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Permissible level of toxaphene residues in fish from the German market based on *in vivo* and *in vitro* effects to tumor promotion

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ABSTRACT

Toxaphene is a chlorinated pesticide consisting of more than 200 congeners that are mainly chlorobornanes and chlorocamphenes. As the congeners exhibit different stability properties in the environment, only between 20 and 30 compounds can be observed in, e.g., fish, which are represented by technical toxaphene as a mixture. In human body, the congeners Parlar #26, #40, #41, #44, #50, and #62 are detected frequently. Three of them, #26, #50, and #62, pose a potential risk to human health due to their persistent characteristic. By using experimental results of a European Union study (MATT, 2000. Investigation into the Monitoring, Analysis and Toxicity of Toxaphene in Marine Foodstuffs, European Union, Brussels, Final report, FAIR CT PL.96.3131. Investigation into the Monitoring, Analysis and Toxicity of Toxaphene in Marine Foodstuffs), a reference dose related to tumor promotion was calculated for these representative persistent toxaphene congeners. In Germany, the sum of the congeners #26, #50, and #62 is defined as the official standard for toxaphene residues in food. In this work, different fish samples obtained from German markets were studied regarding their contamination with toxaphene congeners, presented either in sum, or as single constitutes. The obtained data were used to define the acceptable total concentration of the sum of Parlar #26, #50, and #62 with regard to prevention of tumor promotion in human. The results showed that the currently existing permissible level of the sum of these congeners (0.1 mg/kg) is higher than the acceptable concentration in fish samples determined by this work and calculated at ca. 0.090 mg/kg. It is therefore recommended to improve the permissible level of toxaphene in German food samples.

1. Introduction

Technical toxaphene is a complex mixture of more than 200 polychlorinated bornanes and camphenes (Anagnostopoulos et al., 1974; ATSDR, 1997; Burhenne et al., 1993; Casida et al., 1974; Hainzl et al., 1995; Oehme and Vetter, 1999; Parlar, 2006; Parlar et al., 1997; Purdue et al., 2007; Saleh and Casida, 1977). Formerly, it has been one of the most applied pesticides in different countries (Anon, 1975; Durant and Reimold, 1972; Edwards and Adams, 1970; FAO; Schafer et al., 1969). Approximately 1.3 million tons were applied between 1950 and 1993 (Voldner and Li, 1993), and covered almost all areas of agriculture. Recommended concentrations were between 0.5 and 10 kg per hectare, depending on the types of plant culture (Guyer et al., 1971). Toxaphene is produced by UV-chlorination of technical camphene in carbon tetrachloride. The end product contains more than 67% of organic chloride by weight, conforming to the empirical formula $C_nH_{2n-2-x}Cl_x$ (Landrum et al., 1976; Matsumura et al., 1975; Turner et al., 1975). Due to the chlorination of chlorobornane precursors, numerous of isomers with different numbers of congeners exist in the technical mixture (Chiurdoglu et al., 1957; Jennings and Herschbach, 1965; Nelson and Matsumura, 1975a; Nelson and Matsumura, 1975b; Parlar et al., 1976; Parlar et al., 1977; Saleh et al., 1977; Tishchenko and Uvarov, 1953). Most of the toxaphene congeners are unstable under certain environmental conditions and can degrade to different metabolites and abiotic conversion products (Angerhöfer et al., 1999; Clark and Matsumura, 1979; Fingerling et al. 1997; Lach and Parlar, 1990; Maruya et al., 2005; Mirsatari et al., 1987; Murthy et al., 1984; Parlar et al. 1999; Parlar et al. 2001a,b; Parr and Smith, 1976; Ruppe et al., 2003; Ruppe et al., 2004; Saleh and Casida, 1978; Skopp et al., 2002a; Veith and Lee, 1971; Vetter et al., 2005). The process of reductive dechlorination leading to lower chlorinated bornanes assumes that only congeners with an alternated chlorine substitution at the 6-member ring system (isomers

Abbreviations: RfD, reference dose; PL, permissible level; CLE, cod liver extract; NOAEL; NOAEL, no observed adverse effect levels; AHF, altered hepatic foci; GSTp-AHF, placental glutathion-*S*-transferase; LOEC, lowest observed effect concentration; HRGC–MS, high resolution gas chromatography–mass spectrometry; ECNI–SIM, electron capture negative ion–select ion monitoring.

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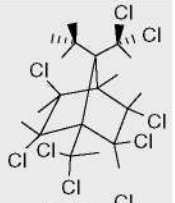
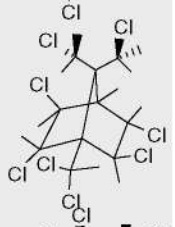
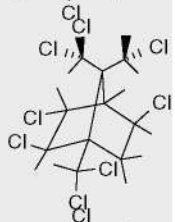
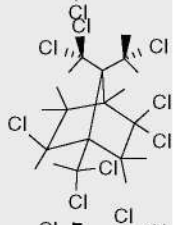
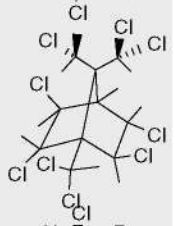
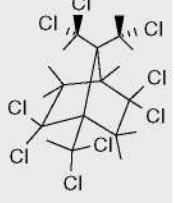
Parlar #26 and #50), or with isolated geminal chlorine groups in the 2- and 5-position (Parlar #62), are stable and can be enriched in mammals after entering the food chain (Gill et al., 1996; Parlar et al., 2001a,b).

The major toxaphene congeners persisting in fish, marine mammals, human serum and milk are therefore Parlar #26, #50, and #62, but the congeners #40, #41, and #44 can also be detected in low amounts. Table 1 shows their codes, names and structures as to IUPAC and Andrews/Vetter (Coelhan and Parlar, 1996; Ekici et al., 2005; Ekici, 2005; Hamed et al., 2005; Kallenborn et al., 1998; Kosubova et al., 2005; Oehme and Baycan-Keller, 2000; Thron et al., 2004; Vetter et al., 2006). Simon and Manning (2006) have recently reported the development of a reference dose (RfD) for the persistent congeners #26, #50, and #62, after animal *in vivo* and *in vitro* studies with relation to tumor promotion. The determined RfD values were based on no observed adverse effect levels (NOAEL) in Sprague-Dawley rats, which were administered with weathered toxaphene via weekly subcutaneous injections. In addition, weathered toxaphene was isolated from farmed cod liver extracts after fish were subjected to pellets spiked with technical toxaphene for two month. The effects observed were altered hepatic foci (AHF) expressing placental glutathion-*S*-transferase (GSTp-AHF), which is an indicator for tumor promotion.

Additional support for the tumor promotion endpoint comes from an *in vitro* study, in which the disruption of gap junctional intercellular communication in Hepa 1c1c7 mouse liver cell lines was observed upon exposure to weathered toxaphene (Investigation into the Monitoring, Analysis and Toxicity of Toxaphene in Marine Foodstuffs; MATT, 2000). Because the RfD values deriving from the MATT study were based on the toxicity of Parlar #26, #50, and #62, they can be compared with other toxicity criteria for toxaphene and weathered toxaphene. The EPA's cancer slope factor for technical toxaphene has different units than those of the MATT study. The tolerable daily intake (TDI) values for weathered toxaphene which also derived from the MATT study were based on the entire weathered toxaphene mixture, and on the percentage of the sum of Parlar #26, #50, and #62 (Besselink et al., 2000; McHugh et al., 2004).

Table 1
Toxaphene congeners according to Parlar and Andrews/Vetter code, including IUPAC nomenclature and structure

Parlar	Andrews/Vetter code	IUPAC	Structure
#26	B8-1413	2-*endo*,3-*exo*,5-*endo*,6-*exo*,8,8,10,10-Octachlorobornane	
#40	B8-1414	2-*endo*,3-*exo*,5-*endo*,6-*exo*,8,9,10,10-Octachlorobornane	
#41	B8-1945	2-*exo*,3-*endo*,5-*exo*,8,9,9,10,10-Octachlorobornane	
#44	B8-2229	2-*exo*,5,5,8,9,9,10,10-Octachlorobornane	
#50	B9-1679	2-*endo*,3-*exo*,5-*endo*,6-*exo*,8,8,9,10,10-Nonachlorobornane	
#62	B9-1025	2,2,5,5,8,9,9,10,10-Nonachlorobornane	

The aim of this study was to determine the recent concentration of toxaphene congeners (either in single form, or as a sum) in different fish samples from different origins obtained from German markets, and thereby to estimate the acceptable daily intake (ADI) of these congeners for the German population. The obtained data were used to evaluate whether the currently existing permissible level (PL) of toxaphene, represented by the sum of Parlar #26, #50, and #62, being 0.1 mg/kg, can still be regarded valid. Toxaphene congeners were analysed using high resolution gas chromatography/high resolution mass spectrometry (HRGC/HRMS), coupled with electron capture negative ionization (ECNI) in select ion monitoring (SIM) mode.

2. Materials and methods

2.1. Materials

The single toxaphene components Parlar #26, #40, #41, #44, #50, and #62 as external standards and aldrine as internal standard came from Ehrenstorfer GmbH, Analytical Standards, Germany. Organic solvents (n-hexane, cyclohexane, ethyl acetate, and toluene) were of analytical grade. Na_2SO_4 was obtained from Merck, Darmstadt, Germany, and the Bio-Beads SX3 from BioRad, Germany.

2.2. Extraction and fractionation of fish samples

Fish samples were obtained from German markets in 2005 and 2006. From every sort of fish, three samples were collected to a pool and kept under −12 °C until use. Five gram of the tissue were dissolved in 25 m1 cyclohexane/ethyl acetate (1:1). Toxaphene congeners were separated from fat by gel permeation chromatography (GPC) performed according to the DFG Method S 19, 1998 (column length: 40 cm, I.D.: 2.5 cm, with Bio-Beads SX3 as the packing material and cyclohexane/ethyl acetate (1:1) as the eluting solvent). One gram of cod liver oil or fish oil, equal to 5 ml dissolved sample, was placed on the GPC column. Toxaphene was recovered in 115 ml (125–240 m1) of the subsequent eluate. The resulting elution speed was ca. 5 ml/min. The cleanup with GPC reached recoveries of 86%.

For the elimination of interfering substances and of the rest of oil (about 5%), simple column chromatography was applied. For this, the columns were filled with l g deactivated silica gel 60 (70–230 mesh, activated at 140 °C for 24 h and then deactivated with 1.5% water), and filled with a layer (0.5 cm) of anhydrous Na_2SO_4. The toxaphene fraction was eluted with 8 ml of *n*-hexane/toluene (65:35), and thereafter with 8 ml of toluene. These two fractions were combined, reduced to 10 ml and stored at −12 °C. Prior to gas chromatography and mass spectrometry analysis, the extracts were reduced to 250 ml.

2.3. HRGC–HRMS/ECNI–SIM analysis

Chlorobornane standards and samples were analyzed using a Hewlett-Packard 5890 Series II gas chromatograph, coupled with a Finnigan 8200 high resolution mass spectrometer as detector, in the following operating conditions: DB-5 column (J&W Scientific) with 30 m length, 0.25 mm ID. and 0.25 μm film thickness, carrier gas: He (1.2 mL/min), splitless injection, injector and transfer line temperature: 280 °C, column temperature program: start with 140 °C; hold for 1 min; increase to 250 °C (4 °C/min). MS detector conditions: ion source temperature for ECNI: 100 °C, methane as reagent gas (99.98% purity), electron beam energy: 120 eV, multiplier voltage: 2100 V; reagent gas pressure: 2×10^{-4} Torr, emission current: ca. 200 μA. The congeners #40 and #41 were analyzed in sum as they cannot be separated under the described conditions.

3. Results

3.1. Quantification of toxaphene congeners

The following ions as mass-to-charge ratios were selected for quantification, as they correspond to the most abundant isotope peaks of the $[M-Cl]^-$cluster of octa- and nonachlorobornanes, respectively: 376.85727 for octachlorobornanes, 412.81534 for nonachlorobornanes (selected for quantification), as well as 378.85434 for octachlorobornanes and 410.81830 for nonachlorobornanes (additionally registered for identification). During the monitoring of selected fragments, many of the chlorinated contaminants could not interfere. Only some substances exist that may cause problems for the correct quantification, as they can form ions with masses similar to toxaphene fragments. These include the cyclodiene insecticides, such as dieldrine, *cis*- and *trans*-chlordane, heptachlor, and their photo-degradation products.

However, these substances can effectively be eliminated by capillary chromatography due to their different retention times at the applied chromatographic conditions. Furthermore, these compounds show rather small molecular ion clusters and almost no $[M-Cl]^-$-ion clusters during ECNI–MS measurements. The resulting ions differ sufficiently from those produced by the chlorobornane standards to avoid interferences or analytical mistakes. Other possibly interfering pesticides, including *p,p′*-DDT, *p,p′*-DDE, and *p,p′*-DDD, show no signals in the masses used for toxaphene quantification. Previous results have shown that a maximum reproducibility of the analysis of toxaphene standard can be achieved using a pressure of 2×10^{-4} Torr and a temperature of 100 °C in the Finnigan MS ion source (Burhenne et al., 1993).

The different ECNI–SIM response factors of chlorobornane standards, lying between 0.45 and 1.99, relative to #50 (1.0), reveal the problem being posed in the quantification of toxaphene residue by ECNI using the technical standard mixture (Alawi et al., 1994). Only the use of pure, isolated standards leads to satisfactory results upon the quantification process. The ECNI responses of the congeners are linear over four orders of magnitude. The detection limits under these conditions are between 0.3 and 7.0 pg absolute, depending on the ion-source pressure and temperature and on the substance's degree of chlorination.

3.2. Toxaphene concentration in fish samples

In Table 2, the concentration of the congeners #26, #40+41, #44, #50, and #62, and of their respective sums, in different fish pool samples available on German markets are shown. The highest concentrations of toxaphene congeners, which are between 46.5 and 107.7 μg/kg, were observed in the species halibut, herring and salmon. The determined high levels of Parlar #50 in all samples show that this congener is the most important toxaphene contaminant, followed by #26. In contrary, the congener #62

Table 2
Concentration (μg/kg) of toxaphene congeners in fresh fish pool samples obtained from German markets, determined with HRGC–HRMS/ECNI–SIM

Fish species	#26	#40+41	#44	#50	#62	∑#26/#40+41/#44/#50/#62	∑#26/#50/#62
Alaska Pollock	0.57 ± 0.01	n.d.	0.26 ± 0.01	1.72 ± 0.02	n.d.	2.6	2.3
Bonitos	n.d.	n.d.	n.d.	0.26	n.d.	0.3	0.3
Cod	2.21 ± 0.67	2.00 ± 0.60	n.d.	4.30 ± 0.40	1.00 ± 0.20	9.5	7.5
Eel	3.14 ± 1.04	1.05 ± 0.55	0.16 ± 0.07	4.95 ± 1.23	n.d.	9.3	8.3
Hake	1.43 ± 0.39	n.d.	n.d.	4.00 ± 0.75	n.d.	5.4	5.4
Halibut	22.78 ± 3.50	10.10 ± 2.20	27.46 ± 4.02	42.51 ± 4.27	4.81 ± 1.25	107.7	70.1
Herring	10.20 ± 1.90	7.10 ± 1.85	9.13 ± 2.01	18.15 ± 2.05	1.93 ± 0.05	46.5	30.3
Mackerel	4.43 ± 1.07	n.d.	2.12 ± 0.57	6.12 ± 0.70	n.d.	12.7	10.6
Redfish	2.15 ± 0.70	n.d.	n.d.	4.95 ± 0.80	n.d.	7.1	7.1
Saith	n.d.	n.d.	n.d.	1.25 ± 0.35	n.d.	1.2	1.2
Salmon	10.90 ± 2.05	11.50 ± 3.70	10.51 ± 3.53	15.55 ± 2.20	1.88 ± 0.42	50.3	27.3
Sardines	1.00 ± 0.20	n.d.	n.d.	1.18 ± 0.22	n.d.	2.2	2.2
Trout	1.95 ± 0.41	n.d.	n.d.	3.16 ± 0.84	0.27 ± 0.05	5.4	5.4

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was detected in negligible concentrations, although it is contained in the official toxaphene standard (∑#26/#50/#62). The sum of the six congeners, ∑#26/#40+41/#44/#50/#62, was observed with significantly higher concentrations in the contaminated fish samples than this of the three congeners ∑#26/#50/#62. For the other samples, the levels of these two standards were nearly the same. The percent contamination with the congener #44 varies from fish to fish. In halibut, the concentration of this contaminant is even higher than with #26, whereas in herring and wild salmon, the concentrations of these two congeners are comparable. In the other samples, the observed concentrations were below 2.1 μg/kg, or even not detectable.

4. Discussion

The consumption trend of the fish products listed in Table 2 has remained positive during the last years. According to the German information center for fish (Fischwirtschaft, 2007), the per capita consumption of whole fish in Germany in 2006 has increased from 14.8 kg in 2005 up to 15.5 kg in 2006. The dominating fish species in this respect are still Alaska-Pollock, herring, bonitos, and salmon, which represent together ca. 65%. Taking only the edible part of whole fish into account, the total per capita consumption of fish in 2006 can be reduced by 50% to obtain 7.8 kg per capita and annum, and conclusively ca. 21.4 g per day (Table 3). From that, the consumption in g per day of the single fish species can be calculated and in turn the daily intake of toxaphene congeners as in sum, correspondingly.

This means for the German population a daily intake of 0.236 μg of ∑#26,#50,#62, or 87.6 μg for the whole year, or 2.63 mg in 30 years. Presuming an average body weight of 60 kg and that toxaphene is stored in human body, then the concentration of toxaphene in the body, when uptaken only via fish, remains at 37.6 μg/kg. Assuming 10% of body fat related to a person weighing 60 kg, the value increases up to 375.7 μg/kg fat. All investigations performed until now concerning toxaphene occurrences in human samples have focused on human milk fat. In Germany, for example, toxaphene concentrations between 7–24 μg/kg in milk fat were observed (Skopp et al., 2002b), which is 15- to 50-fold less compared to body fat.

4.1. Toxaphene toxicity criteria

The cancer slope factor (CSF) for technical toxaphene, based on Litton Bionetics B6CJFI mouse studies and on NCI-Osbourne-Mendel rat studies, is calculated at 1.1 mg/kg/day (Crump, 1984). This value was reduced to 0.1 mg/kg/day in 2000 (Goodman et al., 2000), fixed to 1.2 mg/kg/day in 2003 by CAL-EPA (OEHHA, 2003), and again reduced to 0.86 mg/kg/day by Buranatrevedh, 2004. The RfD determined by Simon and Manning (2006) from an *in vivo* study with rats is based on NOAEL. Weathered toxaphene was studied in cod liver extract (CLE) and significant effects could be observed such as the occurrence of AHF-expecting placental GSTp–AHF. These foci can be related to inducing tumor promotion. The MATT-study provided additional support for the tumor promotion assumption after having performed an *in vitro* study of the disruption of the gap junctional intercellular communication in the Hepa 1c1c7 mouse liver cell line upon exposure to weathered toxaphene (MATT, 2000). The RfD values obtained were based on the toxicity of ∑#26,#50,#62 and, thus, they can be compared with some restriction to those from other studies laying down different toxicity criteria for the toxaphene mixture.

Table 3
Percentage market shares of different fish and per day consumption in 2006 (source: Fischwirtschaft, 2007), including the daily intake of toxaphene congeners as sum

Fish species	Market share (%)	Consumption[a] (g/day)	Daily intake of congeners (μg/day)	
			∑#26/#40+41/#44/#50/#62	∑#26/#50/#62
Alaska Pollock	25.90	5.54	0.014	0.013
Bonitos	10.70	2.28	0.000	0.000
Halibut	0.90	0.19	0.020	0.013
Herring	17.50	3.75	0.170	0.110
Red fish	3.80	0.81	0.000	0.000
Salmon	11.30	2.41	0.120	0.065
Others (incl. saith and cod)	29.90	6.40	0.049	0.035
∑	100	21.38	0.373	0.236

[a] Whole fish, edible part.

The TDI for weathered toxaphene in the MATT study is based on the entire weathered toxaphene, and the percentage of ∑#26/#50/#62 may differ according to different weathered toxaphene mixtures. The lower doses in CLE (0.5, 1.4, and 4.2 mg/ml of corn oil) differed not significantly from the control experiments in terms of the number of GSTp–AHF. In liver, only the congeners #50 and #62 could be detected with concentrations ranging between 2 and 3 μg/kg net weight. Parlar #26 could not be found in liver. Because of problems in interpreting these findings correctly, the next highest dose in CLE (4.2 mg/kg/day of ∑#26/#50/#62) was considered as NOAEL, corresponding to 0.002 mg/kg/day of ∑#26/#50/#62.

In the *in vitro* study, an effect was observed at a concentration of 1 mg/ml, which therefore represents the lowest observed effect concentration (LOEC) in CLE. The corresponding LOEC for ∑#26/#50/#62, based on the concentration of 1 mg/ml in CLE, would be 2.4 μg/ml, considering that the lowest concentration of ∑#26/#50/#62 is 0.24%. From these findings and from the daily intake of weathered toxaphene among the Inuit, it is possible to estimate the daily intake of toxaphene and to compare this to the NOAEL observed in rats, based on GSTp–AHF. The estimated intake for the Inuit is 2.6 μg/kg/day. By considering the average amount of ∑#26/#50/#62 in fish, which is between 25–30% (Xu et al., 1994), the daily intake of these three congeners is calculated at 0.6 μg/kg/day, which is about one-third of the NOAEL of 2 μg/kg/day. Simon and Manning (2006) have used these results and estimated the risk after the consumption of fish containing weathered toxaphene. By using the TDI developed from the MATT study, the acceptable concentration in CLE of fish can be determined via Eq. (1) (IR refers to the ingestion rate of fish).

$$\text{Acceptable toxaphene conc.}_{\text{CLE of Fish}} = \frac{\text{TDI}_{\text{CLE}}}{\text{IR}_{\text{Fish}} \times 0.001\ \text{kg/g}} \qquad (1)$$

In general, the whole toxaphene concentration in fish from Germany is represented by the ∑#26/#50/#62. Therefore, Eq. (1) can be computed as follows to calculate the acceptable concentration of toxaphene in human body, assuming an average body weight of 60 kg (hereby, the acceptable toxaphene concentration in fish is based on ∑#26/#50/#62):

$$\text{Acceptable toxaphene conc.}_{\text{CLE of Fish}} = \frac{\text{RfD}_{\Sigma\#26/\#50/\#62}}{\text{IR}_{\text{Fish}} \times 0.001\ \text{kg/g} \times \%\Sigma\#26/\#50/\#62} = \frac{2\text{E}-05\ \text{mg/kg/day}}{0.214\ \text{g/kg/day} \times 0.001\ \text{kg/g} \times 100\%} = 0.090\ \text{mg/kg}$$

As a consequence, the obtained value of 0.09 mg/kg should be taken for the end-assessment of the acceptable toxaphene concentration in German food samples.

5. Conclusions

The toxaphene burden in edible parts of consumed fish, under consideration of the MATT study and of the determined acceptable concentration, is still a problem in Germany. Because of the fact

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that a North-South divide exists in Germany regarding fish consumption, the toxaphene intake is considerably higher in the North than in the South. Toxaphene residues quantified with HRGC–HRMS/ECNI–SIM show that the components #26, #40+41, #46, #50, and #62 are present with high concentrations in single representative fish samples. For residue analysis of fish, the concentration of these six standard toxaphene substances as standard seems to be better suited than this of the normally used ∑#26/#50/#62 standard. The acceptable toxaphene concentration in fish of 0.090 mg/kg, based on ∑#26/#50/#62, is lower than the currently existing permissible level of 0.10 mg/kg.

Considering the average toxaphene concentration of ∑#26/#50/#62 in the fish pool determined at this level (0.24 μg/day, Table 2), it can generally be assumed that the consumption of toxaphene via contaminated fish does not pose a human health risk. Leonards et al. (2006) have come to a similar conclusion in their study after having assessed the toxicological risk of toxaphene to humans in different Northern European countries. Their average daily intake of toxaphene by fishery products was estimated at 0.4 μg/day related to 20.4 g/day of consumed fish. Not only for Germany, but also for Ireland, Norway and the Netherlands, the risks associated with fish consumption were assumed negligibly small. However, toxaphene still belongs to the most important contaminants contained in fish. The average consumption of fish is about seven times higher in Schleswig-Holstein, Hamburg and the Lower Saxony compared to Bavaria and Baden-Württemberg. Therefore, toxaphene levels should be monitored frequently, especially in the Northern part of Germany.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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