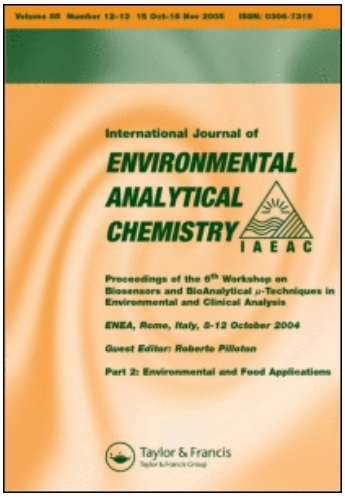

International Journal of Environmental Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713640455

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To cite this Article Stebbins, R. G. , Roy, T. and Doane, T. R.(1989) 'Analysis of Malathion and its Breakdown Products in Aquaria Containing Golden Shiners (*N. CHRYSOLEUCAS*)', International Journal of Environmental Analytical Chemistry, 35: 3, 127 — 137
To link to this Article: DOI: 10.1080/03067318908028387
URL: http://dx.doi.org/10.1080/03067318908028387

Intern. J. Environ. Anal. Chem., Vol. 35, pp. 127–137
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ANALYSIS OF MALATHION AND ITS BREAKDOWN PRODUCTS IN AQUARIA CONTAINING GOLDEN SHINERS (*N. CHRYSOLEUCAS*)

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(*Received 5 February 1988; in final form 8 August 1988*)

The 1-ethylmonocarboxylic acid of Malathion (MCA) was identified as a metabolite of Malathion in the gut of golden shiners (*N. Chrysoleucas*) ($7.11 \times 10^2 \pm 0.66 \times 10^2$ ng/g tissue). As noted in the literature the rate of hydrolysis of Malathion above pH 7 is first order with respect to Malathion and pH dependent. In addition, the rate of hydrolysis of Malathion in aquaria housing these fish was found to be dependent on the concentration of Ca^{+2}; sparging the aquaria with air affected the rate. Some of the hydrolysis products of Malathion were identified by gc/ms and found to be pH dependent above pH 7. The half-life of Malathion under different conditions, the identification of some hydrolytic products and a general scheme for the analysis of Malathion, along with some of its metabolic and hydrolytic products are included in this paper.

KEY WORDS: Malathion, metabolites, golden shiners, hydrolysis, rate constants.

INTRODUCTION

O,o-dimethyl-S-(1,2 dicarboxyethyl) phosphorodithioate (Malathion) is a heavily used organophosphorous pesticide[1] the structure and mass spectrogram of which are shown in Figure 1. It is used by commercial agricultural concerns, by those interested in residential lawn and garden care, and by companies specializing in mosquito control. This heavy use makes it a common environmental pollutant. Both the behavioral[2] and physiological[3,4] effects of this compound to piscine species have been noted. Analysis of this pesticide and assessment of its impact on the aquatic environment are complicated by the fact that Malathion breaks down (hydrolyzes) in water and is also metabolized by aquatic organisms.[1,3] Figure 2 shows the structure of the more common metabolic and hydrolytic products of Malathion. O,o-dimethylphosphorothioic acid (DMTP) and o,o-dimethyl-phosphorodithioic acid (DMDTP) have been identified as metabolic products in mammals, while o,o-dimethyl-S-(1,2 carboxy) phosphorodithioate (DCA), o,o-dimethyl-S-(1,2 dicarboxyethyl) phosphorothioate (Malaoxon) and the mono-carboxylic acid of Malathion (MCA) (α or β isomer unspecified) have been

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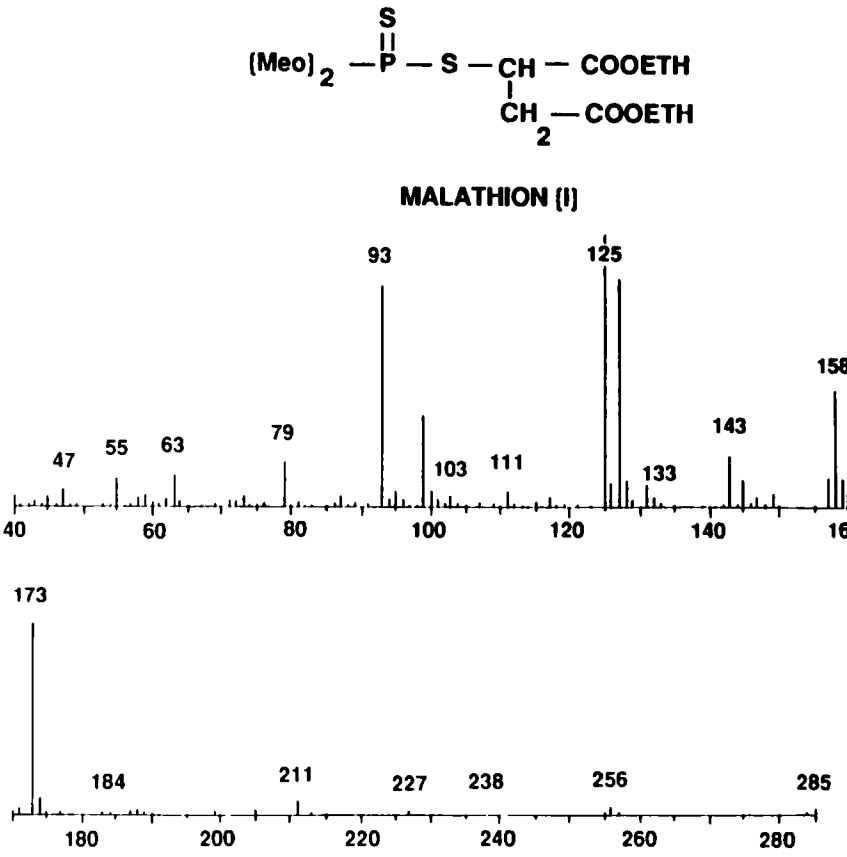

Figure 1 Malathion and its mass spectrogram.

identified as metabolic products in mammals and some species of saltwater fish.[1,3,6,7] No references identifying the metabolic pathways of Malathion in fresh water fish were found in the literature. Since MCA, DCA and Malaoxon have already been identified as metabolic products in salt water fish, it seemed reasonable to search for these metabolites in fresh water species.

EXPERIMENTAL

Materials

Boron trifluoride 14% in methanol (Varian, Sunnydale, CA, USA), calcium nitrate (100.9% Baker Reagent Grade, VWR, Boston, MA, USA) and n-methyl-n'-nitro-n-nitrosoguanadine (97% Aldrich, Milwaukee, WI, USA), were used without further purification. Malathion (99%), Malaoxon (98%), the dicarboxylic acid of Malathion (DCA) (98%), o,o-dimethyl-S-(1-carboxy-2-carbethoxy) phosphorodithioate (the α isomer of MCA) (97%), o,o-dimethyl-S-(1,2 dicarboxymethyl)

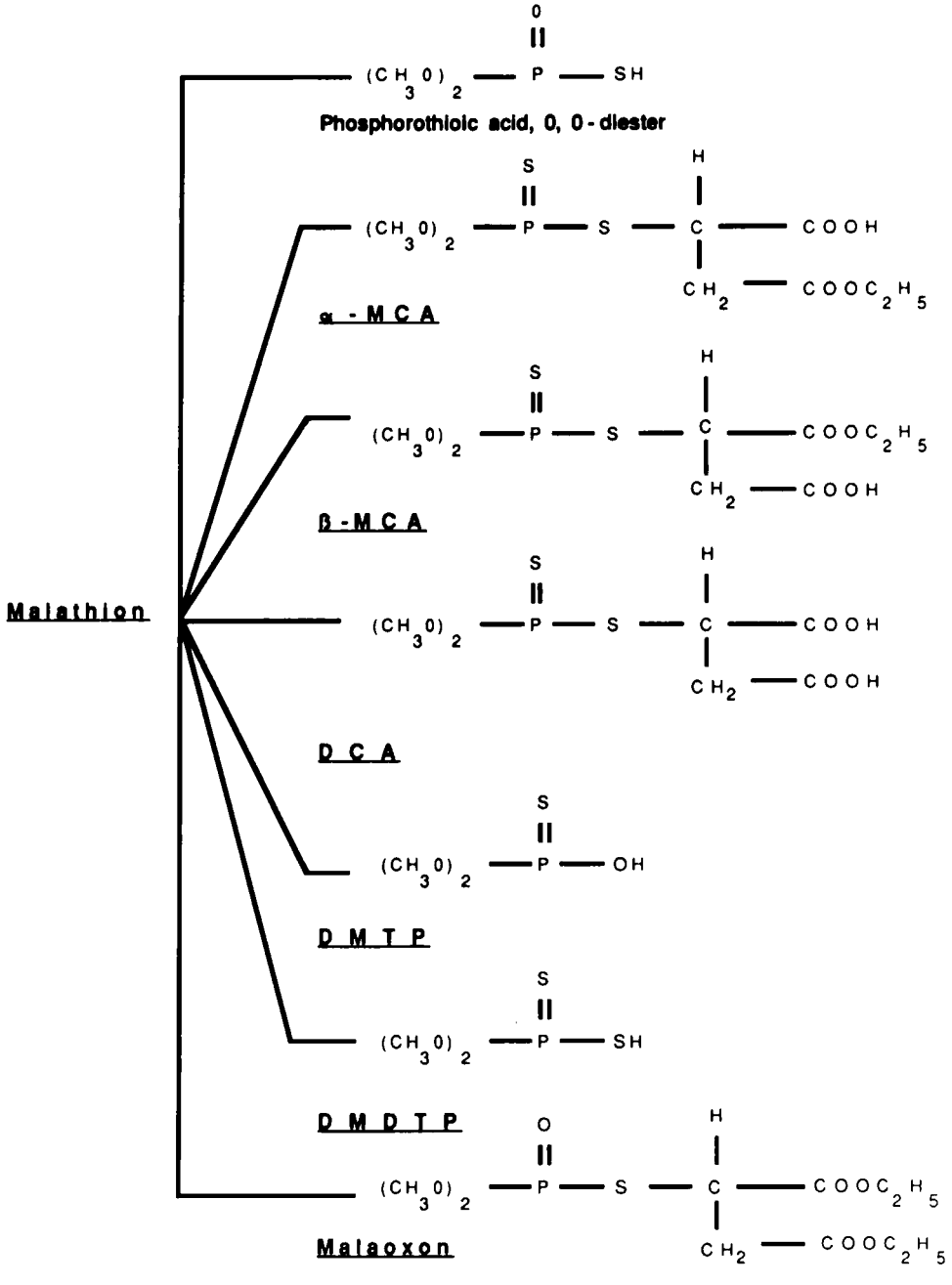

Figure 2 Metabolic and hydrolytic products of Malathion.

phosphorodithioate (the dimethylester of DCA 98%) and o,o-dimethyl-S-(1-carboxy-2-carbethoxy) phosphorodithioate (97%) (all supplied by American Cyanamid of Princeton, NJ, USA) were all used without purification and were stored in a refrigerator at 5 °C. The solvents hexane, methanol, diethyl ether, ethyl acetate and acetonitrile were all Baker Resi-Analyzed or Pesticide Grade (VWR).

Instrumentation

Chromatograms were obtained using a Varian 3700 gas chromatograph with a ^{63}Ni electron capture detector. Two columns were used: a 2m × $\frac{1}{8}$″ glass column with a 4% OV-101 + 6% OV-210 on 80/100 mesh gas chrom Q and a 25 m × 0.02 mm with a film thickness of 0.25 micron BP-1FSOT capillary column (both Varian, Sunnydale, CA, USA). The packed column was run isothermally: the temperature of the injection port, column and detector were 200 °, 200 ° and 300 °C respectively. The carrier gas was grade 5 nitrogen (Airco, Murry Hills, NJ, USA) with a flow rate of 24 cc/min. The capillary column was temperature programmed from 60 ° to 200 °C at 20 °C per minute in the splitless mode. The injection port and detector were 250 °C and 300 °C respectively. The nitrogen carrier gas flow rate was 1.39 cc/min. A Varian 4270 integrator recorded and quantified the detector response. Gc mass spectrograms were obtained on an HP 5840A/HP 5987-MS/HP 100-DS system with a 25 meter × 0.22 mm with a film thickness of 0.15 micron capillary column (Hewlett-Packard, Palo Alto, CA, USA). The carrier gas was helium flowing at 1.0 cc/min. Ionizing voltage was 70 ev in the electron impact mode with the source temperature at 200 °C. Runs were temperature programmed from 35 ° to 290 °C at 6 °C per minute. Occasionally an HP 3760 A-GC/5970 A mass selective detector/HP 9825 B data collector was used under similar conditions.

Analytical methods

Standard solutions of Malathion and related compounds (about 10^{-5} M) were prepared by serial dilutions from stock solutions of the pure compounds dissolved in hexane. It was found that these standard solutions decompose slowly as a function of time even when refrigerated and must be replaced periodically (every three months). All standard solutions were refrigerated between use at 5 °C. Triplicate analysis of Malathion in water was performed on 125 cc samples collected and stored in glass bottles with teflon coated screw tops. The samples were refrigerated at 5 °C and processed within 24 hours of collection. All glassware used in the analysis, including the collection bottles, was cleaned in accordance with EPA method 608 and rinsed three times with Resi-Analyzed hexane. Each sample was acidified with 1 ml of 6 N HCl and extracted with 25, 15, and 10 ml portion of 15% diethylether/hexane v/v. The combined volumes were dried over anhydrous sodium sulfate until ready for gc analysis. Per cent recovery of

Malathion by this method from aqueous solutions of known concentration was 96%.

Rate studies were performed by triplicate analysis of 125 cc water samples containing Malathion (1 ppm) as a function of time. The pH was adjusted with a dilute solution of 1 N HCl. For studies involving calcium, the calcium ion concentration was set by adding the appropriate volume of calcium stock solution (1,000 ppm) to the volumetric flask and diluting to the mark with distilled water.

Florosil columns were prepared by placing a loose plug of glass wool in the tip of a chromoflex column (Kontes no. 42100 22–7 mm, Vineland, NJ, USA). The column was packed with 1.6 g of Florosil which was previously activated at 650 °C. Granular sodium sulfate (1.6 g) was then added to the top of the column. The column was washed with 50 ml of nanograde hexane followed by 50 ml of nanograde methanol and dried overnight at 130 °C in an oven. The columns were cooled to room temperature and pre-wet with 10 ml of hexane prior to elution of the sample.

Duplicate analysis of Malathion in tissue was performed on freshly sacrificed fish. At sacrifice, the appropriate tissue (ca. 10–20 mg of liver and 0.4–0.8 g gut) was immediately removed (within 20 minutes), weighed and ground for 15 minutes with a 5 ml portion of 15% diethylether/hexane in an all glass tissue grinder (Lurex Mfg. Co., Vineland, NJ, USA). The liquid portion was decanted into a glass collection tube and a second 5 ml portion of solvent was added to the tissue grinder and ground for an additional 10 minutes. This liquid portion was combined with the first, refrigerated at 5 °C and dried over anhydrous sodium sulfate until analysis. Each sample was evaporated with nitrogen to 0.3 ml and placed on a Florosil column prepared as above. The sample was eluted with 12 ml of hexane and 12 ml of 1% methanol/hexane v/v. The eluates were discarded and further elution with a second 12 ml portion of 1% methanol/hexane was performed. The eluate, containing Malathion, was evaporated to 1 ml with nitrogen and stored over sodium sulfate until gc analysis. Per cent recovery of Malathion by this method from clean fish tissue was 74%. This was determined by spiking clean tissue with 50 ng of Malathion and treating the tissue as described above.

Methylation of standard solutions of DCA and MCA was accomplished by reaction with BF_3/methanol and by reaction with diazomethane. For the former method, 1 ml of freshly opened BF_3/MEOH was added to about 50 mg of compound dissolved in dry acetonitrile/diethyl ether in a clean, dry glass flask. The contents were heated to 60 °C for 40 minutes, and allowed to cool. Two ml of water and 2 ml of saturated sodium sulfate were added to the flask, and the mixture was extracted 3 times with 5 ml portions of 15% diethylether/hexane v/v. The extracts were combined, evaporated to 1 ml under a stream of nitrogen and stored over anhydrous sodium sulfate prior to analysis. The time and temperature for this procedure were optimized in an attempt to improve the efficiency.

Diazomethane was generated using a procedure published by Aldrich.[9] Derivatization of standard solutions of DCA and MCA was accomplished by adding 3 ml of the freshly prepared diazomethane to about 50 mg of compound dissolved in 50% acetonitrile/diethylether in a clean dry glass flask. The resulting solution was allowed to react for 30 minutes at room temperature, and the excess diazomethane

was purged by bubbling dry nitrogen through the solution for 10 minutes. The resulting solution was evaporated to 0.3 ml with nitrogen and immediately analysed by gc.

Duplicate analysis of possible Malathion breakdown products in water was performed on 125 ml samples; before extraction the water was adjusted with 1 ml of 6 N HCl. Each sample was extracted with 25, 15 and 10 ml portions of 15% diethylether/hexane v/v to extract Malathion. The sample was then extracted with 15, 10 and 10 ml portions of 50/50 v/v acetonitrile/diethylether. These latter extracts were combined, evaporated to dryness under nitrogen gas and derivatized with diazomethane as described above. Per cent recovery of DCA and MCA from aqueous solutions of known concentration was 73% and 87% respectively.

Triplicate analysis of Malathion metabolites was performed on freshly sacrificed fish tissue. At sacrifice, appropriate tissue was immediately removed (within 10 minutes), weighed, and ground for 15 minutes with a 5 ml portion of 50/50 acetonitrile/diethylether v/v in an all glass tissue grinder. The resulting mixture was centrifuged and the supernatant liquid transferred to another flask. The remaining tissue was ground again for 10 minutes with a second 5 ml portion of solvent, centrifuged, and combined with the first 5 ml of liquid. The combined extracts were evaporated with nitrogen to 1.0 ml and derivatized with diazomethane as above. The derivatized sample was placed on a Florosil column and eluted as above. The second 1% methanol/hexane fraction contained the derivatized MCA and DCA. Per cent recovery of MCA and DCA was 67% and 81% respectively. This was determined by spiking clean fish tissue with 50 ng each of DCA and MCA and treating as described above.

Fish

For exposure to Malathion, golden shiners (*N. Chrysoleucas*) were used. The shiners were obtained from Carroll Cutting of Standish, Maine, USA. Three fish each comprised the control and exposed group. The shiners were housed in 20 liter glass aquaria containing 14 liters of dechlorinated, gently aerated water at 21°–24°C. The pH of the water was 7.10 before adding the fish and 7.70 upon completion of the exposure. The aquaria were spiked prior to the introduction of fish with Malathion by the following procedure: Malathion was weighed in a 10 ml volumetric flask, and deionized water added to it. The flask was allowed to stand overnight, and then emptied into the aquarium. The flask was flushed several times with water, and the aquarium water stirred before introduction of the fish. The final concentration of Malathion was 137 ppb. Because Malathion hydrolizes in water, additional Malathion was added once a day to return the concentration to its original value. This static exposure was continued for 96 hours. At that time, both groups of fish were sacrificed and their liver and gut immediately removed.

RESULTS AND DISCUSSION

Standard solutions of Malathion, Malaoxon and the methylated derivatives of possible metabolites and hydrolytic products were chromatographed under the

Table 1 GC/ECD characteristics of organophosphorous compounds

Compound	*RT (min)*	*SEN (R/pg)*[b]	*MDL (pg)*[c]
Malathion	12.90	112	38.0
MCA (4-ethyl-1-methyl)	10.86	228	18.0
DCA (dimethyl)	9.10	926	4.4
Malaoxon	13.02		
DMDTP (methyl)	5.36		
Phosphorothioic acid (methyl thioester)	4.58		

[a]Obtained on a 2 M × ¼" glass column packed with 4% OV-101 + 6% OV-210 on 80/100 mesh Gas Chrome Q. Column T = 200 °C.
[b]Integrator response units per picogram.
[c]Minimum detector limits in picograms.

conditions specified in the experimental section. Retention times, sensitivities and the minimum detection limits associated with the compounds are given in Table 1. Only the ∝ isomer of MCA was available to us, and the retention time cited is for the methyl derivative of this isomer. Because of this we have not attempted to distinguish between the two structural isomers of MCA. If the β-isomer was a metabolic product, our column was unable to distinguish it from the α form.

Methylation of standard solutions of MCA and DCA, a procedure we thought to be well defined by the literature,[6,7,8] was somewhat problematic. Kadoum reported that methylation of MCA and DCA with BF_3/methanol yielded the methyl esters of these two compounds.[8] We found that methylation by this method under a variety of conditions yielded neither of these methyl esters, but did yield the methyl derivative of DMDTP (RT = 5.36 min on the packed column, and confirmed by gc/ms on a separate column). Methylation with diazomethane as reported by Bradway and Shafik[6] yielded the monomethyl ester of MCA and the dimethyl ester of DCA respectively, both at 70% yield. Since these latter compounds were of primary interest all further samples were methylated with diazomethane.

Although it is well known that the rate of hydrolysis of Malathion is a function of pH, temperature and the concentration of some metal ions, there is a large range of values reported for similar conditions in the literature.[10-17] The results of Table 2 were obtained by analysis of aliquots of water containing Malathion as a function of time. The natural log of the chromatographic response was plotted as a function of time and the slope of the line calculated by linear regression analysis. These results show the half-lives for the hydrolysis of Malathion in aqueous solution under varying conditions, confirming that the hydrolysis is first order with respect to Malathion.[16,17] The half-life of 1.79 weeks at pH 7.0 and no added calcium supports the values predicted by Wolfe *et al.*; the half-life of 10.72 weeks at pH 6.8 and no added calcium is greater than Wolfe's by a factor of three.[16,17] Although no rate data was recorded above pH 7.00, it was observed that hydrolysis at or above pH 8.00 was rapid and that the rate of hydrolysis increased with increasing pH and temperature. Breathing air sparged through the system at 147 cc/min greatly increased the rate of breakdown. This suggests that Malathion is oxidized by air. From Table 2 we can also see that at a pH 6.80 an increase in the concentration of divalent calcium increases that rate of hydrolysis. Some of the

Table 2 Half-life of Malathion in aqueous systems at 25 °C

Run	*pH*	$[CA^{+2}]$ *ppm*	$t_{1/2}$ (*weeks*)[e]	R[d]
1[a]	7.0	0	1.79 ± 0.08	−0.9914
2[a,c]	7.0	0	0.10 ± 0.02	−0.9863
3[b]	6.8	0	10.72 ± 0.91	−0.9914
4[b]	6.8	1	10.74 ± 0.89	−0.9823
5[b]	6.8	10	7.66 ± 2.60	−0.9528
6[b]	6.8	50	3.84 ± 0.33	−0.9889
7[b]	6.8	100	2.05 ± 0.17	−0.9861

[a]Initial concentration of Malathion was 1 ppm.
[b]Initial concentration of Malathion was 10 ppm.
[c]Sample sparged with air at 147 cc/min.
[d]R is the linear least squares correlation coefficient.
[e]Uncertainty limits are standard deviation.

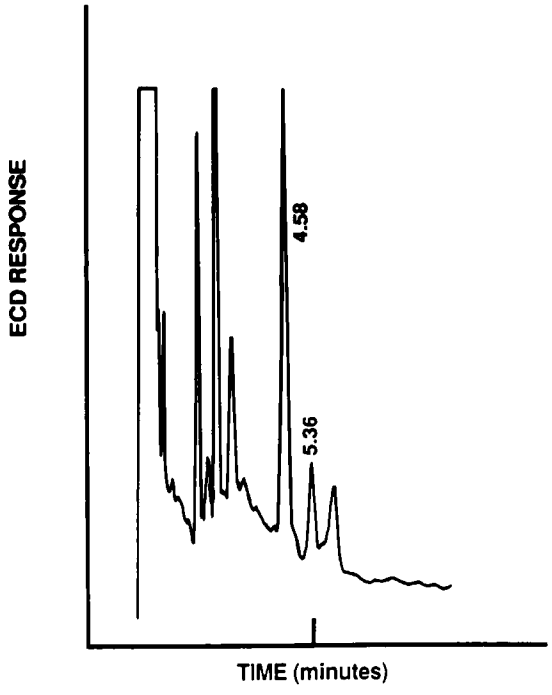

Figure 3 Chromatogram of derivatized water extract, pH 8.00.

hydrolysis products of Malathion (initial concentration 1 ppm) were identified at pH 8.00 and 11.46. Identification at lower pHs (6.8 to 7.7) was not attempted due to the much lower rate of hydrolysis at these pHs and the low initial concentration of Malathion. Analysis of the aqueous Malathion solutions at pH 8.00 gave no evidence of DCA or MCA but did give evidence of DMDTP (RT 5.36 min) and the phosphorothioic acid o,o-dimethylphosphorothioic acid (the P=O oxygen analog of the P=S containing DMDTP) (RT 4.58 min). A chromatogram of the derivatized extracts appears in Figure 3 (no further chromatographic peaks occur

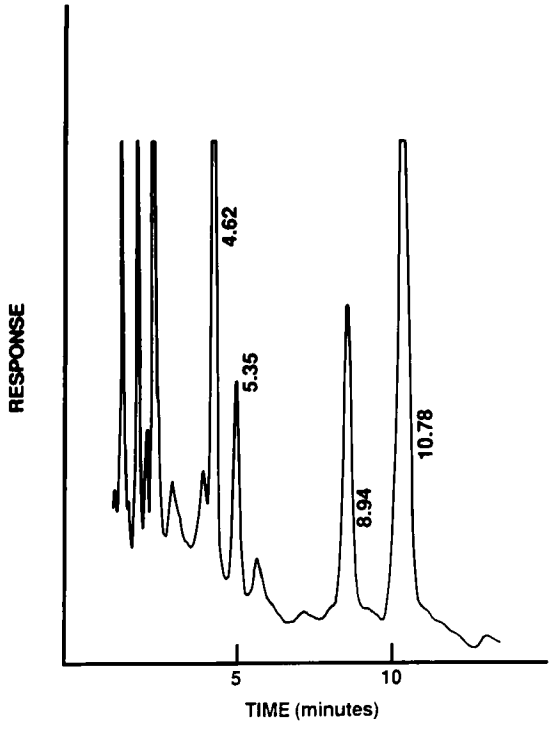

Figure 4 Chromatogram of derivatized water extract (pH = 11.46).

after 6 min in this chromatogram). Gc/ms analysis of these two peaks confirms our identification and resulted in responses at m/e 125, 93, 126, 109 and 79 with relative intensities of 100:83:37:33:33 for DMDTP and responses at m/e 110, 109, 156, 79 and 126 with relative intensities of 100:69:56:51:24 for the P=O analog of DMDTP. These results differ from those of Wolfe;[16,17] in addition to DMDTP, he observed MCA as a minor product at 27 °C and pH 8.00, and did not observe the o,o-dimethylphosphorothioic acid mentioned. That we did not observe MCA as a hydrolysis product is probably due to the much greater initial concentrations of Malathion used by Wolfe's group.

Analysis of aqueous Malathion solutions at pH 11.46 gives evidence of MCA, DCA DMDTP and the previous P=O analog of DMDTP (see peak at RT 4.62 min). Figure 4 shows a chromatogram of the derivatized extract. Except for the P=O analog of DMDTP these results are consistent with those of Wolfe.[15,16]

Golden shiners (*N. Chrysoleucas*) were exposed for 4 days to water containing 137 ppb Malathion at pH 7.02 and a temperature of 23 °C. Analysis of liver tissue after 96 hours was positive for Malathion. Analysis of liver for metabolites was negative. Analysis of gut tissue gave positive evidence of MCA. Figure 5 shows a chromatogram of the derivatized gut extract of an exposed shiner *vs* a control. The peak at 10.92 min is associated with the methyl-MCA. Table 3 shows the results of these analyses. The three gut samples from fish exposed to Malathion yield an average MCA level of $7.11 \times 10^2 \pm 0.66 \times 10^2$ ng/gram. Analysis of aquaria water

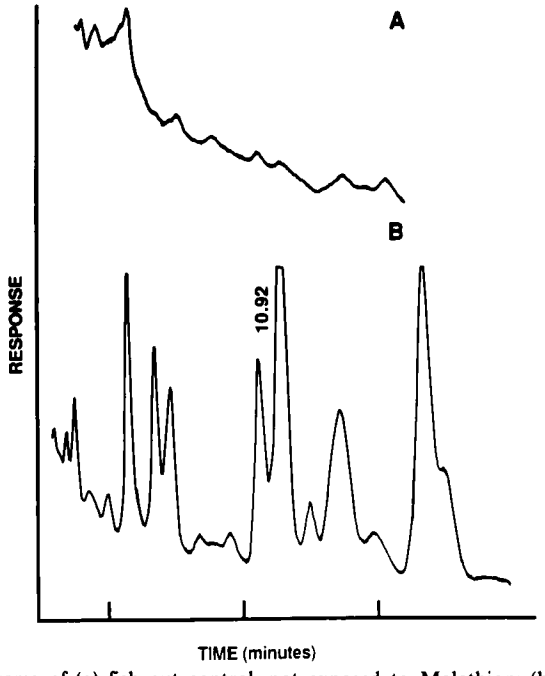

Figure 5 Chromatograms of (a) fish gut control, not exposed to Malathion; (b) fish gut exposed to 137 ppb Malathion.

Table 3 Analysis of *N. Chrysoleucas* (golden shiners) upon exposure to Malathion

Sample	*Tissue mass (mg)*	*Metabolite ng metabolite/g tissue*
Liver controls		
1	50.5	ND[a]
2	111.8	ND
Exposed livers		
D1	96.7	ND
D2	111.9	ND
D3	108.0	ND
Gut controls		
G1	176.4	ND
G2	38.3	ND
Exposed gut		
GD1	158.2	MCA (7.33×10^2)
GD2	150.8	MCA (6.36×10^2)
GD3	174.2	MCA (7.63×10^2)

[a]ND means not detected.

containing 137 ppb Malathion at pH 7.02 at 24, 48, 72 and 96 hours was negative for MCA and DCA. Since we were unable to detect MCA as a hydrolysis product in the aquaria up to time of sacrifice we conclude that MCA is of metabolic origin and not simply ingested from the water.

Acknowledgement

This material is based on work supported by USAFOSR RIP grant no. F 49620-85-C-0013/SB 8551-0360.

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