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INTERUNIVERSITY MASTER PROGRAM IN ECOLOGICAL MARINE MANAGEMENT

“Techniques in ecotoxicology and their application in metal-exposed marine bivalves”



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ABSTRACT

Two standard laboratory protocols for two techniques used in marine ecotoxicology, were applied to the marine bivalve *Crassostrea gigas*. The two different techniques: Embryo Acute Toxicity Test and Quantification of Metallothionein were used to assess the toxicity of cadmium – zinc metal mixtures.

In the Embryo Acute Toxicity Test, 10 adult bivalves were induced to spawning by thermal stimulation in order to obtain alive embryos and these were exposed to 25 cadmium (Cd) – zinc (Zn) metal mixtures combination. These combinations were based on a full factorial design in which 5 concentrations of Zn (0, 11, 39, 66 and 77 µg/L) and other 5 of Cd (0, 124, 422, 721 and 845 µg/L) were combined. After 24 hours, the reading of the multiwell under the microscope took place. The results provided less than 70% live larvae with completely developed shells at the end of the test. Thus, the test was considered unacceptable and the data not valid for further use. This big amount of abnormal larvae is due to the bad quality of the gametes, mainly because gametogenesis was incomplete.

Metallothionein (MT) biosynthesis in gills of 30 adult *Crassostrea gigas* were measured by Differential Pulse Voltammetry (DPV). Oysters were exposed for 7 days to 10 Cd - Zn metal mixture combinations that follow a central composite design. Concentrations combine 5 levels of Zn (0, 11, 39, 66 and 77 µg/L) and other 5 of Cd (0, 124, 422, 721 and 845 µg/L). After the time of exposure, separation of cytosolic fraction, determination of cytosolic total protein and heat treatment, in order to remove other cytosolic proteins of the gills, took place. Quantification by voltammetry of metallothioneins gave no significant difference between treatments, oscillating in a range of 2.789 and 10.323 µg MT/mg Pt.

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1. INTRODUCTION

In the ecotoxicological field, oysters like mussels are widely used as bioindicators in monitoring programs in coastal areas, especially in relation to their strong capacity of heavy metal accumulation (Goldberg et al., 1983).

In this context, we have set up an experimental study with larvae and adult oysters to investigate Cd and Zn effects on their growth and development and MT biosynthesis in gills, respectively. Cadmium and Zn were selected because they are toxic elements, and they are often present simultaneously in the environment. Metal mixtures of these two elements were added to the water medium to study their potential interactive effects.

In the present study, fertilized *Crassostrea gigas* eggs were exposed to zinc and cadmium for 24 h, the period required to complete embryogenesis, obtaining D-shaped larvae. The effects of metals were measured at the end of this exposure period by the counting of abnormal larvae, i.e. larvae with not complete developed shells.

In addition, adult oysters, *Crassostrea gigas*, was exposed for 7 days to 10 of the 25 metal mixtures previously used in larvae test. Metal concentrations in the gills were determined by the quantification of metallothionein expressed in terms of total cytosolic protein (mg MT / mg Cytosolic protein). Metallothionein is used because it is often used as a biomarker and it plays a potential key role in the adaptation to metal exposure of important groups of invertebrates.

The general objective was to develop standard laboratory protocols for 2 techniques commonly used in marine ecotoxicology, i.e. the acute embryo test and the quantification of MT. More specifically these two techniques were applied to the marine bivalve *Crassostrea gigas* exposed to single and binary metal mixtures of Cd and Zn. The sensitivity and usefulness of the two techniques will be compared..

2. LITERATURE REVIEW

2.1 Aquatic toxicology

Aquatic toxicology, a subdiscipline of ecotoxicology, is defined as the study of the effects of chemicals and other toxic agents on aquatic organisms with special emphasis on adverse or harmful effects (Janssen, 2006).

Toxicity tests are used to evaluate the concentration of the chemical and the duration of exposure required to produce the criterion effect. The basic paradigm of aquatic toxicology has been the concentration – response relationship, most of the toxicity testing protocols try to solve this in order to generate useful data. Aquatic toxicity tests, frequently called a bioassay, are used to detect and evaluate the potential toxicological effects of chemicals on aquatic organisms. In aquatic toxicity testing, the test organism is exposed to the chemical indirectly by mixing the chemical into the water in which the animal lives, thus producing the test concentration. The test organisms are supposed to build up an internal concentration through the skin and particularly through the gills by equilibrium by partitioning between water and organism. Bioaccumulation is a dynamic process. Because the internal concentration of the toxicant is not always known, toxicity is expressed as the external concentration in the exposure medium, rather than as the internal concentration (Janssen, 2006).

Acute toxicity tests are designed to evaluate the relative toxicity of a chemical to selected aquatic organisms upon short-term exposure to various concentrations of the test chemical. These usually are conducted for a predetermined length of time (time-dependent test) to estimate the 24, 48 or 96 hours LC50 or EC50 (Janssen, 2006).

2.2 Metal mixtures

The majority of mixture toxicity studies in aquatic toxicology have dealt with mixtures of heavy metals. The pollution of surface waters is not a matter of a single chemical acting on a single species; aquatic organisms are typically exposed to numerous substances simultaneously or in sequence (Altenburger et al., 1996).

A joint action is defined as similar or dissimilar depending on whether the sites of primary action of the two chemicals are the same or different, and as interactive or non-interactive depending on whether one chemical does or does not influence the biological action of the other (Janssen, 2006).

Methods to assess the joint action of components in a mixture of toxicants largely are based on the conceptual groundwork laid by Bliss (1939) and are mathematical rather than biological in nature. Plackett and Hewlett (1952) expanded the scheme of Bliss with the possible types of interactions, joint actions, that can occur between chemical components of mixtures (Table 1).

Table 1. The four possible types of joint action for mixtures

	Similar joint action	Dissimilar joint action
Noninteractive	Simple similar action (concentration addition)	Independent joint action (response addition)
Interactive	Complex similar action	Dependent joint action

Source: Plackett and Hewlett (1952)

If the mixture components are known, the mixture toxicity can be predicted from the toxicity of the individual compounds. In the context of aquatic toxicology, mostly the concept of Concentration Addition (introduced by Loewe and Muischnek date ?) is used for the predictive assessment of combination effects. This concept is based on the idea that the components of a given mixture have a common site of primary action (Plackett et al., 1963). Concentration addition is mathematically expressed as:

$$\sum_{i=1}^n \frac{c_i}{ECx_i} = 1 \quad (1)$$

Where:

n = number of mixture components

C_i = the concentration of the respective component in the mixture

ECx_i = the concentration of the i th mixture component that provokes $x\%$ effect when applied singly

Each fraction c_i / ECx_i represents the concentration of a mixture component scaled for its relative toxicity and is generally termed the toxic unit of that component. Consequently, each compound in the mixture can be replaced by another without changing the overall toxicity as long as the sum of toxic units remains unchanged. If, at a total concentration of the mixture provoking $x\%$ effect, the sum of the toxic units equals one, concentration addition holds. Because of that, the sum of toxic units has been frequently used as a measure for comparing the observed toxicity with the predictions made by concentration addition (Könemann 1981).

In contrast to the concept described above, the concept of independent action (also known as response addition, Bliss independence, or effect multiplication) is based on the assumption that the compounds of a given mixture act on different physiological systems within the exposed organisms (Bliss, 1939). The mathematical formulation is as follows:

$$E(c_{\text{Mix}}) = E(c_1 + \dots + c_N) = 1 - \prod_{i=1}^n [1 - E(c_i)] \quad (2)$$

Where:

c_i = is again the concentration of the i th compound,

$E(c_i)$ = is the effect of that concentration if the compound is applied singly.

$E(c_{\text{Mix}})$ = denotes the predicted effect (scaled from 0–1) of an n -compound mixture,

The concept of independent action has a coefficient of correlation (denoted by r) included. Originally only formulated for binary mixtures, this coefficient varies from -1

(completely negative correlation) to +1 (completely positive correlation). If r equals zero, no correlation exists, which leads to the basic situation described in Equation 2. If the susceptibilities are completely positively correlated ($r = +1$), the effect provoked by the given mixture equals the effect of the most toxic compound (the so-called no addition case). If $r = -1$, the mixture effect equals the sum of the effects of the individual mixture components (effect summation). In the basic case ($r = 0$), the predicted mixture toxicity is higher than in the case of no addition but lower than calculated using the sum of the effects of the individual components. So far all the situations described are more theoretical constructs (Backhaus et al., 2000).

All combinations of a mixture caused a more severe (Synergism) or less severe (Antagonism) effect than calculated from either reference model (Jonker et al., 2005). In other words Synergy is when one toxin may increase the mortality caused by the other, and Antagonism when one toxin may reduce the mortality caused by the other (Clark, 1992). In Figure 1 the straight line connects the effect concentrations of the individual compounds and represents the isobole for concentration-additive behavior of binary mixtures of the components S1 and S2 in varying mixture ratios. The upward-bent, dotted isobole illustrates mixtures that require higher concentrations to exert the effect (i.e., less than concentration additive, CA, or antagonistic). The downward-bent, dashed curve represents mixtures with lower concentrations needed to provoke a combined effect (i.e., more than CA or synergistic effects) (Loewe et al., 1926)

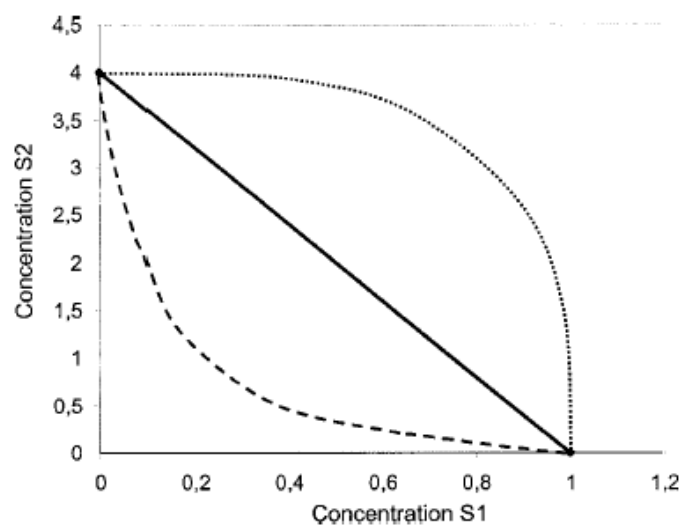


Fig. 1. A schematic isobologram. (Source: LOewe et al., 1926)

2.3 Zinc and Cadmium in aquatic media

Environmental pollution by metals is one of the most important problems nowadays in the world. Environmental pollution by heavy metals has increased in the last decades due to extensive use them in agriculture, chemical and industrial processes, becoming a threat to living organisms (Mule and Lomte, 1994).

Considering the fact that heavy metal concentrations in the environment can change frequently under natural conditions (irregular pollution, dilution by rainfall), the accumulation pattern in animals is dependent not only on uptake but also on depuration rates (Salfinki and V.-Balogh 1985, 1989; V.-Balogh and Salfinki 1984).

Cadmium is not an essential element for any marine organism although, for unknown reasons, it enhances phytoplankton photosynthesis and growth at concentrations up to 100 ppm. Because of its associations with phosphates, cadmium is assumed to be taken up to phytoplankton but, except in two instances, it does not appear to accumulate in the food chain. Mollusks particularly bivalves accumulate large concentrations of cadmium. At higher levels in the food web, fishes and sea mammals have low concentrations of cadmium, at most a few ppm, stored chiefly in the kidney (Clarck, 1992).

Cadmium can penetrate into organisms and produce adverse effects, reason why is important to study its mechanisms of toxicity to obtain a better understanding of its mode of action (Zyadah and Abdel-Baky, 2000; Olsson et al., 1998). Cadmium passes cellular membranes through calcium channels. Once it has passed through the cellular membrane, Cd can bind to biomolecules, accumulate or deposit in subcellular compartments and it may undergo metabolic transformation or elimination (Roesijadi and Robinson, 1994; Di Giulio et al., 1995). The presence of high affinity Cd ligands such as glutathione (GSH), sulfhydryl-rich soluble proteins and metallothioneins within the cell should provide for the continuous removal of the metal, thus reducing the concentration of the free Cd cations in the cell and therefore maintaining a permanent gradient with the external medium (Viarengo, 1989).

Zn has a wide spectrum of biological activities and its deficiency has been related to various tissue dysfunctions and alterations of normal cell metabolism (Fabio et al., 1999). Several studies have reported that Zn deficiency is associated with increased reactive oxygen induced damage in various tissues (Taylor et al., 1990; Parsons and Di Silvestro, 1994; Oteiza et al., 1995; Kraus et al., 1997).

2.4 Oyster used in acute tests

Marine pollution has been traditionally documented in terms of chemical concentrations of contaminants; however, these measurements do not provide estimations of the deleterious effects upon living organisms and should be complemented with biological criteria obtained by means of bioassays (Chapman and Long, 1983).

Nowadays, several biological assays exist to determine the toxicity of sediments using different organisms such as amphipods, polychaetes and bivalves (Geffard et al., 2004). Eggs, embryos and larvae of marine bivalves (mainly of *Crassostrea gigas*, oysters, and *Mytilus* spp., mussels), are among the organisms most commonly used in acute tests to assess the toxicity of micropollutants such as heavy metals detergents and to evaluate the biological quality of marine, coastal or estuarine seawaters and sediments in coastal areas subjected to anthropogenic effects (His et al., 1997).

Oysters present several advantages like: they withstand a broad range of environmental conditions, temperatures from 18 to 28°C, salinity from 25 to 35 (His et al., 1989) and pH from 7 to 8.5 (Calabrese and Davis, 1966); they have a rapid embryonic development; and they are very sensitive to many contaminants (Geffard et al., 2004).

The assessment of toxicity using bivalves is based on the percentage of abnormal D-larvae after the fertilized eggs have been incubated for one or two days in the water under investigation, as originally proposed by Woelke (1972) and reviewed by Calabrese (1984). Intercalibration exercises have demonstrated a strong need to

standardize embryo-larval bioassay procedures (His et al., 1997). The criterion of abnormal shell development in bivalve larvae represents a sublethal response that leads to certain non-recruitment into the population due to increased predatory pressure from longer pelagic existence. Furthermore, morphological deformities in the larvae of broadcast fertilizers can only result in severely decreased chance of survival for the offspring (Martin et al. 1981).

The effects of zinc and cadmium on the embryonic development, larval growth and settlement of the oyster *Crassostrea gigas* have been studied individually in some detail (Brereton et al. 1973; Boyden et al. 1975; Watling 1978). Boyden et al. (1975) reported that settlement of *C. gigas* larvae was both reduced and delayed in the presence of zinc but that those larvae which settled were as viable as controls when on-grown in clean water. The results reported by Watling (1978) indicated the extreme toxicity of cadmium to *C. gigas* larvae and spat but contrasted with those results obtained from the two studies on the effects of zinc (Brereton et al., 1973; Boyden et al., 1975). Watling et al (1983) report that cadmium appeared to be more toxic than zinc to both larvae and spat; in his assay the effect of cadmium was longer lasting than that of zinc; and cadmium appeared to induce early settlement and the numbers settling and their viability were markedly reduced.

2.5 Metallothionein (MT): definition, biology and its implications

Metallothioneins (MTs) are non-enzymatic proteins with a low molecular weight, high cysteine content, no aromatic amino acids and a high heat stability. The thiol groups (–SH) of cysteine residues enable MTs to bind heavy metals. Important functions have been reported to them, such as metal regulation and detoxification (Cherian et al., 1993) and free radical scavenging or immune response (Coyle et al., 2002). MTs have been reported in many vertebrates and aquatic invertebrates, like mollusks (Langston et al., 1998; Isani et al., 2000) and crustaceans (Roesijadi, 1992; Engel and Brouwer, 1993; Barka, 2000).

Two classifications for the MTs exist nowadays. In the first one they have been divided into three classes (Fowler et al., 1987). Class I comprises all proteinaceous

MTs with locations of cysteine closely related to those in mammals. Some molluscan and crustacean MTs belong to this class, among them oysters (Roesijadi et al., 1989). Class II includes proteinaceous MTs and Class III consists of non-proteinaceous MTs also known as phytochelatins. The second and recent classification of MTs (Binz and Kägi, 1999) takes into account phylogenetic features as an additional classification criterion. Class I and Class II MTs known so far are distinguishable into 15 families. According to this MT classification, molluscan MTs belong to family 2 whereas crustacean MTs in family 3 (Amiard et al., 2006).

Mason and Jenkins (1995) proposed two roles for MTs. The first one refers to the fact that these proteins comprise a non-toxic zinc and copper reservoir available for the synthesis of metalloenzymes, allowing the homeostasis of many cellular processes (Brouwer et al., 1989; Viarengo and Nott, 1993; Roesijadi, 1996). Secondly, MTs can reduce the nonspecific binding of non-essential metals (Cd, Hg or Ag) within cells, and so restrict their toxic potential (Roesijadi, 1992, 1996; Zarogian and Jackim, 2000).

While low basal levels of MT are generally present in animals not subjected to elevated metal concentrations, the synthesis of MT is greatly induced as a direct response to exposure to metals Cd, Cu, Zn, and Hg (Hamer, 1986; Palmiter, 1987). The ability of organisms to express the MT gene is associated with the ability to resist metal toxicity (Unger et al., 1996).

The toxicological significance of MT induction has been supported by the numerous studies that have been conducted with aquatic organisms. Metal-binding by MT is protective of cellular function, although the specific intracellular interactions remain to be clarified (Roesijadi, 1992). The involvement of MTs in metal accumulation has led to associate metal tolerance with MT induction and to conclude that these proteins are very likely to be involved in detoxification processes. Moreover, it has been established that a rise in MT concentration can be linked to a decrease of the sensitivity of an organism to metals in excess (Pavicic et al., 1994; Roesijadi and Fellingham, 1987). It has been noticed that in a single species, the populations that live in a medium polluted by metals have higher concentrations of MT (Stuhlbacher et al., 1992; Ross et al., 2002).

Apart of the usefulness of MT as a biomarker, it plays a potential key role in the adaptation to metal exposure of three important groups of invertebrates: mollusks, crustaceans and annelids, which contribute greatly to estuarine and coastal ecosystems, in terms of biomass, and ecosystem structure and functioning (Amiard et al., 2006).

The MTs of aquatic animals have been reported for at least in 80 species of fish and invertebrates. To date, MTs of only eight species have been characterized to the extent that the primary structure has been determined. The occurrence of multiple forms of MTs in a single species lead to a better understanding of their regulation and function as individual forms, because specific biological functions seems to be attribute to each form. Molecular biological advances using MT gene amplification or duplication have confirmed that functions of different isoforms are different, some of them being involved in metal homeostasis and others in nonessential metal detoxification (Tanguy and Moraga, 2001).

In *C. gigas*, three MTs isoforms have been reported to date (Tanguy et al., 2001 and Tanguy et al., 2001). The *CgMT1* and *CgMT2* genes have been characterized, whereas *CgMT3* is only available as a complementary DNA cDNA sequence. From the three isoforms genes from *C. gigas*, *CgMT2* is the most expressed. *CgMT1* is present at a lower copy number, and *CgMT3* is present in minimum concentrations. The same order of expression level ($CgMT2 > CgMT1 > CgMT3$) was conserved after induction by Cd. From this analysis *CgMT2* has been hypothesized to have a greater detoxification capacity than *CgMT1*. The expression level of *C. gigas CgMT3* appeared to be induced only in response to Zn exposure; and Cd-Zn mixture had no effect. Low expression of *CgMT3* suggests that probably MT has no significant physiological functions under metal exposure or could be only expressed in particular developmental stages in oysters. Another observation was that Zn did not induce *C. gigas CgMT1* or *CgMT2* expression (Marie et al., 2006).

Some studies of the expression of MT genes in the presence of heavy metals, new nuclear genetic markers, and polymorphism of selected genes in relation to environmental stress factors such as pollution; are taken place in MT subject (Tanguy et al., 2002). Low levels of polymorphism in the coding sequences of MT

genes have been observed in *C. gigas*. Increases frequencies of types C in exon 2 and B in exon 3 in CgMT1 were observed in Cd-resistant populations. CgMT1 and CgMT2 genes had been therefore envisaged as MTs essentially involved in detoxification and CgMT2 as activated by increased metal exposure (Tanguy and Moraga, 2001). These two MT types in *C. gigas* are now considered as appropriate genetic indicators for monitoring metal polluted sites (Tanguy et al., 2002). However, in another oyster *Ostrea edulis*, Tanguy et al. (2003) observed no significant induction of MTs, either in the gills or the digestive gland, nor MT-RNA expression. These results led to the conclusion that MTs are not involved in metal detoxification in this oyster, consistent with the lack of genetic diversity in field populations (Tanguy et al., 2003).

Tissues directly involved in metal uptake, storage and excretion have a high capacity to synthesise MTs (Amiard et al., 2006). In aquatic organisms MTs and related proteins have been detected and identified in the gills, digestive gland, kidneys and intestines of numerous molluscan and crustacean species (Roesijadi, 1992). In all studied species, MT concentrations were higher in the digestive gland of bivalves than in the gills (Amiard et al., 2006); nevertheless the gills have proved to be the most suitable for analysis because they represent the main barrier involved in metal uptake via the direct exposure route, with a large exchange area in contact with the surrounding environment (Bebiano et al., 2003). The ability of oysters to store metals to a considerably higher degree than can mussels is well documented in biomonitoring programs (Beliaeff et al., 1998).

The relationship between metal bioaccumulation and MT concentrations were studied in larvae of *Mytilus galloprovincialis* (Geffard et al., 2002) and of *C. gigas* (Geffard et al., 2003) exposed to contaminated estuarine sediment elutriates. MT induction in larvae was always strongly correlated with increasing metal content (Cd, Cu and Zn) in the cytosolic fraction of samples. Geffard et al. (2003) hypothesized that MT induction in oyster larvae provides an enhanced early-warning tool for evaluating environmental contamination by metals, demonstrating that oyster larvae may be used to evaluate MTs as biomarkers of metal exposure (Damiens et al., 2006). However the understanding of MT expression during early development is still at an early stage (Roesijadi et al., 1997).

3. METHODOLOGY

3.1 Oyster Embryo Test

The following toxicity test is conducted to obtain information concerning the acute effects of metal mixtures (zinc and cadmium) on growth and development oyster's embryos. Results of acute tests are often considered to be a good indication of the acceptability of pollutant concentrations of saltwater mollusk species in general. Results of acute toxicity tests might be used to predict acute effects likely to occur to aquatic organisms in field situations as a result of exposure under comparable conditions.

3.1.1 General equipment and chemicals

3.1.1.1 Equipment

- A. Fridge 4°C
- B. Freezer -20°C
- C. Inverted microscope
- D. Plastic or glass chambers (5 -10 L)
- E. Thermostates
- F. Water-bath
- G. Shaker Vortex Ika
- H. Analytical scales
- I. Multitip pipette (Finn) + reservoir:
- J. 24 multiwell plates of 3 ml of volume
- K. Thermometer
- L. Oxigen meter
- M. Salinity meter
- N. pH-meter
- O. Kit for dissection
- P. Glass material (erlenmeyer, glass cylinder and glass containers)
- Q. Pipettes (Finn, Eppendorf)

- R. Multipipette
- S. Tips for pipettes
- T. Deionized water
- U. Instant Ocean synthetic sea salt (Mentor, OH, USA)
- V. Paper
- W. Gloves (latex or nitril) (VWR)

3.1.1.2 Chemicals:

- A. $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$ (Fluska)
- B. H_3BO_3 (Merck)
- C. KBr (VWR)
- D. KCl (Merck)
- E. $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (VWR)
- F. Na_2SO_4 (VWR)
- G. $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (VWR)
- H. NaCl (Sigma)
- I. $\text{Na}_2\text{SiO}_3 \cdot \text{H}_2\text{O}$ (Fluska)
- J. NaHCO_3 (Vel)
- K. CdCl_2 (VWR)
- L. ZnCl_2 (VWR)
- M. NaOH 1M (37% formaldehyde)

3.1.2 Animal Supply

10 Adult Pacific oysters (5 females and 5 males), *Crassostrea gigas*, were delivered in March 2007 from Guernsey Sea farms (Park Lane, Vale, Guernsey, UK) on the French atlantic coast (Appendix 1). Guernsey Sea Farms, specialist hatchery and nursery for seed shellfish, supplies oysters, *Crassostrea gigas*, for use in oyster embryo bioassays for monitoring marine disposal sites, estuarine effluents or toxicity testing of chemicals (<http://www.guernseyseafarms.com/>).

Experiments were conducted at the Laboratory of Environmental Toxicology and Aquatic Ecology, Ghent University, Belgium.

3.1.3 Set up and experimental conditions

3.1.3.1 Reconstituted Water:

Following the ASTM standards for reconstituted salt water described in their guidelines of 1995 besides the requirements of being available in adequate supply, the dilution water should: be acceptable to adult bivalve mollusks and their embryos and larvae, be of uniform quality, and not unnecessarily affect results of the test. Quality of the dilution water should be uniform enough that the brood stock is held and conditioned and the test is conducted in water of the same quality.

Reconstituted salt water is prepared by adding a sea salt or specified amounts of reagent-grade chemicals to high quality water with conductivity $\leq 1 \mu\Omega/\text{cm}$ and total organic carbon $\leq 2\text{mg/L}$ or chemical oxygen demand (COD) $\leq 5 \text{mg/L}$. Acceptable water for dissolution of sea salts can usually be deionized or distilled water. A formula for reconstituted water acceptable for use with bivalves is given in **Table 2**. (Zaroogian et al., 1969).

According to the measurements and quantities needed for performing the bioassay, Reconstituted Salt water (also called Artificial Sea Water) was prepared. Add the following reagent-grade chemicals in the amount and order listed in the **Table 3** to deionised water. Each amount of salt must be diluted with a little bit of deionized water before adding it to the total volume in the erlenmeyer, in this way we will have a better dilution of them. Each chemical must be dissolved before the next one is added. While dissolving and mixing the salts use a magnetic stirrer. If the salts are not well dissolved increasing temperature can help. The final reconstituted water should be free of particulated salts.

Table 2. Reconstituted Salt Water

Chemical	Artificial Sea Water	
	1 L	5L
SrCl ₂ *6H ₂ O	0.02 g	0.10 g
H ₃ BO ₃	0.03 g	0.15 g
KBr	0.10 g	0.50 g
KCl	0.70 g	3.50 g
CaCl ₂ *2H ₂ O	1.47 g	7.35 g
Na ₂ SO ₄	4.00 g	20.00 g
MgCl ₂ *6H ₂ O	10.78 g	53.90 g
NaCl	23.50 g	117.50 g
Na ₂ SiO ₃ *H ₂ O	0.02 g	0.10 g
NaHCO ₃	0.20 g	1.00 g

The salinity of the resulting solution diluted to 1L, according to the ASTM protocol, should be 34 ± 0.5 g/kg. If necessary, the water should be diluted to the desired salinity at time of use by adding deionised water. Measure and record the salinity of the Artificial Sea Water (ASW). Due to the biological exigencies of the species, *C. gigas*, the salinity was lowered to 33 ± 0.5 g/Kg.

Cover the erlenmeyer that contains the ASW with parafilm (to prevent evaporation) and leave it under aeration for one night in order to have 100% of oxygen saturation and stabilized pH and salinity. It's better to prepare fresh medium one day before the start of the oyster acute toxicity test. Take into consideration that due to the oxygenation during the night some ASW could be lost by evaporation. The ASW can be stored in darkness at 4°C for one week but it must be aerated before using it again.

3.1.3.2 Metals dilutions

To develop a second-order mathematical model relating chronic toxicity to metal concentrations a full factorial design, a design in which every setting of every factor appears with every setting of every other factor, was used. In other words all factors are tested at all possible combinations of settings. The 5 levels determined for the 2 metals cadmium (Cd) and zinc (Zn) are shown in the **Table 3** and detailed explanation can be found in the section 2.2.3 *Set-up and experimental conditions*.

Table 3. Metal Concentration (ug/L) Tested in the Full Factorial Design within the Binary Mixtures

Metal	Design Point				
	-1.41	-1	0	1	1.41
Zinc	0	11	39	66	77
Cadmium	0	124	422	721	845

Stock solutions of trace metals CdCl₂ and ZnCl₂ (500 mg Cd/L and 500 mgZn/L, respectively) were prepared in Artificial Sea Water. Test solutions were done by appropriate volumetric dilution of stock solution with ASW. The 25 concentrations of the metals to combine (Cd and Zn) are shown in the **Table 4**. The containers were these binary mixtures were put were either sterilised or rinsed with deionised water. The pH of all 25 combinations were between 7.5 ± 0.5. The metal combinations can be stored at 4°C until needed to perform the experiment.

Table 4. Metal Concentration (ug/L) added in each combination tested

Combination #	Concentration of Metals	
	Zn (ug/L)	Cd (ug/L)
1	0	0
2	0	124
3	0	422
4	0	721
5	0	845
6	11	0
7	11	124
8	11	422
9	11	721
10	11	845
11	39	0
12	39	124
13	39	422
14	39	721
15	39	845
16	66	0
17	66	124
18	66	422
19	66	721
20	66	845
21	77	0
22	77	124
23	77	422
24	77	721
25	77	845

3.1.3.3 Instant Sea Water

Instant Ocean Sea Water was prepared with Instant Ocean synthetic sea salt (Mentor, OH, USA) in deionised water and adjusted to 33 ± 0.5 ‰. It should be aerated for at least one night to have 100% of oxygen saturation and stabilized pH and salinity. It can be stored for longer periods (even some months) at 4°C in darkness.

3.1.3.4 Glassware material

Test chambers, aquariums and equipment used to prepare and store dilution water, stock solutions, and test solutions should be cleaned before use. New items should be washed with detergent and rinsed with water, a water miscible organic solvent, water acid (as 10% concentrated hydrochloric acid), and at least twice with deionized, distilled or dilution water. All glassware material must be sterilized in the oven for 45 minutes at 150°C the day before or just before starting the test. Test chambers should be rinsed with dilution water just before use.

At the end of the test, all items that are to be used again should be immediately emptied, rinsed with water, cleaned by a procedure appropriate for removing the test material from the item (for example HCl acid for removing metals dilutions) and rinsed at least twice with deionized, distilled, or dilution water.

3.1.3.5 Artificial Sea Water parameters

Monitoring the parameters of Artificial Sea Water should take place at all times during the test. The dissolved oxygen concentration must be between 60 and 100% of saturation as growth of larvae is reduced at oxygen concentrations below 4.2 mg/l (58.8% of saturation) (Morrison et al., 1971). The pH should be stable $7.5 - 8.0 \pm 0.5$. The testing temperatures for *C.gigas* are shown in **Table 5**, but usually are in the range of the 20°C. The salinity in particular should always be between 18 and 32 g/kg and within the test should not vary by more than 1g/kg among treatments or any

renewals during the test (ASTM, 1995). For this assay we consider the concentration of $33 \pm 0.5 \text{ ‰}$.

Table 5. Recommended Temperature (°C)

Species	Holding	Conditioning	Induction	Never to be Exceeded	Test
<i>Crassostrea gigas</i>	14-15	20	25-32	32	20
<i>Crassostrea virginica</i>	14-15	20 -25	25-32	32	25

3.1.4 Bioassay procedure

Three aquariums of $\square 50 \times 30$ cm well cleaned and filled with Instant Ocean Sea Water were thermostatically controlled at 18, 24 and 28°C. Glass chambers, previously cleaned and rinsed thoroughly, of $\square 5$ to 10 L of capacity containing 1 to 2 L of Artificial Sea Water were placed inside these three aquariums. The aquariums with 28 and 18°C were used for oysters' thermal shock spawn stimulation and the one at 24°C for spawning, gametes collection and fertilization. Temperature in all of them must be controlled.

The assay was performed immediately after arrival of the oysters by DHL from Guernsey Sea Farms. Ten mature *Crassostrea gigas*, 5 females and 5 males, were placed in the glass chambers (males and females mixed together). They were induced to spawn by thermal stimulation, alternating immersion in Artificial Sea Water at 28°C first and then at 18°C for 30 minutes each time (His et al., 1997). Usually after two or three water bath changes we will have them spawning. If no animal spawns within 90 minutes the water bath should be drained, refilled with Instant Ocean Sea Water at the brood stock holding temperature and the stimulation process repeated (ASTM, 1995).

To obtain gametes you can induce the animals by ozone, temperature or another way is liquefying the gonads and then extracting some gametes. The last one should

always be the last option because it induces a stress situation to the animal (the opening of its valves using the force); it might only be used if the thermal stimulation does not work after 3 hours. Another possibility to enhance spawning, used in combination with thermal stimulation, is the addition of gametes/feromones of the same species diluted in ASW, These gametes can be obtained at the end of each experiment and stored at 4°C until the next opportunity.



Figure 2. Females and males oysters spawning. Left: individual female oyster, Right: three male oysters spawning.

Oysters that are about to spawn will clap their valves together violently once or twice. Spawning can be recognized by the appearance of a whitish stream of gametes emerging from the animal (ASTM, 1995). Recognized female spawners were transferred individually in a sterile beaker, while many spawning male oysters can be put together as much as they fit in a container (**Figure 2**); for both cases they have to be with approximately 100 to 150 ml of 24°C ASW and submerged in water bath with the same temperature (**Figure 3**). Generally, males tend to spawn earlier than females. When males start spawning separate them of the rest and stop their emission closing their valves with an elastic band and take them out of the chambers until you have some females also spawning.

Females are allowed to spawn for 30 minutes. In the meanwhile egg quality of each female was checked before using it in the test under a 400x magnification microscope. Eggs about 15 minutes after spawning should have round shape with

regular margins as shown in **Figure 4**. Eggs were sieved through a 100 μm screen to remove impurities and collected in a 500 ml glass cylinder. To ensure a homogeneous suspension of eggs a perforated plunger of PVC was used to suspend the eggs all the time. The concentration of eggs should be determined by counting a sample of egg suspension. A 1 ml sample was taken, placed on a slide and the number of rounded eggs was counted under the microscope. The egg density should be in the range of 20 to 50 eggs/ml (preferable 60 eggs/ml) before adding the sperm.



Figure 3. Female oysters spawning in thermostatic bath at 24°C

Males when spawning discharge a dense milky sperm solution. It takes about 15 min for sperm-cells to activate from the moment they get in contact with sea water, after this time motility must be verified under the microscope. Once the sperm-cell suspension was ready, it was filtered through a 32 μm screen in order to remove extraneous material (Damiens et al, 2006).



Figure 4. Oyster unfertilized egg

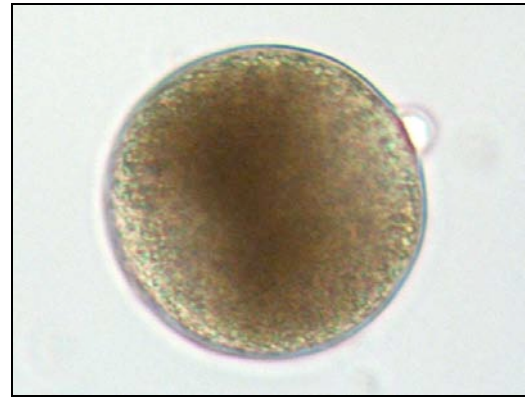


Figure 5. Oyster Fertilised egg

When having both gametes fertilization was proceeded by adding 10 ml of sperm-cell suspension to the 500 ml egg suspension and wait for 20 minutes always resuspending the ASW with the gametes. After this time eggs should be fertilized (**Figure 5**). Although the fertilization membrane is not evident, a sort of spot on the egg surface is visible; also you should be able to count 10 to 20 spermatozoa attached to or around each egg membrane cell (His et al., 1997). If this is the case continue the test. If not, add 5 ml more of the sperm cell suspension to improve fertilization, wait 20 minutes more and then continue the test.

The number of zygotes/ml was counted in order to determine the aliquot needed to inoculate in the plate's wells. The counting was developed on 4 replicates of 1 ml of the zygote suspension; two samples were taken when the plunger was up the glass cylinder and 2 when it was down. Samples were placed on a slide and counted under the microscope. The concentration in the test solutions should be 15 to 30 zygotes per ml, although concentrations up to 100 zygotes per ml do not impair normal development of Pacific oyster embryos (Staeger et al. 1976).

The final zygote suspension (generally from 10 μ l to 70-80 μ l) that must be transferred into each well should contain a number of \approx 60 zygotes/ml (around 200 zygotes/well). The volume to add to the well depends on the density presented in the experiment and must be calculated in order to reach this concentration.

The metal mixtures to test and volume of fertilized eggs required were put in a 24 multiwell plate of 3 ml of volume. The 25 metal mixtures were added in the morning of the testing day and incubated at 24°C. For each metal mixture combination three replicates were used. In each well it was tried to incubate from 200 to 300 fertilized eggs. Once filled the plates were stored in the 24°C thermostatic chamber to reach the right temperature.

After 24 hours of exposure at 24°C, 10 µl of 37% formaldehyde was added to end the test. The multiwell can be stored at 4°C for 1 year if not all the reading of the test can be performed immediately.

3.1.5 Reading of Results under Microscope

Approximately 100 larvae per metal mixture test combination were counted by direct observation of the individuals under 100x magnification of a inverted microscope. If the number of larvae is too low count all the ones present in the well and transform it to percentage values.

For the purposes of the assay, three categories were determined:

- D-shape = only D-shape
- Abnormal (AB) = D-shape deformations / Abnormal Prodissoconch I
- Not Developed (ND) = Eggs, Trochophore larvae and Normal prodissoconch

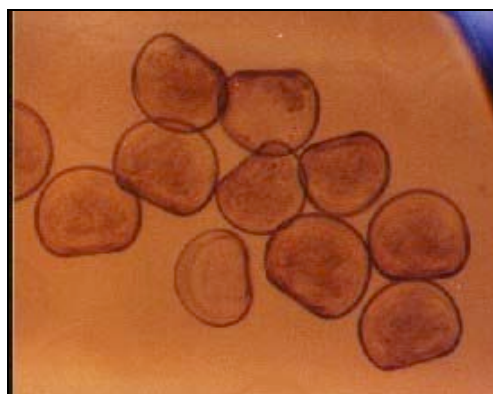


Figure 6. D - shape oyster larvae stage

As is already defined D-shape only considers larvae which get transformed to the shelled, hinged D - shaped veliger (**Figure 6**). Abnormal (AB) criterion was constituted by larvae that included: D-shape larvae with either convex hinge, indented shell margins, incomplete shell or protruded mantle (**Figure. 7**); also abnormal Prodissoconch I was considered (**Appendix 2 and Appendix 3**). While Not develop larvae (ND) included normal or segmented eggs and normal Prodissoconch I (**Appendix 2**). Exploited eggs or dead particulate material were not considered in the counting.

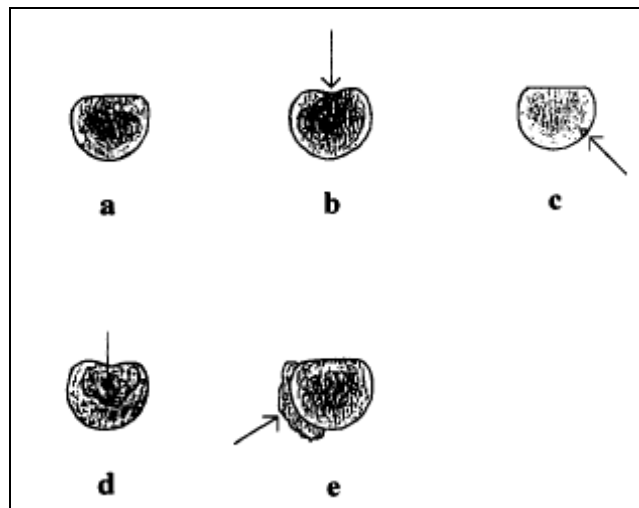


Figure 7. Abnormalities observed in D-larvae of oysters, *Crassostrea gigas*: (a) normal larva; (b) convex hinge; (c) indented shell margin; (d) incomplete shell; (e) protruding mantle.

3.1.6 Statistical Analysis

Depending on the purpose of the test, the experimental design, and the observed results of the embryo test; it might be or not necessary to transform the data set prior to statistical analysis.

After ocular counting of D-shape, abnormal (AB) and not developed (ND), the total number of embryos tested (D-shape + AB + ND = 100%) and the percentage of

embryos that did not result in live larvae with completely developed shells, called also A, (percentage of the sum of AB and ND embryos) was calculated for each test chamber in each treatment.

When having all the A values, the data obtained were transformed and adjusted for control mortality following the equation 1 mention below.

$$E = (100 (A - M)) / (100 - M) \quad (1)$$

Where:

E = Percentage of introduced live embryos that did not result in live larvae with completely developed shells adjusted for the controls.

A = Percentage of embryos that did not result in live larvae with completely developed shells.

M = The average percentage of the embryos that did not result in live larvae with completely developed shells for the test chambers in the control treatment. M should be calculated as an average of A's.

ASTM standards said that the test is unacceptable if M is > 30% for oysters. It also proposes that calculation of an EC50 should be usually considered unacceptable if one or both of the following occurred:

- a. No treatment other than a control treatment resulted in an average E less than 37%.
- b. No treatment resulted in an average E greater than 63%.

The Trimmed Spearman-Kärber Method was used for calculating the EC50 of single metals (Hamilton et al., 1977) and the 95 % confidence interval.

For metal mixtures, the calculated effect (E %) was also used to create surface response relationships between the two metals, using equation 2 in Non Linear Estimations, User-specified regression Least Squares in Statistica software.

$$V3 = A * V1 + B * V2 + C * (V1)^2 + D * (V2)^2 + E * V1 * V2 + G \quad (2)$$

With V3 (E %), V1 (metal 1) and V2 (metal 2)

Terms are considered significant at ($P < 0.05$). Non-significant terms should not be included in the final surface response equation.

3.2 Quantification of Metallothionein (MT) from *Crassostrea gigas* (Japanese oyster)

We have set up an experimental study with oysters to investigate Cd and Zn effects on MT biosynthesis in gills. These two metals were added to Artificial Sea Water in combination to study their potential interactive effects. Measuring MT includes separation of cytosolic fraction, heat treatment to remove other cytosolic proteins and quantification by voltammetry of metallothioneins.

The following methodology described is for gills of *Crassostrea gigas*, but can also be used for other tissues and other organisms as long as sufficient biomass is available.

3.2.1 General equipment and chemicals

3.2.1.1 Equipment

- A. Fridge 13°C where photoperiod can be set
- B. Freezer -20°C
- C. Freezer -80°C
- D. Fridge for storing solutions: Fridge A Ultracentrifuge L755 and rotor SW55Ti (Beckman) or Refrigerated centrifuge-supra speed (RCF of 50 000 x g).
- E. Hettich Microcentrifuge “Mikro 200R” Plate reader (Thermo Labsystems, Multiskan Ascent) with Ascent software Voltammetry instrument with the option to measure in differential pulse mode. Hanging mercury drop electrode (HMDE) “All-in-one” type of instrument: 797 VA Computrace, Metrohm

- F. Homogeniser with Teflon head
- G. Water-bath with a support designed for Eppendorf tubes
- H. Shaker Vortex Ika
- I. Analytical scales
- J. Multitip pipette (Finn) + reservoir:
- K. 96 well microtitre plate-NUNCLON surface (Nunc, VWR)
- L. Schott Duran flask
- M. 50 mL polyethylene cups
- N. Centrifugation tubes for ultracentrifuge (Thickwall polyallomer, size 13x51 mm; Beckman).
- O. Microcentrifuge tubes of 1.5 and 0.5 mL (e.g. Eppendorf)
- P. Thermometer
- Q. Oxigen meter
- R. Salinity meter
- S. pH-meter
- T. Kit for dissection
- U. 10 plastic aquariums of 2L of volume
- V. Pipettes (Finn, Eppendorf)
- W. Tips for pipettes
- X. Styrofoam box
- Y. Deionized water
- Z. Instant Ocean synthetic sea salt (Mentor, OH, USA)
- AA. Ice cubes and ice crusher ("Waring")
- BB. Paper
- CC. Gloves (latex or nitril) (VWR)

3.2.1.2 Chemicals

- A. CdCl_2 (VWR)
- B. ZnCl_2 (VWR)
- C. Liquid nitrogen
- D. TRIZMA-Base (Sigma) or TRIZMA-HCl (Sigma)
- E. HCl

- F. Dithiothreitol (DTT) (Sigma) or β -mercaptoethanol (Freezer -20°C)
- G. Leupeptin (Sigma)
- H. Phenylmethylsulphonylfluoride (PMSF) (Sigma)
- I. Anhydrous ethanol
- J. Bovine Serum Albumin BSA (Sigma P5619)
- K. Bradford reagent (Sigma B6916).
- L. Ammonium chloride, NH_4Cl (Sigma)
- M. Ammonium hydroxide, NH_4OH , puriss.pa. min. ~35% in H_2O (NH_3)
- N. Hexamminecobalt(III) chloride, $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ (Sigma H-7891)
- O. Metallothionein standard, rabbit liver (IKZUS Proteomics MT-95-L). 5 mg/mL; 200 μL
- P. NaCl
- Q. HNO_3
- R. Ethanol (Merck 1.08543 “for molecular biology”)

3.2.2 Animal collection and maintenance

50 Adult pacific oysters, *Crassostrea gigas*, were sampled from Heist Beach, Belgium (**Appendix 3**) and transported to the Laboratory in natural sea water. Experiments were conducted at the Laboratory of Environmental Toxicology and Aquatic Ecology, Ghent University, Belgium.

3.2.3 Set-up and experimental conditions

A Central Composite Design was used to relate toxicity to metal concentrations (Statistica, 1994). Center points are added to the cube design points of a factorial design to allow explicit testing for curvature. In a second-order CCD, the cube is enhanced by a star, these are design points that allow one to estimate the quadratic components of the relationship between the factors and the dependent variable (Lock and Janssen, 2002).

The CCD with two independent variables, which were used for the binary mixtures, consisted of 10 design points: two central points, four cube points and four star points. In the CCDs, metals were tested at five concentrations (**Table 6**). The design points were chosen in such a way that the metals were tested at the EC₅₀ value previously reported for oyster larvae in the laboratory (during the period of October to December) at the higher cube points and at zero concentrations at the lower star points. Because of necessity of a real control one of the central points was omitted and instead an additional point was added where both metals were at zero concentration. Following the design of the CCD the metal concentrations to be added to each aquarium is described in the **Table 7**.

Table 6. Metal concentrations (ug/L) tested in the Central Composite Design with the binary mixtures.

Metal	Design Point				
	-1.41	-1	0	1	1.41
Zinc	0	11	39	66	77
Cadmium	0	124	422	721	845

Table 7. Combinations of metals (Zn and Cd) per aquarium

Aquarium	Concentration of metals ug/L	
	Zn	Cd
Control	0	0
1	0	422
2	11	124
3	11	721
4	39	0
5	39	422
6	39	845
7	66	124
8	66	721
9	77	422

After preparing the concentrations used for the experiment a total of 50 adult individuals (7.5 ± 1.5 cm shell length) were acclimated for 2 days before the experiment began in aquariums containing aerated Instant Sea Water at 13°C and $\text{pH } 7.5 \pm 0.5$; prepared with Instant Ocean synthetic sea salt (Mentor, OH, USA) in deionised water and adjusted to 33.5 ± 0.5 ‰.

Subsequently, the water of the 10 plastic aquariums was changed for 2 L of Artificial Sea Water (**Table 2**. Reconstituted Salt Water) adjusted to 33.5 ± 0.5 ‰, 13°C and $\text{pH } 7.5 \pm 0.5$. A density of 5 animals per aquarium was used. In 9 of these aquariums, the oysters were exposed for 7 days to different metal combinations of Cd and Zn (**Table 7**) using stock solutions of CdCl_2 and ZnCl_2 (500 mg Cd/L and 500 mg Zn/L, respectively). A tenth aquarium contained clean Artificial Sea Water and was used as the Control.

Taking into consideration the analysis developed by Marie et al. (2005) experiments were performed at 13°C , $\text{pH } 7.5 \pm 0.5$, with continuous aeration in order to have dissolved O_2 saturation, and under a 12:12 h light:dark photoperiod. Water in each

tank was renewed every 2 days to maintain contamination pressure throughout the experiment and to avoid changes in the physicochemical parameters. Metal concentrations in water samples of each aquarium collected at the end of the renewal cycle will be measured at the Department of Applied Analytical and Physical Chemistry (Ghent University). Oysters were not fed during the experiment.

Oysters were collected and gills excised on day 7, from 3 of the 5 oysters of each aquarium (**Figure 8**. Soft tissue anatomy of the European flat oyster, *Ostrea edulis*). Excised gills were put individually in eppendorf tubes, frozen in liquid nitrogen and transferred to -80°C freezer for storage. Gills were subsequently analysed for total cytosolic proteins and MT determination as described below.

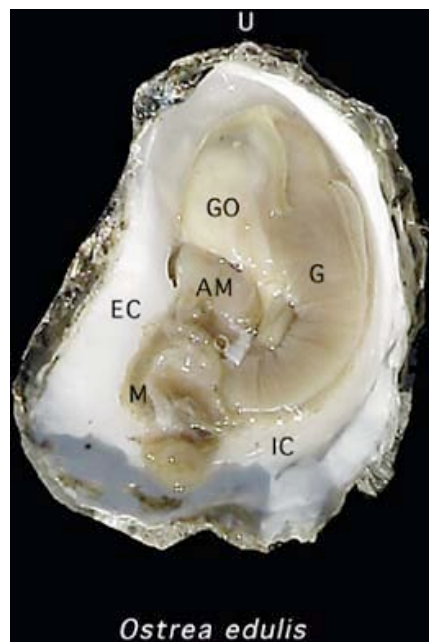


Figure 8. Soft tissue anatomy of the European flat oyster, *Ostrea edulis*. Key: AM - adductor muscle, G – gills, GO – gonad, M - mantle and U - umbo. The inhalant and exhalant chambers of the mantle cavity are identified as IC and EC respectively.

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3.2.4 Preparation of cytosolic fraction and total cytosolic proteins analysis

Principle: Cytosolic fraction is separated by high-speed centrifugation. Total proteins are measured using Bradford reagent. The reaction between the proteins present in the solution and the dye Brilliant Blue G (in Bradford reagent) causes a shift in colour from 465 to 595 nm (blue colour).

3.2.4.1 Solutions

- A. Homogenising buffer 20 mM Tris-HCl/Base pH 8.6 at 4°C (pH 8.1 at 25°C): dissolve 0.48456 g of Tris-Base in 150 mL deionised water, adjust pH to 8.1 (25°C) by adding HCl and fill with deionised water to total volume of 200 mL (glass volumetric flask). Store in Schott Duran flask in Fridge A .
- B. 1 mM dithiothreitol (DTT) (Sigma) (or 0.01% β -mercaptoethanol). Prepare a 10x concentrated stock solution by dissolving 0.01543 g of DTT (Freezer -20°C) in 1 mL of 20 mM Tris-HCl/Base buffer. Store in eppendorf tube in Fridge A.
- C. 0.006 mM leupeptin. Prepare a 200x concentrated stock solution by dissolving 0.005707 g of leupeptin (Sigma) in 5 ml of 20 mM Tris-HCl/Base buffer. Make aliquots of 50 μ L of stock solution in eppendorf tubes and store them at Freezer -20°C.
- D. 0.5 mM phenylmethylsulphonylfouride (PMSF). Prepare a 20x concentrated stock solution by dissolving 0.01742 g of PMSF in 1 ml of anhydrous ethanol. Store in glass tube at Fridge A, 4°C. These have a lifespan of about a couple of weeks.
- E. Homogenization buffer to be used for samples: Take 100 μ L of 1 mM DTT (or 0.01% β -mercaptoethanol), 25 μ L of 0.006 mM leupeptin and 50 μ L of 0.5mM PMSF and add them to 10 ml homogenization buffer. This new solution is only

active for a couple of hours, so it should be made immediately before the homogenization.

- F. Protein standard Bovine Serum Albumin BSA (Sigma P5619). Add 5 mL of deionised water to the vessel (contains 2 mg BSA), resulting in 0.4 mg/mL. Take 200 μ L samples to freeze (-20°C ; see 3.1) in 1 mL eppendorfs for future use. These have a lifespan of about 6 months.
- G. Bradford reagent (Sigma B6916, Fridge A).

3.2.4.2 Methods

A. Samples

Dissect the gills from the oysters (**Figure 9**), weigh them in previously weighed empty eppendorf tubes and store them at -80°C . A weight of 100-150 mg is recommended.

Take the samples (in eppendorf tubes) out of the -80°C freezer and put them in a styrofoam box with crushed ice to thaw slowly. Procedure should be performed at $0-4^{\circ}\text{C}$.

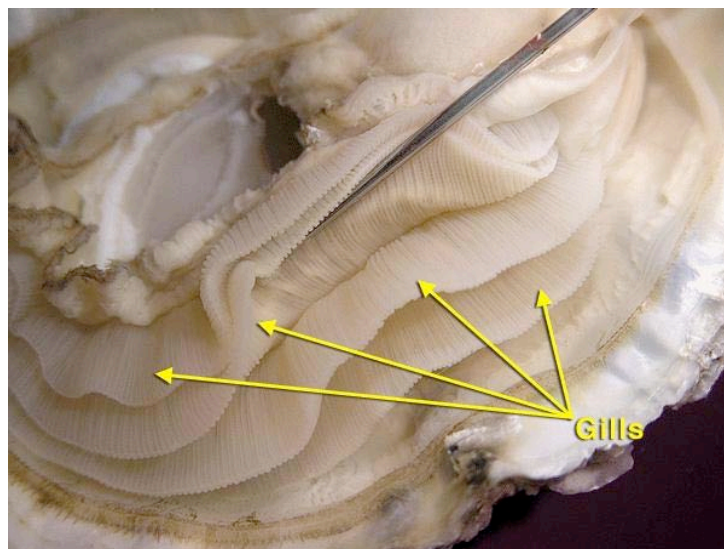


Figure 9. Oyster, *Crassostrea gigas*, gills

Maryland Sea Grant, University of Maryland

(http://www.mdsg.umd.edu/issues/chesapeake/oysters/education/anatlab/lab_i.htm)

B. Cell lysis and homogenization

Contact Professor Van Oostveld or Geert Meesen and make an arrangement for using the ultracentrifuge at the Department of Molecular Biotechnology (Coupure links 653, Ghent University).

From the registered sample weight calculate the volume of homogenization buffer to add. For 100 mg of tissue add 500 μ L of homogenization buffer.

Homogenize the samples using a motorized homogenizer. The duration of homogenization depends on the tissue used, for gill samples count 2 minutes. The eppendorf tube should be immersed in 50 mL polyethylene cups filled with crushed ice. Rinse teflon head with ethanol and deionised water between samples and dry with paper. Homogenization should be performed in the same way for all the samples.

C. Sub-cellular separation

Switch the power and vacuum on of the Beckman ultracentrifuge. Set the temperature at 4°C and the rotation speed should be correlated with the diameter of the rotor to obtain 50,000 g. With the SW55Ti rotor setting the rotation speed at 25,000 RPM will correspond to 60,000 g. Set the centrifugation speed at 60 min. Put the hold off and brake to 800 RPM.

The rotor is cooled in the refrigerator next to the centrifuge. Take it out and wipe the rotor with paper. Transfer the homogenate samples into the centrifugation tubes and place these tubes in the marked metal buckets. Pay attention to the pairs-tubes position that should be in weight-balance (1-4, 2-5 and 3-6). Screw the corresponding cap on the buckets and hang the buckets in their correct position onto the rotor.

After temperature has reached 4°C and vacuum is obtained, turn the vacuum off, put the rotor with the samples in, being careful not to turn or move it in order not to imbalance it. Turn again the vacuum on and after it has been reached push the start button. The RPM will be at 15000 until the vacuum is complete and then it will rise

quickly to 25000 RPM. Make sure it is cooled to 4°C before starting the centrifugation.

When centrifugation is finished turn the vacuum off, take the rotor out and then turn the centrifuge off. Remove the supernatant carefully from the centrifuge tubes (take care not to resuspend the pellet), transfer it over to new labelled Eppendorf tubes of 1.5 ml. The resulting supernatant is the cytosolic fraction.

Three aliquots of 100 µL should be separated for MT analysis and two aliquots of 50 µL for analysis of total cytosolic proteins. Samples of cytosolic fraction should be stored at -80°C until further analysis (avoid repeated thawing and freezing of the samples).

D. Determination of total cytosolic proteins

Standards

Make a standard concentration range of 0.4, 0.2, 0.1, 0.05, 0.025 mg/mL and 3 blanks (deionised water only). Carry out the 1:2 serial dilutions in a 96 multiwell plate: place 50 µL in the first well, remove 25 µL and place it in the next well. Add 25 µL of deionised water to this last one, mix well by pipetting up and down and repeat the procedure. Remove 25 µL from the final dilution to end up with 25 µL. Make 2 sets of standards and 3 blanks.

Standards and samples

Take the samples of total cytosolic proteins (eppendorf tubes of 50 µL) out of the -80°C freezer and put them in a styrofoam box with crushed ice to thaw slowly.

Take 3 replicates of 25 µL from each sample and place in the 96 multiwell plate. Depending on the amount of tissue used, it might be necessary to dilute the samples at this point. For adult *C. gigas* samples, it is necessary to dilute 10 µL of sample in

15 μ L of deionised water; making a total volume of 25 μ L. Samples should be vortexed before placing them in the multiwell plate.

Pour the approximate amount of Bradford reagent needed for the analysis (250 μ L x number of wells to be filled) in a glass beaker (if using a normal pipette) or a special reservoir to use with the multtip pipette. This step is performed in order to avoid contamination of the Bradford reagent in the original bottle.

Add 250 μ L of Bradford reagent (normal pipette or multtip pipette) to each well. Do it quickly but avoid contact between the pipette and the samples in the multiwell plate.

Incubate the plate in the dark (e.g. in a drawer) for 15 minutes at room temperature.

In the meanwhile start the spectrometer and computer:

Switch on the plate reader

Switch on the computer

Select Ascent software

Select: Session – open

Select Bradford

Select Measure

Select folder to save results, e.g. choose the date as a file name

Press START

This program will shake the plate for 5 seconds and then measure the optical density at 595 nm.

Check if the optical density of the samples falls within the range of the standards. If not, repeat this protocol with diluted samples. Remainders of the samples can be stored at -80°C for future use.

Use the results of the standards to calculate the regression line between standard concentration (mg/ml on X-axis) and measured absorbance (on Y-axis). Use this regression to calculate mg/ml or mg/L in the sample according to the absorbances measured. Take into account possible dilution steps.

E. Finishing the test

Pour the content of the multi-well plate in a recipient and discharge it in the correct waste container ('Waterige chemische oplossingen'). The plate itself has to be put in the container with contaminated waste.

3.2.5 Quantification of metallothionein using differential pulse voltammetry (DPV)

This is an electrochemical method for quantification of metallothioneins. The electrochemical response is not specific to a certain type of protein, but to a certain type of functional groups, i.e. SH-groups, which are common to different proteins.

3.2.5.1 Reagents and solutions

- A. Stock solution will last for ~6 months if stored at 4°C, but it is preferred to prepare it each time the assay is run with a new set of samples. The quantity is dependent on the number of samples (**Table 8**). The same solutions for the whole set of samples should be used in order to minimize the possibility of errors.
- B. Solution A: 2 M ammonium chloride + 2 M ammonium hydroxide. Dissolve the required quantity of NH₄Cl in distilled water and add the required volume of concentrated NH₄OH, min 35% and complete to the desired volume in a volumetric flask (all in one flask). *Work in a protected area with ventilation, use gloves and wear protection.*
- C. Solution B: 1.2 mM hexamminecobalt chloride. Dissolve the required quantity of Co(NH₃)₆Cl₃ in distilled water; complete to desired volume in a volumetric flask. *(Avoid light contact storing it in a dark bottle or cover the bottle with aluminium foil).*

Table 8. Volumes of 2 M NH_4Cl + NH_4OH and of $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ to be diluted for preparing solutions A and B.

Solutions for Brdicka reaction	Solution A		Solution B
	NH_4Cl (g)	NH_4OH (mL)	$\text{Co}(\text{NH}_3)_6\text{Cl}_3$ (mg)
for 0.1 L	10.698	15.0	32.10
for 0.2 L	21.396	30.0	64.20
for 0.25 L	26.745	37.5	80.25
for 0.3 L	32.094	45.0	96.30
for 0.4 L	42.792	60.0	128.40
for 0.5 L	53.490	75.0	160.50

Equal volumes of each of these two solutions should be mixed in a voltammetric cell immediately before measurement. Always add the solution A (ammonium buffer) first, and then the solution B (cobalt[III] solution). The final concentrations in the cell are: 1M NH_4Cl + 1M NH_4OH , and 0.6 mM $\text{Co}(\text{NH}_3)_6\text{Cl}_3$.

- D. 0.9% NaCl: Dissolve 0.9 g NaCl in 100 mL De- H_2O
- E. Diluted HNO_3 : Dilute 15 mL of concentrated HNO_3 in 250 mL of deionised water. First add water into the bottle and afterwards the acid. Be careful with concentrated acid. *Use gloves, wear protection and work in the hood, put the ventilation on maximum before you start.*
- F. Metallothionein standard, rabbit liver, IKZUS Proteomics MT-95-L (5 mg/mL; 200 μL). Store in a fridge for maximum \pm 1 year.

3.2.5.2 Methods

A. *Sample preparation (heat treatment)*

Cytosol tissue prepared as described in the “Preparation of cytosolic fraction” is used for MT analysis. Take the samples for MT analysis (three replicates of an aliquot of 100 μ L in eppendorf tubes) out of the -80°C freezer and put them in a styrofoam box with crushed ice to thaw slowly.

Turn on the heater on the water-bath; adjust the temperature to 85°C . Keep in mind it takes ~ 30 minutes to reach the temperature wanted.

Dilute the cytosolic fraction 10 times with 0.9% NaCl solution: 100 μ L of cytosolic fraction + 900 μ L 0.9% NaCl; and vortex the samples for ~ 30 -60 seconds.

Denature the cytosol at 85°C for 10 minutes, transfer immediately on ice and leave it in a refrigerator (at 4°C) for 30 minutes.

Centrifuge the samples at $10,000\times g$ (10,080 RPM on Hettich Microcentrifuge “Mikro 200R”) for 30 min at 4°C , keep in mind to start cooling the centrifuge ~ 5 -10 minutes before use. Transfer the supernatant, as much as will be used later, approximately 500 μ L is enough, to a new marked Eppendorf tube. The resulting supernatant is the metallothionein-rich fraction. Samples should be stored at -80°C until further analysis.

B. *General requirements when using 797 VA Computrace*

The Hardware Manual 8.797.1023 (Metrohm Ion Analysis 07.2005) of the Metrohm machine used, define the 797 VA Computrace like a PC controlled system for voltammetry that is composed by three parts mainly: VA Computrace Stand with accessories, Connecting Cable and 797 VA Computrace Software 1.2. The operation of the 797 VA Computrace Stand follows the potentiostatic 3-electrode principle in which the voltage of the working electrode is controlled by means of a virtually

currentless reference electrode to the preset desired value and the current flows across a separate auxiliary electrode (**Appendix 4**)

797 VA Computrace Software 1.2 can be used in two different operating modes: the exploratory mode for qualitative analysis and the determination mode for quantitative analysis of inorganic or organic substances. In the last one calibration can be done via standard addition or calibration curves; signal evaluation and concentration calculation are automatic; and all voltammograms and calibration curves plus the results can be transferred to other Windows applications or exported in ASCII format and also with measurement reports, can be compiled and printed out if desire.

Every time before starting a new calibration curve or samples analysis we may follow all the parameters and steps that this section is going to describe. The instrument operating parameters are:

Switch on the computer

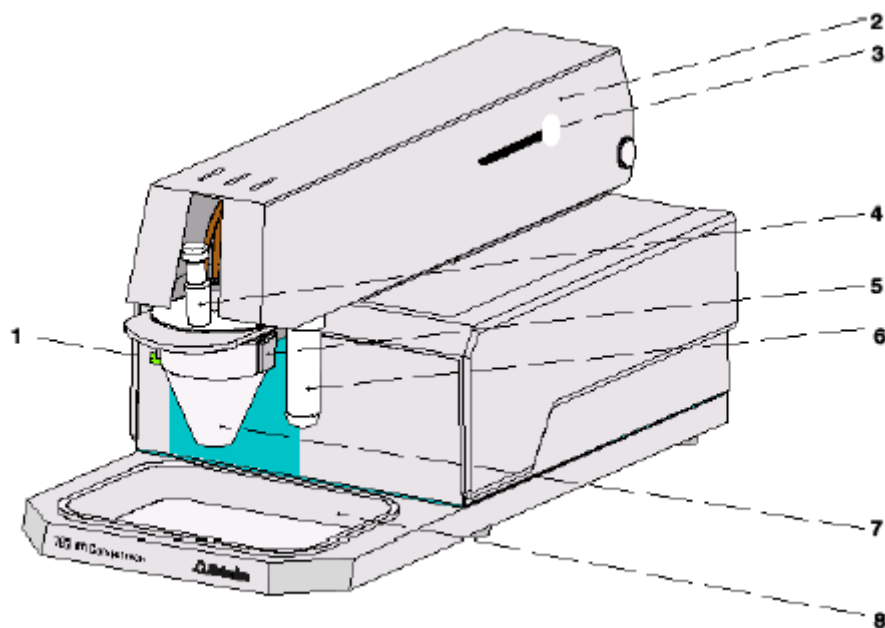
Switch on the Methrom machine

Switch on the circulation system. Temperature required is 20°C, if necessary turn on the heater or cooling.

Clean the electrode and the measuring vessel (**Figure 10**. Front of the 797 VA Computrace Stand) with diluted HNO₃, then with deionised water and finally wipe it dry with paper.

Select 797 VA Computrace (Metrohm) 1. 2 software

Select Start measurements



1 Mains pilot lamp lit up when instrument switched on	6 Gas wash bottle (6.2405.030) for inert gas supply (filling with dist. water, see section 2.2.5)
2 Cover of measuring head arm hinged	7 Measuring vessel when measuring head arm is fully raised, the measuring vessel can be pulled forward out of the holder 5
3 Release slide to release fixture of the lifted measuring head arm	8 Drip pan (6.2711.040)
4 Stopper (6.2709.080) to close the pipetting opening	
5 Holder for measuring vessel	

Figure 10. Front of the 797 VA Computrace Stand

Every time when starting to work with a new set of samples, we should change the solution in the Reference electrode and perform a mercury drop test. Verify that the compartment Ag/AgCl Filling is wet (**Figure11**), if this is not the case replace the Reference System for the spare part or call the maintenance manager of the machine. If it is still wet but there is no solution in the Electrolyte Compartment with Internal Electrolyte, fill it with 3 M KCl solution. Secondly renew the solution of 1 M ammonia buffer contained in the Electrolyte Vessel and introduce on it the Reference System. Take into consideration that the diaphragm must be submerged in the ammonia. And by last see that the Electrode Cable RE is well connected (**Appendix 5**).

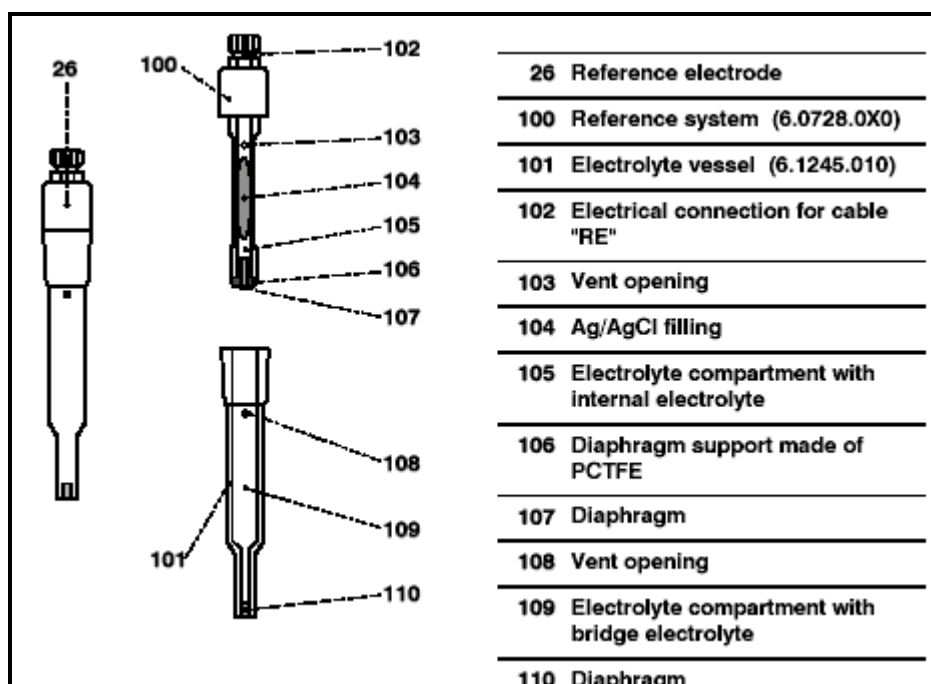


Figure 11. Compartments of the reference electrode

Prepare solutions A and B or take them out of the fridge. They should be at room temperature, $\sim 20^{\circ}\text{C}$, before working. Fill the solutions A and B into the voltammetric cell, also called measuring vessel (**Figure 10**. Front of the 797 VA Computrace Stand). The volume of electrolyte depends on the cell volume (the electrodes should be well immersed in the electrolyte). For this case the cell volume is 10 mL (5 ml of Solution A + 5 ml of Solution B).

Test the mercury drop in order to see if the system is working properly, all mercury drops must have the same size and dimensions. Test the system by selecting in the tool bar the following:

Utility

Computrace control

Parameters:

- Potencial (V): 0.152
- Current (mA): 0.0000
- 100 μA is selected
- HMDE

- RDE/Stirrer speed (rpm): 2000
- Drop Size: 4

Purge, Stirrer/RDE, New drop or Electron test

Close

The program part "Exploratory" is designed for qualitative voltammetric analysis. It shows, record and displays the voltammograms associated with the determined parameters defined in the exploratory specifications.

Switch to Exploratory Mode

Select Exploratory Specifications

The following settings must be reported (**Figure 12. Exploratory Specifications**):

- Mode: DP - Differential Pulse
- Electrode: HMDE (Hanging Mercury Drop Electrode)
- Hg drop size: drop size 4
- Stirrer/RDE (rpm): 1000
- Voltammetric analysis differential pulse, Initial purge time (s): 10
- Start potential (V): -0.9 V
- End potential (V): -1.65 V
- Pulse amplitude (V): 0.02502 V
- Pulse time (s): 0.057 s
- Voltage step (V): 0.002594 V
- Voltage step time (s): 0.2 s
- Sweep rate (V/s): 0.0130 V s⁻¹
- Cell off after measurement: selected (✓)
- Potentiostat: Highest current range 10mA and Lowest current range 100nA
- All conditioning cycles and Pretreatment values leave it on zero (0).
- MT-peak potential (V): from -1.46V to -1.50V
- Electrolyte concentration: 1M NH₄Cl + 1M NH₄OH, and 0.6 mM Co(NH₃)₆Cl₃
- Electrolyte temperature: 20°C

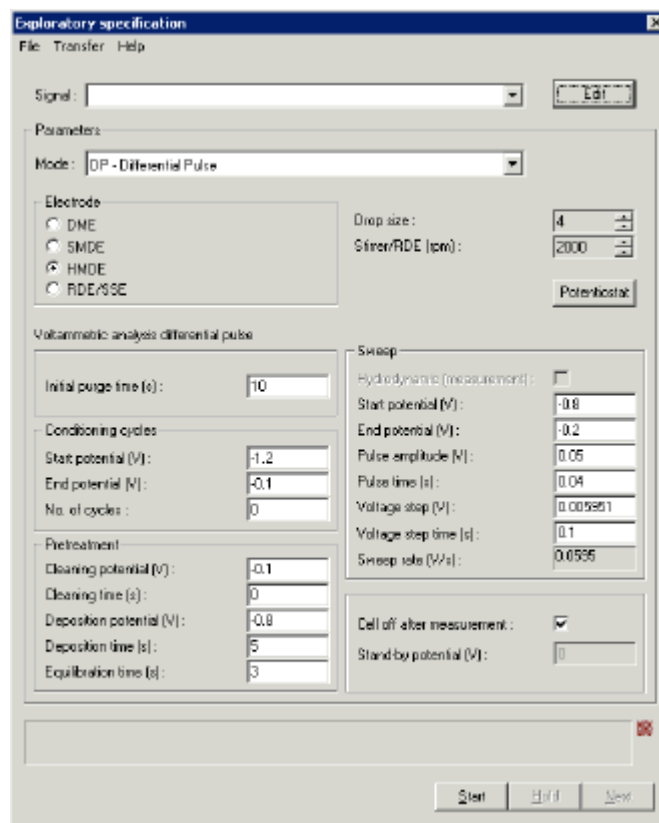


Figure 12. Exploratory specifications

After having all the parameters recorded, press start. When finishing the display of the exploratory curves automatic save will be asked, press OK. The automatic save will be take place to C:\Program Files\Metrohm\797 VA Computrace\ Data.

Example: 797 VA Computrace

Automatic save: '08091714_Signal0001.sig'

Select OK

Peaks of the measured curves (**Figure 13**) can be evaluated automatically or manually, the second one after setting the baseline range of the U(V), approximately Begin: -1.09 or -1.10 and End: -1.38 or -1.39. Tracing the curves with a cursor allows the measured current and voltage values to be accessed. The steps are mentioned as:

Select Signal,

Select Peak search

Select Automatic or manual option, when using the last one define begin and end of the baseline.

Select Close

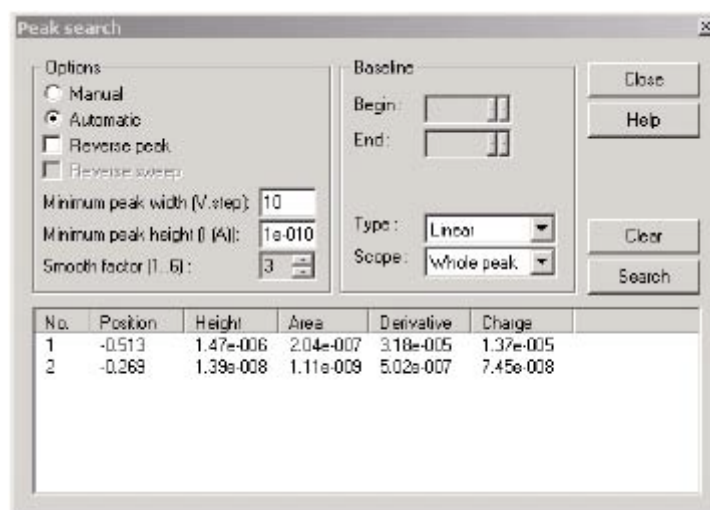


Figure 13. Peak search window

C. Standard and calibration curve construction

For preparing 1 mg/mL solution of Rabbit liver metallothionein standard (IKZUS Proteomics MT-95L) dilute 20 μ L MT-95-L (5 mg/ml solution of rabbit liver MT-95-L) with 80 μ L 5 mM 20 mM Tris (pH 8.1). Make 20 μ L aliquots in eppendorf tubes and store at -80°C for using them when required.

Take out of the -80°C freezer one aliquot of 20 μ L (solution 1 mg/mL MT), immediately prior to calibration measurement. Dilute it in the following way: 20 μ L MT-95-L (1 mg/mL) + 1000 μ L deionised water. Final concentration of the rabbit liver MT standard solution is 0.0196 mg/mL. Use this solution for 4-5 days (keep it in a fridge at 4°C).

Follow all steps mentioned already in the previous section. After finishing with the Exploratory Mode switch to Determination Mode.

Select Working Method Specifications (**Figure 14**)

- Method: Method for MT determination.mth
- Title: Determination of metallothionein

- Remark 1: 5 ml ammonia buffer + 5 ml Co (NH₃)₆Cl₃ (pH=9.5)
- Mode: DP – Differential Pulse
- Calibration: Record calibration curve
- Technique: Batch
- Addition: Manual
- Electrode: HMDE
- Drop size: 4
- Stirrer/RDE (rpm): 800

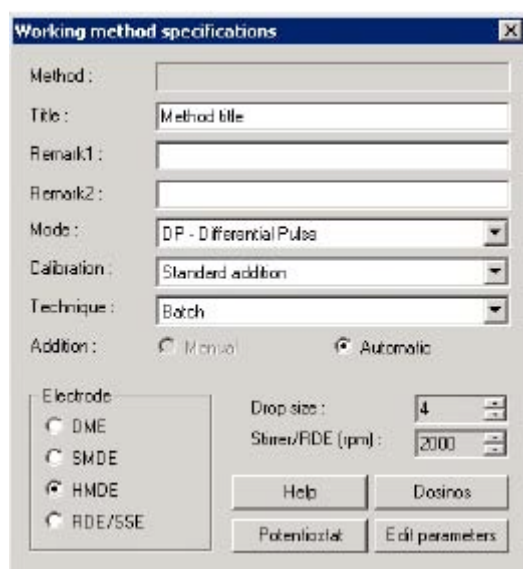


Figure 14. Working method specifications

The following parameters must be reported:

Determination:

- Sample identifier: (*Name of the sample to be measure*)
- Cell volume (ml): 10 ml
- Addition purge time (s): 10
- Standard additions: 6
- No. Of replications: 4

Voltammetric:

- Initial purge time (s): 10
- Start potential (V): -0.9 V

- End potential (V): -1.65 V
- Pulse amplitude (V): 0.02502 V
- Pulse time (s): 0.057 s
- Voltage step (V): 0.002594 V
- Voltage step time (s): 0.2 s
- Sweep rate (V/s): 0.0130 V s⁻¹
- Cell off after measurement: selected (√)

Substances:

- Substance: MT selected (√)
- Peak pos.: -1.46
- +/- (V): 0.04
- Standard solution No.: 1
- Standard solution Conc.: 0.0196
- Standard solution Unit: mg/mL
- Volume (mL): 0.01
- Regression technique: Linear Regression
- Peak evaluation: Height
- Smooth factor (1..6): 4
- Minimum peak width (V.steps): 5
- Minimum peak height (A): 1e-010
- Eliminate spikes: selected (√)

Calculations:

- Substance: MT
- Name: MT
- Formula: (1000/1) + 0 - 0
- Unit: mg/L
- Used: yes

Documentation

- Save final results as ASCII file: selected (√)

Path: MT staal 1 (Browse for folder liked or preferred)

Select "OK"

Use rabbit liver MT standard to create the calibration curve, which should serve to quantify the MT concentration in a sample. The calibration range should cover the

concentrations from about 39 µg/L MT to about 97 µg/L MT. Construct the calibration curve (relationship should be linear, $y=ax+b$) with the following standard MT additions in the voltammetric cell.

$$1^{\text{st}} \text{ addition: } (10 \cdot 10^{-6} \text{ L} \times 0.0196 \text{ g/L}) / 10.01 \cdot 10^{-3} \text{ L} = 19.58 \text{ } \mu\text{g/L}$$

$$2^{\text{nd}} \text{ addition: } (20 \cdot 10^{-6} \text{ L} \times 0.0196 \text{ g/L}) / 10.02 \cdot 10^{-3} \text{ L} = 39.12 \text{ } \mu\text{g/L}$$

$$3^{\text{rd}} \text{ addition: } (30 \cdot 10^{-6} \text{ L} \times 0.0196 \text{ g/L}) / 10.03 \cdot 10^{-3} \text{ L} = 58.62 \text{ } \mu\text{g/L}$$

$$4^{\text{th}} \text{ addition: } (40 \cdot 10^{-6} \text{ L} \times 0.0196 \text{ g/L}) / 10.04 \cdot 10^{-3} \text{ L} = 78.09 \text{ } \mu\text{g/L}$$

$$5^{\text{th}} \text{ addition: } (50 \cdot 10^{-6} \text{ L} \times 0.0196 \text{ g/L}) / 10.05 \cdot 10^{-3} \text{ L} = 97.51 \text{ } \mu\text{g/L}$$

$$6^{\text{th}} \text{ addition: } (60 \cdot 10^{-6} \text{ L} \times 0.0196 \text{ g/L}) / 10.06 \cdot 10^{-3} \text{ L} = 116.90 \text{ } \mu\text{g/L}$$

To start constructing the calibration curve select Monitor and press START. It will be asked to add the sample, in each addition 10 µl of MT standard is added to the cell. After scanning End of Determination will appear and automatic save will be asked, accept it selecting OK. The result will be saved in the format predetermined before in the MT staal 1 file. The access to this file can be found in the desktop, the file is called "Shortcut to Data" and its location is: C:\Program Files\Metrohm\797 VA Computrace. The curves can be shown selecting Determination curves bottom and the results selecting Results.

Discharge the content of the measuring vessel, Buffer + Cobalt + Mercury solution, in the correct waste container (Waste Ammonium buffer + Co (NH₃)₆Cl₃ bottle, when full discharge this in Waste Metals). Clean the electrode and the measuring vessel with diluted HNO₃, then with deionised water and finally wipe it dry with paper.

D. Measurement of MT content

The voltammetric instrumentation will vary between laboratories; to detect possible variation due to instrumentation it is advantageous to get all the instrumentation parameters reported.

Follow all the steps mentioned already in the previous section **3.2.5.2. (B)**. To measure MT content in a sample add 60 µL of heat-treated and diluted 10x cytosolic

fraction of oyster gills, purge with nitrogen for 10 sec and start to scan using differential pulse mode. The instrument operating parameters on the Determination Mode are:

Select Working Method Specifications

- Method: Method for MT determination.mth
- Title: Determination of metallothionein
- Remark 1: 5 ml ammonia buffer + 5 ml Co (NH₃)₆ Cl₃ (ph=9.5)
- Mode: DP – Differential Pulse
- Calibration: Sample with calibration curve
- Technique: Batch
- Addition: Manual
- Electrode: HMDE
- Drop size: 4
- Stirrer/RDE (rpm): 800

The following parameters must be reported:

Determination:

- Sample identifier: *(Name of the sample to be measure)*
- Sample amount:, 0.06 ml (oyster's gills)
- Cell volume (ml): 10 ml
- Calibration curve: *Name of the calibration curve to be used*
- No. Of replications: 4

Voltammetric:

- Initial purge time (s): 10
- Start potential (V): -0.9 V
- End potential (V): -1.65 V
- Pulse amplitude (V): 0.02502 V
- Pulse time (s): 0.057 s
- Voltage step (V): 0.002594 V
- Voltage step time (s): 0.2 s
- Sweep rate (V/s): 0,0130 V s⁻¹
- Cell off after measurement: selected (√)

Substances:

- Substance: MT selected (✓)
- Peak pos.: -1.46
- +/- (V): 0.04
- Regression technique: Linear Regression
- Peak evaluation: Height
- Smooth factor (1..6): 4
- Minimum peak width (V.steps): 5
- Minimum peak height (A): 1e-011
- Eliminate spikes: selected (✓)

Calculations:

- Substance: MT
- Name: MT
- Formula: (1000/1) + 0 - 0
- Unit: mg/L
- Used: yes

Documentation:

- Save final results as ASCII file: selected (✓)
- Path: MT staal 1 (Browse for folder liked or preferred)

Select "OK"

To start measurement of MT select Monitor and press START. It will be asked to add the sample. 60 µl of sample is added to the cell. After scanning the software will tell you End of Determination and automatic save will be asked, accept it selecting OK. The result will be saved in the format predetermined before in the MT staal 1 file. The access to this file can be found in the desktop, the file is called "Shortcut to Data" and its location is: C:\Program Files\Metrohm\797 VA Computrace. The curves can be shown selecting Determination curves bottom and the results selecting Results.

Discharge the content of the measuring vessel, Buffer + Cobalt + Mercury solution, in the correct waste container (Waste Ammonium buffer + Co (NH₃)₆Cl₃ bottle, when full discharge this in Waste Metals). Clean the electrode and the measuring vessel with diluted HNO₃, then with deionised water and finally wipe it dry with paper. Add again 5 ml of Solution A and 5 ml of Solution B, for measuring the next sample. Follow the

procedure described in the section **3.2.5.2 (B)**, for testing the mercury drop and for using the Exploratory Mode. Repeat the procedure described in this section above for the Determination Mode and add 60 µl of the new sample in the cell. Follow the next steps for the samples further.

When starting to measure the samples, after every 10 samples (approximately), put standard MT as an unknown sample and check if you obtain expected value (5-10% lower or higher than expected value are acceptable). In this way we check the validity of the calibration curve, it is important to have calibration under control.

When finish working: discharge the content of the measuring vessel, Buffer + Cobalt + Mercury solution, in the correct waste container (Waste Ammonium buffer + Co (NH₃)₆Cl₃ bottle, when the bottle is full discharge this in Waste Metals), and clean the electrode and the measuring vessel with diluted HNO₃, then with deionised water and finally wipe it dry with paper. Add deionised water and some drops of HNO₃ in the measuring vessel. Turn off the heater or the cooler, the circulation system, the software, the Methrom machine and then the computer.

3.2.6 Determination of MT concentration

MT-concentrations given by 797 VA Computrace calculated from the samples using the calibration curve constructed with rabbit liver is automatically given as mg MT/L.

The units should be changed into µg MT/L. Total cytosolic proteins calculated as described in section **3.2.4.2 (D)** is given in mg Total Cytosolic Proteins/ mL, convert them in mg Total Cytosolic Proteins/L.

After doing the calculations mentioned above, the MT concentration should be reported as µg MT/ mg total cytosolic protein.

3.2.7 Statistical Analysis

All analyses were done with the software package Statistica™ (Statsoft, Tulsa, OK, USA). Significant differences of MT concentrations (mg Mt/mg total protein) between control and Cd - Zn metal mixtures concentrations were determined with the nonparametric Kruskal-Wallis ANOVA and the option Comparing multi-independent samples (groups). If it is significant, Mann–Whitney *U* test (comparing two independent samples) must be used to determine which two groups are significant from each other, starting with comparing all groups with the control. For both methods results were considered statistically significant at $p < 0.05$.

The surface response relationship between the concentration of mg MT/mg Total Cytosolic Protein and the Cd - Zn metal mixtures was obtained with the analysis of the Central Composite Design (CCD). The Model follows the equation format:

$$\text{MT (mg MT/ mg protein)} = \text{intercept} + a * x + b * x^2 + c * y + d * y^2 + e * x * y$$

With $x = \text{metal 1}$ and $y = \text{metal 2}$ (follow the same equation as for embryo test)

Non-significant terms should not be included in the final surface response equation.

4. RESULTS

4.1 Oyster Embryo Test

4.1.1 Bioassay procedure

The assay was performed immediately after arrival of the oysters by DHL from Guernsey Sea Farms (Park Lane, Vale, Guernsey, UK). Ten adults in reproductive stage of the oyster *Crassostrea gigas*, 5 females and 5 males, were placed together in glass chambers that contains Artificial Sea Water at 28°C for a period of 30 minutes, in order to induce them to spawn. After this period they were transferred to

others chambers with the same type of water but adjusted at 18°C, for the same period of time. This thermal shock was repeated four times.

When oysters began to spawn the quality of gametes was checked under the microscope. Males were the first ones to spawn, giving good quality of sperm with a lot of motility; while the eggs of females were not mature as wanted; some were not completely rounded with regular margins at the end of 30 minutes. This is the reason why we waited 15 minutes more before performing the fertilization. After this time, the quality of the eggs was checked again and the number of eggs per ml counted. The aliquot of egg suspension taken for analysis contained less than 100% rounded eggs and other ones with deformed shape or exploded, nevertheless the concentration reached 60 zygotes/ ml appropriate for performing the fertilization.

With both gametes ready fertilization was performed. However, after 20 minutes 100% of fertilization has not taken place. 5 ml more of the sperm cell suspension was added and 20 minutes more were waited, in order to have a larger percentage of success. After confirmation under the microscope that the majority were fertilized eggs, 0.4ml of solution were incubated in each well of a 24 multiwell plate of 3 ml of volume previously filled with 25 different metal mixtures combination by three replicates. Multiwell plates were incubated at a 24°C thermostatic chamber for 24 hours. Then 10 µl of 37% formaldehyde was added and the plates were stored at 4°C until the reading of the plates is done.

4.1.2 Reading of Results under Microscope

Each well-plate was read under 100x magnification of an inverted microscope. The number of larvae per well was too low (i.e. below 100). Therefore the complete well was covered in the counting. After counting the number of D-Shape, abnormal (AB) and not developed (ND), the percentage of embryos that did not result in live larvae with completely developed shells per replicate was calculated ($A = AB + ND$). The average and standard deviation of the three replicates was calculated as well as E values (section 2.1.6). Results are shown in the **Table 9**. E values represent the effect percentage for each treatment adjusted for control mortality.

According to the ASTM protocol (1995) and seeing the results obtained we can conclude this test is actually not valid for further use of data. This is because it did not follow one of the most important requirements for the acceptability of the test. It is said that an acute toxicity test having less than 70% of oyster embryos introduced into a required control treatment resulted in live larvae with completely developed shells at the end of the test should usually be considered unacceptable. Our results present only 50.1% of oyster embryos resulted in live larvae with completely developed shells at the end of the test and 49.9 % oyster embryos that did not result in live larvae with completely developed shells. Although the inconsistent results obtained, E values, EC50s and a surface response curve were calculated following the methodology described in section 3.1.6.

Table 9. Metal mixtures with % of embryos that did not result in live larvae with completely developed shells adjusted for the controls (E).

Treatment	Metal		A% in 1R	A% in 2R	A% in 3R	A % Average	SD	E (%)
	Concentration							
	Cd (ug/l)	Zn (ug/l)						
1	0	0	48.8	49.5	51.5	49.9	1.4	-
2	0	11	52	68	69	63	9.6	26.1
3	0	39	100	100	98.6	99.5	0.8	99.1
4	0	66	100	100	100	100	0	100
5	0	77	99	100	100	99.6	0.7	99.2
6	124	0	57.8	62.8	71	63.9	6.7	27.9
7	124	11	86.6	90.4	80.5	85.8	5.0	71.7
8	124	39	100	100	100	100	0	100
9	124	66	100	100	100	100	0	100
10	124	77	100	100	100	100	0	100
11	422	0	56	62	55.7	57.9	3.6	16
12	422	11	87	80	84.7	83.9	3.6	67.8
13	422	39	100	100	100	100	0	100
14	422	66	100	100	100	100	0	100
15	422	77	100	100	100	100	0	100
16	721	0	64.1	67.5	75.3	69	5.8	38
17	721	11	96.8	91.6	87.2	91.8	4.8	83.7
18	721	39	100	100	100	100	0	100
19	721	66	100	100	100	100	0	100
20	721	77	100	100	100	100	0	100
21	845	0	72.6	75.9	71.1	73.2	2.5	46.5
22	845	11	100	100	100	100	0	100
23	845	39	100	100	100	100	0	100
24	845	66	100	100	100	100	0	100
25	845	77	100	100	100	100	0	100

A% = Percentage of embryos that did not result in live larvae with completely developed shells, percentage of the sum of AB (abnormal) and ND (not developed) embryos (2.1.6)

1R, 2R and 3R = First, second and third replicates respectively

E = Percentage of introduced live embryos that did not result in live larvae with completely developed shells adjusted for the controls (2.1.6)

Despite the fact that the test should be considered unacceptable, the data shows an increase of affected embryos in their development while concentrations of metals are increasing. Individual values for Cd and Zn also give the same tendency, the percentage of altered development embryos increase when metal concentrations are raised. The **Table 10** shows us that an EC 50 can be calculated for Zn but not for Cd, due to the fact that E% never above 50% for Cd.

Table 10. Single metal exposures with % of live embryos that did not result in live larvae with completely developed shells adjusted for the controls (E).

Metal	[] µg/l	E (%)
Zn	11	26.1
	39	99.1
	66	100
	77	99.2
Cd	124	27.9
	422	16
	721	38
	845	46.5

4.1.3 Statistical Analysis

The Trimmed Spearman-Kärber Method was used for calculating the 24hEC50 of single metals based on E(%). From Table 10 it can already be observed that single Cd concentrations were not high enough to result in > 50% effect. Therefore an EC50 for Cd could not be calculated. The calculated EC50 for Zn and 95 % confidence interval was 16.7 (15.1-18.5) µg/ L.

The combined effect of Cd on Zn on oyster larvae development (E(%)) could be analysed using a non linear estimation following the equation previously mentioned in 3.1.6 (2):

$$E (\%)=a* [Cd] + b * [Zn] + c * [Cd]^2 + d * [Zn]^2 + e * [Cd] * [Zn] + g \quad (1)$$

The statistical analysis of our data show R = 0.86 and a proportion of variance accounted for 0.74 in a 95.0% (alpha=0.050) level of confidence.

By analyzing the significance of the factors (Table 11), it was showed that all of them except for the factor e were significantly contributing to the observed response. The factor Cd * Zn (factor e) is not important and was therefore eliminated of the final equation (3).

Table 11. Significantly of factors in the Non Linear Model used.

	Estimate	Standard	t-value	p-level	Lo. Conf	Up. Conf
a	-0.15269	0.054398	-2.80688	0.011663	-0.26697	-0.0384
b	0.33019	0.06481	5.09476	0.000076	0.19403	0.46635
c	0.00017	0.000068	2.54495	0.020313	0.00003	0.00032
d	-0.00037	0.000086	-4.26557	0.000465	-0.00054	-0.00019
e	0.00001	0.000055	0.12127	0.904822	-0.00011	0.00012
g	59.34332	8.44406	7.02782	0.000001	41.603	77.08363

$$E (\%)= -0.15269 * [Cd] + 0.33019 * [Zn] + 0.00017 * [Cd]^2 -0.00037) * [Zn]^2 + 59.34332 \quad (2)$$

The surface response relationship between the two metals (using Equation 1 in Non Linear Estimations in Statistica software) is shown in Figure 15.

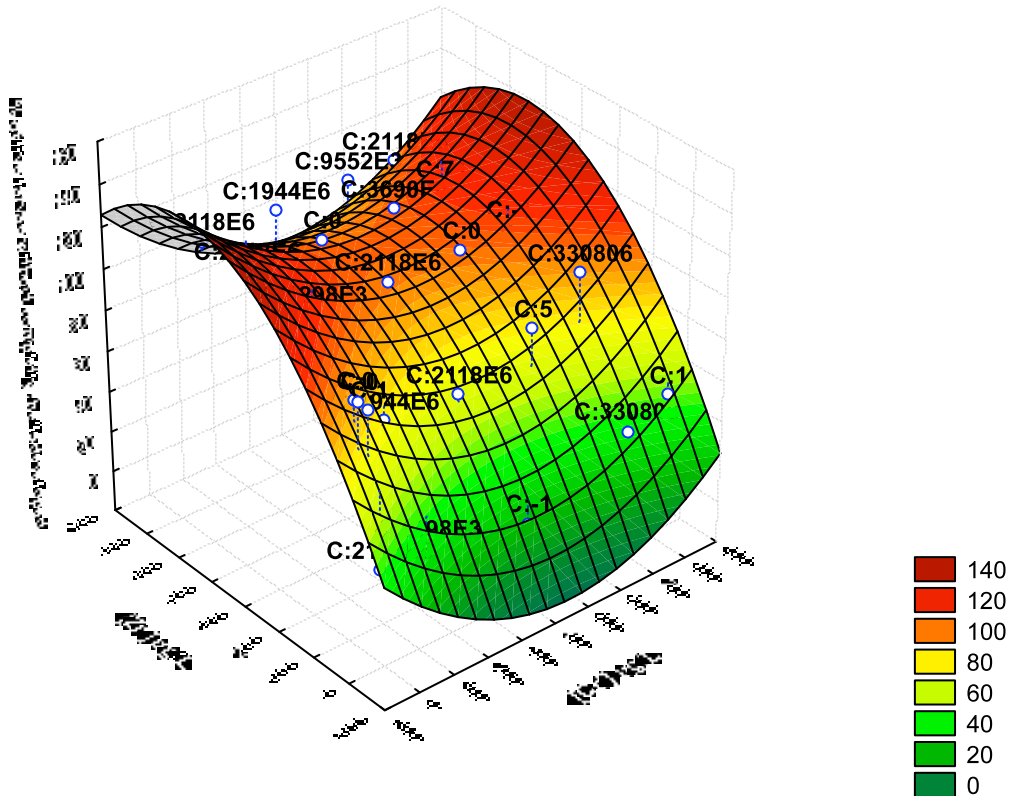


Figure 15. Surface response relationships between the two metals, Zn and Cd, and the % Effect on larval development.

4.2 Quantification of Metallothionein (MT) from *Crassostrea gigas* (Japanese oyster)

4.2.1 Determination of MT concentration

Fifty adult individuals (7.5 ± 1.5 cm shell length) were acclimated for 2 days in aerated Instant Sea Water of 33.5 ± 0.5 ‰, 13°C and $\text{pH } 7.5 \pm 0.5$. Afterwards they were exposed for 7 days in 10 plastic aquariums with different metal combinations of Cd and Zn dissolved in 2L of Artificial Sea Water adjusted to 33.5 ± 0.5 ‰, 13°C and $\text{pH } 7.5 \pm 0.5$. Thirty of them (3 of the 5 animals per aquarium) were selected taking into consideration similar size (Table 12) to avoid physiological differences. To facilitate data handling the 3 oyster sampled per aquarium were called A, B and C.

Table. 12 Length and Weight of Oysters samples per aquarium

Aquarium	Oyster Sample					
	A		B		C	
	Length (cm)	Weight (gr)	Length (cm)	Weight (gr)	Length (cm)	Weight (gr)
Control	10.2	65.5	8	79.58	8	49.23
1	8	47.45	7.8	42.78	7.6	55.4
2	8.5	73.8	9	64.34	8.5	63.27
3	7.8	100.15	7	54.37	6	44.06
4	7.8	94.39	7.5	64.69	8.2	38.28
5	7.9	86.35	7	61.63	6.6	49.27
6	6.9	79.74	8.5	68.14	6.5	33.45
7	7.5	55.74	7	40.48	6.4	29.79
8	8	71.78	7.5	69.07	6.5	49.55
9	7.9	56.8	6.5	36.58	7	47.58

Immediately after finishing recording the length and weight of the 30 samples their gills were excised, transferred individually in eppendorf tubes and stored at -80°C until future analysis. After performing all the procedure for subcellular separation, total cytosolic proteins analysis starts with the building of a standard curve. Standard concentrations in a range of 0.4, 0.2, 0.1, 0.05, 0.025 mg/mL and 3 blanks (deionised water only) were made and read by spectrometer and computer. Results of standards gave a regression line shown in equation 1 with a $R^2 = 0.9758$, where measured absorbance was Y and concentration of protein X. Because a dilution of 2.5 times was made for the cytosolic fraction to analyse, X values had to be multiplied by this factor in order to give real values in mg/ml. Total cytosolic proteins results, repeated twice per sample, can be seen in Table 13 and means of these 2 readings per sample in Table 14.

$$Y = 1.3133 X + 0.3238 \quad \dots\dots\dots (1)$$

For the total cytosolic proteins results we can see that the sample 3A is the one that has the highest concentration and coincidentally is the oyster with more weight. The lowest value of cytosolic protein is present in 8C and again is one of the oysters with

lowest weight and length, nevertheless the sample 3C, the one with the lowest weight and length, presents the second highest cytosolic protein concentration showing that the weight is not a determinate factor to take into account, corroborated with the sample 1A that has the second lowest value and regular size.

Table. 13 Absorbance of spectrometer reading, conversion (X) and total cytosolic proteins (mg pt/ ml) of gills in the two replicates of each of the 3 oysters per aquarium treatment

Aquarium	Metal		Y = Absorbance			X			2.5 X = mg pt/ ml		
	Concentration (ug/L)		A	B	C	A	B	C	A	B	C
	Zn	Cd									
Control	0	0	0.655	1.034	1.271	0.252	0.541	0.721	0.630	1.352	1.803
			1.004	1.218	1.154	0.518	0.681	0.632	1.295	1.702	1.580
1	0	422	1.096	1.23	1.177	0.588	0.690	0.650	1.470	1.725	1.624
			1.244	1.159	1.101	0.701	0.636	0.592	1.752	1.590	1.479
2	11	124	0.995	1.313	0.988	0.511	0.753	0.506	1.278	1.883	1.264
			1.069	1.217	1.197	0.567	0.680	0.665	1.419	1.700	1.662
3	11	721	1.511	1.217	1.358	0.904	0.680	0.787	2.260	1.700	1.969
			1.411	1.131	1.365	0.828	0.615	0.793	2.070	1.537	1.982
4	39	0	0.803	1.387	1.362	0.365	0.810	0.791	0.912	2.024	1.976
			0.862	1.165	1.274	0.410	0.641	0.724	1.025	1.601	1.809
5	39	422	0.776	0.949	-	0.344	0.476	-	0.861	1.190	-
			0.954	1.022	-	0.480	0.532	-	1.200	1.329	-
6	39	845	1.426	0.857	1.256	0.839	0.406	0.710	2.098	1.015	1.775
			1.124	1.289	1.236	0.609	0.735	0.695	1.523	1.837	1.736
7	66	124	0.951	1.304	0.917	0.478	0.746	0.452	1.194	1.866	1.129
			1.163	1.356	1.227	0.639	0.786	0.688	1.598	1.965	1.719
8	66	721	1.289	1.315	-	0.735	0.755	-	1.837	1.887	-
			0.907	1.247	0.749	0.444	0.703	0.324	1.110	1.757	0.809
9	77	422	0.928	1.282	1.149	0.460	0.730	0.628	1.150	1.824	1.571
			0.992	1.035	1.176	0.509	0.542	0.649	1.272	1.354	1.622

Table. 14 Means of total cytosolic proteins (mg pt/ L) in gills of the two replicates of each of the 3 oysters per aquarium treatment.

Aquarium	Metal Concentration (ug/L)		mg pt/ L		
	Zn	Cd	A	B	C
	Control	0	0	962.7	1527.1
1	0	422	1610.8	1657.5	1551.8
2	11	124	1348.1	1791.7	1463.3
3	11	721	2164.8	1618.4	1975.4
4	39	0	968.4	1812.6	1892.6
5	39	422	1030.2	1259.6	-
6	39	845	1810.7	1426.2	1755.5
7	66	124	1395.7	1915.4	1424.3
8	66	721	1473.8	1822.1	809.4
9	77	422	1211.1	1588.9	1596.6

Trying to find a relation between the total protein content in the cytosolic fraction of the samples and the metal mixtures shows that the protein content of control samples are more or less the same as in the first 2 aquariums and that an elevation of the protein concentration is only shown in the aquarium 3 (11 ug Zn/L + 731 ug Cd/L), although not significant. The metal mixture having the second highest cytosolic protein concentration is the aquarium 6 (39 ug Zn/L + 845 ug Cd/L). But then this tendency is no more seen in the aquarium 8 (66 ugZn/L +721 ug Cd/L) demonstrating that a relation between high metal mixtures and high protein concentrations is not consistent.

Subsequent to the measurement of total cytosolic protein, the quantification of metallothionein using the differential pulse voltammetry (DPV) takes place. The calibration curves used to quantify the MT concentration in the samples are shown in Table 15. For each rabbit liver MT standard concentration in each calibration curve, 4 replications were measured; their mean and standard deviation are also shown. The two calibration curves of them have a linear regression ($y = ax + b$), the first one presents a slope of $-3.63e-004$ while the second one $-3.64e-004$. This similarity is

crucial for comparing and analyzing the data. Both of them were used in one half of the set of samples.

Table 15. Calibration Curve for MT quantification using Differential Pulse Voltammetry (DPV)

Rabbit liver MT ($\mu\text{g/L}$)	1st Calibration Curve		2nd Calibration Curve	
	Mean (nA)	Std.Dev.	Mean (nA)	Std.Dev.
19.58	-6.25	0.412	-5.99	0.68
39.12	-12.97	0.392	-11.7	0.422
58.62	-20.69	0.611	-21.32	0.749
78.09	-28.02	0.895	-27.48	1.337
97.51	-34.42	0.859	34.19	4.605
116.9	-40.71	1.92	-42.36	2.093

Metallothionein (MT) concentrations from all the samples given by differential pulse voltammetry (797 VA Computrace) using the calibration curve constructed with rabbit liver MT (as mg MT/L) are shown in Table 16. We must repeat that the analysis of MTs was conducted on three replicates (called also “sub-samples”) of the same gills’ tissue of each of 3 oysters (called “samples”, A, B and C). The mean and the standard deviation of the 3 replicates per sample have been calculated, as well as the coefficient of variation between the 3 replicates. This last step was to determine if the technique was reliable or not (summary of the CV% in Table 17).

The mean MT content from all replicates from all samples ranged from 5.06 to 16.02 mg MT/L. From the 3 replicates per sample usually two of them present similar values and mostly one has a higher or lower value. This fact results in a high percent of coefficient variation between replicates, ranging from 4.4 to 55.5%.

Table. 16 MT concentrations (mg MT/L) from the 3 replicates of each oyster per metal mixture

Aquarium	Metal Concentration		MT (mg/L)		
	Zn (ug/L)	Cd	A	B	C
Control	0	0	7.027	5.572	7.628
			6.338	5.13	6.165
			7.076	5.211	7.249
			6.81	5.30	7.01
			0.41	0.24	0.76
			6.1	4.4	10.8
1	0	422	8.059	6.228	17.31
			12.412	10.413	14.414
			22.58	7.308	16.332
			14.35	7.98	16.02
			7.45	2.17	1.47
			51.9	27.2	9.2
2	11	124	8.806	7.754	4.202
			7.723	8.587	6.301
			11.996	8.242	5.377
			9.51	8.19	5.29
			2.22	0.42	1.05
			23.4	5.1	19.9
3	11	721	8.31	4.415	7.543
			10.74	4.933	16.418
			11.511	5.912	7.54
			10.19	5.09	10.50
			1.67	0.76	5.12
			16.4	14.9	48.8
4	39	0	10.094	5.815	15.197
			10.928	5.181	7.328
			6.373	4.169	7.629
			9.13	5.06	10.05
			2.43	0.83	4.46
			26.6	16.4	44.4
5	39	422	8.128	5.031	-
			7.118	6.347	-
			8.604	4.286	-
			7.95	5.22	-
			0.76	1.04	-
			9.5	20.0	-
6	39	845	5.015	5.855	7.446
			6.686	5.653	4.784
			6.14	13.893	4.837
			5.95	8.47	5.69
			0.85	4.70	1.52
			14.3	55.5	26.8
7	66	124	5.052	14.423	6.168
			4.924	12.488	6.702
			7.175	-	6.899
			5.72	13.46	6.59
			1.26	1.37	0.38
			22.1	10.2	5.7
8	66	721	8.439	12.49	5.72
			8.713	13.629	6.258
			9.295	12.352	7.091
			8.82	12.82	6.36
			0.44	0.70	0.69
			5.0	5.5	10.9
9	77	422	6.856	4.038	14.528
			6.027	6.366	9.471
			8.1	7.391	14.227
			6.99	5.93	12.74
			1.04	1.72	2.84
			14.9	29.0	22.3

The different concentrations of MT between replicates lead to some suppositions of what can be the factor determining this variation, i.e. which step after subcellular separation. Some of them are: dilution after ultracentrifugation was not the same or not accurate or the sub-sample was not well homogenized; denaturalization of the cytosol by heat treatment might have been different in each sub-sample; centrifugation time was not enough; or extraction of the supernatant was not well developed. Other assumptions could be related to the Metrohom machine used for the quantification of MT. The most probable can be that the size of the mercury drop was not the same or the voltage in the recorded moment was different. All these suggestions are not proven; it can be one or a combination of several factors. These technical errors should be taken into account for future analysis.

In Table 19 values of MT concentrations from each of the 3 samples per metal mixture treatments are shown (the mean of the three samples per treatment, the SD and the coefficient of variance between them). Of the values reported the lowest concentration of MT are in control samples, showing that most likely an increase in MT was induced by the presence of metals in the water medium. The highest values of MT per sample were present in the aquarium 1 (0 ug Zn/L + 422 ug Cd/L) coincidentally with literature that describes the induction of MT by cadmium in the gills of *C.virginica* after 4 days of exposure to 50 ug Cd/L (Roesijadi and Klerks, 1989). The other high concentration of MT were present in aquarium 8 (66 ug Zn/L + 721 ug Cd/L) and in the aquarium 3 (11 ug Zn/L + 721 ug Cd/L). Both of them have high concentrations of Cd. The lowest value of mean MT (beside control) is in the aquarium 5 (39 ug Zn/L + 422 ug Cd/L).

Table 17. Concentration of metallothionein (mg MT/L) in each oyster sample exposed to a metal mixture treatment: mean, SD and CV (%) of the samples per treatment.

Aquarium	Metal Concentration (ug/L)		mg MT/ L					
	Zn	Cd	A	B	C	Mean	SD	CV (%)
	Control	0	0	6.81	5.30	7.01	6.38	0.93
1	0	422	14.35	7.98	16.02	12.78	4.24	33.2
2	11	124	9.51	8.19	5.29	7.67	2.16	28.1
3	11	721	10.19	5.09	10.50	8.59	3.04	35.4
4	39	0	9.13	5.06	10.05	8.08	2.66	32.9
5	39	422	7.95	5.22	-	6.59	1.93	29.3
6	39	845	5.95	8.47	5.69	6.70	1.53	22.9
7	66	124	5.72	13.46	6.59	8.59	4.24	49.4
8	66	721	8.82	12.82	6.36	9.33	3.26	35.0
9	77	422	6.99	5.93	12.74	8.56	3.66	42.8

The coefficient of variation between the 3 oysters, exposed to the same metal mixture was calculated in order to determinate if biological factors and aspects are influencing the synthesis of the MT. A high percent of coefficient variation between the samples can be present, ranging from 14.7 to 49.4%, showing that there are others factors that are involved in this synthesis. It has been reported that physiological characteristics, physical parameters of the environment and seasonal variations can affect or define the MT induction. This will be treated in detail in the discussion section.

Finally, MT concentrations are recalculated taking into consideration the total protein content (mg of Total Cytosolic Proteins/L (Table 14)) in order to express them as mg MT/mg total cytosolic protein. Results are presented in Table 18. This conversion is needed to avoid influence of age or general status of oysters.

Table 18. Metallothionein (MT) concentration in oyster gills exposed to the different metal mixtures expressed in ug MT/mg Pt, standard deviation and coefficient of variation between these concentrations.

Aquarium	Metal Concentration (ug/L)		µg MT/ mg Pt					
	Zn	Cd	A	B	C	Mean	SD	CV (%)
	Control	0	0	7.078	3.474	4.146	4.90	1.92
1	0	422	8.909	4.816	10.323	8.02	2.86	35.7
2	11	124	7.053	4.574	3.617	5.08	1.77	34.9
3	11	721	4.706	3.143	5.316	4.39	1.12	25.5
4	39	0	9.430	2.789	5.311	5.84	3.35	57.4
5	39	422	7.717	4.145		5.93	2.53	42.6
6	39	845	3.284	5.937	3.241	4.15	1.54	37.2
7	66	124	4.096	7.025	4.627	5.25	1.56	29.7
8	66	721	5.982	7.038	7.853	6.96	0.94	13.5
9	77	422	5.775	3.733	7.981	5.83	2.12	36.4

In Table 17 the values of MT oscillate in a range of 2.789 and 10.323 µg MT /mg Pt. Unlike the values expressed in mg MT/L (Table 15), here the values from the samples of the control did not differ from the rest and even lower values in some of the samples are present; like the sample B in the aquarium 4 (39 ug Zn/L + 0 ug Cd/L) that presents the lowest mg MT/ mg PT of all the assays, i.e. 2.789 µg MT/mg Pt. Other low values are present in the sample 6A (3.284 µg MT/mg Pt) and in the sample 6C (3.241 µg MT/mg Pt). The tendency that control values are lowest and that MT concentrations increase with the increasing metal concentrations is not present, like it was expected to be.

The highest concentration is in the sample 1C (10.323 µg MT/ mg Pt), and also in this aquarium 1 (0 ug Zn/L + 422 ug Cd/L) the highest mean value (8.02 µg MT/ mg PT) of all the treatments is present coincidentally with the results present in the Table 19 that express the MT as mg MT/L.

Values of MT expressed in terms of total protein are very small, showing how little fraction of the total protein is present as MT, so variations in MT concentrations are difficult to observe.

The CV % obtained ranged between 13.5 and 57.4 % and although we assumed that variation would decrease taking into account total protein concentrations these results are even higher than the ones obtained for the MT concentrations expressed in mg MT/L.

4.2.2 Statistical Analysis

Significant differences of MT concentrations (mg Mt/mg total protein) between control and Cd-Zn-metal mixtures were detected by non parametric statistics Kruskal Wallis ANOVA (tests are significant at $p < .05$), comparing multi-independent samples. No significant differences between the treatments were noted ($p = 0.5201$), meaning that none of the values were significantly different from each other.

Although the previous analysis showed no significant differences a surface response relationship was plotted through the concentration of mg MT/mg Total Cytosolic Protein and the Zn-Cd metal mixtures using the analysis of the Central Composite Design (CCD). The parameters of the surface response are shown in Figure 16 and the response profile in Figure 17. The Model follows the equation:

$$\text{MT (mg MT/ mg protein)} = \text{intercept} + a * \text{Cd} + b * \text{Cd}^2 + c * \text{Zn} + d * \text{Zn}^2 + e * \text{Cd} * \text{Zn}$$

ANOVA Effect Estimates results shown in Table 19 ($R^2 = 0.40369$) indicates that only the intercept is significant, and confirming that Cd and Zn within this range did not have a significant effect on MT concentrations.

Table 19. ANOVA Effect estimates on the fitted response surface of the relationship between the concentration of mg MT/mg Total Cytosolic Protein and the Zn-Cd metal mixtures.

	Effect	Std.Err.	t(4)	p	-95.00%	95.00%	Coeff.	Std.Err.	-95.00%	95.00%
Mean/Interc.	0.00644	0.00118	5.47232	0.00543	0.00317	0.00971	0.00644	0.00118	0.00317	0.00971
(1)Var1 (L)	0.00010	0.00093	0.10634	0.92043	-0.00249	0.00269	0.00005	0.00047	-0.00125	0.00135
Var1 (Q)	0.00008	0.00137	0.05910	0.95571	-0.00373	0.00389	0.00004	0.00069	-0.00186	0.00194
(2)Var2 (L)	-0.00017	0.00093	-0.18468	0.86247	-0.00274	0.00240	-0.00009	0.00046	-0.00137	0.00120
Var2 (Q)	-0.00184	0.00134	-1.37146	0.24213	-0.00556	0.00188	-0.00092	0.00067	-0.00278	0.00094
1L by 2L	0.00068	0.00117	0.58086	0.59249	-0.00258	0.00394	0.00034	0.00059	-0.00129	0.00197

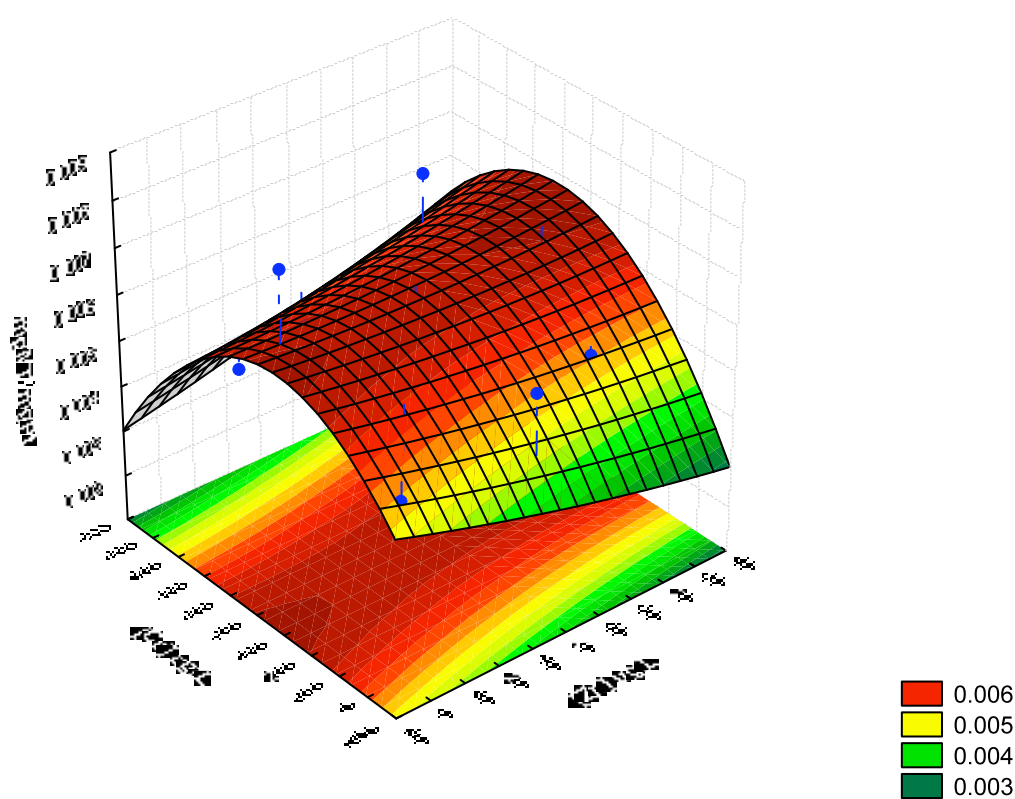


Figure 16. Fitted response surface between the two metals, Cd and Zn, and the concentration of Metallothionein (MT) expressed as mg MT/mg Pt.

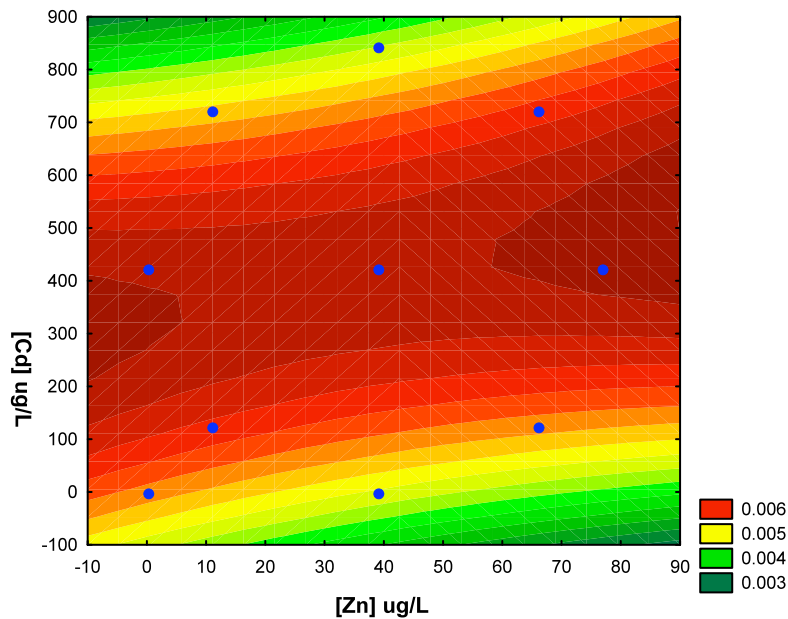


Figure 17. Fitted response profiles between the two metals, Cd and Zn, and the concentration of Metallothionein (MT) expressed as mg MT/mg Pt showing the 10 points used in the Central Composite Design (•).

5. DISCUSSION

5.1 Oyster Embryo Test

C. gigas requires about 20°C or more for spawning and embryonal development. It is a euryhaline species and its embryogenesis is not affected at salinities down to 20 g/kg; this is appropriate for ecotoxicological studies (Geffard et al., 2001). In the assay the salinity was adjusted to 33 ± 0.5 g/kg, the oxygen between 60 and 100% of saturation, the pH stable ($7.5 - 8.0 \pm 0.5$) and the temperature controlled by thermostaters and termometers all the time at required values in each step when performing the bioassay procedure. In that way the measurement and control of physical parameters as stipulated in the protocol were fulfilled (ASTM, 1995). It is well known that the growth of larvae can be reduced if these parameters are not controlled, like at oxygen concentrations below 4.2 mg/l (58.8% of saturation) (Morrison et al., 1971).

The definition of an acceptable level of malformation and mortality in the controls is largely a matter of convention. An acceptability level of more than 20% abnormal controls is questionable because there is always the possibility of either synergistic or compensatory effects between the investigated pollutants and the unknown cause of abnormality in the controls (His et al., 1997). It was found in the controls of the assay done, that the mean percentage of abnormalities was 49.9%. This value does not fulfill the requirement of the ASTM protocol (1995) that said that an acute toxicity test must have more than 70% of oyster embryos introduced into a required control treatment resulted in live larvae with completely developed shells at the end of the test. Therefore this test is invalid and must be repeated.

Contrary to standards mentioned by the ASTM protocol Thain (1991) states that 60% of abnormal larvae in the controls may be acceptable. His bioassay differs from the one presented in this thesis in the method of obtaining the eggs. He resorts to stripping the eggs from adult oysters gonads. This methodology is faster than the thermal stimulation but often results in abnormalities and mortalities exceeding the 20% threshold, and may lead to contradictory results in intercalibration experiments (Butler et al., 1992). This is the reason why this method was not used even when oysters took longer time to spawn than expected.

Mc Fadzen (1992) considers that abnormalities from the fertilized eggs to the prodissoconch (D-shaped) larvae as high as 50% must be because of congenital causes and His et al. (1997) mentions that failure of adult oysters to spawn upon thermal stimulation in the laboratory implies that gametogenesis is incomplete. Considering these assumptions and the fact that: oysters took longer time to spawn than required; eggs obtained were not ready after 20 minutes and after another 20 there were still a lot of them not rounded yet; that fertilization also takes longer than expected; and that when reading the multiwell plates we found a small amount of D-shape and embryos alive; it is found that the gamete quality was not good enough. These could be because the oysters were not totally mature or they have physiological alteration that made them produce bad quality of eggs.

The four different types of abnormalities that can be presented in D-shape stage, mentioned in the section 2.1.5, were found in the samples; as well as normal and

abnormal Prodissoconch I. Prodissoconch I stage was present with more frequency than D-shape. This suggests that maybe 24 hours was not enough time for the embryos to reach the D -shape stage, contrary to what the ASTM protocol mentioned. Geffard et al. (2001) found that fertilized eggs of *M. galloprovincialis* required four days to complete embryogenesis if low ambient temperatures are present in the medium.

Although the test must be considered unacceptable, Cd and Zn concentrations show a tendency of increasing the abnormalities if concentrations of both were increasing. For *C. virginica* embryos several publications with LC50 for Cd and Zn have been reported. Calabrese et al. (1973) reported a 48h LC50 for Zn (310 µg/L) and Cd (3800 µg/L); and MacInnes & Calabrese (1978) show a 48h LC50 for Zn (205 µg/L). For *C. gigas* embryos Woelke (1972) found concentrations of chemicals which did not affect embryonic development at 27°C like Zn (1000 µg/L); even though the criterion for not affected was not described. Martin et al. (1981) report a 48 EC50 for Zn (119 ± 12.0 µg/L) and for Cd (611 ± 195.9 µg/L). In this assay the 24h EC50 found for Zn and 95% confidence interval was 16.7 (15.1-18.5) µg/L, while Cd concentrations up to 845 µg/L were indeed not high enough to cause more than 50 % abnormality. The EC50 found for Zn differs in a big quantity from the ones mentioned before; it can be due to the different time of duration of the assay. Nevertheless the values of Cd that didn't show any 50% of response agree with the literature found. More tests should be done with embryos of oysters. Furthermore Cd alone did not result in considerable abnormality but the combination with Zn did, so it can be concluded that no antagonistic mechanism was present.

5.2 Quantification of Metallothionein (MT)

This assay took place based on the results of the embryo test so in fact an investigation of the same Zn - Cd metal mixtures was performed for both biological stages (larvae and adults) of *C. gigas*. Metal mixtures tolerance and toxicity was measured by MT biomarkers because they are thought to be involved in the detoxification of excess amounts of both essential and non-essential trace metals and enhanced metal tolerance has been associated with MT induction (Roesijadi et

al., 1982; George and Olsson, 1994; Pavicic et al., 1994; Roesijadi, 1996; Wallace and Lopez, 1997; Ritterhoff and Zauke, 1998; Amiard et al., 2006).

Initially, when organisms are exposed to metals these newly accumulated metals will compete and possibly replace constitutive metals in existing MT, according to their different affinities for the SH groups. In this way contrary to the general assumption that metals induce MT synthesis, absence of any induction upon metal exposure can be shown (Amiard et al., 2006). But it is expected - at higher levels of exposure – that when existing MT are saturated new MT will be synthesized.

From the results it appears that the Zn-Cd metal concentrations were too low to induce increases in MT levels in all treatments or the exposure time was too short to induce additional MT. Basal levels may have been sufficient for Cd to replace existing metals (e.g. Zn) bound to MT. These results agree with the ones of Géret et al. (2002). She reported that even though after 4 days of metal exposure, *C. gigas* accumulated cadmium and mercury in the gills and digestive gland, no significant variation of total protein level was occurred. Nevertheless after 21 days of exposure metals were bioaccumulated in both tissues. In addition Marie et al. (2006) report results of no significant difference in terms of MT gene expression, MT protein synthesis and Cd accumulation after juveniles of *C. gigas* were exposed for 14 days to Cd (0.133 μ M), Zn (15.3 μ M) and Cd + Zn. On the contrary Unger et al. (1996) noted an increase of MT mRNA in gills of juvenile *C. gigas*, at all pre-exposure concentrations during 21 and also after days 1, 2 and 3 of the challenge. The individuals were exposed 0.0044, 0.044 and 0.44 μ M Cd during the pre-exposure treatment (Géret et al., 2002).

Two of the highest MT concentrations 10.3 and 8.9 μ g MT/ mg Pt determined in this study were found in Aquarium 1 (0 μ g Zn/L + 422 μ g Cd/L) agreeing with the results observed by Roesijadi and Klerks (1989) in gills of *C. virginica* that present induction of MT after 4 days of exposure to a lower concentration, 50 μ g Cd/L. In laboratory tests, *C. gigas* exposed to 200 μ g/l of Cd were noted to induce MT in the gills while Zn concentrations of 1000 μ g/l did not (Géret et al., 2000). Furthermore, it has been reported that MT is induced in *C. gigas* by Cd in the whole soft tissue (Frazier and George, 1983; Fowler et al., 1986; Roesijadi et al., 1989) and in gills (Geffard et al.,

2001); and Zn in the gills (Geffard et al., 2001). Mouneyrac et al. (1999) showed that *C. gigas* translocated from a reference site to a Cd-contaminated estuary, increased its percentage of Cd linked to MT from 5% at day 0 to 21% after 1 month and 67% after 3 months, the latter being equal to the percentage of MT-bound to Cd in resident oysters from the contaminated site.

Basal MT concentrations determined by Differential Pulse Polarography (DPP) in *C. gigas* from sites considered to be uncontaminated were: in whole animal 0.1 ww (wet weight) (Amiard -Triquet et al., 1998) and in gills 0.12 - 0.30 ww (Geffard et al., 2002). Amiard et al. (2006) suggest that data should be compared with other studies only when they have been obtained with the same analytical technique because it has been proven that various techniques produce concentration values that do differ and the results are often expressed in different units.

Analyzing the set of data obtained it is remarkable to find that there were no significant differences between concentrations of MT of control samples and treatments samples. Physiological mechanisms or abiotic parameters could be responsible for this. The complexity of responsive elements and regulatory factors in MT gene expression and the fact that any variable which is able to influence protein metabolism will be able to influence MT directly (e.g. size, sex, sexual maturity, age, etc.) may be taken into consideration in future assays because they also influence indirectly variations in MT concentrations (Serafim et al., 2002; Bebianno et al., 2003; Duquesne, 1992; Hamza-Chaffai et al., 1995). Although size (weight and length) was measured in all samples (sex could not be differentiated because they were not in reproductive stage and it was not possible to identify gonads), no significant relationship with MT values was found. On the other hand many studies have demonstrated that MT synthesis can be induced by other causes unrelated to metal contamination such as handling, starvation, anoxia and freezing (Templeton and Cherian, 1991; English and Storey, 2003). However the level of induction is usually lower than that caused by metals (Kägi, 1993).

From literature it is shown that parameters of the surrounding medium influence MT induction in mollusks: temperature (Serafim et al., 2002), salinity (Leung et al., 2002) and season, in field studies only, (Baudrimont et al., 1997b; Cotou et al., 2001;

Dragun et al., 2004; Raspor et al., 2004). On the other hand Damiens et al. (2006) noted that salinity has no influence on MT concentrations measured in *C. gigas* larvae whereas temperature significantly increases the concentrations of MT (higher MT concentrations at higher temperature). In this assay these factors can not have influenced MT synthesis because temperature of 13°C was adjusted in a thermostatic fridge and its correct function was checked every moment. The salinity of the artificial sea water was always the same.

Although the next consideration is not applicable for this case, it has been reported in field studies that seasonality seems to influence greatly the synthesis of MTs in specific organs of adult bivalves (Cosson, 2000). Besides the physiological status of parents giving rise to larvae can influence MT induction on the last ones (MT induction in oyster larvae exposed to Cd when sexual maturity of parents is maximal and vice versa). Moreover not only, larvae from parents living in polluted areas are less sensitive to pollution (Damiens et al., 2006) but also adults. The origin of the individuals named as “environmental history” (De Kock and Kramer, 1994) should be considered in future analysis of oyster larvae and adults no matter what kind of metal mixtures are tested (Marie et al., 2006).

The toxic unit approach is usually used to evaluate mixtures toxicity. However this approach is allowed only when the concentration –response curves of the separate compounds and of the mixtures have identical slopes (Chen and Chiou, 1995). These means that the toxic unit approach is only applicable in equitoxic mixtures and not to the prediction of other partial responses, as well as the two models available for describing observed combined effects based in the toxicity of the individual components: Concentration addition (CA) and Independent action (IA). Based on these concepts and the fact that our treatments are not equitoxic mixtures, we apply the surface response modeling for the evaluation of the binary mixtures. Surface response models have the advantage that they can be used to predict the toxic effect of any given concentration of the mixture compounds used, in this particularly case, of Cd and Zn.

6. CONCLUSIONS

- The two new techniques Embryo Acute Toxicity Test and the Quantification of Metallothionein introduced to the Laboratory of Environmental Toxicology and Aquatic Ecology, Ghent University by foreign research visitors during the academic year of 2006-2007 were fitted into standard laboratory protocols by performing, reporting and submitting it.
- The Embryo Acute toxicity test took place with *Crassostrea gigas* embryos. They were exposed to 25 Zn - Cd metal mixtures treatments but the test resulted in more than 30% abnormal larvae in the controls; rendering the test unacceptable.
- Quantification of MT using the differential pulse voltammetry (DPV) was performed with the gills of adults' oysters, *Crassostrea gigas*, after 7 days of exposure in a media with 10 different treatments of Zn - Cd metal mixtures. All treatments showed no significant increases in MT levels compared to the control.
- The results of the two different techniques could not be compared, as the acute embryo test was unacceptable for this specific experiment. Thus, no correlation of the two techniques could be examined.
- The two techniques, mentioned before, could be used in a monitoring programs when assessing toxicants in mollusks. Further research about the advantages of the combination of them should be conducted.

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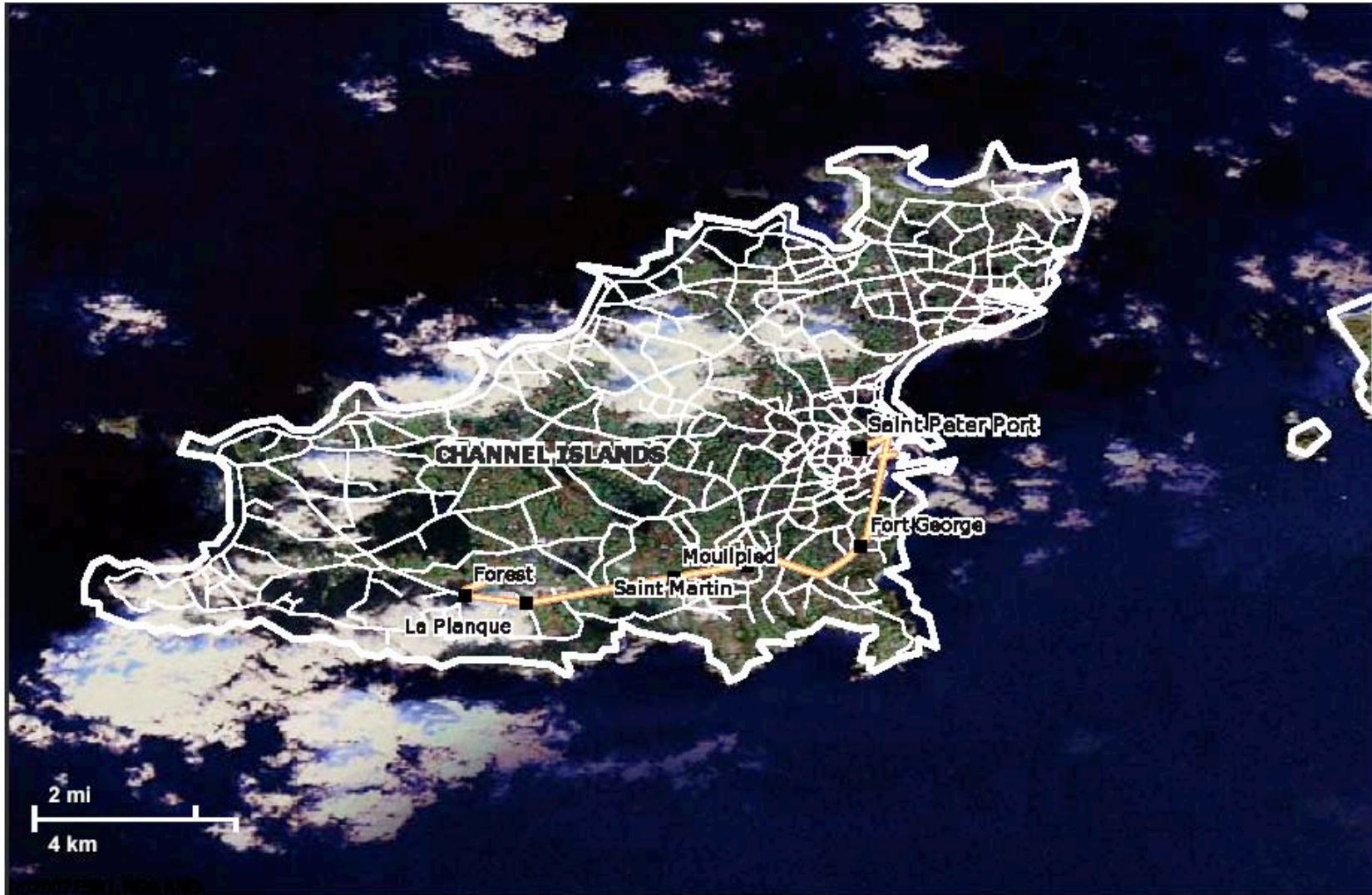
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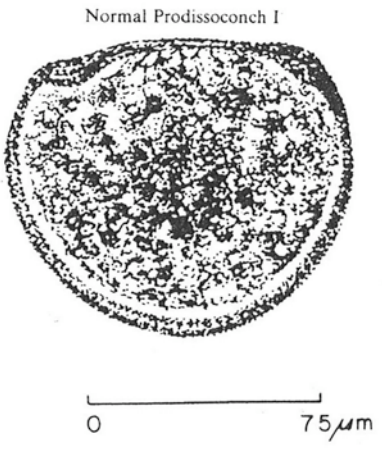
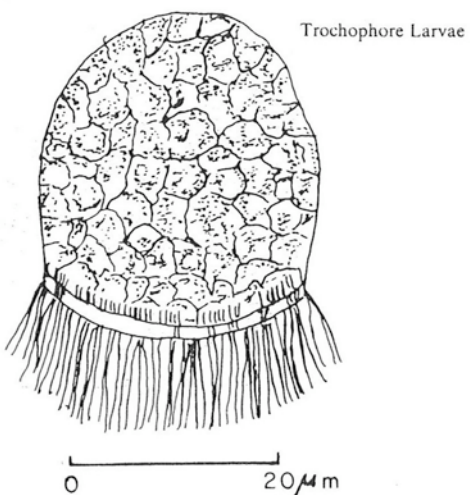
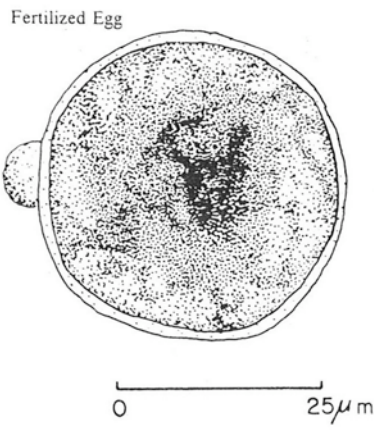
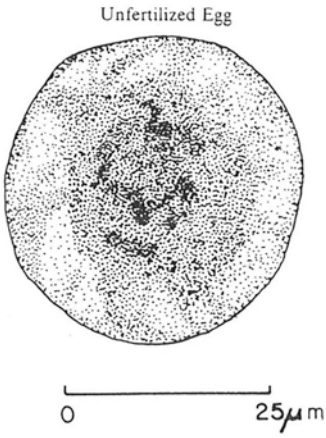
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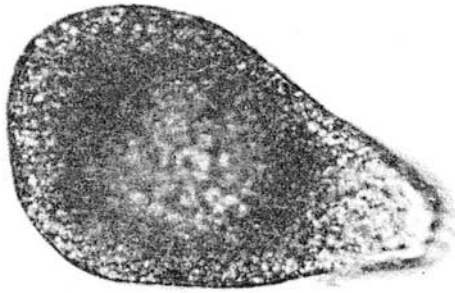
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APPENDIX I Guernsey Sea farms (Park Lane, Vale, Guernsey, UK) Channel Islands - French atlantic coast
(www.nationalgeographic.com)

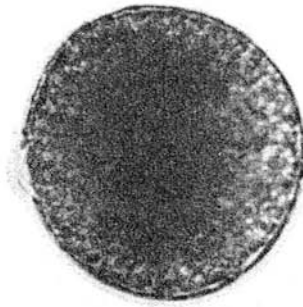


APPENDIX II Drawings and Photomicrographs (x450) Exemplifying Five key Developmental Stages of Bivalve Larvae Occurring during the first 48 h of Development. ASTM 1995, E724, p.223-224.

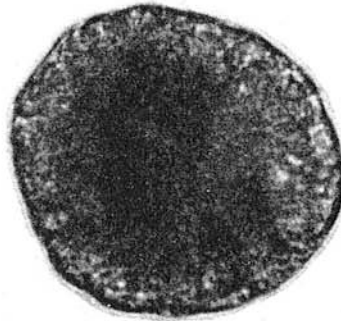




Unfertilized Egg



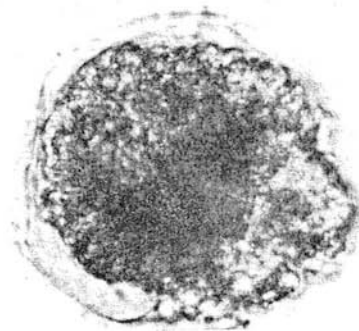
Fertilized Egg



Trochophore Larvae (Cilia will be apparent around margin)

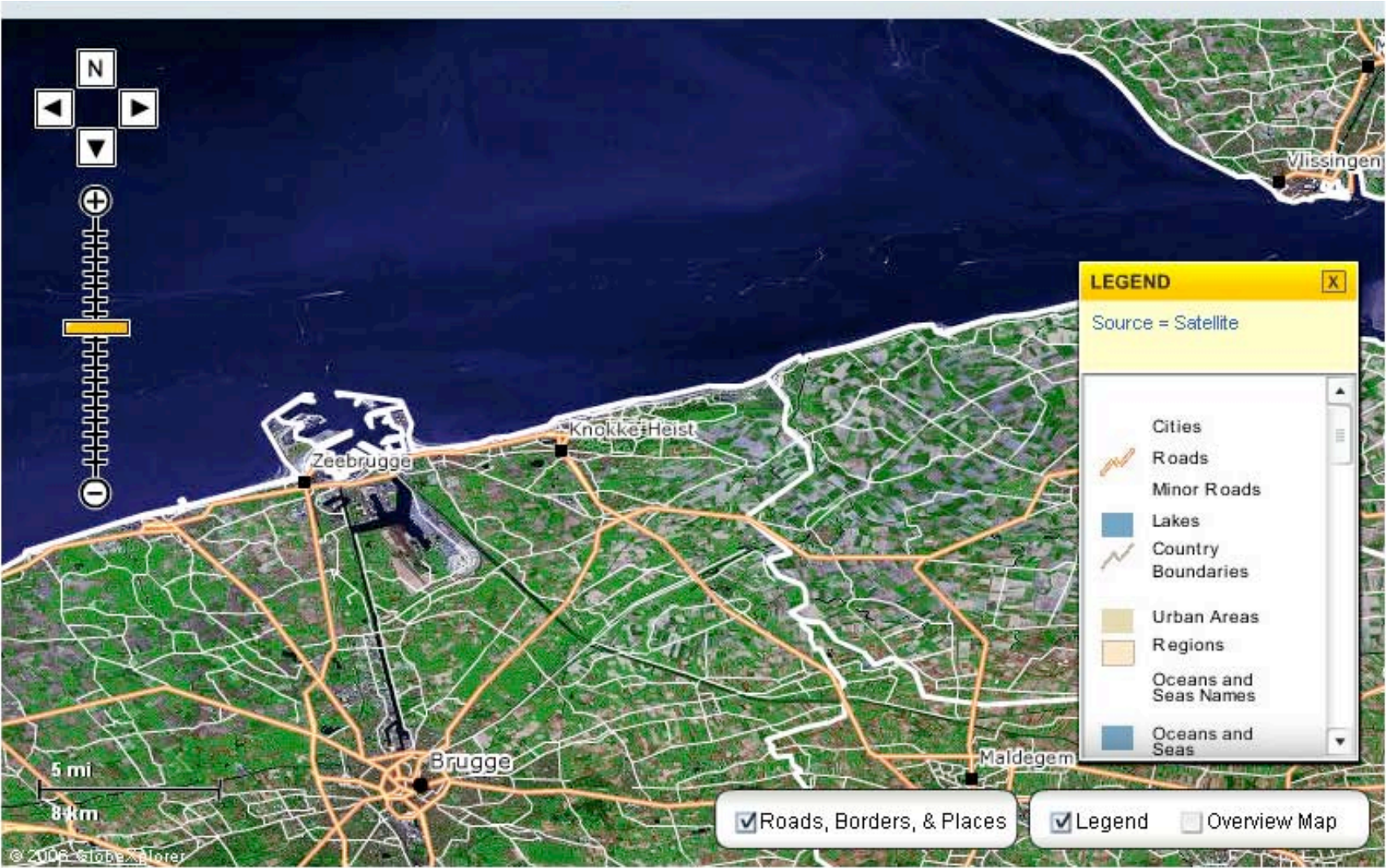


Normal Prodissoconch I

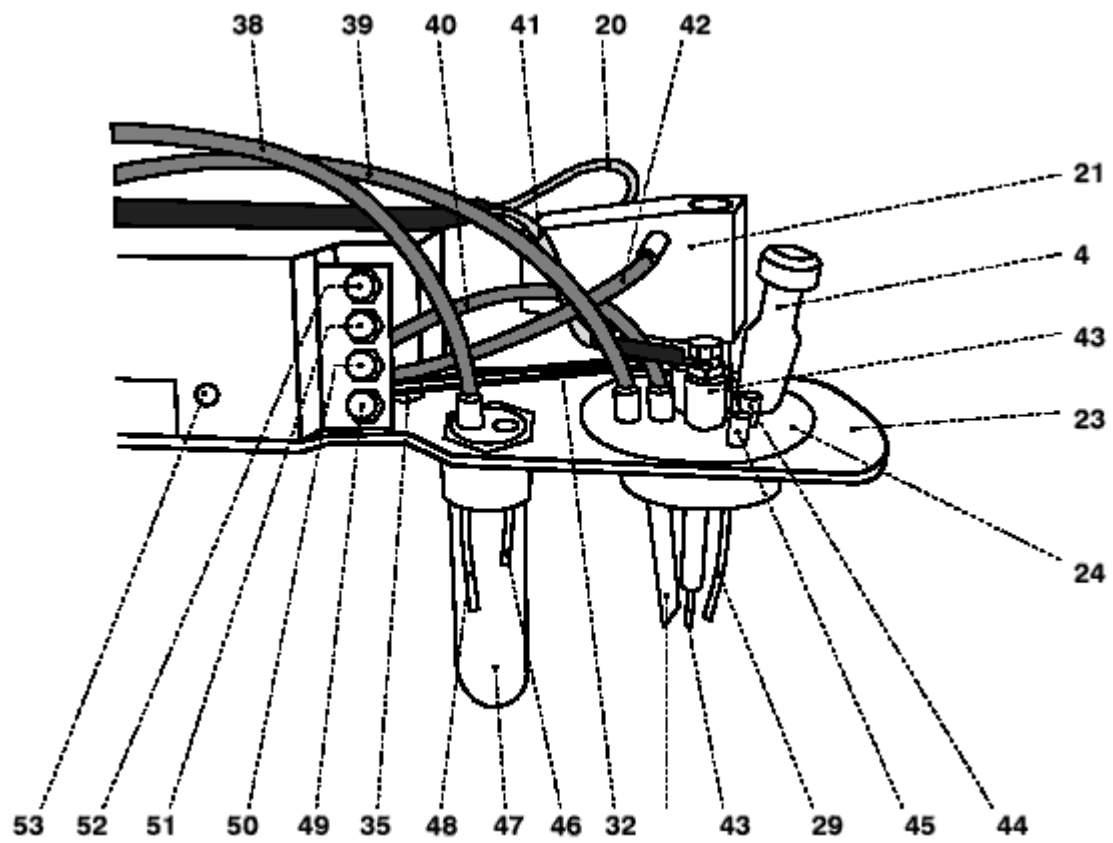
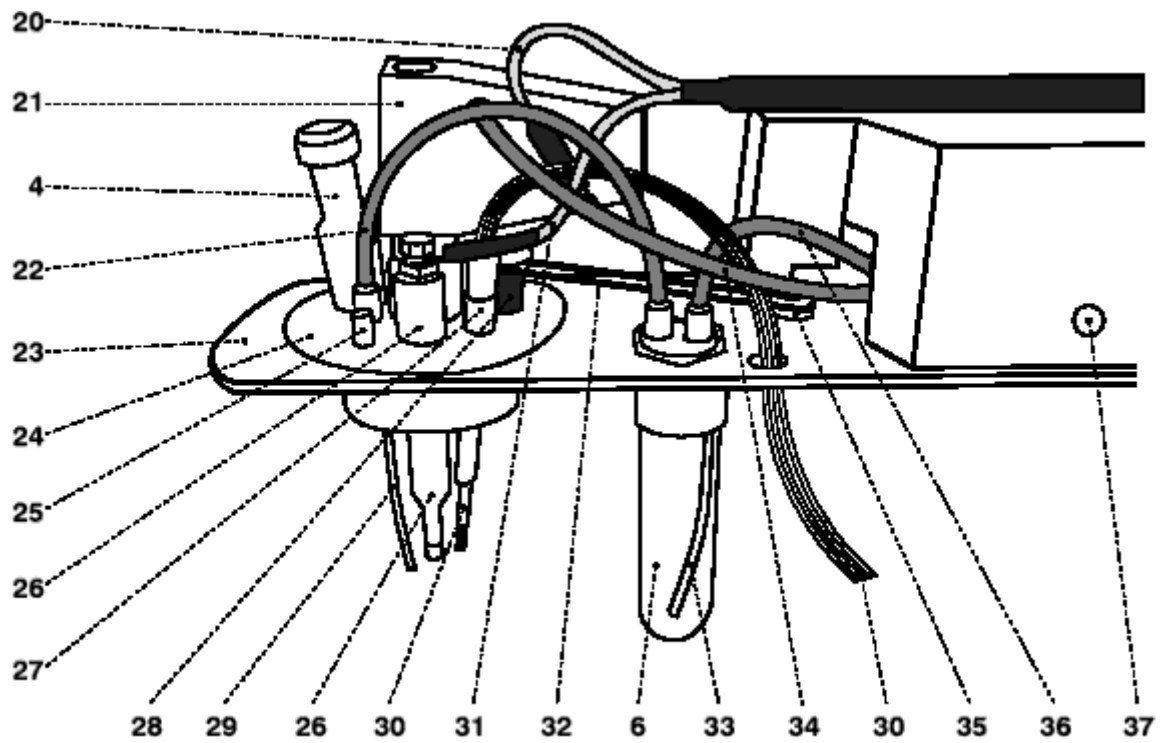


Abnormal Prodissoconch I

APPENDIX III - Heist Beach, Belgium (Source: www.nationalgeographic.com)



APPENDIX IV. Right and Left side view of the 797 VA Computrace Stand (fully equipped)



4 Stopper (6.2709.080) to close the pipetting opening	31 Electrode cable "RE" connection for reference electrode 26
6 Gas wash bottle (6.2405.030) for inert gas supply (must be filled halfway with dist. H ₂ O, see section 2.2.5)	32 Drive belt (6.1244.020) connection between drive wheel 35 and stirrer 28
20 Electrode cable "WE" connection for working electrode (MME or RDE)	33 PTFE tube (6.1819.000) for inert gas delivery to gas wash bottle 6 (attached)
21 Multi-Mode Electrode (MME) (6.1246.020) details, see section 2.3	34 FEP tubing (6.1805.180) for inert gas supply to MME 21
22 FEP tubing (6.1805.180) for inert gas supply to measuring vessel (attached)	35 Drive wheel of drive motor
23 Measuring head arm carrier plate with permanently attached measuring head, raisable	36 FEP tubing (6.1805.040) for inert gas delivery to gas wash bottle 6 attached.
24 Measuring head measuring vessel upper half made of PTFE; with openings for electrodes, stirrer, gas and liquid supply lines	37 Slotted screw for controlling the inert gas flow <i>Note: The factory setting of ca. 20 L/h should not be changed without good reason!</i>
25 Dummy stopper (6.1446.040)	38 FEP tubing (6.1805.020) for inert gas lead-off (attached)
26 Reference electrode comprising 6.0728.020 Ag/AgCl Refer- ence system and 6.1245.010 Electrolyte vessel (details, see section 2.5)	39 FEP tubing (6.1805.090) for inert gas lead-off (attached)
27 Nipple (6.2730.030) for mounting the 4-way microtip 30 or a dummy stopper	40 FEP tubing (6.1805.180) for inert gas supply to tapping mecha- nism (attached)
28 Stirrer (6.1204.200)	41 Electrode cable "AE" connection for auxiliary electrode 43
29 PTFE tube (6.1819.000) (attached)	42 FEP tubing (6.1805.180) for inert gas supply to MME 21
30 4-way microtip (6.1824.000) for delivery of solutions; with 4 lengths of PTFE tubing with connecting nipples for Dosing devices	43 Auxiliary electrode for details see section 2.6
	44 Dummy stopper (6.1446.040)
	45 Dummy stopper (6.1446.040)

46 PTFE tube (6.1819.010)
for optional supply of the waste solution
to gas wash bottle **47** (attached)

47 Gas wash bottle (6.2405.030)
for separating mercury from the waste
solution (attached)

48 PTFE tube (6.1819.010)
for optional siphoning off the waste
solution from gas wash bottle **47**
(attached)

49 Dummy cell connection "WE-D"
differential mode simulation
(peak/wave)

50 Dummy cell connection "WE-L"
linear mode simulation (RC element)

51 Dummy cell connection "RE"

52 Dummy cell connection "AE"

**53 Slotted screw for controlling the
tapping power in the DME or SMDE
case**

*Note: The factory setting should not be
changed without good reason!*