PERSISTENCE AND DIOXIN-LIKE TOXICITY ASSESSMENT OF BROMO- AND CHLOROCARBAZOLES IN SOIL – ARE THEY A POTENTIAL CLASS OF PERSISTENT ORGANIC POLLUTANTS?

Mumbo J[1,2], Henkelmann B[1*], Lenoir D[1], Abdelaziz A[1,2], Pfister G[1], Schramm K-W[1,2]

[1]Helmholtz Zentrum München, German Research Center for Environmental Health, Molecular EXposomics (MEX), Ingolstädter Landstraße 1, 85764 Neuherberg, Germany; [2]Department für Biowissenschaftliche Grundlagen, Technische Universität München, Weihenstephaner Steig 23, 85350 Freising, Germany

1.0 Introduction

Bromo- and chlorocarbazoles are an emerging category of environmental contaminants whose occurrence in water[1], soil[2,3] and sediment[4] presents the necessity for research with respect to their sources, formation, fate, transport and transformation. They possess attributes similar to persistent organic pollutants (POPs) specifically the tendency to persistence and toxicity. 3-mono-, 3,6-dichlorocarbazole are not readily degradable in soil while the later has dioxin-like toxicological potential[3]. Tetrabromocarbazole has also been detected in sediment many years after deposition[4]. Carbazole and 3,4,5,6-dibenzocarbazole are carcinogenic with the latter also being mutagenic[5]. Organic compounds have recorded enhanced toxicity, increased bioaccumulation and lipophilicity potentials after transformation to their respective halogenated products[6]. Bromo- and chlorocarbazoles could possess these properties. Their environmental and human health effects could therefore be significant given that the scale of their distribution is unknown. They have natural and anthropogenic sources. However, there is limited information in literature on their environmental fate. They can be synthesized by an enzymatically catalyzed process in water[7,8]. A similar haloperoxidase mediated oxidative halogenation of carbazole could be responsible for the formation of halogenated carbazoles in soil. Tetrabromocarbazole is the halogenated carbazole reported in literature to be produced by combustion. We report the persistence to natural breakdown and dioxin-like toxicity of carbazole, chloro- and bromo-carbazoles in soil in long term study of 15 months under controlled environment of fluctuating soil moisture conditions. Fluctuating humidity increases bioavailability and degradation of pesticides[9]. Dioxin-like toxicity was determined by EROD induction in H4IIA rat hepatoma cells assay and multi-dimensional quantitative structure- activity relationships (mQSAR) modelling.

2.0 Materials and methods

2.1 Soil physical properties, homogenization and soil water potential determination

Strongly humic, dark brown, Calcic Gleysols was used in this study. Three different soil samples were obtained from A horizon 24-39 cm with a bulk density of 1.04 g/cm^3, pH ($CaCl_2$) 7.62, total carbon 4.17%, organic carbon 2.57%, nitrogen 0.29% and sulfur < 1%. The horizon had the highest concentration of chlorocarbazoles. Soil homogenization was done using Retsch ZM1 ultra centrifugal mill equipped with an integrated 2mm mesh. After soil homogenization, the pF curve was determined at a -15 kPa soil water potential using kaolin box. Water content at -15 kPa and actual soil water content were then used to calculate the amount of water to be added on soil samples to achieve a water potential of -15kPa before and during incubation.

2.2 Experimental design

2.2.1 Temperature

Incubation was conducted under two temperature conditions. Forty two soil samples in triplicates were incubated at a temperature of 15±3ºC while another 42 incubated at a temperature of 20±3ºC also in triplicates. The set-up was such that 42 soil samples were initially at 15°C then moved to 20°C temperature conditions to investigate the influence of change in temperature on dissipation and dioxin-like toxicity. Each jar contained 29g wet weight equivalents to 20g dry weight soil.

2.2.2 Wet and dry cycles

Fluctuating soil humidity conditions of wet and dry cycles were used in this experiment. The wet cycle was characterized by addition of distilled water to the soil sample followed by the dry phase during which water not added to respective water potentials at 15°C and 20°C conditions. Soil samples were rewetted in the wet cycle to maintain water potential at -15kPa.

2.3 Sample collection, extraction and clean-up

Soil samples were collected initially after two weeks for the first 12 weeks to monitor any significant rapid change in the concentration of compounds under study during this period. Given that the rate of dissipation was significantly slow ($p<0.05$), sample collection was eventually increased to 4 weeks. Accelerated solvent extraction (ASE) was performed on 29g wet weight of soil sample by Dionex ASE 300 Sunnyvale, CA, USA. Deuterated PAH (phenanthrene D10, benzo(a)anthracene D12, pyrene D10, benzo(b)fluoranthene D12) was used as the internal standard due to lack of in availability of isotope labelled halogenated carbazoles. A tenth (1/10) of the diluted extract was then subjected to clean-up by column chromatography using silica gel (SiO_2), alumina (Al_2O_3 + 3% H_2O) and sodium sulphate (Na_2SO_4) to remove any material that could cause interference. Columns were eluted with 100mL hexane: DCM (1:1). The eluate was subjected to another clean-up by elution through C18 column using acetonitrile as the eluent. The final eluate was concentrated under a steady but gentle flow of nitrogen gas to a final volume of 20µl. The amber vials were sealed for analysis using high resolution GC-MS.

2.4 EROD induction bioassay and REP calculations

An aliquot of the soil extract without clean-up and pure compounds of carbazole, 3-chloro- and 3,6-dibromocarbazole were assessed for EROD induction EC50 as an indicator for cytochrome P450 IA1 (CYP1A1) activity. Simplified EROD assay (micro-EROD) using rat hepatoma cell line (HII4E) expressing CYP1A1 upon exposure to aryl hydrocarbon receptor (AhR) agonists was performed. HII4E were seeded at a density of 1×10^4 cells/well and exposed to increasing concentrations of soil extract and the pure compounds in the culture medium alongside the controls of TCDD, also in increasing concentrations. After 24 and 72 hrs respectively, the cell culture medium was removed and the cells were exposed to 100 µL fresh medium containing 8 µM 7-ethoxyresorufin and 10 µM dicumarol added to each well. After incubation for 30 min at 37 °C, the medium was transferred to another 96-well plate containing 100 µL ethanol. The conversion of ethoxyresorufin to resorufin was measured fluorometrically at 535 nm excitation and 590 nm emission using a plate-reading spectrofluorometer, SPECTRAFluor XFLUOR4 version V4.51. Resorufin (cytotoxicity) was measured using the same cell cultures at 535 nm excitation and 590nm emission fluorescence wavelengths immediately after EROD activity determination. Protein measurement was done using BSA as protein standard (0-200 µg/ml) in separate wells at absorption wavelength of 540nm. Results were given as pgTCDD toxicity equivalent values (TE values)/g dry sample. Dioxin-like toxicity was then evaluated based on relative potency (REP) values with respect to 2,3,7,8-TCDD (TEQ_{TCDD}). The equivalent concentration of halogenated carbazole in each soil extract was converted into TEQ_{TCDD} using the corresponding REP according to the following equation where C_i is the concentration and i is the number of halogenated carbazole compounds.

$$TEQ_{TCDD} = \sum(C_i \times REP_i)$$

2.5 AhR Affinity calculations

Modelling was used to estimate aryl hydrocarbon receptor (AhR) affinity for carbazole, bromo-, chloro- and iodo-carbazole congeners that could not be done by EROD test due to lack of their pure compounds. This was achieved using the software Open VirtualToxLab version 4.8. The calculation was done with an automated flexible docking combined with multi-dimensional QSAR protocol that calculates binding affinities of molecules towards the AhR receptor and estimates their toxic potential. The binding was then visually inspected in real-time 3-dimensional (3D) and atomic resolution. All chemical structures for input were prepared as standard data files (SDF) using Marvin Sketch software package and 3D optimization was performed.

2.6 GC/MS analysis

Identification of halogenated carbazoles was carried out by high resolution gas chromatography coupled to a mass spectrometer (GC-MS). The criteria for the identification of individual compounds involved the use of retention times (RT), mass spectra and isotope ratio**.** All samples were analysed in single ion monitoring (SIM) mode, whereby the two most intensive masses of the molecular ion cluster were registered. In addition full scan analyses were carried out for identification of halogenated carbazoles by mass spectra specifically for the mono-, tri-, tetra-, and penta- halogenated carbazoles.

2.7 Statistical analysis

Data in tables and figures were presented as the mean ± SD. Pearson correlation, analysis of variance (ANOVA) and regression analysis were performed at $p<0.05$ (95%) confidence using SAS version 9.3.

3.0 Results and Discussion

3.1 Identification and dissipation of carbazole and its chlorocarbazoles congeners

Carbazole, 3-chloro- and 3,6-dichlorocarbazole were detected including trichlorocarbazole not reported previously in soils. The order of concentration beginning with the highest was, 3,6-dichlorocarbazole > 3-chlorocarbazole > carbazole > trichlorocarbazole. 3,6-dichlorocarbazole recorded consistently approximately 10 times higher concentrations in comparison to carbazole and 3-chlorocarbazoles during the study period. Carbazole and 3-chlorocarbazole showed significant dissipation at 15°C but not at 20°C incubating conditions indicating that low temperature could be suitable for dissipation of carbazole and 3-chlorocarbazoles (Fig. 1 and 2). 3,6-dichlorocarbazole was resistant at both conditions. Trichlorocarbazole however exhibited a tendency to increase in concentration with time (Fig 3).

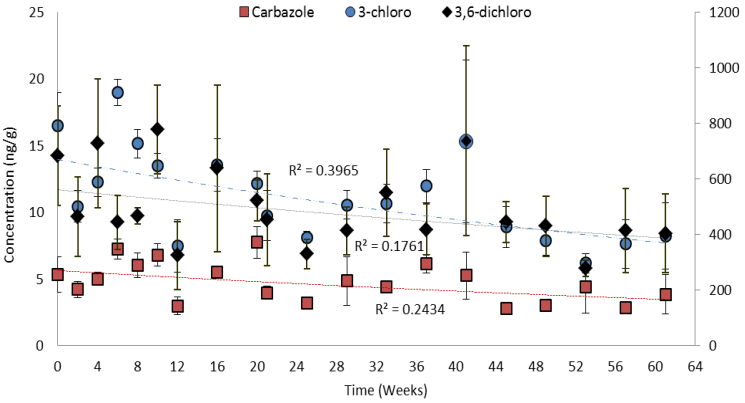

Fig 1. Mean concentrations of carbazole, 3-chloro- and 3,6-dichlorocarbazoles in soil samples at 15°C conditions. 3,6-dichlorocarbazole concentration are on right y-axis.

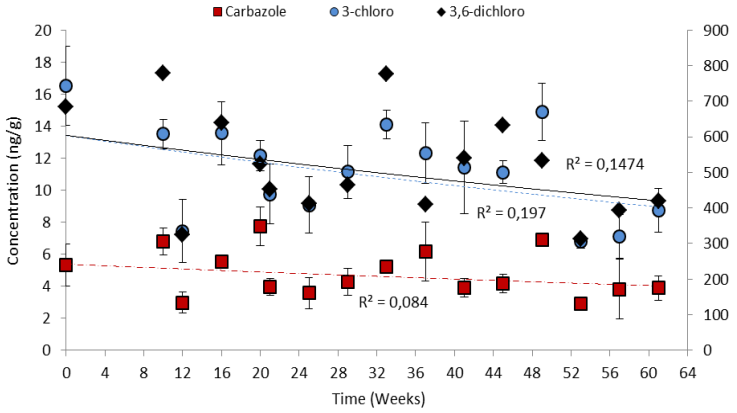

Fig 2. Mean concentrations of carbazole, 3-chloro- and 3,6-dichlorocarbazoles in soil samples at 20°C conditions. 3,6-dichlorocarbazole concentration are on right y-axis.

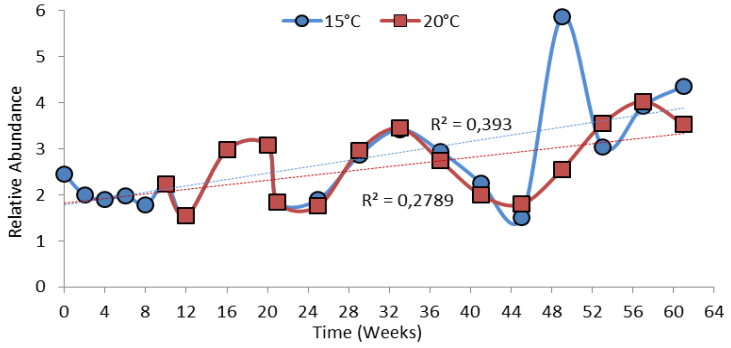

Fig 3. Relative abundance of trichlorocarbazole at 15°C and 20°C soil conditions.

The degree of halogenation could explain the dissipation of carbazole and 3-chlorocarbazole at 15°C given that 3,6-dichlorocarbazole with more chlorine atoms resisted breakdown under the same condition. Low solubility of these compounds renders them less available to biodegradation, leaching, volatilization, and plant uptake[10]. Given that persistence of halogenated aromatic compounds in soil linearly correlates with water partition coefficient of its compounds and it increases with increase in ring complexities, the number and variation in the size of the halogens therefore suggest that the persistence of halogenated carbazoles congeners follows the order, mono < di < tri < tetra < penta.

The very high concentration of 3,6-dichlorocarbazole relative to other compounds in our soil samples could be attributed to its resistance to dissipation as observed from the results of the two temperature conditions. Temperature change did not affect the rate of dissipation. There was no significant difference ($p<0.05$) in the concentration of compounds in soil samples moved from 15° to 20°C to those maintained at 15°C was observed. Trichlorocarbazole is not mentioned here due to its relatively very low concentrations.

3.2 Dioxin-like toxicity assessment

Pure compounds of 3-chlorocarbazole, 3,6-dibromocarbazole, and selected soil extracts exhibited EROD activity. The toxic equivalents (TCDD-EQ) ranged from 220 to 470 pgTCDD-EQ/g representing not only dioxin-like toxicity of halogenated carbazoles but also AhR affinity of other natural occurring compounds. The ∑TEQ for 3-chloro- and 3,6-dichloro-carbazole ranged from 0.025 to 0.069 pg/g. Dioxin-like toxicity did not decrease significantly with time whereas the sum chlorocarbazole toxic equivalence concentrations (∑TEQ) did not contribute significantly to the soil assay dioxin-like toxicity equivalent concentrations (Fig. 4).

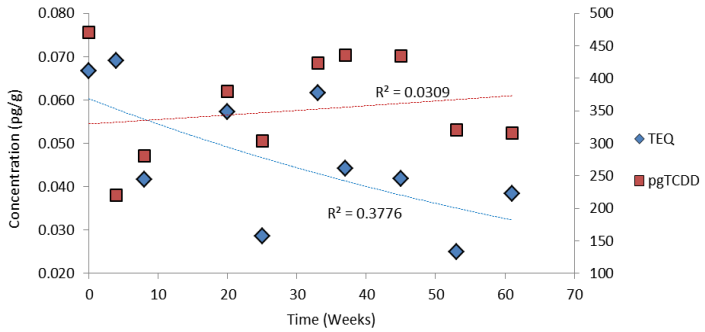

Fig 4. TEQ and EROD bioassay TCDD equivalent concentrations for soil sample extracts against time shown on the respective left and right y-axis.

Based on mQSAR modelling, mono-, di- and tri-halogenated congeners of carbazole expressed a binding affinity to AhR but not the tetra-congeners for bromo-, chloro- and iodo-carbazole. However, it cannot be concluded that tetra-congeners do not possess dioxin-equivalent toxicity. This may be possibly due to the huge size of the molecules that makes it difficult to predict their toxicity through modelling.

3.3 Implications of halogenated carbazoles in the environment

Polychlorinated aromatic hydrocarbons and nitrogen-containing heterocycles are among the extremely resistant compounds to natural degradation requiring many years to degrade under natural conditions[11]. It is evident from this study that halogenated carbazoles are persistent compounds. Coupled with low solubility and dioxin-like toxicity, this provides an opportunity to categorize these compounds as persistent organic pollutants (POPs), persistent, bioaccumulative and toxic (PBT) and very persistent and very bioaccumualtive (vPvB) substances or classified in a suitable category for better assessment.

Screening criteria have been developed for this purpose. For example, the European Commission chemical legislation on registration, evaluation, authorization and restriction of chemicals (REACH) have a criterion for PBT assessment. A persistence of > 120 days recorded for carbazole and chlorocarbazoles in this study puts them in the category of potential priority PBT substances. Carbazole and 9-ethylcarbazole have been identified as a potential PBT. 9-butyl-9H-Carbazole has been classified as high priority PBT substance. An evaluation of the less reported halogenated carbazoles but more potent compounds is required.

4.0 Acknowledgements

This research was supported by Helmholtz Zentrum München, Deutscher Akademischer Austauschdienst (DAAD) and National Council for Science and Technology-Kenya (NCST).

References:

1. Grigoriadou A, Schwarzbauer J (2011); *Water Air Soil Pollut*. 214(1-4):623–643
2. Reischl A, Joneck M, Dumler-Gradl R (2005); *UWSF - Z Umweltchem Ökotox*. 17(4):197–200
3. Tröbs L, Henkelmann B, Lenoir D, Reischl A, Schramm K (2011); *Environ Sci Pollut Res*. 18(4):547–555
4. Zhu L, Hites RA (2005); *Environ. Sci. Technol*. 39(24):9446–9451
5. Lauby-Secretan B, Baan R, Grosse Y, Ghissassi FE, Bouvard V, Benbrahim-Tallaa L, Guha N, Galichet L, Straif K (2011); *The Lancet Oncology*. 12(13):1190–1191
6. Bulloch DN, Lavado R, Forsgren KL, Beni S, Schlenk D, Larive CK (2012); *Environ. Sci. Technol*. 46(10):5583–5589
7. Mumbo J, Dieter L, Henkelmann B, Schramm K-W (2013a); *Environ Sci Pollut Res*. 20:8996-9005
8. Mumbo J, Dieter L, Henkelmann B, Schramm K-W (2013b); *Organohalogen Compounds*. 75:902-905
9. Ngigi A, Dörfler U, Scherb H, Getenga Z, Boga H, Schroll R (2011); *Chemosphere*. 84(4):369–375
10. Providenti MA, Lee H, Trevors JT (1993); *Journal of Industrial Microbiology*. 12(6):379–395
11. Lundstedt S, Haglund P, Öberg L (2003); *Environ Toxicol Chem*. 22(7):1413–1420