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In vitro synthesis and structural elucidation, occurrence and distribution, soil dissipation, aqueous photolysis and toxicity evaluation of bromo- and chlorocarbazoles

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“He gave knowledge and proficiency
in all literature and science.”

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Abstract

Bromo- and chlorocarbazoles have been detected recently in water, sediments and soil samples. They share attributes similar to persistent organic pollutants however their sources, occurrence and fate in the environment have not been explained. Information of these compounds is also limited. In this study, the enzymatic synthesis of these compounds in water, the determination of their stable congeners, their occurrence, spatial variation and vertical distribution in soil was investigated. Also assessed were their dissipation in soil, photolysis in water and dioxin-like toxicity.

Carbazole and chloroperoxidase from the fungus *Caldariomyces fumago* were assayed in vitro in the presence of hydrogen peroxide, bromide and chloride ions in different reaction ratio treatments against constant and varying enzyme concentrations. Halogenated carbazoles formed were identified by high resolution gas chromatography coupled to high resolution mass spectrometry. Mono-, di-, tri- and tetra-substituted bromo- and chlorocarbazoles were synthesized. The relative abundance of 3-substituted and 3,6-disubstituted congeners were higher in comparison to the others. Congeners with bromine and chlorine at position of C-3; C-3,6; C-1,3,6; and C-1,3,6,8 were calculated as the stable intermediate sigma complexes by density functional theory (DFT) method.

The occurrence and distribution of bromo- and chlorocarbazoles was studied in 11 forest soil samples obtained from different uniformly distributed regions in the Federal Republic of Germany. Carbazole, 3-bromocarbazole, 3-chlorocarbazole, 3,6-dibromocarbazole and 3,6-dichlorocarbazole were detected in the forest soil samples by HRGC/HRMS. Individual compound concentrations per sample ranged from 21 to 289 ng/g drw weight (DW) (carbazole); 0.2 to 9 pg/g DW (3-bromocarbazole); 0.5 to 10 ng/g DW (3-chlorocarbazole); 0.3 to 28 ng/g DW (3,6-dibromocarbazole) and 3 to 162 ng/g DW (3,6-dichlorocarbazole) with the concentrations decreasing from humic layer to the 0-5 cm and 5-10 cm mineral soil horizons. However when the dry weight concentrations (pg/g DW) of the compounds were converted to total organic carbon concentrations (pg/g TOC), an overall increase in concentration with increase in soil depth was observed suggesting the different forest soil samples had similar characteristics. Deposition and subsequent

leaching or natural formation of these compounds in soil could be responsible for increased concentration with soil depth.

The dissipation of carbazole and chlorocarbazoles in soil was assessed in a soil lysimeter under controlled conditions for 15 months in 84 soil samples obtained from a site with no recorded history of pollution. Soil samples were divided into two temperature conditions, 15°C and 20°C, both under fluctuating soil moisture conditions comprising 19 and 44 drying-rewetting cycles respectively. Carbazole, 3-chloro- and 3,6-dichlorocarbazole were detected including trichlorocarbazole not previously reported in soils using HRGC/HRMS. Carbazole and 3-chlorocarbazole showed significant dissipation ($p < 0.05$) at 15°C but not at 20°C incubating conditions indicating that low temperature could be suitable for dissipation of carbazole and chlorocarbazoles. 3,6-Dichlorocarbazole was resistant in both conditions. However, trichlorocarbazole exhibited a tendency to increase in concentration with time.

Photolysis of 3-bromo-, 3-chloro-, 3,6-dibromo- and 3,6-dichlorocarbazoles dissolved in ultra-pure water contained in a Pyrex cylindrical glass tube reactor (>290 nm wavelength) was performed by a high pressure mercury lamp. The decline in concentration and photoproducts was monitored by HRGC/HRMS and ultra high performance liquid chromatography (UHPLC). Photolysis obeyed first order degradation kinetics. The maximum wavelength of absorption and rate constant were determined as 296, 295, 301 and 299 nm; and 0.382, 0.344, 0.038 and 0.442 hr^{-1} for 3-bromo-, 3-chloro-, 3,6-dichloro- and 3,6-dibromocarbazole respectively. The half-lives fell within 1.61 to 2.01 hr except for 3,6-dibromocarbazole. Photoproducts were detected but could not be identified due to lack of standards and their concentrations were also too low for nuclear magnetic resonance (NMR) analysis.

Dioxin-like toxicity was determined by Ethoxyresorufin-O-deethylation (EROD) induction in H4IIA rat hepatoma cells assay. 3-Chlorocarbazole, 3,6-dibromocarbazole, and selected soil extracts exhibited EROD activity. Dioxin-like toxicity of these compounds in untreated soil extracts did not decrease significantly ($p < 0.05$) with time during the degradation period. The sum 3-chloro- and 3,6-dichlorocarbazole toxic equivalence concentrations (ΣTEQ) in the soil sample

that ranged from 0.025 to 0.069 pg/g did not contribute significantly to the overall soil sample dioxin-like toxicity equivalent concentrations (TCDD-EQ) which ranged from 220 to 470 pgTCDD-EQ/g. The presence of other EROD inducing compounds may have contributed to the observed TCDD-EQ since crude soil extracts were used.

Bromo- and chlorocarbazoles can be synthesized naturally with 3-mono-, 3,6-di-, 1,3,6-tri- and 1,3,6,8-tetra- substituted congeners predicted as the stable structures in the environment. They are ubiquitous, persistent and exhibit weak dioxin-like toxicity. Halogenated carbazoles should be categorized either as POPs or categorized in suitable class to enable identification of their likely sources, pathways, impacts and fate in the environment.

Zusammenfassung

Brom- und Chlorcarbazol sind jüngst in Wasser-, Sediment- und Bodenproben detektiert worden. Ihre Eigenschaften ähneln denen von persistenten organischen Schadstoffen, allerdings ist deren Herkunft, Vorkommen und Verbleib in der Umwelt noch nicht beschrieben. Darüber hinaus sind dafür notwendige Informationen über diese Verbindungen limitiert. In dieser Studie wurde die enzymatische Synthese dieser Verbindungen in Wasser und ihre stabilen Kongenere untersucht. Des Weiteren wurden das Vorkommen und die Verteilung in Deutschland und mit der Bodentiefe betrachtet. Auch die Abnahme im Boden, die Photolyse im Wasser und die dioxinartige Toxizität wurden untersucht.

Carbazol und Chlorperoxidase aus *Caldariomyces fumago* wurden in vitro in der Gegenwart von Wasserstoffperoxid, Bromid- und Chloridionen in unterschiedlichen Reaktionsverhältnissen mit konstanten und variablen Enzymkonzentrationen untersucht. Gebildete halogenierte Carbazole wurden mit Hilfe eines hochauflösenden Gaschromatographen gekoppelt an ein hochauflösendes Massenspektrometer (HRGC/HRMS), identifiziert. Mono-, di-, tri- und tetra-substituierte Brom- und Chlorcarbazole konnten in vitro synthetisiert werden. 3- sowie 3,6-substituierte Kongenere kamen mit größerer relativer Häufigkeit vor als andere. Kongenere mit Brom und Chlor an C-3, C-3,6, C-1,3,6 und C-1,3,6,8 Position sind nach der Dichtefunktionaltheorie stabile Intermediate des Sigma-Komplexes.

Das Vorkommen und die Verteilung von Brom- und Chlorcarbazolen wurde in 11 Waldbodenproben mit unterschiedlicher aber in der Bundesrepublik Deutschland gleichmäßig verteilter Herkunft untersucht. Carbazol, 3-Bromcarbazol, 3-Chlorcarbazol, 3,6-Dibromcarbazol und 3,6-Dichlorcarbazol konnten in den Waldbodenproben durch HRGC/HRMS nachgewiesen werden. Individuelle Konzentrationen der Substanzen pro Probe lagen im Bereich von 21 bis 289 ng/g Trockengewicht (DW) (Carbazol); 0,2-9 pg/g DW (3-Bromcarbazol); 0,5 bis 10 ng/g DW (3-Chlorcarbazol); 0,3-28 ng/g DW (3,6-Dibromcarbazol) und 3-162 ng/g DW (3,6-Dichlorcarbazol) mit den abnehmenden Konzentrationen von der Humusschicht (0-5 cm) zu Mineralbodenhorizonten (5-10 cm). Wenn die Trockengewichtskonzentrationen (pg/g DW) der Verbindungen aber auf den gesamten

organischen Kohlenstoff bezogen werden (pg/g TOC), konnte ein Anstieg der gesamten Konzentrationen mit zunehmender Bodentiefe beobachtet werden, was für ähnliche Charakteristika der verschiedenen Waldproben spricht. Deposition, nachfolgende Auswaschung oder natürliche Bildung dieser Verbindungen im Boden könnte für eine ansteigende Konzentration mit der Bodentiefe verantwortlich sein.

Die Abnahme von Carbazol und Chlorcarbazolen in Boden wurde unter kontrollierten Bedingungen für 15 Monate anhand von 84 Bodenproben untersucht. Diese stammten von einem Standort ohne aufgezeichnete Umweltverschmutzung. Die Bodenproben wurden verschiedenen Umwelteinflüssen unterzogen, es wurden zwei Temperaturen gewählt, 15°C und 20°C, beide unter fluktuierenden Bodenfeuchtigkeitsbedingungen bestehend aus 19 beziehungsweise 44 Trocknungs- und Befeuchtungszyklen. In diesem Testprozedere konnten Carbazol, 3-Chlor- und 3,6-Dichlorcarbazol mittels HRGC/HRMS detektiert werden, darüber hinaus Trichlorcarbazol, welches zuvor nicht in Boden gefunden werden konnte. Carbazol und 3-Chlorcarbazol zeigten signifikante Abnahme ($p < 0.05$) bei 15°C Inkubation, jedoch nicht bei 20°C, was darauf hinweisen könnte, dass niedrige Temperaturen für den Abbau von Carbazol und Chlorcarbazolen ein geeigneteres Reaktionsmilieu schaffen. 3,6-Dichlorcarbazol war gegenüber beiden Bedingungen resistent. Trichlorcarbazol zeigte jedoch die Tendenz sich im Laufe der Zeit im Boden geringfügig anzureichern. Die Konzentrationen waren für die Kernspinresonanzanalyse (NMR) jedoch zu gering.

Die Photolyse von in Reinstwasser gelösten 3-Brom-, 3-Chlor- und 3,6-Dibrom- und 3,6-Dichlorcarbazolen in einer zylindrischen Pyrex-Glasröhre reaktor (>290 nm Wellenlänge) wurde mit einer Quecksilberhochdruckdampfampe durchgeführt. Die Abnahme der Konzentration und die Produkte des Photoabbaus wurden mit HRGC/HRMS sowie Ultrahochleistungsflüssigchromatographie (UHPLC) verfolgt. Die Geschwindigkeitskonstante, Quantenausbeuten und Halbwertszeiten wurden bestimmt. Die Photolyse folgte einer Abbaukinetik erster Ordnung. Die Wellenlänge des Absorptionsmaximums und die Geschwindigkeitskonstante wurden zu 296, 295, 301 und 299 nm, und 0,382, 0,344, 0,038 und 0,442 hr^{-1} für 3-Brom-, 3-Chlor-, 3,6-Dichlor- bzw. 3,6-Dibromcarbazol bestimmt. Die Halbwertszeiten lagen zwischen

1,61 und 2,01 hr, außer für 3,6-Dibromcarbazol. Photoabbauprodukte konnten detektiert aber aufgrund von fehlenden Standardsubstanzen nicht sicher identifiziert werden.

Dioxin analog wirkende Substanzen wurden mittels der Induktion von Oxygenase CYP450 1A1, gefolgt von der Deethylierung des Substrates 7-Ethoxyresorufin zu fluoreszierendem Resorufin (EROD-Aktivität) in Rattenleberzellen (H4IIE) bestimmt. Die dioxinähnliche Toxizität von unbehandelten Bodenextrakten reduzierte sich nicht signifikant ($p < 0.05$) über Zeit während der Abbauperiode. Die Summe der toxische Äquivalenzkonzentrationen von 3-Chlor- und 3,6-Dichlorcarbazol (Σ TEQ) in der Bodenprobe (im Bereich von 0,025 bis 0,069 pg/g) hat nicht signifikant zur gesamt dioxintoxischen Äquivalenzkonzentration (TCDD-EQ), welche im Bereich von 220 bis 470 pgTCDD-EQ/g lag, beigetragen. Die Präsenz von EROD-aktivierenden Substanzen könnte zum TCDD-EQ beigetragen haben, da Bodenrohextrakte für den Test verwendet worden sind.

Brom- und Chlorcarbazole können auf natürlichem Wege als 3-mono-, 3,6-di-, 1,3,6-tri- und 1,3,6,8-tetra- substituierte Kongenere entstehen und deren Strukturen können als stabil in der Umwelt angesehen werden. Brom- und Chlorcarbazole sind ubiquitär zu finden und besitzen eine hohe Persistenz. Halogenierte Carbazole sollten daher entweder als persistente organische Schadstoffe deklariert werden oder in einer entsprechende Substanzklasse zugeordnet werden, die die Identifikation ihrer möglichen Quellen und die Untersuchung ihres Umweltverhaltens sowie Einflusses auf die Umwelt erlauben.

Publications

1 Persistence and dioxin-like toxicity of carbazole and chlorocarbazoles in soil.

Mumbo J., Henkelmann B., Abdelaziz A., Pfister G., Nguyen N., Schroll R., Munch J.C., Schramm K.W. (2014)

Environment Science and Pollution Research. Electronic publication ahead of print. doi: 10.1007/s11356-014-3386-6

2 Enzymatic synthesis of bromo- and chlorocarbazoles and elucidation of their structures by molecular modeling.

Mumbo J., Lenoir D., Henkelmann B., Schramm K.W. (2013)

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Oral presentations

- 1. Persistence and dioxin-like toxicity assessment of bromo- and chlorocarbazoles in soil. Are they a potential class of persistent organic pollutants?**

Mumbo J., Henkelmann B., Abdelaziz A., Pfister G., Schramm K.W.
The 34th International Symposium on Halogenated Persistent Organic Pollutants-Dioxin MADRID 2014. August 31st – 5th September, Madrid, Spain

- 2. Persistent bromo- and chlorocarbazoles: enzymatic synthesis and elucidation of their structures by molecular modeling.**

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Statements

Most parts of the sections on enzymatic synthesis, DFT calculations, soil dissipation and EROD toxicity assessment have been used in the following publications:

1. **“Enzymatic synthesis of bromo- and chlorocarbazoles and elucidation of their structures by molecular modeling.”** Mumbo John, Lenoir Dieter, Henkelmann Bernhard, Schramm Karl-Werner (2013). Environment Science and Pollution Research 20(12):8996–9005.” doi: 10.1007/s11356-013-1823-6
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Table of Contents

Acknowledgement.....	iii
Abstract.....	v
Zusammenfassung.....	viii
Publications.....	xi
Oral Presentation.....	xii
Statements.....	xiii
List of Tables.....	xvii
List of Figures.....	xviii
1.0 Introduction.....	20
1.1 Bromo- and chlorocarbazoles.....	20
1.2 Enzymatic oxidative halogenation.....	22
1.3 Density functional theory.....	23
1.4 Occurrence and distribution.....	25
1.5 Dissipation in soil.....	27
1.6 Photodegradation in water.....	27
1.7 Toxicity evaluation.....	30
1.8 Objectives.....	31
1.8.1 Main Objective.....	31
1.8.2 Specific Objectives.....	31
2.0 Materials and Methods.....	32
2.1 In vitro enzymatic synthesis of bromo- and chlorocarbazoles.....	32
2.1.1 Chemicals and reagents.....	32
2.1.2 Enzyme assay and water samples and preparation.....	32
2.1.3 Enzymatic halogenation of carbazole by chloroperoxidase (CPO).....	32
2.1.4 Extraction and clean-up.....	34
2.1.5 GC/MS analysis and quantification.....	34
2.2 Computational DFT calculations.....	35
2.3 Occurrence and distribution of bromo- and chlorocarbazoles in soil.....	36
2.3.1 Chemicals.....	36
2.3.2 Study area and soil sampling.....	36
2.3.3 Extraction and clean-up.....	38
2.3.4 GC/MS analysis and quantification.....	38
2.3.5 Quantification criteria.....	39
2.3.6 Recovery rates.....	39
2.3.7 Blank samples.....	39

2.3.8 Control samples	40
2.3.9 Statistical analysis	40
2.4 Dissipation of bromo- and chlorocarbazoles in soil	40
2.4.1 Chemicals	40
2.4.2 Soil physical properties.....	40
2.4.3 Soil homogenization and bacterial cell count.....	41
2.4.4 Soil water potential at -15kPa and soil mass for incubation experiment.....	42
2.4.5 Experimental design.....	43
2.4.6 Sample collection, extraction and clean-up.....	44
2.4.7 GC/MS analysis and quantification	45
2.4.8 Criteria for selecting compounds for dissipation.....	46
2.4.9 Method validation	46
2.4.10 Statistical analysis	46
2.5 Photodegradation of bromo- and chlorocarbazoles.....	46
2.5.1 Chemicals	46
2.5.2 Maximum Wavelength of Absorbance	47
2.5.3 Photodegradation experiments.....	47
2.5.4 Sample extraction.....	51
2.5.5 GC/MS analysis and quantification	51
2.5.6 UHPLC-MS analysis.....	51
2.6 Toxicity evaluation	52
2.6.1 Reagents and chemicals	52
2.6.2 Cell culture	53
2.6.3 EROD induction bioassay.....	53
2.6.4 REP calculations	54
3.0 Results and Discussions.....	55
3.1 In vitro enzymatic synthesis of bromo- and chlorocarbazoles	55
3.1.1 Enzymatic Bromination Reactions	55
3.1.2 Enzymatic Chlorination Reactions	56
3.1.3 Enzymatic halogenation in the environment	59
3.2 DFT calculations.....	60
3.2.1 Structure elucidation.....	60
3.2.2 Role of enzymes and regioselectivity in halogenation reactions	62
3.3 Occurrence and distribution of bromo- and chlorocarbazoles in soil	64
3.3.1 Occurrence and concentrations.....	64
3.3.2 Relationship between organic matter and halogenated carbazoles conc.....	66
3.3.3 Possible role of industrial emissions	68

3.3.4 Haloperoxidase-like natural formation of halogenated carbazoles	69
3.3.5 Halogenated dyes and <i>p</i> -chloroaniline herbicides.....	70
3.4 Dissipation of bromo- and chlorocarbazoles in soil	71
3.4.1 Isolation, identification and concentration	71
3.4.2 Dissipation of carbazole and its chlorocarbazoles congeners	74
3.5 Photodegradation of bromo- and chlorocarbazoles in water	78
3.5.1 Determination of Wavelength of Maximum Absorption	78
3.5.2 Photodegradation rates and quantum yields.....	80
3.5.3 Effect of hydrolysis and volatilization	84
3.5.4 Photoproducts	84
3.6 Dioxin-like toxicity assessment	86
Conclusions.....	88
Recommendations	89
Appendix	90
References.....	109

List of Tables

Table 1	Information describing forest types, quantity of soil samples collected per region in Germany and the number of samples.	37
Table 2	Summarized results of DFT calculations of the intermediate sigma complexes of bromocarbazoles.	60
Table 3	Summarized results of DFT calculations of the intermediate sigma complexes of chlorocarbazoles.	61
Table 4	Percentage total organic carbon (TOC %) and concentrations (pg/g dry weight) of carbazole and bromo- and chlorocarbazoles.	65
Table 5	Comparison of concentrations of carbazole and halogenated carbazoles recorded in this study to those in selected previous studies.	66
Table 6	Carbazole and halogenated carbazoles concentration ratios based on dry weight (DW) concentrations (pg/g DW).	67
Table 7	Carbazole and halogenated carbazole concentration ratios expressed according to percentage total organic carbon in each soil horizon.	68
Table 8	Rate constant, half-lives and molar absorbtivity of bromo- and chlorocarbazole.	81
Table 9	TEQ values for 3-mono- and 3,6-dichlorocarbazole calculated from respective REP values from selected 10 soil samples extracts.	87
Table 10	Criteria for categorization of halogenated carbazoles as POPs.	89
Table 11	Determination of the intensity of irradiation lamp.	103
Table 12	Quantum yield determination of bromo- and chlorocarbazoles.	103
Table 13	Solar rate constant determination of 3-bromocarbazole.	105
Table 14	Solar rate constant determination of 3-chlorocarbazole.	106
Table 15	Solar rate constant determination of 3,6-dibromocarbazole.	107
Table 16	Solar rate constant determination of 3,6-dichlorocarbazole.	108

List of Figures

Figure 1	Congeners of bromo- and chlorocarbazole detected in the environment.	20
Figure 2	Hammonds postulate illustrated.....	24
Figure 3	A map showing the site where soil samples used in the incubation experiment were collected, in the State of Bavaria, Germany.	40
Figure 4	A picture illustrating how moist soil sample was compacted in the incubation flasks to achieve the required density of 0.97 g/cm ³	42
Figure 5	Normalised relative abundances of bromocarbazoles formed in different reaction mixtures.....	55
Figure 6	Gas chromatograms of bromocarbazoles	56
Figure 7	Chlorocarbazoles synthesized in different reaction mixtures.....	57
Figure 8	Gas chromatograms of chlorocarbazoles	58
Figure 9	Compounds identified in the environment with similar substitution positions predicted here.....	62
Figure 10	The formation of bromocarbazoles (A) and chlorocarbazoles (B) in different reaction treatments.....	63
Figure 11	Ortho and para positions on carbazole.	64
Figure 12	Averaged concentrations of carbazole, 3-chloro- and 3,6-dichlorocarbazoles detected in soil samples at 15°C conditions.....	73
Figure 13	Averaged concentrations for carbazole, 3-chloro- and 3,6-dichlorocarbazoles detected in soil samples at 20°C temperature conditions.....	73
Figure 14	The relative abundance of trichlorocarbazole with time for 15°C and 20°	74
Figure 15	Effect of temperature change from 15° to 20°C	77
Figure 16	Absorption spectrum of 3-bromocarbazole.	78
Figure 17	Absorption spectrum of 3,6-dibromocarbazole.....	79
Figure 18	Absorption spectrum of 3-chlorocarbazole.....	79
Figure 19	Absorption spectrum of 3,6-dichlorocarbazole.....	80
Figure 20	Photodegradation of 3-bromocarbazole in water	82
Figure 21	Photodegradation of 3,6-dibromocarbazole in water	82
Figure 22	Photodegradation of 3-chlorocarbazole in water.....	83
Figure 23	Photodegradation of 3,6-dichlorocarbazole in water.....	83
Figure 24	Photodegradation experiment set-up.....	90
Figure 25	An illustration of the cylindrical glass tube reactor on a magnetic stirrer.	91
Figure 26	Mass spectrum of monobromocarbazole	92
Figure 27	Mass spectrum of dibromocarbazole	92
Figure 28	Mass spectrum of tribromocarbazole	93

Figure 29	Mass spectrum of tetrabromocarbazole.....	93
Figure 30	Mass spectrum of dichlorocarbazole.....	94
Figure 31	Mass spectrum of trichlorocarbazole	94
Figure 32	Mass spectrum of tetrachlorocarbazole	95
Figure 33	Combined mass spectrum of the suspect MH^+ 198.0644.....	96
Figure 34	Combined mass spectrum of the suspect MH^+ 216.0725.....	97
Figure 35	Combined mass spectrum of the suspect MH^+ 363.0997	98
Figure 36	Combined mass spectrum of the suspects MH^+ 198.0625 and MH^+ 213.0828	99
Figure 37	Combined mass spectrum of the suspect MH^+ 216.0650	100
Figure 38	Combined mass spectrum of the suspect MH^+ 363.1183	101
Figure 39	Combined mass spectrum of the suspect MH^+ 216.0659	102
Figure 40	Molar extinction coefficient determination of bromo- and chlorocarbazoles.....	104

Introduction

1.1 Bromo- and chlorocarbazoles

Recent studies have shown the presence of halogenated carbazoles in sediments, soils and water. 1,3,6,8-Tetrabromocarbazole including other bromocarbazole congeners were found for the first time in the environment of the sediment cores of Lake Michigan, USA (Zhu and Hites 2005). 3-Chlorocarbazole and 3,6-dichlorocarbazole were isolated in Bavarian soils (Tröbs et al. 2011, Reischl et al. 2005) and the latter in sediments of Lippe River in Germany (Kronimus et al. 2004). Dibromo-, 3-chloro- and 3,6-dichlorocarbazoles were also detected in the sea and soil samples of the Kavala city of Greece (Grigoriadou and Schwarzbauer 2011). Polychlorinated carbazoles were also detected in soil samples from sites contaminated by chloralkali processes in Japan and from a historic site of chlorine production in Germany (Takasuga et al. 2009). Halogenated carbazoles belong to the group of heterocyclic aromatic hydrocarbons some of which are known hazardous environmental pollutants (Seo et al. 2009). Halogenated carbazoles containing both bromine and chlorine halogens (mixed forms) have recently been detected in sediments (Guo et al. 2014). The frequency of their detection in environmental samples provides the need to understand their origin and occurrence given their potential for persistence and toxicity in the environment.

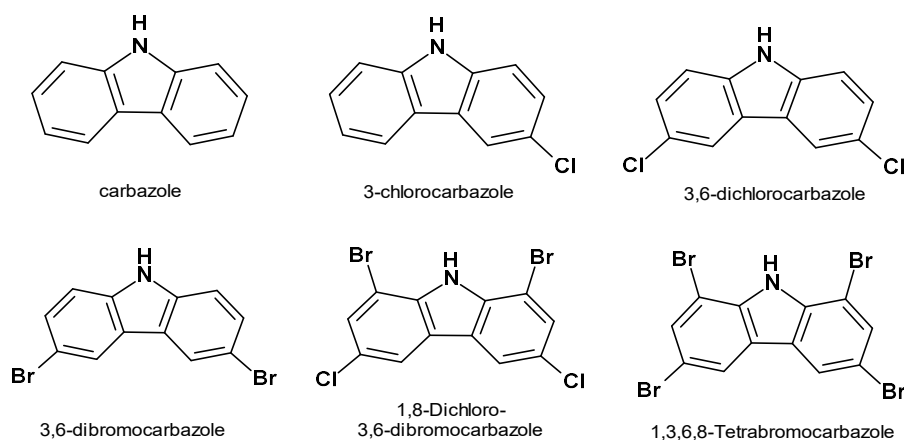


Figure 1 Congeners of bromo- and chlorocarbazole that have been detected in the environment.

3,6-Dichlorocarbazole has dioxin-like toxicological potential (Tröbs et al. 2011). Carbazole in itself is carcinogenic and toxic (Tsuda et al. 1982). Its derivatives are toxic (Sverdrup et al. 2002) and mutagenic (Jha and Bharti 2002). They undergo further transformation generating more toxic hydroxynitro-carbazoles (Benedik 1998). 8-Chlorocarbazole-1-acetic acid, a photodegradation intermediate product of diclofenac, was found to cause cell lysis (Encinas et al. 1998). Just like the polycyclic aromatic hydrocarbons (PAHs) (Seo et al. 2009), they have petrogenic, pyrogenic and biogenic sources. Carbazoles are commonly found in environmental matrices contaminated by coal tar, crude oil and creosote, (Kilbane et al. 2002) and co-exist with other aromatic compounds including PAHs. Dibenzocarbazole, a carcinogen, including other heterocycles such as dibenzofurans and dibenzothiophenes are emitted during combustion processes (Junk and Ford 1980). It has been suggested that halogenated carbazoles could also be of natural origin. (Grigoriadou and Schwarzbauer 2011) reported that 3-chlorocarbazole and 3,6-dichlorocarbazole in soil and sediment samples could be the result of xenobiotic formation while (Zhu and Hites 2005) alluded that bromocarbazoles in sediments could be naturally derived.

Sources and environmental fate of bromo-, chloro- and iodocarbazoles have not been well documented. Tetrabromocarbazole is produced during the combustion of 2,4,6-tribromoaniline, a known flame retardant (Kaur Bindra and Narang 1995). Bromo-, chloro-, and iodocarbazoles have been reported from natural sources such as the marine heterocystous cyanobacterium, *Kyrtuthrix maculans* (Lee et al. 1999) and bovine urine (Luk et al. 1983). Similarly, carbazole and its alkaloids such as 3-methylcarbazole (Schmidt et al. 2012) have natural sources. At the moment, sediments (Grigoriadou and Schwarzbauer 2011), (Zhu and Hites 2005) are the known sinks for bromocarbazoles and its congeners. According to available information, bromo- and iodocarbazoles have not been detected in soil. Sediments (Grigoriadou and Schwarzbauer 2011), (Kronimus et al. 2004) and soils are known sinks for chlorocarbazoles. The highest concentrations of 3-chloro- and 3,6-dichlorocarbazoles in soil have been reported in the E-horizon but not in the organic layer (Reischl et al.

2005). The fate of these compounds in soil, sediments and in other environmental compartments is however not known.

1.2 Enzymatic oxidative halogenation

Chlorination of carbazole proceeds most likely by a type of electrophilic chlorination of the aromatic moiety that is an enzymatically controlled oxidative chlorination. Bromination and chlorination of natural aromatic structures are enzymatically controlled oxidation processes of the bromide and chloride ions yielding electrophilic bromine (Br^+) and chlorine (Cl^+) as reactive species (Neidleman and Geigert 1986). Haloperoxidases are peroxidase enzymes known to catalyze the incorporation of halogen atoms into organic molecules in the presence of halide ions and peroxides such as H_2O_2 (Neidleman and Geigert 1986, Hofmann et al. 1998). Bromoperoxidase, chloroperoxidase and iodoperoxidase are the known haloperoxidases. Chloroperoxidase (CPO) has several species of enzymes some of which are the heme CPO of *Caldariomyces fumago* and the vanadium CPO of *Curvularia inaequalis*. The reaction mechanism involves formation of a halogenium ion (X^+) or hypohalous acid (HOX) intermediate by the CPO (van Schijndel et al. 1994, Wagenknecht and Woggon 1997) to effect electrophilic substitution with electron-rich aromatic substrates (Libby et al. 1992).

Halogenated metabolites are frequent in nature. They are produced by many organisms and have been isolated from bacteria, fungi, marine algae, lichens, higher plants, mammals and insects (Gribble 2003). Bromine-containing metabolites are the most abundant naturally synthesized organohalogens by organisms living in marine environments due to the relatively high bromine concentration in sea water compared to soil (Neidleman and Geigert 1986). In contrast chlorinated metabolites are preferentially produced by terrestrial organisms (de Jong et al. 1992). The first chlorinating enzyme was isolated from the fungus *Caldariomyces fumago* (Morris and Hager 1966). Bromoperoxidase has also been isolated from different algae and sea urchins eggs (Deits et al. 1984). It has been reported that haloperoxidase enzymes lack both substrate and regio-specificity in comparison to flavin-dependent halogenases (Pée and Patallo 2006,

van Pée 1996). However, *Caldariomyces fumago* has been able to chlorinate aromatic hydrocarbon including PAHs yielding mono-, di- and tri-chlorinated compounds (Vázquez-Duhalt et al. 2001, Wannstedt et al. 1990, Dembitsky 2003).

1.3 Density functional theory

Molecular modelling based on density functional theory (DFT), a quantum mechanical calculations method, has been used previously to calculate the most stable products of the chlorinated carbazoles within the mono- and di- homologue groups (Tröbs et al. 2011). The determination of substitution positions on aromatic compounds based on this method follows the general principle of electrophilic substitution of aromatic system where the intermediate sigma (σ) complex is the rate and product determining step. It is not the stability of the formed products but the stability of the intermediate σ -complex (Smith and March 2007). According to Hammond's postulate, the rate-determining step involves the formation of a transition state that closely resembles the intermediate referred to as the σ -complex (Carey and Sundberg 2008). The rate-determining step's activation energy can be estimated according to the energy of the intermediate σ -complex. A more stable low-energy intermediate σ - complex results in low activation energy and a high reaction rate, while a less stable high-energy intermediate σ - complex results in a high activation energy and a low reaction rate. The more stable intermediate mechanism with the lowest activation energy also determines regioselectivity. It is the fastest rate determining intermediate yielding the major product (Fox and Whitesell 2004).

Carbazole undergoes electrophilic aromatic substitution reaction with various chlorinating agents. Halogenation oxidation via an electrophilic bromine (Br^+), chlorine (Cl^+) or iodine (I^+) is also the more common mechanism for enzymatic halogenation of natural products (Fujimori and Walsh 2007). DFT method is therefore more appropriate compared to calculations based on the charge densities of the product at ground state (Bonesi and Erra-Balsells 1997). DFT determined halogenation substitution is also

consistent with electrophilic substitutions on carbazole showing strong activation at ortho and para positions to nitrogen on the compound (Katritzky and Taylor 1990).

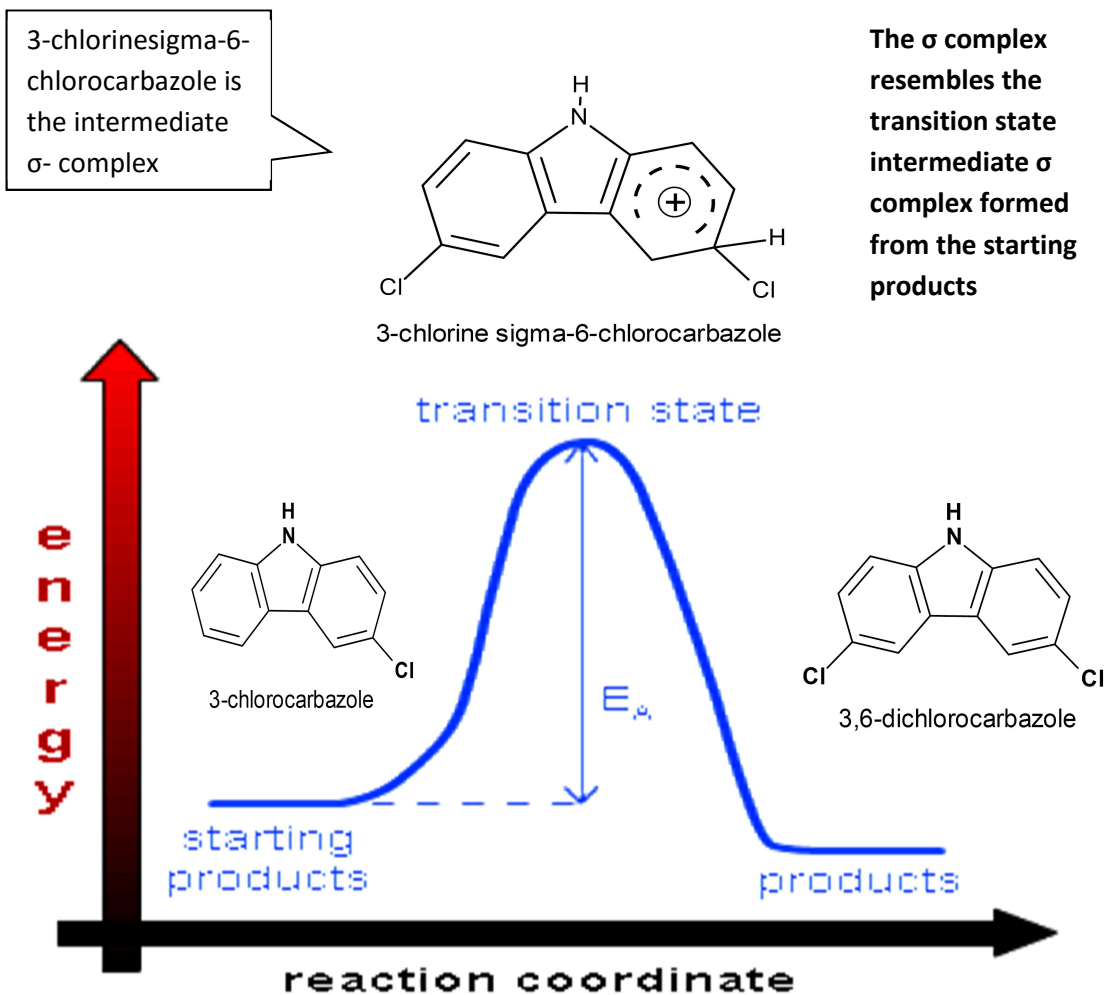


Figure 2 Hammonds postulate illustrated.

1.4 Occurrence and distribution

The environmental trends of bromo- and chlorocarbazoles are not known but they could be widespread given that 3,6-dichlorocarbazole has been detected in soil from 200 locations of different land-uses (Reischl et al. 2005). Bromo- and chlorocarbazoles have been detected in sediments (Grigoriadou and Schwarzbauer 2011) with the latter also in soil (Tröbs et al. 2011). Bromocarbazoles have not been reported in soil. They have not been categorized into a specific group of environmental pollutants but they possess attributes similar to persistent organic pollutants (POPs) and / or persistent bioaccumulative and toxic (PBTs) substances. At the moment sediments have been reported as the major sink of the bromo- and chlorocarbazoles. According to literature, 3-chlorocarbazole and 3,6-chlorocarbazole (Reischl et al. 2005) are the exceptions which have been detected in soil. It is not known whether sediments and soils influence preferential formation of the halogenated carbazole congeners recipient or resident in their matrices or they simply act as sinks. According to previous studies, tetra-substituted halogenated congeners have been detected only in sediments while the mono-, di- and tri-substituted halogenated congeners have been detected in both soil and sediments. 1,3,6,8-Tetrachlorocarbazole detected in the sediment cores of Buffalo River, New York, USA is suggested to be of anthropogenic origin. The river is considered to be heavily polluted by industrial and municipal sewage discharges (Kuehl et al. 1984). In a recent study (Pena-Abaurrea et al. 2014), 3,6-dibromo-1,8-dichlorocarbazole was detected in the sediment extracts but no mono- and di-substituted bromo- and chlorocarbazoles were detected. On the other hand, bromocarbazoles, have been reported in aquatic sediments (Pena-Abaurrea et al. 2014), (Grigoriadou and Schwarzbauer 2011), (Zhu and Hites 2005) but not in soil. It is not known whether this could be indicative of their preferred sinks.

In the study by Reischl et al. (2005) maximum concentrations of chlorocarbazoles were observed to occur in the A- (Ap 0-24 cm) and upper-B (Go-Ah 24-39 cm) horizons of forest soils rather than in the organic layers. It is suggested that the mediation of haloperoxidase catalyzed halogenation of organic matter by inorganic chlorine could be

responsible for this pattern (Latumus et al. 1995). A halogenating chloroperoxidase-like activity has been observed in soil extracts (Asplund et al. 1993). The highest oxidation activity of chloride and bromide was reported in the organic layer and it decreases with depth being lowest at A and E horizons (Latumus et al. 1995). A recent study has also shown that Fe (III), an electron acceptor, without sunlight or micro-organisms, can catalyze the halogenation of organic matter in soil and sediment (Keppler et al. 2000). It has however not been shown that these kind of formative mechanisms could be responsible for the presence of halogenated carbazoles in forest soils considered not exposed to anthropogenic sources of pollution.

Based on information available in literature, there are no studies on the trends of halogenated carbazoles in the environment. Previous studies have reported halogenated carbazoles as the unknown, non-target or unintentional compounds (Pena-Abaurrea et al. 2014), (Grigoriadou and Schwarzbauer 2011), (Zhu and Hites 2005). Their occurrence and distribution beyond the sampling point is often not reported. Therefore limited information is available on the extent of exposure and their effects in the environment given their persistence and dioxin-like toxicities. Since the locations of the sampling points are isolated, compounds detected may vary. Knowledge of their patterns and trends in the environment would provide indicators to predict the most likely sources, formation mechanisms, pathways and ultimately their sinks. Germany has experienced previous historic industrial activities whose emissions have been suggested to be responsible for occurrence of environmental contaminants and forest acidification in some federal states (Gocht et al. 2007), (Steinbrecher et al. 2000), (Jüttner 1997), (Steinberg et al. 1988). Direct evidence for long range atmospheric transport of POPs from the source to deposition on pine needle has been reported (Chen et al. 2012), (Klánová et al. 2009), (Eriksson et al. 1989). However this correlation has not been done with regard to halogenated carbazoles.

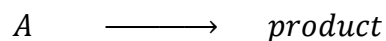
1.5 Dissipation in soil

A resistance to degradation of 1,3,6,8-tetrabromocarbazole in the sediment was observed by (Zhu and Hites 2005). Biodegradation is the dominant process of PAHs dissipation in soil (Lundstedt et al. 2003). Its however highly dependent on abiotic soil factors such as pH, temperature (Leahy and Colwell 1990), moisture content (Davis and Madsen 1996) and organic matter (Manilal and Alexander 1991). Optimal pH conditions in the neutral region (6.5 to 8.0) are suitable for PAHs and petroleum hydrocarbons degradation (Kästner et al. 1998). Bacterial degradation is dominant between pH 6 and pH 8 while for fungi at pH below 5.5 (Eweis and Eweis-Ergas-Chang-Schroeder 1998). A soil water potential of -15 kPa has been identified as suitable for optimal pesticide mineralization (Schroll et al. 2006). Fluctuating soil humidity was found to increase bioavailability and degradation of pesticides (Ngigi et al. 2011; Sebai et al. 2010; Alletto et al. 2006). Generally, a temperature range between 15°C and 45°C is considered ideal with maximum degradation occurring between 25°C to 35°C (Sims et al. 1990). However, *Pseudomonas species* strain C3211 isolated from temperate climate soil was able to degrade carbazole, dibenzothiophene and dibenzofuran faster at 10°C than 25°C (Jensen et al. 2003) illustrating the significant role of microorganisms in biodegradation in soil with some adapted to low temperature conditions. The fate of halogenated carbazoles in soil has not been reported most likely since they are emerging compounds with limited information in literature.

1.6 Photodegradation in water

Photodegradation is the major pathway through which aquatic contaminants are removed from surface water. This may occur by direct or indirect photolysis whereby in the former, a photon is absorbed by a compound, leading to bond cleavage or rearrangement to form a new stable product in the former. In the latter, the interaction of sunlight with organic matter generate photochemically reactive intermediates such as singlet oxygen (1O_2), hydroxyl radical ($\cdot OH$) or triplet state natural organic matter (3NOM) that react with pollutants (Werner et al. 2005). Photodegradation of organic compounds in water obeys either first-order kinetics (equation 1) (Kochany and Maguire

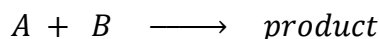
1994) or pseudo first-order kinetics (equation 3) (Filipe et al. 2013), (Yang et al. 2010), (Konstantinou et al. 2001). Photochemical reactions have been suggested to follow the latter (Larson et al. 1981), (Peiró et al. 2001).



$$\text{rate} = \frac{\Delta [A]}{\Delta t} = k[A] \quad (1)$$

$$t = \frac{1}{k} \ln \frac{[A]_0}{[A]_t}$$

where t is time, k is the rate constant (s^{-1}), \ln is the natural logarithm, $[A]_0$ and $[A]_t$ are the concentrations of A at time $t = 0$ and $t = 1$, respectively. However, in the case of two reactants $[A]$ and $[B]$;



$$\text{rate} = k[A][B] \quad (2)$$

if $[B] \gg [A]$ then $[B]$ will be constant during the reaction, the rate law shall be;

$$\text{rate} = k[A][B] = k_{pseudo} [A] \quad (3)$$

The rate constant k_{pseudo} , called the pseudo-first order rate constant, is given by $k_{pseudo} = k[B]$ where k is second-order rate constant. The first order kinetic is given by;

$$\frac{dc}{dt} = kc$$

where c is concentration, t is time and k the rate constant ($\text{Lmole}^{-1}\text{sec}^{-1}$).

The photolysis and photodegradation kinetics of halogenated carbazoles in water has not been reported. Carbazole and dibenzo-(c,g)carbazole obey the first order kinetics in pure and natural water. Their half-lives in pure water are 2.7 hr and 51 min respectively under natural sunlight (Smith et al. 1978). Carbazole undergoes direct photolysis in distilled water (Zika and Cooper 1987), (Picel et al. 1987). Its derivatives undergo photolysis in organic solvents (Bonesi and Erra-Balsells 1997), (Bonesi and Erra-Balsells 1991), (Picel et al. 1987). Due to poor solubility in water, bromo- and chlorocarbazole are liable to sorption (absorption, adsorption and ion exchange) and biodegradation processes. In this case, direct photolysis can occur when these compounds are attached to suspended solids, sediments or organic exposed to sunlight (Chen et al. 2011). They can be adsorbed to organic matter that accelerate (Gerecke 2001) or decrease photodegradation (Bertilsson and Widenfalk 2002), (Oliver et al. 1979). Equally, indirect photolysis may take place through reaction with $\cdot\text{OH}$ generated when nitrate (NO_3^-) is photolyzed in water (Remucal 2014), (Kochany and Maguire 1994), (Zepp et al. 1987). They may also react with reactive oxygen species formed upon photolysis of dissolved organic matter (DOM) or dissolved humic substances (DHS). In some cases, this may occur by direct reaction with photochemically generated excited states of DOM or DHS (Miller and Chin 2005), (Gerecke 2001).

The formation of bromo- and chlorocarbazole derivatives and photoproducts with enhanced toxicity may occur during the photolysis of organic compounds (Sarakha 1993). 8-Chlorocarbazole-1-acetic acid, carbazole-1-acetic acid and 8-hydroxycarbazole-1-acetic acid, photoproducts of the pharmaceutical drug diclofenac, are suggested to be toxic (Svanfeldt et al. 2010). Hydroxycarbazole is mutagenic with activity reportedly depending on the substitution position of the hydroxyl group, the relative order being $1 \geq 4 > 2 = 3 >$ carbazole (Rasmussen et al. 1991). Nitrocarbazoles are also mutagenic (LaVoie et al. 1981). 3,6-Dinitrocarbazoles and the mixed nitrate-hydroxy forms such as 2-hydroxy-3-nitrocarbazole have been shown to be the most mutagenic towards *Salmonella typhimurium* strain TA98 (Holloway and Ball 1993). 1-Chlorocarbazole, a photoproduct of diclofenac and meclofenamic acid, has been

suggested to be responsible for the phototoxicity of these drugs as it is active photobiologically and able to generate radicals upon photolysis (Encinas et al. 1998).

1.7 Toxicity evaluation

Carbazole and 3,4,5,6-dibenzocarbazole are carcinogenic with the latter also mutagenic (Lauby-Secretan et al. 2011). Not much has been reported on the toxicity and bioaccumulative potentials of bromo-, chloro- and iodocarbazoles although 3,6-dichlorocarbazole has dioxin-like toxicity and persistent to degradation (Tröbs et al. 2011). It is also not known whether degradation decreases or enhances toxicity of carbazole and that of its halogenated congeners such as 3,6-dichlorocarbazole and 3,4,5,6-dibenzocarbazole. Belkin et al. (1994) demonstrated that biodegradation of PAHs led to an enhanced toxicity and genotoxicity even though it led to the elimination of original compounds. Biodegradation caused a decrease in toxicity in the bioremediation of a crude oil polluted soil (Chaîneau et al. 2003). Planar halogenated hydrocarbons such as polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) or structurally similar compounds are toxic and bioaccumulate in fish, as well as in the aquatic food chain through to the wildlife and humans. They cause cytochrome P450 1A1 (CYP1A1) induction. Ethoxyresorufin-o-deethylase (EROD) activity, used as an indicator for cytochrome P450 1A1 activity, has only been observed in 3,6-dichlorocarbazoles (Tröbs et al. 2011) indicating the potential toxic potency of halogenated carbazoles besides other attributes they possess that could be similar to PCBs and dioxins.

1.8 Objectives

1.8.1 Main Objective

To study the formation, occurrence, distribution, degradation and toxicity of bromo- and chlorocarbazoles in water and soil.

1.8.2 Specific objectives

1. To conduct enzymatic synthesis of bromo- and chlorocarbazoles in water.
2. To determine the stable isomers of the synthesized bromo- and chlorocarbazoles using density function theory (DFT) calculations.
3. To assess the occurrence and distribution of bromo- and chlorocarbazoles in soil.
4. To set-up soil incubation experiment to assess the degradation of bromo- and chlorocarbazoles in soil under different temperature conditions.
5. To perform direct photodegradation of bromo- and chlorocarbazoles in water.
6. To determine the dioxin-like toxicity of bromo- and chlorocarbazoles.

Materials and Methods

2.1 In vitro enzymatic synthesis of bromo- and chlorocarbazoles

2.1.1 Chemicals and reagents

Pure compounds and standards of carbazole $\geq 95\%$ (GC), 3-bromocarbazole $\geq 97\%$ (GC), 3-chlorocarbazole and 3,6-dibromocarbazole ($\geq 97\%$) were purchased from Sigma Aldrich (Taufkirchen, Germany). Hydrogen peroxide, potassium phosphate buffer salt, dichloromethane, purified chloroperoxidase (CPO) from *Caldariomyces fumago*, potassium chloride and sodium bromide were supplied by Sigma Aldrich (Taufkirchen, Germany). All chemicals were of analytical grade and solvents of pico grade quality.

2.1.2 Enzyme assay and water samples and preparation

0.1 M Potassium dihydrogen phosphate (KH_2PO_4) buffer solution of pH 3.0 at 25°C was prepared in distilled water. $11.96\ \mu\text{M}$ Carbazole solution was then prepared by dissolving carbazole in 0.1 M KH_2PO_4 buffer solution prepared earlier. 13.5% Ethanol ($\text{C}_2\text{H}_5\text{OH}$) was used to increase the solubility of carbazole in water. $11.96\ \mu\text{M}$, $119.6\ \mu\text{M}$ and $11.96\ \text{mM}$ potassium chloride (KCl) solutions were prepared separately by dissolving KCl in buffer solution prepared earlier while ensuring the pH 3 at 25°C was maintained. $5.98\ \mu\text{M}$, $35.88\ \mu\text{M}$ and $0.598\ \text{mM}$ hydrogen peroxide (H_2O_2) solutions were prepared separately by diluting 30% (w/w) H_2O_2 in distilled water. To have enough enzyme material for the analysis, 200 mL CPO enzymatic assay was prepared separately by dissolving 0.03 mL CPO, approx 1263.6 units, in 200 mL cold buffer solution of 0.1 M KH_2PO_4 buffer solution of pH 3.0 at 25°C equivalent to 6.345 units/mL.

2.1.3 Enzymatic halogenation of carbazole by chloroperoxidase (CPO)

In order to find out parameters that influence formation of halogenated carbazoles, the concentration of carbazole was kept constant against varying concentrations of the enzymes, bromides, chlorides and hydrogen peroxide. Temperature of the buffer solution, pH=3.0, was also kept constant at 25°C . NaBr and KCl were used in bromination and chlorination reactions respectively. H_2O_2 was kept low due to its

potential inhibitory effect at high concentrations (above 25 mM during biocatalysis) (Nicell and Wright 1997) mostly when used as a co-substrate for peroxidase activity.

In vitro enzymatic reactions of different reactant ratio treatments were carried out. The halides were dissolved in 0.1 M KH_2PO_4 buffer solution containing 11.96 μM of the substrate (carbazole) and $\text{C}_2\text{H}_5\text{OH}$, followed by the addition of *Caldariomyces fumago* CPO at intervals of 5 min for the next 20 min. The reaction was started with the addition of H_2O_2 semi-continuously at every interval during incubation. The reaction mixture was stirred on a water bath at 25 °C and stopped after 20 min. The reaction mixture was then removed from the water bath and stored under -28°C in a refrigerator. Enzyme concentration was however kept constant for all bromination reactions treatments.

2.1.3.1 Bromination reaction mixtures: *Rx.1 (2:2:1 ratio)*, *Rx.2 (1:10:3 ratio)* and *Rx.3 (1:1000:50 ratio)* treatments

CPO concentration was kept constant in all reaction mixtures. However, the reaction mixtures composition described by *Rx.1*, *Rx.2* and *Rx.3* treatments varied. *Rx.1* contained 11.96 μM carbazole, 11.96 μM NaBr, 5.98 μM H_2O_2 ; *Rx.2* contained 11.96 μM carbazole, 119.6 μM NaBr, 35.88 μM H_2O_2 ; while *Rx.3* contained 11.96 μM carbazole, 11.96 mM NaBr, 0.598 mM H_2O_2 , all in separate solutions each of 20 mL 0.1 M potassium phosphate buffer at pH 3.0 and 2.1% ethanol. CPO was added to each stirring mixture at 25°C at 5 min intervals in 2 units aliquot during the 20 min incubation period (10 units in total). The blank did not contain CPO enzyme.

2.1.3.2 Chlorination reaction mixtures: *Rx.4 (2:2:1 ratio)*, *Rx.5 (1:10:3 ratio)*, *Rx.6 (1:1000:1 ratio)* and *Rx.7 (1:1000:50 ratio)* treatments

Under chlorination reaction, CPO concentration was not constant but varied. The reaction mixtures composition described by *Rx.4*, *Rx.5*, *Rx.6* and *Rx.7* treatments also varied. *Rx.4* contained 11.96 μM carbazole, 11.96 μM KCl, 5.98 μM H_2O_2 , and 32 units aliquots CPO (6.4 units of CPO added at 5min intervals); while *Rx.5* contained 11.96 μM carbazole, 119.6 μM KCl, 35.88 μM H_2O_2 and 159 units aliquots CPO (31.8

units of CPO added at 5 min intervals); *Rx.6* contained 11.96 μM carbazole, 11.96 mM KCl, 11.96 μM H_2O_2 and 10 units (2 units of CPO added at 5 min intervals) and finally *Rx. 7* with 11.96 μM carbazole, 11.96 mM KCl, 0.598 mM H_2O_2 and 317 units aliquots CPO (63.4 units of CPO added at 5 min interval) all in separate solutions of 20 mL 0.1 M KH_2PO_4 buffer pH=3.0 and 2.1% ethanol. CPO was added to the stirring mixture at 25°C during the 20 min incubation period. Blank did not contain CPO enzyme.

2.1.4 Extraction and clean-up

Dichloromethane, DCM (CH_2Cl_2) was used to extract the synthesized halogenated carbazoles from the reaction mixtures with the use of a separation funnel. 300 μL of the extracted solution was then subjected to a clean-up using column chromatography packed with silica gel, deactivated alumina and sodium sulphate to remove any material that could cause interference. The extract was eluted using hexane: DCM (1:1). The eluted extract was then concentrated by rotary evaporation to approximately 1 mL. 0.5 mL acetonitrile was added to the concentrate and then reduced under a steady flow of gentle nitrogen gas to approximately 0.5 mL. The sample was subjected to another clean-up through C18 column using acetonitrile as the eluent. This was followed by concentration of the eluted extract under a steady flow of gentle nitrogen to approximately 0.2 mL before being transferred to amber vials. Syringe standards (Pentachlorotoluene and 1,2,3,7,8,9-Hexachlorodibenzo-p-dioxin- $^{13}\text{C}_{12}$) were added to the transferred cleaned extract in the vials. The extract and standard were concentrated thrice using hexane under a steady but gentle flow of nitrogen gas to a final volume of 20 μL . The amber vials were sealed and then taken for analysis using high resolution gas chromatography coupled to high resolution mass spectrometry.

2.1.5 GC/MS analysis and quantification

Identification of bromo- and chlorocarbazoles was carried out by high resolution gas chromatography coupled to high resolution mass spectrometry. GC, Agilent 6890 Series II; column, Rtx-Dioxin2 column, 40 m, 0.18 mm ID, 0.18 μm film thickness (Restek); carrier gas, helium with a constant flow of 1.3 mL min^{-1} was

used. Samples were injected using cold injection system, CIS 4 (Gerstel) connected to an autosampler, A200S (CTC). The injector was held at 120°C then heated at 12°C s⁻¹ to 280°C for 5 min. Injection volume, 0.5 µL splitless. The GC oven was held at 60°C for 1.5 min. The column temperature raised steadily at 25°C min⁻¹ until 140°C, then increased by 8°C min⁻¹ to 300°C and held at 300°C for 20 min. Temperature of transferline was 300°C. The GC eluent was registered on MS, high resolution sector field MAT 95S (Thermo), using electron impact ionisation (EI⁺, 50 eV, 260°C) operated in SIM mode at resolution >8500 or full scan mode at resolution 1000.

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. Only carbazole, 3-chloro-, 3,6-dichloro- and 3,6-dibromocarbazole were available as neat compounds. Thus, exact quantification was possible only for these substances. For quantification, isotope labelled internal standard compounds was used. Here deuterated PAH (phenanthrene D10, benzo(a)anthracene D12, pyrene D10, benzo(b)fluoranthene D12) were chosen due to lack in the availability of isotope labelled halogenated carbazoles. The PAH exhibit similar physico-chemical properties during sample preparation and GC/MS analysis.

2.2 Computational DFT calculations

The electronic structure calculations of the intermediate sigma complexes of the halogenated carbazoles were performed on an Intel®Core(TM) i7-3520 CPU 2.90 GHz 2.90 GHz personal computer using Density Functional Theory, incorporated in Gaussian 03W program package (Frisch et al. 2004). The geometries of halogenated carbazoles in gas phase were carried out using the Becke 3-Lee-Yang-Parr (B3LYP) functional method. All geometry optimizations and frequency calculations of *intermediate sigma complex* were performed as *singlet* using *opt=gdiis freq=noraman*

rb3yp/6-31+G(d,p) basis set to obtain the zero point vibrational energy. This value was multiplied by the scaling factor of 0.9804 in order to compute thermal energy corrections. Single point energy (SPE) calculations were carried out using the unrestricted functional with mixing orbitals: *uB3LYP/6-311+G(2d,p)guess=mix* basis set. SPE enables the computation of very accurate energy values and other properties for an optimized geometry at a lower level of theory. Stability tests on all calculated structures were performed using *stable B3LYP/6-311+G(2d,p)* basis set to ensure that the wave function computed is stable and that the calculations corresponds to the ground state of the molecule (Foresman and Frisch 1996).

2.3 Occurrence and distribution of bromo- and chlorocarbazoles in soil

2.3.1 Chemicals

Pure compounds and standards of carbazole, bromo- and chlorocarbazoles described in section 2.1.1 were used. Silica gel (SiO₂), sodium sulphate (Na₂SO₄) and all organic solvents used were obtained from LGC Standards (Wesel, Germany). Alumina B (Al₂O₃) was supplied by MP Biomedicals (Eschwege, Germany) while octadecyl-C18-modified silica gel was supplied by Macherey-Nagel (Düren, Germany). All reagents were of analytical grade and solvents were of picograde quality.

2.3.2 Study area and soil sampling

As part of the Second National Forest Soil Inventory in Germany (BZE II), a subset of 86 forest soil samples was selected from a set of 474 samples taken from a 16 km x 16 km grid covering the Federal Republic of Germany (Aichner et al. 2013) in a sampling campaign that was carried out in the years 2006 to 2008. These samples did not include sampling points out of forest areas. Consequently, a qualitative analysis overview revealed the presence of carbazole and halogenated carbazoles in 59 soil samples out of the selected 86 samples. One soil sample was randomly selected for investigation. In total 11 soil samples collected uniformly from forests in different regions of Germany identified as G1, G2, G3, G4, G5, G6, G7, G8, G9, G10 and G11 were subjected to quantitative analysis for carbazole and halogenated carbazoles.

The samples were taken from the organic layer (Of/Oh horizons), excluding the litter horizon (Oi). Additionally, 11 soil samples representing 11 Federal states of Germany were obtained from 0-5 cm and 5-10 cm mineral soil horizons. The soil samples were not subjected to drying to avoid loss of volatile contaminants. Data on total organic carbon (TOC %), and forest type were also collected (Table 1) whereby the overall percentage composition by forest type was 61:18:21, coniferous: deciduous: mixed, respectively. The mean TOC % was 35.87. The soil material was transferred into brown glass bottles immediately after sampling. They were kept at 0°C before being transferred for storage at -20°C awaiting further treatments. The samples were subsequently sieved through a 4 mm cross diameter stainless steel sieve, then homogenized by a Tyler divider and stored in brown glass bottles. Pretreatment of the soil samples was performed at about -10°C. Thereafter, the laboratory samples were stored at > -20°C at the Federal Institute for Materials Research and Testing, Berlin. The samples were then transported to Helmholtz Zentrum München where they were stored at -28°C temperature conditions before analysis.

Table 1 Information describing forest types, quantity of soil samples collected per region in Germany and the number of samples in which carbazole and halogenated carbazoles were detected.

Regions in Germany	Forest type	Quantity of soil samples	Number of samples in which carbazoles and halogenated carbazoles were qualitatively detected
G1	Coniferous	9	7
G2	Coniferous	17	9
G3	Coniferous	7	5
G4	Coniferous	10	8
G5	Coniferous	10	6
G6	Coniferous	4	3
G7	Coniferous	6	4
G8	Coniferous	3	2
G9	Coniferous	7	6
G10	Deciduous	2	2
G11	Mixed	4	3

2.3.3 *Extraction and clean-up*

5 to 10 g fresh weight (fw) soil sample was extracted using Accelerated Solvent Extractor (ASE 300, Dionex GmbH, Idstein, Germany) with a mixture of n-hexane:acetone (75:25, v/v) at a temperature of 120°C and pressure of 12 megapascal (MPa). Two static cycles of 10 min were applied for a complete extraction. A flush volume of 70% of cell volume and a nitrogen purge time of 5 min was applied. The extract was concentrated by rotary evaporation to 1 mL and subjected to a clean-up by column chromatography using silica gel (SiO₂), deactivated alumina (Al₂O₃ + 3% H₂O) and sodium sulphate (Na₂SO₄) to remove any material that could cause interference. Columns were eluted with 100 mL hexane: DCM (1:1, v/v). The eluate was concentrated by rotary evaporation to 1 mL. 0.5 mL Acetonitrile was added to the concentrate and then reduced under a gentle stream of nitrogen to 0.5 mL. The sample was subjected to another clean-up by means of elution through C18 column using acetonitrile as the eluent. Eluate was concentrated under a steady flow of gentle nitrogen before being transferred to amber vials. Syringe (internal) standards (pentachlorotoluene and 1,2,3,7,8,9-hexachlorodibenzo-p-dioxin-¹³C₁₂) were added after which the extract and standard were concentrated under a steady but gentle flow of nitrogen gas to a final volume of 20 µL. The amber vials were sealed and then taken for analysis using high resolution gas chromatogram / high resolution mass spectrometer.

2.3.4 *GC/MS analysis and quantification*

Identification of halogenated carbazoles was carried out by high resolution gas chromatography coupled to high resolution mass spectrometry described in section (2.1.5). Only carbazole, 3-chlorocarbazole, 3-bromocarbazole, 3,6-dichlorocarbazole and 3,6-dibromocarbazole were available as neat compounds. Thus, exact quantification was possible only for these substances. With exception of the analyses of the humic layer, where 3-bromocarbazole was not yet available, the results of other four compounds were only reported.

For quantification, labelled internal standard compounds were used. Here deuterated PAHs [(phenanthrene D10, benzo(a)anthracene D12, pyrene D10, benzo(b)fluoranthene D12)] were chosen due to lack in availability of isotope labelled halogenated carbazoles. The PAH exhibit similar physico-chemical properties during sample preparation and GC/MS analysis. The limit of detection (LOD) was set to three times the signal-to-noise ratio (S/N 3:1) while the limit of quantification (LOQ) was set at nine times (S/N 9:1) of the chromatographic peak. Carbazole, 3-chlorocarbazole, 3-bromocarbazole, 3,6-dibromocarbazole and 3,6-dichlorocarbazole, were identified and reported in pg/g dry weight and the results were blank corrected.

2.3.5 Quantification criteria

An individual halogenated carbazole isomer was identified by comparing the GC retention time with the corresponding retention time of the labeled standard and by acquiring the ion-abundance ratio of the two exact atomic mass number to charge numbers (m/z 's). The relative retention time between analytes and the labeled standard was determined by injecting a mixture of both. In the sample these relative retention time was allowed to vary in the range of ± 3 sec for a positive detection. In addition, the ratio of the two exact m/z 's shall not vary by more than $\pm 20\%$ of the theoretical value and the signal/noise ratio for the particular analyte must be at minimum 3:1. If one part of the criteria is not met, the analyte is identified as not detectable.

2.3.6 Recovery rates

Recoveries rates of the internal standards were determined to control the performance of the sample preparation step (2.3.3 extraction and clean-up). The recovery rates should range between 40-130%.

2.3.7 Blank samples

Three blank samples were performed and prepared for analysis of carbazole and halogenated carbazoles in a manner identical to the soil samples. The samples results were corrected by blank values if detected. In this case, the mean and the

corresponding standard deviation of all blanks were calculated. Then the blanks were subtracted from sample results. The residue had to be greater than 3 times the standard deviation, which is defined as the limit of detection (LOD), otherwise the result was expressed as not detectable. In the case where no blank values were detected for an analyte, the LOD was calculated as three times the signal-to-noise ratio of the related mass trace registered by the MS.

2.3.8 Control samples

A control soil sample was included in the study. Two individual sample preparations and analysis of halogenated carbazoles were performed on the control soil sample.

2.3.9 Statistical analysis

SAS version 9.3 for Windows was used to perform statistical analysis. Pearson correlation was used and significant differences were performed using two way analysis of variance (ANOVA) with factorial treatment design without replication. For multiple comparison tests, least square difference (LSD) was used when the variance was significant. The level of significance was set at $p < 0.05$ (95%).

2.4 Dissipation of bromo- and chlorocarbazoles in soil

2.4.1 Chemicals

Pure compounds of carbazole, bromo- and chlorocarbazoles, reagents, and solvents mentioned in section 2.1.1. were used in this experiment.

2.4.2 Soil physical properties

Strongly humic, dark brown, Calcic Gleysols was used in this study. Three soil samples were obtained randomly on the same site in the state of Bavaria in Germany (Fig. 3) from the A horizon 24-39 cm with a bulk density of 1.04 g/cm^3 , pH (CaCl_2) 7.62, total carbon



Figure 3 A modified map of Germany showing the site where soil samples used in incubation experiment were collected, in the State of Bavaria.

4.17%, organic carbon 2.57%, nitrogen 0.29% and sulfur < 1%. The A horizon had the highest concentration of chlorocarbazoles to be used in the study. The concentrations of 3-chloro- and 3,6-dichlorocarbazole are approximately 35 ng/g and 800 ng/g (fresh weight) respectively in this horizon (Tröbs et al. 2011). The soils samples were provided by the Bayerisches Landesamt für Umwelt. They were kept under refrigeration at 4°C before the start of experiment at their field moisture content.

2.4.3 Soil homogenization and bacterial cell count

Soil homogenization was done using Retsch ZM1 ultra centrifugal mill equipped with an integrated 2 mm mesh. Initially, lumpy soil clumps were broken down manually to approx. 1 cm diameter then mixed manually to achieve uniform homogenization. Debris and stone pebbles were removed in the process. To facilitate homogenization while maintaining the soil moisture content, liquid nitrogen was used to freeze dry the moist soil lumps during milling. This was followed by manual mixing of the milled soil to achieve further homogenization.

Bacterial cell count was performed to count the cultivable bacteria cells. Soil bacteria were extracted from soil by mixing 1 g fresh soil with 99 mL of extraction solution in 200 mL jar. The extraction solution contained 0.1 g NaCl, 0.02 g CaCl₂·2H₂O, 0.2 g MgSO₄·7H₂O and 5.0 g Tween 80 which was adjusted to 1 L using distilled water . Prior to use the solution was autoclaved for 20 min at 121°C. The mixture was shaken vigorously for 1 hour at 150 rpm. The soil particles were then allowed to sediment for 10 min after which a total of four dilutions (10⁻¹ to 10⁻⁴) were prepared. Then, 0.1 mL from each dilution was spread in duplicate on plates with lysogeny broth (LB) medium. The LB was prepared by mixing 10 g trypton enzymatic digest from casein, 5 g yeast extract, 5 g NaCl, 15 g agar and 0.1 mg cycloheximide in 1 L of distilled water. The medium was sterilized for 20 min at 121°C before use. The number of colony forming units (CFU) was determined after 3 days of incubation at 25°C by counting colonies in the plates containing 10⁻² dilutions.

2.4.4 Soil water potential at -15kPa and soil mass for incubation experiment

After soil homogenization, the water tension (pF) at -15 kPa soil water potential was determined using sand/kaolin box (08.02 Eijkelkamp, Netherlands) for pF determination (pF 2.0 to 2.7). A soil density of 0.97 g/cm^3 was achieved by compressing the sieved soil sample into a small metal ring (10 cm^3) and then rewetted to achieve water holding capacity. The soil sample was placed in the sand/kaolin box and the water potential determined at -15 kPa. The sample was allowed to equilibrate under pressure, after which gravimetric water content was determined by weighing.



Figure 4 A picture illustrating how moist soil sample was compacted in the incubation flasks to achieve the required density of 0.97 g/cm^3 . The required volume of water (20.62 mL) equivalent to 20 g (dry weight) soil of 0.97 g/cm^3 density was poured in an empty flask to the Left of the picture. The soil density was determined using the sand / kaolin box. The actual volume of wet soil equivalent to 20 g dry weigh soil was determined based on the actual (%) soil water content and the mass of dry soil. The water the level was marked round the flask as shown on the flask in the Middle. It was then numbered. The wet soil equivalent to 20 g dry weight soil was then compacted to this mark to achieve the required soil density (0.97 g/cm^3) (Right). 2.05 mL of water added to achieve at water soil tension of -15 kPa. The flask was covered loosely with a foil paper to allow aeration and prevent contamination before being placed in the dark under the respective 15°C and 20°C incubation conditions.

Using the water content obtained by sand/kaolin box and actual soil water content of the soil sample, the amount of water to be added to achieve a water potential of -15 kPa was calculated. Distilled water was used as an alternative to rain water. An equivalent volume of the mass of soil (dry weight) to be occupied in the incubation jars (Fig. 4) was

then determined and marked round all incubation jars. The moist soil samples were then compressed to this mark (Fig. 4) and the volume of water estimated earlier was added to achieve a -15 kPa soil water tension. The incubation jar was covered loosely with foil paper to allow aeration and prevent contamination after which the soil sample was placed in incubation in the dark.

2.4.5 Experimental design

2.4.5.1 Temperature

Incubation was conducted under two temperature conditions of $15\pm 3^{\circ}\text{C}$ and $20\pm 3^{\circ}\text{C}$ each comprising of 14 treatments of the same soil sample corresponding to 42 soil samples arranged in triplicates. To investigate the influence of temperature change on dissipation, the soil samples under $20\pm 3^{\circ}\text{C}$ incubating conditions were initially placed under $15\pm 3^{\circ}\text{C}$ incubating conditions and then moved to $20\pm 3^{\circ}\text{C}$ incubating conditions after 10 weeks. Each jar contained 29 g wet weight equivalent to 20 g dry weight soil.

2.4.5.2 Wet and dry cycles

Fluctuating soil humidity conditions of wet and dry cycles were used in this experiment. The soil samples retained moisture during the two cycles. They were not left to get waterlogged or to dry completely. The wet cycle was characterized by addition of distilled water on the soil samples to replenish water lost through evaporation to maintain water potential at -15 kPa determined previously in section 2.4.5. This was done after every dry cycle to replenish water lost naturally by evaporation under both incubating conditions then followed by the dry phase during which water not added. On average, the dry and wet cycles were 2 and 4 weeks respectively. The rate of water loss at 20°C was higher compared to 15°C . Replacing the same volume of soil water lost ~ 2.0 mL, was therefore not the same for the two cycles requiring more cycles at 20°C compared to 15°C . This was done by weighing the incubation jars gravimetrically after every dry phase to replenish the equivalent amount of water lost. After watering, all jars were placed in the dark and covered loosely with foil paper to allow aeration and prevent contamination in their respective constant temperature chambers.

Approximately 10% soil water content was lost in dry phase which was however slow under 15°C condition culminating in 19 and 44 dry-wet cycles for 15°C and 20°C temperature conditions respectively. The objective was to ensure as much as possible the initial soil moisture was maintained while at the same time allow for wet dry cycles. Since soil samples were obtained from a site with perennial wet conditions, excessive water loss was not allowed during the dry phase.

2.4.6 Sample collection, extraction and clean-up

Soil samples were collected initially after 2 weeks for the first 3 months and then after 4 weeks for the rest of the study period. 20 g dry weight soil sample was extracted by accelerated solvent extraction (ASE) method performed on by Dionex ASE 300 Sunnyvale, CA, USA with a mixture of hexane and acetone (3:1) as extraction solvents at a pressure of 120 bar, a static extraction phase of 10 min, temperature of 120°C in two static cycles, a flush volume of 70% of cell volume and a nitrogen purge time of 5 min. The final volume of the extract was 150 mL. The ratio of soil to extraction solvent was 1:7.5 (w/v). 20 g of the soil sample (dry weight) was mixed with hydromatrix. 20 µL D-PAH internal standard was injected on the filter paper placed at the bottom of the metal column (cell). The cell was filled with the soil sample previously mixed with hydromatrix and compressed ensuring it was completely full using hydromatrix as filler. Another filter was placed on the filler at the other end of the cell then closed tightly. The filters were cleaned before use in ASE 300 using the same extraction method. No clean-up of hydromatrix was required. After sample extraction, a funnel was used to transfer the extracted sample into round bottom flask (250 mL). Enough Na₂SO₄ was placed in the funnel equivalent to the amount of water present in the extracted solvent. The sample was then transferred from the vial through the funnel into the flask. The vial was rinsed 3 times with n-Hexane and the residuals made to a constant volume of 100 mL. The sample was then concentrated using rotatory evaporator to 2 mL at 60°C, 120 rpm and 600 mbar. The concentrate was then diluted to 10 mL in 10 mL graduation flask using hexane to enable analysis within the GC/MS detection limits. In case extraction was not done immediately, samples were stored under refrigeration at -28°C.

1 mL, a tenth (1:10, v/v) of the diluted extract, was then subjected to clean-up by column chromatography using silica gel (SiO_2), deactivated alumina ($\text{Al}_2\text{O}_3 + 3\% \text{H}_2\text{O}$) and sodium sulphate (Na_2SO_4) to remove any material that could cause interference. Columns were eluted with 100 mL hexane: DCM (1:1, v/v). The eluate was concentrated by rotary evaporation to 1 mL. 0.5 mL acetonitrile was added to the concentrate and then reduced under a gentle stream of nitrogen to 0.5 mL. The sample concentrate was subjected to another clean-up by means of elution through C18 column using 5 mL acetonitrile as the eluent. The eluate was concentrated under a steady flow of gentle nitrogen to 0.5 mL before being transferred to amber vials containing 20 μL syringe standards (pentachlorotoluene and 1,2,3,7,8,9-hexachlorodibenzo-p-dioxin- $^{13}\text{C}_{12}$). The extract and standard were concentrated under a steady but gentle flow of nitrogen gas to a final volume of 20 μL . The amber vials were sealed and then taken for analysis using high resolution gas chromatography coupled to high resolution mass spectrometry.

2.4.7 GC/MS analysis and quantification

Identification and quantification of halogenated carbazoles was carried out by high resolution gas chromatography coupled to high resolution mass spectrometry described in section 2.1.5. In addition to the above mentioned halogenated carbazoles, only trichlorocarbazole could be identified by exact mass measurement, isotope ratio and retention time. The retention time was compared to enzymatically synthesized trichlorocarbazole (Section 3.1) where this compound was identified by use of its mass spectrum. Only the exact substitution was not known. A semi-quantitative approach was applied to be able to monitor the amount of trichlorocarbazole formed over time. Here the ratio of signals of trichlorocarbazole and internal standard, benzo(b)fluoranthene D12, was calculated for the samples analysed. The recoveries were between 58% and 73%. The limit of detection (LOD) was set to three times the signal-to-noise ratio (S/N 3:1) while the limit of quantification (LOQ) was set at nine times (S/N 9:1) of the chromatographic peak. All concentrations were given on a dry weight basis.

2.4.8 Criteria for selecting compounds for dissipation

Mono-, di-, tri- and tetra- congeners of bromo-, chloro and iodo-carbazoles were assessed in addition to 3-chloro- and 3,6-dichlorocarbazoles studied previously (Tröbs et al. 2011). Carbazole and 3-methylcarbazole were included to represent alkaloids of carbazole based on their natural occurrence (Schmidt et al. 2012). Also included were bromochlorocarbazole to assess the presence of mixed halogenated congeners, and 3,4,5,6-dibenzocarbazole, a known carcinogen and mutagen, for correlation analysis.

2.4.9 Method validation

Due to the long-term nature of the experiment, the methodology was subjected to test in order to validate the results that will be generated using this method. Four soil samples obtained randomly from the batch soil sample that was to be used in the experiment were homogenized thoroughly manually then mechanically using Retsch ZM1 ultra centrifugal mill equipped with a 2 mm mesh. Each soil sample was then taken through all the steps of the methodology as outlined in 2.4.6 and 2.4.7. The soil samples were analyzed for four days, each sample per day assuming the same conditions for all soil samples. The results were then subjected to statistically analysis $p < 0.05$.

2.4.10 Statistical analysis

Data in tables and figures were presented as the mean \pm SD. Pearson correlation, analysis of variance (ANOVA) specifically one and two way factor without replication and regression analysis were performed at $p < 0.05$ (95%) confidence using SAS version 9.3. The t-test least square difference (LSD) was performed in one way ANOVA.

2.5 Photodegradation of bromo- and chlorocarbazoles

2.5.1 Chemicals

Pure compounds and standards of 3-bromocarbazole, 3-chlorocarbazole, 3,6-dibromocarbazole ($\geq 97\%$) and 3,6-dichlorocarbazole were purchased from Sigma Aldrich (Taufkirchen, Germany) including sodium acetate trihydrate ($\geq 99\%$) and 1,10-Phenanthroline ($\geq 99\%$). Crystalline potassium trioxalato ferrate (III) trihydrate was

supplied by Alfa Aesar (Germany). Dichloromethane and sodium sulphate (Na_2SO_4) were obtained from LGC Standards (Wesel, Germany). All reagents were of analytical grade and solvents were of picograde quality.

2.5.2 *Maximum Wavelength of Absorbance*

Determination of maximum wavelength (λ_{max}) of absorbance was done using an Ultrospec 3300 pro UV/Visible spectrophotometer. This achieved by scanning a known concentration of each halogenated carbazoles through a selected wavelength range (180 to 800 nm) in the ultraviolet and visible (UV-Visible) region. The resulting absorption spectrum was then used to locate the wavelength at which absorbance was highest within the Pyrex filtered spectrum (>290 nm). This wavelength was then used for the photodegradation experiment.

2.5.3 *Photodegradation experiments*

2.5.3.1 Photolysis

Irradiation experiments were carried out in a cylindrical glass tube reactor (Appendix Fig. 24-25) containing 450 mL aqueous sample that was exposed to polychromatic radiation without filtration. The radiation source was a high pressure mercury lamp (HPK 125 W Phillips). The lamp was placed in a Pyrex cylindrical glass tube (>290 nm wavelength) which was used as a dive lamp. The halogenated carbazole solution was filled in the cylindrical glass tube reactor and stirred during irradiation. The wavelength of maximum absorption (λ_{max}) was then used to monitor degradation before analysis by gas chromatography. Agitation of the reaction mixture was done using a magnetic stirrer. The temperature of the solution was maintained at $25 \pm 2^\circ\text{C}$ by a cooling jacket surrounding the lamp. The temperature of the solution was also monitored continuously during irradiation. Ultra pure (Milli-Q) water was used as the solvent with acetonitrile as a co-solvent at <1% volume of the halogenated carbazole solution. The solvent was degassed before the irradiation experiment. Additionally, the oxygen concentration was measured before and after irradiation. pH was set between 7 and 8 within the range (pH 4-9) normally found in the environment (OECD 2004). In addition dark controls were

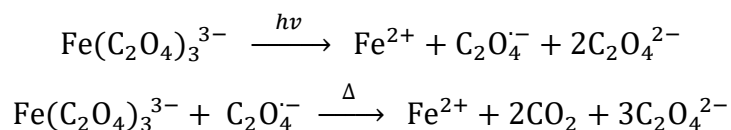
set-up in parallel to all direct photolysis studies at the same temperature conditions. Aliquots of water samples (2 mL) were collected in duplicates before irradiation and then after in predetermined intervals depending on the rate of the degradation. Method blanks of the solvent were also collected in duplicate.

2.5.3.2 Actinometer preparation

The actinometer was prepared according to (Kuhn et al. 2004) and (Kuhn 1989) with slight modifications. A 0.006 M solution was prepared by dissolving 2.947 g of $K_3[Fe(C_2O_4)_3] \cdot 3H_2O$ crystals in 100 mL 0.5M H_2SO_4 followed by dilution with distilled water to 1 litre. Actinometer solution (450 mL) was irradiated with stirring. 1 mL of the irradiated solution was given in to a 5 mL volumetric flask containing a mixture of 1.5 mL 0.1% 1,10-phenanthroline solution and 0.5 mL sodium acetate (NaOAc) buffer. The mixture was diluted to mark with distilled water. A reference was prepared in the same way except that it was not irradiated. The solutions were kept in the dark until full colour development was achieved and the absorbance of the first minus the second sample was measured at 510 nm (1 cm path length, $\epsilon = 11100 \text{ dm}^{-3} \text{ mol}^{-1} \text{ cm}^{-1}$). 0.1% 1,10-phenanthroline was prepared by dissolving 0.1001 g in 0.5 mL 0.05 M H_2SO_4 and diluted to 25 mL with distilled water while NaOAc was prepared by dissolving 4.1 g in 10 mL, 0.05 M H_2SO_4 and diluted to 50 mL with distilled water.

2.5.3.3 Light intensity

The incident light intensity (I_o) determination of the high pressure mercury polychromatic irradiation lamp (HPK 125 W Phillips) used in the irradiation experiment was evaluated by iron (III) potassium oxalate trihydrate [potassium tris(oxalate)ferrate (III) trihydrate] actinometer. It is reliable for UV and visible light applicable over a wide range of wavelengths up to 500 nm (Montalti et al. 2006), (Hatchard and Parker 1956). Potassium ferrioxalate decomposes under light excitation as shown in equations below:



The quantum yield (quantum efficiency) of the actinometer is defined by expression (4):

$$\Phi = \frac{\text{number of reacted molecules per time unit}}{\text{number of photons absorbed per unit time}} \quad (4)$$

The number of ferrous ions (Fe^{2+}) formed in the reaction was monitored by the conversion of 1,10-phenanthroline to the red coloured tris-phenanthroline complex at 510 nm. This was given by equation 5:

$$\text{Number of reacted molecules } (\sum \text{Fe}^{2+}) = \frac{6.022 \times 10^{23} A V_1 V_3}{V_2 l \varepsilon} \quad (5)$$

where 6.022×10^{23} is the avogadro constant, A is the optical difference in absorbance between the irradiated solution and non-irradiated put in the dark, V_1 is the irradiated volume in mL, V_2 is the aliquot of the irradiated solution for determination of ferrous ions in mL, V_3 is the final volume after dilution of irradiated aliquot solution in mL, l is the cell path length, ε is the extinction coefficient of ferrous phenanthroline complex at 510 nm ($1.11 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ or $\text{Lmol}^{-1} \text{ cm}^{-1}$).

Number of quanta absorbed by the actinometer was calculated based on equation 6:

$$\text{Number of quanta (photons) absorbed } (Nh\nu) = \frac{\sum \text{Fe}^{2+}}{\Phi_\lambda} \quad (6)$$

where Φ_λ is the quantum yield of ferrous production at the wavelength of light used. The number of quanta (photons) absorbed by the actinometer per unit time t , in photons per litre per second was given by equation 7:

$$\text{Number of quanta absorbed per unit time } \left(\frac{Nh\nu}{t} \right) = \frac{\sum \text{Fe}^{2+}}{\Phi_\lambda t}$$

$$= \frac{6.022 \times 10^{23} A V_1 V_3}{\Phi_\lambda V_2 l \epsilon t} \quad (7)$$

The light intensity of the high pressure mercury lamp was estimated as 6.821×10^{20} photons per cm^{-2} per second (photons $\text{cm}^{-2} \text{s}^{-1}$) assuming total absorption of irradiated light and also the quantum yield of polychromatic light was the same for all wavelength (Appendix Table 11). The recommended value of quantum yield at 365.6 nm (Φ_λ) 1.21 (Hatchard and Parker 1956) at 365.6 nm used in our calculations is equivalent to the wavelength (365 nm) of maximum energy of the lamp used in this study. Quantum yields of ferrous ion production from potassium ferrioxalate as a function of excitation wavelength is also relatively the same over a wide range of excitation wavelength (254 to 416 nm) (Montalti et al. 2006).

2.5.3.4 Rate of photodegradation and Quantum yield

The half-life ($t_{1/2}$), for first-order reaction was determined according to equation (8) while the quantum yield (Φ) for polychromatic light sources was based on equation (9). $k_{d(solar)}$, the estimated direct photolysis rate constant in near surface clear natural water exposed to average daily solar irradiances (L_λ) at latitude 50° in summer, was determined according to equation (10) as reported by (OECD 2008).

$$t_{\frac{1}{2}} = \frac{1}{k} \ln \frac{[A]_0}{[A]_0/2} = \frac{1}{k} \ln 2 = \frac{0.693}{k} \quad (8)$$

$$\Phi = \frac{D_{sys}}{2.3 \cdot l} \cdot \frac{k_d}{\sum_{290}^{800} \epsilon_\lambda I_{0\lambda} (light\ source)} \quad (9)$$

$$k_{d(solar)} = \Phi \sum_{\lambda=290}^{\lambda=800} \epsilon_\lambda \cdot L_\lambda \quad (10)$$

where k_d is the direct photolysis rate constant (d^{-1}), ε_λ is the molar absorptivity at wavelength λ , l is the light pathlength (cm), $I_{0\lambda}$ is photon irradiance ($\text{mmol cm}^{-2} d^{-1}$) over a 1 nm interval centered at wavelength λ , L_λ is the average daily solar photon irradiance, on amount basis ($\text{mmol cm}^{-2} d^{-1}$) over wavelength interval $\Delta\lambda$ centered at wavelength λ , D_{sys} is the depth of irradiated system (cm) = volume of irradiated system \div incident area. In general, the set-up followed the OECD guidelines on phototransformation of chemicals in water (OECD, 2008).

2.5.4 Sample extraction

0.5 mL of the irradiated solution was diluted in 9.5 mL distilled water to achieve a final volume of 10 mL. Internal standards were then added. The diluted solution was extracted twice with 5 mL dichloromethane (DCM) in a separating funnel. The extract was passed through Na_2SO_4 in funnel to remove water and concentrated with a rotary evaporator to a volume of ~ 0.5 mL. Recovery standard was added to the concentrate which was reduced further to 50 μL under clean nitrogen for analysis by high resolution gas chromatography coupled to high resolution mass spectrometry.

2.5.5 GC/MS analysis and quantification

The decline in halogenated carbazoles concentration was monitored by high resolution gas chromatography coupled to high resolution mass spectrometry described in section 2.1.5. The criterion for identification of individual compounds was also the same. 3-Bromocarbazole, 3-chlorocarbazole, 3,6-dichlorocarbazole and 3,6-dibromocarbazole were available as neat compounds.

2.5.6 UHPLC-MS analysis

In addition, analyses of the photoproducts in irradiated water samples without further treatment, were performed with a Waters nanoAcquity ultra high performance liquid chromatography (nano-UHPLC) system equipped with an autosampler and a binary pump system, coupled either to a photodiode array (PDA)-detector and a quadrupole time-of-flight mass spectrometer (Q-TOF MS) with collision cell (Waters Micromass

Q-TOF2). The chromatographic separation was performed on a Acquity HSS T3 C-18 reversed phase separation column (Waters, 300 μm \times 150 mm; particle size 1.8 μm). A sample loop with a size of 5 μL was used and the flow rate of the mobile phase was 4 μLmin^{-1} . The mobile phase consisted of solvent A (weak eluting force): 0.1% formic acid in water and solvent B (strong eluting force): 0.1% formic acid in acetonitrile. The analytical gradient elution was set up as follows: isocratic gradient at 100% solvent A from 0-1.5 min; linear increase of the concentration of solvent B from 1.5-25 min; isocratic hold at 100% solvent B 25-30 min; reequilibration of the column at 100 % A from 30.1-35 min. The column and autosampler were maintained at 30°C and 10°C, respectively. After each injection, the autosampler needle was washed with 300 μL strong wash solvent (pure acetonitrile) followed by 900 μL of weak wash solvent (99.9:0.1, v:v, water:acetonitrile). UV chromatograms were monitored in the range 190-400 nm with a resolution of 1.2 nm. The MS detector was connected to the LC system through a standard electrospray source operated in positive ion mode with a scan time of 1 s. Nitrogen was used as the drying gas. Non-target analysis was performed by detecting masses from 50 to 950 Da simultaneously in TOF-MS mode at a resolution of 6000. Each chromatogram was blank corrected prior to non-target analysis. The mass spectrometer was initially calibrated using dilute phosphoric acid solution before the experiment infused with a syringe pump at a flow rate of 4 μLmin^{-1} . Data analysis was conducted using Waters MassLynx 4.1 software shipped with the mass spectrometer and the OpenSource Software MZmime 2.11.

2.6 Toxicity evaluation

2.6.1 Reagents and chemicals

Resazurin sodium salt ($\geq 85\%$ dye content) and dicumarol ($\geq 98\%$) were purchased from Sigma Aldrich (Taufkirchen, Germany). Phosphate buffered saline (PBS) reagents; monobasic sodium sulphate (NaH_2PO_4) and monobasic potassium phosphate (KH_2PO_4) were supplied by the same company. The BCA protein assay kit consisting of bicinchoninic acid (BCA) solution, bovine serum albumin (BSA) standard solution and 4% cupric sulphate solution (Cu_2SO_4) was bought from Novagen (Darmstadt,

Germany). 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) standard, silica gel (SiO₂), sodium sulphate (Na₂SO₄) and all organic solvents were obtained from LGC Standards (Wesel, Germany). Glutaraldehyde solution and dimethyl sulfoxide (DMSO) were bought from AppliChem (Darmstadt, Germany); Trypsin/EDTA (0.05%/0.02% w/v) solution from Biochrom AG (Berlin, Germany); ChemTube-hydromatrix, Agilent Technologies (USA); alumina B (Al₂O₃) from MP Biomedicals (Eschwege, Germany); octadecyl-C18-modified silica gel from Macherey-Nagel (Düren, Germany) while calcium chloride (CaCl₂) and sodium chloride (NaCl) from Merck.

2.6.2 Cell culture

Hepatoma rat cells H4IIEC3/T (H4IIE) were obtained from Deutsche Sammlung von Microorganismen (DSMZ, Braunschweig, Germany). H4IIE cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% penicillin/streptomycin, 1% glutamine, and 10% fetal bovine serum (FBS) supplied from Biochrom AG (Berlin, Germany) in a humidified atmosphere of 95% humidity, 7% CO₂ at 37°C set in a Heraeus, gassed incubator, series 6000, BB 6060, 6220.

2.6.3 EROD induction bioassay

An aliquot of the soil extract (section 2.4.6) without clean-up was assessed for EROD induction half maximal effective concentration (EC₅₀) as an indicator for CYP1A1 activity. CYP1A1 is the major enzyme that catalyses the deethylation of 7-ethoxyresorufin to resorufin. Simplified EROD assay (micro-EROD) using rat hepatoma cell line (H4IIE) expressing cytochrome P450 1A1 upon exposure to aryl hydrocarbon receptor (AhR) agonists was performed according to Donato et al. (1993) with modifications (Schwirzer et al. 1998). H4IIE were seeded at a density of 1×10⁴ cells/well. They were then exposed to increasing concentrations of carbazole, 3,6-dibromocarbazole and 3-chlorocarabazole in the culture medium alongside the controls of TCDD also in increasing concentrations. After 24 and 72 hr 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. Dicumarol prevents the reduction of resorufin by cytoplasmic enzyme DT-diaphorase (Sidhu et al. 1993). 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. Protein measurement was done using BSA as protein standard (0-200 µg/mL) in separate wells at absorption wavelength of 540 nm instead of the optimal 562 nm due to lack of this filter.

2.6.4 REP calculations

Data generated by the spectrofluorometer were imported electronically into Excel spreadsheet program for calculations. EC50 values were calculated by transforming the dose-response data to produce Woolf and Scatchard plots. By comparing slopes of halogenated carbazoles curves to the slope of the TCDD standard, TCDD-EQs (toxic potency) were estimated as described by (Tillitt et al. 1993). Blanks, samples and standards were performed in quadruplets. Results were given as pgTCDD toxicity equivalent values (TEQ values)/g dry sample. Dioxin-like toxicity was then evaluated based on Relative Effect 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:

$$TEQ_{TCDD} = \sum_{i=1}^n (C_i \times REP_i) \quad (11)$$

where C_i is the concentration of a halogenated carbazole and i is the number of halogenated carbazole compounds.

Results and Discussions

3.1 In vitro enzymatic synthesis of bromo- and chlorocarbazoles

3.1.1 Enzymatic Bromination Reactions

Mono-, 3,6-di-, tri- and tetra-bromocarbazoles were synthesized in the enzymatic controlled bromination of carbazole. The presence of these compounds in the assay was confirmed by their retention times using reference standards and their individual mass spectra (Fig. 5, 6, Appendix Fig. 26-29). Mono- and 3,6-di-bromocarbazole were the most abundant compounds in *Rx.1* and *Rx.2* treatments with the mono-isomer being more in *Rx.1* compared to 3,6-dibromocarbazole (Fig. 5). With increasing amount of bromide in the reaction mixture, an increased abundance of the higher brominated isomers could be observed. Tribromocarbazole was the most abundant compound in *Rx.3* treatment (Fig. 5).

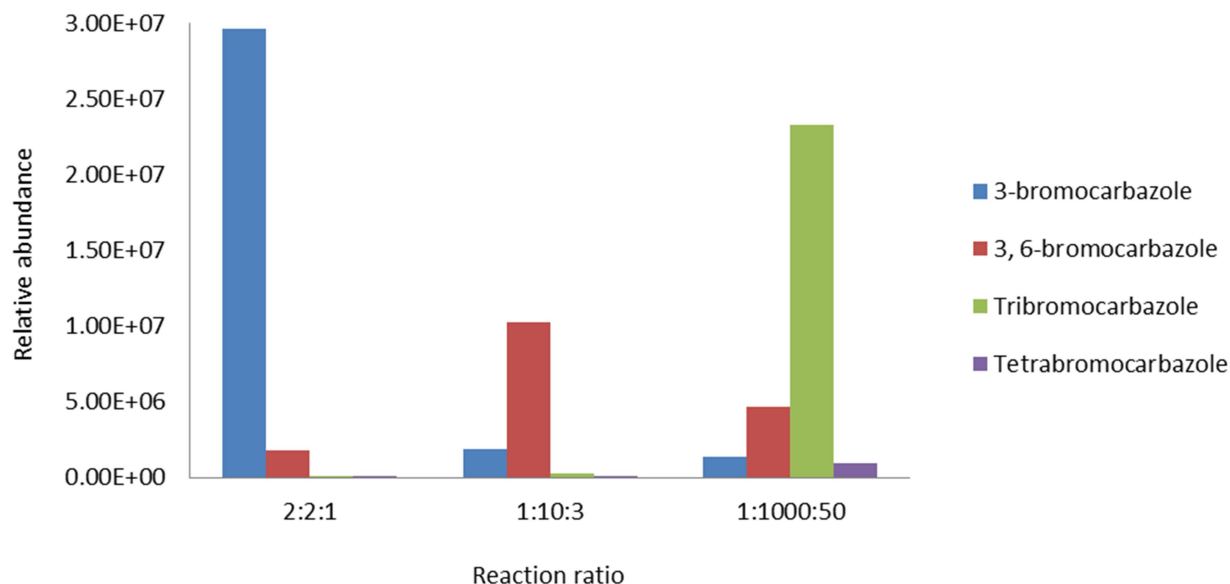


Figure 5 Normalised relative abundances of bromocarbazoles formed in different reaction mixtures. *Rx.1*, *Rx.2* and *Rx.3* are represented here as 2:2:1; 1:10:3 and 1:1000:50 respectively.

Dibromocarbazole and tetrabromocarbazole previously detected as unknown environmental contaminants by (Tröbs et al. 2011, Grigoriadou and Schwarzbauer 2011, Zhu and Hites 2005) were synthesized in this study. The presence of

bromocarbazoles in seawater samples can therefore be explained based on probable enzymatic catalysed synthesis of these compounds in the sea. This could be attributed to the fact that the bromide concentration is higher in seawater than in ground and river waters coupled with the fact that there exist sources of bromo-peroxidase enzymes in the sea (Deits et al. 1984). Hypobromous acid is also a more reactive halogenating agent towards aromatic species than hypochlorous acid in water (Voudrias and Reinhard 1988). Bromocarbazoles concentration in aquatic environment can therefore be concluded to be higher compared to chlorocarbazoles.

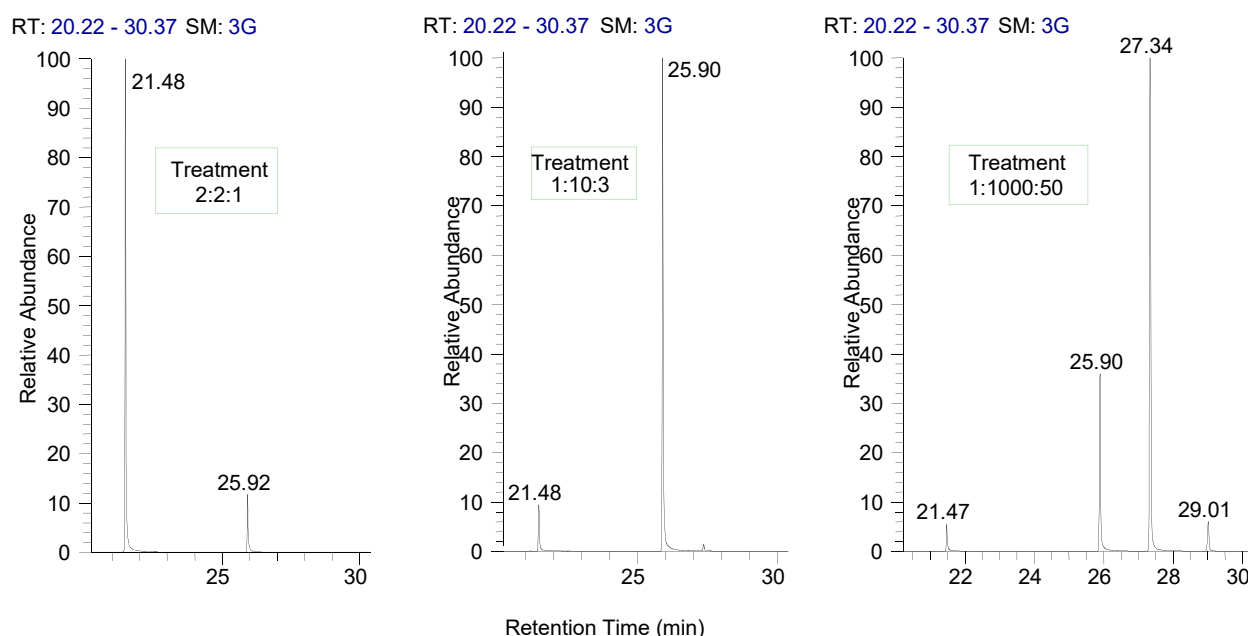


Figure 6 Gas chromatograms of bromocarbazoles from left to right. The mono- and dihalogenated carbazoles were formed in the 2:2:1 and 1:10:3 reaction ratio treatments while mono-, di-, tri- and tetrahalogenated carbazoles were all formed in 1:1000:50 reaction ratio treatments.

3.1.2 Enzymatic Chlorination Reactions

3-Mono-, 3,6-di-, tri- and tetra-chlorocarbazoles were synthesized in the enzymatic controlled chlorination reactions of carbazole (Fig. 7) They were confirmed by reference materials and the mass spectra of individual chlorocarbazoles (Fig. 8, Appendix Fig. 30-32). 3-Chlorocarbazole and 3,6-dichlorocarbazole were the abundant compounds in

Rx.4 and *Rx.5* treatments. However, all four compounds namely 3-mono-, 3,6-di-, tri- and tetra-chlorocarbazoles, were formed in *Rx.6* and *Rx.7* treatments (Fig. 7). Similarly, the type of the compound formed was found to be influenced by halide concentration. When the halide concentration was minimal as in *Rx.4* and *Rx.5* treatments, 3-chlorocarbazole and 3,6-dichlorocarbazole were preferentially formed. With increased halide concentration in *Rx.6* and *Rx.7* treatments, all four chlorocarbazoles were formed (Fig. 7). The blanks did not show chlorination. Terrón et al. (1998) recorded no oxidation of chlorine even at higher H₂O₂ concentrations without the use catalyst.

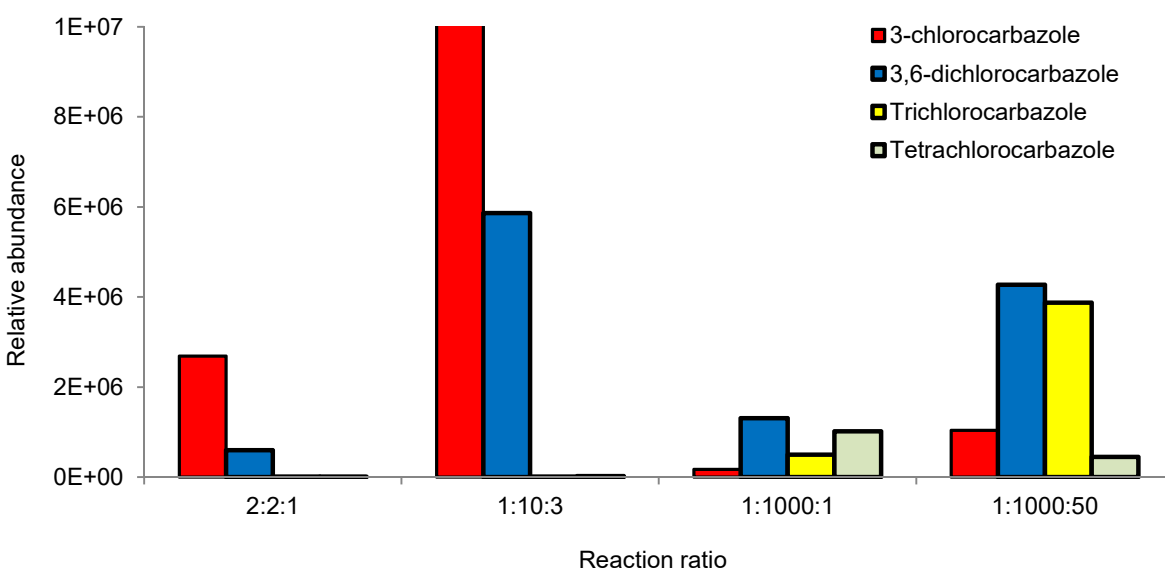


Figure 7 Chlorocarbazoles synthesized in different reaction mixtures. *Rx.4*, *Rx.5*, *Rx.6* and *Rx.7* are represented as 2:2:1, 1:10:3, 1:1000:1 and 1:1000:50. Increasing H₂O₂ concentration results in increased concentration of the compounds in *Rx.7* relative to those in *Rx.6*.

Enzyme concentration neither influenced the formation nor the concentration of chlorocarbazoles. The variation in enzyme concentrations in *Rx.4*, *Rx.5*, *Rx.6* and *Rx.7* treatments did not cause any significant difference in comparison to bromination reactions where the enzyme concentration was kept constant. The results from *Rx.4* and *Rx.5* treatments (Fig. 7) supports suggestions that 3-monochloro- and 3,6-dichlorocarbazoles detected in most sampling campaigns as the stable forms of chlorocarbazoles in environment (Reischl et al. 2005) assuming the halide

concentration does not occur in excess on the sampling site. Increase in hydrogen peroxide was found to cause an increase in concentration of compounds formed as was revealed in *Rx.6* and *Rx.7*. However, this is attributable to enzyme activity as elsewhere (Terrón et al. 1998) recorded no halogenation even at high concentrations of the oxidant in the absence of enzymes or catalysts.

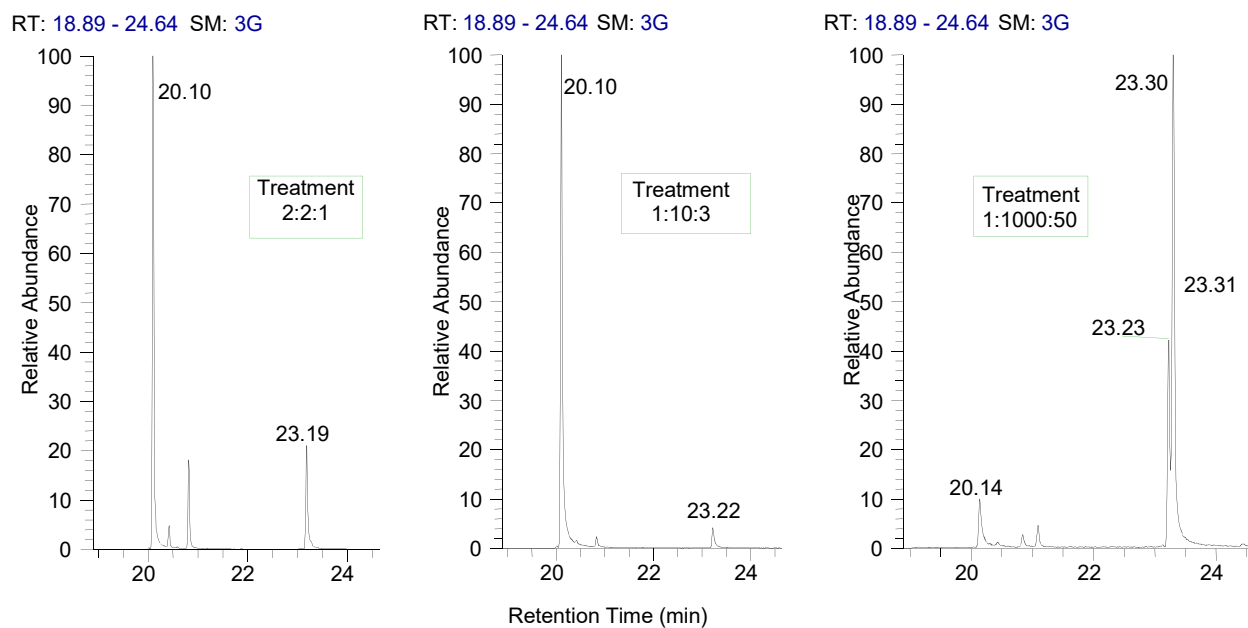


Figure 8 Gas chromatograms of chlorocarbazoles from left to right. The mono- and dihalogenated carbazoles were formed in the 2:2:1 and 1:10:3 reaction ratio treatments while mono-, di-, tri- and tetrahalogenated carbazoles were all formed in 1:1000:50 reaction ratio treatments.

The blanks did not show bromination. This is almost similar to results from a previous study where H_2O_2 oxidation of bromine did not take place at 1 mM concentration (Terrón et al. 1998). On the contrary, results in this study were obtained at H_2O_2 concentrations below 1 mM. Enzyme concentration which was kept constant in all treatments against varying halide concentration did not influence the preferential formation of a specific bromocarbazole isomer with respect to another or their concentration. This confirms the role of enzymes in facilitating oxidation of the bromide to effect electrophilic substitution with the electron rich carbazole (Silverthorn 2004,

Stenesh 1998) irrespective of the treatment composition. Terrón et al. (1998) reported bromine oxidation but this was done at very high H₂O₂ concentration and under long incubation periods. At 1 mM, under similar conditions, no oxidation took place therefore confirming the role of enzymes in facilitating oxidation reactions even at very low or environmental H₂O₂ levels.

3.1.3 Enzymatic halogenation in the environment

Enzyme catalyzed halogenation is not unique to carbazole. Many organohalogen compounds with bromine and chlorine are produced by living organisms or by natural abiotic processes (Gribble 2003). Enzymatic synthesis which we propose is also not the only pathway to halogenation. Carbazole can react photochemically to form halogenated carbazoles (Metcalf and Eddy 1991). Indirect photolysis and photochemical transformation by bromine and chlorine radicals in aquatic environment are possible (Calza et al. 2008, Kochany and Maguire 1994). However, volatilization and oxidation processes involving carbazole are very slow (Kochany and Maguire 1994) requiring long incubation periods. The formation of only one isomer in the mass spectra within each of the four homologue groups also shows that the process in this study is stereoselective making abiotic radical halogenation highly unlikely.

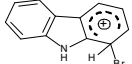
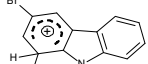
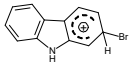
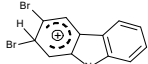
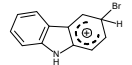
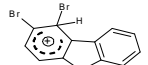
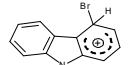
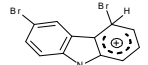
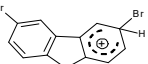
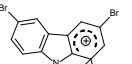
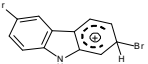
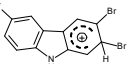
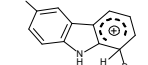
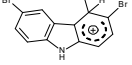
Enzymatic synthesis is highly possible in natural conditions. Environmental concentrations of carbazole in contaminated sites are significant ranging from 0.066 g/L to 1.08 g/L in wastewater effluents and 0.52 to 2.77 mg/L in waste water treatment sludge but quite high in oil and creosote. Bromide is high in seawater while chloride levels in rivers, oceans and waste water are equally high. H₂O₂ is available in freshwater (0.001- 0.109 mg/L) and seawater (0.001 to 0.0136 mg/L). Its production is higher in polluted (Cooper et al. 1988) and eutrophic waters. Chloroperoxidase halogenation has been demonstrated at 1 μM H₂O₂ (Hoekstra et al. 1995). Considering that most halogenated carbazoles reported in literature were isolated in sites with carbazole contaminants, concentrations used in this study were in close comparison to environmental levels recorded in polluted sites making this pathway possible in nature.

3.2 DFT calculations

3.2.1 Structure elucidation

The most stable isomers in the mono-, tri- and tetra- homologue groups of bromocarbazoles were not identified due to lack of reference materials. However, through DFT calculations, 3-bromo-, 1,3,6-bromo-, 1,3,6,8-bromocarbazoles were calculated as the most stable intermediate sigma complexes of the mono-, tri- and tetra- isomers respectively (Table 2).

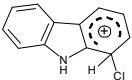
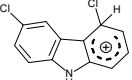
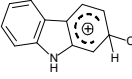
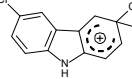
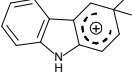
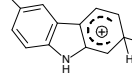
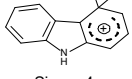
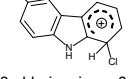
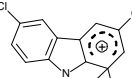
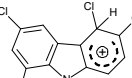
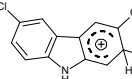
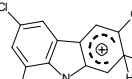
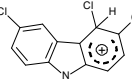
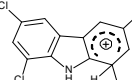
Table 2 Summarized results of DFT calculations of the intermediate sigma complexes of bromocarbazoles synthesized including their energies in atomic units and kcal/mol. A, B and C indicate intermediate sigma complexes of the respective mono-, di- and tri- bromocarbazoles.

Intermediate Sigma Complex	Br-C	Energy in atomic units (au)	Relative energy (kcal/mol)	Intermediate Sigma Complex	Br-C	Energy in atomic units (au)	Relative energy (kcal/mol)
A				B			
	1	-3091.2928	3.89		1	-5664.8315	5.46
Sigma-1-bromocarbazole				3-brominesigma-1-bromocarbazole			
	2	-3091.2913	4.82		2	-5664.8329	4.58
Sigma-2-bromocarbazole				3-brominesigma-2-bromocarbazole			
	3	-3091.2990	0.00		4	-5664.8301	6.29
Sigma-3-bromocarbazole				3-brominesigma-4-bromocarbazole			
	4	-3091.2886	6.53		5	-5664.8293	6.82
Sigma-4-bromocarbazole				3-brominesigma-5-bromocarbazole			
C					6	-5664.8402	0.00
	1	-8238.3729	0.00	3-brominesigma-6-bromocarbazole			
3,6-brominesigma-1-bromocarbazole					7	-5664.8317	5.33
	2	-8238.3729	0.01	3-brominesigma-7-bromocarbazole			
3,6-brominesigma-2-bromocarbazole					8	-5664.8341	3.79
	4	-8238.3718	0.67	3-brominesigma-8-bromocarbazole			
3,6-brominesigma-4-bromocarbazole							

Br-C : position of brominated carbon

1,3,6-Trichloro- and 1,3,6,8- tetrachlorocarbazoles were calculated as the stable intermediate sigma complexes of the tri- and tetra-isomers by DFT method (Table 3).

Table 3 Summarized results of DFT calculations of the intermediate sigma complexes of chlorocarbazoles synthesized including their energies in atomic units and kcal/mol. A, B, C and D indicate intermediate sigma complexes of the respective mono-, di-, tri- and tetra-chlorocarbazoles.

Intermediate Sigma Complex	Cl-C	Energy atomic units (au)	Relative energy (kcal/mol)	Intermediate Sigma Complex	Cl-C	Energy atomic units (au)	Relative energy (kcal/mol)
A				B			
 Sigma-1-chlorocarbazole	1	-977.3747	3.50	 3-chlorinesigma-5-chlorocarbazole	5	-1436.9961	7.23
 Sigma-2-chlorocarbazole	2	-977.3715	5.46	 3-chlorinesigma-6-chlorocarbazole	6	-1437.0076	0.00
 Sigma-3-chlorocarbazole	3	-977.3802	0.00	 3-chlorinesigma-7-chlorocarbazole	7	-1436.9982	5.91
 Sigma-4-chlorocarbazole	4	-977.3695	6.72	 3-chlorinesigma-8-chlorocarbazole	8	-1437.0021	3.44
C				D			
 3,6-chlorinesigma-1-chlorocarbazole	1	-1896.6264	0.00	 1,3,6-chlorinesigma-5-chlorocarbazole	5	-2356.2522	1.24
 3,6-chlorinesigma-2-chlorocarbazole	2	-1896.6261	0.19	 1,3,6-chlorinesigma-7-chlorocarbazole	7	-2356.2528	0.84
 3,6-chlorinesigma-4-chlorocarbazole	4	-1896.6249	0.74	 1,3,6-chlorinesigma-8-chlorocarbazole	8	-2356.2542	0

Cl-C : position of chlorinated carbon

The results were similar to those obtained by semi empirical PM3 method based on electron charge density on carbazole at the ground state (Bonesi and Erra-Balsells

1997). They also seem to follow electrophilic aromatic substitution pattern that favours halide substitution at the *ortho*- and *para*- positions relative to nitrogen on the carbazole (Bonesi and Erra-Balsells, 1997; Katritzky and Taylor 1990) implying that the formation mechanism in enzymatic synthesis of chlorocarbazoles proceeds in a similar manner as chemical synthesis of the same compounds.

3.2.2 Role of enzymes and regioselectivity in halogenation reactions

Enzyme concentration was found not to affect either the type of isomer or the concentration of the bromo- and chlorocarbazoles formed. The hypothesis that enzyme concentration could play a role in determining the type of compound to be synthesized and then proceed to influence its concentration was therefore ruled out. This shows that enzyme activity is generally limited by substrate concentration (Stenesh 1998). Increase in concentration only influences the rate of reaction through increase in the number of the enzyme active binding sites, at optimal temperatures specific to that enzyme (Silverthorn 2004). In the absence of enzymes, reactions may take place slowly or may not at all depending on other environmental variables.

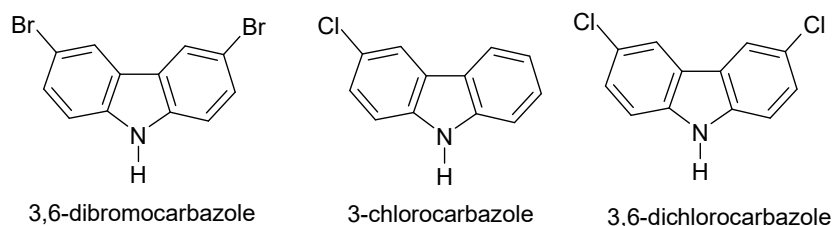


Figure 9 Compounds identified in the environment with similar substitution positions predicted here.

The preferred substitution positions obtained in this study were 3-; 3,6-; 1,3,6- and 1,3,6,8- for the respective mono-, di-, tri- and tetra-isomers of the two congeners. They were similar to substitutions patterns on isomers of compounds (Fig. 9) isolated from environmental samples (Grigoriadou and Schwarzbauer 2011, Zhu and Hites 2005). Stereo-selectivity in substitution at 3- and 3,6- positions was consistent with heterocyclic compounds (Sumpter and Miller 1954). The same was for tri- and tetra-isomers but not distinctively due to very close relative energies to reveal the difference (Tables 2&3).

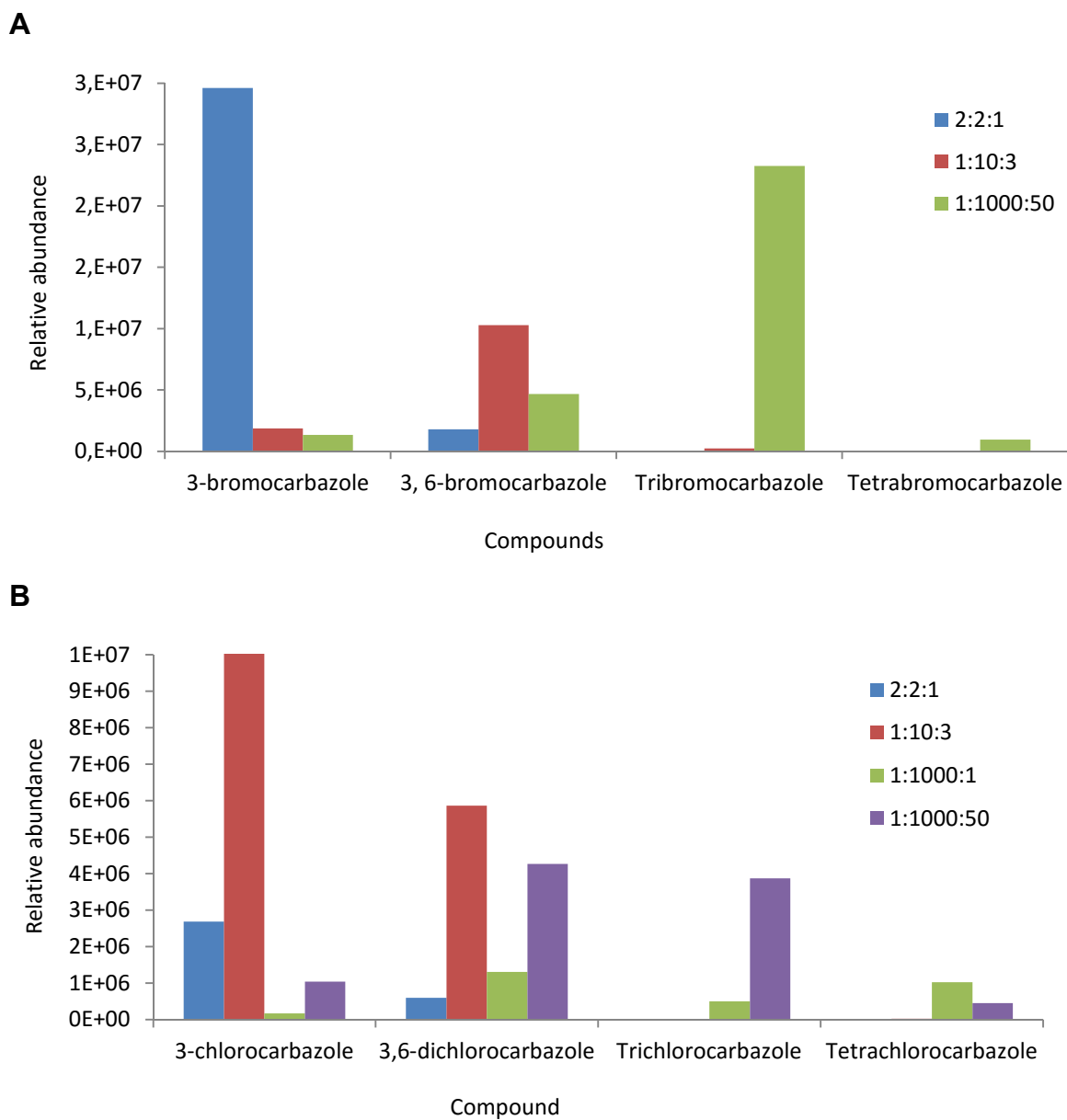


Figure 10 The formation of bromocarbazoles (A) and chlorocarbazoles (B) in different reaction treatments. 3-substituted and 3,6-substituted congeners were synthesized in all treatments and in relatively higher concentration in relation to the others.

3-Mono- and 3,6-di-substituted isomers were the dominant isomers in almost all treatments (Fig. 10) seemingly to explain their occurrence and wide distribution in some of the reported samplings (Grigoriadou and Schwarzbauer 2011, Tröbs et al. 2011). Their dominance in environmental samples can be attributed to the high charge density

at the *para* positions relative to the *ortho* positions (Fig. 11) and the effect of the lone pair of electrons in NH₂ moiety that enhances the amount and stability of para-products (Effenberger and Maier 2001, Smith and March 2007).

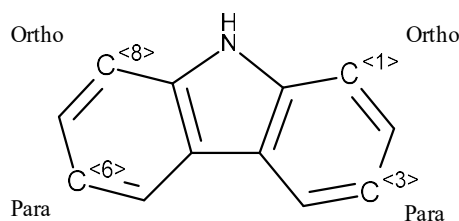


Figure 11 Ortho and para positions on carbazole.

3.3 Occurrence and distribution of bromo- and chlorocarbazoles in soil

3.3.1 Occurrence and concentrations

Carbazole, 3-bromocarbazole, 3-chlorocarbazole, 3,6-dibromocarbazole and 3,6-dichlorocarbazole were detected in most soil samples collected from different regions in Germany. They were qualitatively detected in the humic layer of 59 soil samples of which 11 soil samples, representing different regions of Germany, were selected for quantitative analyses of the humic layer and the mineral soil horizons at 0-5 cm and 5-10 cm depth. Carbazole recorded consistently the highest concentration per sample followed by 3,6-dichlorocarbazole then 3,6-dibromocarbazole. 3-Bromocarbazole and 3-chlorocarbazole were within the same range though the latter was slightly but significantly ($p < 0.05$) higher (Table 4). Individual compound concentrations per sample ranged from 21 to 289 ng/g (carbazole); 0.2 to 9 pg/g (3-bromocarbazole); 0.5 to 10 ng/g (3-chlorocarbazole); 0.3 to 28 ng/g (3,6-dibromocarbazole) and 3 to 162 ng/g (3,6-dichlorocarbazole) with the concentrations decreasing from humic layer to 0-5 cm and 5-10 cm mineral soil horizons. 3-Bromocarbazole and 3,6-dibromocarbazole which have not been reported previously in soil were detected in this study. Concentrations recorded here were generally low compared to those recorded in previous studies (Table 5).

Table 4 Percentage total organic carbon (TOC %) and concentrations (pg/g dry weight) of carbazole and bromo- and chlorocarbazoles analysed in 11 soil samples collected from different regions in Germany.

	Depth	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11
Total Organic Carbon (TOC) %	Humic layer	44	37	36	40	40	39	25	36	35	5	38
	0-5 cm depth	2	5	5	2	6	1	5	2	6	6	5
	5-10 cm depth	2	2	2	1	5	1	2	4	4	5	2
Carbazole	Humic layer	44720	42986	267600	16382	23321	33308	7740	42053	7674	31954	20079
	0-5 cm depth	175380	7941	21689	2494	15296	n.d.	23490	36543	18135	18857	16107
	5-10 cm depth	31663	817	n.d.	2459	5107	n.d.	3549	9963	1585	4374	2093
3-Chlorocarbazole	Humic layer	0	1821	9061	4337	1023	1323	1588	1394	345	2714	298
	0-5 cm depth	72	246	1425	113	388	22	1087	428	179	528	143
	5-10 cm depth	n.d.	40	117	85	135	63	530	100	21	207	80
3,6-Dichlorocarbazole	Humic layer	10375	5991	22774	21493	1996	4061	46284	3946	2236	4978	2006
	0-5 cm depth	839	1808	26298	410	2001	1045	67551	3521	1094	4503	1849
	5-10 cm depth	n.d.	529	3764	n.d.	2430	3259	48365	2198	n.d.	4207	1551
3-Bromocarbazole	0-5 cm depth	465	1742	7236	1074	2667	74	2282	1918	227	5933	2525
	5-10 cm depth	242	357	1342	441	1481	144	711	1588	157	3466	1122
3,6-Dibromocarbazole	Humic layer	9141	3136	19846	2490	806	2786	820	3159	n.d.	3722	2419
	0-5 cm depth	210	767	6545	489	1195	519	1812	669	324	2232	1110
	5-10 cm depth	413	471	1597	308	3408	1497	1533	802	n.d.	1503	627

n.d. – not detected

Table 5 Comparison of concentrations of carbazole and halogenated carbazoles recorded in this study to those in selected previous studies. Concentrations are in ng/g (DW) except for the study by Tröbs which are in fresh weight indicated by (FW). Potential anthropogenic sources are suspected in samples by (Guo et al. 2014), (Grigoriadou and Schwarzbauer 2011), (Kronimus et al. 2004) and (Heim et al. 2004). The others are samples not exposed to pollution.

Carbazole	3-Bromo-carbazole	3-Chloro-carbazole	3,6-Dibromo-carbazole	3,6-Dichloro-carbazole	Matrix
0.5-130	-	370	93	9-100	Sediment (Grigoriadou and Schwarzbauer 2011)
-	-	-	1-41	-	Sediment (Guo et al. 2014)
-	-	-	-	50	Sediment (Kronimus et al. 2004)
11-171	-	-	-	8-32	Sediment (Heim et al. 2004)
0.5-44	0.09-12	0.003-9	0.3-20	0.6-68	Soil - This study
4-12	-	9-29	-	606-1167	Soil - Section 3.4.1
-	-	35 fw	-	800 FW	Soil (Tröbs et al. 2011)
0.5-121	-	5-110	-	14-3500	Soil (Grigoriadou and Schwarzbauer 2011)
-	-	-	-	13.5	Soil (Reischl et al. 2005)

3.3.2 *Relationship between organic matter and halogenated carbazole concentrations*

In most soil samples, a positive correlation ($r > 0.90$, $p \leq 0.05$) between TOC and carbazole, bromo- and chlorocarbazoles concentrations (pg/g DW) could be observed in soil samples in G2, G3, G4, G8, G10 and G11 (Table 4). TOC concentrations decreased with increase in depth except in G10 (Table 4). This is consistent with a previous study where organically-bond halogens were found to be directly related to organic matter and they decreased with increase in depth (Yu et al. 2005). This could also be attributed to retention of deposited carbazole and halogenated carbazoles from atmospheric emissions by TOC in the organic soil layer (Nam et al. 2008) followed by possible formation of halogenated carbazoles. It has been suggested that formation of halogenated compounds is highest within the organic soil layer as highest activity for oxidation of bromide and chloride exists in this layer (Latumus et al. 1995). Subsequent leaching results in decreased concentration with increase in depth due to retention by organic matter in the soil profile (Petruzzelli et al. 2002, Krauss et al. 2000).

However, when ratios were used for comparison of soil dry weight (DW) concentrations, it was observed that more soil samples exhibited an increase in concentration with increase in depth. Soil samples from G1, G5, G6 and G7 (Table 6) however exhibited a decrease in concentration (pg/g DW) including specific compounds such as 3-chloro-, 3,6-dichloro- and 3,6-dibromocarbazole suggesting retention of deposited organic carbon in soil organic layer (Nam et al. 2008) followed by leaching resulting in decreased concentration with increase in soil depth.

Table 6 Carbazole and halogenated carbazoles concentration ratios based on dry weight (DW) concentrations (pg/g DW). The concentration ratios were calculated using data from Table 4 where the first ratio was obtained by dividing concentrations of humic layer by concentrations of 0-5 cm depth layer while the second ratio was obtained by dividing concentrations of 0-5 cm depth layer by concentrations of 5-10 cm depth layer. If the ratio was less than 1, then there is an increase in concentration with increase in depth. In general, there was an increase in concentration with increase with depth except for soils from region G1, G5, G6 and G7. Only one ratio could be obtained for 3-bromocarbazole since concentrations in the humic layer was not quantified due to lack of standards at that the time of analysis. (n.d. - not detected).

Region in Germany	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11
Carbazole	0.3	5.4	12.3	6.6	1.5	n.d.	0.3	1.2	0.4	1.7	1.2
	5.5	9.7	n.d.	1.0	3.0	n.d.	6.6	3.7	11.4	4.3	7.7
3-Chlorocarbazole	0.0	7.4	6.4	38.5	2.6	60.3	1.5	3.3	1.9	5.1	2.1
	n.d.	6.2	12.2	1.3	2.9	0.3	2.1	4.3	8.6	2.5	1.8
3,6-Dichlorocarbazole	12.4	3.3	0.9	52.4	1.0	3.9	0.7	1.1	2.0	1.1	1.1
	n.d.	3.4	7.0	n.d.	0.8	0.3	1.4	1.6	n.d.	1.1	1.2
3-Bromocarbazole	1.9	4.9	5.4	2.4	1.8	0.5	3.2	1.2	1.4	1.7	2.3
3,6-Dibromocarbazole	43.6	4.1	3.0	5.1	0.7	5.4	0.5	4.7	n.d.	1.7	2.2
	0.5	1.6	4.1	1.6	0.4	0.3	1.2	0.8	n.d.	1.5	1.8

When concentrations were based on TOC % per soil sample (pg/g TOC) was used (Table 7), an overall trend in increase in carbazole, bromo- and chlorocarbazole concentration (pg/g TOC) with increase in depth with a few exceptions was observed in most soil samples from the different regions in Germany suggesting that soil type could be responsible in enabling percolation and accumulation of these compounds in the lower soil horizons. Soils samples from G2, G5, G7, G8 and G11 exhibited consistently similar characteristics exhibiting an increase in concentration with depth

suggesting the soil types had similar characteristics. G1, G4 and G10 could be also be classified in different category since the soil type seem to share similar properties.

Table 7 Carbazole and halogenated carbazole concentration ratios expressed according to percentage total organic carbon (TOC %) in each soil horizon (pg/g TOC). The ratios in the table below were obtained using data from Table 4 by converting data in pg/g dry weight to pg/g TOC using TOC % of each soil sample. The first ratio was obtained by dividing concentrations of humic layer by concentrations in 0-5 cm depth layer while the second ratio was obtained by dividing concentrations of 0-5 cm depth layer by concentrations of 5-10 cm depth layer. If the ratio was less than 1, then there is an increase in concentration with increase in depth. Only one ratio could be obtained for 3-bromocarbazole since concentrations in the humic layer was not quantified due to lack of standards at that the time of analysis. (n.d.- not detected).

Region in Germany	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11
Carbazole	0.3	0.7	1.4	0.3	0.2	n.d.	0.1	0.1	0.1	2.0	0.2
	5.5	3.9	n.d.	0.5	2.5	n.d.	2.6	7.3	7.6	3.6	3.1
3-Chlorocarbazole	5.8	1.0	0.7	1.9	0.4	1.5	0.3	0.2	0.3	6.2	0.3
	n.d.	2.5	4.9	0.7	2.4	0.3	0.8	8.5	5.8	2.1	0.7
3,6-Dichlorocarbazole	0.6	0.4	0.1	2.6	0.1	0.1	0.1	0.1	0.4	1.3	0.1
	n.d.	1.4	2.8	n.d.	0.7	0.3	0.6	3.2	n.d.	0.9	0.5
3-Bromocarbazole	1.9	2.0	2.2	1.2	1.5	0.5	1.3	2.4	1.0	1.4	0.9
3,6-Dibromocarbazole	2.0	0.6	0.3	0.3	0.1	0.1	0.1	0.3	n.d.	2.0	0.3
	0.5	0.7	1.6	0.8	0.3	0.3	0.5	1.7	n.d.	1.2	0.7

Another factor which cannot be ignored is the role of natural formation of halogenated carbazoles in soil similar to haloperoxidase-like catalyzed natural formation of halogenated compounds in soil which could contribute to the increase in concentration (pg/g TOC) with increase in depth. (Hjelm et al. 1995) observed an increase in the ratio of organic chlorine to organic matter was found to increase with depth. A pronounced increase was observed in the transition between O-horizon and A-horizon indicating organically bond halogens formation occurs in soil. 3,6-Dichlorocarbazole was detected in many topsoil (A-horizon) and subsoil (B-horizons) in 200 locations of different land uses (Reischl et al. 2005).

3.3.3 Possible role of industrial atmospheric emissions

High concentrations could be related to historic deposition of essential substrates necessary for the formation of halogenated carbazoles from atmospheric industrial emissions in forests. High concentrations of chlorocarbazoles were recorded in the

industrial area of Kavala city, in Greece (Grigoriadou and Schwarzbauer 2011) showing the link between industrial emissions and local pollutant concentrations. In addition, coal is a known source of halogens specifically bromine, chlorine and iodine which are released during combustion (Peng et al. 2012; Vassilev 2000; Finkelman 1993; Block and Dams 1976). It is suggested that during combustion of coal fired power plants they are converted to gaseous Cl_2 and Br_2 (Germani and Zoller 1988), (Klein et al. 1975). Coal-tar pitch fumes, carbazole and other PAHs are also released (Sawicki et al. 1962). Besides coal, other natural sources of carbazole and its derivatives exist that include carbonate and clastic rocks (Clegg et al. 1997), natural alkaloids of carbazoles (Metcalf and Eddy 1991), oil (Frolov et al. 1989) and petroleum (Dorbon et al. 1984), (Vassilaros et al. 1982). It is important to note that petrochemical industries, the likely sources of carbazole and halogenated carbazoles, were activities present in the industrial site of Kavala city where chlorocarbazoles and dibromocarbazole were reported. In recent studies, carbazole has been detected in traffic (Liu et al. 2007), (Moriske and Ruden 1988), agricultural (Masih et al. 2010) and forest aerosol particles (Ladji et al. 2009), (Rissanen et al. 2006). In addition, most of the forest tree species in this study are coniferous with needle leaves (Table 1). Deposition of PAHs and DDT on coniferous pine needles has been reported. Coniferous substrates are rich in wax, resins and lignin (Eriksson et al. 1989), (Swift et al. 1979). Carbazole and halogenated carbazoles have not been reported on coniferous trees but the presence of bromo- and chlorocarbazoles in coniferous forest soils of wide distribution seems to show the role of coniferous tree species in enabling deposition of these compounds emitted most likely during combustion processes into the atmosphere.

3.3.4 Haloperoxidase-like natural formation of halogenated carbazoles

Previous studies have shown the link between halogenated carbazoles and anthropogenic sources (Guo et al. 2014), (Kuehl et al. 1984), (Parette et al. 2014). However, they could be derived naturally in the environment, as there are numerous natural occurring organohalogens (Gribble 1998). Natural formation of trichloromethane has been reported in forest soils (Laturnus et al. 2002). Chlorinated phenols, PCDDs and PCDFs formation has been reported in a douglas fir forest (Hoekstra et al. 1999). The forests that have reported natural formation of

halogenated compounds are similar to coniferous trees in this study (Table 1). Notably, haloperoxidase activity has been detected in spruce forest soil which was suggested to be responsible for the formation of volatile halogenated organic compounds in the same forest soil. Latumus et al. (1995) demonstrated chlorinating and brominating activity in soil samples from the podzol profile of uncontaminated spruce forest soil which was pH dependent. Coniferous litter is more acidic than broad leaves litter and acidification of soil is more pronounced in the former (Staaf and Olsson 1991), (Swift et al. 1979). This environment is suitable for haloperoxidase-activity as the optimum pH for chloroperoxidase activity was found to be at 3.0 and 3.5 while peroxidase activity increased with pH (Asplund et al. 1993). Occurrence of a chloroperoxidase-like catalyst was demonstrated in the organic layer of a spruce forest soils (Asplund et al. 1993). Hjelm et al. (1995) also observed in situ production of organically bound halogens in coniferous forest. Notably, bromocarbazoles and chlorocarbazoles can be naturally formed by haloperoxidase enzymatic reactions (Section 3.1). Considering that most of the forests in this study were coniferous, a similar process utilizing haloperoxidase could be responsible for natural formation of bromocarbazoles and chlorocarbazoles in situ.

3.3.5 *Halogenated dyes and p-chloroaniline herbicides*

Recently, it has been suggested that the historic production of 5,5',7,7'-tetrabromoindigo in United States could be related and corresponded well with environmental concentrations of 1,3,6,8-tetrabromocarbazole (Parette et al. 2014). It is also being suggested that *p*-chloroaniline based herbicides used for weeds control such as monuron [3-(*p*-chlorophenyl)-1,1-dimethylurea] in newly planted trees could be responsible for the observed 3-chloro- and 3,6-dichlorocarbazoles concentrations in forests soils in Germany. Monuron was first used publicly in 1952 (Timmons 2005). Chlorinated carbazoles were hypothesized to occur as an impurity in monuron (Parette et al. 2014). However, more research is required for definitive conclusion.

3.4 Dissipation of bromo- and chlorocarbazoles in soil

3.4.1 Isolation, identification and concentration

Carbazole, 3-chloro- and 3,6-dichlorocarbazole were isolated and positively identified by high resolution gas chromatography coupled to high resolution mass spectrometry using their pure standard compounds as references. Their concentrations ranged from 4 to 12 ng/g, 9 to 29 ng/g and 606 to 1167 ng/g dry weight for carbazole, 3-chloro- and 3,6-dichlorocarbazole respectively. Trichlorocarbazole not previously reported in soil was detected. The order of concentration beginning with the highest was, 3,6-dichlorocarbazole > 3-chlorocarbazole > carbazole > trichlorocarbazole. 3,6-Dichlorocarbazole recorded consistently approximately 100 and 40 times higher concentrations in comparison to carbazole and 3-chlorocarbazoles respectively (Fig.12 & 13). Trichlorocarbazole concentration was very low (Fig. 14) though with recoveries from 58 to 79% and a precision range (uncertainty) of 4 to 32% relative standard deviation (RSD, n=3). Since the uncertainty of the semi-quantification was relatively high, the data presented here can only be considered as tendencies but of which they are worth mentioning. Bromo, iodo- and bromochlorocarbazoles were not detected as well as methylcarbazole a carbazole alkaloid, and dibenzocarbazole. PCBs and the total sum of PCDDs and PCDFs were significantly low with TEQ values of 0.002 (WHO 2005) and 0.40 (WHO 2005) respectively.

3,6-Dichlorocarbazole concentration recorded in this study was high compared to those reported by (Kronimus et al. 2004) and (Reischl et al. 2005) in sediments (50 ng/g) and in soils (10 ng/g), respectively. They were comparably higher in soils reported by (Tröbs et al. 2011) (~800 ng/g fresh weight) but lower to what was reported by (Grigoriadou and Schwarzbauer 2011) (3,500 ng/g). 3-Chlorocarbazole was slightly less in the former study (35 ng/g fresh weight) but higher in the latter (110 ng/g). Anthropogenic influences (Kronimus et al. 2004), (Takasuga et al. 2009) and xenobiotic formation (Grigoriadou and Schwarzbauer 2011) have been suggested as possible sources of chlorocarbazoles in the environment. However, the linkage between the 3-mono- and 3,6-dichloro- isomers in the environment and anthropogenic sources has not been demonstrated. In fact, chlorocarbazoles of anthropogenic origin possess different substitution patterns such as the photodegradation products of the pharmaceutical drug diclofenac namely

1-chlorocarbazole and 8-chlorocarbazole-acetic acid (Poiger et al. 2001), and octachlorocarbazole (Takasuga et al. 2009). It can be suggested that since dibenzocarbazole (Heim et al. 2004) and tetrabromocarbazole (Kaur Bindra and Narang 1995) generated by combustion but not detected in this study confirms that the soil samples were not contaminated and consequently chlorocarbazoles were not of anthropogenic origin.

Natural formation of chlorocarbazoles is the most likely pathway given that 3-chloro- and 3,6-dichlorocarbazole detected in the soil samples can be synthesized enzymatically (Section 3.1). Chlorinated phenols, dibenzo-*p*-dioxins, dibenzofurans (Hoekstra et al. 1999), and adsorbable organic halogens (AOX) (Asplund et al. 1989) have been reported to be formed naturally in soil. Organically-bound chlorine can be produced by white-rot-fungi (Öberg et al. 1997). Organo-chlorine are the dominant forms of Cl in organic fractions of soil, sediments, aquatic systems and humified organics, estimated to be in the range of ~0.5 to 2 mM Kg⁻¹ in humified plant material (Myneni 2002). In addition, aromatic organo-Cl compounds are the most abundant forms of Cl in weathered plant material and their concentration increases with humification (Myneni 2002). The A-horizon (24-39 cm) where soil sample in this study was collected had the highest organic chlorine concentration (Hjelm et al. 1995). The contribution of microbial enzymatic formation processes in the A-horizon characterized by haloperoxidase-like bromination and chlorination activity (Latumus et al. 1995) to chlorocarbazoles concentrations recorded in this study therefore may be significant.

Abiotic oxidation processes play a significant role in formation of organo-chlorine compounds. Fe-oxyhydroxides commonly found in soil and sediments can catalyze the reactions of Cl⁻ and organic substrates. Naturally formed volatile halogenated organic halogens of bromide, chloride, and iodide ions were detected in organic rich peat bog waters and soils (Keppler et al. 2000). Since bromo- and iodocarbazoles were not detected could indicate very low concentrations or lack of their halide ions in the organic matter in soil sample used in this study which was strongly humic, waterlogged and tends to peat formation. PCDDs and PCDFs detected are most likely natural formed compounds (Hoekstra et al. 1999), (Fiedler et al. 1996).

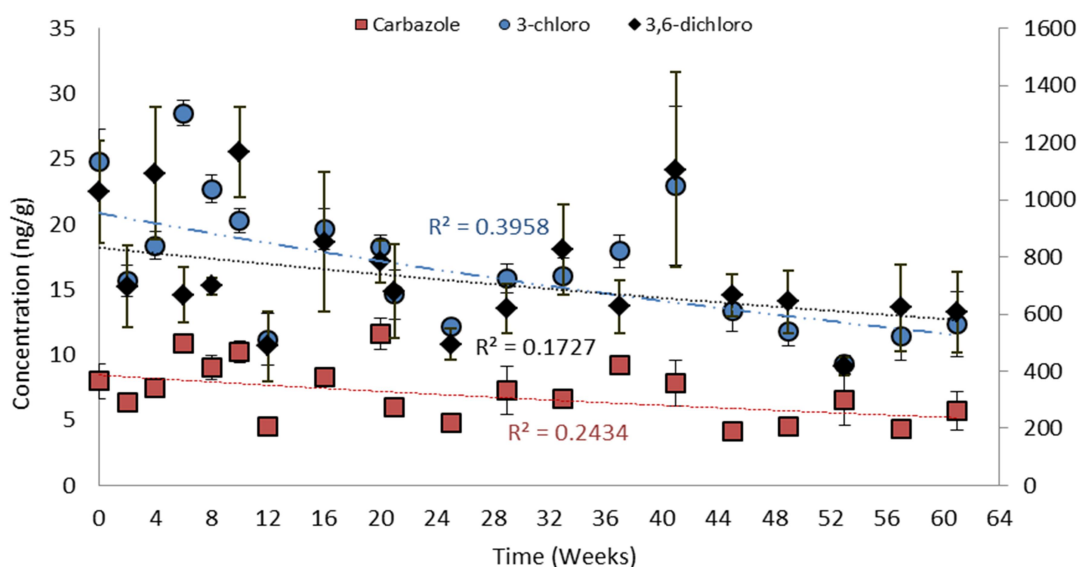


Figure 12 Averaged concentrations of carbazole, 3-chloro- and 3,6-dichlorocarbazoles detected in soil samples at 15°C conditions during the study period. The left y-axis represents concentrations for carbazole and 3-chlorocarbazole, while those on the right axis represent 3,6-dichlorocarbazole. Data are the mean and standard deviation of independent triplicate samples.

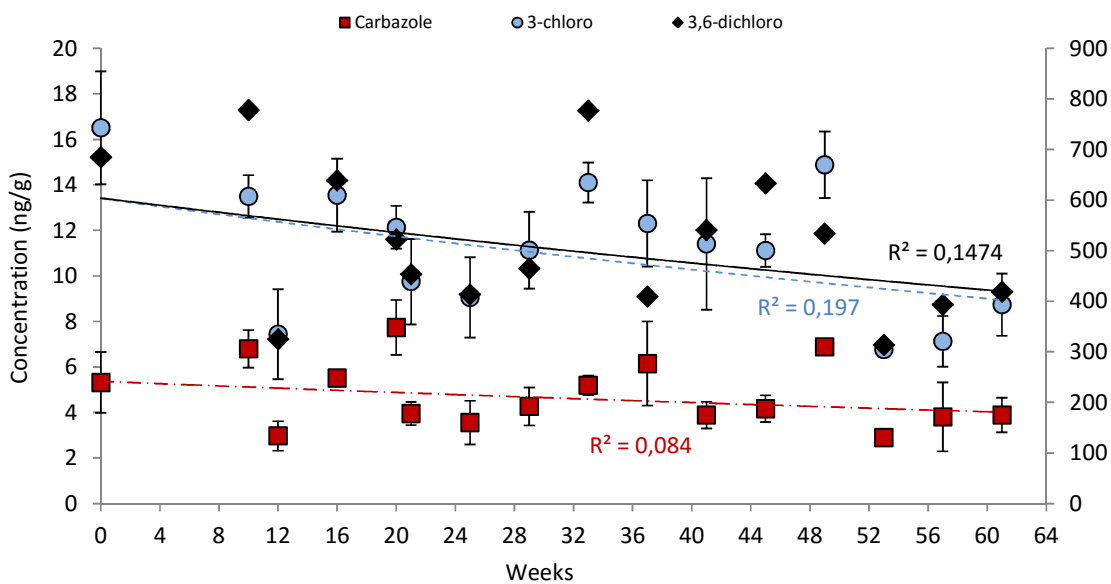


Figure 13 Averaged concentrations for carbazole, 3-chloro- and 3,6-dichlorocarbazoles detected in soil samples at 20°C temperature conditions. The left y-axis represents concentrations for carbazole and 3-chlorocarbazole, while those on the right axis represent 3,6-dichlorocarbazole. Data are the mean and standard deviation of independent triplicate samples.

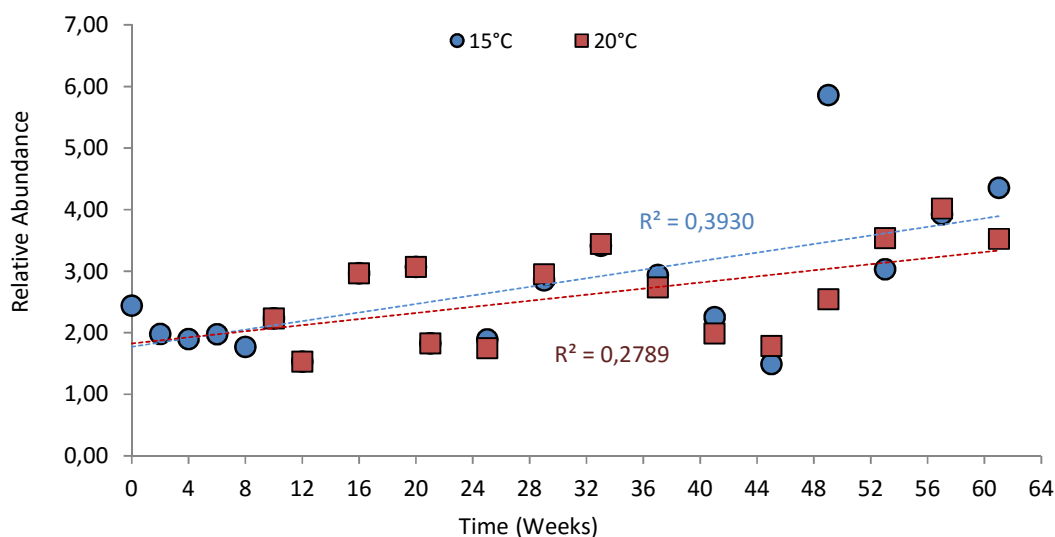


Figure 14 The relative abundance of trichlorocarbazole with time for 15°C and 20°C conditions. No values were recorded between the 8th and 20th for samples at 15°C conditions and between 0 and 8th for samples at 20°C. Data are the mean and standard deviation of independent triplicate samples.

3.4.2 Dissipation of carbazole and its chlorocarbazoles congeners

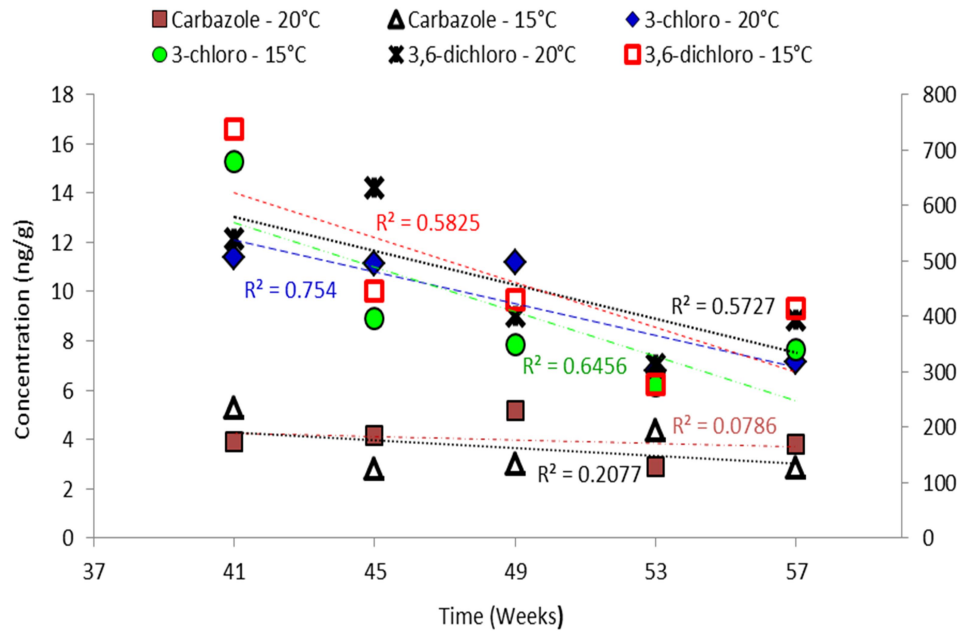
Carbazole, 3-chloro- and 3,6-dichlorocarbazole were monitored in this study due to their significant concentrations in the soil samples. Trichlorocarbazole available in very low concentration was included since it had not been reported previously in soil. Significant dissipation ($p < 0.05$) was observed in carbazole and 3-chlorocarbazole for samples at 15°C but not with 3,6-dichlorocarbazole (Fig. 12). However none of these compounds exhibited significant dissipation at 20°C temperature conditions (Fig. 13). Interestingly, a tendency to increase ($p < 0.05$) in trichlorocarbazole concentration was observed under both temperature conditions (Fig. 14). The characteristic initial lag period followed by a rapid decline in the concentration observed in first order degradation studies of compounds in soil was not witnessed in this study. Instead a slow and uneven decline was observed most likely due to uneven distribution in soil sampling that affected precision (25.3 to 40.6% RSD). Increasing sampling frequency, number of replicates or elongating the study period due to the resistance of the compounds to dissipation could improve precision. Homogeneity and variability were not significant ($P < 0.05$).

The degree of halogenation may have played a significant role in the dissipation of carbazole and 3-chlorocarbazole at both 15°C and 20°C given that 3,6-dichlorocarbazole with more chlorine atoms resisted breakdown under both conditions. Halogenated carbazoles resistance to dissipation has been observed in 1,3,6,8-tetrabromocarbazole, estimated to have been in the sediments of Lake Michigan from early 19th century when their concentrations were believed to be highest (Zhu and Hites 2005). Low water solubility of these compounds renders them less available to biodegradation, leaching, volatilization, and plant uptake (Providenti et al. 1993). Given that persistence of halogenated aromatic compounds in soil has a positive linear correlation with water partition coefficient and it increases with increase in ring complexities, the number and variation in the size of the halogens (Wild et al. 1991), (Birnbaum 1985), it can therefore be suggested that the persistence of halogenated carbazoles congeners follows the order, mono < di < tri < tetra < penta. A similar sequence has been reported with respect to chlorinated dioxins (Kearney et al. 1972), chlorinated benzenes (Brown and Wania 2008), chlorinated phenols (Liu et al. 1991) and chlorinated biphenyls (Birnbaum 1985).

Temperature played a significant role in the dissipation of chlorocarbazoles in soil samples at 15°C condition but not at 20°C. At 15°C temperature conditions, carbazole and 3-chlorocarbazole exhibited significant negative correlation with time ($r \geq 0.50$, $p < 0.05$). Aerobic degradation at low temperature may have been responsible for the dissipation as degradation of PAHs has been reported at lower temperatures relative to higher temperatures. This depends on the abundance of psychrotolerant PAH degraders in the soil responsible for degradation at low temperature (Eriksson et al. 2003). Aerobic degradation of a culture from creosote-contaminated soil was more at 7°C relative to 20°C. A carbazole degrader, *Pseudomonas sp. strain C3211*, isolated from a temperate climate soil contaminated with creosote caused faster degradation of carbazole at 10°C relative to 25°C (Jensen et al. 2003). This implies the aerobic degradation pattern of carbazole could be similar to that of chlorocarbazoles since they seem to prefer low temperature conditions for degradation.

3,6-Dichlorocarbazole did not show significant dissipation at 15°C temperature conditions (Fig. 15) in contrast to carbazole and 3-chlorocarbazoles. No significant dissipation ($p < 0.05$) was observed in soil samples at 20°C temperature conditions for the all compounds (Fig. 15a). A weak but negative correlation with time ($r < 0.50$) indicated dissipation was present but slow. The very high concentration of 3,6-dichlorocarbazole relative to other compounds 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 (Fig. 15a and 15b). Increase in trichlorocarbazole concentration may be attributed to enzymatic oxidative catalyzed chlorination (Hoekstra et al. 1999) of carbazole and chlorocarbazoles by chloride ions possibly made available during dissipation. Chloride ion concentration has been observed to increase during the degradation of polychlorinated biphenyl (Di Toro et al. 2006) which make chloride available for natural formation of halogenated carbazole compounds since chlorocarbazoles can be synthesized enzymatically (Section 3.1).

A



B

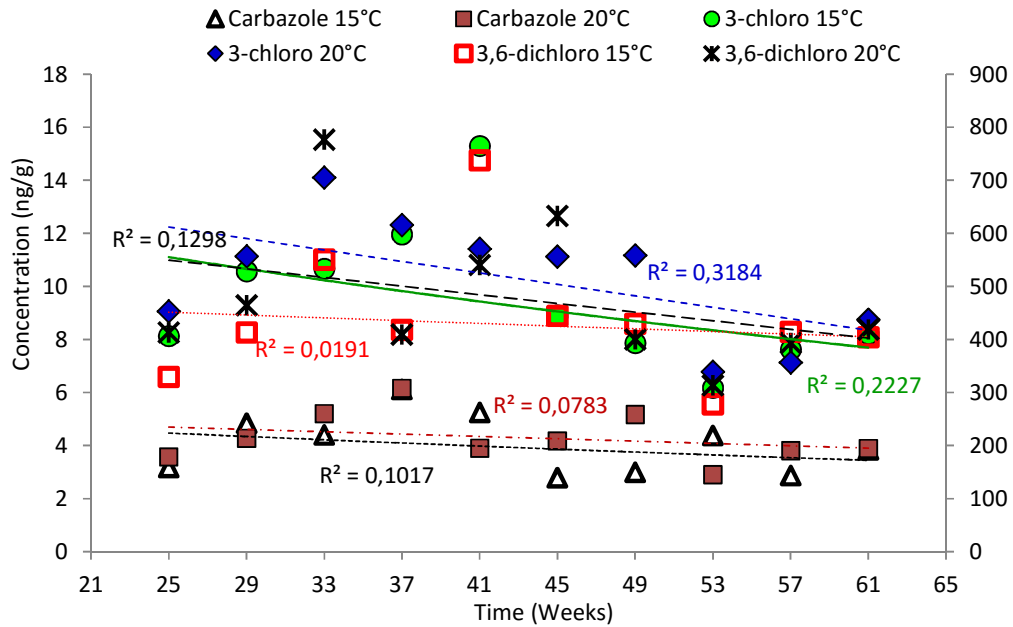


Figure 15 Effect of temperature change from 15° to 20°C is shown in (A). In this case comparison is made between soil samples in these two temperature conditions. A comparison between dissipation under the two temperature conditions is shown in (B). The right axis represents 3,6-dichlorocarbazole concentrations. Data are the mean of independent triplicate samples.

3.5 Photodegradation of bromo- and chlorocarbazoles in water

3.5.1 Determination of Wavelength of Maximum Absorption

The maximum wavelength (λ_{\max}) of absorption for each of the halogenated carbazole under investigation was in the near UV region (Pyrex filtered spectrum) (>290 nm). λ_{\max} recorded were 295 nm, 296 nm, 299 nm and 301 nm for the 3-chloro-, 3-bromo-, 3,6-chloro- and 3,6-dibromocarbazole respectively (Fig. 16-19). A bathochromic shift was observed in the two congeners with the addition of a halogen in their respective mono-substituted congeners which are electron-donating groups. Electron donating groups increase both λ_{\max} and molar absorptivity (ϵ_{\max}) because of their conjugation effects with the π -electrons of the aromatic rings. Bromo-, iodo-, methyl- and nitrated-derivatives of vinylcarbazole are carbazole congeners that are known organic photoconductive compounds.

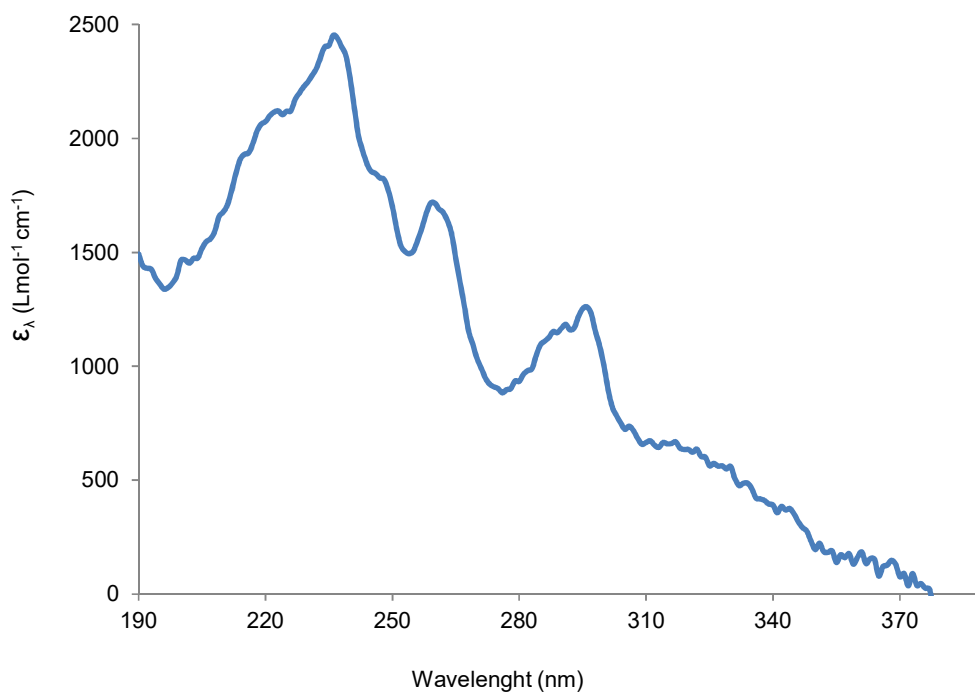


Figure 16 Absorption Spectrum of 3-bromocarbazole. Maximum wavelength of absorption in the Pyrex filtered spectrum (>290 nm) was recorded at 296 nm.

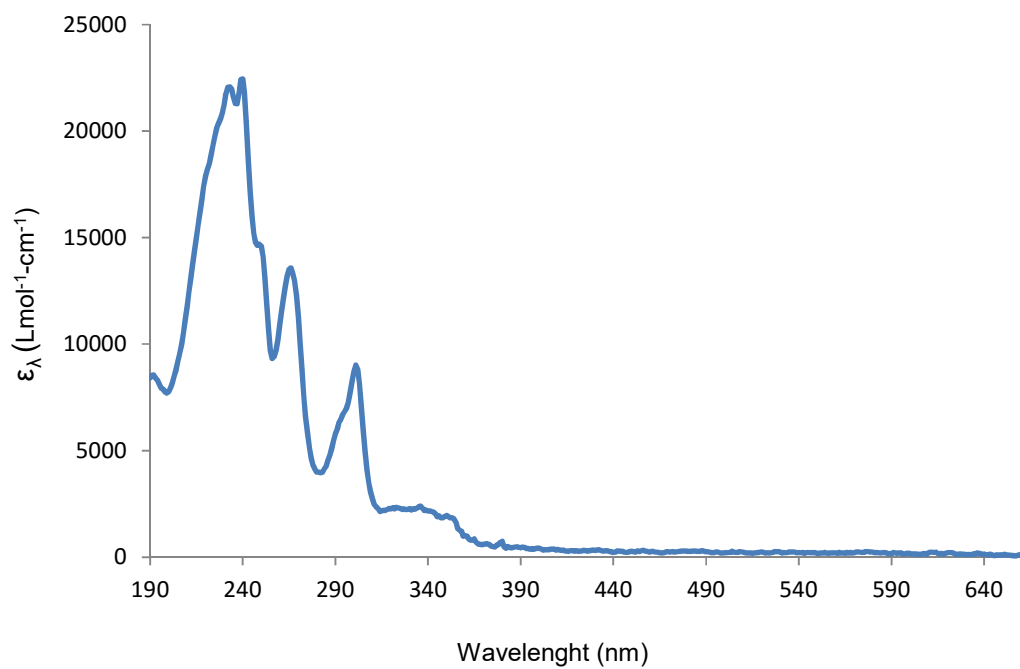


Figure 17 Absorption spectrum of 3,6-dibromocarbazole. Maximum wavelength of absorption in the Pyrex filtered spectrum (>290 nm) was recorded at 301 nm.

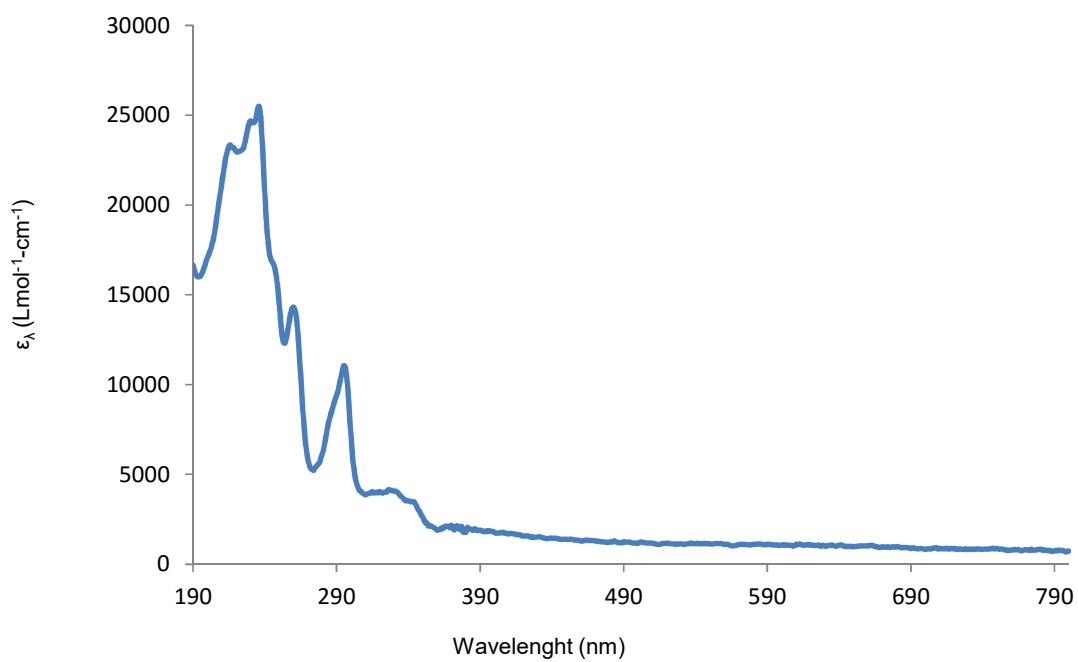


Figure 18 Absorption spectrum of 3-chlorocarbazole. Maximum wavelength of absorption in the Pyrex filtered spectrum (>290 nm) was recorded at 295 nm.

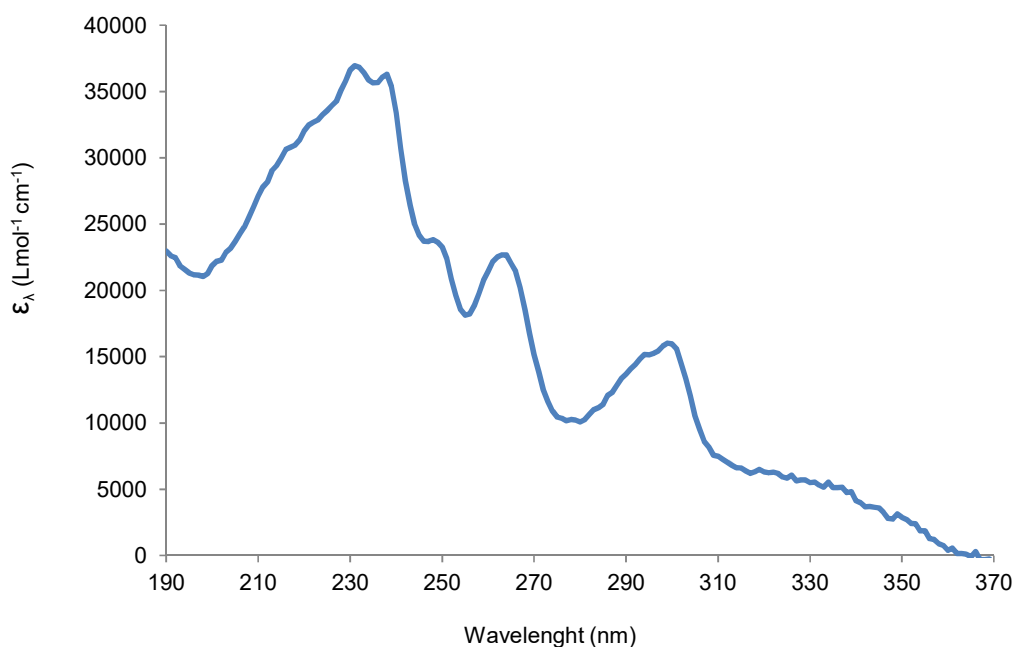


Figure 19 Absorption spectrum of 3,6-dichlorocarbazole. Maximum wavelength of absorption in the Pyrex filtered spectrum (>290 nm) was recorded at 299 nm.

3.5.2 Photodegradation rates and quantum yields

Photodegradation was observed in all compounds under study. The rates of photolysis in milli-Q water/acetonitrile (1%) was faster for the mono-substituted congeners in relation to the di-substituted congeners with first-order degradation rate constants (k) of 0.3822 ($r^2 = 0.8387$) hr⁻¹, 0.3442 ($r^2 = 0.9774$) hr⁻¹, 0.0386 ($r^2 = 0.9763$) hr⁻¹ and 0.4422 ($r^2 = 0.8913$) hr⁻¹ corresponding to half-lives of 1.81, 2.01, 17.95 and 1.62 hr for 3-bromo-, 3-chloro-, 3,6-dibromo- and 3,6-dichlorocarbazole respectively (Fig. 20-23). The rate of photolysis of 3,6-dichlorocarbazole was faster than 3-chlorocarabzole. This is consistent with other studies that report the rate of photodegradation being slower in the less halogenated compounds in relation to those that are highly halogenated (Chen et al. 2010), (Fang et al. 2008), (Eriksson et al. 2004), (Friesen et al. 1996). Higher halogenated compounds absorb at slightly longer wavelengths however the ability decreases with decrease in number of the halogen atoms (Sánchez-Prado et al. 2006). Molar extinction coefficient (ϵ) and quantum yield (ϕ) of 3-bromo-, 3-chloro-, 3,6-dibromo-

and 3,6-dichlorocarbazole were determined (Table 8, Appendix Table 12, Fig. 40) including solar rate constants [$k_{d(solar)}$] and estimated direct photolysis $k_{d(solar)}$ in the surface water (top layer) of the northern hemisphere at latitude 50°N (OECD, 2002) (Table 8, Appendix Table 13-16). Germany lies on latitude 48°N to 52°N. The photolysis of 3,6-dibromocarbazole was however slower in comparison to that of its mono-substituted bromocarbazole. The laboratory estimated half-lives were lower but within the range of carbazole with a half-life 2.9 hr in water. Carbazole and halogenated carbazoles in this study absorb light strongly in the Pyrex filtered spectrum (>290 nm) up to 400 nm (Smith et al. 1978). The effect of conjugation is less in carbazole due to the absence of the halogens causing it to be slower to photolysis in relation to the halogenated carbazoles. The effect of pH may have not been significant as it was maintained in the range (pH 7.5 to 8.0) at 25±2°C. Previous studies have shown pH as having no effect on rate of photolysis of organic compounds (Piscopo et al. 2001), while in another study, photodegradation rate was faster in acidic than in the basic pH even though higher degradation were observed at pH 6.5 (Rakesh M. Pawar et al. 2013).

Table 8 Rate constant k in the laboratory and estimated $k_{d(solar)}$ in the surface water (toplayer) of the northern hemisphere, respective half-lives and molar absorptivity of bromo- and chlorocarbazoles

Compound	Rate constant k (hr^{-1})	Half-life lamp [$t_{1/2}$ (hr)]	Rate constant $k_{d(solar)}$ (d^{-1})		Half-life solar [$t_{1/2}$ (d)]		Quantum yield (Φ)	Molar extinction coefficient in acetonitrile [ϵ ($Lmol^{-1}cm^{-1}$)]
			Summer	Winter	Summer	Winter		
3-Bromocarbazole	0.3822	1.8	1.622	0.274	0.4	2.5	0.184	18,573
3-Chlorocarbazole	0.3442	2.0	2.564	0.453	0.3	1.5	0.180	17,028
3,6-Dibromocarbazole	0.0386	18.0	0.129	0.022	5.4	31.5	0.026	14,010
3,6-Dichlorocarbazole	0.4422	1.6	1.785	0.293	0.4	2.4	0.281	13,385

The type of halogen in comparison to its rate of photodegradation could be observed with respect to the mono-substituted congeners. 3-Chlorocarbazole was observed to be more resistant to photolysis than 3-bromocarbazole and vice versa for the di-substituted congeners. However, these results cannot be conclusive given that the compounds used in this study were not 100% pure (see 2.1.1).

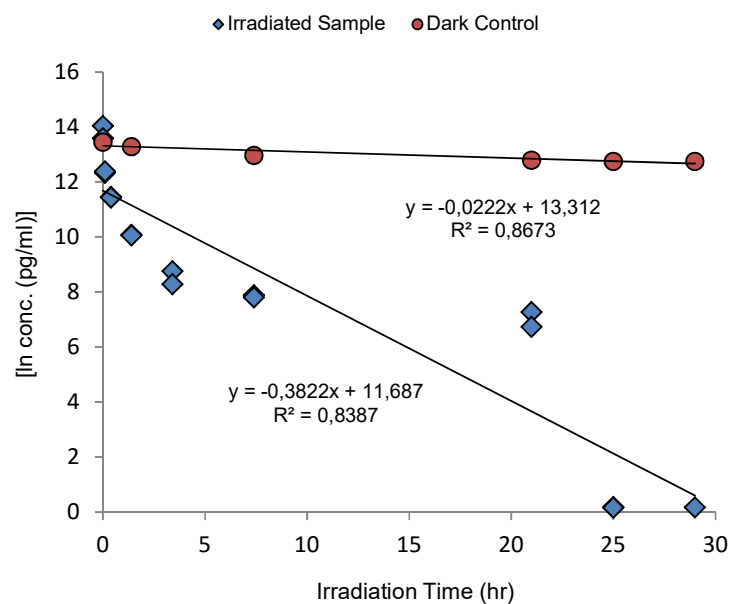


Figure 20 Photodegradation of 3-bromocarbazole in water

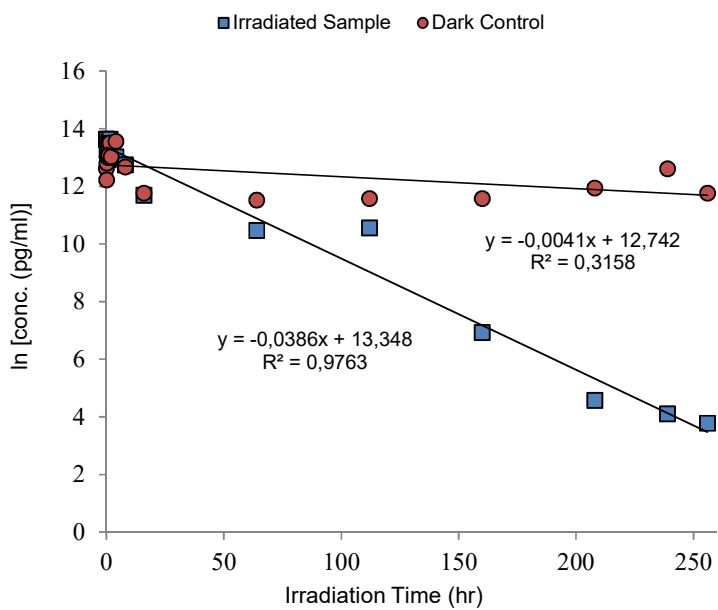


Figure 21 Photodegradation of 3,6-dibromocarbazole in water

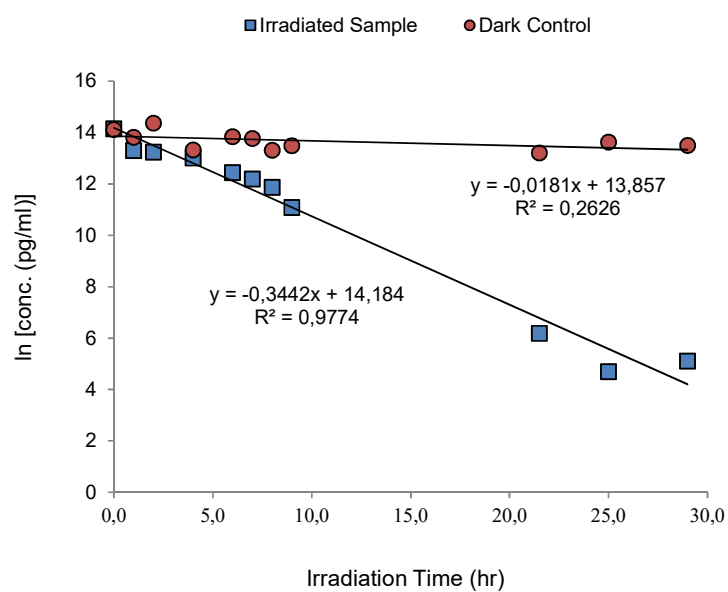


Figure 22 Photodegradation of 3-chlorocarbazole in water.

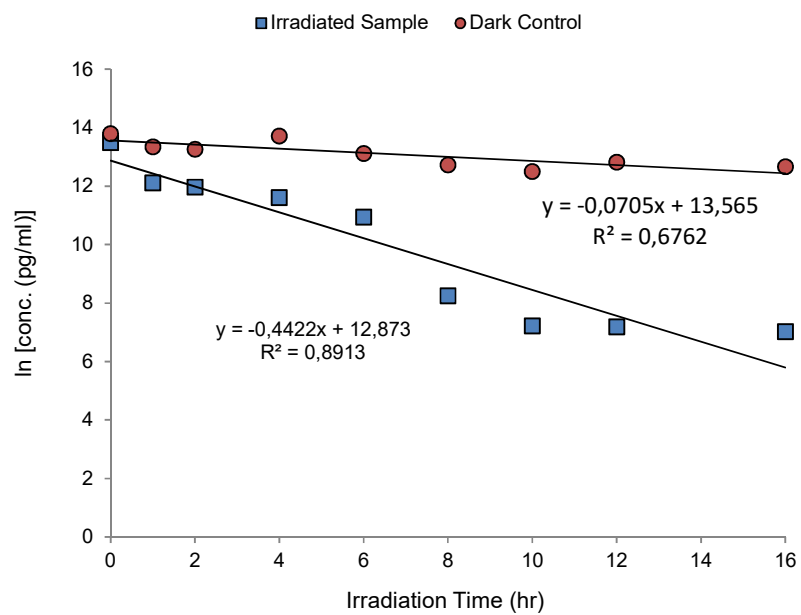


Figure 23 Photodegradation of 3,6-dichlorocarbazole in water.

3.5.3 *Effect of hydrolysis and volatilization*

Quantitative recoveries of bromo- and chlorocarbazoles from dark controls in water / acetonitrile solutions sampled over the entire exposure period confirmed that the observed decrease in concentrations were due to photodegradation. Dark control experiments showed (Fig. 20-23) these compounds as being relatively stable to hydrolysis with time in water. This suggests in the absence of light, sorption to sediment substrates plays an important role in their transport and distribution. PAHs tend to adsorb onto solid phases in aquatic environment because of their hydrophobic nature and low water solubilities (Smith et al. 1978). Previous studies have reported halogenated carbazoles in soil and sediments (Grigoriadou and Schwarzbauer 2011), (Reischl et al. 2005). These results suggest that photodegradation is an important degradation pathway on the fate of halogenated carbazoles in aquatic environment based on their short half-lives in water under irradiation. However, the presence of hydrolyzable halogen groups (Larson and Weber 1994) causes them to be liable to hydrolysis as observed in the gradual but slow degradation of the halogenated carbazole in the dark control experiments. The trend of hydrolysis reactivity reflects the strength of carbon-halogen bond which is in the order F>Cl>Br. Volatilization has been suggested as being insignificant for PAHs with three or more aromatic rings (Park et al. 1990). Temperature was kept constant 25±2°C and therefore this effect was not significant. However, further research is required for conclusive results since in another study substantial amounts of three to five aromatic rings were detected in vapour phase at ambient temperature (Valerio and Pala 1991). Equally, the effect of hydroxyl radical on indirect photolysis of halogenated carbazoles is essential since nitrates have been associated with hydroxyl radical formation in natural water (Takeda et al. 2004).

3.5.4 *Photoproducts*

Several compounds were detected by ultra high performance liquid chromatography-mass spectrometry (UHPLC-MS) from direct photolysis of halogenated carbazoles and subjected to mass spectrometry. Employing positive ionization mode, compounds with molecular ion with m/z MH^+ 175.0996, MH^+ 198.0646, MH^+ 203.1084, MH^+ 216.0659, MH^+ 221.1186, MH^+ 277.1833, MH^+ 363.0996 were detected (Appendix Figures 33-39) The protonated molecular ion signals correspond

to compounds with exact masses of 174.0966, 197.0646, 202.1084, 215.0659, 220.1186, 276.1833, 362.0996 respectively. Halogenated carbazole photoproducts were not detected suggesting dehalogenation was one of the photolysis pathways. The protonated molecular ion signals MH^+ 198, MH^+ 216 and MH^+ 363 were observed in 3-bromo- and 3-chlorocarbazole irradiated solutions, while MH^+ 175.0996 and MH^+ 216.0659 in 3,6-dibromocarbazole solution. In irradiated solutions of 3,6-dichlorocarbazole only very weak signals were observed and therefore not considered for further analysis. MH^+ 216.0675 appeared to correspond to trihydroxycarbazole (molecular weight 215) suggesting hydroxyl (OH) substitution. There is no information in literature on the toxicological properties of this compound. Monohydroxycarbazole (molecular weight 183.21) known to be mutagenic (He et al. 2011) was not isolated in this study. It was therefore not possible to determine whether photolysis of halogenated carbazoles results in compounds of enhanced toxicity. However, these results are not conclusive since the identity of the photoproducts could not be confirmed due to the lack of standards or pure reference compounds. Since lower and higher molecular ion peaks than the compounds (m/z 201.6515, 236.102, 246.10, 325.00) under study were generated, cleavage and coupling might be other important photodegradation processes present during photolysis. MH^+ 363.0996 could be a coupling product of bromoaniline (molecular weight 172.02) with hydration (Fig. 35). Carbazole and halogenated carbazole photoproducts (bromo- and chlorocarbazoles) were detected in irradiation experiments of all compounds. However, their concentration decreased with time as that of the main compound indicating that these could have been impurities in the original compound. In most photodegradation studies there is usually an increase in concentration of the photoproducts as the main compound concentration decreases over time (Calza et al. 2012).

3.6 Dioxin-like toxicity assessment

Pure compounds of carbazole, 3-chloro- and 3,6-dibromocarbazole, and 10 soil samples extracts, one without incubation and the others with different incubation periods, were analyzed for CYP1A1 induction using H4IIE-EROD bioassay. The pure compounds and all soil extracts elicited significant activity for 24 and 72 hrs EROD bioassay. EROD was strongly induced by 3,6-dibromo- and 3-chlorocarbazole, with both exhibiting relative potencies (REPs) of approximately 10^{-10} relative to TCDD (Table. 9). Carbazole showed no affinity with EROD bioassay. The limitation of EROD bioassay provides the need for more studies to confirm the affinity of carbazole to AhR. The observed REP was in the order of carbazole < 3-chlorocarbazole < 3,6-dibromocarbazole.

Untreated extracts from the soil samples elicited EROD activity within the same range but it was highest for the soil sample not subjected to incubation. The toxic equivalents (TCDD-EQ) ranged from 220 to 470 pgTCDD-EQ/g (Table 9) representing not only dioxin-like toxicity of halogenated carbazoles but also AhR affinity of other natural occurring compounds. There was no significant ($p < 0.05$) decline in 72hr EROD bioassay toxicity with time but a fluctuating trend was observed during the study period. The \sum TEQ for 3-chloro- and 3,6-dichlorocarbazole ranged from 0.025 to 0.069 pg/g. A significant negative correlation between \sum TEQ and time was observed which is most likely due to the presence of compounds such as biphenyls, chlorophenols, dioxins and furans able to induce EROD activity that are more resistant to dissipation in soil. Recent studies suggest these compounds can be formed naturally in sediments and clays (Fiedler et al. 1996), also in peat and forest soil by enzymatic oxidative processes (Hoekstra et al. 1999). There was no correlation ($r = 0.11$, $p < 0.05$), between the calculated \sum TEQ and the bioassay-derived TCDD-EQ, though the later was higher by a factor of 10^4 (Table 9). 3-Mono- and 3,6-dichlorocarbazole were only used in \sum TEQ calculations making these results not conclusive to show this correlation but useful in predicting toxicity pattern for emerging category of environmental contaminants. For example, TEQ of carbazole, other halogenated carbazole congeners and other CYP1A1-inducing compounds were not determined. Given the negative correlation between \sum TEQ and the bioassay-derived TCDD-EQ, it is very likely chlorocarbazoles contributed in part but

not significantly to the dioxin-like toxicity in soil extracts. Naturally occurring compounds able to cause EROD induction should therefore be factored in this correlation given the soil extracts used to perform EROD bioassay had not been subjected to clean-up. More data is required to establish the correlation between Σ TEQ and bioassay TCDD-EQ.

Table 9 TEQ values for 3-mono- and 3,6-dichlorocarbazole calculated from respective REP values from selected 10 soil samples extracts obtained during the study period and compared to EROD, H4IIE cell bioassay TCDD-EQ. Since 3,6-dibromocarbazole was not detected in the soil samples, it was not factored in the Σ TEQ. Pure compounds of 3-chloro- and 3,6-dibromocarbazoles were used to calculate their respective REPs with the latter then used to calculate TEQ values for each soil extract.

Incubation Time (Weeks)	Halogenated carbazole in soil extracts (pg/g)		Concentration of soil extract × Chlorocarbazole Relative Effect Potency (REP) (pg/g)		Σ TEQ (pg/g)	24hr bioassay TCDD-EQ (pg/g)	72hr bioassay TCDD-EQ (pg/g)	TEQ ≈ TCDD-EQ?
	3-Chloro	3,6-Dichloro	3-Chloro REP	3,6-Dichloro REP				
0	15019	704220	0.00	0.067	0.067	809	470	No
4	12251	728280	0.00	0.069	0.069	468	220	No
8	13909	439212	0.00	0.042	0.042	561	281	No
20	12470	603885	0.00	0.057	0.057	760	380	No
25	7908	300611	0.00	0.028	0.028	584	303	No
33	11145	649865	0.00	0.062	0.062	662	423	No
37	11995	465064	0.00	0.044	0.044	723	436	No
45	9698	441030	0.00	0.042	0.042	679	434	No
53	5654	262538	0.00	0.025	0.025	689	321	No
61	8209	403916	0.00	0.038	0.038	670	315	No
	3,6-Dibromocarbazole				-	-	343	N/A
NB	3-Chlorocarbazole calculated REP					-	1.94E-10	
	3,6-Dichlorocarbazole pgTCDD obtained from (Tröbs et al. 2011)					-	9.48E-08	
	3,6-Dibromocarbazole calculated REP					-	3.43E-10	
	Σ TEQ (pg/g) = (REP for 3-Chloro + REP for 3,6-Dichloro)							

Conclusions

Bromo- and chlorocarbazoles were synthesized enzymatically in this study therefore demonstrating the role of natural sources to their occurrence and concentrations in the environment. Halogenated carbazoles detected previously (Tröbs et al. 2011, Grigoriadou and Schwarzbauer 2011, Zhu and Hites 2005) were all synthesized by environmentally abundant enzymes in this study. These results provide evidence that bromocarbazoles and chlorocarbazoles can be formed naturally in the environment. The stable structures of bromo- and chlorocarbazoles were determined. The structural position of the halogen at 3-, 3,6-, 1,3,6- and 1,3,6,8- on the carbazole structure were consistent with halogenated carbazoles detected in the environment. Bromo- and chlorocarbazoles have a wide distribution being found to occur in forest soil samples in randomly collected from different regions in Germany. Their distribution is suggested to be governed by natural and anthropogenic sources. Equally, their concentrations vary with increase in soil depth and are related to soil organic carbon. 3-Bromocarbazole and 3,6-dibromocarbazole not reported previously in soil were detected in soil samples. It was also determined that 3-mono- and 3,6-dichlorocarbazoles are persistent to degradation in soil. 3,6-Chlorocarbazole was the most resistant under the two temperature conditions which may be due to halogenation effect hence the consistently high concentrations of 3,6-dichlorocarbazole recorded in all soil samples relative to other compounds. Their photolysis in water follows first-order kinetics. 3,6-Dibromo-, 3-mono- and 3,6-dichlorocarbazoles were also determined to exhibit weak dioxin-like toxicity. Bromo- and chlorocarbazoles share similar attributes to persistent organic pollutants (POPs) given that they degrade slowly in soil and have some degree of toxicity. Since there is limited information on halogenated carbazoles, an opportunity is here presented either to be categorized with POPs or another group of chemicals to enable detailed research and effective management in the environment.

Recommendations

It is evident from this study that halogenated carbazoles are persistent compounds. Coupled with the fact they have low water solubility and possess dioxin-like toxicity, this provides an opportunity to categorize these compounds as persistent organic pollutants (POPs); persistent, bioaccumulative and toxic (PBT); very persistent and very bioaccumulative (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 (Zarfl and Mattheis 2013), (ECHA 2012). The Stockholm Convention on Persistent Organic Pollutants (POPs) has also developed criteria for classification of POPs (UNEP 2001). A persistence of > 120 days was recorded for carbazole and chlorocarbazoles in this study which was more than what is in the set criteria (Table 10) putting them in the category of potential priority PBT substances. Experimental data on bioaccumulative potential is necessary given the level of toxicity these compounds possess. Carbazole toxicity may have been significantly enhanced after halogenation as toxicity of organic compounds is enhanced when transformed to its halogenated products (Wittsiepe et al. 1999). Carbazole and 9-ethylcarbazole have been identified as potential PBT by the Federal Environmental Agency of Germany (Böhnhardt 2013). 9-Butyl-9H-carbazole has been categorized as high priority PBT substance for further investigations (Brooke and Burns 2010). An evaluation of the less known halogenated carbazoles is required.

Table 10 Criteria for categorization of halogenated carbazoles as persistent organic pollutants.

Compounds	Potential POPs or PBT	Status	Criteria
3-Chlorocarbazole, 3-Bromocarbazole, 3,6-Dichlorocarbazole	Criteria met for POPs and PBT	Not categorized	REACH >120 days UNEP-POP > 180 days
Carbazole, 9-Ethylcarbazole	Identified as potential PBT	Not categorized	QSAR methods Böhnhardt A (2013)
9-Butyl-9H-carbazole	Identified as Persistent and very Bioaccumulative (PvB)	High priority PvB for further investigation	REACH Environment Agency, UK. (Brook and Burns, 2010)

Appendix

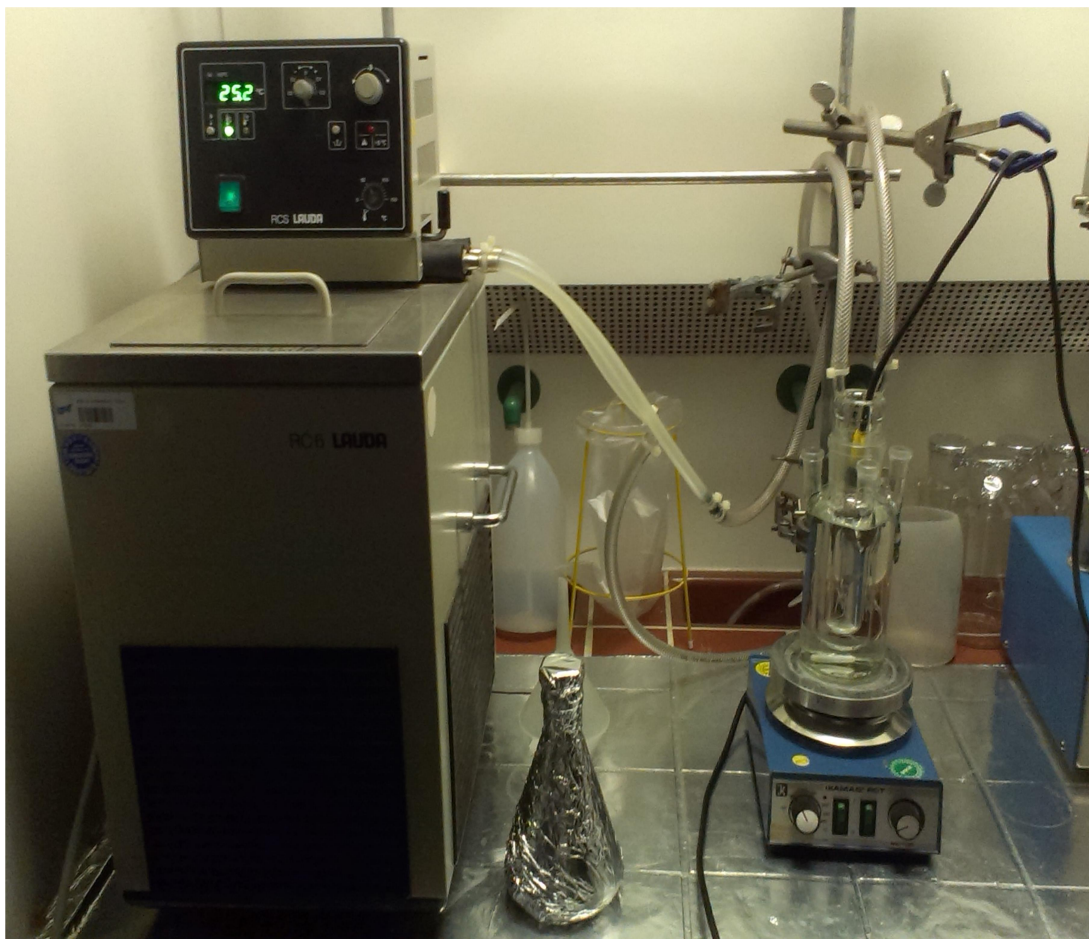


Figure 24 Photodegradation experiment set-up. The cylindrical glass tube reactor (right) on a stirrer is connected a cooler (left) which set to maintain the reactor temperature at $25\pm 2^{\circ}\text{C}$. The irradiation lamp (with a yellow mark) was inserted from the top in water jacked glass tube.



Figure 25 An illustration of the cylindrical glass tube reactor on a magnetic stirrer. The two inlets were sealed during irradiation.

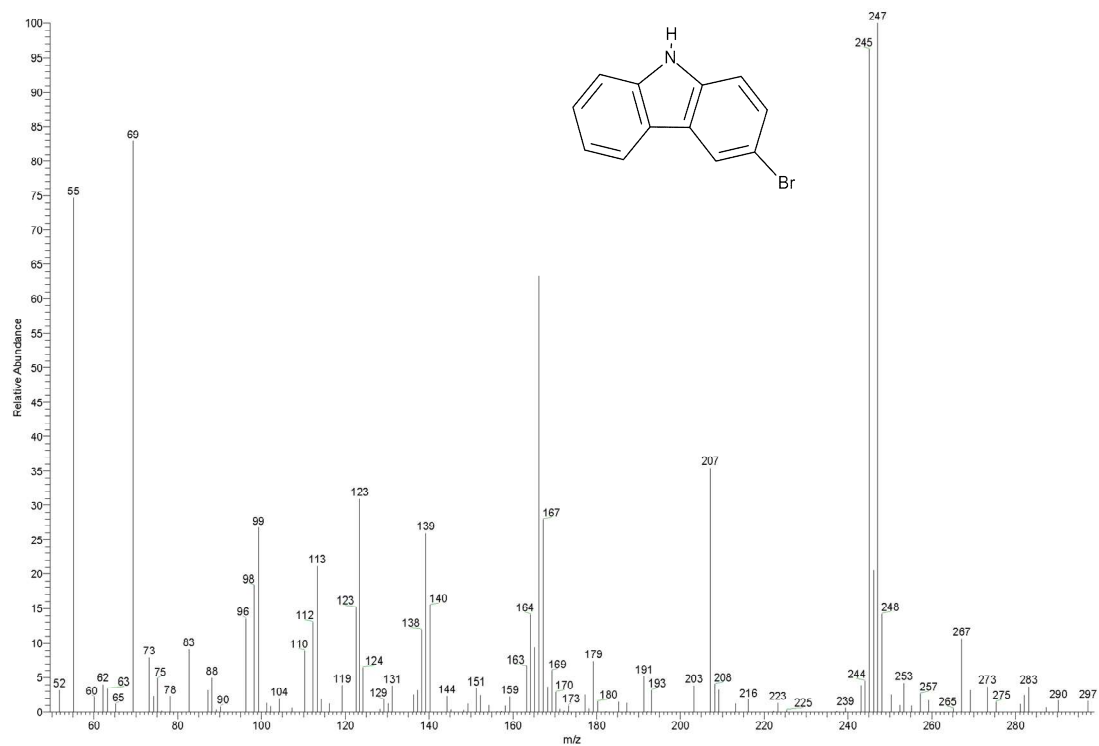


Figure 26 Mass spectrum of monobromocarbazole

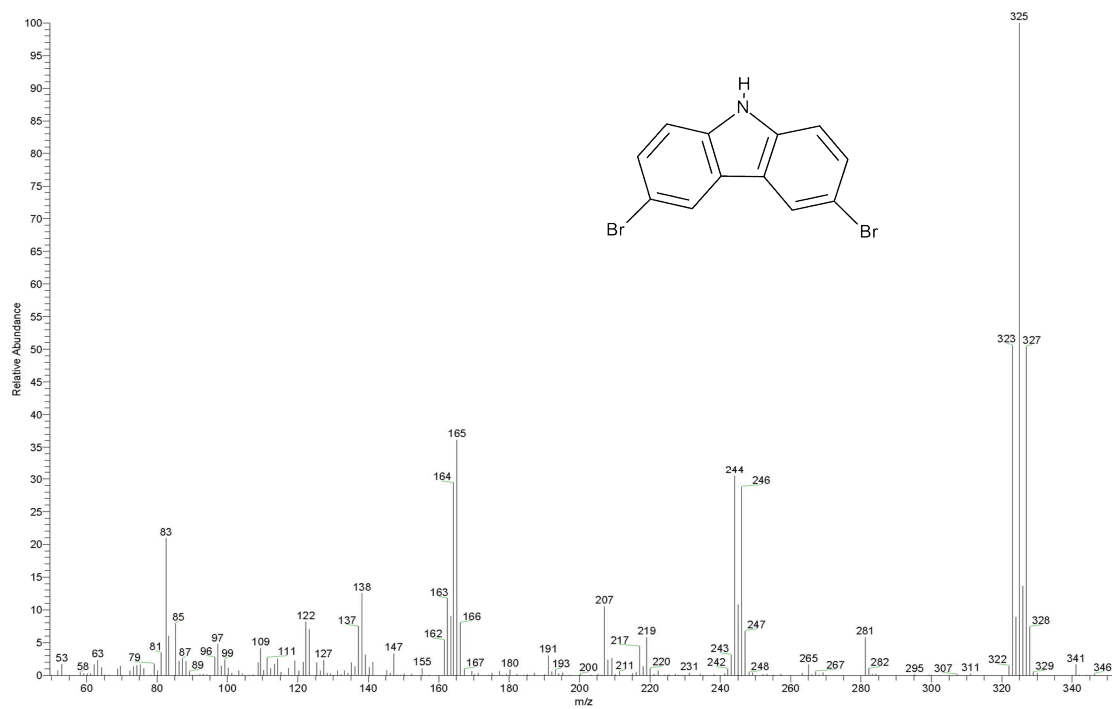


Figure 27 Mass spectrum of dibromocarbazole

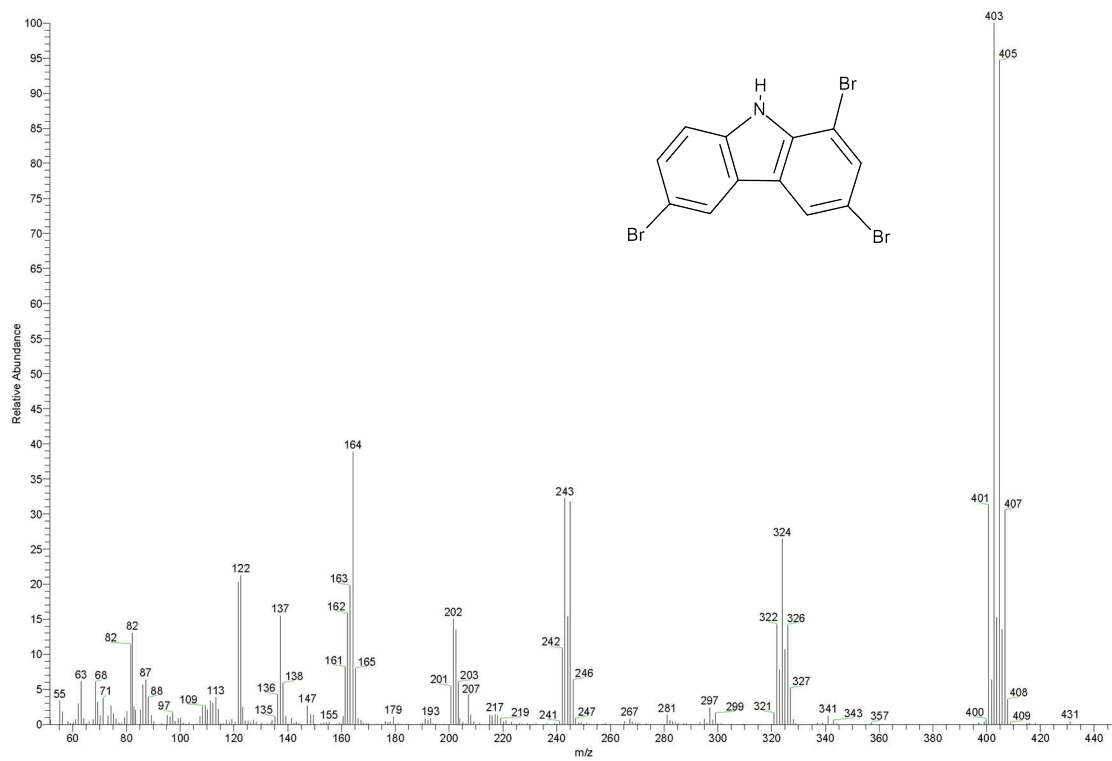


Figure 28 Mass spectrum of tribromocarbazole

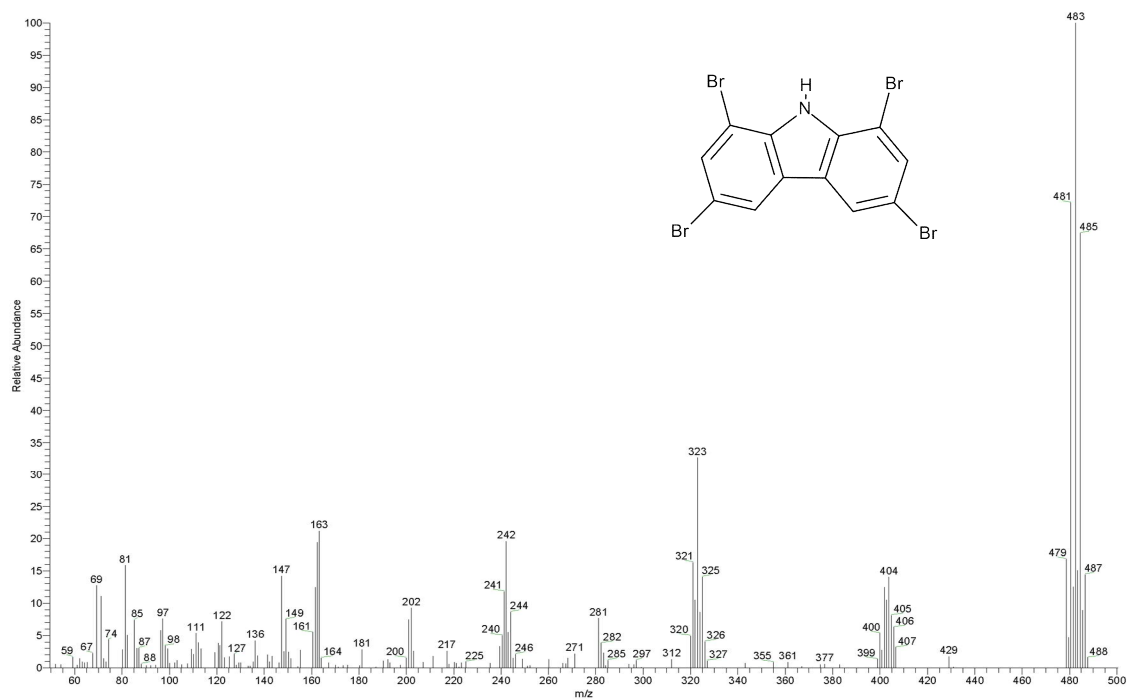


Figure 29 Mass spectrum of tetrabromocarbazole

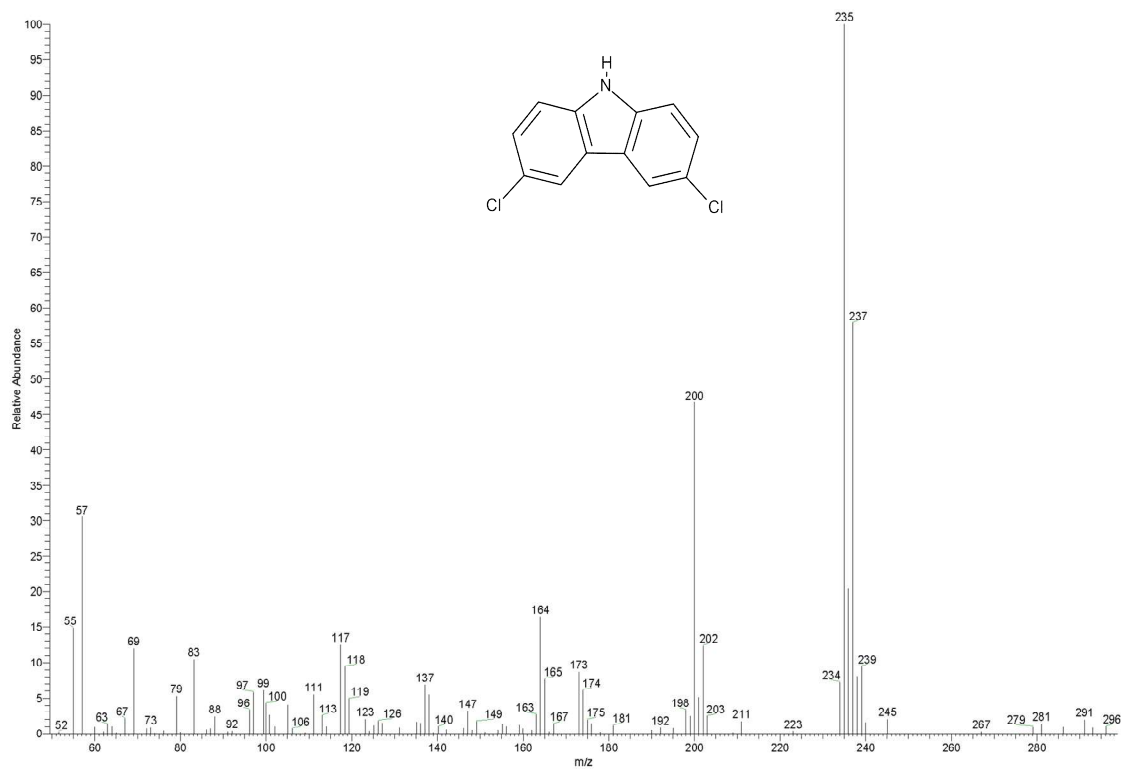


Figure 30 Mass spectrum of dichlorocarbazole

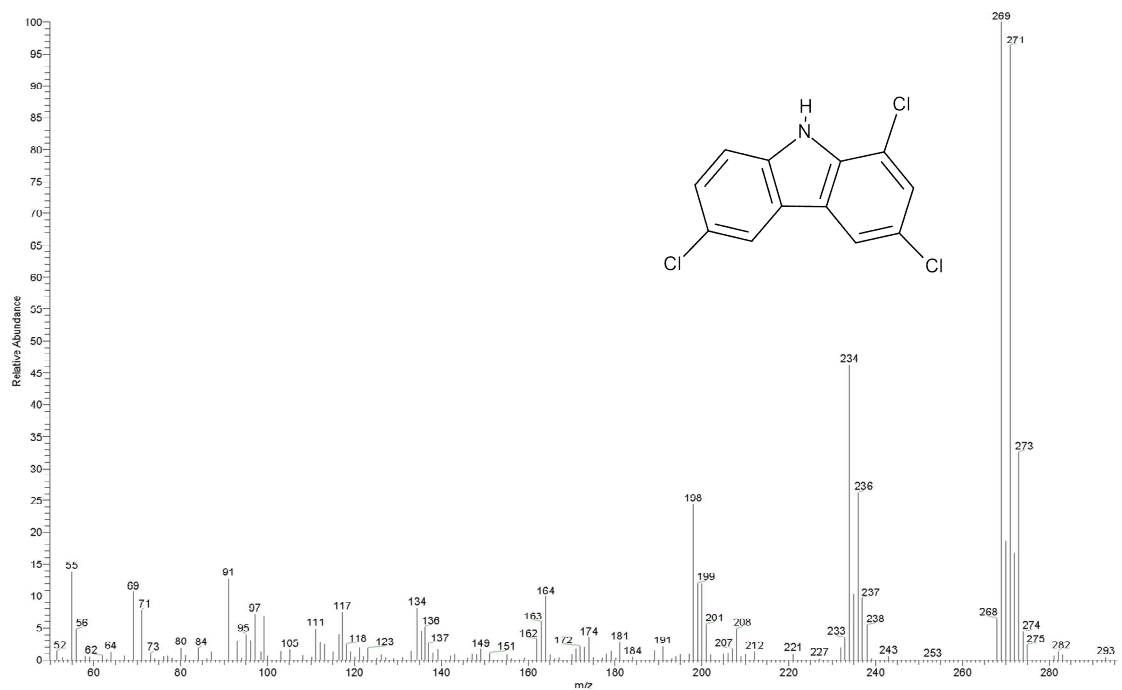


Figure 31 Mass spectrum of trichlorocarbazole

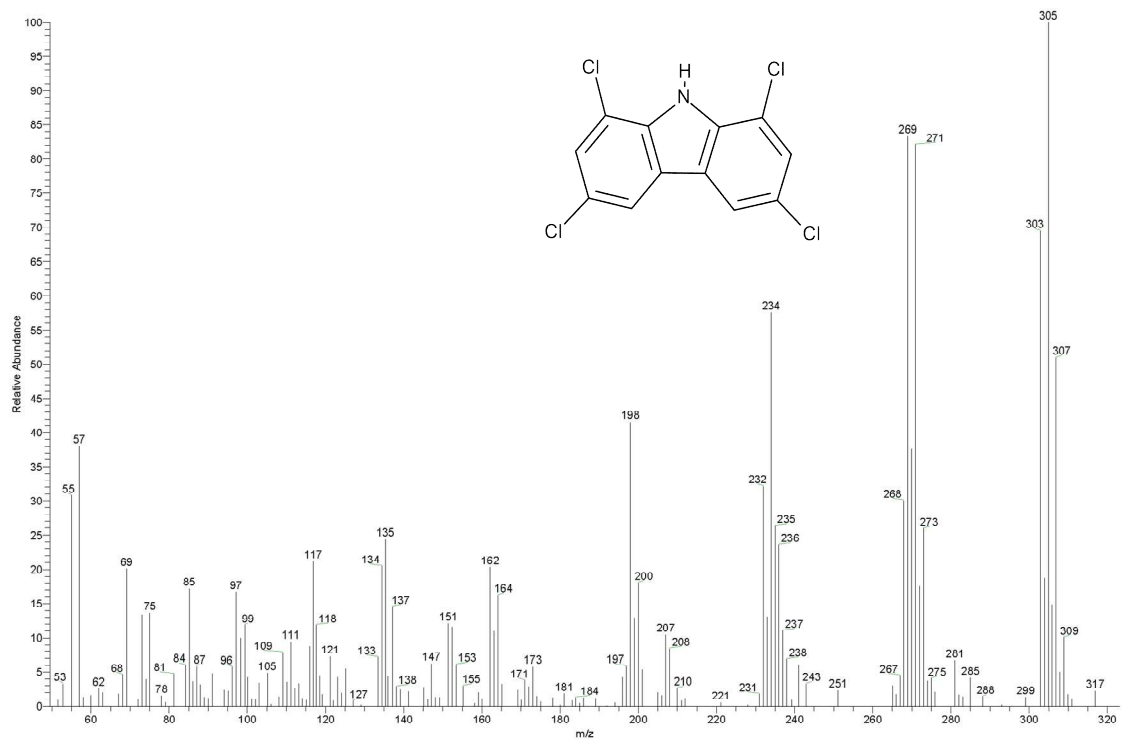


Figure 32 Mass spectrum of tetrachlorocarbazole

30 min 3-Bromocarbazole

20141010_Carbazoles_05 727 (13.481) AM (Cen,5, 80.00, Ar,7000.0,0.00,0.70); Cm (719:737)

TOF MS ES+
1.55e3

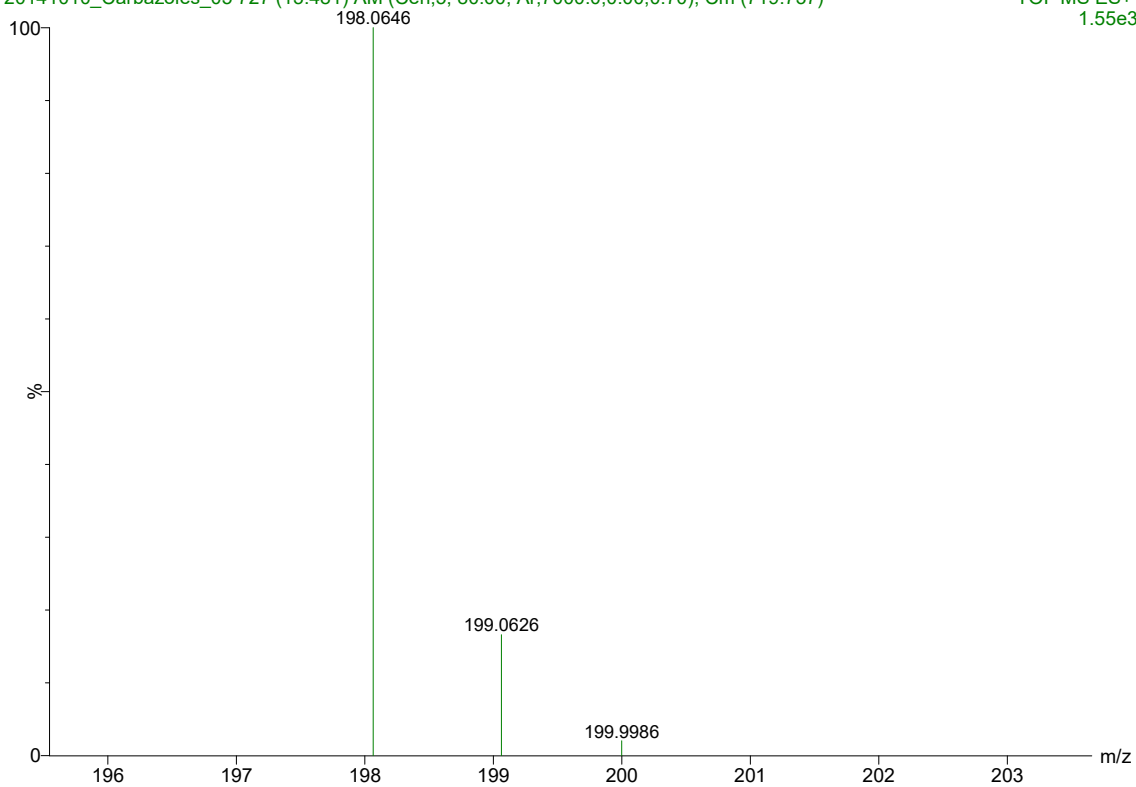


Figure 33 Combined mass spectrum of the suspect MH^+ 198.0646 at retention time 13.44 min detected after 30 min irradiation of 3-bromocarbazole. The isotopic distribution suggests the loss of the halogen atom.

3 hr 3-Bromocarbazole

20141010_Carbazoles_08 622 (11.535) AM (Cen,5, 80.00, Ar,7000.0,0.00,0.70); Cm (616:632)

TOF MS ES+
3.66e3

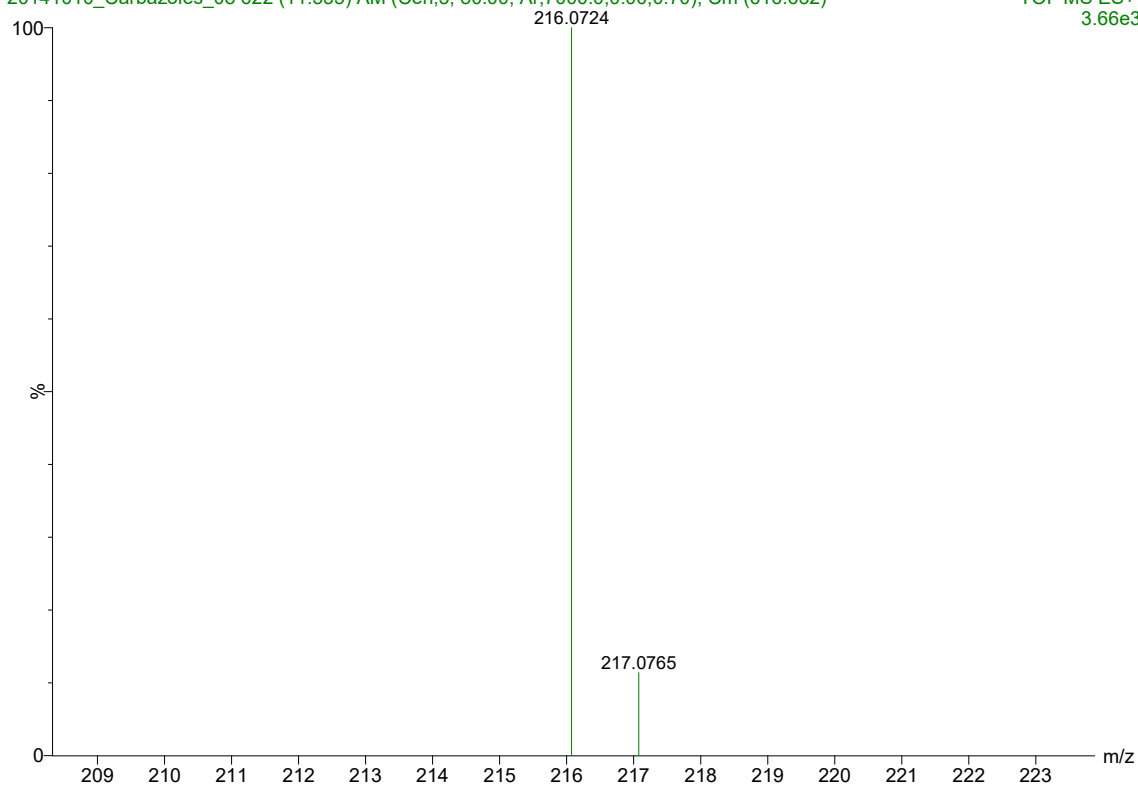


Figure 34 Combined mass spectrum of the suspect MH^+ 216.0724 at retention time 11.53 min detected after 3 hr irradiation of 3-bromocarbazole. The isotopic distribution suggests the loss of the halogen atom.

3-Bromocarbazole 10 min

20140820_TunedAllPos_14 937 (17.373) AM (Cen,5, 80.00, Ar,7000.0,0.00,0.70); Cm (928:945)

TOF MS ES+
1.36e3

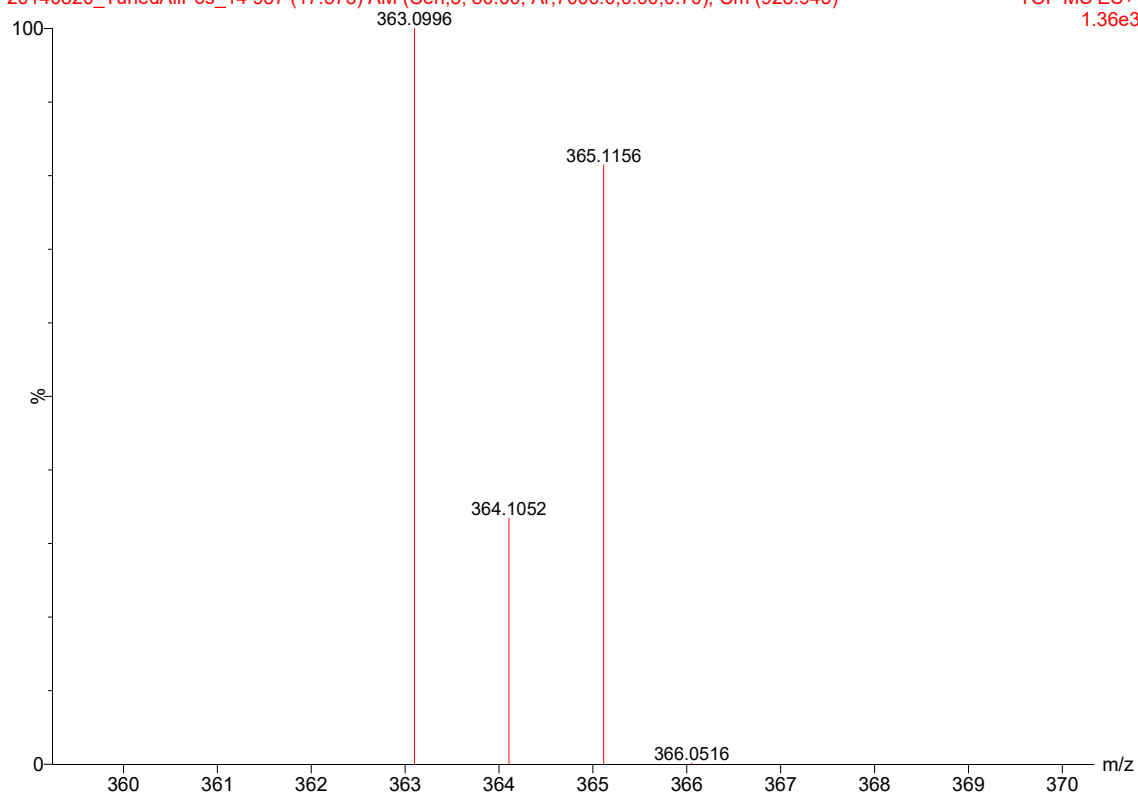


Figure 35 Combined mass spectrum of the suspect MH^+ 363.0996 at retention time 17.35 min detected after 10 min irradiation of 3-bromocarbazole. The isotopic distribution suggests there is one bromine atom since there is a peak at 363 and nearly one at 365.

3-Chlorocarbazole 30 min

20140814_TunedAllPos_13 710 (13.464) AM (Cen,5, 80.00, Ar,7000.0,0.00,0.70); Cm (704:728)

TOF MS ES+
1.10e3

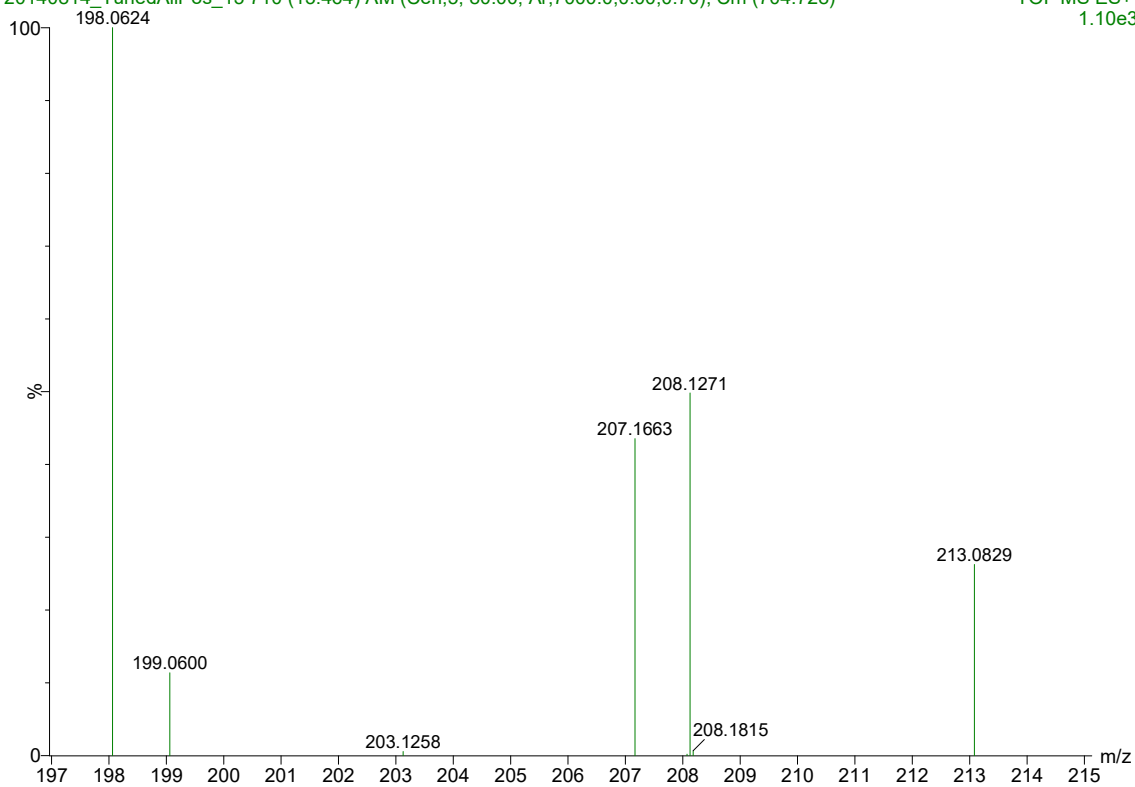


Figure 36 Combined mass spectrum of the suspects MH^+ 198.0624 and MH^+ 213.0829 at respective retention times of 13.43 min and 13.48 min detected after 30 min irradiation of 3-chlorocarbazole. The isotopic distribution suggests the loss of the halogen atom. MH^+ 207.1663 and 208.1271 are background signals.

3-Chlorocarbazole 8 h

20140814_TunedAllPos_20 608 (11.528) AM (Cen,5, 80.00, Ar,7000.0,0.00,0.70); Cm (591:641)

TOF MS ES+
5.01e3

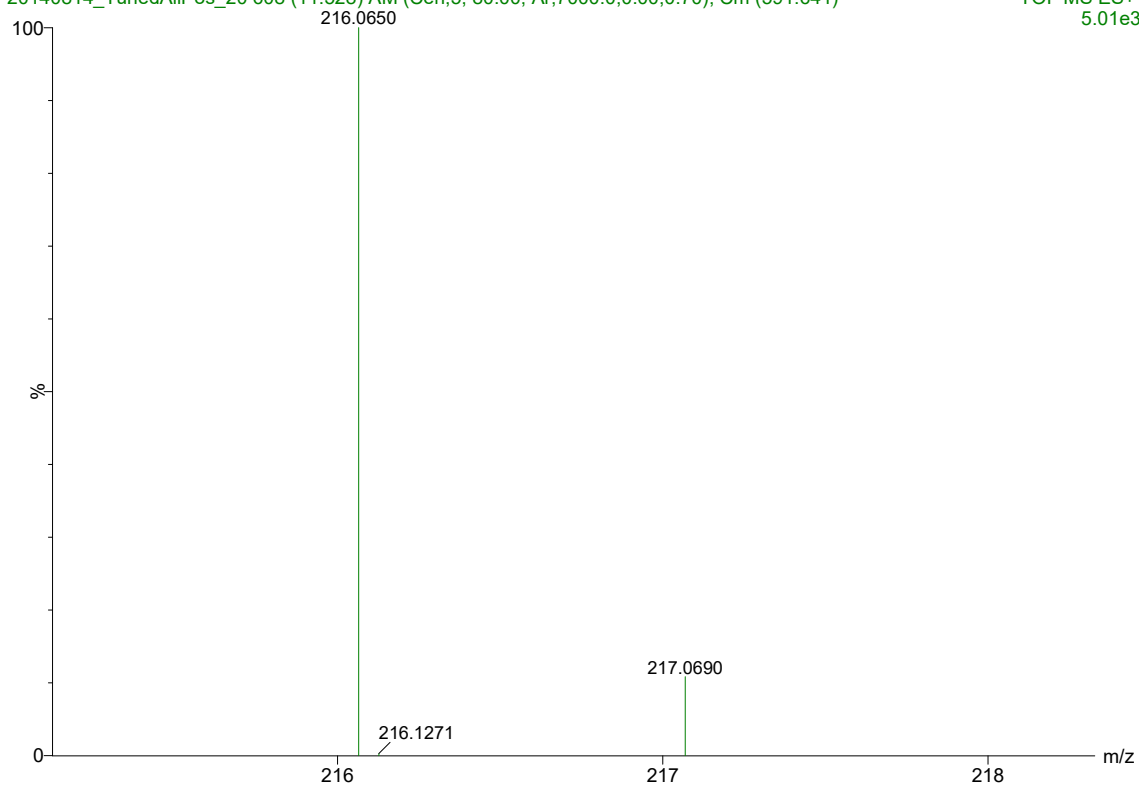


Figure 37 Combined mass spectrum of the suspect MH^+ 216.0650 at retention time 11.55 min detected after 8 hr irradiation of 3-chlorocarbazole. The isotopic distribution suggests the loss of the halogen atom.

3-Chlorocarbazole 10 min

20140814_TunedAllPos_11 918 (17.407) AM (Cen,5, 80.00, Ar,7000.0,0.00,0.70); Cm (901:934)

TOF MS ES+
1.24e3

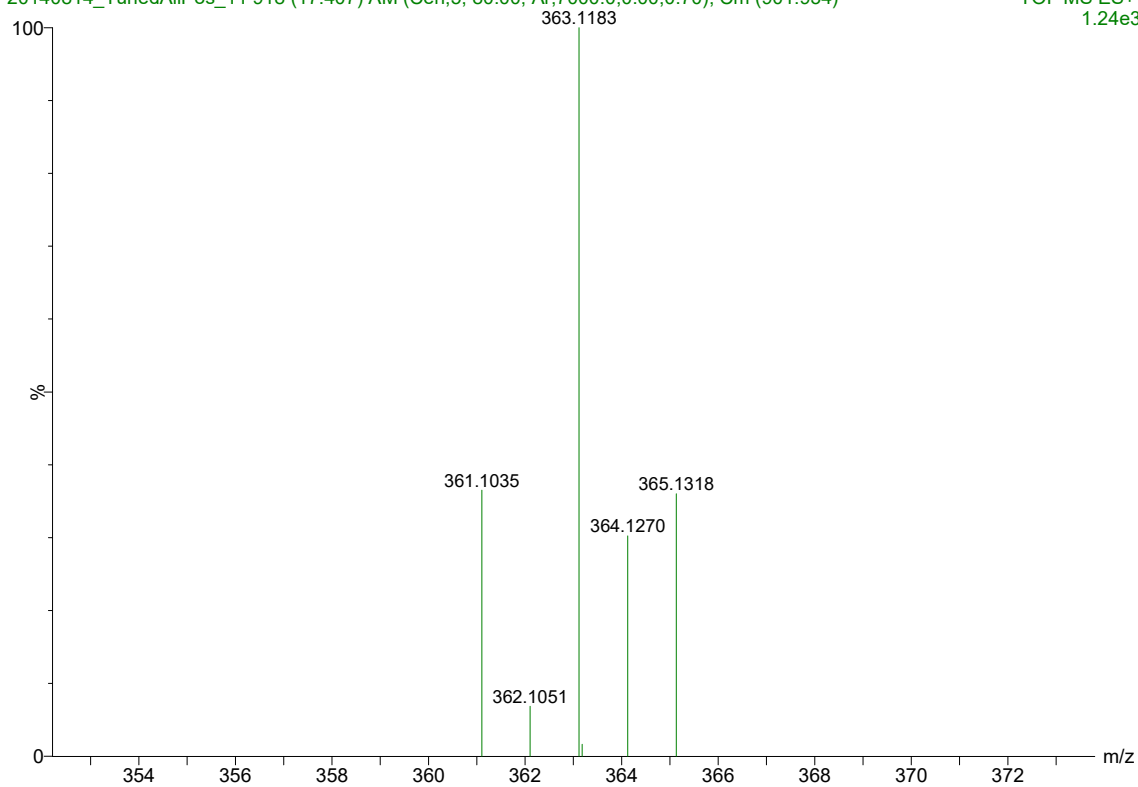


Figure 38 Combined mass spectrum of the suspect MH^+ 363.1183 at retention time 17.41 min detected after 10 min irradiation of 3-chlorocarbazole. The isotopic distribution which is 70% 363 and 30% 365 suggests there is a chlorine atom.

3,6-Dibromocarbazole NaNO3 2 h

20140814_TunedAllPos_32 608 (11.529) AM (Cen,5, 80.00, Ar,7000.0,0.00,0.70); Cm (605:615)

TOF MS ES+
1.61e3

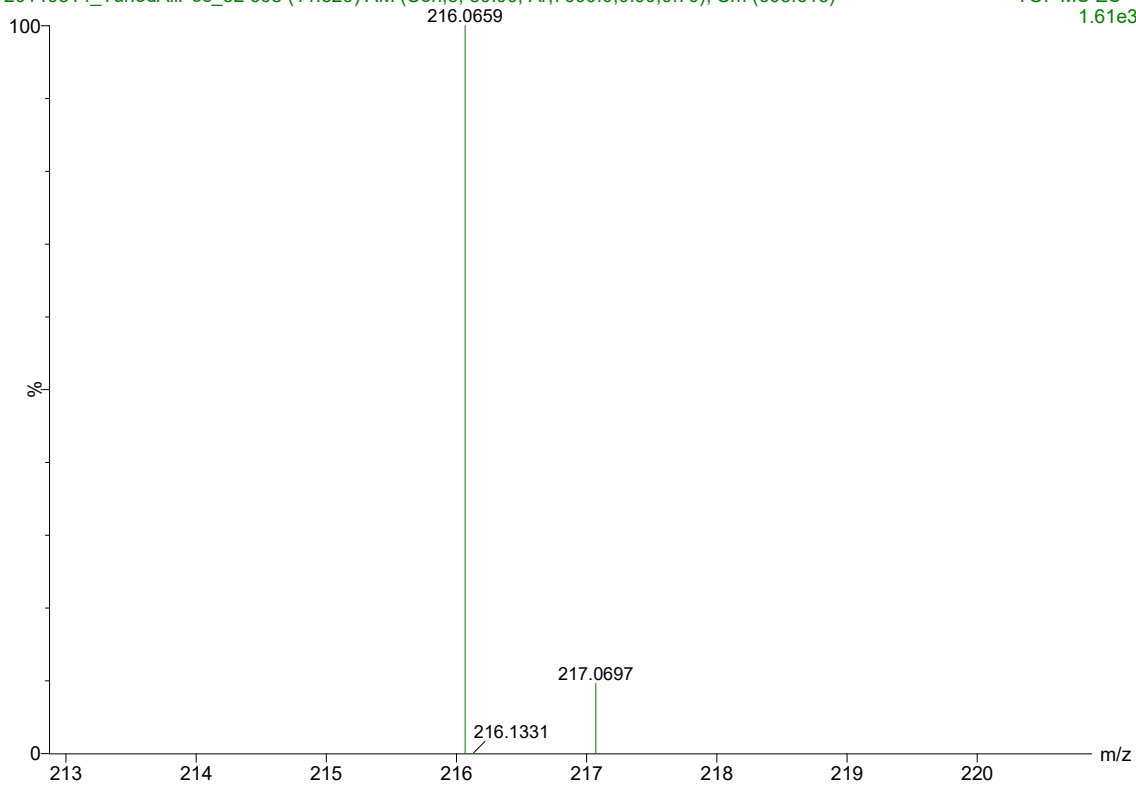


Figure 39 Combined mass spectrum of the suspect MH^+ 216.0659 at retention time 11.55 min detected after 3 hr irradiation of 3,6-dibromocarbazole. The isotopic distribution suggests the loss of the halogen atom.

Table 11 Determination of the intensity of irradiation lamp.

$$\text{Number of quanta absorbed per unit time } \left(\frac{N h \nu}{t} \right) = \frac{\sum F e^{2+}}{\Phi_{\lambda} t}$$

$$= \frac{6.022 \times 10^{23} A V_1 V_3}{\Phi_{\lambda} V_2 l \epsilon t}$$

Avogadro No.	ΔA	V_1	V_2	V_3	Φ_{λ}	l	ϵ	t	Photons
		mL	mL	mL		cm	$\text{mmol}^{-1} \text{L}^{-1}$	s	$\text{quanta s}^{-1} \text{l}^{-1}$
6.02E+23	1.217	450	1	5	1.21	1	11100	180	6.821E+20

Table 12 Quantum yield determination of bromo- and chlorocarbazoles.

$$\Phi = \frac{D_{\text{sys}} \cdot k_d}{2.3 \cdot l \cdot \sum_{290}^{800} \epsilon_{\lambda} I_{0\lambda} (\text{light source})}$$

Compound	D_{sys}	k_d (hr)	k_d (s)	2.3	l	ϵ_{λ}	$I_{0\lambda}$	Moles of photons	$\sum_{290}^{800} \epsilon_{\lambda} I_{0\lambda}$	Φ
	cm	hrs	seconds		cm	$\text{Lmol}^{-1} \text{cm}^{-1}$	$\text{photons cm}^{-2} \text{s}^{-1}$	$\text{nmol cm}^{-2} \text{d}^{-1}$		
3-Bromocarbazole	13	0.3822	1376	2.3	2	18573	6.87E+20	1.14E+00	2.12E+04	1.84E-01
3-Chlorocarbazole	13	0.3442	1239	2.3	2	17028	6.87E+20	1.14E+00	1.94E+04	1.80E-01
3,6-Dibromocarbazole	13	0.0386	139	2.3	2	13385	6.87E+20	1.14E+00	1.53E+04	2.57E-02
3,6-Dichlorocarbazole	13	0.4422	1592	2.3	2	14010	6.87E+20	1.14E+00	1.60E+04	2.81E-01

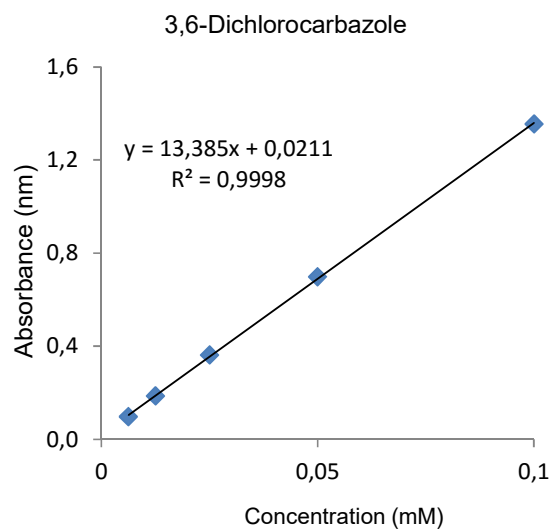
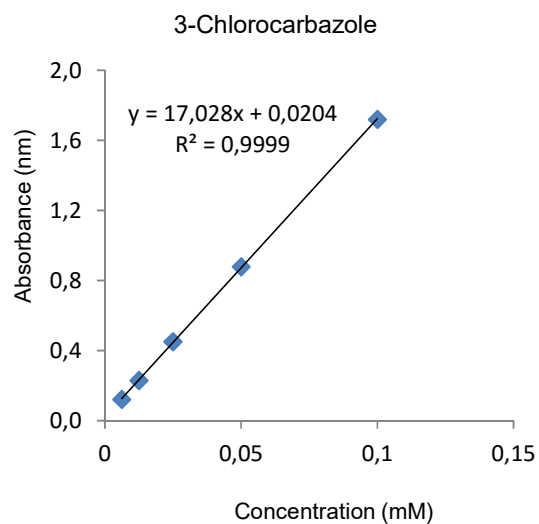
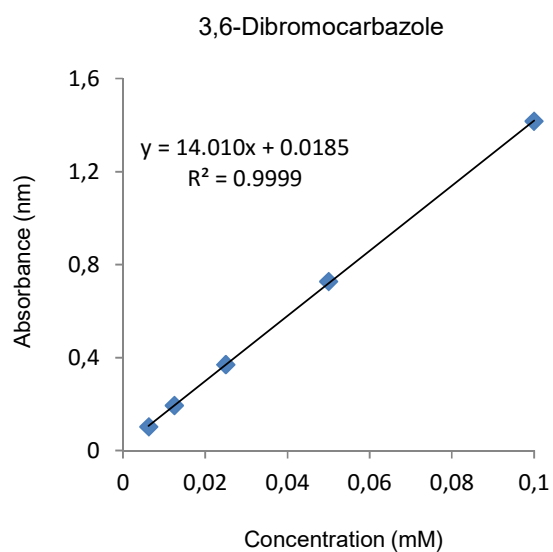
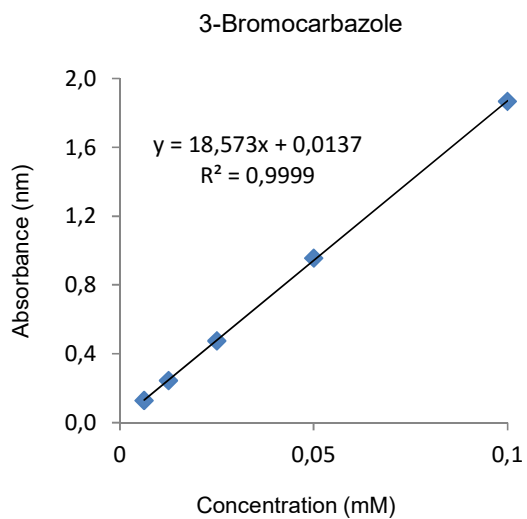


Figure 40 Molar extinction coefficient determination of bromo- and chlorocarbazoles.

Table 13 Solar rate constant determination of 3-bromocarbazole

Adopted L_{λ} values for latitude 50° N based on GC SOLAR Program (OECD, 2002)											
λ centre (nm)	Summer Average daily solar photon irradiance	Winter Average daily solar photon irradiance	ϵ_{λ}	$\sum_{\lambda=29}^{\lambda=80} \epsilon_{\lambda} L_{\lambda}$		Φ	$\Phi \sum_{\lambda=290}^{\lambda=800} \epsilon_{\lambda} L_{\lambda}$		$t_{\frac{1}{2}}$		
	10^3 Einsteins $\text{cm}^{-2} \text{d}^{-1}$	10^3 Einsteins $\text{cm}^{-2} \text{d}^{-1}$		$\text{Lmol}^{-1} \text{cm}^{-1}$	Summer		Winter	Summer	Winter	Summer	Winter
297.5	2.86E-05	5.47E-08	12314	3.52E-01	6.74E-04	0.184	1.6	0.3	0.4	2.5	
300	1.50E-04	4.17E-07	10040	1.51E+00	4.19E-03						
302.5	5.33E-04	2.62E-06	8209	4.38E+00	2.15E-02						
305	1.39E-03	1.34E-05	7233	1.01E+01	9.69E-02						
307	2.89E-03	5.14E-05	7172	2.07E+01	3.69E-01						
310	5.05E-03	1.49E-04	6653	3.36E+01	9.91E-01						
312.5	7.75E-03	3.43E-04	6531	5.06E+01	2.24E+00						
315	1.08E-02	6.52E-04	6592	7.12E+01	4.30E+00						
317.5	1.40E-02	1.07E-03	6683	9.36E+01	7.15E+00						
320	1.71E-02	1.57E-03	6348	1.09E+02	9.97E+00						
323.1	3.12E-02	3.39E-03	6042	1.89E+02	2.05E+01						
330	1.10E-01	1.45E-02	5600	6.16E+02	8.12E+01						
340	1.40E-01	2.12E-02	3906	5.47E+02	8.28E+01						
350	1.57E-01	2.53E-02	1953	3.07E+02	4.94E+01						
360	1.74E-01	2.96E-02	1617	2.81E+02	4.79E+01						
370	1.86E-01	3.30E-02	763	1.42E+02	2.52E+01						
380	1.99E-01	3.65E-02	0	0.00E+00	0.00E+00						
390	1.87E-01	3.49E-02	1907	3.57E+02	6.66E+01						
400	2.69E-01	4.98E-02	1862	5.01E+02	9.27E+01						
410	3.55E-01	6.54E-02	1846	6.55E+02	1.21E+02						
420	3.65E-01	6.71E-02	1419	5.18E+02	9.52E+01						
430	3.52E-01	6.47E-02	1389	4.89E+02	8.98E+01						
440	4.17E-01	7.66E-02	1236	5.15E+02	9.47E+01						
450	4.69E-01	8.62E-02	1114	5.22E+02	9.60E+01						
460	4.75E-01	8.74E-02	977	4.64E+02	8.54E+01						
470	4.91E-01	8.95E-02	1038	5.09E+02	9.29E+01						
480	5.03E-01	9.15E-02	732	3.68E+02	6.70E+01						
490	4.76E-01	8.62E-02	443	2.11E+02	3.81E+01						
500	4.85E-01	8.77E-02	549	2.66E+02	4.82E+01						
525	1.28	2.28E-01	305	3.91E+02	6.96E+01						
550	1.33	2.32E-01	275	3.65E+02	6.37E+01						
575	1.34	2.28E-01	0	0.00E+00	0.00E+00						
600	1.35	2.32E-01	31	4.12E+01	7.08E+00						
625	1.37	2.42E-01	137	1.88E+02	3.32E+01						
650	1.38	2.53E-01	0	0.00E+00	0.00E+00						
675	1.39	2.61E-01	0	0.00E+00	0.00E+00						
700	1.38	2.66E-01	0	0.00E+00	0.00E+00						
750	2.66	5.22E-01	0	0.00E+00	0.00E+00						
800	2.57	5.11E-01	0	0.00E+00	0.00E+00						
				8.84E+00	1.49E+00						

Table 14 Solar rate constant determination of 3-chlorocarbazole

Adopted L_{λ} values for latitude 50° N based on GC SOLAR Program (OECD, 2008)											
λ centre (nm)	Summer Average	Winter Average	ϵ_{λ}	$\sum_{\lambda=290}^{\lambda=800} \epsilon_{\lambda} L_{\lambda}$		Φ	$\Phi \sum_{\lambda=290}^{\lambda=800} \epsilon_{\lambda} L_{\lambda}$		$t_{1/2}$		
	daily solar photon irradiance	daily solar photon irradiance		Summer	Winter		Summer	Winter	Summer	Winter	
	10^{-3} Einsteins $\text{cm}^{-2} \text{d}^{-1}$	10^{-3} Einsteins $\text{cm}^{-2} \text{d}^{-1}$	$\text{Lmol}^{-1} \text{cm}^{-1}$								
297.5	2.86E-05	5.47E-08	13992	4.00E-01	7.65E-04	0.180	2.6	0.5	0.3	1.5	
300	1.50E-04	4.17E-07	8850	1.33E+00	3.69E-03						
302.5	5.33E-04	2.62E-06	6241	3.33E+00	1.64E-02						
305	1.39E-03	1.34E-05	4578	6.36E+00	6.13E-02						
307	2.89E-03	5.14E-05	4166	1.20E+01	2.14E-01						
310	5.05E-03	1.49E-04	3830	1.93E+01	5.71E-01						
312.5	7.75E-03	3.43E-04	4120	3.19E+01	1.41E+00						
315	1.08E-02	6.52E-04	4211	4.55E+01	2.75E+00						
317.5	1.40E-02	1.07E-03	4395	6.15E+01	4.70E+00						
320	1.71E-02	1.57E-03	4318	7.38E+01	6.78E+00						
323.1	3.12E-02	3.39E-03	4578	1.43E+02	1.55E+01						
330	1.10E-01	1.45E-02	4944	5.44E+02	7.17E+01						
340	1.40E-01	2.12E-02	4227	5.92E+02	8.96E+01						
350	1.57E-01	2.53E-02	3052	4.79E+02	7.72E+01						
360	1.74E-01	2.96E-02	1877	3.27E+02	5.56E+01						
370	1.86E-01	3.30E-02	2487	4.63E+02	8.21E+01						
380	1.99E-01	3.65E-02	1389	2.76E+02	5.07E+01						
390	1.87E-01	3.49E-02	1434	2.68E+02	5.01E+01						
400	2.69E-01	4.98E-02	1480	3.98E+02	7.37E+01						
410	3.55E-01	6.54E-02	1221	4.33E+02	7.98E+01						
420	3.65E-01	6.71E-02	1022	3.73E+02	6.86E+01						
430	3.52E-01	6.47E-02	1205	4.24E+02	7.80E+01						
440	4.17E-01	7.66E-02	1053	4.39E+02	8.06E+01						
450	4.69E-01	8.62E-02	854	4.01E+02	7.37E+01						
460	4.75E-01	8.74E-02	763	3.62E+02	6.67E+01						
470	4.91E-01	8.95E-02	702	3.45E+02	6.28E+01						
480	5.03E-01	9.15E-02	549	2.76E+02	5.03E+01						
490	4.76E-01	8.62E-02	565	2.69E+02	4.87E+01						
500	4.85E-01	8.77E-02	595	2.89E+02	5.22E+01						
525	1.28	2.28E-01	473	6.05E+02	1.08E+02						
550	1.33	2.32E-01	549	7.31E+02	1.27E+02						
575	1.34	2.28E-01	397	5.32E+02	9.05E+01						
600	1.35	2.32E-01	534	7.21E+02	1.24E+02						
625	1.37	2.42E-01	473	6.48E+02	1.14E+02						
650	1.38	2.53E-01	458	6.32E+02	1.16E+02						
675	1.39	2.61E-01	443	6.15E+02	1.15E+02						
700	1.38	2.66E-01	351	4.84E+02	9.34E+01						
750	2.66	5.22E-01	397	1.06E+03	2.07E+02						
800	2.57	5.11E-01	336	8.63E+02	1.72E+02						
				1.42E+01	2.51E+00						

Table 15 Solar rate constant determination of 3,6-dibromocarbazole

Adopted L_{λ} values for latitude 50° N based on GC SOLAR Program (OECD, 2002)										
λ centre (nm)	Summer	Winter	ϵ_{λ}	$\sum_{\lambda=290}^{\lambda=800} \epsilon_{\lambda} L_{\lambda}$		Φ	$\Phi \sum_{\lambda=290}^{\lambda=800} \epsilon_{\lambda} L_{\lambda}$		$t_{\frac{1}{2}}$	
	Average daily solar photon irradiance	Average daily solar photon irradiance		Summer	Winter		Summer	Winter	Summer	Winter
	10^3 Einsteins $\text{cm}^{-2} \text{d}^{-1}$	10^3 Einsteins $\text{cm}^{-2} \text{d}^{-1}$	$\text{Lmol}^{-1} \text{cm}^{-1}$							
297.5	2.86E-05	5.47E-08	7252	2.07E-01	3.97E-04	0.026	0.13	0.02	5.4	31.5
300	1.50E-04	4.17E-07	8747	1.31E+00	3.65E-03					
302.5	5.33E-04	2.62E-06	8816	4.70E+00	2.31E-02					
305	1.39E-03	1.34E-05	6020	8.37E+00	8.07E-02					
307	2.89E-03	5.14E-05	4112	1.19E+01	2.11E-01					
310	5.05E-03	1.49E-04	2758	1.39E+01	4.11E-01					
312.5	7.75E-03	3.43E-04	2369	1.84E+01	8.13E-01					
315	1.08E-02	6.52E-04	2182	2.36E+01	1.42E+00					
317.5	1.40E-02	1.07E-03	2209	3.09E+01	2.36E+00					
320	1.71E-02	1.57E-03	2277	3.89E+01	3.58E+00					
323.1	3.12E-02	3.39E-03	2350	7.33E+01	7.97E+00					
330	1.10E-01	1.45E-02	2285	2.51E+02	3.31E+01					
340	1.40E-01	2.12E-02	2182	3.05E+02	4.63E+01					
350	1.57E-01	2.53E-02	1968	3.09E+02	4.98E+01					
360	1.74E-01	2.96E-02	1015	1.77E+02	3.00E+01					
370	1.86E-01	3.30E-02	614	1.14E+02	2.03E+01					
380	1.99E-01	3.65E-02	748	1.49E+02	2.73E+01					
390	1.87E-01	3.49E-02	458	8.56E+01	1.60E+01					
400	2.69E-01	4.98E-02	423	1.14E+02	2.11E+01					
410	3.55E-01	6.54E-02	362	1.29E+02	2.37E+01					
420	3.65E-01	6.71E-02	317	1.16E+02	2.12E+01					
430	3.52E-01	6.47E-02	332	1.17E+02	2.15E+01					
440	4.17E-01	7.66E-02	221	9.23E+01	1.69E+01					
450	4.69E-01	8.62E-02	286	1.34E+02	2.47E+01					
460	0.475	8.74E-02	263	1.25E+02	2.30E+01					
470	0.491	8.95E-02	256	1.25E+02	2.29E+01					
480	0.503	9.15E-02	286	1.44E+02	2.62E+01					
490	0.476	8.62E-02	271	1.29E+02	2.33E+01					
500	0.485	8.77E-02	210	1.02E+02	1.84E+01					
525	1.28	0.228	214	2.73E+02	4.87E+01					
550	1.33	0.232	191	2.54E+02	4.43E+01					
575	1.34	0.228	233	3.12E+02	5.31E+01					
600	1.35	0.232	179	2.42E+02	4.16E+01					
625	1.37	0.242	126	1.72E+02	3.05E+01					
650	1.38	0.253	114	1.58E+02	2.90E+01					
675	1.39	0.261	191	2.65E+02	4.98E+01					
700	1.38	0.266	168	2.32E+02	4.46E+01					
750	2.66	0.522	15	4.06E+01	7.97E+00					
800	2.57	0.511	46	1.18E+02	2.34E+01					
				5.01E+00	8.55E-01					

Table 16 Solar rate constant determination of 3,6-dichlorocarbazole

Adopted L_{λ} values for latitude 50° N based on GC SOLAR Program (OECD, 2002)											
λ centre (nm)	Summer Average	Winter Average	ϵ_{λ}	$\sum_{\lambda=290}^{\lambda=800} \epsilon_{\lambda} L_{\lambda}$		Φ	$\Phi \sum_{\lambda=290}^{\lambda=800} \epsilon_{\lambda} L_{\lambda}$		$t_{\frac{1}{2}}$		
	daily solar photon irradiance	daily solar photon irradiance		Summer	Winter		Summer	Winter	Summer	Winter	
	10^3 Einsteins $\text{cm}^{-2} \text{d}^{-1}$	10^3 Einsteins $\text{cm}^{-2} \text{d}^{-1}$	$\text{Lmol}^{-1} \text{cm}^{-1}$								
297.5	2.86E-05	5.47E-08	15442	4.42E-01	8.45E-04	0.281	1.8	0.3	0.4	2.4	
300	1.50E-04	4.17E-07	15961	2.39E+00	6.66E-03						
302.5	5.33E-04	2.62E-06	14511	7.73E+00	3.80E-02						
305	1.39E-03	1.34E-05	10529	1.46E+01	1.41E-01						
307	2.89E-03	5.14E-05	8606	2.49E+01	4.42E-01						
310	5.05E-03	1.49E-04	7492	3.78E+01	1.12E+00						
312.5	7.75E-03	3.43E-04	7034	5.45E+01	2.41E+00						
315	1.08E-02	6.52E-04	6607	7.14E+01	4.31E+00						
317.5	1.40E-02	1.07E-03	6195	8.67E+01	6.63E+00						
320	1.71E-02	1.57E-03	6317	1.08E+02	9.92E+00						
323.1	3.12E-02	3.39E-03	6210	1.94E+02	2.11E+01						
330	1.10E-01	1.45E-02	5493	6.04E+02	7.97E+01						
340	1.40E-01	2.12E-02	4135	5.79E+02	8.77E+01						
350	1.57E-01	2.53E-02	2884	4.53E+02	7.30E+01						
360	1.74E-01	2.96E-02	397	6.90E+01	1.17E+01						
370	1.86E-01	3.30E-02	0	0.00E+00	0.00E+00						
380	1.99E-01	3.65E-02	0	0.00E+00	0.00E+00						
390	1.87E-01	3.49E-02	1801	3.37E+02	6.28E+01						
400	2.69E-01	4.98E-02	1434	3.86E+02	7.14E+01						
410	3.55E-01	6.54E-02	1434	5.09E+02	9.38E+01						
420	3.65E-01	6.71E-02	1282	4.68E+02	8.60E+01						
430	3.52E-01	6.47E-02	1083	3.81E+02	7.01E+01						
440	4.17E-01	7.66E-02	1083	4.52E+02	8.30E+01						
450	4.69E-01	8.62E-02	885	4.15E+02	7.63E+01						
460	4.75E-01	8.74E-02	763	3.62E+02	6.67E+01						
470	4.91E-01	8.95E-02	610	3.00E+02	5.46E+01						
480	5.03E-01	9.15E-02	290	1.46E+02	2.65E+01						
490	4.76E-01	8.62E-02	443	2.11E+02	3.81E+01						
500	4.85E-01	8.77E-02	137	6.66E+01	1.20E+01						
525	1.28	2.28E-01	0	0.00E+00	0.00E+00						
550	1.33	2.32E-01	0	0.00E+00	0.00E+00						
575	1.34	2.28E-01	0	0.00E+00	0.00E+00						
600	1.35	2.32E-01	0	0.00E+00	0.00E+00						
625	1.37	2.42E-01	0	0.00E+00	0.00E+00						
650	1.38	2.53E-01	0	0.00E+00	0.00E+00						
675	1.39	2.61E-01	0	0.00E+00	0.00E+00						
700	1.38	2.66E-01	0	0.00E+00	0.00E+00						
750	2.66	5.22E-01	0	0.00E+00	0.00E+00						
800	2.57	5.11E-01	0	0.00E+00	0.00E+00						
				6.34E+00	1.04E+00						

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