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Review on Methods for Determination of Metallothioneins in Aquatic Organisms

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Abstract One aspect of environmental degradation in coastal areas is pollution from toxic metals, which are persistent and are bioaccumulated by marine organisms, with serious public health implications. A conventional monitoring system of environmental metal pollution includes measuring the level of selected metals in the whole organism or in respective organs. However, measuring only the metal content in particular organs does not give information about its effect at the subcellular level. Therefore, the evaluation of biochemical biomarker metallothionein may be useful in assessing metal exposure and the prediction of potential detrimental effects induced by metal contamination. There are some methods for the determination of metallothioneins including spectrophotometric method, electrochemical methods, chromatography, saturation-based methods, immunological methods, electrophoresis, and RT-PCR. In this paper, different methods are discussed briefly and the comparison between them will be presented.

Keywords Toxic metal · Pollution · Aquatic organisms · Biomarker · Metallothionein

Introduction

Metallothioneins (MTs), first described by Margoshes and Vallee [1], are small (6–7 kDa) cytosolic proteins involved in metal homeostasis and detoxification processes that contain about 60 amino acids (none of which aromatic) and a high content of cysteine residues. MTs have two globular subunits, each comprising about ten cysteine residues that do not form disulfide bonds and are responsible for sequestering metals with their sulfhydryl (thiolic) groups.

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Four isoforms of metallothioneins (MT1, MT2, MT3, and MT4) have been known until now according to the Expert Protein Analysis System Proteomics Server. Their molecular weight varies from 6.0 to 6.9 kDa, the number of amino acids is about 61, and their pI is about 8.3. Only MT3 group [2, 3] differs from the others because it contains 68 amino acids and its pI is about 4.8. MT occurs mainly in the cytosol and is also present in the nucleus and lysosomes [4].

Although the members of the MT family were discovered nearly 40 years ago, their functional significance remains obscure. MTs are involved in many pathophysiological processes such as metal ion homeostasis and its detoxification, scavenging of reactive oxygen species, cell proliferation and apoptosis, chemo-resistance, and radiotherapy resistance [5–8]. Considering the heavy metal detoxification significance of MTs, these proteins can serve as biomarkers of heavy metal pollution of the environment [9–12]. On the other hand, a comparison between the content of heavy metals and determined MTs could be very useful not only from toxicological aspects but also from biochemical aspects due to the better understanding of different functions of MTs in an organism [13].

These proteins are very conservative even between distinct animal groups and have been widely used as biomarkers of metal exposure in aquatic organisms since there is induction of MT synthesis resulting from exposure to metallic pollutants [14]. MTs constitute between 5 and 7 mol of group IIB heavy metals (such as Cd, Cu, Hg, and Zn) per mole of protein. MTs are induced in the cells as the result of elevated metal concentrations, either essential for cell growth and development (such as Zn and Cu) or non-essential (toxic metal ions such as Cd and Hg among others) to the organism. It is now thought that MT plays an important role in the handling and detoxification of this class of metal ions and in regulating the concentration of the essential metal ions in the cells. The metal content of purified MT is highly variable and depends on the organism, tissue, and history of metal exposure [15].

Generally, MT expression increases with the elevation of tissue concentrations of MT-inducing metals, reflecting metal bioavailability in the environment [16, 17]. For example, Pedersen et al. [18] reported a clear induction of MT in the gills of the crab *Carcinus maenas* related to the presence of copper in the field; whereas Schlenk and Brouwer [19] demonstrated that copper induced MT synthesis in the hepatopancreas of the blue crab *Callinectes sapidus* both in the field and in the laboratory. In fact, it has been established that increases in MT concentrations are associated with decreases in the sensitivity of an organism to metals [20]. Some authors have noted that, in a single species, the populations that live in a medium polluted by metals have higher concentrations of MTs [17, 21, 22]. Among molluscs, they were mainly present in filter feeders, such as mussels and oysters, but also in the whole soft tissues of the limpets *Crepidula fornicata*, *Patella aspera*, *Patella granularis*, *Patella intermedia*, as well as in the whole soft tissues, viscera, and foot of *Patella vulgate* [15, 23].

In the limpets *P. vulgate* collected from the field or exposed in the laboratory (500 mg l^{-1} Cd), MT (with a molecular weight of 10,000 Da) was shown to bind apart from Cd, small amounts of Cu, and Zn [24]. MT induction is variable within metals. Cadmium, for instance, is one of the strongest known MT inducers [25, 26]. MTs are present in all tissues but gills and liver are the most usually surveyed organs for MT induction in fish, due to their role in metal uptake and bioaccumulation/detoxification, respectively [27].

These data demonstrate the necessity to use analytical techniques not only for determination of MTs but also for detection of heavy metals in organisms of interest [13].

Different Methods for Determination of Metallothionein

There are different analytical methods for the determination of MTs like electrochemical methods, metal saturation, spectrophotometry, chromatography, immunological methods, electrophoresis, and quantification of MT mRNA. Here, these methods are briefly reviewed.

Electrochemical Methods

Different electrochemical methods are widely used for the determination of MTs in environmental studies. Also, in some studies, MTs were used as ionophores to design metal-sensitive potentiometric biosensors. In these biosensors, MT as a metal ion ionophore was incorporated into a porous polymer (such as polysulfone). As a model, Ag^{+}-ISEs were successfully developed using polysulfone matrix-embedding metallothioneins. The constructed ISEs respectively have a long lifetime. The reason for this is the lack of leaking processes due to the polymeric nature of the ionophore as well as the fact that the use of fully metallated metalloproteins ensures their resistance to air oxidation. These can cause lower detection limits, as a function of the incubation time, but contrarily show poor selectivity and require regeneration processes [28].

Some different electrochemical methods for the determination of MTs are discussed in the following paragraphs.

Differential Pulse Polarography

In one study on the determination of MTs [29] in the intestine of red mullet *(Mullus barbatus*), MTs were determined in heat-treated cytosolic fractions of intestine tissue by differential pulse polarography [30]. MTs were quantified from the calibration straight line which was obtained with MT I + II from rabbit liver as a calibrant [31]. In another study, for modeling MT induction in the liver of fish *Sparus aurata* exposed to metal-contaminated sediments, DPP method was used and the electrode system consisted of a mercury capillary working electrode, an Ag/AgCl reference electrode, and a platinum auxiliary electrode. The supporting electrolyte was (1 M NH_4Cl, 1 M NH_4OH, and 2 mM $[Co(NH_3)_6]Cl_3$) [27].

Differential Pulse Anodic Stripping Voltammetry

Krizkova et al. developed a method based on voltammetry for the determination of MTs in fish tissues [13]. In this method, heat-denatured cytosol was utilized. Electrochemical measurements were performed using a standard cell with three electrodes. The working electrode was a hanging mercury drop electrode with a drop area of 0.4 mm^2. The reference electrode was an Ag/AgCl electrode and the auxiliary electrode was a graphite stick electrode. An adsorptive transfer stripping technique [32] in connection with chronopotentiometric stripping analysis (CPSA) was used for the determination of metallothionein by recording the inverted time derivation of potential $(dE/dt)^{-1}$ as a function of potential (E).

Cathodic Stripping Voltammetry

In the study done by Hourch et al., in 2003, a new procedure for the quantification of MT by square wave cathodic stripping voltammetry has been developed and optimized [33]. The determination was based on the complexation of cisplatin and MT and the subsequent reduction of the complexes at the electrode. In order to achieve the highest sensitivity and

resolution of the peak, an optimization of the experimental parameters has been carried out using experimental design methodology (response surface). The detection limit was 6×10^{-12} mol l^{-1}. This sensitivity is comparable to that of an immunoassay, inducing that this procedure may also be used for MT quantification in tissue or serum. Some recent works on electrochemical detection of metallothionein are summarized in Table 1.

Metal Saturation

This method is based on saturation with metal ions such as silver, cadmium or mercury for binding to MTs in sample and then removal of excess metal with a protein like hemoglobin. Afterwards, this solution is heated and metal MT is separated by centrifuge. Then, the content of metal is determined by AAS or ICP and corresponds to MT concentration.

Table 1 Application of Electrochemical Methods for Determination of Metallothioneins in Aquatic Organisms

Organism	Method	Determined range	Year	Ref.
Oyster (*Crassostrea gigas*)	Polarography	0–2,000 mg/kg dw, 200–1,600 μg/g dw	2003, 2006	[79, 80]
Shrimp (*Penaeus semisulcatus*)	DPP[a]	–	2005	[81]
Shrimp (*Penaeus indicus*)	DPP	0–25 mg/g dw	2006	[82]
Polychaete (*Hediste diversicolor*)	DPP	–	2003	[83]
Sea bass (*Dicentrarchus labrax*)	DPP	0–2,500 μg/g ww	2008	[84]
Crab (*Carcinus maenas*), clam (*Ruditapes philippinarum*)	ASV[b]	–	2008	[85]
Mussel (*Mytilus galloprovincialis*)	DPP	5–25 mg/g, 0.4–1 mg/mL	2004, 2006	[9, 86]
Crustacean (*Neomysis integer*)	DPV[c]	1–2.5 mg/g ww	2008	[87]
Eel (*Anguilla anguilla*)	DPP	0–20 mg/g pr, 300–700 μg/g ww	2007, 2008	[88–90]
Clam (*Scorbicularia plana*)	DPP	20–200 mg/g pr	2008	[78]
Fish megrim (*Lepidohumbos bascii*), pouting (*Trisopterus luscus*)	DPP	1,000–10,000 μg/g dw	2007	[91]
Perch (*Perca fluviatilis*)	CPSA[d]	1.5–50 ng/mL	2007	[92]
Gammarus pulex	DPP	–	2007	[93]
Red mullet (*Mullus barbatus*)	DPP	8–20 mg/g dw, 0.5–3 mg/g ww	2006, 2007	[94, 95]
Mussel (*Bathymodiolus thermophilus*)	DPP	100–900 μg/g ww	2006	[96]
Sponge (*Spongia officinalis*)	DPP	–	2005	[97]
Mussel (*Mytilus edulis*)	DPP	1,000–7,000 mg/kg ww	2005	[98]
Shrimp (*Penaeus merguiensis*, *Penaeus semisulcatus*)	DPP	–	2005	[99]
Clam (*Ruditapes decussates*)	DPP	0–2 mg/g ww	2003, 2004	[100, 101]
Wrasse (*Symphodus melppis*)	DPP	1–10 mg/g pr	2008	[102]
Scabbard fish (*Aphananopus carbo*)	DPP	–	2007	[103]

dw dry weight, *ww* wet weight, *pr* protein

[a] Differential pulse polarography

[b] Anodic stripping voltammetry

[c] Differential pulse voltammetry

[d] Chronopotentiometric stripping voltammetry

Cadmium Saturation

Cd saturation assay can be carried out as follows: high molecular weight Cd-binding compounds are denatured with acetonitrile, Cu bound to MT is removed with ammonium tetrathiomolybdate, excessive tetrathiomolybdate and its Cu complexes are removed with DEAE-Sephacel, apothionein is saturated with Cd, and excessive Cd is bound to Chelex 100. The thiomolybdate assay is capable of reliably detecting 14 ng MT and thus is particularly suitable for measuring MT in small tissue samples (e.g., biopsies), in extrahepatic tissues, and in cultured cells. Moreover, the combination of the thiomolybdate assay with the recently developed Cd–Chelex assay also makes it possible to determine the portion of MT which binds Cu (Cu load of MT), provided that the amount of non-Cu–thionein exceeds 100 ng, the detection limit of the Cd–Chelex assay [34].

In another study, ^{109}Cd and bovine hemoglobin were added to scavenge Cd not bound to MT. The hemoglobin and excess Cd were separated from the ^{109}Cd–MT by heating. The Cd remaining in the supernatant was a quantitative measure of the MT present. Analysis of Sephadex G-75 and DEAE ion exchange fractions of liver supernatants from Cd- and Zn-treated rats indicated that the assay is specific for MT. Because of its speed and sensitivity, this assay may be useful for measuring MT in large studies where time and expense are limiting factors [35].

Silver Saturation

A silver saturation method was well established for measuring MT concentration in bivalves and was used to measure the MT induced by Cd in the mussel tissue [36]. The tissue was then homogenized in Tris–base buffer with mercaptoethanol (antioxidant) and phenylmethylsulfonyl fluoride (protease inhibitor). The homogenate was centrifuged and the soluble fraction was used for the MT assay. Briefly, stable Ag with 20 kBq ml^{-1} ^{110m}Ag in glycine buffer was added to a homogenate soluble fraction to saturate the MT binding sites. Then, excess Ag was removed by adding rabbit blood cell hemolysate followed by heat treatment and centrifugation. The addition of hemolysate and heat treatment was repeated. Then, the supernatant was analyzed for ^{110m}Ag. MT concentration (μg g^{-1} wet wt.) was calculated as 3.55 times the Ag concentration as in the mammalian tissue. MT recovery was at >70%, using standard MT from rabbit liver [37].

Mercury Saturation

Cadmium and zinc bioaccumulation and MT response in two freshwater bivalves (*Corbicula fluminea* and *Dreissena polymorpha*) transplanted along a polymetallic gradient MT concentration were determined using the mercury saturation assay [38]. This technique was modified slightly as the fresh pig blood hemolysate was replaced by purified and lyophilized pig hemoglobin prepared in a Tris–HCl buffer. Table 2 shows some of recent works on the metal saturation.

Spectrophotometry

The application of spectrophotometry in MT determination was introduced for the first time in 1997 [39]. MT concentration was evaluated utilizing a partially purified metalloprotein fraction obtained by acidic ethanol/chloroform fractionation of the tissue homogenate. The procedure takes into account precautions to obtain a complete MT precipitation and to

Table 2 Application of Metal Saturation Methods for Determination of Metallothioneins in Aquatic Organisms

Organism	Method of saturation/ instrument	Determined range	Year	Ref.
Crab (*Eriocheir sinensis*)	Cd-Sat/ICP-AES[a]	0–7 mg MT/g ww	2005	[104]
Eel	Cd-thiomolybdate Sat/gamma counter	1–100 nmol/g ww	2008	[47]
Rainbow trout (*Oncorhynchus mykiss*)	Hg-Sat/gamma counter	0–500 μg MT/g ww	2005	[105]
Freshwater prawn (*Macrobranchium rosenbergii*)	Cd-Sat/ICP-AES	–	2007	[106]
Zebrafish (*Denario rerio*)	Hg-Sat/AAS	–	2006	[107]
Bivalve (*Dreissna polymorpha*)	Hg-Sat/CV-AAS[b]	–	2006	[108]
Clam (*Corbicula fluminea*)	Hg-Sat/AAS, CV-AAS	–	2006, 2005	[108, 109]
Snail (*Lymnea stagnalis*)	Ag-Sat/AAS	250–1,750 μg/g dw	2003	[110]
Polychaete (*Perinereis aibuhitensis*)	Ag-Sat/radioactivity counter	10–150 μg/g ww	2008	[111]
Daphnid (*Daphnia magna*)	Ag-Sat/radioactivity counter	10–100 μg/g ww	2006	[112]
Eel (*Anguilla anguilla*)	Cd-Sat/gamma counter	0–100 nmol/g ww, 0–350 μg/g ww	2008, 2001	[47, 113]
Oligochaete (*Tubifex tubifex*)	Cd-Sat/gamma counter	0–3 nmol/g ww	2007	[114]
Gudgeon (*Gobio gobio*)	Cd-Sat/gamma counter	0–45 nmol/g ww	2007	[71]
Bivalve (*Pyganodon grandis*)	Hg-Sat	0–400 nmol Hg binding sites/g	2006	[115]
Crab (*Charybdis japonica*)	Hg-Sat/AAS	0–120 nmol Hg binding sites/mg	2006	[116]
Gibel carp (*Carassius auratus gibelio*)	Cd-Sat/gamma counter	01–80 nmol/g	2006, 2003	[45, 117]
Brown trout (*Salmo tratta*)	Cd-Sat	–	2006, 2001	[118, 119]
Carp (*Cyprinus carpio*), barbel (*Barbus graellsii*)	Ag-Sat/AAS	200–1,200 μg/g ww	2006	[120]
Black sea bream (*Acanthopagrus schlegali*), grunt (*Terapon jarbua*)	Ag-Sat/radioactivity	0–160 μg/g	2005	[121]
Bivalve (*Pyganodon grandis*)	Hg-Sat	0–700 nmol Hg binding site/g dw	2005, 2004	[122–124]
Shrimp (*Litopenaeus vannamei*)	Ag-Sat	0–1 mg bounded Ag/g ww	2005	[125]
Chinese crab (*Eriocheir sinensis*)	Ag-Sat	0–100 μg/g ww	2005	[126]
Oligochaete (*Tubifex tubifex*)	Hg-Sat	0–10 nmol/g ww	2004	[127]
Bivalve (*Dreissna polymorpha*)	Ag-Sat	0–450 nmol binding sites/g ww	2004	[128]
Rainbow trout (*Oncorhynchus mykiss*)	Cd-Sat	0–350 μg/g ww	2003, 2001	[117, 129]
Common carp (*Cyprinus carpio*)	Cd-Sat	1–100 μg/g ww	2000, 2001, 2003	[117, 130–132]

Table 2 (continued)

Organism	Method of saturation/ instrument	Determined range	Year	Ref.
Zebrafish (*Danio rerio*)	Ag-Sat	LOD, 9 nmol MT/L	2003	[133]
Dogwhelk (*Nucella lapillus*)	Ag-Sat	10–40 μg MT/g ww, 10–2,100 μg MT/g ww	2001, 2002	[16, 134]
Bottlenose dolphin (*Tursiops aduncus*)	Cd-Sat/gamma counter	2–1,500 nmol Cd bound/g	2009	[135]

ww wet weight, *dw* dry weight

[a] Inductively coupled plasma-atomic emission spectrometry

[b] Cold vapor atomic absorption spectroscopy

avoid the oxidation of sulfhydryl groups (SH), the contamination by soluble low molecular weight thiols, and enzymatic protein degradation which can occur during sample preparation. In the extracts, the concentration of MT, denatured by low pH and high ionic strength, was quantified spectrophotometrically utilizing the Ellman's SH reagent.

In conclusion, this spectrophotometric method allows the simple, repeatable, and low-cost detection of minimal concentrations (nmol) of MT in biological samples, and therefore it is suggested as a tool for MT quantification in eco-toxicological investigations and biomonitoring programs [39]. Table 3 presents some spectroscopy methods for the determination of metallothionein.

Chromatographic and Electrophoretic Methods

Classical techniques, such as metal saturation assays, enzyme-linked immunosorbent assays, and pulse polarography, provide information on total MT levels but do not provide information on the induction of different MT isoforms and the differential binding of metals to these and other metal-binding proteins [40, 41]. Various separation methods were described for MT and metallothionein-like proteins (MTLPs) purification. They generally include several combined chromatographic steps, such as gel filtration, ion exchange, and high-performance liquid chromatography (HPLC). A relatively improved sensitivity has been achieved using fluorescence detection following derivatization of MTs [42, 43]. In a derivatization reaction, MTs are labeled with a fluorogenic reagent such as monobromobimane (mBBr) by nucleophilic substitution of the MT thiolate [44].

Today, most of the works have been led to obtain reliable separations of the different MT/MTLP isoforms and subisoforms, and investigations have been focused to test and optimize different hyphenated chromatographic and electrophoretic techniques with sensitive atomic and mass spectrometric detectors. The coupling of high-resolution techniques, such as HPLC coupled on-line to inductively coupled plasma mass spectrometry (ICP-MS), provides a powerful tool for this purpose [45, 46]. The major virtues of these coupled HPLC and ICP-MS techniques are selectivity, sensitivity, and multi-element capability [47]. Size exclusion chromatography (SEC)–ICP-MS is useful for a first fractionation of the sample before subsequent separation steps. SEC has been used in conjunction with ICP-MS with magnetic sector and quadrupole mass analyzer. Compared to quadrupoles, sector instruments provide an extremely low instrumental background, improved sensitivity in low resolution for isotopes that are not prone to spectral

Table 3 Application of Spectroscopy Methods for Determination of Metallothioneins in Aquatic Organisms

Organism	Method	Determined range	Year	Ref.
Sea bream (*Sparus aurata*)	Spectrophotometry	50–450 μg/g ww	2007	[136]
Fish (*Seriola dumerilli*)	Spectrophotometry	200–1,800 ng/g ww	2006	[137]
Fish (*Oreochromis niloticus*)	Spectrophotometry	50–250 μg/g	2008	[138]
Mussel (*Mytilus galloprovincialis*)	Spectrophotometry	10–180 μg/g ww	2006, 2008	[139, 140]
Fish (*Geophagus brasiliensis*)	Spectrophotometry	10–60 μg/g	2008	[141]
Carp (*Cyprinus carpio*)	Spectrophotometry	5–500 μg/g	2008	[142]
Sea bass (*Dicentrarchus labrax*)	Spectrophotometry	10–60 μg/g pr	2008	[137]
Polychaete (*Perinereis muntia*)	Spectrophotometry	20–180 μg/g	2008	[143]
Mussel (*Mytilus galloprovincialis*)	Spectrophotometry	50–300 μg/g, 40–160 μg/g	2006, 2007	[11, 144–147]
Blue mussel (*Mytilus edulis*)	Spectrophotometry	20–140 μg/g ww, 200–600 μg/g ww, 10–40 μg/g ww	2004, 2007	[148–150]
Antarctic clam (*Laternula elliptica*)	Spectrophotometry	–	2007	[151]
Isopod (*Asellus aquaticus*), Zebra mussel (*Dreissna polymorpha*)	Spectrophotometry	–	2006	[152]
Eelpout (*Zoarces viviparus*)	Spectrophotometry	50–300 μg/g ww	2006	[153]
Perch (*Perca fluviatilis*)	Spectrophotometry	–	2006	[153]
Bivalve (*Macoma balthica*)	Spectrophotometry	200–600 μg/g	2005	[149]
Polychaete (*Laeonereis acuta*)	Spectrophotometry	0.01–2 μmol; GSH/g ww	2004	[154]
Carb (*Carcinus maenas*), Limpet (*Patella vulgate*)	Spectrophotometry	10–40 μg/g ww	2004	[150]
Tood fish (*Halobatrachus didactylus*)	Spectrophotometry	5–70 nmol/g ww	2003	[155]
Mangrove oyster (*Crassostrea rhizophorae*)	Spectrophotometry	50–400 pmol GSH/g ww	2006	[156]
Bivalve (*Laternula elliptica*)	Spectrophotometry	–	2007	[157]

interference, and very good detection limits (0.1 to 1 ppt) for nearly all elements [48]. Also, speciation of some metals (Cd, Cu, and Zn) in different liver MT isoforms was carried out by means of hyphenated techniques, in particular size exclusion-fast protein liquid chromatography (SE-FPLC) followed by anion exchange (AE)-FPLC, both coupled with the ICP-(Q)MS detection [49]

The chemical composition and structure of the MT isoforms in most fish are not exactly known. Therefore, internal calibration carried out either by the standard addition method or mass spectrometric isotope dilution analysis (IDMS) with species-specific spiking mode cannot be applied for direct metal quantification of these species. On-line ID with species-unspecific spiking, in combination with capillary electrophoresis (CE) or reverse-phase (RP)-HPLC to ICP-MS has proved to be a reliable method for quantitative metal speciation of MT isoforms [50, 51]. Partial purification of the MT cytosolic fraction using SEC was found to be necessary prior to analysis. The simultaneous ion extraction and fast full-spectra coverage capabilities of inductively coupled plasma time-of-flight mass spectrometry (ICP-TOF-MS) make it especially attractive for multi-isotope analysis of transient signals and for transient isotope ratios or isotope dilution measurements. Based on the

advantages of ICP-TOF-MS, the combination of species-unspecific isotope dilution methods on-line with liquid chromatography (LC)–ICP-TOF-MS has been exploited for the quantitative determination of Cu, Zn, and Cd associated with the MT fraction in fish cytosolic extracts [52]. Recently, fast protein LC (FPLC) in combination with IDICP-QMS was applied to the metal fractionation of MT isoforms in hepatic cytosols of eels [45, 53].

Nevertheless, the lack of standards for the different metal-binding proteins present makes on-line quantification of metal species a difficult task. The development of an on-line isotope dilution method in combination with the coupling of SE-HPLC to an ICP-TOF-MS makes the on-line multi-element quantitative speciation of metal-binding proteins possible [45]. This method has proven to be a powerful technique to perform metal binding and speciation studies of complex mixtures. The cytosolic metal partitioning was determined by SE and AE-HPLC directly coupled to an ICP-TOF-MS [41, 45, 47].

In some studies, protein identification has been accomplished by means of two-dimensional polyacrylamide gel electrophoresis separations followed by MS analysis [54]. However, due to the intrinsic limitations of gel-based separation methods, a considerable effort has been focused on the development of integrated capillary separation technology through the combination of various separation mechanisms with inorganic and organic mass spectrometric techniques. These separation techniques provide the opportunity to analyze samples of small volumes with high throughput and sensitivity, good dynamic range, and minimal sample handling [55]. Two of popular capillary separation techniques used in proteomics are capillary liquid chromatography (cLC) and CE. These two separation methodologies have been coupled to elemental detection methods such as ICP-MS [55, 56].

cLC and CE have been used for the separation of metalloproteins when using collision cell inductively coupled plasma mass spectrometry (ICP-CC-MS) as a detection system. However, the most important limitations in connection with the coupling of capillary separation techniques to ICP-MS derive from the requirement of using suitable interfaces to maximize analyte transport to the ion source and to minimize post-column peak broadening effects. Up to now, CE and cLC both on-line coupled to ICP-CC-MS have been used for the analysis of certain metalloproteins containing Cd, Cu, and Zn (metallothioneins). A limiting factor in connecting CE techniques to ICP-MS is the use of suitable interfaces to maximize analyte transport to the ion source and to minimize post-column broadening effects that, in this case, some authors have studied different interfaces and compared their efficiencies with like Lobinski and Todoli [56–58].

The results of a study done to compare cLC and CE coupled with ICP-MS for MT isoforms measurement that can be observed in Fig. 1 show that several species can be separated by cLC between 20 and 45 min, while in the case of using CE these fractions elute in a main peak at a migration time of 9.5 min [56]. Several advantages of connection with separation efficiency for high molecular mass species are the small sample volume required (typically 1–30 nl), the analysis time, and reagent consumption. In addition, the disturbance produced by CE–ICP-MS on the existing metal equilibrium is minimal.

Also, according to Alvarez et al., the limited sensitivity of CE in biological samples because of low levels of metals can be tackled by using a large volume sample stacking methodology for CE–ICP-MS measurements that was utilized in fish samples [59].

The use of new metallomics methods in the characterization of MT like SE-LC-ICP-TOF-MS made possible absolute detection limits ranging from 19.9 pg (114 Cu) to 157 pg (66 Zn) in cytosols from carp liver and kidney [60].

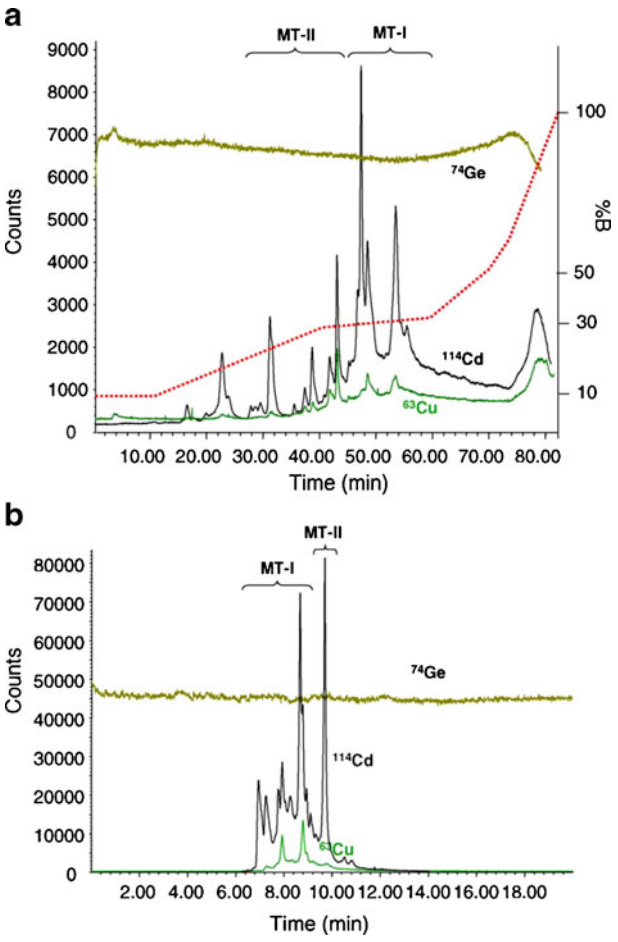

Fig. 1 Separation of MT isoforms containing Cd and Cu (MT-I and -II mixture from rabbit liver) by **a** cLC and **b** CE using ICP-MS detection [56]

Mounicou and co-workers suggested the parallel identification of metallothionein by CE–ICP-MS to detect MT–Cd, Cu, and Zn complexes and CE–ES-MS to identify them [61].

Besides liquid chromatography, capillary zone electrophoresis (CZE) is able to separate different MT isoforms with high resolution. The unfavorable adsorption of proteins on the inner wall of uncoated capillary tubes can be interrupted by modification of the wall by coating with neutral polymers like linear polyacrylamide or neutralizing of charges on the wall with ionic additives like polyamines [62]. CZE-ICP-MS system with post-column isotope dilution was applied for the quantification of MT isoforms. Stable isotopes of ^{34}S, ^{65}Cu, ^{68}Zn, and ^{116}Cd were continuously added via the make-up liquid and mixed with the CZE effluent in the the CZE-ICP-MS interface. By using an ICP sector-field MS instrument in medium mass resolution, mode sulfur detection was also possible, enabling the quantification of the protein via the number of the cysteine and methionine residues in the amino acid sequence. Cd, Cu, and Zn were simultaneously quantified, allowing a determination of the metal–MT complex stoichiometry by the metal-to-sulfur ratios [51, 63, 64].

Besides the above methods, affinity chromatography through metal-chelating columns was utilized for the purification of MTs. The purification of MTs that is done through metal-–cysteine affinity chromatography by utilizing a metal-chelating resin is a simple and rapid

method and, as it is a one-step procedure, the loss of protein mass is greatly reduced during the purification procedure [65]. There are some useful reviews about the application of CE–HPLC coupled to advanced mass spectrometric systems in the determination of biomolecules and their binding to metal species [62, 66–68].

Table 4 summarizes some development on the determination of metallothionein by chromatographic and electrophoretic methods.

MT mRNA

During the last years, the analysis of MT mRNA has been performed basically by means of polymerase chain reaction (PCR). This technique has substituted techniques such as Northern blotting. The latter involves the direct manipulation of mRNA, which is a highly unstable molecule, and causes experimental problems that, in many cases, considerably increase the difficulty of this method. When applying the PCR technique, mRNA, also used as a starting point, is immediately converted into cDNA in a first step, avoiding all problems regarding the continuous use of the mRNA during the entire process. A number of protocols have been developed for the analysis of MT mRNA within different samples by reverse transcription PCR (RT-PCR). Different conditions and isoform-specific MT mRNA primers are employed, including different approaches for the quantification of the PCR products [31].

In addition to the semi-quantitative methods, direct quantification was achieved. Quantification was performed by two different methods: (1) by scintillation counting of the radioactivity of the bands corresponding to target and mimic products labeled to mC of [a-P]dCTP or (2) densitometric analysis of signal intensities of ethidium bromide-stained PCR products in the agarose gel, visualized under UV trans-illumination. Both methods yielded similar results. The detection limits found for these protocols were in the range of 5–10 amol/mg total RNA [31]. Table 5 shows some of the recent works on the PCR methods for the determination of metallothionein.

Immunological Methods

With regard to the immunological reaction of metallothionein with an antibody, techniques like enzyme-linked immunosorbent assay (ELISA) and immunohistochemistry were developed. ELISA assay has been further improved for the determination of MT in plasma and urine. Overestimation of MT by immunochemical methods is expected due to polymerization provoked by oxidation. In order to avoid this effect, mercaptoethanol is added to the samples prior to analysis. The sensitivity reached at 50% of maximal response was 100 ng MT/100 ml and the minimum detectable amount was 10 ng/100 ml sample One advantage of this method is that the total time per assay, 24 h, is shorter than that required by other ELISA and RIA protocols, i.e., 48 or 72 h, respectively. The use of a commercial monoclonal antibody provided a detection limit for MT of 0.8 ng/100 ml, which is lower than previously reported values using the same technique [31].

In general, immunological methods are very sensitive and capable of measuring MT in biological fluids, such as urine and serum, which normally contain low levels of MT. However, the low immunogenicity of MT and the risk of polymerization together with the loss of bound metals could lead to lower detection efficiency. In addition, immunoassays need specific antibodies that do not cross-react [31].

Some of recent works on the electrophoresis and immunological methods of metallothionein are summarized in Table 6.

Table 4 Application of Chromatographic and Electrophoretic Methods for Determination of Metallothioneins in Aquatic Organisms

Organism	Method	Analyte	Year	Ref.
Isopod (*Porcellio scaber*)	Gel chromatography-SEC[a]-HPLC	–	2005	[68]
Fish (*Oreochromis niloticus*)	Gel chromatography-FAA	Metal species (μg metal/mg)	2005	[158]
Cod (*Gadus morhua*)	SE-IE HPLC[b]	Amino acids, metals	1994	[159]
Sea bass (*Dicentrarchus labrax*)	RP-HPLC-FL[c]	MT (ng/mg Pr)	2008	[77]
Clam (*Scorbicularia plana*)	RP-HPLC-FL (after labeling with mBBr)	Total MT (mg/g Pr)	2008	[78]
Clam (*Chamaelea gallina*)	RP-HPLC-FL	MT (mg/g Pr)	2006	[160]
Periwinkle (*Littorina brevicula*)	IE[d]-RP-HPLC-FL	Metal speciation of MT (μmole/g) amino acids	2003	[161]
Eel	On-line ID-SE-HPLC-ICP-TOFMS[e]	Speciation of metal binding MTs	2008	[47]
Mussel (*Mytilus galloprovincialis*)	AE[f] -HPLC-UV (at 254 nm) after PLE[g] AE-HPLC-ICP-OES	Isoforms MLP1 and MLP2 Speciation of metal binding to MTLPs	2007	[162]
Oligochaeta(*Tubifex tubifex*)	Off-line gel chromatography-UV (210 nm)	Total MT (Linearity, 25–75 μg ml^{-1})	2007	[163]
Fish (*Oreochromis niloticus*)	Gel chromatography-UV (254, 280 nm)	MTLPs and sulfhydryl groups	2007	[165]
Oligochaeta(*Tubifex tubifex*)	Gel chromatography-UV (210 nm)	Total MT (Linearity, 25–75 μg ml^{-1})	2006	[164]
fish (*Colossoma macropomum*)	Affinity chromatography (with Ni^{2+} or Cu^{2+} loaded column)	Metal species (μg ml^{-1})	2005	[60]
Oligochaeta(*Tubifex tubifex*)	Gel chromatography-DAD (254 nm)	Total MT	2005	[166]
seals (*phoca vitulina*)	cLC[h] - ICP-MS , CE-ICP-MS	Speciation of Cd, Cu and Zn bounded to MT I , II (LOD, in the range of 0.4–2 ng ml^{-1})	2006	[56]

gibel carp (*Carassius auratus gibelio*)	AE-HPLC-ICP-isotope dilution TOFMS	Speciation of Cd and Zn (at μg g^{-1} wet tissue)	2006	[45]
white sea catfish, *Netuma barba*, pearl cichlid, *Geophagus brasiliensis*	SEC-ICP-MS , AE-FPLC[i] SE-FPLC-ICP-MS	Speciation of Cd, Cu, Zn and MTs	2006	[49]
Blue mussel (*Mytilus edulis*)	Electrophoresis	MT(μg g^{-1})	2008	[167]
Fish (*Atherinops affinis*)	Electrophoresis	MTLPs (DL, 0.12 μg L^{-1})	2006	[168]
Fish (*Prochilodus scrofa*)	Electrophoresis	Total MT (μg g^{-1})	2004	[169]
Elbe-bream (*Abramis brama*)	CE-ICP-MS [j]	Speciation of metal binding to MTLPs and MT isoforms	2003	[170]
Bivalve (*Mercenaria mercenaria*)	CE-UV	MT isoforms	2002	[171]

[a] Size exclusion chromatography

[b] Size exclusion–ion exchange high performance liquid chromatography

[c] Reverse-phase high-performance liquid chromatography–florescence

[d] Ion exchange

[e] On-line isotope dilution–size exclusion–high-performance liquid chromatography–inductively coupled plasma–time of flight mass spectrometry

[f] Anion exchange

[g] Pressurized liquid extraction

[h] Capillary liquid chromatography

[i] Fast protein liquid chromatography

[j] Capillary electrophoresis–inductively coupled plasma–mass spectrometry

Table 5 Application of PCR Methods for Determination of Metallothioneins in Aquatic Organisms

Organism	Method	Year	Ref.
Lake trout (*Salvelinus namaycush*)	RT-PCR[a]	2007	[170]
Japenese medaka (*Oryzias javanicus*)	RT-PCR	2006	[172]
Goldfish (*Carassius auratus*)	RT-PCR	2004	[173]
Zebra fish (*Denario rerio*)	RT-PCR	2008	[174]
Silver sea bream (*Sparus sarba*)	RT-PCR	2008	[175]
Scallop (*Agropectin irradians*)	RT-PCR	2008	[176]
Bivalve (*Dreissna polymorpha*), clam (*Corbicula fluminea*)	RT-PCR	2006	[108]
Antarctic notothenioides (*Chionodraco hamatus*)	RT-PCR	2006	[177]
Zebrafish (*Denario rerio*)	RT-PCR	2004, 2006, 2008	[178–180]
Common carp (*Cyprinus carpio*)	RT-PCR	2008	[181]
Bivalve (*Corbicula fluminea*)	RT-PCR	2008	[182]
Mandarin fish (*Siniperca chuatsi*)	Tail-PCR[b]	2008	[183]
Mussel (*Mytilus galloprovincialis*)	RT-PCR	2008	[184]
Tilapia (*Oreochromis mossambicus*)	RT-PCR	2008	[185]
Nematode (*Caenorhabditis elegans*)	RT-PCR	2004	[186]
Ciliate (*Tetrahymena pigmentosa*)	RT-PCR	2008	[187]
Sea bream (*Sparus aurata*)	RT-PCR	2008	[188]
Pufferfish (*Takifugu obscunus*)	RT-PCR	2008	[189]
Olive flounder (*Paralichthys olivaceus*)	QPCR[c]	2008	[190]
Antarctic clam (*Laternula elliptica*)	RT-PCR	2007	[191]
Rainbow trout (*Oncorhynchus mykiss*)	QPCR, RT-PCR	2004, 2007,2006,2008	[192–195]
Silver barb (*Puntius gonionotus*)	RT-PCR	2007	[196]
Crucian carp (*Carassius cuvieri*)	RT-PCR	2007	[197]
Black goby (*Gobius niger*)	RT-PCR	2005	[198]
Gudgeon (*Gobio gobio*)	RT-PCR	2005	[199]
Fish (*Lithognathus mormyrus*)	RT-PCR	2004	[200]
Brown Trout (*Salmo trutta*)	RT-PCR	2006	[201]
Common sole (*Solea solea*)	EPIC-PCR[d]	2008	[202]
Tilapia (*T. aurea* T. nilotica*)	RT-PCR, mimic PCR	2004	[203]

[a] Real time-polymerase chain reaction

[b] Thermal asymmetric interlaced-polymerase chain reaction

[c] Quantitative real time-polymerase chain reaction

[d] Exon-primed intron crossing-polymerase chain reactions

Discussion

There are several reviews for MT quantification and hyphenated techniques. Therefore, the present review gives only an outline about the general methods of MT quantification in aquatic life.

Table 6 Application of Immunoassay Methods for Determination of Metallothioneins in Aquatic Organisms

Organism	Method	Determined range	Year	Ref.
Rainbow trout (*Oncorhynchus mykiss*)	Radio-immunoassay	1–100 μg/g	2001, 2003	[204, 205]
Mandarine fish (*Siniperca chuatsi*)	Immunohistochemical	–	2009	[183]
Tilapia (*Oreochromis mossambicus*)	ELISA	100–1,600 ng/mg Pr	2006, 2008	[185, 206]
Tilapia (*Oreochromis mossambicus*), milkfish (*Chanos chanos*), fish (*A. Paaradoxus*)	ELISA	–	2006	[207]
Stiped sea bream (*Lithognathus mormyrus*)	cELISA[a]	MT-Pr (nM/nM actin)	2008	[208]
Tilapia (*Oreochromis* sp.)	ELISA	1–6 μg/mg Pr	2007	[209]
Mussel (*Mytilus galloprovincialis*)	ELISA	–	2006	[210]
Lobster (*Homarus americanus*)	ELISA	0.01–0.2 μg/mg	2003	[211]
Pacific oyster (*Crassostrea gigas*)	ELISA	0.01–0.5 mg/g	2009	[212]
Bloch (*Channa punctatus*)	Western blot	–	2006	[213]
Sea bream (*Sparus sarba*)	Dot-blot	–	2006	[214]
Oyster (*Crassostrea gigas*)	Immunohistochemical	–	2005	[215]

[a] Competitive ELISA

The methods used to quantify the total amount of MT in biological materials are divided into two types. One is based on the measurement of the amount of metal bound to MT. As metals bind proportionally to the amount of MT, metal contents indicate the MT content. The affinity to MT differs among metals as per the following order: (Hg (II)>Ag (I)>Cu (I) > Cd(II)>Zn(II)) [69].

The MT produced in response to various stimuli is composed mostly of Zn, though higher-affinity metals form different metal thioneins. Therefore, the simplest method for the quantification of MT is the Cd-hem method [62]. The Cd-hem method utilizes two properties: that MT is a heat- stable protein and that seven atoms of Cd instead of Zn bind 1 M of MT protein when excess Cd is added to the cytosol fraction. Hg and Ag saturation methods apply the same theory [62]. These indirect methods quantify MTs assuming a total saturation of SH groups by metal ions. However, these methods have been shown to present a risk of over-evaluating the quantities of metals bound to MTs due to the presence of other biological metal-binding ligands [44].

The other method of quantifying the total amount of MT is based on the assay of protein content. The quantity of sulfhydryl residues can be used to measure MT protein content like in the spectrophotometric methods. Also, radioimmunoassay (RIA) or ELISA has been developed for this purpose. However, the use of radioisotopes and subsequent heavy metal waste generation has raised some environmental concerns. If the properties of each isoform are to be examined, a separation technique can be used before the detection. For separation, gel permeation chromatography, anion exchange chromatography, HPLC, and CZE are often used, while UV absorption, fluorescence detection, AAS, MS, and ICP-MS are used for the detection of MT and its isoforms as well as metals [62, 70].

Although the abovementioned analytical procedures have been developed for MT determination, the quantitative real-time RT-PCR method is considered simple and accurate. Significant correlations were found among accumulated metal concentrations and MT

protein and mRNA levels [71]. Also, molecular biological advances using MT gene amplification or duplication have confirmed that the functions of different isoforms are different, some of them being involved in metal homeostasis and others in non-essential metal detoxification [22].

It has been extensively used in biomarker studies with a high degree of accuracy. Therefore, taking advantage of this technology application of multi-biomarker approach where MT is also a part of the suite holds promise in biomonitoring and risk assessment [72].

Based on a study about the application of two SH-based methods (spectrophotometry and DPP) for MT determination in mussels, polarographic and spectrophotometric methods gave significantly ($p<0.05$) different results in laboratory and field studies. In the laboratory experiment, MT values detected with DPP were nine times higher than with spectrophotometry. The results obtained by the two methods were significantly correlated. Both methods could discriminate between control and exposed mussels. In field studies, MT values obtained by DPP were 34- to 38-fold higher than with spectrophotometry, and the MT concentrations measured by both methods were not correlated. This discrepancy could be due to several factors, including the low levels of bioavailable metals in the studied areas and the possibility that the different methods can measure MT isoforms differentially [12]. Also, another study showed a good correlation between results obtained, for instance, using DPP and a metal saturation assay [70] and between DPP and spectrophotometric determination [73].

In a research, the comparison of five current methods for measuring MT was done and methods of MT estimation in biological materials (Hg/TCA, Cd/heme, SH assay, G 75 method, and RIA) were compared. Uniform calibration was ensured by measuring the nitrogen content of the standard apoprotein after "Kjeldahl digestion". Known amounts of rabbit standard Cd–MT I were recovered from identical rat S9 samples. In an overall rating, RIA and Cd/heme assay performed best, whereas the other methods either underestimated (SH, G 75) or overestimated (Hg/TCA) the 100% expectation [74].

In another study, the Cd saturation method for MT analysis in tissues was compared with a recently developed RIA. The Cd saturation assay was fast but less specific and accurate than the RIA. The former assay, previously used for MT analysis in urine and plasma, was modified to suit its use in measuring MT concentration in tissues. Liver and kidney from control were assayed and values of 7 and 67 μg g^{-1} were measured in the two tissues, respectively. The detection limit of the assay is 10 ng g^{-1}. A modified version of the Cd saturation assay gave results which were similar to the ones obtained by the RIA [75]. A comparison of the Hg radioassay (i.e., "Piotrowski" assay), the Cd radioassay (i.e., "cadmium–hemolysate" assay), and the RIA for MT in Cd- and Zn-pretreated rats indicated that the Cd and Hg radioassays have similar results in all cases. The RIA values compared very favorably to those obtained by the metal saturation assays for MT from control. The values obtained for Cd- or Zn-treated animals were generally lower in the RIA than in the Hg or Cd radioassay, ranging from 36% to 75% of the metal saturation assays. The various assay systems employed did yield MT concentrations that followed similar intra-assay patterns, being lowest in control, elevated to an intermediate level with Cd treatment, and highest following Zn treatment [76].

In a study to compare the sensitivity of HPLC-FL assay to that of spectrophotometric method, sea bass fish (*Dicentrarchus labrax*) were exposed to Cu, Cd, and Hg. Compared to the spectrophotometric assay, the RP-HPLC-florescence (FL) method detected a significantly higher MT content in all metal-exposed animals. The discrepancy between both methods could be attributed to the underestimation of the MT content due to the use of

GSH as standard instead of MTS and/or to partial co-precipitation of MT with hydrophobic proteins during the solvent extraction required before the spectrophotometric assay. In conclusion, quantification of total MTs by RP-HPLC-FL in unheated fish extracts allows the evaluation of metal effects with higher sensitivity and specificity than the spectrophotometric assay [77].

In another study, MT was determined in clam (*Scorbicularia plana*) by two methods, namely, RP-HPLC-FL assay in unheated samples and DPP. The results show that, in contrast to DPP and other methods based on thiol group detection that rely on heating pre-purification steps, in this method MTs are separated from other cys-containing proteins by HPLC, obtaining a unique intense fluorescent band after chromatography of non-heated clam extracts. In conclusion, quantification of MTs by RP-HPLC-FL in non-heated bivalve extracts allows the assessment of metal contamination with higher sensitivity and specifity than other well-established assays [78].

MTs have a role to play as biomarkers, if used wisely in well-designed sampling programs. Careful choices need to be made on the selection of organism, choice of organ, and method of analysis [22] since MT expression is rapidly induced by a variety of substances including metals, hormones, cytokines, oxidants, stress, and radiation. Also, in natural populations, several confounding factors, such as seasonal variation, water temperature, salinity, tissue, gender, and age can influence both mRNA and protein expression. For example, while MT protein and gene expression give a quantitative picture of metal load at a single time point, quantitative information in natural populations can therefore not always be obtained when different time points (including different years) are compared. In particular, sexual development in female fish can dramatically affect MT metabolism. In biomonitoring studies using MTs, sex differences should therefore be taken into account. Furthermore, monitoring the reproductive status of females, for example, by determining 17-estradiol plasma levels, would be a valuable addition to MT field surveys [71]. So, these confounding factors require attention while standardizing the MT biomarker for field application. A multi-biomarker approach including MT and other specific biomarkers of exposure may provide a better estimate of pollution than using a single biomarker [72].

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