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Bioavailability and mixture effects of metals in different European mussel populations

Thesis submitted in fulfilment of the requirements for the degree of

Doctor (PhD) in Applied Biological Sciences

Proefschrift voorgedragen tot het bekomen van de graad

Doctor in de Bio-ingenieurwetenschappen

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SUMMARY

More than 100 million tons of chemicals that have the potential to pose a risk to the environment are produced in Europe each year. A subset of these chemicals may, intentionally or not, enter and affect the environment. To protect the environment and the diverse services it provides, it is important to know what the impact and risk of a chemical release may be. Underestimating the risk can have harmful effects on the environment and on human health. Overestimating the risks may, unnecessarily, increase the costs of preventing or ameliorating pollution. Hence, accurate knowledge of the effects and the associated risks is essential.

Predicting the effect of a chemical is, currently, primarily based on the results of single-species experiments with freshwater organisms that are exposed to a single stressor in a standardized (laboratory) environment. However, in reality organisms are not exposed to these standardized conditions, but live in and are exposed to a **variable environment**. Furthermore, **inter-population differences** in sensitivity may exist due to differences in local adaptation and even a **single organism's sensitivity** may change during its lifetime. Finally, organisms may be exposed to **multiple stressors**, natural or anthropogenic, simultaneously. Hence, it is suggested that it might not be possible to accurately predict the adverse effects using the currently prescribed methods.

The main objective of this research was to examine the effect of these potential sources of variation on the toxicity of chemicals on marine organisms in order to increase the realism of current environmental risk assessment procedures. This was accomplished by assessing the influence of environmental variation, mixture toxicity, population variability and life-stage variation on the accumulation and toxicity of Cu on a Cu sensitive marine test species, the mussel.

In **chapter 2 and 3,** the combined influence of the two main marine environmental variables, salinity and dissolved organic carbon (DOC), on the distribution, accumulation and toxicity of Cu in mussel larvae (*Mytilus galloprovincialis*) was assessed. By using synchrotron radiation X-ray fluorescence, the distribution and accumulation of Cu in mussel larvae were determined. Cu body burden concentrations varied between 1.1 and 27.6 µg/g DW larvae across all treatments and Cu was homogeneously distributed at a spatial resolution level of 10x10 µm. The 48 h Cu EC_{10} varied between 2.8 and 11.2 µg/L, confirming that mussels are very sensitive to Cu. Cu accumulation and toxicity decreased with increasing DOC concentrations which can be explained by an increase in Cu complexation. In contrast, an increase in salinity increased the Cu toxicity. This change could not be explained by Cu speciation or competition processes and suggests a salinity-induced alteration in physiology, resulting in a changed Cu sensitivity.

In **chapter 4** a similar experiment was performed with two populations of settled mussels (North Sea and Baltic Sea). Baltic Sea mussels were chosen because previous research had indicated that the mussel population in that region is already stressed, due to the low salinity of this marine system. It was hypothesized that environmentally stressed populations would be more sensitive to anthropogenic pollution as they have to allocate more energy towards basic maintenance. The Baltic Sea population did accumulate more Cu compared to the North Sea population (both in the gills and in the total soft tissue). However, both populations exhibited an equal sensitivity to copper. This suggests that environmentally stressed populations are not necessarily more sensitive to anthropogenic pollution and that different populations may have a different way to cope with excess Cu. The influence of salinity and DOC on the accumulation and toxicity of Cu to settled mussels was very limited in both populations. Hence, it is concluded that DOC-Cu complexes are bioavailable to settled mussels. Due to the absence of a protective effect by DOC in settled mussels, implementing a DOC correction factor to determine a Cu environmental quality standard for Cu – as is done for freshwater environments – cannot be proposed for marine environments.

Organisms are not only exposed to a changing environment, but are also frequently exposed to multiple metals simultaneously. In the North Sea, for example, high Cu concentrations frequently coincide with high concentrations of other metals like Ni and Zn. Nevertheless, little information is available on the effect of metal mixtures, certainly of environmentally realistic concentrations, in the marine environment. In **chapter 5** the effect of the Cu-Ni

binary mixture on *Mytilus edulis* larvae was assessed using a full factorial design. The reproducibility of the results was assessed by repeating this experiment 5 times during a 3-year period and having them being performed by different researchers. The data were analyzed using a Markov Chain Monte-Carlo algorithm (MCMC). The use, for the first time for mixture toxicity analysis, of this statistical tool enabled the estimation of both the mixture toxicity deviation from the reference models and the uncertainty on the deviation. The results demonstrated that mussel larvae were about 100 times less sensitive to Ni than to Cu (average Cu EC_{50}: 4.1 µg/L vs Ni EC_{50}: 414.7 µg/L). When mussel larvae were exposed to a mixture of these metals, a reproducible ratio-dependent deviation from the concentration addition reference model was observed. Antagonism was observed at high nickel concentrations (> 200 µg/L) but, more importantly, low concentrations of Ni (as low as 4.9 µg/L) resulted in a synergistic interaction with Cu. To our knowledge this is the first time that synergism (according to the concentration addition reference model) was observed at low, environmentally relevant, metal concentrations. This highlights the need to consider mixture effects in marine environmental risk assessment procedures.

In **chapter 6** mussel larvae from two populations (North Sea and Baltic Sea) were exposed to Cu, Zn, Ni and a Cu-Zn mixture to assess both the influence of the mixture and determine possible inter-population differences in metal (mixture) sensitivity. The Baltic Sea mussel larvae were approximately 20 % smaller and grew slower than North Sea larvae. This agrees with previous research that suggested that settled Baltic Sea mussels are stressed by the low salinity and therefore grow slower. Mussel larvae from the Baltic Sea were three times more sensitive to Zn (as single substance) and Ni, as expected based on the proposed but untested hypothesis that the Baltic Sea mussel population would be more sensitive (due to the environmental stress) to metal exposure. However, both populations had an equal sensitivity to Cu and the effect of the Cu-Zn mixture was also similar in both populations. This indicates that inter-population variability in sensitivity is metal-dependent.

It can be concluded that: all variables investigated in this study changed the accumulation and/or the toxicity of Cu in mussels. The assessed environmental variables, i.e. salinity and DOC, had a strong influence on the accumulation and toxicity of Cu in mussel larvae but not in settled mussels. Furthermore, the influence of salinity on the Cu toxicity in mussel larvae could not be explained based on complexation and competition. Therefore, using the current knowledge, the development of a universal marine BLM based only on the water

chemistry is currently not possible. Next to the influence of environmental factors, we have provided evidence that synergistic metal mixture interactions can occur at concentrations currently measured in marine environments. To adequately protect marine organisms, metal mixture interactions should be included in future environmental risk assessment procedures. Finally, the two assessed populations were equally sensitive to Cu. This suggests that naturally stressed populations are not 'by default' more sensitive to pollution than unstressed populations. However, population differences in organism sensitivity to other metals (Zn and Ni) were observed, indicating that inter-population variability is pollutant-dependent and that this knowledge may need to be included in future ERA procedures.

SAMENVATTING

In Europa wordt per jaar meer dan 100 miljoen ton chemicaliën geproduceerd, die een potentieel risico voor het milieu kunnen vormen. Hiervan kan een deel, met opzet of niet, in het milieu terechtkomen. Om het milieu te beschermen is het belangrijk om de effecten van chemische stoffen te kunnen voorspellen om hieruit de mogelijke risico's te bepalen. Een onderschatting van de risico's kan nadelige effecten hebben op de mens en het milieu. Een overschatting zorgt voor, onnodige, extra kosten om verontreiniging te voorkomen. Een nauwkeurige inschatting van de mogelijke effecten op het milieu en de risico's voor het milieu is dus van essentieel belang.

Het voorspellen van de effecten gebeurt voornamelijk op basis van de resultaten van laboratoriumexperimenten waarbij zoetwaterorganismen worden blootgesteld aan één chemische stof onder gestandaardiseerde condities. In realiteit echter worden organismen niet blootgesteld aan deze standaardcondities, maar leven ze in en zijn blootgesteld aan een **variabele omgeving**. Daarnaast kunnen ook verschillen in gevoeligheid ontstaan tussen **verschillende populaties** door lokale adaptatie of acclimatisatie. Zelfs de **gevoeligheid van één individu** kan veranderen gedurende zijn leven. Ten slotte worden organismen vaak blootgesteld aan **meerdere chemische stoffen** op hetzelfde moment. Het is duidelijk dat deze variabelen het accuraat voorspellen van de effecten van een chemische stof bemoeilijken en, wanneer gebruik gemaakt wordt van de huidige gestandaardiseerde methoden, de voorspellingen af kunnen wijken van de realiteit.

Het doel van deze thesis was dan ook om het effect van deze mogelijke bronnen van variatie op de toxiciteit van chemicaliën bij mariene organismen te onderzoeken om zo meer realistische milieurisicoschattingen te bekomen. Dit werd verwezenlijkt door de invloed na te gaan van omgevingsvariatie, mengseltoxiciteit, populatievariabiliteit en levensstadiumvariabiliteit op de accumulatie en toxiciteit van Cu op de mossel.

In **hoofdstuk 2 en 3** werd de invloed van twee belangrijke mariene variabelen, saliniteit en opgelost organisch koolstof (DOC, dissolved organic carbon), op de accumulatie, distributie en toxiciteit van Cu onderzocht bij mossellarven. De Cu distributie en accumulatie werd bepaald met behulp van synchrotron X-stralen fluorescentie. Op een schaal van 10 bij 10 µm was Cu homogeen verdeeld in de larve met een interne concentratie tussen 1.1 en 27.6 µg/g larve (drooggewicht). De 48-uur Cu EC_{10} (Cu concentratie waarbij 10 % van de larven niet of slecht ontwikkelde) varieerde tussen 2.8 en 11.2 µg Cu/L, wat de extreme Cu gevoeligheid van mossellarven bevestigde. Zoals verwacht daalden de Cu accumulatie en toxiciteit met een stijgende DOC concentratie door een stijging in Cu complexatie. Een stijging in saliniteit resulteerde in een hogere Cu toxiciteit. Dit kan niet worden voorspeld op basis van veranderingen in de waterchemie, maar is waarschijnlijk het gevolg van veranderingen in de fysiologie van de larve.

In **hoofdstuk 4** werd een gelijkaardig experiment uitgevoerd met twee populaties (Noordzee en Oostzee) gesettelde mosselen. Er werd gekozen voor mosselen van de Oostzee, omdat voorgaande studies hebben uitgewezen dat deze mosselpopulatie al, negatief, beïnvloed wordt door de lage saliniteit in die regio (o.a. een lagere groeisnelheid). Door de aanwezigheid van deze natuurlijke stressor werd verwacht dat deze populatie gevoeliger zou zijn voor antropogene pollutie. In lijn met deze verwachtingen accumuleerde de Oostzeemosselpopulatie meer Cu dan de Noordzeepopulatie. Echter, beide populaties waren even gevoelig voor Cu. Dit betekent dat populaties die in suboptimale condities leven, niet noodzakelijk gevoeliger zijn voor pollutie en dat verschillende populaties alternatieve methoden kunnen hebben om met Cu blootstelling om te gaan. De invloed van saliniteit en DOC op de accumulatie en toxiciteit van Cu was minimaal in beide populaties. Dus, het lijkt erop dat DOC/Cu-verbindingen beschikbaar zijn voor opname door gesettelde mosselen.

Naast een variabele omgeving worden organismen vaak simultaan blootgesteld aan verhoogde concentraties van verschillende metalen, niet enkel Cu. In de Noordzee, bijvoorbeeld, komen hoge Cu concentraties vaak samen voor met verhoogde concentraties van Ni en Zn. Er is echter weinig informatie beschikbaar over het effect van metaalmengsels in het mariene milieu. Daarom hebben we in **hoofdstuk 5** het effect van Cu/Ni-mengsels op mossellarven onderzocht. Hierbij werd gebruik gemaakt van een full-factorial design, waarbij milieurelevante concentraties en ratio's werden getest. Om de reproduceerbaarheid van de resultaten te testen, werd het experiment vijfmaal herhaald,

gespreid over drie jaar en uitgevoerd door drie onderzoekers. De data werden geanalyseerd via een nieuw ontwikkelde Markov keten Monte Carlo algoritme. Deze aanpak heeft ervoor gezorgd dat zowel de modelparameters als de onzekerheid op de schattingen accuraat kon worden bepaald. De resultaten tonen aan dat mossellarven ongeveer 100 maal gevoeliger zijn aan Cu dan aan Ni (Cu EC_{50}: 4.1 µg/L; Ni EC_{50}: 414.7 µg/L). Wanneer mossels worden blootgesteld aan een mengsel van beide metalen, treedt een ratio-afhankelijke afwijking op van het concentratie additie-referentiemodel. Antagonisme treedt op bij hoge Ni concentraties (>200 µg/L), maar synergisme bij lage concentraties (4.9 µg/L). Het is de eerste keer dat synergisme werd aangetoond bij lage, milieurelevante metaalconcentraties in het mariene milieu. Deze uitkomst benadrukt de noodzaak om rekening te houden met mogelijke mengseleffecten in toekomstige milieurisicoschattingen.

In **hoofdstuk 6** werden mossellarven van twee populaties (Noordzee en Oostzee) blootgesteld aan Cu, Zn, Ni en een Cu/Zn-mengsel om zo verder de invloed van metaalmengsels en mogelijke interpopulatievariabiliteit na te gaan. De Oostzeemossellarven waren ongeveer 20 % kleiner en groeiden langzamer dan de Noordzeelarven. Dit bevestigt de resultaten van vorige studies, die aantoonden dat volwassen Oostzeemossels trager groeien door de lagere saliniteit in de regio. De Oostzeemossellarven waren ook een factor 3 gevoeliger aan Zn of Ni. Echter, beide populaties waren even gevoelig voor koper en het effect van het Cu/Zn-mengsel was gelijkaardig voor de twee populaties. Dit toont aan dat interpopulatie variatie in gevoeligheid metaal afhankelijk is.

Conclusie: Alle onderzochte variabelen in deze scriptie hadden een invloed op de accumulatie en/of toxiciteit van Cu bij mossels. Omgevingsfactoren hadden een sterk, maar levensstadia afhankelijk, effect op de Cu accumulatie en toxiciteit. De Cu gevoeligheid van mossellarven was afhankelijk van de saliniteit, maar werd waarschijnlijk veroorzaakt door een verandering in de fysiologie niet door een verandering in waterchemie. Daarenboven daalde de Cu-toxiciteit niet met stijgende DOC bij gesettelde mosselen, maar wel bij mossellarven. Het opstellen van een mariene BLM, gebaseerd op complexatie en competitie (zoals het zoetwater BLM), lijkt met de huidige kennis dan ook niet mogelijk. In deze scriptie hebben we voor het eerst aangetoond dat, synergistische metaalmengselinteracties in het mariene milieu voorkomen en dit bij milieurelevante

concentraties. Hierdoor is het aangewezen om in toekomstige milieurisicoschattingen metaalmengsels in beschouwing te nemen, om zo de mariene organismen adequaat te beschermen. Ten slotte werd geen verschil in Cu gevoeligheid gevonden tussen de twee geteste populaties. Echter, de populaties verschilden wel in Zn en Ni gevoeligheid, waardoor interpopulatie variabiliteit in toekomstige ERA best in rekening wordt gebracht.

LIST OF ABBREVIATIONS

µg	microgram
µm	micrometer

A

a	synergism-antagonism deviation parameter
AIC	Akaike information criterion
AXIL	analysis of X-ray spectra by iterative least squares

B

b	ratio or concentration dependent deviation parameter
BB	body burden
BCE	before common era
BLM	biotic ligand model
BS	Baltic Sea

C

C_0	initial concentration
C_1	final concentration
CA	concentration addition (mixture reference model)
CCD	central composite design
Cd	cadmium
CD	concentration dependent synergism/antagonism
CE	common era
CI	confidence interval
cm	centimetre
CR	clearance rate
Cu	copper

D

d	day
DDT	dichlorodiphenyltrichloroethane
DESY	Deutsches Elektronen Synchrotron
DNA	deoxyribonucleic acid
DOC	dissolved organic carbon
DW	dry weight

E

EC_x	x % effective concentration
EDTA	ethylene diamine tetracetic acid
Eq.	equation
EQS	environmental quality standard
EQSD	environmental quality standard directive
ERA	environmental risk assessment
EU	European Union

F

FAV	final acute value
FWHM	full width at half maximum

G

g	gram
GAM	generalized additive model
GB	gill burden
GeV	giga electronvolt
GOE	great oxygenation event

H

h	hour
$HC_{5\text{-}50}$	median of 5 % hazardous concentration
HMDS	hexamethyldisilazane
HTCO	high-temperature catalytic oxidation

I

IA	independent action (mixture reference model)
ICES	international council for the exploration of the sea
ICP-MS	inductively coupled plasma mass spectrometry
ICP-OES	inductively coupled plasma optical emission spectrometry

K

keV	kilo electronvolt

L

L	litre
LM	linear model
LOD	limit of detection

M

MCMC	Markov chain Monte Carlo
MDL	minimum detection limit
MEC	measured environmental concentration
MFD	marine framework directive
mg	milligram
mL	millilitre
MoA	mode of action

N

n	number
ng	nanogram
NH_4	ammonium
NH_4-P	ammonium production rate
Ni	nickel
NIST	national institute of standards and technology
nm	nanometre
NOEC	no observed effect concentration
NS	North Sea

P

PEC	predicted environmental concentration
PNEC	predicted no-effect concentration
psu	practical salinity unit

R

R^2	coefficient of determination
RD	ratio dependent synergism/antagonism
REACH	registration, evaluation, authorisation and restriction of chemicals
ROS	reactive oxygen species
RQ	risk quotient

S

s	second
S/A	synergism/antagonism
SCHER	scientific committee on health and environmental risks
SHM	stockholm humic model
SIT	specific interaction theory
SRM	standard reference material
SR-XRF	synchrotron radiation x-ray fluorescence
SSD	species sensitivity distribution

T

t	time
T_0	start time
T_1	end time
TBT	tributyltin
TC NES	technical committee on new and existing substances
TU	toxic unit

U

US	United States
US EPA	United States environmental protection agency
USGS	United States geological survey

V

V	volume
VO_2	oxygen consumption rate
VRA	voluntary risk assessment
VRAR	voluntary risk assessment report

W

WFD	water framework directive
WFD-UKTAG	water framework directive - United Kingdom technical advisory group

Z

Zn	zinc

I

GENERAL INTRODUCTION AND CONCEPTUAL FRAMEWORK

1 ENVIRONMENTAL RISK ASSESSMENT

There are currently more than 100,000 different commercial chemicals registered in Europe[1] with a total annual production of 322 million tons (2013) [2]. About 40 % of the produced chemicals are classified as harmful to the environment (i.e. 135 million tons). These chemicals may intentionally (e.g. pesticides) or unintentionally (e.g. spills) end up in the environment. When this happens, previous experience has thought us that chemicals can have a detrimental impact on the environment and human health, certainly when the chemicals are used without a full understanding of all risks. Some notable examples are: dichlorodiphenyltrichloroethane (DDT)[3], tributyltin (TBT)[4] and methylmercury[5]. It is clear that the potential risks of chemicals to the environment need to be known accurately and managed appropriately to avoid environmental damage and the loss of biodiversity or ecosystem services.

The goal of environmental risk assessment (ERA) is to quantify the risk that a certain chemical poses to the environment [6]. Typically, this is a two stage process: exposure assessment and the effect assessment. The exposure assessment is used to predict or measure the concentration of the chemical in the environment (predicted environmental concentration (PEC), or measured environmental concentration (MEC)). The effect assessment aims to determine a threshold concentration below which no (adverse) effects on the environment are expected (predicted no effect concentration (PNEC)). In its simplest form, risks are characterized by dividing the PEC by the PNEC i.e. the risk quotient (RQ; Figure 1.1). Hence, a value lower than one indicates no risk and a value higher than one indicates a potential risk [7].

An accurate risk assessment is essential. Underestimating the risks may cause harm to the environment and adversely affect human health. Overestimating the risk may, unnecessarily, increase the costs to prevent or ameliorate pollution and reduce the resources available for other environmental protection actions (in 2014 the cost to protect the environment was estimated at 297 billion euro in Europe[8]). However, the risks are only accurately assessed if the PNEC is properly derived. Due to the many variables that can influence the PNEC this process may be the Achilles' heel of ERA.

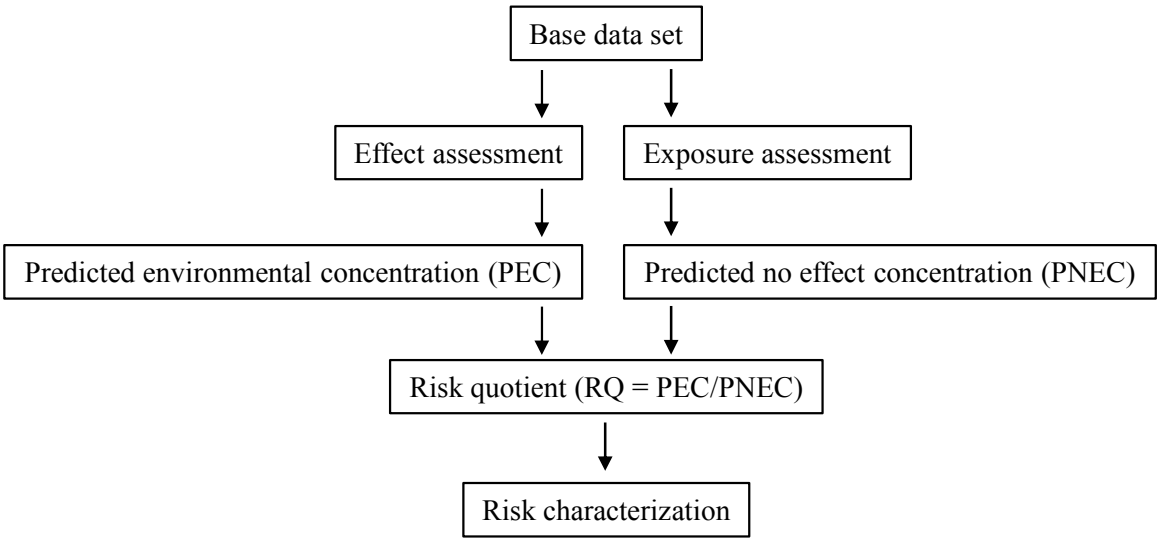


Figure 1.1. Schematic overview of a typical ecological risk assessment, redrafted from van Leeuwen and Vermeire (2007) [9]

2 VARIABLES AFFECTING EFFECT ASSESSMENT

Current effect assessment practices are, for the aquatic environment, mainly based on the results of standardized toxicity tests in which the effect of single substances are evaluated in standardized experiments using freshwater organisms. The underlying idea is that standardized tests increase the comparability between species or chemicals and increase the reproducibility of the results. However, this approach does not necessarily reflect reality [10, 11]. **Different populations** of the same species may be exposed (and acclimated or adapted) to different environments depending on the geographical location (e.g. North vs South). **A population** may experience both short- and long-term temporal variation in the environment (e.g. rainfall or seasons). **Individuals** themselves might go through different life stages, each with a different anatomy and physiology (e.g. caterpillar to butterfly, larvae to fish). Finally, organisms may be **exposed to a mixture** of chemicals instead of a single substance. All these sources of environmental or biological variation can either alter the toxicity of chemicals or alter the sensitivity of the exposed organism [12-16]. Hence, the effect of a pollutant might be over or under predicted via the current standardized methods and may therefore affect the ERA outcome. There is a clear need to increase the environmental realism in risk assessment.

In the following sections the current state of knowledge about several potentially important variables is discussed in detail. The variables are grouped in abiotic and biotic variables.

Abiotic variables are variables that affect the (chemical) environment in which the organism lives. In this context environmental variation (e.g. changes in salinity) and exposure to a mixture of anthropogenic pollutants are discussed. Biotic variables are organisms-dependent and have no effect on the environment or availability of the pollutant in the water but may alter the sensitivity of the organism to the pollutant. In this context inter-population variability and life-stage variability are discussed. It is not possible to discuss all chemicals or exposure scenarios. Therefore, we focussed on metal toxicity as a result of the selected model stressor (Cu; section 3) and the marine environment as the mussel was chosen as model organism (section 4).

2.1 Abiotic variables

Environmental variation

Ion composition

In freshwater the concentration of certain ions may have a major influence on the toxicity of a metal[17-19]. Anions (e.g. OH^-, CO_3^{2-}) can bind to metal ions to form metal species that are not or less available for uptake (e.g. $CuOH^+$, $Cu(OH)_2$, $CuCO_3$ and $Cu(CO_3)_2$) by the organism thereby reducing the toxicity of the metal[20]. Cations can also reduce the toxicity by competing at the site of uptake if they use the same transmembrane transporters. For example, previous research suggest that Na competes with Cu[21], Mg competes with Ni[22] and Ca with Cd[23] for uptake by the organism.

In contrast to freshwater, the relative chemical composition of the major ions (e.g. Na, Mg, Ca, Cl) in saltwater is constant and the pH is buffered. However, evaporation or dilution (e.g. estuaries) can change the overall ion concentration or salinity of the water [24, 25]. Therefore, there is a need to understand the influence of salinity (rather than a change in the individual elements) on the uptake, accumulation and toxicity of metals to marine organisms. Based on the knowledge we have from freshwater studies (see above), a reduced toxicity with increased salinity is expected due to the higher cation and anion concentration resulting in an increased complexation and increased competition. In some cases, an increase in salinity can indeed explain the observed differences in toxicity [26, 27]. However, a changing salinity might also have a profound effect on the physiology of the organism [28-30]. Furthermore, organisms may have to allocate a higher proportion of their energy budget

to osmoregulation when the salinity deviates from the iso-osmotic point resulting in less energy available to detoxify metals or repair any damage [16]. Therefore, a change in salinity may not only alter the toxicity of the metal but also alter the intrinsic sensitivity of the organism [15, 16, 31-33].

Dissolved organic carbon

Dissolved organic carbon (DOC) can bind with several metals due to the presence of thiol groups (R-S-H → R-S-Me). In freshwater, a considerable number of studies have shown that DOC-metal complexes are not available for uptake and, as a result, reduce the accumulation and toxicity of metals [34-37]. To improve the EU environmental quality standards (EQS), the EQS of several metals is now corrected based on the DOC concentration of the receiving surface water (e.g. Cu[38], Zn[39]) [7]. This ensures that waters with a low DOC concentration are sufficiently protected while the EQS for waters with a high DOC concentration can be higher.

Although the research performed in saltwater is much scarcer current research indicates that DOC can also significantly reduce the accumulation and toxicity of metals in a similar manner as that observed in freshwater systems [40-42]. However, several studies with marine bivalves have indicated that DOC and DOC-Cu complexes might be (at least partially) available for uptake, thus decreasing or eliminating the protective action of DOC [43-48]. It should be stressed, however, that all cited studies used short-term exposures. The possible availability of DOC or DOC metal complexes in bivalves has not been investigated in chronic experiments. Furthermore, there is evidence that DOC is an important constituent of the diet of (zebra)mussels [47, 49]. If DOC-metal complexes are indeed (partially) available for some marine organisms (e.g. mussels) this may prohibit the implementation of a DOC correction factor on the EQS or PNEC for the marine environment.

Mixture toxicity

Organisms can be, and regularly are, exposed to elevated concentrations of several metals simultaneously. For example, a strong positive correlation between the concentration of different metals in the North Sea was observed (Box 1.1). Each of these metals may elicit an adverse effect on the organism, however it is important to know the overall influence of the mixture on the organism.

Mixture reference models

Two reference models are frequently used to predict the combined effect of multiple non-interacting stressors: concentration addition (CA) and independent action (IA). Both models are based on the mode of action (MoA) of the chemicals in the mixture, but assume either a different MoA (IA) or a similar MoA (CA).

According to the IA reference model, first proposed by Bliss (1939)[50], the mixture toxicity of pollutants with a different MoA can be predicted based on the statistical concept of independent random events. Hence, by multiplying the unaffected fraction of each single constituent of a mixture one can predict the overall unaffected fraction of a mixture of pollutants that have a different mode of action. The IA model implies that when the concentration of each chemical in a mixture is lower than the concentration needed to elicit an effect individually, no adverse effect will be observed. The IA model can be mathematically described using equation 1.1.

$$E(\text{mix}) = 1 - \prod_{i-1}^{n}(1 - E(Ci)) \qquad \text{(Eq. 1.1)}$$

Where E(mix) is the proportional effect of the mixture of n compounds and E(Ci) is the proportional effect of substance i when applied singly.

The concentration addition (CA) reference model was first proposed by Löewe and Muischnek (1926)[51] to assess a mixture of chemicals that act through a similar MoA. CA states that the different components in a mixture are identical but only differ in potency and therefore can be replaced by (a dilution of) the other component without affecting the overall toxicity as long as the sum of the toxic units (TU) remains 1 (Eq. 1.2).

$$\sum_{i=1}^{n} TU = 1 \text{ with } TUi = \frac{Ci}{ECxi} \rightarrow E(\text{mix}) = x \qquad \text{(Eq. 1.2)}$$

With n is the number of mixture components, c_i the concentration of chemical i in a mixture with n pollutants, $EC_x i$ the concentration of chemical i that results in x % effect when the organism is exposed only to this chemical.

The model implies that even if the concentration of an individual chemical is too low to have an adverse effect on the organism, this chemical increases the overall toxicity of the mixture. So, even if the concentration of all individual constituents in a mixture is below the no observed effect concentration the entire mixture may cause an adverse effect. The CA model is usually more conservative than the IA model [52].

Deviations from the reference models

In theory these two reference models should be able to predict the effect of all mixtures. In reality deviations from these reference models are frequently observed [53, 54]. On the one hand, this may be because the MoA's partially (dis)similar (e.g. Cu has multiple MoA's; see section 3.2) and therefore neither CA nor IA is fully applicable. On the other hand, this may be because the assumption of non-interaction, a vital assumption in both models, is violated. Interactions can occur when a component influences the accumulation or toxicity of the other component(s) in a mixture [55]. Due to these interactions the effect of a mixture may be less than (antagonism) or more than (synergism) expected compared to the reference models. In addition, several studies have indicated that the presence of antagonism/synergism may depend on the ratio or concentration of the components in the mixture[56]. In the context of environmental risk assessment, the possibility of synergism is especially important as it may result in underprotection of the environment.

Jonker et al. (2005)[55] proposed to add one or two additional deviation parameters to the reference models to increase the complexity of the reference models and thereby the ability to describe the reality (Figure 1.2). By adding a deviation parameter "a" to either the CA or IA reference model it was possible to describe synergism and antagonism (S/A deviation model). The addition of a second deviation parameter "b" enabled the assessment of ratio dependent (RD) or concentration dependent (CD) synergism/antagonism. For details see Jonker et al. (2005).

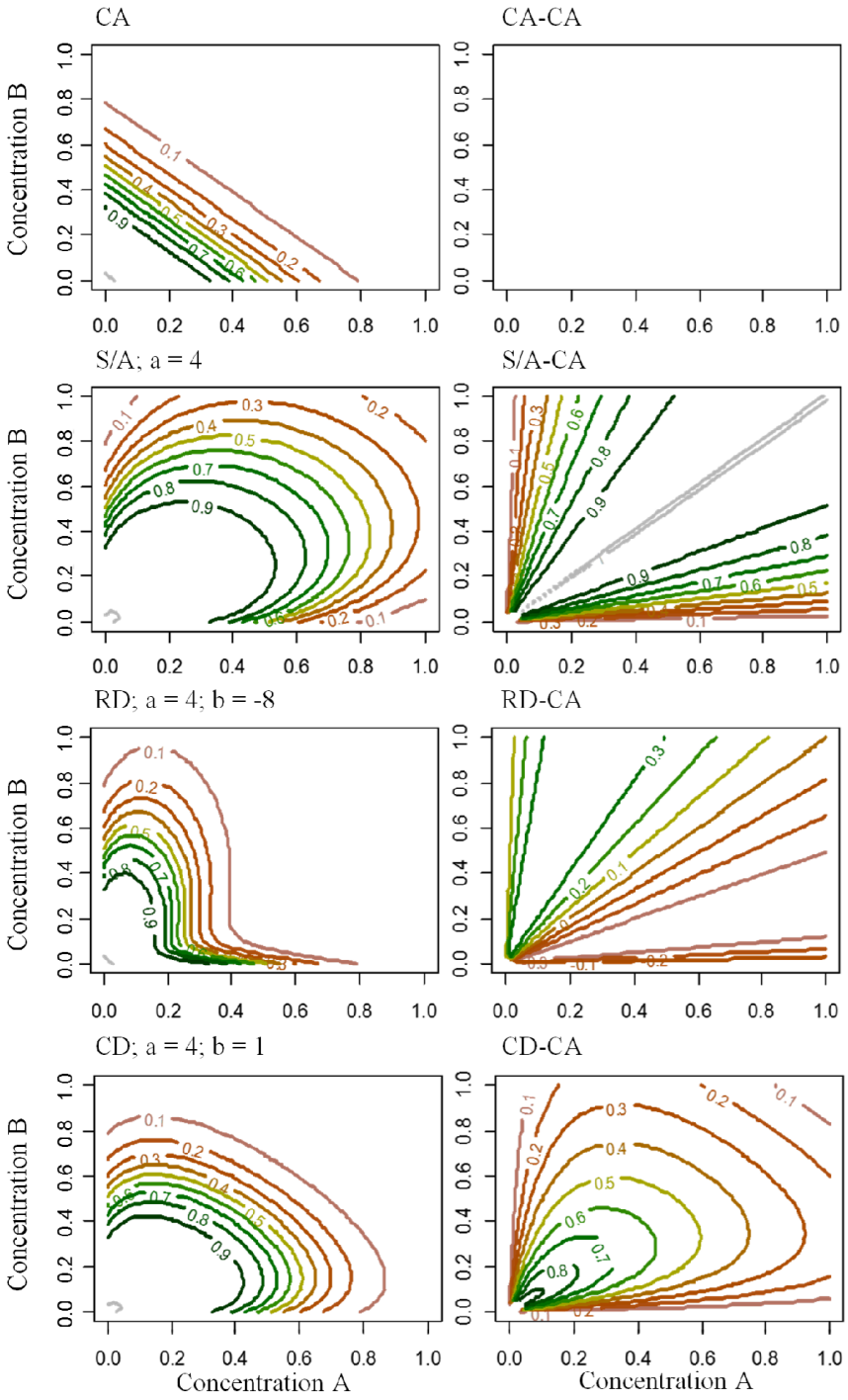


Figure 1.2. A graphical illustration of the reference model (CA) and the three deviation models proposed in Jonker et al. (2005). Both toxicants (A and B) have a maximum of 1, a minimum of 0; a slope of 5 and an EC_{50} of 0.5. From top to bottom: the original concentration addition (CA) reference model; the synergism/antagonism (S/A) deviation model; the ratio dependent deviation model (RD); the concentration dependent deviation model. Left: the original concentration response curves (green = no effect; red = high effect); Right the difference between the deviation model and the reference model (e.g. S/A – CA) to indicate how the original (CA) model is modified by the deviation function.

2.2 Biotic variables

Population variability

Every species can survive and reproduce within a certain range of environmental conditions, i.e. their "environmental envelope". These conditions are not always optimal and organisms living in suboptimal conditions might be physiologically stressed (Figure 1.3). Indeed, environmentally stressed organisms tend to allocate more energy towards basic maintenance, potentially reducing the energy available for detoxification and therefore increase their sensitivity [57-59]. It is hypothesized that this might increase their sensitivity to other stressors such as anthropogenic pollution either because less energy is available for detoxification and repair processes or because pollution reduces the amount of, the much needed, energy that is assimilated [60-62]. This might be especially true for species living at the very edge of their environmental envelope. On the other hand, populations might acclimate or adapt over time to better deal with the challenges of their specific local environment (optimal condition shifts left or right on the x-axis of figure 1.3) [63, 64]. Local adaptations can include changes in the physiology and/or morphology which, as a side effect, can alter the sensitivity towards other stressors such anthropogenic pollutants [65, 66]. However, while knowledge on inter-population variability is important for an adequate ERA, the empirical evidence is scarce.

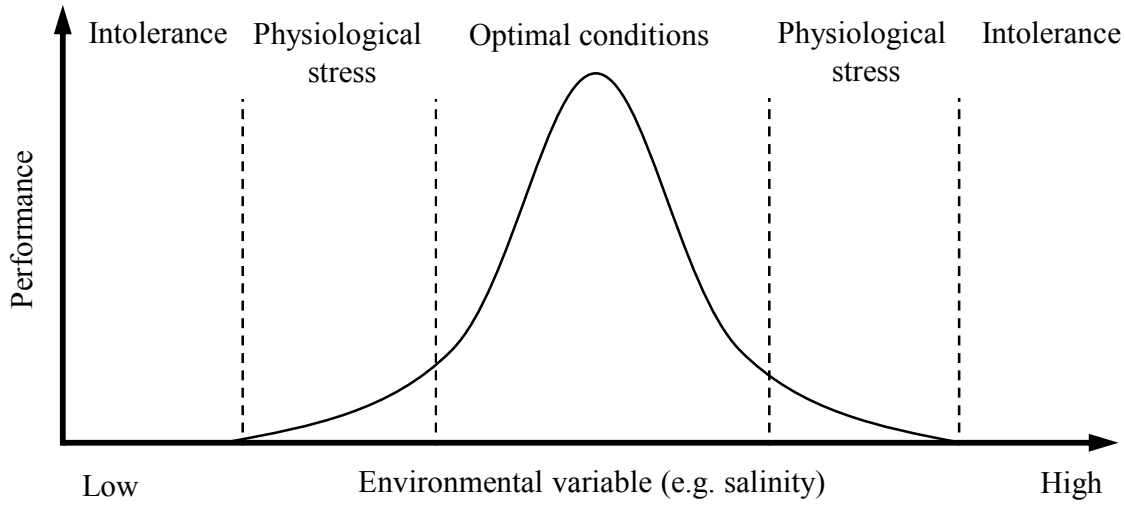


Figure 1.3. Organisms can survive within a certain range of an environmental conditions. Individuals can experience physiological stress when the local environmental conditions deviates from the optimal conditions [67, 68].

Life stage variability

Many organisms go through a succession of life stages, often including multiple larval stages, before reaching maturity. Each life stage can be vastly different from the others in morphology, physiology and gene expression[69], but also the habitat preference and behavior can differ. This is certainly so for many invertebrates (e.g. caterpillar to a butterfly). As a result, the sensitivity to pollution might change throughout the lifetime of an organism [70]. Although exceptions exist [71], the early life stages are commonly considered to be more sensitive than the adult stage(s) [72-74]. However, as described above changes in the environment such as salinity or DOC may affect the toxicity of the pollutant or sensitivity of the organism. Currently little is known on how different life stages respond to the same stressor in combination with changes in the environment.

Box 1.1 The reality of metal mixtures: A North Sea case study

In the context of this thesis, a case study was performed to assess the co-occurrence of Cu with Ni, Zn, Pb and Cd in the North Sea (Figure Box 1, see also Box 1.2). The data was provided by ICES (n = 2879), the Belgian Marine Data Centre (BMDC; n = 375) and the British Oceanographic Data Centre (BODC; n = 6373) and was collected between 1993 and 2013. For all metals concentrations higher than the proposed EU PNEC or EQS have been measured. Furthermore, linear regression (log-log scale) showed that for all assessed metals there was a significant positive correlation with the Cu concentration. Hence, if organisms are exposed to elevated Cu concentrations they are very likely also exposed to higher concentrations of Ni, Zn and/or Cd. In conclusion: organisms in the North Sea are likely to be exposed to a mixture of metals. These mixture effects should be assessed in order to ensure an adequate protection of the marine environment.

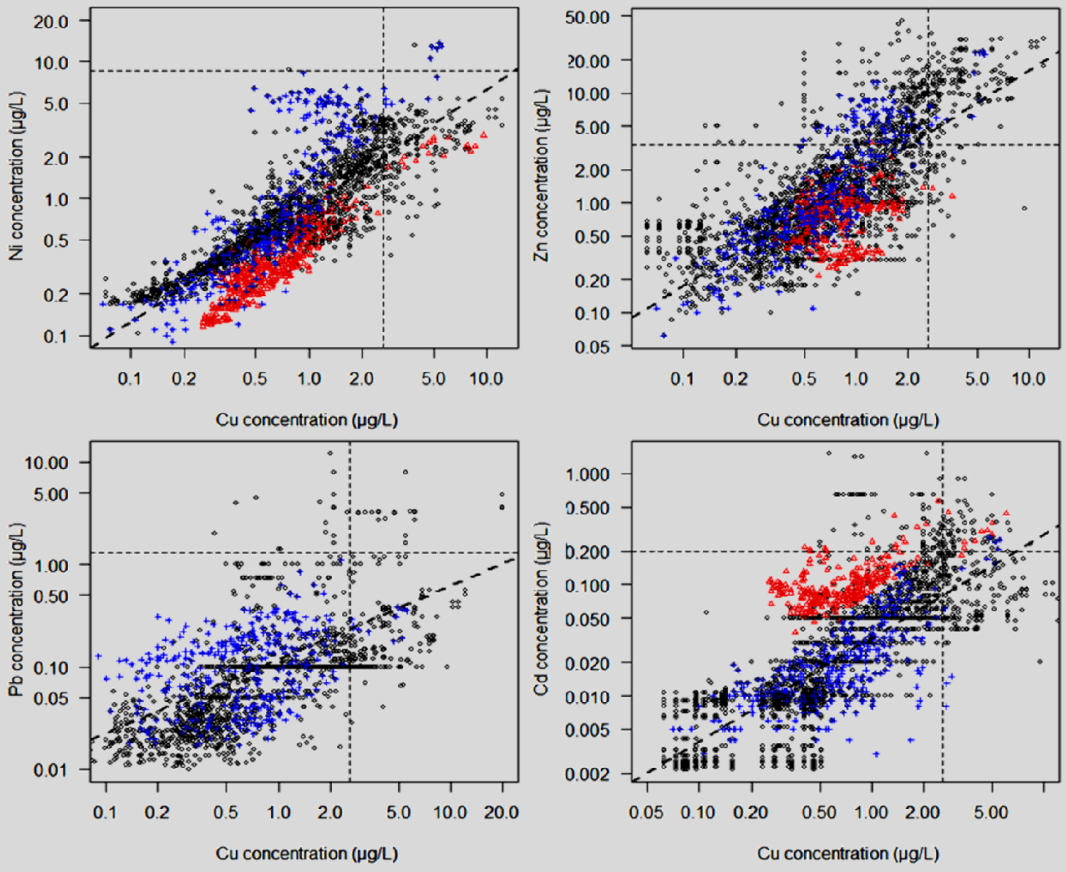


Figure Box 1. The Ni, Zn, Pb or Cd concentration versus the Cu concentration in the North Sea. Dotted lines = proposed European PNEC (Ni and Cu), annual average EQS (Pb and Cd) or WFD-UKTAG proposal (Zn) [38, 75-78] ; dashed line = linear relation between the logaritmic concentration of the metals; black circles = data ICES; blue crosses = data BMDC ; red triangles = data BODC (not available for Pb). All available data was used without distinction in respect to geographical location and or time of sampling (1993-2013).

3 COPPER AS MODEL STRESSOR

Copper was used as model stressor in this thesis. It is both an essential trace element as well as a highly potent toxicant when present at elevated levels. The production and use of Cu has increased rapidly since the 1950's. Furthermore, with the increasing development of China and India it is not likely that this trend will change in the near future [79-81]. Therefore, accurate knowledge on the toxicity of Cu in combination with abiotic and biotic variables is required.

3.1 Historic and current use

Copper is one of the oldest metals known to man with evidence of Cu smelting dating back as far as 7000 years ago [82, 83]. Ever since, Cu has been used for ornaments, coins, weapons, etc. It is clear that Cu has had a profound influence on human history. However, until 150 years ago, the estimated Cu production never exceeded 20,000 metric tons per year with a production peak at the height of the Roman Empire and during the Sung Dynasty in China [84]. After the onset of the Industrial Revolution, Cu production started to increase exponentially up to 18.7 million tons in 2015 (Figure 1.4). Currently, it is the third most used metal in the world and is only surpassed by iron and aluminium. Cu is mainly used for electricity and energy applications (58 %) due to its excellent conductivity [85].

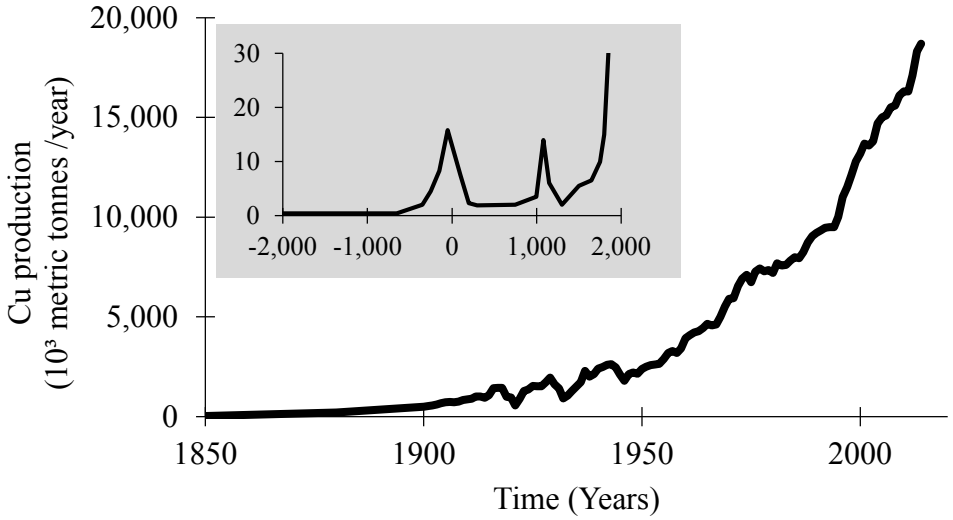


Figure 1.4. The global Cu production between 2000 BCE and 1850 CE (inset), and 1900 to 2014 CE [84, 86].

3.2 Essentiality and toxicity of Cu

Life evolved in an anaerobic world, rich in iron but devoid of Cu as it was only present as the insoluble Cu_2S. It is only after the great oxidation event (GOE; 2.45 to 2.22 billion year ago[87]) that Cu^{1+} was oxidized to the soluble Cu^{2+} and became bioavailable ($2\ Cu_2S + 3\ O_2 \rightarrow 2\ Cu_2O + 2\ SO_2$). Due to the potential of Cu to oxidize (or reduce) substances it was, over time, included as an essential co-factor in numerous enzymes. Some of the key processes in which Cu now plays a role are: oxygen transport (haemocyanin), immune response (phenoloxidase), crosslinking collagen and chitin and electron transport (cytochrome c-oxidase) [88].

The ability of Cu to oxidize biomolecules and its affinity for thiolates implies that it is highly toxic when the intracellular concentration is not properly regulated. Three main modes of action (MoA) have been proposed, but most adverse effects are probably due to a combination of the different MoAs (Figure 1.5). MoA 1: free Cu ions can produce reactive oxygen species (ROS; e.g. OH°) when they react with hydrogen peroxide via the fenton like reaction[89]. In turn, ROS can cause lipid peroxidation resulting in cell wall damage, DNA mutations and oxidation of enzymes and thereby reducing their activity [90, 91]. MoA 2: the change in activity of many enzymes by the direct binding of Cu with the SH-groups of these enzymes. Previous research has indicated that this could be the case for two key enzymes: Na/K-ATPase and carbonic anhydrase [92, 93]. A change in the activity of one of these enzymes may disturb the ion homeostasis of Na and K, the intercellular pH and the cellular resting potential. MoA 3: is the replacement of Fe in several key enzymes (mainly dehydratases) reducing/altering their activity, disturbing the Fe homeostasis and additional ROS production due to the increase in free Fe ions [94, 95].

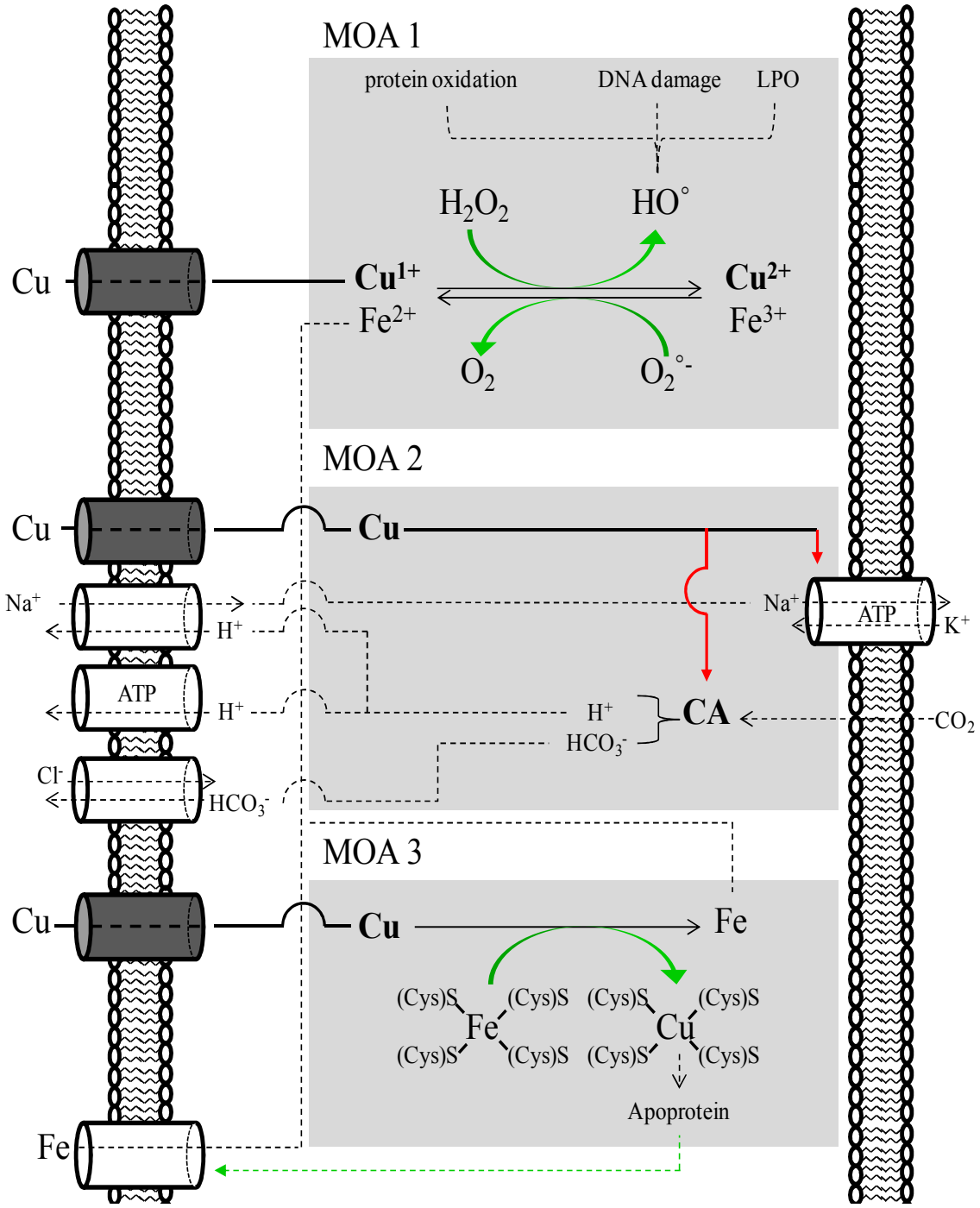


Figure 1.5. A schematic overview of possible modes of action by Cu. MoA 1: production of reactive oxygen species (ROS) via a fenton like reaction; MoA 2: altering the enzyme activity by binding with the SH-groups resulting in a change in ion homeostasis, intracellular pH and resting potential; MoA 3: the displacement of the Fe ion cofactor with a Cu ion resulting in both an altered enzyme activity and increased ROS production. Red = decreased activity; Green = increased activity

3.3 Cu exposure

Cu is a natural element occurring in the marine environment with an estimated average global oceanic natural background concentration of 155 ng/L [96]. Whenever Cu is smelted or used the (local) environment can be contaminated. Evidence for Cu contamination dates back from 2000 years ago as increased Cu concentrations were found in rivers, sheep and humans [97-99]. Cu production at that time was only 0.1 % of today's production and although the modern production processes have less Cu emissions the thousand-fold increase in Cu production and use may be a threat to local or regional environments. Copper may enter the environment via intentional release such as pesticides, anti-fouling paints, animal feed, fertilizers and via unintentional release such as corrosion, brake pads, leaching chromate copper arsenate wood, mining leachates, etc. Lifset et al. (2012)[100] estimated a total release to the water compartment in the USA of 12,590 tons/year in 2000. This is an 60 % increase compared to 1970. In the same period the estimated Cu release from anti-fouling boat paints increased 235 %. Due to the ever increasing Cu production and use in combination with possible environmental contamination it is important to assess and understand the possible (adverse) effects of Cu on the environment.

Based on the data of the Belgian Marine Data Centre, the mean and median Cu concentration in the Belgian part of the North Sea is 0.85 and 0.66 µg/L with 5 % of the values exceeding 1.98 µg/L (n = 374) and a maximum measured concentration of 5.5 µg/L. Not surprisingly, the highest Cu concentrations are found near the port of Antwerp (Westerscheldt) and near the coast where human influence is the highest (Box 1.2). The Belgian data is comparable to the overall Cu concentration in the North Sea area (mean: 0.96 µg/L; median: 0.74 µg/L; 95-percentile: 2.13 µg/L; highest concentration: 39.2 µg/L; n = 8890). Other high quality datasets were not available, but a literature search indicates that high Cu concentrations were found near industrial areas around the globe such as: China (9.26-14.2 µg/L[101, 102]), Spain (up to 11 µg/L[103]) and Chile (48 µg/L[104]).

Box 1.2 Copper distribution along the Belgian Coast and North Sea

In the context of this thesis, a case study was performed to assess the distribution of Cu along the Belgian coast and the North Sea (Figure Box 2). The data was provided by ICES, the Belgian Marine Data Centre and the British Oceanographic Data Centre. The distribution analysis was performed with Ocean Data View using DIVA gridding. The distribution pattern clearly shows that the highest Cu concentrations are found near the coast, coinciding with the habitat of mussels. For the Belgium, the highest concentrations were found near the harbor of Antwerp and Ostend. When the whole North Sea is considered, copper concentrations along the coast line are always slightly higher than more offshore. However, based on the data at hand, the highest Cu concentrations do not always coincide with areas with large cities or harbors.

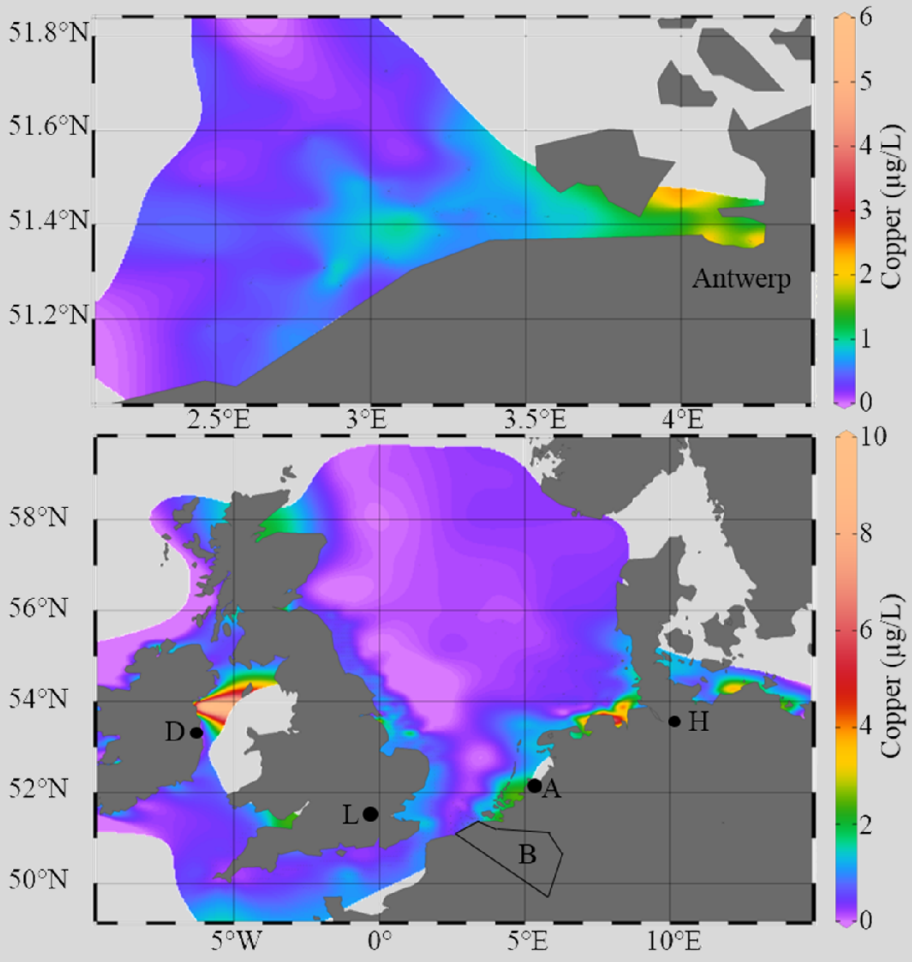


Figure Box 2. The modelled Cu concentration (µg/L) in the Belgian part of the North Sea (top) and the North Sea (bottom) via ocean data view. The concentrations are modelled based on data from the Belgian marine data centre, ICES and the British Oceanographic Data Centre. The analysis was performed with Ocean Data View in combination with DIVA gridding. D = Dublin; L = London; A = Amsterdam; H = Hamburg; B = Belgium

3.4 Current Cu risk assessment

Currently there are four important legislations in Europe that relate to Cu in the marine environment: The EU Registration, Evaluation and Authorization of Chemicals directive (REACH[105]), the water framework directive (WFD[106]), the marine framework directive (MFD[107]) and the environmental quality standard directive (EQSD[108]).

The EU REACH legislation of 2007 transferred the responsibility for assessing risks from the government to the industry. In order to comply to this and previous legislation the European Copper Institute performed a voluntary risk assessment (VRA) [109]. Based on 24 species a species sensitivity distribution (SSD) was constructed and a marine Cu the median fifth percentile of the species sensitivity distribution ($HC_{5\text{-}50}$) of 5.2 µg Cu/L (for a DOC concentration of 2 mg/L) was derived. Because, at the time, a reliable Cu mesocosm experiment was not available at the time a safety factor of 2 was applied resulting in a PNEC of 2.6 µg Cu/L. The proposed PNEC was supported by SCHER[110] (Scientific Committee on Health and Environmental Risks) and TC NES (Technical Committee on New and Existing Substances)[111]. Both committees agreed that the assessment factor could be reduced in the future if the $HC_{5\text{-}50}$ could be validated with reliable mesocosm data. The in 2000 the WFD did not include Cu in the list of priority substances, neither did the 2008 MFD or the 2008 EQSD. However, the WFD and the MFD both strive for a good chemical status of the water, but without specifying a marine Cu EQS. The WFD-UKTAG (Water Framework Directive- United Kingdom Technical Advisory Group) did proposed an Cu EQS_{marine} based on the VRA and literature published thereafter [112] of a $HC_{5\text{-}50}$ of 2.64 µg Cu/L with an assessment factor of 1 (i.e. PNEC = 2.64 µg/L). The advisory group did also propose a DOC correction using the following equation (Eq. 1.3):

$$\text{PNEC site specific} = 1.34 * (\text{DOC} - 1) + 2.64 \qquad \text{(Eq. 1.3)}$$

Lower marine environmental criteria values were established in other countries. In Australia and New Zealand marine water quality guidelines have set the trigger value at which 95 % of the species is protected at 1.3 µg/L [113]. The US EPA (United States Environmental Protection Agency) has proposed a new EQS for Cu in 2016 based on the BLM with an acute (1 h) EQS of 2.0 µg/L and chronic (4 d) EQS of 1.3 µg/L (at 22 °C; pH 8; 1.0 mg DOC/L and 32 psu) [114].

4 MUSSELS AS MODEL ORGANISMS

Mussels were used as model organisms in this research. From a practical, scientific, ecological and economic perspective they are the excellent test animals to assess the toxicity of Cu in the marine environment.

The **practical perspective:** adults of mussels are easy to collect, sessile organisms and can be maintained easily in the lab. Mussel larvae are straightforward to breed when ripe adults are collected [115]. Mussels do have some disadvantages compared to some other model organisms. They do not reproduce via clones (e.g. *Daphnia spp.*) and therefore genetic variability may increase the overall variability in experiments. Furthermore, a whole life cycle tests is nearly impossible to conduct as it may take several months for a mussel to mature.

The **scientific perspective:** Mussels are amongst the most Cu sensitive marine species and the influence of a multitude of variables on Cu toxicity can be assessed: (1) mussels go through a series of pelagic larval stages before settling and acquiring the final (adult) form. Therefore, differences in sensitivity between different life stages can be evaluated easily. In the first 48 h after fertilization the larvae are the most sensitive to Cu (embryo to the D-shaped veliger larvae) [115]. The subsequent larval stages are less sensitive [116]. However, after settling the Cu sensitivity increases again so that chronically exposed settled mussels are only slightly less sensitive to Cu compared to the initial 48 h of their life (EC_{50} growth: 6 µg/L vs EC_{50} on larval development as low as 4.1 µg/L) [117-119]. Eventhough the two life stages have a similar sensitivity, the D-larvae and settled mussels have a vastly different anatomy. Furthermore, evidence for the protective effect of DOC on Cu toxicity has been provided for mussel larvae but two recent studies have indicated that DOC might be (partially) taken up by settled mussels [45, 46]. (2) mussels have populations that are adapted to different geographical regions (e.g. North Sea vs Baltic Sea) and they live near the coast where changes in salinity and DOC occur naturally. The Baltic Sea population is particularly interesting as mussels that live there are adapted to a low salinity environment (down to 6 psu). Although mussels from the Baltic region are adapted they are still stressed by the low salinity as reflected by their lower metabolism and growth [65, 120-123]. This makes this organism ideal to assess the influence of Cu (an anthropogenic stressor) in combination with a natural stressor and inter-population variability in sensitivity.

The **ecological perspective:** mussels are a key species in the marine environment. They are the dominant species in many coastal habitats around the world and have a major influence on the local biodiversity [124, 125]. They provide a hard substrate, shelter and/or food for a range of species thereby increasing the abundance and biomass of the macrofauna [126-128]. Mussels are also capable of changing the phytoplankton, protozoa and copepod community due to their capacity to filter large amounts of water [129, 130].

The **economic perspective:** mussels have an important economic function (Figure 1.6). They have been harvested for millennia as a source of raw material [131] and have been cultured since the 13th century (France) [132]. Since 1970 the global production increases on average with 36,000 tonnes/year and reached a record production of 1,900,000 tonnes in 2014 (estimated value of > 4 billion euros). Beside the use as a food source, mussel farms may be used to mitigate the effects of eutrophication and (harmful) algal blooms in the future. Several studies already indicated that bioextraction of nutrients via mussels may be a viable option [133, 134].

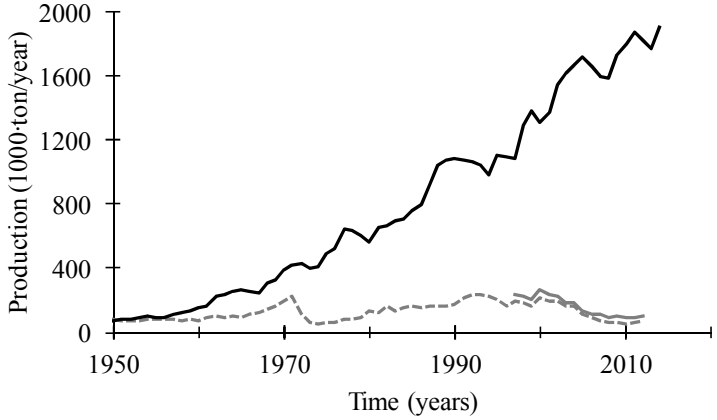


Figure 1.6. The global production and capture of mussels from 1950 up to 2014. Solid black line = aquaculture production, solid grey line = total mussel capture (no data before 1997), dashed grey line = combined capture of *M. edulis*, *M. galloprovincialis* and *Perna viridis* (data up to 1950) [135].

5 PROBLEM FORMULATION AND OBJECTIVES

To protect the environment, an accurate assessment of the potential risks posed by a contaminant is essential. From section 2 it is clear that a multitude of (a)biotic variables may affect the results of the toxicity experiments which form the basis of and the subsequent effect and risk assessment.

The **main objective of this research was to examine the effect of (a)biotic variables on the toxicity of chemicals on marine organisms in order to increase the realism of current environmental risk assessment procedures.** This was achieved by conducting an integrated assessment of the effect of a range of variables on the toxicity of a single chemical using a single, high sensitive marine model species. Copper was chosen as model stressor due to the extensive knowledge on Cu toxicity in freshwater, enabling a good comparison with the marine environment. The mussel was chosen as model organism. As mussels are amongst the most Cu sensitive marine species environmentally realistic concentrations could be used in the experiments enabling the use of the results to improve current Cu risk assessment procedures. Based on the main objective **4 research questions** were formulated aimed at to assessing **4 potential sources of variation** (see also Figure 1.7):

1) **Environmental variability:** Do salinity and DOC affect Cu accumulation and toxicity?
2) **Mixture toxicity:** Do metals in a mixture interact and does this occur at environmentally realistic concentrations?
3) **Population variability:** Are there inter-population differences in Cu accumulation and sensitivity?
4) **Life stage variability:** How does the effect of salinity and DOC on Cu toxicity differ between mussel larvae and settled mussels?

6 CONCEPTUAL FRAMEWORK OF THE THESIS

The research performed in this thesis is described in 5 research chapters (chapters 2-6). The conclusions and research perspectives are summarized in chapter 7.

In **Chapter 2** the combined influence of the two main marine environmental variables (salinity and DOC) on the distribution and accumulation of Cu in mussel larvae was assessed. This was done by exposing larvae to different concentrations of Cu, DOC and salinity and measuring the accumulation using X-ray fluorescence techniques.

In **Chapter 3** the accumulation data from Chapter 2 was combined with toxicity data to explore the relation between Cu accumulation and Cu toxicity and to evaluate the influence of salinity and DOC on the toxicity to mussel larvae.

In the first two experimental chapters only mussel larvae were considered. However, previous studies have indicated that settled mussels exhibit a similar sensitivity although the two life stages have a very different morphology and physiology. Therefore, the combined influence of salinity and DOC on the chronic toxicity and accumulation of Cu in settled mussels was assessed in **Chapter 4**. Furthermore, in this chapter possible inter-population variability was investigated by using two different populations (Baltic Sea and North Sea).

A strong positive correlation between environmental concentrations of Cu and Ni in the North Sea was observed (see box 1.1). Therefore, the influence of this binary metal mixture on mussel larvae was assessed in **Chapter 5**. The experiments focused on evaluating the mixture toxicity effects at low (environmentally relevant) Ni concentrations dosed at various Cu-Ni ratios. A new statistical method was implemented to determine the optimal mixture model parameters and to simultaneously determine the uncertainty associated with the mixture parameter estimates.

In **Chapter 6** the influence of zinc, the metal reaching the highest metal concentration in the North Sea on Cu toxicity was assessed. Simultaneously inter-population variation in metal and metalmixture toxicity was assessed by performing the experiments on two populations (Baltic Sea and North Sea).

In the final **Chapter 7**, the information obtained in the different experimental chapters was combined and reviewed to evaluate the influence of the different variables on the Cu toxicity and implications for risk assessment.

Below is a schematic overview of the research performed in this thesis (Figure 1.7). The arrows indicate the different interactions/relations that were assessed in this thesis. This scheme will be shown at the start of each following chapter, highlighting the specific research objectives of that chapter.

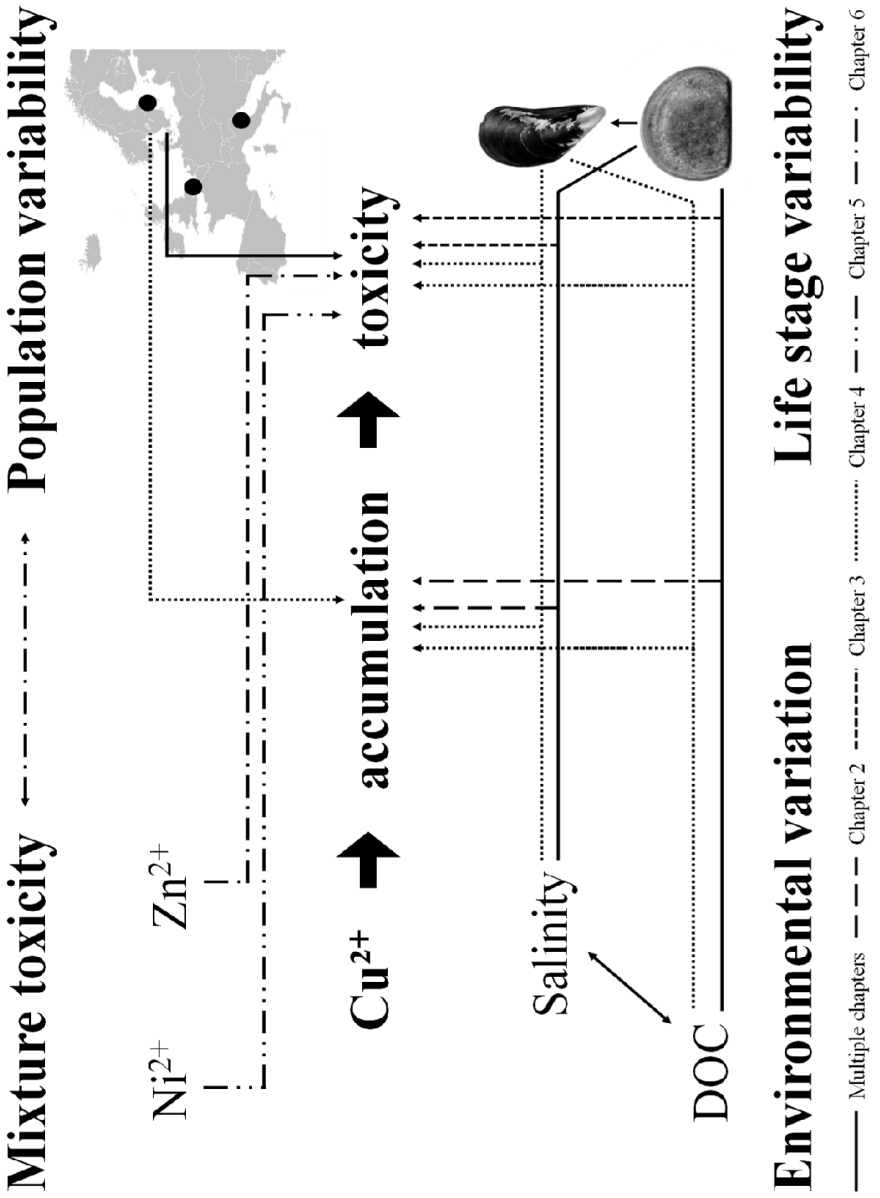
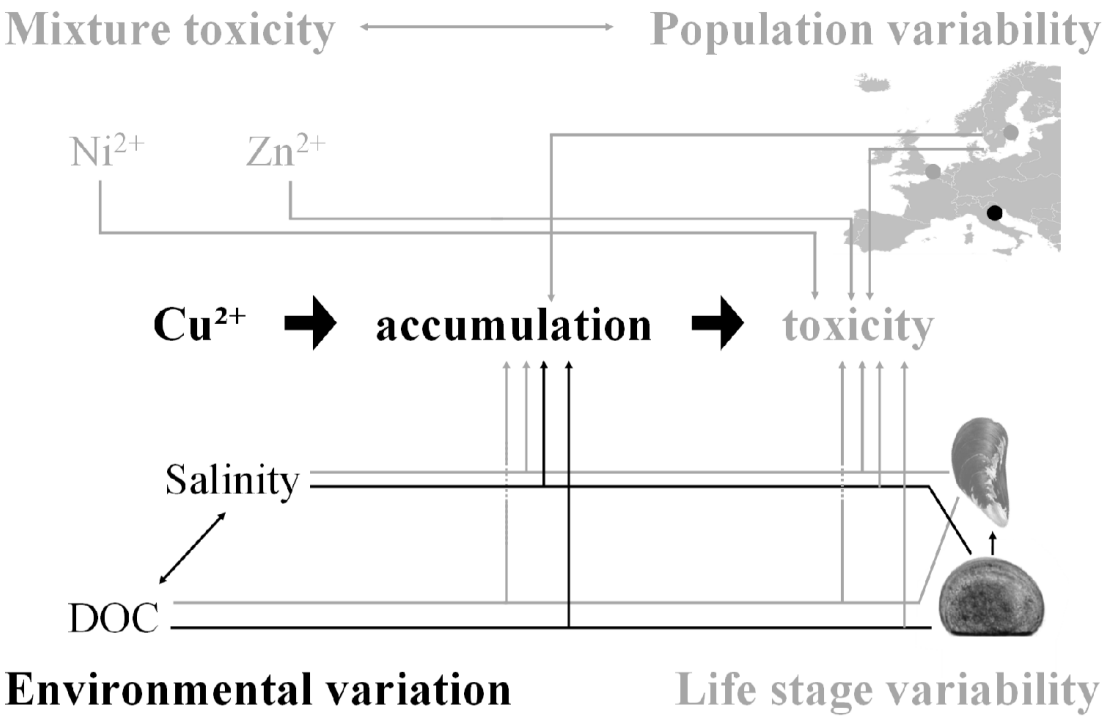


Figure 1.7. Schematic representation of the research performed in this thesis. In each corner the assessed variables are depicted (environment, mixture toxicity, population and life-stage for details see section 2). The arrows indicate the different interactions that were investigated, the type of line indicates the chapter in which the interaction was assessed.


Mixture toxicity
Population variability
Ni2+
Zn2+
Cu2+
accumulation
toxicity
Salinity
DOC
Environmental variation
Life stage variability

II

THE COMBINED EFFECT OF DISSOLVED ORGANIC CARBON AND SALINITY ON THE BIOACCUMULATION OF COPPER IN MARINE MUSSEL LARVAE

Redrafted from:

Deruytter, D., Garrevoet, J., Vandegehuchte, M.B., Vergucht, E., De Samber, B., Vekemans, B., Appel, K., Falkenberg, G., Delbeke, K., Blust, R., De Schamphelaere, K.A.C., Vincze, L. and Janssen, C.R. (2014). The combined effect dissolved organic carbon and salinity on the bioaccumulation of copper in marine mussel larvae. Environmental science and technology 48 (1): 698-705.

ABSTRACT

Larvae of *Mytilus* spp. are among the most Cu sensitive marine species. In this chapter we assessed the combined effect of salinity and dissolved organic carbon (DOC) on Cu accumulation on mussel larvae. Larvae were exposed for 48 hours to three Cu concentrations in each of nine salinity/DOC treatments. Synchrotron radiation X-ray fluorescence was used to determine the Cu concentration in 36 individual larvae with a spatial resolution of 10×10 µm. Cu body burden concentrations varied between 1.1 and 27.6 µg/g DW larvae across all treatments and Cu was homogeneously distributed at this spatial resolution level. Our results indicate decreasing Cu accumulation with increasing DOC concentrations which can be explained by an increase in Cu complexation. In contrast, salinity had a non-linear effect on Cu. This cannot be explained by copper speciation or competition processes and suggests a salinity-induced alteration in physiology.

1 INTRODUCTION

Copper concentrations can be elevated in the aquatic environment both due to natural and anthropogenic influences and this can result in adverse effects on aquatic organisms. However, the total dissolved Cu concentration is, by itself, an inefficient predictor of those effects as they may be influenced by environmental variables [18]. Considerable efforts have been made to improve the prediction of toxic effects by taking into account the aquatic concentration of other elements and dissolved organic carbon (DOC). One, in freshwater, widely used model to predict the accumulation and toxicity of metals including Cu is the Biotic Ligand Model (BLM) [136].

A first BLM principle states that the bioavailability of Cu, which determines Cu accumulation at the biotic ligand, depends on the water chemistry [18]. This can occur through complexation with organic or inorganic substances (e.g. DOC or inorganic anions) or by competition with cations (H, Na, Ca, etc.[2]) for binding with the biotic ligand [18]. According to a second BLM principle, the Cu concentration at the biotic ligand is directly related to the magnitude of the adverse effects of Cu [18]. This implies the existence of a critical Cu biotic ligand concentration, independent of the water chemistry or the organisms' physiology. In the first BLMs developed for freshwater fish, the gills represented the biotic ligand [136]. However, experimentally evaluating the accumulation of metals at the biotic ligand of much smaller animals can be problematic as it is difficult to determine concentrations in different tissues and the actual site of toxic action is often not known.

In a freshwater environment, alterations in the concentration of DOC, Na, Ca etc. can all alter the toxicity of Cu [18, 36]. In marine and estuarine environments, research to elucidate the influence of a changing ion or DOC concentration on metal bioavailability is more scarce. From the available literature it seems that at least DOC can significantly reduce the accumulation and toxicity of Cu in a similar manner as that observed in freshwater systems [40-42]. In contrast to freshwater, in natural seawater the ratios between the different ion concentrations are relatively constant. Although the ion concentration ratios in seawater are constant, the absolute ion concentrations change with changing salinities. According to the BLM concept, a higher salinity (ion concentration) should reduce metal accumulation at the biotic ligand due to the effect of ionic strength on the activity of the free metal ion and others species, increased complexation with inorganic anions (e.g $CuCl_2$ and $CuCO_3$) and increased competition with other cations (e.g. Na) at the biotic ligand. In some cases, this

can indeed explain the salinity-dependent differences in toxicity [33, 42]. However, there is substantial evidence that, besides bioavailability, alterations in the organism's physiology caused by salinity differences are important to explain changes in Cu accumulation and/or toxicity [15, 16, 31-33]. Currently the BLM does not account for changes in physiology [137].

Mytilus spp. are very sensitive to Cu. This makes them ideal species for the further development of a marine/estuarine copper BLM. By using a freshwater BLM (version AP08 build 2001-10-02) the protective effect of DOC on the toxicity of Cu in mussel larvae has successfully been predicted by Arnold et al. [138]. Rosen et al.[42] found a clear relationship between the external Cu concentration and Cu accumulation in *M. galloprovincialis* larvae, demonstrating a decrease in accumulation and toxicity with increasing DOC, confirming the findings of Arnold et al.[138, 139]. Rosen et al.[42] found a critical body Cu residue of 49 µg/g DW larvae at which 50 % of the larvae was deformed after 48 h, independent of the DOC concentration. However, differences in salinity between tested media were small or absent in both studies. As such, to date the effect of a broad range of salinities (e.g. 4-38 psu in the Adriatic sea [140]) on the accumulation of Cu in mussel larvae is still unknown. Moreover, it is unknown whether salinity affects the influence of DOC on Cu accumulation.

Measuring the copper body burden and distribution in mussel larvae is challenging due to their small size (approximate shell length 106 µm [141]). However, this information is necessary to develop a BLM. In this study, we used synchrotron radiation X-ray fluorescence (SR-XRF) micro-spectroscopy to quantify the copper concentration and its spatial distribution in individual larvae. SR-XRF microanalysis has been successfully used to determine the concentration and distribution of metals in various biological tissues such as plants (*Iberis intermedia*), animals (*Daphnia magna*) and humans (breast tumors) [142-145]. Although this technique can analyze the concentration and distribution simultaneously at low concentrations (ng/g), at a high resolution (a few hundred nm) [146, 147] and in a non-destructive manner, the use of SR-XRF microanalysis in ecotoxicology is scarce [148, 149].

The primary goal of research described in this chapter was to assess concurrently the influence of DOC and salinity on the accumulation and distribution of copper in mussel larvae (*Mytilus galloprovincialis*). With this knowledge we evaluated whether the observed accumulation results were in agreement with the BLM principles and if whole larvae can be used as biotic ligand.

2 MATERIALS AND METHODS

2.1 Adult mussel collection and maintenance

Adult *Mytilus galloprovincialis* (n = 150; 5 cm) were collected in the bay of Venice (Italy; March 2012). The ambient salinity was 35 psu and seawater temperature was 8°C. The mussels were transported overnight by plane in an insulated box. The box was filled with ice to keep the mussels cool and avoid spontaneous spawning due to a temperature rise. Upon arrival in the laboratory the mussels were randomly divided in three groups, cleaned and placed in recirculating, aerated, artificial seawater (Instant Ocean®) at a temperature between 8 and 10 °C, a salinity of 35 psu and fed *ad libitum* with Shellfish Diet 1800® (Reed Mariculture Inc.). Salinity was altered in the first week after arrival at a rate of 1 to 2 psu/day until the desired salinity for the spawning experiment was reached (24, 30 or 36 psu; Figure 2.1). The mussels were left to acclimate to the final conditions for another week.

2.2 Experimental design

The experiment was designed to simultaneously assess the effect of salinity and DOC concentration on Cu accumulation in mussel larvae. Mussel embryos were exposed for 48 h to nine combinations of salinity and DOC, based on a central composite design. In that design there is a middle point, four corner points and four star points as extremes (Table 2.1). The measured salinity ranged between 22.9 and 36.8 psu and the DOC concentration between 0.56 and 4.66 mg/L. Exposure media were prepared by combining natural estuarine water, artificial seawater [115] and deionised water to achieve the required salinity/DOC combination. Natural estuarine water (filtered 0.2 µm) was collected in Nieuwpoort (Belgium) and used as a source of natural DOC. A preliminarily test demonstrated that this water did not cause adverse effects on the larval development (> 95 % normal development). In every salinity/DOC treatment, embryos were exposed to a control without added Cu and five different Cu concentrations. The Cu concentrations were chosen based on literature data on Cu toxicity in mussel larvae [139, 150] and are environmentally realistic [151]. Two Cu concentrations were selected out of the five tested concentrations based on visual observation of the larval development.

Table 2.1. The different combinations of salinity, dissolved organic carbon (DOC), Cu and the day they were tested and their position in the central composite design; S = star point, C = corner point, M = middle point

Day	Place	Salinity (psu)	DOC (mg/L)	Cu 1 (µg/L)	Cu 2 (µg/L)	Cu 3 (µg/L)
1	S	22.9	3.0	0.9	3.9	12.4
2	C	24.7	1.2	0.8	3.7	9.0
2	C	25.1	3.9	1.8	4.3	12.2
1	M	29.5	3.0	1.0	4.2	11.6
1	S	29.8	0.6	0.0	2.2	5.3
1	S	29.8	4.7	1.7	4.5	12.6
2	M	30.0	2.8	Lost	3.4	11.5
2	C	34.7	4.6	1.3	3.5	11.0
2	C	34.9	1.5	0.8	3.1	5.4
1	S	36.8	3.0	1.0	7.7	10.9

To ensure equilibrium between Cu and seawater, poly-ethylene vials (50 ml) were filled with 40 ml of the appropriate medium and spiked with copper (as dissolved $CuCl_2$) one day prior to use [152]. Each treatment was replicated four times. Due to logistic restrictions the experiment was split in two groups consisting of five salinity/DOC combinations and set up on two consecutive days (Table 2.1). The middle point (± 30 psu; 2.5 mg DOC/L) was replicated each day to account for possible day to day variation.

2.3 48 hour embryo exposures

Spawning was performed according to the ASTM protocol E724-98 [115] with the following adjustment: to determine the influence of salinity on the copper accumulation, spawning of the mussels and exposure or the embryos was performed in seawater with the desired

salinity and not at the proposed salinity of 34 psu. Embryos from adults acclimated to 24 psu were used for the lowest salinities. Similarly, embryos from adults acclimated to 36 psu were used for the highest salinities (Figure 2.1).

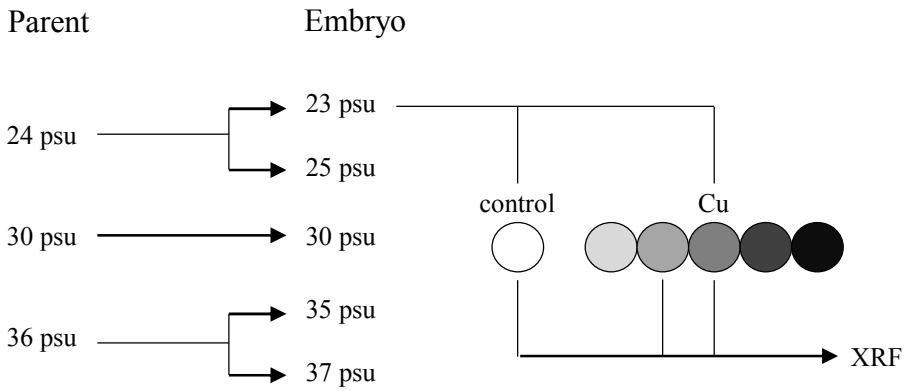


Figure 2.1. A schematic overview of the experimental setup and the nominal salinities

2.4 Analytical chemistry

Water samples for chemical analyses were taken at the start the experiment. Samples were also collected at the end for part of the Cu concentrations (control, middle and highest exposure concentration) to determine the change in Cu and DOC over time. Samples from the 4 replicates were pooled and filtered (0.45 µm). The samples for metal analysis were acidified with 0.14 mol/L analytical grade HNO_3 and stored in polypropylene tubes at 4 °C before analysis. DOC samples were stored in glass tubes at 4 °C ensuring no air was left in the tube. DOC analysis was performed with a Shimadzu TOC-5000 analyzer using the high-temperature catalytic oxidation (HTCO) technique [153, 154]. The statistical analysis was performed on the mean DOC concentration of each salinity/DOC combination. At the end of the test, salinity was measured with a WTW cond 315i (tetracon 325 electrode) and the pH was measured with a Knick portamess (InPro 4260 electrode).

Measurement of dissolved Cu was performed using ICP-MS. A high resolution ICP-MS instrument (Thermo Element 2 XR) was used to determine the metals after appropriate dilution and acidification of the water to reduce salt levels. Instrument settings were used that provided optimal resolution and sensitivity for the given matrix and elements. The Cu concentration at the start of the 48 h exposure period was used for further statistical analysis as recommended by the ASTM [115]. After 48 h on average 32 % of the Cu was present at a

mean initial concentration of 1.00 µg Cu/L (control), 66 % at a mean initial concentration of 6.85 and 92 % at a mean initial concentration of 21.4.

The activity of Cu^{2+} and other Cu species were calculated using Visual MINTEQ 3.0 [155] with the specific interaction theory (SIT) activity correction. Input values were based on measured salinity, pH, DOC and measured total dissolved copper concentration. The Stockholm Humic Model (SHM) was used as model for the Nieuwpoort estuary DOC according to Ndungu [156] with an active $\frac{Dissolved\ Organic\ Matter}{Dissolved\ Organic\ Carbon}$ ($\frac{DOM}{DOC}$) ratio of 2 with 100 % of the active DOM as fulvic acid.

2.5 Synchrotron radiation X-ray fluorescence (SR-XRF) microanalysis

One or two larvae exposed to the control or the two Cu concentrations of each salinity/DOC combination were analysed using microbeam SR-XRF. The larvae were prepared according to the following procedure.

Exposed, live 48 h old D-larvae were dehydrated using a graded acetone/water series (70 %, 80 %, 90 %, 2×98 %, 2×100 % acetone) by submersing a number of larvae consecutively in the different solutions in an acid-washed (15 % HCl) watch glass for five minutes. Subsequently, the larvae were submersed in hexamethyldisilazane (HMDS) for 30 minutes and left to dry overnight in a dessicator [157]. One well developed and well preserved D-larva was and glued on the sharpened tip of a 0.5 mm carbon rod and preserved in a closed dust free container at room temperature (Figure 2.2A). This was done for a total of 36 larvae. One for each Cu/salinity/DOC combinations (n = 29; Table 2.1) and a replicate for seven combinations to assess the variability. Due to time restrictions not all combinations could be tested twice. Previous experiments had indicated that it was impossible to use SR-XRF on undeveloped or heavily deformed larvae due to a poor preservation of the samples.

SR-XRF microanalysis was performed during two experiments at Beamline L of the DORIS-III (HASYLAB) storage ring (4.45 GeV positron ring) at the Deutsches Elektronen Synchrotron (DESY) facility in Hamburg, Germany. During the micro-XRF experiments a Ni/C multilayer monochromator was used to obtain quasi monochromatic excitation energies of 12.5 and 12.7 keV with a spectral bandwidth of $\Delta E/E = 1.83$ %. The primary beam was focussed using a polycapillary based optic, providing a beam size of 36 and 23

µm FWHM (full width at half maximum) respectively. Parameter settings for the synchrotron micro-XRF measurements during the two beam times are listed in Table 2.2.

Table 2.2. The XRF parameters during the two beamtimes

XRF parameters	First beam time	Second beam time
Excitation	12.7 keV	12.5 keV
Beam diameter (FWHM)	23 µm	36 µm
Scanning step-size	10 µm	20 µm
Measuring time/scanning step	15 s	30 s

Each larva was mounted on a goniometer head (device used to correct for sample mounting errors in XYZθφ) to position it vertically with respect to the central axis of the X-ray micro-beam. To minimize self-absorption effects, the sample was placed under a 45° angle with respect to the primary beam. Spectral deconvolution (background removal, elimination of peak overlap and peak-area determination) of each individual XRF spectrum was done using AXIL (Analysis of X-ray spectra by Iterative Least squares) [158]. Elemental distribution images were obtained based on the fitted net-peak intensities, subsequently analysed by K-means clustering algorithms [159] using the in-house developed software MICROXRF2. Before cluster-analysis could be performed, a square root data pre-treatment was performed to take into account Poisson counting statistics with a normalisation to give an equal weight to each elemental constituent included in the K-means clustering algorithm. This resulted in a correct classification of air, halo, larva, carbon tip or a combination of previously mentioned areas (Figure 2.2E). The halo, which is proportional to the concentration in the larvae and clearly visible in figure 2.2D, originates from the beam profile and the 45-degree geometry. The sum-spectra of the obtained clusters were normalised by their respective Compton scattering signal to account for differences in illuminated sample mass. For quantification purposes, a pressed pellet of NIST SRM 1577C (areal density 21.9 mg/cm^2) was measured and the obtained reference spectra were analysed using the same data treatment method as that applied for the unknown samples to ensure a correct quantification. Furthermore, the SRM measurements are used for the determination of the minimum detection limits (MDL, Appendix A: Figure A1). A MDL is defined as the amount of analyte that causes a net peak intensity equal to three times the standard counting error of the background intensity. The Cu body burden is defined as the

total concentration of Cu present in the soft tissues and/or shell of the larvae. No distinction could be made between these two compartments. Although some other studies used EDTA to remove loosely bound Cu on the shell, we assume that eight washing suffice steps as other studies on mussel larvae used a single washing step with deionized water [42, 141].

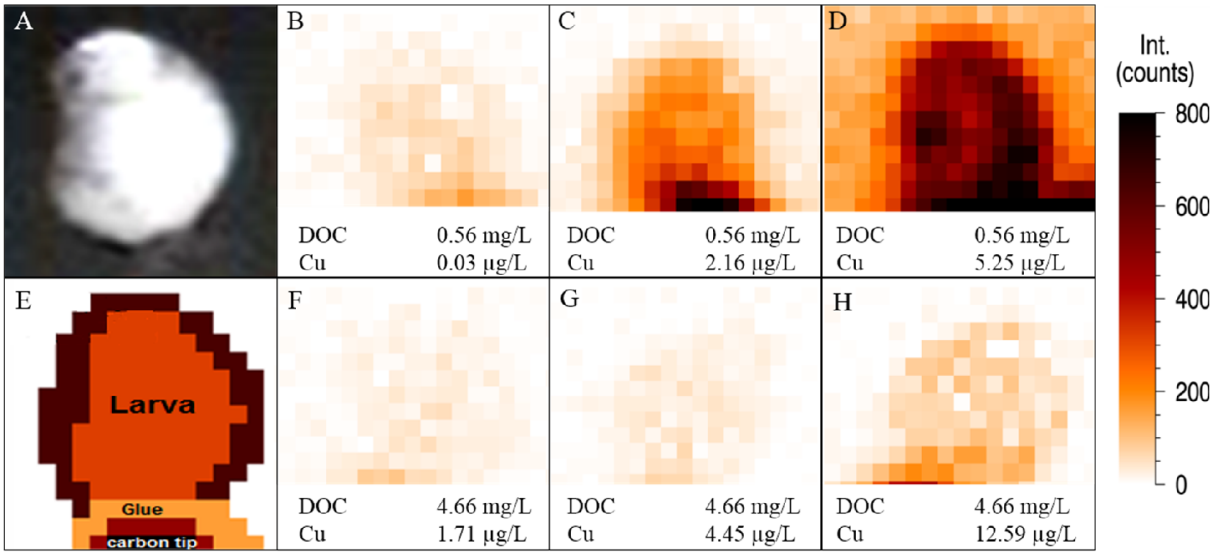


Figure 2.2. Examples of the µSR-XRF data at a resolution of 10×10 µm. A: optical image of the larva; B-D: Cu concentration image of mussel larvae exposed to a salinity of 29.8 psu, a low DOC concentration (0.56 mg/L) and different dissolved Cu concentrations (increasing from left to right); E: result of the clustering analysis with larva, glue, carbon tip and mixture regions; F-H: Cu concentration of mussel larvae exposed to the same salinity (29.8 psu) but a higher DOC concentration (4.66 mg/L) and different dissolved Cu concentrations (increasing form left to right). Darker pixels indicate a higher Cu body burden (see legend on the right). The high Cu concentration at the bottom (dark region) of each image is the result of Cu in the carbon tip which held the larvae.

2.6 Synchrotron radiation X-ray fluorescence (SR-XRF) nanoanalysis

To determine the elemental distribution in mussel larvae at a (sub) µm scale, an additional experiment was performed by exposing mussel larvae (*M. edulis*) for 48 h to a nominal concentration of 10 µg/L Cu in artificial seawater at 32 psu [115], without added DOC. The SR-XRF nanoanalysis was performed at the Micro/Nanoprobe Beamline P06 of the third-generation synchrotron radiation facility PETRA-III (DESY, Germany). This undulator beamline is equipped with a Si111 crystal monochromator providing a spectra bandwidth of $\Delta E/E = 0.014$ % and was set at an energy of 10 keV. The primary x-ray beam was focused using Fresnel Zone plates, providing a beamsize of 50 (H) by 50 (V) nm FWHM. Due to the limited available measurement time, which is typical at synchrotron sources, and the drastic increase in measurement time when measuring samples with nanometer

resolution only one larva could be measured. It was not feasible to measure all 29 larvae with this technique.

2.7 Statistical analysis

Statistical analysis was performed using R 2.12.1 statistical software [160]. There were no significant differences between the two parts of the experiment, therefore the random variable 'day' was not included in further statistical analysis. A linear accumulation model was constructed starting from the full model (Eq. 2.1) with total dissolved Cu, salinity, and DOC.

$$E[\text{Cu accumulation}|Cu, S, DOC] = Cu + S + [DOC] + Cu \cdot S + Cu \cdot [DOC] + S \cdot [DOC] + Cu^2 \cdot S^2 + [DOC]^2 \quad \text{(Eq. 2.1)}$$

E[Cu accumulation|Cu,salinity,DOC] in model 2.1 stands for the expected accumulation of a Cu on a given set of Cu concentration, S (salinity) and [DOC] (DOC concentration). This model was reduced via backward selection. Two commonly used approaches were assessed to determine the optimal model, i.e. selecting the model with only significant factors remaining or the model with the lowest Akaike information criterion (AIC). The latter measures the goodness-of-fit and model complexity and can include non-significant terms [161]. Cu body burdens and Cu^{2+} activity data were $\log_{10}$ transformed to normalize the data. A Shapiro Wilk test confirmed that both datasets were normally distributed after transformation (P-value > 0.05). In the analysis based on Cu^{2+} activity, DOC was not included due to the strong influence of DOC on Cu^{2+} activity and therefore cannot be seen as an independent variable. Although we realize that salinity can influence Cu^{2+} activity there was no correlation between the two variables in our experiment (Appendix A: Figure A2), therefore salinity was not excluded from the model.

3 RESULTS

K-means clustering of the µSR-XRF results successfully segregated the background, the larva, the glue, the carbon tip and larvae/air mixture pixels (Figure 1E). Additional SR-XRF measurements indicate that the Cu concentration in the glue used to hold the larvae onto the carbon rod was below the MDL (Appendix A: Figure A3).

At a spatial resolution level of 10×10 µm the Cu concentrations in the larvae were homogenously distributed in the control organisms and no consistent spatial distribution could be observed in the exposed larvae (Figure 2.2). Therefore, all further analyses are based on the mean total Cu body burden of the whole larva, excluding larva/air mixture pixels. Due to this homogenous Cu distribution, the Cu concentration in the larvae measured at a lower resolution (20×20 µm) could be used without bias. The mean absolute difference in Cu concentration between two larvae exposed to the same conditions was 1.5 µg/g DW larvae (SD: 0.86; n = 7). When data was available for two larvae of the same treatment, the average was taken for further analysis. All Cu body burdens ([Cu_{bb}]) are reported in the supporting information (Appendix A: Table A1). The Cu body burden varied between 1.1 and 27.6 µg Cu/g DW larvae across all treatments with a mean control value over all DOC/salinity combinations of 2.5 µg Cu/g DW larvae (SD: 0.9; n=9). In the larva measured at (sub-)micron resolution level, Cu was spatially heterogeneously distributed at a resolution of 1×1 µm and 200×200 nm as can be seen in Figure 2.3. The Cu accumulation was higher along the edges of the larva and a clear Cu hotspot in the center of the hinge could be observed.

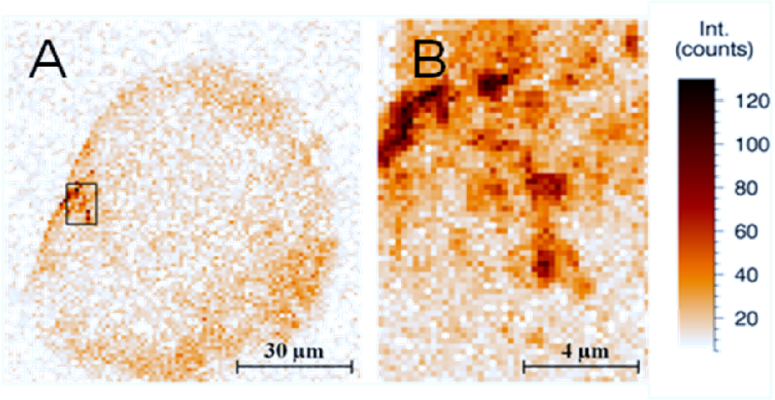


Figure 2.3. High resolution SR-XRF image of Cu distribution in a mussel larva exposed to 10 µg Cu/L, darker regions indicate a higher Cu concentration (see also legend on the right); A: a whole larva at a pixel resolution of 1×1 µm; B: a detail of the same larva at a pixel resolution of 200×200 nm identical as the rectangle indicated in A).

Based on the statistical analysis it is concluded that the total external dissolved aqueous copper concentration ([Cu_{ext}]) alone can explain 46 % (Appendix A: Table A2) of the variation in the observed [Cu_{bb}] values. Backward selection from the full model including [Cu_{ext}], [DOC], salinity, interactions and quadratic effects resulted in equation 2.2 (significant factors only; R^2 adj. = 0.70; n = 29; AIC = -6.3) and equation 2.3 (lowest AIC; R^2 adj. = 0.72; n = 29; AIC = -7.5). Both equations (Eq. 2.2 and Eq. 2.3) can accurately predict the Cu body burden (Figure 2.4A) based on total external dissolved aqueous copper concentration. An increase in [Cu_{ext}] significantly increases [Cu_{bb}]. Addition of DOC significantly reduces Cu accumulation in mussel larvae (Figure 2.4B) and salinity has a non-linear effect on Cu accumulation with a maximum Cu accumulation at 32 psu (Figure 2.4C). The standard error, P-value and proportion explained variation of the individual terms is provided for each model in appendix A (Table A2).

$$\log_{10}[Cu_{bb}] = -3.46 + 6.83\cdot10^{-2}[Cu_{ext}] - 0.127\cdot[DOC] + 0.275\cdot S - 4.39\cdot10^{-3}\cdot S^2 \quad \text{(Eq. 2.2)}$$

$$\log_{10}[Cu_{bb}] = -3.56 + 0.13\cdot[Cu_{ext}] - 4.66\cdot10^{-3}\cdot[Cu_{ext}]^2 - 0.123\cdot[DOC] + 0.276\cdot S - 4.43\cdot10^{-3}\cdot S^2 \quad \text{(Eq. 2.3)}$$

With [Cu_{bb}] as µg/g dry weight larvae, [Cu_{ext}] as µg/L, DOC as mg/L and salinity (S) as psu.

The logarithm of Cu^{2+} activity ($\log_{10}\{Cu^{2+}\}$) by itself explains 70 % of the variation in Cu body burden. All terms in the equation (Eq. 2.4) with the lowest AIC were significant (Table A2; R^2 adj. = 0.74; n = 29). An increase in $\{Cu^{2+}\}$ is associated with an increase of the Cu body burden ($P < 0.001$), the salinity (psu) has a similar effect on Cu accumulation as the models based on total dissolved Cu. 90 % of the predicted Cu body burdens are within a factor 2 of the observed Cu concentrations (Appendix A: Figure A4).

$$\log_{10}[Cu_{bb}] = -0.163 + 0.237\cdot\log_{10}\{Cu^{2+}\} + 0.251\cdot S - 4.04\cdot10^{-3}\cdot S^2 \quad \text{(Eq. 2.4)}$$

With [Cu_{bb}] as µg/g dry weight larvae, $\{Cu^{2+}\}$ as mol/L and salinity (S) as psu.

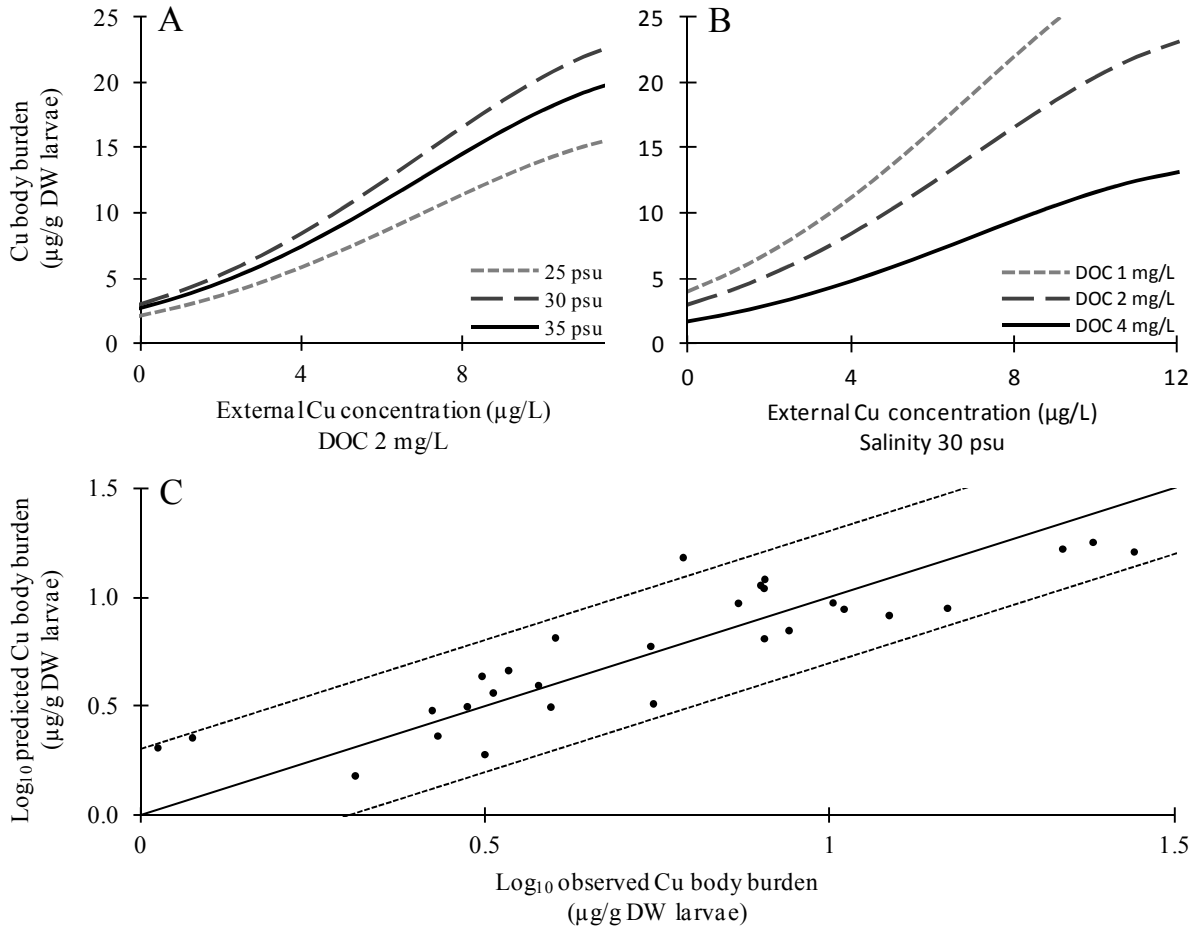


Figure 2.4. A: the predicted effect of a changing salinity (25, 30 and 35 psu) and [Cu_{ext}] on [Cu_{bb}] at a fixed [DOC] of 2 mg/L according to model 2.3; B: the predicted effect of a change in DOC (0.8, 2 or 3.2 mg DOC/L) and [Cu_{ext}] on [Cu_{bb}] at a fixed salinity of 30 psu according to model 2.3; C: predicted [Cu_{bb}] (Equation 2.3: $\log_{10}[Cu_{bb}] = -3.56 + 0.129\cdot[Cu_{ext}] - 4.66\cdot10^{-3}\cdot[Cu_{ext}]^2 - 0.123\cdot[DOC] + 0.276\cdot S - 4.43\cdot10^{-3}\cdot S^2$) compared to observed [$Cu_{bb}$] (n=29), all but one value differ less than a factor 2 (dotted outer lines) between predicted and observed [Cu_{bb}]

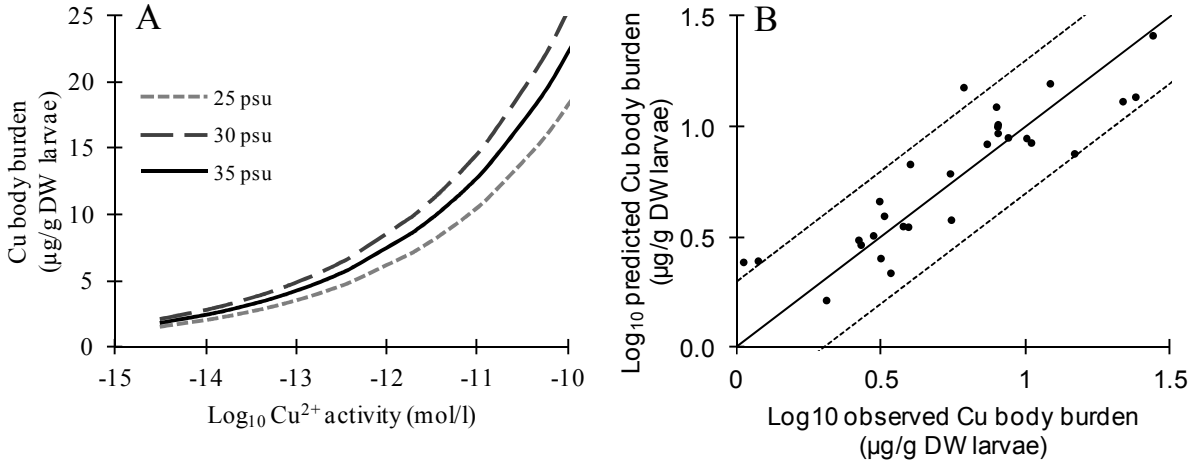


Figure 2.5. A: The modelled effect (Eq. 2.4) of salinity and Cu^{2+} activity on the Cu body burden; B: The predicted versus observed Cu body burdens.

4 DISCUSSION

This is, to our knowledge, the first study to measure the concentration and spatial distribution of Cu in individual mussel larvae. The present study indicates that synchrotron radiation X-ray fluorescence (SR-XRF) spectroscopy is a useful tool to determine the concentration and distribution of Cu in small organisms with a sub-micron spatial resolution.

The Cu distribution in the larvae revealed no consistent spatial variation at a resolution level of 10×10 µm (Figure 2.1). Therefore, the statistical analyses were performed on the mean Cu concentration in the whole larvae and the entire larva was considered as potential biotic ligand. However, at high spatial resolution level (1 µm or less; Figure 2.2), Cu in an exposed larva seemed to be heterogeneously distributed. At the moment it is unclear if this heterogeneity is consistent over several larvae. Because of the exponential increase in measuring time when the resolution is increased, it was at the moment not feasible to perform these high resolution measurements on all larvae in our experimental design. The sub-micrometer scale XRF measurements offer unprecedented possibilities for future research regarding the biological implications of spatially resolved metal accumulation in small biological samples like these mussel larvae and smaller certainly if technological advances reduce the measuring time.

Cu accumulation increased with increasing total external dissolved aqueous Cu concentration. However, the Cu body burdens in the present study were a factor 2.9 lower than the concentrations found by Rosen et al. (2008)[42] (calculated based on the mean ER_{50}: 49.2 µg Cu/g DW) compared to calculations made by equation 2.3 (17.1 µg Cu/g DW). The dry weight of the larva used in that study ranged approximately between 40 and 70 ng. This is a factor 1.7 to 4.5 lower compared to the larva dry weight values found in other studies of 134 ng[162], 182 ng[163] (at 100 µm shell length, which is approximately the length of a 48 h old D-larva) or between 120 to 160 ng [164]. The Cu body burdens found by Geffard et al. (2002)[164] are also lower than those found by Rosen et al. (2008)[42] but comparable to the concentrations found in our study. The reason behind this difference is unclear but may be due to differences in the drying protocol (air drying compared to freeze-drying). This difference in dry weight may have resulted in a much higher Cu concentration per unit of larval dry weight reported in their study [42]. In the present study, mussel larvae were immediately dehydrated after the exposure and subsequently embedded in HMDS. This

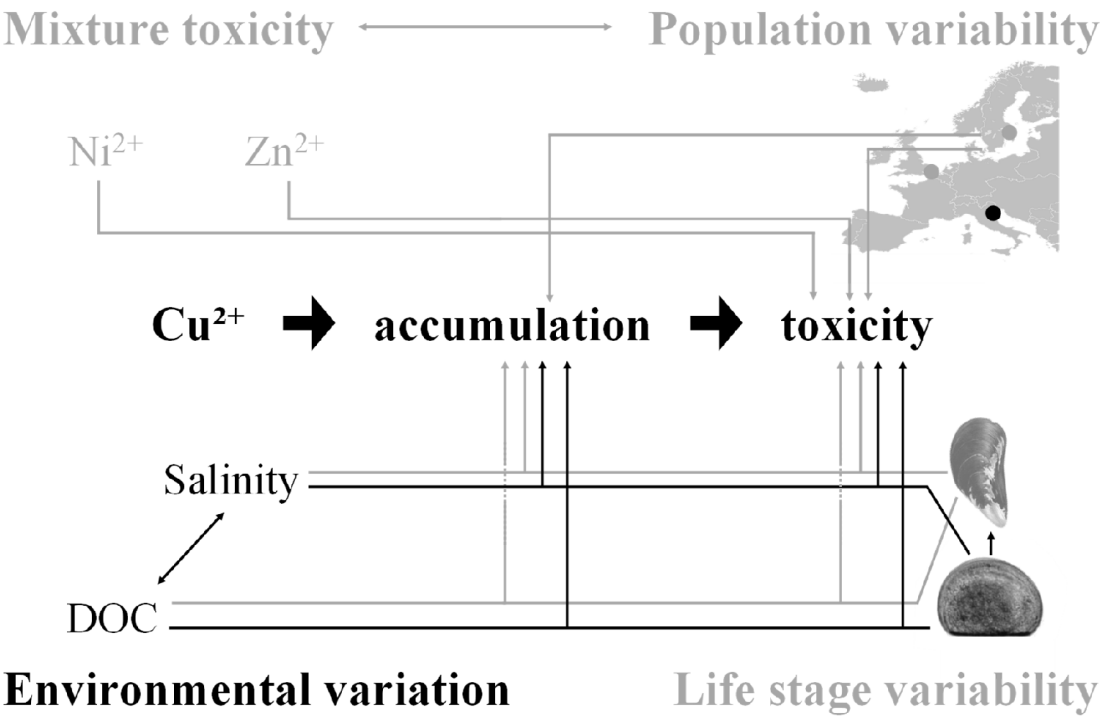
allowed, to our knowledge for the first time, measurements of Cu accumulation in individual mussel larvae. Additional experiments have confirmed that this sample preparation technique does not alter the Cu body burden in mussel gills when the Cu body burdens are compared to conventionally oven dried gills (and ICP-MS; appendix A: Figure A5).

Models based on total dissolved copper indicate that both [DOC] and salinity have a significant effect on Cu accumulation (Eq. 2.2 and 2.3). Reduced Cu accumulation with increasing DOC concentrations was expected, as DOC is known to reduce the accumulation and toxicity of Cu through the formation of Cu-DOC complexes [42, 139]. For example, equation 2.3 predicts a 43 % decrease in Cu accumulation when [DOC] increased from 1 to 4 mg DOC/L at a dissolved Cu concentration of 8 µg/L. According to the BLM, an increase in salinity should increase the complexation of Cu with anions in the water, increase the ionic strength of the water and increase the competition with cations at the biotic ligand, therefore reducing the bioavailability of Cu. However, in the present study a non-linear effect of salinity on Cu accumulation was observed with a steep increase in Cu accumulation up to 32 psu. Exposure to higher salinities reduced Cu accumulation in the larvae. This suggests that a change in larval physiology rather than a change in competition from cations caused the salinity-dependent alterations in Cu accumulation, certainly in the 23 to 32 psu range. According to Grosell et al. (2007)[16], physiology is important as changing salinity alters the osmolarity of the water and Cu toxicity is minimal at iso-osmotic conditions for osmoregulating organisms. However, mussel larvae are osmo-conforming; therefore salinity should influence their physiology in a different way. The salinity where Cu accumulation is the highest corresponds closely to the salinity of 34.8 psu at which *M. galloprovincialis* larvae have been reported to develop optimally [165, 166]. This could indicate that a better development, and presumably higher metabolism, increases Cu uptake. Further research is needed to elucidate this relation. Although salinity explained only 7.1 % of the variation in our experiment and the effect of salinity on Cu accumulation is less pronounced than the effect of DOC (21 % of the variation explained), salinity should not be neglected when studying Cu accumulation in mussel larvae.

Cu^{2+} activity is, according to the BLM principles, the key determinant of Cu accumulation and adverse effects to aquatic organisms [136]. Considering the whole mussel larva as a biotic ligand, our accumulation data can indeed be modeled more accurately using only Cu^{2+} activity instead of only total dissolved Cu (R^2 adj. increased from 0.46 to 0.70). This

indicates that, to some extent, the variation in accumulation is due to variations in Cu bioavailability. This is most probably the case for DOC as this effect is eliminated when modeling with Cu^{2+} activity instead of total dissolved Cu. Some studies suggest that $Cu(CO_3)_2^{2-}$, $Cu(OH)^+$ or Cu-DOC complexes can also be, partly, responsible for copper toxicity [15, 18, 45, 46]. In the present study, although Cu accumulation in mussel larvae is best predicted by Cu^{2+} activity, an influence of $Cu(CO_3)_2^{2-}$ or $Cu(OH)^+$ on Cu accumulation cannot be excluded due to high covariation between the Cu species.

Including salinity as predictor improves the model fit (significant and lower AIC; model 2.4). According to speciation calculations in Visual Minteq, the effect of salinity on the Cu^{2+} activity is small in the concentration range studied, with an increase in Cu^{2+} activity between 5 and 20 % between the lowest and highest salinity depending on [DOC]. This increase in Cu^{2+} activity may be the result of an increased competition between Cu and cations (e.g. calcium) at DOC binding sites [167]. According to model 2.4 a 20 % increase in Cu^{2+} activity would result in an increased accumulation of maximum 4 % and cannot account for the 46 % increase in Cu accumulation that was observed between 25 and 32 psu (based on equation 2.3; at $[Cu_{ext}]$ = 8 µg/L). Therefore it is not surprising that salinity reduced the AIC of the regression model (Eq. 2.4) and is considered important for predicting the copper accumulation based on Cu^{2+} activity. This model (Eq. 2.4) indicates that salinity altered Cu accumulation in a similar way as model 2.2 or 2.3 (37 % increase in Cu accumulation between 25 and 32 psu). Contradictory to our results, Cu accumulation should, according to the BLM, decrease with increasing salinities at the same Cu^{2+} activity and [DOC] due to increased competition at the biotic ligand. Thus, competitive effects cannot explain the observed changes in Cu accumulation in the present study. An altered physiology due to salinity changes is probably at the basis of the observed effect of salinity on Cu accumulation.


Mixture toxicity
Population variability
Ni2+
Zn2+
Cu2+
accumulation
toxicity
Salinity
DOC
Environmental variation
Life stage variability

III

SALINITY AND DISSOLVED ORGANIC CARBON BOTH AFFECT COPPER TOXICITY IN MUSSEL LARVAE: COPPER SPECIATION OR COMPETITION CANNOT EXPLAIN EVERYTHING

Redrafted from:

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ABSTRACT

Predicting copper toxicity in marine and estuarine environments is challenging due to the influence of anions on Cu speciation, competition between Cu^{2+} and other cations at the biotic ligand and the effect of salinity on the physiology of the organism. In this paper the combined effect of salinity and dissolved organic carbon (DOC) on Cu toxicity to larvae of *Mytilus galloprovincialis* was assessed. Two statistical models were developed and used to elucidate the relationship between Cu toxicity, salinity and DOC. All models based on dissolved Cu indicate a decrease in Cu toxicity with increasing DOC concentrations, which can partly be explained by complexation of Cu^{2+} ions with DOC. These models also indicate an increase in Cu toxicity (modeled with dissolved Cu or Cu^{2+} activity) with increasing salinity, suggesting a salinity-induced alteration in the physiology of the mussel larvae. When based on Cu body burdens, neither of the models indicate an effect of salinity or DOC. This shows that the Cu body burden is a more constant predictor of Cu toxicity, regardless of the water chemistry influencing Cu speciation or competition and possible physiological alterations or changes in Cu speciation or competition.

1 INTRODUCTION

Copper (Cu) is present in all aquatic environments as a natural element. It is essential for all organisms as a cofactor in numerous enzymes [168]. Due to this essentiality, organisms have developed mechanisms to regulate their internal Cu concentration when the environmental Cu availability changes. However, when the external concentration increases beyond a certain threshold (e.g. through human activity), Cu homeostasis can no longer be maintained and deleterious effects may occur.

To predict and assess possible adverse effects of elevated aquatic Cu concentrations, the Cu biotic ligand model (BLM) is a widely used tool which was first described by Di Toro et al. [17] and further refined by other authors [18, 20]. According to this model, Cu toxicity can be predicted by assessing Cu accumulation at a discrete site of action or a biotic ligand [169]. This Cu accumulation at the biotic ligand can be altered due to complexation with anions (e.g. to $CuCO_3$) or with dissolved organic carbon (DOC) and due to competition with other cations at the biotic ligand. The Cu BLM has proven to be accurate in freshwater environments where it can predict Cu toxicity under different conditions of dissolved organic carbon concentration ([DOC]), Na concentration, pH, etc. [170].

Salt water is substantially different from freshwater: its pH is well buffered and major ions always occur in approximately the same ratios. Cu speciation or competition may be influenced by changes in the salinity or DOC concentration. The freshwater BLM has been used in salt water with some success by Arnold et al. [138] who accurately predicted the effect of DOC on Cu toxicity. However, potential effects of changing salinity were not explicitly investigated in that study due to the narrow salinity range (28-32 psu) used. There is substantial evidence that when assessing the influence of salinity on metal toxicity, not only metal speciation and competition should to be taken into account but also changes in the physiology of the exposed organisms have to be considered. Several experiments have demonstrated increased Cu toxicity with increasing salinity or a nonlinear effect of salinity on Cu toxicity instead of the toxicity decrease that is expected based on speciation and competition [16]. Currently, the BLM does not account for changes in physiology. Arnold et al. (2009) found that DOC reduced Cu toxicity to larvae of both *Mytilus edulis* and *Mytilus galloprovincialis* [139]. Nadella et al. (2009) assessed the effect of DOC and salinity (independently) on Cu toxicity to *Mytilus trossulus* larvae [150]. In that study, the effect of DOC was similar to that observed by Arnold et al. [139]. However, Nadella et al. (2009) did

not find a significant effect of salinity on Cu toxicity [150]. The latter is not in agreement with the BLM principles, according to which an increase in salinity (from 60 to 100 % of full strength seawater) should result in an increased competition at the biotic ligand and a reduction in Cu^{2+} activity due to complexation with anions.

Mussels, especially the embryo-larval stages of development, are among the most Cu sensitive marine species. This fact, combined with their ecological [124, 127] and economic [171] importance make *Mytilus* spp. very appropriate to further develop the marine Cu BLM. In the present chapter, the combined effects of changes in DOC concentration and salinity on Cu toxicity to *M. galloprovincialis* larvae were assessed. Testing both factors simultaneously has the advantage that interactions can be modeled and allows a direct comparison of DOC concentration and salinity changes. Results were combined with the data from chapter 2 on Cu accumulation to evaluate if whole mussel larvae can be regarded as biotic ligand and if Cu accumulation is a good predictor of Cu toxicity.

2 MATERIAL AND METHODS

The effect of changes in DOC concentration and salinity on Cu toxicity to Mytilus galloprovincialis larvae was assessed in the same experiment as described in chapter 2. A summary of the materials and methods used, and a more extensive description of the determination of toxic effects is given below.

2.1 48 h mussel larvae toxicity test

The simultaneous effects of natural DOC and salinity on Cu toxicity were assessed using nine salinity/DOC combinations based on a central composite design (Chapter 2: Table 2.1) testing a salinity range between 22.9 and 36.8 psu and a [DOC] range between 0.56 and 4.66 mg/L (measured values). Treatments were replicated four times. The test was set up on two consecutive days, each day including the middle point, with twice as many replicates as the other treatments. The different mixtures were made by combining natural estuarine water rich in DOC (Nieuwpoort; Belgium), artificial seawater [115] and deionised water to achieve the desired salinity/DOC combination. For every combination, a toxicity test with a control and five different Cu concentrations was set up. The control and two Cu concentrations of this experiment were used in chapter 2 to assess Cu accumulation in developing larvae. Cu (as a $Cu(II)Cl_2$ solution; VWR international; Analytical Grade) was spiked directly into poly-ethylene vials 24 h before exposure of the embryos [152, 172].

Adult mussels (*M. galloprovincialis*) were collected in the bay of Venice (Italy) and acclimated in the lab for 14 days. Spawning was performed according to the ASTM Standard Guide E724-98 with minor adjustments [115]. Spawning was induced by administering a heat shock. Spawning mussels were removed from the batch and the gametes were collected and checked for quality and quantity. Eggs and sperm of sufficient quality, quantity and no older than 30 minutes were combined to initiate fertilization. After one hour the quality and quantity of the embryos (most of them at the two or four cell stage) was assessed again. Finally, the embryos were transferred to the test vials (density 80 individuals/mL; total volume 40 mL) and left undisturbed at 16 °C for 48 h to develop into D-larvae.

After 48 h the vials were gently stirred to homogenize the distribution of the larvae. Three samples of 1 mL per replicate were transferred to a 24-well plate. The larvae were killed and preserved by adding formaldehyde to a final concentration of 2 % (vol:vol). Multiwell plates were covered and stored at 4 °C until counting using an inverse microscope (10×10× magnification). Larvae were manually counted according to the ASTM Standard Guide E724-98 [115].

2.2 Analytical chemistry

Pooled, filtered (0.45 µm) water samples were taken for chemical analyses at the start the experiment. Samples were also collected at the end for the control, middle and highest Cu exposure concentration to determine the change in Cu and DOC over time. Dissolved Cu concentrations were determined by ICP-MS analysis (LOD = 0.1 µg Cu/L). DOC concentrations were measured with a Shimadzu TOC-5000 analyzer using the high-temperature catalytic oxidation (HTCO) technique (LOD = 0.25 mg/L) [153, 154]. The Cu concentration at the start of the 48 h exposure period was used for further statistical analysis as recommended by the ASTM [115]. After 48 h on average 32 % of the Cu was present at a mean initial concentration of 1.0 µg/L (control), 66 % at a mean initial Cu concentration of 6.9 µg/L and 92 % at a mean initial Cu concentration of 21.4 µg/L. At the end of the test, salinity was measured with a WTW Cond 315i (tetracon 325 electrode) and the pH was measured with a Knick portamess (InPro 4260 electrode). All equipment was acid washed (1.8 mol HCl/L) before use.

The Cu^{2+} activity was calculated using Visual MINTEQ with Specific Interaction Theory (SIT) activity correction based on the salinity, pH, DOC and dissolved copper concentration [155]. The Stockholm humic model (SHM) was used as model for the Nieuwpoort estuary DOC according to Ndungu with an active $\frac{Dissolved\ Organic\ Matter}{Dissolved\ Organic\ Carbon}$ ($\frac{DOM}{DOC}$) ratio of two and 100 % of the active DOM as fulvic acid [156]. Additional speciation calculations were made to understand the influence of salinity on Cu^{2+} activity in the studied range.

2.3 Statistical analysis

Statistical analysis was performed using R 2.12.1 [160]. The analysis was performed using dissolved Cu concentration, Cu^{2+} activity or Cu body burden when appropriate. To determine the effect of Cu, salinity and DOC on larval development two statistical approaches were used. In a first approach generalized additive modeling (GAM) was applied. This technique has the advantage that all concentration-response data (n = 72) can be used because GAMs do not a priori assume a linear relationship between predictor and response variables. GAMs are therefore able to model effects similar to a concentration response curve but taking into account two independent variables (DOC and salinity). A binomial distribution was used with a logit link function and the data were normalized to 100 % larvae development at the Cu concentration with the highest proportion of developed larvae (maximum 106 % of the control). This was necessary to avoid fractions of developed larvae higher than 1. The following full model (Eq. 3.1) was used for the GAM:

$$E[\text{Fraction}|\text{Cu, salinity, DOC}] = \text{intercept} + f(\text{Cu, salinity, DOC}) \quad \text{(Eq. 3.1)}$$

With E[Fraction|Cu,salinity,DOC] the expected fraction of developed larvae at a given combination of Cu concentration (as dissolved Cu; µg/L), salinity (psu) and DOC concentration (mg/L). The smoothing function f denotes the combined effect of Cu, salinity and DOC. This full model was reduced via backward selection to find the model with the lowest Akaike information criterion (AIC). The latter measures the goodness of fit and model complexity and can include non-significant terms [161]. DOC and the Cu^{2+} activity are correlated with each other and therefore cannot be used both as independent variables (Pearson correlation coefficient = 0.42). Hence a GAM model with only salinity and Cu^{2+} activity as predictors was constructed. This could not accurately predict the fraction developed larvae and the residuals were not randomly distributed around zero when plotted against the independent variables (Appendix B: Figure B1-B2). Therefore no conclusions could be made from this model.

As a second approach, the more classic concentration-response models were used and combined with linear modeling (LM) of obtained Cu EC_x values (Cu concentration at which X percent is adversely affected). Although this technique does not use all data points, it allows one to create a mathematical model to predict the Cu EC_x. EC_{50}s and EC_{10}s, based on dissolved Cu, for larval development were derived for each salinity/DOC combination

using the 3-parameter log-logistic function in the concentration-response curve analysis (DRM) R-package [173]. Visual MINTEQ was used, as described above, to calculate the Cu^{2+} activity at the EC_{50}s and EC_{10}s. A LM was constructed to predict the EC_{50}s or EC_{10}s as a function of salinity (S) and DOC concentration ([DOC]). The full model (Eq. 3.2) contained the individual terms, all two way interactions and quadratic effects. This model was reduced via backward selection. Two methods were used to determine the optimal model, i.e. selecting the model with only significant factors remaining ($p < 0.05$) or the model with the lowest AIC.

$$E[ECx|\ salinity, DOC] = S + [DOC] + S\cdot[DOC] + S^2 + [DOC]^2 \quad \text{(Eq. 3.2)}$$

To assess the relation between the Cu body burden (Cu_{bb}) and the larval development both a GAM (identical to the equation described above (Eq. 3.1) and a linear model were constructed (Equation 3.3).

$$E[Fraction|Cubb, salinity, DOC] = S + [DOC] + Cubb + Cubb\cdot S + Cubb\cdot[DOC] + S\cdot[DOC] + Cubb^2 + S^2 + [DOC]^2 \quad \text{(Eq. 3.3)}$$

This equation was reduced according to the selection criteria described above. Because neither the GAM or LM indicated any effect of salinity or [DOC] the drm-package in R was used (with the 3-parameter Weibull function) to construct a concentration-response curve of normal larval development (corrected to 100 % normal control development) versus Cu accumulation after 48 h exposure (Chapter 2; Appendix A: Table A1) to determine the EC_{50} and EC_{10}, expressed as a Cu body burden.

Finally, the accumulation equation described in Chapter 2 (Eq. 2.2) was combined with the internal Cu EC_{50} and EC_{10} to assess if these predicted body burdens can be related to the external Cu EC_{50}s or EC_{10}s. The accumulation model based on dissolved Cu was used because this analysis is more robust when based on measured Cu concentrations versus modeled Cu^{2+} activity. Furthermore the model including only the significant factors was selected for this assessment, since the absence of a quadratic Cu term allows a straightforward inverse use of the equation.

$$[Cu_{ext}EC_{50}] = (\log_{10}[Cu_{int}EC_{50}] + 3.46 + 0.127\cdot[DOC] - 0.275\cdot S + 4.39\cdot10^{-3}\cdot S^2) / 6.83\cdot10^{-2} \quad \text{(Eq 3.4)}$$

3 RESULTS

The fraction of normally developed larvae was above 80 % in all control treatments after 48 h with the exception of the 34.7 psu/4.56 mg DOC/L treatment, where it was 66 %. There was no significant effect of a changing salinity or DOC concentration on the control larval development. The individual concentration-response data and fitted curves for each combination salinity/[DOC] combination can be found in appendix B (Figure B3). Dissolved Cu EC_{50}s ($EC_{50[Cu]}$), EC_{10}s and their standard errors are summarized in Table 3.1.

Table 3.1. Summary of the different salinity and dissolved organic carbon concentrations at which *M. galloprovincialis* larvae were tested with corresponding $EC_{50[Cu]}$ and $EC_{10[Cu]}$ values for larval development and their standard error (SE) in µg dissolved Cu/L.

Salinity (psu)	DOC (mg/L)	$EC_{50[Cu]}$ (µg/L)	$EC_{50\ [Cu]}$ SE (µg/L)	$EC_{10[Cu]}$ (µg/L)	$EC_{10[Cu]}$ SE (µg/L)
22.9	3.0	12.2	1.1	10.0	8.0
24.7	1.2	10.1	0.1	8.5	0.1
25.1	3.9	13.4	0.3	9.4	0.5
29.3	3.0	12.6	0.5	10.1	1.1
29.6	3.0	12.2	1.5	10.4	1.6
29.8	0.6	4.1	0.3	2.8	0.5
29.8	4.7	13.6	0.6	11.2	0.7
29.9	2.8	10.3	0.4	8.1	1.1
30	2.8	11.0	2.2	9.6	6.5
34.7	4.6	10.7	0.4	8.2	1.7
34.9	1.5	5.0	0.1	2.9	0.1
36.8	3.0	8.3	0.3	6.0	0.4

DOC = Dissolved organic carbon; SE = Standard Error

3.1 Analysis: dissolved Cu

The GAM indicates that both [DOC], salinity alter the effect of dissolved Cu on larval development. An increase in [DOC] decreases Cu toxicity whereas an increase in salinity increases toxicity (Figure 3.1). Eliminating salinity or [DOC] from the model resulted in a higher AIC (AIC respectively 782 or 1556) compared to the full model (AIC: 445) indicating that all three predictors are important in predicting larval development. The residuals were randomly distributed around zero when plotted against the independent variables (Appendix B: Figure B4) or in the predicted vs observed values graph (Figure 3.1C), indicating a good model fit.

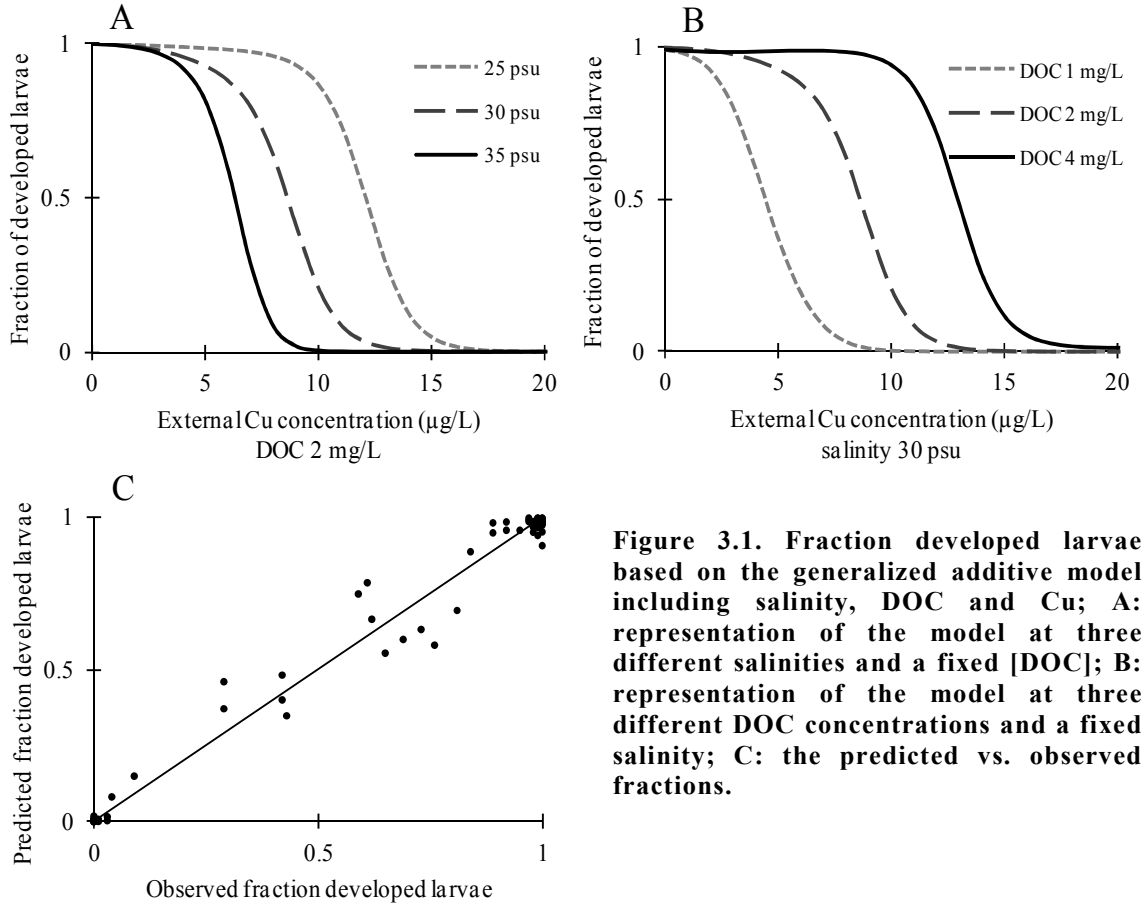


Figure 3.1. Fraction developed larvae based on the generalized additive model including salinity, DOC and Cu; A: representation of the model at three different salinities and a fixed [DOC]; B: representation of the model at three different DOC concentrations and a fixed salinity; C: the predicted vs. observed fractions.

A similar and significant effect of [DOC] and salinity was found when the Cu EC_{50}s and EC_{10}s were analyzed based on a LM (Figure 3.2), i.e. increasing EC_{50} values with increasing DOC and decreased EC_{50}s with increasing salinity. Backward selection of the full model resulted in two models: equation 3.5 (only significant terms; R^2 adj. = 0.89; AIC = 38.9; n = 12) and equation 3.6 (lowest AIC; R^2 adj. = 0.90; AIC = 38.7; n = 12). The

variability, *p* value and explained variation (individual R^2) of each term can be found in appendix B (Table B1) together with the analysis based on the $EC_{10[Cu]}$.

$$EC_{50[Cu]} = 8.03 + 4.19 \cdot [DOC] - 0.414 \cdot [DOC]^2 - 6.33 \cdot 10^{-3} \cdot S^2 \quad \text{(Eq. 3.5)}$$

$$EC_{50[Cu]} = -8.54 + 4.40 \cdot [DOC] - 0.454 \cdot [DOC]^2 + 1.11 \cdot S - 2.47 \cdot 10^{-2} \cdot S^2 \quad \text{(Eq. 3.6)}$$

With $EC_{50[Cu]}$ in µg/L, [DOC] in mg/L and salinity (S) in psu

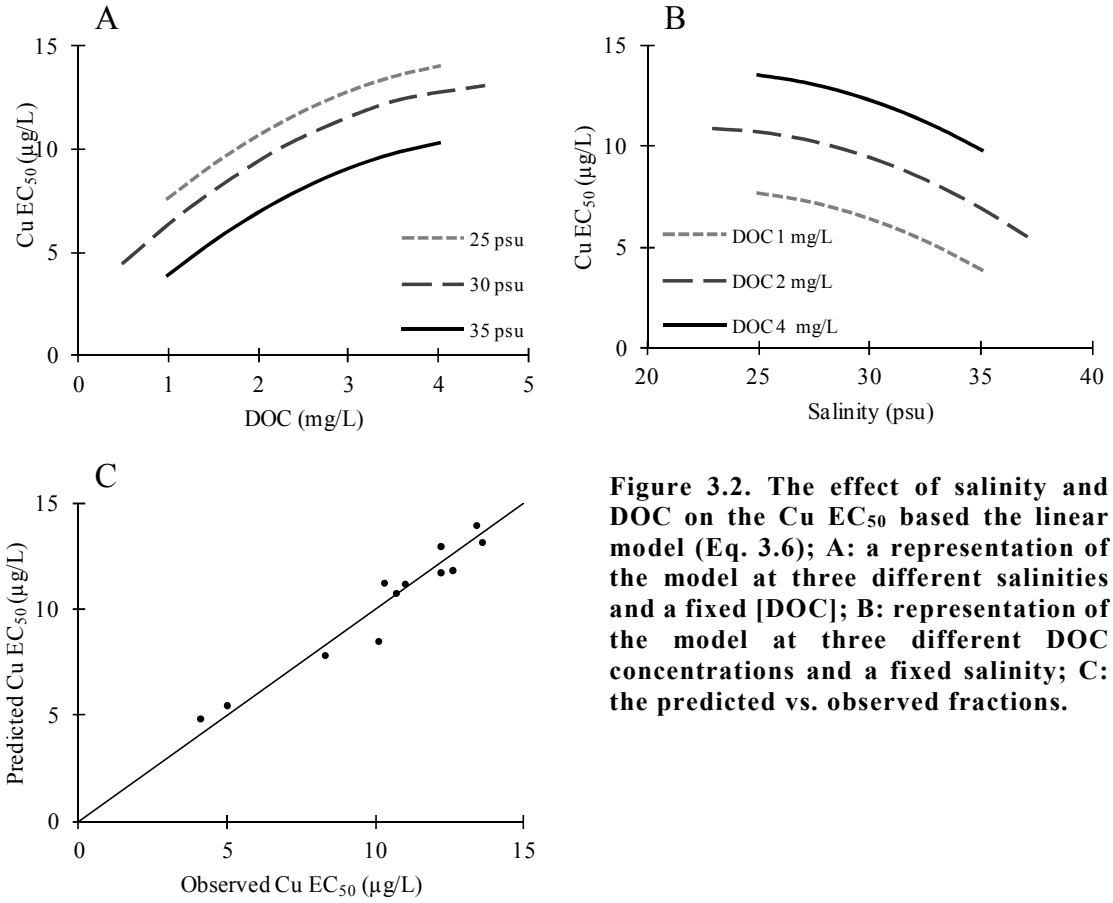


Figure 3.2. The effect of salinity and DOC on the Cu EC_{50} based the linear model (Eq. 3.6); A: a representation of the model at three different salinities and a fixed [DOC]; B: representation of the model at three different DOC concentrations and a fixed salinity; C: the predicted vs. observed fractions.

3.2 Analysis based on Cu^{2+} activity

The EC_{50}s base on Cu^{2+} activity ($EC_{50\{Cu2+\}}$) ranged from $2.05 \cdot 10^{-12}$ mol/L to $5.68 \cdot 10^{-11}$ mol/L. The model (Eq. 3.7) based on significant factors only did not differ from the model with the lowest AIC (R^2 adj. = 0.96; n = 12). As in the models based on dissolved Cu, an increasing salinity resulted in a decreased $EC_{50\{Cu2+\}}$ (Figure 3.3). A higher DOC concentration, however, lead to in a lower $EC_{50\{Cu2+\}}$ values. The analysis based on the Cu^{2+} activity EC_{10}s resulted in similar conclusions and are given in appendix B.

$$\text{Log}_{10}\, EC_{50\{Cu2+\}} = -9.66 - 0.498\cdot[DOC] + 3.86\cdot10^{-2}\cdot[DOC]^2 - 4.58\cdot10^{-4}\cdot S^2 \quad \text{(Eq. 3.7)}$$

With $EC_{50\{Cu2+\}}$ in mol/L, [DOC] in mg/L and salinity (S) in psu.

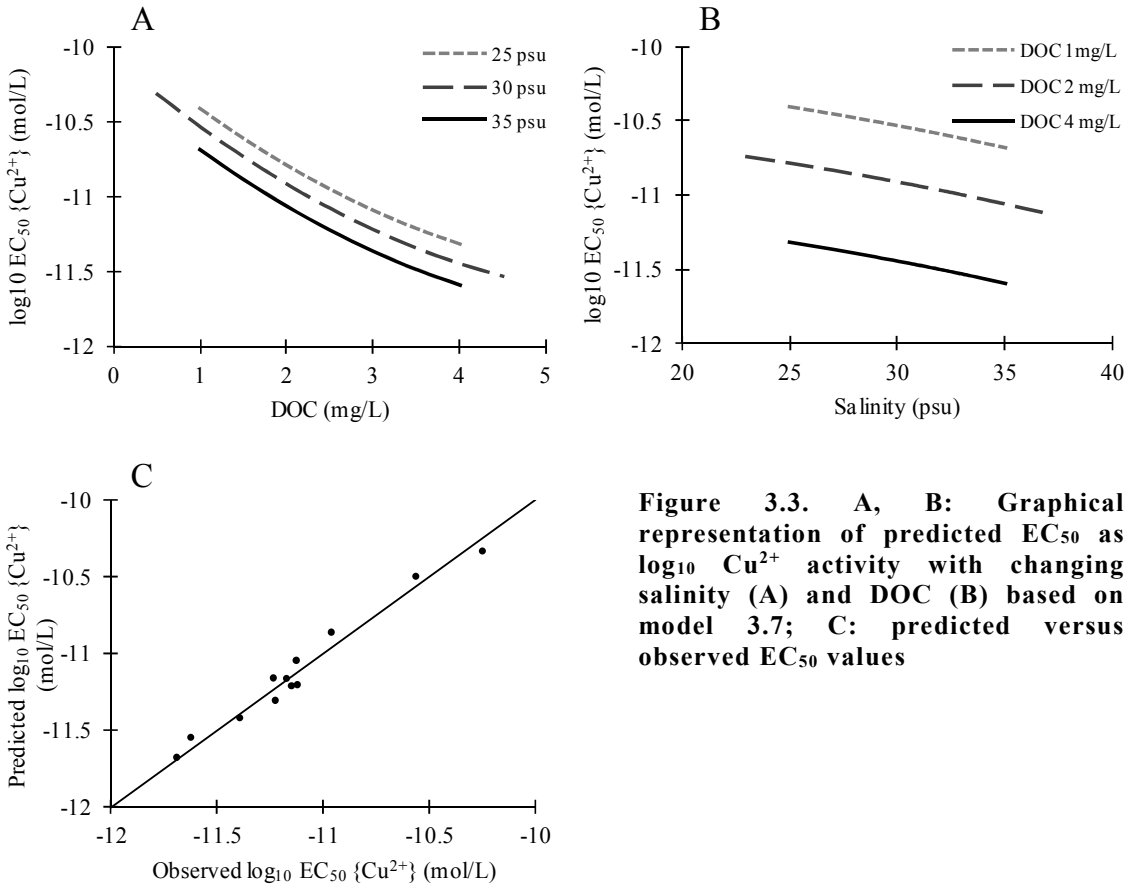


Figure 3.3. A, B: Graphical representation of predicted EC_{50} as $\log_{10}$ Cu^{2+} activity with changing salinity (A) and DOC (B) based on model 3.7; C: predicted versus observed EC_{50} values

3.3 Analysis based on Cu body burdens

The Cu body burden [Cu_{bb}] previously determined in Chapter 2 were also used to predict the percentage of normal larval development. Salinity and [DOC] did not significantly affect larval development and did not reduce the AIC based on LM or GAM analysis. A concentration-response curve of normal larval development was constructed based on Cu body burden (Figure 3.4), resulting in equation 3.8 with an $EC_{50[Cubb]}$ of 16.6 µg Cu/g DW larvae (95 % confidence interval: 11.2-22.0 µg Cu/g DW) and an $EC_{10[Cubb]}$ of 5.3 µg Cu/g DW larvae (95 % confidence interval: 3.5-7.0 µg Cu/g DW).

$$\%\ \text{normal development} = 100 - 100 \times \exp(-\exp(-1.046(\text{LN}(Cu_{bb}) - \text{LN}(11.67)))) \quad \text{(Eq. 3.8)}$$

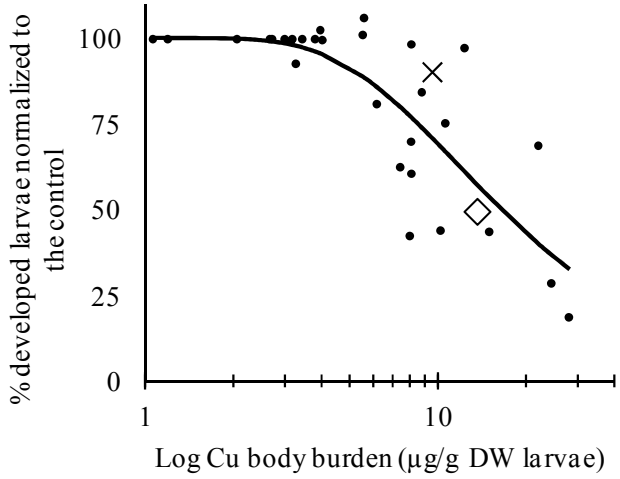


Figure 3.4. Concentration-response data and fitted curve based on internal Cu concentrations, with an EC_{50} of 16.6 µg Cu/g DW. The cross and diamond indicate respectively the $EC_{10[Cubb]}$ and $EC_{50[Cubb]}$ based on model 2.2 from chapter 2.

Under the assumption that there is a critical body burden, the $EC_{50[Cubb]}$s and $EC_{10[Cubb]}$s were also directly calculated from model 2.2 (chapter 2) by using each $EC_{50[Cu]}$ or $EC_{10[Cu]}$ and the associated salinity and DOC. This resulted in a mean $EC_{50[Cubb]}$ of 13.4 µg/g DW larvae with a 95 % CI between 5.2 and 21.7 µg/g DW larvae (range between 8.0 and 20.2) and a mean $EC_{10[Cubb]}$ of 9.6 µg/g DW larvae with a CI between 3.1 and 16.1 (range between 5.6-14.5). These values are not significantly different from the values obtained by the concentration-response curve (t-test *p* value: 0.5) but the higher EC_{10} and lower EC_{50} indicate that the slope of the concentration-response curve might be steeper (Figure 3.4).

3.4 Combining water chemistry, Cu accumulation and Cu toxicity

Combining the accumulation model based on the dissolved Cu concentration with the $EC_{50[Cubb]}$ or $EC_{10[Cubb]}$ (Eq. 3.8) resulted in predicted dissolved Cu EC_{50} and EC_{10} values that closely match the observed values. However, changing the EC_{10} and EC_{50} body burden to 9.6 and 13.4 µg Cu/g dry body weight respectively increased the fit (reduced sum of squares errors) between predicted and observed values (Figure 3.5; Appendix B: Figure B7).

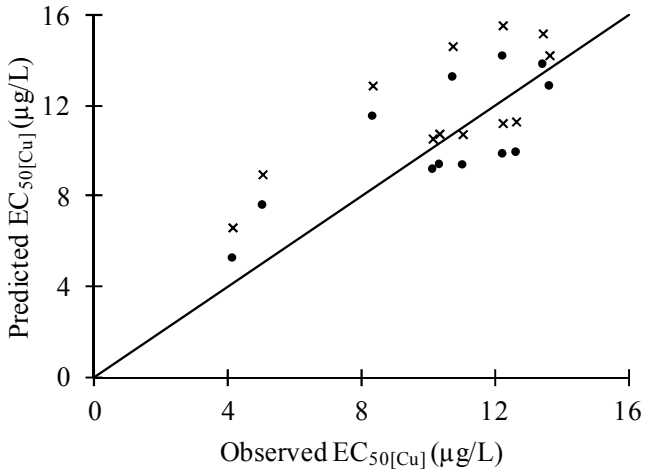


Figure 3.5. Predicted versus observed EC_{50} values, crosses with a $EC_{50[Cubb]}$ of 16.6 (sum of squares = 78) and circles with a $EC_{50[Cubb]}$ of 13.4 (sum of squares = 47).

4 DISCUSSION

The linear model (LM) with dissolved copper EC_{50}s and EC_{10}s and the general additive model (GAM) based on dissolved Cu all indicate similar trends for the effect of DOC and salinity on Cu toxicity. Therefore, the different models (LM and GAM) will not be discussed separately, unless needed.

According to the BLM principles a decrease in toxicity based on dissolved Cu is expected with increasing salinity and/or DOC concentrations. In our study, an increase in [DOC] indeed significantly reduced Cu toxicity based on dissolved Cu in all models (LM and GAM). This is in accordance with previous studies on the effect of DOC on Cu toxicity in mussel larvae and is related to the binding of ionic Cu to organic ligands [42, 139, 150]. The increase in $EC_{50[Cu]}$ with increasing DOC as observed in the present study (Eq. 3.2) is almost identical to the results obtained with the model proposed by Arnold et al. (2009) and derived for waters with a salinity of 28-32 psu (Figure 3.6) [139].

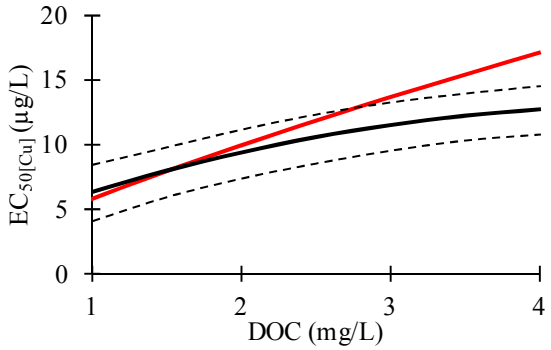


Figure 3.6. A comparison between the models of Arnold et al. (2009) (solid red line) and the developed model in this study (Eq 3.2), the solid black line indicates the predictions at a salinity of 30 psu. The dashed lines indicate the 95 % confidence interval including variations between 28-32 psu.

According to the BLM, apparent differences in Cu toxicity due to complexation should disappear if one models using Cu^{2+} activity as the predictor instead of with the dissolved Cu concentration as this removes any effect of DOC on Cu toxicity. However, our LM (Eq. 3.7) indicates that DOC still has a significant effect when the EC_{50}s are expressed as Cu^{2+} activity. Indeed, when expressed on a Cu^{2+} activity basis Cu toxicity increased with increasing DOC (Figure 3.3). This observation suggests that Cu-DOC complexes may be partially bioavailable and contribute to Cu toxicity. It is known that humic acid can be

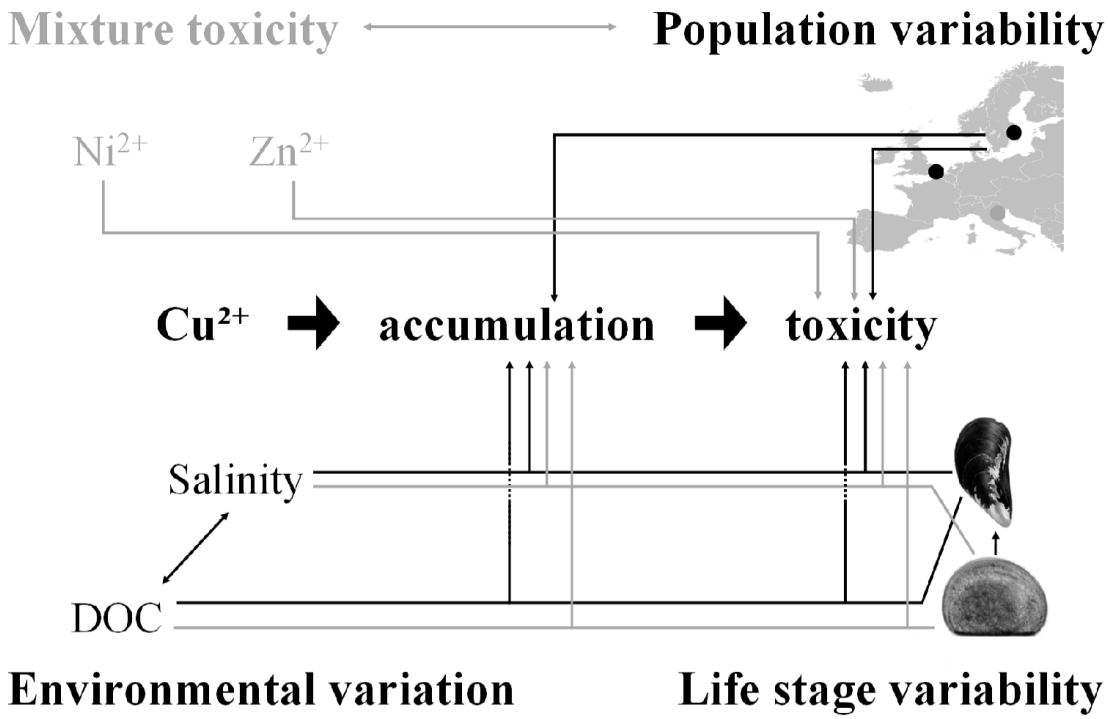
partially available for uptake in the gills of juvenile *Mytilus edulis* and *Perna viridis* [45, 46]. Another explanation for the calculated decrease in $EC_{50\{Cu2+\}}$ with increasing [DOC] may be a nonlinear decrease in Cu^{2+} activity with increasing DOM at a fixed dissolved Cu concentration in seawater as multiple binding sites (weak and strong) may exist [174]. However, according to Chadwick et al. an increase in the dissolved Cu/DOC ratio should result in a linear increase of Cu^{2+} in the range used in this study ($3.7 \cdot 10^{-8}$ – $1.3 \cdot 10^{-7}$ mol dissolved Cu/mg DOC). Therefore, non-linearity should not be an issue in this study. Furthermore, our calculated concentration of Cu^{2+} (not activity) ranges between $9.9 \cdot 10^{-12}$ and $2.6 \cdot 10^{-10}$ which is similar to the predicted Cu^{2+} concentrations made by Chadwick et al. (2008) [174].

The data presented clearly indicate that salinity influences Cu toxicity in mussel larvae. Including salinity in the developed models significantly reduced the variation in the observed effects (e.g. Figure 3.1). In contrast to the BLM principles an increase in salinity resulted in an increase in Cu toxicity. According to theoretical speciation calculations, the effect of salinity on the Cu^{2+} activity is small in the studied range, with an increase in Cu^{2+} activity between 5 and 20 % when the salinity is increased from 23 to 37 psu, depending on [DOC]. This increase in Cu^{2+} activity may be the result of an increased competition between Cu and cations (e.g. calcium) at DOC binding sites [167]. However, these changes cannot explain the 60 % differences in $EC_{50\{Cu2+\}}$ between the lowest and highest salinity. Furthermore, according to the BLM an increased cation concentration in the medium should increase competition with Cu^{2+} ions and decrease Cu toxicity when expressed as Cu^{2+} activity. However, this is opposite of what we observed. The results cannot be explained by speciation in the water or competition at the biotic ligand and indicate that a change in physiology rather than a change in Cu speciation or competition is causing the changes in Cu toxicity at different salinities. The increase in toxicity with higher salinities largely coincided with enhanced Cu accumulation discussed in chapter 2 (up to 32 psu). A non-linear change or increasing sensitivity to Cu with increasing salinity has also been observed in other animals like killifish (*Fundulus heteroclitus*) and ragworm (*Hediste diversicolor*) [16]. According to Grosell et al. (2007) an increased transcellular Na^{+} gradient leads to an increased Cu sensitivity in osmoregulating animals when the salinity deviates from the iso-osmotic point [16]. Changes in physiology (e.g. hemocyanin concentration and osmolarity of the hemolymph) with salinity changes have also been observed in osmoconforming invertebrates [33]. However, the mechanisms behind this remain unclear.

Nadella et al. (2009) did not find an influence of salinity on Cu toxicity in mussel larvae [150]. Potential reasons why our results differ from that study may be the use of a different (sub)species (*M. trossolus*) [175], the lack of statistical power to detect the patterns with three different salinities in a narrower range in their study or the difference in acclimatization time of the adult mussels (24 h vs 2 weeks).

When comparing the variation in Cu EC_{50} in our dataset explained by including salinity (8-34 %) and/or DOC (48-88 %) in the model, it is clear that DOC is the most important factor to predict Cu toxicity although salinity should not be neglected. This also becomes apparent when examining the magnitude of the effect of salinity and DOC on Cu toxicity. Based on equation 3.6 the $EC_{50[Cu]}$ decreased with 35 % over a salinity range between 25 and 35 psu (at [DOC] of 2 mg/L), while the $EC_{50[Cu]}$ doubled over the 1.0 to 4.0 mg DOC/L (Figure 3.1 at 30 psu). This means that there is a higher probability for effects at a given dissolved Cu concentration in *M. galloprovincialis* larvae in areas with a high salinity and low DOC concentration.

The Cu body burden is a more constant predictor of Cu toxicity and is independent of the water chemistry influencing complexation and competition, possible bioavailability of Cu-DOM complexes or physiological influences of salinity. The $EC_{50[Cu]}$ can be relatively accurately predicted based upon the critical body burden calculated from the concentration response curve. Nonetheless, the actual slope of the body burden – response curve might be steeper than what is depicted in Figure 3.4, since the recalculation of critical body burdens based on the accumulation model and the $EC_{x[Cu]}$ values observed in each toxicity test resulted in a higher EC_{10} and a lower EC_{50}. This uncertainty stems from the inability to determine the Cu concentration in malformed larvae at higher Cu concentrations. Combining the observation of a critical Cu body burden with our previous research that indicated the absence of a spatially consistent Cu distribution in mussel larvae up to a resolution of 10x10 µm (Chapter 2), the whole larva can be regarded as the biotic ligand in a marine BLM for mussel larvae.


Mixture toxicity
Population variability
Ni2+
Zn2+
Cu2+
accumulation
toxicity
Salinity
DOC
Environmental variation
Life stage variability

IV

SALINITY, DISSOLVED ORGANIC CARBON AND INTER-POPULATION VARIABILITY HARDLY INFLUENCE CU ACCUMULATION AND TOXICITY IN *MYTILUS EDULIS*

Redrafted from:

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ABSTRACT

To improve the ecological relevance of environmental risk assessment (ERA) we need to improve our understanding of: (1) the influence of environmental conditions on the toxicity of pollutants, and (2) the effect of these factors in combination with possible inter-population variability. In this study the influence of salinity and dissolved organic carbon (DOC) on the accumulation in, and toxicity of Cu to settled mussels was investigated. The experiments were performed with mussels obtained from a North Sea and a Baltic Sea population. We found that both populations were equally sensitive to copper even though the Baltic Sea population lives in suboptimal conditions. The Baltic Sea mussels, however, accumulated more Cu. This suggests that these two populations may have different ways to cope with excess Cu. The influence of salinity on the Cu toxicity to settled mussels was limited for both populations. An increase in DOC concentration did not decrease the Cu accumulation or toxicity in either population. This suggests that DOC-Cu complexes are bioavailable for settled mussels. These findings are in contrast with previous research which indicated that DOC decreased the toxicity and accumulation of Cu in the larval stage. As a consequence, mussel larvae are not the most Cu sensitive life stage at high DOC concentrations. Furthermore, a DOC correction factor for Cu toxicity cannot be used for settled mussels. This should be accounted for in future marine Cu ERA.

1 INTRODUCTION

Every species can survive and reproduce within a certain range of environmental conditions. As these conditions are not always optimal, a population can adapt over time to better deal with the specific challenges of its local environment [63, 64]. These local adaptations can include changes in the physiology and morphology of the organism which may alter its sensitivity towards other (anthropogenic) stressors [65, 66, 176]. *Mytilus edulis*, for example, thrives in the full strength seawater of the North Atlantic, but a population has adapted to the brackish water of the Baltic Sea. It has been observed that the Baltic Sea mussel population is physiologically stressed under the ambient conditions of the Baltic Sea as they have a lower metabolism and have to allocate more energy to basic maintenance, resulting in a lower growth rate [65, 120-123]. It has been suggested that Baltic mussels, due to the salinity stress, could be more sensitive to (anthropogenic) pollution compared to mussels that live in and originate from oceanic seawater [58, 59].

Environmental conditions may change the sensitivity of different populations to metals, but can also change the bioavailability of metals as a result of a changing water chemistry (e.g. salinity and dissolved organic carbon (DOC)). It has been demonstrated for both the freshwater and marine environment that an increase in [DOC] can decrease the bioavailability of Cu due to complexation and therefore ameliorate Cu accumulation and toxicity [31, 119, 177, 178]. In freshwater, a change in ion concentration has also been known to affect the toxicity of Cu due to complexation (anions) or competition at the site of uptake (cations) [17, 18, 170]. Likewise, in the marine environment a change in salinity (ion concentration) can also alter Cu toxicity. However, salinity may also have a strong direct influence on the physiology of the organism and change the sensitivity of the organism [16]. Therefore the relationship between the water chemistry and the toxicity is not always fully described by changes in Cu speciation or competition at the site of uptake [16].

In conclusion: the environment may affect the toxicity of a metal by: 1) directly changing the bioavailability and 2) indirectly by changing the sensitivity or physiology of the organism. It is possible that organisms from different populations may have a different response to changes in the environment and therefore respond differently to copper pollution, but this is currently unknown.

The goal was to address the possible inter-population differences in Cu sensitivity of *Mytilus edulis* in combination with changes in Cu toxicity due changes in salinity and DOC. To achieve this goal, settled mussels were exposed to different combinations of salinity, DOC and Cu. The experiments were performed with two mussel populations: 1) a population at optimal salinity conditions (North Sea; 30 psu) and 2) a population at the lower limit of the species' salinity range (Baltic Sea; 6 psu). The results were compared the results of chapter 2 and 3 and with previously published research[150, 179] on the effect of DOC and salinity on Cu toxicity to mussel larvae.

2 MATERIAL AND METHODS

2.1 Collection and maintenance

The "North Sea (NS) *Mytilus edulis*" specimens were collected at the Belgian coast (N 51° 11' 45'', E 2° 49' 38''; salinity 32 psu), "Baltic Sea (BS) *M. edulis*" specimens were collected near Stockholm (N 58° 44' 40'', E 17° 28' 09''; salinity 6 psu). Upon arrival in the laboratory the mussels were placed in a tank containing recirculating, artificial seawater (Instant Ocean®) at a temperature of 15 °C. The mussels were fed *ad libitum* and kept in the lab for 3 weeks before the start of the experiments. In the first two weeks, the salinity was modified (1 to 2 psu/day) from their local salinity (6 and 32 psu) to the salinity used in the experiments. During the last week, the mussels were left to acclimate to the final experimental conditions.

2.2 Mussel experiments

Three experiments were performed to assess the influence of salinity and DOC on the Cu toxicity in combination with inter-population variability. The first experiment was designed to assess the influence of environmental variation (salinity and DOC) on the toxicity of Cu for the two populations. In the second experiment both populations were exposed simultaneously in an identical salinity range (fixed [DOC]) to assess inter-population differences in intrinsic Cu sensitivity. In the third experiment North Sea mussels were exposed to two different DOC concentrations with a fixed salinity to confirm the DOC results of the first experiment. An overview of the experimental salinity and DOC ranges and the measured endpoints can be found in Table 4.1. The experiments are described in detail below; a schematic representation can be found in appendix C (Figure C1).

Table 4.1. The DOC and salinity ranges and measured endpoints for the three experiments performed in this study.

	North Sea population		Baltic Sea population		Endpoints				
Exp.	DOC (mg/L)	salinity (psu)	DOC (mg/L)	salinity (psu)	CR	VO_2	NH_4-P	BB	GB
1	2.6-7.6	17.2-44.3	3.1-9.4	4.0-17.1	x	x	x	x	x
2	4.9	16.9-34.2	4.9	16.7-33.5	x	NA	NA	x	x
3	1.8-6.1	30	NA	NA	x	NA	NA	x	NA

Exp.: experiment; DOC: dissolved organic carbon; CR: Clearance rate; VO_2 = oxygen consumption; NH_4-P: ammonium production; BB: body burden; GB: gill burden; x = assessed endpoints; NA = not applicable

Experiment 1

This experiment was performed (non-simultaneously) with both North Sea and Baltic Sea mussels. Mussels were exposed to 15 combinations of Cu, salinity and DOC, based on a three-factor central composite design (CCD; Appendix C: Table C1). In this design there was a central point, 8 corner points and 8 star points as extremes. We used ten replicates for the central point and five for each other combination. The DOC and Cu concentration range used was almost identical for both populations (DOC between 2.6-9.4 mg/L, dissolved Cu between 1.5 and 56.3 µg/L), however, the salinity range used for the Baltic Sea mussels (to include their native salinity) was lower than that used for the North Sea population (Table 4.1).

The test media were prepared by combining natural estuarine water, artificial seawater (Instant Ocean) and deionised water to achieve the required salinity/DOC combination. Natural estuarine water (filtered 0.2 µm) was collected in the harbour of Nieuwpoort (Belgium) and used as a source of natural DOC. Previous experiments indicated that this water was not toxic to mussel larvae (Chapter 3). Copper (as $CuCl_2$, VWR international, Analytical Grade) was added one day prior to test initiation to ensure chemical equilibrium [152].

Due to the size of the experiment (110 aquaria), it was set up on three consecutive days with on each day a block of the CCD (Appendix C: Table C1). Three mussels were placed in a glass aquarium (0.5 L) filled with 250 mL of test medium. All aquaria were placed at random in a temperature controlled room at 15 °C. The mussels were fed daily with $6 \cdot 10^4$ cells/(mL·mussel) of Shellfish diet 1800®. The medium was renewed every two or three

days, after which the aquaria were re-randomized. Exposure duration was 14 days. A preliminary experiment had indicated that this is a suitable duration for chronic toxicity testing as the Cu toxicity became time independent after nine days (for details see Appendix C: Figure C2).

The following physiological endpoints were measured after 14 days: Clearance rate (CR), oxygen consumption (VO_2) and ammonium production (NH_4-P). To assess the CR the mussels were transferred to a clean aquarium (with the same medium) and left to acclimate for 30 minutes. Food was added (shellfish diet 1800®, $6 \cdot 10^4$ cells/(mL·mussel)), the water was gently stirred to ensure a homogenous distribution of the algae and water samples were taken to determine the initial algae concentration (C_0). After 30 minutes new samples were taken to assess the final algae concentration (C_1). The algae concentration was determined using a Z1 coulter particle counter (Beckman Coulter™). The CR was calculated using the following formula (Equation 4.1):

$$CR = (V \cdot (\log_e(C_0) - \log_e(C_1)))/(t \cdot n \cdot DW) \quad \text{(Eq. 4.1)}$$

With CR in L/ (g DW·h), V = exposure volume (L), C_0 = initial algae concentration, C_1 = final algae concentration, t = time (h), n = number of mussels in per aquarium, DW = average dry weight of the soft tissue per mussel (g).

The VO_2 and NH_4-P were measured by placing the three mussels in a 250 mL glass vessel which was completely filled with air-saturated medium and closed airtight for three hours. The O_2 concentration was measured at the start (T_0) and end (T_1) using a WTW oxi 3210 (with a Cellox 325 electrode) and the NH_4 concentration with the Merck spectroquant® NH_4^+ testkit in combination with the Thermo Aquamate spectrometer. To ensure that the filtration by the mussel continues, algae were added (Shellfish Diet 1800®; $6 \cdot 10^4$ cells/(mL·mussel)) to these vessels. Blanks were used to correct for any change in the background O_2 and NH_4 concentration in the test vessel. The VO_2 and NH_4-P were calculated using the following formula (Equation 4.2):

$$VO_2 \text{ or } NH_4\text{-P} = (V \cdot (T_1 - T_0))/(t \cdot n \cdot DW) \quad \text{(Eq. 4.2)}$$

With VO_2 in ml/(g DW·h), NH_4-P in mg/(g DW·h), V = exposure volume (L), T_0 concentration at the start (ml/L for O_2, mg/L for NH_4), T_1 concentration at the end (ml/L

for O_2, mg/L for NH_4), t = time (h), n = number of mussels per aquarium, DW = average soft tissue dry weight per mussel (g).

After test termination, the soft tissue of two mussels from each replicate was removed subsequently to assess Cu accumulation, further referred to as the body burden (BB). The dry weight was determined after drying the mussels for three days at 60 °C. The tissue was digested in 0.5 mL analytical grade HNO_3 (68 %; 80 °C; 3 h) for metal analysis. The Cu concentration was determined via ICP-OES (Thermo scientific, iCAP 7000 series). The protocol used resulted in a mean Cu recovery of 107 % using NIST standard reference material 2977 (mussel tissue). Due to the differential accumulation between different tissues and the key role of the gills in Cu accumulation, gills from two mussels per Cu-salinity-DOC combination were dissected and processed as described above (with 0.3 mL HNO_3) to determine the copper concentration in the gill, further referred to as gill burden (GB). A preliminary analysis X-ray fluorescence (XRF) was performed to assess the distribution of Cu in the gill. Cu is homogeneously distributed at a resolution of 100 by 100 µm, further dissection of the gill was therefore deemed unnecessary. For details on the XRF analysis see Appendix C (Figure C3).

Experiment 2

In this experiment the test design was identical as that used in the first experiment, however, here simultaneous for the two populations (to avoid temporal bias) using an identical salinity range (16.7 – 34.2 psu; see Appendix C: Table C2), but without variations in DOC concentration (4.9 mg/L). The salinity range did not include the native salinity of the Baltic Sea population due to the inability of the North Sea mussels to survive in a salinity lower than 15 psu. In this experiment only the CR was assessed as most sensitive Cu toxicity endpoint in combination with the BB and GB to assess the Cu accumulation (three gills/combination).

Experiment 3

To confirm the results of experiment 1, the influence of DOC on Cu toxicity was tested using mussels from the North Sea population by testing a full concentration response curve with a control and seven Cu concentrations for two DOC levels (1.8 – 6.1 mg DOC/L) at a fixed salinity of 30 psu. The experimental method was identical to that of the first experiment described above. At the end of the test the CR and BB were determined.

2.3 Physicochemical parameters

From each aquarium, four water samples (two just before medium renewal two just after renewal) were taken during the experiment to determine the copper and DOC concentration. The salinity was measured concurrently using a WTWCond 315i (Tetracon 325 electrode). The samples from the different replicates were pooled and filtered (0.45 μm). Samples for metal analysis were acidified (HNO_3 0.14 mol/L; analytical grade) and stored in polypropylene tubes at 4 °C. DOC samples were stored in glass tubes at 4 °C. The dissolved Cu concentration was determined via ICP-OES (Thermo scientific, iCAP 7000 series). The DOC concentration measurements were performed with a Shimadzu TOC-5000 analyzer using the high temperature catalytic oxidation technique [153, 154]. The mean dissolved Cu and DOC concentration of the four samples was used in the subsequent statistical analysis. On average the dissolved Cu concentration in the aquaria decreased with 31 % between two media renewals for the Belgian population and with 42 % for the Baltic population. Due to technical problems, some DOC measurements for the Baltic Sea mussels in the first experiment could not be performed. Therefore, the average DOC concentration was used of all combinations with the same nominal DOC concentration in this experiment.

Changes in the DOC concentration during the experiment could not be prevented due to the required food additions. On average the DOC concentration increased with 2.6 mg DOC/L (North Sea) and 2 mg DOC/L (Baltic Sea) between two media renewals in the first experiment. By subsequently slightly adjusting the feeding time (not the amount of food) in experiment 3 the variability was limited to an increase of 0.8 mg/L.

2.4 Statistical analysis

General Cu toxicity

All statistical analyses were performed using R 2.12.1 statistical software [160]. To assess the general toxicity of Cu, the EC_{50} (always 14 days unless stated otherwise) for each endpoint was estimated via the DRC package using a log-logistic concentration response model [180]. In this analysis the overall Cu toxicity was assessed and the possible influence of salinity or DOC was not accounted for.

Experiment 1

To assess the influence of salinity and DOC on Cu toxicity a general linear mixed model was applied to the results starting from the following full model (Eq. 4.3):

$$\text{Endpoint} = \alpha + \beta1 \cdot [Cu] + \beta2 \cdot [Cu]^2 + \beta3 \cdot [Cu]^3 + \beta4 \cdot S + \beta5 \cdot S^2 + \beta6 \cdot [DOC] + \beta7 \cdot [DOC]^2 + \beta8 \cdot [Cu] \cdot S + \beta9 \cdot [Cu] \cdot [DOC] + \beta10 \cdot [DOC] \cdot S \quad \text{(Eq. 4.3)}$$

The full model predicts the expected endpoint value when exposed to a given combination of Cu, DOC and salinity (S) with [Cu] in µg/L, salinity in psu and [DOC] in mg/L. To account for possible temporal variation of treatments that were set up at different consecutive days a "Day" variable was added as random variable. The statistical analysis was performed independently for both populations. If necessary, the data were transformed (square root or $\log_{10}$) to achieve normally distributed residuals or to avoid heteroskedasticity. To determine the optimal model, the full model (Eq. 4.3) was reduced via backward selection until all remaining parameters in the model were significant. To avoid large type I errors a Bonferroni correction was used resulting in a cutoff P-value of 0.005 (P-value < 0.0045 / number of parameters in the full model (11)).

Experiment 2

A similar approach was used as that applied in experiment 1. However, both populations were tested simultaneously. Hence, the results could be assessed in one model thereby explicitly assessing the population variable (Pop.; factorial variable; full model: Eq. 4.4).

$$\text{Endpoint} = \alpha + \beta1 \cdot [Cu] + \beta2 \cdot [Cu]^2 + \beta3 \cdot [Cu]^3 + \beta4 \cdot S + \beta5 \cdot S^2 + \beta6 \cdot \text{Pop.} + \beta7 \cdot [Cu] \cdot S + \beta8 \cdot [Cu] \cdot \text{Pop} + \beta9 \cdot S \cdot \text{Pop} \quad \text{(Eq. 4.4)}$$

Experiment 3

Due to the different experimental design a log-logistic concentration response curve was constructed to assess the effect of Cu on the CR. Significant differences between the EC_{50}'s and overall fit were assessed via a Wheeler-ratio test and an Anova respectively. The influence of dissolved Cu and [DOC] on the Cu body burden was evaluated via linear regression (full model: Eq.4.5; DOC as factorial variable).

$$\text{Cu body burden} = \alpha + \beta1 \cdot [Cu] + \beta2 \cdot [Cu]^2 + \beta3 \cdot [Cu]^3 + \beta4 \cdot [DOC] + \beta5 \cdot [Cu] \cdot [DOC] \quad \text{(Eq.4.5)}$$

3 RESULTS

3.1 General Cu toxicity

In Table 4.2 the individual EC_{50}'s for the CR, VO_2 and NH_4-P for the three experiments are listed. From experiment 1 it is clear that the CR is the most sensitive endpoint measured in this study with an average Cu EC_{50} of 15.8 µg/L for the North Sea population and 16.5 µg/L for the Baltic Sea population. There was no significant effect of Cu on the VO_2 of the Baltic Sea population and therefore no EC_{50} could be calculated. For details on the concentration response models and visual representation see Appendix C (Table C3 and Figure C4).

Table 4.2. The average Cu EC_{50}s (±SE) for the different endpoints measured in this study.

	endpoint	North Sea pop. EC_{50} (µg/L)	Baltic Sea pop. EC_{50} (µg/L)
exp. 1	CR	16.4 (1.0)	11.1 (2.5)
	VO_2	33.6 (2.3)	NS
	NH_4-P	34.3 (4.7)	32.2 (8.9)
exp. 2	CR	18.4 (2.1)	21.9 (1.6)
exp. 3	CR	12.5 (0.4)	NA

Exp. = experiment; pop. = population; CR = clearance rate; VO_2 = oxygen consumption; NH_4 = ammonium production; exp. = experiment; NS = not significant; NA = not available

3.2 Experiment 1

No mortality was observed in the lowest Cu concentrations (up to 20 µg Cu/L) but up to 50 % mortality occurred in the highest Cu concentration, mainly for the North Sea population (Appendix C: Figure C5). Due to the poor model fit or lack of a Cu effect on the oxygen consumption (VO_2; R^2: 0.5) and ammonium production (NH_4-P; R^2: 0.25) the

details for these two endpoints are given in the supplementary information (Appendix C: Table C4 and Figures C6-10). Briefly, an increase in Cu significantly decreased the NH_4-P in both populations. The VO_2 decreased for the North Sea population with increasing Cu concentration, but Cu had no significant effect on the VO_2 of the Baltic Sea population within the investigated range. An increase in salinity had no effect on the North Sea population but it did increase the VO_2 and NH_4-P of the Baltic Sea population. A change in the [DOC] (DOC, DOC^2 or DOC · Cu) did not result in a significant change in VO_2 or NH_4-P.

The clearance rate (CR), Cu body burden (BB) and gill burden (GB) could be modeled well with an estimated $R^2 > 0.8$ in five of the six models (Table 4.3; for details see Appendix C: Table C5) with the residuals randomly distributed around zero when plotted against the predictor variables (Appendix C: Figures C11-C16). The CR was significantly reduced with increasing dissolved Cu for both populations. The increase in CR at the highest Cu concentration (Figure 4.1) is probably an artifact due to the use of a cubic model. An increase in salinity had no effect on the CR of the North Sea population, but significantly increased the CR of the Baltic Sea population. However, the increase in salinity also resulted in a steeper slope of CR versus Cu for the Baltic Sea population (significant interaction effect between Cu and salinity; Table 4.3). Overall, salinity could explain 22.4 % of the observed CR variation (Appendix C: Table C5). A change in the [DOC] (DOC, DOC^2 or DOC · Cu) did not result in a significant change in CR.

Table 4.3. The parameter values of the predictor variables in the reduced models of the first experiment from the initial full model: Endpoint = $\alpha + \beta1 \cdot [Cu] + \beta2 \cdot [Cu]^2 + \beta3 \cdot [Cu]^3 + \beta4 \cdot S + \beta5 \cdot S^2 + \beta6 \cdot [DOC] + \beta7 \cdot [DOC]^2 + \beta8 \cdot [Cu] \cdot S + \beta9 \cdot [Cu] \cdot [DOC] + \beta10 \cdot [DOC] \cdot S$ (Eq. 4.3).

Pop.	Endpoint	α	β_1	$\beta_2 \cdot 10^{-3}$	$\beta_3 \cdot 10^{-5}$	β_4	$\beta_5 \cdot 10^{-3}$	β_8	R^2
North Sea	√(CR)	4.72	0.110	-17.3	28.6				0.92
	$\log_{10}$(BB)	-0.420	0.109	-2.16	1.23	0.0657	-1.12		0.93
	$\log_{10}$(GB)	1.11	0.104	-2.23	1.53				0.92
Baltic Sea	√(CR)	-1.36	0.0275	2.14		0.683		-0.0220	0.66
	$\log_{10}$(BB)	0.872	0.0731	-0.782		0.0125			0.96
	$\log_{10}$(GB)	0.703	0.121	-1.67					0.87

S = salinity (psu); Pop. = population; CR = Clearance rate (L/(g DW·h)); BB = Cu Body Burden (µg/g DW); GB = Cu Gill Burden (µg/g DW);

An increase in dissolved Cu increased the GB and BB in both populations. There was no significant effect of salinity on the GB. The BB was significantly affected by salinity, but could only explain between 1.1 % (Baltic Sea) and 3 % (North Sea) of the observed variation. A change in the [DOC] (DOC, DOC^2 or DOC · Cu) did not result in a significant change in Cu accumulation. The final reduced models can be found in Table 4.3 and are depicted in Figure 4.1.

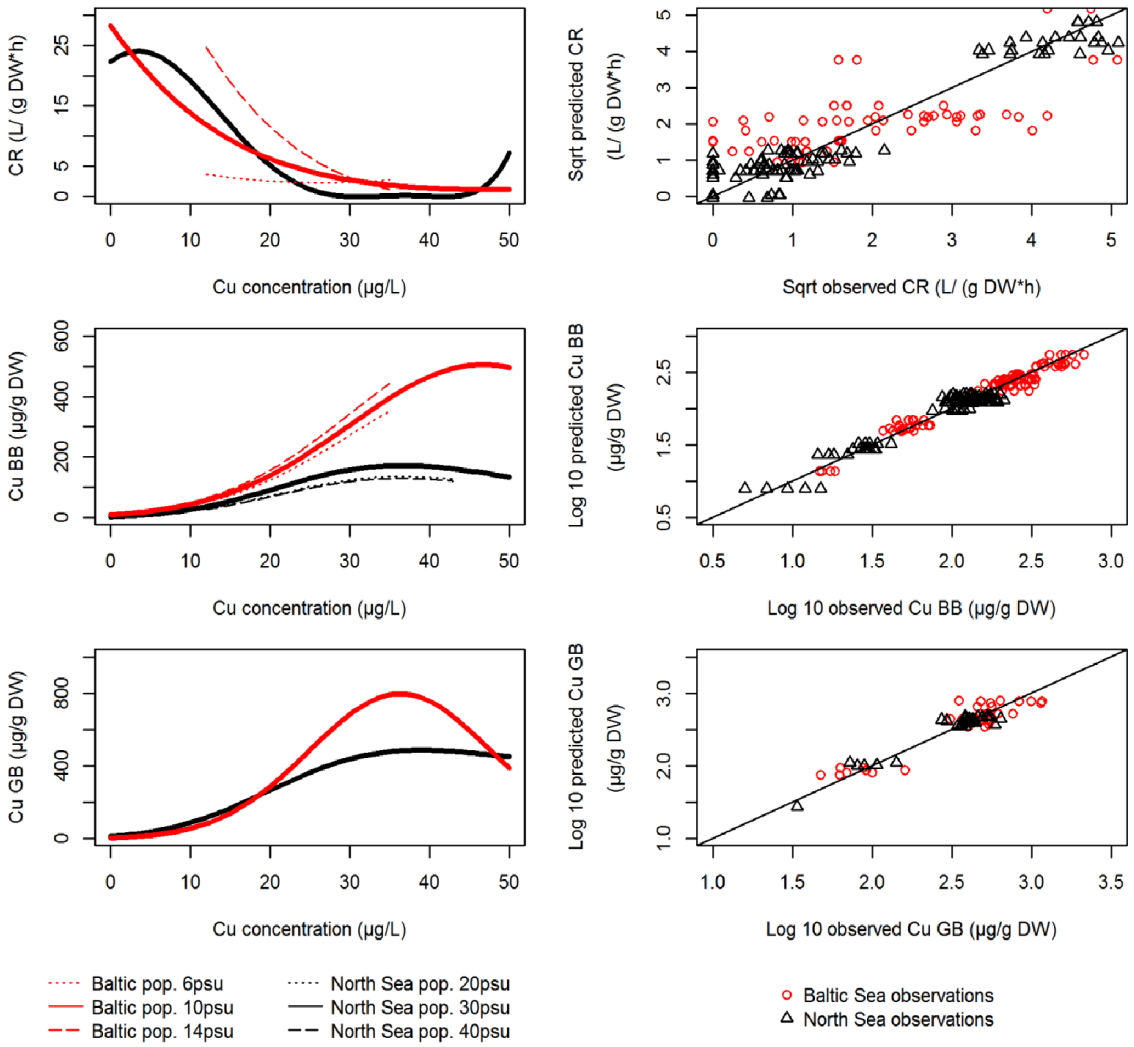


Figure 4.1. Left: model predictions of the first experiment for the two *M. edulis* populations (North Sea in black and Baltic Sea in red) and three salinities (see also Table 4.3). top: Clearance rate (CR), middle: Cu body burden (BB), bottom: Cu gill burden (GB). Right: the matching predicted versus observed values. If dashed lines are not visible, salinity had no significant effect on the endpoint.

3.3 Experiment 2

The overall survival rate was 98 %. The models were able to fit the data with an estimated $R^2 \geq 0.8$ and with residuals that are randomly distributed around 0 when plotted against the independent variables (see Appendix C: Figures C17-19). As observed in the first experiment, the CR decreased with an increasing dissolved Cu concentration. The estimated CR EC_{50}s based on the concentration response curve (Table 4.4) are not significantly different between the two populations (North Sea EC_{50}: 18.4 ± SD 2.1 µg/L; Baltic Sea EC_{50}: 21.9 ± SD 1.6 µg/L). For both populations the CR model indicates a significant salinity and population effect with a maximum CR at 25 psu, which was significantly higher for the Baltic Sea population (Figure 4.2).

The Cu accumulation (BB and GB) was significantly higher (factor 1.6-3) in the Baltic Sea population compared to the North Sea population. An increase in salinity significantly reduced the Cu accumulation in both populations but including salinity could only reduce the observed variability by 0.4 % (BB) to 3.3 % (GB) (details Appendix C: Table C6). The final reduced models can be found in Table 4.4 and are depicted in Figure 4.2.

Table 4.4: The parameter values of the predictor variables in the reduced models of the second experiment from the initial full model: Endpoint = $\alpha + \beta1 \cdot [Cu] + \beta2 \cdot [Cu]^2 + \beta3 \cdot [Cu]^3 + \beta4 \cdot S + \beta5 \cdot S^2 + \beta6 \cdot Pop. + \beta7 \cdot [Cu] \cdot S + \beta8 \cdot [Cu] \cdot Pop. + \beta9 \cdot S \cdot Pop.$ (population: North Sea = 1, Baltic Sea = 0)

endpoint	α	$\beta_1 \cdot 10^{-2}$	$\beta_2 \cdot 10^{-4}$	$\beta_3 \cdot 10^{-4}$	$\beta_4 \cdot 10^{-2}$	$\beta_5 \cdot 10^{-2}$	$\beta_6 \cdot 10^{-1}$	$\beta_8 \cdot 10^{-3}$	R^2
CR	-2.59	7.31	-83.6	1.11	54.8	-1.09	- 4.21		0.80
$\log_{10}$(BB)	1.18	6.85	-7.16		-0.664		- 2.35	- 4.55	0.97
$\log_{10}$(GB)	1.47	8.24	-9.31		-2.31		-1.97		0.90

Pop. = population; S = Salinity (psu) CR = Clearance rate (L/(g DW·h)); BB = Cu Body Burden (µg/g DW); GB = Cu Gill Burden (µg/g DW)

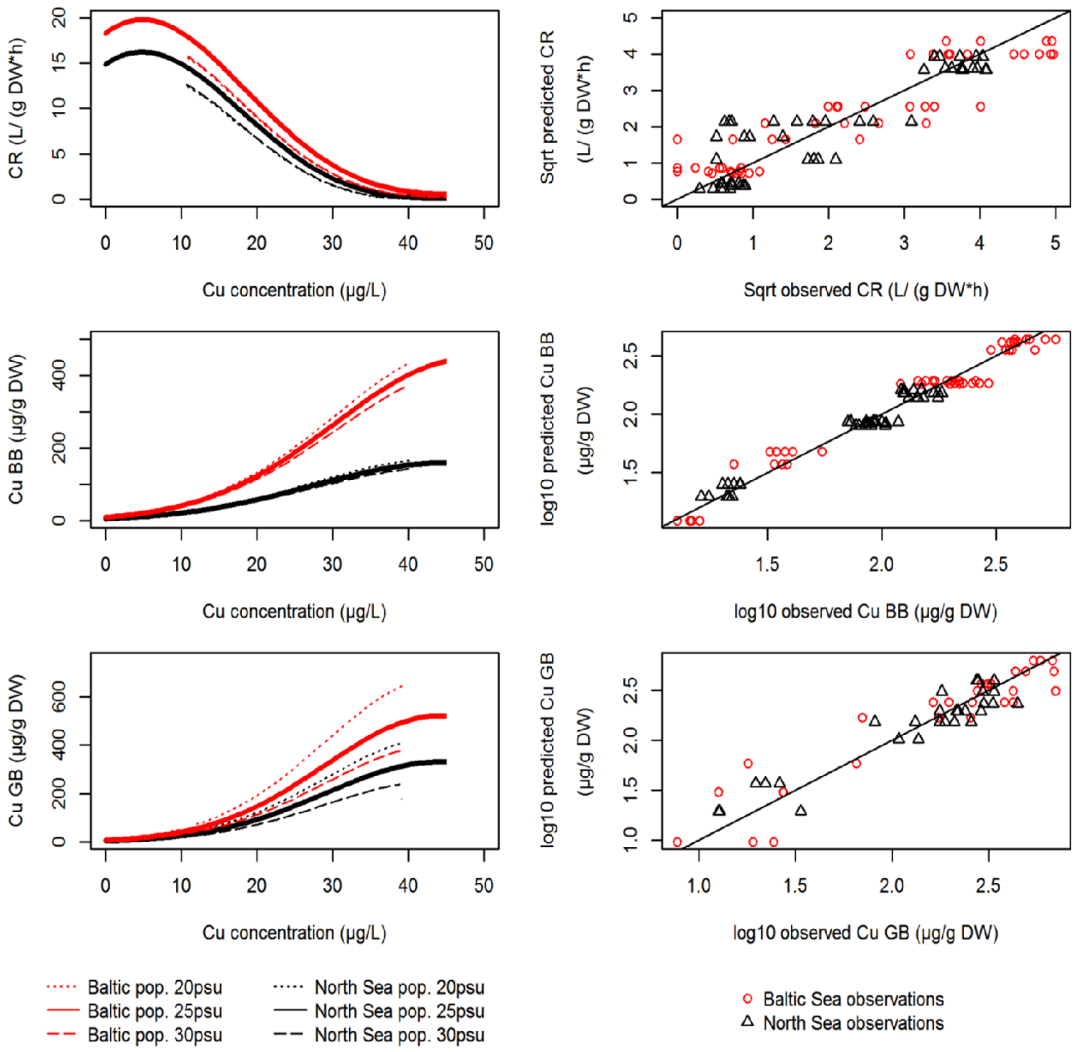


Figure 4.2. Left: model predictions of the second experiment for both *M. edulis* populations (North Sea in black and Baltic Sea in red) and three salinities (see also Table 4.4). Top: clearance rate (CR), middle: Cu body burden (BB), bottom: Cu gill burden (GB). Right: the matching predicted versus observed values.

3.4 Experiment 3

An increase in DOC had no significant effect on the Cu clearance rate EC_{50} (at 1.7 mg/L DOC: EC_{50} = 12.4 ± SE 0.5 µg Cu/L; at 6.2 mg/L DOC, EC_{50} = 13.2 ± SE 0.6 µg Cu/L). Furthermore, including DOC in the model did not significantly improve the overall model fit (Figure 4.3). When all data is combined the Cu EC_{50} = 12.5 ± SE 0.4 µg/L and Cu EC_5 = 8.8 ± SE 0.5 µg/L. Similar to the CR, the BB model indicates no significant difference between the low and high DOC treatment and the Cu accumulation could best be predicted via Equation 4.6 (R^2 = 0.93).

$$\log_{10} \text{Cu BB} = 0.638 + 4.55 \cdot 10^{-2} \cdot \text{Cu} + 2.81 \cdot 10^{-3} \cdot \text{Cu}^2 - 9.89 \cdot 10^{-5} \cdot \text{Cu}^3 \quad \text{(Eq. 4.6)}$$

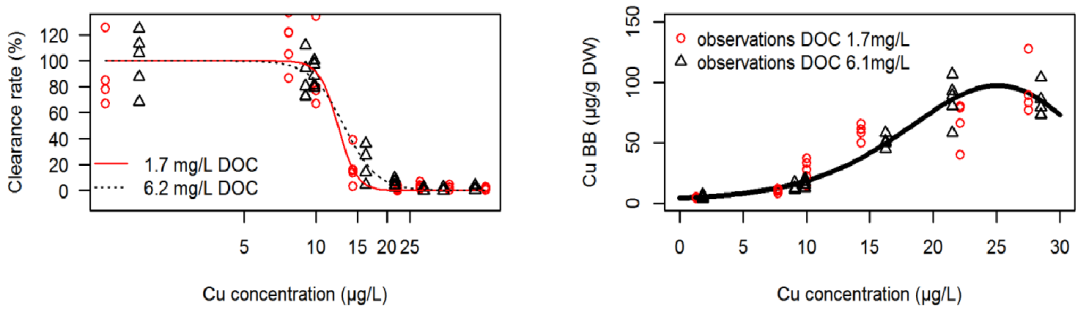


Figure 4.3. The influence of DOC and Cu on the clearance rate (left) and the Cu accumulation in the total soft tissue (right) for the North Sea population.

4 DISCUSSION

An increase in dissolved Cu resulted in a decreased clearance rate and energy consumption (VO_2, NH_4-p) indicating an overall decline in metabolic activity. CR was the most sensitive endpoint measured in this study with an EC_{50} range between 11.1 and 21.9 µg Cu/L (Table 4.2).

The Baltic Sea mussel population is able to survive in brackish water but previous research has indicated that these organisms have to allocate more energy to general maintenance such as osmoregulation, resulting in a reduced growth rate compared to the North Sea *M. edulis* population [65, 120, 122]. It was expected that mussels from the Baltic Sea, which live near their environmental salinity limit, would be intrinsically more sensitive to pollution. The results of experiment 2 reject this hypothesis. Both populations have the same intrinsic Cu sensitivity when exposed to the same environmental conditions (Experiment 2; Figure 4.2). Furthermore, in experiment 1 the Cu EC_{50} of VO_2 and NH_4-P of Baltic Sea mussels was equal to or higher than the EC_{50} of the North Sea mussels (Table 4.2). Only the influence of Cu on the CR is slightly, but not significantly, higher for Baltic mussels (Cu EC_{50} 11.1 µg/L) in a low saline environment compared to the North Sea population (Cu EC_{50} 16.4 µg/L; experiment 1). However, a direct comparison at the same, low, salinity was not possible as North Sea mussels do not survive at these low salinities. Compared to organisms collected from the North Sea population, Baltic Sea mussels do accumulate significantly more Cu in both the gills (GB) and total soft tissue (BB). A similar result was found for Cd, i.e. comparable toxicity but higher accumulation, by Tedengren et al. (1999) [59]. Because the difference in Cu accumulation is still present when both populations are exposed to the same salinity (Figure 4.2), the similar toxicity cannot be explained by changes in Cu speciation or competition at the site of uptake, but have to result from changes in the physiology of Baltic Sea mussels. It has been shown that Baltic Sea mussels have an increased ion exchange capacity due to the increased need for osmoregulation (compared to North Sea mussels) to maintain homeostasis in the brackish water [57]. This may cause an increase in Cu accumulation.

In conclusion, our results suggest that adaptation to local environments might alter the accumulation of pollutants even if the toxicity remains equal. This implies that different populations may differ in how they cope with pollution. Therefore, extrapolating

accumulation or detoxification mechanisms observed in one population to another should not be done.

Predicting the possible influence of salinity on Cu accumulation or toxicity is not straightforward since a changing salinity might affect both the bioavailability of Cu (e.g. via complexation) and the physiology of the organism (e.g. osmoregulation) [16]. In this study, the most pronounced effect of an increased salinity was an increase in the metabolism (CR, VO_2 and NH_4-P) of the Baltic Sea population, certainly between 4 and 10 psu. This further supports previous observations that this population lives in suboptimal conditions [65, 120, 122]. The influence of salinity on the CR, VO_2 or NH_4-P of the North Sea population was limited or absent. Our results, for both populations, corroborate these found by Maar et al. (2015) where the specific growth rate, shell growth rate and the condition index were highest and near constant between 20 and 30 psu [57]. Even though the salinity had a pronounced effect on the metabolism of the Baltic Sea population, an increase in salinity had only a limited effect on the toxicity of Cu because a higher salinity also resulted in a steeper slope of the concentration response curve. In contrast to the toxicity, the influence of salinity on the Cu accumulation is small (max. explained variation < 5 %) and complex. In general, a higher salinity slightly decreased the Cu accumulation in both populations with the highest accumulation in the (gills of) Baltic Sea mussels exposed to a low salinity. It is unclear why the salinity effect was not seen in the gills of experiment 1. This might be due to a lower statistical power (two replicates) compared to experiment 2 (three replicates). The limited to absent effect of salinity on Cu toxicity in settled mussels is in contrast with the effect observed in mussel larvae. In Chapter 3 we reported a large and continuous increase in Cu toxicity to *M. galloprovincialis* larvae with increasing salinity.

It has been shown that DOC generally reduces Cu toxicity due to the complexation of Cu with DOC resulting in a decreased bioavailability. This has been frequently confirmed for both freshwater and saltwater species [35, 36]. However, in experiment 1 and 3 no effect of DOC was observed on both Cu accumulation and toxicity in both populations. This indicates that Cu bound to DOC is bioavailable for uptake by settled mussels when chronically exposed. Several studies have already reported that DOC or DOC-Cu complexes are (partially) available for uptake in settled mussels during acute exposure [43-48]. Furthermore, there is evidence that DOC is an important constituent of the diet of (zebra)mussels [47, 49]. Currently it is not known why mussels deviate from the frequently

observed protective effect of DOC to Cu toxicity. Given this information and our observations, we suggest the following hypotheses: (1) DOC-Cu complexes are bioavailable due to the direct uptake of (small) DOC-Cu complexes through the gill or because these complexes are used as food source, (2) there are changes in the Cu binding capacity of DOC in the gill micro environment, e.g. a change in pH [181], (3) the Cu diffusion rate into the gill boundary layer is the limiting step, and not the Cu uptake rate, therefore Cu-DOC complexes might dissociate in the boundary layer and become bioavailable [182]. These hypotheses are not contradicted by the fact that DOC reduces both Cu accumulation and toxicity in mussel larvae, which do not feed or possess gills [119, 139].

Our results imply that: (1) the Cu toxicity DOC correction for mussel larvae proposed in previous studies[119, 150, 179] and chapter 3 cannot be extrapolated to the settled life stage, (2) the first 48 h (up to D-larvae stage) are the most sensitive life stage [115] but only up to ± 2.5 mg DOC/L (Figure 4.4). At higher DOC concentrations settled mussels can be more sensitive. Hence, the suggested DOC correction factor to determine a Cu environmental quality standard proposed in the Cu EU voluntary risk assessment report (VRAR)[38] or by the Water Framework Directive- United Kingdom Technical Advisory Group (WFD-UKTAG)[112] and the United States Environmental Protection Agency (US-EPA)[183] may not adequately protect settled mussels at higher DOC concentrations. Based on our results this would mean that the EC_5 of 8.8 µg Cu/L (experiment 3) is lower than the currently proposed $HC_{5\text{-}50}$ at 5 mg DOC/L (VRAR and WFD-UKTAG) and lower than the FAV (final acute value) of the US-EPA at 2.2 mg DOC/L (Figure 4.4).

In conclusion, changing the water chemistry (salinity or DOC) had little to no effect on the toxicity or accumulation of Cu in settled mussels within the evaluated range. Because there is a complete lack of any DOC effect on the accumulation or toxicity of Cu, the construction of a universal marine biotic ligand model with a universal effect of DOC is, based on current knowledge, not possible [138]. Further research is needed to elucidate if (settled) mussels are exceptions or if this is a common phenomenon in marine environments. Finally, the results of this study indicate that different life stages of the same species may not react in a similar way to a changing environment (DOC effect, Figure 4.4). Therefore, when an organism goes through vastly different life stages (in morphology, anatomy and/or physiology), it may not always be possible to extrapolate the results from one life stage to another.

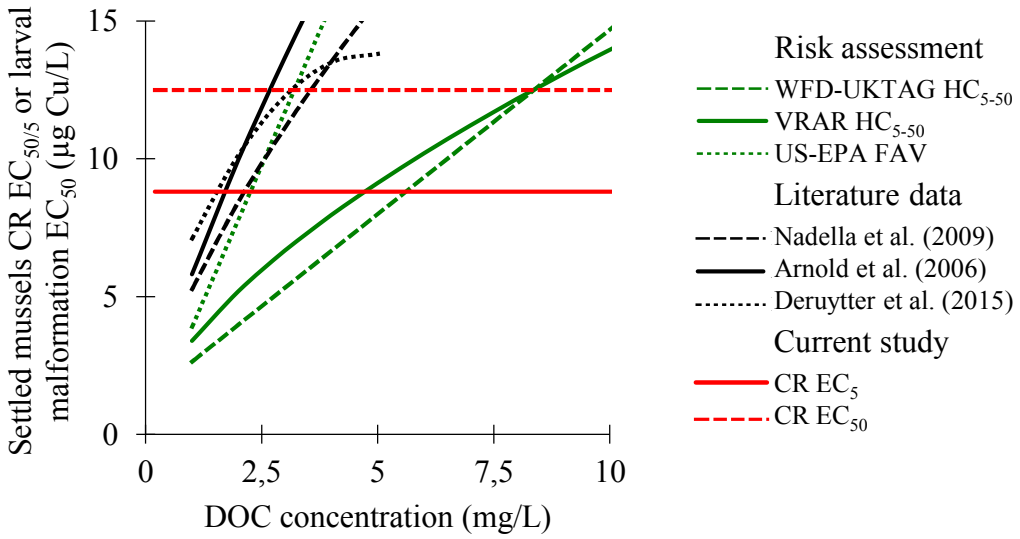
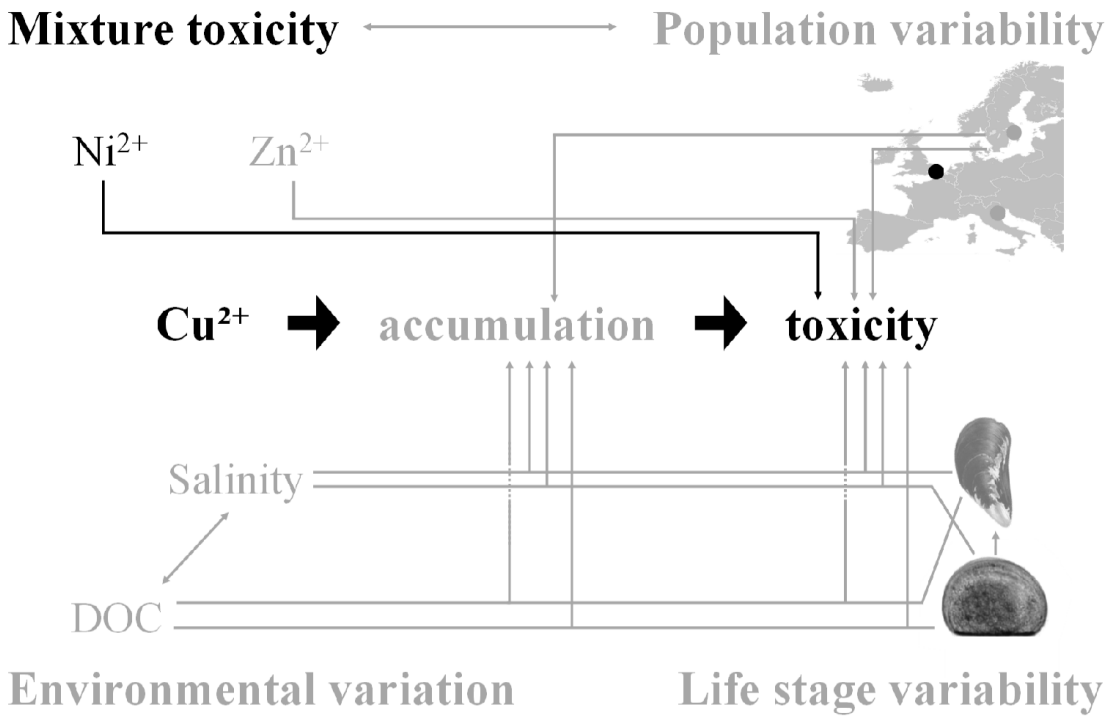


Figure 4.4. The effect of DOC on the settled mussels clearance rate (CR) 14 d $EC_{50/5}$ of experiment 3 (red), larval malformation 48 h EC_{50}'s reported in previous studies and chapter 3 [119, 150, 179]. In black the proposed $HC_{5\text{-}50}$ or FAV in relation to the DOC concentrations (EU VRAR, WFD-UKTAG and US-EPA).


Mixture toxicity
Population variability
Ni2+
Zn2+
Cu2+
accumulation
toxicity
Salinity
DOC
Environmental variation
Life stage variability

V

COPPER-NICKEL MIXTURE TOXICITY IN THE MARINE ENVIRONMENT: MODEL DEVELOPMENT AND EVIDENCE FOR SYNERGISM AT ENVIRONMENTAL CONCENTRATIONS

Redrafted from:

Deruytter, D., Baert, J.M., Nevejan, N., De Schamphelaere, K.A.C. and Janssen, C.R. (2016). Copper-nickel mixture toxicity in the marine environment: model development and evidence for synergism at environmental concentrations. Submitted to Environmental toxicology and chemistry (3-11-2016).

ABSTRACT

Little is known about the effect of metal mixtures on marine organisms, especially when exposed to environmentally realistic concentrations. This information is, however, required to evaluate the need to include mixtures in future environmental risk assessment (ERA) procedures. Here, the effect of Cu-Ni binary mixtures on *Mytilus edulis* larvae was assessed using a full factorial design that included environmentally relevant metal concentrations and ratios. The reproducibility of the results was assessed by repeating this experiment 5 times. The data was compared to predictions of the concentration addition (CA) reference model. Deviations from the CA were estimated using a Markov Chain Monte-Carlo algorithm (MCMC). This enabled the accurate estimation of the deviations and their uncertainty. The results demonstrated, reproducibly, that the type of interaction – synergism or antagonism – mainly depended on the Ni concentration. Antagonism was observed at high Ni concentrations while synergism occurred at Ni concentrations as low as 4.9 µg Ni/L. The latter, low and realistic Ni concentration, was 1 % of the individual Ni EC_{50} or 57 % of the PNEC (predicted no effect concentration in the EU ERA). It is concluded that results from mixture studies should not be extrapolated to other concentrations or ratios and mixture interactions can occur at environmentally realist concentrations and therefore should be accounted for in (marine) ERA of metals.

1 INTRODUCTION

Current environmental risk assessment (ERA) procedures and regulations in most countries are almost exclusively based on a substance-by-substance approach. In reality, however, organisms are often exposed to multiple stressors simultaneously. Understanding how mixture effects can reliably be predicted from single substance data is therefore a major challenge in ecotoxicology. Two reference models are commonly applied to predict mixture toxicity effects: Concentration Addition (CA) [51] for similarly acting chemicals, and Independent Action (IA) [50] for dissimilarly acting chemicals [55]. However, dissimilarly acting chemicals are rare when considering complex biological processes. Empirical studies consequently demonstrated that the CA model often provides a good estimate of mixture effects. Hence, the more conservative CA model has been suggested as the default model (or Tier 1 approach) in risk assessment [52, 184, 185].

The CA model assumes that there are no interactions between the different mixture components. Synergistic and antagonistic interactions, however, are frequently observed and thereby cause the CA model to under- or overestimate the mixture effect, respectively. Playle's theoretical metal mixture biotic ligand model predicts a concentration-dependent effect of metal mixtures: low concentrations are expected to result in synergistic effects, whereas high concentrations are expected to cause additive or antagonistic mixture effects [186]. This is a consequence of the non-linear relation between metal accumulation and the concentration in the medium, for details see Playle et al. (2004) [178, 186, 187]. Several studies found experimental evidence that (metal) mixture effects may indeed depend on the metal concentration or ratio to which the organisms are exposed. The type of the interaction found in these studies, however, sometimes differ from Playle's theoretical model predictions [56, 188, 189]. Still, most (marine) studies have tested unrealistically high metal concentrations [190]. It is therefore unclear if effects observed at such high concentrations can be extrapolated to the low, environmentally realistic concentrations required for ERA. Hence, there is a need to assess mixture effects at low (environmentally relevant) concentrations to improve our understanding of mixture toxicity and how to apply this in future ERA procedures.

Next to assessing mixture effects at environmentally relevant concentrations, evaluation of the overall reproducibility, significance and variability of observed deviations from the CA

model in mixture experiments is also needed [191-193]. Reproducibility has been shown to decrease when the response variability increases or due to the non-simultaneous testing of single substances and their mixture [191, 193]. The high resource investment, also, limits most mixture studies to a single experiment, and simultaneous testing is still not always done [192]. If mixture interaction effects are truly non-reproducible – especially at environmental concentrations – this would reduce the scientific value of a single mixture experiment. Furthermore, the uncertainty on the estimated deviation from the CA model yields important information on the significance of the deviation, but is not formally tested in the majority of mixture studies [194-197]. Knowledge on the reproducibility and uncertainty of mixture interactions at environmentally relevant concentrations is therefore essential if mixture effects are to be included in ERA.

In this study the occurrence and reproducibility of deviations from the CA model in Cu-Ni mixtures with a concentration range that includes both high and environmentally relevant concentrations was assessed using a full factorial test design. Furthermore, the experiment was repeated 5 times over 3-year period by 3 different researchers to assess the reproducibility of the test results. In addition, the use of a, new, Markov-Chain Monte Carlo algorithm enabled a full quantification of parameter uncertainty and deviations from the CA model. Mussel larvae (*Mytilus edulis*) were used as test organism as they are amongst the most copper sensitive marine organisms (larval development 48 h EC_{10} 2.8 µg/L [119] ; 0.6 mg dissolved organic carbon (DOC)/L) [119, 139, 150]. Hence, adverse effects may already occur near environmentally relevant concentrations (EU predicted no effect concentration (PNEC) = 2.6 µg Cu/L).

2 MATERIALS AND METHODS

2.1 Adult mussel collection and maintenance

Adult *Mytilus edulis* were collected along the Belgian North Sea coast (Middelkerke) in spring 2013, 2014 and 2015 (when gravid mussels were available). The ambient salinity was 34 psu and seawater temperature between 8 and 10 °C. Before each experiment the mussels were kept in a holding tank (100 L) containing recirculating, aerated, artificial seawater (Instant Ocean®) at a temperature of 8 °C and a salinity of 34 psu. They were fed *ad libitum* with Shellfish Diet 1800® (Reed Mariculture Inc.). The experiments with the larvae were performed within two weeks after collection of the adults.

2.2 Mixture experiments with mussel larvae

Mussel larvae were exposed to a range of concentrations and ratios of Cu and Ni in a full factorial design with 4 replicates per treatment. The single metals and metal mixtures were tested simultaneously. The test concentrations were selected based on literature data, previous experiments and environmentally relevant concentrations/ratios for the North Sea [119, 139, 150, 198]. The selected environmental concentration range for Cu and Ni tested was obtained from monitoring datasets of ICES (n = 2380), the British Oceanographic Data Centre (n = 457) and the Belgian Marine Data Centre (n = 373) (Figure 5.1).

Experiments were performed according to: 'the ASTM standard guide for conducting static acute toxicity tests starting with embryos of four species of saltwater bivalve molluscs (E724-98) [115]' (see chapter 3). In summary, the mussel larvae were exposed in 50 mL vials filled with 40 mL of ASTM seawater, which was spiked with Cu, Ni or a mixture one day prior to the experiment to ensure an equilibrium [152]. Embryos were obtained from heatshock-induced spawning events, after which fertilization took place within 30 minutes. The embryos were subsequently added to the vials and exposed for 48 h. At the end of the test the larvae were killed and preserved using formaldehyde and the development of the larvae was determined via microscopy. The entire experiment was repeated 5 times with a full factorial setup with 6 to 8 Cu concentrations and 6 to 13 Ni concentrations (Appendix D: Table D1 and Figure D1). Three independent researchers carried out this protocol in the course of three years (2013-2015).

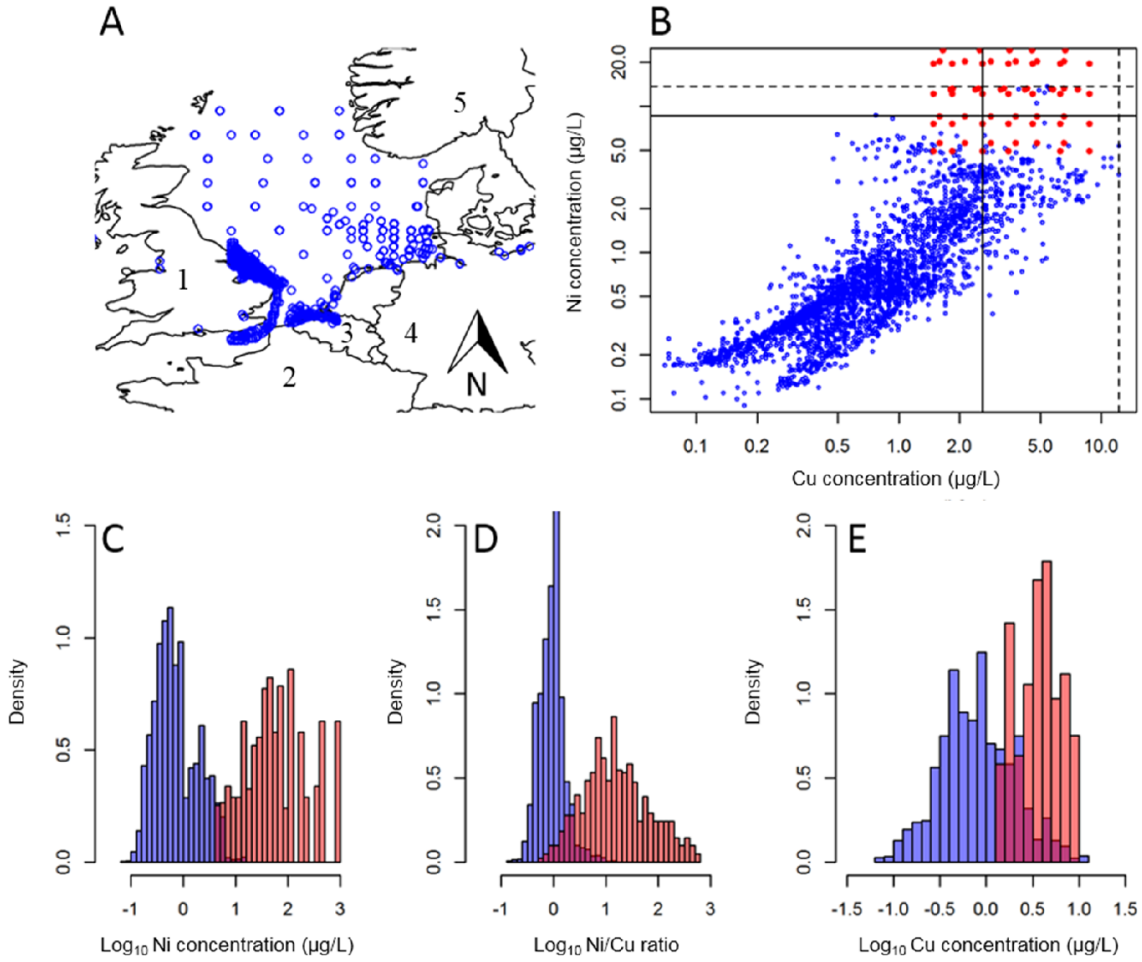


Figure 5.1. Comparison between North Sea Cu and Ni concentrations and the test concentrations in this study. A: geographic location of the samples with 1: United kingdom, 2: France, 3: Belgium, 4: Germany, 5: Norway; B: correlation between the Ni and Cu concentration in the environment (blue) and assessed mixtures (red), the EU PNEC values (solid lines and the maximum environmental concentrations (dashed lines); C-E: histogram of the environmental concentrations/ratios (blue), the tested concentrations/ratios (red), the overlap between the environmental concentrations and tested concentrations (dark red).

2.3 Analytical chemistry

Water samples were taken at the start of the experiments [115]. Samples from the four replicates were pooled and filtered (0.45 µm). DOC samples were stored in glass tubes at 4 °C, ensuring no air was left in the tube and analysed with a Shimadzu TOC-5000 analyser using the high-temperature catalytic oxidation (HTCO) technique [153, 154]. The samples for metal analysis were acidified with 0.14 mol/L analytical grade HNO_3 and stored in polypropylene tubes at 4 °C. Dissolved Ni and Cu concentrations were determined via ICP-OES (Thermo scientific iCAP 7000 series). The average measured metal concentration per treatment was used for further statistical analysis.

2.4 Data analysis

Three different methods were used to test if the Cu-Ni mixture effects significantly deviated from those predicted by the CA reference model. First, the observed mixture effects were compared with CA model predictions based on the single metal concentration response curves (assuming additivity). A linear model was used to test if deviations from the CA model depended on the single metal concentrations or ratio. Secondly, the CA model and 3 more complex models (synergism/antagonism, ratio dependent and concentration dependent) were fitted to the whole dataset. The uncertainty of parameter estimates and model predictions was fully quantified using a Markov Chain Monte-Carlo (MCMC) algorithm, and it was tested which model explained deviations from the CA model best. Thirdly, concentration response curves were fitted for each metal, for a constant concentration of the other metal, and it was tested if the sensitivity to one metal (i.e. EC_{50}) depended on the concentration of the other metal. These three approaches are described in detail hereunder.

Assessment of deviations from the CA model based on single concentration response curves

Three parameter log-logistic single metal concentration response curves were fitted using the DRC package [180]. The obtained control development, single metal EC_{50} and slope were used to predict the larval development in the mixture treatment according to the CA model. Next, the deviation of the residuals was modelled as a function of Cu and Ni concentrations using a linear regression model (full model Eq. 5.1) with zero intercept (no deviation in the control), to test if a systematic deviation from the CA model occurred. Data that corresponded to Cu concentrations higher than 5 µg/L and Ni concentrations higher than 450 µg/L were excluded because no larvae developed at these concentrations. Including them would therefore zero-inflate the data. The optimal model was selected via backward selection until all parameters were significant.

$$\Delta = \beta_1 \cdot [Cu] + \beta_2 \cdot [Cu]^2 + \beta_3 \cdot \log_{10}[Ni] + \beta_4 \cdot \log_{10}[Ni]^2 + \beta_5 \cdot [Cu] \cdot \log_{10}[Ni] \quad \text{(Eq. 5.1)}$$

with deviation (Δ) in % larval malformation, Cu and Ni in µg/L.

Assessment of deviations from the CA model based on the full dataset

The CA model (Eq. 5.2) and three models that represent different deviations from additivity were fitted to each dataset [55]. Deviations were modelled as function of the toxic units of the mixture compounds. Synergism or antagonism mixture effects (S/A) can be modelled by a single parameter *a* (Eq. 5.3; note that *a* equals 0 for CA). Modelling ratio-dependent (RD; Eq. 5.4) or concentration-dependent (CD; Eq. 5.5) synergistic or antagonistic effects, however, requires the introduction of a second deviation parameter *b* (for details see Jonker et al. 2005 [55]). The interpretation of the sign of the deviation parameters *a* and *b* is explained in Table 5.1.

$$TUx_1 + TUx_2 = 1 \quad \text{(Eq. 5.2)}$$

$$TUx_1 + TUx_2 = \exp(\mathbf{a} \cdot z_1 \cdot z_2) \quad \text{(Eq. 5.3)}$$

$$TUx_1 + TUx_2 = \exp(\mathbf{a} \cdot z_1 \cdot z_2) \cdot \exp(\mathbf{b} \cdot z_1 \cdot z_1 \cdot z_2) \quad \text{(Eq. 5.4)}$$

$$TUx_A + TUx_B = \exp(\mathbf{a} \cdot z_1 \cdot z_2) \cdot \exp(-\mathbf{a} \cdot \mathbf{b} \cdot z_1 \cdot z_2 \cdot (TU_{501} + TU_{502}) \quad \text{(Eq. 5.5)}$$

With $z_i = TUx_i / \sum_{j=1}^{n} TUx_j$ and $TUx_i = c_i / ECx_i$

The optimal parameter estimates and the uncertainty on the parameter estimates (called the credibility interval) was estimated for all four models and all experiments were estimated using a Markov Chain Monte Carlo (MCMC) algorithm [199]. This is in contrast to the routinely used minimization of the least squared error objective function, which only yields an optimal set of parameter estimates [200]. In addition, the MCMC readily enables a Bayesian approach to parameter estimation, including prior knowledge on the optimal parameter values. For example, a negative EC_{50} is deemed impossible since negative concentrations do not have any physical meaning. Hence, this can easily be included in the model by setting the prior probability for negative values of the EC_{50} to zero. Although this is a rather trivial example, it demonstrates that properly chosen priors can result in more realistic models. Here, the normal distributions of the single metal concentration-response curves were used as prior distributions for the control larval development, slope and EC_{50}. Normal distributions were truncated to the 99.999% confidence interval and to positive values as negative values are biologically irrelevant. The upper limit of the Ni EC_{50} normal distribution was truncated at 1000 µg/L for all datasets as higher values are improbable.

The slopes for Ni for experiment 1 and 2 were restricted between 0 and 5 due to difficulties in fitting the single metal response curve as a result of limited Ni effects (See appendix D). Uninformative uniform distributions [-20,20] were used as prior distributions for *a* and *b*. The relative support for each of the four models was quantified by the Akaike weights based on the optimal parameter estimates, i.e. the median of the posterior likelihood distribution [201]. All calculations were done in R 2.12 [160] using the drm, GenSa and nleqslv packages [173, 202, 203].

Table 5.1. The possible combinations of the 2 deviation parameters (a and b) and the interpretation of the sign of the deviation parameters. For more detailed information, see Jonker et al. (2005) [55].

Equation	a	b	interpretation of the sign
2 (S/A)	+	NA	Antagonism
	−		synergism
3 (RD)	+	+	Antagonism
	−	−	Synergism
	+	−	Ratio dependent antagonism/ synergism
	−	+	Ratio dependent antagonism/ synergism
4 (CD)	−	>1	From antagonism to synergism at concentration below EC_{50} metal 1
	−	0-1	From antagonism to synergism at concentration above EC_{50} metal 1
	−	<0	Antagonism at all concentrations but change in magnitude
	+	>1	From synergism to antagonism at concentration below EC_{50} metal 1
	+	0-1	From synergism to antagonism at concentration above EC_{50} metal 1
	+	<0	synergism at all concentrations but change in magnitude

+: positive; −: negative; NA: not available for this model; S/A = Synergism antagonism; RD = ratio dependent synergism/antagonism; CD = concentration dependent synergism/antagonism

EC_{50} mixture analysis

The influence of Ni on the EC_{50} Cu was calculated via an approach similar to the method used by Traudt et al. (2015) [204]. Briefly, for each Ni concentration, the corresponding Cu EC_{50} was calculated using the DRC package in R using the three parameter log-logistic model. With the Cu EC_{50} at a given Ni concentration defined as: the Cu concentration at which 50% of the larvae develop compared to the control at this Ni concentration. Significant differences between the Cu EC_{50} with and without Ni present were assessed. As proposed by Julious (2004) and Meyer et al. (2015), two EC_{50}'s were significantly different at the 95% confidence level when the 84% confidence intervals did not overlap [205, 206]. Next, a log-logistic function was used to model the effect of Ni on the Cu EC_{50} and the effect of Cu on the Ni EC_{50}.

3 RESULTS

3.1 Single metal analysis

In the control treatments the % normal developed larvae after 48 h ranged between 73.5 and 95.3 %. The single metal EC_{50}'s and slopes are listed in table 5.2 with a mean Cu EC_{50} of 4.1 µg/L (SE: 0.2 µg/L) and a mean Ni EC_{50} of 414.7 µg/L (SE: 78.1 µg/L). The Ni EC_{50} and slope of experiment one could not be determined as the tested concentrations were too low (maximum tested concentration: 91.5 µg Ni/L). The concentration response curves are shown in appendix D (Figure D2).

Table 5.2. The single metal log-logistic concentration response curve parameter estimates (EC_{50}, slope and control development). Standard errors are given between parentheses.

Exp.	Year	Control %	EC_{50} Cu µg/L (± SE)	EC_{10} Cu µg/L (± SE)	Cu slope	EC_{50} Ni µg/L (± SE)	EC_{10} Ni µg/L (± SE)	Ni slope
1	1	73.5 (± 7.8)	4.2 (± 0.1)	3.3 (± 0.1)	9.4 (± 1.2)	ND	ND	ND
2	1	75.7 (± 1.1)	4.5 (± 0.1)	3.5 (± 0.1)	8.8 (± 0.6)	607.2 (± 159.9)	43.2 (± 20.0)	0.8 (± 0.2)
3	2	92.0 (± 3.4)	4.4 (± 0.1)	3.4 (± 0.1)	8.1 (± 0.9)	332.7 (± 6.0)	254.7 (± 16.9)	8.2 (± 2.2)
4	2	95.3 (± 2.8)	4.0 (± 0.1)	3.2 (± 0.1)	9.7 (± 1.5)	251.7 (± 8.3)	145.8 (± 12.4)	4.0 (± 0.5)
5	3	94.2 (± 1.4)	4.1 (± 0.0)	3.4 (± 0.1)	11.1 (± 0.8)	467.2 (± 25.6)	370.4 (± 21.9)	9.5 (± 4.6)
mean		86.8 (± 4.3)	4.1 (± 0.2)	3.4 (± 0.0)	9.4 (± 1.1)	414.7 (± 78.1)	203.5 (± 70.4)	5.6 (± 2.0)

Exp. = Experiment; Control =control larval development control treatment; SE = standard error; ND = not determined; Year 1-3 = 2013-2015

3.2 Mixture assessment based on single concentration response curves

The observed Cu-Ni mixture effects deviated from the predictions based on the CA reference model (Figure 5.2 and 5.3). The optimal model explained deviations from the CA model as a function of both Cu and Ni concentrations (Eq. 5.6) with the deviation calculated as % observed larval development minus the % predicted larval development ($R^2 = 0.6$).

$$\Delta = -9.1 \cdot [Cu] + 44.6 \cdot \log_{10}[Ni] - 24.7 \cdot \log_{10}[Ni]^2 + 6.6 \cdot [Cu] \cdot \log_{10}[Ni] \quad \text{(Eq. 5.6)}$$

with deviation (Δ) in % larval malformation, Cu and Ni in µg/L.

Positive deviations (i.e. an underestimation of larval malformation) were observed at the lowest Ni concentration suggesting synergism relative to the CA reference model (as low as 4.9 µg/L). In contrast, at higher Ni concentrations, antagonistic interactions (i.e. overestimation of larval malformation) were observed (Figure 5.2B and 5.3). Cu concentration had only a minor effect on deviations from the CA model and did not show a strong, directional deviation from the CA model (Figure 5.2A and 5.3).

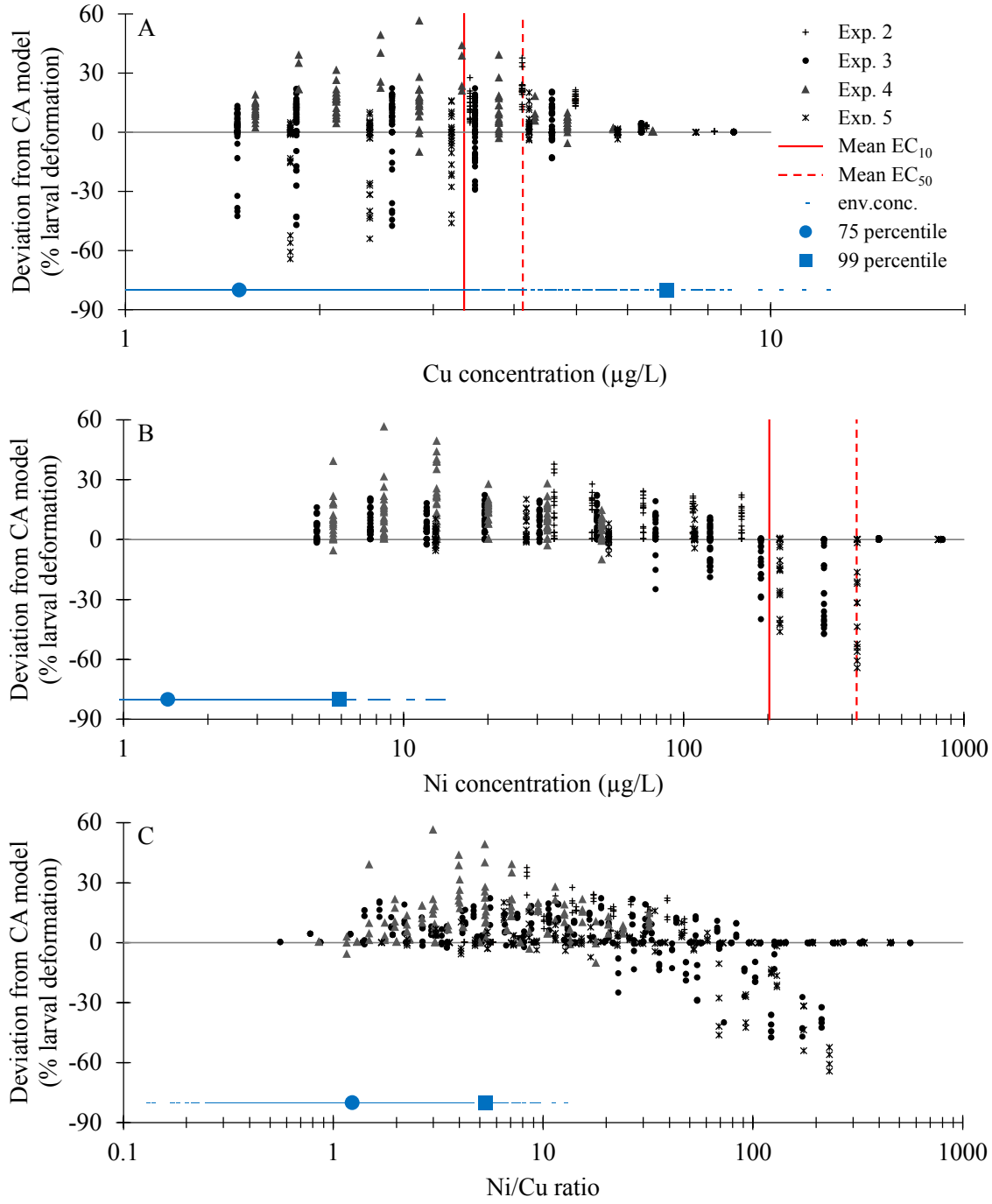


Figure 5.2. Deviations of the observed larval development from the development predicted with CA from the single concentration response curves (Table 5.2), as a function of increasing concentration of Cu (A), Ni (B) or Ni-Cu ratio (C) for all mixture observations (n = 728). Positive values indicate synergism, negative values antagonism. Solid red line = average EC_{10}; dashed red line = average EC_{50}; blue dots = environmental concentrations or ratio; blue circle/square = 75 and 99 percentile of the environmental concentrations/ratio

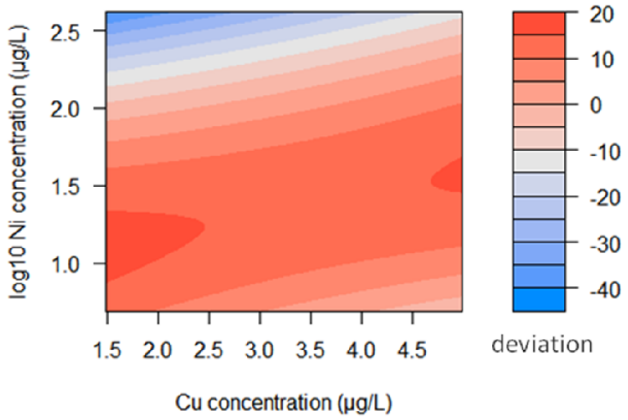


Figure 5.3. Modelled deviation from the CA model predictions based on the single concentration response curves and the observed data (all data combined, Eq. 5.5). Positive values indicate synergism, negative values indicate antagonism.

3.3 Assessment of deviations from the CA model based on the full dataset

All experiments supported a metal ratio-dependent (RD) deviation from the CA reference model (Table 5.3). The CA reference model explained the least amount of variance in all experiments (i.e. highest sum of squared error (SSE)). By including the deviation parameter *a* to allow for synergistic or antagonistic mixture effects (S/A; Eq. 5.3), the model fit improved in four of the five experiments (Table 5.3). On average, the SSE decreased with 15.4% in the S/A model compared to the CA model. However, the S/A mixture interaction effect was not reproducible between experiments. The negative estimate for the deviation parameter *a* indicated significant synergistic mixture effects in experiment one and two, whereas positive estimates suggested an antagonistic effect in experiment three and five, and no deviation occurred in experiment four ($a = 0$).

The incorporation of the ratio- or concentrations-dependent deviation parameter (*b*; Eq. 5.4) improved the model fit significantly in all experiments. On average, the SSE decreased in the RD model with 34.1% compared to the S/A model (Eq. 5.3) and 42.7% compared to the CA model. Based on AICc weights, the RD model was the most probable model in all experiments, with a support of 100% in four of the five experiments. The credibility interval of the estimated values of deviation parameters *a* and *b* deviation parameters was significantly different from 0 and was identical in sign for all experiments. Hence, mixture effects were reproducible among studies. The positive value of *a* and negative value of *b* indicate synergism at low Ni-Cu ratios and antagonism at high Ni-Cu ratio (see also Figure

5.2 C). The switch from synergism to antagonism occurred on average at a Ni-Cu ratio of 32.6 (range 14.0 to 74.1; calculations based on Jonker et al. (2005) [55]).

The credibility intervals for the concentration-dependent synergism/antagonism model (CD; Eq. 5.5) could not be calculated. We believe that this is due to the specific structure of the deviation parameter, which can result in multiple similar local optima in the objective function, making it impossible for the MCMC to reliably sample the posterior distributions. Therefore, the least square estimators obtained from the simulated annealing algorithm were used. Including the CD decreased the overall SSE on average by 4.4% compared to the SA model and never predicted the mixture better than the ratio-dependent model.

Note, that the parameter estimates based on the new MCMC approach are a match to the predictions based on the more classic least square estimator approach (e.g. method described in Jonker et al. (2005)[55]; Figure 5.4). Furthermore the overall conclusion (ratio dependent mixture toxicity) is the same for both methods (Appendix D: Table D2-D3).

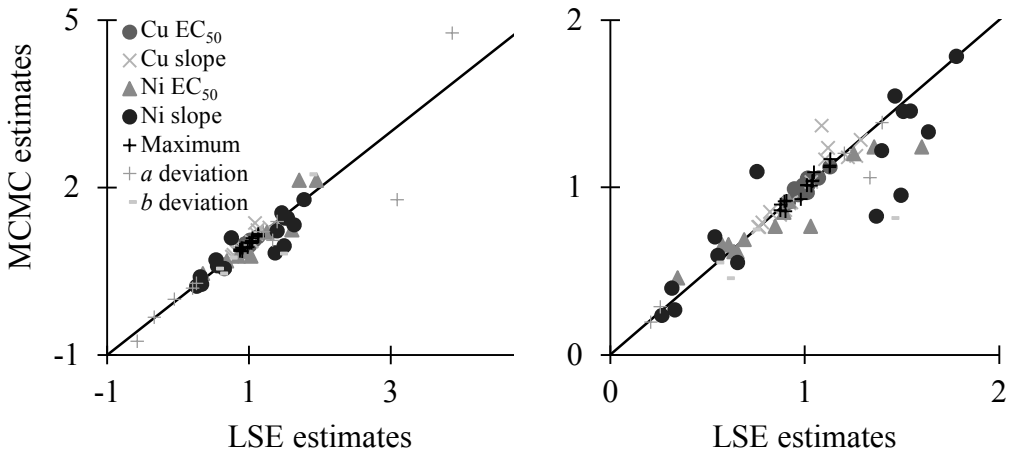


Figure 5.4. Comparison between the optimal least square estimator parameter estimates (LSE) and the parameters obtained from the Markov Chain Monte Carlo (MCMC) algorithm (left: all parameters; right: detail of the values between 0 and 2). The estimates are rescaled to an average of 1, the original values can be consulted in appendix D (Table D2-D3).

Table 5.3. The optimal deviation parameter estimates and 95% credibility interval (between brackets), sum of squared error (SSE) and AICc weight (AICcW in %) for each experiment and each deviation model (Eq. 5.1-5.4).

	Model	*a*	*b*	SSE	AICcW
Exp. 1	CA	x	x	5153	0
	S/A	-2.05 (-2.11- -1.98)	x	4901	0
	RD	13.03 (11.83-14.16)	-16.96 (-18.22- -15.63)	4396	64
	CD	3.25	1.66	4433	35
Exp. 2	CA	x	x	6716	0.0
	S/A	-0.88 (-0.93- -0.84)	x	5746	0.0
	RD	3.79 (3.71-3.88)	-5.67 (-5.83- -5.54)	2301	100.0
	CD	-0.06	-20.00	5494	0
Exp. 3	CA	x	x	29383	0.0
	S/A	0.78 (0.7-0.793)	x	20230	0.0
	RD	2.89 (2.87-2.92)	-3.46 (-3.50- -3.42)	14557	100.0
	CD	0.14	-0.39	19562	0
Exp. 4	CA	x	x	16498	0.0
	S/A	0.002 (-0.027-0.026)	x	16498	0.0
	RD	4.88 (4.81-4.95)	-6.20 (-6.30- -6.10)	14431	100.0
	CD	2.41	1.03	16369	0
Exp. 5	CA	X	x	16278	0.0
	S/A	0.53 (0.52-0.55)	x	11944	0.0
	RD	3.29 (3.25-3.33)	-4.17 (-4.23- -4.10)	4800	100.0
	CD	0.02	-19.38	11470	0

***a* = *a* deviation parameter; *b* = *b* deviation parameter; SSE = sum of squared error; AICcW = support for the different models; Exp. = experiment; CA = concentration addition reference model (Eq. 5.2); S/A = synergistic or antagonistic deviations (Eq. 5.3); RD = ratio dependent synergism or antagonism (Eq. 5.4); CD = concentration dependent synergism/antagonism (Eq. 5.5)**

3.4 EC_{50} mixture analysis

Increasing the Ni or Cu concentration had a significant negative effect on the EC_{50} of the other metal in all experiments (Figure 5.5, details appendix D: Tables D4-5). Increasing Ni reduced the Cu EC_{50} starting from the lowest assessed Ni concentration (0.013 TU_{50} or 4.9 µg/L in experiment three based on the 84 % CI [205]). Furthermore, in line with a ratio-dependent deviation of the CA model, the negative influence of Ni on the Cu EC_{50} was higher than expected based on the CA reference model predictions for lower Ni concentrations. Cu also significantly decreased the Ni EC_{50} starting at 0.58 TU_{50} (or 2.4 µg/L in experiment 5), and this decrease was stronger at higher Cu concentrations. There were no significant differences in Cu EC_{50} response in respect to a changing Ni concentrations between the experiments. Therefore a single concentration response curve could be constructed (Eq. 5.7; R^2 = 0.81). The Ni EC_{50} response to a changing Cu concentration could not be fitted based on the individual experiments due to the low number of observations, therefore a single concentration response curve was fitted (Eq. 5.8; R^2 = 0.98; with the Cu or Ni EC_{50} as % of the control EC_{50} and Ni or Cu in µg/L).

$$\text{Cu } EC_{50} = 62.6 + (100 - 62.6) / (1 + (\text{Ni}/0.11)^{0.74}) \quad \text{(Eq. 5.7)}$$

$$\text{Ni } EC_{50} = 3.3 + (100 - 3.3) / (1 + (\text{Cu}/0.68)^{10.58}) \quad \text{(Eq. 5.8)}$$

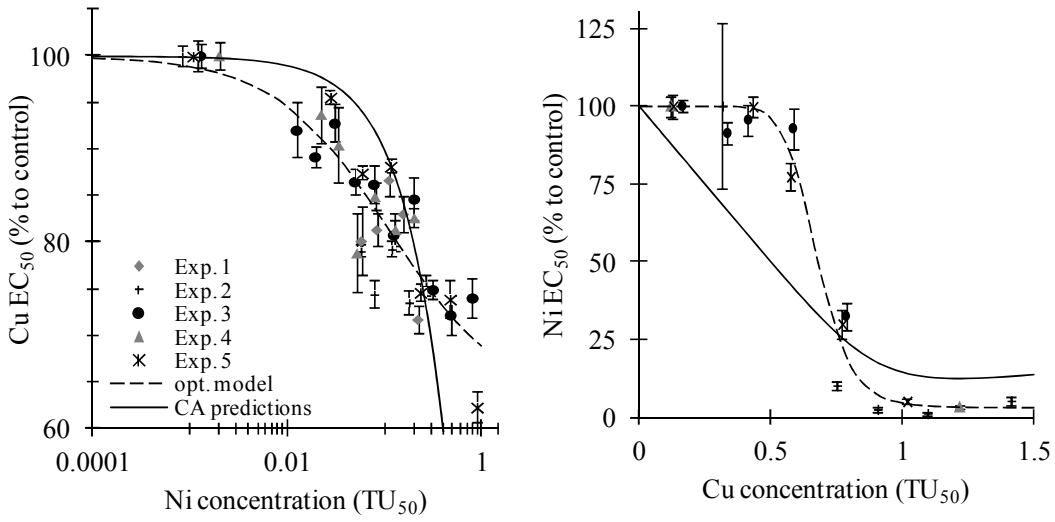


Figure 5.5. Left: the influence of Ni on the Cu EC_{50} (% to the control); Right: the influence of Cu on the Ni EC_{50} (% to the control). The black solid line indicates the predicted decrease in EC_{50} according to the CA reference model based on the average EC_{50}'s and slopes (Table 5.2), the black dashed line represents the optimal model predictions (Eq. 5.7 and 5.8).

4 DISCUSSION

4.1 Implications for risk assessment

Mussel larvae were, as expected, rather insensitive to Ni. The high Ni EC_{50}'s observed in this study (between 251.7 and 607.2 µg/L) were in line with previously reported Ni EC_{50}'s for *Mytilus* spp. (150[150] - 891[198] µg/L). Even the lowest observed Ni EC_{10} (43.2 µg/L) is still a factor 7 higher than 99 % of the Ni concentrations measured in the North Sea (99 % percentile: 5.9 µg/L). Therefore, it is unlikely that Ni, as single metal, has a significant adverse effect on mussel larval development in the environment. In contrast, mussel larvae were very sensitive to Cu with an average Cu EC_{50} of 4.1 µg/L and EC_{10} of 3.4 µg/L. These values are similar to the EC_{50}'s found in previous studies and chapter 3 with low DOC concentrations (EC_{50}: 4.9[139] - 4.6 µg/L). This confirms the high sensitivity of mussel larvae to Cu with possible adverse effects occurring at environmentally realistic concentrations (95 % quantile: 4.1 µg/L). Therefore, any change in the Cu sensitivity due to the presence of other stressors (e.g. nickel) might have implications in nature.

Significant ratio-dependent deviations from additivity, i.e. the CA reference model, were observed in all experiments. Antagonistic interactions were observed at (unrealistically) high Ni concentrations. However, synergistic interactions were found at low concentrations at which Ni as a single metal had no effect, much lower than the individual Ni EC_{50} or Ni EC_{10} (Figure 5.2). Furthermore, the Ni and Cu concentrations and the Ni-Cu ratios at which synergism was observed do occur in the environment or are lower than the proposed EU PNEC (Figure 5.1). The Cu EC_{50} is predicted to decrease with 9 % when the mussel larvae are exposed to a Ni concentration at European PNEC level (8.6 µg/L or 0.02 TU), and the Ni EC_{50} is predicted to decrease 34 % when larvae are simultaneously exposed to the European Cu PNEC (2.6 µg/L).

Although synergism in Cu-Ni mixtures has been reported [207, 208], here we demonstrate for the first time synergistic interaction effects at environmentally relevant concentrations for a binary metal mixture. Therefore, the effect of mixtures and possible interactions (including ratio dependent synergism/antagonism) should not be neglected in future marine metal risk assessments. Including the CA model would already greatly improve the ERA even for (partially) dissimilar acting chemicals such as Cu and Ni. However, even the CA model would not fully protect the mussel larvae.

How Cu and Ni interact to cause a ratio-dependent effect is unclear. Traudt et al. (2015) suggested that when the Ni concentration is high enough, free Ni ions could displace Cu at the DOC binding sites [204]. However, synergism is only observed when the Ni-Cu ratio is low and significant Cu displacement is therefore unlikely. Playle et al. (2004) proposed an alternative hypothesis and suggested that synergism occurs at low concentrations and antagonism at high concentrations due to the non-linear relationship between metal accumulation and concentration [186]. However, the accumulation dynemics of Cu and Ni (or the mixture) in mussel larvae is unknown.

4.2 Implications for future mixture experiments

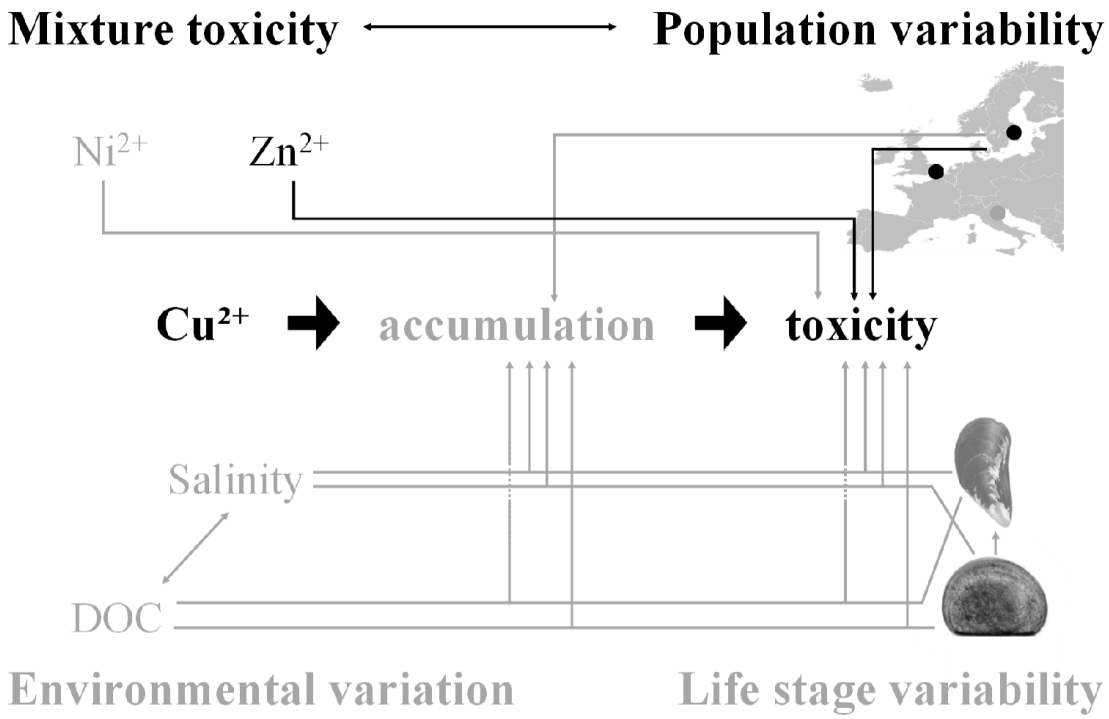
Previous research has indicated that: (1) mixture experiments with high endpoint variability have a reduced reproducibility, but reducing this variability is difficult [191], (2) mixture experiments that do not test the single metals simultaneously with the mixtures, are prone to false positive/ negative results (up to 85%) [193]. The present study provides clear evidence that reproducible effects can be obtained when using a biomarker with a low variability (larval development) in combination with an extensive setup ($144 < n < 400$) and simultaneous testing of the single metals with the mixtures. Furthermore, our results indicate that a full set of models describing all possible deviations from the CA model need to be assessed to assess reproducibility. Indeed, not assessing ratio dependency would have led to the different conclusions depending on the experiment. When only synergism/antagonism would have been assessed, the conclusion would have been synergism for two experiments, antagonism for two experiments and additivity for one leading to the conclusion that the results are not reproducible. When the assessed Ni concentrations were low the model indicated synergism but when the emphasis was on the higher Ni concentrations, antagonism was concluded. Hence, the results indicate that mixture interaction results cannot be extrapolated from high to low metal concentrations or vice versa.

Deviation parameters have a direct interpretation (e.g. synergism if $a < 0$ for the S/A model). Therefore, estimating the uncertainty on these estimates is important to understand reproducibility of empirical results and for their use in ERA. The original method developed by Jonker et al. (2005)[55] involved likelihood estimation. However, optimization algorithms only yield an optimal set of parameters [200]. Therefore, calculation of the

likelihood profile has been proposed as a way to quantify parameter uncertainty and to test significance [55]. Nonetheless, this is currently not routinely done in mixture toxicity tests [194-197]. Here, we introduced a Metropolis-Hastings Markov chain Monte Carlo algorithm to obtain posterior likelihoods of all parameters. This Bayesian approach offers two advantages over the likelihood profile. First, likelihood profiles are calculated from the likelihood function by changing 1 or a set of parameters while keeping all other parameters constant at their optimal value. MCMC algorithms, in contrast, sample the full marginal distributions, i.e. integrating the (posterior) likelihood over all possible values of all other parameters. Therefore, MCMC yields a true probability distribution. The profile likelihood, in contrast, only approaches this distribution using a transect through parameter space (i.e. because likelihoods are only evaluated at the optimal values of the other parameters). Hence, the estimated uncertainty and confidence intervals of the parameter estimate may differ between both approaches when the profile likelihood provides a poor approximation of the true likelihood distribution [209]. Second, the MCMC algorithm readily allows including any prior knowledge parameters into the model. Fitting the model to a mixture dataset using a least square estimator yields values for the EC_{50} and slope that result in an optimal overall fit to the data. However, EC_{50} and slopes can therefore strongly support from the estimates obtained from the single metal experiments or previous experiments. Prior distributions can be used to express strong belief in values of the EC_{50}, resulting in a set of parameter estimates that will more closely match the single metal EC_{50}s and slopes and therefore reality.

4.3 CONCLUSION

In this study we demonstrated a reproducible ratio-dependent effect of Cu-Ni mixtures on mussel larval development. The results indicate that synergism according to the CA model can occur in the marine environment at environmentally relevant concentration. This should be accounted for in future ERA methods and procedures. Our results also highlight that extrapolating mixture toxicity results (e.g high to low mixture concentrations) may result in erroneous toxicity predictions.


Mixture toxicity
Population variability
Ni2+
Zn2+
Cu2+
accumulation
toxicity
Salinity
DOC
Environmental variation
Life stage variability

VI

POPULATION DEPENDENT METAL AND METAL MIXTURE TOXICITY IN *MYTILUS EDULIS* LARVAE

ABSTRACT

Populations living in suboptimal conditions are assumed to be more sensitive to (mixtures of) pollutants as they are already stressed by the environment. This hypothesis was tested by investigating the influence of Cu, Zn, Ni and of Cu-Zn mixtures on larvae of *Mytilus edulis* on two populations: A North Sea population and a, salinity stressed, Baltic Sea population. Larvae from the Baltic Sea population were indeed smaller, grew more slowly and were more sensitive to Zn and Ni. However, organisms from both populations were equally sensitive to Cu and responded similar to a Cu-Zn mixture. This demonstrates that organisms living in suboptimal conditions are not necessarily more sensitive to pollution or that an environmentally stressed population is more affected by mixture toxicity even if they have a different sensitivity to the individual components of the mixture.

1 INTRODUCTION

Different populations may have a different sensitivity to pollution. This could result from differences in acclimation or adaptation to the local environment which, as a side effect, can result in a different sensitivity [210, 211] or because organisms that live in suboptimal conditions (Figure 1.3) experience environmental stress and may therefore be more sensitive to anthropogenic pollution [58, 59]. Furthermore, because mixture toxicity effects may depend on the ratio or concentration of the individual pollutants (see chapter 5 and Nys et al. (2015)[56]) any changes in sensitivity to individual pollutants may also result in an altered response to the whole mixture. Therefore, knowledge on inter-population differences in pollution sensitivity and the possible consequences for mixture toxicity is needed to accurately and realistically predict the possible adverse effect of pollution. For the marine environment, however, only few studies have assessed the influence of inter-population variability on the toxicity of pollutants and their mixtures [212].

In this study *Mytilus edulis* larvae from two populations (North Sea and Baltic Sea) were used as model organisms. Mussels can live in environments with different salinities ranging from the typical seawater in the North Sea to the brackish water of the Baltic Sea. Previous research has suggested that adult Baltic Sea mussels experience environmental stress due to the low salinity [58, 59]. Several studies showed that these mussels have a slower metabolism and higher basic energy needs resulting in a reduced growth rate compared to mussels that live in full strength seawater [122, 213]. It has been hypothesized that Baltic Sea mussels could therefore be more sensitive to other stressors (e.g. Cu or Zn) [58, 59]. It is currently not known if the Baltic Sea mussel larvae are also experiencing salinity stress and if they are indeed more sensitive to metals or their mixtures compared to mussels that live in typical (full strength) seawater.

The goal of this study was to assess if there are differences in the sensitivity to single and mixtures of metals between two mussel populations originating from different (salinity) environments. This was accomplished by exposing mussel larvae from two populations (North Sea and Baltic Sea) to three metals (Cu, Zn and Ni) and to a binary mixture (Cu-Zn) in a full factorial design.

2 MATERIALS AND METHODS

2.1 Adult mussel collection and maintenance

Adult *Mytilus edulis* were collected in the intertidal zone of the Belgian coast (April 2013 and 2015; Middelkerke) and, subtidal, in the Baltic Sea near Stockholm (June 2014; Askö). The ambient salinity was 32 psu in the North Sea and 6 psu in the Baltic Sea with a seawater temperature between 8 and 10 °C at both locations. Prior to the toxicity experiments the mussels were kept in a 100 L aquarium containing recirculating, aerated, artificial seawater (Instant Ocean®) at a temperature of 8 °C, a salinity of 32 psu (North Sea) or 6 psu (Baltic Sea) and fed *ad libitum* with Shellfish Diet 1800® (Reed Mariculture Inc.). The toxicity experiments were performed within two weeks after collection and new adult mussels were collected for each experiment.

2.2 Mixture experiments

The individual Cu, Zn and Ni sensitivity was assessed in standard concentration response tests with minimum 1 control and 5 treatments (four replicates/treatment). The toxicity of the Cu-Zn mixture was assessed using a full factorial design experiment (6 to 7 Cu concentrations and 6 to 11 Zn concentrations; Appendix E: Table E1) with four replicates per treatment. All experiments (both single metals and mixtures) were performed according to the *ASTM standard guide for conducting static acute toxicity tests starting with embryos of four species of saltwater bivalve molluscs* (E724-98) [115] as described in Chapter 3. Briefly: Gametes were obtained after a heat shock induced spawning of the adult mussels. Within 30 minutes fertilization took place and thereafter the embryos were added to the test vials (50 mL poly-ethylene vials filled with 40 mL of ASTM seawater) and placed in a temperature controlled room at 15 °C for 48 h. Test vials were prepared one day prior to ensure a chemical equilibrium [152]. At the end of the experiment the larvae were killed and preserved with formaldehyde and their development was determined using a microscope (10x10x magnification). The size of 15 developed larvae in the control treatment of the mixture experiments were measured (length and width) via light microscopy. Each experiment was repeated (at least) twice. Ni toxicity data for the North Sea population was described in chapter 5 and reused here.

Two changes to this workflow were made for the Baltic Sea population. Preliminary experiments had shown that after 48 h none of the Baltic Sea embryos had reached the D-larvae stage in the control treatment. The exposure time was thus prolonged from the recommended 48 h to 60 h after which the majority of the control organisms reached the D-larvae stage. Secondly, Baltic Sea embryos did not develop successfully in the artificial seawater described in the ASTM protocol (four attempts with different batches and different sources of deionized water). So natural, filtered seawater from the Baltic Sea was used in all subsequent tests. We are aware that the use of different seawater sources can result in additional variability in the results. Especially DOC (0.8 ± 0.1 mg/L for ASTM seawater and 5.3 ± 0.2 mg/L for the natural Baltic Sea seawater) may confound the results as DOC can complex Cu and thereby reduce the Cu toxicity to mussel larvae (see chapter 3). However, several specific models are available to correct the Cu toxicity to mussel larvae in the presence varying dissolved DOC concentrations ([139, 150] and chapter 3). This issue will be assessed in depth in the discussion.

2.3 Analytical chemistry

A water sample was taken from each vial at the start the experiment [115]. Samples from the four replicates were pooled and filtered (0.45 µm). The samples for metal analysis were acidified with 0.14 mol/L analytical grade HNO_3 and stored in polypropylene tubes at 4 °C before analysis. Cu, Zn and Ni concentrations were determined via ICP-OES (Thermo scientific iCAP 7000 series). Water samples for DOC analysis were stored in glass tubes at 4 °C. The DOC analysis was performed via a Shimadzu TOC-5000 analyzer using the high-temperature catalytic oxidation technique [153, 154].

2.4 Data analysis

Larval development and single metal toxicity

Differences in larval length and width were assessed both between experiments and between populations using ANOVA. The single metal toxicity data was evaluated by fitting the data to a 3-parameter log-logistic concentration response model using the DRC-package in R [173]. Differences in EC_{50} were considered significant if 95% confidence intervals (CI) of the EC_{50} did not overlap.

Cu-Zn mixture toxicity

First, six types of mixture models were considered. The two commonly used reference models independent actions (IA[50]) and concentrations addition (CA[51]), as well as two frequently used deviations from both IA and CA: synergistic/antagonistic (SA) and dose ratio dependent (DR) deviations (for details see Jonker et al. (2005)[55]). Each of the six models was fitted to the data obtained in each experiment using a Markov Chain Monte Carlo (MCMC) algorithm. This technique offers the advantages over routinely used least square estimates[55] that prior knowledge on parameters values can easily be included into the algorithm and that the parameter uncertainty is fully quantified. For a more in depth discussion see Chapter 5. Parameter estimates of the maximum development, EC_{50} and slope were restricted to the positive values of the 99.999% CI of the parameter estimate obtained from the single metal concentration-response curve (see above). Maximum development was further restricted to 100 %. Deviation parameters were restricted using a uniform uninformative prior distribution [-20, 20]. MCMC algorithms were implemented in R using the method described in chapter 5 for the CA models and using the JAGS[214] package for the IA model. For IA, implementation of the MCMC using JAGS was preferred over the method provided in Chapter 5 because of computational efficiency (note that CA cannot be implemented in JAGS as numeric approximation is required to solve the model equations). The support for each model was calculated based on the AIC weights. Concentration dependent deviations from both the CA and IA model, as proposed by Jonker et al. (2005)[55], could not be fitted via the MCMC approach.

Second, mixture effects were investigated by testing if the EC_{50} of each metal depended on the concentration of the other metal (e.g. Traudt et al. (2015)[204] and Chapter 5). Briefly: Cu EC_{50} was determined for each Zn concentration, and vice versa, using a 3-parameter log-logistic concentration response models. Next we tested if the EC_{50} of one metal dependet on the concentration of the other metal by fitting the data to a 3-parameter log-logistic model. Hence, this is similar to evaluating if the mixture response deviates from the IA reference model as the IA model assumes a constant EC_{50} (see also Chapter 1). Because differences in individual metal toxicity were not the focus of this analysis the EC_{50}'s were rescaled to the control (with control as 100 % EC_{50}) and the concentrations rescaled to toxic units (TU, concentration/control EC_{50}). We simultaneously assessed if the mixture effect differed between the two populations via an ANOVA analysis between the model with and without population as parameter.

3 RESULTS

3.1 Larval development and single metal toxicity

North Sea embryos developed faster and were significantly larger (length 102.3 ± 2.4 µm, width 72.9 ± 2.4 µm after 48 h) compared to the Baltic Sea embryo's (length 87.2 ± 5.0; width 67.7 ± 4.2 µm after 60 h; Figure 6.1). There were no significant differences in length or width between the two experiments of the same population;

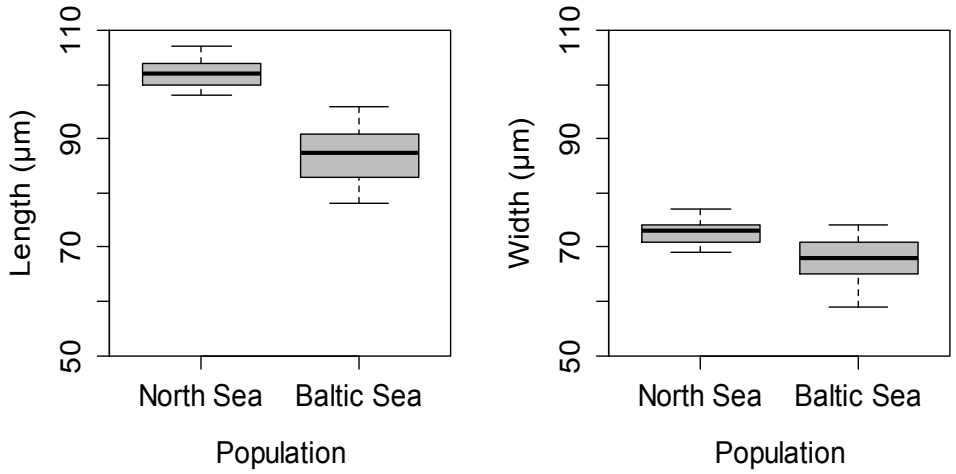


Figure 6.1. Boxplots (25 % - median - 75 %, whiskers 1.5 x interquartile distance) of the length and width of the North Sea (NS, n = 30, 48 h) and Baltic Sea (BS, n = 30, 60 h) larvae.

For all three tested metals (Cu, Zn and Ni) there was a significant difference in toxicity between the two populations (table 6.1). Copper was significantly less toxic to the Baltic Sea population compared to the North Sea populations (EC_{50}: factor 2.8) while for both Zn and Ni the Baltic Sea population was significantly more sensitive than the North Sea population (by a factor 2.7 to 4.4 for Zn and 1.7 to 4.8 for Ni). No significant differences in toxicity of Cu, Zn and Ni were observed between different experiments of the Baltic Sea population. For the North Sea population significant differences between the different experiments were detected (e.g. experiment I and M1 for Zn; Table 6.1). Yet, for all three metals there was no overlap in the 95% confidence intervals of the estimated EC_{50} values between the two populations (Figure 6.2).

Table 6.1. The single metal concentration response curve parameters (Control development, EC_{50}, EC_{10}, slope) and SE (between brackets) for all experiments.

Pop.	Exp.	C. %	Cu EC_{50} µg/L	slope	Zn EC_{50} µg/L	slope	Ni EC_{50} µg/L	slope
NS	I	96.8 (2.1)	NA	NA	141.3[a] (1.5)	15.4 (1.1)	NA	NA
	M1	90.0 (5.0)	4.0[a,c] (0.1)	9.3 (1.0)	97.6[b] (4.4)	2.9 (0.4)	NA	NA
	M2	94.1 (1.4)	3.8[a] (0.0)	9.4 (0.8)	133.1[a,b] (23.8)	18.0 (17.4)	NA	NA
BS	I	91.3 (2.8)	11.0[b] (0.2)	9.9 (2.0)	NA	NA	NA	NA
	M1	94.9(5.0)	11.2[b] (0.3)	12.7 (2.9)	32.5[c] (7.9)	25.9 (47.2)	NA	NA
	M2	86.1 (1.3)	10.9[b] (0.1)	12.2 (2.1)	36.3[c] (0.4)	7.6 (0.8)	NA	NA
BS	I	86.3 (3.2)	NA	NA	NA	NA	126.8[a] (12.0)	1.8 (0.3)
	I	91.3 (2.8)	NA	NA	NA	NA	145.0[a] (3.9)	3.1 (0.2)
NS	M1	73.5 (7.8)	4.2[c,d] (0.1)	9.4 (1.2)	NA	NA	ND	ND
	M2	75.7 (1.1)	4.5[d] (0.1)	8.8 (0.6)	NA	NA	607.2[b] (159.9)	0.8 (0.2)
	M3	92.0 (3.4)	4.4[d] (0.1)	8.1 (0.9)	NA	NA	332.7[b,c] (6.0)	8.2 (2.2)
	M4	95.3 (2.8)	4.0[c] (0.1)	9.7 (1.5)	NA	NA	251.7[d] (8.3)	4.0 (0.5)
	M5	94.2 (1.4)	4.1[c] (0.0)	11.1 (0.8)	NA	NA	467.2[b] (25.6)	9.5 (4.6)

Pop. = population (NS = North Sea, BS = Baltic Sea); Exp. = Experiment; C. = control larval development; I = single metal experiment; M = mixture experiment; NA = not applicable; ND = not determined (not significant); [a,b,c,d] indicate significant differences between EC_{50}'s

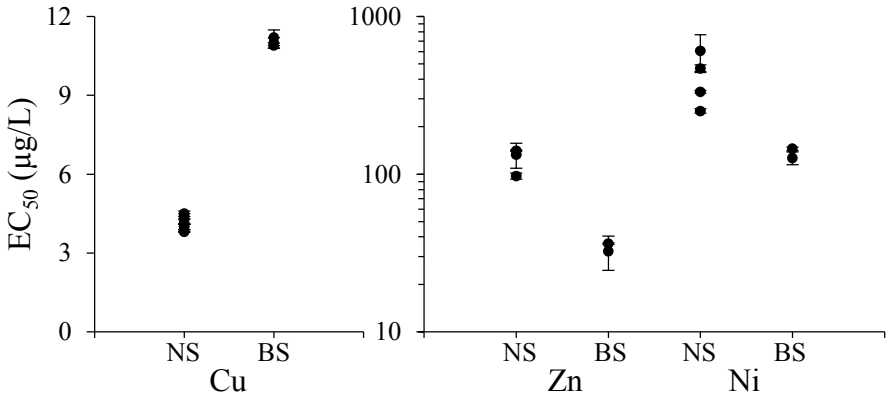


Figure 6.2. A visual representation of the single metal (Cu, Zn and Ni) EC_{50}'s for both populations (NS = North Sea; BS = Baltic Sea).

3.2 Cu-Zn mixture toxicity

A consistent synergistic interaction between Cu and Zn according to the IA reference model (*a* deviation < 0; 37 % reduction of the residual variation) and antagonistic interaction according to the CA reference model (*a* deviation > 0; 77 % reduction of the residual variation) was supported for both populations (Table 6.2). However, the Cu-Zn mixture had, on average, more synergistic (IA) and less antagonistic (CA) effect on the North Sea population compared to the Baltic Sea population (Table 6.2, magnitude of the *a* deviation). Further expanding the mixture model to include ratio dependent mixture interactions (RD) could only explain 6 (IA) to 8 % (CA) more of the observed variation. The RD model also supports a consistent synergism (CA) or antagonism (IA) (i. e. consistent sign for the 'a' deviation parameter). However, the sign of the *b* deviation parameter (indicating the ratio at which the change occurs) was not reproducible between experiments or populations. For more detailed information on the models appendix E (Tables E2 and E3).

Similar results were observed when the data was evaluated via the EC_{50} analysis (Figure 6.3). With the exception of two Cu EC_{50}s, the decrease in the EC_{50} is situated between the IA and CA predictions for both populations with a similar trend in both populations. There was a significant difference in the magnitude of decrease of both the Zn EC_{50} and Cu EC_{50} due to an increase in respectively Cu or Zn between the two populations (see Eq. 6.1 - 6.4). More specifically, the Baltic Sea mussel larvae Zn or Cu EC_{50} was less affected by high Cu or Zn concentration than the North Sea mussels EC_{50} although the difference was only present between 0.5 and 1 TU (for both metals). For details on the EC_{50}s see appendix E (Tables E4-5).

Baltic $\%\ Cu\ EC_{50} = 86.9 + (100-86.9) / 1 + (ZnTU/0.288)^{46.6}$ (Eq. 6.1)

North Sea $\%\ Cu\ EC_{50} = 69.7 + (100-69.7) / 1 + (ZnTU/0.488)^{3.76}$ (Eq. 6.2)

Baltic $\%\ Zn\ EC_{50} = 100 / 1 + (CuTU/1.04)^{24.0}$ (Eq. 6.3)

North Sea $\%\ Zn\ EC_{50} = 100 / 1 + (CuTU/0.928)^{4.23}$ (Eq. 6.4)

With % EC_{50} as percentage of the control EC_{50} (see table 6.1) and TU as toxic unit (concentration/control EC_{50}). The lower bound of the Zn EC_{50} models was fixed at 0 as lower values are nonsensical.

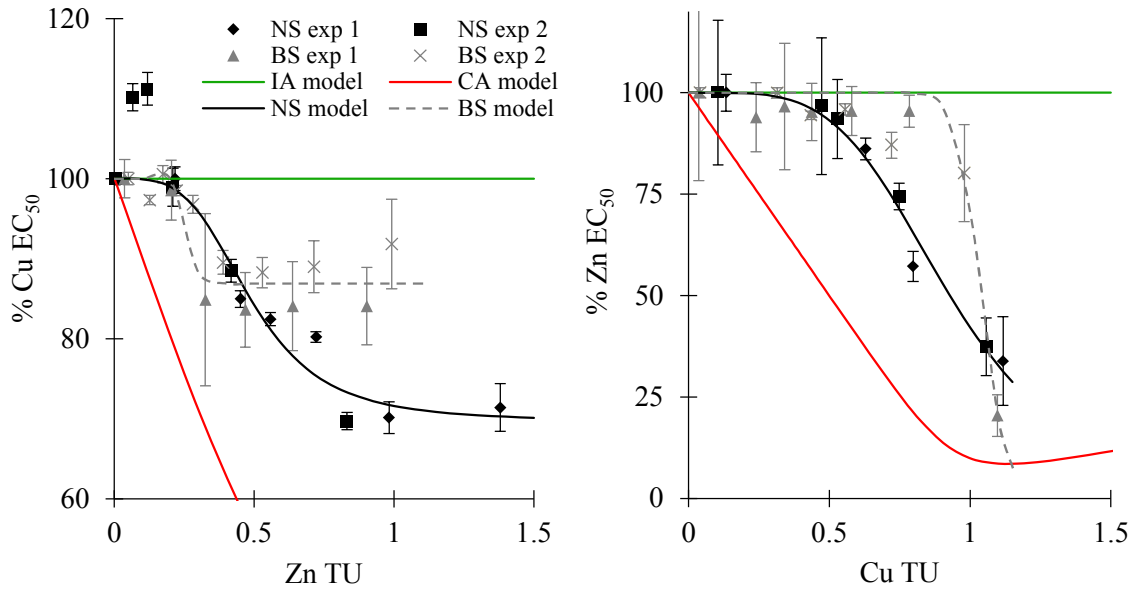


Figure 6.3. The influence of Zn on the Cu EC_{50} (left, Eq. 6.1 for BS and Eq. 6.2 for NS) and Cu on the Zn EC_{50} (right, Eq. 6.3 for BS and Eq. 6.4 for NS) compared to the reference models (IA (green), CA(red)). Predictions of the reference models are based on average the single metal concentration response curves (Table 6.1). % EC_{50} = EC_{50} at TU_x/ EC_{50} at TU_0; NS = North Sea; BS = Baltic Sea; IA = independent action reference model; CA = concentration addition reference model; TU = toxic unit (concentration / EC_{50}); error bars = SE

Table 6.2. The optimal deviation parameter estimates and 95 % credibility interval (between brackets), sum of squared error (SSE) and AICc weight (AICcW in %) for each experiment and each deviation model (see appendix E and Jonker et al. (2005) for details).

Exp.	Mod.	Dev.	a	b	SSE	AICcW
NS exp. 1		-	NA	NA	17485	0
	CA	S/A	1.39 (1.36- 1.42)	NA	5613	63
		RD	2.03 (1.94- 2.10)	-1.47 (-1.59- -1.30)	5490	37
		-	NA	NA	10219	0
	IA	S/A	-1.66 (-2.08- -1.29)	NA	5935	86
		RD	-0.90 (-1.94- 0.17)	-1.87 (-4.74-0.41)	5909	14
NS exp. 2		-	NA	NA	22495	0
	CA	S/A	1.09 (1.07- 1.11)	NA	6370	85
		RD	0.67 (0.53- 0.79)	0.48 (0.34-0.65)	6348	15
		-	NA	NA	19609	0
	IA	S/A	-3.32 (-3.85- -2.79)	NA	11916	0
		RD	-7.83 (-9.01- -6.66)	8.18 (6.13-10.41)	9036	100
BS exp. 1		-	NA	NA	49055	0
	CA	S/A	1.95 (1.94- 1.97)	NA	10131	0
		RD	4.31 (4.26- 4.37)	-4.71 (-4.82- -4.60)	8162	100
		-	NA	NA	9188	0
	IA	S/A	-4.92 (-5.51- -4.28)	NA	4612	88
		RD	-5.47 (-7.01- -3.91)	1.37 (-2.16-4.93)	4608	12
BS exp. 2		-	NA	NA	86963	0
	CA	S/A	1.63 (1.62-1.64)	NA	9428	0
		RD	2.30 (2.28-2.32)	-1.01 (-1.08- -0.95)	8491	100
		-	NA	NA	9047	0
	IA	S/A	-2.00 (-2.41- -1.56)	NA	7432	10
		RD	-0.19 (-1.34-1.05)	-3.68 (-5.61- -1.73)	7222	90

Mod. = reference model; dev. = deviation model; *a* = *a* deviation parameter; *b* = *b* deviation parameter; SSE = sum of squared error; AICcW = support for the different models; Exp. = experiment; CA = concentration addition reference model; IA = independent action model S/A = synergistic or antagonistic deviations; RD = ratio dependent synergism or antagonism; NA = not applicable for this model; NS = North Sea population; BS = Baltic Sea population.

4 DISCUSSION

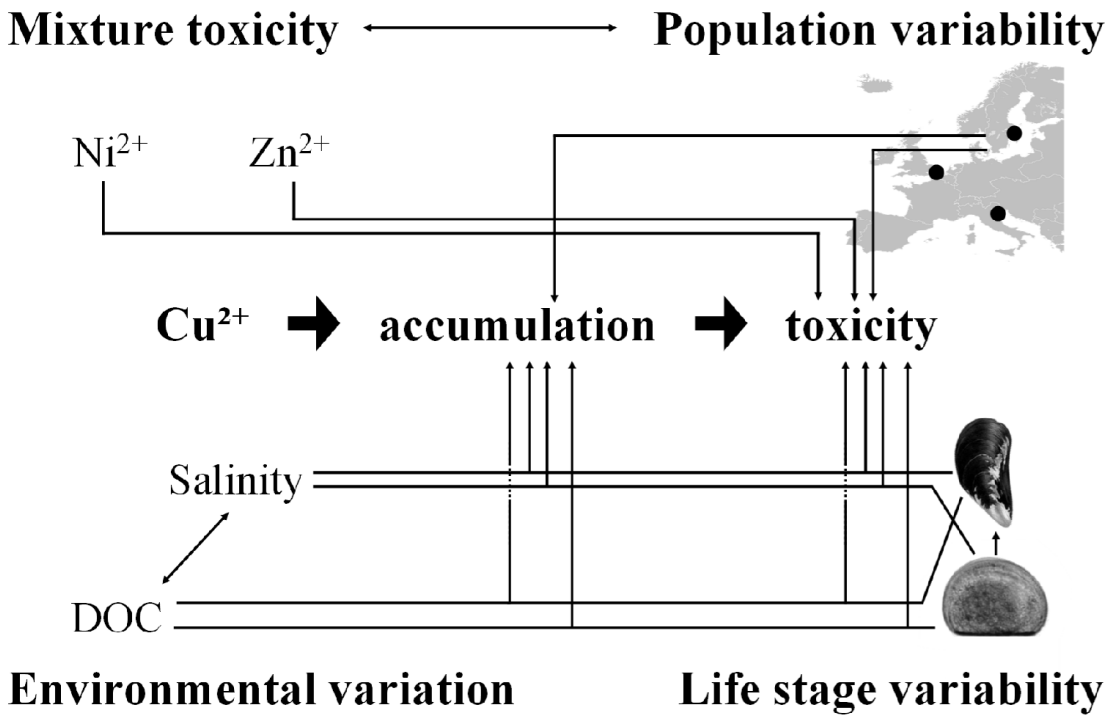
It has been hypothesized that mussels that live in the brackish water of the Baltic Sea could be more sensitive to anthropogenic stressors compared to mussels that live in the North Sea, because they already experience environmental stress due to the low salinity [58, 59]. Previous research has provided evidence that adult mussels from the Baltic Sea have a lower metabolism and growth rate in their natural environment compared to North Sea mussels [122, 213]. In this study Baltic Sea larvae developed slower (> 48 h) compared to North Sea larvae (< 48 h) and were 20 % smaller, indicating that the larvae life stage is also affected by the lower salinity. The possible adverse influence of size on mussels larval settlement and survival is currently unknown.

Besides the possibility that Baltic mussels are more sensitive to anthropogenic stressors due to the salinity stress, the biotic ligand model predicts an increase in metal availability due to a decrease in complexation and competition with a decreasing salinity (decreased ion concentration) [17, 136]. Therefore, one could assume that the Baltic Sea mussel population would be more sensitive to metals either directly due to the low salinity itself or due to the suboptimal living conditions (by the low salinity). Because the goal of the study was to assess differences in toxicity between populations at their natural salinity the experiments both differed in salinity and population (North Sea and Baltic Sea). Hence, it is not possible to disentangle the effect of salinity and the effect of possible local adaptation at this stage.

The Zn and Ni results of this study do support the assumption that Baltic mussels are, at their natural salinity, more sensitive to anthropogenic pollution. Baltic mussel larvae are a factor 2.7 to 4.4 more sensitive to Zn compared to the North Sea larvae and a factor 1.7 to 4.8 more sensitive to Ni. Even in the presence of a higher DOC concentration which may reduce the toxicity of metals by forming metal-DOC complexes that are not or less available for uptake [215, 216]. In contrast to Zn and Ni, the larval Cu EC_{50} was between 2.4 and 2.9 times higher for the Baltic Sea compared to the North Sea population. However, due to the difference in DOC concentration, and the profound effect of DOC on Cu toxicity, these values are not directly comparable. Yet, several equations have been proposed to adjust the Cu toxicity to mussel larvae for the DOC concentration in seawater. Based on these equations a very similar difference in Cu toxicity is predicted (factor 2.1 (chapter 3), 3.6 [150] or 3.1 [179]). Combined with the fact that previous research has not found a difference

in Cu sensitivity between North Sea and Baltic Sea settled mussels (Chapter 3) it seems that Baltic Sea and North Sea mussels have an equal sensitivity to Cu.

Baltic mussel larvae are smaller, grow slower and are more sensitive to Zn and Ni compared to North Sea mussel larvae. Nevertheless, the overall observed Cu-Zn mixture interaction is very similar between the two populations: synergism according to the CA reference model and antagonism according to the IA reference model. Baltic Sea mussel larvae are even (slightly) less affected by the Cu-Zn mixture compared to the North Sea mussels. This result demonstrates that organisms living in suboptimal conditions do not need to be more sensitive to pollution in terms of mixture interactions even if they have a different sensitivity to the individual components of the mixture.


Mixture toxicity
Population variability
Ni2+
Zn2+
Cu2+
accumulation
toxicity
Salinity
DOC
Environmental variation
Life stage variability

VII

GENERAL CONCLUSIONS AND RESEARCH RECOMMENDATIONS

1 INTRODUCTION

In ecotoxicology, experiments are preferably performed in optimal conditions with as little variation as possible to increase reproducibility and inter-experiment comparability. The data may subsequently be used in environmental risk assessment to calculate the predicted no effect concentration (PNEC) and determine environmental quality standards (EQS). However, this approach might not reflect reality. Both biotic and abiotic variables may affect the outcome of an ecotoxicity experiment and therefore the risk assessment process [17, 18, 169]. **The main objective of this research was to examine the effect of possible sources of variation on the toxicity of chemicals on marine organisms in order to increase the realism of current environmental risk assessment procedures**. To address this issue, we examined the influence of **environmental variability, mixture toxicity, population variability** and **life stage variability** on the accumulation and/or toxicity of Cu on the mussel. In this chapter the main conclusions of this dissertation are summarized and possible future research perspectives are suggested based on each of the four original research questions.

2 ENVIRONMENTAL VARIABILITY

Do salinity and DOC affect Cu accumulation and toxicity? (Chapter 2,3 and 4)

In Chapter 2 and 3 the influence of salinity and DOC on the accumulation and toxicity of Cu to mussel larvae was assessed. An increase in DOC had – as expected – a protective effect on the Cu accumulation and toxicity in mussel larvae similar to observations made in several previous studies and as predicted by the BLM predictions [41, 217]. An increase in salinity should, according to the freshwater BLM, decrease the toxicity of Cu due to an increased complexation of Cu with the anions in the water and increased competition with cations at the biotic ligand [17, 18, 169, 170]. However, an increase in salinity resulted in an increased Cu accumulation and toxicity to the mussel larvae. Most likely a changing salinity alters the physiology of the larvae and thereby the accumulation rate and toxicity of Cu [16].

In Chapter 4 the influence of the same variables to settled mussels was evaluated. In contrast to the mussel larvae, salinity and DOC did not or barely affect the accumulation

and toxicity of Cu to settled mussels. To our knowledge this is the first time that no protective effect to Cu toxicity or accumulation of DOC was observed in a chronic exposure experiment. The absence of a protective DOC effect contradicts with the BLM concept and indicates that, for some lifestages of some organisms, DOC-Cu complexes can be bioavailable.

In conclusion: a changing salinity may alter the physiology and consequently the sensitivity of an organism and DOC does not always protect against Cu toxicity. The construction of a marine BLM to determine a environment-dependent Cu PNEC or EQS (similar to the freshwater BLM [17, 169]) based only on complexation and competition alone is currently not possible.

Future research recommendations: The absence of any effect of DOC on the toxicity of Cu to settled mussels was one of the main findings in this thesis and one of the most unexpected. It remains possible that this is a local phenomenon restricted to *Mytilus edulis* and the DOC in the North Sea. Further research is recommended to assess whether or not this is a more widespread phenomenon. Possible species to consider for analysis are *M. californianus* (genus specific response), *Perna viridis* (family specific response) and oysters (class specific response). Besides investigating different species, it would be very useful to investigate the mechanistic reason why we do not observe the protective effect of DOC in settled *M. edulis* for example via stable isotope analysis. Knowing this would allow a biology-based prediction of animals or life-stages for which Cu-DOC complexes could also be available.

3 MIXTURE TOXICITY

Do metals in a mixture interact and does this happen at environmentally relevant concentrations? (Chapter 5 and 6)

In Chapter 5 the effects of binary Cu-Ni mixtures on mussel larvae development were assessed. A new statistical analysis method was implemented to analyze the data. The framework of the analysis was based on the models in Jonker et al. (2005)[55] but through the use of Markov Chain Monte Carloc algorithms (MCMC) we ensured that optimal parameter estimates were obtained and that the variability on the parameter estimates could

be derived. A reproducible concentration-ratio dependent mixture toxicity effect was observed according to the CA reference model. Antagonism was observed at high Ni concentrations (high Ni-Cu ratio) and synergism at lower Ni concentrations. Providing evidence that synergistic effects can occur at concentrations and metal ratio's that are relevant for the marine environment.

In Chapter 6 the effect of the binary Cu-Zn mixture on mussel larvae was assessed. The effect was antagonistic according to the concentration addition model but synergistic according to the independent action model. The interaction between Cu and Zn was less strong compared to the interaction between Cu and Ni.

In conclusion: both studies indicate that metal mixture toxicity interactions occur in the marine environment and that this may occur at concentrations currently measured in -polluted- waters. Importantly, there is a positive correlation between the concentration of Cu and other metals in the environment (for the North Sea, chapter 1 box 1.1). Therefore, we suggest that future marine ERA should take into account mixture toxicity. This could be based on the CA reference model as most conservative approach, although even this is not protective enough in some cases.

Future research recommendations: The results of this study have also indicated that an extrapolation of the mixture toxicity results to untested concentrations or ratios may lead to the wrong effect assessment. Therefore, future mixture experiments should (also) focus on evaluating environmentally relevant concentrations and ratio's and not only the concentrations were an effect is anticipated.

4 INTER-POPULATION VARIABILITY

Are there inter-population differences in Cu accumulation or sensitivity? (Chapter 4 and 6)

In Chapter 4 and 6 two *M. edulis* populations were assessed, a North Sea population and a Baltic Sea population. It was hypothesized that the Baltic Sea population would be more sensitive to anthropogenic pollution (compared to the North Sea population) as they experience natural stress due to the low salinity in the Baltic Sea. Previous research has indicated that they have to allocate more energy to overall basic maintenance, resulting in

a reduced growth rate of settled mussels compared to mussels of the North Sea population [65, 120, 122]. Our studies (1) confirmed that settled mussels from the Baltic Sea have a lower feeding rate compared to North Sea mussels (Chapter 4) and (2) provided evidence larval growth is also reduced in the Baltic Sea mussel larvae (Chapter 6). Baltic Sea mussels accumulated significantly more Cu compared to their North Sea counterparts (Chapter 4). This might be due to an increased ion exchange capacity of the Baltic mussels to maintain cellular homeostasis. However, in contrast to the initial hypothesis, no major differences were found in sensitivity to Cu toxicity or the influence of other variables on the toxicity of Cu (salinity, DOC, Zn). Although no differences were observed for Cu, Baltic mussels were more sensitive to Zn and Ni indicating that inter-population variability in sensitivity to anthropogenic pollution does occur but is metal-dependent.

In conclusion: environmentally stressed populations are not necessarily - by default - more sensitive to anthropogenic pollution. Inter-population variability in sensitivity is pollutant dependent. Furthermore, organisms from different populations with a similar sensitivity may have a different strategy to deal with pollution. This also means that the body burden may not be a good predictor of exposure and should not be extrapolated between populations.

Future research recommendations: Although no difference in Cu sensitivity was observed between the two populations used in this study, the Baltic Sea population was more sensitive to Zn and Ni. Therefore, more research is needed to assess why different populations may have a different sensitivity and why this is pollutant-dependent. Eventhough the Cu sensitivity was similar, major differences were observed in the Cu body and gill burdens. Further research to have a better (mechanistic) understanding of the relation between the accumulation and toxicity of pollutants and the possible different mechanisms to deal with pollution in different populations of a single species is recommended.

5 LIFE STAGE VARIABILITY

How does the effect of salinity and DOC on Cu toxicity differ between mussel larvae and settled mussels? (Chapter 2, 3 and 4)

In chapter 2 and 3 the effect of salinity and DOC on Cu toxicity was assessed on mussel larvae, while similar experiments were performed on settled mussels in chapter 4. As demonstrated for most species, the larval stage of the mussel is the most Cu sensitive life stage when exposed under standard laboratory conditions [115] although the Cu sensitivity of settled mussels is only marginally lower. However, there are major differences in their response to Cu in combination with environmental variation. Salinity and DOC affected the Cu accumulation and sensitivity of mussel larvae but not that of settled mussels. As a consequence, the settled mussels are more Cu sensitive than the larvae when water has a high DOC concentration.

In conclusion: our results imply that: 1) it is not possible to extrapolate the results of one life stage to another and 2) the most sensitive life stage to a pollutant may depend on the laboratory or environmental conditions.

Future research recommendations: Due to the variability in life stage sensitivity in combination with environmental variation it is recommended that, certainly for sensitive species, not only the larval stage is investigated in ecotoxicology or included in ERA.

6 OVERALL CONCLUSION

All variables investigated in this study changed the accumulation and/or the toxicity of Cu in mussels. The assessed environmental variables, i.e. salinity and DOC, had a strong influence on the accumulation and toxicity of Cu to mussel larvae but not to settled mussels. Furthermore, the influence of salinity on the Cu toxicity in mussel larvae could not be explained based on complexation and competition. Therefore, using the current knowledge, a marine BLM based only on the water chemistry seems implausible. Besides the influence of the environment, we have provided evidence that synergistic metal mixture interactions can occur at concentrations currently measured in the marine environment. To adequately protect marine organisms, metal mixture interactions should be included in future

environmental risk assessment procedures. Finally, the two assessed populations were equally sensitivity to Cu. This suggests that naturally stressed populations are not 'by default' more sensitive to pollution than unstressed populations. However, population differences in organism sensitivity to other metals (Zn and Ni) were observed indicating that inter-population variability is pollutant-dependent and that this knowledge may need to be included in future ERA procedures.

A visual representation of the influence of the different variables (and interactions) that were assessed in this thesis is presented in Figure 7. The thickness of the lines indicates the relative magnitude of the effect of the variable on the Cu accumulation or toxicity. The color indicates if it is a positive (green), negative (red) or no/limited (black) influence on the accumulation or toxicity of Cu. For example: Ni had a more severe effect on the toxicity of Cu than Zn (thicker line), or DOC has a protective effect on the accumulation and toxicity of Cu to mussel larvae (green) but not to settled mussels (black).

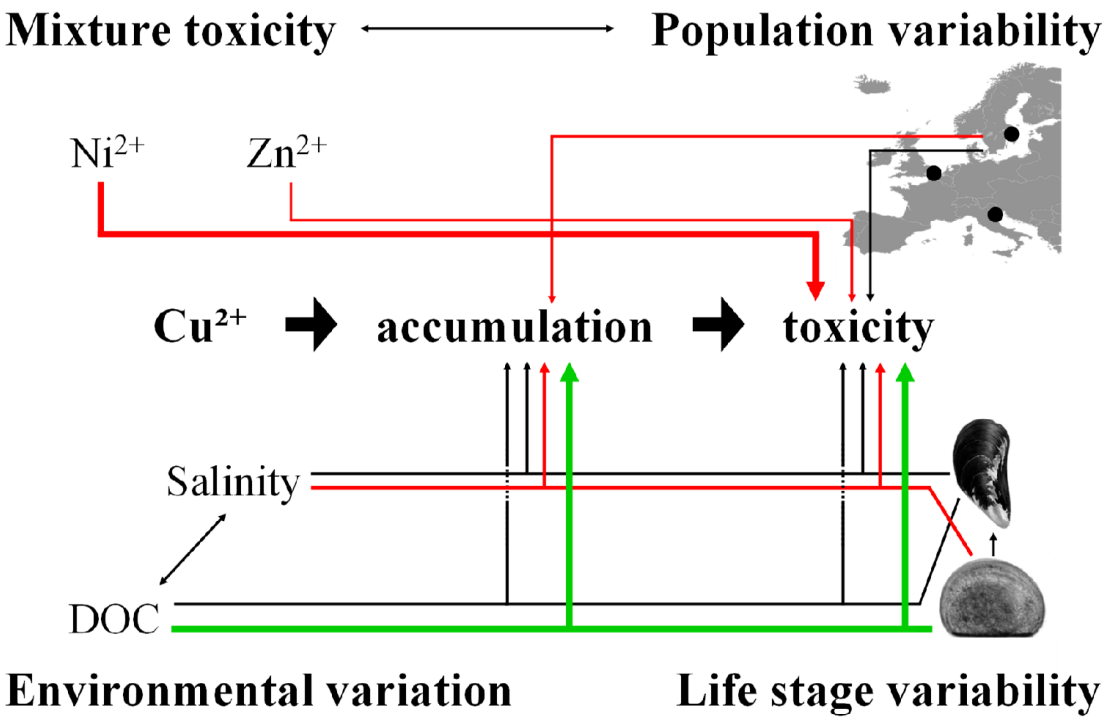


Figure 7.1. A schematic overview of all variables and interactions that were assessed in this thesis. Black: no/limited interaction; Red: mainly increased Cu accumulation/toxicity; Green: mainly decreased Cu accumulation/toxicity; thickness of the lines indicates relative importance of the variable

A

APPENDIX A: SUPPORTING INFORMATION CHAPTER II

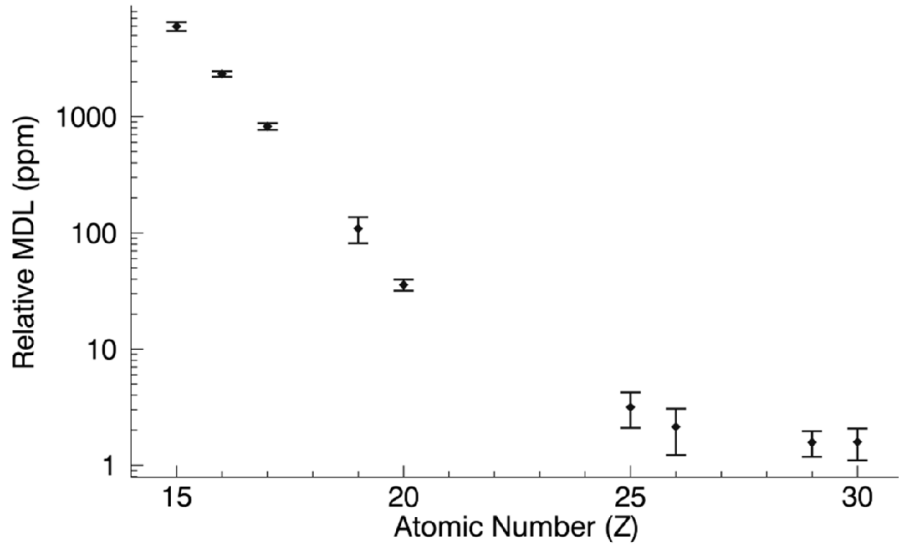


Figure A1. The minimum detection limit for the micro SR-XRF based on a bovine liver sample (NIST 1577b)

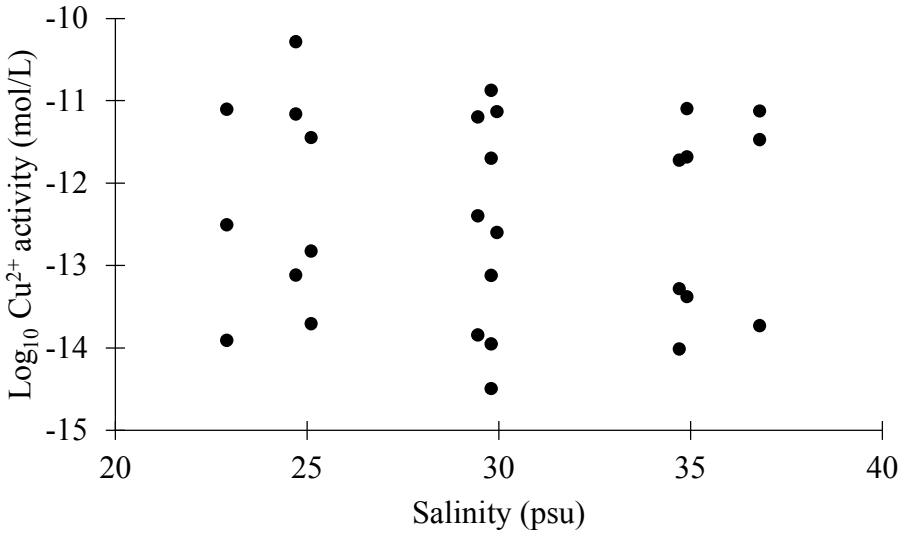


Figure A2. The effect between salinity and Cu^{2+} activity in this study calculated via Visual Minteq.

The elemental composition of the glue was measured to assess if there was copper present in the glue which could interfere with the measurements of Cu in the larva. If copper was present in the glue a Cu peak would be visible in between the Ni and Ta-L peak. The absence of this peak indicates the absence or a concentration of Cu below the MDL in the glue and eliminates a risk of Cu contamination and erroneous measurements.

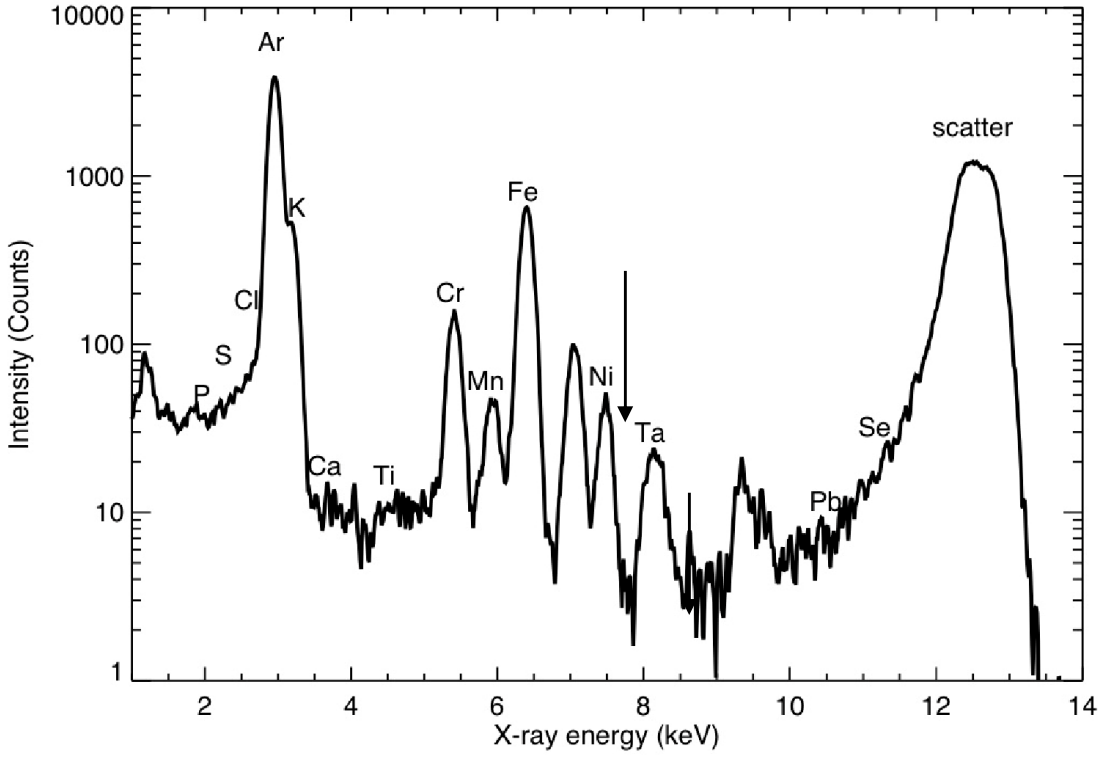


Figure A3. XRF spectrum of the glue used to mount the mussel larvae, if Cu was present a peak should be visible between Ni and Ta peak (black arrow)

Table A1. The measured internal copper concentration in mussel larvae for different combinations of external dissolved Cu, [DOC] and salinity

dissolved copper (µg/L)	Cu^{2+} activity (mol/L)	Salinity (psu)	DOC (mg/L)	internal Cu concentration (µg/g DW larvae)
0.9	1.24E-14	22.9	3.0	2.1
3.9	3.13E-13	22.9	3.0	3.9
12.4	7.91E-12	22.9	3.0	14.8
0.8	7.64E-14	24.7	1.2	3.0
3.7	6.92E-12	24.7	1.2	8.0
9.0	5.21E-11	24.7	1.2	6.1
1.8	1.97E-14	25.1	3.9	1.1
4.3	1.50E-13	25.1	3.9	3.3
12.2	3.56E-12	25.1	3.9	7.4
1.0	1.43E-14	29.45	3.0	2.7
4.2	4.00E-13	29.45	3.0	4.0
11.6	6.39E-12	29.45	3.0	21.8
0.03	3.21E-15	29.8	0.6	3.4
2.2	1.34E-11	29.8	0.6	12.2
5.3	1.11E-10	29.8	0.6	27.7
1.7	1.11E-14	29.8	4.7	2.7
4.5	7.55E-14	29.8	4.7	3.1
12.6	2.00E-12	29.8	4.7	8.0
3.4	2.51E-13	29.95	2.8	5.5
11.5	7.37E-12	29.95	2.8	24.1
1.3	9.68E-15	34.7	4.6	3.2
3.5	5.23E-14	34.7	4.6	5.6
11.0	1.90E-12	34.7	4.6	10.1
0.8	4.19E-14	34.9	1.5	3.8
3.1	2.08E-12	34.9	1.5	8.7
5.4	8.01E-12	34.9	1.5	7.9
1.0	1.86E-14	36.8	3.0	1.2
7.7	3.39E-12	36.8	3.0	10.5
11.0	7.54E-12	36.8	3.0	8.1

Table A2. Summary of the terms in Eq. 2.2, 2.3 and 2.4 with units, parameter estimates, standard error (SE), significance (P-value) and explained variation (R^2)

	Predictor	Unit	Parameter estimate	SE	P-value	R^2
Eq. 2.2	$[Cu_{ext}]$	µg/L	$6.83 \cdot 10^{-2}$	$8.9 \cdot 10^{-3}$	<0.001	0.46
	[DOC]	mg/L	-0.127	$2.8 \cdot 10^{-2}$	<0.001	0.21
	Salinity	psu	0.275	0.12	0.030	0.03
	Salinity²	psu^2	$-4.39 \cdot 10^{-3}$	$2.0 \cdot 10^{-3}$	0.038	0.05
Eq. 2.3	$[Cu_{ext}]$	µg/L	0.129	$3.8 \cdot 10^{-2}$	0.002	0.46
	$[Cu_{ext}]^2$ *	$(µg/L)^2$	$-4.66 \cdot 10^{-3}$	$2.8 \cdot 10^{-3}$	0.110	0.03
	Salinity	psu	0.276	0.12	0.026	0.03
	Salinity²	Psu^2	$-4.43 \cdot 10^{-3}$	$1.9 \cdot 10^{-3}$	0.031	0.05
Eq 2.4	DOC	mg/L	-0.123	$2.7 \cdot 10^{-2}$	<0.001	0.21
	$\log_{10}\{Cu^{2+}\}$	mol/L	0.237	0.027	<0.001	0.70
	Salinity*	psu	0.251	0.11	0.034	0.02
	Salinity² *	Psu²	$-4.04 \cdot 10^{-3}$	$1.9 \cdot 10^{-3}$	0.041	0.04

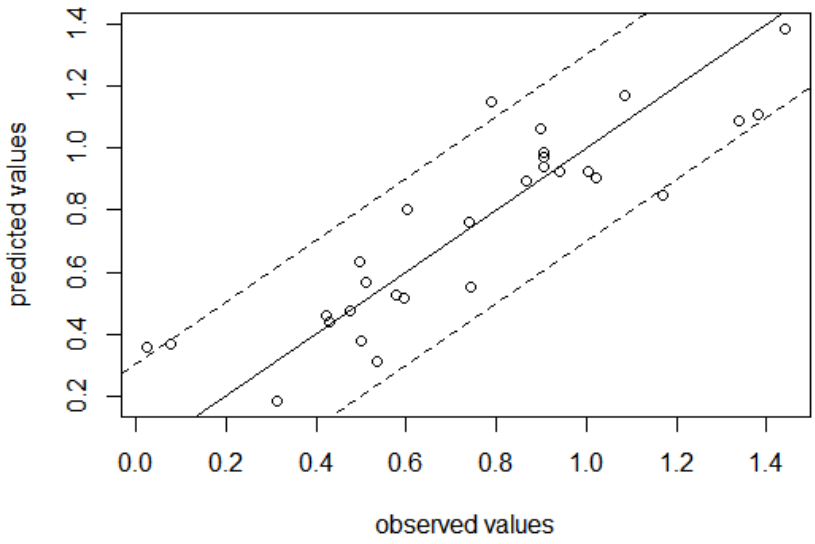


Figure A4. The predicted internal Cu concentrations of equation 2.4 compared to the observed Cu body burden

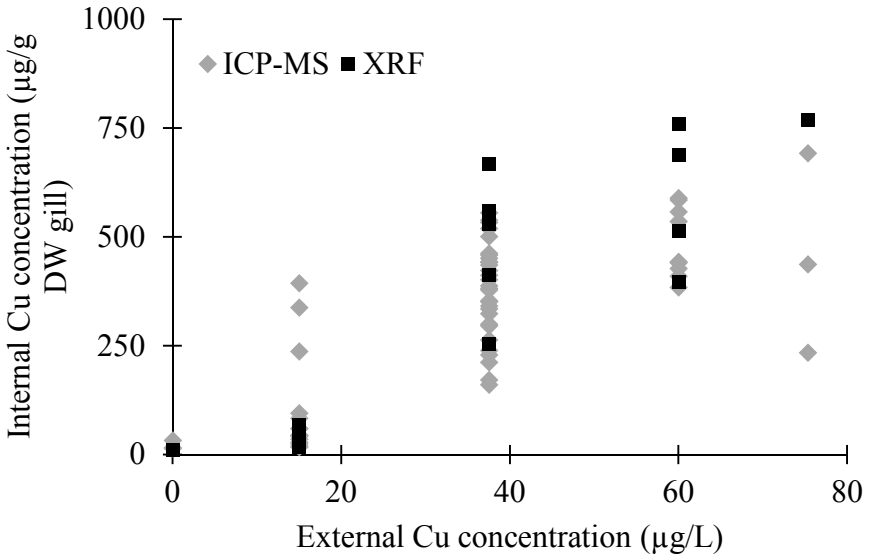


Figure A5. The measured internal Cu concentration in the gill via ICP-MS and XRF

B

APPENDIX B: SUPPORTING INFORMATION CHAPTER III

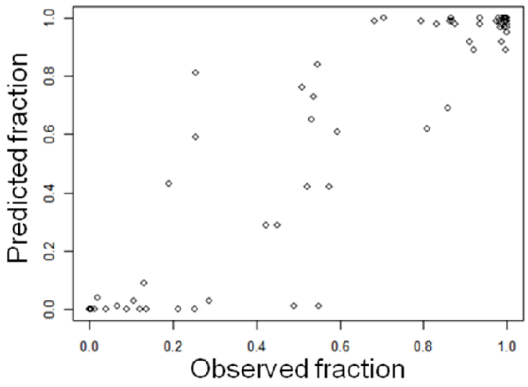


Figure B1. The observed vs predicted fraction of developed larvae based on a GAM analysis with Cu^{2+} activity as independent variable

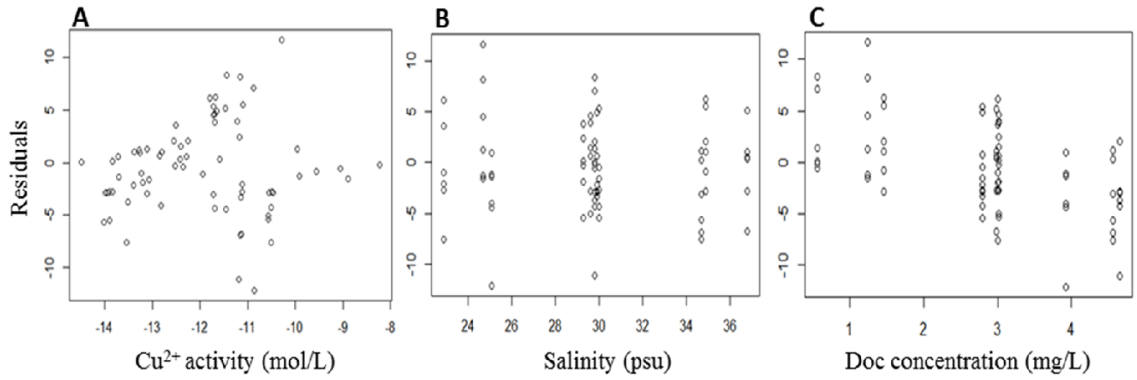


Figure B2. The residuals vs the independent variables for the GAM based on Cu^{2+} activity. A: plot for Cu^{2+} activity, B: salinity and C: DOC.

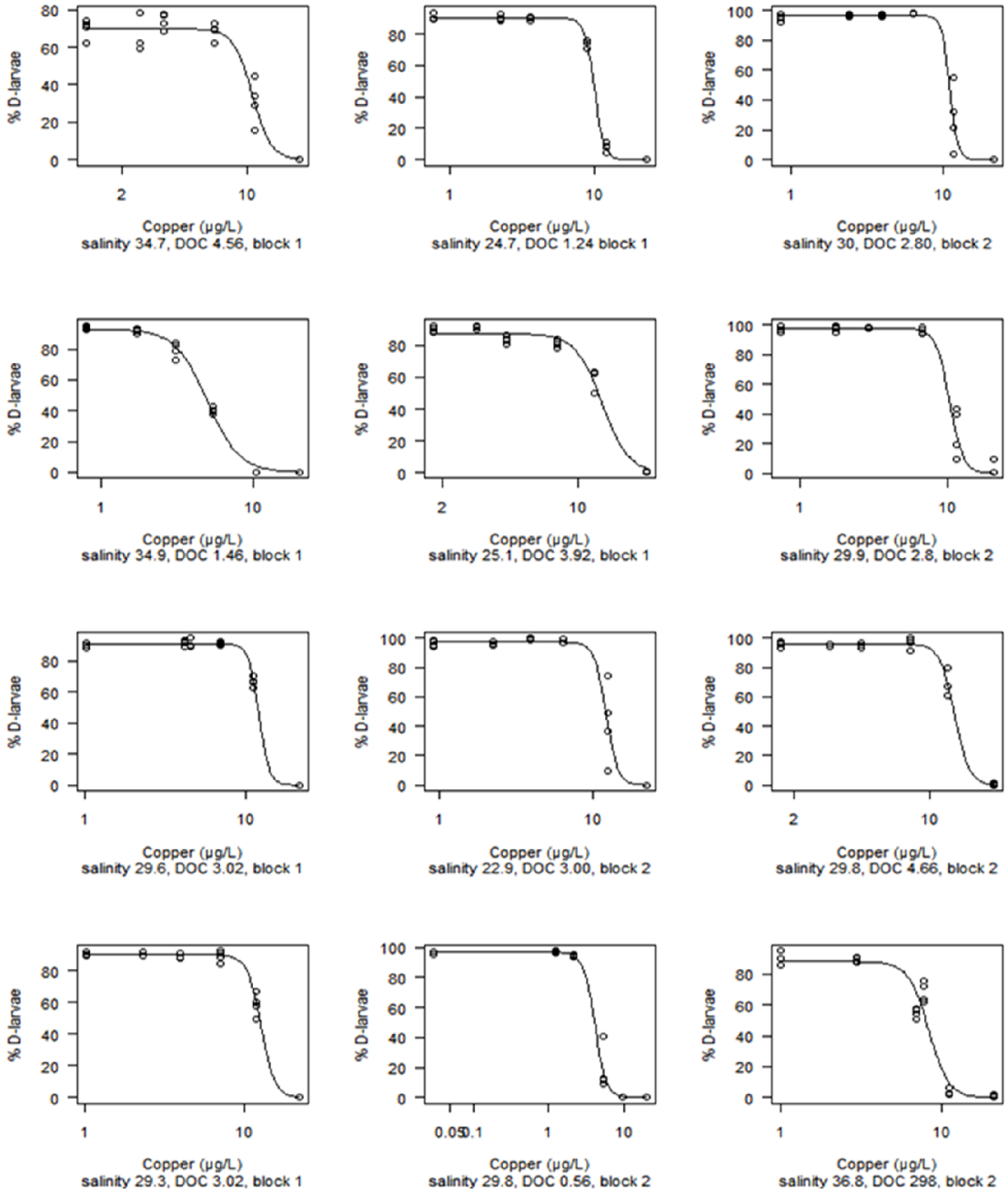


Figure B3. The individual concentration response curves for each salinity/DOC concentration.

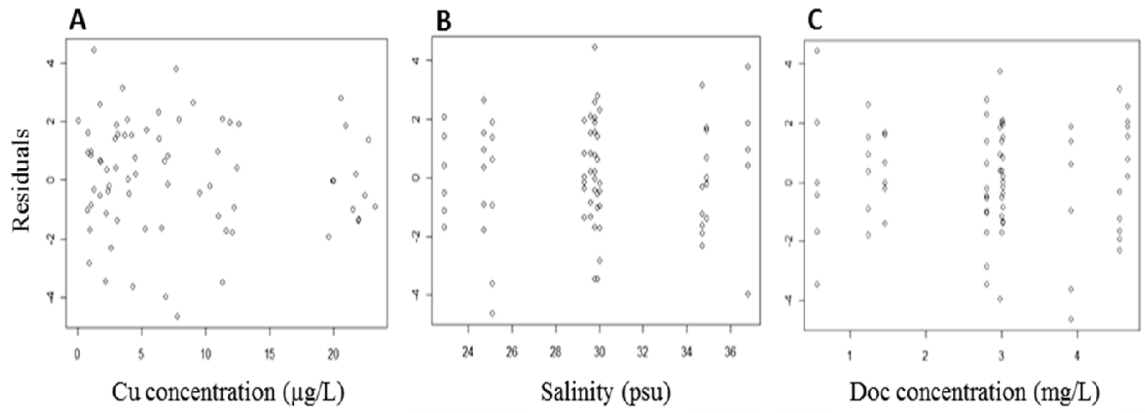


Figure B4. The different residual plots for the GAM model based on total dissolved Cu. A: residual plot for total dissolved Cu, B: plot for salinity, C: plot for DOC.

Table B1. Summary of the terms in the different EC_{50} models with the units, parameter estimates, standard error (SE), P-value and the explained variation for the individual terms (R^2)

	Predictor	Unit	Parameter estimate	SE	P-value	R^2
Eq. 3.5	[DOC]	mg/L	4.19	0.97	0.002	0.59
	[DOC]²	mg/L²	-0.414	0.18	0.047	0.07
	salinity²	psu²	$6.33 \cdot 10^{-3}$	$1.2 \cdot 10^{-3}$	<0.001	0.27
Eq. 3.6	[DOC]	mg/L	4.40	0.97	0.003	0.58
	[DOC]²	mg/L²	0.454	0.18	0.279	0.05
	Salinity	psu	1.11	0.94	0.037	0.28
	salinity²	psu²	$2.47 \cdot 10^{-2}$	$1.6 \cdot 10^{-2}$	0.160	0.02
Eq 3.7	[DOC]	mg/L	-0.498	0.082	<0.001	0.86
	[DOC]²	mg/L²	$3.87 \cdot 10^{-2}$	$1.5 \cdot 10^{-2}$	0.034	0.02
	Salinity²	psu²	$-4.58 \cdot 10^{-4}$	$1.0 \cdot 10^{-4}$	0.002	0.08

EC_{10} calculations based upon total dissolved Cu

As in the EC_{50} model, both [DOC] and salinity remain highly significant ($P < 0.01$, Table B2) and backward selection from the full model resulted in equation B3.1 (only significant terms, R^2 adj.= 0.73, AIC = 47.7) and equation B3.2 (lowest AIC (38.8), R^2 adj. = 0.88).

$$EC_{10[Cu]} = 9.01 + 1.63 \cdot [DOC] - 6.12 \times 10^{-3} \cdot S^2 \quad \text{(Eq. B3.1)}$$

$$EC_{10[Cu]} = -7.74 - 0.304 \cdot [DOC] - 0.599 \cdot [DOC]^2 + 1.39 \cdot S - 3.70 \cdot 10^{-2} \cdot S^2 + 0.170 \cdot S \cdot [DOC] \quad \text{(Eq. B3.2)}$$

With $EC_{10[Cu]}$ in µg/L, [DOC] in mg/L and salinity (S) in psu.

Table B2. Summary of the terms in the EC_{10} model based on total dissolved Cu with the units, parameter estimates, standard error (SE), P-value and the explained variation for the individual terms (R^2)

	Predictor	Unit	Parameter estimate	SE	P-value	R^2
Eq. B 3.1	[DOC]	mg/L	1.63	0.35	0.001	0.48
	Salinity	psu	$-6.12 \cdot 10^{-3}$	$1.7 \cdot 10^{-3}$	0.007	0.30
Eq. B 3.2	[DOC]	mg/L	-0.304	2.04	0.886	0.48
	$[DOC]^2$	mg/L^2	-0.599	0.183	0.017	0.08
	Salinity	psu	1.39	0.945	0.192	0.29
	$Salinity^2$	psu^2	$3.70 \cdot 10^{-2}$	$1.57 \cdot 10^{-2}$	0.057	0.03
	[DOC] ·salinity	mg/L· psu	0.170	$6.93 \cdot 10^{-2}$	0.049	0.06

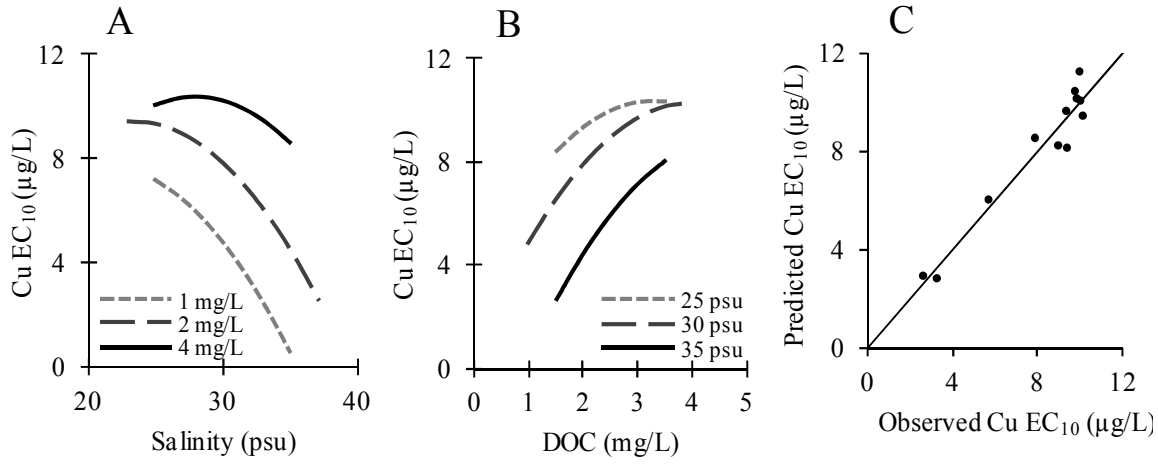


Figure B5. Graphical representation of predicted EC_{10} as dissolved Cu with: a changing [DOC] (A) or salinity (B) based on equation B3.2; C: predicted versus observed values

EC_{10} calculations based on Cu^{2+} activity

[DOC] and salinity remained significant and backward selection from the full model resulted in equation B3.3 (only significant terms, R^2 adj.= 0.88, AIC = 47.7) and model B3.4 (lowest AIC =38.8, R^2 adj. = 0.93).

$$Log_{10}EC_{10\{Cu^{2+}\}} = -10.0 - 0.258 \cdot [DOC] - 7.35 \cdot 10^{-4} \cdot S^2 \quad \text{(Eq. B3.3)}$$

$$log_{10}EC_{10\{Cu^{2+}\}} = -12.1 - 0.724 \cdot [DOC] + 0.186 \cdot S - 4.56 \cdot 10^{-3} \cdot S^2 + 0.0155 \cdot S \cdot [DOC] \quad \text{(Eq. B3.4)}$$

With $EC_{10\{Cu^{2+}\}}$ in µg/L, [DOC] in mg/L and salinity (S) in psu.

Table B3. Summary of the terms in the EC_{10} model based on Cu^{2+} activity with the units, parameter estimates, standard error (SE), P-value and the explained variation for the individual terms (R^2)

	Predictor	Unit	Parameter estimate	SE	P-value	R^2
Eq. B3.3	[DOC]	mg/L	0.258	0.034	0.001	0.69
	Salinity²	psu	$-7{\cdot}35\times10^{-4}$	$1.7\cdot10^{-4}$	0.002	0.21
Eq. B3.4	[DOC]	mg/L	-0.724	0.22	0.013	0.69
	Salinity	psu	0.186	0.10	0.112	0.20
	Salinity²	psu²	$-4.56\cdot10^{-3}$	$1.7\cdot10^{-2}$	0.031	0.04
	[DOC] ·salinity	mg/L· psu	0.0155	$7.3\cdot10^{-3}$	0.070	0.03

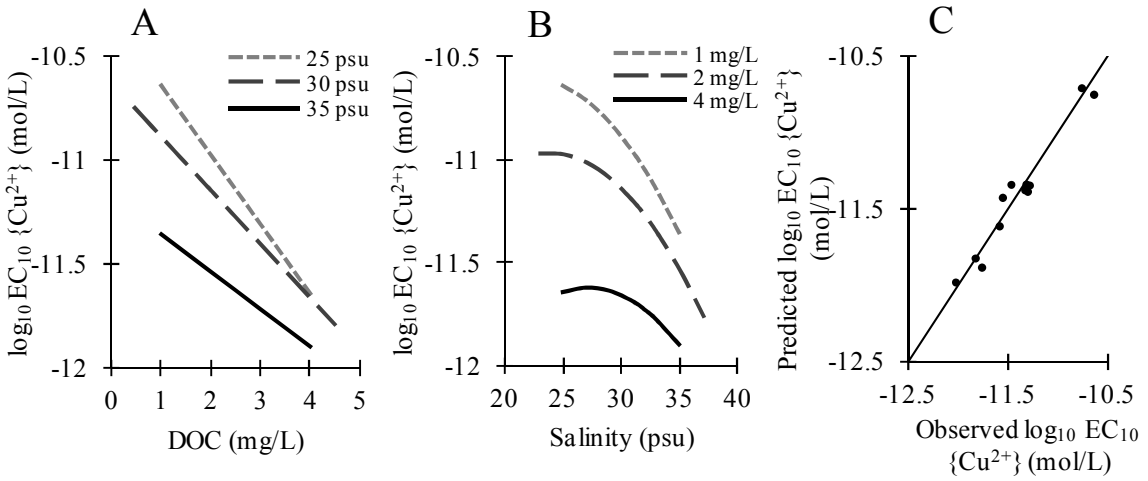


Figure B6. A, B: Graphical representation of predicted log_{10} of the EC_{10} as Cu^{2+} activity with changing [DOC] (A) and changing salinity (B); C: predicted versus observed log_{10} EC_{10} values as Cu^{2+} activity

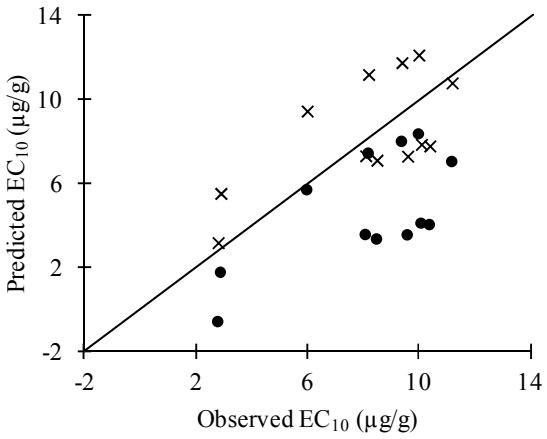


Figure B7. Predicted compared to observed EC_{10} values, blue diamonds with a $EC_{10[Cuint]}$ of 5.3 (sum of squares residuals = 197) and green squares with a $EC_{10[Cuint]}$ of 9.6 (sum of squares residuals= 58).

C

APPENDIX C: SUPPORTING INFORMATION CHAPTER IV

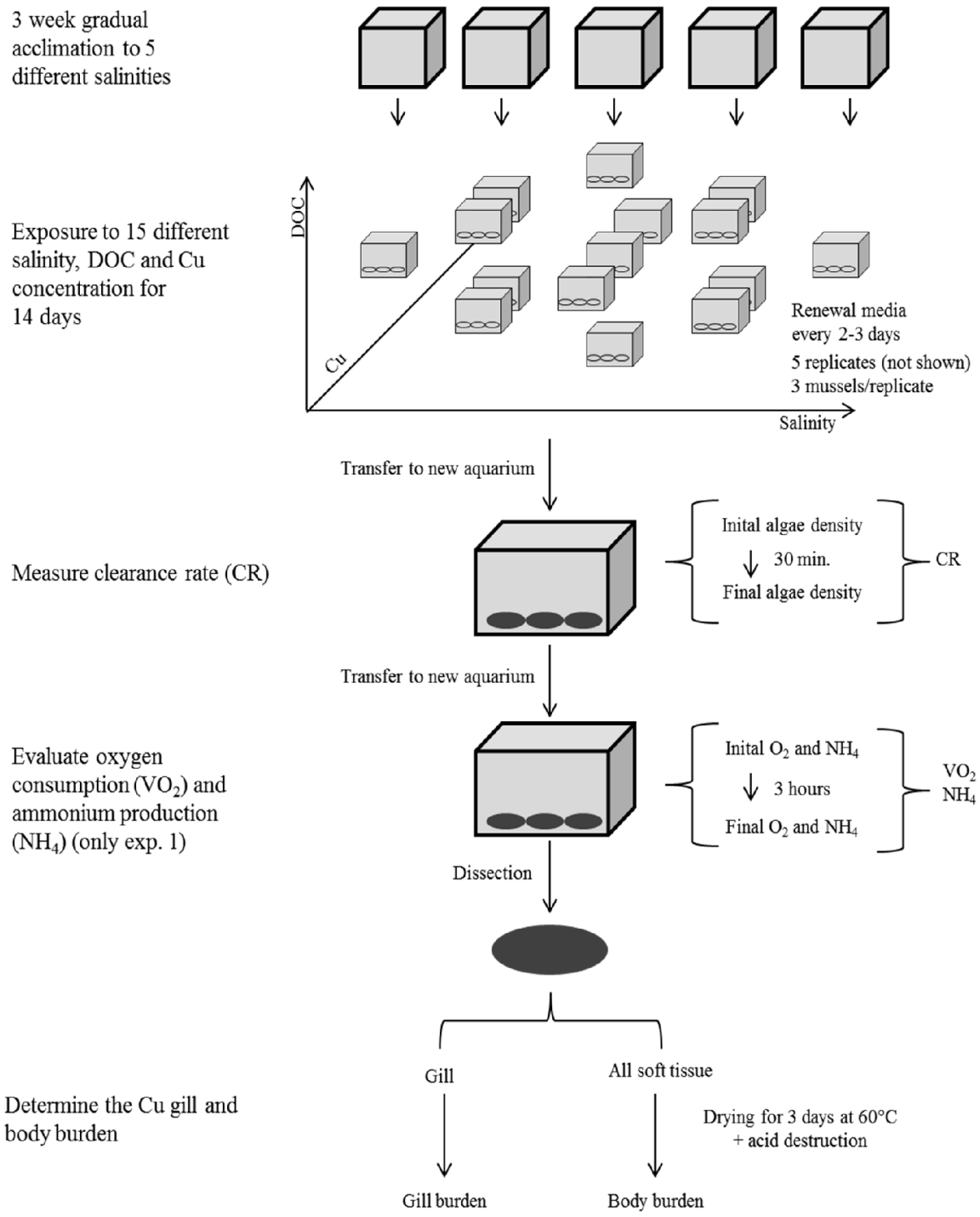


Figure C1. A simplified representation of the experimental design and workflow from the acclimation to the salinity after collection of the animals to the dissection of the gill or soft tissue to determine the internal Cu concentration.

Table C1. The measured salinity, DOC and Cu concentrations for the first experiment according to a 3-factor central composite design (n = 20, 5 replicates per combination). For each population, the experiment was set-up on three consecutive days, the populations were not simultaneously tested.

Baltic Sea population				North Sea population			
day of setup	salinity psu	DOC mg/L	Cu µg/L	day of setup	salinity psu	DOC mg/L	Cu µg/L
1	6.6	3.8	12.3	1	22.4	3.6	11.7
1	6.6	7.3	34.6	1	22.5	6.3	45.4
1*	10.5	6.0	27.5	1*	30.3	4.9	26.1
1*	10.6	6.0	23.5	1*	30.4	4.9	26.1
1	14.6	3.8	31.5	1	38.2	4.1	42.9
1	14.7	7.3	11.9	1	38.5	6.7	11.1
2	4.0	6.0	22.2	2	17.2	5.5	25.3
2*	10.5	6.0	21.5	2	30.2	2.6	23.9
2*	10.5	6.0	23.9	2	30.3	6.2	56.3
2	10.5	3.1	24.2	2	30.4	5.5	3.0
2	10.6	6.0	1.5	2*	30.4	5.1	26.4
2	10.6	9.4	25.7	2*	30.4	5.1	26.4
2	10.7	6.0	46.8	2	30.5	7.6	25.0
2	17.1	6.0	21.6	2	44.3	5.1	27.4
3	6.3	3.8	34.5	3	22.2	7.2	12.4
3	6.4	7.3	12.8	3	22.5	4.2	40.6
3*	10.4	6.0	29.0	3*	30.2	4.8	25.9
3*	10.4	6.0	29.5	3*	30.4	4.8	25.9
3	14.3	3.8	11.6	3	38.1	4.5	11.5
3	14.5	7.3	37.7	3	38.3	6.2	44.8

DOC = Dissolved Organic Carbon; Cu = Copper; *middle point of the central composite design

The influence of exposure time on the clearance rate EC_{50}

A pilot experiment was performed to assess the change in clearance rate with exposure time from 0.5 h up to 27 days. The analysis was performed on nominal Cu concentrations using R in combination with the DRC package and the log logistic model. The results indicate an initial decrease in EC_{50} during the first days but from day 9 onwards there is no significant difference anymore in the dose-response curve (P-value = 0.12) with an average Cu EC_{50} of 31.4 (± 0.9) µg/L (nominal concentration) (Figure C2).

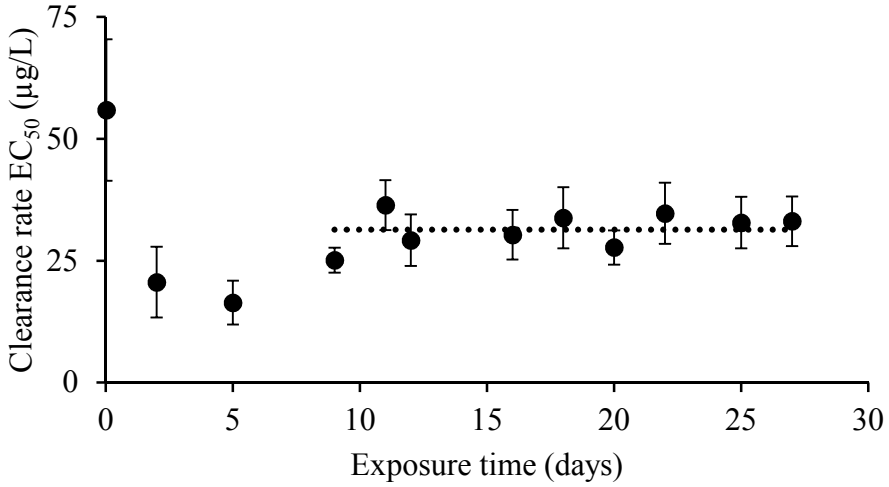


Figure C2. The change in the clearance rate EC_{50} with exposure time

Cu distribution in the gill

The X-ray Fluorescence (XRF) measurements were performed under vacuum using an Edax Eagle 3 equipped with a rhodium anode X-ray tube using an acceleration voltage of 40 kV and a current of 650 µA. The produced X-rays are focused on the sample using a poly capillary optic, obtaining a resolution of 105 µm at 6.4 keV. The samples were raster scanned with a step size of 100 µm and an acquisition time per scan point of 100 s live time. The obtained spectra were deconvoluted using AXIL1 and element distribution images were obtained using in-house written routines.

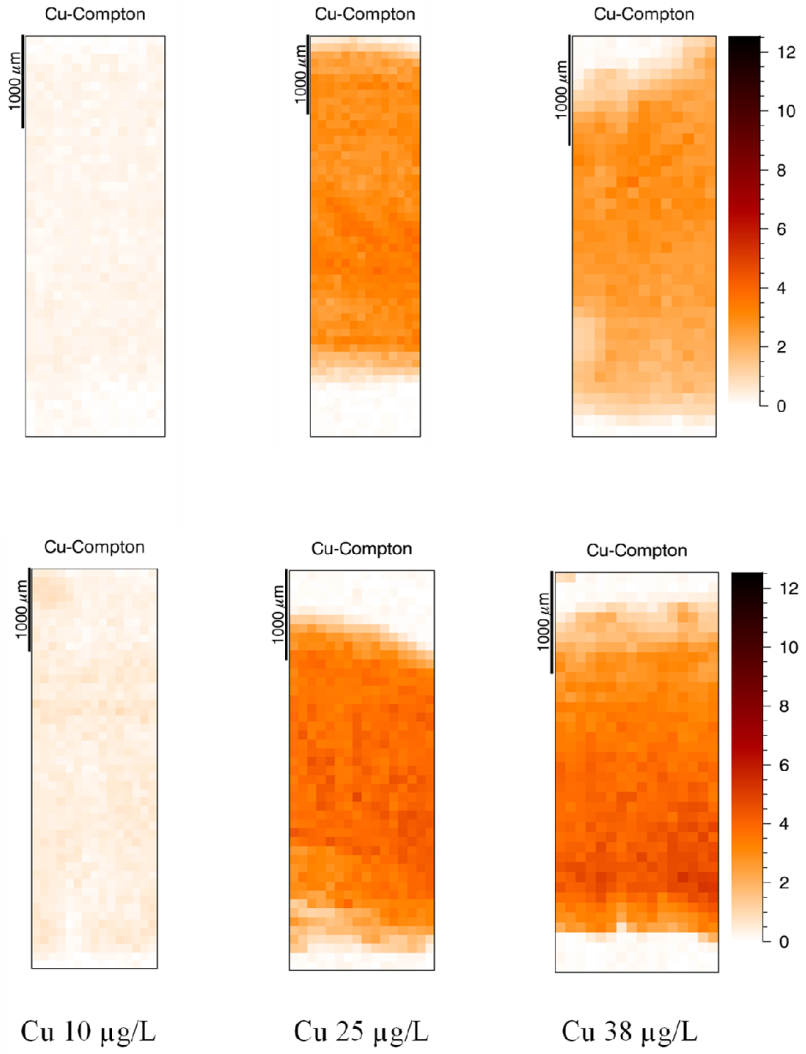


Figure C3. The distribution of Cu in the gill (in counts). Corrected for tissue density via the Compton scatter.

Table C2. The measured salinity and Cu concentration of the second experiment (5 replicates per combination), the experiment was set-up in 1 day and both populations were assessed simultaneously.

Baltic Sea		North Sea	
Salinity	Cu	Salinity	Cu
psu	µg/L	psu	µg/L
16.7	23.1	16.9	23.1
20.2	10.5	20.2	10.5
20.3	38.7	20.4	38.7
25.2	1.15	25.3	1.15
*25.4	25.4	*25.3	25.4
*25.4	25.4	*25.3	25.4
25.3	48.9	25.5	48.9
30.4	9.7	30.1	9.7
30.4	38.8	30.4	38.8
33.5	26.4	34.2	26.4

Cu = Copper; *middle point of the central composite design

Individual EC_{50}'s for the different endpoints

The EC_{50}'s for the different endpoints. A lower limit of 0 was assumed for the clearance rate, oxygen consumption and ammonium production. In this analysis the possible influence of salinity or DOC is not included. All data was used to construct a single concentration response curve.

Table C3. The EC_{50}s for the different biomarkers, for both populations

	endpoint	North Sea pop.			Baltic Sea pop.		
		Max.	EC_{50}	slope	Max.	EC_{50}	slope
exp. 1	CR	20.7 (1.2)	16.4 (1.0)	6.3 (0.9)	33.0 (3.8)	11.1 (2.5)	1.9 (0.5)
	VO_2	4.9 (0.3)	33.6 (2.3)	2.5 (0.5)	NS	NS	NS
	NH_4-P	137.1 (12.0)	34.3 (4.7)	1.5 (0.3)	175.2 (33.8)	32.2 (8.9)	1.5 (0.9)
exp. 2	CR	14.6 (1.1)	18.4 (2.1)	4.8 (1.6)	18.2 (1.1)	21.9 (1.6)	4.9 (1.7)
exp. 3	CR	100	12.5 (0.4)	8.6 (1.4)	NA	NA	NA

pop = population; CR = clearance rate; VO_2 = oxygen consumption; NH_4-P = ammonium production; exp. = experiment; NS = not significant; NA = not available

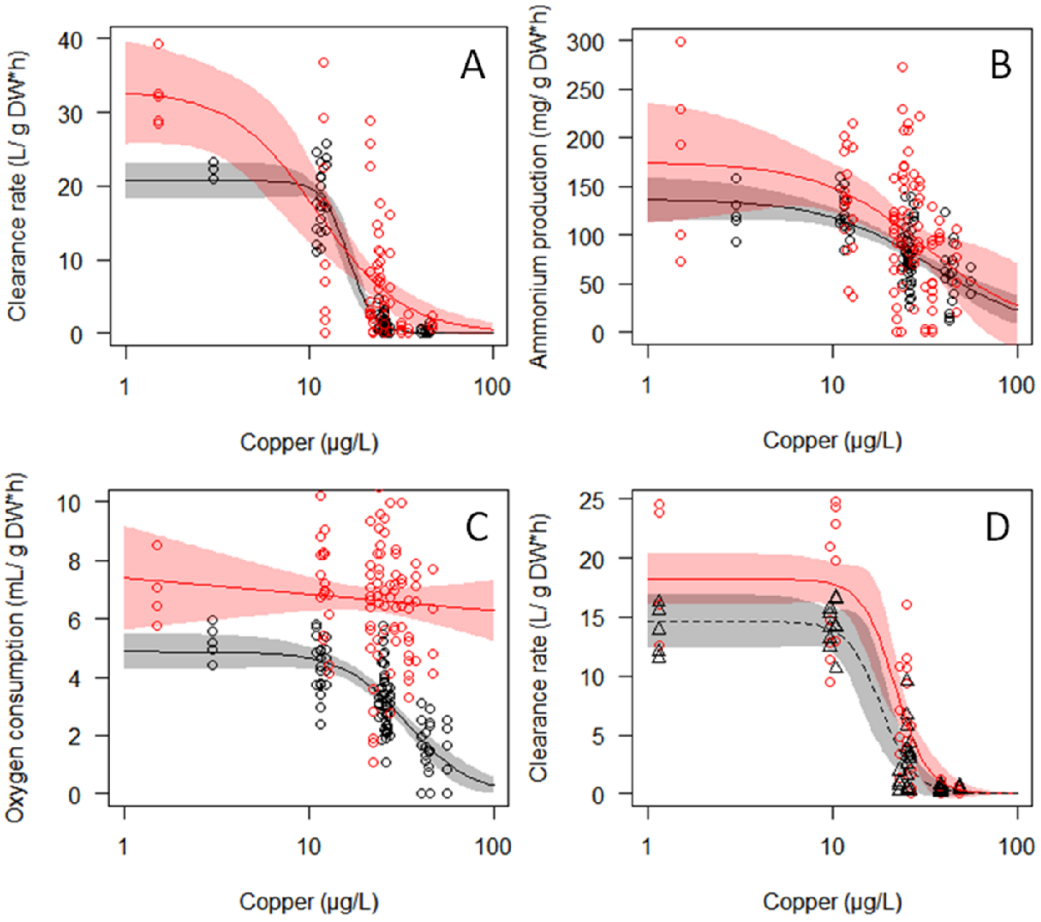


Figure C4. The concentration response curves for the different endpoints. A-C: experiment 1; D: experiment 2; Black = North Sea population; Red: Baltic Sea population; circles = observed data; shaded areas indicate CI

Mortality in the first experiment

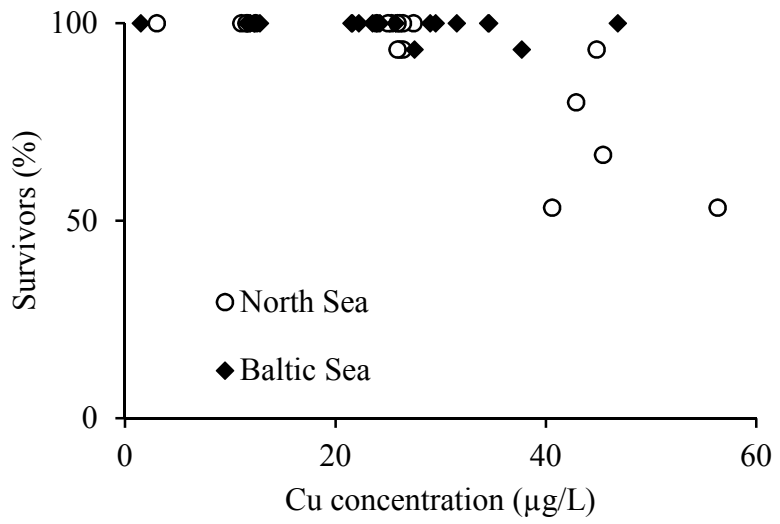


Figure C5. The percentage survivors as a function of the copper concentration

Modeling the effect of Cu, salinity and DOC on the oxygen consumption and ammonium production in experiment one

The influence of Cu, salinity and DOC on the oxygen consumption (VO_2) and ammonium production (NH_4-P) could not be modeled accurately, certainly for the Baltic Sea population. The VO_2 is significantly reduced when North Sea mussels are exposed to elevated Cu levels. However, this is not the case for the Baltic Sea population. In both populations the NH_4 decreases significantly with increasing Cu. A change in DOC concentration did not alter the VO_2 or the NH_4 or change the sensitivity to Cu. An increase in salinity increased both the VO_2 and NH_4 for the Baltic Sea population.

Table C4. The parameter values of the predictor variables in the reduced models of the first experiment from the initial full model: Endpoint = $\alpha + \beta1 \cdot [Cu] + \beta2 \cdot [Cu]^2 + \beta3 \cdot [Cu]^3 + \beta4 \cdot salinity + \beta5 \cdot salinity^2 + \beta6 \cdot [DOC] + \beta7 \cdot [DOC]^2 + \beta8 \cdot [Cu] \cdot salinity + \beta9 \cdot [Cu] \cdot [DOC] + \beta10 \cdot [DOC] \cdot salinity$ (Eq. 4.3).

		α	β_1	β_2	β_3	β_4	β_5 10^{-3}	β_8	R^2
North Sea	VO_2	5.35	-0.0822						0.59
	NH_4-P	130	-1.70						0.40
Baltic Sea	VO_2	-1.46				1.39	-53.2		0.31
	NH_4-P	-46.4	-2.60			40.4	-174		0.20

int. = intercept, sal = salinity, VO_2 = oxygen consumption, NH_4-P = ammonium production

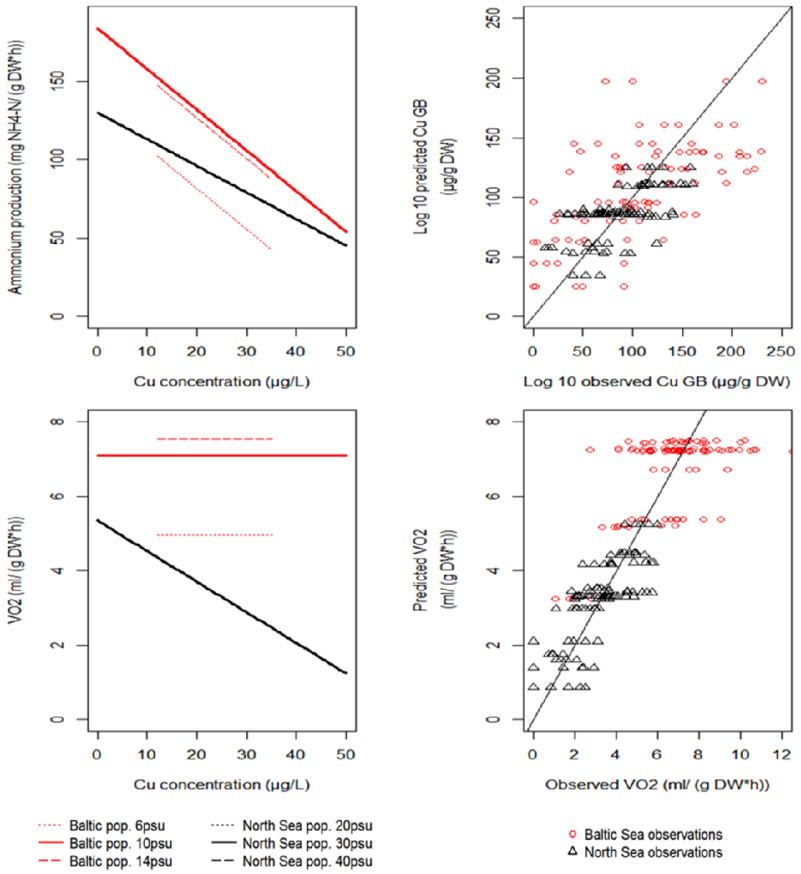


Figure C6. Left: the model predictions of the main experiment for ammonium production (top) and VO_2 (bottom); right the matching predicted versus observed values

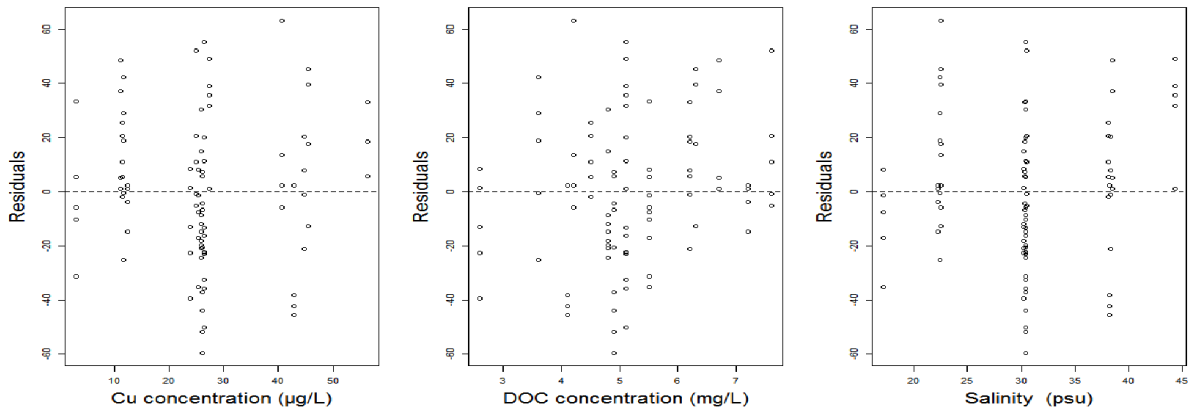


Figure C7. The residual distribution of the ammonium production model for the North Sea population

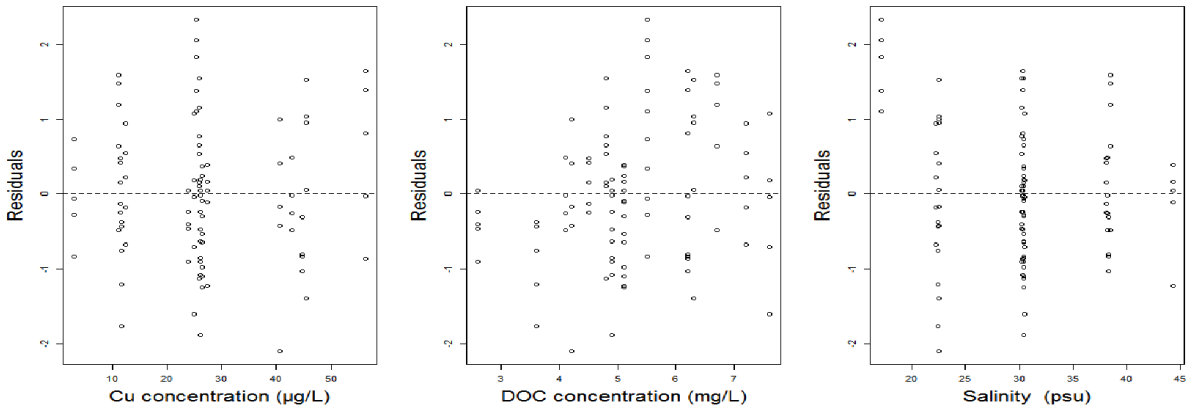


Figure C8. The residual distribution of the oxygen consumption model for the North Sea population

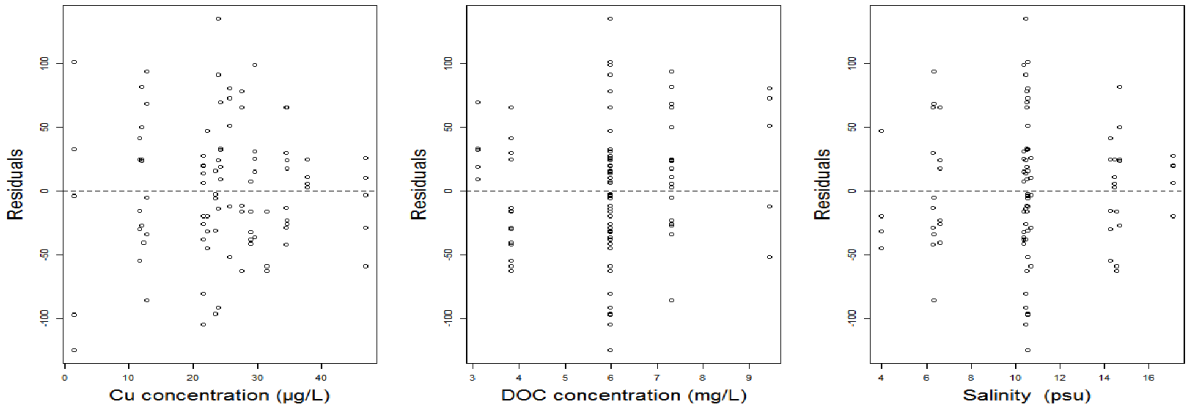


Figure C9. The residual distribution of the ammonium production model for the Baltic Sea population

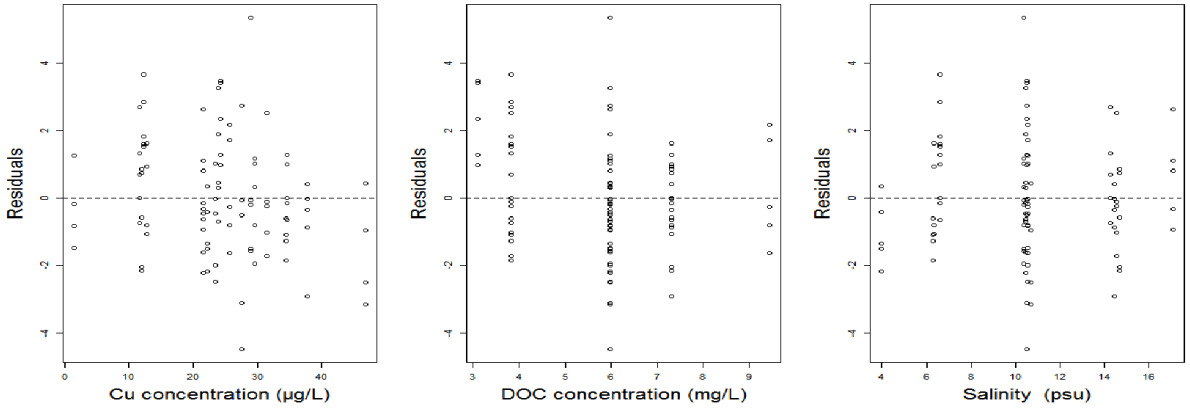


Figure C10. The residual distribution of the oxygen consumption model for the Baltic Sea population

Details of the first experiment: CR, BB and GB

Table C5. Details on the final (reduced) models of the first experiment, including: the parameter estimate, SE and percentage variance that the parameter explains. The initial full model Endpoint = $\alpha + \beta1 \cdot [Cu] + \beta2 \cdot [Cu]^2 + \beta3 \cdot [Cu]^3 + \beta4 \cdot salinity + \beta5 \cdot salinity^2 + \beta6 \cdot [DOC] + \beta7 \cdot [DOC]^2 + \beta8 \cdot [Cu] \cdot salinity + \beta9 \cdot [Cu] \cdot [DOC] + \beta10 \cdot [DOC] \cdot salinity$ (Eq. 4.3)

		α	SE	β1	SE	%VE	$\beta2 \cdot 10^{-3}$	$SE \cdot 10^{-3}$	%VE	$\beta3 \cdot 10^{-5}$	$SE \cdot 10^{-5}$	%VE	β4	SE	%VE	$\beta5 \cdot 10^{-3}$	$SE \cdot 10^{-3}$	%VE	β8	SE	%VE	R^2
North Sea	√(CR)	4.72	0.380	0.110	0.0634	74.9	-17.3	3.02	12.9	28.6	4.05	4.3										0.92
	$\log_{10}$(BB)	-0.420	0.186	0.109	0.00816	53.6	-2.16	0.330	35.2	1.23	0.383	1.1	0.066	0.0114	0.3	-1.12	0.185	2.8				0.93
	$\log_{10}$(GB)	1.11	0.101	0.104	0.0123	54.4	-2.23	0.459	35.5	1.53	0.506	2.2										0.92
Baltic Sea	√(CR)	-1.36	1.36	0.0275	0.0651	39.1	2.14	0.674	4.9				0.683	0.124	13.8				-0.022	0.00531	8.6	0.66
	$\log_{10}$(BB)	0.872	0.0592	0.0731	0.00276	86.6	-0.782	0.0556	8.2				0.0125	0.00250	1.1							0.96
	$\log_{10}$(GB)	0.703	0.142	0.121	0.0111	63.1	-1.67	0.203	24.4													0.87

CR = Clearance rate (L/(g DW*h)); BB = Cu Body Burden (µg/g DW); GB = Cu Gill Burden (µg/g DW); SE = standard error, %VE = % variance explained

Residual pattern analysis for the main experiment

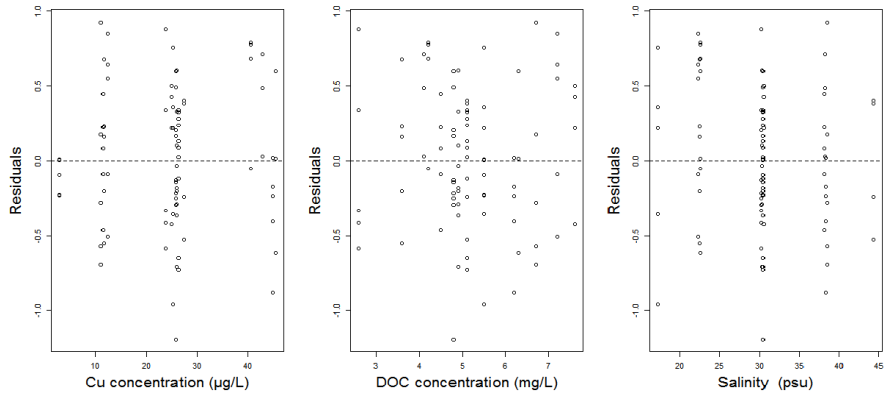


Figure C11. The residual distribution of the clearance rate model for the North Sea population

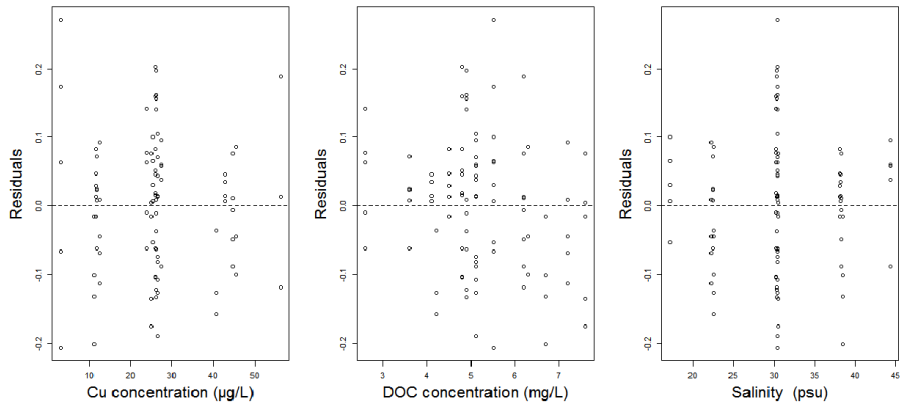


Figure C12. The residual distribution of the Cu body burden model for the North Sea population

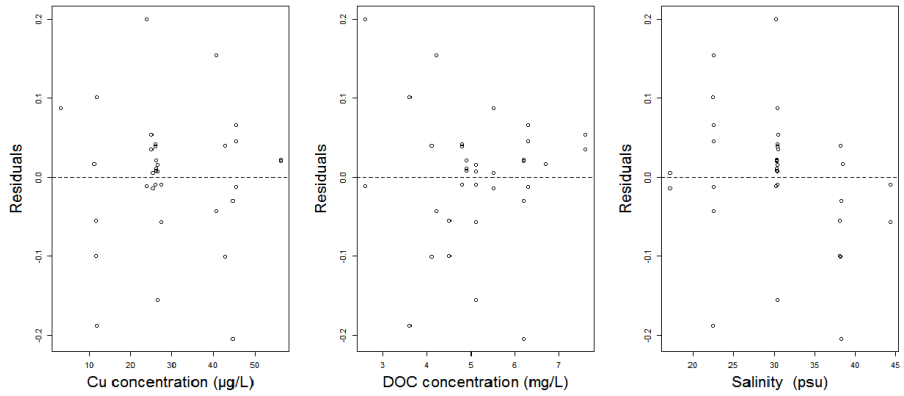


Figure C13. The residual distribution of the Cu gill burden model for the North Sea population

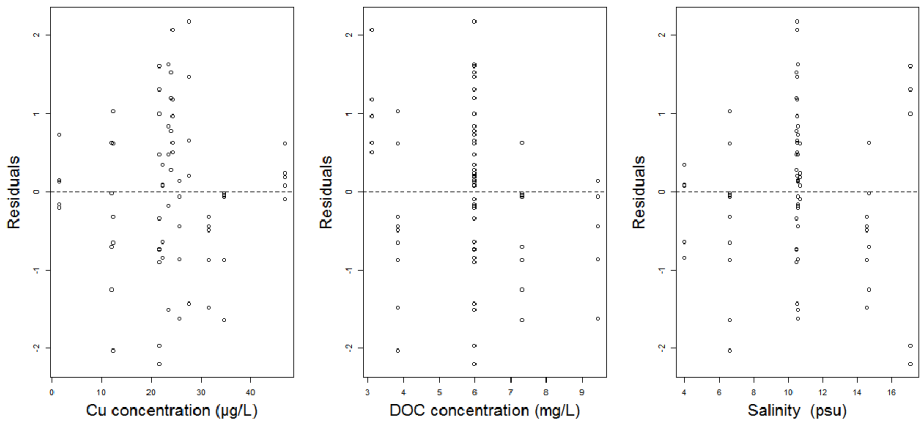


Figure C14. The residual distribution of the clearance rate model for the Baltic Sea population

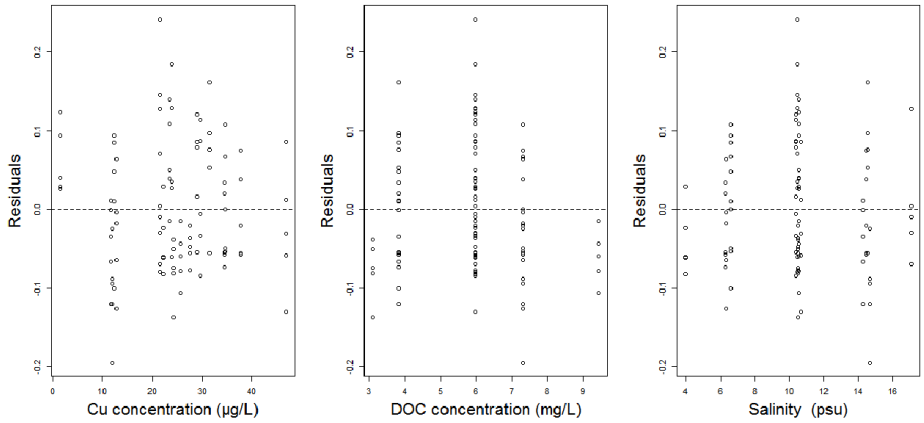


Figure C15. The residual distribution of the Cu body burden model for the Baltic Sea population

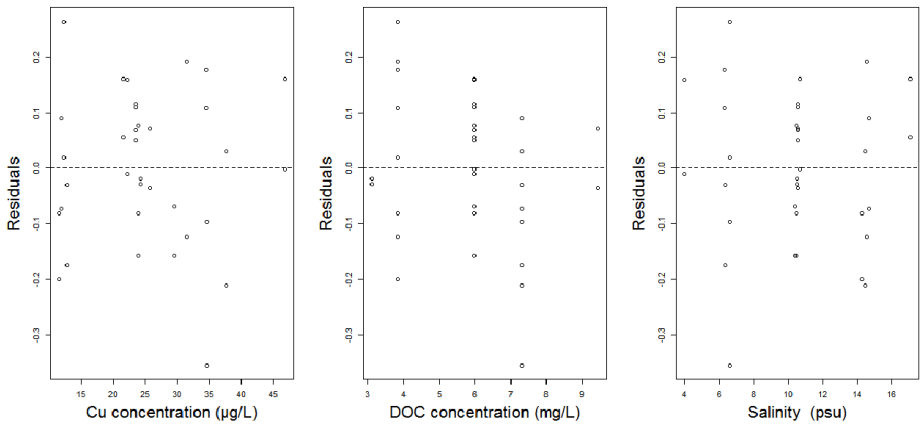


Figure C16. The residual distribution of the Cu gill burden model for the Baltic Sea population

Details on the additional salinity experiment

Table C6. Details on the final (reduced) models of the additional salinity experiment, including: the parameter estimate, SE and percentage variance that the parameter explains. The initial full model: Endpoint = $\alpha + \beta1 \cdot [Cu] + \beta2 \cdot [Cu]^2 + \beta3 \cdot [Cu]^3 + \beta4 \cdot salinity + \beta5 \cdot salinity^2 + \beta6 \cdot population + \beta7 \cdot [Cu] \cdot salinity + \beta8 \cdot [Cu] \cdot population + \beta9 \cdot salinity \cdot population$ (population: North Sea = 1, Baltic Sea = 0, Eq. 4.4)

	α	SE	$\beta1 \cdot 10^{-2}$	$SE \cdot 10^{-2}$	%VE	$\beta2 \cdot 10^{-4}$	$SE \cdot 10^{-4}$	%VE	$\beta3 \cdot 10^{-4}$	$SE \cdot 10^{-4}$	%VE	$\beta4 \cdot 10^{-2}$	$SE \cdot 10^{-2}$	%VE	$\beta5 \cdot 10^{-2}$	$SE \cdot 10^{-2}$	%VE	$\beta6 \cdot 10^{-1}$	$SE \cdot 10^{-1}$	%VE	$\beta8 \cdot 10^{-3}$	$SE \cdot 10^{-3}$	%VE	R^2
√(CR)	-2.59	1.95	7.31	0.836	72.2	-83.6	2.07	1.1	1.11	0.267	3.5	54.8	15.0	0.1	-1.10	0.293	2.8	-4.21	1.35	1.9				0.80
Log_{10}(BB)	1.18	0.0490	6.85	0.207	75.2	-7.16	0.383	9.1				-0.664	0.162	0.4				-2.35	0.327	12.4	-4.55	1.14	0.4	0.97
Log_{10}(GB)	1.47	0.147	8.24	0.603	72.6	-9.31	1.17	11.98				-2.31	0.489	3.3				-1.97	0.488	2.8				0.90

CR = Clearance rate (L/(g DW·h)); BB = Cu Body Burden (µg/g DW); GB = Cu Gill Burden (µg/g DW); SE = standard error, %VE = % variance explained

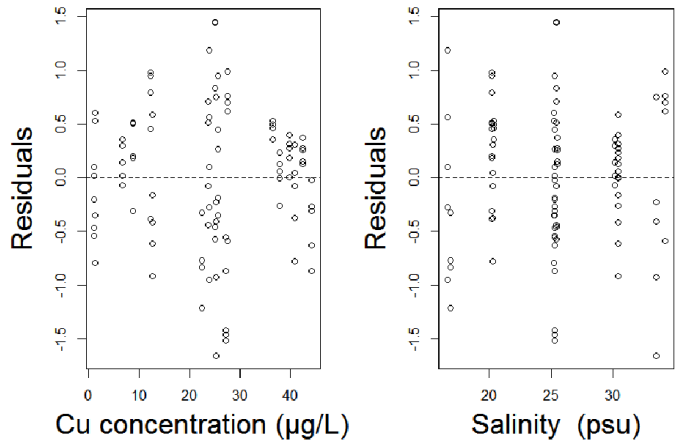


Figure C17. The residual distribution of the clearance rate model

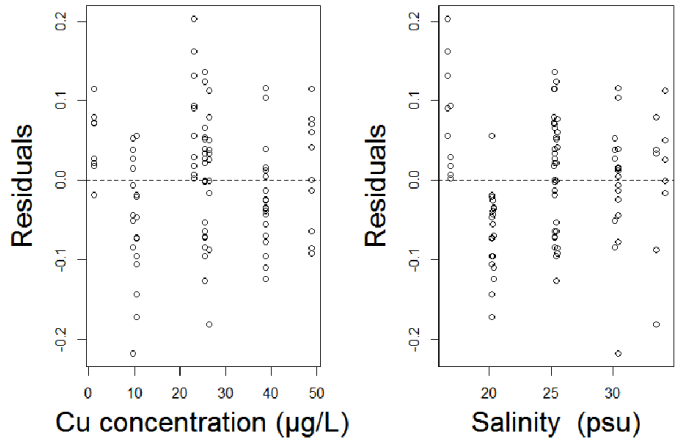


Figure C18. The residual distribution of the Cu body burden model

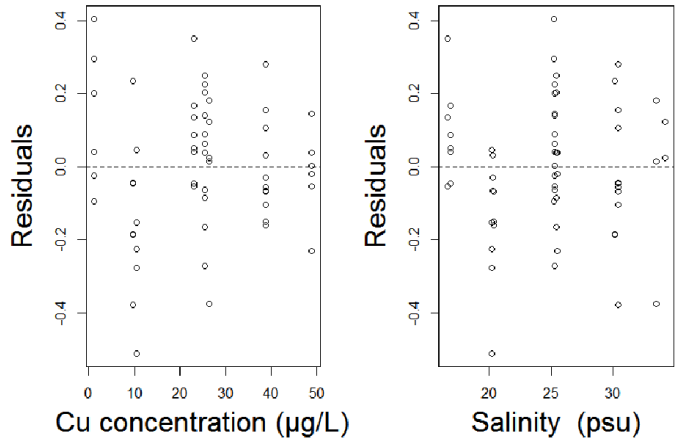


Figure C19. The residual distribution of the Cu gill burden model

D

APPENDIX D: SUPPORTING INFORMATION CHAPTER V

Table D1. The dissolved metal concentrations for the five Cu-Ni mixture experiments. * indicates an extension to the full factorial design, no mixtures were tested at this concentration.

Concentration	Experiment 1		Experiment 2		Experiment 3		Experiment 4		Experiment 5	
	Cu	Ni	Cu	Ni	Cu	Ni	Cu	Ni	Cu	Ni
1	0.8	0.5	1.4	0.5	0.73	0.5	0.5	0.4	0.5	0.5
2	1.7	23.9	3.4	34.5	1.5	4.9	1.6	5.6	1.8	13.0
3	2.5	34.5	4.1	47.2	1.8	7.6	2.1	8.5	2.40	27.4
4	3.5	46.1	5.0	71.7	2.6	12.0	2.9	13.1	3.2	54.0
5	4.6	64.7	6.4	107.9	3.5	19.5	3.8	20.1	4.2	109.3
6	6.7	91.5	8.2	161.0	4.6	30.6	4.8	32.6	5.8	220.6
7			11.5 *	240.1 *	6.3	49.0	6.6	51.0	7.7	417.0
8					8.8	79.4	8.6*	81.8*		811.8
9					12.2	124.3		130.1*		
10					16.2	188.7		207.3*		
11						316.7		332.7*		
12						497.5		530.5*		
13						838.1		848.9*		

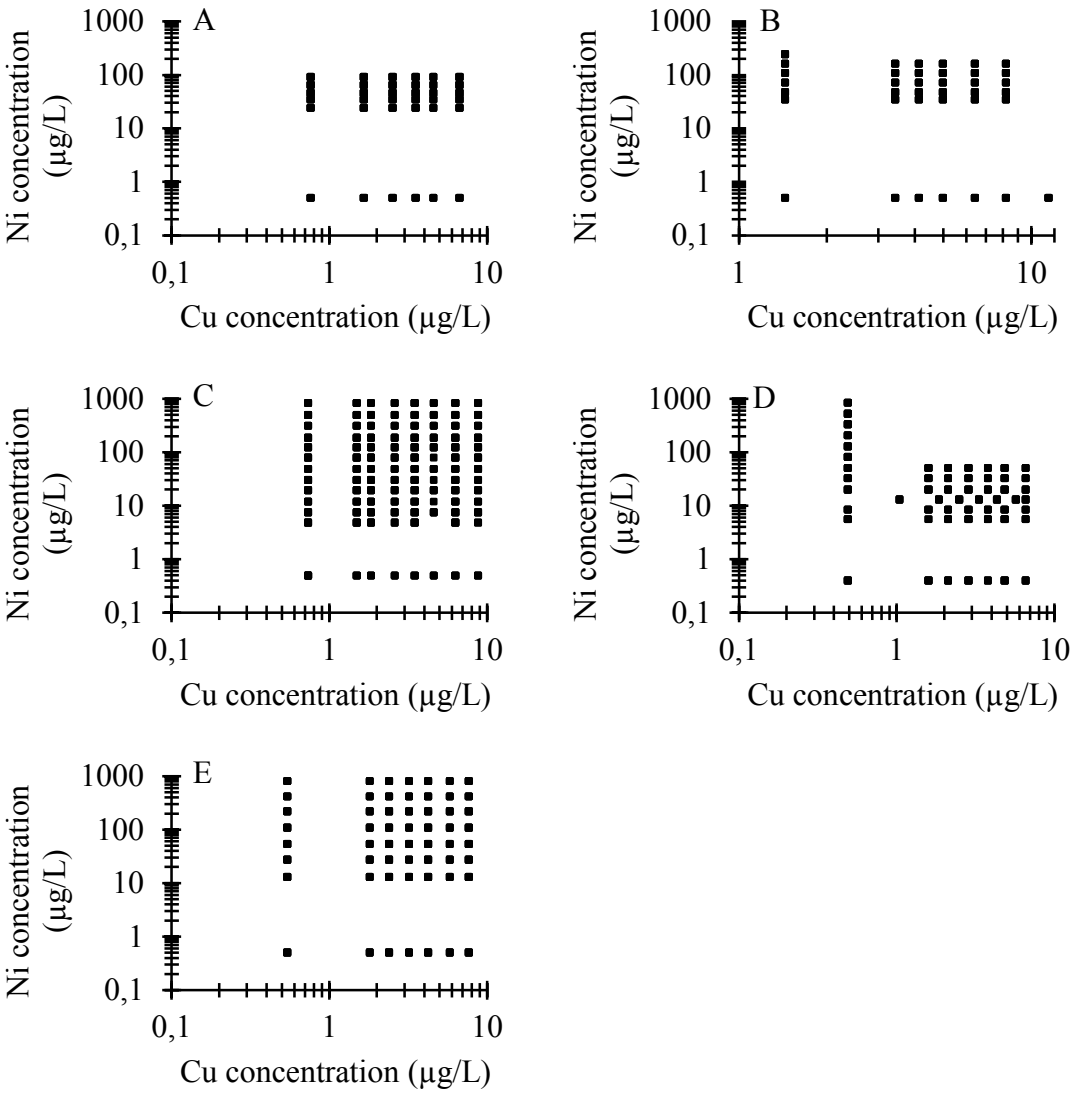


Figure D1. The design of the different experiment. A: experiment 1 performed in 2013 by researcher 1 (6·6 full factorial design); B: experiment 2 performed in 2013 by researcher 1 (6·6 full factorial design + additional single Ni concentrations); C: experiment 3 performed in 2014 by researcher 2 (8·13 full factorial design); D: experiment 4 performed in 2014 by researcher 2 (7·7 full factorial design + additional single Ni concentrations); E: experiment 5 performed in 2015 by researcher 3 (8·7 full factorial design);

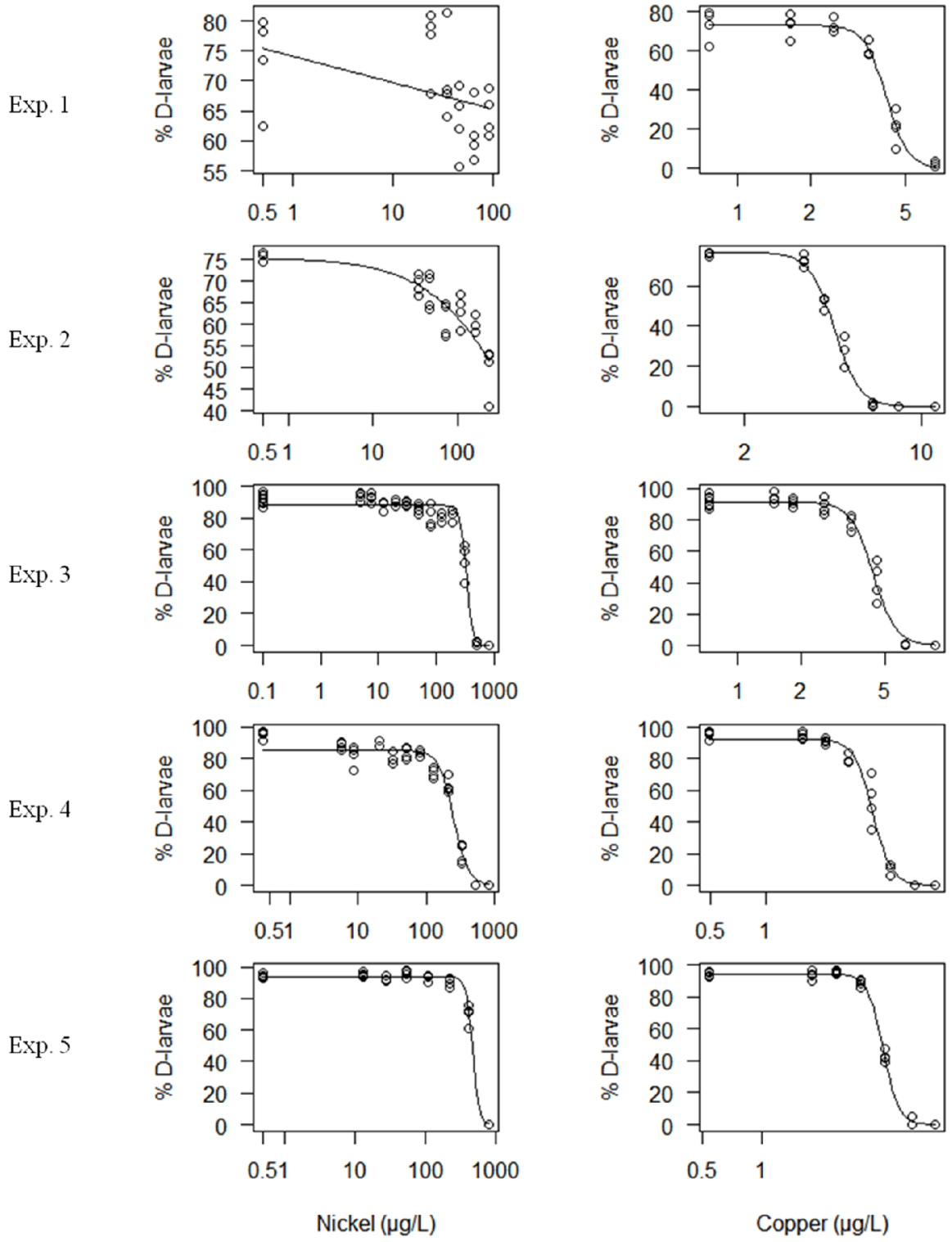


Figure D2. The single concentration response curves for the 5 experiment

Table D2. The optimal parameter values according to the method proposed by Jonker et al. (2005), sum of squared error (SSE) and AICc weight (AICcW in %) for each experiment and each deviation model (Eq. 5.2-5.5). Max. = maximum larval development, a = a deviation parameter; b = b deviation parameter; SSE = sum of squared error; AICcW = support for the different models; Exp. = experiment; CA = concentration addition reference model (Eq. 5.2); S/A = synergistic or antagonistic deviations (Eq. 5.3); RD = ratio dependent synergism or antagonism (Eq. 5.4); CD = concentration dependent synergism/antagonism (Eq. 5.5)

		Cu EC_{50} µg/L	Cu Slope	Ni EC_{50} µg/L	Ni Slope	Max. %	a	b	SSE	AICcW %
Exp. 1	CA	3.98	10.06	321.32	1.80	71.56			5143	0
	S/A	3.96	9.06	798.09	1.08	73.42	-1.57		4865	1
	RD	4.07	8.95	910.85	0.86	74.18	10.57	-14.11	4397	92
	CD	3.97	7.75	1382.75	0.70	75.02	-0.13	-29.77	4558	7
Exp. 2	CA	4.16	12.32	417.09	1.75	73.82			6287	0
	S/A	4.17	10.74	584.43	1.03	80.37	-0.91		5505	0
	RD	4.63	11.90	283.90	2.13	71.78	3.82	-5.59	2295	100
	CD	4.17	9.56	663.65	0.86	80.59	-0.014	-88.58	5288	0
Exp. 3	CA	4.22	7.60	481.34	4.45	85.76			21872	0
	S/A	4.06	7.99	396.23	4.54	85.31	0.70		19137	0
	RD	4.39	8.75	268.56	4.76	85.14	3.65	-4.57	13798	100
	CD	4.05	8.17	391.21	6.21	85.10	0.03	-22.28	18359	0
Exp. 4	CA	3.72	7.41	297.13	4.90	82.87			16461	0
	S/A	3.75	7.45	304.48	5.02	82.84	-0.137		16432	0
	RD	4.15	8.49	161.67	2.45	84.68	8.44	-10.99	12679	100
	CD	3.76	7.41	306.82	4.90	82.83	-0.0007	-278.54	16411	0
Exp. 5	CA	4.01	10.90	747.96	4.86	92.66			12229	0
	S/A	3.88	10.58	633.63	5.32	92.48	0.57		10235	0
	RD	4.17	12.53	428.03	5.79	92.60	3.29	-4.15	4801	100
	CD	3.88	10.85	624.92	6.42	92.36	0.008	-63.57	9874	0

Table D3. The optimal parameter estimates with the MCMC method and 95 % credibility interval (between brackets), sum of squared error (SSE) and AICc weight (AICcW) for each experiment and each deviation model (Eq. 5.2-5.5). Max. = maximum larval development, a = a deviation parameter; b = b deviation parameter; SSE = sum of squared error; AICcW = support for the different models; Exp. = experiment; CA = concentration addition reference model (Eq. 5.2); S/A = synergistic or antagonistic deviations (Eq. 5.3); RD = ratio dependent synergism or antagonism (Eq. 5.4); CD = concentration dependent synergism/antagonism (Eq. 5.5)

		Cu EC_{50} µg/L	Cu Slope	Ni EC_{50} µg/L	Ni Slope	Max. %	a	b	SSE	AICcW %
Exp. 1	CA	3.98 (3.96-3.99)	10.06 (9.85-10.26)	321.41 (314.76-328.15)	1.93 (1.84-2.02)	70.74 (70.31-71.18)	x	x	5153	0
	S/A	3.92 (3.91-3.94)	8.86 (8.70-9.02)	994.63 (993.92-999.78)	0.87 (0.84-0.89)	75.08 (74.66-75.49)	-2.05 (-2.11- -1.98)	x	4901	0
	RD	4.05 (4.03-4.06)	8.82 (8.66-8.98)	995.06 (975.14-999.80)	0.76 (0.74-0.78)	75.40 (74.97-75.85)	13.03 (11.83-14.16)	-16.96 (-18.22- -15.63)	4396	64
	CD	4.02	7.52	716.29	0.74	75.22	3.25	1.66	4433	35
Exp. 2	CA	4.32 (4.32-4.32)	11.57 (11.51-11.59)	397.60 (391.96-403.40)	2.29 (2.22-2.36)	70.18 (69.77-70.56)	x	X	6716	0
	S/A	4.32 (4.32-4.32)	11.36 (11.15-11.56)	560.92 (544.78-578.92)	1.29 (1.24-1.34)	76.09 (75.50-76.70)	-0.88 (-0.93- -0.84)	x	5746	0
	RD	4.60 (4.57-4.62)	11.48 (11.26-11.58)	307.54 (294.98-320.39)	1.79 (1.67-1.93)	73.43 (72.70-74.14)	3.79 (3.71-3.88)	-5.67 (-5.83- -5.54)	2301	100
	CD	4.32	10.21	654.25	1.04	76.57	-0.061	-20.00	5494	0

Continuation of Table D3

		Cu EC_{50} µg/L	Cu Slope	Ni EC_{50} µg/L	Ni Slope	Max.	a	b	SSE	AICcW
Exp. 3	CA	4.29 (4.28-4.30)	7.69 (7.59-7.79)	358.88 (358.86-358.89)	2.69 (2.66-2.73)	89.36 (89.21-89.51)	x	X	29383	0
	S/A	4.15 (4.15-4.30)	8.30 (8.19-8.40)	358.87 (358.79-358.89)	3.97 (3.91-4.03)	85.25 (85.15-85.43)	0.78 (0.77-0.79)		20230	0
	RD	4.33 (4.33-4.34)	8.43 (8.36-8.49)	306.56 (306.54-306.65)	5.03 (4.95-5.11)	84.90 (84.79-84.99)	2.89 (2.87-2.92)	-3.46 (-3.50- -3.42)	14557	100
	CD	4.15	8.28	358.88	5.20	85.08	0.14	-3.90	19562	0
Exp. 4	CA	3.73 (3.72-3.74)	7.44 (7.35-7.55)	288.33 (287.97-288.42)	4.73 (4.57-4.87)	83.06 (82.86-83.25)	x	x	16498	0
	S/A	3.73 (3.72-3.74)	7.44 (7.35-7.54)	288.33 (287.97-288.42)	4.74 (4.60-4.88)	83.04 (82.86-83.21)	-0.00 (-0.03-0.03)	x	16498	0
	RD	3.97 (3.96-3.99)	8.14 (8.01-8.23)	214.90 (214.89-214.98)	3.56 (3.44-3.65)	82.73 (82.55-82.88)	4.88 (4.81-4.95)	-6.20 (-6.30- -6.10)	14431	100
	CD	3.75	6.90	288.42	3.25	82.98	2.41	1.03	16369	0
Exp. 5	CA	4.07 (4.06-4.08)	12.02 (11.77-12.29)	580.22 (580.17-580.24)	3.10 (3.05-3.14)	95.88 (95.70-96.07)	x	x	16278	0
	S/A	4.06 (4.06-4.06)	13.32 (13.04-13.63)	580.20 (580.05-580.24)	4.33 (4.25-4.41)	91.95 (91.47-91.83)	0.53 (0.52-0.55)	x	11944	0
	RD	4.17 (4.16-4.18)	12.50 (12.26-12.74)	427.88 (424.75-431.05)	5.80 (5.68-5.92)	92.64 (92.46-92.82)	3.29 (3.25-3.33)	-4.17 (-4.23- -4.10)	4800	100
	CD	4.06	13.49	580.19	5.22	91.36	0.02	-19.38	11470	0

Appendix D

Table D4. The Cu EC_{50} and slope for the different experiments and Ni concentrations. Exp. = experiment; SE = standard deviation; TU = Toxic unit (Concentration / EC_{50}); * significant at the 84 % confidence interval; ** significant at the 95 % confidence interval

	Ni conc. µg/L	Ni TU	Cu EC_{50} µg/L	SE µg/L	Slope	SE
Exp. 1	0.5	0.00	4.2	0.07	9.4	1.24
	23.9	0.06	3.3**	0.15	4.7	0.92
	34.5	0.08	3.4**	0.07	5.7	0.60
	46.1	0.11	3.6**	0.07	6.6	0.84
	64.7	0.16	3.5**	0.08	8.9	1.90
	91.5	0.22	3.0**	0.06	5.5	0.45
Exp. 2	0.5	0.00	4.5	0.05	8.8	0.64
	34.5	0.06	3.6**	0.03	12.5	0.95
	47.2	0.08	3.4**	0.07	6.0	0.59
	71.7	0.12	3.6**	0.04	9.0	0.73
	107.9	0.18	3.3**	0.06	6.7	0.72
	161.0	0.27	3.4**	0.05	7.9	0.76
Exp. 3	0.5	0.00	4.4	0.06	8.1	0.85
	4.9	0.01	4.1*	0.13	8.5	1.77
	7.6	0.02	3.9**	0.05	9.1	0.73
	12.1	0.03	4.1**	0.09	9.6	1.57
	19.5	0.05	3.8**	0.06	6.8	0.70
	30.6	0.08	3.8**	0.09	5.8	0.80
	49.0	0.12	3.6**	0.07	6.9	0.96
	79.4	0.20	3.7**	0.10	6.3	1.27
	124.3	0.32	3.3**	0.05	6.9	0.64
	188.7	0.48	3.2**	0.10	5.6	0.88
	316.7	0.80	3.3**	0.09	9.4	2.37
Exp. 4	0.5	0.00	4.0	0.06	9.7	1.47
	5.6	0.02	3.7*	0.11	6.0	0.99
	8.5	0.03	3.60*	0.16	5.6	1.16
	13.1	0.05	3.1**	0.17	4.7	0.84
	20.1	0.08	3.4**	0.06	8.6	0.94
	32.6	0.13	3.2**	0.07	6.9	0.75
	51.0	0.20	3.3**	0.04	8.0	0.64
Exp. 5	0.5	0.00	4.1	0.02	14.0	0.95
	13.0	0.03	4.0	0.03	14.8	1.24
	27.4	0.06	3.6**	0.03	14.4	0.86
	54.0	0.11	3.7**	0.03	11.9	0.69
	109.3	0.23	3.1**	0.04	8.3	0.94
	220.6	0.47	3.1**	0.09	7.3	1.46
	417.0	0.89	2.6**	0.07	6.9	0.86

Table D5. The Ni EC_{50} and slope for the different experiments and Cu concentrations. Exp. = experiment; SD = standard deviation; TU = Toxic unit (Concentration / EC_{50}); * significant at the 84 % confidence interval; ** significant at the 95 % confidence interval

	Cu conc. µg/L	Cu TU	Ni EC_{50} µg/L	SD µg/L	Slope	SD
Exp. 2	1.4	0.32	600.00	159.90	0.83	0.20
	3.4	0.75	61.29**	7.48	0.39	0.16
	4.1	0.91	15.46**	3.79	0.35	0.04
	5.0	1.10	7.74**	3.44	0.56	0.09
	6.4	1.41	32.03**	7.45	10.13	26.71
Exp. 3	0.7	0.17	332.70	5.93	8.23	2.23
	1.5	0.34	303.70*	12.35	3.95	0.59
	1.8	0.42	317.90	16.94	4.69	1.37
	2.6	0.59	308.60	22.59	3.81	1.09
	3.5	0.79	108.30**	14.40	1.23	0.17
	4.6	1.04	7.79**	4.28	0.61	0.12
Exp. 4	0.5	0.12	251.65	8.32	4.03	0.51
	4.9	1.22	8.41**	0.53	23.38	104.00
Exp. 5	0.5	0.13	470.45	18.01	8.90	2.81
	1.8	0.43	469.53	15.50	7.37	1.94
	2.4	0.58	363.80**	21.50	2.74	0.40
	3.2	0.77	141.01**	21.11	1.33	0.19
	4.2	1.02	24.16**	3.64	1.09	0.13
	5.8	1.40	0.20**	1.17	0.20	0.13

E

APPENDIX E: SUPPORTING INFORMATION CHAPTER VI

Table E1. The dissolved metal concentrations for the four mixture experiments. * indicates an extension to the full factorial design, no mixtures were tested at this concentration.

	NS exp. 1		NS exp. 2		BS exp. 1		BS exp. 2	
	Cu	Zn	Cu	Zn	Cu	Zn	Cu	Zn
1	0.5	21.2	0.5	0.5	0.4	1.2	0.5	1.8
2	2.5	44.0	1.8	8.7	2.7	6.4	3.4	4.6
3	3.2	54.5	2.0	15.7	3.8	10.3	4.5	6.3
4	4.4	73.3	2.9	27.8	4.9	14.7	6.1	8.1
5	6.6	95.8	4.0	55.6	6.5	20.1	7.9	10.2
6	9.6	134.6	5.9	101.7	8.8	28.5	10.7	14.1
7			8.27	202.0	12.3	42.6	14.7	192.0
8				373.5*		65.2		25.9
9				906.9*		97.8		36.0
10								52.2
11								69.0

Table E2. The optimal parameter estimates with the MCMC methods and 95 % credibility interval (between brackets), sum of squared error (SSE) and AICc weight (AICcW) for each experiment and each deviation model. NS = North Sea; Max. = maximum larval development, a = a deviation parameter; b = b deviation parameter; SSE = sum of squared error; AICcW = support for the different models; Exp. = experiment; CA = concentration addition reference model; S/A = synergistic or antagonistic deviations; RD = ratio dependent synergism or antagonism

		Cu EC_{50}	Slope	Zn EC_{50}	Slope	Max.	a	b	SSE	AICcW
		µg/L		µg/L		%				%
NS exp. 1	CA	4.24 (4.24-4.24)	13.45 (13.25-13.50)	117.10 (117.09-117.11)	1.68 (1.66-1.70)	100.00 (100.00-100.00)			17485	0
	S/A	3.83 (3.80-3.86)	13.50 (13.48-13.50)	103.68 (102.73-104.55)	4.10 (4.03-4.17)	88.39 (87.94-88.81)	1.39 (1.36- 1.42)		5613	63
	RD	4.22 (4.18-4.24)	13.50 (13.48-13.50)	98.76 (97.76-99.92)	4.45 (4.39-4.46)	87.80 (87.44-88.16)	2.03 (1.94- 2.10)	-1.47 (-1.59- -1.30)	5490	37
NS exp. 1	CA	3.72 (3.72-3.75)	8.00 (6.98-9.16)	97.11 (96.19-98.05)	2.64 (2.27-3.06)	90.43 (89.40-91.45)			10219	0
	S/A	3.75 (3.72-3.86)	8.93 (8.01-9.99)	97.76 (96.82-98.68)	3.47 (3.09-3.91)	91.34 (90.30-92.37)	-1.66 -2.08- -1.29)		5934	86
	RD	3.82 (3.72-4.05)	9.18 (8.16-10.35)	97.69 (96.76-98.62)	3.54 (3.14-3.40)	91.27 (90.24-92.30)	-0.90 (-1.94- 0.17)	-1.87 (-4.74-0.41)	5909	14
NS exp. 2	CA	3.96 (3.96-3.96)	6.03 (6.03-6.04)	216.15 (215.99-216.31)	94.93 (94.70-94.99)	97.97 (97.96-97.97)			22495	0
	S/A	3.96 (3.96-3.96)	7.22 (7.10-7.34)	170.24 (168.30-172.11)	33.58 (30.41-37.59)	93.83 (93.65-94.00)	1.09 (1.07- 1.11)		6371	85
	RD	3.96 (3.96-3.96)	7.20 (7.09-7.33)	184.70 (180.23-190.16)	48.38 (39.99-62.28)	93.76 (93.58-93.94)	0.67 (0.53- 0.79)	0.48 (0.34-0.65)	6348	15
NS exp. 2	IA	3.74 (3.71-3.82)	6.63 (6.08-7.71)	133.08 (132.67-133.48)	17.95 (17.48-18.42)	93.56 (92.00-95.15)			19608	0
	S/A	3.96 (3.92-3.96)	7.19 (6.44-8.09)	133.10 (132.70-133.50)	17.96 (17.49-18.43)	95.68 (94.16-97.20)	-3.32 (-3.85- -2.79)		11916	0
	RD	3.93 (3.84-3.96)	7.02 (6.42-7.72)	133.22 (132.82-133.62)	18.23 (17.76-18.70)	95.73 (94.34-97.12)	-7.83 (-9.01- -6.66)	8.18 (6.13-10.41)	9036	100

Appendix E

Table E3. The optimal parameter estimates with the MCMC methods and 95 % credibility interval (between brackets), sum of squared error (SSE) and AICc weight (AICcW) for each experiment and each deviation model. BS = Baltic Sea; Max. = maximum larval development, a = a deviation parameter; b = b deviation parameter; SSE = sum of squared error; AICcW = support for the different models; Exp. = experiment; CA = concentration addition reference model; S/A = synergistic or antagonistic deviations; RD = ratio dependent synergism or antagonism

		Cu EC_{50} µg/L	Slope	Zn EC_{50} µg/L	Slope	Max. %	a	b	SSE	AICcw %
BS exp.1	CA	12.37 (12.37-12.37)	16.07 (15.55-16.59)	49.24 (49.09-49.37)	6.06 (5.97-6.15)	96.31 (96.14-96.49)			49055	0
	S/A	10.00 (10.00-10.01)	14.14 (13.80-14.48)	33.10 (32.95-33.25)	15.96 (15.45-16.52)	92.94 (92.78-93.11)	1.95 (1.94- 1.97)		10131	0
	RD	11.81 (11.75-11.88)	11.40 (11.16-11.64)	28.02 (27.86-28.18)	51.77 (46.95-57.31)	91.72 (91.55-91.88)	4.31 (4.26- 4.37)	-4.71 (-4.82- -4.60)	8162	100
BS exp.1	IA	10.09 (10.00-10.29)	12.81 (11.87-13.87)	31.31 (31.01-31.61)	32.95 (32.87-33.04)	93.61 (92.53-94.70)			9188	0
	S/A	11.46 (11.24-11.68)	13.67 (12.85-14.53)	31.59 (31.35-31.82)	32.95 (32.87-33.04)	94.76 (93.84-95.69)	-4.92 (-5.51- -4.28)		4612	88
	RD	11.37 (11.01-11.69)	13.56 (12.69-14.46)	31.62 (31.37-31.87)	32.95 (32.87-33.04)	94.78 (93.86-95.71)	-5.47 (-7.01- -3.91)	1.37 (-2.16-4.93)	4608	12
BS exp.2	CA	11.28 (11.28-11.28)	5.70 (5.65-5.75)	38.21 (38.21-38.21)	4.21 (4.21-4.21)	89.44 (89.44-89.44)			86963	0
	S/A	10.53 (10.53-10.53)	10.99 (10.75-11.22)	36.80 (36.69-36.91)	6.29 (6.19-6.39)	85.72 (85.59-85.85)	1.63 (1.62-1.64)		9428	0
	RD	10.59 (10.55-10.65)	11.36 (11.10-11.64)	34.35 (34.34-34.37)	6.21 (6.12-6.31)	85.93 (85.80-86.06)	2.30 (2.28-2.32)	-1.01 (-1.08- -0.95)	8491	100
BS exp.2	IA	10.56 (10.53-10.65)	10.52 (9.45-11.82)	35.09 (34.54-35.68)	6.41 (5.83-7.11)	85.18 (84.27-86.09)			9047	0
	S/A	11.04 (9.27-11.27)	10.17 (9.27-11.27)	37.98 (37.30-38.20)	7.13 (6.47-7.93)	85.69 (84.80-86.57)	-2.00 (-2.41- -1.56)		7432	10
	RD	11.25 (11.12-11.28)	10.84 (9.77-12.07)	37.12 (35.91-38.09)	6.85 (6.29-7.53)	85.76 (84.89-86.64)	-0.19 (-1.34-1.05)	-3.68 (-5.61- -1.73)	7222	90

Table E4. The Zn EC_{50} and slope for the different experiments and Cu concentrations. Exp. = experiment; SE = standard deviation; TU = Toxic unit (Concentration / EC_{50}); * significant at the 84 % confidence interval; ** significant at the 95 % confidence interval

Exp.	Cu concentration µg/L	TU	Zn EC_{50} µg/L	SE µg/L	Slope	SE
NS1	0.5	0.13	97.6	4.4	2.9	0.4
	2.5	0.63	84.1*	2.6	2.9	0.2
	3.2	0.80	57.8**	3.6	3.0	0.4
	4.4	1.10	33.1**	10.6	3.9	2.1
NS2	0.4	0.11	133.1	23.8	18.0	17.4
	1.8	0.47	128.8	22.4	15.9	18.4
	2.0	0.53	124.5	12.9	13.2	11.6
	2.8	0.74	99.1	4.4	3.8	0.6
	4.0	1.05	49.8**	9.5	2.8	0.9
BS1	0.4	0.04	32.5	7.9	25.9	7.9
	2.7	0.24	30.4	2.8	20.0	26.5
	3.8	0.34	31.3	5.1	20.7	34.7
	4.9	0.44	30.9	2.3	16.2	14.6
	6.5	0.58	30.9	2.0	16.0	12.0
	8.8	0.79	30.9	1.3	10.7	5.6
	12.3	1.10	6.6**	1.7	15.0	118.5
BS2	0.5	0.05	36.3	0.4	7.6	0.8
	3.4	0.31	36.3	0.5	8.1	1.0
	4.5	0.41	34.3**	0.4	6.5	0.4
	6.1	0.56	34.8*	0.5	5.8	0.5
	7.9	0.72	31.6**	1.2	4.3	0.6
	10.7	0.98	29.1*	4.3	2.8	1.0

Table E5. The Cu EC_{50} and slope for the different experiments and Zn concentrations. Exp. = experiment; SE = standard deviation; TU = Toxic unit (Concentration / EC_{50}); * significant at the 84 % confidence interval; ** significant at the 95 % confidence interval

Experiment	Zn concentration µg/L	TU	Cu EC_{50} µg/L	SE µg/L	Slope	SE
NS1	21.2	0.22	4.0	0.1	9.3	1.0
	44.0	0.45	3.4**	0.0	9.6	1.0
	54.5	0.56	3.3**	0.0	9.8	1.1
	70.3	0.72	3.2**	0.0	14.7	6.0
	95.8	0.98	2.8**	0.1	6.8	1.1
	134.6	1.38	2.8**	0.1	22.7	8.0
NS2	0.5	0.00	3.8	0.3	9.4	0.8
	8.7	0.07	4.2	0.7	7.8	1.1
	15.7	0.12	4.3	0.8	10.2	3.1
	27.8	0.21	3.8	0.1	7.7	1.4
	55.6	0.42	3.4**	0.1	6.9	0.6
	110.7	0.83	2.7**	0.0	6.1	0.4
BS1	1.2	0.04	11.2	0.3	12.7	2.9
	6.4	0.20	11.0	0.4	16.4	5.5
	10.3	0.32	9.5	1.2	23.3	36.9
	14.7	0.45	9.4**	0.5	20.9	17.7
	20.1	0.62	9.4**	0.6	18.8	17.5
	28.4	0.87	9.4**	0.5	15.4	12.9
BS2	1.8	0.05	10.9	0.1	12.2	2.1
	4.6	0.13	10.6	0.1	12.9	2.3
	6.3	0.17	11.0	0.1	10.9	2.0
	8.1	0.22	10.8	0.1	11.4	1.6
	10.2	0.28	10.6	0.1	10.5	1.9
	14.1	0.39	9.8**	0.2	7.9	0.8
	19.2	0.53	9.6**	0.2	6.5	0.7
	25.9	0.71	9.7**	0.4	5.7	0.9
	36.0	0.99	10.0	0.6	5.8	1.7
	52.2	1.44	8.9	3.2	24.3	61.0

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2010-2011	Master in Milieusanering en Milieubeheer	Ghent University
	Thesis: *Interactie van Prorocentrum lima met Mytilus edulis*	
2008-2010	Master in Biology	Ghent University
	Thesis: Juveniele dispersie en habitatselectie bij *Atypus affinis*	
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Publications (Newest to oldest)

Deruytter, D., Baert, J.M., Nevejan, N., De Schamphelaere, K.A.C. and Janssen, C.R. (2016) Copper-nickel mixture toxicity in the marine environment: model development and evidence for synergism at environmental concentrations. Environmental toxicology and chemistry (submitted 26-10-2016).

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Poster Presentations (Newest to oldest)

Deruytter, D., De Schamphelaere, K.D.S. and Janssen, C. (2016). Does the dose make the poison? The influence of biotic and abiotic factors on the toxicity of copper in mussels. VLIZ marine scientist day, Bruges, Belgium, 12th February 2016

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Deruytter, D., Vandegehuchte, M. and Janssen, C.R. (2012) Physiological effects of a marine algal toxin on a primary consumer: tales of the unexpected. SETAC 6th world Congress, Berlin, 22-24th May 2012

Deruytter, D., Vandegehuchte, M. and Janssen, C.R. (2012) Physiological effects of a marine algal toxin on a primary consumer: tales of the unexpected. VLIZ young marine scientists' day, Bruges, Belgium, 24th February 2012

Attended conferences (Newest to oldest)

VLIZ marine scientist day,	16th edition, 20 February 2016, Bruges, Belgium
SETAC Europe,	25th edition, 3-7th may 2015, Barcelona, Spain
VLIZ young marine scientists' day,	15th edition, 20 February 2015, Bruges, Belgium
SETAC Europe,	24th edition**,** 11-15 may 2014, Basel, Switzerland
VLIZ young marine scientists' day,	14th edition, 7 March 2014, Bruges, Belgium
Doctoriales de la mer,	1st edition, 18th October 2013, Boulogne-sur-Mer, France
SETAC Europe,	23th edition, 12-16 May 2013, Glasgow, United Kingdom
VLIZ young marine scientists' day,	13th edition, 15 February 2013, Bruges, Belgium

National Symposium on Applied Biological Sciences, 18th edition, 8 February 2013, Ghent, Belgium

SETAC World Congress,	6th edition, 22-24 May 2012, Berlin, Germany
VLIZ young marine scientists' day,	12th edition, 24 February 2012, Bruges, Belgium