BIOMARKERS, 2001, VOL. 6, NO. 1, 7–14

Quantitative competitive (qc) RT-PCR as a tool in biomarker analysis

CLIVE W. EVANS[1]*, DAVID A. WILSON[1] and GEOFFREY N. MILLS[2]

[1] Molecular Genetics and Development, School of Biological Sciences, University of Auckland, Private Bag 92019, Auckland, New Zealand.

[2] National Institute of Water & Atmospheric Research, PO Box 11-115, Hamilton, New Zealand

All inducible proteins which respond to known pollutants are potential molecular biomarkers. Quantitative competitive (qc) RT-PCR represents a uniquely sensitive tool for measuring the extent of induction of molecular biomarkers such as metallothionein, which is responsive to inducers that include a range of heavy metals. Using the yellowbelly flounder *Rhombosolea leporina* collected from sites in the Manukau Harbour and Hauraki Gulf (Auckland, New Zealand) as an indicator species, we describe the methodology underpinning the use of qcRT-PCR as a tool in biomarker analysis with reference to the induction of metallothionein. The results show reasonable correlation between the extent of metallothionein induction and the liver burden of Cu and Zn.

Keywords: flounder, biomarker, metallothionein, RT-PCR, heavy metals, pollution.

Introduction

Potential biomarkers may be defined as all those biological processes which can provide sensitive, reliable and quantitative indices of the extent of biotic impact arising from environmental stress. The relevant biological processes can extend from molecular events at the level of the single cell through to events occurring at the level of the ecosystem. Events at the molecular level represent an important subset of biomarkers since they can act as early warning indicators of environmental stress. Changes at the molecular level, for example, may pre-empt changes at other levels where significant (possibly lethal) impacts could result. Furthermore, their response reflects the bioavailable fraction of the inducing agents and a variety of analytical tools are available to quantify the extent of change.

The metallothionein protein family is recognized widely as a group of potential molecular biomarkers. The family represents a related group of low molecular weight, cysteine-rich cytosolic proteins which have high affinity for divalent metal ions. The promoter region of the metallothionein gene contains multiple metal-responsive elements (MREs) which mediate induction of the gene by heavy metals such as Cu, Cd and Zn (Hamer 1986, Roesijadi 1996). This observation underlies both the use of metallothionein gene induction as a biomarker of metal exposure (Chan *et al*. 1989) and the application of the reverse-transcriptase polymerase chain reaction (RT-PCR) as a tool for quantifying the response (Schlenk *et al*. 1997).

The present study focuses on the use of the yellowbelly flounder (*Rhombosolea leporina*) as an indicator species for pollution assessment and utilizes the extraordinary sensitivity of RT-PCR amplification in the quantitative assessment

* Corresponding author: Clive W. Evans, Molecular Genetics and Development, School of Biological Sciences, University of Auckland, Private Bag 92019, Auckland, New Zealand. e-mail: c.evans@auckland.ac.nz

Biomarkers ISSN 1354-750X print/ISSN 1366-5804 online © 2001 Taylor & Francis Ltd
http://www.tandf.co.uk/journals

of metallothionein induction. Quantitative competitive (qc) RT-PCR is one of the most sensitive techniques for detecting changes in the levels of specific mRNA species (Wang *et al*. 1989). It can be performed with minute amounts of tissue and is applicable to many inducible molecular biomarkers. In this study we compare metallothionein mRNA levels in *R. leporina* collected from two different harbour localities bordering the city of Auckland, New Zealand, and correlate the data with the burden of specific metals in the liver of each fish examined.

Materials and methods

Sampling

Yellowbelly flounder were collected using gill nets from Mangere Inlet in the Manukau Harbour which borders the western side of metropolitan Auckland, New Zealand. Flounder were also collected from one site (Te Matuku Bay, Waiheke Island) in the inner Hauraki Gulf which is to the eastern side of the city. Nets were set for a maximum of 3 h and fish < 20 cm fork length were returned live to the water. Remaining flounder (only females were collected) were returned to the laboratory for immediate processing where they were first weighed and measured and then autopsied. The excised liver was then freed of the gall bladder and weighed, and a fragment transferred to Trizol (Gibco-BRL) for total RNA extraction. The remaining portion of the liver was transferred to an acetone-washed glass vial and stored at −20 °C for metal analysis.

Extraction of total RNA

Fresh liver samples (20 mg) collected as above were homogenized in 500 µl Trizol (Gibco-BRL) and the RNA was then extracted according to the manufacturer's instructions. Total RNA was resuspended in 25 µl 0.1 % diethyl pyrocarbonate (DEPC)-treated H_2O containing 33U RNAsin (Promega) and 1 mM dithiothreitol (DTT).

Metallothionein sequence analysis

Metallothionein sequences from the plaice *Pleuronectes platessa* (GenBank accession number X56743) and the winter flounder *Pseudopleuronectes americanus* (Chan *et al*. 1989) were used in the design of two primers MT.F1 (5′GACATGGATCCYTGCGAATGCTCC3′) and MT.R2 (5′TCCA-CAGTGCATCAGGCTCCTCTC3′) for RT-PCR amplification of *R. leporina* RNA.

Oligo dT primed reverse transcription was carried out in a total volume of 50 µl which contained 75 mM KCl, 3 mM $MgCl_2$, 50 mM Tris–HCl (pH 8.3), 25 mM DTT, 20 mM dNTPs, 500 ng oligo dT_{15} and 200 U Moloney murine leukaemia virus reverse transcriptase (Gibco-BRL). The cDNA products of this reaction were then amplified in a 50 µl reaction mix which contained 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl, pH 8.3, 25 mM dNTPs, 50 pmol each of MT.F1 and MT.R2, and 1 U *Taq* polymerase (Boehringer Mannheim). The reaction was set up on ice, overlaid with mineral oil (Sigma) and amplified with an initial denaturing step of 94 °C for 2 min followed by 35 cycles involving denaturation at 94 °C (30 s), annealing at 60 °C (30 s) and extension at 72 °C (30 s). The resulting product was separated on a TAE-buffered 0.7 % agarose gel and extracted using a Micron 100 microconcentrator (Amicon). The purified cDNA was then cloned into the pGEM-T vector (Promega) and transfected into competent JM109 *E. coli* cells (Stratagene). The bacteria were plated overnight and standard blue/white selection procedures were used to select colonies containing successfully ligated cDNA as confirmed by subsequent PCR amplification. One positive colony was then expanded in Luria-Bertani (LB) medium overnight and the plasmid DNA isolated using a Wizard mini-prep DNA purification kit (Promega). Purified plasmid DNA was subsequently sequenced on an automated sequencer (ABI 373A) and the information used in the design of primers MT.F3 (5′AAGACTGGCACCTGCAACTGC3′) and MT.R4 (5′TTGCAGCAAATTGTGTCGCACGTC3′) which amplify a 158 bp fragment of *R. leporina* metallothionein cDNA under the conditions described above.

Construction of the RNA competitor

An RNA competitor template for quantitative RT-PCR was constructed from the zebrafish (*Danio rerio*) β2 microglobulin gene sequence (GenBank accession number L05383). Zebrafish β2 microglobulin cDNA was made by Oligo dT primed reverse transcription of total liver RNA essentially as described above. A 180 bp competitor with MT.F3 and the reverse compliment of MT.R4 at the 3′ and 5′ ends respectively of a nested zebrafish β2 microglobulin sequence was then constructed using the extended primer method of Celi *et al*. (1993). This cDNA competitor was then ligated into pGEM-T,

grown in JM109, plated and screened, and a single colony expanded from which purified plasmid DNA was isolated. The plasmid DNA was then linearized with *Sal 1* (Gibco-BRL) and transcribed *in vitro* using Ampliscribe (Epicentre Technologies) to yield the RNA competitor. The transcription reaction mixture was subsequently treated with DNase (Boehringer Mannheim) to remove plasmid DNA, and the remaining RNA was then precipitated, washed, resuspended in DEPC–H_2O containing 33 U RNAsin and 1 mM DTT, and quantified using a GeneQuant spectrophotometer (Pharmacia). The resulting RNA competitor (180 bp) was 22 bp larger than the target mRNA and since it was tagged with primers MT.F3 and MT.R4 it could be reverse transcribed and subsequently amplified in the same PCR reaction used to amplify target *R. leporina* metallothionein mRNA.

Quantitative competitive RT-PCR

Quantitative competitive RT-PCR was undertaken using the standard curve method (Tsai and Wiltbank 1996, Miller *et al.* 1999). The standard curve was produced by co-reverse transcription and co-amplification of a constant amount of competitor RNA with a dilution series of *R. leporina* metallothionein RNA transcribed *in vitro* from the pGEM-T sequencing plasmid referred to previously. Competitor RNA (1 fmol) was added to a tube containing 200 ng random hexamers, 33 U RNAsin, 1 mM DTT, metallothionein RNA (10.0–0.078 fmol, prepared by a 2× dilution series) and made to a total volume of 50 µl with DEPC–H_2O. Following reverse transcription and PCR amplification using the conditions described above, the reaction products were separated on a 10% polyacrylamide gel, stained with 1.0 µg ml^{-1} ethidium bromide, visualized over UV light and photographed for measurement of band intensities using an image analysis programme (NIH Image 1.54). The standard curve was then plotted from the log concentration of target metallothionein RNA against the log transformed ratio of the target RNA concentration to the competitor RNA concentration (which is represented by the ratio of band intensities). In order to obtain an accurate curve the full procedure was carried out in triplicate (figure 1).

Levels of metallothionein transcripts in unknown samples were quantified by co-reverse transcription and co-amplification of total RNA with 1 fmol of competitor RNA under the same conditions as used to construct the standard curve except that the PCR buffer contained 2 mM Mg^{2+} and the reaction was carried out over 30 cycles. Quantification of metallothionein mRNA levels in the unknown samples was then undertaken by interpolating the log transformed ratio of the target to the competitor band intensities on the standard curve. All measurements were carried out in duplicate.

Analysis of liver metal burden

Liver samples stored frozen at −20 °C in acetone-washed glass vials were digested with *aqua regia* and analysed for Cu and Zn by induction coupled plasma-mass spectrometry (ICP-MS) under commercial contract. Detection limits were 0.03 and 0.05 mg kg^{-1} wet weight for Cu and Zn respectively.

Results

RT-PCR of *R. leporina* total liver RNA using primers MT.F1 and MT.R2 and subsequent cloning and sequencing of a positive colony yielded a product with extensive homology to plaice and winter flounder metallothionein cDNA (table 1). The *R. leporina* sequence was then used to design the primers MT.F3 and MT.R4 which amplified a 158 bp fragment of the cDNA as shown (table 1).

Metallothionein mRNA in sampled flounder livers was measured by qcRT-PCR as described using the standard curve presented in figure 1. A summary of the results, converted to fmol mRNA per µg total RNA, is presented in table 2. Flounder from Te Matuku Bay had significantly higher mean liver metallothionein mRNA levels than did flounder collected from the Manukau Harbour ($p < 0.01$, Student's *t*-test).

The levels of Cu and Zn determined in flounder liver samples by ICP-MS are shown in table 2. In concordance with the higher mean level of metallothionein mRNA detected in Te Matuku Bay fish, flounder from this locality had significantly more Cu and Zn in their livers than did fish from the Manukau Harbour ($p < 0.05$). The levels of Cu and Zn determined in the livers of individual fish from both sites were then plotted against the extent of metallothionein

Table 1. Comparison of sequence data for metallothionein cDNA from the yellowbelly flounder (*R. leporina*), the plaice (*P. platessa*) and the winter flounder (*P. americanus*). Components of the primer sequences are in italics. Differences are underlined. The sequences for the cysteines of the characteristic Cys-X-Cys repeat of the metallothioneins are conserved (bold).

R. leporina	ATG	GAT	CCT	**TGC**	GAA	**TGC**	TCC	*AAG*	*ACT*	*GGC*	*ACC*	***TGC***	*AAC*
P. platessa	ATG	GAT	CCT	TGC	GAA	TGC	TCC	AAG	ACT	GGA	ACC	TGC	AAC
P. americanus	ATG	GAT	CCT	TGC	GAA	TGC	TCC	AAG	ACT	GGA	ACC	TGC	AAC
R. leporina	***TGC***	GGC	GGA	TCC	**TGC**	AGC	**TGC**	ACC	AAC	**TGC**	GCC	**TGC**	ACC
P. platessa	TGC	GGA	GGA	TCT	TGC	ACC	TGC	AAG	AAC	TGC	AGC	TGC	ACC
P. americanus	TGC	GGA	GGA	TCT	TGT	ACC	TGC	AAG	AAC	TGC	AGC	TGC	ACC
R. leporina	TCA	TGC	AAG	AAA	AGC	TGC	**TGC**	CCA	**TGT**	TGC	CCA	ACC	GGC
P. platessa	ACC	TGC	AAC	AAG	AGC	TGC	TGC	CCA	TGC	TGC	CCA	TCC	GGC
P. americanus	ACC	TGC	AAC	AAG	AGC	TGC	TGC	CCA	TGC	TGC	CCA	TCC	GGC
R. leporina	TGC	ACT	AAT	TGC	GCC	TCT	GGC	**TGC**	GTG	**TGC**	AAA	GGG	*AAG*
P. platessa	TGC	CCC	AAG	TGC	GCC	TCT	GGC	TGC	GTG	TGC	AAA	GGG	AAG
P. americanus	TGC	CCC	AAG	TGC	GCC	TCT	GGC	TGC	GTG	TGC	AAA	GGG	AAG
R. leporina	*ACG*	*TGC*	*GAC*	*ACA*	*ATT*	*TGC*	*TGC*	*AAN*	NGA	(183)			
P. platessa	ACA	TGC	GAC	ACC	AGC	TGC	TGT	CAG	TGA				
P. americanus	ACA	TGC	GAC	ACC	ACT	TGC	TGT	CAG	TGA				

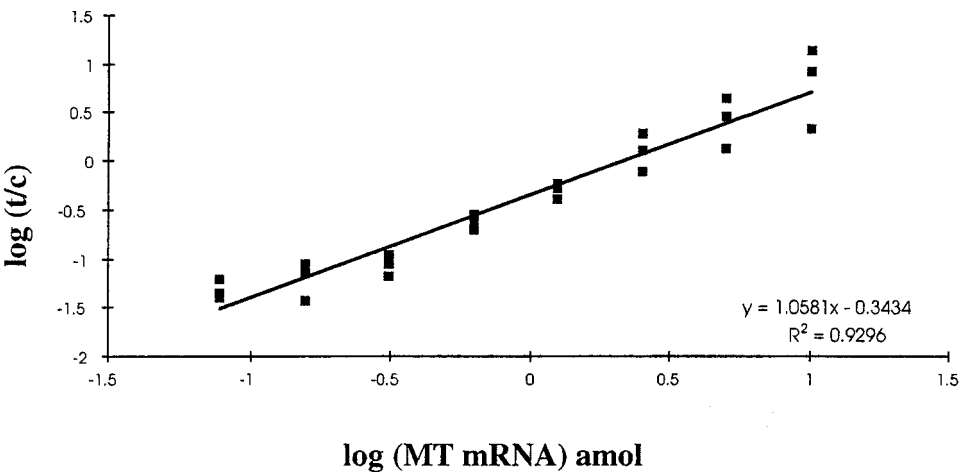

Figure 1. Standard curve for metallothionein competitive RT-PCR. The line of best fit was derived from three independent experiments. t = target; c = competitor; MT = metallothionein.

induction as measured by qcRT-PCR for the same fish (figure 2). Regression analysis showed a linear relationship for both metals with the extent of metallothionein induction (Cu: $y = 24.141x + 501.25$, $R^2 = 0.7285$; Zn: $y = 19.891x - 1350$, $R^2 = 0.7209$). Pearson's correlation analysis confirmed that the liver burden of both Cu and Zn covaried with hepatic metallothionein mRNA as determined by qcRT-PCR ($r = 0.8536$ and 0.8491 respectively). Thus not only are metallothionein mRNA levels significantly different between the two populations of fish, but they correlate with the liver tissue burden of two major heavy metals inducers of the metallothionein gene.

Table 2. Levels of hepatic metallothionein mRNA, Cu and Zn in *R. leporina*. Data are expressed as means ± SD (*N*).

Sample site	Metallothionein mRNA (fmol mRNA μg^{-1} total RNA)	Copper (μg g^{-1})	Zinc (μg g^{-1})
Manukau Harbour	1.204 ± 0.750 (14)	15.8 ± 9.89 (8)	105.54 ± 18.13 (8)
Te Matuku Bay	1.785 ± 0.543* (13)	39.9 ± 20.97* (5)	143.2 ± 21.92* (5)

* $p<0.05$ (Student's *t*-test).

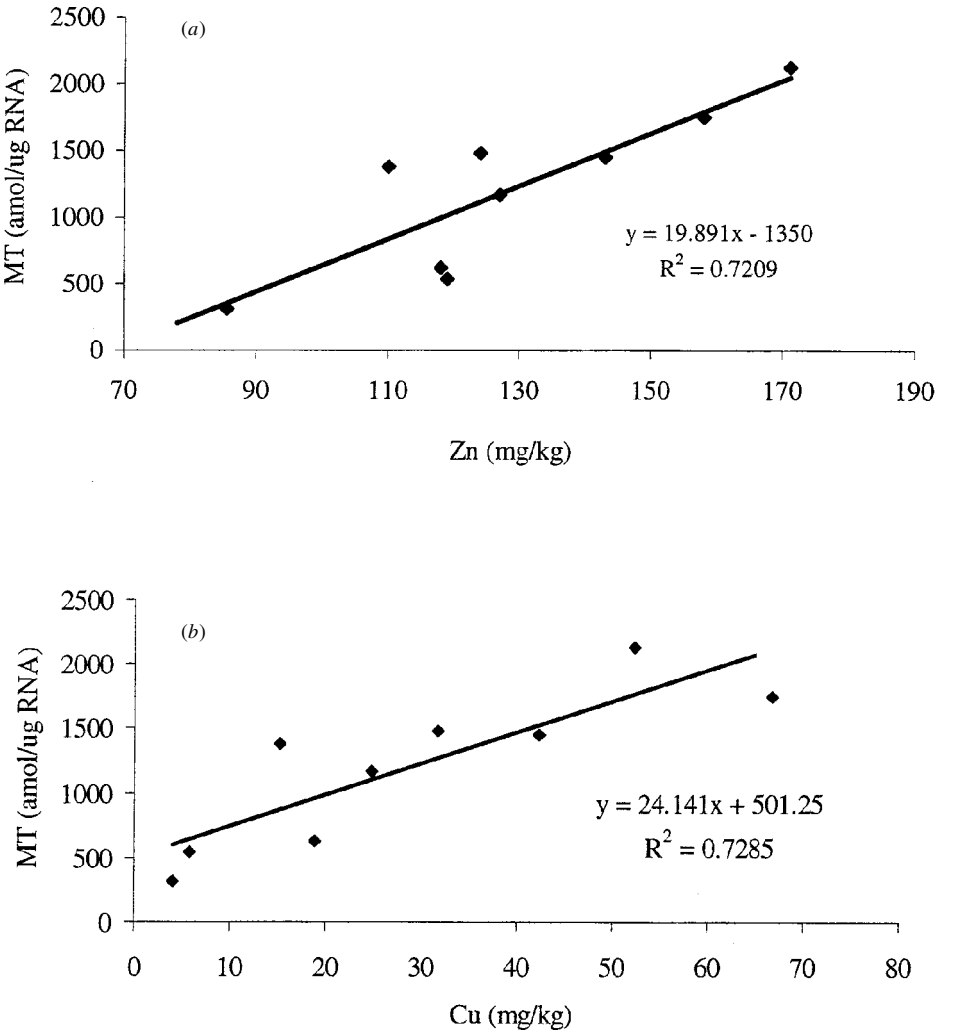

Figure 2. Correlation between metallothionein induction and hepatic metal levels. (a) Zinc; (b) copper.

Discussion

Extensive research utilizing a wide variety of fish species and other organisms has been undertaken to demonstrate the induction of metallothionein by heavy metals under laboratory controlled conditions. Relatively few studies have used the

measurement of metallothionein induction in fish as an ecotoxicological tool for assessing sublethal exposure to heavy metals in the field (reviewed in Goksøyr *et al.* 1996). Those studies that have been done have yielded mixed results which may reflect response differences in the indicator species as well as differences in metal bioavailability and other factors (George *et al.* 1996). Nevertheless, a number of studies have shown that levels of hepatic metallothionein correlate with the levels of trace metals in feral or caged fish. Thus Sulaiman *et al.* (1991), for example, measured metallothionein protein levels in the livers of flounder (*Platichthys flesus*) caught along a pollution gradient in the Forth Estuary, Scotland. They reported a 19-fold increase in metallothionein levels in fish caught from near a large petrochemical complex relative to controls. This presumably was due largely to the effects of Cu, since of the four metals (Cu, Cd, Zn and Pb) these authors measured, only Cu showed significantly raised levels in the livers of fish caught near the pollution source. Mean hepatic levels of Cu and Zn ranged from 10 to 63 and 64 to 117 μg g^{-1} (dry weight) respectively. Hylland *et al.* (1992) measured levels of metallothionein protein in livers from dab (*Limanda limanda*) caught at seven stations along a transect within the German Bight. They found that liver metallothionein levels in female dab correlated with hepatic Zn, while that in males correlated with a combination of Cu and Cd. Interestingly, neither hepatic metallothionein nor liver metal levels correlated with metal concentrations in the sediment.

In the present study, quantification of metallothionein mRNA by qcRT-PCR showed that there was significantly less in the livers of flounder from the Manukau Harbour than there was in that of flounder from Te Matuku Bay. Analysis of Cu and Zn levels in flounder livers paralleled these results and the amounts present of both metals showed reasonable correlation with metallothionein gene induction. The Manukau Harbour has been recognized as one of the most polluted harbours in New Zealand, receiving significant domestic and industrial inputs which include the main sewage system for the central and southern parts of greater Auckland. According to Glasby *et al.* (1988) sediments from in-shore regions of both harbours contain moderate to high levels of heavy metals such as Zn, Cu and Pb from anthropogenic sources, but Te Matuku Bay (which lies further into the Hauraki Gulf) can be considered to be remote from these impacts. Our observations that the liver burdens of both Cu and Zn in Te Matuku Bay flounder exceed that in fish from the more polluted Manukau Harbour emphasizes the importance of bioavailability in biomarker analysis (George *et al.* 1996). Interestingly, experiments undertaken by Berge and Brevik (1996) with caged flounder (*P. flesus*) indicate that this species does not necessarily accumulate considerable amounts of metals when exposed to contaminated sediments for a short time (4–12 weeks). Their results confirm other studies with flounder that high metal levels in contaminated sediments do not necessarily result in high tissue burden in resident fish (Beyer *et al.* 1996).

A variety of parameters other than bioavailability are likely to impact on metallothionein response levels and thus have potential to compromise its use as a biomarker. These include exogenous factors such as water temperature and hardness, and endogenous factors such as sex and reproductive state (Olsson 1996). The flounder used in this study were all females at early but different stages of gonadal maturation, the males presumably having already migrated to deeper water prior to spawning. According to Olsson *et al.* (1990) metallothionein levels increase to peak in female rainbow trout at the time of spawning as a result of hepatic Zn

accumulation. However, there was no evidence in the present study of any correlation between hepatic metallothionein mRNA content and reproductive state as indicated by gonadal size (data not shown). This result is not surprising since relative levels of both Cu and Zn were elevated in Te Matuku Bay flounder indicating that reproductive state (as reflected in hepatic Zn) is only one parameter in the response analysed.

We conclude that qcRT-PCR is a useful tool in analysing the induction of molecular biomarkers such as metallothionein in response to specific inducers. The universality of this approach remains to be fully tested, however, and many of the limitations attached to the use of other molecular biomarkers in assessing environmental impact (such as antagonistic or synergistic responses arising from complex mixtures of pollutants in the environment, differences in tissue pharmacokinetics and bioavailabilty, and differences attributable to sex and reproductive state) require further elucidation.

Acknowledgements

We thank the University of Auckland Research Committee for financial support; Dr Dave Bembo, Dr Leon Huynen, and Jenny Rains for their practical advice; Beryl Davy for histology; and Dave Todd and David Mutoro for their support in the field.

References

Berge, J. A. and Brevik, E. M. 1996, Uptake of metals and persistent organochlorines in crabs (*Cancer pagurus*) and flounder (*Platichthys flesus*) from contaminated sediments: mesocosm and field experiments. *Marine Pollution Bulletin*, **33**, 46–55.

Beyer, J., Sandvik, S., Hylland, K., Fjeld, E., Egaas, E., Aas, E., Skare, J. U. and Goksøyr, A. 1996, Contaminant accumulation and biomarker responses in flounder (*Platichthys flesus* L.) and Atlantic cod (*Gadus morhua* L.) exposed by caging to polluted sediments in Sorfjorden, Norway. *Aquatic Toxicology*, **36**, 75–98.

Celi, F. S., Zenilman, M. E. and Shuldiner, A. R. 1993, A rapid and versatile method to synthesise *internal* standards for competitive PCR. *Nucleic Acids Research*, **21**, 1047.

Chan, K. M., Davidson, W. S., Hew, C. L. and Fletcher, G. L. 1989, Molecular cloning of metallothionein cDNA and analysis of metallothionein gene expression in winter flounder tissues. *Canadian Journal of Zoology*, **67**, 2520–2527.

George, S. G., Todd, K. and Wright, J. 1996, Regulation of metallothionein in teleosts: induction of MT mRNA and protein by cadmium in hepatic and extrahepatic tissues of a marine flatfish, the turbot (*Scophthalmus maximus*). *Comparative Biochemistry and Physiology*, **113C**, 109–115.

Glasby, G. P., Stoffers, P., Walter, P., Davis, K. R. and Renner, R. M. 1988, Heavy-metal pollution in Manukau and Waitemata Harbours, New Zealand. *New Zealand Journal of Marine and Freshwater Research*, **22**, 595–611.

Goksøyr, A., Beyer, J., Egaas, E., Grosvik, B. E., Hylland, K., Sandvik, M. and Skaare, J. U. 1996, Biomarker response in flounder (*Platichthys flesus*) and their use in pollution monitoring. *Marine Pollution Bulletin*, **33**, 36–45.

Hamer, D. H. 1986, Metallothionein. *Annual Review of Biochemistry*, **55**, 913–951.

Hylland, K., Haux, C. and Hogstrand, C. 1992, Hepatic metallothionein and heavy metals in dab *Limanda limanda* from the German Bight. *Marine Ecology Progress Series*, **91**, 89–96.

Miller, H. C., Mills, G. N., Bembo, D. G., Macdonald, J. A. and Evans, C. W. 1999, Induction of *cytochrome P4501A* (*CYP1A*) in *Trematomus bernacchii* as an indicator of environmental pollution in Antarctica: assessment by quantitative RT-PCR. *Aquatic Toxicology*, **44**, 183–193.

Olsson, P.-E. 1996, Metallothioneins in fish: induction and use in environmental monitoring. In *Toxicology of Aquatic Pollution,* E. W. Taylor, ed. (Cambridge: Cambridge University Press), pp. 197–203.

Olsson, P.-E., Zafarullah, M., Foster, R., Hamor, T. and Gedamu, L. 1990, Developmental regulation of metallothionein mRNA, zinc and copper levels in rainbow trout, *Salmo gairdneri*. *European Journal of Biochemistry*, **193**, 229–235.

ROESIJADI, G. 1996, Metallothionein and its role in toxic metal regulation. *Comparative Biochemistry and Physiology*, **113C**, 117–123.

SCHLENK, D., CHELIUS, M., WOLFORD, L., KHAN, S. and CHAN, K. M. 1997, Characterisation of hepatic metallothionein expression in channel catfish (*Ictalurus punctatus*) by reverse-transcriptase polymerase chain reaction. *Biomarkers*, **2**, 161–167.

SULAIMAN, N., GEORGE, S. and BURKE, M. D. 1991, Assessment of sublethal pollutant impact on flounders in an industrialised estuary using hepatic biochemical indices. *Marine Ecology Series*, **68**, 207–212.

TSAI, S.-J. and WILTBANK, M. C. 1996, Quantification of mRNA using competitive RT-PCR with standard-curve methodology. *BioTechniques*, **21**, 862–866.

WANG, A. M., DOYLE, M. V. and MARK, D. F. 1989, Quantitation of mRNA by the polymerase chain reaction. *Proceedings of the National Academy of Sciences, USA*, **86**, 9717–9721.