Sublethal Toxic Effects and Induction of Glutathione S-transferase by Short Chain Chlorinated Paraffins (SCCPs) and C-12 alkane (dodecane) in *Xenopus laevis* Frog Embryos

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Abstract

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Short chain chlorinated paraffins (SCCPs) are important industrial chemicals with high persistence in the environment but poorly characterized ecotoxicological effects. We studied embryotoxic effects of commercial mixture of SCCP (carbon length C-12, 56% of chlorine; CP56-12) and non-chlorinated n-alkane (dodecane, C-12) in the 96h Frog Embryo Teratogenesis Assay – *Xenopus* (FETAX). Only weak lethal effects were observed for both substances (the highest tested concentration 500 mg/L of both chemicals caused up to 11% mortality). On the other hand, we observed developmental malformations and reduced embryo growth at 5 mg/l and higher concentrations. However, the effects were not related to chlorination pattern as both SCCPs and dodecane induced qualitatively similar effects. SCCPs also significantly induced phase II detoxification enzyme glutathione S-transferase (GST) in *Xenopus laevis* embryos even at 0.5 mg/L, and this biomarker might be used as another early warning of chronic toxic effects. Our results newly indicate significant developmental toxicity of both SCCPs and n-dodecane to aquatic organisms along with inductions of specific biochemical toxicity mechanisms.

Developmental toxicity, FETAX, frog embryo, SCCPs, glutathione S-transferase

Short chain chlorinated paraffins (SCCPs) are chlorinated derivatives of n-alkanes with carbon chain length ranging from 10 to 13, and a chlorine content between 30% and 70%, by weight. They are important industrial chemicals with the main applications as metal working fluids, plasticizers and flame retardants in paints, rubber and textiles, coatings and sealants. SCCPs have low vapour pressure (2.8×10^{-7} to 0.5 Pa), which is in the range of some persistent organic pollutants (POPs) known to undergo long-range atmospheric transport. SCCPs have low solubility in water (up to 994 µg/l; D r o u i l l a r d et al. 1998), and due to extremely low biodegradability (S t r e e t et al. 1983) and high hydrophobicity (T o m y et al. 1998) they concentrate in organic matter-rich matrices such as sediments and soils (B a l l s c h m i t e r 1994; D r o u i l l a r d et al. 1998) and they also tend to bioaccumulate in living organisms (F i s k et al. 1996).

Available monitoring data document widespread contamination of water, sediments, aquatic and terrestrial organisms and even commercial foods by SCCP residues (Government of Canada 1993; S t e j n a r o v a et al. 2005). Concentrations in the U.K. marine and fresh waters remote from industry ranged 0.1 - 1 µg/l, but increased levels are expected in urban or industrial areas (C a m p b e l l and M c C o n n e l l 1980). Bioaccumulation in marine mammals has been documented by T o m y et al. (2000) who reported concentrations of SCCPs ranging 110-770 ng/g wet weight in the blubber of ringed seal

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(*Phoca hispida*), beluga whales (*Delphinapterus leucas*), and walrus (*Odobenus rosmarus*).

Although the EU Directive 2002/45/EC restricts the use of SCCPs in Europe (especially in metal working fluids and leather finishing products), SCCPs remain of environmental relevance and scientific interest. SCCPs are listed as priority hazardous substances within the Water Framework Directive of EU (EU Directive 2000/60/EC), and are considered "toxic" pursuant to Decision 1998/2, Paragraph 1(b) of the UNECE POPs Protocol.

In spite of the wide use and distribution of SCCPs, there are gaps in understanding their toxicity and ecotoxicity. Professional exposures as well as secondary poisoning in the food chain might be of special concern to human health. Some of the documented SCCPs effects with laboratory animals include hepatocellular hypertrophy, increase of thyroid weight and follicular cell hypertrophy, depletion of thyroxin (T4) and inductions of uridin diphosphate glucuronosyl transferase - UDPGT (European Commission 2000).

Available ecotoxicity data indicate low acute lethality of SCCPs (Thompson and Madeley 1983a; Linden et al. 1979) but severe negative effects in aquatic biota were observed after chronic exposures (Bengtson and Ofstad 1982; Madeley and Maddock 1983a). For example in fish, significant decrease in larval growth of sheepshead minnow (*Cyprinodon variegatus*) was recorded after 32 day exposure to SCCPs (Hill and Maddock 1983b). Lowest observable effects concentrations (LOECs) in the embryolarval assays with Japanese medaka (*Oryzias latipes*) ranged from 55 to 460 µg/l (Fisk et al. 1999). Cooley et al. (2001) reported behavioural effects (absent startle response and reduced feeding) and histopathological changes in thyroid gland and liver of juvenile rainbow trout exposed dietary.

However, there is a substantial lack of data on SCCPs' effects on other organisms than fish such as amphibians, which are of particular concern due to a world-wide population decline (Kiesecker et al. 2001). The standardized 96h assay Frog Embryo Teratogenesis Assay – *Xenopus* (FETAX) is often used as a screening test to determine embryotoxicity and the teratogenic risk of chemical agents (Dumont et al. 1983). The aim of this study was to provide first experimental data on sublethal toxic effects of SCCPs (CP-12 mixture, 56% of chlorine) to frog embryos. In order to evaluate the role of chlorination in toxicity, we compared the effects of SCCPs with parent non-chlorinated n-alkane (dodecane, C-12). We studied mortality, malformations and growth inhibitions as well as biochemical changes (activities of glutathione S-transferase, GST) in *Xenopus laevis* embryos exposed to tested chemicals. Induction of GST activity is often used as a biomarker of chronic toxic effects of organic xenobiotics (Livingstone 1998). In this study we newly describe significant non-lethal effects of SCCPs and n-dodecane to frog embryos, and we demonstrate methodological improvement of the FETAX by determining GST as an early warning biomarker of non-lethal toxicity.

Materials and Methods

Commercial mixture of SCCPs, CP56-12 (56% of chlorine by weight, chain length C12) was supplied by Novácke Chemicke Závody, Slovakia, n-dodecane (99% purity) was from Sigma (Sigma-Aldrich, Prague). Reduced glutathione (GSH) and 1-chloro-2,4 dinitrobenzene (CDNB) were purchased from Sigma (Sigma-Aldrich, Prague). Other chemicals used for preparation of FETAX exposure media as well as solvent (dimethylsulfoxide, DMSO) were of p.a. quality.

Frog embryos were obtained from adult pairs of *Xenopus laevis* injected with human chorionic gonadotropin (HCG; N.V. Organon, Oss, Holland) in the dorsal lymph sac (females: 300 IU; males: 150 IU). Amplexus normally ensued within 2 to 6 h and the deposition of eggs took place from 9 to 12 h after injection. Embryo tests were conducted using the standard guide for conducting the Frog Embryo Teratogenesis Assay – *Xenopus* (ASTM 1998). Only mid-blastula (stage 8) to early gastrula (stage 11) embryos (Nieuwkoop and Faber 1994) were selected for testing.

Groups of 25 embryos were randomly placed in covered 60-mm plastic Petri dishes in 10 ml of standard FETAX solution (625 mg NaCl, 96 mg NaHCO$_3$, 30 mg KCl, 15 mg CaCl$_2$, 60 mg CaSO$_4$·2H$_2$O, and 75 mg MgSO$_4$ per litre of distilled water, pH was 7.6-7.9). Stock solutions of tested compounds and appropriate dilutions were

prepared in DMSO and 50 µl per 10 ml of exposure media were added (0.5% v/v final concentration of solvent), final nominal tested concentration ranged 0.2 - 500 mg/l. Each concentration was tested in two parallels, four dishes with embryos in FETAX solution only served as controls. The assay was performed at 23 °C ± 1 °C for 96 h, exposure solutions were changed every 24 h and dead embryos were recorded and removed. Embryos were also examined for behavioral abnormalities such as twisting. At the end of the assay (96 h) surviving embryos were fixed in formaldehyde (3% v/v) and assessed for morphological abnormalities under a dissecting microscope, and the head-tail length of fixed embryos was determined. For the biochemical determinations, embryos were frozen at -80 °C until further processing.

Glutathione S-transferase activity (GST) was measured spectrophotometrically using 1-chloro-2,4 dinitrobenzene (CDNB) as a substrate, according to the method of Habig et al. (1974). Specific activity was expressed as nanomoles of formed product per minute per milligram protein. The protein concentration was determined according to the method of Lowry (1951).

Independent experiments using two different pairs of adults were performed. Since 50% lethality was not observed even at the highest tested doses, concentrations causing 25% effect - LC_{25} (for lethality) and EC_{25} (for malformations) - were estimated as endpoints using a probit model. Differences among the total embryo lengths were evaluated by ANOVA and LSD post-test, and the MCIG (minimal concentration significantly inhibiting the growth) was estimated. Differences in frequencies were compared by chi-square test. The 95% confidence intervals were used as the measure of variability. The modified teratogenic index (TI) was calculated as a ratio of LC_{25} and EC_{25} for tested compounds.

Results

Our study revealed low acute lethality of both SCCP and dodecane to frog embryos. The highest tested concentrations (500 mg/l) resulted in weak 11% mortality (Table 1). On the

Table 1. Effects of short chain chlorinated paraffins (mixture C-12, 56% chlorine) and n-dodecane on the survival and growth of *Xenopus laevis* embryos in 96h FETAX

	Concentration(mg/l)	N	% mortality		head-tail length (mm)		
			Mean	SD	Mean	SD	P
SCCPs	500	40	11.00	8.49	8.18	0.64	< 0.001*
	50	40	11.50	9.19	8.85	0.94	0.0487*
	5	40	5.00	0	9.08	0.45	> 0.05
	1	40	4.50	6.36	9.01	0.62	> 0.05
	0.2	40	2.50	3.54	9.21	0.48	> 0.05
C12	500	40	7.00	2.83	8.87	0.73	0.025*
	50	40	12.50	2.12	9.03	0.37	> 0.05
	5	40	10.50	3.54	9.14	0.72	> 0.05
	1	40	2.00	2.83	9.41	0.43	> 0.05
	0.2	40	0	0	9.15	0.41	> 0.05
control		40	3.50	4.95	9.18	0.45	

Asterisks (*) indicate statistically significant difference from the control (ANOVA plus LSD post-test)

other hand, both non-chlorinated n-dodecane and polychlorinated paraffins induced morphological malformations at concentrations as low as 5 mg/l (Fig. 1). More than 50% of embryos were malformed at the highest tested concentration of SCCPs, non-chlorinated dodecane induced malformations in about 30% of surviving embryos. Oedema, abnormal gut development, adverse curving of backbone and microphthalmia were the most often observed malformations at both substances (Plate VI, Fig. 2). The differences between the mean head-tail length of controls and exposed embryos were statistically evaluated and estimated experimental minimum concentrations inhibiting growth (MCIG) were 50 and 500 mg/l for SCCPs and n-dodecane, respectively (Table 1). Animals exposed to SCCP (≥ 50 mg/l) and n-dodecane (500 mg/l) exhibited also apparent changes in behavior as non-coordinated twisting and writhing of embryos after 72 and 96 h of exposure. Newly introduced assessment of GST in frog embryos revealed inductions of enzyme activity in all tested concentrations (Fig. 3).

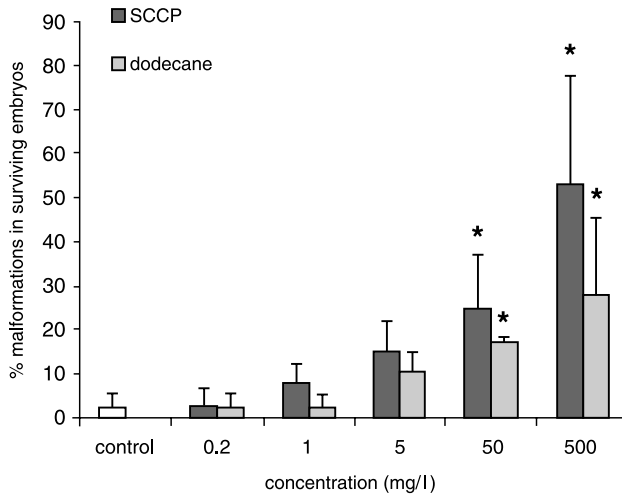To evaluate teratogenic risk, modified calculation of TI was used. For SCCP, the

Fig. 1. Effects of SCCPs and n-alkane on development of frog embryos. Bars represent means ± standard deviation of mean of independent experiments each performed in two parallels. Asterisks (*) indicate statistically significant increase in malformations in comparison with the control (mean ± SD; Chi-square, $P < 0.01$)
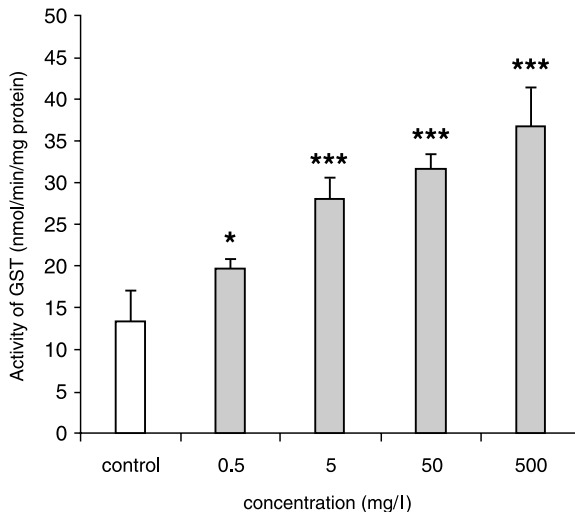

Fig. 3. Activity of glutathione S-transferase (GST) in tissue homogenate from *Xenopus laevis* after 96h exposure to commercial mixture of SCCP (CP56-12). Asterisks (*) indicate statistically significant differences from the control (mean ± SD; ANOVA, LSD-test, * $P < 0.05$; *** $P < 0.001$)

malformation EC_{25} as well as MCIG were 50 mg/l. Comparable lethal effect (25% mortality, LC_{25}) was not observed even at the 10-times higher concentration (500 mg/l). Therefore, teratogenic index for SCCPs (TI = LC_{25} / EC_{25}) could be expressed as TI > 10. In case of n-dodecane, EC25 and MCIG corresponded to the highest tested concentration (500 mg/l) in which only minor lethality was observed (7%) and evaluation of TI was not possible.

Discussion

The results presented in this report show significant growth inhibition and increase in numbers of malformed frog embryos exposed to both chlorinated technical mixture of SCCPs (56% chlorine by weight) and non-chlorinated parent compound (n-dodecane). Our study indicates significant embryotoxic and teratogenic potential of SCCPs (estimated TI >10) as TI >1.5 is considered significantly teratogenic in standardized FETAX protocol (ASTM 1998). To our best knowledge, there is surprisingly limited information on the developmental toxicity of these priority persistent compounds to aquatic biota. Our investigations with frog embryos complement the previous study of Fisk et al. (1999) who evaluated toxicity of C-10-, C-11-, C-12-, and C-14- polychlorinated alkanes (40-70% of chlorine) to embryos of Japanese medaka fish (*Oryzias latipes*). Fisk et al. (1999) reported 20 day LOECs ranging 55 to 460 µg/l. The differences in effective concentrations (LOEC ~ 50 mg/l in our study) can be explained either by prolonged exposures in the study of Fisk et al. (1999) but also by interspecies variability as the comparative study of Sparling et al. (2000) demonstrated relatively lower sensitivity of *Xenopus laevis* embryos to various xenobiotics.

Other studies documented significant ecotoxicity of SCCPs to aquatic biota. In rainbow trout, 48 and 96 hour LC_{50} were above 100 mg/l but chronic effects were observed (60 day $LC_{50} = 0.34$ mg/l and NOEC < 0.040 mg/l; Madeley and Maddock 1983a). Also aquatic invertebrates seem to be sensitive to SCCPs as 21 day tests with *Daphnia magna* showed EC_{50} values ranging 0.101-0.228 mg/l (European Commission 2000; Thompson and Madeley 1983a). Significant effects of SCCPs were also observed at both acute and chronic exposures with mysid shrimp *Mysidopsis bahia* (96 hour mortality LC_{50} ranged 14.1-15.5 µg/l, chronic 28-day LOEC = 7.3 µg/l; Thompson and Madeley 1983d), and significant growth reductions were recorded in common mussel (*Mytilus edulis*) exposed to SCCP (58% Cl) at 9.3 µg/l (Thompson and Shillabeer 1983).

In spite of existing toxicological and ecotoxicological studies with SCCPs, there is only limited experimental evidence on possible biochemical toxicity mechanisms of these compounds. The thyroid and liver are target organs in repeated dose studies with rats and mice. The liver damage is associated with peroxisome proliferation, whereas the thyroid effects are correlated to altered thyroid hormone status and uridin diphosphate glucuronosyl transferase (UDPGT) induction (Brucker-Davis 1998; Wyatt et al. 1993; European Commission 2000). Recent study also showed comparable effects in fish (Cooley et al. 2001). Humans would be much less sensitive to peroxisome proliferation and thyroid hormone perturbation than rats and mice (European Commission 2000). There is adequate evidence for the carcinogenicity of SCCPs in rodents. Neoplastic effects were seen in the liver, thyroid and kidneys in rats and mice (Brucker-Davis 1998; Wyatt et al. 1993). However the dose-related increases in the incidence of adenomas and carcinomas observed in these organs are not relevant to human health, considering the likely underlying mechanisms for these tumors (European Commission 2000). Significant growth inhibitions and morphological malformations observed in our study could reflect possible effects of SCCPs on thyroid regulations affecting embryonic development but further research in this area is needed.

Surprisingly, the observed non-lethal embryotoxic effects of the tested compounds seem to be independent of chlorine content as both SCCP and n-dodecane induced qualitatively comparable effects (Fig. 1, Table 1). This observation is of particular interest as higher chlorination is traditionally linked to higher toxicity of organic compounds. Interestingly, also the study of Fisk et al. (1999) with medaka fish did not report significant differences in toxicities of SCCPs varying in the chain length and chlorine content. Only limited data exist on acute and chronic toxicities of non-

chlorinated parent alkane, n-dodecane. In a study of Sice (1966), it was proposed to act as tumor promoter, possibly via peroxisome proliferation. Toxicological studies with structurally related n-alkanes showed effects on neural regulation (CNS depressants) or general irritation toxicity. Acute ecotoxicities (48h LC_{50}) of n-octane and n-decane to *Daphnia magna* were 3.3 and 0.2 mg/l, respectively (Irwin 1997) but we have observed only minor lethality at these concentrations after 96 hours. On the other hand, n-dodecane significantly induced developmental malformations and inhibited growth of frog embryos (Fig. 1, Table 1). Taken together, comparisons of our study with literature data indicate significant effects of both chlorinated and non-chlorinated aliphatic compounds to aquatic biota but further research is needed to fully understand consequences of possible chronic toxicities.

FETAX evaluating morphological malformations in frog embryos is an established and standardized assay (ASTM 1998). In our study we also assessed biochemical determination of GST activities as a biomarker of chronic stress (Livingstone 1998). SCCPs significantly induced GST after 96 h exposure and the modulations were observed at concentrations about an order of magnitude lower than those resulting in morphological alterations (Fig. 3). Although the embryonic development of *Xenopus laevis* is generally well understood, there is surprisingly limited information on profiles and activities of detoxification enzymes. Some recent studies focused on isolation and principal characterization phase II enzymes in *Xenopus laevis* (Angelucci et al. 2002), but to our best knowledge, biochemical responses in *X. laevis* embryos exposed to xenobiotics were not studied so far. Our study demonstrates good suitability of GST determination in FETAX as sensitive early warning parameter of chronic toxicities. Our results also correspond to the study of Brunstrom (1985) that described inductions of cytochrome-P-450 (detoxification phase I enzymes) after 4 days exposures of chick embryos to SCCPs (C10-13, Cl 70%).

To evaluate environmental risks of SCCPs, no observable effect concentrations (NOECs) are often derived and compared with the environmental levels. In our study with *Xenopus laevis* embryos, experimental LOECs for SCCP (C-12, 56% chlorine) were about 50 mg/l for morphological malformations and 0.5 mg/l for biochemical response (GST induction). Due to methodological limitations, there is only limited information on concentrations of SCCPs in the environmental compartments. Levels of SCCPs between 5 – 180 ng/g dry weight were recently reported in river sediments from the Czech Republic (Stejnarova et al. 2005), and these concentrations are within the range observed in Germany (< 5 to 83 ng/g, Ballschmiter 1994) or Canada (5.9 to 290 ng/g dry weight, Muir et al. 2000). However, concentrations of SCCPs as high as 448 ng/l were observed in sewage treatment plant effluents in Ontario, Canada, and the accumulation of SCCPs in the common carp up to 2.6 µg/g wet weight was documented (Muir et al. 2000). Although the NOEC values derived in our study with FETAX are above the actual concentrations found in the environment, other studies with aquatic organisms (*Daphnia magna* 21 day chronic NOEC= 5 µg/l, Thompson and Madeley 1983a) indicate certain environmental hazards of SCCPs.

In conclusion, our study provides first experimental evidences on SCCPs- and dodecane-induced morphological malformations in frog embryos. Suitability of GST assessment as early warning biomarker in FETAX was also demonstrated. Although the effects were observed in relatively high concentrations, the teratogenic index for SCCPs (TI > 10) indicates substantial embryotoxicity and teratogenic potential of SCCPs. With respect to high environmental persistency and bioaccumulation of these priority pollutants (EU Directive 2000/60/EC, Water Framework Directive), further research is needed to fully understand their chronic environmental hazards.

Subletální toxické efekty a indukce glutathion S-transferázy po expozici krátkořetězcovým chlorovaným parafínům a dodekanu v testu FETAX s embryi drápatky vodní (*Xenopus laevis*)

Chlorované parafíny s krátkým řetězcem (SCCPs – Short Chain Chlorinated Paraffins) patří mezi významné průmyslové chemikálie s vysokou perzistencí v životním prostředí a bioakumulačním potenciálem, ale málo prostudovanou ekotoxicitou. V naší práci byly studovány neletální efekty komerční směsi SCCP (délka řetězce C12, procento chlorace 56 %, CP56-12) a nechlorované parentální látky (dodekan) v testu embryotoxicity a teratogenity s pulci drápatky *Xenopus laevis* (test FETAX - Frog Embryo Teratogenesis Assay – Xenopus). U obou hodnocených vzorků byla pozorována jen nízká letalita, která potvrdila malou akutní toxicitu těchto látek (v nejvyšší testované koncentraci 500mg/l byla letalita 11%). Na druhé straně byl zaznamenán významný nárůst v počtu vývojových defektů ve všech testovaných koncentracích (až 50 % embryí s morfologickými malformacemi). Současně byla pozorována také inhibice růstu embryí. Naše výsledky prokazují významnou embryotoxicitu studovaných prioritních polutantů; účinek však nebyl závislý na stupni chlorace vzhledem k relativně srovnatelným výsledkům obou testovaných látek. Vedle klasických parametrů (mortalita, malformace) byl hodnocen také vliv chlorovaných parafínů na aktivitu detoxifikačního enzymu glutathion S-transferázy (GST). Byla zjištěna významná stimulace GST aktivity po expozici SCCP již v koncentracích 0.5 mg/l. Ačkoliv efektivní toxické koncentrace chlorparafínů jsou vyšší než udávají informace o koncentracích v prostředí, významná bioakumulace a perzistence těchto látek může vést k negativním chronickým účinkům u vodních organismů.

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Plate VI
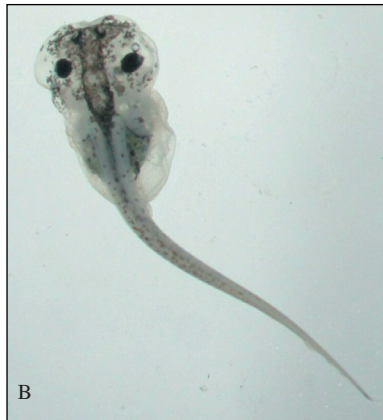Burýšková B. et al.: Sublethal Toxic Effects ... pp. 115-122

Fig. 2. Developmental malformations observed in *Xenopus laevis* embryos after 96 h exposure to commercial mixture of SCCP (CP56-12, Novácke Chemické závody).
A - control embryo, B - oedema and microphthalmia, C - skeletal malformation (adverse backbone curving) at SCCPs-exposed embryos