BIOMARKERS, 2001, VOL. 6, NO. 4, 274–288

Brown trout (*Salmo trutta*) metallothioneins as biomarkers for metal exposure in two Norwegian rivers

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Received 20 July 2000, revised form accepted 10 October 2000

The potential use of the metal binding protein metallothionein (MT) as a biomarker for trace metal exposure has been evaluated in brown trout (*Salmo trutta*) from the Cu-contaminated Rugla and the Cd/Zn-contaminated Naustebekken Norwegian rivers, as well as in hatchery control trout. Metal concentrations were measured in gills, liver and kidney as well as in ambient water, and compared with Cd/Zn MT and Cu MT levels, measured by two Cd-saturation techniques. In addition haematocrit, plasma chloride and condition factors (weight × 100/length^3) were measured, and genetic diversity determined. A negative correlation was found between the Cd/Zn MT content and the condition factor in the different trout populations, and Naustebekken trout, having the lowest condition factor, were less heterozygous than Rugla trout, indicating that Naustebekken trout have adapted to the metal in their environment. Significant positive correlations were found between accumulated levels of Cd and Cd/Zn MT in liver and kidney, suggesting that liver and kidney, but not gill, Cd/Zn MT can be used as a biomarker for chronic Cd exposure. Cu MT seems less suitable as a biomarker for Cu exposure. These data together further emphasize that MTs must be applied with caution, when assessing these proteins in biomonitoring of the natural environment.

Keywords: brown trout, metallothionein; biomarker, trace metals, freshwater.

Introduction

An ecotoxicological biomarker can be defined as a xenobiotic-induced alteration in biochemical or cellular components or processes, structures, or functions that is measurable in a biological system or sample (Depledge 1994). The concept of biomarkers is still controversial because it is often difficult to establish a clear relationship between the level of exposure and the magnitude of the biomarker response, and to separate signal from background noise (Huggett *et al*. 1992). Normally biomarkers do not provide information regarding the physiological impact of the pollutant exposure, and provide no information regarding population, community, and ecosystem effects. Depledge *et al*. (1995) suggested that biomarker responses should be related to a given degree of impairment of growth, reproductive output, or energy utilization that directly affects the survivorship and fertility of organisms. This approach may, however, be difficult to apply in field studies. Tests for biomarkers and other stress indicators at various biological levels can still give valuable information regarding monitoring of the natural environment.

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Biomarkers ISSN 1354-750X print/ISSN 1366-5804 online
http://www.tandf.co.uk/journals
DOI: 10.1080/13547500010014522

Many studies have shown that gross analysis of water and sediments for toxic metals cannot be used to predict or assess environmental impact since the major proportions of these metals are present in forms not directly available to biota (Campbell 1995). Organisms are capable of accumulating concentrations of metals many thousand times higher than those present in their surrounding waters, and numerous studies have focused on metal accumulation in aquatic animals from polluted systems (Spry and Wiener 1991, Mason and Jenkins 1995). Since such studies have focused on metal accumulation alone, it is difficult to interpret toxicological impact. Metallothionein (MT) protects tissues from metal damage, and may be used as a direct measure of the biochemical state of the affected tissue. The use of this intracellular protein as a biomarker for elevated levels of trace metals in aquatic environments has therefore recently been proposed as a powerful tool in ecotoxicological studies (Benson *et al*. 1990, Depledge 1994, George and Olsson 1994). Changes at the biochemical level offer distinct advantages as biomarkers, since molecular alterations are normally the first detectable, quantifiable responses to environmental changes, and may therefore serve as markers of both exposure and effect (Huggett *et al*. 1992).

Metallothionein induction can be considered as a biochemical biomarker of exposure, and be used to signal trace metal exposures in the field. It is well established that exposures to elevated levels of both waterborne and dietary metals such as Cd, Cu and Zn can lead to induction of MT in fishes (Kito *et al*. 1982, McCarter *et al*. 1982, Olsson and Hogstrand 1987). Induced MT bind and thereby immobilize trace metals after exposure, counteracting intracellular effects. Metals bound to MT are only displaced by metals of higher affinity with binding affinities declining in the order of Cu > Cd > Zn (being the metals studied here) (Kägi 1993). It is therefore important to differentiate between the various forms of MT, i.e. which metals that are bound to the protein at a given time. Half-lives of Cu MT and Zn MT are estimated to be 1 month at temperatures of 11–12¡C in fishes (McCarter and Roch 1984, Overnell *et al*. 1987), whereas Cd MT probably lasts much longer, as shown for other vertebrates (Squibb 1996). In salmonids the kidney normally contains the highest Cd levels (Olsson and Hogstrand 1987, Dallinger *et al*. 1997, Olsvik *et al*. 2000). Intracellular levels of essential metals such as Cu and Zn are normally closely regulated through homeostatic mechanisms, making it more difficult to evaluate MT as biomarkers for these metals. Earlier the emphasis was mainly focused on the development of biomarkers in the laboratory rather than direct application in field assessments (Huggett *et al*. 1992). In recent studies, however, focus has been put on salmonid MTs in these respects (Farag *et al*. 1995, Dallinger *et al*. 1997, Linde *et al*. 1999), but extensive studies are still needed before MT routinely can be applied as a biochemical biomarker for trace metal exposures in environmental monitoring.

The aim of the present study was to investigate whether the MTs of brown trout (*Salmo trutta*) can be used as biomarkers for Cd, Cu and Zn exposures under natural environmental conditions in the Cu-contaminated Rugla River and in the Cd/Zn-contaminated Naustebekken River. For this reason the levels of Cd, Cu and Zn in water and in gills, liver and kidney of three different brown trout populations, one from a laboratory, low-metal environment, and the other two from Rugla and Naustebekken, were measured. The levels of both Cd/Zn MT and Cu MT were determined in the same tissues with two Cd-saturation techniques. Rugla and Naustebekken trout were analysed for the amount and distribution of genetic

variation by enzyme electrophoresis, in order to test whether they belong to different populations and possible effects of metal pollution on allele frequencies. In addition, possible relationships between physiological conditions (haematocrit, plasma chloride, condition factors and accumulated levels of metals) and MT levels were investigated in the trout populations. Finally, the usefulness of the different MT forms as biomarkers is discussed.

Methods

Study sites

The Rugla and Naustebekken rivers are situated in the County of Sùr-Trùndelag, Central Norway. Rugla belongs to the Gaula drainage system, whereas Naustebekken belongs to the uppermost part of the Glomma River drainage system. Even though they have neighbouring catchments, the topographic divide separates them completely and fish have no natural possibility to migrate from one river to the other. As a result of earlier mining activities, Rugla is mainly Cu-contaminated, whereas Naustebekken is Cd- and Zn-contaminated (Traaen *et al*. 1987, Iversen 1994).

Water chemistry

Laboratory water from the Lake Jonsvatnet (which provides tap water for the city of Trondheim, Norway, referred to as control water) was analysed for trace elements (five water samples) and other limnochemical parameters (up to 12 water samples) during one week of February 1998. Five water samples from both Rugla and Naustebekken rivers were analysed for the same parameters during the first week of September 1998. Samples for total metal determination were collected on acid-washed (6 M HNO_3) 125 ml polyethylene bottles by a standardized procedure involving three refillings with river water. The samples were then acidified to yield a total concentration of 0.1 M HNO_3. Ca and Zn concentrations were determined by fiame atomic absorption spectrophotometry (AAS), while Cd and Cu were determined by graphite AAS (Perkin Elmer 2100 AAS, equipped with a graphite furnace (Model HGA-700) and an autosampler (Model AS-70)). Re-slope was performed for every 10 samples, and two internal standards were analysed between each re-slope. Values within −10% from a long time average were accepted for these metals. pH was determined with a Radiometer PHM 80 portable pH meter. Alkalinity measurements were done by a Hach Digital Titrator Model 16900, while *in situ* conductivity measurements were done by a WTW Microprocessor Conductivity Meter LF 196.

Fish treatment, sampling and analysis

Brown trout reared at Lundamo Hatchery in Sùr-Trùndelag were used as a control population (referred to as hatchery trout). They were transferred to and kept in laboratory water at Brattùra Research Center in Trondheim for several months before sampling of biological tissues. During this period they were fed a commercial salmon food (Skretting Royal Redline 6 mm). Brown trout from Rugla and Naustebekken were captured at low water in summer of 1997 and on 3rd September 1998 by electric fishing and by fish traps. They were killed by a blow to the head, blood samples were collected from the caudal vessels into heparinized tubes by cutting the caudal fin, length and weight determined, and immediately frozen on dry ice for transport to the laboratory to be stored at –80 °C before further processing. Haematocrit was determined immediately after blood collection by a Compur M 1100 microcentrifuge. Blood samples were centrifuged for $12\,000 \times g$ at room temperature for 10 min and plasma frozen for later determinations of Cl^- concentrations. Blood plasma Cl^- concentrations were assayed using a Radiometer CTM chloride titrator. Condition factors (c.f.) were determined by the equation: c.f. = weight (g)/(length^3 (cm)) × 100.

After thawing, gills, liver and kidney were dissected out, washed in ice-cold distilled water to remove blood remnants and kept on ice. The filaments were cut off from the gill arches before further processing. For direct metal quantification the wet weights of tissue samples were determined, and the samples were stored at –80 °C. The samples were then lyophilized for 24 h and the dry weights obtained. Samples of approximately 50 mg were digested in 1.8 ml polypropylene tubes by 1 ml of 65% HNO_3 (suprapur grade, Merck), boiled until all the liquid had evaporated and finally 1 ml of 0.1 M HNO_3 was added to the digests. Whenever necessary the samples were diluted in 0.1 M HNO_3 before AAS analysis. Re-slope with two internal standards was performed for every tenth sample, and values within 10% from the long-term average of the standard solutions were accepted for metal analysis. Quality assurance for metal analysis was achieved by the use of standard metal solutions (Cu and Zn: Spectrosol, BDH Laboratory Supplies, Poole, England; Cd: Spectroscan, Teknolab A/S, Drùbak, Norway) and standard reference material (Bovine liver SRM 1755b, National Institute of Standards and Technology, Gaithersburg, MD, USA).

After thawing, gill, liver and kidney tissues were transferred to ice-cold deoxygenated 1:4 w/v 5 mM Tris–HCl, pH 8.5, and homogenized with a Glas-Col Homogenizer (Potter Elvehjem). Then 2-mercaptoethanol to yield a total concentration of 5 mM was added to avoid oxidation of MT. Homogenates were centrifuged at 12 000 × *g* for 10 min, and supernatant aliquots kept at –80 °C in 4.5 ml polypropylene cryo tubes before further use. The Cd-chelex assay was used to quantify Cd/Zn MT (Bartsch *et al*. 1990). This radiometric Cd-saturation assay is based on ^{109}Cd for MT quantification. High molecular weight proteins in the supernatants were denatured by treatment with acetonitrile, and excess amounts of Cd bound to Chelex-100. Remaining ^{109}Cd in the supernatant solution was then measured (Packard Cobra Gamma Counter). The concentrations of total MT (including Cu MT) were determined by the radiometric thiomolybdate assay (Klein *et al*. 1990). In this method Cu was removed from MT by ammonium tetrathiomolybdate, and excessive tetramolybdate and its complexes removed with DEAE-Sephacel. Apothionein was then saturated with Cd, and excessive Cd removed by Chelex-100. The ^{109}Cd bound to MT in the supernatant solution was then measured. The concentrations of Cd/Zn MT and total MT were determined by assuming the molecular weight of MT of 7000 and a molar ratio of 7 gram-atom of Cd per mole of protein (Roesijadi 1992). Analysis of water samples from the two rivers by ICP-MS and Neutron Activation Analysis methods revealed very low concentrations of other metals (P. Gundersen, pers. comm.). The assumption that total MT minus Cd/Zn MT equals Cu MT was therefore considered valid, since no other metals could interfere with the MT quantification methods. To test the linearity of these assays we used commercial MT from Sigma (MT rabbit liver, lot 56H9500). We also used dilution series obtained from samples of Naustebekken trout liver, and linearity was found down to approximately 1 μg MT g^{-1} fresh weight for the Cd-chelex assay and to approximately 10 μg MT g^{-1} fresh weight for the thiomolybdate assay. At Cd/Zn MT concentrations above approximately 150 μg MT per gram fresh weight the Cd-chelex assay gave a small underestimation of the calculated values. This was found to be 9 % for the rabbit liver sample of 500 μg MT, calculated as the deviation from the regression line for nine samples between 1 and 500 μg MT (each measured three times, $r = 0.993$). The linearity of the thiomolybdate assay was found satisfactory throughout the measured value range ($r = 0.998$). The reproducibility errors for the Cd-chelex and the thiomolybdate assay were less than 4 and 10%, respectively.

Statistical methods

One-way analysis of variance (ANOVA) and Tukey's multiple-means comparison test were used to evaluate differences between limnochemical data, and between physiological parameters obtained from the brown trout populations. Differences between the populations in tissue metal concentrations and MTs were tested by Kruskal–Wallis one-way analysis of variance on ranks. Multiple comparisons were made with Dunn's test. Because of non-normal distribution of these data, median values and quartiles (1. and 3.) are given. Associations between MT levels and condition factors/metal concentrations in tissues were tested using Spearman rank correlation. Statistical significance was assigned at $p = 0.05$.

Genetic methods

To test whether trout from Rugla and Naustebekken rivers belong to different populations, 35 and 33 individuals, respectively, from the two populations were collected and analysed for genetic variation by enzyme electrophoresis (Aebersold *et al*. 1987). The buffer systems used, the proteins examined, and the genetic interpretations of the electrophoretic banding patterns follow Allendorf *et al*. (1977), with additional systems and overall nomenclature given by Jorde (1994). We screened all individuals for 26 enzyme-coding genes (loci), of which the following showed genetic variation: aspartate aminotransferase (*sAAT-4**, Enzyme Commission no. 2.6.1.1), β-*N*-acetylgalactosaminidase (*bGALA-2**, EC 3.2.1.53), *N*-acetyl-β-glucosaminidase (*bGLUA**, E.C. 3.2.1.30), glucose-6-phosphate isomerase (*GPI-3**, EC 5.3.1.9), L-iditol (sorbitol) dehydrogenase (*IDDH-1**, EC 1.1.1.14), $NADP^+$-dependent isocitrate dehydrogenase (*sIDHP-1**, EC 1.1.1.42), L-lactate dehydrogenase (*LDH-1**, *LDH-5**, EC 1.1.1.27), and malate dehydrogenase (*sMDH-2**, *sMDH-3,4**, EC 1.1.1.37).

Allele frequencies were estimated by direct allele counts. Tests of Hardy–Weinberg genotypic equilibrium within population, and of allele frequency homogeneity between populations, were performed using exact tests for each locus, and by using Fisher's combined probability test for multiple loci. Differences between populations in average observed heterozygosity across variable loci were analysed by ANOVA according to Weir (1990). All genetic tests were performed using the GENEPOP (Raymond and Rousset 1995) or SPSS for Windows program packages.

Results

The metal compositions of laboratory water on one side and the Rugla and Naustebekken rivers on the other are quite different (table 1). Laboratory water

Table 1. Chemistry of laboratory, Rugla and Naustebekken waters. Data are expressed as means – SD. Number of observations in parentheses. The different parameters were analysed by one-way analysis of variance (ANOVA), with Tukey's post test to compare means (considered significantly different at $P < 0.05$).

Water	pH	Alk [μEq^{-1}]	Cond. [$\mu S\ cm^{-1}$]	Ca [mg^{-1}]	Cd [ng^{-1}]	Cu [μg^{-1}]	Zn [μg^{-1}]
Laboratory	6.8 – 0.1 (8)	232.0 – 21.0 (7)	56.5 – 0.3 (12)	5.2 – 0.1 (6)	n.d. (5)	1.5 – 0.5 (4)	3.5 – 2.5 (4)
Rugla	7.1 – 0.2* (3)	330.0 – 26.0* (3)	46.1 – 4.6* (6)	5.7 – 0.1* (6)	8.0 – 13.0 (5)	10.5 – 1.0* (5)	4.3 – 2.2 (5)
Naustebekken	6.9 – 0.1 (3)	106.0 – 21.0*† (3)	18.6 – 0.6*† (6)	2.2 – 0.0*† (6)	40.0 – 40.0 (5)	5.1 – 0.3*† (5)	48.7 – 10.2*† (5)

* Significant differences between laboratory and river water;

† Significant differences between river waters. Alk: Alkalinity; Cond.: Conductivity; n.d.: non detectable (considered as zero when group means were compared).

contained only low Cu and Zn levels, while Cd could not be detected by the AAS technique used here. Cu mainly contaminates Rugla water, while Naustebekken water has elevated levels of Cd and Zn. The Ca concentration and alkalinity were significantly lower in Naustebekken water than in laboratory and Rugla waters.

Wild trout populations have significantly lower condition factors than the hatchery control trout ($P < 0.01$), as could be expected due to the rich food supply for the reared fish (table 2). When this parameter was compared for the wild trout populations, significantly higher condition factors were found for Rugla trout than for Naustebekken trout ($P < 0.001$). Plasma chloride concentration were significantly lower in the Naustebekken trout population than in the hatchery trout population ($P < 0.05$). No differences could be seen in haematocrit levels for the three populations.

Accumulated levels of Cd, Cu and Zn in gills, liver and kidney of the wild trout populations, but not for the hatchery population, reflected the environmental concentrations of these metals (table 3). The concentrations of these metals were found to vary significantly between the three trout populations in all the investigated tissues (Kruskal–Wallis test, $P < 0.01$). The highest Cd levels were found in kidney, but also liver contained a considerable amount of this metal. The Cd levels in Naustebekken trout tissues were significantly higher than in Rugla trout tissues; kidney and liver Cd concentrations were 24 and 18 times higher in Naustebekken trout than in Rugla trout, respectively. Only low levels of Cd were measured in tissues of hatchery trout. For Cu the results were not so obvious, even though Rugla trout tissues contained the highest levels of this metal as suggested from the water concentrations.

Liver from the hatchery trout contained the same amounts of Cu as Naustebekken trout, with Naustebekken water containing 3.4 times higher levels than laboratory water. The accumulated levels of Zn were highest in Naustebekken trout tissues, in accordance with the levels measured in the water. All tissues of hatchery trout had significantly higher Zn levels than Rugla trout, even though the concentration of this metal was 1.5 times higher in Rugla water.

The concentrations of both Cd/Zn MT and total MT in gills, liver and kidney were found to vary significantly between the three trout populations (Kruskal–Wallis test, $P < 0.01$) (table 4). Surprisingly high Cu MT levels were

Table 2. Physiological parameters in hatchery, Rugla and Naustebekken brown trout *Salmo trutta* ($n = 10$). Data are expressed as means – SD. Condition factor (c.f. = weight (g)/(length^3 (cm)) × 100), plasma chloride and haematocrit were analysed by one-way analysis of variance (ANOVA), with the Tukey's HSD test for post-hoc comparisons of means (considered significantly different at $P < 0.05$). Data were transformed when necessary to meet the normality and homogeneity assumptions of an ANOVA.

Trout population	Weight (g)	Length (cm)	Condition factor	Chloride (mM)	Haematocrit (%)
Hatchery	266 – 76	28 – 2	1.16 – 0.10	128 – 6	37 – 8
Rugla	52 – 24	17 – 3	1.00 – 0.06*	121 – 8	42 – 7
Naustebekken	173 – 50	26 – 3	0.90 – 0.04*†	113 – 13*	42 – 4

* Significant differences between hatchery and wild trout;
† Significant differences between wild trout.

Table 3. Tissue concentrations of Cd, Cu and Zn (μg g^{-1} tissue fresh weight) in hatchery, Rugla and Naustebekken brown trout *Salmo trutta* ($n = 6$, #$n = 5$, ″$n = 4$).Values are given as medians, first and third quartiles (in parentheses). The Kruskal–Wallis one-way analysis was used to test whether Cd, Cu and Zn concentrations were different between the three groups of trout, with Dunn's post test for median comparisons between the different populations when the Kruskal–Wallis test gave a significant result ($P < 0.05$).

Trout population	Tissue	Cd	Cu	Zn
Hatchery	Gills	0.02 (0.02/0.02)	0.47 (0.39/0.68)	94.20 (65.17/107.64)
	Liver	0.03 (0.03/0.03)	102.89 (62.02/112.81)	22.15 (19.61/23.51)
	Kidney	0.07 (0.07/0.08)	0.66 (0.63/0.80)	32.17 (29.18/33.45)
Rugla	Gills	0.12 (0.10/0.13)	4.88 (3.57/5.70)*	63.09 (44.04/84.33)
	Liver	0.30 (0.27/0.30)	242.93 (236.41/336.85)*	16.71 (16.31/17.45)
	Kidney	0.44 (0.39/0.45)	3.85 (3.51/4.90)*	24.74 (24.27/25.84)
Naustebekken	Gills	0.78 (0.70/1.13)*	1.92 (1.48/2.01)	186.55 (152.60/241.11)*†
	Liver	5.48 (4.70/5.64)*	103.42 (87.72/122.98)†	47.99# (40.61/54.69)†
	Kidney	10.48 (9.14/12.55)*	1.97″ (1.67/2.38)	73.71# (65.92/76.00)†

* Significant differences between hatchery and wild trout;
† Significant differences between wild trout.

found in gills of hatchery trout. In fact, the levels of both Cd/Zn MT and Cu MT in gills of hatchery trout resembled those of Naustebekken trout, whereas the corresponding levels in Rugla trout were significantly lower. In liver, however, the Cd/Zn MT and Cu MT levels in hatchery trout resembled that of Rugla trout. Naustebekken trout had significantly higher Cd/Zn MT and Cu MT levels in this tissue. In kidney, the Cu MT levels were much lower in hatchery trout compared with Rugla and Naustebekken trout.

When condition factors were plotted against Cd/Zn MT levels in various tissues, associations tested with the Spearman rank correlation method were found to be significantly different in liver and kidney in the three trout populations (figure 1). No clear patterns could be seen between the condition factor and Cd/Zn MT in gills or between the condition factor and Cu MT in any of the investigated tissues (data not shown). When the accumulated levels of Cd, Cu and Zn were compared with gills, liver and kidney levels of Cd/Zn MT and Cu MT, we only found positive

Table 4. Metallothionein concentrations (μg MTg^{-1} fresh weight) in gills, liver and kidney of hatchery, Rugla and Naustebekken brown trout *Salmo trutta*. Concentrations are given as medians, first and third quartiles (in parentheses) ($n = 10$, #$n = 9$, ″$n = 6$, ′$n = 5$). The Kruskal–Wallis one-way analysis was used to test whether total (Tot) MT and Cd/Zn MT concentrations were different between the three groups of trout, with Dunn's post test for median comparisons between the different populations when the Kruskal–Wallis test gave a significant result ($P < 0.05$).

Tissue	MT	Hatchery trout	Rugla trout	Naustebekken trout
Gills	Tot MT	355 (303/558)	89 (50/101)*	401 (300/433)†
	Cd/Zn MT [%]	57 (52/63) [16]	35 (32/41) [39]*	61 (56/66) [15]†
	Cu MT [%]	298 [84]	54 [61]	340 [85]
Liver	Tot MT	401 (344/530)	410# (361/497)	901 (793/115)*†
	Cd/Zn MT [%]	120 (114/134) [30]	147 (126/152) [36]	363 (253/631) [40]*†
	Cu MT [%]	281 [70]	263 [64]	538 [60]
Kidney	Tot MT	64′ (35/63)	308″ (248/380)	463 (355/532)*
	Cd/Zn MT [%]	47 (45/50) [73]	67 (55/71) [22]	197 (137/235) [43]*†
	Cu MT [%]	17 [27]	241 [78]	266 [57]

* Significant differences between hatchery and wild trout;

† Significant differences between wild trout. The Tot MT and Cd/Zn MT concentrations were determined by the thiomolybdate assay and the Cd-chelex assay, respectively. Cu MT was calculated as the difference between these two methods (see text for further explanation), while % Cd/Zn MT and Cu MT are shown in brackets.

correlations between the accumulated levels of Cd and hepatic and renal levels of Cd/Zn MT (figure 2). No clear patterns could be seen when accumulated levels of Cu were compared with Cu MT levels (data not shown). When the MT data used here, obtained from trout captured at low water levels, were compared with results from trout captured during a runoff episode with elevated metal levels in the same rivers (Olsvik *et al*. 2000), it became evident that the composition of metals bound to MT changes during runoffs (figure 3).

Genetic variation was found in 10 out of 26 loci in the Rugla trout population and in six of these in the Naustebekken population. No locus within either population showed significant deviation from Hardy–Weinberg proportion of genotypes (Fisher's combined probability for Rugla, $\chi^2 = 9.6$, d.f. = 20, $P = 0.975$ and Naustebekken, $\chi^2 = 6.4$, d.f. = 12, $P = 0.896$). Significant allele frequency differences were found between Rugla and Naustebekken trout at five of the 10 variable loci, resulting in a highly significant genetic differentiation between the two populations ($P < 0.001$). Levels of genetic variation, estimated as the average observed heterozygosity at the 10 variable loci, also differed between populations ($F_{1,621} = 4.20$, $P = 0.041$), with the Rugla population being more heterozygous (average – standard error = 0.273 – 0.022) than the Naustebekken population (0.209 – 0.022).

Discussion

The reported concentrations of metals in Rugla and Naustebekken rivers (table 1) represent measurements of water samples collected when the water levels in these rivers were low. When water samples collected during a runoff episode in October 1997 were analysed for the same metals, the Cu concentration in Rugla

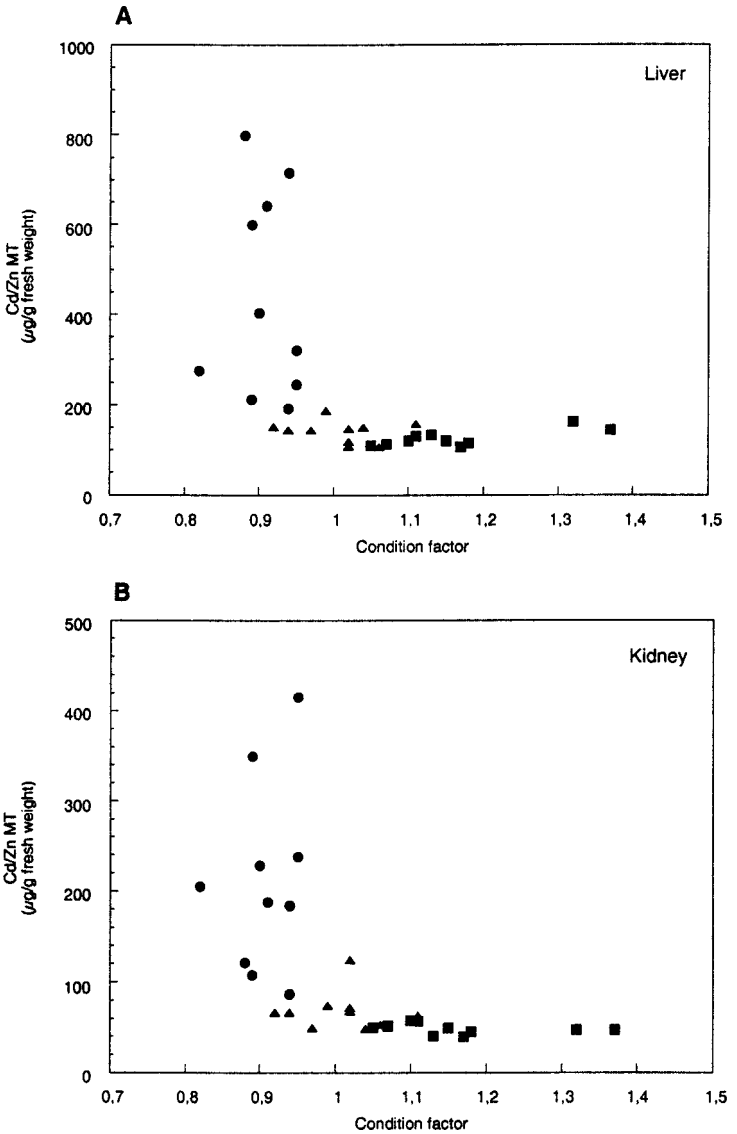

Figure 1. Proportionality between liver (A) and kidney (B) Cd/Zn MT concentrations and the condition factors in hatchery, Rugla and Naustebekken brown trout *Salmo trutta*. The Spearman rank correlation coefficient was considered significant ($P < 0.01$) both in liver and kidney. Individual values are shown in the plot. Hatchery trout (n), Rugla trout (s) and Naustebekken trout (l). Note the different *y*-axes in the figures. $n = 10$.

River was four times higher, while the Cd and Zn levels in Naustebekken River were about twice as high as reported here (Olsvik *et al*. 2000). The metal levels in the river water obviously fiuctuate considerably throughout the year, and the accumulated levels of metals in the trout may therefore refiect higher ambient metal concentrations than is shown in table 1. However, the waterborne levels of metals in these rivers have been monitored for years, and the concentrations shown in table 1 are in accordance with these results (Traaen *et al*. 1987, Iversen 1994,

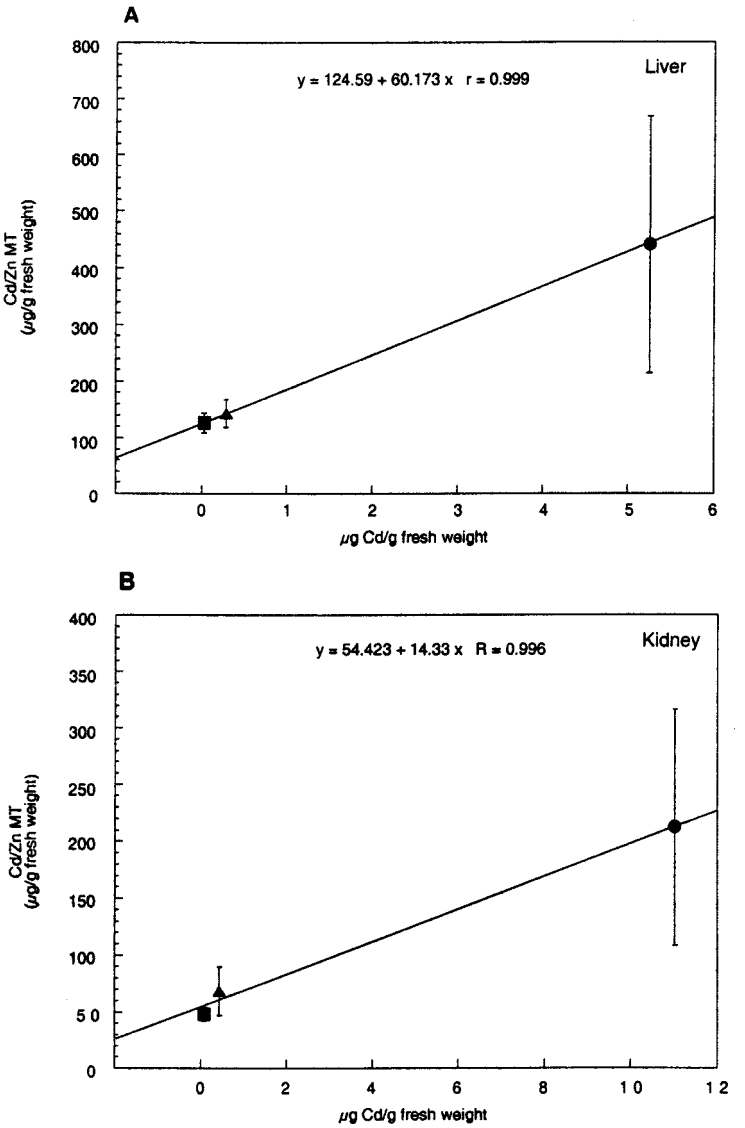

Figure 2. Proportionality between liver (A) and kidney (B) Cd/Zn MT concentrations and Cd concentrations in hatchery, Rugla and Naustebekken brown trout *Salmo trutta*. The Spearman rank correlation coefficient was considered significant ($P < 0.01$) both in liver and kidney. Best fit line presented as the regression line. Each population is presented as means and SD, since Cd concentrations only were measured in six individuals, as compared with the 10 individuals measured for Cd/Zn MT concentrations. Hatchery trout (n), Rugla trout (s) and Naustebekken trout (l). Note the different axes in the figures.

Olsvik *et al*. 2000). The average dissolved fractions of Cd, Cu and Zn (potentially available for biological action) were higher in Naustebekken (82, 59 and 82%, respectively) than in Rugla (64, 55 and 69%, respectively) (P. Gundersen, pers. comm.). The values were estimated from dialysis experiments performed *in situ* in Rugla and Naustebekken throughout 1997. The metals were considered to be dissolved when able to pass through a dialysis membrane with a molecular weight

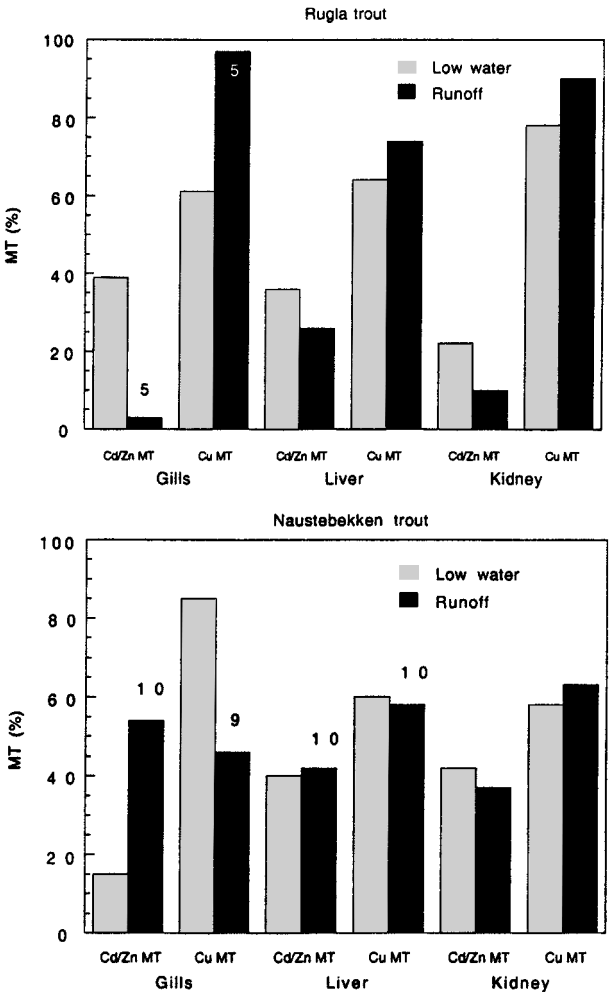

Figure 3. Brown trout *Salmo trutta* Cd/Zn MT and Cu MT (%) in various tissues measured at low water levels compared with the corresponding levels during a runoff episode (the runoff data were obtained from Olsvik *et al*. (2000)). The highest Cd, Cu and Zn concentrations during the runoff were 0.025, 41.2 and 9.3 μg l^{-1} in Rugla, and 0.170, 4.4 and 91.0 μg l^{-1} in Naustebekken, respectively. Low water: $n = 10$, runoff: $n = 6$ (n other than 6 marked in the plot).

cut-off of 10 000–20 000 daltons or allowing particles smaller than 5 nm to pass. Such parameters are known to influence metal speciation and thereby the toxicological impact of Cd, Cu and Zn in natural waters (Davis *et al*. 1993, Campbell 1995). Fish from Naustebekken may therefore be more susceptible to metal exposures, as also suggested by the lower Ca and alkalinity levels in this river (Luoma 1995).

The genetic analyses demonstrate that the Rugla and Naustebekken trout populations are genetically different, and that they do not represent mixtures due to transfer of individuals. Brown trout is considered to be one of the most polymorphic vertebrates (Ferguson 1989), and many papers have been published

on the genetic variation of this species (Skaala 1992). One reason for checking the wild trout populations for genetic differences in this work was that stocking attempts have been done for decades in rivers and lakes throughout Norway, for the purpose of increasing the catches. Biomonitoring is more complicated when genetically mixed populations are involved. Theoretically, exposures to elevated levels of metals over many generations can impose a selective pressure on trout populations, resulting in selection of a more metal-tolerant strain (McDonald and Wood 1993). Differences in metal accumulation, and subsequently in MT levels, may be the result of either genotypically- or phenotypically-based sensitivity, and these may have adaptational or acclimational explanations (Klerks and Weis 1987). However, based on the current data it is difficult to conclude whether the observed differences in metal accumulation and MT levels between the two wild populations have an adaptational basis, but the lower heterozygosity in the Naustebekken than the Rugla population indicates that the Naustebekken trout have adapted to the metals in their environment.

The higher heterozygosity in the Rugla than the Naustebekken population is interesting from at least two perspectives. One relates to the causes of the difference in heterozygosity, the other to the consequences for future generations of trout. In the uppermost parts of the Gaula River, into which Rugla drains, no fish stocks could survive for decades because of the adverse metal pollution. The same situation is valid for Naustebekken trout, because the Lake Orvsjùen, into which Naustebekken drains, has been without fish stocks since 1830, according to local people. The trout captured at both river locations thus represents isolated populations originating from unpolluted parts lying above the sites where the mines discharge their effiuents. The higher heterozygosity in Rugla trout could be due to a number of reasons (cf. Hedrick 1983), including higher heterozygosity in the source population, a larger number of founders, or a more relaxed selection pressure due to metals than in Naustebekken. If metal exposure in these rivers affects the trout populations in adverse ways, one would suspect that fish from the most hostile environment (i.e. Naustebekken) should be more homozygotic due to stronger natural selection (Hoffmann and Parsons 1991). Whatever the causes for different levels of genetic variation, the lower heterozygosity in the Naustebekken population is likely to make it more susceptible to the effects of additional metal exposures. More heterozygous individuals are expected to have a higher mean fitness than homozygous individuals, according to general hypotheses regarding evolutionary genetics and environmental stress (Hoffmann and Parsons 1991). However, further studies are needed to elucidate the possible relationship between heterozygosity and the effects of metal stress in these trout populations.

A large number of parameters have been used as indicators of stress in fin fishes (Barton 1997). Haematological and hydromineral secondary stress parameters such as haematocrit and plasma chloride may change rapidly in minutes and hours, and they are therefore generally not suited as indicators for long-term stress. The observed plasma chloride differences between hatchery and the wild trout in this work could represent a temporary response due to stress during collection, because these parameters may change rapidly after electroshock treatment (Barton and Grosh 1996). However, secondary responses such as reduction in condition factor may well refiect physiological impairment as a result of chronic trace metal exposure (figure 1). When the gross number of trout collected from these rivers over the years 1996–98 are compared, the mean condition factor of Rugla trout

(0.88, $n = 77$) and Naustebekken trout (0.82, $n = 99$) were significantly different ($P < 0.01$). However, the observed variation in condition factor can have a number of reasons (i.e. abiotic: temperature, biotic: genetic, feeding, behavioural), and therefore not solely be the result of trace metal exposures.

The high Cu MT levels in gills of hatchery trout are difficult to interpret. MT is, however, known to be induced by a wide range of stressors in vertebrates (Kägi 1993). In salmonids it has been shown that elevated levels of corticosteroids may induce MT synthesis (Hyllner *et al*. 1989). Increased levels of corticosteroids are the product of the hypothalamo-pituitary-interrenal response to stress in fin fishes (Sumpter 1997), suggesting that MT induction may occur after a general stress response also in fin fishes, as shown for mammals (Kägi 1993). It is therefore possible that the high levels of Cu MT in gills of hatchery trout reflect repeated acute stress (e.g. handling), triggering the 'simple' stress response, as described by Sumpter (1997). Because MT acts as a strong scavenger of oxygen free radicals, MT synthesis by oxidative stress is also documented (Sato and Bremner 1993, Stohs and Bagchi 1995), but currently the mechanisms for such interactions are largely unknown. It is difficult to interpret whether MT induction is a result of trace metal exposure alone, especially in field studies, where it is impossible to evaluate variations in all environmental or biological stress factors. However, trace elements are generally the most powerful inducers of MT in vertebrates (Klaassen and Lehman-McKeeman 1989). The paucity of data concerning the induction of MT following stresses other than trace metals in fishes makes it difficult to interpret the observed Cu MT levels in trout gills. Surprisingly high Cu MT levels were also found in tissues of Naustebekken trout, especially in the liver, containing twice the amount found in Rugla trout liver. MT accounted for more Cu in Naustebekken trout liver (33 %) than in Rugla trout liver (7 %), even though Rugla water and trout tissues contained higher Cu concentrations (tables 3 and 4). The data therefore suggest that Cu MT is of lower importance in Cu-acclimated trout, also supported by findings in rainbow trout *Oncorhynchus mykiss* by Grosell *et al*. (1997). It can also be speculated that MT induced by Cd and Zn may later be saturated by Cu, because of the higher affinity for this metal (Kägi 1993). However, further studies are needed to interpret the reasons for the high Cu MT levels in Naustebekken trout, but these findings clearly indicate the difficulties using Cu MT as a biomarker for Cu exposure.

In non-contaminated environments, liver levels of Zn and basal levels of MT concentrations display a linear relationship, as shown for plaice, *Pleuronectes platessa* (Overnell *et al*. 1988). This proportionality may, however, vary with age and sex, and it may also vary between season and populations (George and Olsson 1994). In rainbow trout, the basal levels of MT may vary during the annual reproductive cycle (Olsson *et al*. 1987). Thus, it can be difficult to establish a baseline of MT concentrations in groups of trout. Such natural variations in MT levels should therefore be examined before MT can be used as a biomarker for trace metal exposures in a species. Since Cu and Zn normally are closely regulated in biological systems, the accumulated levels are not supposed to reflect environmental levels. The MT levels may neither reflect accumulated levels of these metals during chronic exposures, because the metals may be bound to other proteins or be stored in compartments, hiding an eventual positive correlation with MT induction. For the non-essential Cd the accumulated levels are supposed to be positively correlated with MT levels, as was also found in liver and kidney of the

currently investigated brown trout populations (figure 2). Gills can be considered to be the organs most vulnerable during acute exposures (McDonald and Wood 1993), whereas liver and kidney often reflect chronic exposures (Spry and Wiener 1991). Cd in gills may be rapidly cleared via the circulation system to the liver and kidney, where it can be retained for a long time, and this may explain why the Cd/Zn MT levels in gills, being generally low, do not reflect the accumulated Cd levels.

The composition of metals bound to MT may obviously change after runoff episodes (figure 3). Three important points can be deduced from these results:

1. The composition of metals bound to MT changes in accordance to fluctuations of metal levels in the ambient water, as shown for all studied tissues, except for kidney of Naustebekken trout.
2. The gills show the most dramatic changes in metal composition, whereas smaller changes appear in liver and kidney.
3. Cu seems to be the most potent trigger for alterations in metals bound to MT in gills, in accordance to the high MT affinity for this metal.

When trout were transferred to and kept in cages in the other river during the runoff episode (Olsvik *et al*. 2000), the highest MT induction was found in gills and liver of Naustebekken trout kept in Rugla, suggesting that Rugla trout have acclimated to the higher Cu levels in their own river. Accumulated metal levels in tissues might not reflect rapid and transient environmental changes, but rather the cumulative concentrations of metals in the water over a longer period that may last for months. The correlation between accumulated metal levels and the corresponding MT levels in tissues may therefore not display a clear linear relationship in trout captured in field studies, making the interpretation of such result more difficult. Overall, runoff episodes with elevated metal levels may have a great toxicological impact on brown trout inhabiting Cd, Cu and Zn contaminated rivers.

To conclude, the data reveal that it may be difficult to apply gill MTs as biomarkers for prolonged trace metal exposure in brown trout. The application of gill Cd/Zn MT as a biomarker for acute changes in river Cd concentration seems to be valid as long as the river Cu concentration is low. Liver and kidney Cd/Zn MT can be used as a biomarker for chronic Cd exposure in brown trout exposed to elevated concentrations of this metal. Cu MT, on the other hand, seems less suitable as a biomarker for Cu exposure in gills, liver and kidney of brown trout inhabiting Cu-contaminated freshwater environments. It is therefore important to differentiate between Cd/Zn MT and Cu MT when these proteins are used as biomarkers for trace metal exposure in various tissues. More investigations are, however, needed to reveal how high environmental levels of Cu affect MTs in aquatic animals under field conditions. This study further emphasizes that application of MTs as biomarkers for trace metal exposures must be done with caution.

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