PRELIMINARY ASSESSMENT OF DIOXIN-LIKE COMPOUNDS IN/FROM CHLORPYRIFOS - A POTENTIAL PRECURSOR OF THE PYRIDINE ANALOGUE OF 2,3,7,8-TCDD

Sakiyama T[1], Weber R[2], Behnisch P[3], Nakano T[4]

[1]Osaka City Institute of Public Health and Env. Sciences, Tohjo-cho 8-34, Tennoji-ku, Osaka 543-0026, Japan;
[2] POPs Environmental Consulting, Ulmenstrasse 3, 73035 Göppingen, Germany
[3]BioDetection Systems BV (BDS), Kruislaan 406, 1098 SM Amsterdam, The Netherlands,
[4]Osaka University, Research Center for Environm. Preservation 2-5 Yamadaoka, Suita, Osaka 565-0871, Japan

Introduction

Pesticide production, use and disposal have contributed significantly to polychlorinated dibenzo-*p*-dioxins and dibenzofuran (PCDD/F) emissions in the past[1-4]. However also in a recent monitoring of current used pesticides in Australia in all assessed formulations PCDD/PCDF were detected with high concentrations in some pesticide formulation (in particular pentachloronitrobenzene/Quintozene)[4]. From historic perspective the two pesticides with the highest Dioxin levels and release were 2,4,5-trichlorophenoxy acetic acid (2,4,5-T) and pentachlorophenol (PCP)[1,5]. The production and use of 2,4,5-T and 2,4,5-Trichlorophenol - the precursor of 2,3,7,8-TCDD - resulted in large contamination during the Vietnam War (366 kg TEQ release from Agent Orange spray)[6] and had a long series of dioxin contamination in the factories[3] with a last accident in Seveso where about 30 kg TEQ have been released[7].

While today 2,4,5-T seems not be available on the market and the use of 2,4,5-trichlorphenol seems to be largely restricted to the synthesis of the still available hexachlorophen (e.g. skin cleanser PhisoHex) a major pesticide used today - Chlorpyriphos[A] - has as chlorinated aromatic moiety the pyridine-analogue of 2,4,5-trichlorophenol (3,5,6-trichloro-2-pyridinol) (Figure 1). Therefore the pesticide is a potential direct precursor of the pyridine-analogue of 2,3,7,8-TCDD (Figure 1). While in the recent study of Holt et al some PCDF have been detected in a Chlorpyrifos sample[4], the presence of this 2,3,7,8-TCDD analogue has to our knowledge not been assessed yet. Therefore we analysed in a preliminary study the presence of these potentially dioxin-like compound in a Chlorpyrifos formulation. In addition we performed thermal treatments of Chlorpyrifos at moderate temperatures to assess it's precursor potential and the first condensation steps.

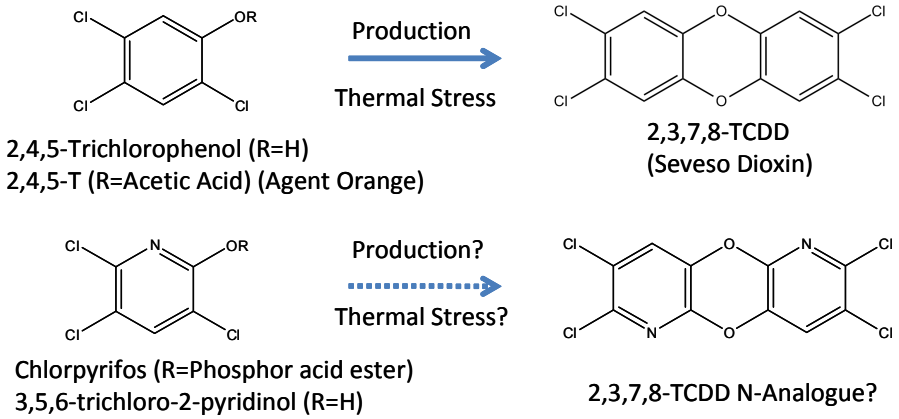

Figure 1: Formation of 2,3,7,8-TCDD from 2,4,5-T/2,4,5-TCP and potential formation of the 2,3,7,8-TCDD pyridine analogue from Chlorpyrifos or 3,5,6-trichloro-2-pyridinol (used in Chlorpyrifos production).

[A] Chlorpyrifos (*O,O*-diethyl *O*-3,5,6-trichloropyridin-2-yl phosphorothioate) is a organophosphate insecticide that inhibits acetylcholinesterase and is used to control insect pests. Chlorpyrifos is moderately toxic and chronic exposure has been linked to neurological effects, developmental disorders, and autoimmune disorders. (http://en.wikipedia.org/wiki/Chlorpyrifos)

Materials and methods

Thermal treatment

Chlorpyrifos was bought as chemical standard (Wako Pure Chemicals, Osaka Japan).
All pyrolysis experiments were carried out in sealed brown glass ampoules (10 ml) with about 2 mg of Chlorpyrifos at temperature between 200ºC and 300ºC in a GC-oven (Agilent). After cooling to room temperature the ampoules were opened carefully. The reaction products were extracted with toluene (pesticide grade; Wako Pure Chemicals, Osaka, Japan). The toluene was concentrated to 1 ml under gentle nitrogen stream.

GC/MS analysis

For the analysis a GC-TOF-MS:7890A (Agilent) and a JMS-T100GC (JEOL) GC-MS/MS : 450-GC/320-MS (Bruker). The TOF-MS was used with a 30 m DB-5MS (ID: 0.25 mm df:0.25um).for accurate mass analysis and unidentified substance assessment.
GC settings were: Oven Temp: 130ºC (1 min); 10ºC/min to -320ºC (10 min hold) ; Injector Temperature: 280ºC
As scan range 50-800 Amu was selected with a scan cycle of 0.4s/scan.
As carrier gas Helium was used with constant flow (1.0 ml/min).
The MS was operated at a resolution: >5,000 (50% valley)
The 320-MS was used with a 10 m Rapid-MS column (ID: 0.25 mm; df: 0.25um) for MS spectra analysis and for rapid screening.

DR CALUX® bioanalysis

The procedure for the DR CALUX® by BDS bioassay is described in detail previously[8]. Briefly, the bioassay is performed using a rat hepatoma H4IIE cell line stably transfected with an AhR-controlled luciferase reporter gene construct. Cells were cultured in α-MEM culture medium supplemented with 10% ($^{v}/_{v}$) FCS under standard conditions (37^{o}C, 5% CO_2, 100% humidity). Cells were exposed in triplicate on 96-well microtiterplates containing the standard 2,3,7,8-TCDD calibration range, the additional 2,3,7,8-TCDD calibration concentrations, a DMSO blank, an internal reference material and various samples extracts at multiple dilutions (e.g. sediment, foodstuffs, feeding stuffs). Following a 24 hour incubation period, cells were lysed, a luciferine containing solution was added and the luciferase activity was measured using a luminometer equipped with 2 dispensers.
For this procedure the samples were transferred from the toluene fraction to DMSO and applied in the assay without further clean-up.

Results and discussion

In the scanning of the raw Chlorpyrifos sample by GC/MS the pyridine analogue of 2,3,7,8-TCDD has not been detected. However the detection limit of this screening method might roughly be estimated in the ng/g range. This indicate that no 2,3,7,8-TCDD analogue was present in the crude samples at ppb levels. For trace analysis in the ppt and sub ppt level a trace analysis for this compound class need to be developed[B].

Table 1: Accurate mass and related intensity of isotope cluster of TCDD and the pyridine analogue of TCDD

	PCDD	Intensity	Pyridin-Analogue of PCDD	Intensity (%)
Tetrachloro (M)	319.8965	76.4	321.8870	77.6
Tetrachloro (M+2)	321.8936	100	323.8842	100
Tetrachloro (M+4)	232.8906	49.4	325.8813	48.6
Tetrachloro (M+4)	325.8877	11.0	327.8786	10.6

Thermal treatment of Chlorpyrifos

Chlorpyrifos was thermally treated in closed glass ampoules at 200ºC (30 minutes and 15 hours), 250ºC (45 minutes) and 300ºC (5 minutes) in glass ampoule sealed under atmosphere. At these temperatures the phosphorester was expected to degrade and Under these relatively mild conditions for phenol condensation the intermediate of the first condensation steps should be detected as has been found for pyrolysis of chlorophenols[9].

[B] The nitrogen analogues of TCDD might behave quite differently on the various clean-up columns compared to PCDD/PCDF and therefore can not be used for trace analysis for these compounds without further detailed assessment.

GC/MS screening of formed compounds

The crude toluene extracts (from 1 mL) were injected into the GC/MS without further clean-up step. The samples were analysed in SCAN mode and in Select Ion Monitoring (SIM mode). Table 1 shows the accurate mass of the pyridine analogue of TCDD and the native TCDD with the relative intensities.

In this first experimental series relative low pyrolysis temperature were chosen to detect the first condensation products and assess the potential reaction pathway[9]. As major condensation product a dimer with a mass of either the pyridine analogue of hydroxyl-biphenyl or the diphenyl ether was detected (see mass spectra in Figure 2). In these preliminary low temperatures experiments we did not detect the 2378-TCDD pyridine analogue however the potential precursor 3,5,6-trichloro-2-pyridinol.was detected in the experiment as degradation product of Chlorpyrifos.

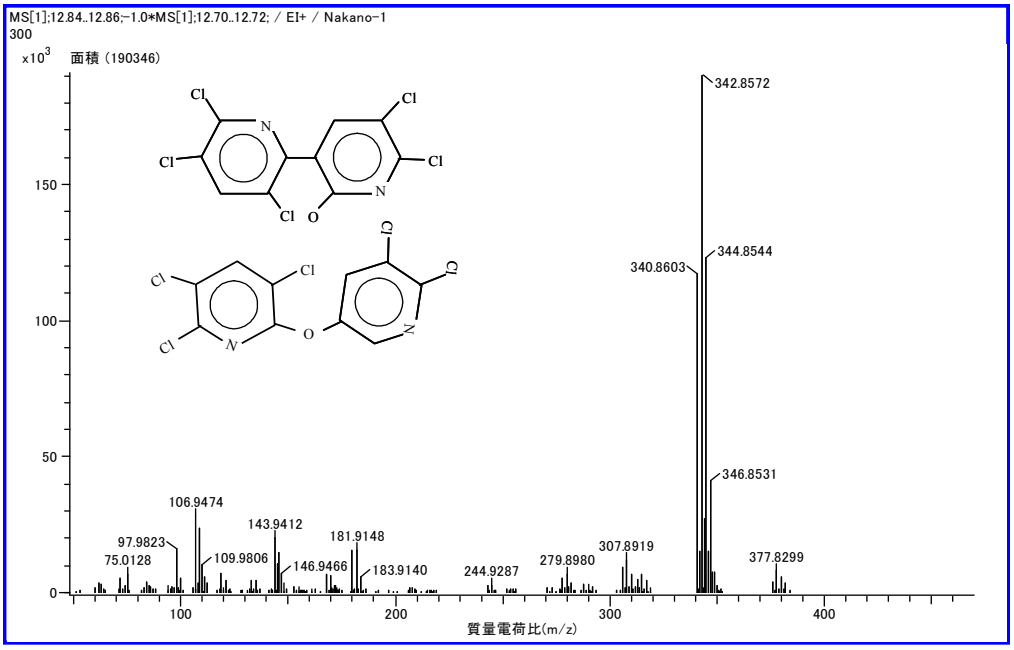

Figure 2: Mass spectra of condensation products of Chlorpyrifos at 300ºC. The accurate mass was in agreement with pentachloro hydroxybiphenyl derivative of pyridine and the pentachloro hydroxydiphenyl ether derivative of pyridine.

Screening of dioxin like toxicity by DR-CALUX

The raw pesticide formulation and the thermally treated samples where sent to Bio Detection Systems (Amsterdam, Netherlands) for analysis of dioxin-like toxicity.

The raw pesticide did not show measurable dioxin-like TEQ at a limit of quantification (LOQ) of 20 ppb. The limit of quantification was relatively high since in this first screening only a fraction of the dissolved 2 mg have been sent to BDS as a "blank" for the thermal treatments. For a through assessment of dioxin-like toxicity of Chloryrifos higher volumes for lower detection limits need to be analysed.

Also at the two experiments at 200ºC (30 minutes and 15 hours) no dioxin-like activity was detected (at a LOQ of 3.6 and 8.1 ppb). However, at 250ºC (45 minutes) 38 pg bio-TEQ/mg treated Chlorpyrifos (ppb) was detected and at the experiment at 300ºC (5 minutes) 116 pg bio-TEQ/mg treated Chlorpyrifos (ppb) was measured (Table 2).

These data indicate that condensation products with high dioxin-like activity have been formed in the experiment. Since in our GC/MS analysis the 2,3,7,8-TCDD analogue was not detected, rather the main condensation product(s) tentatively identified as pentachloro hydroxybiphenyl derivative of pyridine or the pentachloro hydroxydiphenyl ether derivative of pyridine (see figure 2).

Also in a study of another research group comparing dioxin-like toxicity of some pesticides (not including Chlorpyrifos) with GC/MS measurements it was found that for some pesticide formulations considerably higher dioxin-like toxicity was detected in the bio-assay compared to the PCDD/F TEQ measured with GC/MS.[10]
This finding needs further assessment considering that Chlorpyrifos is a major used pesticide worldwide and can be involved in fires in pesticide productions & storages[11-13] and in the combustion of post harvest residues[14] with associated thermal stress for present dioxin and dioxin-like substance precursors.
Further assessment at higher temperature is therefore needed to assess also such high temperatures processes.

The current used Chlorpyrifos was not an actual pesticide formulation but an analytical standard. It is known that the dioxin content in pesticide formulations that dioxin levels in the same type of pesticide can vary orders of magnitudes. Therefore the current analysed analytical standard does not give any indication on possible dioxin-like contaminants in actual Chlorpyrifos pesticide formulations. According to the current data of the combined instrumental analysis and the DRCALUX assay of the thermal treatments, the assessment for dioxin-like activity of Chlorpyrifos (and other pesticides)[10] should in addition to instrumental analysis also be assessed with bio-assays measurements. .

Table 1: Results from DR CALUX (24 hrs kinetic, direct exposure in 0.8% DMSO)

Experiment	2,3,7,8-TCDD-TEQ (by DR CALUX)		Unit
Chlorpyrifos (crude)	**LOQ (20)**		pg 2378-TCDD TEQ/ mg chlorpyrifos (ppb)
200ºC (30 minutes)	**LOQ**		pg 2378-TCDD TEQ/ mg chlorpyrifos (ppb)
200ºC (15 hr)	**LOQ**		pg 2378-TCDD TEQ/ mg chlorpyrifos (ppb)
250ºC (45 min)	**38**		pg 2378-TCDD TEQ/ mg chlorpyrifos (ppb)
300ºC (5 minutes)	**116**		pg 2378-TCDD TEQ/ mg chlorpyrifos (ppb)

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