

PERSISTENT ORGANIC POLLUTANTS IN
FISH - IMPROVING THRESHOLD VALUES
FOR MARINE RISK ASSESSMENT

Dissertation zur Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen
Fakultät der Christian-Albrechts-Universität zu
Kiel

vorgelegt von
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Kiel, 01. Dezember 2015

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Tag der mündlichen Prüfung:	10. Februar 2016
Zum Druck genehmigt:	10. Februar 2016
gez.	Prof. Dr. Wolfgang J. Duschl, Dekan/in

Summary

Since 2008, the Marine Strategy Framework Directive (MSFD) has coordinated the monitoring and integrated assessment in European marine waters in order to protect marine ecosystems and simultaneously guarantee the sustainable use of marine goods and services. So far, many progresses have been made to maintain or achieve a 'good environmental status' by the year 2020. However, many gaps and open questions still exist in regard to the risk assessment of persistent organic pollutants (POPs).

The general aim of this thesis was to contribute to the implementation of the MSFD at different levels. Primary focus was set on methodical issues, especially the validation of toxicity tests through the definition of sound dose-response relations, which are the basis for inferring unambiguous threshold values. Additionally, it was tested if microinjection could be a valuable tool for the development of reliable threshold values for biological effect monitoring. Secondly, this thesis also contributed to the discussion whether contaminants in gonads of fish are a valuable indicator for healthy marine wildlife in marine risk assessment. Many contaminants, especially POPs are suspected to impair reproduction and therefore to adversely affect the fitness of a species. Hence, information on POP concentrations in native fish species as well as their potential transfer rates from liver and muscle to gonad tissue during maturation in spawners and from spawners to their offspring is crucial to evaluate the contaminants with regard to potential embryotoxic effects.

In **Chapter I** the statistical tool '*toxtestD*' is presented, which offers a user-friendly way to appropriately design toxicity studies aiming for binary response data, as for instances the Fish Embryo Toxicity (FET) test. The developed tool advances the quality of dose-response data by focussing on adequate numbers of test subjects, on optimized dose allocation, and on inclusion of a baseline for toxicity tests that aim for small target concentrations to provide an optimal basis for the definition of threshold values as well as other purposes.

The FET test is a potent way to identify and quantify embryotoxic effects of contaminants. However, this test is limited to the application of polar substances. Non-polar substances cannot be applied. A potent way to identify and quantify the embryotoxic potential of lipophilic POPs offers the direct application of contaminants into Zebrafish embryos by simultaneously mimicking a maternal pollutant transfer via microinjection. In **Chapter II** water, methanol, DMSO, and triolein were directly administered into the yolk sac of freshly fertilized Zebrafish embryos to validate microinjection as tool in ecotoxicology studies. High volume oscillations between consecutive injections into several fish eggs question this method as adequate tool to produce unambiguous

dose-response relations in order to infer threshold values. However, microinjection is still a potent way to identify toxicities of lipophilic substances to fish development.

In **Chapter III & IV** contaminant concentrations of several POPs were determined in muscle, liver, and gonads of Baltic herring, *Clupea harengus*, and in muscle, gonads, and eggs of European eel, *Anguilla anguilla*. Remarkable concentrations were detected in any tested tissue including gonads of fish during maturation. POPs, accumulated in gonads, possibly result in impaired reproduction by a general decrease in gamete quantity and quality or end up in spawned fish eggs after maternal transfer processes where they potentially develop embryotoxic effects. Both may result in a reduced fitness. Hence, including content loads of gonads as indicator into the current monitoring seems promising to assess healthy marine wildlife. The results of this thesis help to improve the current situation of data quality and quantity for the derivation of threshold values in marine risk assessment. It revealed microinjection as a potent method to identify toxicity of lipophilic substances and determined content load in gonads as a potential new indicator for healthy marine wildlife.

Zusammenfassung

Im Jahr 2008 wurde die Meeresstrategie- Rahmenrichtlinie (MSRL) mit dem Ziel ins Leben gerufen, die marinen Ökosysteme der Europäischen Gemeinschaft zu schützen und gleichzeitig eine nachhaltige Nutzung mariner Ressourcen innerhalb dieser Regionen sicher zu stellen. Der 'gute Umweltzustand soll dabei bis zum Jahr 2020 erreicht bzw. erhalten werden. Im Rahmen der Umsetzung der MSRL ist bereits viel erreicht worden, doch existieren weiterhin zahlreiche Lücken und offene Fragen besonders im Hinblick auf die Bewertung der Schadstoffbelastung mariner Ökosysteme.

Ziel dieser Arbeit war es, zur Umsetzung der MSRL an verschiedenen Stellen beizutragen. Zunächst stand die Validierung von Toxizitätstests durch die Definition von aussagekräftigen Dosis-Wirkungsbeziehungen durch eine angemessene Versuchsplanung im Mittelpunkt. Eindeutige Dosis-Wirkungsbeziehungen sind zur Ableitung von aussagekräftigen Schwellenwerten unbedingt erforderlich. Weiterhin wurde die Mikroinjektion als Methode zur Ermittlung von Schwellenwerten für das Monitoring biologischer Effekte, im Besonderen für lipophile Substanzen und als Ergänzung für den klassischen FET Test, überprüft.

Darüber hinaus beschäftigte sich diese Arbeit mit der Schadstoffbelastung in verschiedenen Gewebetypen heimischer Fischarten. Viele Substanzen, v. a. persistente organische Stoffe, stehen im Verdacht, den Reproduktionserfolg und damit die Fitness einer Population zu verringern. In diesem Zusammenhang standen sowohl die Schadstoffkonzentrationen in den Gonaden selber, als auch die Transferraten zwischen den Gewebetypen im Fokus. Die Kenntnis über den potentiellen Schadstofftransfer von Muskel und Leber zu Gonaden und Eiern in Wildpopulationen ist besonders wertvoll für die Bewertung von Substanzen im Hinblick auf mögliche embryotoxische Effekte im marinen Ökosystem. Im Zusammenhang mit dem Reproduktionserfolg kann darüber hinaus die Schadstoffbelastung in den Gonaden von Fischen als möglicher Indikator für die Gesundheit von marinen Wildpopulationen dienen.

In **Kapitel I** wird das Tool '*toxtestD*' zur Planung von Toxizitätsuntersuchungen am Beispiel des Fischembryo Toxizitäts- (FET) Test erläutert. Die Erstellung eindeutiger Dosis-Wirkungsbeziehungen erfordert, neben einer adäquaten Anzahl an Untersuchungsobjekten, eine Auswahl möglichst optimaler Testkonzentrationen und die Einführung einer Basislinie. Letztere gewinnt besonders bei der Abgrenzung kleiner Effekte an Bedeutung, die für die Ableitung von Schwellenwerten besonders wertvoll sind. Das Tool '*toxtestD*' bietet eine benutzerfreundliche Möglichkeit Toxizitätstests mit binären Antwortvariablen optimiert für die Ableitung von verlässlichen Schadstoffschwellenwerten zu planen.

Eine etablierte Methode embryotoxische Effekte im Labor zu identifizieren und zu quantifizieren bietet der FET Test. Aufgrund seines experimentellen Aufbaus ist der FET auf die Applikation polarer Substanzen limitiert. Nicht-polare Schadstoffe können nicht getestet werden. Eine Möglichkeit die embryotoxische Wirkung von lipophilen Substanzen zu untersuchen bietet die direkte Applikation einer Substanz in den Fischembryo via Mikroinjektion. Gleichzeitig können dabei ein maternaler Schadstofftransfer vom Muttertier in die Eier simuliert und so aufwendigere Tierversuche vermieden werden. Um die Mikroinjektion als Methode zur Ermittlung von Schwellenwerten zu bewerten, wurden in **Kapitel II** Wasser, Methanol, DMSO und Triolein direkt in die Dotterzelle von Zebraäbrblingseiern im Ein- bis Zweizellstadium injiziert. Im Verlauf von mehreren, aufeinander folgenden Injektionen schwankte das Injektionsvolumen so stark, dass die Mikroinjektion als Methode zur Ermittlung von eindeutigen Dosis-Wirkungsbeziehungen nicht empfohlen werden konnte. Trotzdem stellt die Mikroinjektion eine Möglichkeit dar, embryotoxische Effekte lipophiler Substanzen zu identifizieren.

In den **Kapiteln III** und **IV** wurde die Belastung durch verschiedene Schadstoffe in Muskeln, Lebern und Gonaden des Ostseeherings, *Clupea harengus*, und in Muskeln, Gonaden und Eiern des Europäischen Aals, *Anguilla anguilla*, gemessen. In allen Gewebetypen konnten Schadstoffe nachgewiesen werden. Substanzen, die während der Reifung in den Gonaden akkumuliert werden, stehen im Verdacht den Reproduktionserfolg zu verringern. Einerseits können Schadstoffe in den Gonaden die Qualität und Quantität der Gameten vermindern. Andererseits können Schadstoffe vom Muttertier auf den Nachwuchs übertragen werden, wo sie ihre embryotoxische Wirkung entfalten. Beides hat direkten Einfluss auf die Fitness einer Spezies. Die Schadstoffbelastung in den Gonaden von Fischen kann demnach möglicherweise ein adäquater Indikator für die Gesundheit von marinen Wildpopulationen sein und deshalb als Parameter in das obligate Monitoring biologischer Effekte aufgenommen werden.

Die Ergebnisse dieser Arbeit tragen dazu bei, die gegenwärtige Situation der Datenqualität und -quantität für die Ableitung von Schadstoffschwellenwerten in der marinen Risikobewertung zu verbessern. Darüber hinaus konnte gezeigt werden, dass die Toxizität von lipophilen Substanzen mittels Mikroinjektion nachgewiesen werden kann und dass die Schadstoffkonzentrationen in den Gonaden von Fischen als Indikator zur Bewertung der Gesundheit von marinen Wildpopulationen dienen können.

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General Introduction

Contaminants in the seas

Chemical pollution threatens the marine environment and is caused by manifold human activities like ship traffic, as well as riverine inputs and land runoffs. Assessing the risk arising to marine ecosystems after the introduction of chemical substances and contaminants is required to protect and conserve the marine environment as well as the many goods and services it offers to humans [EU, 2008b; Roose et al., 2011].

Chemical substances and contaminants are released from various land based sources as well as from vessels travelling the seas [Rieser, Gray Hudson, and Roady, 2005]. They accumulate *inter alia* in biota and linger in the marine environment for a long period of time. These contaminants are referred to as persistent organic pollutants (POPs). Once accumulated within biota, POPs can cause harmful effects on marine species. Altering mobility, reproduction, migration, or survival of single species POPs concomitantly may affect the biodiversity of marine ecosystems [Fent, 2013].

Maintaining healthy marine ecosystems requires an adequate risk assessment of the pollution of the seas and oceans. This includes determining critical values of contamination, tracking of chemical pollution in marine wildlife, and, if critical values are exceeded, taking adequate measures to reduce the harmful concentration.

Assessment of chemical pollution in European Seas

Assessing the chemical pollution of the marine environment incorporates three individual steps, which in the best case scenario cooperate closely with each other. At first, hazardous substances should be monitored on a regular basis to detect temporal and spatial trends of pollution. At second, assessment criteria need to be developed to assess the pollution status of the environment. At third, screening for 'new' substances is required to regularly update the list of substances with potential harm to the environment.

Owing to an increased awareness of chemicals released from multiple sources into the marine environment and their potentially adverse effects to marine wildlife, several regional and national monitoring initiatives have been evolved during the last decades. Their procedures include generally the monitoring and assessment of contaminant concentrations in water, sediments, and biota in the concerned sea regions or subregions. An overarching marine strategy governing the progress on a European level has long been absent [Roose et al., 2011], but exists as Marine Strategy Framework

Directive since 2008 [EU, 2008b].

The Marine Strategy Framework Directive

In 2008, the Marine Strategy Framework Directive (MSFD) was established to coordinate the monitoring and the integrated assessment in the European marine waters considering the whole ecosystem (ecosystem approach). The Directive aims for the development and the progressive implementation of policies and measures to protect the marine environment from pollution and to guarantee the sustainable use of marine goods and services. This is ensured by the integration of environmental concerns into policies, such as the Common Fisheries Policy under the perspective of maintaining or achieving a 'good environmental status' (GES) by the year 2020.

Marine waters of a GES are defined as seas and oceans that are ecologically diverse and dynamic. They are clean, healthy, and productive within their intrinsic conditions. Currently, eleven qualitative descriptors (D) have been identified in order to assess GES addressing contaminants (D8, D9), but also encompass varying aspects of biodiversity (D1, D2, D6), the status of commercially exploited fish and shellfish (D3, D4), eutrophication (D5), alteration of hydrographical conditions (D7), marine litter (D10), and underwater noise (D11) [EU, 2008b; EU, 2010a].

The implementation of the MSFD involves all member states of the European Union (EU) with direct access to the marine environment and neighbouring states, regardless whether those are EU members or not. These countries are obligated to develop marine strategies for protecting and using the marine environment in a sustainable way according to the intrinsic characteristics of the marine region or subregion. These regions encompass waters in the Baltic Sea, the North-east Atlantic Ocean including the waters surrounding the Azores, Madeira and the Canary Islands, as well as the Mediterranean and the Black Sea.

The MSFD is assisted by the activities and developed strategies of Regional Sea Conventions (RSCs) like the Helsinki commission (HELCOM) and the Oslo and Paris commission (OSPAR) [EU, 2008b; EU, 2010a]. Both commissions coordinate an extensive monitoring programme in their area of responsibility, which is implemented by adjacent states to the North-east Atlantic (OSPAR) and the Baltic Sea (HELCOM).

Contaminants - hazardous substances within descriptor 8 MSFD

The introduction of hazardous contaminants has been identified as pressure and impact on the marine environment within descriptor 8 MSFD. Among these chemicals are synthetic compounds as e.g. priority substances defined under the Water Framework Directive (WFD), which are also relevant for the marine environment, non-synthetic substances and compounds like heavy metals and hydrocarbons, as well as radionuclides. Priority substances are classified as hazardous contaminants with proven

aquatic ecotoxicity or with proven human toxicity via aquatic exposure. Widely distributed contaminants and substances potentially widespread due to their production or use are also listed [EU, 2000]. Among the priority substances are pesticides, antifouling agents, and pharmaceuticals [EU, 2008b; EU, 2010a].

Persistent organic pollutants

Contaminants of particular concern are persistent organic pollutants mostly with lipophilic characteristics. Due to their persistence and potential to bioaccumulate, these chemical substances and compounds are measurable in remarkable concentrations in sediment and biota such as fish even in offshore regions.

POPs are suspected to affect organisms directly by reducing their general fitness by influencing mobility, growth, reproduction, or survival. This in consequence may affect the ecosystem functioning in the marine environment. However, the effects of most chemical substances in wild living organisms have not or only rarely been identified so far [Fent, 2013].

The current monitoring of contaminants

Besides the initial assessment of the environmental status of the European waters, the MSFD called also for an 'establishment and implementation of a monitoring programme for an ongoing assessment'. Where possible, existing monitoring programmes have been adapted and if necessary, were updated for the purposes of the MSFD (EU 2008a). Today, European monitoring of contaminants in the marine environments is mainly carried out at national level with international agreements compiled by RSCs [EU, 2008b; OSPAR, 2012; HELCOM, 2015].

The marine monitoring of contaminants according to the requirements of MSFD covers the three matrices water, sediment, and biota and is carried out on a regular basis. Chemical monitoring comprises the major tasks of detecting the regional pollution patterns, identifying time trends of pollution, and assessing the potential risk for the monitored species as well as species of higher trophic level. Especially, fish that serves as food source for marine mammals, sea birds, and humans is a common indicator for the last purpose.

Indicator species are recommended by RSCs with regard to the intrinsic characteristics of the ecosystem. In open sea regions, only fish is monitored while in coastal zones also algae and invertebrates are of interest. Species used as indicators for monitoring purposes are mostly keystone species within the ecosystem. They comprise a broad distribution, a high accessibility, and they reflect changes in the concentration of contaminants in the surrounding environment. A common indicator fish is herring (*Clupea harengus*), as are also diverse round and flat fishes [OSPAR, 2012; HELCOM, 2015]. In addition, the RSCs define the sampling station, the substances to be monitored as well as the target tissue according to the characteristics of the contaminant and the available

analytical procedure [OSPAR, 2012; HELCOM, 2015].

Bio-effects: reproductive success as an example

Understanding the toxicity of a chemical on a molecular or organism level is a crucial step for the assessment of the potential risk this chemical may cause to an ecosystem. Effects on organisms, such as loss of successful reproduction or increase in diseases or mortality, also influence the ecosystem probably by reducing biodiversity [Fent, 2013]. So far, reproductive success is not considered as bio-effect for healthy marine wildlife within the monitoring program of the MSFD. A loss in reproduction equals a loss in the general fitness of a population or species [Fent, 2013]. Among other environmental effects, pollution may play a significant role in causing impaired reproduction. Many contaminants either act as endocrine disruptors [Scholz and Mayer, 2008], reduce parental gonad development [Deng et al., 2010; He et al., 2011], or cause malformation or mortality in the offspring after parental pollutant transfer [Nyholm et al., 2008]. Contaminant effects on reproduction differ between males and females as has been shown at least for Zebrafish (*Danio rerio*) [Nyholm et al., 2008; Norman et al., 2010; He et al., 2011]. Hence, knowledge on gonad burden of both sexes of marine fish may be necessary to estimate reproductive success. While few studies so far concentrate on the extend of maternal transfer in wild living fish such as Chinese sturgeon (*Acipenser sinensis*) [Peng et al., 2010; Peng et al., 2012], data on contamination by POPs in male gonads are still absent.

Combining data from in situ observations with dose-response relations obtained from laboratory tests may lead to a potent way to infer the putative risk for the reproductive success of a species. In particular, species with high fat content, such as Baltic herring and European eel, may benefit from this approach as effects are more likely to appear in these species compared to lean fish.

Screening for new relevant contaminants

Beyond monitoring of known substances with identified adverse effects to the marine environment MSFD also calls for a regular updating of targets [EU, 2008b]. This includes the screening for 'new' substances with a potential risk to marine ecosystems. The application of more and more new substances and materials by a still increasing chemical industry offers an immense source for pollution by pesticides, antifouling agents, flame retardants and pharmaceuticals, or their degradation products [Roose et al., 2011]. Once introduced into the environment, adverse effects on marine ecosystems may emerge. These effects of 'new' substances have currently not been examined although essential for marine risk assessment. Further on, screening for 'new', potentially hazardous substances is mostly not part of the current marine monitoring and still imposes a major task for the MSFD.

Assessment of contaminants

The implementation of the MSFD is still in state of development. Many gaps and open questions can be identified [Law et al., 2010; OSPAR, 2013a]. Table 1 gives an overview of deficiencies concerning the risk assessment of contaminants in European marine waters.

In general, the environmental risk of contaminants is assessed based on threshold values that are derived from dose-response relations obtained from acute and/or chronic toxicity tests. Threshold values have been adopted from OSPAR and WFD for the initial assessment within the MSFD. These are 'Environmental Assessment Criteria' (EACs) and 'Environmental Quality Standards' (EQS), respectively [OSPAR, 1997; EU, 2000; EU, 2013; EU, 2008b; OSPAR, 2009]. Even though several EQS have been developed during the last couple of years, assessment criteria for priority substances are still incomplete (Table 1).

This deficiency is caused by difficulties in transferring data from single species laboratory studies to a multi-species ecosystem as it is requested by the ecosystem approach stated in the MSFD. Such a transfer is crucial and generally requires certain assumptions and extrapolation of the toxicity test results. However, an overarching concept to solve these difficulties has not yet been developed [OSPAR, 2010; OSPAR, 2013b].

While the derivation concepts differ between EAC and EQS, their purpose is similar. Both values are considered as transition points; concentrations below are not of significant risk to the environment while concentrations above are of concern and require direct measures for reduction [Roose et al., 2011].

Toxicity tests

Toxicity tests are powerful tools to detect and quantify adverse properties of chemical contaminants. The resulting dose-response relations can be used as basis to derive threshold values used in the risk assessment of priority substances [OSPAR, 2009; OSPAR, 2010]. Toxicity tests are laboratory studies conducted under controlled conditions with model organisms. These individuals are cultured in the laboratory facilities, comprise short generation cycles, and are therefore available in high numbers, which is advantageous for the statistical security of results [Scholz et al., 2008]. The further use of consistent test settings as governed by the guidelines of the Organisation for Economic Co-operation and Development (OECD) ensures the comparability of the test procedure and results, which is another important prerequisite for setting threshold values.

Many different test procedures are available spanning from acute to chronic tests, involving varying species and life-stages of test organisms [OECD, 2015]. Early life stages tests have been preferred over tests using adults in recent years due to several reasons [Braunbeck et al., 2004; Lammer et al., 2009]. First, early life stages are either similarly sensitive or more sensitive compared to adult life stages [Hutchinson, Solbe, and Kloepper-Sams, 1998; Lammer et al., 2009]. Second, early life stage tests operate faster

than tests on full-grown parental fishes as toxic effects emerge rapidly during the development of an embryo. Third, the use of embryos instead of adults in toxicity tests is also in line with current animal welfare legislations [EU, 2010b].

Table 1: Summary of gaps and open questions identified within the European marine risk assessment of contaminants.

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1. List of priority substances and list of priority hazardous substances need an update.
Are substances relevant for WFD^a equally relevant for MSFD? Are there further substances released to the environment potentially causing adverse effects to marine wildlife?
 2. Non-target screenings for 'new' substances are lacking.
 3. High-resolution analytical methods are required.
 4. Most sensitive species is unknown.
Determination of the most sensitive species, its most sensitive life stage as well as the related effects appearing in the marine environment is hardly feasible.
 5. Bio-effects are not included into obligatory monitoring.
Bio-effects are only voluntarily measured by regional monitoring programs.
 6. The list of current threshold values is still incomplete.
WFD provided 3 EQS^b or whole fish and OSPAR published 8 EACs^c or fish. The number of substances with potential harm to the marine environment is certainly higher.
 7. Current threshold values are questionable.
Values have been adopted from WFD or OSPAR:
 - a EQS in WFD: Used data basis differ in quality and quantity. Assessment factors (AF) account for uncertainties in data basis arisen from intra- and inter-laboratory variation, intra- and inter-species variation, and from extrapolation of short-term to long-term toxicity as well as from laboratory data to field impacts. AFs increase with increasing uncertainties in data basis. The applicability of an EQS derived on such a basis is generally ambiguous.
 - b EACs from OSPAR are partly adopted from foodstuff directives and were initially not developed for the risk assessment of the marine environment.
 8. Overarching concept for deriving threshold values is lacking.
Sound dose-response data is required but lacking for most substances. Data transfer from single species laboratory studies to multi-species ecosystems is difficult as species sensitivities may differ between fresh- and saltwater organisms, as well as between laboratory and wild living species, or between different taxa.
 9. Sound dose-response data is lacking for most chemical substances.
Toxicity tests are generally tailored for the half maximal effective concentration (EC₅₀) or half maximal lethal concentration (LC₅₀), which is too high to reliably infer threshold values. Aiming for small effect sizes requires some adjustments in experimental design.
 10. Toxicity tests such as the FET test are limited by technical difficulties.
Within a FET test only polar substances comprising molecular weights below 3 kDa can be applied. For the application of other substances further application routes need to be developed and evaluated.
 11. Toxicity of mixtures of contaminants is mostly unknown so far.
Effects of chemical mixtures may be either additive, synergistic or antagonistic and can only hardly be predicted from investigations with single contaminants.
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a WFD: Water framework directive (2000/60/EG, 2008/105/EG, 2013/39/EU)

b EQS: Environmental Quality Standard derived by WFD published in 2008 (2008/105/EG), 11 EQS have been published in 2013 (2013/39/EU)

c EAC: Environmental Assessment Criteria derived by Oslo and Paris Commission (OSPAR).

The Fish Embryo Toxicity Test - a classical test

A famous example among toxicity tests is the Fish Embryo Toxicity (FET) test, which replaced the Acute Fish Toxicity test in recent years [DIN EN ISO, 2009; Lammer et al., 2009]. The FET test uses fish embryos, preferably those of the Zebrafish (*Danio rerio*), to investigate adverse effects emerging after waterborne exposure of embryos to increasing concentrations of a contaminant. The resulting adverse effects, in the following also referred to as responses, are examined after 48 or 96 hours (h) and can be lethal and sublethal malformations, teratogenic effects, death, or coagulation [DIN EN ISO, 2009; OECD, 2013a].

The FET test offers the opportunity of detecting and quantifying the adverse effects of applied substances in an easy way. However, two major restrictions arise, when resulting dose-response relations shall be used for the derivation of threshold values as requested by OSPAR, WFD, or MSFD. First, FET is restricted to the application of polar substances with molecule sizes below 3 kDa [OECD, 2013a]. Each test substance is applied into the aqueous environment of the FET test. Hence, non-polar substances comprising a octanol-water partition coefficient ($\log K_{ow}$) above 4 like polybrominated biphenyl ethers cannot or can only rarely be solved in water and are generally not applicable through the FET test [United States Environmental Protection Agency (U.S. EPA), 2010]. Large molecules, regardless if polar or not, can presumably not pass through the pores of the egg envelope (chorion) of the fish embryo. Consequently, a potential risk appears to generate false negative results in common FET studies due to limited permeability of the egg envelope for some contaminants [Henn and Braunbeck, 2011].

The second restriction is described by the experimental design. In literature, FETs generally aim for the half maximal effect concentration (EC_{50}) or half maximal lethal concentration (LC_{50}) [OECD, 2013a] while smaller effect concentrations are required for the derivation of threshold values [OSPAR, 2010]. Smaller effect sizes, such as LC/EC_{10} , are computed by extrapolation of existing dose response data accepting the possibility of overlooking potential errors or emerging uncertainties by this procedure. Assuring the reliability of threshold values is only possible by an implementation of a proper experimental design of toxicity tests aiming for the EC_{10} instead of EC_{50} [OSPAR, 2013b]. In fact, this requires some adaptations in the experimental design, which will be outlined within the scope of this thesis.

Microinjection - a new method in aquatic toxicity testing?

Microinjection allows the direct injection of substances into single cells under a microscope (Figure 1) and is already a common tool in many disciplines of biology [Wittbrodt, 2010]. It has not been established as a standard technique in ecotoxicology yet but offers the opportunity to administer harmful substances directly into the yolk cell of fish embryos to investigate their mode of action or toxic potential [Dodd et al.,

2000; Spitsbergen and Kent, 2003].

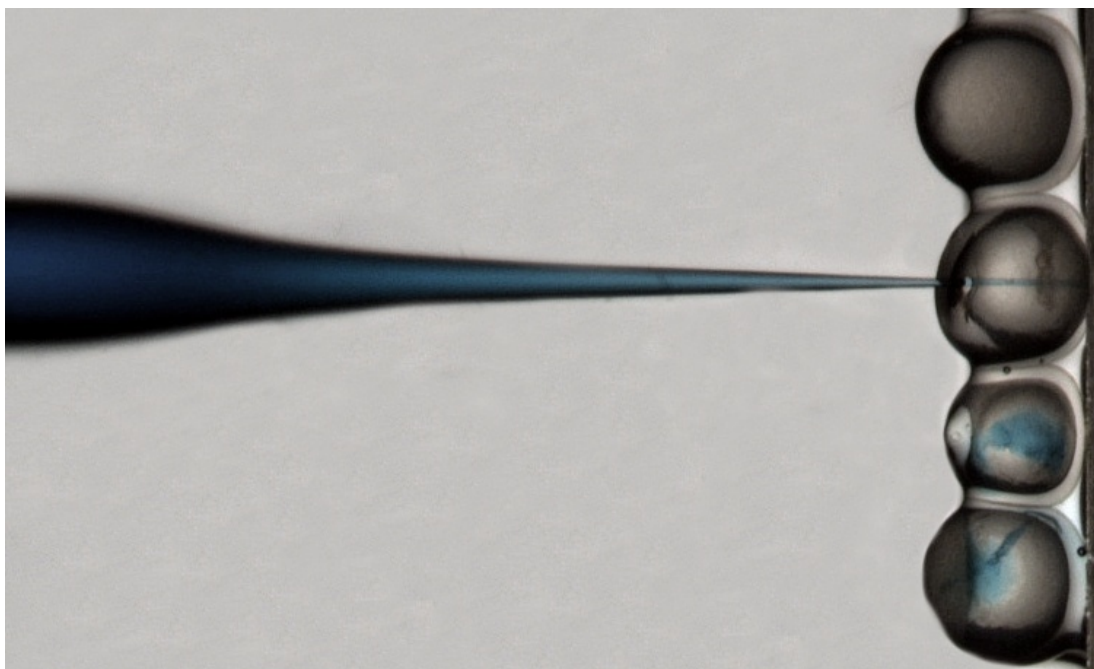


Figure 1: Microinjection into yolk cell of an early Zebrafish embryo.

It can potentially be used for the development of dose-response relations when increasing concentrations of a contaminant are administered into fish eggs of similar stages of cell divisions. Both polar and non-polar substances can presumably be administered. The direct application additionally offers the advantage that chorion and embryo envelope, both acting as natural barriers to protect the embryo from contaminants, can be overcome. Hence, it is likely that smaller toxicant concentrations can be applied to cause a response. This in turn avoids the production of huge amounts of test waters as necessary for the FET test, which have to be disposed correctly afterwards. Microinjection is also a novel approach that allows mimicking maternal transfer of contaminants from the adult into the egg. Particularly lipophilic substances may be transferred into the fish egg together with parental fat reserves as shown for Zebrafish [Nyholm et al., 2008]. After fertilization the lipophilic compounds can presumably be stored in the egg yolk. Injection of contaminants into the yolk cell of the freshly fertilized fish egg seems to be most promising to mimic this natural maternal transfer process. However, the feasibility of microinjection within this context needs to be tested as well as appropriate carrier (=vehicle) for the application of lipophilic contaminants need to be identified.

Aim and outline of this thesis

The general aim of this thesis was to contribute to the implementation of the Marine Strategy Framework Directive (MSFD) at different levels. Primary focus was set on methodical issues, especially the validation of toxicity tests through the definition of sound dose-response relations, which are the basis for inferring unambiguous threshold values. Additionally, it was tested if microinjection could be a valuable tool for the development of reliable threshold values for biological effect monitoring. Secondly, this thesis also contributed to the discussion whether contaminants in gonads of fish are a valuable indicator for healthy marine wildlife in marine risk assessment. Many contaminants, especially POPs are suspected to impair reproduction and therefore to adversely affect the fitness of a species. Hence, information on POP concentrations in native fish species as well as their potential transfer rates from liver and muscle to gonad tissue during maturation in spawners and from spawners to their offspring is crucial to evaluate the contaminants with regard to potential embryotoxic effects.

This thesis is divided into the following chapters:

CHAPTER I

Optimal test design for binary response data: the example of the fish embryo toxicity test.

This study aimed at the presentation of a new R package called *'toxtestD'* that assists the user with developing an adequate design of toxicity tests with binary response data. The tool focuses on sample size, dose allocation, and the inclusion of a baseline. The latter is especially interesting for toxicity tests that aim for small target concentrations in order to provide an optimal database for inferring threshold values.

CHAPTER II

Microinjection into Zebrafish Embryos (*Danio rerio*) - A useful tool in aquatic toxicity testing?

This study focuses on the direct application of putative vehicles (=carriers) for potentially hazardous substances into the yolk sac of Zebrafish embryos via microinjection. Benefits and limitations of this application are outlined and microinjection as a putative tool for ecotoxicology studies is discussed with special emphasis on data quality.

CHAPTER III

Persistent organic pollutants in Baltic herring (*Clupea harengus*) - an aspect of gender.

In this study, the environmental status of the Baltic herring, *Clupea harengus*, is assessed with regard to contamination with POPs. Three tissue types of this indicator species are investigated to elucidate the distribution of POPs throughout the fish body with

special emphasis on gonad contamination. POPs accumulating in gonads during maturation may affect the reproduction of a species including effects on the gonad quality and quantity as well as on the embryonic development of the next generation. Measuring content loads of POPs in gonads as indicator for reproductive success is discussed.

CHAPTER IV

Maternal transfer of emerging brominated and chlorinated flame retardants in European eels.

In Chapter IV, emerging brominated and chlorinated flame retardants are analysed in eggs, gonads, and muscle tissue of European eel, *Anguilla anguilla*. Maternal transfer of contaminants in eel should be demonstrated for the first time. Additionally, toxicity tests have been carried out in order to assess concentrations found in eel tissues as potentially harmful or not-harmful to fish development.

Material & Methods

Materials and methods have been described in detail within the corresponding sections of the single chapters and are not repeated here.

Chapter I

Optimal test design for binary response data: the example of the fish embryo toxicity test

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Abstract

Background The fish embryo toxicity test (FET) is an established method in toxicology research for quantifying the risk potential of environmental contaminations and other substances. Typical results of the method are the half maximal effective concentration (EC_{xx}) or the no observed effect concentration (NOEC). However, from an environmental perspective, it is most important to safely identify the concentration of the substance effect which lies above the effect under control condition (spontaneous effect). The common FET is not optimal to detect ECs for small target effects. This paper shows how to optimize the efficiency and consequently the benefit of the FET for small effects by using an adequate experimental design. The approach presented here can be carried over to all test systems generating binary (yes/no) outcomes.

Results The experimental design has three components in this context: determination of spontaneous response, sample size calculation, and dose allocation. A strategy for all three components is proposed from which a design is given including precision requirements and makes the most effective use of the experimental effort. This strategy amounts to expanding the usual FET guidelines of Organisation for Economic Co-operation and Development, German Institute for Standardization, or American Society for Testing and Materials by adding a planning step that adapts the test to the specific user's need.

Conclusions For the practical calculation of an adapted design, a newly developed software is presented as R package '*toxtestD*'. It provides a user-friendly way of developing an optimal experimental design for the FET without in-depth statistical knowledge. The programme is suited for all experimental problems involving a binary outcome and a continuous concentration.

Keywords

Toxicity test planning; R statistic package; Zebrafish embryo; Spontaneous lethality; Sample size; Dose design

Background

Toxicity tests in ecotoxicology serve to detect and quantify toxic properties of chemical substances. Typically, a toxicity test is a laboratory test, which means that the experimenter chooses the test procedure, the number of subjects to test, the doses or concentrations to apply, as well as the appropriate way to quantify toxicity. Such quantifications are used to set thresholds for allowable concentrations in the environment. Choices in experimental design should ensure high quality of the results in terms of precise and unbiased toxicity quantification and of statistical decisions with controlled error rates. The design should also make optimal use of the experimental effort. A proper experimental design is frequently demanded, but only a few publications on risk assessment deal with this aspect in detail. In this paper we use the fish embryo toxicity test (FET) as an example to demonstrate how to design a toxicity experiment attaining the required precision of results. We also include considerations on how to quantify toxicity using the FET results. The procedure proposed involves a four-parameter logistic dose-response model, which allows incorporating spontaneous effects as well as non-effects due to an insusceptible subpopulation. For the numerical operations of planning and analysis, we provide the R package *toxtestD*, whose reference manual can be downloaded from the CRAN homepage [R Core Team, 2014; Keddig and Wosniok, 2014].

In recent years, FET has predominantly replaced the fish acute toxicity test [Lammer

et al., 2009; DIN EN ISO, 2009]. Research projects like *DanTox* favour the embryos of the model organism *Danio rerio* to identify toxicity processes [Keiter et al., 2010]. The classical version of the FET is established in research laboratories as well as in service laboratories [Research Centre for Toxic Compounds in the Environment, 2014; Fraunhofer-Gesellschaft, 2014; Microtest Laboratories, 2014].

A core component of the FET is exposing fertilized eggs, preferentially from zebrafish (*D. rerio*), in an early stage of cell division to an aquatic compound, which is charged with harmful substances. Responses to the tested substance can be death, coagulation, lethal or sublethal malformations, or teratogenic effects. The presence of effects is examined after 48 or 96 h post fertilization (hpf) [OECD, 2013b; DIN EN ISO, 2009]. This test setup is used because early life stages are more sensitive than the adult life stage. In addition, early life stage tests operate faster than tests on full-grown parental fishes [Hutchinson, Solbe, and Kloepper-Sams, 1998]. Following the norm of the German Institute of Standardization (DIN), ten fertilized, normally developed eggs per concentration and a negative control should be tested [DIN EN ISO, 2009]. The Organisation for Economic Co-operation and Development (OECD) guideline recommends 20 eggs per test concentration and positive control, respectively, and 24 eggs per negative control [OECD, 2013b]. Both guidelines accept up to 10 % spontaneous deaths among negative controls [DIN EN ISO, 2009; OECD, 2013b].

Effect quantification

Effect quantification means expressing the toxicity of a substance by a single number. The full information about the relation between concentration and toxicity (effect) is described by the concentration-response relation (see example in Figure I.1a).

A major concept of effect quantification is the no observed effect concentration (NOEC). It is the result of comparing observed effects in treated groups to the effects observed in the control group. The other major effect quantification is the effective concentration value (EC_{xx}). It denotes the concentration which causes an effect of xx %. Depending on its application, EC_{xx} has been varyingly labelled as effective dose (ED_{xx}), lethal concentration/dose (LC_{xx} / LD_{xx}), benchmark dose (BMD), or virtual safe dose (VSD, for very small xx) [Crump, 1995, OECD 2013]. Both concepts differ clearly with regard to their properties and interpretation.

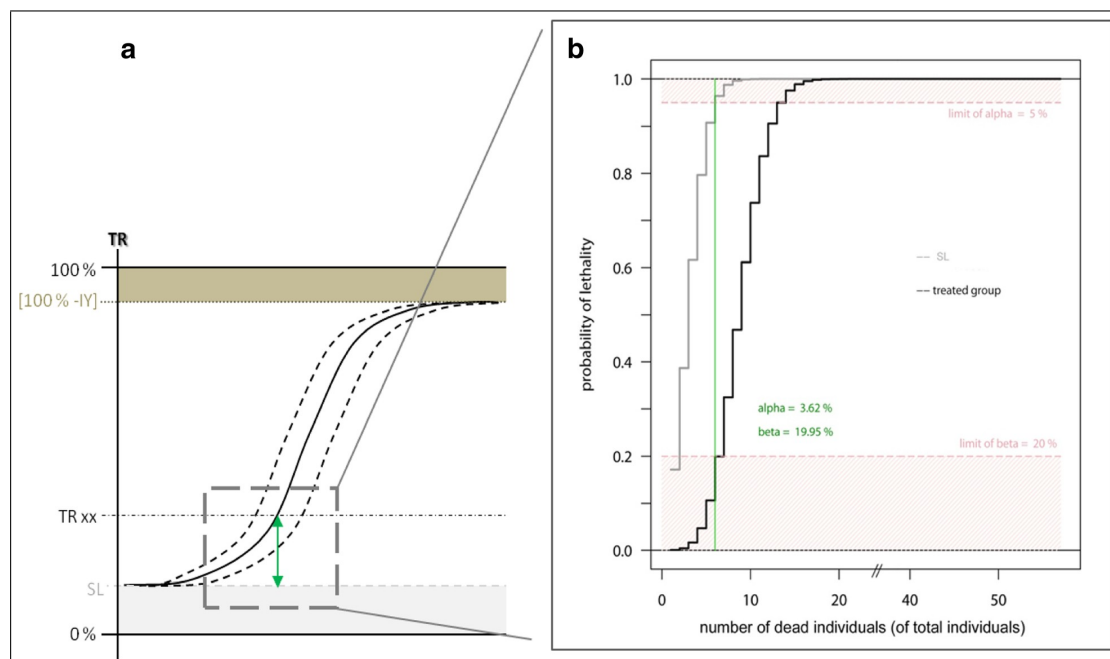


Figure I.1: Sample size calculation. Logistic distribution of a concentration-response relationship (line) with a confidence interval (dotted lines around). **a** Difference between SL and TR xx at the dose-response curve (green arrow) marks the basic distance for calculation of the sample size. The confidence interval of SL is not shown. **b** Calculation of the sample size with the two different distributions of SL (light grey line) and a treated group (dark grey line). Under the restrictions of alpha and beta (red marked areas) will the optimal number of individuals be estimated (green line). IY immunity, SL spontaneous lethality, TR total risk, xx target value

NOEC, the controversial legacy

As stated by the guidance for the implementation of REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals), the NOEC is '*the highest tested concentration for which there is no statistical significant difference of effect when compared to the control group*' [ECHA, 2008]. Similar to other statistical tests, the test sequence leading to the NOEC will detect a substance-related effect with a given safety only if it has a certain size. The detectable size and the probability of detection depend on sample size, the number of concentration points, and their allocation. Changing the sample size may shift the NOEC value over the whole range of tested concentrations, e.g. if a test sequence is repeated with the identical set of concentrations, but with a different number of replicates per concentration, the highest concentration tested (for few replicates) or the smallest concentration (for many replicates) or a concentration somewhere in between may result as NOEC [Van Der Hoeven, 1997]. The high importance of sample size becomes evident when looking at a simple example: if a concentration causes one of 20 organisms to show an effect, but only 10 organisms are tested, it is not very likely that the experimenter will see an effect at all. Generally, an existing effect may be accidentally missed due to a too small sample size, an unfortunate choice of concentrations or just by chance [Chapman, Caldwell, and Chapman, 1996].

The abbreviation NOEC contains the wording of a 'no observed effect' [Crane and

Newman, 2000], and the NOEC only indicates a concentration which could not be shown to cause a response. However, in some cases, NOEC seems to be misunderstood as indicating a concentration that produces generally 'no effect', particularly when no effect was observed in the actual experiment. But a true effect greater than zero may be undetected in an experiment simply because of a small sample size. It would be seen in an experiment with larger sample size. A power calculation would unveil this situation. In a NOEC analysis involving only few replicates, the detection of a small effect cannot be expected due to the small statistical power of a statistical test on binary effects with few replicates [Chapman, Caldwell, and Chapman, 1996]. As a NOEC is typically reported without the circumstances of its genesis, a user cannot comprehend whether a high NOEC is either due to weak toxicity or to an experiment with few replicates [Crane and Newman, 2000]. Moreover, NOECs from two experiments with different concentration patterns and varying replicate numbers can hardly be compared. Guidelines try to establish a minimum of experimental standards; nevertheless, resulting NOECs fluctuate still between concentrations generating 10 and 30 % effect [Moore and Caux, 1997]. NOECs are therefore considered as highly problematic in the scientific discussion [Crane and Newman, 2000; Warne and Dam, 2008; Landis and Chapman, 2011].

Parametric Modelling

The EC_{xx} calculation relies on a parametric assumption about the concentration-response relation (concentration response curve) underlying the data EC_{xx} links the pre-specified effect level to the effective concentration [Grasso, 2003]. Presumably, the most frequently used target effect value is the mean effective concentration (EC_{50}), which relates to a mean response of 50 % [Ewijk and Hoekstra, 1993]. Different from NOEC, a confidence interval (CI) can be calculated for both the whole curve and for every EC_{xx} value. The width of the CI for EC_{xx} is affected by the number of replications and the concentration allocation pattern. This can be exploited to set up an optimal experimental design that makes best use of the experimental effort. The EC_{xx} concept is commonly preferred over NOEC because of its fewer problematic attributes [ECHA, 2008], in particular, for the fact that the expected value of EC_{xx} does not depend on sample size and that a confidence interval can be given.

Target shift to small effect sizes for threshold calculations

The effects of much smaller sizes than 50% need to be detected to determine concentrations acceptable for health level and environmental conservation. Therefore, the target of the experiment is shifted towards smaller effects. The detection of small effects is necessary for employment and environmental protection to define concentration thresholds that should not be exceeded in order to keep the amount of adverse effects (response) due to exposure below the tolerable level [EU, 2008a; Federal Institute for Occupational Safety and Health (BAuA), 2010]. All ECs should be calculated from

a concentration-response curve fitted to observed response data. Approximate calculations of EC_{xx} for small xx lead to diverging results as a consequence if controversial safety or assessment factors become necessary to apply [Institute for Health and Consumer Protection, 2003]. Concluding the EC_{xx} from EC_{50} is an unsafe operation, as the difference between EC_{xx} and EC_{50} depends on the slope of the concentration-response curve, which is unknown and cannot be concluded from EC_{50} . The NOEC is by definition neither related to the size of an effect nor to a concentration-response curve; therefore, no EC_{50} can reasonably be concluded from the NOEC. Small responses just above zero are generally hard to detect by a statistical test and proving the probability of a detectable response at zero requires an infinitely large number of biological objects in the test. As an example, if the substance effect increases and consequently the effect rate rises by 0.000001 (= 0.0001%), the experimental group must contain at least $1/0.000001 = 1,000,000$ objects to make the expected increase in response (by only one object) visible. In reality, experiments are designed with much smaller sample sizes simply for logistic reasons. This means that only concentrations with an effect clearly above zero can be detected, whereas the exact meaning of 'clearly above zero' needs to be calculated during planning and design of the experiment. It depends on sample size as well as on concentration-response relation. The required effect size, in order to fulfill the aim of the experiment, needs to be assessed for each particular problem. It defines the tolerable level mentioned above. Typically, a substance effect in the range of 1 to 10% is set as tolerable level. This directs the focus on effect concentrations like EC_{01} , EC_{05} , and EC_{10} .

Changes in experimental design as a first step

In the discussion about how thresholds should be derived, the danger of using an insufficient data set has been identified as a basic point [Warne and Dam, 2008]. Actual norms and guidelines are optimized in regard to economic advisements [EU, 2006]. Reducing time and equipment-dependent costs (including the number of organisms) when estimating a concentration effect relationship seems to be more honoured than safely protecting the environment [Frayse, Mons, and Garric, 2006]. An example for a questionable proceeding is designing an experiment with high concentrations causing high effects and extrapolating the obtained data to the low effect situation. As fewer biological objects are needed, this approach has the advantage of being easier and more cost-efficient than an experiment with low doses, in which a higher number of objects is needed to generate effects [Piegorsch et al., 2013]. The extrapolation strategy increases the random error of the estimated EC [Warne and Dam, 2008]. An adjustment for low risk effects is not considered in the procedure of OECD guidelines, which is typically proposed only for the optimal determination of EC_{xx} .

We recommend determining EC_{xx} for small xx by organizing the FET according to the purpose of detecting small effects and then to estimate EC_{xx} from a fitted concentration-response curve. When developing an optimal design for small effect detection, it

should be recalled that the FET is used in laboratory experiments, which gives full control over the experimental conditions, i.e. the number of different concentrations, the concentrations themselves, and the number of biological objects per concentration. This freedom will be exploited when developing an optimal design. Only small modifications of the standard FET are necessary to adjust to the shifted target question. The main steps in designing a FET experiment are choosing appropriate effect quantification, followed by setting up an optimal plan for the sample size, the number of concentrations, and the concentration allocations. In this context, optimal means determining the concentration of interest with a given precision while using as few organisms as possible.

Results and discussion

Before developing an experimental design for a toxicity test, a decision must be made on how to quantify the toxic effect. Both concepts presented, the NOEC and the EC_{xx} , have their merits and disadvantages. The interpretation of a NOEC without additional information is not statistically sound. The NOECs state that when comparing the response of a control group to that of a group exposed to the NOEC concentration, no significant difference in response could be found. This may have two reasons: either there was really no difference in responses, or there was a difference in responses that could not be detected by the test due to the (too small) group size. As the number of cases per group is typically neither reported nor generally standardized, the effect size that may have been undetected is unknown and cannot be calculated. Therefore, there is a danger of underestimating the effect potential when using the NOEC compared to an effect-based analysis [Warne and Dam, 2008]. EC_{xx} has a clear interpretation as it is always an estimate of the concentration which causes a response of $xx\%$.

The major criticism regarding the EC_{xx} concept is the need of specifying a mathematical model for the concentration-response relation. Such a model is not needed for the NOEC. However, a library of standard concentration-response models exists, from which an appropriate problem-specific model can be selected. For the example of the FET, a binary four-parameter logistic model (see (Figure I.1a and Appendix) has been found suitable [DeLean, Munson, and Rodbard, 1978; Ratkowsky and Reedy, 1986; Kammann et al., 2009]. The EC_{xx} concept does not rely on using the logistic model; it can be adapted to every other strictly monotone concentration-response model. Also, non-parametric approaches can be used [Piegorsch et al., 2013].

Different from the NOEC approach, a CI can be calculated for EC_{xx} as a measure of precision. The width of the EC_{xx} confidence interval depends, among others, on the value of xx . In contrast to the NOEC, EC_{xx} itself does not depend on the design of the experiment, which makes interlaboratory comparisons of EC_{xx} more consistent than comparing NOEC values [Chapman, Caldwell, and Chapman, 1996], even if different experimental designs are involved.

When setting up a design for an experiment to determine acceptable concentrations in health prevention and environmental conservation, the main insight is that concentrations of small effects such as EC_{01} , EC_{05} , EC_{10} are relevant. Designs optimized for detecting EC_{50} are not suitable, but it is straightforward to build a design optimized for any specified effect size xx . There is no way to do so if a NOEC is used as risk quantification, because the NOEC concept means to search for an effect of zero, not for an effect of size $xx > 0$. Considering the advantages and disadvantages of both risk quantification concepts, we concluded using the EC_{xx} concept. NOECs are still used and generated by other authors [Landis and Chapman, 2011; Carlsson et al., 2013] despite their adverse properties and the debate to abandon them, which has been ongoing for more than 30 years. NOECs are not generated in this package because of the described reasons above.

The procedure for designing an experiment according to the strategy outlined in the 'Methods' section is implemented in the open-source statistic software R as the software package *toxtestD*, which is described below. Power consideration is part of the package, as requested since quite some time [Hayes, 1987].

The need of a good experimental design is a well-communicated issue, but only a few publications on the FET made reference to this [Wedekind, Siebenthal, and Gingold, 2007], as well as current publications to concentration-response relationships [Kent et al., 2014]. The chosen procedure affects the sample size and the selection of concentrations in the experiment. Sample size will be a balance between contrasting interests: a high precision of the estimated EC, which requires a high number of biological organisms in the test, and the ethical and the economic aspects, which require using few objects. Even though embryos are not considered to be living organisms and are therefore not protected by animal welfare regulations [EU, 2010a], they are animals by ethical considerations [Braunbeck et al., 2004]. Both interests ask for avoiding experiments which are uninformative because of too few test organisms. Following the suggested design, the experiments should be organized such that the effect of interest can be detected with reasonable precision without involving more biological organisms than necessary. We explicitly recommend following the suggested steps. Especially, the first step should be designed as single experiment for determining the spontaneous lethality (SL). The SL is an indication for the health of the breed and describes the response rate under control conditions. It should be determined with precision because it serves as a baseline for subsequent calculations. As the health status of breed may depend on lab conditions, the design of the experiment may be lab-specific. This gives a serious baseline for the further experiments, the detection of the group size per concentration, and the allocation of concentrations for the main experiment. The consideration of SL is precisely important in FET. With the general approach described in this paper, the user is free to choose the necessary adjustments depending on the purpose and object. The methods implemented in the package apply not only for the analysis of FET data but also to all other doseresponse analysis tasks involving a binary target quantity. In

all these cases, test designs can be developed which include a properly defined EC_{xx} by specifying the risk type and a reasonable power by regarding the error types I and II (see 'Methods' section for more explanations).

Conclusions

The quality of biological test procedures like the FET relies on using it in an appropriate experimental design. Test results are used for risk assessment and risk management. It is highly desirable that underlying tests are run transparently, with a sufficient number of objects warranting small error rates. Ethical considerations require concomitantly that samples larger than required to attain the accepted error rates should be avoided. This paper discusses standard approaches of risk quantification, concluding that the effect-oriented EC_{xx} concept for effect quantification is more favourable than the test-oriented NOEC concept. We therefore propose the effect size-oriented approach adjusted to small target effects. We suggest a way to organize an experiment according to this conclusion. Realizing such an experimental design is facilitated by the R software package *toxtestD*, which has been introduced in this paper. It organizes the design process in three steps. Being an open-source product, it is available for everybody and allows designing proper experiments also for non-statisticians. The procedure will be specific for the target quantity to be determined with the user-required precision and safety for the quantities of interest.

Methods

Basic considerations for an optimal test design

Error types

For calculation of the optimal test conditions, it is necessary to consider different error types as mathematical principals such as significance, power, and precision. In general, considering two concentrations (i.e. negative control and a test concentration >0), the associated observed effects are random values from two different distributions with concentration-specific mean values (Figure I.2).

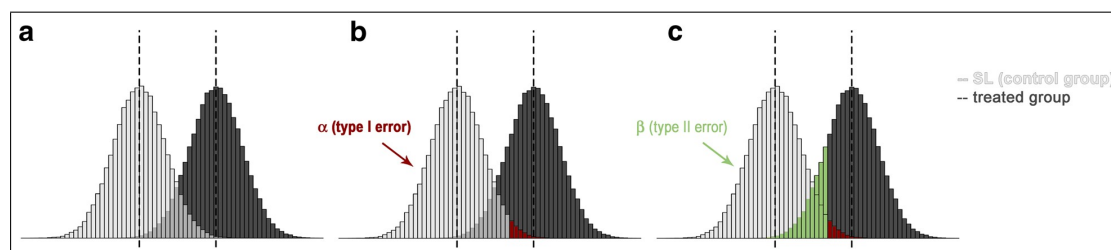


Figure I.2: Error type I and type II: Two distributions with an overlapping area a, red marked area of type I error b, green marked area of type II error c. The dotted lines mark the mean values of each distribution

If the test concentration has no effect, both distributions and their means are identical. If the test concentration has an effect, the associated mean is higher than the control group mean. The two distributions will in general show a certain overlap. If the test concentration in an experiment generates a result in the overlapping range, it cannot safely be concluded from this result that the test concentration has a systematic effect greater than the control group. The observed value has a considerable probability also under control conditions (Figure I.2a). This means that two different errors may occur when assessing response data from control and test concentrations. A type I error occurs if an effect is interpreted as a concentration effect although it is an effect of the negative control (Figure I.1b). The probability α of a type I error, also known as the level of significance (the p value), reflects the risk of the producer [Gad, 2005]. To keep the danger of a type I error small, the empirical significance level is computed in an actual statistical test procedure, and only if this probability is small (smaller than or equal to a prespecified α), it is concluded that the test concentration had a systematically higher effect than the control condition. The default value for α is 0.05 or 5%. The other error that might occur in a statistical test is that a systematically higher response from the test concentration is not recognized as such, so that an existing effect remains undetected (Figure I.2c). This is the type II error, the probability of which should also be restricted to a reasonable value. It reflects the risk of the consumer. A typical value for the accepted type II error is $\beta = 0.20$ or 20%. The complementary probability of the type II error ($= 1 - \beta$), which is the probability of detecting a systematic difference between responses, is also known as the power or quality of the test. Consideration of the type II error seems to be much less common than considering the type I error [Fairweather1991], possibly because it demands an extra effort, but it is a constitutional component of experimental design. The probabilities of both errors depend (among others) on the group sizes involved. Increasing group sizes is the only way to reduce both error probabilities, and consequently, the crucial step in sample size planning is finding the minimal group sizes for which the accepted sizes of both errors are not exceeded (Figure I.1b). The distributions shown in Figs. 2 and 1b get narrower with increasing group size, which decreases the zone in which values from both groups overlap so that observations can more safely be attributed to one of the groups. The present proposal uses $\alpha = 5\%$ and $\beta = 20\%$ as defaults for accepted errors. Both values are not fixed but are frequently used in experimental design. They may be changed, but the user should be careful when relaxing these defaults, as too liberal requirements make the test procedure ineffectual. It should be kept in mind that with a β close to 50%, the user will declare a truly existing effect with a probability of 50% as not existing. This situation is equivalent to tossing a coin to obtain the test result. Obviously, a power analysis compliant to the individual target is a fundamental component in experimental design [Warne and Dam, 2008].

Risk types

There are various ways of defining the response percentage (the xx part in EC_{xx}), differing by the way how the spontaneous and the immune level are incorporated. Three different common types of risks are considered here. They differ in the way of how the concentration-related increase in the response is expressed. They will further be named as risk types. Each risk type (Figure I.3) has its own interpretation of xx and its specific value for EC_{xx} , and it may also require its own specific experimental design. The risk types used here are extended versions of the US Environmental Protection Agency (EPA) definitions [United States Environmental Protection Agency (U.S. EPA), 1992], whereas the extension consists in the additional consideration of immunity (IY) [DeLean, Munson, and Rodbard, 1978; Ratkowsky and Reedy, 1986]. Immunity describes the phenomenon that within a population, a subpopulation shows by chance no reaction at all. The EPA definitions result by setting $IY = 0\%$.

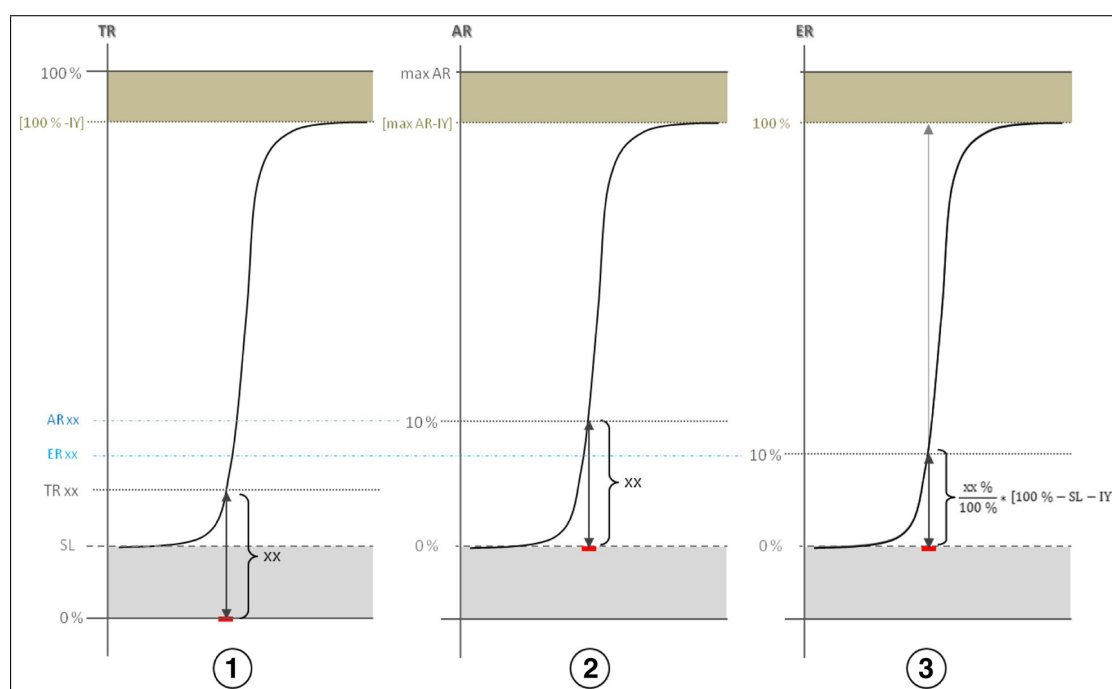


Figure I.3: Risk types and their conversions: 1 total risk (TR), 2 added risk (AR), 3 extra risk (ER). IY immunity, SL spontaneous lethality, xx target value (shown example = 10%); converted values, $\max AR = [100 - SL]$; $TR_{xx} = xx = 10\%$; $AR_{xx} = [SL + xx] = SL + 10\%$; $ER_{xx} = xx/100 \cdot [100 - SL - IY] = 0.1 \cdot [100 - SL - IY]$

1. Total risk (TR): The total risk is the total response expressed as percentage of affected biological units among all treated units. Spontaneous lethality and immunity are ignored. Example: A desired $xx = 10\%$ will force estimation of the concentration that generates an effect of 10% (Figure I.3 (1)).
2. Added risk (AR): The reference frame is restricted below and above by spontaneous lethality and immunity. Only the response above the SL counts as an effect.

Using AR, the total response associated with a target effect of size xx and a spontaneous lethality SL is $xx + SL$. Example: A desired AR of $xx = 10\%$ and $SL = 3.5\%$, $IY = 7\%$, prompts estimating the concentration producing an effect of 10% above the SL , equivalent to a total response of 13.5% . The immunity parameter does generally not affect the EC_{xx} value but restricts its possible maximum to $[100\% - SL - IY]$ (Figure I.3 (2)).

3. Extra risk (ER): The reference frame is the interval from SL to $[100\% - IY]$. Example: A desired ER with $xx = 10\%$ and $SL = 3.5\%$, $IY = 7\%$, will force estimating the concentration which generates a total response of $[SL + 0.01 \times xx \times (100\% - SL - IY)] = 3.5\% + 0.01 \times 10 \times (100\% - 3.5\% - 7\%) = 12.45\%$ (Figure I.3(3)).

The total response associated with a target effect of xx and using ER as risk type is smaller than or equal to the total response associated with the same xx , but with risk type AR.

Proposed software solution (*toxtestD*) for an optimal test design

To consider all our proposals, we implemented a package with a set of functions in R code [R Core Team, 2014], which do experimental design as outlined above in a user-friendly way. The R software is an open-source software. The package requires only a few inputs by the user. Sophisticated statistical understanding or modelling experiences are not necessary (though useful). Even though the concept is drafted for the fish embryo toxicity assay, it is possible to transfer the procedure in principle to all other toxicological questions, in which a continuous concentration equivalent generates a yes/no (binary) response per single study object. The package *toxtestD* should already be consulted during the planning phase of a test series. It contains the functions *spoD*, *setD*, and *doseD* which cover identification of the spontaneous lethality, the estimation of the necessary number of test organisms, and a concentration design according to the user's requirements. Examples for the application of all these functions are available after installation of the package by the command `help('toxtestD')` [Keddig and Wosniok, 2014].

Determination of the spontaneous lethality by *spoD*

The first task when designing the experiment is calculating the sample size for determining the SL . The necessary sample size depends on the required precision of the estimated SL . It should be recalled that because SL will be calculated from data containing random variation, the resulting SL will also be a quantity with random error. The function *spoD* offers two services. In the planning process, the total number of individuals or eggs to test is calculated, together with a proposal for partitioning the total data set into subgroups in order to identify the amount of biological variation in the separated tests. The calculations will be done for the denoted rate and additionally for the worst case in the interval given for the predicted SL . The optimal number

of biological units is calculated by using an exact binomial test with α and β as specified. The previously mentioned random error can be quantified by a CI (see Appendix for calculation details), which contains the true value of SL (the response that would hold if no random fluctuation were present) with large probability, typically 95%. We propose that as a default requirement, the limits of the CI for the SL should differ from the estimated SL by no more than $\pm 2.5\%$ (further on denoted by max CI).

In the analysis process (initiated by setting analysis = TRUE), the spontaneous lethality together with its 95% confidence interval and the biological variation are computed from the user's data. Biological variation becomes visible when comparing spontaneous rates from several experiments under control conditions. A χ^2 test is applied to check whether these rates vary according to binomial variation under the hypothesis of the same true spontaneous rate for all experiments. A significant result signals the presence of biological variation between experiments. If present, its standard deviation is reported. It is recommended to determine the spontaneous lethality very early under separate test conditions.

Specifications by the user

Subprocess planning

n: The maximal number (integer) of test organisms, with which the laboratory is willing to cope. Limiting the number is necessary to avoid non-essential calculations and thereby save computing time. The programme will invite the user to increase the number if the number is not high enough to estimate the SL with the given precision requirement.

SL.p: (optional SLmin, SLmax): To gain an optimal number of test objects for the determination of the true spontaneous lethality, the user needs at least a rough idea about the SL prior to test planning. This estimate is inserted in SL. It is possible to specify the SL either as single number or as an interval between 0 and 100%. The maximum tolerated spontaneous lethality by OECD is 10%. Datasets with higher SL should be discarded [OECD, 2013b].

bio.sd.p (optional): The standard deviation of SL consists of normal random variation and a biological variation due to biological effects like season, daytime, or well-being. The default value of 2.008% for bio.sd.p was determined from empirical data sets collected over 10 years by the Thünen Institute, Hamburg, Germany [U. Kammann, personal communication), Kammann, Vobach, and Wosniok, 2006]. The value refers exclusively to dead eggs and lethal malformations (pursuant to the definition in DIN EN ISO 15088) after 96 hpf in water in per cent [DIN EN ISO, 2009; OECD, 2013b]. If not specified otherwise, this default will be used for determining the optimal number of partitions.

max CI (optional): It is the maximally accepted absolute difference in per cent between mean SL and its confidence limits; default, 2.5%.

print.result: If omitted, the result is written to a text file called '01_spontaneous lethality.txt' in the calling directory. If a file name is given in double quotes, the result is written to that file. Nothing is written if FALSE is chosen.

Subprocess analysis

analysis: The default value is FALSE, indicating that the function does planning. To analyse the own dataset, choose analysis = TRUE.

SLdataset: This is the R data frame containing the experimental data. It needs two columns titled 'n' and 'bearer'. In column n, the total number of observations of each single experimental run is listed. The column bearer comprises the number of organisms which are carriers (in the case of FET the counts of dead or lethal malformed eggs) within each single experimental run. Each row contains the outcome from one single experimental run.

Determination of the optimal number for each experimental run by *spoD*

The second task should be the calculation of the optimal number per experimental run. The proposed calculations in *setD* involve a robustness consideration as it is done in the third task. We propose for every concentration in the FET a sample size such that a test for a concentration effect of size xx at the sought concentration EC_{xx} would detect this effect with a high (pre-specified) probability. Requiring a specified quality of the test leads to the necessary sample size per concentration. Two distributions will be estimated, one assuming SL as true response level, the other using SL + xx defined above (Figure I.1). The estimation of these two distributions bases on binomial probability. The default of the distance is $xx\%$ in respect to a posterior target of EC_{xx} . Additionally the distance depends on the reference frame. In consequence, it is necessary to choose the convenient risk type (see section 'Risk types'). The procedure increases the number of cases per concentration until the overlapping area of the two distributions corresponds to the specified levels of error types I and II (Figure I.1b)

Specifications by the user

nmax: Number (integer) of the maximum available number of organisms that can be tested in each treatment within an experimental run. The estimation of the optimal number will only start when this number is high enough to generate the response of at least one organism ($nmax \times p > 1$). If the chosen nmax is too small, a warning message is issued

SL.p: SL is calculated in per cent from own experimental data by the process *spoD*

immunity.p: A population of biological objects might contain a subpopulation which shows no reaction at all. Consequently, a reaction of 100% will never be reached

[Dinse, 2011]. We call this effect immunity in our procedure. To account for this kind of non-response, the size of the immune subpopulation can be specified as percentage of the total population. The concentration-response curve then has $[100\% - IY]$ as maximum. Please choose `risk.type = 3` to include immunity in all calculations

`risk.type`: Please choose one of three risk types. Each type defines a specific reference frame for the concentration-response curve (for detailed information see the 'Risk types' section)

`target.EC.p`: The target response in per cent (e.g. 10%, to calculate EC_{xx}). Note that the interpretation of `target.EC` depends on the `risk.type` setting.

`plot`: There are three possibilities:

`plot = FALSE`: no plots

`plot = 'single'`: creates only one plot showing the two distributions under SL and under treated conditions with the optimal number of cases. Additionally, the real rates of error type one and two are given (see Figure I.1b). The special setting for `risk.type` is not included into this plot

`plot = 'all'`: In addition to the single plot, this option provides an estimation for all possibilities of target values. This gives an impression which possibilities of detection exist under the chosen conditions. This option may need a lot of computer capacity and time. It should not be activated in general.

`alpha.p` & `beta.p`: See explanations in 'Error types' section.

`print.result`: If omitted, the result is written to a text file called '02_sample size.txt' in the calling directory. If a file name is given in double quotes, the result is written to that file. Nothing is written if `FALSE` is chosen.

Allocating concentration points by *doseD*

The third task, defining the concentration allocation for the main experiment, is guided by an idea of robustness similar to the second task. From a formal point of view, only as many concentration points as unknown parameters in the dose-response model are needed. It would however be unwise to involve only this minimum because it would not allow model checks. Concentration allocation needs at least a vague idea about position and scale of the concentration-response curve. Concentration-finding experiments, pilot studies, literature data, and similar sources are used to obtain these planning assumptions. Given an initial assumption, we propose the following concentration allocation strategy: calculate EC_{10} , EC_{50} , EC_{90} from the planning assumptions, assuming a logistic concentration-response relation and involving SL and IY, if the selected risk type requires so. The control concentration of zero and these three concentrations plus target concentrations, given by the EC_{xx} values of the experimenter's

interest, constitute the concentration allocation pattern for the main experiment. Two-sided CIs with 95 and 99% coverage probability will be calculated for the concentration-response curve. If more than four concentration points are chosen and there is an even number of free points, these will be allocated symmetrically around the chosen EC_{xx} value. If two free points are available, these are located at the limits of the 95% CI. If an even number of 4 or more free points is available, these are allocated equidistantly in the 99% CI. For an odd number of free points, 1 point is located at EC_{xx} and the others are allocated according to the rule for an even number. Note that the 1 of EC_{10} , EC_{50} , EC_{90} can be used twice as an experimental concentration, if the user's target coincides with one of these. The strategy prefers low concentrations if several targets are specified by the user. This pattern is a robust strategy which focuses on the main interest of finding EC_{xx} but does not rely too strongly on the planning assumptions. If previous experience suggests that the planning assumptions are realistic, concentrations could be allocated more closely around the presumed EC_{xx} .

Specifications by the user

DP: The results from pre-tests must be given as a data frame with the columns 'name', 'organisms', 'death', 'concentration' and 'unit', which will be needed for the calculations of the dose scheme.

immunity.p: immunity in per cent (see also settings of *spoD*).

SL.p: SL is calculated in percent from the user's experimental data by the function *spoD*.

target.EC.p: effect in %, which is of special interest. It is possible to denote more than one target in the same calculation. For example: if EC_5 and EC_{10} are of special interest, then `target.EC = c(5,10)` may be chosen, and the dose points will be allocated around both targets.

nconc: number of different concentrations the user is willing to test in each cycle.

text: `text = TRUE` adds extended information in the plot.

risk.type: Please choose one of three possible risk types. Each type defines another reference frame for concentration-response curve and target estimation (for detailed information see the 'Risk types' section). A plot for each risk type will be created separately.

print.result: If omitted, the result is written to a text file called '03_dosestrategy.txt' in the calling directory. If `filenameisgivenindoublequotes`, the result is written to that file. Nothing is written if `FALSE` is chosen.

Abbreviations

AR: Added risk; ASTM: ASTM International, known until 2001 as the American Society for Testing and Materials; BMD: Benchmark dose; DIN: German Institute for Standardization; EC: Effective concentration; ED: Effective dose; EPA: US Environmental Protection Agency; ER: Extra risk; FET: Fish embryo toxicity test; hpf: Hours post fertilization; IY: Immunity; LC: Lethal concentration; LD: Lethal dose; NOEC: No observed effect concentration; OECD: Organisation for Economic Co-operation and Development; REACH: Registration, Evaluation, Authorisation and Restriction of Chemicals; SL: Spontaneous lethality; TR: Total risk; VSD: Virtual safe dose.

Acknowledgements

The authors thank Malte Damerau, Ulrike Kammann, Michael Haarich and Norbert Theobald for their thematic support. This study was incorporated into 'MERIT-MSFD: Methods for detection and assessment of risks for the marine ecosystem due to toxic contaminants in relation to implementation of the European Marine Strategy Framework Directive'. It was supported by a grant (grant number 10017012) from the German Federal Ministry of Transport and Digital Infrastructure (BMVI) and the German Maritime and Hydrographic Agency (BSH).

Chapter II

Microinjection into Zebrafish embryos (*Danio rerio*) - a useful tool in aquatic toxicity testing?

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Abstract

Background Microinjection was tested as a potentially powerful tool to introduce natural and anthropogenic pollutants directly into fish eggs to determine their toxicological impact on fish. With this technique, parental transfer of lipophilic contaminants may be mimicked. Here, we investigated the applicability of pollutant injection into the yolk of early Zebrafish (*Danio rerio*) eggs with special regard on survival after vehicle injection. Tested vehicles were autoclaved tap water, dimethyl sulfoxide (DMSO), methanol, and triolein.

Results Highest mortality occurred after the injection of DMSO and methanol. The lethality rates were up to 40% higher than under control conditions. Best survival rates

were obtained after triolein and water injections. However, the triolein droplet was not assimilated by the embryo within 96 h post fertilization suggesting an incomplete uptake of triolein-solubilized chemicals. Technical aspects concerning microinjection in Zebrafish eggs are discussed with special emphasis on quantitative injection.

Conclusions Microinjection into the yolk cell of Zebrafish eggs is feasible, but the application of exact volumes appears problematic. However, microinjection is a powerful tool for studies without the demand for high volume accuracy. Adopting microinjection for pollutant research requires further investigation.

Keywords

Toxicity testing; Microinjection; Zebrafish embryo; Vehicle injection; Triolein; Dimethyl sulfoxide; Methanol

Background

Early life stages often show a greater sensitivity towards contaminants than adults [Russell, Gobas, and Haffner, 1999a; Hutchinson et al., 2006]. Hence, for environmental risk assessment, it is specifically important to determine the influence of contaminants during embryonic development. Early life stages of aquatic species including fish face different pollutant exposure routes. Besides maternal transfer, in which contaminants are mobilized during gametogenesis together with parental fat reserves to build up ovaries [Russell, Gobas, and Haffner, 1999b; Serrano, Blanes, and López, 2008; Merwe et al., 2011a], they experience waterborne exposure or get in direct contact to the sediment, immediately after the embryo is released into the environment.

In wild fish, significant pollutant concentrations have been found in both, oocytes [Serrano, Blanes, and López, 2008; Westernhagen and Rosenthal, 1981] and spawned eggs [Russell, Gobas, and Haffner, 1999a; Peng et al., 2010] giving evidence that maternal pollutant transfer in fish cannot be neglected. The pollutant transfer from adults to offspring was also investigated in laboratory studies with Medaka [Merwe et al., 2011a] and Zebrafish [Nyholm et al., 2008; King Heiden et al., 2008].

In these studies, fish were exposed to pollutants and observed for several weeks. A potent way to mimic maternal pollutant transfer with the advantage to shorten the experimental duration offers the direct substance administration into the early fish egg via microinjection.

During the last decades, microinjection has been widely used in experimental biology. Microinjection allows, e.g., the production of transgenic cell lines or animals. It also offers the direct administration of supporting or harmful substances into cells to investigate their mode of action or toxic potential [Dodd et al., 2000; Spitsbergen and

Kent, 2003]. Unlike the classical Fish Embryo Toxicity Test, both polar and nonpolar substances can be administered and natural barriers, i.e., the chorion [Mizell and Romig, 1997] and embryonic envelope, can be overcome. However, microinjection as a tool for the administration of contaminants has only rarely been tested. In the early 1990s, xenobiotics [Norrgrén, Andersson, and Björk, 1993] and different organochlorines [Walker et al., 1991; Walker et al., 1992; Zabel, Cook, and Peterson, 1995] were injected into rainbow trout eggs. These studies resulted in relatively high mortalities even for the control groups ($\geq 30\%$) questioning the reliability of the method [Braunbeck et al., 2004; ASTM Standard, 2007; OECD, 2013b]. Since the year 2000, Japanese medaka became the most favorable species for toxicant application via microinjection [Edmunds, McCarthy, and Ramsdell, 1999; Villalobos et al., 2000; Villalobos et al., 2003; Colman and Ramsdell, 2003; Colman et al., 2004; Colman et al., 2005; Escoffier et al., 2007; Hano et al., 2007; Nassef et al., 2010].

Medaka is a common model organism for many laboratory purposes. It is easy to maintain, comprises a fast development, and its embryos are moderately transparent and therefore an adequate choice for developmental studies [Braunbeck et al., 2004]. The yolk of medaka embryos has additional oil droplets comprising lipid reserves necessary as nutritional resources during development [Dodd et al., 2000; Spitsbergen and Kent, 2003; Iwamatsu, 2004; Gonzalez-Doncel et al., 2005]. Prior to injection, pollutants were diluted into triolein to mimic one of the natural oil droplets. Other fish species were rarely tested in microinjection studies linked to toxicity evaluation so far. However, investigating the effects of injected toxicants to species other than medaka is important to develop more generalized toxicity levels.

Even though *D. rerio* is a common vertebrate model, it was so far not used for direct pollutant administration directly into the egg yolk to mimic maternal transfer. Due to the phylogenetic divergence of medaka and Zebrafish, a comparative approach seems appropriate to test, whether the injection procedure itself is equally harmless to the embryos of Zebrafish as compared to medaka. Thereby, we aim for basic parameters that are essential for a reliable microinjection procedure into the yolk cell of one-cell staged zebrafish embryos.

In a second step, we tested the effect of four different vehicle substances, i.e., water, dimethyl sulfoxide (DMSO), methanol, and triolein on Zebrafish embryos. Water, DMSO, and methanol are regularly used as solvents in bioassays like the Fish Embryo Toxicity (FET) Test. Salts, alcohols, and acids are easily solved in water. Methanol is often used to extract other polar substances as secondary metabolites of plants and other biomaterials [Maes et al., 2012]. DMSO is known to improve the solubility of less polar contaminants and is accepted not to be harmful to the developing individuals as long as their concentrations were kept below 2.5% v/v in the FET [Maes et al., 2012]. In contrast, triolein is presumably an excellent carrier for lipophilic substances. It was found to be the most promising vehicle administered in medaka embryos causing a very low mortality among them. Identifying a variety of vehicle substances offering

the possibility to administer a wide range of polar and nonpolar substances directly into the yolk of eggs with one-cell staged Zebrafish embryo is one major aim of our study. This will add basic knowledge to the field of environmental toxicity testing.

Methods

Maintenance and egg production of Zebrafish

Wild-type Zebrafish brood stock was held in breeding groups of about 20 females and 30 males in the facilities of the Thünen Institute of Fisheries Ecology in Hamburg, Germany. Fish were kept in three glass aquaria (160 L) at $26\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ and a light/dark period of 14 h/10 h in tap water. Water quality was maintained by external bioactive filter devices. Filter material and aquarium water were changed twice a week. Fish were fed ad libitum twice a day with dry flake food (Tetramin, Tetra Werke, Melle, Germany).

Embryos were obtained from mass spawning. Eggs were collected 30 min after the light was switched on and rinsed in aquarium water. Embryos were inspected under an inverted microscope and staged according to Kimmel et al. [1995].

Assurance of egg quality/validation criteria

For valid experiments, eggs were obtained only from spawns with a fertilization rate higher than 70% according to the OECD guideline for fish-egg assays with Zebrafish embryos [OECD, 2013b]. Spontaneous lethality (SL) of the fish breed was determined on a regular basis and used as a measure of egg quality. In sterile 24-well plates, embryos were kept in groups of five eggs per well under standard test conditions without the influence of any toxicant for 96 h. Each well contained 1 mL of autoclaved tap water. The plates were kept at $26\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ and a light/dark period of 14 h/10 h. Dissolved oxygen was not measured as no severe oxygen stress for the embryo was expected during the test procedure. Braunbeck et al. [2004] reported that Zebrafish eggs are capable to tolerate oxygen concentrations of 2 mgL^{-1} without the development of malformations. Furthermore, even $100\text{ }\mu\text{L}$ water per egg was tested indicating no appearing oxygen stress. Here, the used water volume per egg was at least $200\text{ }\mu\text{L}$.

In three independent tests, each with 120 Zebrafish eggs, mean SL was determined. Additionally, each microinjection test comprised a negative control containing 60 eggs, which were neither treated with any pollutant nor subjected to the microinjection procedure itself. In general, for valid test procedures the control group needed to have a survival rate higher than 90% [ASTM Standard, 2007; OECD, 2013b].

Chemicals and substances

Injection vehicles were purchased and prepared as follows: methanol (99,9 %) was obtained from Merck KGaA, Darmstadt, Federal State of Hesse, Germany. Triolein

($\geq 99\%$) and DMSO (CHROMASOLV, 99.7%) were purchased from Sigma-Aldrich, Seelze, Federal State of Lower Saxony, Germany. All substances were used undiluted. High-quality tap water ($\leq 8^\circ\text{dH}$) was autoclaved prior to experiments. Aliquots of vehicles were kept in the fridge at 4°C prior to experiments.

Microinjection

Preparation

Injection needles (needle types: O.D. = $20\ \mu\text{m}$; BM100T-10, ends fire polished, beveled, Biomedical Instruments, Germany or Femtotip II, Eppendorf, Germany) were backfilled with $10\ \mu\text{L}$ substance by a Microloader (Eppendorf, Germany). The needle was placed in the microinjection manipulator (Narishige MN-151, Narishige International Limited, London, United Kingdom) connected to a pneumatic microinjection pump (FemtoJet from Eppendorf, Germany). Injections were made with $20\times$ magnification under an inverted microscope (Nikon MSZ800, Nikon GmbH, Düsseldorf, Federal State of North Rhine-Westphalia, Germany). Injection volume was determined according to Sive et al. [2010] by an object micrometer (Bresser 1/10 mm, Bresser GmbH, Rhede, Federal State of North Rhine-Westphalia, Germany). Briefly, for the three vehicles, DMSO, methanol, and water, a mineral droplet was mounted on the scale of the object micrometer. For triolein, a drop of water was used as matrix for measurements. The arising vehicle sphere was measured with the scale of the object micrometer. Each vehicle was administered several times into the oil droplet until the target injection volume was achieved. According to the sphere volume formula ($V = \frac{1}{6}\Pi d^3$), a sphere diameter of 1 bar corresponded to an injection volume of 0.5 nL. Two bars corresponded to 4.2 nL. Injection volume needed to be measured and adjusted for every solution, concentration as well as for every control prior and past injections due to the putative variations at the needle tip during injection procedure.

Procedure

One-cell stadium Zebrafish embryos were stringed at the edge of a microscope slide placed in a petri dish. Surplus water was removed with a paper towel such that the eggs were immobilized during the injection procedure. Per treatment between 40 and 60 eggs was consistently injected with triolein, DMSO, methanol, or autoclaved water, into the yolk. Each treatment was tested in triplicate.

To avoid needle clogging by any of the injection substances, capillaries were cleaned at frequent intervals. Post injection eggs were rinsed with autoclaved tap water ($26^\circ\text{C} \pm 2^\circ\text{C}$) into a petri dish. After 2 h, viable eggs were separated from coagulated and/or non-fertilized eggs and transferred in groups of two to five individuals into the wells of a 24-well plate. Viable eggs were kept in 1 mL autoclaved tap water at $26^\circ\text{C} \pm 2^\circ\text{C}$ and a 14 h/10 h-light/dark rhythm. Eggs were checked for coagulation and malformation every 24 h. Experimentation was terminated at latest 96 h post fertilization (hpf).

Statistics

Survival rates of 24-h-old Zebrafish embryos injected with 4.2 nL of each vehicle were normally distributed (p value = 0.69). A Fligner-Killeen test of homogeneity of variances yielded not significant ($df = 3$, p value = 0.50). As a consequence, we performed a one-way analysis of means not assuming equal variances. It ended up in a significant differences between treatment groups (p value = 0.003). To identify the location of these differences, we chose a pairwise t-test which was bonferroni corrected ($p \leq 0.008$). The entire statistic was performed with the freeware 'R' [R Core Team, 2014]. All data are represented as means \pm standard deviation (SD).

Results

The survival after vehicle injection ranged from 100% to 60% depending on the injected substance as well as the injection volume within 24 hpf. Between 24 and 96 hpf, survival of injected embryos decreased not significantly (Table II.1). Individuals injected with 4.2 nL DMSO or methanol showed significantly lower survival rates than Zebrafish eggs after injection with similar volumes of autoclaved tap water or triolein (Figure II.1 A,B, Table II.2). In general, the strength of an effect depends on injection volumes. Smaller injection volumes caused comparably less, but not significantly less mortality.

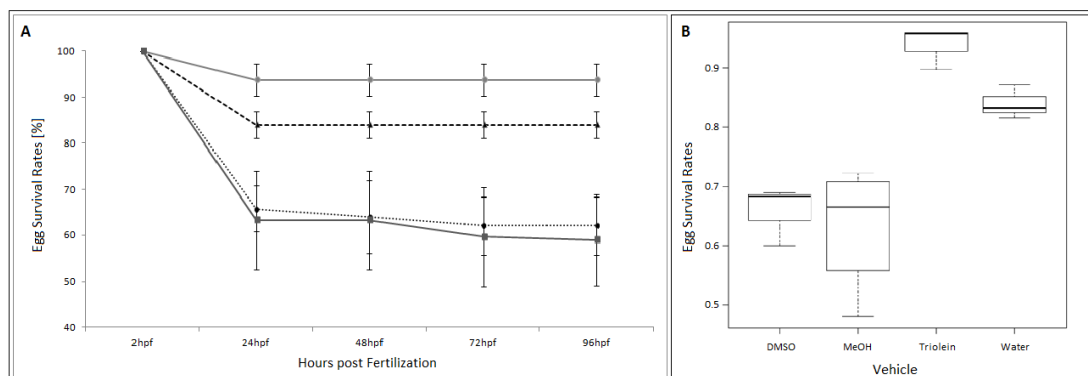


Figure II.1: Survival rates of Zebrafish eggs after vehicle injection and 24 h post injection with 4.2 nL vehicle. (A) Survival rates [%] of Zebrafish eggs after vehicle injection into the yolk sac of the one-cell stages ($V = 4.2$ nL, respectively) differed depending on substance: highest survival rates after (solid grey) triolein injection, followed by (dotted black) autoclaved tap water. Individuals injected with (dotted grey) dimethyl sulfoxide (DMSO) or (solid black) methanol showed lower survival rates. (SD = standard deviation). (B) Survival rates of Zebrafish eggs 24 h post injection with 4.2 nL vehicle, respectively, showing differences in survival. Highest survival rates were obtained after triolein injection, followed by water, DMSO, and methanol (MeOH) injection.

Table II.1: Survival rates (SR) of Zebrafish (*Danio rerio*) eggs after vehicle injection.

Substance	Eggs	Replicates	Volume [nL]	24h SR [%]	SD	96h SR [%]	SD
Untreated	718	6	-	100	0	98	3.6
Autoclaved water	121	3	0.5	100	0	95	0
	155	3	4.2	84	2.9	84	2.9
DMSO	158	3	0.5	88	5.9	88	8.7
	90	3	4.2	60	5.0	55	6.2
Methanol	125	3	0.5	97	0.9	96	1.6
	92	4	4.2	63	10.8	59	9.9
Triolein	94	3	0.5	97	2.4	96	0.4
	110	3	4.2	94	3.5	94	3.5

Untreated (pooled data) and after the pure injection of autoclaved tap water, DMSO, methanol, and triolein 24 and 96 h post fertilization (hpf), given with standard deviation (SD), respectively, total number of injected eggs, number of replicates, and injection volumes.

A DMSO injection of 0.5 nL resulted in 12% mortality 24 hpf. All other vehicle substances caused less mortality. However, high visible volume alterations occurred especially during DMSO and methanol injections. No secure statement of the real volume in place of the nominal volume can be made especially for methanol. Highest volume constancy was obtained after triolein injection. A distinct oil droplet appeared in the yolk sac, which was still visible 96 hpf (Figure II.2).

Table II.2: Pairwise comparison of survival rates of Zebrafish eggs 24 h after vehicle injection.

	DMSO	MeOH	Triolein
MeOH	0.65795	-	-
Triolein	0.00084*	0.00029*	-
Water	0.01097**	0.00379*	0.12088

One-cell staged eggs were injected with 4.2 nL of either dimethyl sulfoxide (DMSO), methanol (MeOH), autoclaved tap water, or triolein. Differences between survival rates were *significant after Bonferroni correction ($p \leq 0.008$) and ** significant without Bonferroni correction ($p \leq 0.05$).

Discussion

The experiments show that the microinjection of substances into the yolk of early egg stages of the Zebrafish embryo is a feasible tool even though survival rates differ depending on the injected vehicle. Methanol and DMSO caused higher mortalities after

injection into egg yolk than triolein or water. From this result, DMSO and methanol cannot be recommended as putative vehicles for microinjection into the yolk of one- to two-cell staged Zebrafish eggs. The high mortality rate after DMSO injection matches previous results for rainbow trout (*Oncorhynchus mykiss*) eggs [Walker et al., 1991, 1992]. For *O. mykiss* mortality rates after the injection of DMSO, acetone, and dioxane were found to be higher than 60%. Even though dose-dependent survival rates could be shown, the high mortalities in the control groups challenged the reliability of the method [Braunbeck et al., 2004; ASTM Standard, 2007; OECD, 2013b].

Triolein was chosen for the application of lipophilic substances to mimic a maternal pollutant transfer. It has already been successfully used for microinjection into early egg stages of medaka (*Oryzias latipes*) carrying ciguatoxin [Edmunds, McCarthy, and Ramsdell, 1999; Colman et al., 2004], type B brevetoxin [Colman and Ramsdell, 2003], and azaspiracid-1 as well as anthropogenic substances as tributyltin, a DDT metabolite [Villalobos et al., 2003], pharmaceuticals [Nassef et al., 2010], crude oil extracts [Escoffier et al., 2007] and polychlorinated naphthalenes [Villalobos et al., 2000]. In contrast to Zebrafish embryos, eggs of medaka naturally contain oil droplets which are involved in embryo developmental processes [Dodd et al., 2000; Spitsbergen and Kent, 2003; Iwamatsu, 2004; Gonzalez-Doncel et al., 2005]. Mortality rates in vehicle controls tend to zero after the pure injection of triolein [Edmunds, McCarthy, and Ramsdell, 1999; Villalobos et al., 2000; Villalobos et al., 2003; Colman and Ramsdell, 2003; Colman et al., 2004; Colman et al., 2005; Escoffier et al., 2007; Hano et al., 2007; Nassef et al., 2010].

Our results concerning low mortalities post oil injection into the embryos of *D. rerio* are in accordance with the results for medaka. However, triolein was not assimilated by the Zebrafish embryo until the termination of the experiment 96 hpf. We assume that a lipophilic pollutant dissolved in such a triolein droplet will not substantially affect the development of a Zebrafish embryo during this time. It may be possible that an injected triolein droplet is assimilated later than 96 hpf, as Zebrafish embryos completely consume their yolk sac during 165 ± 12 hpf [Jardine and Litvak, 2003]. To clarify whether the injected oil droplet is generally assimilated at a later point in development of Zebrafish embryos, a prolongation of the experiment may be considered. However, in this case, the whole experiment needs permission by the Animal Welfare Act which would be contradictory to the general idea of the microinjection to be an alternative to experiments with adult animals concerning maternal transfer.

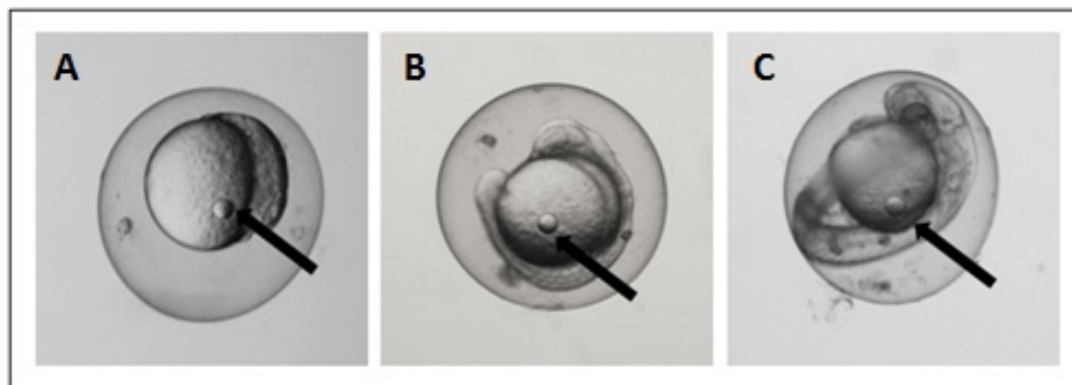


Figure II.2: Developmental stages of a Zebrafish embryo after injection of 4.2 nL triolein into the one-cell stage. Injected triolein droplet remains visible inside the yolk sac at (A) 2 hpf, (B) 24 hpf, and (C) 48 hpf.

Water could be shown to be an adequate vehicle causing mortalities below 20% in Zebrafish embryos when low volumes were injected (≤ 4.2 nL). The injection volume seems to be an important factor for egg survival post injection. High injection volumes (≥ 4.2 nL) cause yolk sac swelling. Consequently, yolk sac content leaks through the injection piercing if it is not sealed. Survival rates can be enhanced by 14% by sealing immediately after vehicle injection as has been shown for salmonid eggs [Walker et al., 1992]. However, sealing of any injection hole is a time consuming procedure. Instead of sealing, we decided to use small injection volumes. The chosen volume of 4.2 nL does not exceed 10% of Zebrafish egg volume, which was recommended to avoid egg trauma by Walker et al. [1992]. In our experiments, no yolk sac leaking post egg injection was observed after the injection of 4.2 nL.

Survival of triolein-injected individuals showed no remarkable difference between the tested injection volumes. For water, methanol, and DMSO, we found higher survival rates for smaller injection volumes. In general, the injection volume is directly linked to the applied concentration. An increase in volume resulted in a simultaneous increase in dose. However, this is only true for methanol and DMSO, which are known to be toxic to the fish embryo in higher concentrations [Maes et al., 2012], but cannot be assumed for water. Water is generally nontoxic to the fish embryo. Hence, smaller injection volumes seem to cause less mortality [Zabel, Cook, and Peterson, 1995]. However, a smaller injection volume needs less injection pressure and/or a shorter injection time, which appears advantageous at the first sight. But the risk of needle clogging is enhanced at the same time. Volume constancy over a series of injections may therefore not be warranted when low volumes are injected. We observed this effect especially during the injection of low methanol volumes. The injection needle was successively clogging, and an injection droplet was not always visible. Hence, the high survival rate after the injection of 0.5 nL methanol is possibly overestimated. Methanol as a putative vehicle was not tested before in any of the prior studies concerning microinjection as tool for pollutant administration. However, it is used in a few cases as substance carrier

for, e.g., perfluorooctane sulfonate (PFOS) [Sharpe et al., 2010] and was shown to be tolerated up to a concentration of 2% by early Zebrafish embryos in the Fish Embryo Toxicity Test [Maes et al., 2012].

Generally, volume fluctuations may be due to changes on needle tip diameters or depend on the viscosity of the cytoplasm of the injected cell [Minaschek, Bereiter-Hahn, and Bertholdt, 1989]. During the experiments, volume variations appeared on a regular basis. As these volume differences result in concentration shifts, the nominal and the real concentrations differ from each other by an unknown dimension. In ecotoxicology, high-quality data are essential and concentration variation as a consequence of volume oscillations affect data reliability. According to the OECD guideline for the Fish Embryo Toxicity Test, nominal and real concentrations should not differ more than $\pm 20\%$ from each other to assure data reliability [OECD, 2013b]. As volume variations occur regularly and partly unnoticed throughout a series of injections, it is not warranted that the real concentration deviates maximal $\pm 20\%$ from the nominal concentration. Hence, the use of data derived from microinjection is presumably not feasible for the risk assessment of pollutants.

Furthermore, volume determination in general is difficult as injection volumes are in pico- to nanoliter range. Here, an object micrometer was used for the volume identification. Depending on the micrometer scale, volume determination is more or less accurate. It comprises a high-error risk. Differences in droplet diameter of $10 \mu\text{m}$ lead to approximately 10% differences in calculated injection volumes. To further reduce the high-error risk, it may be reasonable to use a micrometer with an even smaller scale. Due to the rapid development of a Zebrafish egg [Kimmel and Law, 1985] and therefore for time saving as well as practical reasons, we determined the injection volume prior and past a series of 40 to 60 injections of one vehicle in a droplet of mineral oil spotted on the micrometer scale [Sive, Grainger, and Harland, 2010]. Hence, volume oscillations between single injections were not observed. Probably the best way to circumvent this effect is to measure the diameter of the injected droplet inside the egg. But this is not feasible for any vehicle. A distinct injection droplet in the shape of a sphere was only visible after triolein administration. The droplets appearing after DMSO, methanol, or water injection were rather diffuse. They had either no distinct shape or appeared as several small injection droplets within the yolk. Volume determination after each injection was not feasible in any of the described scenarios.

Diameters of injected triolein have previously been determined for medaka. Due to the volume oscillations between single injections, the authors reported a volume range instead of a distinct injection volume for dose-response relationships [Edmunds, McCarthy, and Ramsdell, 2000; Colman et al., 2004]. Nominal and real concentrations are not identical for any single injection. As mentioned above, this approach may lead to uncertain data not feasible for any effect concentration (EC) calculation or to estimate the risk potency of a pollutant.

Recommendations for the use of microinjections as tool in toxicity testing

In general, toxicity studies with fish eggs require a sufficient number of healthy and freshly fertilized eggs for each concentration and control treatment to assure the gain of reliable dose-response relationships of the tested contaminant. Therefore, the use of laboratory fish as, e.g., Zebrafish or medaka has the major advantage of constant egg supply throughout the year independent from seasons and environmental disturbances. In general, for Zebrafish two spawning routes are established within different laboratories. These are mass and group spawning [Westerfield, 2000; Braunbeck et al., 2004; OECD, 2013b]. During mass spawning, relatively high numbers of eggs are produced once a day. Due to the rapid development of the Zebrafish embryos [Kimmel, Spray, and Bennett, 1984, Westerfield, 2000] gaining a high number of eggs within a single egg stage is rather difficult and, therefore, laborious pre-selecting of egg stages may be necessary. Contrary, group spawning offers a smaller but constant egg supply throughout the day, when groups are assembled consecutively and small fish groups can spawn one after another [Braunbeck et al., 2004; OECD, 2013b]. Due to the rapid development of the Zebrafish eggs and the demand to inject in at least similar egg stages to assure similar distribution patterns of the contaminant within the embryo, we advise to favor group spawning over mass spawning for further applications.

The use of laboratory fish assures a high value of reproducibility, even if a constant egg supply may experience variations in egg quality and quantity due to variations in the breed. To identify variations in egg quality, it is recommended to determine the spontaneous lethality rate of the fish breed. This rate is a measure for all mortality events putatively occurring during embryonic development without any influence of a harmful substance or microinjection. The spontaneous lethality rate is needed to keep tests reproducible and reliable throughout parallel and repeated tests [Keddig, Schubert, and Wosniok, 2015]. It can be determined in short examinations prior to experiments. To distinguish already low contaminant effects from background mortality, we propose a correspondingly low spontaneous lethality rate ($\leq 2.5\%$), which is in line with the results from Kammann et al. [2006; 2009]. In comparison, the OECD recommends to discard datasets with a lethality equal or above 10% under control conditions [ASTM Standard, 2007; OECD, 2013b]. Ali et al. [2011] found a spontaneous lethality for Zebrafish ELS test to be 9% in control treatments. Due to the purpose of a study, a small spontaneous lethality rate may be essential especially when the authors are aiming for EC10 instead of EC50. In this case, distinguishing a substance effect from the background mortality is more crucial [Keddig, Schubert, and Wosniok, 2015].

We further recommend for *D. rerio* injecting substances during the earliest developmental stages. The most homogeneous distribution of injected substances throughout the addressed compartment was achieved by keeping track of a GFP protein distribution during the initial developmental stages, i.e., the one-cell stage (data not shown). This strategy offers another advantage. Piercing the chorion by an injection needle may

become more difficult with ongoing development of fish eggs due to the chorion hardening [Robles et al., 2007; Henn and Braunbeck, 2011]. It is recommended that injection needles should be mostly inflexible and sharp to pierce a chorion even though it is already hardening and to avoid unwanted egg or needle damage [Pase and Lieschke, 2009].

Besides, the usage of appropriate needles, a sufficient supply of healthy and fertilized eggs, as well as the determination of the spontaneous lethality rate, we recommend examining the success rate. It describes the number of times when the contaminant is successfully injected into the addressed compartment. However, we propose that the success rate should be above 90%. Accepting a rate below this recommendation leads to the detection of either false-positive or even no results.

Conclusion

Microinjection is an easy way to administer substances into newly fertilized fish eggs comprising many advantages. Once established in the lab, it can be applied for many fish species with only minor modifications. Effects on embryonic development become visible almost immediately after injection. Even small contaminant effects can be distinguished from background mortality. In contrast to the classical Fish Embryo Toxicity Test, both polar and nonpolar substances can be administered and natural barriers, i.e., the chorion [Mizell and Romig, 1997] and embryonic envelope, can be overcome. However, preventing volume oscillations across consecutive injections remain difficult. Therefore, the usage of microinjection as a tool for the calculation of dose-response relationships in terms of environmental risk assessment may be problematic.

Abbreviations

ANOVA, analysis of variances; DDT, dichlorodiphenyltrichloroethane; DMSO, dimethyl sulfoxide; EC, effect concentration; hpf, hours post fertilization; MeOH, methanol; SD, standard deviation; SL, spontaneous lethality; SR, survival rate; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin

Acknowledgements

The authors thank Manfred Trenk and Marc Willenberg for their skillful technical assistance as well as Malte Damerau, Wolfgang Gerwinski, Michael Haarich, Norbert Theobald, Jochen Trautner, and Werner Wosniok for their thematic support. This study was incorporated into 'MERIT-MSFD: Methods for detection and assessment of risks for the marine ecosystem due to toxic contaminants in relation to implementation of the European Marine Strategy Framework Directive'. It was supported by a grant (grant number 10017012) from the German Federal Ministry of Transport and Digital Infrastructure (BMVI) and the German Maritime and Hydrographic Agency (BSH).

Chapter III

Persistent organic pollutants in Baltic herring (*Clupea harengus*) - an aspect of gender

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Abstract

Persistent organic pollutants (POPs) are monitored regularly in water, sediment, and biota in the Baltic Sea. Thereby, lipophilic substances are measured in remarkable concentrations especially in the fatty parts of fish, such as herring (*Clupea harengus*). However, less lipophilic POPs, e.g. perfluorinated compounds (PFCs), can also be detected. To our knowledge, this study provides for the first time a broad range of contaminant concentrations simultaneously measured in filet, liver, and gonads of both sexes of Baltic herring. We analysed organochlorines, polybrominated diphenyl ethers (PBDEs), and PFCs in mature autumn-spawning individuals and found distinct organ

pollutant pattern for all POPs in both sexes. However, POP concentrations found in gonads of both sexes indicate that not only females but also males tend to reduce contaminants via reproduction. Thereby sex-dependent differences could be identified for hexachlorobenzene, PBDEs, and were most remarkable for PFCs. The extent and effect of such a reproductive loss in both sexes of herring may have been underestimated so far as it may directly affect the general reproduction success as well as the healthy development of the next generation. Hence, gonad burdening should be considered as one possible threat to a healthy wildlife as its achievement is stated by the Baltic Sea Action Plan. Inclusion of a periodic monitoring of POP concentrations in gonads of fish may be an important bio-effect measure to assess the environmental status of biota in the Baltic Sea.

Keywords

Baltic Sea, perfluorinated compounds, organochlorines, polybrominated diphenyl ethers, tissue distribution

Introduction

The Baltic Sea environment has long been ranked among the most polluted marine areas worldwide [Koistinen et al., 2008; HELCOM, 2010]. The concentrations of persistent organic pollutants (POPs) are annually observed in an extensive Baltic Sea monitoring program [Bignert et al., 1998; Strandberg et al., 1998a; Isosaari et al., 2006; Pikkarainen and Parmanne, 2006; Karl and Ruoff, 2007; Szlinder-Richert et al., 2008; Szlinder-Richert et al., 2009; Karl et al., 2010; Roots et al., 2010; Reindl et al., 2013; Airaksinen et al., 2014; Koponen et al., 2014]. For the environmental assessment concentrations of POPs are compared to existing threshold values in water, sediment, and biota. One of the applied threshold values, the 'Environmental Assessment Criteria' (EAC), is defined as the 'contaminant concentration in the environment below which no chronic effects are expected to occur in marine species' [OSPAR, 2009]. Among the possible consequences of an accumulation of POPs could be an impairment of reproduction leading to a reduced spawning success or a reduction of the general fitness of the following generation [Rolland, 2000; He et al., 2011; Papoulias et al., 2014]. Such a significant decline in body condition was reported for autumn-spawning Baltic herring (*Clupea harengus*) during the last decade [HELCOM, 2007b]. Even though the decline depends on several factors, such as food availability, hydrothermal conditions, and selective fishing [Casini et al., 2011], high POP concentrations may also negatively influence the survival of a population especially if the population is already under threat.

The Baltic herring has become one of the keystone species in Baltic Sea monitoring because of its broad geographical distribution, its high abundance, and its high body

fat content [Bignert et al., 1998; Kiviranta et al., 2003; Parmanne et al., 2006; Bignert et al., 2007; Szlinder-Richert et al., 2008; Koistinen et al., 2008; Szlinder-Richert et al., 2009; HELCOM, 2010; Airaksinen et al., 2014]). Generally, the body burdening of bioaccumulating POPs in herring varies between the regions of the Baltic Sea. The concentration of POPs in open sea areas can be lower compared to coastal zones [Galassi et al., 2008] because most sources for contaminants are located near-shore. Nonetheless, the degree of pollution is still remarkably in offshore regions, such as the Arkona Basin. Monitoring of this sea basin was established in the 1990ties. Since then, chlorinated POPs such as polychlorinated biphenyls (PCB) are annually recorded in the filets of herring to assess the pollution status of the Arkona Basin [HELCOM, 2010]. However, information on the concentrations of brominated and fluorinated compounds in herring from this region are lacking in the literature so far but are demanded for assessing the current status of this region [HELCOM, 2010].

In many studies, only one tissue type is investigated for its pollutant content [Strandberg et al., 1998a; Pikkarainen and Parmanne, 2006; Parmanne et al., 2006; Karl and Ruoff, 2007; Szlinder-Richert et al., 2008; Szlinder-Richert et al., 2009; Karl et al., 2010]. Herring due to the relatively high body fat content, accumulates high concentrations of lipophilic POPs in liver and filet. The filet of herring has been found most appropriate for monitoring lipophilic POPs, such as PCBs and polybrominated diphenyl ethers (PBDE), and was therefore recommended as target tissue by HELCOM [2015]. Nonetheless, POPs distribute throughout the whole body and accumulate in various tissues according to organ-specific characteristics (e.g. fat content) and to the physico-chemical characteristics of the POPs (e.g. octanol-water partition coefficient) themselves [Fent, 2013]. Knowledge on the inter-organ distribution of POPs in herring is scarce [Hansen, Westernhagen, and Rosenthal, 1985; Reindl et al., 2013] and needs further attention to investigate possible effects of high POP concentrations on reproduction for evaluating POP-mediated effects on the next generation as contaminants can be transferred from adults to the offspring [e.g. Rolland, 2000; Dabrowska et al., 2009; Peng et al., 2010; Sühling et al., 2015].

One of the ecological objectives of the Baltic Sea Action Plan (BSAP) considers the conservation of 'healthy wildlife' to 'restore the good environmental status of the Baltic marine environment by 2021' [HELCOM, 2007a]. Even though reproduction success is so far not considered as bioeffect in the BSAP, it is one important trait of healthy wildlife. POPs have the potential to impair reproduction leading to reduced numbers and/or health of the next generation [He et al., 2011; Papoulias et al., 2014]. The burdening of gonads of fish could therefore be used as putative bioeffect measure for the reproduction success of a species to assess the healthiness of marine wildlife. As, furthermore, sex-dependent accumulations were reported from Baltic cod and North Sea dab [Kammann, Landgraff, and Steinhart, 1993; Dabrowska et al., 2009], investigating gonads of both sexes may be necessary.

In the present study we provide new data for the current assessment in the Arkona

Basin by measuring a broad range of POPs in Baltic herring and compare our results to existing threshold values. We bridge a gap of knowledge by presenting measurements of PBDEs and perfluorinated chemicals (PFCs) in herring from Arkona Basin for the first time. Additionally, we elucidate organ pollutant patterns and sex-specific differences to investigate the potential impairment of the reproduction processes by POPs. We discuss our results in the light of the ecological objective 'healthy wildlife' as it is demanded in the BSAP.

Material & Methods

Sampling

Autumn spawners (gonadic maturity stage IV-V) of Baltic herring ($n = 30$) were collected from Arkona Basin ($54^{\circ}34.00'N - 55^{\circ}00.00'N$, $13^{\circ}55.00'E - 14^{\circ}20.00'E$) in August 2012. On board individuals were examined for sex and sexual maturity [Bucholtz, Tomkiewicz, and Dalskov, 2008]. In total 20 females and 10 males were measured for weight and length prior to the dissection of liver, gonads and filets for contaminant and age determination by otolith reading [Peltonen, 2002]. In the present study, filet refers to a piece of muscle tissue without skin and subcutaneous fat. Condition factor (cf; $[(100 \times \text{total weight})/(\text{total length}^3)]$), gonadosomatic index (GSI; $[(100 \times \text{gonad wet weight})/(\text{body weight})]$) and hepatosomatic index (HSI; $[(100 \times \text{liver wet weight})/(\text{body weight})]$) of each individual were calculated.

Chemical analysis

Storage and preparation of samples were performed according to procedures as described in the technical annexes of the OSPAR JAMP guideline for contaminants in biota [OSPAR, 1999; HELCOM, 2015] and in ICES publications [Webster et al., 2009; Ahrens and Ebinghaus, 2010; Webster et al., 2013]. All solvents used were pesticide grade or better. In particular, following procedures were applied: for analysis of organochlorine compounds and brominated diphenyl ethers (PBDEs): drying in a microwave, manual homogenisation, extraction with toluene by accelerated solvent extraction (DIONEX AS 200), lipid removal by gel permeation chromatography (cyclohexane/ethyl acetate 1:1; Biobeds SX3); clean-up by fractionation on silica gel with hexane and hexane/toluene. Determination of organochlorine compounds was performed by GC-ECD (HP 6890 with micro-ECD) on JW DB5 and JW DB1701 (60 m, 0.25 mm i.d., 0.25 μm film thickness); PBDEs were measured by GC-MS (JW DB-5ms [30 m, 0.25 mm i.d., 0.25 μm film thickness] on Agilent 6890N GC with 5973 MSD detector) in NCI single ion mode.

For PFCs sample preparation and lipid reduction were performed by extracting the chopped tissue with acetonitrile and separation of fat after deep freezing, as described by Theobald et al. [2007].

PFCs were separated and detected by HPLC-MS-MS tandem mass spectrometric (Dionex/Thermo Scientific Ultimate 3000 and AB Sciex 5500QTrap) with Phenomenex Synergi Polar-RP column (50 mm x 2 mm, particle size 4 μm) and Phenomenex Kinetex C18 column (100 mm x 2.1 mm, particle size 2.6 μm) with water and methanol as mobile phases.

Quality assurance

The analytical laboratories are working according to DIN/EN ISO/IEC 17025 and are participating regularly in an international laboratory performance testing scheme (QUASIMEME) for all methods applied in the present study. Appropriate certified reference materials have been investigated for organochlorines and PBDEs. Detection limit of GC-MS was between 0.0018 and 0.125 ng/g wet weight (ww), for GC-ECD ranged from 0.02 to 0.12 ng/g ww. For HPLC-MS-MS, recovery rates and internal standards were used for quality assurance. Its detection limit ranged between 0.02 and 0.09 ng/g ww.

Expression of results

All results are expressed on a wet weight basis (ww) to maintain the comparability of the broad range of POPs we measured in different tissues. Organochlorines and PBDEs can be expressed on both, ww and lipid weight basis (lw); PFCs should be expressed on ww as PFCs bind on proteins. Single substances were combined in different sums for PCBs as listed in Table III.1.

Table III.1: Congeners included in sums: polychlorinated biphenyls (e.g. \sum PCBs), dichlorodiphenyltrichloroethanes (\sum DDTs), hexachlorocyclohexanes (\sum HCHs), polybrominated diphenyl ethers (\sum PBDEs/ \sum BDEs), and perfluorinated compounds (\sum PFCs).

Sums	Congeners/substances
\sum PCBs	CB28, 31, 52, 101, 105, 118, 129, 138, 149, 153, 156, 170, 180, and 187
\sum PCB ₆	CB28, 52, 101, 138, 153, and 180
\sum PCB _{s6} + CB118	CB28, 52, 101, 118, 138, 153, and 180
\sum DDT	<i>o'</i> , <i>p'</i> -DDT, <i>p'</i> , <i>p'</i> -DDT, <i>p'</i> , <i>p'</i> -DDD, and <i>p'</i> , <i>p'</i> -DDE
\sum HCHs	α , β , γ , and δ - HCH
\sum PBDEs	BDE28, 47, 66, 99, 100, 85, 154, 153, and 183
\sum BDE _{s6}	BDE28, 47, 99, 100, 153, and 154
\sum PFCs	perfluorodecanoic acid (PFDA), perfluorododecanoic acid (PFDOA), perfluorohexane sulfonate (PFHxS), Perfluorononanoic acid (PFNA), perfluorooctanoic acid (PFOA), linear and branched perfluoro-1-decanesulfonate (PFOS), perfluorooctane sulfonamide (PFOSA), perfluorotetradecanoic acid (PFTeDA), perfluorotridecanoic acid (PFTrDA), and perfluoroundecanoic acid (PFUDA)

Pollutant transfer rates

Concentration ratios between gonads and filet (GFR) and between gonads and liver (GLR) can be used as indicator for a putative redistribution of POPs within an organism during maturation and as hint for a putative POP reduction from adults [Serrano, Blanes, and López, 2008; Peng et al., 2010; Sühring et al., 2015]. However, none of the ratios do provide an absolute evidence for the reduction rate or redistribution rate of POPs from or within an organism. The transfer rates were calculated using the following equations:

$$GFR = \frac{c_{\text{gonad}}}{c_{\text{filet}}} \quad (1)$$

$$GLR = \frac{c_{\text{gonad}}}{c_{\text{liver}}} \quad (2)$$

where c is the concentration [$\mu\text{g}/\text{kg ww}$] in gonads, liver, or filet, respectively.

Statistics

The importance of gender (male/female) or tissue (filet/liver/gonads) was considered by determining linear mixed effect models (linear mixed effect (lme)-function within the linear and nonlinear mixed effect model (nlme)-package) for each contaminant with the statistic software R [R Core Team, 2014]. The most complex model form included interactions between tissue and sex with the maximization of log-likelihood as chosen method (in R terminology):

$$\text{pollutant content} \sim \text{sex} \bullet \text{tissue} \quad \text{random} = \sim 1 | \text{individual} \quad (3)$$

All different models were compared by ANOVA (anova-function) to investigate the impact of the two parts tissue and sex.

Results

Constitutional fish parameter

Investigated fishes were in similar biological conditions considering age, length, weight, and condition factor (cf) (Table III.2). However, the gonad weight was higher in males than in females resulting in a higher gonadosomatic index (GSI) in males. Livers of male fish were smaller resulting in lower hepatosomatic index (HSI) in males compared to females (Table III.2).

Contaminants in herring tissues

In filets of both sexes the highest mean concentration was found for \sum DDTs, closely

Table III.2: Measured and calculated constitutional parameters of Baltic herring (*Clupea harengus*) from Arkona Basin. All values are given as means with standard deviation (sd): cf - condition factor, GSI - gonadosomatic index, HSI - hepatosomatic index.

parameters	all fish	Males	females
age [yr]	3.93 ± 0.98	3.89 ± 0.93	3.93 ± 1.03
length [mm]	235.86 ± 10.36	231 ± 12.87	238.42 ± 8
weight [g]	102.1 ± 12.53	97.69 ± 13.37	104.42 ± 11.77
cf [g/cm ³]	0.78 ± 0.05	0.79 ± 0.04	0.77 ± 0.05
GSI	20.45 ± 4.13	23.76 ± 1.36	18.71 ± 4.04
HSI	1.52 ± 0.81	0.68 ± 0.62	1.96 ± 0.48

followed from \sum PCBs. Three different sums of PCBs were given (Table III.3) representing the sum of all fourteen measured PCBs (\sum PCBs), the six indicator PCBs (\sum PCBs₆), and the sum of \sum PCBs₆ and CB118. \sum PCBs₆ is generally monitored in the Baltic Sea because it is reliably found in fish. CB118 is the most dominant dioxin-like PCB and therefore often combined with \sum PCBs₆.

The mean concentration of hexachlorobenzene (HCB) in herring filet was lower by one order of magnitude than \sum PCBs. Lowest mean concentrations in filets have been found for \sum PBDEs, \sum PFCs, and \sum HCHs, respectively (Table III.3).

Organ related distribution of contaminants

The concentrations of chlorinated contaminants were highest in filets, mediate in liver, and lowest in gonads (Table III.3; Figure III.1). In contrast, \sum PBDEs was highest in liver, mediate in filets, and lowest in gonads. \sum PFCs was highest in liver, mediate in gonads and lowest in filets of Baltic Herring.

Sex dependent POP distribution in Baltic herring

The mean lipid contents of both sexes were almost similar in filets of Baltic herring but differed in liver and gonads. Male liver and gonads exhibited twice as much fat as female liver and gonads (Table III.1). The distribution of \sum PCBs, \sum DDTs, and \sum HCHs in filets was similar in both sexes, but concentrations of HCB, \sum PBDE, and \sum PFC differed in liver and gonads (Figure III.1; Table III.3). Using ANOVA we found significant influences of the tissue type for all tested contaminants ($p < 0.0001$). An additional significant effect of sex was determined for HCB ($p < 0.001$) and \sum PFCs ($p < 0.001$). Furthermore, mutual interaction effects between sex and tissue were found significantly for \sum PFCs ($p < 0.001$), HCB ($p < 0.001$), and \sum PBDEs ($p < 0.001$).

Table III.3: Contaminant concentration [$\mu\text{g}/\text{kg ww}$] and lipid contents [%] of Baltic herring (*Clupea harengus*) from Arkona Basin. Contents were measured in filets, liver, and gonads of 10 females and 5 males, respectively. Mean values are given with standard deviation (sd).

Parameters	Filets			Liver			Gonads		
	total	males	females	total	males	females	total	males	females
Lipid content [%]	7.24 \pm 3.51	7.03 \pm 3.74	7.34 \pm 3.59	3.15 \pm 2.05	5 \pm 2.74	2.26 \pm 0.72	2.3 \pm 1.10	3.6 \pm 0.28	1.7 \pm 0.79
\sum PCBs ^a	21.84 \pm 8.62	25.9 \pm 10.5	19.8 \pm 7.32	12.7 \pm 3.42	11 \pm 3.04	13.5 \pm 3.50	3.2 \pm 1.11	2.9 \pm 0.61	3.3 \pm 1.31
\sum PCBs ₆ ^b	13.75 \pm 5.62	16.3 \pm 6.77	12.5 \pm 4.66	7.63 \pm 2.33	6.6 \pm 1.79	8.17 \pm 2.18	2 \pm 0.89	1.8 \pm 0.39	2.1 \pm 0.99
PCBs ₆ +CB118	15.81 \pm 6.28	18.7 \pm 7.61	14.1 \pm 4.65	8.61 \pm 2.45	7.4 \pm 2.07	9.78 \pm 2.61	2.3 \pm 0.94	2.1 \pm 0.45	2.5 \pm 1.44
\sum DDTs ^c	24.12 \pm 8.91	26.6 \pm 10	22.9 \pm 8.59	10.7 \pm 3.63	8.9 \pm 3.25	11.7 \pm 3.60	2.9 \pm 1.08	2.6 \pm 0.45	3.1 \pm 1.27
HCB	2.37 \pm 1.16	2.54 \pm 1.39	2.29 \pm 1.11	1.6 \pm 0.57	1.1 \pm 0.43	1.83 \pm 0.50	0.8 \pm 0.51	0.4 \pm 0.03	1 \pm 0.51
\sum HCHs ^d	0.79 \pm 0.37	0.85 \pm 0.45	0.76 \pm 0.34	0.44 \pm 0.16	0.3 \pm 0.11	0.5 \pm 0.16	0.1 \pm 0.07	0.1 \pm 0.01	0.1 \pm 0.08
\sum PBDEs ^e	0.86 \pm 0.32	0.95 \pm 0.31	0.82 \pm 0.34	1.58 \pm 1.26	2.8 \pm 1.22	0.95 \pm 0.70	0.2 \pm 0.03	0.2 \pm 0.03	0.2 \pm 0.04
\sum BDE ₆ ^f	0.83 \pm 0.31	0.32 \pm 0.3	0.79 \pm 0.33	1.55 \pm 1.24	2.79 \pm 1.19	0.93 \pm 0.68	0.14 \pm 0.03	0.14 \pm 0.03	0.14 \pm 0.04
\sum PFCs ^g	0.84 \pm 0.43	1.15 \pm 0.47	0.67 \pm 0.32	22 \pm 16.33	42 \pm 8.02	10.8 \pm 2.35	5 \pm 1.96	7.1 \pm 1.75	3.8 \pm 0.73

^a PCBs: CB28, 31, 52, 101, 105, 118, 129, 138, 149, 153, 156, 170, 180, 187

^b PCBs₆: CB28, 52, 101, 138, 153, 180

^c DDTs: o',p'-DDT, p',p'-DDT, p',p'-DDD, and p',p'-DDE.

^d HCHs: α , β , γ , and δ - HCH.

^e PBDEs: BDE28, 47, 66, 99, 100, 85, 154, 153, and 183.

^f BDEs₆: BDE28, 47, 99, 100, 153, and 154.

^g PFCs: PFDA, PFDOA, PFHxS, PFNA, PFOA, linear and branched PFOS, PFOSA, PFTeDA, PFTrDA, and PFUDA.

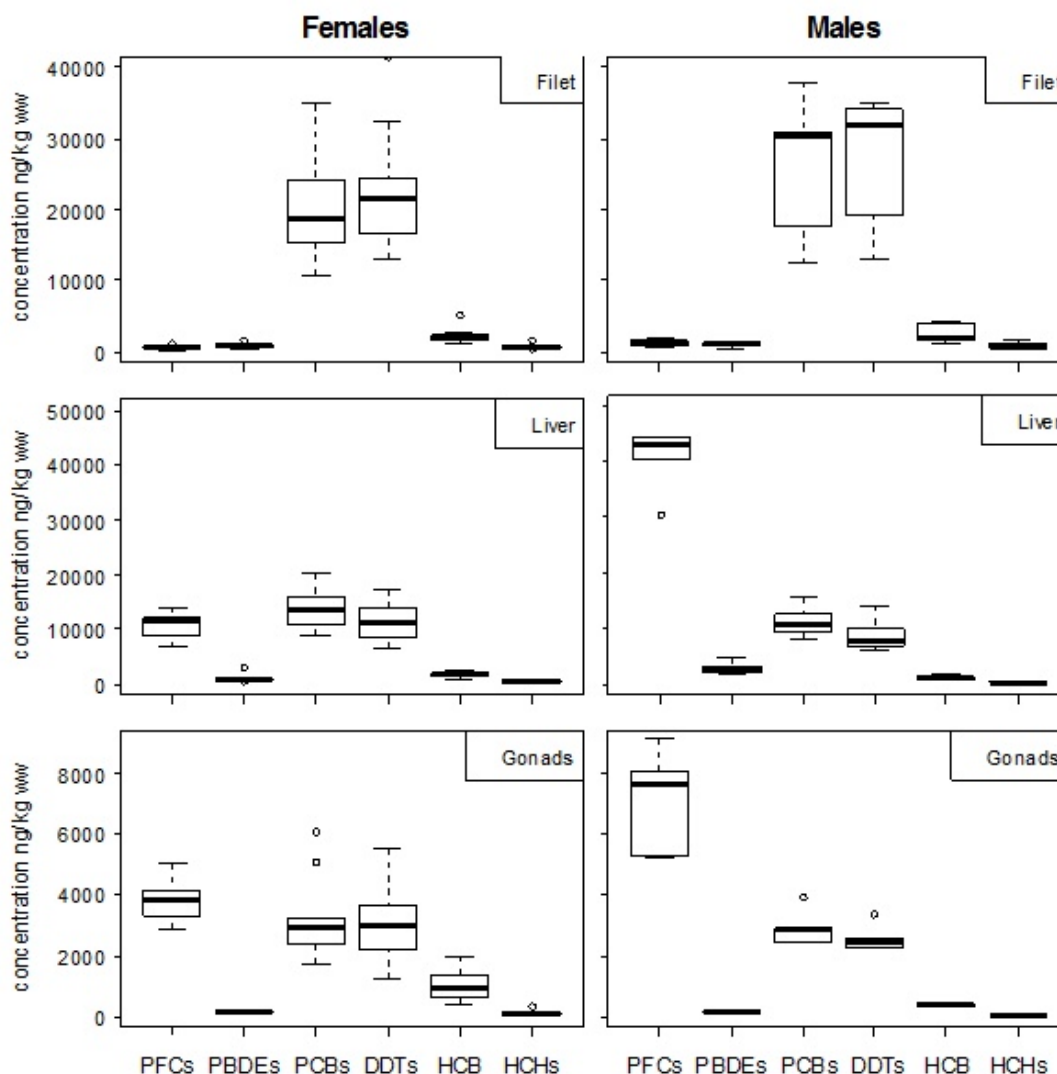


Figure III.1: Boxplots of contaminant concentration [ng/kg ww] in Baltic herring (*Clupea harengus*) from Arkona Basin. Contents of all 14 polychlorinated biphenyls (PCBs: CB28, 31, 52, 101, 105, 118, 129, 138, 149, 153, 156, 170, 180, 187), dichlorodiphenyl-trichloroethanes (DDTs: *o*′,*p*′-DDT, *p*′,*p*′-DDT, *p*′,*p*′-DDD, and *p*′,*p*′-DDE.), hexachlorocyclohexanes (HCHs), polybrominated diphenyl ethers (PBDEs: BDE28, 47, 66, 99, 100, 85, 154, 153, and 183), and perfluorinated compounds (PFCs: PFDA, PFDOA, PFHxS, PFNA, PFOA, linear and branched PFOS, PFOSA, PFTeDA, PFTrDA, and PFUDA) were measured in filet, liver, and gonads of 5 males and 10 females, respectively.

Discussion

Contaminants in herring filets of Arkona Basin

The examined filet burdening with POPs was generally in accordance with concentrations in herring from many different location in the Baltic Sea [Strandberg et al., 1998b; Kiviranta et al., 2003; Isoaari et al., 2006; Parmanne et al., 2006; Karl and Ruoff, 2007; Szlinder-Richert et al., 2008; Roots et al., 2009; Szlinder-Richert et al., 2009; Karl et al., 2010; Airaksinen et al., 2014] but was higher than concentrations measured in

filets of herring from Arkona Basin before: HELCOM reported concentrations of 1 μg CB153/kg ww and 5 μg DDE/kg ww [HELCOM, 2010]. In comparison, we found approximately four times higher median concentrations for CB153 (4.2 $\mu\text{g}/\text{kg}$ ww) and three times higher median concentration for DDE (15.2 $\mu\text{g}/\text{kg}$ ww). Fishes of the present study were larger and presumably older than fish used by HELCOM [2010]. In general, the age of a specimen is positively correlated to the content of organochlorins [Larsson, Okla, and Collvin, 1993; Kiviranta et al., 2003]. Sampling time in year was in accordance with the samples reported by HELCOM [2010].

Concentrations of PBDEs and PFCs from herring filets of Arkona Basin were lacking in literature thus far. However, content of Σ PBDEs in herring filets (Table III.3) are comparable with concentrations reported from specimens of different locations of the Baltic Sea [Isosaari et al., 2006; Roots et al., 2009; Airaksinen et al., 2014]. The median concentration of Σ PFCs reported in filets and subcutaneous fat of Baltic herring from the Finnish coast line was 1.7 $\mu\text{g}/\text{kg}$ ww [Koponen et al., 2014]. In contrast, lower median concentrations (0.72 $\mu\text{g}/\text{kg}$ ww) were recorded in filets in the present study which is presumably a consequence of the general lower concentration level of POPs in offshore regions [Galassi et al., 2008].

However, higher concentrations of PFCs were determined in livers of herring from various regions of the Baltic Sea [Berger et al., 2009; Ullah et al., 2014]. PFCs are less lipophilic than organochlorines or PBDEs and have a higher tendency to bind to blood or liver proteins rather than in fatty filets. Furthermore an enterohepatic cycle favor the presence of PFCs in liver tissue [Fent, 2013]. From various regions of the Baltic Sea liver concentrations of perfluorooctane sulfonate (PFOS) were reported to range around 10 $\mu\text{g}/\text{kg}$ ww [HELCOM, 2010]. This concentration is generally in accordance with our findings for PFOS for pooled data of both sexes (data not shown).

Comparisons with threshold values

Currently used threshold values in the Baltic Sea were either derived from existing EACs for roundfish determined by OSPAR [OSPAR, 1997; OSPAR, 2009; HELCOM, 2010] or adopted from 'Environmental Quality Standards' (EQS) for fish from the 'Water framework directive' (WFD) [EU, 2013].

EACs exist for the most relevant PCBs in filets of fish, among them are the six indicator PCBs (CB28, CB52, CB101, CB138, CB153, CB180) and three dioxin-like PCBs, i.e. CB105, CB118, and CB156. Further threshold values are available for p', p' -DDE, HCB, and γ - HCH [OSPAR, 1997; OSPAR, 2009; HELCOM, 2010]. EQS exist for PFOS and all its derivatives as well as for the sum of six PBDEs, which are BDE28, BDE47, BDE99, BDE100, BDE153, and BDE154 (Σ BDEs₆) [EU, 2013].

In the present study, concentrations of most chlorinated compounds found in herring filets varied around the thresholds and only occasionally exceeded the existing EACs (Table III.3). The situation was different for CB118 which exceeded the threshold

value of $24 \mu\text{g}/\text{kg lw}$ [OSPAR, 2009] in 12 out of 15 samples (80%) in filet. Furthermore, the concentrations of p',p'-DDE in herring filets from Arkona Basin, $16.65 \pm 5.98 \mu\text{g}/\text{kg ww}$, noticeably exceeded the threshold value for roundfish filets at $5 \mu\text{g}/\text{kg ww}$ [OSPAR, 1997]. Concentrations below threshold values were found for HCB and ΣHCHs .

EQS for PFOS and its derivatives ($9.1 \mu\text{g}/\text{kg ww}$) was exceeded in liver tissues of the Baltic herring but not in gonads or filet (Table III.3). In contrast, EQS for ΣBDE_6 ($0.0085 \mu\text{g}/\text{kg ww}$) derived by the WFD was exceeded in any of the tested tissues.

Even though a decreasing trend for most POPs was reported from the Baltic Sea during the last decades [HELCOM, 2010] we found that some organochlorines exceed the existing threshold values. Hence, the body burdening of herring is remarkable in Arkona Basin and should further be investigated and observed.

Organ related distribution of contaminants

According to their physical and chemical characteristics chlorinated and brominated POPs are generally analysed in the filet of herring. In contrast, perfluorinated compounds are measured in livers [Bignert et al., 2007; HELCOM, 2010; HELCOM, 2015]. Hence, monitoring programs are not focused on tissue-specific distributions even though POPs disperse throughout all tissues of the whole body.

Herring as rather fatty fish comprises a high lipid content in filets (Table III.3) which was therefore recommended as target tissue for the environmental monitoring of organochlorines and PBDEs in this species [HELCOM, 2015]. Our results mainly contribute to this assumption. Concentrations of organochlorines were generally higher in filets compared to liver tissues (Table III.3; Figure III.1). In contrast, higher concentrations of PBDEs were found in liver compared to filet (Table III.3) despite a lower fat content in this compartment (Table III.3). This result indicates that not only the content but rather the composition of fat influence the accumulation potential of PBDEs in herring as it is also hypothesized to occur in Baltic flounder (*Platichthys flesus*) [Waszak, Dabrowska, and Góra, 2012]. However, a higher PBDE content in liver could also be a consequence of metabolic action in the liver as it was suggested for pike [Bureau, Broman, and Örn, 2000]. This point needs further investigations to adapt the monitoring and assessment program.

Caught herring were preparing for maturity which is indicated by a high GSI (Table III.2). During vitellogenesis proteins, lipids, and vitamins are transferred via the blood stream from the liver into the developing gonads [Lubzens et al., 2010]. Pollutants that are stored in the energy reserves of adults are concomitantly transferred to the offspring and can also be measured in gonads (Table III.3) [Ungerer and Thomas, 1996]. The ratio of POPs measured in gonads and liver (GLR) can be seen as one possible measure of the pollutant transfer during vitellogenesis [Serrano, Blanes, and López, 2008]. We found GLRs ranging between 0.14 for ΣPBDEs and 0.5 for HCB (data not shown) indicating that especially HCB is partly eliminated from the herring body by

reproduction which was also reported for dab [Kammann, Landgraff, and Steinhart, 1993]. The ratio of POPs measured in gonads and filets (GFR) ranged from 0.11 for Σ DDTs and Σ HCHs to 0.42 for HCB. Only Σ PFCs exceed this value (Table III.4). Gonad to filet ratios of Σ PFCs in herring was slightly higher than values reported for the egg to filet ratio (EMR) in Chinese sturgeon (*Acipenser sinensis*) [Peng et al., 2010] which may be due to species dependent differences as a result of varying life-histories or simply a consequence as two different ratios were investigated. However, all ratios should only be considered as a hint for the putative redistribution of POPs in fish and as a measure for differences appearing among species and target substances. The varification of parental transfer processes on a molecular basis offer still scope for further investigation.

In conclusion, we found an organ-specific distribution of all measured POPs in herring of Arkona Basin. The dynamics of bioaccumulation among tissues in herring need further investigation especially with view on the fat content in combination with the organ-specific fat composition as major force for the accumulation potential of lipophilic POPs.

Sex dependent POP distribution in Baltic herring

Besides the inter-organ distributions of all POPs we found additional sex-dependent differences which were most remarkable in livers and gonads. In filets, males showed slightly higher burden than females even though their fat content was smaller in this compartment (Figure III.1; Table III.3). These findings again contribute to the assumption that not only the fat content [Dabrowska et al., 2009] but also the fat composition [Waszak, Dabrowska, and Góra, 2012; Lana et al., 2014] in Baltic herring may be one major force for the accumulation of lipophilic substances. Thereby, differences in pollutant accumulation between males and females could also be identified in liver and gonads. Here, concentrations of organochlorines were higher in liver and gonads of females compared to males. In contrast, higher concentrations in liver tissues of males were found for Σ PBDEs and Σ PFCs and also higher contents of Σ PFCs were measured from gonads of males compared to females (Table III.3). The latter sex-dependent and inter-organ distribution for Σ PFCs and Σ PDBEs were found to be significant.

Both sexes of herring invest approximately the same amount of energy reserves for maturation which can be concluded from similar GSI (Table III.2). However, physiological differences in gonadal composition do exist, a fact that is partly reflected in the different lipid composition of female and male gonads. For testis of Atlantic herring cholesterol was found as main component. In oocytes of the same species phosphatidylcholine constituted the main proportion. Furthermore gonads of females showed higher proportions of triacylglycerols than gonads of males [Henderson and Almatar, 1989]. The percentage of triacylglycerols was positively correlated with POP concentration in Atlantic bluefin tuna (*Thunnus thynnus*, L.) [Sprague et al., 2012].

Hence, the higher proportion of triacylglycerols in females gonads of herring may simultaneously be responsible for the higher accumulation of lipophilic POPs compared to male gonads as our results indicate (Figure III.1). Comparing all GFRs of tested contaminants, lipophilic substances showed higher ratios in females compared to males even though the sex dependent differences for \sum PCBs, \sum DDTs, and \sum HCHs were rather small (data not shown). Differences between sexes were found for \sum PBDEs and HCB (Table III.4) indicating that those two presumably tend to be eliminated partly in adults via maternal transfer processes as it was reported before for HCB from dab [Knickmeyer and Steinhart, 1989; Kammann, Landgraff, and Steinhart, 1993] and penta- and octa-PBDEs from european eel with higher GFRs for lower brominated congeners [Sühring et al., 2015].

Table III.4: Gonad to filet ratio (GFR) of persistent organic pollution in Baltic herring (*Clupea harengus*) from Arkona Basin. Values are means with standard deviation (sd), given for 10 females and 5 males, respectively, and pooled for both sexes.

Contaminant	all fish n = 15	Males n = 5	females n = 10
\sum PBDEs ^a	0.20 ± 0.10	0.18 ± 0.08	0.21 ± 0.11
HCB	0.42 ± 0.36	0.20 ± 0.12	0.54 ± 0.39
\sum PFCs ^b	6.30 ± 3.22	6.59 ± 1.62	6.15 ± 3.86

^a \sum PBDEs: BDE28, 47, 66, 99, 100, 85, 154, 153, and 183.

^b \sum PFCs: PFDA, PFDOA, PFHxS, PFNA, PFOA, linear and branched PFOS, PFOSA, PFTeDA, PFTrDA, and PFUDA.

The putative redistribution of POPs from liver to gonads or from filets to gonads has only been investigated as maternal pollutant transport in many fish species such as cod [Dabrowska et al., 2009], sturgeon [Peng et al., 2010], and eel [Sühring et al., 2015] so far. Our data indicate that such a redistribution of POPs exists in both sexes as remarkable contaminant concentrations were found in female and also male gonads. Considering the GFR as measure of a putative way to loose pollutants, females eliminate higher proportions of organochlorine and \sum PBDEs than males which is presumably due to the higher content of triacylglycerols in female gonads. In contrast, the elimination routes for PFCs may differ from other POPs. Perfluorinated substances are less lipophilic and proportions of \sum PFCs eliminated in males are higher than in females (Table III.4). In general, the transfer of POPs into the gonads is described as one of six possible elimination routes of contaminants from an organism [Mackay and Fraser, 2000] and such a reproductive loss is mostly investigated in females only [Dabrowska et al., 2009; Peng et al., 2010; Sühring et al., 2015], but seems to occur also in males. Its extend and effect might have been underestimated so far and should be considered especially for less lipophilic POPs.

Synopsis and consequences for marine monitoring

The results of the present study indicate that the body burden of herring from Arkona Basin is remarkably high. The exceedance of threshold values by CB118, DDE, Σ BDE6, and PFOS are reasons of concern and may impair the 'good environmental status' of the Baltic Sea as it is targeted by the European Marine Strategy Framework Directive (MSFD). Therefore monitoring of chlorinated POPs in herring should be continued and the monitoring of brominated and fluorinated compounds should be initiated in Baltic Sea offshore regions like the Arkona Basin. However, the validity of threshold values used for the assessment of the environmental status of the Baltic Sea needs to be reviewed again. Especially, the EQS which have been adopted from the WFD are questionable to apply in the marine environment as marine fish may have different sensitivities than fresh water species. Applying assessment factors just by chance can lead to pointless threshold values for the marine environment.

The recommended monitoring tissue for most POPs in herring is the filet [HELCOM, 2015]. Due to the high concentrations of PBDEs and PFCs, the liver may be an appropriate target tissue as well and could be incorporated into the current monitoring procedure. Additionally, we suggest to monitor the POP concentrations of gonads on a regular basis and use it as bioeffect measure in the assessment of healthy wildlife as it is demanded by the BSAP. This adds the possibility to assess the potential risk for the next generation and therefore the whole population to the current Baltic Sea monitoring program which is mainly focussed on the assessment of the current status as well as time trends of the environmental pollution.

Conclusion

In conclusion, the results of our study clearly show that: (1) Body burden of POPs in Baltic herring from the Arkona Basin are remarkable high and exceeds in some cases the threshold levels for unacceptable effects. (2) Accumulation of POPs in Baltic herring differed depending on the target tissue as well as (3) the sex. (4) Reproductive loss occur in both sexes whereas the extend differs depending on the sex and on the substance. Females reduce a higher content of lipophilic substances, like HCB, whereas males reduce higher concentrations of less lipophilic substances, like PFCs, by reproduction. As both processes directly affect reproduction success and therefore the healthy development of offspring, gonad burdening monitored on a regular basis in both sexes could be used as putative and additional bioeffect measure to assess the healthy wildlife as it is demanded by the BSAP.

Abbreviations

ANOVA: analysis of variances, POPs: persistent organic pollutants, PCBs: polychlorinated biphenyls, DDTs: dichlorodiphenyltrichloroethane, HCHs: hexachlorocyclohexanes, HCB: hexachlorobenzene, PBDEs: polybrominated diphenyl ethers, PFCs:

perfluorinated compounds, PFOS: perfluorooctane sulfonate, PFDA: perfluorodecanoic acid, PFDOA: perfluorododecanoic acid, PFHxS: perfluorohexane sulfonate, PFNA: Perfluorononanoic acid, PFOA: perfluorooctanoic acid, PFOSA: perfluorooctane sulphonamide, PFTeDA: perfluorotetradecanoic acid, PFTrDA: perfluorotridecanoic acid, PFUDA: perfluoroundecanoic acid, EAC: environmental assessment criteria, ww: wet weight, lw: lipid weight, lme: linear mixed effect models in R, nlme: linear and nonlinear mixed effect model in R, cf: condition factor, GSI: gonadosomatic index, HSI: hepatosomatic index, sd: standard deviation, GLR: gonad/liver ratio

Acknowledgements

The authors thank Elke Hammermeister, Dagmar Korte and Helga Wolter for their skilful technical assistance as well as Werner Wosniok for his thematic support. This study was incorporated into the research project 'MERIT-MSFD: Methods for detection and assessment of risks for the marine ecosystem due to toxic contaminants in relation to the implementation of the European Marine Strategy Framework Directive'. It was supported by a grant (grant number 10017012) from the German Federal Ministry of Transport and Digital Infrastructure and the German Maritime and Hydrographic Agency.

Chapter IV

Maternal transfer of emerging brominated and chlorinated flame retardants in European eels

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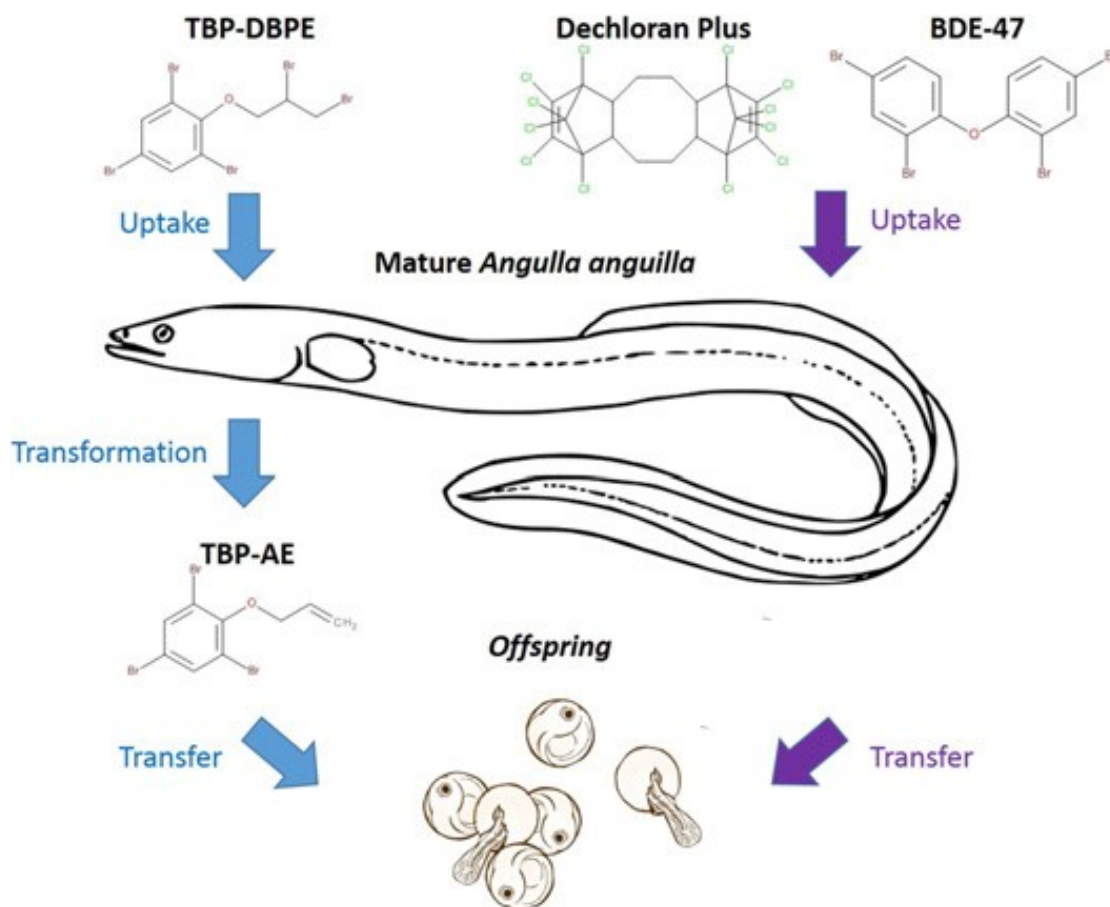
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Graphical Abstract



Abstract

The European eel (*Anguilla anguilla*) is regarded as a critically endangered species. Scientists are in agreement that the 'quality of spawners' is a vital factor for the survival of the species. This quality can be impaired by parasites, disease and pollution. Especially endocrine disrupting organic chemicals pose a potential threat to reproduction and development of offspring.

To our knowledge, the findings in this publication for the first time describe maternal transfer of contaminants in eels. We analysed the concentrations of in total 53 polybrominated diphenyl ethers (PBDEs) and their halogenated substitutes in muscle, gonads and eggs of artificially matured European eels and in muscle and gonads of untreated European eels that were used for comparison. We found evidence that persistent organic pollutants such as PBDEs, as well as their brominated and chlorinated substitutes are redistributed from muscle tissue to gonads and eggs. Concentrations ranged from 0.001 ng g⁻¹ ww for sum Dechlorane metabolites (DPMA, aCL₁₀ DP, aCL₁₁ DP) to 2.1 ng g⁻¹ ww for TBA in eggs, 0.001 ng g⁻¹ ww for Dechlorane metabolites to 9.4 ng g⁻¹ ww for TBA in gonads and 0.002 ng g⁻¹ ww for Dechlorane metabolites to

54 ng g⁻¹ ww for TBA in muscle tissue. Average egg muscle ratios (EMRs) for compounds detectable in artificially matured eels from both Schlei Fjord and Ems River ranged from 0.01 for Dechlorane 602 (DDC-DBF) to 10.4 for PBEB. Strong correlations were found between flame retardant concentrations and lipid content in the analysed tissue types, as well as transfer rates and octanol-water partitioning coefficient, indicating that these parameters were the driving factors for the observed maternal transfer. Furthermore, indications were found, that TBP-DBPE, TBP-AE, BATE and TBA have a significant uptake from the surrounding water, rather than just food and might additionally be formed by metabolism or biotransformation processes. Dechloranes seem to be of increasing relevance as contaminants in eels and are transferred to eggs. A change of the isomer pattern in comparison to the technical product of Dechlorane Plus (DP) was observed indicating a redistribution of DP from muscle tissue to gonads during silvering with a preference of the syn-isomer. The highly bioaccumulative DDC-DBF was the most abundant Dechlorane in all fish of the comparison group even though it is not produced or imported in the EU. The aldrin related 'experimental flame retardant' dibromoaldrin (DBALD) was detected for the first time in the environment in similar or higher concentrations than DP.

Highlights

- Investigation of maternal transfer of halogenated flame retardants (HFR) in eels.
- Indication for metabolism or biotransformation of HFRs during maturation of eels.
- The syn Dechloran Plus isomer is preferably transferred into gonads and eggs.
- First detection of the experimental HFR dibromoaldrin in the environment.

Introduction

The survival of any species highly depends on its ability to produce healthy, fertile offspring. A failure to do so will substantially affect the overall population or even lead to its extinction. In case of the European eel (*Anguilla anguilla*) the strong decline of glass eel recruitment during the last 30 years [Fisheries Forum, 2003; Ices, 2012] has led to the classification 'critically endangered' by the International Union for Conservation of Nature (IUCN).

As of today, the reason for this drastic decline has not been ultimately determined. A variety of factors have been postulated, including overfishing, obstruction of migration, parasitism, predation, and pollution as well as climatic changes that might affect larval transport and survival [Ices, 2006]. However, scientists are in agreement, that the 'quality of spawners' (the fitness of mature silver eels migrating back to their

spawning ground in the Sargasso Sea) is vital for the survival of the species [Ices, 2012]. This quality seems to be seriously impaired by e.g. pollution, parasites and disease [Kirk, 2003; Ginneken, 2006; Geeraerts and Belpaire, 2010]. The high body fat content of eels (up to 40% of total body weight [Svedäng and Wickström, 1997] and their longevity favour the accumulation of lipophilic contaminants [Geeraerts and Belpaire, 2010]. Since fat reserves are the primary energy source during spawning migration and gonad development [Boetius and Boetius, 1985] it must be considered that the accumulated contaminants reach their toxic potential maximum during this crucial lifehistory phase and might affect egg and embryo development. Because eels only reproduce once in their life this could be especially problematic. The accumulated contaminants of the entire lifetime could therefore potentially transferred at once to their gonads and offspring. Contaminants might, furthermore, weaken the eel during its migration back to the supposed spawning grounds in the Sargasso Sea, which means the spawner might not even be able to complete its journey.

Among these, halogenated contaminants have been postulated to be of major concern [Palstra et al., 2006]. They are suspected to affect the eel's lipid metabolism, thereby lowering its chance to migrate back to the spawning grounds in the Sargasso Sea, to decrease its ability to reproduce or affect the viability of offspring [Palstra et al., 2006]. To be able to test these hypotheses and assess the impact of halogenated contaminants on the quality of spawners it is vital to measure the transfer rates from mother into eggs, determine the decisive factors for these transfer rates and find contaminant patterns, as well as study the toxic effects on eels and their eggs. However, since the European eel spawning grounds have still not been definitively located the actual levels of contaminants in eel eggs could, so far, not be studied. The impact of halogenated contaminants could therefore only be estimated based on concentrations found in muscle or gonad tissue. With advances in the artificial reproduction of the European eel, it is now and for the first time possible to measure potentially hazardous compounds directly in eggs.

Eels are not only exposed to contaminants including legacy POPs, such as Polybrominated Diphenylethers (PBDEs), but also to substitutes for these banned compounds [Geeraerts and Belpaire, 2010]. Many of these substitutes have structures and properties similar to the replaced compounds and can therefore be expected to have similar adverse effects. In case of PBDEs, brominated (alternate BFRs) as well as chlorinated (Dechloranes) substitutes are in use. Many of them are now detected in similar or higher concentrations than PBDEs in the environment [Harju et al., 2009]. There is little information available on production, usage, persistence, or toxicity of these substitutes, yet many are suspected to at least partially fulfill the criteria for POPs or have endocrine disrupting properties [Harju et al., 2009; Sverko et al., 2011].

In this study we analysed the contamination patterns of flame retardants (FR) in muscle and gonads of artificially matured silver eels (mature eels that would naturally be on the migration back to their spawning grounds and have stopped feeding) and their stripedeggsaswellasinmuscleandgonadsofuntreatedeels that were kept for

comparison. The aim was to better understand mobilization and redistribution of contaminants during maturation and to investigate the impact of the maturation process on FR patterns (Table IV.1).

An additional important factor determining potential negative impacts of contaminants on *A. anguilla* is the degree of interaction of the compound and the eel's metabolism. It is therefore important to assess whether a compound was distributed throughout the body during uptake and 'merely' stored, or whether it was redistributed specifically during maturation and lipid metabolism. In order to address this question, we compared the contamination patterns detected in artificially matured eels with patterns found in muscle and gonads of yellow and silver eels collected in a previous study [Sühling et al., 2013]. Yellow eels are mostly sedentary in their habitats (mostly rivers), where they build up high lipid reserves in preparation of the maturation to silver eel and the migration back to the spawning grounds in the Sargasso Sea. As silver eels they stop feeding and use their stored lipid as energy reserve for the journey as well as the development of gonads and eggs. A detection of compounds in muscle as well as in gonads of yellow eels therefore indicates their distribution throughout the different tissue types during uptake rather than during maturation.

The aim of this investigation was, to determine if and to what extent PBDEs and their halogenated substitutes are transferred from parent eel through gonads to eggs, identify processes driving the transfer of these compounds and investigate their relevance. To the best of our knowledge, no data on maternal transfer of contaminants in eels and on levels of the here analysed compounds in eel eggs are available.

Materials and methods

Experimental design

Between October and November 2012, a total of 16 female European eels were caught in two German drainage systems (Ems River, Schlei Fjord) at the onset of spawning migration and held in freshwater tanks for up to seven days. 11 individuals were sacrificed and used as a comparison group to determine the contaminant load before the onset of artificial maturation. Five specimens were transferred to a 1500 L saltwater recirculation system and kept in a moderate circular current. The system was equipped with a trickle filter for mechanical filtration and denitrification and with aquarium bubblers for the supply of oxygen. To simulate seasonal variability as well as temperature changes based on e.g. change in water depth water temperature was varied between 15.4 °C and 22.0 °C during weeks 1 - 11. From week 11 onwards temperature was controlled and kept between 18.1 °C and 18.8 °C until week 17. Afterwards temperature was increased and varied between 21.4 °C and 22.2 °C for the remaining time of the experiment. Salinity was kept between 34 and 37.

After an acclimatization phase of 11 days a weekly dose of 20 mg kg⁻¹ salmon pituitary extract (SPE) (Argent Aquaculture, Redmond, USA) was injected intramuscularly

for up to 20 weeks to induce gonad maturation and ovulation. Prior to injections eels were anaesthetised with 2-Phenoxyethanol (Carl Roth, Karlsruhe, Germany). Body weight, total length (L_T) and body girth (B_G) were recorded to calculate the Body Girth Index ($BGI = B_G \cdot L_T^{-1}$) [Palstra and Thillart, 2009]. From week 16 onwards, egg samples were taken by biopsy and staged according to Palstra and van den Thilart [2009] to document oocyte maturation. 48 h after the sudden increase of body weight and BGI, final oocyte maturation and ovulation were induced by an additional SPE injection (20 mg kg^{-1}) and a subsequent intraperitoneal injection of 2 mg kg^{-1} 17, 20/3-dihydroxy-4-pregnen-3-one (DHP) (Sigma-Aldrich, St. Louis, USA) another 10 h later.

In case of two eels the additional SPE injection was waived because egg development was already advanced. One eel did not respond to the SPE treatment after 22 weeks and neither the additional SPE nor the DHP injection was applied. Eels were killed by an overdose of 2-Phenoxyethanol and the remaining gonadal tissue was removed from the peritoneal cavity. Muscle samples were taken from the epaxial muscle. All samples were stored in aluminium foil at -20°C until further analysis.

Muscle and gonad samples from 10 yellow and 10 silver eels from a sampling station near the city of Cuxhaven in the Elbe River in 2012 originated from a previous study [Sühling et al., 2013]. Since then samples were stored at -20°C in aluminium containers.

Extraction and clean-up

The frozen egg, muscle and gonad samples were homogenised with anhydrous Sodium sulphate (Na_2SO_4) (Merck) using a stainless steel/glass 1 L laboratory blender (neoLab Rotorblender). Prior to extraction all samples were spiked with mass labelled surrogate standards ^{13}C -BDE-28, ^{13}C -BDE-47, ^{13}C -BDE-99, ^{13}C -BDE-153, ^{13}C -BDE-183, ^{13}C -MeOBDE-47, ^{13}C -MeOBDE-100, ^{13}C -HBB, ^{13}C -synDP and ^{13}C -PBBz (Wellington Laboratories, Cambridge Isotopes).

Extraction and clean-up were performed in accordance with the method described in Sühling et al. [2013], using accelerated solvent extraction with subsequent gel permeation chromatography and silica gel clean-up. 500 pg (absolute) ^{13}C -PCB-141 and ^{13}C -PCB-208 was added as an injection standard to each sample. The lipid content of samples was determined gravimetrically from separate aliquots following a method described in Sühling et al. [2013].

$2 \times 200 \text{ L}$ tank water of the recirculation tank for the hormone treated eels was enriched on PAD3 sorbent filled glass cartridges at 1 mL per minute. Surrogate standards were added (see above) prior to extraction. Extraction and clean-up was performed using a method by Möller et al. [2011]. The water samples were analysed for all studied compounds.

The aqueous salmon pituitary extract (SPE) was ultrasonic extracted with 2:1 hexane: SPE (v/v) for 2×15 min and analysed.

Instrumental Analysis

In order to obtain maximum sensitivity as well as selectivity all extracts were analysed by gas chromatography/mass spectrometry (Agilent QQQ 7000) in electron capture negative ionisation mode (ECNI) with single MS (GC-MS) as well as in electron ionisation mode (EI) with tandem mass spectrometry GC-MS/MS. Results for target analytes in both methods were statistically indistinguishable (MEMO Test). Concentrations were therefore calculated as the average of the four measurements per sample (both analytical methods of each two aliquots).

For analysis in EI the instrument was fitted with a Restek 1614 column (15 m \times 0.25 mm i.d. \times 0.10 μ m film thickness, Restek) with Helium (purity 99.999%) as carrier gas and Nitrogen as collision gas. The instrument was operated in multiple reaction monitoring mode (MRM) at 70 eV. Samples were analysed for nine PBDE congeners (BDE-28, -47, -66, -85, -99, -100, -153, -154, -183), eight methoxylated PBDEs (5MeOBDE-47, 6MeOBDE-47, MeOBDE-49, -68, -99, -100, -101, -103), twenty four alternate BFRs (2,4,6-tribromophenol (2,4,6-TBP), 2,4,6-tribromophenyl allylether (TBP-AE), 2-bromoallyl 2,4,6-tribromophenyl ether (BATE), 1,2-bis(2,4,6-tri-bromophenoxy)ethane (BTBPE), Decabromodiphenylethane (DBDPE), 2,3-dibromopropyl-2,4,6-tribromophenyl ether (TBP-DBPE), 2-ethyl-1-hexyl 2,3,4,5-tetrabromobenzoate (EH-TBB), Hexabromobenzene (HBB), Hexachlorocyclopentadiene (HCCPD), Hexachlorocyclopentadienyldibromocyclooctane (DBHCTD), Pentabromobenzyl acrylate (PBBA), Pentabromobenzylbromide, 1-bromoethyl-2,3,4,5,6-pentabromobenzene (PBBB), Pentabromobenzene (PBBz), Pentabromoethylbenzene (PBEB), Pentabromotoluene (PBT), Tetrabromo-p-xylene (TBX), 2,4,6-tribromoanisole (TBA), Tris(2,3-dibromopropyl) isocyanurate (TBC), Tetrabromo-o-chlortoluene (TBCT), Tetrabromophthalic anhydride (TEBP-Anh), Bis(2-ethyl-1-hexyl)tetrabromophthalate (TBPH), α/β -tetrabromoethylcyclohexane (α/β -DBE-DBCH), α/β -1,2,5,6-tetrabromocyclooctane (α/β -TBCO)), a 12 Dechloranes (Dechlorane Plus (DP)), the one- and two-fold dechlorinated DP species (aCl11DP [-1Cl + 1H], aCl10DP [-2Cl + 2H]), 1,5-Dechlorane Plus monoadduct (DPMA), Dechlorane 601, 602 (DDC-DBF), 603 (DDC-Ant) and 604 (HCTBPH), Chlordene Plus (Cplus), Dibromochlordene (DBCD), Dibromoaldrin (DBALD), Hexachlorocyclopentadiene (HCCPD) and Hexachloro(phenyl)norbornene (HCPN).

ECNI analysis was based on a method developed by Möller et al. [2011]. The method was extended to include further analytes and a backflush system. The instrument operated in selected ion monitoring mode (SIM) with methane as reactant gas. It was fitted with a HP5MS column (30 m \times 0.25 mm i.d. \times 0.25 μ m film thickness, JW Scientific). In both EI and ECNI a restriction capillary (0.8 m \times 0.1 mm i.d., deactivated) with a backflush system was used. In ECNI eels were analysed for fourteen alternate BFRs,

eight Dechloranes and three PBDE congeners.

A detailed list of standards, MRM transitions as well as commonly used acronyms and acronyms used in this paper are presented in supplement information Tables S3 and S4.

Peak areas of the obtained chromatograms were integrated using Agilent Technologies MassHunter Workstation Software Quantitative Analysis B.06.00. Further data analysis was performed with Microsoft Office Excel 2010. Statistical analysis, including normality test, outlier test, and t-test were performed using Origin Lab 9.1 Pro software. T-test was only applied for normally distributed data.

Table IV.1: Analysed eel samples including information on treatment, life cycle phase, habitat, tissue types and sample number (n).

Name	Hormone treated [yes/no]	Life Cycle Stage	Habitat	Tissue type	Lipid [%]	Weight [g]	Stage
Yellow Elbe	No	Yellow	Elbe	Muscle	27 ± 8	412 ± 160	2 and 3
n = 10				Gonads	n.a.	n.a.	
Silver Elbe	No	Silver	Elbe	Muscle	25 ± 4	655 ± 125	5
n = 10				Gonads	n.a	84	
comp Ems	No	Silver	Ems	Muscle	26 ± 5	684 ± 129	5
n = 10				Gonads	22 ± 5	10 ± 3	
comp Schlei	No	Silver	Schlei	Muscle	22 ± 4	611 ± 121	5
n = 10				Gonads	24 ± 4	10 ± 2	
ht Ems	Yes	Silver	Ems	Muscle	28 ± 6	1014 ± 403	5
n = 10				Gonads	24 ± 13	256 ± 262	
				Eggs	18 ± 11	238 ± 81	
ht Ems	Yes	Silver	Schlei	Muscle	15-35	567-1177	5
n = 10				Gonads	33	131-161	
				Eggs	4-8	194-498	

QA/QC

Extraction and clean-up were conducted in a clean lab (class 10,000). Materials containing FRs were avoided during sample preparation and analysis.

Surrogate recoveries were determined for every eel sample. Mean recoveries were $116 \pm 27\%$ for ^{13}C -BDE-47, $90 \pm 20\%$ for ^{13}C -BDE-28, $134 \pm 25\%$ for ^{13}C -BDE-99, $136 \pm 47\%$ for ^{13}C -BDE153, $125 \pm 58\%$ for ^{13}C -BDE-183, $73 \pm 38\%$ for ^{13}C -MeOBDE-47, $139 \pm 24\%$ for ^{13}C -MeOBDE-100, $87 \pm 35\%$ for ^{13}C -HBB, $112 \pm 38\%$ for ^{13}C -synDP and $98 \pm 36\%$ for ^{13}C -PBBz. All concentrations were recovery corrected.

A blank test, using Na_2SO_4 treated similar to real samples, was conducted with every extraction batch (five samples). Concentrations of FR in blanks were between 1 pg absolute for HBB and 136 pg absolute for TBP. Average blank values were subtracted from concentration found in the samples.

The limit of detection (LOD) was calculated from a signal to noise ratio of three or by using the average blank + three times the standard deviation (if the analyte was present in the blanks). The limit of quantification (LOQ) was calculated from a signal-to-noise ratio of ten or using the average blank + ten times the standard deviation (if the analyte was present in the blanks). LODs in ECNI ranged from 0.17 pg absolute for DDC-DBF to 190 pg absolute for HCTBPH. In EI LODs ranged from 0.34 pg absolute for DPMA to 25 ng absolute for BDE-183.

Recoveries of target analytes and ^{13}C -standards were tested with and without matrix during method validation of both used analytical methods. The reproducibility was good, with an average of <10% deviation for five measurements.

The salmon pituitary extract (SPE) as well as the water of the recirculation tanks were analysed as described above. In SPE none of the target analytes could be detected. In water of the recirculation tanks TBA, TBP-DBPE as well as trace amounts (<1 pg L^{-1} after blank subtraction) of TBP-AE and BATE were detected.

A detailed list of blank values, LOD and LOQ is presented in supplement information Table S5.

Table IV.2: Overview results of all discussed samples in ng g⁻¹ wet weight as well as fsyn [g/g], lipid content [%] and number of samples (n). Data marked with * was published in Sühning et al., 2013, data marked with ** was published in Sühning et al., 2014.

Location	Water system	Sample type [n]	\sum PBDEs	\sum MeOBDEs	BDE-47	TBA	Potential BFR metabolites	TBP- DBPE
Germany	Ems	Eggs (n = 3 x 10 g)	0.16 ± 0.05	<LOD -0.02	0.13 ± 0.04	0.88 ± 0.55	0.12 ± 0.03	0.68 ± 0.11
Germany	Ems	Gonads hormone treated						
		Silver eels (n = 3)	0.64 ± 0.34	<LOD - 0.25	0.51 ± 0.26	2.7 ± 0.47	0.42 ± 0.2	2.1 ± 0.86
Germany	Schlei	Gonads hormone treated						
		Silver eels (n = 2)	1.26 ± 0.89	<LOD - 0.03	0.84 ± 0.66	9.4 ± 12.2	0.34 ± 0.16	1.7 ± 0.32
Germany	Schlei	Muscle hormone treated						
		Silver eels (n = 3)	1.56 ± 1.08	0.29 ± 0.29	1.03 ± 0.90	5.1 ± 4.7	<LOD - 1.4	5.2 ± 0.81
Germany	Schlei	Muscle hormone treated						
		Silver eels (n = 2)	1.07 - 26.4	0.6 ± 0.47	0.60 - 12.2	54 ± 55	1.9 ± 0.79	13.6 ± 10.0
Germany	Ems	Gonads hormone treated						
		Silver eels (n = 7)	1.0 ± 0.60	<LOD - 2.4	0.71 ± 0.40	0.80 ± 0.27	<LOD - 0.05	0.34 ± 0.17
Germany	Ems	Muscle hormone treated						
		Silver eels (n = 7)	1.4 ± 0.83	<LOD - 8.6	0.76 ± 0.45	1.6 ± 0.80	<LOD - 0.05	0.23 ± 0.24
Germany	Schlei	Gonads comparison group						
		Silver eels (n = 4)	0.23 ± 0.11	<LOD	0.12 ± 0.04	2.0 ± 1.6	<LOD	0.08 ± 0.08
Germany	Schlei	Muscle comparison group						
		Silver eels (n = 4)	0.83 ± 1.1	<LOD	0.06 - 1.4	4.2 ± 0.96	<LOD - 0.09	0.14 ± 0.04
France	Estuary	Glass eels** (n = 100)	1.8 ± 0.89	n.a.	<LOD	n.a.	n.a.	0.22 ± 0.08
Germany	Vida	Elvers (n = 20)	0.22 ± 0.042	n.a.	<LOD - 0.088	n.a.	n.a.	0.20 ± 0.10
Germany	Elbe	Yellow Eels* (n = 10)	8.9 ± 3.4	n.a.	6.0 ± 2.2	n.a.	n.a.	0.19 ± 0.18
Germany	Elbe	Silver Eels* (n = 10)	8.3 ± 3.7	n.a.	5.9 ± 2.9	n.a.	n.a.	2.3 ± 2.8
Germany	Elbe	Gonads Yellow els* (n = 10)	0.62 - 7.64	n.a.	0.91 ± 0.55	n.a.	n.a.	<LOD - 0.63
Germany	Elbe	Gonads Silver eels						
		(n = 10)	4.5 ± 2.8	n.a.	<LOD -4.4	n.a.	n.a.	<LOD - 0.018

EH-TBB	Further alternate BFRs	DBALD	\sum DP	\sum DDC-DBF	Dechlorane metabolites	Further Dechloranes	f (syn)	Lipid [%]
<LOD - 0.012	<LOD - 0.031	<LOD	0.009 ± 0.005	<LOD -0.003	<LOD -0.001	<LOD	0.7 ± 0.1	18
0.005 ± 0.003	<LOD - 0.10	<LOD	0.026 ± 0.025	0.0005 ± 0.00001	<LOD - 0.06	<LOD	0.6 ± 0.1	6
0.02 ± 0.001	0.054 ± 0.0045	<LOD	0.024 ± 0.016	<LOD - 0.00005	<LOD - 0.015	<LOD	0.7 ± 0.1	24
0.04 ± 0.02	<LOD - 0.003	<LOD	0.011 ± 0.0097	<LOD - 0.0002	<LOD - 0.001	0.012 ± 0.017	0.9 ± 0.02	33
<LOD - 0.004	<LOD - 0.093	<LOD - 0.47	0.007 ± 0.006	0.016 ± 0.003	<LOD - 0.002	<LOD	0.3 ± 0.02	28
<LOD - 0.09	<LOD - 0.91	0.53 ± 0.63	0.096 ± 0.1	0.028 ± 0.002	<LOD - 0.030	<LOD - 0.001	0.4 ± 0.1	25
<LOD	<LOD - 0.26	<LOD - 0.11	0.12 ± 0.06	0.055 ± 0.064	<LOD - 0.033	<LOD - 0.07	0.4 ± 0.2	22
<LOD	<LOD - 0.53	<LOD - 0.18	0.08 ± 0.06	0.093 ± 0.10	<LOD - 0.034	<LOD - 0.03	0.2 ± 0.2	26
<LOD	<LOD - 0.010	<LOD	0.015 ± 0.011	0.068 ± 0.023	0.066 ± 0.052	<LOD	n.a.	24
<LOD	<LOD - 0.24	<LOD - 0.13	0.018 ± 0.005	0.11 ± 0.039	<LOD	<LOD	0.1 ± 0.2	22
n.a.	<LOD -0.1	n.a.	<LOD - 0.46	<LOD - 0.66	<LOD	<LOD	0.9 ± 0.1	1
n.a.	<LOD	n.a.	<LOD - 0.46	<LOD - 0.66	<LOD	<LOD	0.8 ± 0.1	1.4
n.a.	<LOB - 0.042	n.a.	0.41 ± 0.027	<LOD - 0.25	<LOD	<LOD	0.97 ± 0.1	27
n.a.	0.022 ± 0.012	n.a.	0.028 ± 0.015	0.017 ± 0.009	<LOD	<LOD	0.4 ± 0.1	25
n.a.	0.17 ± 0.21	n.a.	<LOD	<LOD	<LOD	<LOD	n.a.	n.a.
n.a.	n.a.	<LOD - 0.018	0.017 ± 0.0083	0.055 ± 0.038	<LOD	<LOD.	0.6 ± 0.4	n.a.

Results and discussion

The maternal transfer of 53 FRs of the three compound groups PBDEs, alternate BFRs and Dechloranes was investigated. 32 of these compounds were detectable in muscle tissue of hormone treated silver eels. 29 compounds could additionally be detected in eggs, indicating a further maternal transfer. Within the maternally transferred contaminants three types of maternal transfer were observed.

1) DDC-DBF, PBT, PBEB, BDE-28, BDE-47, BDE-66, BDE-99, BDE-100, BDE-153 and BDE154 were detected in all tissue types of hormone treated silver eels as well as the comparison group from all sampling sites and yellow and silver eels from the Elbe. The detection in all tissue types of eels from various life stages indicated that these compounds were distributed into various tissue types during uptake and also redistributed into eggs during artificial maturation.

2) EH-TBB, HBB, synDP, antiDP, aCl₁₀DP and aCl₁₁DP were not detected in yellow eel gonads and showed increasing concentrations in gonads and eggs of hormone treated eels compared to the comparison group. This indicates that these compounds were not distributed throughout the body during uptake, but transferred or redistributed into gonads and eggs specifically during the artificial maturation process.

3) TBA and TBP-DBPE were present in various tissue types of yellow eels, untreated silver eels and hormone treated eels, but additionally displayed a high continued uptake from the water phase during the artificial maturation process. This resulted in a strong increase of these substances in hormone treated silver eels compared to the comparison group, indicating a high bioaccumulation as well as transfer rate for these compounds.

BATE, BTBPE, CPlus, DDC-Ant, HCCPD, TBP-AE, 5MeOBDE47, 6MeOBDE47, MeOBDE49 and MeOBDE68 were detected in various tissue types of hormone treated eels and the comparison group. In eels from Elbe River these compounds were not detectable. It could therefore not be determined whether these compounds were distributed throughout the body during uptake or exclusively during the maturation process. However, as will be discussed later, a change in the MeOBDE congener pattern between comparison group and hormone treated eels indicated metabolism or transformation processes for this compound group specifically during artificial maturation. For BDE-85, DBALD, DBCD, DBE-DBCH, DBHCTD, DPMA, TBP, TBX, MeOBDE99, MeOBDE100, MeOBDE101 and MeOBDE103 no maternal transfer into eggs could be observed, even though many of the compounds were detected in comparably high concentrations in muscle and gonad tissue.

Results on detected concentrations, patterns, maternal transfer and observed decisive processes are described in detail in the following sections.

PBDEs were the predominant contaminants in muscle tissue as well as gonads of all non-hormone-treated eels, regardless of developmental stage (yellow or silver) and origin with average concentrations for total PBDEs between $0.23 \pm 0.11 \text{ ng g}^{-1}$ wet

weight (ww) in gonads of silver eels from Schlei Fjord to $8.9 \pm 3.4 \text{ ng g}^{-1}$ ww in muscle tissue of yellow eels from Elbe River (Table IV.2). BDE-47 was the predominant congener in all analysed samples, with an average contribution of 64% to total PBDEs. Sum concentrations of alternate BFRs, Dechloranes and methoxylated BDEs (MeOBDEs) in these eels were below 1 ng g^{-1} ww in all analysed tissue types. In hormone treated silver eels, on the other hand, alternate BFRs were found in significantly higher concentrations than PBDEs (t-test at level 0.05). Average total alternate BFR concentrations were between $0.70 \pm 0.10 \text{ ng g}^{-1}$ ww in eel eggs from Ems River and 7.4 ng g^{-1} ww in muscle tissue from Ems River, whereas total PBDE concentrations ranged between $0.16 \pm 0.05 \text{ ng g}^{-1}$ ww and 1.07 ng g^{-1} ww in the same samples (Table II.2, Figure IV.1). This significant increase could be an indication, that alternate BFRs either have a higher uptake through skin and gills than PBDEs or are remobilised from other tissue types during artificial maturation. Another significant (t-test at level 0.05) difference between hormone treated eels and the comparison group was the increase of potential TBP-DBPE metabolites/transformation products (TBP-AE and BATE) with median contribution to sum contamination of 1% in silver eels of the comparison group from Ems River and 12% in hormone treated eels from the same habitat; correspondent to the high observed concentrations of alternate BFRs. This trend was less distinct, but still existent in eels from Schlei Fjord, with 3% in the comparison group and 7% in hormone treated eels (Table IV.2, Figure IV.2). Dechloranes had the lowest average concentrations in all analysed specimens. Interestingly, Dechlorane contribution to sum FR concentration was higher in eels of the comparison group than hormone treated eels from both Ems River and Schlei Fjord (Table IV.2). This decrease could be caused by a removal via e.g. excretion, metabolism or redistribution into other lipid rich tissues such as the liver. Overall concentrations of MeOBDEs differed largely between individual samples of hormone treated eels as well as the comparison group. However, only low brominated MeOBDEs (up to MeOBDE-68) were detected in hormone treated eels whereas low and high brominated MeOBDEs (up to MeOBDE-103) were detectable in the comparison group. All analysed contaminant groups, observed trends and patterns will be discussed individually in the next sections.

PBDEs

The detection of PBDEs in yellow as well as silver eel gonads indicated a distribution of contaminants into various tissues during uptake but could also be an indication of a redistribution of contaminants during maturation. The latter assumption was supported by the observed pattern of PBDE congeners in the analysed eels. Yellow eels were contaminated with congeners of both the technical Penta- (BDE-47 (24-38%), BDE-82, BDE-85, BDE-99, BDE-100 (50-62%), BDE-153 and BDE-154 (4-8%)) and technical Octa-BDE (BDE-153, BDE-154 (10-12%), BDE-183 (43-44%)) mixtures while all analysed silver eels showed an increase of congeners attributed to the technical PentaBDE mixture to up to 98% of the total PBDE contamination. Additionally an increase of lower

brominated BDE congeners could be observed in gonads and eggs of hormone treated eels compared to the pattern in muscle tissue. In muscle tissue of hormone treated eels BDE congener distribution was similar to profiles previously reported by Belpaire [2008] with BDE47 > BDE-100 > BDE-153 > BDE-99. In gonads and eggs however, BDE99 had a similar contribution to total PBDEs as BDE-100, followed by low brominated congeners such as BDE-28 and BDE-66. PBDEs are known to undergo enzymatic debromination [Eljarrat and Barceló, 2011], which could explain the change in the contamination pattern. However, in the comparison group, PBDE congener profile in gonads remained similar to the profiles in muscle tissue, indicating, that the changes were caused by the artificial maturation process.

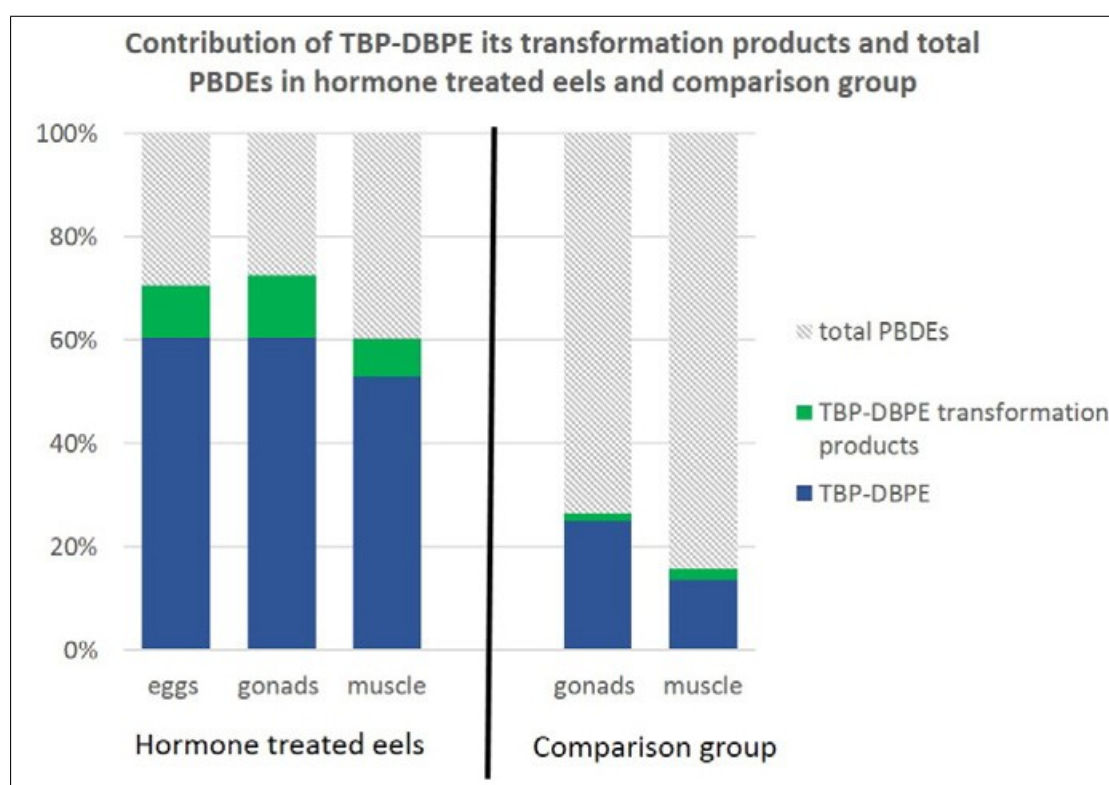


Figure IV.1: General contamination pattern. Average concentrations of total PBDEs in grey, MeOBDEs in dark green, total alternate BFRs in blue, TBP-DBPE transformation products (TBP-AE, BATE) in light green and total Dechloranes in red in ng g^{-1} wet weight in eggs, gonads and muscle tissue of hormone treated silver eels and muscle tissue and gonads of comparison group silver eels from Ems River (left) and Schlei Fjord (right).

Interestingly, MeOBDE concentrations were about tenfold higher in comparison group silver eels from Ems River than hormone treated eels from the same habitat and differed significantly (t-test at level 0.05) concerning the congener pattern. Only low brominated MeOBDE congeners up to MeOBDE-68 could be found in hormone treated eels, whereas muscle as well as gonad tissue of silver eels of the comparison group from the same habitat were contaminated with MeOBDE congeners up to MeOBDE-103 (Figure IV.1, Table IV.2).

This change in the contamination pattern could be an indication that MeOBDEs undergo similar debromination process observed for PBDEs, leading to an increase of lower brominated congeners over time. This would again imply that contaminants are not merely redistributed, but subjected to metabolism. However, metabolism studies have to be conducted to confirm this hypothesis. In eels from Schlei Fjord no MeOBDEs could be detected.

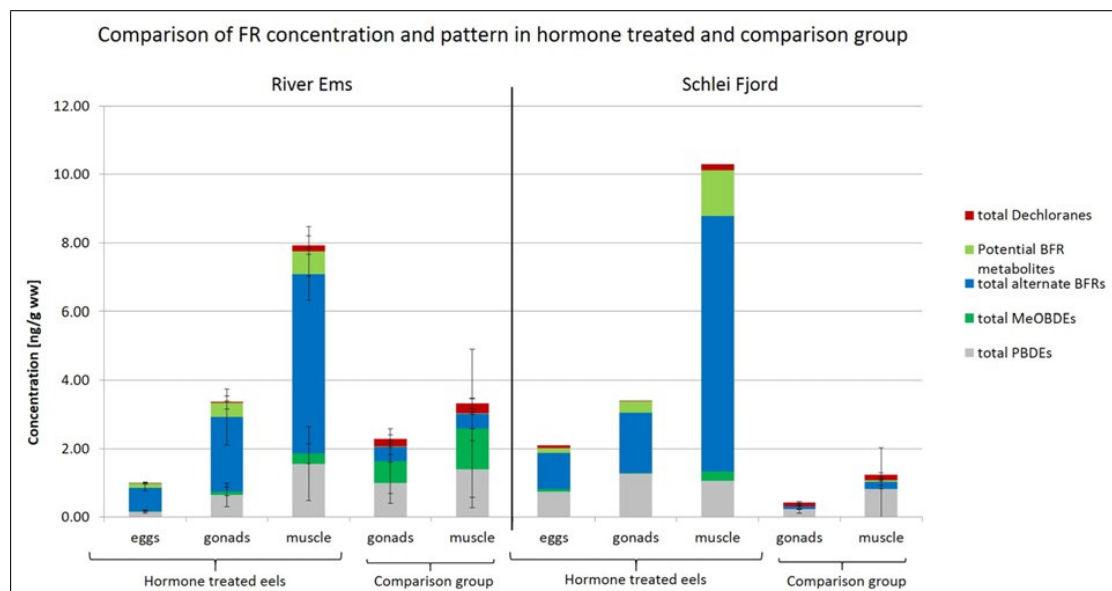


Figure IV.2: Average contribution to total PBDEs, TBP-DBPE and TBP-TBPE transformation products (TBP-AE, BATE in % in hormone treated eels (left) and comparison group (right)).

Alternate brominated flame retardants

A higher number of alternate BFRs were detected in the comparison group, compared to hormone treated eels. However, detection frequencies and overall concentrations were up to fifty times higher in all tissue types of hormone treated eels (Figure IV.1, Table IV.2).

1,3,5-tribromo-2-(2,3-dibromopropoxy)-benzene (TBP-DBPE) was the most abundant with the highest concentration of alternate BFR in all analysed eels (Figure IV.1, IV.2). It was detected in all analysed tissue types of yellow and silver eels indicating distribution of this compound into various tissues during uptake, rather than just remobilisation during artificial maturation. Interestingly, concentrations in hormone treated eels were significantly (t-test at level 0.05) higher than concentrations found in the comparison group; with $0.85 \text{ ng g}^{-1} \text{ ww}$, $2.0 \text{ ng g}^{-1} \text{ ww}$ and $8.6 \text{ ng g}^{-1} \text{ ww}$ in eggs, gonads and muscle tissue of hormone treated eels and $0.21 \text{ ng g}^{-1} \text{ ww}$ and $0.18 \text{ ng g}^{-1} \text{ ww}$ in gonads and muscle tissue of the comparison group. The increase cannot have been caused by ingestion, because both groups had stopped feeding.

To assess potential sources for this increase in TBP-DBPE concentration, the water from

the tanks of the hormone treated group was analysed to confirm if changes in contamination patterns between hormone treated eels and comparison groups were caused by uptake of contaminants through the surrounding water or the artificial maturation process. TBP-DBPE concentrations higher than 1 ng L^{-1} were detected in the tank water, suggesting that the increased concentrations in hormone treated eels might have indeed been caused by a continued uptake from TBP-DBPE leaching out of the recirculation system into the tank water during the maturation process. The repeated detection of TBP-DBPE in eels that had not been kept in tanks prior to sampling tissue remained inexplicable, because there is no report on current production or use and the only known producer, Chemische Fabrik Kalk, Germany, ceased its production in the 1980s [Recke and Vetter, 2007]. Another potential source of TBP-DBPE could be remobilisation from other tissues during the artificial maturation process. Corresponding TBP-DBPE transformation products 1,3,5-tribromo-2-(2-propen-1-yloxy)-benzene (TBP-AE) and 2-bromoallyl-2,4,6-tribromophenyl ether (BATE) [Recke and Vetter, 2007] had the second and third highest concentration and detection frequency (>90%) of alternate BFRs in all tissue types of hormone treated eels, while detection frequencies in the comparison group were below 50% and TBP-AE could not be detected in gonads (Table IV.2). The transformation products were also found in water samples, however in much lower concentrations ($<1 \text{ pg L}^{-1}$). The detection of transformation products in eggs and gonads of hormone treated eels rather than the comparison group could be an indication that TBP-DBPE might not just be redistributed into gonads and eggs. TBP-DBPE seems to be subjected to metabolism or biotransformation during maturation resulting in the increase of its transformation products in gonads and eggs of hormone treated eels (Figure IV.2). A continuous uptake as well as a potential metabolism of TBP-DBPE during maturation are reason for concern, because TBP-DBPE as well as its transformation products TBP-AE and BATE are known endocrine disrupters and able to penetrate the brain-blood-barrier [Recke and Vetter, 2007], making them a potential danger to the healthy development of offspring.

EH-TBB was only detected in two muscle samples but in all gonad samples and all but one of the egg samples of the hormone treated eels, making it the most abundant alternate BFR after TBP-DBPE and its metabolites in hormone treated silver eels (Table IV.2). EH-TBB is the principal component in the additive flame retardant Firemaster 550 (FM 550) produced since 2003 by Chemtura as a replacement for PentaBDE in polyurethane foam (PUF) applications [Covaci et al., 2011]. The high contribution of EH-TBB in gonads and eggs of hormone treated eels rather than muscle tissue could be the result of redistribution from tissue types other than muscle during the artificial maturation. The high lipid content of the liver in eels [Lewander et al., 1974; Dave et al., 1975] could make the liver a storage medium for BFRs, including EH-TBB. During maturation the eel uses its stored lipid to develop gonads and eggs, resulting in high lipid contents in these tissue types (as discussed above). This lipid metabolism pathway is driven by processes in the liver [Boetius, Boetius, and Hansen, 1991]. Contaminants

stored in the liver could be remobilised during the process. This is very likely in case of EH-TBB, which is known to be biologically metabolised [Barr, Stapleton, and Mitchellmore, 2010].

Further alternate BFRs detected in hormone treated eels were concentration wise TBP > BTBPE > PBEB > HBB > PBT. In eels of the comparison group additional alternate BFRs were TBP > DBE-DBCH > HBB > PBT > PBEB > BTBPE > TBX > HCCPD > DBHCTD. The noticeable fewer compounds in hormone treated eels might be an indication of a removal of the contaminants from muscle as well as gonad tissue during the artificial maturation process. The removal cannot be explained by redistribution into eggs, yet substances could be either excreted, distributed into other tissue types, or transformed.

Dechloranes

The Aldrin-related experimental flame retardant Dibromoaldin (DBALD) was found to be the highest concentrated Dechlorane in muscle tissue of hormone treated eels (up to 0.98 ng g⁻¹ ww) and the third highest in the comparison group (up to 0.18 ng g⁻¹ ww). This is, to our best knowledge, the first time that DBALD has been reported in the environment. DBALD was first mentioned in the US patent 3941758 as a fire retardant additive for polymers [Maul and Carlson, 1976]. Recently, Riddell et al. (n.d.) conducted a research project on the 'structural confirmation of legacy halogenated flame retardants derived from hexachlorocyclopentadiene', naming DBALD as one of the relevant monoadducts. However, there is no information available on current use or production. DBALD is structurally similar to the banned insecticide Aldrin, with two chlorine atoms substituted by bromine. The presence of an Aldrin-related contaminant could be problematic due to the potentially very high toxicity for fish (the LC₅₀ of Aldrin is 0.006- 0.01 mg kg⁻¹ for trout and bluegill [Metcalf, 2000]). However, DBALD could not be detected in gonads or eggs of hormone treated eels and was detected in only two gonad samples of the comparison group. It therefore seems that it is not readily distributed into these tissue types during uptake or maturation (Table IV.2).

The most frequently detected and highest concentrated Dechlorane in muscle as well as gonads of the comparison group was DDC-DBF. Whereas the antistereoisomer of Dechlorane Plus (DP) was more abundant in tissue of hormone treated eels. Σ DP as well as DDC-DBF could be found in all samples of the comparison group from Ems River and Schlei Fjord with Σ DP concentrations up to 0.079 ± 0.064 ng g⁻¹ ww in muscle tissue and 0.12 ± 0.064 ng g⁻¹ ww in gonads. DDC-DBF concentrations reached up to 0.11 ± 0.039 ng g⁻¹ ww in muscle tissue and 0.068 ± 0.022 ng g⁻¹ ww in gonads. In hormone treated eels Σ DP was in the 10-100 pg g⁻¹ ww range as well, with the highest concentrations in muscle tissue and < 30 pg g⁻¹ ww in eggs. DDC-DBF in hormone treated eels was low, compared to the comparison group with maximum concentrations of 30 pg g⁻¹ ww in muscle tissue from Schlei Fjord (Table IV.2) and concentrations below the limit of quantification in gonads or eggs. The similar or even higher concentrations of DDC-DBF in eels of the comparison group compared to DP

remained inexplicable, because DDC-DBF is, other than DP, not produced or imported to the EU. However, DDC-DBF is known to be highly bioaccumulative [Shen et al., 2011]). Small amounts, below the registration limit of the REACH legislation, leaching out of imported products could therefore potentially be the origin of the observed contamination in eels. The decrease of DDC-DBF in hormone treated eels indicates excretion, redistribution or metabolism/ biotransformation of DDC-DBF during the artificial maturation process. None of the observed DDC-DBF concentration levels induced effects in mutagenic and genotoxicity tests (see supplement information 2.6).

In contrast to results of all earlier life stages, e.g. yelloweels [Sührling et al., 2013] the anti-isomer of DP was predominant in muscle tissue and gonads of silver eels of the comparison group from both Ems River and Schlei Fjord with $\text{synDP}/\sum\text{DP}$ ratio (f_{syn}) of as low as 0.09 ± 0.18 in muscle and 0.44 ± 0.23 in gonads from Ems River. SynDP could not be detected in gonads of comparison group eels from Schlei Fjord. This low f_{syn} ratio in silver eels had already been reported in silver eels from Elbe River [Sührling et al., 2013], however it remained surprising, because in all other analysed eels from both this study and Sührling et al. [2013], synDP was clearly predominant with f_{syn} of up to 0.9. In Sührling et al. [2013] it was concluded, that this observed change in the isomer contributions had probably been caused by either excretion or redistribution into other tissue types of synDP. A selective uptake of antiDP was unlikely, because the overall concentrations of antiDP were similar in yellow and silver eels. Gonads were postulated as one of the possible tissue types into which redistribution might occur, which would indicate a selective redistribution of the DP isomers with a preference of the syn-isomer. This hypothesis was supported by the higher contribution of synDP in silver eel gonads (as shown above), but especially by the results of the hormone treated eels. In hormone treated eels syn- and antiDP were detected in 93% of the samples. The overall concentrations were similar in hormone treated eels and comparison group (not significantly different, t-test at level 0.05). The f_{syn} ratio however, differed strongly from the comparison group as well as between the different tissue types (Table IV.2). In muscle tissue of hormone treated eels f_{syn} was as low as 0.3 ± 0.04 . Contributions in gonads on the other hand were similar to previously observed levels in yellow eel muscle tissue with up to 0.9 ± 0.02 . SynDP was also predominant in eggs with up to 0.7 ± 0.1 . In yellow eel gonads from Elbe River no DP could be detected, indicating a preferred distribution of synDP into gonads and eggs specifically during maturation (Table IV.2).

Apart from DBALD, DDC-DBF and DP a variety of other Dechloranes and metabolites could be detected in individual fish. Interestingly, the DP metabolites aCl10DP, aCl11DP and DPMA were primarily detected in muscle samples of the comparison group, rather than gonads or tissue of hormone treated eels (Table IV.2), indicating, that DP is not subjected to additional metabolism during maturation. Further Dechloranes that could be detected in individual samples were DDC-Ant and CPlus in one sample of the hormone treated eels and 29%, 18% of the samples from the comparison

group, respectively.

Maternal transfer to eggs

The ratio of FR concentrations between maternal muscle tissue and eggs (EMR) as well as between muscle tissue and gonads (GMR) was calculated to assess maternal transfer efficiencies.

The transfer rates were calculated using the following equations:

$$EMR = \frac{C_{\text{egg}}}{C_{\text{muscle}}} \quad (4)$$

$$GMR = \frac{C_{\text{gonad}}}{C_{\text{muscle}}} \quad (5)$$

where c is the concentration [$\text{ng g}^{-1} \text{lw}$] in paired egg and muscle or gonad and muscle tissue.

Average EMRs for compounds detectable in artificially matured eels ranged from 0.01 for DDC-DBF to 10.4 for PBEB. The higher EMRs matched the general higher FR concentrations in fish from this habitat, indicating that transfer efficiencies increase with tissue concentration.

EMRs and GMRs could provide further indications for potential metabolism or transformation processes e.g. in the case of the observed increase of the relative contribution of BDE congeners attributed to technical PentaBDE (mostly BDE-47) in silver eels. The redistribution of Penta and OctaBDE congeners in silver eel gonads of the comparison group was similar, with GMRs of 0.5 and 0.4, respectively. In hormone treated eels on the other hand, PentaBDEs had significantly higher maternal transfer efficiencies than OctaBDEs, with GMRs for PentaBDE of 0.7 and 0.4 for OctaBDEs and EMRs of 0.08 and 0.05 for Penta- and OctaBDEs, respectively. The increase of the relative contribution of PentaBDEs could be caused by either selective redistribution or metabolism processes such as the enzymatic debromination. As with every metabolism process this would imply an interaction of contaminant and organism, potentially inducing adverse effects, especially in case of known reproduction toxicants such as the technical OctaBDE mixture [Wit, 2002].

Driving factors for maternal transfer

A major observed difference between the analysed eel groups was the lipid content in different tissue types. Yellow eels had very high lipid contents of up to 35% in muscle tissue, while the lipid content of the scarcely developed gonads was only around 1% (Table IV.2). Silver eels from the comparison group had slightly lower lipid contents in muscle (around 25%) and larger gonads with lipid contents similar to the muscles. During the maturation process eels use the lipid stored in their muscle to develop

gonads and eggs [Boetius, Boetius, and Hansen, 1991]. An increase of lipid content in gonads and eggs along with a decrease in muscle is therefore an indication for the progress of maturation. This change was observed in artificially matured eels with a significant negative correlation between lipid content in muscle tissue, eggs and gonads of $r = -0.73$. The lipid content in muscles was in some cases as low as 15%, while lipid content in gonads reached up to 35% and up to 29% in eggs.

This change in pattern of lipid distribution throughout the body can be expected to highly impact the distribution of BFRs and Dechloranes, because both groups are lipophilic.

As expected significant positive correlation ($r = 0.82$) was found between lipid content and absolute FR load for all tissue types (Figure IV.3). The correlation between lipid and total FRs for eggs was above average with $r = 0.86$. The decreasing lipid content in muscle and the increase of lipid in eggs and gonads represent the use of lipids for development of gonads and eggs during the maturation process. These observed changes in lipid and contaminant distribution give a strong indication that the lipid content in muscle as well as the therein-stored contaminants are transferred into eggs specifically during the maturation process. As eels are undergoing a similar starvation and lipid metabolism process during the natural maturation process, a similar transfer of contaminants is likely to occur in naturally maturing eels.

The lipid driven transfer will be impacted by the physical-chemical properties of different compounds and especially their ability to bind to lipids. The octanol-water partition coefficient ($\log K_{OW}$) can be used as a proxy to describe and quantify this ability.

Positive correlations between $\log K_{OW}$ and $\log EMR$ were observed for all analysed eels (r up to 0.47). Recent studies found similar correlations for PCB EMRs in drum (*Aplodinotus grunniens*) [Russell, Gobas, and Haffner, 1999b] with $r = 0.41$ and zebrafish (*Danio rerio*) exposed to BFRs, with $r = 0.89$ [Nyholm et al., 2009] (Figure IV.4). Peng et al. [2012], on the other hand, observed strong negative correlation between $\log K_{OW}$ and EMRs for Dechloranes in Chinese sturgeon (*Acipenser sinensis*), indicating potentially high inter-species differences in the maternal transfer mechanism.

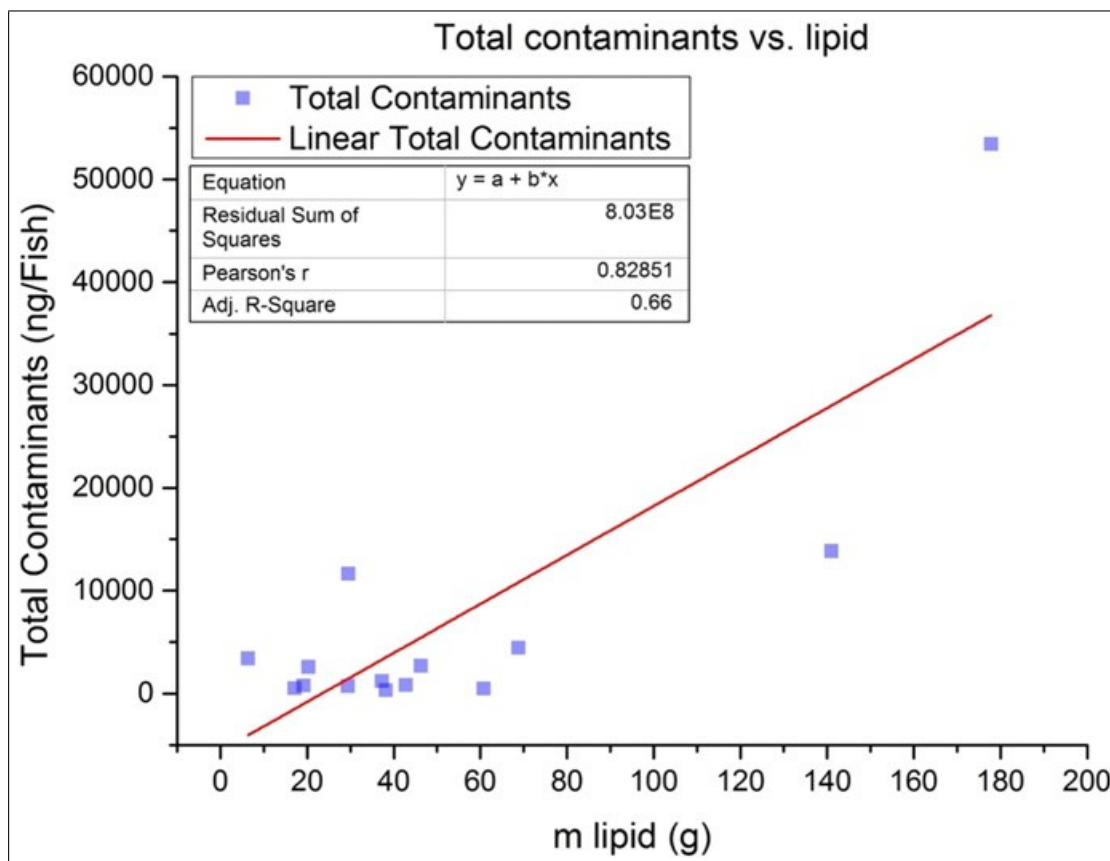


Figure IV.3: Correlation of lipid mass in gram per fish and sum contaminants of halogenated flame retardants in ng per fish.

The difference could be caused by differences in lipid metabolism during gonad development in different fish species.

The correlation between $\log k_{OW}$ and transfer rates explains the higher transfer rates into eggs of e.g. DP (average EMR in analysed eels: 3.5, $\log k_{OW}$: 11.27) compared to DDC-DBF (average EMR in analysed eels: 0.01, $\log k_{OW}$: 8.05), which was rarely detectable in any eggs, even though the concentrations in muscle were higher than DP and it is known to be a highly bioaccumulative and bioavailable substance [Shen et al., 2011].

Combining $EMR = \frac{C_{egg}}{C_{muscle}}$ with the observed relation for $\log k_{OW}$ of a compound x and its EMR in artificially matured silver eels $EMR = 0.9801 \cdot \log k_{OW} - 4.3303$ provides a first estimate for the maternal transfer of a compound x based on its $\log k_{OW}$ and the concentration found in muscle tissue; with:

$$C_{egg} = 0.981 \cdot \log k_{OW} \cdot C_{muscle} - 4.3303 \cdot C_{muscle} \quad (6)$$

Plotting the concentration in eggs against the lipid content showed the following relationship for the artificially matured eels: $C_{egg} = 1.5 \cdot \% lipid + 1.6$. As discussed above the lipid content in gonads and eggs increases during the maturation process, as lipid stored in muscles is used to develop gonads and eggs. The lipid content in muscle of

the analysed eels was in some cases as high as 35%. To assess the potential concentrations in eel eggs, assuming a complete transfer of lipids, a lipid content of 35% was used for calculation. The average weight of a single egg was 0.07 g ww or 0.025 g lw. The average total FR load per egg after a complete transfer of lipids from muscle to eggs would therefore theoretically be

$$Total_{egg} = C_{egg} \left[\frac{ng}{g} lw \right] \cdot [g lw] = 1.5 \cdot 35 + 1.6 \left[\frac{ng}{g} lw \right] \cdot 0.025 [g lw] = 13ng \quad (7)$$

with varying contributions of individual compounds based on their $\log k_{OW}$.

The observed relations are just a first and rough estimate, describing overall trends rather than exact transfer rates for individual compounds. They do, for example, not explain the observed difference in EMR of the stereoisomers of DP, with average EMR of 3 for the syn-isomer and 0.5 for the anti-isomer. Despite the overall different trends Peng et al. [2012] reported similar observations for the maternal transfer of the DP isomers in Chinese sturgeon, providing further indications that additional factors to lipid content and k_{OW} might affect the maternal transfer of BFRs and Dechloranes.

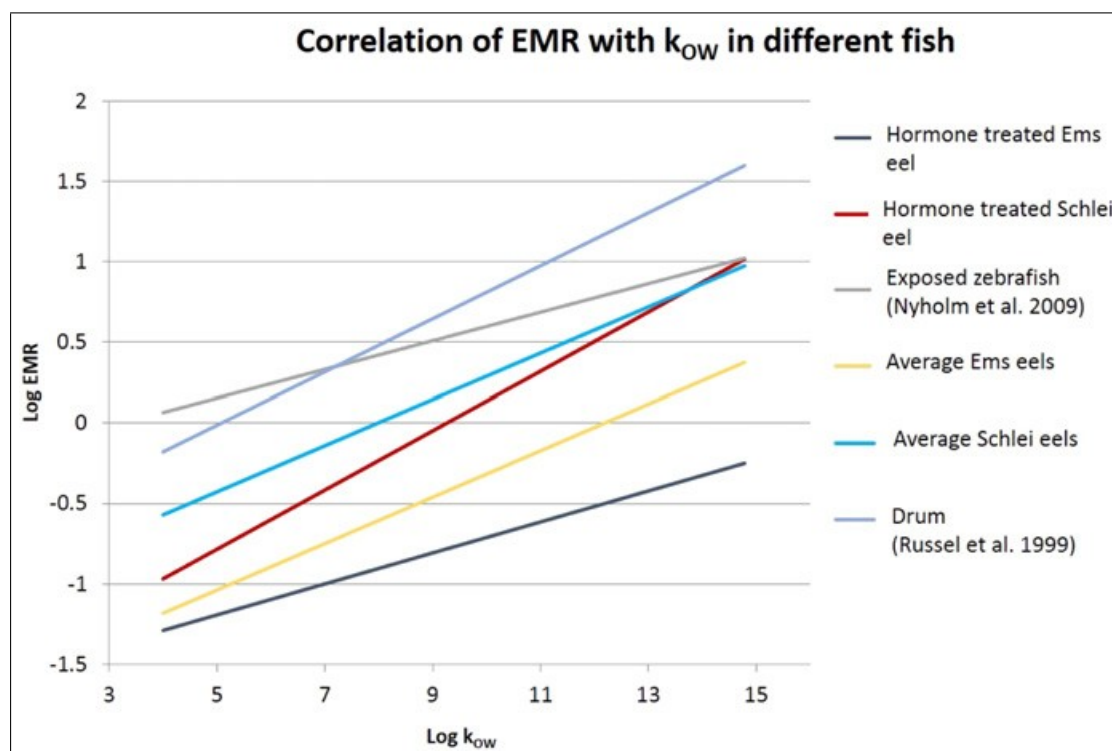


Figure IV.4: Correlation of log EMR (egg muscle ratio) and log k_{OW} (octanol-water partitioning coefficient) of halogenated flame retardants and polychlorinated biphenyls (PCBs) in different fish species.

Metabolism and continued uptake

Lipid metabolism and subsequent metabolism of stored contaminants during maturation could potentially have a high impact on contamination patterns throughout the body. Especially in eels, different uptake pathways of contaminants could also lead to

major changes in contamination patterns during maturation. Eels stop feeding during that period, which could increase the relative contribution of contaminants with continued uptake through gills, skin or the ingestion of water compared to contaminants with primary uptake through food.

As shown above indications were found that several compounds might not only be re-distributed into gonads and eggs, but could be continuously absorbed from the water as well as subjected to metabolism or biotransformation during the maturation process. This leads to a significant change in the contamination pattern between hormone treated eels and comparison group from the same habitat as well as hormone treated eels from different habitats. Especially in the case of TBP-DBPE and its transformation products the significant increase in hormone treated eels (Figure IV.2), along with high concentrations in the water phase indicated a high continued uptake from the water phase.

Further investigations are necessary to determine which compounds are accumulated through the water phase and whether observed changes were caused by metabolism processes or other physical or habitat based changes during the maturation process. Especially because the increase of potential metabolites such as PentaBDE, low brominated MeOBDEs as well as TBP-AE and BATE in gonads of hormone treated eels indicate metabolism processes.

Another potential BFR and PBDE metabolite is tribromoanisole (TBA) [Nyholm et al., 2009]. It was detected in all analysed eels and tissue types with the highest average concentrations of several ng g^{-1} ww (Table IV.2). Determining the potential origin of this contamination proved difficult, because TBA is also a naturally occurring substance and was found in the water samples from the tanks. However, a significant increase (t-test at level 0.05) of the TBA concentration was observed in hormone treated eels from Ems River, compared to the comparison group from the same habitat, indicating either continued uptake during the artificial maturation (i.e. tank water) or formation through metabolism of other brominated compounds. TBA did not induce effects at any detected concentrations in a standardised fish embryo toxicity test (see supplement information 2.5. for details).

The difference between the artificial and natural maturation was too high to draw conclusions regarding effects on eels in general. The repeated observation of increased levels of potential BFR metabolites as well as contamination of the tank water in hormone treated eels compared to the comparison groups from the same habitat call for further investigation of uptake pathways during the silver eel life stage as well as BFR metabolism or transformation in eels. This is especially important regarding the essential process from maturation to reproduction, where the quality of spawners might be particularly at risk due to endocrine disrupting or in general toxic substances.

Conclusion

This study provided evidence that PBDEs as well as their brominated and chlorinated substitutes are redistributed to gonads and eggs during maturation. The driving factors for this maternal transfer seem to be primarily the transfer through lipid dependent on the $\log K_{OW}$ of the individual compound. Based on these observed correlations a contaminant load of >1 ng per egg was estimated for sum brominated and chlorinated flame retardants. Correlations were also found for the maternal transfer and the concentration in muscle tissue, which provides a potential possibility to assess the maternal transfer of halogenated flame retardants in eels without actually having to sample eggs. Further studies should be conducted to verify these correlations for other compounds potentially affecting the quality of spawners, especially information on PCBs and Dioxins in eel eggs are needed to assess whether the critical concentrations for impairment of embryo development reported by Palstra et al. [2006] are reached in the environment.

Additionally, indications were found that the brominated flame retardant TBP-DBPE and potentially other BFRs are not merely redistributed to gonads and eggs, but continuously absorbed from the surrounding water and potentially subjected to metabolism or transformation processes, resulting in the increase of transformation products such as low brominated MeOBDEs, PentaBDE, TBP-AE and BATE. Studies regarding the potential impact of this continued exposure, metabolism or transformation processes on the maturation and reproduction success of eels or fish in general are needed. Especially considering that the release of stored chemicals in eels occurs during their maturation phase and that not only a fraction, but a lifetime worth of accumulated contaminants are potentially released, affecting the quality of spawners.

The results of this study also emphasize the necessity to further increase the research on emerging brominated and chlorinated flame retardants. A variety of potentially hazardous non-PBDE flame retardants were detected, such as the aldrin related DBALD and the highly bioaccumulative DDC-DBF. Even though neither are officially produced nor imported into the EU. The observed maternal transfer of potentially hazardous and endocrine disrupting contaminants could impair 'quality of spawners', reproduction success and development of offspring.

Abbreviations

BFRs: brominated flame retardants, BG: body girth, BGI: body girth index, FRs: flame retardants, LOD: Limit of detection, LOQ: Limit of quantification, LT: total lengths, Na₂SO₄: Sodium sulphate, PBDEs: polybrominated diphenyl ethers, PCBs: polychlorinated biphenyls, POPs: persistent organic pollutants, SPE: salmon pituitary extract, ww: wet weight, lw: lipid weight

Acknowledgements

We'd like to thank Udo Koops for the technical support as well as Nadine Griem for her help with the sample preparation.

The artificial maturation of eels was funded by the German Federal Ministry of Food and Agriculture through the project 'Overcoming the difficulties of European eel reproduction. Optimization of artificial maturation, eel husbandry and breeding conditions' (313-06.01-28-173.034-10).

General Discussion

Development of sound dose-response relations as a prerequisite for the derivation of threshold values

Among others, the lack of unambiguous dose-response relations for most chemical substances has been identified as one gap for a proper risk assessment according to the MSFD (Table 1). Dose-response relations are the basis for the definition of threshold values. For many contaminants threshold values are either of low quality or completely missing. Low quality thresholds are often a result of an inadequate test designs, while a complete lack of thresholds is mostly a consequence of the absence of adequate test systems, often observed for lipophilic substances. Both methodical issues have been considered within this thesis and are discussed in the next sections.

Planning and Design of toxicity tests with binary response data

The European MSFD calls for an overarching mathematical concept to derive threshold values [EU, 2008b; EU, 2010a; OSPAR, 2010; OSPAR, 2013b]. The development of such a concept relies on a sound database, which is obtained from reliable and reproducible toxicity tests. Such a database is either incomplete in or absent from the literature so far. Further on, most toxicity tests aim for the LC/EC50, which is not appropriate for the derivation of threshold values that base on small target effects like EC10 [OSPAR, 2010; OSPAR, 2013b]. An adequate experimental design for this purpose requires identification of the baseline ensuring a clear distinction between substance effects and background noise (Chapter I). It is assisted by an appropriate number of test subjects and a certain dose allocation adjusted to small target effects [Harris et al., 2014]. This in consequence helps to generate sound dose-response curves with small confidence intervals (CIs) appropriate for the derivation of threshold values [OSPAR, 2010; OSPAR, 2013b]. An adequate experimental planning is assisted by the statistical tool '*toxtestD*' implemented in R code, which has been newly developed within MERIT-MSFD [Keddig and Wosniok, 2014; R Core Team, 2014].

In general, the concepts of dose-response relations assumes that an increase in contaminant concentration is followed by an increase in response [OSPAR, 2010; OSPAR, 2013b]. According to the defined endpoint (response) a baseline should be evaluated as additional quality measure of the result. Knowledge on background noise provides

a better understanding of study results and allows the interpretation of their environmental significance [Harris et al., 2014]. Spontaneous lethality (SL) represents an adequate baseline for FET tests aiming for lethal concentrations. It is determined by a FET test under controlled conditions without the application of any substance. Adequate planning of these pre-tests is incorporated as the first step in *'toxtestD'*. So far most studies in literature do not consider an adequate baseline such as SL (Table 2).

The determination of SL is particularly important when small toxicity effects are investigated. Otherwise results could be biased by natural variation. Additional to the knowledge on SL an adequate number of test individuals are required to safely differentiate between background noise and effects of contaminants [Keddig and Wosniok, 2014]. Adequate numbers of test individuals restrict the probabilities of type I and type II error to an acceptable level [Gad, 2005]. The determination of sample size with regard to the prior determined SL including the choice of one out of three common risk models with immunity as extension [DeLean, Munson, and Rodbard, 1978; Ratkowsky and Reedy, 1986; United States Environmental Protection Agency (U.S. EPA), 1992] is also part of *'toxtestD'*. Risk models govern the procedures behind the discrimination between SL and effect and are explained in detail in Chapter I (Figure I.3).

Besides the determination of SL as well as sample size, an appropriate dose allocation is the next step in *'toxtestD'* to improve the robustness of the resulting dose-response relation [Keddig and Wosniok, 2014]. Concentrations for the development of dose-response relations depend upon the results obtained from pilot studies or concentration range findings or upon data obtained from current literature. *'toxtestD'* sets doses to fixed points, i.e. 0, EC_{10} , EC_{50} , EC_{90} as well as to the target value. Free dose points are set according to certain rules explained in Chapter I. This procedure results in dose-response relations with small confidence intervals (Figure 2) leading to robust target values.

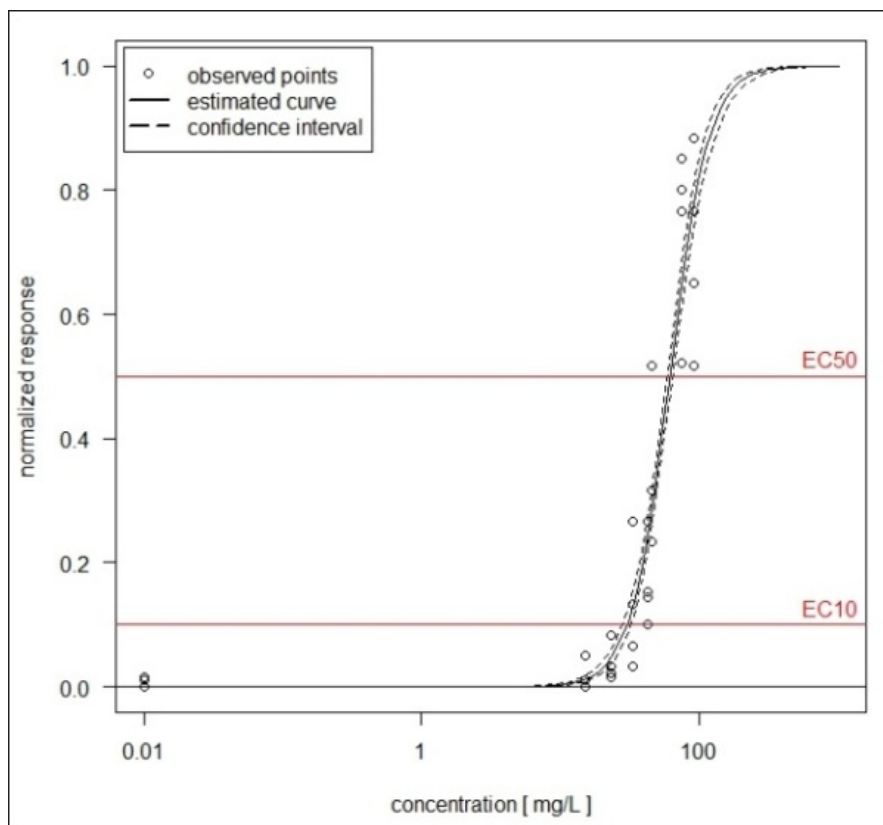


Figure 2: Example for a dose-response curve developed with the tool 'toxtestD'

None of the listed studies is adequately designed for the derivation of proper threshold values as requested for MSFD. With only one exception, all studies aim solely for the LC_{50} . Additional information like the shape of the dose-response curves is not provided. However, the slope of such a relation would be necessary to safely derive LC_{10} from LC_{50} . Further on, reported LC_{50} deviate severely between studies. Due to the lack of SL in most of the studies it cannot be clarified if this difference may be the consequence of varying healthiness among fish breeds (=natural variation) or instead be caused by other reasons (=variation in handling) [Harris et al., 2014]. The provided confidence intervals in the literature are larger than those obtained from data within this thesis. This is presumably a consequence of too small sample sizes (≤ 25 individuals per replicate). An increase in sample size is among others one option to keep type I and type II errors small, while increasing the power of the analysis and concomitantly the reliability of the obtained data [Harris et al., 2014].

Hence, using 'toxtestD' to improve the current guidelines for conducting FET tests [OECD, 2013b] by including a target specific experimental design will help to create a sound data base for the derivation of threshold values. Unnecessary extrapolation of data can be avoided and lethal concentrations can be safely determined.

Table 2: Summary of Fish embryo toxicity tests with Zebrafish (*Danio rerio*) with CdCl₂. SL - spontaneous lethality, LC₅₀ - half maximal lethal concentration; LC₁₀ - concentration with 10% lethal effects, hpf-hours post fertilization.

Study aim	SL	Replicates	Individuals / Replicate	Concentration [mg/L]	Stage at start [hpf]	Duration [h]	endpoint	mg/L	Confidence intervals	Source
mode of action	nd	15	20	0-2193.4	5	24	LC ₁₀	120.4	-	[Cheng and Wai, 2000]
						24	LC ₅₀	368.5	-	
effects of temperature and cadmium	nd	3	20	0-100	<2	48	LC ₅₀	4.75 ^a	-	[Hallare et al., 2005]
							LC ₅₀	30.1 ^b	-	
							LC ₅₀	46.8 ^c	-	
Developmental toxicity	9%	3	16	0.1-1000	24	24	LC ₅₀	255.9	± 64.0	[Ali, Champagne, and Richardson, 2012]
						48	LC ₅₀	407.3	± 45.2	
						72	LC ₅₀	327.0	± 55.1	
						96	LC ₅₀	27.9	± 0.1	
Developmental toxicity	nd	3	25	0-205.4	72	96	LC ₅₀	42.9	25.1-168.5	[Blechinger et al., 2002]
Developmental toxicity	2.4	4	60	0-90	<2	48	LC ₁₀	30.9	28.8-32.6	Results from this thesis
						48	LC ₅₀	62.3	60.2-64.6	
						96	LC ₁₀	27.5	25.4-29.1	
						96	LC ₅₀	56.2	54-58.6	

^a Test temperature: 21 °C

^b Test temperature: 26 °C

^c Test temperature: 33 °C

Microinjection as method to develop sound dose-response relations

The FET test is a common tool to detect and quantify the adverse effect of chemical substances on organism levels. However, it is limited to the application of polar substances with a molecular weight below 3 kDa as has been outlined before. For non-polar substances and contaminants of higher molecular weight new methods have to be evaluated for ecotoxicology purposes. Amongst others, microinjection has been identified as a potent tool to administer polar and non-polar substances, which cannot be applied within a FET test. With this approach the two natural barriers, chorion and embryo envelope, can be overcome and maternal transfer of contaminants from adults to the offspring can be mimicked. Besides these distinctive advantages compared to the FET test several technical difficulties have been identified, which have already been outlined in detail (Chapter II). Briefly, two most striking methodological uncertainties appeared during test evaluation. These are the encapsulation of injected substances and the considerable variation of injection volumes.

Contaminants have been administered into the yolk cell of freshly fertilized fish eggs. Variations in injection volumes appeared while several microinjections were administered successively into the yolk of a series of eggs. As the injected volumes are very small (≤ 4.2 nL) their variation directly influences the concentration of the injected substance within the yolk. High variations in dose lead to severe uncertainties in the predicted model describing the data. In consequence the derivation of reliable threshold values becomes questionable. Aiming for small effect sizes (EC_{10}) as it is requested for deriving assessment criteria [OSPAR, 2010; OSPAR, 2013b], complicates the clear distinction between substance effects and background noise. The use of microinjection as a tool in ecotoxicology to generate dose-response relations as sound base for the derivation of threshold values is problematic. However, microinjection could be used as a method for studies without the demand on high volume accuracy.

The phenomenon of substance encapsulation has been shown for triolein but was also detectable for water and DMSO (Figure 3). Due to the regulations of the European animal welfare legislation [EU, 2010b] all experiments have been terminated after 96 to 120 hours post fertilization (hpf). A putative assimilation of the microinjected substances may have occurred later in embryonic development when the fish larvae completely consumed the yolk. Zebrafish was shown to deplete all yolk until 165 ± 12 hpf [Jardine and Litvak, 2003]. Elongation of the test duration may therefore be necessary to elucidate potential effects of toxicity appearing in advanced stages of fish development. In this regard microinjection may be a powerful tool to investigate the mode of action of chemical substances that are conveyed through maternal transfer from parental individuals to the offspring. The information on delayed substance effects emerging in advanced stages of fish development may be essential for understanding the mechanisms behind pollution and reproductive success.

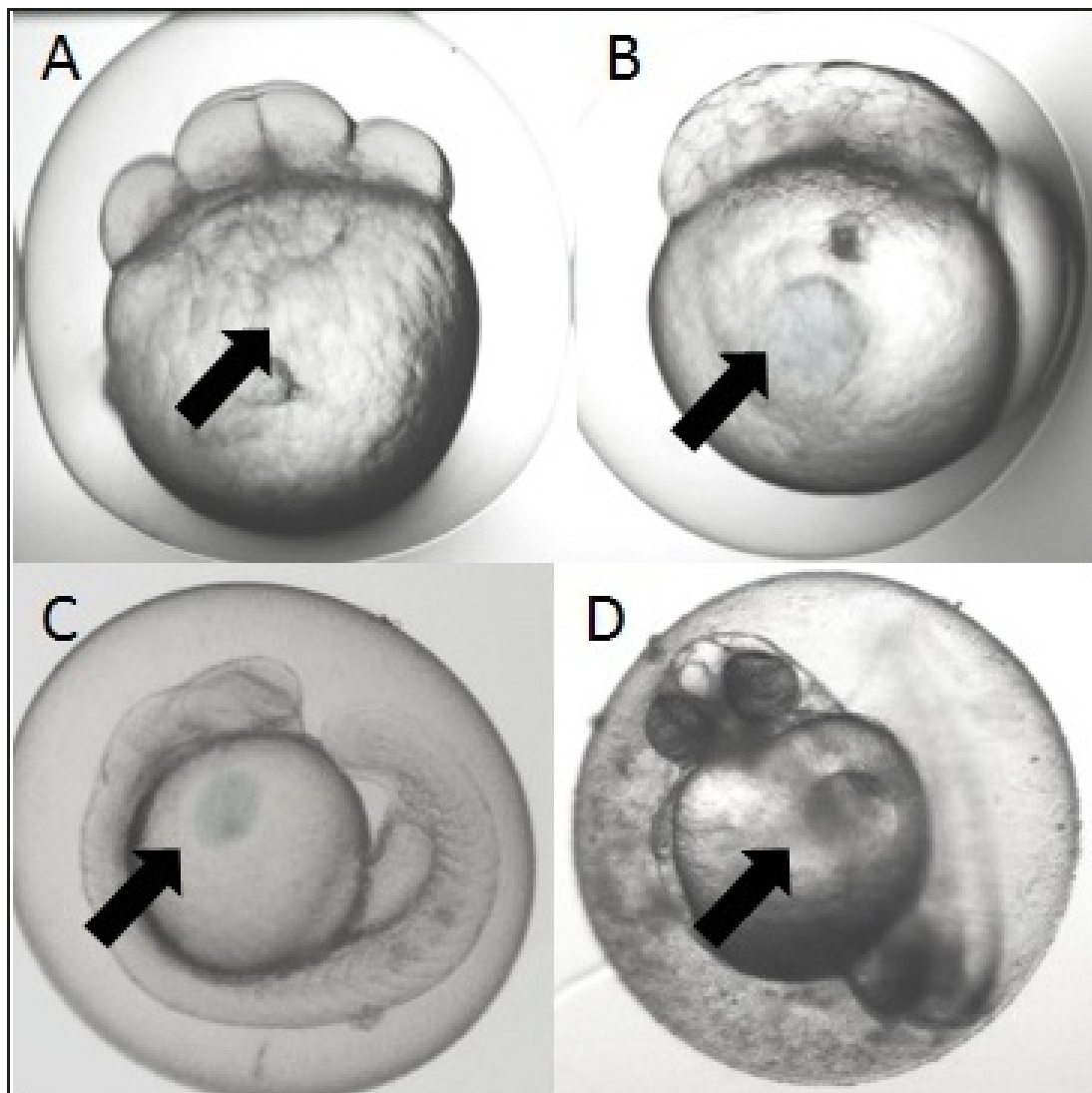


Figure 3: Zebrafish embryos after yolk sac injection of water A 1.5 hours post fertilization (hpf) and B 24 hpf, and after injection of DMSO C 2hpf and D 48 hpf.

The marine risk assessment

Assessing the environmental status of biota requires the investigation of content loads in wild living individuals as well as corresponding assessment criteria. As adequate threshold values for the marine environment are still absent, the marine assessment has been carried out on basis of existing threshold values, i.e. EQS and EACs (Table 4) [EU, 2000; EU, 2008b; EU, 2010a; OSPAR Commission, 2010; EU, 2013].

Persistent organic pollutants in fish

Contaminant concentrations of Baltic herring and European eel have been investigated (Chapters III & IV). Both are rather fat rich species. In consequence they accumulate higher contents of POPs compared to lean fish and may therefore be at higher risk to

Table 3: Environmental Quality Standard (EQS) for polybrominated diphenylethers (PBDEs) and perfluorooctane sulfonic acid (PFOS) derived by the Water Framework Directive (2013/39/EU) (EU 2013) and Environmental Assessment Criteria (EAC) for congener 118 of polychlorinated biphenyls (CB118) derived by OSPAR's Coordinated Environmental Monitoring Programme (CEMP) (OSPAR Commission 2010). Both assessment criteria shall be applied for whole fish samples.

Contaminant	Assessment Criteria	[$\mu\text{g}/\text{kg ww}$]
PBDEs ^a	EQS	0.0085
PFOS and its derivatives	EQS	9.1
CB118	EAC	0.65

^a BDE 28, 47, 99, 100, 153, 154

suffer from POP mediated effects. Concentrations of POPs were remarkable in Baltic herring (Chapter III) and European eel while content loads occasionally exceeded the currently used threshold values (Table 3). In particular for POPs whose concentra-

Table 4: Contaminant concentration [$\mu\text{g}/\text{kg ww}$] of Baltic herring (*Clupea harengus*) and European Eel (*Anguilla anguilla*) from different locations. Mean values are given with standard deviation for polybrominated diphenylethers (PBDEs), perfluorooctane sulfonic acid (PFOS), and congener 118 of polychlorinated biphenyls (CB118).

Substance	Tissue	Sex	Baltic herring Arkona Sea n=10	European eel Ems n=7	European eel Schlei n=4	European eel Elbe n=10
PBDEs ^a	Filet	female	0.82 \pm 0.34	0.79 \pm 0.45	0.06 - 1.4	8.3 \pm 3.7
PBDEs ^b	Liver	female	0.95 \pm 0.70	n.d.	n.d.	n.d.
PBDEs ^c	Gonad	female	0.15 \pm 0.04	0.71 \pm 0.40	0.12 \pm 0.04	n.a.
CB118	Filet	female	1.89 \pm 0.68	n.d.	n.d.	n.d.
PFOS ^d	Liver	female	6.34 \pm 1.45	n.d.	n.d.	n.d.
PFOS ^e	Liver	male	27.0 \pm 5.43	n.d.	n.d.	n.d.

^a BDE 28, 47, 66, 99, 100, 85, 153, 154, and 183

^b linear + branched PFOS, n=9

^c linear + branched PFOS, n=5

tions exceed EQS or EACs adequate measures are required to minimize the risk for marine wildlife to be adversely affected. In general, the use and production of harmful substances need to be banned or at least restricted in order to reduce environmental concentrations. Although polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) have already been banned in Europe in 1996 and 2004, respectively, remarkable concentrations are still present in marine biota. The use and production of perfluorooctane sulfonate (PFOS) has been restricted in 2008. However, contents in marine biota showed a general increasing trend [HELCOM, 2010]. POPs are transported over long distances from various sources worldwide and are introduced into European waters due to atmospheric deposition [Roose et al., 2011].

Bio-effects: Gonad burden as indicator for reproductive success

POPs are suspected to affect the overall fitness of a species by altering inter alia survival, growth, behaviour, or reproduction [Fent, 2013]. Contaminants tend to accumulate organ-specific in Baltic herring and European eel (Chapters III & IV). Such a distribution is driven by the physico-chemical characteristics of POPs in relation to the intrinsic characteristics of tissue types [Peng et al., 2012; Waszak, Dabrowska, and Góra, 2012]. Certain contaminant concentrations could be detected in gonad tissue of both species (Table III.2& IV.2 4) and in case of PBDEs exceeded the current threshold value (Table 3). In theory, especially contamination of gonads affect the reproductive success, which can itself be seen as measure for the general fitness of a species [Schaefer, 1992].

A reduction in quantity and quality of gametes of both sexes was shown in laboratory experiments with contaminated Zebrafish. Additionally, sex-related effects, like the opposed up- or down- regulations in vitellogenin expression, have been identified [Nyholm et al., 2008; Deng et al., 2010; Norman et al., 2010; He et al., 2011]. Although, the mechanisms of gonad development have so far not been clarified in detail, it was assumed that differences arise during maturation of males and females [Gillis, McKeown, and Hay, 1990]. Consequently, not only the composition of gonad tissue [Henderson and Almatar, 1989] but also the composition and concentration of contaminants accumulating in gonads during maturation differ. Accordingly, POP concentrations accumulated in gonad tissues of Baltic herring differed between sexes (Table III.2; Figure III.1). Hence, investigations of both ovaries and testis are required to include gonad burden as an indicator for reproductive success in the current monitoring progress.

In addition to direct effects of POPs on reproduction, an existing maternal pollutant transfer from females to eggs has been shown in Zebrafish to occur in feeding experiments [Sharpe et al., 2010; Merwe et al., 2011b]. In wild living individuals a maternal pollutant transfer was found in Chinese sturgeon (*Acipenser sinensis*) [Peng et al., 2010; Peng et al., 2012] and European eel (Chapter IV). The extend of contaminant transfer depends upon the fat content of the gonads [Peng et al., 2012] as well as upon the physico-chemical characteristics of the POP itself. To what extend this transfer is also present in other fish species needs to be further investigated. Similarly, the contaminant effects of transferred POPs on the developing embryo also offer scope for further research. Toxicity tests, like the FET test, are potential tools to help identifying adverse effects and are necessary to assess the potential risk of POP contents in gonads and their effects on the next generation.

In European eel (Chapter IV), 2,4,6-tribromoanisol (TBA) was found in remarkable concentrations in different tissues including gonads (Table IV.2). Although TBA is a naturally occurring substance it is also a potential PBDE metabolite [Nyholm et al., 2009]. A FET test with TBA detected no effects in developing Zebrafish embryos at the applied concentrations, which were kept beneath 10 µg/mL due to solubility problems (Chapter IV). If effects would have appeared at higher concentrations could not be clarified

here. Alternative exposure routes like direct application via microinjection are required to circumvent the solubility issue in the future.

In general, recording reproductive success on behalf of the general fitness of key species may be a suitable way to assess the health of marine wildlife and the integrity of marine ecosystems. Therefore, including gonad burden as an indicator for reproductive success into the current monitoring requires a newly developed mathematical concept that is based on knowledge of POP concentrations in gonads present in wild living fish and their direct effects on maturation of both sexes as well as their indirect effects on the development of the next generation after maternal transfer including pollutant transfer rates.

A general discussion on risk assessment of contaminants

The results obtained in this thesis lead to a critical view on the current risk assessment (Table 1). The list of priority hazardous substances contains 21 contaminants and is presumably incomplete considering the number of new contaminants synthesized each year all over the world. The extensive use of chemical substances and compounds in various fields lead to an enormous release of components into the marine environment. Once introduced many substances accumulate in sediments and/or biota, are less biodegradable and persistent in the environment [EU, 2000], and may cause adverse effects to ecosystem functioning [Roose et al., 2011]. Even though the MSFD calls for a regular update of the list of hazardous substances it is not achieved by member states. This relies basically upon the lack of adequate screenings for new contaminants incorporated into current monitoring as well as upon difficulties in detecting and quantifying the potential harm of new contaminants to marine ecosystems.

For an improved risk assessment of the chemical pollution high-resolution screening methods for new contaminants are needed. Furthermore, the potential harm of these substances to the marine ecosystem should be assessed and quantified through adequate tests identifying the mode-of-action of a chemical on an organism level. From here the potential risk for the ecosystem can be predicted.

Substances found in high concentration but with no detected adverse effect to the marine environment should be included into a target-screening to keep track of their spatial distribution over time. However, monitoring on an annual basis may not be necessary. Contaminants found in remarkable concentrations causing adverse effects to marine wildlife should be added to the list of hazardous substances. Here, monitoring on an annual basis may be necessary to identify spatial and temporal trends as well as to assess the potential effects to the marine ecosystem. In this regard, analytical methods need to be improved for detecting a broad range of POP concentrations within the three matrices water, sediment, and biota.

At present about 30,000 chemicals are on the EU market comprising a production volume of higher than one tonne by year. Although not all substances end up in rivers,

estuaries, and seas it is presumably still a high number of contaminants causing potentially damage to marine organisms, ecosystems, and services [Roose et al., 2011]. However, most substances that are released into the environment are not observed or even recorded.

For the assessment of contaminations reliable threshold values are required. The use of current EQS is not recommended due to severe uncertainties arising when high assessment factors (AFs) are applied in order to adopt EQS for the purposes of MSFD [OSPAR, 2013b]. AFs account for uncertainties in data basis arisen from variations between laboratory studies as well as between species and from extrapolation of short-term to long-term toxicity as well as from single species laboratory studies to a multi-species or even ecosystem levels. AFs increase with increasing uncertainties in data basis. The applicability of an EQS derived from a basis of low quantity and/or quality is generally ambiguous. EACs are also not reliable as some of them are adopted from food safety standards [OSPAR, 2013b], which are obviously not adequate for the protection and conservation of the marine environment. Hence, new threshold values have to be developed.

The MSFD calls for threshold values that are based on the sensitivity of the most sensitive species [EU, 2008b; EU, 2010a; EU, 2013]. Identifying this species in the field is difficult [OSPAR, 2013a]. Hence, adequate model systems need to be developed to assess the risk for a whole ecosystem. Model systems are generally based on laboratory studies testing the effects of one component to a model organism such as Zebrafish, oyster, Blue mussel or algae [OECD, 1998; OECD, 2006; OECD, 2012; OECD, 2013b; OECD, 2015]. Accounting for varying sensitivities among species the development of species sensitivity distribution (SSD) curves as basis for the derivation of threshold values seems most promising. From SSD curves threshold values can be derived that ensure that the contaminant causes no adverse effect in 95% of the tested species (HC5) [OSPAR, 2013a]. Whenever possible, the use of saltwater species should be preferred over freshwater species to avoid uncertainties in data transfer from fresh- to saltwater organisms. However, this may not be possible for each contaminant and mathematical models may be the only option to transfer the obtained data from fresh- to saltwater. At present, these models are still lacking.

Many guidelines for the implementation of model systems concerning different taxa have been published by the OECD [OECD, 1998; OECD, 2006; OECD, 2012; OECD, 2013b; OECD, 2015] and can be used for the development of SSD curves as already been recommended for the probabilistic approach to derive EACs [OSPAR, 2013a]. An adequate experimental design for each single test is essential to obtain statistically sound data regardless, which model is applied to develop dose-response relations of contaminants. For binary response data, this can easily be achieved by '*toxtestD*' as has already been described for the FET test in a previous section [Keddig and Wosniok, 2014]. Additionally, the package offers a possibility to include immunity, which describes a proportion of test individuals not affected by the test contaminant. In current

toxicity no attention has been paid to immunity so far although it is likely to occur in the environment.

Further on, classical toxicity tests consider only single components [OECD, 1998; OECD, 2006; OECD, 2012; OECD, 2013b; OECD, 2015]. In the marine environment substances do not occur separately but in contaminant mixtures. Their effects can be simply additive, but also antagonistic, or synergistic [Fent, 2013]. The interaction between substances is hard to predict from tests with single components. Hence, additional toxicity tests with chemical mixtures are necessary to improve both the prediction of the interaction between substances and the assessment of the risk for the marine environment.

Model systems currently in use in ecotoxicology are fraught with methodological limitations. In toxicity tests with waterborne POP exposure non-polar substances cannot be administered. Due to low water solubility these contaminants precipitate in water. In consequence no effect is observed. The use of solvents like dimethyl sulfoxide, methanol, and acetone is only a partial solution for this solubility problem. For all these putative solvents FET tests have been conducted that resulted in acceptable concentrations under which no effects of the solvent on the development of a Zebrafish embryo appear [Maes et al., 2012]. However, the interaction of low POP concentrations with increasing concentrations of solvents has never been investigated. Thus, a remaining risk for biasing dose-response relations by the use of solvents exists. In consequence, the use of solvents in toxicity tests should be avoided. If this is not feasible mixture effects caused by the substance together with the applied solvent need to be eliminated. Simple tests with low POP concentrations and increasing contents of solvents can help elucidating this issue and should be included into the current toxicity testing.

Further on, new model systems need to be evaluated offering the exposure to non-polar substances or to contaminants with molecular weights above 3 kDa without the use of solvents. Although every model system comprises methodological difficulties and requires certain assumptions, some promising methods exist. Among them are microinjection (Chapter II), passive dosing via a silicon ring or sea sand [Butler et al., 2013], dechoriation of fish eggs [Henn and Braunbeck, 2011], as well as feeding experiments with adults [Nyholm et al., 2008; Deng et al., 2010; Norman et al., 2010; He et al., 2011]. In fact, the right choice of model system individually adjusted to the characteristics of the POP of interest is essential to produce sound data.

The recommendations listed above are hardly to achieve, since a lack of money and manpower challenge their implementation. However, there are options possibly resolving the financial issue e.g. by expanding the costs-by-cause-principle that has already been incorporated into the MSFD. This principle implies that the polluters pay up for the costs arising during the removal of a hazard. Unfortunately, the identification of the perpetrator is not always possible. In this case the producer of a chemical, i.e. the synthesizing factory, should be partly in charge for the arising costs. This in consequence would presumably incentivise the industry to develop chemicals that are

non-toxic to the environment, that comprise low potential for bioaccumulation and that have only short half life periods.

Further on, newly synthesized contaminants could be assessed for their potential risk before they are potentially released to the environment by an extended approval process. Such a process could include the development of adequate screening methods, the development of dose-response relations, especially for substances that potentially end up in the marine environment, as well as the identification of specific effect concentrations. This in consequence may restrict the use of potential harmful substances. However, these measures are only meaningful in case an international institution is established governing the single steps behind a global risk assessment.

Conclusions

This thesis was designed to contribute the implementation of the Marine Strategy Framework Directive (MSFD) at different levels.

First, a new concept has been developed to advance the experimental design for toxicity tests with binary response data, which is available in R code (*'toxtestD'*). It offers a user-friendly way to an optimized planning procedure without the demand of deep statistical knowledge. The tool can easily be added to the current recommendation of OECD by closing gaps that have recently been identified in many studies in ecotoxicology. These are amongst others a good planning and adequate design, e.g. choosing reasonable targets and numbers of test subjects, the definition of a base line, e.g. spontaneous lethality, and the allocation of relevant doses [Harris et al., 2014].

Second, the development of new model systems for ecotoxicology has revealed the applicability of microinjection as powerful tool for studies without volume accuracy that help identify potential effects caused by POPs. By microinjection polar and non-polar contaminants can be applied directly into the fish egg. The two natural barriers, chorion and embryo envelope, can be overcome. Further on, microinjection is a potent way to mimic maternal pollutant transfer. However, several methodological drawbacks appeared and the applicability of microinjection as tool for the development of sound dose-response relations stays problematic.

Third, the assessment of POPs in herring and eel has revealed the importance for monitoring contaminants that have already been banned in Europe but are still present in remarkable concentration in marine biota. A reduction of these contaminants is only possible when existing measures taken in the EU will be expanded to a world-wide scale.

Additionally, the introduction of gonad burden as indicator for reproductive success into the current monitoring may be promising to assess the health of marine wildlife. An organ-specific distribution of POPs was shown to occur in Baltic herring and European eel and maternal pollutant transfer seems to be evident in wild living species as was shown in hormone treated eel. However, there are still open questions concerning the risk of impaired reproduction after the contamination of gonads and eggs. This still offers scope for future investigations.

The implementation of given recommendations are best approachable by a close cooperation of the three main parts of marine risk assessment, which are monitoring, screening, and the development of proper assessment criteria.

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Optimal test design for binary response data: the example of the fish embryo toxicity test

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This appendix summarizes the main formulae that are proposed as part of the design procedure and are also incorporated in the R package.

The basic data in a concentration-response experiment is the number n of examined objects and the number r of responding objects. The empirical response rate is the ratio $p = r/n$. It is frequently expressed as a percentage by multiplying p by 100. This appendix, however, uses only rates, not percentages, for easier notation.

A CI is a way to express how precisely the response rate p_{true} of the whole population is estimated by an empirical rate. A confidence interval $\text{CI} = (p_{\text{low}}, p_{\text{high}})$ for an empirical rate is obtained from observed n and r by [Johnson and Kotz, 1969]:

$$p_{\text{low}} = \frac{r \cdot F_{2(n-r+1), 2r, 1-\alpha/2}}{r + (n-r+1) \cdot F_{2(n-r+1), 2r, 1-\alpha/2}}$$

$$p_{\text{high}} = \frac{(r+1) \cdot F_{2(r+1), 2(n-r), 1-\alpha/2}}{n-r+(r+1) \cdot F_{2(r+1), 2(n-r), 1-\alpha/2}}$$

The F terms in both equations are the quantiles of the F distribution with degrees of freedom and associated probability as given in the subscripts. The value of α controls the coverage probability. The calculated interval contains the value p_{true} , which holds for the whole population under study, with probability $1 - \alpha$. This statement must be understood in a strategic sense: If the actual experiment is replicated many times and the CI is calculated for each replicate, then the population rate p_{true} will be contained in $(1 - \alpha) \cdot 100\%$ of the calculated CIs.

The equation for the CI makes use of the fact that the number r of responses has a binomial distribution, which means that the probability of observing r responses among n examined subjects is as follows:

$$\Pr(r, n, p_{\text{true}}) = \binom{n}{r} \cdot p_{\text{true}}^r \cdot (1 - p_{\text{true}})^{n-r}$$

Different concentrations x_i of a substance generate their specific $p_{\text{true}}(x_i)$ values. A concentration-response (or dose-response) curve relates the probability p_{true} to the dose x involved. This relation will always be a non-linear one, because the concentration or dose may be any value ≥ 0 and the associated p_{true} must lie in the interval $[0, 1]$. The present proposal uses the fourparameter logistic curve as concentration-response curve:

$$p_{\text{true}}(x) = SL + \frac{1 - SL - IY}{1 + e^{-(a + b \cdot x)}}$$

The terms a and b in this equation control location and scale (slope) of the concentration-response relation. The values for these terms are estimated from I experiments with doses x_i and numbers (n_i, r_i) of examined and responding objects by a maximum likelihood approach by maximizing

$$\log l(a, b | n, r, x) = \sum_i^l n_i \cdot \log p_{\text{true}}(a, b | x) + (n_i - r_i) \cdot \log(1 - p_{\text{true}}(a, b | x))$$

The optimization is done iteratively by a NewtonâRaphson approach. EC_{xx} is obtained while using the estimates for (a, b) by

$$EC_{xx} = -\frac{1}{b} \left[\ln \left(\frac{1-SL-IY}{xx-SL} - 1 \right) + \alpha \right]$$

All calculations listed here are contained in the R package described.

Maternal transfer of emerging brominated and chlorinated flame retardants in European eels

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Table S.1: Biodata of hormone treated eels.

Nr.	Habitat	M after egg removal (g)	M before egg removal (g)	Length (cm)	Liver (g)	M gonads (g)	Eye \varnothing (mm)	Stage (s.i.)	Pectoral fin length (mm)	Lipid (%)	Sex
VV1	Schlei	669	1177	80	14.3	161	12.5	4	33.3	36	f
VV2	Ems	1200	1385	81	18.4	558	14.4	4	37.9	19	f
VV3	Ems	377	584	65	6.4	85	11.4	5	35.9	20	f
VV4	Ems	696	1073	79	16.6	126	12.2	4	43.9	17	f
VV6	Schlei	328	567	63	6.38	131	12.6	5	37.9	19	f

Table S.2: Biodata of comparison group eels.

Nr.	Habitat	M (g)	Length (cm)	Liver (g)	M gonads (g)	Eye \varnothing (mm)	Pectoral fin length (mm)	Stage (s.i.)	Lipid [%]	Sex
1637	Ems	627	68	11.0	8.6	10.20	28.50	5	23	f
1640	Ems	753	76	10.2	10.5	9.80	40.30	5	31	f
1644	Ems	611	70	12.1	9.3	9.60	38.00	5	20	f
1645	Ems	875	74	14.9	15.4	10.50	41.80	5	27	f
1651	Ems	553	65	11.2	6.8	8.05	38.00	5	31	f
1748	Schlei	332	65	5.4	1.5	6.50	24.30	2	25	f
1749	Schlei	578	71	8.0	9.0	9.15	29.40	3	15	f
1750	Schlei	766	69	9.7	12.3	9.40	36.70	5	26	f
1751	Schlei	627	70	8.4	10.0	8.00	32.50	3	23	f
1752	Schlei	474	62	6.9	7.3	10.15	31.60	5	22	f

Material and Methods

Sample collection and holding conditions

Between October and November 2012 female eels were caught in German waters (River Ems, Schlei Fjord) with fyke nets at the onset of spawning migration. They were kept in freshwater up to 10 weeks before the start of the experiment.

Six females were transferred to a recirculation system and acclimatized to experimental conditions for another 2 weeks. The recirculation system consisted of a 1000L circular tank in which females were kept in a moderate circular current, and another 500L water reservoir. It was equipped with a trickle filter for mechanical filtration and denitrification and with aquarium bubblers for the supply of oxygen.

Water temperature varied between 15.4 °C and 22.0 °C during weeks 1-11. From week 11 onwards temperature was controlled by a heating-cooling unit (Titan 4000, Aqua Medic, Bissendorf, Germany) and kept between 18.1 °C and 18.8 °C until week 17. Afterwards temperature was increased and varied between 21.4 °C and 22.2 °C for the remaining time of the experiment. Salinity was kept constantly between 34 and 37.

Artificial maturation

Salmon pituitary extract (SPE) (Argent Aquaculture, Redmond, USA) in aqueous solution was injected at a dose of 20 mg kg⁻¹ body weight once a week into the dorsal muscle of eels. Prior to injections all individuals were anaesthetised with 2-Phenoxyethanol (Carl Roth, Karlsruhe, Germany). They were weighted and total length (LT) and body girth (BG) was measured to calculate the Body Girth Index (BGI = BG LT⁻¹) [Palstra and Thillart, 2009]. From week 16 eggs were removed by biopsy and staged according to Palstra & Van den Thillart [2009] to document oocyte maturation. As soon as body weight and BGI increased significantly final oocyte maturation and ovulation was induced by an additional SPE injection (20 mg kg⁻¹) after 48 hours and an intraperitoneal injection of 2 mg kg⁻¹ 17, 20/3-dihydroxy-4-pregnen-3-one (DHP) (Sigma-Aldrich, St. Louis, USA) another 10 hours later. In case of two eels the additional SPE injection was waived because egg development was already advanced. One eel did not respond to the SPE treatment after 22 weeks and neither the additional SPE nor the DHP injection was applied. 13 to 16 hours after the DHP injections eggs were striped and gonads and muscle tissue were removed. Egg, gonad and muscle samples were stored in aluminium foil at -20 °C.

Extraction and clean-up

The frozen egg, muscle, and gonad samples were homogenised with anhydrous Na₂SO₄ (Merck) using a stainless steel/glass 1 L laboratory blender (neoLab Rotorblender). All samples were spiked with mass labelled surrogate standards ¹³C-BDE-28,

^{13}C -BDE-47, ^{13}C -BDE-99, ^{13}C -BDE-153, ^{13}C -BDE-183, ^{13}C -MeOBDE-47, ^{13}C -MeOBDE-100, ^{13}C -HBB, ^{13}C -synDP, and ^{13}C -PBBz.

Extraction was performed by accelerated solvent extraction with dichloromethane (DCM) as solvent, using the method described in Sühling et al. 2013. Extracts were purified by gel permeation chromatography (GPC) and silica gel clean-up as described by Sühling et al. 2013. Finally, each 500 pg (absolute) ^{13}C -PCB-141 and ^{13}C -PCB-208 was added as an injection standard to each sample. The lipid content of samples was determined gravimetrically from separate aliquots.

Instrumental analysis

For analysis in EI mode a Restek 1614 column (15m x 0.25 mm i.d. x 0.10 μm film thickness, Restek) and a restriction capillary (0.8m x 0.1 mm i.d., deactivated) was used with Helium (purity 99,999%) as carrier gas and a constant column flow of 2.5 mL/min. The injector was operated in pulsed-splitless mode (injection pulse 25 psi for 2 min) with an inlet temperature program: 60 °C for 0.3 min, 300 °C min^{-1} until 280 °C and held for a final 10 min. The GC oven program was as follows: initial 60 °C for 1 min, 10 °C min^{-1} until 280 °C and held for 10 min, 40 °C min^{-1} until 300 °C and held for 2 min. A 5 min backflush was conducted as post-run at 300 °C with a flow of 5.1446 mL/min to reduce analysis time and increase working life of the column.

The instrument was operated in multiple reactions monitoring mode (MRM) at 70 eV. The mass range was scanned from 70 to 900 m/z at 1 s/scan for the full-scan mode. General parameters for MRM were as follows: Gain factor 50, filament current 35 μA , dwell time 50 ms. The MS transfer line was held at 280 °C, the ion source temperature was 230 °C and quadrupole temperatures were 150 °C. In the collision cell Nitrogen was used as collision gas at a flow of 2.25 mL/min and Helium as quench gas at 1.5 ml/min.

Samples were analysed for nine PBDEs (BDE-28, -47, -66, -85, -99, -100, -153, -154, -183), eight methoxylated PBDEs (5MeOBDE-47, 6MeOBDE-47, MeOBDE-49, -68, -99, -100, -101, -103), twenty four alternate BFRs (2,4,6-tribromophenol (2,4,6-TBP), 2,4,6-tribromophenyl allylether (TBP-AE), 2-bromoallyl 2,4,6-tribromophenyl ether (BATE), 1,2-bis(2,4,6-tribromophenoxy)ethane (BTBPE), Decabromodiphenylethane (DBDPE), 2,3-dibromopropyl-2,4,6-tribromophenyl ether (TBP-DBPE), 2-ethyl-1-hexyl 2,3,4,5-tetrabromobenzoate (EH-TBB), Hexabromobenzene (HBB), Hexachlorocyclopentadiene (HCCPD), Hexachlorocyclopentadienyl-dibromocyclooctane (DBHCTD), Pentabromobenzyl acrylate (PBBA), Pentabromobenzylbromide, 1-bromoethyl-2,3,4,5,6-pentabromobenzene (PBBB), Pentabromobenzene (PBBz), Pentabromoethylbenzene (PBEB), Pentabromotoluene (PBT), Tetrabromo-p-xylene (TBX), 2,4,6-tribromoanisole (TBA), Tris-(2,3-dibromopropyl) isocyanurate (TBC), Tetrabromo-o-chlortoluene (TBCT), Tetrabromophthalic anhydride (TEBP-Anh), Bis(2-ethyl-1-hexyl)tetrabromophthalate (TBPH), α/β -tetrabromoethylcyclohexane (α/β -DBE-DBCH), α/β -1,2,5,6-tetrabromocyclooctane (α/β -TBCO)), Dechlorane Plus (DP), the one- and two-fold dechlorinated DP species

(aCl11DP [-1Cl+1H], aCl10DP [-2Cl+2H]), DPMA, and Dechlorane 602, 603 and 604 (see Table 1 for MS/MS parameters and instrumental detection limits).

NCI analysis was based on a method developed by Möller et al. [2010]. The method was extended to include further analytes and a backflush system. The instrument operated in single ion monitoring mode (SIM) with methane as reactant gas. It was fitted with a HP-5MS column (30m x 0.25mm i.d. x 0.25 μm film thickness, JW Scientific) and a restriction capillary (0.8m x 0.1 mm i.d., deactivated). The injector was operated in pulsed-splitless mode (injection pulse 20 psi for 2 min) with an inlet temperature program: 60 °C for 1 min, 500 °C/min until 28 °C and held for a final 20 min. The GC oven program was as follows: initial 60 °C for 2 min, 30 °C/min until 180 °C, 2 °C/min until 280 °C, 30 °C/min until 300 °C and held for 5 min. A 15 min backflush was conducted as post-run at 300 °C with a flow of 5.1446 mL/min. General parameters for SIM were as follows: Gain factor 50, filament current 35 μA , dwell time 50 ms. The MS transfer line was held at 280 °C, the ion source temperature and quadrupole temperatures were 150 °C. In NCI eels were analysed for 14 alternate BFRs (TBP-AE, PBB, TBCT, BATE, PBEb, PBT, HBB, TBP-DBPE, PBBA, DBHCTD, EH-TBB, BTBPE, TBC and TBPH), syn and anti-DP, aCl11DP, aCl10DP, 1,5-DPMA, Dechlorane 602, 603 and 604, as well BDE-66, BDE-100 and BDE-154.

Fish egg toxicity test

Due to the high concentrations found in all analysed tissue types and the lack of information regarding the toxicity of TBA a fish embryo test was performed for zebrafish (*Danio rerio*). The solubility limit was reached at 10 $\mu\text{g mL}^{-1}$ as highest test concentration. An effect of TBA on the development of zebrafish was not observed after 96 h indicating that the LC50 is greater than the solubility limit of TBA in DMSO. To be able to assess the effect of TBA on the European eel, a standardized production of fertilized eel eggs is needed to perform additional methods, such as nano-injections and tests on chronic effects.

Maintenance and egg production of Zebrafish

Wild-type Zebrafish brood stock was held in breeding groups of about 20 females and 30 males in the facilities of the Thuenen Institute of Fisheries Ecology in Hamburg, Germany. Fish were kept in three glass aquaria (160 L) at 26 °C \pm 2 °C and a light/dark period of 14 h/ 10 h in tap water. Water quality was maintained by external bioactive filter devices. Filter material and aquarium water were changed twice a week. Fish were fed ad libitum twice a day with dry flake food (Tetramin, Tetra Werke, Melle, Germany). Embryos were obtained from mass spawning and collected 30 minutes after the light was switched on. Eggs were rinsed with aquarium water and staged in accordance with Kimmel et al. [1995] under an inverted microscope.

Egg quality and validation criteria

In accordance with the OECD Guideline for fish-egg assays with Zebrafish embryos [OECD, 2013a] only eggs from spawns with a fertilization rate higher than 70% were used for the test. Additionally, the general test design was supported by the R-package 'toxtestD' [Keddig and Wosniok, 2014; Keddig, Schubert, and Wosniok, 2015]. Accordingly, the spontaneous lethality (SL) of the fish breed was determined as a measure of egg quality. In sterile 24-well plates embryos were kept in groups of five eggs per 1mL autoclaved tap water under standard test conditions ($26^{\circ}\text{C} \pm 2^{\circ}\text{C}$, 14h/10h light/dark period) without the influence of any toxicant for 96 hours. In nine independent test runs SL was found to be 3.05%.

Test procedure

The Fish Embryo Toxicity (FET) test was conducted according to the recommendations of the OECD Guideline for fish egg assay with Zebrafish embryo [OECD, 2013a] with minor modifications. We used 60 instead of 20 eggs per treatment and control, respectively. Stock solution of 1 mg TBA/mL was prepared in DMSO. Nominal test concentrations were 0.01 $\mu\text{g}/\text{mL}$, 0.1 $\mu\text{g}/\text{mL}$, 1 $\mu\text{g}/\text{mL}$, 10 $\mu\text{g}/\text{mL}$. Individuals were checked 48 and 96 hours post fertilization (hpf) for coagulation, lethal malformation such as non-detachment of tail, lack of heart beat or somite formation, and sub-lethal malformations as e.g. edema, spinal curvatures, and eye deformations.

Mutagenicity, Gentoxicity test

Genotoxic and mutagenic effects of DDC-DBF

So far no information describing toxic effects such as mutagenicity or genotoxic effects of DDC-DBF in concentrations relevant for eels at different stages of development were available. Considering the repeated detection of this compound throughout different life stages as well as habitats of eels (Sühring et al. 2014), mutagenic and genotoxic effects of DDC-DBF were tested. The test concentrations were based on concentrations measured in different life stages of eels.

Neither genotoxic effects of DDC-DBF from 0.01 to 10 ng ml^{-1} , which corresponds to these of the different development stages of eels, were detected in the umuC test nor mutagenic effects was obtained with the Ames fluctuation test in the investigated concentration.

In the umuC test the Induction Ratio (IR) were between 0.88 and 1.10 independent of the test concentration or the presence or absence of S9-mix. The growth factors were between 0.91 and 1.07. Therefore these values were similarly to the negative control and a cytotoxic effect of the compound could be excluded. In the Ames fluctuation test the highest increase over the baseline was 0.99 at 0.1 ng ml^{-1} DDC-DBF for TA98 and 1.1 at 5 ng ml^{-1} DDC-DBF for TA100. Therefore all values were under the threshold of

2.

Because of the low tested concentration it cannot be fully excluded that at higher test concentrations DDC-DBF may have DNA damaging potential thereby posing a risk for other environmental species. However, the substance does not seem to have a DNA damaging potential in concentrations similarly to these found in different development stages of eels.

Further investigations, especially concerning potential endocrine disrupting properties and fish egg toxicity, are necessary to assess the potential adverse effects of this highly bioaccumulative compound on eels and other biological species.

A stock solution of DDC-DBF (1 mg/ml) was sterile filtered and stored at -20 °C until using. The Dec-602 solution was diluted with sterile ultrapure water to the required test concentration and the pH was adjusted to 7 ± 0.2 before testing. Every test was conducted under sterile conditions.

umuC-test (ISO 13829)

The umuC-test was used to evaluate the genotoxic effects of DDC-DBF and was done according to ISO 13829 [DIN EN ISO, 2000]. In brief, the test was performed with and without metabolic activation system. Aroclor-1254 induced rat liver homogenate were obtained from Xenometrix AG, Switzerland. *Salmonella typhimurium* TA1535/pSK 1002 were obtained from the DSMZ (German collection of microorganisms and cultures, Braunschweig, Germany.)

At first, the bacteria were cultivated over night until 800 FNU (approx. $OD_{600} \geq 1.0$). Then the culture was diluted ten times with 1x TGA medium and incubated for an additional growth until approx. 350 FNU (~ 1.5 h). Afterwards Dec-602 (final concentrations from 10 to 0.01 ng/ml) was incubated with fresh medium, bacterial solution and in the absence and presence of S9 mix on a 96-well plate for 2 h (37 °C, 250 rpm). After the incubation time an aliquot of each sample was transferred to a new 96-well plate and incubated with fresh 1x TGA medium for additional 2 h (37 °C, 250 rpm) followed by the measurements of the optical density (OD_{600}). An aliquot of the test solutions was transferred to a third 96-well plate and was incubated (30 min, 28 °C) together with ONPG (o-Nitrophenyl- β -D-galactopyranoside)-solution, the substrate for the β -galactosidase and B-buffer. Afterwards the β -galactosidase activity was measured at OD_{420} by the release of the yellow colored product of the enzymatic reaction, o-nitrophenol. During all incubation steps the plates were covered with aluminum foil to avoid any changings of the samples from natural light. 4-Nitroquinolineoxide (4-NQO, final conc.: 0.05 μ g/ml, -S9) and 2-Aminoanthracene (2-AA, final conc.: 0.25 μ g/ml, +S9) were used as positive controls.

The growth factor (G), the β -galactosidase activity (UT) and the Induction Ratio (IR) were calculated according to the ISO guideline [DIN EN ISO, 2000]. The test substance

was classified as genotoxic, if the IR was ≥ 1.5 and a clear concentration-response relationship was observed. Validity criteria was according to the ISO guideline: (1) IR of the positive control >2.0 ; (2) G of all samples > 0.5 ; (3) minimum growth in the negative control should be 140 FNU.

Ames fluctuation test (ISO 11350)

The Ames fluctuation test was prepared with TA 98 and TA 100 and in accordance with ISO 11350 [DIN EN ISO, 2012]. S9-mix (Aroclor-1254 induced rat liver homogenate), bacterial strains, exposure medium and reversion indicator medium were obtained from Xenometrix AG, Switzerland. Growth medium were prepared according to the ISO guideline [DIN EN ISO, 2012].

The bacterial solution were cultivated overnight (37 °C, 250 rpm) and diluted with exposure medium to a final density of 1800 FAU (OD600 2.4) for TA 98 and 450 FAU (OD600 0.6) for TA 100. The bacteria were transferred to a 24-well plate and were exposed with different DDC-DBF concentrations (10.00 - 0.01 ng/ml) for 100 min (37 °C and 250 rpm). After incubation the half volume of the samples were transferred to a new 24-well plate and the OD600 was measured to calculate the cytotoxicity of DDC-DBF in accordance with ISO 11350 [ISO, 2012]. Afterwards the reversion indicator medium was added in each well of the 24-well plate and the samples were transferred to 384-well plates. During the following incubation time for 48 h (37 °C) the pH indicator in the reversion indicator medium change the color if metabolic active bacterial colonies are present in the wells.

For the analysis at first the number of negative wells (purple colored) and positive wells (yellow colored) were scored. The baseline (mean of NC \pm standard derivation of NC) of each bacterial strain was calculated. Afterwards the fold increase over the baseline was calculated by the mean number of positive wells for the each sample divided by the negative control. The test substance was classified as mutagenic if the fold increase over the baseline was ≥ 2 and a significant concentration-response was determined.

The test was valid if the mean of positive wells for the negative controls was ≥ 0 and ≤ 10 wells per 48 well area and the mean for the positive control was ≥ 25 wells per 48 well area. A mixture of 4-NQO (final conc.: 0.125 $\mu\text{g}/\text{ml}$) and 2-Nitrofluorene (final conc.: 2.5 $\mu\text{g}/\text{ml}$) were used as positive controls for testing without S9. 2-AA in a final concentration of 4 $\mu\text{g}/\text{ml}$ was used as positive control for testing with metabolic activation.

Further supplemented material to this article can be found online at <http://dx.doi.org/10.1016/j.scitotenv.2015.05.094>.

Acknowledgements

Zu Beginn möchte ich mich bei PD. Dr. Reinhold Hanel für die Betreuung der Arbeit, deine Unterstützung, sowie für die Möglichkeit zur Promotion bedanken.

Im gleichen Zusammenhang möchte ich mich bei Prof. Dr. Oscar Puebla für die Begutachtung der vorliegenden Dissertation bedanken.

Besonders danken möchte ich Dr. Ulrike Kammann und Dr. Michael Haarich für die Betreuung und Unterstützung in den vergangenen Jahren und dafür, dass ich meine Promotion im Rahmen des Projektes "Methoden zur Erfassung und Bewertung der Risiken von toxischen Schadstoffen im marinen Ökosystem im Rahmen der Umsetzung der Meeresstrategie-Rahmenrichtlinie" anfertigen konnte.

Des Weiteren danke ich allen Projektmitarbeitern und -partnern für die gute Zusammenarbeit, sowie die anregenden Fachgespräche und konstruktiven Kritiken. Besonders dir, Nadia danke ich für die wunderbare Zusammenarbeit während der gesamten Zeit sowie die immerwährende Unterstützung in den schwierigen Phasen. Danke für deine Freundschaft und all die kleinen und großen Ablenkungen rund um meine Dissertation.

Allen Mitarbeitern des Bibliotheksteams danke ich für die stets herzliche und schnelle Unterstützung. Besonders bei Heike möchte ich mich bedanken, für die zahlreichen gemeinsamen Waffelevents, die Ausflüge in die Welt der Literatur und des Tees und für dein immer offenes Ohr.

Ebenso möchte ich mich bei meinen Kollegen vom Thünen-Institut für Fischereiökologie für das angenehme Arbeitsklima und ihre Unterstützung bedanken. Helga Wolter, Dagmar Korte, und Jan Neukirchen danke ich besonders für die Unterstützung bei der Schadstoffanalytik. Danke auch für die anregenden Fachgespräche und zahlreichen kleinen Hinweise und Tipps. Im gleichen Zusammenhang möchte ich mich bei Elke Hammermeister und Wolfgang Gerwinski vom Bundesamt für Seeschifffahrt und Hydrographie bedanken. Danke auch an Dr. Thomas Lang für die Möglichkeit an einer Seereise mit dem Forschungsschiff Walther Herwig III teilzunehmen und die Proben für die Schadstoffanalytik selber an Bord zu bearbeiten.

Besonderer Dank gilt Vicky und Malte für eure vielen hilfreichen Anmerkungen und Kommentare beim Zusammenschreiben der Arbeit sowie eure aufmunternden Worte in dieser Zeit. Malte, dir möchte ich besonders für die herzliche Aufnahme in deinem Büro danken, für Kaffee und Schokolade, sowie für zahlreiche Gespräche über Gott und die Welt.

Den Bergedorfer Fußballjungs danke ich für die vielen Traumtore und -pässe während unserer gemeinsamen Spiele und der anschließenden biergetrübten Spielauswertung

im DIMA-Sportzentrum in Bergedorf. Besonderer Dank gilt Jorg und Niels, die mich darüber hinaus so schnell aus der Steckdosen-Misere befreit haben.

Bei Gertrud und Sakis vom Institut für Seefischerei bedanke ich mich für eure Hilfe rund um die Altersbestimmung der Heringe. Danke auch an Christina und Birgit vom Institut für Seefischerei für die herzliche Aufnahme in eurem Büro und die Möglichkeit dort ungestört am Mikroskop zu arbeiten. Für gemeinsames Grillen am Teich und dessen Organisation danke ich Eckhart, Sakis, Christian, Thilo, Timo und Jule, ebenfalls vom Institut für Seefischerei.

Mein besonderer Dank an meine Eltern für all eure Unterstützung, Rückhalt, Vertrauen und Geduld in den letzten Jahren! Ohne Euch wäre das alles hier nicht möglich gewesen. Vielen Dank auch an meine Familie und Freunde. Danke an Marcel, dass du immer eine Quelle der Erholung und des Ausgleichs bist.

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List of Publications

The chapters of this thesis are either published (Chapter I, II, IV) or submitted for publication (Chapter III) to peer-reviewed scientific journals. Journal-specific format styles were applied to the published and submitted manuscripts and may vary to the format used in this thesis. The individual chapter references are combined in the final references of this thesis.

Chapter I: Optimal test design for binary response data: the example of the Fish Embryo Toxicity Test.

Authors: Nadia Keddig, Sophia Schubert, Werner Wosniok

Published in *Environmental Sciences Europe* 2015, 27:15. doi: 10.1186/s12302-015-0046-5

Chapter II: Microinjection into zebrafish embryos (*Danio rerio*) - a useful tool in aquatic toxicity testing?

Authors: Sophia Schubert, Nadia Keddig, Reinhold Hanel, Ulrike Kammann

Published in *Environmental Sciences Europe* 2014, 26:32 doi: 10.1186/s12302-014-0032-3

Chapter III: Contaminants in *Clupea harengus* - an aspect of gender.

Authors: Sophia Schubert/Nadia Keddig, Wolfgang Gerwinski, Jan Neukirchen, Ulrike Kammann, Michael Haarich, Reinhold Hanel, Norbert Theobald

Submitted for publication to *Archives of Environmental Contamination and Toxicology*

Chapter IV: Maternal transfer of emerging brominated and chlorinated flame retardants in European eels.

Authors: Roxana Sühling, Marko Freese, Mandy Schneider, Sophia Schubert, Jan-Dag Pohlmann, Mehran Alaei, Hendrik Wolschke, Reinhold Hanel, Ralf Ebinghaus, Lasse Marohn

Published in *Science of the Total Environment* 2015, 530-531:209-218, doi: 10.1016/j.scitotenv.2015.05.094

Contribution of Authors

The following list clarifies my personal contribution to the manuscripts presented in this thesis.

Chapter I: Optimal test design for binary response data: the example of the Fish Embryo Toxicity Test.

Nadia Keddig devised and realized the R package '*toxtestD*' and wrote the manuscript. Sophia Schubert has participated in the conception and design of the approach, performed all test runs (computer based as well as the FET), and has been involved in drafting the manuscript. Werner Wosniok has made substantial contributions to the conception and design of the R package and revised the manuscript critically for important intellectual content. All Co-Authors substantially contributed to the finalization of the manuscript prior to submission.

Chapter II: Microinjection into zebrafish embryos (*Danio rerio*) - a useful tool in aquatic toxicity testing?

Sophia Schubert designed the study, performed all laboratory tests, statistical analysis, and wrote the manuscript. All Co-Authors substantially contributed to the finalization of the manuscript prior to submission.

Chapter III: Contaminants in *Clupea harengus* â an aspect of gender.

Sophia Schubert and Nadia Keddig designed and coordinated the study, performed sampling in 2012, sample preparation, and selection. Wolfgang Gerwinski assisted with the HPLC-MS-MS; Jan Neukirchen performed GC-MS and GC-ECD. Nadia Keddig performed data evaluation and analysis and wrote the corresponding parts of the manuscript. Ulrike Kammann has been involved in drafting the manuscript and revising it critically for important intellectual content. Sophia Schubert was involved in data evaluation and wrote most parts of the manuscript. All Co-Authors substantially contributed to the finalization of the manuscript prior to submission.

Chapter IV: Maternal transfer of emerging brominated and chlorinated flame retardants in European eels.

The study was designed by Roxana Sührling and Mehran Alaei and primarily executed by Roxana Sührling. Marko Freese and Jan-Dag Pohlman performed sampling, and assisted with sampling concept. Sophia Schubert and Mandy Schneider designed and performed laboratory toxicity tests and wrote toxicological tests section in the

manuscript. Lasse Marohn performed artificial maturation of eels and wrote the corresponding section in the manuscript. Mehran Alaei participated in data evaluation. Roxana Sühling wrote the manuscript. All Co-Authors substantially contributed to the finalization of the manuscript prior to submission.

List of Abbreviations

AFs	Assessment Factors
CdCl₂	Cadmium (II) Chloride
CIs	Confidence Intervals
D	Descriptors
EACs	Environmental Assessment Criteria
EC	effective concentration
EQS	Environmental Quality Standards
EU	European Union
FET	Fish Embryo Toxicity test
GES	good environmental status
h	hours
HC₅	5th percentile of the SSD, no adverse effect in 95% of tested species
HELCOM	Helsinki Commission
hpf	hours post fertilization
K_{ow}	octanol-water partition coefficient
LC	lethal concentration
MERIT-MSFD	Methods to measure and Evaluate the Risk of Toxic pollutant in the marine ecosystem in relation to the Marine Strategy Framework Directive
MSFD	Marine Strategy Framework Directive
MSRL	Meeresstrategie Rahmenrichtlinie
OECD	Organisation for Economic Co-operation and Development
OSPAR	Oslo and Paris commission
PBDEs	Polybrominated diphenylethers

PCBs	Polychlorinated biphenyls
PFOSs	Perfluorootane sulfonate
POPs	Persistent Organic Pollutants
RSCs	Regional Sea Conventions
SL	Spontaneous Lethality
SSD	Species Sensitivity Distribution
TBA	2,4,6 -Tribromoanisol
toxtestD	R Package: Experimental design for binary toxicity tests
WFD	Water Framework Directive

Eidesstattliche Erklärung

Hiermit erkläre ich, dass die vorliegende Dissertation selbstständig von mir angefertigt wurde. Die Dissertation ist nach Form und Inhalt meine eigene Arbeit und es wurden keine anderen als die angegebenen Hilfsmittel verwendet. Diese Arbeit wurde weder ganz noch zum Teil einer anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegt. Die Arbeit ist unter Einhaltung der Regeln guter naturwissenschaftlicher Praxis der deutschen Forschungsgemeinschaft entstanden. Dies ist mein einziges und bisher erstes Promotionsverfahren. Die Promotion soll im Fach Biologische Meereskunde erfolgen.

Sophia Schubert:

Kiel, den: 01.12.2015
