Polybrominated dibenzo-\(p\)-dioxins –
Natural formation mechanisms and biota retention, maternal transfer, and effects

Kristina Arnoldsson
Kristina Arnnoldsson received her B.Sc. in Chemistry from Stockholm University. She began her graduate studies in Environmental Chemistry at the Department of Chemistry, Umeå University in 2007.

Polybrominated dibenzo-\(p\)-dioxins (PBDD) and dibenzofurans (PBDF) are a group of compounds of emerging interest as potential environmental stressors. Their structures as well as toxic responses are similar to the highly characterized toxicants polychlorinated dibenzo-\(p\)-dioxins. High levels of PBDDs have been found in algae, shellfish and fish, even from remote areas in the Baltic Sea. The geographical and temporal variations of PBDD in biota samples suggests natural rather than anthropogenic origins.

The work underlying this thesis investigated the retention and transfer behavior, as well as health and reproductive effects, of PBDD/Fs in fish, to further increase the knowledge on persistency, retention and effects of PBDD/Fs, specifically concerning influence of substitution pattern and physico-chemical properties. In addition, biotic and abiotic formation of PBDDs from naturally abundant phenolic precursors were explored, to evaluate whether the PBDD profiles found in Baltic Sea biota can be explained by natural formation processes.
Polybrominated dibenzo-\(p\)-dioxins –
Natural formation mechanisms and biota retention, maternal transfer, and effects

Kristina Arnoldsson

Akademisk avhandling

som med vederbörligt tillstånd av Rektor vid Umeå universitet för avläggande av filosofie doktorsexamen framläggs till offentligt förvar i hörsal KB3B1, KBC-huset, fredagen den 3 februari, kl. 10:00. Avhandlingen kommer att förvaras på engelska.

Fakultetsopponent: Professor Walter Vetter,
Institute of Food Chemistry, University of Hohenheim,
Stuttgart, Tyskland.
Polybrominated dibenzo-p-dioxins (PBDD) and dibenzofurans (PBDF) are a group of compounds of emerging interest as potential environmental stressors. Their structures as well as toxic responses are similar to the highly characterized toxicants polychlorinated dibenzo-p-dioxins. High levels of PBDDs have been found in algae, shellfish, and fish, also from remote areas in the Baltic Sea. This thesis presents studies on PBDD behavior in fish and offspring, and natural formation of PBDDs from naturally abundant phenolic precursors.

The uptake, elimination, and maternal transfer of mono- to tetraBDD/Fs were investigated in an exposure study reported in Paper I. The effects of PBDDs in fish were examined in a dose-response study (Paper II). It was shown that fish can assimilate PBDD/Fs from their feed, although non-laterally substituted congeners were rapidly eliminated. Laterally substituted congeners were retained as was congeners without vicinal hydrogens to some extent. PBDD/Fs were transferred to eggs, and congeners that were rapidly eliminated in fish showed a higher transfer ratio to eggs. Exposure to the laterally substituted 2,3,7,8-TeBDD had significant effects on the health, gene expression and several reproduction end-points of zebrafish, even at the lowest dose applied.

The geographical and temporal variations of PBDD in biota samples from the Baltic Sea suggest biogenic rather than anthropogenic origin. In Paper III, bromoperoxidase-mediated coupling of 2,4,6-tribromophenol yielded several PBDD congeners, some formed after rearrangement. The overall yield was low, but significantly higher at low temperature, and the product profile obtained was similar to congener profiles found in biota from the Swedish West Coast. In Paper IV, photochemically induced cyclization of hydroxylated polybrominated diphenyl ethers under natural conditions produced PBDDs at percentage yield. Rearranged products were not detected, and some abundant congeners do not seem to be formed this way. However, the product profile obtained was similar to congener profiles found in biota from the Baltic Proper.

Since the PBDD congeners found in biota have a high turn-over in fish, the exposure must be high and continuous to yield the PBDD levels measured in wild fish. Thus, PBDDs must presumably be formed by common precursors in general processes, such as via enzymatic oxidations, UV-initiated reactions or a combination of both. The presented pathways for formation of PBDDs are both likely sensitive to changes in climatic conditions.
Polybrominated dibenzo-\textit{p}-dioxins –
Natural formation mechanisms and biota retention, maternal transfer, and effects

Kristina Arnoldsson
Till Joel och Linnea
– ni gör livet och arbetet värt!
Abstract

Polybrominated dibenzo-\textit{p}-dioxins (PBDD) and dibenzofurans (PBDF) are a group of compounds of emerging interest as potential environmental stressors. Their structures as well as toxic responses are similar to the highly characterized toxicants polychlorinated dibenzo-\textit{p}-dioxins. High levels of PBDDs have been found in algae, shellfish, and fish, also from remote areas in the Baltic Sea. This thesis presents studies on PBDD behavior in fish and offspring, and natural formation of PBDDs from naturally abundant phenolic precursors.

The uptake, elimination, and maternal transfer of mono- to tetraBDD/Fs were investigated in an exposure study reported in \textbf{Paper I}. The effects of PBDDs in fish were examined in a dose-response study (\textbf{Paper II}). It was shown that fish can assimilate PBDD/Fs from their feed, although non-laterally substituted congeners were rapidly eliminated. Laterally substituted congeners were retained, as was congeners without vicinal hydrogens to some extent. PBDD/Fs were transferred to eggs, and congeners that were rapidly eliminated in fish showed a higher transfer ratio to eggs. Exposure to the laterally substituted 2,3,7,8-TeBDD had significant effects on the health, gene expressions and several reproduction end-points of zebrafish, even at the lowest dose applied.

The geographical and temporal variations of PBDD in biota samples from the Baltic Sea suggest biogenic rather than anthropogenic origin. In \textbf{Paper III}, bromoperoxidase-mediated coupling of 2,4,6-tribromophenol yielded several PBDD congeners, some formed after rearrangement. The overall yield was low, but significantly higher at low temperature, and the product profile obtained was similar to congener profiles found in biota from the Swedish West Coast. In \textbf{Paper IV}, photochemically induced cyclization of hydroxylated polybrominated diphenyl ethers under natural conditions produced PBDDs at percentage yield. Rearranged products were not detected, and some abundant congeners do not seem to be formed this way. However, the product profile obtained was similar to congener profiles found in biota from the Baltic Proper.

Since the PBDD congeners found in biota have a high turn-over in fish, the exposure must be high and continuous to yield the PBDD levels measured in wild fish. Thus, PBDDs must presumably be formed by common precursors in general processes, such as enzymatic oxidations, UV-initiated reactions or a combination of both. The presented pathways for formation of PBDDs are both likely sensitive to changes in climatic conditions.
Sammanfattning (summary in Swedish)

Bakgrund
Östersjön är ett av jordens största brackvattenhav, med mycket speciell miljö, men det är också en av de mest förorenade havsmiljöerna i världen. Ett av de allvarligaste miljögiften i Östersjön, som egentligen består av en grupp ämnen, är polyklorerade dibenso-p-dioxiner och dibensofuranner (PCDD/Fs). På senare tid har man hittat ämnen med liknande struktur och kemiska egenskaper; polybromerade dibenso-p-dioxiner (PBDDs), i bl.a. fisk och musslor från Östersjön. Halterna är högst i Egentliga Östersjön, framförallt vid kusten och i samma nivå som halterna av PCDD/Fs. I mussla har hittats 4 ng PBDDs/g färskvikt från en opåverkad lokal. Den toxiska mekanismen är sammarr för PCDD/Fs och PBDD/Fs och de ger samma toxiska effekter. Därfor är det viktigt ur risksynpunkt att öka kunskapen om PBDD/Fs för att utröna om de kommer att ha en påverkan på miljö och/eller hälsa.

Den geografiska spridningen av PBDDs tyder på att källorna är lokala i motsats till PCDD/Fs, och en hypotes är att PBDDs är naturligt producerade. En bildning av bromerade dioxiner skulle kunna ske genom reaktioner med enklare byggstenar (precursorer). Föreslagna precursorer är bromfenoler och bromerade hydroxy-difenyletrar, som båge har hittats i marin miljö.

Målet med avhandlingsarbetet har varit att öka kunskapen om PBDD/Fs främst inom två områden: upptag av PBDD/Fs i fisk från föda samt bildning av PBDD/Fs från enklare molekyler under naturliga förhållanden. Jag hoppas kunna svara på följande frågor:

Hur mycket PBDD/Fs tas upp i fisk från föda och vad händer med PBDD/Fs i fisken?

Kan PBDD/Fs bildas naturligt - enzymatiskt i organismer och/eller foto-kemiskt med hjälp av UV-ljus från solen?

Egenskaper hos bromerade dioxiner och relaterade ämnen
PBDD/Fs är uppbyggda med samma typ av kolskelett som PCDD/Fs men är substituterade med brom i stället för klor. Hur många brom, och i vilka positioner på molekylen de sitter (kongener), avgör vilka egenskaper den får, och även toxicitet. Liksom PCDD/Fs är PBDD/Fs mycket lipofila, d.v.s. de fördelas sig i högre grad till fet t än till vatten, vilket gör att de har möjlighet att tas upp av organismer och lagras i fett. De föreslagna precursorerna (bromfenoler och bromerade hydroxy-difenyletrer) är också lipofila, men hydroxy-gruppen gör att de kommer att vara deprotonerade vid Östersjöns pH och därigenom vara mer vattenlösliga.
Förekomsten av dioxiner i naturen är låg, men eftersom toxiciteten är väldigt hög för vissa kongener är ändå betydelsen av dessa ämnen stor.
Hittills vet man bara toxiciteten för några få kongener av PBDD/Fs, därför är det svårt att veta vilken påverkan de har, med de halter som finns i Östersjön.

**Upptag och eliminering av PBDD/Fs**

Vilka ämnen som tas upp i fisk och till vilken grad bestäms i första hand av ämnenas förmåga att fördela sig mellan vatten och fett (lipofilicitet). I studierna som redovisas här, visas att också vattenlöslicheten för ämnena har betydelse för upptaget. De ämnen som tagits upp i fisk kan elimineras på olika vägar, t.ex. genom metabolism. Andra vägar är genom gälar och avföring. En del av ämnet kan överföras från fiskens kropp till ägg under rombildningen, "maternal transfer", och hamnar då i fiskembryot.

I de presenterade studierna exponerades zebrafisk för mono- till tetra-bromerade PBDD/Fs tillsatt i fodret. Det visas att PBDD/Fs kan tas upp och bli kvar i fisken, och överföras till ägg. De kongener som är substituerade med brom i de yttre, laterala (2,3,7,8-), positionerna har högst retention, d.v.s. blir kvar längst. Högst retention har 2,3,7,8-tetabrormerad dioxin (2,3,7,8-TeBDD). Kongener med lägre bromeringsgrad, och med närliggande (vicinala) osubstituerade positioner har högre elimineringar, troligen pga metabolism. Ämnen som har låg vattenlösighet hade lägre upptag, förmodligen pga lägre biotillgänglighet. Alla kongener med retention i fisken förs över till ägg. Vid högre doser är överföringen till ägg relativt sett större än vid lägre doser. Det kan bero på att fisken har en högre grad av metabolism vid högre doser. Reproduktionseffekter på fisk och ägg kunde ses i de grupper som fått 2,3,7,8-TeBDD.

**Bildning av PBDD/Fs**

Många alger och andra marina organismer producerar bromerade föreningar. Några är tänkbara precursorer för att bilda PBDD/Fs. I det här arbetet har två vägar för att bilda PBDD/Fs undersömts; dels en biotisk med enzymatisk koppling av bromfenoler och dels en abiotisk med fotokemisk inducerad koppling av polybromerade hydroxy-difenyletrar (OH-PBDE).

I den enzymatiska bildningen användes bromperoxidas från en rödalg, *Corallina officinalis*, och 2,4,6-tribromfenol (TrBP) som substrat. TrBP produceras av alger i relativt höga halter. I studien visas att det bildades PBDD, men inte PBDF, i låga halter (nmol/mol TrBP), och att bildningsvägarna var dels direkt kondensation och dels omlagringsprodukter. Halten av bildade dioxiner var ungefär lika hög vid olika pH (pH 5.5–7.5) men högre vid 4°C än vid rumstemperatur. Alla bildade PBDD kongener har hittats i biologiska prover från Östersjön. Mönstret av PBDD i enzyminkubationen liknade det som återfinns i mussla på Västkusten.

Vid de abiotiska bildningsförsöken användes UV-Ljus för att inducera fotokemisk cyclisering av OH-PBDE. Vanligt förekommande OH-PBDE löstes i vatten, med samma salthalt och pH som i Östersjön, och belystes
med antingen artificiellt UV-ljus eller solljus. Alla kongener cykliserade till den förväntade PBDDn, inga omlagringsprodukter kunde detekteras, men bland produkterna återfanns många debromerade kongener. Utbytet var relativt högt (procent). I studien visades också att humushalten i vattnet till viss del påverkade utbytet av PBDD för vissa kongener. Mönstret av PBDD i de fotokemiska försöken liknade det som återfinns i mussla i Egentliga Östersjön och från samma lokaler har man också hittat höga halter av OH-PBDE i mussla och alger.

**Diskussion**

Förekomsten av PBDD/Fs i Östersjön är förvånansvärt hög, i vissa fall i nivå med PCDD/Fs. Generellt är förekomsten av di- och tribromerade dioxiner högst och mönstret av kongener är likartat mellan sediment, alger, mussla och fisk, vilket tyder på att substanserna transporteras i näringskedjan. Eftersom omsättningen av flera kongener är hög enligt försöken, då de metaboliseras lätt i fisk, måste exponeringen för dessa ämnen vara hög för att ge de halter som man kan återfinna. Bildningen av dessa ämnen bör därför också vara hög och kontinuerlig. Även om dessa föreningar bildas naturligt kan de, eftersom de har liknande toiska egenskaper som PCDD/Fs, påverka organismer genom den samlade belastningen som uppstår. Dessutom kan den kemiska belastningen öka högre upp i näringskedjan. För närvarande finns inga belägg för att de PBDD/Fs som återfinns i Östersjön överförs till människa.

De undersökta processerna för bildning av PBDD/Fs är båge känsliga för klimatrelaterade förändringar. Det framtida klimatet för Östersjön förutsågs ge ökad temperatur och ökad nederbörd. Ändringar i temperaturen och salthalt kommer att påverka artsammansättningen, speciellt som många arter i Östersjön lever nära sin toleransnivå för salthalt. En förändrad artsammansättning kan ge en ändrad produktion av precursorer och PBDD. Även förändringar i solinstrålning skulle kunna ändra produktionen.

Baserat på de presenterade resultaten föreslås några framtida studier:

- **Exponeringsvägar:** Analys av PBDD kongener och nivåer i vatten från Östersjön kan avgöra om exponeringen sker via vatten eller föda för, i första hand, fisk. Analys av sediment kan belysa om det kan vara en källa för exponering av t.ex. mussla.

- **Bildning:** Undersökning av halter av precursorer kan avgöra om och hur det finns en koppling mellan biotisk och abiotisk bildning av PBDD. Ytterligare studier av andra peroxidaser och bromfenoler kan eventuellt visa på flera produkter.

- **Riskvärdering:** Studier av de kongener som ännu saknar toxisk utvärdering skulle ge en bättre uppskattning av risknivån. Hur PBDD transportereras i näringsväven och om exponering kan ske för människor, kan studeras med analys av arter på olika trofisk nivå.
# Table of Contents

Abstract i
Sammanfattning (summary in Swedish) ii
Table of Contents v
List of papers vi
Abbreviations and definitions vii

1 Background 1
   1.1 The Baltic Sea 1
   1.2 Levels of PBDD/Fs in Baltic biota 2
   1.3 Aims of the studies 5

2 PBDDs – their properties, precursors, effects, and analysis 7
   2.1 Structure and physico-chemical properties 7
   2.2 Possible precursors of PBDD/Fs 10
   2.3 Biological effects of PBDD/Fs 13
   2.4 Analytical aspects 17

3 Dietary exposure studies of PBDD/Fs in zebrafish 19
   3.1 Dietary uptake, retention and maternal transfer of PBDD/Fs – exposure study Paper I 22
   3.2 Effects of PBDDs in zebrafish – dose-response study Paper II 26
   3.3 Uptake and transfer – discussion related to properties of PBDD/Fs and PCDD/Fs 30
   3.4 Retention and effects — comparison with Baltic Sea data 32

4 Biotic and abiotic formation of PBDD/Fs 35
   4.1 Bromoperoxidase-mediated formation of PBDD/Fs Paper III 35
   4.2 Photochemical formation of PBDD/Fs Paper IV 39
   4.3 Congener profiles 43

5 Exposure situation and scenarios 45
   5.1 Present PBDD/F exposure in the Baltic Sea 45
   5.2 Future scenarios with a changing climate 46

6 Conclusions and future perspectives 49

Acknowledgements 51
References 53
Electronic sources and programs 65
List of papers

This thesis is based on the following papers, which are referred to in the text by their respective Roman numerals I-IV. Paper I and II are reproduced with the permissions of John Wiley & Son and Elsevier, respectively. Some unpublished results are also included in the thesis.

I Retention and maternal transfer of environmentally relevant PBDD/Fs, PCDD/Fs and PCBs in zebrafish (*Danio rerio*) after dietary exposure.


II Retention and maternal transfer of brominated dioxins in zebrafish (*Danio rerio*) and effects on reproduction, aryl hydrocarbon receptor-regulated genes, and ethoxyresorufin-O-deethylase (EROD) activity.


III Formation of environmentally relevant brominated dioxins from 2,4,6-tribromophenol via bromoperoxidase-catalyzed dimerization.


IV Photochemical formation of polybrominated dibenzo-\(p\)-dioxins (PBDDs) from environmentally abundant hydroxy polybrominated diphenylethers (OH-PBDEs).


Contribution by the author of this thesis to the papers

I The author was involved in the planning of the experiment, performed the analysis and wrote the manuscript.

II The author was involved in the planning of the experiment, performed the chemical analysis and wrote relevant parts of the manuscript.

III The author was highly involved in the planning of the experiment and performed parts of the experimental work, performed the analysis and wrote the manuscript.

IV The author was highly involved in the planning and performing of the experiment, performed the analysis and wrote the manuscript.

- vi -
## Abbreviations and definitions

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3,7,8-TeBDD</td>
<td>2,3,7,8-tetrabrominated dibenzo-p-dioxin</td>
</tr>
<tr>
<td>2,4,6-TrBP</td>
<td>2,4,6-tribromophenol</td>
</tr>
<tr>
<td>ACW</td>
<td>artificial coast water</td>
</tr>
<tr>
<td>AHH</td>
<td>aryl hydrocarbon hydroxylase</td>
</tr>
<tr>
<td>AhR</td>
<td>aryl hydrocarbon receptor</td>
</tr>
<tr>
<td>BCF</td>
<td>bioconcentration factor</td>
</tr>
<tr>
<td>BFR</td>
<td>brominated flame retardants</td>
</tr>
<tr>
<td>BPO</td>
<td>bromoperoxidase</td>
</tr>
<tr>
<td>BP</td>
<td>bromophenol</td>
</tr>
<tr>
<td>CYP1A</td>
<td>cytochrome P450 1A</td>
</tr>
<tr>
<td>DOC</td>
<td>dissolved organic carbon</td>
</tr>
<tr>
<td>EOM</td>
<td>extractable organic material</td>
</tr>
<tr>
<td>EROD</td>
<td>ethoxyresorufin-O-deethylase</td>
</tr>
<tr>
<td>GC-HRMS</td>
<td>gas chromatography-high resolution mass spectrometry</td>
</tr>
<tr>
<td>GR</td>
<td>glutathione reductase</td>
</tr>
<tr>
<td>H</td>
<td>Henry’s law constant, air-water partitioning constant</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>Ka</td>
<td>acid dissociation constant</td>
</tr>
<tr>
<td>Kow</td>
<td>octanol-water partitioning coefficient</td>
</tr>
<tr>
<td>MeO-PBDE</td>
<td>methoxylated polybrominated diphenyl ether</td>
</tr>
<tr>
<td>Mw</td>
<td>molecular weight</td>
</tr>
<tr>
<td>NOEL</td>
<td>no observed effect level</td>
</tr>
<tr>
<td>OH-PBDE</td>
<td>hydroxylated polybrominated diphenyl ether</td>
</tr>
<tr>
<td>PBDD</td>
<td>polybrominated dibenzo-p-dioxin</td>
</tr>
<tr>
<td>PBDD/F</td>
<td>polybrominated dibenzo-p-dioxin and dibenzofuran</td>
</tr>
<tr>
<td>PBDF</td>
<td>polybrominated dibenzofuran</td>
</tr>
<tr>
<td>PCB</td>
<td>polychlorinated biphenyl</td>
</tr>
<tr>
<td>PCDD/F</td>
<td>polychlorinated dibenzo-p-dioxin and dibenzofuran</td>
</tr>
<tr>
<td>Psu</td>
<td>practical salinity units</td>
</tr>
<tr>
<td>PXDD/F</td>
<td>polyhalogenated (brominated and/or chlorinated) dibenzo-p-dioxin and dibenzofuran</td>
</tr>
<tr>
<td>R²</td>
<td>coefficient of determination</td>
</tr>
<tr>
<td>REP</td>
<td>relative effect potency</td>
</tr>
<tr>
<td>TeCDD</td>
<td>2,3,7,8-tetrachlorodibenzo-p-dioxin</td>
</tr>
<tr>
<td>TEF</td>
<td>toxic equivalent factor</td>
</tr>
<tr>
<td>TEQ</td>
<td>toxic equivalent</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet (light)</td>
</tr>
</tbody>
</table>
Vp        vapor pressure  
ww        wet weight     
Ws        water solubility

lateral   sideways (positions 2,3,7 and 8 in PXDD/F structure)  
vicinal   adjacent
congeners Substances with the same backbone structure but different numbers and placements of the halogen atoms
congener profile Relative abundance between congeners
precursor Starting substance in the formation of another substance
primary producer organism that can produce organic compounds from inorganic carbon through photosynthesis, mainly plants, algae and cyanobacteria

Words and abbreviations for numerals

<table>
<thead>
<tr>
<th>mono</th>
<th>M</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>di</td>
<td>D</td>
<td>2</td>
</tr>
<tr>
<td>tri</td>
<td>Tr</td>
<td>3</td>
</tr>
<tr>
<td>tetra</td>
<td>Te</td>
<td>4</td>
</tr>
<tr>
<td>penta</td>
<td>Pe</td>
<td>5</td>
</tr>
<tr>
<td>hexa</td>
<td>Hx</td>
<td>6</td>
</tr>
<tr>
<td>hepta</td>
<td>Hp</td>
<td>7</td>
</tr>
<tr>
<td>octa</td>
<td>O</td>
<td>8</td>
</tr>
<tr>
<td>nona</td>
<td>No</td>
<td>9</td>
</tr>
<tr>
<td>deca</td>
<td>De</td>
<td>10</td>
</tr>
</tbody>
</table>
1 Background

Halogenated chemicals began to be produced on large-scale for industrial and household use in the second half of the 20th century. However, the benefits of their intentional uses where soon accompanied by often unexpected undesirable environmental effects (Carson, 1962; Jensen et al., 1969). Since then, environmental chemists have been engaged in the study of fate, distribution and effects of halogenated substances released into natural environments as a result of human activities. The presence of structurally similar halogenated compounds of natural origin has also gained attention in recent years and raised questions about their additional possible impact (Haglund et al., 2007; Vetter and Gribble, 2007). Brominated analogues of the widely dispersed, mainly anthropogenically released, toxic polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/Fs) are the polybrominated dibenzo-p-dioxins and dibenzofurans (PBDD/Fs), of which the polybrominated dibenzo-p-dioxins (PBBDs), have been found in high concentrations in the Baltic Sea increasing the need for knowledge of their sources and possible environmental impact.

1.1 The Baltic Sea
The Baltic Sea is one of the largest bodies of brackish water, although as a sea it is small. It has unique properties due to its geographical location, climate, and oceanographic conditions. It consists of several basins, between which exchange of water is restricted. From north to south the main basins are the Bothnian bay, Bothnian Sea, and Baltic Proper. The sea is connected through the Danish Straits via the water bodies outside the West Coast of Sweden (Kattegatt and Skagerrack), and thence to the North Atlantic. As the connection is very narrow, the exchange of water between the Baltic and the ocean is limited, and residence time of the water in the Baltic Sea can be up to 30 years. The catchment area encompasses 12 countries and a population
of 85 million people, hence the inflow from rivers carries high inputs of nutrients and hazardous substances.

The Baltic Sea is a geographically young sea that has changed from lake to sea and vice versa several times since the last glacial period. Its connection to the North Sea was established approximately 10 000 years ago and its salinity has slowly decreased due to uplift of the land and consequently increasing restriction of inflow of ocean water. Because the ocean water is diluted with runoff from rivers, there is currently a salinity gradient from the oceanic 35 psu (practical salinity units) through around 20 and 6 psu in the Kattegat and the Baltic Proper, respectively, to just 1–2 psu in the northernmost Bothnian Bay.

Most of the animal and plant species present in the Baltic Sea are sea water species that have adapted to the low salinity, but some fresh water species have invaded (Johannesson et al., 2011; Ojaveer et al., 2010). The challenges for these organisms adapting to either lower or higher salinity conditions has resulted in a low biodiversity, which has been exacerbated by eutrophication, the high pollutant levels and other pressures. For example, the cod population is declining mainly due to eutrophication and overfishing, and declines in population of coastal fish like perch and pike, accompanied by changes in species composition of fish communities have been observed along the coast of the Baltic Sea (Nilsson et al., 2004). The low biodiversity, the high exploitation, and high pollutant pressure collectively make the Baltic Sea an ecologically vulnerable sea.

1.2 Levels of PBDD/Fs in Baltic biota
PBDDs have been identified in several marine organisms from the Baltic Sea, including blue mussels (Mytilus edulis), sponge (Ephydatia fluviatilis), the red alga (Ceramium tenuicorne), the brown alga (Dictyosiphon foenicolaceus), cyanobacteria and several fish species, e.g. perch (Perca fluviatilis) (Haglund et al., 2007; Haglund et al., 2010; Löfstrand et al., 2010; Malmvärn et al., 2005b; Malmvärn et al., 2008; Unger et al., 2009). Analyzed fish from nearby fresh water lakes do not reportedly contain PBDDs, thus the source seems to be marine. Further, surprisingly high levels of total PBDDs have been found in mussels from a remote area in the Baltic Proper, at levels exceeding 4 ng/g wet weight (ww) (Haglund et al., 2007). As the levels of PCDD/Fs in fatty fish from the Baltic Sea already are near the maximum residue level for food established by the European Comission, an additional load of high levels of PBDDs may amplify the toxic impact of PCDD/Fs in the Baltic Sea. From north to south in the Baltic Sea, levels of PBDDs in fish increase, from non-detectable to 75 ng/g ww. Littoral fish, like perch (Perca fluviatilis) a coastal, resident species (Nilsson et al., 2004), generally have higher levels than pelagic fish, indicating that the source of PBDDs is located in the coastal zone (Haglund et al., 2007) (Figure 1.1).
Figure 1.1  Total concentrations of PBDDs (filled bars) and PCDD/Fs (dotted bars) in pg/g ww in biota from indicated locations in the Baltic Sea and an inland lake in the Baltic catchment in southern Sweden. Samples represent littoral fish (perch, coast eel), pelagic fish (herring), primary producers (algae), and filter feeders (mussels). (a) Mussel and herring from the West Coast, b) coast eel and herring from the south Baltic Proper, c) perch, mussel and algae from the north Baltic Proper (PCDD/F data lacking for mussel and algae), d) perch from an inland lake, e) perch from Bothnian Sea. Note the different scales. Data from Haglund et al. (2007).
Background

PBDD congeners (i.e. PBDDs differing in substitution positions and bromination degree) identified in Baltic biota are reportedly limited to lower brominated congeners, di- to tetraBDDs. Presence of polybrominated dibenzofurans (PBDFs) have been indicated but congeners were not structurally identified (Malmvärn et al., 2005b; Malmvärn et al., 2008; Unger et al., 2009). Samples from specific geographical locations show typical PBDD congener profiles (i.e. the relative abundance of different congeners), and both congener profiles and relative levels were concordant between biota samples from different trophic levels collected at the same geographical location. The differences in PBDD congener profiles between different locations indicate that sources of PBDDs, and/or their relative importance may vary between locations (for further discussion see section 4.3).

1.2.1 Origin of PBDDs

Several findings indicate that the PBDDs found in the Baltic Sea have natural rather than anthropogenic origins (Gullett et al., 2010; Haglund et al., 1988; Haglund et al., 2007; Löfstrand et al., 2010; Söderström and Marklund, 2002);
- the congener distribution of PBDDs: the PBDD/Fs formed in anthropogenically related (combustion) processes are mostly PBDFs and highly brominated (tetra- to hepta-) congeners, whereas Baltic biota have very low PBDF content and PBDDs are mainly less brominated (mono- to tetra-) congeners
- the spatial and temporal distributions of PBDDs: PBDDs have only been found in marine environments, levels of PBDDs differ between different locations and fluctuate over time; and congener profiles also vary between locations, notably between the Baltic Proper and West Coast
- the congener profiles of PBDDs: biota from different trophic levels (cyanobacteria, mussels, and fish) collected at the same location have similar congener profiles, indicating a common source

In conclusion, sources of PBDDs in the Baltic Sea are probably local and their inputs probably change over time. Primary producers such as cyanobacteria or algae have been proposed as possible sources of the PBDDs, suggesting a biogenetic formation from simple substances e.g. phenolic compounds (Haglund et al., 2007; Haglund, 2010; Löfstrand et al., 2010; Malmvärn et al., 2008). Changes in conditions, both short-term (e.g. fluctuations in levels of nutrients and temperature) and long-term, (e.g. climate changes and changes in biodiversity) could thus be reflected in changes in PBDD production levels and/or congener profiles.
1.2.2 Risk perspectives

Halogenated (i.e. brominated and/or chlorinated) dibenzo-\(p\)-dioxins and dibenzofurans (PXDD/Fs) consist of many congeners with different chemical properties and biological activity and thus different toxic potency. However, as their toxicity is mediated through a common mechanism of action, the toxic potency of individual congeners can be compared and a total toxic value given based on the respective relative values. The toxic potency for PXDD/Fs is related to that of the most potent PCDD congener, 2,3,7,8-tetrachlorodibenzo-\(p\)-dioxin (TeCDD), and expressed as pg TeCDD toxic equivalents (TEQ). No values for the toxicity of PBDD/Fs relative to TeCDD (so called toxic equivalent factors; TEFs) have been established as yet, hence TEFs for chlorinated analogues or singly determined relative potencies (REPs) are generally used at present, where available. However, neither TEFs nor REPs have been determined for the most abundant PBDD/F congeners found in Baltic Sea samples.

For comparison, a very rough estimate of the toxicity based on available REPs can be made for Baltic Sea mussels, calculated from concentrations from a high level location in the Baltic Proper and a moderate level location from the West Coast (Haglund et al., 2007) (for a detailed discussion see section 2.3). Using REPs from two different types of bioassays (Mason et al., 1987a; Olsman et al., 2007) available for the lateral substituted 2,7-/2,8-DBDD and 2,3,7-TrBDD, and for 1,3,6,8-/1,3,7,9-TeBDD, estimated TEQ values are 9 – 110 pg TEQ/g ww in mussels from the Baltic Proper, and 0.01 – 0.2 pg TEQ/g ww in mussels from the West Coast, depending on type of assay. This can be compared to the total PCDD/F and dioxin-like polychlorinated biphenyl (PCB) limits set by the European Commission for food: 8 pg TEQ/g fresh weight (European Commission, 2006). Thus, levels from the Baltic Proper would exceed those limits, although the contribution of the most abundant congeners in mussels (1,3,7-/1,3,8-TrBDD) have not been accounted for because of the lack of determined REPs. Mussels account for more than 90% of the total animal biomass in the Baltic Sea, and are key components of the littoral food web, as they feed on plankton and are in turn food for fish (Gilek et al., 1997; Haglund et al., 2007). Thus, the high PBDD levels found in mussels could have potentially profound ecological effects.

1.3 Aims of the studies

The aims of the studies presented in this thesis were to increase the knowledge of PBDD/Fs within two areas; the retention characteristics and biological effects of PBDD/Fs in fish; and the formation of PBDD/Fs from structurally related molecules under natural conditions.

More specifically I hope to answer these questions:
How are PBDD/Fs from feed taken up, retained and transferred in zebrafish, and what biological effects do they have? (Papers I and II)

Can PBDD/Fs be formed naturally from bromophenolics via either enzymatic or photochemical mechanisms? (Papers III and IV)

In this thesis the methodology applied and the results obtained in the four studies are summarized and the implications of the results are discussed.
2 PBDDs – their properties, precursors, effects, and analysis

The long-lived idea that halogenated compounds are mostly of anthropogenic origin has been challenged by the increasing number of halogenated compounds of natural origin that has been found, mainly in the marine environment (Gribble, 2000; Gribble, 2003; Vetter, 2006). Up to date about 4000 naturally produced halogenated compounds have been identified, most of these containing chlorine and/or bromine (Gribble, 2003). The possibility of these as new sources of medical drugs has increased the interest in these compounds and extended our knowledge. However, the similarities in structure and properties between certain natural halogenated compounds and known persistent organic pollutants have also raised concerns about possible ecotoxicological effects (Birnbaum et al., 2003; Haglund et al., 2007; Vetter, 2006). The PBDD/Fs and the precursors studied in this thesis could be both of anthropogenic and natural origin, and have structural and physico-chemical similarities with other known pollutants (Buser, 1986; Flodin and Whitfield, 1999a; Hakk and Letcher, 2003; Howe et al., 2005; Malmvärn et al., 2005a; Malmvärn et al., 2005b). Thus, the formation and ecological impact of PBDD/Fs are of interest.

2.1 Structure and physico-chemical properties
The general structures of polybrominated dibenzo-\(p\)-dioxins (PBDDs) and polybrominated dibenzofurans (PBDFs) are shown in Figure 2.1. They are coplanar compounds with two aromatic rings coupled with one or two oxygen bridges. There are eight possible positions for bromine substitution which gives 75 different PBDD-congeners and 135 different PBDF-congeners taking all bromination degrees into account. The studies in this thesis deals with mono- to tetraBDD/Fs since these are the most prevailing congeners in Baltic Proper biota, and some of the congeners discussed in the thesis are
shown in Figure 2.2. There is no industrial production of PBDD/Fs, but they are unintentionally formed in production of brominated flame retardants (BFR) (WHO/ICPS, 1998) and during combustion of materials containing BFR or other brominated products (Buser, 1986; Schuler and Jager, 2004; Söderström and Marklund, 2002; Wang and Chang-Chien, 2007). Compared to the chlorinated analogues (PCDD/Fs), the PBDD/Fs have different properties e.g. higher molecular weight, increased lipophilicity, lower water solubilities, and are more sensitive to ultraviolet light (UV) degradation. This is because the bromine atom is heavier and bigger than chlorine, and because the bromine-carbon bond is weaker as compared to a chlorine-carbon bond, thus requires less energy to break. Experimental data on physico-chemical properties of PBDD/Fs are scarce (WHO/ICPS, 1998). Properties of environmental concern, calculated from molecular data, are shown in Table 2.1.

![Figure 2.1 General structure of (a) PBDDs and (b) PBDFs. Numbering denote substitution positions. x and y are 0–4 Br-substituents, x + y = 1–8 Br.](image)

**Table 2.1.** Physico-chemical properties of PBDD/Fs at different bromination degrees, calculated in EpiSuite (US EPA, 2008); molecular weight (Mw), partition coefficient for octanol-water (Kow), water solubility (Ws), vapour pressure (Vp), Henry’s law constant (H), and bioconcentration factor (BCF).

<table>
<thead>
<tr>
<th>Substance</th>
<th>Mw</th>
<th>log Kow</th>
<th>Ws a</th>
<th>Vp b</th>
<th>H c</th>
<th>logBCF d</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBDD</td>
<td>263.09</td>
<td>5.23</td>
<td>5.3e-2</td>
<td>6.8e-3</td>
<td>4.7e-1</td>
<td>3.1</td>
</tr>
<tr>
<td>DBDD</td>
<td>341.99</td>
<td>6.12</td>
<td>3.2e-3</td>
<td>4.5e-4</td>
<td>1.9e-1</td>
<td>3.7</td>
</tr>
<tr>
<td>TrBDD</td>
<td>420.88</td>
<td>7.01</td>
<td>1.8e-4</td>
<td>5.7e-5</td>
<td>7.5e-2</td>
<td>4.1</td>
</tr>
<tr>
<td>TeBDD</td>
<td>499.78</td>
<td>7.90</td>
<td>1.0e-5</td>
<td>6.1e-6</td>
<td>3.0e-2</td>
<td>3.7</td>
</tr>
<tr>
<td>[2378-1]</td>
<td>[6.50f]</td>
<td>[1.6e-4]</td>
<td>[7.5e-8]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>238-TrBDF</td>
<td>404.88</td>
<td>6.38</td>
<td>7.8e-4</td>
<td>4.9e-5</td>
<td>3.3e-1</td>
<td>3.9</td>
</tr>
<tr>
<td>2378-TeBDF</td>
<td>483.78</td>
<td>5.98e</td>
<td>5.5e-4</td>
<td>4.7e-6</td>
<td>1.3e-1</td>
<td>3.7</td>
</tr>
</tbody>
</table>

(a) From Kow  (b) Modified Grain method  (c) Bond contributing method  (d) Regression based method  (e) Experimental value (Jackson et al., 1993).
Figure 2.2 Structures of mono- to tetraBDDs and tri- and tetraBDFs discussed in the thesis. Abbreviations are shown next to each structure.
PBDD/Fs are highly lipophilic compounds with low water solubility, and hence, the octanol-water partition coefficients ($K_{ow}$) are high, with log $K_{ow}$ values ranging from around 5 for monoBDD/Fs up to 7–8 for tetraBDD/Fs (Table 2.1). This will mean that these compounds have a high affinity for lipids and e.g. particles of organic material. They have therefore, the prerequisites for uptake and retention in biota, and possibly bioaccumulation.

2.2 Possible precursors of PBDD/Fs
Natural formation of PBDD/Fs will most likely go via a phenolic precursor, since this would have the structural elements needed to obtain a dioxin-structure; the aromatic ring and an oxygen atom for the ether bond. Production of brominated phenolic substances in the marine environment is widespread, algae are an important source of bromophenols (BPs) (Flodin and Whitfield, 1999a; Whitfield et al., 1999) and hydroxylated polybrominated diphenylethers (OH-PBDE) have been found at substantial levels in algae and mussels in the Baltic Sea (Löfstrand et al., 2011; Malmvärn et al., 2005a). Both groups of substances are suggested as possible precursors for formation of PBDD/Fs, trough biotic and/or abiotic pathways.

2.2.1 Bromophenols (BPs)
The origin of BPs in the marine environment is mainly from algae (Whitfield et al., 1999) but they can be found in fish and shell-fish where they are key flavor components (Whitfield et al., 1998). The formation of BPs has been shown to be catalyzed by bromoperoxidase, and the proposed precursor for 2,4,6-tribromophenol (2,4,6-TrBP) is 4-hydroxybenzoic acid (Flodin and Whitfield, 1999a; Flodin and Whitfield, 1999b). Bromination occurs primarily in the 2, 4, and 6-positions due to the ortho-, para-directing property of bromine, leaving 2,4,6-TrBP (Figure 2.3) as the main product in many algal species. Levels up to several μg 2,4,6-TrBP/g alga ww have been found, but e.g. 2-, 2,4-, and 2,6-BP are also formed (Whitfield et al., 1999). Some of these have also been found in water and sediments in the Baltic Sea, ranging from 1–50 ng/L (water) and 0.3–360 ng/g dry weight (sediments) (Reineke et al., 2006).

Apart from the natural formation of BPs there is also a substantial production and use of these, especially of 2,4,6-TrBP which is used as a wood preservative and intermediate in flame retardant production and has an annual production of 9 500 tonnes (2001) (Howe et al., 2005). High yields (> 50%) from the thermal formation of PBDD/Fs from 2,4,6-TrBP has been shown (Hutzinger et al., 1989; Na et al., 2007; Sidhu et al., 1995). Environmentally important properties of 2,4,6-TrBP are shown in Table 2.2. There is no data on possible release of 2,4,6-TrBP into the environment. However, since it will be mostly in its deprotonated state in marine
environment at natural pH (8.4), and as the bioconcentration factor is relatively low (log BCF 2.46, calculated in EpiSuite [US EPA, 2008]), bioaccumulation of 2,4,6-TrBP is expected to be moderate (Howe et al., 2005).

![Figure 2.3 Structure of 2,4,6-tribromophenol (2,4,6-TrBP).](image)

**Table 2.2** Physico-chemical properties of 2,4,6-tribromophenol (2,4,6-TrBP) used in Paper III, and hydroxylated polybrominated diphenyl ethers (OH-PBDEs) used in Paper IV; molecular weight (Mw), octanol-water partition coefficient (Kow), water solubility (Ws), Henry’s law constant (H), and acid dissociation constant (Ka). Data for 2,4,6-TrBP from Kuramochi et al. (2004), data for OH-PBDEs estimated in EpiSuite (US EPA, 2008), except Ka estimated using SPARC On-line calculator.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Mw</th>
<th>log Kow</th>
<th>Ws (^{a})</th>
<th>H (^{b})</th>
<th>pK(_a) (^{c})</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4,6-TrBP (^{d})</td>
<td>263.09</td>
<td>4.24</td>
<td>61</td>
<td>3.2e-2</td>
<td>6.08</td>
</tr>
<tr>
<td>OH-BDE 47</td>
<td>501.80</td>
<td>6.29</td>
<td>0.011</td>
<td>3.1e-5</td>
<td>7.22</td>
</tr>
<tr>
<td>OH-BDE 68</td>
<td>501.80</td>
<td>6.29</td>
<td>0.011</td>
<td>3.1e-5</td>
<td>6.86</td>
</tr>
<tr>
<td>OH-BDE 85</td>
<td>580.69</td>
<td>7.18</td>
<td>6.0e-4</td>
<td>1.2e-5</td>
<td>6.58</td>
</tr>
<tr>
<td>OH-BDE 90</td>
<td>580.69</td>
<td>7.18</td>
<td>6.0e-4</td>
<td>1.2e-5</td>
<td>6.04</td>
</tr>
<tr>
<td>OH-BDE 99</td>
<td>580.69</td>
<td>7.18</td>
<td>6.0e-4</td>
<td>1.2e-5</td>
<td>5.83</td>
</tr>
<tr>
<td>OH-BDE 123</td>
<td>580.69</td>
<td>7.18</td>
<td>6.0e-4</td>
<td>1.2e-5</td>
<td>6.00</td>
</tr>
</tbody>
</table>

(a) From log Kow
(b) Bond contributing method
(c) Estimated using SPARC On-line calculator.
(d) Values from Kuramochi et al. (2004)

### 2.2.2 Hydroxylated polybrominated diphenyl ethers (OH-PBDEs)

The OH-PBDEs are derivatives of polybrominated diphenylethers (PBDE) and the abbreviation numbering system used for both groups of substances is based on the numbering system for PCBs (Ballschmiter and Zell, 1980). PBDEs are widely used as flame retardants, with an estimated yearly
production of 67,000 tonnes (2000) (Birnbaum and Staskal, 2004). Despite the high use of PBDEs and the possible formation of OH-PBDEs as metabolites from PBDEs (Hakk and Letcher, 2003; Malmberg et al., 2005), most OH-PBDEs found in environmental samples will likely be natural products (Gribble, 2000; Kelly et al., 2008; Löfstrand et al., 2011; Malmvärn et al., 2005a). Other suggested routes of OH-PBDE formation from anthropogenic PBDEs include atmospheric transformation through hydroxyl radical reactions (Raff and Hites, 2006; Ueno et al., 2008) and oxidation in sewage treatment plants (Hua et al., 2005; Ueno et al., 2008). Photochemical formation of OH-PBDEs from 2,4-BP has also been reported (Liu et al., 2011). The structurally related methoxyolated polybrominated diphenylethers (MeO-PBDEs) are primarily of natural origin (Haglund et al., 1997; Teuten and Reddy, 2007; Teuten et al., 2005). The interconversion of MeO-PBDEs to OH-PBDEs has been shown both in vitro and in vivo (Wan et al., 2009; Wan et al., 2010). Thus, they could be additional sources of OH-PBDEs in the environment. OH-PBDEs of metabolic origin often have the hydroxy group in meta- or para-position to the ether bond, whereas naturally produced OH-PBDEs have the hydroxyl group ortho to the ether bond (Kelly et al., 2008; Marsh et al., 2003). The naturally produced OH-PBDEs thus have a favorable sterical configuration for PBDD formation. OH-PBDEs used in the study described in Paper IV are shown in Figure 2.4.

OH-PBDEs have been found in a variety of species and locations, but are believed to be mainly produced by primary producers, such as cyanobacteria and algae (Haraguchi et al., 2010; Malmvärn et al., 2005a; Malmvärn et al., 2008; Unson et al., 1994). Reported concentrations in Baltic biota of OH-PBDEs are in the range of 12–23 μg/g extractable organic material (EOM) for the red alga (Ceramium tenuicorne) and 1–170 ng/g lipid for blue mussel (Mytilus edulis) (Löfstrand et al., 2010; Malmvärn et al., 2008). The most abundant congener in red alga is 6-hydroxy-2,2′,4,4′-pentabDE (OH-BDE 99), at 4.4 μg/g EOM, while 6-hydroxy-2,2′,4,4′-tetraBDE (OH-BDE 47) dominates in mussel, at 70 ng/g lipid. Some environmentally important properties of the OH-PBDEs discussed in this thesis are shown in Table 2.2. As for 2,4,6-TrBP the pKa value of the studied OH-PBDEs show that these compounds will mostly be in their deprotonated state in marine environments at natural pH (8.4). Although their log Kow values are high, OH-PBDEs generally do not bioaccumulate in lipid tissue but can be found in blood, due to their phenolic character (Asplund et al., 1999; Athanasiadou et al., 2008). OH-PBDEs are susceptible to UV-light and have been shown to be transformed and degraded in aqueous media, some of the transformation products being PBDDs (Bastos et al., 2009; Steen et al., 2009) (see section 4.2 and Paper IV).
2.3 Biological effects of PBDD/Fs

PBDD/Fs have diverse toxicological effects, including *inter alia* wasting, thymic atrophy, teratogenesis, immunotoxicity and adverse reproductive effects (Birnbaum et al., 2003; Weber and Greim, 1997; WHO/ICPS, 1998), similar to PCDD/Fs. In Wistar rats PBDDs have typical dioxin-like effects, e.g. body weight loss, thymic atrophy and induction of cytochrome P450-dependent enzymes aryl hydrocarbon hydroxylase (AHH) and ethoxyresorufin-O-deethylase (EROD). The toxicity of tetra- and pentaBDDS
in these respects is similar to that of the highly toxic TeCDD (Mason et al., 1987a). Similarly, tetraBDDs, tetraBDFs and mixed tetrahalogenated dibenzo-\(p\)-dioxins show early life stage mortality in rainbow trout at comparable or higher levels to TeCDD (Hornung et al., 1996b) and effects were shown to be additive for pairs of PBDD/Fs (Hornung et al., 1996a). Apart from the latter studies, little is known about effects of PBDD/Fs on fish health and reproduction, but they are likely to have similar effects generally to those of chlorinated analogues. TeCDD has been shown to cause effects in zebrafish including induction of cytochrome P450 1A (CYP1A), alteration in gonad morphology, reduced spawning and developmental toxicity (King Heiden et al., 2005; King Heiden et al., 2009; Wannemacher et al., 1992; Zodrow et al., 2004).

2.3.1 Toxicity of PBDD/Fs compared to PCDD/Fs

Halogenated dibenzo-\(p\)-dioxins and dibenzofurans (PXDD/Fs) mediate their toxicity by binding to the aryl hydrocarbon receptor (AhR), and both halogenation degree and substitution pattern affects the strength of the binding, \textit{i.e.} the affinity for the AhR (Mason et al., 1986; Mason et al., 1987a; Mason et al., 1987b). The binding site of AhR is hydrophobic and especially planar non-polar ligands, like PXDD/Fs, have a high affinity for it (Landers and Bunce, 1991). Once formed, the receptor-ligand complex triggers a cascade of biochemical and toxic events. Furthermore, it has been shown that the effects mediated by AhR are additive (Hornung et al., 1996a; Zabel et al., 1995).

Since the toxic effects of PXDD/Fs have a common mechanism of action, and the severity of their effects is related to the structure and degree of halogenation, a system for comparing the toxicity of different congeners has been established. These so-called toxic equivalency factors (TEFs) are consensus values based on \textit{in vivo} and \textit{in vitro} data from several studies. The World Health Organization (WHO) has agreed on a TEF scale that includes PCDD/F and a number of PCBs (Van den Berg et al., 2006). The relative fraction of toxic potency, TEF, for each congener compared to the most toxic congener, TeCDD, is determined, and the value multiplied with the concentration of that congener to get the TeCDD equivalents concentration, TEQ. This enables a toxicity value to be assigned to any sample, regardless of which congeners are present.

For PBDD/Fs no TEF values have yet been established, because too little data are available, but studies of singly determined relative potencies (REPs) from several types of bioassays (Behnisch et al., 2003; Mason et al., 1987a; Olsman et al., 2007; Samara et al., 2009) and from \textit{in vivo} data (Hornung et al., 1996b) have been published (Table 2.3). In general, 2,3,7,8-tetraBDD (2,3,7,8-TeBDD) and 2,3,7,8-tetraBDF have comparable REPs to TeCDD while TrBDD/Fs and non-2,3,7,8-substituted TeBDDs have two to three
magnitudes lower REPs. However, lateral substituted DBDD and TrBDD have shown high binding affinity in a competitive AhR binding assay (Mason et al., 1987a). In addition, certain mixed PXDD/Fs congeners, i.e. congeners with both chlorine and bromine substituents, may have higher REPs than TeCDD (Behnisch et al., 2003; Hornung et al., 1996b; Olsman et al., 2007; Samara et al., 2009).

Table 2.3. Relative effective potencies (REPs) of PBDD/Fs compared to TeCDD. REPs determined in three bioassays (TV101L, human hepatoma cells; DR-CALUX and CALUX, rat hepatoma cells), and for comparison receptor binding affinities (hydroxyapatite receptor binding, HAP), in vivo data (early life stage mortality of rainbow trout), and corresponding PCDD/F toxic equivalent factor (TEF). ECxx (measured at the effective concentration of xx% of the maximum effect level). [13C] denote use of labeled compound.

<table>
<thead>
<tr>
<th>Compound</th>
<th>TV101L EC25a</th>
<th>DR-CALUX EC25a</th>
<th>DR-CALUX EC20b</th>
<th>CALUX EC50c</th>
<th>HAP EC50d</th>
<th>trout LD50e</th>
<th>TEF TeCDDf</th>
</tr>
</thead>
<tbody>
<tr>
<td>27/28DBDD</td>
<td></td>
<td></td>
<td></td>
<td>2e-4</td>
<td>6.5e-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>237TrBDD</td>
<td>1.3e-2</td>
<td>8.1e-2</td>
<td>6.2e-2</td>
<td>6.0e-4</td>
<td>0.85</td>
<td>1.7e-2</td>
<td></td>
</tr>
<tr>
<td>1368/1379TeBDD</td>
<td>8.6e-4</td>
<td>6.2e-4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1378TeBDD</td>
<td>1.0e-3</td>
<td>1.1e-2</td>
<td></td>
<td></td>
<td>0.50</td>
<td>1.3e-2</td>
<td></td>
</tr>
<tr>
<td>2378TeBDD</td>
<td>0.60</td>
<td>0.42</td>
<td>0.87</td>
<td>0.99</td>
<td>0.67</td>
<td>1.1</td>
<td>1</td>
</tr>
<tr>
<td>1234TeBDD</td>
<td>8.1e-4</td>
<td>1.4e-4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>228TeBDF</td>
<td>7.8e-4</td>
<td>4.9e-4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2378TeCDF</td>
<td></td>
<td>0.86</td>
<td>0.41</td>
<td>0.25</td>
<td>0.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Br-378TrCDD</td>
<td>0.42</td>
<td>1.9</td>
<td>0.44</td>
<td>0.72</td>
<td>0.71</td>
<td>0.65</td>
<td></td>
</tr>
<tr>
<td>23-Br-78DCDD</td>
<td>0.49</td>
<td>1.0</td>
<td>1.15</td>
<td>0.43</td>
<td>0.71</td>
<td>0.68f</td>
<td></td>
</tr>
</tbody>
</table>

(a) Olsman et al. (2007)  (e) Hornung et al. (1996b)
(b) Behnisch et al. (2003)  (f) Van den Berg et al. (2006)
(c) Samara et al. (2009)  (g) 28-Br-37DCDD
(d) Calculated from Mason et al. (1987a)

2.3.2 Toxicity estimate of PBDDs in Baltic biota
PBDD/Fs have been found in both marine and terrestrial matrices, generally at low levels. Because of lack of established TEF-values for PBDDs, levels are usually reported in TEQs using the TEFs for chlorinated analogues. In Japan, fish from an industrialized area have been found to contain up to 0.2 pg TEQ/g ww of mainly heptaBDF, with some 2,3,7,8-TeBDD/F (Ashizuka et al., 2008), and shellfish from around the Scottish coastline reportedly contain tri- and tetraBDD and PBDF, at levels ranging from 0.02 to 0.2 pg TEQ/g ww (Fernandes et al., 2008). Adipose tissue from the general Swedish human population reportedly contain PBDFs at levels ranging from 0.1 to 1.8 pg
TEQ/g lipid, but no detectable PBDDs (Jogsten et al., 2010). The presence of predominantly PBDFs in the samples in the cited studies indicates anthropogenic sources.

The established TEF values for PCDD/Fs matches very few of the detected PBDD congeners found in Baltic biota. In order to estimate the toxic potency of the PBDD levels in Baltic mussels, a rough estimate of the TEQ value based on the PBDD concentrations from two different locations (Haglund et al., 2007) and available REPs for PBDDs is presented here (Table 2.4). The REPs used have been determined using two types of bioassays: the DR-CALUX assay, which provided REPs for TeBDD and 2,3,7-TrBDD (Olsman et al., 2007), and a receptor binding assay, which provided REPs for 2,7-/2,8-DBDD and 2,3,7-TrBDD (Mason et al., 1987a).

Table 2.4. Estimated toxicity equivalents (pg TEQ/g ww) in mussels from two locations: high level location (Baltic Proper) and moderate level location (West Coast). TEQs are based on relative effective potencies of PBDDs from studies of receptor binding affinities\(^b\) and from DR-CALUX bioassay\(^c\), see Table 2.3.

<table>
<thead>
<tr>
<th></th>
<th>Baltic Proper(^a)</th>
<th>West Coast(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pg/g</td>
<td>pgTEQ/g(^b)</td>
</tr>
<tr>
<td></td>
<td>pg/g ww</td>
<td>pgTEQ/g(^b)</td>
</tr>
<tr>
<td>13DBDD</td>
<td>32</td>
<td>0.33</td>
</tr>
<tr>
<td>27/28DBDD</td>
<td>250</td>
<td>16</td>
</tr>
<tr>
<td>17DBDD</td>
<td>26</td>
<td>0.18</td>
</tr>
<tr>
<td>18DBDD</td>
<td>44</td>
<td>1.1</td>
</tr>
<tr>
<td>137TrBDD</td>
<td>1400</td>
<td>3.4</td>
</tr>
<tr>
<td>138TrBDD</td>
<td>2100</td>
<td>15</td>
</tr>
<tr>
<td>237TrBDD</td>
<td>110</td>
<td>94</td>
</tr>
<tr>
<td>1368TeBDD</td>
<td>0.36</td>
<td>2e-4</td>
</tr>
<tr>
<td>1379TeBDD</td>
<td>0.53</td>
<td>3e-4</td>
</tr>
<tr>
<td>pg PBDD/g ww</td>
<td>4000</td>
<td></td>
</tr>
<tr>
<td>pg TEQ/g ww</td>
<td>110</td>
<td>9</td>
</tr>
</tbody>
</table>

(a) Haglund et al. (2007)  
(b) Mason et al. (1987a)  
(c) Olsman et al. (2007)

The differences in total and relative levels of congeners between samples are clearly reflected in the TEQ values and potential toxic levels in both Baltic Proper and West Coast mussels are clearly at least equal to those of other matrices, although the contribution of the most abundant congeners (1,3,7-/1,3,8-TrBDD) have not been accounted for because of the lack of determined REPs. TEQ-levels of PBDDs in the Baltic Proper are definitively at or above
the dioxin-limits for food (8 pg TEQ/g fresh weight) (European Commission, 2006), which should raise concerns since mussels are an important part of the marine food web.

2.4 Analytical aspects
The studies included in this thesis have investigated levels of PBDD/Fs in the picogram (pg) range, which placed high demands on the analytical methods used. To obtain the sensitivity and specificity needed, both work-up procedures and detection methods have to be optimized for the purpose. Matrix and interfering compounds have to be sufficiently removed in the work-up step to obtain high sensitivity in the detection step, where high specificity is obtained by efficient separation and selective detection. In all of the studies gas chromatography – high resolution mass spectrometry (GC-HRMS) was used for the separation and detection of the substances of interest.

2.4.1 Extraction and clean-up
Since PBDD/Fs are lipophilic compounds they are dissolved in the lipid phase of the materials investigated. Thus, the extraction step must isolate the lipid fraction efficiently. To obtain optimal yields of lipids from biota samples, cell walls must be broken and water must be removed. This was done in the studies reported in Papers I and II by grinding the samples with dehydrated sodium sulfate before extraction with suitable organic solvents. After extraction lipids were removed using sulfuric acid – silica columns, which provide a convenient means of removing both lipids and interfering material since PBDD/Fs (like PCDD/Fs and PCBs) are stable under very harsh conditions, such as sulfuric acid treatment (Haglund et al., 2007). To further enhance the sensitivity of subsequent analyses, fractionation using carbon columns can be applied, especially for environmental samples. The coplanarity of PBDD/Fs and PCDD/Fs gives them high affinity to carbon, and other groups that sorb less strongly to carbon, e.g. PCBs, can be eluted while PXDD/Fs are retained on the column. The PXDD/Fs can then be back-flushed from the column with toluene (Haglund et al., 2007). However, for most of the analyses reported in this thesis no fractionation on carbon was applied as samples were obtained from laboratory controlled exposure experiments with adjusted levels of substances, instead care was taken to obtain sufficient separation in the detection step.

For clean water samples regulation of pH and extraction with suitable organic solvents are sufficient to obtain high recoveries of lipophilic compounds. In the studies described in Papers III and IV, polar and non-polar substances in the samples were fractionated after extraction to remove phenolic precursors and break-down products (Hovander et al., 2000).
Thereafter the samples were cleaned with sulfuric acid-silica columns as mentioned above, prior to analysis.

To account for losses during extraction and clean-up, internal standards were added at the start of work-up and recovery standards were added at its final stage to determine the efficiency of the procedure. These standards were usually $^{13}$C-labelled PCDD/F and PCB congeners that were similar or identical to the analytes, and thus had similar or identical properties and behavior during work-up procedure.

2.4.2 Separation and detection

Mono- to tetraBDD/F compounds were separated by gas chromatography on highly polar cyanosiloxane columns (Supelco SP 2330 or SP 2331), which are well suited for analyzing dioxins as they have both polar and polarizable features that interact with polarizable compounds like dioxins. Differences in overall effects of interactions, e.g. strong dispersive and dipole-dipole interactions, determine the elution order of congeners, and provide high degrees of separation between them. The required detection specificity for PBDD/Fs was obtained by using a high-resolution (magnetic sector) mass spectrometer (GC-HRMS), where high resolution between different mass over charge ratios (m/z) can be obtained. In the presented studies the resolution was generally 10 000 which gives a mass peak width of 0.05 amu (atomic mass unit) for a compound with molecular weight of 500. To achieve the high sensitivity needed only selected ions are monitored, usually the two most abundant isotopic molecular ions, and the resulting detection limit is in the low pg range, often 0.1–10 pg injected substance, depending on e.g. the matrix used. Use of GC-HRMS allows confirmation of congeners at given bromination degrees, by comparing the relative abundances of specific mass fragments to expected ratios based on the natural isotopic abundance of bromine ($^{79}$Br to $^{81}$Br ratio: 50.5/49.5), which will give a specific “fingerprint” pattern of isotopic ions for each bromination degree. Chlorinated compounds can be confirmed likewise (natural $^{35}$Cl to $^{37}$Cl abundance ratio: 75.5/24.5).
3 Dietary exposure studies of PBDD/Fs in zebrafish

Many effects of chemical substances have been demonstrated in the marine environment, which continuously receives inputs of natural and synthetic chemicals from both point and diffuse sources. Exposure of marine organisms to these chemicals occurs via water and food, in which their concentrations can be substantial, and as there are often more steps in marine food webs than in terrestrial food webs, this might lead to high degrees of bioaccumulation of chemicals in marine organisms, especially higher organisms like fish. The fate of a substance and its effects on fish are thus important aspects to consider when assessing their environmental impact.

Exposure of aquatic organisms to chemicals from food or surrounding media can lead to uptake, at rates dependent on the concentrations of the substances in the feed or water and the organism’s ability to assimilate the substance. Retention is the net result of uptake and elimination of a given substance in the organism. Elimination may occur through a number of routes, notably by excretion, diffusion through gills, and metabolism in fish (Figure 3.1). Elimination may also occur through reproductive (maternal) transfer of some of the load of a substance from females to eggs during development of the eggs. Growth dilution, the reduction in concentration of a substance by growth rather than through reduction in its amount, will also affect tissue concentrations, but this is not, strictly, a form of elimination.

Both uptake and elimination rates and pathways will be affected by the properties of the substance. The toxicokinetics of PCDD/Fs and PCBs in fish have been well studied, providing a substantial body of information for comparison with those of similar chemicals (Andersson et al., 2001; Elonen et al., 1998; Fisk et al., 1998; Fox et al., 1994; Gobas and Schrap, 1990; Jones et al., 2001; Kleeman et al., 1988; Loonen et al., 1993; Nichols et al., 2004;
Dietary exposure studies of PBDD/Fs in zebrafish

Opperhuizen and Sijm, 1990; Opperhuizen and Schrap, 1988; Örn et al., 1998; Sijm et al., 1993). The maternal transfer of PCDD/Fs and PCBs in fish of several species has been thoroughly studied (Delorme et al., 1998; Fisk and Johnston, 1998; King Heiden et al., 2005; Monteverdi and Di Giulio, 2000a; Monteverdi and Di Giulio, 2000b; Nichols et al., 1998; Sundberg et al., 2007). In contrast, the retention and toxic effects of PBDD/Fs in fish have been much less intensively examined; effects of, primarily higher (tetra- to hepta-) brominated, PBDD/Fs are reported in rainbow trout and zebrafish (early effects on survival, histopathological effects) and of AhR-mediated responses (Hornung et al., 1996a; Hornung et al., 1996b; Kuiper et al., 2006; Olsman et al., 2007). The studies presented in this thesis included an exposure study in which zebrafish (selected as a model for aquatic organisms) were concurrently exposed to dietary PBDD/Fs, PCDD/Fs and PCBs to assess their uptake, retention and maternal transfer (Paper I), and a dose-response study in which dose-effects of selected PBDDs on different end-points, connected with reproduction, in zebrafish were investigated (Paper II).

![Figure 3.1 Uptake and elimination routes in fish. 1. Dietary uptake. 2. Uptake and elimination via gills. 3. Exchange in the gastro-intestinal tract: uptake from feed and elimination to faeces. 4. Elimination via metabolism and reproduction, dilution via growth. 5. Excretion.](image-url)

The physico-chemical properties of a substance govern its uptake, transfer, and elimination rates. In fish the relative importance of uptake of a substance from food and from water via the gills is dependent on the substance’s lipid/water partitioning properties (lipophilicity and water solubility), i.e., its log $K_{OW}$ (Qiao et al., 2000). The uptake of a substance both in the gut and through the gills proceeds via passage through membranes by passive diffusion. The partitioning process, i.e. diffusion, through the membranes is affected by the substance’s lipophilicity, and for uptake from feed the substance must migrate through the unstirred water layer near the
intestinal membranes, thus it is also affected by the substance’s hydrophobicity (Arnot et al., 2010; Hayton, 1980; Kelly et al., 2004; Loonen et al., 1993). Hence, superhydrophobic compounds, with log $K_{OW}$ values $> 6$–$7$, will have lower rates of transfer from feed to blood than others, due to their low water solubility (Arnot et al., 2010; Gobas et al., 1989). Molecular size may also influence substance’s uptake via membranes (Opperhuizen et al., 1985) but no definitive molecular size cut-off value has been established, and the validity of defined cut-off values has been questioned (Arnot et al., 2010). Nevertheless, large molecules are likely to have low uptake rates, due to the high energy required for them to cross the membrane. The same physico-chemical properties will also affect the reverse process, i.e. elimination from the body via gills and the exchange in the gut to faeces (Gobas et al., 1989).

Metabolism of PXDD/Fs occurs principally through their oxidation by CYP1A enzyme systems at two vicinal (adjacent) hydrogens, thus congeners lacking vicinal hydrogens tend to be most persistent, especially congeners with substituted lateral (2,3,7,8-) positions because of the associated sterical hindrance (Hu and Bunce, 1999). However, metabolic oxidations of PCDDs lacking vicinal hydrogens have been shown (Petroske et al., 1997), as well as epoxidation of the carbons adjacent to the central ether groups of TeCDD and subsequent ring opening, indicating that metabolic elimination of PXDD/Fs without vicinal hydrogens is possible (Hu and Bunce, 1999). In rats, metabolites of 2,3,7,8-TeBDD have been shown to form by debromination and hydroxylation (Dejongh et al., 1993). The reported elimination rates in rats of 2,3,7,8-TeBDD and TeCDD are similar, whereas 2,3,7,8-TeBDF was found to be more resistant to metabolism than its chlorinated analogue, probably due to the steric hindrance of the larger bromine atom (Birnbaum et al., 2003).

Maternal transfer of substances from fish to eggs is generally assumed to be driven by chemical partitioning between lipid pools, which should theoretically yield an egg/fish concentration ratio close to unity, on a lipid weight basis (Russell et al., 1999). However, transfer ratios smaller than one have been reported in some cases, possibly due to effects of the substances’ physico-chemical properties on the partitioning process (Serrano et al., 2008). The maternal transfer ratio of 2,3,7,8-TeCDD in zebrafish, following 10 ng/g feed exposure, has been estimated to be approximately 0.9 using lipid-adjusted values of egg and carcass concentrations (King Heiden et al., 2005; lipid contents derived from Nyholm et al.[2008]). However, at a high (270 ng/g) dose level the transfer ratio seemed to decrease.

Zebrafish (Danio rerio) has long been an important model organism in genetics, toxicology, and developmental biology and thus abundant information regarding effects and toxic responses on various chemicals are available (Hill et al., 2005; Hutchinson et al., 2006). The zebrafish has several
advantages for chemical testing, and is a recommended species for testing biological effects of chemicals (OECD, 2008). It is a small freshwater fish that can be easily bred and readily thrives, making it highly convenient for experimental studies. It also has a short generation time, and is thus suitable for reproductive and developmental studies. Frequent reproduction during an extended spawning season enables monitoring of chemical transfer from mother to egg more or less continuously during an exposure period, and rapid embryonic development makes zebrafish suitable for early-life stage development studies. Toxic and developmental effects are defined by specific endpoints (OECD, 2006). Endpoints related to reproductive output and early-life stage development that were monitored in the response study included spawning success (i.e. percentage of days with egg production), fertilization success (i.e. percentage of embryos reaching the four-cell stadium), and hatching success. Other markers of biological effects that were monitored included changes in gene expressions, e.g. for AhR regulated genes and enzyme inducions of ethoxyresorufin-O-deethylase (EROD; connected to detoxification) (Förlin et al., 1992; Örn et al., 1998) and glutathione reductase (GR; connected to oxidative stress) (Stephensen et al., 2002).

3.1 Dietary uptake, retention and maternal transfer of PBDD/Fs – exposure study Paper I

The uptake, elimination, and maternal transfer of mono- to tetraBDD/F congeners were investigated in the exposure study reported in Paper I. The study also included simultaneous exposure to a selection of mono- to octaCDD/F congeners and tetra- to octaPCB congeners for comparison, as these compounds have been thoroughly studied in exposure studies. However the observed dietary uptake, elimination and maternal transfer rates of PBDD/Fs will be mainly compared to those of their chlorinated analogues, PCDD/Fs, and PCB data will only be discussed briefly in this context.

Two groups of fish were exposed to spiked feed: one for six weeks (after which there was a six-week elimination period) while the other was exposed for 12 weeks to allow steady-state conditions to be reached for slowly equilibrating (highly lipophilic) compounds. A spiking level in feed below the no observed effect level (NOEL), calculated as toxic equivalents, for the total amount of spiking compounds (King Heiden et al., 2005) was selected, and a parallel control group was fed control (unspiked) feed throughout the 12-week period.

The change in concentration of a substance in fish after dietary exposure depends on the uptake rate from feed and the combined contributions of
elimination pathways, and can be expressed by the following equation:

\[ \delta C_{\text{fish}} / \delta t = k_1 * C_{\text{feed}} - k_2 * C_{\text{fish}} \]  

(1)

where \( C_{\text{fish}} \) is the concentration in fish, \( C_{\text{feed}} \) is the concentration in feed and \( t \) is time. The rate constants \( k_1 \) and \( k_2 \) are for uptake and elimination rates respectively. At steady-state \( k_1 \) and \( k_2 \) are equal. Integrated forms of Equation 1 were used to estimate the uptake rate constant (Equation 2), and the elimination rate constant (Equation 3) in the study. The data were fitted simultaneously by the least-squares method according to Nyholm et al. (2009):

\[ C_{\text{fish}} = C_{\text{feed}} \left( \frac{k_1}{k_2} \right) (1 - e^{-k_2 t}) \]  

(2)

\[ C_{\text{fish}} = C_0 e^{-k_2 t} \]  

(3)

The uptake efficiency in fish was calculated from \( k_1 \) using \( k_1 = u_{\text{eff}} * F \), where \( u_{\text{eff}} \) is the uptake efficiency and \( F \) is the feeding rate (here 2% of body weight/day). The half-life was calculated from \( k_2 \) using \( t_{1/2} = -\ln 2 / k_2 \).

3.1.1 Dietary uptake and elimination of PBDD/Fs compared to PCDD/Fs

The retention of PBDD/Fs congeners varied greatly and was generally lower than that of analogous PCDD/Fs. Uptake and retention rates of PCBs were generally high (>80% of dose), with some exceptions. There was considerable variation in concentrations between individual fish sampled at the same time point, in general concentrations differed by 10 to 40%, which may be attributed to individual differences in food consumption and/or pollutant uptake, metabolism, and excretion. Due to these variations, uptake efficiency and half-life rates were only calculated for congeners for which uptake and elimination rates had a coefficient of determination \( (r^2) > 0.7 \) to avoid over-interpreting the results (Paper I; Supplemental Data, Table S2).

For PXDD/F congeners, the highest net retention within the respective groups was seen for laterally substituted congeners, 2,3,7,8-TeBDD and 2,3,7,8-TeCDD. The concentrations in fish of 2,3,7,8-TeBDD and 2,3,7,8-TeCDD on the last day of the prolonged exposure period (day 87) were 260 and 1700 pg/g ww, equivalent to 30 and 100% retention of the given dose, respectively. Retention of less halogenated congeners was low, and retention of more highly chlorinated PCDD/Fs (penta- to octa-) decreased with chlorination degree. The period required to obtain 95% of the modeled steady-state concentration was estimated to be more than 200 days for most PCBs, approximately 100 days for 2,3,7,8-substituted PCDDs, and approximately 50 days for 2,3,7-TrBDD and 2,3,7,8-TeBDD. Thus, PXDD/Fs
were mostly at or close to steady-state by the end of the prolonged exposure period (87 days). Non-laterally substituted congeners showed no or only initial retention; of the PBDD/Fs only 1,3,6,8-TeBDD was considerably retained during the first weeks of exposure. Initial uptake of several other PXDD/Fs, both with and without vicinal hydrogens, was also detected, with body concentrations decreasing within two weeks, probably due to induction of detoxification enzyme systems (Figure 3.2).

Figure 3.2  Different uptake and elimination patterns observed in the study. (a) Uptake curve of 2,3,7,8-TeBDD (solid line) showing that steady-state concentrations were reached within the prolonged exposure period, and elimination curve (stripe-dotted line) showing exponential decrease in body concentration after 42 days; (b) Uptake curve of 1,3,6,8-TeBDD, showing initial uptake during the first weeks of exposure followed by a rapid elimination. Cross (+) denote concentrations during exposure; triangles (△) denote concentrations during the elimination phase. Curves in (a) modeled from Equations 2 and 3. From Paper I.

Half-lives of retained PXDD/Fs ranged from 5 to 36 days and were generally lower or much lower than those of PCBs (12–160 days). Laterally substituted 2,3,7,8-TeCDD and 2,3,7,8-TeBDD had the longest half-lives, 36 and 12 days, respectively, of their respective PXDD/F groups. The fact that the bromine-carbon bond is weaker than the chlorine-carbon bond may explain the faster elimination of 2,3,7,8-TeBDD. However, as also observed in mammalian elimination studies (Birnbaum et al., 2003), laterally substituted 2,3,7,8-TeCDF and 2,3,7,8-TeBDF showed the opposite pattern, as only 2,3,7,8-TeBDF was retained throughout the exposure period, with a half-life of five days. Slower metabolism due to steric hindrance caused by the large bromine atom may explain this difference.

As metabolic transformation of PXDD/Fs occurs principally through oxidation by CYP1A enzyme systems at two vicinal hydrogens (Hu and Bunce, 1999), congeners with this structural feature should theoretically be
more rapidly eliminated than other congeners. This was found to be true for mono- to triBDD/Fs, except for laterally substituted triBDD/Fs (2,3,7-TrBDD and 2,3,8-TrBDF), and for mono- and triCDDs (for which no uptake was observed), and for mono- to triCDF (for which only initial uptake was observed). However, increased elimination after initial uptake was also observed for congeners lacking vicinal hydrogens (1,3,6,8-TeBDD, 1,3,7,8-TeBDD, 1,3,6,8-TeCDF, 2,3,7,8-TeCDF), suggesting that metabolic processes are involved in their elimination too. A probable transformation pathway is epoxidation of the carbons adjacent to the central ether groups of PXDD/Fs and subsequent ring opening (Hu and Bunce, 1999). The relatively high elimination of peri-substituted 1,3,7,9-TeBDD, and laterally substituted 2,3,7,8-TeBDF/-TeCDF observed, may also indicate transformation mediated via this metabolic pathway, facilitated by lesser steric hindrance of the ether bond by halogen substitution in these compounds, in comparison with the more strongly retained 1,3,6,8-TeBDD and laterally substituted PXDDs, respectively. It has been suggested that differences in the metabolic pathways affecting 2,3,7,8-TeCDF and 2,3,7,8-TeCDD may be due to electronic factors (Veerkamp et al., 1983).

Several possible metabolites of PBDD/Fs were sought in the tissue and gasto-intestinal tract of exposed zebrafish. Hydroxylated and methylsulphonyl-metabolites were analyzed using the method of Hovander et al. (2000) modified for hydroxylated polybrominated diphenylethers (Löfstrand et al., 2011), and possible conjugates were analyzed using the method of Söderström et al. (1994). However, no metabolites were detected in these analyses.

The results show that laterally substituted PXDD/Fs are preferentially taken up and retained, that PBDD/Fs generally show lower retention than PCDD/Fs, and that analogous congeners show similar uptake and elimination patterns.

### 3.1.2 Maternal transfer of PBDD/Fs and PCDD/Fs

For all but a few assimilated compounds, transfer of the substance to eggs was observed. The changes in the internal dose of the fish during the elimination phase could be followed in the eggs because of the short interval between spawnings (Paper I, Figure 4). A weak linear relationship between fish and egg concentrations was found for 2,3,7,8-TeCDD ($r^2=0.64$) and 2,3,7,8-TeBDD ($r^2=0.60$). Transfer factors (egg to fish concentration ratios) based on lipid weight-adjusted concentrations in egg and fish, could be determined for 2,3,7- and/or 8-substituted congeners of PBDD/Fs and they were highly variable, ranging from 0.18 to 1.3 (Paper I, Table 2). Transfer factors for PCDD/Fs were determined for tetra- to hexaCDD/Fs and were slightly less variable, ranging from 0.25 to 1. Most transfer factors were < 1, with lower transfer factors for more highly halogenated congeners. The
transfer factors were similar for tetraBDD/Fs (0.18–0.25) and hexaCDD/Fs (0.25–0.35), which have similar log $K_{ow}$ values, suggesting that transfer rates of the compounds are mainly affected by their lipophilicity and water solubility.

The results indicate that most compounds used in the exposure study were transferred from fish to eggs through passive diffusion and that the partitioning (tissue/blood and blood/egg) of highly halogenated congeners seems to be too slow (due to their low aqueous solubility) for body-to-egg equilibrium to be reached before spawning.

3.2 Effects of PBDDs in zebrafish – dose-response study  Paper II

The effects of PBDDs in fish were examined in a dose-response study at three exposure levels. The PBDDs investigated were chosen because of their presence in Baltic biota, and the levels of individual congeners were added to feed in proportions measured in biota, ranging from 1 to 50 (Paper II, Table 1) (Haglund et al., 2007). 2,3,7,8-TeBDD was used as a positive control since its uptake in zebrafish is high (as stated in section 3.1.1) and it was expected to have effects because of its structural similarity to 2,3,7,8-TeCDD, which has been shown to be maternally transferred and to have reproductive effects in zebrafish (King Heiden et al., 2005; King Heiden et al., 2006). Dietary exposure of zebrafish to either the mixture of PBDDs (Baltic Sea mixture), or 2,3,7,8-TeBDD (positive control) were performed during 9 weeks with doses one log unit apart. Concentrations of PBDDs in eggs were followed during exposure, and retention in female fish was measured from the end of exposure for up to two weeks after exposure. The reproductive effects of the test compounds were followed by monitoring spawning success, fertilization of eggs, offspring early life-stage development, vitellogenin gene expression, and histopathological inspection of ovaries (Selman et al., 1993). Effects connected to the AhR pathway were investigated by measuring EROD activity and expression of AhR-related genes, and potential stress effects were monitored by measuring GR activity and induction of genes responsive to intracellular stress.

3.2.1 General effects of PBDDs

General toxic effects were detected in the high dose, positive control group, where mortality was increased (23% compared to <2% in all other groups), body weight decreased, and spawning ceased after one week. Because of the health effects this group was sampled before the end of the exposure period (at six weeks). Spawning and body weight were also affected in the medium dose, positive control group, but the exposure of this group was maintained for the full nine weeks. All other groups showed good health.

Although relative concentrations of PBDDs in the Baltic Sea mixture were meant to reflect concentrations in Baltic biota, the net retention pattern in
zebrafish was different; the most strongly retained congener in zebrafish was 1,3,6,8-TeBDD whereas concentration profiles in biota are dominated by di- and triBDDs. Nevertheless, all congeners were detected in fish after nine weeks (Paper II, Table 3). Retention of substances supplied at low dose with low propensity for metabolism (2,3,7-TrBDD and 2,3,7,8-TeBDD) was comparable to their retention in the exposure study. However, substances that showed low retention in the exposure study, possibly due to metabolism (1,3,7/-1,3,8-TrBDD and 1,3,6,8/-1,3,7,9-TeBDD) were more highly retained, or much more highly retained in this study, probably because of weak induction of detoxification systems, since substances with high capability of inducing these systems (PCDD/Fs and PCBs) were not included. In groups exposed to the Baltic Sea mixture, dose-related concentrations of 2,3,7,8-TeBDD were detected, and the feed was found to contain trace levels of 2,3,7,8-TeBDD (approximately 0.3% of the positive control level), probably originating from impurities in standards used in the feed preparation.

**Table 3.1** Differences in enzyme activities and gene expressions of AhR-regulated genes compared to control group. 0 denote no difference, ** and *** denote significant (p ≤ 0.01 and p ≤ 0.001, respectively) difference. Distinct difference in activity or expression between sexes is denoted as female / male. Δ sex: difference in vtg1 expression between female/male, where difference in control = ***. Adapted from Paper II.

<table>
<thead>
<tr>
<th></th>
<th>Mix-L</th>
<th>Mix-M</th>
<th>Mix-H</th>
<th>TBDD-L</th>
<th>TBDD-M</th>
</tr>
</thead>
<tbody>
<tr>
<td>EROD activity</td>
<td>0</td>
<td>0/**</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>GR activity</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0 / **</td>
<td>***</td>
</tr>
<tr>
<td>cyp1a1</td>
<td>-</td>
<td>0</td>
<td>**</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>ahr2</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>*** / 0</td>
</tr>
<tr>
<td>ahrr2</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>0 / **</td>
<td>***</td>
</tr>
<tr>
<td>vtg1</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>*** / 0</td>
</tr>
<tr>
<td>Δ sex</td>
<td>-</td>
<td>-</td>
<td>***</td>
<td>**</td>
<td>0</td>
</tr>
</tbody>
</table>

*: not determined

**EROD**: ethoxyresorufin-O-deethylase

**GR**: glutathione reductase

**cyp1a1**: cytochrome P450 1A

**ahrr2**: aryl hydrocarbon receptor 2

**ahrr2**: aryl hydrocarbon receptor repressor 2

**vtg1**: vitellogenin 1

The retention of PBDDs was dose-related, the percentage of the dose retained decreasing with increases in the level of exposure, previously observed for TeCDD and 2,3,7,8-TeBDD in rat (Birnbaum et al., 2003). This may be attributed to the induction of metabolic enzyme systems, a hypothesis supported by the increased activity of EROD and up-regulation of some AhR-related genes observed in positive control groups and the high dose group of Baltic Sea mixture. Increased GR activity and some up-regulation of stress-responsive genes were also observed in positive control groups, but not in Baltic Sea mixture groups (Table 3.1; Paper II, Figures 5-7).
The reduction of concentrations in fish during the elimination phase after nine weeks exposure was followed for one week in the Baltic Sea mixture groups (two sampling points) and for two weeks in the positive control groups (three sampling points) (Table 3.2). In the low and medium dose Baltic Sea mixture groups all of the retained compounds had half-lives of 1-3 days except 1,3,6,8-TeBDD (4-8 days). In the high dose group only the elimination of mono- and diBDDs followed an exponential decline (2-4 days). Among the positive controls elimination was insignificant in the low dose group, which may be attributed to a lower degree of induction of detoxification enzymes, but at medium dose the half-life was 17 days, comparable to the half-life obtained in the exposure study.

Table 3.2 Half-lives (t½) in fish and transfer factors from egg to fish. Half-lives calculated from the exponential decline of fish concentrations followed for one week (Baltic Sea mixture), or two weeks (positive control) during the elimination phase. Values for t½ are not given for cases where the r² value of the exponential curve is <0.7. Transfer factors are lipid adjusted concentration ratios of egg to female fish, after 9 weeks exposure. Adapted from Paper II.

<table>
<thead>
<tr>
<th>Baltic Sea mixture</th>
<th>Low Half-life, t½ (days)</th>
<th>Transfer factor</th>
<th>Medium Half-life, t½ (days)</th>
<th>Transfer factor</th>
<th>High Half-life, t½ (days)</th>
<th>Transfer factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-MBDD</td>
<td>1.2</td>
<td>0.10</td>
<td>1.1</td>
<td>0.12</td>
<td>1.6</td>
<td>0.41</td>
</tr>
<tr>
<td>2,7/2,8-DBDD</td>
<td>1.6</td>
<td>0.30</td>
<td>1.7</td>
<td>0.27</td>
<td>4.0</td>
<td>2.4</td>
</tr>
<tr>
<td>1,3,7-TrBDD</td>
<td>2.0</td>
<td>0.29</td>
<td>2.5</td>
<td>0.25</td>
<td>-</td>
<td>1.8</td>
</tr>
<tr>
<td>1,3,8-TrBDD</td>
<td>2.2</td>
<td>0.40</td>
<td>3.1</td>
<td>0.31</td>
<td>-</td>
<td>2.5</td>
</tr>
<tr>
<td>2,3,7-TrBDD</td>
<td>1.8</td>
<td>&lt;LOD</td>
<td>2.7</td>
<td>0.49</td>
<td>-</td>
<td>4.4</td>
</tr>
<tr>
<td>1,3,6,8-TeBDD</td>
<td>4.4</td>
<td>0.07</td>
<td>7.8</td>
<td>0.08</td>
<td>-</td>
<td>0.08</td>
</tr>
<tr>
<td>1,3,7,9-TeBDD</td>
<td>2.0</td>
<td>&lt;LOD</td>
<td>-</td>
<td>0.41</td>
<td>-</td>
<td>1.7</td>
</tr>
<tr>
<td>1,2,4,7/1,2,4,8-</td>
<td>1.4</td>
<td>0.57</td>
<td>3.5</td>
<td>1.1</td>
<td>-</td>
<td>7.2</td>
</tr>
<tr>
<td>TeBDD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,2,3,7-TeBDD</td>
<td>&lt;LOD</td>
<td>nd</td>
<td>-</td>
<td>&lt;LOD</td>
<td>-</td>
<td>14</td>
</tr>
<tr>
<td>1,2,3,8-TeBDD</td>
<td>&lt;LOD</td>
<td>nd</td>
<td>-</td>
<td>2.4</td>
<td>-</td>
<td>17</td>
</tr>
<tr>
<td>positive control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,3,7,8-TeBDD</td>
<td>-</td>
<td>0.26</td>
<td>17</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

<LOD : concentration below level of detection
- : r² for elimination curve <0.7
nd : not determined
NA : not analyzed

Health was thus significantly affected in the 2,3,7,8-TeBDD (positive control) groups, but only affected to a limited extent in the Baltic Sea mixture groups. In contrast to the exposure study, retention of all congeners in the Baltic Sea mixture groups was seen throughout the study period.

3.2.2 Reproductive effects of PBDDs

All substances that were retained in the fish were transferred to eggs, and the maternal transfer factor of 2,3,7,8-TeBDD was comparable to that derived from the exposure-study. Transfer factors increased with increasing
Dietary exposure studies of PBDD/Fs in zebrafish

dose, and hence were highest for fish exposed to the high dose, Baltic Sea mixture (Table 3.1). Since metabolism in egg and embryo is low to non-existent (Petersen and Kristensen, 1998), this would also support the theory of induced metabolic elimination of PBDDs in the fish at higher doses. In addition, concentration ratios between dose levels (i.e. 1:10:100) of Baltic Sea mixture were reflected in the eggs to a higher degree (average 1:5:86), than in the fish (average 1:6:17), further strengthening the hypothesis.

Effects connected to reproduction could be seen primarily in the 2,3,7,8-TeBDD exposed groups (positive control). Spawning was affected in the positive control groups, in which a dose-related spawning response was observed; the high dose group stopped spawning after one week and the medium dose group after five weeks. In contrast, no dose-related effect of the Baltic Sea mixture was observed. Histopathological examination also revealed significant dose-related effects on ovarian morphology in the positive control groups; the percentage of mature eggs (vitellogenic oocytes) decreasing and frequencies of degenerated eggs (atretic follicles) increasing with increasing dose (Figure 3.3). Again no significant effects were seen for the Baltic Sea mixture.

![Figure 3.3](image)

**Figure 3.3** Reproductive effects on eggs and offspring. Proportions (%) of mature eggs (white bars) and degenerated eggs (dotted bars) after 9 weeks exposure to control, Baltic Sea mixture (Mix) or positive control (TBDD) feed at low (L), medium (M), or high (H) dose. Proportions (%) of unfertilized or abnormal offspring (striped bars) at 5 h post fertilization in exposure week 8–9. Values are presented as mean ± standard deviation. * and *** denote significant (p≤0.05 and p≤0.001, respectively) difference to control (eggs) or pre-exposure offspring development (offspring). Adapted from Paper II.
Increased numbers of unfertilized and abnormal offspring at exposure week 8–9 were observed in both the positive control and in the high dose, Baltic Sea mixture groups. However, the results for the Baltic Sea mixture group may have been affected by the low levels of 2,3,7,8-TeBDD present in the Baltic Sea mixture feed; for the high dose feed this would have been equivalent to approximately 1/3 of the 2,3,7,8-TeBDD concentration of the low dose, positive control feed. Thus, the detected effect, at comparable levels for both groups, might have been due to the presence of 2,3,7,8-TeBDD. Hence, the number of unfertilized and abnormal offspring seems to be the most sensitive endpoint for reproductive effects of PBDDs, as exposure to the high dose of the Baltic Sea mixture also significantly affected this variable.

### 3.3 Uptake and transfer – discussion related to properties of PBDD/Fs and PCDD/Fs

In the exposure study described in Paper I, a clear relationship between the retention of specific PXDD/Fs congeners and their halogenation degree was found (Figure 3.4).

![Graph of PXDD/F muscle concentrations](image)

**Figure 3.4** Graph of PXDD/F muscle concentrations in pg/g wet weight vs. number of halogens, showing major processes influencing the retention of less to highly halogenated congeners. From Paper I.

For both PCDD/F and PBDD/F congeners, retention in zebrafish was low for mono- to trihalogenated congeners, presumably due to high metabolic and excretion rates. Laterally substituted PXDDs were highly retained and the highest retention of all PXDD/F congeners was seen for 2,3,7,8-TeCDD, thereafter concentrations of more highly chlorinated (penta- to octa-) PCDD/Fs decreased with increasing degree of chlorination (Paper I;
Supplemental Data, Table S2). A similar trend was observed in the uptake efficiencies, suggesting that the lower tissue concentrations of highly chlorinated PCDD/Fs were due to limited uptake. The uptake of PCDD/Fs was inversly correlated to their respective water solubilities and log $K_{\text{OW}}$ values showing a clear negative correlation of water solubility with increasing chlorination (Figure 3.5).

Of interest is also the significant difference in uptake between the most strongly retained congeners of the two halogen groups: 2,3,7,8-TeCDD and 2,3,7,8-TeBDD, which share the same backbone structure, substitution pattern, and have similar molecular volumes (SPARC On-line calculator). These congeners have significantly different log $K_{\text{OW}}$ values (6.9 and 7.9, respectively) reflecting their difference in water solubility of approximately one order of magnitude. Notably, in this context, 1,2,3,4,7,8-HxCDD, which has a similar log $K_{\text{OW}}$ value to 2,3,7,8-TeBDD (estimated log $K_{\text{OW}}$ 8.2 vs. 7.9), seemed to be retained to a similar extent as 2,3,7,8-TeBDD (Figure 3.4). The relation between uptake and water solubility may be understood in terms of factors affecting the diffusion of the substance from feed to cells (Dulfer et al., 1996), since it must be transferred through the unstirred water layers adjacent to the cell membranes before absorption into the cells, and low water solubility of the substance will affect the overall membrane transport. Therefore, the low bioavailability of highly chlorinated PCDD/Fs is probably the rate-limiting factor for their uptake.

Different TeBDD congeners may have the same (calculated) log $K_{\text{OW}}$ value, but their retention in zebrafish may vary substantially, reflecting differences in their propensity for metabolic transformation. The combined
effects of propensity for metabolism transformation and hydrophobicity can thus explain the uptake patterns observed in the reported studies.

The transfer of substances from mother to egg is generally considered to be a lipid-lipid partitioning process, where lipid-adjusted egg/fish concentration ratios will be close to one at equilibrium. However, transfer ratios may be affected by physico-chemical properties of the substances and ratios < 1 have been reported for highly chlorinated PCBs, *i.e.* substances with high K\textsubscript{OW} values (Serrano et al., 2008). Transfer factors < 1 were also seen for most substances in the exposure study and for all PBDD congeners in the dose-response study, at low PBDD-dose exposure. However, at higher exposure doses significantly higher transfer factors (> 1) were seen for congeners with low retention in fish, while congeners that were moderately or strongly retained in fish still had transfer factor ratios < 1. This suggests that egg concentrations reflect the actual exposure to these congeners whereas the concentrations in fish are affected by metabolism, and that monitoring of intermittent exposure, like dietary exposure, of easily metabolized substances, will give a more complete estimate of the actual exposure using egg concentrations as well.

Since metabolism in egg and embryo is low to non-existent (Petersen and Kristensen, 1998), this also supports the hypothesis that metabolic elimination is induced in the fish at higher doses since the transfer ratios increase with increasing dose. It also implies that exposure to substances that are metabolized in fish may be higher for vulnerable life stages (eggs and embryos).

### 3.4 Retention and effects — comparison with Baltic Sea data

The congener pattern obtained from the exposure of zebrafish to PBDD/Fs and the PBDD congener profiles from Baltic Sea biota have striking differences (Figure 3.6). The zebrafish study confirmed that congeners with vicinal hydrogens, *i.e.* mono- to triBDD/Fs and certain TeBDDs, indeed were quickly eliminated as expected, and did not reach high concentrations. In contrast, the most abundant congeners in marine organisms from the Baltic, *e.g.* perch, are DBDDs and TrBDDs.

As relatively high concentrations of these easily metabolized congeners are found in Baltic fish, the fish would be continuously exposed to high or very high concentrations of PBDDs. The exposure could arise from either feeding or diffusive uptake from water over the gills. Water concentrations of PBDDs have not been determined to my knowledge. A further contribution to the differences in congener pattern between perch and zebrafish may be differences in metabolic rates between zebrafish, as tropic species, compared to cold water fish (Sobek et al., 2010).

The results of the dose-response study indicate that the biological significance of PBDD exposure for marine organisms in the Baltic Sea may
be small, but possibly significant. As high doses seem to induce metabolic systems, continuous exposure to high concentrations of PBDDs may lead to overall increases in the activity of metabolic systems. In addition, as eggs seem to receive higher internal doses compared to fish due to a lack of metabolic capacity, relatively high exposure may occur at vulnerable life stages.

Figure 3.6  Comparison of PBDD/F congener patterns observed in the exposure study (upper panel), and biota (perch) from the Baltic Sea (lower panel). The congener concentrations of compounds spanned by the brackets are magnified 10 times (10 X). Perch data from Haglund et al. (2007). Figure from Paper I.
6BDietary exposure studies of PBDD/Fs in zebrafish
4 Biotic and abiotic formation of PBDD/Fs

The high levels of PBDD/Fs found in biota from the Baltic Sea have raised the question whether the sources of these compounds are of anthropogenic or natural origin. PBDD/Fs can be unintentionally produced by various high-temperature processes involving brominated material, e.g. incineration of products containing brominated flame retardants, analogously to the formation of PCDD/Fs (Gullett et al., 2007; Sidhu et al., 1995; Söderström and Marklund, 2002; Weber and Kuch, 2003). The PBDD/Fs formed in those processes are predominantly PBDFs, and overall more highly brominated congeners than those found in marine biota (Hayakawa et al., 2004; Ren et al., 2011). Furthermore, the geographical and temporal variations in the concentrations of PBDD/Fs in biota are indicative of diffuse, local sources (Haglund et al., 2007). Thus, the PBDD/Fs seems to have natural origins (Haglund et al., 2010; Malmvärn et al., 2008), but the processes involved are uncertain. Two possible formation pathways were primarily considered in the studies in this thesis: biotic formation via dimerization of bromophenols by bromoperoxidase (BPO) and abiotic formation via UV-initiated cyclization of hydroxylated polybrominated diphenyl ethers.

4.1 Bromoperoxidase-mediated formation of PBDD/Fs Paper III

Peroxidases comprise a large family of enzymes that are widely dispersed in the cellular compartments, of primarily marine organisms (Butler and Walker, 1993; Pedersén et al., 1996). Algae, marine sponges and bacteria produce a broad range of structurally different brominated substances (Gribble, 2000; Vetter, 2006), and BPOs are likely involved in their biosynthetic production (Butler and Carter-Franklin, 2004; Soedjak et al., 1995; Winter and Moore, 2009). BPOs scavenge peroxide formed during photosynthesis and in responses to various stresses in cells, brominating and oxidizing suitable substrates in the process (Butler and Carter-Franklin,
2004; Littlechild and Garcia-Rodriguez, 2003). The halogenated products formed in the scavenging process could be regarded as waste materials (Collén and Pedersén, 1994; Pedersén et al., 1996) but the halometabolites have also a proposed function as predator-deterring agents (Johnson et al., 2011; Whitfield et al., 1999). Peroxidases also have demonstrated involvement, inter alia, in phenolic coupling reactions of several enzyme-substrate systems (Berglin et al., 2004; Dec and Bollag, 1994; Eickhoff et al., 2001; Henriksen et al., 1999; Laurenti et al., 2003; Salgado et al., 2009), and peroxidase-mediated formation of PCDD/Fs from chlorophenols has been shown, albeit in low yields (Öberg et al., 1990; Silk et al., 1997; Svenson et al., 1989; Wittsiepe et al., 1999; Wittsiepe et al., 2000). The PCDD/F products formed in peroxidase-mediated dimerization has been attributed to the coupling of a phenoxy radical to a phenol or the coupling of two phenoxy radicals, followed by cyclization with or without loss of a hydroxy group (Hoekstra et al., 1999). A similar formation pathway involving BPO has been suggested for the formation of PBDD/Fs (Haglund et al., 2007).

The production of high levels of bromophenols in algae (Flodin and Whitfield, 1999a; Whitfield et al., 1999) and the widespread occurrence of BPOs in algae (Moore and Okuda, 1996) makes this an interesting possible formation pathway of PBDD/Fs. This is thought to proceed through enzymatic-mediated oxidative coupling of bromophenols, analogously to the peroxidase-mediated formation of PCDD/Fs. The proposed mechanism for general oxidation of phenolic substrates by horseradish peroxidase (HRP) involves hydrogen abstraction from the hydroxyl group and subsequent electron transfer to the enzyme, yielding a phenoxy radical (Henriksen et al., 1999). No mechanism for oxidation of phenols by BPO has been described, but formation of a phenoxy radical could involve hydrogen abstraction during the formation of a peroxy-intermediate, and electron transfer, analogously to the proposed one-electron transfer step in BPO sulfide oxidation of sulfides (ten Brink et al., 2001). BPOs have been shown to have hydrophobic residues at a possible substrate binding site (Littlechild et al., 2009) and adduct formation of substrate and enzyme has been proposed to control the rate of coupling reaction as the coupling presumably occurs in close proximity to the enzyme (Berglin et al., 2004).

4.1.1 Experimental
In the study reported in Paper III, BPO from the red alga Corallina officinalis, which is known to have a high BPO content (Moore and Okuda, 1996), was incubated in buffer solutions with 2,4,6-TrBP as substrate. 2,4,6-TrBP was selected as a model compound for the enzymatic coupling experiments, because of its high abundance in algae and other marine organisms. The rate of coupling was monitored over time, and the influence of pH on product formation was evaluated by examining BPO activity at its optimum pH (6.5)
and both one pH unit higher and lower (representing different physiological pH). To mimic natural conditions an experiment was also conducted at low temperature (4°C and pH 6.5). Each experiment was performed in triplicate except for the time series, in which there were only triplicates of samples incubated for 24 h, all the others were single samples. The amounts of mono- to tetraBDD/Fs formed were analyzed in all samples.

4.1.2 Enzymatic formation of PBDD/Fs

In the incubations of BPO with 2,4,6-TrBP, all experiments showed the formation of the direct condensation product 1,3,6,8-TeBDD (Figure 4.1). In the time-series experiments, the formation rate remained constant throughout the investigated period of 2 to 48 h.

![Figure 4.1 Scheme of the direct condensation of 2,4,6-TrBP to 1,3,6,8-TeBDD via (2’-OH-2,3′,4,5′,6′) phenoxyphenol radical, putatively formed during dimerization of 2,4,6-TrBP.](image)

Formation of 1,3,8-TrBDD and 1,2,4,7- and/or 1,2,4,8-TeBDD was also detected in the pH dependence experiment, in which higher phenol and enzyme concentrations were used, with no significant pH-related differences in yields (Table 4.1). The latter two TeBDD congeners cannot be separated chromatographically, thus they could not be unambiguously identified. Nevertheless, both congeners are possible products of the enzymatic coupling, as illustrated in Paper III, Figure 2. Since the binding site of the enzyme has hydrophobic residues the enzyme could possibly discriminate between the phenol and the phenolate, giving a lower reaction rate when the phenolate dominates. With a pKa of 6.08 for 2,4,6-TrBP (Table 2.2), the substrate will span from mostly protonated to mostly deprotonated specie in the pH range used; with dissociation fractions of 21% at pH 5.5, 72% at pH 6.5 and 96% at pH 7.5. However, the yields were not shown to be significantly influenced by the fraction of phenolate, although yields were lower at pH 7.5.

The most complex congener pattern was obtained in the low temperature experiment, where the previously mentioned congeners were found together with the minor components 1,3,7,9-TeBDD, 1,3,7-TrBDD, 2,3,7-TrBDD and 2,7/-2,8-DBDD (which co-elute) (Table 4.1). The minor components were detected in all three replicates, but were rarely observed in the other experiments. The identified di- and tribrominated congeners
were formed by debromination of more highly brominated congeners. Occasional traces of PBDFs were detected in a few samples. All of the PBDDs that were formed by the BPO-mediated dimerization of 2,4,6-TrBP have been found in biota samples from the Baltic Sea, and the congener profile resembles the profile obtained from analysis of mussel from the Swedish West Coast (Haglund et al., 2007) (see section 4.3).

Table 4.1 Amounts of PBDDs (nmol PBDD/mol TrBP) formed during incubations of 2,4,6-TrBP with BPO at ambient temperature and varied pH, and low temperature (4°C) at optimal pH 6.5. Figures are mean values ± 1 standard deviation obtained from triplicate incubations. ** denote significantly (p<0.01) higher PBDD formation at low temperature than at ambient temperature.

<table>
<thead>
<tr>
<th>nmol/mol TrBP</th>
<th>pH 5.5</th>
<th>pH 6.5</th>
<th>pH 7.5</th>
<th>pH 6.5 4°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>27/28-DBDD</td>
<td></td>
<td></td>
<td></td>
<td>10 ± 2.3</td>
</tr>
<tr>
<td>137-TrBDD</td>
<td></td>
<td></td>
<td></td>
<td>27 ± 6.3</td>
</tr>
<tr>
<td>138-TrBDD</td>
<td>25 ± 4.4</td>
<td>16 ± 4.4</td>
<td>8.9 (n=1)</td>
<td>59 ± 9.3 **</td>
</tr>
<tr>
<td>237-TrBDD</td>
<td></td>
<td></td>
<td></td>
<td>5.3 ± 1.1</td>
</tr>
<tr>
<td>1368-TeBDD</td>
<td>90 ± 19</td>
<td>100 ± 41</td>
<td>65 ± 32</td>
<td>650 ± 110 **</td>
</tr>
<tr>
<td>1379-TeBDD</td>
<td></td>
<td></td>
<td></td>
<td>79 ± 20</td>
</tr>
<tr>
<td>1247/1248-TeBDD</td>
<td>140 ± 150</td>
<td>130 ± 110</td>
<td>45 ± 13</td>
<td>280 ± 360</td>
</tr>
</tbody>
</table>

The yields in all incubations were low, but significantly higher in the low temperature experiment, which yielded 650 nmol/mol of 1,3,6,8-TrBDD compared to 100 nmol/mol in the corresponding room temperature experiment. Since the same products were formed at both low and room temperatures the difference is probably not related to the formation mechanism of PBDD but rather the relative rates of competing coupling, oxidation and debromination reactions (Laurenti et al., 2003). For example, the relatively low formation of 1,3,8-TrBDD at 4°C may be due to a reduced rate of debromination.

The formation of 1,3,7,9- and 1,2,4,8-TeBDD presumably involves a Smiles rearrangement *via* a spiro-intermediate (Sidhu et al., 1995) (Figure 4.2). Spiro coupling would be promoted by the bromine substitution in position 6 (Bernasconi and Fairchild, 1988). The ether bonds of this putative intermediate are connected with the dibrominated phenyl ring perpendicular to the other (tribrominated) phenyl ring; thus rearrangement to the adjacent carbon with bromine loss could proceed to either position leading to 1,3,6,8- or 1,3,7,9-TeBDD. However, since the yield of 1,3,7,9-TeBDD is much lower than that of 1,3,6,8-TeBDD, this formation pathway would not be dominating (Sidhu et al., 1995).
4.2 Photochemical formation of PBDD/Fs Paper IV

For photochemical reactions to occur, one of the molecules involved must absorb energy in the emission spectrum of the light source. The ability to absorb light by a molecule is determined by its molar absorption coefficient, ε (M⁻¹·cm⁻¹). After absorption of a photon, transition of the molecule to an excited state occurs. The excess energy of the excited molecule is then dissipated through e.g. dissociation or other chemical reactions. The energy of a photon is given by E = h c / λ where h is Planck’s constant, c is the speed of light and λ is the wavelength, thus shorter wavelengths (UV) yield photons with higher energy. Solar light at the Earth’s surface has a wavelength span of approximately 280 nm up to 4 000 nm, and the range of 280–400 nm (shorter wavelengths than visible light) is called UV-light. Halogenated aromatic compounds usually have absorption spectra that reach this range and thus meet the prerequisites for photochemical reactions. Brominated compounds, such as OH-PBDEs, are especially susceptible to UV-irradiation since the bromine-carbon bond is weak (Bastos et al., 2009; Neupert et al., 1988). Although the main process of UV irradiation of OH-PBDEs is debromination to lower brominated congeners (Eriksson et al., 2004; Söderstrom et al., 2004; Watanabe and Tatsukawa, 1987) other reactions, such as cyclization, after UV-induced excitation are possible (Bastos et al., 2009; Steen et al., 2009). Because of their acidity, OH-PBDEs can be deprotonated at higher pH, which results in a shift in spectra towards higher wavelengths (Steen et al., 2009). Thus, the fraction of ionic species in a solution will affect the overall absorption spectrum, and hence the overlap with the emission spectrum of the light source and consequently the possibility of photochemical reactions.
For halogenated diphenylethers substituted with a hydroxyl group ortho to the ether bond and a halogen substituent in the ortho position of the non-hydroxylated phenyl ring, intramolecular coupling to halogenated dibenzo-p-dioxins is possible. The reaction can be initiated by UV-light absorption, as shown for Triclosan, a chlorinated hydroxylated diphenyl ether used as an antibacterial agent in various household products (e.g. toothpaste), for the environmentally abundant tetrabromo OH-BDE 47, and for chlorinated derivatives thereof, all of which cyclize to the corresponding halogenated dioxin after UV-irradiation (Buth et al., 2009; Latch et al., 2005; Steen et al., 2009; Tixier et al., 2002). All reported OH-PBDEs of natural origin are substituted with the hydroxyl group in ortho position and bromine in the 2- or 2,4-position of the other aromatic ring (Kelly et al., 2008; Marsh et al., 2003). They thus fulfill the criteria for formation of PBDDs via photochemically induced intramolecular coupling.

Natural waters contain dissolved organic carbon (DOC), in forms such as fulvic acid, which absorb light and have photo-sensitive properties (Liu et al., 2011; Yu et al., 2010). Fulvic acid can enhance phototransformations of other substances, as it can produce excited species that reacts with them (indirect phototransformations), but also compete for incoming light energy, thereby lowering the direct phototransformation rate of other substances. As the formation of PBDDs is believed to take place in coastal areas where DOC levels are high due to outflow from rivers and high productivity, the possibility that it may enhance or reduce photochemical reaction rates of PBDDs was explored.

4.2.1 Experimental

The process of PBDD formation from OH-PBDEs was studied under conditions that were chosen to mimic the Baltic Sea environment, using tetrabromo OH-BDE 47 and 68, and pentabromo OH-BDE 85, 90, 99 and 123 (Figure 2.4). These are reportedly among the most abundant congeners in Baltic biota, and reported from various trophic levels: algae, shellfish and fish (Malmvärn et al., 2005a). OH-PBDEs were added to petri dishes containing artificial coast water (ACW) (Kester et al., 1967) at pH 8.4, with or without addition of DOC (fulvic acid) giving three different matrices: ACW only, and AWC supplemented with DOC at either 5 mg or 10 mg carbon/L (DOC5 and DOC 10, respectively). Irradiation was performed by using an artificial UV-light source or natural sunlight. The products of each congener were separately examined, and the total congener profile of PBDDs formed was compared to the PBDD congeners found in biota. Product formation over time in mixtures of selected OH-PBDEs was investigated, and the influence of DOC on the product profile was explored. Finally the products formed in artificial and natural sunlight were compared. The concentrations of mono- to tetraBDD/Fs were analyzed for all samples.
4.2.2 Formation of PBDD/Fs by UV-light

Following UV-irradiation, all OH-PBDEs yielded their corresponding cyclized PBDD product with bromine-loss, which was the main product obtained for tetrabromo OH-PBDEs and pentabromo OH-BDE 90 and 99 (≥ 66% of total PBDDs formed). Other products detected were debrominated congeners from the cyclized product, and the primary debromination product was the main product for OH-BDE 85 and OH-BDE 123 (≥ 48% of total PBDDs formed). Over time the relative yields of products changed, as debromination of the formed products continued (Paper IV, Figure 3). The respective product formation pathways are presented in Paper IV; Figure 2 and Supplemental information Figure S2.

The total yields of the products were in the percent range, and highest for tetrabromo OH-BDEs 47 and 68 (13 and 10 mmol/mol, respectively) (Figure 4.3). The major products from these two congeners were 1,3,7-TrBDD and 1,3,8-TrBDD, respectively, which are highly abundant in the environment (Haglund et al., 2007). PBDFs were only found at 0.1 to 1% yields relative to total PBDDs. As Smiles rearrangement products could possibly be formed (Haglund, 2010), rearrangement products were specifically sought (1,2,3,8-TeBDD from OH-BDE 85; 1,2,3,7-TeBDD from OH-BDE 123; 1,3,7-TrBDD from OH-BDE 90 and 1,3,8-TrBDD from OH-BDE 99, respectively). Non of these products were detected after UV irradiation of the corresponding OH-PBDE for 2 h. Prolonged UV-exposure of OH-BDE 99 for 24 h also failed to yield formation of the rearrangement product. This is in contrast with results from the enzymatic mediated coupling reactions where rearrangement products were found from a presumably phenoxyphenol intermediate (see section 4.1.2). However, as the 6-position in the phenoxyring is unsubstituted for the investigated OH-PBDEs, spiro-coupling may be disfavored for natural occurring OH-PBDEs (Bernasconi and Fairchild, 1988).

The influence of DOC on product yield depended on the bromination degree of the substrate (Paper IV, Figure 1). For tetrabromo OH-BDE 47, the shielding effect of DOC, (i.e. absorption of incoming light), resulted in a lower product yield with increasing concentration of DOC. In contrast, the yield of pentabromo OH-BDE 90 increased with increases in DOC concentration, although the total yield of PBDD products remained similar. A possible explanation for this difference is that DOC may partially sorb the 1,2,4,7-TeBDD (which would be less water-soluble than 1,3,7-TrBDD) thereby stabilizing and protecting it from further break-down, i.e. debromination to TrBDDs. This hypothesis is supported by the reductions in levels of primary debromination products with increases in DOC-concentration, as also observed for pentabromo OH-BDE 85, although the low yield of the initial cyclized product of this compound, 1,2,3,7-TeBDD, did not show any significant effect of DOC. This may however, depend on
the presumed shortlived existence of the 1,2,3,7-TeBDD, as this congener with congested bromines is more prone to debromination. DOC appears to provide approximately 10% shielding (based on total product yield) at 10 mg C/L, corresponding to concentrations of DOC in coastal regions where most PBDD formation is presumed to occur (Haglund et al., 2007; Kuivikko et al., 2010).

Figure 4.3 Scheme of PBDD formation from OH-PBDEs used in the study. Main product formed after 2 h UV exposure of artificial coast water solutions of each OH-PBDE is shown, together with total PBDD yields (mmol/mol; in boldface) and percentage yields of main product (in italics).

To confirm product formation, OH-BDE 47 and OH-BDE 90 were exposed to natural solar irradiation. The same products were obtained, but the relative yields of the cyclized products differed. In artificial light (2 h irradiation) 1,3,7-TrBDD was formed from OH-BDE 47 in approximately 3 times higher yield compared to the yield of 1,2,4,7-TeBDD from OH-BDE 90, whereas in sunlight (3 h irradiation) only half the yield of 1,3,7-TrBDD was obtained compared to the yield of 1,2,4,7-TeBDD. The steady formation of 1,3,7-TrBDD up to 9 h in artificial light, changed to a clear decrease in formation

- 42 -
between 3 and 9 h of irradiation in natural solar light. This may be explained by the higher irradiance in the solar spectrum at shorter wavelengths than that of the artificial UV-light used in the experiments, which also had a cut-off at 305 nm (Paper IV; Supporting Information, Figure S1a). As the maximum absorption wavelength for OH-BDE 47 is shorter than that of OH-BDE 90 and its quantum yield (number of excited molecules undergoing reaction divided by the number of absorbed photons) is higher (Bastos et al., 2009), this may lead to a higher rate of degradation of OH-BDE 47 in sunlight.

All of the PBDDs formed in the UV-irradiation experiments have been found in biota samples from the Baltic Sea, and the congener profiles resemble those obtained from analysis of mussels from the Baltic Proper (Haglund et al., 2007) (see section 4.3). However, no formation were shown of some of the frequently identified congeners in biota with 1,3-substitution. A substitution pattern of OH-PBDEs consistent with one that would lead to the formation of PBDDs substituted in such manner, have not been detected among OH-PBDEs considered to be of natural origin (Kelly et al., 2008; Marsh et al., 2003).

### 4.3 Congener profiles

The profiles of PBDDs from both the enzyme-catalyzed and UV-induced formation experiments, together with PBDD profiles from Baltic biota, are shown in Figure 4.4. The profiles from mussels show distinctive location-dependent differences. As mussels are filter feeders and have low metabolic capacity, they should reflect the isomer pattern of PBDD congeners found in water, sediments, and plankton in the surrounding area. The differences in profiles imply that sources of the PBDDs differ, or differ in relative importance, between the saltwater (35 psu) West Coast and brackish (6 psu) Baltic Proper environments. The PBDD profile obtained from the UV-irradiation of OH-PBDEs matches that of biota from the Baltic Proper, where considerable levels of PBDDs have been found in primary producers and organisms of low trophic levels (mussels) (Löfstrand et al., 2011; Malmvärn et al., 2008) as well as high levels of OH-PBDEs (Löfstrand et al., 2010). In contrast, the PBDD profile of mussels from the Swedish West Coast, where significant levels of TBPs have been reported in mussels (Löfstrand et al., 2010), has striking similarities to the congener distribution observed in the enzymatic-catalyzed BP couplings. The source of the BPO used in the study, the red alga Corallina officinalis, is frequently found on the Swedish West Coast but is not found in the Baltic Proper because of the low salinity. The large difference in profiles may thus be at least partly due to variations in species composition related to salinity. Other explanations could be differences in species composition due to eutrophication of the Baltic Proper.
or the specificity of the BPOs of the primary producers between these locations, but possibly also to local differences in levels of precursors.

The rapid formation of PBDDs at percent yields, from OH-PBDEs under simulated natural conditions, implies that this could be an important formation route for the PBDDs found in the environment, especially during periods with moderate to intense sunlight; in the Baltic region sunlight durations exceed 100 h per month from approximately April to September (STRÅNG; SMHI, 2011). The enzymatic pathway gave much lower yields, but still formed significant levels of PBDDs even at low temperature, implying that they may be formed in the sea throughout the year, including during cold periods. The formation of PBDDs substituted in the 1,3-positions, which will not form from naturally abundant OH-PBDEs in UV-induced cyclization, also points to this pathway as a plausible and possibly important route of formation for the PBDDs found in the environment. Since the PBDD congeners found in biota have relatively short retention times in fish after dietary exposure and most are prone to metabolic transformations (Papers I and II), the exposure must be very high and continuous to yield the PBDD levels measured in wild fish. For such continuous PBDD exposure to be maintained, PBDDs must presumably be formed by general processes involving common precursors, such as enzymatic oxidations, UV-initiated reactions or a combination of both.
5 Exposure situation and scenarios

The high levels of PBDDs found in algae, shellfish and fish in the Baltic Sea, especially in the coastal zone, are concerning. There seems to be a high turnover of PBDDs in this natural environment, given that there are high levels of PBDD congeners in organisms that show low retention (high elimination). This raises questions about how turnover of these substances is maintained and how present concentrations may affect the biological systems in the Baltic Sea.

Although PBDDs are probably natural products, they may still affect wildlife and cause ecological effects. Periods of high exposure to PBDDs may result in increases in toxic load that could exacerbate exposure to anthropogenic substances like PCDD/Fs through additive or synergistic effects.

Further, as the Baltic Sea is an extremely vulnerable marine ecosystem, there may be clear effects of changes in exposure levels and substance compositions in it that would have more subtle effects in other seas. Thus, knowledge gained from studies of the Baltic Sea may help attempts to detect and elucidate environmental effects of chemicals elsewhere.

5.1 Present PBDD/F exposure in the Baltic Sea

The production of possible PBDD precursors and degree to which PBDD formation is favored are likely to depend on geographical and biological conditions and may vary over time. Nevertheless, high productivity in the coastal zone can be assumed, with production of possible precursors, such as BPs and OH-PBDEs, in algal communities. The presence of high levels of possible precursors (BPs, OH-PBDEs, and MeO-PBDEs) and PBDDs in algae and cyanobacteria coincides with high levels of the same substances in mussels from the same locations (Löfstrand et al., 2010), although concentrations of more lipophilic compounds, e.g. MeO-PBDEs and PBDDs, are reportedly higher than those of OH-PBDEs in mussels.
The total OH-PBDE, MeO-PBDE and PBDD concentrations in blue mussels co-vary with season, implying a connection between the formation and sources of these compounds (Löfstrand et al., 2011). Accordingly, interconversions of OH-PBDEs and MeO-PBDEs in fish (Wan et al., 2010), and methylation of phenols by bacteria (Allard et al., 1987), have been reported. Levels of MeO-PBDE are higher than levels of OH-PBDE in Philippine macroalgea (Haraguchi et al., 2010), whereas the opposite was found in the Baltic Sea (Malmvärn et al., 2008). Thus, the variations in relative levels of these compound groups may be a result of differing factors affecting the sources.

The toxic potential of many of the most abundant PBDD congeners present in Baltic Biota have not been evaluated. Hence, it is difficult to predict the overall toxic impact of PBDDs in the Baltic. The most potent and persistent compound, 2,3,7,8-TeBDD, has rarely been found in biota. Of the congeners that are abundant in wild fish, mainly di- and triBDDs, the laterally substituted 2,3,7-TrBDD is likely to be the most potent, in analogy with chlorinated dioxins. However, the potency of the two most abundant compounds (1,3,7-TrBDD and 1,3,8-TrBDD) is currently unknown, and it is possible that these congeners, though not persistent, may cause problems if exposure to them is continuous and high. The possibility of exposure to metabolites of PBDDs, though probably more hydrophilic, may also be a source of concern.

The possibility of continuous exposure to PBDDs via water cannot be ruled out; based on the concentrations in mussels of the most abundant di- and triBDD congeners (Haglund et al., 2007) and their estimated log K\textsubscript{OW} values (EpiSuite; US EPA, 2008), water concentrations could be ca. 10^{-8} \text{mg/L}; well below their estimated water solubility (10^{-4} – 10^{-3} \text{mg/L}). This may be of particular concern for eggs and embryos as it would be a route of direct exposure for PBDDs at vulnerable life stages, thereby possibly increasing risks of developmental effects.

It is also possible that the substances are transferred to higher organisms in the food web, and accordingly PBDDs have been found at considerable levels in seal from the Baltic Sea (P. Haglund, personal communication). At present there is, however, no evidence that PBDDs from the Baltic Sea are transferred to man.

### 5.2 Future scenarios with a changing climate

The presented pathways for formation of PBDDs (by enzymatic and photo-induced couplings of phenolic precursors) are both sensitive to changes in climatic conditions. Under greenhouse gas emission scenarios proposed by the IPCC (Intergovernmental Panel on Climate Change; 2001), the climate in the Baltic Sea region is expected to become warmer and wetter during the coming century (Kjellström and Ruosteenoja, 2007; Meier, 2006; Neumann,
If so, this will certainly affect the formation and production of both PBDDDs and possible precursors. Annual and, in particular, winter precipitation over the Baltic Sea catchment area are expected to increase by 5–15% and 20–35%, respectively (Kjellström and Ruosteenoja, 2007). Thus, increasing inflow to the Baltic Sea is expected, with anticipated reductions in salinity and increases in DOC levels (Löptien and Meier, 2011), although with differences between basins as the highest increase in inflow will be in the north (Meier, 2006). The salinity of the total central Baltic Sea water column is projected to decrease by approximately 2‰ (Neumann, 2010), though changes in coastal areas are expected to be lower (Meier, 2006). Sea surface temperature is expected to increase with 3°C (Meier, 2006), and this change will have substantial effects, *inter alia*, on the ice-coverage of the Baltic Sea; by 2100 the ice cover is projected to decline by two-thirds (Neumann, 2010).

All of these changes would have effects on Baltic Sea ecosystems. Changes in salinity and temperature would affect the species composition, especially as many species are already at, or close to, their salinity tolerance limits (Jaanus et al., 2011; Mūren et al., 2005). Increasing eutrophication, due to increased inflow of nutrients, would also lead to changes in species composition; in particular primary producers would increase. Primary producers are the most plausible sources of PBDDs and precursors, but no specific species have been identified as sources (Löfstrand et al., 2010; Malmvärn et al., 2008). Thus, changes in species composition may affect the absolute and relative levels of PBDDs and precursors, with shifts depending on the species present and their abundance under the new conditions. Effects on abiotic processes are also likely to occur. Despite the increased precipitation, cloudiness is expected to decrease, resulting in increased solar radiation into the sea (Meier et al., 2006). This, and the predicted decrease in ice cover and increase in turbidity due to increased inflow of DOC, may alter UV-influenced processes like UV-induced cyclization of OH-PBDEs.

In conclusion, the present PBDD concentrations and congener patterns in the Baltic Sea will possibly alter in the future due to climate changes. This may also alter effects on the marine life. Raised general levels of PBDDs could affect biota *e.g.* with increased activity of metabolic systems. Changes in congener pattern would be of particular concern if the production of laterally substituted congeners, 2,3,7-TrBDD and 2,3,7,8-TeBDD, was increased. Even a small production of 2,3,7,8-TeBDD would be concerning because of its effects and persistence in biota.
Exposure situation and scenarios
6 Conclusions and future perspectives

The main conclusions from the studies this thesis is based upon are:

- fish can assimilate PBDD/Fs from their feed, although non-laterally substituted congeners are rapidly eliminated. Laterally substituted congeners are retained, and non-laterally substituted congeners without vicinal hydrogens are retained to some extent. In addition, PBDD/Fs are less strongly retained at higher doses, indicating the induction of metabolic systems.

- PBDD/Fs that are retained in fish are transferred to eggs. Congeners that are rapidly eliminated in fish show a higher transfer ratio to eggs, probably because of low to non-existent metabolism in eggs.

- exposure to the laterally substituted 2,3,7,8-TeBDD has significant effects on the health, gene expressions and several reproduction end-points of zebrafish, even at the lowest dose applied. The Baltic Sea mixture also had some effects at high dose, though they may have been due to traces of 2,3,7,8-TeBDD present in the feed.

- enzymatic catalyzed coupling of 2,4,6-TeBP yields several congeners, some formed after rearrangement. The overall yield was low, but significantly higher at low temperature, and the product profile obtained is similar to congener profiles found in biota from the Swedish West Coast.

- photochemically induced cyclization of OH-PBDEs under natural conditions gives the corresponding PBDD at percentage yield. Rearranged products were not detected, and some abundant congeners do not seem to be formed this way. However, the product profile obtained is similar to congener profiles found in biota from the Baltic Proper. In addition, DOC levels in water were found to have some influence on formation rates of PBDDs.
Based on the above results some future lines of research are suggested here.

Routes of exposure:

Determination of PBDD levels and congener profiles in water may reveal whether the exposure of Baltic biota to PBDDs is mainly from their diet or via the bioconcentration of PBDDs from water. Additional input of PBDDs, adsorbed to DOC or sediment particles, may be of particular concern for filter feeders like mussels.

In studying uptake and elimination of PBDDs in endemic Baltic fish species, e.g. three spined stickleback, possible differences compared to zebrafish in PBDD retention patterns would be discerned.

Formation process:

Analysis of intermediates, initially OH-PBDEs, in enzymatic coupling reactions may establish connections between biotic and abiotic formation routes. Further studies on enzymatic formation of PBDDs or OH-PBDEs may involve other algal peroxidases and other bromophenols as substrates, to possibly broaden the product spectrum. Investigations of the influence of temperature on formation may reveal climatic conditions for the process.

The study of OH-PBDE levels and their state (dissolved, adsorbed) in the water column may establish the possible extent of photochemical formation of PBDDs in the Baltic Sea.

The generality of the PBDD and/or precursor formation may be investigated by studies of several primary producer species or species groups, to establish if formation is dependent on one or a few specific species or groups.

Risk estimation:

To provide better estimates of the risks connected with exposure, there is a need for evaluation of the toxic potency of congeners abundant in Baltic biota. Evaluation of the effect of metabolites may also be of interest as turnover of abundant PBDDs is high. The study of food web transfer of PBDDs may reveal the extent of exposure to higher organisms, and if transport to man is likely.

Analysis of biota from similar environments to the Baltic Sea, i.e. brackish and eutrophic, such as the Black Sea (an inland sea connected to the Mediterranean Sea), and Chesapeake Bay (a large estuary between Maryland and Virginia), may establish to what extent PBDD formation is general globally, and under what environmental conditions there is substantial formation.
Acknowledgements

The financial support for research and travels is acknowledged: FORMAS (The Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning), Ångpanneföreningen’s Foundation for Research and Development, Strömpilen Environmental Grant and the Kempe Foundations.

No (wo)man is an island, not even a scientist, so my thanks are due to all those around me, who has supported me with small and big thoughts and things during this work.

First of all, my main supervisor Peter Haglund and my co-supervisor Patrik Andersson who have stood by me with cheers and critical eyes on timeplans, ideas, experimental designs, conclusions, and manuscripts. You have been a really good supervisor team, with complementary skills both concerning organic chemistry and toxicology, but also concerning calendars and red marks in my drafts. All in all, I would not have made it without you!

My co-authors Anna Norman Haldén and Leif Norrgren, SLU Uppsala, for giving me insights into a very tiny (zebrafish) world. You made even me, although chemist, interested in these little fishes. And I was always impressed by the skills behind those neatly packed samples of eggs and fish that arrived to my lab!

My PhD-project reviewers Agneta Andersson and Bertil Eliasson, always having a keen eye on my results and one even keener on the time schedule. Thank you for your help!

I would also like to thank those who put me on the track of environmental chemistry research in the first place (long time ago!); Carl Axel Wachtmeister and Åke Bergman and the rest of the staff at Environmental Chemistry, Stockholm University. Little did I know what kind of journey I was going to embark on when I stepped inside the doors of Wallenberg laboratory that summer day. Circumstances made the journey quite long – but I’m finally here!

……….ora et labora…………..

Som färsk doktorand kommer en glad i hägen och med fyra år (en evighet!) framför sig – efter ett antal labtimmar, analyser, avbrunna filament, kriser, gråa hår, hp’ar, studenter, upptjipade dataark, fredagsfikan, manusversioner, ännu fler manusversioner och (till slut) artiklar har tiden gått – aldeles för fort! TACK alla ni nuvarande och gamla miljökemister som gör miljökemiska laboratoriet till en så trevlig plats att jobba på, fyra år går snabbt när en har roligt!
Några särskilda tack till:

Mina PhD-students kamrater i forskningsgruppen: Tomas, Ulrika, Lan, Kicki, Matyas. Kämpa på! Don’t Panic! Och stort TACK till Patricia för genomläsningen av kappan 😊

Min rumskamrat Chau för att du inte klagar på mina pappershögar och för att jag fick ha det stora skrivbordet

Jenny för att du också är intresserad av zebraﬁskar

Sarah för att du också gillar sushi och ﬁlm, med eller utan nakna män - bara de är från Finland, Italien eller Grekland förstås (ja ﬁlmerna alltså!)

Maria, Anna, Rolﬁ, och Sture för goda råd, lån av natriumsulfat, värmeugnar, korkringar mm mm, och för att ni alltid försöker svara på alla konstiga frågor som dyker upp hos en doktorand. Ett speciellt tack till Sture för Queen!

Per för all hjälp med A2an – när en prövat allt, då har du ytterligare ett knep att ta till, och sen funkar det 😊 Och tack för lånet av fönstret!

Alla övriga på miljökemi, doktorander och seniorer - ingen nämnd och ingen glömd!

Ett stort tack till släkt och vänner som hejat på mig i mina studier men också sett till att jag har kommit i hag att världen består av andra saker än att plugga och jobba. Jag tror det kallas livet.....


Tack till min systers Gunilla för hjälp med hundvakt när jag varit på resor och under den sista hektiska tiden med avhandlingsskrivande.

Till mina barn - för att ni finns! Fortsätt med det.

Slutligen ett tack till Jonas som visade mig annonsen; utan den ingen PhD!

Och allra, allra sist – den verklige hjälten som stått ut med mig med sena dagishämtningsar, semestrar på lab och ständigt denna matte fjätttrad vid datorn – Hamlet, en Welsh i sina bästa år, som sannerligen lever upp till devisen To err is human, to forgive is canine. Utan din glada svans och ständiga beredskap på ett kli bakom örat skulle livet vara betydligt mer stådat, men definitivt tråkigare och mindre fyllt av skogspromenader!

so………………Phinally Done……………
References


Asplund, L., Athanasiadou, M., Sjödin, A., Bergman, Å., Börjeson, H., 1999. Organohalogen substances in muscle, egg and blood from healthy Baltic salmon (Salmo salar) and Baltic salmon that produced offspring with the M74 syndrome, Ambio 28, 67-76.


Flodin, C., Whitfield, F.B., 1999b. 4-Hydroxybenzoic acid: a likely precursor of 2,4,6-tribromophenol in Ulva lactuca, Phytochemistry 51, 249-255.


Hayakawa, K., Takatsuki, H., Watanabe, I., Sakai, S., 2004. Polybrominated diphenyl ethers (PBDEs), polybrominated dibenzo-p-dioxins/dibenzofurans (PBDD/Fs) and monobromo-polychlorinated dibenzo-p-dioxins/dibenzofurans (MoBPXDD/Fs) in the atmosphere and bulk deposition in Kyoto, Japan, *Chemosphere* 57, 343-356.


Johannesson, K., Smolarz, K., Grahn, M., André, C., 2011. The future of Baltic Sea populations: Local extinction or evolutionary rescue?, *Ambio* 40, 179-190.


King Heiden, T.C., Carvan, M.J., Hutz, R.J., 2006. Inhibition of follicular development, vitellogenesis, and serum 17 beta-estradiol concentrations in zebrafish following chronic, sublethal dietary exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin, Toxicol. Sci. 90, 490-499.

King Heiden, T.C., Spitsbergen, J., Heideman, W., Peterson, R.E., 2009. Persistent adverse effects on health and reproduction caused by exposure of zebrafish to 2,3,7,8-tetrachlorodibenzo-p-dioxin during early development and gonad differentiation, Toxicol. Sci. 109, 75-87.


Liu, H., Zhao, H., Quan, X., Zhang, Y., Chen, S., Zhao, H., 2011. Formation of 2'-hydroxy-2,3',4,5'-tetrabromodipheyl ether (2'-HO-BDE68) from 2,4-dibromophenol in aqueous solution under simulated sunlight irradiation, Chemosphere 84, 512-518.


OECD 2008. Revised draft OECD Guideline for the testing of chemicals. The Fish Screening Assay for endocrine active substances.


**Electronic sources and programs**


STRÅNG, SMHI (Swedish Meteorological and Hydrological Institute). [http://strang.smhi.se/](http://strang.smhi.se/)