Original Article

Investigation on the Contamination of Freshwater Fish with Herbicides (CNP, Chlomethoxynil, Benthioncarb and Molinate)

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Investigations were made on whether or not freshwater fishes in the Sagami River were contaminated by the herbicides CNP (chloronitrofen), chlomethoxynil, molinate and benthioncarb (thiobencarb). Application of these herbicides to paddy fields is restricted during the rice planting season, so this investigation was carried out in July and the contamination levels of other months were compared. CNP was detected in all four species of fishes (pale chub, pike gudgeon, carp and crucian carp), and benthioncarb was detected in two species in July, 1980. The residual levels of CNP were from 0.055 ppm to 0.61 ppm, and those of benthioncarb were 0.02 ppm and 0.10 ppm. These values were similar to those found in pale chub, ayu, crucian carp and large mouth bass in July, 1981 tests; the residual levels of CNP were from 0.046 ppm to 0.88 ppm and those of benthioncarb from 0.04 ppm to 0.11 ppm, respectively. But in September, 1981, the residual levels were below a detectable level or detected only in very low concentrations. The bioconcentration ratio (concentration level in fish/concentration level in water) of CNP was supposed to be 420–8,000 and that of benthioncarb 20–100, when estimated from the results of this investigation. But it was thought that the residual period in fish did not continued too long. Chlomethoxynil and molinate were not detected in any fish examined.

INTRODUCTION

It has been feared that herbicides applied to paddy fields flow out gradually with effluent water, causing contamination of rivers and organisms in the river. Nowadays in Japan, herbicides such as CNP (chloronitrofen, 2,4,6-trichlorophenyl 4'-nitrophenyl ether), chlomethoxynil (2,4-dichlorophenyl 3'-methoxy-4'-nitrophenyl ether), molinate (S-ethyl *N,N*-hexamethylenethiocarbamate) and benthioncarb (thiobencarb, S-4-chlorobenzyl *N,N*-diethylthiocarbamate) are used extensively and heavily in paddy fields^{1,2)} but their application is restricted during the rice planting season. There have been several reports on the contamination levels of certain kinds of herbicides in the aquatic environment,^{3–5)} and recently

it was reported that CNP was detected in fish and shellfish.^{6–8)} In Kanagawa Prefecture there are only about 6,000 ha of paddy fields (about 1/400 of the country's total paddy area),²⁾ and these are located mostly in the central and western parts of the prefecture.

For the surveillance of residual levels of herbicides (CNP, chlomethoxynil, molinate and benthioncarb) in fish, freshwater fish were caught in July of 1980 and 1981 in the Sagami River, which flows through central Kanagawa Prefecture. The residual levels of herbicides were measured and those levels were compared with September, 1981 levels to ascertain whether or not contamination was continuous. The level of contamination of these herbicides in river water and a comparison with the levels found in the fish were also studied.

MATERIALS AND METHODS

1. Chemicals and Reagents

CNP (purity 99%) and benthocarb (purity above 98%): Wako Pure Chemical Ind., Ltd.; chlomethoxynil (purity 99%): supplied by Ishihara Ind., Ltd.; molinate (purity above 99%): supplied by Yashima Chemical Ind., Ltd. These chemicals were used as analytical standards.

Aceton, *n*-hexane, acetonitrile and anhydrous sodium sulfate: nano-grade for pesticide residue analysis, Wako Pure Chemical Ind., Ltd. Florisil: activated for 10 hours at 130°C (Florisil Co., U.S.A.)

2. Instruments

N-P FID Gas Chromatograph (N-P FID GC): Hewlett Packard 5880A (equipped with nitrogen-phosphorus detector), column; 2% OV-101 on Uniport HP 100/120 mesh, 0.5 m × 2 mmφ conditions; Col. Temp. 180°C, Inj-Det. Temp. 230°C, carrier gas He 50 ml/min.

FPD-Gas Chromatograph (FPD-GC): Shimadzu GC-5A (equipped with S filter), column; 5% OV-101 on Gaschrom Q 80/100 mesh, 1.5 m × 3 mmφ conditions; Col. Temp. 180°C, Inj-Det. Temp. 210°C, carrier gas N₂ 60 ml/min.

ECD-Gas Chromatograph (ECD-GC): Shimadzu GC-3BE (equipped with ⁶³Ni detector), column; 5% OV-17 on Gaschrom Q 80/100 mesh, 1.7 m × 3 mmφ conditions; Col. Temp. 230°C, Inj-Det. Temp. 260°C, carrier gas N₂ 90 ml/min.

Gas Chromatograph-Mass Spectrometry (GC-MS): Shimadzu LKB-9000

Homogenizer: Biotrone BT 20S (Biotrona, Switzerland)

3. Materials

The freshwater fishes were obtained with a casting net at a point about 10 kilometers up the stream from the mouth of river on July 9 and December 9, 1980, and July 13 and September 18, 1981. Figure 1 shows the sampling point.

4. Analytical Method of Fish

Dozens of each species of fish were collected

Fig. 1 Securing point of freshwater fish.

except only a few ayu and large mouth bass were caught. The edible part of the fish (only the meat) was used for analysis. Twenty grams of the meat was homogenized with a homogenizer (Biotrone), then cleaned up by following Scheme 1, and **F1** was used as a test solution. CNP and chlomethoxynil were measured with N-P FID GC and ECD-GC; benthocarb and molinate were measured with N-P FID GC and FPD-GC.

5. Analytical Method of Water

Two liters of water was taken as a sample from the Sagami River near the point the fish were taken from late May to August and the herbicidal content was analyzed. Five grams of sodium chloride was added to 500 ml of the sample and the sample was extracted successively with 100 ml and 50 ml of *n*-hexane. Then, the *n*-hexane layer was dried with anhydrous sodium sulfate, concentrated to 5 ml, and herbicides were measured with a gas chromatograph.

RESULTS

1. Recovery Tests

Twenty grams of homogenized fish meat (crucian carp) or 500 ml of water were fortified with herbicides dissolved in acetone at levels shown in Table 1, and recovery tests were carried out by the described method. Good results were obtained as shown in Table 1.

Scheme 1 Method of analyzing herbicides in fish.

2. Residual Levels of Herbicides in Freshwater Fish

Figure 2 shows the gas chromatograms of herbicide standards and Fig. 3 shows the typical gas chromatograms of refined test solution (**F1**) obtained with N-P FID GC, FPD-GC and ECD-GC.

N-P FID GC has a specific selectivity for nitrogenous and phosphorus compound, and FPD-GC (equipped with S filter) has a similar specific selectivity for sulfur compound; clear chromatograms could thus be obtained because they accepted little interference by other organic substances. ECD-GC has a higher sensitivity for the measurement of CNP

and chlomethoxynil compared to N-P FID GC, but its selectivity is not as good as N-P FID GC.

Table 2 shows typical sizes of samples used and residual levels of herbicides in fish. CNP was detected in all four species, carp (*Cyprinus carpio*), crucian carp (*Carassius gibelio*), pale chub (*Zacco platypus*) and pike gudgeon (*Pseudogobio esocinus*), secured on July 9, 1980 and their residual levels were from 0.055 ppm to 0.61 ppm. But CNP was not detected in crucian carp or pale chub which were caught on December 9, 1980. On the other hand, benthocarb was only detected in two kinds of fishes, crucian carp and pale chub, secured in

Table 1 Recovery rate of herbicides from water and fish.

	Water 500 ml		Fish 20 g	
	Fortified level (μ g)	Recovery rate (%)	Fortified level (μ g)	Recovery rate (%)
CNP	10	97	10	94
Chlomethoxynil	10	96	10	89
Benthiocarb	10	94	10	88
Molinate	10	93	10	85

Table 2 Residual levels of herbicides in freshwater fish and typical fish sizes analyzed.

	July 9, 1980						December 9, 1980						(ppm)
	Length (cm)	Weight (g)	CNP	Benthio- carb	Chlometho- xynil	Molinate	Length (cm)	Weight (g)	CNP	Benthio- carb	Chlometho- xynil	Molinate	
Crucian carp	9	25	0.22	0.02	nd*	—	13	37	nd**	nd***	nd	—	
Pale chub	13	23	0.61	0.10	nd	—	10	9.5	nd	nd	nd	—	
Pike gudgeon	11	18	0.11	nd	nd	—	—	—	—	—	—	—	
Carp	20	250	0.055	nd	nd	—	—	—	—	—	—	—	
	July 13, 1981						September 18, 1981						
	Length (cm)	Weight (g)	CNP	Benthio- carb	Chlometho- xynil	Molinate	Length (cm)	Weight (g)	CNP	Benthio- carb	Chlometho- xynil	Molinate	
Crucian carp	9	22	0.046	0.04	nd	nd****	11	40	nd	nd	nd	nd	
Ayu	12	24	0.42	0.11	nd	nd	17	70	0.082	nd	nd	nd	
Pale chub	12	26	0.88	0.09	nd	nd	11	25	0.005	nd	nd	nd	
Large mouth bass	19	145	0.19	0.04	nd	nd	—	—	—	—	—	—	

* $p < 0.005$, ** $p < 0.005$, *** $p < 0.01$, **** $p < 0.01$.

Fig. 2 Gas chromatograms of herbicide standards obtained with (a) N-P FID GC, (b) FPD-GC and (c) ECD-GC.

Peak 1: molinate, 2: benthocarb, 3: CNP, 4: chlomethoxynil.

Fig. 3 Typical gas chromatograms of test solution (F1) obtained with (a) N-P FID GC, (b) FPD-GC and (c) ECD-GC.

Peak 1: benthocarb, 2: CNP.

July, 1980 and their levels were 0.02 ppm and 0.10 ppm. As in 1980, CNP and benthocarb were detected in all four species, carp, pale chub, large mouth bass (*Micropterus salmoides*) and ayu (*Plecoglossus altivelis*), secured on July 13, 1981. Their residual levels of CNP were from 0.046 ppm to 0.88 ppm and those of benthocarb from 0.04 ppm to 0.11 ppm. In September, 1981, however, their residual levels were below a detectable level or detected only at very low concentration. Chlomethoxynil and molinate were not detected in any samples examined. Accurate determination was carried out as follows. After measuring the GC-MS

spectra of CNP and benthocarb standards (Fig. 4), CNP in the extracted test solution from fish was determined by mass fragmentography of m/z 319, 238 and benthocarb by m/z 257.

3. Contamination Levels of Herbicides in Water

The herbicidal contamination levels in water of the Sagami River near the point where the fish were taken were investigated from late May to August, and Fig. 5 shows the results. CNP and benthocarb were detected in water and their detected levels from late June to early July were about 0.1 ppb and 1.4–2.3 ppb,

Fig. 4 GC-MS spectra of (a) CNP and (b) benthocarb standards and indication of mass number for measurement of mass fragmentography.

Fig. 5 Contamination levels of CNP and benthocarb in water of the Sagami River.
■: CNP, ▲: benthocarb.

respectively. Chlomethoxynil and molinate were not detected throughout the examining period.

DISCUSSION

CNP and benthocarb were detected in freshwater fish but it was thought that their residual period did not continue too long. It especially became clear that even if CNP or benthocarb existed at an extremely low concentration level in water, fishes were contaminated at a high concentration level. Generally, fish take in contamination substances dissolved in water not only directly by gill breathing, but also indirectly through contaminated feed.

It was reported that each bioconcentration

ratio (concentration level in fish/concentration level in water) of CNP and benthocarb was examined in the freshwater fish, topmouth gudgeon, in a breeding tank, and the ratio of CNP was 1,160 while that of benthocarb 170.⁹⁾ The bioconcentration ratio of CNP was supposed to be 420–8,000 and that of benthocarb to be 20–100 when estimated from the results of this investigation, simply by comparing residual levels in fish with those in water. The range of residual levels of CNP and benthocarb in fish was roughly the same during the two years of investigations. The contamination level differed in the species of fish and pale chub was contaminated with CNP at the highest concentration both years. Chlomethoxynil and molinate were not detected in water nor in fish. It was confirmed that both molinate and chlomethoxynil flow out with the effluent water when used on paddy fields.^{10,11)} Therefore it was assumed that the contamination of these substances did not come up to a detectable level because the amount of their application is small in Kanagawa Prefecture.²⁾ Degradation of CNP in soil is rapid¹²⁾ and it has a very low solubility in water, so it has been thought that the possibility of environmental contamination with this substance is very low. But judging from this investigation, the bioconcentration ratio of CNP in fish is extremely high in field samples as well as in the results of the breeding tank, so the contamination of CNP to fish and shellfish needs to be carefully surveyed. CNP has low toxicity,¹³⁾ but Miyachi *et al.*¹⁴⁾ reported that it was reduced to the amine derivative by carp and rat intestinal microorganisms, and its derivative causes mutagenesis when treated with S-9.¹⁵⁾ It is thus necessary to investigate the contamination of fish and shellfish not only with CNP, but also with its metabolites.

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要 約

除草剤 (CNP, クロメトキシニル, ベンチオカーブおよびモリネート) の淡水魚汚染調査

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国内で広範囲に多用されている水田除草剤 (CNP, クロメトキシニル, ベンチオカーブ, モリネート) の汚染実態調査を, 相模川 (神奈川県) で採取した淡水魚について実施した。これら除草剤の水田への使用は, 田植時期に限定されるため, 調査を7月に行ない, 他の時期の汚染レベルと比較した。昭和55年7月に採取した4魚種 (オイカワ, カマツカ, コイ, フナ) すべてから CNP が, また2魚種からベンチオカーブが検出された。CNP の残留レベルは 0.055 ppm から 0.61 ppm の範囲で, ベンチオカーブは 0.02 ppm および 0.10 ppm であった。昭和56年7月採取した4魚種 (オイカワ, アユ, フナ, ブラックバス) に, CNP が 0.046 ppm から 0.88 ppm, ベンチオカーブは 0.04 ppm から 0.11 ppm の範囲で検出され, 昭和56年の調査結果とほぼ同程度であった。しかし, 昭和56年9月採取した魚には, これら除草剤はいずれも不検出あるいは微量しか検出されなかった。今回の調査結果から, CNP の生物濃縮係数は 420~8,000, ベンチオカーブは 20~100 と想定された。しかし, これら除草剤の淡水魚への蓄積残留は比較的短期間であると考えられた。クロメトキシニルおよびモリネートは, いずれの検体からも検出されなかった。