AN INTEGRATED MULTIDISCIPLINARY APPROACH TO STUDY THE EFFECTS OF COPPER AND OSMOTIC STRESS IN FISH

A Thesis submitted for the degree of Doctor of Philosophy

by

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DECLARATION

The work submitted in this Thesis was carried out between 2010 and 2014 at Brunel University, Uxbridge, UK. This work was carried out independently and has not been submitted for any other degree.

ABSTRACT

Since many estuarine zones are impacted by copper contamination, there is an on-going effort to develop Biotic Ligand Models (BLMs) predicting copper toxicity in transitional environments. In the first stage of this project, a critical analysis of the BLM framework identified some aspects of the model that required further investigation. In particular, a BLM for estuaries needed *(a)* a better characterization of the dissolved organic matter (DOC) and its effect on copper availability, and *(b)* the inclusion in the model's equation of a salinity-correction factor modulating the relationship between copper accumulation on the biotic ligand and toxicity. The first issue was addressed by modelling the data produced using a Chelex resin method to determine the labile fraction of copper in samples of mixed riverine and estuarine waters. A refined and simplified BLM equation was then presented, accounting for both the DOC characteristics and the relevance of the osmotic gradient in modulating the relationship between copper accumulation and toxicity.

A critical analysis of the literature on copper toxicity and salinity led to the hypothesis that copper-exposed fish are more sensitive to osmotic stresses, as copper interferes with their osmoregulatory pathways. In particular, the cytosolic isoform-2 of the enzyme carbonic anhydrase (CA2) was identified as an osmotic effector protein targeted by copper and involved in osmotic stress response pathways, hence representing a mechanistic link between the combined effects of copper exposure and osmotic stress. To test this hypothesis, two *in vivo* studies were performed, using the euryhaline fish sheepshead minnow (*Cyprinodon variegatus*) and applying different rates of salinity changes as a way of dosing osmotic stress. The results showed a disturbance in plasma ion homeostasis after the salinity transitions, but notably the magnitude of the disturbance was greater in the copper-exposed individuals, suggesting a sensitizing effect of copper on the responses of fish to osmotic stress. Gene expression data demonstrated that CA2 is targeted by copper and confirmed the role of the enzyme in osmoregulatory pathways, as further supported by a promoter analysis of the gene coding for zebrafish CA2, which revealed the presence of osmotic-stress related elements. Overall, these results suggest that CA2 is an osmotic effector protein whose response can be activated by a medium level of osmotic stress through a combination of transcriptional and post-translational control circuits.

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PUBLICATIONS

Chapter One reports the following peer reviewed paper:

- **de Polo A** and Scrimshaw MD (2012). Challenges for the development of a biotic ligand model predicting copper toxicity in estuaries and seas. *Environmental Toxicology and Chemistry* 31: 230–238.

The results reported in Chapter Four were published in the following peer reviewed paper and platform presentation:

- **de Polo A**, Margiotta-Casaluci L, Lockyer AE, Scrimshaw, MD (2014). A new role for carbonic anhydrase 2 in the response of fish to copper and osmotic stress: implications for multi-stressor studies. *PlosOne* (in press)
- **de Polo A**, Scrimshaw MD, Jobling S (2014). Interactions between copper and salinity in the teleost fish sheepshead minnow: a multi-stressor approach. *SETAC European 24th Annual Meeting*, Basel, Switzerland (Platform presentation)

THESIS OVERVIEW AND PROJECT RATIONALE

This research project started with the aim to develop a refined version of the Biotic Ligand Model (BLM) able to predict copper toxicity in estuaries. As a first step, a thorough study of the relevant literature on this subject was performed and resulted in a critical review paper, published in the journal ET&C in 2012 and here presented in Chapter Two. The main objective of the review was to highlight the "weaknesses" of the BLM and particularly of its two key parts, namely the *chemistry-based part* and the *physiology-based part* (see Outline Figure on page xvii), if the model was to be applied in estuaries. From the screening of the literature it emerged that the *chemistry-based part* needed to better describe the characteristics of the dissolved organic matter (DOC) and its interactions with copper, given the high affinity of this metal for the DOC; whilst the *physiology-based part* should put more emphasis on the sensitivity of the organism and, in particular, on the osmotic gradient between the internal fluids of the organism and the external environment (i.e. the "iso-osmotic point hypothesis"). Furthermore, a detailed analysis of the literature on the physiology of osmoregulation, in parallel with the mechanisms of action of copper, led to the hypothesis that copper could affect fish responses to a change in salinity (i.e. an osmotic stress) by interacting with some osmotic effector proteins and particularly with the enzyme carbonic anhydrase, isoform two (CA2), which was therefore identified as a copper target linking fish responses to both copper exposure and osmotic stresses (i.e. the "CA hypothesis").

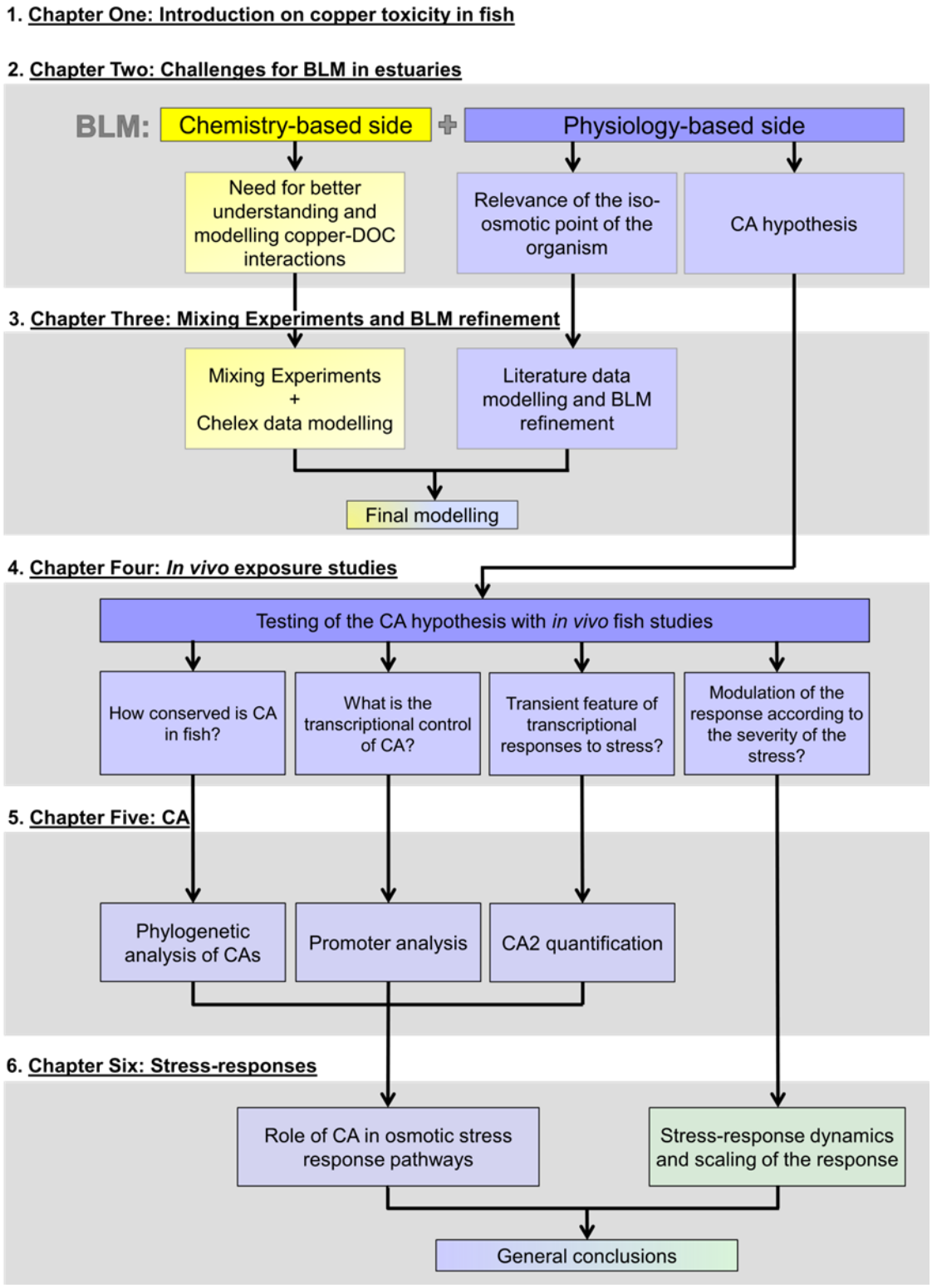
The first two issues – characterization of DOC and modelling of the iso-osmotic point – were experimentally addressed in the second stage of this research project, here presented in Chapter Three, and led to a refined-BLM algorithm stating the relevance of salinity, physiology and organic matter in determining copper toxicity in estuaries.

As for the CA hypothesis, it was tested experimentally in the third stage of the project, by performing two *in vivo* studies using the euryhaline fish sheepshead minnow (*Cyprinodon variegatus*) as test species and administrating copper and salinity as two combined stressors. The outcomes of these studies are illustrated in a paper now in press in the journal PlosOne and presented in Chapter Four. Measured endpoints included plasma ion concentrations and gene expression of CA2 and the enzyme Na^+/K^+ ATPase. The results suggested a sensitizing effect of copper on the responses of fish to osmotic

stress, in line with the original hypothesis, and demonstrated that CA2 played a role in the combined effects of copper and osmotic stress on ion homeostasis. Among the many aspects that emerged from the analysis of the results, four issues in particular needed more investigation (see Outline Figure). First, in light of the different transcriptional responses displayed by CA2 in different fish tissues, a comparative analysis of the CA isoforms present in different species could help understanding the tissue-specificity of its response. Second, since the gene expression results supported the role of CA2 in osmotic stress response pathways, a better understanding of the transcriptional control of CA2 was required. Third, the gene expression data needed to be integrated with more downstream data (i.e. enzyme activity and/or protein quantification) in order to produce a more comprehensive picture of the responses of CA2 to copper and salinity exposure. And fourth, some disagreements between the results of the two *in vivo* studies hinted at a modulation of the response according to the severity of the stress (i.e. osmotic stress) to which the fish were exposed.

The first three issues are discussed in Chapter Five and were addressed by performing, respectively: *(a)* a computational and phylogenetic analysis of CAs across different species; *(b)* a promoter analysis of the gene coding for zebrafish CA2; and *(c)* a quantification of CA2 protein using a human ELISA kit, which was optimized for fish tissues. The phylogenetic analysis showed that function and cellular localization are stronger discriminators than species-specificity in driving CAs classification, demonstrating that CA isoforms are highly conserved across species and taxa. The promoter analysis of zebrafish CA2 revealed the presence of transcription elements that suggest a control of the gene by osmotic stress response pathways similar to those present in yeast, plant and humans. Finally, the results of CA2 protein levels in fish gills were in line with the gene expression results, hence stressing the relevance of an accurate dosing of the stress to elicit a response of a comparable magnitude.

The forth aspect that emerged from the two *in vivo* studies, i.e. the scaling of the biological response according to the degree of the stress applied, is discussed in Chapter Six, where the role of CA as osmotic effector protein, along with the architecture of osmotic stress pathways in euryhaline fish, are put in the wider context of stress response dynamics.



Outline Figure. Scheme of the rationale followed in the research project and the corresponding structure of the Thesis.

CHAPTER ONE - INTRODUCTION

COPPER IN FISH

Don’t call me chemist

If there is a common thread in this otherwise very diverse thesis it is copper – copper and its chemical behaviour in the water, when entering a fish and then inside a fish, in its blood and organs. Copper as a toxicant, whose toxicity is modelled and predicted; copper as a xenobiotic to administer to fish under varying conditions; copper as an enzyme inhibitor and pathway disruptor; finally, copper as a stressor and homeostasis perturbator, which is probably the ultimate conceptualization of a chemical entity interfering with a biological system, such as a fish.

But more than anything, copper in this thesis is an excuse, a tool I used to navigate through the many aspects that are part of the field of environmental toxicology: analytical chemistry, toxicity modelling, physiology, molecular biology and even a bit of systems biology. Having got to the end of this journey I certainly cannot say I master all of them – probably none of them – but at least I have a fairly comprehensive idea of the complexity of this field and its intrinsic multidisciplinary feature.

So, given the diversity of my “PhD journey”, I decided to structure this thesis in chapters that are quite independent from each other: in short, you are not about to read a story, with a classical introduction, a traditional M&M chapter and then the fun parts, results and discussion. However, I think that the reader deserves at least a general introduction on copper and its toxicity, since it constitutes the common underlying theme of the whole work. So here it is: an overview of copper and its deeds in the aquatic environment.

1.1. Introduction

Copper is ubiquitous in the aquatic environment, where it occurs naturally at concentrations ranging between 0.2 and 30 µg/L in freshwater systems [1] and between 0.06 and 17 µg/L in coastal systems [2]. However, anthropogenic inputs (e.g. mining activities, antifouling paintings, municipal waste waters, leather and electronic industry) can raise copper concentrations up to 100 µg/L [1]. In fact, copper is, after mercury, the metal that is most frequently reported to impair water quality [3], a fact that explains the extensive scientific effort to better understand copper toxicity and the regulatory interest to set proper water quality standards, both in the USA and EU (see Chapter Two for an overview of metals regulation).

From a biological viewpoint, the effect that copper can have on organisms is both concentration and condition dependant. Low levels of copper are vital for living organisms, since copper acts as a cofactor for many enzymes, such as the mitochondrial cytochrome *c* oxidase, which exploits its redox potential for oxidative phosphorylation and protection against oxidative stress [4]. At higher concentrations, copper can become harmful for aquatic organisms; although many of them, when the stress is mild, have evolved cellular and organismal mechanisms to cope with this stress and preserve their homeostasis. These mechanisms of response can lead to an acclimation of the organisms, whose copper-disrupted physiological functions display a compensatory response that allows the organisms to survive by re-establishing their homeostasis. However, elevated levels of copper constitute a severe stress for the organism: in this case, the homeostasis of the system cannot be re-established, resulting in toxic effects and ultimately leading to death. This dual feature as either essential element or toxicant makes copper a tricky chemical to handle and organisms have to balance between copper deficiency and excess [5].

An important aspect of copper exposure and its effects on the aquatic biota is the relevance of water chemistry in determining its toxicity. The chemical features of the water can on the one hand affect copper speciation and bioavailability [6-8] and, on the other hand, influence organisms' physiology and hence their sensitivity to the metal [9]. It is difficult to determine the general sensitivity of a given biological endpoint to copper, for example, because the observed response is often so condition-specific that

cross extrapolating between different exposure conditions can be misleading. This is especially true when the water chemistry strongly affects the physiology of the organisms: in this case, at different conditions copper can target different molecules and hence act through different mechanisms of action, as occurs in freshwater and saltwater [10].

It is therefore apparent that copper toxicity constitutes a complex issue that needs to be addressed integrating different perspectives and approaches. In particular, chemistry and physiology are the two major factors controlling copper toxicity and their intertwined interactions are extremely important in understanding the responses of the organisms to copper exposure. Since copper speciation and bioavailability will be discussed in Chapters Two and Three, the focus of this introduction will be on the physiological aspects of copper toxicity, especially those that are relevant for the subject of this Thesis and with particular regard to fish.

1.2. Uptake routes

Given that the majority of studies on copper homeostasis in fish are performed in freshwater, the following discussion is mainly focused on freshwater fish, unless otherwise stated.

1.2.1. Gills

Fish can uptake copper via two routes, the gastrointestinal tract and the gills, with uptake through each of them being modulated by the system according to exposure conditions and basal copper status (i.e. internal copper levels). Under normal exposure conditions, gills account for only 10% of the whole-body copper uptake, the rest being provided by dietary uptake; however, when dietary uptake is reduced, gills may contribute more than 60% of the total copper uptake. Interestingly, gill uptake seems to be finely modulated according to the basal copper status, whereas dietary uptake is less dynamic and is not reduced under elevated waterborne copper conditions [11, 12].

The onset of waterborne copper exposure produces a rapid accumulation of copper in the branchial tissue, followed by a steady state with elevated but stable gills copper levels and constant rates of gill copper uptake. This transient accumulation in the gills is explained by the fact that the basolateral membrane of branchial cells is rate-limiting for

gill copper uptake, at least early in the exposure, whereas afterwards copper entry through the apical membrane is balanced by its transfer across the basolateral membrane and into the circulatory system [13-15].

Considering the mechanisms of copper uptake through the apical membrane of branchial cells, in freshwater fish gills three possible apical copper uptake pathways have been described [16, 17]: one *sodium-sensitive* uptake pathway, which relies on the apical sodium channel [16, 18], and two *sodium-insensitive* pathways, represented by the copper transporter Ctr1 [19, 20] and the divalent metal transporter DMT1 (Figure 1.1.) [21]. These two mechanisms seem to be differentially modulated according to sodium and copper levels in the external medium. At low sodium concentrations, the sodium-sensitive pathway is responsible for the majority of copper uptake, whereas at higher sodium concentrations the sodium-insensitive pathways (via Ctr1 and DMT1) dominate copper uptake [16].

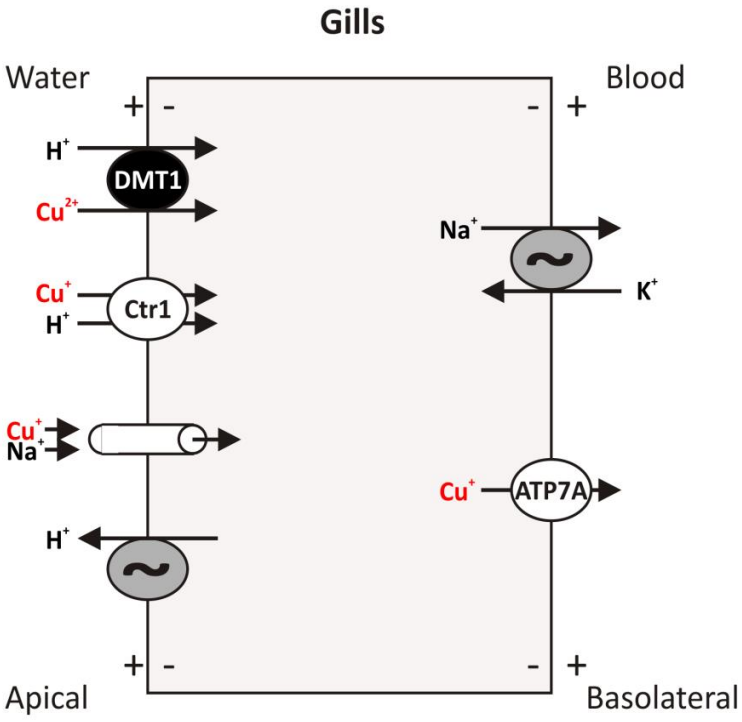


Figure 1.1. Copper uptake routes across (freshwater) teleost fish gills. On the apical membrane, the high-affinity copper transporter Ctr1 and the divalent cation transporter DMT1 transport copper into the cells, favoured by the acid layer provided by the H^+ extrusion via the proton pump. Also, copper uptake can occur via the sodium channel. On the basolateral membrane, sodium is exported by the Na^+/K^+ ATPase, while copper is likely to be transported via the fish orthologues of the human ATP7A. Both Ctr1 and the sodium channel transport reduced Cu^+, whereas DMT1 facilitates Cu^{2+} uptake. Since free copper in natural waters is present predominantly as Cu^{2+}, the presence of a metal reductase at the apical membrane has been hypothesized [5], but it is yet to be identified in fish gills.

As for the basolateral copper uptake pathway, this seems to rely on a copper specific transporter, as suggested by the fact that it is rate-limiting for copper uptake via the gills and exhibits saturation kinetics [22, 23]. A possible candidate for this role of specific transporter is the copper P-type ATPase, atp7a, the fish orthologue of the mammalian copper ATP7A, which plays a key role in copper trafficking in mammalian cells, delivering copper into the Golgi for the incorporation into cuproenzymes and participating to the secretion of copper from the cells [24]. The hypothetical role of atp7a in copper transport across the basolateral gill membrane, based on cross-species extrapolation, is further supported by the observation that fish atp7a has a similar tissue distribution to mammalian ATP7A and serves similar functions, including facilitating copper basolateral excretion in polarized cells, such as branchial and intestinal cells [25].

1.2.2. Intestine

Under normal conditions the intestinal tract accounts for most of whole-body copper uptake. The rapid copper accumulation in intestinal tissues during dietary copper exposure preceding copper accumulation in internal tissues demonstrates that, as for gill copper uptake, the rate-limiting step in intestine copper uptake is represented by the basolateral membrane [5]. Again, a candidate copper transporter across the basolateral membrane of intestinal cells is protein atp7a, which has been shown to respond to waterborne and dietary copper exposure respectively with an increase and a decrease in expression levels in the intestine [25, 26].

Considering the mechanisms of copper uptake through the apical membrane of intestinal cells (Figure 1.2.), there appear to be at least three uptake pathways: the copper transporter Ctr1, the divalent metal transporter DMT1 and a peptide/histidine transporter (PHT1) [27, 28]. Contrary to the copper uptake pathways through the gills, which are either sodium-insensitive or sodium-sensitive (i.e. sodium either does not affect or reduces copper uptake), intestine pathways of copper uptake seem to be positively affected by high luminal sodium levels, which have been demonstrated to stimulate copper uptake both in trout and in mammals [29]. In brief, elevated luminal sodium facilitates apical proton extrusion, and elevated proton concentrations near the apical surface stimulates copper uptake via Ctr1 or DMT1, both of which are fuelled by

protons [30]. These copper uptake pathways are therefore pH-dependant, as is the one occurring via the PHT1 transporter and involving the absorption of a histidine-copper complex. The pH dependence of intestinal copper uptake pathways is particularly relevant when considering marine fish, where the pH in the intestinal lumen is considerably higher than in freshwater teleost fish.

Once copper has entered the organism, either via the gills or the intestine, it is then transported to the liver by the bloodstream, where most copper is bound to different types of proteins and only a negligible fraction is present in the unbound form [5].

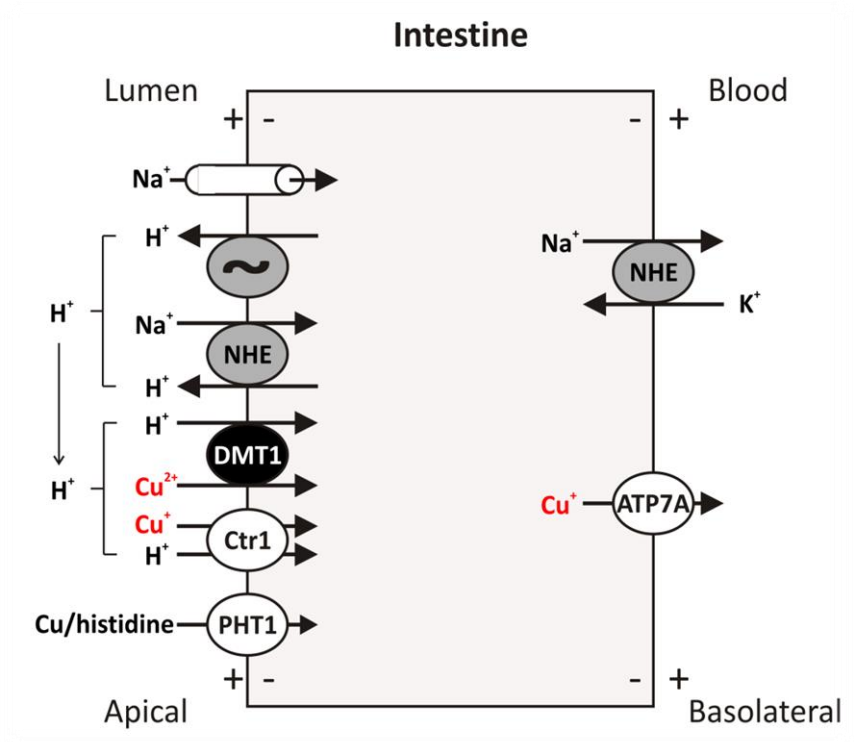


Figure 1.2. Copper uptake route across teleost fish intestinal cells. On the apical membrane, the high-affinity copper transporter Ctr1 and the divalent cation transporter DMT1 transport copper into the cells, favoured by the acid layer provided by the H^+ extrusion via the proton pump and the Na/H exchanger (NHE). Ctr1 transport copper in its reduced form, Cu^+, whereas DMT1 facilitates the uptake of Cu^{2+}. In addition to these two pathways, a peptide/histidine transporter (PHT1) has been suggested to be responsible for uptaking copper-histidine complexes. On the basolateral membrane, copper is likely to be transported across the membrane via the fish orthologues of the human ATP7A.

1.3. Internal handling

Much information on the internal handling of copper in fish is extrapolated from mammalian systems in order to fill knowledge gaps regarding copper transport, metabolism and excretion. However, the conservation in fish of human molecules involved in copper homeostasis, as well as similar tissue distribution and function of these molecules are considered good evidence of pathway conservation.

The cross extrapolation between fish and mammals used in this section to fill knowledge gaps regarding copper homeostasis in fish can be symmetrically exploited to gain insight into vertebrate copper homeostasis. More importantly, although many aspects of copper handling in fish appear to resemble the mammalian system, they also present differences that could be useful for comparative copper physiology studies in vertebrates.

1.3.1. Blood transport and plasma protein association

The majority of blood copper is associated with the plasma fraction, which accounts for 90% of whole-blood copper concentrations. Plasma copper levels in fish are elevated by waterborne copper exposure, but in some studies this elevation has been shown to be transient and followed by either normalization or a second modestly elevated steady state, suggesting that plasma copper is under a tight homeostatic control [14, 15]. Dietary copper uptake, on the other hand, seems to have no effect on plasma copper levels, despite contributing more significantly to whole-body copper accumulation [31]. This observation might be due to the fact that copper entering the bloodstream from the intestine flows directly into the liver, where it is efficiently cleared.

Little is known about protein association of copper in the bloodstream of fish, but much information can be drawn from studies on mammals. According to these studies, approximately 80% of plasma copper is tightly bound to ceruloplasmin, a plasma protein that is synthesized in the liver and released to the blood for delivering copper to peripheral tissues [32, 33]. The same protein is present in fish too, where it is likely to have similar function [34]. Another fraction of plasma copper (around 18%), in particular that which has been recently uptaken, is associated with albumin and other amino acids [33]. It is in this form that copper is then transferred into the liver.

1.3.2. Accumulation, metabolism and excretion

Once it enters into the bloodstream, copper is transported to the liver, where it is stored in copper-protein complexes, excreted via the bile or incorporated into ceruloplasmin and then released into the bloodstream again to be delivered to extrahepatic tissues. The liver is therefore the main homeostatic organ in copper metabolism, displaying a very efficient clearance of plasma copper, as well as a high rate of copper accumulation [35]. The high rate of hepatic accumulation suggests that the liver possesses copper-specific carriers, though these are yet to be identified in fish. An obvious candidate carrier would be the copper transporter Ctr1 [36], but studies on mice demonstrated that its deletion did not significantly affect hepatic copper accumulation, hinting at the presence of alternative pathways of copper transport in hepatic tissues [37].

Once into the liver cells, copper remains largely active. Fish studies on hepatic speciation of copper demonstrated that hepatic cellular copper in controls and copper-exposed fish is present in similar forms, with only 40% copper being associated with metallothioneins (MTs) and the majority of it present in the metabolically active fraction [38], in agreement with what is known on mammalian cellular copper metabolism [39].

While a fraction of copper is complexed by ceruloplasmin and delivered to other organs, excess of copper is excreted via the hepatobiliary route [14, 15, 40], in line with the role of the liver as the main site of copper homeostasis. In fish, biliary copper excretion is stimulated by both waterborne [15, 41] and dietary borne copper [26], with gall bladder bile copper reaching levels as high as 20 μg/mL [41]. In mammals, hepatobiliary excretion of copper occurs via two main routes: the ATP7B protein (also known as Menkes disease protein) and the canicular multiorganic anion transporter (cMOAT) coupled with lysomal excretion [42]. Similar mechanisms seem to be present in fish too, where expression levels of the fish atp7b in liver samples have been shown to be increased by elevated copper status [25]. There is also evidence of a copper excretion route via lysomal excretion in fish [43]. As for urinary copper excretion, this route seems to have little role in freshwater teleost fish [15], in agreement with the tight association of plasma copper with relatively large proteins, rendering copper unavailable for glomerular filtration [5]. However, studies on marine fish demonstrated

that waterborne copper exposure produced elevated copper levels in the kidney [40], suggesting that in marine fish the urinary excretion route may play a role in copper elimination.

Studies with continuous infusion of radiolabelled copper in trout provided some evidence of a possible role of the gills in copper excretion [41], further supported by the presence in gill tissue of atp7b, which is regulated according to copper status [25]. However, more research is needed to confirm these observations in other species and under different exposure conditions.

1.4. Mechanisms of toxicity

Most of the following discussion is focused on copper toxicity in freshwater fish, unless otherwise stated, since further description of copper mechanisms of action in marine fish will be provided in Chapter Two. Also, the described mechanisms of toxic action regard waterborne acute copper exposure, whilst dietary and truly chronic exposures (i.e. full life-cycle or early-life stages exposures) are not covered, since they are not relevant in the context of the Thesis.

1.4.1. Olfaction

The olfactory system is critical for population health and its impairment can affect the ability of fish to avoid predators, locate prey, socially interact with other individuals and successfully reproduce. Copper has been demonstrated to inhibit this system at concentrations as low as few micrograms per litre (2-3 µg/L increase in total dissolved copper over background levels [44]), with effects persisting for weeks or longer, making the olfactory inhibition one of the most sensitive endpoints for copper toxicity [45]. Studies based on behavioural tests and on recording of electro-olfactograms from the olfactory epithelia revealed that copper affects most of fish behavioural endpoints, including fright responses [45, 46] and feeding behaviour [47]. In accordance with the broad range of stimuli affected, copper mechanism of action in this case seems to be of a general nature rather than target-specific, as supported by the wide range of genes responding to copper exposure in the olfactory epithelium [48].

An interesting aspect of olfactory inhibition by copper is the fact that ambient water hardness and particularly calcium do not seem to offer consistent protection on copper-

induced olfactory effects [44], in contrast to what we would expect based on copper and calcium competition for uptake. This observation is consistent with the low calcium accumulation observed in the olfactory epithelium and in the gills under increased ambient calcium concentrations [47]. However, calcium does offer protection against acute copper toxicity (i.e. mortality). The lack of a consistent correlation between calcium accumulation and protective effects supports the argument that the protection offered by calcium is of a physiological nature rather than simply the product of cation competition at the binding sites [5].

1.4.2. Respiratory distress

Copper-induced reduction in oxygen consumption has been shown to be transient at low copper and persistent at high copper concentrations [49]. The observed respiratory distress in response to sublethal copper exposure could be due to the fact that copper impairs the ability of fish to either sense reduced oxygen or regulate oxygen consumption, or both. In both cases, it implies that copper can have a sensitizing effect on fish response to environmental hypoxia.

At high concentrations, copper causes hypoxic hypercapnia as a result of gill histopathologies, such as cell swelling and lamellae thickening, leading to impaired gas transfer across the gills and respiratory acidosis [50]. However, the mortality observed at high copper concentrations does not occur as a result of hypoxemia but rather as a result of ion homeostasis impairment and, ultimately, cardiovascular collapse, since the increase of blood-water diffusive distance caused by damage of the branchial epithelium affects not only gas transfer but also ion transport.

1.4.3. Oxidative stress

Copper exposure can cause oxidative stress by *(1)* inhibition of antioxidant enzymes; *(2)* alteration in the mitochondrial electron-transfer chain; *(3)* ROS (reactive oxygen species) formation and *(4)* depletion of cellular glutathione [51-55].

In general, ROS are generated continuously during aerobic metabolism; however, under normal conditions, protection against oxidative stress resulting from high ROS levels is offered by a complex system of proteins, including metallothioneins and antioxidant enzymes, such as catalase, glutathione peroxidase and copper/zinc superoxidase

dismutase (SOD). When the combined cellular antioxidant capacity of these enzymes is exceeded by the rate of ROS production, oxidation of DNA, proteins and lipids occurs [5].

Copper has been demonstrated to induce ROS formation in fish gill [56] and liver cells [57] and also to affect either gene expression and/or enzymatic activities of antioxidant enzymes, including catalase, glutathione peroxidase and SOD, in the gills, liver and kidney [58, 59]. However, some disagreements exist between transcriptional and enzymatic responses of these enzymes, hinting at the fact that these enzymes might be under a complex control system. Additionally, their responses to copper exposure seem to be transient, a fact that could be interpreted as the outcome of a complex interaction between a need to respond to ROS accumulation and a direct inhibition of copper on these enzymes.

In mammals, oxidative stress has been shown to induce increased expression of metallothioneins, which are generally known as metal scavenger proteins responsible for complexing excess metal in cells. This response is mediated by both antioxidant response elements and metal response elements (MRE) present in the 5' regulatory region of metallothionein genes, a fact that provides evidence for a direct role of metallothioneins in cellular response to oxidative stress [60, 61]. This observation is particularly interesting because, along with the observation that only a minor fraction of cellular copper is present in the non-complexed form [38], it suggests that the antioxidant function of metallothioneins plays a more prominent role in the response to copper exposure than their function as copper storage proteins.

1.4.4. Ammonia excretion

Elevated plasma ammonia is the most consistent response observed in fish exposed to copper both in freshwater and saltwater [62-65] and it is the result of two mechanisms [5]: copper-induced elevated plasma cortisol [66], which stimulates protein catabolism and hence ammonia production, and impairment of ammonia excretion, which results in elevated plasma ammonia [10].

Ammonia excretion takes place in the gill and relies on the diffusion trapping mechanism at the boundary layer, which facilitates ammonia excretion by converting ammonia into ammonium at the boundary surface [67]. The enzyme carbonic anhydrase

(CA) seems to play an important role in this mechanism and its possible sensitivity to copper may explain copper effects on this endpoint. This mechanism of action of copper will be discussed in more detail in Chapter Five, Section 5.1.3.1.

1.4.5. Acid-base balance

Sublethal copper exposure can result in elevation of extracellular pH as a result of elevated plasma bicarbonate levels [64, 68], an effect that could be due to copper interaction with branchial Cl^-/HCO_3^- exchanger and with cytosolic CA [5].

Copper interaction with CA could also explain copper-induced impairment of the compensatory increase of extracellular bicarbonate in response to elevated ambient CO_2 (i.e. hypercapnia), thereby affecting the ability of freshwater and seawater fish to respond to hypercapnia. Indeed, combined exposure of marine fish to hypercapnia and copper results in an impaired ability of the fish to compensate for hypercapnia-induced respiratory acidosis [69], suggesting that one or more of the exchangers, cotransporters and/or CA enzymes involved in the process are targeted by copper.

In marine fish, the intestine also plays a key role alongside the gills in maintaining acid-base balance through net base secretion [70], a mechanism that can be impaired by copper exposure.

1.4.6. Sodium chloride transport

Osmoregulatory disturbances displayed by freshwater fish exposed to sublethal copper concentrations usually result in decreased plasma sodium and chloride levels, an effect that can be due to either a *decrease in ion uptake* and/or an *increase in diffusive loss* [5]. The relative importance of these two mechanisms depends on the level of copper exposure. At low concentrations, copper acts mainly by inhibiting the mechanisms of branchial sodium and chloride uptake, whilst at higher copper concentrations, the observed impairment in ion homeostasis is the product of both copper-induced inhibition of ion uptake and increased sodium loss, the latter being caused by the displacement of calcium by copper in tight junction proteins, thereby increasing paracellular permeability [62]. Severe loss of sodium and chloride results in reduced plasma osmolarity, which in turn results in movement of fluids from plasma to tissues,

hence causing increased blood viscosity and, ultimately, cardiovascular collapse [16, 50, 62].

As for the decrease in ion uptake and particularly sodium uptake, this effect occurs as a result of copper acting at both the apical and the basolateral membranes of freshwater fish branchial epithelium [71]. The copper-impaired sodium uptake at the apical membrane seems to be the product of a competition between copper and sodium for entry through the sodium channels (see Figure 1.1), whilst basolateral uptake of sodium can be impaired by copper as a result of copper-inhibited activity of the Na^+/K^+ ATPase enzyme, which is responsible for sodium transport across the basolateral membrane and into the bloodstream of freshwater fish. Additionally, apical sodium uptake also occurs through apical Na^+/H^+ exchangers, a pathway that could be inhibited by copper either directly (i.e. through enzyme activity inhibition) or indirectly [5].

A possible role of CA in the mechanisms of copper-induced decrease of sodium uptake will be discussed in Chapter Five, Section 5.1.3.1. In brief, both mechanisms of apical sodium entry, either via sodium channels or via Na^+/H^+ exchangers, rely on the availability of cellular protons, which are produced by the catalytic activity of CA [5]. In the case of the sodium channels, this is because H^+ excretion contributes to the hyperpolarization of the electrochemical gradient across the apical membrane, an effect that facilitates sodium entry. As for the Na^+/H^+ exchangers, they obviously depend on H^+ cellular substrate. Inhibition of CA by copper could therefore lead to indirect impairment of these two pathways of apical sodium entry.

Additionally, copper inhibition of CA activity may also lead to decreased chloride uptake, since the bicarbonates produced by CA hydration of CO_2 are required by Cl^-/HCO_3^- exchangers for chloride uptake at the apical membrane [5].

The osmoregulatory effects of copper in marine fish, as well as the role of the osmotic gradient in copper toxicity mechanisms at intermediate salinities, will be discussed in Chapter Two, Sections 2.4.1 and 2.3.4.

1.5. References

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CHAPTER TWO

CHALLENGES FOR THE DEVELOPMENT OF A BLM PREDICTING COPPER TOXICITY IN ESTUARIES AND SEAS

An anadromous PhD student

If you want to get yourself into troubles as a PhD student, choose estuaries as your study area. Only the pragmatic mind of the Latins could name *aestuarium* (literally and straightforwardly "channel")* such a complex and dynamic ecosystem, characterized by strong chemical and physical gradients as well as by some of the highest biodiversity in the world. In my case, this masochistic choice was explained by the simple fact that, being a freshly graduated marine environmental scientist interested in BLMs, I soon realized that there were no available BLMs for estuaries and seas, and yet estuaries (more than seas) are highly impacted ecosystems, especially by metals, as I had learnt in my previous research on metal accumulation in estuarine sediments.

Having scaled down my overambitious list of six metals to one – copper – I set out to dissect the BLM framework in search for those parameters that would play major roles in determining copper toxicity in an estuary. I shall say that by that time my idea of estuaries had become progressively more abstract, till it vanished into a purely chemical mixing between fresh and sea water. I was in the midst of those changing environmental conditions (varying salinities, different types of organic matter and fluctuating pH) when it occurred to me that an estuarine version of BLM had to look at what is going on inside a fish, not just outside, and possibly before the fish is dead (mortality is the favourite endpoint of a chemist). It is from this realization that this paper really started to take off, leading me ever deeper into the fascinating world of aquatic toxicology.

*An alternative translation is "tidal inlet", from *eastus*, "tide".

Abstract

There is an on-going effort to develop a BLM that predicts copper toxicity in estuarine and marine environments. At present, the BLM accounts for the effects of water chemistry on copper speciation, but it does not consider the influence of water chemistry on the physiology of the organisms. We discuss how chemistry affects copper toxicity not only by controlling its speciation, but also by affecting the osmoregulatory physiology of the organism, which varies according to the salinity. In an attempt to understand the mechanisms of copper toxicity and predict the impacts, we explore the hypothesis that the common factor that linking the main toxic effects of copper is the carbonic anhydrase enzyme (CA), since it is a copper target with multiple functions and salinity-dependant expression and activity. According to this hypothesis, the site of action of copper in marine fish may be not only the gill, but also the intestine, since this is where the CA plays a role in ion transport and water adsorption. Thus, the BLM of copper toxicity to marine fish may also consider the intestine as a biotic ligand. Finally, we underline the need to incorporate the osmotic gradient into the BLM calculations to account for the influence of physiology on copper toxicity.

2.1. Introduction

Historically, the environmental quality criteria/standards (Environmental Quality Standards, EQSs, in the EU and Water Quality Criteria, WQC, in the U.S.) for metals have been based on total, or total dissolved, metal concentrations, despite the fact that many physiological and toxicological studies have demonstrated that water chemistry affects both bioavailability and toxicity of metals to aquatic organisms [1-4]. The effect of water chemistry began to be incorporated into regulations from the 1970s onwards, when the European Agencies and the U.S. Environment Protection Agency (EPA) set higher environmental quality standards for metals in fresh waters than for those in marine waters [5]. The threshold values in freshwaters were also adjusted for hardness, in order to generate site-specific WQC/EQS [6]. This represented a breakthrough because, for the first time, the regulatory authorities acknowledged the relevance of water chemistry on metal bioavailability and toxicity, even though other critical water chemistry variables, such as dissolved organic matter (DOM), were still overlooked. A further step in accounting for bioavailability was represented by the implementation in the U.S. of the water effects ratio (WER) procedure [7], which accounted for the difference between the toxicity of the metal in laboratory water and its toxicity in the water at the site. The WER is determined by dividing the endpoint (i.e. LC50: the concentration that is lethal to the 50% of the test organisms) obtained in the site water by the endpoint obtained in the laboratory diluted water. This procedure, although effective, implies a number of site specific tests and analyses, while the regulatory authorities have a need for easy-to-use tools to assess site-specific water quality standards for metals [8].

Speciation and toxicity models can respond to the need for ease of use because they incorporate the complexity of the environment into simplified systems based on specific assumptions. Their value does not depend on their capability to reproduce reality in the most exhaustive way, but rather on their usefulness and effectiveness. Their theory, along with the calibration data on which they are founded, has to be robust enough to give realistic output data over a wide range of conditions. In this respect, one of the most relevant systems developed for the aquatic environment is the Biotic Ligand Model (BLM) [9], originally conceived to predict metal availability and toxicity to

freshwater fish and daphnids [10, 11]. The model is essentially composed of two parts, a *chemistry-based part* and a *physiology-based part* (Figure 2.1).

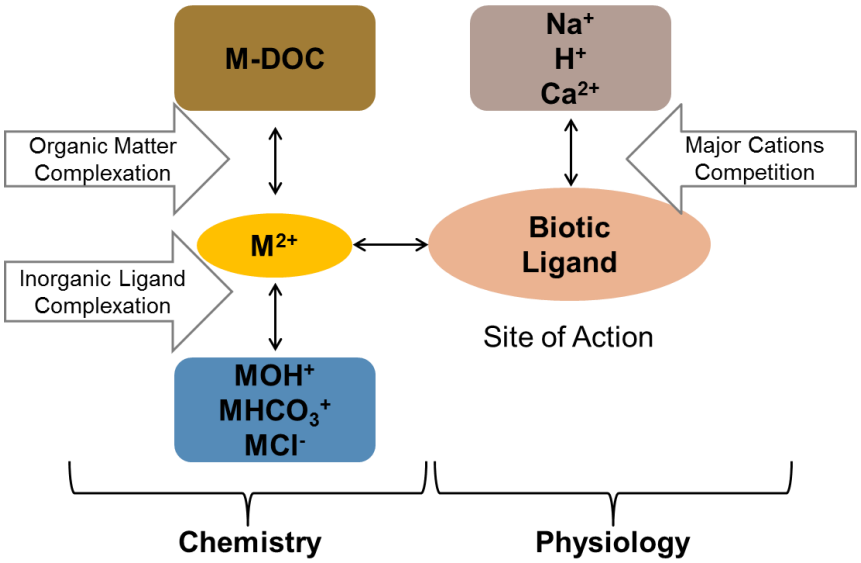

Figure 2.1. Schematic diagram of the BLM conceptual framework.

The first is represented by the chemical speciation computations, both inorganic and organic: the inorganic speciation is relatively simple and is performed by the Chemical Equilibria in Soils and Solution (CHESS) model [12], while the organic speciation is computed by the Windermere Humic Acid Model (WHAM), developed by Tipping and co-workers [13, 14]. Modelling the complexation of metal cations by organic matter is challenging, because of the uncertainties regarding the characteristics of the ligands and their binding constants. The second part of the BLM accounts for the effect of the metal binding to the biotic ligand and it is based on the concept that toxic effects occur when the metal-biotic ligand complex reaches a critical concentration.

The BLM has gained acceptance in the scientific and regulatory communities over the last decade, with the U.S. EPA publishing, in 2007, its Framework for Metal Risk Assessment [15], which introduced the BLM as a tool to derive criteria for protection of aquatic life. The BLM has successively been adopted by the Australian and New Zealand Environmental Protection Agencies as a tool for regulating metal concentrations in the aquatic environment [16] and it is in the process of being adopted by Environment Canada and the EU. In 2008 the UK Environment Agency published a

report on EQSs for trace metals in the aquatic environment [17], which recommended the use of BLM-type models to assess compliance with EQSs. In particular, they proposed a "field" BLM, the Toxicity Biotic Model (TBM), which may in the future be optimized with field data and includes the effect of metal mixtures, which is considered an outstanding issue that needs to be addressed. They also developed and presented a tiered assessment approach [18] that takes into account background metal concentrations. Through this approach it would be possible to prioritise sites where the concentration of a metal exceeds the EQS expressed in terms of total dissolved concentration, allowing focusing resources on sites at most risk.

In 2009 a European workshop discussed the incorporation of metal bioavailability into the regulatory framework* [19] and concluded that the application of the BLM in estuarine and marine environments should be carefully evaluated. In the marine environment, work should be focused on the DOM variable, given the different characteristics of riverine, marine and estuarine organic matter, while in estuaries the complexity of the geochemical dynamics and the osmoregulatory responses of the estuarine organisms should be accounted for, otherwise the effectiveness of the model might be compromised.

The BLM framework is based on both water chemistry and physiology. The former factor greatly influences copper bioavailability and thus its toxicity: alkalinity, water hardness, pH, DOM and salinity are all variables that affect copper speciation [1-4]. High salinity, in particular, has a protective effect against copper toxicity, since it reduces the availability of the metal as a consequence of both increased inorganic metal complexation and increased competition for binding sites by cations such as Na^{+}, Mg^{2+} and Ca^{2+} [9, 20]. But in addition, salinity can also affect the physiology of aquatic organisms by influencing the mechanisms they adopt to maintain an osmotic balance and thus their responses to metal exposure [21, 22]. Copper toxicity can vary depending on osmoregulatory strategies adopted by the organisms: osmoregulators and osmoconformers display different patterns of sensitivity to copper, as well as differing

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* *Text update: In the Directive 2013/39/EU on priority substances, the EU recommended revising some EQSs to take into account bioavailability [DIRECTIVE 2013/39/EU OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 12 August 2013. Official Journal of the European Union L 226/1. Brussels]*

physiological responses as salinity changes. The iso-osmotic point of the organism and, in general, the relative Na^+ gradient between the internal fluids and the external environment, particularly the electrochemical gradient across the gill epithelium, can affect the osmoregulatory strategy of the organism and consequently its sensitivity to the metal [21, 23]. The BLM does account for the effects of water chemistry on metal speciation, but it does not explicitly consider the influence of water chemistry on the physiology of the organisms. For this reason, although both physiology and water chemistry are likely to be critical in determining copper toxicity, there is a need for research on the BLM to shift from the *chemistry-based side* to the *physiology-based side* of the model, so that understanding of the mechanism of toxicity may underpin predicting the effects of acute exposure. The aim of the present review is to discuss both these aspects, with particular regard to the effect of water chemistry on physiology, underlining the main issues that need to be addressed in future research, with a view to the development of a marine and estuarine version of the model.

2.2. Copper speciation in estuarine and coastal environments

Many estuarine and coastal zones are areas of special scientific interest and their habitats are under some kind of protection, but they are also among the ecosystems at greatest risk from anthropogenic impact, because they are affected by many human activities, including shipping, urbanization and industrialization, which are leading to their degradation [24]. One of the main concerns about these environments is the presence of metals in general and particularly copper, since its usage as a biocide in antifouling painting coatings has considerably increased after the banning of TBT (tributyltin) [25]. A survey of UK harbours, marinas and estuaries has observed that only between 10 and 30% of total dissolved copper in these areas is present in the labile fractions (i.e. free copper ion and inorganically bound copper), which are believed to be the toxic forms, whereas most of the dissolved copper is present in the non-toxic form (i.e. organically bound copper) [26]. These results suggest that the use of total copper concentrations as an indicator of toxicity may lead to an overestimation of the environmental risk. For this reason, there are ongoing efforts to incorporate copper speciation in EQS/WQC and to develop a BLM model more suitable to estuarine and marine environments [26].

Even though the BLM has shown some potential in predicting copper toxicity to marine organisms, it still has some limitations. Considering only the chemistry-based side of the model, one of the main issues regards the metal complexation with organic matter, which is calculated using WHAM. This model has been developed and validated on data obtained at concentrations of metals and humic substance appropriate for freshwaters [13, 27]. However, in estuarine and marine waters the concentrations are generally lower. Organic matter in these environments is also likely to have different characteristics and in particular different complexation capacities, compared to fluvial material [28, 29]. Thus, data on copper speciation, as well as DOM concentrations and complexation capacity are necessary for the development of a marine version of the BLM. Furthermore, some studies have shown that the source of DOM has a significant effect on acute copper toxicity [29-33]. This effect, not specifically considered by the BLM at present, may be accounted for by refining either the percentage of active humic acid (%AHA) [34] or the DOM ligand density [30], which both affect organic matter complexation capacity in the model.

Since correct modelling of copper complexation by the organic matter appears to be critical in estuaries, where most of the metal is complexed by organic ligands [35], it may be worthwhile considering possible alternatives to WHAM, although this model has been shown to be the most robust in predicting organic complexation with metals in estuaries [35]. Among the models that describe the interaction of organic matter with metals, two of them have been applied to estuaries with some success: the Stockholm Humic Model (SHM) [36] and the Nica-Donann Model [37], which is part of the Visual MINTEQ Model. The main difference between them is in the modelling of the binding sites of the organic matter: WHAM and SHM consider discrete binding sites on the organic matter surface, whereas the Nica-Donann model uses a bimodal, continuous distribution of binding affinity values for protons and metal ions.

From a purely chemical perspective, an estuary represents a zone where seawater of high ionic strength is diluted by river water, resulting in a linear salinity gradient. The two main driving forces are the river and the tidal flows: the relative intensities of one or the other control the vertical stratification of the estuary and, ultimately, the mixing of river and sea water [38]. On the basis of their vertical mixing, estuaries can be classified into three categories: partly-mixed, well-mixed and salt-wedge. Even though

this classification is often rather theoretical, it can still be useful in designing an experiment with the aim of predicting and modelling the fate and behaviour of a chemical contaminant, such as copper. In this context, dissolved metal species vary mainly as a function of salinity and, to a lesser extent, of pH and redox conditions. In an estuary, pH generally shows significant variation, which can produce a critical change in copper speciation. Model calculations have indicated that, in the absence of organic ligands, copper speciation is dominated by carbonate complexation [39], which has a strong influence on the concentration of Cu^{2+}, as an increase in pH from 7.6 to 8.2 results in a 3-fold reduction of the Cu^{2+} concentration [40].

In summary, the main issues that should be addressed in the development of a BLM for estuaries and sea are the interaction between copper and organic matter, with particular regard to the different DOM sources, and the effect of pH on copper speciation, considering the relevance of carbonate complexation at high salinity.

2.3. Effects of salinity on copper toxicity responses

2.3.1. Osmoregulatory strategies and copper toxicity in saltwater

All freshwater (FW) organisms osmoregulate to maintain the internal ion concentration above that of the external environment. They do this by an active uptake of Na^{+}, which can be inhibited by copper exposure [41-42]. As this is true for both fish and invertebrates, it is one reason why in FW the BLM is relatively successful in predicting acute copper toxicity in both groups of organisms, although the sensitivity to copper displayed by FW organisms varies by more than three orders of magnitude among different taxa [21]. On the other hand, saltwater (SW) organisms present wider inter-taxa variability of osmoregulatory strategies. In particular, SW teleost fish (bony fish) are osmo- and iono-regulators, as they maintain extracellular ion concentrations much below that of the surrounding medium. For this reason they need to continuously intake seawater and excrete the excess of salts to compensate for the water loss due to the osmotic gradient and the diffusion of salts into their tissues [21]. Elasmobranch (cartilaginous) fish are iono-regulators and osmo-conformers, because they regulate the ion concentration at approximately 50% of that in the SW, but they maintain the osmotic pressure of extracellular fluids similar to the surrounding medium by reabsorbing and retaining urea and other organic osmolytes in their tissues. This allows

them to drink less seawater than teleosts, but they still face the problem of the diffusion of salts from the external SW, where the ion concentration is higher, into their body. They compensate for this by salt excretion in urine, by secretion from the rectal gland and by salt transfer at the gill epithelium [43]. Considering finally marine invertebrates, most of them are both iono- and osmoconformers, because the ionic composition and osmotic pressure of their internal fluids are very close to those of the surrounding seawater [21].

Since acute copper toxicity is mainly a consequence a disturbance of ion transport [44-48], it is plausible that differences in osmoregulatory strategies may result in differences in copper sensitivity among species and also, as the salinity changes, within the same species. If we assume that copper exerts its toxic effect by disrupting the maintenance of a net Na^+ gradient, we may hypothesize that osmoregulators would be more sensitive to copper than osmoconformers. As the available data do not support this expectation (see Grosell et al. [21] for a review), the hypothesis that copper acts as an osmoregulatory toxicant in seawater clearly needs further discussion.

In marine osmoconformers exposed to copper, the principal cause of mortality appears to be either a disturbance in acid-base balance, related to impaired respiratory gas exchange, and/or an effect on ammonia excretion [49-52], although there is also evidence for copper toxicity via oxidative stress [53], DNA damage [54] and metabolic inhibition as a secondary effect of oxygen uptake inhibition [55]. As for marine osmoregulators, despite some disagreements between studies, there is a general agreement in recognizing impaired ion regulation as the main effect of copper exposure both in fish and invertebrates [45-48, 56].

Acute and chronic copper exposure of the gulf toadfish *Opsanus beta* induced an increase in plasma Na^+ and Cl^- concentrations, due to impaired osmoregulatory capacity both in the gill and in the intestine, resulting in fluid loss from muscle tissue [45]. These results are in accordance with previous studies on the seawater-adapted flounder *Platichthys flesus* and the rainbow trout *Oncorhynchus mykiss* [57, 58]. However, some exceptions exist: in other studies on the killifish *Fundulus heteroclitus* and the cod *Gadus morhua*, disturbances in acid-base balance and ammonia excretion were the main observed effects [23, 59]. In the experiments performed on the killifish, copper toxicity was studied across a range of salinities, from freshwater to seawater [23], testing the

hypothesis that copper act via the same general mechanism, regardless of salinity. However, the results did not support this hypothesis, showing instead that copper had different modes of action. In FW, a decrease in plasma Na^+ concentration occurred, probably as a result of copper inhibition of the Na^+/K^+ ATPase activity, whilst in SW no effect was observed either on Na^+ homeostasis or on Na^+/K^+ ATPase activity, and the only parameter affected was ammonia excretion, a very consistent endpoint in copper exposure [58, 59].

Taken together, these observations not only demonstrate the complex pattern of physiological responses to copper exposure, but also suggest the presence of a copper-targeted common factor, controlling ion transport as well as acid-base balance and nitrogenous waste excretion. Assuming this is true, we can try to give an overall explanation for the main reported effects of copper, both in osmoconformers and osmoregulators. In the former, acid-base and ammonia excretion disturbances are the main causes of copper-induced mortality because the maintenance of an osmoregulatory gradient is not an issue for this group of animals, whilst in osmoregulators the disturbance of ion transport is the main effect, as the maintenance of a Na^+ gradient is critical for them, but acid-base balance and ammonia excretion are also affected and in some cases more sensitive endpoints [23] (Table 2.1).

Table 2.1. Main copper effects in osmoregulators and osmoconformers

Main effects	**Osmoregulatory strategies**	
	Osmoregulators	Osmoconformers
Acid-base disturbance, caused by impaired gas-exchange	•	•
Increased plasma ammonia concentration	•	•
Impaired intestine and/or branchial ion transport	•	
Drinking rate disturbances	•	

2.3.2. Carbonic anhydrase enzyme: a copper target

One common denominator of the previously described mechanisms is the carbonic anhydrase (CA), an enzyme whose activity can be inhibited by metals, including copper, as has been observed both *in vivo* and *in vitro* in crustaceans [60, 61], and *in vitro* in fish [62]. This enzyme is present in a range of tissues, including branchial and intestinal epithelia, and is involved, either directly or indirectly, in a number of

physiological processes, including gas-exchange, acid-base balance, Na^+ and Cl^- transport and ammonia/ammonium excretion [63, 64], which are all reported to be affected by copper exposure. The catalytic function of CA is to facilitate the conversion of carbon dioxide and water to bicarbonates and protons [65]. In the gill of FW fish, the protons produced by the hydration of the carbon dioxide are involved in Na^+ transport at the apical membrane, while the bicarbonates are exchanged for chloride. The inhibition of CA, besides influencing these mechanisms, can also affect ammonia excretion, by reducing the diffusive trapping mechanism [66-68]. Hence the multiple functions of CA make it a candidate as the common factor that links together ion transport as well as acid-base balance and nitrogenous waste excretion and thus offer a possible explanation of the complex pattern of physiological responses to copper exposure. This hypothesis can be supported by giving further consideration to marine osmoregulators, with some distinctions between fish and crustaceans.

In marine fish there are two sites of ion transport, the gill and the intestine, which are both relevant in the maintenance of the osmotic balance. In contrast to FW fish, ion transport in SW fish is not believed to be directly associated with CA in the gill [69], but it is in the intestine, which is responsible for absorbing water to compensate for the diffusive loss of water to the concentrated external environment [69, 70]. Water absorption is driven by the uptake of Na^+ and Cl^-, which are then excreted at the gill. Ions are moved actively through the intestinal epithelium and water follows passively along the generated osmotic gradient, from the intestinal lumen into the blood. The presence of copper (and also silver [71-73], which has similar toxic effects) in seawater can reduce drinking rates, depending on the exposure time, and interferes with the intestinal uptake of water [46] through the inhibition of the active ion uptake processes that drive water flux by osmosis. Although the driving force for the movement of ions across the intestinal epithelium is provided by the enzyme Na^+/K^+ ATPase, to date the inhibition of its activity by metal exposure has not been demonstrated to be responsible for the impaired uptake processes in the intestine of marine fish. It is therefore plausible that one or several other proteins involved in ion transport in this tissue are more sensitive targets. One of them could be CA, which has been shown to play a key role in osmoregulation [74]. It has been demonstrated that both gene expression and enzymatic activity of CA can be modulated by salinity changes, similarly to the enzyme Na^+/K^+

ATPase, and that their response is tissue-specific, with significant differences between gills and intestine.

Considering the gill, the salinity-dependence of CA expression and activity has been shown in several studies, but this pattern still needs to be fully elucidated, since some discrepancies exist among species. In killifish, an increase in CA gene expression was observed after transfer from intermediate salinity to freshwater [75]; in the coho salmon *Oncorhynchus kisutch* and in the Mozambique tilapia *Oreochromis mossambicus*, CA activity increased with increasing salinity [76, 77], while flounders kept at different salinities did not show any significant differences in CA activity [78].

As for the intestine, CA expression and activity increased 2- to 4-fold in killifish [23] after transfer to seawater. In the rainbow trout, a salinity change induced a response that involved two isoforms of the enzyme CA: the cytosolic CA (CAc) and the extracellular isoform membrane-bound CA type 4 (CA4), localized at the apical region of the intestinal epithelium [79]. The former usually displays the majority of the CA activity in the intestinal epithelium and provides the cellular substrate for the Cl^-/HCO_3^- anion exchanger on the apical membrane [80]. The latter contributes to the deposition of $CaCO_3$ in the intestinal lumen, a process that reduces the osmotic concentration of the intestinal fluid and thus facilitates ion transport and water absorption through the epithelium [81]. An osmotic stress, such as caused by an abrupt transfer from freshwater to 65% saltwater, induced in the rainbow trout a transient increase of the mRNA expression and of the enzymatic activity both of CAc and membrane-bound CA4 [79]. A study on CA expression and activity, both in the intestine and in the gills of the gulf toadfish following transfer to salinity of 60 ppt, showed that the CAc was important for tolerance to hypersalinity [74].

What emerges from these studies is that CA plays a key role in osmoregulation and the modulation of its activity and expression appears to be a response to osmotic stress. If we hypothesize that CA is the main copper target in saltwater, and that in fish it displays its osmoregulatory functions mainly in the intestine, then we might assume that the copper target in SW is not (or not only) in the gill, but (also) in the intestine, and so consider copper speciation and bioavailability in this medium. In this regard, it has been observed that copper becomes less bioavailable as it moves along the intestine of the gulf toadfish; this may be due to the fact that the absorption/excretion mechanisms at

the intestinal epithelium progressively modify the chemical composition of the intestinal fluid and thus the speciation and availability of copper [46].

Marine crustaceans are metabolically less complex than fish, since the only tissue where the enzyme CA may be involved in copper-sensitive functions is the gill. However, despite being generally osmoconformers, several species of marine crustaceans can shift from osmoconformity to osmoregulation below a critical salinity, typically around 25 ppt [82, 83]. In this transition the enzyme CA could play a key role, since it represents a central component of ion uptake in the branchial epithelium of crustaceans [83], its expression is salinity sensitive and its inhibition has been linked to the disruption of ion transport and regulation [84]. The increase in CA activity in response to low salinity appears to be a central feature of the transition from osmoconformity to osmoregulation and this is believed to be a common adaptive characteristic of all euryhaline crustaceans capable of osmotic- and iono-regulation [63, 85]. If copper exposure affects CA activity, it may interfere with their ability to respond to osmotic stress and thus result in osmoregulatory imbalance.

Conceptually, from the modelling perspective, the validation of the hypothesis presented here would imply that the biotic ligand in marine fish is not only the gill, but also the intestine (as suggested also for silver [72-73]), and the relationship between toxic effect and metal accumulation at the binding site is modulated by salinity and in particular by the osmotic gradient.

2.3.2. Branchial permeability and mucus secretion

An alternative explanation for the toxic effects of copper is a general alteration of the epithelial function of the gill. Since most of the processes that have been reported to be affected by copper toxicity are associated either directly or indirectly with ion transport across the gill, a generalized mode of action has been hypothesized, possibly caused by mucus secretion [56]. Mucus is thought to act as a buffer, preventing the metal from interacting with the site of toxicity, but, if a thick layer of mucus is produced, it may increase the diffusive distance at the gill surface and thus affect branchial processes by reducing branchial permeability. However, most studies on the effects of copper exposure reported an increase, not a decrease, in branchial permeability, due to a displacement of Ca^{2+} at the gill surface, which controls the passive diffusion of Na^{+}, Cl^{-}

and also Mg^{2+}, all ions that demonstrated an increase in plasma concentrations after copper exposure [45, 58].

2.3.3. Na^+/K^+ ATPase activity: why not a target in saltwater?

An issue that may need to be elucidated is why the Na^+/K^+ ATPase activity is one of the targets of copper in FW [86], while it is reported to be unaffected in SW [87]. Since this enzyme is central to ion transport across the gills of both freshwater and marine fish, it may be expected to respond similarly to copper in the two environments. An explanation of these apparently puzzling observations can be found in a study conducted on the gill of the SW-adapted flounder [57], to which copper was applied in both *in vitro* and *in vivo* experiments. The application of copper to gill homogenates during the *in vitro* experiments caused a marked reduction in Na^+/K^+ ATPase activity, but the *in vivo* experiments showed that copper exposure induced an increase in the number of Na^+/K^+ ATPase units in the gill. This increase counterbalanced the reduced activity of each Na^+/K^+ ATPase site and thus resulted in an overall unchanged Na^+/K^+ ATPase activity in the gill tissue, as reported in most studies on marine fish gill physiology. Furthermore, the increase of Na^+/K^+ ATPase units is controlled by the production of cortisol [88], a hormone responsible for osmoregulatory balance in SW and the concentration of which is enhanced by copper exposure. Another effect of high levels of cortisol is the enhancement of protein catabolism [88], which increases ammonia production and thus plasma ammonia concentration, a reported effect of exposure to copper. A further explanation of the apparent insensitivity of the Na^+/K^+ ATPase to copper at high salinity is provided by the observation that rapid salinity change can induce the expression of different isoforms of the enzyme [89]. It is plausible that different isoforms may display differential sensitivity to copper [46].

2.3.4. Salinity changes: relevance of the osmotic gradient

With the development of a BLM for marine and transitional waters in mind, the previous considerations imply that an estuarine/marine version of the BLM should give more relevance to the physiology, as it has been shown to be important in marine and transitional environments. At present, species differences in sensitivity are addressed through adjustments of the stability constants characterizing the metal-gill interaction.

This may be sufficient in FW, but in SW it cannot explain the variation in sensitivity to copper shown by SW and estuarine organisms. The physiological mechanisms underlying the osmoregulatory system are more various and variable in marine organisms than in freshwater ones and thus result in a large inter-species variation in copper sensitivity and an intra-species variation in copper sensitivity when salinity changes [21]. The second point is particularly relevant if we consider estuaries, transitional waters or other environments characterized by fluctuating salinities.

Estuarine invertebrates display a wide range of osmoregulatory strategies. In general, at their iso-osmotic point they are osmoconformers. Above it, they are still generally osmoconformers, although a few (particularly decapods crustaceans) are able to osmoregulate [90]. Below the iso-osmotic point they weakly osmoregulate up to a certain salinity level, after which they start strongly osmo- and iono- regulating [83, 91]. Only a few estuarine invertebrates are osmo- and iono- regulators in the full tolerance range [92, 93]. A good example of the transition from osmotic and ion conformity to osmoregulation is provided by the euryhaline green crab *Carcinus maenas*, which is an osmoconformer in full strength seawater but, at a critical salinity of 26 ppt, starts to actively uptake ions across the gill. The activation of the mechanisms of ion transport is correlated with an 8-fold induction of the enzyme CA. The role of CA in ion transport and regulation in this species is confirmed by the observation that the ability of green crabs to regulate their hemolymph osmotic and ion concentrations is disrupted when branchial CA activity is inhibited [84]. A similar mechanism was also observed in the blue crab *Callinectes sapidus* [94]. If copper exposure affects CA activity, which is a key feature of the acclimation of euryhaline organisms to salinity changes, it might be deduced that copper exposure can theoretically affect the ability of euryhaline species to respond to osmotic stress.

Reviewing eight studies on the influence of salinity on acute copper toxicity, Grosell et al. [21] pointed out that none of the studies showed a linear increase in tolerance with increasing salinity, as competitive interactions among cations would have suggested, and the displayed tolerance variations were not fully explained on the basis of copper speciation. A non-linear trend of LC50 values as a function of salinity has also been reported in a study of copper toxicity on the estuarine copepod *Eurytemora affinis* [20], where the lowest toxicity was observed around the iso-osmotic point of the organism, a

fact observed also by Grosell et al. [21]. The trend of copper toxicity related to salinity gradient displayed in this study (Figure 2.2) suggests that the physiology of the organism was the driving factor influencing toxicity, hence supporting the observation that euryhaline species are more tolerant to metal exposure at iso-osmotic salinities, due to the minimization of osmotic stress [95]. By comparing measured and predicted values (calculated using the main equation of the BLM, as modified by De Schampheleare et al. [96]), Figure 2.2 also demonstrates that at present BLM predictions are inaccurate across such a wide salinity range (from 0 to 35 ppt), although, as indicated by the good agreement at high salinities, it may accurately predict toxicity in some limited situations [97].

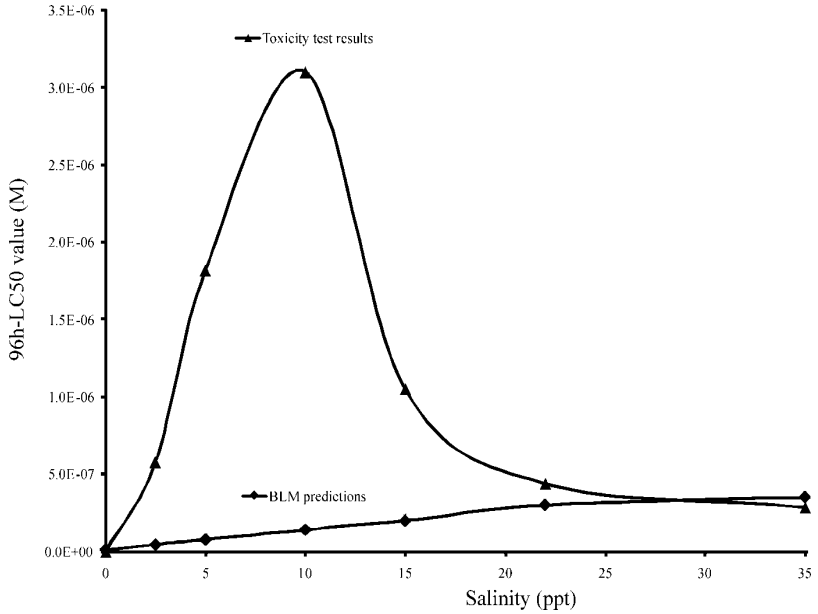

Figure 2.2. Model predictions versus toxicity data. Acute copper toxicity to juvenile killifish, *Fundulus heteroclitus*, as a function of ambient salinity (ppt), compared to BLM predictions. Data from Grosell et al. [21], and values predicted by the BLM equation as modified by De Schamphelaere et al, (2006) [96]. LC50 values are expressed as free metal ion activity (M).

Since copper disrupts the ability to sustain the osmotic gradient between the internal fluids and the external medium, the greater the gradient, the higher is the toxic effect. Therefore, including a parameter which accounts for the equilibrium potential across the gill epithelium (E_p) may reduce the bias between the predictions of the model and the observed toxicity, since the E_p undergoes a wide variation with salinity (Figure 2.3). Water and ions (including metals) are exchanged between the internal fluids and the external environment at different rates, in relation to the osmotic gradient and, ultimately, to the external salinity [21, 98]. This means that metal uptake and toxicity -

assuming a direct relationship between metal accumulation at the biotic ligand, uptake rate and toxic response - is modulated by the osmotic gradient. In fact, the mechanism is even more complex, since the mode of action of copper changes according to the osmoregulatory physiology, which in turn varies with salinity. Therefore, the relationship between copper exposure and toxic response is influenced by salinity through a "double" interaction: on the one hand, copper inhibits the ability of the organism to respond to an osmotic stress, by interfering with its osmotic mechanisms (i.e. CA), and on the other hand, a salinity variation produces an increase in copper uptake, due to a general increase in ion uptake rate, as well as, in some cases, a change in the copper biotic ligand. At present, this complex relationship is clearly overlooked by the BLM, which assumes that the toxic response is directly related to the amount of copper bioavailable and to its affinity for the binding sites, regardless of the water chemistry. Conceptually, the challenge is to introduce the effect of water chemistry (i.e. salinity) on the physiology-based part of the model, on the basis that – as I hypothesize – at varying salinities (i.e. in an estuary), the relationship between the [Cu]EC50 and the fraction of binding sites that need to be occupied by the metal to observe a toxic effect in 50% of the test organisms ($f_{CuBL}^{50\%}$) is not linear and constant, but variable and modulated by the osmotic gradient.

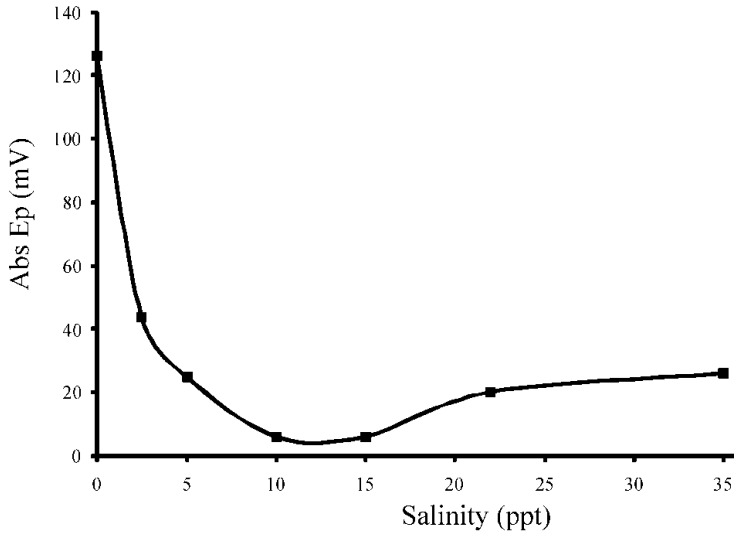

Figure 2.3. Absolute equilibrium potential (Ep) at different salinities. Data from Grosell et al. [21].

2.4. Conclusions

In conclusion, considering the chemistry-based part of the model, it is necessary to improve the ability of the BLM to describe the interaction between copper and organic matter: further investigations should be addressed at the different sources of DOM in estuaries and sea, with particular regards to their stability constants. Copper speciation studies in estuaries should also take into account the effect of pH, since it displays a significant change in this environment and it influences the carbonates, which are thought to dominate inorganic copper speciation in estuarine and sea waters.

As for the physiology-based part of the model, this presents the main issues of interest. I suggest that, in estuaries and sea, the water chemistry affects copper toxicity not only by controlling its speciation, but also by affecting the osmoregulatory physiology of the organism, which in turn varies according to the salinity. I present the hypothesis that the common factor that links together the main observed effects of copper, both in osmoregulator and in osmoconformer organisms, is the CA enzyme, given its multiple functions and its salinity-dependent expression and activity. According to this hypothesis, I also suggest that the site of action of copper in marine fish is not only the gill, but also the intestine, since this is where CA plays a role in ion transport and water adsorption. Thus, a BLM for marine fish should also consider the intestine as a biotic ligand. In conclusion, there is a need to incorporate the osmotic gradient, and possibly the organisms' osmoregulatory strategy, into BLM calculations, in order to use a mechanistic understanding of copper modes of action to predict its toxicity in a wide range of salinities.

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CHAPTER THREE

BLM REFINEMENT AND CHELEX TITRATION EXPERIMENTS AT VARYING SALINITIES

On exactitude in metal toxicity modelling

The first evidence of the strong role played by the free metal ion in determining metal toxicity dates back to the early 70's, but it took nearly thirty more years of research before the relevance of water chemistry in determining metal toxicity was eventually incorporated into a model used for regulatory purposes. And yet, it is not uncommon to come across a newly published paper that argues the role of the free ion concentrations in predicting metal toxicity. Such a never aging scientific interest shows how metals are an evergreen in ecotoxicology, probably for few fairly simple reasons:

- They are virtually everywhere;
- Unlike other chemicals in the environment, they have environmental concentrations that are not so far from their effect concentrations and therefore they can always be defined as "a matter of concern";
- Their study is great fun for scientists, since they are simple molecules yet interacting in very complex ways with both abiotic and biotic factors, i.e. living organisms.

The third point – which is also the reason I personally got interested into this class of chemicals – naturally leads to the need of modelling metal toxicity, since complex, multi-variable systems require equally complex multi-variable approaches, such as mathematical models.

The first versions of what was then baptized Biotic Ligand Model (BLM) were born some twenty years ago; since then there has been a great deal of effort in developing, refining, improving and validating new versions of BLMs. "More research is needed" is the explicit or implicit conclusion of almost any paper on toxicity models, including BLMs. Naturally; otherwise how could we justify our next grant? However, the main goal of our research, as environmental scientists, should be to understand and possibly predict, rather than just ever better describe the reality of natural systems. The latter often representing a fatal attraction for scholars, as effectively captured by Borges in his short story "On Exactitude in Science"*:

In that Empire, the Art of Cartography attained such Perfection that the map of a single Province occupied the entirety of a City, and the map of the Empire, the entirety of a Province. In time, those Unconscionable Maps no longer satisfied, and the Cartographers Guilds struck a Map of the Empire whose size was that of the Empire, and which coincided point for point with it. The following Generations, who were not so fond of the Study of Cartography as their Forebears had been, saw that that vast Map was Useless, and not without some Pitilessness was it, that they delivered it up to the Inclemencies of Sun and Winters. In the Deserts of the West, still today, there are Tattered Ruins of that Map, inhabited by Animals and Beggars; in all the Land there is no other Relic of the Disciplines of Geography.

**Jorge Luis Borges: Collected Fictions. Translated by A Hurley, Penguin Books Ltd., New York, 1998.*

Abstract

Regarding the BLM theory and the challenges it faces when it is to be applied in saline and transitional environments, the key points discussed in the first part of the chapter (Section 3.2) are the following:

- The sensitivity of the tested organism cannot be assumed constant across varying salinities: a more complex relationship between salinity and toxicity should be assumed by the model;
- In saline environments, other forms of copper, besides the free metal ion, should be considered toxic, especially those weakly bound to organic matter;
- Given the key role played by DOC in affecting copper toxicity in estuaries, it is important to better model its complexation capacity, considering for instance the different binding capacities of different types of DOC;

In the second part of the chapter (Section 3.3), a refinement of the BLM is proposed to improve the physiology-based part of the model. Published data on acute copper toxicity were used to test the predictive power of the BLM at different salinities. Model output showed that the BLM in its current form failed to predict the biphasic pattern displayed by the biological data, overall supporting the hypothesis that the linear relationship assumed by the model between copper toxicity and salinity does not reflect the complexity of the biological reality. The main equation of the model was then modified to include a salinity-correction factor, based on the absolute equilibrium potential E_P, to account for the effect of osmoregulatory physiology on copper toxicity across different salinities. Modelling results using the refined-BLM demonstrated an improvement in the predictive power of the model and highlighted the importance of the physiology-based part of BLMs in modelling toxicity under complex environmental conditions. The last part of the chapter is focused on the chemistry-based part of the BLM framework and particularly on the role of DOC in determining metal availability in estuarine waters. The results of the experiments performed with water mixing and varying salinities suggested that salinity may reduce the binding capacity of riverine DOC. It was also observed that estuarine DOC was a less strong ligand than riverine DOC, although further investigations are required to confirm this observation.

3.1. Introduction

Over recent decades, the study and regulation of metal toxicity to aquatic biota has undergone an evolution that was punctuated by a series of scientific and regulatory milestones [1], such as the introduction, in the 1970s, of a water-hardness correction factor applied to the total metal concentration in order to generate site-specific environmental quality standards (EQS) [2]. This advancement in metals regulation represented the first acknowledgment of the relevance of water chemistry on metal bioavailability and toxicity [3], which was previously based solely on total, or total dissolved metal levels. In the US, the hardness-based approach to calculating water-quality criteria was replaced in 2007 by the introduction of the Biotic Ligand Model (BLM) [4], a toxicity model that accounts for the influence of chemical binding and interactions on metal bioavailability [5, 6]. In particular, the BLM predicts metal speciation and toxicity on the basis of ten water quality inputs: temperature, pH, alkalinity, dissolved organic carbon (DOC), major cations (Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}), and major ions (Cl^{-}, SO_4^{-}).

This more comprehensive approach adopted by the US and subsequently by other countries, including the UK [7, 8], represented another important step forward towards a science-based regulation of metal toxicity. This is significant from both an environmental and an economic point of view, since overlooking speciation and bioavailability issues could lead in some cases to costly overprotection of aquatic biota [9], e.g. where natural organic matter decreases metal bioavailability. This aspect is particularly relevant in the UK, where EQSs failures based on total metal concentrations for copper and zinc would not be uncommon, even though there is little evidence that this results in adverse effects on the ecosystem. In other contexts, the application of metal EQSs that do not consider chemical parameters such as pH may lead to damaging underprotection of the environment [8]. In either case, it is now well established that best-practise metal toxicity assessment and regulation must account for the influence of the water chemistry parameters.

Despite the fact that the BLM concept has improved our understanding of metal bioavailability, adding new emphasis on chemical speciation, at present the application of BLMs requires the acquisition of large amount of chemical data, which can be costly

if not impractical [10]. It is therefore sensible to question the relevance of the model input factors and assess whether these chemical data are all equally relevant to the accuracy of the model predictions, or if instead we should focus our attention only on some key parameters. For example, Arnold et al. [11] suggested that a marine version of the BLM should give more weight to the DOC factor, given its influence on metal toxicity, and in so doing allowing excluding other, less relevant chemical parameters. The same approach could be applied to other environments, such as estuaries and coastal zones, where DOC and pH appear to be the driving factors controlling metal speciation and hence toxicity [12].

Another, more fundamental observation to the BLM concept is that it is essentially "chemistry-oriented", with little or no emphasis on the physiological mechanisms of toxic action displayed by the metal when entering an organism. If we consider the deep understanding reached on the chemistry-based part of BLMs, it is even more striking to see the lacking of mechanistic understanding displayed by the physiology-based part of the model, where toxicity is directly related to the amount of metal ions accumulated at the biological site of action, typically the gills of the organism, regardless of the toxicodynamic processes underlying that relationship (see Figure 3.1.).

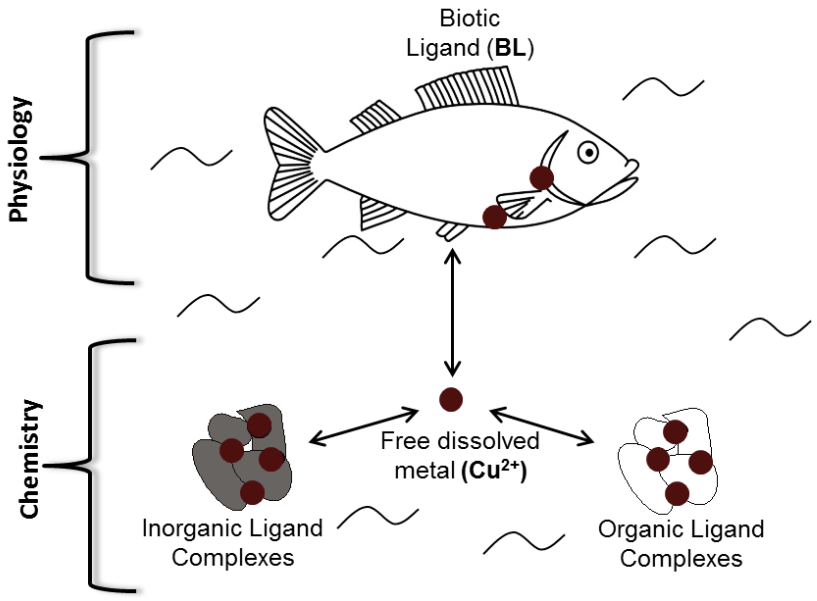

Figure 3.1. Schematic representation of the BLM framework, highlighting its *physiology-based part* and *chemistry-based part*. The former includes the interactions between the free metal ion (Cu^{2+}) and the fish, whose branchial and intestinal surfaces constitute the so-called biotic ligands; the latter includes the interactions between the metal and either inorganic ligands, such as bicarbonates, chloride ions or hydroxides, or organic ligands (i.e. organic matter).

Even though in many contexts BLMs have been proved robust and effective tools to predict metal toxicity, there are cases where their effectiveness and accuracy are challenged by the complexity of the system. In some studies, the lack of model fit has been addressed by empirically adjusting model parameters, a practice that can weaken the mechanistic basis of the model itself, reducing it to a data-fitting exercise with little biological meaning [1]. Most of these cases share one common feature: they all involve interactions of the metal in question with other chemical stressors, usually metals [13, 14], or non-chemical stressors, such as salinity [11]. Put another way, it is in multi-stressor scenarios that the BLM concept is likely to show its weaknesses, presumably because it fails to account for the physiological mechanisms and toxicodynamics processes underpinning the complexity of many biological responses [15].

Although BLMs were originally developed for single metals, rather than mixtures, and under constant exposure conditions, there is now a generally recognized need to take toxicity models one step forward, from single to multiple metal scenarios and from constant to variable environmental conditions. Regarding mixed-metals exposures, one approach that has been applied with some success is based on the concept of toxicity units and on the assumption of strict additivity among metals [16]. Even though the admitted intent of the study was solely to test the ability of the BLM concept in handling multiple metals, it made it nonetheless apparent that, when faced with the multi-stressor challenge, BLM development often applies a purely mathematical approach.

As for the interaction between metals and non-chemical stressors, studies are scarce and usually inconclusive from the modelling standpoint, but often rich in mechanistic information and biologically relevant interpretations (see [17] for a review). Given the rising interest of both the EU and US EPA to incorporate the interactions between environmental chemicals and natural stressors into cumulative risk assessment procedures (see, for example, the EU *NoMiracle* project [18], and the 2013 workshop of the Society of Toxicology [19]), it is timely for the scientific community to rethink toxicity models such as BLMs and apply them to more complex scenarios. In order to rise to this challenge of including aspects of combined stressors into metal toxicity models, it is crucial to first achieve an understanding of mechanisms of toxic action by applying mechanistic physiology-based approaches, and then fit the acquired knowledge into more complex systems through the use of pathway-centric approaches.

Therefore, a possible way to re-think the BLM concept in order to improve its predictive power, especially in complex scenarios such as metal mixture or multi-stressor studies, would be to develop its physiology-based part, taking into account the effects that some factors may have on the physiological response of the organisms to metal exposure in specific conditions or environments [15]. Although this appears to be a promising approach, at least for BLMs applied in saline environments, it requires an understanding of the physiological mechanisms of action that at present is available only for a limited number of metals and/or conditions. However, given the rising interest to apply BLMs to complex scenarios [18], it is worth maximizing the information available in the literature to try and broaden the applicability of the model to variable conditions, changing environments and multi-stressor contexts.

This study is focused on the case of a BLM predicting copper toxicity in estuaries, which are environments highly impacted by metal pollution and particularly by copper, given its use as a biocide in antifouling painting coats [20]. From the more theoretical point of view of testing the ability of BLMs to cope in complex scenarios, estuaries represent a challenge for toxicity modelling, as the biota they host is subjected to a combination of anthropogenic impacts and variable abiotic factors [21]. The following section outlines the two models from which the current forms of BLMs originated, with the aim of highlighting the complexity of the chemistry-based part of the model, as in contrast with its physiology-based side. The main chemistry parameters presently included in BLMs are then analysed (Section 3.2) to identify the factors that should be further investigated in the context of a BLM development for estuaries. The third part of the chapter (Section 3.3) illustrates a possible way to refine the BLM algorithm to include a factor accounting for the effect of the osmotic gradient on the physiology of organisms exposed to metals under different salinities. The results presented here are just preliminary and aimed at proving the concept of the relevance of physiology in modelling metal toxicity in complex environments. Finally, the last part of the chapter presents the experimental work done to test the influence of DOM type and salinity on copper availability when riverine and estuarine waters are mixed. The results are modelled to identify chemical features (i.e. binding capacity and ligand strength) that explain the observed difference between riverine and estuarine DOM.

3.1.1. BLM ancestors: the WHAM-SCAMP and GSIM-FIAM models

The BLM framework is essentially composed of two parts: a *chemistry-based part*, including inorganic and organic speciation computational models, and *a physiology-based part* (Figure 3.1), based on the concept that toxicity occurs when the fraction of binding sites occupied by the metal ions reaches a critical concentration, mathematically expressed as $f_{CuBL}^{50\%}$ [5, 6]. The chemistry-based part of the BLM framework derives from the incorporation of two speciation models: the Windermere Humic Aqueous Model (WHAM), originally developed by Tipping and Hurely [22, 23] to compute the chemical distribution of dissolved metals, and the Surface Chemistry Assemblage Model for Particles (SCAMP) [24, 25], modelling the interactions of metals with the particulate and colloidal phases (Figure 3.2.). WHAM describes the interactions of metals with *(1)* inorganic ligands (OH^-, HCO_3^-, CO_3^-, SO_4^-, Cl^-), using equilibrium constants from the literature; and *(2)* humic substances, using the Humic Ion-Binding Model (HIBM) [22, 26].

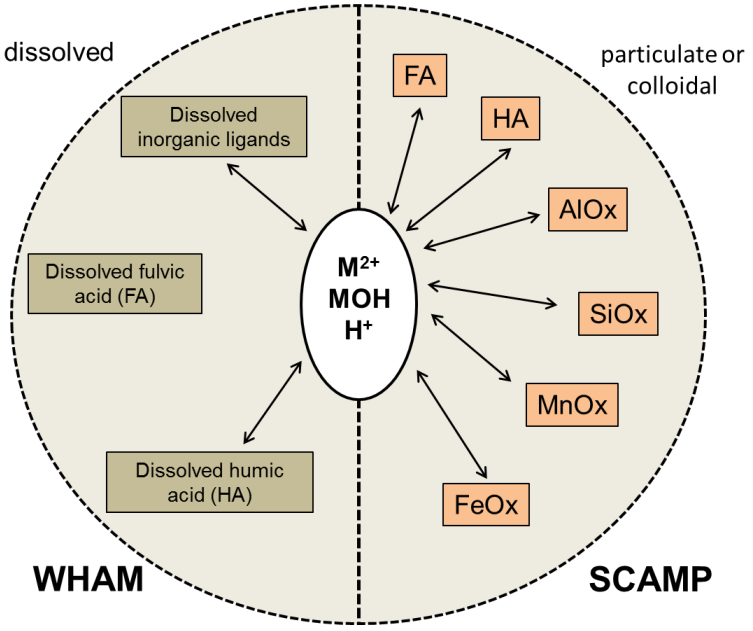

Figure 3.2. Schematic representation of the chemistry-based part of the BLM, which includes two models: the WHAM and the SCAMP. The WHAM computes for the interaction of metal ions with the dissolved fraction, constituted by dissolved inorganic ligands (OH^-, HCO_3^-, CO_3^-, SO_4^-, Cl^-) and dissolved organic ligands (fulvic acid, FA, and humic acid, HA). The SCAMP describes the interactions of metals with the particulate and colloidal fractions, which include both particulate organic matter (FA and HA) and aluminium, silicium, manganese and iron oxides.

The humic substances are described as humic acids (HA) and fulvic acids (FA) possessing hydroxyl groups that can interact with protons and metal ions. Metal binding takes place

at proton-dissociating sites carried on the surface of the humic compounds and the interactions between the metal ions and the humic substances are described by intrinsic equilibrium constants. The model permits the binding of the first hydrolysis product (e.g. $CuOH^+$ in the case of copper), as well as the free metal ion, Cu^{2+}, both species having the same intrinsic equilibrium constant, pK_{MHA}. The smaller is the value of the pK_{MHA}, the stronger is the binding of the metal [24, 27]. As for SCAMP, it calculates the binding of metals to suspended particulate matter on the basis of its chemical composition and accounting for the effects of pH, ionic strength and competition with major cations. It includes a sub-model for oxides, which considers the interactions with aluminium, silicium, manganese and iron oxides, and a sub-model for cation exchange, which simulates the interactions with an idealized clay cation exchanger. The two sub-models are in their essence similar to the HIBM: the binding sites are represented by hydroxyl groups carried on the surface of the oxides (or of the clays) and the metal binding is expressed in terms of metal-proton exchange reactions [24].

While WHAM and SCAMP provided the benchmark from which the chemistry-side of BLMs originated, the "closest ancestors" of the BLM framework in its more comprehensive formulation are the Gill Surface Interaction Model (GSIM) and the Free Ion Activity Model (FIAM), formulated respectively by Pagenkopf [28] and Morel [29], both in 1983. In the GSIM, toxicity is modelled as a simple, empirical correlation between accumulation of metal ions at the gill surface and observed toxic effects, where accumulation is reduced by both metal-complexing ligands and other cations. Both the GSIM and the FIAM assume chemical equilibrium conditions.

Pagenkopf was the first, followed by Playle and co-workers [30, 31], to hypothesize that the reactions taking place at the gill surface, which he called *critical sites*, occurred much faster than the physiological response elicited in the organism. Such an observation was an important assumption underling the use of the equilibrium modelling principles. Both models also recognized that the degree of toxic response was directly related to the fraction of critical sites impacted by the metal species, which is another key assumption at the basis of the BLM theory. The term "critical site", originally conceived by Pagenkopf as a generalization of the fish gill surface, was renamed *biotic ligand* in the BLM framework to expand the modelling concept beyond the gills and also to stress that toxicologically active sites on the organisms are modelled as biotic ligands competing

with abiotic ligands in the exposure water for binding the metal ion. Although there are many different definitions of biotic ligand, the term is now commonly used to indicate every site in which the metal interacts with the organism; this broad definition is applicable to other tissues besides gills and other taxa besides fish. Indeed, in the two decades following its birth in 2001 [5, 6] the BLM approach has been further developed beyond its original scope to include other taxa, endpoints and exposure conditions, and significant BLM development research is still on-going. The basis of the BLM remains, however, in modelling an accumulation of metal on a biologically active surface and linking this concentration to toxicity.

3.2. BLM theory and challenges for modelling copper toxicity in estuaries

The theory of BLMs in general and copper BLMs in particular is essentially based on a few key assumptions [5, 6, 32], each of which can be critically discussed in the perspective of a BLM version for transitional environments.

1) The first assumption is that toxic effects occur when the metal-biotic ligand complex reaches a critical concentration, determined by the number of binding-sites occupied by the metal and thus, since the binding-site density is assumed constant, correlated only to the concentration of metal available to bind. The model assumes a direct correlation between metal bioavailability in the exposure medium, metal accumulation on the biotic ligand and metal toxicity.

Most of the original development work for modern BLMs has been done on freshwater fish [28, 30], while the parameters of BLMs applied to other freshwater organisms (e.g. daphnids) were derived by recalibrating the fish-based constants of the original model using acute toxicity data, regardless of the physiological mechanisms behind the observed toxicity and with no account for the sensitivity of the test species [33]. This simplification of the mechanisms of toxic action is questionable, in the context of an estuarine BLM, because in marine fish and invertebrates the mechanisms of copper toxicity have been shown to display more complex patterns [34, 35]. In particular, some studies of chronic copper exposure on marine fish do not support a simple correlation between copper concentration in osmoregulatory tissues and observed effects at organism level [35, 36],

overall supporting the idea that the relationship between copper exposure and toxic response in transitional waters and at varying salinities is not simple and direct, but complex and modulated by salinity.

2) The most toxic form of copper is the free metal ion, Cu^{2+}, which interacts with the binding site of the organism. Copper toxicity is thus largely dependent on the free metal ion activity.

Although it is generally accepted that copper toxicity is largely related to the concentration of free copper ions (Cu^{2+}), other forms of copper, such as copper hydroxides ($CuOH^{+}$ and $Cu(OH)^{2}$) and carbonates (i.e. $CuCO_3$) can also be toxic. Although the affinities of $CuOH^{+}$ and $CuCO_3$ for the biotic ligand are respectively 5- and 10-fold lower than that of Cu^{2+} [37], some authors suggest that these should still be considered in toxicity modelling [38, 39]. The possible toxic effects of copper hydroxides were first suggested by Meador [40] and later acknowledged by De Schamphelaere [33, 41], who included $CuOH^{+}$ and $Cu(OH)^{2}$ (and also $CuCO_3$) in his refined version of the BLM as other active forms of copper. Meador observed that the percentage of variation in the mortality data explained by Cu^{2+} decreased at increasing pH, suggesting that this was due to the increasing toxicity of other forms of copper and particularly copper hydroxides at increasing pH [40].

The toxicity of copper hydroxides and carbonate might be especially relevant in saltwater, where the average pH (8.1) is higher than in freshwater (7.6). If both pH and salinity are increased simultaneously, as can occur in an estuary, copper carbonate also becomes a relevant fraction of the total copper speciation and should therefore be taken into account in toxicity modelling. In the estuary mixing modelled by Mantoura et al. [42], when salinity is gradually increased from 0 to 35 ppt and particularly above 10 ppt, most of the total copper is represented by $Cu(OH)_2$ (~80% at 10 ppt), followed by $CuCO_3$ (~8%) and $CuOH^{+}$ (~1%), leaving only < 1% in the form of Cu^{2+}, while the fraction of copper complexed with organic matter, which was the dominant form at salinity below 10 ppt, decreases to ~10%.

Another form of copper that has been shown to be bioavailable to some species is the copper fraction weakly bound to organic matter. When the binding affinity of a biotic ligand to copper is greater than that of some organic ligands, organic copper can become

bioavailable and thus bind to the biotic ligand [12, 43]. Put another way, if gills and organic matter are seen as two binding factors competing for the copper ions, the one with the highest affinity for copper will prevail, forming the stronger complex with the metal. The potential bioavailability and hence toxicity of some forms of organic copper is particularly relevant in riverine and estuarine zones, where most of the copper is complexed by organic matter, with stability constants ranging from $10^{6.6}$ and $10^{15.6}$ [44]. For instance, a study on the effect of organic complexation on copper toxicity to the estuarine red macroalga *Ceramium tenuicore* showed that copper accumulation and its growth inhibitory effects in artificial seawater at DOC concentrations typical of estuaries (2-4 mg/L) are better correlated to weakly complexed copper concentration rather than to Cu^{2+} [45].

3) Organic ligands compete with the biotic ligand for binding the free metal ion; natural dissolved organic matter (DOM) can hence complex copper ions, reducing copper bioavailability and ultimately toxicity.

Although the protective effect of DOM on metal toxicity is generally widely acknowledged [3, 40, 46], some studies have shown that DOM source has a significant effect on copper toxicity [47, 48]. From the modelling standpoint, this effect could be accounted for by refining either the percentage of active fulvic acid (%AFA) [49] or the DOM ligand density [48], as both parameters affect DOM complexation capacity. Evidence exists that not only riverine and marine DOMs, but also estuarine DOM has specific characteristics, being a mixture of various sources of organic matter, partly autochthonous (mainly due to estuarine phytoplanktonic blooms) and partly allochthonous, with a dominance of terrestrial organic matter [50]. Since DOMs from different sources are composed of molecules with quantitatively and/or qualitatively different copper-binding functional groups, it is plausible that their complexation capacity would be different [47, 51] and this should therefore be accounted by BLMs.

4) The major cations present in solution have a protective effect on copper toxicity, because they compete with the metal ions for binding to the biotic ligand.

Among the major cations considered in BLMs (Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}), sodium is the trickiest to parameterize, as it has a double protective effect on copper toxicity: on the one hand it competes with copper for the binding sites, but on the other hand it also

provides “assistance” in sodium uptake, whose impairment is one of the main effects of acute copper toxicity [52]. Whilst the “competition effect” is expected to reach a plateau at increasing salinity, the “assistance effect” is not only salinity-dependent, but more importantly is “saturable”. The salinity-dependent feature of the sodium effect on copper could be expressed by different stability constant values at different salinity levels, provided that we first identify the threshold concentrations of sodium at which it switches from one mode of protection to another.

Another issue regarding the parameterization of the sodium effect is that the variation in copper toxicity observed at varying salinities cannot be fully explained by the competitive effects of the major cations (and neither by copper speciation) [53], suggesting instead that physiology is critical in modelling the relationship between salinity and acute copper toxicity. In particular, as highlighted in Chapter Two [15], at varying salinities the relationship between copper toxicity and copper accumulation at the binding sites is not linear and constant, as predicted by the model in its current form, but variable and in particular modulated by the osmotic gradient between the internal fluids of the organism and the external medium.

5) Water chemistry parameters affect copper toxicity by affecting copper speciation and accumulation at the binding sites; additionally, they affect the capacity of the organic and inorganic ligands to form complexes with both copper and the organism itself.

One of the most important chemical factors controlling copper speciation is water hardness, which refers to the concentration of the multivalent cations, Ca^{2+} and Mg^{2+}. In general, calcium and magnesium ions affect copper toxicity by competing for either the biotic ligand or the binding sites at the organic matter surface, in one case decreasing copper toxicity and in the other case increasing it by reducing the complexation capacity of the organic matter. These two mechanisms, resulting in opposite effects on copper toxicity, may explain the disagreeing results reported in the literature [46, 48, 54-56]. In addition to this already complex scenario, the final outcome of the combined effects of hardness and organic matter on copper toxicity depends on the hardness level: the higher the hardness, the weaker is the protective effect of DOC on copper toxicity and the stronger is the competition of calcium and magnesium ions at the binding sites. However, the latter effect is saturable and reaches a plateau at high hardness levels [3]. Hence there

seems to be a reinforcing effect of the two factors at low hardness and a counteracting effect at high hardness [55], suggesting a non-linear relationship between hardness and copper toxicity in the presence of organic matter.

Another important factor controlling copper speciation is pH, whose final effect on copper toxicity is highly debated and still not fully understood [3, 40]. In general, pH can influence copper toxicity either by affecting its speciation or by interfering at the biological surface through the competition of hydrogen ions with metal ions at the binding sites [57]. When pH increases, the concentration of free copper ions decreases, as more copper hydroxides are formed, leading to a decreased copper toxicity, as free copper ions are more toxic than copper hydroxides. However, the amount of hydrogen ions competing with cupric ions for the biotic ligand decreases at increasing pH, thus enhancing copper toxicity. This implies that, even if overall less free copper is available, a given amount of free copper ions has more toxic effect at higher pH levels, eventually resulting in a "cancelling effect" that might explain why in some studies copper toxicity was reported to be unaffected by pH.

Furthermore, pH can theoretically affect the complexation capacity of DOC and hence its protective effect on copper toxicity, by either altering the stereochemistry of the DOC molecules or by competing for its binding sites with the metal ions. However, statistical analyses on toxicity and chemistry data haven't revealed a significant interaction of DOC and pH [40].

3.3. BLM refinement

3.3.1. Introduction and aims

In the main equation of the BLM (see below Equation 1), toxicity is determined by the concentration of copper bound to the binding sites of the biotic ligand ($CuBL^+$) and is expressed as a function of Cu^{2+}, Ca^{2+}, Mg^{2+}, Na^+ and H^+ concentrations [5, 6, 41]. The ratio between the concentration of binding sites occupied by copper ($CuBL^+$) and the total site density, which describes the complexation capacity of the biotic ligand (CC_{BL}), expresses the fraction of binding sites occupied by copper when a toxic effect occurs (f_{CuBL}). This fraction f_{CuBL} determines the magnitude of the toxic effect and is constant at 50% effect ($f_{CuBL}^{50\%}$), regardless of the water chemistry and its effect on the physiology of

the test species. The general BLM equation has been refined by De Schamphelaere et al. [33] to account for the effects of copper hydroxides and carbonates (see Equation 2).

(1)

$$\mathbf{f_{CuBL}} = \frac{[\mathrm{CuBL^{+}}]}{\mathrm{CC_{BL}}} = \frac{[\mathrm{K_{CuBL}} * (\mathrm{Cu^{2+}})]}{\{1 + \mathrm{K_{CuBL}} * (\mathrm{Cu^{2+}}) + \mathrm{K_{NaBL}} * (\mathrm{Na^{+}}) + \mathrm{K_{MgBL}} * (\mathrm{Mg^{2+}}) + \mathrm{K_{CaBL}} * (\mathrm{Ca^{2+}}) + \mathrm{K_{HBL}} * (\mathrm{H^{+}})\}}$$

(2)

$$\mathbf{EC_{50}} = \frac{\mathrm{f_{CuBL}^{50\%}} * \{1 + \mathrm{K_{NaBL}}(\mathrm{Na^{+}}) + \mathrm{K_{MgBL}}(\mathrm{Mg^{2+}}) + \mathrm{K_{CaBL}}(\mathrm{Ca^{2+}}) + \mathrm{K_{HBL}}(\mathrm{H^{+}})\}}{(1 - \mathrm{f_{CuBL}^{50\%}}) * \{\mathrm{K_{CuBL}} + \mathrm{K_{CuOHBL}} * \mathrm{K_{CuOH}} * (\mathrm{OH^{-}}) + \mathrm{K_{Cu(OH)2BL}} * \mathrm{K_{Cu(OH)2}} * (\mathrm{OH^{-}}) + \mathrm{K_{CuCO3BL}} * \mathrm{K_{CuCO3}} + [\mathrm{CO_3^{2-}}]\}}$$

In both equations, the fraction of binding sites occupied by copper when a toxic effect occurs in 50% of the test organisms ($\mathrm{f_{CuBL}^{50\%}}$) is intrinsically independent from the water chemistry and does not account for the effect that this can have on the sensitivity of the organism to the metal. This formulation therefore assumes a direct and linear relationship between metal accumulation at binding sites and the toxic effects.

It is the linearity of this relationship that is questionable, from a biological viewpoint and particularly at varying salinities, as occurring in an estuary. Indeed, exposure studies on both fish and crustaceans (see for example [53, 58]) performed across different salinities demonstrated that the sensitivity of the organism depends on the iso-osmotic point of the organism and, in particular, on the osmotic gradient between the internal fluids and the external exposure media. Taking account of these observations, metal toxicity should be modulated by the osmotic gradient.

An example of the non-linear relationship between metal accumulation and toxic response is provided by Grosell and co-workers [53], who tested copper toxicity across a full range of salinities. Their results showed a significant correlation between acute copper toxicity and the sodium gradient, the latter being represented by the energy required from the organism to sustain this gradient across the gill membrane. In order to account for this salinity-gradient effect, Grosell suggested using the Nernst equation

(Equation 3), which is based on electrochemical gradients and estimates the energy required to sustain a given ion gradient across an epithelium or a membrane, expressed as the absolute equilibrium potential, E_P [59]:

$$E_P = (\frac{RT}{zF}) * 2.303 * \log\frac{[Na^+_{blood}]}{[Na^+_{water}]} \qquad (3)$$

Where $[Na^+_{blood}]$ and $[Na^+_{water}]$ are the concentrations of sodium in the blood and in the water, respectively, *z* the valence of the ion in question (e.g. copper) and *R*, *T* and *F* the gas constant, the absolute temperature and the Faraday's constant, respectively. Since all of these parameters are constants, the equilibrium potential E_P varies only according to the sodium gradient across the epithelium (i.e. the gills), which is in turn dependent on the external concentrations of sodium (and ultimately on the salinity), given that the internal levels are relatively constant under normal conditions. Regression of copper toxicity, expressed as log[Cu^{2+}]EC50, versus the equilibrium potential E_P (Figure 3.3), revealed that around 93% of the variation in toxicity can be explained by the osmotic gradient, in accordance with the hypothesis that copper toxicity is correlated with osmoregulatory physiology of the test species.

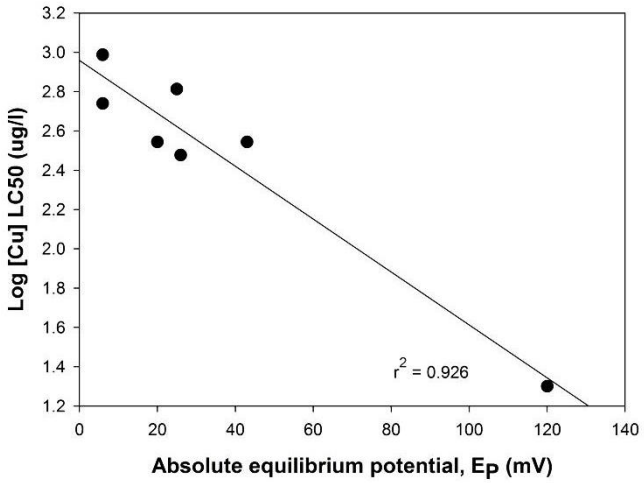

Figure 3.3. Log LC50s as a function of the absolute equilibrium potential E_P

In this context, the aim of this study was dual and consisted in two steps:

- First, modelling the toxicity data presented by Grosell et al. [53] to test the ability of the BLM to predict the observed toxicity;

- Second, modifying the general BLM equation in order to fit model predictions with biological observations; in particular, this data fitting was based on an osmotic-gradient modulation of the toxic response, as discussed in Chapter One.

3.3.2. Results and discussion

To perform the modelling of Grosell's data with the BLM, input values were constructed from the data provided in the paper [53] and included Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, pH, CO_3 and DOC concentrations. Ion concentrations were transformed into free ion activities using the WHAM model before being input in the software; binding constants and the $f_{CuBL}^{50\%}$ value were the ones provided by De Schamphelaere [49]; temperature was assumed constant (25 ºC) and a 50% active fraction of DOC was used in all simulations. Model outputs are plotted in Figure 3.4, together with measured toxicity results. As it can be seen, measured and predicted data follow two different trends: observed toxicity data display a biphasic pattern, with decreasing copper toxicity until 10 ppt and then increased toxicity at salinities above 10 ppt.

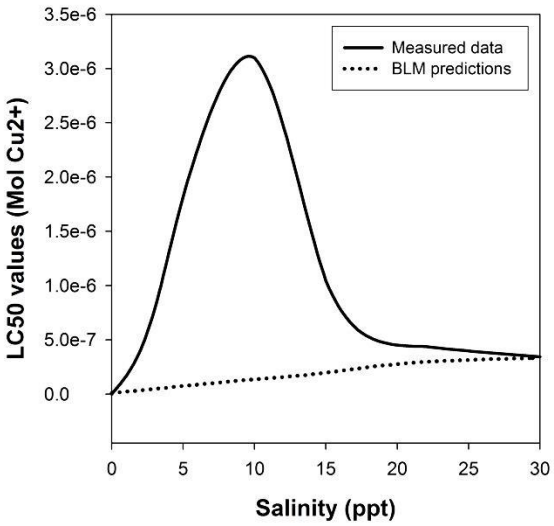

Figure 3.4. LC50 values (expressed as moles of free copper) across a full range of salinities, respectively observed (black line) and predicted (dotted line) by the standard BLM. Data from [53].

In contrast to measured values, BLM predictions follow a trend of decreasing copper toxicity at increasing salinity, in line with the general pattern of reduced copper bioavailability at higher ionic strength. According to this analysis, it appears that salinity has an effect on copper toxicity that it is not accounted for by the model, probably because the BLM considers the effect of salinity only on copper speciation and not on the

organism's sensitivity to copper. In order to include species sensitivity into the BLM algorithm and modulate the relationship between [Cu]EC50 and the $f_{CuBL}^{50\%}$ on the basis of the osmotic gradient, the equilibrium potential E_P was added into the general equation and a *salinity correction factor* was applied to the $f_{CuBL}^{50\%}$. The so corrected parameter, defined as $\left(f_{CuBL}^{50\%}\right)^*$, was re-calculated as showed in Equation 4.

$$\left(f_{CuBL}^{50\%}\right)^* = \begin{cases} f_{CuBL}^{50\%} * \left(\frac{100}{E_p}\right)^{1.5}, & E_p \neq 0 \\ f_{CuBL}^{50\%}, & E_p = 0 \end{cases} \qquad (4)$$

In this way, at the iso-osmotic point of the organism, $f_{CuBL}^{50\%}$ remains unchanged, while it decreases when the absolute equilibrium potential E_p increases. This means that the number of binding sites that need to be occupied by the metal ion to elicit a toxic effect increases at increasing osmotic gradient. Mathematically, when $E_p = 0$ the $f_{CuBL}^{50\%}$ parameter is to be used, instead of the modified $\left(f_{CuBL}^{50\%}\right)^*$.

The second step in this study was to re-analyse Grosell's data using the BLM equation corrected for the osmotic gradient. As showed by Figure 3.5A, the refined BLM demonstrated an overall better predictive power, with model predictions (represented by the dashed line) displaying a biphasic pattern, in line with the biological data. However, at salinities above the iso-osmotic point of the tested organism (10 ppt), BLM-predicted LC50 values were above the observed data, a fact that could represent a potential risk of underprotection. This overestimation could be explained by an inaccurate modelling of the sodium effect. In particular, this refined version of the model accounted for the effect of sodium twice: first, directly, through the sodium factor, $K_{NaBL}(Na^+)$, and second, indirectly, through the *salinity correction factor*, which in its formulation includes the external sodium concentrations. The sodium factor $K_{NaBL}(Na^+)$ was therefore removed from the algorithm and the [Cu]LC50s were re-calculated using Equation 5. Output data (Figure 3.5B) displayed an improvement in the predictive power of the modified-BLM above 25 ppt, as demonstrated by the overlapping of predicted and observed LC50 values, although the new BLM-predictions were still skewed at salinities between 15 and 25 ppt,

demonstrating that further model fitting will be required to refine the model and especially to validate it with data obtained from other *in vivo* studies.

(5)

$$\mathbf{EC_{50}} = \frac{(f_{CuBL}^{50\%})^{*} * \{1 + \cancel{K_{NaBL}(Na^{+})} + K_{MgBL}(Mg^{2+}) + K_{CaBL}(Ca^{2+}) + K_{HBL}(H^{+})\}}{(1 - (f_{CuBL}^{50\%})^{*}) * \{K_{CuBL} + K_{CuOHBL} * K_{CuOH} * (OH^{-}) + K_{Cu(OH)2BL} * K_{Cu(OH)2} * (OH^{-}) + K_{CuCO3BL} * K_{CuCO3} + [CO_3^{2-}]\}}$$

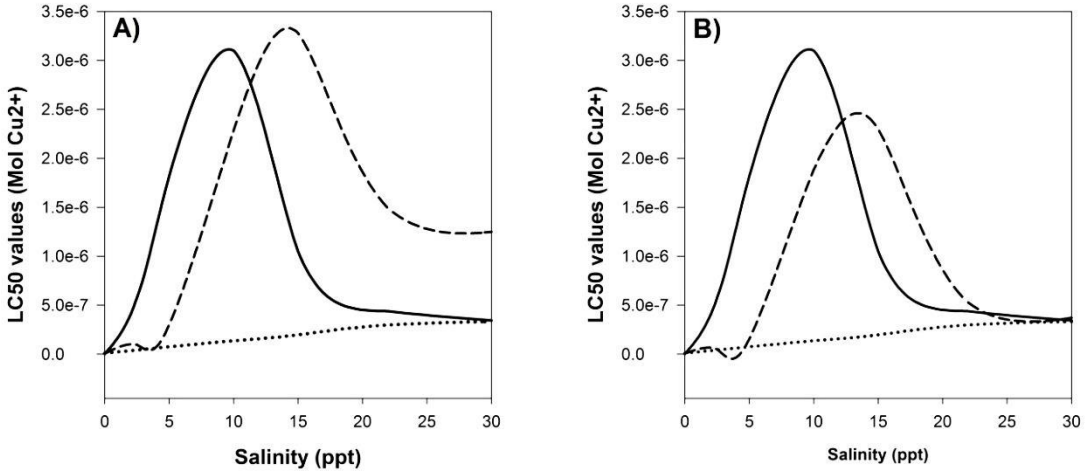

Figure 3.5. LC50 values (expressed as moles of free copper) across a full salinity range. Black lines represent observed values, dotted lines are values predicted by the standard BLM and dashed lines represents model predictions using the refined-BLM, respectively with (**A**) and without (**B**) the sodium factor $K_{NaBL}(Na^{+})$. Data from [53].

This first section of the study was aimed at refining the *physiology-based part* of the BLMs in order to include an iso-osmotic point factor into the model and so account for the effect of water chemistry on the physiology of the test organism. However, the *chemistry-based part* of the BLMs also requires some refinement, if the model has to be applied to estuarine and transitional environments. Therefore, the second section of this study was focus on understanding how BLMs could be improved in order to better predict copper toxicity in estuaries.

3.4. Mixing Experiments with Chelex column method

3.4.1. Introduction and aims

As discussed in Section 3.1.1, one important aspect of modelling metal speciation to predict toxicity is the relevance of the dissolved organic matter (DOM) in determining metal bioavailability [40, 46, 49]. This is especially true for metals like copper, which

exhibits a high affinity for the organic matter, and in environments such as rivers and estuaries, where the majority of the total dissolved metal is bound to the DOM [12, 60]. Two aspects of the copper-DOM interaction issue are worth particular consideration, in the context of a BLM refinement:

- First, the possible availability of the metal fraction weakly bound to organic matter [12, 43];
- Second, the relevance of the DOM types and characteristics in determining its binding affinities and complexation capacities, hence resulting in different levels of protection against metal toxicity [48-51].

In the context of this study and its focus on modelling copper toxicity in estuaries, the first issue translates into a need to redefine the fraction of metal available to the organisms, accounting also for the weak copper-ligand complexes, whilst the second one can be reformulated as:

- Is estuarine-derived DOC more or less effective in binding copper than riverine-derived DOC?
- How does the mixing of river and estuary waters affect the overall capacity of the DOC present in solution to bind copper and hence reduce its bioavailability?

Both aspects can be theoretically addressed by picturing the organic ligand and the biotic ligand as two chemical entities competing for that fraction of metal available to be bound, which is generally assumed to be represented by the free metal ion (i.e. Cu^{2+}) [29]. However, if we assume that other forms of metal can also bind to the biotic ligand, this available fraction needs to be experimentally re-defined according to its affinity respectively for the biotic and the organic ligands (i.e. the two strongest "competitors"; inorganic ligands are also competing to bind metal ions). The concentration of this experimentally-defined fraction of metal, called the *labile fraction*, depends on the binding affinity and complexation capacity of the organic matter present in solution [61]. Therefore, in order to better characterize the interaction between copper and DOC, it is first necessary to characterize DOC itself and particularly estuarine-derived DOC, as in contrast with the type of DOC that can be found in a typical river.

From a modelling perspective, DOC is considered to be composed of an active fraction to which ions bind, and another fraction which is inert. The active fraction determines the

DOC concentration that is used by the model to calculate metal speciation and toxicity and therefore has a significant effect on the BLM forecast. Since the actual active fraction values are rarely known, a default value of 50% is often applied in the model. However, the extent by which the default active fraction values, which are assumed to be representative of the ion binding characteristics of naturally derived DOC, may be representative of estuarine-derived DOC is uncertain. In order to identify a value that would be more appropriate for estuarine DOC, the active fraction can be treated as an adjustable parameter and optimised according to that which results in agreement between observed and predicted toxicity. Two types of approaches can be used to estimate active fraction values in the context of biotic ligand modelling: one approach implies using *in vivo* exposure studies, where organisms are used as a biological speciation probe [41, 49]. An alternative approach is based on the use of the *Chelex column ion exchange technique*, which estimates the labile fraction of metal using chemical binding sites that mimic the biological active sites on the surface of a cell [62].

The Chelex method has been selected for this study and used to characterize estuarine-derived DOC, investigating the interaction between copper and DOC in the simulated water mixing of an ideal estuary. Before getting into the details of the method, however, it is worth explaining a key assumption of the Chelex method that differs from the BLM framework, i.e. the equilibrium assumption.

The BLM framework considers the metal bound to the cell surface of the organism to be in pseudo-equilibrium with the metal in solution. This assumption allows the model describing all the interactions between the metal ion and the ligands, including the biotic ligand, as chemical reactions controlled by stability constants [28, 63]. A common criticism of BLMs and equilibrium models in general is that in the aquatic environment equilibrium conditions hardly ever exist. Although this is generally true, the equilibrium assumed by the model is relative, because it is based on the assumption that the timescale of diffusion of metal species to the cell surface is relatively fast, compared to the rate of metal internalization across the cell membrane. This is particularly relevant not only from a theoretical point of view, but also from a practical one, when an analytical technique has to be established to mimic the interactions between the metal and the organism.

In respect to the equilibrium issue, the Chelex method applies a different approach, because it assumes that the diffusion rate of metal species to the cell surface is slow,

compared to the rate of metal internalization. In this situation, a concentration gradient develops at the biological site, between the external medium and the internal fluids, allowing metal complexes (e.g. metal-organic complexes) to dissociate in response to the localized gradient. In this way, the labile metal, including the metal fraction complexed by weak ligands, has enough time to dissociate and thus contribute to the free metal ion flux from the external medium to the organism [62].

The Chelex method mimics this situation by using the Chelex 100 resin (in the calcium form). The active sites of the resin can be looked at as the chemical counterpart of the active sites of the biotic ligand, since they compete with the organic and inorganic ligands to bind the free metal ion. If the metal is weakly bound to ligands present in solution (i.e. organic matter), the metal-complex dissociates and thus the metal ion binds to the active sites of the resin, as shown in Equations 6 and 7.

$$ML \overset{K_d}{\leftrightarrow} M^{2+} + L \qquad (6)$$

Where $K_d = [M][L]/[ML]$

$$M^{2+} + CaR \leftrightarrow MR + Ca^{2+} \qquad (7)$$

If the rate of calcium exchange is fast, the rate-limiting factor will be the dissociation rate constant (K_d), which is inversely proportional to the stability constant K of the metal-ligand complex. Also, the K_d value allows estimating the lability of the complex and thus indirectly quantifying the labile fraction of the metal. Taking into account not only the free metal ion, but also the labile fraction in modelling metal bioavailability, represents a more conservative approach from a regulatory point of view. However, its application for the development of new BLMs requires some considerations, given the discrepancies regarding the equilibrium issue.

In the following experiments the Chelex method was used to determine the labile fraction of copper when mixing riverine and estuarine samples of water in different proportions. A statistical method has then been applied to the results of the Chelex analysis in order to determine complexation capacities and dissociation rate constants of the organic matter in the analysed samples. These parameters were used as descriptors respectively of estuarine-derived and riverine-derived DOC to assess the overall binding capacity of the solution when mixing river and estuary waters, with the final aim to understand whether

a BLM version for estuaries should consider DOC as composed of a different percentage of active fractions.

3.4.2. Materials and Methods

3.4.2.1. Samples collection and treatment

For each experiment, two samples of water (25 L each), one riverine and one estuarine, were collected (at high tide) at two sites along the course of the river Itchen, a chalk river that flows for 45 km from the mid-Hampshire to Southampton and has been designated as a Special Area of Conservation for the presence of several species of ecological interest (http://jncc.defra.gov.uk/protectedsites). The riverine sampling site was upstream of the Woodmill tidal barrier, while the estuarine site was at the Mayflower Park (Figures 3.6 and 3.7).

Figure 3.6. Pictures of the two sampling sites, respectively at the Woodmill tidal barrier (left) and at Southampton (right). Student and supervisor collecting water samples and measuring salinity (centre).

The salinity measured *in situ* with a digital salinometer was respectively 0 and 28 ppt in the river and estuary end member. Samples were collected just below the water surface, stored in plastic bottles previously rinsed with 1% HNO_3 and filtered on arrival in the laboratory with glass microfiber paper filters (range MF200, size 125mm, Fisher). After filtration, samples were mixed in the proportions showed in Table 3.1 and stored in the dark at 4 ºC.

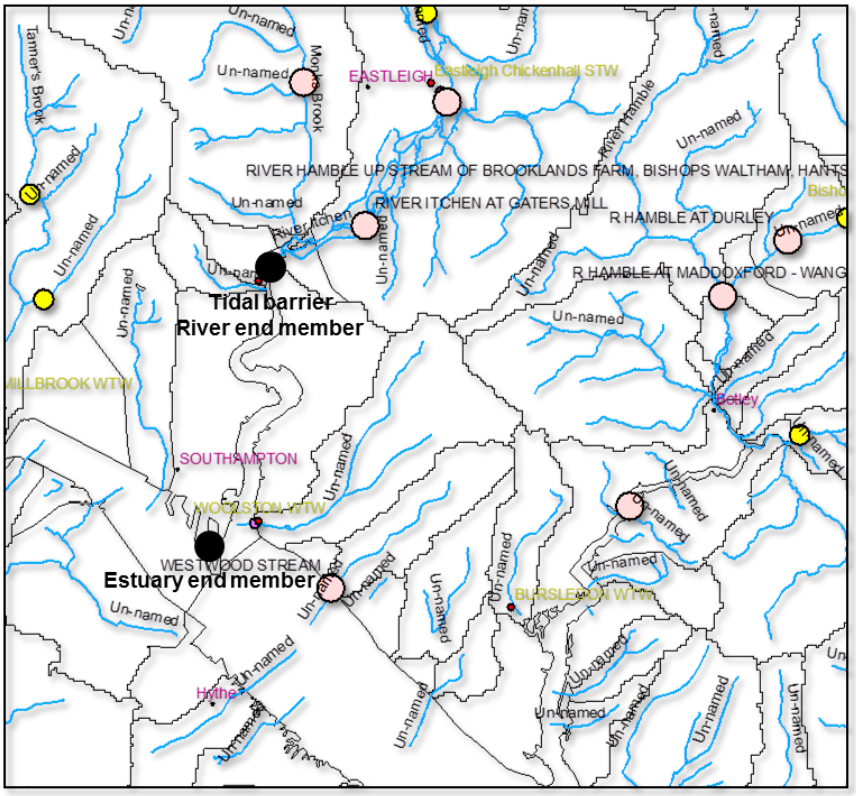

Figure 3.7. Map reporting the two sampling sites where the water samples were collected. The river end member was just upstream of the Woodmill tidal barrier, whilst the estuary end member was located at the Mayflower Park in Southampton. Each sampling trip was arranged during high tide time, in order to ensure the maximal inflow of seawater at the estuary end member.

3.4.2.2. Experimental design

Two Mixing Experiments (sampling times, respectively: July 2011 and March 2012) and one experiment with varying salinities were performed in order to mimic in laboratory conditions the mixing of river and estuary waters occurring in a model estuary. The Salinity Experiment, in particular, was designed to test the effect of varying salinities on the binding capacity of riverine-derived DOC.

In the case of the two Mixing Experiments, two samples of water, collected respectively along the river course, upstream of the tidal barrier, and downstream of it, in the estuary, were filtered and mixed according to the proportions indicated in Table 3.1. This resulted in five sub-samples of mixed water, each named according to the proportion of riverine/estuarine water used. Each of the sub-samples was split in two aliquots and then each aliquot was spiked with eight increasing concentrations of copper standard to give: 50, 100, 150, 200, 250, 300, 400, 500, 600 and 700 µg/L total dissolved copper. Half of

each copper-spiked sub-aliquot was acidified with 1% HNO_3 (Optima® grade, Fisher Scientific) and stored at 4 ºC for subsequent copper analysis, whilst the other half was passed through two Chelex columns as described in Section 3.4.2.5, then with 1% HNO_3 and stored in the dark at 4 ºC. The difference between the concentration of copper measured in the pre- and post-column water aliquots represented the labile fraction.

Table 3.1 Mixing Experiments design - Relative proportions of riverine and estuarine waters when mixed to obtain five sub-samples of mixed water to be spiked with copper standards.

Sample ID	Mixing
R100	river
70/30	70% river -30% estuary
50/50	50% river -50% estuary
30/70	30% river -70% estuary
E100	estuary

As for the Salinity Experiment, a sample of river water (collected in March 2012) was divided in four sub-samples, which were then added with increasing amount of NaCl (ACS reagent, Sigma-Aldrich) in order to obtain the following salinity points: 0 (100% river water), 9, 15 and 21 ppt. Each of these sub-samples was split in two aliquots and then each aliquot was spiked with increasing concentrations of copper standard as described above. Half of each copper-spiked sub-aliquot was added with 1% HNO_3 and stored at 4 ºC for copper analysis, whilst the other half was passed through the Chelex columns, added with 1% HNO_3 and stored in the dark at 4 ºC. The difference between the concentration of copper measured in the pre- and post-column water aliquots represented the labile fraction.

3.4.2.3. Total organic carbon analysis

Total organic carbon (TOC) was determined as the Non-Purgeable Organic Carbon fraction (NPOC) by high-temperature catalytic oxidation using a Shimadzu total organic carbon-V CPN Analyzer.

A preliminary method optimization analysis was conducted to select the most appropriate technique for analysing these water samples. Briefly, total organic carbon (TOC) can be determined either as a difference between total and inorganic carbon (TC – IC) or as Non-

Purgeable Organic Carbon (NPOC). The former method consists of two separate measurements, whilst the latter uses only a single step analysis, in which organic carbon is measured after removing inorganic carbon by acidification and purging of the sample.

For this study, the NPOC was selected, because the analysed water samples had a fairly high content of inorganic carbon (approximately 56.3 mg/L in the river sample and 28.1 mg/L in the estuary sample), relatively to the total carbon (around 59.6 and 32.7 mg/L, respectively) and hence this could have resulted in higher analytical error in the measurements. To verify that the NPOC method was indeed removing all the inorganic carbon, a solution containing 50 mg/L of total organic standard was spiked with 50 mg/L of inorganic carbon standard (sodium carbonate and sodium hydroxide carbonate) and run using the NPOC method. Measured TOC concentration was 49.96 ± 0.23 mg/L, confirming the effective elimination of the inorganic carbon and hence the validity of the method.

3.4.2.4. Chelex-100 resin method

Reagents and materials: All glassware and plasticware was cleaned by soaking in 10% HNO_3 for a minimum of 24 h followed by rinsing with Milli-Q water. Buffer solutions of 0.1 M and 1 M concentrations were prepared using 3-(N-morpholino) propanesulfonic acid (MOPS). The pH of the MOPS buffers was stabilized at 6.5 using 1 M and 0.1 M NaOH solutions and 1 M and 0.1 M of HCl solutions. Copper standards for spiking experiments were prepared from 1000 mg/L spectroscopic standards as 50 mL solutions in 1% HNO_3. A quality control solution for testing method performance was made up of 1 mM trisodium nitriloacetate (NTA) buffered to pH 7 with MOPS.

Procedure: Chelex-100 resin (sodium form, 200-400 mesh, Bio-Rad) was soaked in Milli-Q water overnight. A small disposable plastic syringe was used to transfer 1 mL of wet Chelex slurry into the exchange column. Milli-Q water was passed through the column to help the resin to settle and a plug of glass wool was inserted over the top of the resin. Plastic end caps were fitted to each end of the column to prevent leaking during storage. The resin was converted from the sodium to the calcium form by pumping at least 5 mL of 2 M $Ca(NO_3)_2$ solution through the column at a flow rate of ~ 20 mL/min, followed by at least 10 mL of Milli-Q water at the same flow rate. Approximately 50 mL of 0.1 buffer solution (MOPS, pH 7.0 ± 0.2) was pumped through the column in order to stabilize the

pH. Flow rate was checked at the start of each analysis. Before ion exchange, the pH of each sample was adjusted with the MOPS buffer at 7.0 ± 0.2 and checked before the analysis. The sample was then divided into 10 sub-samples (plus 1 blank) and spiked every 5 min with copper standard solution at increasing concentrations (50, 100, 150, 200, 250, 300, 400, 500, 600, 700 µg/L). After one hour, the first spiked sample was passed through the column following the procedure previously described. Each spiked sample was passed through two different columns simultaneously (see Figure 3.8). Sample solution was pumped through the column at 60 ± 2 mL/min. At least 10 mL of sample was allowed to flow before collecting a sub-sample in a plastic centrifuge tube (post-column sample). An aliquot of the original sample was also collected in a separate tube (pre-column sample). Both the pre and post column samples were acidified with HNO_3.

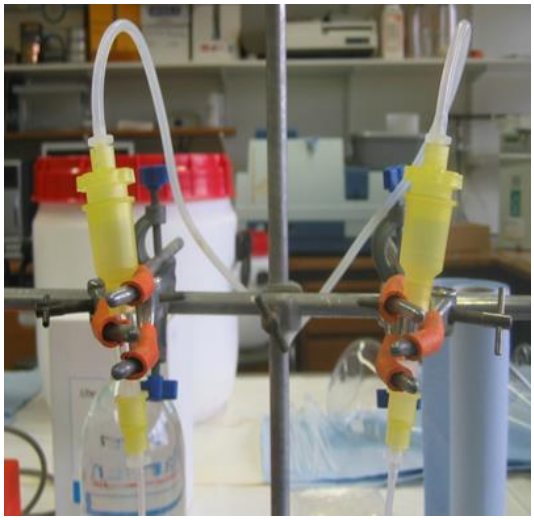

Figure 3.8. Picture of Chelex-100 resin ion exchange columns. Each copper-spiked sample was passed simultaneously through two separate columns in order to have two independent replicates of post-column sample. Samples were pumped through the column at a 60 ± 2 mL/min rate and at least 10 mL of sample was allowed to flow to waste before collecting the post-sample in a plastic vial.

MOPS buffer solution was pumped through the column between samples. At the end of the analysis, which took approximately 2 h overall for each set of samples, the pre and post column samples were collected in 50 mL vials, acidified with HNO_3 and stored at 4 ºC for the copper concentration analysis by flame atomic absorption spectrometry (F-AAS).

3.4.2.5. Copper analysis by F-AAS

Total copper concentrations were determined by flame atomic absorption spectroscopy, F-AAS (AAnalyst100 AAS, Perkin Elmer), using standard operating conditions. For analytical procedures, all acid was nitric acid (Optima® grade, Fisher Scientific). Copper standards were run every 6 samples to check measurement accuracy.

3.4.2.6. *Statistical Model*

The equilibrium dissociation constant or *affinity constant for the ligand*, **K_d,** and the *maximal number of binding sites*, **B_{max}**, were estimated using a non-linear regression analysis. A spreadsheet model was used to calculate best-fit metal binding parameter values (B_{max}, K_d) for titration data produced using the Chelex column method. The model applies a Monte Carlo methodology with 1,000 iterations to estimate confidence interval values for the binding parameters. While Monte Carlo simulations often apply a random error value with a Gaussian distribution to the sample data for which new best-fit parameters are estimated, this model applies a bootstrap method whereby forecast residuals were applied as the random error values. The rationale is that the bootstrap approach requires no assumptions about the distribution of the error in the data.

The relationship between the titration data and the parameters values is described by the following equation (8):

$$y = \frac{Bmax * x}{Kd + x} \qquad (8)$$

Where *x* is the pre column copper concentration and *y* the post column.

Also, the K_d value is inversely proportional to the stability constant value (K) of the metal-ligand complex, which thus can be derived as follow:

$$K = \frac{1}{Kd} \qquad (9)$$

3.4.3. Results and discussion

3.4.3.1. *Dissolved organic matter concentrations*

Average concentrations of dissolved organic matter measured in the river and estuary water samples (Table 3.2) were approximately 2.5 mg/L and did not differ significantly between the river end and the estuary end members, within each Experiment. However, DOC concentrations in the first Mixing Experiment were higher than in Mixing Experiment 2 and Salinity Experiment, possibly because those samples were collected in the summer period (July), when rainfalls are generally lower and therefore the organic matter transported by the river could be less diluted.

However, for the scope of this study the most important issue was to have similar DOC concentrations within the same experiment, in order to allow comparing ligand strength and complexation capacity of DOC at different mixing points but within the same sampling

Table 3.2. DOC concentrations - Organic carbon concentrations (mg/L) measured in filtered samples of river and estuary water collected respectively in July 2011 (Mixing Experiment 1) and March 2012 (Mixing Experiment 2 and Salinity Experiment). Values are means ± SE (n = 3).

	Dissolved organic carbon (mg/L)		
	July 2011	**March 2012**	
Sample ID	**Mix Exp.1**	**Mix Exp.2**	**Salinity Exp.**
R100	3.3 ± 0.1	1.59 ± 0.01	1.467 ± 0.02
E100	4.6 ± 0.3	2.33± 0.02	-

3.4.3.2. Labile and non-labile fraction of copper

Table 3.3 reports the percentages of labile copper calculated as the relative difference between the copper concentrations measured before and after passing through the ion exchange columns. Considering for simplicity only the river (R100) and estuary (E100) values, it can be noted that, at similar copper concentrations, the percentages of labile copper in the estuary were lower, although the difference tended to smooth at increasing copper concentrations.

Table 3.3. Labile fractions in Mixing Experiment 1. Pre- and post-column copper concentrations (expressed as µg/L) and labile fractions, represented by the pre-post difference in copper concentration (expressed as %). Comparison between the riverine and the estuarine samples (R100 and E100, respectively).

R100			**E100**		
Pre	**Post**	**%labile**	**Pre**	**Post**	**%labile**
63.0	29.1	54	68.8	64.8	6
63.0	23.8	62	68.8	68.4	1
111	33.1	70	118	70.2	40
111	36.0	68	118	67.2	43
150	38.6	74	163	71.0	57
150	40.4	73	163	68.0	58
209	43.5	79	207	74.0	64
209	43.5	79	207	73.4	64
256	53.7	79	263	76.8	71
256	57.7	77	263	72.2	73
307	60.6	80	305	82.1	73
307	64.0	79	305	73.2	76

This pattern of decreasing lability of copper, when moving from the river end towards the estuary end, was observed in both Mixing Experiments (Figure 3.9 A and B), although the labile fraction was relatively higher in the second experiment, possibly because of seasonal variations in the DOC characteristics. This observation hinted at the presence of strong ligands from unknown sources in the estuarine sample which reduced the labile fraction and thus the bioavailability of the metal. Alternatively, however, it could be explained by the effect of increasing salinity on the ligand strength of the organic matter. And indeed, the Salinity Experiment supported the latter interpretation (Figure 3.9 C), showing the same trend of decreasing percentages of labile copper at increasing salinities.

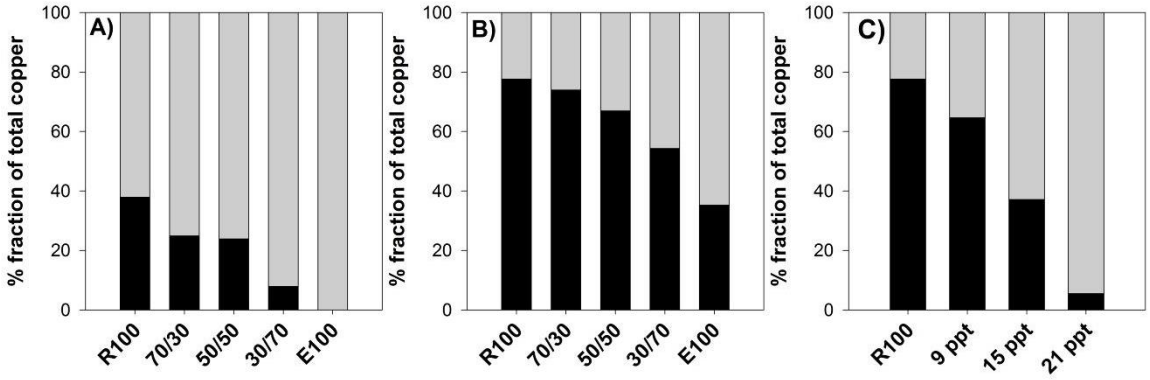

Figure 3.9. Relative fraction (%) of labile (black) and non-labile (grey) copper (50 µg/L) in samples of Mixing Experiment 1 (graph **A**) and 2 (**B**) and of the Salinity Experiment (**C**). The labile fraction corresponds to the difference between pre and post column copper concentrations, whilst the non-labile fraction is experimentally defined as the post column copper concentration.

3.4.3.3. Binding capacity and ligand strength

Table 3.4 reports the organic ligand parameters calculated by the spreadsheet model as described in Section 3.2.3.6, using the pre and post column copper concentrations measured in Mixing Experiment 1.

Table 3.4. B_{max} (maximal number of binding sites) and K_d (dissociation constant) values calculated by the spreadsheet model using titration data obtain by Chelex analysis on samples of Mixing Experiment 1. Upper and lower 25th percentile (CI) of the distribution are reported for each parameter. LogK values, derived by the K_d values, are also indicated for each sample.

Sample ID	Parameter	Parameter values	Upper CI	Lower CI
R100	B_{max}	592	670	534
	K_d	4883	6544	4000
	logK	-3.7		
70/30	B_{max}	296	351	258
	K_d	1326	2449	698
	logK	-3.1		
50/50	B_{max}	271	342	224
	K_d	1577	3544	747
	logK	-3.2		
30/70	B_{max}	352	403	314
	K_d	970	1952	475
	logK	-3		
E100	B_{max}	296	351	258
	K_d	1326	2449	698
	logK	-3		

Among the calculated parameters, the ligand strength values, expressed by the dissociation rate constants (K_d), are noticeably higher in the river end member, compared to the other mixing points and especially to the estuary end member, suggesting that copper in the estuary is more stably complexed than in the river. The same trend of increasing ligand strength (which is inversely proportional to the dissociation rate constant of the metal-ligand complex) from the river towards the estuary end member was observed also in the Mixing Experiment 2 (Figure 3.10 A).

The stability constants (K) of the riverine and the estuarine samples, derived from the K_d values according to Equation 8, are respectively $10^{-3.7}$ and 10^{-3}. The calculated value for the river is within the range reported in literature for the Suwannee River (around $10^{-3.4}$), though close to the value reported for a wastewater treatment plant ($10^{-3.5}$) [64]. This may be due to an upstream input of organic matter from the Eastleigh Chickenhall treatment plant, along the river Itchen.

Considering the complexation capacity values, expressed by the B_{max} parameter (Figure 3.10 B), they show no significant change among the different mixing points in both experiments, suggesting that the decreased lability of copper towards the estuary end

member observed in the Chelex analysis could be more probably attributed to an increased affinity for the organic ligand and therefore a higher stability of the metal-ligand complex, rather than an increased complexation capacity of the ligand itself.

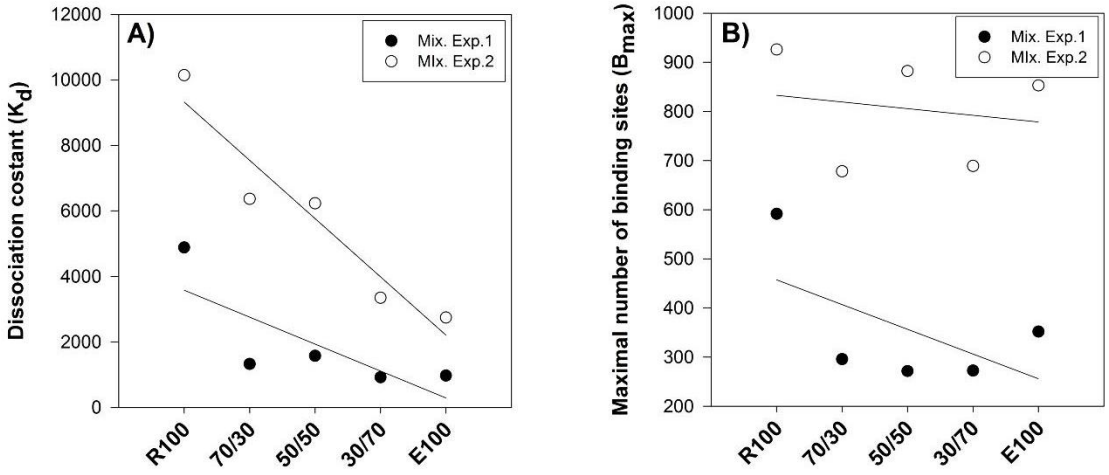

Figure 3.10. K_d (dissociation constants) (**A**) and B_{max} (maximal number of binding sites) (**B**) estimated by the spreadsheet model using titration data from Chelex analysis performed on samples from the two Mixing Experiments (respectively black and white dots). Dissociation constant display a significant trend of decreasing values from the river to the estuary end member ($r^2 = 0.60$ and 0.91 in Exp.1 and 2, respectively), whilst no trend was observed in the maximal number of binding sites.

When considering both B_{max} and K_d parameters together, the calculated constants displayed a clear difference only for the river end member, whereas the intermediate samples were all grouped close to the estuarine end member, as shown in Figure 3.11. This clustering might be explained by the fact that the high strength ligands in the estuary were dominant when mixed with the river and thus the riverine sample behaved, in regard to copper binding, as a full strength saline sample when just 30% of salt water was added. This would imply that there is no gradual change in the binding capacity of the ligands when riverine and estuarine waters are mixed, suggesting that salinity is not the driving factor controlling the copper binding process.

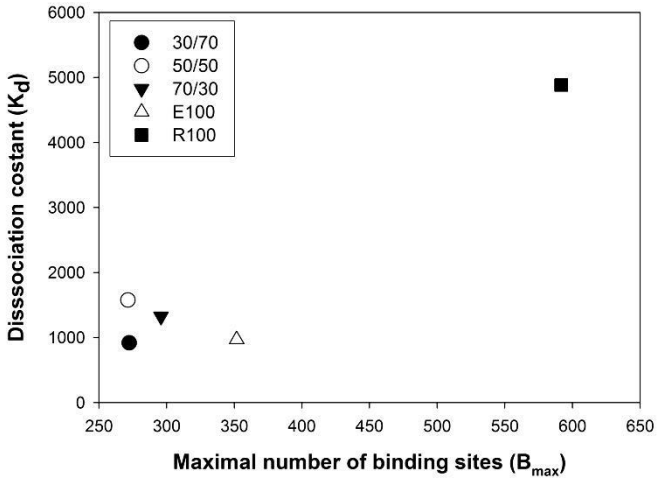

Figure 3.11. K_d (dissociation constant) vs. B_{max} (maximal number of binding sites) values in the Mixing Experiment 1 samples, as calculated by the model applying a Monte Carlo analysis.

However, this interpretation was not supported by the analysis of the K_d and B_{max} values calculated using the Chelex results from the Salinity Experiment (Figure 3.12 A), which showed that the stability of the metal-ligand complex tended to be higher at increasing salinities (i.e. 15 and 21ppt). This result was in line with what observed in the analysis of the labile fraction of copper in both the Mixing Experiments, whereas the complexation capacity, expressed by the B_{max} values, showed a trend of increasing complexation capacity at increasing salinities (Figure 3.12 B), in contrast with that observed in the two Mixing Experiments (Figure 3.10 B). This disagreement between the complexation capacities estimated in the Mixing Experiments and in the Salinity Experiment is actually interesting, since it suggests that increased salinity could generate a change in the chemical characteristics of the riverine organic matter that renders the DOC a stronger ligand for copper at higher ionic strength. But, when both riverine and estuarine derived organic matters are mixed, the effect of the ionic strength on the binding capacity of the riverine DOC is cancelled out, possibly because the estuarine organic matter has an intrinsically lower complexation capacity than its riverine counterpart. The fact that estuarine-derived DOC is a weaker ligand than the riverine-derived DOC is in accordance with the literature [50], since it is partly composed of autochthonous organic matter, mainly derived from estuarine phytoplanktonic blooms, which is less "aged" and therefore has a higher percentage of inactive fraction, compared to allochthonous DOC of terrestrial origin.

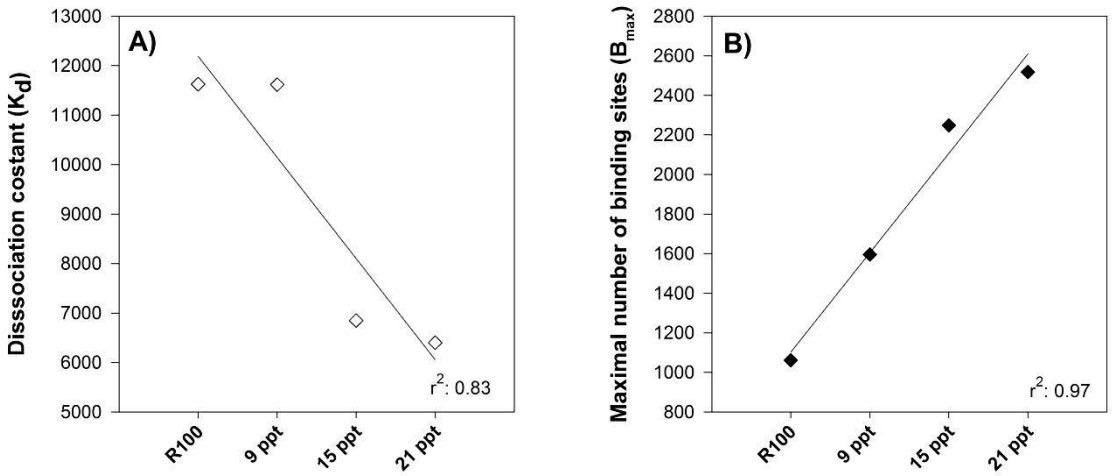

Figure 3.12. Dissociation constants (**A**) and maximal number of binding sites (**B**) estimated by the spreadsheet model using titration data from Chelex analysis performed on samples from the Salinity Experiment.

Figure 3.13 reports the titration data plots of the 5 sub-samples of the two Mixing Experiments, compared to the best-fit forecast of the spreadsheet model. Considering first Mixing Experiment 1, the trend of the data set in the first graph (R100) shows that the complexing ligands present in the sample require higher copper concentration to be saturated and thus are "weaker" ligands, compared to the ligands in the estuary (E100), confirming the hypothesis of strong ligands in the estuary end member.

Titration data from the second Mixing Experiment showed a slightly better trend of saturation in the estuary end member, suggesting the presence of weaker ligands in estuarine waters. However, a bias between estimated and actual post-column copper concentrations was observed in the last three mixing points at very low copper concentrations, hinting instead at the presence of a strong ligand that binds a proportionally greater fraction of copper than that predicted by the model.

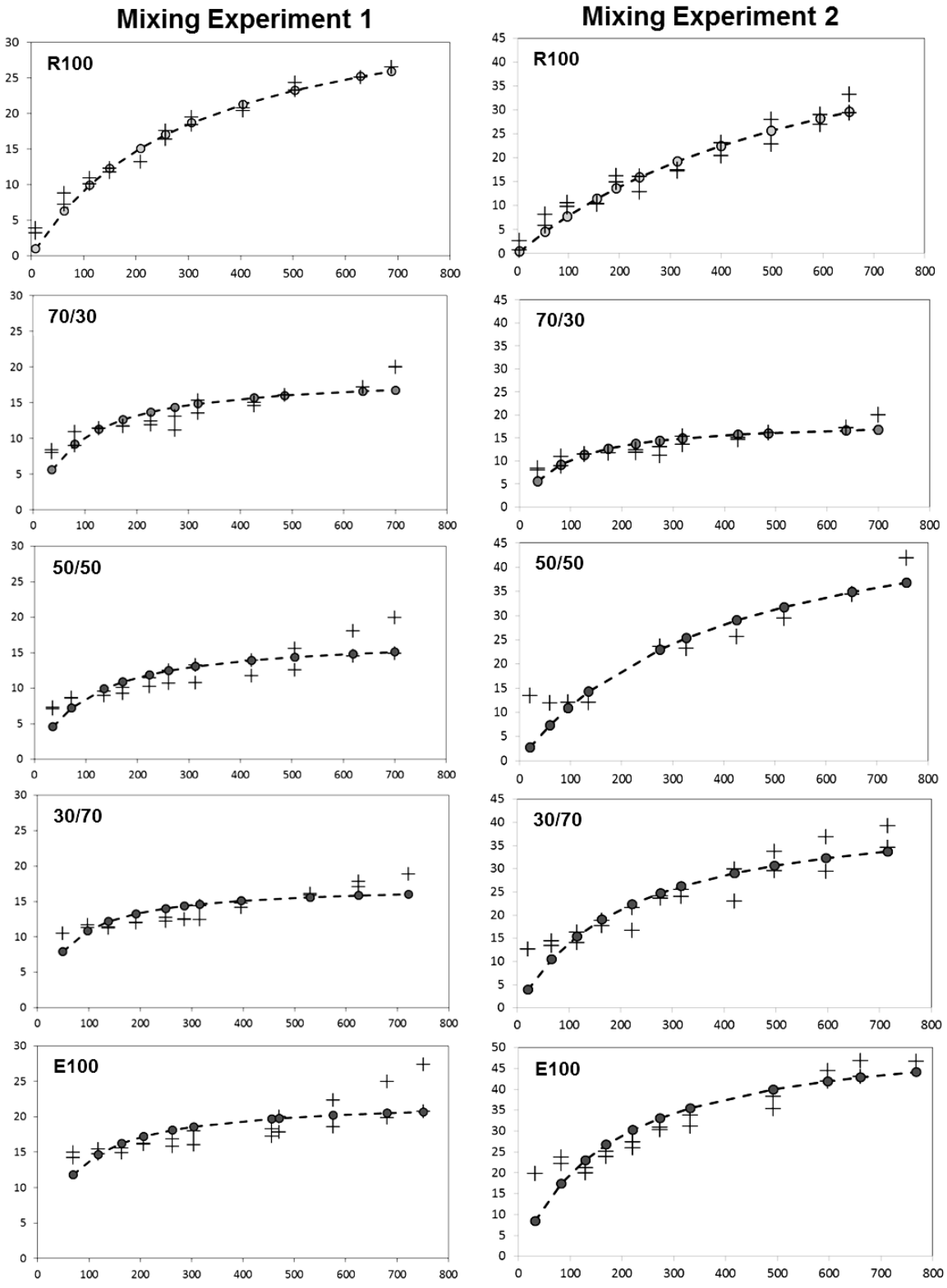

Figure 3.13. Titration data plot and best-fit forecast for the samples from the two Mixing Experiments. The x values represent the pre column copper concentrations, and the y values the post-column concentrations. Crosses: actual (µg/L copper per mg of DOC); circles and dots lines: best-fit forecast.

3.4.3.4. Summary of the results

- Mixing Experiment 1:
  - The **labile fraction** of copper decreased at increasing percentages of estuarine water, suggesting that copper was more strongly bound to organic ligands in estuarine waters;
  - The **dissociation rate constants** (K_d) were lower in the estuary than in the river end member, suggesting that the metal-ligand complexes were more stable in estuarine waters;
  - The **maximal numbers of binding sites** (B_{max}) were higher in the river than in the estuary, suggesting a higher complexation capacity of riverine-derived organic matter.

- Mixing Experiment 2:
  - The **labile fraction** of copper decreased at increasing percentages of estuarine water, in line with the results from the first experiment, although the percentage of labile copper was proportionally higher within each mixing point;
  - The **dissociation rates constants** (K_d) followed the same trend displayed by the values calculated for the first experiment, confirming the higher stability of the metal-ligand complexes in estuarine waters;
  - The **maximal numbers of binding sites** (B_{max}) of the solution were overall unchanged by the mixing, suggesting that the complexation capacity of the organic ligands did not change appreciably between the river and the estuary or with the water mixing, in contrast with what observed in the first experiment.

- Salinity Experiment:
  - The **labile fraction** of copper showed a clear trend of decreasing percentages at increasing salinity, suggesting that the salinity was probably the driving factor controlling the decreased availability of the metal in estuarine waters observed in the other two experiments;
  - The **dissociation rates constants** (K_d) followed the same trend displayed by the values calculated in both Mixing Experiments, suggesting that the higher stability of the metal-ligand complexes observed in the Mixing Experiments were

probably due to an effect of the saltwater on the binding affinities of the organic ligands, rather than to the presence of stronger ligands in the estuarine waters;

- The **maximal numbers of binding sites** (B_{max}) increased with salinity, suggesting that higher salinities affected the complexation capacity of the organic ligands present in the river waters, resulting in more stable metal-ligand complexes.

To summarize, the higher strength of the ligands observed in the estuarine samples could be explained by the effect of salinity on the riverine ligands, rather than by the mixture of different types of organic matters. As a consequence of mixing the samples, there might be some change in the nature of the DOC, which generates a new category of binding site with a higher binding affinity. This is strongly suggested by the salinity experiment.

The differences in complexation characteristics observed in the Salinity Experiment could be attributed to the effect of ionic strength on complexation. As a consequence of increasing the salinity of the river water sample, the ionic strength of the solution increased and therefore the activity of the copper added as part of the titration decreased, which might have rendered some of the weaker binding ligands ineffective. This could explain why, *on average*, the binding affinity in the dilution samples was higher, since only the stronger ligands were exerting an influence.

From a wider perspective, despite some discrepancies between the different experiments that could be due to seasonal variability in the DOC characteristics, the general conclusion that can be drawn from these results is that copper in estuaries seems to be less available. Moreover, the Salinity Experiment suggests that the lesser availability of the metal can be at least partly explained by the effect of salinity (i.e. ionic strength). Further investigations should be performed to try and understand if this is due an effect of salinity on either the nature of the ligand (i.e. ligand strength), the activity of the free copper ion (i.e. complexation capacity), or a combination of the two. In either case, recalling the points made in Section 3.2.2, it can be concluded that estuarine-derived DOC is less effective in binding copper than riverine-derived DOC, but the mixing of river and estuary waters increased the overall capacity of the DOC present in solution to bind copper and hence reduce its bioavailability.

3.5. Conclusions and final modelling

The results of the experiments performed with water mixing and varying salinities suggested that salinity may reduce the binding capacity of riverine DOC, thereby reducing its protective effect on copper toxicity. It was also observed that estuarine DOC was a less strong ligand than riverine DOC, although further investigations are required to confirm this observation. One thing that clearly emerged from the analyses of the data produced from all the three experiments, however, was the importance of DOC in determining the labile fraction of copper. The challenge is then to translate this aspect into modelling.

Since DOC is a major component in estuarine waters and apparently a driving factor in determining copper toxicity, Arnold et al. [11] suggested predicting copper toxicity in marine environments solely on the basis of the organic matter, using the following Equation:

$$EC50 = 11.22 * DOC^{0.6} \qquad (10)$$

However, in order to correlate the EC50 values only to the DOC concentrations, it is necessary to better characterize the nature of the organic matter, as was done in Section 3.4 using the ligand strength and complexation capacity parameters.

As suggested by the results of the Chelex experiments, DOC ligand capacity can vary at varying salinity and, also, estuarine and riverine organic matter can have different strength and hence bind copper in complexes of different stabilities [58]. In BLM model terms, copper complexation capacity is expressed as percentage of active fulvic acid (%AFA), as described by Equation 11:

$$DOC_{imput} = (\%AFA/100) * DOC_{test} \qquad (11)$$

Where DOC_{imput} is the DOC concentration that needs to be put into the model and DOC_{test} the DOC concentration measured in the test solution. Following the work of Dwane and Tipping [65], in the BLM framework organic matter is often assumed to behave as 50% active fulvic acid with regard to copper speciation. However, this parameter could be adjusted to better describe the complexation capacity of DOC, but this requires a relatively fast and easy-to-use tool to quantitatively define the activity of organic matter

derived from different sources, if it has to be applied routinely. One approach was proposed by De Schamphelaere [49] and colleagues, who observed a linear relationship between the $\%AFA_{opt}$ (calculated by fitting predicted and observed EC50s) and the UV absorbance coefficient measured at 350nm (ε_{350}) in different types of DOM, as described by Equation 12:

$$\%AFA_{opt} = \text{slope} * \varepsilon_{350} \qquad (12)$$

This relationship could allow calculating the actual %AFA of a sample, and thus the DOC_{imput}, on the basis of a relatively simple measurement such as UV-absorbance. If we combine Equations 11 and 12, the DOC_{imput} can be calculated as follow:

$$DOC_{imput} = \text{slope} * \varepsilon_{350}\,[DOC_{test}] \qquad (13)$$

Adding the so-obtained DOC_{imput} in Equation 10 would then allow calculating the EC50 values using Equation 14:

$$EC50 = k\,(\text{slope} * \varepsilon_{350}\,[DOC_{test}])^{q} \qquad (14)$$

where *k* and *q* are constants that are determined mathematically.

Combining Equation 14 with Equation 5 would lead to a simplified BLM equation that includes and expresses mathematically the refinements suggested in this Chapter for both the *physiology-based* and the *chemistry-based part* of the model. Having argued that in transitional waters the main parameters controlling copper toxicity are the osmotic gradient between the internal fluids and the external medium, and the complexation capacity of the DOC, the conclusions of this chapter can be summarized by the following equation:

$$\mathbf{EC50 = (f_{CuBL}^{50\%})^{*} * k\,(slope * \varepsilon_{350}\,[DOC_{test}])^{q}} \qquad (15)$$

Equation 15 is a mathematical statement of the relevance of salinity, physiology and organic matter in determining copper toxicity in estuaries and transitional environments.

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CHAPTER FOUR

ROLE OF CARBONIC ANHYDRASE 2 IN THE RESPONSE OF FISH TO COPPER AND OSMOTIC STRESS

From pickle-jar ecology to stress response dynamics: a brief history of ecotoxicology

The origins of aquatic toxicology can be traced to a Spaniard from the Island of Minorca, M.J.B. Orfila, who in 1815 published the first book devoted to the harmful effects of chemicals on organisms. However, it wasn't until the early 1920's that the systematic study of toxic effects in laboratory animals began, though mostly as mammalian toxicology studies arising from activities in clinical toxicology and pharmacology. In the early years prior to World War II, concerns about the effects of chemicals on nonhuman biota led to the first efforts to standardize techniques for investigating acute toxicity of various chemicals, including metals, to aquatic organisms, i.e. cladocerans, water fleas and fish. It was the end of the era of the "pickle-jar ecology" (Doundoroff, 1976)*, when toxicity tests with aquatic organisms were performed in jars of 5-20 litres.

A cornerstone in the history of environmental toxicology is the classic book "Silent Spring", by Rachel Carson (1962), who "almost single-handedly created modern society's fears about synthetic chemicals in the environment" (Rodricks, 1992)**, thereby generating a burst of public and governmental interest that led to the establishment of some major laboratories in UK, US and Canada. The 60's and 70's saw the biggest advancements in the performance and design of the exposure studies, i.e. improvements in static and flow-through exposure techniques, the birth of multifactorial experimental designs and a new interest in chronic sublethal effects. If the 60's and 70's were the "years of the exposure", the 80's and 90's represented the "era of the endpoints"; in the 1990's in particular, great advances were made for example in organismal responses (thanks to inputs from studies on human health), measurement of genotoxic and mutagenic effects and automation of many techniques for measuring toxicity.

In the first decade of the 21st century, the field of ecotoxicology has broadened its horizons to include natural systems and develop new testing strategies, valid on an ecosystem scale and not just within the limits of a pickle jar. The inclusion of more and more natural factors affecting organisms in the real environment merges with a wider vision of the test organism as a complex multi-levelled system. This "holistic" approach is also promoted by the new availability of great amount of data, e.g. from omics studies, combined with a more extensive use of bioinformatics tools to dissect biological information at systemic level.

Ecotoxicological studies are going beyond exposing fish to a single chemical and testing few single endpoints: in a non-distant future there will be stresses instead of chemical *a* or *b*, biological systems instead of fish and pathway responses rather than survivorship tests. Although the fundamental principles of ecotoxicology, as well its main goals, will probably remain unchanged in the future as they have essentially done in the last century, the field of ecotoxicology will have to shift from a dose-response paradigm to a stress-response paradigm, if it is to face the challenges posed by this new data-rich and hypothesis-poor era of science.

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Abstract

The majority of ecotoxicological studies are performed under stable and optimal conditions, whereas in reality the complexity of the natural environment faces organisms with multiple stressors of different type and origin, which can activate pathways of response often difficult to interpret. In particular, aquatic organisms living in estuarine zones already impacted by metal contamination can be exposed to more severe salinity variations under a forecasted scenario of global change. In this context, the present study aimed to investigate the effect of copper exposure on the response of fish to osmotic stress by mimicking in laboratory conditions the salinity changes occurring in natural estuaries. I hypothesized that copper-exposed individuals are more sensitive to osmotic stresses, as copper affects their osmoregulatory system by acting on a number of osmotic effector proteins, among which the isoform two of the enzyme carbonic anhydrase (CA2) was identified as a novel factor linking the physiological responses to both copper and osmotic stress. To test this hypothesis, two *in vivo* studies were performed using the euryhaline fish sheepshead minnow (*Cyprinodon variegatus*) as test species and applying different rates of salinity transitions as a controlled way of dosing osmotic stress. Measured endpoints included plasma ions concentrations and gene expression of CA2 and the α1a-subunit of the enzyme Na^+/K^+ ATPase. Results showed that plasma ions concentrations changed after the salinity transition, but notably the magnitude of change was greater in the copper-exposed groups, suggesting a sensitizing effect of copper on the responses to osmotic stress. Gene expression results demonstrated that CA2 is affected by copper at the transcriptional level and that this enzyme might play a role in the observed combined effects of copper and osmotic stress on ion homeostasis.

4.1. Introduction

Ecotoxicological studies on environmental chemicals are usually performed under optimal exposure conditions, whereas aquatic organisms in their natural settings have to cope with additional stressors, such as variations in temperature, oxygen levels or salinity, which can affect the way they respond to chemical stressors [1]. Conversely, exposure to chemical stressors could impair organisms' responses to changes in environmental factors. In either cases, this two-way interaction may or may not lead to more adverse effects on the organisms and ultimately on the population, since the correlation between tolerance to chemical and non-chemical stressors can be positive or negative, depending on the type and level of the stressor in question (e.g. [2, 3]).

The potential interactions between toxic substances and environmental factors represent one of the main challenges for ecotoxicologists. The importance of refining toxicity studies and applying them to more complex scenarios has been recognized by both the EU and US EPA, who have expressed an increasing interest in studying the responses of biological systems to a combination of stressors, both chemical and environmental, rather than to single chemical in stable conditions [4, 5]. An integrated examination of chemical and non-chemical stressors is especially pertinent when considering chemical pollution in the context of global change scenarios, where more fluctuating environmental variables are plausible to influence the responses of biological systems to chemical exposure [6, 7]. This is again a two-way interaction, because global change can make organisms more sensitive to chemical stressors as well as exposure to chemical stressors can make organisms more sensitive to changes in environmental stressors caused by global change.

In this context, the challenge is to identify physiology-based interactions between non-chemical and chemical stressors affecting key physiological processes in an organism. As a first step to applying a multi-stressor approach into ecotoxicological studies, it is crucial to acquire an understanding of the mechanisms of action of the stressors in question, by dissecting the biological pathways through which they exert their effects [8]. One possible approach in this sense is the application of the adverse outcome pathway (AOP) concept, a unified framework that links molecular initiating events with the cascade of responses occurring across all levels of biological organization [9]. Such

approach, despite being mainly qualitative [10], does highlight the importance of dissecting the physiological mechanisms and toxicodynamic processes underpinning the complexity of many biological responses, which is a key step in interpreting the stress response dynamics displayed by any biological system in the complexity of the real world.

With this aim in mind, the present study examined the mechanisms of interaction between one chemical stressor, i.e. copper, and one environmental stressor, i.e. osmotic stress, which was applied in the form of salinity transitions from either freshwater to saltwater or from saltwater to freshwater. In order to put this laboratory-based study into an environmentally realistic context, the copper-salinity interactions were investigated by mimicking under laboratories conditions the salinity changes occurring in estuaries, which are environments where the combination of anthropogenic impacts and fluctuating abiotic factors represents an ideal context to study the interactions between chemical and non-chemical stressors. Considering future global change scenarios and their impacts on estuarine and coastal pollution [11], aquatic organisms inhabiting transitional environments are likely to be exposed to more severe and/or more frequent salinity fluctuations in the future, as a result of increased frequency of extreme events [12]. Therefore salinity changes, also defined as osmotic stress, were chosen as the non-chemical stressor to test in this study. As for the chemical stressor, a metal was selected (i.e. copper) because in general metal contamination is of particular concern in estuarine and coastal environments, where the historically high anthropogenic impacts, mainly due to shipping, urbanization and industrialization, often lead to elevated concentrations of metals both in the water column and in the sediments [13]. In particular, one of the main concerns about transitional zones is represented by copper, whose use as biocide in antifouling painting coatings has increased since TBT (tributyltin) and other organic biocides has been phased out [14]. I therefore focused on the interactions between copper and salinity changes, given their theoretical as well as applicative relevance for multi-stressor studies applied to transitional environments in a global change perspective. However, since this topic has been almost exclusively addressed from the point of view of the effects of different salinities on copper toxicity [15-18], this study put instead more emphasis on the effects of copper exposure on the response of aquatic organisms, i.e. fish, to salinity transitions. In accordance with the mechanistic intent of

the study, it was hypothesized that, given that acute copper toxicity is mainly a consequence of osmotic disruption [19-21], copper-exposed fish are more sensitive to osmotic stresses, as copper can affect their osmoregulatory system by interacting with a number of osmotic effector proteins, such as the enzyme Na^+/K^+ ATPase (e.g.[22]).

As discussed in Chapter Two [23], a critical screening of the ecotoxicological data available in the literature on copper toxicity and salinity led to the hypothesis that one factor linking the main physiological responses to both copper and osmotic stress is the cytosolic isoform-2 of the enzyme carbonic anhydrase (CA2), potentially a copper target [23-25] with salinity dependent expression and activity [26-28]. Because CA2 displays its osmoregulatory functions not only in the gills, but also in the intestine of fish [29], it was argued that in saline environments the intestine should be considered alongside the gills as a site of action for copper [30]. It was hypothesized that the combined effect of copper and osmotic stress is not the overall product of two independently acting factors, but rather the outcome of a mechanistic interaction between two stressors that act through similar pathways and affect similar effector proteins.

The *in vivo* experiments reported here were aimed at testing that hypothesis, using as test species the teleost fish sheepshead minnow (*Cyprinodon variegatus*), an euryhaline fish found in saltwater bays, estuaries and coastal inland areas along the Atlantic Coast and the Gulf of Mexico. This euryhaline fish can tolerate salinities ranging from 0 up to 140 ppt, but normally is found at salinities between 10 and 20 ppt [31]. It is a standard test species used by the US EPA for toxicity tests in saltwater conditions (reported copper 96h-LC50 is 2.5 mg/L [32]).

4.2. Materials and methods

4.2.1. Ethics statement

These studies were carried out under project and personnel licences granted by the Home Office under the United Kingdom Animals Act (Scientific Procedures).

4.2.2. Experiment 1

4.2.2.1. Test species

Adult male and female sheepshead minnows (*Cyprinodon variegatus*) were obtained from the Brixham Environmental Laboratory (Brixham, UK) and acclimated to freshwater for approximately three weeks under flow-through conditions. Fish age was 4 month post-hatching and average wet weight was 4.7 ± 1.1 g.

4.2.2.2. Experimental design and protocol

The experiment was carried out using a continuous flow-through system. Thermostatically heated dechlorinated carbon filtered tap water, from a header tank, flowed through 6 flow-meters into 6 mixing chambers via silicon tubing (medical grade, VWR) at a rate of 120 ml/min. From each mixing chamber the water flowed into the fish tanks through silicone tubing. In total there were 6 glass fish tanks each with a working volume of 11 L. Each tank was aerated and received approximately 15 tank volume renewals per day. During the exposure period, copper was added as $CuSO_4 \cdot 5H_2O$ dissolved in 1% HNO_3 (Optima® grade, Fisher Scientific) and dosed into each individual mixing chamber via a peristaltic pump at a rate of 0.12 ml/min. The stock solutions and mixing regime were designed to yield final nominal copper concentrations of 10 and 100 µg/L. Dilution water and chemical flow rates were checked twice per day and adjusted if necessary. Temperature, pH, oxygen, total alkalinity (see Table 4.1), nitrate and ammonia were monitored and recorded daily. Water samples for total organic carbon (TOC), copper and major constituents analyses were collected in 50 ml centrifuge tubes on days 3, 6, 8 and 9. Samples for TOC analysis were stored at – 20 ºC after collection, whereas samples for copper and major constituents analysis were acidified with 1% HNO_3 and stored at 4 ºC.

Table 4.1. Water parameters in fish tanks – Exp.1. Water parameters in fish tanks during Experiment 1. Reported values are means ± SD of the measurements taken daily in all 6 groups (n = 6) respectively before (PRE) and after (POST) the salinity switch

	PRE	POST
Salinity (ppt)	0	20
Temperature (°C)	25.8 ± 0.23	25.3 ± 0.25
pH	7.9 ± 0.08	8.4 ± 0.06
Oxygen (mg/L)	7.2 ± 0.34	7.9 ± 0.06
Alkalinity (mg/L $CaCO_3$)	220	260

The 9-day exposure experiment consisted of 3 treatments: 0, 10 μg/L and 100 μg/L nominal copper concentrations. All treatments were in freshwater (FW) for the first 8 days of exposure. Each treatment was composed of 2 single-sex tanks, each tank containing 6 fish (12 fish per treatment, 6 males and 6 females). Fish were maintained in a photoperiod of 16 h of light followed by 8 h of dark, with 20 min dawn/dusk transition, and were fed *ad libitum* three times per day: twice with flake food (King British Tropical flake food, Lillico, Surrey) and once with brine shrimp (Tropical Marine Centre, gamma irradiated). Food was withheld 20 h prior to each sampling. After 8 days of exposure in FW, 3 fish per tank (6 fish per treatment) were humanely sacrificed according to UK Home Office procedures, using an overdose of buffered ethyl 3-aminobenzoate methanesulfonate (300 mg MS222/L, adjusted to pH 7.8, the average pH throughout the study). Blood samples were collected from the caudal peduncle using heparinised capillary tubes, transferred into Eppendorf tubes and kept on ice until plasma was separated by centrifugation at 14,000 *g* for 5 min, removed and stored at − 20 °C. Fish were weighed and measured (fork length). Tissue samples (gills, liver and intestine) were collected by dissection. The intestine was divided into three segments (anterior, mid and posterior intestine) and sub-samples of each segment were kept for gene expression analysis. This procedure was chosen in order to account for potential differences in gene expression along the intestinal tract. All tissue samples were immediately frozen in liquid nitrogen after collection and stored at − 80 °C.

4.2.2.3. Salinity transition

After the first sampling on day 8, the salinity in all treatments was increased from 0 to 20 ppt over a time of 4 h by dosing hyper-concentrated (200 ppt) saltwater (SW) prepared by dissolving and mixing synthetic seasalts (Tropic Marin) in three glass tanks of 40 L volume each, filled with dechlorinated carbon filtered tap water. The brand of synthetic seasalts (Tropic Marin) used to prepare the saline stock solutions was selected as it has been shown to have a low content of trace elements, particularly copper, compared to other commercially available brands [33]. The hyper-saline stock solution was prepared 24 h in advance and left overnight with strong aeration to allow the pH to stabilize. SW was dosed from the stock solution into the mixing chambers at a rate of 12 ml/min to yield a final salinity of 20 ppt in all fish groups. Salinity was monitored every 15 min with a refractometer throughout the salinity transition period. After reaching 20

ppt, fish in all treatments were held in SW for 24 h, until the second sampling on day 9. Copper dosing was maintained constant during the entire study. On the 9th day of exposure, the remaining fish (6 per treatment) were sampled following the same procedure described above, with the exception of the MS222 solution, which was prepared in SW instead of FW and buffered at a pH of 8.4 (instead of 7.8), consistent with the salinity and average pH of the tanks after the salinity transition.

4.2.3. Experiment 2

4.2.3.1. Test species

Adult male and female sheepshead minnows (*Cyprinodon variegatus*) were obtained from a livestock bred and maintained at Brunel University (UK) in a saltwater re-circulated system supplied with UV-sterilizer, sand biofilter and protein skimmer (Marine Compact Filtration System, Tropical Marine centre). The synthetic seasalts (Tropic Marin) used for maintaining the system were the same used for the preparation of the artificial seawater (SW) during the two experiments.

Approximately one month before the start of the experiment, a sub-stock of fish has been gradually moved to freshwater and then kept under these conditions for three weeks before starting the exposure. Fish age at the beginning of the experiment was approximately 6 months post-hatching and average wet weight was 2.7 ± 0.7 g.

4.2.3.2. Experimental design and protocol

The experiment was carried out using a continuous flow-through system and artificial SW dosed from a concentrated stock for the groups held in SW. Thermostatically heated dechlorinated carbon filtered tap water, from a header tank, flowed through 16 flow-meters into 16 mixing chambers via silicon tubing (medical grade, VWR) at a rate of 60 ml/min. This final flow of 60 ml/min was half the rate of the one used in the Experiment 1 (120 ml/min). Thus, a full replacement was now reached within double the time, which implied that the salinity switch on day 20 would then be performed over 8 h instead of 4, hence representing a milder osmotic stress, but still within the time range of a tidal change in an average estuary. From each mixing chamber the water flowed into the fish tanks through silicone tubing. In total there were 16 glass fish tanks with a working volume of 11 L. Each tank was aerated and received approximately 7

tank volume renewals per day. During the exposure period, copper was added as $CuSO_4 \cdot 5H_2O$ dissolved in 1% HNO_3 (Optima® grade, Fisher Scientific) and dosed into each individual mixing chamber via a peristaltic pump at a rate of 0.06 ml/min. The copper stock solutions and mixing regime were designed to yield final nominal copper concentrations of 32, 100 and 320 µg/L. Dilution water and chemical flow rates were checked twice per day and adjusted if necessary. Temperature, pH, oxygen, total alkalinity (see Table 4.2), nitrate and ammonia were measured and recorded daily. Water samples for TOC, copper and major constituents analysis were collected in 50 ml centrifuge tubes on days 1, 4, 8, 12, 16, 19 and 21. Water samples for TOC analysis were stored at – 20 ºC after collection and water samples for copper and major constituents analysis were acidified with 1% HNO_3 and stored at 4 ºC.

Table 4.2. Water parameters in fish tanks – Exp.2. Water parameters measured in fish tanks during Experiment 2. Reported values are means ± SD of the measurements taken daily over the exposure period, respectively in the freshwater (FW) groups and in the saltwater (SW) groups.

	FW	SW
Salinity (ppt)	0	19.9 ± 0.3
Temperature (°C)	24.8 ± 0.4	24.4 ± 0.5
pH	7.57 ± 0.01	7.59 ± 0.06
Oxygen (mg/L)	6.00 ± 2.3	5.76 ± 2.2
Alkalinity (mg/L $CaCO_3$)	120/180	180/240

The 21-day exposure experiment consisted of 4 treatments: 0, 32, 100 and 320 µg/L nominal copper concentrations (refer to Figure 4.1. for an outline of the experimental design and set-up). Each treatment consisted of 4 tanks, 2 in FW (0 ppt) and 2 in SW (20 ppt), of which one replicate tank contained 10 males and one 10 females (*n=12* in control tanks). SW conditions were maintained by dosing hyper-concentrated SW (100 ppt) from a dosing stock prepared daily by dissolving and mixing synthetic seasalts (Tropic Marin) in two fibreglass tanks of 80 L volume each, filled with dechlorinated carbon filtered tap water. The hyper-saline stock solution was made at least 24 h in advance and left overnight with strong aeration to allow the pH to stabilize. SW was transferred into two intermediate dosing stocks (40 L volume each) and from there dosed into the mixing chambers at a rate of 12 ml/min to yield a final salinity of 20 ppt in the SW groups (2 tanks per treatment). Throughout the experiment, fish were maintained under the same photoperiod and food regime as in the Experiment 1 (see Section 4.2.2.2).

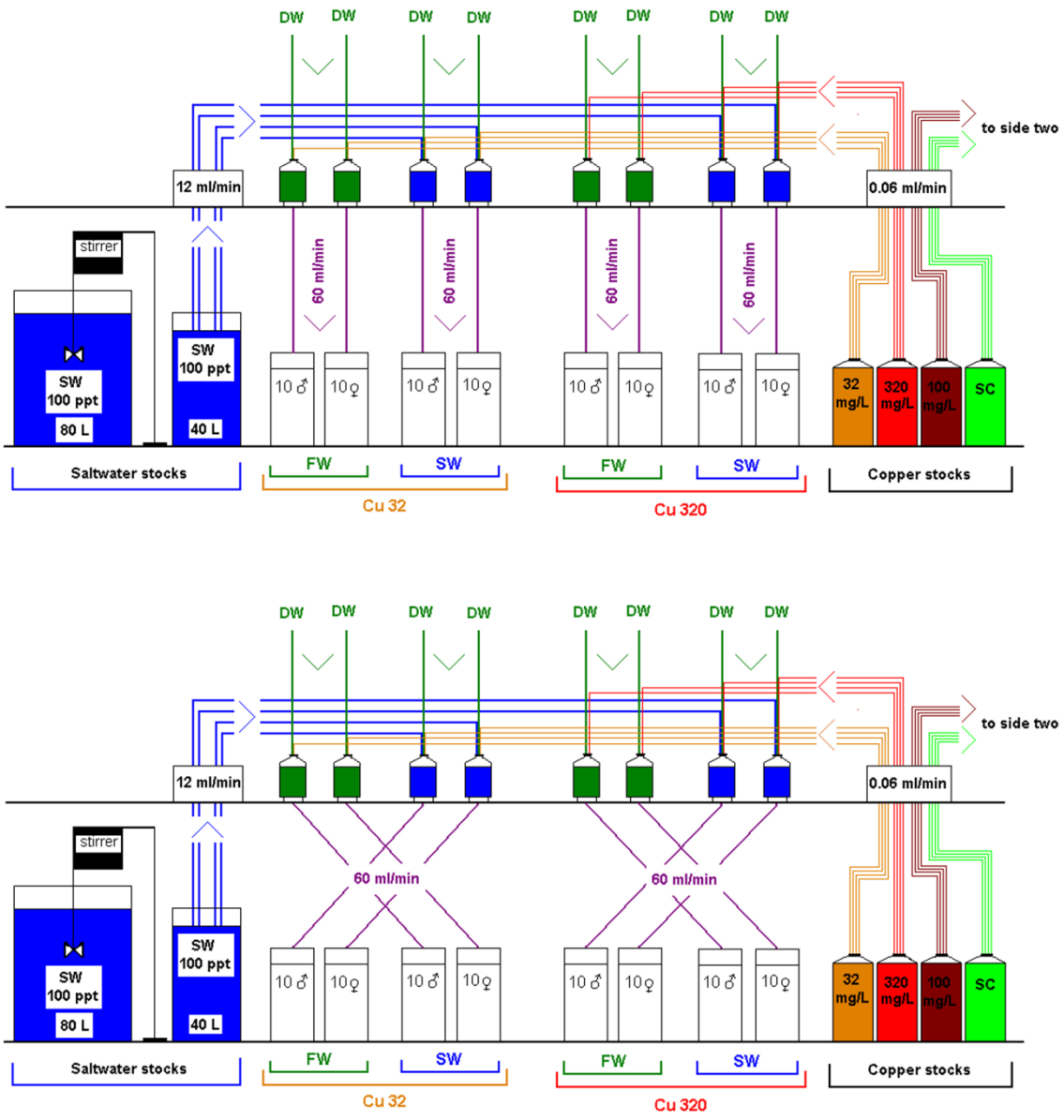

Figure 4.1. Exposure set-ups – Exp.2. Exposure set-ups one on one side of the exposure room (the other was symmetrical), respectively before (top figure) and after (bottom figure) the salinity switch. Blue colour represents the saltwater (SW) supply and dark green colour the dilution water (DW) supply. The SW stock was mixed with a RZR 2052 overhead stirrer (Heidolph) in two 80L tanks (one per side) and then transferred into two 40L tanks, from which the SW was dosed at a rate of 12 ml/min via a peristaltic pump into the mixing chambers of the SW groups. Copper stock solutions were dosed at a rate of 0.06 ml/min via one single peristaltic pump that fed both sides of the room.

After 19 days of copper exposure in either FW or SW, half of the fish (10 per treatment, 12 from controls) were humanely sacrificed according to UK Home Office procedures, using an overdose of ethyl 3-aminobenzoate methanesulfonate (300 mg MS222/L, buffered at pH 7.6, the average pH throughout the study, and adjusted to a salinity of either 0 or 20 ppt, consistently with the exposure conditions). Blood samples were collected and stored as indicated in Section 4.2.2.2. Fish were weighed and measured (fork length). Tissue samples of gills and intestine were collected by dissection. The intestine was divided into two segments (mid-anterior and posterior) and only sub-samples of the mid-anterior segment were kept for gene expression analysis, since preliminary gene expression analyses had shown that the mid-anterior segment displayed slightly higher expression levels of the measured genes, compared to the posterior segment of the intestinal tract (data not shown). All tissue samples were immediately frozen in liquid nitrogen after collection.

4.2.3.3. Salinity transition

On day 20 of exposure (after the first sampling), the salinity in all treatments was either increased from 0 to 20 ppt in the groups previously held in FW or decreased from 20 to 0 ppt in the SW groups, over a time of 8 h. This was achieved by swapping the final tubing between the mixing chambers and the fish tanks (Figure 4.1), with a time lag of two hours between treatments, in order to assure that each group would be held in the new conditions for the same time (24 hours), assuming a sampling rate of 10 fish per hour on the following day.

Salinity was monitored at least every hour with a refractometer throughout all the salinity switch period (Figure 4.2). After reaching either 0 or 20 ppt in all groups, fish were held in the new conditions for 24 h until the second sampling. Copper dosing was maintained constant during the entire study. On day 21 of exposure, the remaining fish (8 to 10 per treatment, 12 from controls) were sampled following the same procedure described in Section 4.2.3.2.

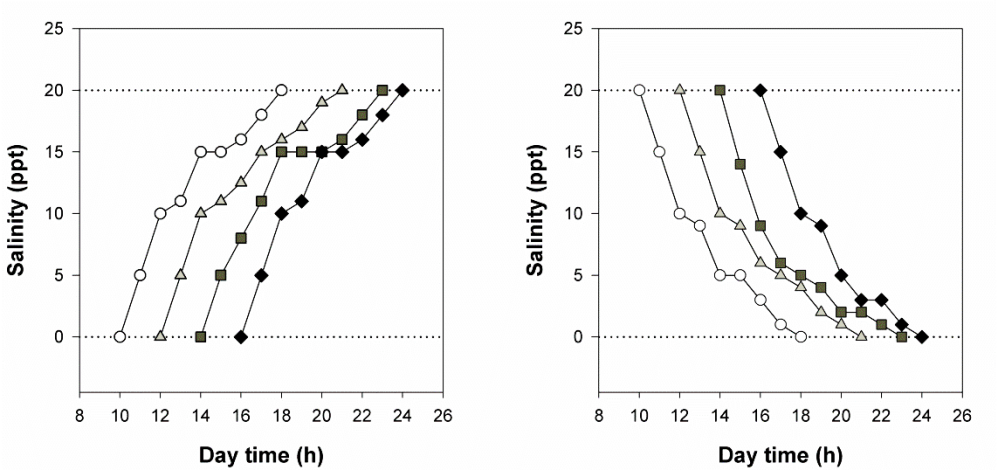

Figure 4.2. Salinity switch - Exp.2. Salinity measurements (ppt) throughout the salinity transition period (8 h) of Experiment 2. The graph on the left side represents the FW groups in the 4 treatments (Controls, Cu32, Cu100 and Cu320) that were moved towards SW conditions, whilst the graph on the right represents the SW groups moved towards FW. Control groups (white circles) were started at 10am, Cu32 (grey triangles) at 12am, Cu100 (dark grey squares) at 14pm and Cu320 (black diamonds) at 16pm. A 2h-lag was chosen to ensure that all treatments were held in the new conditions for exactly 24 h prior to sampling on the following day.

4.2.4. Chemical analyses and speciation modelling

4.2.4.1. Copper, total organic matter and major cations in water

Total copper concentrations in fish tank water were determined by either graphite furnace, GF-AAS (4100ZL Zeeman Atomic Absorption Spectrometer, Perkin Elmer) or flame atomic absorption spectroscopy, F-AAS (AAnalyst100 AAS, Perkin Elmer), depending on the expected range of concentrations, using standard operating conditions and dilutions when necessary. For analytical procedures, all acid was nitric acid (Optima® grade, Fisher Scientific). In order to account for sodium interference in the SW series, calibration standards were prepared in acidified Milli-Q water, with the addition of artificial seasalts to yield a final salinity of 20 ppt (or lower, when samples were diluted). Copper standards were run every 6 samples to check measurement accuracy. Total organic carbon (TOC) was determined as the Non-Purgeable Organic Carbon fraction (NPOC) by high-temperature catalytic oxidation using a Shimadzu total organic carbon-V CPN Analyzer. Major cations (Na^+, Mg^{2+} and Ca^{2+}) concentrations in water samples were determined by F-AAS after appropriate dilution.

4.2.4.2. Copper speciation modelling

Based on water parameters, measured TOC, cations and total dissolved copper concentrations (Table 4.1, 4.4 and 4.5), inorganic and total speciation of copper in exposure conditions (both in FW and SW) was calculated using the Visual MINTEQ 3.0 software [34]. For the total speciation calculations, charges on dissolved organic matter were calculated based on speciation and the % of organic C was set at 50.

4.2.4.3. Copper burden in liver

Six individual 0.2 g aliquots of certified standard DOLT-4 (dogfish liver certified reference material for trace metals, NRC, Canada) were pre-digested overnight in 4 ml of 100% HNO_3, digested in a Microwave Accelerated Reaction System (MARS X, CEM Corporation) following the heating programme recommended by the manufacturer for fish tissue digestion (15 min ramp-to-temperature time and 15 min hold time at 200 ºC), diluted appropriately and analysed by F-AAS. The average wet weight of the liver samples was 0.1 ± 0.06 g. For each batch of samples, one blank and one certified standard were analysed and the average percent recovery was 119 ± 19% (n = 5).

4.2.4.4. Copper content in fish food

Copper content in fish food was analysed by GF-AAS after pre-digestion with HNO_3, microwave digestion (15 min ramp-to-temperature time and 15 min hold time at 200 ºC) and appropriate dilution with Milli-Q water.

4.2.4.5. Copper, chloride and major cations in plasma

Plasma copper concentrations were determined by GF-AAS after dilution in acidified Milli-Q water (1% HNO_3). Plasma Na^+, Mg^{2+} and Ca^{2+} concentrations were measured by F-AAS and plasma Cl^- concentrations by ion chromatography (DIONEX DX, Dionex Corp.), in both cases after appropriate dilution.

4.2.5. Bioinformatics and molecular analyses

4.2.5.1. RNA extraction and cDNA synthesis

Total ribonucleic acid (RNA) was isolated from individual gills and intestine samples (mean tissue weights were respectively 25.8 ± 8.6 and 17.7 ± 9.2 mg) using the RNeasy Fibrous Tissue Mini Kit (Qiagen), according to the manufacturer's instructions, which included tissue homogenization in buffer RLT using a TissueLyser II (Qiagen) for 3 min at maximum speed. The protocol also included a DNAse step to eliminate contaminating genomic DNA. The extracted RNA was resuspended in 50 µl of RNase-free water. Quantification and purity of each RNA sample were determined by spectrophotometry (Nanodrop, Fisher Scientific), and RNA integrity was visually checked by agarose gel electrophoresis. Complementary DNA (cDNA) was synthesised from 2 µg total RNA using Invitrogen SuperScript III First-Strand Synthesis System for reverse transcription-PCR kit according to the manufacturer's protocol, using random hexamers to prime synthesis. Diluted (1:5) cDNA samples were assessed for carbonic anhydrase isoform-2 (CA2) and Na^+/K^+ ATPase α1.a5-isoform (NKA) expression using quantitative real-time PCR (qPCR).

4.2.5.2. NKA and CA2 primer design

Sheepshead minnow specific qPCR primers were developed for both NKA and CA2 from partial *C. variegatus* mRNA sequences identified from GenBank using basic local alignment search tool (BLAST) searches. For NKA, no previously characterized sequence of *C. variegatus* NKA was available in the National Centre for Biotechnology Information (NCBI) databases (http://www.ncbi.nlm.nih.gov/). However, the screening of NCBI Expressed Sequence Tags (ESTs) database led to the identification of four highly similar *C. variegatus* ESTs (GenBank Acc. No. GE337281.1, GE337212.1, GE334919.1 and GE336240.1). GE337281.1 demonstrated 79% identity with the 3' region of the NKA sequence expressed in *Oncorhynchus mykiss* and in BlastX searches it identified NKAα1-isoforms from several species, including *Danio rerio* (Acc. No. AAH90285), which showed high similarity with *C. variegatus* EST and hence confirmed its identity. For CA2, a partial mRNA sequence of *C. variegatus* putative cytosolic carbonic anhydrase (cCA) (Acc. No. HM142344.1) had been previously identified. HM142344.1 showed 81% similarity with the 5' region of the EST of CA2

expressed in *Pimephales promelas* (Acc. No. DT261041.1), which in turn identified *Oncorhynchus mykiss* carbonic anhydrase isoform-2 (NP_954685) in BlastX searches, confirming its identity and hence that of the original *C. variegatus* sequence. These identified sequences were used to design the primers for NKA and CA2 (Table 4.3), with the assistance of PRIMER3 web software (http://bioinfo.ut.ee/primer3-0.4.0/).

Table 4.3. Primers sequences. Primers used for qPCR and respective optimal annealing temperatures.

Gene	Forward Primer	Reverse Primer	T (°C)
CA2	5'- GAAGGTTCTGGATGCTTTGG - 3'	5'- AGTTGGAGAAGGTGGTCTGC - 3'	59
NKA	5'- GCCACACAGCCTTCTTCAC – 3'	5'- ACAATAGAGTTCCTCCTGGTCTTG – 3'	59
18S	5'– GCTGAACGCCACTTGTCC – 3'	5'– CTCAGAGCAAGCAATAGCCTTA – 3'	57

4.2.5.3. NKA and CA2 gene expression

The qPCR primers designed for *C. variegatus* CA2 and NKA were verified using PCR. Reactions contained diluted gill/intestine cDNA (1: 5), 1× Buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs mix, 0.5 μM forward and reverse primer and 0.1 μM *Taq* Polymerase. Cycling conditions were 2 min initial denaturation at 95 °C, 35 cycles at 95 °C for 30 sec, annealing at 58/62 °C for NKA/CA2 respectively, then 72 °C for 20 sec and final extension of 5 min at 72 °C. PCR products were electrophoresed on 2% agarose gel containing GelRed™ (Biotium) to verify a single product and correct amplicon size. qPCRs for CA2, NKA and the reference gene 18S were performed in triplicate on cDNA from individual fish using a CFX96 Real-Time PCR detection system (Bio-Rad) and Fast SYBR® Green Master Mix (Invitrogen) as per instructions of the manufacturer. Reactions were optimized for annealing temperature (see Table 1) and run for 2 min at 50 °C followed by 10 sec at 95 °C, then 40 cycles of 10 sec at 95 °C, 30 sec at optimal annealing temp °C and then 1 min ramp from 55 to 65 °C. Finally a dissociation curve was obtained (melt curve: 65 to 95 °C, increment 0.5 °C for 0.05 sec) to confirm single products in each reaction. The relative expression of the target genes were normalized to the expression of the housekeeping gene 18S using the Excel-based software qGENE [35], which takes into account the amplification efficiency of both the target genes and the reference gene to calculate the mean normalized expression (MNE) of each target gene.

4.2.6. Statistical analysis

Numerical data are presented as means ± SD throughout. Statistical analyses were conducted using SigmaStat software (version 3.5 Systat Software Inc.). Data were analysed for normality (Kolmogorov-Smirnov test) and variance homogeneity (Levene's test). Where assumptions of normality and homogeneity were met, one-way analysis of variance (ANOVA) was performed, followed by all pairwise comparison using Dunn's *post-hoc* test. Where the assumptions were not met, data were analysed using a non-parametric test, Krustal-Wallis ANOVA on ranks, followed by Dunn's *post-hoc* test. Significant differences between pre- and post-salinity transition data were assessed by the Tukey *t*-test. In all cases, values were considered significantly different at $P < 0.05$.

4.3. Results

4.3.1. Experiment 1

4.3.1.1. Water chemistry and copper speciation modelling

Average copper concentrations in the water during the exposure period were < 1 µg/L in the controls, 10.04 ± 0.77 in the low-copper and 115.5 ± 9.97 µg/L in the high-copper treatment (Table 4.4).

The average concentration of organic matter in the water was 2.6 ± 0.5 mg/L and remained stable throughout the exposure period, ranging between 1.8 and 3.9 mg/L (Table 4.5).

Table 4.4. Copper concentrations in the water – Exp.1. Experiment 1 water concentrations (µg/L) of copper during the exposure period, analysed by GF-AAS (controls and 10µg/L) and F-AAS (100µg/L). Reported values are means ± SD (*n* = 3 for each given time point and concentration).

Exposure day	Replicate tank	Nominal copper concentrations (µg/L)		
		Control	10	100
3	A	<1µg/L	10.7 ± 5.7	103.5 ± 3.70
	B	<1µg/L	8.74 ± 3.5	115.7 ± 0.80
6	A	<1µg/L	11.2 ± 3.7	110.6 ± 1.96
	B	<1µg/L	9.34 ± 2.1	123.2 ± 1.19
8	A	<1µg/L	9.70 ± 0.2	120.4 ± 7.33
	B	<1µg/L	10.5 ± 0.7	125.8 ± 2.83
9	A	<1µg/L	10.5 ± 5.7	98.09 ± 0.19
	B	<1µg/L	9.63 ± 2.3	127.1 ± 0.23
mean values (µg/L)			10.04 ± 0.77	115.5 ± 9.97

Table 4.5. TOC and major cations concentrations in the water – Exp.1. Experiment 1 water concentrations (µg/mL) of Total Organic Carbon (TOC) and Na^+, Ca^{2+} and Mg^{2+} analysed respectively by Shimadzu total organic carbon-V CPN Analyzer and F-AAS. Reported FW and SW values are means ± SD of all 6 groups (n = 6) respectively before (PRE) and after (POST) the salinity switch

	PRE	POST
TOC	2.61 ± 0.55	
Na^+	32.50 ± 0.42	6367 ± 211
Mg^{2+}	4.580 ± 0.04	497.9 ± 50.6
Ca^{2+}	101.7 ± 1.63	258.0 ± 18.6

Among the water parameters (Table 4.1), alkalinity and pH underwent the most significant increase following the salinity switch, probably as a result of the increased amount of calcium carbonate in the water due to the dissolved sea salts used to prepare the SW stock solutions [33]. Higher calcium carbonate concentrations shifted the carbonate equilibria towards more basic conditions and this change, along with the increased water concentrations of cations and chloride, affected the inorganic speciation of copper, as it can be observed in Table 4.6 A. The most relevant difference in copper speciation between pre and post salinity switch was the decreased percentage fraction of free copper ions (from around 28% to 14% of the inorganic speciation), which are considered the most bioavailable and therefore toxic form. This fraction and the other percentage fractions of copper forms reported in Table 4.6 A are calculated without including organic matter among the input parameters. When factoring it into the calculations, the model predicted that, given the same average amount of organic matter, around 90% and 84% of the total copper was bound to it, respectively in FW and SW (Table 4.6 B).

Table 4.6. Copper speciation. Inoganic (A) and total (B) copper speciations. Most relevant copper forms are reported (expressed as % of total dissolved copper concentrations), repectively in FW and SW, as calculated by Visual MINTEQ using measured water chemistry parameteres of Experiment 1.

A) Inorganic copper speciation

	% of total copper	
Species	FW	SW
Cu^{2+}	28.2	14.2
$Cu(OH)^+$	55.3	44.5
$Cu(OH)^2$	7.44	14.07
$Cu_2(OH)_2{}^{2+}$	4.44	4.53
$CuCl^+$	0.02	2.28
$CuCO_3$	3.75	18.26

B) Total copper speciation

	% of total copper	
Species	FW	SW
Cu^{+2}	3.08	2.46
$Cu(OH)^+$	6.02	7.71
$Cu(OH)^2$	0.81	2.44
$Cu_2(OH)_2{}^{2+}$	0.05	0.14
$CuCO_3$	0.41	3.17
Organic fraction	89.6	83.6

Hence, although it decreased in SW, the organic fraction of copper remains the most represented fraction. As this form of copper is considered not bioavailable, internal copper concentrations, both in the liver and in the plasma, were measured in order to confirm that copper was indeed uptaken by the fish and its internal levels displayed a dose-response trend.

4.3.1.2. Liver copper burden and copper content in food

Hepatic copper levels in the high-copper treatment were significantly elevated after 9 days of exposure (Figure 4.3 A). However, the relatively high copper levels measured in controls fish (around 110 µg/g wet weight), given the low concentrations of copper in the water (<1 µg/L), led us to investigate fish food as a potential source of copper in control fish. Analysis showed that copper concentrations were 9.1 ± 3.2 µg/g wet weight in the flakes and 1.5 ± 0.8 µg/g wet weight in the brine shrimps, thus providing a possible explanation for the elevated hepatic copper levels in control fish.

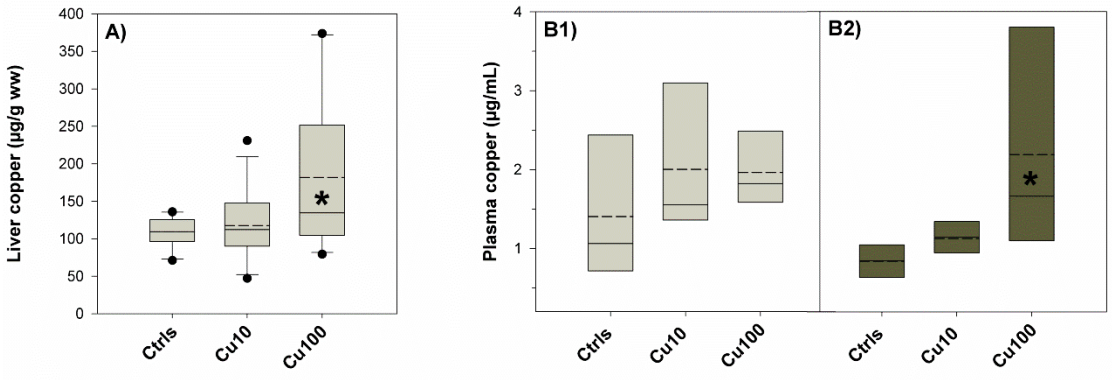

Figure 4.3. Copper in liver and plasma – Exp.1. (A) Copper liver burden (expressed as µg/g wet weight) in fish exposed to 0 (Ctrls), 10 and 100 µg/L copper for 9 days. Values are means ± SE (n = 10/13). The asterisk denotes statistically significant difference from the control value ($P < 0.05$, Tukey t-test) (B1-2) Plasma copper levels (µg/ml) in fish exposed to 0, 10 and 100 µg/L copper and sampled either before (B1) or after (B2) the salinity transition. Values are means ± SE (n = 4/7). The asterisk denotes statistically significant difference from the control value post switch ($P < 0.05$, Tukey t-test). Solid horizontal lines represent median values and dashed lines represent mean values.

4.3.1.3. Plasma copper

Despite model predictions indicating that most of the total dissolved copper was probably complexed by organic matter, 8 days of copper exposure resulted in an increase in plasma copper levels detectable at 10 µg/L and statistically significant at 100

µg/L post salinity switch (Figure 4.3 B). Within the same treatment, plasma copper levels did not show a significant difference pre and post switch.

4.3.1.4. Plasma ions

Plasma sodium levels were around 3000 µg/ml in control fish after 8 days of exposure in FW and did not appear to be significantly affected by copper exposure in a concentration-dependant manner. However, the transition to SW resulted in an average 30% increase of sodium levels in the high-copper treatment, whilst only a 20% increase was detected in the controls.

Table 4.7. Degree of change in plasma ions concentrations – Exp.1. Differences (expressed as percentage) in plasma sodium, chloride, magnesium and calcium levels before and after the salinity transition in fish exposed to 0 (Ctrls), 10 and 100 µg/L copper.

	Sodium	Chloride	Magnesium	Calcium
Ctrls	20	38	11	19
Cu10	16	50	-15	13
Cu100	30	56	21	-12

Plasma chloride levels followed a similar trend, displaying a 38, 50 and 56% increase after the salinity transition respectively in controls, Cu10 and Cu100 (Table 4.7). Plasma magnesium levels underwent a similar though smaller change, whereas calcium cations appeared to follow an opposite trend, showing a greater degree of change in the controls compared to the copper-exposed fish. However, none of the changes in plasma ions levels were statistically significant (Figure 4.4).

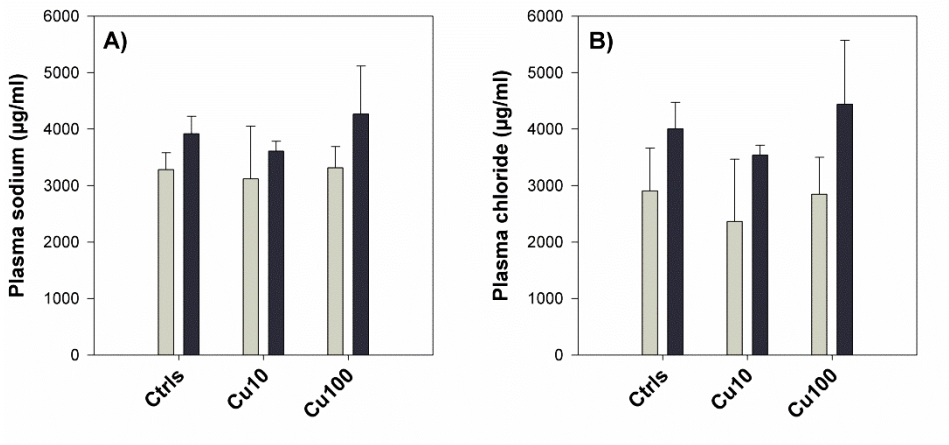

Figure 4.4. Plasma sodium and chloride – Exp.1. Plasma sodium (A) and chloride (B) levels (µg/ml) measured in fish exposed to 0 (Ctrls), 10 and 100 µg/L copper before (light grey bars) and after (dark grey bars) the salinity transition. Values are means ± SE (n = 6/7).

4.3.1.5. NKA and CA2 gene expression

NKA gene expression did not appear to be significantly affected by copper exposure in a concentration-dependant fashion, either in the gills or in the intestine. However, when comparing NKA gene expression in controls before and after the salinity switch (Figure 4.5 A and B), the change in salinity resulted in a significant down-regulation of NKA expression in the gills and a significant up-regulation (~ 3.5-fold change) in the intestine. The same trend of down-regulation in the gills and up-regulation in the intestine was detected in the copper-exposed groups and in both tissues.

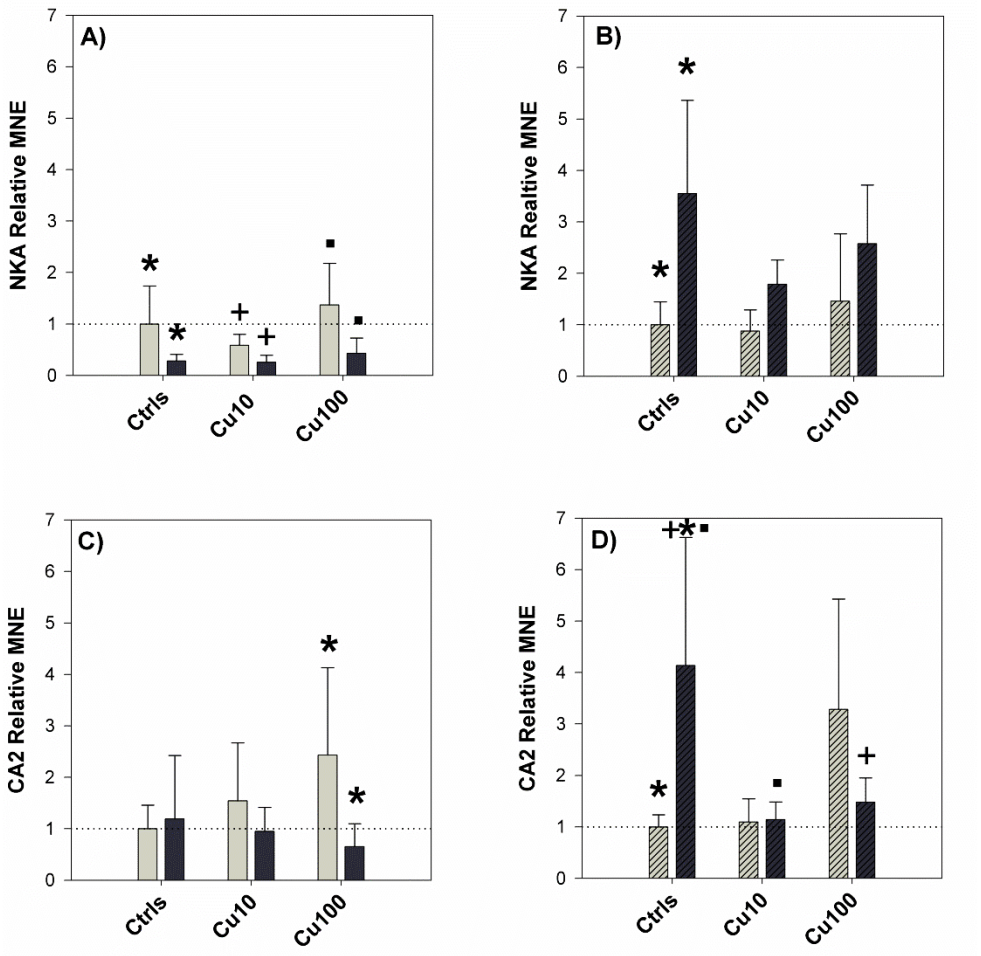

Figure 4.5. **Pre-post change in NKA and CA2 expression – Exp.1**. Relative Mean Normalized Expression (MNE) levels of NKA (graphs A and B), and CA2 (graphs C and D) measured in the gills (A and C) and in the mid-anterior tract of the intestine (B and D, striped bars) of sheepshead minnows exposed to 0 (Ctrls), 10 and 100 µg/L copper. Within each copper treatment, the pair of bars represents expression levels before (left side) and after (right side) the salinity transition. Relative MNE levels were determined by qPCR, normalized to control gene (18S) and expressed as fold change relative to pre-switch control value, which was set at 1 (dotted horizontal line). Values are means ± SE (n = 6/7). In each graph, bars sharing the same symbol (asterisk, cross or dot) are significantly different one from the other ($P < 0.05$, Tukey t-test).

CA2 gene expression was affected by copper exposure in a concentration-dependant manner, both in gills and intestine, displaying in the high treatment a ~ 2-fold up-regulation in the gills and a ~ 3-fold up-regulation in the intestine. CA2 expression in the gills of the controls did not exhibit any substantial difference after the salinity switch, whereas in the intestine CA2 displayed a ~ 4-fold up-regulation in the controls following the switch (Figure 4.5 C and D).

4.3.2. Experiment 2

4.3.2.1. Mortality

Table 4.8. Mortality observed in the freshwater groups.

Treatment	Males	Females
Ctrl FW	1	1
Cu32 FW	2	0
Cu100 FW	0	1
Cu320 FW	4	11

4.3.2.2. Water chemistry

Concentrations of copper in the water were in the expected range for all treatments. Mean measured concentrations in the 32, 100 and 320 µg/L groups were, respectively, 31.82 ± 9.1, 116.6 ± 6.6 and 375.2 ± 21 µg/L in the FW groups, and 29.01 ± 4.7, 81.20 ± 16 and 317.4 ± 52 µg/L in the SW groups. Copper concentrations were < 1 µg/L in all the control groups. Water chemistry parameters (Table 4.2) were stable over the exposure period. In the SW groups, mean measured salinity was 19.9 ± 0.3 ppt, mean pH was only slightly higher than in FW and mean alkalinity was 180/240 mg/L $CaCO_3$.

4.3.2.3. Plasma copper and sodium

The volume of plasma collected from the fish used in this experiment was lower than in the first experiment, due to the smaller fish size. Therefore, copper levels in plasma could be measured only where possible, given the dilution factor applied and the minimal sample volume required for the instrumental analysis. For this reason, results from fish sampled before and after the salinity switch within the same groups were pooled and reported graphically as one group (Figure 4.6), after verifying that there was no detectable difference between pre and post values. In the FW groups, plasma copper

levels were slightly but not significantly elevated by copper exposure, whereas in the SW groups the mid- and high-copper treatments (Cu100 and Cu320) displayed significantly higher copper levels than controls. Given the limited amount of plasma sample available in the second experiment, I chose to measure only sodium as the other plasma endpoint besides copper. Plasma sodium levels in FW and SW controls were very similar, despite the very different osmotic conditions, measuring respectively 2932 ± 343.8 and 2952 ± 459.2 µg/ml (equivalent to, respectively, 127.5 ± 14.9 and 128.3 ± 21.5 µmol/ml). Both in FW and SW conditions, the low- and mid-copper treatments (Cu32 and Cu100) displayed elevated sodium levels before the salinity transition, though this increase was statistically significant only in the SW Cu100 group (Figure 4.7). In the Cu320 treatments, a markedly different type of response was observed between the two salinity groups: in the SW one, sodium levels were significantly higher than controls, even though not higher than the Cu100 group, whereas in the FW group a 50% drop in plasma sodium levels was detected, although here *n* equalled only 3, because in the last days of exposure 6 fish where left in that group (out of 10), of which just 3 provided sufficient volume of plasma for the analysis. However, despite the poor statistical power of the FW Cu320 group, the drastically lower levels measured in those samples were still significantly different from the control value. Unfortunately, in that treatment no fish survived the salinity transition, hence preventing any plasma analysis in that treatment following the switch.

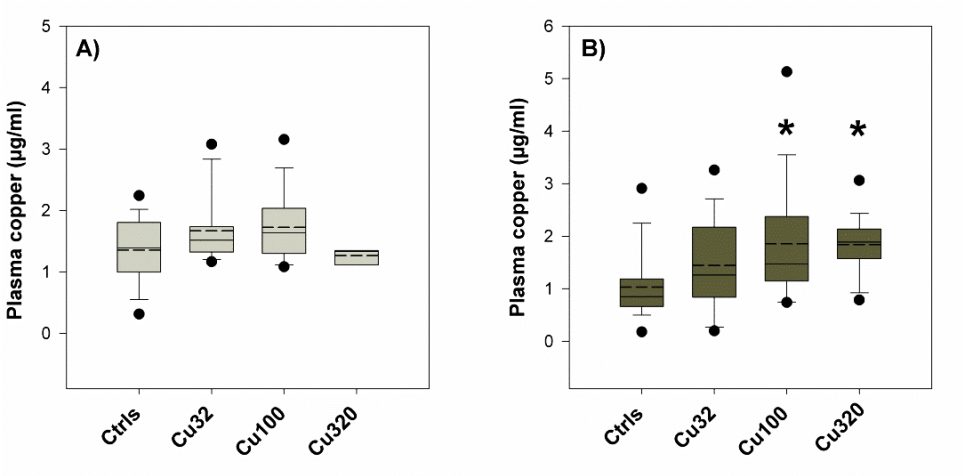

Figure 4.6. Plasma copper – Exp.2. Plasma copper levels (µg/ml) measured in fish exposed to 0 (Ctrls), 32, 100 and 320 µg/L copper in either FW (A) or SW (B). Values are means ± SE (*n* = 18/20, except for FW Cu320, where *n* = 3). The asterisk denotes statistically significant difference from the control value ($P < 0.05$, Tukey t-test). Solid horizontal lines represent median values and dashed lines represent mean values.

As for the pre-post switch effect on plasma sodium levels in the other treatments, a trend of response in ion homeostasis was observed. In the FW control group, mean sodium levels exhibited a 584 µg/ml increase after the salinity change, and in the SW control group almost exactly the same delta of plasma sodium (587 µg/ml) was detected before and after the salinity change (Figure 4.8), although in this case it represented a decrease. Hence, the degree of change in the controls was + 20% for the FW groups and – 20% for the SW ones. An almost equally symmetrical delta change was observed in the low-copper groups (Cu32), where the degree of change was around 22% (of either increase from FW to SW or decrease from SW to FW), and in the mid-copper groups, with a 34% increase in one direction and 32% decrease in the other.

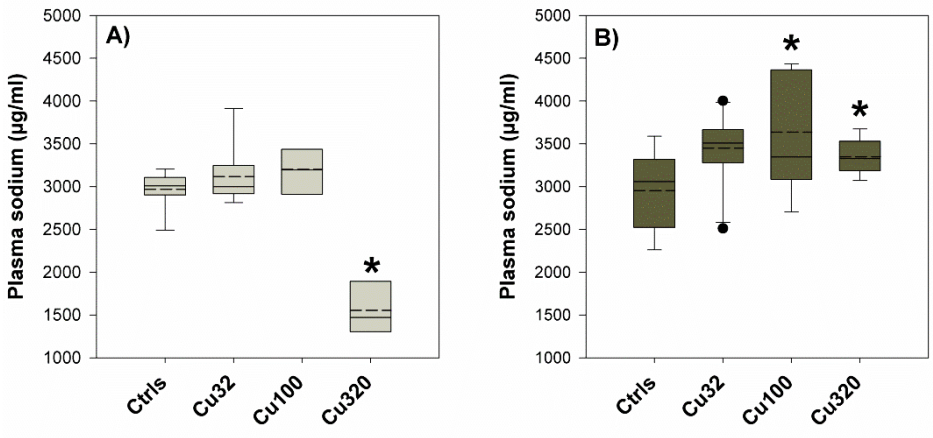

Figure 4.7. Plasma sodium – Exp.2. Plasma sodium concentrations (µg/ml) measured in fish exposed to 0 (Ctrls), 32, 100 and 320 µg/L copper in either FW (A) or SW (B). Values are means ± SE (n = 10, except for FW Cu320, where n = 3). The asterisk denotes statistically significant difference from the control value (P < 0.05, Tukey t-test). Solid horizontal lines represent median values and dashed lines represent mean values.

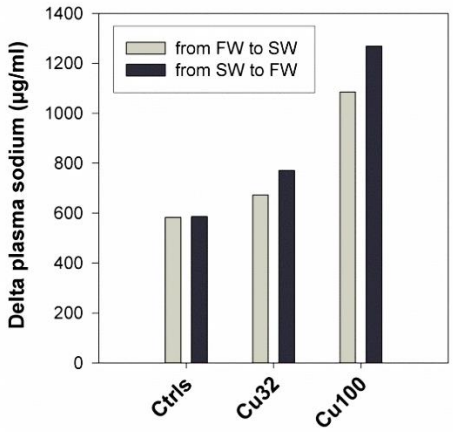

Figure 4.8. Degree of change in sodium levels. Difference (delta) in plasma sodium levels (µg/ml) before and after the salinity transition in fish exposed to 0 (Ctrls), 32 and 100 µg/L copper. Light grey and dark grey bars represent the delta change in the transition respectively from FW to SW and from SW to

FW. No values are reported for the high-copper group as no fish survived after the salinity change. Values are calculated as difference between means and as such have no SE.

4.3.2.4. NKA gene expression

NKA gene expression was significantly up-regulated by copper exposure in the gills of both FW and SW groups, exhibiting a 2 to 3-fold change relative to control values (Figure 4.9 A and B). In samples of intestine from FW groups, the low- and mid-copper groups displayed increased NKA expression, whilst in the high-copper group expression levels were down to control values (Figure 4.9 C). No appreciable changes of NKA expression were detected in the intestine of SW groups (Figure 4.9 D).

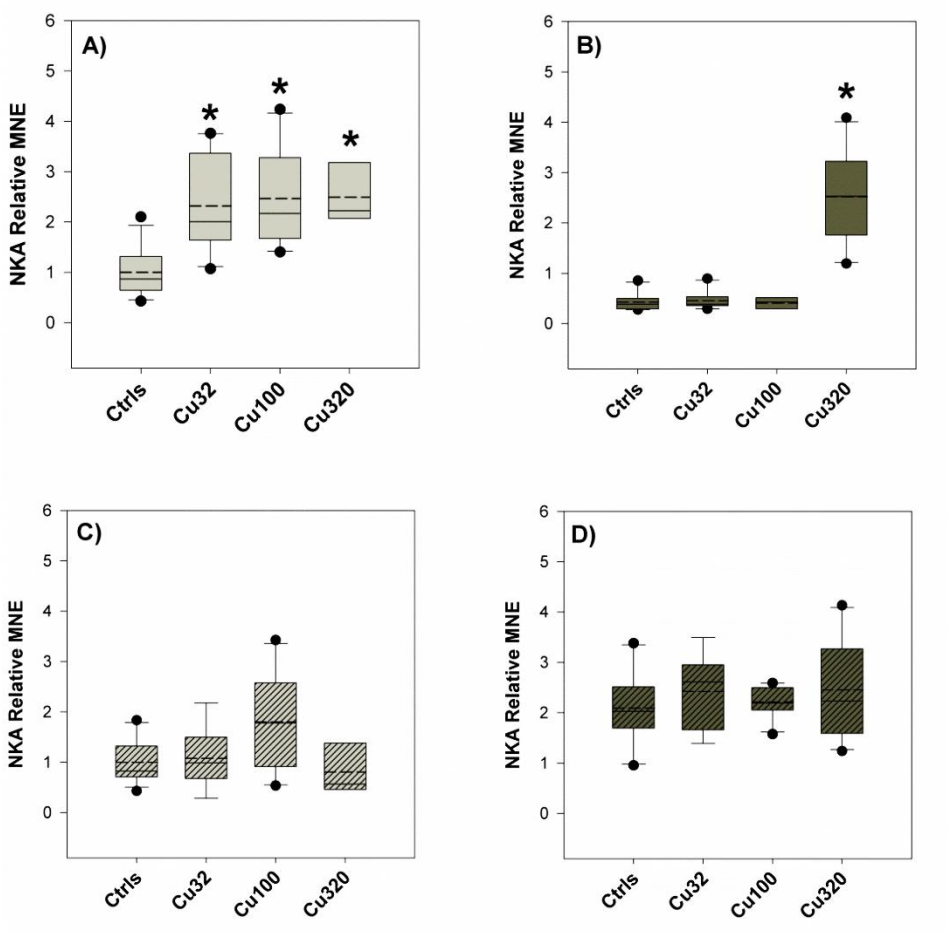

Figure 4.9. NKA expression – Exp.2. Relative Mean Normalized Expression (MNE) levels of NKA measured in the gills (A and B) and in the mid-anterior tract of the intestine (C and D, striped bars) of sheepshead minnows exposed to 0 (Ctrls), 32, 100 and 320 µg/L copper for 19 days. Light grey boxes (graphs A and C) and dark grey boxes (B and D) represent NKA levels respectively in FW and SW groups. Relative MNE levels were determined by qPCR, normalized to control gene (18S) and expressed as fold change relative to FW control value, which was set at 1. Values are means ± SE (n = 10/12, except for the FW Cu320 group, where n = 6). The asterisk denotes statistically significant difference from the control value (P < 0.05, Tukey t-test). Solid horizontal lines represent median values and dashed lines represent mean values.

Considering the effects of the salinity change on NKA expression levels (Figure 4.10), a general trend of up-regulation from FW to SW and down-regulation from SW to FW was displayed in the intestine samples, whereas in the gills NKA expression did not change appreciably. However, when comparing NKA levels of expression in the gills of FW controls versus the SW controls, both of them before the salinity change, the FW gills exhibited a higher level of expression. The opposite was observed when comparing FW with SW controls in the intestine: in this tissue, the SW controls were the ones displaying higher expression levels.

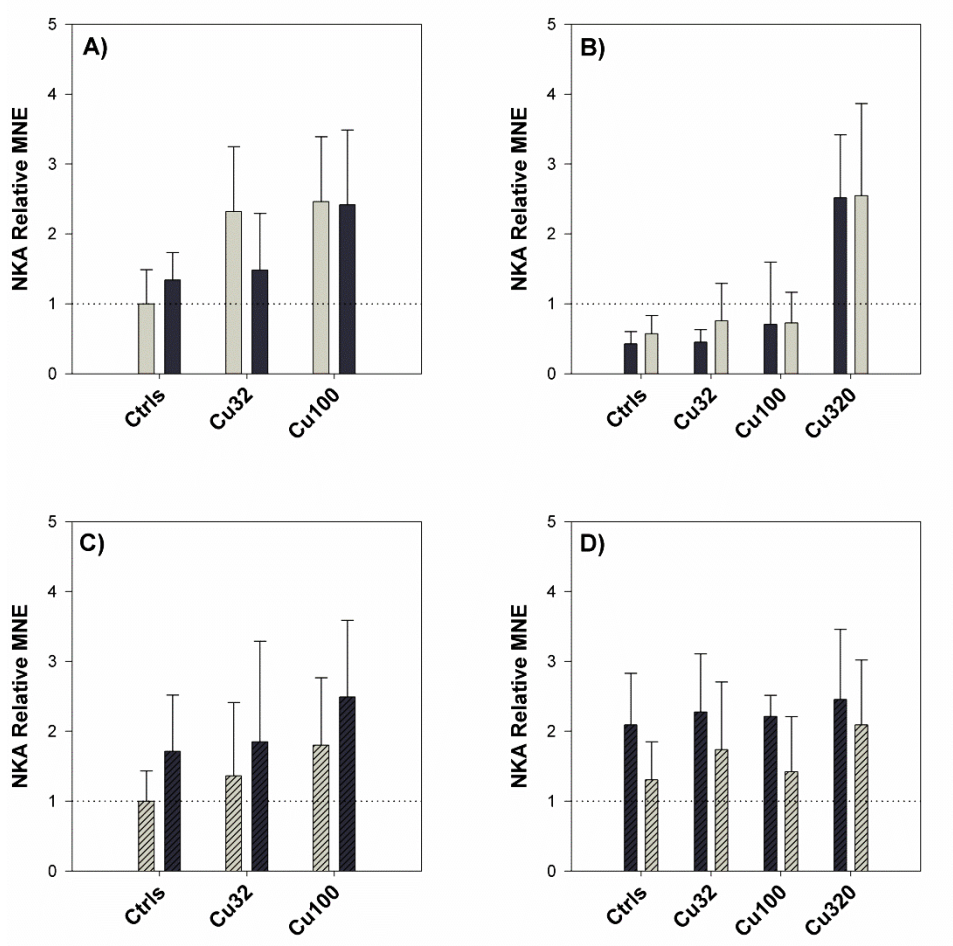

Figure 4.10. Pre-post change in NKA expression – Exp.2. Relative Mean Normalized Expression (MNE) levels of NKA measured in the gills (A and B) and in the mid-anterior tract of the intestine (C and D, striped bars) of sheepshead minnows exposed to 0 (Ctrls), 32, 100 and 320 µg/L copper. Within each copper treatment, the pair of bars represents NKA expression levels before (left side) and after (right side) the salinity transition. The colour of the bars represents the salinity conditions: light-grey for FW and dark-grey for SW. Relative MNE levels were determined by qPCR, normalized to control gene (18S) and expressed as fold change relative to pre-switch FW control value, which was set at 1 (dotted horizontal line). Values are means ± SE (n = 10/12). No values are reported for the high copper dose as no fish survived after the salinity change.

4.3.2.5. CA2 gene expression

CA2 gene expression was up-regulated in response to copper exposure in the gills of both FW and SW groups, exhibiting a ~ 2-fold change in the FW Cu100 group and a ~ 12-fold change in the SW Cu320 group (Figure 4.11 A and B), whereas in the FW Cu320 group CA2 levels dropped down to control values. Similarly, CA2 expression in intestine samples of FW groups was affected by copper in a concentration-dependant manner up to the mid-copper treatment, whilst the high-copper group broke the trend, displaying similar expression levels to the low-copper group (Figure 4.11 C).

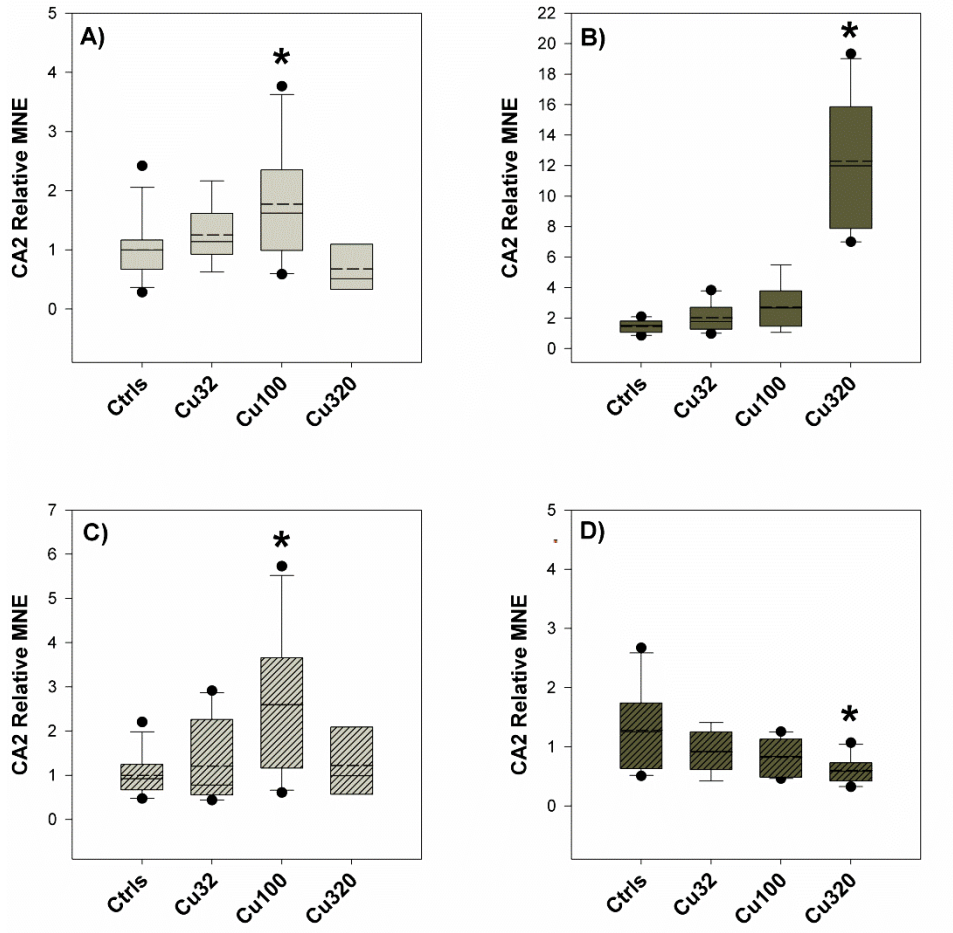

Figure 4.11. CA2 expression – Exp.2. Relative Mean Normalized Expression (MNE) levels of CA2 measured in the gills (A and B) and in the mid-anterior tract of the intestine (C and D, striped bars) of sheepshead minnows exposed to 0 (Ctrls), 32, 100 and 320 µg/L copper for 19 days. Light grey boxes (graphs A and C) and dark grey boxes (B and D) represent NKA levels respectively in FW and SW groups. Relative MNE levels were determined by qPCR, normalized to control gene (18S) and expressed as fold change relative to FW control value, which was set at 1. Values are means ± SE (n = 10/12, except for the FW Cu320 group, where n = 6). The asterisk denotes statistically significant difference from the control value (P < 0.05, Tukey t-test). Solid horizontal lines represent median values and dashed lines represent mean values.

CA2 expression in intestine samples of the SW groups displayed a trend of down-regulation in response to copper exposure, in contrast with the general up-regulation observed in the gills of both groups and in the intestine of the FW groups (Figure 4.11 D). No statistically significant change in CA2 expression was detected either in the gills or in the intestine in response to the salinity change, nor was it possible to observe any appreciable effect of copper on the response to the osmotic stress (Figure 4.12), in contrast to what was detected in the first experiment. However, a comparison between CA2 levels in controls of FW and SW revealed that the SW controls had a significantly higher expression levels, compared to the FW ones, whereas no appreciable difference was seen between FW and SW controls in the intestine.

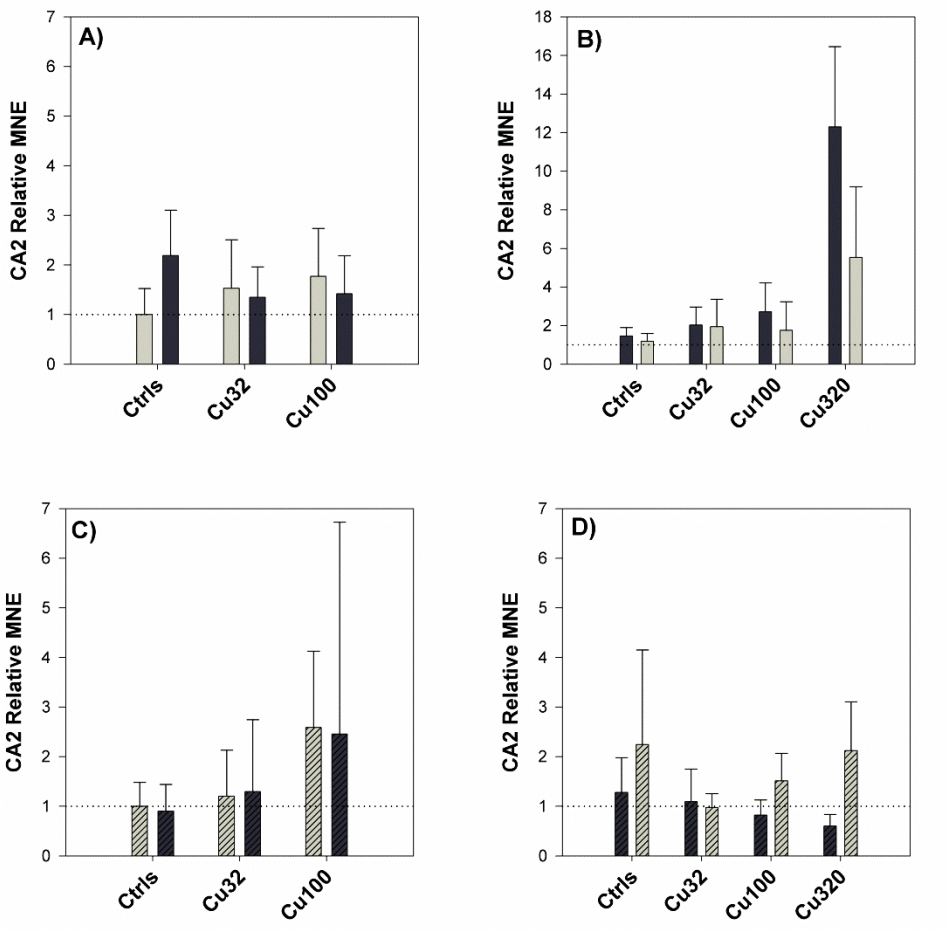

Figure 4.12. Pre-post change in CA2 expression – Exp.2. Relative Mean Normalized Expression (MNE) levels of CA2 measured in the gills (A and B) and in the mid-anterior tract of the intestine (C and D, striped bars) of sheepshead minnows exposed to 0 (Ctrls), 32, 100 and 320 µg/L copper. Within each copper treatment, the pair of bars represents CA2 expression levels before (left side) and after (right side) the salinity transition. The colour of the bars represents the salinity conditions: light-grey for FW and dark-grey for SW. Relative MNE levels were determined by qPCR, normalized to control gene (18S) and expressed as fold change relative to pre-switch FW control value, which was set at 1 (dotted horizontal line). Values are means ± SE (n = 10/12).

4.4. Discussion

Modelling of copper speciation in the exposure conditions revealed that a high proportion of the total dissolved copper was probably bound to organic matter and only a small percentage remained in the most bioavailable and hence toxic form of free metal ion (Table 4.6). Nevertheless, analyses of copper content in plasma showed that copper was still uptaken by fish in a concentration-related manner (Figure 4.3 B and 4.6). One of the few studies that measured copper concentrations in the plasma reported only a small and transient increase of plasma copper levels after 24 hours of exposure, followed by a return to control values from day two onward [36]. This lack of a clear and consistent relationship between external concentrations and internal accumulation may appear to undermine the soundness of any biological effect attributed to water-borne metal exposure. However, metals are highly mobile chemical entities whose mode of action is intrinsically dynamic, in accordance with the high degree of complexation and compartmentalisation they undergo when entering the organism [37]. It is therefore their passing through some biological compartments, such as gills, rather than their accumulation, that bears most of their toxicological meaning and potential adverse effect, especially in short/mid-term exposures at low concentrations. They do accumulate in some tissues, mainly liver and bile [19, 36], but often in complexed and hence relatively inactive forms [38], whose toxicological relevance depends to the type of study and the endpoints of interest. This observation is supported by the high hepatic copper levels detected in this study, possibly as a result of a copper-rich diet rather than of water-borne exposure (Figure 4.3 A).

In the context of this study and its aims, the anchor point from where to start dissecting the results and build up their discussion is constituted by plasma ion homeostasis, as represented by plasma levels of sodium, chloride, calcium and magnesium, and their perturbation in response to copper and osmotic stress. All these four parameters responded to the salinity transition in the first experiment (Table 4.7); therefore, in the second experiment I chose to focus only on sodium (Figures 4.7 and 4.8), which was used as a proxy of plasma ion homeostasis (or internal osmotic pressure). Given the complex experimental designs of this study, it may be useful to bear in mind three categories of questions in order to navigate the patterns of responses displayed by plasma sodium levels and the other endpoints: *(a)* Did copper affect endpoint *x*? *(b)* Did

salinity (i.e. the osmotic stress, administrated in the form of a salinity transition) affect endpoint *x*? And *(c)* did copper exposure affect the response of fish to the osmotic stress? These three questions should guide the analysis and interpretations of each dataset, with the final goal of addressing the main hypothesis, which is itself composed of three key concepts: *(1)* copper-exposed fish struggle to adapt to new salinity conditions since copper affects their osmoregulatory response to an osmotic stress; *(2)* this copper-salinity interaction is explained by an effect of copper on some osmotic effector proteins, among which the CA2 enzyme is a factor linking the physiological responses to both copper and osmotic stress; *(3)* since CA2 displays its osmoregulatory functions also in the intestine, the effects of copper-salinity interaction should also be manifested in the intestine, as suggested by Grosell et al. [20, 29].

With all this in mind, we can go back to analysing the results of plasma sodium levels (Figure 4.7 and 4.8). First of all, it is interesting to note that in both experiments and regardless of the exposure conditions (either FW or SW), control values of sodium in plasma before the salinity transition were all extremely close, around 3000 µg/ml, confirming that this parameter is very tightly and actively regulated by fish.

According to the literature, the iso-osmotic point of sheepshead minnow, meaning the salinity point at which the internal osmotic pressure equals that of the external medium, is around 10 ppt [39]. Based on this information, we should expect that both the fish held in FW and those held in SW (20 ppt) actively regulate their internal plasma concentrations against the same gradient of 10 ppt. However, it must also be considered that my stocks of fish were bred and kept at 20 ppt for most of their life, before being adapted to FW conditions for a month. If we take into account this aspect, it is plausible to assume that strict FW conditions require a higher energetic cost to maintain the internal plasma ion homeostasis. Bearing this in mind, the first question is whether the plasma sodium parameter was affected by copper exposure, in FW and in SW, irrespective of the salinity switch. Since copper is commonly considered an osmoregulatory disrupter [19, 40], displaying its osmoregulatory effects mainly through the disruption of sodium excretion mechanisms, we should expect that this impairment results in either increased sodium levels, when the gradient between internal and external plasma ion levels is negative (i.e. SW), or decreased sodium levels, when the gradient is positive (i.e. FW) [41]. And indeed, SW copper-exposed fish had higher

levels of sodium in their plasma, compared to control ones, in so proving that copper exerted its osmoregulatory disruption, whereas in FW sodium levels displayed an increase in response to copper exposure, contrary to what we would expect given the positive osmotic gradient between internal and external osmotic pressure in these conditions. However, this small increase was observed only in the low- and mid-copper treatments, whereas in the high-copper treatment, Cu320, a dramatic drop of ~ 50% in sodium levels was detected. It is important to stress here that in that particular group only 6 fish had survived after three weeks of copper exposure in FW: this in itself showed how those fish, exposed to the "double stress" of copper and FW conditions, were physiologically struggling, as confirmed by the 50% decrease in sodium levels. Given the tight regulation of this parameter under normal conditions, such a change can be considered as the effect of acute toxicity, similarly to other plasma parameters, such as human plasma glucose concentration [42]. This is an important element to consider when analysing this plasma sodium dataset with the aim of answering question *(1)*, which is at the heart of the hypothesis tested in this study. To address this question we should look at the degree of change in plasma sodium levels before and after the salinity transition (Figure 4.8). If the degrees of change (or delta sodium) calculated for the controls are equal or similar to those calculated for the copper-exposed groups, then copper did not affect fish response to the osmotic stress. Alternatively, if the degrees of change, from FW to SW and from SW to FW, calculated for the copper-exposed groups are higher than those of the controls, we could conclude that copper did have an effect on the fish response to the osmotic stress and impaired their adaptation to the new conditions [20, 21]. Results presented in Figure 6 not only support the latter option by showing a higher delta change in the copper treatments, but also display a concentration-dependent trend of increasing degrees of change at increasing copper concentrations, further supporting the hypothesis of a copper-disrupted osmoregulatory response to the salinity change. This was true in both directions of salinity transitions, from FW to SW and from SW to FW. One possible weakness of this set of results is the narrow range of change in plasma levels, i.e. few percentage points. However, this observation should be put in the context of this particular endpoint and its physiological regulation: as on the one side a 20% change was the "normal" degree of change observed in the controls, we know that on the other side a 50% change in plasma sodium is highly toxic for the organisms (see results in the Cu320 FW group). Framed

in such context, we can consider a 22 or a 34% delta change as very physiologically significant.

Having addressed the question of whether or not copper affected the physiological response of fish to osmotic stress, the next step is to understand if the observed interaction is the combined effect of two independent effects, or if it is the outcome of a mechanistic interaction between the two stressors, i.e. copper and osmotic stress. According to the reasoning presented in Chapter Two [30], I hypothesize that the interaction between copper exposure and osmotic stress is indeed of a mechanistic nature. I also speculate that it is indirect, rather than direct, and that, in the exposure conditions applied in this study, it takes place mainly at the transcriptional level. In brief and according to my hypothesis, it should be an interaction rather than a combined effect because the pathways activated by both of them are similar (i.e. osmotic stress pathways) and they all share some common elements, one of which is the enzyme CA2. Given the numerous and diverse functional roles of CA2 [42, 43], it is more plausible to think that copper affects the transcription of CA2 not directly, such as through the binding of some metal-responsive elements upstream of the gene coding for CA2, but rather indirectly, through the activation of osmotic-stress-related factors, which in turn regulate CA2 transcription.

In order to interpret the results of the molecular analyses, it can be useful to apply the same set of questions used to interpret the results of plasma sodium levels, particularly *(a)* and *(c)*. The first question can be re-formulated as to whether copper exposure affected the transcriptional levels of CA2 and NKA, whereas the second one addresses the effect of copper on the transcriptional response to the osmotic stress. The expression of NKA was not significantly affected by copper exposure in the first experiment, in contrast with the second one, where there was a significant induction in response to copper in both FW and SW (Figure 4.9). CA2 expression was affected by copper in a dose-dependent manner, in both experiments and in both gills and intestine (Figure 4.11), demonstrating a maximum 12-fold up-regulation in the gills in response to copper (320 µg/L) in SW. As for the second question, i.e. whether copper affected the transcriptional response of fish to the salinity challenge, the two experiments provided similar results for NKA, whereas CA2 responses were somehow different between experiments. Considering the effect of copper on the regulation of NKA in response to

the salinity transition, according to the results of both experiments (Figure 4.5 and 4.10) copper did not affect the response of NKA to the salinity change, as its regulation was not impaired by copper exposure. However, if we consider the results of the first experiment and compare the response of NKA in the two tissues, gills and intestine, it is interesting to note that the down-regulation of NKA caused by the salinity change in the gills was accompanied by an almost symmetrical up-regulation in the intestine, a pattern that might suggest a "deactivation" of NKA in one tissue and a parallel "activation" in the intestine as a result of the transition to SW conditions. This is in line with the expression patterns displayed by this isoform of NKA in rainbow trout during salinity transfer [44]. Considering the effect of copper on CA2 and its regulation in response to the salinity change, this is where some disagreements between the two datasets emerge. In the first experiment, CA2 expression in the intestine displayed a clear up-regulation in the controls following the salinity change (Figure 4.5 C and D) and, notably, this up-regulation in the controls was not detected in the low-copper treatment and was even replaced by a down-regulation in the high-copper treatment. This opposite trend of response suggests that, under normal conditions, an osmotic stress induces the expression of CA2, at least in the intestine, but this response is disrupted by copper. Overall, these results support the original hypothesis that copper-exposed fish struggle to adapt to new salinity conditions because copper affects their osmoregulatory response to osmotic stress, and it does so by interfering with the regulation of osmotic effector proteins such as CA2. However, contrary to what was observed in the first experiment, the results from the second experiment did not show any significant effect of copper on the regulation of CA2 either in the gills or in the intestine (Figure 4.12).

The disagreement between the two datasets can be explained by addressing the interaction of copper and salinity from a multi-stressor perspective, where copper exposure and osmotic stress are regarded as a chemical and an environmental stressor that disrupt the homeostasis of the system, i.e. the fish. In this systemic context, it is plausible to assume that the organism responds to the perturbation of its homeostasis by activating responses that are of a magnitude and complexity appropriate to the degree of perturbation, i.e. level of stress. Put another way, if the organism responded to a mild stress with a disproportionally complex response, it would waste its cellular resources, whereas underestimating the level of stress may compromise its cellular function.

Therefore, it is energetically sensible for the organism to size its adaptive response to the severity of the perturbation. Applying this concept to the case of osmotic stress responses, we should expect that different doses of osmotic stress elicit different magnitudes of response, as was indeed shown by the results on CA2 transcriptional levels in the two experiments. Since osmoregulatory mechanisms are finely modulated according to varying degrees of osmotic stress [45], the different rate at which the salinity transition was performed in the two exposures (4 hours in the first and 8 hours in the second one) could explain the activation of different transcriptional responses, resulting in a significant induction of CA2 in the first experiment and in an overall unaffected response of the same enzyme in the second one. Additionally, the responses of some endpoints at high copper doses were completely different than those displayed at low and mid doses, suggesting that somewhere between the mid and the high dose a threshold of different pathways activation was passed, as the dose of chemical stress applied went from mild to severe. Of course this is another way to formulate the classical concept of chronic and acute dose, but this may actually need to be reformulated, when multiple stressors of different source and nature are studied in atypical combinations. Although these arguments are admittedly speculative, they in any case hint at the complexity of multi-stressor studies. Since such a complexity lies at the level of biological responses to different stressors, it cannot be overlooked when it comes to modelling metal toxicity in multi-stressor scenarios.

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CHAPTER FIVE

A COMPARATIVE APPROACH TO STUDY THE ROLE OF CARBONIC ANHYDRASES IN FISH RESPONSE TO COPPER AND OSMOTIC STRESS

A brief history of carbonic anhydrases

The first carbonic anhydrases were discovered simultaneously by two laboratories in Philadelphia and Cambridge (UK) in 1933 and until 1945 there were thought to be only two isoforms, CA1 and CA2 (the latter being the enzyme with the highest turnover rate ever found in nature). Incidentally, it seems that the Cambridge paper was the most frequently cited among the two because it had a shorter and more elegant title…or so the story goes.

In 1940 it was discovered that some sulphonamide drugs are specific CA inhibitors and acetazolamide has been successfully used to treat glaucoma since the early 50's. Many other clinical applications for CA inhibitors have been discovered since then, as well as another 14 mammalian isoforms of CA, all coded by different genes.

The fact that each isoform is the product of a different gene is itself a demonstration of the huge physiological importance of these enzymes, since nature could not afford to spend so much energy on a family of unnecessary enzymes. This importance lies in the reaction that they catalyse, which is so fundamental that, without it, life would probably be impossible. In fact, carbonic anhydrases are extremely convergent and they may have evolved as many as six times.

In her preface to the book "The Carbonic Anhydrases: Cellular Physiology and Molecular Genetics" [1], the editor S.J. Dodgson wrote: "*this is an anthology of the wonderful things that can happen throughout the body when a gas becomes a salt, with recipes.*"

Abstract

The general intent of this chapter was to apply a comparative approach to study CA enzymes from different aspects (phylogenetic, genetic and enzymatic), with particular focus on those isoforms that are involved in metal toxicity and osmoregulatory responses. The introduction to this chapter highlights the diversity of function and localization that characterizes CA enzymes and, at the same time, the high level of conservation that they display, as illustrated by examples from human, fish and invertebrates studies. The phylogenetic analysis described in Section 5.2 showes that function and cellular localization are stronger discriminators than species-specificity in driving CAs classification, demonstrating that CA isoforms are highly conserved across species and taxa. The promoter analysis of zebrafish CA2, described in Section 5.3, shows that zebrafish CA2 enhancer region contains transcription elements that suggest a control of the gene by osmotic stress response pathways similar to those present in yeasts, plants and humans. The last section of the chapter describes the overall successful validation of a human CA2 assay on fish samples and its use to assay the samples of the second *in vivo* experiment. The results of CA2 protein levels in the gills were in line with gene expression results, hence stressing the relevance of an accurate dosing of the stress. Overall, this chapter illustrates the importance of studying a biological factor like CA2 from different angles, integrating "wet biology" with bioinformatics and using a comparative approach to fill those knowledge gaps that can be encountered when working with non-model species such as sheepshead minnow.

5.1. Introduction

5.1.1. Carbonic anhydrase enzymes: general features

Carbonic anhydrases (CAs, EC 4.2.1.1.) are ubiquitous zinc enzymes, present in prokaryotes and eukaryotes and encoded by four distinct gene families: the α-CAs (present in vertebrates, *Bacteria*, algae and cytoplasm of green plants), the β-CAs (predominantly found in *Bacteria* and algae), γ-CAs (present in *Archaea* and some *Bacteria*) and the δ-CAs (present in some marine diatoms) [1, 2].

Considering the α-CAs family, so far 16 different isoforms of α-CAs have been characterized in mammals and several novel isozymes have also been identified in non-mammalian vertebrates, all differing in their subcellular localization, tissue distribution, catalytic activity and susceptibility to different inhibitors. There are three groups of mammalian CA isozymes within the α-CAs gene family:

- the cytosolic isozymes (CA1, CA2, CA3, CA7 and CA13);
- membrane-bound isozymes (CA4, CA9, CA12, CA14 and CA15);
- mitochondrial isozymes (CA5A and CA5B);
- secreted isozymes (CA6).

Three acatalytic isozymes, CA8, CA10 and CA11, called carbonic anhydrase-related proteins, have also been identified. These isozymes have lost classical CA activity and have no known physiological function [3].

Except for these few isoforms, however, most CAs act as very efficient catalysts for the reversible hydration of carbon dioxide to bicarbonate and protons,

$$CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$$

by means of a metal-hydroxide mechanism [$Lig^3M^{2+}(OH)^-$], where Lig^3 is constituted by three histidine residues and M^{2+} is ZnII. The zinc ion is in the +2 state and is located in a cleft near the centre of the enzyme. Its role is to facilitate the deprotonization of water with the formation of the hydroxide ion, which in turn can attack the carbonyl group of the carbon dioxide to convert it into bicarbonate [4]. Besides zinc, other metals have been shown to act as cofactors for some CAs (e.g. FeII for γ-CAs [5]). It is the active site of the enzyme that is so highly conserved among all the isoforms and across

so many phyla, making CAs an excellent example of molecular convergence. The molecular architecture of the rest of the protein, on the contrary, is much less conserved, demonstrating that these enzymes, rather than having evolved from a common ancestor, are the products of a convergent evolution, driven by the need to rapidly hydrate carbon dioxide.

In vertebrates, α-CAs are distributed in several tissues, including brain, liver, kidney, erythrocytes, gills and intestine, and are involved in many key physiological functions, such as gustation and olfaction, secretion of electrolytes, muscle function and others [2]. In particular, isozymes 1, 2 and 4 are involved in respiration and acid-base regulation; isozymes 2, 4 and 9 in vision (their malfunctioning leading to glaucoma); and CA2 in bone development and function, among others [3].

Given their multiple functions and relatively simple biochemistry, CA isozymes are important targets in the design of drugs used as antiglaucoma agents, diuretics and antiepileptics, and in the treatment of ulcers, neurological disorders, osteoporosis and many other diseases. Among the clinical application of CA isoforms as drug targets, recent studies have demonstrated that the inhibition of the mitochondrial isoforms CA5A and B, in conjunction with that of the cytosolic isoform CA2, can be clinically exploited to treat obesity. Interestingly, this was one of the cases where an isoform of CA that was originally an off target of a given drug was then exploited as primary target of a newly developed drug used to treat a new disease. In the case of anti-obesity CA inhibitors, the discovery came from the search for a pharmacological explanation of a side effect of an anti-epileptic drug – Topiramate – whose administration to obese patients had been shown to cause loss of body weight [6-8]. A similar case of an off target CA with new potential clinical applications of its own is the finding that human CA1 and 2 are off targets of the selective serotonin reuptake inhibitors (SSRI) fluoxetine, sertraline and citalopram, which act as brain CA activators, hence suggesting that CA activation could constitute a possible therapeutic approach for the enhancement of synaptic efficacy [9].

Among the many clinical applications of CA inhibitors, cancer treatment is one of the main areas of research and some isoforms of CA represent a promising alternative to the classical anticancer drugs.

4.1.1.1. Tumour-associated carbonic anhydrases

Two carbonic anhydrase enzymes – CA9 and CA12 – are overexpressed in several types of tumours and are associated with cancer progression and response to therapy [6]. The transmembrane isoform CA9 is normally expressed in the stomach and in the peritoneal lining, but is ectopically induced by hypoxia in human cancer cells [10, 11]. Its role in cancer cell metabolism makes it a potential target for anticancer drugs. Like CA9, CA12 is also a transmembrane isoform with an extracellular active site; it is expressed in several tumours but also diffused in many healthy tissues [12]. Also the cytosolic CA2 plays an indirect role in oncogenesis metabolism, but its subcellular location as well as its virtual ubiquity makes it a less interesting target for anticancer drug discovery.

Because many CA isoforms are associated with anion exchangers or sodium bicarbonate cotransporters, they play an important role in the transport of ions across biological membranes and in the secretion of electrolytes in many tissues or organs [3]. It is this function in cell ion homeostasis and pH regulation that constitutes, in extreme synthesis, the functional link between CAs, hypoxia and tumorogenesis. In brief, the high metabolic rate that characterizes tumour cells often leads to enhanced acidification of the tumour microenvironment, which is accompanied by various degrees of hypoxia (leading to a metabolic shift towards glycolysis and lactate production). Tumour cells, however, have evolved the ability to function in a more acidic environment than normal cells and this feature gives them a selective advantage over normal cells, which undergo apoptosis under these conditions [13]. The ability of tumour cells to cope with acidic environments relies on the functioning of some key pH regulators (Figure 5.1), which include isoforms 2, 9 and 12 of CA enzymes, various isoforms of ion exchangers, Na^+/HCO_3^- co-transporters, Na^+/H^+ exchangers and the vacuolar ATPase [14]. Considering CA isoforms in particular, they contribute to maintaining the pH homeostasis of the tumour cells by keeping in equilibrium the excess of protons and carbon dioxide generated by the oncogenesis metabolism. In simple terms, selectively inhibiting these CA isoforms would deprive tumour cells of their Darwinian growth advantage, hence limiting the neoplastic growth and the metastatic spread of cancer cells to distant sites [14, 15]. For instance, genetic silencing of both CA9 and CA12 led to an 85% reduction in tumour growth, demonstrating that CA9 and CA12 are major

tumour prosurvival pH-regulating enzymes [16]. There are currently a number of CA9 and CA12 inhibitors that are in clinical development or in use, but much more needs to be understood about the contribution of pH regulators to cancer proliferation before these antitumour mechanisms could be fully exploited.

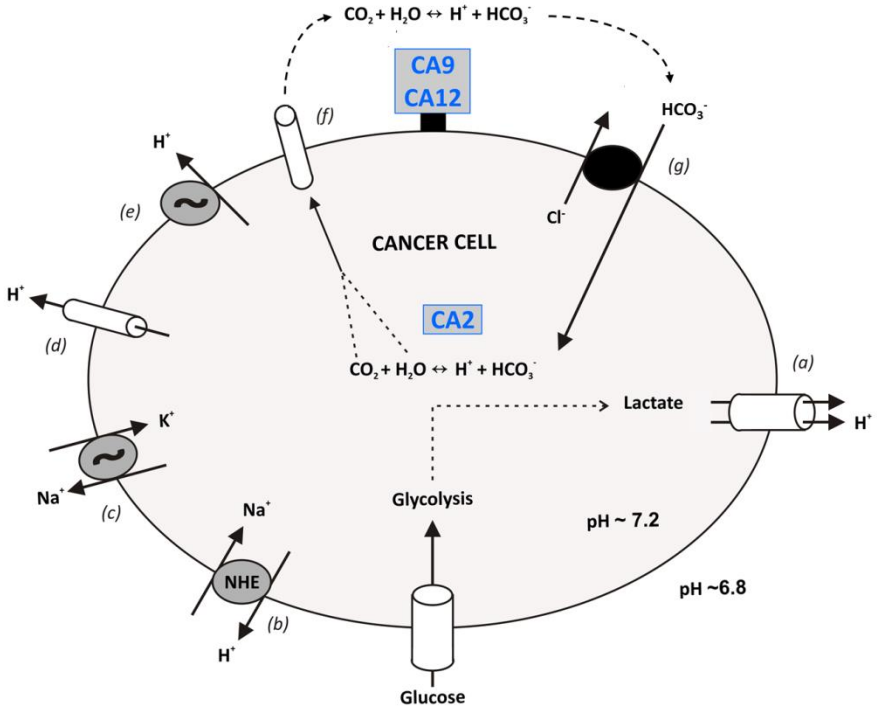

Figure 5.1. Molecular mechanisms involved in pH regulation and ion transport in cancer cells. Lactic acid is produced from glucose by glycolysis. The lactate and H^+ ions are exported from the extracellular fluid by the monocarboxylate carrier **(a)**. The Na^+–H^+ exchanger **(b)** exports H^+ and imports Na^+, contributing to high levels of intracellular Na^+, a common feature in tumor cells. Other protein machines used by cancer cells to regulate intracellular pH include ATP-dependent Na^+–K^+ antiporters **(c)**, H^+ channels **(d)**, and the plasma membrane proton pump H^+–ATPase **(e)**. H_2O and CO_2 provided by the cytosolic CA2 are exported through aquaporins and used by cell-surface CAs to produce HCO_3^-**(f)**. Conversely, CA9 and CA12 produce HCO_3^- ions outside the cell, which are transported inside by HCO_3^-–Cl^- anion exchangers **(g)** and used by the cytosolic CA2. (Adapted from [17])

For the purpose of this Thesis, exploring the clinical applications of CA inhibitors and activators and understanding the mechanisms of action of anticancer drugs targeting CA isoforms is not just an information compiling exercise. Instead, it helps shed light onto the multiple pathways in which CAs are involved, putting into a wider perspective the role of these enzymes in maintaining cell homeostasis. There is a fascinating parallelism between the molecular mechanisms involved in pH regulation and ion transport in human cancer cells and those of the ionocytes in the branchial epithelium of teleost fish. This parallelism can be exploited to extrapolate across humans and other vertebrates the cellular mechanisms in which CAs are involved and, in particular, to use the

information available on human studies to better understand the responses observed in fish. However, there are some key differences between mammalian and fish CA isoforms, and between isoforms of different fish species, that must be taken into account when applying a cross-species extrapolation on CA enzymes, especially because the information on fish CAs is still relatively limited.

5.1.2. Carbonic anhydrases in fish

Analysis of genomic databases has indicated that many mammalian CA isoforms have fish orthologues, but relatively little is known about most of them, including their interspecific diversity and tissue distribution [18]. For instance, CA4 is notably different between fish and mammals: whilst in mammals only one isoform of CA4 is present, some teleost fish seem to express as many as nine CA4-like isoforms, all exhibiting different tissue distribution and therefore presumably different functions [19]. Also cytosolic CA isoforms and particularly CA2, the most active and nearly ubiquitous isoform of CA, have been shown to differ between fish and mammals. In mammals, CA2 displays its catalytic activity both in the red blood cells, where it regulates CO_2 excretion, and in the kidney, where it accounts for 95% of renal activity [20]. In contrast to mammals, fish have two isoforms of CA2, CA2-like b (CAb) and CA-like c (CAc), both displaying different functions and tissue distribution [19, 21, 22]. This differentiation was probably the result of the whole genome duplication of the teleost common ancestor and the consequent uneven gene duplication, which in turn led to the acquisition of new functions (sub-functionalization). The CAb isoform is expressed predominantly in the blood of zebrafish and trout and seems to be specialized in CO_2 excretion [19, 21], whereas CAc is more widely distributed, with high expression in the gills and kidney and almost no expression in the red blood cells, in accordance with its main function of acid-base regulation [22].

5.1.2.1. Carbonic anhydrases and fish osmoregulation

Several studies have shown that CA isoforms are involved in osmoregulatory responses of teleost fish, crustaceans and other aquatic invertebrates to various types of osmotic stress (e.g. transfer from fresh to saltwater or vice versa), as demonstrated by changes in expression levels and enzymatic activity of CA observed mainly in branchial, but also intestinal epithelia (see Chapter Two). The patterns of response observed in these

studies differ among each other and present an apparent species-specificity that could be attributed to several elements, including differences in experimental design, exposure conditions, salinity range or endpoint selected: all factors that can result in different response patterns. However, these discrepancies among different studies could also be at least partly explained by differences in the tissue distribution, subcellular localization and functional differentiation of CA isoforms in different species, because the specificity of a response to a stress is also related to the functional specificity of the cellular elements involved in the response itself.

Considering teleost fish, it is well documented that cytoplasmic CA in fish gills display high levels of activity [18], but it is still unclear which CA isoform is responsible for this activity. As discussed in the previous section, fish CAs appear to have a higher degree of sub-functionalization, compared to mammalian CAs, a feature that in turn is closely related to their different tissue distribution. If this is also the case for those CA isoforms involved in osmoregulation, it is plausible to hypothesize that CAs expressed in different tissues, such as gills and intestine, are specialized in different functions and therefore represent different sub-isoforms of CAs, having different response patterns to the same type of stress (i.e. osmotic stress).

The cellular localization of the enzyme within the same tissue can play a role in its function and hence in its response to a stress. For instance, considering the main site of osmoregulation in fish, the branchial epithelium is composed of different types of cells, each having different specific functions and enzymatic make-ups [23]. This as well may be a factor affecting the pattern of response to an osmotic stress, if, as it is plausible to assume, different branchial cells also have different levels of CA expression or even different isoforms of CAs. CA-like enzymes have been localized in most cell types of teleost fish gills, including ionocytes (also called “chloride cells” or “mitochondrion-rich cells”), pavement cells and mucus cells, where they have primarily an apical localization [18]. However, their characterization and subcellular distribution still remain objects of investigation, especially in light of the high diversity and inter-species variation of the ionocytes present in the branchial epithelium [23, 24]. This already diverse cellular and molecular landscape is further complicated by the inter-species variation in ionocytes with different salinities [25].

Finally, a third factor that can theoretically affect the response of CAs to a stress and, even more so, to a xenobiotic, is their subcellular localization. Intra or extracellular CA isoforms as well as cytosolic or membrane-bound isoforms are involved in different cellular processes [26, 27], albeit their general catalytic function is the same, and they can be differentially affected by a chemical stressor such as a metal [28-30], depending on the ability of the xenobiotic to reach that cellular compartment. This is for example why CA9 and CA12 are potentially better anticancer drug targets than CA2, since they have an extracellular active site, "more easily reachable" by a drug molecule than the other isoform [14].

Whereas differential centrifugation of gill homogenates to isolate subcellular fractions has indicated that the vast majority of CA activity is associated with the cytoplasmic fraction, mainly involved in acid-base regulation [18], studies on rainbow trout have demonstrated that part of the total CA activity (13-20%) in the intestine is attributable to an extracellular membrane-bound CA4-like isoform that contributes to the deposition of $CaCO_3$ in the lumen of the intestine [27]. These findings suggest that CA isoforms expressed in branchial and intestinal epithelia might have different subcellular distribution, corresponding to partly different functions.

Whether this sub-functionalization is coupled or not with the presence of different isoforms of CA is a matter of relative importance, from a toxicological viewpoint; more relevant is to understand if this fine tuning of the enzymatic function has an effect on the response of the enzyme to a stressor, such as an osmotic stress or a xenobiotic like copper. Given the high inter-species diversity of the osmoregulatory systems and their functioning in different tissues at different environmental salinities, it is not surprising that studies on the response of fish CAs to salinity changes often appear to be in disagreement [31].

5.1.3. Carbonic anhydrases and metal toxicity

Essential metals and particularly zinc play a crucial role in the functioning of many enzymes, including CAs, since their binding to the active site allows the enzyme to display its normal catalytic function (Figure 5.2). However, besides their role as cofactors in enzymes catalytic activity, metals can also inhibit enzyme activity by displacing the metal (i.e. zinc) associated with it and so produce an inactive metal-

substituted enzyme (see [30] for a review). The first evidence of an *in vitro* inhibition of fish CA enzymes by water toxicants dates back to the late 70's [32]. Since then, several metals have been demonstrated to inhibit CA activity both in vertebrates and invertebrates, often showing a species-specific as well as isoform-specific and tissue-specific inhibition [30].

An example of tissue- and isoform-specific inhibition is provided by the works of Lionetto et al. [28, 29], who investigated the effect of cadmium on CA activity in the gills and in the intestine of the European eel (*Anguilla anguilla*). Their results revealed that the gills were more sensitive to cadmium than the intestine, possibly because in the intestine part of the total CA activity is displayed by the membrane-bound isoform of CA, which is less sensitive to metals than the cytosolic isoform.

An excellent example of species-specificity of CA inhibition is provided by the study of Skaggs and Henry [33], who tested four metals (silver, cadmium, copper and zinc) on branchial samples of two species of euryhaline crustaceans and found that the binding affinity of one species was a thousand time lower than the other. Moving from aquatic organisms to humans, some *in vitro* studies have demonstrated the competitive and/or non-competitive inhibition of some metals (i.e. lead, cobalt and mercury) on human CA isoforms (see for example [34]).

The species-specificity observed in the inhibition of CA by metals is in line with what discussed in Section 5.1.2 and probably relates to the variability among different CA isoforms. In particular, it can be due to the structural differences among CA protein isoforms, hence explaining the differences in metal-binding affinities observed in different tissues and different species [30].

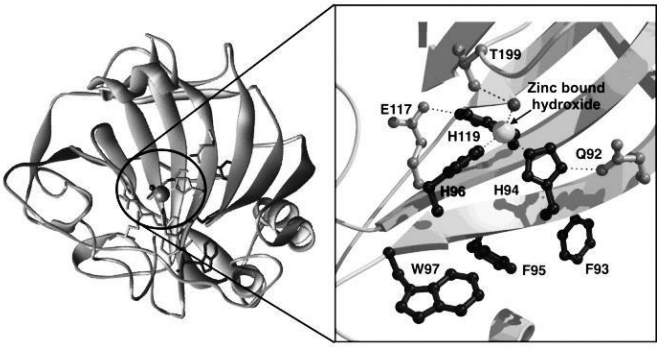

Figure 5.2. Ribbon diagram of human CA2: Metal binding site highlighting: the zinc ion as a sphere; the direct ligand histidines, H94, H96, H119. From [35]

5.1.3.1. Carbonic anhydrases and mechanisms of acute copper toxicity

Among the different mechanisms through which copper can exert its toxic action [36], those that are either directly or indirectly involved in ionoregulation are the most likely to be linked to the activity of CA enzymes and their function in maintaining cellular and organismal homeostasis. In particular, the three main mechanisms that can be impaired by copper through CA disruption are:

- Ammonia excretion;
- Acid-base balance;
- Ion transport.

Ammonia excretion: Sublethal copper exposure has been shown to produce elevated plasma ammonia and ammonium (NH_3 and NH_4^+) through the disruption of the mechanisms of ammonia excretion. The branchial extracellular CA isoform participates in the boundary layer acidification and diffusion trapping mechanism, by contributing to the conversion of NH_3 to NH_4^+ at the apical membrane of branchial cells [37] (Figure 5.3). This mechanism controls the process of NH_3 excretion, whose impairment, combined with elevated NH_3 production, results in overall increased plasma NH_3 levels, which represents the most consistently observed response to sublethal copper exposure in fish [37-40].

Acid-base balance: Another copper effect in which CA can play a role is acid-base balance disturbance, which can be induced by low levels of copper exposure and result in metabolic alkalosis [41, 42]. An increase in intracellular pH, accompanied by increased bicarbonate concentrations, could be caused by copper through the direct and/or indirect impairment of the branchial Cl^-/HCO_3^- exchanger, which relies on the cytosolic CA for the "production" of bicarbonate ions [36] (Figure 5.3). In marine fish, acid-base balance is also maintained by a net bicarbonate excretion across the intestinal epithelium [27, 36]. This mechanism relies on the activity of a number of enzymes and transmembrane transporters, including the branchial Cl^-/HCO_3^- exchanger, fuelled by the activity of the cytosolic CA [43, 44], and on the mechanism of $CaCO_3$ deposition in the intestinal lumen, which is favoured by the activity of the extracellular CA (Figure 5.3).

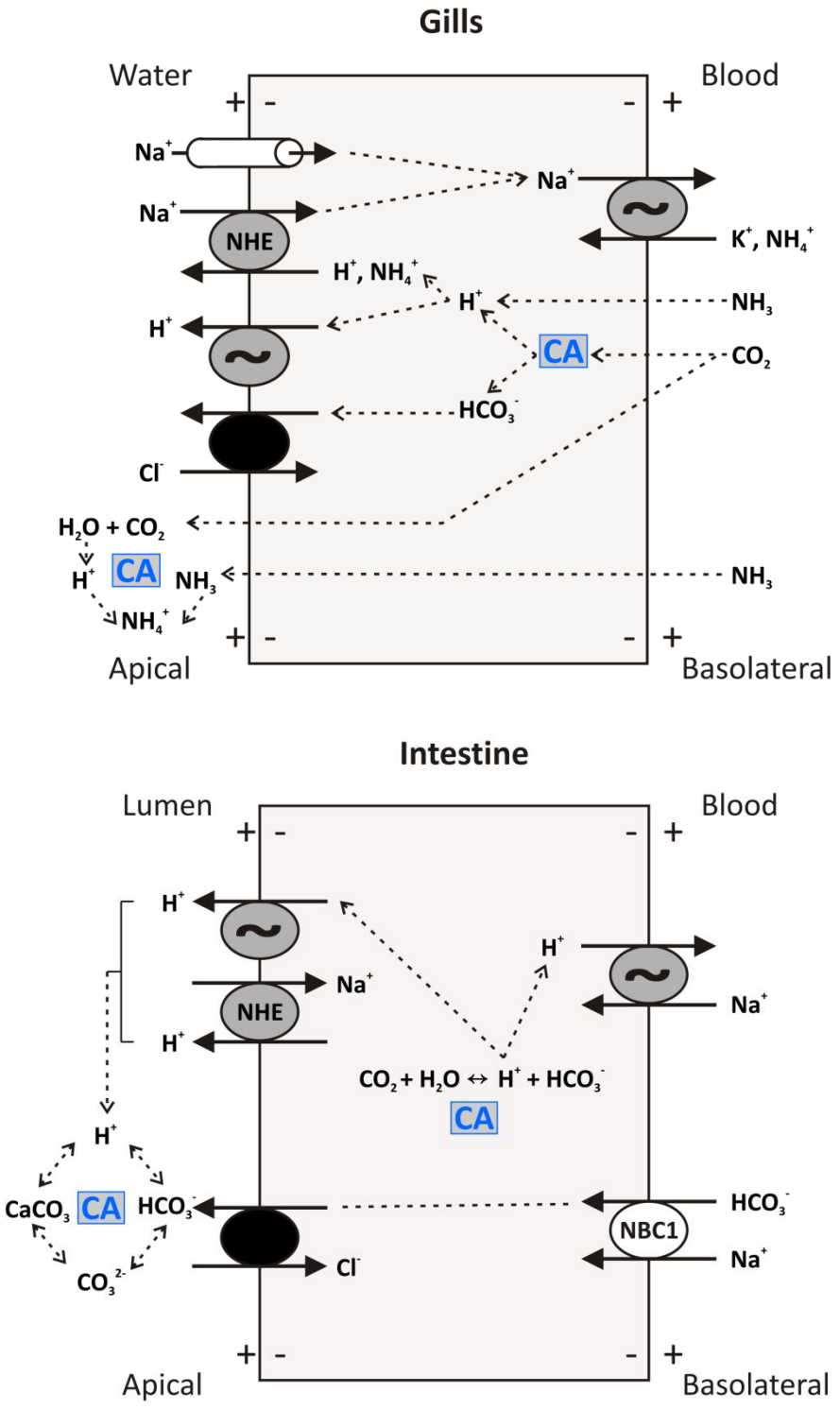

Figure 5.3. Role of CA in molecular mechanisms of branchial and intestinal epithelia in fish. **(Top)** Schematic representation of the branchial epithelium of FW fish, highlighting the role of CA in the mechanisms of ammonia excretion, acid-base balance and sodium transport. Dotted lines represent diffusive transport events, while solid lines depict all other events. Modified from [45]. **(Bottom)** Schematic representation of the intestinal epithelium of SW fish, highlighting the role of CA in the processes of HCO_3^- secretion, $CaCO_3$ precipitates formation and CO_2 hydration. Modified from [43]

Sodium chloride transport: Copper exposure in freshwater can affect osmoregulation mechanisms and in particular sodium chloride transport. The impairment of plasma sodium homeostasis can be the result of two direct mechanisms of action of copper: reduced sodium uptake, at low copper doses, and a combination of reduced uptake and increased sodium loss at high copper doses, due to increased paracellular permeability caused by the displacement of calcium in tight junction proteins [38]. The reduction of sodium uptake is the combined product of a decreased transport capacity (i.e. NKA inhibition) and a competitive inhibition between copper and sodium [46]. Besides these direct mechanisms of action of copper, however, other osmoregulatory processes can be indirectly affected by copper, resulting in the perturbation of the plasma sodium homeostasis. A limiting factor for the transport of sodium through the apical membrane is the availability of protons, which are required by Na^+/H^+ exchangers and also, indirectly, by the sodium channels, which rely on the hyperpolarization of the transmembrane electrochemical gradient maintained by the apical proton pump. The cellular substrate necessary for these mechanisms is fuelled by the hydration of carbon dioxide, a reaction catalysed by intracellular CA (Figure 5.3). Therefore, the inhibition of CA, as a result of copper exposure, could indirectly perturbate sodium homeostasis by depleting the mechanisms of apical sodium uptake of their cellular substrates [36]. Indeed, the link between CA and sodium uptake is illustrated by pharmacological studies, where the pharmacological inhibition of CA resulted in inhibited sodium uptake [47].

In contrast with most of the classical enzymes directly involved in osmoregulation, represented by either transmembrane proteins or ion-transporters that actively or passively transport ions across the cell membrane, CA participates in osmoregulation indirectly, by fuelling the ion transport mechanisms across the cellular membrane. This indirect role of the enzyme in controlling ion homeostasis implies that its activation, e.g. in response to osmotic stress, or inhibition, e.g. by metal exposure, can trigger a cascade of multiple effects, involving different cellular processes and functions that may be partially overlapping and generating an overall complex pattern of response.

5.2. Computational and phylogenetic analysis of fish CAs

5.2.1. Aims

In the context of the theoretical framework discussed in Chapter Two and the experimental work described in Chapter Four, this study is focused on those CA isoforms that are involved in ion transport mechanisms and whose impairment by copper exposure could underlie the osmoregulatory response of fish to osmotic stress.

According to Grosell and co-workers [27], the two main isoforms involved in fish (i.e. trout) adaptation to different salinities are the membrane-bound isoform CA4 and the cytosolic isoform CAc, also defined as CA2. Given that CA4 apparently explains only 30% of the total CA activity observed by Grosell in response to a salinity change, whilst the majority of the activity is displayed by CA2, it is appropriate to focus on the cytosolic isoform CA2, whose gene expression, activity and/or quantification were therefore selected as the main endpoints of the exposure study.

In this context and *before* the start of the *in vivo* exposure experiments, the first questions that had to be answered were:

- Is there an available full and/or partial sequence of CA2 for my test species, sheepshead minnow (*Cyprinodon variegatus*)?
- If yes, how similar is sheepshead minnow CA2 to either other CA isoforms or to CA2 isoforms of different species or taxa?

Answering these questions had both practical and theoretical implications. From the practical side, the identification of a sheepshead minnow putative sequence of CA2 was the starting point for designing the primers to use for the gene expression analysis, as outlined in Section 4.2.5.2 of Chapter Four. From a more theoretical standpoint, a phylogenetic analysis of CAs across different taxa could help in understanding the sub-functionalization and level of differentiation of CA enzymes, as discussed in the Introduction of this chapter. More specifically, the aim was to verify whether the phylogenetic analysis supported the argument that fish orthologues of CA are highly different from mammalian CA isoforms, and if fish CAs are indeed so highly differentiated, both in terms of function and cellular localization.

A third implication of the bioinformatics analyses on CA regarded the interpretation of the results, *after* performing the experiments and the gene expression analysis. In particular, according to the data presented in Chapter Four, the CA2 measured in the intestine samples appeared to behave differently from its branchial counterpart, both in terms of basal level of expression (which was lower in the intestine than in the gills) and of response pattern to copper and osmotic stress, particularly in the saltwater groups. For instance, CA2 expression in intestine samples of the saltwater groups displayed a pattern of down-regulation in response to copper exposure, in contrast with the general up-regulation observed in the gills of both groups and in the intestine of the freshwater groups (sees Figure 4.11 D). How to interpret the different trend displayed by those data has proved challenging, especially in the absence of further data on other factors involved in the regulation of the observed interaction between copper and osmotic stress. One possible explanation is that the regulation of osmotic effector proteins such as CA2 and NKA (among others) is tissue-specific, and therefore their transcriptional response to an osmotic stress is different. Alternatively, the observed up-regulation of CA2 in the gills as a result of copper exposure may be a form of compensatory response to a copper-inhibited activity of the enzyme that might have occurred in the gills, but not in the intestine. These and other possible mechanistic explanations can be found to those results; however, the observed tissue-specific and condition-specific response displayed by CA2 also offers fascinating evidence supporting the argument of high specificity and differentiation of CA enzymes. Following this thinking, a comparative analysis of CA across different species was performed using various bioinformatics tools.

5.2.2. Methods

5.2.2.1. Sequence alignments and database screening

The following online NCBI databases (http://www.ncbi.nlm.nih.gov/) and tools were used to collect information on CA genes and proteins:

- nucleotide, protein, expressed sequence tags (ESTs) databases;
- nucleotide basic local alignment search tool (BLAST);
- BLASTn (search nucleotide database using a nucleotide query);

- BLASTx (search protein database using a translated nucleotide query);
- tBLASTx (search translated nucleotide database using a translated nucleotide query);
- tBLASTn (search translated nucleotide database using a protein query).

Nucleotide and aminoacid sequences were analysed for similarity to other CA sequences from other species using the software ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

5.2.2.2. Phylogenetic analysis

Phylogenetic trees were constructed using the software ClustalX, using as source data the multiple sequence alignment file obtained by ClustalW2, and applying the neighbour-joining (NJ) method. Trees were visualized using the software NJplot [48] and the results were presented in the form of rectangular trees.

5.2.3. Results and discussion

5.2.3.1. Sequence alignments and database screening

The screening of NCBI databases provided both full length and partial mRNA sequences coding for several isoforms of CA in human, house mouse, frog, zebrafish (see Table 5.1 for database accession numbers), and other teleost fish, including Atlantic salmon (*Salmo salar;* CA1 and CA12), rainbow trout (*Oncorhynchus mykiss;* CA1, CA2, CA4a and CA8) and channel catfish (*Ictalurus punctatus*; CA1 and CA7). No full mRNA sequences were available for sheepshead minnow (*Cyprinodon variegatus*).

However, the screening of NCBI database by both basic local alignment search tool (BLAST search, NCBI) and word search led to the identification of one partial mRNA sequence of sheepshead minnow putative cytosolic carbonic anhydrase CA2 (GenBank Acc. No. HM142344.1), which showed 81% similarity with the 5' region of EST of CA2 expressed in fathead minnow (Acc. No. DT261041.1).

Table 5.1. Accession numbers of CA isoforms used for sequence alligments and phylogenetic analysis.

	Human (*Homo sapiens*)	House mouse (*Mus musculus*)	Frog (*Xenopus tropicalis*)	Zebrafish (*Danio rerio*)
CA1	M33987.1	BC110681.1	BC118911.1	NM_131110.1
CA2	J03037.1	BC055291.1	BC160798.1	NM_199215.1
CA3	NM_005181.3	M27796.1		
CA4a	BC057792.1	BC012704.1		NM_001114407.1
CA4b				NM_001166211.1
CA5a	BC137405.1	BC030174.1		BC153994.1
CA5b		BC034413.1	NM_001045690.1	
CA6	M57892.1	BC055437.1	BC121907.1	
CA7	AY075019.1	BC094913.1	NM_001015903.1	NM_200813.1
CA8	BC069744.1	BC010773.1	NM_001011213.1	NM_001017571.1
CA9	NM_001216.2	BC120546.1	XM_002944046.1	
CA10	BC047456.1	BC066111.1		NM_001037121.1
CA11	BT007265.1	BC019393.1		
CA12	BT006656.1	BC031385.1	NM_001016431.2	
CA13	BC052602.1	BC064050.1	NM_001078980.1	
CA14	BT020054.1	BC046995.1	NM_001110051.1	NM_001037693.1
CA15a		BC019975.1	BC123069.1	NM_001081689.1
CA15b				NM_213182.1
CA15c				NM_001077333.1

To verify that fathead minnow 5'EST was indeed a CA2, the sequence was used in a basic local alignment search tool using a nucleotide query (BLASTn, NCBI) and identified the CA2 sequence of zebrafish, showing that fathead minnow 5'EST fell in the 5' region of zebrafish CA2 with 80% similarity (Table 5.2). Sheepshead minnow partial sequence of CA2 was then aligned with zebrafish CA2 and fathead minnow EST (Figure 5.4) and the most conserved regions were used to design the primers for gene expression analysis.

Table 5.2. Percentage similarity scores of sheepshead minnow CA2 sequence vs other species of fish.

Species	Scores
Zebrafish	82
Rainbow trout	80
Gulf toadfish	80
Mozambique tilapia	78
Common carp	76
Fathead minnow (clone)	81

```
Danio_rerio_CA2              TGGAGGCCCGGTCACAGGCACATTCAGACTCAAACAGTTTCACTTCCACT 321
Cyprinodon_variegatus_CA2    ---------------------------------------------------
Pimephales_promelas_clone    AGAAGGCCCGGTCTCAGGTAAATACAGGCTCAAACAGCTTCACTTCCACT 350


Danio_rerio_CA2              GGGGATCTGCAGACGACAAGGGCTCCGAACACACGGTCAATGGCAAATGT 371
Cyprinodon_variegatus_CA2    -------------CGGCA------------------TCAG--------GT 11
Pimephales_promelas_clone    GGGGGGCCAGTGACGACAAAGGCTCCGAGCACACAGTCGATGGGAAATGC 400
                                        ** **                 **          *

Danio_rerio_CA2              TACCCAGCCGAGCTCCATCTGGTCCACTGGAACACTAAATATCCCAGTTT 421
Cyprinodon_variegatus_CA2    T-CCCATGTGAGCTCCACCTGGTCCACTGGAACACCAAGTACCCCAGCTT 60
Pimephales_promelas_clone    TATCCAGCCGAGCTCCATCTGGTCCACTGGAACACAAAATATCCCAGCTT 450
                             *  ***   ******** ***************** ** ** ***** **

Danio_rerio_CA2              TAAGGACGCAGTTGATAAGCCTGATGGTCTTGCTGTGGTTGGGATTTTTC 471
Cyprinodon_variegatus_CA2    CGGGGAGGCGGCCGATAAGCCCGACGGACTGGCTGTGGTGGGGATCTTCC 110
Pimephales_promelas_clone    TGGGGAAGCAGCCGATAAGCCTGATGGTCTTGCTGTGGTTGGAGTTTTTC 500
                                *** ** *  ******** ** ** ** ******** **  * ** *

Danio_rerio_CA2              TGAAGATTGGTGCAGACAACCCTAAACTCCAGAAAATTCTGGATGCTATG 521
Cyprinodon_variegatus_CA2    TGAAGATCGGTGCAGCCAACCCCCGCCTCCAGAAGGTTCTGGATGCTTTG 160
Pimephales_promelas_clone    TGACGATCGGTGAAGACAATCCTAAGCTTCAGAAGGTTCTGGATGCTATG 550
                             *** *** **** ** *** **    ** *****  *********** **

Danio_rerio_CA2              GATGCTATCAAGTCCAAGGGAAAGCAAACCCCCTTCCCAAACTTTGACCC 571
Cyprinodon_variegatus_CA2    GACGCCATTAAGACCAAGGGGAAGCAGACCACCTTCTCCAACTTTGATGC 210
Pimephales_promelas_clone    GATGCTATCAAGTCCAAGGGAAAGCAGACCTCATTCACAAACTTTGACCC 600
                             ** ** ** *** ******* ***** *** * *** * ********  *

Danio_rerio_CA2              AAGTGTGTTGCTGCCCAGTTCTCTGGACTACTGGACATACCTGGGCTCTC 621
Cyprinodon_variegatus_CA2    AAG-------------AACTCTC--------------------------- 220
Pimephales_promelas_clone    AACCTGCCTGCTCCCGAAATCTCTGGAGTATTGGACGTACCCTGGGTCTC 650
                             **             *  ****
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Figure 5.4. CA2 sequences alignments - ClustalW alignment of the partial sequence of sheepshead minnow putative cytosolic CA2 against the sequences of CA2 in fathead minnow and zebrafish. Asterisks indicates identical nucleotide in all three species of fish.

5.2.3.2. Phylogenetic analysis

In order to broaden the range of comparison to other taxa, a phylogenetic analysis was performed, using the teleost fish sequences found on NCBI databases, along with sequences of human, frog, mouse and zebrafish CAs (Table 5.1). In particular, the intent was to compare different CA isoforms of different species to try and understand whether the level of similarity within species and across different isoforms was higher or lower than the similarity within isoforms across different species.

The analysis of the nucleotide sequences lead to the phylogenetic tree shown in Figure 5.5. It must be mentioned that the length of the analysed sequences was not the same in all the species: some sequences, such as zebrafish CA2, were particularly long and this might have affected the classification, with a consequent loss of evolutionary information. This is also the reason for not including the fathead minnow EST and the putative sheepshead minnow CA2 sequences, as their length would have been the driving factor affecting the clustering. However, the analysis did give a very good

classification of CA isoforms, with a clear dichotomy between extracellular and intracellular isoforms.

The first thing that can be noticed is the overall good clustering of similar isoforms and – even more so – of the six groups of CAs that have been classified on the basis of their subcellular localization, tissue distribution and catalytic activity. As listed in the Introduction of this chapter, these classes are:

- the cytosolic isozymes (CA1, CA2, CA3, CA7 and CA13),
- membrane-bound isozymes (CA4, CA9, CA12, CA14 and CA15),
- mitochondrial isozymes (CA5A and CA5B),
- secreted isozymes (CA6),
- acatalytic isozymes (CA8, CA10 and CA11), also called carbonic anhydrase-related proteins (CA-RP).

Incidentally, a further observation that arises from this phylogenetic tree is that the analysis tended to discriminate (with few exceptions) between transmembrane (CA9, CA14 and CA12) and membrane-bound isoforms (CA4 and CA15). This might be explained by the fact that a defining characteristic of the latter is the presence of a GPI (glycosylphosphatidylinositol) anchorage to the plasma membrane, a feature that is exploited to selectively separate membrane-bound enzymes from cytosolic enzymes [26]. The presence of a sequence region coding for this anchorage could have contributed to the observed different clustering.

More in general, the main message that emerged from this phylogenetic analysis is the clustering of similar isoforms and groups of CAs of different species, showing that the cellular localization and catalytic activity of the enzymes have a stronger impact on CAs classification than the species and/or taxon specificity. This observation is in line with the hypothesis that some features and functions of CAs are highly conserved across species. This characteristic of CA enzymes might also have applicative implications, as addressed later in this chapter (Section 5.4).

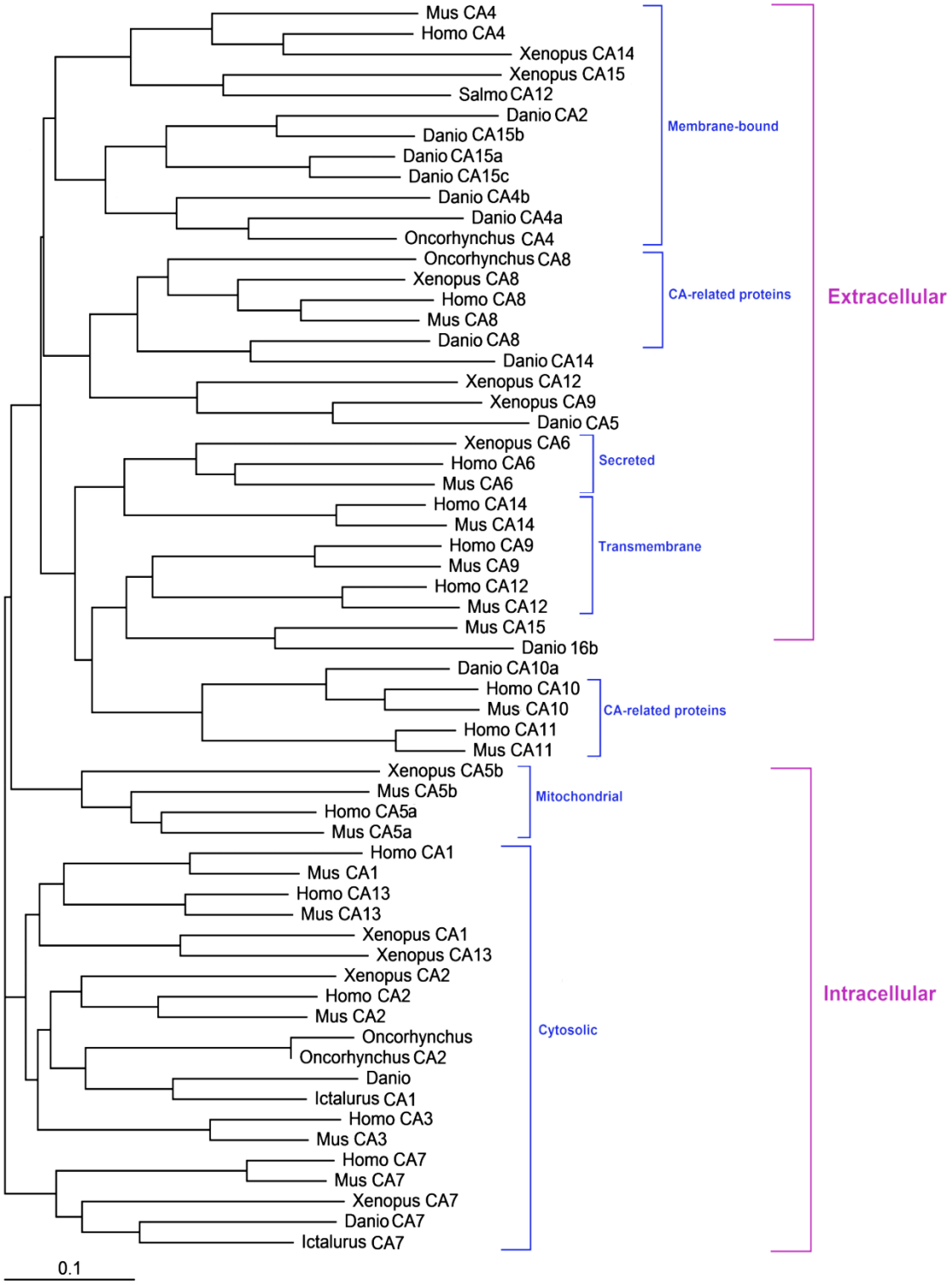

Figure 5.5. Phylogenetic tree of the genes coding for different isoforms of CA in human (abbreviated as "Homo" in the tree), house mouse (Mus), frog (Xenopus), zebrafish (Danio), trout (Oncorhynchus), salmon (Salmo) and catfish (Ictalurus). The tree was generated using ClustalX. Database accession numbers are provided in Table 4.1. CA genes were classified into two main categories, intracellular and extracellular isoforms.

Additionally, the importance of cellular and tissue distribution of CA isoforms that has been shown by the phylogenetic analysis can also help interpreting the gene expression results of the copper exposure experiments, and particularly those regarding CA2 expression in the intestine of saltwater-acclimated fish.

According to the literature, an important role played by the intestine in the acclimation to higher salinities is a net bicarbonate secretion, which leads to rectal base excretion in hyperosmotic environments and ultimately contributes to the whole animal acid-base balance [27, 36]. This system involves several transmembrane transporters, including the branchial Cl^-/HCO_3^- exchanger, whose provision of bicarbonate ions relies on the activity of the cytosolic CA2 [43, 44]. Another important mechanism contributing to the net base excretion across the intestinal epithelium is the formation of $CaCO_3$ precipitates in the intestinal lumen of saltwater-acclimated fish. Similarly to the bicarbonate excretion system, this biomineralization system also seems to involve CA activity, as seen in many other biomineralization processes, such as bone development [49] and shell deposition [50].

Given the high degree of tissue-specificity and sub-functionalization of CA enzymes, as supported by the phylogenetic analysis, and considering the functional difference between intestinal epithelia of freshwater and marine fish [23], it cannot be ruled out that marine fish might have evolved an intestinal isoform of cytosolic CA specialized in bicarbonate excretion, somehow functionally different from its branchial equivalent and resembling its human orthologue, which plays a role in biomineralization processes. This hypothesis could provide an explanation for the lower affinity displayed by the primers in the intestine samples, resulting in seemingly lower expression levels. But more importantly, the hypothetical presence of a sub-isoform of cytosolic CA2 in the intestine of marine fish could also help in interpreting the different trend of downregulation in response to copper exposure displayed by CA in the intestine of the saltwater groups (Figure 4.11 in the Results section of Chapter Four). Since copper exposure can cause increased intracellular bicarbonate concentrations, ultimately resulting in acid-base imbalance [41], it is plausible – at least theoretically – that an enzyme specialized in fuelling intestinal base excretion by converting CO_2 into bicarbonates (and protons) would be “shut down” by the cellular system when faced

with a chemical stress, such as copper, that induces an increase in intracellular bicarbonate concentrations.

It would be interesting to follow up experimentally this theoretical idea, starting with a full sequencing of the CA2 gene in both the branchial and intestinal samples of sheepshead minnow, followed by alignments with human CA orthologues. However, this would have gone beyond the scope of this study. Instead, another aspect that I wanted to investigate using the bioinformatics was the transcriptional control of fish CA2, particularly in regard to osmotic stress response pathways.

5.3. Promoter analysis of zebrafish CA2

5.3.1. Aim

When discussing the experimental work presented in Chapter Four, it was hypothesized that the interaction between copper and osmotic stress is a form of mechanistic interaction taking place (also, but not necessarily only) at transcriptional level. Moreover, it was argued that this interaction is indirect, rather than direct, because it seems unlikely that the expression of such a ubiquitous enzyme could be under a *direct* transcriptional control of a metal like copper, as it would expose the cellular system to a risky toxicological modulation of a key biological factor. Instead, it seems more plausible that copper affects CA2 expression not by binding a metal-responsive element (MRE) upstream of the gene coding for CA2, as it does for example in the case of the genes coding for metallothionein [51], but rather through the activation of osmotic-stress related factors, which in turn regulate CA2 transcription. Following this reasoning, the promoter region of zebrafish CA2 was searched for osmotic-stress related elements, since the full genome of sheepshead minnow is not available. In synthesis, the questions to address were:

- Does the enhancer region of CA2 contain any transcription element suggesting the involvement of this gene in osmotic stress response pathways?
- How these elements and the corresponding binding proteins correlate to other transcription factors controlling osmoprotective genes in other taxa?

5.3.2. Methods

Using 1kb of the known 5' UTR of zebrafish CA2 (GenBank Accession no. NM_199215.1), a computational search for transcription factor binding sites was performed, using the public version of TFSearch (http://www.cbrc.jp/research/db/TFSEARCH.html) [52] with all the available matrices and applying a threshold score up to 85.

5.3.3. Results and discussion

The computational analysis of the 5' UTR of zebrafish CA2 revealed the presence of several putative transcriptional factor binding sites (Figure 5.6). Among them, 10 repeats of the stress-responsive-element STRE (CCCCT or AGGGG), and 4 repeats of the tonicity-responsive element TonE (TGGAAA) were identified.

```
AACTATTATGTTGAGAAATGTGTCTAAAAAATCTTCTTTCCGTTAAACATAAACTGGGAG
                STRE                STRE
AGAAAAAAAATAAACAGGGGGCTAATAATTCAGGGGGTTTAATAACTCTGAGTCATGTAC -2089
ATATTTATATAGTGCATTCATTGTGTATGGCCATATCAATCATGAGAGGCGGGTCTCCCC
ACTCCACCACCAGTATGCAGCATCCACTTGAAGGATGAGACGGCAGCCACAGGAAACGGC
ACTATAGTGCACTCACCACACACCAATAGATGGAGTGGAGAGACAGTGATAAAGCCATTT
CGGTAGATGGGGATGTTTGGGAGGCCATAATGGGTGAGGGACGATGAAGACAATTTGGCC
AGGACACCAGGGTTATACCCCCACTCTTGTGTAAGGGTAACCCTGAGCTCCATCTTTTGT
GGAGAGAGAAGAAACTTACAGAAGAACAACCATGAATTTAACCAACCATGAAGTAATGAA
                                    TonE
TTTAAACCATTTTGGATTAAGGCTGTAACATGAACAAATGTGGAAAAAGTGAAGCGCTAT -1669
GAATACTTTCTGGAAGCAAAATATATTATTAAAGTCTGTAATATATTTTTTATGTCATTT
TAAAAAATAATTTTTATAAAGTGGACAAAGAAATTGGTGAAAATCAGGCTTACAAATATA
```

Figure 5.6. Snapshot of the enhancer region of zebrafish CA2, analysed using TFSearch. Stress-responsive elements (STRE) and tonicity-responsive elements (TonE) are highlighted respectively in yellow and green. Negative numbers on the right represent the distance (in bp) from the start of the coding region.

The STRE motifs have been reported to be essential in gene expression responses to osmotic stress and heat shock in yeast [53]. They have been identified in the 5'-flaking region of a *Saccharomyces cerevisiae* heat shock protein (HSP12), which is activated by the high-osmolarity glycerol (HOG) response pathway, a circuit triggered by hyperosmotic stress and involving a number of mitogen-activated protein kinases (MAPK) [54]. Similar stress response pathways to those involving the STRE

transcription factor have also been shown to be activated by osmotic stress in fish and plants. In fish, MAPK are key players during osmotic stress responses [55], regulating cell proliferation, growth and differentiation. The MAPK signalling pathway influences the activity of the osmotic stress transcription factor 1 (OSTF1), a transcriptional regulator that is transiently induced in fish gill cells exposed to hyperosmotic stress [55, 56]. Considering the role of MAPK in regulating cell turnover and the function of OSTF1 in osmotic stress response, it is likely that exposure to acute hyperosmotic stress, such as abrupt transfer to higher salinities, may involve the activation of pathways controlling cell cycle and cell differentiation, i.e. the proliferation of ionocytes in branchial epithelia of fish acclimated to saltwater.

In plants and more specifically in *Arabidopsis*, it has been demonstrated that there is an osmotic stress MAPK cascade that suppresses salt-induced cell growth inhibition [57] in response to osmotic stress, drought and also to cold stress. The genes involved in this response are regulated by the C-repeat/dehydration responsive element binding factor (CBF1), which is up-regulated during hyper-osmotic stress in plant cells [58].

The other identified motif, TonE, is the binding site of the tonicity-responsive element binding protein TonE-BP, also called ORE-BP or NFAT5, an important transcription factor responsible for regulating osmolyte transporters genes in mammalian cells during hyperosmotic stress [58]. Its osmoprotective function has been investigated both *in vitro* and *in vivo*, where ORE-BP has been demonstrated to be essential for urine concentration in renal epithelia [59]. The ORE binding protein has been identified also in fish (i.e. rainbow trout, *Oncorhynchus mykiss*, and mummichog, *Fundulus heteroclitus*), where it shows an interesting bi-directional shuttling between the nucleus and the cytoplasm and a dual activation by both hypo- and hyper-osmotic stress [60].

The remarkable conservation of the factors controlling osmoprotective genes and, in general, of the osmoregulatory responses across all biological kingdoms is perhaps not surprising, if we consider their importance in maintaining cell homeostasis for any biological system. For the same reason, however, it is plausible to expect that many genes involved in ionoregulation are related to osmoregulatory response pathways, which does not necessarily imply that they have a specific osmoprotective function. Equally, the presence in the genome of sequences coding for osmosensitive proteins does not imply that the osmotic stress response pathway in its whole, including

osmosensing and signal transduction circuits, is conserved and functioning in that particular species. Stenohaline fish, for example, possess the genes encoding for most of the transducers and effector proteins, but have lost the ability to sense and transduce osmotic cues and hence to actively modulate osmotic effector proteins [61].

In this context, although the performed promoter analysis provided some interesting evidence supporting a control of CA2 by the osmotic stress response pathway, further and more detailed analysis of the enhancer region not only of zebrafish but also of sheepshead minnow CA2 should be performed before coming to any conclusion about the regulatory circuits controlling the transcription of CA2 in relation to osmotic stress. In particular, it would be interesting to clone an extended 5'-flaking region of the sheepshead minnow CA2 gene in order to identify other elements regulating this multifunctional gene. Furthermore, its role in osmotic stress responses could be further assessed by disrupting its coding gene and testing its expression under varying osmotic conditions. However, this would have represented a whole new project of its own.

Having tried to understand first how fish CA2 correlated to other isoforms of CA in other taxa, and second what the upstream region of the gene could teach us about the transcriptional control of this enzyme, I then went on looking "downstream", i.e. at the protein level.

5.4. CA2 quantification in fish samples

5.4.1. Aims

Going back to the results of the two *in vivo* experiments, the analyses of gene expression levels of CA2 revealed that this enzyme responded to *in vivo* copper exposure both in freshwater and in saltwater and both in branchial and intestinal epithelia, demonstrating that CA2 gene expression could be used as endpoint for *in vivo* toxicity studies on euryhaline fish.

As illustrated in Section 5.1.3, so far the majority of ecotoxicology studies that have tested the effects of metals on aquatic organisms using CA enzymes as targets have been performed *in vitro*, rather than *in vivo*, and almost exclusively using enzymatic activity as endpoint. Despite its informative power, enzymatic activity does not provide a comprehensive picture of the molecular mechanisms behind an observed apical

response, whilst for this purpose gene expression is a more powerful endpoint. On the other hand though, in some cases gene expression in itself can fail to match apical biological effects and provide conclusive and reproducible results, stressing the need for integrating both endpoints in order to try and reconstruct the mechanisms of action of a tested chemical. So ideally both gene expression and enzymatic activity should be tested, which is what was decided at the beginning of the exposure study.

However, after a more thorough reading of the literature it emerged that the enzymatic assay commonly used to measure CA activity (determined using an electrometric delta pH method [62]) could underestimate metal inhibitory effect, since the dilution of the samples required by the assay can lead to the release of the metal ion from the protein molecule and therefore to false negative results [36].

In light of these and other more practical reasons (e.g. expertise available, costs and time) it was decided to measure instead CA2 protein abundance, using the commercially available human CA2 enzyme-linked immunosorbent assay (ELISA; Uscn Life Science). This assay has been developed and optimized for human samples, whilst it has never been used before with fish tissues. However, the 80% similarity between nucleotide sequences of fish and human CA2 demonstrated by the previously described sequence alignments meant that theoretically there were 80% chances that the kit would have worked on fish samples too.

The validation of the ELISA kit required before assaying the samples from the *in vivo* study represented an opportunity to develop an easy-to-use tool to assess physiological responses involving CA2, thereby overcoming the analytical issues related to the enzymatic assay and paving the way toward the development of a new copper biomarker. Furthermore, the validation of a human assay on fish samples represented an excellent example of how comparative approaches can be put into practise.

5.4.2. Materials and methods

5.4.2.1. *Sample preparation*

For the validation of the CA2 kit, samples of gills and intestine tissues from the first *in vivo* experiment were used. Once the kit was tested and validated for fish tissues, the analysis was performed on gills and intestine samples from the second *in vivo*

experiment. Where the individual amount of tissue was not sufficient to perform the analysis, samples within treatment were pooled together.

Gills and intestine samples (mean tissue weight, respectively, 24.1 ± 8 and 30.7 ± 9 mg) were homogenized using a TissueLyser II (Qiagen) for 3 min at maximum speed after adding appropriate volume of chilled 0.01 M buffer solution prepared using PBS (phosphate buffered saline) tablets (Fisher Bioreagents), buffered at pH 7.4. The homogenate was centrifuged at 14 000 *g* for 20 min at 4°C to pellet nuclei and large cellular fragments. Approximately 100 µl of supernatant was transferred to 0.5 ml tubes and stored at – 20 °C for CA2 ELISA assay, whereas 5 µl was transferred to 0.2 ml tubes, with the addition of 35 µl PBS buffer (dilution factor 1:8) and used for total protein quantification.

5.4.2.2. Total protein quantification

Protein concentrations were determined with Quick Start Bradford Protein Assay Kit (Bio-Rad) [63], according to the manufacturer's instructions, using bovine serum albumin (BSA) as a standard. Total protein content of homogenates was then used to normalize sample measurements of CA2 protein levels.

5.4.2.3. ELISA for CA2

Homogenized tissue samples were diluted using PBS buffer and then assayed directly on the CA2 enzyme-linked immunosorbent assay (ELISA) kit (Cat. Number SEA782Hu, Uscn Life Science), following manufacturer's instruction. Briefly, 100 µl of standards and samples were added to the microtiter plate wells, which contained a biotin-conjugated antibody specific to CA2. After 2 hours of incubation, Avidin conjugated to Horseradish Peroxidase (HRP) was added to each microplate well and incubated for 1 hour at 37 ºC. After the addition of a substrate solution, only those wells that contained CA2, biotin-conjugated antibody and enzyme-conjugated Avidin would exhibit a change in colour. The enzyme-substrate reaction was terminated by the addition of sulphuric acid solution and the colour change was measured spectrophotometrically at a wavelength of 450 nm.

5.4.2.4. Validation of CA2 assay

The CA2 ELISA kit was assayed for accuracy in two steps:

- Step 1: Assessment of fish samples concentration range and identification of the appropriate dilution factor;
- Step 2: Assessment of assay linearity, reproducibility and specificity.

For the preliminary step, 6 samples (3 gills and 3 intestines; average weight 0.064 ± 0.02 mg) of control fish from the first *in vivo* experiment were used; 0.01 M PBS buffer was added to each sample, applying 3 different dilution factors: 1:3, 1:5 and 1:10. Samples were then treated as described in Section 5.4.2.1 and assayed in duplicates on the ELISA kit. According to the results, a 1:5 dilution factor was selected as the most appropriate for the studied samples and it was therefore used for the following analyses.

In the second step of the validation, a dilution series (100%, 50%, 25% and 12.5%) of pooled samples of gill tissue was prepared by adding increasing amount of PBS buffer and then assayed using the kit. The results were plotted on the same graph as the standard curve to determine if the curves were parallel. The specificity of the assay was determined by standard additions using the standards provided with the kit. Measured and expected concentrations were plotted together to assess the fitting between predicted and measured data. The kit was also tested for intra-assay reproducibility by assaying 6 replicates of the same sample on a single assay kit. The coefficient of variation (CV) was then calculated among the 6 replicates.

5.4.2.5. Data analysis

Data are reported as means ± standard errors. Sample size for all treatments was 4–7 animals, since samples pooling was necessary, due to the limited amount of tissue available. For the same reason, measurement of each sample was made in duplicate for total protein quantification and singlet for CA2 quantification, after having assessed the accuracy of the method. Statistical analyses were conducted using SigmaStat software (version 3.5 Systat Software Inc.). Results were analyzed by one-way analysis of variance (ANOVA) followed by all pairwise comparison using Dunn's *post-hoc* test. Significant differences between pre- and post-salinity transition data were assessed by the Tukey *t*-test and values were considered significantly different at $P < 0.05$.

5.4.3. Results and discussion

5.4.3.1. Assay validation

The pooled sample used for the dilution series had a relatively low CA2 concentration (27.4 ng/ml): this meant that the points of the dilution series were outside the linearity range of the standard calibration curve (20-200 ng/mL), hence preventing the assessment of kit linearity (Figure 5.7).

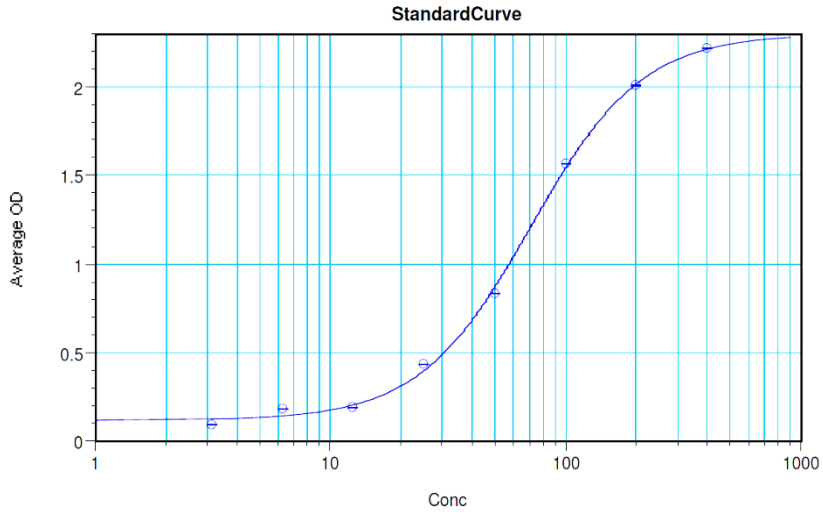

Figure 5.7. Four-parametric standard curve of the CA2 ELISA assay, reporting the average optical density (OD) measured at a wavelength of 450 nm versus the nominal standard concentrations of CA2. A non-linear tail can be noted below 20 ng/ml.

The method of the standard additions demonstrated the specificity of the kit, as shown by the good fitting of the curves reported in Figure 5.8. The coefficient of variation calculated among the 6 replicates was <10% (CV = 8.4%), demonstrating the good reproducibility of the assay.

As stated in Section 5.4.1., the first purpose of this work was to validate a commercially available rapid ELISA human CA2 kit for the quantification of CA2 in samples of fish tissues. For this scope, these data overall illustrated that human CA2 ELISA kit can be used to assay samples of fish branchial and intestinal tissues with high specificity and minimal intra-assay variation, although further analyses are required to complete the kit validation by testing the assay for linearity and inter-assay reproducibility. However, if CA2 has to be used as a copper target in future studies on fish, it is essential to integrate gene expression data with data on protein abundance in order to have a more comprehensive picture of the enzyme response to the stress.

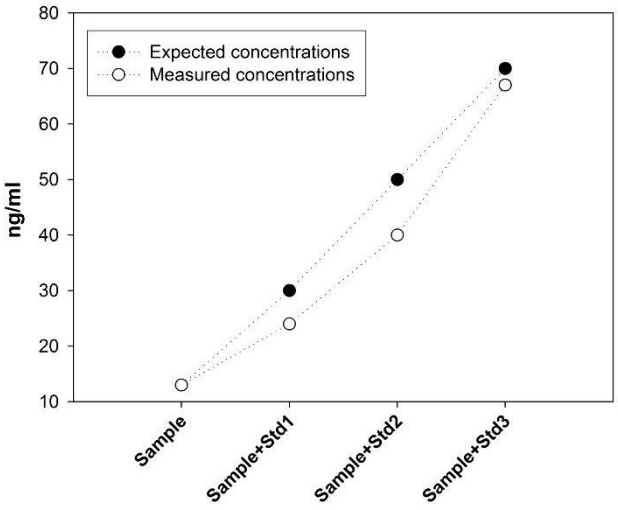

Figure 5.8. Standards additions: measured vs. expected concentrations - Protein concentrations (ng/ml) in a pooled sample spiked with increasing concentrations of standards. White dots represent the assayed concentrations and black dots the expected concentrations.

5.4.3.2. Quantification of CA2 levels in fish samples of Experiment 2

The second aim of this work was to use the kit to assay samples from the *in vivo* experiment, in order to complement the gene expression results with data on protein levels. In particular, the quantification of CA2 in the fish samples had two goals: firstly, linking the copper effect observed at mRNA level with a more downstream endpoint such as protein level; secondly and more importantly, understanding whether the timescale of the salinity change and the time-window chosen for the sampling could have affected the absence of a pre-post change effect on CA2 mRNA levels. The relevance of the time factor is due to the fact that gene regulation in response to a stress is often transient, unless the stress is acute/severe, reaching a maximum in the response around 12-24 hour post-stress, followed by a decrease until the system homeostasis is re-established. According to previous studies, CA2 is not an exception to this rule, as demonstrated by Grosell et al. [27]: it is on the basis of their work that 24 hours post salinity switch was selected as the most appropriate timescale. However, this choice was just theoretical and could have still been disproved by the experiment, especially if we consider all the possible variables that could have affected fish response to the salinity change: hence the interest in testing CA2 protein abundance. Unfortunately, the results of CA2 protein quantification could only provide partial answers to these questions.

The mean protein level of CA2 in samples of branchial tissue was 261.2 ± 94.6 ng/ml, which was within the standard range of the assay (3.12-400 ng/ml), whereas CA2 protein level in most of the samples of intestine tissue was below the standard range (3.12 ng/ml), considering the applied dilution factor. Therefore, no statistical analysis could be performed on this dataset. As for CA2 levels in gills samples, the statistical analysis did not show any significant difference between controls and copper-exposed samples. Also, no consistent pattern of response was observed before and after the salinity changes (Figure 5.9), if not a faint tendency of pre-post increase from FW to SW and decrease from SW to FW, confirming the results of CA2 gene expression. This result could be due to the fact that CA2 response to the osmotic stress was transient and occurred earlier than 24 hour post-switch. In this case, designing a time course experiment to assess the time-sensitivity of this endpoint would allow testing this interpretation. Alternatively, both gene expression and protein levels results are "true negatives" and can be explained by the rate at which the salinity switch was performed, which did not produce a stress of a degree sufficient to trigger the activation of the osmotic stress response pathway. This correlation between the degree of a stress applied to a biological system and the magnitude of the response will be further discussed in Chapter Six.

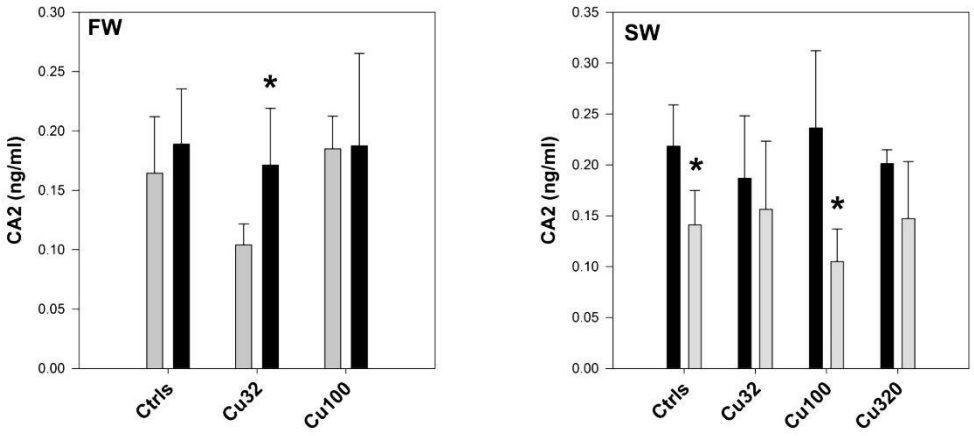

Figure 5.9. Mean normalized protein levels of CA2 measured in the branchial tissue of sheepshead minnows exposed to 0 (Ctrls), 32, 100 and 320 µg/L copper, respectively in FW (left graph) and saltwater (right graph). Within each copper treatment, the pair of bars represents CA2 protein levels before (left bars) and after (right bars) the salinity transition. The colour of the bars represents the salinity conditions: grey for FW and black for SW. CA2 protein levels were assayed using CA2 ELISA assay, adjusted to tissue weight, normalized to total protein levels and expressed as ng/mg tissue. Values are means ± SE (n = 4/7). The asterisk denotes statistically significant difference from the pre-switch vale ($P < 0.05$, Tukey t-test). No values are reported for the high copper FW dose as no fish survived after the change to SW.

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CHAPTER SIX

OSMOTIC STRESS RESPONSES IN THE CONTEXT OF STRESS RESPONSE DYNAMICS

"*This result is too beautiful to be false*" (Paul Dirac, 1963)*

In 1939, Paul Dirac observed that "*the physicist, in his study of natural phenomena, has two methods of making progress: experiment and observation, and mathematical reasoning. Although there is no logical reason why the second method should be possible, nevertheless it works, and to great effect*." The key, according to Dirac, was beauty, which he considered as a method to discover truth. Even though this approach based on mathematical beauty sometimes led him astray, it also resulted in predictions that were confirmed after his death.

The idea of beauty as method, though quite provocative for a scientist, may have some basis if we consider that humans have "learnt" beauty – as harmony, equilibrium and symmetry – from the natural world. It is not, therefore, an innate concept, a platonic form that we forcefully fit to nature, but rather a mental archetype induced by the observation of natural phenomena. Therefore, the apparently top-down approach of Dirac is in fact the second phase of a cognitive process started with a bottom-up acquisition of the idea of Beauty. Hence, its application in science is justified, although only as complement and not replacement to experiment and observation.

The beauty seen by Dirac in his mathematical equations is certainly not missing in the biological systems and their ways of functioning and responding to external stimuli. The apparently chaotic complexity of the signalling networks triggered by a stress in a cell, for example, reveals patterns of astonishing harmony, if looked at from a wider perspective.

In the end, seeing patterns is a tendency of all scientists, who on the other hand tend to be upset when reality doesn't fit with their expectations. If this occurs, however, it doesn't mean that beauty is lost. Instead, reality is telling them that something in their theory is missing, inaccurate or simply wrong. But if they are ready to dismiss their original ideas and embrace the unknowns and the apparent ugliness of a not fully shaped theory, even more beautiful places will be disclosed to them, because – at least according to Dirac's principle – the more fundamental and powerful the theory, the more beauty there is in it.

* *Dirac P (1963).The evolution of the Physicist's Picture of Nature*. Scientific American ***208****(5)*

6.1. Background

Recalling what discussed in Chapter Four (Section 4.4.), some of the results from the second *in vivo* experiment failed to reproduce what was observed in the first one. In brief, data from Experiment 1 showed that CA2 expression in the intestine of the controls was up-regulated following the salinity change, but this up-regulation was not detected in the low-copper treatment and was even "replaced" by a down-regulation in the high-copper treatment (Figure 6.1 A). This opposite pattern of response was considered as supportive evidence to the hypothesis that copper-exposed fish struggle to adapt to new salinity conditions because copper affects their osmoregulatory response to osmotic stress. However, contrary to what was observed in Experiment 1, the results from Experiment 2 did not show any significant effect of copper on the regulation of CA2 either in the gills or in the intestine (Figure 6.1 B).

Even taking into account the different statistical power of the two experiments (n = 6/7 in Experiment 1 and 10/12 in Experiment 2) this disagreement between the two datasets was still puzzling, especially considering that both the exposure conditions and the procedures used to analyse the samples were all the same. All except one, i.e. the rate of the salinity change, which is the factor I focused my attention on. The underlying questions were then why a different rate of salinity change should result in a different biological response? Is it possible to see the rate of change as a dose of osmotic stress and, if yes, is this dose of stress proportional to the magnitude of the biological response? Clearly, these questions had wider implications that the specific case of copper and salinity interactions and, symmetrically, answering to them required a step back and an observation of the issue from the perspective of systems biology.

Following this line of thinking, the two datasets were interpreted by regarding copper exposure and osmotic stress as a chemical and an environmental stresses that disrupt the homeostasis of the system, i.e. the fish. In this systemic context, the organism is expected to respond to the perturbation of its homeostasis by activating responses that are of a magnitude appropriate to the degree of perturbation, i.e. level of stress. We should therefore expect that different levels of osmotic stress elicit different magnitudes of response, since osmoregulatory mechanisms are finely modulated according to varying degrees of osmotic stress.

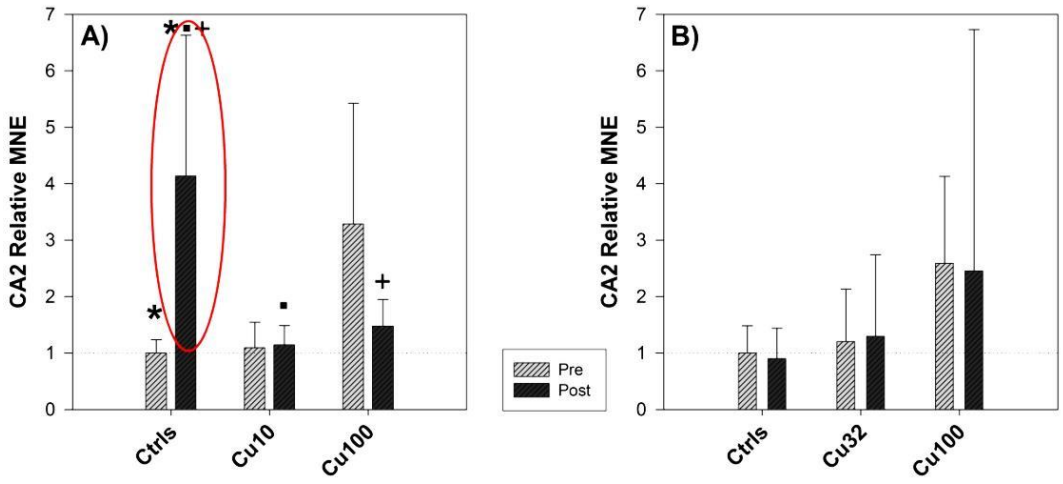

Figure 6.1. Pre-post change in CA2 expression – Exp.1 versus Exp.2. Relative Mean Normalized Expression (MNE) levels of CA2 measured in the intestine of sheepshead minnows exposed respectively to 0 (Ctrls), 10 and 100 µg/L copper (A) and to 0, 32 and 100 µg/L copper (B). Within each copper treatment, the pair of bars represents expression levels before and after the salinity transition. Relative MNE levels were determined by qPCR, normalized to control gene (18S) and expressed as fold change relative to pre-switch control value, which was set at 1. Values are means ± SE (n = 6/7 in A and 10/12 in B). Bars sharing the same symbol (asterisk, cross or dot) are significantly different one from the other ($P < 0.05$, Tukey t-test).

In this context, the different rate at which the salinity transition was performed led to the activation of different transcriptional responses. Additionally, the responses of some endpoints at high copper doses differed from those displayed at low and mid doses, suggesting that a threshold of different pathways activation was passed, as the dose of chemical stress applied went from mild to severe.

This interpretation of the data related to a different way of viewing and analysing the combined effects of chemical and non-chemical stressors, along with the corresponding pattern of response in biological systems: the so-called stress response dynamics. In the following sections, some key aspects of stress response systems are described, using cancer mode of actions as an example, and then applied to the case of osmotic stress responses in fish. What is discussed in this Chapter represents the main theoretical outcome of this research project, because it would not apply only to the specific case of copper toxicity and osmotic stress in fish, but could also be extended and used as a mind-set to interpret virtually any case where a stressor interact with a biological system. It is, in other words, potentially universal.

6.2. Stress response dynamics

6.2.1. Core stress response versus stress-specific response

It is from the universality of the stress response processes that arises their high biological relevance, since every living organism needs to sense and respond to external conditions that perturbate its homeostasis; otherwise, in the absence of an appropriate response, external stressors can damage or kill it. To minimize stress-related damage, organisms have evolved systems to detect and respond to stress, both at cellular and at systemic level. Most of the studies performed on stress-response processes have focused on unicellular organisms, where the responses are relatively easier to interpret [1, 2]. However, when analysed from a global standpoint, some common features in the architecture of stress response dynamics, both in unicellular and in multicellular organisms, start to emerge. Probably the most relevant of these shared features is a dichotomy between stress-specific versus stress-aspecific sets of responses, the formers being generally more represented in complex organisms and the latter more developed in unicellular organisms.

6.2.1.1. *Unicellular organisms and core stress response*

Unicellular organisms, particularly those which are free living and not hosted by another organism, are most exposed to external stressors and have therefore evolved both specific and global stress response programs that display considerable sophistication and fine tuning [3]. The global stress response shown by unicellular organisms such as the yeast *Saccharomyces cerevisiae* is common to most stress conditions and involves genes whose expression is regulated in a stereotypical manner [1]. This so-called core stress response is characterized by the induction of a typical set of stress-related genes, accompanied by the suppression of growth-related genes [4], the expression of the former being more intrinsically variable, stochastic and extensively regulated than that of the latter [3]. One of the physiological implications of this general stress response is the phenomenon of cross-protection, by which cells treated to low levels of one stress become more resistant to another stress [5]. This represents a great evolutionary advantage as it can cross protect the organism against multiple environmental stresses.

6.2.1.2. Multicellular organisms and stress response pathways

In cells of multicellular organisms, the core stress response involves the activation of a smaller set of genes, compared to unicellular organisms, and growth-related genes appear to be only weakly downregulated in response to environmental stresses [6]. For example, in human cell lines exposed to different types of stress, the proportion of gene sets that are commonly activated by all stresses is much smaller than in yeast, whereas most of the observed responses at gene level are stress-specific [7]. This is in line with the fact that individual cells in a multicellular organism are normally not subjected to the variety of environmental conditions that unicellular organisms are exposed to. Instead, complex organisms have evolved more sophisticated and systemic mechanisms of stress response to buffer and minimize environmental variations within the organism.

However, even in multicellular organisms there appears to be a limited number of core stress response pathways activated by cells in response to external stimuli, both environmental and chemical, in order to maintain cellular homeostasis and make specific cell fate decisions [5]. These stress response pathways (e.g. oxidative stress, DNA-damage and hypoxia responses) share a common architecture, based essentially on three tires:

- Signal sensors
- Signal transducers
- Effectors.

Each pathway level is in turn made up of a core set of functional regulatory motifs [8], including negative or positive feedbacks and coherent or incoherent feed-forward loops, and the combined activation of these motifs generates complex regulatory circuits. Stress response pathways can also cross-talk with one another, particularly under exposure to multiple stresses. For example, inflammation induces stress responses, but chronic sources of stress, such as oxidative stress and heat shock, also induce inflammation, and both these stresses can cause DNA damage or other types of cellular damage. Alongside these core stress response pathways, multicellular organisms also display specific endogenous pathways, making the overall organism response to stress highly complex and diverse [9].

Considering only the core stress response pathways, their conserved architecture appears to match conserved sets of response patterns commeasured to the level of stress that caused the pathway activation. So, discrete levels of stress elicit discrete degrees of pathway perturbation across the different levels of biological organization, starting from the perturbation of basal functions and the upregulation of adaptive/homeostatic gene sets up to occurrence of phenotypical adverse effects. To illustrate this concept we can consider the p53-mediated perturbation of DNA damage response pathway.

6.2.1.3. Cancer mode of action and DNA damage pathways

Carcinogenicity is commonly believed to result from a progressive accumulation of mutations and epimutations that select for a growth/survival advantage in affected cells. The two major categories of carcinogens are genotoxic carcinogens and non-genotoxic carcinogens. Genotoxic carcinogens are usually electrophiles that can directly react with DNA and thus initiate the neoplastic transformation of a normal cell, whilst non-genotoxic carcinogens alter DNA indirectly by acting on pathways associated with the modulation of reactive oxygen species (ROS), cell proliferation and apoptosis, among others [10]. From a stress response perspective, these two categories of carcinogens exemplify the dichotomy between specific versus aspecific stresses, as ontologically different stressors that elicit phenotypically similar cellular responses. To simplify, the homology of the response they activate allows unifying these stressors under the category of DNA damage/repair pathway perturbators, regardless of their source or nature. Within the organism, however, the degree of perturbation has a profound effect not only on the magnitude, but also on the type of response triggered in the system, as exemplified by the pathways activated downstream DNA damage.

Under normal conditions, DNA damage activates DNA repair pathways, but epigenetic mutations and/or DNA repair deficiency can cause more DNA damage to accumulate and lead to increased mutation frequency and hence cancer progression. In mammalian cells, cellular stress and DNA damage activate the tumour suppressor p53, which transcriptionally regulates several key pathways that cooperatively suppress cell proliferation [11]. The slowdown or blockade of cell proliferation reduces the probability of mutation, because DNA replication is almost always required for DNA damage to become a heritable mutation. Therefore, even though the tendency to mutate

increases as a result of increased DNA damage, if cell proliferation rate is also reduced accordingly, the overall mutation frequency may remain unchanged. However, if the degree of DNA damage in the cell is too severe to be effectively repaired, strongly activated p53 triggers the apoptosis pathway to eliminate the cell, which could otherwise mutate at a potentially dangerous high frequency [12]. Therefore, to understand the changes in mutation frequency it is necessary to examine the degree of activation of the pathways underlying DNA repair, cell cycle arrest, and apoptosis [9]. In stress-response terms, the higher the degree of DNA damage, the higher is the degree of activation of the signal transduction pathways regulating DNA repair and programmed cell death.

DNA damage can be caused by a number of factors, or agents, some of which are endogenous, such as the production of ROS, and some exogenous, such as radiation, viruses or genotoxins. The temporal and quantitative modulation of these stresses can lead to either an adequate repair function or an excessive perturbation that result in increased mutation frequency, or apoptosis, depending on the degree of pathway activation. This modulation of the responses to DNA damage is also time-dependent, as demonstrated by the dynamic feature of the p53 pathway [13]. Since activated p53 can upregulate murine double minute 2 (MDM2), a regulatory protein that in turn suppresses p53 activity [14], this negative feedback motif generates oscillatory behaviour of p53 and MDM2 in response to DNA damage. Prolonged oscillations can act as a signal for persistent damage, triggering the activation of the apoptotic pathway, whilst acute/short term oscillations can stimulate cell proliferation through p53 inhibition [15].

Another possible example of time-dependent pathway modulation is represented by the hypoxia stress pathway [16]. Cellular responses to hypoxia usually involve the transcription of hypoxia-induced genes such as cytokines, growth factors and angiogenic factors, leading to angiogenesis, cell proliferation and, if hypoxic conditions are prolonged, apoptosis. Hypoxia can also activate the transcription factor NFkB, which regulates the induction of p53 and so causes cell cycle arrest (cooperative interaction). On the other hand, NFkB can also suppress p53 activity by increasing the levels of MDM2, hence resulting in angiogenesis and cell proliferation (antagonistic interaction). Applying the concept of stress response dynamics to the case of NFkB and

p53, we can hypothesize that low levels of hypoxia activate the antagonistic circuit of MDM2-p53, whereas prolonged hypoxia triggers the cooperative, transcriptional interaction between NFKB and p53, one leading to cell proliferation and the other to cell cycle arrest. Since the first type of response acts mainly at protein-protein level, it is more rapid and therefore more suitable for a transient stress, whilst a transcriptional response is slower but also more systemic, hence suitable for prolonged/severe conditions of stress.

This type of mechanism, where a stress-induced destabilization of a protein-protein interaction results in the activation of one of the involved proteins and hence triggers the stress response, resembles a mechanism occurring in the context of cellular responses to osmotic stress in fish. Briefly, the protein damage caused by an osmotic stress triggers an increased requirement for molecular chaperones, one of which is the translationally controlled tumour protein (TCTP) [17]. Under normal conditions, this protein forms a complex with the NKA enzyme, but can be removed in response to protein damage, hence releasing NKA and allowing it to display its osmoregulatory functions. This chain of interactions taking place in fish is also qualitatively similar to signalling pathways displayed by plants in response to salt stress, suggesting that it represents a response pattern that may be conserved across taxa.

The following section describes the response patterns to osmotic stress displayed by fish and in particular by euryhaline fish, as they are the most studied when it comes to responses to salinity changes.

6.2.2. Osmotic stress response pathways in fish

Euryhaline fish can acclimate to a range of salinities through complex adaptive processes whose scale and magnitude are as variable as the degree and severity of salinity changes they have evolved to respond to. Different degrees of osmotic stress trigger a spectrum of different adaptive responses ranging from small scale changes in ion transport protein activity during periods of minor osmotic stress to large scale changes such as the increased differentiation and turnover of particular gill cell types during more severe and/or prolonged osmotic stress [18]. Given the high energetic cost of osmoregulatory processes (consuming 20-68% of the total energy expenditure in fish [19, 20]), selective pressure has led euryhaline fish to evolve optimized osmoregulatory

mechanisms allowing them to co-ordinate a response of a magnitude appropriate to the severity of the stress.

This ability of euryhaline fish to scale their adaptive response according to the magnitude of osmotic change is displayed mainly in the fine tuning of the upstream mechanisms that regulate effector proteins expression and orchestrate adaptive processes. In this symphony of interconnected events, the regulation of the abundance and activity of osmotic stress effector proteins - typically ion transporters and organic osmolyte supplying enzymes - is the apical outcome of a cascade of cellular and physiological processes that integrate molecular osmosensing, signal transduction and translational stabilization into a complex framework of multi-level responses.

6.2.2.1. Sensors-Effectors coupling

If the osmotic stress is of a small scale and/or temporally acute, it triggers a cellular response where osmosensing molecules are coupled to osmotic stress effector proteins through direct, protein-protein interactions (Figure 6.2). This direct coupling of osmosensors to osmotic effectors also allows the fish to rapidly re-establish cellular homeostasis while more comprehensive mechanisms of adaptation take place; in so doing they minimize the time that cells are exposed to adverse conditions and hence reduce the probability of cellular damage. One example of protein involved in this type of protein-protein interaction response is the TCTP protein [17], whose control circuit has been described in the previous Section.

6.2.2.2. Sensors-Transducers-Effectors

In addition to these rapid means of initiating adaptive responses through direct protein-protein interactions, euryhaline fish have evolved non-linear circuits of response that are activated when more comprehensive osmoregulatory adaptations are needed. Besides being directly coupled to some effector proteins, osmosensing molecules are also linked to cellular signalling pathways that are activated by ligand-receptor binding and propagated through a number of transducer proteins *via* progressive phosphorylation and dephosphorylation events. One example of a factor involved in this type of response is the serum and glucocorticoid-regulated kinase isoform 1 (SGK-1), a signalling molecule that controls sodium transport by modifying the activity of sodium channels through phosphorylation [21-23].

Each transducer molecule is often controlled by multiple signalling inputs, just as each effector protein often has multiple phosphorylation sites: in this way, each molecule is the convergent point of multiple regulators. The non-linearity of the osmoregulatory cascade, besides representing a sort of "buffer" for the system, ensures that the osmosensory signal is modulated, amplified and distributed to a variety of downstream effectors.

6.2.2.3. Sensors-Transducers-Transcription Factors-Effectors

This set of cellular responses, along with the direct coupling of osmosensors to osmotic effectors, is suitable for relatively minor or slow salinity changes, whereas more severe changes in environmental salinity require more profound osmoregulatory strategies that involve genomic-based responses such as an increase in gene expression and change in cell proliferation and turnover.

A key step is constituted by the transcriptional regulation of effector protein expression during changes in environmental salinity. Interestingly, transcription factors involved in osmoregulatory processes appear to be exclusively regulated by osmotic stress, more so than the effector proteins whose expression they control, which suggests that they have been uniquely evolved to re-establish the homeostasis of cells facing osmotic stress. Two examples of osmotic stress-related transcription factors are the osmotic stress transcription factor 1 (OSTF1) [24, 25] and the osmotic response element-binding protein transcription factor (ORE-BP) [26, 27].

In some euryhaline fish, adaptive changes in gene expression of osmotic effector proteins are coupled with the stabilization of their transcripts by osmotic-stress specific RNA-binding proteins (e.g. [28]), which represent a further mean of modulating the magnitude of adaptive responses according to the degree of osmotic stress posed by a change in environmental salinity.

6.2.2.4. Sensors-Transducers-Transcription Factors-Endocrine System-Effectors

Large-scale adaptations require the involvement of the endocrine system, which acts at cellular and organismal level through hormone-receptor interactions. The major players in endocrine control of osmoregulatory responses include corticosteroids (i.e. cortisol), prolactin, growth hormone and insulin-like growth factor 1 (IGF-1) [29]. These

hormones exert their physiological actions by binding to their corresponding receptors, which in turn regulate gene expression of effector proteins by acting as ligand-dependent transcription factors [30, 31]. A key feature of this set of hormones is to operate in synergy with each other and this characteristic, alongside precise osmosensing and signalling events, allows the refinement and stabilization of the downstream responses. Also at this more systemic level, where a set of hormones are associated with a number of signalling cascades to regulate an array of cellular processes, euryhaline fish have evolved a way to modulate and scale the apical responses to osmotic stress, which is achieved by regulating the availability of both the hormones and their receptors. This regulation takes place *via* a protein-protein type of interaction that usually involves chaperone proteins, when it comes to complexing hormone receptors (e.g. FK506-binding protein 51) controlling the availability of glucocorticoid receptor [21], or specific high-affinity binding proteins, when it comes to hormones (e.g. IGF binding proteins) [30, 31].

To summarize, one feature that emerges from what described here is the importance of the signalling cascade in osmoregulatory mechanisms, a fact further supported by the observation that the limited osmoregulatory abilities of stenohaline species is due to their inability to actively control and modulate the expression of osmotic effector proteins, despite possessing the genes encoding for all of them.

A second important feature of osmoregulatory mechanisms is their being finely modulated according to varying degrees of osmotic stress. Although hints of this characteristic seem to be a recurring note all along the cascade of adaptive responses, the actual functional explanation of it can only be speculative. One plausible hypothesis is that osmoregulatory cues emanate from multiple cellular osmosensors, each with different sensitivity ranges [18]. As stress levels increase, more and more osmosensors are triggered, each of them being coupled to a specific set of signal transducers and effector proteins that function during particular magnitudes of osmotic stress. In this way, the adaptive responses to the stress are both modulated in their intensity and functionally distinct, depending on the time frame and the scale of the stress itself. This specific modulation appears to take place at the upstream level of osmosensing and signalling events, making of them potential markers of early-stage responses to osmotic disruption.

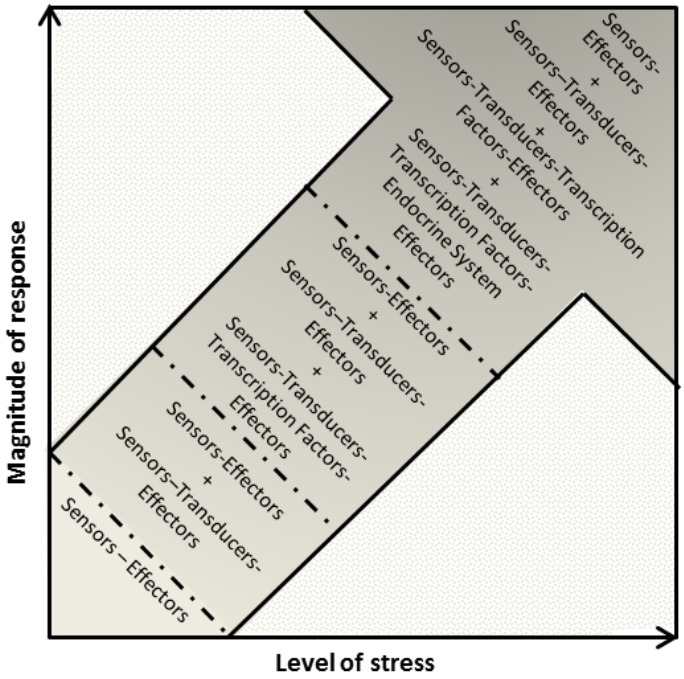

Figure 6.2. Schematic representation of the response of euryhaline fish to osmotic stress. The magnitude of the response is commeasured to the level of stress. If the organism responded to a mild stress with a disproportionally complex set of response mechanisms, it would waste its cellular resources, whereas underestimating the level of stress may compromise its cellular function. Modified from [18]

6.3. Carbonic anhydrase as an osmotic effector protein?

In the context of the osmotic response pathway described and considering the data presented in this Thesis, the questions to answer are:

- Is CA an osmotic effector protein?
- If yes, how is it controlled?
- Given the evidence provided by the gene expression data and the protein levels, where is CA placed along the range of "magnitude of response"? Is it activated by mild, medium or severe levels of osmotic stress?

As discussed in Chapters Two and Five, there is recurring evidence in the literature supporting a role of CA as osmotic effector protein, ranging from its detection in fish ionocytes to the changes displayed by both CA gene expression and enzymatic activity in response to salinity changes, both in fish and in other aquatic organisms, i.e. crustaceans. Also the responsiveness of CA to metal exposure could be considered partly supportive of its role in maintaining ion homeostasis, since osmoregulatory

disturbances are among the effects of metal exposure, as demonstrated in a number of studies, and therefore the perturbation of ion homeostasis caused by metal exposure could be related to CA expression.

The results presented in Chapter Four are overall in line with the literature. In particular, the similar pattern of response displayed by CA expression and plasma sodium levels in the FW treatments of Experiment 2 (Figure 6.3) suggests that the interaction between copper and CA relates to the disturbance of ion homeostasis. However, whether plasma sodium imbalance is the cause or the effect of copper interaction with the enzyme is something that requires some further thinking, especially because the two interpretations hold different implications for understanding the transcriptional control of CA.

So, having observed an interaction between copper, CA and plasma ion levels, how can we explain it mechanistically? Is it because copper inhibits CA activity, thus causing a compensatory upregulation (positive feedback) of the gene expression and, "at the same time", a disruption of plasma ion levels? Or rather, is copper disrupting plasma sodium transport "first", through other mechanisms of action (see Chapter One for details), hence inducing CA expression *indirectly*, through an osmotic cue that triggered a transcriptional signal activated by the osmotic stress?

If the first hypothesis of mechanism was true, the observed drop in plasma sodium levels in the FW group of the high copper treatment (Figure 6.3 A) should be due to the inhibition of CA activity, which in turn should be accompanied by a compensatory upregulation of CA. However, it is difficult to imagine that the stereochemical interaction between the metal ion and the enzyme is not dose-dependent, as would be suggested by the response pattern shown in Figure 6.3 A. Furthermore, the gene expression of CA measured in the same group (Figure 6.3 B and C) is not upregulated in either of the tissues.

Considering these arguments, we should therefore deduce that the second mechanistic explanation of the copper-CA-salinity interaction is more plausible, at least for the specific case of this experiment. According to this interpretation, copper should have caused the observed pattern of response in the plasma sodium levels through other mechanisms of action.

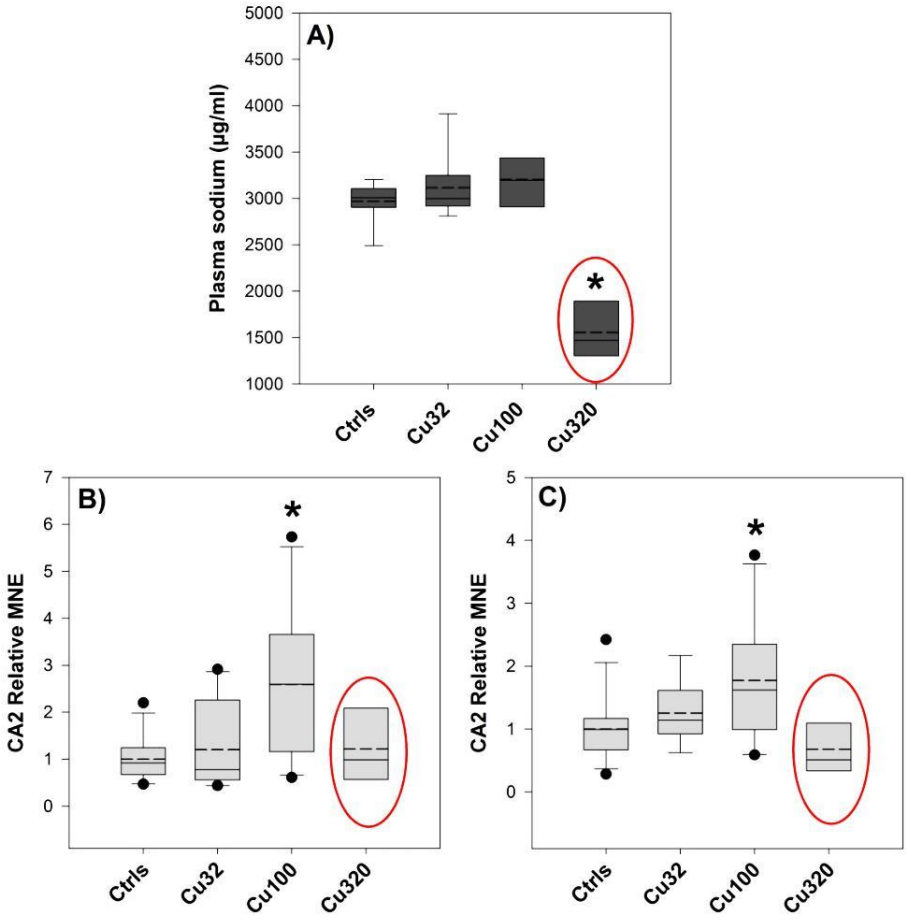

Figure 6.3. (A) Plasma sodium levels in FW groups – Exp.2. Plasma sodium concentrations (µg/ml) measured in fish exposed in FW to 0 (Ctrls), 32, 100 and 320 µg/L copper. Values are means ± SE (n = 10, except for FW Cu320, where n = 3). **(B and C) CA2 expression – Exp.2.** Relative Mean Normalized Expression (MNE) levels of CA2 measured in the gills (B) and in the intestine (C) of sheepshead minnows exposed in FW to 0 (Ctrls), 32, 100 and 320 µg/L copper for 19 days. Relative MNE levels were determined by qPCR, normalized to control gene (18S) and expressed as fold change relative to FW control value, which was set at 1. Values are means ± SE (n = 10/12, except for the FW Cu320 group, where n = 6). The asterisk denotes statistically significant difference from the control value ($P < 0.05$, Tukey t-test). Solid horizontal lines represent median values and dashed lines represent mean values.

And indeed, the copper-level dependence of the mechanisms of ion transport, when considered in their whole, offers a much more satisfying explanation for the observed pattern of response. Recalling what described in Chapter One, the osmoregulatory disturbances displayed by freshwater fish exposed to copper are due to a decrease in ion uptake at low copper concentrations and a combination of decreased uptake and increased diffusive loss at high copper concentrations. At low concentrations, copper inhibits branchial sodium transport, mainly by inhibiting the activity of osmotic effector proteins, whilst at higher levels this effect is combined with increased paracellular

permeability and hence increased sodium loss. The copper-level dependence of this mechanism of action seems more in agreement with the observed non-linear response of the plasma sodium levels to copper exposure. In any case, the most probable conclusion is that the observed osmoregulatory disturbances, alongside the changes in CA expression levels, were the result of a combination of complex and non-linear interactions and therefore none of the mechanisms could provide, by itself, the sole mechanistic explanation.

Further evidence in support of the role of CA as osmotic effector protein is provided by the statistically significant difference between the basal expression levels of gills CA respectively in the FW and in the SW control groups of Experiment 2 (Figure 6.4 A), which are interestingly specular to the pattern of expression displayed by another osmotic effector protein, NKA (Figure 6.4. B).

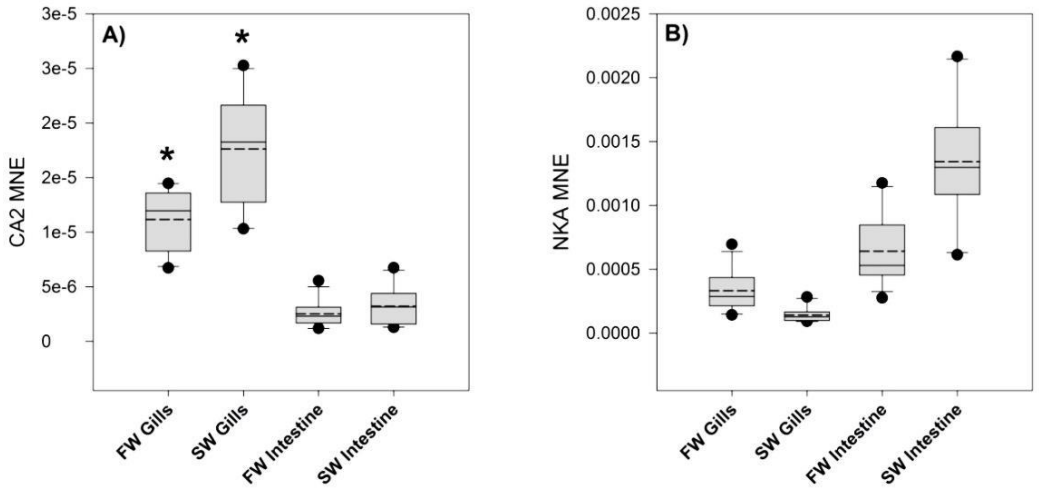

Figure 6.4. Baseline expression levels of CA2 and NKA – Exp.2. Mean Normalized Expression (MNE) levels of CA2 **(A)** and NKA **(B)** measured in the gills and in the intestine of sheepshead minnows maintained in either FW or SW for 19 days. Values are means ± SE (n = 12). The asterisk denotes statistically significant difference ($P < 0.05$, Tukey t-test). Solid horizontal lines represent median values and dashed lines represent mean values.

The last of the three questions regarding CA and its role in osmoregulation refers to the magnitude of response as commeasured to the level of stress. Is CA activated by mild, medium or severe levels of osmotic stress? Answering this question would require more *in vivo* experiments performed using different rates of salinity changes. However, the comparison between CA responses observed in Experiment 1 and 2 (Figure 6.1) suggests that a too mild stress (i.e. the one applied in Experiment 2) does not

transcriptionally activate CA, whilst what we could consider as a medium osmotic stress (Experiment 1) does, although it remains to be seen whether a stronger stress would still elicit a response in CA expression levels and, if yes, of which magnitude. Additionally, in light of what is described in Section 6.3 (Figure 6.2), if we assume that CA is under the transcriptional control of osmotic-stress related factors, this fact should further support the conclusion that CA is part of a response activated by a medium level of osmotic stress.

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CHAPTER SEVEN – CONCLUSIONS

The initial aim of this research project was to develop a refined version of the Biotic Ligand Model (BLM) able to predict copper toxicity in estuaries. A thorough study of the relevant literature on this subject was therefore performed, with the main objective to highlight the weaknesses of the BLM and particularly of its two key parts, the chemistry-based part and the physiology-based part. It emerged that the chemistry-based side of the BLM needed to better describe the characteristics of the dissolved organic matter (DOC) and its interaction with copper, given the high affinity of copper for DOC. As for the physiology-based side of the BLM, it should put more emphasis on the physiology of the organisms and include a factor accounting for the osmotic gradient between the external environment and the internal fluids of the organism (i.e. the iso-osmotic point hypothesis). Furthermore, an analysis of the mechanisms of action of copper led to the hypothesis that copper could affect fish responses to a change in salinity (i.e. an osmotic stress) by interacting with some osmotic effector proteins and particularly with the enzyme carbonic anhydrase, isoform two (CA2), which was therefore identified as a copper target linking fish responses to both copper exposure and osmotic stresses (i.e. the CA hypothesis). According to this hypothesis, not only should salinity affect the toxicity of copper, but also copper exposure should affect the adaptive responses of aquatic organisms to salinity changes**.** The CA hypothesis also implied that a BLM version for estuaries and seas should consider the intestine as a biotic ligand, alongside the gills, since in marine fish this tissue plays a crucial role in acid-base balance and ion transport.

The second stage of the project was designed to experimentally address the first two issues – characterization of DOC and modelling of the iso-osmotic point – and led to a refined-BLM algorithm stating the relevance of salinity, physiology and organic matter in determining copper toxicity in estuaries. In particular, the inclusion of a salinity-correction factor in the main equation of the BLM, based on the absolute equilibrium potential E_P across the epithelial membrane, led to an improvement in the predictive power of the model, highlighting the importance of physiology in modelling copper toxicity under variable environmental conditions. The results of the experiments performed with water mixing and NaCl-spiked riverine water suggested that increased ionic strength reduces the binding capacity of riverine DOC, thereby reducing its

protective effect on copper toxicity. It was also observed that estuarine DOC appears to be a less strong ligand than riverine DOC. One aspect that clearly emerged from all the experiments was the importance of DOC in determining the labile fraction of copper.

As for the CA hypothesis, it was tested experimentally in the third stage of the project by performing two *in vivo* studies using the euryhaline fish sheepshead minnow (*Cyprinodon variegatus*) as test species and administrating copper and salinity as two combined stressors. Measured endpoints included plasma ion concentrations and gene expression of CA2 and the enzyme Na^+/K^+ ATPase. The results suggested a sensitizing effect of copper on the responses of fish to osmotic stress, in line with the original hypothesis, and demonstrated that CA2 played a role in the combined effects of copper and osmotic stress on ion homeostasis. Among the many aspects that emerged from the analysis of the results, some issues in particular needed more investigation and were addressed by performing, respectively: *(a)* a computational and phylogenetic analysis of CAs across different species; *(b)* a promoter analysis of the gene coding for zebrafish CA2; and *(c)* a quantification of CA2 protein using a human ELISA kit, which was optimized for fish tissues. The phylogenetic analysis of CAs across different species and taxa showed that function and cellular localization are stronger discriminators than species-specificity in CAs classification, demonstrating that CA isoforms are highly conserved across species and taxa. The promoter analysis of zebrafish CA2 revealed the presence of stress-responsive-elements (STRE) and tonicity-responsive elements (TonE), suggesting a control of the gene by osmotic stress response pathways, similar to yeasts, plants and humans. The human CA2 ELISA kit was successfully validated on fish samples and used to assay samples of gills and intestine from the second *in vivo* experiment. Most of the results of CA2 protein levels in the intestine were below the detection range of the assay, whereas CA2 protein levels in the gills fell within the range and appeared in agreement with the pattern displayed by the gene expression results.

Another aspect that emerged from the two *in vivo* studies was the scaling of the biological response according to the degree of the stress applied, as discussed in Chapter Six, where the role of CA as osmotic effector protein, along with the architecture of osmotic stress pathways in euryhaline fish, were put in the wider context of stress response dynamics. Like other types of stress response mechanisms, also

osmoregulatory mechanisms are finely modulated according to the degree of stress; euryhaline fish, in particular, have the ability to scale their adaptive response to the magnitude of osmotic stress. In this context, CA2 can be considered as an osmotic effector protein whose response is activated by a medium level of osmotic stress, possibly through a combination of transcriptional and post-translational control circuits.

From the wider perspective of structuring the project in its whole, the most general conclusion that emerged from this research study was the pivotal role of a multidisciplinary approach to investigate the effects of a chemical on aquatic biota. This is especially true when the chemical in question is examined in combination with other, non-chemical variables, such as copper with salinity in the case of this project. The complexity of the dynamics controlling the availability of the chemical *outside* the organism and its interactions with environmental factors is matched with an equally complex system of responses *inside* the organism. Neither of these aspects can be overlooked, if the aim is to obtain a comprehensive and accurate picture of the overall system, a goal that can only be achieved by integrating different disciplines and techniques, as it was done throughout this research project. A particular application of integrated and comparative approaches is represented by the unified stress response framework outlined in the last chapter. This conceptual tool can be used to frame the interactions between virtually any stressor and any biological system into a wider context of conserved pathways and cross-extrapolated mechanisms of response.

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I dedicate this Thesis to my grandmother, Alma, and my parents, Mario and Loredana, for enduring being thousands of kilometres away from their little girl. My achievements shall be your reward.

Appendix: upstream region of zebrafish CA2

>24:33869300..33879300

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