Original Article

Fate of Fenitrothion in Eel Gills

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Eels of species *Anguilla anguilla* were exposed to sublethal concentrations of fenitrothion in a flow-through test system. Bioaccumulation and elimination of the toxicant in the branchial tissue of the eels were studied. Steady-state was reached after 48 hr of exposure to 0.02 ppm of toxicant, and earlier (24 hr) exposed to 0.04 ppm. Steady-state was maintained until the end of the exposure in both experiments. The highest bioconcentration factor (BCF) value was calculated in animals exposed to 0.04 ppm of toxicant, showing the relationship between the bioconcentration into gills and the water disponibility. When the animals were transferred to clean water (depuration phase) they showed a rapid tendency to eliminate the pesticide. Pharmacokinetics parameters for fenitrothion in eel gills (K_1 , K_2 and $T_{1/2}$) were calculated. They showed relatively low K_2 (0.0014 and 0.029 hr⁻¹) and higher half-lives. Paralelly, total protein content in the gills was monitored during exposure and depuration phases. A depletion of total proteins of a 70% was observed in gill tissue at the end of the pesticide exposure. Although a tendency to recover protein levels was observed during the depuration phases, the depletion was maintained until the end of the experiments. The results showed a stress situation as a consequence of fenitrothion exposure which indicates the use of proteins as response to the increased energy requirements in this tissue.

INTRODUCTION

It is often assumed that bioconcentration of organophosphate insecticides (OP) poses no risk for the ecosystem. Exposures, if they occur, would almost invariably be sublethal concentrations of pesticide. However, a number of physiological and behavioral responses can have a direct bearing on the survival and/or reproductive potential of individuals of a species. The significance of these effects is the latitude with which any level of exposure can be interpreted.¹⁾ Any of these effects can influence the ability of species to adapt and respond to other environmental stresses and community interactions. An important part of predicting accumulation is understanding and quantifying the exchange of organic chemicals across fish gills. Gill epithelium is the dominant site of gas exchange, ionic-regulation, acid-base balance, and nitrogenous waste excretion for fishes, thereby serving a multitude of vital functions for these aquatic animals. It represents the major portion of the body surface area of fish and also presents only at 5 to 10 μ m barrier between water and blood²⁾ consequently most of the chemical transferred between the fish and the environment occurs across the gills. The possibility exists that agricultural use of a pesticide or animal health

product could result in introduction of the agent to nearby bodies of water, leading to exposure of aquatic organisms. Numerous chemicals have been found to bioconcentrate in the aquatic organisms.³⁾ The extent to which an organic compound may accumulate in aquatic organisms such as fish is often predicted to assess the potential of a compound to be present in the associated food chain.

Fenitrothion is employed mainly for elimination of rice stem bores and due to its low persistence in aquatic environment.⁴⁾ Sublethal levels of fenitrothion (0.02 μ l/l) have been found in water-ways following spray operations.^{4,5)} Since normal organic constituents acts as the key substrates for intermediary metabolism, a study of these compounds is essential to understand the changes brought about by the treatment with pesticides. Lipid and protein metabolism, tissue glycogen and glucose and lactate blood levels are usually altered after exposure to a pesticide and can be used as good indicators of low pollutant levels in the aquatic environment.

To assess the bioconcentration of fenitrothion by a fish, a dynamic study was undertaken using the european eel (*Anguilla anguilla*). Specifically, the uptake and depuration of fenitrothion by gill eel tissue were measured to determine the corresponding rate constants and

the steady-state bioconcentration factor (BCF), total proteins in gill tissue were also estimated.

MATERIALS AND METHODS

1. Test Chemical

Technical grade fenitrothion [O,O-dimethyl-O-(4-m-tolyl) phosphorothioate] (96% of purity) was purchased from AFRASA Company (Spain). Two sublethal test concentrations (0.04 and 0.02 mg/l) used for this study were based on 96-hr static LC_{50} (0.2 mg/l) values of this insecticide for *A. anguilla*.⁶⁾

2. Test Animals

Individuals of *A. anguilla* (flesh weight, 20–30 g; length, 16–20 cm) were collected from Albufera Lake (Valencia, Spain). They were acclimatized to laboratory conditions for one week in 300 l glass tanks before the start of the experiments. The tanks were supplied with a continuous flow of tap water (temperature: $20 \pm 2^\circ\text{C}$; total hardness: 240 ± 10 ppm as CaCO_3 according to the Merck classification, Aquamerck® 8039; pH: 7.9 ± 0.2 using a Crison pH meter®; alkalinity: 4.1 ± 0.5 mmol/l, Aquamerck® 11109, Germany). A 12-hr photoperiod (light on from 8.0 to 20.0 hr) was maintained. Eels did not respond to feeding attempts in our laboratory, but all animals were healthy.⁶⁾ No mortality was observed during the acclimation period.

3. Test System

Experiments were carried out in a continuous flow-through system. In this system, fenitrothion was dissolved in acetone (17 $\mu\text{l/l}$) and the solution was supplied to a glass mixing chamber with tap water and connected to a perfusor pump (Gilson minipulse 3) that generated a constant solution flow of 1.46 ml/min diluting to the desired concentration (0.02 and 0.04 mg/l) by a constant water flow of 18 l/hr, the outlet was connected to a 300-l glass test aquarium. In this way, the aqueous test solution was renewed approximately 3.6 times a day. Forty-eight hours before starting the experiences the system was connected to reach a balance of fenitrothion contaminated water. Every 24 hr the fenitrothion mixture stock was renewed.

Eels were exposed to fenitrothion for a period of 96 hr. After 2, 8, 24, 48, 56, 72 and 96 hr four eels were removed, rinsed with tap water, anaesthetized with 100 mg/l of MS222,⁶⁾ weighted and dissected out quickly on an ice cold glass plate. Gill samples were removed quickly to analyze fenitrothion bioconcentrated. In a second experiment, eels previously exposed to fenitrothion during 96-hr were rapidly transferred to a 300-l glass aquarium with a flow of 300 ml/min of clean tap water (recovery period) in the above described conditions but without toxicant. This system was maintained for 72 hr more. At 12, 24, 48 and 72 hr of experience four eels were

removed. Gills were taken out and stored to further analyses of fenitrothion content and total protein determination.

Finally, another experiment was performed to test if the solvent (acetone) had any effect on branchial protein metabolism.

4. Fenitrothion Residues Determination

Gas chromatography analysis of the concentration used confirmed the presence of fenitrothion in the water over the entire exposure period. Water pesticide extraction was done based on the method of Zweigh and Sherma.⁷⁾ The determination of fenitrothion in the gill samples was carried out based on the method of Richardson and Seiber⁸⁾ and adapted to our samples as following: gill tissue was lyophilized and homogenized with 50% HCl (0.01 ml) and 0.5% ethanol-ethyl acetate (20 ml). This homogenate was centrifuged at 1200 rpm (5 min). After centrifugation a drop of 5% decanol in acetone was added to the supernatant. This mixture was dried on a rotatory vacuum evaporator. The dried residue was redissolved in 3 ml of a mixture 1:1 cyclohexane-ethyl acetate and ultrasonized (1 min). Then, it was filtered through a nylon filter (45 μm diameter) by using a Varian Vac-elut. This volume was redissolved in 3:1 ethyl acetate-cyclohexane (6 ml) and introduced in a Gas Permeation Chromatograph. The obtained eluate was dried and redissolved in ethyl acetate (1 ml). This final volume was injected in a Fisons Instruments Gas Chromatograph equipped with a FPD 700 photometric detector and a SSL injector type. The detector and injector temperatures were 280 and 240°C . Helium was used as gas carrier with a pressure of 145 kPa. The detection limit was $<0.001 \mu\text{g/g}$. All solvents used were pesticide residue analysis grade. Fenitrothion recovery from gill samples was $>85\%$.

5. Pharmacokinetic Parameters

A first-order one-compartment model was assumed to estimate the different pharmacokinetic parameters of fenitrothion in the branchial tissue⁹⁾. One compartment model is useful, when an apparent steady-state is reached and all residues in the tissues are assumed to belong to a common pool, where they are equally available for depuration¹⁰⁾. In a steady-state situation, the bioconcentration factor (BCF) can be calculated from the ratio between the concentration of the chemical in the fish and the concentration in the water (C_f/C_w). During the first part of the exposure period, elimination of the chemical can be neglected and the uptake rate constant (K_1) can be calculated from: $K_1 = C_f/(C_w \cdot t)$, where t is the time. When the fish are transferred to clean water, the elimination rate constant (K_2) can be derived from the slope of the first-order elimination equation¹¹⁾: $\ln C_f(t) = \ln C_f(0) - K_2 \cdot t$, where $C_f(0)$ is the concentration of the chemi-

cal in the fish at the beginning of the elimination period. The half-life of fenitrothion in the gill tissue was estimated according to the equation: $T_{1/2} = -\ln 0.5 / K_2$.

6. Total-Protein Determination

Total proteins in gill tissue of animals exposed to fenitrothion were estimated by the method of Lowry *et al.*¹¹⁾ using the kit Protein assay Sigma Diagnostics®n°P 5656. Tissue homogenates were prepared in 0.1 M phosphate buffer, pH 7.2 at 0°C, and centrifuged at 3500 rpm (7 min). To the supernatant an equal volume of trichloroacetic acid was added to precipitate the soluble proteins and centrifuged at 3500 rpm (10 min). The supernatant was discharged and the pellet was dissolved in Lowry reagent solution (1 ml). The tubes were kept at room temperature for 20 min. Then Folin-phenol reagent was added (0.5 ml), and the color after 30 min was read at 750 nm in a spectrophotometer against a reagent blank. Bovine serum albumin was used to construct the standard curve.

7. Statistical Analysis

Gills were analyzed individually, this gave a sample size of six gills for each exposure time. Mean values and standard deviations were calculated for each test group based on the values obtained for each individual gill tissue from six fishes. These values were compared by analysis of variance (ANOVA) and Duncan's multiple range test. All statistical analyses were performed on an IBM computer using SPSS+ Programme. The significance level was set at 0.05.

RESULTS AND DISCUSSION

As we can see in Table 1 the bioaccumulation of fenitrothion in eel gills was apparent after exposure to both pesticide concentrations. The results showed a linear correlation ($r=0.8$) between the exposure concentration and the bioconcentrated fenitrothion in gills of the european eel until the steady-state was reached. Bioaccumulation was higher in those animals exposed to the highest pesticide concentration. The rapid fenitrothion accumulation observed was in agreement with those works reported by several authors in other fish species.^{9,12-13,15)}

An uptake rate constant (K_1) of $0.037 \text{ ml g}^{-1} \text{ hr}^{-1}$ was calculated from the data set obtained in the experiment

with 0.02 ppm of fenitrothion. This value was bigger when the animals were exposed to 0.04 ppm ($0.058 \text{ ml g}^{-1} \text{ hr}^{-1}$) and this would be related with the less time required to reach the steady-state (Table 2). That fact denotes, the fate that OP insecticides bioaccumulate in fish tissues depending on their properties but also on the type of organism or tissue, physiological condition and environmental factors as for example water concentration. BCF and internal bioconcentration factor (IntBCF) calculated in this study were quite similar from both experiments (Table 2). This could mean that a balance was reached between the fenitrothion concentration in the blood and in the water during the uptake phase. The highest K_1 during exposure to 0.04 ppm induced an earliest steady-state. So, after 24 hr a plateau was reached and thereafter it was maintained during the rest of the exposure period (Fig. 1). That fact, may lead to high accumulation rates in organs highly irrigated as brain or liver. Similar values of BCF and IntBCF were observed in other fish species as *Cyprinus carpio* exposed to different compounds.¹⁵⁾

The constant sublethal concentration of fenitrothion in the surrounding water during 96 hr appears to be physiologically stressful to the european eel. The most obvious sign of fenitrothion intoxication was restlessness, erratic swimming, convulsions and lost of balance, some

Table 1 Fenitrothion content ($\mu\text{g/g}$) in gills of the eel (*Anguilla anguilla*) during the exposure to fenitrothion in a flow-through test system and after the recovery period.

Time (hr)	0.02 ppm	0.04 ppm
Exposure period		
2	0.006 ± 0.000	0.470 ± 0.110
8	0.009 ± 0.006	0.530 ± 0.010
24	0.017 ± 0.007	1.705 ± 0.105^a
48	0.033 ± 0.015^a	1.305 ± 0.725
56	0.027 ± 0.012	1.055 ± 0.065
72	0.023 ± 0.006	1.763 ± 0.429
96	0.033 ± 0.006	1.445 ± 0.828
Recovery period		
104	0.057 ± 0.006	1.740 ± 0.430
108	0.030 ± 0.010	0.855 ± 0.050
120	0.025 ± 0.005	1.365 ± 0.475
144	0.023 ± 0.002	0.485 ± 0.125
168	0.030 ± 0.011	0.175 ± 0.035

^{a)} Bold = steady-state.

Table 2 Fenitrothion pharmacokinetics in the gills of the european eel (*Anguilla anguilla*).

	K_1 ($\text{ml g}^{-1} \text{ hr}^{-1}$)	Steady-state (hr)	BCF ^{a)}	IntBCF	K_2 (hr^{-1})	$T_{1/2}$ (hr)
0.02 ppm	0.037	48-96	1.65	0.524	0.0014	495
0.04 ppm	0.058	24-96	36.36	34.77	0.029	23.90

^{a)} Bioconcentration factor.

Fig. 1 Fenitrothion content ($\mu\text{g/g}$) and total protein levels (%) compared to controls in the gills of the european eel during a 96 hr exposure to 0.02 ppm of fenitrothion in a flow-through system and after transferred to clean water.

Table 3 Total protein content (mg/g) in gills of the eel (*Anguilla anguilla*) during the experiments.

Time (hr)	Acetone ^{b)} 17 $\mu\text{l/l}$	Fenitrothion	
		0.02 ppm ^{b)}	0.04 ppm
Contaminated water			
0	12.78 $\pm$ 1.96	17.29 $\pm$ 5.10	37.12 $\pm$ 9.26
2	10.75 $\pm$ 3.19	16.62 $\pm$ 4.34	26.59 $\pm$ 5.53
8	14.41 $\pm$ 4.17	12.47 $\pm$ 4.90 ^{a)}	18.00 $\pm$ 7.39 ^{a)}
24	17.50 $\pm$ 4.43	9.37 $\pm$ 3.74 ^{a)}	12.74 $\pm$ 4.22 ^{a)}
48	15.16 $\pm$ 4.01	7.19 $\pm$ 1.41 ^{a)}	12.44 $\pm$ 1.93 ^{a)}
56	14.85 $\pm$ 1.44	7.98 $\pm$ 2.95 ^{a)}	10.28 $\pm$ 1.25 ^{a)}
72	16.74 $\pm$ 5.08	6.64 $\pm$ 2.09 ^{a)}	10.27 $\pm$ 3.39 ^{a)}
96	14.40 $\pm$ 5.16	5.76 $\pm$ 1.96 ^{a)}	10.13 $\pm$ 2.98 ^{a)}
Clean water			
104	10.51 $\pm$ 1.51	8.25 $\pm$ 3.00 ^{a)}	6.42 $\pm$ 3.07 ^{a)}
108	12.87 $\pm$ 4.19	8.20 $\pm$ 0.21 ^{a)}	10.76 $\pm$ 2.41 ^{a)}
120	13.90 $\pm$ 2.76	10.35 $\pm$ 3.64 ^{a)}	12.15 $\pm$ 3.93 ^{a)}
144	15.83 $\pm$ 4.12	11.04 $\pm$ 4.48 ^{a)}	11.18 $\pm$ 3.89 ^{a)}
168	13.93 $\pm$ 4.87	12.85 $\pm$ 5.12 ^{a)}	16.24 $\pm$ 4.02 ^{a)}

^{a)} Significant differences ($p < 0.05$). ^{b)} From Sancho *et al.* 1997.

of the experimental fishes showed diminished motor and sensory activity but all of them survived the stipulated exposure period. These symptoms had been reported in the european eel under sublethal exposure to other organophosphate insecticides.^{16–18)}

Total protein content on branchial tissue of the eels exposed to the solvent (acetone) did not show any significant difference ($p > 0.05$) from control animals as was previously reported.²⁰⁾ However, fish exposed to fenitrothion showed a significant ($p < 0.05$) depletion in total proteins. This depletion in protein content was observed throughout the exposure period (Table 3, Fig. 2). A maximum depletion was observed after 8 hr of exposure and was constantly maintained until the end of

the exposure. These findings were related with the duration of the steady-state for fenitrothion into branchial tissue of the eels (Tables 1 and 2). We found a good linear correlation ($r = 0.9$) between fenitrothion bioconcentration and protein depletion in eel gill tissue during the uptake period in animals exposed to 0.04 ppm and this correlation was maintained until the 96 hr exposure. A 70% of reduction was observed in total proteins of the gills after 96 hr. Total protein depletion was also observed in gill tissue during the exposure of the animals to 0.02 ppm of fenitrothion as was reported in a previous work.¹⁹⁾ Comparing pesticide bioconcentration during the exposure to 0.02 ppm with the results obtained in this work, a good correlation between both also occurs ($r =$

Fig. 2 Fenitrothion content ($\mu\text{g/g}$) and total protein levels (%) compared to controls in the gills of the european eel during a 96 hr exposure to 0.04 ppm of fenitrothion in a flow-through system and after transferred to clean water.

0.9). So we can assume the same behaviour for fenitrothion during the exposure period to both fenitrothion concentrations. A similar decrease in total proteins in branchial tissue was reported previously in other fish species exposed to several pesticides.^{20,21)}

A reduction in the total protein content indicates that tissue proteins may undergoes proteolysis which results in the production of free amino acids used in the TCA cycle for energy production during stress conditions.²¹⁾ But also for tissue regeneration and compensatory production of enzymes lost as a result of cellular damage or enzyme induction in an effort to detoxify the pesticide.²²⁾ In fact, proteins are the major energy resources in fish.²³⁾ It is known the capacity that the european eel has to mobilize and metabolize proteins for glucose resource under starvation situations before use glycogen stores.²⁴⁾ Fish with a trophic/migratori live-cycle have strong tendency to protect stored glycogen in liver and muscle until a physiological metamorphose appears from a steady to another.²⁵⁾

A rapid tendency to the elimination of the toxicant accumulated was observed when the animals were transferred to clean water. A passive elimination may be done across the gills by the same partitioning process involved in the uptake, owing to their physical characteristics.¹⁰⁾ Elimination of bioconcentrated fenitrothion from branchial tissue could be explained by a one-compartment first-order kinetic process (Table 2) with a K_2 of 0.029 hr^{-1} confirming its rapid tendency to elimination. That would be related with a low half-life in the gill tissue (23.90 hr) during the experiment with 0.04 ppm of toxicant. Although this toxicant was detected during the entire depuration phase, a reduction of 88% was reached at the end of the test confirming the

ability of the parent compound to cross branchial barrier in both directions water/blood and indicating that the elimination would be also limited. That could explain the lowest value of K_2 and the highest half-life of fenitrothion in gills during the experiment with 0.02 ppm (Table 2).

Presence of fenitrothion in gills of the eel during the recovery period would indicate a branchial elimination of the toxicant from the organs via blood. That in fact provides availability for metabolism or excretion of fenitrothion coming from organs with a high pesticide "affinity" as brain, as well as highly irrigated.²⁶⁾ This tendency to eliminate fenitrothion via gills could be related directly with a protection of the animals against many transformation products. Fenitrothion metabolites, as fenitro-oxon, are known as even more toxic than the parent compound.²⁷⁾ Our results are in agreement with those found by Dauble and Curtis²⁸⁾ who found a rapid branchial elimination of dietary quinoline by the fish *Salmo gairdneri*, showing the branchial excretion as primary route of excretion because all of the other possible routes of elimination contributed to <5% of the total eliminated dose.

As indicated by Nimmo²⁹⁾ various organs apart liver can be involved in elimination process as gills, kidneys and integument. Although some fish species appears to eliminate pesticides through biliary excretion while others eliminate those compounds primarily through branchial excretion. Differences in detoxification systems occurs among species, the physico-chemical properties of the insecticide should act allowing and supplying some detoxification routes.³⁰⁾

Other fish species show a high tendency to eliminate fenitrothion.³¹⁾ These authors ajusted the elimination of

the bioconcentrated fenitrothion in the fish *Poecilia reticulata* to a first-order, one-compartment model; in that case a K_2 of 0.047 hr^{-1} was calculated for this insecticide in the full organism, indicating that a rapid elimination of fenitrothion had been done. Moreover, these authors suggested that *in vivo* biotransformation occurred at a rate that was relatively slower than the elimination rate of the parent compound. Elimination of fenitrothion from fish tissues also seems to be not influenced by the presence in a mixture with other organophosphates.¹³⁾ On the other hand, as indicated Dauble and Curtis²⁸⁾ rapid excretion of a xenobiotic by fish can occur across the gills because this organ has a high surface area, receives the entire cardiac output, and is ventilated by large volumes of water. Other teleost excretory routes are more limited in their ability to rapidly excrete xenobiotics because they are dependent upon active processes.

During recovery period a tendency to recuperate total protein levels in branchial tissue was observed (Table 2, Fig. 2). At the end of this period, recovery of protein content was upon 50%. These data are in agreement with those found in the eel exposed to 0.02 ppm.¹⁹⁾ In that work, authors reported metabolic disorders during the exposure to the toxicant however they did not persist when the fish were transferred to clean water. Those findings are very important in evaluations of the ecotoxic potential of pesticides in the aquatic environment and of possible implications for fish populations. Data obtained from fenitrothion bioconcentration in the European eel bring information about real situation in the important branchial tissue. However, in both cases, the authors are unable to correlate these findings due to a lack of information in the literature regarding the recovery aspect. Although morphological and structural damage were not estimated in the present study, it is known the ability of OP as other pesticides to cause lesions in the gills of exposed fish, including hypertrophy, hyperplasia, edema and necrosis³²⁾; a study of the gill ATPase activity, parallelly to the present work, showed how fenitrothion induced a significant inhibition of both Mg^{2+} and Na^+K^+ ATPase activities in branchial tissue and a tendency to recover the normal function was observed when a recovery period was allowed³³⁾.

In summary, fenitrothion shows to be rapidly pass branchial barrier and a high tendency to bioconcentrate in it but, in the same way, eels showed a high capacity to eliminate this toxicant across gills. The constant depuration of parent fenitrothion from gills could suggest a low biotransformation rate. Gill bioconcentration produced an eel stress situation and then, a protein depletion parallelly to fenitrothion uptake. When a steady-state was reached for the toxicant a tendency to stop depletion also was observed. During the entire recovery period metabolism tendency was to recuperate

total protein levels for regeneration and repair of damaged tissue, and restored energy and normal function in gills. That would allow survival of the animals in the nature if concentrations of fenitrothion rest in the water after spray operation.

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要 約

ウナギのエラにおけるフェニトロチオンの消長

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ウナギ (*Anguilla anguilla*) を致死濃度以下のフェニトロチオン (以下薬物) を溶解した流水試験系に放ち, 生物濃縮および排泄を調べた。0.02 ppm 区で 48 hr 後, 0.04 ppm 区で 24 hr 後にそれぞれ安定状態になった。生物濃縮係数は 0.04 ppm 区で大きかった。ウナギを清浄水に移すと速やかに薬物を排泄した。ウナギのエラにおける薬物動態パラメーター, K_1 , K_2 , $T_{1/2}$ を算出したところ, 0.02 ppm, 0.04 mmp 区でそれぞれ K_1 : 0.037, 0.058 ml g⁻¹ hr⁻¹; K_2 : 0.0014, 0.029 hr⁻¹; $T_{1/2}$: 495, 23.9 hr であった。並行して総タンパク濃度を調べたところ, 0.04 ppm 区で 9.6 hr 後に 70%減少していた。タンパク濃度の回復傾向は清浄水に移された後みられたが, その程度は大きくなかった。これらの結果から, 薬物に接触するというストレス状態がウナギのエラにおけるエネルギー要求に応じてタンパクの利用を必要とすることが示唆された。