Environmentally relevant chemical disruptors of oxidative phosphorylation in Baltic Sea biota
Exposure and toxic potentials

Anna-Karin Dahlberg
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Exposure and toxic potentials

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Abstract

This thesis focuses on toxicity and occurrence of hydroxylated polybrominated diphenyl ethers (OH-PBDEs) in Baltic Sea biota. The aims were to assess OH-PBDEs potency for disruption of oxidative phosphorylation (OXPHOS) and determine their and related compounds exposure in Baltic blue mussel, herring and long-tailed duck. A method for analysis of OH-PBDEs in herring and long-tailed duck plasma was also evaluated. Relevant OH-PBDEs were tested in vitro for OXPHOS disruption, using a classic rat mitochondrial respiration assay and a cell mitochondrial membrane potential assay. All compounds were found to disrupt OXPHOS either by protonophoric uncoupling and/or via inhibition of the electron transport chain. 6-OH-BDE47 and 6-OH-BDE85, were identified as particularly potent OXPHOS disruptors. Strong synergism was observed when OH-PBDEs were tested as a mixture corresponding to what is present in Baltic blue mussels.

Baltic blue mussel is main feed for several species of mussel feeding sea ducks which have decreased dramatically in numbers. To assess long-tailed ducks exposure to brominated substances, liver tissue from long-tailed ducks wintering in the Baltic Sea and blue mussels were analysed. The result confirms that long-tailed duck are exposed to OH-PBDEs via their diet. However, low concentrations were found in the duck livers, which suggest low retention of these compounds despite daily intake. How the nutritional value of blue mussels as feed for sea ducks are affected by OH-PBDE exposure still needs further studies. Other species of sea ducks foraging on Baltic blue mussels during summer months can also be more exposed due to seasonal variation in primary production.

Herring sampled in the Baltic Proper and Bothnian Sea, were found to contain OH-PBDEs and high levels of their methylated counterpart, MeO-PBDEs. As demethylation of MeO-PBDEs is known to occur in fish, MeO-PBDEs may pose as additional source for more toxic OH-PBDEs in herring and their roe.
Abstract

In dit proefschrift is gekeken naar de toxiciteit en de aanwezigheid van ge-hydroxyleerde polybroomdifenylethers (OH-PBDE’s) in biota van de Baltische Zee. De doelstellingen van dit proefschrift waren het bepalen van het vermogen van OH-PBDE’s om oxidatieve fosforylatie (OXPHOS) te kunnen verstoren en het vaststellen van de blootstelling aan OH-PBDE’s en gerelateerde stoffen in mosselen, haring en ijseenden uit de Baltische Zee. Er is ook een methode getoetst voor de analyse van OH-PBDE’s in plasma van haring en ijseenden.

In twee standaard in vitro testen, de klassieke mitochondriale respiratietest met mitochondrie van ratten en de mitochondriale membraanpotentietest met cellen van de zebravis, zijn relevante OH-PBDE’s onderzocht op het verstoren van OXPHOS. Alle stoffen bleken OXPHOS te verstoren via protonophore ontkoppeling en/of via remming van de elektronentransportketen. Met name 6-OH-BDE47 en 6-OH-BDE85 zijn geïdentificeerd als sterke OXPOS verstoorders. Verder werd in sterke mate synergisme waargenomen bij een mengsel van OH-PBDE’s die qua samenstelling overeenkomt met de gevonden hoeveelheden OH-PBDE’s in de Baltische blauwe mossel.

De Baltische blauwe mossel is de belangrijkste voedselbron voor meerdere soorten mosseletende zee-eeenden. De aantallen mosseletende zee-eeenden zijn dramatisch gedaald. Om de blootstelling van ijseenden aan gebromineerde stoffen vast te stellen, zijn zowel de lever van ijseenden die overwinteren in de Baltische Zee als de blauwe mosselen gemanalyseerd. Hierdoor werd bevestigd dat ijseenden via hun voedsel aan OH-PBDE’s worden blootgesteld. Alleen zijn de gevonden concentraties OH-PBDE’s in de lever van de ijseenden laag en dit suggereert dat, ondanks dagelijkse inname, de retentie van de stoffen laag is.

Op welke manier de voedingswaarde van blauwe mosselen als voedsel voor zee-eeenden beïnvloed is door de blootstelling aan OH-PBDE’s moet nog verder onderzocht worden. Andere soorten zee-eeenden die zich bevoorraden met Baltische blauwe mosselen tijdens de zomermaanden kunnen bijvoorbeeld meer blootgesteld worden vanwege seizoensgebonden variatie in de primaire productie.

In haring uit het centrale deel van de Baltische Zee en uit de Botnische Zee zijn zowel OH-PBDE’s als grote hoeveelheden van de gemethyleerde tegenhanger, MeO-PBDE’s, gevonden. Omdat in vissen demethylering van MeO-PBDE’s kan optreden, kunnen MeO-PBDE’s een extra bron vormen voor de blootstelling aan toxische OH-PBDE’s in haring en hun kuit.
To my beloved parents
List of papers

This thesis is based on the following articles and manuscripts, referred to in the text by their roman numerals (I-IV). The published articles are reproduced here with kind permission of the publisher. Co-shared first authorship is indicated with an asterisk.

I  Disruption of oxidative phosphorylation (OXPHOS) by hydroxylated polybrominated diphenyl ethers (OH-PBDEs) present in the marine environment.

II  Hydroxylated and methoxylated polybrominated diphenyl ethers in Long-tailed duck (Clangula hyemalis) and their main food, Baltic blue mussels (Mytilus trossulus x Mytilus edulis)
    Anna-Karin Dahlberg, Vivian Lindberg Chen, Kjell Larsson, Åke Bergman and Lillemor Asplund.
    Manuscript

III  Anthropogenic and naturally produced brominated substances in Baltic herring (Clupea harengus) from two sites in the Baltic Sea.
    Anna-Karin Dahlberg, Anders Bignert, Jessica Legradi, Juliette Legler and Lillemor Asplund.
    Manuscript

IV  Recovery discrepancies of OH-PBDEs polybromophenols in human plasma and cat serum versus herring and long-tailed duck plasma.
    Anna-Karin Dahlberg*, Jessica Norrgran*, Lotta Hovander, Åke Bergman and Lillemor Asplund.
    Chemosphere. 2014, 94, 97-103.
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<tbody>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AhR</td>
<td>Aryl hydrocarbon receptor</td>
</tr>
<tr>
<td>APPI</td>
<td>Atmospheric pressure photo ionization</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BAs</td>
<td>Brominated anisoles</td>
</tr>
<tr>
<td>BPs</td>
<td>Brominated phenols</td>
</tr>
<tr>
<td>CA</td>
<td>Concentration addition</td>
</tr>
<tr>
<td>4,4’-DDE</td>
<td>1,1-dichloro-2,2-bis(4-chlorophenyl) ethene</td>
</tr>
<tr>
<td>4,4’-DDT</td>
<td>1,1,1-trichloro-2,2-bis(4-chlorophenyl) ethane</td>
</tr>
<tr>
<td>DNP</td>
<td>2,4-dinitrophenol</td>
</tr>
<tr>
<td>EC</td>
<td>Effect concentration</td>
</tr>
<tr>
<td>ECNI</td>
<td>Electron capture negative ionization</td>
</tr>
<tr>
<td>EI</td>
<td>Electron ionization</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>FCCP</td>
<td>4-(trifluoromethoxy)phenylhydrazone</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>GM</td>
<td>Geometric mean</td>
</tr>
<tr>
<td>GPC</td>
<td>Gel permeation chromatography</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>HOCs</td>
<td>Halogenated organic compounds</td>
</tr>
<tr>
<td>HPCs</td>
<td>Halogenated phenolic compounds</td>
</tr>
<tr>
<td>IA</td>
<td>Independent action</td>
</tr>
<tr>
<td>LLE</td>
<td>Liquid-liquid extraction</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of detection</td>
</tr>
<tr>
<td>LOEC</td>
<td>Lowest observed effect concentration</td>
</tr>
<tr>
<td>LOQ</td>
<td>Limit of quantification</td>
</tr>
<tr>
<td>MeO-PBDEs</td>
<td>Methoxylated polybrominated diphenyl ethers</td>
</tr>
<tr>
<td>HRMS</td>
<td>High resolution mass spectrometry</td>
</tr>
<tr>
<td>LRMS</td>
<td>Low resolution mass spectrometry</td>
</tr>
<tr>
<td>NOEC</td>
<td>No observed effect concentration</td>
</tr>
<tr>
<td>OH-PBDEs</td>
<td>Hydroxylated polybrominated diphenyl ethers</td>
</tr>
<tr>
<td>OXPHOS</td>
<td>Oxidative phosphorylation</td>
</tr>
<tr>
<td>PBDEs</td>
<td>Polybrominated diphenyl ethers</td>
</tr>
<tr>
<td>PCBs</td>
<td>Polychlorinated biphenyls</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>PCP</td>
<td>Pentachlorophenol</td>
</tr>
<tr>
<td>PFBCl</td>
<td>Pentafluorobenzoyl chloride</td>
</tr>
<tr>
<td>POPs</td>
<td>Persistent organic pollutants</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per million</td>
</tr>
<tr>
<td>SF6847</td>
<td>3,5-di(tert-butyl)-4-hydroxybenzylidenemalononitrile</td>
</tr>
<tr>
<td>SIM</td>
<td>Single ion monitoring</td>
</tr>
<tr>
<td>T₃</td>
<td>3,3',5-triiodo-L-thyronine</td>
</tr>
<tr>
<td>T₄</td>
<td>3,3',5,5'-tetraiodo-L-thyronine</td>
</tr>
<tr>
<td>TBG</td>
<td>Thyroid binding globulin</td>
</tr>
<tr>
<td>TMRM</td>
<td>Tetramethylrhodamine methyl ester perchlorate</td>
</tr>
<tr>
<td>TPP⁺</td>
<td>Tetraphenylphosphonium ion</td>
</tr>
<tr>
<td>TR</td>
<td>Thyroid hormone receptor</td>
</tr>
<tr>
<td>TTR</td>
<td>Transthyretin</td>
</tr>
<tr>
<td>TU</td>
<td>Toxic unit</td>
</tr>
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</table>
1 Introduction

During the 20\textsuperscript{th} century the chemical industry has developed new products and materials that have considerably improved the life of mankind. However, the awareness of the risks anthropogenic chemicals can pose has also grown over the recent half century. Today several halogenated organic compounds (HOCs) of anthropogenic origin are known to be harmful for humans and wildlife due to their persistent, toxic and bioaccumulative properties, and their production and use is nowadays banned or restricted\cite{1}. At the same time, the knowledge regarding natural/biogenic production of HOCs has increased tremendously\cite{2}.

With seawater being rich in halides (i.e. chloride, bromide and iodide) it is not surprisingly that a large number of HOCs are naturally produced by marine biota. The chemical structures produced ranges from simple haloalkanes to complex halogenated aromatic structures\cite{2}. In the Baltic Sea a large number of brominated phenolic compounds, including hydroxylated polybrominated diphenyl ethers (OH-PBDEs) have been identified. The Baltic Sea is a unique brackish water body with a sensitive ecosystem. During the last half century the Baltic Sea has suffered from chemical pollution and eutrophication due to human activities, which have severely affected the ecosystem\cite{3,4}.

In recent years have the toxicology sciences done a lot of research on HOCs and an extensive knowledge base has been created. Many studies have confirmed that phenolic metabolites of HOCs in fact can be more biological active than their neutral parent compounds\cite{5}. OH-PBDEs of both natural and anthropogenic (metabolic) origin have been linked to several toxicological effects, including effects on the endocrine- and nervous systems\cite{6,7}. OH-PBDEs have also been reported to be acutely toxic and induce developmental arrest in zebrafish\cite{8,9}. The underlying mechanism for this toxicity was suggested by van Boxtel and co-workers to be due to disruption of mitochondrial oxidative phosphorylation (OXPHOS)\cite{9}. OXPHOS is the metabolic pathway used by cells to produce energy (i.e. adenosine triphosphate, ATP) under aerobic conditions. Exposure to compounds which disturb this mechanism may thus lead to severe biological consequences as shown for several “classical” uncouplers of OXPHOS\cite{10}.
1.1 Aim of thesis

This thesis focuses on brominated organic chemicals of natural and/or anthropogenic origin, with special emphasis on OH-PBDEs present in Baltic biota. The work presented herein comprises both toxicological and analytical studies with the overall aim of assessing OH-PBDEs potency for OXPHOS disruption and to determine the exposure of these compounds in Baltic wildlife (i.e. in mussel, fish and waterfowl). The specific objectives of each paper are presented as follows:

**Paper I:** OH-PBDEs share many structural characteristics with potent disruptors of OXPHOS (i.e. being weak lipophilic acids). The objective of this study was to determine the OXPHOS disrupting potential of several OH-PBDEs present in Baltic biota, using two *in vitro* bioassays. Single OH-PBDE congeners as well as mixtures were tested to study potential mixture effects.

**Paper II:** The objective was to determine sea ducks exposure to brominated organic substances by analysing livers from long-tailed duck (*Clangula hyemalis*) wintering in the Baltic Sea. Further to assess their dietary intake prior to migration and reproduction, the concentration of brominated substances in Baltic blue mussels collected from sites used by sea ducks for foraging were analysed.

**Paper III:** The condition and fat content in Baltic herring has shown decreasing trends since the 1980’s. Meanwhile has the concentrations of OH-PBDEs increased in herring livers. Given the toxicity of the OH-PBDEs to zebrafish, the aim of this study was to further assess the environmental concentrations of these compounds in Baltic herrings by analyse OH-PBDEs and related brominated organic compounds in fish sampled from the northern Baltic Proper and the southern Bothnian Sea.

**Paper IV:** The aim of this paper was to evaluate the applicability of a liquid-liquid extraction method used for analysing HPCs in human plasma, for OH-PBDE analysis in blood matrices from mammal, bird and fish.
2 Background

2.1 The Baltic Sea

The Baltic Sea is a semi-enclosed brackish water body located in the northern hemisphere (Figure 1.). The sea is connected to the North Sea via the three narrow and shallow Danish straits and consists of several deep basins separated by narrow sills. Large input of freshwater from the catchment area surrounding the Baltic Sea together with limited water exchange with the North Sea, results in a salinity gradient ranging from 30‰ down to 3‰ with increasing distance from the North Sea. Further are the salt- and freshwater masses in the Baltic Sea vertically stratified and separated by a halocline. The stratification is caused by colder saltwater having higher density than freshwater. The stratification prevents vertical mixing of the water and oxygenation of deep bottoms. Seasonally a thermocline is also present in the Baltic Sea which further prevents vertical mixing of the water during summer[3].

Figure 1. The Baltic Sea with the salinity gradients depicted.
Being neither a true marine nor a freshwater sea, the number of species living in the Baltic Sea is limited. The few marine and freshwater species which have adapted to the brackish water environment is under constant physiological stress due to the osmotic pressure caused by the low salinity of the sea water. The low biodiversity makes the ecosystem sensitive to external pressures such as eutrophication, extensive fishing and hazardous substances[11].

Today nearly the entire Baltic Sea is eutrophicated. Agricultural land use and other human activities in the surrounding catchment area results in riverine and atmospheric discharges of nutrients (e.g. inorganic nitrogen and phosphorus) which together with the limited water exchange in the Baltic Sea have resulted in elevated nutrient levels. Despite actions to reduce inflow of nutrients into the Baltic Sea, the concentrations of nitrogen and phosphorous are still too high. High nutrient concentrations favor growth of phytoplankton which results in increased sedimentation of organic matter. During degradation of organic matter large quantities of oxygen is consumed. Together with the vertical stratification of the water column which prevents oxygenation at greater depths, this has resulted in prevalent anoxia in the deep sea bottoms of the Baltic Proper and occasional hypoxia in more shallow coastal areas. Anoxic sediments contribute to increased phosphorous levels in the Baltic Sea by release of inorganic phosphate. In the Baltic Sea growth of nitrogen fixating bacteria is limited by the availability of phosphorous. As a result of high phosphorous levels, large summer blooms of nitrogen fixating bacteria (e.g. *Aphanizomenon flos-aquae* and *Nodularia spumigena*) are now a reoccurring phenomenon in the Baltic Sea. Increased production of phytoplankton and nitrogen fixating bacteria also reduces the light penetration depth, which affects the distribution and specie composition of the macro algae community in favor of fast growing filamentous macro algae[3,12].

In addition to eutrophication the Baltic Sea is heavily polluted by anthropogenic substances, including persistent organic pollutants (POPs) of which some have caused severe adverse effects on the Baltic marine wildlife[13,14]. POPs are chemically stable organic compounds, toxic for humans and wildlife. Due to their chemical stability these chemicals are only transformed very slowly in the environment. Further can POPs undergo long-range transport and thus become wildly distributed. POPs are highly lipophilic and able to bioaccumulate and biomagnify in the food web, with the risk of reaching high concentrations in top predators[15]. Given the threat these chemicals pose on humans and wildlife, 179 parties have signed a global treaty to eliminate or reduce release of POPs listed in the Stockholm Convention[16]. Today this list consists of 23 halogenated compounds, and additionally four halogenated substances are under consideration for inclusion[15].
In the Baltic Sea, the concentrations of several POPs listed in the Stockholm Convention are continuously measured in marine biota by the National Swedish Contaminant Monitoring Programme. POPs included in the monitoring programme are for example 1,1,1-trichloro-2,2-bis(4-chlorophenyl) ethane (4,4’-DDT) and polychlorinated biphenyls (PCBs)[17], former used as insecticide and heat exchange fluids in electrical appliances, respectively[18,19]. The concentrations of polybrominated diphenyl ethers (PBDEs), used as brominated flame retardants, are also continuously measured. PBDEs are further discussed in section 2.4.1.

In addition to halogenated substances of anthropogenic origin, a large number of organohalogens are also known to be naturally biosynthesized in the marine environment[2]. Natural production of brominated substances is more thoroughly discussed in section 2.3.

2.2 Adverse effects in Baltic marine wildlife

That the Baltic ecosystem is sensitive to external pressures such as hazardous substances becomes evident in the 1960-70’s when the Swedish population of white-tailed sea eagle (Haliaeetus albicilla) faced extinction, due to reproductive failure[20]. This adverse effect was later found to be primarily caused by 1,1-dichloro-2,2-bis(4-chlorophenyl) ethene (4,4’-DDE), the main metabolite and degradation product of technical DDT. The concentration of 4,4’-DDE was strongly correlated with eggshell thinning, which was found to be the underlying cause for the population decline[13]. At the same time, other adverse effects such as uterine lesions, sterility and skeletal deformations were observed among Baltic Sea populations of grey seal (Halichoerus grypus), harbor seal (Phoca vitulina) and ringed seal (Phoca hispida), effects likely caused by high concentrations of chlorinated compounds[21].

An extensive conservatory program to save the Swedish population of white-tailed sea eagle and declining concentrations of DDE and PCB has resulted in recovery of population[22]. However, white-tailed sea eagles living in the southern Bothnian Sea still show signs of impaired reproduction, for which the cause is yet unknown[22]. The grey seal and harbor seal populations have increased after the population decline in the 1960’s and 1970’s. Whereas ringed seal and the Kalmarsund subpopulation of harbour seal is still classified as vulnerable species[23]. Although the reproductive health has improved, pathological changes such as colonic ulcers and thickening of the adrenal cortices (Adrenocortical hyperplasia) are still observed in Baltic grey seals[24].
Today several adverse effects of unknown cause/s are being observed Baltic Sea wildlife of which some will be discussed in the following sections.

In grey seals, decreasing trends in blubber thickness has been observed since year 2000[25]. The fat content in Baltic herring (Clupea harengus) from the Baltic Sea has also decreased in the Baltic Proper since the 1980’s[25].

For several sea duck species, the Baltic Sea is an important wintering and breeding area. In 2009 the populations of five of the most common sea duck species were observed to have declined dramatically (by 4.2 million birds or 60%) since the early 1990’s when the first large survey on waterfowls in the Baltic Sea was performed. For common eider (Somateria mollissima) and long-tailed duck (Clangula hyemalis) the population showed decreases of 51% and 65%, respectively[26]. There are various pressures such as predation, oil pollution, by-catches, hazardous substances, diseases and larger ecosystem changes, potentially affecting the food quality of sea ducks, which may have contributed to the decline[26].

Further has common eider and herring gull (Larus argentatus), living in the Baltic Sea been reported to be suffering from a mortal paralytic syndrome with clinical symptoms such as paralysis of wings and legs, diarrhea, stargazing and ataxia[27], symptoms which were found to be remedied by injections of thiamine (vitamin B<sub>1</sub>)[27].Thiamine deficiency has also been reported in Baltic salmon (Salmo salar) and is commonly referred to as the M74 syndrome and affects the development of the salmon fry at the yolk-sac stage[28]. The cause of M74 is still unresolved but it has been shown to be associated with thiamine deficiency[29]. In a study determining the concentration of organohalogens in muscle, blood and roe from healthy Baltic salmon and salmon producing offspring with M74, no association was found for e.g. DDT, PCBs or PBDEs. However presence of a large number (>100) of halogenated phenolic compounds (brominated and/or chlorinated), including OH-PBDEs were found in the salmon blood[30]. Further work resulted in structural identification of several OH-PBDEs and methoxylated polybrominated diphenyl ethers (MeO-PBDEs) of which eight compounds had not previously been reported in the environment[31].

High mortality in herring roe has also been observed in the Baltic Sea. The mortality was found to change during the spawning season and follow the life cycle of filamentous brown algae (i.e. Pilayella littoralis)[32]. Results from further field and laboratory experiments in which herring roe was exposed to algae exudates, suggests that the increased mortality was due to toxic substances released by the filamentous brown algae[33].
2.3 Natural production of brominated substances

Today more than 4000 halogenated organic compounds are known to be naturally produced in terrestrial and marine environments by living organisms or formed in natural abiotic processes (e.g. in volcanoes and forest fires). The chemical structures ranges from simple haloalkanes to complex halogenated aromatic structures. The majority of these compounds contain chlorine and/or bromine substituents, but a few organoiodine and organofluorine compounds have also been identified as natural products[2]. For a thorough review of the diversity of naturally produced organohalogen the book “Naturally occurring organohalogen compounds – a comprehensive update” by Gordon Gribble[2] is recommended for further reading.

The marine environment is likely the single largest source for biogenic organohalogens and there is a plethora of species such as bacteria, algae, sponges, corals and sea squirts producing them[2]. Sea water is rich in chloride ions (~0.5M), whereas bromide (~1mM) and iodide (~1µM) is present in only smaller amounts[34]. The high halogen content makes sea water a natural substrate reservoir for haloperoxidases involved in the biosynthesis of organohalogenes in biota. Haloperoxidases are enzymes that catalyze oxidation of halides (i.e. chloride, bromide and iodide) using hydrogen peroxide, resulting in subsequent halogenation of organic compounds[34]. As bromine is less electronegative than chlorine, bromine is more easily oxidized, which likely explains the abundance of organobromine compounds in the marine environment despite bromide ions being present at lower concentration than chloride ions in sea water[34].

Bromoperoxidases (which can catalyze oxidation of bromide and iodide) have been found in all classes of marine algae and in other marine organisms[34]. It has been suggested that formation of brominated compounds can be a pathway for marine algae to scavenge hydrogen peroxide formed during photosynthesis, which at high concentrations can be harmful to the cell[35]. It has also been suggested that the halogenated organic compounds play a role as chemical defense in algae against grazers[36] and epiphytic settlement[37].
2.4 Brominated substances discussed in this thesis

Among the various chemical classes of brominated substances naturally produced in the marine environment this thesis focus on a few groups thereof i.e. simple brominated phenols (BPs), simple brominated anisoles (BAs), OH-PBDEs and MeO-PBDEs, with special focus on OH-PBDEs. PBDEs of anthropogenic origin are also discussed as an additional source for OH-PBDEs via e.g. metabolic transformation. General chemical structures of the compounds discussed in the thesis are given in Figure 2.

![Figure 2 General chemical structures and abbreviations of the compounds discussed.](image-url)
2.4.1 Polybrominated diphenyl ethers

PBDEs have been industrially produced since the early 1970’s and used as additive flame retardants in various polymers (e.g. polyurethanes, polyamides, polystyrene and styrene copolymers) used in textile, furniture and electronic equipment[38]. PBDEs and other bromine containing flame retardants, prevent fire to propagate by scavenge the free radicals formed during combustion[38]. However, since PBDEs are not chemically bound to the material, they are prone to migrate out of the material and subsequently leak into the environment.

Commercially PBDEs have been produced in three technical mixtures known as PentaBDE, OctaBDE and DecaBDE, classified according to their average bromine content[39]. The PBDE congener composition of some commercial PentaBDE, OctaBDE and DecaBDE products are presented elsewhere[40]. Since 2009, Penta- and OctaBDE are included in the Stockholm Convention on Persistent Organic Pollutants (POPs) under Annex A[1], and the use of DecaBDE is restricted within the European Union by the RoHS directive[41]. In the United States companies committed to phase out DecaBDE by the end of 2013[42]. In 2001 the estimated global use of PentaBDE, OctaBDE and DecaBDE were 7,500, 3,800 and 56,000 metric tonnes, respectively[39]

2.4.1.1 Physicochemical properties

PBDEs are lipophilic compounds with low vapor pressures. PBDEs physicochemical properties are influenced by their degree of bromination. For example does their lipophilicity (log K_{ow}) increase with increasing number of bromine substituents, i.e. from 5.9-6.2 for tetraBDEs, 6.5-7.0 for pentaBDEs, 8.4-8.9 for octaBDEs and 10 for decaBDE[43]. On the opposite, their vapor pressure decrease with increasing number of bromine substituents. Conformational studies of PBDEs have shown that they exist in twist to skewed conformations where increasing number of bromine substituents ortho to the diphenyl ether linkage force the molecule to adopt a more skewed conformation[44,45]

2.4.1.2 Environmental occurrence – with focus on the Baltic Sea

Today PBDEs have become ubiquitous environmental contaminants found also in remote areas like the Arctic. For levels and trends of PBDEs in the environment several reviews are recommended[46-48]. PBDEs have shown to bioaccumulate in lipid rich tissue and biomagnify in marine food webs[46].
In the Baltic Sea, PBDEs have been reported in marine top predators such as ringed seal (liver)[49] and white tailed sea eagle (egg and serum)[50,51]. Increasing concentrations of PBDEs (i.e. BDE-47, BDE-100, BDE-99) were observed in Guillemot (Uria aalge) eggs from the 1960’s until early 1990’s, when the concentrations started to decrease[17]. Similar trends with increasing concentrations from the 1970’s to the mid 1980’s, were also observed in cod (Gadus morhua) liver in the Baltic Sea[17]. In herring (Clupea harengus) muscle, decreasing concentrations are seen at most sampling sites included in the Swedish Monitoring Program since the late 1990’s, but the concentrations are generally higher in the Baltic Proper compared to the Swedish west coast[17].

2.4.1.3 Metabolic transformation of PBDEs
There are several studies on metabolic transformation of PBDEs which have been reviewed elsewhere[39,52]. For highly brominated PBDEs reductive debromination has been suggested to be the first metabolic step, followed by hydroxylation (inserted either directly or formed via an arene oxide intermediate) by cytochrome P450 enzymes. Formation of OH-PBDEs metabolites have been confirmed in several studies[39]. In one study, sixteen OH-PBDEs and two di-OH-PBDEs were identified in rat plasma five days after the rats were given an intraperitoneal dose of equimolar concentrations of seven PBDEs (i.e. BDE-47, BDE-99, BDE-100, BDE-153, BDE-154, BDE-183 and BDE-209)[53]. The metabolites formed had the hydroxyl group inserted in either meta or para position to the diphenyl ether bond[53]. Formation of meta and para substituted OH-PBDEs metabolites have also been reported by others and seem to be a structural characteristic for OH-PBDEs originating from metabolic transformation[52]. However, it should be stated that a few ortho substituted OH-PBDEs have also been reported as metabolites[31,54]. OH-PBDE metabolites have also been confirmed in fish i.e. northern pike (Esox lucius) and common sole (Solea solea), after PBDE dietary exposure[55,56]. However, in common sole OH-PBDE formation was found to be a minor transformation route compared to metabolic transformation via debromination[56].

2.4.1.4 Toxicological effects
Several experimental animal studies have shown that prenatal or neonatal exposure to PBDEs has effects on thyroid homeostasis, neurobehavioral and reproductive systems. Although the thyroid hormone system seem to be the main target by which PBDE elicit its toxicity, reproductive effects associated with estrogenic and androgenic processes have also been reported. For a more comprehensive understanding of the toxicological effects of PBDEs, the following articles are recommended[6,39,57]
Disruption of thyroid hormone system has been studied in rodents and found to be associated with reduction in thyroxine (T₄) concentrations in the blood[39]. 3,3’,5,5’-tetraiodo-L-thyronine (T₄) is a prohormone produced by the thyroid gland and a precursor for formation of the active thyroid hormone, 3,3’,5-triiodo-L-thyronine (T₃). The thyroid hormone plays an essential role for normal fetal development and for regulation of growth and basal metabolism throughout life. The T₃ hormone acts by binding to the nuclear thyroid hormone receptor (TR) which initiates transcription of thyroid hormone responsive genes[58]. Metabolites of PBDEs (i.e. OH-PBDEs) have shown strong affinity to both the thyroid hormone receptor[59] and thyroid hormone transporting proteins; transthyretin (TTR) and thyroid binding globulin (TBG)[60,61]. Interestingly, PBDEs show no binding affinity to TTR and no or only weak affinity to TBG[60,61], which indicate that metabolic transformation is important for PBDEs to elicit this effect in vivo.

Thyroid hormones play an important role during early brain development[58], and accordingly behavioral effects of PBDE exposure have been studied. In one of the first studies published, Eriksson et al (2001) exposed 10 day old mice to a single oral dose of BDE-47 and BDE-99 and reported dose-dependent permanent aberrations in spontaneous behavior (i.e. locomotion, rearing and total activity) in 2- and 4- month old mice[62]. Effects also observed after exposure of other PBDE congeners using similar experimental design, as reviewed elsewhere[6]. Together these studies strongly indicate that PBDE causes adverse neurobehavioral effects. Further, PBDEs have been shown to affect neuronal cell viability, cell differentiation and migration and neuronal signaling in vitro, as reviewed elsewhere[6]. For some neurotoxic endpoints such as altered calcium homeostasis and induced vesicular neurotransmitter release (exocytosis), 6-OH-BDE47 showed a higher potency than BDE-47[63].

PBDEs have also shown to affect other endocrine pathways than the thyroidal. For example were anti-androgenic, estrogenic and anti-estrogenic activities observed in vitro[64]. A recent publication also reported altered expression of genes regulated by the aryl hydrocarbon receptor (AhR), estrogen receptor (ER) and glucocorticoid receptor (GR) in zebrafish larvae exposed to BDE-47[65].
2.4.2 Hydroxylated polybrominated diphenyl ethers

OH-PBDEs may originate from metabolic transformation of PBDEs as discussed in chapter 2.4.1.3 or from natural production. Naturally produced OH-PBDEs are widespread in the marine environment and have been reported in various marine species such as sponge[66,67], sea squirt[68], macro algae[69] and cyanobacteria[69,70]. Biogenic demethylation of naturally produced MeO-PBDEs has also been confirmed as a pathway for formation of OH-PBDEs in fish[71,72].

2.4.2.1 Physicochemical properties

OH-PBDEs are weak lipophilic acids and as for PBDEs their physicochemical properties are dependent on the degree of bromination. For the OH-PBDEs studied in paper I, the log $K_{ow}$ and pKa ranged between 5-8 and 5-9, respectively. The acidity of OH-PBDEs makes them depending on pKa fully or partly deprotonated in sea water which result in higher water solubility of the compounds.

2.4.2.2 Biosynthesis in marine bacteria and algae

OH-PBDEs are believed to be biosynthesized via formation of BPs and free radical dimerization[73](Figure 3). 4-Hydroxybenzoic acid has been proposed as precursor for formation of 2,4-dibromophenol (2,4-diBP) and 2,4,6-tribromophenol (2,4,6-triBP) in both algae[74] and marine bacteria[75]. Bromination at the 2 and 4 position on the phenolic ring is likely favored due to the ortho and para directing property of the hydroxyl group. The precursor, 4-hydroxybenzoic acid can be formed from chorismate, an intermediate in the shikimate acid pathway[73] which is the common anabolic pathway in bacteria and plants for formation of the aromatic amino acids phenylalanine, tyrosine and tryptophan[76]. Hydroxybenzoic acid has also been proposed to be formed from degradation of tyrosine in marine algae[74].
Figure 3 Proposed biosynthetic pathway for formation of hydroxylated polybrominated diphenyl ethers. Modified figure adopted from [73,76]. 3-deoxy-D-arabino-heptulosonate (DAHP), 3-dehydroquinic acid (DHQ), 5-enolpyruvylshikimate-3-phosphate (EPSP)

Dimerization of BPs to OH-PBDEs has been shown to be catalyzed by bromoperoxidase in algae[77] and by cytochrome P450 in marine bacteria[75]. Theoretically, dimerization via radical formation can result in OH-PBDEs having the hydroxyl group in either ortho and para position to the diphenyl ether bond as shown in Figure 3. However, naturally produced OH-PBDEs identified in the marine environment are almost exclusively ortho substituted. However, three para-substituted OH-PBDEs were recently reported to be formed in vitro, by enzymes from marine bacteria[75].

2.4.2.3 Environmental occurrence – with focus on the Baltic Sea
OH-PBDEs are naturally produced in the Baltic Sea, by cyanobacteria (Aphanizomenon flos-aquae, Nodularia spumigena)[70,78], red algae (Ceramium tenuicorne, Furcellaria lumbricalis)[69,70], brown algae (Pilayella littoralis, Dictyosiphon foeniculaceus)[69,78] and green algae (Cladophora glomerata, Enteromorpha intestinalis)[69]. The concentration of OH-PBDEs in Ceramium tenuicorne and Cladophora glomerata have been shown to vary seasonally, with highest concentrations in the summer[69]. Several ortho substituted OH-PBDEs have also been found in blue mussels (Mytilus edulis) in high concentrations (50ng/g f.w.)[79]. Furthermore have the concentration of OH-PBDE in blue mussels been observed to mimic the seasonal variation in algae[69,79].
Several OH-PBDEs have also been confirmed or tentatively identified in Baltic salmon (Salmo salar) plasma[31]. The structures of the major compounds found suggest that they originate from natural sources rather than PBDE metabolism[31]. OH-PBDEs have also been found in ringed seal plasma[49], common guillemot eggs[80] and in serum from white-tailed sea eagle nestlings[51]. A recent study has also shown that the concentrations of 2’-OH-BDE68 and 6-OH-BDE47 in Baltic herring has increased from 1980 to 2010[25,81].

2.4.2.4 Toxicological effects
The toxicity of OH-PBDEs is not as well studied as for PBDEs. However, the scientific interest has grown over the last decade as more studies indicate that OH-PBDEs are highly biological active, having both endocrine and neurotoxic effects[6,7].

OH-PBDEs have potential to disrupt the thyroid hormone system and action. OH-PBDEs have shown strong binding affinity to both the thyroid hormone receptor[59] and thyroid transporting proteins (TTR and TBG)[60,61]. The high affinity are likely due to OH-PBDEs structural resemblance to the thyroid hormones T₃ and T₄, as exemplified with 4’-OH-BDE121 in Figure 4. Effects on other endocrine pathways than the thyroidal have also been reported for OH-PBDEs. Low brominated OH-PBDEs have for example been reported to show estrogenic activity whereas higher brominated OH-PBDEs showed anti-estrogenic activity in vitro[82]. The difference in estrogenic activity between high and low brominated OH-PBDEs was found to correlate with the observation of two different binding modes to the estrogen receptor which were dependent on the molecular size of the OH-PBDE[82]. Further, 6-OH-BDE47 has shown anti-androgenic activity in vitro, having similar potency as the anti-androgenic drug flutamide[64].

![Figure 4. Chemical structures of 4’-OH-BDE121 and the thyroid hormones T₃ and T₄.](image-url)
6-OH-BDE47 is acutely toxic to adult and developing zebrafish when exposed to concentrations in the nanomolar range[9]. Developmental defects such as pericardial edema, yolk sac malformations, reduced pigmentation and lowered heart rate and developmental arrest were observed in the zebrafish embryo[9]. Microarray analysis of gene expression in the zebrafish embryonic fibroblast cells (PAC2) led to identification of two functional gene groups involved in carbohydrate metabolism and proton transport. Further work measuring respiration and membrane potential in isolated zebrafish mitochondria showed that 6-OH-BDE47 inhibits complex II in the mitochondrial electron transport chain[9].

Developmental arrest in zebrafish has also been observed for 3-OH-BDE47, 5-OH-BDE47[8]. In this study genes involved in thyroid hormone regulation, neurodevelopment and stress response were found to be upregulated after exposure to 6-OH-BDE47[8]. Others have reported altered expression of genes controlled by ER, mineralocorticoid receptor and AhR in zebrafish larvae exposed to 6-OH-BDE47[65]. 6-OH-BDE47 have also been reported to cause cytotoxic effects (i.e. increased apoptosis, cell cycle block and cause DNA damage) and oxidative stress in human hepatoma cells[83]

2.4.3 Methoxyalted polybrominated diphenyl ethers
MeO-PBDEs are naturally produced in the marine environment and to the best of the author’s knowledge there are no known anthropogenic sources for MeO-PBDEs.

2.4.3.1 Physicochemical properties
MeO-PBDEs are neutral lipophilic compounds and their physicochemical properties are influenced by their degree of bromination. The methoxylated analogues to the OH-PBDEs studied in paper I have log Kow values ranging from 6 to 8, based on computer modeling calculations using ACD/Labs software, version 11.02.

2.4.3.2 Environmental occurrence – with focus on the Baltic Sea
MeO-PBDEs have been found in cyanobacteria (Aphanizomenon flos-aquae, Nodularia spumigena)[70,78], red algae (Ceramium tenuicorne, Furcellaria lumbricalis)[69,70], brown algae (Pilayella littoralis, Dictyosiphon foeniclaceus)[69,78] and green algae (Cladophora glomerata, Enteromorpha intestinalis)[69] from the Baltic Sea. The concentration of MeO-PBDEs has shown to vary seasonally in blue mussel, but with increase in summer being less pronounced as for OH-PBDEs[79]. MeO-PBDEs have also been reported in Baltic herring[84], common guillemot eggs [80], white-tailed sea eagle eggs[50] and in serum from white-tailed sea eagle nestlings[51].
2.4.3.3 Toxicological effects

Information regarding MeO-PBDEs toxicity is limited. However one study reports teratogenic and morphological effects after exposing zebrafish embryo to 5-MeO-BDE47[85]. Another study showed no significant developmental alterations in zebrafish development with 6-MeO-BDE47 during early development. However, pericardial edema and curved spine was observed in exposed larvae after 120 hours post fertilization[65]. Biotransformation of 6-MeO-BDE47 in the zebrafish embryo/larvae was also studied and interestingly the 6-OH-BDE47 concentration increased in a time dependent manner[65]. This may be an indication that the effect observed is caused by the more potent 6-OH-BDE47 rather than 6-MeO-BDE47.

MeO-PBDEs potential to disturb thyroid hormone homeostasis is likely limited since MeO-PBDEs (i.e. 6-MeO-BDE47 and 2’-MeO-BDE68) have shown only slight or no binding affinity to TTR and TBG[60].

2.4.4 Polybrominated phenols and anisoles

BPs found in the environment can originate from both natural and anthropogenic sources[2,86]. 2,4-diBP, 2,4,6-triBP and pentabromophenol (PentaBP) are industrially produced chemicals and used as reactive intermediates in production of brominated resins and polymers[86]. 2,4,6-TriBP is the most widely used simple brominated phenol[87] and has also been used as a wood preservative[86]. Simple brominated phenols can also be formed from degradation of brominated benzenes and PBDEs in the environment[86]. To the best of the author’s knowledge, no anthropogenic source for BAs is known.

2.4.4.1 Physicochemical properties

BPs are lipophilic acids with low vapor pressures. Their log Kow range from 3 (2,4-diBP) to 5 (pentaBP) and their pKa range from 8 (2,4-diBP) to 4 (pentaBP)[86]. Corresponding anisoles lipophilicity range from log Kow 4 (2,4-diBA) to 6 (pentaBP) based on computer modeling calculations using ACD/Labs software, version 11.02.

2.4.4.2 Environmental occurrence – with focus on the Baltic Sea

The concentration of 2,4,6-triBP in herring (liver) have increased with 6% during 1980-2010 in southern Baltic Proper[25,81]. Whereas no such trend is visible in the northern Baltic Proper and likewise, not in the southern Bothnian Sea either[25,81].
2.4.4.3 Toxicological effects

The toxicity BPs have been reviewed by others [86,88], while data on toxicity of BAs seem to be lacking. Dietary exposure to 2,4,6-triBP have been reported to affect reproduction (i.e. reduced fertilization success and disturbed gonad morphology) in zebrafish [89]. Altered sex ratio towards males has also been observed in zebrafish after chronic exposure to environmental levels of 2,4,6-triBP during embryo and larvae development [90]. Further, were offspring to exposed fish found to be affected by malformations, retarded growth and reduced survival [90]. 2,4,6-triBP have also been reported to bind to TTR with 10 times higher binding affinity to TTR than T4 [64].
3 Oxidative phosphorylation

Mitochondria are intracellular organelles that play a pivotal role in energy production in eukaryotic cells as the majority of all adenosine triphosphate (ATP) is produced there. A mitochondrion is composed of two lipid membranes. The outer mitochondrial membrane separates the organelle from the cytosol whereas the inner mitochondrial membrane encloses the mitochondrial matrix. The mitochondrial matrix contains enzymes involved in key catabolic processes such as the tricarboxylic acid cycle (TCA cycle) and fatty acid (β) oxidation. The inner mitochondrial membrane is impermeable to ions and contains the membrane bound proteins involved in oxidative phosphorylation (OXPHOS)[10,91].

OXPHOS is the main metabolic pathway used by cells to produce ATP under aerobic conditions. The process of OXPHOS involves the electron transport chain (ETC) and the ATP synthase (Figure 5). The ETC consist of four membrane bound complexes (I-IV) which transfer electrons from NADH and Succinate (derived from catabolism of nutrients) via series of redox-reactions to the final electron acceptor, molecular oxygen. The energy released during these redox-reactions is used by complex I, III and IV to create a proton gradient (ΔpH+) and a separation of charge (ΔΨ) across the inner mitochondrial membrane. The ATP synthase use the energy stored in this electrochemical proton gradient (i.e. the proton-motive force) to fuel phosphorylation of adenosine diphosphate (ADP) to ATP[10]. Under normal conditions the proton motive force is approximately 180 mV[92]. The rate of substrate oxidation and electron transfer in the ETC is tightly coupled to the electrochemical proton gradient, a process known as respiratory control. When the cellular energy (ATP) demand is high, the electrochemical gradient decrease which stimulates substrate oxidation by the ETC[10].
Figure 5. Simple representation of the complexes involved in the electron transport chain (ETC) and the ATP synthase located in the inner mitochondrial membrane. The electron flow and proton transfer is depicted with red and orange arrows, respectively.

NADH dehydrogenase (Complex I), succinate dehydrogenase (Complex II), Ubiquinone (Q), ubiquinone:cytochrome c oxidoreductase (Complex III), cytochrome c (Cyt c) and cytochrome c oxidase (Complex IV)

3.1 Disruption of oxidative phosphorylation

The tight control between ETC substrate oxidation, the electrochemical proton gradient and ATP production, depends greatly on the structural integrity of the inner mitochondrial membrane and its impermeability to ions. An uncontrolled transfer of ions across the inner mitochondrial membrane against the proton gradient created by the ETC will affect the electrochemical proton gradient negatively and result in increased substrate oxidation and oxygen consumption. In such case, the mitochondrion is utilizing energy (metabolic substrates) without it being coupled to ATP formation. Instead the energy from substrate oxidation is released as heat. Exposure to agents dissipating the electrochemical potential in this manner will result in increased substrate oxidation but reduced ATP production. Rapid depletion of ATP can be acutely toxic, whereas low dose exposure over a prolonged time can lead to less efficient energy metabolism (i.e. body weight loss) and increased thermogenesis[10,93].
Some xenobiotics cause such uncontrolled proton leak by damaging the structural integrity of the inner mitochondrial membrane. Other xenobiotics act as proton carriers across the membrane, translocating protons from the intermembrane space to the mitochondrial matrix[10]. Generally, so called protonophoric uncouplers share common structural characteristics such as being lipophilic weak acids (pKa 5-7) with an acid dissociable group, a strong electron withdrawing moiety and bulky lipophilic groups[94]. Substituted phenols are a well-studied class of protonophoric uncouplers and both mono-molecular and bi- molecular models have been proposed to describe their proton translocation in the mitochondrial membrane[10] (Figure 6). Examples of potent protonophoric uncouplers are e.g. 2,4-dinitrophenol (DNP) and pentachlorophenol (PCP)[94].

![Figure 6 Molecular models of proton translocation across the inner mitochondrial membrane by substituted phenols. a) Monomolecular model b) bimolecular model. The protonated (HA) and deprotonated (A⁻) forms of the substituted phenol are depicted.](image)

Other mitochondrial toxins exert their toxicity by inhibiting the protein complexes involved in the ETC. Dependent on which complex is inhibited this can hamper or stop the electron and proton transfer by the ETC, resulting in suppressed substrate oxidation, oxygen consumption and ATP production[10]. Examples of potent inhibitors are rotenone (which inhibits Complex I), Antimycin A (inhibits complex III), potassium cyanide (inhibits complex IV)[95]. There are also compounds which inhibit the ATP synthase (e.g. Oligomycin[95]), resulting in decreased ATP formation[10].

Other compounds (e.g. Valinomycin[96]) depolarize the membrane potential by facilitate transfer of small ions across (e.g. K⁺) the inner mitochondrial membrane. These so called ionophores can either form ion channels or lipid soluble ion complexes which mediate ion transport across the membrane[10].
3.2 Methods to study OXPHOS in vitro

OXPHOS can be studied in vitro in both isolated mitochondria and in cells. Usually measurement of both mitochondrial respiration and membrane potential provides the best mechanistic information. The methods using isolated mitochondria are generally well established and the experimental settings can be modified (e.g. by using inhibitors) to provide mechanistic insight how xenobiotic disturb OXPHOS. However, the drawback of using isolated mitochondria is the lack of the cellular context. Hence, cell assays provides a more physiological relevant test condition as all cellular functions are preserved in the cell. However, the impermeability of the cell membrane to some commonly used substrates and inhibitors used to study OXPHOS can be problematic. Nevertheless, new techniques to study bioenergetics in intact cells are increasing as one advantage is the possibility for high throughput screening[92].

In paper I the OXPHOS disrupting potential of 18 OH-PBDE congeners of both natural and anthropogenic (metabolic) origin, were determined using a classic liver mitochondrial assay (TPP assay) and a mitochondrial potential cell assay (TMRM assay).

3.2.1 The TPP assay

The TPP assay is a well-established assay in which respiration (O$_2$) and membrane potential ($\Delta\Psi$) is measured simultaneously in isolated mitochondria[95,97]. The experiment was performed in a closed vessel and the oxygen level was measured using a Clarke-type electrode[95]. The membrane potential was assessed by help of a tetraphenylphosphonium ion (TPP$^+$) electrode. TPP$^+$ is a lipid soluble cation which is transferred electrophoretically into the mitochondria where it accumulates in proportion to the membrane potential[97]. Hence, the amount of free TPP$^+$ in the reaction medium is a relative measurement of $\Delta\Psi$. The test compound was added during titration and by recording changes in respiration and membrane potential simultaneously, effects on OXPHOS could be studied. 6-OH-BDE47 has previously been reported to be a potent inhibitor of complex II of the ETC[9]. By adding rotenone to inhibit complex I, substrate oxidation could only be mediated by complex II. This allowed us to study inhibitory effects as well as protonophoric uncoupling in the same experiment. A decrease in membrane potential and respiration after addition of test compound is indicative for inhibition of ETC, whereas a decreased membrane potential and increased respiration is symptomatic for protonophoric uncoupling.
3.2.2 The TMRM assay

Tetramethylrhodamine methyl ester (TMRM) is a fluorescent lipophilic rhodamine dye which accumulates in the mitochondrial matrix in proportion to the mitochondrial membrane potential ($\Delta\Psi$)[98]. In paper I, TMRM was used to study changes in mitochondrial membrane potential in zebrafish embryonic fibroblast (PAC2) cells after addition of test compound. The zebrafish cell line was chosen as it provides an outlook for future toxicity studies using zebrafish and zebrafish embryo. By measure the TMRM fluorescence in the cell spectrophotometrically a relative measurement of the mitochondrial membrane potential was obtained. The TMRM method is described in detail in paper I. The bioassay responds to compounds which alter the mitochondrial membrane potential but provides no detailed mechanistic information.

3.2.3 Cytotoxicity

Two bioassays (LDH- and MTT assay) were used in paper I to detect potential cytotoxic effects to confirm that the altered mitochondrial membrane potential observed in the TMRM assay was not due to nonspecific cytotoxicity caused by other mechanism than OXPHOS disruption, mitochondrial toxicity or disturbed mitochondrial membrane integrity.

3.3 Mixture toxicity

OH-PBDEs are found as mixtures in environment. The toxicity of chemicals in combination can differ from what predicted from the toxicity of single compounds as the compounds can interact which may either increase or decrease the toxic effect of the mixture. There are in principle two concepts which are used to assess combined effects of chemicals (i.e. concentration addition and independent action). Both models describe the quantitative relationship between the toxicity of simple compounds and their mixture, but are based on very different theories on how to assess mixture toxicity[99].

Concentration addition (CA) is built on the assumption that the compounds included in the mixture share a common mode of action and behave as they are simple dilutions of one another, showing no interactions. In such case, the effect exerted by one compound could be replaced by another compound contributing to an equal toxic effect without changing the overall mixture toxicity. The mixture toxicity can thus be described by summarizing each compounds effect contribution to the mixture, also called toxic unit (TU) summation. The effect contribution is based on the single compounds individual potencies (e.g. EC$_{50}$ or EC$_{10}$) and their concentrations in the mixture.
An important feature with the CA model is that it takes into account all compounds in the mixture and their effect contribution to the complete mixture toxicity, including compounds which are below their No observed effect concentration (NOEC) when tested as single compounds[99].

The model of independent action (IA) on the other hand is a probability based method using the statistical concept for independent random events to calculate the combined effects of chemicals. In the model of IA the effect contribution of the single compounds making up the mixture are assessed separately, assuming no interactions. Compounds which are below NOEC will not contribute to the calculated mixture effect using IA. The model of independent action is used to assess mixtures which contain compounds which exert their toxicity by different modes of action[99].

As both the CA and IA model assume no interactions amongst the chemicals in the mixture. Observed deviations from the predicted effect value by either method can thus be used to describe and quantify interactions (i.e. synergism or antagonism) within a mixture[99]. In paper I mixture effects of OH-PBDEs potency to disrupt OXPHOS were studied in the TMRM assay. An artificial mixture (composed of four OH-PBDEs) was tested and the compounds were combined so that each compound contributed equally to the mixture’s toxicity at each dose level (for details see paper I). The choice of using equitoxic concentrations of OH-PBDEs was derived from the TU concept in the CA model, using altered mitochondrial membrane potential as shared toxic endpoint. If the OH-PBDEs act as dilutions of each other (i.e. show additive effects but no interactions) the mixtures effect concentration (EC$_{50}$ or EC$_{10}$) should be one TU.

In addition to artificial mixtures, a mixture composed of seven OH-PBDEs reported in Baltic blue mussels were assessed in paper I. The seven compounds (i.e. 6-OH-BDE47, 2’-OH-BDE68, 6-OH-BDE85, 6-OH-BDE90, 6-OH-BDE99, 2-OH-BDE123 and 6-OH-BDE137) were combined at fixed ratios to mirror the internal exposure in blue mussel. The mixture was tested at dose levels 1 - 1000 times above the reported environmental concentration. Details regarding concentrations and compound ratios tested are given in paper I.
4 Chemical analysis

4.1 Description of species studied

4.1.1 Blue mussel (*Mytilus trossulus x Mytilus edulis*)

The Baltic blue mussel is a marine suspension feeding bivalve that successfully has adapted to the brackish water environment in the Baltic Sea. The Baltic blue mussel differ genetically[100], physiologically[101] and morphologically (i.e. being smaller in size and more elongated) from marine living blue mussels[102]. Since predation pressure and competition with other species are negligible in the Baltic Proper, blue mussels have become a biomass dominant that colonize hard bottoms down to approximately 30 m depths[103]. The distribution of blue mussels in the Baltic Sea is restricted by the salinity gradient, and their abundance decrease rapidly north of the Åland Sea, due to lowered salinity[103].

4.1.2 Long-tailed duck (*Clangula hyemalis*)

The long-tailed duck is a small mussel feeding sea duck (Figure 7) found in the northern hemisphere. The long-tailed duck breeds in fresh water habitats in northern Europe and western Siberia but migrate to ice-free brackish and marine waters in the winter to forage[26]. The Baltic Sea is the most important wintering area for the European population of long-tailed ducks. The majority of these sea ducks breeds in western Siberia but migrate to the Baltic Proper during October-December where they stay until mid-May. During winter and spring, the birds are found foraging on mussel banks located offshore and along the coasts of the Baltic Proper[26]. Long-tailed ducks dive and forage down to 35 m depths and in the Baltic Sea their diet consists mainly of bivalves, supplemented with other benthic organisms, crustaceans and small fish and fish egg[104-106].
In 2009, the population of long-tailed duck was surveyed and found to consist of 1,482,000 birds in the Baltic Sea. This represents a population decline by 65% (or 2.8 million birds) since the early 1990’s[26] and long-tailed duck is now classified as a vulnerable species on the IUCN global red list of threatened species[107]. Dramatic population declines have also been observed for other mussel feeding sea duck species e.g. common eider (*Somateria mollissima*) and velvet scoter (*Melanitta fusca*), in the Baltic Sea[26].

![Figure 7 Long-tailed ducks, one female and four males in flight. Photo by: Kjell Larsson](image)

4.1.3 Herring (*Clupea harengus*)

Baltic herring and sprat (*Sprattus sprattus*) are the two dominant pelagic zooplanktivorous species in the Baltic Sea and prey for e.g. cod (*Gadus morhua*), salmon (*Salmo salar*) and seals (e.g. *Halichoerus grypus*)[108]. From 1980’s to 2010, decreasing trends in condition (weight versus length) and fat content in Baltic herring has been observed in the Bothnian Bay (Harufjärden), the northern Baltic Proper (Landsort) and in the southern Baltic Proper (Utlängan)[17]. During the last three decades, dramatic changes in the fish community has been observed with a large decrease in population of cod and an increased abundance of sprat[109]. As sprat and herring to some extent share the same feeding preferences[110], an increased competition with sprat over food resources can have affected the condition of the herring negatively.
High mortality of herring roe has been observed in the Baltic Sea as mentioned in chapter 2.2. Baltic herring spawn in coastal areas and depending on region, the spawning season varies from March to June[111]. Herring place their roe on the sea bottom in shallow coastal waters and a study of the spawning bed selection of herring has shown that herring prefer hard bottoms with rich vegetation[112]. Algae species used by herring as substrate for their roe are for example Pilayella sp. and Cladophora sp[112]. Both these species from the Baltic Sea have shown to contain OH-PBDEs, and especially Pilayella can contain high concentrations in June[69].

4.2 Samples and sampling

The sampling sites of blue mussel, long-tailed duck and herring analysed in paper II-IV are presented in Figure 8.

![Figure 8. Sampling sites of blue mussel, long-tailed duck and herring.](image-url)
4.2.1 Blue mussels

The blue mussels analysed in paper II were collected during March, May and June (2011/12) from areas used by sea ducks for foraging. The sampling sites included both coastal areas as well as mussel banks in open waters (Figure 8). The sampling depth ranged from 6-19 m, depending on site. The mussels were collected using a bottom scraper dragged from a boat. The mussels (1-2 cm of length) were dissected and the mussel soft body was removed from the shell by tweezers. Mussels collected from the same sampling site and sampling time, were pooled and the tissue homogenized to reduce the effects between individuals.

4.2.2 Long-tailed duck

The long-tailed duck (n=10) analysed in paper II were collected from two locations in the Baltic Sea in February 2000 and May 2009 (Figure 8). The ducks collected in February (n=8) were from an offshore mussel bank (Hoburgs bank) located south of Gotland. The birds had drowned in fishnets and were collected by local fishermen. Two birds from Åland archipelago were shot by authorised hunters according to permit. The liver was dissected and the tissue homogenised, each bird was analysed individually.

Plasma from long-tailed duck was used in paper IV. The blood was collected from birds post mortem and stored in heparin treated tubes. The blood was separated into plasma and red blood cells by centrifugation.

4.2.3 Herring

Herring analysed in paper III were collected from two sites in the Baltic Sea (Figure 8). The fish (n=12) from Askö (May 2012) were collected using gillnets according to ethical permit. The fish from Ångskärsklubb (June 2012) (n=12) were sampled within the Swedish Environmental Monitoring Program and kindly provided by the Swedish Museum of Natural History. The fish were sexed post mortem and found to be sampled prior to spawning.

Plasma from herring was used in paper IV. The blood was sampled from the caudal vein with a syringe treated with heparin. The fish were euthanised directly after blood sampling according to ethical procedures and permit. The blood was separated into plasma and red blood cells by centrifugation.
4.3 Analytical methods

A simple schematic presentation of extraction and sample clean-up applied in paper II, III and IV are presented in Figure 9.

Figure 9. Scheme of the extraction and sample clean-up used in paper II, III and IV.
4.3.1 Extraction

Over the years several extraction methods have been developed for extraction of lipids and lipophilic compounds in biological tissues[113-117]. The biological samples analysed in paper II (i.e. blue mussel and liver from long-tailed duck) and paper III (i.e. herring) were extracted using the liquid-liquid extraction (LLE) method described by Jensen et al.[118], with the minor modification that normal-hexane (n-hx) was replaced with iso-hexane (iso-hx) as solvent. This method is based on the former method by Jensen et al.[115] which were modified to make the extraction faster and simpler by removing the filtrating step. The solvent ratio between n-hx and diethyl ether (DEE) was also changed from 9:1 (v/v) to 3:1 (v/v) in the new method. The method by Jensen et al[118] has been shown to extract lipids more efficiently than other methods (i.e. Bligh and Dyer[113] and Smedes[116]) and to give satisfactory recovery of phenols (e.g. simple chlorinated/brominated phenols, OH-PCBs and OH-PBDEs) in herring and blue mussel[118].

The extraction procedure is described in detail paper II and paper III. In short, the sample is denaturated with 2-propanol and extracted after addition of iso-hx:DEE (3:1, v/v) under natural pH. The extraction is repeated once more and the combined extracts is thereafter washed with a weak aqueous solution of hydrochloric acid (0.2 M) to remove 2-propanol and any unwanted water soluble co-extracts from the organic phase.

In paper IV the applicability of a LLE method developed by Hovander et al. for analysis of HPCs (i.e. OH-PCBs) in human plasma[119], was evaluated for OH-PBDE analysis in blood matrices (serum/plasma) from mammal (human, cat), bird (long-tailed duck) and fish (herring). The method applied in paper IV is the same as described by Hovander et al. [119], with the minor modification that n-hx was replaced with cyclo-hexane (c-hx) as solvent. Briefly described, the sample (serum/plasma) is denaturated with hydrochloric acid (6M) and 2-propanol and extracted after addition of c-hx:MTBE (1:1, v/v) under acidic pH. The extraction is repeated once more with c-hx:MTBE (1:1, v/v) and the combined extract is then washed with an aqueous solution to remove 2-propanol and water soluble co-extracts.

4.3.2 Separation of neutral and phenolic compounds

Neutral and phenolic compounds in paper II-IV were separated into two fractions by potassium hydroxide partitioning. The phenolic compounds are deprotonated by the potassium hydroxide and partition into the alkaline phase whereas the neutral lipophilic compounds remain in the organic phase. The phenolic compounds can then be retrieved from the alkaline phase by acidify the water phase and re-extract it with organic solvent.
Sample matrix (e.g. free fatty acids) can interfere with the potassium hydroxide partitioning and result in low recovery of OH-PBDEs as shown in shark (liver)[120] and herring (unpublished data). To avoid matrix effects gel permeation chromatography (GPC), a non-destructive clean-up method, was used in paper II to remove most of the lipids in the long-tailed duck liver samples prior to the potassium partitioning step. This clean-up step was introduced due to the high lipid content (mean: 5.8%) in the liver samples.

In paper IV, the herring samples were divided prior to the potassium partitioning step to reduce the lipid content in each fraction to avoid matrix effects.

4.3.3 Derivatization

The phenolic compounds analysed in paper II-IV were methylated prior to instrumental analysis to enable analysis using gas chromatography-mass spectrometry (GC-MS). The derivatization was performed using diazomethane, synthesized in house from N-methyl-N-nitroso-p-toluene-sulfonamide according to the method described by Vogel[121]. The methoxy-derivatives formed after derivatization with diazomethane are stable i.e. can be stored and tolerate destructive clean-up methods such as sulfuric acid treatment. However, diazomethane is carcinogenic and explosive and must thus be handled with great care. The laboratory work with diazomethane was approved by the Swedish work environment authority.

Acetylation and silylation agents can also be used as derivatizing agents. However, their main disadvantage is that their products generally are less stable than methoxy-derivatives[122]. However, pentafluorobenzoyl chloride (PFBCl) has successfully been used to acetylate halogenated phenolic compounds (HPCs) in biological samples with help of tetrabutylammonium as a phase transfer catalyst, yielding products (PFB acetyl derivatives) able to withstand concentrated sulfuric acid treatment[118].

4.3.4 Lipid removal

Both non-destructive and destructive clean-up methods were used to clean the samples from lipids which would otherwise obstruct the instrumental analysis.

Partitioning with concentrated sulfuric acid is an efficient but destructive method that was used in paper II, III and IV. However care should be taken when applying this method since some compounds are degraded by concentrated sulfuric acid. For the phenolic compounds to withstand the sulfuric acid treatment, the phenolic compounds were methylated.
The mussel and fish samples in paper II, III and IV were cleaned up on silica gel columns impregnated with sulfuric acid to remove the last remaining lipids in the samples.

Gel permeation chromatography (GPC), a non-destructive clean-up method, was used in paper II to remove most of the lipids in the long-tailed duck liver samples prior to the potassium partitioning step. The stationary phase used consisted of small neutral porous beads of styrene divinylbenzene (with 3% cross linkage). Large compounds with a molecular size larger than the exclusion limit of the stationary phase (i.e. ≥2000 daltons) will pass the beads unhindered, whereas small compounds diffuse into the pores of the beads where they are retained. Hence, small compounds will take longer time to pass through the GPC column than larger compounds. Further, the choice of mobile phase influence the retention time of the analytes as the solvent will affect swelling of the beads and the adsorption of the analytes to the stationary phase[123]. In paper II, a mixture composed of iso-hx:dichloromethane (1:1 v/v) with 0.5% formic acid (v/v) was used as mobile phase. The formic acid was used to facilitate elution of OH-PBDEs, by increasing the polarity of the mobile phase and more importantly ensure that the OH-PBDEs remained protonated during clean-up.

4.3.5 Instrumental analysis

The brominated substances discussed in this thesis were analyzed using gas chromatography-low resolution mass spectrometry (GC-LRMS) operated in electron capture negative ionization (ECNI) mode. The analyses were performed using single ion monitoring (SIM), scanning the bromide ions, m/z 79 and 81. This method gives a limit of quantification (LOQ) in the same order of magnitude as gas chromatography-high resolution mass spectrometry (GC-HRMS) operated in electron ionization (EI) mode for the brominated compounds analysed, but provides no structural information. Hence identification is based on the retention times of the authentic reference standards employed. The authentic reference standards used were either purchased or synthesized in house.

Lack of available reference standards are often a limiting factor for identification of new compounds, as true identification requires e.g. matching retention times and full scan spectra of the unknown compound and the reference compound. The OH-PBDEs and MeO-PBDEs quantified in this thesis have previously been identified in Baltic biota (i.e. red algae, blue mussel and salmon) by comparing relative retention times (on both polar and nonpolar columns) and ECNI and EI full scan spectra, against synthesized reference compounds[31,124].
Liquid chromatography-mass spectrometric techniques (LC-MS) using electro spray (ESI) and atmospheric pressure photo ionization (APPI) have been used by several others for OH-PBDEs analysis[125-129]. However, a slightly better chromatographic separation and probably higher LOQ using GC compared to LC have resulted in preference of the former technique[130].

4.3.6 Quality assurance/Quality control

Measures were taken to limit sample contamination during extraction and clean-up. Hence, all solvent, acids and salt used in the sample work up in paper II-IV were of highest purity available. All glass equipment were also washed and heated at 300 °C prior to use to avoid contamination. To measure any potential background contamination during the analysis, procedural solvent blanks were prepared in parallel with each batch of samples. Blank contamination was mainly a problem with the long-tailed duck liver samples (paper II) were small amounts of PBDEs, MeO-PBDEs and OH-PBDEs were found in the procedural solvent blanks. Likely these residues originate from sample cross contamination in the GPC injection vault.

The limit of detection (LOD) was set to three times the background noise (signal to noise ratio = 3). The limit of quantification (LOQ) was set as three times the LOD or five times the mean concentration in procedural solvent blanks. All samples were blank subtracted when blank contamination was present. The efficiency of the extraction and clean-up method was assessed by the use of surrogate standards. The surrogate standard should share structural and chemical properties as the analytes studied (to mimic their behavior in the sample work up) and the compound of choice must not be present as an environmental contaminant in the sample.

Very low recoveries of the surrogate standard (4’-OH-BDE121) were unexpectedly observed when OH-PBDEs were analysed in herring plasma (unpublished data). The method applied for the analyses has been developed by Hovander et al.[119], and is well established. The low recovery of 4’-OH-BDE121 pointed out the need to evaluate this method for analysis of OH-PBDEs in fish plasma. The method was evaluated, as described in paper IV using nine OH-PBDEs, three simple bromophenols and three MeO-PBDEs as test compounds. The recoveries of the analytes were tested in plasma from herring, long-tailed duck, humans as well as in cat serum. The results are further discussed in chapter 4.4
4.4 An analytical challenge

The results of the recovery study and the set up are presented in paper IV and indicated satisfactory recoveries of MeO-PBDEs across all species. High and reproducible recoveries were also obtained for BPs and OH-PBDEs in human plasma and cat serum. On the contrary, poor recoveries and large variations were obtained for plasma samples from herring and long-tailed duck. This initiated an extensive and tedious work to trace the reason(s) for this significant obstacle in the analysis of such samples.

4.4.1 Further method evaluation (additional results)

The observation of poor OH-PBDEs recoveries, called for some in-depth method evaluation using herring plasma. A structured evaluation scheme was set up using nine OH-PBDEs (5 ng/compound) which were added to herring plasma (0.5 g) at different steps in the sample work up as shown in Figure 10. As control OH-PBDEs were spiked to solvent which were extracted and clean-up along with the serum samples. The tests were performed in duplicate and the recoveries determined in relation to three references samples (solvent spiked with the same amount of OH-PBDEs as each sample). The references phenols were methylated with diazomethane and partitioned with sulfuric acid to remove impurities from the derivatization agent, but were otherwise handled as little as possible. A volumetric standard (BDE-138, 5 ng) was added to references and samples prior to instrumental analysis by GC/MS (ECNI). The recoveries of the references were set to 100%. The recovery of each OH-PBDE congener added to serum and solvent at different steps of the work up is presented in Figure 10. The samples spiked prior to denaturation (B) showed the poorest recoveries but also samples spiked prior to derivatization (D) gave very low recoveries for some OH-PBDEs (i.e. 2’-OH-BDE68, 4’-OH-BDE49 and 4’-OH-BDE121). While other OH-PBDEs were recovered in fairly good yields. The low recoveries of 2’-OH-BDE68, 4’-OH-BDE49 and 4’-OH-BDE121 are indicated to be matrix dependent since the spiked, extracted and cleaned-up solvent samples (A) had satisfactory recoveries.
Differences in recovery between sample B and C (Figure 10) were tested for pH dependency i.e. three extractions of herring plasma were performed under acidic, neutral and alkaline conditions. Briefly, three herring plasma samples (0.5 ng/sample) were spiked with 4’-OH-BDE121 (5ng) and 2-propanol (2 mL) and c-hx:DEE (3:1, v/v) was added. One sample was acidified by adding hydrochloric acid (6M, 0.3mL) whereas trimethylamine (99%, 0.1 mL) was added to another sample to increase the pH. The three samples were vortexed and after centrifugation the liquid phase was transferred to new test tubes. The remaining pellet was re-extracted with 2-propanol (2 mL) and c-hx:DEE (3:1, v/v, 3 mL). The pooled liquid phase which contained triethylamine was acidified by adding a few droplets of hydrochloric acid (6M) before all samples were partitioned with hydrochloric acid (0.2 M, 5 mL) and further treated with potassium hydroxide partitioning, derivatization (diazomethane) and sulfuric acid partitioning. A volumetric standard (BDE-138) was added prior to instrumental analysis and the recovery was determined against a reference, prepared as previously described. The recovery of 4’-OH-BDE121 was found to be poorest when extracted under acidic conditions (3%), whereas neutral (25%) and alkaline extraction (45%) gave slightly better yields.
The poor recovery of OH-PBDEs in sample E (Figure 10) was hypothesized to be due to co-extractives which obstructed the derivatization of some of the OH-PBDEs.

Since both the herring and the long-tailed duck plasma samples were red stained by haemolysis, potential effects of hemin/hematin on the derivatization efficiency was further investigated by a colleague (Dennis Lindqvist, Personal communication). In this experiment, hemin (0.2 mg) was added to aqueous sodium chloride solution (2.5 mL), spiked with 6-OH-BDE47 and 4’-OH-BDE121 and the sample extracted according to Hovander et al.[119]. After potassium hydroxide partitioning to isolate the phenolic fraction, the sample was divided in two subsamples and the phenols were methylated with either diazomethane or pentafluorobenzoyl chloride (PFBCl). The test was performed in duplicates and the recoveries of 6-OH-BDE47 and 4’-OH-BDE121 after derivatization with diazomethane were 56±2% and 19±1%, respectively, whereas PFBCl yielded good recoveries of both 6-OH-BDE47 (95±1%) and 4’-OH-BDE121 (96±4%). The result indicate that co-extracted hemin/hematin in blood samples can affect diazomethane methylation efficiency of OH-PBDEs. However, the use of PFBCl as derivatisation agent in biological samples is problematic since the efficiency of this derivatisation agent is known to be sensitive to other potential co-extractables (i.e. free fatty acids), as discussed elsewhere[118].

These findings emphasises the importance of proper method evaluations, especially when working with complex biological matrices. Most importantly, care needs to be taken when analysing OH-PBDEs in plasma or serum. Samples with haemolysis should be avoided as it can have profound effects on the result as shown here and in Paper IV. Since blood is a favorable matrix to work with for analysing OH-PBDEs, due to their specific retention to blood proteins (e.g. transthyretin). The need to develop a reliable extraction-, derivatization- and clean-up method for OH-PBDEs which are insensitive to hemolysis and preferable also could be applied to whole blood is most needed.
5 Results and Discussion

This chapter is aimed to highlight and discuss major findings of paper I-III.

5.1 OH-PBDEs potency to disrupt OXPHOS in vitro

In paper I, the OXPHOS disrupting potential of 18 OH-PBDEs were determined in vitro. The compound studied included both OH-PBDEs of natural and anthropogenic (metabolic) origin, and comprised compounds previously reported in biota from the Baltic Sea (table S1, paper I).

5.1.1 TPP data on OH-PBDEs

In the TPP assay, all OH-PBDEs tested were found to disrupt OXPHOS either by protonophoric uncoupling and/or via inhibition of the ETC at micro Molar (µM) concentrations in isolated rat liver mitochondria (Table 1). The lowest observed effect concentration (LOEC) was found to differ with a factor of 100 within the group. Three compounds (i.e. 3-OH-BDE47, 6-OH-BDE47, 6-OH-BDE85) were found to be as potent (LOEC=0.01 µM) as the model uncouplers; SF6847 (LOEC= 0.02µM), FCCP (LOEC=0.05µM) and PCP (LOEC=0.05µM), used as positive controls. Nearly all compounds tested were found to act via both uncoupling and inhibition within the concentration range tested (including the positive controls; FCCP, SF6847, PCP and DNP). Only 6-OH-BDE47 and 3’-OH-BDE154 did solely act as inhibitors whereas 5-OH-BDE47 and 6-OH-BDE90 did only act via protonophoric uncoupling.
Table 1. OH-PBDEs potency to uncouple and/or inhibit oxidative phosphorylation in the TPP- and TMRM assay. The compounds occurrence in Baltic biota is indicated. Table adopted from paper I.

<table>
<thead>
<tr>
<th>Compound</th>
<th>TPP assay</th>
<th>TMRM assay</th>
</tr>
</thead>
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<tr>
<td></td>
<td>LOEC (µM)</td>
<td>LOEC (µM)</td>
</tr>
<tr>
<td>2'-OH-BDE28</td>
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<td>5.0</td>
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<tr>
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</tr>
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</tr>
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</tr>
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</tr>
<tr>
<td>BDE-47</td>
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</tr>
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</table>

no effect (n.e.), not detected (n.d.), cyanobacteria (C), algae (A), blue mussel (B), herring (H), salmon (S), long-tailed duck (L), common guillemot (G), ringed seal (R). For references please see table S1, paper I and paper II and III.

5.1.2 TMRM data on OH-PBDEs

In the TMRM assay, 13 out of 18 OH-PBDEs were found to disrupt OXPHOS in zebrafish embryonic fibroblast cells (Table 1). 6-OH-BDE47 (LOEC=6.0µM) and 6-OH-BDE85 (LOEC=8.0µM) were the two most potent OH-PBDEs tested with effect concentrations in the same magnitude as the model uncouplers, FCCP (LOEC=0.75µM) and SF6847 (LOEC=2.5 µM). Five compounds (i.e. DNP, 2'-OH-BDE28, 2'-OH-BDE68, 3-OH-BDE47 3-OH-BDE155 and 5-OH-BDE47) which showed effect in the TPP assay did not disrupt OXPHOS in the TMRM assay. The negative response might be due to insufficient uptake by the cell/mitochondria, interaction with
the cell media or possible that these compounds are unable to disturb OXPHOS in zebrafish embryonic fibroblast cells, at least within the concentration range tested.

For the other compounds, a positive correlation (Pearson’s product moment correlation, $r=0.68$, $P=0.004$) between the TPP and TMRM LOEC values was obtained which indicate that the TMRM assay well predicts the effects of OXPHOS disruption. Since we were not able to pin point why some compounds showed a negative response in the TMRM assay compared to the TPP assay, the use of both assays is recommended also in the future when assessing chemicals potential for OXPHOS disruption.

5.1.3 Cytotoxicity

None of the compounds tested showed sign of cytotoxicity in the LDH assay. In the MTT assay, a decrease in formazan production was observed for some compounds at 50µM whereas other showed no effect or an increased formazan production compared to control (indicative of increased mitochondrial metabolism). However, altogether the results show that the observed disruption in mitochondrial membrane potential in the TMRM assay is likely not caused by confounding cytotoxicity.

5.1.4 Synergistic effects

When the OH-PBDEs were assessed as mixtures in the TMRM assay, synergistic effects were observed. In toxicology and ecotoxicology, synergistic effects are rarely observed and if they occur they are usually very specific for the chemical mixture tested and end-point studied[99]. For the four compound mixture (composed of 6-OH-BDE47, 6-OH-BDE99, 6-OH-5-Cl-BDE47, 2’-OH-6’-Cl-BDE68) the obtained TUs were 0.55 TU (EC$_{50}$) and 0.34 TU (EC$_{10}$), hence showing strong synergism (Figure 11a). However, when the potent inhibitor 6-OH-BDE47 was removed from the mixture, the three compound mixture (combined at equitoxic concentrations) showed additive effects (0.96 TU) when derived from EC$_{50}$ values, and synergism (0.22 TU) was only observed at lower concentrations (EC$_{10}$ values) as shown in Figure 11b. The four compound mixture was also assessed using the IA model, assuming that OH-PBDEs act independently, because OH-PBDEs were found to act both as protonophoric uncouplers and inhibitors of the ETC as shown in the TPP assay. The IA model predicted a lower effect than observed, again indicating synergism.
Figure 11 Concentration response curves in the TMRM assay for a four compound mixture (a) and a three compound mixture (b). The concentrations in graph a and b are expressed as toxic units (TU) calculated based on EC50 and EC10 concentrations derived from the individual OH-OPBDEs dose-response curves depicted in graph c. Figures adopted from paper I.

Valmas et al. (2008) have previously reported strong synergism when combining protonophoric uncouplers (FCCP and PCP) and inhibitors of the ETC (phosphine, azide)[131]. The synergistic effect was suggested to be caused by simultaneous inhibition of the ETC and depletion of the proton gradient by uncoupling, causing a rapid collapse of the mitochondrial membrane potential[131]. As the OH-PBDEs tested in paper I were found to act via protonophoric uncoupling and/or inhibition, this combined effect on the mitochondrial membrane potential, could likely explain the synergism observed. This hypothesis is also strengthened by the observation in paper I that the mixture toxicity decreased when the potent inhibitor 6-OH-BDE47 was removed from the mixture.
Strong synergism (0.22 TU, EC₅₀) was also observed when the seven compound mixture, mimicking the internal exposure in Baltic blue mussels, was studied. The synergism was even stronger than for the four compound mixture (0.55 TU, EC₅₀), indicating that the synergistic effects increase with increasing number of compounds. Further, the mixture were found to disrupt the mitochondrial membrane potential at dose levels only 100 to 1000 times higher than reported in Blue mussels from the Baltic Sea (Σ₇OH-PBDEs: 50 ng/g f.w.) (Figure 12).

![Figure 12. Dose response curve of a seven compound mixture added to mimic the exposure of OH-PBDEs in Baltic blue mussel. The concentration factor of one represent the environmental concentration in blue mussels and a factor of 100 a concentration 100 times more concentrated. Figure adopted from paper I.](image)

5.2 Blue mussels

The blue mussel is a key species in the Baltic Sea ecosystem[103] and the main food source for long-tailed ducks while wintering in the Baltic Sea[106]. The concentrations of brominated substances (i.e. BPs, BAs, OH-PBDEs, MeO-PBDEs and PBDEs) were assessed in blue mussels, collected in March and May from nine sites in the Baltic Proper used by long-tailed ducks for foraging (Paper II). The geometric mean (GM) concentrations of ΣOH-PBDEs, ΣMeO-PBDEs and ΣPBDEs are presented in Figure 13.

On average the Blue mussels collected in spring were found to contain analytes in the following decreasing order, with geometric means (GM) presented on fresh weight basis in parenthesis: ΣMeO-PBDEs (GM 1.3 ng/g f.w.), ΣBPs (GM 0.87 ng/g f.w.), ΣOH-PBDEs (GM 0.82 ng/g f.w.), ΣBAs (GM 0.20 ng/g f.w.) and ΣPBDEs (GM 0.13 ng/g f.w.). 2,4,6-triBP, 6-OH-BDE47 and 6-OH-BDE85 were the three dominating compounds in the phenolic fraction, where 6-OH-BDE47 and 6-OH-BDE85 comprised as much as 50% and 21% of the sum OH-PBDEs, respectively.
The mean concentration of ΣOH-PBDEs (GM 0.82 ng/g f.w.) in blue mussel collected during March-May is approximately 60 times lower than concentration measured in the Blue mussels sampled in June (ΣOH-PBDEs: 53 ng/g f.w.) in paper II. The higher exposure seen in summer is expected since concentrations of OH-PBDEs in the Baltic Sea vary seasonally with an increase during summer in both algae[69] as well as in blue mussels[79]. Further, blue mussels collected in June contained similar OH-PBDE concentrations as previously reported from the same site in June (ΣOH-PBDEs (50 ng/g f.w.))[79]. The results confirm that the high exposure to these compounds in the Baltic blue mussel most likely is an annually reoccurring summer phenomenon.

It is not yet understood how blue mussels are affected in vivo by long time exposure to environmental levels of OH-PBDEs, a fact that requires further research. Exposure studies with the potent protonophoric uncoupler pentachlorophenol (PCP) have shown to affect the metabolic rate in blue mussel[132] and fresh water clam (Pisidium amnicum)[133]. In the study with blue mussels, the lowest water concentration tested (50 µg/L PCP) were found to enhance the metabolic rate (measured as oxygen uptake and heat output) by 35% after two days exposure under normal aerobic conditions. The effect concentration corresponded to a tissue concentration of 2.9 µg/g f.w. in blue mussel[132]. The measured effect concentration is approximately 50 times higher than the concentration of ΣOH-PBDEs measured in blue mussels collected in June (Paper II).

Others have also reported PCP to affect the anaerobic metabolism in blue mussels[134]. During prolonged period of anaerobiosis blue mussels mainly derive their energy from malate, which originate from phosphoenolpyruvate derived in the glycolysis. In the mitochondria, part of the malate is oxidized to pyruvate and acetate which yield NADH whereas another portion of malate is converted to fumarate and reduced to succinate by fumarate reductase. The electrons to fuel the reduction of fumarate is derived from the NADH formed from the oxidation of malate and purvate which are funnelled via complex I of ETC and rhodoquinone to the fumarate reductase[135]. The ratio of fumarate:succinate were found to increase with increasing PCP exposure as reported in a study with blue mussels exposed to PCP under anoxic conditions[134]. The authors of the study suggest a metabolic block in the electron transfer system as potentially responsible for the observed effect[134].

PCP was also found to inhibit ETC at higher concentration in the TPP assay in paper I. However, inhibition of complex I could not be studied in the experimental setup used in the TPP assay in paper I, as rotenone was used to block this complex to enable simultaneous studies of protonophoric un-
coupling and inhibition of complex II, III and IV. Hence, further studies are needed to determine if OH-PBDEs also have potential to affect the anaerobic metabolism in blue mussels.

As OH-PBDEs showed to disrupt OXPHOS in paper I the effect of OH-PBDEs on aerobic metabolism in blue mussels can potentially affect the condition of the blue mussel, and their nutritional value for mussel feeders such as sea ducks.

5.3 Long-tailed duck

Long-tailed ducks need to digest large quantities of mussels each day to meet their daily energy requirement. Their estimated daily intake of blue mussel meat was assessed in paper II, based on the assumption that wintering long-tailed ducks have a daily energy expenditure of 1500kJ/day[105]. Long-tailed ducks average daily consumption was estimated to be approximately 450 g mussel meat (calculations given in paper II). Based on the mean fresh weigh concentrations of brominated substances in blue mussels collected in March and May, this correspond to a daily intake of 390 ng ΣBPs, 90ng ΣBAs, 370ng ΣOH-PBDEs, 590ng ΣMeO-PBDEs and 59ng ΣPBDEs for the long-tailed ducks. Although this is an estimate, it provides a first assessment of long-tailed duck dietary intake of these substances while foraging in the Baltic Sea.

Dietary exposure to protonophoric uncouplers (i.e. PCP) has been reported to significantly decrease the body weight and reduce the lipid content in mallard ducklings (Anas platyrhynchos)[136]. The lowest observed adverse effect level (LOAEL) after 11 day exposure was found for birds feed a diet with a concentration of 961 µg/g PCP (which corresponded to a liver tissue concentration of 31µg/g).

Concentration of brominated substances in livers of long-tailed ducks wintering in the Baltic Sea is presented in paper II. The geometric mean (GM) concentrations of ΣOH-PBDEs, ΣMeO-PBDEs and ΣPBDEs are shown in Figure 13. The liver concentration of ΣOH-PBDEs (GM 0.34 ng/g f.w.) is approximately 5 order of magnitude below LOAEL for PCP (31µg/g f.w.) reported to cause a 50% reduction in lipid content in Mallard ducklings after 11 day exposure[136]. How adult long-tailed ducks are affected by long term exposure to low doses of OH-PBDEs while foraging in the Baltic Sea (from October to mid-May) is not known. As mussel feeding sea ducks have potential to be affected by compounds disturbing OXPHOS directly and/or indirectly via reduced food quality of their feed, further studies on the in vivo effect of OH-PBDE exposure in sea ducks and mussels are needed.
The significantly lower concentrations of ΣBPs, ΣBAs, ΣOH-PBDEs and ΣMeO-PBDEs in long-tailed duck liver tissue compared to blue mussel (Figure 13) despite a constant daily intake, suggest that these compounds are rapidly excreted in long-tailed duck liver tissue whereas PBDEs seem to be retained.

The PBDE congener profile in long-tailed duck were found to be similar as previously reported in liver tissue from common eider (Somateria mollissima) and white-winged scoter (Melanitta deglandi) from the Canadian arctic[137] (Figure 14). The result indicate that the PBDE congener profile in benthic feeding sea ducks differ from what is seen in sea birds[138], fish and fish feeding mammals[46], where BDE-47 usually dominate.
5.4 Baltic herring

Baltic herring is one of the dominant pelagic zooplanktivorous species in the Baltic Sea. Concentrations of brominated substances (i.e. BPs, BAs, OH-PBDEs, MeO-PBDEs and PBDEs) were assessed in Baltic herring as described in paper III. The herring, collected in May and June, were from the northern Baltic Proper (Askö) and the southern Baltic Sea (Ängskärsklubb).

The geometric mean (GM) concentrations of ΣOH-PBDEs, ΣMeO-PBDEs and ΣPBDEs are presented in Figure 15 showing no significant differences in ΣOH-PBDEs levels between Askö (GM 9.4 ng/g l.w.) and Ängskärsklubb (GM 10 ng/g l.w.). 6-OH-BDE47 dominated the OH-PBDE congener profile in herring from both locations, where it comprised 87% (Askö) and 91% (Ängskärsklubb) of the sum OH-PBDEs.

6-OH-BDE47 was one of the toxicologically most potent OH-PBDEs tested in paper II. This compound has also been found to be acutely toxic to adult and developing zebrafish[8,9]. In adult zebrafish lethality was observed after 12 min when exposed to a water concentration of 1µM 6-OH-BDE47 (corresponding to a liver concentration of 3.5 µg/g f.w.)[9]. The mean (min-max) concentration of 6-OH-BDE47 in herring liver from the northern Baltic Proper (Landsort) sampled in autumn during 1980-2010 were 2.0 (0.89-3.8) ng/g f.w.[81].

Figure 14. PBDE congener profile in blue mussels, sea ducks and herring compared to a technical PentaBDE mixture (Bromkal 70-5DE).
OH-PBDEs have shown to be taken up in fish after both water and dietary exposure[72,139]. 6-OH-BDE47 has also been reported to be formed as a metabolite of BDE-47 in Northern pike (Esox lucius)[55] but little or no transformation has been seen in zebrafish (Danio rerio)[9,72] or Japanese medaka (Oryzias latipes)[71]. As the metabolic capacity of herring is not fully understood, the metabolic contribution to the here reported 6-OH-BDE47 concentration cannot be excluded, although natural production is most likely the major source in the Baltic Sea. However, metabolic interconversion of 6-MeO-BDE47 and 6-OH-BDE47 has been observed in zebrafish[72] and Japanese medaka[71], which indicate that metabolic de-methylation of MeO-PBDEs can play a role as source for OH-PBDEs in fish.

![Figure 15](image_url) Geometric mean concentration (95% confidence intervals) of ΣOH-PBDEs, ΣMeO-PBDEs and ΣPBDEs in Baltic herring from Askö (n=12) and Ängskärsklubb (n=12) presented on a) lipid weight basis and b) fresh weight basis. Figure adopted from Paper III.

At Ängskärsklubb, the concentration of ΣMeO-PBDEs (GM 150 ng/g l.w.) was 15 times higher than the concentration of ΣOH-PBDEs, whereas the concentration of ΣMeO-PBDEs (GM 42 ng/g l.w.) at Askö was lower. 6-MeO-BDE47 dominated the MeO-PBDE congener profile contributing to 64% (Askö) and 77% (Ängskärsklubb) of the sum MeO-PBDEs. The major source for MeO-PBDEs, as reported herein, is most likely natural production. Metabolic conversion of PBDE to OH-PBDE followed by in vivo O-methylation in herring is a less likely source, as neither OH-PBDEs nor MeO-PBDEs were found as transformation products of BDE-47 in either Japanese medaka or zebrafish[71,72]. The high ratio between 6-MeO-
BDE47 (GM 116 ng/g l.w.) and BDE-47 (GM 10 ng/g l.w.) in herring from Ängskärsklubb further supports that the MeO-PBDEs originate from natural sources rather than from PBDEs.

Substantial maternal transfer of 6-OH-BDE47 and 6-MeO-BDE47 to the roe has also been observed in zebrafish. For 6-OH-BDE47 the ratio between egg and liver concentrations were 0.8, and an even higher ratio (2.9) was observed for 6-MeO-BDE47[72]. In Japanese medaka have similar ratio been observed for 6-OH-BDE47 (0.59) while the ratio for 6-MeO-BDE47 was lower (0.74)[71]. Maternal transfer of OH-PBDEs and MeO-PBDEs (as precursor for OH-PBDEs) may be of concern, due to OH-PBDEs high toxicity to the developing fish embryo[8,9]. 6-OH-BDE47 has shown to cause a wide range of developmental effects such as pericardial edema, yolk sac deformations and developmental arrest in zebrafish embryo when exposed to low water concentrations (EC50=25 nM)[9]. In Baltic herring, high mortality of herring roe has been observed both in field[33] and in controlled experiments when herring egg were exposed to algae[140], now known to contain OH-PBDEs[69]. The low effect concentration for 6-OH-BDE47 and the higher production of OH-PBDEs in algae during the summer months, may explain the observed herring egg mortality.

Furthermore, as one of the consequences of disturbed OXPHOS is altered metabolism, potentially leading to weight loss [10,93]. More research is needed to determine if the decreasing trends in condition and fat content observed in Baltic herring [17] is associated with their exposure to chemicals with OXPHOS disrupting properties, such as OH-PBDEs.
6 Concluding remarks and future perspective

The thesis main results are that naturally produced OH-PBDEs act as potent disruptors of OXPHOS in vitro and that these compounds act synergistically. However, further investigations on the in vivo effects at the environmental levels determined in Baltic biota (algae, cyanobacteria and in wildlife) are needed. Especially the effect of chronic low dose exposure to OH-PBDEs needs to be assessed. This should preferentially be done in controlled experiments and with field studies. Efforts should also be made to investigate potential biomarkers to study OXPHOS in vivo. Biological factors reported to be significantly altered in fish exposed to PCP are for example; body weight, plasma inorganic phosphate, blood glucose, lactate and plasma protein[141].

Given the high toxicity of OH-PBDEs in zebrafish, and the high exposure seen in Baltic blue mussels during summer, future studies of in vivo effects in fish and mussels are warranted. The long-tailed duck are constantly exposed to OH-PBDEs via their mussel diet while wintering in the Baltic Sea, but the low levels observed in long-tailed duck (liver) compared to their food, indicate that OH-PBDEs and MeO-PBDEs have low retention in ducks. However, more knowledge regarding OH-PBDEs toxico-kinetics is needed. If blue mussels exposure to OH-PBDEs affect their nutritional value as food source for sea ducks also needs to be further assessed. Since we see a clear seasonal variation of OH-PBDEs and MeO-PBDEs in algae and blue mussels, with higher levels in summer[69,79], studies of in vivo effects in mussels should be carried out during summer. Further, studies with mussel feeding sea ducks should preferentially be focused on species feeding on blue mussels during the exposure peak of OH-PBDEs and MeO-PBDEs (e.g. the Baltic breeding common eider).

Further, OH-PBDEs also have potential to disturb the thyroid hormone system, which can affect the metabolism. Hyperthyroidism (i.e. elevated thyroid homone levels) stimulates a hypermetabolic state with increased energy expenditure and weight loss[142]. Thiamine deficiency has also been reported in both fish (i.e. Baltic salmon)[29] and waterfowl (e.g. Common eider) from the Baltic Sea[27]. Thiamine (Vitamin B1) is in its phosphorylated form an essential cofactor for several enzymes involved in catabolism and in extension in cellular energy production. For example is thiamine crucial for nor-
mal function of α-ketoglutarate dehydrogenase, an enzyme involved in the citric acid cycle[143]. As thiamine deficiency, altered thyroid homoestasis and disturbed OXHOS have potential to affect catabolism and thereby ultimately the energy (ATP) production and ATP expenditure in the organism. Studies of combined effects of low thiamine levels and OH-PBDE exposure should be prioritised in the future.

The effect of eutrophication in the Baltic Sea can have affected the production of OH-PBDEs as cyanobacteria and macroalgae species are primary producers of these compounds. The temporal trend of 6-OH-BDE47 and 2’-OH-BDE68 in herring liver tissue, indicate that the exposure has increased from 1980 to 2010 in the southern and the northern Baltic Proper[81]. However, temporal trends from other species such as blue mussel would be beneficial to better assess the situation. As the concentration in algae and blue mussels varies seasonally, care should be taken to ensure that samples have been collected under similar conditions to avoid drawing erroneous conclusions.

In addition to the compounds discussed in this thesis, several yet unidentified halogenated phenols have been observed in algae, blue mussels[78] and salmon[30] from the Baltic Sea. Hence, further efforts must be made to identify these compounds and their potential toxicity should be investigated.

This thesis emphasis the fact that naturally produced compounds, in addition to anthropogenic substances, have the potential to pose a significant stress on the already sensitive Baltic Sea ecosystem.
Många antropogena halogenerade organiska ämnen har visats vara skadliga för miljö och djurliv och är därför idag reglerade på grund av dess toxiska, persistenta och bioaccumulerande egenskaper. Polybromerade difenyletrar (PBDEer), i form av tekniska blandningar såsom PentaBDE, OctaBDE och DecaBDE, är en grupp bromerade organiska ämnen som sedan 1970-talet använts för att flaskydda bland annat textilier och elektronik men vars användning idag är förbjuden (PentaBDE och OctaBDE) eller begränsad (DecaBDE) inom EU.

Samtidigt har kunskapen om att halogenerade ämnen även bildas naturligt i miljön ökat. En stor mängd bromerade organiska ämnen produceras naturligt i våra hav. Hydroxylerade polybromerade difenyletrar (OH-PBDEer) är en grupp ämnen som i Östersjön bildas av cyanobakterier och olika arter av makroalger, men som även kan bildas in vivo via metabolism av PBDEer. Östersjön är ett av världens största brackvattenhav som på grund av hög tillförsel av näringsämnen från omgivningen idag lider av utbredd eutrofiering. Höga halter av näringsämnen främjar tillväxt av fytoplankton och cyanobakterier men påverkar även makroalgsamhället, genom att tillväxt av snabbväxande fintrådiga alger gynnas.

Den avhandlingen som här presenteras har haft fokus på förekomsten av bromerade ämnen av både naturligt och antropogen ursprung i Östersjöns flora och fauna, med speciellt focus på OH-PBDEer. Avhandlingen omfattar både toxikologiska och kemiskt analytiska studier med det övergripande målet att bestämma OH-PBDEers förmåga att störa oxidativ fosforylering (OXPHOS) in vitro, samt att bedöma exponeringen av dessa ämnen i olika arter från Östersjön (dvs. i blåmussla, strömming och alfågel).

I Paper I beskrivs två metoder (s.k. bioassays) vilka användes för att studera 18 olika OH-PBDEers potential för OXPHOS-störning in vitro. OXPHOS är den biokemiska process som producerar huvuddelen av all energi (ATP) i eukaryota celler under aeroba förhållanden (se Figur 5, sid. 19). OH-PBDEerna testades enskilt men även i blandningar för att studera eventuella synergieffekter. Det visade sig att alla de testade ämnena frikopplade och/eller inhiberade elektrontransportkedjan då isolerade mitokondrier exponerades. Exponering av zebrafiskceller (PAC2) visade att 13 av de 18
OH-PBDEerna störde OXPHOS-processen. Två ämnen, 6-OH-BDE67 och 6-OH-BDE85 befanns vara lika potenta som modell-substanserna FCCP och SF6847, vilka används vid studier av effekter på OXPHOS. Dessutom visade sig blandningar av OH-PBDEer ge upphov till synergistiska effekter. En blandning bestående av sju OH-PBDEer, som adderats i samma proportioner som de förekommer i blåmusslor från Östersjön, gav effekt redan vid koncentrationer som endast var 100-1000 gånger högre än vad som uppmätts i blåmusslorna.

Flera arter av musselätande dyänder i Östersjön, inklusive alfåglar (Clangula hyemalis), har minskat dramatiskt i antal de senaste 20 åren. Östersjön är ett av de viktigaste över-vintringsområdena för den europeiska populationen av Alfåglar. Från oktober till mitten av maj kan man observera alfåglar i Östersjön, innan de migrerar norrut till sina häckningsplatser i bland annat västra Sibirien. Då alfåglars kost till övervägande del består av blåmusslor i Östersjön, som kan innehålla höga halter av OH-PBDEer var det angeläget att bedöma alfåglars exponering för dessa ämnen.

I Paper II analyserades halterna av bromerade ämnen av både naturlig och antropogent ursprung i lever från tio alfåglar. Även blåmusslor från platser där alfåglar födosöker analyserades för att jämföra halter och kongenmönster mellan arterna. Resultatet visade att alfåglar är exponerade för flera olika naturligt producerade bromerade ämnen via födan. Men att änderna tycks utsöndra dessa ämnen snabbt då halterna i levern var betydligt lägre än i blåmusslorna, trots ett relativt högt estimerat dagligt intag hos änderna. Dock tycks de mer lipofila PBDEerna ha högre retention i alfågeln än t.ex. MeO-PBDEer. Även kongenmönstret mellan alfåglar och musslor skiljde sig åt.

OH-PBDEer har i kontrollerade exponeringsförsök visat sig vara mycket giftiga för zebrafisk (Danio rerio) och zebrafiskembryo vid mycket låga vatten koncentrationer av OH-PBDE. Det är därför viktigt att bedöma exponeringen av dessa ämnen i fisk från Östersjön.

I Paper III, analyserades strömming från norra egentliga Östersjön (Askö) och Södra Bottenhavet (Ängskärsklubb). Den naturliga produktionen av OH-PBDEer i alger varierar under året, men troligen är som störst under sommarhalvåret. Fiskarna (n=12/lokalt) fångades därför i maj/juni strax före deras reproduktion. Halterna (analyserade som helfiskhomogenat) av ΣOH-PBDEer skiljde sig inte signifikant åt mellan Askö (9.1 ng/g fett vikt) och Ängskärsklubb (10 ng/g fettvikt). 6-OH-BDE47 dominerade och utgjorde 87% (Askö) och 91% (Ängskärsklubb) av den total mängden OH-PBDEer. I Ängskärsklubb uppmättes höga halter (150 ng/g fettvikt) av naturligt producerade metoxylerade polybromerade difenyletrar (MeO-PBDEer). I strömming kan dessa ämnen vara en källa till mer giftiga OH-PBDEer då
andra fiskarter visats kunna omvandla MeO-PBDEer till OH-PBDEer in vivo. Även PBDEer uppmättes i strömming från båda lokalerna.

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9 Appendix A

Contribution to Paper I-IV

I. Performed part of the laboratory work and together with my co-author Jessica Legradi, was responsible for writing the manuscript.

II. Assisted the laboratory work and was responsible for the data evaluation and manuscript writing.

III. Performed all laboratory work and was responsible for data evaluation and writing the manuscript.

IV. Performed major part of the laboratory work and together with my co-author Jessica Norrgran, was responsible for writing the manuscript.
10 References


