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The toxicity of iron to brown trout and effects on the gills: a comparison of two grades of iron sulphate

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The 96-h LC_{50} on brown trout *Salmo trutta* of a commercial iron (III) sulphate liquor, used for treating reservoirs to reduce algal growth, was 28 mg total Fe l^{-1} (0·05 mg soluble Fe l^{-1}). The 96-h LC_{50} for analar grade iron (III) sulphate was 47 mg total Fe l^{-1} (0·24 mg soluble Fe l^{-1}). Lethal and sublethal exposure to both grades of iron resulted in accumulation on the gill, which appears to be the main target for iron toxicity. Greater iron accumulation occurred during exposure to commercial iron sulphate liquor. Physical clogging of gills and gill damage was seen during lethal and sublethal exposure to iron. Gill tissue analysis showed no evidence of iron uptake into gill tissues during lethal or sublethal exposure to iron. Iron did not accumulate in plasma of fish exposed to iron compared to controls. Respiratory disruption due to physical clogging of the gills is suggested as a possible mechanism for iron toxicity.

Key words: iron sulphate; *Salmo trutta*; toxicity; gills; respiration.

INTRODUCTION

The water companies of south-east England exploit the complexing behaviour of iron to combat algal blooms in reservoirs. Iron binds to negatively charged phosphate ions and reduces soluble phosphate available to algae (Hayes *et al.*, 1984). Iron has been used widely in the U.S.A., U.K. and Holland to reduce available phosphate in fresh waters (Welch, 1980; Hayes *et al.*, 1984). Typically iron is added to a reservoir in the form of an acidic ferric sulphate liquor. The liquor is a by-product of a titanium extraction process and is contaminated with other trace metals, including titanium, manganese, zinc, and nickel (Table I). It is also extremely acidic (pH 0·6).

There is a lack of information on the possible toxic effects of this treatment process on fish. There is also little information on the specific action of iron. However, there is general agreement that the gills are the main site of iron toxicity. Several authors report the deposition of iron flocs on to the gill epithelium, resulting in gill clogging and damage (Larson & Olsen, 1950; Kinne & Rosenthal, 1967; Brenner *et al.*, 1976). Iron sulphate forms an insoluble precipitate of iron hydroxide in water at near neutral pH. Lehtinen & Klingstedt (1983) exposed perch *Perca fluviatilis* L., to an effluent from a titanium dioxide producing plant. The main constituent of the effluent was iron in the ferrous

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TABLE I. Composition of commercial grade iron (III) sulphate liquor and analar grade iron (II) sulphate

Parameter	Commercial liquor				Analar grade	
	Analysis 1*		Analysis 2†		Based on a 180 g l^{-1} stock‡	
Total iron	174·8	g Fe l^{-1}	180	g Fe l^{-1}	180	g Fe l^{-1}
Fe^{3+}	11·3	% w/w	NM		NM	
Fe^{2+}	0·16	% w/w	NM		NM	
Free acid	0·37	as H_2SO_4 w/w	NM		NM	
Copper	0·5	mg l^{-1}	3·2	mg l^{-1}	1·8	mg l^{-1}
Silver	NM		0·02	mg l^{-1}	NM	
Zinc	80	mg l^{-1}	47·5	mg l^{-1}	9	mg l^{-1}
Cadmium	2	mg l^{-1}	0·05	mg l^{-1}	NM	
Mercury	<0·05	mg l^{-1}	0·005	mg l^{-1}	NM	
Titanium	600	mg l^{-1}	1490	mg l^{-1}	NM	
Lead	5	mg l^{-1}	0·38	mg l^{-1}	0·9	mg l^{-1}
Chromium	3	mg l^{-1}	6·3	mg l^{-1}	NM	
Manganese	700	mg l^{-1}	850	mg l^{-1}	90	mg l^{-1}

*Typical analysis from supplier: E&A West, Derbyshire.
†Analysis from the Environment Agency, Anglian Region, Peterborough.
‡Typical analysis from Merck Ltd.
NM, Not measured.

state, but it also contained a considerable amount of titanium dioxide particles. Brown precipitates containing relatively large amounts of iron and titanium were observed on the gills of fish. Although zinc, copper, lead and cadmium could also be found in this effluent (Larsson *et al.*, 1980) these metals were not detected accumulating on the gill surface.

Gonzalez *et al.* (1990) suggested that respiratory distress was a significant factor in the mortality of brook charr *Salvelinus fontinalis* (Mitchill) on exposure to iron, and Grobler-van Heerden *et al.* (1991) observed a decrease in activity, coughing, yawning, spasmodic movements and an increase in opercular movements in iron exposed *Tilapia sparrmanii* (Smith). Peuranen *et al.* (1994) agreed with earlier research, observing iron deposits on the surface of gill epithelia in brown trout *Salmo trutta* L., exposed to iron. They reported gill damage during exposure to 0·8–1·7 mg l^{-1} iron at pH 5 and 6. They suggested that as iron had been detected only on the surface, and not inside gill epithelia, it exerted its toxicity through action on the gill surface. Exposure to sublethal levels of acidity, without the modifying effects of trace metals will cause morphological changes to gill tissue (McDonald, 1983; Wood, 1989) but will not cause substantial structural damage. Lethal acid exposure causes significant structural damage to gill tissues, such as lamellar epithelial exfoliation, lamellar deformation and chloride cell damage (Perry & Laurent, 1993). Such morphological changes include hypertrophy of filaments and lamella chloride cells, and filament mucus cell stimulation (Perry & Laurent, 1993).

The current study aimed to establish the possible target of iron toxicity by investigating gill tissues. The investigation included both morphological analysis of gills and chemical analysis of gill and plasma iron content to reveal sites of

TABLE II. Composition of supply water used during acclimation and exposure with toxicant

Parameter	Units	Results	Parameter	Units	Results
pH	—	7·62 (7·39–7·85)	Sulphate	mg l^{-1}	96 (83–107)
Total hardness	mg $CaCO_3$ l^{-1}	287 (250–375)	Iron	µg l^{-1}	<20 (<20–<20)
Alkalinity	mg $CaCO_3$ l^{-1}	132 (82–173)	Zinc	µg l^{-1}	10 (<10–20)
Conductivity	µS cm^{-1}	617 (514–679)	Aluminium	µg l^{-1}	<20 (<20–<20)
Free chlorine	mg l^{-1}	<0·02 (<0·02–<0·02)	Cadmium	µg l^{-1}	<0·2 (<0·2–<0·5)
Calcium	mg l^{-1}	81 (66–99)	Chromium	µg l^{-1}	<2 (<2–<5)
Magnesium	mg l^{-1}	15·6 (11–19)	Copper	µg l^{-1}	<20 (<20–30)
Sodium	mg l^{-1}	46 (35–58)	Lead	µg l^{-1}	8 (<2–100)
Potassium	mg l^{-1}	4·7 (3·6–5·7)	Manganese	µg l^{-1}	<10 (<10–<10)
Chloride	mg l^{-1}	65 (56–76)	Nickel	µg l^{-1}	7 (<2–40)

Values are means, figures in parentheses represent minimum and maximum values. Metal analysis supplied by Severn Trent Water plc.

iron deposition. There is no available evidence in the literature to suggest that iron (III) will be taken across the gill epithelium. This phenomenon was investigated during this study. There is also no evidence of removal mechanisms at the gills in the form of mucus production, as has been suggested for other trace metals, including aluminium (Handy & Eddy, 1989). Any appearance of mucus formation was noted during exposure to iron. Fish exposed to both lethal and sublethal concentrations of iron as both the commercial liquor and analar grade were studied to determine the acute toxicity of this treatment process.

MATERIALS AND METHODS

Commercial grade iron (III) sulphate was obtained from E&A West, Derby, England (Table I). It was compared to an analar grade iron (III) solution produced by oxidizing iron (II) sulphate (Table I) obtained from Merck Ltd to iron (III) in 15 ml of 1 M analar sulphuric acid by adding a stoichiometric quantity of analar hydrogen peroxide. The stock was then diluted to the required volume with distilled water and mixed for 24 h. The use of iron (II) sulphate was necessary due to the unavailability of analar grade iron (III) sulphate.

Brown trout fingerlings (15–30 g) were collected from Leadmill trout farm, Derbyshire. Before testing, all fish were acclimated for 14 days to dechlorinated Nottingham mains water (Table II) at 15° C, pH 7·2, and to a 12 L : 12 D photoperiod. In all experiments 10 fish were used per individual test concentration and controls. Toxicity tests were carried out in an automatic siphon-dosing apparatus. The dosing apparatus supplied the same aerated water as that used for acclimation at 15° C to six separate 120-l polythene tanks. The average volume of exposure medium was 100 l. The flow rate was set at 500 ml min^{-1}, ensuring a 90% replacement of exposure medium within 9 h. Toxicant was supplied from storage tanks via a peristaltic pump (Watson & Marlow, model 502S) and mixed with mains supply water in a mixing chamber prior to being siphoned into the appropriate exposure chamber. The pH of exposure water was measured but not controlled (Table III). The apparatus was run for 24 h before the test to enable iron concentrations to equilibrate. All containers were composed of polyethylene and tubing used was made of silicone rubber. Samples were taken at regular intervals over the testing period for total and soluble iron determination by flame atomic absorption spectrophotometry (AAS) using a Perkin–Elmer Model 1100 (detection limit 30 µg Fe l^{-1}). Total iron samples were acidified immediately using concentrated

TABLE III. Composition of exposure water during lethal tests using commercial and analar grade iron sulphate

	Nominal iron concentration mg Fe l^{-1}					
	Control	10	16	22	28	32
Commercial grade						
Total iron (mg Fe l^{-1}) (14)	$0{\cdot}01^{<0{\cdot}01}$	$12{\cdot}2^{0{\cdot}2}$	$19{\cdot}5^{0{\cdot}8}$	$25{\cdot}9^{0{\cdot}5}$	$33{\cdot}5^{1{\cdot}8}$	$36{\cdot}9^{0{\cdot}8}$
Soluble iron (mg Fe l^{-1}) (6)	$<0{\cdot}01^{<0{\cdot}01}$	$0{\cdot}02^{<0{\cdot}01}$	$0{\cdot}03^{0{\cdot}01}$	$0{\cdot}06^{0{\cdot}02}$	$0{\cdot}12^{0{\cdot}03}$	$0{\cdot}14^{0{\cdot}04}$
pH (14)	$7{\cdot}36^{0{\cdot}01}$	$6{\cdot}56^{0{\cdot}01}$	$6{\cdot}34^{0{\cdot}01}$	$6{\cdot}11^{0{\cdot}01}$	$6{\cdot}02^{0{\cdot}01}$	$5{\cdot}73^{0{\cdot}01}$
Analar grade						
Total iron (mg Fe l^{-1}) (13)	$0{\cdot}19^{0{\cdot}05}$	$13{\cdot}0^{0{\cdot}7}$	$17{\cdot}0^{0{\cdot}6}$	$28{\cdot}2^{1{\cdot}3}$	$32{\cdot}3^{1{\cdot}1}$	$42{\cdot}4^{3{\cdot}1}$
Soluble iron (mg Fe l^{-1}) (6)	$0{\cdot}06^{0{\cdot}02}$	$0{\cdot}02^{0{\cdot}01}$	$0{\cdot}03^{0{\cdot}02}$	$0{\cdot}07^{0{\cdot}03}$	$0{\cdot}13^{0{\cdot}06}$	$0{\cdot}16^{0{\cdot}08}$
pH (13)	$6{\cdot}23^{0{\cdot}07}$	$6{\cdot}27^{0{\cdot}03}$	$6{\cdot}26^{0{\cdot}04}$	$6{\cdot}07^{0{\cdot}03}$	$5{\cdot}98^{0{\cdot}04}$	$5{\cdot}74^{0{\cdot}04}$

Values are means $^{S.E.}$ The number of samples n in parentheses.

hydrochloric acid to a final concentration of 1% (v/v). All iron analyses were carried out using recommendations from HMSO (1983). Soluble iron was defined as the fraction passing through a 0·45-μm filter. Samples were collected, filtered immediately and acidified with concentrated hydrochloric acid, as above.

LETHAL TESTS

Fish were starved for 24 h prior to testing, and throughout the test. Fish were exposed for 96 h to a lethal concentration range of iron sulphate as commercial grade liquor and analar grade iron sulphate. Death was defined at the point where ventilation had ceased, the fish were overturned and unresponsive to external stimuli. At death fish were removed from the exposure tank, rinsed in tap water and weighed. Those surviving the toxicity test were killed by a blow to the head. Further investigations were then carried out immediately after measurements were taken. Fish that had been dead for an indeterminate length of time were not analysed for gill iron determinations as they may have accumulated more iron after death.

IRON ACCUMULATION BY GILLS

Fish were analysed for accumulated iron on gill surfaces using a semi-quantitative filter paper technique employed by Playle & Wood (1991). Filter paper discs (Whatman No. 1) of 13 mm diameter were placed between the first and second gill hemibranchs on the left side (dorsal view) of test and control fish and left for 30 s. Discs were then placed in 10 ml 1% HCl and shaken vigorously for 5 s. Samples were then left at 4° C overnight, and stored at − 20° C until required for analysis. After thawing, filter discs were removed and solutions analysed for iron by AAS. Results were calculated as mg Fe kg^{-1} dry weight. During sublethal tests fish were fed with pellets which may have contained iron, however, the iron content of control exposure water remained close to detection limits of the AAS instrument.

TISSUE DIGESTION

The third and fourth gill hemibranchs were removed from the right side (dorsal view) only of test and control fish. Tissues were placed on filter paper discs and dried in an oven to constant weight at 80° C. Dried tissue was then digested completely in 5 ml concentrated nitric acid, diluted 1 : 10, and analysed for iron by AAS. Results were expressed as the iron content of the right side third and fourth gill hemibranchs in mg Fe kg^{-1} dry weight.

TABLE IV. Composition of exposure water during sub-lethal tests using commercial and analar grade iron sulphate

	Control	Commercial	Analar
Duration of test=3 days			
Total iron mg Fe l^{-1} (n=10)	$0{\cdot}08^{(0{\cdot}03)}$	$12{\cdot}31^{(0{\cdot}56)}$	$10{\cdot}97^{(0{\cdot}33)}$
pH (n=7)	$6{\cdot}57^{(0{\cdot}21)}$	$6{\cdot}38^{(0{\cdot}13)}$	$6{\cdot}53^{(0{\cdot}12)}$
Duration of test=14 days			
Total iron mg Fe l^{-1} (n=16)	$0{\cdot}04^{(0{\cdot}01)}$	$6{\cdot}74^{(0{\cdot}45)}$	$7{\cdot}4^{(0{\cdot}15)}$
pH (n=15)	$7{\cdot}29^{(0{\cdot}02)}$	$7{\cdot}07^{(0{\cdot}01)}$	$7{\cdot}1^{(0{\cdot}02)}$

Values are means $^{(S.E.)}$ taken over the whole testing period.

HISTOLOGICAL PREPARATIONS

The first and second gill hemibranchs of test and control fish were removed from the right side and fixed in buffered neutral formaldehyde (40% w/v). An automatic tissue processor, model SE400 (Shandon Scientific) dehydrated tissues by rinsing in 50%, 70% and 90% alcohol, followed by four successive rinses in absolute alcohol. Alcohol was removed by three rinses in toluene and tissues were embedded finally in paraffin wax (*c.* 55° C). Wax blocks were then sliced using a Leitz rotary microtome to give sections of *c.* 5 µm. After mounting, the sections were stained using haematoxylin and eosin (H&E) to determine gill structural changes, and Perls' Prussian blue stain to reveal deposition of ferric iron (Bancroft & Stevens, 1982).

The 96-h LC_{50} values were calculated using graphical methods recommended by Litchfield & Wilcoxon (1948) for both total and soluble iron (Dalzell, 1996).

SUBLETHAL TESTS

Two series of sublethal exposures of fish to commercial and analar grade iron sulphate were carried out using the dosing equipment constructed for lethal toxicity tests. In the first series, fish were exposed for 3 days to 12·3 mg total Fe l^{-1} as commercial grade iron or 11 mg total Fe l^{-1} as analar grade iron (Table IV). At the beginning of the test 10 fish were placed in each exposure concentration, with a further 10 fish as controls. Control water was adjusted to equivalent acidity as the test waters by the addition of analytical grade sulphuric acid to toxicant stocks. The sublethal dose was based on the results obtained from lethal tests. As death occurred in the commercial grade exposure tank after 3 days, the test was terminated. A second series of sublethal tests was carried out using the lower doses of 6·7 mg Fe l^{-1} as commercial grade iron or 7·4 mg Fe l^{-1} as analar grade iron (Table IV). No mortality occurred over the 14 days of testing. As before, the control supply water was adjusted in pH. Fish were fed using fingerling ration size 1 trout pellets (Trouw Aquaculture) daily during each set of tests. On completion of each test, fish were removed from exposure water and killed by a blow to the head, rinsed in tap water and weighed. Total gill iron was determined together with histological studies as described for lethal tests.

PLASMA IRON ANALYSIS

Plasma iron determinations were carried out on fish exposed to the 14-day sublethal exposure to iron only. Blood samples of 0·5 ml were taken by caudal puncture using heparinized syringes and placed in microcentrifuge tubes. Tubes were then spun in a Decaspeed MH-2 centrifuge for 3 min. Plasma was extracted using a Pasteur pipette acidified with concentrated hydrochloric acid, as above, and samples stored at −20° C until required. Samples of 100 µl were diluted with 10 ml deionized water and analysed for iron using AAS.

TABLE V. Toxicity data for commercial grade and analar grade iron (III) sulphate in brown trout

	Total iron	Soluble iron
Commercial grade		
96 h LC_{50}, mg Fe l^{-1}	28	0·05
95% confidence interval of LC_{50}	21·5–36·4	0·03–0·14
Slope	1·68	NC
Analar grade		
96 h LC_{50}, mg Fe l^{-1}	47	0·24
95% confidence interval of LC_{50}	36·2–61·1	0·12–0·38
Slope	1·96	NC

NC, Not calculated.

STATISTICAL ANALYSIS

Statistical analyses recommended by Fowler & Cohen (1993) were carried out where appropriate using Student's *t*-test and two-way analysis of variance (ANOVA) for lethal tests, and the Mann–Whitney *U*-test for sublethal tests.

RESULTS

LETHAL TESTS

During lethal exposure, symptoms of intoxication included gasping, coughing and heightened respiratory rate, a lack of response to stimuli, and loss of equilibrium. Commercial grade iron was lethal at lower concentrations than was analar grade iron (Table V).

Gill iron accumulation

Results were gathered from both surviving fish and those recently killed. Exposure of fish to both commercial grade and analar grade iron resulted in an accumulation of iron on the gills and iron accumulation increased generally with exposure concentration (Fig. 1).

The iron removed from gill surfaces of fish exposed to both sources of iron using the filter paper technique also showed iron accumulation on the gill, which increased with dose (Fig. 2). When removed from the gills of iron exposed fish, discs were coated with orange/brown iron flocs. Results presented are again for surviving fish, and those recently killed. Results revealed an accumulation of iron on gills of fish exposed to both grades of iron.

Gill morphology

H&E staining revealed extensive damage to gill tissues on exposure to commercial iron sulphate. Secondary lamellae of control gills were thin and well spaced with no lifting of the epithelial cell layer (Fig. 3). Gills from fish exposed to commercial grade iron sulphate showed disruption of the secondary lamellae, subepithelial spaces created by epithelial lifting and an increase in the diffusion distance due to hypertrophy of epithelial cells. At concentrations above 12·2 mg Fe l^{-1} gills showed widespread epithelial rupture, necrosis of

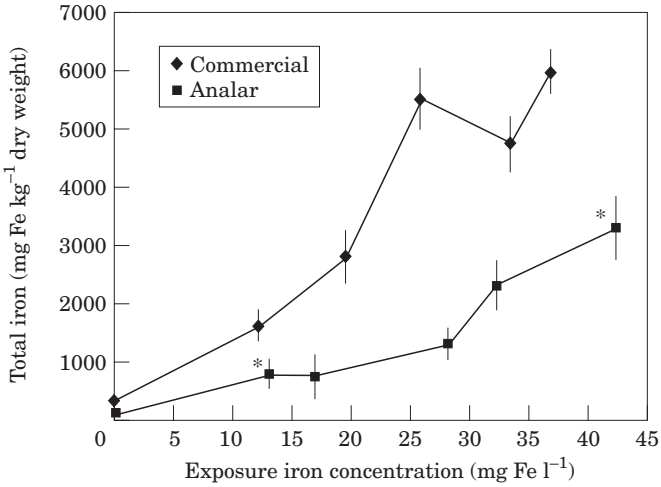

FIG. 1. Concentration of total iron in gill hemibranchs of control fish and fish exposed to a lethal concentration range of commercial and analar grade iron after 96 h. Values are means ± S.E. (n=10, except * where n=9). All test results were significantly different from the controls (P<0·05, Student t-test). There was also a disproportionately greater build-up of iron on gills of fish exposed to commercial grade iron sulphate compared with analar grade iron (P<0·05, two-way ANOVA).

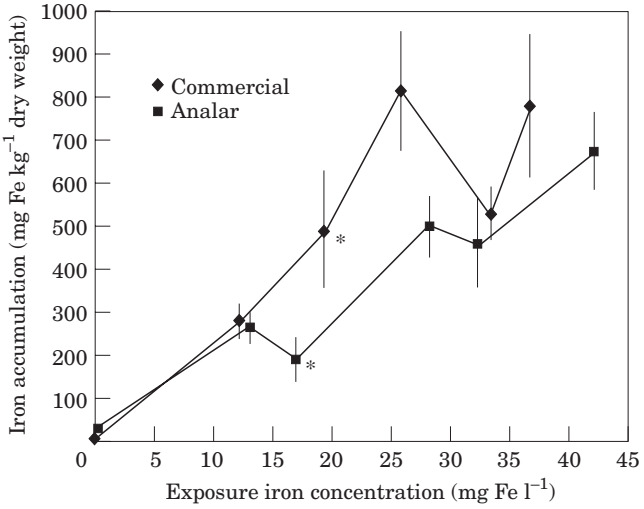

FIG. 2. Iron accumulation on gill surfaces of control fish and fish exposed to a lethal concentration range of commercial and analar grade iron after 96 h using a semi-quantitative filter paper technique. Values are means ± S.E. (n=10, except * where n=9). All test results were significantly different from the controls (P<0·05, Student's t-test). Results for iron removed from fish exposed to commercial grade iron appeared to be greater compared with fish exposed to analar grade iron, however, this was not significant (Student's t-test).

epithelial cells and fusion of secondary lamellae. Perls staining revealed no evidence of iron precipitating on control gill surfaces. Staining showed progressively greater build-up of iron floc with exposure concentration, to the point

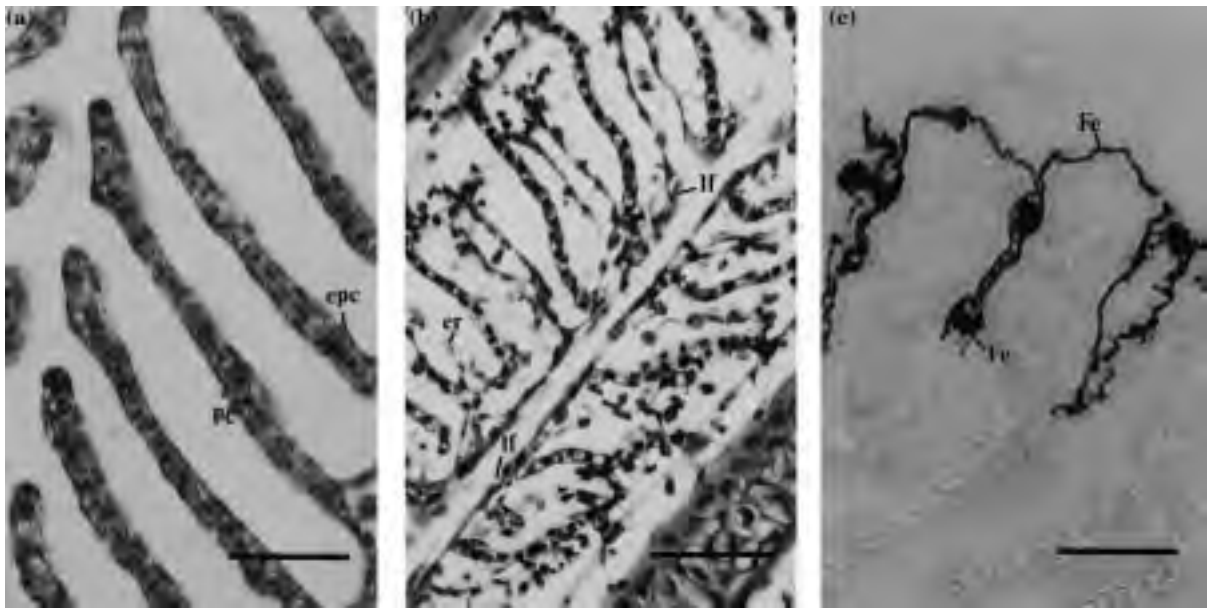

FIG. 3. Response of fish gills exposed to commercial grade iron. (a) Normal gills of control fish. (b) Haematoxylin and eosin stained gills exposed to 36·9 mg Fe l^{-1} for 96 h. Note widespread disruption including lamellar fusion (lf) and epithelial rupture (er). (c) Perls stained gills exposed to 25·9 mg Fe l^{-1} for 96 h. Note dark stained iron floc (Fe) continuously coating epithelial layer. Gill sections were of 4–5 μm thickness. epc, Epithelial pavement cell; pc, pillar cell; Bar = 50 μm.

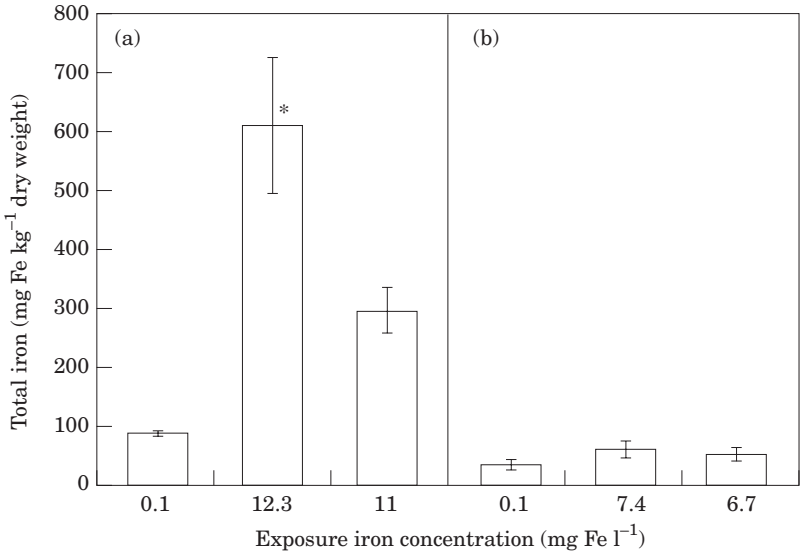

FIG. 4. (a) Total iron concentrations in gill hemibranchs of control fish and fish exposed to 12·3 mg Fe l^{-1} as commercial grade iron and 11 mg Fe l^{-1} as analar grade iron after 3 days. Both treatments were significantly higher than the controls ($P<0{\cdot}05$, Mann–Whitney *U*-test but there was no significant difference between fish exposed to commercial and analar grade iron (Mann–Whitney *U*-test); (b) total iron concentrations in gill hemibranchs of control fish and fish exposed to 7·4 mg Fe l^{-1} as commercial grade iron and 6·7 mg Fe l^{-1} as analar grade iron after 14 days. There was no significant difference between test fish and controls (Mann–Whitney *U*-test). Values are means ± S.E.; $n=10$, except * where $n=9$.

where the floc was forming a continuous layer between filaments and lamellae (Fig. 3). No evidence of staining was seen within the gill tissues.

Gills taken from fish exposed to the lethal concentration range of analar grade iron sulphate showed consistently less damage compared with gills from commercial grade iron exposure (data not shown). On exposure to analar grade iron sulphate epithelial lifting and cell hypertrophy were observed most frequently. Secondary lamellar structure was maintained, with less tissue fusion apparent. The Perls staining procedure showed that iron floc did not accumulate on gill surfaces to as great an extent as on gills exposed to commercial grade iron, and did not appear to form a complete covering on the epithelial surfaces (data not shown). No evidence of extensive mucus formation was seen during lethal tests.

SUBLETHAL TESTS

Gill iron accumulation

Exposure of fish for 3 days to 12 mg Fe l^{-1} as commercial grade iron and 11 mg Fe l^{-1} as analar grade iron resulted in accumulation of iron on the gills compared to controls using total tissue digestion [Fig. 4(a)]. Iron accumulation was significantly greater in fish exposed to both grades of iron compared with controls ($P<0{\cdot}05$, Mann–Whitney *U*-test). There was no significant difference between the gills of fish exposed to the two grades of iron (Mann–Whitney *U*-test). Fish exposed for 14 days to 7·4 mg Fe l^{-1} as commercial grade and

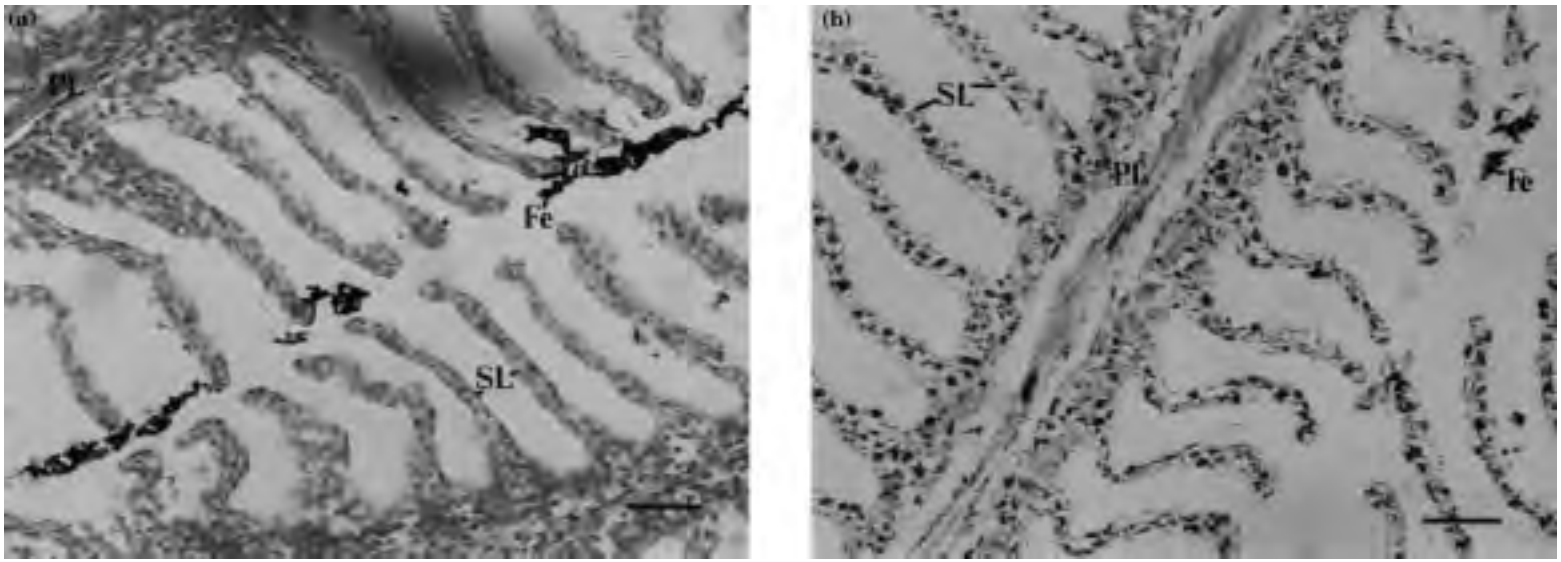

FIG. 5. Perls-stained sections of fish gills exposed to (a) 7·4 mg Fe l^{-1} as commercial grade iron (III) sulphate for 3 days, and (b) 11 mg Fe l^{-1} as analar grade iron (III) sulphate for 14 days. Note majority of iron floc accumulation between primary lamellae (PL), rather than between secondary lamellae (SL). Gill sections were of 4–5 μm thickness. Fe, Iron floc. Bar = 50 μm.

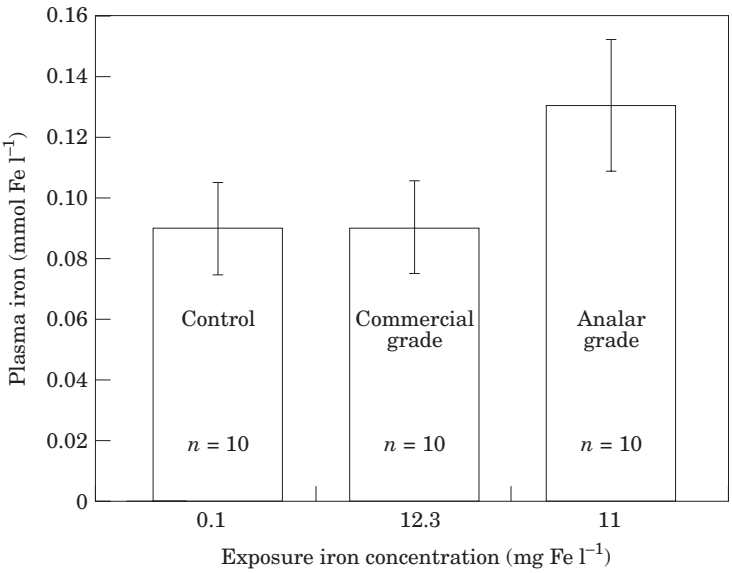

FIG. 6. Plasma iron concentrations of fish exposed to 7·4 mg Fe l^{-1} as commercial grade iron (III) sulphate and 6·7 mg Fe l^{-1} as analar grade iron (III) sulphate after 14 days. Values are means ± S.E. There was no sigificant difference between test fish and controls (Mann–Whitney *U*-test).

6·7 mg Fe l^{-1} as analar grade showed no significant difference in total iron levels on gills compared to control gills [Fig. 4(b)]. No evidence of mucus formation was seen during sublethal tests.

Gill morphology

Using H&E staining, gills exposed to 12 mg Fe l^{-1} as commercial grade iron and 11 mg Fe l^{-1} as analar grade iron showed little difference in appearance to control gills, except for a slight hypertrophy of terminal epithelial cells of the secondary lamellae (data not shown). The accumulation of iron floc was more noticeable on gills of fish exposed to commercial grade iron (data not shown) compared to analar grade iron (Fig. 5). The results of Perls staining revealed no blue stain on control gills (data not shown).

During the second series of tests, fish were exposed to 7·4 mg Fe l^{-1} as commercial grade iron, and 6·7 mg Fe l^{-1} as analar grade iron for 14 days. On H&E sections gills exposed to analar grade iron sulphate were similar to control gills (data not shown). After 14 days of exposure to commercial iron sulphate, the thickness of secondary lamellae had increased due to widespread epithelial cell hypertrophy. Some subepithelial spaces appeared. Perls stained sections showed that more iron accumulation during exposure to commercial grade iron (Fig. 5) compared with analar grade iron (data not shown).

Plasma iron

Plasma iron values in fish exposed to 7·4 mg Fe l^{-1} as commercial grade and 6·7 mg Fe l^{-1} as analar grade showed no significant difference compared with controls (Mann–Whitney *U*-test, Fig. 6) after 14 days.

DISCUSSION

Exposure of fish to iron either as the commercial grade or analar grade iron sulphate resulted in accumulation on the gill which was dose dependent. Gills exposed to the commercial iron showed widespread damage. This included epithelial lifting with the formation of subepithelial spaces, hypertrophy and necrosis of epithelial cells, lamellar rupture and fusion; symptoms consistent with exposure to gill irritants (Mallatt, 1985). Histology showed consistently more damage in gills exposed to commercial grade compared with analar grade iron. This greater damage was accompanied by more accumulation on the gill. During exposure to analar grade iron there was little or no cell necrosis, lamellar disruption or fusion, and the lamellar structure was retained throughout the exposure range. Perls staining revealed that iron tended not to accumulate to as great an extent on the gill surface during exposure to analar grade iron. With less iron build-up on gill surfaces and generally less gill damage, this may have been responsible for the reduction in toxicity during lethal tests.

The damage after iron exposure would lead to a reduction in effective surface area, and an increase in diffusion distance for respiratory exchange. Perls staining revealed iron generally coating the epithelial surfaces of gills during exposure to high concentrations. A mechanism of acute toxicity for iron in fish might therefore be respiratory disruption, resulting from gill clogging and damage. This hypothesis is supported by the symptoms exhibited by fish during exposure to lethal concentrations of commercial iron sulphate, namely coughing, gasping and increased amplitude and frequency of ventilation, seen often when fish are exposed to compounds containing iron (Larson & Olsen, 1950; Lehtinen, 1980; Grobler *et al*., 1989).

The trend of iron accumulation for whole gill tissue was reflected using the filter paper technique. The former represents a quantitative method of estimating total iron content of the gill, whilst the latter is only a semi-quantitative method. The filter paper technique removed iron from between the first and second gill hemibranchs. As only a proportion of the total iron was removed it does not rule out the possibility that iron crosses the gill epithelium and accumulates in tissues. However, results showed that plasma iron remained constant after 14 days exposure to sublethal concentrations of both grades of iron. This supports the suggestion that iron accumulates only on gill surfaces. Generally, iron accumulation on fish gills is viewed as purely physical clogging, and not one of chemical binding (Peuranen *et al*., 1994). Results from Perls staining of gill tissues showed iron clogging gill surfaces, but with no iron taken up into gill tissues. There was no visual evidence of mucus formation during the testing period. This might have been expected if the iron had been binding chemically to gill epithelia, as in the case of aluminium which binds chemically to gills and induces mucus formation (Handy & Eddy, 1989). It therefore seems unlikely that iron was binding chemically to the gill epithelium. Sublethal exposure to commercial and analar grade iron resulted in noticeable reductions in gill damage and the incidence of gill lesions coupled with a decrease in iron accumulation. Results from the sublethal tests suggest that fish are able to survive a concentration of 7 mg Fe l^{-1} for a period of 14 days. They do not show a proportionate increase in gill iron content with increase in exposure time,

therefore iron appears to be removed effectively from gill surfaces. Possibly the only mechanism required would be mechanical (i.e. exaggerated opercular movement and coughing) supporting the view of a physical rather than a chemical binding of iron.

Previous workers had concluded that other trace metals present with iron in effluents from the TiO_2-producing industry may contribute to toxicity (Lehtinen, 1980). The evidence from this study shows that the toxic action of the commercial iron sulphate was due mainly to the iron, however, some trace metals, i.e. Zn and Mn, were present in higher concentrations in the commercial preparation compared to the analar grade source. The reduction in toxicity in fish exposed to analar grade iron may be due to a reduction in these trace metals. The gills of fish exposed to commercial grade iron had a disproportionately greater total iron content compared to the gills of fish exposed to analar grade iron. A change in floc formation could alter the physical characteristics which might in turn affect the ability of the fish to remove iron floc from the gill. Sykora *et al.* (1972) have reported that differences in the size of iron (III) flocs might influence gill respiration. When soluble iron (III) ions are added to fresh water they undergo dissolution and hydration to form multivalent iron hydroxide species (Rubin & Kovac, 1974). This process was described by Hutchinson & Healey (1990) who separated it into coagulation, where components in a stable suspension or solution are destabilized by overcoming forces which maintain stability, and flocculation, a process where destabilized particles join together to form large stable particles or agglomerates. The presence of other contaminating trace metals may change the nature of floc formation. This change in floc formation may also be due to the differences in chemical preparation of the two grades. The commercial source was obtained as an iron (III) liquor, whereas the analar grade stock was prepared by oxidizing iron (II) sulphate to iron (III) in a sulphuric acid solution before use.

Abrams & Collins (unpubl. data) determined the 96-h LC_{50} for iron in brown trout as 8 mg Fe l^{-1}, much lower than the 96-h LC_{50} for commercial grade iron sulphate found during this project. However, they used general purpose grade iron (III) sulphate as their iron source. This may have accounted for the difference in toxicity. Also, their test water had a total hardness of 250 mg $CaCO_3$ l^{-1} compared with 287 mg $CaCO_3$ l^{-1} used during this study. The increase in hardness may aid iron coagulation and increase gill accumulation.

From this study it appears that the gill is the main target for iron toxicity, and a possible mode of action may be one of respiratory disruption caused by physical gill clogging. The commercial grade iron sulphate liquor was more toxic compared with an analar grade iron preparation, however as the toxicity values obtained were relatively similar, the majority of toxicity appeared to be due to iron. The present results support the general hypothesis that iron is relatively non-toxic to fish (96 h LC_{50} in brown trout of 28–47 mg Fe l^{-1}) compared with other trace metals with regard to direct acute toxicity, i.e. 96 h LC_{50}s in rainbow trout *Oncorhynchus mykiss* (Walbaum) of 20 µg Cu l^{-1} (Howarth & Sprague, 1978); 34 µg Cd l^{-1} (Cusimano *et al.*, 1986); 92 µg Zn l^{-1} (Bradley & Sprague, 1985). However, as some of these metals are present as contaminants in the commercial iron liquor in mg l^{-1} concentrations (Table I), the long-term ecological impact of this treatment process requires further investigation.

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