ISSN 0145-5680

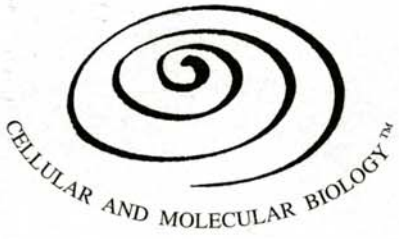
March
Vol. 46/ No. 2 2000

CELLULAR AND MOLECULAR BIOLOGY™

Editor

R. Wegmann, Paris

OFFPRINT



8 Issues published per year: February, March, May, June, July, September, November and December
Publishing, Subscription and Advertising: C.M.B. Association, Editorial Office, 1 avenue du Pavé Neuf, 93160 Noisy-le-Grand, France. Tel.: +33 (0)1 45 92 37 19, Fax: +33 (0)1 43 04 20 30, e-Mail: cmbass@club-internet.fr

Annual Subscription Rate (2000)
Annual Institutional Rate (2000): Fr. Frs. 3029,50 or US$ 598.
Special Annual Personal Rate and Members of the World Society of CMB™ (confidential).
Price include economical postage and are guaranteed to not change for the subscription inbetween the subscription year.

Cellular and Molecular Biology™ **46** (2), 283-294
Printed in France.

0145-5680/00
2000 Cell. mol. Biol.™

Review

METALLOTHIONEINS IN MARINE MAMMALS

K. DAS, V. DEBACKER and J.M. BOUQUEGNEAU✍

✍ Department of Oceanology, University of Liège, B6 Sart-Tilman, B-4000 Liège, Belgium
fax: +32 4 366 4521; e-mail: jm.bouquegneau@ulg.ac.be

Received September 22, 1999; Accepted October 14, 1999

***Krishna DAS**, graduated in 1996 in Zoology and in 1997 in Oceanology (University of Liège, Belgium) has undertaken a Ph.D. on heavy metal contamination and detoxification processes of stranded marine mammals from European coasts.*

***Virginie DEBACKER**, graduated in 1993 from University of Liège, Belgium with a degree in Zoology after presenting a study dedicated to the contamination and speciation of heavy metals of wintering common guillemots (Uria aalge) at the Belgian coast. She further continued her toxicological studies on seabirds and marine mammals stranded at the Belgian coast, in view to present her PhD thesis at Liège University.*

***Jean-Marie BOUQUEGNEAU**, is full Professor at the University of Liège (Belgium) and Head of Oceanology Laboratory. He is teaching biological oceanography, chemical oceanography and marine ecotoxicology. His research experience concerns heavy metal ecotoxicology including metallothioneins and other detoxification mechanisms, as well as Posidonia seagrass beds ecology in the Mediterranean Sea (Corsica).*

Abstract - Metallothioneins (MTs) have been detected in livers and kidneys of 10 marine mammals species (Pinnipeds and Odontocetes). Characterization of renal MTs of striped dolphin has shown that the protein has two isoforms (MT-1 and MT-2) with a molecular weight estimated around 6800. MT concentrations also vary widely in marine mammals tissues (from 58 to 1200 $\mu g.g^{-1}$ ww) underlying the numerous parameters involved: physiological status, pregnancy, age, diet. The participation of this protein in metal detoxification has been investigated since high levels of cadmium (Cd) and mercury (Hg) have been measured in livers and kidneys of marine mammals. It has been suggested that those animals can mitigate at least in part, the toxic effects of Cd and Hg through binding to MTs. The percentage of the cytosolic Cd bound to MTs can reach almost 100%. On the contrary, the percentage of hepatic and renal Hg bound to MT is very low (generally less than 10%) and this metal is mainly associated with selenium (HgSe) under a detoxified form in the insoluble fraction of the tissues. MTs appear to play a minor role in the binding and detoxification of Hg by marine mammals. On the contrary, close and dynamic interactions occur between Cd and MTs. Cytosolic MTs appear as a potential short term way of detoxification of Cd accumulated from

diet. Long-term detoxification would imply a sequestration of the metal under a precipitated form (e.g. in lysosomes).

Key words: Metallothioneins, marine mammals, cadmium, mercury

INTRODUCTION

Many areas have emerged in metallothionein (MT) researches since their first discovery (Margoshes and Vallee, 1957), but metallothionein natural function remains elusive (Palmiter, 1998). Previous studies on structure, function and molecular regulation have established a central role for these molecules in the homeostatic regulation of essential metals as zinc (Zn) and copper (Cu). Thus, it is not surprising that MTs have been detected in both prokaryotes and eukaryotes including marine mammals (reviewed by Roesijadi, 1992, 1996). Indeed, the use of metals as cofactors in biochemical reactions and their toxicity associated with their affinity for S, N and O (the predominant ligands in biomolecular structures), represent dual aspects of metal-biological interactions. Therefore, mechanisms that regulate the intracellular availability of essential metals and protect against inappropriate and potentially deleterious intracellular interactions, are primordial for efficient biochemical function. MTs work as Zn or Cu donors to other metalloproteins. They are induced by and bind excesses of these metals. Another function postulated for MTs is the detoxification of non-essential metals like cadmium (Cd) and mercury (Hg). As a result of its capacity to bind cations, MTs are able to bind non-essential metals as Cd^{2+} Ag^{+}, Hg^{2+} and Pb^{2+} and, in this way, reduce the bioavailability of these toxics (reviewed by Webb, 1987; Roesijadi, 1992).

It has been suggested that Cd toxicity occurs when available MTs are insufficient to bind all the Cd. Recent experiments with mice genetically deprived of MTs due to the loss of functional MT-1 and MT-2 genes (coding for the 2 main isoforms of MTs involved in the detoxification process) confirm the protective role of these proteins against cellular damages from metals such as Cd or inorganic Hg (Satoh *et al.*, 1997; Klaassen and Liu, 1998). This leads to consider that these proteins prevent organisms from toxic hazards that could occur following the high exposure to Cd and Hg. Recently, Klaassen *et al.* (1999) reviewed the protective action of MTs against Cd toxicity.

So far, there are few data on MTs in marine mammals. Nevertheless, in the framework of heavy metal ecotoxicology, marine mammals appear as a choice material, since high levels of Hg and Cd can be naturally encountered in these animals. While plankton-eating Mysticetes are generally weakly contaminated by heavy metals, fish-eating and squid-eating Odontocetes and Pinnipeds are heavily contaminated by Hg and Cd, respectively (Bouquegneau and Joiris, 1992). Heavy metals in marine mammals have been recently reviewed by Das *et al.* (1999a).

A maximal Hg concentration of about 13 $mg.g^{-1}$ dry weight has been reported in the liver of one bottlenose dolphin stranded on the Italian coast (Leonzio *et al.*, 1992). Such high levels of Hg without overt evidence of deleterious effects may only occur if Hg is detoxified. Koeman *et al.* (1973, 1975) have first reported a strong correlation between Hg and selenium in livers of marine mammals. A molar ratio Hg: Se of approximately 1 has been observed suggesting Hg detoxification mechanisms in presence of selenium. The fate of Hg has been mainly elucidated by histological studies carried out in livers from different marine mammals species as Cuvier's beaked whale *Ziphius cavirostris* and bottlenose dolphins *Tursiops truncatus* (Martoja and Viale, 1977; Martoja and Berry, 1980; Nigro and Leonzio, 1996). These authors have observed mercuric selenide granules (HgSe) located mainly in the liver macrophages, the Kupffer cells, and in the proximal tubules of the kidney. The transformation

of assimilated Hg in tiemmanite (HgSe) appears to be the last step of detoxification leading to formation of inert et non-toxic Hg compounds.

In the same way, the Cd renal concentrations can reach levels as high as 2 $mg.g^{-1}$ dry weight in some Arctic ringed seals without any pathological effects (Dietz *et al.,* 1998). This is much higher than the critical concentrations of approximately 800 µg/g dry weight (200 µg/g wet weight) associated with kidney damage in mammals (WHO, 1992). Moreover, following Elinder and Järup (1996), this critical concentration has been largely overestimated as Cd-induced renal dysfunctions have been observed at kidney cortex concentrations of 200 µg/g dry weight (50 µg/g wet weight). For comparison, in human adults, the renal Cd concentrations amongst non-smokers is about 1 $\mu g.g^{-1}$ wet weight (Pesch *et al.,* 1989). These investigations indicate that marine mammals are able to maintain considerable concentrations of Cd without showing renal damages. It has been postulated that marine mammals mitigate the toxic effects of Cd through binding to MTs. So far, MTs and metallothionein-like proteins (MT-LP) have been described in 10 marine mammal species (Table 1) with concentrations ranging from 58 to 710 $\mu g.g^{-1}$ and 140 to 1200 $\mu g.g^{-1}$ fw in the liver and kidneys respectively.

CADMIUM BINDING

As quoted above, the renal concentrations of cadmium in marine mammals can reach levels much higher than the critical concentrations associated with kidney damage in terrestrial mammals. The question has therefore been raised about animals so heavily contaminated with Cd. Dietz *et al.* (1998) have compared low and high concentrations of cadmium in the kidney of ringed seals (*Phoca hispida*) from Northwest Greenland in an attempt to do macroscopic and light microscopic examinations. No differences in renal morphology could be observed between experimental groups. These investigations indicate that marine mammals appeared able to maintain considerable concentrations of Cd without showing renal damage. Dietz *et al.* (1998) have therefore postulated that ringed seals were adapted to the naturally high Cd levels of the Greenland Arctic regions.

The role of MTs in binding Cd present in the tissues may vary widely between different species as well as between different individuals from the same species (Table 2). This leads to more or less important spillage of Cd to other metalloproteins. It is interesting to note that the low values of 5, 17 and 18% have been measured in the liver of three highly debilitated sperm whales found stranded on the Belgian coast (Bouquegneau *et al.,* 1997; Holsbeek *et al.,* 1999). Moreover, a spillage of Cd from MTs to other soluble components has been observed: 34 to 38% of the total Cd is bound to soluble proteins other than MTs (calculated from Holsbeek *et al.,* 1999). This suggests that Cd was not in a detoxified form, either on MT or in lysosomes. These animals were seriously debilitated as indicated by their reduced blubber thickness and body weight (Jauniaux *et al.,* 1998). Cd which is known to induce debilitation in mammals can be considered as one of the factors responsible for the condition of these animals, which in addition to stress and starvation, could have resulted in their stranding (Bouquegneau *et al.,* 1997).

However, high levels of Cd with low binding to MTs have been reported in healthy animals. Amiard-Triquet and Caurant (1997) have reported that 51% of total Cd were bound to MTs in the livers of pilot whales caught in July 1986 whereas individuals caught in November from the same year displayed only 6%. Moreover, individuals caught in winter have low plasma Cd levels. According to these authors, Cd in the plasma resulted from a recent Cd contamination. These results are in agreement with the seasonal availability of preys: squids which are known to concentrate Cd in their tissues, are more abundant in summer. So it seems that only recently assimilated Cd is bound to MTs, the others being stored in the insoluble fraction (Amiard-Triquet and Caurant, 1997). In contrast to pilot whales, in the narwhal, more than 70% of the cytosolic Cd is

Table 1 *Metallothionein and metallothionein-like protein detection and quantification (metal-free protein) in marine mammals*

Species	Geographic Location	Tissue	Concentration (µg/g ww)	Isoforms detected	Method	References
Pinnipeds						
Zalophus californianus californianus (California sea lion)	Oregon (USA)	liver kidney	nd 140 (n=1)	MT-1 MT-2	Sephadex G-75 or Sephadex G-200 Whatman DE 32-column	Lee *et al.*, 1977
Histriophoca fasciata (ribbon seal)	Japan	liver	nd	MT-1 MT-2	Sephadex G-75 HPLC-AAS DEAE-3SW column	Mochizuki *et al.*, 1985
Phoca vitulina (harbour seal)	Japan	liver	nd	MT-1 MT-2	Sephadex G-75 HPLC-AAS DEAE-3SW column	Mochizuki *et al.*, 1985
	Japan	liver kidney	240 ± 139 (n=15) 343 ± 219 (n=15)	nd nd	Radioimmunoassay	Tohyama *et al.*, 1986
Halichoerus grypus (grey seal)	Canada	liver	70 (n=1)	nd	Sephadex G-75 followed by G-50	Olafson and Thompson, 1974
Callorhinum urisnum (Pacific fur seal)	Canada	liver	90 (n=1)	nd	G-75 followed by G-50	Olafson and Thompson, 1974
Odontocetes						
Globicephala melas (pilot whale)	Faroe Islands	liver kidney	167 ± 36 (n=7; july 1986) 592 ± 200 (n=7; november 1986) 751 ± 213	nd nd	Polarography	Caurant *et al.*, 1996 Amiard Triquet and Caurant, 1997
Stenella coeruleoalba (striped dolphin)	NorthWestern Pacific	kidney	314[1] (n=1)	MT-1 MT-2	Sephadex G-75 Sephadex G25 DEAE Sephadex- A25 HPLC on GS-320 column Amino acid characterization and primary structure	Kwohn *et al.*, 1986, 1988
Monodon monoceros (narwhal)	Arctic	liver kidney	710[1] (n=1) 1200[1] (n=1)	MT-1 MT-2 MT-1	Sephadex G-75 Sephadex G-50 Polyacrylamide gel electrophoresis DEAE Sephadex A25	Wagemann *et al.*, 1984 Wagemann and Hobden, 1986
Phocoena phocoena (harbour porpoise)	North Sea	liver	nd	nd	AcA-54	Antoine *et al.*, 1992
Physeter macrocephalus (sperm whale)	Oregon (USA)	liver	nd	nd	Sephadex G-75 and DEAE Sephadex gel	Ridlington *et al.*, 1981
	North Sea	liver kidney	58[1] (n=3) 468-951[1] (n=2)	nd nd	AcA-54	Bouquegneau *et al.*, 1997 Holsbeek *et al.*, 1999

Data about MT concentrations are either single value or in range, or mean ± standard deviation) [1]Metallothionein concentrations are estimated assuming 7 gram-atoms (Cu, Zn, Cd, Hg) per mole and a molecular weight of 7 kDa. nd: not determined

located in the MT fraction of the liver, indicating low spillage of Cd to other metalloproteins (Wagemann *et al.*, 1984; Wagemann and Hobden, 1986). This could be an adaptation of this Arctic species to the high Cd concentrations present in its environment. However, more data on Cd speciation in marine mammals are needed to get a better comprehension of the precise role of MTs in detoxification processes.

MERCURY BINDING

Hg also has a strong affinity for MTs and high levels of this metal can be associated with elevated levels of Cd in marine mammal tissues (Caurant *et al.*, 1996). However, studies conducted on dolphins (Kwohn *et al.*, 1986), California sea lions (Lee *et al.*, 1977), pilot whales (Caurant *et al.*, 1996), narwhals (Wagemann *et al.*, 1984) and sperm whales (Bouquegneau *et al.*, 1997; Holsbeek *et al.*, 1999) demonstrated that only a small part of the total Hg was bound to MTs (Table 3). A significant amount of Hg measured in the cytosol of a narwhal liver was found to be associated with the high molecular weight fractions (Wagemann *et al.*, 1984). According to these authors, such a spill over of Hg to the high molecular weight components would be a normal occurrence in marine mammals and is not related to the saturation of MTs.

Even though there is a high affinity of Hg for MTs, most of the metal is bound to components other than MTs. These results are quite different from those deriving from studies carried out on terrestrial mammals, in which Hg is shown to be particularly bound to MTs (Whanger and Deagen, 1983). This striking difference between terrestrial and marine mammals is mainly due to differences in Hg speciation in the diet. In the marine environment,

Table 2 *Cadmium speciation in the tissue, the cytosolic fraction and metallothioneins*

Species	Tissue	N	Total Cd ($\mu g.g^{-1}$ dw)	% of Cd in cytosolic fraction	% cytosolic Cd bound to metallothioneins	References
Stenella coeruleoalba (striped dolphin)	kidney	n=4	87	58	98	Kwohn *et al.*, 1986
Zalophus californianus (California sea lion)	kidney	n=1	37	68	nd	Lee *et al.*, 1977
		n=5	65 ± 30	63	71	Ridlington *et al.*, 1981
	liver	n=1	<dl	<dl	<dl	
		n=5	11 ± 7	60	55	
Globicephala melas (pilot whale)	kidney	gestating females n=7	548 ± 164	nd	54 ± 6	Amiard-Triquet and Caurant, 1997
		foetus n=5	1 ± 0.8	nd	44 ± 41	
	liver	gestating females	312 ± 124	nd	51 ± 20	
		foetus	0.6 ± 0.7		25 ± 34	
Physeter macrocephalus (sperm whale)	liver	n=1	50	92	100	Ridlington *et al.*, 1981
		n=1	64	53	18	Bouquegneau *et al.*, 1997
		n=1	71	55	17	
		n=1	103	39	5	Holsbeek *et al.*, 1999
	kidney	n=1	225	81	21	
		n=1	316	85	66	
Monodon monoceros (narwhal)	kidney	n=1	332	92	72	Wagemann *et al.*, 1984 Wagemann and Hobden, 1986
	liver	n=1	176	88	77	

Cd concentrations are estimated in $\mu g.g^{-1}$ dry weight; nd: not determined, dl: detection limit

Table 3 *Distribution of mercury within cellular fractions*

Species	Tissue	Total Hg ($\mu g\ g^{-1}$ fw)	% Hg in the insoluble fraction	% Hg associated with MTs	Reference
Globicephala melas	kidney (n=7)	6	79	7	Caurant *et al.*, 1996
Zalophus californianus	kidney (n=5)	10	54	22	Lee *et al.*, 1977
	liver (n=5)	61	93	2.6	
Monodon monoceros	liver (n=1)	9	88	5	Wagemann *et al.*, 1984
	kidney (n=1)	2	73	10	
Physeter macrocephalus	liver (n=3)	2	85	<1	Bouquegneau *et al.*, 1997
		15	95	<0.3	
		15	84	<3	Holsbeek *et al.*, 1999
	kidney (n=2)	2	72	<2	
		5	70	<6	
Stenella coeruleoalba	kidney (n=4)	16	83	6	Kwohn *et al.*, 1986

almost all the Hg present in fish is methylated (Svensson *et al.*, 1992). Methylmercury, whose affinity for MTs is low, cannot be detoxified by this process. In marine mammals, it has been shown that the relative MeHg levels decreased from 100% (of the total Hg level) in juveniles to only 2 or 3% in the liver of adults (Joiris *et al.*, 1991). This reflects the existence of a slow mineralisation process without formation of free Hg^{++} ions which can bind to MTs.

The observed high percentage of Hg bound to the insoluble fraction of the liver results from the formation of tiemannite (HgSe) These dense intracellular granules have been observed in the liver macrophages and Kuppfer cells, the proximal tubules of the kidney, the lung and hilar lymph nodes (Martoja and Viale, 1977; Martoja and Berry, 1980; Augier *et al.*, 1993; Rawson *et al.*, 1995; Nigro and Leonzio, 1996). By this way, in marine mammals, selenium plays a key role in methylmercury detoxification processes, and hence MTs would play a minor role, probably limited to the detoxification of Hg^{2+}.

STRUCTURE AND CHARACTERIZATION

Comparative sequence studies of MTs from different species and organs have revealed remarkable similarities among mammalian species (see Binz and Kägi, 1999). About 56% of the 61 amino acid residues are conserved in mammals, among them all the 20 cysteine and nearly all lysine and arginine residues (Kojima *et al.*, 1999).

Characterization of marine mammal metalloproteins were first attempted on sea lion and sperm whale kidney and liver (Ridlington *et al.*, 1981). Metal binding proteins isolated from liver sperm whale eluted in a manner similar to rat MTs, but the amino acid analyses yielded only 12% cysteine residues whereas all mammalian MTs contain approximately 30%. According to the authors, this protein was not likely a metallothionein but rather a type of Cu-chelatin. Similar conclusions were drawn for sea lion liver and kidney metal binding proteins as the amino acid analysis for the different metal-binding fractions contained 2 to 15% cysteine residues. It must be noted however, that general re-examination of copper chelatin has resulted in its designation as a MT (Winge *et al.*, 1981 quoted by Roesijadi, 1992). However, results reported by Ridlington *et al.* (1981) are inconsistent with other marine mammal MT characterization. Indeed, striped dolphin MT-1 and MT-2 isolation and characterization have been performed (Kwohn *et al.*, 1986, 1988). According to these authors, 61 amino acid residues are present per mole of each MT, including 20-21 cysteine residues (Table 4). The absence of aromatic amino acids

agrees with the lack of absorbance at 280 nm. MT amino acid composition of striped dolphin compared with rabbit for both isoforms is quite similar. According to Kwohn *et al.* (1988), 89% of the MT-2 amino acid sequence is conserved between rabbit and dolphin. Neither valine nor leucine were detected in rabbit both isoforms, which suggests microheterogeneity between the two species.

This discrepancy observed between the two characterizations of marine mammal MTs might be explained, at least in part, by technical evolution. Indeed, Roesijadi (1992) has underlined early technical difficulties associated with purifications of MTs. Studies that have attempted to characterise metal-binding proteins have often been influenced by the presence of impurities or the isolation of proteins onto which metals could be redistributed during sample preparation.

Kwohn *et al.* (1986) have detected two isoforms in the dolphin kidney identified as MT-1 and MT-2. According to its absorbance at 254 nm, the MT-2 isoform is much more abundant than MT-1 with a ratio 1/16 while, for example, it is *e.g.* only 1/6.2 in equine tissues (Kojima *et al.*, 1976 quoted by Kwohn *et al.*, 1986). Most of the metals are therefore bound to MT-2 except Cu which is preferentially bound to MT-1 (Kwohn *et al.*, 1986). The same tendency is also observed for horse renal MTs (Kojima *et al.*, 1976 quoted by Kwohn *et al.*, 1986) indicating different cellular functions for each renal MT of striped dolphins. However, it is worth noticing that Wagemann and Hobden (1986) reported MT-1 as the major form of MTs in the liver and kidney from a narwhal. The functional significance of multiple isoforms of MTs has yet to be demonstrated for any mammalian species. The different roles of MTs in the regulation of metals are probably critical to cell functions but these have yet to be studied in any marine mammal. It is clear, however, that understanding the MT function will need to consider the functionality and structure of each MT isoform.

Table 4 *Amino acid composition of striped dolphin renal MT-1 and MT-2* (after Kwohn *et al.*, 1986) *and rabbit hepatic MT-1 and MT-2* (after Nordberg *et al.*, 1972)

Amino acid	Dolphin MT-1	Dolphin MT-2	Rabbit MT-1	Rabbit MT-2
Asp	3	3	5	5
Thr	3	2	3	4
Ser	7	8	7	9
Glu	2	2	1	3
Gly	6	6	4	4
Ala	6	6	8	8
Cys	21	20	15	19
Val	1	1	/	/
Met	1	1	1	1
Ile	1	1	1	1
Leu	1	/	/	/
Phe	/	/	/	/
Lys	6	8	7	9
Arg	/	/	/	/
Pro	3	3	5	3
Tyr	/	/	/	/
His	/	/	/	/
Total	61	61	57	66
Molecular weight*	5986	6013	5608	6616

*Calculated as metal free molecular weight

FACTORS MODULATING METALLOTHIONEIN CONCENTRATIONS

The range of MT concentrations in marine mammal kidneys and liver is widely extended (from 58 to 1200 μg/g fw). Their concentrations appear to be high compared with those in rats. Indeed, hepatic levels in normal rats are about 1 $\mu g.g^{-1}$ while it can reach more than 200 $\mu g.g^{-1}$ in Cd-induced animals (Eaton and Toal, 1982). Maximal concentrations encountered in marine mammals are much higher (Table 1). However, the modulation of MT concentrations in marine mammals is poorly understood due to a lack of extensive quantifications. In most cases, the factors influencing MT concentrations in the wild are assumed after experimental studies on other mammals. Till now, only two studies have attempted to correlate MTs with ecological or ecotoxicological factors (Tohyama *et al.,* 1986; Amiard-Triquet and Caurant, 1997). According to these authors, the MT concentrations depend more on particular tissues, the heavy metal concentration, the age and the diet.

The tissue

MT concentrations are always higher in the kidneys than in the livers (Table 1). Few data are available to allow for comparison, but MT level in the kidney appears to be1.2 to 1.6 fold higher than in the liver (Table 5) except for sperm whales stranded on the Belgian coast in which the ratio may be related with the debilitated status of these animals.

The metal level

A study performed on harbour seals (*Phoca vitulina*) caught on Japanese coasts show that MT concentrations are significantly correlated with the level of Cd and Zn in the livers and with the level of Cd, Zn and inorganic Hg in the kidneys (Tohyama *et al.,* 1986). These authors suggest that inorganic Hg and Cd could be sequestered in MT and that Hg toxicity could also be lessened by this protein.

The age

Tohyama *et al.* (1986) have pointed out that higher MT levels have been quantified in the liver and kidney of a seal pup as compared to adults. Neonates of various mammalian species are known to have relatively high levels of MTs with associated Zn and Cu in the liver (Bakka and Webb, 1981, quoted by Tohyama *et al.,* 1986). Amiard-Triquet and Caurant (1997) have quantified hepatic MTs in gestating female pilot whales and their foetuses. MT mean concentrations were always lower in the foetus than in mother's livers (mean: 73 mg/kg and 167 mg/kg, respectively). However, the ratio MT / total proteins remained unchanged. In harbour seals, Tohyama *et al.* (1986) found a correlation between MT levels and age. The observed age-dependant changes in renal and hepatic MTs are associated with Cd accumulation as it has been shown recently in human tissue (Yoshida *et al.,* 1998).

The diet

As quoted above, Caurant *et al.* (1996) and Amiard-

Table 5 *Comparison of the ratio of MT concentrations in the kidney* (K) *and liver* (L)

Species	n	Sampling status	Ratio $K.L^{-1}$	Reference
Globicephala melas	7	by-caught	1.2	Amiard-Triquet and Caurant, 1997
Phoca vitulina	15	by-caught	1.4	Tohyama *et al.,* 1986
Monodon monoceros	1	by-caught	1.6	Wagemann *et al.,* 1984
Physeter macrocephalus	2	stranded	8.7-12.4	Holsbeek *et al.,* 1999

Triquet and Caurant (1997) have compared metallothionein-like protein (MT-LP) concentrations between pilot whales caught in July and November 1986 (Fig. 1).

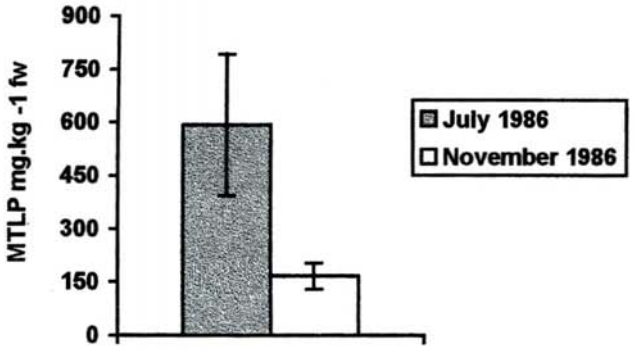


Fig. 1 *Mean concentrations and standard deviations of metallothionein-like proteins* (MT-LP) *calculated in the livers of pilot whales* (*Globicephala melas*) *caught in the Faroe Islands* (data adapted from Caurant *et al.,* 1996; Amiard-Triquet and Caurant, 1997).

MT mean concentrations in the livers of pilot whales caught in the summer 1986 are more than three fold higher than those encountered in winter caught individuals, while Cd concentrations are similar for both groups. Squid is the major food item in pilot whales and is considered as a significant source of Cd for several predators (Das *et al.,* 1999b). The lower level of MTs in whales caught in November could be related to lower Cd assimilation in winter. Indeed, the summer diet of pilot whales consists mainly on cephalopods whereas the winter diet is characterized by a higher fish input (Caurant *et al.,* 1996; Amiard-Triquet and Caurant, 1997). According to the authors, the elevated MT concentrations would reflect a induction by a recent Cd assimilation. The metal could be sequestered later under another detoxified form (*e.g.* precipitated within lysosomes).

MTs AS BIOCHEMICAL INDICATOR OF METAL EXPOSURE AND TOXICITY

Soon after their discovery in aquatic species, the induction of MT and the analyses of its capacity to bind metals were proposed as candidates for biochemical monitoring of metal pollution in the aquatic environment (Roesijadi, 1996). Heavy metal analysis in the tissues reflects the level of contamination of a population but not its response to metal exposure, as these can be detoxified, at least in part, through the binding to MTs. The induction of MTs by non-essential metals as Cd has been considered as an asset in biomonotoring studies. Moreover, with current methodologies, MT induction can be measured at several levels: increase in metal content in the MT pool, increase in MT and increase in MT mRNA (*e.g.* Suzuki, 1992; Caurant *et al.,* 1996; Tom *et al.,* 1998). Each reflects a different level of cellular regulation and function, and provides complementary information. Arguments against application of MT analysis in environmental studies resides mainly in our current lack of detailed understanding of basal MT function and its relationship to induction by metals (Roesijadi, 1992; Cosson and Amiard, 1998). Through forty years of MT research, the different interacting processes appear numerous and complex. Cosson and Amiard (1998) have recently reviewed the utilisation of MTs as potential biomarkers of metal contamination in aquatic animals including marine mammals and have underlined the difficulty to correlate metal bioaccumulation and increased MT level. Thus proposal for the use of MTs as biochemical indicators of metal pollution in marine mammals merits continued considerations, as further investigations are needed to understand the processes involved in detoxification.

LIMITS TO DETOXIFICATION

Drawing conclusions concerning the potential role of MTs in marine mammals is not an easy task due to an obvious lack of recent published studies and extrapolation from other mammals are often advanced. Concerning potential role of these proteins in Cd detoxification, advises are mitigated. The rates of MT synthesis can become limiting as exposure concentrations increase. The related

decreasing metal binding to MTs could result in the spillage of metals to other structures that would include target sites for metal toxicity (Roesijadi, 1992). Any detoxification process has a cost for the cell or the organism involved and might have a limit. This threshold cannot be fully defined in terms of tissue metal or MT concentrations because of the number of parameters that can interact to limit physiological pathways that lead to detoxification. For example, gender and hormonal activity can modulate the synthesis of MTs (Blazka and Shaikh, 1991). Moreover, binding to MTs might not be a final step, and the formation of secondary components with toxic effects have been demonstrated in other mammals. The accumulation and degradation of Cd-metallothionein complex (Cd-MT) in the renal tubular epithelial cells can induce nephrotoxicity in mice counteracted by Zn which has a protective effect against this Cd-MT-induced nephrotoxicity (Liu *et al.,* 1996; Tang *et al.,* 1998). As a result of their physiological function in the homeostasis of essential metals, MTs could be involved in many cellular pathways. Thus, they could modulate physiological processes as an indirect effect of heavy metal exposure. For example, MTs have been demonstrated as potential modulators of some parameters of the immune response (Liebbrandt *et al.,* 1994; Borghesi *et al.,* 1996). Detoxification pathways could therefore lead to more subtle toxic effects underlying the complexity to approach toxic effects of heavy metals.

CONCLUSION

Compared with other animals, liver and kidneys of marine mammals display high MT concentrations, related with their high levels of contamination by heavy metals (mainly Cd and Hg). These high heavy metal concentrations result from both their homeothermy (which requires large food consumption) and their position at the top of marine food webs. Data about MTs remain scarce and, untill now, discussion is only possible about their potential role in the detoxification of Hg and Cd. Obviously, MTs play a minor role (if any, when considering methylmercury) in the binding and detoxification of Hg by marine mammals. On the contrary, close interactions occur between Cd and MT dynamics. Cytosolic MTs appear as a potential short term way of detoxification of Cd accumulated from diet. Long-term detoxification however would imply a sequestration of the metal under a precipitated form (e.g. in lysosomes). So many parameters are likely to modulate the MT concentration in marine mammals tissues that its use as a biomarker of heavy metals pollution in the marine environment remains debatable.

Acknowledgments – The research work was supported by a Grant from the Belgian Office for Scientific, Technical and Cultural Affairs (Contract MN/DD/50). We are grateful to Prof. D. Cyr (from INRS-Santé, Montréal, Québec, Canada) and Prof. R.P. Cosson (from Service d'Ecotoxicologie, Université de Nantes, France) for their constructive and useful comments on a previous version of this paper.

REFERENCES

Amiard-Triquet, C. and Caurant, F., Adaptation des organismes marins à la contrainte métallique: l'exemple du cadmium chez *Globicephala melas*, mammifère Odontocète. *Bull. Soc. zool. Fr.* 1997, **122**: 127-136.

Antoine, N., Jansegers, I., Holsbeek, L., Joiris, C. and Bouquegneau, J.M., Contamination par les métaux lourds des oiseaux marins et des marsouins de la Mer du Nord. *Bull. Soc. roy. Sci.* Liège 1992, **61**: 163-176.

Augier, H., Benkoël, L., Brisse, J., Chamlian, A. and Park, W.K., Microscopic localization of mercury-selenium interaction products in liver, kidney, lung and brain of Mediterranean striped dolphins (*Stenella coeruleoalba*) by silver enhancement kit. *Cell. mol. Biol.* 1993, **39**: 765-772.

Binz, P.A. and Kägi, J.H.R., Metallothionein: Molecular evolution and classification. In: *Metallothionein IV,* Klaassen, C.D. (ed.), Birkhaüser, Basel, 1999, pp. 7-14.

Blazka, M.E. and Shaikh, Z.A., Sex difference in hepatic and renal cadmium accumulation and metallothionein induction. *Biochem. Pharmacol.* 1991, **41**: 775-780.

Borghesi, L.A., Youn, J., Olson, E.A. and Lynes, M.A., Interactions of metallothionein with murine lymphocytes: plasma membrane binding and proliferation. *Toxicology* 1996, **108**: 129-140.

Bouquegneau, J.M., Debacker, V., Gobert, S. and Nellissen, J.P., Toxicological investigations on four sperm whales stranded on the Belgian coast: inorganic contaminants. In: *Sperm Whale Deaths in the North Sea*: *Science and Management*. Jacques, T.G. and Lambertsen, R.H. (eds). *Bull. Inst. roy. Sci. natn. Belg*. 1997, **67**(Suppl.): 75-78.

Bouquegneau, J.M. and Joiris, C., Ecotoxicology of stable pollutants in cetaceans: organochlorines and heavy metals. In: *Whales: Biology - Threats - Conservation.* Symp. Proc., Brussels, 5-7 June 1991, Symoens, J.J. (ed.), Royal Academy of Overseas Sciences, Brussels, 1992, pp. 247-250.

Caurant, F., Navarro, M. and Amiard, J.C., Mercury in pilot whales: possible limits to the detoxification process. *Sci. tot. Environm.* 1996, **186**: 95-104.

Cosson, R.P. and Amiard, J.C., Utilisation des métallothionéines comme biomarqueurs d'exposition aux métaux. In: *Utilisation de Biomarqueurs pour la Surveillance de la Qualité de l'Environnement.* Lagadic, L., Caquet, T., Amiard, J.C. and Ramade, F. (eds.), Masson, Paris, 1998, pp. 77-109.

Das, K., Debacker, V., Pillet, S. and Bouquegneau, J.M., Heavy metals in marine mammals. In: *Toxicology of Marine Mammals,* Taylor and Francis Publ. Washington, D.C., 1999a, invited paper.

Das, K., Lepoint, G., Loizeau, V., Debacker, V., Dauby, P. and Bouquegneau, J.M., Tuna and dolphin associations in the Northeast Atlantic: Evidence of different ecological niches from stable isotope and heavy metal measurements. *Mar. Poll. Bull.* 1999b, in press, accepted June 9, 1999.

Dietz, R., Nørgaard, J. and Hansen, J.C., Have arctic mammals adapted to high cadmium levels? *Mar. Pollut. Bull.* 1998, **36**: 490-492.

Eaton, D.L. and Toal, B.F., Evaluation of the Cd/hemoglobin affinity assay for the rapid determination of metallothionein in biological tissues. *Toxic. appl. Pharmacol.* 1982, **54**: 148-155.

Elinder, C.J. and Järup, L., Cadmium exposure and health risks: recent finding. *Ambio* 1996, **25**: 370-373.

Holsbeek, L., Joiris, C.R., Debacker, V., Ali, I.B., Nellissen, J.P., Gobert, S., Roose, P., Bouquegneau, J.M. and Bossicart, M., Heavy metals, organochlorines and polycyclic aromatic hydrocarbons in sperm whales stranded in the Southern North Sea during the 1994/1995 winter. *Mar. Poll. Bull.* 1999, **38**: 304-313.

Jauniaux, T., Brosens, L., Jacquinet, E., Lambrigts, D., Addink, M., Smeenk, M. and Coignoul, F., Postmortem investigations on winter stranded sperm whales from the coasts of Belgium and the Netherlands. *J. Wild. Dis.* 1998, **34**: 99-109.

Joiris, C.R., Holsbeek, L., Bouquegneau, J.M. and Bossicart, M., Mercury contamination of the harbour porpoise *Phocoena phocoena* and other cetaceans from the North Sea and the Kattegat. *Water, Air Soil Poll.* 1991, **56**: 283-293.

Klaassen, C.D. and Liu, J., Metallothionein transgenic and knock-out mouse models in the study of cadmium toxicity. *J. toxicol. Sci.* 1998, **2**: 97-102.

Klaassen, C.D., Liu, J. and Choudhuri, S., Metallothionein: an intracellular protein to protect against cadmium toxicity. *Annu. Rev. Pharmacol. Toxicol.* 1999, **39**: 267-294.

Koeman, J.H., Peeters, W.H.M., Koudstaal-Hol, C.H.M., Tjioe, P.S. and De Goeij, J.J.M., Mercury-selenium correlations in marine mammals. *Nature* 1973, **245**: 385-386.

Koeman, J.H., Van de Ven, W.S.M., Goeij, J.J.M., Tjioe, P.S. and Van Haaften, J.L., Mercury and selenium in marine mammals and birds. *Sci. tot. Environm.* 1975, **3**: 279-287.

Kojima, Y., Binz, P.A. and Kägi, J.H.R., Nomenclature of metallothionein: Proposal for a revision. In: *Metallothionein IV.* Klaassen, C.D. (ed.), Birkhäuser, Basel, 1999, pp. 3-6.

Kwohn, Y.T., Okubo, A., Hirano, H., Kagawa, H., Yamazaki, S. and Toda, S., Primary structure of striped dolphin renal metallothionein II. *Agric. Biol. Chem.* 1988, **52**: 837-841.

Kwohn, Y.T., Yamazaki, S., Okubo, A., Yoshimura, E., Tatsukawa, R. and Toda, S., Isolation and characterization of metallothionein from kidney of striped dolphin, *Stenella coeruleoalba. Agric. Biol. Chem.* 1986, **50**: 2881-2885.

Lee, S.S., Mate, B.R., Von Der Trenck, K.T., Rimerman, R.A. and Buhler, D.R., Metallothionein and the subcellular localisation of mercury and cadmium in the California sea lion. *Comp. Biochem. Physiol.* 1977, **57**C: 45-53.

Liebbrandt, M.E.I., Khokha, R. and Koropatnick, J., Antisense down-regulation of metallothionein in a human monocytic cell line alters adherence, invasion, and the respiratory burst. *Cell Growth Differ.* 1994, **5**: 17-25.

Leonzio, C., Focardi, S. and Fossi, C., Heavy metals and selenium in stranded dolphins of Northern Tyrrhenian (NW Mediterranean). *Sci. tot. Environm.* 1992, **119**: 77-84.

Liu, J., Liu, Y., Michalska, A.E., Choo, K.H. and Klaassen, C.D., Metallothionein plays less of a protective role in cadmium-metallothionein-induced nephrotoxicity than in cadmium chloride-induced hepatotoxicity. *J. Pharmacol. exp. Ther.* 1996, **276**: 1216-1223.

Margoshes, M. and Vallee, B.L., A cadmium protein from equine kidney cortex. *J. am. Chem. Soc.* 1957, **79**: 4813-4814.

Martoja, R. and Berry, J.P., Identification of tiemannite as a probable product of demethylation of mercury by selenium in cetaceans. A complement to the scheme of the biological cycle of mercury. *Vie et Millieu* 1980, **30**: 7-10.

Martoja, R. and Viale, D., Accumulation de granules de séléniure mercurique dans le foie d'Odontocètes (Mammifères, Cétacés): un mécanisme possible de détoxication du méthylmercure par le sélénium. *C.r. Acad. Sc. Paris* 1977, **285**: 109-112.

Mochizuki, Y., Suzuki, K.T., Sunaga, H., Kobayashi, T. and Doi, R., Separation and characterization of metallothionein in two species of seals by high performance liquid chromatography-atomic absorption spectrophotometry. *Comp. Biochem. Physiol.* 1985, **82**C: 249-254.

Nigro, M. and Leonzio, C., Intracellular storage of mercury and selenium in different marine vertebrates. *Mar. Ecol. Prog. Ser.* 1996, **135**: 137-143.

Nordberg, G.F., Nordberg, M., Piscator, M. and Versterberg, O., Separation of two forms of rabbit metallothionein by isoelectric focusing. *Biochem. J.* 1972, **126**: 491-498.

Olafson, R.W. and Thompson, J.A., Isolation of heavy metal binding proteins from marine vertebrates. *Mar. Biol.* 1974, **28**: 83-86.

Palmiter, R.D., The elusive function of metallothioneins. *Proc. natn. Acad. Sci.* USA 1998, **95**: 8428-8430.

Pesch, H.J., Palesch, T. and Seibold, H., The increase of the Cd-

burden in man. Post-mortem examinations in Franconia by absorption spectroscopy. In: *Heavy Metals in the Environment*. Vernet, J.P. (ed.), vol. **2**, CEP Consultants, Edinburgh, 1989, pp. 111-114.

Rawson, A.J., Bradley, J.P., Teetsov, A., Rice, S.B., Haller, E.M. and Patton, G.W., A role for airborne particulates in high mercury levels of some cetaceans. *Ecotoxicol. environm. Saf.* 1995, **30**: 309-314.

Ridlington, J.W., Chapman, D.C., Goeger, D.E. and Whanger, P.D., Metallothionein and Cu-chelatin: characterization of metal-binding proteins from tissues of four marine animals. *Comp. Biochem. Physiol.* 1981, **70B**: 93-104.

Roesijadi, G., Metallothioneins in metal regulation and toxicity in aquatic animals. *Aquat. Toxicol.* 1992, **22**: 81-114.

Roesijadi, G., Metallothionein and its role in toxic metal regulation. *Comp. Biochem. Physiol.* 1996, **113C**: 117-123.

Satoh, M., Nishimura, N., Kanayama, Y., Naganuma, A., Suzuki, T. and Tohyama, C., Enhanced renal toxicity by inorganic mercury in metallothionein-null mice. *J. Pharmacol. exp. Ther.* 1997, **283**: 1529-1533.

Suzuki, K.T., Metallothionein: analysis in tissues and toxicological significance. *IARC Sci. Pub.* 1992, **118**: 211-217.

Svensson, B.G., Schütz, A., Nilsson, A., Åkesson, I., Åkesson, B. and Skerfving, S., Fish as a source of exposure to mercury and selenium. *Sci. tot. Environm.* 1992, **126**: 61-74.

Tang, W., Sadovic, S. and Shaikh, Z.A., Nephrotoxicity of cadmium-metallothionein: protection by zinc and role of glutathione. *Toxicol. appl. Pharmacol.* 1998, **151**: 276-282.

Tom, M., Moran, O., Jakubov, E., Cavari, B. and Rinkevich, B., Molecular characterization of metallothionein-cDNA of *Sparus aurata* used for detecting heavy metal pollution along the Mediterranean coast of Israel. *Mar. Pollut. Bull.* 1998, **36**: 131-137.

Tohyama, C., Himeno, S.I., Watanabe, C., Suzuki, T. and Morita, M., The relationship of the increased level of metallothionein with heavy metal levels in the tissue of the harbour seal (*Phoca vitulina*). *Ecotoxicol. environm. Saf.* 1986, **12**: 85-94.

Wagemann, R. and Hobden, B., Low molecular weight metalloproteins in tissues of the narwhal *(Monodon monoceros)*. *Comp. Biochem. Physiol.* 1986, **84C**: 325-344.

Wagemann, R., Hunt, R. and Klaverkamp, J.F., Subcellular distribution of heavy metals in liver and kidney of a narwhal whale (*Monodon monoceros*): an evaluation for the presence of metallothionein. *Comp. Biochem. Physiol.* 1984, **78C**: 301-307.

Whanger, P.D. and Deagen, J.T., Effect of dietary mercury levels and cadmium on rat tissue metallothionein: mercury binding and influence on zinc. *Environm. Res.* 1983, **30**: 372-380.

Webb, M., Toxicological significance of metallothionein. *Experientia* 1987, **52**: 109-134.

WHO, Cadmium. *Environmental Health Criteria*, 134. World Health Organization, Geneva, 1992, 280 p.

Yoshida, M., Ohta, H., Yamauchi, Y., Seki, Y., Sagi, M., Yamazaki, K. and Sumi, Y., Age-dependent changes in metallothionein levels in liver and kidney of the Japanese. *Biol. Trace elem. Res.* 1998, **63**: 167-175.

Continued from the outside backcover

M.A. MORCILLO, M.I. RUCANDIO and J. SANTAMARÍA — 435 Effect of gamma irradiation on liver metallothionein synthesis and lipid peroxidation in rats

T. MINAMI, Y. SAKITA, Y. OKAZAKI, M. TSUTSUMI and Y. KONISHI — 445 Lack of involvement of metallothionein expression in pancreatic carcinogenesis by n-nitrosobis (2-oxopropyl) amine in syrian hamsters

M. NORDBERG and G.F. NORDBERG — 451 Toxicological aspects of metallothionein. A review

C.O. SIMPKINS — 465 Metallothionein in human disease. A review

489 Erratum

Announcement 3d World-Congress CMB, Oct. 2000

CELLULAR AND MOLECULAR BIOLOGY™

Volume 46, Number 2 2000

METALLOTHIONEIN: A REMARKABLE MOLECULE FOR MULTIDISCIPLINARY APPROACH

Content

G. BORDIN	iii	Foreword
K. POŁEĆ, S. MOUNICOU, H. CHASSAIGNE and R. ŁOBIŃSKI	221	Probing metal-complexes with metallothioneins by reversed phase microbore chromatography and capillary zone electrophoresis coupled with inductively coupled plasma and electrospray mass spectrometry
A.R. RODRÍGUEZ and M. ESTEBAN	237	Application of electroanalytical methods to the characterization of metallothioneins and related molecules. A review
I. ŠESTÁKOVÁ and P. MADER	257	Voltammetry on mercury and carbon electrodes as a tool for studies of metallothionein interactions with metal ions
M. ERK and B. RASPOR	269	Advantages and disadvantages of voltammetric method in studying cadmium-metallothionein interactions
K. DAS, V. DEBACKER and J.M. BOUQUEGNEAU	283	Metallothioneins in marine mammals. A review
R.P. COSSON	295	Bivalve metallothionein as a biomarker of aquatic ecosystem pollution by trace metals: limits and perspectives. An overview
G. ISANI, G. ANDREANI, M. KINDT and E. CARPENÈ	311	Metallothioneins (MTs) in marine molluscs. A review
R. DALLINGER, B. BERGER, C. GRUBER, P. HUNZIKER and S. STÜRZENBAUM	331	Metallothioneins in terrestrial invertebrates: structural aspects, biological significance and implications for their use as biomarkers
M.G. CHERIAN and M.D. APOSTOLOVA	347	Nuclear localization of metallothionein during cell proliferation and differentiation. A review
Y. OGRA and K.T. SUZUKI	357	Nuclear trafficking of metallothionein: possible mechanisms and current knowledge. A review
J.M. DEMOOR and D.J. KOROPATNICK	367	Metals and cellular signaling in mammalian cells. A review
T. HE, D. WEI, D. FABRIS and C. FENSELAU	383	Intracellular sequestration of anti-tumor drugs by metallothionein
G. ROESIJADI	393	Metal transfer as a mechanism for metallothionein-mediated metal detoxification. A review
A. VIARENGO, B. BURLANDO, N. CERATTO and I. PANFOLI	407	Antioxidant role of metallothioneins: a comparative overview
N. VANDEGHINSTE, P. PROOST and M. DE LEY	419	Metallothionein isoform gene expression in zinc-treated human peripheral blood lymphocytes

Continued on the inside backcover

Indexed/Abstracted in:
Current Contents, Index Medicus, MEDLINE, BIOSIS Database, SUBIS, PASCAL/CNRS Database, Cam. Sci. Abstr., CAB Inter., Chem. Abstr. Service, RIS in Reference

ISSN 0145-5680
CMBIDI 46(2) 219-490 (2000)

Published by C.M.B. ASSOCIATION
Editorial Office: 1, Avenue du Pavé Neuf
F-93160 Noisy-le-Grand (France)

Printed by PEETERS - FRANCE S.A.R.L.
52, Boulevard Saint-Michel
F-75006 Paris (France)