INVESTIGATION OF BIOTIC DEGRADATION OF HEXABROMOCYCLODODECANE (HBCDD)

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I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

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ABSTRACT

INVESTIGATION OF BIOTIC DEGRADATION OF HEXABROMOCYCLODODECANE (HBCDD)

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Hexabromocyclododecane (HBCDD), a brominated flame retardant, is used in heat insulation materials, and in furniture, textile, etc. for incombustibility. HBCDD is listed among the persistent organic pollutants (POPs) by the Stockholm Convention in 2013. In this study, anaerobic biodegradation of HBCDD was investigated in laboratory sediment microcosms and mesocosms. The microcosms set up as natural attenuation, biostimulation, contaminant control and sterile set were operated for 20 days. HBCDD biodegradation rate tripled (degradation rate constants were 0.069 day^{-1} vs. 0.221 day^{-1}) with the addition of a carbon source and electron donor in biostimulation (100% removal), when compared to natural attenuation (75% removal). No HBCDD was detected in contaminant control and no trend observed in sterile microcosms, though lower than target concentration was measured initially. Larger scale mesocosm reactors were set up similarly as four sets and operated for 49 days. Biodegradation rate observed for biostimulation was more than triple that of the natural attenuation (rate constants of 0.048 day^{-1} vs. 0.157 day^{-1}). The order of diastereomer degradation rates was found as β-HBCDD > γ-HBCDD > α-HBCDD. Mesocosm results indicate biotransformation of γ- and/or β- into α-HBCDD at some stage in incubation. Degradation was observed in sterilized mesocosms, indicating a breach of sterility. Sterilization method was found to affect HBCDD, both in terms of loss of total-HBCDD and shift in diastereomers. Results of sterilization control microcosms indicated that addition of mercuric chloride and autoclaving result in unwanted

degradation of HBCDD at initial time, with no further degradation taking place throughout incubation period.

Key words: HBCDD, anaerobic biodegradation, brominated flame retardant, microcosm, mesocosm

ÖZ

HEKZABROMOSİKLODODEKANIN (HBCDD) BİYOTİK PARÇALANMASININ İNCELENMESİ

Karahan, İrem
Yüksek Lisans, Çevre Mühendisliği Bölümü
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Bromlu bir alev geciktirici olan hekzabromosiklododekan (HBCDD) ısı yalıtımı malzemelerinde ve yanmazlık için mobilya, tekstil vb. alanlarda kullanılmaktadır. HBCDD, 2013 yılında Stockholm Sözleşmesi kalıcı organik kirleticiler (KOK) listesine eklenmiştir. Bu çalışmada, laboratuvar sediman mikrokozm ve mezokozmlarında HBCDD'nin anaerobik biyodegradasyonu araştırılmıştır. Mikrokozmlar doğal giderim, biyostimülasyon, kirlilik kontrol ve steril set olmak üzere 20 gün boyunca çalıştırılmıştır. HBCDD bozunma hızının karbon kaynağı ve elektron sağlayıcı eklenen biyostimülasyonda (%100 giderim) doğal giderime oranla (%75 giderim) üç katından hızlı gerçekleştiği (bozunma hız sabitleri 0.069 gün^{-1} ve 0.221 gün^{-1}) gözlemlenmiştir. Kirlilik kontrol setinde hiçbir HBCDD saptanmazken, steril mikrokozmlarında hedeflenenden daha düşük bir derişimle başlanmış olmasına karşın bir bozunma eğilimi gözlenmemiştir. Daha büyük ölçekli mezokozmlar da dört set olarak kurulmuş ve 49 gün boyunca işletilmiştir. Bozunma hızının, biyostimülasyon için doğal giderimin üç katından fazla olduğu bulunmuştur (bozunma hız sabitleri 0.048 gün^{-1} ve 0.157 gün^{-1}). Ayrıca, diastereomer bozunma hızları sırası β-HBCDD>γ-HBCDD>α-HBCDD olarak bulunmuştur. Diastereomerler arasında α-HBCDD'ye doğru β- ve/veya γ-HBCDD'den biyotransformasyon gerçekleştiğine dair bulgular gözlemlenmiştir. Steril mezokozmlarda HBCDD giderimi gözlenmiştir, bu da sterilliğin bozulduğunu göstermiştir. Sterilizasyon yönteminin HBCDD'nin hem

toplam miktar hem de diastereomer oranı olarak bozunmasına sebep olduğu belirlenmiştir. Kurulan sterilizasyon kontrol mikrokozm sonuçları, cıva klorür ve otoklavlamanın HBCDD'nin ilk anda istenmeyen şekilde bozunmasına sebep olduğunu, ancak kalan inkübasyon sırasında bozunma gerçekleşmediğini göstermiştir.

Anahtar kelimeler: HBCDD, anaerobik biyobozunma, bromlu alev geciktirici, sediman, mikrokozm, mezokozm

To my family

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TABLE OF CONTENTS

ABSTRACT v

ÖZ vii

ACKNOWLEDGMENTS x

TABLE OF CONTENTS xi

LIST OF TABLES xiv

LIST OF FIGURES xvi

CHAPTERS

1. INTRODUCTION 1

2. LITERATURE REVIEW 5

2.1. Brominated Flame Retardants 5

2.2. Hexabromocyclododecane (HBCDD) 9

2.2.1. Chemical Structure and Properties 9

2.2.2. Toxicity of HBCDD 14

2.2.3. Environmental Levels 17

2.3. Degradation of HBCDD 19

2.3.1. Abiotic Degradation of HBCDD 20

2.3.2. Biotic Degradation of HBCDD in Solid Media 22

2.3.3. Definition of Remediation Technologies Used in This Study 26

3. MATERIALS AND METHODS 27

3.3. Reagents and standards 27

3.4. Extraction methods 27

3.5. Instrumental Analysis 30

3.6. Sediment used in experiments 31
3.7. QA/QC Protocols 33
3.8. Experimental setup 37
3.9. Determination of Biodegradation Reaction Rate Constants 44
4. RESULTS AND DISCUSSION 47
4.1. HBCDD Method Validation 47
4.1.1. Laboratory Control Sample Results 47
4.1.2. Sediment Contamination Test Results 48
4.2. Results of Microcosm Studies 49
4.2.1. Degradation of total-HBCDD in sediment 49
4.2.2. Total-HBCDD degradation rate 52
4.3. Results of Mesocosm Studies 53
4.3.1. Degradation of total-HBCDD in sediment 53
4.3.2. Degradation of HBCDD diastereomers in sediment 55
4.3.3. Total- and Diastereomer Specific HBCDD degradation rate constants observed in mesocosms 60
4.4. Results of experiments regarding sterilization 62
4.4.1. Sterilization control microcosms pre-set 63
4.4.2. Degradation of HBCDD in Sterilization Experiment 66
4.4.3. HBCDD Degradation Rate Constants in Sterilization Control Microcosms 73
4.4.3. Overall Evaluation and Comparison of Degradation Rates 74
5. CONCLUSIONS 77
6. RECOMMENDATIONS 79
REFERENCES 81
APPENDICES
A.LC-MS/MS CHROMATOGRAM OF TOTAL-HBCDD 89

B.LC-MS/MS TOTAL HBCDD RESULTS 91

C.AVERAGE ALPHA , BETA, AND GAMMA HBCDD CONCENTRATIONS IN MESOCOSM SETS 93

D.PORTION OF DIASTEREOMER OF HBCDD IN MESOCOSMS 95

E. PERCENTAGE CHANGES OF DIASTEREOMERS IN MESOCOSMS 97

LIST OF TABLES

TABLES

Table 2-1 CAS Numbers of HBCDD main diastereomers (European Commission, 2008)..... 12
Table 2-2 Physico-chemical properties of HBCDD (Demirtepe,2017). 13
Table 2-3 Persistence, bioaccumulation and long-range transport potential characteristics of HBCDD (Marvin et al.,2011). 14
Table 2-4 Half-lives of HBCDD in aerobic environment with different media. 23
Table 2-5 Half-lives of HBCDD in anaerobic environment with different media..... 25
Table 3-1 Methods used in extraction and analysis of HBCDD. 28
Table 3-2. Wet sediment particle size distribution analysis results (Demirtepe,2017). 33
Table 3-3 MDL and LOQ for total and diastereomer specific HBCDD analysis. 35
Table 3-4 Ingredients of ECl medium for 1000 mL total volume. 37
Table 3-5 HBCDD sediment microcosm reactors setup summary 38
Table 3-6 Summary of HBCDD sediment mesocosms..... 39
Table 3-7 HBCDD sediment sterile microcosm reactors setup summary 43
Table 3-8 Relevant information about sterilization methods. 44
Table 4-1 Recoveries of LCS analyses. 47
Table 4-2 Total-HBCDD results of SCT..... 48
Table 4-3 BDE-99 results of CSS analysis. 48
Table 4-4 Summary recovery and RSD of LCS and SCT..... 49
Table 4-5 HBCDD degradation rate constants and half-lives of microcosms. 52
Table 4-6 Total-HBCDD degradation rate constant and half-life in sediment mesocosms. 60
Table 4-7 Alpha (α-), beta (β-) and gamma (γ-) HBCDD degradation rates and half-lives in sediment mesocosms. 62

Table 4-8 Summary of relevant information on sterilization 64
Table 4-9 Summary of degradation rate constants and half-lives in sterilization control microcosms. 74
Table 4-10 Summary results of this study and studies in literature 74

LIST OF FIGURES

FIGURES

Figure 2-1 POPs list 7
Figure 2-2 Structure of HBCDD 9
Figure 2-3 Schematic representation of all 16 possible 1,2,5,6,9,10-hexabromocyclododecanes (Heeb et al., 2005). 11
Figure 2-4 Hazard profile of HBCDD. 15
Figure 2-4 Hazard profile of HBCDD. 16
Figure 2-5 The proposed degradation pathway of HBCDD (Peng et al., 2015). 25
Figure 3-1 a) Ultrasonic bath b) top view during extraction. 29
Figure 3-2 Rotary evaporator 29
Figure 3-3 Photograph of the pond at Çamkoru Natural Park where sediments are collected. 31
Figure 3-4 Satellite view of Çamkoru Natural Park (Demirtepe, 2017). 32
Figure 3-5 GC-MS calibration curves for a) BDE-99 b) HBCDD 34
Figure 3-6 Picture of microcosm reactors. 38
Figure 3-7 Picture of the anaerobic glovebox. 39
Figure 3-8 Picture of a) Biostimulation set (day-0) b) Biostimulation set (day-28) c) Biostimulation set (day-49) 41
Figure 3-9 Picture of depicting sampling from mesocosm reactors 42
Figure 4-1 a) The time dependent HBCDD concentration changes in the microcosm sets b) HBCDD remaining in the microcosm sets (average) c) HBCDD remaining for each microcosm parallel reactor set. 51
Figure 4-2 Total-HBCDD Concentration b) HBCDD remaining for each mesocosm parallel reactor set 54
Figure 4-3 Alpha (α-), beta (β-), and gamma (γ-) HBCDD concentrations in mesocosm 57
Figure 4-4 Diastereomer portion of total-HBCDD in mesocosms 58
Figure 4-5 Results of sterilization control microcosms pre-set 64

Figure 4-6 The time dependent a) HBCDD concentration changes b) HBCDD remaining..... 66
Figure 4-7 Diastereomer abundance in sterile control microcosms. 68
Figure 4-8 Diastereomer portion of total-HBCDD 69
Figure 4-9 Percentage of HBCDD diastereomers in sterilization microcosms..... 70
Figure 4-10 Distribution of diastereomers in different sampling times. 71

ABBREVIATIONS

BFRs: Brominated flame retardants

BS: Biostimulation

BDL:Below detect limit

EI: electron impact

GC-MS: Gas chromatography coupled with mass spectrometry

HBCDD: Hexabromocyclododecane

K_{oc}: Organic carbon-water partitioning coefficient

K_{ow}: Octanol-water partitioning coefficient

LC-MS/MS: Liquid chromatography tandem mass spectrometry

LCS: Laboratory control sample

LOQ: Limit of quantitation

MDL: Method detection limit

MONET: Global passive air monitoring network

NA: Natural attenuation

BDEs: Polybrominated diphenyl ethers

POPs: Persistent organic pollutants

QA/QC: Quality assurance/quality control

SIM: selected ion monitoring

US EPA: United States Environmental Protection Agency

CHAPTER 1

INTRODUCTION

Numerous synthetic chemicals are produced as a consequence of increasing civilization and industrialization. However, they can reach high concentrations and result in pollution of natural sources by entry into the environment via various pathways, Also, they could have toxic effects to animals and humans. Brominated flame retardants (BFRs) are such synthetic chemicals which include different types of chemicals produced for preventing ignition of materials. Environmental fate of BFRs has recently become a topic of concern (Gerecke et al., 2006). Prior to its regulation by the Stockholm Convention, hexabromocyclododecane (HBCDD) was one of the commonly used BFRs. Therefore, elevated concentrations of HBCDD is expected in natural environments.

HBCDD was used as an alternative to polybrominated diphenyl ethers (PBDEs), mainly as an insulation material in the buildings. In the extruded polystyrene foams (XPS) and expanded polystyrene foams (EPS), HBCDD was used as an additive BFR. Concentration of HBCDD in EPS and XPS is typically lower than 3% (weight/weight). Also, HBCDD was used in upholstery material, plastics and electric-electronic devices. In 2001, annual HBCDD demand was 16700 metric tons, and the large part of this demand belonged to the European Union with 9500 metric tons (Covaci et al., 2006).

HBCDD is a bioaccumulative, toxic and persistent chemical, hence listed as a persistent organic pollutant (POP) in Stockholm Convention under Annex-A Elimination List in 2013 (Stockholm Convention, 2013). This development influenced the production and use of HBCDD. There are 182 countries party to the Stockholm Convention where 152 of them are signatory countries. As being party and signatory

of the Convention, Turkey has ratified the convention in 2010. However, Turkey has filed only one exemption until 2016, and it was for HBCDD use. Due to its high consumption in the building industry, it will take some time to change the HBCDD with new alternative chemicals. Therefore, Turkey applied for HBCDD exemption until 2019. Also, in 2011, Turkish Ministry of Environment and Urbanization had HBCDD in their list for the chemicals produced and/or imported above 1 tone or more per year in Turkey, though the exact amount is not reported. Hence one can say that there is a potential for HBCDD exposure in Turkey and that there may be soil or sediments contaminated with this chemical.

HBCDD was used in a number of different ways in various products, which caused its release into different types of environmental media. Because of its hydrophobic characteristics, HBCDD is expected to strongly be bound to a solid matrix, such as sediments, solids and digester sludges (Covaci et al., 2006). Also, due to its bioaccumulative properties, HBCDD can accumulate in the fatty tissues of living creatures, especially in animals at higher levels of the food chain. However, HBCDD has many toxic effects. It can cause neurotoxicity, and disruption in hormonal development such as reproductivity problems (European Commission, 2008).

Initially, studies in the literature were focused on degradation of HBCDD as one specie. However, HBCDD commercial mixture consists of different diastereomers and each of have slightly different physicochemical properties. After realizing that, HBCDD studies were concentrated on the three main diastereomers of HBCDD, which are alpha (α-), beta (β-) and gamma (γ-) HBCDD. Marvin et al. (2011), in their detailed review on HBCDD, made recommendations for future studies on HBCDD, and they emphasized the requirement for the isomer specific degradation of HBCDD for improvement of knowledge on the fate of HBCDD in the environment.

Although there are studies on biotic and abiotic degradation of HBCDD, these are very limited for HBCDD in soil and sediment (Davis et al., 2005, 2006; Gerecke et al., 2006). Half-life and degradation rates of anaerobic biodegradation are given in the literature, but their range is very wide, e.g. the minimum and maximum half-lives reported are 0.66 day to 115.5 days, respectively (Gerecke et al., 2006; Davis et al., 2005, 2006). Furthermore, there are conflicting findings in the literature regarding the order of degradation rates of main diastereomers of HBCDD.

HBCDD is found in environment compartments, so it is important to know more about possible degradation mechanisms and kinetics. Although the studies on the fate of HBCDD is gaining more importance in the last decade, information is still very limited on HBCDD, especially when it comes to difference between diastereomers. Hence, it is crucial to obtain comprehensive information on HBCDD and its diastereomers, in regard to remediation technologies so that its toxic effects can be reduced. As focusing on the biodegradation of HBCDD and its individual diastereomers, this study will contribute to the relevant literature.

The overall aim of this study is investigation of anaerobic degradation of HBCDD. Specifically, this study aims:

1) To investigate anaerobic degradation of HBCDD in microcosm (small scale) and mesocosm (large scale) sediment reactors,
2) To determine degradation rates and order of degradation for α-, β- and γ-HBCDD diastereomers,
3) To investigate the effect of sterilization on degradation of HBCDD.

CHAPTER 2

LITERATURE REVIEW

2.1. Brominated Flame Retardants

In the last decades, increasing human population and industrialization have brought many environmental problems. To meet increasing demands of civilization, synthetic chemicals were produced widely. However, natural resources on earth are depleting continuously, and remains of these chemicals are polluting the existing natural sources. Therefore, countries have decided to take several national and international measures to sustain the natural resources and make them available for both today's and future generations. Both international and national contracts and regulations have entered into force to control the impact of these chemicals that may be released a result of production, use or disposal phases.

Flame retardants are chemicals used for delaying the ignition and preventing the spread of the flame on the combustible substances. Production of flame retardants may result with different properties. Therefore, they can be classified depending on their chemical composition or on the interaction methods to polymers. Chemical composition differs as halogenated, inorganic, phosphorus based, or nitrogen based, while interaction methods into polymers separate as reactive and additive. This study is about the additive flame retardant HBCDD, a brominated organic compound regulated by many countries. (US.EPA,2014). This study is about hexabromocyclododecane (HBCDD and it is one of the flame retardant regulated by many countries. It is a brominated, organic compound used as an additive flame retardant.

One of the international key movements on a group of toxic chemicals named as persistent organic pollutants (POPs) has been launched under the Stockholm Convention.

Once released to the environment, POPs may show different physical and chemical properties such as:

- Stay intact for extremely long periods
- Being widely distributed around the environment as a result of natural processes involving soil, water and most important air
- Accumulate in the fatty tissues of humans, including those in higher levels of the food chain
- Toxic for both humans and wildlife

POPs are now widely distributed including places where POPs have never been used. This widespread contamination of environmental media and living organisms involves many foodstuffs and leading to both acute and chronic toxic effects for several species including humans. Also, POPs concentrate in living creatures by bioaccumulation process. Not being soluble in water, POPs are easily absorbed in fatty tissue, where concentrations can be increased up to 70,000 times their background level. Predatory birds, fishes, mammals and humans place at the high level in the food chain and they absorb POPs at the highest concentrations. When they travel, POPs travel with them, and POPs can be found even at Arctic, where is far away from any POP source. POPs can cause problems including cancer, allergies and hypersensitivity, central and peripheral nervous system damage, reproductive defects and impaired immune system. Some POPs are considered as endocrine disruptors, which is altering the hormonal system, damaging the reproductive and immune systems of exposed creatures. Also, POPs could cause developmental impacts or carcinogenic effects (Stockholm Convention, 2018).

By contracting, party countries are obliged to fulfill the measures of referred chemicals in the Convention in their land. Measures could contains reducing the release, eliminating the production and usage, and ending imports and exports. By 2018, a total of 182 countries have become parties to the Stockholm Convention and this agreement has entered into force in 152 countries. Turkey signed this contract in 2001, the

legislature (TBMM) ratified the agreement in 2009 (Official Gazette No. 27304, 2009) so Turkey became a formal party, responsibilities began as of 12 January 2010.

HBCDD used in various industries as flame retardants, and as having the properties of persistent organic pollutants, it was recommended to regulate under the Stockholm Convention. In 2013, at the sixth Stockholm Convention meeting in Geneva, Switzerland, HBCDD was included in the Annex-A Elimination list (Stockholm Convention, 2013). POPs listed in the annexes of Stockholm Convention as of May 2018 are presented in Figure 2-1.

As requirement of the Stockholm Convention, Turkey prepared the first National Implementation Plan on POPs between 2004-2007, and delivered to Secretariat in 2011, and this plan was updated in 2014-2015. In the National Implementation Plan, country profile, information on the institutions and their responsibility areas related with POPs, and the environmental policy issues related to POPs was given. The plan also includes research on the monitoring of POPs, impacts on human and environment (T.C. Ministry of Environment and Urban Planning, 2014). HBCDD was placed at the updated plan of Turkey, and Sectoral Impact Assessment and Regulatory Impact Analysis has been included in the study (T.C. Ministry of Environment and Urbanization, 2014).

The Water Framework Directive (WFD), which was signed in Europe in 2000, is the basic legal framework for the protection and improvement of all water bodies in the European Union in terms of quality and quantity. According to the Environmental Quality Standards Directive added in 2008, hazardous and priority hazardous environmental pollutants as substances or groups that create risks for surface waters and aquatic environment were given. Environmental quality standards of them were determined, and HBCDD was listed among the new priority hazardous substances and its EQS value set as 0.0016 μg/L.

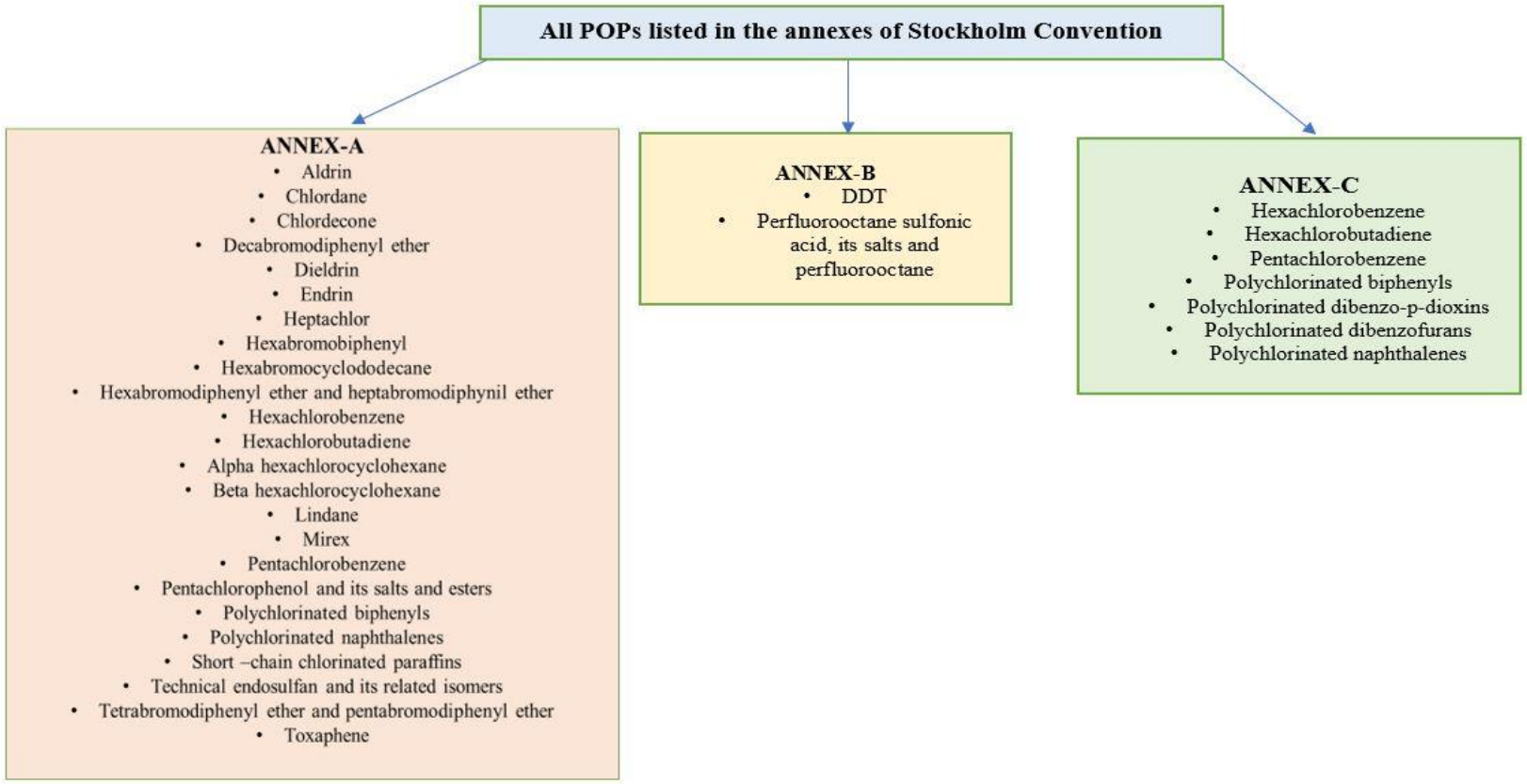


Figure 2 1 POPs listed in the annexes of Stockholm Convention as of May 2018

2.2. Hexabromocyclododecane (HBCDD)

2.2.1. Chemical Structure and Properties

HBCDD is used primarily in the buildings as insulation materials (i.e. extruded or expanded polystyrene foam boards), it is also used as upholster of furniture, in textile material of automobiles, car cushions, and electric-electronic devices. HBCDD is produced since 1960s and in 2007, HBCDD use of EU was 11000 ton (Marvin et al., 2011).

Hexabromocyclododecane (HBCDD) is a brominated aliphatic cyclic hydrocarbon. It consists of 18 hydrogen atoms bonded to 12 carbon atoms, and 6 brome atoms in the positions 1,2,5,6,9 and 10. Its molecular formula is $C_{12}H_{18}Br_6$ with a molecular weight of 641.70 g/mole. Its CAS number is 3194-55-6 and the general molecular structure of the HBCDD is shown as in Figure 2-2.

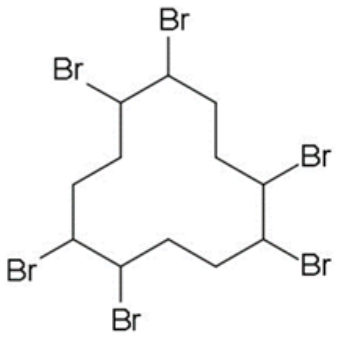


Figure 2-2 Structure of HBCDD

Considering the chemistry of isomers, molecular structure of HBCDD could be understood better. There are two types of isomerism, (i) structural or constitutional isomerism, (ii) and stereo isomerism. While structural isomers differ in connectivity between the atoms (i.e. how atoms are connected to each other), stereoisomers differ in their attachment space, yet their connectivity is the same.

Stereogenic center is a location in the molecule where interchange of any two group happens. When a carbon atom is bonded with four different atoms or groups, it loses

its symmetry and be a stereogenic center. By having stereogenic center, if two stereoisomers are mirror-image of each other, they are called enantiomers.

The achiral properties of enantiomers are identical such as, melting point and solubility in water. However, their chiral properties like optical rotation are different. Enantiomers commonly have different biological activity affinity, biodegradation patterns and rate, depending on the spaces of the substituents in their compounds.

If enantiomer nomenclature is done according to the relative priorities of the four substituents (atoms or groups) configuration of around the stereogenic center, it is called the R-S system. Priority of the substituents is given depending on the atomic numbers. In this system, lowest priority substituent is placed the opposite side of the stereogenic center. The symbol R or S is labeled whether the substituent configuration is clockwise or counter-clockwise. If they are in clockwise array labeled as R, otherwise it is S.

Also, the enantiomers of optically active compounds are differentiated according to their optical rotation. The optical activity is analyzed by measuring the linear polarized light with a polarimeter. In the measurements, the rotation of the polarization is checked. If the rotation happens to the right, enantiomers is dextrorotary (+), otherwise, it is levorotary (-).

Diastereomers are stereoisomers that are not enantiomers of each other, in other words they are not the mirror images of each other, and their chiral and achiral properties are different. Meso compounds are achiral diastereomer compounds with multiple stereogenic centers. They are optically inactive despite their stereocenters.

1,5,9-cyclododecatriene (CDT) is the precursor molecule for the technical grade HBCDD synthesis, by brominating of 1,5,9-cyclododecatriene, six stereocenters formation happens. There are also four known cis- and trans- isomers of 1,5,9-cyclododecatriene (CDT) and during the production of the technical grade HBCDD, isomeric arrangement and the purity of the 1,5,9-cyclododecatriene (CDT), and the industrial processes affect the stereoisomeric composition of HBCDD (Heeb et al., 2005).

In Figure 2-3, all the theoretic stereoisomers are presented. There are totally 16 theoretically possible stereoisomers: 6 pair of enantiomers that are showed in 1(a-b), 2(a-b), 5(a-b), 6(a-b), 7(a-b), 8(a-b) and 4 meso formation are demonstrated in 3, 4, 9, 10 (Heeb et al., 2005).

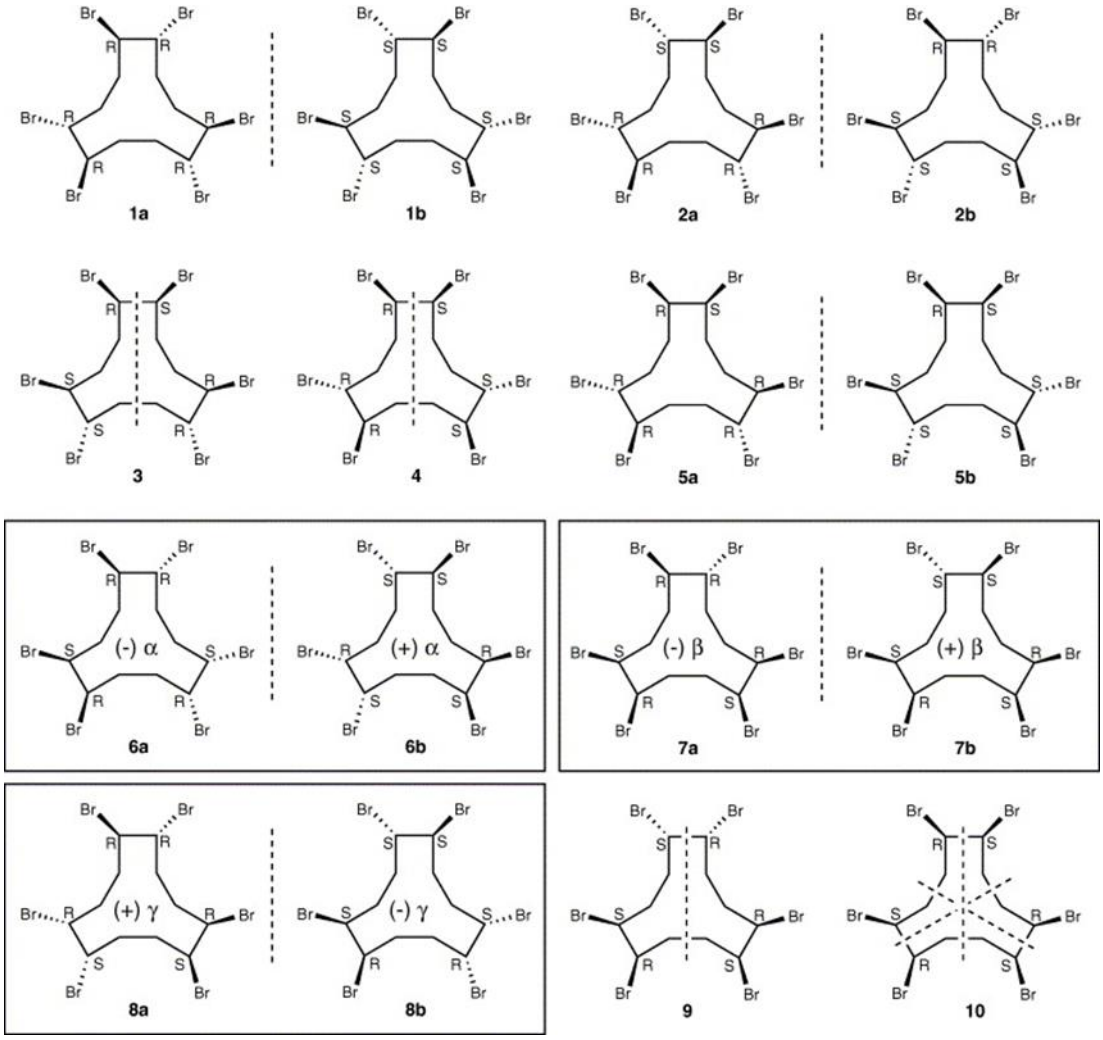


Figure 2-3 Schematic representation of all 16 possible 1,2,5,6,9,10-hexabromocyclododecanes (Heeb et al., 2005).

Five diastereomers, named as alpha (α-), beta (β-), gamma (γ-), delta (δ-) and epsilon (ε-) HBCDD, could be identified in the HBCDD mixtures, but in the commercial HBCDD 3 main diastereomeric enantiomer pairs, which are alpha (α-), beta (β-), and gamma (γ-) HBCDD are present mainly. The other delta (δ-) and epsilon (ε-) HBCDD diastereomers could be present in very small amounts (Covaci et al., 2006). HBCDD

consists 70-95% gamma (γ-), 5-30% alpha (α-), and beta (β-) HBCDD (European Commission, 2008). CAS numbers of the alpha (α-), beta (β-), and gamma (γ-) HBCDD diastereomer are showed in Table 2-1.

Table 2-1 CAS Numbers of HBCDD main diastereomers (European Commission, 2008).

CAS Number	Name of Diastereomer
134237-50-6	Alpha (α-) Hexabromocyclododecane
134237-51-7	Beta (β-) Hexabromocyclododecane
134237-52-8	Gamma (γ-) Hexabromocyclododecane
3194-55-6	Total- Hexabromocyclododecane

Optical rotation measurements showed that three enantiomers pairs, which are alpha (α-), beta (β-), and gamma (γ-) HBCDD can be observe, but there is no optical rotation detected for delta (δ-) and epsilon (ε-) HBCDD diastereomers and these two are assigned as meso forms (Heeb et al., 2005).

In Demirtepe's study (2017) , physicochemical properties of HBCDD were collected from Marvin et al. (2011), also provided in the European Commission Risk Assessment Report (European Commission, 2008). The physical and chemical characteristics of the HBCDD are given in . The physical state of the HBCDD is given as a white odorless solid, with a density between 2.24 and 2.38 g/cm^3 (European Commission, 2008). Some of the data in were derived from measurements, while some are based on model estimates, i.e. modelling software like COSMOtherm, SPARC, EPI Suite.

When logk_{ow} values given in is viewed, it can be seen that there is no significant difference between the diastereomers of the HBCDD. However, they have relatively high logk_{ow} values around 5-7. When logk_{ow} is greater than 4, it indicates hydrophobicity (USEPA, 2016). Hence diastereomers of HBCDD individually has potential to bioaccumulate in fatty tissues. Also, as can be seen from , the solid and liquid state vapor pressures are very low for each diastereomer.

Table 2-2 Physico-chemical properties of HBCDD (Demirtepe,2017).

	technical-HBCDD	α-HBCDD	β-HBCDD	γ-HBCDD
Melting Point (°C) [a]	172-184 to 201-205	179 – 181	170 – 172	207 – 209
Boiling point (°C) [b]	Decomposes at >190°C			
Water Solubility at 20°C (mg/L)	0.0656 [c]	0.0488 [c]	0.0147 [c]	0.0021 [c] 0.0034 [d] (@25°C)
Solid state vapor pressure (Pa)	$6.3 * 10^{-5}$ @ 21°C [e] $7.23 * 10^{-7}$ [f]	$2.72 * 10^{-10}$ [g]	$3.58 * 10^{-11}$ [g]	$1.86 * 10^{-11}$ [g]
Liquid state vapor pressure (Pa)	-	$2.93*10^{-4}$ [g]	$3.86*10^{-5}$ [g]	$2.00*10^{-5}$ [g]
Henry's Law Constant ($atm.m^3/mol$)	$7.4*10^{-6}$ [h] $1.72*10^{-6}$ [i]			
Log Kow	5.62 [j] 6.73 [k]	5.07 [j] 5.59 [g]	5.12 [j] 5.44 [g]	5.47 [j] 5.53 [g]
Log Koa	10.46 [l]	9.96 [l]	10.47 [l]	10.40 [l]

[a] Smith et al. 2005 cited in EC 2008, [b] Peled et al. 1995 cited in EC 2008, [c] MacGregor and Nixon 2004 cited in EC 2008, [d] Stenzel and Markley 1997 cited in EC 2008, [e] Stenzel and Nixon 1997 cited in EC 2008, [f] Wania et al 2002 cited in Marvin et al., 2011, [g] COSMOtherm estimates given in Marvin et al., 2011, [h] EC 2008, [i] EPI Suite estimate (bond method) given in Marvin et al. 2011, [j] MacGregor and Nixon 1997, Hayward et al. 2006 cited in EC 2008, [k] Average of model estimates given in Marvin et al. 2011., [l] thermodynamically consistent estimates given in Marvin et al. 2011.

Also, characteristic of HBCDD on its persistence, bioaccumulation and long-range transport are summarized in Table 2-3.

Table 2-3 Persistence, bioaccumulation and long-range transport potential characteristics of HBCDD (Marvin et al.,2011).

Characteristic	**Regulatory Threshold**	**HBCDD**
Persistence		
overall persistence (Pov, days)	-	120 (12-1200)
half-life in air (days	>2	0.4-5.2
half-life in water (days)	>60	no obs. deg (60-130)
half-life in sediment (days)	>60	no obs. deg (60-130)
detected in remote region	(yes/no)	yes
Bioaccumulation		
[1]log BCF ($L\ kg^{-1}$ wet weight)	3.7	3.9-4.3
[2]log BAF ($L\ kg^{-1}$ wet weight)	3.7	3.7-6.1
Long-Range Transport Potential (LRTP)		
persistence in air (days)	>2	inconclusive
detected in remote region	(yes/no)	yes
[3]CTD (km)	-	600 (200-1500)

[1]BCF = bioconcentration factor, [2]BAF = bioaccumulation factor, [3]CTD = characteristic travel distance

2.2.2. Toxicity of HBCDD

Living creatures may be exposed to HBCDD as a result of its production, industrial use, or during use of products including HBCDD. HBCDD is hazardous because of its

persistency, and due to its bioaccumulative properties. It is causing high hazard in the means of environmental fate. As being a toxic chemical, HBCDD exposure can cause different levels of hazard for humans or environmental media. The hazard profile of HBCDD, as prepared by USEPA (2014) is given in Figure 2-4.As can be seen from the table, HBCDD is denoted as having "very high hazard" for chronic and acute toxicity in the aquatic environment. Also, for humans, HBCDD is denoted to have "high developmental hazard". Developmental hazard includes "death of developing individual, abnormalities, transformed growth and lack of functions" (USEPA, 2014).

Moreover, individual diastereomers show different effects on living creatures. For example, in Hamer et al.'s study (2006), HBCDD diastereomers impact on the degradation of the estrogen receptors were at different levels, for γ- and β-HBCDD, IC_{50} (inhibition concentration) was found to be 4.9 μM and 11.0 μM, respectively, but α-HBCDD was found to have no effect. Also, in another study, heart rate of zebrafish larvae was decreased due to α- and β- HBCDD at 1 mg/L concentration, and γ-HBCDD was found to increase the heart rate (Du et al., 2012). Therefore, Hamer et al. (2006) and Du et al. (2014) concluded that it was difficult to figure out the diastereomer with the highest toxicity.

VL = Very Low hazard L = Low hazard M = Moderate hazard H = High hazard VH = Very High hazard -- Endpoints in colored text (VL, L, M, H, and VH) were assigned based on empirical data. Endpoints in black italics (*VL, L, M, H, and VH*) were assigned using values from predictive models and/or professional judgment.
This table contains hazard information for HBCDD; evaluation of risk considers both hazard and exposure. Variations in end-of-life processes or degradation and combustion byproducts
are discussed in the report but not addressed directly in the hazard profiles. The caveats listed below must be taken into account when interpreting the information in the table.
d This hazard designation would be assigned MODERATE for a potential for lung overloading if >5% of the particles are in the respirable range as a result of dust forming operations.
§ Based on analogy to experimental data for a structurally similar compound.
¥ Aquatic toxicity: EPA/DfE criteria are based in large part upon water column exposures which may not be adequate for poorly soluble substances such as many flame retardants that may partition to sediment and particulates.

Chemical	**CASRN**	Human Health Effects											Aquatic Toxicity		Environmental Fate	
		Acute Toxicity	Carcinogenicity	Genotoxicity	Reproductive	Developmental	Neurological	Repeated Dose	Skin Sensitization	Respiratory Sensization	Eye Irritation	Dermal Irritation	Acute	Chronic	Persistence	Bioaccumulation
HBCDD	25637-99-4; 3194-55-6	L	*M*	L	M	H	*M*	M	L		VL	VL	VH	VH	H	VH

Figure 2-5 Hazard profile of HBCDD (USEPA, 2014).

2.2.3. Environmental Levels

HBCDD enters the environment with different pathways. During the production of HBCDD and leaching or disposal of the manufactured products that are exposed HBCDD can cause entrance of HBCDD to the environment. Especially few decades ago, HBCDD was used widespread, and it was transferred to environmental systems such as soil, sediment, air and water bodies, and due to its physiochemical properties, it became widespread. Commonly, higher concentrations of HBCDD were recorded around the point sources, where HBCDD is produced or processed, in other words around the places, where urbanized and industrial areas. However, even in the places, where no obvious HBCDD source, HBCDD concentration was detected in low concentrations. Diffusing from sources and long-range transport are the predicted reasons of detecting HBCDD in these places (Covaci et al., 2006).

HBCDD is firstly detected in fish and sediment samples collected from possible point sources, where a textile industry wastewater discharge was taking place, along the Viskan River in Sweden. Due to the hydrophobic character of HBCDD, it was revealed that some industries in this place have used HBCDD instead of decabromodiphenyls since the 1990s. Also, fish to sediment ratios are found and large values are obtained, so this shows that HBCDD is highly bioavailable and bioaccumulates in fish (Sellström et al., 1998).

When sediment samples were taken and analyzed, and their HBCDD contribution was also not similar to the technical mixture. In a study done with the sediment samples, it was found that gamma (γ-) HBCDD contribution lowered, whereas alpha (α-) HBCDD contribution increased comparing with the total HBCDD mixture contributions (Lee et al., 2015). In another study, distribution of three HBCDD diastereomer were also examined in sediments of Haihe River in China, and alpha (α-) HBCDD was founded the dominated stereoisomer in most sample sites controversy to the total HBCDD mixture distribution (Zhao et al., 2017). The reason behind this difference is not known clearly, but it is predicted that stereoisomer-specific processes happened in the environment or thermal isomerization occurred through the production of the HBCDD (Covaci et al., 2006). Besides, in other studies, the reason for the favored bioisomerization of beta (β-) and gamma (γ-) to alpha (α-) HBCDD in-vivo is predicted

as the inter-transformation and different degradation rate constants of diastereomers (Marvin et al., 2011; Zhao et al., 2017).

In the literature, high concentrations are found at the top predators due to increasing HBCDD in the food chain (i.e. due to the biomagnification property of HBCDD), 9600 and 19200 ng/g lipid weight was determined in marine mammals and birds of prey, respectively. Also, some lower concentrations were recorded from the human studies, and their values were between 0.35 and 1.1 ng/g lipid (Covaci et al., 2006).

Humans are exposed to HBCDD through touching, inhaling and eating the foods contain HBCDD. As hydrophobic chemical, HBCDD accumulates especially in the fat tissues. Therefore, animal foods and oily foods are the sources for the spreading HBCDD (Marvin et al., 2011). HBCDD can be found in serums and breast milk of humans. In one study, HBCDD was detected in the serum from the consumers of fish angled from the highly HBCDD polluted lake. By founding the concentration of HBCDD for men 4.1 and for women 2.6 ng/g lipids, consumption of fish from the lake was related with the concentration of HBCDD in the serum of human beings (Thomsen et al., 2008) Also, in another study HBCDD concentrations in human milk was detected from 1980 to 2004 in Stockholm, and it is found that the concentrations have increased four to five times through the years (Fängström et al., 2008).

The exposure of HBCDD affects negatively the immunity and reproduction systems. Also, HBCDD neurotoxic effects and causing problems in endocrine system is predicted (Schecter et al., 2012). HBCDD toxicity tests are made on the rats, and they revealed that HBCDD can cause distraction in thyroid hormone system and neurotransmitter uptake, and can have the brain neurotoxic effects (Covaci et al., 2006).

In the study that is done for determination of the diastereomeric composition of HBCDD was revealed that α- HBCDD the most abundant diastereomer at the samples of the harbor porpoise and dolphin blubber, due the biotransformation β- and γ-HBCDD (Zegers et al., 2005). Also, another study was done on the lipid-rich food samples such as fish, peanut butter, poultry, pork, and beef in Dallas, Texas. As a result of the study, α- HBCDD was detected as most abundant diastereomer followed by γ-,

and then β- HBCDD, and food was though as substantial contributor of α- HBCDD level in humans (Schecter et al., 2012).

The solubility of γ- HBCDD is one magnitude lower than the other two diastereomers, and α-HBCDD has the higher solubility by comparing with others. While doing measurements, γ-HBCDD had reached its the maximum solubility at 0.0021 mg/L, as the total HBCDD dissolved concentration was 0.0024 mg/L, β-HBCDD at 0.0147, as the total HBCDD dissolved concentration was 0.039 mg/L, and α-HBCDD was 0.0488 mg/L, as the total HBCDD concentration was 0.0656 mg/L. Therefore, the total dissolved HBCDD concentration is 0.0656 mg/L and, and for example, if 0.61 mg/L of total HBCDD is discharged into the water, 0.5444 mg/L HBCDD present in the water as non-dissolved (European Commission, 2008).

By knowing the partition of the diastereomers in the mixture total HBCDD, it can be found that about all the α-HBCDD in the mixture was dissolved, and most of the non-dissolved diastereomers was expected as γ- and β- HBCDD. This higher water solubility of the α-HBCDD results the higher bioavailability in aquatic systems when 0.0147 mg/L, which is the solubility limit of β-HBCDD solubility, is exceed but stayed in the range of relevant environment concentrations (not very high concentration exposures); because bioavailability of the HBCDD in the water is function of partitioning concentrations in water and the solid phases (Marvin et al., 2011).

2.3. Degradation of HBCDD

As being one of the brominated flame retardants, HBCDD has limited degradability because of its persistency and tendency to accumulation. However, when certain conditions are achieved, its degradation could occur through biotic or abiotic mechanisms.

During degradation of HBCDD, the main step is dehalogenation. There are different mechanisms of dehalogenation in aerobic and anaerobic conditions, and as a result of these mechanisms, lower brominated products or hydroxylated derivatives are produced. Mineralization should not be expected all the time, and although the resistance of biodegradation is decreased by the debromination, there is a possibility of producing toxic intermediates during these mechanisms (Peng et al., 2015). Because of its physical and chemical characteristics, HBCDD is mainly associated with solid

matrices in the environment, so in this study degradation of HBCDD is investigated in sediments.

2.3.1. Abiotic Degradation of HBCDD

In theory, HBCDD is expected to debrominate abiotically, because it has lower thermo- stability when compared with other aromatic brominated flame retardants. However, in practice, abiotic degradation of HBCDD is not important portion due to the its rigid ring shape and low water solubility. There are theoretically three possible abiotic mechanism for the degradation of the HBCDD, which are exposing the sunlight and air, elimination process catalyzed Lewis base and nucleophilic substitution reactions (European Commission, 2008).

1) Exposure to sunlight and air

$H\text{-}R\text{-}Br + h\nu \rightarrow H\text{-}R^{\cdot} + Br^{\cdot} \rightarrow R' + HBr$

H-R-Br represents HBCDD and R´ is unsaturated substance here.

2) Elimination process catalyzed Lewis base

$H\text{-}R\text{-}Br + L \rightarrow L\text{-}H^{+} + R' + Br^{-}$

3) Nucleophilic substitution reactions

$H\text{-}R\text{-}Br \rightarrow H\text{-}R^{+} + Br^{-} + Nu^{-} \rightarrow H\text{-}R\text{-}Nu + Br^{-}$

$Nu^{-} + H\text{-}R\text{-}Br \rightarrow Nu\text{---}HR\text{---}Br^{-} \rightarrow Nu\text{-}R\text{-}H + Br^{-}$

For these reactions, Nu represents a nucleophile and solvent for solvatization of ions is needed (European Commission, 2008).

In the literature, very limited study is done with soil and sediments on the abiotic degradation of HBCDD, therefore abiotic degradation of HBCDD is explained in all kind of media. Other than the mechanisms mentioned above, HBCDD abiotic degradation could also happen through the thermal degradation, photodegradation by UV-C irradiation, zero-valent iron, and with Sulphur species which are reduced.

Thermal degradation of HBCDD was measured by using thermogravimetric analyzers. Also, during the debromination of HBCDD lab-scale fixed bed reactor, furnace, condenser, absorbers, and adsorber is used, and identification of decomposition

products, which are hydrogen bromide and hydrocarbon compounds, are made. Decomposition of HBCDD occurs mainly mono-, bi-, or tribrominated, but non-, tetra- and pentabrominated forms also exist (Barontini et al., 2001a,2001b).

By using the UV-C irradiation, photodegradation of HBCDD achieved with 29% - 35.6% for 4-hour irradiation and hydroxylated products are identified (Zhou et al., 2012). In another study, the degradation of HBCDD is examined under the simulated sunlight with the presence of the Fe(III)-carboxylate complexes and H_2O_2. By the increase in the concentration of carboxylate complexes, which are Fe(III)-oxalate and Fe(III)-citrate, photodegradation is increased. Also, adding H_2O_2 into the Fe(III)-citrate solutions approximately doubled the degradation of HBCDD, but H_2O_2 is useful concentrations from 200 to 600 μM (Zhou et al., 2014). Besides, direct and indirect (with HO and 1O2-singlet oxygen) photolysis was studied by using UV-C lamps in the range between 220-260nm, in acetonitrile-water solution degradation of HBCDD and photolysis debromination products were identified (Yu et al., 2015).

Diastereomeric conversion is possible at the elevated temperatures, γ-HBCDD transformation to α-HBCDD at temperatures between 140–160°C was studied (Heeb et al., 2010). However, debromination and isomerization between the diastereomers of HBCDD is studied under the sunlight, and there was no notable loss reported. It is explained as incorporated HBCDD to the treated products is not affected from the photodegradation, but if it releases to air and dust, it is susceptible to the photodegradation (Kajiwara et al., 2013).

By using ultrasonic irradiation, HBCDD degradation was studied on α-, β-, γ- HBCDD in Argon (Ar) with the arrangement of pH and H_2O_2 concentration, and at the end of 1-hour, α-, β- and γ-HBCDD HBCDD were degraded 100%, 98.0% and 60.0%, respectively (Ye et al., 2014). Also, nanoscale zerovalent iron (NZVI) was used to degrade HBCDD, with an increase of iron dosage and temperature. The removal of HBCDD was also increased (Tso & Shih, 2014).

Polysulfide and bisulfide, which are from the reduced sulfur species, can be found in the anoxic parts of the coastal subsurface waters/sediments, and they are reacting with HBCDD, and playing a significant role on the degradation of HBCDD (Lo et al., 2012). Degradation of HBCDD is favored in γ- and β-HBCDD comparing to α-

HBCDD in anoxic conditions with polysulfides and bisulfide, so α-HBCDD concentration was increased when compared to the other diastereomers. It is suggested that this could be the reason of observing high concentration of α-HBCDD in biological samples (Lo et al., 2012). In another study, iron monosulfide (FeS) is used as reactive under anoxic conditions. Reductive transformation of HBCDD happened by FeS, and it is found that 90% of HBCDD could be transformed in 24 hours. Calculation of the rate constants depend on the initial concentration of FeS and HBCDD, and it is found that β- and γ-HBCDD were showed faster degradation than α-HBCDD (Li et al., 2016).

In soil, HBCDD mechanochemical degradation was studied for different co-milling substances to see the total HCBDD transformation. At the end of the study it was found that presence and absence of co-milling resulted in 99% and 75% degradation of HBCDD in 2 hours, respectively (Zhang et al., 2014)

2.3.2. Biotic Degradation of HBCDD in Solid Media

Biodegradation of HBCDD can be achieved under two conditions in sediments: aerobic and anaerobic. In the literature, there are limited number of studies on the biodegradation of HBCDD in sediment and/or soil, so all relevant studies about aerobic biodegradation of HBCDD and anaerobic biodegradation of HBCDD are explained in detail below.

Aerobic Biodegradation of HBCDD. The biodegradation of HBCDD was examined in the aerobic microcosms with soil and aquatic sediments (Davis et al., 2005). In soil microcosms, sewage sludge (i.e. activated sludge) was added to soil in the concentration 5 mg/g soil, to mimic land application. Also, HBCDD degradation was observed in two different sediment, which comes from two different rivers. For both soils and sediments, in biologically viable environment, degradation of HBCDD was found faster than the biologically inhibited environment (i.e., sterilized with autoclave). It was predicted that aerobic degradation of the HBCDD in the soil was observed due to the microbial biotransformation. In viable soil (i.e. natural attenuation) microcosms, 75% of the HBCDD was degraded while in abiotic control (i.e. sterile) microcosm showed only 3% degradation in 119 days. Also, HBCDD was decreased to the non-detectable level in one river's natural attenuation sediment microcosms, and

in sterile microcosm, HBCDD was decreased 31% within 64 days. In the natural attenuation microcosm with other sediment sample, 93% of HBCDD degraded and in sterile microcosm, 62% of HBCDD degraded within 21 days. Half-lives of the aerobic degradation of HBCDD are given in Table 2-4 (Davis et al., 2005).

Table 2-4 Half-lives of HBCDD in aerobic environment with different media.

Media	Half-life	Reference
Soil	63 days*	Davis et al., 2005
Sediment (Schuylkill River)	11 days*	Davis et al., 2005
Sediment (Neshaminy Creek)	32 days*	Davis et al., 2005

* Pseudo first order reaction

After that study, Davis and colleagues (2006) observed the aerobic degradation of HBCDD in soil and activated sludge. In soil, the degradation trend of HBCDD was similar for both natural attenuation and abiotic control. However, in the activated sludge, while natural attenuation HBCDD biodegradation was observing about 21%, abiotic control microcosm, which was sterilized by using mercuric chloride, showed approximately 60% degradation HBCDD (Davis et al., 2006).

In another study, humic acid and glucose existence effect on the aerobic HBCDD degradation was examined, and by the presence of them, HBCDD degradation efficiency and microbial diversity was increased in rhizosphere and non-rhizosphere soils. Also, in the microbial community, gram-positive bacteria population decreased and gram positive bacteria like *Brassia rhizosphere, Sphingomonas* sp. increased through the aerobic degradation of HBCDD (Le et al., 2017). Besides, 13 bacterial strains were isolated from a contaminated soil sample and tested for the degradation of γ-HBCDD in aerobic environment. It was found that *Pseudomonas* sp. (HB01 strain), degraded more than 70% of γ-HBCDD within 5 days (Yamada et al., 2009).

Anaerobic Biodegradation of HBCDD. The anaerobic degradation of HBCDD was examined in different studies, and they were mentioned in below.

HBCDD anaerobic degradation was observed in microcosms with soil, aquatic sediments and digester sludge (Davis et al., 2006, 2005). Also, in a study, anaerobic degradation was examined in the digested sewage sludge by adding primers and nutrients, which are starch and yeast. As a result of the study, it was found that there

was HBCDD degradation by approximately more than 35 days half-life in the heat sterilized set, and in the biostimulation set (with primers and nutrients) HBCDD degradation half-life was found as 0.66 day (Gerecke et al., 2006). In all these mentioned studies, known amount of HBCDD were analyzed by spiking of HBCDD onto media in the environment of laboratory, but there is another study that HBCDD analysis was done with the contaminated samples. By knowing contamination level, two sewage sludge samples were taken and incubated in the anaerobic conditions. After incubating for 3 months without taking sample, HBCDD concentration was found below the detect limit, and kinetic degradation of HBCDD could not be calculated (Stiborova et al., 2015a).

Biodegradation rate constants of HBCDD diastereomers were found as β->α->γ- HBCDD in digester sludge and in anaerobically prepared aquatic sediments microcosms degradation rate constants were found as β->γ-> α- HBCDD (Davis et al., 2006). However, in the digested sewage sludge, the anaerobic biodegradation rates of HBCDD diastereomers were given as γ- ≈ β-> α- HBCDD (Gerecke et al., 2006). In all studies, generally, it was observed that alpha (α-) HBCDD degrades slower compared to other diastereomers. The anaerobic degradation of HBCDD was also tested in rhizosphere and non-rhizosphere soils, and an increase in the proportion of the α-HBCDD was observed (Le et al., 2017).

Furthermore, the degradation of HBCDD was studied in the mixed liquor batch reactors, by the addition of two pure culture strain, which are called HBCD-1 and HBCD-2 (Peng et al., 2015). These two pure culture strains were isolated from an anaerobic reactor prepared for the degradation of tetrabromobisphenol A, which was studied previously (Peng et al., 2012). HBCD-1 strain, which was identified as *Achromobacter* sp, showed 90% removal within 8 days, and it was more effective on the degradation of alpha (α-) HBCDD especially. The degradation of diastereomers was observed as α- > β-> γ- HBCDD for HBCD-1 strain, and as α-> γ- ≅ β-HBCDD for HBCD-2 (Peng et al., 2015). All these studies showed different order for the degradation of the HBCDD diastereomers due to the differences in media and the experimental conditions, and microbial community. Half-lives of the anaerobic degradation of HBCDD, obtained from different studies, are given in the Table 2-5.

Table 2-5 Half-lives of HBCDD in anaerobic environment with different media.

Media	Half-life	Reference
Soil	6.9 days*	Davis et al., 2005
Sediment	1.5 day*	Davis et al., 2005
Sediment	1.1 day*	Davis et al., 2005
Sludge	5.4 days	Davis et al., 2006
Sludge	0.66 day*	Gerecke et al., 2006
Mixed liquor	5.4 days (α-), 8.2 days (β-), 8.8 days (γ-)	Peng et al., 2015

* Pseudo first order reaction

The degradation products of HBCDD was also studied and tetrabromocyclododecene (TBCD), 1,2-dibromocyclododecadiene (DBCD), and 1,5,9-cyclododecatriene (CDT) were observed following removal of two bromines at each reaction step (Peng et al., 2015; Davis et al., 2006). Additionally, Peng and colleagues (2015) detected 2-dodecene as the last product during the analysis. This is a novel product which is revealed by cleavage of HBCDD cycloaliphatic ring. The proposed degradation pathway of HBCDD is presented in Figure 2-6.

Figure 2-6 The proposed degradation pathway of HBCDD (Peng et al., 2015).

2.3.3. Definition of Remediation Technologies Used in This Study

Monitored Natural attenuation: is the natural process for decreasing contaminant concentrations in soil/solid media. Contaminated environment clean-up in nature happens in five ways: biodegradation, sorption, dilution, evaporation and chemical reactions. In this study, natural attenuation occurs via biodegradation. Contaminant concentrations and other site properties are analyzed regularly to make sure that natural attenuation is working properly, and the technical term used by USEPA (2012a) for this process is monitored natural attenuation. When compared to other clean-up processes, monitored natural attenuation is a reasonable method because it requires less equipment and labor. However, monitoring for many years can be costly (USEPA, 2012a).

Biostimulation: is stimulation of the natural environment to enhance bioremediation of contaminants. By adding limiting nutrient and electron acceptors for microorganisms, their growth is supported (USEPA, 2012b). Also, in anaerobic environments, bioremediation of halogenated contaminants is stimulated because they act as the electron acceptor for the externally added electron donor, consequently biodegradation of contaminants is achieved (USEPA, 2008).

CHAPTER 3

MATERIALS AND METHODS

All the methodology, materials, equipment, methods used during analysis and laboratory set-ups are described in this chapter for:

- Sampling of clean sediments from Çamkoru Natural Park
- Laboratory set-up for investigation of the degradation of HBCDD (in total and three individual diastereomers) in sediment microcosms and mesocosms
- Analysis of HBCDD and its 3 individual diastereomers in sediment

Firstly, followed extraction methods and instrumental analysis for HBCDD are explained. Then, quality assurance/quality control protocols are presented. Lastly, detail of the experimental set-up of microcosms and mesocosms is explained.

3.3. Reagents and standards

All solvents, namely n-hexane (HEX), dichloromethane (DCM), acetone(ACE), used for analysis, anhydrous sodium sulfate (granular), copper fine powder (<63 μm), and aluminum oxide (0.063-0.200 mm) were supplied from Merck KGaA (Darmstadt, Germany). Total HBCDD (1,2,5,6,9,10-HBCDD) and internal standard PCB-209 (2,2',3,3',4,4',5,5',6,6'-CB) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Surrogate standard BDE-99 (2,2',4,4',5-BDE) obtained from CPA (Bulgaria).

3.4. Extraction methods

Methods used for the analysis of HBCDD from sediment are published by United States Environmental Protection Agency (US EPA), and listed in Table 3-1 In this

study, for extraction and cleanup procedures, the methods of US EPA are used as a guide, but they are not entirely followed.

Table 3-1 Methods used in extraction and analysis of HBCDD.

Method Number– Name	**Purpose of method**	**Reference**
8000D – Determinative chromatographic separations	Guidance on analytical chromatography and QA/QC requirements	(USEPA, 2014b)
3550C – Ultrasonic extraction	Ultrasonically extraction of nonvolatile and semivolatile organic compound from solid matrices	(USEPA, 2007)
3665A – Sulfuric acid/ permanganate cleanup	Cleanup of concentrated extracts	(USEPA, 1996c)
3660B – Sulfur cleanup	Cleanup of elemental sulfur from extracts	(USEPA, 1996b)
3610B – Alumina cleanup	Column cleanup of sample extracts for purification	(USEPA, 1996a)
Chapter 4 – Organic analytes	Sample collection, preservation techniques, and sample preparation methods	(USEPA, 2014a)
40 CFR Appendix B part 136-Definition and procedure for the determination of the method detection limit	Estimation of method detection limit (MDL) for physical and chemical methods	(USEPA, 2016)

Ultrasonic extraction of HBCDD from sediment was depend on US EPA method 3550C with minor modifications (USEPA, 2007). For each one gram of sample, one gram of anhydrous sodium sulfate was taken and mixed in 40 mL vials. Samples were soaked into the 30 mL hexane:dichloromethane:acetone mixture (7:7:1 v/v) overnight. Ultrasonic extraction is done in an ultrasonic bath for 30 minutes two consecutive times by adding solvent mixture into the vials (Figure 3-1).

Figure 3-1 a) Ultrasonic bath b) top view during extraction.

Copper powder is added into the solvent mixture to achieve sulfur removal before starting extraction (USEPA, 1996b). The two extracts come from ultrasonic bath were collected and concentrated to 2-5 mL by using rotary evaporator (Heidolph, Hei-Vap Advantage HL/G1) shown in the Figure 3-2. Removal of possible interfering organic compounds is achieved by adding concentrated sulfuric acid (1:1) into the colored extract (U.S. EPA Method 3665A). Clear extract accumulates at the top layer in the vial. The top was taken and purified by passing through the column prepared with 0.5 g of alumina (deactivated to 3%) topped with anhydrous sodium sulfate. Then, 5 mL of n-hexane followed with 2 mL of n-hexane:dichloromethane mixture (1:1 v/v) was used for elution. The collected extract from column was concentrated to 2 mL by using rotary evaporator.

Figure 3-2 Rotary evaporator

3.5. Instrumental Analysis

Analysis of HBCDD was done with gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS/MS) to determine concentration in the sediment samples taken from reactors. Total-HBCDD analysis is done with GC-MS in Department of Environmental Engineering, METU. The instrumental analysis of α-, β- and γ-HBCDD diastereomers for the same extracts were performed using LC-MS/MS analysis was done in Central Laboratory of METU.

For total HBCDD analysis, Agilent 7890A GC 5975C inert mass spectrometry (GC-MSD) in EI mode with DB5-MS column (15 m x 0.25 mm ID x 0.10 μm) was used. Injection temperature, ion source temperature and quadrupole temperature were set at 200°C, 230°C and 150°C, respectively. Splitless injection was done with 1 μL sample. The carrier gas was helium with a constant rate of 1.5 mL/min. Oven program starts with holding 60°C for 1 min, elevated to 200°C with 15°C/min, then elevated to 310°C at 10°C/min and was held for 5 min.

The internal standard was PCB-209, while the surrogate standard was BDE-99. Analysis made in scan mode has showed that primary/secondary ions (m/z), to confirm HBCDD, PCB-209 and BDE-99, primary/secondary ions (m/z) was 79/159.1, 497.8/427.8 and 403.8/563.6, respectively. Then, the analysis is made in SIM mode by using these ions.

α-, β- and γ-HBCDD diastereomers could not be separated in GC-MS analysis because all diastereomers were present in the standard mix solution and they leave the column at very close retention times. Therefore, in GC-MS analysis, sum of three diastereomers can be determined. A chromatogram of LC-MS/MS for total-HBCDD was given in Appendix A.

The instrumental analysis of α-, β- and γ-HBCDD diastereomers was performed using Agilent 6460 triple quadrupole with Jet Stream Technology (LC-MS/MS) Agilent 1200 liquid chromatography and nitrogen generator, equipped with zorbax SB-C18 (2.1 x 50 mm x 1.8 μm) column. Injection was done as 2 μL. For mobile phase gradient, (A) water/acetonitrile (95:5) and (B) methanol/acetonitrile (95:5) was used. As the initial composition of 50:50 A/B (v/v), the elution program started, and it was raised

to 60% B in 1 min, 95% B in 5 min, 90% B in 1 min and 60% B in 1 min. After that, it was back to beginning conditions in 2 min.

3.6. Sediment used in experiments

Sediment used in this study was taken from the pond at Çamkoru Natural Park under the Kızılcahamam Forestry Management Directorate in Çamlıdere. Pond is located 110 km to the northwest direction of Ankara, Turkey and its altitude is about 1350 m. The pond is isolated from the residential areas and it is showed in the Figure 3-3.

No HBCDD contamination was expected in the pond. A passive air POPs monitoring station of MONET (global passive air monitoring network) has been in operation in the close vicinity of the pond since December 2009. A satellite view of MONET POPs air sampling station and Çamkoru Natural Park Pond is given in the Figure 3-4. According to the results of year-round atmospheric monitoring (samplers deployed every three months) and soil samples (collected on a yearly basis), POPs concentrations are very low, i.e. levels observed are similar in magnitude or lower to other rural background stations monitored as a part of MONET (RECETOX, 2018).

Figure 3-3 Photograph of the pond at Çamkoru Natural Park where sediments are collected.

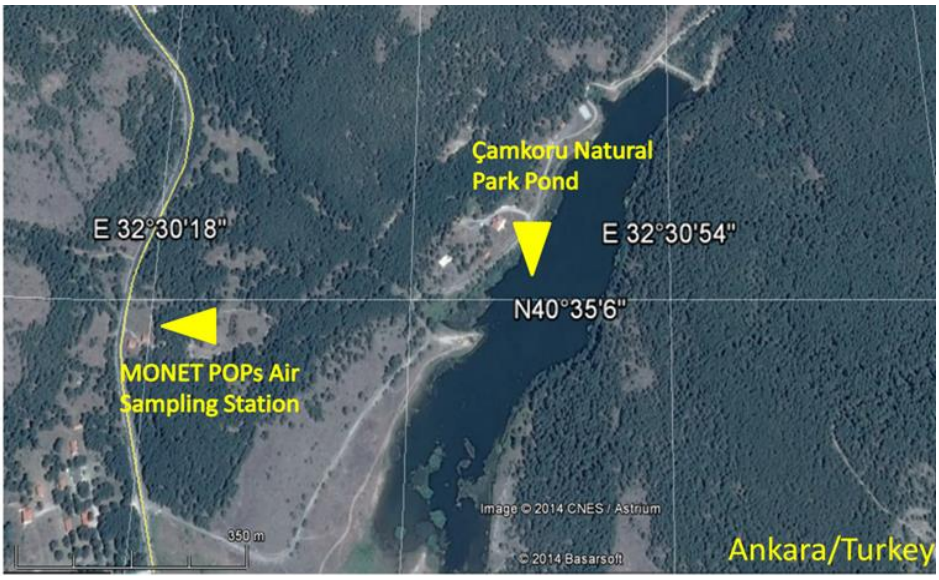


Figure 3-4 Satellite view of Çamkoru Natural Park (Demirtepe, 2017).

Sediment samples were collected under 70 cm water depth from surface and from five different spots of the pond. It was wet sieved with a 2 mm sieve on site to remove large particles and stones from the sediment according to paper on chemical monitoring in Water Framework Directive (European Commission, 2010). Collected sediments were put in glass jars, and after transport to the laboratory, stored at 4°C in the dark until use. For the sediment used in the microcosm and mesocosm study, moisture content analysis was done by taking 10 g of sediment sample and drying at 105°C oven for 24 hours. Sediment moisture content is then calculated via formula (1) given below.

$$Sediment\ Moisture\ Content(\%) = \frac{sample\ weight - dried\ sample\ weight}{sample\ weight} \times 100 \qquad (1)$$

Then, total organic content analysis was done by following the procedure of loss of ignition, so the sample was put in the furnace at 550°C for 4 hours to ignite (Heiri et al, 2001), and calculated according to formula (2) given below.

$$Organic\ content\ (\%) = \frac{dry\ sample\ weight - ignited\ sample\ weight}{dry\ sample\ weight} \times 100 \qquad (2)$$

Sediments used in microcosms had 36.5±1.53% (n=3) moisture content and total organic content of 1.43±0.16% (n=3). The moisture content analysis and total organic

content analysis of sediments used in mesocosms were 29.64±0.31% (n=3) and 1.06±0.12% (n=3), respectively.

The pond water was also taken by sampling the sediments because it is recommended that some parameters should be measured to characterize the sediment and water collected (OECD, 2002). So, temperature and pH were measured during sampling. The mean temperature in the first sampling was 8°C and pH was 8.38. In the second sampling the average temperature and pH were 18.3°C and 7.85, respectively. Wet sediment particle size distribution analysis was done, and results are given in Table 3-2

Table 3-2. Wet sediment particle size distribution analysis results (Demirtepe,2017).

Parameter	Value
Specific surface area	0.653 m^2/g
Size range	0.02 to 2000 μm
Surface weighted mean	9.186 μm
Volume weighted mean	295.933 μm
d(10%)	4.103 μm
d(50%)	28.644 μm
d(90%)	1251.751 μm

3.7. QA/QC Protocols

Quality assurance/control protocols (QA/QC) include cleaning of laboratory equipment, standardized instrumental analysis via calibration curves and establishing detection limits, as well as analysis of blank and laboratory control samples.

For the purpose of cleaning of laboratory equipment, glassware and syringes, the Organic Analytes Chapter 4 of US EPA was followed (USEPA, 2014a). Before use, any clean glassware was rinsed with n-hexane.

Five-point internal calibration was performed for total HBCDD mix standard from 300 ppb to 1300 ppb in GC-MS. For surrogate standard BDE-99, calibration was also performed. In the calibration, RSD values are 5.10 for HBCDD and 5.06 for BDE-99, which confirm to the 20% limit stated by USEPA, and R^2 values are greater than 0.99 for BDE-99 and HBCDD (USEPA, 2014b). GC-MS calibration curves for BDE-99

and HBCDD are drawn by proportioning their area; and concentration to PCB-209 (internal standard) area and concentration and shown in Figure 3-5.

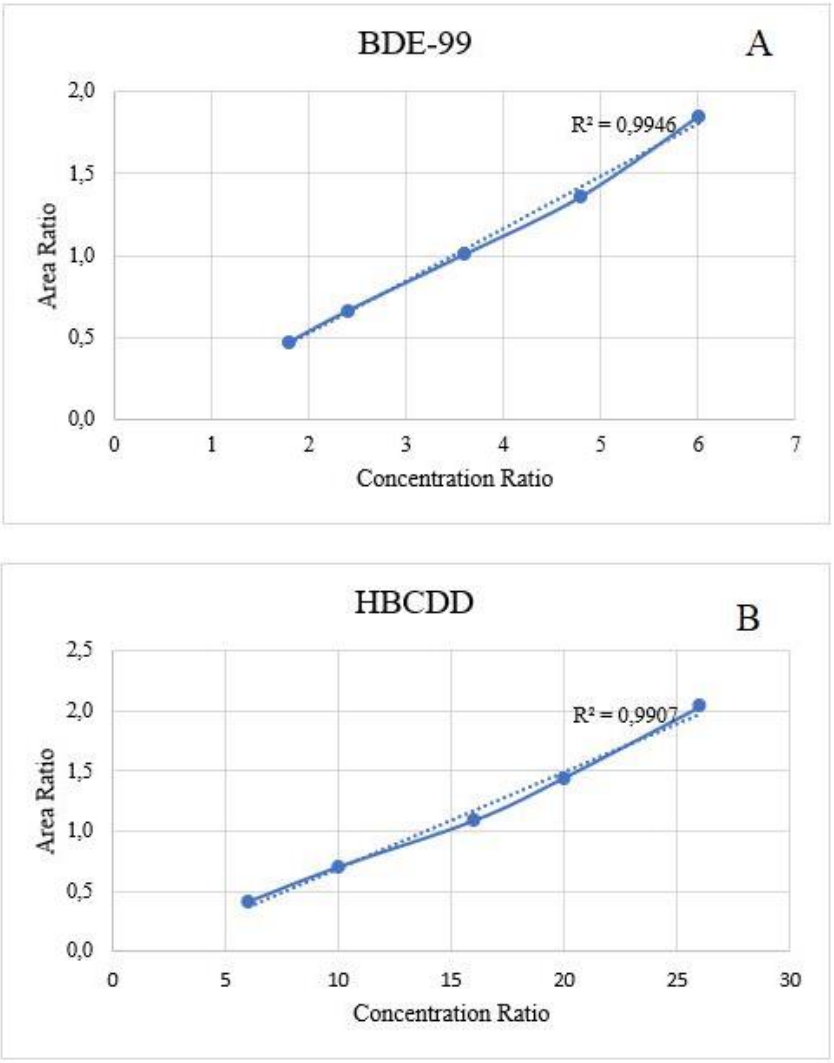


Figure 3-5 GC-MS calibration curves for a) BDE-99 b) HBCDD

Method detection limit (MDL) and limit of quantitation (LOQ) were established by following US EPA 40 CFR Appendix B part 136 (USEPA, 2016). MDL is calculated by multiplying the appropriate single tailed 99% t value (with n-1 degrees of freedom) by the standard deviation replicate sample analysis (equation 3).

$$MDL = t_{(n-1,0.99)} * S_n \qquad (3)$$

For this purpose, 10 analyses were done at the lowest point of the calibration standard (i.e. 300 ppb). The t value for 10 sample is 2.82. At first, for microcosm measurements,

MDL was found as 105.9 ppb for HBCDD and 18.68 ppb for BDE-99, LOQ was calculated as 336.76 ppb for HBCDD and 59.40 ppb for BDE-99. Before the mesocosm and sterile experimental sets, a universal gas trap was inserted to the gas line for the GC-MS. Hence, MDL was re-established as 59.39 ppb for HBCDD and 8.25 ppb for BDE-99. LOQ was calculated as 188.87 ppb for HBCDD and 26.23 ppb for BDE-99 by multiplying MDL with 3.18 (USEPA, 2016).

For LC-MS/MS analysis, five external calibration point was used for each alpha (α-), beta (β-), and gamma (γ-) HBCDD diastereomer from 50 to 1000 ppb. Method detection limit for alpha (α-), beta (β-), and gamma (γ-) HBCDD were determined as 3.72, 2.23 and 10,70 ppb, respectively, and LOQ were calculated as 11.83, 7.08 and 34.02 ppb, respectively. The concentration of each diastereomer in the samples were made according to the relative areas and proportion of the diastereomers in the 1000 ppb total-HBCDD mixture. All MDL and LOQ values are presented in Table 3-3. uring data analysis, i.e. HBCDD removal percentage and rate constant calculations, below LOQ (but above MDL) measurements were also included in order to have a larger data set.

Table 3-3 MDL and LOQ for total and diastereomer specific HBCDD analysis.

Compound	MDL (ppb)	LOQ (ppb)
BDE-99[1]	8.25	26.23
Total-HBCDD[1]	59.39	188.87
Alpha (α-) HBCDD[2]	3.72	11.83
Beta (β-) HBCDD[2]	2.23	7.08
Gamma (γ-) HBCDD[2]	10.70	34.02

[1] For GC-MS analysis [2] For LC-MS/MS analysis

Method blanks samples were analyzed in every 10 – 15 samples set. There were no peaks detected in blanks during analysis of microcosm or mesocosm sediment samples, hence, blank correction was not performed.

Laboratory control samples (LCS) were prepared and analyzed regularly. Preparation of LCSs was done by spiking a predetermined amount of HBCDD mixture to the sediment. Concentration spike to clean matrix was done around the middle of the

calibration range for the HBCDD and BDE-99 (USEPA, 2014b). Accordingly, in 4 different sample, spike was done with 750 ppb for HBCDD mixture and 150 ppb for BDE-99. Results are provided in the Results and Discussion chapter. In addition, before preparation of the microcosms, a sediment contamination test set is prepared with 5 samples by targeting 1000 ppb HBCDD, and average recovery was 98% and 92% for HBCDD and BDE-99, respectively. Analysis of certified reference material could not be done as a part of QA/QC procedures because, currently, a certified reference material does not exist for HBCDD.

HBCDD was not detected in the sediment taken from the pond. Hence sediments were used as is, during preparation of microcosms and mesocosms.

To ensure the accuracy of the results, identifying the performance of analytical methods was made by calculating the HBCDD (analyte) and BDE-99 (surrogate standard) recoveries of LCS.

Recovery is calculated by the following formulas:

$$BDE-99\ (surrogate)\ recovery(\%) = \frac{Concentration\ measured}{Concentration\ added} \times 100 \quad (4)$$

$$HBCDD\ recovery\ (\%) = \frac{C_s - C_u}{C_n} \times 100 \quad (5)$$

where,

C_s is the measured concentration of the spiked sample,

C_u is the concentration of unspiked sample and it is taken zero for LCS,

C_n is the theoretical (nominal) concentration of the sample (USEPA, 2014b).

According to the US EPA 8000D procedure, the acceptable analyte and surrogate recovery is in the range of 70 – 130% (USEPA, 2014b), in this study, in order to obtain a better performance, the acceptable analyte (HBCDD) and surrogate (BDE-99) recovery range was used as 80 – 120%.

For precision of analysis, relative standard deviation (RSD) is calculated as coefficient of variation by the following formula:

$$RSD\ (CV) = \frac{S}{\bar{X}} \times 100 \quad (6)$$

In the formula, S is the standard deviation of the measurement results, and $\bar{X}$ is the arithmetical mean of measurement results.

3.8. Experimental setup

HBCDD microcosms. Initially a microcosm set was prepared to get an initial idea of about the rate of HBCDD degradation and provide the basis for mesocosm sets. The microcosm was run for 20 days, as 20 mL volume duplicate reactors. The sediment/liquid ratio in all reactors was kept constant at 3 g/3.5 mL. Distilled water was added to all sets except the biostimulation set, where organic medium is added instead of distilled water. Organic medium (fresh ECl medium) was prepared by adding vitamins, minerals and various salts listed at Table 3-4 into water under a N_2:CO_2 atmosphere and pH is adjusted to 6.8 (Berkaw et al.,1996). The medium also contained 50 mM sodium formate and ethanol as carbon source and electron donor, respectively.

Table 3-4 Ingredients of ECl medium for 1000 mL total volume.

Ingredient	Amount	Ingredient	Amount
H_2O	996 ml	Trace mineral solution (1000x)	1.0 ml
NaCl	8.4 g		
$MgSO_4 * 7H_2O$	4.8 g	Vitamin solution (1000x)	1.0 ml
KCl	0.27 g	HCl	0.5 g
$CaCl_2 * 2H_2O$	0.05 g	$Na_2HPO_4 * 7H_2O$	1.12 g
NH_4Cl	0.5 g	Cysteine	0.25 g
Resazurin (0.1%)	1.0 ml	Na_2CO_3	3.0 g

Two types of control set were set up: (i) Sterile set was prepared by adding mercuric chloride (0.5 mg $HgCl_2$/g sediment) and also autoclaving for 20 minutes at 1.1 atm pressure at 120 °C for three consecutive days to prevent microbial activity in the sediment, (ii) contaminant control set was prepared without adding HBCDD. A summary of HBCDD sediment microcosm reactors is shown in Table 3-5. Target HBCDD concentration was 1000 ng/g dry weight. HBCDD was spiked over the dry sediments and mixed until the solvent evaporated. Then, wet sediment was added over the dry spiked sediment and mixed together. This method of contaminating the

sediment was adopted from Tokarz III et al., (2008).Unspiked contaminant control reactor was prepared by spiking with n-hexane.

Table 3-5 HBCDD sediment microcosm reactors setup summary

Reactor Type	**Reactor**	**HBCDD spike**	**Sediment (3 g)**	**Overlying Liquid Content (3.5 mL) (Total volume – 20 mL vial)**
Test Reactors	Natural Attenuation	+	+	Distilled water
	Biostimulation	+	+	e- source (ethanol) & carbon source (sodium formate) added rich organic medium
Control reactors	Contaminant Control	-	+	Distilled water
	Sterile	+	+	Distilled water

Reactor vials were closed by crimping the Teflon lined septa caps, then high-purity nitrogen gas was circulated into the vials to enable anaerobic conditions. Reactors were incubated at 25°C in the dark. During sampling, duplicate reactors were opened and all sediments in the vials were analyzed. In Figure 3-6, microcosm reactor samples from a sampling day is demonstrated. Sampling from microcosms was done on days 0, 8, 13 and 20. This study does not cover monitoring of the products of HBCDD.

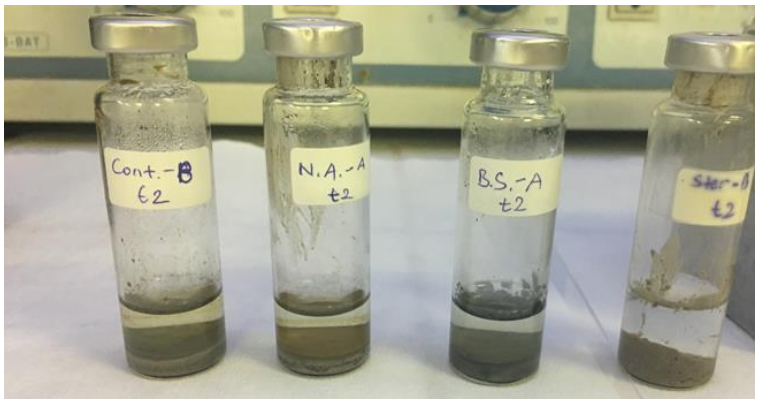


Figure 3-6 Picture of microcosm reactors.

HBCDD mesocosms. Larger scale reactors were operated to observe HBCDD biodegradation in sediments. Special made glass reactors (approximate volume of 2400 mL) made out of seamless solid glass was used during the mesocosm study. The length, width and height were 21 cm, 5.7 cm, and 20.4 cm, respectively. Duplicate mesocosms were prepared. These reactors were prepared entirely in the N_2:CO_2:H_2 environment of an anaerobic glovebox (PlasLabs 818GB/Exp) showed in Figure 3-7. The details of sediment mesocosm are presented in Table 3-6.

Figure 3-7 Picture of the anaerobic glovebox.

Table 3-6 Summary of HBCDD sediment mesocosms.

Reactor Type	**Reactor**	**HBCDD spike**	**Sediment (685 g)**	**Overlying Liquid Content (550 mL) (Total volume~2400 L)**
Test Reactors	Natural Attenuation	+	+	Distilled water
	Biostimulation	+	+	e- source (ethanol) & carbon source (sodium formate) added rich organic medium
Control reactors	Contaminant Control	-	+	Distilled water
	Sterile	+	+	Distilled water

Similar to the preparation of the microcosm reactors, HBCDD spike was done over dry sediments, after the solvent evaporation by mixing for 15 minutes, wet sediment was added and then all sediments were mixed together for 45 minutes. In sterile sets, mercuric chloride (0.5 mg $HgCl_2$ per g sediment) was added to reactor after wet sediment, then all were mixed together for 45 minutes. Sterile sets were autoclaved at 1.1 atm pressure for 20 min at 120°C on three consecutive days to prevent any biological activity in reactors. Similar to the contaminant control set in microcosms, an unspiked control set was prepared by adding n-hexane only. The target HBCDD concentration was 1000 ng/g dry weight for all mesocosms.

Each mesocosm reactor were prepared containing 685 g of wet sediments, and a total of 550 mL liquid. Distilled water was added into the reactors as overlying liquid, except the biostimulation set. For biostimulation set, organic medium was added on top of the sediments, similar to the biostimulation microcosm set. All of the overlying liquid was purged with nitrogen before taken into the anaerobic glovebox.

After sediments were transferred into mesocosm reactors, overlying liquids of reactors were poured over sediments. Rich organic medium added into the biostimulation reactors contains resazurin to observe the color change (i.e. pink color indicates the presence of oxygen in the reactor) during the incubation time. Pictures of biostimulation sediment mesocosms from different sampling days are shown in Figure 3-8.

Upon preparation, the sediment mesocosms (with a glass lid) were placed in an incubator at 25°C in the dark. Despite setting up the reactors in an anaerobic glovebox, anaerobic environment was not provided during incubation in order to simulate environmental conditions. While sampling, glass lids of the reactors were opened, and the overlying liquid was exposed to the atmosphere.

Figure 3-8 Picture of a) Biostimulation set (day-0) b) Biostimulation set (day-28) c) Biostimulation set (day-49)

During sampling, triplicate samples were taken from each duplicate reactor. While sampling, tip of glass pipettes was cut-off and submerged into the reactors, and sediment was taken with minimum disturbance. For all sets, sampling of sediments was done on the days 0,7,14,21,28,35,42 and 49, except for the sterile set. A problem was encountered during autoclaving of the sterile mesocosms, therefore the first sampling was performed on day 8 instead of day 7, but the rest were taken at same intervals, i.e. days 14,21,28,35,42,49. Photographs from a sampling day is presented in Figure 3-9.

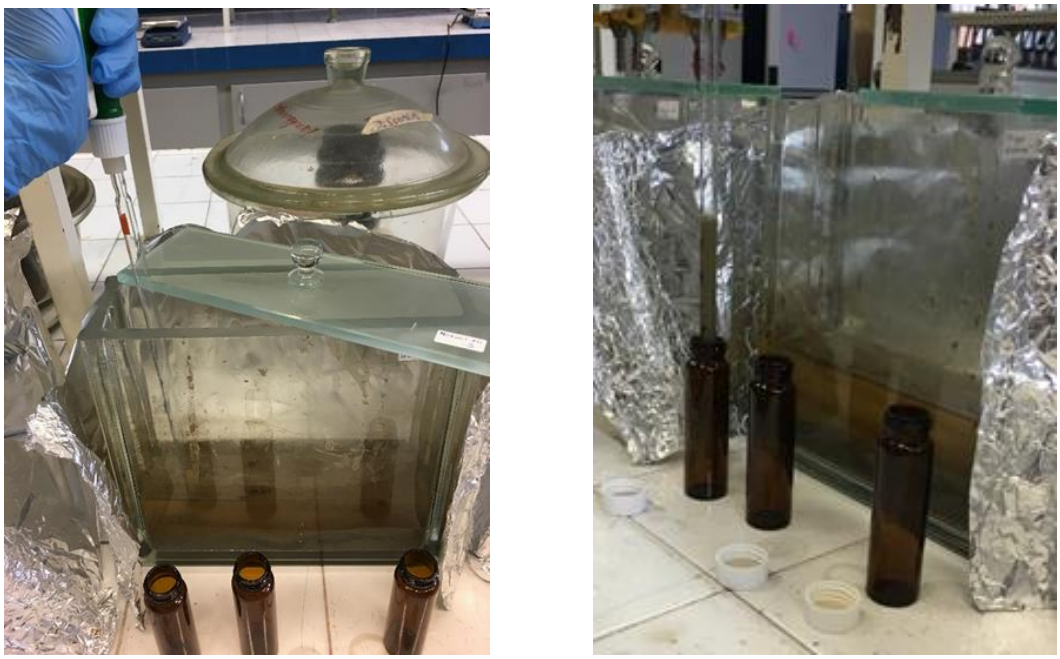


Figure 3-9 Picture of depicting sampling from mesocosm reactors

Sampling of headspace to monitor gas arisen from the biodegradation was not done. Also, the overlying liquid of the reactor was not analyzed because vapor pressure of HBCDD is between the 1.86×10^{-11} Pa and 7.23×10^{-7} Pa (Marvin et al., 2011), and water solubility is between 2.1 µg/L and 66 µg/L (Marvin et al., 2011) so dissolving in the overlying liquid and vaporization is not expected for HBCDD and its diastereomers.

Sterilization control microcosms pre-set: A microcosm pre-set (20 mL volume vials) was prepared to get an initial idea about the HBCDD degradation during sterilization of reactors. Hence microcosms were prepared by spiking sediments with the same concentration of HBCDD, but triplicate reactors were sterilized using only mercuric chloride (0.5 mg $HgCl_2$/g sediment), autoclaved 20 minutes at 1.1 atm pressure at 120 °C for three consecutive days with added mercuric chloride (0.5 mg $HgCl_2$/g sediment), and no sterilization (i.e. same as natural attenuation). Sediment/liquid ratio in all reactors was kept constant at 3 g/3.5 mL. These reactors were not incubated, that is, only time zero is sampled.

Sterilization control microcosms: In order to investigate further the effect of sterilization on HBCDD degradation, a new microcosm set was prepared. HBCDD sediment sterile microcosm reactors setup summary is shown in Table 3-7. The

purpose of this set was to examine the effect of sterilization on HBCDD concentration change at the initial time as well as the change of concentration over time. The target HBCDD spike concentration was 1200 ng/g dry weight. As was performed for all previous sets, HBCDD was spiked over the dry sediments and mixed until the solvent evaporated. Then, wet sediment was added over the dry spiked sediment and mixed together for 45 minutes. Sterilization was performed by two means: (i) by autoclaving for 20 minutes at 1.1 atm pressure at 120 °C for three consecutive days, (ii) by adding mercuric chloride (0.5 mg HgCl2/g sediment) and then autoclaving for 20 minutes at 1.1 atm pressure at 120 °C for three consecutive days. After that, mercuric chloride was added over wet sediment and mixed for 30 minutes. Unspiked contaminant control reactor is prepared by adding the same amount of n-hexane as those spiked with HBCDD.

Table 3-7 HBCDD sediment sterile microcosm reactors setup summary

Reactor Type	**Reactor**	**HBCDD spike**	**Sediment (3 g)**	**$HgCl_2$**	**Overlying Liquid Content (3.5 mL) Total volume (20 mL vial)**
Test Reactors	Sterile-No1	+	+	-	Distilled water
	Sterile-No2	+	+	+	Distilled water
Control Reactors	Natural Attenuation	+	+	-	Distilled water
	Contaminant Control	-	+	-	Distilled water

Reactor vials were closed by crimping the Teflon lined septa caps, then high-purity nitrogen gas was circulated into the vials to establish anaerobic conditions. Reactors were incubated at 25°C in the dark. During sampling, triplicate reactors were opened and all sediments in the vials were analyzed. Sampling from microcosms was done on days 0, 8, 14 and 21. This study does not cover monitoring of the products of HBCDD, however diastereomers of HBCDD was monitored.

Summary of relevant information about sterilization method for all experimental setups given in Table 3-8.

Table 3-8 Relevant information about sterilization methods.

Set Name	$HgCl_2$ addition	$HgCl_2$/g Added		$HgCl_2$ Mixing with sediment	Autoclaved
		More than 0.5 mg	Exactly 0.5 mg		
Microcosm	✓	✓	x	x	✓
Mesocosm	✓	x	✓	✓	✓
Sterilization control microcosm pre-set	✓	x	✓	x	x
	✓	x	✓	x	✓
Sterilization control microcosm	x	x	x	x	✓
	✓	x	✓	✓	✓

3.9. Determination of Biodegradation Reaction Rate Constants

The order of HBCDD biodegradation rate constants was determined trying zero, first (pseudo) and second order and their equations are given below (Mihelcic, 1999) :

Zero order equation:

$$C_t = -kt + C_0 \quad (7)$$

First (Pseudo) order equation:

$$\ln\left(\frac{C_t}{C_0}\right) = -kt \quad (8)$$

Second order equation:

$$\frac{1}{C_t} = \frac{1}{C_0} + kt \quad (9)$$

Where,

C_t is the HBCDD concentration at a specific time,

C_0 is the HBCDD concentration at time zero,

t is time,

k is the rate constant.

CHAPTER 4

RESULTS AND DISCUSSION

4.1.HBCDD Method Validation

4.1.1. Laboratory Control Sample Results

Laboratory control samples (LCS) are prepared to verify that analytes can be measured within the acceptable recovery ranges. The target analytes in this study were total-HBCDD and BDE-99 (surrogate standard), and they were prepared at a concentration of 750 ppb and 150 ppb, respectively, according to the level of spiking to be used in following micro and mesocosms. Blank analysis was also performed together with each batch of LCS. Results are presented in Table 4-1.

Table 4-1 Recoveries of LCS analyses.

Sample	Total-HBCDD Recovery (%)	BDE-99 Recovery (%)
LCS-1	99.54	94.55
LCS-2	96.95	94.81
LCS-3	101.93	87.57
LCS-4	95.85	93.95
Blank	no peak detected	92.50

According to LCS analysis recoveries given in Table 4-1, average recovery of total-HBCDD and BDE-99 was calculated as 98.57±2.36% and 92.68±2.99%. RSD values for HBCDD and BDE-99 was calculated as 2.39% and 3.23%. Also, as seen in Table 4-1, no HBCDD was detected in the blank sample, and BDE-99 recovery in this sample was 92.50%. As mentioned in Section 3.5, the acceptable analyte and surrogate

recovery is between 70 – 130% in US EPA 8000D procedure (USEPA, 2014b), in this study to control the analysis performance analyte (HBCDD) and surrogate (BDE-99) recovery range was adopted as 80 – 120%. Hence, the LCS analysis recoveries were in the acceptable range.

4.1.2. Sediment Contamination Test Results

The purpose of the contamination test was to make sure that each microcosm would contain sediment HBCDD levels the same or very similar to each other. For this purpose, a large amount of clean sediment is spiked with HBCDD, then separated into 5 different sample vials with the intention of attaining 1000 ng/g concentration HBCDD in each vial. BDE-99 was added to the sediments by targeting 150 ppb. Blank analysis was also performed with this batch.

Table 4-2 Total-HBCDD results of SCT.

Sample	Measured Total-HBCDD Concentration (ng/g)	Targeted HBCDD Concentration (ng/g)	Recovery (%)
SCT-1	953.67	1000	95.37
SCT -2	983.91	1000	98.39
SCT -3	1009.67	1000	100.97
SCT -4	987.37	1000	98.74
SCT -5	984.04	1000	98.40
Blank	no peak detected	0	0

Table 4-3 BDE-99 results of CSS analysis.

Sample	BDE-99 Detected Concentration(ppb)	BDE-99 Targeted Concentration (ppb)	Recovery (%)
SCT -1	130.11	150	86.74
SCT -2	136.19	150	90.79
SCT-3	137.27	150	91.51
SCT-4	139.72	150	93.15
SCT-5	142.95	150	95.30
Blank	143.86	150	95.91

Total-HBCDD was detected as indicated in Table 4-2, and average total-HBCDD was found as 983.73±16.28%. Total-HBCDD recovery was calculates as 98.37±1.78% with RSD being 1.81%. According to the results given in Table 4-3, average recovery for BDE-99 was found as 91.50±2.59%, and RSD calculated as 2.83%. In the blank sample, no HBCDD was detected and BDE-99 recovery was calculated as 95.91%. As a result, SCT sample recoveries were also in the acceptable range. These results show that when sediment microcosms are prepared, the concentration of analyte in each bottle is expected to show no more than a few percentages of variation from each other. This means that any change in analyte concentration that will be observed in microcosms/mesocosms can be attributed to the tested parameters, i.e. biodegradation. For LCS and SCT analysis, recovery and RSD values are summarized in Table 4-4.

Table 4-4 Summary recovery and RSD of LCS and SCT.

Analysis Name	n	HBCDD recovery (%)	BDE-99 Recovery (%)	HBCDD RSD (%)	BDE-99 RSD (%)
LCS	4	98.57±2.36%	92.68±2.99%	2.39	3.23
SCT	5	98.37±1.78%	91.50±2.59%	1.81	2.83

4.2. Results of Microcosm Studies

4.2.1. Degradation of total-HBCDD in sediment

In the microcosms, the initial concentrations of total-HBCDD were 699.46 and 703.94 ng/g dry weight for a and b parallel of natural attenuation set, respectively, and 969.26 and 914.62 ng/g dry weight for a and b parallel in biostimulation set, respectively. Although the initial concentrations of HBCDD in natural attenuation and biostimulation sets were aimed to be the same, the concentrations in natural attenuation were measured to be lower than those in biostimulation. Since no problems are expected in the sample extraction process, due to high recoveries, the dissimilarity was estimated to be due to incomplete mixing of the sediments during preparation. Nevertheless, since there is no significant difference inbetween the concentrations of the parallels (i.e. parallels of natural attenuation and biostimulation have very close concentrations in themselves), it is predicted that this will not have a significant effect

on the results. The time dependent HBCDD concentration changes in the microcosm sets are shown as grouped bars in Figure 4-1(a), while HBCDD remaining in the reactors is shown in Figure 4-1(b), and HBCDD remaining for each microcosm parallel reactor set is shown in Figure 4-1(c).

As can be seen from Figure 4-1, a rapid decrease was observed in natural attenuation and biostimulation sets within the first 8 days, then the decrease slowed down but continued in both sets. At the end of 20 days, in the natural attenuation set, there were 77.5% and 72.13% decrease of total-HBCDD in a and b parallels, respectively, whereas in biostimulation set, HBCDD was below detection limit in both a and b parallels at the end of 20 days.

Sterile set reactors were established with the aim of understanding whether there is a change due to abiotic reactions depending on time, in other words, as a control set for biodegradation of HBCDD. Total-HBCDD concentration in sterile reactors were measured to be almost half of that of the natural attenuation and biostimulation sets - even though sediment spiking was performed as one batch and separated out equally into each vial. No remarkable decrease is observable in sterile reactors throughout the operation period. This finding suggests that HBCDD does not break down abiotically. Even though there is no decreasing trend with time, the fact that the concentration in sterile sediments were almost half of that of the intended concentration was noted as an issue to be further investigated. No HBCDD peak was found in the sediments analyzed from contaminant control reactors, so it was testified that no HBCDD contamination occurred during sampling, extraction or analysis stages.

Findings related to HBCDD degradation were compared with the current, albeit limited literature. Davis and colleagues (2005) reported that the commercial HBCDD mixture in anaerobic aquatic sediments with HBCDD concentrations of 30-40 ng/g dry weight decreased to non-detectable levels within 7 days. However, in aquatic sediments having two orders of magnitude higher initial HBCDD concentration (i.e. approximately 5650 ng/g) showed 61.5% reduction in 113 days without any extraneous substance (Davis et al., 2006).

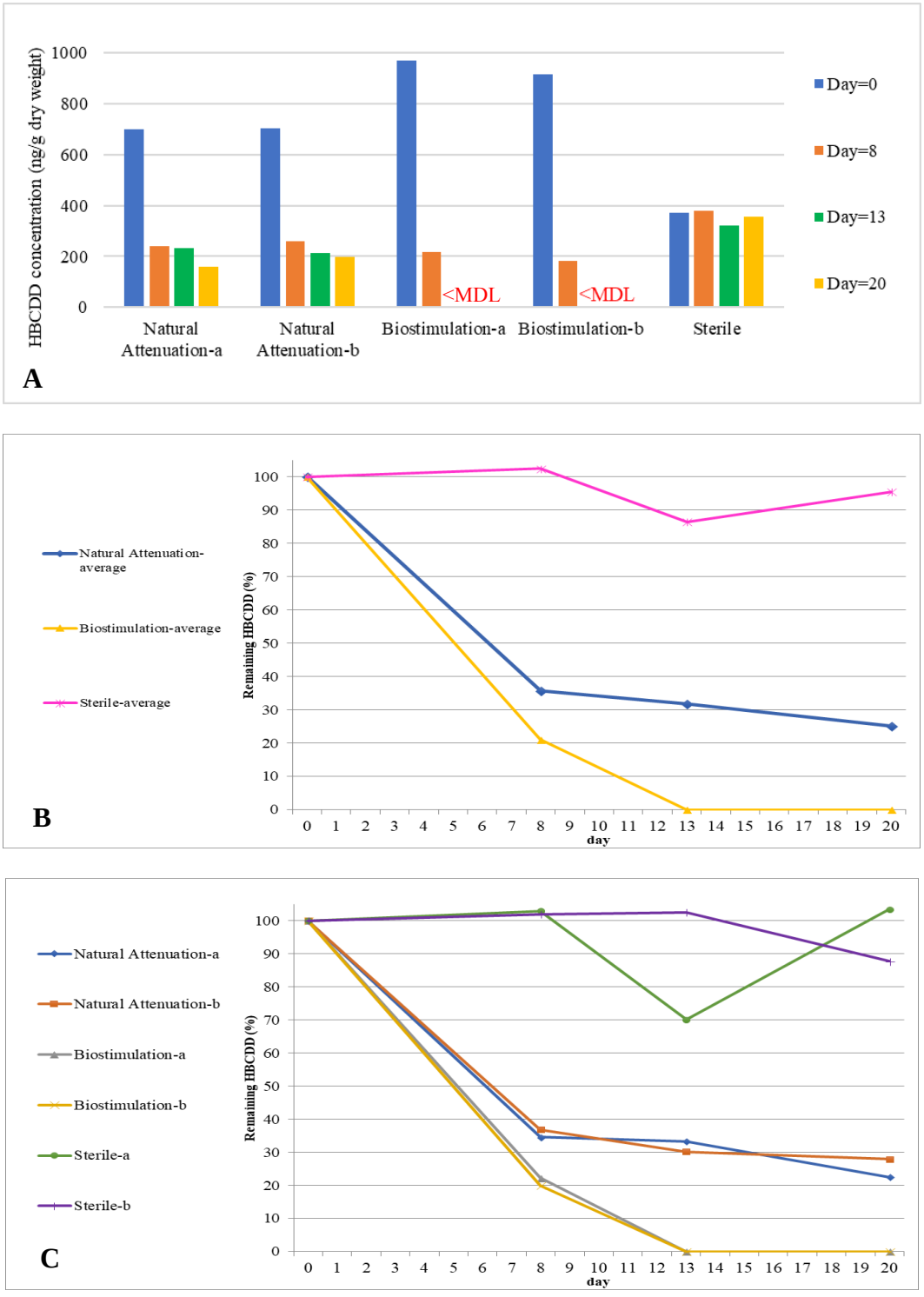


Figure 4-1 a) The time dependent HBCDD concentration changes in the microcosm sets b) HBCDD remaining in the microcosm sets (average) c) HBCDD remaining for each microcosm parallel reactor set.

In this study, natural attenuation sediment reactors, which are prepared without any extraneous substance (i.e. via addition of spiked sediment and distilled water only), with initial total-HBCDD concentrations of approximately 700 ng/g dry weight showed 72-78% reduction in 20 days. As will be discussed in the upcoming section, although the removal percentage is similar, the rate of degradation of HBCDD in the current study is much greater when compared to that of Davis et al., (2006). Similar to the biostimulation set of this study, which showed 100% total-HBCDD reduction in 20 days, a mineral salts medium added into digester sludge reactors resulted in approximately 90% reduction in 28 days (Davis et al., 2006).

4.2.2. Total-HBCDD degradation rate

The order of biodegradation rate constants was determined by trying zero, first (pseudo) and second order equations. As data fitted best, HBCDD decay rate in sediment microcosms could explain by pseudo first order reaction kinetics. The calculated HBCDD degradation rate constants and half-lives are presented in Table 4-5. There was a difference in the total-HBCDD levels of biostimulation parallel reactors at the 13th day of incubation. While no HBCDD was observed in biostimulation-a microcosm (as was shown in Figure 4-1), albeit small, HBCDD remained in biostimulation-b reactor, but it was below MDL value Therefore, half of the method detection limit was used as the 13th day data in degradation rate calculation for the biostimulation-b reactor. As can be seen from the table, in this microcosm study, total-HBCDD decay rate of biostimulation set was more than three times that of the natural attenuation set. A higher rate would be expected since more suitable conditions for microbial growth were provided in the biostimulation microcosms via the addition of a carbon source and electron donor (i.e. 50 mM sodium formate and ethanol).

Table 4-5 HBCDD degradation rate constants and half-lives of microcosms.

Name of reactor set	k **(day^{-1})**	$t_{1/2}$ **(day)**
Natural Attenuation	0.069	10.1
Biostimulation	0.221	3.13

Interestingly, in the literature different half-lives were reported for the degradation of HBCDD. Gerecke et al. (2006) found that 50% of HBCDD degradation happened in less than a day (0.66 day) in digester sludge under anaerobic conditions, but Davis et al. (2006) reported a half-life of 5.4 days. Also, Davis et al. (2005) previously reported anaerobic HBCDD degradation half-lives in sediments as 1.1 to 1.3 days. The difference between these studies were attributed to the initial HBCDD concentration, Davis et al. (2006) used approximately two orders of magnitude greater initial HBCDD concentration than Gerecke et al. (2006), and discussed that the greater the initial concentration, the slower the rate of degradation (i.e. the greater the half-life). At higher concentrations, for poorly soluble substances such as HBCDD, biodegradation rates are stated to be more affected by mass transfer limitations than on true biodegradation kinetics (Davis et al., 2006).

4.3. Results of Mesocosm Studies

4.3.1. Degradation of total-HBCDD in sediment

The HBCDD concentration in sediments were monitored as total-HBCDD via GC-MS and via individual diastereomers via LC-MS/MS. The total-HBCDD from GC-MS and the total-HBCDD as the sum of three diastereomers from LC-MSMS do not exactly give the same concentration (i.e. 885.64 ng/g from GC-MS vs. 1172.87 ng/g from LC-MSMS, please see Appendix B for concentration changes with time using total-HBCDD data from LC-MS/MS. It is estimated that the difference between these results are due to different measurement sensitivities of the instruments. All discussions on total-HBCDD degradation will be made using GC-MS results, and LC-MSMS results are only used to provide information regarding the relative abundance of α-, β- and γ-HBCDD diastereomers.

Although the initial concentrations of HBCDD in natural attenuation and biostimulation sets were aimed to be the same, the concentrations in natural attenuation were lower than those in biostimulation, similar to the case observed in the microcosm study. The difference between concentrations of these two sets and parallels of natural attenuation and biostimulation have close concentrations within themselves, therefore it is predicted that this will not have a major impact on the results. Time dependent total-HBCDD concentration changes are shown in Figure 4-2.

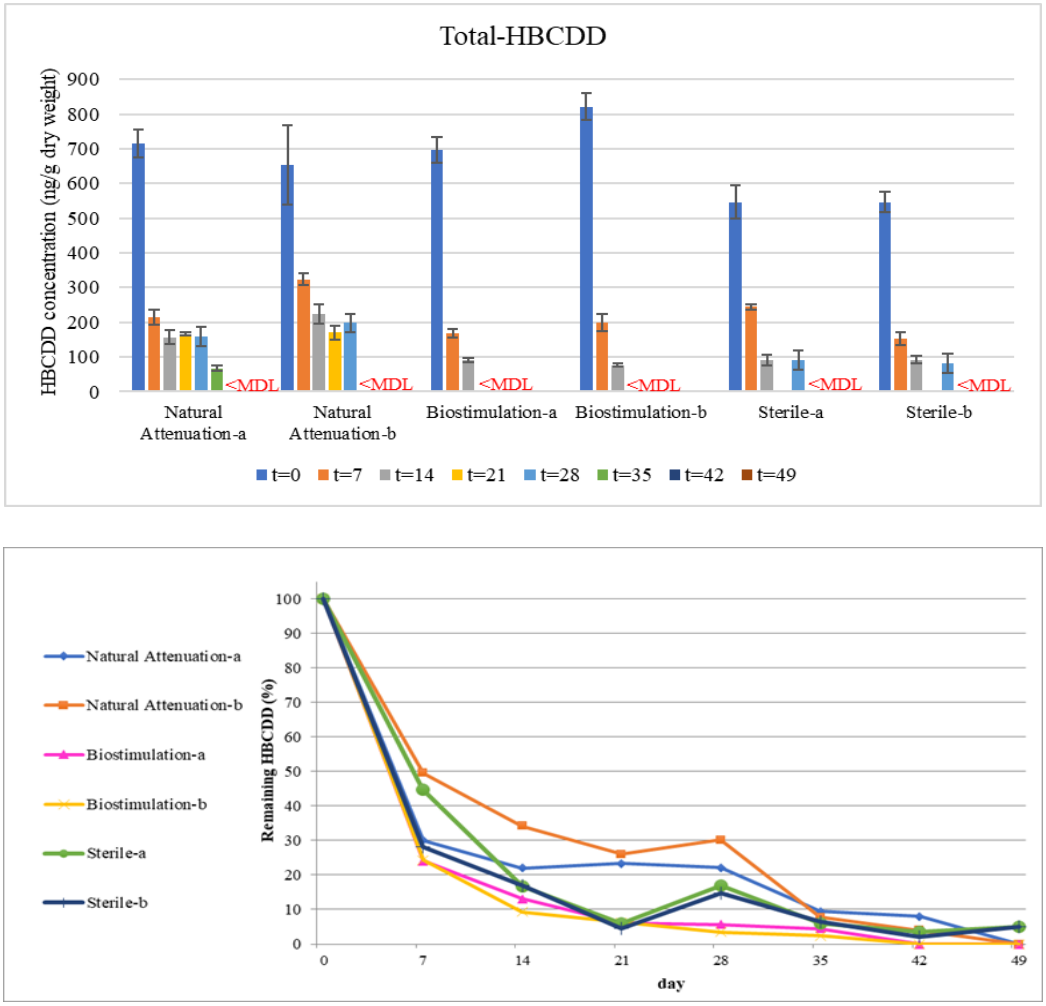


Figure 4-2 Total-HBCDD Concentration b) HBCDD remaining for each mesocosm parallel reactor set

As can be seen from Figure 4-2, a rapid decrease was observed in natural attenuation and biostimulation set within the first 7 days. The decrease in concentration continued although slower in both sets. At the end of 35 days in natural attenuation set, HBCDD was below MDL for b parallel; and after 42 day, HBCDD peak could not be measured in either a or the b parallel. In biostimulation set, after 21 days, HBCDD was observed below MDL for both a and b parallels. No HBCDD peak was observed in the sediments analyzed from contaminant control reactors. However, the change in total-HBCDD concentration of sterile reactors was an unexpected result.

Similar to the microcosm study, the initial concentration of sterile set reactors was measured to be lower than those of the natural attenuation or biostimulation sets – though not as low as those observed for microcosms. The variation observed in initial concentrations of all sets are greater than any measurement uncertainty observed during extraction of laboratory control samples. Not only did the initial total-HBCDD concentration in sterile reactors were less than that of the natural attenuation and biostimulation reactors, but also a very fast degradation trend was observed in both sterile parallel mesocosms. This was not the case for microcosms, although the initial concentration was almost half of that of the other test reactors, no decreasing trend (i.e. degradation of HBCDD) was observed in the sterile sets.

One possible explanation for this observation in the sterile mesocosms is a potential breach of sterilization. Microcosms established in 20mL closed vials, operated via sacrificing a vial at each time step, is much easily maintained as a sterile reactor. The mesocosms, on the other hand, just has a glass lid which is periodically opened for sediment sampling. The reactors were exposed to the atmosphere which perhaps led to the possible activation of aerobic microorganisms in the mesocosms. Mesocosm reactors, similar to the natural conditions, are set-up in such a way that physicochemical decomposition via volatilization following solubilization could take place. But HBCDD has a very low vapor pressure and solubility as given in Table 2-2 at section 2.2.1., therefore, physicochemical weathering is not expected.

Sterilization problem in HBCDD studies were also mentioned in the literature. Davis et al. (2006) reported 60% degradation in mercuric chloride treated abiotic control microcosms (as opposed to 21% in natural attenuation microcosms), while Gerecke et al (2006) mentioned HBCDD degradation in a heat sterilized (autoclaved) set, with a half-life of 35 days (as opposed to 0.66 days in the biostimulation set). Moreover, complete degradation of HBCDD took place after 60 days in anaerobic sterile sediment reactors (Davis et al., 2005). This issue was also a concern in Demirtepe's (2017) study, hence a more detailed investigation was done and explained in Section 4. 4..

4.3.2. Degradation of HBCDD diastereomers in sediment

Time dependent α-, β- and γ-HBCDD concentration changes in each mesocosm reactor are shown as individual diastereomers in Figure 4-3, and as portion of the total in

Figure 4-4, respectively. Also, time dependent α-, β- and γ-HBCDD concentration changes as average of the a and b parallels of mesocosms given in Appendix C and separated portion of diastereomers in total-HBCDD for each mesocosm given in Appendix D. The distribution of diastereomers in the mixture is such that approximately 21% is α-HBCDD, 12% is β-HBCDD and 67% is γ-HBCDD. The relative abundance of diastereomers show a few percentages of variation, resulting from LC-MSMS sensitivity. Nevertheless, some clear trends can be observed in the data. In Appendix E, time depended percentage changes of diastereomers in mesocosms were given.

As seen in Figure 4-3 and Figure 4-4, γ-HBCDD and β-HBCDD show consistently declining trends in all sets. On the other hand, α-HBCDD levels show a fluctuation, they decline until the third time period, after which the concentration increases and then decreases or stays relatively constant, depending on the mesocosm set. Most abundant diastereomer, γ-HBCDD is observed to degrade very easily, which conforms to the findings reported in the literature (Davis et al., 2006; Gerecke et al. 2006).

Among the three diastereomers, α-HBCDD was the only one that was above detection limit until the end of the 49-day incubation period. This finding is also in line with those cited in the literature (Davis et al., 2006; Gerecke et al., 2006; Le et al., 2017). Trends discussed above appear to be consistent within parallel mesocosms. Since there is no other source of HBCDD in the mesocosms other than the spiked amount (i.e. contaminant control and blanks consistently show no contamination during laboratory handling, extraction or analysis stages), the fluctuation of α-HBCDD can be explained by its transformation from β- and/or γ-HBCDD. This is the first study that report such an observation of inter-transformation of HBCDD diastereomers in sediments. Such transformation from other diastereomers to α-HBCDD was previously reported to be in biota (Zegers et al., 2005; Schecter et al., 2012).

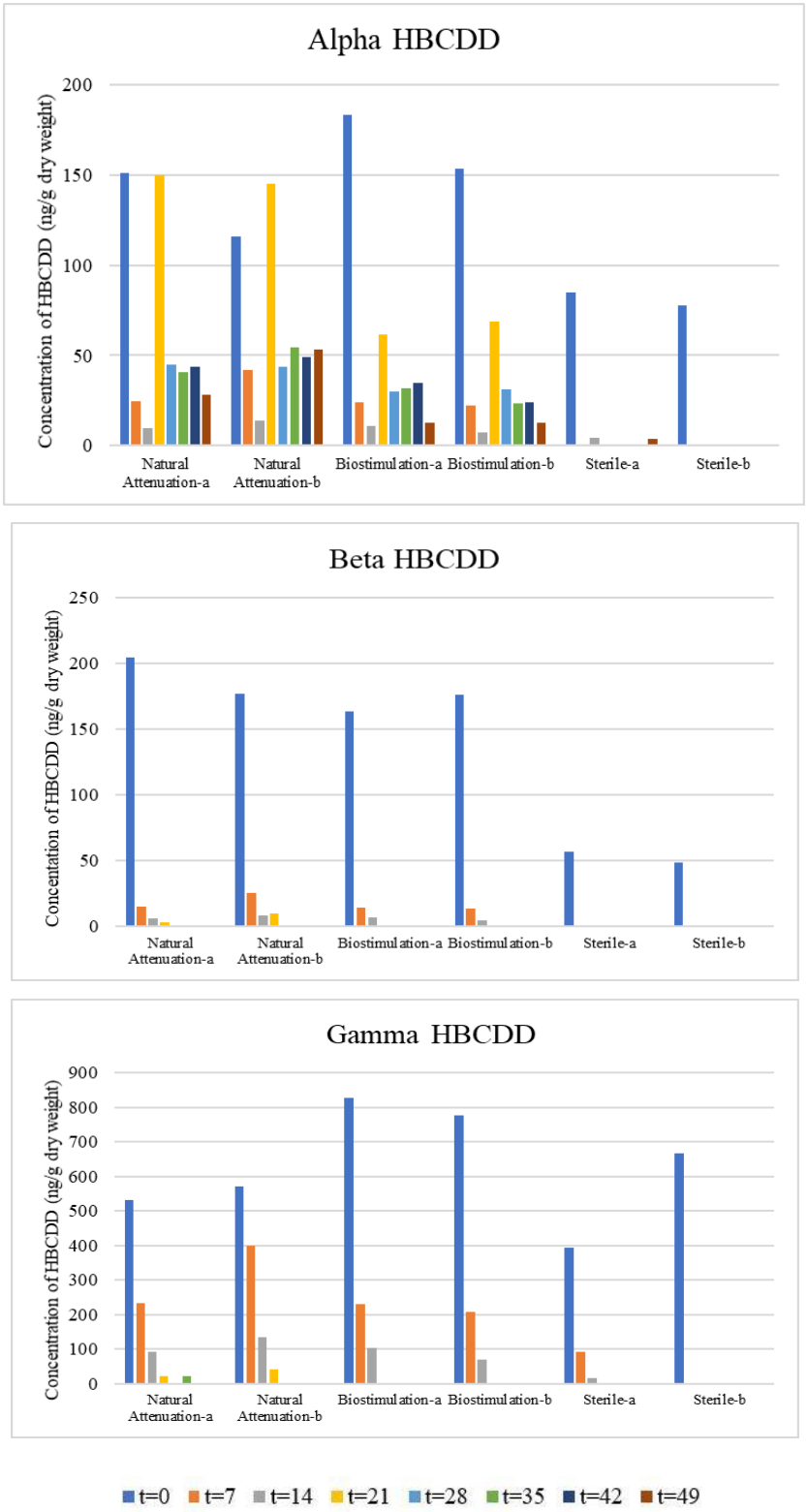


Figure 4-3 Alpha (α-), beta (β-), and gamma (γ-) HBCDD concentrations in mesocosm

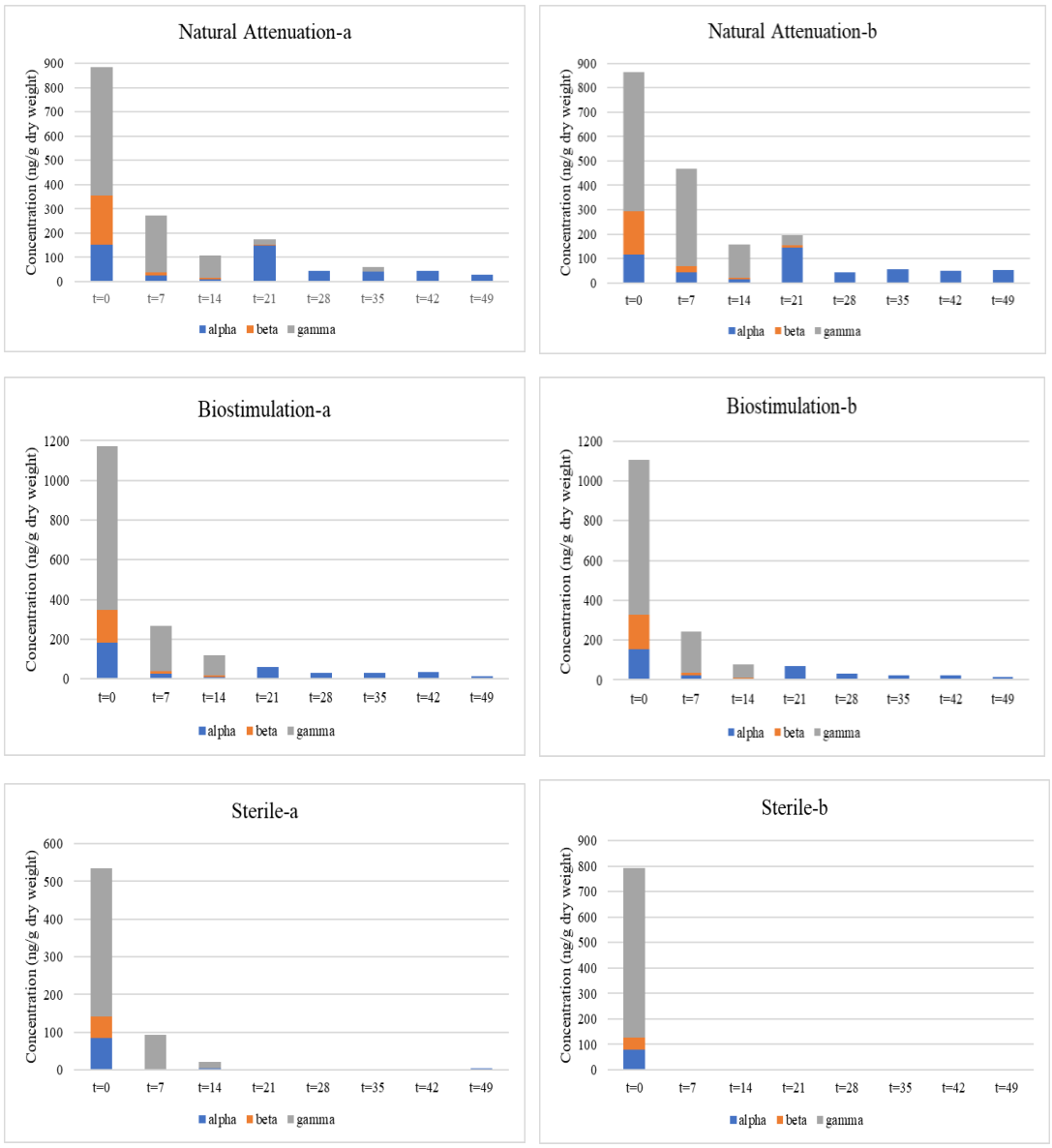


Figure 4-4 Diastereomer portion of total-HBCDD in mesocosms

Potential transformation of one diastereoisomer to the other may be explained using thermodynamics. Thermodynamic properties of α-, β- and γ-HBCDD were investigated by Zhao et al. (2010). The thermodynamic properties of three HBCDD diastereoisomers indicate that α-HBCD is the most stable one.The Gibbs free energies of β- and γ-HBCDD were determined by comparing with α-HBCDD, the most stable diastereoisomer. Authors state that the relative energies calculated from polarizable continuum model PCM-B3LYPsuggest γ-HBCDD's poor water solubility compared with α- and β-HBCDD (Zhao, 2010).

When natural attenuation and biostimulation are compared with each other, a distinctly slower degradation rate is observed for α- and γ-HBCDD in the former when compared to the latter. β-HBCDD degradation, on the other hand, seem to be very fast for both types of mesocosms. A more quantitative discussion of degradation rates is provided in the next section.

In the sterile mesocosms, β- and γ-HBCDD was not detected after the initial day (except in sterile-a at t=7 days). One reason could be the low initial HBCDD concentration of sterile sets, resulting in below detection levels quickly after incubation. But in sterile-b, the γ-HBCDD concentration is greater than that of the natural attenuation sets, yet still no γ-HBCDD could be detected at the first sampling date, i.e. day 8. Measurement uncertainty is expected to be a factor in these findings, as LC-MSMS measurements are quite sensitive, yet complicated. Throughout analysis of samples of the mesocosm sets, the α-, β-, and γ-HBCDD abundance varied as 21±6%, 12±5% and 67±10%, respectively. So, some variation is to be expected as a result of analytical uncertainty. Even so, trends discussed above which are consistent within parallel sets point to the presence of some other mechanism. There is also some possibility that perhaps the mercuric chloride addition and autoclaving somehow has an impact on the degradability of HBCDD. The α-HBCDD levels observed in sterile sets may also support this hypothesis. It is typically stated that α-HBCDD is the most resistant diastereomer among to rest to degradation (Davis et al., 2006; Gerecke et al., 2006; Le et al., 2017), yet, similar to the other diastereomers, α-HBCDD is not detected (or detected at very low concentrations) in sterile mesocosms after the first day. This is in contrast to the levels of α-HBCDD observed in natural attenuation and biostimulation sets, where α-HBCDD is detected until the last sampling day. These findings imply that a removal or transformation mechanism other than biodegradation (e.g. binding of Cl to HBCDD, or debromination followed by chlorination of HBCDD during autoclaving, etc.) could be playing part in the disappearance of HBCDD diastereomers in sterile mesocosms. One such comment, although not elaborated, is present in the HBCDD Risk Profile document of the Stockholm Convention, where the following statement is made in one of the summary tables describing HBCDD biodegradation studies: “Larger decrease of HBCDD than in the viable test indicating transformation. However, it is possible that the $HgCl_2$ is by some way involved in this transformation.”

4.3.3. Total- and Diastereomer Specific HBCDD degradation rate constants observed in mesocosms

The order of biodegradation rate constants was determined by trying zero, first (pseudo) and second order equations. As data fitted best, rate of HBCDD decay in sediment mesocosms could explain by pseudo first order reaction kinetics. Total-HBCDD degradation rates and half-lives in mesocosm for each parallel of reactors were given in Table 4-6. Similar to microcosm reactors, it was observed that total-HBCDD decay rate in biostimulation set is calculated to be faster than that of the natural attenuation set. It can be said that rates calculated for parallel microcosms do not differ from each other much. By using average concentration of a and b parallel reactors, degradation rate and half-life were found as 0.048 day^{-1} and 14.4 days for natural attenuation, 0.157 day^{-1} and 4.4 days for biostimulation and 0.127 day^{-1} and 5.4 days for sterile mesocosms, respectively. The fact that sterile set outperformed natural attenuation in terms of total-HBCDD degradation rate was also reported previously (Davis et al. 2006).

Table 4-6 Total-HBCDD degradation rate constant and half-life in sediment mesocosms.

	Reactor Type					
Parameter	**NA[1]-a**	**NA[1]-b**	**BS[2]-a**	**BS[2]-b**	**ST[3]-a**	**ST[3]-b**
Rate constant(d^{-1})	0.067	0.042	0.145	0.170	0.128	0.127
Half-life (day)	10.28	10.20	4.77	4.09	5.42	5.45
R^2	0.78	0.80	0.95	0.99	0.99	0.94

[1]NA: Natural Attenuation [2]BS: Biostimulation [3]ST: Sterile

Natural attenuation degradation rate constant was 0.069 day^{-1} in microcosm reactors of this study, so there is no major difference between the total-HBCDD rate constant calculated for microcosm and that of the mesocosm. Also, for biostimulation microcosm reactors, which were prepared by adding organic medium with 50 mM sodium formate and ethanol, degradation rate constant was found as 0.221 day^{-1}, and in mesocosm biostimulation reactors, which were prepared by adding organic medium

with 10 mM sodium formate and ethanol, the degradation rate constant of total-HBCDD was found as 0.157 day^{-1}. Therefore, it can be said that the concentration of sodium formate and ethanol has an effect on the rate of degradation. The higher their concentration, the faster the degradation of HBCDD. A similar finding was also observed by Demirtepe (2017) during biodegradation of PBDEs in sediment microcosms and mesocosms.

The HBCDD decay rate constants in sediment mesocosms were also calculated for α-, β- and γ-HBCDD diastereomers and given in Table 4-7. For natural attenuation set, the degradation rates for α-, β- and γ-HBCDD were calculated as 0.024 day^{-1}, 0.161 day^{-1} and 0.138 day^{-1}, respectively. So, the order of degradation rate was found as beta > gamma > alpha HBCDD. In the literature, the same order was given by Davis et al. (2006) in anaerobic sediment microcosms. Also, for the biostimulation set, the degradation rate constants for α-, β- and γ-HBCDD were calculated as 0.053 day^{-1}, 0.248 day^{-1} and 0.160 day^{-1}, respectively. Hence the same degradation order with natural attenuation was observed for the biostimulation set. Moreover, as expected, the degradation rates of each diastereomer were increased (approximately doubled) in biostimulation set compared to the natural attenuation set. As mentioned in Section 2.3.1, in the literature generally, it was observed that alpha (α-) HBCDD has the slowest degradation in the solid media among three main diastereomers, and the results of this study also confirms this finding. If there is inter-transformation among diastereomers towards conversion into α-HBCDD, then such low degradation rate as well as low R^2 associated with this diastereomer, when compared to the others would be expected, which is the case here.

Table 4-7 Alpha (α-), beta (β-) and gamma (γ-) HBCDD degradation rates and half-lives in sediment mesocosms.

Diastereomer of HBCDD	Parameter	Reactor Type			
		NA-a	NA-b	BS-a	BS-b
α-	Rate constant (d^{-1})	0.034	0.016	0.054	0.051
	Half-life (day)	20.24	43.76	12.79	12.59
β-	Rate constant (d^{-1})	0.196	0.139	0.231	0.2655
	Half-life (day)	3.53	4.99	3.00	2.61
γ-	Rate (d^{-1})	0.153	0.126	0.149	0.174
	Half-life (day)	4.52	5.48	4.66	3.99

*NA: Natural Attenuation BS: Biostimulation ST: Sterile

4.4.Results of experiments regarding sterilization

As was discussed in microcosm and mesocosm set-up results, unexpected results were obtained in sterile sets, such that initial analyte concentration was measured to be much less than the prepared concentration and/or degradation of the analyte was observed through incubation time. Similar problems were discussed in the literature (Davis et al., 2006; Gerecke et al., 2006) as well as during studies in our laboratories. During Demirtepe's (2017) study, in the pre-set microcosm established to investigate general degradability of HBCDD via the *Dehalobium chlorocoercia* strain DF-1, an 89% removal was observed in the sterile set, which was prepared by only autoclaving the HBCDD spiked sediment microcosms (for three consecutive days). However, there, samples were extracted before autoclaving the sediments therefore the effect of sterilization on HBCDD concentration could not be observed. Later, Demirtepe (2017) established another set of HBCDD spiked sediment microcosms, but this time not only sterilized the microcosm via autoclaving, but also poisoned it using mercuric chloride. The assumption was that presence of coenzymes, e.g. Vitamin B12, might cause degradation of HBCDD in autoclaved sterile microcosms. In her study, she was able to successfully eliminate microbial activity and/or any reactivity from enzymes via this procedure. She was also able to measure much closer to the target concentration of HBCDD spike, i.e. target being 1000ppb vs the measured 800 ppb. Therefore, the same

procedure was adopted in this study. However, the results were not similar. In her study, Demirtepe (2017) used pure γ-HBCDD, but in this study mixture-HBCDD was used. Hence, it may be speculated that presence of other diastereomers, such as in the HBCDD mixture used in this study, the inter-transformation mechanism of HBCDD could be triggered.

The problem with sterilized reactors were therefore investigated further in this study. For this purpose, two experiments were carried out. The first is a sterilization control microcosm pre-set which aimed to investigate the effect of sterilization on initial HBCDD concentration, and the second is sterilization control microcosms, which aimed to investigate the change of HBCDD concentration with time following two different sterilization applications. Results are presented under corresponding sub-headings.

4.4.1. Sterilization control microcosms pre-set

This set of microcosms were prepared to understand the effect of sterilization steps on HBCDD initial concentration, since this was a repeating problem in microcosm and mesocosms. For this purpose, HBCDD spiked sediments were prepared as one batch and separated out into nine 20mL vials (the same ones previously used in microcosms). Three vials were left as is to represent natural attenuation. Mercuric chloride was added into six of them, where three were left as is, and three were autoclaved (for three consecutive days). The solid/liquid ratio and everything else were kept the same as the previous microcosm experiment. Degradation of HBCDD in time was not aimed to be investigated in this study. The results of this experiment are given in Figure 4-5.

Although the target concentration was 1000 ppb, it was observed that none of the microcosms reached that level. But interestingly, in the set with no sterilization and the one where sterilization was performed by only-mercuric addition, similar concentrations where obtained. On the other hand, as can be seen from the figure, in the microcosms where sterilization was performed by mercuric chloride addition and autoclaving, significantly lower concentrations were obtained.

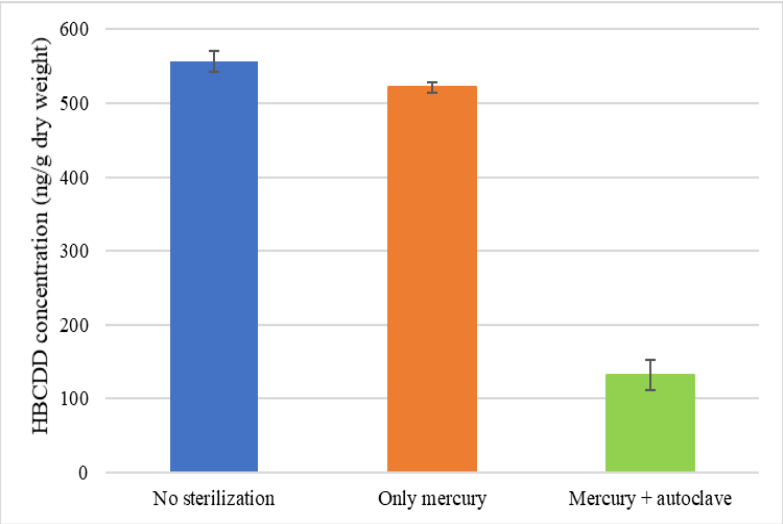


Figure 4-5 Results of sterilization control microcosms pre-set.

Since there is no notable difference between no-sterilization and only mercuric sterilization samples, mercuric chloride is decided not to cause degradation of total-HBCDD at initial time. However, Davis et al. (2016) applied only mercuric chloride to for their abiotic control sediment reactors and observed 60% degradation of HBCDD after 56 days of incubation. Therefore, use of only mercuric chloride for sterilization may not be preferable. When mercuric chloride and autoclave were applied to sediment samples together, however, the initial HBCDD concentration was observed to decrease to almost 15% of the target concentration. It can be speculated that autoclaving somehow induces some form of degradation of γ-HBCDD in the presence of mercuric. It was unfortunately not possible to identify any transformation products due to analytical limitations. Potential mechanisms could be debromination or replacement of bromines with chlorines in the dodecane structure since Cl^- is abundant in the sediments due to mercuric chloride addition. No explicit study could be found in the literature on the effect of sterilization on analyte degradation, regarding HBCDD or other hydrophobic organic chemical. A summary of relevant information obtained in this and Demirtepe's (2017) study regarding the effect of sterilization on initial HBCDD concentration is presented in Table 4-8 to aid in the evaluation of different factors affecting the results.

Table 4-8 Summary of relevant information on sterilization

Set Name	Measured HBCDD (ng/g) at t=0	Measured/target HBCDD	HBCDD Degradation (%)	Incubation Duration (day)	0.5 mg $HgCl_2$/g Added		$HgCl_2$ Mixing with sediment	Autoclaved
					over	exact		
Initial Microcosm (Demirtepe, 2017)	790	0.79	0	36		✓	x	✓
Microcosm	371.81±2.71	0.37	4.46	20	✓		x	✓
Mesocosm	546.11±39.85	0.55	95.05	49		✓	✓	✓
Sterilization control microcosm pre-set	523.39±4.23	0.52	-	-		✓	x	
	132.09±16.87	0.13	-	-		✓	x	✓
Sterilization control microcosm	123.05±26.71	0.10	28.10	21	-		-	✓
	437.17±34.83	0.36	17.32	21		✓	✓	✓

4.4.2. Degradation of HBCDD in Sterilization Experiment

The measurement of total-HBCDD was done both with GC-MS and LC-MS/MS, and for alpha (α-), beta (β-) and gamma (γ-) HBCDD only LC-MS/MS was used. According to GC-MS measurements, the initial concentrations of total-HBCDD were 727.29, 798.2 and 782.64 ng/g dry weight for a, b and c parallels of natural attenuation set, respectively, and 405.41, 420.43 and 485.67 ng/g dry weight for a, b and c parallels in mercury and autoclave set, respectively. However, in only autoclaved samples the initial concentrations were found much lower as 157.33, 92.18 and 119.65 ng/g dry weight for a, b and c parallels. The progression of total-HBCDD concentrations in each microcosm is presented as a function of time in Figure 4-6.

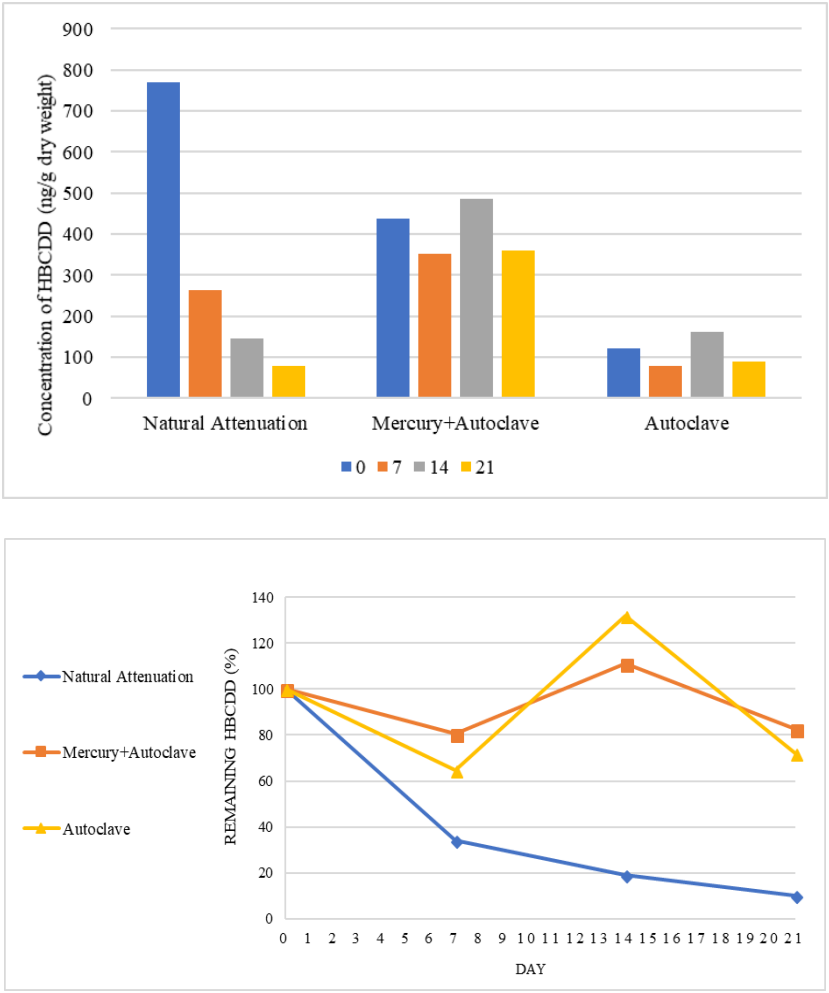
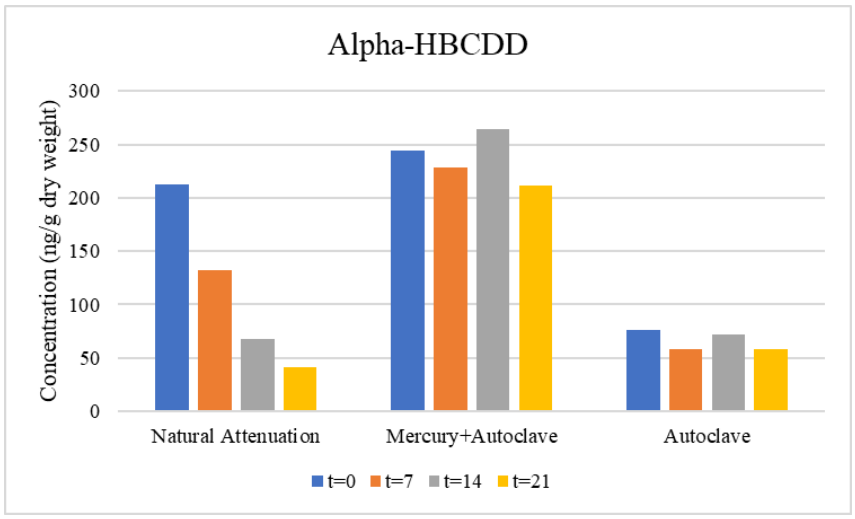
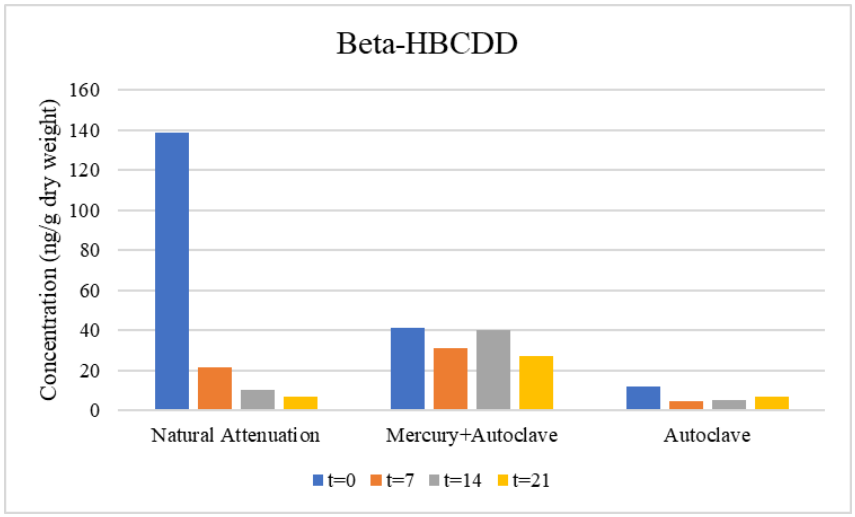


Figure 4-6 The time dependent a) HBCDD concentration changes b) HBCDD remaining.

As seen in Figure 4-6, the degradation in the natural attenuation set was found as 89.9% at the end of the 21-day incubation. The initial concentrations in mercuric chloride plus autoclaved set, and only autoclaved set were about 30% and 8% of the target concentration (i.e.1200ppb), respectively. Although this is the case, sterilization seems to be successful such that no degradation trend can be observed in these sets. On the other hand, non-sterilized set shows consistent decrease in HBCDD concentration with time. When this set is compared with the initial microcosm set discussed previously in Section 4.4 (i.e. Natural Attenuation), a better removal (90%) was observed in this set when compared to the previous (75%). Although every effort is made to keep consistent conditions in the laboratory in terms of extraction and analysis, such a variation was obtained. A possible explanation for this variation in removal percentage can be attributed to the sediment samples used in microcosms. The sediments were collected from the same pond at Çamkoru Natural Park, however, they belong to two different sampling times. The organic matter content and perhaps season might have had some impact on the sediments and/or microbial activity in the sediments.

The change of HBCDD with time, in terms of the diastereomer abundance is shown in Figure 4-7, and diastereomer portion of total-HBCDD given in Figure 4-8.As can be seen, there is a decreasing trend for all diastereoisomers of HBCDD in the no-sterilization microcosms, while no particular decreasing trend can be observed for sterilized microcosms. This is similar to the observations made regarding total-HBCDD concentrations.





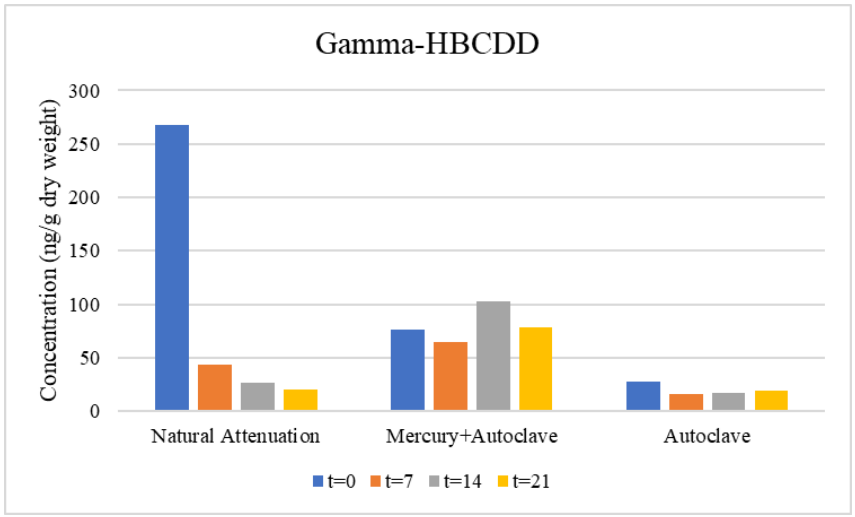


Figure 4-7 Diastereomer abundance in sterile control microcosms.

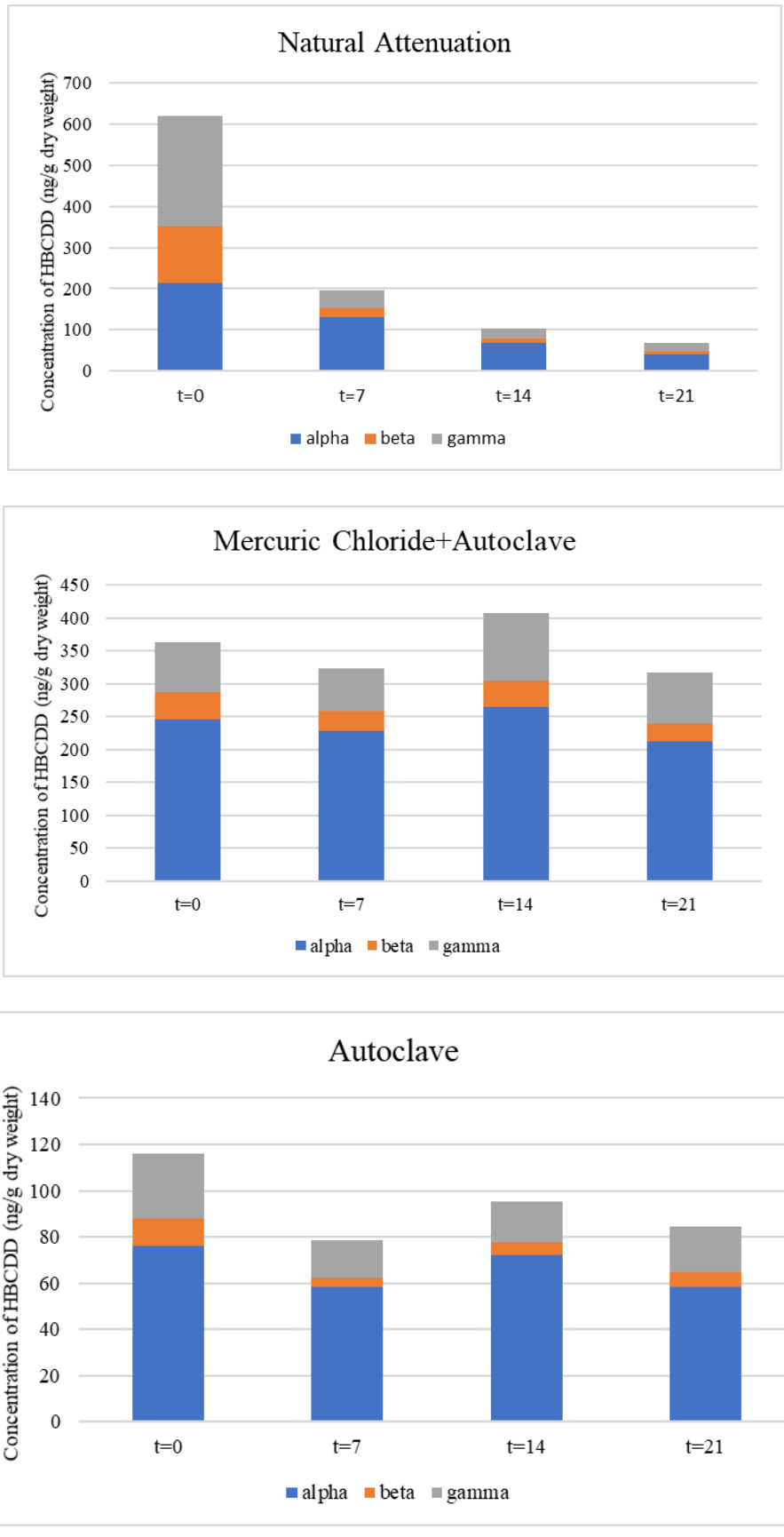


Figure 4-8 Diastereomer portion of total-HBCDD

No decreasing trend over time is observed for the sterilized sets. This indicate that HBCDD is initially transformed during sterilization (as a result of physicochemical changes taking place, perhaps as a result of high temperature and pressure environment of the autoclave, and/or presence of Hg and Cl^- in the medium), yet no impact on remaining HBCDD occur in terms of inducing further degradation/transformation. Microcosms with only mercuric chloride addition was not prepared because the aim was to explain the trends of sterile reactors observed in previous microcosms and mesocosms. Also, it was proven that mercuric chloride was not successful for abiotic control (Davis et al., 2006). In those experiments sterilization was performed via mercury poisoning and autoclaving, or only autoclaving in the case of the pre-set in Demirtepe's (2017) study.

Another depiction of the change of HBCDD with time, in terms of the diastereomer abundance is shown in Figure 4-9.

Figure 4-9 Percentage of HBCDD diastereomers in sterilization microcosms.

The diastereomer ratios for the total-HBCDD standard was reported to have, alpha (α) 21, beta (β-) 13%, while gamma to be 66% of the total, during sterilization control microcosms. The variation observed in the diasteromer ratios of the same total-HBCDD standard with mesocosm measurement, but calculations were made taking into account of this difference. Therefore, it is predicted that these variations will not affect the results.

Distribution of diastereomers in different sampling times are given in Figure 4-10. Firstly, the t=0 distribution of diastereomers show a distinct difference between the non-sterilized (i.e. natural attenuation) and sterilized (i.e. mercury+autoclave and autoclave) microcosms.

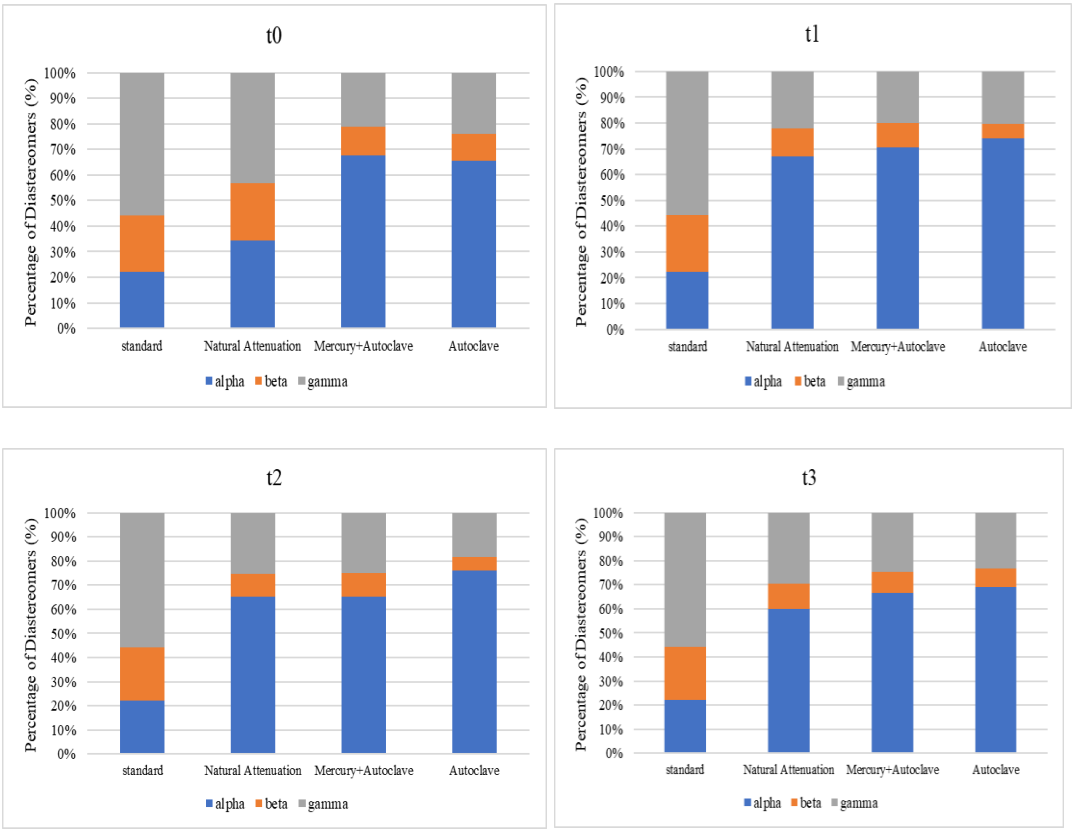


Figure 4-10 Distribution of diastereomers in different sampling times.

The percent of alpha (α-) diastereomer is observed to be 67% and 65% of the total in the sterilized microcosms (in mercury+autoclave and autoclave microcosms, respectively), whereas it was observed to be 34% for the non-sterilized microcosms. It is expected for the relative ratio of alpha (α-) diastereomer to increase in time. This is mainly because gamma (-γ) and beta (-β) diastereomers are amenable to degradation more easily when compared to their alpha- counterpart, which results in a relative increase in abundance of alpha-. Another reason is the potential of other diastereomers to be transformed into alpha- through the course of incubation. i.e. as biodegradation takes place. This is because gamma (γ-) and beta (β-) diastereomers are amenable to degradation more easily when compared to their alpha (α-) counterpart, which results in a relative increase in abundance of alpha-. However, yet, this shift in the stereomer ratios only due to sterilization was never reported before.

Secondly, the relative ratio of diastereomers change relative to time in non-sterilized microcosms (i.e. a change being greater than the variation observed in LC-MSMS measurements of the standard), whereas they stay within the analytical variation (Figure 2-b and c) in both types of sterilized sets throughout the operation of the microcosms (i.e. 21 days). These findings all indicate that any degradation that is caused by sterilization (whether via mercuric chloride or autoclaving or both) happens at t=0 with no further change being observed in time observable after that time. This supports the hypothesis that high temperature and pressure environment of the autoclave, in the presence or absence of mercuric chloride, results in physicochemical changes/shifts in the diastereomer ratios of HBCDD as well as total concentration of HBCDD, with no further change happening throughout the rest of the incubation time.

Any abiotic degradation of HBCDD in sediment microcosms was ruled out in the conditions of this laboratory during Demirtepe's (2017) study. She investigated HBCDD degradation in microcosms set-up using kaolinite spiked with HBCDD as the solid medium, instead of sediments, without application of sterilization. No degradation was observed, indicating that abiotic degradation of HBCDD is not expected in microcosms.

In microcosms, mercuric chloride mixing was done to sediment by only applying vortex. However, vortex was not able for mesocosm, so mercuric chloride was added to sediment with HBCDD and mixed together for 45 minutes. After observing

unexpected degradation in mesocosms, in sterilization control microcosms, mixing with HBCDD was also applied. By not observing a notable degradation trend in these microcosms, it was proven that mixing is not causing the degradation of HBCDD.

4.4.3. HBCDD Degradation Rate Constants in Sterilization Control Microcosms

As discussed previously, no decreasing trends in time were observed in sterilized microcosms, hence no rate constants were calculated for those. The degradation rate constants for HBCDD degradation in the non-sterilized sterile control microcosm was was found as 0.109±0.011 day^{-1} (as average ± standard deviation of triplicate microcosms). Half-life of total-HBCDD was calculated as 6.36±1.71 days. The degradation rate of the non-sterilized (i.e. natural attenuation) set in this sterile control experiment was the highest rate among all-natural attenuation sets of this study, i.e. initial microcosms and mesocosms. Although not very different, the concentration in these microcosms were higher than those of the microcosm and mesocosm study (i.e. 650 ppb vs. 750 ppb) presented in the previous sections. Yet this contradicts with Davis et al.'s (2006) comment regarding degradation rate increasing as concentrations decrease – although orders of magnitude differences were present in that comparison with Gerecke et al.'s (2006) study.

HBCDD decay rates in natural attenuation were also calculated for alpha (α-), beta (β-) and gamma (γ-) HBCDD diastereomers. For natural attenuationnon-sterilization set, the degradation rates for alpha (α-), beta (β-) and gamma (γ-) HBCDD were calculated as 0.0784 day-1, 0.1420 day-1 and 0.1231 day-1, respectively. So, the order of degradation rates was found as beta (β-) HBCDD > gamma (γ-) HBCDD > alpha (α-) HBCDD, and this is the same with the order found for mesocosms and given in study of Davis and colleagues (2006) for anaerobic sediment microcosm. Also, half-lives of alpha (α-), beta (β-) and gamma (γ-) HBCDD were calculated as 8.84, 4.88 and 5.63 days, respectively. Summary of degradation rates and half-lives of sterile experiment are given in Table 4-9.

Table 4-9 Summary of degradation rate constants and half-lives in sterilization control microcosms.

Name	Degradation Rate (day^{-1})	Half-life (day)
Total-HBCDD	0.109	6.36
alpha (α-) HBCDD	0.078	8.84
beta (β-) HBCDD	0.142	4.88
gamma (γ-) HBCDD	0.123	5.63

4.4.3. Overall Evaluation and Comparison of Degradation Rates

Sterilization control microcosms revealed that an initial HBCDD degradation takes place during autoclaving and mercuric chloride addition. Yet, sterilization can be sustained such that no further degradation took place. However, this was not the case for mesocosms, due to the nature of operation of these large-scale sediment reactors. Mesocosm results show total-HBCDD at measurable levels until 21 days incubation, while diastereomers are measurable until only 7 days of incubation. The rates observed in mesocosms hence may be as a result of anaerobic activity as well as possibly aerobic activity.

Summary results of this study and relevant literature studies are given in Table 4-10. As seen in the table, range for HBCDD degradation and half-lives is very large. Different factors, such as initial concentration of HBCDD, media type and conditions, can cause such a variation in results.

Table 4-10 Summary results of this study and studies in literature.

Media & Conditions	Isomer	Initial HBCDD concentration (ng/g)	k (day^{-1})	$t_{1/2}$ (day)	R^2	Reference
Lake sediment microcosms without any substrates	Total	701.70	0.069	10.1	0.86	This study
Lake sediment microcosms with organic medium (50 mM sodium formate+ethanol)	Total	941.94	0.221	3.1	0.99	
Lake sediment mesocosms without any substrates	Total α β γ	684.72±90.65 133.42±22.09 190.21±32.18 551.26±132.41	0.048 0.024 0.161 0.138	14.4 28.6 4.3 5.0	0.84 0.02 0.84 0.97	
Lake sediment mesocosms with organic medium (10 mM sodium formate+ethanol)	Total α β γ	759.17±72.67 168.58±27.33 169.56±11.39 800.83±178.28	0.157 0.053 0.247 0.160	4.4 13.2 2.8 4.3	0.97 0.21 0.94 0.99	
Lake sediment microcosms without any substrates (sterile control microcosms)	Total α β γ	769.38±30.43 212.75±14.62 138.67±13.14 268.03±7.57	0.109 0.078 0.142 0.123	6.36 8.84 4.88 5.63	0.97 0.99 0.88 0.84	
Lake sediment microcosms without any substrates	γ	~810	0.015	44.7	0.71	(Demirtepe,2017)
Lake sediment microcosms with organic medium (sodium formate+ethanol)	γ	~820	0.054	12.8	0.96	
Lake sediment microcosms with *Dehalobium chlorocoercia* strain DF-1	γ	~860	0.012	56.3	0.96	

Table 4-10 Summary results this study and studies in literature. (continued)

Media & Conditions	Isomer	Initial HBCDD concentration (ng/g)	k (day^{-1})	$t_{1/2}$ (day)	R^2	Reference
Anaerobic soil microcosms with activated sludge simulating land application of sludge	Total	30-40	0.100[a]	6.9	-	(Davis et al., 2005)
River sediment microcosms without any substrates	Total	30-40	0.450[a]	1.5	-	
Creek sediment microcosms without any substrates	Total	30-40	0.610[a]	1.1	-	
Digester sludge microcosms with mineral salts medium	Total α β γ	~5650	0.128 0.133 0.152 0.125	5.4 5.2 4.6 5.6	-	(Davis et al., 2006)
Freshwater sediment microcosms without any substrates	Total α β γ	~5650	0.011 0.006 0.014 0.011	63.6 115.5 49.5 61.3	-	
Digester sludge microcosms with nutrients (starch+yeast) and primers	Total	60	1.1	0.66	-	(Gerecke et al., 2006)

[a]Calculated from the half-lives given.

CHAPTER 5

CONCLUSIONS

Anaerobic biodegradation of HBCDD was investigated in sediments under various conditions to evaluate potential application of bioremediation alternatives, which are natural attenuation and biostimulation. Investigation of HBCDD anaerobic biodegradation is done in two scale laboratory reactors: microcosm and mesocosms. In the literature, HBCDD degradation studies in the microcosm scale are available. However, to the best of our knowledge, mesocosm reactors in this study are the largest scale reactors used to investigate HBCDD degradation in the literature.

Degradation studies are important for devising bioremediation strategies for HBCDD contaminated areas. Degradation of HBCDD was investigated under laboratory conditions, therefore, it should be considered that the degradation rate of HBCDD could decrease under environmental conditions.

Main conclusions of the study can be listed below:

1) Anaerobic degradation rate of HBCDD can be increased by biostimulation, i.e. addition of an organic medium rich with a carbon source and electron donor. The observed degradation rate constants for biostimulation were more than three times that of the natural attenuation, as observed in sediment microcosms. The observed rates decreased in mesocosms, when compared to the microcosms. However, the conclusion remained the same, such that the biodegradation rate of HBCDD observed in biostimulation mesocosms was more than twice that of the natural attenuation mesocosms.
2) The scale of the sediment reactor may have an impact on the anaerobic degradation of HBCDD in sediments. The rate constant for microcosm and

mesocosm was similar in natural attenuation sets (i.e.0.048 day^{-1}), however it was much higher (i.e. 0.109 day^{-1}) in the sterile control microcosm. These results were in a way consistent with the wide variation of degradation rate constants reported in the literature.

3) Degradation of HBCDD for main diastereomers, which are α-, β-and γ-HBCDD, was studied and α-HBCDD showed the lowest degradation rate, while β-HBCDD had the highest degradation rate. The degradation order of diastereomer degradation was found as β-HBCDD > γ-HBCDD > α-HBCDD and this is the same with the order found for mesocosms and given in study of Davis and colleagues (2006) for anaerobic sediment microcosm. Also, half-lives of alpha (α-), beta (β-) and gamma (γ-) HBCDD were calculated as 8.84, 4.88 and 5.63 days, respectively.
4) In this study, for the first time – to the best of our knowledge - transformation of one diastereomer to another was observed in sediments during anaerobic degradation. Alpha-HBCDD concentration was observed to fluctuate through incubation time.
5) Degradation was observed in sterilized mesocosms. Since the same sterilization method was successful in both microcosms, this degradation was explained by a breach in sterilization due to sampling from large scale sediment reactors exposed to the atmosphere.
6) Sterilization was shown to have an impact on total-HBCDD concentration, as well as the relative ratio of diastereomers. Sterilization control microcosms were set-up to further understand the reason behind this. Firstly, it is revealed that only adding mercuric chloride into the reactors for sterilization have no significant effect on total-HBCDD concentration. Then, sterilization control microcosms were prepared to test mercuric chloride added and autoclaved and only autoclaved effect on the degradation and sterilization of the HBCDD. Although, no degradation trend was observed throughout the incubation period, HBCDD was observed to degrade significantly (30% to 8% of the target concentration) at the initial time. Diastereomer ratio was observed to shift significantly as a result of sterilization but stay constant throughout the incubation time.

CHAPTER 6

RECOMMENDATIONS

HBCDD is being one of the POPs, degradation and fate of HBCDD in the environment is important, so to increase the knowledge in this area and improvement of the literature is crucial. For future studies the itemized recommendation in the below can be taken into consideration.

- Examination of degradation of HBCDD at different concentrations, and observing the effect of concentration on the degradation rate
- Examination of degradation of HBCDD main diastereomers individually for determining the rate of degradation each of them and making comparison with standard mixture degradation rates
- Making mass balance and modelling of HBCDD and its main diastereomers for the contaminated areas of the environment
- Further study on the abiotic control of HBCDD in the large scale (mesocosm) reactors for achievement of sterilization in the laboratory control reactors
- Isolation of microorganisms for degradation of HBCDD and remediation

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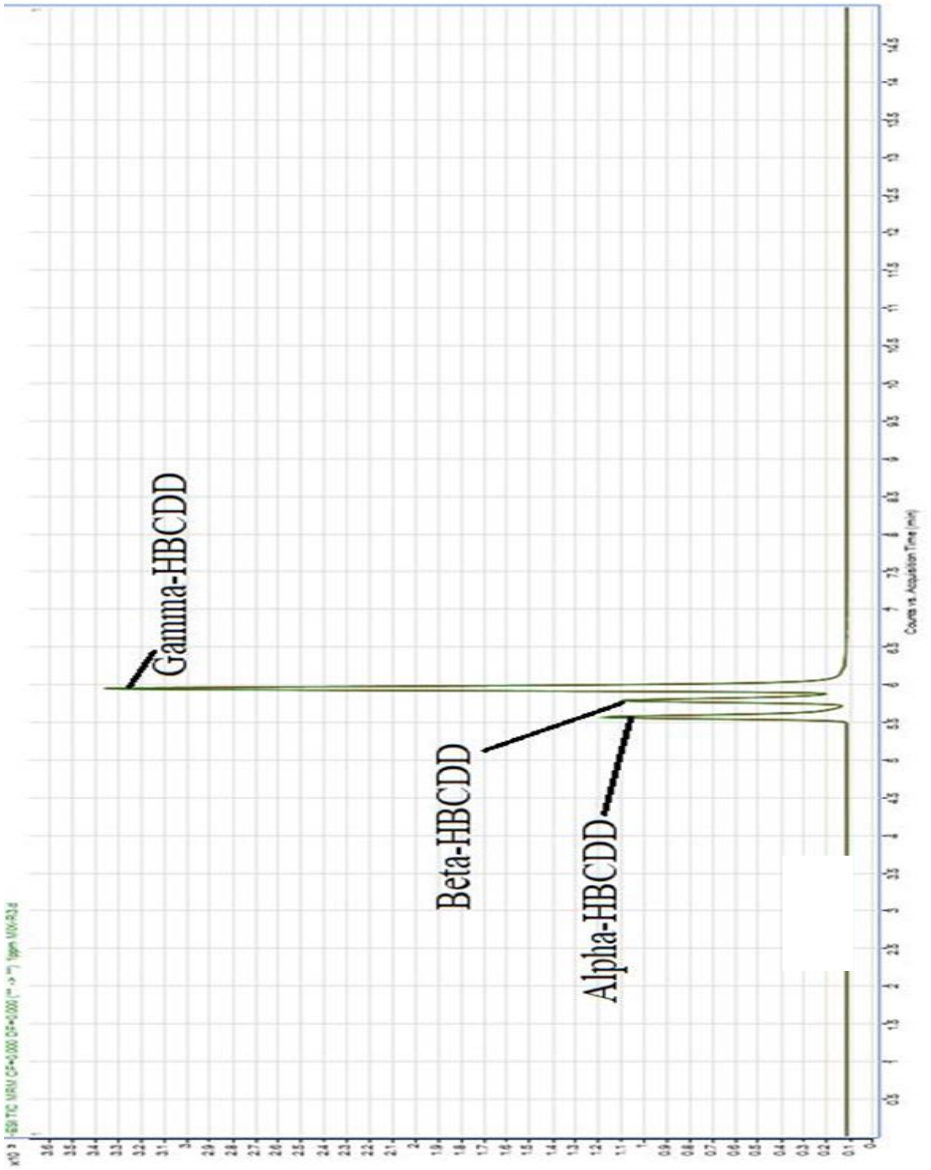
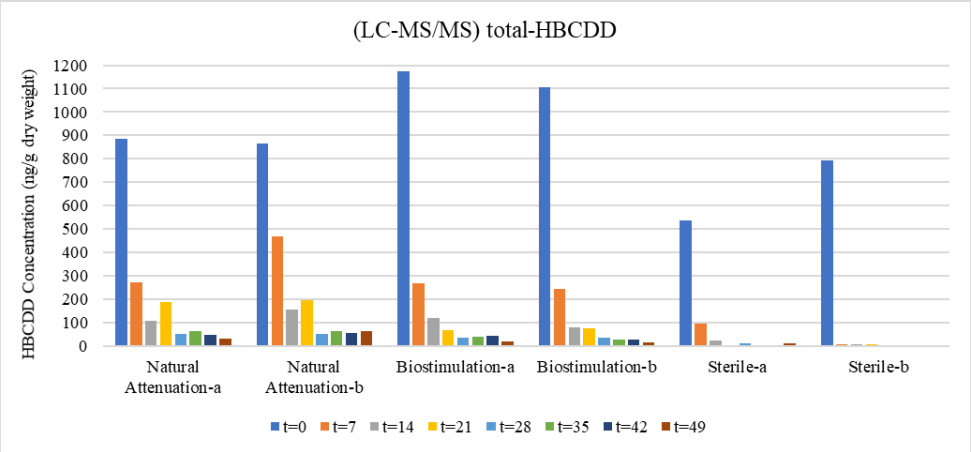
APPENDIX A

LC-MS/MS CHROMATOGRAM OF TOTAL-HBCDD



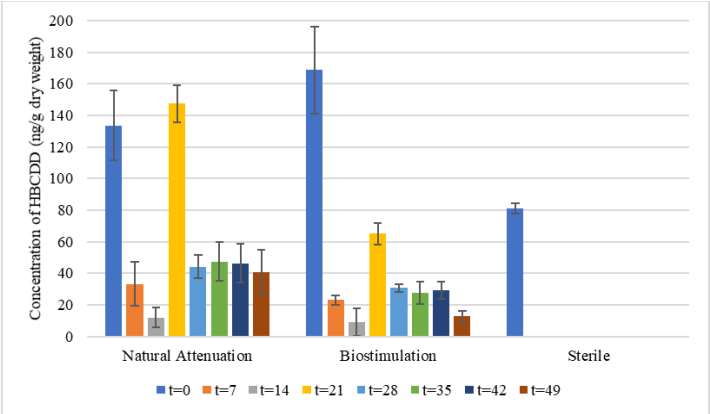
APPENDIX B

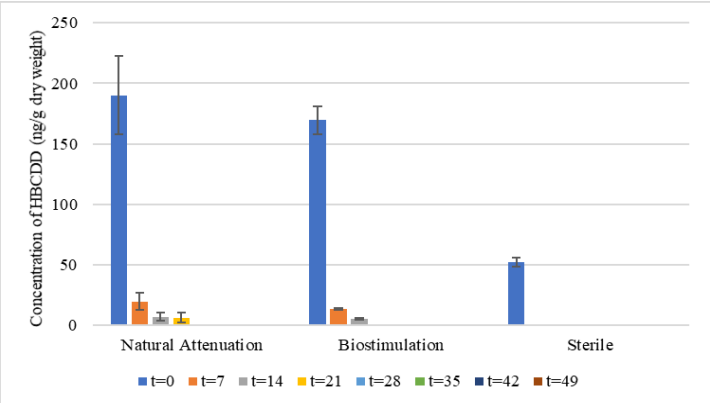
LC-MS/MS TOTAL HBCDD RESULTS

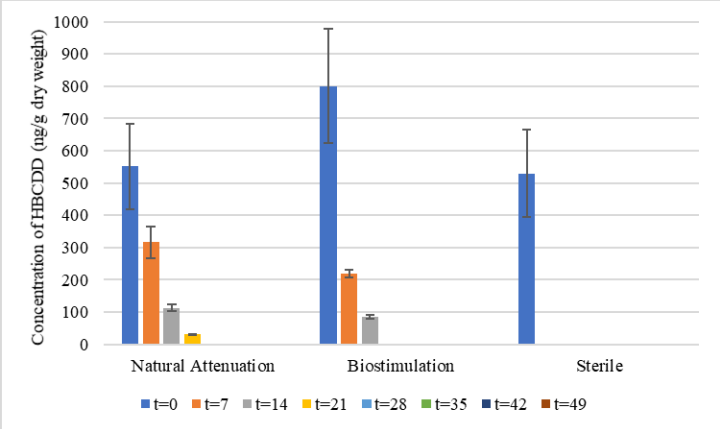


APPENDIX C

AVERAGE ALPHA , BETA, AND GAMMA HBCDD CONCENTRATIONS IN MESOCOSM SETS

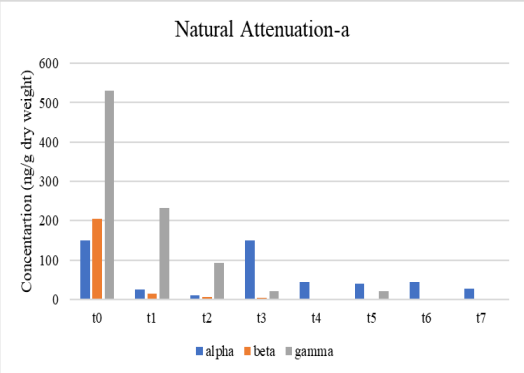


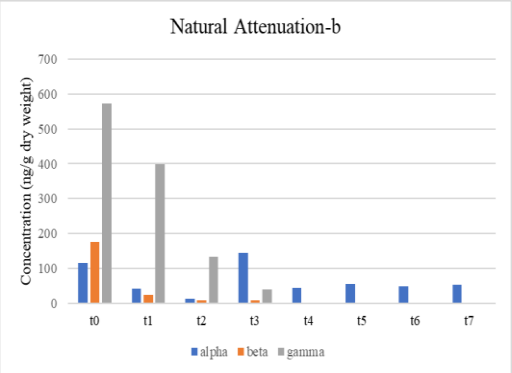


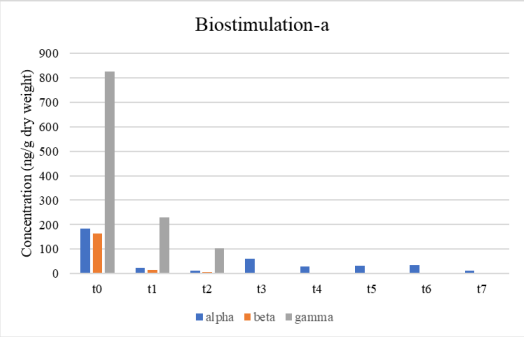


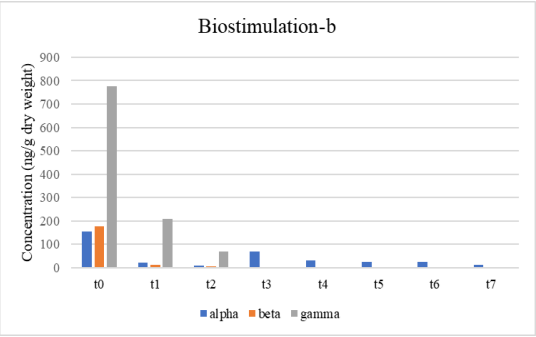
APPENDIX D

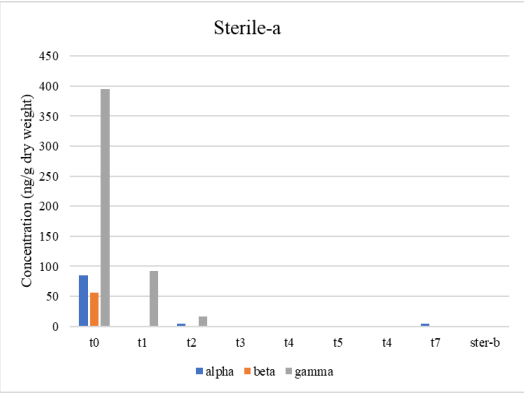
PORTION OF DIASTEREOMER OF HBCDD IN MESOCOSMS

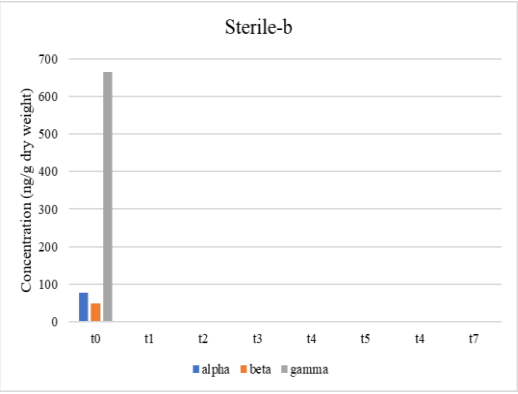












APPENDIX E

PERCENTAGE CHANGES OF DIASTEREOMERS IN MESOCOSMS