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EFFECTS OF WATER TEMPERATURE AND pH ON TOXICITY OF TERBUFOS, TRICHLORFON, 4-NITROPHENOL AND 2,4-DINITROPHENOL TO THE AMPHIPOD *GAMMARUS PSEUDOLIMNAEUS* AND RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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Abstract—Acute toxicity tests were conducted to determine (a) the individual and interactive effects of water temperature (7, 12, 17°C), pH (6 5, 7 5, 8 5, 9 5), and time on the toxicity of terbufos, trichlorfon, 4 nitrophenol, and 2,4-dinitrophenol to rainbow trout (*Oncorhynchus mykiss*) and the amphipod *Gammarus pseudolimnaeus*, and (b) the individual and interactive effects of water temperature and pH on chemical bioconcentration during acute tests with rainbow trout and *Gammarus* exposed to terbufos, 4 nitrophenol, and 2,4 dinitrophenol The toxicity of all four chemicals was significantly affected by pH in all tests, except for *Gammarus* exposed to terbufos The toxicity of terbufos to rainbow trout and *Gammarus* was less at pH 7 5 than at higher or lower pH The toxicity of both nitrophenols decreased as pH increased, whereas the toxicity of trichlorfon increased with pH The effect of pH on trichlorfon toxicity decreased with temperature Temperature significantly affected the toxicity of all four chemicals to both species Toxicity increased with temperature in all tests, except for rainbow trout exposed to nitrophenols, toxicity decreased as temperature increased for rainbow trout Chemical bioconcentration was also significantly affected by temperature and pH and was directly related to toxicity in most tests Significant interactive effects between toxicity modifying factors were also frequently observed Temperature and pH effects on chemical toxicity need to be considered in chemical hazard assessment to ensure adequate protection of aquatic organisms

Keywords—Temperature pH Amphipods Rainbow trout Toxicity

INTRODUCTION

The Federal Insecticide, Fungicide, and Rodenticide Act (PL 80 104) and the Toxic Substances Control Act (PL 94-469) authorize the U S Environmental Protection Agency (EPA) to acquire data from industry on human health and environmental effects of pesticides and industrial chemicals Standard aquatic toxicity tests provide much of the toxicity information required for both laws and are useful in evaluating potential chemical hazards to aquatic life Managers assessing chemical risk often extrapolate from a toxicity data base generated in the laboratory under standard, controlled conditions to the natural environment, which is much more complex and unpredictable This extrapolation assumes that standard laboratory toxicity tests can reasonably predict effects of toxicants in the field

At present, aquatic toxicity tests are performed according to established standard physicochemical conditions, such as temperature and pH, for given test species [1,2] However, physicochemical changes that occur in natural aquatic systems may alter the physiological condition of biota and the interactions between organisms and toxicants [3] The toxicity of many chemicals to aquatic organisms can be affected by numerous environmental factors such as pH, temperature, salinity, dissolved oxygen, and sediment interaction, depending on the chemical nature of the toxicant and the environmental conditions [4–8] Current standards for toxicity tests do not require determination of chemical toxicity over a range of temperature or pH The effects of physicochemical factors on the toxicity of chemicals must be considered in hazard assessment to assess risk accurately and protect aquatic organisms

Mayer and Ellersieck [8] reviewed the effects of pH and other physicochemical factors on chemical toxicity and concluded that the toxicity of weak organic acids and bases is greatly affected by pH Decreases in pH increase the toxicity of weak acids but decrease the toxicity of weak bases by changing the proportion of ionized to un ionized chemical They reported that of any toxicity-modifying factor tested, pH caused the greatest average change in chemical toxicity In a similar review of pH effects on chemical toxicity, Sprague [7] stated that un ionized organic molecules may be more toxic than ionized molecules because they more readily penetrate cell membranes, and that pH is the major factor determining the extent of ionization and subsequent membrane penetration Conversely, some cationic metals such as copper are typically more toxic in ionic form [7]

The toxicity of many pesticides is affected by pH For example, Hunn and Allen [4] identified pH as the environmental variable with the greatest influence on the toxicity of the

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lampricide 3-trifluoromethyl-4-nitrophenol (TFM). They demonstrated that un-ionized TFM (found at low pH) is the form most readily transported across the gill membrane. In laboratory tests, the toxicity of TFM increased 20 to 50 times as pH decreased from 9.5 to 6.5 [9]. The toxicity of ionic herbicides such as diquat and paraquat is also influenced by pH [10].

Holcombe et al. [11] investigated the effects of pH on the lethal and sublethal toxicity of 2,4-dichlorophenol. They found that the schooling and spawning behavior of fathead minnows (*Pimephales promelas*) exposed to 7.4 mg/L 2,4-dichlorophenol was disrupted at pH 7.6, but no effect was observed at pH 8.7 and 9.0. Acute and chronic toxicity and bioaccumulation also increased as pH decreased.

The effects of temperature on the toxicity of chemicals to aquatic organisms have been reviewed by Cairns et al. [5,6], Johnson [12], Sprague [13], and Tucker and Leitzke [14]. The toxicity of most chemicals is positively correlated with temperature. However, the toxicity of some organochlorine pesticides such as DDT, DDD, and Methoxychlor is negatively correlated with temperature [14]. The toxicity of some pyrethrins and pyrethroids to fish is also negatively correlated with temperature [15].

The effects of temperature on chemical toxicity have been attributed to changes in respiration rates [16], chemical absorption [17], and chemical excretion and detoxification [13] by organisms. Other physiological mechanisms such as peripheral nerve sensitivity [18] may also be altered by temperature.

Relatively few empirical studies have adequately examined the effects of pH and temperature on chemical toxicity to aquatic organisms. In some studies, the reliability of toxicity data is questionable because of improper test design and the use of nonstandardized test procedures. Sprague [7], for example, noted that the effects of physicochemical factors on chemical toxicity should be cautiously interpreted within experiments and not among them, because repeated toxicity tests seldom yield identical results. Interlaboratory comparisons of toxicity values may vary 10-fold for the same chemical, and intralaboratory comparisons can vary fivefold [7]. In addition, most studies determining the effects of physicochemical factors on toxicity evaluate only one factor at a time, which has minimal relationship to the way natural processes influence toxicity. Therefore, an evaluation of variables that modify toxicity should be based on results of experiments specifically designed to test such effects.

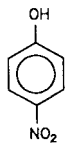
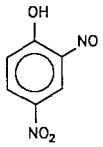
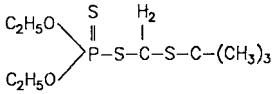
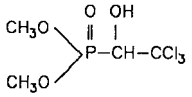
The purpose of this study was to evaluate the effects of water temperature and pH on the toxicity of four chemicals to two species of freshwater organisms. The specific objectives were to determine (a) the individual and interactive effects of water temperature (7, 12, 17°C), pH (6.5, 7.5, 8.5, 9.5), and time on the acute toxicity of terbufos, trichlorfon, 4-nitrophenol, and 2,4-dinitrophenol to rainbow trout (*Oncorhynchus mykiss*) and the amphipod *Gammarus pseudolimnaeus*, and (b) the individual and interactive effects of water temperature and pH on the bioconcentration of terbufos, 4-nitrophenol, and 2,4-dinitrophenol in rainbow trout and *Gammarus* during acute exposures.

Terbufos and trichlorfon are common organophosphate (OP) insecticides that are frequently detected in surface-runoff water from agricultural fields [19]. They act as cholinesterase inhibitors that can alter organism behavior and cause death [19]. Trichlorfon is also used in aquaculture as a treatment for fish infested with external parasites.

The nitrophenols (4-nitrophenol and 2,4-dinitrophenol) are common components of industrial effluents and have been detected in urban and agricultural waste [20]. Nitrophenols are intermediates in pesticide and dye synthesis, are applied as herbicides and insecticides, and are found in urban rain water as a result of the tropospheric transformation of alkylbenzenes [20]. Nitrophenols uncoupled oxidative phosphorylation and can affect cellular metabolism at concentrations <10 μM [20]. Chemical and physical properties for these nitrophenols and OPs are listed in Table 1.

Rainbow trout and *Gammarus* were chosen as test organisms because they are standard species used in aquatic toxicity tests, are sensitive to most toxicants, have broad geographic distributions, are easily obtained and cultured,

Table 1. Chemical and physical properties of 4-nitrophenol, 2,4-dinitrophenol, terbufos, and trichlorfon

Property	4-Nitrophenol	2,4-Dinitrophenol	Terbufos	Trichlorfon
Molecular structure				
Chemical Abstracts Service no.	100-07-7	51-28-5	13071-79-9	52-68-6
Molecular wt.	139.11	184.11	288	257.44
Melting point (°C)	114.9	114	−29.2	81–82
Boiling point (°C)	279	Unavailable	312	100
Purity (%)	98	85	96	98
pK_a[a]	7.15	4.09	Unavailable	Unavailable
Log K_{ow}[b]	1.34	−0.233	0.832	0.304
Water solubility (mg/L)	16,000	5,000	0.1	9,000

[a]pH at which 50% of chemical is ionized.
[b]Determined in reconstituted test water at pH 7.5 and 12°C.

and tolerate the water conditions used in this study The 12 temperature and pH regimes are representative of those in aquatic systems in temperate regions of North America

MATERIALS AND METHODS

Test organisms

Rainbow trout were obtained as eggs from Trout Lodge, Inc (McMillin, WA, and Erwin (TN) National Fish Hatchery and reared at the National Fisheries Research Center (La Crosse, WI) Fish were held in flowing well water at 12°C and pH 7 9 and maintained according to standard procedures for handling bioassay fish [21] Fish were fasted 96 h before testing and gradually acclimated to test conditions 24 h before testing, according to standard procedures [1,2] The rainbow trout used in tests weighed 0 6 to 1 0 g and were of the same production lot for each tested chemical

Gammarus pseudolimnaeus was obtained from Seas Branch Creek near Viroqua, Wisconsin, transported to the laboratory, and gradually acclimated to the dilution water for 48 h before testing according to standard procedures [1,2] During this period *Gammarus* were fed maple leaves (*Acer* sp) and watercress (*Nasturtium officinale*), and mortality observations were made to determine suitability for testing Randomly selected *G pseudolimnaeus* from each collection were identified to species Additional specimens were sent to the National Museum of Natural Sciences (Ottawa, Canada) for species verification

Test chemicals

Test chemicals were purchased by the U S Environmental Protection Agency, Environmental Research Laboratory (Gulf Breeze, FL), checked for purity, and shipped to the National Fisheries Research Center, La Crosse Terbufos (86 0% a i) and trichlorfon (98 0% a i) were provided by American Cyanamid Co (Wayne, NJ) and Mobay Chemical Co (Kansas City, MO), respectively The 4 nitrophenol and 2,4-dinitrophenol (98 and 85% a i , respectively) were purchased from Aldrich Chemical Co (Milwaukee, WI) The test chemicals were solubilized terbufos in purified triethylene glycol (Fisher Scientific, Fair Lawn, NJ), both nitrophenols in pesticide grade acetone (Fisher Scientific), and trichlorfon in deionized water Radiolabeled (^{14}C) chemicals were used for the third replicate only in terbufos, 4-nitrophenol, and 2,4-dinitrophenol tests Radiolabeled terbufos (specific activity 7 17 mCi/mmol) was provided by American Cyanamid Co , ^{14}C-labeled 4-nitrophenol (specific activity 6 90 mCi/mmol) and 2-4-dinitrophenol (specific activity 10 20 mCi/mmol) were purchased from Sigma Chemical Co (St Louis, MO) Radiolabeled trichlorfon was unavailable

Dilution water

Dilution water for acute toxicity tests were prepared by deionizing and reconstituting well water according to standard procedures [1,2] The pH of exposure water was maintained (±0 1 unit) during tests by the addition of buffer, base, and acid and was adjusted before tests, after toxicant addition, and daily thereafter The pH was measured with a Beckman Instruments model (Fullerton, CA) Theta II pH meter that was calibrated daily with Fisher certified buffers Total water hardness and alkalinity were measured before tests and after toxicant addition, according to standard methods [22] Water hardness ranged from 40 to 48 mg/L as $CaCO_3$, and alkalinity ranged from 30 to 35 mg/L as $CaCO_3$ Dissolved oxygen concentrations were monitored daily with a Yellow Springs Instrument (Yellow Springs, OH) model 58 dissolved oxygen meter Minimum oxygen concentrations exceeded 5 mg/L for all test exposures Water temperature was regulated (±0 5°C) by immersing test jars in constant-temperature water baths and was measured daily with a calibrated mercury thermometer

Acute toxicity tests

Acute toxicity tests were conducted for 96 h, according to procedures prescribed by the American Society for Testing and Materials [2] and by the Committee on Methods for Toxicity Tests with Aquatic Organisms [1] We exposed 30 organisms (three replicates of 10 organisms each) to selected concentrations of each test chemical in glass jars containing 15 L oxygen saturated dilution water Exposure concentrations were selected from a logarithmic scale within the test range determined from range finding tests Range-finding tests consisted of a control, a solvent control, and five chemical concentrations (0 01, 0 1, 1, 10, and 100 mg/L)

Each test series, except those containing trichlorfon, consisted of eight exposure concentrations, a control, and a solvent control that contained the same amount of acetone or triethylene glycol needed to solubilize the highest chemical concentration Tests with trichlorfon consisted of nine concentrations and a control because no solvent was necessary for solubilization Each test series was performed in triplicate with the third replicate receiving a calculated proportion of ^{14}C-labeled toxicant (except for trichlorfon) Radiolabeled terbufos test stock was formulated to contain 0 035% radiolabeled chemical Both nitrophenol compounds were tested using a 0 020% radiolabeled test stock We conducted 96 acute tests with rainbow trout and *Gammarus* exposed to the four chemicals at temperatures of 7, 12, and 17°C and pH values of 6 5, 7 5, 8 5, and 9 5 in a factorial design (four chemicals × two test species × three temperatures × four pH values) Tests for all three temperatures at a given pH were conducted simultaneously

Mortality of test organisms was recorded at 1, 3, 6, 24, 48, 72, and 96 h after test initiation, and dead organisms were removed daily Dead organisms from test solutions containing ^{14}C radiolabeled isotopes were blotted dry and weighed daily, then pooled with remaining survivors for each exposure concentration at 96 h After weighing, organisms were dried at 40°C for 24 h and frozen until sample oxidation

Chromatography analysis

Concentrations of test chemicals were determined by either GC or HPLC at the beginning of each test Samples from the water control, solvent control, a spiked solvent control, and high- and low-exposure concentrations were analyzed from each replicate to verify exposure concentrations Randomly selected samples were analyzed in triplicate for 10% of the test concentrations Chemical stocks were analyzed to confirm proper preparation Spiked solvent control samples were

prepared by removing an exact volume of solution from the solvent control (0.5 or 1 L) and adding an appropriate amount of stock to produce a desired concentration.

Terbufos concentrations were determined by GC analysis. One-liter water samples were taken from test jars, extracted twice with petroleum ether, concentrated on a steam table, and analyzed with a Hewlett Packard (Avondale, PA) 570A GC with an electron-capture detector. The method used a 1.83-m × 2-mm-i.d. glass column packed with 3% SP2100 on 100/200 mesh Supelcoport® (Supelco, Bellefonte, PA). Other GC characteristics were as follows: column temperature 200°C; detector temperature 300°C; injection temperature 200°C; carrier gas, nitrogen; injection volume 5 μl. Peak heights for each sample were measured and quantified by comparison with a four-point standard calibration curve with a LOTUS™ spreadsheet program.

Concentrations of trichlorfon, 4-nitrophenol, and 2,4-dinitrophenol in exposure water were determined by HPLC analysis on a Waters Associates, Inc. (Milford, MA) HPLC system that consisted of a model 712 Waters intelligent sample processor (WISP) auto sampler, a model 484 absorbance detector, two model 510 pumps, a model 680 gradient controller, and a model 745B data module integrator. The analytical column was a Waters μBondapak™, C_{18}, 30 cm- × 3.9-mm-i.d. column. A 0.45-μm disposable filter assembly was used to filter samples before injection. Peak areas were quantified by the integrator with an external standard quantification program. Three-point calibration curves were used with both linear and nonlinear plots, as appropriate.

Operating conditions for HPLC analysis of trichlorfon samples were adapted from Slahck [23] as follows: mobile phase, 30% acetonitrile, 70% HPLC water, one vial of Waters low-UV B8 PIC reagent per liter; flow rate 1.5 ml/min; chart speed 2.0 cm/min; wavelength 210 nm. Injection volume and spectrophotometer attenuation varied with test concentration. The pH of all test water samples containing trichlorfon at test pH values of 8.5 and 9.5 was adjusted to pH 6.5 with 0.1 N HCl to retard chemical degradation after sampling.

Operating conditions for HPLC analysis of 4-nitrophenol and 2,4-dinitrophenol were as follows: mobile phase, 60% HPLC-grade methanol; 40% 0.05 M H_3PO_4/KH_2PO_4 buffer; flow rate 1.5 ml/min; chart speed 1.0 cm/min; wavelength 260 nm. The buffer was prepared by dissolving 28.6 ml of 1.0 M H_3PO_4 and 21.4 ml KH_2PO_4 in 1 L of HPLC-grade water. Mobile phase was prepared daily, degassed, and filtered with a 0.45-μm Millipore® (Millipore Corp., Bedford, MA) filter. Injection volume and spectrophotometer attenuation varied with test concentration.

Radiometric analysis

All test solutions of the third replicate containing ^{14}C-labeled chemical were sampled in triplicate and analyzed to determine the ^{14}C chemical concentrations. Concentrations of ^{14}C-labeled terbufos were below detection limits and required extractions of aliquots and sample concentration to produce detectable quantities. Both the ^{14}C-labeled 4-nitrophenol and the ^{14}C-labeled 2,4-dinitrophenol water samples from test jars were analyzed directly. Water samples (10-ml aliquots) and concentrated extracts (terbufos samples only) were combined with 10 ml Aquasol® 2 (DuPont/New England Nuclear Products, Boston, MA) liquid scintillation cocktail, thoroughly mixed, allowed to sit overnight in the dark (to reduce chemiluminescence), then counted on a Beckman LS5801 scintillation counter. Counts for each replicate sample were automatically averaged and reported. Spiked solvent control aliquots and dilutions of radiolabeled chemical stock were analyzed for ^{14}C content in triplicate in the same manner. Activity in water samples were analyzed for 10 min or until a 2-SD counting error of 2% was achieved (10,000 accumulated counts).

Fish and invertebrate tissues were also analyzed for ^{14}C content. Frozen organisms from each test concentration were pelletized and oxidized to $^{14}CO_2$ in a Packard Instrument Co. (Downers Grove, IL) model B306 Tri-Carb® sample oxidizer. The oxidizer, which was calibrated every 20 sample burns by combustion of [^{14}C]methacrylate chips (Amersham Radiochemicals, Arlington Heights, IL), was 95.3% (SD 2.6%) efficient. Radioactivity in oxidized tissue samples was also analyzed for 20 min or until a 2-SD counting error of 2% was achieved (10,000 accumulated counts).

Activity in water and oxidized tissue samples was calculated as disintegrations per minute (dpm) by a stored internal computer program that automatically corrected for sample quench and background counts. The dpm data for water and tissue samples were stored on a Beckman data transporter and transferred via data capture software to LOTUS files on an IBM personal computer. Total body concentration residue values were calculated for each exposure from total-exposure wet-tissue weight, total-exposure dpm data, and specific activity of compound, according to the following equation:

$$\text{Body concn.} = \frac{\dfrac{\text{tissue (dpm)}}{\text{specific activity (dpm/}\mu\text{g compound)}}}{\text{body weight (g)}}$$

$$= \mu\text{g compound/g tissue}$$

BCFs were calculated by dividing body concentration by exposure concentration as follows:

$$\text{BCF} = \frac{\text{Body concn. (}\mu\text{g compound/g tissue)}}{\text{exposure concn. (mg/L)}}$$

Liquid scintillation counting data were also used to calculate measured ^{14}C test chemical concentrations and total test chemical concentrations in all exposures of the third replicate receiving radiolabeled toxicant.

Statistical analysis

For each test, the trimmed Spearman-Karber method [24] was used to calculate median lethal chemical concentrations (LC50s) and 95% C.I.s at 24, 48, 72, and 96 h, based on calculated exposure concentrations. In instances where the Spearman-Karber method could not be used because of insufficient mortality data, the method of Litchfield and Wilcoxon [25] was employed. Mortality data were pooled from

each of the three replicates at each concentration to calcu late the LC50s and 95% confidence limits

Individual and interactive effects of pH, temperature, and time on chemical toxicity were assessed using LC50 values calculated from individual test replicates in a three way fac torial ANOVA The ANOVAs were performed on LC50 data for each chemical and species combination with the Statistical Analysis System (SAS®) [26] Duncan's multiple-range test was used to assess differences among means

Individual and interactive effects of pH and temperature on chemical bioconcentration were assessed using analysis of covariance (ANCOVA) The BCFs from the three lowest exposure concentrations of each test (exposures with 100% survival at 96 h) were used in this analysis so that exposure time was equal among the compared tests For each test, total body concentration values for each exposure concentration were also plotted against respective chemical exposure concentrations These plots, along with regression analyses, were used to determine factors affecting body concentration values

All data were tested for normality and homogeneity of variance before statistical analysis Data were found to be normally distributed with homogeneous variance A signif icance level of $p \leq 0\ 05$ was used to judge the results of all statistical comparisons

Test condition problems

In several tests with trichlorfon, 4-nitrophenol, and 2,4-dinitrophenol, we could not obtain 96 h LC50s for *Gamma rus* due to unacceptably high mortality (>10%) in control exposures at pH 6 5 and 9 5 and temperature of 17°C In tests with rainbow trout exposed to 2,4-dinitrophenol at pH 6 5 and a temperature of 17°C, some control fish died be fore 96 h due to a bacterial bloom that depleted the dissolved oxygen Bacteria were presumably living on the acetone solvent present in the jars All of these tests were repeated, however, the problems recurred

RESULTS

Chemical analyses

Mean percentages of differences between calculated and measured concentrations for HPLC and GC analyses of test chemicals in exposure water ranged from 0 1 to 9 2% (Table 2) Percentages of differences for terbufos exposures were higher than average (5 8–8 8%), possibly because of sample degradation or loss of sample in the concentration procedure The largest differences between calculated and measured concentrations in trichlorfon tests occurred at higher pH (8 5 and 9 5) and in *Gammarus* exposures, where concentrations were relatively low Trichlorfon was probably less stable at higher pH, higher temperature, and lower exposure concentration

The quality of chromatographic analysis and dosing techniques was assured by analyses of controls, solvent controls, spiked-solvent controls, triplicate analyses, and stock checks No test chemicals were detected in the analysis of control and solvent-control samples Mean recovery percentages of the spiked solvent controls ranged from 92 to 109% (Table 2) Mean relative standard deviations for triplicate analysis of selected concentrations ranged from 1 3 to 5 6% (Table 2) The mean percentages of differences between calculated and measured concentrations for all stock checks was 2 0%, with a standard deviation of 3 2% Overall, the results of GC, HPLC, and radiometric analysis were adequate to verify cal-

Table 2 Mean percentages of differences between calculated and measured test concentrations, mean recovery percentages for spiked solvent control samples, and mean relative standard deviations for triplicate analyses of randomly selected samples

Chemical and test species[a]	Analytical[b] method	Mean percent differences and SD (%)	Mean percent recoveries and SD (%)	Mean relative SD (%) (N = 9–12)
4 Nitrophenol				
RBT	HPLC	2 6 ± 11	101 + 4 6	1 3
GAM	HPLC	0 1 + 5 1	99 ± 5 9	2 2
RBT	RAD	1 1 ± 3 2	99 ± 2 7	1 1
GAM	RAD	1 4 ± 3 2	104 ± 3 5	1 2
2,4 Dinitrophenol				
RBT	HPLC	1 3 ± 5 0	101 ± 2 7	1 6
GAM	HPLC	3 3 ± 8 2	99 ± 4 0	1 4
RBT	RAD	7 8 ± 6 1	109 ± 7 6	1 0
GAM	RAD	6 1 ± 4 6	105 ± 5 3	1 1
Trichlorfon				
RBT	HPLC	0 8 ± 9 1	96 ± 11	5 6
GAM	HPLC	9 2 ± 13	92 ± 8 2	2 6
Terbufos				
RBT	GC	5 8 ± 12	100 ± 8 9	2 6
GAM	GC	8 4 ± 15	96 + 0 2	2 2
RBT	RAD	8 7 ± 4 8	100 ± 8 9	1 1
GAM	RAD	8 8 ± 7 6	90 ± 5 6	1 3

[a]Rainbow trout (RBT) and *Gammarus* (GAM)
[b]High performance liquid chromatography (HPLC), gas chromatography (GC), and radiometric (RAD) analyses

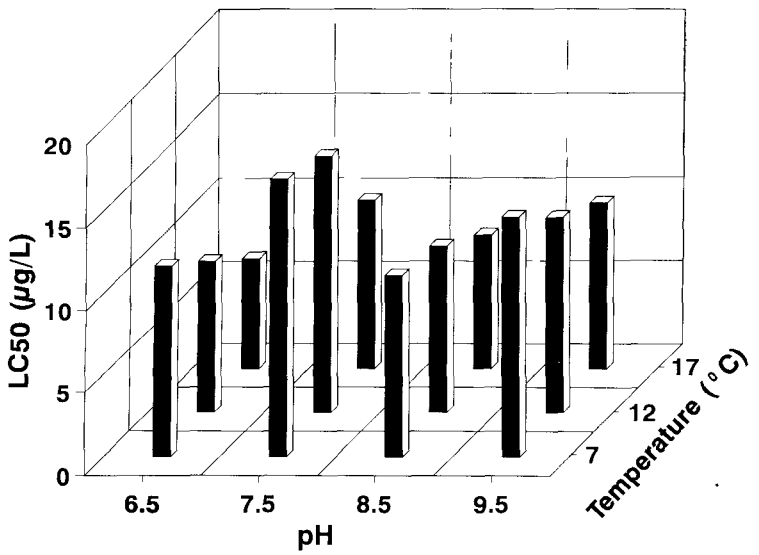


Fig 1 Toxicity (LC50) of terbufos to rainbow trout at 96 h for selected temperature and pH regimes.

culated exposure concentrations and the accuracy of dosing techniques.

Effects of pH

The toxicity of all four chemicals to *Gammarus* and rainbow trout was significantly affected by pH, except for *Gammarus* exposed to terbufos (Tables 3 and 4). Terbufos was least toxic at pH 7.5, and more toxic at higher and lower pH (Figs. 1 and 2). Terbufos was considerably more toxic to *Gammarus* than to rainbow trout; the 96-h LC50s ranged from 0.08 to 1.24 µg/L for *Gammarus* and from 6.7 to 16.8 µg/L for rainbow trout (Figs. 1 and 2). Although significant, the effect of pH on terbufos toxicity to rainbow trout was less than that observed for trichlorfon or the nitrophenols.

The toxicity of trichlorfon increased with pH (Figs. 3 and 4). The effect of pH on toxicity was greatest at 7°C. The 96-h LC50s at 7°C and pH of 6.5 and 9.5 were 40.9 and 0.52 mg/L for rainbow trout and 10.9 and 0.07 mg/L for *Gammarus*. Trichlorfon was approximately four times more toxic to *Gammarus* than to rainbow trout.

Unlike trichlorfon, the toxicities of 4-nitrophenol and 2,4-dinitrophenol increased as pH decreased. For example, the 96-h LC50s for 4-nitrophenol at 12°C and pH values of 6.5 and 9.5 were 3.8 and 78.9 mg/L for rainbow trout and 2.8

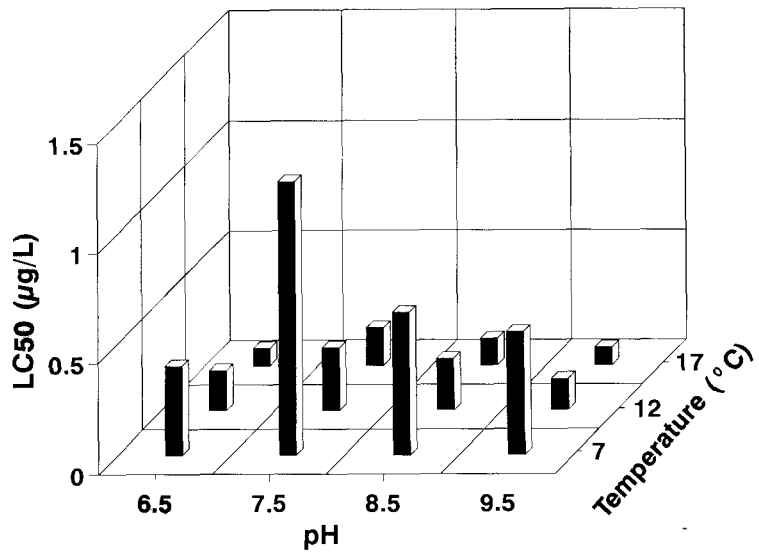


Fig. 2. Toxicity (LC50) of terbufos to *Gammarus* at 96 h for selected temperature and pH regimes

Table 3 Values of p for three way ANOVA identifying individual and interactive effects of pH, temperature, and time on toxicity of terbufos, trichlorfon, 4 nitrophenol, and 2,4-dinitrophenol to rainbow trout and *Gammarus*

		Rainbow trout				*Gammarus*			
Effect	*d f*	Terbufos	Trichlorfon	4 Nitrophenol	2,4 Dinitrophenol	Terbufos	Trichlorfon	4 Nitrophenol	2,4-Dinitrophenol
Individual effects									
pH	3	0 0001*	0 0001*	0 0001*	0 0001*	0 0603	0 0001*	0 0001*	0 0001*
Temp	2	0 0001*	0 0001*	0 0001*	0 0001*	0 0001*	0 0001*	0 0001*	0 0001*
Time	3	0 0001*	0 0001*	0 0001*	0 0001*	0 0001*	0 0001*	0 0001*	0 0001*
Interactive effects									
pH × time	9	0 1135	0 0001*	0 0001*	0 0001*	0 0423*	0 0001*	0 0001*	0 0001*
Temp × time	6	0 0001*	0 0001*	0 0001*	0 0044*	0 0001*	0 0001*	0 0001*	0 0001*
pH × temp	6	0 0173*	0 0001*	0 0001*	0 0001*	0 0344*	0 0001*	0 0001*	0 0001*
pH × temp × time	18	0 3644	0 0001*	0 0001*	0 0001*	0 0002*	0 0001*	0 0001*	0 0001*
Error	79–96								

*Significant effect ($p \leq 0\ 05$)

Table 4 Results of Duncan's multiple-range test for mean 24-, 48 , 72 , and 96-h LC50s (mg/L, μg/L for terbufos) averaged by pH, temperature, and time

	Rainbow trout				*Gammarus*			
	Terbufos	Trichlorfon	4-Nitrophenol	2,4 Dinitrophenol	Terbufos	Trichlorfon	4 Nitrophenol	2,4 Dinitrophenol
Temp. (°C)								
7	18 16 A	12 84 A	25 19 A	9 05 A	2 00 A	15 66 A	30 04 A	21 20 A
12	16 96 B	11 49 B	33 03 B	10 02 B	0 63 B	6 40 B	26 81 B	16 35 B
17	12 30 C	3 67 C	31 75 C	12 17 C	0 37 B	1 26 C	19 01 C	9 74 C
pH								
6 5	12 54 A	30 61 A	3 94 A	0 42 A	0 84	18 67 A	3 97 A	1 16 A
7 5	19 50 B	6 10 B	6 39 B	1 82 B	0 89	10 74 B	8 39 B	7 43 B
8 5	14 29 C	1 90 C	21 80 C	6 74 C	1 08	2 56 C	27 62 C	19 53 C
9 5	16 04 D	0 68 D	87 82 D	32 68 D	1 08	0 68 C	65 01 D	46 84 D
Time (h)								
24	23 74 A	15 96 A	30 96 A	13 20 A	1 79 A	17 55 A	37 43 A	27 08 A
48	17 34 B	8 90 B	31 34 A	10 36 B	1 19 B	9 17 B	27 65 B	14 72 B
72	12 66 C	7 30 C	30 58 A	9 28 C	0 59 C	3 83 C	19 36 C	12 80 C
96	11 35 D	5 59 D	27 08 B	8 83 C	0 45 D	1 65 D	16 50 D	10 11 D

Means with same letter are not significantly different within species–chemical combinations ($\alpha = 0\ 05$)

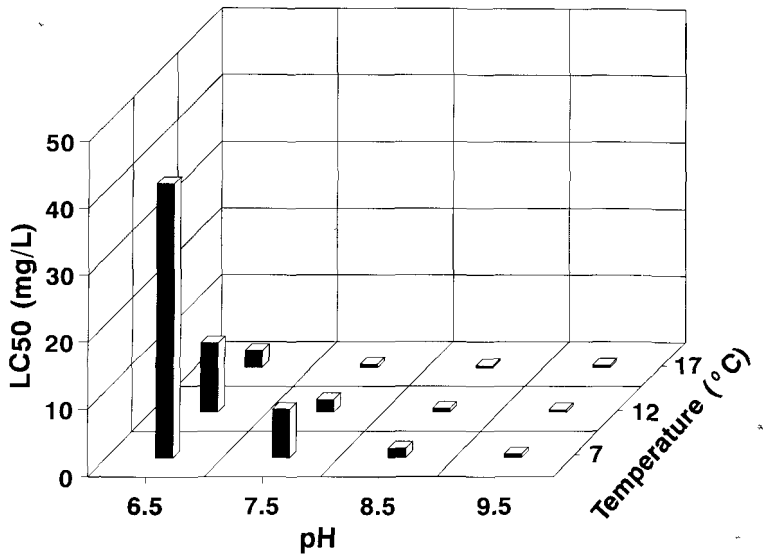


Fig. 3. Toxicity (LC50) of trichlorfon to rainbow trout at 96 h for selected temperature and pH regimes.

and 42.5 mg/L for *Gammarus* (Figs. 5 and 6). The sensitivity of rainbow trout and *Gammarus* to 4-nitrophenol was generally similar at all pH values except 9.5, where *Gammarus* was more sensitive than rainbow trout. The 96-h LC50s for 2,4-dinitrophenol at 12°C and pH of 6.5 and 9.5 were 0.39 and 27.1 mg/L for rainbow trout and 0.60 and 25.6 mg/L for *Gammarus* (Figs. 7 and 8). Rainbow trout were more sensitive to 2,4-dinitrophenol than *Gammarus* at all pH values except 9.5, for which the toxicities were similar. Overall, 2,4-dinitrophenol was approximately three times as toxic as 4-nitrophenol to rainbow trout and nearly twice as toxic as 4-nitrophenol to *Gammarus*.

Effects of temperature

The toxicity of all four test chemicals to both species was significantly affected by temperature (Tables 3 and 4). The toxicity of terbufos increased with temperature (Figs. 1 and 2). For example, the 96-h LC50s at pH 7.5 and temperatures of 7 and 17°C were 16.8 and 10.2 μg/L for rainbow trout and 1.25 and 0.17 μg/L for *Gammarus*.

The toxicity of trichlorfon to both species increased with temperature, but these temperature effects were less pronounced at high than at low pH (Figs. 3 and 4). For example, at pH 6.5, the 96-h LC50s at 7 and 17°C were 40.9 and

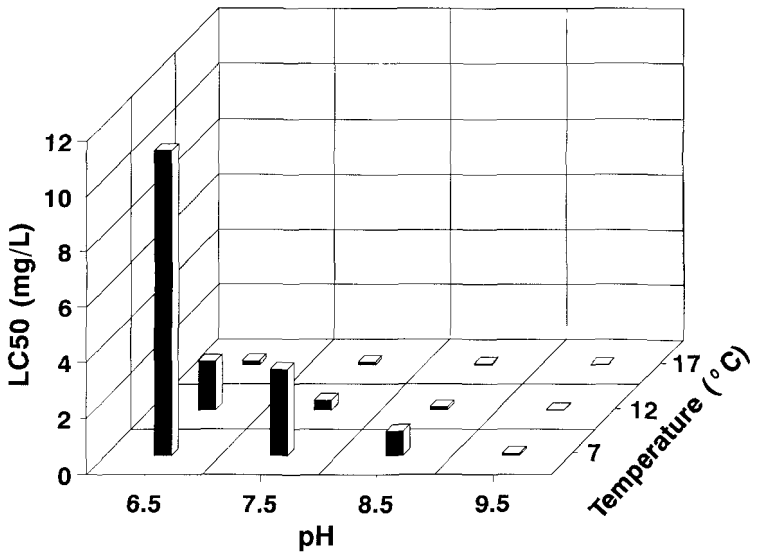


Fig. 4. Toxicity (LC50) of trichlorfon to *Gammarus* at 96 h for selected temperature and pH regimes.

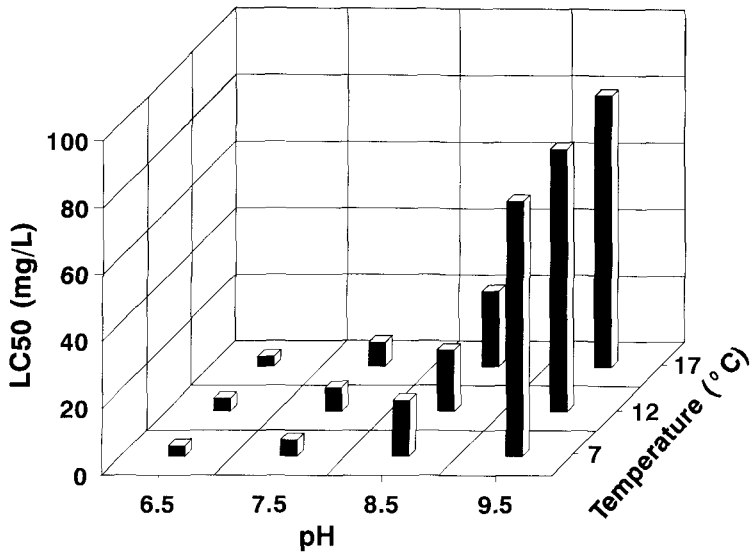


Fig. 5. Toxicity (LC50) of 4-nitrophenol to rainbow trout at 96 h for selected temperature and pH regimes.

2.50 mg/L for rainbow trout and 11.0 and 0.14 mg/L for *Gammarus*. At pH 9.5, the 96-h LC50s at 7 and 17°C were 0.52 and 0.33 mg/L for rainbow trout and 0.07 and 0.02 mg/L for *Gammarus*.

Temperature had a significant but lesser effect on the toxicity of the two nitrophenols (Figs. 5–8). Temperature effects on nitrophenol toxicity were opposite for rainbow trout and *Gammarus*. For rainbow trout, toxicity decreased with temperature. Conversely, nitrophenol toxicity to *Gammarus* increased with temperature (Table 4). The 96-h LC50s for 4-nitrophenol at pH 7.5 and temperatures of 7 and 17°C were 4.82 and 7.07 mg/L for rainbow trout and 7.54 and 6.55 mg/L for *Gammarus* (Figs. 5 and 6). The 96-h LC50s for 2,4-dinitrophenol at pH 7.5 and temperatures of 7 and 17°C were 1.50 and 1.78 mg/L for rainbow trout and 4.44 and 3.08 mg/L for *Gammarus* (Figs. 7 and 8).

Effects of time

The toxicity of all four chemicals to both species increased with exposure duration for all temperature and pH treatments. Multiple comparisons among means indicated that toxicity increased with time for nearly all tests and time intervals, except from 24 to 72 h in 4-nitrophenol tests with rainbow trout and from 72 to 96 h in 2,4-dinitrophenol tests

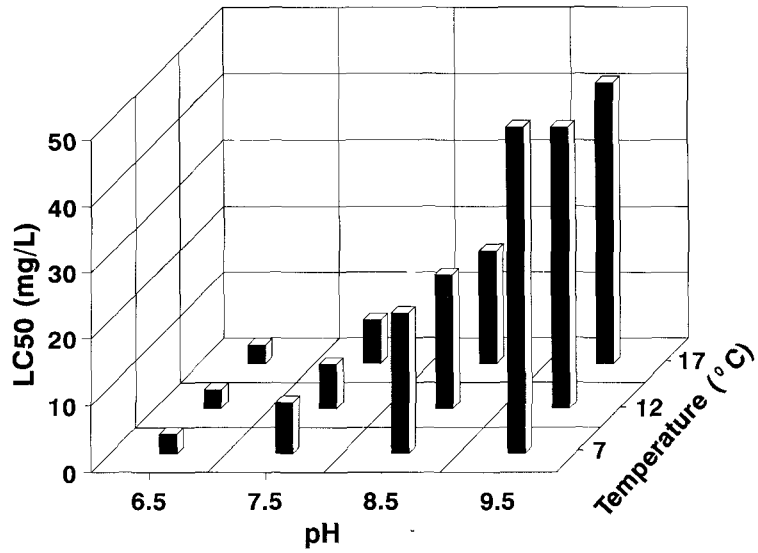


Fig. 6. Toxicity (LC50) of 4-nitrophenol to *Gammarus* at 96 h for selected temperature and pH regimes.

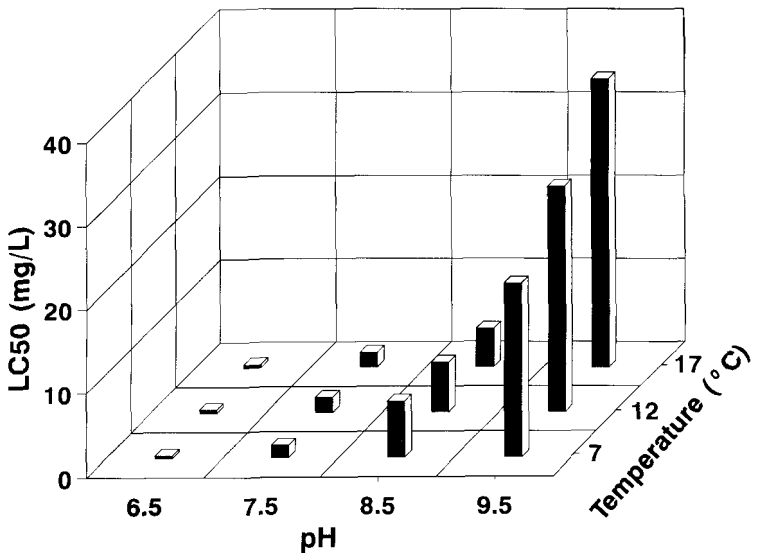


Fig. 7. Toxicity (LC50) of 2,4-dinitrophenol to rainbow trout at 96 h for selected temperature and pH regimes.

with rainbow trout (Table 4). Overall, exposure duration had the least effect on nitrophenol toxicity to rainbow trout and the greatest effect on OP toxicity to *Gammarus*. Data for the 24-, 48-, and 72-h LC50s and 95% C.I.s for all LC50s are available in Howe [27].

Interactive effects

Interactive effects between factors modifying toxicity were observed in most tests for both species and the four chemicals tested (Table 3). Three-way ANOVA for interactive effects indicated that most combinations of treatment factors (pH × time, temperature × time, pH × temperature, and pH × temperature × time) significantly affected chemical toxicity. There were two exceptions: the effect of pH on terbufos toxicity to rainbow trout was not altered by time (pH × time), and the effects of pH and temperature on terbufos toxicity to rainbow trout were not altered by time (pH × temperature × time). The most significant interactive effect observed was between pH and temperature in trichlorfon tests for both species. Trichlorfon toxicity increased with pH; however, this trend was much more prominent at the low (7°C) than at the high (17°C) temperature. Although other interactive effects were detected, no clear trends were evident. Interactive effects between factors modifying chemical bio-

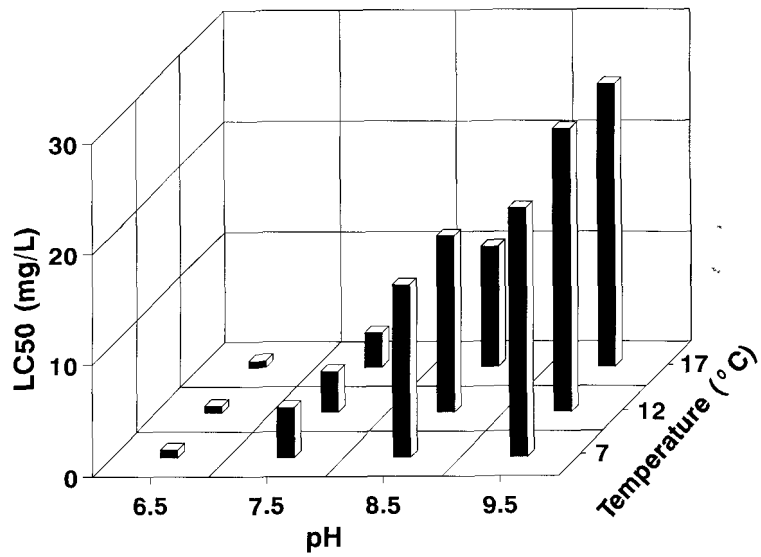


Fig. 8. Toxicity (LC50) of 2,4-dinitrophenol to *Gammarus* at 96 h for selected temperature and pH regimes.

Table 5. Values of p from ANCOVA identifying individual and interactive effects of pH and temperature on bioconcentration (BCFs) of terbufos, 4-nitrophenol, and 2,4-dinitrophenol in rainbow trout and *Gammarus*

		Rainbow trout			*Gammarus*		
Effect	*d.f.*	Terbufos	4-Nitrophenol	2,4-Dinitrophenol	Terbufos	4-Nitrophenol	2,4-Dinitrophenol
Individual effects							
pH	3	0.0009*	0.0001*	0.0001*	0.0001*	0.0052*	0.0091*
Temp.	2	0.0039*	0.0086*	0.0086*	0.0074*	0.0201*	0.0001*
Concn.	14	0.0504	0.9543	0.8610	0.1256	0.2301	0.1203
Interactive effects							
pH × temp.	6	0.1428	0.2087	0.0073*	0.1775	0.488*	0.0028*
Error	24						

*Significant effect ($p \leq 0.05$).

concentration were also observed for some tests (Table 5). A significant interaction between temperature and pH was observed in tests with rainbow trout and *Gammarus* exposed to 2,4-dinitrophenol and in tests with *Gammarus* exposed to 4-nitrophenol.

Bioconcentration of test chemicals

Bioconcentration of test chemicals in most tests appeared to be directly related to mortality (Figs. 1–14). Water temperature, pH, chemical type, species type, chemical exposure concentration, and duration of exposure affected chemical bioconcentration for most tests (Table 5, Figs. 9–14).

Both pH and temperature significantly affected bioconcentration of test chemicals by rainbow trout and *Gammarus* in all tests (Table 5). Bioconcentration of terbufos in rainbow trout generally increased with pH and decreased with temperature (Fig. 9). Bioconcentration of terbufos in *Gammarus* generally increased with temperature, and was greater at pH 7.5 and 8.5 than at the more extreme pH values of 6.5 and 9.5 (Fig. 10). Bioconcentration of nitrophenols in both species increased with temperature and decreased with pH for most tests (Figs. 11–14).

Species and chemical type also significantly affected chemical bioconcentration. Among chemicals, BCFs were generally higher for *Gammarus* than for rainbow trout (Figs. 9–14). Bioconcentration of terbufos was approximately 10 times higher than bioconcentration of nitrophenols (Figs. 9–14).

Chemical exposure concentration and the duration of exposure influenced bioconcentration and resulting body concentrations. This was determined by regression analysis performed on body concentration data and was evident upon examination of body concentration vs. chemical exposure concentration plots. Body concentrations of terbufos in rainbow trout and *Gammarus* increased with chemical exposure concentration; length of exposure had less of an effect. The evidence was that organisms exposed to high concentrations for very short periods had much higher body concentrations

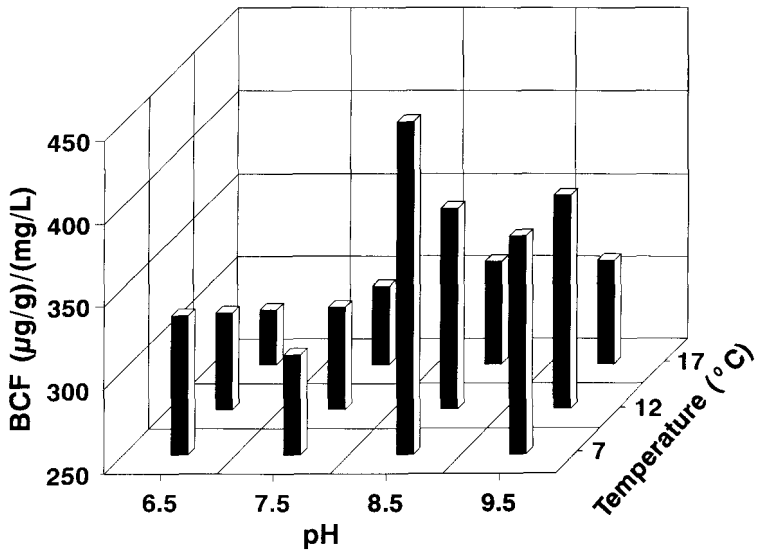


Fig. 9. Bioconcentration (mean BCFs) of terbufos in rainbow trout at selected temperature and pH regimes.

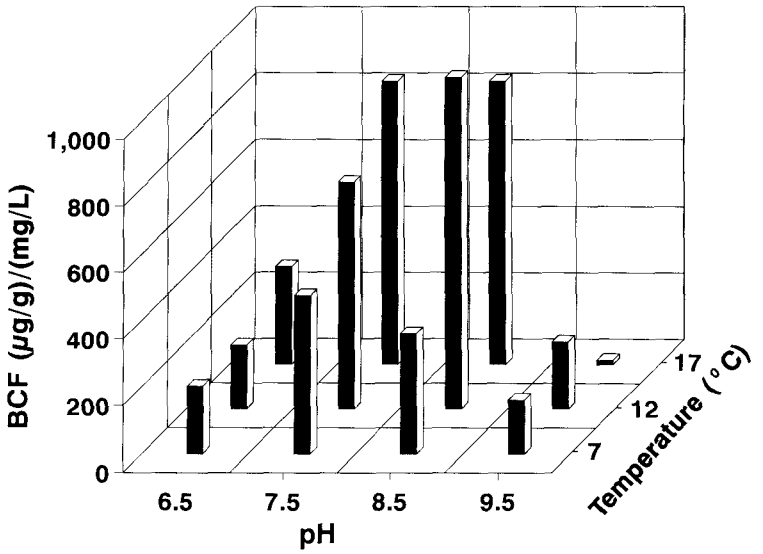


Fig. 10. Bioconcentration (mean BCFs) of terbufos in *Gammarus* at selected temperature and pH regimes.

than those exposed to lower concentrations for longer periods. The opposite was true, however, for nitrophenol tests, in which body concentrations increased with length of exposure and exposure concentration had less of an effect. Organisms at lower test concentrations had higher body concentrations than those at higher test concentrations, probably because of a longer exposure to test chemicals.

DISCUSSION

pH effects

The effects of pH on chemical toxicity observed in this study generally agree with other reports. Mayer and Ellersieck [8] reported similar 96-h LC50s of 11.40 and 0.21 mg/L, respectively, for rainbow trout exposed to trichlorfon at pH 6.5 and 9.0. They also concluded that trichlorfon toxicity increased with pH. They suggested that, in alkaline conditions, trichlorfon rapidly hydrolyzes to dichlorvos, which is 2.6 to 350 times more toxic than trichlorfon. Our results were similar. This mechanism was also supported by our HPLC analytical results, which showed greater chemical degradation and subsequent lower recovery percentages of trichlorfon at high pH. Trichlorfon is one of the few OPs that hydrolyzes to a more toxic compound. For most OPs, hydrolysis products are poor inhibitors of acetylcholinesterase [28] and are, therefore, less toxic than the parent compound.

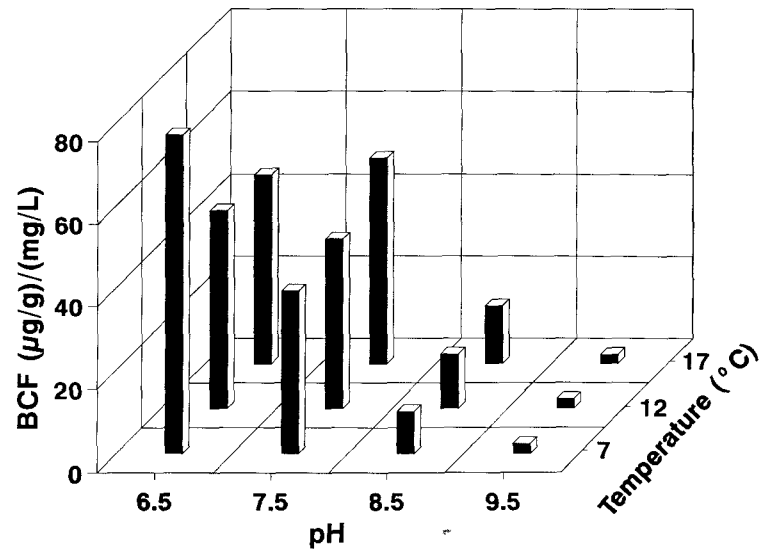


Fig. 11. Bioconcentration (mean BCFs) of 4-nitrophenol in rainbow trout at selected temperature and pH regimes.

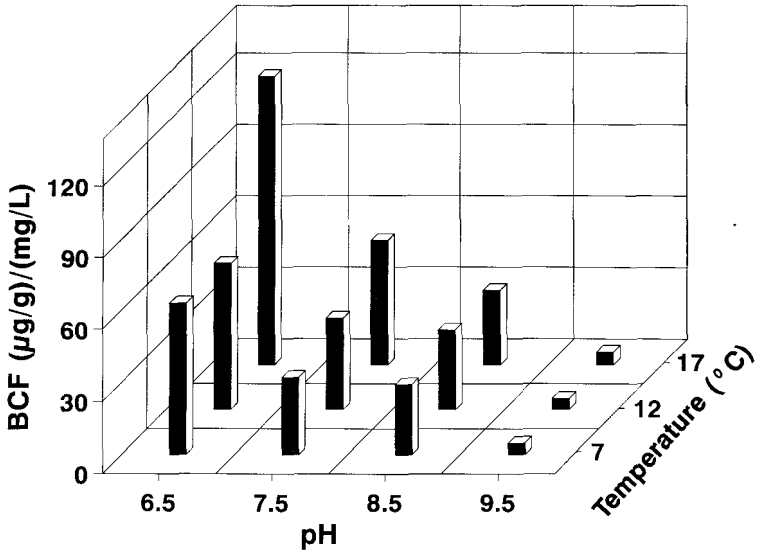


Fig. 12. Bioconcentration (mean BCFs) of 4-nitrophenol in *Gammarus* at selected temperature and pH regimes.

The relatively minor effects of pH on terbufos toxicity observed in this study agree with results of previous studies [8]. Terbufos is a thionate (sulfur-containing OP; Table 1). Thionate toxicity is not affected by high pH as is trichlorfon because thionates do not hydrolyze readily in alkaline conditions; therefore, thionates do not degrade to less toxic hydroxylated products [8].

Both nitrophenols were 25 to 50 times more toxic at low pH than at high pH. These compounds are ionizable weak acids and should be more toxic at low than at high pH [8]. Based on the pK_a values (Table 1), both nitrophenols should become increasingly ionized as pH increases. Sprague's [7] contention that un-ionized organic molecules are more toxic because they more readily penetrate cell membranes would explain the effect of pH on toxicity observed in our study.

There is additional evidence that the un-ionized forms of most drugs penetrate cell membranes more readily than ionized forms. For example, laboratory studies by Hunn and Allen [4] showed that residues of the lampricide TFM in fish tissue were correlated with TFM dissociation at different pH values; as pH increased, TFM tissue residues and toxicity decreased. The pK_a of TFM is 6.07; therefore, above pH 6.07 a greater proportion of the chemical becomes ionized and less readily crosses gill membranes. Laboratory toxicity tests also indicated that TFM toxicity increased by a factor of 20 to 50 times as pH decreased from 9.5 to 6.5 [9]. The two nitrophe-

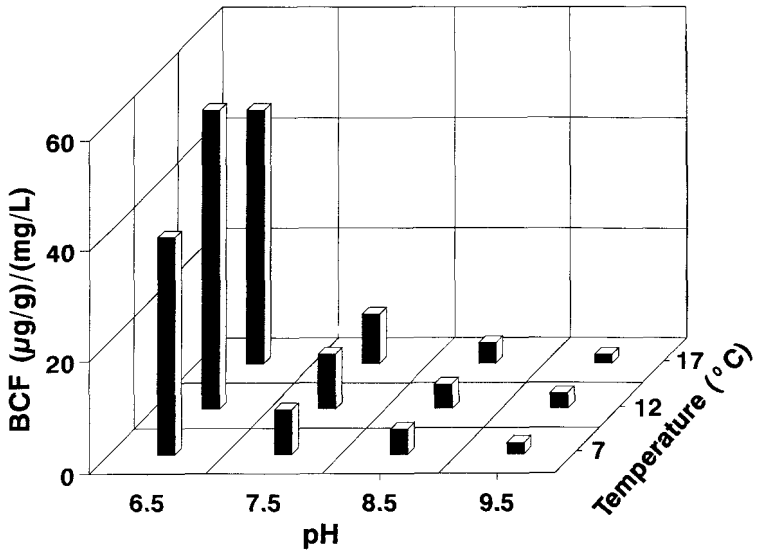


Fig. 13. Bioconcentration (mean BCFs) of 2,4-dinitrophenol in rainbow trout at selected temperature and pH regimes.

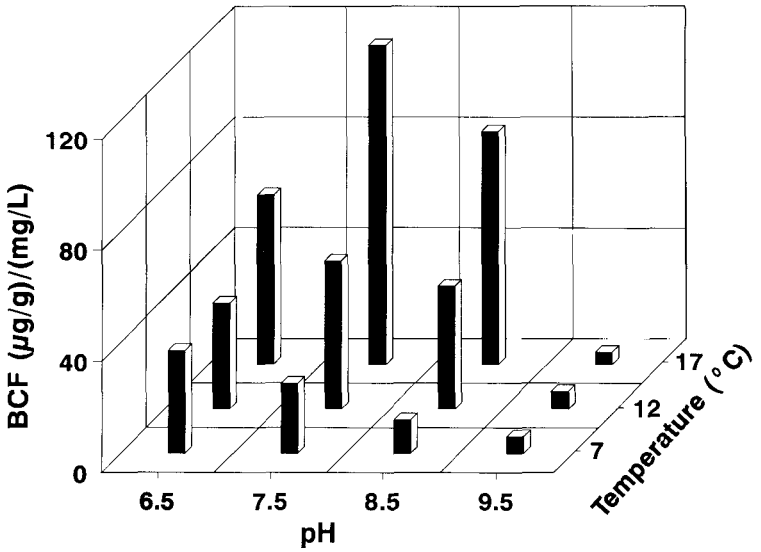


Fig. 14. Bioconcentration (mean BCFs) of 2,4-dinitrophenol in *Gammarus* at selected temperature and pH regimes.

nols 4-nitrophenol and 2,4-dinitrophenol, which are chemically similar to TFM, were probably similarly affected by pH.

Temperature effects

Temperature and toxicity were positively correlated in most tests. This generally agrees with the conclusions of others who have reviewed the effects of temperature on acute toxicity of similar chemicals to aquatic organisms [5–8]. According to Mayer and Ellersieck [8], studies that address the effects of temperature on chemical toxicity to fish report that most chemicals exhibit a two- to fourfold change in toxicity (96-h LC50s) for each 10°C change in water temperature. This observation conforms to the Q_{10} concept that ectothermic metabolism increases approximately twofold for every 10°C increase in temperature [29]. As metabolism increases, so does chemical uptake. However, biochemical detoxification and elimination of the chemical may also increase with temperature. Therefore, an increase or decrease in chemical toxicity may be observed with an increase in temperature, depending on organismal physiology.

A negative correlation between chemical toxicity and temperature was observed in tests with rainbow trout exposed to 4-nitrophenol and 2,4-dinitrophenol. The decrease in toxicity with temperature was small but significant. Higher temperatures may have increased fish metabolism and allowed for increased biochemical detoxification and elimination. A similar response was not observed with *Gammarus*, which may lack physiological mechanisms for similar toxicant elimination.

Bioconcentration

Bioconcentration was generally affected by the same factors that affected toxicity; however, several differences were observed. Test pH did not affect toxicity of terbufos to *Gammarus* but did affect the bioconcentration of terbufos by *Gammarus*. An interactive effect between pH and temperature on toxicity was observed in all tests; however, the same interactive effect on bioconcentration was observed only in tests with *Gammarus* exposed to nitrophenols or rainbow trout exposed to 2,4-dinitrophenol.

Species differences in bioconcentration were not large, but *Gammarus* appeared to bioconcentrate chemicals more than rainbow trout. Rainbow trout may be better able to detoxify and eliminate chemicals than *Gammarus*, which might explain why *Gammarus* was more sensitive than rainbow trout to all chemicals tested.

Different chemicals bioconcentrated in test organisms to different degrees. Bioconcentration of terbufos was much higher than the bioconcentration of nitrophenols, which would be expected upon examination of the octanol/water partition coefficient (K_{ow}) of each chemical (Table 1). Terbufos, with a higher log K_{ow} and very low water solubility, is much more lipophilic than the very water-soluble nitrophenols and thus would be expected to bioconcentrate more.

Interactive effects

Analysis of interactive effects indicated that factors modifying toxicity can interact in various ways, depending on the toxicant, the test organism, and the test conditions. For example, pH may affect chemical toxicity only within a particular temperature range (pH × temperature interaction), and time may also affect this interaction (pH × temperature × time interaction).

The interactive effect between pH and temperature in trichlorfon tests for both species was presumably due to the differential degradation of trichlorfon to a more toxic form at different water temperatures and pH values. The apparent degradation of trichlorfon and subsequent increased toxicity at higher pH was much more prominent at the low temperature (7°C). Trichlorfon degradation was sufficiently

enhanced at the high temperature (17°C) to nearly mask the pH effect on degradation

The observed interactive effects of pH, temperature, and exposure duration on toxicity would not have been evident without a factorial design Nearly all the interactive effects were significant, indicating that chemical toxicity and modifying factors must be examined from a factorial approach to understand chemical toxicity fully and to relate laboratory data to field situations, where interactions between factors affecting toxicity are even more complex and unpredictable

SUMMARY AND RECOMMENDATIONS

This study demonstrated that temperature and pH can significantly affect chemical toxicity Other studies and reviews have drawn similar conclusions, however, this is one of few freshwater studies specifically designed to determine factors modifying toxicity for such an extensive matrix of species, toxicants, and conditions The significant effects of 12 pH and temperature regimes on the toxicity of four chemicals were determined for two species of aquatic organisms

If toxicity tests are to yield useful information for evaluating chemical hazards to aquatic biota, they should accurately assess chemical toxicity in aquatic environments Routine toxicity tests, conducted at one standard exposure-water condition, do not accurately portray chemical toxicity in the environment because they do not identify toxicity-modifying factors and interactive effects

Development of hazard assessment protocols and water-quality criteria should include consideration of the potential effects of factors that modify toxicity to adequately protect valuable aquatic resources Factorial toxicity tests are expensive and time consuming, but in some cases there is no substitute for the type of information that they provide Whenever possible, existing data on physical and chemical characteristics, chemical toxicity, and factors that alter chemical toxicity should be organized, studied, and used In some cases, adequate risk assessment can be made from existing data, and adjustments to safety margins may compensate for inadequate data. We recommend that factorial-designed toxicity studies be included in risk assessment protocols when necessary and feasible Chronic studies should also be used appropriately because acute studies cannot always predict the effects of certain modifying factors and do not provide data concerning sublethal and long term effects

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