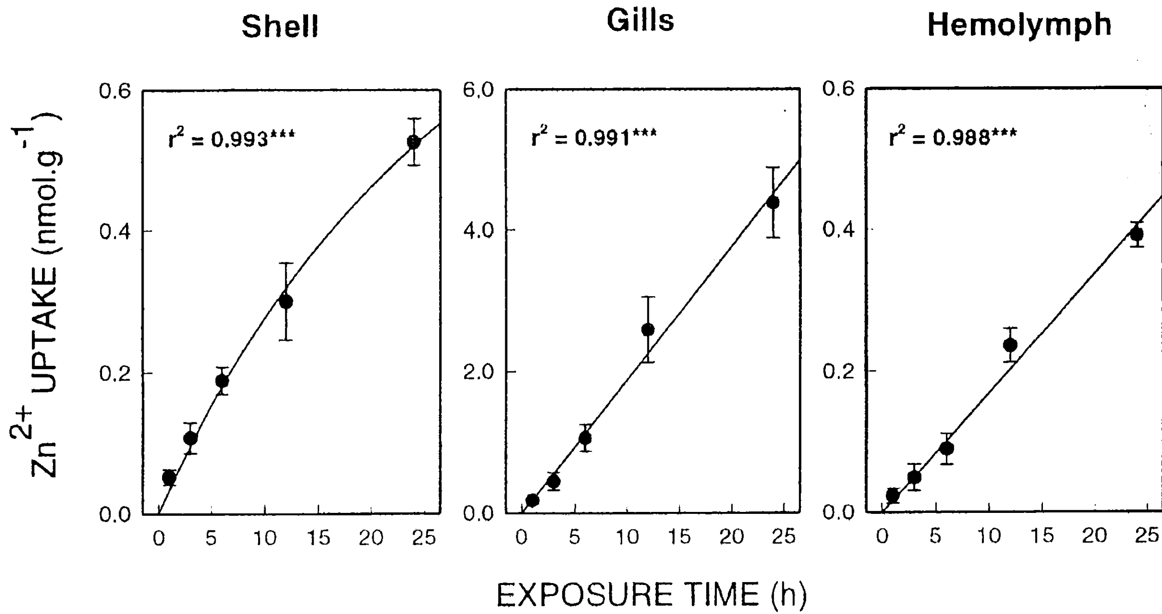

Figure 1: Mytilus edulis. Uptake of zinc by shell, gills and hemolymph as function of exposure time (Zn_T : 5 µmol.l^{-1}). Data points represent means for 7 replicates with standard deviations. *** = $P \leq 0.001$.

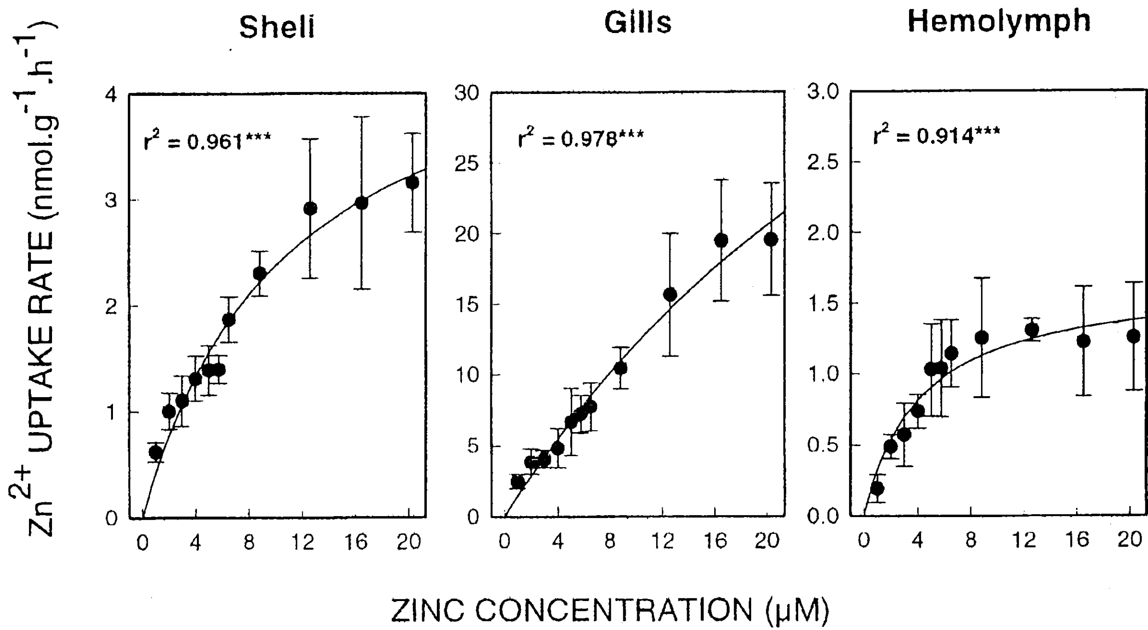

Figure 2: Mytilus edulis. Uptake of zinc by shell, gills and hemolymph as function of the total zinc concentration (exposure time: 24 hours). Data points represent means for 7 replicates with standard deviations. *** = $P \leq 0.001$.

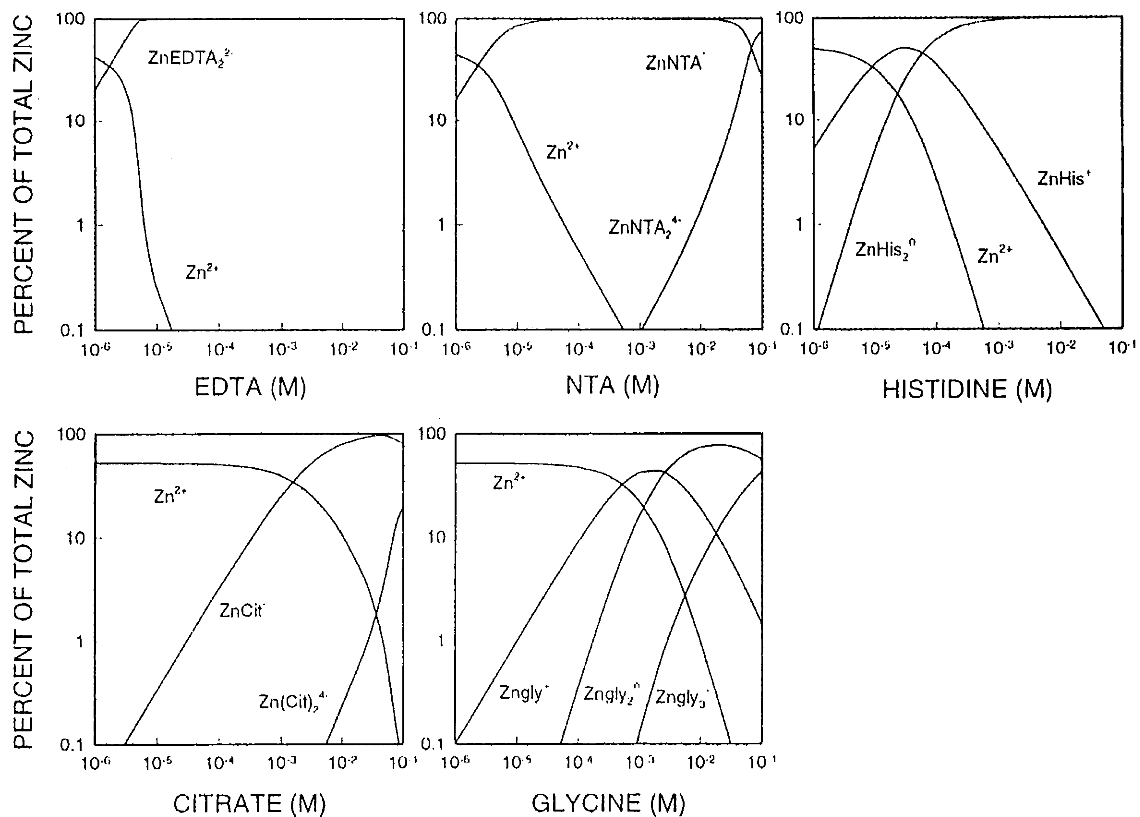

Figure 3: Effect of complexation by five organic ligands (EDTA, NTA, histidine, citrate and glycine) on the chemical speciation of zinc in seawater containing 5 µmol.l^{-1} zinc (sal: 35 ‰; pH: 8.00; T:15 °C).

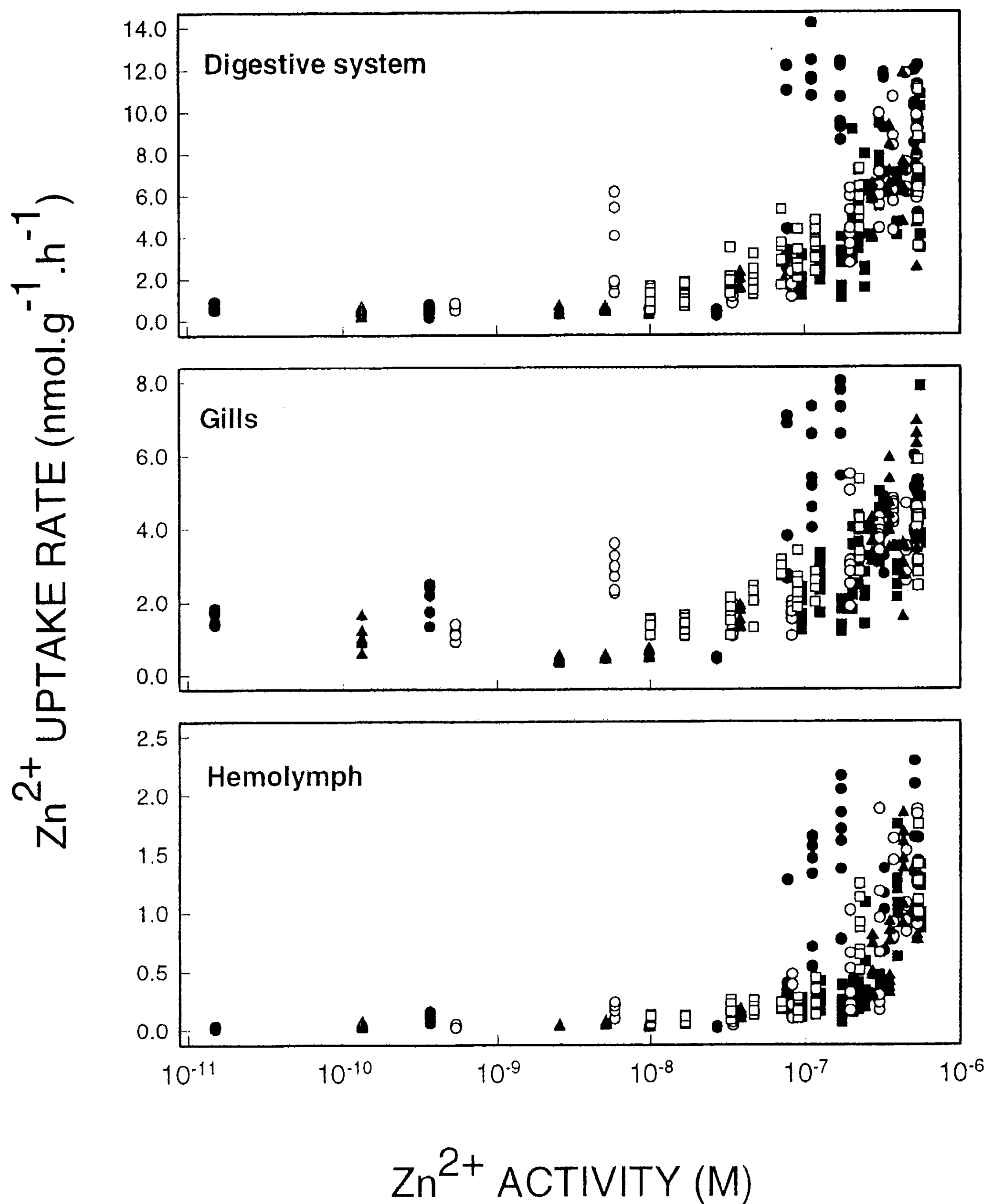

Figure 4: Mytilus edulis. Uptake of zinc by digestive system, gills and hemolymph. Effect of organic complexation on the zinc uptake rate in function of the free zinc ion activity in a solution containing 5 $\mu mol.l^{-1}$ zinc. (sal 35 ‰; pH:8.00; T:15°C). (solid triangles: EDTA; open circles: NTA; solid circles: histidine; open squares: glycine; solid squares: citrate).

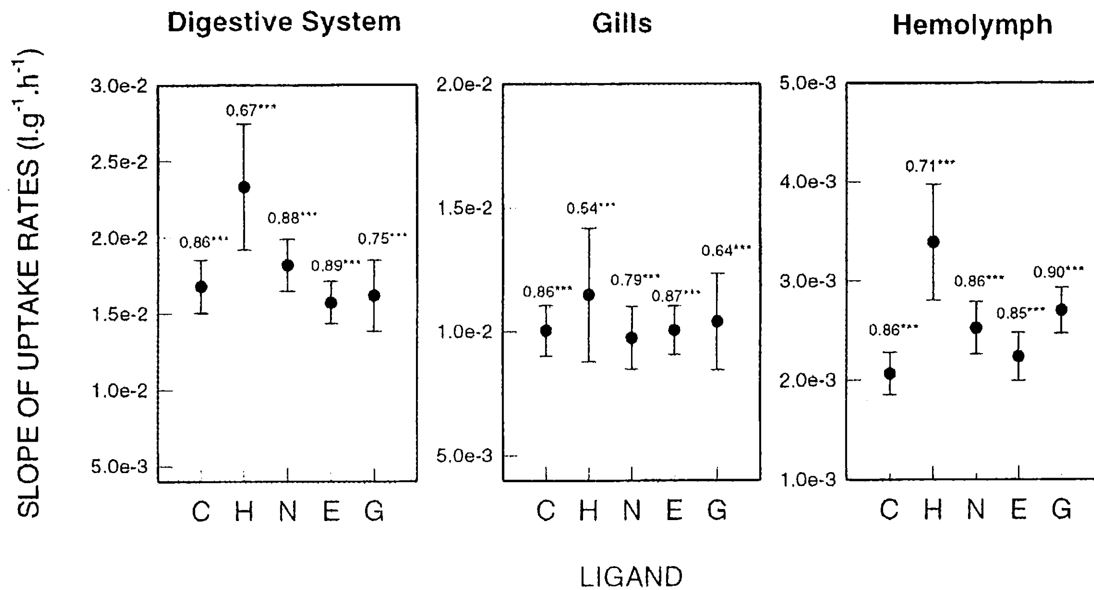

Figure 5: Mytilus edulis. 95% comparison intervals for the slopes of zinc uptake rate on free zinc ion activity for digestive system, gills and hemolymph.
Regression coefficients whose intervals do not overlap are significantly different. The coefficients of determination (r^2) for the linear regression through the origin between the free zinc ion activity (nM) and the zinc uptake rate ($nmol.g^{-1}.h^{-1}$) are given for each ligand. (E:EDTA; N:NTA; H:histidine; C:citrate; G:Glycine). *** = $P \leq 0.001$.

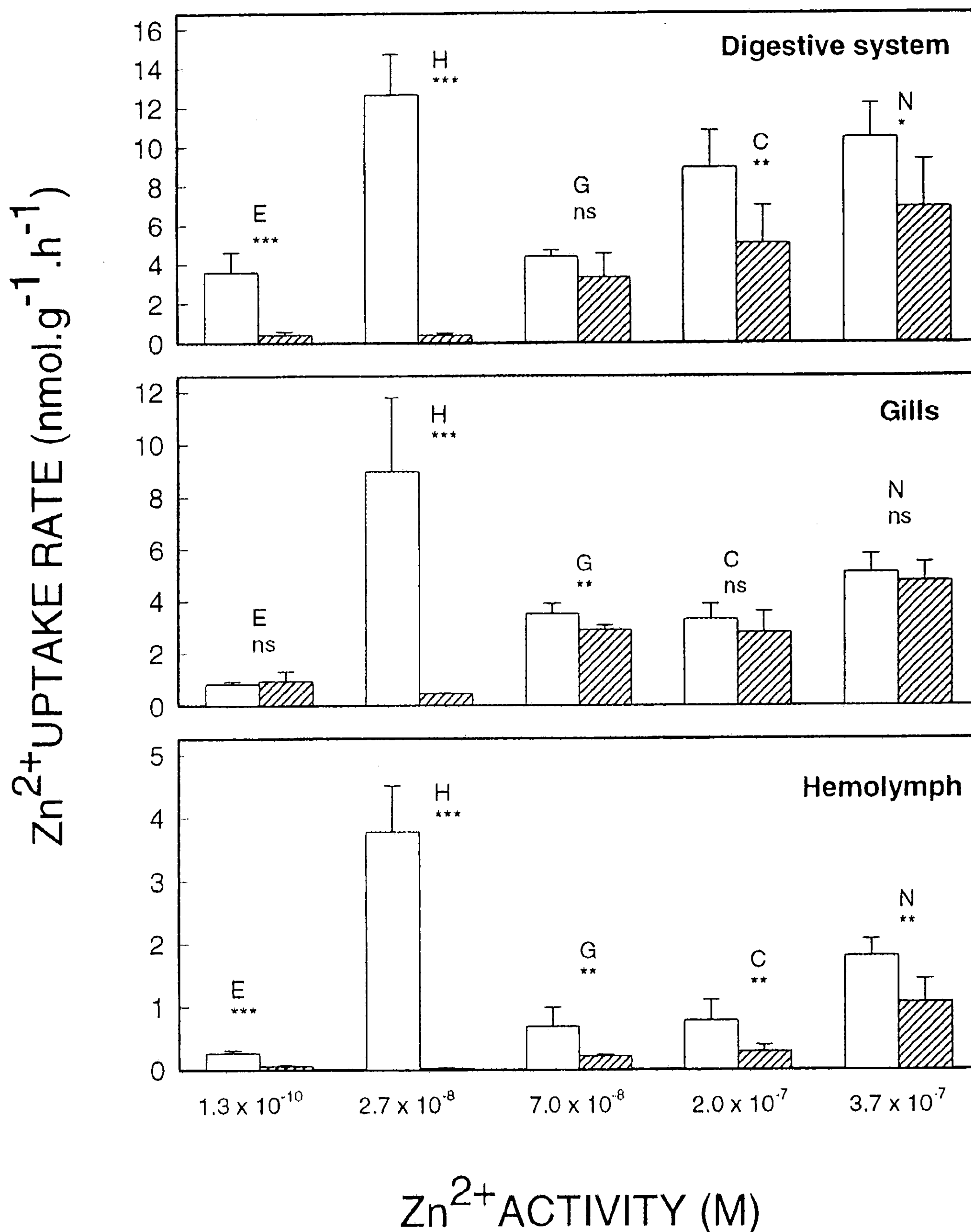

Figure 6: Mytilus edulis. Comparison of the zinc uptake rate by digestive system, gills and hemolymph in metal buffered (open bars) and non metal buffered (hatched bars) solutions at the same free zinc ion activities. (E:EDTA; N:NTA; H:histidine; C:citrate; G:Glycine). Significance levels indicated by asteriks according to the following probability ranges: ns = $p>0.05$; * = $0.05 \geq P > 0.01$, ** = $0.01 > P > 0.001$, *** = $P \leq 0.001$.

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The effect of salinity on the uptake of zinc and cadmium by the common mussel, *Mytilus edulis* and the relative importance of the calcium channel

INTRODUCTION

The uptake and toxicity of metals in aquatic organisms strongly depends on the biological availability of the metals. The biological availability in its turn depends on the chemical speciation of the metals and the organisation of the exchange surfaces which separate the animal from its environment (Simkiss and Taylor, 1995). A widely accepted mechanism for metal uptake is the binding of the free metal ion to a membrane embedded channel type protein which mediates the transport of the metal ion across the membrane. Uptake of metals is therefore usually explained on the basis of the free ion activity of the metals rather than the total concentration of the metals (Sunda et al., 1978; Blust et al. 1992, 1995). The uptake of heavy metals from solution into marine invertebrates at permeable surfaces is generally considered to be a passive process not requiring metabolic energy, a situation constrasting with that for metals such as sodium, potassium and calcium (Carpene and George, 1981; Simkiss and Taylor, 1989).

Salinity is one of the most important factors which varies in aquatic environments, starting at very low levels in freshwaters and reaching extremely high levels in some hyper saline lagoons (Kennish, 1989). In estuaries, animals are exposed to tidal and seasonal fluctuations in salinity causing changes in metal exposure and uptake.

One of the most important effects of salinity on the chemical speciation of metals is the change in ligand concentration (e.g. chloride, carbonate, sulphate) which controls the free metal ion concentration (De Lisle and Roberts, 1988; Blust et al., 1992; Rainbow et al., 1993; Bjerregaard and Depledge, 1994). So the bioavailability of metals increases at lower salinities due to the increased free metal ion concentration. Salinity also influences the ionic strength of the water, which determines the activity coefficient of the chemical species (activity=activity coefficient x concentration) (Millero, 1990). In addition, salinity also has an important effect on the physiological condition of the animal. The filtration rate can change, the permeability can increase or decrease or there can be an increased competition with other divalent ions, such as calcium or magnesium, for the same uptake site at higher salinities (Wright et al 1977, Roesijadi and Unger, 1993, Bjerregaard and Depledge, 1994).

During the last few years, there is strong evidence that heavy metal ions are capable of interacting with ionic channels, receptors, and/or ionic-channel-receptor complexes. Earlier workers demonstrated the interaction between the uptake of calcium and other divalent ions (Wright, 1977; Hinkle et al., 1987; Verbost et al., 1988,1989a&b; Blazka and Shaikh, 1991; Chow, 1991; Roesijadi and Unger, 1993), suggesting that calcium and metal ions are taken up through the same channel, namely the calcium channel. The mechanism of metal ion interaction can be quite diverse. Metal ions may compete with essential ions (such as Ca^{2+}) for uptake sites or they may react with sulphydryl groups of enzymes altering the enzyme function.

Different types of Ca channels are known to exist and are characterised by fundamental differences in the mechanisms concerning their opening and closing (Hosey and Lazdunski, 1988).

Some Ca channels are voltage dependent and open in response to an appropriate membrane depolarisation. Within this category there are subclasses that differ in their voltage sensitivities, kinetic properties, pharmacological sensitivities, etc. Three types of voltage dependent Ca channels are known to exist. They are referred as: (i) the L-type, high-voltage activated which conducts a long lasting current of

large conductance; (ii) the T-type, low -voltage activated which is activated at low voltages and is characterized by transient currents with small conductance and (iii) the N-type channel which is neither T nor L, but is activated at relatively high voltages and conducts a relatively transient current of intermediate size. Voltage dependent Ca^{2+} channels found in a variety of cells are blocked more or less effectively by di- or trivalent cations such as Co^{2+}, Cd^{2+}, La^{3+}, Mg^{2+} (Tsien et al. 1989). Other Ca channels are receptor operated because they are operated through receptor dependent mechanisms and open in response to activation of an associated receptor (Hosey and Lazdunski, 1988). Drugs that specifically interact with Ca channels (voltage sensitive and receptor operated) were initially developed because of their potential usefulness in cardiovascular research. Drugs that have received most attention belong to three distinct classes: (i) 1,4-dihydropyridine derivates such as nifedipine, (ii) phenylalkylamines such as verapamil and (iii) benzothiazepines such as diltiazem.

It should be stressed that it remains to be clarified whether there is any link between the uptake of particular heavy metals and that of calcium and whether physiological responses of estuarine invertebrates to reduced salinities are more significant to heavy metal uptake than associated physicochemical changes in the surrounding seawater.

The present work was performed to study the effect of acclimation and exposure salinity and the effect of calcium on the uptake of zinc and cadmium by the common mussel, *Mytilus edulis*. To determine whether the calcium transport system is involved in the uptake of zinc and cadmium and if the uptake of these metals is ATP dependent, different inhibitors were used. This to identify and quantify the relative importance of the different chemical and biological factors which contribute to the observed changes in metal uptake by mussels exposed to different salinities.

MATERIALS AND METHODS

Mytilus edulis

Mussels of approximately 6-8 cm in length, were collected at low water sites at Westkapelle (The Netherlands) and were kept in a seawater aquarium containing 350 litres of synthetic seawater at a salinity of 35 ‰ (pH 8.0, 15°C ± 1°C). The aquarium water was filtered over a trickling filter and weekly tested for NH_4^+ (< 0.25 $mg.l^{-1}$), NO_2^- (< 0.1 $mg.l^{-1}$) and NO_3^- (< 10 $mg.l^{-1}$l) (Hawkins 1981). The mussels were fed with a mixture of dried *Spirulina platensis* and *Saccharomyces cerevisiae* every 2 days (8 mg dry weight per animal). Under these conditions the animals could be maintained for several months. Zinc uptake rates for the mussels did not change during this period.

Experimental procedures

Experiments were performed as described previously (Vercauteren and Blust, 1996). For the acclimation experiments, the organisms were acclimated for at least 28 days to different salinities. These salinities were obtained by diluting the chemically defined seawater with deionised water. The solutions were aerated for 24 hours to establish equilibrium with the atmosphere and pH stabilisation (8.0-8.2).
The uptake of zinc and cadmium was followed by means of the radioactive tracers ^{65}Zn and ^{109}Cd (Amersham International). Twenty four hours prior to the start of the experiments, the exposure medium was spiked with 296 $kBq.l^{-1}$ ^{65}Zn and 296 $kBq.l^{-1}$ ^{109}Cd. Diluted stocks were prepared in 1mmol HCl. Uptake of the metals by the mussels was followed over a 24 hours period in chemically defined solutions to which 1 $\mu mol.l^{-1}$ Zn and 0.1 $\mu mol.l^{-1}$ Cd were added.

Speciation of zinc and cadmium as function of salinity

The equilibrium activitities of the different species were calculated using the computer program SOLUTION (Blust and Van Ginneken, unpubl.) an adaptation of the program COMPLEX (Ginzburg, 1976) This speciation model allows the calculation of the composition of solutions in equilibrium with the atmosphere. The model uses the ion-association concept which invokes the existence of molecular species like free ions, ionpairs and complexes. Stability constants used in the calculations are based on the data of Dickson & Whitfield (1981) for the major components, and on the data of Smith & Martell (1976, 1989) and Martell & Smith (1974, 1977, 1982) for the zinc and cadmium species. For each species the stability constants at different ionic strengths were fitted to an interpolation function that has the form of an extended Debye-Hückel equation (Turner et al. 1981).

Experiments

In a first experiment, the effect of the salinity of acclimation on the uptake of zinc and cadmium, was determined. Mussels were acclimated to 6 different salinities. Organisms of each acclimation group were exposed to solutions of the salinity of acclimation.

In a second experiment, organisms of the lowest and the highest salinity acclimation groups (10 and 35 $^0/_{00}$) were exposed to solutions of differing salinity (10-15-20-25-30-35 $^0/_{00}$).
In a third experiment the uptake of the metals was measured in calcium free seawater. These experiments were performed with mussels acclimated to a salinity of 35 $^0/_{00}$. For these experiments the chemically defined seawater was prepared by replacing all the calcium ions by sodium and potassium ions so that the osmolarity of this solution was the same as normal seawater of 35 ppt. This calcium free seawater was further diluted to obtain lower salinities. The mussels were exposed to solutions of differing salinity (10-15-20-25-30-35 $^0/_{00}$).
To determine the effect of salinity on pumping rate, a filtration experiment was performed. The filtration rate was determined by determining the rate at which yeast (*Saccharomyces cerevisae)* cells were removed by the mussels from the exposure system. These yeast cells were chemically treated with sulphydryl compounds to improve its digestibility (Coutteau et al., 1990). Each mussel was placed in a separate beaker containing 500 ml chemically defined seawater of the specified salinity. After mussels opened their valves, yeast cells were added to each beaker to give an initial concentration of 25000 cells.ml^{-1}. The water was aerated to maintain a homogeneous distribution of the cells. Every 15 minutes a 10 ml aliquot from each beaker was taken and the cell concentration was counted using an electronic particle counter (Coulter Counter Model ZF). The filtration constant was determined by measuring the removal of yeast cells from the seawater as function of time. The results were fitted to the equation $C = IC \times e^{-k.t}$ with C the concentration of cells in cells.ml^{-1}, IC the initial cell concentration in cells.ml^{-1} and t the filtration time in minutes. In this equation k is the filtration rate constant which is estimated by least square regression.
In a subsequent experiment we examined the effects of the following calcium channel inhibitors : nifedipine(100 µM), verapamil (250 µM), diltiazem (250 µM), La^{3+}(250 µM); the effects of the metabolic inhibitors: sodium cyanide (1 mM) and 2,4-dinitrophenol (1 mM) and the effect of the Na-K ATP-ase inhibitor ouabain (1mM) on the uptake of zinc and cadmium by the common mussel. The inhibitors were purchased from Sigma Chemical Co. (St Louis, MO).

Statistical analysis

Multiple analysis of variance followed by a post hoc Newman Keuls test were performed with STATISTICA (StatSoft Inc.,Tulsa). Significance levels of tests are indicated by asterisks according to the following probability ranges (ns: $p>0.05$, *: $0.05 \geq p > 0.01$, **: $0.01 \geq p > 0.001$, ***: $p \leq 0.001$).

RESULTS

Chemical speciation

The inorganic speciation of cadmium in seawater is controlled by the formation of chloride-complexes. The free cadmium ion becomes only a major species at low salinity. At a salinity of 35 $^{0}/_{00}$ the free cadmium ion constitutes 3.0 % and at a salinity of 10 $^{0}/_{00}$ the free cadmium ion constitutes 13.4 % of the total cadmium concentration.The inorganic speciation of zinc in seawater is less controlled by chloride complexes. Although zinc-chloride complexes are formed with increasing salinity, free zinc ion concentration does not change to such a great extent with salinity. At a salinity of 35 $^{0}/_{00}$ the free zinc ion constitutes 52.1 % and at a salinity of 10 $^{0}/_{00}$ the free zinc ion constitutes 65.7 % of the total zinc concentration (figure 1). Results of the chemical speciation calculations were expressed on the molar concentration scale and multiplied by the appropriate activity coefficients to obtain species activitites. The activity coefficients were based on the data of Millero (1982) for the free zinc ion and on the data of Sposito and Traina (1987) for the free cadmium ion.

Salinity experiments

Upake experiments were conducted in chemically defined seawater for 24 h to which 1 $\mu mol.l^{-1}$ Zn and 0.1 $\mu mol.l^{-1}$ Cd were added. During this time period and at these metal concentrations the metal uptake by *Mytilus edulis* remained linear. Hence a constant concentration gradient is maintained across the exchange surfaces. (Carpene and George, 1981; Vercauteren and Blust, 1996). In figure 2 and 3 it appears that the uptake of zinc and cadmium does not change considerably with salinity when mussels are exposed to the salinity of acclimation (10-35$^{0}/_{00}$). Generally, a significant relation between the free metal ion activity and metal uptake could not be demonstrated. Such effect is not expected for zinc since the free zinc ion activity does not change considerably in the range of salinities used. For cadmium the free cadmium ion activity changes considerably but an effect on uptake was not oberved. Only the adsorption of cadmium on the shells increased with decreasing salinity. The observed increase in cadmium binding to the shell with decreasing salinity is less than the increase in the free cadmium ion activity with decreasing salinity.
The effect of exposing mussels to a range of salinities on metal uptake strongly depended on the salinity of acclimation (fig. 4-5). Again, a clear speciation effect could not be demonstrated. The filtration rates for mussels from different salinity groups increased with increasing salinity (figure 6). When the salinity of exposure differed more than 15 $^{0}/_{00}$ from the salinity of acclimation, filtration rates dropped very significantly. The increase in filtration rate may partially compensate for a decreased availability of the metal ion with increasing ionic strength and complexation. Within the range of 15 to 30 $^{0}/_{00}$ metal uptake rates were usually not significantly different between the two acclimation groups. This suggests that, between these salinity range, the salinity of acclimation has no important effect on the activity of the metal uptake systems. The uptake of

cadmium and zinc does not increase when mussels are exposed to a calcium free seawater in the salinity range 10 to 35 $^{0}/_{00}$ (figure 7 and 8). Comparison with the results for calcium containing seawater shows that uptake of the metals is in fact lower in the absence of calcium. This suggest that calcium influences metal uptake by altering the physiology of the organisms rather than competing with cadmium and zinc for the same uptake sites.

Inhibition experiments

Figure 9 and 10 give the results of the effect of different inhibitors on the uptake of zinc and cadmium. In most cases the inhibitors inhibit the uptake of zinc and cadmium significantly but it is remarkable that nifedipine seems to activate the uptake of zinc in the gill and has no effect on the uptake of cadmium in the gill. Even lanthanium has no significant effect on the uptake of zinc and cadmium. Although our data do not exclude metal uptake by other routes, they show that zinc and cadmium uptake can be reduced by calcium channel blockers. Even the metabolic inhibitors, sodiumcyanide and 2,4-dinitrophenol inhibit the uptake of zinc and cadmium. These reagents inhibit the cellular energy production and hence the formation of ATP. Ouabain inhibits the enzyme (Na^{+}-K^{+})ATP ase which is responsible for Na^{+} pumping and Na^{+} cotransport systems.

DISCUSSION

The effect of salinity on the uptake of zinc and cadmium by the mussel, *Mytilus edulis*, was studied in chemically defined seawater. Although the activity of the free metal ions decreases with increasing salinity, it appears that the uptake of zinc and cadmium is not influenced by the salinity of the ambient solution.

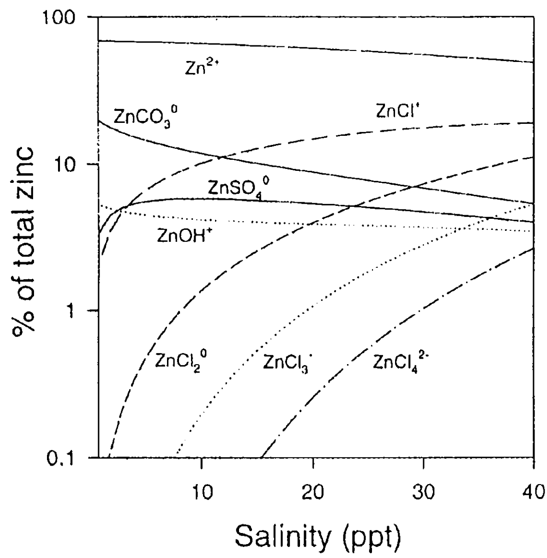
Phillips (1976) suggested that low salinities do not effect the net uptake of zinc by mussels, but increase the net uptake of cadmium. The only explanation he gave was that the flux of cadmium across the mussel gill could be linked to that of calcium. These two ions can be treated as analogues for the same uptake site (Part et al, 1985).

Bjerregaard and Depledge (1994) designed an experimental matrix to investigate salinity and calcium effects independently on cadmium uptake by *Mytilus edulis*, *Littorina littorea* and *Carcinus maenas*, and found that the uptake of cadmium was controlled by salinity and calcium concentrations for *Littorina littorea* and *Carcinus maenas,* but calcium concentration had no effect on the uptake of cadmium for *Mytilus edulis.* The possible role of calcium as a modulator of general permeability might explain why calcium concentration affected cadmium uptake in *Littorina littorea* and *Carcinus maenas* but not in *Mytilus edulis. Mytilus edulis* is an osmoconformer (Conklin and Krogh, 1938) and generally osmoconformers have higher permeabilities than osmoregulators (Rudy, 1967). As calcium plays a role in the regulation of permeability, it is also possible that calcium may affect cadmium uptake in osmoregulators by modulating the permeability of the general surface.

Chan et al. (1992) reported that the zinc accumulation rate in the shore crab, *Carcinus maenas*, from high salinity waters (33 $^0/_{00}$) decreased as salinity was lowered and that the zinc accumulation rate in the same crab from low salinity waters (15 to 25 $^0/_{00}$) showed the same trend although uptake rates were not significantly different. Thus, although zinc bioavailability increased with a decline in salinity, the crabs became increasingly impermeable to zinc to such an extent that overall uptake of zinc fell. Depledge (1990) suggested that many euryhaline invertebrates are able to survive in brackish waters by virtue of well developed physiological control systems and that such systems might also confer some degree of protection against exposure to trace metals by limiting uptake. Rainbow and Kwan (1995) reported that the rates of uptake of cadmium and zinc by the amphipod crustacean, *Orchestia gammarellus*, increased with a decrease in salinity up to 25 $^0/_{00}$. Between 15 and 25 $^0/_{00}$ cadmium and zinc uptake reached a plateau, and the cadmium uptake rate fell at 12 $^0/_{00}$. They concluded that at low salinities the amphipods effect one or more physiological responses that offset any increase in cadmium and zinc uptake rates expected from an increase in the free metal ion activity at low salinities. Although, it seems that the presence or absence of calcium in the exposure medium does not influence the metal uptake, the results of this study demonstrate that calcium inhibitors like nifedipine, verapamil and diltiazem, inhibit zinc and cadmium uptake in hemolymfe and digestive system and verapamil and diltiazem, inhibit zinc and cadmium uptake in the gill. Nifedipine, verapamil and diltiazem are known as antagonists of voltage-sensitive (Hosey and Lazdunski, 1988) and receptor-operated (Hughes et al., 1986) calcium

channels. Each of the reagents binds a different site in the channels (Hosey and Lazdunski, 1988). Hinkle et al. (1987) demonstrated cadmium inhibition through voltage dependent calcium channels in a pituitary cell line and Blazka and Shaikh (1991) reported inhibition by cadmium through receptor operated channels in rat hepatocyte cultures. Roesijadi and Unger (1993) demonstrated that verapamil, nifedipine and diltiazem inhibit the uptake of cadmium in *Crassostrea virginica*. But since the same reagents inhibit calcium uptake in *Crassostrea virginica* to a much lesser extent in comparison with cadmium, it appears that cadmium uses only a part of the uptake routes for calcium. In our experiments, uptake of zinc and cadmium in the gills is inhibited by diltiazem and verapamil, but nifedipine stimulates zinc uptake and has no effect on cadmium uptake in the gills. There can be different explanations for this observed stimulation. First it is possible that nifedipine blocks the channel at the basolateral site in the gills but not at the apical site. If the metals are taken up from solution via the gills, the metals accumulate and are unable to reach the hemolymph and digestive system. An other explanation can be that the calcium channels in the gills of *Mytilus edulis* are of a different type. The N and T types of the voltage dependent calcium channels are insensitive to dihydropyridines such as nifedipine (Hinkle et al., 1987). So it is possible that the calcium channels in the gills are of one of these two types. The class of dihydropyridines (e.g. nifedipine, nitrendipine, PN 200-110, Bay K 8644) is a class of drugs which bind to recepors associated with calcium channels. Nifedipine and nitrendipine are calcium channel inhibitors because they obstruct calcium channels, Bay K 8644 activates calcium channels and is referred to as a calcium channel activator. So structurally similar dihydropyridines can produce opposite effects on calcium channels. Therefore it is possible that some structures have intermediate effects instead of a pure inhibitory or activator effect (Hess et al., 1984). Instead of the inhibitory effect of nifedipine, it could be that nifedipine activates the channel in the gills of *Mytilus edulis*.
Our results also show that the metabolic inhibitors, sodiumcyanide and 2,4-dinitrophenol, inhibit the uptake of zinc and cadmium indicating that ATP-dependent processes are involved in the uptake. Inhibition of mercury by 2,4-dinitrophenol was also reported for the gill of *Mytilus edulis* (Roesijadi, 1982). The inhibition of zinc and cadmium uptake may be explainable by the fact that ATP-dependent mechanisms facilitate the movement through the channels, or that ATP is required for the ciliary activity which provides a waterflow through the mantle cavity. Ouabain inhibits the enzyme (Na^+-K^+)ATPase which is responsible for Na^+/ K^+ pumping across the cell membrane to maintain electrolyte balance (Mathews, 1990) . It is possible that ouabain inhibits the uptake of zinc and cadmium by a disturbance the total osmo-and ionregulation.
Although La^{3+} is known to block calcium channels, in our experiments we did not observe a great inhibition on the uptake of zinc or cadmium by mussels exposed to lanthanium. This is probably due to the fact that lanthaan is complexed by carbonate in seawater so very little of free lanthaan is present.
This study has demonstrated that the effect of salinity on the uptake of metals is not only due to the free ion activity but that the physiology of the organisms plays an important role often obscuring the speciation effect. The study provided evidence that cadmium and zinc uptake by the common mussel,

Mytilus edulis, occurs via channels which are sensitive to calcium channel blockers but the absence of a calcium effect on the metal uptake remains to be explained.



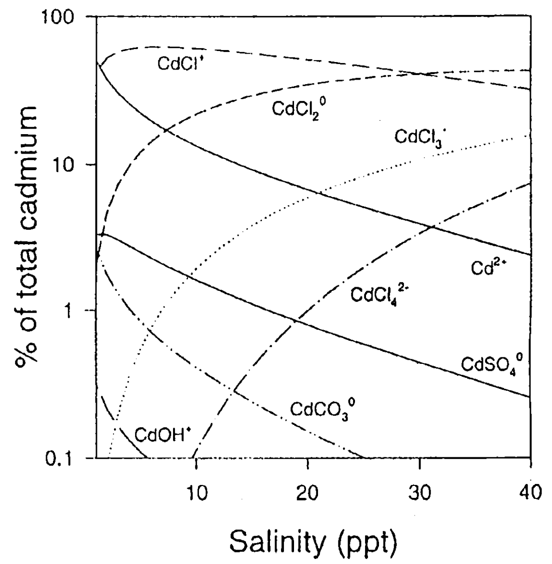


Figure 1: Speciation of zinc and cadmium as a function of the salinity of the solution (pH 8.0, temperature: 15°C)

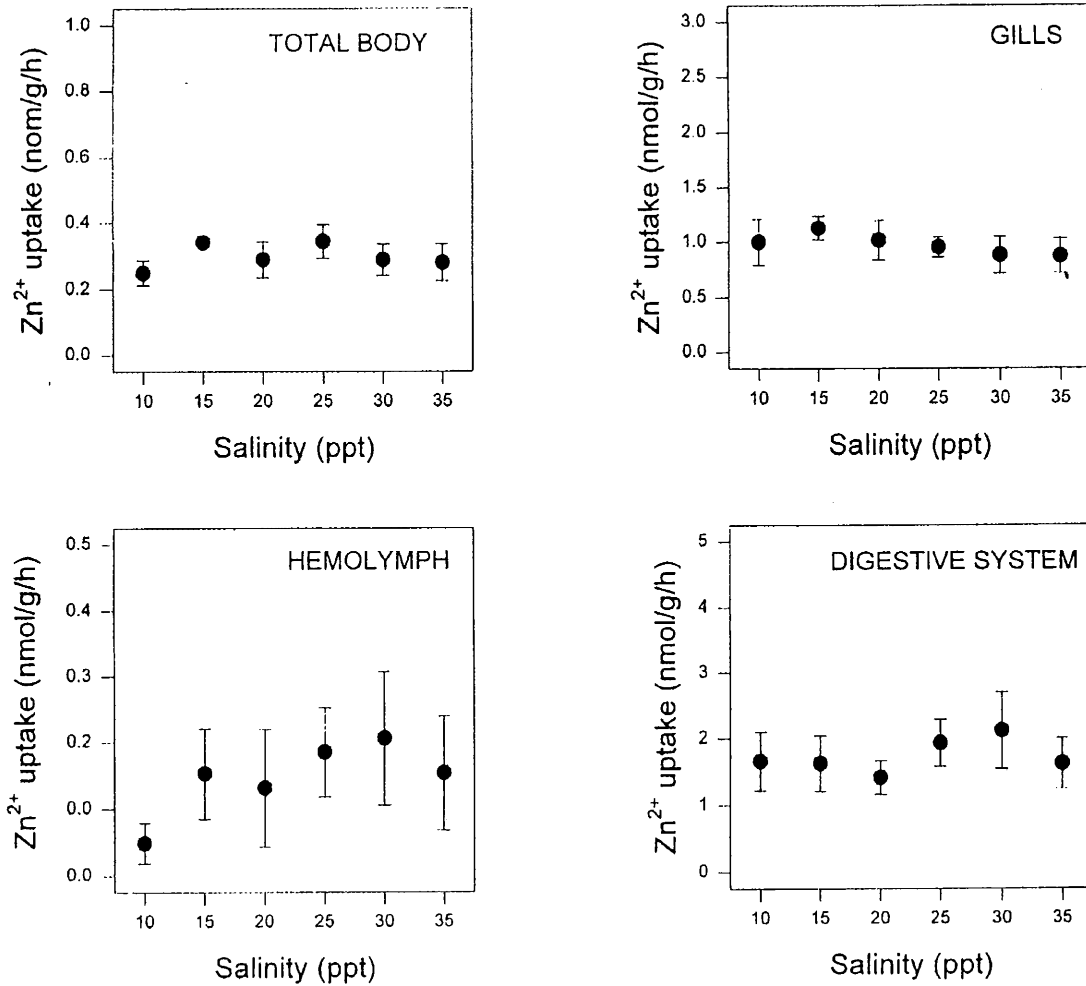


Figure 2: *Mytilus edulis*. Effect of the salinity of acclimation on uptake of zinc over a 24h period for total body, gill, hemolymph and digestive system. Zinc concentration: 1μM. Data points represent means for 7 replicates with standard deviations.

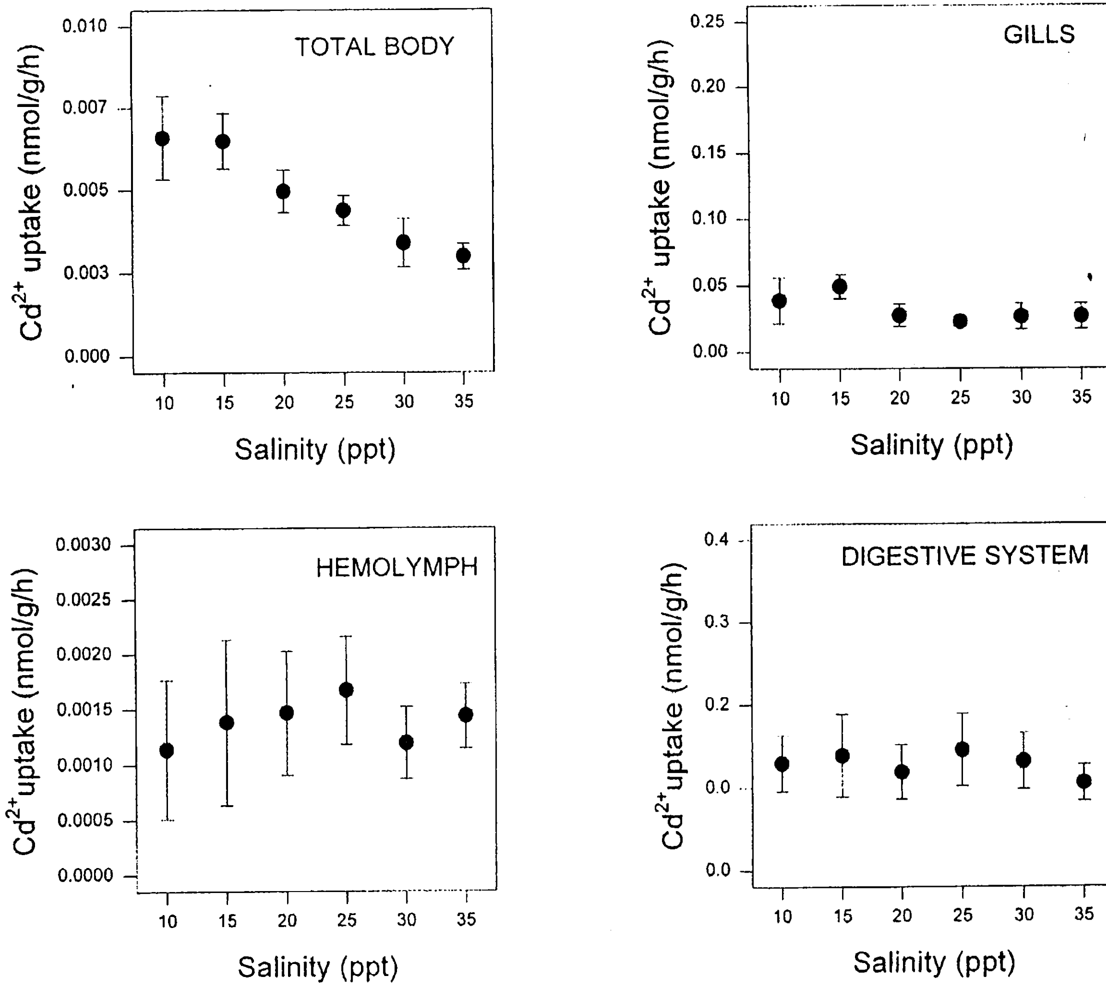


Figure 3: *Mytilus edulis*. Effect of the s alinity of acclimation on uptake of cadmium over a 24h period for total body, gill, hemolymph and digestive system. Cadmium concentration: 0.1μM. Data points represent means for 7 replicates with standard deviations.

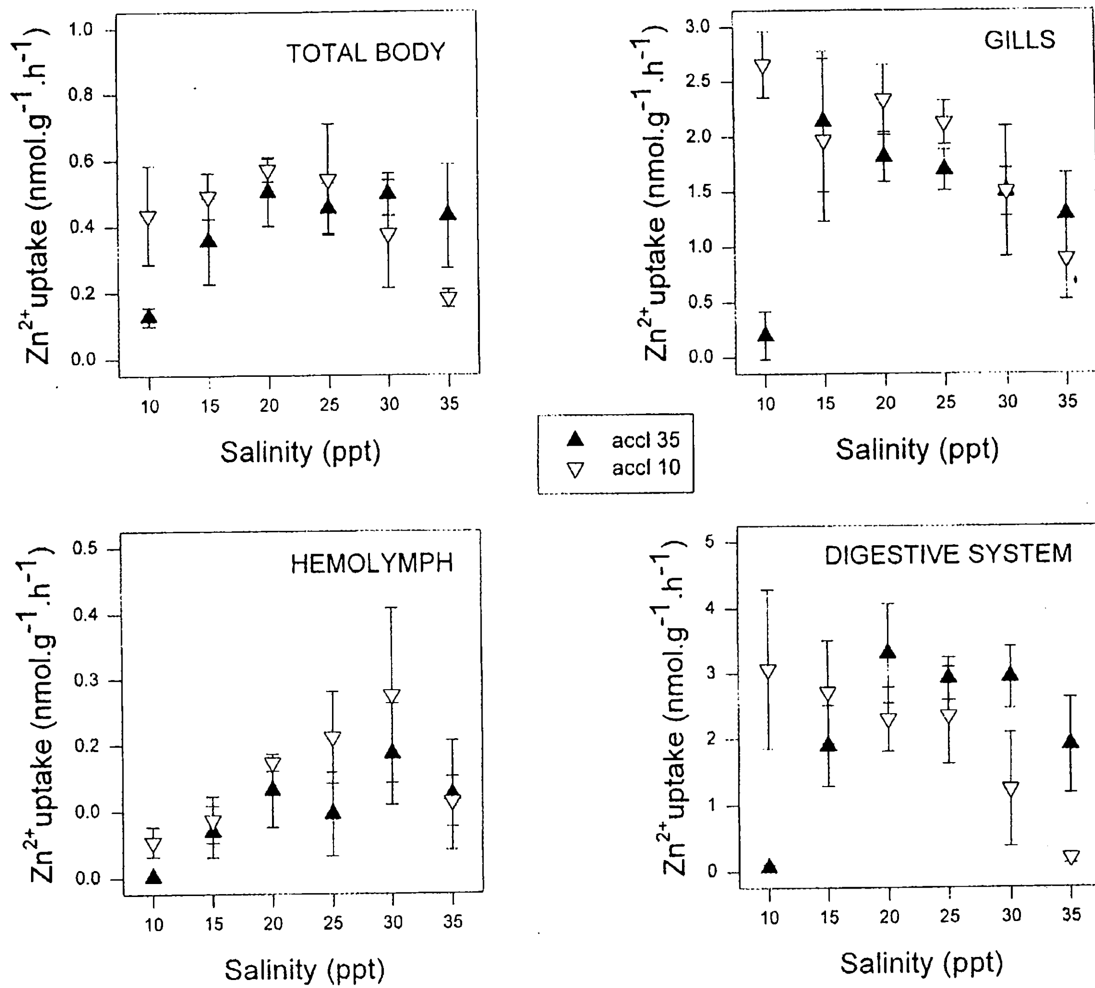


Figure 4: *Mytilus edulis*. Effect of the salinity of exposure on uptake of zinc over a 24h period for acclimation groups 35 and 10 ppt for total body, gill, hemolymph and digestive system. Zinc concentration: 1μM. Data points represent means for 7 replicates with standard deviations.

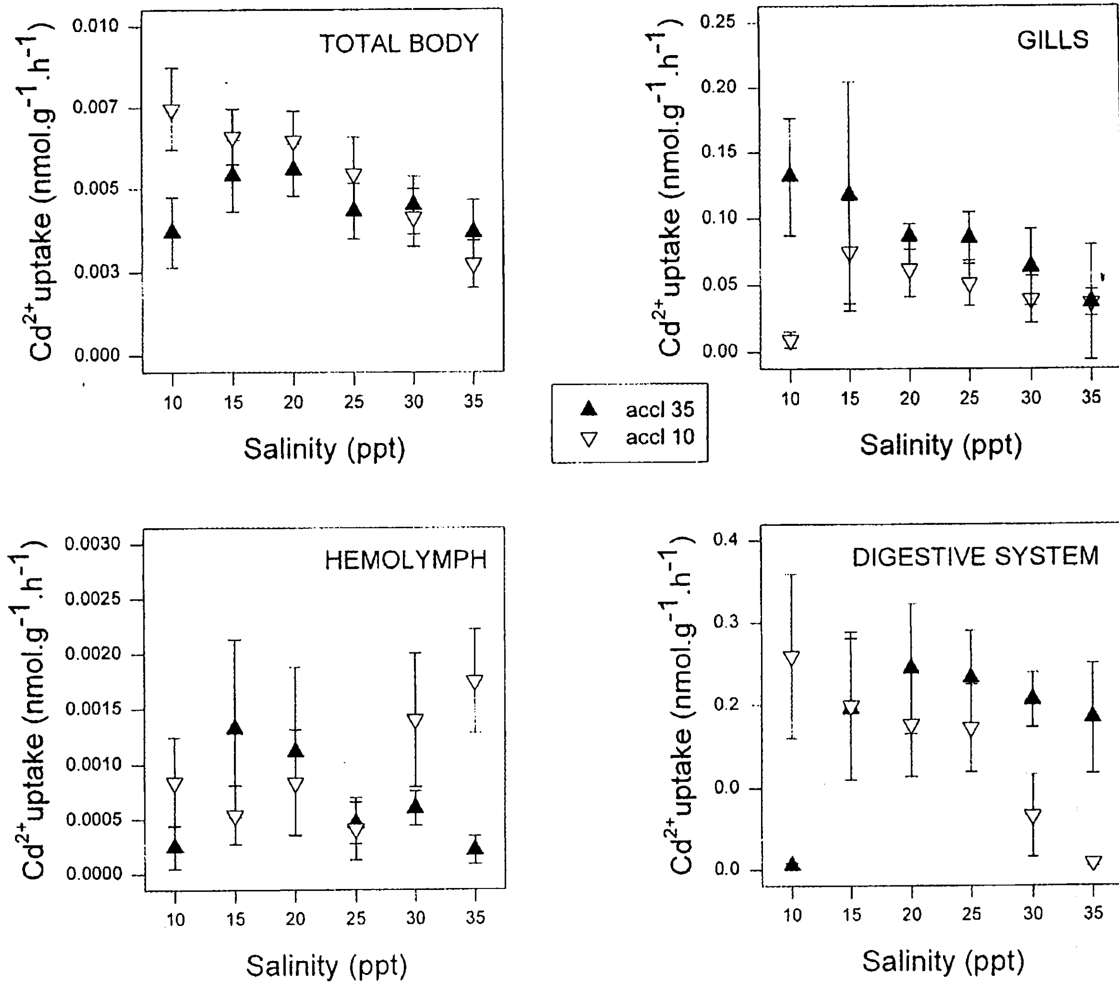


Figure 5: *Mytilus edulis*. Effect of the salinity of exposure on uptake of cadmium over a 24h period for acclimation group 35 and 10 ppt for total body, gill, hemolymph and digestive system. Cadmium concentration: 0.1μM. Data points represent means for 7 replicates with standard deviations.

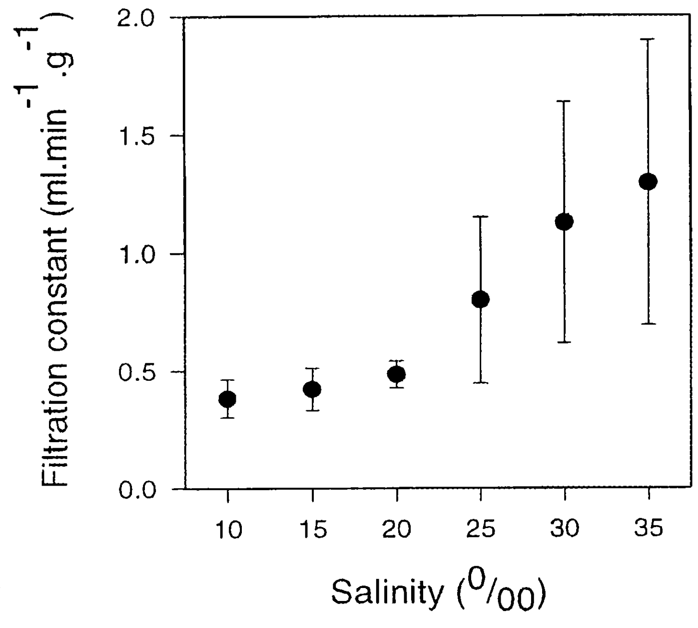


Figure 6: *Mytilus edulis*. Effect of the salinity on the filtration constant. Data points represent means for 5 replicates with standard deviations.

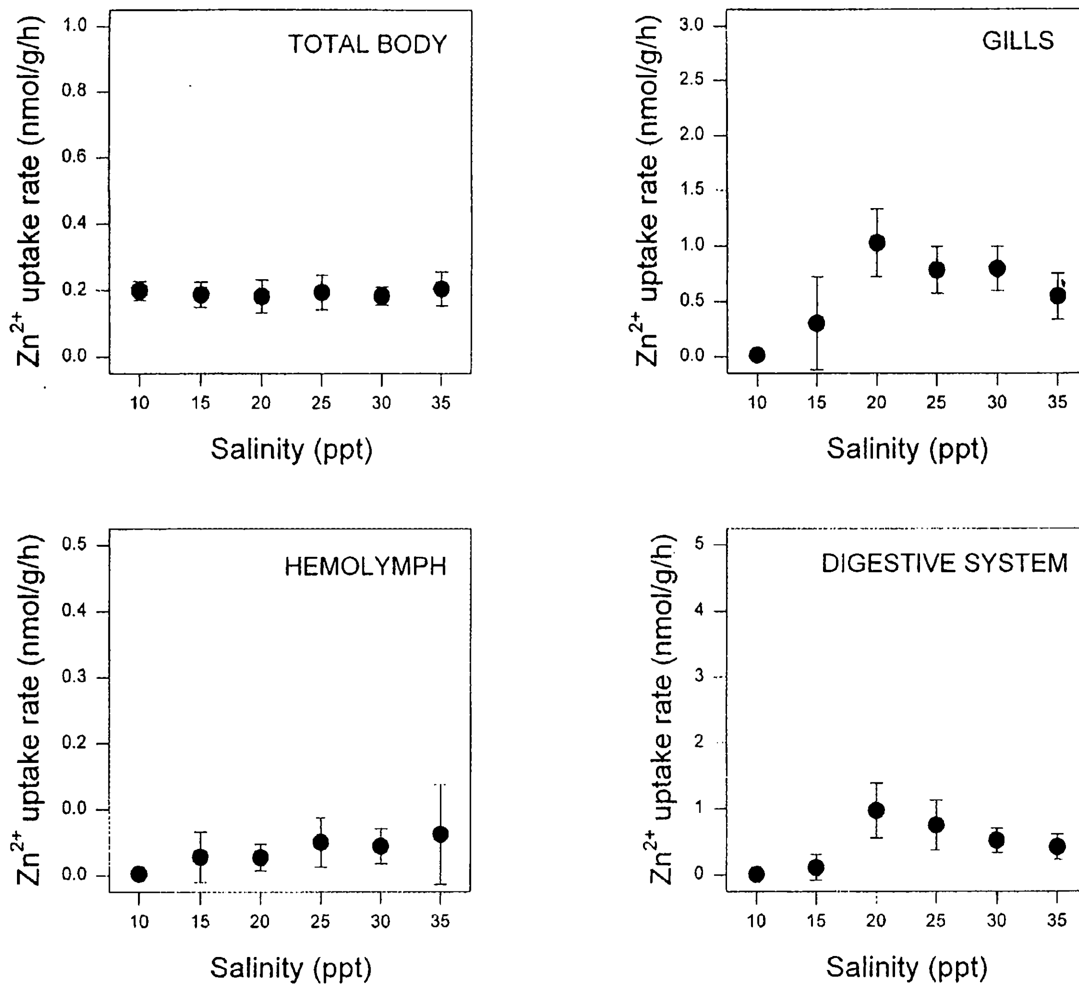
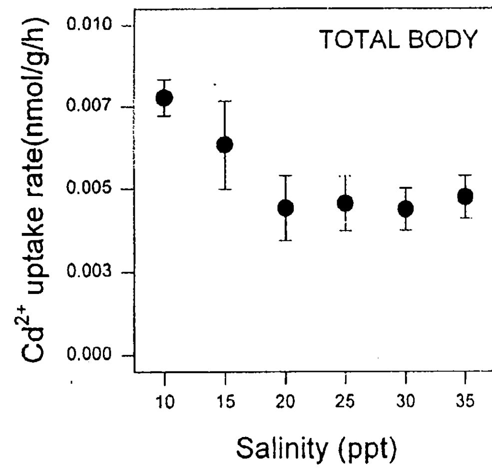
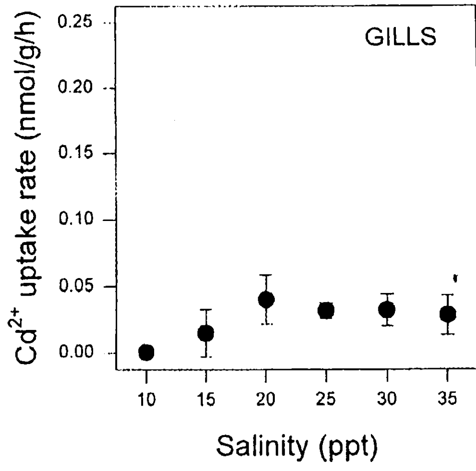
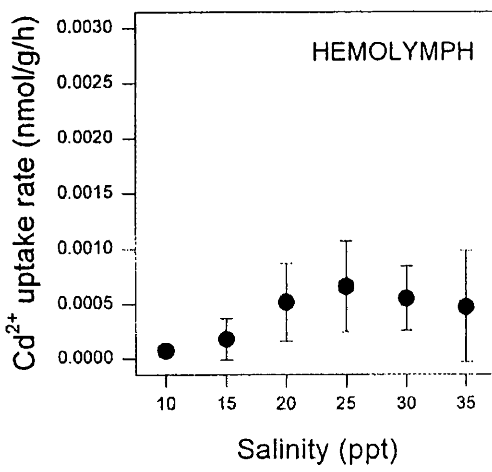


Figure 7: *Mytilus edulis*. Effect of the salinity of exposure to calcium free seawater on the uptake of zinc over a 24h period for acclimation groups 35 ppt for total body, gill, hemolymph and digestive system. Zinc concentration: 1μM. Data points represent means for 7 replicates with standard deviations.







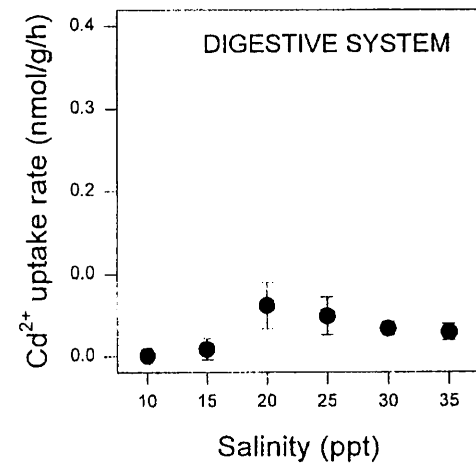


Figure 8: *Mytilus edulis*. Effect of the salinity of exposure to calcium free seawater on the uptake of cadmium over a 24h period for acclimation group 35 ppt for total body, gill, hemolymph and digestive system. Cadmium concentration: 0.1μM. Data points represent means for 7 replicates with standard deviations

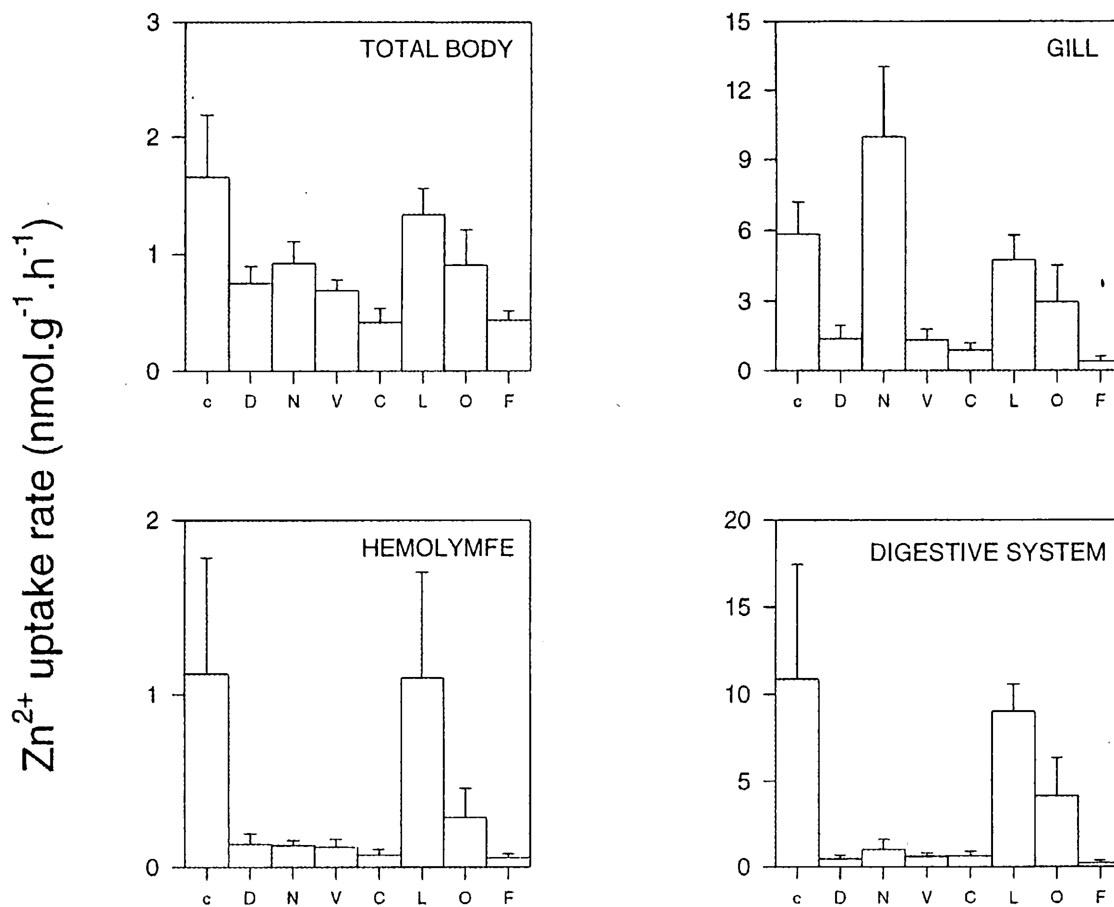


Figure 9: *Mytilus edulis*. Effect of different inhibitors on the uptake of zinc for total body, gill, hemolymfe and digestive system. c: control; D: diltiazem; N: Nifedipine; V: verapamil; C: sodiumcyanide; L: lanthanium; O: ouabain; F: 2,4-dinitrophenol.

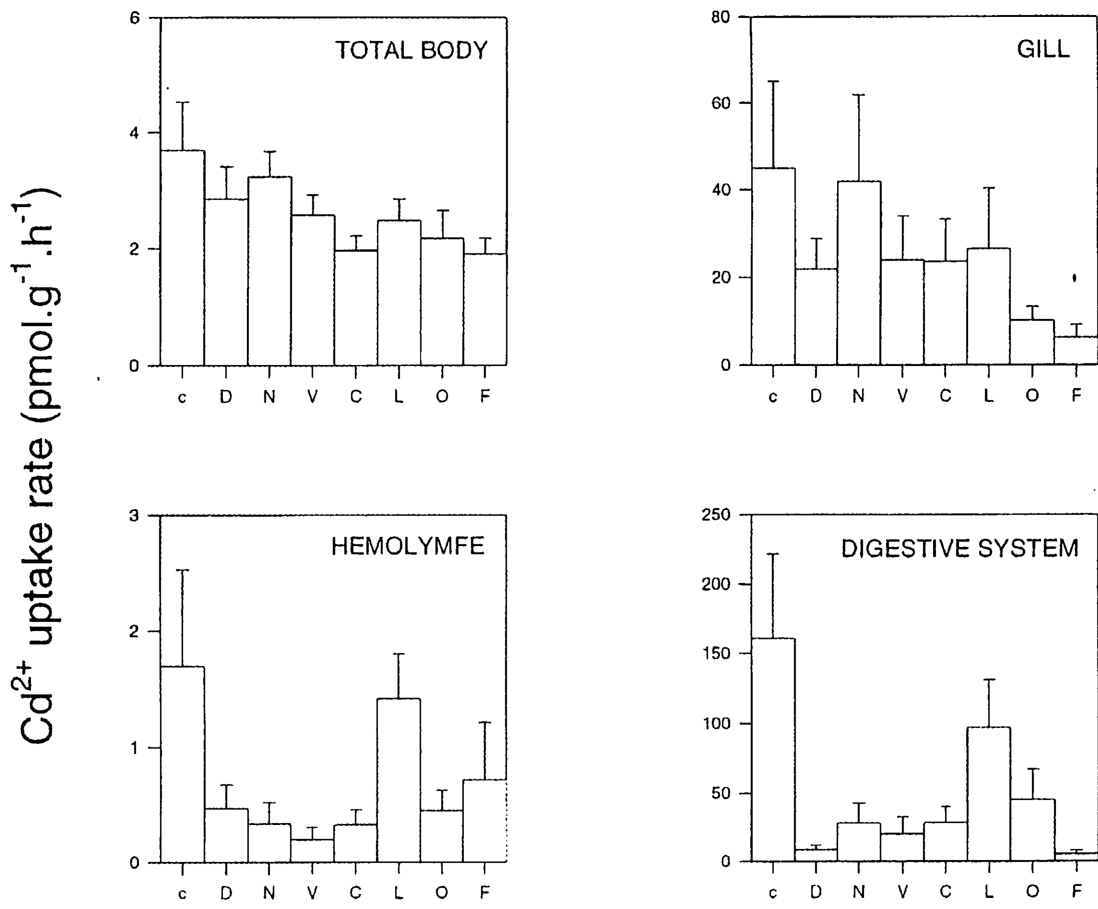


Figure10: Mytilus edulis. Effect of different inhibitors on the uptake of cadmium for total body, gill, hemolymfe and digestive system. c: control; D: diltiazem; N: Nifedipine; V: verapamil; C: sodiumcyanide; L: lanthanium; O: ouabain; F: 2,4-dinitrophenol.

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