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Increase of metallothionein-immunopositive chloride cells in the gills of brown trout and rainbow trout after exposure to sewage treatment plant effluents

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Summary

Metallothionein, a biomarker of exposure and toxicity of heavy metals, has been detected in the gills of brown trout (*Salmo trutta fario* L.) and rainbow trout (*Oncorhynchus mykiss* Richardson) by means of immunohistochemistry. A very prominent labelling of chloride cells was found after exposure to diluted sewage plant effluents. No significant increase was observed in either the number of labelled cells or their labelling intensity after exposure to water of a polluted river compared to fish kept in tap water. These results do not correlate with findings of a histopathological study, suggesting that the metal levels at the sewage treatment plant were too low to produce gross histopathology. A comparison between the species indicated that the rainbow trout showed a generally higher metallothionein expression than the brown trout.

Introduction

The river Langeten, district of Berne, Switzerland, is known for its decrease in fish populations for many years. However, possible causative agents have either not been detected or already been eliminated (Ochsenbein 1997). Since heavy metals are known to affect fish in many ways (see Olsson *et al*. 1998), metal pollution could be one factor in the assessed impairment of fish in this river (Frick *et al*. 1998, Schmidt *et al*. 1999). At the lower stretch of the river Langeten, elevated zinc concentrations in the river sediment have been found, but the cause could not be detected. As possible sources, sewage treatment plant effluents are of interest since these sites are often the only point sources discharging concentrated waste water into rivers. However, analysis of heavy metal concentrations in the water and sediments alone gives little information about the actual exposure situation and the bioavailability for aquatic organisms (Hodson 1988). In contrast, metallothionein is a reliable tool for providing an answer whether or not an organism is affected by heavy metals.

Metallothionein is a low-molecular weight protein, rich in cysteine, which is induced by cadmium, zinc, copper and silver and is found in all vertebrates. Even if the detailed functions of metallothionein are not clearly established, its involvement in the metabolism of zinc and copper is generally accepted (Maret & Vallee 1998, Vallee 1995). Metallothionein is believed to play a protective role against the toxic effects of metals by sequestering and reducing the amount of freely available, or bioreactive, metals in tissues thereby reducing potential toxicity. Tissue concentrations of metallothionein are often increased after exposure to heavy metals as a function of increased levels of bioreactive metal (Olsson *et al*. 1998). Since it is this bioreactive pool of the accumulated metals that induces metallothionein synthesis and since it is the same fraction that is likely to react with other biomolecules, metallothionein can be considered not only as a biomarker of exposure, but also of toxicity. This notion is supported by carefully conducted multi-level case studies of metal impacted areas in which liver metallothionein concentrations were found to correspond well to metal-induced pathological aberrations and changes at higher levels of biological organization (Farag *et al*. 1995, Hogstrand & Haux 1990a). For the above reasons, metallothionein is one of the most commonly used biomarkers of heavy metal impact on aquatic animals. In mammals, many other exogenous and endogenous factors are known to induce metallothionein synthesis (Klaasen & Lehman-McKeeman 1989). Of these, only free radicals seem to be of significance for metallothionein gene activation in salmonids (reviewed by Olsson *et al*. 1998). However, even free radicals are weak inducers in a comparison with metals, such as zinc (Kling *et al*. 1996, Olsson & Kille 1997).

The highest metallothionein concentrations in environmentally-exposed fish are usually found in liver and kidney, which are major accumulatory organs for metals (Dallinger *et al*. 1997, Hogstrand *et al*. 1991). Data concerning the

increase of metallothionein in gills after exposure to heavy metals are inconsistent. In many studies no increase of branchial metallothionein has been found although other tissues showed significant increases of metallothionein (Farag *et al*. 1995, Wicklund Glynn *et al*. 1992). Other reports do show elevated metallothionein levels in response to metal exposure (Benson & Birge 1985, Dang *et al*. 1998, Stagg *et al*. 1992). The gill is the major target organ for acute copper, zinc, cadmium, and silver toxicity to freshwater fish (Hogstrand & Wood 1998, Karlsson-Norrgren *et al*. 1985, Olsson *et al*. 1998). In freshwater fish, the gill is the dominant uptake site for waterborne metals and it has been suggested that many metals are specifically absorbed by the ion-transporting chloride cells (reviewed by Olsson *et al*. 1998). Furthermore, recent data suggest that metallothionein is differentially expressed in chloride cells during exposure to waterborne copper (Dang *et al*. 1998). The chloride cells are characterized by numerous mitochondria and an extensive tubular system equipped with a high copy number of Na/K-ATPase (see review by Perry 1997). They are located mostly in the interlamellar regions and at the junctions between the filaments and the lamella. Normally, there is only a sparse population of chloride cells on the lamellae (Perry 1997). The relative density of chloride cells is low, contributing to only 5–10% of all gill epithelial cells in freshwater fish (Mueller *et al*. 1991, Perry & Laurent 1993). Thus, an increase of metallothionein restricted to the chloride cells may not be detectable in whole gill tissue homogenates. Consequently, cell-selective immunohistochemistry could be a useful approach. Antibodies against fish metallothionein are available from several sources (Hogstrand & Haux 1990b, Hylland *et al*. 1994, Norey *et al*. 1990) but rarely reported to be used in immunohistochemistry (Dang *et al*. 1998, Kito *et al*. 1986).

Two salmonid species were of interest from the perspectives of the present study. The brown trout (*Salmo trutta*), the native species in middle European river systems, is reported to be more sensitive to pollutants than the rainbow trout (*Oncorhynchus mykiss*) which was introduced at the end of the last century (Peter 1995, Schmidt *et al*. 1999). The rainbow trout has been more intensely investigated and, moreover the legislation of many countries declares it to be a model species for toxicity testing.

The objectives of the present study were to investigate if (i) metallothionein can be localized in the gill of brown and rainbow trout by means of immunohistochemistry; if (ii) sewage treatment plant effluent or polluted water from the river Langeten induces metallothionein in trout species; and if (iii) there are differences between brown trout and rainbow trout in respect to metallothionein expression in gills.

Materials and methods

Exposure sites

The river Langeten is located in the Northwest of Switzerland, district of Berne, on calcareous subsoil. Its water quality as defined by the saprobial index, has been declared to be moderately to heavily polluted (Aquaplus 1994, Vokos 1997). The river receives effluents from several sewage treatment plants. One of the plants, ARA Lotzwil, was chosen for our experiments due to its overload and because the fish density was extremely low downstream of this plant. Waste water originated mainly from small industries and several villages. The plant had both biological and chemical cleaning steps, the latter precipitated phosphorous by the addition of chloride sulphate with 2% aluminium since 1996. In 1996, the plant served a population of 7700 inhabitant equivalents, with 33% industrial wastewater. Nitrification was about 77%. Regular measurements of heavy metals in the sewage sludge revealed no episodic event exceeding the consent limits during the experimental period (H. Bürgy, personal communication). A second experimental site was located 8 km downstream of the sewage treatment plant, at the village of Roggwil.

Fish and experimental design

Sixty brown trout (*Salmo trutta*) and 60 rainbow trout (*Oncorhynchus mykiss*) from the culture stock located in the Centre of Fish and Wildlife Health were randomly assigned to groups of 30 fish per species. They were transferred to round 2000 L fibreglass tanks, one of each located at the two experimental sites. Brown and rainbow trout were kept separated in the same tanks by a perforated steel sheet (diameter of perforations: 1 cm). The tanks were covered partly by a lid, the remaining part covered by a net. At the sewage treatment plant (Lotzwil site), the tank received a constant flow-through (20 L/min) of aerated effluent water diluted with commercial tap water in a ratio 1 : 10. This dilution corresponds to the dilution in the river directly downstream of the point of discharge into the river. At Roggwil, the fish were kept in water from the river Langeten (temperature 3–16 °C), which was mechanically filtered (mesh size: 2 cm) to remove coarse plant material. A further 10 brown trout and 10 rainbow trout were held as controls at the Centre of Fish and Wildlife Health in slightly chlorinated (< 0.01 mg/L) municipial tap water at a water temperature ranging between 5 °C in January and 17 °C in July and an oxygen concentration of ≥ 8 mg/L. Here, the two species were kept in separate 2000-litre fibreglass tanks receiving a constant flow-through of 26 l/s (1560 L/min).

At the beginning of the experiment, the length of brown trout ranged from 13 to 17 cm and that of rainbow trout from 19 to 22 cm; the weight of the brown trout ranged from 20 to 50 g and that of rainbow trout from 70 to 110 g. All fish were fed commercial trout pellets (HOKOVIT, Switzerland) with a daily food ratio equal to 1–2% of the body weight.

Sampling

First sampling was performed in July 1996, two months after stocking. Further samplings took place in January 1997. Exposure at the sewage treatment plant was terminated at the end of April 1997 and remaining fish were transferred to the tapwater at the Centre of Fish and Wildlife Health and

were sampled in July 1997 after three months of recovery. At Roggwil, another sampling was performed in July 1997. The controls were sampled in January 1997 and November 1997.

Due to unexpected high mortalities which were caused by a technical accident at the sewage plant effluent during the summer months (Schmidt *et al*. 1999), unexposed control animals were inserted into the tanks (17 brown trout and 27 rainbow trout) marked by a fin cut.

At each sampling, five fish were euthanized in buffered 3-aminobenzoic acid ethyl ester 100 mg/L (Finquel; Argent Chemical Labs., Redmond, USA), the second gill arch of the left body side fixed in Bouin's fluid for 24 h, paraffin wax embedded, and sections cut at a thickness of 6 µm.

Immunohistochemistry

Endogenous peroxidase of the unstained sections was quenched by treatment with 3% H_2O_2 in methanol for 15 min at room temperature (RT). After rinsing in water and 50 mM Tris-buffered saline (TBS) pH 7.6, the sections were immunohistochemically labelled with the polyclonal antibody against perch metallothionein (Hogstrand and Haux 1990b) for 12 h in a moist chamber (4 °C). The antibodies were used in a dilution of 1 : 300 as determined by serial dilution for optimal target-background ratio. After rinsing, sections were incubated with a link serum diluted 1 : 20 (Swine anti-rabbit IgG, Dako A/S, Glostrup, Denmark) for 1 h at RT and rinsed thereafter. Then, the sections were incubated with rabbit peroxidase anti-peroxidase (PAP; 1 : 100; Dako) for 1 h at RT. After a further wash in TBS for 2 × 5 min and subsequent incubation with freshly prepared 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma) for 7 min, the sections were rinsed again, dehydrated and mounted in Entellan. Controls included replacement of specific antisera by the buffer or by the diluted metallothionein antiserum pre-incubated overnight at 4 °C with 210 ng/µl of purified rainbow trout metallothionein. Both controls resulted in the lack of labelling. The absence of non-specific binding of the monoclonal antibodies was shown by using the inappropriate polyclonal antibody anti-vitellogenin (AA-1; Biosense Laboratories AS, Bergen, Norway). This latter antibody is of the same Ig subclass as the anti-metallothionein polyclonal antibody used.

The monoclonal antibody alpha 5 directed against the alpha subunit of a Na^+/K^+-ATPase (alpha 5, Developmental Studies Hybridoma Bank, University of Iowa, Department of Biological Sciences, Iowa City, USA) was applied on several consecutive sections for comparison with the metallothionein-labelled cells.

For the semiquantitative analysis of each animal three filaments per gill arch were evaluated for metallothionein-positive chloride cells. These were defined by their localization at the filament, and their shape as well as the shape of the nuclei. At each filament, the base, the middle part and the tip of the filament were considered. For the quantification, only the chloride cells at the filament were counted (and not those located at the lamellae). The number of metallothionein-positive chloride cells per 0.05 mm length of the gill filament regions (base, middle, tip) were counted and recorded. The mean of three counts per fish was used to calculate the number of labelled chloride cells. A Zeiss Axioskope microscope with a 20× objective was used.

Statistics

The data were checked for assumptions of normality. Differences between the different sites were tested using Kruskal–Wallis one-way analysis of variances. Differences between different exposure trials at a given site were tested using Kruskal–Wallis one-way analysis of variances with Bonferroni adjustments. Mann–Whitney *U*-Test with Bonferroni adjustments were used to test fish of the sewage treament plant group against the Roggwil group of the river Langeten at each exposure interval. A p-value of <0.05 was regarded as significant. Statistical evaluations were done with SYSTAT statistical program (SPSS Inc. Chicago, USA).

Results

Controls

In both species, immunohistochemistry with anti-perch metallothionein serum produced a metallothionein-specific labelling in the gill. The metallothionein staining was predominantly found in cells located in the inter-lamellar regions of the filaments. Scrutiny of adjacent sections indicated that the metallothionein-positive cells were also Na/K-ATPase positive. This strong Na/K-ATPase staining combined with their morphology and location identified the metallothionein-positive cells as chloride cells. In addition, a very slight labelling of the superficial respiratory gill epithelial cells and of single cells in the connective tissue was observed.

The following results were presented in this order: For each species, differences in the number of labelled cells after the exposure regimes are described. Then, observations on labelling intensity were recorded.

Exposure of rainbow trout

In rainbow trout, significant differences between treatment groups were evident (Kruskal–Wallis; $H = 28.29$; $p < 0.001$) (Figure 1A). The number of labelled chloride cells was significantly higher in the groups exposed to the sewage treatment plant effluent and their 'recovery' counterparts than in the Roggwil group and in the control fish. Gill metallothionein expression in fish from the latter two sites were not significantly different. Whilst there was considerable heterogeneity in the number of labelled cells observed between different filaments there were no differences in the number of labelled chloride cells ($p > 0.05$) between the two samplings at the sewage treatment plant effluents. At each sampling occasion in July 1996, January and July 1997, fish exposed to sewage treatment plant effluents and their 'recovery' counterparts showed significantly higher numbers

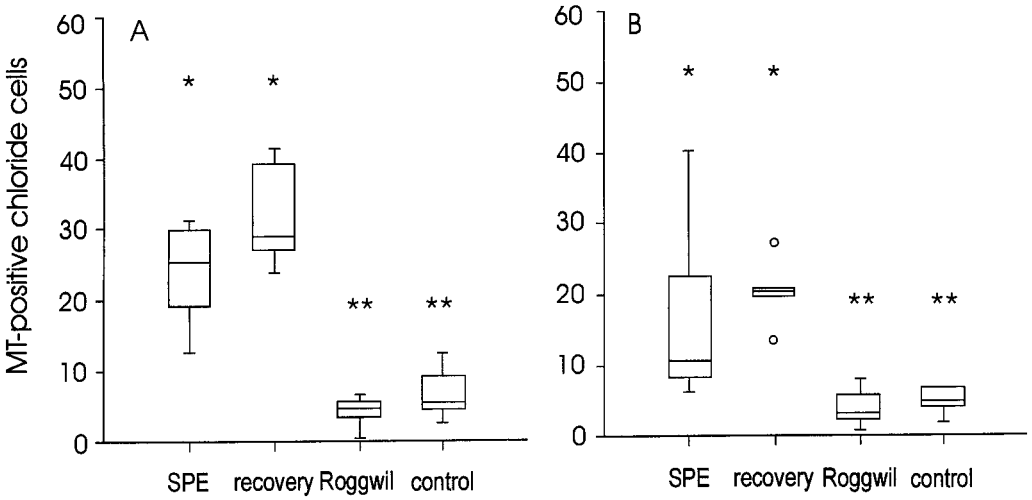

Figure 1. Number of metallothionein (MT)-positive chloride cells of rainbow trout (A) and brown trout (B) exposed in diluted sewage treatment plant effluents (SPE) and following recovery period in tap water (recovery), as well as exposed in water of the river Langeten (Roggwil) and tap water (control). There are no significant differences between groups signed with the same symbol (Kruskal–Wallis). The figure includes data from all samplings conducted at the respective sites, i.e.: SPE: 10 animals of each species (5 of July 1996 and 5 of January 1997); recovery: 5 animals of each species; Roggwil: 15 animals of each species (July 1996, January 1997, July 1997); control: 10 animals of each species (January 1997, November 1997).

Table 1. Metallothionein-immunopositive chloride cells in rainbow trout and brown trout after different times and sites of exposure. Values represent medians ± MAD (median absolute deviation). Numbers in brackets: exposure time in months.

Species	Site	July 1996 $n = 5$	January 1997 $n = 5$	July 1997 $n = 5$
Rainbow trout	Sewage treatment plant effluent	29.0 ± 1.3 (2)	21.0 ± 7.3 (8) (2)**	29.0 ± 5.3 (5/3)*
	Langeten river (Roggwil)	3.0 ± 0.7[1] (2)	6.0 ± 0.7 (2)	4.7 ± 1.0 (8)
Brown trout	Sewage treatment plant effluent	22.3 ± 7.0 (2)	8.3 ± 1.0[2] (8) (2)***	20.3 ± 0.6 (5/3)*
	Langeten river (Roggwil)	2.0 ± 0.7[3] (2)	7.0 ± 1.0 (2)	4.3 ± 1.7 (8)

*5 months exposure and 3 months of recovery in tap water.
**One rainbow trout was exposed for two months and four rainbow trout were exposed for 8 months.
*** Two brown trout were exposed for 2 months and three animals for 8 months, respectively.
[1]Significantly different from the group January 1997 of the same exposure site (Kruskal–Wallis; $H = 7.692$; $p < 0.025$).
[2]Significantly different to the other groups of the same exposure site (Kruskal–Wallis; $H = 7.609$; $p < 0.025$).
[3]Significantly different to the other groups of the same exposure site (Kruskal–Wallis; $H = 8.68$; $p = 0.013$).

of metallothionein-positive chloride cells than fish of the Roggwil group (Mann–Whitney U-test; $U = 25.0$; $p = 0.009$). In the latter group, the numbers of metallothionein-positive labelled chloride cells were significantly elevated in January 1997 compared to animals sampled in July 1996 (Kruskal–Wallis; $H = 7.7$; $p < 0.022$) whereas no significant differences were found between the samplings of January 1997 and July 1997. The number of metallothionein positive chloride cells did not change with increasing time period of exposure (cf. Table 1, January 1997).

Chloride cells at the base of the lamellae were most intensively labelled (Figure 2A,C), especially in the basal part of the filament, but sometimes also at the tip of the filament. However, the latter were restricted to a smaller area. The labelling intensity was similar in all rainbow trout sampled directly from the sewage treatment plant effluent and after a

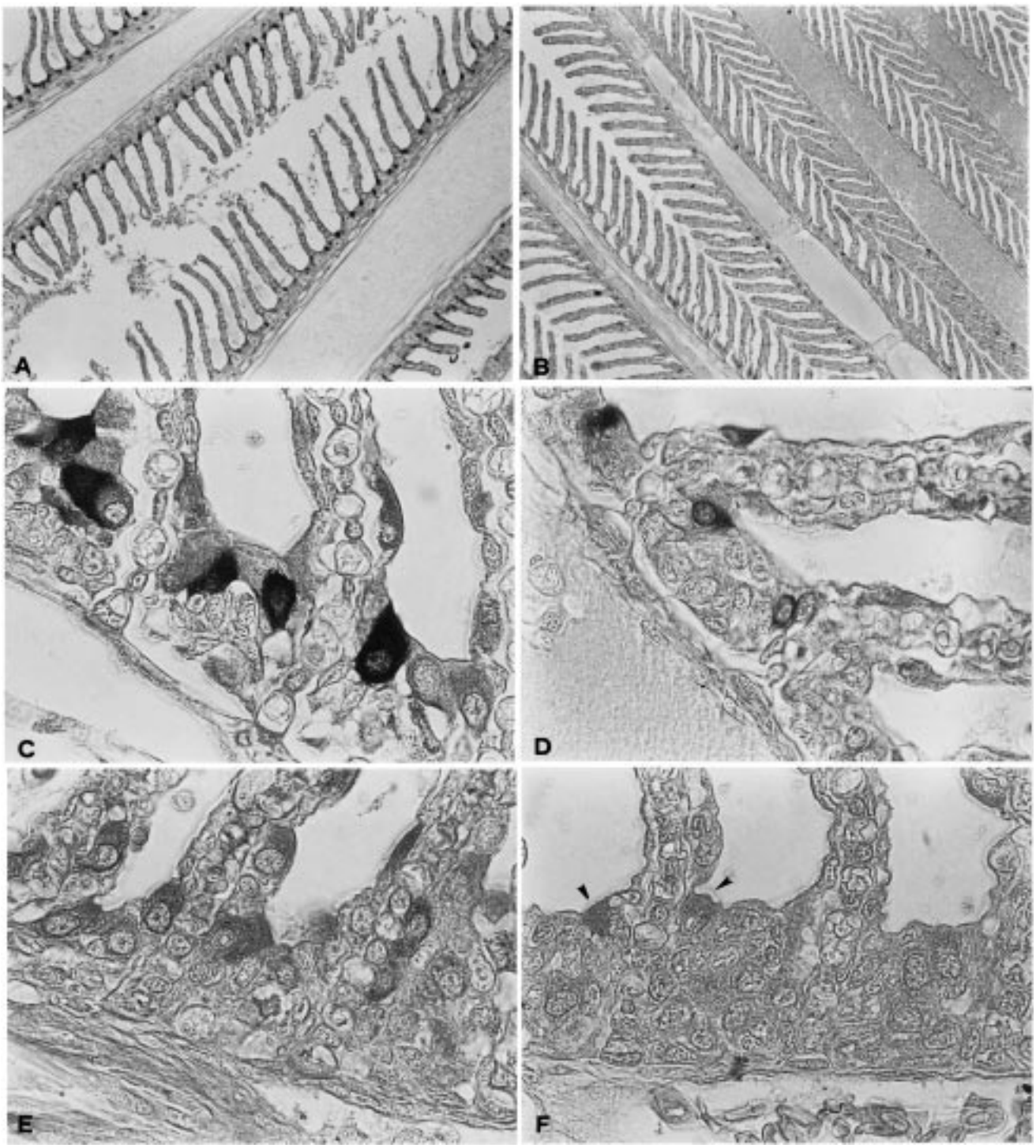

Figure 2. Immunohistochemical detection of metallothionein in the gill of rainbow trout (A,C,E) and brown trout (B,D,F). A: Intensely labelled chloride cells after exposure to sewage treatment plant effluents and three months recovery in tap water are located predominantly in the interlamellar region of the gill filament (80×) B: After exposure to sewage treatment plant effluents, chloride cells labelled with anti-metallothionein in brown trout were less numerous than in the rainbow trout after recovery (cf. Figure 2A) (80×). C: Rainbow trout exposed to sewage treatment plant effluents and sampled in January revealed metallothionein-labelled chloride cells. The labelling intensity is variable as well as the labelling of the nuclei (830×). D: Gills of brown trout exposed to sewage plant effluents sampled in January showed metallothionein positive chloride cells, however, of slightly lower labelling intensity than in the rainbow trout (cf. Figure 2C) (830×). E: After exposure to river water and sampled in January, several chloride cells showed a moderate labelling intensity in rainbow trout (830×). F: Control brown trout kept in tap water revealed a weak labelling of few chloride cells (arrowheads) (830×).

post-exposure recovery period of three months, but slightly less intense in the Roggwil group (Figure 2E).

Exposure of brown trout

Significant differences were also evident between brown trout treatment groups (Kruskal–Wallis; $H = 24.78$; $p < 0.001$) (Figure 1B). Again, the number of labelled chloride cells was significantly higher in fish exposed to sewage treatment plant effluent and the post-exposure 'recovery' group than in the Roggwil and control groups; differences between the latter two sites were not significant. Brown trout, sampled at the sewage treatment plant effluent in January 1997, showed significantly lower numbers of metallothionein-positive chloride cells compared to the other sampling occasions at the same site (Kruskal–Wallis; $H = 7.6$; $p = 0.022$, Table 1). In July 1996 and 1997, the amount of metallothionein-positive chloride cell numbers were significantly higher in sewage plant effluent fish than in the Roggwil group sampled in July 1996 and July 1997 (Mann–Whitney U-test; $U = 25.0$; $p = 0.009$), whereas there was no significant inter-site difference in January 1997 (Mann–Whitney U-test; $U = 20.0$; $p > 0.1$). As in rainbow trout, there was no obvious correlation between time period of exposure and the number of labelled chloride cells (two versus eight months). The lamellar distribution of labelled chloride cells was similar in brown trout as in the rainbow trout, but immunostaining was slightly lower than in rainbow trout (Figure 2B,D). However, there was still a clear difference between the sewage treatment plant groups (exposure and recovery groups) and the other groups (downstream and controls) in respect to labelling intensity. Brown trout from Roggwil exhibited clearly lower intensity of labelled chloride cells in July 1996 when compared to either of the other two samplings at this location. However, metallothionein labelling intensity was still higher than the staining in chloride cells of the controls kept at the Centre of Fish Health at Bern (Figure 2F).

Discussion

In the present study, we demonstrate that metallothionein is expressed differentially among cells in gill tissue from two salmonid species. This, and the inducibility of metallothionein can be detected by immunohistochemistry. Thus, reports from previous studies of no or low metallothionein induction in gills during metal exposure may be misleading because whole-gill homogenates were analyzed, as opposed to metallothionein expression in individual cell types within the gill. The differential expression of metallothionein in chloride cells was concluded from a comparison of consecutive histological sections labelled with metallothionein-antibody or a specific antibody against Na/K-ATPase, which is a chloride cell marker enzyme (Witters *et al*. 1996). These results are totally in line with previous evidence suggesting that many metals specifically cross the gill epithelium through the chloride cells (reviewed by Olsson *et al*. 1998, Wood 1992). Interestingly, labelled chloride cells were mainly located in the inter-lamellar space, whereas chloride cells at the lamellae were mostly metallothionein-negative. It is believed that lamellar and inter-lamellar chloride cells are of different sub-types (Perry 1997). The lamellar, or α chloride cells may be precursors of seawater-type chloride cells, whereas the inter-lamellar, or β chloride cells are present only in freshwater-dwelling teleosts (Pisam *et al*. 1995, Pisam & Rambourg 1991). The selective metallothionein-staining in the inter-lamellar chloride cells found in the present study supports the view of functionally distinct chloride cell subtypes and suggests that the inter-lamellar chloride cells are involved in metal uptake. Strong pharmacological and correlative evidence exists that waterborne zinc and cadmium enter the branchial epithelium via the same apical transporter of the gill epithelium as calcium (Hogstrand *et al*. 1996, 1998, Verbost *et al*. 1987, 1989). Transport of calcium across the branchial epithelium most likely takes place across chloride cells (see Perry 1997) but there is conflicting evidence as to which of the two sub-types of chloride cells transport calcium. Payan *et al*. (1981) presented evidence that the chloride cells on the gill filaments are the sites of branchial calcium uptake whereas experiments conducted by Perry & Wood (1985) indicated that the lamellar chloride cells are more important sites of calcium uptake. Since uptake of zinc and cadmium likely occurs in the same cell type as that of calcium and since both these metals are strong inducers of metallothionein, one approach to identify the calcium transporting chloride cell sub-type could be to expose trout to zinc or cadmium with subsequent immunohistochemical localization of metallothionein.

Although immunohistochemistry is limited in the quantification of effects (Van Veld *et al*. 1997), we were able to show a significant increase in the number and in the labelling intensity of metallothionein-positive chloride cells in both trout species after exposure to diluted sewage treatment plant effluents. The causative agent(s) for this elevated metallothionein-level has not been identified, although one or several of the potent inducers of fish metallothionein (Cu, Zn, Ag, Cd, Hg) are likely candidates. Some of the industries discharging their sewage water into the sewage plant at Lotzwil use several of these metals, including zinc, cadmium, mercury, and copper. The chemical analysis of the sewage sludge revealed metal concentrations clearly below the pollutant limits ordered by legislation for Switzerland (StoV 1986). Of the metal concentrations measured in the sediment downstream of the sewage effluent, only the zinc value (230 µg/g dry weight; Bürgy, personal communication) exceeded the sediment criterion in Switzerland (VSBo 1986; zinc limit 200 µg/g). No levels exceeding the limits for heavy metals in the river (AbwV 1975) are known. Thus, the results suggest that chloride cell metallothionein expression is a very sensitive indicator of metal exposure, signalling effects at levels below or at current regulatory criteria.

An alternative explanation to the elevated metallothionein expression in fish from the sewage treatment plant location is that factors other than metals induced metallothionein synthesis. This has been firmly established for a number of noxious stimuli and agents (Klaasen & Lehman-McKeeman 1989) although such factors generally seem to be of lesser importance in fish (reviewed by Olsson *et al*. 1998). Stress seems to induce metallothionein synthesis in some fish species (Baer & Thomas 1990, Sabourin *et al*. 1986) whereas neither stress nor injection with glucocorticoids or inflammatory agents remarkably seem to affect metallothionein levels in others (Baer & Thomas 1990, Overnell *et al*. 1987). Laboratory experiments and numerous field studies as well as *in vitro* studies suggest that salmonids belong to the latter category (Olsson *et al*. 1998, Hogstrand, unpublished data). In summary, non-metal agents are not very effective in inducing metallothionein in rainbow trout. If the sewage sludge contained a non-metal component that was responsible for the increased expression of metallothionein in chloride cells it probably acted either via the *jun-fos* pathway or by indirectly increasing the metal activities in the chloride cells. Everything considered, we favour the explanation that metallothionein was induced by metals in the present study.

It was surprising to us that the chloride cells of fish from the sewage treatment plant effluent stained heavily for metallothionein after three months of recovery. These results would suggest that a significant amount of metal still was active in the chloride cells at this point. However, the average generation time of gill epithelial cells was demonstrated to be in the range of about two weeks (Conte & Lin 1967). This would raise the question whether it is possible that newly developed chloride cells are able to 'inherit' the induction of metallothionein from the old dying ones or whether there is another pool of metals from which metals flow to the newly synthesized chloride cells and stimulate metallothionein induction long after the actual exposure was finished. Studies on zinc and cadmium in minnows and rainbow trout have shown that there are rapid and a slow turnover pools of these metals in the gills. However, the rapidly eliminated pool, which typically has a half-life of a week or less, constitutes most of the zinc and cadmium in gills of minnows during chronic exposure (Wicklund Glynn 1991, Wicklund Glynn *et al*. 1992).

The comparatively low numbers of metallothionein-positive chloride cells in the fish exposed to the river water were unexpected. It is especially interesting that the liver, kidney, gill and skin from rainbow and brown trout showed pronounced histological alterations when exposed to the Langeten water at Roggwil, but, in comparison, fish held in the sewage treatment plant effluent did not demonstrate significant histopathological changes (Schmidt *et al*. 1999). These results, together with the ones in the present study, suggest that the agent that causes histopathology to fish in the river Langeten may not be a metallothionein inducing metal, and that the metal levels that resulted in chloride cell metallothionein induction at the sewage treatment plant were too low to produce gross histopathology.

Comparisons between species

The ratio of metallothionein-positive cells in rainbow trout to that in brown trout ranged between 1.1 and 2.5. It was interesting to find that metallothionein expression generally was higher in rainbow trout than in brown trout subjected to the same conditions. These results are in accordance with previous observations. When comparing the cadmium content in the gills of rainbow trout with that of the brown trout, the latter always showed a lower content, in different exposure regimes (Roberts *et al*. 1979). Thus, the stronger metallothionein response in rainbow trout could be due to a higher branchial metal accumulation than in brown trout.

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