The Toxicity of MCPA to Fish. Light and Electron Microscopy and the Chemical Analysis of the Tissue

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INTRODUCTION

The use of MCPA (4-chloro-2-phenoxyacetic acid) has increased extensively in Finland during the last few years. The raise from 1974 to 1975 was 39% (TIITTANEN and BLONQVIST 1976). If assumed that only 1% of the herbicides used in Finland (mainly phenoxyacetic acid derivatives) get to the water ecosystem the annual load is approximately of the order of 10 tons (PAASIVIRTA et al. 1976). The toxicity of MCPA to fish has been earlier studied by BANDT (1957), BODENSTEIN and MÜLLER-BAGSTEN (1960) and PRAVDA (1973). According to BANDT (1957) the LD_{50}-value for the Northern pike was 200 ppm in 3-4 days whereas PRAVDA reported that 100 ppm caused the death of the same species in 20 hours. According to BODENSTEIN and MÜLLER-BAGSTEN the LD_{50}-value for guppy was between 30 and 40 ppm.

In our study two species were used for the experiments e.g. sea trout (Salmo trutta) both for chemical and histological analyses and goldfish (Carassius auratus) for histological analyses only. The LD_{50}-value was determined in pilot experiments after which the experiment for the subchronic toxicity study was started. The experiment lasted 30 days and the chemical analyses and histopathological analyses by light and electron microscopy were carried out thereafter.

MATERIALS AND METHODS

Fish

The trout used were obtained from a central fish hatchery of Middle-Finland. The average weight of the fish was 3.5 g. The fish (20 in each group) were kept in 20 l glass aquaria and the aeration occurred by pressure air. The temperature 10±1°C was maintained by circulating cold water in a cement bucket in which the aquaria were set. The goldfish, average weight 4.5 g, were maintained in 20 l glass aquaria at room temperature 22-24°C and the aeration occurred by pressure air. The fish food used was Biorell (Biorell Heimtiercost, Bremen) which did not contain MCPA.

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MCPA

The chemical used in most of the experiments was the commercial product of the Na-salt of MCPA (Kemira Co., Finland) containing 88% MCPA of which one batch was analyzed for 4-chlorocresol and the technical product contained approximately 4% cresol as byproduct. MCPA Na-salt (Kemisk Værk, Køge, Denmark), which contained 85.2% MCPA, was used in one experiment (cresol contents $<0.5\%$). The stock solutions were made in distilled water (1 mg/ml) and the concentrations of the technical product applied in the subchronic experiments were 10, 30 and 100 ppm for the trout and 40 and 60 ppm for the goldfish. In one experiment MCPA (85.2%) was applied 100 ppm for the trout.

Experimental

The fish were placed in the aquaria which were covered by glass plates to evade the loss of MCPA and only a small space was left free for the aeration. The solutions were renewed daily to keep the concentration of MCPA even. Firstly the LD_{50}-value was determined by the method of HORN (1956). The fish were observed for 24 hours. Secondly a subchronic experiment was carried out and the fish from the concentrations of 10 and 30 ppm were killed after 28 days and the goldfish from 40 and 60 ppm after 42 days. The trout which were kept in the commercial product (100 ppm) died in 5 days whereas the trout which were kept in 100 ppm of the 85% purity MCPA were killed after 10 days.

The chemical analysis was carried out from all groups of trouts and 10 fish from each group were wrapped in aluminium foil and frozen in $-25^{\circ}C$ until analyzed. The histopathological samples were taken from the fish which had been kept in concentrations 10-60 ppm. Of the trout the samples were taken from the whole fish, gills, kidney and liver. Only liver was applied for electron microscopy. Of the goldfish only the whole body samples were made.

Preparing the histopathological samples

The trout were fixed in buffered formalin and the paraffin sections were stained with haematoxylin-eosin for the light microscopy. For the electron microscopy small spieces of liver tissue were fixed in buffered 4% formaldehyde and 1% glutaraldehyde according to McDOWELL and TRUMP (1976) for 3-4 months. After fixation they were postfixed in 1% osmium-tetroxide in s-collidine buffer for one hour, stained with uranyl-acetate, dehydrated and embedded in epon 812. Semi-thin sections were cut with glass knives and stained with toluidine-blue for light microscopy. Selected areas were cut with glass knives on a Sorvall Porter-Blum MT 1 ultramicrotome and ultra-thin sections were stained with lead citrate (REYNOLDS 1963) and examined with a JEM 100-U electron microscope. Of the goldfish only the light microscopy was carried out. The specimens were fixed in Bouin's fluid and the paraffin sections were stained haematoxylin-eosin.

Chemical analysis

The fish were weighed and grounded in a mortar with anhydrous Na_2SO_4 (Merck, p.a.) and kept at room temperature for 24 hours. The dry sample was extracted 3 x 0.5 h with 50 ml chloroform-diethylether 1:1 (v/v) made acidic with dry HCl (pH < 3) in a 100 ml bottle equipped with a glass stopcock in a shaker. The extracts were evaporated in a Büchi-evaporator and the residue was transferred quantitatively by ether in a 10 ml volumetric flask of which 1 ml was taken into glass tube with a sharp end and evaporated carefully with nitrogen (99.999% purity).

Clean up

Because unexpected difficulties were encountered in separating MCPA from the fish fat a new TLC-method was developed by which both MCPA and cresols can be separated quantitatively by one process.

The thin-layer plates (24 x 24 cm) were made of Kieselgel G nach Stahl and the layer thickness was 1 mm. The plates were dried in a draft oven in 120°C for 24 hours until used.

The cooled plate was then devided by a pencil in five sections by upright lines and the material analyzed was applied at the distance of 1 cm from the lower end in the middle of the section.

The evaporated residue was applied in 100 µl ether on the plate and the tube was carefully rinsed with ether. On the last section only the standard solutions were applied (10 µl solution which contained two standards 1 mg/ml). The plate was developed in dichloromethane (Merck, p.a., redistilled) and dried at room temperature. The plate was then covered with aluminium foil, the standard section was left uncovered and the plate was sprayed with 2% 2,6-dichlorochinonchlorimid (Merck, zur Analyse) in benzene to detect cresols. MCPA could be made visible only by spraying the plate with 0.2% bromophenol (Merck, zur Analyse) in water.

The Rf-value for cresol in this method was 0.45 and 0 for MCPA which stayed at the applying point. Part of fat and fat soluble compounds moved to the upper end of the plate.

After removing the foil the fractions were marked according to visible standard spots and the fractions were scraped with razor blade in a glass stoppered centrifuge tube (MCPA) or into a glass column (10 x 1 cm) with a pipette end and stoppered acid washed glass wool (cresols).

MCPA was extracted with 2 x 1.5 ml acidic chloroform-ether (pH 3) for 5 min ultrasonically, and the combined extracts were evaporated in a glass tube with a sharp end with nitrogen. The residue was dissolved in 0.5 ml ether and treated with fresh diazomethane for 15 minutes after which the solvent was evaporated carefully with nitrogen and dissolved in 1 ml redistilled n-hexane (May & Baker) which was applied with Carlsberg-pipette. The recovery for MCPA in this method was 82.8% (s_d 25.7). The cresol-fraction was eluted with diethylether into a 1 ml volumetric tube. The recovery of 4-chloro-o-cresol was 83.7% (s_d 13.4).

Two different concentrations (10 and 20 µg/ml of both standards were chromatographed daily by the TLC-method. After the clean-up process there remained some fatty acids which, however, did not disturb the quantitative analysis by GLC. When chloroform-ether 1:1 (v/v) was used instead of dichloromethane in developing the plate the fats were better removed but for some unknown reason the recovery of MCPA did not exceed 50%.

Standards

The standards used were MCPA 99.99% purity (Kemisk Værk, Denmark) and 4-chloro-o-cresol which was purified from the commercial product of Fluka to 100% purity which was checked by IR-, NMR- and mass-spectrometry.

Quantitative analysis

The final analysis was carried out by glass capillary GLC. The gas chromatograph used was Carlo Erba Model Fractovap 2300 equipped with FID-detector and a Grob-type split-less injection system. The column used was 15 m glass capillary (ϕ 0.35 mm) of FFAP. The residues were applied at 60°C and chromatographed to 200°C (program 30°C/min). The quantitative determination was made by calculating the peak heights.

RESULTS

The results of the chemical study

The concentrations of MCPA and 4-chloro-o-cresol mg/kg in wet tissue after different exposures of MCPA are presented in Table 1.

TABLE I

MCPA and 4-chloro-o-cresol and the standard deviations in the trout tissue (mg/kg).

	Concentration in water ppm commercial product of MCPA			purum grade MCPA
	10	30	100[x]	100
Tissue conc. mg/kg				
MCPA	3.08	4.06	4.11	3.96
s_d	0.36	0.31	0.48	0.69
4-o-cresol	0.91	1.67	3.98	0.81
s_d	0.14	0.25	0.62	0.14

[x] analyses of dead fish

The results show that the tissue concentrations of MCPA were of the same order in spite of the dose of application. The trout to which the technical product was applied contained 4-o-cresol a considerable amount, approximately 4 ppm after five days, wheras the fish in the solution containing purum-grade MCPA contained only 0.8 ppm cresol.

The results of the histopathological study

The light microscopic observations showed that in the two exposure levels 10 and 30 ppm MCPA no changes were observed in any of the tissues studied. The whole body samples of the goldfish after 40 and 60 ppm exposure did not differ from those of the controls.

In the electron microscopic studies no obvious changes were observed in the livers of the experimental fish when they were compared to controls.

DISCUSSION

The LD_{50}-value for the trout in this experiment 147 ppm is between the values obtained by PRAVDA (1973) and BANDT (1957) for the Northern pike.

The results showed that the tissue concentration of MCPA was of the same order in spite of the different concentrations in water. The tissue concentrations may be regarded low which indicates that MCPA is not heavily concentrated in fish tissue. The histopathological studies showed no changes in fish tissue at the exposure levels of 10-60 ppm MCPA in water. However, the fish which lived only 3-5 days in 100 ppm of the commercial mixture lived over 10 days in the solution of the pure grade compound. The tissue concentration of 4-o-cresol was five times higher in the fish obtained from the commercial product and gives the indication that this impurity and possible other impurities, too, cause the earlier death because all other experimental conditions were similar. The preliminary investigations carried out in our laboratory concerning the toxicity of 4-o-cresol to fish give support to the fact that cresols are far more toxic to trout than pure MCPA. MCPA is water soluble whereas 4-o-cresol is fat soluble which might explain the rapid enrichment of the compound into the tissues.

Because the concentrations used in the experiment hardly are met in the environment and no changes in tissues were observed MCPA as pure compound can be regarded relative safe to fish.

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