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The identification of Metallothioneins in the bivalve *Macoma balthica* from the Western Scheldt Estuary.

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Abstract :

Macoma balthica have been sampled every two months for two years at two different locations, one brackish and one marine, from the Western Scheldt Estuary and submitted to *in vitro* short term exposure to Cd, Cu and Zn. Total and heat stable cytosolic metal concentrations have been measured before and after exposure to study the effects of the sampling season on the metal uptake by the bivalve. Along with this, concentrations of metal-binding proteins, exhibiting the characteristic properties of metallothioneins (MTs) have been measured in the entire cytosol and the chromatographically separated cytosol to see if they increase after the metal exposure of the clams and to determine their molecular weight.

Much higher uptakes of Cd and, to a lesser extent, Cu occurred in Winter while Zn appears to be constantly regulated. These phenomena are amplified in the cytosol, especially for Cd. The respective contents of the various elements, either at the total or at the cytosolic level, are not constant, showing that the body weight seasonal fluctuations of the clams are not the only parameter responsible for the metal uptake variability. As well as this, the proportion of cytosolic metal was found to vary up to maxima of 50 % depending on the absolute cytosolic concentration. MT or MLP concentrations have been shown to increase on metal exposure and are found in the cytosolic chromatographic fractions in the molecular weight range of 6000-12000 Da. Metal maxima, after the exposure were found in these same fractions suggesting that the cytosolic phase, with its heat stable proteins, plays an important role in trace metal detoxification mechanisms of clams. The use of MT seasonal "norm" charts has been investigated for their suitability to be used as indicators of trace metal pollution.

1.0 Introduction :

The primary aim of this study was to clarify whether, or not, metallothioneins (MTs) are involved in heavy metal (HM) regulation and detoxification mechanisms in the marine bivalve *Macoma balthica* from the Western Scheldt Estuary. This point has, in fact, been the subject of some controversy as to whether, or not, intraspecific variations exist between N.American and European populations (Johansson et al., 1986; Langston and Zhou, 1987) regarding their ability to produce MTs and/or metallothionein-like proteins (MLPs) and therefore demands further investigation. The secondary aim was to establish "seasonal norms" for MT concentrations, if present, in these bivalves at two different stations, Baalhoek (B) and Paulinapolder (PP), providing natural uncertainties with these values over a two year period (March 1993 to March 1995) as well as Upper Warning (UWL) and Upper Action Limits (UAL) to be used as indicators of heavy metal pollution when an MT value goes above these differing seasonal limits.

Needless to say, when one begins to study the specific detoxification mechanisms of one bivalve species, in particular, of an entire ecosystem, the question of "Why bivalves and why specifically *Macoma balthica* ?" has to be asked. We chose bivalves because, being heavy metal accumulators, they are ideal indicators of heavy metal pollution, if and when pollution is present, and they facilitate analysis by this natural process of metal preconcentration. This bioaccumulation also gives a good idea of the amount of metal that is available for uptake in the system as metal concentrations in seawater or sediment alone do not give a true indication of what is actually available for uptake. Bivalves are generally consumed by waterfowl and fish and so are a source of heavy metals to higher trophic levels (Luoma and Phillips, 1988; Johns et al., 1988). Bivalves, of many types, are also known producers of MTs and/or MLPs and are therefore of great interest when one is studying detoxification mechanisms. Another reason for choosing the *M. balthica* species was because it occurs in adequate abundance in both of our sampling stations even though the degree of metal pollution in both stations is quite different.

Before launching into a detailed description of the properties of metallothioneins and their effect on heavy metals, some background information will ge given on the estuary, the bivalve, the use of bivalves in monitoring environmental contamination, factors affecting bioaccumulation of chemical contaminants in bivalves and contaminant interactions. Once these subjects have been covered, in as much relevant detail as possible, the topic of heavy metal metabolism in bivalves will be covered. A section on invertebrate MTs, as opposed to mammalian MTs, is provided to shed some light on the range of invertebrates known to produce MTs or MLPs and also to describe the nutritional and physiologic significance of such proteins. Finally, the principles of the analytical techniques used will be given but again including only relevant subject matter.

So, the research involved parallel experiments on both natural and *in vitro* metal exposed bivalves. After each sample collection, half of the total amount of clams, from each station, were exposed to a mixed metal spike in aquaria while the other half were immediately deshelled. The methods involved will be described in full in the Methodology section but the overall plan was to measure total metal concentrations in the natural bivalves and the uptake of Cd, Cu and Zn in the spiked bivalves. Along with this, the natural partitioning of cadmium, copper, zinc and iron was determined as well as the influence of the mixed metal exposure on this partitioning. In conjunction with the metal measurements, the natural levels of MTs, in the clams, were measured before and after metal exposure. Enhanced MT concentrations, in the spiked clams, would suggest the fact that production of such proteins is induced by heavy metal exposure.

1.1 The Western Scheldt Estuary.

The Scheldt Estuary region is a densely populated and industrialised area in W-Belgium and SW-Netherlands. The estuary is 70 km long and fed by the rivers Scheldt, Rupel and Durme with an average fresh water discharge of ca. 100 $m^3.s^{-1}$, varying from 20 $m^3.s^{-1}$ in Summer to 400 $m^3.s^{-1}$ in Winter (Stronkhorst, 1993). The estuary can be divided into a marine zone (salinity > 18 $g.kg^{-1}$), a mesohaline (brackish) zone (5-18 $g.kg^{-1}$) and an oligohaline-fresh water zone (< 5 $g.kg^{-1}$). Fig.1 shows a map of the Western Scheldt Estuary, indicating the three zones as well as our two sampling stations, Baalhoek (B) and Paulinapolder (PP).

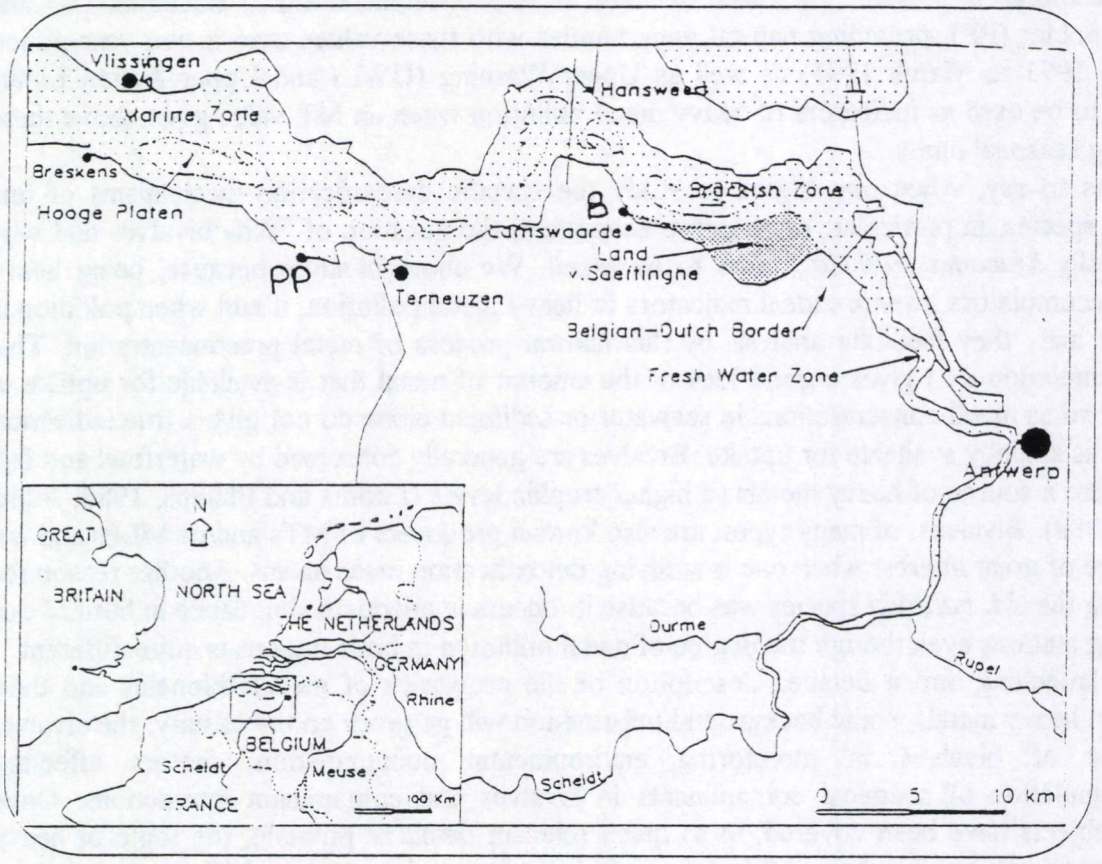

Figure 1 : The Western Scheldt Estuary.

The tidal amplitude in the estuary varies between 4 m near Vlissingen, 5 m near Antwerp and 2 m near Gent. Since the average river discharge (100 $m^3\ s^{-1}$) is rather small, the influence of the tide and salt intrusion is very important in this estuary (Panutrakul and Baeyens, 1991). Tidal flats have developed along the entire length of the estuary because of weak wave action and the moderate to large tidal range. In most tidal flats, such as in the Western Scheldt Estuary, there is a seaward progression in sediment grain size from mud-dominated sediments at the landwards end to sand-dominated sediments at the seawards end. Both of our sampling stations are tidal flats with mud-dominated sediments, ideal habitats for the mud burrowing bivalve, *Macoma balthica*. Paulinapolder differs from Baalhoek in that it has a high tidal flat which has been almost totally colonised by plants, forming a salt-marsh which is usually only flooded during high spring tides.

The water quality in the Western Scheldt Estuary is adversely affected by the riverine input (Holland et al., 1991) but improves in the seaward direction. This means, that for our choice of stations, Paulinapolder, in the marine zone, is the cleaner of the two, with Baalhoek, in the brackish zone, being more heavily polluted, having less species diversity and on average,

smaller bivalves. The seaward water quality improvement is the result of dilution with relatively uncontaminated seawater and marine sediment, of the self-purification capacity of the tidal system and of sedimentation on salt marshes with the "Land van Saeftinge" being a major sink (Van Eck et al., 1991). The estuary has an anoxic region in the low salinity part near Antwerp where the behaviour of metals in exchange processes may be different from those in oxic zones. In a strong anoxic sediment, where sulphate reducing bacteria are active, the heavy metals are trapped as poorly soluble metal sulphide complexes, while in an oxic or suboxic sediment, metals tend to be redissolved due to the oxidation of organic matter and the reduction of particulate Fe and Mn oxyhydroxides.

Between the mid-1970s and mid-1980s a significant reduction in water pollution took place (Stronkhorst, 1992) but in recent years, monitoring with biota revealed that concentrations of trace metals and most organic micro-contaminants remain constant and are still at a substantial level (Stronkhorst, 1993). Besides trends and gradients in concentrations other factors may also influence the exposure of organisms to the estuarine pollution. In the aquatic environment the dissolved fraction of a compound is generally regarded as the biologically available fraction that leads to bioconcentration. However, the associated levels in sediment or food may also determine the concentration in an organism (Connell, 1988; Langston and Burt, 1991). Factors such as dissolved oxygen concentration, pH and salinity may also influence the biologically available fraction. The fact that the bioavailability of several trace metals in the Scheldt Estuary is affected by these variables has been reported by Zwolsman and Van Eck (1990) and this accounts for the spatial and temporal variations in the degree of exposure of, for instance, *Macoma balthica*, to these toxicants. Table 1 lists values for some of the physicochemical variables in the brackish zone of the Western Scheldt Estuary during the period of January 1993 to December 1993 (Courtesy of RIKZ/RIZA, NL : "Jaarboek Monitoring Rijkswateren - 1993").

Table 1 : Physicochemical variables in the Western Scheldt Estuary, 1993 (brackish zone).

	Variable	Highest	Median	Lowest
General	Water temperature (°C)	19.5	11.6	1.8
	Dissolved O_2 (mg/l)	10.9	8.4	6.8
	Salinity (‰)	27.9	22.1	10.5
	Suspended solids (mg/l)	184	43	8
	Dissolved Organic Carbon (DOC) (mg/l)	4.9	3.1	1.4
Nutrients	Nitrate, Nitrite & Ammonium (mg N/l)	**Winter 3.45 ± 0.12 ; Summer 3.01 ± 0.13**		
	Ortho-phosphate (µg P/l)	**Winter 147 ± 9 ; Summer 168 ± 10**		
	Silica (mg Si/l)	**Winter 2.43 ± 0.10; Summer 0.85 ± 0.16**		
Primary production	Chlorophyll a (µg/l) - Winter	3.3	2.1	1.4
	- Summer	60.9	10.5	4.6
Total Inorganic Pollutants (Dissolved + Particulate)	Total Cd (µg/l)	0.46	0.16	0.06
	Total Cu (µg/l)	8.6	2.9	1.6
	Total Ni (µg/l)	8.0	4.2	2.3
	Total Zn (µg/l)	68	11	4
	Total Hg (µg/l)	0.50	0.03	<0.01

A concentration of 2 mg O_2 l^{-1} is regarded as a critical minimum for marine fish (Rosenberg, 1984) and 5 mg O_2 l^{-1} is regarded as a desirable average level so there appeared to be no reason for concern in 1993, although it must be remembered that the values in this table only

hold for the brackish area. Most trace metals show a downstream decrease in toxicity because of increasing salinity, however, this is not the case for Cu and Cd in the Western Scheldt Estuary as conditions can be more anoxic at the Belgian-Dutch border than downsteam, thus reducing the dissolved concentrations of these metals. This could explain why *M. balthica* are more abundant in B than PP, although it has to be pointed out that the concentrations of Cd and Cu in clams from Baalhoek are always higher than in clams from Paulinapolder (see Results section).

Zinc continues, in 1993, to be a critical metal in the Western Scheldt Estuary. It has an effect on a wide range of taxonomical groups, with lowest reported NOEC (No Observed Effect Concentration) of 50 µg/l for algae, 100 µg/l for annelida , 18 µg/l for crustacea , 19µg/l for mollusca and 10 µg/l for echinodermata (Stronkhorst, 1993). It is clear that the 1993 Zn levels could have had an effect on *Macoma balthica*. Constant et al. (1987) found that the dissolved Zn concentration varied with the Zn complexing ligand concentration throughout the estuary. It is perhaps interesting to note that a field study on the blue mussel *Mytilus edulis* in the Western Scheldt showed that increasing contents of micro-contaminants (e.g. trace metals), caused an increased vulnerability to natural stress (e.g. changes in temperature and salinity) or anaerobic survival time (Veldhuizen-Tsoerkan, 1991). Stronkhorst (1993) clearly identifies that in the Western Scheldt Estuary (1987-1989) the concentration of Cd in suspended matter (6.370 µg/g) was much higher than in water (as is the case in most estuaries), this being attributed to sorption. The average level of Cd in intertidal macrozoobenthos *(Nereis diversicolor, Arenicola marina, Macoma balthica, Mytilus edulis, Cerastoderma edulis* and *Crangon crangon)* was high i.e. 1.520 µg/g d.w., this being logical as these macrozoobenthos feed on suspended matter. General relationships between levels of contaminants in sediments and their toxicity to macrozoobenthos may be obscured by the uncertainty of bio-availability, the occurrence of combination toxicity and the presence of unknown toxic substances, making a complete understanding of routes of contamination, for a bivalve, such as *Macoma balthica*, difficult.

1.2 The bivalve *Macoma balthica.*

Macoma balthica is a Tellinid bivalve of the class Lamellibranchia. Bivalves are laterally compressed and possess a shell with two valves, hinged dorsally, that completely enclose the body. The foot, like the remainder of the body is also laterally compressed and the head is very poorly developed. The gills are usually very large assuming, in most species, a food collecting function in addition to that of gas exchange. They live in the first 14 cm of the sediment, as shown in Figure 2 (courtesy of Barnes, 1987) exploiting the protection offered by a sub-terranean life in marine mud while utilising food suspended in water brought in from above the surface.

The majority of bivalves are dioecious. The gonoducts are simple since there is no copulation and the gonads emcompass the intestinal loops. In most bivalves, fertilisation occurs in the surrounding water; the gametes are shed into the suprabranchial cavity and then swept out with the exhalant current. The development of a free-swimming trocophore, succeeded by a veliger larva, is typical in marine bivalves. The veliger is symmetrical and eventually becomes enclosed within the two valves characteristic of bivalves. *Macoma balthica* have a life span of about 3-4 years and can reach approximately 20 mm shell length at this age.

Macoma balthica is a deposit feeding species with suspension feeding capabilities (Harvey and Luoma, 1984; Hummel, 1985). Data from Hummel (1985) suggest a deposit feeding rate of 0.5g of sediment (g of tissue)$^{-1}$ day^{-1} for *M. balthica* of 12-22 mm shell length. Their principal sources of nutrition are benthic/suspended microalgae (diatoms) and the microbial biomass and non-living organic materials associated with fine-grained (<100 µm) surficial sediments (Fenchel, 1972; Tunnicliffe and Risk, 1977). As already mentioned, *Macoma balthica*, among

other bivalves, are a source of heavy metals to higher trophic levels. Comparisons of heavy metal bioaccumulation from sediment and suspended matter would have expanded the scope of this work too much, so attention was given only to suspended matter in the estuarine water as a source of these contaminants and no sediment was used in the *in vitro* spiking experiments in the laboratory. Anyway, Luoma et al. (1992) found that *Macoma balthica* could reach the same Se tissue concentration by either suspension feeding or deposit feeding, despite the fact that suspended concentrations were often lower than sediment concentrations.

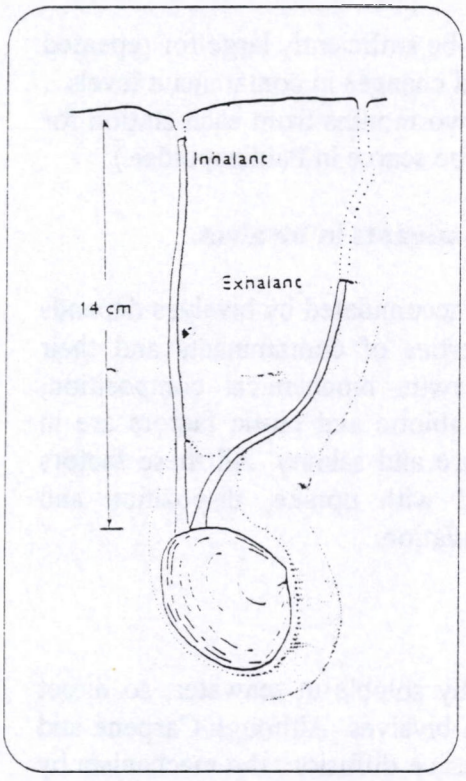

Figure 2a : *Macoma balthica* in burrow with inhalant siphon withdrawn.

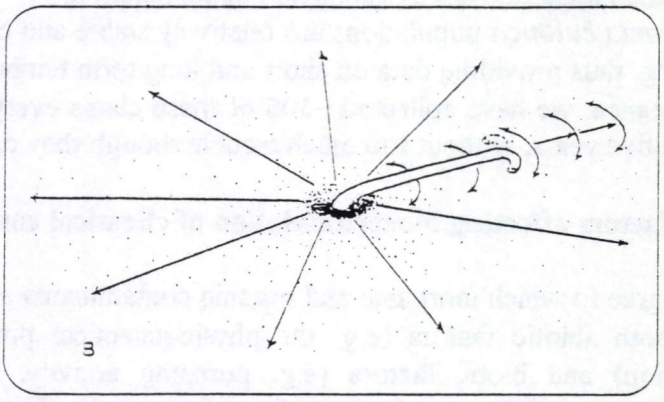

Figure 2b : Feeding movements of inhalent siphon at low tide.

1.2.1 The use of bivalves in monitoring environmental contamination.

The following attributes have led to the use of bivalves, particularly mussels, as "sentinel" or "indicator" organisms in environmental monitoring programmes throughout the world (Widdows and Donkin, 1992) :

1) Bivalves, such as *M. balthica*, are dominant members of coastal and estuarine communities and have a wide geographical distribution. This minimises the problems inherent in comparing data for markedly different species.
2) They are mostly sedentary and are therefore better than mobile species as integrators of chemical contamination in a given area.
3) They are relatively tolerant of (but not insensitive to) a wide range of environmental conditions, including moderately high levels of many types of contaminants.
4) They are deposit feeders but have suspension feeding capabilities, meaning they can pump relatively large volumes of water and concentrate many chemicals in their tissues. This often makes measurement of trace contaminants easier to accomplish in their tissues than in seawater.

5) The measurement of chemicals in bivalve tissue provides an assessment of biological availability which is not apparent from measurement of contaminants in environmental compartments (water, suspended particulates and sediment).
6) In comparison to fish and crustacea, bivalves have a very low level of activity of those enzyme systems capable of metabolising organic contaminants, such as aromatic hydrocarbons and PCB's. Therefore, contaminant concentrations in the tissues of bivalves more accurately reflect the magnitude of environmental contamination.
7) *Macoma balthica* populations are relatively stable and can be sufficiently large for repeated sampling, thus providing data on short and long term temporal changes in contaminant levels (for instance, we have collected ~300 of these clams every two months from each station for the last five years, without too much trouble though they can be scarce in Paulinapolder.)

1.2.2 Factors affecting bioaccumulation of chemical contaminants in bivalves.

The degree to which inorganic and organic contaminants are accumulated by bivalves depends upon both abiotic factors (e.g. the physicochemical properties of contaminants and their speciation) and biotic factors (e.g. pumping activity, growth, biochemical composition, reproductive condition and metabolism/elimination). These abiotic and biotic factors are in turn affected by environmental variables, such as temperature and salinity. All these factors can influence the rates of dynamic processes concerned with uptake, disposition and depuration which together determine the degree of bioaccumulation.

- Abiotic factors :

a) Solubility : Most metals occur in forms which are highly soluble in seawater, so direct uptake from solution is a quantitatively important process in bivalves. Although Carpené and George (1981) provided evidence that Cd enters gills by passive diffusion, the mechanism by which bivalves accumulate heavy metals directly from water is still unclear (Viarengo, 1989). Metals in saline waters occur either as free ion or as inorganic and organic complexes, some of which are uncharged (Davenport and Redpath, 1984; Viarengo, 1989). Simkiss (1983, 1984) has suggested that metal uptake is the result of partitioning of these relatively hydrophobic complexes into the lipid membrane, a mechanism analogous to the uptake of hydrophobic organic contaminants.
Metals can also be absorbed directly from seawater and incorporated into the shell of the bivalve, thus providing the potential for long-term records of changes in environmental contamination. While there is no apparent relationship between metal levels in the whole shells and bivalve tissues (Bryan and Uysal, 1978), primarily due to shell surface adsorption of metals, recent studies have shown significant relationships between Pb in the inner nacreous layer (i.e. the layer most recently deposited) and mussel tissue concentrations (Bourgoin, 1990).

b) Dissolved organic matter : Saline waters contain dissolved organic material (DOM) composed of small molecules and macromolecules of natural origin. DOM concentrations are highest in estuaries (Mantoura and Woodward, 1983) and can influence the solution behaviour of both metals and organic contaminants, with concomitant effects on bioavailability (Farrington, 1989; Suffet and MacCarthy, 1989). The influence of DOM on the bioaccumulation of metals by bivalve molluscs is quite complex, with both increases and decreases having been reported. For example, chelators of both large and small molecular weight (e.g. humic acid and EDTA, respectively) increase the bioaccumulation of Cd by the mussel *Mytilus edulis* (George and Coombs, 1977; Pempkowiak et al., 1989), which, compared to dissolved inorganic Cd complexes, possibly enhance the rate of Cd exchange

with membrane ligands. In contrast, structurally similar dissolved organic compounds decrease the uptake of Cu by the oyster *Crassostrea virginica* due to its strong binding to DOM which makes the Cu less bioavailable (Zamuda and Sunda, 1982; Zamuda et al.,1985). Absil et al.(1993) used ^{64}Cu to assess the influence of natural organic ligands on the bioavailability of copper to *Macoma balthica*. They found that, in the Western Scheldt estuary, dissolved copper uptake by these clams is determined mainly by the ligand concentration in the overlying water i.e. complexation by natural ligands caused a large reduction of biologically available ^{64}Cu. At low ligand concentrations, salinity was shown to have the major influence.

c) Particulates/Food : Though primarily deposit feeders, *Macoma balthica* are capable of suspension feeding and can therefore remove and concentrate particles from the water column as well as from the sediment. This is therefore a potentially important route of entry of contaminants into the bivalve. The significance of particulates in bioaccumulation is dependant on both the nature of the contaminant and the particulates. Uptake from food appears to contribute little to the bioaccumulation of Cd by *Mytilus edulis* in experimental systems (Borchardt, 1983; Riisgard et al., 1987) but contributes significantly to the uptake of Am and Pu (Bjerregaard et al., 1985) and Pb (Schulz-Baldes, 1974). In the environment, particulates may be the major source of bioaccumulated Pb (Loring and Prossi, 1986; Bourgoin, 1990), and are also of paramount importance in the process of Fe accumulation (George et al., 1976). In experimental studies, the relative bioavailability of inorganic Hg in water, phytoplankton, and sediment is in the approximate ratio of 10:5:1 (King and Davies, 1987). However, because of differences in their relative abundance within a mercury-contaminated estuary, mussels accumulated mainly inorganic Hg from suspended particulates at the seaward end, whereas both dissolved inorganic Hg and methylmercury compounds became more important further up the estuary. Although this study showed that Hg in highly organic particles (such as phytoplankton) is readily available, the bioavailability of this metal to the deposit feeding bivalves *Scrobicularia plana* and *Macoma balthica* is reduced with increasing organic content of the sediment due to preferential uptake of sediment organic matter as opposed to using the phytoplankton source.

- Biotic Factors :

a) Biochemical composition / Physiological condition : Once absorbed, the physiological condition and the biochemical composition of the bivalve are important in determining the contaminant's tissue distribution and whole-animal retention. Since metal levels are actively controlled by physiological and biochemical factors, there can be considerable individual animal variability in bioaccumulation (Lobel, 1986). The tissue disposition of metals can also be modified as a result of seasonal reproductive cycles and major alterations in biochemical composition (Cossa et al., 1980; Simpson, 1989; Coimbra and Carraca, 1990). The concentration of metal within the whole body can be strongly influenced by growth and weight loss (Simpson, 1979; Cossa et al., 1980; Fischer, 1988; Tusnik and Planinc, 1988; Borchardt et al., 1989). However, Cain and Luoma (1990), suggest that metal content in *Macoma balthica* is related more to age than shell length. This was supported in the work of Bordin et al. (1995) who found clam metal burden maximum in winter and minimum in summer, resulting from higher metal uptakes by smaller animals in winter and vice versa in summer.

b) Metabolism / Storage / Elimination : The fate of metals absorbed by mussels is largely determined by a complex set of binding and sequestration processes, involving specialised proteins (metallothioneins and ferritin), lysosomes and inorganic granules (Bootsma et al., 1988; Viarengo, 1989). The distribution of these agents of metal ion homeostasis within tissues influences their capacity to accumulate and remobilize metals, as well as their

bioavailability at sites of toxic action within the tissues. Depuration curves for both metals and organic contaminants often approximate to a biphasic relationship, indicating accumulation into, and release from, at least two "compartments", often termed "fast" and "slow" compartments. Possible "slow" release compartments for metals include specific metal-binding proteins (MTs), lysosomes and inorganic granules (Viarengo, 1989). In the case of MTs , their importance can increase with time since they are inducible (Roesijadi, 1982; Viarengo et al., 1985).
If MT does have a storage function, it is relatively transient insofar as the protein has a short half-life and the metals are rapidly released when exposure to the metals is reduced.
Unfortunately, little is known of the fate of metals after binding to MT. It has been reported that direct transfer (donation) of zinc and copper can occur from MT to apoenzymes, such as alkaline phosphatase and superoxide dismutase, implying that MT might be involved in regulation of the activation of these and other enzymes (Geller and Winge, 1982; Krezoski et al., 1988 and Udom and Brady, 1980). Alternatively the protein may be secreted in intact form from the cell or it may, in some cases, accumulate in lysosomes or other organelles in the form of insoluble aggregrates of metal-MT.

Typically, **abiotic** and **biotic factors** can modify bioaccumulation by ca. 2-fold and consequently, are of less importance when detecting and comparing marked (i.e. > 5-10 fold) spatial and temporal differences in contaminant concentrations in bivalves (Phillips, 1980).

1.2.3 Contaminant interactions.

There have been several reports of interactions between bioaccumulating metals. Bivalves exposed to mixtures of Cu, Cd and Pb at 20µg.l^{-1} showed a reduction in the uptake of Cd and Pb (Theede and Jung, 1989), probably as a result of the relatively rapid uptake of Cu. Exposure of some bivalve species to Cu or Cd can induce MTs which then serve to "protect" the animal during subsequent exposure to Hg (Roesijadi and Fellingham, 1987). In addition, Cu can displace Zn from the thionein pool, thus affecting the partitioning of the latter element in the tissue. Zn can also alter the bioaccumulation of Cd when the Zn/Cd ratio falls outside the range typical of "natural coastal environments" (Fischer, 1988).

1.3 Heavy metal metabolism in bivalves.

Once inside the cell, heavy metals are primarily complexed by thiol-containing molecules such as amino acids, glutathione and, in particular, the metal binding detoxication proteins, metallothioneins. In addition, part of the metal is compartmentalised in the lysosomal vacuolar system, or trapped in different types of specialised inorganic granules.

a) Metallothioneins : Historically, the term metallothionein was introduced to designate the cadmium-, zinc- and copper-containing sulphur-rich protein from equine renal cortex. This protein was characterised as follows (Kägi et al., 1974; Kojima et al., 1976; Vasák, 1980) :

1. Low molecular weight (6000-7000 Daltons) proteins found in the cytoplasm.
2. High metal content (7-10g atoms of metal/mol).
3. Characteristic amino acid composition (high cysteine content, no aromatic amino acids nor histidine).
4. Soluble and heat stable.
5. Spectroscopic features characteristic of tetrahedral metal-thiolate (-mercaptide) complexes i.e. $A_{254}:A_{280} >> 1$.
6. Metal-thiolate clusters.

Some of the possible biological roles of metallothioneins are : metal storage (e.g. Zn^{2+} and Cu^{2+}), detoxification of metals (e.g. binding of xenobiotic metals such as Hg^{2+}, Cd^{2+}, Au^{2+} and Ag^{2+}), metal transport, participation in immune response and metabolism of essential metals. Therefore, metallothioneins mainly function to maintain low levels of free heavy metal cations in cells, initially through the displacement of Zn in existing MT and the binding of the xenobiotic metal, and then through induction by the synthesis of increased amounts of metallothionein. Thus, their primary function is to regulate heavy metals in the cell.
It has been suggested that MTs provide metal where and when needed for many roles (Vallee, 1979, 1987). To achieve this, a process would be needed that would not run contrary to the high stability of the metal mercaptides. Metallothionein could be a quick storage and delivery system pressed into service when zinc absolutely, positively has to function and to be there at a specific time. Thionein could be stored so that its oxidoreductive SH groups are available for that particular potential. It must also ensure, protect and defend against all intruders, including heavy metal imposters, oxidisers and alkylating agents as well as anticipating both short-term and long-term needs and rapidly respond to environmental changes.
MTs have a wide tissue distribution in mussels e.g. gills, mantle and digestive gland in *M. galloprovincialis* (Viarengo et al., 1981). Copper-binding (Viarengo et al., 1984), Cd-binding (George et al., 1979) and Hg-binding (Roesijadi and Hall, 1981) proteins (molecular weight 10,000 to 15,000 daltons, or more) have been isolated and characterised from *M. edulis* or *M. galloprovincialis.* Copper displaced Zn (but not Cd) from Cd, Zn -Thioneins in digestive gland of *M.galloprovincialis* (Viarengo, 1989). Elevation of MT levels have been seen in various tissues of mussels exposed to Cu (Viarengo et al., 1981), Cd (Noël-Lambot, 1976) and Hg (Roesijadi, 1982) and in other molluscs (Viarengo, 1989). Enhanced Hg-tolerance in *M. edulis* was related to the induction of Hg-binding proteins (Roesijadi et al., 1982a). Mercury-tolerance was also enhanced by pre-exposure to other heavy metals capable of inducing metallothioneins (Roesijadi and Fellingham, 1987). In agreement with this, Cosson (1994) found that, in carp liver, pretreatment by zinc improved cadmium binding with MT and *de novo* synthesis of MT.

b) Lysosomes : Lysosomes are present in considerable numbers in digestive gland and kidney of bivalves. Tertiary lysosomes accumulate undegradable end-products of lipid peroxidation (oxidised lipid and protein polymers), so-called lipofuschin. In kidneys, lipofuschin granules have been shown to bind metals in two ways, viz. (a) metals weakly bound by acidic groups in the outer region of the granules, and thus able to dissociate and be in equilibrium with cations in the cytoplasm, (b) metals, sterically "trapped" in a non-toxic form in the centre of the developing granules (George, 1983). Active excretion of these residual bodies by exocytosis leads to metal elimination.

c) Inorganic granules and vesicles : Two major types of metal-containing granules, involved in heavy metal detoxification, are found, viz. Cu-sulphur-containing granules and calcium-containing granules. An association of Cu with sulphur has been seen in membrane-limited vesicles of oyster granular amoebocytes (Viarengo, 1989). Heavy metal cations are trapped in calcium-insoluble concretions as ortho- and pyrophosphates, e.g. in the kidney of scallops (Fowler, 1987). Kinetic relationships are likely to exist between these concentrations of metals and those of lysosomes and metallothioneins.

1.4 Metallothioneins in invertebrates.

The ubiquitous nature of metallothionein or MT-like metal-binding proteins (MLPs) in almost all major animal groups studied thus far is widely recognized (Roesijadi, 1981; Kägi and

Kojima, 1987; Engel and Brouwer, 1989). First reported to occur in invertebrates in a study on the American oyster *Crassostrea virginica* (Casterline and Yip, 1975), these proteins have been identified in most major invertebrate groups, including nematodes, annelids, molluscs, arthropods, pogonophora and echinoderms (Table 2). Most of these proteins in

Table 2 : Invertebrate Species for which MTs or MLPs have been reported (Riordan and Vallee, 1991).

Phylum	Class	Species and common name
Echinoderms	Echinoids	*Strongylocentrotus purpuratus* (sea urchin embryo)[b,c]
		Anthocidaris crassispina (sea urchin egg)[d]
	Asteroids	*Asterias rubens* (starfish)[e]
Pogonophora		*Riftia pachyptila* (hydrothermal vent worm)[f]
Arthropods	Insects	*Drosophila melanogaster* [g,h]
		Sarcophaga peregrina (fleshfiy)[i]
		Baetis thermicus (mayfly)[j]
	Crustaceans	*Scylla serrata* (crab)[k,l]
		Cancer magister (crab)[k]
		Cancer pagurus (crab)[m]
		Carcinus maenus (crab)[n]
		Callinectes sapidus (crab)[o]
		Rithropanopeus harrissii (crab larvae)[p]
		Homarus americanus (Amencan lobster)[q]
		Austropotamobius pallipes (crayfish)[r]
		Palaemon elegans (shrimp)[s]
		Acetes sibogae (shrimp)[k]
Molluscs	Bivalves	*Crassostrea virginica* (American oyster)[t]
		Crassostrea gigas (Japanese oyster)[u]
		Ostrea edulis (European oyster)[u]
		Ostrea lutaria (New Zealand oyster)[v]
		Mytilus edulis (blue mussel)[w]
		Mytilus galloprovincialis (Mediterranean mussel)[x]
		Macoma balthica (clam)[y]
		Protothaca staminea (clam)[z]
		Mercenaria mercenaria (clam)[aa]
		Calyptogena magnifica (clam)[ab]
		Placopecten magellanicus (scallop)[ac]
		Anodonta cygnea (freshwater clam)[ad]
		Unio elongatus (freshwater clam)[ad]
	Gastropods	*Crepidula fornicata* (limpet)[ae]
		Patella vulgata (limpet)[af]
		Patella intermedia (limpet)[af]
		Littorina littorea (marine snail)[ag]
		Helix pomatia (land slug)[ah]
		Cepaea hortensis (land slug)[ah]
		Arianta abustorum (land slug)[ah]
		Arion lusticanus (land slug)[ai]
Annelids	Polyplacophora	*Cryptochiton stelleri* (chitron)[k]
	Oligochaetes	*Eisenia foedita* (earthworm)[aj]
		Monopylephorus cuticulatus (marine worm)[ak]
		Limnodrilus hoffmeisteri (freshwater worm)[al]
	Polychaetes	*Alvinella pompejana* (hydrothermal vent worm)[f]
		Neanthes arenaceodentata (bristle worm)[am]
		Eudystylia vancouveri (fanworm)[an]
Nematodes		*Caenorhabditus elegans* (soil nematode)[ao,ap]

Note : [a, b, c,] etc. denote references.

invertebrate species remain uncharacterised. With the exception of a relatively early study by Olafson et al. (1979), which resulted in the purification of Cd-induced crab MTs and subsequent characterisation of their primary and secondary structures (Lerch et al., 1982; Otvos et al., 1982), procedures that have suitably purified invertebrate MTs for biochemical characterisation have only recently been reported (Overnell, 1986; Roesijadi et al., 1989; Brouwer et al., 1989; Slice et al., 1990 and Imagawa et al., 1990). Thus only a few invertebrate metal-binding proteins have been purified to the extent that their characterisation as MTs (criteria of Fowler et al., 1987) has been possible. As a result, the progress on the biochemistry and structure-function relationships for invertebrate MTs has occurred at a slower pace than that seen with higher animals. To date, MTs considered to belong to both class I and class II MTs have been identified among the invertebrates. Class I MTs are polypeptides with locations of cysteine closely related to those in equine renal metallothionein and class II are polypeptides with locations of cysteine only distantly related to those in equine renal metallothionein, such as yeast metallothionein. However, phylogenetic comparisons based solely on the amino acid sequences should be regarded with caution because apparently nonhomologous MTs may have a high degree of similarity in the metal responsive elements in noncoding sequences in the genome, e.g. comparisons with vertebrate class I MTs and sea urchin class II MTs (Harlow et al., 1989). Invertebrate MTs, as well as mammalian MTs, have also been reported to occur as isoforms. Isoforms contain the same number of cysteinyl residues, occupying the same positions along the polypeptide chain but differ in their electric charge (due to small variations of their amino acid composition) and hydrophobicity. They are named in the order of their elution from an anion-exchange column e.g. lobster MTs exist as three isoforms (I, II and III) (Brouwer et. al, 1989), two that are inducible and involved in metal detoxification and one that is not induced and can function as a copper donor for hemocyanin, the oxygen carrier protein in these animals. This is one of the few examples of direct evidence for MT involvement in a physiologically relevant function.

1.5 Nutritional and Physiologic Significance of Metallothionein.

Apart from metal control, synthesis of MT can also be induced, by many physiological and nutritional factors, including starvation and imposition of various types of physical or inflammatory stress. This has implied that the protein could have other physiological roles, such as in the acute phase response, the scavenging of free radicals, the regulation of cell differentiation and the storage of sulphur. However, as the list of proposed functions of MT grows, it becomes increasingly difficult to believe that any one protein, even one with such unique properties, could be so versatile. It seems more likely therefore that MT has some relatively basic functions, consistent with its highly conserved structure, the existence of an MT "housekeeping" gene, and the ease with which its synthesis can be induced by a plethora of metals, hormones and related factors.

Although MT binds to and its synthesis is induced by many metals, copper and zinc are the only ones of nutritional importance. The others, including cadmium and mercury, are nonessential metals , the cytotoxic effects of which appear to be reduced by binding to MT. Reduction of food intake increases liver MT concentrations, probably because of the influence of glucagon and other "stress" factors on MT synthesis (Bremner, 1987). Since the imposition of stress increases the circulating levels of glucocorticoids, they have been proposed as inducing agents for MT synthesis (Hager and Palmiter, 1981). Protein deficiency also increases liver MT concentrations, even though liver zinc concentrations are decreased, but its effects on kidney MT concentrations are variable and depend on the degree of protein deprivation (Bremner, 1990). Because of the high cysteine content of MT there has been some interest in the effects of dietary sulphur supply on MT production. Surprisingly, liver MTs

were increased in rats given sulphydryl-deficient diets, probably because of the reduction in their food intake, indicating that sulphur is not a limiting factor for MT synthesis (Sendelbach et al., 1990). Considerable interest has been shown in the possible role of MT as a free radical scavenger (Thornally and Vasak, 1985). Zinc-MT has been shown to scavenge hydroxyl radicals *in vitro* and to be more effective than glutathione in preventing DNA degradation by hydroxyl radicals (Abel and de Ruiter, 1989). However, although MT synthesis is induced in different types of oxidant stress, it does not prevent the oxidative damage to tissues (Arthur et al., 1987). Aerobic radiolysis of an MT solution induces metal loss and thiolate oxidation.

1.6 General Precautions during Purification of Metallothioneins.

a) Protection from oxidation : Although sulphydryl groups of the MT's cysteinyl residues are stabilised by co-ordination with heavy metals, this functional group is easily oxidised to form disulphide bonds intra- and intermolecularly. A reducing agent such as 2-mercaptoethanol, at a concentration of 0.5% (v/v), can be used to protect samples from air oxidation . It can also be used to reduce not only intermolecular but also intramolecular disulphide bonds in MTs because this agent is small in size and can reach the folded disulphide bonds to which other reducing agents, such as glutathione and cysteine, do not have access (Suzuki et al., 1980; 1981). Furthermore, 2-mercaptoethanol does not remove metals bound to MTs.

b) Displacement of metals in metallothioneins : Metals bound to MT are easily displaced by metals with higher affinities. Zinc in zinc-containing MT is replaced with Cd at the same molar ratio by adding Cd into the MT solution. Cd and Zn bound to MT are replaced by Cu.
Cupric ions are reduced to cuprous ions at the expense of sulphydryl groups, indicating that copper-containing MT produced by replacement of Cd and/or Zn with Cu is partially oxidised and contains disulphide bonds. Unintended replacement of Cd and Zn often occurs during isolation procedures due to the presence of contaminating cupric ions in buffer solutions and columns and also due to the presence of endogenous copper in biological samples.
MT binds heavy metals through the mercaptide bond (metal-sulphur bond) and heavy metals bound to MT are fully co-ordinated with sulphydryl groups. The disulphide bond does not serve as a ligand for heavy metals in MTs; therefore, formation of disulphide bonds means that the capacity of MT to bind heavy metals is decreased. When, for example, Cd- and Zn-containing MT is partially oxidised , Zn is always liberated from the oxidised MT as a result of its lower affinity for MT than Cd , thus giving an MT with a higher Cd:Zn ratio than the original one. The relative ratio of metals, such as the Cd:Zn ratio, seems to be related to the synthesis of MT in the organ (Suzuki, 1982) and may have other biological significance not yet understood.

c) Multiplicity of isoforms : MT is well separated from high- and low- molecular weight substances on a size-exclusion column, such as Bio-Gel P-10 but only elutes as a single peak. However, anion-exchange chromatography can then be used to separate the isoforms because of the differences in their electric charge and this can be followed by reverse-phase high performance liquid chromatography (RP-HPLC) for even further purification of sub-isoforms.

1.7 Principle of Size-Exclusion Chromatography.

The actual experimental conditions will be given in Section Two, so only the principle of the cytosolic separation technique will be given here.

One of the most useful and powerful tools for separating proteins from each other on the basis of size is *molecular-exclusion* chromatography, also known as *gel-filtration* or *molecular-sieve* chromatography. In size-exclusion chromatography, the mixture of proteins, dissolved in a suitable buffer (eluent), is pumped through a column packed with beads of an inert, highly hydrated polymeric material that has previously been washed and equilibrated with the buffer alone. Common column materials are Sephadex, the commercial name of a polysaccharide derivative; Bio-Gel, a commercial polyacrylamide derivative (which we used) and agarose, another polysaccharide - all of which can be prepared with different degrees of internal porosity. In the column, proteins of different molecular size penetrate into the internal pores of the beads to different degrees and thus travel down the column at different rates (Figure 3). Very large protein molecules cannot enter the pores of the beads ; they are said to be excluded and thus remain in the excluded volume of the column, defined as the volume of the aqueous phase outside the beads (Void volume). On the other hand, very small proteins can enter the pores of the beads freely. Small proteins are retarded by the column while large proteins pass through rapidly, since they cannot enter the hydrated polymer particles. Proteins of intermediate size will be excluded from the beads to a degree that depends on their size ; hence the term *exclusion* chromatography. An "unknown" eluting MT can be identified, when standards of known molecular weight are used for column calibration since each standard has a characteristic elution volume (Ve). The resolving power of molecular-exclusion chromatography is so great that this simple method is now widely used as a way of determining the molecular weight of proteins (Lehninger, 1975) and is used in this case to identify whether, or not, the proteins in the heat-treated *Macoma balthica* cytosol fall within the typical size range of metallothioneins (~ 6000-12000 Daltons).

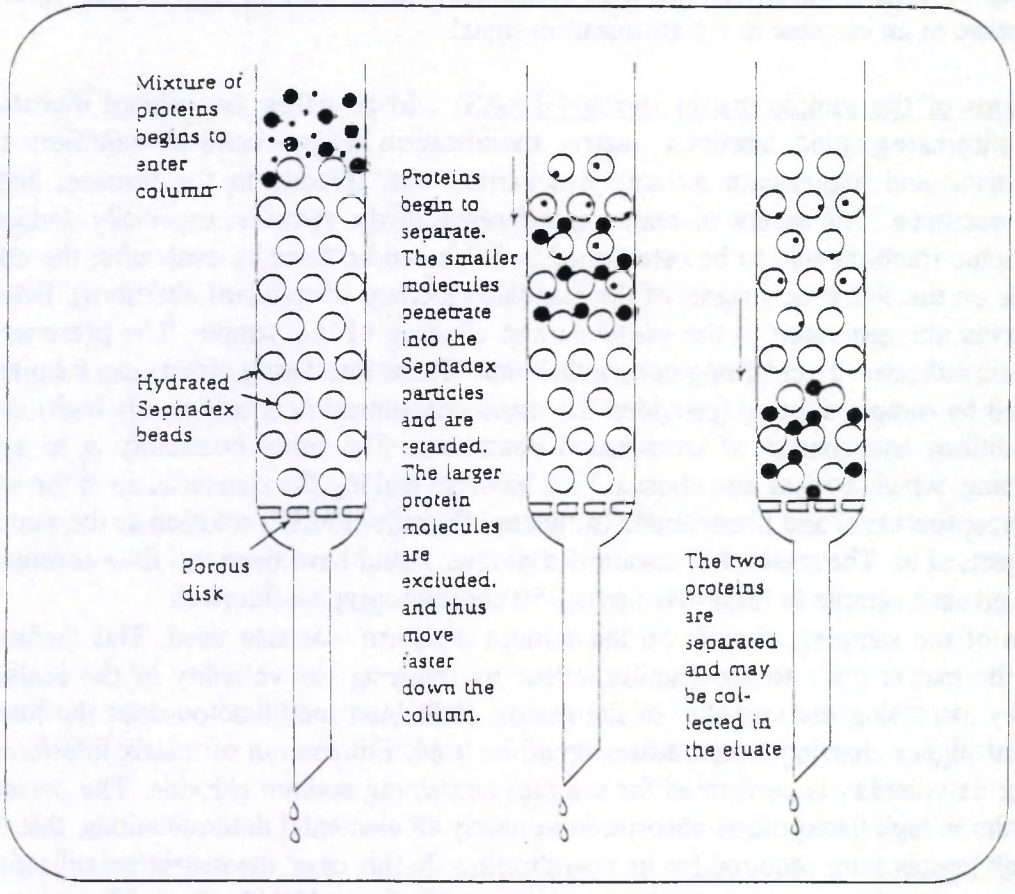

Figure 3 : Separation of two proteins of different size on a Sephadex column.

1.8 Background on the metal quantitation procedures.

The principle of atomic absorption spectrometry itself will not be covered here, however the possible problems associated with such measurements and ways of dealing with them will be covered. The measurement of metals in MT preparations in various stages of purification can be carried out using either flame (FAAS) or more sensitive electrothermal atomic absorption spectrometry (ETAAS). To decide which of these methods is more suitable for a given determination it is necessary to consider such factors as the expected concentration of the metal, the amount of sample available and the nature of the matrix. The FAAS technique is less susceptible to various interference effects but requires considerably larger volumes and is ~100-fold less sensitive than ETAAS. Although there are a number of potential interference effects in metal analysis by ETAAS, most of them can be avoided by appropriate sample preparation and chemical modifications of the sample matrix.

-Chemical and matrix interferences : The ETAAS technique is highly sensitive to chemical and matrix interference effects. Chemical interference occurs when the metal ion of interest combines with some other component(s) of the sample matrix to form a compound of high boiling point and low volatility. As a result the number of neutral metallic atoms generated during atomization under standard conditions is reduced and there is a decrease in signal peak height. Matrix interferences arise when the physical and chemical characteristics of the sample differ considerably from those of the reference solution, causing a change in the metal atomization rate. This type of interference is most commonly observed in samples containing large amounts of organic materials and high concentrations of salts or acid. It can result in either a decrease or an increase in the atomization signal.

- Modifications of the sample matrix (using ETAAS) : In all cases, i.e. diluted digests or undigested chromatographic fractions, matrix modification using chemical modifiers (i.e. palladium nitrate and magnesium nitrate) was carried out, directly in the furnace, before atomization occurred. The extent of matrix interference in the samples, especially undiluted chromatographic fractions had to be established and this can be done by evaluating the effect of the sample on the absorption signal of the standard (method of standard additions). Briefly, standard curves are generated in the presence and absence of the sample. The presence of interferences is indicated by obtaining nonparallel lines. These interfering effects can frequently be eliminated by sample dilution (provided the metal concentration is sufficiently high) or by chemical additions and change of atomization conditions. The other possibility is to apply matrix matching, which is what was chosen. This involves making the standards up in the same buffer, 2-mercaptoethanol and phenylmethylsulphonid fluoride (PMSF) solution as the samples were homogenised in. The method of standard additions would have been too time consuming to carry out on each sample as there were over 350 chromatographic fractions.
Modification of the samples, directly on the furnace platform was also used. This facilitates removal of the matrix prior to atomisation, either by reducing the volatility of the analyzed element or by increasing the volatility of the matrix. Palladium modification does the former for Cd so that higher charring temperatures could be used. Elimination of matrix interference by increasing its volatility is performed for samples containing sodium chloride. The presence of NaCl results in high background absorption in nearly all elemental determinations, due to a relatively high temperature required for its volatilisation. In this case, the matrix volatilisation temperature can be decreased by adding ammonium nitrate, which leads to formation of volatile sodium nitrate and ammonium chloride.

Once the molecular size of the MT has been estimated, by size-exclusion chromatography and once the quantities of metals bound to the MT have been obtained, by ETAAS analysis of the

chromatographic fractions, the process of MT quantitation in the fractions and in the unseparated cytosol can be carried out.

1.9 Electrochemical Detection of Metallothionein.

Due to a lack of a measurable biological activity the quantitation of MT has proven to be problematical and investigators have been forced to consider unique structural aspects of the molecule in order to provide analytical procedures. Thus efforts to detect MT have relied heavily on estimates of metal binding and sulphydryl content, or on immunological assays. Electrochemical quantitation of MT by differential pulse polarography (DPP) is a technique that takes advantage of the high cysteine content of MT. Capable of pico-mole sensitivity , the technique is very rapid, highly reproducible and species independent, providing a useful procedure for following MT concentrations in the cytosol (whole or fractionated).
First described by Brdicka in 1933 and further investigated by Palecek and Pechan (1971), the method was originally adopted for MT analysis by Kehr (1973). The technique was subsequently refined by Olafson (1979;1981), Thompson and Cosson (1984), Olsson and Haux (1986) and I have used the method of "simplex optimisation" to obtain conditions giving best combined sensitivity, resolution and reproducibility while sacrificing individual optimums of each of these parameters.

- **Principles :** Voltammetry comprises a group of electrochemical procedures based on the potential current behaviour of a polarizable electrode in an analyzed electrolyte. Theoretically any species can be analysed if it undergoes oxidation or reduction within the working potential range of the electrode system employed.The redox reaction taking place at the electrode is therefore controlled by variation in the applied electrode potential. The DPP procedure involves the use of a dropping mercury electrode (DME) where the difference between two current measurements taken at two times is repetitively made at a renewed mercury droplet while the instrument scans a potential range.
The basis of the Brdicka DPP assay for protein is the generation of catalytic double waves from SS/SH-containing proteins in a hexamminecobalt chloride supporting electrolyte. Although the complex electrochemical reactions at the electrode have not been explained in detail, it is generally accepted that cobalt complexes with ionized sulphydryl groups catalyzing the evolution of protons (Kuik and Krassowski, 1982). Brdicka (1965) has reported that on decomposition of metallic cobalt from the complex into cobalt amalgam, the ionised sulphydryl groups take up protons from the supporting electrolyte. It is proposed that the co-ordination bond to the sulphur anion remains preserved on deposition of cobalt, facilitating proton reduction. The wave height is therefore a function of the number of thiol groups adsorbed on the electrode surface and is controlled by the recombination rate of ionized sulphydryl groups and protons (Figure 4). It is significant to note that the electrochemical reactions occur regardless of whether the sulphur-containing side chains are thiols, disulphides or complexed with metals.

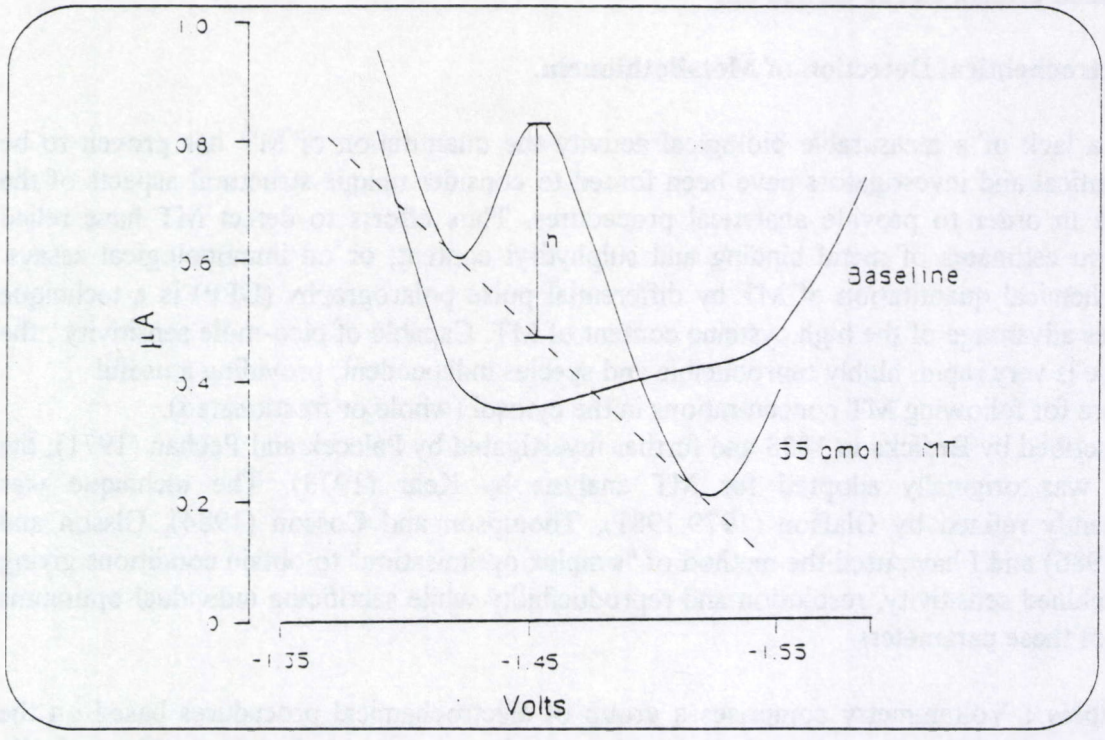

Figure 4 : Polarographic scan showing an electrolyte baseline and the peak resulting from 55 pmol of purified rainbow trout MT-I. [Reprinted from P -E. Olsson, 1987].

Since DPP quantitation of MT is not a specific procedure, as in the case of Radioimmunoassay (RIA), it is critical that the contribution of other cysteine-containing proteins be determined and eliminated before making determinations from complex samples. In our case, as in most cases, this was achieved by heat denaturation, leaving the heat-stable MT in solution (Figure 5). Although MT is not the only heat-stable protein remaining in solution, it is usually the only species left having significant polarographic activity.

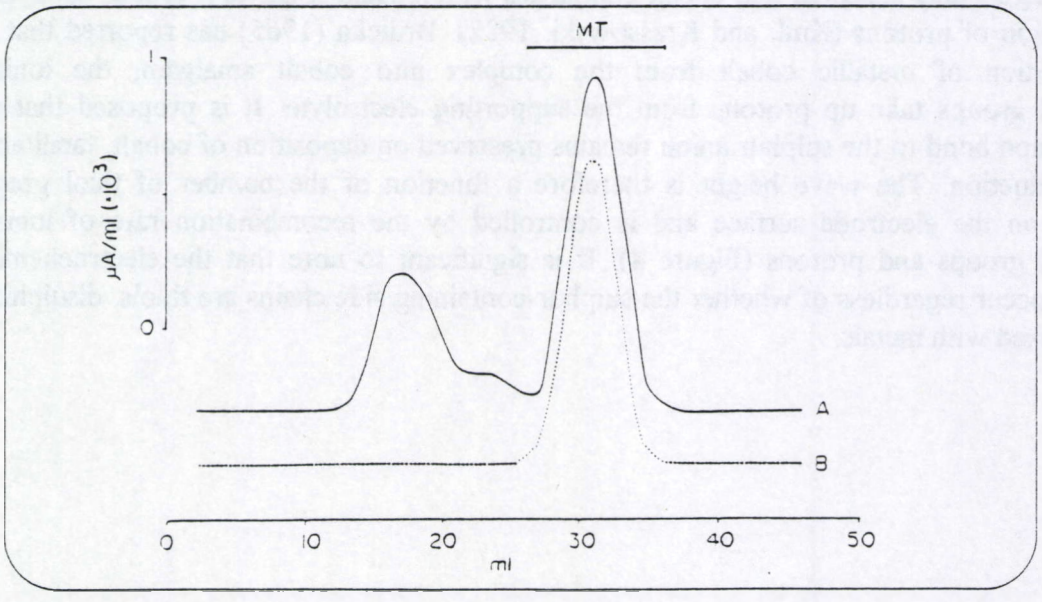

Figure 5 : Sephadex G-75 chromatography of rainbow trout supernatant before (A) and after (B) heat denaturation at 95° for 5 minutes. [Reprinted from P -E.Olsson, 1987].

As with all non-specific methods, further verification of the identity of uncharacterised MTs may require ion-exchange chromatography and amino acid analysis. Members of our Analytical Chemistry group, such as Dr. Vesa Virtanen is presently working on the optimisation of an electrophoretic method for MT isoform isolation ; Dr. Adela Rodriguez has been considering the idea of using Liquid Scintillation Counting (LSC) for the determination of the number of sulphydryl groups (LSC involves the counting of light flashes, produced in a scintillator crystal e.g. sodium iodide, when radiation strikes a metal-sulphide) and Dr. Guy Bordin and Fernando Cordeiro Raposo are working on the HPLC method of MT isoform isolation.

The presence of low-molecular weight sulphydryl-containing species, such as glutathione and free cysteine in tissue samples, does not interfere with the DPP assay, as concentrations of the former compound an order of magnitude above that found in tissues had no effect on the polarographic response to standard MT (Olafson, 1991). This appears to be due to an unusually low response to low-molecular weight compounds (Table 3).

Table 3 : Wave potentials and heights for selected sulphydryl compounds.

Compound	Wave potential (V vs. $Ag^+/AgCl$	Wave height (µA/µmol)
Crab[a] Cd - MT-I	-1.47	555
Bovine serum albumin	-1.44	975
Synthetic peptide[b]	-1.43	7.86
Glutathione	-1.41	0.17
Insulin	-1.52	15.31
2-Mercaptoethanol	-1.46	0.01
Cysteine	-1.41	1.13
Cystine	-1.39	1.92

Note : [a] *Scylla serrata* ; [b] Ser-Cys-Thr-Cys-Thr-Ser-Ser-Cys-Ala.

- Principle of Simplex Optimisation :

Since analytical techniques, such as DPP, are concerned with detecting minute traces of analyte, it is usually important that the levels of the factors on which the response depends are chosen so as to maximise this response. The process of finding these optimum factor levels is known as optimization. The first step is to determine which factors and which interactions between them are important in affecting the response. Simplex optimization may be applied when all the factors are continuous variables. This is the case in our DPP method (Brdicka electrolyte). [It is perhaps important to point out, at this stage, that the choice of name for this method of optimization is unfortunate since the method is different from the simplex methods used in linear programming]. The variables I considered are : Electrolyte concentration ; pH ; Temperature ; Pulse height ; Drop interval and Scan increment.

A **simplex** is a geometrical figure which has $n+1$ vertices when a response is being optimized with respect to n factors. For example, for two factors, the simplex will be a triangle. The method of optimization is illustrated by Figure 6. The initial simplex is defined by the points labelled 1, 2 and 3. In the first experiment the response is measured at each of the combinations of factor levels given by the vertices of the simplex. The worst response would

be found at point 3 and it would be logical to conclude that a better response would be obtained at a point which is the reflection of 3 with respect to the line joining 1 and 2, i.e. at 4. The points 1, 2 and 4 form a new simplex and the response is measured for the combination of factor levels given by 4. Comparing the responses for the points 1, 2 and 4 will show that 1 gives the worst response. The reflection procedure is repeated to give the simplex defined by 2, 4 and 5. The continuation of this process is shown in Figure 6. It can be seen that no further progress is possible beyond the stage shown, since points 6 and 8 both give a worse response than 5 and 7.

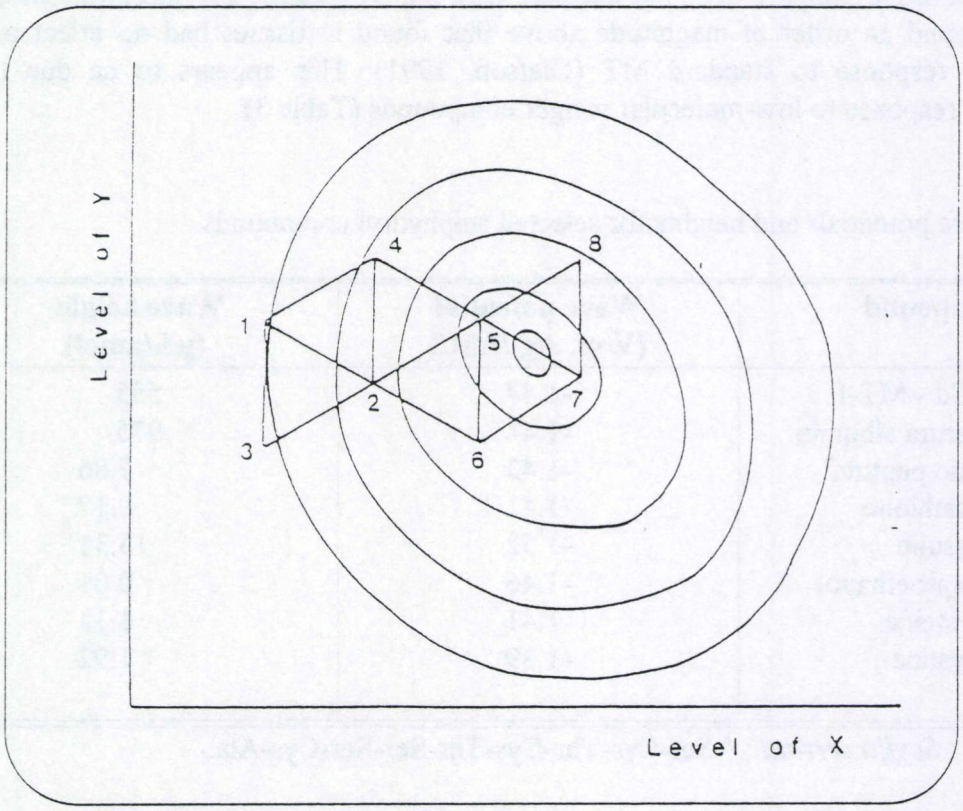

Figure 6 : Simplex optimization.

In order to improve the performance of the simplex method various modifications have been (and are still being) proposed (Miller and Miller, 1993). The advance towards the maximum can be accelerated by using a simplex which can vary in size according to how the response for the new vertex in a simplex compares with the other vertices. Initially the simplex is large to give rapid progress towards the the maximum; near the maximum it contracts to allow the maximum to be found accurately and to avoid the situation shown in Figure 6, where further reflection points of the same magnitude, were only going further away from the optimum.
The position of the new vertex of a simplex can be found by calculation rather than drawing - this is essential when there are more than two factors. The calculation is most easily set out in a table, as shown in Table 4. In this example there are 5 factors and hence the simplex has 6 vertices. The response for vertex 4 is lowest and so this vertex is to be rejected.

Table 4 : An example of the method of Simplex Optimization.

Vertices	Factors					
	A	**B**	**C**	**D**	**E**	**Response**
Vertex 1	1.0	3.0	2.0	6.0	5.0	7
Vertex 2	6.0	4.3	9.5	6.9	6.0	8
Vertex 3	2.5	11.5	9.5	6.9	6.0	10
Vertex 4	*2.5*	*4.3*	*3.5*	*6.9*	*6.0*	*6*
Vertex 5	2.5	4.3	9.5	9.7	6.0	11
Vertex 6	2.5	4.3	9.5	6.9	9.6	9
(i) Sum (excluding vertex 4).	14.50	27.40	40.00	36.40	32.60	
(ii) Sum/n (excluding vertex 4).	2.90	5.48	8.00	7.28	6.52	
(iii) Rejected vertex (i.e. 4).	2.50	4.30	3.50	6.90	6.00	
(iv) Displacement = (ii) - (iii).	0.40	1.18	4.50	0.38	0.52	
(v) Vertex 7 = (ii) + (iv).	3.30	6.66	12.50	7.66	7.04	

The co-ordinates of the centroid of the vertices which are to be retained are found by (i) summing the co-ordinates for the retained vertices and (ii) dividing by the number of factors, *n*. The displacement of the new point from the centroid is given by (iv) = (ii) - (iii) and the co-ordinates of the new vertex, vertex 7, by (v) = (ii) + (iv). If the simplex is to be varied in size then the values in row (iv) are multiplied by a suitable scaling factor.

An obvious question in using the simplex method is the choice of the initial simplex. If this is taken as a *regular* figure in *n* dimensions, then the positions taken by the vertices in order to produce such a figure will depend on the scales used for the axes. These scales should be chosen so that unit change in each factor gives roughly the same change in response. If there is insufficient information to achieve this, the difference between the highest and the lowest feasible values of each factor can be represented by the same distance. The choice of the size of the initial simplex is discussed in a paper by Yarbro and Deming (1974), who show that the size of the initial simplex is not critical if it can be expanded or contracted as the method proceeds. Yarbro and Deming quote an algorithm which can be used to calculate the initial positions of the vertices; one vertex is normally positioned at the currently accepted levels of the factors.

Once an optimum has been found, the effect on the response when one factor is varied while the others are held at their optimum levels can be investigated for each factor in turn. This procedure can be used to check the optimization. It also indicates how important deviations from the optimum level are for each factor: the sharper the response peak in the region of the optimum the more critical any deviation in the factor level.

The values obtained for the simplex optimization of the Brdicka method will be given in the Results section.

2.0 Methodology.

2.1 Sample Collection.

Samples of *Macoma balthica* were collected every two months, between March 1993 and March 1995 (i.e. Set 19 to 31) from two locations, Baalhoek (B) and Paulinapolder (PP), (see Figure 1). Each set comprised of two sub-sets, i.e. natural and spiked. See section 2.4 for a description of sample labelling. Baalhoek, being more upstream, has higher metal

concentrations in the water column, in the suspended matter and in the sediments than Paulinapolder, which is closer to the mouth of the estuary (Van den Berg et al., 1987 ; Regnier and Wollast, 1993).

M. balthica can be found at low tide, by shovelling some of the top 14 cm of the mud onto a net of the correct mesh size (5 mm diameter) and then washing this in the estuary. After some minutes, one is left with the bivalves (among other organisms, such as annelids, nematodes, juvenile crabs etc.). The *Macoma balthica* can then be sorted from the other bivalves on a sieve (5 mm pore diameter) and can be easily recognised because of their size (12 mm to 22 mm), shape and variety of colours. The number of *Macoma balthica* collected from each station is confined for practical reasons (quite a lot of work is required to collect 300 individuals from each station) and environmental reasons (not wishing to disturb the bivalve balance in the mudflats). Thirty litres of Scheldt water are also collected and the water temperature measured. The clams are then placed in a plastic bag and later transferred to a plastic container filled with Scheldt water. A "cool box" is used for overnight storage in the summer.

2.2 Sample preparation.

Having allowed the *M. balthica* to depurate undigested matter in filtered estuarine water for 24 hours the clams are either immediately deshelled and undergo procedure 2.2.1 and 2.2.2 or are placed in aquaria for short-term exposure to a mixed metal spike.

- *In vitro* exposure of bivalves to heavy metals :

Sets of 100 individuals were kept in plastic tanks containing 500 ml estuarine water previously spiked with cadmium (100 $\mu g.l^{-1}$ added as $CdCl_2$), copper (100 $\mu g.l^{-1}$ added as $CuCl_2$) and zinc (600 $\mu g.l^{-1}$ added as $ZnCl_2$). The clams remain exposed for 3 days in a static system with the water being totally replaced every day. They are then deshelled and divided into two groups, one group being treated as described in 2.2.1 and the other being treated as described in 2.2.2.

2.2.1 Pooled bivalves for "total" metal analysis.

This procedure was described by Bordin et al. (1992) giving full detail on the optimisation of the various parameters.

- Deshelling :

This procedure is carried out the morning after collection, for the unspiked bivalves and after three days exposure to the mixed spike, for the others. To remove the tissue, the shell is prised open, with a plastic knife and then the tissue is placed in a pre-weighed plastic petri dish, with lid, in a laminar flow bench (Woerden Clean Air - Type CLF 490). As well as the wet weight of clams in the dish, the number of clams that this involves, must be recorded to enable the calculation of the average body weight of the bivalves for each sampling period and at each sampling location.

- Freezing :

A petri dish, containing a minimum of 4 g (usually ~8 g) of wet *Macoma balthica* tissue from the relevant station i.e. one dish from Baalhoek and another from Paulinapolder, are placed in a deep freeze (AEG Arctis Jumbo) at ~-35 °C for a minimum of 24 hours. This is a necessary step in the preparation for freezedrying.

- Freezedrying :

Lyophilisation is one of the most suitable methods for removing moisture from biological samples without losses of trace metals. This is carried out in a Leybold-Heraeus Lyovac GT2 freezedryer at 36 °C for 72 hours. On completion of the drying process, the vacuum is broken by using dry N_2 to avoid moisture uptake from the air. The dishes are then once again weighed to allow the estimation of the loss of moisture on freezedrying.

- Homogenisation :

This is carried out in a Spex 8000 Mixer/Mill. A fine homogenous powder results after mixing in a teflon vial with ceramic balls for 10 minutes. The powder is then transferred to brown glass bottles (10 ml volume) on the clean bench and stored in a cupboard with an overpressure of nitrogen (Laflow) awaiting pressurised digestion to enable "total" metal analysis by AAS.

- Pressurised digestion :

Approximately 0.1 g (precisely weighed) of the dry powder is placed in an acid cleaned teflon cup with lid.

Three ml concentrated HNO_3 (suprapur) are then added and the mixture left to stand for 2 hours.

A reagent blank is digested as well as the samples, every time.

The teflon cup is then placed in its Parr bomb housing and the bomb lid is firmly secured.

Finally a pressurised digestion, in a conventional laboratory oven (Heraeus T6030), is carried out under the following conditions :

130 °C for 3 hours followed by 150 °C for 7 hours.

The resulting solution is then transferred, with bidistilled water, to a 10 ml. volumetric flask, made up to volume and stored, for eventual AAS analysis, in a plastic bottle (acid cleaned polyethylene).

The experiments carried out to select the best digestion method (i.e. whether conventional oven or microwave and under which conditions (time/temperature)) were described in our earlier paper (Bordin et al., 1992). The digestion efficiency was checked by submitting a Community Bureau of Reference (BCR) certified reference material (CRM 278, *Mytilus edulis*) to different digestion procedures until an optimum recovery (96-99 %) was obtained. The procedure chosen leads to highly satisfactory data for the four elements, as shown in Table 5. The validity of this sample preparation method was regularly checked (for most sets of *Macoma balthica* digested thereafter).

Table 5 : Comparison between certified and experimental metal concentrations in the CRM 278 *Mytilus edulis* reference material (Bordin et al., 1992).

Element	Copper	Cadmium	Iron	Zinc
Certified value (µg/g)	9.60 ± 0.16	0.34 ± 0.02	133 ± 4	76 ± 2
Experimental value (µg/g)	9.51 ± 0.12	0.33 ± 0.03	130 ± 3	73 ± 9
Accuracy (%)	99.1	97.1	97.7	96.1

2.2.2 Extraction of the soluble phase of the tissues.

This procedure, also referred to as the partitioning procedure, leads to the separation of the bivalve tissues into three phases: the insoluble fraction, the high molecular weight proteinic fraction (HMWP) and the heat stable cytosol (Bordin et al., 1994).

- Partitioning :

The first step is to homogenise the wet bivalve tissue and since the eventual purpose is to extract MTs, if present, this homogenization is carried out in an atmosphere of nitrogen or else in a reducing medium. Relative ratios of buffer volumes to sample weights depend on the organs and contents of MT; 4 volumes (v/w) of buffer / mercaptoethanol / phenylmethylsulphonid fluoride (PMSF) solution are used in our case. Also, the recovery of MT is reduced if low volumes of buffer solution are used for homogenization because of the risk of loss of MT by coprecipitation in the heat-denaturing step, if too much tissue and not enough homogenisation solution are used. As MTs in homogenates are easily degraded enzymatically, homogenization must be carried out using ice-water cooling. PMSF is used as a protease inhibitor (Ridlington and Fowler, 1979).

Therefore, approximately 5 g of pooled bivalve tissue (either "natural" or after "spiking") are introduced into a centrifuge tube placed in an ice bath and homogenised by ultrasound (Vibra Cell 72454) in an extracting solution containing 5 mM 2-mercaptpethanol and 0.1 mM PMSF. The homogenate is then centrifuged at 5000xg for 2 hours in order to remove particulate matter. The resulting supernatant is then transferred into a new centrifuge tube and heated in a water bath at 80 °C for 20-30 minutes to precipitate the heat-unstable proteins, leaving the heat-stable MT intact in the soluble fraction. This is followed by a cooling off period in an ice-bath. A second centrifugation step of 5000xg for 1.5 hours is then performed in order to remove the high molecular weight (HMWP) heat-unstable proteins and lipids making it easier to use the final supernatant as a source for further purification (i.e. on a size-exclusion column). Better chromatographic separation of MT is assured for samples of lower protein content (i.e. of a simpler matrix), such as a heat treated supernatant.

The final supernatant (the cytosol) and the two precipitates, resulting from the two centrifugation steps, are then frozen at -30 °C. The precipitates (pellets) then undergo freezedrying and digestion, as already described, before metal analysis by AAS. The cytosol is thawed and divided into two, with metal (by AAS) and MT (by DPP) analyses being performed on one part while the other is separated into 14 fractions by size-exclusion chromatography for eventual metal analysis by AAS and MT analysis by DPP.

Figure 7 provides an overview of what is involved once the *M. balthica* have been collected from the estuary and deshelled.

Procedure 2.2.1 :

- Total Heavy Metal Analysis.

- Freeze
- Freezedry
- Homogenise
- Digest
- AAS metal analysis

Procedure 2.2.2 :

- Partitioning.

- Ultrasonic homogenisation
  - Centrifuge
  - Freeze 1st. precipitate (insoluble fraction)
  - Heat treat the supernatant
    - Centrifuge
    - Freeze 2nd. precipitate (incl. soluble proteins)
    - Freeze cytosol

Cytosol ..... + ... 1st.&2nd.ppts.

- AAS analysis
- DPP analysis
- Chromatographic separation
  - AAS analysis
  - DPP analysis

- Freezedry
- Homogenise
- Digest
- AAS analysis

Figure 7 : Summary of the entire laboratory method.

2.2.3 Fractionation of the cytosol by Size-Exclusion Chromatography (SEC).

a) Instrumentation : Gel filtration chromatography of the bivalve cytosol was carried out using an automated Econo-System (Bio-Rad Laboratories) consisting of a model EP-1 peristaltic pump, a model EM-1 UV monitor, a model 2110 fraction collector, a dual pen recorder (model 1327), an ES-1 peak collection controller, an EG-1 gradient elution monitor, buffer selector and a low pressure econo-column, from Bio-Rad (28 x 1 cm i.d.). A 1000 µl injection loop was used.

b) Reagents : The column was filled with a hydrophilic polymer, polyacrylamide P-10 medium particle size gel (obtained from Bio-Rad) with size exclusion limits of 1500 to 20000 Daltons. Column calibration was carried out using proteins of known molecular weight, namely aprotinin from bovine lung (Mw : 6500 Da ; Lot 21H9472), cytochrome C from horse heart (Mw : 12400 Da ; Lot 11H9451), both obtained from Sigma Chemical Co., equine myoglobin (Mw : 17000 Da) and cyanocobalamine (Mw : 1350 Da), both obtained from Bio-Rad. Blue dextran (Mw : 2,000,000 Da ; Lot 62H0796), from Sigma, was used to calculate the void volume. The eluent used was a mixture of Na_2HPO_4 (0.1 M) and NaH_2PO_4 (0.1 M) ; pH = 6.8.

c) Procedure : A volume of 1 ml of filtered (0.45 µm) cytosol is injected into the loop and eluted with the buffer at a speed of 0.3 $ml.min^{-1}$. Fractions of 2 ml are collected, with their absorbance at 254 nm being recorded. This procedure is repeated three times, so as to have enough volume for eventual metal analysis by AAS and MT analysis by DPP. The fractions, including a blank, which is simply the eluent (buffer) after having passed through the column, are stored at ~ 5°C while awaiting measurement. UV spectra of some sets of fractions were recorded on a Perkin-Elmer Lambda 7 spectrophotometer.
Selecting the proper column size and flow rate yields a better separation of MT. Overloading and faster flow rate generally result in poorer separation. The gel should be packed after a complete degassing procedure: the gel, swollen in an elution buffer, is degassed completely in a round flask under reduced pressure, using an aspirator at 80°. The elution buffer should also be degassed to keep the column material free from air and to protect MT from oxygen.

2.3 Measurements.

- Metal analysis by Atomic Absorption Spectroscopy (AAS).

Total metal in the pooled bivalves as well as metal concentrations in the three phases resulting from the soluble fraction extraction were measured by AAS.

a) Instrumentation : This was accomplished using a Perkin-Elmer Model 5100 PC AAS equipped with a Zeeman Furnace (model 5100 ZL), with L'vov platform, as well as a conventional dual-option (air acetylene and nitrous oxide acetylene) burner system. An optical interface connects the conventional AAS to the Zeeman furnace module. Two ways of correcting for background absorption are available, namely zeeman-effect and continuum source background correction. Our Perkin-Elmer zeeman module employs a magnetic field strength of approximately 0.9 tesla, which shifts the lines about 0.01 nm to each side of the original wavelength thereby allowing the discrimination between atomic and non-atomic background absorption, as it only affects atomic species. Measurements by flame involved

Continuum source background correction, with a deuterium arc being used for the far UV region and a tungsten-iodide lamp for the near UV and visible regions of the spectrum.

b) Reagants : Titrisol (Merck) metal standards were used for establishment of calibration curves. A multielement standard, CRDL, from Perkin-Elmer was used as a reference for accuracy control and the CRM 278, *Mytilus edulis*, was also used for this purpose (see Table 5). $Pd(NO_3)_2$ and $Mg(NO_3)_2$ matrix modifiers, obtained from Perkin-Elmer, were also used for stabilisation of the analyte on the furnace platform.

Table 6 : Optimised parameters for ETAAS analysis.

Instrument conditions	**Element**	
	Cu	**Cd**
Wavelength (nm)	324.8	228.8
Spectral band pass (nm)	0.70 Low	0.70 Low
Hollow cathode lamp current (mA)	15	4
Background correction.	Zeeman	Zeeman
Matrix modification (µg)	5µg Pd + 3µg Mg	10 µg Pd
Sample injection volume (µl)	15	15
Furnace programmes :		
Dry 1		
Ramp (s)	1	1
Hold (s)	20	20
Temperature (°C)	110	110
Gas (ml min^{-1})	250	250
Dry 2		
Ramp (s)	5	5
Hold (s)	35	35
Temperature (°C)	130	130
Gas (ml min^{-1})	250	250
Char		
Ramp (s)	10	10
Hold (s)	20	20
Temperature (°C)	1200	600
Gas (ml min^{-1})	250	250
Atomise		
Ramp (s)	0	0
Hold (s)	5	3
Temperature (°C)	2100	1450
Gas (ml min^{-1})	0	0
Clean		
Ramp (s)	1	1
Hold (s)	3	3
Temperature (°C)	2400	2400
Gas (ml min^{-1})	250	250

*c) **Procedure** :* Copper and cadmium measurements were carried out using Zeeman electro-thermal AAS at 324.8nm and 228.8nm respectively ($Pd(NO_3)_2$ and $Mg(NO_3)_2$ matrix modification used), while Zn and Fe measurements were done in an air-acetylene flame (using a flow spoiler for Zn and an impact bead for Fe) at 213.9nm and 248.3nm respectively.
The detection limit, calculated according to the IUPAC definition (Long and Winefordner, 1983), is expressed as $3S_B/m$, where S_B is the absolute standard deviation of blank measurements (in our case 15 repetitions were used) and m the analytical sensitivity. Our achieved detection limits of Cu, Cd, Zn and Fe were therefore 0.51, 0.05, 9.0 and 34.0 $ng.g^{-1}$ respectively. Table 6 above shows the instrument conditions for optimum analysis of Cd and Cu by ETAAS.

Table 7 shows the standard conditions used for flame AAS of iron and zinc, in both cytosolic and chromatographic fraction samples as well as in the whole tissue digests.

Table 7 : Standard conditions for FAAS

Instrument conditions	Element	
	Fe	**Zn**
Wavelength (nm)	248.3	213.9
Spectral band pass (nm)	0.20 High	0.70 High
Hollow cathode lamp current (mA)	30	15
Background correction	Deuterium Arc	Deuterium Arc
Flame Type	Air / Acetylene	Air / Acetylene
- Air flow (L/min.)	10.0	10.0
- Acetylene flow (L/min.)	2.0	2.0
Flame Temperature (°C)	2125-2400	2125-2400
Impact device	Impact bead	Flow spoiler

- Metallothionein (MT) analysis by Differential Pulse Polarography (DPP).

Differential pulse polarographic assay for -SH compounds based on the Brdicka reaction (Brdicka, 1933), was used in the determination of MT in all 13 sets of heat-treated cytosol. An estimate of the relative thiolic content of individual chromatographic fractions (14 in each set) of the cytosol of Set 19 (March 1993) was also carried out. In the absence of a *Macoma balthica* metallothionein standard, quantitation of MT in the cytosol was based on rabbit liver MT-I and MT-II (Sigma), using the method of standard additions for each of the 52 cytosol samples. Concentrations of MT were determined as $mg.g^{-1}$ dry weight of tissue initially homogenised.
The thiolic content of individual column fractions of chromatographed cytosol from Set 19 (March 1993) have been expressed as $\mu g.ml^{-1}$ MT.

*a) **Instrumentation** :* The assay was accomplished using an EG&G Model 384B Polarographic Analyzer, an EG&G Model 303A Electrode housing and a Houston DMP-40 Plotter.
The three electrode system consists of a bevelled capillary working electrode, a platinum counter electrode and a Ag/AgCl reference electrode. Thermostatic control is obtained using an EG&G water jacketed cell bottom with cooling water being supplied by a temperature controlled circulating bath (Ministat, Huber).

b) Reagents : The Brdicka electrolyte, consisting of 0.86 mM Hexamminecobalt(III)chloride ($Co(NH_3)_6Cl_3$) obtained from Sigma and buffered to pH 9.5 with 1 M solution of NH_4OH and NH_4Cl, obtained from Merck Suprapur, is prepared weekly and kept immersed in the cooling bath during experimental work or refrigerated at 3-5°C while not being used.
Triton X-100 (Merck pro analyse) is used to suppress Co maxima and minima and generally 700 µl of a $2.5x10^{-2}$ % (v/v) working solution is required, resulting in $2.5x10^{-2}(0.7) = X(10.7)$ i.e. a concentration of $1.6x10^{-3}$ % Triton X-100 in 10 ml electrolyte. Hexadistilled mercury (Grade 9N; Rhône-Alpes Mercure) is used in the mercury reservoir. Purging is achieved with 99.9999% N_2 (Air Liquide N60). Protein standards are prepared from commercially available RL MT II (Sigma Lot 9049605) with a working standard concentration of 7261 $\mu g.l^{-1}$ (or 1.19 µM MT given that Mw MT = 6100 Da) and this was used for standard additions to samples from Set 19 to 27. RL MT I (Sigma Lot 93H9559) with a working standard concentration of 7014 $\mu g.l^{-1}$ (or 1.15 µM MT) was used for standard additions to samples from Set 28 to 31. The standard used was changed for practical reasons only as when the RL MT II was used up, the RL MT I was still fresh and since these solutions were prepared by our metrology department (weighings within ± 0.003 mg for the MT powder and within ± 0.05 g for the diluent) and since the peak potential did not change, it was considered "safe" to use it.

c) Procedure : 10.0°C is established in the cell cooling system.
Instrument settings are as given in Table 8, however the initial potential is only kept at -1.000V while checking that the Co maxima and minima have disappeared (i.e. that enough Triton X-100 has been added to the 10 ml of electrolyte). Once the blank has been "run" and appears satisfactory and reproducible, the initial potential should be set to -1.250 V and the blank run again, as if this isn't done, the scale to accomodate the high Co peak at -1.16 V would mask the MT peaks (standards at -1.50 V and filtered cytosol MT peaks at -1.47 V and chromatographic fraction MT peaks between -1.47 and -1.48 V).
Therefore the blank contains 10 ml fresh cool electrolyte and 0.700 ml of the Triton X-100 working solution.
Standards : Successive additions of 50 µl of the RL MT (I or II) working solution are made to the blank in the same cell. This results in a linear calibration curve between ~30 $\mu g.l^{-1}$ and 100 $\mu g.l^{-1}$ MT with ~2.0 nA per ppb MT.
Samples : The blank should be run, every time, with extra purging (4 min.). The cytosols should be filtered using 0.45 µm syringe top filters before cell introduction. Prior dilutions are made if necessary. Additions of 30 µl of filtered cytosol gives adequate signal intensity, in fact considerable dilution is usually necessary, while 50 µl of the cytosolic chromatographic fraction (no need to filter) is required for good peak resolution.

Table 8 : Simplex optimised instrument settings for the DPP assay of MTs.

Polarographic analyzer - EG&G Model 384B	**Polarographic cell - EG&G Model 303A**
Potential scan : - Initial : - 1.000 V (*) - Final : - 1.700 V Scan rate : 2 mV/sec. - Drop interval : 2.0 sec. - Scan increment : 4 mV Pulse height : 100 mV Operation mode : DPP	Cell temperature : 10 °C Purge time : 2 min. - samples 4 min. - blanks Mode : DME Drop size : Medium.

(*) refers to the fact that this is decreased to -1.300 V after the blank has been checked for disappearance of Co maxima and minima, bearing in mind that the blank has to be re-run under the shorter potential scan range also.

2.4 Labelling :

Set Nr. e.g. 19 = March 1993; Set 20 = May 1993 etc.
aB = Natural state / Baalhoek. bB = Mixed spike / Baalhoek.
aPP = Natural state / Paulinapolder. bPP = Mixed spike /Paulinapolder.

Therefore "a" stands for the bivalve in its natural state and "b" stands for the bivalve after spiking.

"b" = Mixed spike of 100 ppb Cd, 100 ppb Cu and 600 ppb Zn.

1st.ppt. = First precipitate = Insoluble fraction.
2nd.ppt. = Second precipitate = High molecular weight protein fraction = HMWP.

3.0 Results.

The results will be given in as much detail as possible but where one example serves to illustrate the point of interest, better than a combination of all values, then the extra data will be presented in an annex. Before reading this section please refer to the "labelling" description given in paragraph 2.4.

3.1 Total metal concentrations.
The metals Cd, Cu, Zn and Fe were measured, in the whole soft tissue of the clams, both in their natural state and after spiking.The results of these AAS analyses are presented in Table 9.

Table 9 : Whole tissue metal concentrations ($\mu g.g^{-1}$ dry weight) in *Macoma balthica*.

Element	Sample i.d.	Concentrations			
	Month, Year, Set.	Natural state		After spiking	
		aB	aPP	bB	bPP
Cd	Mar '93-19	1.270 ± 0.016	0.383 ± 0.009	10.717 ± 0.314	12.770 ± 0.431
	May '93-20	0.924 ±0.090	0.287 ± 0.026	6.046 ± 0.495	4.090 ± 0.318
	Jul '93-21	0.393 ± 0.034	0.124 ± 0.028	3.191 ± 0.322	3.865 ± 0.133
	Sep '93-22	0.575 ± 0.077	0.155 ± 0.023	5.750 ± 0.001	3.135 ± 0.170
	Nov '93-23	0.605 ± 0.075	0.106 ± 0.033	10.251 ± 0.705	6.737 ± 0.174
	Jan '94-24	0.613 ± 0.044	0.238 ± 0.017	9.321 ± 0.172	6.834 ± 0.321
	Mar '94-25	0.616 ± 0.011	0.261 ± 0.010	8.629 ± 0.426	6.715 ± 0.230
	May '94-26	0.545 ± 0.069	0.196 ± 0.017	5.063 ± 0.554	5.261 ± 1.396
	Jul '94-27	0.491 ± 0.019	0.202 ± 0.011	5.310 ± 0.332	4.502 ± 0.348
	Sep '94-28	0.720 ± 0.033	0.223 ± 0.012	9.299 ± 0.136	8.543 ± 0.305
	Nov '94-29	0.547 ± 0.015	0.200 ± 0.008	9.296 ± 0.113	10.607 ± 1.361
	Jan '95-30	0.777 ± 0.014	0.199 ± 0.020	7.868 ± 0.176	6.869 ± 0.430
	Averages	**0.673 ± 0.150**	**0.215 ± 0.048**	**7.562 ± 1.547**	**6.661 ± 1.841**

Cu	Mar '93-19	25.5 ± 0.14	27.3 ± 0.13	34.1 ± 0.27	38.1 ± 1.04
	May '93-20	20.4 ± 0.62	18.6 ± 0.50	28.8 ± 0.42	28.5 ± 0.72
	Jul '93-21	22.2 ± 0.27	15.2 ± 1.90	22.0 ± 0.46	19.4 ± 0.76
	Sep '93-22	14.6 ± 0.28	16.3 ± 0.17	31.7 ± 0.33	20.5 ± 0.32
	Nov '93-23	18.7 ± 0.23	31.1 ± 1.74	39.3 ± 1.10	46.3 ± 0.99
	Jan '94-24	23.0 ± 0.34	20.8 ± 0.27	28.2 ± 0.36	34.5 ± 1.15
	Mar '94-25	29.5 ± 0.40	25.8 ± 0.36	38.5 ± 0.79	47.6 ± 0.80
	May '94-26	30.9 ± 1.50	25.8 ± 1.17	49.0 ± 4.39	43.0 ± 3.02
	Jul '94-27	37.1 ± 2.01	21.5 ± 0.19	41.5 ± 2.6	37.1 ± 1.82
	Sep '94-28	34.2 ± 1.01	21.6 ± 0.14	70.8 ± 1.69	48.2 ± 1.46
	Nov '94-29	31.4 ± 0.68	22.3 ± 0.38	75.8 ± 1.95	49.6 ± 1.87
	Jan '95-30	31.8 ± 0.78	26.4 ± 1.38	46.5 ± 2.14	42.4 ± 1.22
	Averages	**26.61 ± 4.44**	**22.73 ± 3.04**	**42.18 ± 10.62**	**37.93 ± 6.72**
Zn	Mar '93-19	716 ± 12	791 ± 18	742 ± 6	725 ± 3
	May '93-20	402 ± 8	418 ± 2	500 ± 10	749 ± 13
	Jul '93-21	326 ± 5	374 ± 9	367 ± 6	394 ± 10
	Sep '93-22	372 ± 8	376 ± 3	393 ± 6	423 ± 4
	Nov '93-23	461 ± 16	414 ± 6	482 ± 6	436 ± 3
	Jan '94-24	490 ± 2	450 ± 2	506 ± 2	489 ± 6
	Mar '94-25	518 ± 4	434 ± 6	580 ± 4	429 ± 3
	May '94-26	463 ± 2	396 ± 6	535 ± 4	479 ± 6
	Jul '94-27	410 ± 3	408 ± 12	434 ± 12	454 ± 7
	Sep '94-28	511 ± 7	400 ± 5	502 ± 6	418 ± 2
	Nov '94-29	555 ± 6	415 ± 8	576 ± 6	424 ± 9
	Jan '95-30	633 ± 5	376 ± 7	627 ± 12	433 ± 6
	Averages	**488 ± 71**	**438** ± 73	**520** ± 66	**488 ± 77**
Fe	Mar '93-19	1644.4 ± 26.5	2671.4 ± 11.3	1688.0 ± 28.9	1994.7 ± 92.1
	May '93-20	1251.8 ± 23.0	1723.3 ± 33.8	1550.1 ± 6.2	2268.9 ± 18.6
	Jul '93-21	576.7 ± 23.6	1081.2 ± 20.1	710.8 ± 13.9	1005.0 ± 23.5
	Sep '93-22	590.4 ± 8.5	732.9 ± 8.7	357.0 ± 10.0	448.7 ± 8.5
	Nov '93-23	1935.0 ± 9.6	993.0 ± 3.9	1869.0 ± 24.6	597.0 ± 13.0
	Jan '94-24	1448.0 ± 10.8	960.5 ± 50.8	1491.0 ± 12.6	1460.0 ± 15.9
	Mar '94-25	1376.0 ± 17.8	1606.0 ± 23.9	1414.0 ± 17.9	642.0 ± 25.1
	May '94-26	1331.0 ± 10.9	1344.0 ± 6.0	1049.0 ± 2.9	1461.0 ± 34.1
	Jul '94-27	792.0 ± 15.7	764.0 ± 20.3	736.0 ± 8.8	687.0 ± 6.7
	Sep '94-28	1910.0 ± 32.7	687.0 ± 19.8	1231.0 ± 10.7	625.0 ± 16.5
	Nov '94-29	2059.0 ± 15.8	906.0 ± 20.0	1674.0 ± 21.2	581.0 ± 12.7
	Jan '95-30	1679.0 ± 9.6	1274.0 ± 15.8	946.0 ± 20.9	1074.0 ± 23.5
	Averages	**1382.8 ± 327**	**1228.6 ± 363**	**1226.3 ± 301**	**1070.4 ± 387**

Table 9 continued.

The uncertainty is calculated by $(s/n^{½})t$, where s = absolute standard deviation, n = the number of repetitions i.e. 3 and t = students value for n-1 degrees of freedom at 95 % confidence interval. A graphical representation of metal concentration as a function of time is shown in Figures 8a,b,c,and d. A worked example of this is given in Annex 1b.

As shown in Table 10, the **Cd** concentration varies between 0.39 and 1.27 $\mu g.g^{-1}$ at B and between 0.11 and 0.38 $\mu g.g^{-1}$ at PP, for the natural clams, which shows that there is a significant difference (P = 0.05) [*see annex 1 for a worked example of this test - Cu data used*] between the Cd levels in *M. balthica* from Baalhoek and *M. balthica* from Paulinapolder. The is reflected in the spatial factor of 3.13, indicating this difference.

The short-term mixed metal spiking procedure results in very high average concentration factors (C.F.) {where CF = [Cd] spiked / [Cd] natural} of 11.6 at B and 32.7 at PP (Table 11). However on inspection of Figure 8a, one can see that these increases are more visible in Autumn / Winter than in Spring / Summer, in any one station. For example [Cd] at Baalhoek rose from 0.55 $\mu g.g^{-1}$ to 9.30 $\mu g.g^{-1}$ in Nov. '94 whereas [Cd] only rose from 0.92 $\mu g.g^{-1}$ to 6.05 $\mu g.g^{-1}$ in May '93, i.e. the CF values being 16.9 in Nov. '93 as opposed to 6.5 in May '93. This phenomenon is even more pronounced at Paulinapolder, with the CF values being 53.0 in Nov.'94 and 14.3 in May '93. The two monthly concentration factors for each metal in bivalves from both stations are also presented in Table 11. The seasonal factors, presented in Table 10, show that for Cd, annual fluctuations can be quite significant but are more or less the same for each station (seasonal factors of 3.32 and 3.61 at B and PP respectively).

Total **Cu** concentrations varied between 14.6 and 37.1 $\mu g.g^{-1}$ at B and between 15.2 and 31.1 $\mu g.g^{-1}$ at PP, for the natural clams and there is not a significant difference (P=0.05) between the [Cu] values for each station. It is however worth pointing out that [Cu] is almost always slightly higher at B than at PP (Figure 8b) but this difference is not high enough to be reflected in the spatial factor (only 1.2). The annual variation for copper is moderate, with seasonal factors being between 2.1 and 2.5 at both stations.

The spiking results in significant increases (P=0.05) from the natural levels, with the average CF values being 1.6 and 1.7 at B and PP respectively, again taking the average two year values into account. Examination of the individual CF values (Table 11) of each set show that minimum values are found in Summer, e.g. 0.99 in July'93 and 1.12 in July'94 compared to 2.10 in November '93 and 2.41 in November '94 (Baalhoek).

Table 10 : Seasonal and spatial concentration factors over the entire sampling period for metal concentrations ($\mu g.g^{-1}$ d.w.) in *Macoma balthica*.

Metal	Seasonal factor at Baalhoek			Seasonal factor at Paulinapolder.			Spatial factor
	Highest conc. - B $\mu g.g^{-1}$ d.w.	Lowest conc. - B $\mu g.g^{-1}$ d.w.	Highest conc./ Lowest conc.	Highest conc. - PP $\mu g.g^{-1}$ d.w.	Lowest conc. - PP $\mu g.g^{-1}$ d.w.	Highest conc./ Lowest conc.	Average conc. at B / Average conc.at PP
Cd	1.27	0.39	**3.32**	0.38	0.11	**3.61**	**3.13**
Cu	37.1	14.6	**2.54**	31.1	15.2	**2.05**	**1.17**
Zn	716	326	**2.20**	791	374	**2.11**	**1.11**
Fe	2059.0	576.7	**3.57**	2671.4	687.0	**3.89**	**1.13**

On examination of Figure 8c, one can see that the natural **Zn** concentrations are very similar in *Macoma balthica* from Baalhoek and Paulinapolder, with no significant difference in the comparison of the "two year" averages at the 99% confidence level (P=0.01). Seasonal differences in the natural [Zn] concentrations can be observed, with lowest values in Summer

(326 $\mu g.g^{-1}$ in 21aB and 374 $\mu g.g^{-1}$ in 21aPP) and higher values in Winter/early Spring (716 $\mu g.g^{-1}$ in 19aB and 791 $\mu g.g^{-1}$ in 19aPP), with seasonal factors being between 2.1 and 2.2 at both stations.
In fact the range of natural [Zn] concentrations in both B (326 $\mu g.g^{-1}$ to 716 $\mu g.g^{-1}$) and PP (374 $\mu g.g^{-1}$ to 791 $\mu g.g^{-1}$) do not differ significantly (P=0.01) from the values obtained after exposure to the mixed spike i.e. 367 $\mu g.g^{-1}$ to 742 $\mu g.g^{-1}$ at B and 394 $\mu g.g^{-1}$ to 749 $\mu g.g^{-1}$ at PP, with average CF values being 1.1 for both stations.

The case of **Fe** is very interesting and not quite clarified. Inspection of Figure 8d shows that there is an obvious seasonal pattern and when one looks at this pattern over the five year period (Figure 8e), one can see that the seasonal fluctuations are sinusoidally shaped. Lowest natural [Fe] values are found in Summer/early Autumn (576.7 $\mu g.g^{-1}$ in 21aB and 687.0 $\mu g.g^{-1}$ in 28aPP) and highest values in Winter/early Spring (2059.0 $\mu g.g^{-1}$ in 29aB and 2671.4 $\mu g.g^{-1}$ in 19aPP). This results in high seasonal factors of 3.57 and 3.89 for B and PP respectively. Location, however, does not appear to play a role (spatial factor of 1.13).

Another interesting, if confusing fact, is that there appears to be a reduction in the total Fe concentration in *M. balthica* after spiking. The spike does not contain Fe, so an increase in the exposed organisms is naturally not expected. However, the apparent reduction, with average CF values being 0.9 in both stations, was also not expected, even if the differences between the "aB" and the "bB" two-year averages and the "aPP" and "bPP" averages are not significant (P=0.05). It is not clear yet if the variation comes from imprecision, intermetallic interferences, or losses during the various procedures.

Table 11 : Concentration factors (CF) as a function of time and where CF $= [metal]_{spiked} / [metal]_{natural}$

Set	Concentration factors							
	Cd		Cu		Zn		Fe	
	B	PP	B	PP	B	PP	B	PP
Mar'93-19	8.4	33.3	1.34	1.40	1.04	0.92	1.03	0.75
May'93-20	6.5	14.3	1.41	1.53	1.24	1.79	1.24	1.32
Jul'93-21	8.1	31.2	0.99	1.28	1.13	1.05	1.23	0.93
Sep'93-22	10.0	20.2	2.17	1.26	1.06	1.13	0.60	0.61
Nov'93-23	16.9	63.6	2.10	1.49	1.05	1.05	0.97	0.60
Jan'94-24	15.2	28.7	1.23	1.66	1.03	1.09	1.03	1.52
Mar'94-25	14.0	25.7	1.31	1.84	1.12	0.99	1.03	0.40
May'94-26	9.3	26.8	1.59	1.67	1.16	1.21	0.79	1.09
Jul'94-27	10.8	22.3	1.12	1.73	1.06	1.11	0.93	0.90
Sep'94-28	12.9	38.3	2.07	2.23	0.98	1.05	0.64	0.91
Nov'94-29	17.0	53.0	2.41	2.22	1.04	1.02	0.81	0.64
Jan'95-30	10.1	34.5	1.46	1.61	0.99	1.15	0.56	0.84
Averages	**11.6**	**32.7**	**1.60**	**1.66**	**1.07**	**1.13**	**0.91**	**0.88**

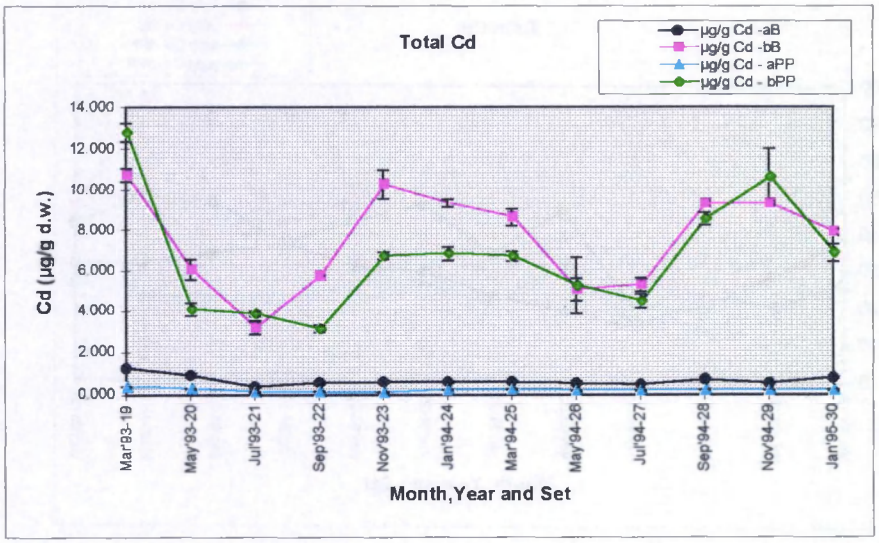

Figure 8a : Seasonal fluctuations of whole body cadmium concentrations in *Macoma balthica* before and after metal mixture exposure.

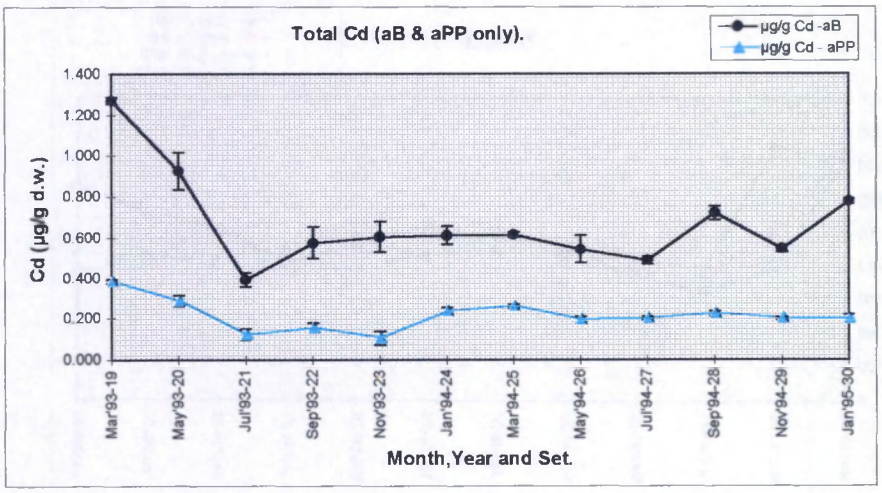

Figure 8a Zoom : Seasonal fluctuations of whole body cadmium concentrations in natural *Macoma balthica* i.e. without spiking.

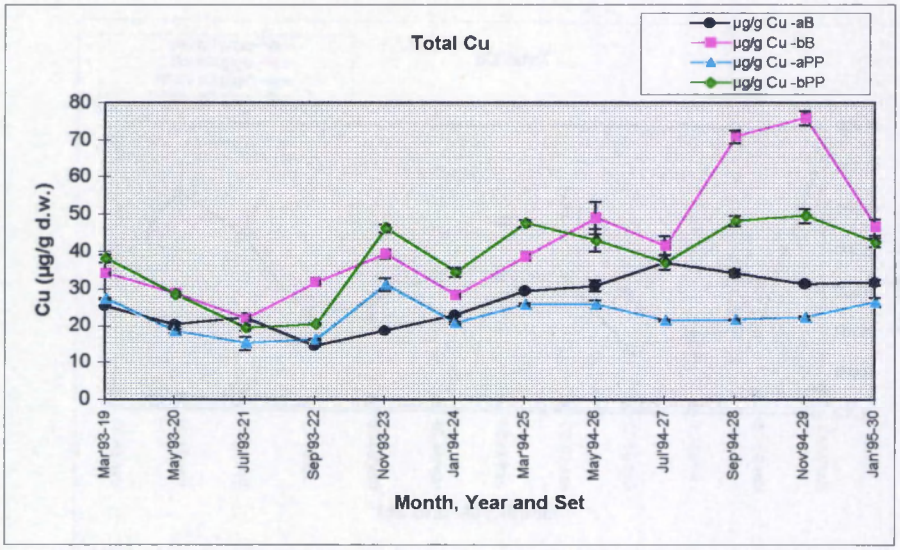

Figure 8b : Seasonal fluctuations of whole body copper concentrations in *Macoma balthica* before and after metal mixture exposure.

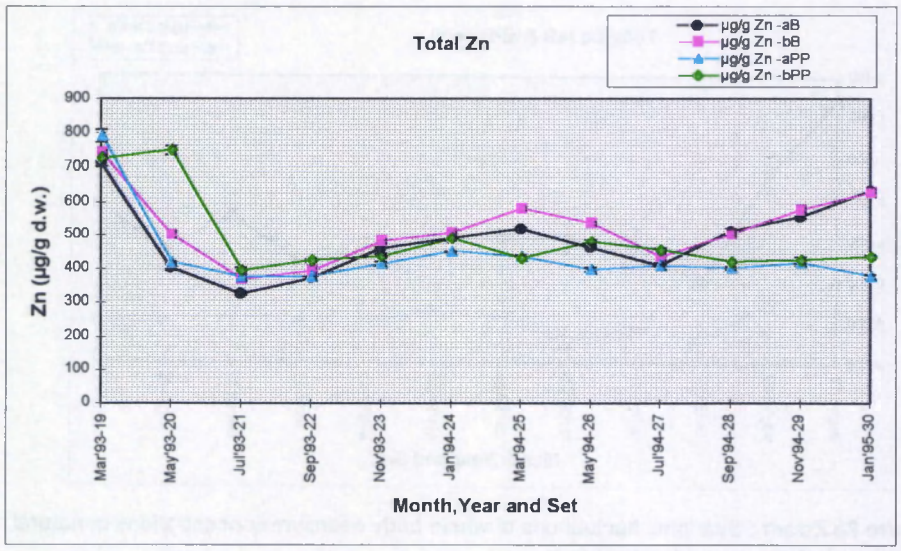

Figure 8c : Seasonal fluctuations of whole body zinc concentrations in *Macoma balthica* before and after metal mixture exposure.

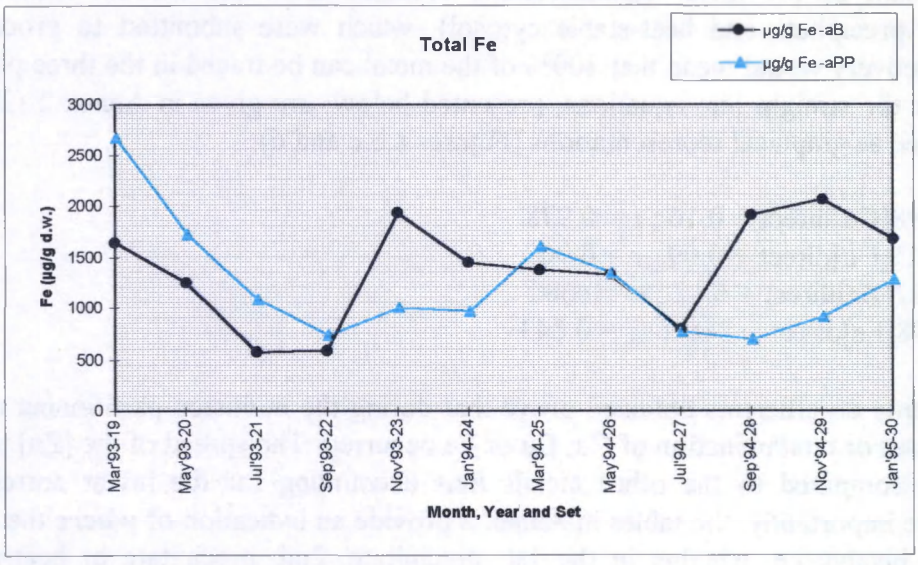

Figure 8d : Seasonal fluctuations of whole body iron concentrations in natural *Macoma balthica* i.e. without spiking.

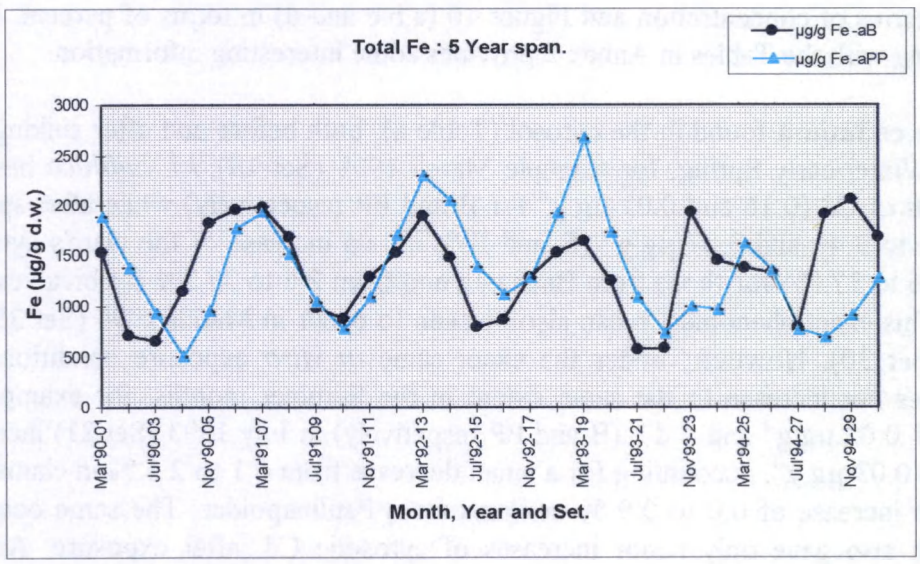

Figure 8e : Seasonal fluctuations of whole body iron concentrations in natural *Macoma balthic* over a five year period and without spiking (black and blue series).

3.2 The intracellular partitioning of metals.

The results of these metal assays serve to show the difference, if any, between the direct measurement of the metal concentration of whole bivalve tissue (submitted to procedure 2.2.1) and the sum of the metal concentrations obtained from the indirect route (1st. precipitate, 2nd. precipitate and heat-stable cytosol), which were submitted to procedure 2.2.2. Optimal recovery would mean that 100% of the metal can be traced in the three phases. The raw data for the straight line equations, presented below, are given in Annex 2 (Tables a,b,c and d) as well as graphical representations (Figures a,b,c and d).

[Cd]indirect = 0.98[Cd]direct + 0.16 ; r = 0.978
[Cu]indirect = 1.15[Cu]direct + 0.09 ; r = 0.909
[Zn]indirect = 1.12[Zn]direct + 6.12 ; r = 0.680
[Fe]indirect = 0.78[Fe]direct + 248.2 ; r = 0.843

The high correlation co-efficients obtained prove that during the multistep partitioning route, no significant losses or contamination of Cd, Cu or Fe occurred. The spread of the [Zn] values is quite narrow, compared to the other metals thus accounting for the lower correlation co-efficient. More importantly, the tables in Annex 2 provide an indication of **where** the metal is stored by the bivalve i.e. whether in the 1st. precipitate, 2nd. precipitate or heat-stable cytosol. Please note that wherever zero occurs, a value lower than the detection limit was found.

The effect of the season on how *M. balthica* deal with excess metal is illustrated in Figure 9 (a,b,c and d) in terms of concentration and Figure 10 (a,b,c and d) in terms of percent. Using these figures along with the Tables in Annex 2 provides some interesting information.

Focussing on the **cadmium** found in the cytosol (Table a), both before and after spiking, one can see that in Winter/early Spring, for example March 1993 (Set 19), *M. balthica* has very low natural levels of Cd (0.16 and 0.01 $\mu g.g^{-1}$ for B and PP respectively) while after spiking, these levels rose to 3.40 and 3.07 $\mu g.g^{-1}$ (B and PP), i.e. an increase of the clam's cytosolic burden from 10.6 to 37.8% for those from Baalhoek and from 2.6 to 37.2% for bivalves from Paulinapolder. This exact phenomenon can also be seen to occur in March 1994 (Set 25) and January 1995 (Set 30). However, under the exact same *in vitro* exposure conditions, the cytosolic Cd does not increase to the same extent in the Summer months, for example the natural values of 0.02 $\mu g.g^{-1}$ and < d.l. (B and PP respetively) in July 1993 (Set 21) increased to only 0.08 and 0.03 $\mu g.g^{-1}$, accounting for a small decrease from 6.1 to 2.1 % in clams from Baalhoek and an increase of 0.0 to 0.9 % in clams from Paulinapolder. The same occurs in July 1994 which also gave only minor increases of cytosolic Cd, after exposure. Another interesting fact is that the proportion of Cd in the HMWP pool (2nd.ppt.) decreases strongly, after exposure to the metal mixture, accounting for the increases in the cytosol, described above. This is particularly obvious in March and May '93 (Sets 19 and 20) with decreases of ~34% in clams from Baalhoek and ~44% in clams from Paulinapolder. The linear relationship between the cytosolic cadmium concentration and the total Cd concentration in *Macoma balthica* is shown in Figure 11a.

The case of **copper** is similar (Table b, Annex 2) with clams apparently having the same mechanism for dealing with excess copper (as shown in Figures 9b and 10b), which is a higher Cu uptake in the cytosol in Winter/early Spring and a less noticeable uptake in the Summer months.The natural copper concentrations in the cytosol of clams sampled in March 1993 were 7.5 and 4.9 $\mu g.g^{-1}$ (B and PP), which, on *in vitro* exposure, rose to 15.2 and 10.7 $\mu g.g^{-1}$

(B and PP respectively) i.e. increases of 16 and 13%. This is repeated in March '94 (Set 25), with increases of ~9% and ~14% for B and PP respectively. The opposite trend occurs in Summer months (e.g. Sets 20, 21 and 26) with decreases of ~ 9% in clam cytosolic Cd proportions after *in vitro* exposure in May '93 (Baalhoek); ~ 8% in July '93 (Baalhoek) and ~ 4 % in May '94 (Paulinapolder). However, it is important to note that the relationship between cytosolic copper concentration and total Cu concentration is a straight line (as shown in Figure 11b) indicating that even though the % Cu in the cytosol can fall on *in vitro* exposure, the concentration almost always increases.

Zinc, an essential element (Table c, Annex 2), appears to be dealt with in the same way as Cd and Cu, with cytosolic zinc increases, on spiking, being greatest in Winter/early Spring. For example, a rise of 23.9 to 47.3 $\mu g.g^{-1}$ (B) and 28.4 to 51.4 $\mu g.g^{-1}$ (PP) was found in the cytosol of clams sampled in January '94 after *in vitro* exposure, accounting for percentage increases of 8% and 5% (B and PP respectively). This phenomenon was equally obvious in January 1995, as illustrated in Figure 9c. The relationship between the cytosolic zinc concentration and the total [Zn], though not as clear as the Cd and Cu relationships, nevertheless appears to follow a linear trend. Another effect of the spiking procedure, on the clams, is a slight shift of the Zn burden from the insoluble fraction to both the HMWP pool and the cytosol (Table c). The results of Set 19 to 22 were deliberately omitted from Figures 9c, 10c and 11c and as there was an inconsistency in the Zn measurements in clams from these sets i.e. different instruments were used, one instrument having only ETAAS equipment while the new instrument has both FAAS (much more suitable for Zn measurements as Zn is generally present in high concentrations) and ETAAS facilities.

The most striking thing about Table d (Annex 2) is the illustration that almost all of the clam burden of **iron** is in the insoluble fraction (79-98%) with more or less equal amounts in the soluble HMWP pool and the (heat-stable) cytosol. Examination of Figures 9d and 10d shows that there doesn't seem to be a particular time of the year when the [Fe] in the cytosol noticeably rises. The relationship between cytosolic iron ($\mu g.g^{-1}$) and total [Fe], as depicted in Figure 11d, shows that there is not a matching increase in the cytosol for increases in the Total [Fe].

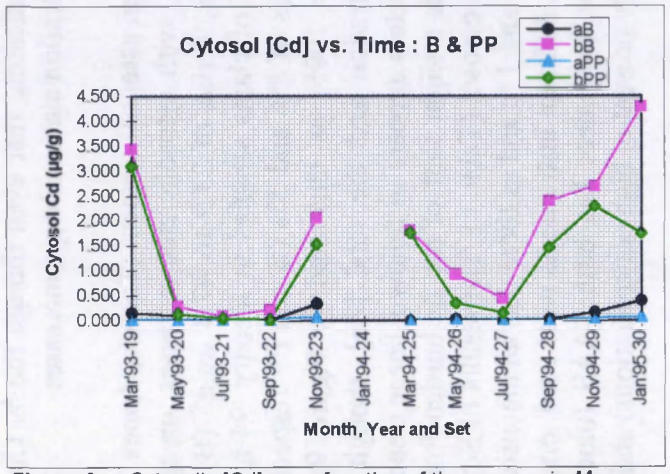

Figure 9a : Cytosolic [Cd], as a function of the season, in *Macoma balthica* from Baalhoek and Paulinapolder (natural and spiked).

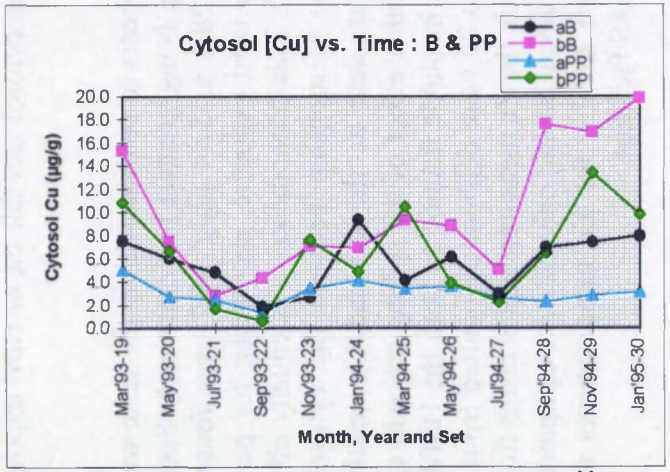

Figure 9b : Cytosolic [Cu], as a function of the season, in *Macoma balthica* from Baalhoek and Paulinapolder (natural and spiked).

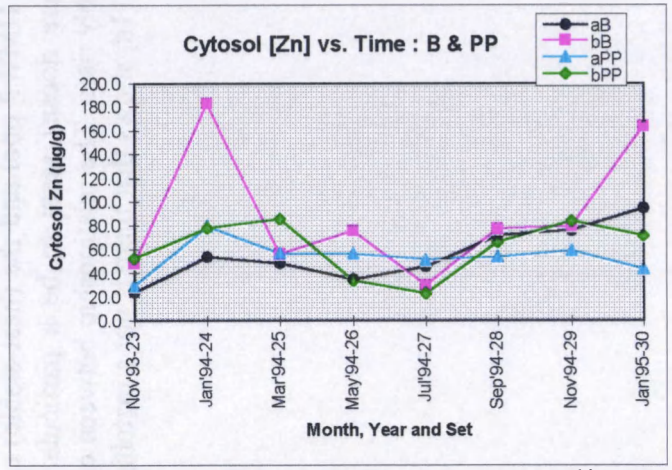

Figure 9c : Cytosolic [Zn], as a function of the season, in *Macoma balthica* from Baalhoek and Paulinapolder (natural and spiked).

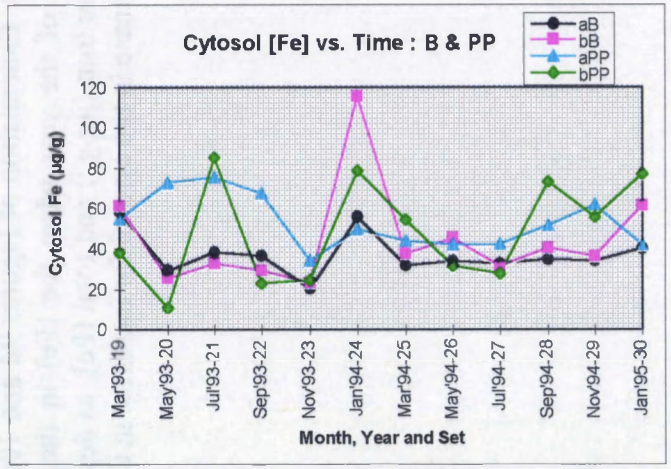

Figure 9d : Cytosolic [Fe], as a function of the season, in *Macoma balthica* from Baalhoek and Paulinapolder (natural and spiked).

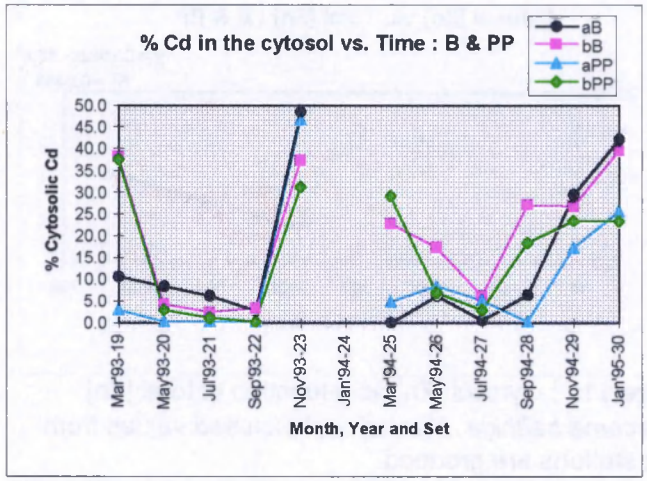

Figure 10a : % Cytosolic Cd , as a function of the season, in *Macoma balthica* from Baalhoek and Paulinapolder (natural and spiked).

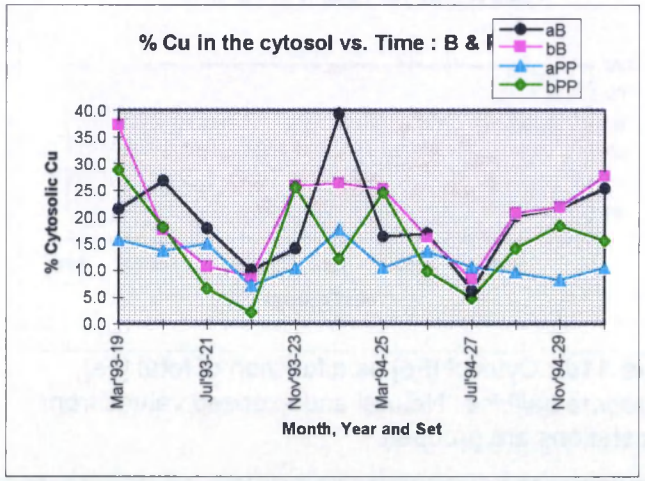

Figure 10b : % Cytosolic Cu , as a function of the season, in *Macoma balthica* from Baalhoek and Paulinapolder (natural and spiked).

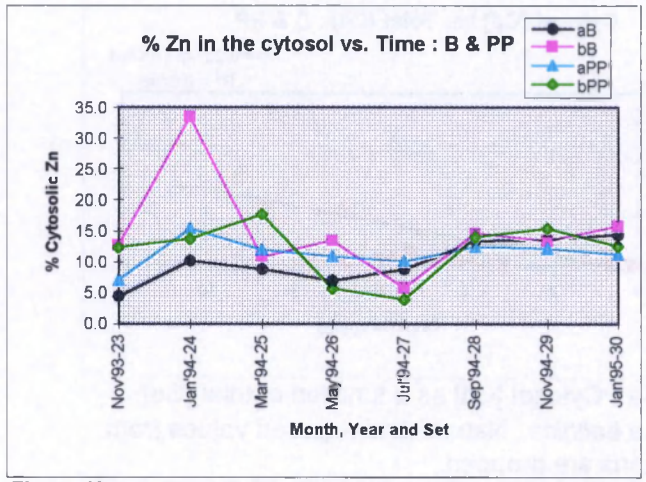

Figure 10c : % Cytosolic Zn , as a function of the season, in *Macoma balthica* from Baalhoek and Paulinapolder (natural and spiked).

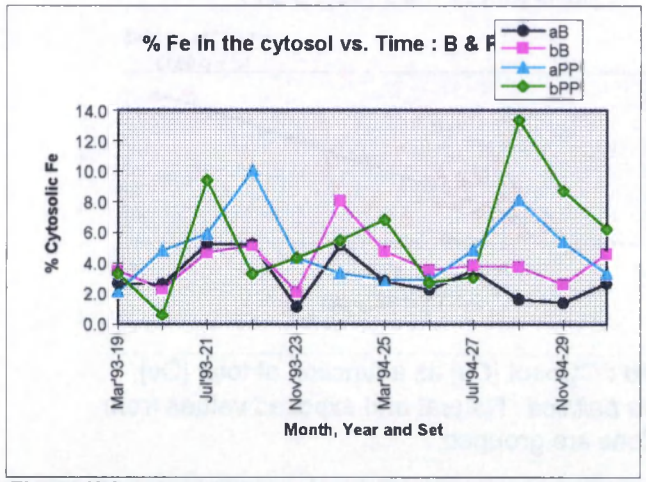

Figure 10d : % Cytosolic Fe , as a function of the season, in *Macoma balthica* from Baalhoek and Paulinapolder (natural and spiked).

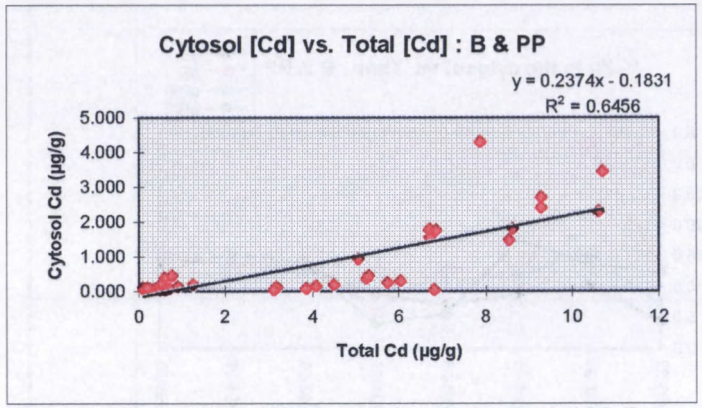

Figure 11a : Cytosol [Cd] as a function of total [Cd], in *Macoma balthica* . Natural and exposed values from both stations are grouped.

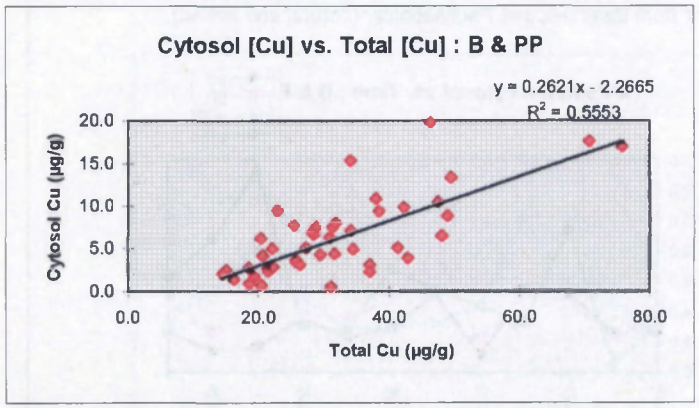

Figure 11b : Cytosol [Cu] as a function of total [Cu], in *Macoma balthica* . Natural and exposed values from both stations are grouped.

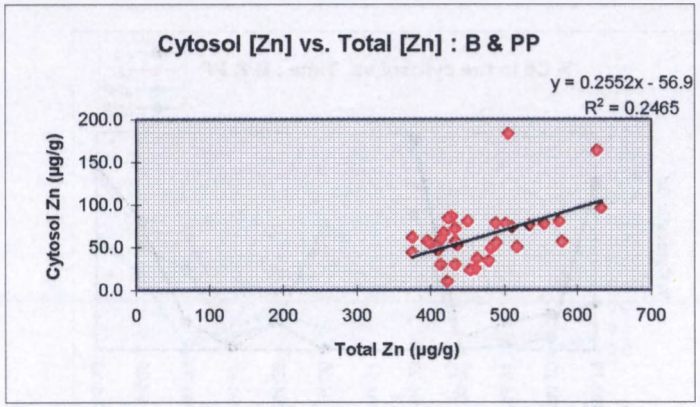

Figure 11c : Cytosol [Zn] as a function of total [Zn], in *Macoma balthica* . Natural and exposed values from both stations are grouped.

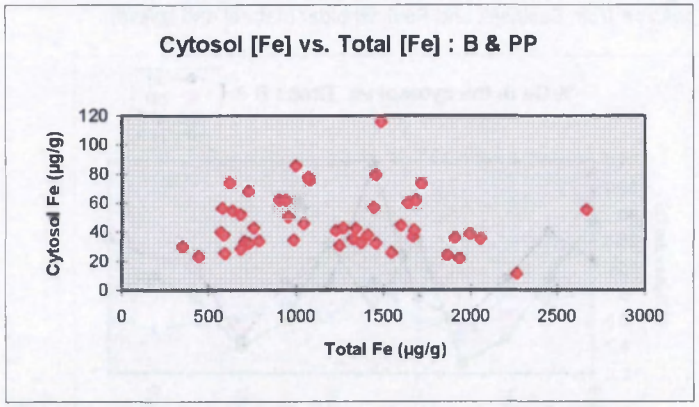

Figure 11d : Cytosol [Fe] as a function of total [Fe], in *Macoma balthica* . Natural and exposed values from both stations are grouped.

3.3 Metallothioneins in the heat-stable cytosol .

3.3.1 Optimisation :

The results of the method of simplex optimisation are given in Table 12.

Table 12 : Results of the Simplex optimisation for the analysis of MTs by DPP.

Vertices	Factors						Response
	A	B	C	D	E	F	(n=3)
Vertex 1	0.6	9.53	5	50	2.0	4	198.5
Vertex 2	1.0	9.46	20	25	0.5	2	24.0
Vertex 3	3.0	9.49	15	75	1.0	6	68.8
Vertex 4	2.0	9.52	10	50	1.0	4	543.3
Vertex 5	2.5	9.43	10	50	1.0	4	-
Vertex 6	2.0	9.52	10	100	2.0	2	989.7
Vertex 7	1.5	9.39	10	75	2.0	6	207.9
(i) Sum (excluding vertex 5).	10.1	56.91	70	375	8.5	24	
(ii) Sum/n (excluding vertex 5).	1.68	9.49	11.67	62.5	1.42	4	
(iii) Rejected vertex (i.e. 5).	2.5	9.43	10	50	1.0	4	
(iv) Displacement = (ii) - (iii).	-0.82	0.06	1.67	12.5	0.42	0	
(v) **Vertex 8** = (ii) + (iv).	**0.86**	**9.55**	**13.34**	**75.0**	**1.84**	**4**	**429.5**

"Factors" A, B, C, D, E and F are : Electrolyte concentration (mM) ; pH ; Temperature (°C) ; Pulse height (mV); Drop interval (s) and Scan increment (mV) respectively. The response is recorded in nA and the optimised conditions are those derived by Vertex 8. Further refinement of these conditions led to those given in Table 8 (section 2.3), there being only two changes i.e. a slight decrease in temperature to 10°C and an increase in the pulse height to 100 mV. The value for the drop interval was naturally rounded off to 2.

3.3.2 MT concentrations :

Using the optimised DPP method, providing the best **combined** sensitivity, resolution and reproducibility, the concentrations of MTs in the cytosol are presented in Table 13. Examples of typical standard addition calibration curves will be given in Annex 3 as well as some polarograms. Since there were 52 curves in total, with accompanying polarograms (260 in all), it is not possible to provide all of the raw data.

Table 13 : Metallothionein concentrations ($mg.g^{-1}$ dry weight).

Sample i.d.	Concentrations			
Month, Year and Set	Natural state		After spiking	
	aB	aPP	bB	bPP
Mar '93-19	5.41 ± 0.35	1.69 ± 0.05	6.15 ± 0.33	4.65 ± 0.13
May '93-20	4.89 ± 0.18	2.79 ± 0.17	5.86 ± 0.30	3.22 ± 0.06
Jul '93-21	1.79 ± 0.07	3.28 ± 0.32	2.81 ± 0.06	4.81 ± 0.15
Sep '93-22	3.70 ± 0.27	2.11 ± 0.08	3.68 ± 0.27	4.41 ± 0.08
Nov '93-23	5.47 ± 0.11	4.45 ± 0.30	5.11 ± 0.16	8.45 ± 0.15
Jan '94-24	6.41 ± 0.41	8.79 ± 0.42	6.06 ± 0.46	8.93 ± 0.35
Mar '94-25	5.89 ± 1.13	9.11 ± 0.32	7.33 ± 0.50	8.93 ± 0.11
May '94-26	5.69 ± 0.14	3.94 ± 0.29	6.93 ± 0.25	6.53 ± 0.11
Jul '94-27	3.22 ± 0.20	1.23 ± 0.10	5.92 ± 0.37	10.98 ± 0.37
Sep '94-28	1.70 ± 0.17	0.85 ± 0.28	1.40 ± 0.35	1.77 ± 0.24
Nov '94-29	3.37 ± 0.22	2.18 ± 0.12	1.11 ± 0.32	1.42 ± 0.05
Jan '95-30	7.65 ± 0.25	3.38 ± 0.05	11.05 ± 0.58	9.13 ± 0.50
Mar 95-31	7.81 ± 0.19	6.33 ± 1.00	11.29 ± 0.52	10.83 ± 0.67
Averages	**4.85 ± 1.15**	**3.86 ± 1.56**	**5.75 ± 1.81**	**6.47 ± 1.92**

The uncertainties are calculated as described in Annex 1 (b). Graphical representations of these results are given in Figures 12a and 12b.

The most obvious trend in these figures is that MT concentrations are lowest in the Summer/early Autumn months in natural state bivalves e.g. 1.79 $mg.g^{-1}$ in July '93 (Set 21) and 1.70 $mg.g^{-1}$ in September '94 (Set 28) for Baalhoek and 2.11 $mg.g^{-1}$ in September '93 (Set 22) and 0.85 $mg.g^{-1}$ in September '94 for Paulinapolder. Highest natural state values are found in Winter/early Spring e.g. 7.81 $mg.g^{-1}$ in March '95 (Baalhoek) and 9.11 $mg.g^{-1}$ in March '94 (Paulinapolder). This is reflected in the substantial seasonal factors of 10.7 at Paulinapolder and 4.6 at Baalhoek (Table 14). Comparing this trend with the metal fluctuations (Figures 8a,b,c,d), one can see the parallel i.e. metal minima in *Macoma balthica* also occur in Summer and maxima in Winter. Comparing average MT values for each station, following the method described in Annex 1 (a), one does not find a significant difference (P=0.05) for aB vs. aPP or for aB vs. bB. However, comparing the average aPP MT concentration with the average bPP MT concentration, one finds that they do differ significantly (P=0.05).

Table 14 : Seasonal and spatial concentration factors over the entire sampling period for natural MT concentrations ($mg.g^{-1}$ d.w.) in *Macoma balthica*.

Seasonal factor at Baalhoek			Seasonal factor at Paulinapolder.			Spatial factor
Highest conc. - B $mg.g^{-1}$ d.w.	Lowest conc. - B $mg.g^{-1}$ d.w.	Highest conc./ Lowest conc.	Highest conc. - PP $mg.g^{-1}$ d.w.	Lowest conc. - PP $mg.g^{-1}$ d.w.	Highest conc./ Lowest conc.	Average conc. at B / Average conc.at PP
7.81	1.7	**4.59**	9.11	0.85	**10.72**	**1.26**

Table 15 : Concentration factors (CF) as a function of time and where CF = [MT]spiked / [MT]natural.

Set	Mar'93-19	May'93-20	Jul'93-21	Sep'93-22	Nov'93-23	Jan'94-24	Mar'94-25
CF - Baalhoek	1.14	1.20	1.57	0.99	0.93	0.95	1.24
CF - Paulinapolder	2.75	1.15	1.47	2.09	1.90	1.02	0.98

Set	May'94-26	Jul'94-27	Sep'94-28	Nov'94-29	Jan'95-30	Mar'95-31	Averages
CF - Baalhoek	1.22	1.84	0.82	0.33	1.44	1.44	**1.16**
CF - Paulinapolder	1.66	8.96	2.09	0.65	2.70	1.71	**2.24**

The most interesting fact is that the CF values are highest in July '94 for both stations (i.e. 1.84 and 8.96 for B and PP respectively), indicating perhaps that when the natural metal burden is low and the natural MT level is low, then exposure to a high concentration of heavy metals leads to an induction of MTs. That is not to say that this induction doesn't occur in other months as quite substantial CF values were found for January and March e.g. 1.44 in Baalhoek (Sets 30 and 31) and 2.70 and 2.75 in Paulinapolder (Sets 30 and 19).

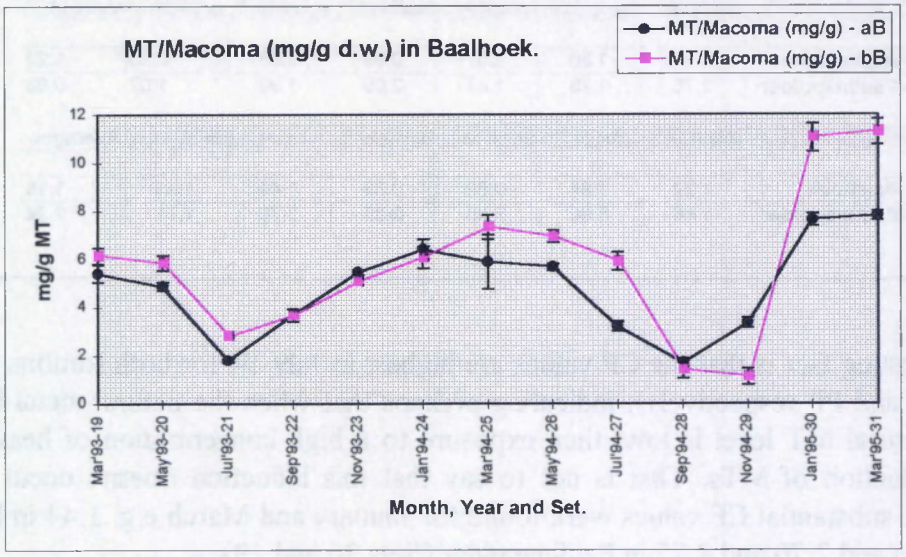

Figure 12 a : Seasonal fluctuations of MT in *Macoma balthica* from Baalhoek, before and after metal spiking.

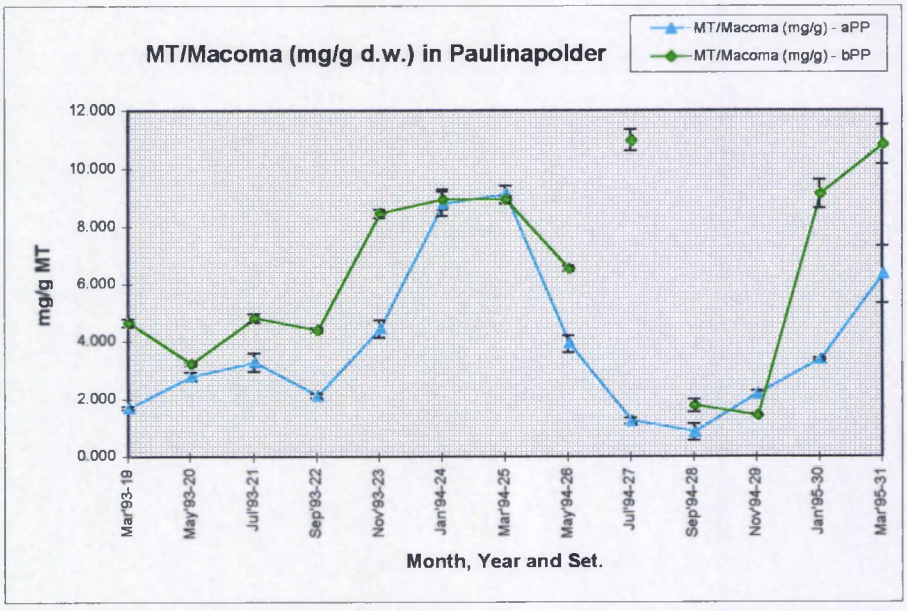

Figure 12 b : Seasonal fluctuations of MT in *Macoma balthica* from Paulinapolder, before and after metal spiking.

Note : *The value of 10.98, for Set 27bPP is thought to be erroneously high.*

3.4 Metals and Metallothioneins in the fractions of the chromatographically separated heat-stable cytosol :

Having established that whenever metal concentrations rise, the MT concentration also rises (Figures 8 and 12), it was decided to pin point exactly where the metals are found in the cytosol i.e. in which molecular weight range. The results for Set 19 (March 1993) are presented in Table 16.

Table 16 : Metal ($ng.ml^{-1}$) and MT ($\mu g.ml^{-1}$) concentrations in Set 19 chromatographic fractions of the heat stable cytosol, both before (a) and after (b) metal exposure of *M. balthica* from both stations.

Fraction Nr.	Metal concentrations (ng/ml).			MT conc.	Fraction Nr.	Metal concentrations (ng/ml).			MT conc.
	Cd	Cu	Zn	(µg/ml)		Cd	Cu	Zn	(µg/ml)
19aB-01	Same as	26.40	18		19bB-01		22.00	34	
19aB-02	eluent	10.90	17	5.168	19bB-02		32.50	40	5.352
19aB-03		4.50	19	5.375	19bB-03	0.660	39.40	45	6.449
19aB-04		10.50	11	1.947	19bB-04	2.060	72.60	69	11.271
19aB-05		2.90	10		19bB-05	1.740	46.70	72	3.374
19aB-06		5.50	24		19bB-06	3.730	23.90	60	
19aB-07		5.00	150		19bB-07	0.200	13.70	67	
19aB-08		4.50	243		19bB-08		9.60	138	
19aB-09		1.30	134		19bB-09		4.70	313	
19aB-10			74		19bB-10		3.10	336	
19aB-11			15		19bB-11			144	
19aB-12			9		19bB-12			102	
19aB-13			8		19bB-13			78	
19aB-14			8		19bB-14				
19aPP-01	Same as		11		19bPP-01		3.90	45	
19aPP-02	eluent	21.60	13	1.598	19bPP-02	1.780	6.00	47	3.354
19aPP-03		4.20	16	2.634	19bPP-03	2.820	12.60	60	6.242
19aPP-04		6.00	39	1.795	19bPP-04	0.529	17.00	64	9.832
19aPP-05		2.40	11		19bPP-05		8.80	58	2.341
19aPP-06		7.00	10		19bPP-06		4.00	46	
19aPP-07		4.50	49		19bPP-07		0.50	90	
19aPP-08		5.00	161		19bPP-08		1.00	183	
19aPP-09		5.30	197		19bPP-09			265	
19aPP-10		1.80	101		19bPP-10			200	
19aPP-11			20		19bPP-11			77	
19aPP-12			11		19bPP-12			52	
19aPP-13			14		19bPP-13			30	
19aPP-14			8		19bPP-14			24	

Graphical representations of these results are presented in Figures 13 a, b, c, and d.

Using a calibrated SEC column, the fractions corresponding to the Mw of MTs or MLPs have been found. This region lies between 6000 and 12000 Daltons i.e. between fraction numbers 2 and 5. Examination of Table 16 and Figure 13a show that there is no Cd in the fractions of natural *M. balthica* cytosol as values of 0.34 and 0.55 $ng.ml^{-1}$ (not given in the Table) (B and PP respectively) are also found in the blank (eluent only). However, on exposure to the mixed metal spike, appreciable quantities of Cd are found in fractions 3 to 6 (i.e. 0.66-3.73 $ng.ml^{-1}$) for Baalhoek and fractions 2 to 3 (1.78-2.82 $ng.ml^{-1}$) for Paulinapolder. Copper appears in fractions 1 to 10 in natural cytosolic fractions (ranging from 1.3-26.4 $ng.ml^{-1}$ (B) and from 1.8-21.6 $ng.ml^{-1}$ (PP). The copper taken up by the bivalve, on *in vitro* exposure, can be found in the same range of fractions, with highest increases in fractions 2-6. Paulinapolder samples show highest increases in fractions 3-5. Before exposure, almost all zinc was found in fractions 7-10 (both stations), a region corresponding to low molecular weight proteins (~1000 Da) however, after exposure Zn concentrations rose in every fraction, highest values still being in fractions 7-10 but with notable increases in fractions 2-5. Natural Zn values ranged between 8

and 243 ng.ml^{-1} (B) and between 8 and 197 ng.ml^{-1} (PP) while values after exposure ranged from 34-336 ng.ml^{-1} at B and 24-265 ng.ml^{-1} at PP.

Looking at Figure 13d, one can see that the thiolic proteins are found in fractions 2-5, exactly the region of highest metal concentrations and the region characteristic of MTs and /or MLPs. In samples from both stations, the highest protein increases are to be found in fraction 4. For example, 1.95 mg.ml^{-1} is found in sample 19aB-04 i.e. before exposure while being significantly different (P=0.05) after exposure i.e. 11.27 mg.ml^{-1} (19bB-04). The same is true of samples from PP, showing a rise from 1.80 mg.ml^{-1} in fraction 19aPP-04 to 9.83 mg.ml^{-1} in fraction 19bPP-04.

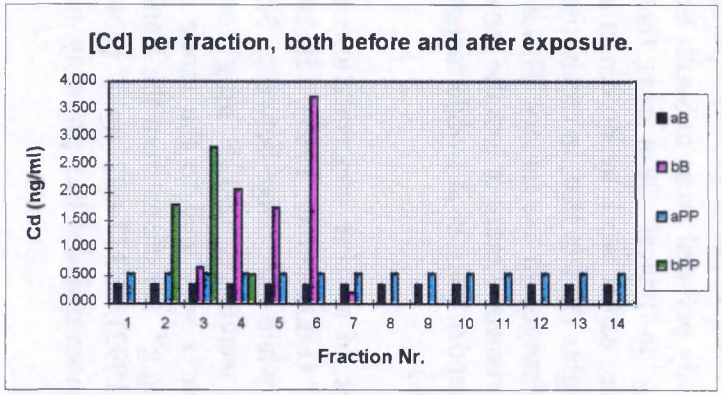

Figure 13a : Cd concentration per cytosolic fraction of Set 19. Data from each station were used.

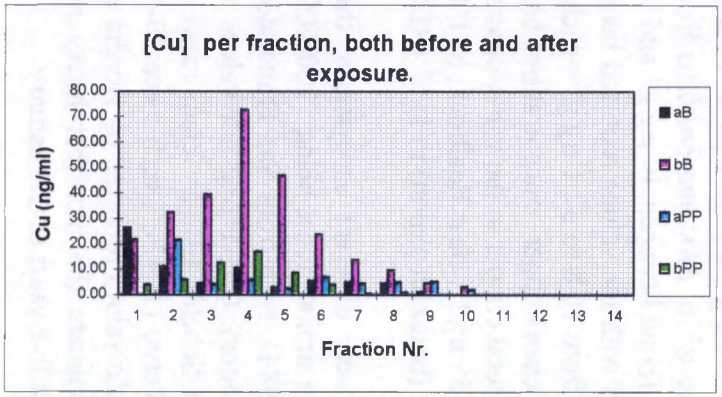

Figure 13b : Cu concentration per cytosolic fraction of Set 19. Data from each station were used.

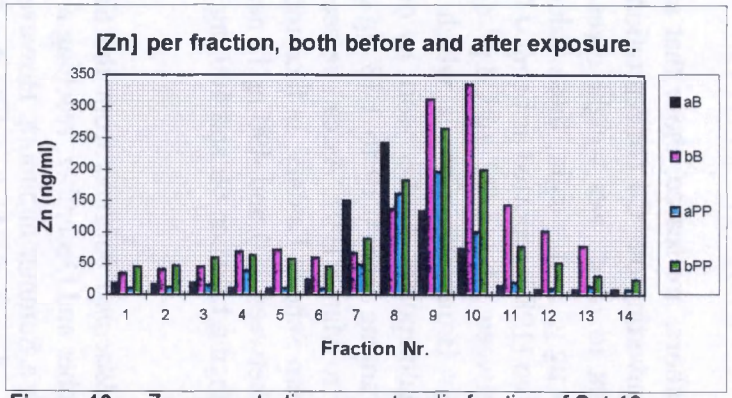

Figure 13c : Zn concentration per cytosolic fraction of Set 19. Data from each station were used.

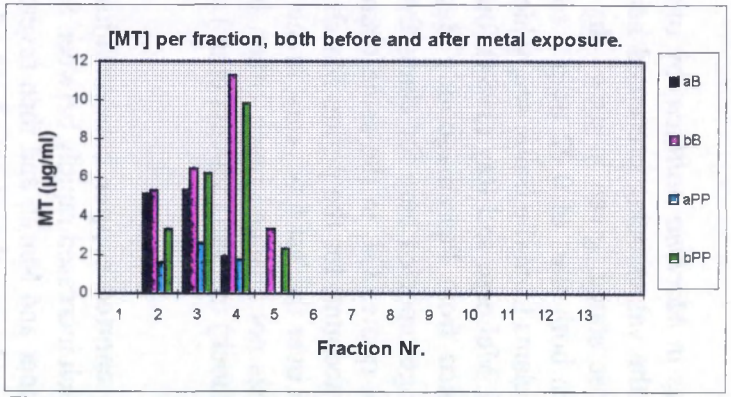

Figure 13d : MT concentration per cytosolic fraction of Set 19. Data from each station were used.

4.0 Discussion :

4.1 Metal concentrations : seasonal and spatial fluctuations:

Investigation of the literature shows that the temporal fluctuation patterns we observed, during this period, compare well with previous works on *Macoma balthica* from different ecosystems.

Although **cadmium** values in *Macoma balthica*, by other authors, are scarce, those that are available agree well with the values being presented here. Converting our Cd concentrations, which are, unless otherwise stated, given in $\mu g.g^{-1}$ dry weight to $\mu g.g^{-1}$ wet weight gives a range of Cd in clams from both sites of 0.02 $\mu g.g^{-1}$ to 0.20 $\mu g.g^{-1}$ wet weight, these values comparing very well with clams from the Baltic sea, which Tervo (1987) reported as being 0.03 to 0.12 $\mu g.g^{-1}$ wet weight. McLeese and Ray (1984) found between 0.1 and 0.3 $\mu g\ Cd.g^{-1}$ dry weight in *Macoma balthica* from Passamaquoddy Bay, New Brunswick, Canada which is almost identical to the range reported here for clams from Paulinapolder. Earlier work by our team (Bordin et al.,1992), quotes Cd, in the same clam, as ranging from 0.19 to 1.13 $\mu g.g^{-1}$ (taking both stations into account) for the period March 1990 to March 1991. As can be seen, this has not changed much over the last few years, in the Western Scheldt Estuary. In fact, total Cd in the water column has not changed much either, being between 120 and 490 $ng.l^{-1}$ near Hansweert (opposite Baalhoek) in 1986 (Valenta et al.) and ranging between 60 and 460 $ng.l^{-1}$ in 1993 (RIZA, NL).

Cain and Luoma (1990) reported **copper** concentrations in *Macoma balthica* from the San Francisco Bay (USA), which increased rapidly between September and December, reaching a maximum between December and March and then reverting to a Summer minimum. However, depending upon location and year, the copper levels they found were far higher than the Cu values being reported here. Their copper concentrations ranged from 50 $\mu g.g^{-1}$ to 500 $\mu g.g^{-1}$ while ours ranged from 14.6 $\mu g.g^{-1}$ to 37.1 $\mu g.g^{-1}$ (Table 10). Thomson et al. (1984) reported copper levels of >1000 $\mu g.g^{-1}$ in *Macoma balthica* from heavily polluted areas in South San Francisco Bay, while they found between 30 $\mu g.g^{-1}$ and 50 $\mu g.g^{-1}$ in "typical" clean areas, these comparing reasonably well with the values reported here. Again, earlier work by Bordin et al. (1992) shows Cu ranging from 16.8 to 32.1 $\mu g.g^{-1}$ which highlights the fact that, in recent time (1990-1995), the range of clam copper concentrations has not changed, in this estuary. This is supported by the fact that total copper in the water column has remained more or less the same, being between 2.65 and 9.65 $\mu g.l^{-1}$, near Baalhoek, in 1987 (Van den Berg et al.) while ranging between 1.6 and 8.6 $\mu g.l^{-1}$ (brackish region) in 1993 (RIZA, NL).

Although collected from very different ecosystems, the range of all **zinc** concentrations in *Macoma balthica* are quite similar; these being : 200-600 $\mu g.g^{-1}$ (Luoma et al., 1985), 150-500 $\mu g.g^{-1}$ (Thomson et al., 1984), both from San Francisco Bay, while ours range between 326 and 791 $\mu g.g^{-1}$ (both stations). Converting our values to wet weight i.e. 52-125 $\mu g.g^{-1}$, one can see that the Western Scheldt Estuary clam range compares very well to the range of concentrations found by Tervo (1987), which were 30-100 $\mu g.g^{-1}$ for clams from the Baltic Sea. Looking back to the Zn values reported by Bordin et al. (1992), of 377-692 $\mu g.g^{-1}$ (d.w.) once again serves to illuminate the fact that heavy metal bioavailability has probably not changed much over the last five years in this estuary.

There is a lack of **iron** data for this species, therefore one can only refer to the 1990-1991 values (Bordin et al., 1992) which were 506-1984 $\mu g.g^{-1}$, comparing quite well with the 1993-1995 values of 577-2671, although there are significantly higher maxima of 2059 $\mu g.g^{-1}$ at B and 2671 $\mu g.g^{-1}$ at PP. The sinusoidal evolution of Fe concentrations vs. time could

possibly be due to corresponding seasonal changes in redox potentials in the river water flowing in to the estuary and hence changing quantities of Fe oxy-hydroxides in suspension and in sediments.

4.2 Possible processes leading to these temporal and spatial metal variations :

The potentially major sources of metal concentration variations in aquatic organisms include seasonal and geographical metal concentration and speciation variability in water and sediment (variable metal discharges of natural and/or anthropogenic origins), changes in hydrologic and chemical characteristics of the ecosystem such as water flow, pH, salinity, suspended matter etc. and temporal evolutions of the biological state, such as fluctuations in tissue weight, in metabolism and in sexual maturity etc.. Some of these processes will now be discussed.

a) Gonad maturation :

Of the many parameters which may influence trace metal variations in bivalves, gonad development and state is commonly thought responsible for the Spring metal decrease (Chu et al., 1990). In many ecosystems, gonad maturation in the autumn and spawning in Spring result in a loss of metal. This is the case in the Western Scheldt estuary, where clam gonads are full in Winter after an autumnal maturation. Spawning then occurs in Spring with clams having empty gonads in Summer, this therefore being a possible reason for low metal levels in the *Macoma balthica* in July.

b)Effect of clam body weight on the absolute metal content and concentration - Natural state :

In order to discriminate between the influence of environmental metal levels and the influence of the various biological factors, mentioned above, on metal fluctuations in marine invertebrates, such as *Macoma balthica*, the relationship between metal concentration in the clam and its body weight, has been investigated. Therefore the terms "content" and "body burden" come into play. They can be defined as the total mass of a metal in the body of an average-length clam ($\mu g.ind^{-1}$). To see if there is a correlation between concentration and/or content and body weight, the concentration and/or absolute amount of metal per individual is plotted against the whole tissue dry weight. To do this the "log-log" format is often used (Boyden, 1974,1977) which follows the metabolic equation $Y=aX^b$, where Y is the concentration or content, X the tissue dry weight, a the intercept and b the regression co-efficient. For convenience the average whole body dry weight of *M. balthica*, from B and PP has been plotted as a function of the season and is shown in Figure 14.
In order to avoid the problem of cases where it is difficult to assess whether observed differences in element tissue concentrations between *M. balthica* reflect real differences in environmental trace element constitution, or are merely due to variations in body size, plots of μg metal.individual^{-1} i.e. content versus dry body weight have been carried out. It is perhaps interesting to note that the regression co-efficient value (b) of 0.75 is generally accepted as being the value which relates many metabolic functions to body weight (Hemmingsen, 1960). Therefore, in cases where an element is related to 0.75 body weight, some aspect of metabolism could well be influencing the final trace element content.

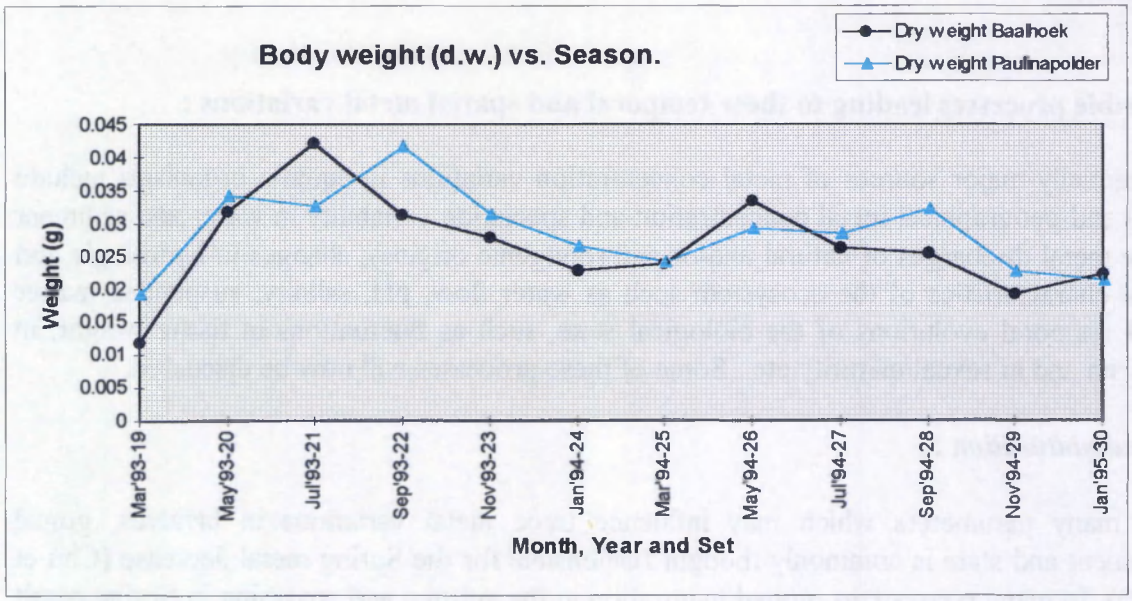

Figure 14 : The average whole body dry weight of *M. balthica,* from Baalhoek and Paulinapolder, as a function of the season.

According to Boyden (1977), plotting element **content** against body weight, on a linear basis gives three possible outcomes :

Case I

- (i) Content (Y) is directly related to weight (X), Y = aX, and is therefore a straight line. A family of lines can be obtained, the slopes of which are determined by the magnitude of metal increase per unit weight (clearly they must all pass through the origin).
- (ii) The relation is curved, with larger individuals containing less metal than would be predicted if content was directly related to weight. Content (Y) is related to weight (X) by the power function $Y = aX^b$ where b, the regression co-efficient, is less than 1.0.
- (iii) The relation is again curved, but with larger individuals containing more metal than if content were directly related to weight. In this case, which is not very common, content (Y) is related to weight by the power function $Y = aX^b$ where b>1.

Plotting element **concentration** against body weight, on a linear basis also gives three possible outcomes :

Case II

- (i) A straight line of slope 0.00 indicating that weight has no influence upon concentration.
- (ii) Since an element content to body weight slope (b) of 1.00 produces an element concentration related to weight by (b-1) and is therefore independent of body weight. Where the content slope (b) < 1.00, then the element concentration to body weight relationship has a slope of (< 1.00 - 1.00), i.e. a negative slope, therefore concentrations will be greatest in the smallest individuals.
- (iii) If content is related to body weight by a slope >1.00 then the concentration to weight relation with a slope of (>1.00 - 1.00) will itself be positive, thus concentrations will be greatest in larger individuals.

The possible outcomes for both content vs. body weight and concentration vs. body weight are depicted in Figure 15. However, even though I go on to plot these relationships for our *M. balthica* data, it must be remembered that only a very small weight range is available in our case (0.01-0.04 g) whereas Boyden used weight ranges of 0-2.0 g. Therefore the figures (16-19) illustrating these relationships can only be considered as an approximation.

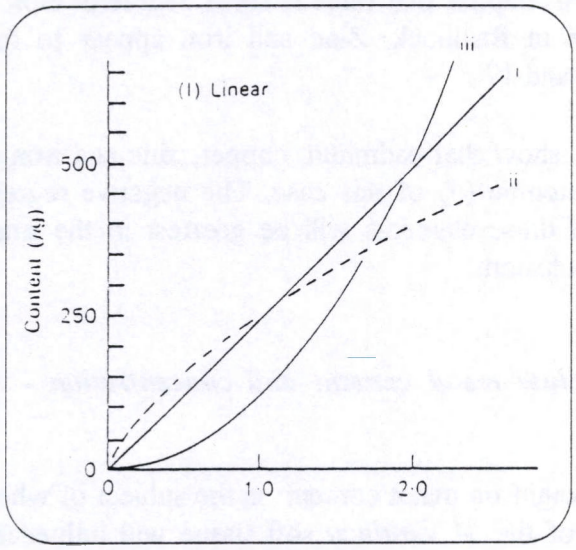

Figure 15a : Content vs. body weight (g). Possible outcomes i, ii and iii.

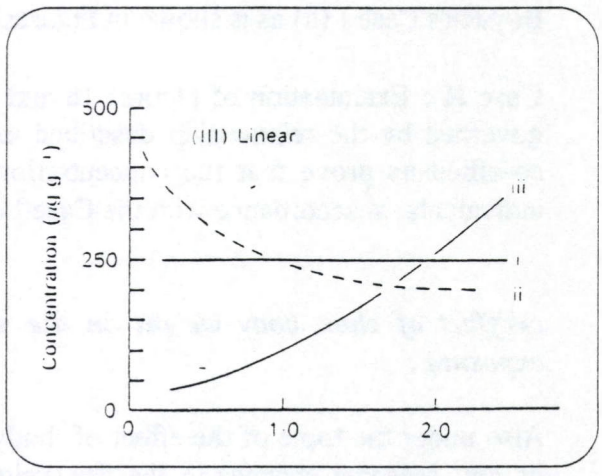

Figure 15b : Concentration vs. body weight (g). Possible outcomes i, ii and iii.

The following equations, using the power function $Y = aX^b$ have been found :

- Case I : using **contents** ($\mu g.ind^{-1}$) : Natural state *M. balthica*.

$[Cd]_B = 0.0574(X)^{0.34}$; r = 0.4391
$[Cu]_B = 10.097(X)^{0.75}$; r = 0.6635
$[Zn]_B = 48.92(X)^{0.38}$; r = 0.7697
$[Fe]_B = 74.816(X)^{0.26}$; r = 0.1949

$[Cd]_{PP} = 0.0102(X)^{0.16}$; r = 0.1196
$[Cu]_{PP} = 2.625(X)^{0.40}$; r = 0.4641
$[Zn]_{PP} = 63.825(X)^{0.47}$; r = 0.5333
$[Fe]_{PP} = 77.994(X)^{0.31}$; r = 0.1304

See Figures 16 and 17 and refer to Tables 17a and 17b for the raw data.

- Case II : using **concentrations** ($\mu g.g^{-1}$) : Natural state *M. balthica*.

$[Cd]_B = 0.0574(X)^{-0.66}$; r = 0.6846
$[Cu]_B = 10.097(X)^{-0.25}$; r = 0.2898
$[Zn]_B = 48.92(X)^{-0.62}$; r = 0.8915
$[Fe]_B = 74.816(X)^{-0.74}$; r = 0.4866

$[Cd]_{PP} = 0.0102(X)^{-0.84}$; r = 0.5257
$[Cu]_{PP} = 2.625(X)^{-0.60}$; r = 0.6146
$[Zn]_{PP} = 63.825(X)^{-0.53}$; r = 0.5831
$[Fe]_{PP} = 77.994(X)^{-0.70}$; r = 0.2869

See Figures 18 and 19 and refer to Tables 18a and 18b for the raw data.

Case I : Following Boyden's example, the relationship of Cd content vs. body weight was plotted as shown in Fig. 16a for B and Fig. 17a for PP. Looking at the natural values only (black series for B and blue series for PP), one can see that the regression co-efficients (b) are less than 1.00 therefore suggesting that larger individuals contain less Cd than would be predicted if Cd content was directly related to weight - (ii). These results are in agreement with

the works of Amiard et al. (1987) on metal bioaccumulation in various estuarine organisms (such as *Nereis diversicolor, Scrobicularia plana, Littorina littorea, Mytilus edulis, Crangon crangon* and *Carcinus maenas*), who showed that their non-essential metal (such as Cd and Pb) levels depended mainly on the environmental levels of these metals. Though being closer to 1.00, the regression co-efficient of 0.75, obtained for copper content vs. clam body weight in Baalhoek (Figure 16b), still follows Case I (ii) but is also the b value found when metabolism is influencing the final trace element content. This implies that there is some degree of biological control of the Cu body burden in clams from Baalhoek. Zinc and iron appear to follow Boyden's Case I (ii) as is shown in Figures 16 and 17.

Case II : Examination of Figures 18 and 19, show that cadmium, copper, zinc and iron are governed by the relationship described in outcome (ii) of this case. The negative regression co-efficients prove that the concentration of these elements will be greatest in the smallest individuals in accordance with the Case I conclusion.

c)Effect of clam body weight on the absolute metal content and concentration - After exposure :

Also under the topic of the effect of body weight on metal content is the subject of whether, or not, seasonal changes in the dry weight of the *M. balthica* soft tissue will influence the variability of the *in vitro* metal uptake by these bivalves. It is for this reason that Figures 16-19 also include concentration and content data (vs. dry body weight) after spiking. Following the same metabolic equation $Y = aX^b$, as previously described, the power functions were calculated and are as follows :

- Case I : using **contents** ($\mu g.ind^{-1}$) : *M. balthica* after spiking.

$[Cd]_B = 0.4178(X)^{0.26}$; r = 0.1822 $\quad$ $[Cd]_{PP} = 0.0269(X)^{-0.52}$; r = 0.4141
$[Cu]_B = 10.427(X)^{0.64}$; r = 0.5057 $\quad$ $[Cu]_{PP} = 1.6372(X)^{0.13}$; r = 0.1122
$[Zn]_B = 77.597(X)^{0.49}$; r = 0.8209 $\quad$ $[Zn]_{PP} = 236.78(X)^{0.80}$; r = 0.6508
$[Fe]_B = 64.177(X)^{0.19}$; r = 0.1616 $\quad$ $[Fe]_{PP} = 34.979(X)^{0.03}$; r = 0.0173

See Figures 16 and 17 and refer to Tables 17a and 17b for the raw data.

- Case II : using **concentrations** ($\mu g.g^{-1}$) : *M. balthica* after spiking.

$[Cd]_B = 0.4178(X)^{-0.74}$; r = 0.4638 $\quad$ $[Cd]_{PP} = 0.0269(X)^{-1.52}$; r = 0.7996
$[Cu]_B = 10.427(X)^{-0.36}$; r = 0.3172 $\quad$ $[Cu]_{PP} = 1.6372(X)^{-0.87}$; r = 0.5932
$[Zn]_B = 77.597(X)^{-0.51}$; r = 0.8347 $\quad$ $[Zn]_{PP} = 236.78(X)^{-0.20}$; r = 0.2042
$[Fe]_B = 64.177(X)^{-0.81}$; r = 0.5832 $\quad$ $[Fe]_{PP} = 34.979(X)^{-0.97}$; r = 0.5360

See Figures 18 and 19 and refer to Tables 18a and 18b for the raw data.

Case I : All elements exhibit regression co-efficients of less than 1.00, after exposure, for content vs. dry weight (Figures 16 and 17). When one compares the season, dry body weight and respective Cu content (Tables 17a and 17b) one can see that a bivalve of a certain weight can contain different metal burdens, depending on the season. For example, in Baalhoek, November '93, a clam of 0.028g carried 0.52 µg Cu in its natural state and 1.09 µg after exposure. A clam of similar weight (0.026g) in July '94 carried 0.97 µg Cu in its natural state

and still only 1.09 µg after exposure. The same occurs in Paulinapolder with a clam of 0.033g carrying 0.50 µg Cu in July '93 (natural state) and 0.63 µg after exposure whereas a clam of 0.031g carried 0.97 µg in November of the same year and 1.45 µg after exposure. See Figures 20a,b,c,and d for an illustration of how the clam metal content can vary with the season. Content values obtained, after spiking, denoted as "Exp.", are also shown in order to prove how the season can be important for copper uptake of equal weight clams. Therefore, it now seems safe to say that for cadmium and copper, temporal fluctuations in total metal uptake are not only due to dilution or concentration effects of their metal loads. The near horizontal regression co-efficients (b) of Figures 16d and 17d (pink and green trend lines) of iron content vs. dry weight, after exposure to a spike, not containing iron, are naturally more or less the same as for the natural state measurements.

Case II : The over-riding conclusion is that since the regression co-efficients are less than 1, this proves that the concentration of these elements will be greatest in the smallest individuals in accordance with the Case I conclusion.

- Conclusion on the effect of body weight on contents and concentrations :

Considering Figures 16 to 19, one can say that there is an overall decrease of total natural and spiked levels of these metals in *Macoma balthica*, with increasing body weight. A possible reason for this could be due to the fact that concentrations of Cu, Cd and Zn are often much lower in gonadal than in somatic tissues of many invertebrate species, such as *Nereis diversicolor* (Howard and Brown, 1983), *Arenicola marina* (Packer, 1979) and *Mytilus edulis* (Latouche and Mix, 1981; Lobel and Wright, 1982; Amiard et al., 1986) taking into account the fact that the period of weight gain in clams also corresponds to the period of their reaching sexual maturity i.e. full gonads.

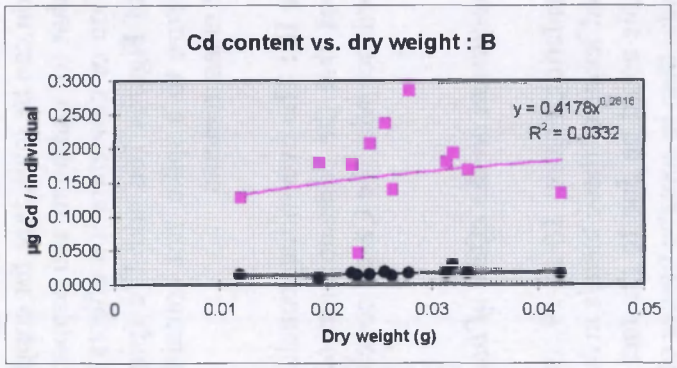

Figure 16a : Cd content (Y) as a function of the dry body weight (X) of *Macoma balthica* (Baalhoek) before (black circles) and after (pink boxes) mixed metal spike.

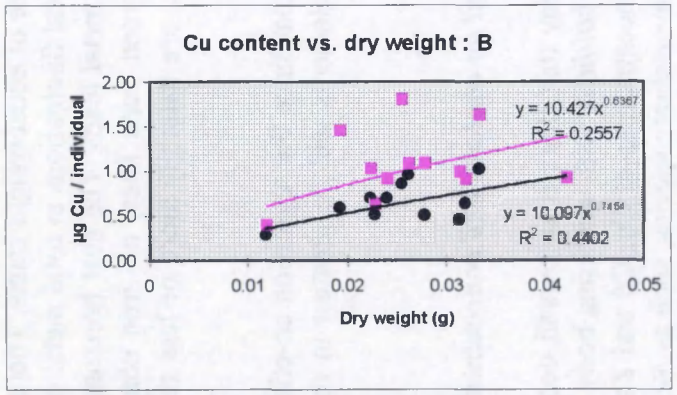

Figure 16b : Cu content (Y) as a function of the dry body weight (X) of *Macoma balthica* (Baalhoek) before (black circles) and after (pink boxes) mixed metal spike.

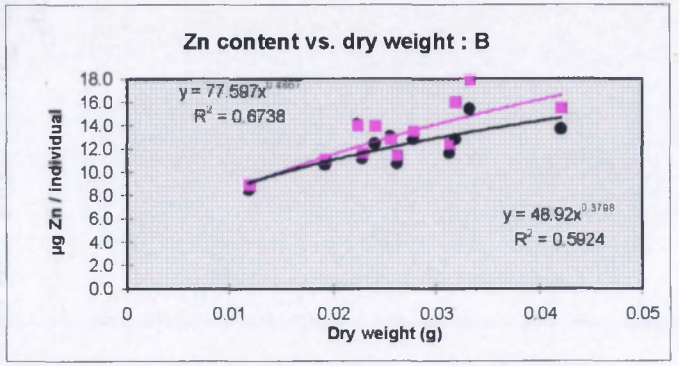

Figure 16c : Zn content (Y) as a function of the dry body weight (X) of *Macoma balthica* (Baalhoek) before (black circles) and after (pink boxes) mixed metal spike.

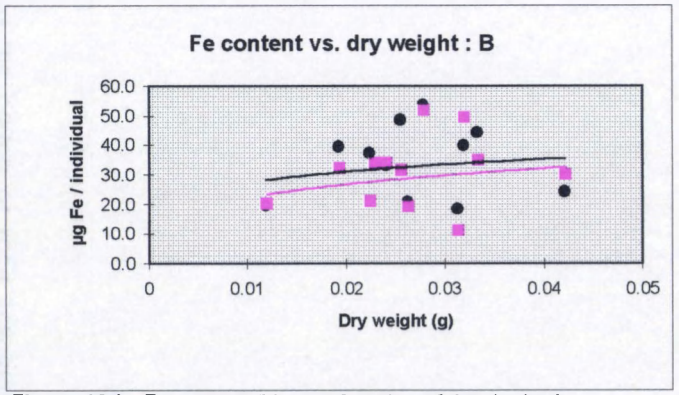

Figure 16d : Fe content (Y) as a function of the dry body weight (X) of *Macoma balthica* (Baalhoek) before (black circles) and after (pink boxes) mixed metal spike.

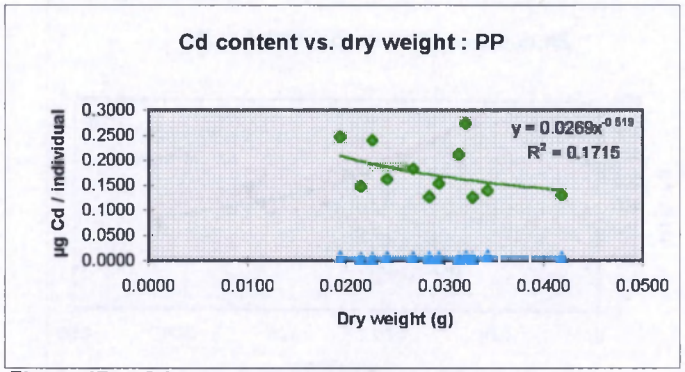

Figure 17a : Cd content (Y) as a function of the dry body weight (X) of *Macoma balthica* (Paulinapolder) before (blue triangles) and after (green diamonds) mixed metal spike.

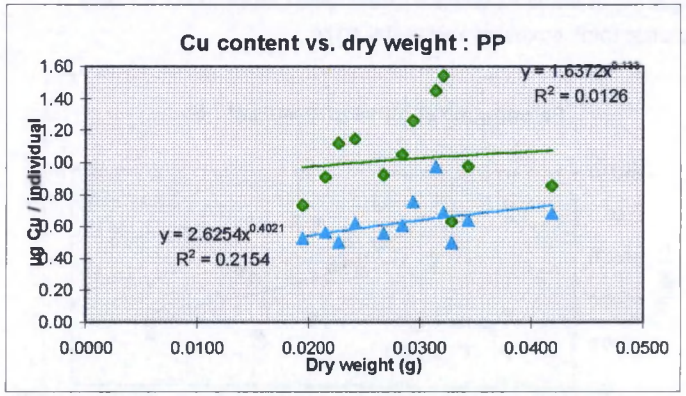

Figure 17b : Cu content (Y) as a function of the dry body weight (X) of *Macoma balthica* (Paulinapolder) before (blue triangles) and after (green diamonds) mixed metal spike.

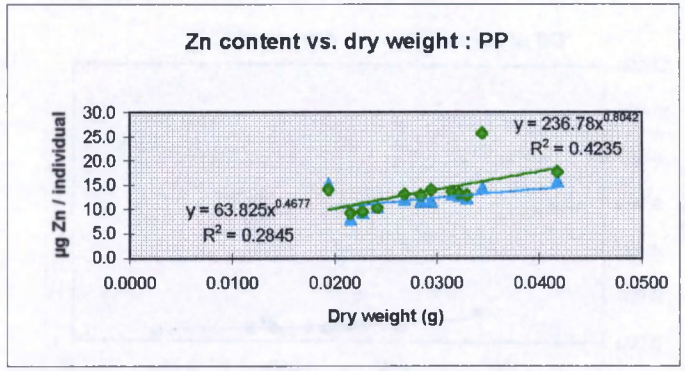

Figure 17c : Zn content (Y) as a function of the dry body weight (X) of *Macoma balthica* (Paulinapolder) before (blue triangles) and after (green diamonds) mixed metal spike.

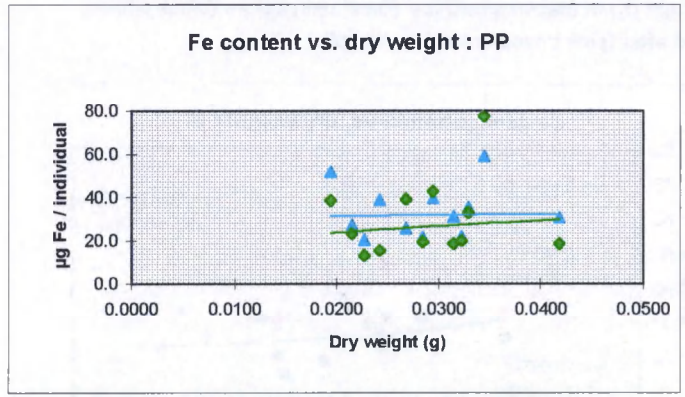

Figure 17d : Fe content (Y) as a function of the dry body weight (X) of *Macoma balthica* (Paulinapolder) before (blue triangles) and after (green diamonds) mixed metal spike.

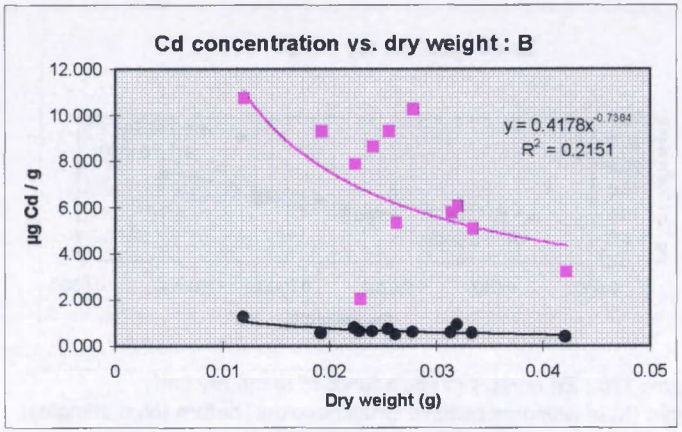

Figure 18a : Cd concentration (Y) as a function of the dry body weight (X) of *Macoma balthica* (Baalhoek) before (black circles) and after (pink boxes) mixed metal spike.

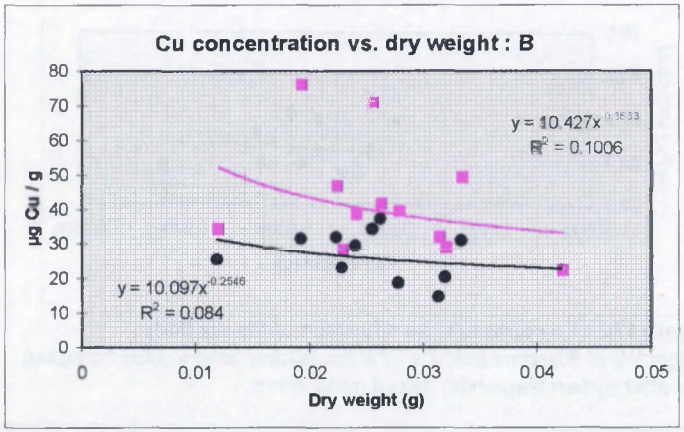

Figure 18b : Cu concentration (Y) as a function of the dry body weight (X) of *Macoma balthica* (Baalhoek) before (black circles) and after (pink boxes) mixed metal spike.

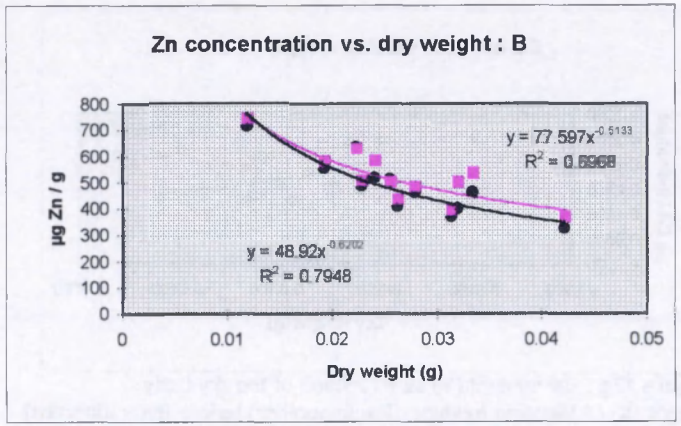

Figure 18c : Zn concentration (Y) as a function of the dry body weight (X) of *Macoma balthica* (Baalhoek) before (black circles) and after (pink boxes) mixed metal spike.

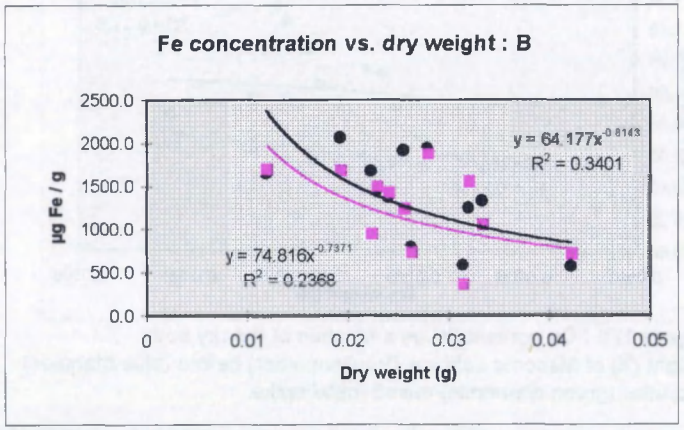

Figure 18d : Fe concentration (Y) as a function of the dry body weight (X) of *Macoma balthica* (Baalhoek) before (black circles) and after (pink boxes) mixed metal spike.

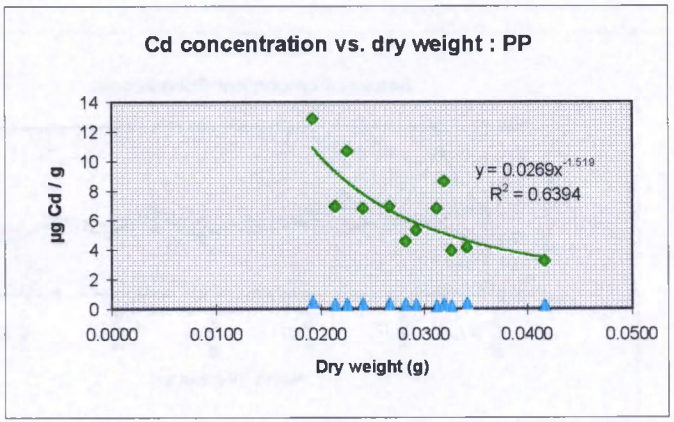

Figure 19a : Cd concentration (Y) as a function of the dry body weight (X) of *Macoma balthica* (Paulinapolder) before (blue triangles) and after (green diamonds) mixed metal spike.

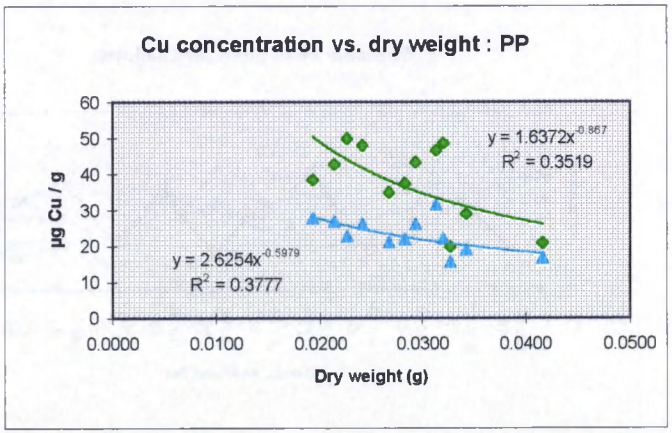

Figure 19b : Cu concentration (Y) as a function of the dry body weight (X) of *Macoma balthica* (Paulinapolder) before (blue triangles) and after (green diamonds) mixed metal spike.

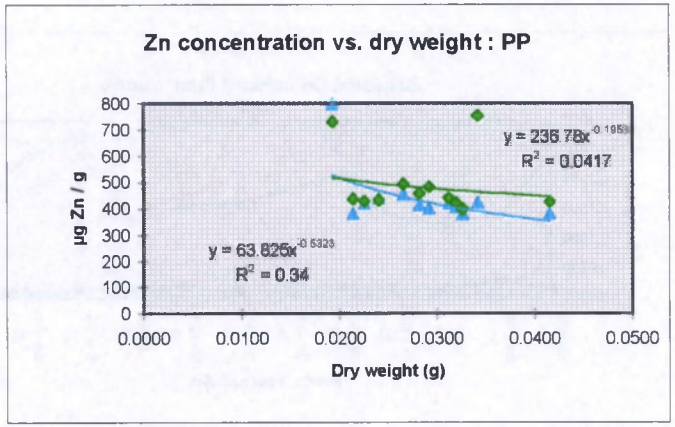

Figure 19c : Zn concentration (Y) as a function of the dry body weight (X) of *Macoma balthica* (Paulinapolder) before (blue triangles) and after (green diamonds) mixed metal spike.

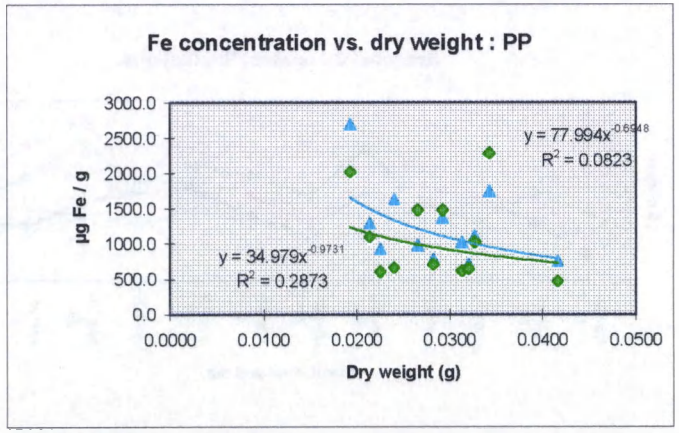

Figure 19d : Fe concentration (Y) as a function of the dry body weight (X) of *Macoma balthica* (Paulinapolder) before (blue triangles) and after (green diamonds) mixed metal spike.

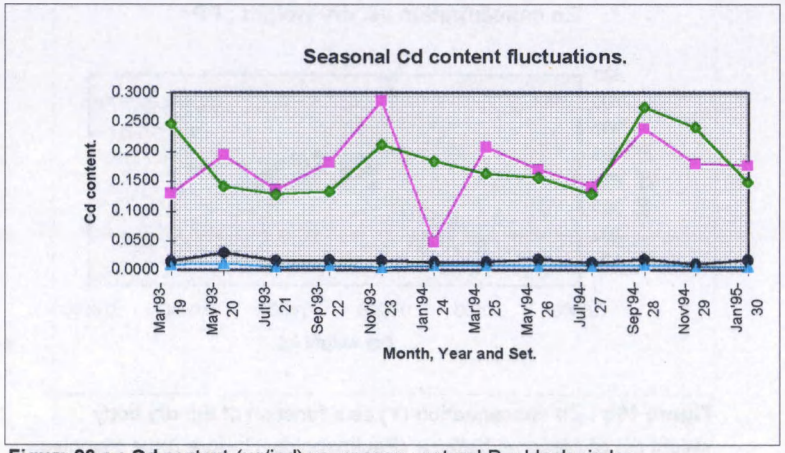

Figure 20 a : Cd content (µg/ind) vs. season ; natural B = black circles, natural PP = blue triangles, Exp.B = pink boxes and Exp. PP = green diamonds

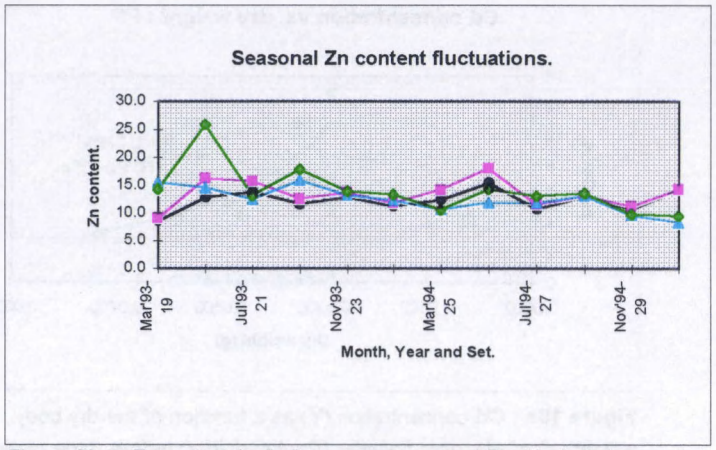

Figure 20 c : Zn content (µg/ind) vs. season ; natural B = black circles, natural PP = blue triangles, Exp.B = pink boxes and Exp. PP = green diamonds

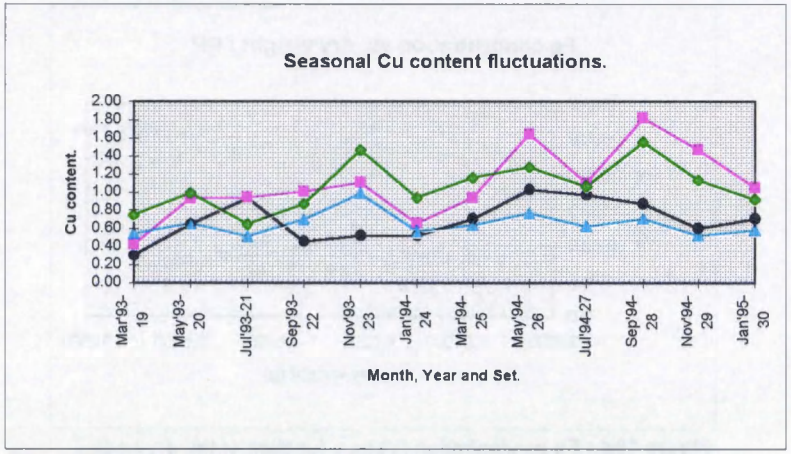

Figure 20 b : Cu content (µg/ind) vs. season ; natural B = black circles, natural PP = blue triangles, Exp.B = pink boxes and Exp. PP = green diamonds

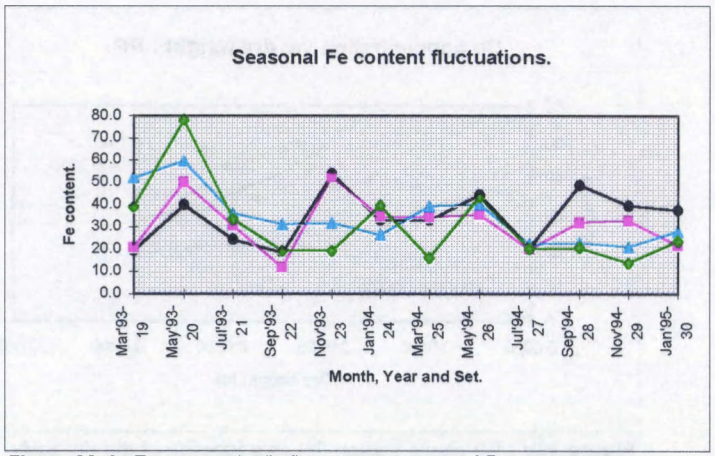

Figure 20 d : Fe content (µg/ind) vs. season ; natural B = black circles, natural PP = blue triangles, Exp.B = pink boxes and Exp. PP = green diamonds

4.3 Metal concentrations : the intracellular partitioning of metals :

The intracellular partitioning of Cd, Cu, Zn and Fe, in *Macoma balthica,* is significant because it can help elucidate how the clam deals with excess metal. Earlier paragraphs in this section, covering whole tissue metal concentrations, serve to explain which metals are governed by biological processes and which by external levels. It is now appropriate to attempt to explain the significance of the actual proportion of soluble metals i.e. the cytosolic metal concentration as the cytosol is the phase most likely to be involved in any metallothionein detoxification mechanism.

To our knowledge, only one paper has been published on metal fractionation in this clam (Johansson et al., 1986). Working on samples collected in South San Francisco Bay, a much more metal enriched ecosystem than the Western Scheldt, the authors found 5-15% cytosolic copper and 25-70% (most values < 40%) cytosolic zinc. Our copper values, as shown in Figure 11b, range from 1-40% but with most natural values being between 5 and 25%, therefore agreeing quite well with the San Francisco Bay situation. However, the % cytosolic zinc results differ greatly from our natural values, which range from 5-15%. Since this type of literature on *Macoma balthica* is very scarce, it could be worthwhile to compare metal partitioning values of other species. The following table (Table 19) summarises the findings of other authors and shows that Cd, Cu and Zn are always present in significant, although variable, proportions in the soluble phase, regardless of the species. In most species, with the exception of *M. balthica*, over 50% of the cadmium burden, appears in the cytosol. The reason for the lower soluble Cd burden in *M. balthica* could be due to the fact that the total Cd concentrations, in the species listed in Table 19, are up to four times higher than the maximum (1.27 $\mu g.g^{-1}$) found in "natural" *M. balthica* from the Western Scheldt. Marine mollusc sensitivity to metals has been well documented (Luoma, 1983) and is also true for *M. balthica*. For instance, after only three days exposure to 100 $\mu g.l^{-1}$ Cd, the concentrations of total and soluble metal increase greatly, the degree of which depends on the season (as shown in Figures 8a, 10a and 11a). The exposure aquaria also contained 100 $\mu g.l^{-1}$ Cu and after the same length of time, noticeable increases of total and soluble copper were seen, the degree of which also depended on the season (as shown in Figures 8b, 10b and 11b). Amiard et al. (1987) found a linear correlation between external Cu levels and internal copper concentrations for the bivalve *Scrobicularia plana*, which supports our findings of higher copper concentrations in *M. balthica* from Baalhoek (Figure 8b), the station with higher external Cu concentrations (Van den Berg et al., 1987).

Table 19 : Cytosolic metal proportions in various marine invertebrate species.

Species	% Cd	% Cu	% Zn	Reference
Littorina littorea	70	34	46	Langston and Zhou, 1986 (1)
Littorina littorea	55-65	29-42	42-50	Noël-Lambot et al., 1978 (1)
Purpura lapillus	23-66	4-37	22-36	idem (1)
Patella vulgata	50-65	61-70	33-45	idem (1)
Ostrea edulis	-	40	40	Coombs, 1974 (1)
Ostrea lutaria	51	81	70	Sharma, 1983 (1)
Mercenaria mercenaria	44	-	27	Carmichael et al., 1980 (1)
Macoma balthica	-	5-15	25-70	Johansson et al., 1986 (2)
Macoma balthica	0-28	10-33	7-17	Bordin et al., 1994 (2)
Macoma balthica	12-77	19-67	21-71	idem (1)
Macoma balthica	0-30	5-25	5-15	This work (2)

(1) Heat un-treated cytosol ; (2) Heat treated cytosol.

Even though the exposure aquaria also contained 600 $\mu g.l^{-1}$ Zn, there were no significant increases in total [Zn] (Figure 8c), proving that *M.balthica* can regulate their internal concentrations of this element. The period around the month of January seems to be the only time when increases of zinc are found in the cytosol (Figures 10c and 11c). A comparative study on bioaccumulation of trace metals in different aquatic organisms, including several molluscs (*Littorina littorea* and *Scrobicularia plana*), Amiard et al. (1987), showed that exposure to a four day contamination of up to 1000 $\mu g.l^{-1}$ Zn and to chronic contaminations of low levels did not change the internal zinc concentration. This metal has been shown to be involved in homeostatic regulation in many species.

a) Effect of body weight on the cytosolic metal content - Natural state :

Contrary to what was found for total whole body element content versus body weight, slightly better correlations were obtained by using linear regression as opposed to a power function regression. The equations for the best straight lines are presented below : $Y = aX + b$

- Case I : using **cytosol contents** ($\mu g.ind^{-1}$) : Natural state *M. balthica.*

$[Cd]_B = -0.012(X) + 0.002$; $r = 0.041$ $\quad$ $[Cd]_{PP} = -0.019(X) + 0.009$; $r = 0.217$
$[Cu]_B = 3.805(X) - 0.001$; $r = 0.544$ $\quad$ $[Cu]_{PP} = 0.163(X) + 0.059$; $r = 0.046$
$[Zn]_B = 45.99(X) - 0.228$; $r = 0.872$ $\quad$ $[Zn]_{PP} = 64.58(X) - 0.322$; $r = 0.683$
$[Fe]_B = 24.99(X) + 0.024$; $r = 0.660$ $\quad$ $[Fe]_{PP} = 103.6(X) - 1.56$; $r = 0.855$

See Figures 21 and 22 and Tables 20a and 20b for the raw data.

Cytosolic Cd contents decrease with increasing weight (Figure 21a and 22a) but this decrease is very slight for the natural state clams because of the initially low cytosolic levels. The other elements show an increase.

b) Effect of body weight on the cytosolic metal content - After exposure :

- Case I : using **cytosol contents** ($\mu g.ind^{-1}$) : *M. balthica* after spiking.

$[Cd]_B = -0.785(X) + 0.041$; $r = 0.372$ $\quad$ $[Cd]_{PP} = -1.200(X) + 0.052$; $r = 0.437$
$[Cu]_B = -0.399(X) + 0.149$; $r = 0.039$ $\quad$ $[Cu]_{PP} = -4.463(X) + 0.225$; $r = 0.477$
$[Zn]_B = -7.230(X) + 1.430$; $r = 0.143$ $\quad$ $[Zn]_{PP} = -28.91(X) + 1.93$; $r = 0.352$
$[Fe]_B = 35.25(X) - 0.228$; $r = 0.872$ $\quad$ $[Fe]_{PP} = 25.6(X) + 0.23$; $r = 0.234$

See Figures 21 and 22 and Tables 20a and 20b for the raw data.

After exposure one can see a very steep decrease in the Cd and Cu content of the bivalves.
The slopes of the iron linear regressions for cytosolic content do not differ greatly from the total plots, so iron regulation is assumed to continue. This is of course normal as iron was absent from the spike.

Grouping these observations, one can say that, on a yearly scale, smaller **exposed** clams tend to have more elevated Cd, Cu, Zn and Fe in their cytosol than larger clams. The opposite is true for whole tissue metal contents. Therefore, considering the general tendancy of clams to totally regulate Cu and Zn, it can be said that the cytosol displays very different behaviour on exposure to these heavy metals.

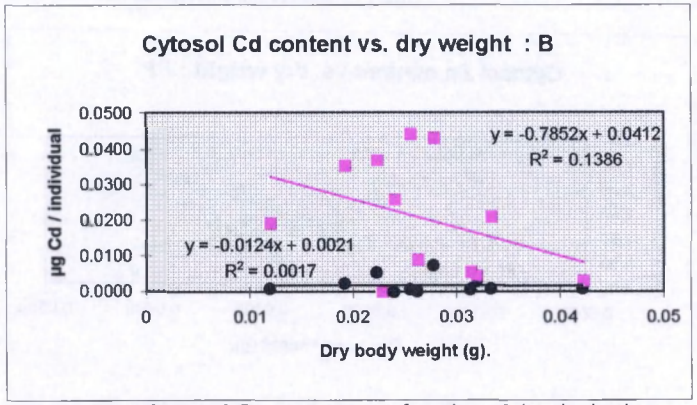

Figure 21a : Cytosol Cd content as a function of the dry body weight of *Macoma balthica* (Baalhoek) before (black spots) and after (pink boxes) mixed metal spike.

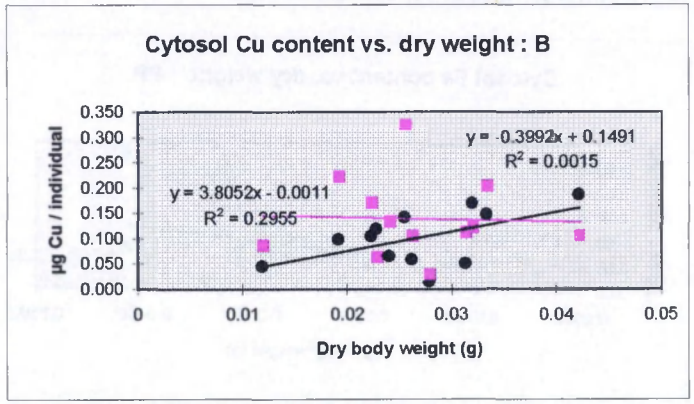

Figure 21b : Cytosol Cu content as a function of the dry body weight of *Macoma balthica* (Baalhoek) before (black spots) and after (pink boxes) mixed metal spike.

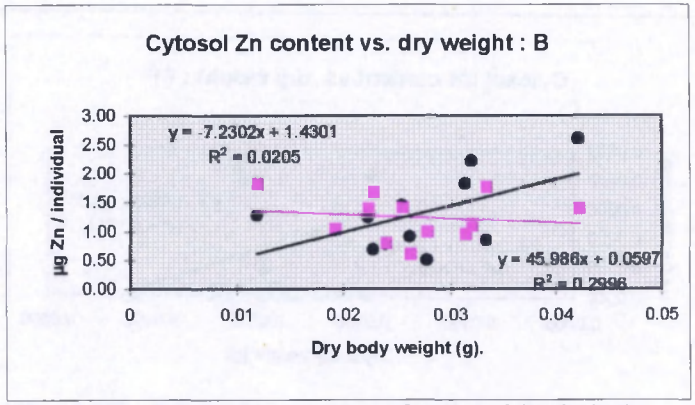

Figure 21c : Cytosol Zn content as a function of the dry body weight of *Macoma balthica* (Baalhoek) before (black spots) and after (pink boxes) mixed metal spike.

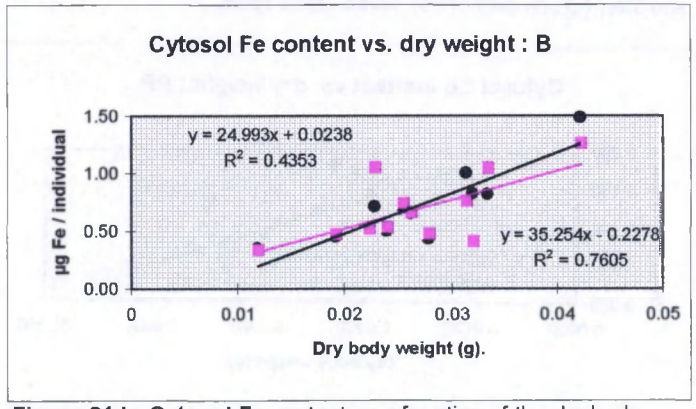

Figure 21d : Cytosol Fe content as a function of the dry body weight of *Macoma balthica* (Baalhoek) before (black spots) and after (pink boxes) mixed metal spike.

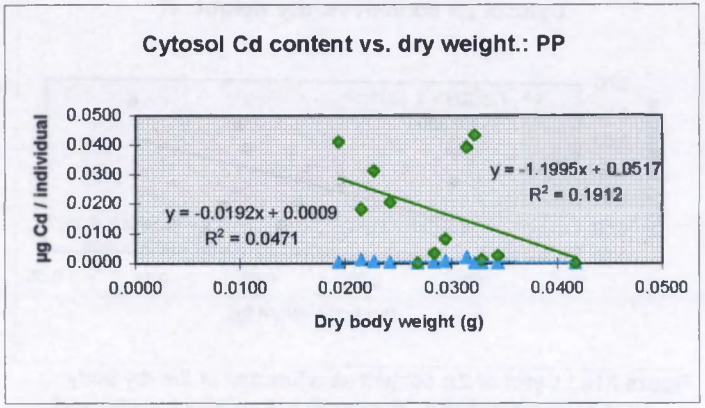

Figure 22a : Cytosol Cd content as a function of the dry body weight of *Macoma balthica* (Paulinapolder) before (blue triangles) and after (green diamonds) mixed metal spike.

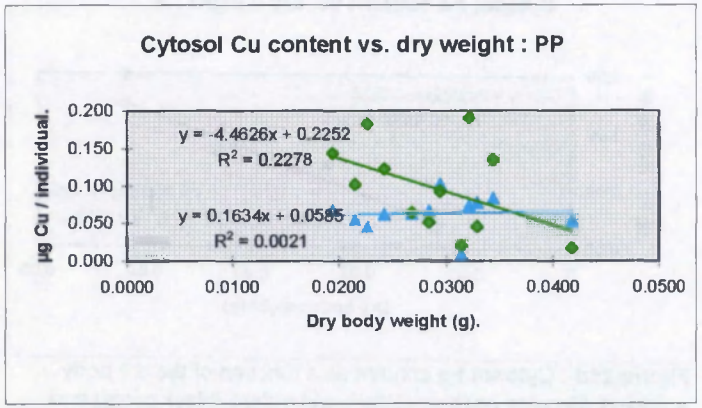

Figure 22b : Cytosol Cu content as a function of the dry body weight of *Macoma balthica* (Paulinapolder) before (blue triangles) and after (green diamonds) mixed metal spike.

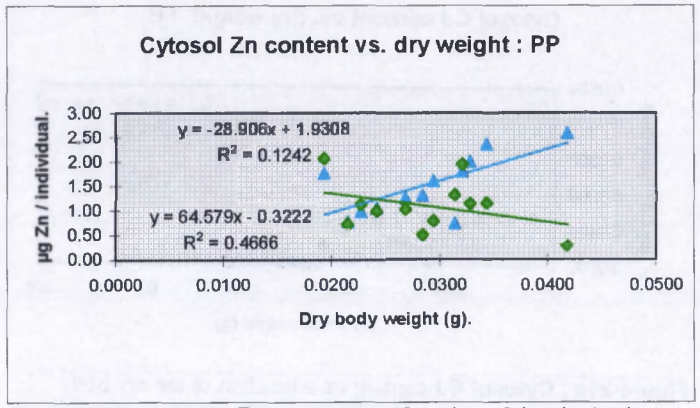

Figure 22c : Cytosol Zn content as a function of the dry body weight of *Macoma balthica* (Paulinapolder) before (blue triangles) and after (green diamonds) mixed metal spike.

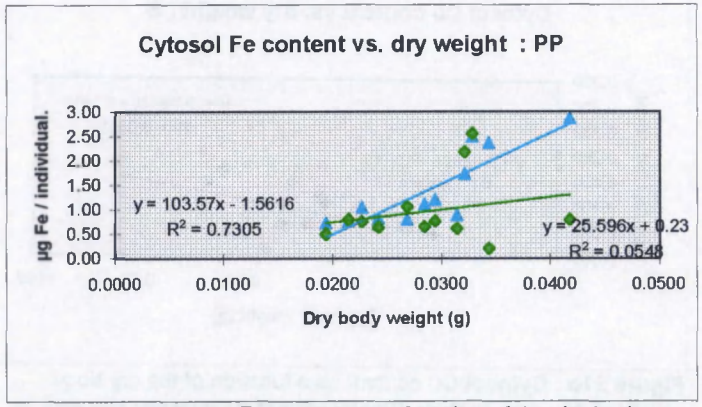

Figure 22d : Cytosol Fe content as a function of the dry body weight of *Macoma balthica* (Paulinapolder) before (blue triangles) and after (green diamonds) mixed metal spike.

c) % Cytosolic metal and cytosolic metal concentration :

Plotting % cytosolic metal vs. the metal concentration in the cytosol (Figures 23a-e) gives one an idea of how the higher the cytosolic concentration is, the higher the proportion it represents in the cytosol. This is observed for all of the elements, taken into consideration in this study, with rather similar trends of variation. A linear region followed by a plateau is observed for Cd, Cu and Fe. The natural data for Cd from both stations were plotted separately as the concentration scale was so much smaller than the exposed data. Examination of these figures show that after a certain metal concentration in the cytosol has been surpassed, the proportion of cytosolic metal reaches a maximum or steady state region. This is approximately 40 % for Cd corresponding to a cytosolic concentration of ~ 2 $\mu g.g^{-1}$, ~ 35 % for Cu corresponding to a cytosolic concentration of ~ 10 $\mu g.g^{-1}$ and ~ 12 % for Fe corresponding to a cytosolic concentration of ~ 70 $\mu g.g^{-1}$. There are two points on the Fig. 23c plot which appear to be spurious but if they are not, then there may be a plateau region for Zn after ~ 25% corres- ponding to a cytosolic concentration of ~ 125 $\mu g.g^{-1}$.

Therefore, for a given series of Cd concentrations, either low as in natural clams or high as after exposure, there is a tendancy for Cd to quickly concentrate in the heat stable cytosol, as soon as the concentration starts to increase. For example, in Fig. 23a one can see that in clams from PP, a cytosolic [Cd] of < 0.10 $\mu g.g^{-1}$ resulted in a 50% cytosolic proportion whereas in clams from Baalhoek, a higher cytosolic [Cd] of < 0.40 $\mu g.g^{-1}$ resulted in the same 50% cytosolic proportion i.e. the point being how little cytosolic [Cd] it takes in order to reach maximum cytosolic proportion.
This rapid response to Cd exposure, remembering that the duration of the exposures was only three days, is significant when one is reminded of the toxicity of Cd to aquatic organisms (Taylor, 1983).

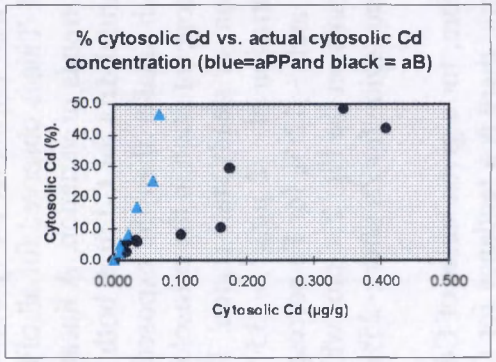

Figure 23a : % Cd in the cytosol as a function of the cytosolic Cd concentration for natural *M. balthica* at B & PP.

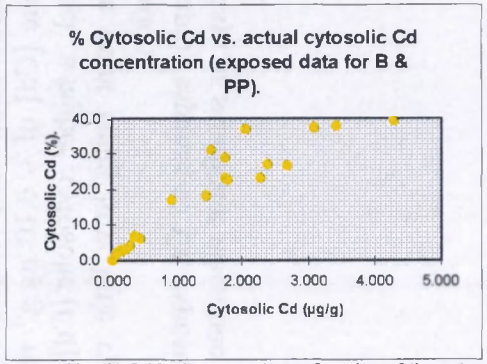

Figure 23b : % Cd in the cytosol as a function of the cytosolic Cd concentration for exposed *M. balthica* at B & PP.

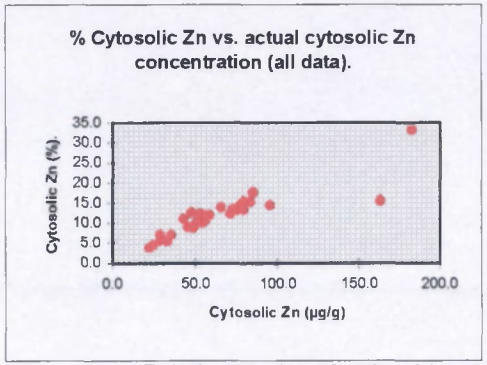

Figure 23c : % Zn in the cytosol as a function of the cytosolic Zn concentration for *M. balthica* at B & PP (nat. and exp.).

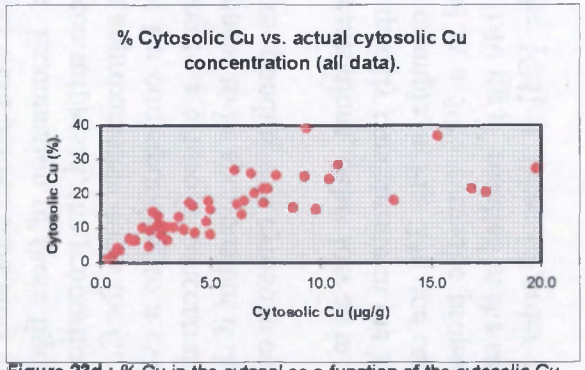

Figure 23d : % Cu in the cytosol as a function of the cytosolic Cu concentration for *M. balthica* at B & PP (natural and exposed).

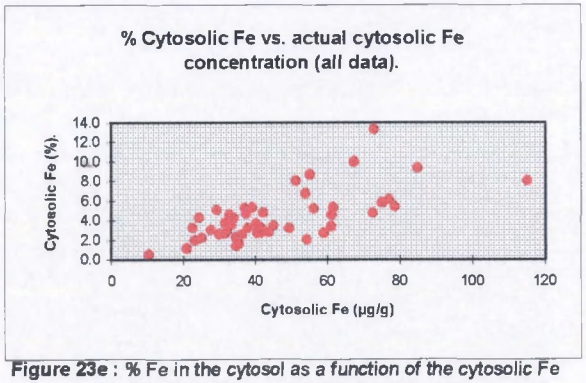

Figure 23e : % Fe in the cytosol as a function of the cytosolic Fe concentration for *M. balthica* at B & PP (natural and exposed).

4.4 Evidence to suggest MT induction :

Having explained how *M. balthica* :
a) deal with excess metal, particularly their intracellular partitioning of Cd and Cu,
b) produce metal-binding proteins in the heat stable cytosol after exposure,
c) have their maximum metal and MT concentrations in the same chromatographic fractions,
one can assume that there must be a very good reason for this.
Since the cytosol is where the metal-binding proteins are induced, storage of a large percentage (up to 50%) of its excess metal in the cytosol would allow the clam to detoxify the metal present in toxic concentrations.

Referring back to Section 1.3, where the characteristics of metallothioneins were listed, one can now say that the metal-binding proteins present in the cytosol of *M. balthica* have the following attributes :
- Low molecular weight (6000-12000 Da) as found by SEC.
- Soluble and heat stable as thiolic proteins were quantified after denaturation of heat un-stable proteins.
- Mainly found in the cytoplasm.
- High content of metal binding sulphydryl groups as found by DPP.
- Induced by metals such as Cd, Cu, Zn, Hg etc. We found increased concentrations of such metal binding proteins in almost all exposed samples over a two year period.
- UV ratio of A_{254} : A_{280} >> 1 suggesting low aromatic amino acid content. This was found in all cases and the results for Set 19 chromatographic fractions (not shown) give ratios of close to 2.
- Metal peaks in chromatographic cytosolic fractions co-incide with the MLP peaks in concentration.

Therefore, one can safely conclude that the proteins induced in *M. balthica* from the Western Scheldt Estuary are MTs or MLPs but since an amino acid analysis of these metal-binding proteins, in order to elucidate their primary structure, has not yet been done, one is reluctant to categorically state that the proteins are MTs and not simply MLPs.

Further research is going on by colleagues at the moment using RP-HPLC and Capillary electrophoresis to refine their isolation and is giving very promising results.

4.5 MT "norm" charts to provide evidence of metal pollution in the indicator organism :

If the natural levels of MTs in *M. balthica* from both Baalhoek and Paulinapolder were to be considered "normal" then the creation of control charts with upper warning (UWL) and upper action limits (UAL) could be a valuable tool for indicating heavy metal pollution, when and if concentrations of MTs rose above either of those differing seasonal limits. Therefore, using the following formulas to calculate the UWL and UAL (and lower WL and lower AL), the MT concentrations ($mg.g^{-1}$) are plotted against the season, as shown in Figure 24 and the values of which are given in Table 21.

- UWL and LWL are calculated by : ± (2 x Pop.SD) / Sqrt (n) i.e. 95 % limit.
- UAL and LAL are calculated by : ± (3 x Pop.SD) / Sqrt (n) i.e. 99.7 % limit.

where Pop.SD is the standard deviation of the population and n is the number of repeat measurements (3). That is to say if 95 % of the sample MT means (the samples being clams after exposure to the mixed metal spike, in this case) were to lie within the overall "natural" MT mean ± [(2 x Pop.SD) / Sqrt (n)] and if 99.7 % were to lie within the overall "natural" MT

mean ± [(3 x Pop.SD) / Sqrt (n)], then there would be no cause for environmental concern. As already stated, due to seasonal fluctuations in natural MT levels, one set of limits would not have been useful so the natural data were used to construct seasonal limits. Examination of Figure 24a and b shows that the MT ranges are quite narrow for the Winter/early Spring months in Baalhoek (e.g. LAL to UAL of 5.26-6.47 $mg.g^{-1}$ MT) but are wider in the Summer with action limit values ranging between for e.g. 1.09 and 4.40 $mg.g^{-1}$ MT. However the opposite is true for Paulinapolder. The values of Set 19 and 20 PP also strangely belong to the Summer grouping.

Having obtained these differing seasonal control charts, the data were grouped to provide average seasonal control limits for the two year period. Time will tell if these change much over a longer period of time. The average seasonal "norm" charts are shown in Figure 25 and the raw data is given in Table 22. The action limits for Baalhoek are 4.42-7.88 $mg.g^{-1}$ MT in Winter/ early Spring months while they are 1.30-4.21 $mg.g^{-1}$ MT in Summer/Autumn months. Using the average data, one finds the same trend in Paulinapolder with wider ranges in Winter/early Spring (2.06-9.95 $mg.g^{-1}$ MT) and narrower in Summer/Autumn (0.66-3.38 $mg.g^{-1}$ MT).

In Figure 25a and b, one can see that the clams exposed to the metal spike often have MT concentrations above the UWL and UAL. It is perhaps worth pointing out that natural MT values in clams from Baalhoek, in January and March 1995, are actually above the seasonal average UWL but below the UAL. Whether this trend shall continue or was just sporadic remains to be seen.

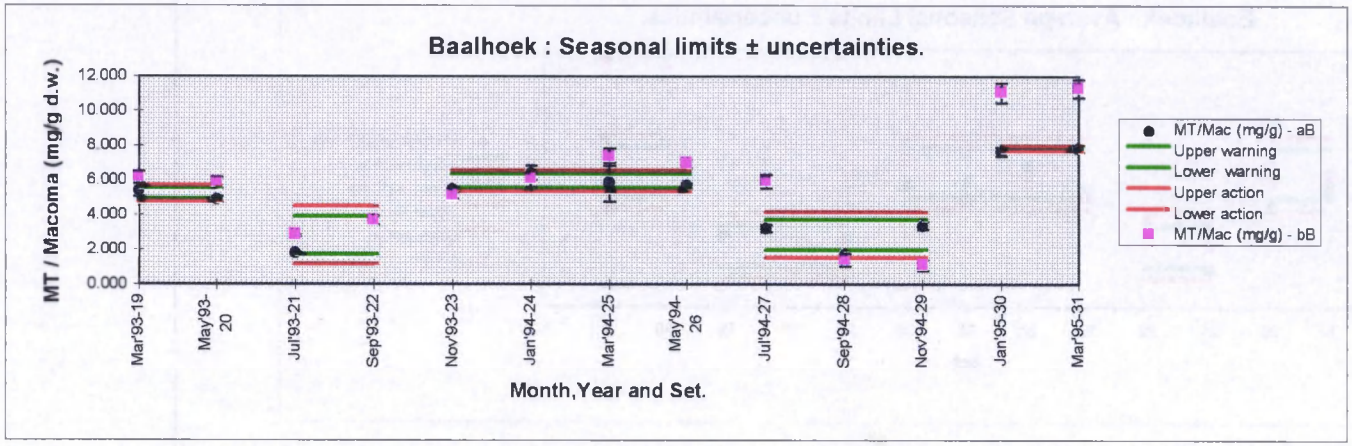

Figure 24 a : Seasonal "norm" chart for MT concentrations in *M.balthica* from Baalhoek.

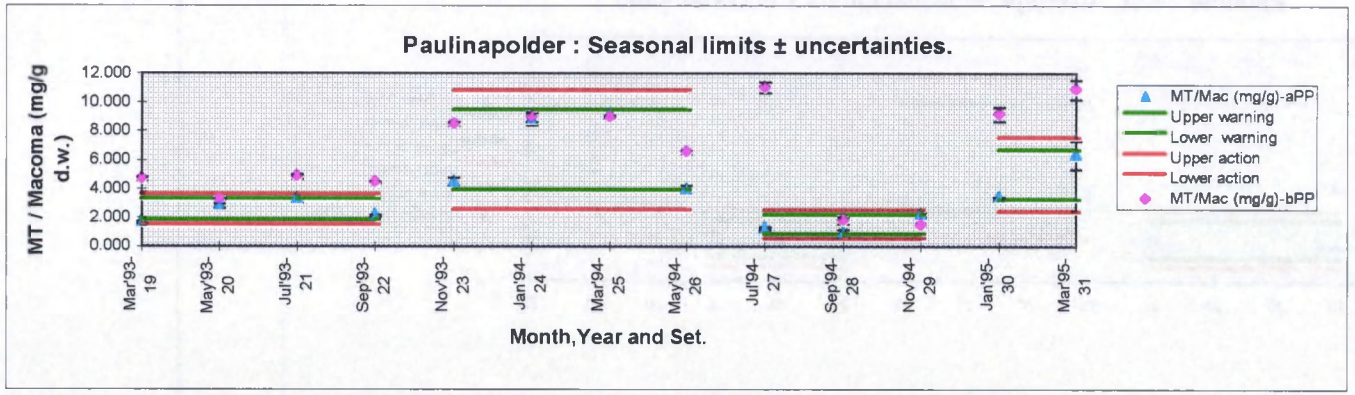

Figure 24 b : Seasonal "norm" chart for MT concentrations in *M.balthica* from Paulinapolder..

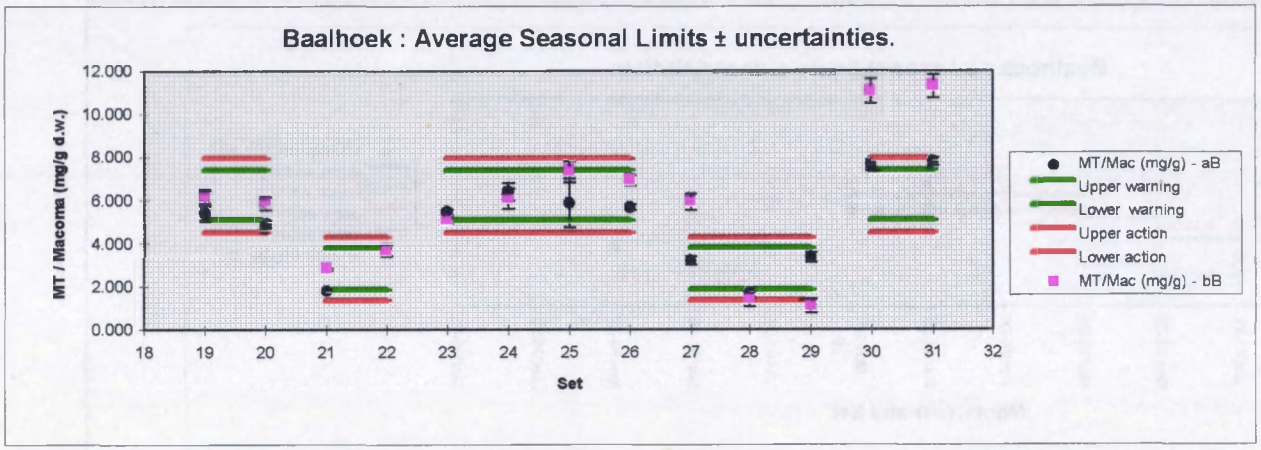

Figure 25 a : Average seasonal "norm" chart for MT concentrations in *M.balthica* from Baalhoek.

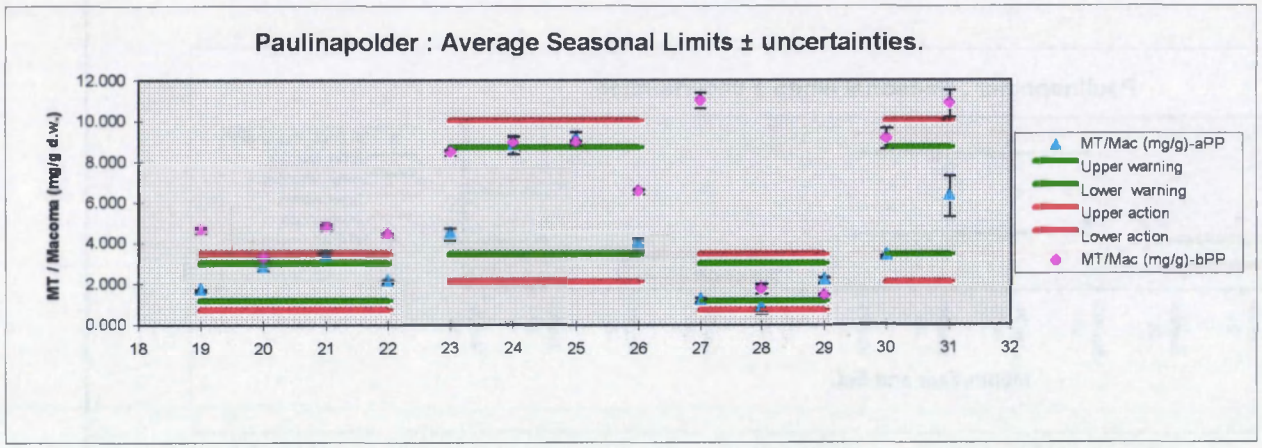

Figure 25 b : Average seasonal "norm" chart for MT concentrations in *M.balthica* from Paulinapolder..

5.0 Conclusion :

➢All four metals show similar seasonal patterns in terms of their concentration in *Macoma balthica*, although naturally of different amplitudes, but all being higher in Winter and lower in Summer. There are relatively weak influences of time and location on copper and zinc concentrations but location plays an important role in cadmium concentrations, while time exhibits a more moderate influence. Contrary to this, iron concentrations are strongly influenced by time but weakly by location.

➢Considering bivalve metal content instead of metal concentration provides more information on metal behaviour. Copper and zinc appear to be primarily controlled by biological processes. The clam Cd burden, however, largely depends on the ecosystem metal level, reflecting the differences, in cadmium pollution of Baalhoek and Paulinapolder quite well. The case of iron seems to be between that of Cu/Zn and that of Cd, with its spatio-temporal fluctuations being dependent on both the biological evolution and the environmental metal level, without any precise discrimination.

➢Of the four metals studied, only Cd, a non-essential element, could be used to detect notable differences in environmental metal contamination in the Western Scheldt estuary, if *Macoma balthica* can be considered as an indicator organism.

➢One can say that there is an overall decrease of total natural and exposed levels of Cd, Cu, Zn and Fe concentrations in *Macoma balthica*, with increasing body weight.

➢ Short term exposures to high cadmium, copper and zinc concentrations results in high accumulation of Cd and significant quantities of Cu *by M. balthica* while it can regulate its zinc and to some extent, its iron concentrations. These phenomena are generally amplified for Cd and Cu in the heat stable cytosol. Naturally, as shown, these statements are subject to season dependancy, with concentration maxima in Winter and minima in Summer, as a result of higher metal uptakes by smaller clams in Winter and vice versa in Summer. These fluctuations do not come from "simple" dilution or concentration effects, since the metal content is not constant.

➢ The behaviour of whole tissue and cytosolic metals are not parallel since the variations of their contents versus body weight are described by different functions. It has also been demonstrated that the proportion of cytosolic metals is not constant but can vary from very little up to a maximum of 35-40% as a function of the concentration of metal partitioned in the cytosol and hence of the levels of whole tissue concentrations. Therefore the cytosolic phase quite obviously displays a growing function in the storage of metals when their concentrations increase. This fact is supported by the presence of metal binding proteins in the cytosol.

➢ MTs or MLPs have been found in *M. balthica* from both stations (the stations not being significantly different to each other (P=0.05)). However increases of MT concentrations are significantly (P=0.05) greater after spiking bivalves from Paulinapolder than those from Baalhoek.

➢ The presence of Cd and Cu peak maxima, after spiking, in the 6000-12000 Da Mw range corresponding to the range of maximum MT concentrations suggests the induction of these proteins on exposure of *Macoma balthica* to these metals.

➢ It is thought that average seasonal MT limits (UAL) in a "norm' chart could be used for indication of metal pollution when MT levels rise above such limits.

6.0 Future work :

- MT is recovered more efficiently with less oxidation using ultracentrifugation so when this instrument is acquired, it will be tested for efficiency in cytosolic preparation.
- Amino acid analyses of the chromatographic fractions of the cytosol will be carried out to determine the primary structure in *M. balthica* MLPs.
- Further sets of chromatographic fractions of the cytosol will be measured for their MT concentrations.
- Exposure of clams to a low Cd only spike is already in progress with these bivalves remaining exposed for ~ 40 days. This is to see the effect of more realistic Cd contamination possibilities.

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Annex 1 :

Statistical Tests - some worked examples.

a) Comparison of the means of two samples : This approach tests whether the difference between two results is significant, or can be accounted for merely by random variations.

Example :

Baalhoek : Mean = 26.61 $\mu g.g^{-1}$ Cu ; Standard deviation = 6.898 $\mu g.g^{-1}$
Paulinapolder : Mean = 22.73 $\mu g.g^{-1}$ Cu ; Standard deviation = 4.720 $\mu g.g^{-1}$
For each station, 12 measurements were made, over the two year period.

Ques. : Do these stations give results having means which differ significantly ?
Answer : Ho (null hypothesis) : The means of the results are equal.

Pooled estimate of the standard deviation :
$S^2 = \{(n_1-1)S_1^2 + (n_2-1)S_2^2\} / (n_1 + n_2 - 2)$
$S^2 = \{(11 \times 6.898^2) + (11 \times 4.720^2)\} / 22 = 34.93$
$S = 5.91$

"**t**" is given by : $\{(Mean_1 - Mean_2) / S\} \times \{Sqrt\ (1/n_1 + 1/n_2)\}$
That implies that **t** = {(26.61 - 22.73) / 5.91} x {0.408} = 1.61
There are 22 degrees of freedom (d.f.), so the critical value of |t| (P=0.05) = 2.07
Since 1.61 < 2.07, the null hypothesis is not rejected at the 5% level of confidence.
Therefore, Baalhoek and Paulinapolder can be considered equal in their average copper concentrations.

b) Evaluation of an uncertainty : The uncertainty is calculated by $(s/n^{1/2})t$; where s = absolute standard deviation; n = the number of repetitions and t = students value for n-1 degrees of freedom at 95 % confidence interval (CI).

Example :

The [Cd] in *Macoma balthica* for Set 19aB gave the following results :

Mean = 1.270 $\mu g.g^{-1}$; Standard deviation = 0.0064 $\mu g.g^{-1}$
Three repeat measurements were made, so there are 2 d.f.
Therefore t = 4.30 at 95 % CI.

$(0.0064 / 3^{1/2}) \times 4.30 = 0.016\ \mu g.g^{-1}$

The result can be expressed as $1.270 \pm 0.016\ \mu g.g^{-1}$

Annex 2 : Tables a,b,c and d show the intracellular partitioning of metals (indirect route) as well as the whole tissue concentration (direct route) in *Macoma baltica* - both natural state and exposed values.

Table a :The distribution of Cd in the insoluble phase (1st.ppt.), the soluble phase (HMWP) (2nd.ppt.) and the heat-stable cytosol. Y = 0.977(X) + 0.16 ; r = 0.978

Note : Omitted from graph and from straight line equation.

	X							Y	
Sample i.d.	Direct route	Quantity recovered (µg/g) "Indirect route"							
	Whole tissue	1st. ppt		2nd. ppt.		Cytosol		Sum of the three phases	Recovery %
	(µg/g)	(µg/g)	%	(µg/g)	%	(µg/g)	%	(µg/g)	
19aB	1.270	0.387	25.3	0.980	64.1	0.162	10.6	1.529	120.4
19bB	10.717	2.889	32.1	2.711	30.1	3.402	37.8	9.002	84.0
19aPP	0.383	0.092	24.8	0.270	72.5	0.010	2.6	0.372	97.1
19bPP	12.770	2.822	34.2	2.355	28.5	3.073	37.2	8.250	64.6
20aB	0.924	0.684	55.6	0.444	36.1	0.102	8.3	1.231	133.2
20bB	6.046	6.051	93.6	0.156	2.4	0.258	4.0	6.465	106.9
20aPP	0.287	0.215	60.6	0.140	39.4	0.000	0.0	0.355	123.8
20bPP	4.090	3.943	91.5	0.253	5.9	0.115	2.7	4.311	105.4
21aB	0.393	0.133	36.5	0.209	57.4	0.022	6.1	0.365	92.8
21bB	3.191	1.717	48.3	1.760	49.5	0.075	2.1	3.552	111.3
21aPP	0.124	0.088	47.8	0.095	52.2	0.000	0.0	0.183	147.6
21bPP	3.865	2.171	54.6	1.769	44.5	0.034	0.9	3.974	102.8
22aB	0.575	0.274	36.5	0.455	60.7	0.020	2.7	0.749	130.3
22bB	5.750	2.501	37.3	4.001	59.7	0.199	3.0	6.702	116.5
22aPP	0.155	0.051	43.0	0.067	57.0	0.000	0.0	0.118	75.8
22bPP	3.135	2.656	76.6	0.810	23.4	0.000	0.0	3.466	110.6
23aB	0.605	0.131	18.6	0.233	32.9	0.343	48.5	0.708	117.0
23bB	10.251	1.502	27.2	1.978	35.8	2.039	36.9	5.519	53.8
23aPP	0.106	0.021	14.7	0.056	38.8	0.068	46.5	0.145	137.1
23bPP	6.737	1.400	28.5	1.995	40.6	1.518	30.9	4.913	72.9
24aB	0.613	0.249	50.0	0.249	50.0	0.000	0.0	0.498	81.2
24bB	9.321	0.730	55.3	0.591	44.7	0.000	0.0	1.320	14.2
24aPP	0.238	0.056	29.1	0.136	70.9	0.000	0.0	0.192	80.8
24bPP	6.834	2.480	35.5	4.516	64.5	0.000	0.0	6.996	102.4
25aB	0.616	0.184	24.6	0.563	75.4	0.000	0.0	0.747	121.2
25bB	8.629	1.804	22.9	4.305	54.6	1.771	22.5	7.880	91.3
25aPP	0.261	0.086	32.7	0.164	62.7	0.012	4.5	0.262	100.5
25bPP	6.715	1.075	17.9	3.198	53.2	1.735	28.9	6.008	89.5
26aB	0.545	0.141	23.9	0.411	69.9	0.036	6.2	0.588	108.0
26bB	5.063	1.785	33.8	2.594	49.2	0.894	17.0	5.274	104.2
26aPP	0.196	0.072	26.5	0.177	65.5	0.022	8.0	0.271	138.2
26bPP	5.261	1.566	31.3	3.102	62.1	0.329	6.6	4.997	95.0
27aB	0.491	0.121	16.4	0.610	83.0	0.004	0.5	0.734	149.5
27bB	5.310	1.594	22.9	4.965	71.2	0.413	5.9	6.972	131.3
27aPP	0.202	0.045	21.8	0.151	73.3	0.010	4.9	0.206	101.9
27bPP	4.502	1.561	29.1	3.671	68.4	0.139	2.6	5.372	119.3
28aB	0.720	0.308	55.8	0.208	37.8	0.035	6.3	0.551	76.5
28bB	9.299	3.190	36.2	3.247	36.9	2.366	26.9	8.803	94.7
28aPP	0.223	0.063	43.3	0.082	56.7	0.000	0.0	0.145	64.9
28bPP	8.543	4.019	50.4	2.513	31.5	1.440	18.1	7.972	93.3
29aB	0.547	0.251	42.3	0.168	28.3	0.175	29.4	0.595	108.7
29bB	9.296	3.591	35.9	3.765	37.6	2.659	26.6	10.015	107.7
29aPP	0.200	0.092	46.4	0.073	36.7	0.034	16.9	0.199	99.4
29bPP	10.607	3.768	38.6	3.740	38.3	2.263	23.2	9.771	92.1
30aB	0.777	0.277	28.7	0.281	29.1	0.407	42.2	0.966	124.3
30bB	7.868	3.144	28.9	3.458	31.8	4.264	39.2	10.867	138.1
30aPP	0.199	0.078	34.4	0.092	40.3	0.058	25.3	0.227	114.2
30bPP	6.869	2.749	36.7	3.014	40.2	1.727	23.1	7.490	109.0

Table b :The distribution of **Cu** in the insoluble phase (1st.ppt.), the soluble phase (HMWP) (2nd.ppt.) and the heat-stable cytosol. Y = 1.152(X) + 0.09 ; r = 0.909

Note : Omitted from graph and from straight line equation.

	X							Y	
Sample I.d.	**Direct route**	**Quantity recovered (µg/g). "Indirect route"**							
	Whole tissue	**1st. ppt**		**2nd. ppt.**		**Cytosol**		**Sum of the three phases**	**Recovery %**
	(µg/g)	**(µg/g)**	**%**	**(µg/g)**	**%**	**(µg/g)**	**%**	**(µg/g)**	
19aB	25.5	15.3	43.5	12.3	35.1	7.5	21	35.09	137.6
19bB	34.1	14.6	35.5	11.3	27.5	15.2	37.0	41.13	120.6
19aPP	27.3	20.0	62.7	7.0	21.9	4.9	15.4	31.96	117.1
19bPP	38.1	19.7	52.6	7.1	18.8	10.7	28.6	37.51	98.5
20aB	20.4	10.1	45.0	6.4	28.2	6.0	26.8	22.53	110.4
20bB	28.8	32.2	76.2	2.7	6.5	7.4	17.4	42.27	146.8
20aPP	18.6	10.9	57.3	5.6	29.3	2.6	13.4	19.03	102.3
20bPP	28.5	27.5	75.9	2.3	6.2	6.5	17.9	36.21	127.0
21aB	22.2	15.5	57.3	6.7	24.8	4.8	17.9	27.03	121.8
21bB	22.0	14.4	55.6	8.8	33.9	2.7	10.5	25.94	117.9
21aPP	15.2	8.0	51.1	5.3	34.2	2.3	14.8	15.60	102.7
21bPP	19.4	13.8	58.4	8.3	35.2	1.5	6.4	23.62	121.8
22aB	14.6	10.0	54.6	6.4	35.3	1.8	10.1	18.22	124.8
22bB	31.7	22.3	46.1	21.9	45.2	4.2	8.7	48.45	152.8
22aPP	16.3	11.2	63.1	5.3	29.9	1.2	6.9	17.76	109.0
22bPP	20.5	19.1	79.4	4.5	18.6	0.5	2.0	24.07	117.4
23aB	18.7	10.7	62.1	5.8	33.8	2.7	14.1	19.17	102.5
23bB	39.3	12.3	56.5	8.1	37.3	7.0	25.5	27.39	69.7
23aPP	31.1	24.1	81.1	5.3	17.8	3.3	10.1	32.75	105.3
23bPP	46.3	14.1	61.6	8.0	34.9	7.5	25.3	29.58	63.9
24aB	23.0	11.3	47.9	3.0	12.7	9.3	39.4	23.63	102.7
24bB	28.2	11.8	45.2	7.5	28.7	6.8	26.1	26.03	92.3
24aPP	20.8	11.8	51.9	7.0	30.7	4.0	17.4	22.74	109.3
24bPP	34.5	22.0	55.2	13.1	32.9	4.8	11.9	39.87	115.6
25aB	29.5	10.9	43.4	10.1	40.2	4.1	16.4	25.21	85.5
25bB	38.5	12.7	34.3	15.0	40.7	9.2	25.0	36.85	95.7
25aPP	25.8	20.7	65.1	7.8	24.7	3.2	10.2	31.78	123.2
25bPP	47.6	16.9	39.8	15.2	35.9	10.3	24.3	42.35	89.0
26aB	30.9	16.0	44.0	14.1	39.0	6.1	17.0	36.23	117.3
26bB	49.0	25.3	47.0	19.9	37.0	8.7	16.1	53.94	110.1
26aPP	25.8	13.0	49.6	9.8	37.1	3.5	13.3	26.28	101.9
26bPP	43.0	16.2	41.9	18.7	48.5	3.7	9.6	38.66	89.9
27aB	37.1	22.2	48.5	20.7	45.1	3.0	6.5	45.88	123.7
27bB	41.5	23.6	39.7	31.0	52.0	4.9	8.3	59.51	143.4
27aPP	21.5	11.6	48.7	9.8	40.9	2.5	10.4	23.90	111.2
27bPP	37.1	19.7	43.1	23.9	52.3	2.1	4.7	45.74	123.3
28aB	34.2	19.2	56.0	8.2	23.8	6.9	20.2	34.32	100.4
28bB	70.8	46.6	54.8	21.0	24.7	17.4	20.5	84.96	120.0
28aPP	21.6	14.5	62.1	6.7	28.5	2.2	9.3	23.34	108.0
28bPP	48.2	28.4	62.1	10.9	24.0	6.3	13.9	45.64	94.7
29aB	31.4	18.1	53.0	8.7	25.5	7.4	21.5	34.23	109.0
29bB	75.8	39.8	51.1	21.2	27.3	16.8	21.6	77.75	102.6
29aPP	22.3	24.9	73.4	6.3	18.7	2.7	7.9	33.92	152.1
29bPP	49.6	39.9	54.3	20.3	27.7	13.2	18.0	73.39	148.0
30aB	31.8	12.7	40.6	10.7	34.1	7.9	25.3	31.22	98.2
30bB	46.5	27.8	38.7	24.4	33.9	19.7	27.4	71.89	154.6
30aPP	26.4	17.6	60.5	8.5	29.2	3.0	10.2	29.00	109.8
30bPP	42.4	30.8	48.7	22.7	35.9	9.7	15.4	63.10	148.8

Table c :The distribution of **Zn** in the insoluble phase (1st.ppt.), the soluble phase (HMWP) (2nd.ppt.) and the heat-stable cytosol. Y = 1.117 (X) + 6.12 ; r = 0.680

Note : Omitted from graph and from straight line equation.

	X							Y	
Sample I.d.	**Direct route**	**Quantity recovered (µg/g). "indirect route"**							
	Whole tissue	**1st. ppt**		**2nd. ppt.**		**Cytosol**		**Sum of the three phases**	**Recovery %**
	(µg/g)	**(µg/g)**	**%**	**(µg/g)**	**%**	**(µg/g)**	**%**	**(µg/g)**	
19aB	716	406.9	42.3	343.3	35.7	212.4	22.1	963	134.4
19bB	742	252.4	24.4	455.5	44.1	326.0	31.5	1034	139.3
19aPP	791	377.8	53.4	201.2	28.4	128.7	18.2	708	89.5
19bPP	725	213.5	29.9	347.0	48.5	154.5	21.6	715	98.6
20aB	402	300.4	47.7	249.7	39.7	79.5	12.6	630	156.6
20bB	500	1127.3	86.0	116.6	8.9	66.5	5.1	1310	262.1
20aPP	418	322.2	48.8	266.5	40.3	72.0	10.9	661	158.1
20bPP	749	1274.8	84.9	170.4	11.3	56.1	3.7	1501	200.4
21aB	326	331.0	52.0	237.5	37.3	67.7	10.6	636	195.1
21bB	367	427.7	53.6	334.1	41.9	35.8	4.5	798	217.3
21aPP	374	457.5	50.5	387.8	42.8	59.9	6.6	905	242.0
21bPP	394	471.5	53.4	374.1	42.3	38.3	4.3	884	224.3
22aB	372	223.1	47.3	182.4	38.7	66.5	14.1	472	126.9
22bB	393	298.0	44.0	343.3	50.7	35.5	5.2	677	172.2
22aPP	376	175.6	42.8	174.5	42.5	60.5	14.7	411	109.2
22bPP	423	411.5	78.6	104.0	19.9	8.1	1.5	524	123.8
23aB	461	409.0	75.3	110.3	20.3	23.9	4.4	543	117.8
23bB	482	235.4	62.0	97.0	25.6	47.3	12.4	380	78.8
23aPP	414	232.4	56.6	150.2	36.5	28.4	6.9	411	99.3
23bPP	436	207.6	48.8	166.7	39.2	51.4	12.1	426	97.6
24aB	490	401.6	75.8	74.5	14.1	53.7	10.1	530	108.1
24bB	506	211.8	38.7	154.0	28.1	182.1	33.2	548	108.3
24aPP	450	199.7	38.5	239.5	46.2	78.9	15.2	518	115.1
24bPP	489	238.8	41.9	253.4	44.5	77.0	13.5	569	116.4
25aB	518	298.1	54.1	204.0	37.1	48.5	8.8	551	106.3
25bB	580	273.6	52.6	191.5	36.8	55.1	10.6	520	89.7
25aPP	434	189.0	39.7	231.0	48.5	56.0	11.8	476	109.7
25bPP	429	149.9	31.1	247.9	51.4	84.5	17.5	482	112.4
26aB	463	274.5	54.4	194.9	38.6	35.0	6.9	504	108.9
26bB	535	247.4	43.6	244.5	43.1	75.1	13.2	567	106.0
26aPP	396	218.8	42.2	244.2	47.1	55.5	10.7	519	130.9
26bPP	479	261.5	43.7	304.8	50.9	32.6	5.4	599	125.0
27aB	410	153.2	29.9	313.0	61.2	45.4	8.9	512	124.8
27bB	434	153.7	30.1	328.4	64.3	28.4	5.6	511	117.6
27aPP	408	147.6	29.2	308.4	60.9	50.2	9.9	506	124.1
27bPP	454	207.0	35.4	355.8	60.9	21.6	3.7	584	128.7
28aB	511	229.9	42.7	236.0	43.9	72.1	13.4	538	105.3
28bB	502	220.3	41.4	235.1	44.2	76.4	14.4	532	105.9
28aPP	400	133.7	30.9	245.8	56.8	53.0	12.3	433	108.1
28bPP	418	233.1	49.5	172.7	36.7	65.2	13.8	471	112.7
29aB	555	212.3	37.9	271.8	48.5	76.0	13.6	560	100.9
29bB	576	229.2	38.4	288.5	48.4	78.8	13.2	596	103.5
29aPP	415	184.0	37.8	244.8	50.3	58.0	11.9	487	117.3
29bPP	424	195.0	35.8	267.4	49.0	82.9	15.2	545	128.6
30aB	633	241.6	36.6	323.9	49.1	94.8	14.4	660	104.3
30bB	627	395.7	38.0	482.9	46.4	162.3	15.6	1041	166.0
30aPP	376	101.9	26.7	238.2	62.3	42.2	11.0	382	101.7
30bPP	433	202.6	35.4	299.7	52.3	70.6	12.3	573	132.3

Table d :The distribution of **Fe** in the insoluble phase (1st.ppt.), the soluble phase (HMWP) (2nd.ppt.) and the heat-stable cytosol. Y = 0.781(X) + 248.2 ; r = 0.843

	X							Y	
Sample I.d.	Direct route Whole tissue (µg/g)	Quantity recovered (µg/g). "Indirect route" 1st. ppt		2nd. ppt		Cytosol		Sum of the three phases (µg/g)	Recovery %
		(µg/g)	%	(µg/g)	%	(µg/g)	%		
19aB	1644	1973	91.3	130	6.0	59	2.7	2161	131.4
19bB	1688	1618	91.1	97	5.5	61	3.4	1776	105.2
19aPP	2671	2424	92.5	143	5.5	54	2.1	2622	98.1
19bPP	1995	1050	90.7	70	6.1	37	3.2	1158	58.0
20aB	1252	1046	92.5	56	4.9	29	2.6	1131	90.3
20bB	1550	1063	96.3	16	1.5	25	2.2	1103	71.2
20aPP	1723	1387	90.9	66	4.3	72	4.7	1525	88.5
20bPP	2269	1852	97.6	35	1.9	10	0.5	1898	83.6
21aB	577	653	89.5	38	5.2	39	5.3	730	126.5
21bB	711	614	87.4	56	8.0	32	4.6	702	98.8
21aPP	1081	1136	88.5	73	5.7	75	5.8	1284	118.7
21bPP	1005	746	82.4	74	8.2	85	9.3	905	90.1
22aB	590	625	89.5	37	5.3	37	5.2	699	118.4
22bB	357	471	83.2	66	11.7	29	5.1	566	158.6
22aPP	733	558	82.8	49	7.2	67	10.0	673	91.9
22bPP	449	628	90.6	43	6.2	22	3.2	693	154.5
23aB	1935	1704	94.9	72	4.0	21	1.1	1796	92.8
23bB	1869	1056	93.0	56	5.0	23	2.0	1135	60.8
23aPP	993	671	85.5	80	10.2	34	4.3	785	79.0
23bPP	597	479	85.4	58	10.3	24	4.3	561	94.0
24aB	1448	952	88.5	68	6.3	56	5.2	1076	74.3
24bB	1491	1221	85.0	101	7.1	115	8.0	1437	96.4
24aPP	961	1383	91.2	84	5.5	49	3.2	1516	157.9
24bPP	1460	1250	86.4	119	8.2	78	5.4	1447	99.1
25aB	1376	990	89.4	85	7.7	32	2.9	1107	80.5
25bB	1414	688	87.8	59	7.5	37	4.7	784	55.5
25aPP	1606	1403	91.1	93	6.0	43	2.8	1540	95.9
25bPP	642	676	85.0	66	8.3	54	6.7	796	124.0
26aB	1331	1309	88.0	144	9.7	34	2.3	1487	111.7
26bB	1049	1123	87.1	122	9.4	45	3.5	1290	123.0
26aPP	1344	1310	88.7	126	8.5	41	2.8	1477	109.9
26bPP	1461	1016	87.0	120	10.3	31	2.7	1168	79.9
27aB	792	756	81.7	136	14.7	33	3.5	925	116.8
27bB	736	664	80.8	126	15.4	31	3.8	821	111.6
27aPP	764	683	78.0	151	17.2	42	4.8	876	114.7
27bPP	687	731	80.9	146	16.1	27	3.0	904	131.5
28aB	1910	1975	93.6	100	4.8	35	1.7	2111	110.5
28bB	1231	973	89.5	75	6.9	40	3.7	1087	88.3
28aPP	687	499	78.8	84	13.2	51	8.1	634	92.3
28bPP	625	433	79.3	40	7.4	73	13.3	546	87.4
29aB	2059	2285	94.2	107	4.4	34	1.4	2426	117.8
29bB	1674	1281	91.5	84	6.0	36	2.6	1401	83.7
29aPP	906	1002	86.6	94	8.1	61	5.3	1158	127.8
29bPP	581	512	80.6	68	10.8	55	8.6	636	109.4
30aB	1679	1307	88.7	126	8.5	40	2.7	1473	87.7
30bB	946	1135	84.8	143	10.7	61	4.5	1339	141.5
30aPP	1274	1103	86.5	130	10.2	41	3.3	1275	100.1
30bPP	1074	1079	86.8	88	7.1	77	6.2	1244	115.8

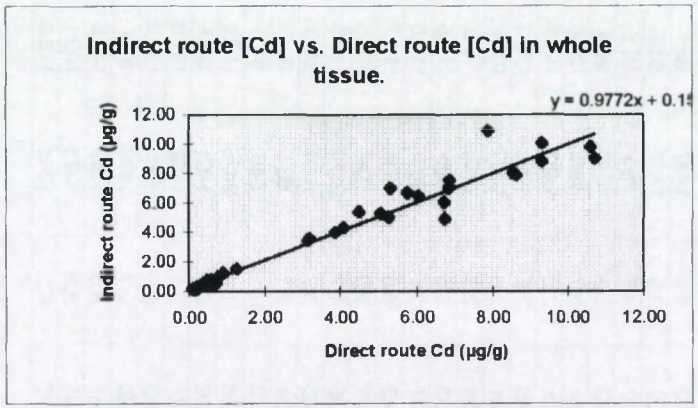


Figure a : Relationship between the [Cd] obtained by the indirect route i.e. by partitioning, and the [Cd] obtained directly in whole tissue *Macoma balthica*.

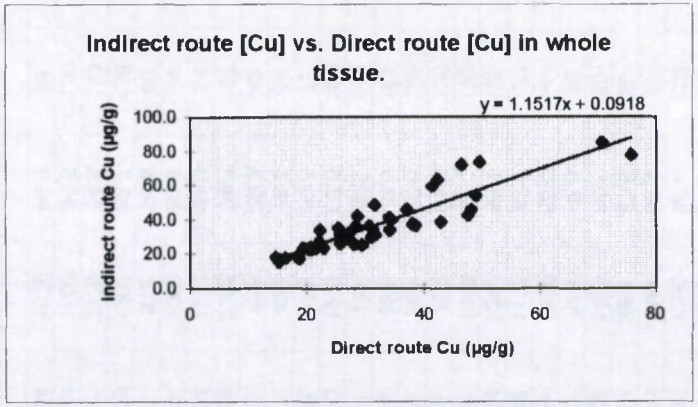


Figure b : Relationship between the [Cu] obtained by the indirect route i.e. by partitioning, and the [Cu] obtained directly in whole tissue *Macoma balthica*.

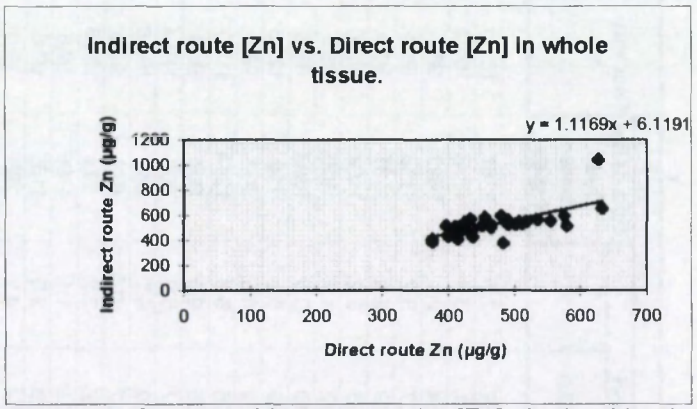


Figure c : Relationship between the [Zn] obtained by the indirect route i.e. by partitioning, and the [Zn] obtained directly in whole tissue *Macoma balthica*.

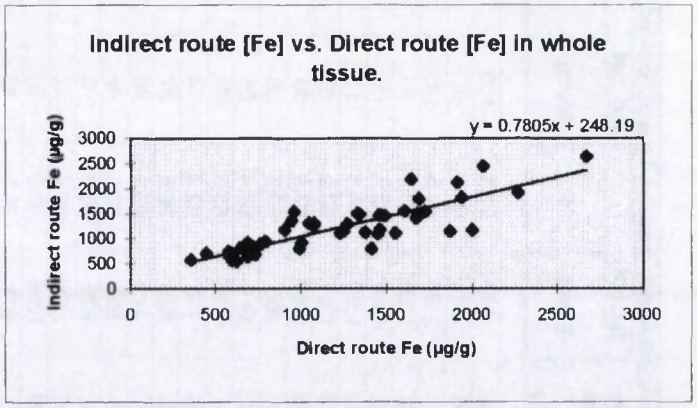
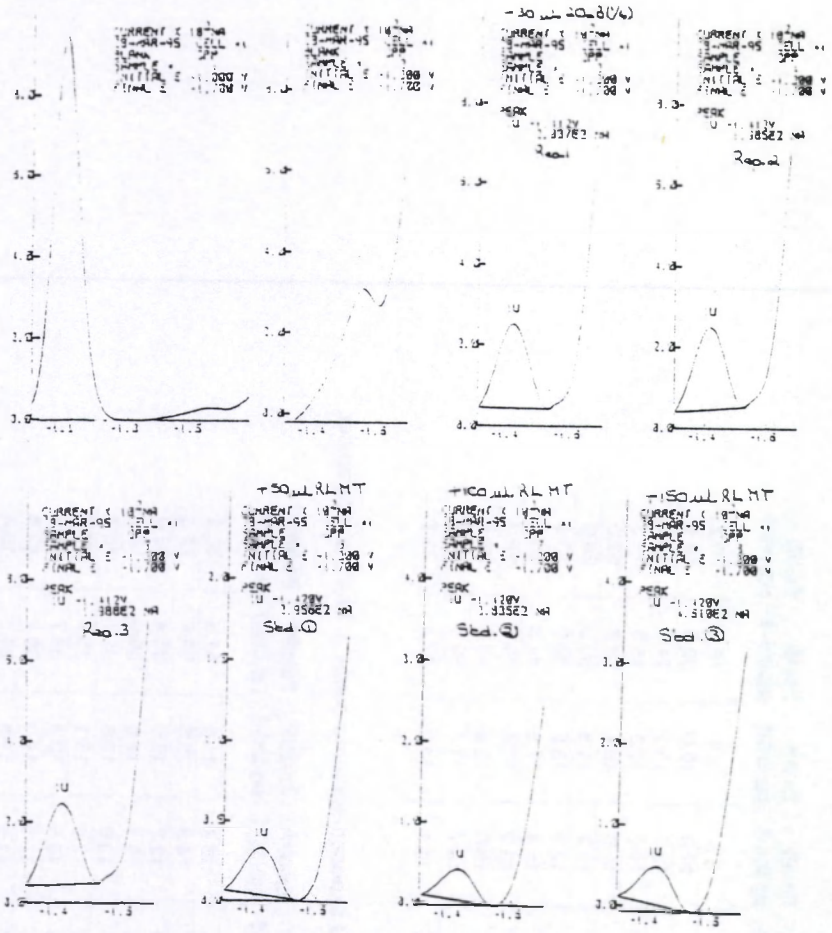


Figure d : Relationship between the [Fe] obtained by the indirect route i.e. by partitioning, and the [Fe] obtained directly in whole tissue *Macoma balthica*.

Annex 3 : Examples of DPP polarograms of Set 20aB, showing the blank, the sample (n = 3) and three standard additions of 50 µl of a 7261 µg.l^{-1} RL MT II (or 1.19 µM) commercial standard. The corresponding standard adddition calibration curve is also provided.

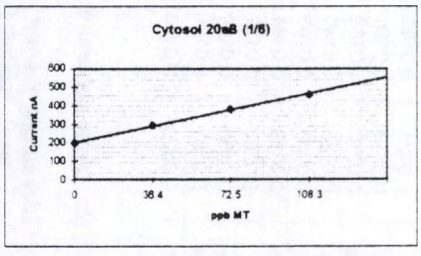
The experimental conditions are as given in Table 8.



Expt.A) Used 30 µl of Cytosol 20aB (1/6 dilution) as a sample and call this "zero cup"
BK cup volume = 10.70 ml
Zero cup volume = 10.73 ml.
Added successive 50 µl additions of RL MTII (22-09-1994) as the std.addns.

	Conc. ppb MT	Ep V	Current nA			Average	SD	%SD
+	0	-1.412	203.7	198.5	198.8	200.3	2.921	1.46
+	36.4	-1.420				295.6		
+	72.5	-1.420				383.5		
+	108.3	-1.420				481.0		

Slope = 2.410
Intercept= 204.2
Correl. = 0.9991
X = -84.75



If X = 84.8 ppb that implies that the concentration in ppm =

((84.8 * 6)(10.73/0.03))/1000 = 182.0 ppm MT

Annex 4 : Tables 17, 18, 20, 21 and 22.

Table 17a : Metal content vs. dry weight for natural and exposed *Macoma balthica* from Baalhoek.

Set	Dry weight (g)	Cd-aB µg.ind-1	Cd-bB µg.ind-1	Cu-aB µg.ind-1	Cu-bB µg.ind-1	Zn-aB µg.ind-1	Zn-bB µg.ind-1	Fe-aB µg.ind-1	Fe-bB µg.ind-1
Mar'93-19	0.0119	0.0151	0.1275	0.30	0.41	8.5	8.8	19.6	20.1
May'93-20	0.0319	0.0295	0.1929	0.65	0.92	12.8	16.0	39.9	49.4
Jul'93-21	0.0421	0.0165	0.1343	0.93	0.93	13.7	15.5	24.3	29.9
Sep'93-22	0.0313	0.0180	0.1800	0.46	0.99	11.6	12.3	18.5	11.2
Nov'93-23	0.0278	0.0168	0.2850	0.52	1.09	12.8	13.4	53.8	52.0
Jan'94-24	0.0228	0.0140	0.0456	0.52	0.64	11.2	11.5	33.0	34.0
Mar'94-25	0.0240	0.0148	0.2071	0.71	0.92	12.4	13.9	33.0	33.9
May'94-26	0.0333	0.0181	0.1686	1.03	1.63	15.4	17.8	44.3	34.9
Jul'94-27	0.0262	0.0129	0.1391	0.97	1.09	10.7	11.4	20.8	19.3
Sep'94-28	0.0255	0.0184	0.2371	0.87	1.81	13.0	12.8	48.7	31.4
Nov'94-29	0.0192	0.0105	0.1785	0.60	1.46	10.7	11.1	39.5	32.1
Jan'95-30	0.0223	0.0173	0.1755	0.71	1.04	14.1	14.0	37.4	21.1

Table 17b : Metal content vs. dry weight for natural and exposed *Macoma balthica* from Paulinapolder.

Set	Dry weight (g)	Cd-aPP µg.ind-1	Cd-bPP µg.ind-1	Cu-aPP µg.ind-1	Cu-bPP µg.ind-1	Zn-aPP µg.ind-1	Zn-bPP µg.ind-1	Fe-aPP µg.ind-1	Fe-bPP µg.ind-1
Mar'93-19	0.0193	0.0074	0.2465	0.53	0.74	15.3	14.0	51.6	38.5
May'93-20	0.0342	0.0098	0.1399	0.64	0.97	14.3	25.6	58.9	77.6
Jul'93-21	0.0327	0.0041	0.1264	0.50	0.63	12.2	12.9	35.4	32.9
Sep'93-22	0.0417	0.0065	0.1307	0.68	0.85	15.7	17.6	30.6	18.7
Nov'93-23	0.0313	0.0033	0.2109	0.97	1.45	13.0	13.6	31.1	18.7
Jan'94-24	0.0267	0.0064	0.1825	0.56	0.92	12.0	13.1	25.6	39.0
Mar'94-25	0.0241	0.0063	0.1618	0.62	1.15	10.5	10.3	38.7	15.5
May'94-26	0.0293	0.0057	0.1541	0.76	1.26	11.6	14.0	39.4	42.8
Jul'94-27	0.0283	0.0057	0.1274	0.61	1.05	11.5	12.8	21.6	19.4
Sep'94-28	0.0320	0.0071	0.2734	0.69	1.54	12.8	13.4	22.0	20.0
Nov'94-29	0.0226	0.0045	0.2397	0.50	1.12	9.4	9.6	20.5	13.1
Jan'95-30	0.0214	0.0043	0.1470	0.56	0.91	8.0	9.3	27.3	23.0

Table 18a : Metal concentrations vs. dry weight for natural and exposed *Macoma balthica* from Baalhoek.

Set	Dry weight (g)	Cd-aB µg.g-1	Cd-bB µg.g-1	Cu-aB µg.g-1	Cu-bB µg.g-1	Zn-aB µg.g-1	Zn-bB µg.g-1	Fe-aB µg.g-1	Fe-bB µg.g-1
Mar'93-19	0.0119	1.270	10.717	25.5	34.1	716	742	1644.4	1688
May'93-20	0.0319	0.924	6.046	20.4	28.8	402	500	1251.8	1550.1
Jul'93-21	0.0421	0.393	3.191	22.2	22.0	326	367	576.7	710.8
Sep'93-22	0.0313	0.575	5.750	14.6	31.7	372	393	590.4	357
Nov'93-23	0.0278	0.605	10.251	18.7	39.3	461	482	1935.0	1869
Jan'94-24	0.0228	0.613	2.002	23.0	28.2	490	506	1448.0	1491
Mar'94-25	0.0240	0.616	8.629	29.5	38.5	518	580	1376.0	1414
May'94-26	0.0333	0.545	5.063	30.9	49.0	463	535	1331.0	1049
Jul'94-27	0.0262	0.491	5.310	37.1	41.5	410	434	792.0	736
Sep'94-28	0.0255	0.720	9.299	34.2	70.8	511	502	1910.0	1231
Nov'94-29	0.0192	0.547	9.296	31.4	75.8	555	576	2059.0	1674
Jan'95-30	0.0223	0.777	7.868	31.8	46.5	633	627	1679.0	946

Table 18b : Metal concentrations vs. dry weight for natural and exposed *Macoma balthica* from Paulinapolder.

Set	Dry weight (g)	Cd-aPP µg.g-1	Cd-bPP µg.g-1	Cu-aPP µg.g-1	Cu-bPP µg.g-1	Zn-aPP µg.g-1	Zn-bPP µg.g-1	Fe-aPP µg.g-1	Fe-bPP µg.g-1
Mar'93-19	0.0193	0.383	12.770	27.3	38.1	791	725	2671.4	1994.7
May'93-20	0.0342	0.287	4.090	18.6	28.5	418	749	1723.3	2268.9
Jul'93-21	0.0327	0.124	3.865	15.2	19.4	374	394	1081.2	1005
Sep'93-22	0.0417	0.155	3.135	16.3	20.5	376	423	732.9	448.7
Nov'93-23	0.0313	0.106	6.737	31.1	46.3	414	436	993.0	597
Jan'94-24	0.0267	0.238	6.834	20.8	34.5	450	489	960.5	1460
Mar'94-25	0.0241	0.261	6.715	25.8	47.6	434	429	1606.0	642
May'94-26	0.0293	0.196	5.261	25.8	43.0	396	479	1344.0	1461
Jul'94-27	0.0283	0.202	4.502	21.5	37.1	408	454	764.0	687
Sep'94-28	0.0320	0.223	8.543	21.6	48.2	400	418	687.0	625
Nov'94-29	0.0226	0.200	10.607	22.3	49.6	415	424	906.0	581
Jan'95-30	0.0214	0.199	6.869	26.4	42.4	376	433	1274.0	1074

Table 20a :Cytosol metal contents vs. dry weight for natural and exposed *Macoma balthica* from Baalhoek.

Set	Dry weight (g)	Cd-aB µg/ind.	Cd-bB µg/ind.	Cu-aB µg/ind.	Cu-bB µg/ind.	Zn-aB µg/ind.	Zn-bB µg/ind.	Fe-aB µg/ind.	Fe-bB µg/ind.
Mar'93-19	0.0119	0.0010	0.0189	0.045	0.084	1.28	1.81	0.35	0.34
May'93-20	0.0319	0.0028	0.0042	0.169	0.121	2.22	1.09	0.82	0.41
Jul'93-21	0.0421	0.0009	0.0029	0.186	0.106	2.60	1.39	1.49	1.25
Sep'93-22	0.0313	0.0006	0.0053	0.051	0.111	1.83	0.94	1.01	0.76
Nov'93-23	0.0278	0.0073	0.0426	0.015	0.028	0.51	0.99	0.43	0.48
Jan'94-24	0.0228	0.0000	0.0000	0.119	0.062	0.68	1.66	0.71	1.05
Mar'94-25	0.0240	0.0000	0.0255	0.066	0.132	0.77	0.79	0.51	0.53
May'94-26	0.0333	0.0009	0.0208	0.147	0.202	0.84	1.75	0.82	1.04
Jul'94-27	0.0262	0.0001	0.0088	0.059	0.104	0.91	0.60	0.66	0.66
Sep'94-28	0.0255	0.0007	0.0438	0.140	0.322	1.46	1.41	0.71	0.74
Nov'94-29	0.0192	0.0023	0.0349	0.098	0.220	1.01	1.03	0.46	0.47
Jan'95-30	0.0223	0.0053	0.0365	0.104	0.169	1.24	1.39	0.53	0.52

Table 20b : Cytosol metal contents vs. dry weight for natural and exposed *Macoma balthica* from Paulinapolder.

Set	Dry weight (g)	Cd-aPP µg/ind.	Cd-bPP µg/ind.	Cu-aPP µg/ind.	Cu-bPP µg/ind.	Zn-aPP µg/ind.	Zn-bPP µg/ind.	Fe-aPP µg/ind.	Fe-bPP µg/ind.
Mar'93-19	0.0193	0.0001	0.0411	0.068	0.143	1.77	2.07	0.74	0.50
May'93-20	0.0342	0.0000	0.0024	0.084	0.135	2.35	1.17	2.36	0.21
Jul'93-21	0.0327	0.0000	0.0010	0.077	0.046	2.00	1.15	2.50	2.55
Sep'93-22	0.0417	0.0000	0.0000	0.053	0.017	2.58	0.29	2.87	0.80
Nov'93-23	0.0313	0.0018	0.0393	0.009	0.021	0.76	1.33	0.89	0.62
Jan'94-24	0.0267	0.0000	0.0000	0.064	0.064	1.27	1.05	0.79	1.06
Mar'94-25	0.0241	0.0002	0.0207	0.062	0.123	1.07	1.01	0.83	0.64
May'94-26	0.0293	0.0006	0.0082	0.101	0.092	1.62	0.81	1.21	0.77
Jul'94-27	0.0283	0.0003	0.0034	0.066	0.052	1.33	0.52	1.10	0.66
Sep'94-28	0.0320	0.0000	0.0433	0.074	0.191	1.80	1.96	1.74	2.18
Nov'94-29	0.0226	0.0006	0.0312	0.046	0.182	1.00	1.14	1.05	0.76
Jan'95-30	0.0214	0.0011	0.0182	0.055	0.102	0.79	0.74	0.77	0.81

Table 21 : Seasonal UWL and UAL for MTs as a function of the season.

Baalhoek :

Set	MT/Mac (mg/g) - aB	S.D. (absol.)	Uncer tainty	95% Upper warning	Lower warning	99.7% Upper action	Lower action	MT/Mac (mg/g) - bB	S.D. (absol.)	Uncer tainty
Mar'93-19	5.412	0.142	0.352	5.453	4.845	5.605	4.693	6.149	0.132	0.327
May'93-20	4.885	0.071	0.176	5.453	4.845	5.605	4.693	5.858	0.122	0.303
Jul'93-21	1.791	0.027	0.067	3.848	1.644	4.399	1.093	2.813	0.023	0.057
Sep'93-22	3.700	0.107	0.265	3.848	1.644	4.399	1.093	3.675	0.107	0.265
Nov'93-23	5.473	0.044	0.109	6.265	5.463	6.466	5.262	5.105	0.064	0.159
Jan'94-24	6.409	0.165	0.409	6.265	5.463	6.466	5.262	6.063	0.184	0.456
Mar'94-25	5.890	0.456	1.131	6.265	5.463	6.466	5.262	7.333	0.202	0.501
May'94-26	5.685	0.055	0.136	6.265	5.463	6.466	5.262	6.934	0.101	0.250
Jul'94-27	3.218	0.081	0.201	3.631	1.893	4.065	1.459	5.920	0.156	0.387
Sep'94-28	1.702	0.070	0.174	3.631	1.893	4.065	1.459	1.403	0.142	0.352
Nov'94-29	3.367	0.088	0.218	3.631	1.893	4.065	1.459	1.112	0.128	0.317
Jan'95-30	7.648	0.102	0.253	7.827	7.635	7.875	7.587	11.049	0.233	0.578
Mar'95-31	7.814	0.077	0.191	7.827	7.635	7.875	7.587	11.287	0.211	0.523

Paulinapolder :

Set	MT/Mac (mg/g)-aPP	S.D. (absol.)	Uncer tainty	95% Upper warning	Lower warning	99.7% Upper action	Lower action	MT/Mac (mg/g)-bPP	S.D. (absol.)	Uncer tainty
Mar'93-19	1.689	0.020	0.050	3.200	1.760	3.560	1.406	4.646	0.052	0.129
May'93-20	2.790	0.067	0.166	3.176	1.760	3.530	1.406	3.223	0.024	0.060
Jul'93-21	3.282	0.129	0.320	3.176	1.760	3.530	1.406	4.810	0.062	0.154
Sep'93-22	2.109	0.034	0.084	3.176	1.760	3.530	1.406	4.405	0.032	0.079
Nov'93-23	4.454	0.121	0.300	9.333	3.815	10.712	2.436	8.454	0.060	0.149
Jan'94-24	8.793	0.168	0.417	9.333	3.815	10.712	2.436	8.931	0.140	0.347
Mar'94-25	9.114	0.129	0.320	9.333	3.815	10.712	2.436	8.933	0.046	0.114
May'94-26	3.935	0.115	0.285	9.333	3.815	10.712	2.436	6.531	0.044	0.109
Jul'94-27	1.226	0.041	0.102	2.069	0.769	2.393	0.445	10.982	0.150	0.372
Sep'94-28	0.847	0.114	0.283	2.069	0.769	2.393	0.445	1.766	0.095	0.236
Nov'94-29	2.184	0.050	0.124	2.069	0.769	2.393	0.445	1.423	0.021	0.052
Jan'95-30	3.384	0.018	0.045	6.556	3.156	7.406	2.306	9.132	0.201	0.498
Mar'95-31	6.328	0.403	0.999	6.556	3.156	7.406	2.306	10.832	0.270	0.670

Table 22 : Average Seasonal UWL and UAL for MTs as a function of the season.

Baalhoek :

Set	MT/Mac (mg/g) - all	S.D. (absol.)	Uncer tainty	95% Upper warning	Lower warning	99.7% Upper action	Lower action	MT/Mac (mg/g) - bB	S.D. (absol.)	Uncer tainty
19	5.412	0.142	0.352	7.304	5.000	7.880	4.424	6.149	0.132	0.327
20	4.885	0.071	0.176	7.304	5.000	7.880	4.424	5.858	0.122	0.303
23	5.473	0.044	0.109	7.304	5.000	7.880	4.424	5.105	0.064	0.159
24	6.409	0.165	0.409	7.304	5.000	7.880	4.424	6.063	0.184	0.456
26	5.890	0.456	1.131	7.304	5.000	7.880	4.424	7.333	0.202	0.501
28	5.685	0.055	0.136	7.304	5.000	7.880	4.424	6.934	0.101	0.250
30	7.648	0.102	0.253	7.304	5.000	7.880	4.424	11.049	0.233	0.578
31	7.814	0.077	0.191	7.304	5.000	7.880	4.424	11.287	0.211	0.523
21	1.791	0.027	0.067	3.725	1.787	4.209	1.303	2.813	0.023	0.057
22	3.700	0.107	0.265	3.725	1.787	4.209	1.303	3.675	0.107	0.265
27	3.218	0.081	0.201	3.725	1.787	4.209	1.303	5.920	0.156	0.387
28	1.702	0.070	0.174	3.725	1.787	4.209	1.303	1.403	0.142	0.352
29	3.367	0.088	0.218	3.725	1.787	4.209	1.303	1.112	0.128	0.317

"Blue" (Winter/Spring values) Average Seasonal conc. = 6.152
"Blue" (Winter/Spring values) Average Seasonal Pop. S.D 0.9979

"Yellow" (Summer/Autumn values) Average Seasonal conc. = 2.756
"Yellow" (Summer/Autumn values) Average Seasonal Pop. S.D. = 0.8390

Paulinapolder :

Set	MT/Mac (mg/g)-aPP	S.D. (absol.)	Uncer tainty	95% Upper warning	Lower warning	99.7% Upper action	Lower action	MT/Mac (mg/g)-bPP	S.D. (absol.)	Uncer tainty
23	4.454	0.121	0.300	8.630	3.372	9.945	2.057	8.454	0.060	0.149
24	8.793	0.168	0.417	8.630	3.372	9.945	2.057	8.931	0.140	0.347
26	9.114	0.129	0.320	8.630	3.372	9.945	2.057	8.933	0.046	0.114
28	3.935	0.115	0.285	8.630	3.372	9.945	2.057	6.531	0.044	0.109
30	3.384	0.018	0.045	8.630	3.372	9.945	2.057	9.132	0.201	0.498
31	6.328	0.403	0.999	8.630	3.372	9.945	2.057	10.832	0.270	0.670
19	1.689	0.020	0.050	2.927	1.109	3.381	0.655	4.646	0.052	0.129
20	2.790	0.067	0.166	2.927	1.109	3.381	0.655	3.223	0.024	0.060
21	3.282	0.129	0.320	2.927	1.109	3.381	0.655	4.810	0.062	0.154
22	2.109	0.034	0.084	2.927	1.109	3.381	0.655	4.405	0.032	0.079
27	1.226	0.041	0.102	2.927	1.109	3.381	0.655	10.982	0.150	0.372
28	0.847	0.114	0.283	2.927	1.109	3.381	0.655	1.766	0.095	0.236
29	2.184	0.050	0.124	2.927	1.109	3.381	0.655	1.423	0.021	0.052

"Blue" (Winter/Spring values) Average Seasonal conc. = 6.001
"Blue" (Winter/Spring values) Average Seasonal Pop. S.D. = 2.2768

"Yellow" (Summer/Autumn values) Average Seasonal conc. = 2.018
"Yellow" (Summer/Autumn values) Average Seasonal Pop. S.D. = 0.7871

Table 22 : Average Seasonal UWL and UAL for MTs as a function of the season.

Baalhoek :

Set	MT/Mac (mg/g) - aB	S.D. (absol.)	Uncer tainty	95% Upper warning	Lower warning	99.7% Upper action	Lower action	MT/Mac (mg/g) - bB	S.D. (absol.)	Uncer tainty
19	5.412	0.142	0.352	7.304	5.000	7.880	4.424	6.149	0.132	0.327
20	4.885	0.071	0.176	7.304	5.000	7.880	4.424	5.858	0.122	0.303
23	5.473	0.044	0.109	7.304	5.000	7.880	4.424	5.105	0.064	0.159
24	6.409	0.165	0.409	7.304	5.000	7.880	4.424	6.063	0.184	0.456
25	5.890	0.456	1.131	7.304	5.000	7.880	4.424	7.333	0.202	0.501
26	5.685	0.055	0.136	7.304	5.000	7.880	4.424	6.934	0.101	0.250
30	7.648	0.102	0.253	7.304	5.000	7.880	4.424	11.049	0.233	0.578
31	7.814	0.077	0.191	7.304	5.000	7.880	4.424	11.287	0.211	0.523
21	1.791	0.027	0.067	3.725	1.787	4.209	1.303	2.813	0.023	0.057
22	3.700	0.107	0.265	3.725	1.787	4.209	1.303	3.675	0.107	0.265
27	3.218	0.081	0.201	3.725	1.787	4.209	1.303	5.920	0.156	0.387
28	1.702	0.070	0.174	3.725	1.787	4.209	1.303	1.403	0.142	0.352
29	3.367	0.088	0.218	3.725	1.787	4.209	1.303	1.112	0.128	0.317

"Blue" (Winter/Spring values) Average Seasonal conc. = 6.152
"Blue" (Winter/Spring values) Average Seasonal Pop. S.D 0.9979

"Yellow" (Summer/Autumn values) Average Seasonal conc. = 2.756
"Yellow" (Summer/Autumn values) Average Seasonal Pop. S.D. = 0.8390

Paulinapolder :

Set	MT/Mac (mg/g)-aPP	S.D. (absol.)	Uncer tainty	95% Upper warning	Lower warning	99.7% Upper action	Lower action	MT/Mac (mg/g)-bPP	S.D. (absol.)	Uncer tainty
23	4.454	0.121	0.300	8.630	3.372	9.945	2.057	8.454	0.060	0.149
24	8.793	0.168	0.417	8.630	3.372	9.945	2.057	8.931	0.140	0.347
25	9.114	0.129	0.320	8.630	3.372	9.945	2.057	8.933	0.046	0.114
26	3.935	0.115	0.285	8.630	3.372	9.945	2.057	6.531	0.044	0.109
30	3.384	0.018	0.045	8.630	3.372	9.945	2.057	9.132	0.201	0.498
31	6.328	0.403	0.999	8.630	3.372	9.945	2.057	10.832	0.270	0.670
19	1.689	0.020	0.050	2.927	1.109	3.381	0.655	4.646	0.052	0.129
20	2.790	0.067	0.166	2.927	1.109	3.381	0.655	3.223	0.024	0.060
21	3.282	0.129	0.320	2.927	1.109	3.381	0.655	4.810	0.062	0.154
22	2.109	0.034	0.084	2.927	1.109	3.381	0.655	4.405	0.032	0.079
27	1.226	0.041	0.102	2.927	1.109	3.381	0.655	10.982	0.150	0.372
28	0.847	0.114	0.283	2.927	1.109	3.381	0.655	1.766	0.095	0.236
29	2.184	0.050	0.124	2.927	1.109	3.381	0.655	1.423	0.021	0.052

"Blue" (Winter/Spring values) Average Seasonal conc. = 6.001
"Blue" (Winter/Spring values) Average Seasonal Pop. S.D. = 2.2768

"Yellow" (Summer/Autumn values) Average Seasonal conc. = 2.018
"Yellow" (Summer/Autumn values) Average Seasonal Pop. S.D. = 0.7871

Annex 5 : ***Abbreviations.***

1st. ppt. : First precipitate
2nd. ppt. : Second precipitate.

aB : unspiked clams from Baalhoek.
aPP : unspiked clams from Paulinapolder.
bB : spiked clams from Baalhoek.
bPP : spiked clams from Paulinapolder.
B : Baalhoek
CI : Confidence interval.
CF : Concentration factor.
CRDL : Contract required detection limit (standard).
CRM : Community reference material.
d.w. : Dry weight.
Da : Daltons.
df : Degrees of freedom.
DME : Dropping mercury electrode.
DOM. : Dissolved organic material.
DPP : Differential pulse polarography.
ETAAS : Electrothermal atomic absorption spectroscopy.
FAAS : Flame atomic absorption spectroscopy.
LSC : Liquid scintillation counting.
HM : Heavy metal
HMWP : High molecular weight protein.
i.d. : Identification.
IUPAC : International union of pure and applied chemistry.
MTs : Metallothioneins
MLPs : Metallothionein-like proteins.
Mw : Molecular weight.
NOEC : No observed effect concentration.
PMSF : Phenylmethylsulphonid fluoride.
PP : Paulinapolder.
RIA : Radioimmunoassay.
RP-HPLC : Reverse phase - High performance liquid chromatography.
SH : Thiol
SEC : Size-exclusion chromatography.
SS : Disulphide
UAL : Upper action limit.
UWL : Upper warning limit.
UV : Ultraviolet spectroscopy.