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Biological effects of contaminants: Quantification of metallothionein (MT) in fish liver tissue

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Abstract

This document describes methods to analyse the protein metallothionein in fish tissues. Metallothionein is induced by and binds essential (Cu, Zn) and non-essential (Cd, Hg) metals and is used in monitoring programmes as a marker for environmental metal exposure. The main focus is on the use and development of immunochemical procedures (ELISA). In addition, two alternative methods, electrochemical and spectrophotometric, are described.

Key words: metallothionein, fish, cod, *Gadus morhua*, biomarker, Cu, Zn, Cd, monitoring

1 INTRODUCTION

The low molecular weight protein metallothionein (MT) is present in most vertebrate tissues. A major role of this protein is regulation of the intracellular availability of zinc (Zn) and/or copper (Cu). Other functions, such as metal detoxification and free radical scavenging, have also been suggested (Kägi and Schäffer, 1988). In addition to Cu and Zn, MT binds non-essential metals such as cadmium (Cd), mercury (Hg), and silver (Ag). The synthesis of the protein is induced by elevated intracellular concentrations of the metals mentioned above. Metallothionein induction is a response to elevated intracellular metal concentrations and the protein has been suggested as a biomarker for environmental metal contamination (Engel and Roesijadi, 1987; Haux and Förlin, 1989; George and Olsson, 1994).

The methods most commonly used to quantify MT in tissues exploit some of the characteristics of this protein: its metal content, the number of sulfhydryl groups, and its size and/or its heat stability. In addition, various immunochemical assays using MT-specific antisera have been developed in the past 10–15 years. Furthermore, metallothionein mRNA expression has been quantified using specific probes in blots or polymerase chain reaction (PCR) techniques (Kille *et al.*, 1992; Olsson, 1993; Jessen-Eller *et al.*, 1994). Quantification of the protein, and not the mRNA, appears most useful for monitoring purposes for two reasons. Firstly, the physiological and toxicological significance of a response will depend upon the concentration of MT, not MT mRNA, present in the cell. Secondly, the half-life of MT mRNA is much shorter than the half-life of MT and the time-span of a measurable response is thus shorter. The existing methods for quantification of MT may conveniently be divided into four categories: (1) quantification by metal content, (2) metal substitution, (3) quantification of sulfhydryl groups, and (4) immunoassays. These four methods have different characteristics, as summarized in Table 1. In addition, MT may be determined semi-quantitatively by immunohistochemistry (Jasani and Elmes, 1991), immunoblots (Aoki *et al.*, 1986) or by polyacrylamide or capillary electrophoresis (Lin and McCormick, 1986). Although capillary electrophoresis is an especially promising technique, this method has not yet been applied to environmental samples to any extent.

Table 1. Overview of methods that have been used to quantify metallothionein (MT) in environmental or medical samples. For comments on the methods, see text. A recent description and protocol for the methods marked with an asterisk (*) can be found in Riordan and Vallee (1991).

Category of Methods	Method	Reference
Metal content	Gel filtration/AAS	Brown, 1977
	HPLC/AAS*	Suzuki, 1980
Metal substitution	Hg saturation	Piotrowski *et al.*, 1973
	Cd saturation*	Chen and Ganther, 1975
	Ag saturation*	Scheuhammer and Cherian, 1986
Sulfhydryl group	Differential pulse polarography*	Olafson and Sim, 1979
	Spectrophotometry	Viarengo *et al.*, 1997
Immunoassay	RIA*	Vander Mallie and Garvey, 1979
	ELISA*	Thomas *et al.*, 1986

AAS = atomic absorption spectrophotometry
HPLC = high performance liquid chromatography
RIA = radioimmunoassay
ELISA = enzyme-linked immunosorbent assay

The methods that depend on the metal content of MT as the means of quantification are time-consuming, as they require an initial chromatographic separation of MT from other proteins. The measurement of metals in the 'MT-peak', generally Cu, Zn, Cd, sometimes Hg, yields information that is not available by any other method. However, this method is not optimal for quantifying the protein, as the concentration of MT is calculated from the concentration of the metals. The measurement error inherent in the determination of each metal will result in a large measurement error for MT. Conceivably, MT could also adsorb or bind non-specifically to the gel filtration column. In addition, it is not known whether MT in fish tissues is saturated with metals under all physiological conditions. Quantification of MT by metal content following ion-exchange chromatography is not recommended as a quantitative method because the recovery of MT from most columns is variable.

The most widely used methods to quantify MT involve the substitution of some or all metals in the protein by a metal added to the sample, generally a radioactive isotope (^{109}Cd, ^{203}Hg, ^{110}Ag) to facilitate quantification. Metal substitution methods are easy to perform and require little equipment, but they exhibit some unsatisfactory aspects. Metal substitution assays do not work well with MT containing Cu (or, for that matter, Hg or Ag). In the Cd-substitution assay, Cd will not replace Cu, whereas Hg and Ag may easily cause oxidation of the protein during the metal-exchange process and may 'over-saturate' MT. Such methods are generally not very suitable when fish liver is the target tissue, as a significant proportion of hepatic MT in many fish species is associated with Cu. Recently, an amendment to the Cd-assay was suggested, to include Cu-thionein in the quantification (Klein *et al.*, 1990). This improved method has not been evaluated for fish tissues.

The third method, quantification of sulfhydryl groups, has also been widely used, primarily with an electrochemical quantification of MT by differential pulse polarography (DPP). This method involves an initial removal of high molecular weight proteins, either by heat denaturation or organic precipitation, followed by quantification of sulfhydryl groups. Sulfhydryl groups are quantified either by DPP or spectrophotometrically, using 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) as a chromophore. There are two problems related to the use of these methods. Firstly, the initial removal of high molecular weight proteins may also remove or modify MT. Secondly, low molecular weight species such as glutathione or free cysteine will not be removed by the initial treatment and may interfere in the subsequent quantification. Both of these problems need to be resolved for each new species and tissue to be studied. Quantification of MT through measurement of sulfhydryl groups may be expected to include all MT in tissues, independent of metal content.

Immunochemical methods have been developed for various mammalian MTs. To date, the only published immunoassay for MT in fish tissues is the radioimmunoassay (RIA) developed by Hogstrand and Haux (1990). There are some major advantages of immunoassays in comparison with the above methods: pre-treatment of samples (heat treatment, precipitation) is unnecessary, and immunoassays have high specificity, low detection limits, and high sample throughput. Enzyme-linked immunosorbent assay (ELISA) is probably better than RIA as an immunoassay for MT because RIA requires the binding of a radioactive label to the competing antigen (i.e., MT), a process that may easily modify major epitopes (especially at the N-terminus).

In the following sections, a protocol for the establishment and use of ELISA to quantify hepatic MT in fish is given, using an ELISA established for Atlantic cod as an example. Protocols for two additional quantification methods for MT can be found in Annex 1, quantification by differential pulse polarography, and Annex 2, spectrophotometric quantification of sulfhydryl groups.

2 TEST METHOD

2.1 Experimental Design

The response in any biological effects method will be modulated by various endogenous and exogenous factors. It is crucial that relevant factors are controlled, either through sampling design or through additional analyses or observations. Contaminated sites should be similar to reference sites with regard to the following: depth, sediment quality (grain size, organic content), oxygen availability, eutrophication status, salinity, and food availability. The same fish species must be used throughout the investigation. Furthermore, all samples must be collected within a period of one month outside the spawning season (preferably in early autumn). Where possible, juvenile fish should be used. If mature fish are used, their sex must be recorded and the sample size increased (see below). Length, weight, liver weight, gonad weight, gross external disease or parasites should always be recorded for each individual. If there is a substantial heterogeneity in any of the above factors (e.g., size differences), the sample size should be increased. The required number of samples will depend on factors such as the objective of the study, local conditions, and analytical procedures. From previous experience, 15–20 juvenile fish (or 10–15 of each sex) would be required to reject H_0: no difference between sites ($\alpha = 0.05$) if the true mean differed by a factor of 2 (i.e., hepatic MT in fish at a contaminated site was double that in fish at a reference site).

2.2 Chemicals

Reagents for the ELISA, including buffer constituents or buffer capsules, secondary antibodies and *o*-phenylenediamine OPD-Tablets, are available from, e.g., Sigma, St. Louis, MO, USA. Microtiter plates, pipetting reservoirs, and sealing tape may be obtained from Nunc, Roskilde, Denmark, or Pierce, Rockford, IL, USA. All other chemicals should be of analytical grade and are available from most major suppliers of laboratory chemicals.

2.3 Equipment

The following equipment is needed to perform the analyses described below: a refrigerated centrifuge with a fixed-angle rotor (capable of at least 10 000 × **g**), a motorized homogenizer, a plate-reader fitted with the appropriate filter, a multichannel pipette (25–250 µl), and a plate-washer. In addition, equipment to refrigerate and store samples is required (a liquid nitrogen thermos, an ultrafreezer). Chromatographic equipment (pump, columns, detector, fraction collector) is needed to purify MT.

2.4 Selection of Tissue and Treatment of Samples

Liver is the recommended tissue. If the entire liver is not sampled, care should be taken to excise the same section of liver from all fish. Livers from different fish species have varying morphology, but the sample should be taken as a slice through the central part of the liver. Immediately following sampling, tissues should be transferred to cryo-vials and frozen in liquid nitrogen. Liver samples may be shipped on dry ice and stored at –80 °C. Samples may be stored for up to one year at –80 °C before processing.

2.5 Preparation of Homogenates

The procedures should be performed at 0–4 °C. Frozen samples should be thawed on ice, weighed, and homogenized in at least 3 volumes (w/v) of 100 mM Tris-HCl, pH 8.1 with 5 mM 2-mercaptoethanol using a Potter-Elvehjem glass-teflon motorized homogenizer. Samples for metal analyses may be taken from the homogenate[1]. Following homogenization, samples should be centrifuged for 30 min at 10 000 × g (at 4 °C). In most marine fish species there will be some fat overlying the supernatant. The supernatant should be removed avoiding the fat. For samples with a high fat content, it will in many cases be necessary to centrifuge the supernatant a second time to remove the fat. The resulting supernatant, hereafter referred to as S9, is used for the measurement of MT and may be stored for up to one year at –80 °C before analysis.

2.6 Protein Determination

Protein in the S9 supernatant may be determined by any protein assay, e.g., Bradford (Bradford, 1976; Read and Northcote, 1981) or Lowry (Lowry *et al.*, 1951). The Bradford assay can easily be adapted to measurement by a plate-reader and is the method chosen by many due to its simplicity. Care should be taken to select the correct protein standard. Although used by many workers, bovine serum albumin (BSA) should be avoided in the Bradford assay because it gives about double the absorbance of most other proteins at any given concentration. Bovine gamma globulin is recommended as a general protein standard if the Bradford assay is used.

2.7 Metallothionein Standards

Regardless of which method is used for MT quantification in a given species, there is a need for a standard from that species. Purified MT is sensitive to oxidation and polymerization, fish MT even more so than MT from mammalian sources. A purified MT preparation for the relevant species is needed for initial work with any technique and as a check on the performance of the assay. It is, however, more convenient to use heat-treated S9 as a standard (and coating antigen) in routine analyses because it is easier to prepare and more stable. Heat treatment is done by diluting the original S9 (or cytosol) 1:10 in 100 mM Tris-HCl, pH 8.1, heating at 95 °C for 4 min, then immediately cooling on ice followed by centrifugation at 5000 × g for 10 min (at 4 °C). The heat-treated S9 (supernatant) is aliquotted and stored at –80 °C. The absolute concentration of MT in the heat-treated sample will need to be established using a purified MT standard.

2.8 Purification of Metallothionein

Hepatic MT can be induced in fish by repeated i.p. (intraperitoneal) injections with Cd, as described in Carpenè *et al.* (1992) or Hylland *et al.* (1994). Following Cd-treatment, metallothionein from most fish species can easily be purified in a three-step procedure using S9 or cytosol (all steps conducted on ice or at 4 °C if not otherwise indicated).

Step 1 Gel filtration on a Sephadex G-75, Superdex G-75, Superose 12 (Pharmacia, Uppsala, Sweden) or equivalent calibrated column using 100 mM Tris-HCl, pH 8.1, as eluting buffer; the peak containing MT can be identified by high absorbance at 250 nm, low absorbance at 280 nm, and an apparent size of 10–15 kDa. This peak should be collected and diluted 1:5 with ice-cold distilled water before ultrafiltration.

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[1] Constituents of the homogenizing buffer need to be checked for metal content.

Step 2 The sample from Step 1 must be concentrated, which is conveniently done using ultrafiltration, e.g., an Amicon stirred-cell ultrafiltration unit with a DP5 filter (Amicon, USA). The ultrafiltration step is done at room temperature, but the sample should be cooled immediately following this step and prior to conducting Step 3.

Step 3 Pure MT can be isolated by ion-exchange chromatography of the concentrated sample from Step 2 using an anion-exchange material, e.g., DEAE or Mono-Q (Pharmacia, Uppsala, Sweden). Metallothionein will elute from the column in a linear gradient from 20 to 400 mM Tris-HCl, pH 8.1. In the most relevant fish species, i.e., Atlantic cod, flounder, and dab, there is presumably only one MT-isoform[2] and there is no need to optimize the ion-exchange system to separate different isoforms. However, sufficiently pure MT for use as a standard can be obtained without a gradient pump system by using small Mono-Q columns (Hi-Q; Pharmacia, Uppsala, Sweden). The sample from Step 2 is then eluted through the equilibrated column (20 mM Tris-HCl, pH 8.1) using a syringe. Following a 10-ml wash with the same buffer, MT can be eluted using stepwise increases in buffer strength, all injected from a syringe. Appropriate concentrations are: 40, 50, 60, 80, 100, and 400 mM Tris-HCl, pH 8.1 (10 ml each). The fraction containing MT can be identified from its 250 nm:280 nm ratio, which should be > 10 for samples from Cd-treated fish.

It is important to note that the above procedures are not quantitative. Metallothionein will be lost at stages in the process, especially during ion-exchange chromatography.

The purified MT may either be aliquotted and frozen directly (–80 °C) or concentrated using ultrafiltration before aliquotting and freezing. At least two aliquots should be used for the analysis of amino acid composition, a check on the purity of the preparation, and an absolute quantification of MT (using the known number of cysteine (20) and methionine (1) residues in fish MTs).

2.9 Production of Polyclonal Anti-Metallothionein Antiserum

Generally, MT from mammalian sources must be modified (polymerized, conjugated) in order to raise antibodies towards it in another species of mammal such as rabbit, goat or sheep. In contrast, fish MTs appear to be sufficiently dissimilar to mammalian MT to be immunogenic in mammals, presumably mainly through the deletion of an amino acid in the N-terminus (Figure 1). As a result, antisera raised against mammalian MT has no (or very low) affinity for fish MTs and vice versa. With MT purified from Atlantic cod, the following procedure yielded specific antisera in immunized rabbits (Chbb: CH, 2–2.5 kg). Rabbits were injected subcutaneously with 200–300 µg purified cod-MT mixed with an equal volume of Freunds incomplete adjuvant in four injections at two-week intervals, followed by a booster injection after an additional two months. One month after the last injection the rabbits were bled and the serum was recovered by allowing the blood to clot.

[2] Recently, Duquesne and Richard (1994) found two or three metal-binding peaks following ion-exchange chromatography of dab liver extracts, but it was not clear whether the peaks were different MT isoforms or breakdown products.

Figure 1. N-terminal sequence of selected vertebrate metallothioneins. Sequences from: Hylland *et al.*, 1994 (cod); Bonham *et al.*, 1987 (rainbow trout MT-A, MT-B); Overnell *et al.*, 1981 (plaice); Kille *et al.*, 1991 (stone loach, pike); Chan *et al*, 1988 (winter flounder); and Winge and Rajagopalan, 1972 (rat).

Residues from N-terminal	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Atlantic cod	M	D	P	-	C	D	C	A	K	T	G	T	C	N	C
Plaice, winter flounder	Ac-M	D	P	-	C	E	C	S	K	T	G	T	C	N	C
Rainbow trout MT-A/ MT-B, pike	Ac-M	D	P	-	C	E	C	S	K	T	G	S	C	N	C
Stone loach	?-M	D	P	-	C	E	C	S	K	T	G	T	C	N	C
Rat MT-2	Ac-M	D	P	N	C	S	C	A	T	D	G	S	C	S	C

A = alanine Ac-M = acetylated methionine C = cysteine D = aspartic acid
E = glutamic acid G = glycine K = lysine M = methionine
N = asparagine P = proline S = serine T = threonine

The affinity of the serum for antigen at stages in the process may conveniently be evaluated using dot-blots as follows. Dilutions of purified MT are blotted onto PVDF-membranes (0.45 µm pore size) with a syringe; the membranes are then rinsed in tris-buffered saline (TBS) (pH 8.5) and blocked for 45 min in 3 % gelatine in TBS (pH 8.5). Membranes are then incubated overnight with dilutions of the serum to be tested (TBS, pH 8.5, with 1 % gelatine; 1:500, 1:1000, 1:2000, 1:4000). Following 5 × 5 min rinses in TBS (pH 8.5), the membranes are incubated for 2–6 hrs with secondary antibodies (e.g., horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG) in TBS (pH 8.5)/1 % gelatine. The membranes are rinsed 5 × 5 min in TBS (pH 7.5) and then developed for 5–30 min. A substrate for the relevant conjugated enzyme is used. All rinses and incubations may be done using glass petri dishes and the incubations preferably conducted on a shaking platform.

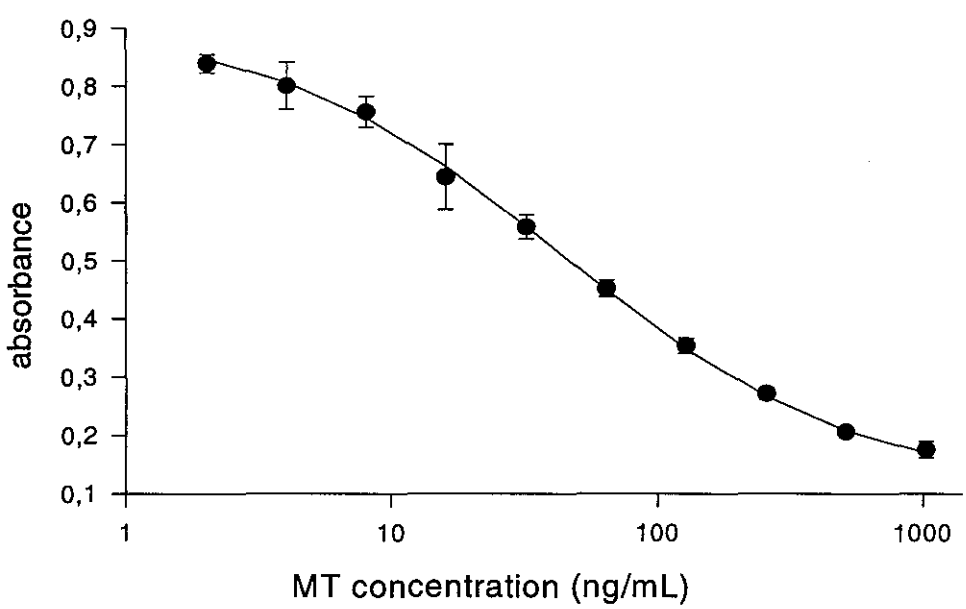
The specificity of the relevant primary antiserum should be evaluated using immunoblots before being used in RIA or ELISA (cf. Hylland *et al.*, 1995).

2.10 Establishing an ELISA

Various procedures exist for ELISAs. The present protocol describes a single-antibody competitive quantitative ELISA (e.g., Garvey *et al.*, 1987; Specker and Anderson, 1994). The optimization procedure consists of two steps: (1) optimization of coating, primary and secondary antibody concentrations, and (2) identification of acceptable working range for MT concentrations. ELISA is performed using 96-well microtiter plates designed for high protein adsorption, e.g., Nunc Immunosorp (Nunc, Denmark). In an initial step, an optimal combination of coating concentration and concentrations of primary and secondary antisera is determined. Buffers, volumes, and washes are described in the following section. Wells A1–H3 (i.e., rows 1–3) are coated with 50 ng MT ml^{-1}, A4–H6 with 100 ng MT ml^{-1}, A7–H9 with 200 ng MT ml^{-1}, and A10–H12 with 400 ng MT ml^{-1}. Following overnight incubation with coating solution, the microtiter plates are washed and dilutions of primary antisera are pipetted into rows A–H (100 µl): row A (A1–A12) 1:500, row B (B1–B12) 1:1000, row C (C1–C12) 1:2000, row D (D1–D12) 1:4000, row E (E1–E12) 1:8000, row F (F1–F12) 1:16 000, row G (G1–G12) 1:32 000, and row H (H1–H12) 1:64 000. The plates are then sealed and incubated overnight at 4 °C. Following washes, dilutions of secondary antiserum may be as follows (100 µl): rows A1–H1, A4–H4, A7–H7, and A10–H10 1:3000; rows A2–H2, A5–H5, A8–H8, and A11–H11 1:9000; rows A3–H3, A6–H6, A9–H9, and A12–H12 1:27 000. The plates are then sealed and

incubated for 6 hrs at 4 °C. The plates are then washed and colour reagent pipetted into all wells. Following colour development for 20–30 min, the reaction is stopped. An optimal concentration of coating, primary and secondary antisera may then be determined by visual inspection of the plate. The working range for the assay may then be determined. One plate is coated with the determined concentration of MT, including wells to determine blank and non-specific binding (see Section 2.11, below). Following washes, a $\log_2$-series of MT-concentrations is pipetted into the wells (each concentration in quadruplicate, 50 µl each): 0.5, 1, 2, 4, ..., 1024 ng ml^{-1}. Wells are also included to determine blank and non-specific binding (see Section 2.11, below). Appropriately diluted primary antiserum (50 µl, double the determined concentration) is then pipetted into all wells except the wells for blanks and non-specific binding. The plate is then incubated overnight at 4 °C. Following washes, appropriately diluted secondary antiserum is pipetted into all wells except blanks; the microtiter plate is sealed and incubated for 6 hrs at 4 °C. Following washes, colour development and absorbance measurement, the appropriate working range for the assay can be determined (cf. Figure 2 for standard curve for gadoid MT using anti-cod MT antiserum).

Figure 2. Typical standard curve for cod MT in ELISA (n = 4; mean ± standard error).



2.11 Protocol for ELISA

Analysis by competitive ELISA consists of the following steps: coating (or sensitization), incubation of primary antibody with sample or standard, incubation with conjugated secondary antibody, colour development, measurement of absorbance, and calculation of concentration. Of the 96 wells, two wells should be used for blanks (no coating antigen, no primary or secondary antisera), two to quantify non-specific binding (NSB) (no coating antigen, no primary antiserum), and the remainder for samples or standards (Figure 3).

Step (1) coating: 100 µl antigen solution (100 ng MT ml^{-1} in carbonate-bicarbonate buffer, pH 9.6) is pipetted into all wells except A1–B1 (blank) and C1–D1 (non-specific binding), which receive carbonate-bicarbonate buffer only. The plates are then incubated in a moist chamber overnight at 4 °C. Following 3 × 1.5 min rinses in TBS with 0.5 % Tween/20 (TTBS) (pH 8.5), blocking with 0.1 % BSA in TTBS for 30 min at room temperature (350 µl/well) and aspiration of blocking solution, plates may be used for analysis immediately or frozen for future use.[3]

Step (2) first incubation: pipette 100 µl TTBS into blank wells, 50 µl TTBS into NSB wells, and 50 µl standard or sample (diluted in 0.1 % BSA in TTBS) into the remaining wells (cf. Figure 3). Then pipette 50 µl anti-fish MT antiserum (appropriately diluted in 0.1 % BSA in TTBS; 1:4000 for anti-cod MT antiserum) into all wells except blanks. Seal and incubate overnight at 4 °C.

Step (3) second incubation: following 3 × 1.5 min washes in TTBS, pipette 100 µl secondary antibody solution (diluted in 0.1 % BSA in TTBS; 1:10 000 for Sigma HRP-conjugated goat anti-rabbit IgG) into all wells except blanks. Pipette 100 µl TTBS into blank wells. Seal and incubate for 6 hrs at 4 °C.

Step (4) colour development: following 5 × 1.5 min washes in TTBS, pipette 100 µl of substrate solution (0.04 % OPD in 150 mM Na-phosphate, 50 mM Na-citrate buffer, pH 5.7, with 0.012 % H_2O_2) into all wells. The substrate solution should be prepared immediately prior to use. Incubate the plates in the dark at room temperature for 5–30 min. Stop the reaction by adding 50 µl 2 N HCl or H_2SO_4 to all wells.

Step (5) measurement of absorbance: absorbance at 490 nm is measured in all wells.

Step (6) calculation of concentration: a computer with the appropriate software to calculate standard curves and sample concentrations is normally used in association with most plate readers, but it can also be done manually. Various models are available to fit a line to the standards. The author has found a four-parameter model useful (Equation 1), but other alternatives are, e.g., log-log (Equation 2), semi-log (Equation 3), and linear (Equation 4):

$$y = (A - D) / (1 + (x/C)\text{^}B) + D \quad (1)$$

$$\log(y) = A + B \times \log(x) \quad (2)$$

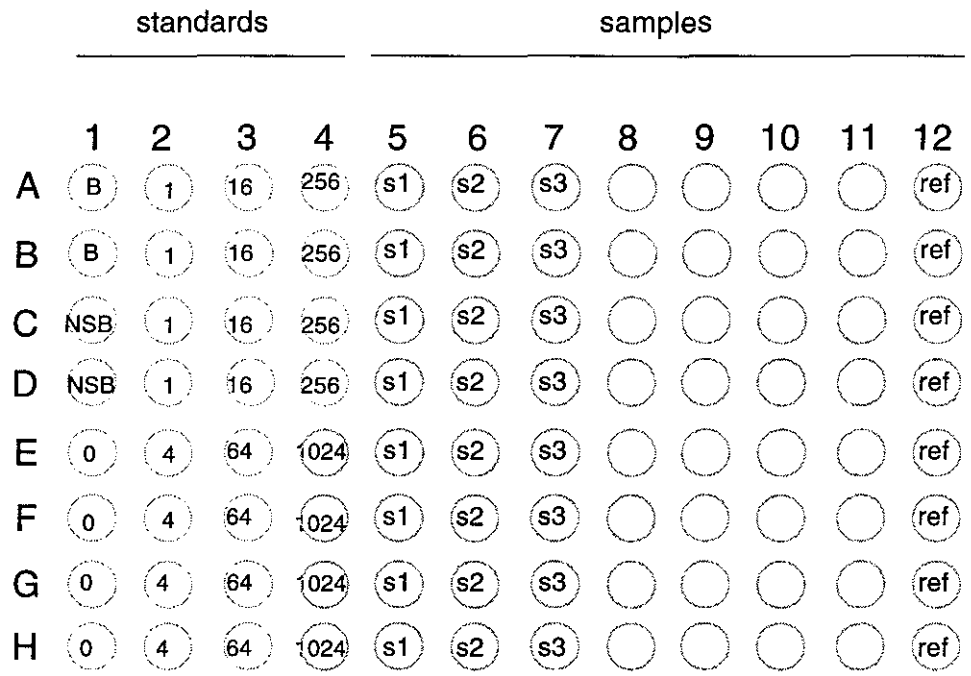
$$y = A + B \times \log(x) \quad (3)$$

$$y = A + Bx \quad (4)$$

A competitive assay will normally produce a sigmoidal curve if concentrations are taken to the low and high ends (cf. Figure 2). It is important to remember that the uncertainty in each determination increases at each end of the curve (as it flattens). Samples should be diluted so that the concentrations are within the linear part of the curve.

[3] Plates can be stored for at least two months at –80 °C, but probably longer and at higher temperatures (–20 °C).

Figure 3. A 96-well microtiter plate showing the layout of blanks, standards, and samples. B = blank; NSB = non-specific binding; 0, 1, 2, 4, 8, etc. = MT standards (ng ml^{-1}); s1 = sample 1; s2 = sample 2; etc.; ref = internal laboratory reference material.



2.12 Additional Analyses

Induction of MT signals intracellular accumulation of metals. The protein may be used for two purposes in monitoring. Metallothionein induction may be used on its own (or as part of a suite of cellular markers) to indicate a potential pollution problem, to be followed by chemical analyses. In an area with a known problem of metal contamination, both MT and metal (at least Cu, Zn, and Cd) analyses should be performed simultaneously (see Section 4 for discussion on interpretation). Depending on the local problem, the analysis of other metals, e.g., Hg, Ag, may be required.

2.13 Expression of Results

Results should ideally be expressed in nmol MT per mg S9 protein, since the use of µg will introduce an added component of uncertainty in that the exact molecular weight of the protein must be estimated (which will vary according to the relative amount of each metal associated with the protein).

2.14 Quality Control

At present, there is no commercially available standard reference material for MT, let alone fish MT. Suitable reference materials (both homogenate and S9 from relevant fish species) should be prepared by each laboratory undertaking MT analyses. This material should be aliquotted and stored at –80 °C for use with each batch of analyses.

3 SOURCES OF ERROR

3.1 Sample Treatment

Although MT in fish liver extracts appears to be stable and not easily degraded, it is recommended that all procedures be done on ice or at 4 °C. Protease inhibitors do not appear to be required to recover MT from fish livers. Metallothionein, including fish MT, is however highly sensitive to oxidation and freezing-thawing cycles. All solutions used for chromatography or ultrafiltration should be degassed and frozen samples or S9 should not be thawed without being processed or analysed.

3.2 Analytical Procedures

The correctness of a given MT standard is obviously crucial to obtaining correct absolute values. As implied above, it is recommended that a standard for the appropriate fish species be used. The quality of this standard should be checked occasionally by an alternative quantification method, absorbance ratios (250:280, 250:220), cysteine/methionine content, gel chromatography or electrophoresis.

4 INTERPRETATION OF RESULTS

A significant increase in hepatic MT is indicative of a change in normal trace metal (Cu, Zn) metabolism. Relationships between hepatic MT concentration and hepatic metal concentrations in each individual fish should be investigated using simple correlation analysis, analysis of covariance or multiple regression (Draper and Smith, 1981; Sokal and Rohlf, 1981). A significant association between hepatic MT and Zn could be indicative of processes not related to metal contamination, but to reproduction or non-metal stressors (cf. Hylland *et al.*, 1992). If the sampled fish or sample sites are heterogeneous with regard to any of the factors indicated in Section 2.1, an appropriate statistical analysis, e.g., analysis of covariance, needs to be performed to identify their influence, if any, on the measured response. In general, hepatic MT in fish does not appear to be strongly influenced by non-metal factors other than gender and, to some extent, nutritional status. If there are no confounding factors, differences between sites should be evaluated using analysis of variance (ANOVA; cf. Underwood, 1981) followed by Dunnett's test (with reference site), Tukey's test or the SNK test (with no reference site) if significant effects are found in the ANOVA (Day and Quinn, 1989).

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ANNEX 1

PROTOCOL FOR POLAROGRAPHIC DETERMINATION OF METALLOTHIONEIN

(Modified from Olafson and Olsson, 1991)

This analytical procedure has been used for both fish (McCarter and Roch, 1983; Hogstrand and Haux, 1992; Cosson, 1994) and invertebrate (Bebianno *et al.*, 1992; Pavicic *et al.*, 1993) environmental samples.

Equipment and Chemicals

The following equipment is needed to perform the assay: homogenization equipment (see main text), a refrigerated centrifuge with a fixed-angle rotor, a heating block, a polarograph (e.g., PARC 174A; Princeton Applied Research Company, Princeton, NJ, USA), an electrode stand with drop dispenser (e.g., PAR model 303A static mercury drop electrode (SMDE)), and an output device (X-Y plotter or computer). Hexamminecobalt chloride may be obtained from BDH, Toronto, Canada. The remaining reagents should all be of analytical grade and are available from most major suppliers of laboratory chemicals.

Sample Preparation

Liver tissue from fish should be homogenized and centrifuged as described in the main text. 100 µl S9 is mixed with 900 µl 0.9 % NaCl solution, heat treated (95 °C for 4 min), and cooled on ice. The heat-treated sample is then centrifuged at 10 000 × **g** for 15 min. The supernatant is used for analysis.

Polarographic Analysis

The quantification of MT by differential pulse polarography may be done using any polarographic system with a dropping mercury electrode. Here, detailed procedures and settings are given for the PARC 174A polarographic analyser with a PAR model 303A SMDE electrode stand and drop timer.

Supporting electrolyte

The optimal composition of the electrolyte depends on the electrode system. For the 303A SMDE, the electrolyte is: 1 M ammonium chloride (NH_4Cl), 1 M ammonium hydroxide (NH_4OH), 1.2 mM hexamminecobalt chloride $[Co(NH_3)_6]Cl_3$. This electrolyte should be freshly made every week and stored in the dark at 4 °C. The electrolyte must be equilibrated to room temperature before analyses are performed (25 °C).

Instrument settings

The following settings are recommended for the PARC 174A:

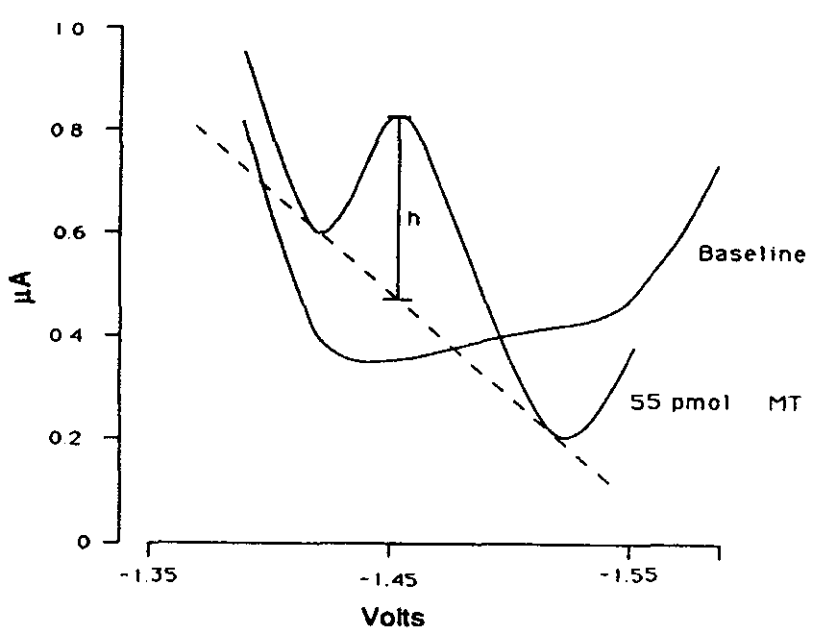
range of scan:	–1.35 V to –1.60 V versus the Ag/AgCl reference electrode;
pulse amplitude:	50 mV;
scan rate:	5 mV sec^{-1};
drop time:	0.5 sec;
display direction:	positive;
output offset:	off;
low-pass filter:	off.

The settings for the PAR model 303A SMDE should be as follows: 'DME' was selected and drop size was set to 'medium'.

Analytical procedure

10 ml electrolyte is dispensed into the polarographic cell and 100 µl 0.0125 % Triton X-100 is added. This solution is bubbled with nitrogen for 2–4 min. Thereafter, the sample is introduced (50–200 µl) and the reaction mixture again purged with nitrogen for 15–30 sec. The reading is then initiated. The peak height, as indicated in Figure A1.1, is proportional to the amount of MT in the sample. The concentration of MT is calculated from a standard curve using appropriate standards. At high concentrations of MT, the curve will flatten due to adsorption of sample on the mercury drop.

Figure A1.1. Result of two typical scans: one baseline (without MT) and one with 55 pmol rainbow trout MT. The concentration of MT in the sample is proportional to the peak height, defined as 'h' (Olsson, 1987).



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ANNEX 2

PROTOCOL FOR DETERMINATION OF METALLOTHIONEIN USING SPECTROPHOTOMETRIC ASSAY

(Adapted from Viarengo *et al.*, 1997)

The method described below was established for molluscs, but has also been evaluated for fish tissues (Viarengo, unpublished).

Materials

The following equipment is needed to perform the assay: homogenization equipment (see main text), a refrigerated centrifuge with fixed-angle and swing-out rotors, and a spectrophotometer (or plate reader). Calf liver RNA is available from Sigma, St. Louis, MO, USA. The remaining reagents should all be of analytical grade and are available from most major suppliers of laboratory chemicals.

Sample Preparation

Liver tissue from fish should be homogenized and centrifuged as described in the main text. One ml of the resulting S9 is transferred to a reaction tube, and 1.05 ml cold (–20 °C) ethanol and 80 µl chloroform are added (Kimura *et al.*, 1979). The mixture is then centrifuged at 6000 × **g** for 10 min (at 4 °C). The supernatant is collected, mixed with 1 mg RNA and 40 µl 37 % HCl, and thereafter with cold ethanol to a final concentration of 87 %. The reaction mixture is kept at –20 °C for 1 hr, then centrifuged in a swing-out rotor at 6000 × **g** for 10 min. The MT-containing pellet is washed in 87 % ethanol/1 % chloroform in homogenizing buffer, centrifuged at 6000 × **g** for 10 min, the supernatant removed and the MT-containing pellet dried under a flow of nitrogen.

Spectrophotometric assay

The assay uses the reaction of DTNB (5,5′-dithio-bis[2-nitrobenzoic acid]) with sulfhydryl groups described by Ellman (1958, 1959). The MT-containing pellet is resuspended in 150 µl 250 mM NaCl. Subsequently, 150 µl 1 N HCl with 4 mM EDTA is added. A volume of 4.2 ml of 2 M NaCl, pH adjusted to 8.0 by 0.2 M Na-phosphate, with 0.43 mM DTNB, is then added to the MT-containing solution at room temperature. Following mixing and centrifugation at 3000 × **g** for 5 min, the absorbance of the supernatant is read at 412 nm. The concentration of MT is calculated from a standard curve using appropriate standards.

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