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Influence of Ca, humic acid and pH on lead accumulation and toxicity in the fathead minnow during prolonged water-borne lead exposure

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Abstract

The present study examines the influence of Ca^{2+} as ($CaSO_4$), dissolved organic carbon (DOC) and pH on chronic water-borne lead (Pb) toxicity to the larval fathead minnow (*Pimephales promelas*) under flow-through conditions. The 30 day LC50 for low hardness basic test water (19 mg $CaCO_3$ L^{-1}) was 39 (range: 27–51) μg dissolved Pb L^{-1} and was greatly increased by increasing concentrations of $CaSO_4$ and DOC to as much as 1903 (range: 1812–1992) μg dissolved Pb L^{-1}. Both reduced and increased pH (6.7 and 8.1, respectively) compared to control pH of 7.4 appeared to increase Pb toxicity substantially. Whole body Pb accumulation did not reflect water chemistry and thus exhibited no correlation with Pb induced mortality. One possible explanation for this lack of correlation is that mortality occurred predominantly during the first 4–6 days of exposure, whereas Pb accumulation was determined in surviving fish at the end of 30 days of exposure. Chronic Pb exposure resulted in a general iono-regulatory disturbance affecting K^+, Na^+ and Ca^{2+} homeostasis. However, recovery of Na^+ and K^+ levels and reversal of effects on Ca^{2+} homeostasis during continued exposure strongly suggest fathead minnow can acclimate to Pb. The gills accumulate the highest Pb concentrations during chronic exposure but the skeleton contains the largest mass of Pb by contributing up to ~80% of whole body Pb. In conclusion, water chemistry characteristics like Ca^{2+} and DOC should be considered for chronic water quality criteria.

Keywords: Chronic Pb toxicity; Calcium; DOM; pH; *Pimephales promelas*

1. Introduction

Lead (Pb) is not required for normal physiology in plants or animals and is potentially toxic. Erosion and volcanic activity contribute to environmental Pb as do anthropogenic sources such as combustion of fossil fuels and mining processes. As a result of these processes, surface waters may contain elevated Pb concentrations, especially in industrialized areas (World Health Organization, 1995). Lead toxicity arising from chronic water-borne exposure has been reported for a number of freshwater species and occurs at concentrations as low as 4 μg/L (Grosell et al., 2005). Such low concentrations are well within the range of concentrations reported from freshwater environments (World Health Organization, 1995) emphasizing the need for further studies of Pb toxicity. It is well established that water chemistry can influence toxicity of water-borne exposure to most metals including copper (Pagenkopf, 1983; Welsh et al., 1993; Erickson et al., 1996), silver (Erickson et al., 1998; Bury, 1998), zinc (De Schamphelaere and Janssen, 2004a) and cadmium (Meinelt et al., 2001). However, less information is available about how water chemistry might influence Pb toxicity arising from acute or chronic water-borne exposure. Exceptions include observations of a strong protective effect of hardness on both acute and chronic Pb toxicity to rainbow trout (Davies et al., 1976) and clear effects of pH on Pb induced chronic toxicity to the common carp (Stouthart et al., 1994) and rainbow trout (Hodson et al., 1978b).

Supporting the effect of pH on Pb induced toxicity are observations of increased Pb accumulation in blood of rainbow trout at reduced water pH (Hodson et al., 1978b). More recently,

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Table 1
Chemistry of test solutions (in μM except for hardness which is expressed as mg/L $CaCO_3$)

	Concentration										pH
	$[Na^+]$	$[K^+]$	$[Ca^{2+}]$	$[Mg^{2+}]$	$[Cl^-]$	$[SO_4^{2-}]$	$[HCO_3^-]$	$[CO_3^{2-}]$	[DOC]	Hardness	
Base water	352±2	27±1	143±5	43±4	404±47	47±1	283±62	0.3±0.07	100±9	19±1	7.4±0.1
0.5 mM Ca^{2+}	351±13	30±4	425±13	44±5	418±66	287±27	269±59	0.2±0.04	–	47±2	7.2±0.1
1.0 mM Ca^{2+}	305±11	14±1	965±27	43±4	380±40	910±75	269±59	0.2±0.04	–	104±3	7.2±0.1
2.0 mM Ca^{2+}	364±3	20±1	2074±90	45±2	282±4	1752±24	276±61	0.2±0.04	–	218±2	7.3±0.1
2 mg humic	419±4	24±1	177±7	48±3	322±7	18±8	302±66	1.3±0.29	168±23	22±1	8.0±0.1
4 mg humic	400±11	22±1	164±3	45±1	294±7	18±7	300±66	1.0±0.22	215±25	21±1	7.9±0.1
8 mg humic	486±10	27±1	178±11	58±2	326±1	25±12	302±66	1.3±0.29	608±215	25±1	8.0±0.1
16 mg humic	515±11	26±1	179±11	55±1	320±17	25±5	300±66	1.0±0.22	878±219	25±1	7.9±0.1
pH 6.3	358±111	222±89	157±34	34±4	541±154	30±10	209±46	0	–	19±4	6.7±0.1
pH 8.3	368±100	1672±535	160±30	33±5	305±47	30±10	303±67	1.6±0.35	–	19±4	8.1±0.1

$[HCO_3^-]$ and $[CO_3^{2-}]$ concentrations were calculated from measured pH and the mean total $[CO_2]$ concentration in the test solutions (310±67 μM).

studies of acute Pb toxicity and gill Pb binding revealed that dissolved organic carbon (DOC) greatly influence short term gill binding and acute toxicity in juvenile rainbow trout (Macdonald et al., 2002). To summarize, only hardness and pH have been demonstrated to influence Pb toxicity during chronic or prolonged exposures. The study examining the influence of water hardness on chronic Pb toxicity varied not just the concentrations of the major hardness ions (calcium and magnesium) but also pH, alkalinity and the concentrations of most other common freshwater inorganic constituents (Davies et al., 1976).

The United States Environmental Protection Agency (USEPA) is currently recognizing the importance of water quality by employing the Biotic Ligand Model (BLM) to establish acute site specific water quality criteria for copper (USEPA, 2003) and the development of acute BLMs for other metals is well underway. However, chronic water quality criteria for metals do not directly consider the influence of water chemistry on chronic toxicity which represents a shortcoming in current environmental regulation of metals. With the long term goal of being able to predict chronic Pb toxicity in freshwaters of different water chemistries we aimed at evaluating the influence of key water chemistry parameters. We chose to investigate the effect of DOC on chronic Pb toxicity because DOC has been demonstrated to potently reduce acute Pb toxicity (Macdonald et al., 2002) and chronic copper toxicity (McGeer et al., 2002; De Schamphelaere and Janssen, 2004b). In addition, the influence of Ca^{2+} was considered, not only because Ca^{2+} is the main hardness ion but also because Pb is a known Ca^{2+} antagonist interrupting Ca^{2+} transport through voltage activated calcium channels (Büsselberg et al., 1991; Audesirk, 1993) and across fish gills (Stouthart et al., 1994; Rogers et al., 2003; Rogers and Wood, 2004). Finally, we included variation in pH as part of the present study since pH has previously been demonstrated to influence chronic Pb toxicity and accumulation (Hodson et al., 1978b; Stouthart et al., 1994).

The present study was performed on the teleos fish, the fathead minnow, *Pimephales promelas* (Cyprinidae), which is commonly used for toxicity testing. Teleosts are well suited test organisms for studies of chronic Pb toxicity because they are among the most sensitive organisms tested to date. Available information about chronic Pb toxicity in freshwater organisms was recently summarized to reveal that teleos fish are among the most Pb sensitive organisms, only exceeded in sensitivity by freshwater pulmonate snails (Grosell et al., 2005).

2. Materials and methods

2.1. Experimental animals

Fathead minnow (*Pimephales promelas*) of <24 h old were obtained from Aquatic BioSystem, Inc. (Fort Collins, CO, USA). Groups of 10 fish were placed in 1 L polyethylene beakers each receiving a flow-through of approximately 20 mL min^{-1} of 2:1 dechlorinated Virginia Key tap water:deionized water and were fed freshly hatched *Artemia* nauplii once daily.

2.2. Chronic Pb exposure

Lead exposures were initiated following 1 week of gradual acclimation to appropriate test media. Exposure media were made up from a base water consisting of 2:1 deionized water: dechlorinated Virginia Key tap water (Table 1) and were kept at 23±1 °C. This medium was modified to achieve the desired experimental test waters as described below.

Deionized water and dechlorinated Virginia Key tap water were supplied via gravity to a series of primary mixing chambers at a 2:1 ratio. Stock solutions of $CaSO_4$, Aldrich humic acid (HA) or MOPS–KOH were added to these primary mixing chambers via Mariotte bottles or peristaltic pumps to achieve the test conditions reported in Table 1. Total water flow into the primary mixing chambers each with a volume of 30 L, was approximately 250 mL min^{-1} with mixing being achieved by vigorous aeration. A constant water level in the primary mixing chambers was achieved by an overflow drain. Each primary mixing chamber fed test water via gravity to a total of six (6) secondary mixing chambers at a constant rate of approximately 30 mL·min^{-1}. Lead stock solutions ($PbNO_3$ in nanopure water) were added to these secondary mixing chambers, each 4 L in volume, from Mariotte bottles at a constant rate of 1 mL·min^{-1}. Each secondary mixing chamber was aerated to ensure mixing and supplied test solution (Pb added) to each of three replicate 1 L test chambers, containing larval fathead minnows, at a flow rate of 6–8 mL·min^{-1}.

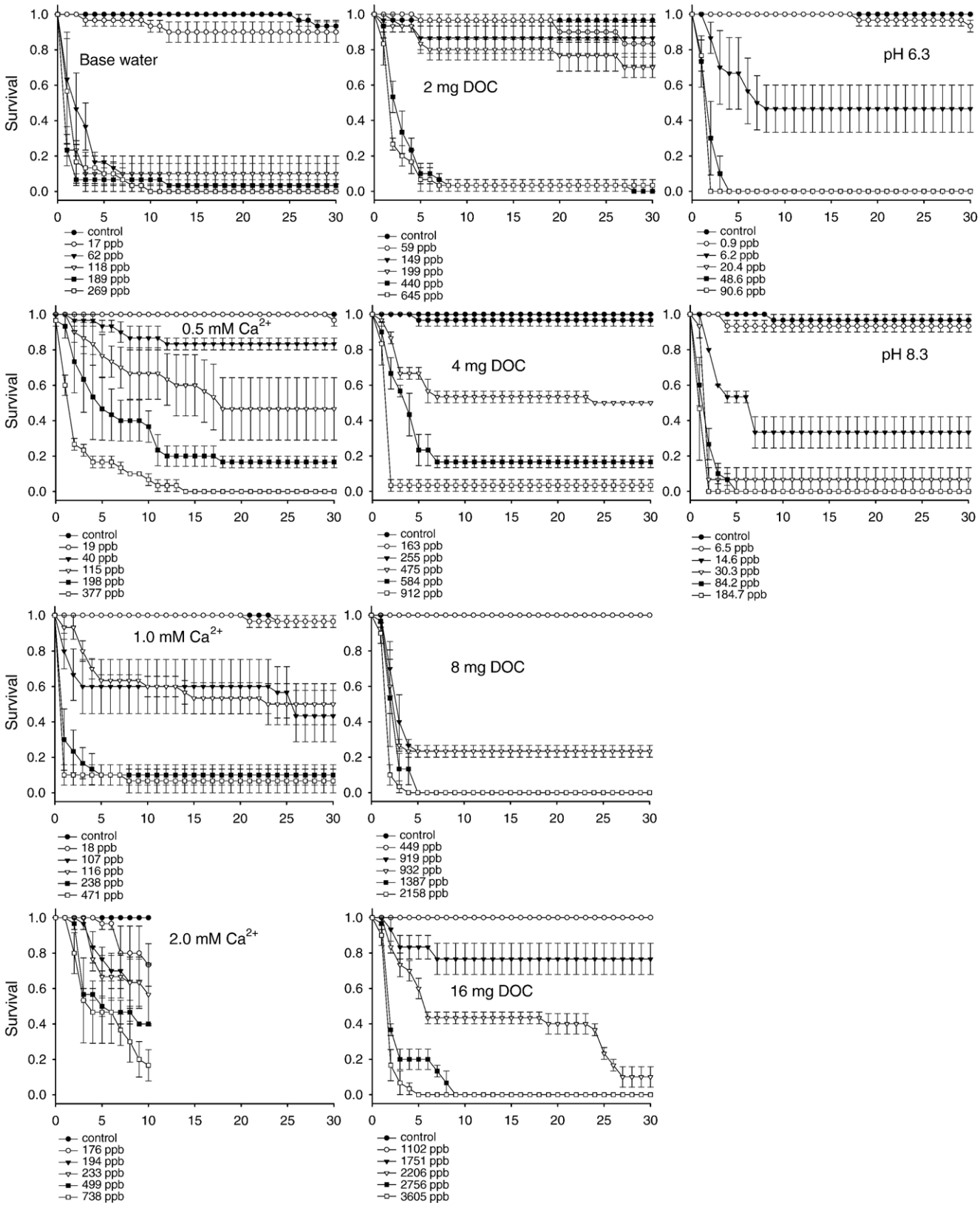

Fig. 1. Cumulative mortality of fathead minnow (8–38 days of age) during 30 days of exposure to a range of Pb concentrations (as dissolved Pb) at different water compositions (see Table 1 for detailed water chemistry).

Table 2
Concentrations ($\mu g \cdot L^{-1}$) at which 50% lethality occurred (LC50s) (95% confidence interval) and Acute to Chronic Ratios (ACR)

Medium	4-day LC50	10-day LC50	30-day LC50	ACR
Base water	52 (0.04–131)	43 (9–83)	39 (27–51)	1.3
0.5 mM Ca^{2+}	206 (170–257)	136 (109–170)	91 (73–113)	2.3
1.0 mM Ca^{2+}	174 (139–208)	155 (126–184)	104 (70–133)	1.7
2.0 mM Ca^{2+}	524 (397–838)	293 (223–382)	N/A	>1.8
2 mg humic	372 (318–432)	273 (235–316)	255	1.5
4 mg humic	544 (487–606)	435 (385–490)	443 (393–495)	1.2
8 mg humic	1094 (749–1187)	932 (619–1007)	832	1.3
16 mg humic	1656 (1140–2779)	1684	1903 (1812–1992)	0.9
pH 6.3	7.9 (7.5–8.3)	7.3 (6.5–7.8)	4.5 (3.1–6.1)	1.8
pH 8.3	15 (3.3–52)	13 (11–16)	13 (10–16)	1.2

Calcium concentrations were manipulated through addition of a 12 mM $CaSO_4$ stock solution (close to the solubility limit for $CaSO_4$) at different rates controlled by peristaltic pumps. Aldrich humic acid was used as an affordable and well characterized dissolved organic carbon (DOC) surrogate and was added at constant rates to the primary mixing chambers via Mariotte bottles. Desired DOC concentrations were achieved by varying the DOC concentrations in the stock solutions. A low (6.7) and a high (8.1) pH in test solutions bracketing the control water pH of 7.4 was achieved by adding a 400 mM MOPS buffer stock solution to the primary mixing chambers to achieve a final concentration of approximately 4 mM. The stock solutions were titrated to pH 6.3 and 8.3 for the low and high pH exposure, respectively through addition of KOH.

The demand for $CaSO_4$ stock solution for the 2 mM $CaSO_4$ exposures was close to 60 L day^{-1} which led us to limit the duration of these exposures to 10 days as opposed to the remaining tests which were all 30 days in duration. The 10-day duration was chosen as a compromise between $CaSO_4$ use and sufficient exposure time to result in Pb induced mortality. Lead exposures at lower $CaSO_4$ concentrations revealed that the majority of the Pb induced mortality occurred within the first 7–10 days of exposure.

2.3. Animal care and sampling protocol

Fish were inspected daily, mortality was recorded and dead fish were removed from exposure chambers. Each exposure chamber was siphoned clean from leftover food and feces prior to feeding. Fish were fed freshly hatched *Artemia* sp. daily for the duration of the exposures and, in addition, were fed Tetramin flake food from day 20 onwards.

Water samples were collected daily from each treatment for the first three days of exposure and once–twice per week for the remaining exposure period. Aliquots of water samples were passed through a 0.45 μm cellulose nitrate syringe filter (Acrodisc, Pall Life Sciences, MI, USA) and then acidified by addition HNO_3 (Fisher Scientific, trace metal grade) to a final concentration of 1%. Remaining water was analyzed for O_2 content, pH, total CO_2 and concentrations of dissolved organic carbon, major cations and major anions as described below. At the end of the Pb exposures, surviving fish were euthanized by an overdose of buffered tricaine methanesulfonate (MS–222) (0.3 g L^{-1}), blotted dry on paper towels and placed in pre-weighed 15 mL Falcon tubes. Mass of individual fish was determined to the nearest milligram by re-weighing the Falcon tubes containing the fish.

2.4. Internal distribution of accumulated Pb

A separate set of experiments were conducted under conditions similar to those described above to follow the time course of Pb accumulation in selected tissues. These experiments were initiated with 20 day old fish (~80–100 mg each) rather than 8 day old fish to allow for dissections of gill, liver, muscle and intestine. Fish were exposed to 26±5 μg L^{-1} in the base test water for up to 30 days with 10 fish being sampled on days 4, 10 and 30 of exposure. Unexposed control fish were sampled on day 30 only. Dissected gills, liver, muscle and intestine as well as the remaining carcasses were placed in pre-weighed 2 mL centrifuge tubes, their mass was determined and samples were prepared for analysis of Pb concentration as outlined below.

2.5. Analytical techniques

Whole surviving fish as well as gill, liver, muscle and intestine of dissected fish were digested in 1 N trace metal grade nitric acid (approximately 5 vol of acid: tissue mass) at 80 °C overnight. Samples were vortexed rigorously and centrifuged at 10,000×*g* for 2 min after which the supernatant was recovered for analysis of Pb concentration. To separate soft tissue Pb from Pb incorporated

Table 3
Concentrations ($\mu g \cdot L^{-1}$) at which 20% mortality occurred (EC20s) and No Observable Effect Concentrations (LOECs) (95% confidence interval)

Medium	4-day EC20	4-day LOEC	10-day EC20	10-day LOEC	30-day EC20	30-day LOEC
Base water	25 (0–59)	69	23 (1.2–45)	68	21.6 (12–31)	62
0.5 mM Ca^{2+}	114 (82–141)	117	68 (47–87)	111	47 (33–60)	40
1.0 mM Ca^{2+}	95 (61–122)	168	90 (58–114)	136	51 (22–75)	107
2.0 mM Ca^{2+}	209 (141–271)	178	111 (48–160)	171	N/A	N/A
2 mg humic	254 (196–300)	451	197.9 (160–230)	466	189	199
4 mg humic	401 (339–451)	414	306 (254–349)	406	318 (262–363)	475
8 mg humic	919 (428–1062)	1237	805 (364–918)	1026	736	919
16 mg humic	1145 (510–1573)	1051	1684	1495	1729 (1591–1816)	1751
pH 6.3	6.6 (6.0–7.5)	7.5	5.7 (5.1–6.2)	7.5	2.1 (1.2–3.1)	6.2
pH 8.3	8.0 (0.03–15)	13	9.3 (6.5–11)	15	8.7 (5.9–11)	15

in skeletal tissue, carcasses from the experiment investigating the internal distribution were subjected first to a digestion in 1 N NaOH at 80 °C overnight to allow for analysis of Pb in soft tissue. The resulting basic digest was subsequently acidified by addition of 2 vol of 2.765 N HCl and left to digest at 80 °C overnight. This later acidic digest allowed for analysis of both soft tissue and skeletal Pb combined. Lead concentrations in tissue digests and water samples were analyzed by graphite furnace atomic absorption (Varian 220Z, Varian, Australia). Test water was analyzed for Na^+, K^+, Ca^{2+} and Mg^{2+} by atomic absorption (Varian 220FS, Varian, Australia), for Cl^- and SO_4^{2-} by anion chromatography (DIONEX DX120, CA), for pH (PHM220 meter, Radiometer, Copenhagen, Denmark) fitted with an Accumet combination glass electrode) and for total CO_2 using a Corning 962 carbon dioxide analyzer (UK). Dissolved oxygen was measured by a handheld dissolved oxygen meter (WTW Oxi 340i, Weilheim, Germany). In addition, the whole fish Na^+, K^+, Ca^{2+} and Pb concentrations in selected groups were determined after appropriate dilution by atomic absorption (instrumentation as above). DOC measurements were performed by high-temperature catalytic oxidation using a Shimadzu total organic carbon-VCSH analyzer (Kyoto, Japan) (Hansell and Carlson, 2001).

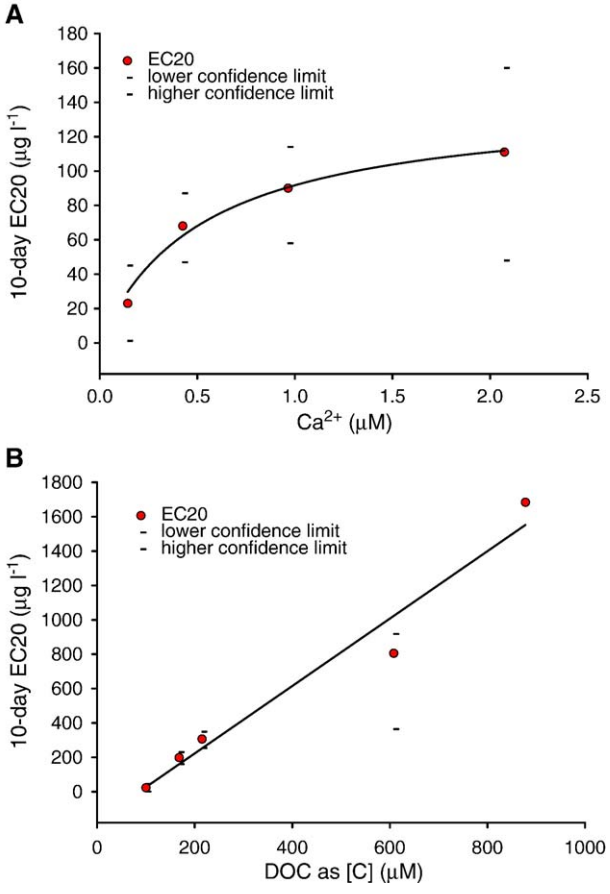

Fig. 2. Dependence of Pb 10-day EC20 on ambient [$CaSO_4$] and [dissolved organic carbon] in test solutions. Solid points represent calculated EC20 values with horizontal bars representing upper and lower confidence limits. Relationship between ambient [$CaSO_4$] and 10-day EC20 is best described as: $EC20 = 140.75 \cdot [CaSO_4]/(0.535 + [CaSO_4])$, $r^2 = 0.98$. The influence of DOC on EC20 adhere to $EC20 = [DOC] \cdot 1.96$, $r^2 = 0.96$.

2.6. Statistical analysis and data presentation

Data are presented as means ± 1 standard error of the mean (SEM) as a function of mean measured dissolved Pb unless otherwise stated. Survival and growth data were evaluated using the statistical computer package ToxCalcTM version 5.0 (Tidepool Scientific, CA, US). In all cases, the lowest observable effect concentration (LOEC) was determined using analysis of variance (ANOVA) and the EC20 and LC50 for days 4, 10 and 30 were determined by linear regression.

Tissue concentrations of Ca^{2+}, Na^+, K^+ and Pb are reported as concentration per unit wet mass while bioconcentration factors are derived from Pb concentrations expressed per unit dry mass. Dry mass was estimated from wet mass assuming 80% water content of the fathead minnows.

Fish size at the end of exposures and tissue concentrations of major cations were compared using Student's *t*-test with multi-sample comparison correction whenever appropriate. In all cases, samples were considered statistically significantly different at $P < 0.05$.

3. Results

3.1. Water chemistry

Generally, the chemical compositions of the test waters were close to the desired profile (Table 1). A range of different calcium concentrations were achieved which were associated with similar sulfate concentrations as a result of $CaSO_4$ addition. Similarly, a range of different DOC concentrations were accomplished by addition of HA. Interestingly, increasing DOC concentrations were associated with slight increases in sodium concentrations and elevated pH from 7.4 in the control base water to 7.9–8.0 in waters supplemented with HA (Table 1). Finally, addition of buffered MOPS stock solutions resulted in reduced and increased pH as desired. However the target pHs of 6.3 and 8.3 were not achieved and resulted in waters with pH of 6.7 and 8.1, respectively. These pH levels, which differed from the control base water at 7.4 were associated with different K^+ concentrations as a result of pH adjustment of MOPS stock solutions by KOH addition (Table 1). Dissolved oxygen remained at > 90% saturation.

3.2. Effects of Pb exposure — mortality

All control groups exhibited limited or no mortality regardless of water chemistry (Fig. 1). Lead induced mortality was observed in all test waters, although at very different dissolved Pb concentrations. Interestingly, for most treatments, most mortality occurred during the first 5–7 days of exposure with almost no mortality occurring after day 10 (Fig. 1). Based on cumulative mortality data, calculated LC50s, EC20s and LOECs are presented in Tables 2 and 3. Acute to chronic ratios (ACR) were determined from the 4 day and 30 day LC50 values and are presented in Table 2. Generally these ACRs are low regardless of

Table 4
Mean±SEM body mass at the end of 30 day exposure (2 mM Ca^{2+} were sampled after 10 days of exposure) to ranges of Pb concentrations in different test media

Medium	[Pb] (μg L^{-1})	Mass (mg)
Base water	Control	96±6 (28)
	17±2	94±6 (27)
	62±5	123±28 (3)
	118±11	100±28 (3)
	189±16	–
	352±90	–
0.5 mM Ca^{2+}	Control	90±7 (30)
	19±4	79±5 (29)
	40±4	103±6 (25)
	115±7	100±11 (14)
	198±12	115±12 (5)
	377±21	–
1.0 Mm Ca^{2+}	Control	142±8 (30)
	18±3	142±7 (30)
	107±16	192±18 (13)*
	116±15	200±13 (15)*
	238±33	237±51 (3)*
	471±47	204±5 (2)*
2.0 mM Ca^{2+}	Control	15±1 (30)
	171±5.2	15±1 (24)
	194±64	16±1 (17)
	233±17	12±1 (19)*
	499±9	9±1 (3)*
	738±64	11±2 (2)
2 mg DOC	Control	120±9n (29)
	59±3	134±9 (25)
	149±13	134±10 (26)
	199±15	150±8 (21)*
	440±40	–
	645±31	–
4 mg DOC	Control	124±10 (30)
	163±7	119±10 (29)
	255±13	113±7 (29)
	475±17	174±21 (15)*
	585±38	176±17 (3)
	912±58	176±60 (2)
8 mg DOC	Control	144±13 (30)
	449±83	134±9 (30)
	919±99	234±18 (7)*
	932±97	229±21 (7)*
	1387±115	–
	2158±170	–
16 mg DOC	Control	138±10 (29)
	1102±87	119±7 (30)
	1751±144	131±12 (24)
	2206±375	138±36 (3)
	2756±183	–
	3605±110	–
pH 6.3	Control	95±6 (30)
	0.9±0.1	92±6 (29)
	6.2±0.9	138±8 (14)*
	20±4	–
	49±7	–
	91±18	–
pH 8.3	Control	89±6 (29)
	6.5±0.8	92±5 (28)
	15±1	148±11 (10)*
	30±2	–
	84±6	–
	185±13	–

Numbers in brackets denotes *n*-numbers and "*" denotes statistical significant difference from corresponding control.

test medium, illustrating that most Pb induced mortality occurred early during the 30-day exposure. However, the ACR from groups exposed in media with elevated calcium (and also the group in low pH) exhibit higher ACRs than observed in control water and waters with added DOC and high pH.

The lowest tolerance of fathead minnow to Pb exposure regardless of exposure time was observed at low pH (6.8) followed by elevated pH (8.1) and control water. Progressive increase of $CaSO_4$ resulted in increased resistance to Pb as indicated by higher EC20s, LC50s and LOECs regardless of exposure time. A point worth noting is that while toxicity thresholds based on 4-day mortality results in as much as a 10-fold difference in sensitivity between the controls and the 2 mM calcium group, this difference is less at 10 days of exposure (Tables 2 and 3).

Addition of DOC (as HA) resulted in as much as a 20-fold increase in tolerance to Pb at the highest concentration employed (16 mg DOC). Unlike calcium, DOC appears to provide the same degree of protection against Pb induced mortality during both short- and long-term Pb exposure (Tables 2 and 3).

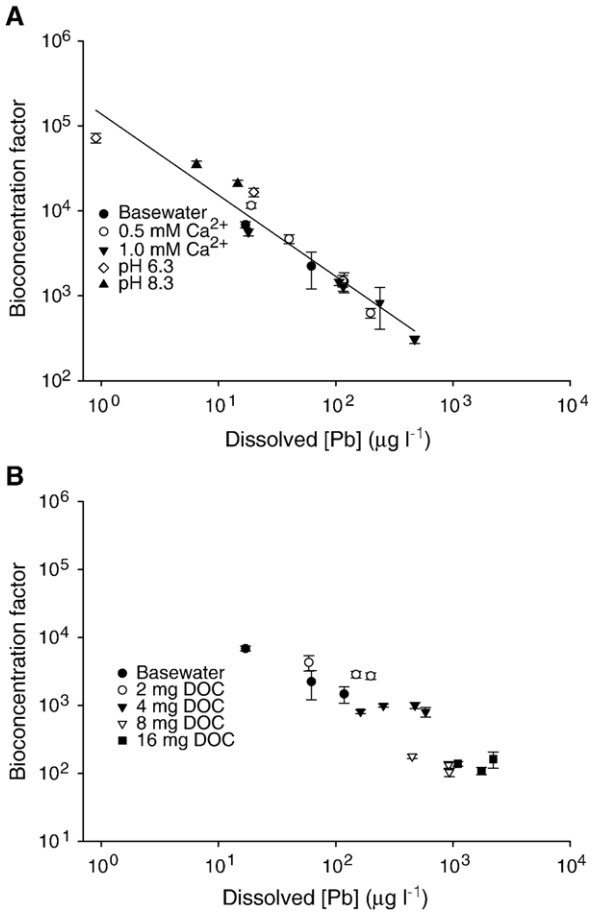

Fig. 3. Bioconcentration of Pb in fathead minnow as a function of dissolved Pb concentration following 30 days of water borne exposure in 9 freshwater media of different chemical composition. Top panel show effect of varying Ca^{2+} and pH while bottom panel show the effect of DOC. Mean±SEM, $N=10$ except for treatments were mortality prevent analysis of 10 individuals. The 10 individual were chosen randomly from all three replicates of a given treatment. For those treatments with less than 10 surviving individuals at the end of the 30-day exposure, all surviving fish were analyzed (see Table 2 for *N*-numbers).

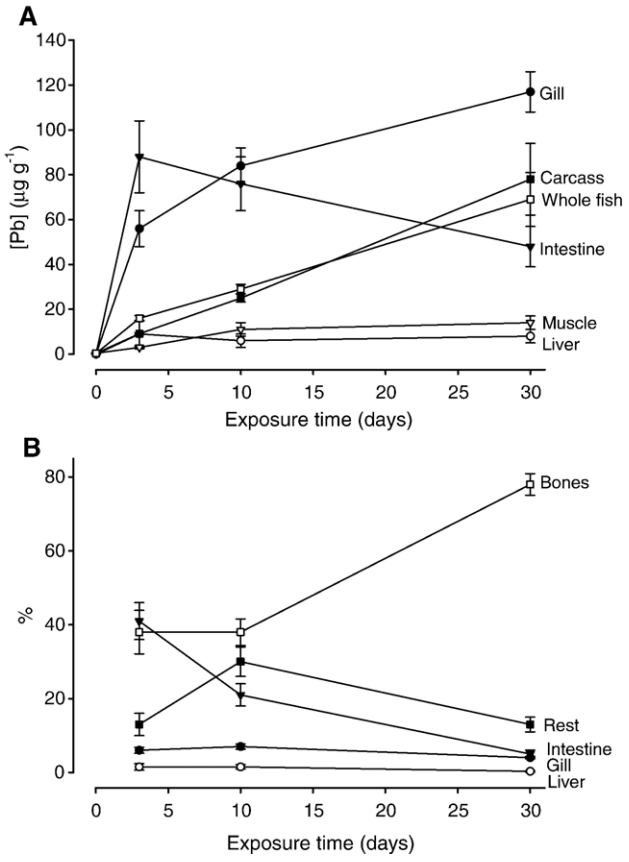

Fig. 4. Lead accumulation (A) and internal distribution among selected tissues (B) in fathead minnow during 30 days of water borne exposure to 26 µg Pb L^{-1}. Mean±SEM, N=9–10 in all cases. All examined tissue exhibited significant Pb accumulation during the 30 days of exposure.

The strong relationship between Pb induced mortality, expressed as the 10-day EC20, and ambient concentrations of $CaSO_4$ and DOC is displayed in Fig. 2.

3.3. Effects of Pb exposure — growth

The control fathead minnow larvae exhibited high growth rates in all waters tested reaching a wet weight of 100–140 mg in mass at the end of the exposures (38 days old). The 2 mM calcium group reached a final wet mass of 15 mg by day 10 (Table 4). For all 30-day exposed fish, no growth inhibition was observed. Rather, in a number of waters, an apparent increased growth was observed in groups exposed to higher Pb concentrations. It should be noted that this apparent stimulated growth occurred in groups where considerable mortality occurred early during exposure (Table 4).

Table 5
Whole body concentration of major cations in control and Pb exposed fathead minnows after 30 days

	Whole body ion concentration (µmol g^{-1})		
	K^+	Na^+	Ca^{2+}
Control	220±10	128±6	208±12
Pb exposed (115 µg L^{-1})	200±18	106±10	266±22*

Two sub-samples of 10 fish each from the 0.5 mM Ca^{2+} trail were selected for this analysis. "*" denotes statistical significant difference from control.

3.4. Effects of Pb exposure — Pb accumulation and internal distribution

Bioconcentration factors exhibited a wide range of over three orders of magnitude from 10^2 to 10^5 and a general strong negative correlation with ambient dissolved Pb concentration (Fig. 3A and B). Surprisingly, treatments with varying Ca^{2+} and pH appear to adhere to the same general relationship between dissolved ambient Pb concentration and whole body Pb accumulation (Fig. 3A). A similar relationship was not seen for DOC treatments; at

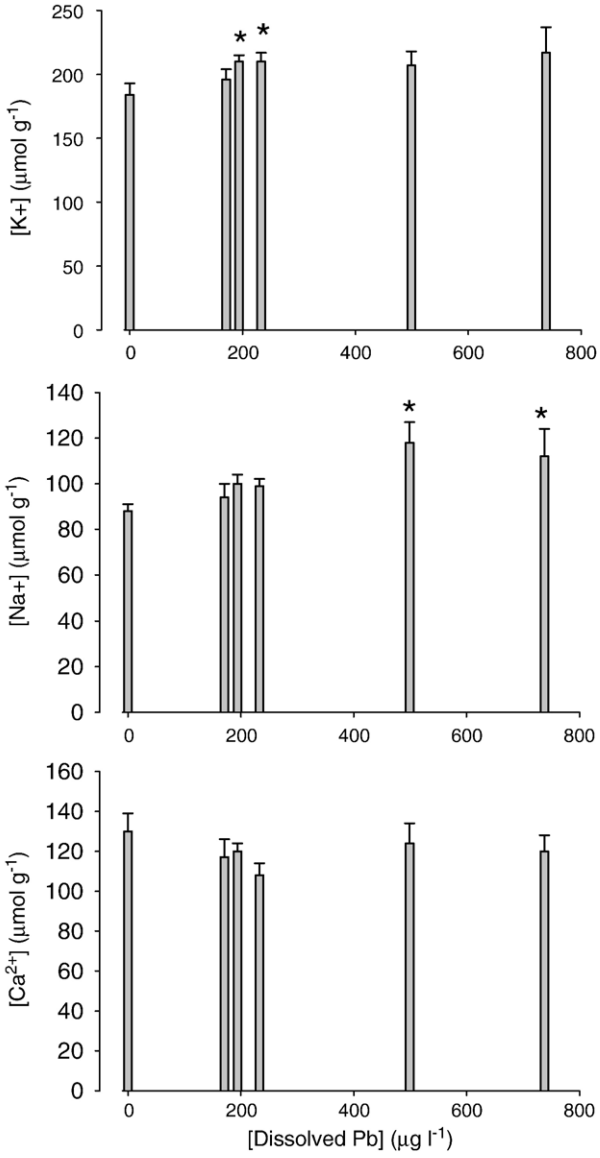

Fig. 5. Whole body [K^+] (top panel), [Na^+] (middle panel) and [Ca^{2+}] (bottom panel) in fathead minnow exposed to a range of dissolved Pb concentrations for 10 days. Mean±SEM, N=10 for controls and the three lowest Pb concentration but only 3 and 2 for the second highest and highest Pb concentration, respectively. "*" denotes statistical significant difference from controls.

any given DOC concentration, Pb accumulation appeared to be independent of ambient Pb concentrations. Considering all DOC treatments, however, it is clear that DOC alters bioaccumulation (Fig. 3B). A separate experiment was performed to evaluate the internal fate in accumulated Pb in fathead minnows. This experiment revealed that whole body Pb concentrations increase linearly with time, reaching approximately 70 μg g^{-1} after 30 days of exposure to 26±5 μg Pb L^{-1} in base water (Fig. 4A). The highest concentrations of Pb were observed in the gill tissue at both 10 and 30 days of exposure while the muscle and liver remained low although slightly above background levels. Unexpectedly, the intestine exhibited rapid and substantial Pb accumulation during the first 3 days of exposure after which levels gradually decreased for the remainder of the 30 days (Fig. 4A). Considering the concentration and mass of individual tissues, the relative contribution of various internal compartments was determined. Despite high absolute Pb concentrations, the gills contributed little to overall Pb accumulation in the fish (Fig. 4B). The intestine accounted for a substantial fraction (~40%) at day 4 but this contribution declined to less than 10% by day 30 as internal Pb pools continued to increase (Fig. 4B). Lead accumulated in the carcass was found predominantly in the bone fraction which accounted for close to 80% of the whole body Pb accumulated by day 30.

3.5. Effects of Pb exposure — whole body Ca^{2+}, Na^+ and K^+

Fish from the 0.5 mM $CaSO_4$ medium exposed to 115 μg Pb L^{-1} were chosen for analysis of whole body Ca^{2+}, Na^+ and K^+. This particular exposure group was chosen because a sufficient *n*-number was available even after substantial Pb induced mortality occurred. Contrary to expectations, Pb exposed fish exhibited an elevated rather than reduced whole body Ca^{2+} concentration after 30 days (Table 5). In contrast, no significant effect was observed on whole body concentrations of K^+ or Na^+.

Fish exposed to Pb for only 10 days exhibit different effects compared to those seen in fish exposed for 30 days. After 10 days of exposure, both whole body K^+ and Na^+ concentrations are elevated at higher Pb exposure concentrations (Fig. 5). For K^+, the effects are seen at intermediate concentrations but not at the highest concentration where *n*-numbers and thereby statistical power is low due to Pb induced mortality. The effects on whole body Na^+, on the other hand are evident only at the highest Pb concentrations despite reduced *n*-numbers (Fig. 5). Whole body Ca^{2+} concentrations exhibit a downward trend with increasing Pb concentrations for the concentrations with high *n*-numbers although this was not statistically significant ($P<0.06$). As for K^+ concentrations, low *n*-numbers at the highest concentrations appeared to mask effects on whole body Ca^{2+} concentrations (Fig. 5).

4. Discussion

4.1. Mortality at different water chemistries

Common for all treatments were early mortalities at higher Pb concentrations with, by far, most mortality occurring prior to day 10 of exposure. Further, it is clear that water chemistry greatly influences Pb induced mortality during 30 day exposures with Ca^{2+}, DOC and pH influencing toxicity. The increased tolerance to Pb could be the result of acclimation as discussed below or could be related to size (or age) of the test organism.

Regardless of the reason for the early, rather than late mortality, both Ca^{2+} and DOC reduce Pb toxicity in a concentration dependent manner as expected. However, interesting differences between Ca^{2+} and DOC induced protection are evident from the saturation type relationship between effect concentrations of Pb and ambient calcium concentrations compared to the linear relationship observed for DOC. These differences can be interpreted to reflect the competitive nature of the Ca^{2+}:Pb relationship at the gill where Pb competes for Ca^{2+} uptake pathways and the complexing nature of the DOC:Pb relationship. Due to finite number of Ca^{2+} uptake proteins at the gill surface and the Ca^{2+} antagonistic characteristics of Pb, two things are to be expected: first, increasing Ca^{2+} will reduce Pb uptake and toxicity and second, that there is a maximal Ca^{2+} concentration at which no further protection can be obtained from Ca^{2+} (all Ca^{2+} uptake proteins are saturated). Presumably, under such Ca^{2+} saturated conditions, Pb toxicity would occur as a result of alternate uptake pathways or direct, non-specific damage to the apical membrane of the gill epithelium. The simple linear relationship between effect Pb concentrations and DOC concentrations is perhaps even more predictable because DOC simply renders Pb unavailable for uptake and toxicity by the formation of Pb:DOC complexes (Macdonald et al., 2002). Thus, for the DOC:Pb interaction, the prediction would be a proportional increase in the amount of Pb required to exert toxicity and the amount of DOC present. Our findings of Pb induced mortality at different Ca^{2+} and DOC concentrations perfectly agree with expected relationships and the DOC results are similar to results obtained for chronic Cu exposures and DOC in rainbow trout (McGeer et al., 2002).

The third water chemistry parameter varied in the present study, pH also influenced Pb induced mortality but in a more complex manner. Lead appeared to be least toxic at circumneutral pH with higher toxicity observed at both higher and lower pH. The observed increased sensitivity at lower pH is in agreement with observations for rainbow trout (Hodson et al., 1978b) and carp (Stouthart et al., 1994) and is easily explained by Pb speciation. As pH decreases, the fraction of Pb attributable to ionic Pb^{2+} dramatically increases (Santore and Driscoll, 1995), which likely account for the increased toxicity at lower pH. However, our finding of apparent increased Pb toxicity at higher pH is in contrast to findings of reduced toxicity at pH above 7 reported for rainbow trout (Hodson et al., 1978b) and cannot be explained simply by speciation. Two possible explanations might be (1) that reduced competition from H^+ renders ionic Pb^{2+} more available for uptake by the gill despite a lower fraction of total Pb being in the ionic form and/or (2) that the high K^+ concentrations associated with the pH 8.1 water in the present study may have exacerbated the Pb effects in some way.

4.2. Sublethal effects

The 30-day Pb exposures did not result in reduced growth as evaluated by final mass of individual fish at the end of the exposure.

Rather, for several experiments an apparent Pb induced increased growth was observed. The apparent increase was, however, only observed in treatments where final n-numbers were low due to Pb induced mortality. The obvious interpretation of these observations is that reduced fish density at higher Pb concentrations allowed remaining individuals to grow faster. In support of this interpretation is the lack of increased growth in the fish exposed to Pb for only 10 days. In fact, the fish exposed for only 10 days (2 mM $CaSO_4$) displayed reduced growth at higher Pb concentrations, despite greatly reduced fish density. The explanation for the difference between Pb effects on growth at 10 and 30 days may be, at least in part, that fish after 10 days of exposure did not experience reduced growth due to density even in treatments with no or limited mortality. The mass of fish at 10 days was approximately 10% of the mass at 30 days, so it is possible that crowding reduced growth at 30 days but not at 10 days.

Our findings contrast with reports of fish exposed to cadmium (Cd), another Ca^{2+} antagonist (Hollis et al., 1999) which has been demonstrated to reduce activity, appetite and growth rates in rainbow trout (McGeer et al., 2000). One possible explanation for the lack of growth effects in the present study may be that fish were fed to satiation. However, the presence of excess food may allow Pb exposed larval fathead minnow to feed at rates high enough to sustain normal growth.

Surprisingly, considering that Pb may act as a Ca^{2+} antagonist (Büsselberg et al., 1991; Audesirk, 1993) and that during acute exposure Pb has been reported to reduce Ca^{2+} uptake in rainbow trout and carp (Stouthart et al., 1994; Rogers et al., 2003; Rogers and Wood, 2004), 30 days of Pb exposure resulted in an increased rather than decreased whole body Ca^{2+} concentration. In contrast, K^+ and Na^+, the major intracellular and extracellular cations, respectively, were not influenced by Pb after 30 days of exposure.

However, considering the 10-day exposed fish, no increase in whole body Ca^{2+} was observed. Rather, a tendency toward reduced whole body Ca^{2+} was seen at Pb concentrations with no, or limited mortality. This observed trend to reduced whole body Ca^{2+} is consistent with reports of reduced Ca^{2+} uptake in rainbow trout and carp during Pb exposure (Stouthart et al., 1994; Rogers et al., 2003; Rogers and Wood, 2004). In contrast to the 30-day exposed fish, an increase in whole body K^+ and Na^+ concentrations was seen in fish exposed to Pb for 10 days.

Taken together, these observations suggest that an acclimation response to Pb exposure occurs in larval fathead minnows during prolonged exposure. The disruption of whole body ion homeostasis, which seems general in nature (K^+, Na^+ and possibly Ca^{2+} are influenced) as previously reported for rainbow trout (Rogers et al., 2003) and carp (Stouthart et al., 1994), appears to be recovered after 30 days despite continuous exposure. Considering that acclimation, either as increased tolerance to acute metal challenge or recovery of normal physiological parameters during continuous exposure, has been reported for Cu (Lauren and McDonald, 1987a,b; Grosell et al., 1997, 1998), Zn (Hogstrand and Wood, 1996; Alsop et al., 1999) and Cd (McGeer et al., 2000; Szebedinszky et al., 2001), it is not surprising to see evidence of acclimation to Pb.

Fascinatingly, considering that Pb is a Ca^{2+} antagonist, whole body Ca^{2+} concentrations not only recover during exposure but exhibit significantly elevated levels at 30 days despite continuous exposure. Recovery of Na^+ during continuous Cu exposure and Ca^{2+} during continuous Zn or Cd exposure in rainbow trout is due to modifications of the branchial Na^+ and Ca^{2+} uptake pathways, respectively (Lauren and McDonald, 1987a,b; Hogstrand et al., 1998; Grosell et al., 1998; Alsop et al., 1999; Niyogi and Wood, 2004). More specifically, these modifications involved an increased transport capacity for Na^+ (Lauren and McDonald, 1987b) and an increased affinity for Ca^{2+} (Hogstrand et al., 1998; Alsop et al., 1999) during copper and zinc or cadmium exposure respectively. Similar modifications could be involved in the recovery of whole body ion homeostasis in fathead minnows exposed to Pb and could also explain the apparent "overshoot" seen for whole body Ca^{2+} concentrations.

4.3. Lead accumulation

Whole body accumulation of Pb expressed as bioconcentration factors displayed a clear negative relationship with ambient Pb concentration in treatments with varying Ca^{2+} and pH which is in agreement with relationships, not just for Pb, but for a number of other metals (McGeer et al., 2003). Such relationships likely reflect that metals from aqueous exposure at least in part are taken up via ion transport pathways with saturable transport kinetics enforcing a limitation on maximal uptake at higher concentrations. In contrast, Pb accumulation in presence of DOC did not exhibit the same strong relationship. At any given DOC concentration in the exposure medium it seems that Pb bioaccumulation factors are independent of ambient Pb concentrations. However, varying DOC concentrations appear to influence Pb bioaccumulation. The difference between the influence of Ca^{2+} and pH on one hand and DOC on the other hand, likely reflects that Ca^{2+} and pH treatments alter competitive interactions between Pb and other cations as well as Pb inorganic speciation while DOC treatments results in Pb-complex formation rendering Pb unavailable for accumulation. This observation of DOC dependent Pb accumulation agree with reduced short term Pb accumulation in presence of dissolved organic matter and Ca^{2+} (Macdonald et al., 2002) but the lack of effect of pH on bioaccumulation in our study contrast pH sensitive blood Pb levels in rainbow trout (Hodson et al., 1978b). Further, it appears the Pb induced mortality and 30-day Pb accumulation are poorly correlated. This lack of relation between Pb accumulation and mortality may be explained by the time course of mortality. Another possible explanation for the lack of apparent relation between effects and accumulation is that we are considering whole body accumulation rather than accumulation in tissues most affected by Pb. The gill is a likely target for at least acute Pb toxicity (Stouthart et al., 1994; Rogers et al., 2003; Rogers and Wood, 2004) but even though Pb concentrations in this organ reach higher levels than any other examined tissues, this gill accumulation attributes very little to whole body Pb accumulation. Whole body Pb accumulation in fathead minnows is clearly dominated by Pb accumulated in bones from which it likely exerts little toxic action.

While the whole body exhibits a continuous accumulation of Pb throughout 30 days of exposure, the same is not true for

individual organs. As expected, the gill displays a rapid accumulation during the initial face of exposure and continues to accumulate Pb throughout the 30 days, although at lower rates. Perhaps more surprising, rapid accumulation was observed in the intestine for the first three days of exposure after which a gradual decline was observed through to the end of the 30 day period. The reason for this early, rapid accumulation is not clear but could be related to ingestion of Pb contaminated *Artemia* nauplii. Earlier studies have found that dietary Pb is largely unavailable for accumulation (Hodson et al., 1978a) but it cannot be excluded that loosely bound Pb on the surface of *Artemia* nauplii could have contributed to intestinal Pb accumulation. It is interesting to note that Pb accumulation as assessed by fraction of whole body Pb found in an acid digestible fraction was relatively slow initially but increased after the first 10 days of exposure. This may reflect that Pb is moving through from uptake sites (gill and intestine) to internal organs or tissues like the blood and with some delay finally accumulates as part of newly generated bone.

4.4. Conclusions

Ambient Ca, DOC and pH clearly influence chronic Pb toxicity to fathead minnow during 30 day exposures with Ca and DOC offering clear protection with increasing concentrations as would be expected. It seems reasonable to incorporate at least these two parameters in site-specific chronic water quality criteria. The situation for pH is less clear and further studies are needed. It remains to be demonstrated that the results obtained during 30 day exposure are representative of even longer exposure times and sublethal endpoints. Our findings suggest that an acclimation response to Pb may occur in fathead minnows during continuous exposure and the exact nature of this response offers an exciting area for further research. Finally, we demonstrate that Pb readily accumulates in the gill tissue, which reach the highest concentrations even during chronic exposure but that the majority of whole body Pb after prolonged exposure is found in bones.

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