Hydrobiologia **306**: 229–240, 1995.

Joint and single toxicity of Cd and Fe related to metal uptake in the mayfly *Leptophlebia marginata* (L.) (Insecta)

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Received 16 December 1993; accepted in revised form 6 July 1994

Abstract

The toxic effects of Cd and Fe on the mayfly *Leptophlebia marginata* were tested during single and joint exposure to the metals. Data on uptake and localization of the metals were related to the following biological parameters: survival, food consumption (% gut contents) and activity (escape from prodding).

The uptake of Cd by *L. marginata* was significantly reduced by the presence of Fe ($p<0.0005$).

Fe^{2+}- and Fe^{3+}-crusts on the gills, body surface and on the gut membrane precipitated when the mayflies were exposed to either Fe alone or to Fe and Cd. This caused decreased food consumption by the mayflies.

In the pigments of the eyes and the cuticle Fe^{2+} was found.

Exposure to Cd led to disturbances in Cl^- and K^+ ion balance, which were ameliorated by additional Fe-exposure due to co-precipitation processes.

Exposure to Cd caused an increase in the concentrations of S and P in the mayflies.

Whereas Fe caused sublethal effects (food consumption) without being taken up into the organism. Cd caused lethal effects (inactivity, death) due to disturbances of the ion balance at the membranes.

Introduction

Even though toxicity studies mostly consider one toxicant, wastewaters often carry mixtures of different toxicants. Few studies have considered the combined effects of metal mixtures on freshwater invertebrates (EIFAC, 1987), whilst fish have been used more often as test organisms (Hutchinson & Sprague, 1986; Spehar *et al.*, 1978; Spehar & Fiandt, 1986; Enserink *et al.*, 1991). In particular chronic toxicity studies on the effects of metal mixtures on aquatic organisms are rare (Enserink *et al.*, 1991; Kraak *et al.*, 1994). The literature on such interactions is unfortunately confusing and contains much contradictory terminology and theory (Haas & Stirling, 1994). No general pattern concerning metal-metal interactions can be discerned from the literature, and conflicting results have been reported (Wang, 1987; Altenburger *et al.*, 1992; Gerhardt, 1993).

Most metals become more soluble and bioavailable at low pH, however toxicity studies with aquatic invertebrates at low pH are rare (Gerhardt, 1993).

In order to explain metal interactions the site and mode of toxic action of the metals has to be known. Data about the localization of metals in the organisms can therefore be very useful, however there are only a few metal-specific histochemical techniques (Cd: Sumi *et al.*, 1984; Prentø, 1991; Fe: Moewis, 1978). Other possible techniques are the use of radioactive tracers and a recently developed microprobe method, which allows for scanning metals in thin histological sections (Tapper & Malmqvist, 1991).

In the present paper, the toxic effects of Cd and Fe, applied singly as well as in mixtures were studied using nymphs of the mayfly *Leptophlebia marginata*. Uptake of the metals was related to toxicity, measured as changes in survival, food consumption and activity. Cd was chosen, because it is one of the most toxic metals. Fe was chosen, because toxicity studies on this essential element are rare.

Material and methods

Experimental design

Nymphs of *L. marginata* were collected from a small forest stream in South Sweden in autumn 1991 and 1992. The streamwater used for the experiments was characterized by pH 5.5–6.5; total hardness 0.27 mmol l^{-1}; Cd<1 $\mu g\ l^{-1}$; Fe_{tot} 1.5 mg l^{-1}.

The animals were kept individually in a static water system with the stream water being renewed twice a week (Gerhardt, 1992 a, b). Fine detritus from the stream was filtered on Whatman GF/C glassfibre filters and served as food. The mayflies were acclimated to laboratory conditions for 72 h. During that time, the pH was decreased in steps of 0.5 pH units down to pH 4.5 ± 0.2 using H_2SO_4. Experiments were performed at low pH in order to keep the metals in solution. Moreover, earlier experiments revealed increased metal toxicity at low pH (Gerhardt, 1992 b). The maximal duration of the experiments was 42 days, depending on survival of the mayflies.

After the acclimation period, groups of 45 mayflies were exposed to different concentrations of Cd, Fe and mixtures of both metals, each treatment in duplicate (Table 1). The metal concentrations were chosen according to the following criteria: (1) observed effects in previous experiments, (2) close to natural concentrations (e.g. 4 mg Fe l^{-1}) and (3) avoid saturation and immediate precipitation of the metal (e.g. 40 mg Fe l^{-1}).

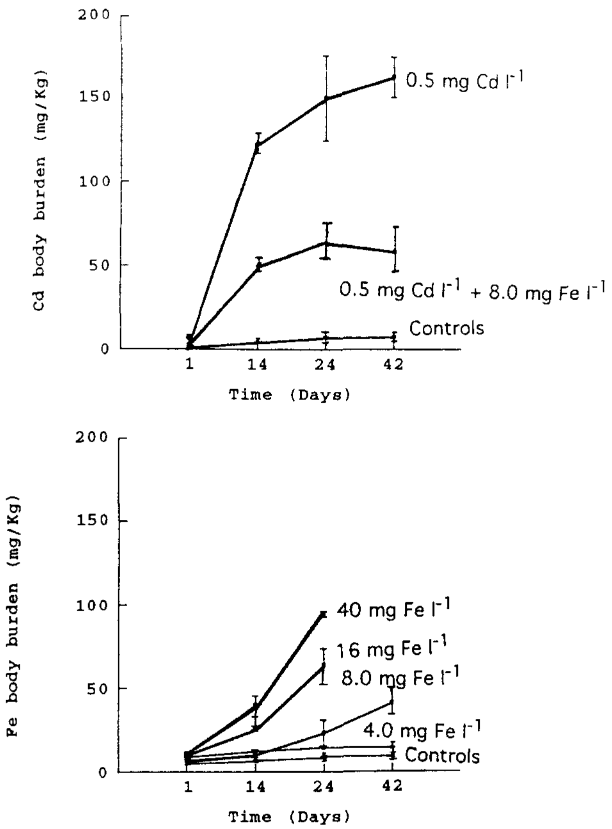

Fig. 1. Uptake of Cd and Fe (mg kg^{-1}) by *Leptophlebia marginata* after single and joint exposure to the metals. All values represent means of 2 replicate experiments.

Chemical measurements

Daily chemical monitoring included the measurement of Fe_{tot} (flame AAS and spectrophotometry), Fe^{2+} (spectrophotometry), Cd_{tot} (AAS) and pH adjustment with 0.1 M H_2SO_4 and 0.1 M NaOH. The spectrophotometric measurements were made at 522 nm with 0.5% bipyridin. Fe_{tot} concentrations were yielded after reduction of Fe^{3+} to Fe^{2+} with ascorbic acid. Fe^{2+} concentrations in the water were determined directly. pH adjustments were made twice a day.

Biological monitoring

The following biological parameters were recorded twice a week: survival, feeding (% gut contents: 0%, <50%, >50%) and locomotory activity (escape from prodding). A positive escape reaction was recorded if the mayflies moved more than one body length away from the stimulus. Biological monitoring was followed by a renewal of the stream water, new addition of the metals and food (fine detritus filtered on Whatman GF/C filters according to Gerhardt (1992 b)).

Every 10 days 4–6 living nymphs were sampled from each treatment and replicate and the amount of metal in the mayflies determined using flame AAS (Gerhardt, 1992 a). At the end of the experiments, one nymph from each treatment and replicate was prepared for the localization of Cd and Fe in the mayflies with staining techniques and PIXE.

Histochemical analysis

The mayflies were dehydrated in an ethanol and xylol gradient before being embedded in paraffin (Moewis, 1978). Transverse sections (8 μm) through the whole animal were mounted on slides which had been pretreated with a gelatin-$CrK(SO_4)_2 \times 12\ H_2O$ solution.

Table 1. pH and metal concentrations in the experiments with *L. marginata* exposed to different concentrations of Cd, Fe and Cd + Fe.

Treatments	pH	n	Cd (mg l^{-1})	n	% of LC_{50}	Fe (mg l^{-1})	n
(1) Control	4.53	82	0.0007	48	0	2.30	46
(2) Cd (0.2)	4.51	32	0.19	6	25	1.36	6
(3) Cd (0.5)	4.54	76	0.46	41	50	2.43	41
(4) Cd (1.0)	4.50	35	0.98	15	100	1.18	15
(5) Cd (2.5)	4.51	21	2.58	8	250	1.32	8
(6) Fe (4.0)	4.42	60	0.0007	26	5	3.55	26
(7) Fe (8.0)	4.36	75	0.0008	41	10	7.82	41
(8) Fe (16)	4.57	59	n.d.		22	10.2	25
(9) Fe (40)	4.42	59	n.d.		55	45.4	25
(10) Cd (0.2)/Fe(4.0)	4.52	75	0.250	48	30	4.43	48
(11) Cd (0.5)/Fe(8.0)	4.39	62	0.491	30	60	8.13	30
(12) Cd(1.0)/Fe(16)	4.57	41	1.009	13	122	17.34	13
(13) Cd(2.5)/Fe(40)	4.32	21	2.36	8	305	43.04	8

Mean ($r = 2$) (mg/l), n: number of analyses during the experiments. n.d.: not determined.

After deparaffination and hydration the slides were stained.

Two blue Fe staining reagents were used, $K_4[Fe(CN)_6]$ for Fe^{3+} and $K_3[Fe(CN)_6]$ for Fe^{2+} (Moewis, 1978; Gerhardt, 1992 b). The slides were washed and dehydrated in an ethanol and xylol gradient and embedded in Eukitt for long-term storage.

Cd was stained with BTAN-b (Benzothiazolylazo-b-naphtol) (Sumi *et al.*, 1982, 1984; Prentø, 1991). The slides were mounted in a water-soluble medium (glycerol), because dehydration would have delocalized BTAN-b (Prentø pers. comm.).

Nuclear microprobe

PIXE (particle induced X-ray emission) is a sensitive multielement technique (Johansson & Campbell, 1988). Proton (2.55 MeV) interaction with target atoms results in ejection of electrons from inner shells of the atom. The excess energy is released as an emitted photon (X-ray emission). Two-dimensional X-ray images for specific atoms heavier than Al can be produced, with varying sensitivity (Lövestam *et al.*, 1990, Tapper & Malmqvist, 1991). Na^+ was not detected with PIXE due to the absorption of soft X-rays in the absorber.

Backscattering spectroscopy (RBS) was performed simultaneously with PIXE. From the intensity of the scattering events the irradiated mass was detected. This mass was used to calculate concentrations (mg kg^{-1}) from the trace element masses obtained by PIXE. As backscattering spectroscopy monitored matrix elements (e.g. C, N, O) during irradiation, possible sample deterioration and element losses could be detected (Themner *et al.*, 1990). For PIXE and RBS analyses, the transverse sections from the abdominal region of the mayflies were mounted directly on thin kimfoil without chemical pretreatment.

Statistical analysis

Differences of the actual pH values between the metal concentration levels were analysed by one-factor ANOVA. Additionally, pairwise comparisons were made using t-tests. Uptake data were compared by two-factor ANOVA (conc. levels and kind of exposure) for different dates (day 14, 24, 42) of the experiments.

The effects of the metals (Cd, Fe, Cd+Fe) and concentration levels on survival, gut contents and activity were analysed by two-factor ANOVA for day 6, 14 and 24. LC_{50} and EC_{50} values were obtained by Maximum Likelihood Estimation from probit transformed data on the concentration-response relationship (Weber, 1986). Metal concentrations were preferred to doses, because a metal such as Fe can exert toxic effects without being taken up by the organism (Gerhardt, 1992 b).

The use of models to determine interactive effects of Cd and Fe was avoided because of several reasons such as the limited data set, the limited knowledge about toxic sites and modes of action and the limited

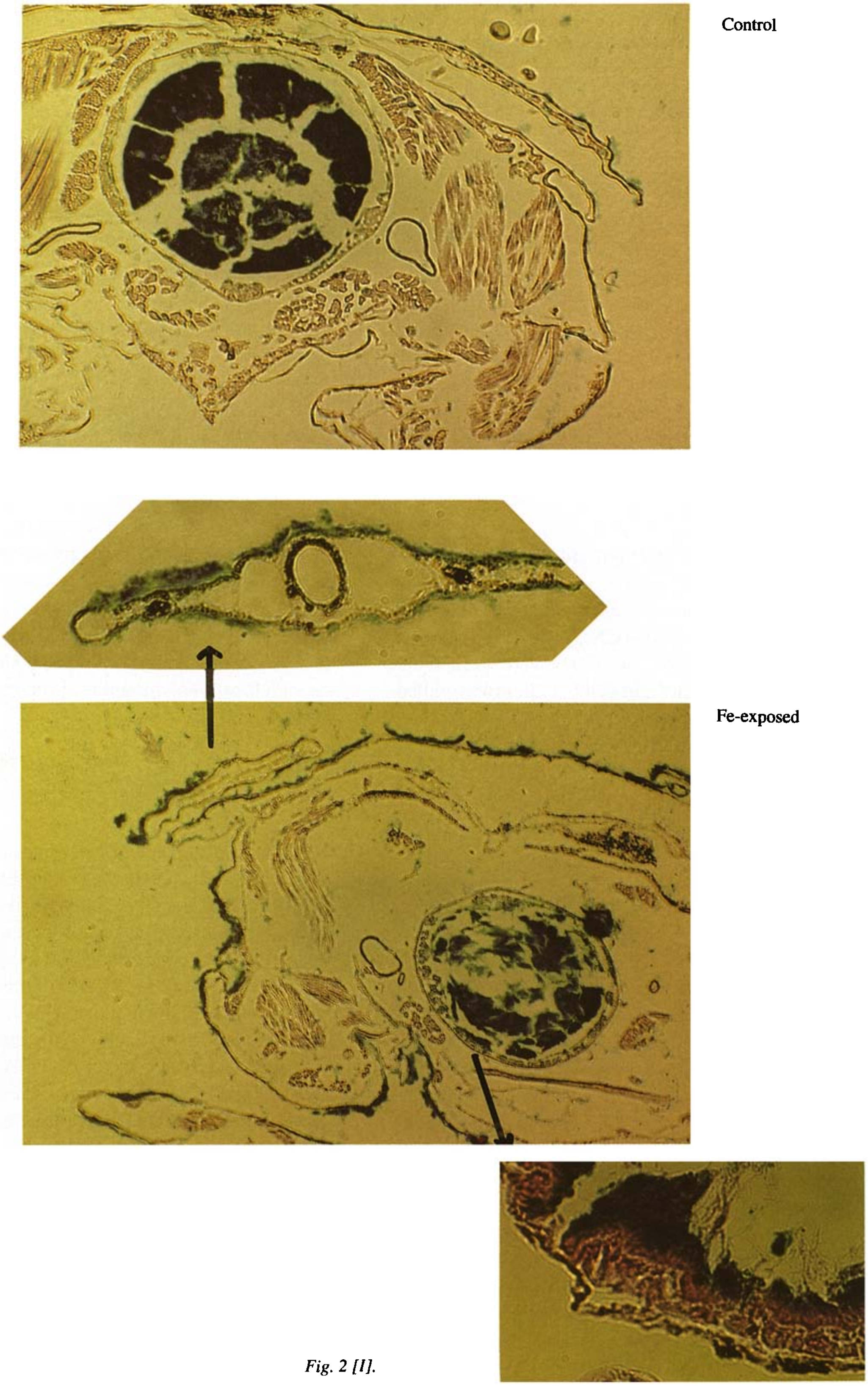

Fig. 2 [I].

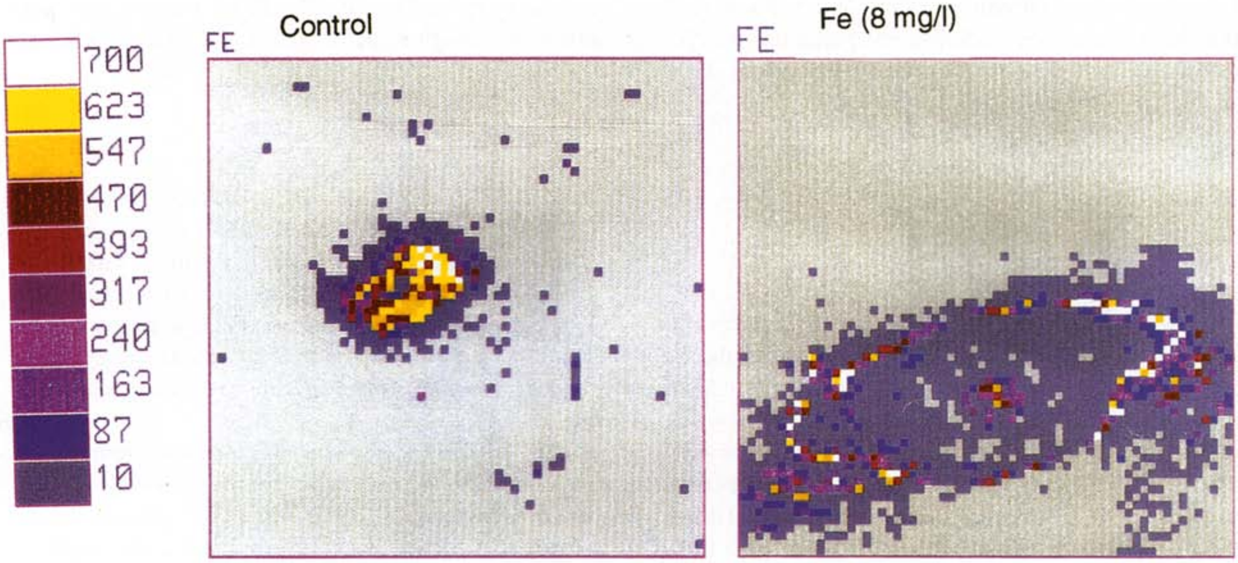

Fig. 2 [II].

Fig. 2 [I] and [II]. Localization of iron in unexposed (a) and Fe-exposed (b) mayflies. [I] Histochemical localization of Fe^{3+} in transverse sections (8 μm) through the thorax region of the mayflies. [II] Fe_{tot}, in transverse sections (8 μm) through the abdominal region of the mayflies (PIXE-analysis).

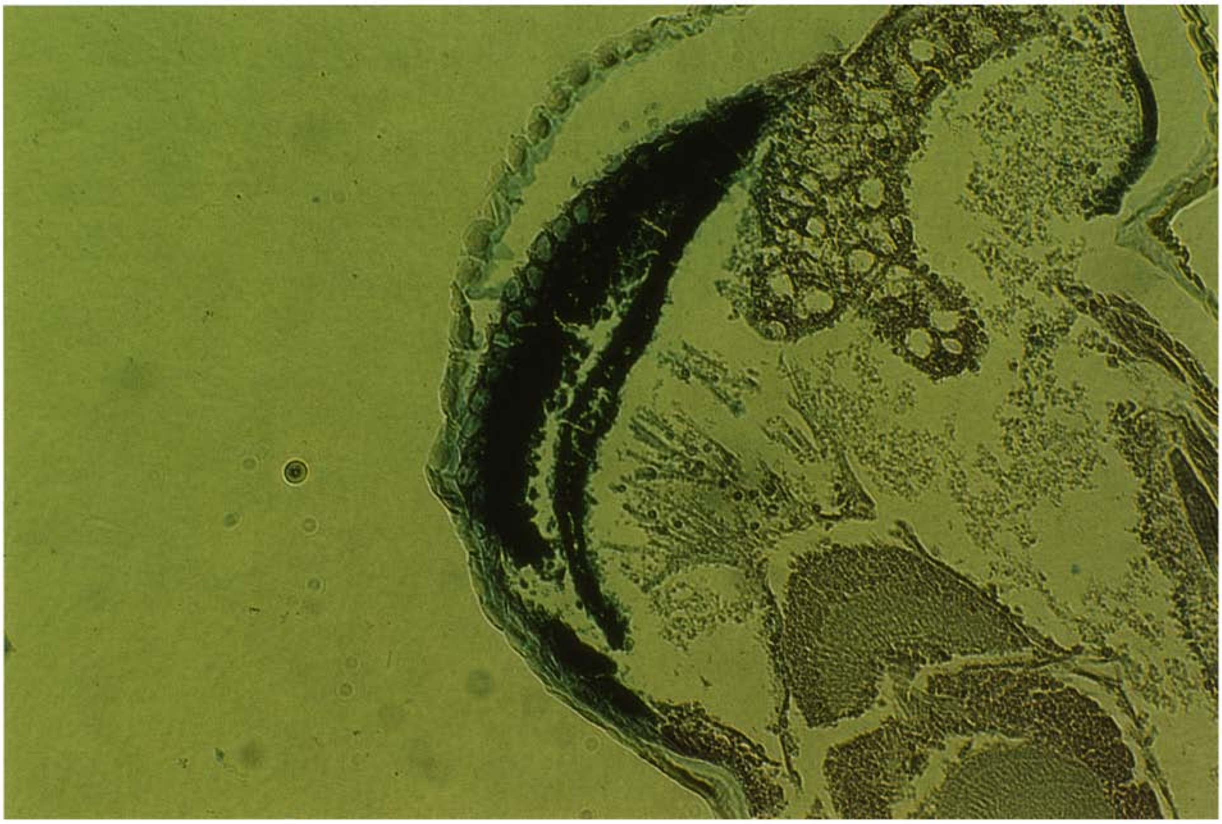

Fig. 3. Histochemical localization of Fe^{2+} in the eyes of *L. marginata.*

knowledge about chronic concentration response realtionships. Moreover, modelling and predicting interactive effects of metals is still under development (Haas & Stirling, 1994).

Results

Chemical parameters

The pH values in the Cd treatments did not differ significantly from the control, whereas those in the Fe and Cd/Fe treatments were significantly lower than in the control (Fe: $p<0.01$, Cd/Fe: $p<0.03$). Moreover, pH values in the Cd treatments differed significantly from those in Fe treatments ($p<0.01$) or Cd/Fe treatments ($p<0.02$). It was difficult to keep the pH at 4.5 in the Fe-exposures, because oxidation of Fe^{2+} to Fe^{3+} and co-precipitation of Fe-hydroxides with humic substances caused a pH decrease.

Metal uptake by L. marginata

Uptake of Cd by nymphs of *L. marginata* exposed to 0.5 mg Cd l^{-1} was significantly reduced by up to 50% in the presence of 8 mg Fe l^{-1} ($p<0.0005$; Fig. 1).

Uptake of Fe by the mayflies increased in a dose-dependent way (p = 0.0025; Fig. 1). No effects of 0.25 mg Cd l^{-1} and 0.5 mg Cd l^{-1} on Fe-uptake were found. Higher concentrations of Cd caused death of the mayflies before a significant Fe-uptake occurred.

Metal localization in L. marginata

Fe^{3+} was found in the gut contents of all nymphs. Mayflies exposed to additional Fe^{2+} contained Fe^{3+} and Fe^{2+} in their guts as well as on the body surface and gills (Fig. 2). Within the organism, only Fe^{2+} occurred and was located in the eyes and tracheae (Fig. 3). PIXE analysis of the microtome sections through the abdominal region of the mayflies (Fig 2) supported the results from the staining procedures. Mayflies exposed to lethal Cd concentrations together with high Fe concentrations did not show Fe-crusts on their body surface. Lethal Cd-concentrations caused death of the mayflies after about 12 days of exposure, which was too short a timc for the formation of Fe-precipitates on the nymphs.

The BTAN-b staining procedure gave no clear facts about the localization of Cd, except for an indication of subcuticular dark colored granula in the sections from mayflies exposed to Cd. The PIXE method was not sensitive enough to detect Cd in the sections after 1 hour's scanning.

Changes in element-composition

The PIXE spectra revealed that mayflies exposed to Cd contained less Cl^- and K^+ than unexposed and Fe-exposed specimens (Fig. 4). In the metal mixtures, the loss of Cl^- and K^+ from the body of the mayflies was less pronounced than in mayflies exposed to Cd alone, but only if Fe was present in sufficiently high concentrations ($\geq$8 mg l^{-1}), thus preventing ion losses.

At 1 mg Cd l^{-1}, the concentrations of sulphur and phosphorus in the sections were almost twice that of all other treatments. As 1 mg Cd l^{-1} caused death of the mayflies, the increase in sulphur and phosphor might be connected with Cd-toxicity in the mayflies. The concentrations of Ca, Mn, Cu, Zn were similar in all sections irrespective of metal-exposure.

Biological parameters

Survival

Survival of *L. marginata* decreased in a concentration-dependent way in the Cd exposures and the metal mixtures (p = 0.001). From day 6 onwards, survival of the mayflies in the Cd-exposures was significantly lower than that in the metal mixtures ($p<0.001$, Fig. 5). Fe (<40 mg l^{-1}) had no severe effect of the survival of the mayflies within 30 days. The LC_{50} values of the metal-mixtures decreased with increasing exposure time (Table 2). This may indicate that during long-term exposure survival is not only decreased by Cd but also by Fe, probably due to precipitation processes, which may prevent oxygen and nutrient uptake by the organisms.

Gut contents

From day 14 onwards, mayflies exposed to Fe became constipated (Fig. 5) as they had significantly more particles in the gut than those exposed to Cd, Cd and Fe or the controls ($p<0.001$). This effect increased with increasing concentration of Fe in the water and with time of exposure (p=0.001; Table 2). Lethal concentrations of Cd ($\geq$1 mg l^{-1}) also caused decreased food intake prior to death.

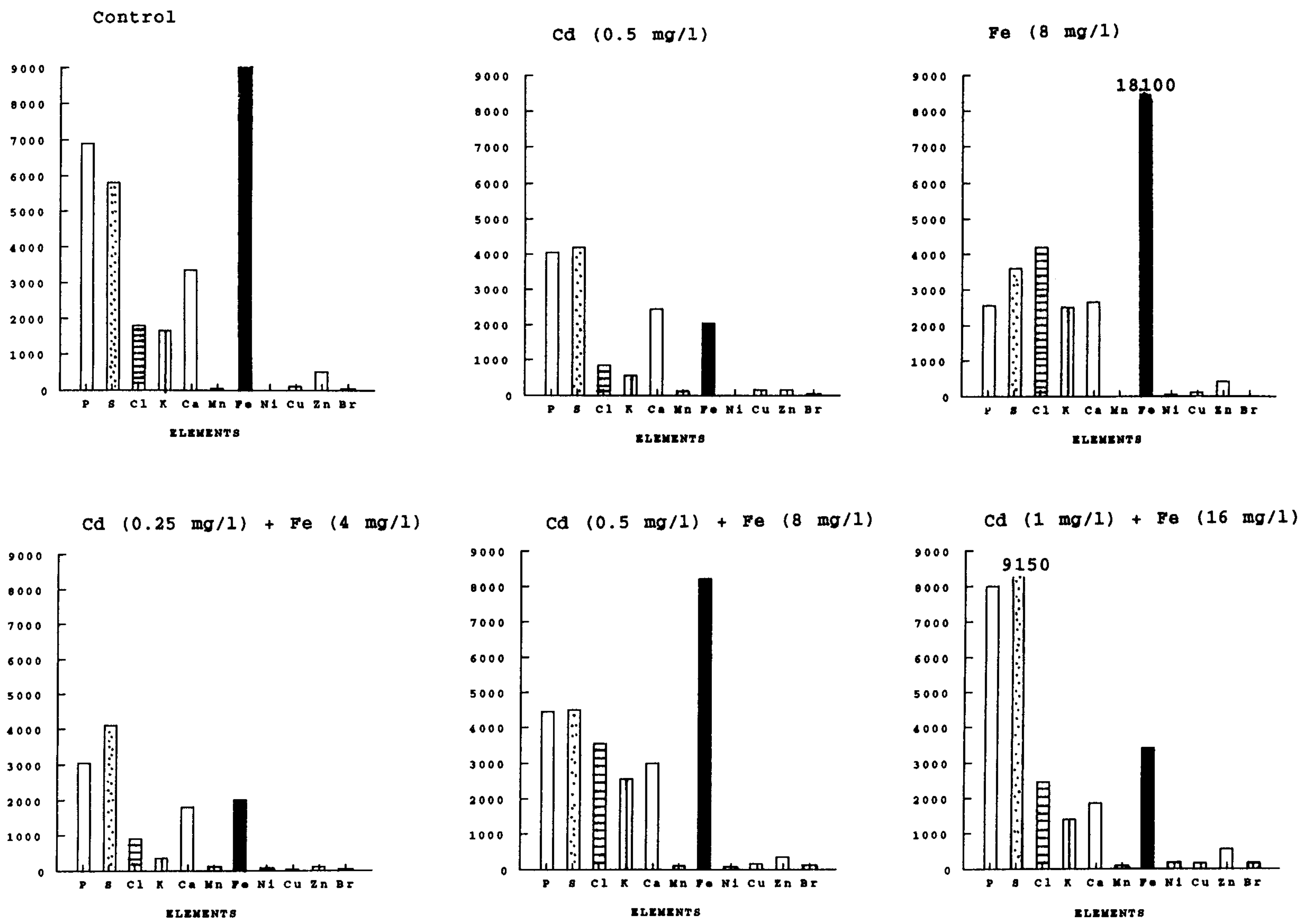

Fig. 4. Element composition of transverse sections (8 μm) through the abdominal region of *L. marginata*, analysed with PIXE and RBS. y-axis units: ng/mg.

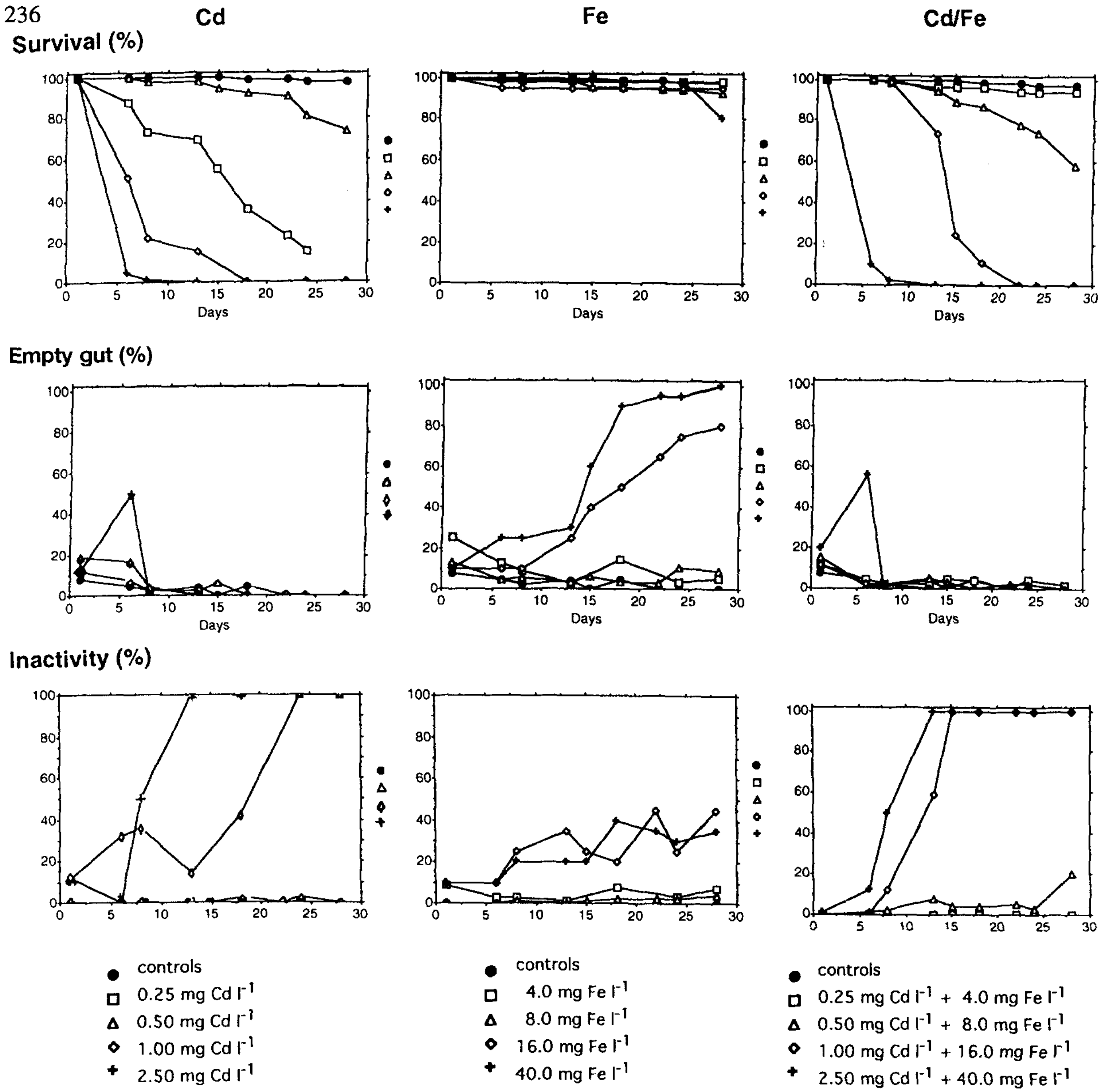

Fig. 5. Changes in the biological parameters of *L. marginata* during single and joint exposure to Cd and Fe:(a) Survival,(b) Gut contents,(c) Behavior. All values are means of 2 replicate treatments with $n = 45$.

Behavior

There were significant differences in the display of escape behavior by the mayflies depending on the kind of metal ($p<0.001$) and concentration levels in the exposures ($p<0.001$; Fig. 5). High inactivity of mayflies exposed to lethal Cd-concentrations preceeded death. From day 14 onwards, inactivity of mayflies exposed to Fe increased in a concentration dependent way. The EC_{50} values for mayflies exposed to Fe were lower than those for nymphs exposed to the metal mixtures indicating an weak antagonistic effect of Cd and Fe (Table 2; $p<0.05$).

Discussion

Metal uptake by L. marginata

The presence of Fe in the gut caused an inhibition of the uptake of Cd by *L. marginata*. Cd might coprecipitate

Table 2. EC_{50} values (mg l^{-1}) for *L. marginata* exposed to Cd, Fe and metal mixtures.

Metal	5 days EC_{50}	95% C.I.	24 days EC^{50}	95% C.I.
Survival				
Cd (mg Cd l^{-1})	1.00	1.02–1.00	1.06	1.11–0.78
Fe (mg Fe l^{-1})	–		73.07	186.2–28.20
Cd/Fe (mg Cd l^{-1})	1.45	2.10–0.90	0.61	0.73–0.51
(mg Fe l^{-1})	12.86	16.2–10.20	8.22	9.44–7.16
Food consumption				
Cd (mg Cd l^{-1})	–		6.45	7.66–5.44
Fe (mg Fe l^{-1})	8.48	9.39–7.64	50.12	51.99–48.31
Cd/Fe (mg Cd l^{-1})	–		1.81	1.82–1.80
(mg Fe l^{-1})	32.76	164.10–6.50	–	
Escape behavior				
Cd (mg Cd l^{-1})	–		1.10	1.21–1.00
Fe (mg Fe l^{-1})	19.84	26.7–14.7	23.47	23.49–23.41
Cd/Fe (mg Cd l^{-1})	–		4.31	4.77–3.81
(mg Fe l^{-1})	–		66.62	159.80–27.77

– Not linear after probit transformation.

with Fe-hydroxides in the gut lumen, thus being less bioavailable. $Fe(OH)_3$ colloids were found to bind to the cell membrane of the algae *Nitzschia closterium*, sorbing metal ions such as Cu and thus preventing Cu uptake (Stauber & Florence, 1985). Antagonistic effects of Cd and Fe on metal uptake in the intestinal tract of mice seemed to be due to competition of the metals for the same uptake sites (Hamilton & Valberg, 1974; Sugawara & Sugawara, 1991).

Zn reduced the uptake of Cd by 50% in the freshwater mussel *Anodonta cygnea*, even at low exposure concentrations (Cd: 0.025 mg l^{-1}, Zn: 2.5 mg l^{-1} (Hemelraad *et al.*, 1987). The antagonism between Cd and Zn was found mutual in algae (Ting *et al.*, 1991). Cd reduced the uptake of Cu by 50%, whereas Cu reduced Cd uptake by up to 90% in *A. cygnea*, exposed to 0.1 mg Cd l^{-1} and 0.1 mg Cu l^{-1} for about 6 weeks (Holwerda, 1991).

Thus, antagonism between a xenobiotic and an essential metal seems to be a common interaction mechanism for metal uptake by freshwater animals. However, the antagonism is not always strictly mutual.

Metal localization in L. marginata

Within the mayflies, iron occurred only as Fe^{2+} and was found in the pigments of the eyes and tracheae. Fe^{2+} is a component of the accessory pigments in the insect eye and in the cuticle, where it is involved in melanization, tanning and hardening (Locke & Nichol, 1992). Moreover, Fe^{2+} is bound to ferritin and transferrin, both proteins being responsible for transport and detoxification of free Fe^{2+} ions in insects (Locke & Nichol, 1992) and marine amphipods (Moore & Rainbow, 1992).

Fe^{3+} was located in the gut, on the surface of the body and on the gills of mayflies exposed to iron alone or together with Cd. Fe-crusts on the gills might also affect oxygen uptake and osmoregulation of aquatic invertebrates (McKnight & Feder, 1984). However, the time taken to build up a Fe-hydroxide layer on the gills was longer than that for Fe-incrustation in the gut, probably due to the acidic conditions and the low particle concentration in the surrounding water compared to the circumneutral pH and high particle concentrations in the gut lumen.

Changes in element composition

Cl^- and K^+ were found in low concentrations in the body sections of mayflies kept at pH 4.5 but not exposed to any metal. In acid soft-waters, insects loose Na^+ and K^+ ions from their body and a compensatory loss of Cl^- occurs in order to maintain the balance of the charges at the membranes (Wood, 1991).

Exposure of the mayflies to Cd resulted in an additional loss of K^+ and Cl^- from the body. This is supported by findings from Hemelraad *et al.* (1987) for the freshwater clam *Anodonta cygnea.* Cd-exposure also caused K^+ efflux from the algae *Scenedesmus quadricauda* and yeast cells and was explained as an exchange for Cd^{2+} uptake (Reddy & Prasad, 1992). Even low concentrations of Cd^{2+} (0.003 mg l^{-1}) led to changes in the chloride cells of fish gills, resulting in an impairment of respiratory, osmoregulatory and excretory functions (Bauer, 1988; Gony, 1989). The toxic mechanism behind these disturbances in ion regulation might be an interaction of Cd with the SH-groups of the enzyme ATPase of the ion pumps in the membranes of fish gills (Shephard & Simkiss, 1978) and in the gut epithelium of fish and humans (Verbost *et al.*, 1987: Hajem *et al.*, 1991).

Concentrations of $\geq$8 mg Fe l^{-1} reduced the pH- and Cd-induced ion losses from the insects. Iron hydroxide crusts on the gill and gut membranes may serve as mechanical protection against ion losses. During the first 10 days of exposure, >0.5 mg Cd l^{-1} might have affected the membranes, as the mayflies died before the protective Fe-crusts had been formed. High concentrations of sulphur and phosphorus in the mayflies exposed to lethal Cd-concentrations might be a sign of the initiation of a detoxification mechanism for Cd. For example, Cd is bound as an insoluble pyrophosphate salt in granules in the gills of freshwater mussels, whilst in marine mussels, Cd is bound to S-groups of metallothionein (Nott & Langston, 1989).

It is obvious, that Cd^{2+} affects ion regulation in aquatic organisms. However, Fe seems to reduce the adverse effects of Cd by the formation of protective Fe-hydroxide crusts on the gill and gut membranes.

Biological parameters

Survival

The effects of mixtures of Cd and Fe on survival of *L. marginata* were mainly caused by Cd. The acute toxicity of Cd on *L. marginata* may obscure chronic effects of Fe. Other studies of chronic exposure of freshwater invertebrates to metal mixtures are rare. Spehar *et al.* (1978) demonstrated that Cd and Zn had a less than additive effect on larval survival of the flagfish *Jordanella floridae.* Comparisons with studies involving experiments with complex metal mixtures (Hutchinson & Sprague, 1986) are difficult, because it is not known which of the components in the metal mixture caused the observed effects. Comparisons with short-term experiments (de March, 1988; Thorp & Lake, 1974; Herkovits & Péres-Coll, 1991) are difficult, as there may be different mechanisms of toxicity during short and long term exposure.

Food consumption

The observed decrease in food consumption of *L. marginata* caused by Fe-exposure, alone or in combination with Cd, might be explained by Fe-hydroxide-precipitation on the gut membrane of the insects, thus preventing the uptake of food. Other studies reported antagonistic as well as synergistic effects of metal mixtures on food consumption. For example, mixtures of Zn and Cd caused a significantly greater reduction of the filtration rate of the bivalve *Anadena granosa* compared to that of the single metal exposures (Patel & Anthony, 1991). The filtration rate of marine bivalves was reduced by 50% in mixtures of Cd and Hg compared to that in the single metal exposures (Mohan *et al.*, 1986). Mixtures of Cu and Zn caused antagonistic effects on the filtration rates of the freshwater bivalve *Dreissena polymorpha,* whilst Cu and Cd acted synergistically after an exposure of *D. polymorpha* for 48 h (Kraak *et al.*, 1994).

Behavior

The activity of the mayflies was reduced by acute toxic levels of Cd. The activity was also slightly decreased by sublethal concentrations of Fe, which might be due to the formation of thick precipitations on the body, which immobilize the mayflies. In the metal mixture exposures, the adverse effect of Cd on activity of the mayflies was reduced by the presence of Fe, probably due to decreased Cd-uptake. The interaction of Cd and Fe might be a weak antagonism. Prakasam *et al.* (1989) measured ciliary activity (time to move a distance of 1 cm) of a marine bivalve under Zn and Hg stress. Both metals increased the activity of the mussel at low concentrations, but decreased it at higher concentrations. In combination, a weak antagonistic effect of the metals was observed.

Conclusions

Mixtures of Cd and Fe acted antagonistically on metal uptake by *L. marginata,* probably due to precipitation processes and competition between the metal ions at the uptake sites. Fe-crusts on the gut membrane impeded uptake of food and nutrients. Fe-crusts on the gills counteract K^+ and Cl^- efflux from the mayflies, which was caused by Cd-exposure. Before that protection layer on the gills had built up, the disruption of ion regulation by Cd led to death of the mayflies exposed to high metal concentrations.

Acknowledgements

I am grateful to C. Becker and S. Hülsman for their assistance during the daily control of some of the experiments in autumn 1991. I wish to thank M. Kaiser for assistance in the histochemical preparations and T. Olsson for the analyses of Cd and Fe with flame AAS. J. Pallon and C-Y. Yang from the Dept of Nuclear Physics at Lund University kindly performed the PIXE and RBS analyses at the Lund Nuclear Microprobe. I am very grateful to Dr M. H. S. Kraak for critical comments on this paper. Dr R. Finlay kindly corrected the English text. I also appreciate the discussion of the histochemical results from the Cd-staining with Dr P. Prentø. This study was supported by grants from the Swedish Environmental Protection Agency and 'OKs Miljöstiftelse'.

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