DIOXIN-LIKE ACTIVITY MEASURED BY DR CALUX™ FROM THERMAL TREATMENT OF CHLORPYRIFOS AND OTHER POTENTIAL PRECURSORS OF THE PYRIDINE ANALOGUE OF 2,3,7,8-TCDD

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Introduction

Pesticide production, use and disposal have contributed significantly to Dioxin emissions in the past[1-5]. However, also in a recent monitoring program of current used pesticide formulations in Australia PCDD/F were detected in all assessed formulations[4] and formations of PCDD/F from sunlight exposure of selected pesticide formulations5. From a historic perspective the two chemicals with the highest dioxin release into the environment were 2,4,5-trichlorophenoxy acetic acid (2,4,5-T) and pentachlorophenol (PCP)[1,2,6]. 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 contaminated area during the Vietnam War (total release of 366 to kg TEQ)[6].

A current used high production volume pesticides – chlorpyrifos and chlorpyrifos-methyl – have as chlorinated aromatic moiety the pyridine-analogue of 2,4,5-trichlorophenol (3,5,6-trichloro-2-pyridinol) (Fig. 1). Therefore, these pesticides are potential precursors of the pyridine-analogues of 2,3,7,8-TCDD (2,3,7,8-TCDD-Py; Fig.1).

At the former DIOXIN conferences we reported on the formation of 2,3,7,8-TCDD-py[7,8] from chlorpyrifos, chlorpyrifos-methyl and the major degradation product of chlorpyrifos, 3,5,6-trichloro-2-pyridinol (TCPy) in a temperature range between 250°C and 380ºC.[7,8] DR CALUX™ measurements showed a dioxin-like activity of the reaction products. In the current study we report further results from thermal experiments (300 to 440 °C) with chlorpyrifos, chlorpyriphos-methyl and the major degradation product of chlorpyrifos, the 3,5,6-trichloro-2-pyridinol (TCPy). For the first time a preliminary estimate of the bio-toxicity (REP) was performed by a semi quantification of 2,3,7,8-TCDD-py via 2,3,7,8-TCDD standard and DR CALUX™ measurements.

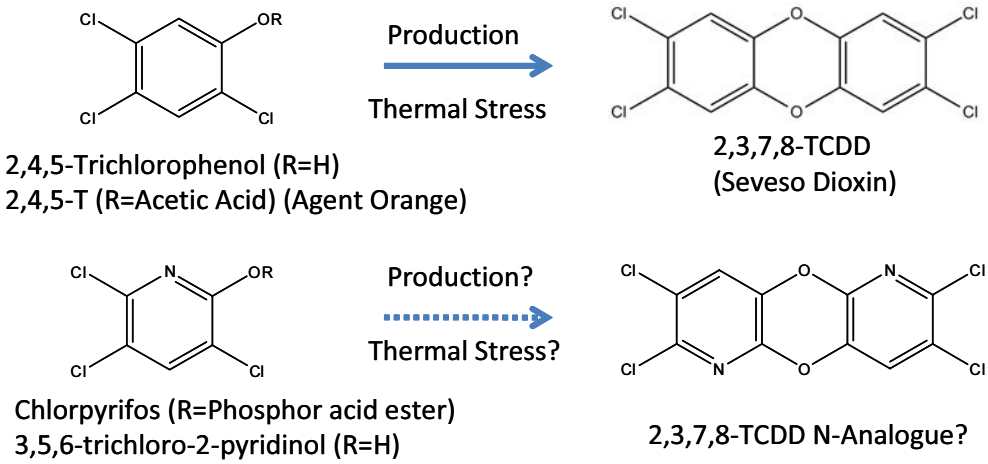

Figure 1: Formation of 2,3,7,8-TCDD from 2,4,5-T/2,4,5-TCP and potential formation of the 2,3,7,8-TCDD pyridine analogue from Chlorpyrifos or 3,5,6-trichloro-2-pyridinol (used in Chlorpyrifos production).

Materials and methods

Chemicals and thermal treatment: Chlorpyrifos and 3,5,6-trichloro-2-pyridinol was purchased as chemical standards from Dr. Ehrenstorfer GmbH (Augsburg, Germany) and chlorpyrifos-methyl from Wako Pure

Chemicals (Osaka Japan). All pyrolysis experiments were carried out in sealed brown glass ampoules (10 ml; air) with about 0.5 mg of respective chemical at temperatures between 300ºC and 440ºC in electric furnace (Eyela TMF-3200; Tokyo Rikakikai, Tokyo, Japan). After cooling to room temperature, ampoules were opened and reaction products extracted with toluene.

GC/MS analysis

The procedure for the GC/HRMS analysis by Osaka City institute is described in detail previously[1,2]. Briefly, a GC-TOF-MS: 7890A (Agilent) with a 30 m DB-5MS (ID: 0.25 mm), a JMS-T100GC (JEOL), a GC/HRMS: 6890GC (Agilent) with a 60 m HT-8PCB (ID: 0.25 mm), a Autospec Ultima (micromass) was used for analysis. Temp. program DB-5MS: 130ºC (1 min); 10ºC min to -320ºC (10 min hold). HT-8PCB: 130°C (1min) - 30°C/min to 200°C; 20°C/min to 310°C (hold). The TOF/MS was operated at a resolution >5,000 (50% valley) and HRMS at a resolution >10,000. Further a 450-GC/320-MS (Bruker) 320-MS was used with a 10 m Rapid-MS column for MS spectra and screening. 2,3,7,8-TCDD-py concentrations were semi-quantitatively determined using a 2,3,7,8-TCDD ^{13}C-standard.

DR CALUX^{TM} bioanalysis:

The procedure for the DR CALUX^{TM} by BioDetection System (BDS) in Amsterdam is described in detail previously[1,2]. 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°C, 5% CO_2, 100% humidity). Cells were exposed in triplicate on 96-well micro-titer plates 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

Screening of dioxin like toxicity by DR-CALUX of thermally treated chlorpyrifos, chlorpyrifos-methyl and 3,5,6-trichloro 2-pyridinol standards

All thermally treated pesticide samples for analysis of dioxin-like toxicity were analysed by DR CALUX^{TM} by BioDetection Systems (Amsterdam, Netherlands).

For an assessment of the formation potential of chlorpyrifos, chlorpyrifos-methyl and 3,5,6-trichloro 2-pyridinol (TCPy) (major degradation product of chlorpyrifos in environment) the chemicals where heated at the respective conditions (temperature, time) in individual ampoule experiments.

Measurable concentrations of DR CALUX^{TM} activities were formed from chlorpyrifos, chlorpyrifos-methyl and 3,5,6-trichloro 2-pyridinol (TCPy) after already 15 minutes at 300°C (table 1). DR CALUX^{TM} activity increased for chlorpyrifos with increasing temperature by ca. 5-times from 300°C to 340°C and than ca. 10-times again to 380°C. The GC/HRMS analysed concentration of the sum of cis/trans 2,3,7,8-TCDD-Py increased by a factor of 25-times from 340°C to 380°C (table 1/table 2).

For chlorpyrifos-methyl the DR CALUX^{TM} activity increased from 340°C to 380°C within 15 minutes reaction time ca. 170-times (table 1/figure 2), while the sum of cis/trans 2,3,7,8-TCDD-Py increased by a factor of ca. 500-times.

For 3,5,6-trichloro 2-pyridinol (TCPy) the DR CALUX^{TM} activity increased from 300°C to 340°C (within 15 minutes reaction time) ca. 60-times. Interestingly, both the DR CALUX^{TM} activity and the sum of cis/trans 2,3,7,8-TCDD-Py increased at the same intensity of ca. 7-times from 340°C to 380°C to a finally stable level of 200 to 400 ng BEQ/mg level. (table 1/figure 2).

In table 1 also the concentrations analysed by GC/HRMS for the sum of cis/trans 2,3,7,8—TCDD-py (in ng/mg) of these thermally treated chlorpyrifos, chlorpyrifos-methyl and 3,5,6-trichloro 2-pyridinol standards are reported. The experiments show that the potential of chloropyrifos and chlorpyrifos-methyl to form the DR CALUX^{TM} activity was considerably lower compared to 3,5,6-trichloro-2-pyridinol (table 1/ figure 2):

From the first semiquantification of 2,3,7,8-TCDD-py using ^{13}C-2,3,7,8-TCDD as standard (with different retention time[7] and possibly differences in the response in the MS): In case we consider that the sum of cis/trans 2,3,7,8-TCDD-py caused all AhR-activity measured by DR CALUX™ and consider a measurement uncertainty from using 2,3,7,8-TCDD as standard to measure 2,3,7,8-TCDD-py of a factor of 3, then a REP-value range between 0.01 to 0.1 can be estimated. This REP would therefore be in the range of PCB 126; with a REP value of 0.08 or the upper estimated range close to the dioxin-toxicity of 2,3,7,8-substituted HexaCDD or HexaCDF[9].

Table 1: Dioxin-like activity measured by DR-CALUX™ (in ng BEQ/mg) and concentrations for the sum of cis/trans 2,3,7,8-TCDD-Py by GC/HRMS (in ng/mg) of thermally treated chlorpyrifos, chlorpyrifos-methyl and 3,5,6-trichloro 2-pyridinol standards (Experiment design: 0.5 mg compound, 15 min heating time; DR CALUX: 24 hrs kinetic, direct exposure in 0.8% DMSO)

Experiment	DR CALUX: ng BEQ/mg	GC/HRMS: ng/mg	Approx. REP**
Chlorpyrifos: 300ºC	**0.057**	**n.a.**	
Chlorpyrifos: 340ºC	**0.26**	**3.9**	**0.067**
Chlorpyrifos: 380ºC	**2.9**	**97**	**0.030**
Chlorpyrifos-Methyl: 300ºC	**0.013**	**n.a.**	
Chlorpyrifos-Methyl: 340ºC	**0.035**	**0.96**	**0.036**
Chlorpyrifos-Methyl: 380ºC	**6.0**	**490**	**0.012**
3,5,6-trichloro 2-pyridinol: 300ºC	**1.3**	**73**	**0.018**
3,5,6-trichloro 2-pyridinol: 340ºC	**60**	**1,950**	**0.031**
3,5,6-trichloro 2-pyridinol: 380ºC	**200-400**	**13,100**	**0.031**
3,5,6-trichloro 2-pyridinol: 400ºC	**190**	**n.a.**	
3,5,6-trichloro 2-pyridinol: 420ºC	**250**	**n.a.**	
3,5,6-trichloro 2-pyridinol: 440ºC	**410**	**n.a.**	
Mean REP value			**0.032****

****Note: The use of an 2,3,7,8-TCDD standard as "internal" standard causes a measurement uncertainty of approx. an factor of 3 since the differences in response in MS of these two compounds are unknown.**

Relevance of the findings

The estimated REP of 0,01 to 0,1 highlight the possible relevance of 2,3,7,8-TCDD-py in respect to Dioxin toxicity when considering the high volume use of these pesticides. This finding needs further assessment considering that chlorpyrifos is a major used pesticide worldwide and can be involved in fires in pesticide productions & storages and in the combustion of post-harvest residues with associated thermal stress for present dioxin and dioxin-like substance precursors[1,2].

Further assessment at higher temperature is therefore needed to assess also such high temperatures processes.

Considering the 2,3,7,8-TCDD contamination in 2,4,5-T formulations (e-g.Agent Orange) possible dioxin-like contaminants in actual chlorpyrifos pesticide formulations should be considered and assessd.

According to the current data of the combined instrumental analysis and the DR CALUX™ assay of the thermal treatments, the assessment for dioxin-like activity of chlorpyrifos (and other pesticides) should in addition to instrumental analysis also be assessed by bio-assays measurements.

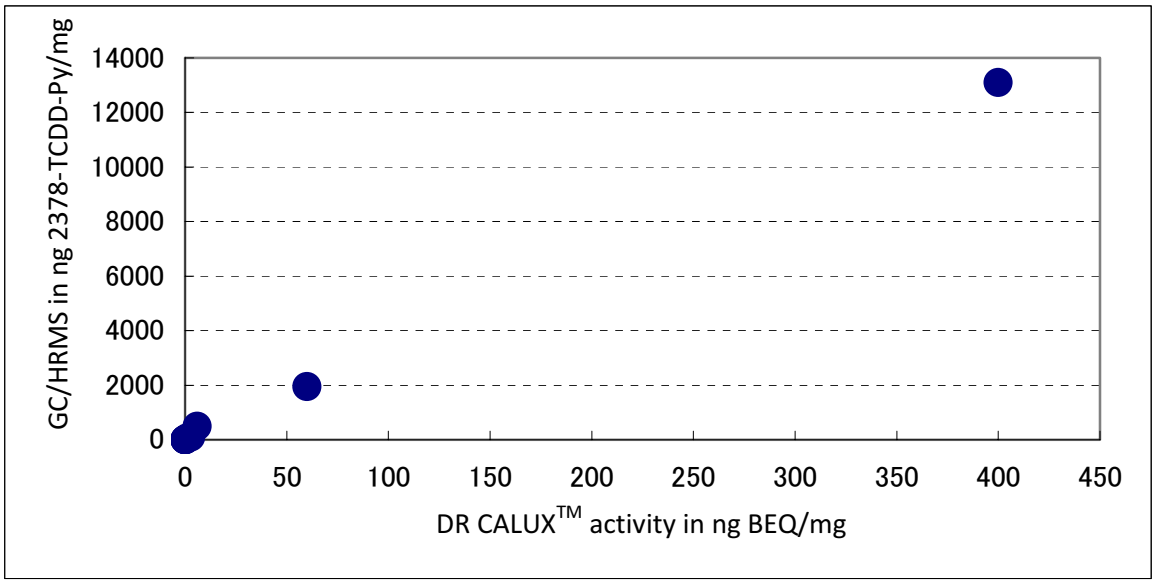

Figure 2: Comparison of dioxin-like activity measured by DR-CALUX™ (in ng BEQ/mg) and concentrations for the sum of cis/trans 2,3,7,8-TCDD-py by GC/HRMS (in ng/mg) of thermally treated chlorpyrifos, chlorpyrifos-methyl and 3,5,6-trichloro 2-pyridinol standards. (**please note:** the uncertainty in the measured 2,3,7,8-TCDD-Py concentration using 2,3,7,8-TCDD standard).

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