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Environmental Contamination and Toxicology

Effect of pH on Pentachlorophenol Toxicity to Embryos and Larvae of Zebrafish (*Brachydanio rerio*)

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The hydrogen ion is an important determinant of toxicity in the aquatic environment, especially for weak acids or bases. Pentachlorophenol is a weak acid (pKa 4.7-5.0; Buikema et al., 1979) and is consequently more toxic at low pH because of increasing proportions of free phenol. For some heavy metals the toxic interaction with ambient pH is complicated by complexation with organic and inorganic compounds. These complexes are affected by pH, and bioassays may be the easiest and most reliable method of determining the influence of pH in such cases.

The objective of this study was to find a simple laboratory procedure for determination of the effect of pH on the toxicity of various chemicals added to or already present in various samples of ground and surface waters. Because of (1) respiratory production of CO_2 (pKa 6.4 for H_2CO_3) and the resulting difficulties to maintain high and low pH in static systems, and (2) the increase in sample volume, and (3) the degree of complexity accompanied with flow-through technology, addition of buffers was the only method left. Since all buffers are weak acids or bases, the problem became to combine a sufficient buffering action with a minimal toxicity. Pentachlorophenol was selected as a reference toxicant and positive control because of its established pH-toxicity interaction in fish (Dalela et al., 1980; Kobayashi and Kishino, 1980; Könemann, 1979).

MATERIALS AND METHODS

The test organisms were embryos (eggs) and larvae of zebrafish (Brachydanio rerio) obtained from our laboratory culture unit. Aged, aerated Göteborg municipal tapwater with a hardness of 45 mg L^{-1} as $CaCO_3$ and a temperature of 25 ± 2 C was used for culture, spawning and as dilution water matrix in all experiments. Adjustments of pH were made with dilute NaOH and H_2SO_4 of analytical grade. Additions of buffer are described below and in Table 1.

After initial semi-static tests without additions of buffer, an acetic acid-sodium acetate buffer was tried. The first system was unable to maintain pH at high and low values, and the acetic acid-acetate system was toxic at concentrations high enough to maintain low pH values. After that ordinary commercial buffers for pH-meter calibration were tested. Addition of 1 mL buffer

solution/50 mL pH-adjusted dilution water was the best compromise between control of pH and survival, and this method was used in the experiments described below. Median survival times were determined by log (time) - probit (mortaliy) analysis on a microcomputer (Davies, 1971) or graphically on paper when not possible.

In experiment 1 ten eggs (3-5 h post-spawning) were exposed in 50 mL pH-adjusted and buffered water (nominal pH 4, 5, 6, 7, 8 and 9) in petri dishes (i.d. 100 mm) at 26 ± 1 C. Control eggs were exposed to the dilution water without addition of acid, base or buffer. All treatments were duplicated. Test solutions were renewed daily and pH was recorded in new and old solutions. Mortality and hatch were recorded once or twice daily until at least 90 % of the larvae had died.

In experiment 2 ten eight-day-old larvae (post-spawning) were exposed in beakers containing 200 mL pH-adjusted, buffered (4 mL buffer/200 mL) water with PCP added to nominal concentrations between 0.3 and 640 $\mu g\ L^{-1}$ (dilution factor 0.5) and a control without PCP. Three series with nominal pHs of 5, 7 and 9 and a control series, without buffer, were tested simultaneously. Mortality was recorded after 24 and 48 h. Initial and final pHs were measured in all beakers. The temperature was 25.2 ± 0.1 C.

In experiment 3 the set up was similar to experiment 1, but no replicates were made, the embryos were 30-32 h old at start (3-5 h in exp. 1), and PCP was added to nominal concentrations of 0, 10, 20, 40, 80, 160, 320 and 640 $\mu g\ L^{-1}$ at each pH (4, 5, 6, 7, 8 and 9) and the dilution water control. The temperature was 25 ± 0.5 C.

RESULTS AND DISCUSSION

The results from experiment 1 are shown in Table 1. The ranges for measured pH values are considered acceptable because higher concentrations of buffer reduced survival. Initial embryo mortality (up to 24 h) was only affected at pH 4. This is consistent with the rapid mortality at pH 3 (not shown in Table 1). An initial embryo mortality of 10 to 30 % is normal. Median survival times for larvae were reduced at pH 4 (expected) and at pH 7 (unexpected). Median survival times at the other pHs were not different from the control. The reduced survival at pH 7 was probably caused by the buffer, because the control without buffer but with the same pH showed a normal survival.

Median survival times in experiment 1 and for controls without PCP in experiment 3 are shown in Figure 1. Control survival times in experiment 3 have been corrected for the longer time after spawning prior to exposure (+ 27 h) in order to be comparable. In spite of this difference in exposure, survival at different pH values was similar. Also the unexpected reduced survival at pH 7 in experiment 1 was confirmed in experiment 3, although it was less pronounced.

Table 1. Survival of embryos and larvae of zebrafish (Brachydanio rerio) at different pH in buffered soft water[a].

Nominal pH	Buffer (final total conc.)[b]	Replicate	Measured pH (range)	Embryo mortality within 24 hours	Survival time for larvae (days)		
					Median	95% CL	$\bar{x}$
4	citrate-HCl	1	3.9 - 4.4	6/10	8.4	8.0 - 8.8	8.1
	(1.1 mM citric acid)	2	3.9 - 4.5	3/10	7.8	7.5 - 8.2	
5	citrate-NaOH	1	4.9 - 5.9	3/10	13.3	13.0 - 13.5	13.8
	(1.9 mM citric acid)	2	4.9 - 5.8	1/10	14.3	14.1 - 14.5	
6	citrate-NaOH	1	6.0 - 7.5	2/10	15.3	15.0 - 15.6	15.4
	(1.2 mM citric acid)	2	6.0 - 7.5	5/10	15.5	-[c]	
7	phosphate	1	7.1 - 7.4	3/10	7.7	-[c]	8.0
	(1.3 mM phosphate)	2	7.1 - 7.5	3/10	8.3	-[c]	
8	borate-HCl	1	7.5 - 8.1	3/10	15.6	-[c]	15.4
	(2.2 mM boric acid)	2	7.1 - 7.5	3/10	15.1	13.9 - 16.5	
9	boric acid, KCl-NaOH	1	7.7 - 8.9	3/10	15.3	14.4 - 16.1	15.1
	(1.0 mM boric acid)	2	7.7 - 8.9	3/10	14.9	14.6 - 15.3	
control	none	1	6.9 - 7.6	3/10	15.4	-[c]	14.0
		2	7.0 - 7.6	3/10	12.7	-[c]	

a/ Aerated, aged Göteborg tapwater (hardness 45 mg L^{-1} as $CaCO_3$; temperature 26 ± 1 C).
b/ Final concentration upon addition of 1 mL ready-for-use buffer solution/50 mL test solution. Ready for use buffer solutions (Merck, Darmstadt, Federal Republic of Germany, Cat. No. 9435-9461) also contains various amounts of chloride, potassium and sodium ions.
c/ Ninetyfive percent confidence limits (95% CLs) not determined due to insufficient partial effects.

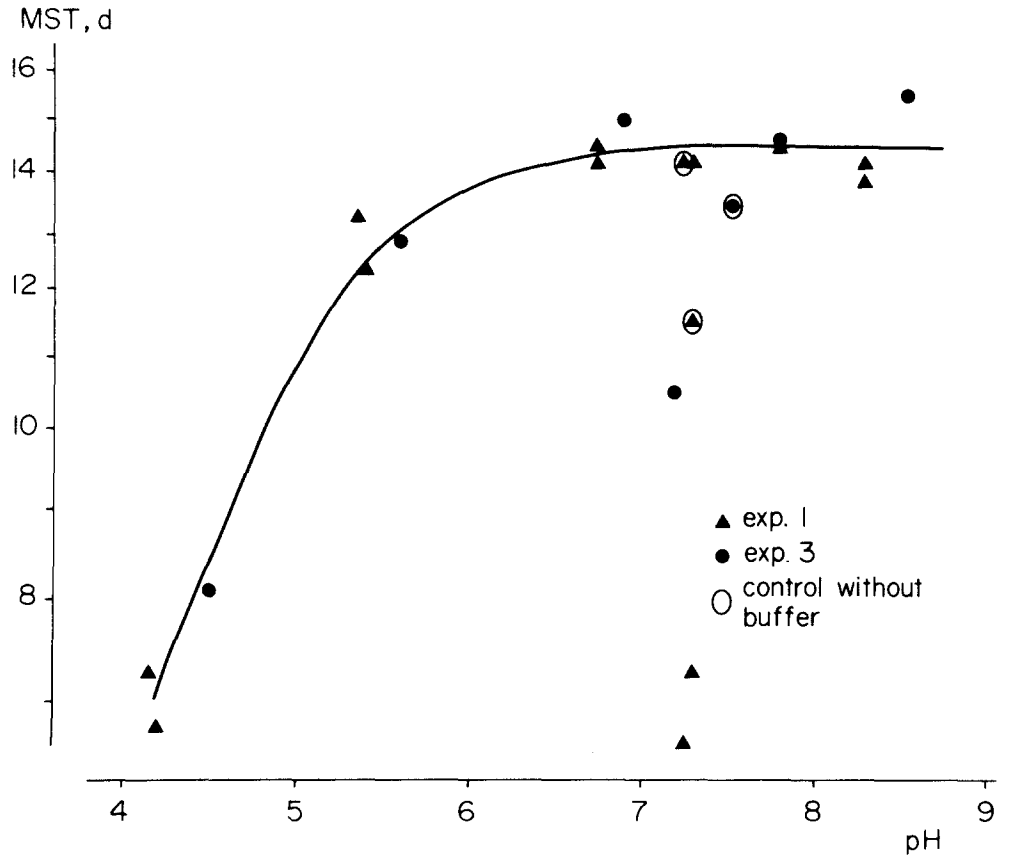


Figure 1. Effect of pH on embryo-larval survival in zebrafish.

Median Survival Times (MSTs) in days in experiments 1 and 3 at corresponding measured average pH values are shown, and the eye-fitted curve is also included. The reduced MSTs around pH 7.3 are discussed in the text.

Earlier studies on effects of pH have established a pH range which is not directly lethal to fish of 5 - 9 (Alabaster and Lloyd, 1982). In the present study median survival times at measured pH values around 5.5 (nominal pH 5) were somewhat lower than those above, but not significantly reduced. The reduced hatching of zebrafish larvae at pH 5.5 reported by Johansson et al. (1973) was not confirmed in the present study. Lillie et al. (1979) mentioned that unpublished results indicated that pH 4.5 did not affect hatching or larval survival in zebrafish but that spawning was inhibited. The present results (Fig. 1) suggest that, with exception for the variable results around pH 7.3, survival of zebrafish larvae was slightly reduced at pH 4.5, which, when compared to the studies mentioned above and to other studies discussed by Lee and Gerking (1980), indicates a "normal" sensitivity to low pH. This suggests that the buffers used at low pH had no significant effect.

The results from experiment 2 (Fig. 2) show that the presence of buffer did not affect the toxicity of PCP. Furthermore, they are consistent with those obtained in unbuffered pH-adjusted water by Dalela et at. (1980) in Notopterus notopterus, and the pH-toxicity correlations for PCP in zebrafish are only quantitatively but not qualitatively different from the correlations calculated for other fish species in Table 2.

The results from experiment 3 are presented in Fig. 3. High concentrations of PCP were lethal within one day post-hatch. This rapid lethal action was correlated to pH. The 48-h LC50s for embryo-larval exposure is described by a similar equation as used above for larvae. The pH-toxicity correlation is shown in Fig. 2 and the equation is given in Table 2.

At lower concentrations of PCP two different time-response patterns were found at pH 6, 7, 8 and 9. The first after two days with a steep slope, and the second after 7 to 10 days with a shallow slope. A similar shallow slope can be seen already after 2 days at pH 4 and 5. Since the proportion of free phenol is higher at low pH it is possible that the shallow slope is due to the toxicity of free phenol and the the steep slope is due to the phenolate ion toxicity. At high pH the proportion of free phenol is low and more time is required for accumulation of a lethal dose. Therefore, at high concentrations the free phenol toxicity may be preceded by phenolate ion toxicity.

The proposed mode of action for PCP is uncoupling of oxidative phosphorylation (reviewed by Buikema et al., 1979). Thus, embryos and larvae exposed to PCP in the absence of food would become less efficient in utilizing their sole source of energy, the yolk. This would result in a shorter period of survival. In addition to this, PCP may have another more rapidly lethal mode of action which is presumably not caused by uncoupling. Therefore, the different slopes in the toxicity curves presented in Fig. 3 may also reflect two different modes of action. One rapidly lethal mode shown by the steep slope and one slowly expressed mode seen after 7 to 10 days and which is presumably caused by uncoupling of oxidative phosphorylation.

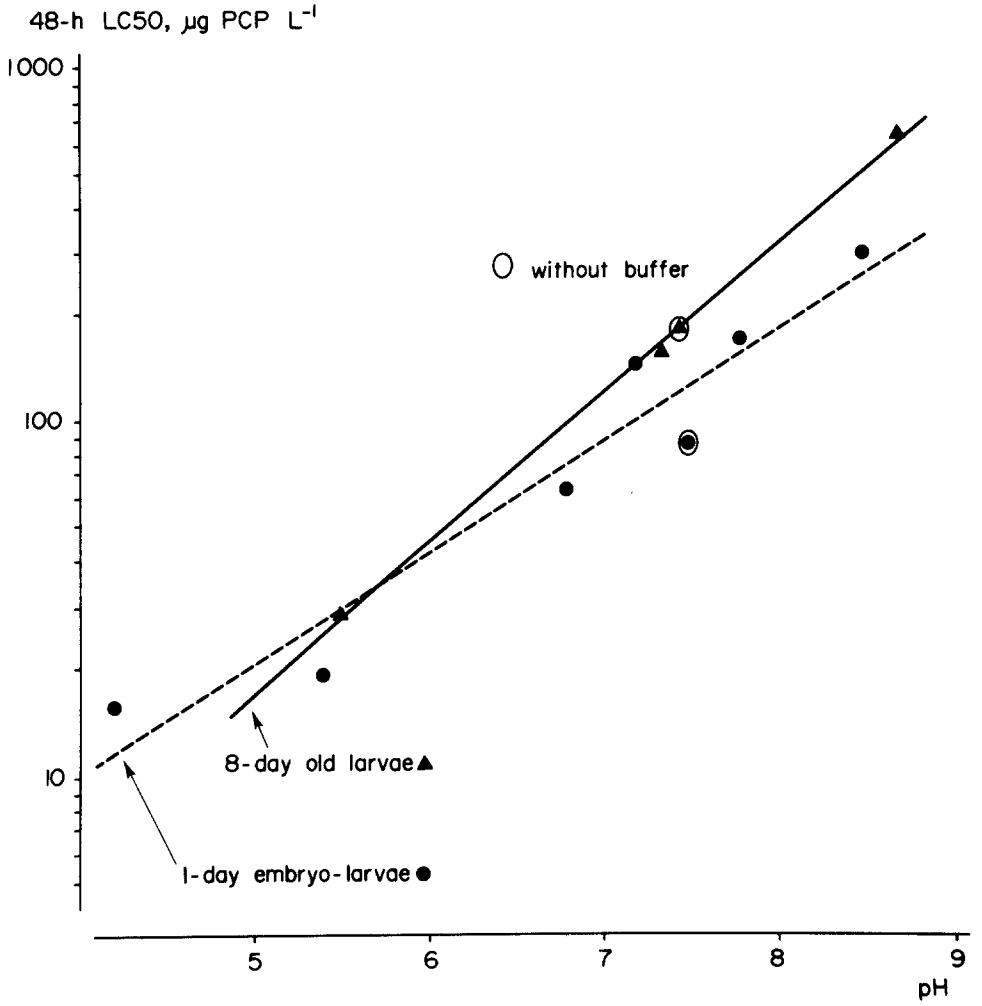


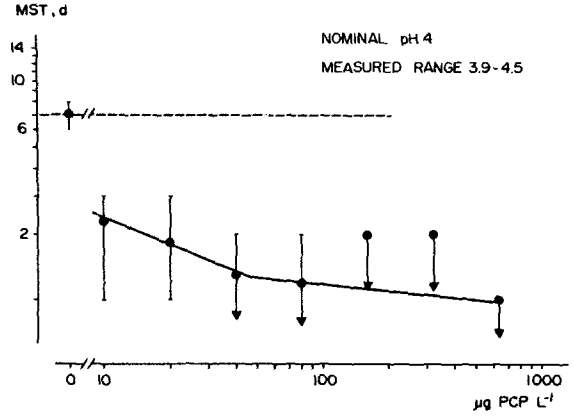
Figure 2. Correlations between pH and 48-h LC50s for PCP in embryo-larval and larval exposures.

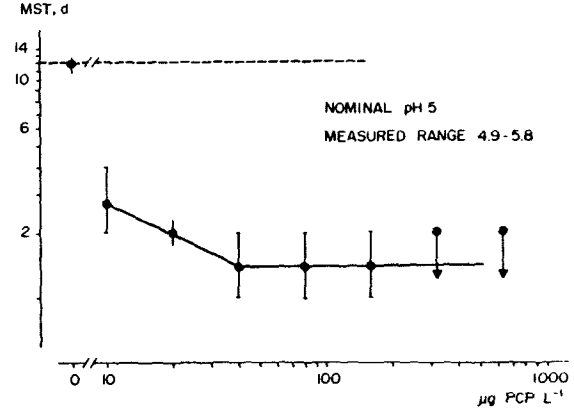
Fortyeight hour LC50s are based on nominal values and corresponding pH values are average measured values.

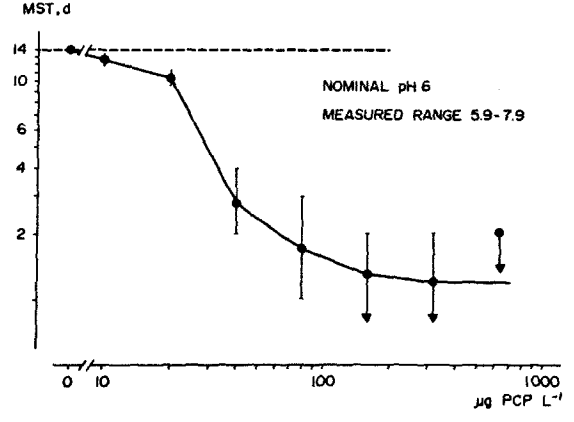
Table 2. Toxicity-pH correlation for PCP in some species of fish as described by the equation: Log(LC50, µg PCP L^{-1}) = a + b x pH.

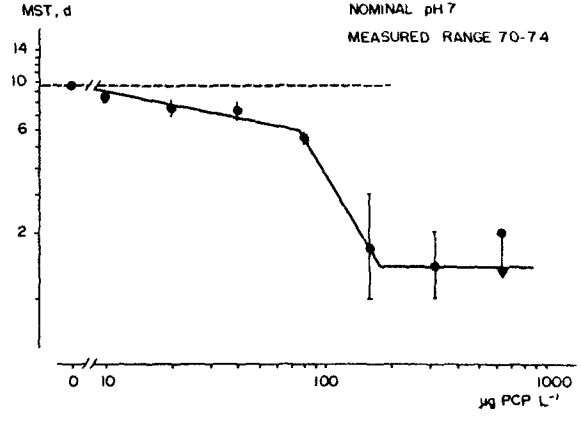
Fish species	Developmental stage, size or age	Period of exposure	a	b	r^{1}	Source of data
Brachydanio rerio	Embryo - larvae	48 h	-0.247	0.312	0.963	Present study
-"-	8-day old larvae	48 h	-0.892	0.423	0.999	-"-
Colisa fasciatus	6.2 - 8.8 cm	48 h	1.11	0.222	0.957	Dalela et al., 1980
Saccobrachus fossilis	11,6 - 14.2 cm	48 h	0.785	0.247	0.989	-"-
Notopterus notopterus	8.5 - 10.2 cm	48 h	-0.798	0.399	0.971	-"-
Poecilia reticulata	2 - 3 months old	7 or 14 days	-0.639	0.447	0.993	Könemann, 1979

1/ Correlation coefficient


MST, d
NOMINAL pH 4
MEASURED RANGE 3.9-4.5
14
10
6
2
0
10
100
1000
µg PCP L⁻¹


MST, d
NOMINAL pH 5
MEASURED RANGE 4.9-5.8
14
10
6
2
0
10
100
1000
µg PCP L⁻¹


MST, d
NOMINAL pH 6
MEASURED RANGE 5.9-7.9
14
10
6
4
2
0
10
100
1000
µg PCP L⁻¹


MST, d
NOMINAL pH 7
MEASURED RANGE 7.0-7.4
14
10
6
2
0
10
100
1000
µg PCP L⁻¹

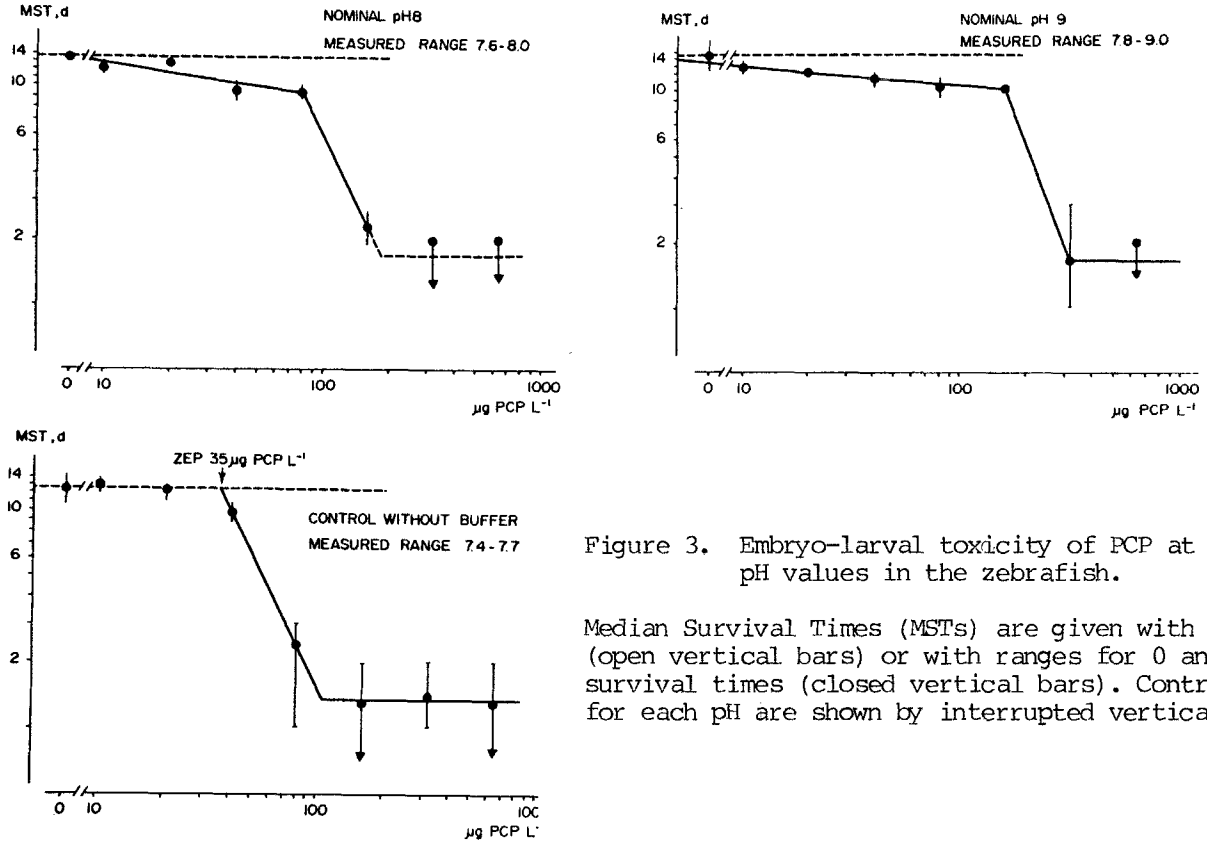


Figure 3. Embryo-larval toxicity of PCP at different pH values in the zebrafish.

Median Survival Times (MSTs) are given with 95% CLs (open vertical bars) or with ranges for 0 and 100% survival times (closed vertical bars). Control MSTs for each pH are shown by interrupted vertical lines.

Except for the control series without buffer, the lowest concentration (10 µg PCP L^{-1}) reduced survival at all pH values as compared to the respective buffer controls without PCP. The NOEC (No Observed Effect Concentration; Maki, 1979) in the control series without buffer was between 20 and 40 µg PCP L^{-1} and the ZEP (Zero Equivalent Point; Luckey, 1975) shown in Fig. 3 was 35 µg L^{-1}. In order to get comparable long-term toxicity values at low and high pH lower concentrations must be tested. The present study have shown that also at high pH values there are long-term effects of PCP resulting in a slightly reduced period of survival in unfed larvae, presumably because of less efficient use of energy through uncoupling of oxidative phosphorylation.

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