Impacts of chemical pollution during the continental life of the European eel (*Anguilla anguilla* L.)

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Für meine Familie

Mephistopheles (Goethe, Faust I, 117):

„Das kommt nur auf Gewohnheit an.
So nimmt ein Kind der Mutter Brust
nicht gleich im Anfang willig an;
doch bald ernährt es sich mit Lust.
So wirds euch an der Weisheit Brüsten
mit jedem Tage mehr gelüsten.“

In Gedenken an Dr. Beate Engling

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SUMMARY

The stock of European eel (*Anguilla anguilla* L.) is considered critically endangered due to severe decline in recruitment in the 1970s. Reasons for the decline are still not fully understood. It is noteworthy, however, that around the same period of time, also the stocks of American eel (*Anguilla rostrata)* and Japanese eel (*Anguilla japonica*) were affected by steep drops in numbers of young-of-the-year eels. Scientists now broadly accept the perception, that multiple stressors are simultaneously in effect and that the situation for the eel stocks was most likely not caused monocausal but additive. Besides fishing pressure on all continental life stages, habitat degradation, mortality caused by hydropower plants, oceanic changes due to climate change, parasites and diseases, also toxic pollution is considered as a possible factor, which may have contributed to the current situation. Until today, no main driver among the discussed reasons has been identified and the contributing share of each known impact to the stock situation is still difficult to assess.

To counteract the observed decline and aid the recovery of the stock, the European Union introduced management plans on an international scale. Management actions under the under EU-Regulation 1100/2007 aim at a long-term increase in escapement of descending silver eels to the sea, supposed to constitute of at least 40% of the number of eels of a given population, without any anthropogenic mortality. This target was set in order to secure a sustainable spawning stock biomass leading to sufficient reproduction and recruitment. Recommended management options to achieve this goal comprise actions such as regulation of fisheries, restoration and passability of habitats and also the translocation of young, wild caught eels into habitats with low natural recruitment. However, no specific policies or regulations have yet been adopted to foster the quality and condition of potential spawners, or to protect designated stocking material from being transferred into contaminated environments even though the majority of inland waters in central Europe are still exceeding critical thresholds for a good chemical status. In a number of connected studies, it was investigated in this dissertation if, how and to what extent eels during their continental lives are affected by different types of chemical contamination mediated through their growth habitats. Additionally, it was experimentally examined and discussed if and how this may influence an eel's individual reproductive success. Findings of this thesis suggest that the quality of a selected habitat in terms of contamination burden has the potential to directly influence the reproductive capacity of a local eel population. Accordingly, the practice of stocking and reallocation of young eels as stock enhancement measures should only be performed in suitable water bodies or sections, provided that these actions prove to actually lead to an increased survival compared to in their original habitats.

ZUSAMMENFASSUNG

Seit einigen Jahrzehnten verfestigt sich die Sorge um den Bestand des Europäischen Aals (*Anguilla anguilla*). Das Maß der Rekrutierung, also der Menge der an den Küsten Europas und Nordafrikas ankommenden Glasaale, dient hierbei als Kenngröße für die Nachwuchssituation der Art. Noch immer ist nicht vollständig verstanden, aus welchen Gründen die Zahlen ankommender Glasaale des Europäischen Aals seit Ende der 1970er Jahre so dramatisch eingebrochen sind. Bemerkenswert ist die Tatsache, dass etwa im gleichen Zeitraum auch die Rekrutierungszahlen für den Amerikanischen Aal (*Anguilla rostrata*) und den pazifischen Japanischen Aal (*Anguilla japonica*) ähnlich starke Rückgänge zu verzeichneten. Mittlerweile herrscht unter Wissenschaftlern weitestgehend ein Konsens darüber, dass nicht eine Ursache alleine, sondern eher eine Verkettung mehrerer gleichzeitig wirkender Faktoren zu diesem Negativtrend beigetragen haben muss. Neben der Befischung aller kontinentalen Lebensstadien werden auch Zerstörung der Lebensräume, Sterblichkeit verursacht durch Wasserkraftturbinen, klimabedingte Ozeanveränderungen, verschiedene Krankheiten & Parasitierung aber auch Umweltverschmutzung durch toxisch wirkende Chemikalien als negativ wirkende Faktoren auf die Bestände der genannten Arten geführt. Bis heute ist es schwierig diese Faktoren zu quantifizieren oder gar zu gewichten, weshalb noch keine Hauptursache für die Bestandseinbrüche identifiziert werden konnte.

Um der negativen Bestandsentwicklung beim Europäischen Aal entgegenzuwirken, wurden in Europa mit der EU-Verordnung 1100/2007 verschiedene Maßnahmen ins Leben gerufen, welche zur Wiederauffüllung des Bestandes beitragen sollen. Ein definiertes Ziel dieser Maßnahmen ist eine Erhöhung der Abwanderung von Blankaalen auf mindestens 40% der angenommenen „ursprünglichen“ Menge, ohne anthropogenen Einfluss. Neben Einschränkungen der Fischerei oder der Wiederherstellung der Durchgängigkeit von verbauten Gewässern ist auch das Besetzen von andernorts gefangenen Glas- und Steigaalen in Regionen mit vergleichsweise niedriger natürlicher Rekrutierung eines der empfohlenen Mittel um dieses Ziel erreichen zu können. Bisher gibt es jedoch noch keine spezifischen Regelungen oder Verordnungen, welche die Qualität der anwachsenden und später abwandernden Laichtiere sichstellt oder schlicht Besatzmaterial vor dem Ausbringen in kontaminierte Gewässer als Aufwuchshabitate schützen soll. In einer Reihe von verknüpften Einzelstudien wurde in dieser Dissertation untersucht, wie und in welchem Maße Aale während ihres kontinentalen Lebensabschnittes durch chemische Schadstoffe in ihren Aufwuchsgewässern beeinflusst werden und wie sich dies ihren individuellen Reproduktionserfolg auswirken könnte. Die Ergebnisse der hier zusammengetragenen Studien suggerieren, dass die Habitatqualität in Bezug auf Schadstoffbelastung durchaus das Potenzial besitzen könnte, einen verheerenden Einfluss auf die reproduktive Kapazität lokaler Aalpopulationen zu haben. Dementsprechend sollte das Umsetzen und Besetzen von jungen Aalen als bestandsfördernde Maßnahme höchstens in geeignete und möglichst unbelastete Gewässer oder Abschnitte durchgeführt werden, sofern gesichert werden kann, dass die Tiere dort eine höhere Überlebenswahrscheinlich bekommen als sie natürlicherweise in ihren Herkunftsgewässern gehabt hätten.

CONTENT

CONTENT

LIST OF FIGURES

PREFACE

According to a report published in 2019 by the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services (IPBES 2019), up to one million plant and animal species currently face extinction, mostly as a result of human activities. The analysis incorporated findings of almost 15000 studies and represents the first single unified statement from the world's governments on the topic. In this report, it is made clear how humankind has altered the Earth's ecosystems with devastating effects for its biodiversity and ecology. As never before, human-driven agricultural activities, but also the exploitation of wild plants and animals through logging, hunting and fishing have led to direct impact on abundance, habitat space and distribution of the world's wildlife. Also presented in the IPBES report are undirected, secondary effects caused by climate change, the spread of invasive species and chemical pollution, all adding up to today's alarming detrimental anthropogenic impacts on the environment.

One of the million threatened species is the critically endangered (IUCN Red List of Threatened Species) European eel *Anguilla anguilla* (Jacoby & Gollock, 2014). This teleost species exhibits a peculiar life cycle and is affected by multiple of the aforementioned anthropogenic impacts. This could be the reason why the eel has already been a showcase organism for the global changes caused by anthropogenic actions (Drouineau *et al.* 2018). The present thesis focusses on one of these impacts and investigates how chemical pollution affects the biology of the European eel, how it may have contributed to the decline of this species and how it possibly impedes its stock recovery by affecting the reproductive capacity of the species on a population and stock level.

GENERAL INTRODUCTION

Contamination and Pollution

Contamination can be defined as the presence of elevated concentrations of a substance or form of energy above the natural background level for the respective area and organism. Pollution, however, rather describes the introduction of a constituent such as chemical or biological matter or energy (e.g. heat, light or noise) into natural environments (Longcore & Rich 2004; Geissen *et al.* 2015; Goines & Hagler 2007; Grimm *et al.* 2008). Consequently, pollution can occur in different forms, originate from diverse sources and lead to different consequences. While the term almost exclusively stands in connection with anthropogenic activities causing adverse effects, some sources also use the term natural pollution, which can be caused for instance by the releases from volcanoes, forest fires and biological decay. Depending on its origin, several types of pollution can be distinguished such as land pollution, water pollution and air pollution. The latter types, from a historical perspective, have probably accompanied humankind already since its controlled use of fire in prehistoric caves several hundred thousand years ago (Spengler & Sexton 1983), while pollution as apparent nowadays, presumably started with anthropogenic activities alongside the industrial revolution in the late eighteenth century (Wijbenga & Hutzinger 1984; Crutzen 2002; Kampa & Castanas, 2008). Waste and wastewater problems associated with the steep population growth as well as air pollution caused by the almost unrestrained utilization of coal during that time could be regarded as the precursors of the issues connected with pollution today.

Chemical pollution of the environment

Chemical pollution can occur when synthetic or natural chemicals are discharged or accumulate to unnatural levels in the environment. Besides air pollution caused by toxic aerosols, for example byproducts of burning coal and oil, a number of technically produced compounds are also known to cause adverse health effects. Especially since World War II, synthetic chemical pollutants have accumulated in the environment, which affects food webs on a global scale, posing direct hazard to environmental as well as human health (Thornton 2000; El-Shahawi *et al.* 2010). Toxic chemicals can enter the environment through different sources: While point sources are single sites with specific origins of discharge such as factories or production sites, nonpoint or diffuse sources are

usually widespread and can include a variety of contaminants with different origins. Diffuse pollution is closely linked to land use, may combine contamination from multiple point sources and transport it due to various ways such as rainfall, water run-off, soil infiltrations or storms. Consequently, nonpoint sources (due to their complexity) are difficult to manage and release natural and human-made pollutants into lakes, rivers wetlands, coastal waters and ground waters. They thus constitute the single largest contributor of water pollution in the USA (Ribaudo *et al.* 1999) and probably most other parts of the world. Water pollution generally is regarded as a serious concern in modern society. Enormous amounts and numbers of different chemical pollutants, originating from various sources, have caused impairment of the quality of water and sediments in watersheds around the world with negative impacts on their ecosystem health (Schwarzenbach *et al.* 2006; Carpenter *et al.* 2011). Some of these compounds are extremely persistent and mobile and thus can today be found even in remote areas with low direct human influence. Due to the severity of this subject, modern-day chemical pollution has been assigned one of nine anthropogenic impacts of global relevance in the concept of planetary boundaries, proposed by a group of scientists in 2009 (Rockström *et al.* 2009a; Rockström *et al.* 2009b). Based on scientific evidence that anthropogenic activity has become the main driver of global environmental change, the proposing authors defined a "safe operating space for humanity" in order to showcase nine planetary life support systems and their boundaries. These boundaries are represented by global conditions: staying within them will allow for continued human survival and sustainable development, while exceeding them will put human existence at risk. But not only in this concept has pollution of the environment been acknowledged as one of the current major threats on Earth. Reduction and avoidance of pollution also constitute a central element among three of the seventeen goals included in the 2030 Agenda for Sustainable Development by the UN General Assembly. This agenda compiles targets to stimulate action in order to support the needs of the present and future generations of humanity and to protect our planet (UN SDGs 2015).

A large number of scientific studies and literature reviews have focused on chemical pollution with emphasis on its effects and interactions on whole ecosystems or on specific organisms in detail (Jones & Voogt 1999; Zala & Penn 2004; Halpern *et al.* 2008; Diamond *et al.* 2015). However, the scale and extent of effects caused by chemical pollution on the science-based limits of the Earth's system has yet to be determined (Rockström *et al.* 2009a, 2009b; Diamond *et al.* 2015). Possible control variables that could help estimate the natural planetary boundaries for chemical pollution were already

suggested in literature and include data on emissions, plastics, endocrine disruptors, heavy metals, nuclear wastes and concentrations or effects of persistent organic pollutants (POPs) (Diamond *et al.* 2015).

Different types of chemical pollution

Metals

Some metals and metalloids, sometimes inaccurately defined as heavy metals, are chemical elements with relatively high atomic weights and densities. Even though naturally present in the environment, metals can be concentrated and mobilized to the state of contamination, which is often caused by anthropogenic activities (Tchounwou *et al*. 2012). While trace amounts of some of these metals such as (among others) iron, copper, cobalt and zinc are required for certain biological processes, metals can also exert adverse effects and thus are noted for their potential toxicity. In biological systems, metals are able to affect cellular entities such as the cell membrane, mitochondria, lysosomes, endoplasmic reticulum, nucleic proteins and others, leading to impaired cell damage repair, DNA damage, carcinogenesis or apoptosis (Tchounwou *et al.* 2012). These "toxic metals" can bind to vital cellular components such as enzymes, structural proteins and nucleic acids, and interfere with their functioning by disrupting endocrine pathways (Landis *et al.* 2000).

Persistent organic pollutants

Persistent organic pollutants (POPs) are a loosely defined group of chemicals, often comprising different chemical "families". POPs have in common, that they can persist in soils, sediments, waste reservoirs, air or biota over long timeframes ranging from decades to centuries or even longer (Jones & Voogt 1999; Weber *et al.* 2008). POPs typically are halogenated and can be characterized as inert, rather stable and non-reactive towards hydrolysis or photolytic degradation. Their stability is rooted in the strong chemical bonds and physicochemical properties of their halogen substituent (F, Cl, Br, or I).

Many POPs represent a legacy from the uprising of industrial production techniques shortly before and after World War II, when thousands of synthetical chemicals were introduced into commercial use (Jones & Voogt 1999; El-Shahawi *et al.* 2010). Most of them, due to their chemical properties, were designed for particular purposes and showed beneficial characteristics useful for crop protection, pest control and industrial

applications. Others were produced unintentionally and can originate spontaneously from certain industrial or combustion processes (Bertazzi *et al.* 1998; Jones & Voogt 1999). POPs have the ability to partition between different phases and among environmental media, as they can evaporate from water and land into the air or adsorb to airborne particles and come back as snow, rain or dust (Wania & Mackay 1995; Jones & Voogt 1999). These properties make POPs widely distributed and subject to long-range air- or water-borne transport; even into areas with little direct anthropogenic influence (Jones & Voogt 1999; Halpern *et al.* 2008). Their typical chemical characteristics also give POPs a tendency to partition into solids, notably organic matter and avoiding the aqueous phase in aquatic systems.

Some of these chemicals have shown unexpected negative effects on the environment and health of animals (including humans) (Jones & Voogt 1999; Li *et al.* 2006; El-Shahawi *et al.* 2010) long after their first applications or introduction into the environment. Toxic effects associated with POPs reach from endocrine disruption, reproductive impairment to damage of the immune system, behavioral effects and cancerogenity (Bosveld & van den Berg 1994; Safe 1994; De Swart *et al.* 1994; Ross *et al.* 1995; Van den Berg *et al.* 1998). By now, most countries have set up rules, restrictions or even bans on their use, trade or production. Besides national actions, also inter-regional and global programs arose with the aim to protect human health and the environment from persistent organic pollutants. The probably best-known example is an international treaty composed at the Stockholm Convention on Persistent Organic Pollutants in 2001, that resulted in a list of banned, mostly halogenated products (Stockholm Convention 2001, 2010).

Dioxins and dioxin-like compounds

Halogenated hydrocarbons (and more specifically organochlorines) make up a large group of POPs that were banned within the Stockholm Convention. Included in this class of organohalogens are polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs), often summarized as “dioxins”.

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Figure 1) is regarded as one of the most toxic manmade substances and, dependent on the dose, can lead to death in certain species already in marginal doses (Schecter *et al.* 2006). Even though formation of dioxins has been observed in small amounts from natural combustion and geological processes, the major share of their global emission originates from unintended byproducts of industrial

processes including smelting, waste incineration, chlorine bleaching and herbicide and pesticide production (Schecter *et al.* 2006; Weber *et al.* 2008; Wong *et al.* 2012).

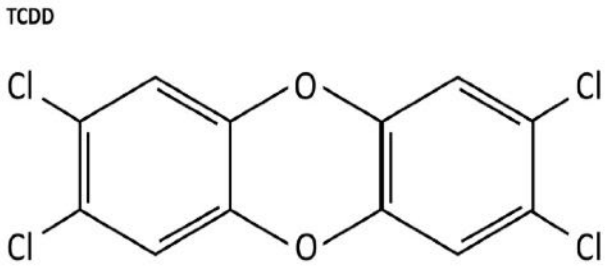

Fig. 1 Chemical structure of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the eponymous congener among the group of dioxin-like compounds. Other DLCs may have from one to eight chlorine atoms attached, the position of the halogen determines the numbers included in the chemical nomenclature developed by the International Union of Pure and Applied Chemistry (IUPAC).

Polychlorinated biphenyls (PCBs) are synthetic chlorinated hydrocarbon compounds that, similar to TCDD, consist of two benzene rings linked by a single carbon-carbon bond with one to all ten of the hydrogen atoms replaced with chlorine (Figure 2).

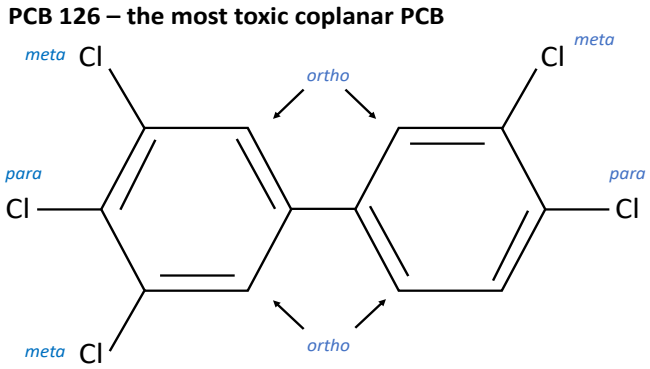

Fig. 2 Chemical structure of 3,3',4,4',5-Pentachlorobiphenyl (PCB126), the most toxic congener among the dioxin-like PCBs. Non-dioxin like PCBs have a non-coplanar 3-dimensional geometry caused by a higher amount of chlorine substitutions in the ortho-positions.

Differing in their molecular structure, a total of 209 different PCBs (congeners) is theoretically possible (Safe 1994). All PCB congeners are lipophilic, which means soluble in non-polar organic solvents such as oils and biological lipids. However, the whole group can be further categorized by means of their physicochemical configuration: The benzene rings of PCBs can rotate around the connecting bond but the rings are forced

towards either the same plane (called coplanar) or perpendicular planes (called non-planar) by the electrostatic repulsion of the highly electronegative chlorine atoms. There are a total of 12 different coplanar PCBs that have structural similarities to TCDD and thus belong to the group of DLCs and can be toxic at low doses.

The collective of the so-called dioxin-like compounds (DLCs) form a large group of hundreds of chemicals, which are structurally related and due to their common mechanism of action, induce a common spectrum of responses (Van den Berg *et al.* 1998). The chemical structure of dioxins is composed of two benzene rings, connected by stable oxygen bridges, and varying degrees of chlorination. Due to their aforesaid similarities, and in order to grasp their differences in toxicity better, DLCs have been assigned with so called toxic equivalence factors (TEFs), based upon their relative potency compared to TCDD with a TEF of 1 (Van den Berg *et al.* 1998). The total toxic equivalent (sum TEQ) thus represents the sum concentrations of each DLC congener multiplied with their individual TEF and expresses the entire toxicity as if the respective mixture was pure TCDD.

Increasing planarity (as explained above for PCBs) of the DLC results in higher susceptibility to bind with a protein called the cytosolic aryl hydrocarbon receptor (AhR), a transcription factor that regulates gene expression (Okey *et al.* 1994). It is believed that most (if not all) of the health issues caused by DLCs are mediated through this. The AhR is a ligand-activated transcription factor responsible for the expression of a collective of genes, including the so-called "AhR gene battery", that control numerous functions including cellular growth and differentiation (Denison & Nagy 2003; Tijet *et al.* 2006). Another function is the regulation of biological responses to planar hydrocarbons and several similar structured compounds (Okey *et al.* 1994; Denison & Nagy 2003). Normally inactively complexed with chaperones, the complex changes its structure upon binding of a ligand, and is then transported into the cellular nucleus by aid of the aryl hydrocarbon nuclear translocator (ARNT) (Reyes *et al.* 1992; Tijet *et al.* 2006). There the resulting transcription factor binds to specific regions of the DNA and promotes transcription of the particular genes with possible negative effects, depending on the respective ligand.

Emerging contaminants

Adding to the already existent chemicals of concern, more and more chemical compounds are continuously being produced and recognized around the globe. Also, with advancing technology and methodology regarding detection and analysis, the occurrence of newly identified contaminants in our environment is of growing official and public concern (Wong *et al.* 2012). As of January 2017, the Chemical Abstracts Registry, a global database maintained by the American Chemical Society (Chemical Abstract Service), counted already more than 126 million registered organic and inorganic substances, of which more than 347000 are authority-regulated chemicals listed in CHEMLIST (CAS 2017). The term "*emerging chemicals*" or sometimes "emerging contaminants of concern" (EEC) is therefore a moving target, dependent on time and location and thus mainly based on novelty, timeliness, or new concern and relates to the new discoveries of adverse effects of some of the man-made chemicals that were previously thought to be safe (Wijbenga & Hutzinger 1984; Wong *et al.* 2012). These include pharmaceuticals, pesticides, personal care products, surfactants and various industrial additives. Sources of these compounds are diverse and range from wastewater and sewage treatment plants (including hospital waste) to agricultural operations, aquaculture discharges and household discharges (Talib & Randhir 2016). One challenge in today's situation is that the collective of EECs are represented by a variety of polar and sometimes ionic compounds, for which many of the exposure, effect and risk models developed for classical non-polar POPs do not apply. Authorities therefor have postulated approaches and projects with the goal to identify and prioritize emerging pollutants and develop criteria and predictive tools for new and unrecognized pollutants with assessments and options for their management (Brack *et al.* 2015).

Pollutants in biota and wildlife

Research on pollution in wildlife can be traced back to the late nineteenth and early twentieth centuries. Early studies reported unintentional poisoning of birds from predator control agents, ingestion of lead shot and alkali poisoning of water birds, and die-offs from maritime oil spills (Calvert 1876; Grinell 1894 cited in Rattner 2009). The scientific field of ecotoxicology evolved further in the second half of the 20st century, after public interest in the potential hazards of chemicals arose after synthetic pesticides such as DDT were investigated in biota and linked with decreasing population sizes for example of

brown pelicans, white-tailed eagles and other birds in Europe and North America. Since then, a variety of contaminants have been associated with population or stock declines of biota including insects (Sánchez-Bayo & Wyckhuys, 2019), birds (Koemann *et al.* 1972), mammals (Atkinson *et al.* 2008) and fish (Hamilton *et al.* 2016). Albeit many of the respective problematic substances have already been banned, various species around the globe are still affected by POPs (Jepson & Law, 2016). This is severely problematic, as even decades after their ban, many of these chemicals persist in the environment and continue to accumulate in wildlife (e.g. PCBs and Dioxins). Banning hazardous substances, however, does not even always help to eliminate the issue, as also newly introduced substitutes often share similar chemical properties due to the nature of their application (e.g. chlorinated flame retardants substituted by brominated flame retardants) and thus presumably cause similar issues. Often the persistent and lipophilic nature of certain contaminants cause them to diversely accumulate (through bioaccumulation, bioconcentration and biomagnification) in living organisms.

As mentioned above, the term lipophilicity describes the tendency of a chemical compound to dissolve in oils, fat and lipids. This ability is often quantified by the octanol/water partition coefficient (K_{ow}) of a compound, which helps to assess water solubility, soil/sediment adsorption and bioconcentration factors for aquatic life (Karickhoff *et al.* 1979; Shiu & Mackay 1986; Thomann 1989). Octanol was proposed as a model of biological partitioning due to its chemical structure, which is considered similar to molecules present in the cell membrane of most biota (Fujita *et al.* 1964). While ‘bioaccumulation’ describes a plain concentration increase of a chemical in an organism, relative to their environment (irrespective of the source), ‘bioconcentration’ is a more specific term, describing the process of magnification of chemicals in certain tissues of an aquatic organism solely through uptake from the water phase. ‘Biomagnification’ is also more specific and refers to a multiplication of compounds in an organism, which typically is an increase in concentration of pollutants when these compounds move up in the food chain. This happens when chemical concentration multiplies in an organism of higher trophic level through the number of ingested food items with each lower levels of contamination. In sum, the here described mechanisms mean that in wildlife, highest concentrations of POPs are found in animals with high body fat ratios, up in the food chain coming from heavily polluted environments.

Pollutants in eel

Freshwater eels of the genus Anguilla represent such lipid-rich, high trophic-level predatory animals, that are particularly prone to the accumulation of especially lipophilic pollutants due to their biology and lifestyle. Research on mechanisms of contaminant uptake and its effects in the European eel *Anguilla anguilla* and the American eel *Anguilla rostrata* developed slowly but steady already in the early 1980s in Europe and North America and further intensified through the 90s (Lopez *et al.* 1981a; Lopez *et al.* 1981b; Hodson *et al.* 1994; Ferrando *et al.* 1987; Bruslé 1991; de Boer *et al.* 1994a, 1994 b). Larsson *et al.* were probably the first authors to directly link the decline in recruitment to possible effects derived from chemical contamination (Larsson *et al.* 1990, 1991). In 2006, Palstra *et al.* further elaborated on this hypothesis and were the first to directly investigate effects of organochlorine toxicity in eel embryos. They concluded that recruitment-impairing effects caused by environmental concentrations of DLCs were realistic, if eels belonged to toxicologically-sensitive species. Another study in 2009 from the Netherlands suggested impairment of lipid metabolism caused by chemical body burdens and thus presented realistic mechanisms linking contamination to impaired reproduction in eels (Van Ginneken *et al.* 2009). ICES (ICES WGEEL 2010) estimated that more than half (>60%) of all European eels from eight different countries were at risk of reproductive impairment based on toxicity thresholds for PCB effects on reproduction of other fish species. Tissue concentrations of DLCs in American and European eels put in relation to threshold concentrations affecting lake trout reproduction lead to similar conclusions (Byer *et al.* 2015). Yet, compared to other fish species, assessment of pollution effects in Atlantic eels can be seen as particularly difficult, since large parts of its lifecycle including aspects of the reproductive biology are still not fully understood.

Biology and lifecycle of the European eel

To be able to understand the special susceptibility of freshwater eels to pollution, it is important to go further into these species' lifecycle and biology. The European eel is part of the highly diverse teleost order *Anguilliformes*, which comprises about 820 species. One symplesiomorphy of this large group of bony fishes is the obligation to reproduce in marine waters. Another shared feature within some of the species is that their reproduction strategy is connected to long and intense spawning migrations. Probably the

most prominent members in this taxon are the freshwater eels, the family Anguillidae. *Anguilla*, the single genus of this family, are the only anguilliform known to regularly inhabit freshwater systems and despite a comparably extensive scientific attention, their whole life history is still regarded as mysterious and unusual (Aoyama & Tsukamoto 1997). Life histories of two species in the genus *Anguilla* are associated with the Atlantic Ocean: the American eel *Anguilla rostrata* and the European eel *Anguilla anguilla* are considered closely related. These two species have evolutionarily diverged some 10 -20 million years ago (Lecomte-Finiger 2003, Lin *et al.* 2001). Under natural conditions, *Anguilla rostrata* can be found in coastal waters, tributaries and freshwater systems from Venezuela, along the eastern coast of North America up to Greenland and Iceland, while *Anguilla anguilla* is distributed along the coastal and freshwater systems from Northern Africa to Scandinavia, including the Baltic-, Black– and Mediterranean Sea and its rivers. Despite their different distribution ranges, these two species share a very similar biology and lifestyle, as both are assumed to start and end their lives in the Sargasso Sea, a 2000-km long spawning area in the western Atlantic (Schmidt 1912, 1922, 1923; Tesch 2003; Miller *et al.* 2019).

Spawning strategy and oceanic life history phase

Albeit up to today no adult eels nor eggs were ever caught in the open sea, evidence and indications are present that this is their only spawning area, as the smallest ever found larvae of both species were found in significant numbers exclusively here (Schmidt 1923; van Ginneken & Maes 2005). There has been some scientific controversy concerning the reproduction strategy and the involved mechanisms for the Atlantic members of the *Anguillidae* family. While some studies, including molecular (Koehn & Williams 1978; Wirth & Bernatchez 2001; Maes and Volckaert 2002) and morphological works (Boëtius 1980; Harding 1985), found indications that speak against a single randomly mating population or panmixia, the majority of recent genetic studies indicate a panmictic lifestyle for both Atlantic eel species. In these publications, no significant genetic differences were found between adult European eels from southern and northern Europe (Palm *et al.* 2009; Als *et al.* 2011; Pujolar *et al.* 2014) and no evidence for geographical distinction among populations in American eels (Pujolar 2013a; Coté *et al.* 2013). Current understanding is that spawning activities of both species take place during an extended timeframe between January and March, with larvae hatching shortly after. Pelagic eel

larvae, called leptocephali, have a different shape than their adult and sub-adult counterparts (Figure 3), even though also with an elongated appearance, leptocephali are laterally flattened and mostly translucent. Their special appearance and shape are believed to be an evolutionary attainment, protecting them from predation and bringing benefits for their larval transport towards their natural distribution ranges (Wang & Tzeng 2000; Miller 2009). Different theories have been suggested in which the larval migration is a result also of passive drifting (McCleave *et al.* 1998; Knights 2003; Bonhommeau *et al.* 2009), or at least in combination with active swimming (Lecomte-Finiger 1992, 1994; Arai 2000).

Continental life history phases

After the transatlantic transport, the larvae undergo metamorphosis and develop into glass eels, another unpigmented, translucent life stage with similar body shape as the pre-adult and adult life-history stages (Figure 3). Typically, the young eels then concentrate in estuaries and river openings of coastal zones and start colonizing coastal areas or ascend rivers and freshwater systems. At this point, the young fish are believed to be sexually undifferentiated still, with environmental factors such as population density regulating further differentiation (Tesch 1977; Krueger & Oliveira 1999; Davey & Jellyman 2005). As a result, European and American eels show differing sex ratios from north to south, with a higher proportion of male eels in the warmer, more southern habitats and predominantly female eels in the colder, more northern habitats (Vladykov 1966; Kuhlmann 1975; Tesch 2003).

At the river openings and coastal zones, the glass eels eventually start to pigment and begin their juvenile growth phase, a life-history stage referred to as yellow eels due to their greenish to yellowish coloration (Fig. 3). Yellow eels are benthic, omnivorous fish with a wide range of accepted food items. Their prey includes a variety of invertebrates such as worms, mollusks, bivalves, crustaceans and insects, as well as aquatic vertebrates such as amphibians and fish (Costa *et al.* 1992; Moriarty 2003). The yellow eels' rather sedentary growth phase, depending on their sex and some regional aspects, lasts for roughly 6-8 years until growing to sizes of 40-50 cm (males) or 8-20 years to reach 50-120 cm (females). When reaching their final size and lipid content, another metamorphosis into the next life-history stage occurs (Larsson *et al.* 1990; Durif *et al.* 2005). During this second metamorphosis called silvering, eels undergo morphological

and physiological changes that prepare them for their once-in-a-lifetime oceanic migration back to their reproduction area, the Sargasso Sea (Fig. 3).

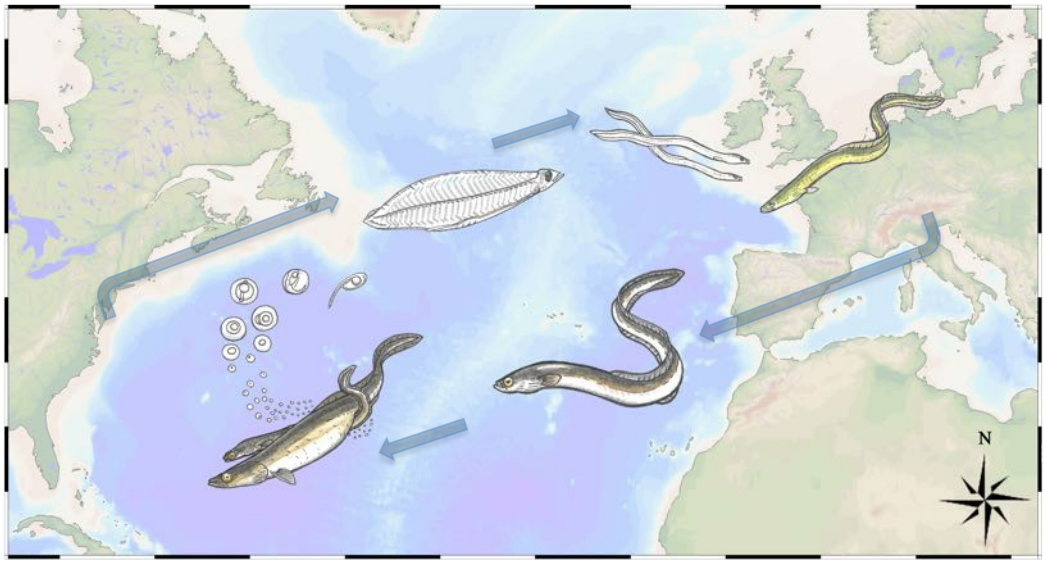

Fig. 3 Schematic lifecycle of the European eel (*Anguilla anguilla*) displaying different life stages in its natural distribution range, the Atlantic Ocean. The lifecycle is displayed in clockwise rotation and begins with spawning silver eels in the Sargasso Sea (left), developing eggs and leptocephalus larva (top), glass eels (top right), the continental yellow eel stage (right) and a migrating silver eel (bottom).

These body changes during silvering involve internal modifications and physiological adaptations such as cessation of food intake with a resulting degeneration of the gastrointestinal tract, a first onset of gonadal maturation as well as transformation of external characteristics. Visible alterations include changes in coloration from brown, green and yellow to black and silver, an increase of pectoral fin length, enlargement of eye diameter with concomitant increase in the number of rods in the retina, development of neuromasts along the lateral line and iono- and osmoregulatory adaptations for a future transition from fresh to saltwater (Pankhurst 1982; Tesch 2003; Durif *et al.* 2005; Righton *et al.* 2012). Once their transition to this final life history stage is completed, silver eels begin their migration back to their spawning grounds with distances as far as 5000-7500 km depending on their starting point. After silvering, eels cease to feed and are then entirely reliant on the already stored energy reserves in their body to provide for migration and their final sexual maturation (Pankhurst & Sorensen 1984; Tesch 2003; Chow *et al.* 2010). Atlantic eels are semelparous, a strategy in which individuals put all available resources into a single reproduction event, resulting in death of the parental animals after

successful spawning. Authors of several studies therefor interpret the comparably high lipid content of these species to be of great importance for the success of their migration (Svedäng & Wickström 1997; Van den Thillart *et al.* 2007; Belpaire *et al.* 2009). The migration itself, however, is still cloaked in mystery as no eel was ever observed nor caught in the open Atlantic or around the assumed spawning besides one described encounter of a submersible near the Bahamas with what apparently was an eel of the genus *Anguilla* (Robins *et al.* 1979). Consequently, this last part of the eel's lifecycle, the open ocean migration, will continue to challenge scientific research in the future.

Stock situation and management

Eels have played a certain role for men for a long time. Most species of the genus *Anguilla* are utilized for human consumption, which is why the catch of eels is an established tradition in inland and coastal fisheries outside and inside Europe. However, large-scale commercial exploitation is mostly found throughout temperate waters (Dekker 2002), even though the majority of *Anguilla* species are found in tropical waters (Tsukamoto & Aoyama 1998). For fisheries in Europe alone, eel has been an important target for centuries now, which is why they are supposed to cover almost the whole distribution range of approximately 90.000 km^2. In this area, all of the eels' continental life stages from ascending glass eels to descending silver eels are targeted by fisheries with spatial differences and highly specialized fishing gear (Dekker 2003).

Besides being marketed for direct human consumption, glass and young yellow eels are also caught in high numbers as seed for aquaculture farming and partially as stocking material meant for fisheries management measures. In the past, a significant share of European glass eel catches was also exported to Asia. However, this is forbidden since 2011, when the European eel became listed in Annex II of the Convention on International Trade in Endangered Species (Anonymous 2007). Illegal exports still remain a massive problem due to the high prices glass eels generate on the market and glass eel smuggling has been denoted as one of the world's biggest wildlife crimes (Gristwood 2019).

Due to their inter-regional importance, historical catch and monitoring data on different European eel life history stages exist and give good insight on the dynamics and development of the stock over the past decades. It was data like these, that revealed vast declines in recruitment of several species of the genus *Anguilla* since the 1980s.

As a consequence, the affected species have been rated as critically endangered (*Anguilla anguilla*) and endangered (*Anguilla rostrata* & *Anguilla japonica*) in the Red List of Threatened Species by the International Union for Conservation of Nature (IUCN) (Jacoby & Gollock 2014). For European eels, the recruitment had decreased by 90-99% in the monitored distribution area compared to a 1960-1979 average (ICES WGEEL 2015). While catches of glass eels have been reported to significantly decline, also international landings of yellow and silver eel fisheries had shown distinct downward trends (ICES WGEEL, 2015). Reasons for the decline are yet not fully understood.

To install counter measures against these alarming trends, the European Union passed Regulation No 1100/2007 (European Commission 2007) with the objective of building up actions for the recovery of the European eel stock. Following the regulation, European member states from there on were obliged to implement special eel management plans (EMPs) aiming at a reduction of anthropogenically caused mortalities and facilitating an increase of silver eel escapement to 40% relative to the best estimate of escaping silver eel's biomass under pristine conditions. Choice and implementations of management measures, however, are fairly arbitrary and in consequence, heterogenous among member states. Measures to increase silver eel escapement carried out in European waters include reduction of fishing mortality (i.e. minimum landing size or closed seasons), habitat restoration and assisted migration programs or stocking (Pohlmann *et al.* 2016). The driving factors behind the devastating collapse are still subject to research, which lead to a number of different hypotheses including overfishing and overexploitation, habitat loss and degradation, oceanic changes, parasitism and pollution (Dekker 2003; Knights 2003; Geeraerts & Belpaire 2010; Wysujack *et al.* 2014, Miller *et al.* 2015). The specific relevancy of each stressor and their respective contribution to the situation remains unclear to this day.

AIM AND OUTLINE OF THIS THESIS

At the starting point of this thesis, the scientific community was alarmed by the decline in eel stocks and no consensus about the driving forces of detrimental impacts on the stock existed. Possible reasons for the decline are diverse and cover a large array of anthropogenic impacts, including impaired reproduction due to toxicological contamination. However, uncertainties around the driving factors for the initial decline remain, and even though the possible reasons are often mentioned together, they have never been quantitively assessed or evaluated comprehensively. Introduced management actions to counteract the negative trend, such as redistribution and stocking of young fish, have to be looked at a bit differently compared to similar actions for other freshwater and saltwater species due to the eels' semelparous and panmictic lifestyle. Even though the production of larvae in captivity is generally possible, the full lifecycle of European eels still cannot be closed in captivity. Thus stocking measures rely on natural sources of young recruits. Contamination by chemical pollution has shown to be capable of significantly altering and influencing stock structures of a number of different invertebrate and vertebrate species and population numbers on local and supra-regional scales of a variety of species have been directly affected in the past. For the European eel however, it is still unclear if and to what extent chemical pollution may have played a role in the devastating stock decline as large parts of the oceanic silver eel migration as well as the actual spawning activities in the wild pose a "black box", regarding the lack of in-depth knowledge about the involved processes.

The goal of this thesis was to investigate aspects of the possible influence of chemical pollution on the stock situation of the European eel. The here included studies were drafted in order to gain knowledge about chemical contamination in European freshwater eels and how the distinct susceptibility of this species towards pollution can be addressed in stock management. During its continental life, the different life history stages of the European eel are exposed to diverse sources and intensities of pollution, depending on the respective habitat. Chapter I addresses this topic, in order to clarify how habitat influences contamination burden and to investigate which life history stage is most suitable to assess the actual impact of DLC pollution of a local population. By further investigating the role of halogenated pollutants during sexual maturation in order to understand whether concentrations found in eggs after spawning may reach concentrations critical for successful reproduction, Chapter II, III and IV had special

emphasis on closing knowledge gaps in maturation physiology and on gaining understanding of the degree of expected maternal transfer of selected pollutants during gametogenesis. To better understand how lipophilic compounds distribute in the eel's body during growth and physiologically-derived body changes (maturation), we created a physiologically-based toxicokinetic model for the eel (Chapter V), that can help estimate concentrations in different body matrices of eels at any given time. In the last chapter (Chapter VI) we used our PBTK model to investigate if found concentrations of the pesticide Fipronil and its metabolites in water samples and eel tissue samples from German rivers, could be explained by waterborne uptake alone or if other pathways of uptake were necessary.

Following the goals of the presented thesis, research questions of the study were addressed in 6 chapters:

Chapter I ("A question of origin: dioxin-like PCBs and their relevance in stock management of European eels")

Due to their specialized biology, European eels are predisposed to chemical contamination. Dioxin-like compounds are toxic lipophilic organic chemicals, that can accumulate to high amounts in eels and thus have been put in connection to the stock decline of this species. The research goal of this chapter was to find out which life-history stages of eels are mostly affected by DLC contamination. To address this, we investigated accumulation intensity and patterns of dioxin-like PCBs in muscle tissue of all continental life history stages of European eels. To further examine how intra-habitat specific differences can affect the contamination status of an eel, we sampled individuals in the yellow eel growth stage along the German river Elbe and compared their contamination levels and patterns. Eel stock management in many countries includes (re-)stocking, a practice in which young eels are caught at a place of high abundance and then transported to habitats of low abundance to increase the number of potential spawners eventually leaving the system. This often happens without consideration of pollution as an aspect of suitability regarding the respective new habitat. To shed light on this issue, another important research question in this chapter was to find out how the respective habitat influences contamination pattern and intensity of silver eels, that are about to leave for their journey back to their spawning grounds, the Sargasso Sea.

Chapter II ("Maternal transfer of dioxin-like compounds in artificially matured European eels")

Results from habitat and life-history stage comparisons in chapter I indicated a strong influence of the respective habitat on the contamination status of an eel. As a consequence, feeding and growth habitats are decisive for the body burdens of descending silver eels, which are about to leave for their reproduction mission in the Sargasso Sea. As the survival of any species depends on its ability to have healthy, fertile offspring, the capability of a descending silver eel to successfully reproduce is an important assessment criterion in the management of this threatened species. For a variety of fish and other wildlife species, it was shown that lipophilic contaminants such as DLCs can be maternally transferred to the offspring, which poses a hazard to their health or may even impede survivability. Due to the limited availability of mature, ready-to-spawn silver eels, empirical evidence and analytical concentration data on the maternal transfer of DLCs in eels are scarce or have never even been published. The goal of this chapter therefor was to investigate how and to what extent DLCs are being transferred from eels' maternal somatic tissues to their eggs as well as to elucidate possible driving factors of the transfer. Moreover, we utilized our findings from the present and from previous studies, in order to develop a tentative approach to benchmark whether a habitat is suitable for stocking measures or not.

Chapter III ("Maternal transfer of emerging brominated and chlorinated flame retardants in European eels")

The Stockholm Convention led to the ban of the so-called "dirty dozen" in 2004. This list of POPs includes pesticides, industrial chemicals and byproducts, that have been recognized to cause adverse effects on humans and the ecosystem. Among these compounds were the group of DLCs (dioxin-like PCBs, PCDDs and PCDFs), the production and use of which was then restricted or eliminated. The industry then developed alternative chemicals with similar chemical properties by using other halogens such as bromine, fluorine and iodine instead of chlorine. Due to the novelty of these chemical constellations, most of these emerging alternatives (including a range of halogenated flame retardants) were widely unregulated and their behavior and possible effects on the environment were unknown and undetermined. This was the case even though comparable negative effects, as caused by PCBs or dioxins, were expectable

according to the generally similar chemical structures of these chemicals. In this chapter, our investigations focused on the possible maternal transfer of emerging halogenated flame retardants (HFRs) such as polybrominated diphenyl ethers (PBDEs) and their brominated and chlorinated substitutes from somatic tissues to the eggs in European eels. Our research goal thus was to find out if, how and to what extent these novel types of compounds were transferred during gametogenesis and how they could impact the species "quality of spawners".

Chapter IV ("Bone resorption and body reorganization during maturation induce maternal transfer of toxic metals in anguillid eels")

The physiological challenges Atlantic eels undergo during spawning migration are accompanied by a peculiar re-organization of their bodies. This shapeshifting involves the degradation of lipids and proteins from muscle stores to fuel the energetic demands of locomotion as well as to supply nutrients for the buildup gonadal tissues. Additionally, the eels' skeleton is known to hold large reserves of calcium and phosphorus, which are also needed to ripen and develop the reproductive glands, that produce the gametes needed for sexual reproduction. In this paper, we approached to understand more about the re-organization of the body during the maturation of European eels, to get an insight how the eel uses its skeleton as a store for minerals for the buildup and ripening of the reproductive glands. By additionally investigating the internal distribution of potentially toxic metals besides the essential minerals along the gonadal buildup, we investigated possible adverse consequences of toxic metals for the reproductive capacity of eels from metal-contaminated waters.

Chapter V ("A PBTK model for moderately lipophilic organic chemicals in the European eel (*Anguilla anguilla*)")

Physiologically based toxicokinetic (PBTK) modeling is used for the prediction of the absorption, distribution, metabolism and excretion of chemical substances in biota for risk assessment and pharmaceutical research. By using mathematical equations, these models help to describe and quantify temporal and spatial change in concentrations of chemicals and / or their metabolites in different biological matrices or compartments of an organism. By using the volume and physicochemical properties of these compartments and certain metabolic rates of processes, toxicokinetic models are usually fitted to experimental data in order to interpolate kinetics and target tissue concentrations after different scenarios and routes of exposure. Due to their extremely specialized biology and high lipid content, eels are particularly prone to bioconcentration of hazardous substances, even though they exhibit low rates of constant uptake due to their respiration physiology and capability of cutaneous respiration. Our goal in this study was to gain knowledge about the kinetics of potentially threatening lipophilic chemicals in eels during their feeding and growth phase. To achieve this, we developed a first PBTK model for moderately lipophilic organic contaminants in yellow eels. With good predictive power, such a model for eel could help to reduce animal experiments with eels and waterborne chemicals and lead to a better assessment of possible effects of lipophilic contaminants during the spawning migration in the future.

Chapter VI ("Fipronil and two of its transformation products in water and European eel from the river Elbe")

Besides the well-studied group of classical lipophilic POPs and alternative halogenated compounds such as BFRs, a growing number of newly developed, emerging contaminants with potentially harmful effects on aquatic species is being detected in significant amounts in the environment. The use of some chemically rather unstable pesticides, such as Fipronil, is often not strictly regulated nor monitored, even though the largely unknown toxicological impacts and interactions resulting from these compounds have been suggested in the literature. The research questions of this study were if we could detect Fipronil and its transformation products in water samples of the German river Elbe and whether (and to what extent) we could find these compounds in body tissues of European eel, which due to its biologically predestined bioaccumulation

potential, is considered a useful bio-indicator for the ecological quality of aquatic environments. To link the water-borne Fipronil and FIP-metabolite concentrations to those measured in eel-tissue samples, we modified and used our earlier developed PBTK model to estimate metabolization pathways of this compound.

Overview of the chapters in this thesis:

CHAPTER I

Dioxin-like PCBs and their relevance in stock management of European eels

CHAPTER II

Maternal transfer of dioxin-like compounds in artificially matured European eels

CHAPTER III

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

CHAPTER IV

Bone resorption and body reorganization during maturation induce maternal transfer of toxic metals in anguillid eels

CHAPTER V

A physiologically based toxicokinetic (PBTK) model for moderately hydrophobic organic chemicals in the European eel (*Anguilla anguilla*)

CHAPTER VI

Fipronil and two of its transformation products in water and European eel from the river Elbe

CHAPTER I

A question of origin – dioxin-like PCBs and their relevance in stock management of European eels

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A question of origin: dioxin-like PCBs and their relevance in stock management of European eels

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Abstract The stock of European Eel (*Anguilla anguilla* L.) has reached an all-time low in 2011. Spawner quality of mature eels in terms of health status and fitness is considered one of the key elements for successful migration and reproduction. Dioxin-like Polychlorinated Biphenyls (dl-PCBs) are known persistent organic pollutants potentially affecting the reproductive capability and health status of eels throughout their entire lifetime. In this study, muscle tissue samples of 192 European eels of all continental life stages from 6 different water bodies and 13 sampling sites were analyzed for contamination with lipophilic dl-PCBs to investigate the potential relevance of the respective habitat in light of eel stock management. Results of this study reveal habitat-dependent and life history stage-related accumulation of targeted PCBs. Sum concentrations of targeted PCBs differed significantly between life stages and inter-habitat variability in dl-PCB levels and -profiles was observed. Among all investigated life stages, migrant silver eels were found to be the most suitable life history stage to represent their particular water system due to habitat dwell-time and their terminal contamination status. With reference to a possible negative impact of dl-PCBs on health and the reproductive capability of eels, it was hypothesized that those growing up in less polluted habitats have a better chance to produce healthy offspring than those growing up in highly polluted habitats. We suggest that the contamination status of water systems is fundamental for the life cycle of eels and needs to be considered in stock management and restocking programs.

Keywords Dioxin like PCB · Eel · Silver eel · Habitat quality · Spawner quality · Stock management

Introduction

The panmictic stock of the European Eel (*Anguilla anguilla L.*) has experienced a drastic decline since the early 1980s when recruitment numbers of arriving glass eels have dropped startlingly (Moriarty 1986, 1996; ICES 2010). Even though slight increases of arriving glass eels have been observed since, the stock is still considered as outside safe biological limits. Reasons for this decline are currently subject to ongoing comprehensive research on a global scale. Apart from natural phenomena such as oceanic factors and predation (Knights 2003; Friedland et al. 2007; Durif et al. 2010; Bevacqua et al. 2011; Wahlberg et al. 2014), a number of anthropogenic influences including habitat loss, overfishing, denaturation of water bodies, the introduction of parasites and pollution are suspected to be contributing factors (Robinet and Feunteun 2002; Dekker 2003; Palstra et al. 2006, 2007; Quadroni et al. 2013; Sühring et al. 2013, 2014; Barry et al. 2014). Their biology as sediment related, bottom dwelling predators with high body fat contents make eels in their growth phase (yellow eels) particularly vulnerable to chemical pollution by a variety of lipophilic bio-accumulating contaminants including metals (Maes et al. 2008), polycyclic aromatic hydrocarbons (Kammann et al. 2014),

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chlorinated and brominated flame retardants (CFRs & BFRs), polychlorinated biphenyls (PCBs) and polychlorinated dibenzo-p-dioxins and furans (PCDDs/Fs) (Geeraerts and Belpaire 2010; Tapie et al. 2011; Sühring et al. 2013, 2014; Szlinder-Richert et al. 2014). Some of these contaminants are known to cause a variety of adverse health effects including cancer, reproductive failure, nervous and endocrine system disorders, and others (Safe 1994; Robinet and Feunteun 2002; Ross 2004; Corsi et al. 2005). Due to their specific predisposition towards xenobiotics, the composition of chemical contamination in eels could be interpreted as a result of environmental imprinting by the local environment (Belpaire and Goemans 2007; Belpaire et al. 2008; Grabowska 2010; Byer et al. 2013). In a Portuguese study by Guimaraes et al. (2009), results indicated that yellow eels originating from stronger polluted habitats showed higher adverse physiological effects determinant for their survival and performance than yellow eels originating from a less polluted habitat. In another study on Japanese eels (*Anguilla japonica)* by Arai and Takeda (2012) from Japan, the authors state that the ecological risks of organochlorine compounds (OC) increase as the freshwater residence period in eel become longer. Therefore individual lipid contents and migratory histories directly affect the accumulation of those OCs in anguillid eels.

One of the probably most prominent groups among these xenobiotics with negative effects on aquatic organisms are dioxin-like polychlorinated biphenyls (dl-PCBs) since these lipophilic and mostly persistent compounds tend to accumulate through the trophic cascade (James and Kleinow 2014). Some congeners, dependent on the number and position of chlorine atoms, have particularly been identified to be capable of causing severe health damages and to possibly influence the ovarian and embryonic development, as shown for different species (Gutleb et al. 1999, 2007; Daouk et al. 2011) as well as specifically for the European eel (Robinet and Feunteun 2002; Corsi et al. 2005; Palstra et al. 2006). The production of PCBs was stopped in the 1980s and PCBs have been considered POPs and listed for a global ban in 2001 by the Stockholm Convention (Stockholm Convention 2001; Porta and Zumeta 2002). However, due to their persistence, they are still found in considerable concentrations in the atmosphere, in soils as well as in aquatic sediments (Nizzetto et al. 2010; Grabowska 2010; Wetzel et al. 2013) which, mainly after flood events or excavation works, play an important role as secondary sources also for the contamination of inland water bodies and flood plains (Stachel et al. 2004; Lake et al. 2014). It has been estimated that the total dioxin-like toxicity in the historically produced 1.3 million metric tons PCB (Breivik et al. 2002) were between 11,000 and 16,000 kg toxic equivalents (TEQ) (Weber et al. 2008). This can be compared to the current global PCDD/F emission inventory of approximately 58 kg TEQ for 68 countries, covering 50 % of the world population (Fiedler et al. 2012). Therefore, dl-PCBs still account for the largest share of dioxin-like toxicity in European rivers, considerably higher than the toxic equivalence (TEQ^{2005}) contribution from PCDDs and PCDFs (Stachel et al. 2007; Blanchet-Letrouvé et al. 2014; Guhl et al. 2014). Furthermore, approximately 3 million metric tons of PCB-contaminated waste oils and contaminated equipment still need to be managed, globally contributing to ongoing environmental pollution (Stockholm Convention 2010; Weber et al. 2013).

The European eel is a species of high economic and ecological value, which is why a number of protection measures have been set up within the European Union, to counter-act against the decline of the stock. These actions include habitat restoration, fisheries and trade restrictions like keeping-size limits, closed seasons and a variety of accessory measures to increase the escapement of silver eels to a given target of 40 % of the pristine biomass (Council Regulation (EC) 1100/2007). A common counter measure against locally dropping numbers of recruits is the catch and reallocation of glass eels for stocking purposes (ICES 2013). The aim of this practice (besides the support of local commercial fisheries) is to harvest individuals from water bodies exceeding their carrying capacity and to distribute them into less recruited habitats and thereby eventually reduce rates of natural mortality. However, until now, very few studies targeted the effectiveness of such management actions. Considering the negative impact of environmental contaminants such as dl-PCBs on the quality of eel spawners, it is vital to assess the contamination status of those water bodies selected for restocking measures as part of management plans that are explicitly aiming at the recovery of the eel stock. Until now, habitat adequacy for stocking programs in terms of the environmental status of targeted rivers has not been taken into account.

The aim of this study was to investigate the influence of different habitats on the quantity and patterns of dl-PCBs in eels throughout their different continental life history stages. Results are supposed to help identify quality indicators for the habitat selection with regards to restocking purposes as well as appropriate life stages for certain monitoring strategies. In addition, findings of this study may lead to an improvement of eel-related management and assessment in line with the European Data Collection Framework (EU DCF) in the future. The DCF is a Community framework for the collection, management and use of data in the fisheries sector and support for scientific advice regarding the common fisheries policy (CFP). Our results give an impression on decisive factors for the

contamination of eels with dl-PCBs and why the selection of habitats for stock management measures should be influenced by their contamination status.

Materials and methods

Samples

A total of 100 European glass eels were obtained from French Atlantic coast glass eel fisheries and 30 young-of-the-year elvers (young, recently ascended yellow eels) from an elver-monitoring site in the river Vidå at the German-Danish border (Fig. 1). In addition, 35 migrating female silver eels were purchased from commercial fishers situated in the potamal sections (lower stretch) of the rivers Elbe, Eider, Ems, the Schlei Fjord and the lower river Rhine close to the German/Dutch border in the frame of the German data collection according to the EU Data Collection Regulation (DCR) (European Commission 2008, 2010) (Fig. 1). In addition, 27 yellow eels were caught at six sampling sites along river Elbe by electrofishing, also in line with the EU DCR. A list with detailed biological parameters of the analyzed eels can be found in Table 1. Eels in this study were killed by decapitation after being anaesthetized with 2-Phenoxyethanol (ROTH, Karlsruhe, Germany). To eliminate sources of contamination, samples were strictly handled with cleaned equipment made of glass, aluminum or steel, preventing any contact with plastics, oils or other possible sources of cross-contamination. For further analyses, between 10 and 25 g muscle tissue of yellow and silver eels was excised from the skeletal muscle just behind the level of the anus. From elvers, whole filets of 3 randomly chosen individuals were pooled and homogenized. For glass eels, 10 randomly chosen individuals were each entirely combined to a pool-sample and homogenized. Age determination of yellow and silver eels was based on otolith readings following the cutting and burning method (Graynoth 1999) as recommended by ICES (2009, 2011). For better comparability, yellow eels were selected to fit in a certain age frame and maturation stage [between eight and twelve years old and growth- & pre-migrating silvering stages I, II or III after Durif et al. (2005)]. All silver eels were in the silvering stage V (migrating phase V after Durif et al. (2005)). Due to low availability of stage V eels in the Schlei fjord, 2 Stage III specimens with similar biological characteristics according to length, weight and age and a Pankhurst stage higher than 7 (migrating stage, Pankhurst 1982) were included in the analysis of this sample group (Table 1).

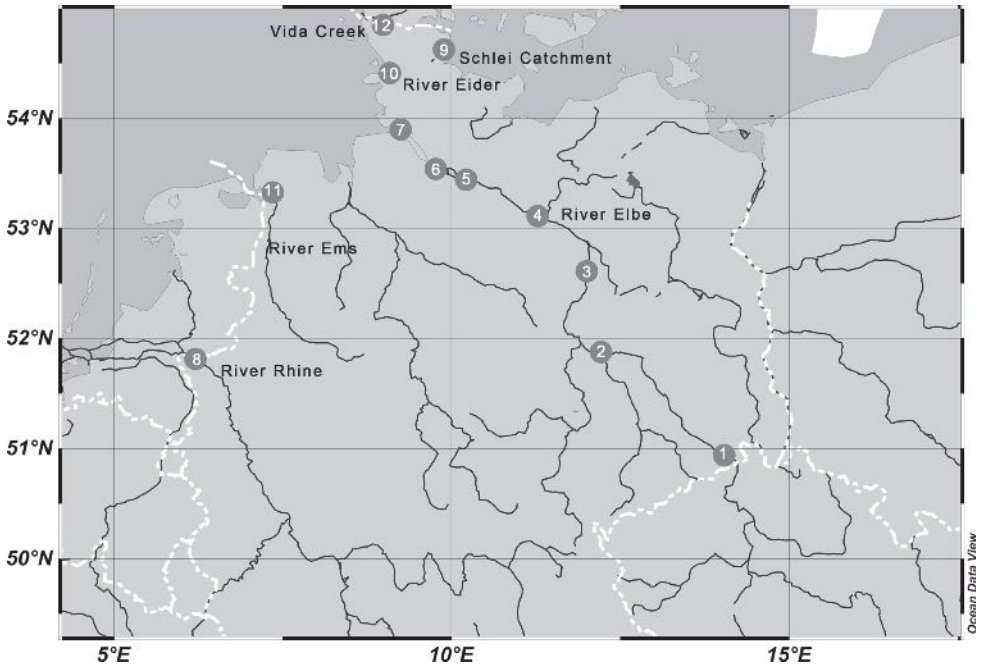

Fig. 1 Sampling positions of all collected continental life stages in the German waterbodies Elbe (*1* Bad Schandau, *2* Dessau, *3* Hohengören, *4* Gorleben, *5* Winsen, *6* Jork, *7* Cuxhaven), Rhine (*8* Kalkar-Grieth), Schlei (*9* Schleswig), Eider (*10* Nordfeld), Ems (*11* Emden), Vidå (*12* Verlath)

Table 1 Summary of amalgamated data (mean ± standard deviation) for different life history stages of European eels collected from different sampling sites and water bodies

Life stage	Sample size (n)	River basin	Sampling location (pos. on map (Fig. 1))	Length (cm) ±sd	Weight (g) ±sd	Age (y) ±sd	Lipid (%) ±sd
Y	1	Elbe	Bad Schandau (1)	67.0 ± N/A	533.3 ± N/A	12 ± N/A	24.5 ± N/A
Y	7	Elbe	Dessau (2)	59.7 ± 12.9	366.0 ± 213.9	8.4 ± 2.2	25.7 ± 6.1
Y	5	Elbe	Hohengoeren (3)	62.4 ± 5.4	406.6 ± 149.4	9.4 ± 1.5	28.4 ± 11.0
Y	4	Elbe	Gorleben (4)	64.3 ± 3.9	416.3 ± 94.8	8.5 ± 1.3	35.3 ± 5.1
Y	5	Elbe	Winsen (5)	61.6 ± 5.0	459.8 ± 129.1	9.4 ± 1.1	25.6 ± 10.1
Y	5	Elbe	Jork (6)	58.6 ± 10.4	362.4 ± 204.2	8.4 ± 0.9	22.0 ± 16.6
S	10	Elbe	Lower stretch (6&7)	69.4 ± 6.7	646.2 ± 154.2	10.6 ± 1.5	27.5 ± 2.0
S	10	Rhine	Kalkar (8)	69.0 ± 7.0	639.2 ± 111.2	13.3 ± 2.8	24.3 ± 3.5
S	5	Eider	Nordfeld (10)	66.6 ± 3.8	530.0 ± 166.3	13.8 ± 2.2	23.6 ± 4.0
S	5	Ems	Emden (11)	70.6 ± 4.5	683.8 ± 129.4	14.6 ± 2.1	26.4 ± 4.9
S	5	Schlei	Schleswig (9)	66.8 ± 4.0	624.8 ± 109.3	10.0 ± 3.2	21.8 ± 4.1
ELV	10 × 3	Vidå	Verlath (12)	12.0 ± 0.9	1.8 ± 0.4	N/A	1-2 ± N/A
GE	10 × 10	Atlantic Coast	France	6.9 ± 0.4	0.2 ± 0.1	N/A	0-1 ± N/A

Data were grouped according to their life stages (*Y* yellow eel, *S* silver eel, *ELV* elver, *GE* glass eel) and sampling locations respectively

Extraction, clean-up and lipid content

Extraction and clean-up were conducted following the protocol as described by Sühring et al. (2013). Frozen yellow and silver eel muscle samples were homogenized with anhydrous Na_2SO_4 (2:1; w/w) for approximately 20 min using a 1 L stainless steel/glass laboratory blender (Rotorblender, neoLab, Heidelberg, Germany). The homogenized samples were extracted by accelerated solvent extraction (ASE-200, Dionex, Sunnyvale, USA) using dichloromethane (DCM, ROTH, Karlsruhe, Germany) at 100 °C and 120 bar. All samples were spiked with 13C mass labeled internal standards (IS) analogous for each analyzed compound (WHO PCB+PCB-170+PCB-180 CLEAN-UP STANDARD (13C12, 99 %), Cambridge Isotope Laboratories (CIL), Tewksbury, USA). Any remaining volume was filled with anhydrous Na_2SO_4 (ROTH, Karlsruhe, Germany). For extraction of the homogenized glass eel samples, a Na_2SO_4–eel–mixture (equal to 3 g eel tissue) for each pool was extracted by Soxhlet filled with 28–60 mm glass–fiber extraction thimbles with DCM at 55 °C for 24 h. After extraction, the samples were reduced to approx. 2 mL using rotary evaporators. For the first clean-up step, a gel permeation chromatography (GPC) was used with 30 g Bio-Beads SX-3 (Bio-Rad Laboratories, Hercules, USA) and DCM:Hexane (1:1; v:v) as eluent. The first fraction (75 mL) was discarded while the second fraction (110 mL), that contained the target substances, was reduced to about 2 mL and then transferred into hexane. A column with 2.5 g 10 % H_2O deactivated silica gel (ROTH, Karlsruhe, Germany). was used as a second clean-up step. Analytes were eluted with 20 mL hexane and the volume concentrated to 150 µL before transferring them to measurement vials. Finally, 10 µL 13C PCB 141/PCB 208 (50 ng mL^{-1}) was added as injection standard to each sample. The lipid content of samples was determined gravimetrically from separate aliquots following a method described in Sühring et al. (2013).

Instrumental analysis

The instrumental analyses were performed on a GC/MS-system (Agilent 6890 GC/5973 MSD, Agilent Technologies, Santa Clara, USA) fitted with a HP-5MS column (30 m × 0.25 mm i.d. x 0.25 µm film thickness, J&W Scientific, Agilent Technologies, Santa Clara, USA) in electron capture negative ionization mode (ECNI) using methane as ionization gas. The instrument was operated in selected ion monitoring mode. Samples were analyzed for dl-PCBs (IUPAC numbering) 77, −81, −105, −114, −118, −126, −156, −157, −167, −169, −189 as well as the non dioxin-like PCBs 170 and −180. PCB 170 and PCB 180 were included in the analysis because of their physiological relevance as active inducers of EROD activity and their quantitative significant presence in environmental samples and thus to provide for better comparability with studies involving non-dl and/or indicator PCBs.

QA/QC

Extraction and clean-up were conducted in a clean lab (class 10,000). Recovery rates of IS were determined for each sample. Mean IS recoveries ranged from 59 ± 24 % for PCB 81 to 77 ± 31 % for PCB 169. A blank test, using Na_2SO_4 treated similar to real samples, was performed with every extraction batch (eleven samples). All blanks were either below MQL or otherwise 1–2 magnitudes lower than lowest samples concentrations. The limits of detection and quantification (LOD/LOQ) were calculated either from the blank or from a signal to noise ratio of 3 and 10. The LOD ranged from 0.003 to 0.012 ng/g wet weight (ww) for PCB 189 to 0.012–0.09 ng/g ww for PCB 81. The LOQ ranged from 0.004 to 0.04 ng/g ww for PCB 189 to 0.032–0.30 ng/g ww for PCB 180. For further quality control, a twofold measurement was conducted for samples from areas with low sample numbers and a threefold measurement was done for PCB 126 from randomly selected samples from remaining areas. Results for PCB 123 were excluded from our results due to incomplete chromatographic separation.

Data processing and statistical analyses

TEQ values were calculated under consideration of the WHO-2005 Toxicity Equivalent Factors (TEFs) (Van den Berg et al. 2006). TEQ concentrations are reported for dl-PCB TEFs solely, thus not PCDD/Fs.

All statistical analyses were performed using R 3.1.2 (R Core Team 2014). Differences in accumulation quantities of targeted dl-PCBs between the respective sample groups were tested on sums of dl-PCB concentration. For more than 2 groups, the Kruskal–Wallis test was used and subsequent post hoc tests were performed with Bonferroni corrected p value adjustment using the package agricolae (de Mendiburu 2014). When testing just 2 groups against each other, the Mann–Whitney *U* test was performed. Nonmetric multidimensional scaling (nMDS) was used to compare congener accumulation patterns of individuals between life-history-stages, intra habitat catch locations and inter habitats (river systems). To avoid zero values, a small constant was added to each congener measure and data was log-transformed subsequently. NMDS was performed using the function metaMDS in the package vegan (Oksanen et al. 2015). Euclidean distance was used to calculate the dissimilarity matrix. Number of dimensions was set to two. Maximum number of random starts was set to 100, and no automatic transformation was used. Permutational multivariate analysis of variance (PERMANOVA) was performed to test whether groups differed significantly. Number of permutations was set to 10,000. Bonferroni correction was used for post hoc tests.

Results

dl-PCB accumulation and patterns in eels of different life history stages

Detailed concentrations for individual detected congeners as well as the resulting TEQs from the investigated compounds can be found in Table 2. Dl-PCB congener patterns of eels of different life history stages are displayed in Fig. 2a. Congeners with highest overall concentrations among targeted dioxin-like PCBs were 118, 105 and 156, summing up for 84.8 % (PCB 118 = 58.5 %; PCB 105 = 13.5 %; PCB 156 = 12.8 %) of dl-PCBs in all samples. Considering the congener concentration patterns of the different stages, nMDS (stress = 1.92 %) led to a clear separation of both glass eels and elvers from all other groups (PERMANOVA, F-model = 147.23, df = 3, $p < 0.001$ (Fig. 3a)). Yellow eels and silver eels did not differ significantly from each other (PERMANOVA, F-model = 0.23, df = 1, $p > 0.05$).

Sum concentrations of the targeted PCBs in the respective sample groups (glass eels, elvers, yellow eels, silver eels) are displayed in Fig. 2b. Glass eels and elvers originating from the Atlantic Coast and the Vidå creek revealed low accumulated concentrations accounting for a median of 0.3 ng/g ww in glass eels and 0.2 ng/g ww in elvers. Yellow and silver eels from river Elbe showed dl-PCB concentrations summing up to a median of 51.4 ng/g ww and 74.1 ng/g ww respectively. Sum concentrations of measured dl-PCBs in yellow and silver eels showed high interindividual variability (Table 2). However, silver eels were tested to have accumulated significantly higher amounts of dl-PCBs than yellow eels (Mann–Whitney-U-Test W = 48, p = < 0.001). Median TEQs resulting from dl-PCB concentrations in glass eel and elver samples both were lower than 0.1 pg/g ww, while they ranged from 65 pg/g ww for for yellow eels to 71 pg/g ww for silver eels (Table 2). All targeted congeners except PCB 169 were detected in ng/g ww range in yellow and silver eels from river Elbe with some individual congeners ranging below or close to the detection or quantification limits.

PCB accumulation in yellow eels from different sampling locations along the river Elbe

Dl-PCB congener patterns of yellow eel samples from different sites along the river Elbe (Fig. 4a) were dominated by congeners 118, 105 and 156 summing up for 80.9 % (PCB 118 = 52.5 %; 105 = 13.0 %; 156 = 15.5 %) of the targeted dl-PCBs in all samples (Fig. 4a). The nMDS (stress 2.41 %) revealed similar dl-PCB congener patterns between individuals of the different

Table 2 PCB concentrations (median values with maximums and minimums) for each targeted congener along with median sum concentrations [wet weight (ww) and lipid weight (lw)] and resulting TEQs (WHO 2005) of each group obtained in this study

Sampling group	Sampling location [Pos. on map (Fig. 1)]	dl-PCBs										
		PCB-81 (pg g^{1} ww)	PCB-77 (pg g^{-1} ww)	PCB-118 (pg g^{1} ww)	PCB-114 (pg/g^{-1} ww)	PCB-105 (pg g^{-1} ww)	PCB-126 (pg g^{-1} ww)	PCB-167 (pg g^{-1} ww)	PCB-156 (pg g^{-1} ww)	PCB-157 (pg g^{-1} ww)	PCB-169 (pg g^{-1} ww)	PCB-189 (pg g^{-1} ww)
Elbe (Y)	Bad Schandau (1)	273	341	38,049	1915	7832	745	6460	11,959	1093	0	1491
	Min/max	±N/A	±N/A	±N/A	±N/A	±N/A	±N/A	±N/A	±N/A	±N/A	±N/A	±N/A
Elbe (Y)	Dessau (2)	376	281	30,782	1991	6856	737	5532	10,150	1121	0	1224
	Min	172	135	12,821	982	3432	379	2340	4615	540	0	623
	Max	431	438	35,936	2349	7766	1014	7113	13,173	1280	0	1560
Elbe (Y)	Hohengoeren (3)	598	353	25,834	2081	5859	633	4917	8824	1095	0	1154
	Min	450	228	14,285	1099	3097	330	1784	3843	538	0	480
	Max	679	702	83,709	3784	27,253	827	9130	18,091	3175	0	1585
Elbe (Y)	Gorleben (4)	279	150	13,528	916	3634	431	2110	3896	535	0	542
	Min	0	0	6091	421	1791	119	963	1730	264	0	145
	Max	652	313	30,602	2020	6327	745	5448	9945	1294	0	1214
Elbe (Y)	Winsen (5)	756	180	27,133	1963	7113	637	4604	8064	1128	0	1098
	Min	505	0	18,519	1313	4903	402	3345	5437	779	0	698
	Max	1017	367	33,103	2438	9423	807	5782	9747	1521	0	1235
Elbe (Y)	Jork (6)	317	134	11,427	790	2928	229	2685	3677	448	0	574
	Min	0	0	2345	146	606	0	296	462	74	0	60
	Max	1244	350	39,945	2926	10,025	755	5504	7408	1273	0	1108
Elbe (S)	Cuxhaven (7)	785	342	38,839	2486	10,651	689	6324	10,095	1317	0	1265
	Min	265	204	18,697	1421	4585	574	5096	7759	1039	0	1011
	Max	1815	451	63,944	4852	14,381	1347	9926	16,518	2262	19	2116
Rhine (S)	Kalkar (8)	533	505	71,168	3603	22,336	921	7456	13,334	2186	0	1454
	Min	0	0	6542	292	2600	75	690	1205	225	0	119
	Max	939	1483	164,603	6580	50,034	2097	17,598	24,687	4323	0	2261
Schlei (S)	Rendsburg (9)	15	19	2472	275	780	53	346	453	100	5	48
	Min	6	9	1187	132	475	24	195	212	53	0	25
	Max	36	117	8201	981	1425	137	2276	2633	287	9	309
Eider (S)	Nordfeld (10)	35	80	5298	752	1355	146	1374	1693	295	10	267
	Min	26	56	3202	300	920	59	571	881	112	0	104
	Max	112	331	18,087	2298	4620	436	4371	5906	859	17	694
Ems (S)	Emden (11)	24	71	1533	143	1501	74	640	929	464	6	146
	Min	21	52	311	0	505	19	11	0	59	0	39
	Max	250	330	7564	640	21,676	211	2746	3852	1086	25	422

Table 2 continued

Sampling group	Sampling location [Pos. on map (Fig. 1)]	dl-PCBs										
		PCB-81 (pg g^{1} ww)	PCB-77 (pg g^{-1} ww)	PCB-118 (pg g^{1} ww)	PCB-114 (pg/g^{-1} ww)	PCB-105 (pg g^{-1} ww)	PCB-126 (pg g^{-1} ww)	PCB-167 (pg g^{-1} ww)	PCB-156 (pg g^{-1} ww)	PCB-157 (pg g^{-1} ww)	PCB-169 (pg g^{-1} ww)	PCB-189 (pg g^{-1} ww)
ELV	Verlath (12)	0	0	140	0	0	0	15	34	0	0	0
(pools)	Min	0	0	74	0	0	0	0	0	0	0	0
	Max	0	0	499	0	97	0	82	163	0	0	32
GE (pools)	French Atl. Coast	0	10	143	7	59	0	14	18	4	0	3
	Min	0	7	90	5	36	0	7	11	3	0	1
	Max	0	19	292	15	113	3	23	37	7	0	6

Sampling group	Sampling location [Pos. on map (Fig. 1)]	Non dl-PCBs		Median Σ dl-PCBs (ng/g ww)	Median Σ dl-PCBs (ng/g lw)	Median Σ -WHO-PCB-TEQ (2005) (pg g^{-1} ww)
		PCB-170 (pg g^{-1} ww)	PCB-180 (pg g^{-1} ww)			
Elbe (Y)	Bad Schandau (1)	48,496	109,965	80.2	268.6	77
	Min/max	±N/A	±N/A	±N/A	±N/A	±N/A
Elbe (Y)	Dessau (2)	36,758	83,533	59.6	208.9	76
	Min	18,218	40,073	26.3	131.6	39
	Max	49,576	109,855	69.9	241.0	103
Elbe (Y)	Hohengoeren (3)	36,764	73,658	51.4	214.2	65
	Min	14,921	32,318	26.1	144.8	34
	Max	41,752	94,734	148.7	338.0	85
Elbe (Y)	Gorleben (4)	14,550	31,553	25.9	80.7	44
	Min	4722	8598	11.7	32.4	12
	Max	35,430	82,655	58.6	139.4	76
Elbe (Y)	Winsen (5)	26,766	59,398	52.9	244.6	66
	Min	19,581	43,313	36.1	100.2	41
	Max	30,123	68,981	63.9	509.2	83
Elbe (Y)	Jork (6)	15,343	31,995	22.9	227.9	23
	Min	1741	3849	4.0	14.2	0
	Max	28,127	66,391	69.2	660.5	78
Elbe (S)	Cuxhaven (7)	32,153	68,343	74.1	265.0	71
	Min	26,734	52,808	41.3	137.6	59
	Max	68,049	159,318	115.4	450.4	138
Rhine (S)	Kalkar (8)	38,553	74,278	127.2	461.4	95
	Min	3772	7413	11.7	51.1	8
	Max	74,651	150,920	266.0	1157.9	215

Table 2 continued

Sampling group	Sampling location [Pos. on map (Fig. 1)]	Non dl-PCBs		Median Σ dl-PCBs (ng/g ww)	Median Σ dl-PCBs (ng/g lw)	Median Σ -WHO-PCB-TEQ (2005) (pg g^{-1} ww)
		PCB-170 (pg g^{-1} ww)	PCB-180 (pg g^{-1} ww)			
Schlei (S)	Rendsburg (9)	1025	1984	4.6	20.8	6
	Min	698	1139	2.3	10.1	3
	Max	16,322	27,920	16.3	70.9	14
Eider (S)	Nordfeld (10)	8805	13,953	11.3	66.8	15
	Min	3782	5024	6.2	23.9	6
	Max	28,138	43,295	37.7	137.5	45
Ems (S)	Emden (11)	5131	7023	6.0	29.8	8
	Min	1422	2090	3.0	11.2	2
	Max	18,247	21,039	31.4	138.6	22
ELV	Verlath (12)	210	425	0.2	13.8	<1
(pools)	Min	0	0	0.1	4.9	0
	Max	854	1558	0.9	58.1	0
GE (pools)	French Atl. Coast	78	169	0.3	25.5	<1
	Min	42	91	0.2	161	0
	Max	159	279	0.5	50.5	0

Data were grouped according to their life stages (Y = Yellow Eel, S = Silver Eel, ELV = Elver, GE = Glass Eel) and sampling locations, respectively

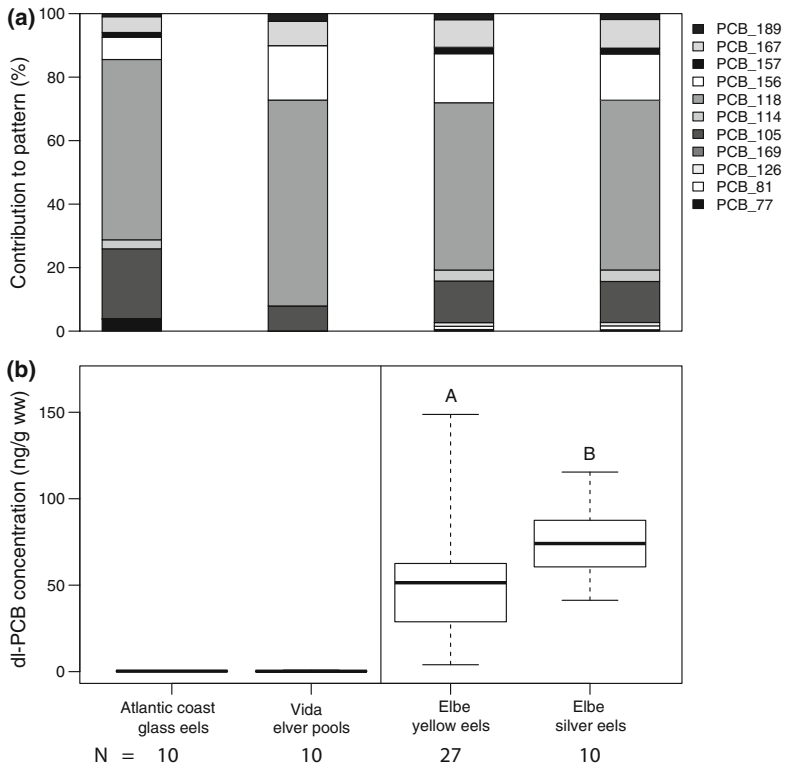

Fig. 2 **a** Median congener patterns of different life history stages (glass eel & elver each pooled (n = 10), yellow eels (n = 27) and silver eels (n = 10)) from river Elbe in percent of total sum dl-PCB. Numbers of the most abundant congeners are given in boxes. **b** Mean sum PCBs in ng/g ww. *Whiskers* represent medium and maximum values, *boxes* represent middle 50 % of data set, *bold line* indicates median value. Significant differences ($P < 0.05$) between groups from the same habitat are indicated by *capital letters*. N indicates the total number of individual eels in the respective groups

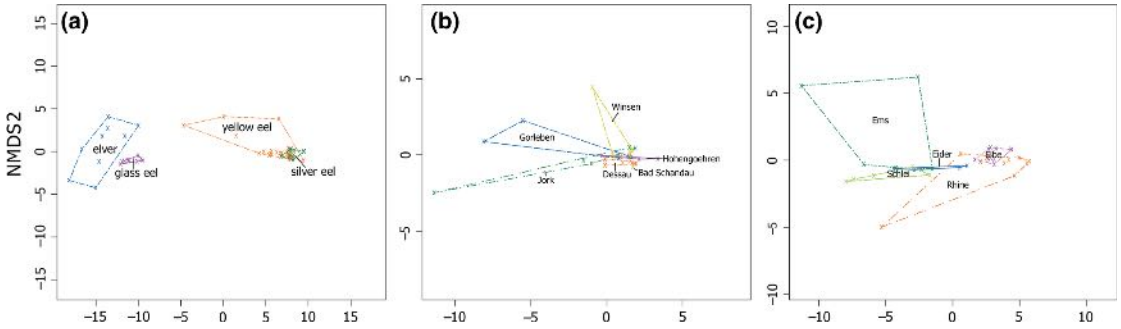

Fig. 3 NMDS Plots of congener patterns displaying **a** different life history stages with **b** yellow eels sampled from different sampling locations along River Elbe **c** silver eels from different German catchments

locations and high variability between individuals for Jork, Gorleben and Winsen (Fig. 3b). No significant differences between the locations along the river Elbe were found (PERMANOVA, F-model = 1.58, df = 5, $p > 0.05$).

Sum concentrations of targeted dl-PCBs in analyzed eels sampled from the respective sampling locations are displayed in Fig. 4b. Yellow eels sampled from different locations along the same habitat showed median concentrations of targeted dl-PCBs ranging from 22.9 ng/g ww in the most downstream sampling location Jork to 80.2 ng/g ww and 59.6 ng/g ww for the most upstream locations Bad Schandau (1 individual) and Dessau, respectively. Although no statistically significant differences were found (Kruskal–Wallis-Test H = 4.92, df = 4, $p = > 0.05$), median sums of dl-PCBs indicated a slight decreasing trend from the Czech Boarder towards the

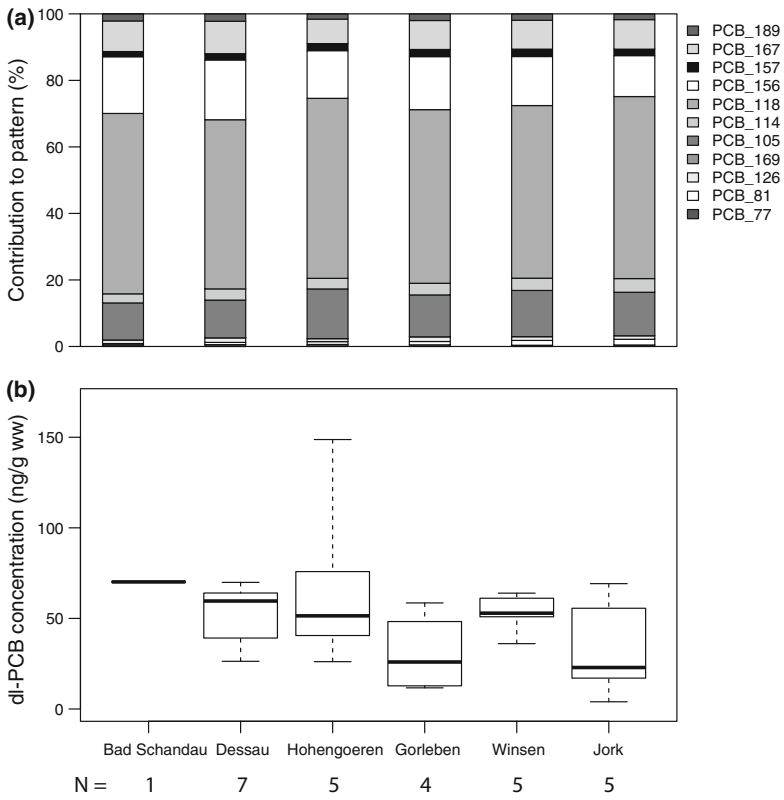

Fig. 4 **a** Median congener patterns and Sum d-PCB of yellow eels sampled along river Elbe in percent of total sum dl-PCB. **b** Sum dl-PCBs of sampled eels given as means arranged in order of distance from the estuary beginning with the location furthest away. N indicates the total number of individual eels in the respective groups (Bad Schandau: n = 1, Dessau: n = 7, Hohengoehren: n = 5, Gorleben: n = 4, Winsen: n = 5, Jork: n = 5)

Estuary of the Elbe River. Mean TEQs resulting from dl-PCBs in yellow eels ranged from 23 pg/g ww (Jork) to 77 pg/g ww (Bad Schandau) (Table 2).

PCB accumulation in silver eels from different German rivers

Congener patterns of silver eels from different German rivers are displayed in Fig. 5a. Strongest represented congeners summing up highest overall concentrations among targeted dioxin-like PCBs in all examined silver eel samples were PCB 118, 105 and 156 accounting for a median sum of 79.5 % (PCB 118 = 48.4 %, 105 = 18.3 %; PCB 156 = 12.8 %). All targeted congeners except PCB 169 were detected in silver eels in ng/g ww range from all investigated habitats.

Silver eels from different rivers could slightly be separated by nMDS (stress: 2.80 %) using their congener concentration patterns (Fig. 3c). Silver eels from the river Elbe differed significantly in their congener patterns from all rivers other than the Rhine, while silver eels from the Rhine displayed congener patterns significantly different from the Schlei and Ems (PERMANOVA, F-model = 11.39, df = 4, $p < 0.05$). Dl-PCB sum concentrations measured in silver eels from different habitats are displayed in Fig. 5b. Concentrations in silver eels from the rivers Rhine and Elbe were significantly higher than in silver eels from Eider, Ems or Schlei (Kruskal–Wallis-Test H = 24.35, df = 4, $p > 0.01$). The highest median sum was found in eels from river Rhine (127.2 ng/g ww) followed by samples from the rivers Elbe (74.1 ng/g ww), Eider (11.3 ng/g ww), Ems (6.0 ng/g ww) and Schlei (4.6 ng/g ww). Median TEQs resulting from dl-PCBs in silver eels summed up to 95 pg TEQ/g, 71 pg TEQ/g, 15 pg TEQ/g, 8 pg TEQ/g and 6 pg TEQ/g ww for silver eels from the rivers Rhine, Elbe, Eider, Ems and Schlei fjord, respectively.

Discussion

PCB congener patterns in eels

PCB patterns in eels analyzed in this study show distinct signs of environmental imprinting with life stage-specific differences among targeted congeners. This is well in line

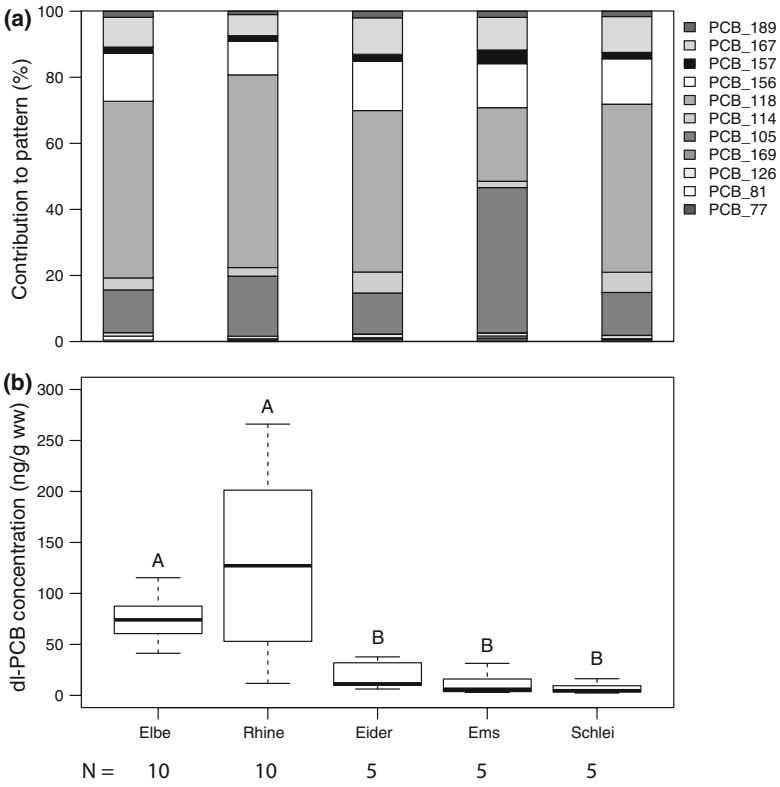

Fig. 5 **a** Median congener patterns of silver eels sampled in different German river bodies given in percent of total sum dl-PCB. **b** Sum dl-PCBs sampled eels in ng/g ww. *Whiskers* represent maximum and minimum values, *boxes* the middle 50 % of the datasets and the *bold line* indicates the median value of each data set. Significant differences in ($p < 0.05$) between groups of fish from different habitats are indicated by *capital letters*. N indicates the total number of individual eels in the respective groups (Elbe: n = 10, Rhine: n = 10, Eider: n = 5, Ems: n = 5, Schlei: n = 5)

with findings of previous studies on eels from Canada, Belgium and France (Tapie et al. 2011; Byer et al. 2013) and should be regarded as crucial for eel management driven stocking measures. While glass eels and elvers represent life stages with no or only short termed influence by their freshwater habitat, yellow and silver eels have been dwelling in their growth habitats for several years resulting in a site-specific alteration of their dl-PCB profile. Possible reasons for occurring differences in congener patterns between life history stages may lie in the phenomenon that highly chlorinated congeners tend to remain in the body longer than less-chlorinated congeners due to their physiological character or they can be result of preferential metabolism (Steele et al. 1986; Hopf et al. 2013). Uptake of lipophilic xenobiotics in water by biota mainly follows three basic pathways: bioconcentration bioaccumulation and biomagnification. While bioconcentration describes the direct uptake from water by diffusion over the body surface (e.g. skin and gills), bioaccumulation is the increase in concentration of a substance in certain tissues within an organism's body due to absorbtion from food and the environment. Biomagnification however, is defined by the increase in concentration of a pollutant from one link in a food chain to another (Kwon et al. 2006; James and Kleinow 2014). This mode of steady and continuous uptake of dl-PCBs and other hazardous, biomagnifying xenobiotics over a longer period of time should be considered in stock management and possible inter-habitat comparisons for restocking measures.

Our results indicate glass eels to be mainly influenced by congeners taken up during their oceanic life, and in contrast, elvers to be already affected by continental pollution impacts, as expressed by higher loads of highly chlorinated dl-PCB congeners as well as higher concentrations of non-dl-PCBs 180 and 170 (Table 2). These congeners occur in higher concentrations in the continental environment due to their widespread anthropogenic use in technical mixtures, their high chlorination degree and resulting persistency. A similar shift in contamination patterns from oceanic to freshwater between glass eels and elvers has previously been reported for PCBs by Tapie et al. (2011), Blanchet-Letrouvé et al. (2014) as well as by Sühring et al. (2013) for brominated and chlorinated flame retardants. The differences in congener patterns despite the similarly low lipid content of glass eels and elvers indicate a rapid uptake of halogenated contaminants by eels as soon as they enter

polluted freshwater habitats during their feeding and growth life history phases. Evidently the growth phase in continental freshwater and coastal systems is the decisive phase for the uptake of contaminants during the eel's life cycle. These findings are in agreement with results from similar studies (Tapie et al. 2011; Arai and Takeda 2012; Byer et al. 2013; Sühring et al. 2013; Blanchet-Letrouvé et al. 2014). Congener patterns of yellow and silver eels from the same habitat in this study showed no significant differences. In addition, congener patterns of targeted PCBs in yellow eels from different sampling sites along the same river system did not differ significantly. These findings indicate either evenly distributed sources for the contaminants or at least the same emission pathways within the same system. Significant differences in congener patterns found in silver eels sampled from different river systems in this study also suggest that sources along the same habitat may play a secondary role compared to the system as a source itself. Since the worldwide ban for PCBs in 2002, active point sources have become increasingly unlikely and recent contamination of biota apparently follows remobilization of PCBs deposited in sediments, soils or suspended particles (Stachel et al. 2004; Wetzel et al. 2013; Lake et al. 2014).

Accumulation and sum concentrations of targeted PCBs in eels

The here measured PCB levels and ranges are well in line with findings from previous studies. Sum concentrations of PCBs measured in glass eels are similar to comparable investigations along the French Atlantic coast (Blanchet-Letrouvé et al. 2014). Results from yellow eels from river Elbe were close to results for yellow eels from the Elbe published by Stachel et al. in 2007 and comparable to yellow eels in similar size and age from the French river Loire (Blanchet-Letrouvé et al. 2014; Couderc et al. 2015). Silver eels from the Rhine and Elbe analyzed in this study however, showed very high concentrations of targeted PCBs, almost up to twice as high as found in silver eels from the Loire Estuary (Blanchet-Letrouvé et al. 2014; Couderc et al. 2015).

Previous studies by Belpaire et al. (2007, 2008), Byer et al. (2013) as well as Sühring et al. (2013) reported high intra-habitat variability in sum concentrations for halogenated contaminants such as PCBs and BFRs in yellow eels, concluding this life history stage to be most suitable for the detection of local point sources due to their sedentary lifestyle (Belpaire and Goemans 2007; Belpaire et al. 2008; Van Ael et al. 2014). In the here presented study, results of sum concentrations of targeted PCBs in yellow eels showed no significant differences along different sampling locations within the same habitat (river Elbe). Nevertheless, a slight decreasing tendency was observed from the upper river towards the tidal zone close to the river's mouth. This may be rooted either in age and lipid content of tested individuals or in local passive sources of the contaminants, which does match with the literature. A previous study by Stachel et al. (2004) reported sediments in the mouth of the Mulde river and a source in the Czech Republic to be the main historical entry paths of PCBs into the Elbe system.

Generally speaking, accumulated sum concentrations of targeted PCBs in eel samples used in this study were correlated to life history stage. Similar to findings concerning the congener patterns, glass eels and elvers show rather low alterations concerning concentrations of targeted PCBs compared to yellow and silver eels. Reasons for this lie in the habitat dwell time of each individual that defines the range and intensity of contamination stress it was exposed to. In a study by Tapie et al. (2011), the authors investigated PCB concentrations in eels and their data revealed a clear rise of accumulated PCBs with age and size of the fish as well. However, this accumulation effect is specifically critical for semelparous species like the European eel but has to be viewed at in context with the fish's life history stage. While studies on yellow eels may allow for a valuable snapshot of their current contamination status, silver eels on their downstream migration form the most representative life history stage to provide information on the health and fitness status of local populations from a certain habitat. Looking at our results of dl-PCB concentrations in muscle tissue of silver eels from different water bodies, it becomes evident that the respective origin of each eel is the most important driving factor for final lipophilic contaminant loads. Silver eels have a lower variation in fat content since they begin the migration to their spawning grounds (Larsson et al. 1990) and are not likely to experience any further influential events with strong impact on their complete and final contamination status. These final amounts of accumulated OCs may be important for future assessments of the contaminants potential risks for the eels offspring after possible maternal transfer (Palstra et al. 2006; Sühring et al. 2015) or for the lipid metabolism of the individual itself during its migration (Corsi et al. 2005). Unfortunately effects of dioxin-like contaminants on eels are yet not entirely understood and (due to a lack of available data) it remains difficult to entirely assess the consequences for the health and reproductive capability of eel stocks caused by dioxin-like contaminants. One possible way to quantitatively facilitate risk assessment and regulatory control of the toxicity of these compounds is the use of TEQs.

TEQ-levels of silver eel samples out of nearly all sampling locations (except fish from the Schlei fjord and River Ems) in this study exceeded the minimum risk levels

(MRLs) of 12 pg/g ww TEQ for human consumption (EC regulation No 1881/2006) and those (less than 4 pg/g ww TEQ), that were held responsible to have impaired normal embryonic development of eels in a study by Palstra et al. (2006). Generally speaking, total sums of PCBs and their resulting TEQs in analyzed silver eels from this study were highly dependent on their provenance and the respective urbanization: While Rhine or Elbe account for industrial rivers with historically higher anthropogenic influence and sediment contamination, more rural rivers such as the Eider, Ems or the Schlei fjord (as an example for Baltic-associated water body) tend to produce silver eels with lower loads of lipophilic contaminants and as a result form more suitable habitats for eels in terms of their contamination-related reproductive capacities. With regard to German and other European national eel management programs which contemplate restocking as a stock enhancement measure, it has to be considered whether restocking is meant to support local utilization and use for commercial interests or if it is done for stock enhancing purposes to increase the escapement of healthy and high-quality spawners.

Conclusions

This study strongly confirms that (dl-)PCB contamination of eels is mainly driven by uptake during their continental growth phase. Eels originating from the here analyzed German river systems differ significantly in their total sum PCB contamination and pollution patterns. The potential negative effects of dl-PCBs on the health and reproductive capability of eels make it crucial to evaluate designated habitats for restocking of eels. We conclude that concentrations of dl-PCBs found in muscle tissue of silver eels can be used along with other crucial indicators to describe the quality of their respective habitat. Considering the high impact of habitat combined with the continuous accumulation of PCBs up to the silver stage, stocking and reallocation of young eels as stock enhancement measures should only be performed in suitable habitats. For this, the contamination levels of the rivers and river sections should be assessed and only the most suitable water bodies or sections should be selected. This implies meeting requirements and conditions for eels to gain an improvement of living conditions in their continental phase and thus, to produce healthy and qualitatively generative silver eels and spawners, which is in accordance with the goals of the eel management plans of the European Union.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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CHAPTER II

Maternal transfer of dioxin-like compounds in artificially matured European eels

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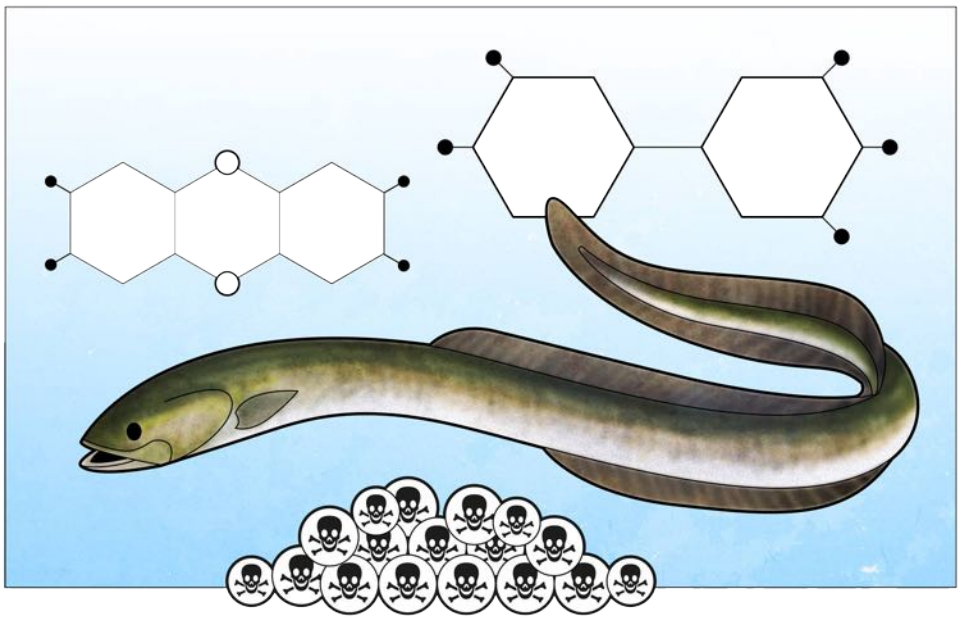

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Maternal transfer of dioxin-like compounds in artificially matured European eels☆,☆☆

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ABSTRACT

Several eel species of the genus *Anguilla* are considered endangered due to a severe decline in recruitment. Up to now, the reasons for this threatening development are not fully understood. The eel's highly specialized biology can lead to explicitly high accumulation of globally distributed organic lipophilic contaminants during its continental life. Because of this and due the particular toxicological sensitivity of early life stages of oviparous organisms towards dioxin-like compounds, it is crucial to improve our understanding concerning toxicokinetics and maternal transfer of organic contaminants in eels.

This study presents analytical data on maternal transfer of dioxin-like (dl) compounds in relevant tissue samples taken from artificially matured and non-matured European silver eels (*Anguilla anguilla*) from German inland waters using gas chromatography coupled with mass spectrometry (GC/MS) and high-resolution mass spectrometry (GC/HRMS). Detected concentrations revealed a lipid-driven transfer of targeted compounds from muscle-fat-reserves to gonads and eggs respectively, with no distinct preferences concerning the chlorination degree of targeted compounds. Dl-PCBs were shown to contribute the major share of toxicity equivalents found in analysed eel tissues. Maternal muscle tissue to egg concentration ratios in wet weight–based samples had a mean of 6.95 ± 1.49 in accordance with the differences in total lipid content in the respective body matrices. Dioxins and furans in analysed samples were (from a toxicological point of view) of less relevance. Furthermore it was shown that muscle concentrations in silver eels could be used in future assessments to make conservative predictions for expected egg concentrations in female eels.

1. Introduction

Since the 1980s, the three of the temperate freshwater eel species European eel (*Anguilla anguilla*), American eel (*Anguilla rostrata*) and Japanese eel (*Anguilla japonica*) have experienced severe declines in glass eel recruitment (Dekker et al., 2003; ICES, 2014). As a consequence, the affected species have been rated as critically endangered (*Anguilla anguilla*) and endangered (*Anguilla rostrata* and *Anguilla japonica*) by the International Union for Conservation of Nature (IUCN). A number of different hypotheses on possible causes have been raised including habitat loss and degradation, overfishing, oceanic changes, parasitism and pollution (Knights, 2003; Geeraerts and Belpaire, 2010; Wysujack et al., 2014; Miller et al., 2015). It is more than likely that only a combination of these impacts has led to the recruitment declines and it is important to identify and evaluate the major drivers in this combination of influential factors.

☆ This paper has been recommended for acceptance by Dr. Harmon Sarah Michele.

☆☆ This work provides novel analytical data on maternally transferred dioxin-like contaminants measured in European eel eggs.

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Anthropogenically introduced chemical pollution especially by halogenated lipophilic persistent organic pollutants (POPs) is believed to be capable of severely impairing the reproductive success of European eels (Palstra and Van den Thillart, 2010, Geeraerts and Belpaire, 2010; Geeraerts et al., 2011; Sühring et al., 2015; Foekema et al., 2016). Dioxin-like compounds such as polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and dioxin-like polychlorinated biphenyls (dl-PCBs) are considered to be among the most toxic manmade substances in the world and constitute a frequently discussed group of hazardous contaminants in scientific literature. Dioxin-like compounds have been shown to cause several health effects on animals including endocrine disruption, terato- & mutagenesis, hepatic damage and impaired reproduction (Cook et al., 2003; Mandal, 2005; Palstra and Van den Thillart, 2010; King-Heiden et al., 2012; Foekema et al., 2014; Rigaud et al., 2016).

The eel's specific predisposition towards lipophilic contamination as semelparous, bottom-dwelling predators with naturally high body fat contents in combination with the chemical properties of dioxin-like substances and their high concentration in sediments and biota of many continental water bodies can lead to comparably high accumulation in muscle tissue of this species (Stachel et al., 2007; Byer et al., 2013; Blanchet-Letrouvé et al., 2014; Freese et al., 2016). A number of studies have made clear that different chemical profiles as well as different concentration ranges of contaminants in eel samples are related to the respective habitat (Belpaire et al., 2008; Sühring et al., 2013; Van Ael et al., 2014; Kammann et al., 2014; Blanchet-Letrouvé et al., 2014; Freese et al., 2016). Nevertheless, with exception of modeled scenarios (Foekema et al., 2016) and a single experimental work by Palstra et al., in 2006, no scientific studies are available in literature, in which the actual transfer of dioxin-like substances from the maternal tissue to eggs or larvae was investigated in eels.

In their study, Palstra et al. (2006) put the survivability of eel embryos in relation with Toxicity Equivalents (TEQs) of dioxin-like compounds (DLCs) determined by the DR-CALUX test in gonad and muscle tissue of artificially matured eels as well as in a control group. Even though the maternal transfer of dioxin-like substances and other POPs have already been described in many other species (Henriksen et al., 1996; Russell et al., 1999; Sühring et al., 2015), a lot of uncertainty about the involved mechanisms and effects of DLCs and their physiological relevance in eels remains. Reason for this may be that large parts of the eel's natural reproduction cycle are still considered a mystery and it is yet not entirely possible to artificially reproduce European and American eels. However, progress on the protocols in the artificial maturation and hatchery design made it possible to shed some light on the reproduction biology of these highly specialized species (*A. anguilla*: Tomkiewicz (2012); *A. rostrata*: Oliveira and Hable, 2010).

The major aim of this study was to get detailed insights into the extent of maternal transfer from body lipid reservoirs into ovarian tissues of dioxin-like substances during maturation of eels from European water bodies and also to gather information on the biological mechanisms and driving factors involved. As a consequence, this study was intended to enable the estimation of the total DLC TEQ-concentrations per egg-mass deriving from dl-PCB contamination in muscle tissue from female silver eels representing *in situ* occurring contamination histories.

2. Material & methods

2.1. Samples

In this study we used female, migrating silver eels caught with fyke nets by commercial fishermen in the potamal sections (lower stretch) of the river Ems and the Schlei fjord in February 2013. From each sampled water body, we bought complete commercial hauls of fish in line with samplings done for the European Data Collection Framework (DCF), as defined by the European Commission (2008, 2010). After acclimatization in flow-through freshwater tanks for seven days, eels were sacrificed and their otoliths excluded for age estimations following an expert protocol for age determination in eels (ICES, 2009, 2011). For possible later use, samples of white muscle and gonadal tissues were taken from each specimen. For this, between 10 and 25 g of fresh gonad- and skin-free muscle tissue taken from the filet between anus and tip of the tail of the eels were sampled and stored at −20 °C until usage. To eliminate sources of contamination, samples were strictly handled with clean equipment made of glass, aluminum or steel, preventing possible sources of cross-contamination. After age reading, samples from five female eels of comparable length, weight, age and migration stage (Durif et al., 2005) from each water body were selected to determine their dioxin-related contamination (See supplement information S1 for a detailed list of individual variables).

For artificial maturation, five fish from each batch were acclimatised to saltwater (20 ± 1 °C; 35 ± 0.5 practical salinity units (PSU)) and held under constant water flow in a round recirculation system equipped with aeration stones and a trickle filter for mechanical filtration and denitrification. All artificially maturated fish were held in the experiment for a timespan of 17–19 weeks until final gonadal maturation. As under natural conditions migrating and maturing silver eels are believed not to feed anymore, maturing eels in this experiment were constantly moving against gentle water flow and received no food. The eels were hormone-treated by one weekly intramuscular injection (20 mg/kg into the dorsal muscle, close to the dorsal fin) of aqueous salmon pituitary extract (SPE, Argent Aquaculture, Redmond, USA) to induce maturation and egg development. With a final injection of 17α,20β-Dihydroxy-4-pregnen-3-one (DHP, Sigma-Aldrich, Taufkirchen, Germany) ovulation was induced and after stripping the eels were sacrificed and dissected for further analyses. Only entirely matured (visually evaluated during dissection) eels (two from Schlei and three from Ems) were selected for chemical analyses. Tissue samples of gonads, eggs and muscle from hormone-treated fish were taken according to the sampling described for the untreated fish. All eels in this study were killed by decapitation after being anaesthetized with 2-Phenoxyethanol (ROTH, Karlsruhe, Germany).

2.2. Total lipid content in organs and tissue groups

Total extractable lipid levels in analysed tissue were determined as described by Smedes (1999) along with methodological alterations introduced by Schlechtriem et al. (2012). Briefly, approximately 100 mg of homogenized, freeze-dried tissue sample was used for lipid extraction with a mixture of cyclohexane (2.50 mL), propan-2-ol (2.00 mL) and water (2.75 mL), followed by a second extraction with cyclohexane (2.175 mL) and propan-2-ol (0.325 mL). The organic phase was collected after each extraction and the solvents were evaporated prior to gravimetric determination of the fat content. All samples were analysed in duplicates.

2.3. Extraction and clean-up

All analysed compounds were prepared the same way before extraction by pressurised liquid extraction: Frozen silver eel tissue samples were homogenized with anhydrous Na_2SO_4 (2:1; w/w) for approximately 20 min using a 1 L stainless steel/glass laboratory blender (Rotorblender, neoLab, Heidelberg, Germany). Then, separate aliquots for analyses of dl-PCBs and PCDDs/PCDFs were spiked with ^{13}C mass labeled surrogate standards analogous for each

analysed compound respectively. (PCBs: WHO PCB + PCB-170+PCB-180 CLEAN-UP STANDARD ($^{13}C_{12}$, 99%), Cambridge Isotope Laboratories (CIL), Tewksbury, USA; PCDD & PCDFS: EPA1613 LCS, Wellington Laboratories, Guelph, Canada). Any remaining volume in the extraction cartridges was filled with anhydrous Na_2SO_4 (ROTH, Karlsruhe, Germany). Spiked, homogenized samples were extracted by accelerated solvent extraction (ASE-200, Dionex, Sunnyvale, USA) using dichloromethane (DCM, ROTH, Karlsruhe, Germany) at 100 °C and 120 bar, following the method described in Sühring et al. (2013).

2.4. PCDD & PCDFs clean-up & analyses

For PCDD and PCDF clean-up, CAPE technology acid silica columns (Cape Technologies L.L.C., South Portland, ME, USA) with carbon mini-columns were used. Each of these columns was conditioned using 10 mL each of acetone and hexane while the carbon mini-column was conditioned with 10 mL each hexane and toluene. The carbon mini-column was attached to the outlet of the acid silica column and the extracts were then applied onto the acid silica column using the CAPE glass-syringe funnel.

First, the targeted analytes were eluted onto the activated carbon mini-column using ten ml of hexane. Subsequently, 20 mL of hexane were used to elute the dl-PCBs from the column. Following that, the mini-column was detached from the acid silica column and connected with a clean, empty CAPE column. Afterwards, five mL of a toluene-n-hexane (v/v 1:1) mixture was used to extract any remaining dl- PCBs from the column. The carbon mini-column was then reversed and the PCDDs/PCDFs were eluted with 30 mL of toluene. Analysis of PCDDs/PCDFs was conducted in accordance with the method previously published by Byer et al. (2013). Briefly, gas chromatography/high-resolution mass spectrometry (GC-HRMS) analyses were performed using a Micromass AutoSpec mass spectrometer (Micromass, Manchester, UK) in electron ionization (EI) and selected ion-monitoring (SIM) modes. The mass spectrometer was coupled with a Hewlett–Packard 6890 gas chromatograph (Hewlett Packard, Palo Alta, CF, USA) fitted with a Restek Dioxin-2, 60 m × 0.25 mm × 0.25 μm column (Restek, Bellefonte, PA, USA) and an CTC A200S autosampler (Leap Technologies, Chapel Hill, NC, USA). Following settings were used: Helium as carrier gas: 1.5 mL min^{-1}; source temp: 280 °C; front Inlet temp: 280 °C; transfer line temp: 280 °C; splitless injection: 1.5 min at 30 mL min^{-1}. The system was tuned using perfluorokerosene as a reference (10,000 resolution at 5% peak height definition) over the mass range of the PCDD/F. To ensure stable conditions, the instrument was calibrated after every batch of five samples and the instrument was re-tuned and re-calibrated daily.

2.5. Dl-PCB clean-up & analyses

As described in Sühring et al. (2013), clean-up of the samples was done by gel permeation chromatography (GPC), using 30 g Bio-Beads SX-3 (Bio-Rad Laboratories, Hercules, USA) and dichlormethane:hexane (1:1; v:v) as eluent. While discarding the first fraction (75 mL), the second fraction (110 mL) containing the target compounds, was reduced to about 2 mL before its transfer into hexane. As a second step, we used a column with 2.5 g 10% H_2O deactivated silica gel (ROTH, Karlsruhe, Germany) and 20 mL of hexane as an eluent, before the samples were narrowed down to a volume of 150 μL and transferred to measurement vials. Finally, 10 μL ^{13}C-PCB 141 and ^{13}C-PCB 208 (50 ng mL^{-1}) was added as injection standard to each sample.

Analyses of targeted PCBs were conducted using a GC/MS-system (Agilent 6890 GC/5973 MSD, Agilent Technologies, Santa Clara, USA) equipped with a HP-5MS column (30 m × 0.25 mm i.d. × 0.25 μm film thickness, J&W Scientific, Agilent Technologies, Santa Clara, USA) operating in electron capture negative ionization mode (ECNI) with methane as ionization gas. Samples in our study were analysed for dl-PCBs (IUPAC numbering) 77, −81, −105, −114, −118, −126, −156, −157, −167, −169 and −189.

3. QA/QC

All samples were handled in a clean-lab class 10000 (United States federal standard 209E).

3.1. PCDD & PCDFs

Analysis was performed in batches of five, in combination with two blanks and one certified reference material (CRM) sample (CARP-2, National Research Council of Canada) per batch. CARP-2 reference values then were compared to the measured results with a paired *t*-test (mean values from five replicates). 1,2,3,7,8-PeCDF and 1,2,3,4,6,7,8-HpCDF were up to 15% lower than the reference values (Student's t-test), while the remaining PCDD/F congeners were statistically indistinguishable from the reference values. Blank values were generally low with “not detected” for most analysed compounds. LODs ranged from 0.55 pg/g wet weight (ww) (1,2,3,4,7,8-HxCDD) to 2.4 pg/g ww (2,3,7,8-TCDF). LOQs ranged from 1.5 pg/g ww (1,2,3,4,7,8-HxCDD) to 5.2 pg/g ww (2,3,4,7,8-PeCDF). Recoveries of ^{13}C isotope marked surrogate standards were good and ranged between 77% and 128% with an average of 101%. For statistical analyses, concentrations below LOD were assigned a value of 1/2 of the LOD (mid-bound), concentrations below LOQ (one sample) were included in calculations.

3.2. PCBs

Recovery rates of isotope labelled (^{13}C) internal standards (IS) were determined for each sample. Mean IS recoveries ranged from 57 ± 26% for PCB 81 to 96 ± 34% for PCB 169. A blank test, using Na_2SO_4 treated similar to real samples was performed with every extraction batch (eleven samples). All blanks were either below the method quantification limit (MQL) or otherwise 1–2 magnitudes lower than lowest samples concentrations. The limits of detection and quantification (LOD/LOQ) were calculated either from the blank plus 3 times or 10 times blank standard deviation, or from a signal to noise ratio of 3 or 10, respectively. LODs ranged from 0.99 ± 0.5 pg/g ww (PCB 189) to 30.9 ± 29.7 pg/g ww (PCB 77). LOQs ranged from 3 ± 1.4 pg/g ww (PCB 189) to 102.3 ± 99.9 pg/g ww (PCB 77). For further quality control, a twofold measurement was conducted for each sample. Results for PCB 123 were entirely excluded from our results due to incomplete chromatographic separation. For statistical analyses, concentrations below LOD were assigned a value of 1/2 of the LOD (mid-bound), concentrations below LOQ were included in calculations.

3.3. Data processing and statistical analyses

To assess toxicological relevance and provide for good comparability of results from our study with literature, World Health Organization Toxic Equivalent values (WHO_{2005} TEQs) were calculated based on re-evaluated Toxic Equivalent Factors (WHO_{2005} TEFs) (Van den Berg et al., 2006). All statistical analyses were performed using GraphPad Prism (Prism 6.0h, October 2015, GraphPad Software Inc., Ca, USA). Differences in accumulation quantities of targeted dl-PCBs between the respective sample groups were tested using the sum concentrations of individuals in each group. When testing two groups against each other, the Mann-Whitney

test was performed. When testing more than two groups against each other, a Kruskal-Wallis-Test was performed. After investigating a possible influence of habitat on relevant characteristics in sampled untreated silver eels from Ems (N = 5) and Schlei (N = 5), we combined all untreated fish to one group (N = 10) to compare them against the group of hormone-treated (N = 5) fish.

4. Results and discussion

4.1. Influence of sampling origin

Eels used in this study were caught in 2 German catchments. Length, weight and muscle lipid content of fish were not statistically different between the untreated groups from the two catchments (Mann-Whitney test of unpaired *t*-test; length: P = 0.73; start weight: P = 0.73; muscle lipid content: P = 0.55) (Table 1). The origin of the sampled individuals also showed no statistical influence on the total concentration of targeted compounds detected in the sampled (untreated) fish (Mann-Whitney test of unpaired *t*-test; P = 0.55). (See supplement information S1 for a detailed list of individual variables and concentrations).

4.2. Lipids and body composition in eels during maturation

Along with the metabolic reallocation of lipid stores from muscle to reproductive tissues, analytical data from our study confirm a transfer of dioxin-like contaminants from maternal somatic to reproductive tissues in European eels. Total extractable lipid content in wet muscle tissue did not differ significantly between hormone-treated and untreated eels (Table 1) (Mann-Whitney test of unpaired *t*-test; P = 0.49). This is well in line with observations made for other artificially matured European eels in studies by Palstra and Van den Thillart in 2010 or Nowosad et al., in 2014 and Japanese eels by Ozaki et al., in 2008, in which artificially matured eels maintained their muscle lipid content and general body composition at the same levels as untreated eels. Nevertheless, total muscle-mass was reduced which indicates, as also suggested by Ozaki et al. (2008) that in addition to lipids, other macronutrients such as proteins/amino acids are being metabolized in maternal muscle tissue during starvation and maturation. In line with these depletions of energy reserves in muscle tissue, gonadal mass in hormone-treated eels multiplied, making up to 51.6 ± 6.1% of total pre spawning body weight compared to 1.4 ± 0.3% in untreated eels. (See supplement information S1 for a detailed list of individual variables).

4.3. Tissue concentrations

DLC concentrations measured in eel muscle tissue in this study are in similar ranges as found in previous studies on European eels. Total WHO_{2005} TEQ concentrations for ΣPCDD/F ranged between 2 and 9 pg WHO_{2005} TEQ/g ww, including estimated middle-bound LOD concentrations for non-detected congeners. These results are in a comparable range as found in other studies on eel from European water bodies (Bordajandi et al., 2003; Stachel et al., 2007; Szlinder-Richert et al., 2010; Byer et al., 2013; Blanchet-Letrouvé et al., 2014).

TEQ concentrations for dl-PCBs in eel muscle tissue ranged from 8.35 to 75.56 pg WHO_{2005} TEQ/g ww in hormonally treated eels and from 1.98 to 40.35 pg WHO_{2005} TEQ/g ww in untreated eels with (by far) highest contribution of congener 126 to total WHO_{2005} dl-PCB TEQs. Also these results were comparable to concentrations found in previous studies for eels muscle from some European water bodies in Belgium, Germany and France (Stachel et al., 2007; Geeraerts et al., 2011; Blanchet-Letrouvé et al., 2014). The high individual variability in tissue concentrations (also from fish within the same water body) reflects the difficulties associated with field studies on fish contamination. Eels obviously are mobile throughout their continental life, which may lead to different contamination ranges due to local differences of pollution within different parts of the habitat (Freese et al., 2016). Concerning dl-PCBs TEQ concentrations in gonads and eggs, we are not aware of many available publications with data on these matrices. Concentrations in eel eggs derived from indirect measurements using DR CALUX bioassay in a study by Palstra et al. (2006) predicted similar concentrations in eel eggs as measured in this study.

4.4. Tissue burden calculations

To depict the physical transfer of muscle (lipid)-bound POPs into the egg mass, we calculated the amounts of total dl-PCBs bound in entire reproductive tissue and put them in contrast to the absolute amount of these compounds calculated in total muscle tissue of the same individuals per group (Fig. 1).

$$B\ REP = m\ (egg)\ {}^{*}c_{\Sigma dl\text{-}PCB}\ (egg) + m\ (gon)\ {}^{*}c_{\Sigma dl\text{-}PCB}\ (gon)$$

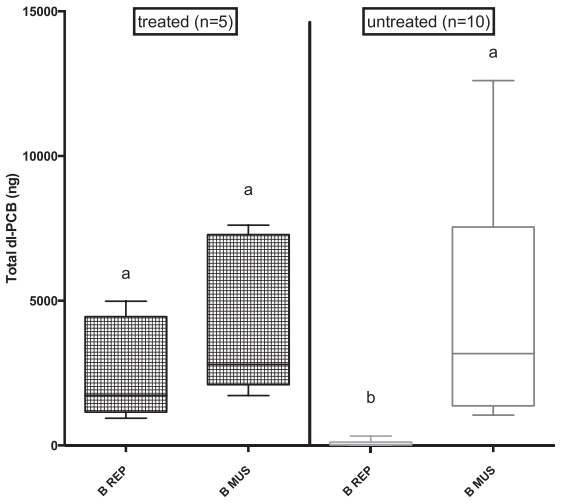

Fig. 1. Tissue burdens (based on wet weight) of dl-PCBs bound in muscle and gonadal tissue in hormone-treated and untreated silver eels. Median values indicated by horizontal lines in boxes, whiskers represent data range.

Table 1
Biometric parameters (if applicable) including bodylength, bodyweight (before and after treatment) and lipid content of eels used in this study.

Life stage	n	Length (cm)	Start weight (g)	End weight (g)	Muscle lipid (%)	Gonad lipid (%)	Egg lipid (%)
Hormone treated	5	73.6 ± 8.8 (63–81)	755.0 ± 294.1 (405–1042)	957.2 ± 336.2 (567–1385)	27.7 ± 6.5 (21.9–35.0)	11.8 ± 4.1 (6.6–15.3)	5.2 ± 0.6 (4.3–5.9)
Untreated	10	69.7 ± 4.1 (62–76)	654.3 ± 117.1 (474–875)	N/A	25.3 ± 3.3 (20.2–30.8)	18.9 ± 5.8 (11.5–26.8)	N/A

Data are given in mean values ± standard deviation (minimum-maximum) where applicable.

B MUS = (m (carc)-m (rest))*c $_{\Sigma dl\text{-}PCB}$ (mus)

where B REP is the total mass of hydrated eggs (*m (egg)*) and the mass of remaining gonadal tissue (*m (gon)*) multiplied with measured dl-PCB concentrations found in respective reproductive tissues ($c_{\Sigma dl\text{-}PCB}$ *(egg)* + $c_{\Sigma dl\text{-}PCB}$ *(gon)*) and B MUS is the dl-PCB concentration found in total muscle tissue ($c_{\Sigma dl\text{-}PCB}$ *(mus)*) calculated by the mass of the eels carcass (*m (carc)*) minus the combined mass of remaining tissue types (m (rest)) including reproductive tissues, intestines, skeletal bones and skin multiplied with measured dl-PCB concentrations found in muscle tissue samples. (See supplement information S1 for a detailed list of individual variables).

Total amounts of dl-PCBs bound in muscle and reproductive tissue of hormone-treated fish compared to amounts bound in gonad-tissue of untreated fish differed significantly (Kruskal-Wallis-Test H = 18.63, p = 0.0003), with gonads of untreated fish having significantly less dl-PCBs bound than any other tested tissues. This finding reflects the change in mass of gonadal products in relation to total body mass between fully matured and non-mature silver eels during maturation. Although no statistically significant difference was found between muscle tissue of untreated fish compared to muscle tissue of hormone-treated fish (Mann Whitney test of unpaired *t*-test; P = 0.86), it is noteworthy that tissue burdens in muscle of untreated fish showed a wider range than concentrations found in muscle tissue of treated fish. As a result, combined muscle and gonad/gonad&egg burdens of both groups sum up to similar concentration ranges (treated = 2661–11944 ng dl-PCB; untreated = 1072–12930 ng dl-PCB) with no significant differences (Mann-Whitney test of unpaired *t*-test; P = 0.24), underlining a statement made in our previous study (Freese et al., 2016), that escaping silver eels have reached their "final contamination status" before spawning migration and at the same time, giving further indication that elimination of higher chlorinated PCBs during starvation and migration is negligible (de Boer et al., 1994).

4.5. Maternal transfer of PCDDs & PCDFs

PCDDs and PCDFs were analysed in a subsample of n = 3 artificially matured and n = 4 non-matured individuals used in this study (Table 2).

Results revealed that total concentrations of PCDDs/PCDFs compared to those of dl-PCBs in eels from the sampled habitats play a secondary role, as most PCDDs/PCDFs congeners were not detected in any of our analysed samples. As a result, non-ortho and mono-ortho PCBs constituted the vast majority in both, total concentration and TEQs of analysed dioxin-like compounds in this study (Fig. 2). Detected PCDDs & PCDFs in all samples had detection frequencies of less than 5% compared to 100% for most analysed dl-PCB congeners. Concentrations of total dl-PCBs were in ng g^{-1} ww range while quantified concentrations of dioxins and furans were much lower, with maximum total PCDD concentrations of 6.5 pg/g ww in gonads of the comparison group fish and maximum total PCDF of 31 pg/g ww in eggs from one hormone treated eel from river Ems. Congruent with the results reported by Byer et al. (2013) for eels from Belgium, 2,3,7,8-TCDF, 1,2,3,7,8-PeCDD and 2,3,4,7,8-PeCDF were the highest concentrated PCDD/PCDFs in muscle and gonad tissue of the European eel comparison group, rather than TCDD reported as the predominant PCDD/PCDF in American eels from the Great Lakes region (Byer et al., 2013). The overall detection frequencies were too small to derive any statistically significant conclusions.

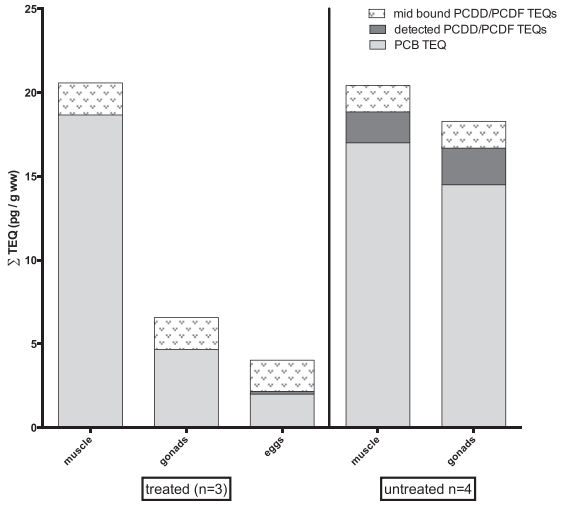

Fig. 2. Mean contributions of dl-PCBs, middle bound LOD and/or detected PCDD & PCDFs to total dioxin TEQ_{2005} concentrations based on wet weight in three matrices (muscle, gonad and eggs) of hormone-treated eels (n = 3) and in two matrices (muscle and gonad) of untreated eels (n = 4). For TEQ calculations, concentrations below LOD were considered as half the LOD (middle bound).

Table 2
Overview of amalgamated data obtained for samples of analysed hormonally treated and untreated eels. Units or sample specifications are indicated in brackets.

		Hormone-treated (n = 5)	Untreated (n = 10)
dl-PCBs	Σdl-PCB in muscle (pg/g ww)	28,500 ± 26,500 (10,609–73808)	14,300 ± 14,550 (2780–46861)
	WHO_{2005}-PCB-TEQ (muscle)	28.0 ± 27.9 (8.35–75.56)	12.2 ± 12.5 (1.98–40.35)
	Σdl-PCB in gonads (pg/g ww)	8400 ± 5800 (4904–18701)	8400 ± 10,900 (2134–37426)
	WHO_{2005}-PCB-TEQ (gonads)	6.5 ± 5.4 (2.53–15.99)	7.5 ± 9.2 (1.64–25.92)
	Σdl-PCB in eggs (pg/g ww)	4450 ± 4862 (1843–13062)	N/A
	WHO_{2005}-PCB-TEQ (eggs)	3.8 ± 4.4 (1.04–11.46)	N/A
	Transfer Ratio muscle/gonads	3.2 ± 1.4 (1.89–5.16)	2.3 ± 1.9 (0.85–6.68)
	Transfer Ratio muscle/eggs	7.0 ± 1.5 (5.27–8.92)	N/A
		Hormone-treated (n = 3)	Untreated (n = 4)
PCDD/PCDFs	ΣPCDD & PCDF in muscle (pg/g ww)	9 ± 0 (9–9)	13 ± 7 (9–25)
	WHO_{2005}-PCDD/F-TEQ (muscle)	1.9 ± 0.00 (1.91–1.91)	3.4 ± 2.9 (1.91–7.77)
	ΣPCDD & PCDF in gonads (pg/g ww)	9 ± 0 (9–9)	14 ± 9 (9–28)
	WHO_{2005}-PCDD/F-TEQ (gonads)	1.9 ± 0.00 (1.91–1.91)	3.8 ± 3.8 (1.91–9.41)
	ΣPCDD & PCDF in eggs (pg/g ww)	23 ± 16 (9–40)	N/A
	WHO_{2005}-PCDD/F-TEQ (eggs)	2.0 ± 0.1 (1.91–2.15)	N/A

Data are given in mean values ± standard deviation (minimum-maximum) where applicable.

It is interesting to note that in hormone-treated eels, eggs were the only tested matrix in which 1,2,3,4,6,7,8-HpCDF was detected. This is especially noteworthy since lipid content in eggs was overall lower than in muscle tissue (Table 1). With respect to the small number of tested individuals, these findings could eventually be an indication for a selected transfer, changes in uptake, distribution or metabolism during the artificial maturation process, as we have previously observed for flame retardants (Sühring et al., 2015). Another influential factor could be the composition of the eggs, including different lipid classes as well as vitellogenin, an egg yolk precursor protein for the lipoproteins and phosphoproteins present in the protein content of yolk. Vitellogenin has been suggested to associate with 2,3,7,8-TCDD and therefore may play an important role as a vector in maternal transfer of dioxin-like substances. Its structure with both, phosphate–rich regions and large non-polar lipid moieties makes it well suited to function as a vessel or vector for maternal transfer of a variety of compounds (Monteverdi and Di Giuio, 2000). Apart from percental lipid content, also lipid composition should be regarded as of importance in the kinetics of lipophilic POPs. The group of lipids is constituted mainly of two slightly different classes: polar and non-polar lipids. While the group of polar lipids consists primarily of phospholipids, the neutral and non-polar lipids are formed essentially by triacylglycerols (TAGs), cholesterol and wax esters (Tocher, 2003; Elskus et al., 2005). While TAGs are the most abundant among the non-polar tissue lipids that are mainly used as energy reserves and storage depots, primarily in liver, muscle and mesenteric fat, phospholipids are the main lipids in cellular membranes and form one of the major fractions of egg yolk (Johnson, 2009) and thus can be found in higher proportions in reproductive glands than in muscle tissue of fish (Kammann et al., 1990; Jobling et al., 1998; Sutharshiny et al., 2013). Different lipid classes may have different binding affinities to lipophilic compounds dependent partly on their octanol-water partitioning coefficient (K_{ow}). Nevertheless, chemical partitioning solely based on log K_{ow} values must be considered with caution, since octanol used as a surrogate for biological lipid cannot simulate barriers to uptake such as molecular configuration or steric hindrance by membranes and functions instead of simple linear partitioning (Elskus et al., 2005). It is therefore likely that the composition of lipid classes as well as the amount of generated and incorporated vitellogenin in the different analysed matrices (muscle, gonads, eggs) has an impact on the concentration as well as the composition of distinctive halogenated congeners.

4.6. Maternal transfer of dioxin-like PCBs

Congener patterns of dl-PCBs did not differ between hormone-treated and untreated fish in our setup (Fig. 3). Different from previous observations made in our study on flame retardants (Sühring et al., 2015), where metabolites from halogenated flame retardants seemed to increase after maternal transfer, the here targeted PCB congeners remain stable and patterns in reproductive glands (gonads and eggs) did not change noteworthy. In future approaches on this topic, it would be interesting to add lower chlorinated PCBs to the targeted compounds to see if the chlorination degree then would have an effect on the found congener patterns.

Induced by hormonal treatment, energy reserves stored in muscle tissue are being reduced by catabolic processes and re-assembled in gonadal tissue during sexual maturation. Generally, the redistribution of lipophilic contaminants in altering body compartments is assumed to be limited by blood flow and diffusion (Nichols et al., 1990). It seems likely that the transportation of stored lipids from the muscle tissue follows a physiological

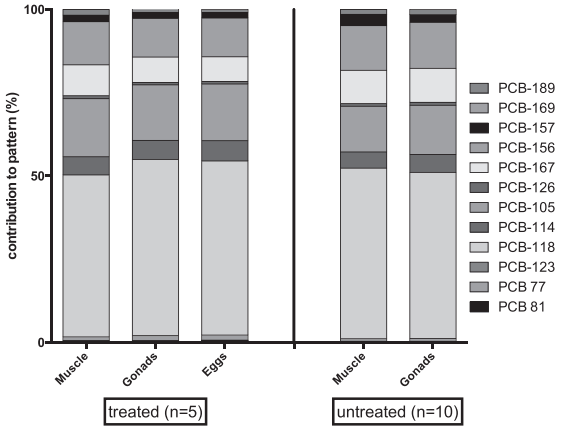

Fig. 3. Percentaged contributions of analysed dl-PCB congeners to total dl-PCB concentration (per wet weight) in different matrices of grouped samples (means) of hormone-treated (n = 5) and untreated eels (n = 10).

pathway over the liver (Nichols et al., 1998). This suggestion is supported by results of Ozaki et al. (2008) in which lipid content in livers of artificially matured Japanese eels increased along with maturation. Moreover, in a study by Okumura et al. (2001), histological examinations showed that size and number of oil droplets in livers of Japanese eels increased during artificial maturation. As a result, it would be interesting to include samples of liver tissue in analyses of future investigations.

In our study, lipid normalized total-concentrations of dl-PCBs showed no significant differences (Kruskal-Wallis-Test H = 7.625, p = 0.1063) among groups or tissue types (Fig. 4). This is well in line with findings by Russell et al. (1999) who confirmed in a number of different fish that transport of hydrophobic organic compounds from maternal tissues to eggs results in equilibrium in concentration, after following a number of passive transport processes.

4.7. Transfer rates

Transfer rates of dl-PCBs from muscle to gonad tissue in treated and untreated fish were heterogeneous (Table 2) and ranged

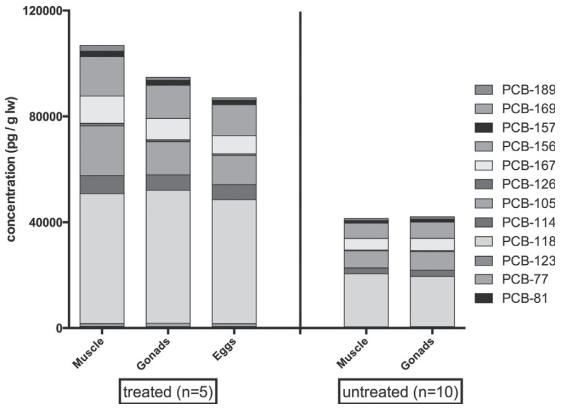

Fig. 4. Means of lipid-normalized contributions of analysed dl-PCB congeners to total dl-PCB concentration in different matrices of grouped samples of hormone-treated (n = 5) and untreated eels (n = 10).

between 0.85 and 6.69 in untreated silver eels compared to 1.89 to 5.16 in treated silver eels. Reasons for this very likely lie in the differences in lipid concentration found in unripe, non-ovulated gonadal tissue as well as in growth dilution as a factor in still growing gonadal tissue of untreated silver eels.

In contrast, the transfer from muscle to eggs in our sampled eels followed a fairly stable ratio (Table 2). After egg release, total dl-PCB concentration based on wet weight in remaining muscle tissue of artificially matured fish was between 5.27- and 9.92- fold higher (average 6.95 ± 1.49) than found in egg tissue. For the most part, this reflects the lipid contents of the matrices, as for lipid-normalized data; concentrations found in the three sampled tissue types were not significantly different (Fig. 4) (although not perfectly even). This observation is in line with findings of a study by Russell et al. (1999), in which the authors investigated the maternal transfer of hydrophobic organic chemicals in 14 different fish and snapping turtle species. One of their central results was that lipid normalization of most of the tested egg and maternal concentrations was not significantly different from 1.0. Mean values of untreated fish compared to the artificially matured individuals however, revealed slightly more balanced concentrations in muscle and gonad tissue. These observations could be explained by expectable differences in the earlier mentioned lipid-composition and vitellogenin content in each matrix along with the toxicokinetics of lipophilic compounds. The kinetics of lipophilic compounds in fish bodies during metabolic changes are believed to be rapid (Nichols et al., 1990) but still require time defined by blood flow, catabolic depletion of reserves and gonadal growth during maturation to reach equilibrium between body compartments.

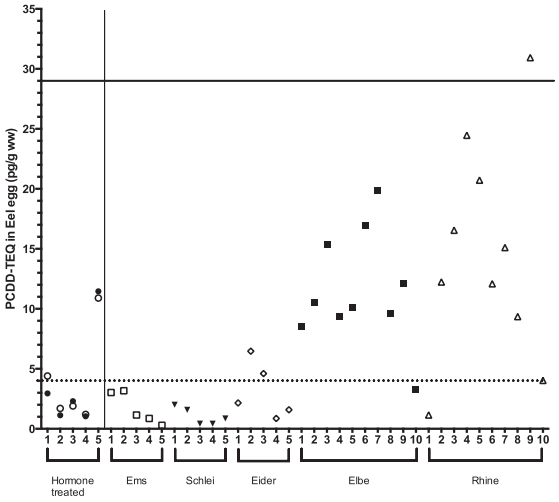

Fig. 5. Measured and estimated (black circles and white circles) TEQ values found in eggs from artificially matured eels. Angled symbols represent estimated concentrations, projected from muscle concentrations found in silver eels from different German water bodies (Freese et al., 2016). Horizontal lines represent different threshold effect concentrations taken from literature. (Thin, dotted line at 4 pg TCDD equivalence/g gonadal mass represents the threshold for occurrence of disrupting effects found in eel embryos presented by Palstra et al., in 2006. The thick line at 29 pg TEQ/g egg represents beginning of direct egg mortality in lake trout King-Heiden et al., 2012.

4.8. Predictions of egg-TEQs based on muscle concentrations and implications for stock management

To help build a better understanding of consequences caused by contamination with dioxin-like substances for reproduction in eels, we used the mean muscle-egg concentration ratio of our hormonally matured silver eels and estimated the same ratio to be applicable for all migrating silver eels. Projected concentrations based on the muscle-egg ratio and measured concentrations in the muscle tissue alone were very close to actually measured concentrations in egg tissues due to the relatively low variability in calculated muscle-egg ratios (Fig. 5, black and white circles). If this ratio of concentration transfer in artificially matured eels is similar to concentration ratios during the eel's natural migration, it can be used to predict the expectable TEQ concentration in eggs from migrating wild silver eels. As a consequence, we estimated expectable egg WHO_{2005} TEQ concentrations derived from silver eel muscle concentrations from different German water bodies (Freese et al., 2016), and put them in relation to threshold values for eel and different fish species, taken from literature. Even though our limited data set has to be regarded with caution, this approach may help to get an idea whether reproduction of eels from German river systems is likely to be impaired through contamination by dioxin-like contaminants and as a consequence, successfully contribute to the European eels spawning stock (Fig. 5). More than 50% of the projected estimates led to values exceeding the threshold of 4 pg WHO_{2005} TEQ/g ww for developmental disruption in eel embryos suggested by Palstra et al. (2006) with some of the examined water bodies being more affected than others. One of our projected concentrations even exceeded a value of 29 pg TEQ/g egg, representing the beginning of direct egg mortality measured in lake trout by King-Heiden et al. (2012). In a different study but also for lake trout, the lethal dose concentration (LD50) for maternally transferred 2,3,7,8-TCDD in eggs was determined at 65 pg/g ww (Walker and Peterson, 1994). In a work on maternal transfer of dioxin in brook trout (*Salvelinus fontinalis*) by Johnson et al. (1998), the authors found that median lethal residue (LR50) values for 2,3,7,8-TCDD were as high as 127 pg/g ww in eggs. For several other fish species, even higher concentrations were needed to reach LR50. Embryos exposed to water concentration of TCDD of the, comparably, non-sensitive zebrafish (*Danio rerio*) or shovelnose sturgeon (*Scaphirhynchus platorynchus*) exhibited a much higher tolerance towards dioxin-like contaminants compared to the previously mentioned salmonid species with LD50s of 2610 and 13,000 pg of TCDD/g ww of egg, respectively (Elonen et al., 1998; Buckler, 2011). Nevertheless, elevated incidences of malformations in embryos in other sturgeon species have been reported at concentrations as low as 50 pg of TCDD/g egg (Chambers et al., 2012). Some of the here mentioned concentrations are considerably higher than expectable concentrations in reproductive tissues from contaminated fish in the wild. In our study, even eels from waters, that have produced eels with comparably high DLC contamination in the past (e.g. Elbe, Rhine), would not reach concentrations of several hundred pg TCDD TEQ, even if TEQ-calculations were not limited on dl-PCBs values alone. However, due to the differing sensitivity among investigated species to the various dioxin-like compounds, there remains uncertainty regarding the risk assessment of DLCs in fishes.

Our here used approach can be regarded as rather conservative, since our predictions are based on dl-PCBs only and do not include TEQs deriving from PCDDs and PCDFs since in the current study as well as other studies from German & European water bodies showed that PCDDs and PCDFs contribute considerably smaller shares of TEQs compared to those driven by dl- PCBs (Stachel et al., 2007; Blanchet-Letrouvé et al., 2014). Also, under a natural scenario it has to be considered that the higher energy costs of locomotion during spawning migration would additionally alter the final contaminant concentrations in lipid rich tissues. In our study, we did not quantify the energetic difference between locomotion of our artificially matured eels during the experiment and the energy

needs for locomotion occurring during natural migration. This gives our projections another level of uncertainty that has to be considered for future experimental works on this topic.

For spawning, eels have to migrate several thousand kilometers and rely on their energy stores, formed mainly by muscle-lipids. In an early work by Böetius and Böetius (1980), the authors estimated that eels would use 75% of their total lipid reserves for spawning activities and their journey, of which 18% are used for gonad development, 27% for basic metabolism and an additional 30% depleted for locomotion. In contrast, Van den Thillart et al. (2004) calculated that 60% of the total fat reserves of silver eels is required for swimming and basic metabolism and concluded in another study (Van Den Thillart et al., 2007) around 36% for incorporation in the eggs. Palstra and Van den Thillart. (2010) estimated that 67% of the total energy stores in eels are spent on spawning migration and oocyte maturation. Since in our experimental setup, fish did not perform similar amounts of locomotion as under natural circumstances, less than the required 60% of their lipid reserves were presumably used for this partial aspect. As a consequence, this could lead to clearly elevated concentrations of lipophilic contaminants in muscle, gonads and eggs at the end of their journey in the field compared to those found in our experiment.

Metabolic elimination as an influential factor on the redistribution and thus concentration ratio of dioxin-like compounds in (newly built) reproductive tissue compared to respective muscle tissue can be disregarded in our case since elimination rates of higher chlorinated PCBs and other organochlorine contaminants in eels have been shown to be very low to not existent at all (de Boer et al., 1994). Also, differences in timespan as an influential factor can be neglected. Depending on the distance from the spawning area, modeled estimations for the duration of natural migration based on average dates of escapement and timing of estimated peak spawning in the Sargasso Sea lie between 63 and 209 days (Righton et al., 2016). This timeframe is well in the range of the time used for the here-applied artificial maturation of the fish (119–135 days).

5. Conclusions & outlook

Results of our study deliver analytical proof of maternal transfer of DLCs from muscle lipids towards ovarian tissues in European eels. Some detected DLC concentrations in eel eggs taken from animals from comparably low contaminated habitats exceeded levels responsible for potentially impairing embryo development and survival. Due to the rather low number of analysed individuals and the high variability of occurring chemical contamination in eels under natural conditions, results of this study must be regarded with caution. Still, the presented findings can now help to further investigate this topic and eventually help improve the management of these endangered species. With reference to the toxicological role of POPs in the reproductive biology of eels, their potential for high accumulation may result in consequences for the success of stock management measures in the long run. Therefore it is crucial to consider contamination of escaping silver eels when identifying and evaluating the suitability of habitats for restocking measures considered for stock enhancement. Our results may bring important new insights to the question whether escaping silver eels are capable of entering the effective spawning stock biomass in the future. Management strategies could use these findings by combining pollution monitoring with protective measures such as harvest restrictions specifically for silver eels escaping habitats of low contamination levels or with regard to site selection for eel stocking programs.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2017.04.096.

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Appendix A. Supplementary Material

Table S1: Detailed biodata and dl-PCB burden calculations of hormone treated and non-treated female silver eels

Alias	Origin	Age (y)	Stage (SI)	Length (cm)	Weight (g)	Weight (After treatment)	Liver (g)	Gonads (g)	Eggs (g)	Bones (g)	Skin (g)	Guts (g)	Gills (g)	Swim-bladder (g)	Calculated total muscle mass (g)	DL-PCB conc in gonads (pg/g ww)	DL-PCB conc in eggs (pg/g ww)	Total dl-PCB burden in rep. tissue (ng)	Total dl-PCB burden in muscle (ng)	Total dl-PCB burden (ng)	Lipid (%) muscle	Lipid (%) gonads	Lpid (%) eggs
HT1	Ems	15	4	81	1042	1385	18,4	558,0	185,0	82,5	131,2	13,2	11,2	4,2	249,7	5895	3408	3920	7592	11512	34,39	8,30	4,80
HT2	Ems	11	5	65	525	584	6,4	84,9	197,4	35,3	69,3	7,6	5,4	2,6	123,7	6747	1843	937	1724	2661	22,32	14,56	4,34
HT3	Ems	14	4	79	871	1073	16,6	125,5	331,7	55,6	107,4	11,9	9,2	3,4	263,6	5599	2011	1370	2796	4165	21,85	14,50	5,54
HT4	Schlei	12	4	80	1032	1177	14,3	161,0	498,0	69,2	112,2	10,4	9,4	4,2	176,5	4905	1890	1731	2456	4187	35,03	15,29	5,94
HT5	Schlei	15	5	63	405	567	6,4	131,0	194,0	23,6	48,3	5,4	7,8	2,0	93,9	18702	13062	4984	6929	11913	25,00	6,55	5,15
E1	Ems	16	5	68	627	N/A	11,0	8,6		86,8	87,3	25,1	9,2	2,8	396,3	37426	N/A	322	12608	12930	22,70	26,60	N/A
E2	Ems	16	5	76	753	N/A	10,2	10,5		105,6	102,6	22,2	10,1	2,9	488,9	14886	N/A	156	7840	7997	27,00	18,00	N/A
E5	Ems	13	5	70	611	N/A	12,1	9,3		90,4	87,6	21,3	8,3	3,1	379,1	N/A	N/A	N/A	2757	N/A	20,20	19,30	N/A
E3	Ems	12	5	74	875	N/A	14,9	15,4		106,6	108,6	22,0	9,6	3,3	594,6	5019	N/A	77	3585	3662	27,10	23,50	N/A
E4	Ems	15	5	65	553	N/A	11,2	6,8		78,9	81,7	19,4	7,8	3,1	344,2	3563	N/A	24	1048	1072	30,80	26,80	N/A
S1	Schlei	15	3	72	679	N/A	12,8	4,4		96,7	76,1	20,2	9,2	3,0	456,6	5022	N/A	22	7450	7472	28,00	12,00	N/A
S2	Schlei	16	3	71	578	N/A	8,0	9,0		80,3	74,1	19,9	7,9	3,2	375,6	2655	N/A	24	4033	4056	26,00	11,50	N/A
S3	Schlei	12	5	69	766	N/A	9,7	12,3		85,6	98,8	18,5	7,2	3,9	530,2	4590	N/A	56	2101	2157	25,90	22,30	N/A
S4	Schlei	10	3	70	627	N/A	8,4	10,0		82,7	89,4	20,8	8,2	3,0	404,6	2721	N/A	27	1127	1154	23,20	12,50	N/A
S5	Schlei	14	5	62	474	N/A	6,9	7,3		70,5	80,6	16,4	10,4	4,1	278,0	2142	N/A	16	1448	1464	21,60	16,00	N/A

CHAPTER III

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

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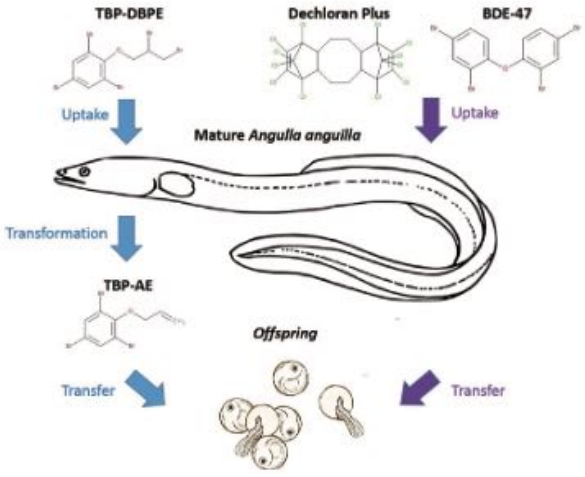

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Maternal transfer of emerging brominated and chlorinated flame retardants in European eels

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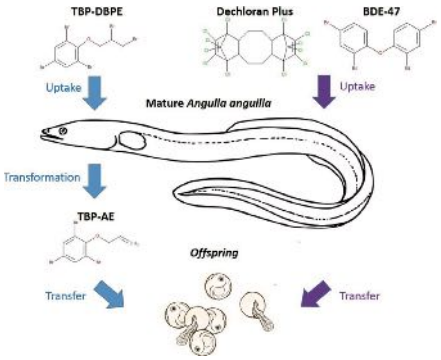
HIGHLIGHTS

- 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

GRAPHICAL ABSTRACT



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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, $aCL_{10}DP$, $aCl_{11}DP$) 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

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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.

1. 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 (Québec Declaration of Concern, 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; Van Ginneken et al., 2005; 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 (Boëtius and Boëtius, 1985) it must be considered that the accumulated contaminants reach their toxic potential maximum during this crucial life-history 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 fulfil 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 striped eggs as well as in muscle and gonads of untreated eels 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 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 (Sühring 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.

2. Materials and methods

2.1. 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 anesthetised 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 ($BGI = B_G\, L_T^{-1}$) (Palstra and van den Thilart, 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 (Sühring et al., 2013). Since then samples were stored at −20 °C in aluminium containers.

2.2. 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 Sühring 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 Sühring et al. (2013).

2 × 200 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.

2.3. 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 × 0.25 mm i.d. × 0.10 μm 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-tribromophenoxy)ethane (BTBPE), Decabromdiphenylethane (DBDPE),

Table 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 n = 10	No	Yellow	Elbe	Muscle Gonads	27 ± 8 n.a.	412 ± 160 n.a.	2 and 3
Silver Elbe n = 10	No	Silver	Elbe	Muscle Gonads	25 ± 4 n.a.	655 ± 125 n.a.	5
comp Ems n = 7	No	Silver	Ems	Muscle Gonads	26 ± 5 22 ± 5	684 ± 129 10 ± 3	5
comp Schlei n = 4	No	Silver	Schlei	Muscle Gonads	22 ± 4 24 ± 4	611 ± 121 10 ± 2	5
ht Ems n = 3	Yes	Silver	Ems	Muscle Gonads Eggs	28 ± 6 24 ± 13 18 ± 11	1014 ± 403 256 ± 262 238 ± 81	5
ht Schlei n = 2	Yes	Silver	Schlei	Muscle Gonads Eggs	15–35 33 4–8	567–1177 131–161 194–498	5

Table 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 Sühring et al., 2013, data marked with ** was published in Sühring et al., 2014.

Location	Water system	Sample type (n)	∑PBDEs	∑MeOBDEs	BDE-47	TBA	Potential BFR metabolites	TBP-DBPE
Germany	Ems	Eggs (n = 3 × 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	Schlei	Eggs (n = 2 × 10 g)	0.74 ± 0.50	0.07 ± 0.10	0.46 ± 0.39	2.1 ± 1.4	0.15 ± 0.01	1.0 ± 0.03
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	0.02 ± 0.001	0.054 ± 0.045
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	0.04 ± 0.02	<LOD − 0.003
Germany	Ems	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	<LOD − 0.004	<LOD − 0.093
Germany	Schlei	Muscle hormone treated						
Silver eels (n = 2)	1.07–26.4	0.60 ± 0.47	0.60–12.2	54 ± 55	1.9 ± 0.79	13.6 ± 10.0	<LOD − 0.09	<LOD − 0.91
Germany	Ems	Gonads comparison group						
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	<LOD	<LOD − 0.26
Germany	Ems	Muscle comparison group						
Silver eels (n = 7)	1.4 ± 0.83	<LOD − 8.6	0.79 ± 0.45	1.6 ± 0.80	<LOD − 0.05	0.23 ± 0.24	<LOD	<LOD − 0.53
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	<LOD	<LOD − 0.010
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	<LOD	<LOD − 0.24
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 eels (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 − 0.37	n.a.	<LOD − 0.018

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-bromoethy-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), Tetrabromophtalic 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 HP-5MS column (30 m × 0.25 mm i.d. × 0.25 μm film thickness, J&W Scientific). In both EI and ECNI a restriction capillary (0.8 m × 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.

2.4. 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 ± 27% for ^{13}C-BDE-28, 134 ± 25% for ^{13}C-BDE-47, 90 ± 20% for ^{13}C-BDE-99, 136 ± 47% for ^{13}C-BDE-153, 125 ± 58% for ^{13}C-BDE-183, 73 ± 38% for ^{13}C-MeOBDE-47, 139 ± 24% for^{13}C-MeOBDE-100, 87 ± 35% for ^{13}C-HBB, 112 ± 38% for ^{13}C-synDP and 98 ± 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 13C-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.

EH-TBB	Further alternate BFRs	DBALD	∑DP	DDC-DBF	Dechlorane metabolites	Further Dechloranes	f (syn)	Lipid [%]
<LOD − 0.012	<LOD − 0.031	<LOD	0.009 ± 0.005	<LOD − 0.0003	<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
<LOD	0.024 ± 0.016	<LOD − 0.00005	<LOD − 0.015	<LOD	0.7 ± 0.1	24		
<LOD	0.011 ± 0.0097	<LOD − 0.0002	<LOD − 0.001	0.012 ± 0.017	0.9 ± 0.02	33		
<LOD − 0.47	0.007 ± 0.006	0.016 ± 0.003	<LOD − 0.002	<LOD	0.3 ± 0.04	28		
0.53 ± 0.63	0.096 ± 0.10	0.028 ± 0.002	<LOD − 0.030	<LOD − 0.001	0.4 ± 0.1	25		
<LOD − 0.11	0.12 ± 0.06	0.055 ± 0.064	<LOD − 0.033	<LOD − 0.07	0.4 ± 0.2	22		
<LOD − 0.18	0.08 ± 0.06	0.093 ± 0.10	<LOD − 0.034	<LOD − 0.03	0.2 ± 0.2	26		
<LOD	0.015 ± 0.011	0.068 ± 0.023	0.066 ± 0.052	<LOD	n.a.	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.	<LOD − 0.042	n.a.	0.041 ± 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.	0.017 ± 0.0083	0.055 ± 0.038	<LOD	<LOD	0.6 ± 0.4	n.a.		

3. 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, $aCl_{10}DP$ and $aCl_{11}DP$ 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 ± 0.11 ng g^{-1} wet weight (ww) in gonads of silver eels from Schlei Fjord to 8.9 ± 3.4 ng g^{-1} ww in muscle tissue of yellow eels from Elbe River (Table 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 ± 0.10 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 ± 0.05 ng g^{-1} ww and 1.07 ng g^{-1} ww in the same samples (Table 2, Fig. 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 2, Fig. 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 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.

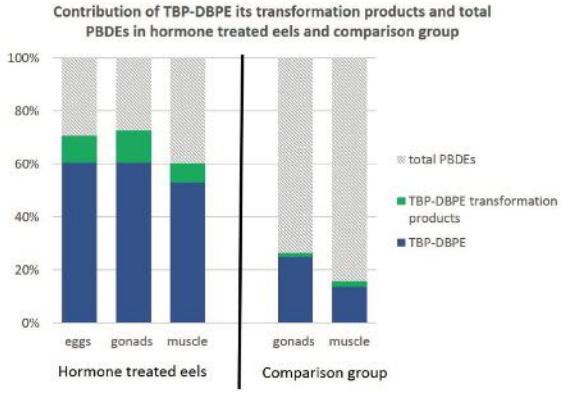

Fig. 2. Average contribution to total PBDEs, TBP-DBPE and TBP-TBPE transformation products (TBP-AE, BATE in % in hormone treated eels (left) and comparison group (right)).

3.1. 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 BDE-47 > BDE-100 > BDE-153 > BDE-99. In gonads and eggs however, BDE-99 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 et al., 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.

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 (Fig. 1, Table 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.

3.2. 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 (Fig. 1, Table 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 (Figs. 1,2). It was detected in all analysed tissue types of yellow and silver eels indicating distribution of this compound into

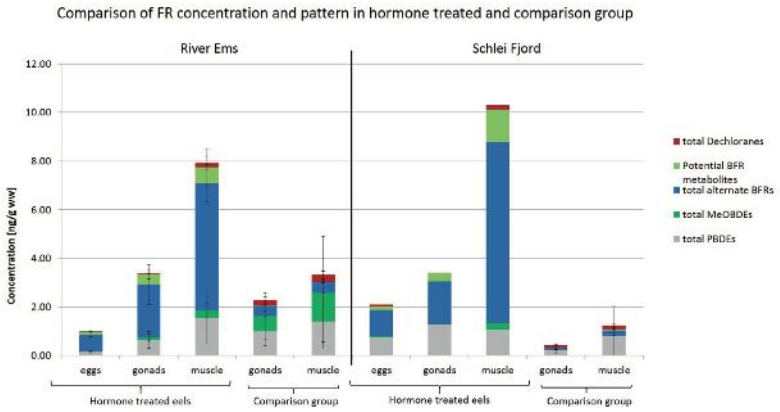

Fig. 1. General contamination pattern. Average concentrations of total PBDEs in grey, MeOBDEs in dark green, total alternate BFRs in blue, TBP-DBPE transformation products (TBP-AE, BATE) in light green and total Dechloranes in red in ng g^{-1} wet weight in eggs, gonads and muscle tissue of hormone treated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

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 ng g^{-1} ww, 2.0 ng g^{-1} ww and 8.6 ng g^{-1} ww in eggs, gonads and muscle tissue of hormone treated eels and 0.21 ng g^{-1} ww and 0.18 ng g^{-1} 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 (von der 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) (von der 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 2). The transformation products were also found in water samples, however in much lower concentrations (<1 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 (Fig. 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 (von der 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 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 (Boëtius and Boëtius, 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 (Bearr et al., 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.

3.3. Dechloranes

The Aldrin-related experimental flame retardant Dibromoaldrin (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 et al., 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 for trout and bluegill (Metcalf, 2002)). 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 2).

The most frequently detected and highest concentrated Dechlorane in muscle as well as gonads of the comparison group was DDC-DBF. Whereas the anti-stereoisomer of Dechlorane Plus (DP) was more abundant in tissue of hormone treated eels. ΣDP as well as DDC-DBF could be found in all samples of the comparison group from Ems River and Schlei Fjord with ΣDP 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 ΣDP 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 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 gentoxicity tests (see supplement information 2.6).

In contrast to results of all earlier life stages, e.g. yellow eels (Sühring 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 synDP/$\sum$ DP ratio (fsyn) 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 fsyn ratio in silver eels had already been reported in silver eels from Elbe River (Sühring et al., 2013), however it remained surprising, because in all other analysed eels from both this study and Sühring et al. (2013), synDP was clearly predominant with fsyn of up to 0.9. In Sühring 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 fsyn ratio however, differed strongly from the comparison group as well as between the different tissue types (Table 2). In muscle tissue of hormone treated eels fsyn 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 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 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.

3.4. 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:

$$EMR = \frac{C_{egg}}{C_{muscle}} \quad (1)$$

$$GMR = \frac{C_{gonad}}{C_{muscle}} \quad (2)$$

where c is the concentration [ng g^{-1} 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 (de Wit, 2002).

3.5. 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 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 (Boëtius and Boëtius, 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 (Fig. 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 ($Logk_{OW}$) can be used as a proxy to describe and quantify this ability.

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

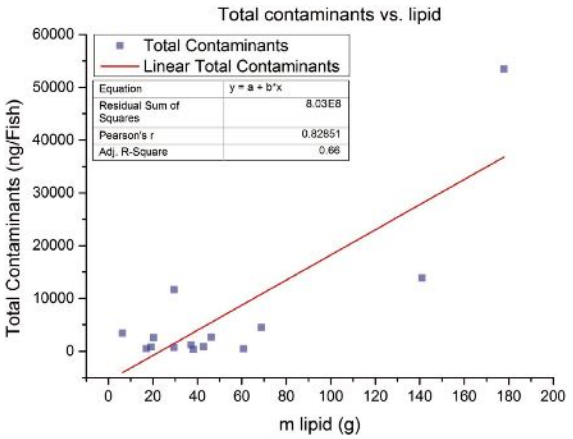

Fig. 3. Correlation of lipid mass in gram per fish and sum contaminants of halogenated flame retardants in ng per fish.

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

The correlation between $logk_{OW}$ and transfer rates explains the higher transfer rates into eggs of e.g. DP (average EMR in analysed eels: 3.5, $logk_{OW}$: 11.27) compared to DDC-DBF (average EMR in analysed eels: 0.01, $logk_{OW}$: 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 $logk_{OW}$ of a compound x and its EMR in artificially matured silver eels $EMR_x = 0.9801 * logkOW_x - 4.3303$ provides a first estimate for the maternal transfer of a compound x based on its $logk_{OW}$ and the concentration found in muscle tissue; with:

$$C_{egg} = 0.981 * \log kOW_x * C_{muscle} - 4.3303 * C_{muscle}.$$

Plotting the concentration in eggs against the lipid content showed the following relationship for the artificially matured eels: $C_{egg} = 1.5 * \%\ lipid_{egg} + 1.6$. 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

$$Total_{egg} = C_{egg}\left[\frac{ng}{g} lw\right] * m_{egg}[g\ \ lw] = 1.5 * 35 + 1.6\left[\frac{ng}{g} lw\right] * 0.025\ [g\ \ lw] = 1.3\ ng,$$

with varying contributions of individual compounds based on their $logk_{OW}$.

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.

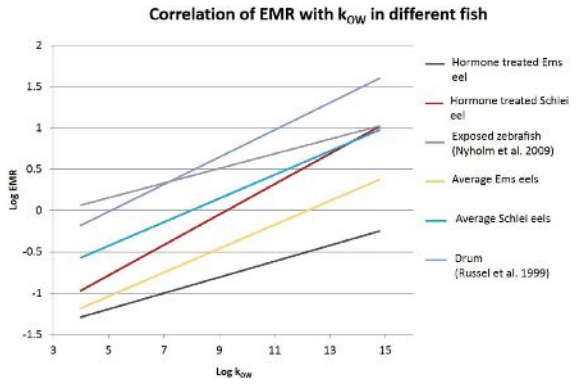

Fig. 4. Correlation of log EMR (egg muscle ratio) and log k_{OW} (octanol–water partitioning coefficient) of halogenated flame retardants and polychlorinated biphenyls (PCBs) in different fish species.

3.6. 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 redistributed 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 (Fig. 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 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.

4. 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 $logk_{OW}$ 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2015.05.094.

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

Table S1a: 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 Ø (mm)	Pectoral fin length (mm)	Stage (s.i.)	Lipid (%)	Sex
VV1	Schlei	669	1177	80	14.3	161	12.5	33.3	4	36	f
VV2	Ems	1200	1385	81	18.4	558	14.4	37.9	4	19	f
VV3	Ems	377	584	65	6.4	85	11.4	35.9	5	20	f
VV4	Ems	696	1073	79	16.6	126	12.2	43.9	4	17	f
VV6	Schlei	328	567	63	6.38	131	12.6	37.9	5	19	f

Table S1b: Biodata of comparison group eels

Nr.	Habitat	M (g)	Length (cm)	Liver (g)	M gonads (g)	Eye Ø (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

Supplement Information

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

S2: Material and methods

2.1. 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.

2.2. 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 anesthetised with 2-Phenoxyethanol (Carl Roth, Karlsruhe, Germany). They were weighted and total length (L_T) and body girth (B_G) was measured to calculate the Body Girth Index (BGI = B_G L_T $^{-1}$) (Palstra & Van den 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.

2.3. Extraction and clean-up

The frozen egg, muscle, and gonad samples were homogenised with anhydrous Na_2SO_4 (Merck) using a stainless steel/glass 1 L laboratory blender (neoLab Rotorblender). 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. Extraction was performed by accelerated solvent extraction with dichloromethane (DCM) as solvent, using the method described in Sühring *et al.* 2013. Extracts were purified by gel permeation chromatography (GPC) and silica gel clean-up as described by Sühring *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.

2.4. 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), Decabromdiphenylethane (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-bromoethy-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), Tetrabromophtalic 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.* (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, J&W 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.

2.5. 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 µg 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.

2.5.1. 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 ± 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.* [1] under an inverted microscope.

2.5.2. Egg quality and validation criteria

In accordance with the OECD Guideline for fish-egg assays with Zebrafish embryos [2] 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”[3][4]. 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 ± 2°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%.

2.5.3. 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 [2] 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 µg/mL, 0.1 µg/mL, 1 µg/mL, 10 µg/mL. Individuals were checked 48 and 96 hours post fertilization (hpf) for coagualation, 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.

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 (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 µg/ml, -S9) and 2-Aminoanthracene (2-AA, final conc.: 0.25 µg/ml, +S9) were used as positive controls.

The growth factor (G), the β-galactosidase activity (U_T) and the Induction Ratio (IR) were calculated according to the ISO guideline (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 (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 (ISO, 2012).

The bacterial solution were cultivated overnight (37°C, 250 rpm) and diluted with exposure medium to a final density of 1800 FAU (OD_{600} ~ 2.4) for TA 98 and 450 FAU (OD_{600} ~ 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 OD_{600} 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 ± 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 µg/ml) and 2-Nitrofluorene (final conc.: 2.5 µg/ml) were used as positive controls for testing without S9. 2-AA in a final concentration of 4 µg/ml was used as positive control for testing with metabolic activation.

Table S3: Analytes

Analytes	Abbreviation	Cas Nr.
BDE-28; 2,4,4'-Tribromodiphenyl ether	BDE-28	41318-75-6
BDE-28; 2,4,4'-Tribromo[13C12]diphenyl ether	13C BDE-28	N/A
BDE-47; 2,2',4,4'-Tetrabromodiphenyl ether	BDE-47	5436-43-1
BDE-47; 2,2',4,4'-Tetrabromo[13C12]diphenyl ether	13C BDE-47	N/A
BDE-99; 2,2',4,4',5-Pentabromodiphenyl ether	BDE-99	60348-60-9
BDE-99; 2,2',4,4',5-Pentabromo[13C12]diphenyl ether	13C BDE-99	N/A
BDE-100; 2,2',4,4',6-Pentabromodiphenyl ether	BDE-100	189084-64-8
BDE-153; 2,2',4,4',5,5'-Hexabromodiphenyl ether	BDE-153	68631-49-2
MBDE-153; MBDE1531199; 13C-2,2',4,4',5,5'-Hexabromodiphenyl ether	13C BDE-153	N/A
BDE-154; 2,2',4,4',5,6'-Hexabromodiphenyl ether	BDE-154	207122-15-4
BDE-183; 2,2',3,4,4',5',6-Heptabromodiphenyl ether	BDE-183	207122-16-5
MBDE-183; MBDE1831105; 13C-2,2,3,4,4,5,6-Heptabromodiphenyl ether	13C BDE-183	N/A
BDE-209; Decabromodiphenyl ether	BDE-209	1163-19-5
MBDE-209; MBDE2090608; 13C-Decabromodiphenyl ether	13C BDE-209	N/A
MPBBZ; MPBBZ0810; 1,2,3,4,5-Pentabromo[13C6]benzene	13C PBBz	N/A
MBDE-MXFR; MBDEFR0809; 13C-PBDE Recovery Soln.; [13C-BDE77, 13CBDE138)	13C-BDE77, -BDE138	N/A
BDE-MXF; Native PBDE-Solution/Mix; BDE 28, 47, 66, 85, 99, 100, 153, 154, 183	native PBDE mixture	N/A
DBDPE; 1,2-bis(pentabromophenyl)ethane	DBDPE, BDPE-209	84852-53-9
BEHTBP; bis(2-ethyl-1-hexyl)tetrabromo-phthalate	BEHTBP, BEH-TEBP	26040-51-7
BTBPE; 1,2-Bis(2,4,6-tribromophenoxy)ethane	BTBPE	37853-59-1
HCDBCO; Hexachlorocyclopentadienyl-dibromocyclooctane	HCDBCO, DBHCTD	51936-55-1
EHTBB; 2-ethylhexyl-2,3,4,5-tetrabromo-benzoate	EHTBB, EH-TBB	183658-27-7
ATE; Allyl-2,4,6-Tribromophenyl ether	ATE, TBP-AE	3278-89-5
HBB; Hexabromobenzene	HBB	87-82-1
pTBX; 2,3,5,6-Tetrabromo-p-xylene	pTBX, TBX	23488-38-2
PBB; 1,2,3,4,5-Pentabromobenzene	PBB	608-90-2
PBT; 2,3,4,5,6-Pentabromotoluene	PBT	87-83-2
PBBA; Pentabromobenzyl acrylate	PBBA, PBB-Acr	59447-55-1
TBCT; Tetrabromo-o-chlorotoluene	TBCT	39569-21-6
s-DP; Syn-Dechlorane Plus	s-DP, DDC-CO	13560-89-9
aCl10DP; Cl10 Dechlorane Plus	aCl10DP	N/A
a-DP; anti-Dechlorane Plus	a-DP, DDC-CO	13560-89-9
DPMA; Dechlorane Plus-Mono Adduct	DPMA	135821-04-4
Dechlorane 604; Component A; (Tetrabromophenyl) hexachloronorbornene	Dec604, HCTBPH	34571-16-9
Dechlorane 603	Dec603, DDC-Ant	13560-92-4
Dechlorane 602	Dec602, DDC-DBF	31107-44-5
2,4,6-Tribromoanisole	TBA	607-99-8
Tetrabromophthalic anhydride	TEBP-Anh, TBPA	632-79-1
Tetrabromobisphenol A	TBBPA	79-94-7
Tetrabromobisphenol A bis(2,3-dibromopropyl ether)	TBBPA-DBPE	21850-44-2
Tetrabromobisphenol A diallyl ether	TBBPE-DE, TBBPA-BAE	25327-89-3

FRS-030S; Pentabromobenzylbromide	PBBB	38521-51-6

Table S3 continued:

Analytes	**Abbreviation**	**Cas Nr.**
BP-246S; 2,4,6-Tribromophenol	TBP	118-79-6
alpha/beta-Tetrabromoethylcyclo-hexane; isomere; □/□-TBECH	a/b- DBE-DBCH	3322-93-8
aTBCO; alpha-1,2,5,6-Tetrabromo-cyclooctane; (1R, 2R, 5S, 6S)-1,2,5,6-Tetrabromo-cyclooctane	aTBCO	3194-57-8
bTBCO; beta-1,2,5,6-Tetrabromo-cyclooctane; (1R, 2R, 5R, 6R)-1,2,5,6-Tetrabromo-cyclooctane	bTBCO	3194-57-8
5-norborne-2,3-dicarbocyclic anhydride, 1,4,5,6,7,7-Hexachloro-	HCBCH-DCAnh	34571-16-9
1,2,3,4,5,5-hexachloro-1,3-cyclopentadiene	HCCPD	77-47-4
1,2,3,4,5-pentabromo-6-ethylbenzene	PBEB	85-22-3
1,3,5-tribromo-2-(2,3-dibromopropoxy)-benzene	DPTE, TBP-DBPE	35109-60-5
5MeOBDE47, 6MeOBDE47, 4MeOBDE49, 2MeOBDE68, 5MeOBDE99, 5MeOBDE100, 4MeOBDE101, 4MeOBDE103	Methoxy-Bromodiphenyl Ethers	N/A
2,2',4,4'-Tetrabromo-6-methoxy[13C12]diphenyl ether	13 C- MeOBDE47	N/A
2,2',4,4',6-Pentabromo-6'-methoxy[13C12]diphenyl ether	14 C- MeOBDE100	N/A

Table S4: MRMs for all analytes

Abbreviation	TS	Transition Quantifier	Transition Qualifier	Transition Qualifier 2	Type	RT [min]	IS
TBA	1	328.7 -> 300.8	343.7 -> 300.8		Target	5.76	13C-PBBz
2,4,6-TBP	1	329.8 -> 140.9	221.8 -> 140.9		Target	5.9	13C-PBBz
TBP-AE	1	330.8 -> 302.6	369.8 -> 209.9		Target	6.56	13C-PBBz
alpha DBE-DBCH	1	187.0 -> 105.1	187.0 -> 105.1		Target	8.03	13C-PBBz
beta DBE-DBCH	1	187.0 -> 105.0	187.0 -> 105.0		Target	8.15	13C-PBBz
pTBX	2	421.7 -> 340.7	421.7 -> 340.7		Target	8.5	13C-PBBz
BATE	2	327.7 -> 140.9	289.6 -> 209.0		Target	8.52	13C-PBBz
beta TBCO	2	187 -> 105.1	266.5 -> 105.2		Target	8.65	13C-PBBz
13C-PBBz	2	479.8 -> 398.8	398.8 -> 319.8		ISTD	8.7	
PBB	2	471.7 -> 311.8	392.6 -> 313.8		Target	8.7	13C-PBBz
HCPN	3	104.1 -> 103	104.1 -> 102.1		Target	9.08	13C-PBBz
alpha TBCO	3	187 -> 105.1	266.5 -> 105.2		Target	9.2	13C-PBBz
TBCT	3	441.8 -> 362.7	362.7 -> 281.8		Target	9.2	13C-PBBz
DPMA	3	263.4 -> 228.9	344.2 -> 231		Target	9.85	13C-PBBz
PBT	4	487.7 -> 406.8	325.6 -> 246.9		Target	10.4	13C-BDE28
13CBDE28	4	259.9 -> 150.0	419.8 -> 260.1		ISTD	10.5	
BDE28	4	247.5 -> 139.0	405.8 -> 246		Target	10.5	13C-BDE28
DBCD	4	264.8 -> 230.05	464.7 -> 264.8		Target	10.6	13C-BDE28
PBEB	4	501.6 -> 486.7	484.9 -> 405.7		Target	10.92	13C-BDE28
TBP-DBPE	5	328.8 -> 140.9	371.8 -> 212		Target	12.3	13C-HBB
13C-HBB	5	559.7 -> 399.7	478.7 -> 399.7		ISTD	12.5	
HBB	5	549.5 -> 389.9	470.6 -> 391.7		Target	12.5	13C-HBB
DBALD	5	262.9 -> 192.3	488.7 -> 262.9		Target	12.75	13C-HBB
13CBDE47	5	497.7 -> 337.9	337.8 -> 230		ISTD	13.35	
BDE47	5	325.5 -> 217.0	485.6 -> 325.8		Target	13.35	13-CBDE47
BDE66	5	325.5 -> 217.0	485.6 -> 325.9		Target	13.8	13-CBDE47

Table S4 continued:

Abbreviation	TS	Transition Quantifier	Transition Qualifier	Transition Qualifier 2	Type	RT [min]	IS
MeOBDE68	6	515.8 -> 419.6	515.8 -> 421.76		Target	14.7	13C-MeOBDE647
13C-PCB208	6	473.3 -> 403.9	405.4 -> 335.9		Surrogate	15.2	
13CMeOBDE647	6	527.8 -> 367.8	527.8 -> 431.9		ISTD	15.22	
6MeOBDE47	6	515.8 -> 419.8	515.78 -> 356		Target	15.25	13C-MeOBDE647
TBPA	6	419.7 -> 391.9	463.7 -> 419.5		Target	15.3	13C-MeOBDE647
PBBA	6	476.7 -> 448.8	556.67 -> 476.7		Target	15.35	13C-MeOBDE647
BDE100	7	403.8 -> 296.9	565.6 -> 405.8		Target	15.7	13C-BDE99
5MeOBDE47	7	515.8 -> 341.08	515.8 -> 355.9		Target	15.9	13C-MeOBDE647
MeOBDE49	7	515.8 -> 340.9	515.8 -> 500.74		Target	16.03	13C-MeOBDE647
DDC-DBF	7	271.8 -> 236.9	236.5 -> 142.9		Target	16.1	13C-DP
13CBDE99	8	575.7 -> 415.7	417.8 -> 308.1		ISTD	16.3	
BDE99	8	565.6 -> 405.8	403.8 -> 296.9		Target	16.3	13C-BDE99
HCDBCO	8	271.8 -> 236.9	308.9 -> 200		Target	16.32	13C-DP
EH-TBB	8	232.8 -> 153.9	420.8 -> 311.8		Target	16.5	13C-BDE99
13CMeOBDE100	9	607.8 -> 514.0			ISTD	16.85	
HCCPD	9	271.8 -> 236.9	236.8 -> 118.9		Target	17.25	13C-DP
Cplus	9	273.8 -> 238.88	238.88 -> 142.93	238.88 -> 118.96	Target	17.25	13C-DP
MeOBDE100	9	593.8 -> 433.7	593.8 -> 418.79		Target	17.3	13C-MeOBDE100
MeOBDE103	9	593.8 -> 390.9	593.8 -> 578.8	593.8 -> 433.9	Target	17.5	13C-MeOBDE100
BDE154	10	483.7 -> 376.8	645.5 -> 485.7		Target	18	13C-BDE153
MeOBDE99	10	593.9 -> 433.9	593.9 -> 418.9		Target	18.25	13C-MeOBDE100
MeOBDE101	10	593.8 -> 418.9	593.8 -> 434.94	593.8 -> 578.8	Target	18.4	13C-MeOBDE100
13CBDE153	10	655.5 -> 495.9	495.9 -> 387.7		ISTD	18.8	
BDE153	10	645.5 -> 485.7	483.7 -> 376.8		Target	18.8	13C-BDE153
DDC-Ant	11	262.4 -> 192.9	236.7 -> 142.9		Target	20.5	13C-DP
HCTBPH	11	419.7 -> 259.9	440.6 -> 280.8		Target	21.16	13C-DP

Table S4 continued:

Abbreviation	TS	Transition Quantifier	Transition Qualifier	Transition Qualifier 2	Type	RT [min]	IS
13CBDE183	11	573.8 -> 413.8	733.4 -> 573.7		ISTD	21.4	
BDE183	11	721.4 -> 561.6	563.6 -> 401.8		Target	21.4	13C-BDE183
aCl10DP	11	203.9 -> 169.0	166.9 -> 82.9		Target	21.8	13C-DP
BTBPE	11	356.8 -> 277.9	277.6 -> 118		Target	22.65	13C-BDE183
TBC	11	249.1 -> 208			Target	22.75	13C-BDE183
13C-DP	12	276.8 -> 241.9	241.7 -> 145.9		ISTD	23.1	
synDP	12	237.8 -> 202.9	271.8 -> 236.8		Target	23.1	13C-DP
Dec-601	12	271.8 -> 236.9	271.8 -> 116.9	236.9 -> 118.97	Target	23.1	13C-DP
BEH-TEBP	12	112.1 -> 70.0	464.7 -> 380.8		Target	23.2	13C-DP
aCl11DP	12	237.8 -> 202.9	202.9 -> 142.9		Target	23.45	13C-DP
antiDP	12	237.8 -> 202.9	271.8 -> 236.8		Target	23.7	13C-DP
BDE209	13	399.6 -> 265.4	799.3 -> 639.5		Target	34.2	13C-BDE209
13C-BDE209	13	463.8 -> 303.8	811.3 -> 651.5		ISTD	34.2	
DBDPE	13	484.5 -> 324.6	405.7 -> 326.6		Target	39.3	13C-BDE209

Table S5: Average Blanks, LODs and LOQs:

Analytes	LOD EI [pg]	LOD ECNI [pg]	LOQ EI [pg]	LOQ ECNI[pg]	Average blank [pg]	stdev blank [pg]
5MeOBDE47	63	n.a.	110	n.a.	43	7
6MeOBDE47	55	n.a.	127	n.a.	24	10
aCl10DP	21	15	34	49	15	2
aCl11DP	28	12	43	39	22	2
aDBE-DBCH (aTBECH)	171	n.a.	497	n.a.	32	46
anti-DDC-CO (antiDP)	59	18	61	61	57	0
BATE	123	n.a.	333	n.a.	0	0
bDBE-DBCH (bTBECH)	99	n.a.	270	n.a.	34	30
BDE100	54	34	89	115	26	24
BDE153	1297	n.a.	4324	n.a.	39	5
BDE154	54	3	91	9	0	0
BDE183	25405	n.a.	84685	n.a.	38	5
BDE28	65	n.a.	86	n.a.	0	0
BDE47	72	n.a.	120	n.a.	56	3
BDE66	73	15	118	51	51	7
BDE-85	n.a.	21	n.a.	70	54	6
BDE99	98	n.a.	141	n.a.	80	6
BEH-TEBP (BEHTBP)	23083	n.a.	68250	n.a.	3725	6453
BTBPE	18	93	55	310	3	5
CPlus	19	n.a.	55	n.a.	4	5
DBALD	244	n.a.	814	n.a.	0	0
DBHCTD (HCDBCO)	1	62	4	207	0	0
DDC-Ant (Dec 603)	52	37	173	124	0	0
DDC-DBF (Dec 602)	13	0	44	1	0	0
Dec-601	15	n.a.	44	n.a.	2	4
DPMA	0	19	1	63	0	0
EH-TBB	n.a.	42	n.a.	138		
HBB	6	14	19	47	1	2
HCCPD	10	n.a.	27	n.a.	2	2
HCTBPH (Dec 604)	355	189	1184	629	0	0
MeOBDE100	314	n.a.	1046	n.a.	0	0
MeOBDE101	784	n.a.	2612	n.a.	0	0
MeOBDE103	659	n.a.	2195	n.a.	0	0
MeOBDE49	181	n.a.	487	n.a.	50	44
MeOBDE68	84	n.a.	140	n.a.	60	8
MeOBDE99	176	n.a.	587	n.a.	0	0
PBB	3176	n.a.	9304	n.a.	550	875
PBEB	18	1	20	3	17	0
PBT	31	1	48	3	24	2
syn-DDC-CO (synDP)	42	14	71	46	29	4

Table S5 continued:

	LOD EI [pg]	LOD ECNI [pg]	LOQ EI [pg]	LOQ ECNI[pg]	Average blank [pg]	stdev blank [pg]
TBA	56	n.a.	116	n.a.	30	9
TBCT	28	n.a.	34	n.a.	25	1
TBP (2,4,6-TBP)	262	n.a.	557	n.a.	136	42
TBP-AE (ATE)	128	n.a.	343	n.a.	36	31
TBP-DBPE (DPTE)	108	48	309	161	22	29
TBX (pTBX)	54	n.a.	148	n.a.	14	13
TEBP-Anh (TBPA)	12700	n.a.	37552	n.a.	2050	3550

Table S6: Results alternate BFRs [ng/g lipid weight]

	habitat	TBA	TBP (2,4,6-TBP)	TBP-AE	aDBE-DBCH	bDBE-DBCH	TBX	BATE	bTBCO	PBB	aTBCO	TBCT	PBT	PBEB	TBP-DBPE	HBB	EH-TBB	PBBA	BTBPE	TDBP-TAZTO	lipid [%]
VV1Egg	Schlei	77	n.d.	0	n.d.	n.d.	n.d.	3.7	n.d.	n.d.	n.d.	n.d.	0.27	0.54	26	0.38	0.075	n.d.	1.146989	n.d.	4
VV6Egg	Schlei	13	n.d.	0.80	n.d.	n.d.	n.d.	1.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	12	n.d.	0.085	n.d.	n.d.	n.d.	8
VV1Gonad	Schlei	451	n.d.	2.9	n.d.	n.d.	n.d.	8.2	n.d.	n.d.	n.d.	n.d.	0.069	0.0044	49	n.d.	1.3	n.d.	n.d.	n.d.	4
VV6Gonad	Schlei	2.3	n.d.	0.19	n.d.	n.d.	n.d.	0.50	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5.9	n.d.	2.1	n.d.	n.d.	n.d.	30
VV1Muscle	Schlei	268	0.28	1.0	n.d.	n.d.	n.d.	2.8	n.d.	n.d.	n.d.	n.d.	n.d.	0.029	20	n.d.	n.d.	n.d.	2.28001	n.d.	35
VV6Muscle	Schlei	102	0.95	6.2	n.d.	n.d.	n.d.	10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	145	0.0180	1.2	n.d.	n.d.	n.d.	15
Average egg	Schlei	45	n.d.	0.40	n.d.	n.d.	n.d.	2.4	n.d.	n.d.	n.d.	n.d.	0.13	0.27	19	0.19	0.080	n.d.	0.573495	n.d.	6
Average gonad	Schlei	227	n.d.	1.6	n.d.	n.d.	n.d.	4.4	n.d.	n.d.	n.d.	n.d.	0.034	0.0022	27	0	1.7	n.d.	0	n.d.	17
average muscle	Schlei	185	0.62	3.6	n.d.	n.d.	n.d.	6.5	n.d.	n.d.	n.d.	n.d.	n.d.	0.015	83	0.0090	0.62	n.d.	1.140005	n.d.	25
total	Schlei	457	0.62	5.6	n.d.	n.d.	n.d.	13.3	n.d.	n.d.	n.d.	n.d.	0.17	0.29	129	0.20	2.4	n.d.	1.7135	n.d.	48
GMR	Schlei	1.2	n.d.	0.43	n.d.	n.d.	n.d.	0.67	n.d.	n.d.	n.d.	n.d.	n.d.	0.15	0.33	n.d.	2.8	n.d.	n.d.	n.d.	0.68
EGR	Schlei	0.20	n.d.	0.26	n.d.	n.d.	n.d.	0.55	n.d.	n.d.	n.d.	n.d.	3.9	122	0.69	n.d.	0.047	n.d.	n.d.	n.d.	0.35
EMR	Schlei	0.24	n.d.	0.11	n.d.	n.d.	n.d.	0.37	n.d.	n.d.	n.d.	n.d.	n.d.	19	0.23	21	0.13	n.d.	0.503063	n.d.	0.24
EGMR	Schlei	**1.5**	**n.d.**	**0.54**	**n.d.**	**n.d.**	**n.d.**	**1.0**	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**19**	**0.56**	**21**	**2.9**	**n.d.**	**0.503063**	n.d.	**0.92**
VV2Egg	Ems	4.8	n.d.	0.16	n.d.	n.d.	n.d.	0.33	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.1	n.d.	0.042	n.d.	n.d.	n.d.	21
VV3Egg	Ems	4.6	n.d.	0.18	n.d.	n.d.	n.d.	0.21	n.d.	n.d.	n.d.	n.d.	n.d.	0.00046	2.0	0.0029	n.d.	n.d.	0.10317	n.d.	29
VV4Egg	Ems	6.0	n.d.	0.54	n.d.	n.d.	n.d.	2.9	n.d.	n.d.	n.d.	n.d.	n.d.	0.0053	15	n.d.	0.23	n.d.	n.d.	n.d.	5
VV2Gonad	Ems	27	1.3	1.2	n.d.	n.d.	n.d.	1.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	20	n.d.	1.3	n.d.	n.d.	n.d.	8
VV3Gonad	Ems	7.9	0.085	0.33	n.d.	n.d.	n.d.	0.78	n.d.	n.d.	n.d.	n.d.	n.d.	0.0077	8.7	n.d.	1.1	n.d.	n.d.	n.d.	35
VV4Gonad	Ems	11	0.081	0.63	n.d.	n.d.	n.d.	1.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	12	n.d.	1.6	n.d.	n.d.	n.d.	33
VV2Muscle	Ems				n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	20	n.d.	n.d.	n.d.	n.d.	n.d.	35
VV3Muscle	Ems	25	0.14	0.70	n.d.	n.d.	n.d.	1.7	n.d.	n.d.	n.d.	n.d.	n.d.	0.0017	26	n.d.	n.d.	n.d.	n.d.	n.d.	24
VV4Muscle	Ems	38	0.37	1.7	n.d.	n.d.	n.d.	4.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	40	n.d.	0.070	n.d.	n.d.	n.d.	25
Average egg	Ems	5	0	0.29	n.d.	n.d.	n.d.	1.1	n.d.	n.d.	n.d.	n.d.	n.d.	0.0019	6.8	0.0010	0.092	n.d.	0.03439	n.d.	18
Average gonad	Ems	15	0.48	0.73	n.d.	n.d.	n.d.	1.4	n.d.	n.d.	n.d.	n.d.	n.d.	0.0026	14	n.d.	1.3	n.d.	n.d.	n.d.	25
average muscle	Ems	31	0.26	1.2	n.d.	n.d.	n.d.	2.9	n.d.	n.d.	n.d.	n.d.	n.d.	0.00083	29	n.d.	0.023	n.d.	n.d.	n.d.	28
total	Ems	52	0.73	2.2	n.d.	n.d.	n.d.	5.4	n.d.	n.d.	n.d.	n.d.	n.d.	0.0053	49	0.0010	1.4	n.d.	0.03439	n.d.	72
GMR	Ems	0.48	1.9	0.60	n.d.	n.d.	n.d.	0.47	n.d.	n.d.	n.d.	n.d.	n.d.	3.1	0.48	n.d.	57	n.d.	n.d.	n.d.	0.90
EGR	Ems	0.34	n.d.	0.40	n.d.	n.d.	n.d.	0.84	n.d.	n.d.	n.d.	n.d.	n.d.	0.74	0.49	n.d.	0.069	n.d.	n.d.	n.d.	0.72
EMR	Ems	0.16	n.d.	0.24	n.d.	n.d.	n.d.	0.40	n.d.	n.d.	n.d.	n.d.	n.d.	2.3	0.24	n.d.	3.9	n.d.	n.d.	n.d.	0.65
EGMR	Ems	**0.64**	**1.88**	**0.84**	**n.d.**	**n.d.**	**n.d.**	**0.87**	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**5.4**	**0.72**	**n.d.**	**61**	**n.d.**	n.d.	n.d.	**1.6**

GMR: Gonad/muscle ratio, EGR: Egg/gonad ratio, EMR: Egg/muscle ratio, EGMR: (Egg+Gonad)/muscle ratio

Table S7: Results PBDEs and MeOBDEs [ng/g lipid weight]

	habitat	BDE28	BDE47	BDE66	BDE85	BDE99	BDE100	BDE153	BDE154	BDE183	MeOBDE49	5MeOBDE47	6MeOBDE47	MeOBDE68	lipid [%]
VV1Egg	Schlei	0.77	4.6	n.d.	n.d.	1.4	1.0	1.0	0.87	n.d.	0.61	0.69	0.78	1.4	4
VV6Egg	Schlei	0.11	9.1	0.015	n.d.	2.6	1.5	0.17	0.17	n.d.	n.d.	n.d.	0.046	n.d.	8
VV1Gonad	Schlei	0.22	9.4	0.14	n.d.	3.6	1.7	0.34	0.49	n.d.	n.d.	n.d.	0.47	0.21	4
VV6Gonad	Schlei	0.040	4.0	0.76	n.d.	1.2	0.85	0.022	0.58	n.d.	n.d.	n.d.	n.d.	n.d.	30
VV1Muscle	Schlei	0.12	1.7	0.13	0.076	0.27	1.1	0.38	0.62	n.d.	n.d.	0.12	0.47	0.17	35
VV6Muscle	Schlei	1.1	82	n.d.	0.042	1.8	21	4.75	4.5	n.d.	n.d.	n.d.	3.1	3.1	15
Average egg	Schlei	0.44	6.9	0.0075	n.d.	2.0	1.3	0.59	0.52	n.d.	0.31	0.35	0.42	0.71	6
Average gonad	Schlei	0.13	6.7	0.451	n.d.	2.4	1.3	0.18	0.53	n.d.	n.d.	0	0.23	0.11	17
average muscle	Schlei	0.59	42	0.067	0.059	1.0	11	2.6	2.5	n.d.	n.d.	0.058	1.8	1.6	25
total	Schlei	1.2	55	0.53	0.059	5.4	14	3.3	3.6	n.d.	0.31	0.41	2.4	2.5	48
GMR	Schlei	0.22	0.16	6.7	n.d.	2.4	0.11	0.070	0.21	n.d.	n.d.	n.d.	0.13	0.065	0.68
EGR	Schlei	3.4	1.0	0.017	n.d.	0.8	0.99	3.3	0.97	n.d.	n.d.	n.d.	1.8	6.7	0.35
EMR	Schlei	0.74	0.16	0.11	n.d.	1.9	0.11	0.23	0.21	n.d.	n.d.	5.9	0.23	0.44	0.24
EGMR	Schlei	**0.96**	**0.32**	**6.9**	n.d.	**4.3**	**0.23**	**0.30**	**0.42**	n.d.	n.d.	**5.9**	**0.36**	**0.50**	**0.92**
VV2Egg	Ems	0.014	0.42	n.d.	n.d.	n.d.	0.05	n.d.	0.0050	n.d.	n.d.	n.d.	n.d.	n.d.	21
VV3Egg	Ems	0.013	0.59	0.0043	n.d.	0.013	0.08	0.0050	0.0094	n.d.	n.d.	n.d.	0.013	n.d.	29
VV4Egg	Ems	0.13	3.0	0.032	n.d.	n.d.	0.42	0.0049	0.10	n.d.	n.d.	n.d.	0.29	0.18	5
VV2Gonad	Ems	0.059	2.6	n.d.	n.d.	n.d.	0.40	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	8
VV3Gonad	Ems	0.059	1.9	0.87	n.d.	0.13	0.34	0.073	0.92	n.d.	n.d.	0.034	0.19	n.d.	35
VV4Gonad	Ems	0.061	2.2	0.74	0.93	0.011	0.31	n.d.	0.050	n.d.	n.d.	n.d.	0.49	0.35	33
VV2Muscle	Ems	n.d.	2.3	n.d.	0.022	0.68	0.60	n.d.	0.20	n.d.	n.d.	n.d.	0.088	0.22	35
VV3Muscle	Ems	0.11	5.8	n.d.	0.17	0.30	1.9	0.54	0.91	n.d.	n.d.	n.d.	1.0	0.070	24
VV4Muscle	Ems	0.17	6.9	n.d.	0.81	0.17	2.2	0.46	0.49	n.d.	n.d.	n.d.	2.3	0.10	25
Average egg	Ems	0.053	1.3	0.012	0	0.0043	0.18	0.0033	0.037	n.d.	n.d.	n.d.	0.10	0.059	18
Average gonad	Ems	0.060	2.2	0.54	0.31	0.046	0.35	0.024	0.32	n.d.	n.d.	0.011	0.23	0.12	25
average muscle	Ems	0.14	5.0	n.d.	0.33	0.38	1.6	0.33	0.53	n.d.	n.d.	n.d.	1.1	0.13	28
total	Ems	0.25	8.6	0.55	0.64	0.43	2.1	0.36	0.89	n.d.	n.d.	0.011	1.5	0.31	72
GMR	Ems	0.44	0.45	n.d.	0.93	0.12	0.22	0.073	0.60	n.d.	n.d.	n.d.	0.20	0.88	0.90
EGR	Ems	0.89	0.60	0.022	n.d.	0.094	0.53	0.14	0.12	n.d.	n.d.	n.d.	0.45	0.51	0.72
EMR	Ems	0.39	0.27	n.d.	n.d.	0.011	0.12	0.010	0.070	n.d.	n.d.	n.d.	0.09	0.45	0.65
EGMR	Ems	**0.82**	**0.71**	**n.d.**	**0.93**	**0.13**	**0.34**	**0.08**	**0.67**	n.d.	n.d.	n.d.	**0.29**	**1.3**	**1.6**

GMR: Gonad/muscle ratio, EGR: Egg/gonad ratio, EMR: Egg/muscle ratio, EGMR: (Egg+Gonad)/muscle ratioTable

S8: Results Dechloranes [ng/g lipid weight]

	habitat	DBALD	DDC-DBF	Cplus	HCCPD	HCPN	DBCD	DDC-Ant	HCTBPH	aCl10DP	syn-DP	aCl11DP	anti-DP	lipid [%]
VV1Egg	Schlei	n.d.	0.012	0.21	0.18	n.d.	n.d.	0.40	0.87	0.72	0.59	0.81	0.50	4
VV6Egg	Schlei	n.d.	0.0058	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.060	n.d.	0.034	8
VV1Gonad	Schlei	n.d.	0.0047	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.40	0.029	0.040	4
VV6Gonad	Schlei	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.79	n.d.	0.0013	30
VV1Muscle	Schlei	0.26	0.44	n.d.	0.017	n.d.	n.d.	n.d.	0.020	0.037	0.039	0.049	0.086	35
VV6Muscle	Schlei	6.5	0.43	n.d.	n.d.	n.d.	n.d.	0.0040	n.d.	n.d.	0.38	n.d.	0.75	15
Average egg	Schlei	n.d.	0.0089	0.10	0.092	n.d.	n.d.	0.20	0.44	0.36	0.33	0.40	0.27	6
Average gonad	Schlei	n.d.	0.0023	n.d.	0	n.d.	n.d.	0	0	0	0.59	0.015	0.021	17
average muscle	Schlei	3.4	0.43	n.d.	0.0084	n.d.	n.d.	0.0020	0.010	0.019	0.21	0.025	0.42	25
total	Schlei	3.4	0.44	0.10	0.10	n.d.	n.d.	0.20	0.45	0.38	1.1	0.44	0.71	48
GMR	Schlei	n.d.	0.0054	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.9	0.59	0.050	0.68
EGR	Schlei	n.d.	3.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.55	28	13	0.35
EMR	Schlei	n.d.	0.021	n.d.	11	n.d.	n.d.	100	42	19	1.6	16	0.64	0.24
EGMR	Schlei	n.d.	**0.026**	n.d.	**11**	**n.d.**	**n.d.**	**100**	**42**	**19**	**4.4**	**17**	**0.69**	**0.92**
VV2Egg	Ems	n.d.	0.0014	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.0020	0.042	0.0028	0.026	21
VV3Egg	Ems	n.d.	0.000060	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.019	n.d.	0.015	29
VV4Egg	Ems	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.063	n.d.	0.018	5
VV2Gonad	Ems	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.82	n.d.	0.70	8
VV3Gonad	Ems	n.d.	0.0053	n.d.	0.0047	n.d.	n.d.	n.d.	n.d.	0.020	0.026	0.024	0.020	35
VV4Gonad	Ems	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.059	n.d.	0.035	33
VV2Muscle	Ems	1.4	0.16	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	35
VV3Muscle	Ems	n.d.	0.15	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.013	n.d.	0.072	24
VV4Muscle	Ems	n.d.	0.30	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.0087	0.015	n.d.	0.097	25
Average egg	Ems	n.d.	0.00049	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.00068	0.041	0.00093	0.020	18
Average gonad	Ems	n.d.	0.0018	n.d.	0.0016	n.d.	n.d.	n.d.	n.d.	0.0066	0.636	0.0078	0.25	25
average muscle	Ems	0.45	0.21	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.0029	0.009	n.d.	0.056	28
total	Ems	0.45	0.21	n.d.	0.0016	n.d.	n.d.	n.d.	n.d.	0.010	0.69	0.0088	0.33	72
GMR	Ems	n.d.	0.0086	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.3	68	n.d.	4.4	0.90
EGR	Ems	n.d.	0.28	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.10	0.065	0.12	0.079	0.72
EMR	Ems	n.d.	0.0024	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.23	4.4	n.d.	0.35	0.65
EGMR	Ems	**n.d.**	**0.011**	n.d.	n.d.	**n.d.**	**n.d.**	n.d.	n.d.	**2.5**	**72**	n.d.	**4.8**	**1.6**

GMR: Gonad/muscle ratio, EGR: Egg/gonad ratio, EMR: Egg/muscle ratio, EGMR: (Egg+Gonad)/muscle ratio

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CHAPTER IV

Bone resorption and body reorganization during maturation induce maternal transfer of toxic metals in anguillid eels

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Bone resorption and body reorganization during maturation induce maternal transfer of toxic metals in anguillid eels

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During their once-in-a-lifetime transoceanic spawning migration, anguillid eels do not feed, instead rely on energy stores to fuel the demands of locomotion and reproduction while they reorganize their bodies by depleting body reserves and building up gonadal tissue. Here we show how the European eel (*Anguilla anguilla*) breaks down its skeleton to redistribute phosphorus and calcium from hard to soft tissues during its sexual development. Using multiple analytical and imaging techniques, we characterize the spatial and temporal degradation of the skeletal framework from initial to final gonadal maturation and use elemental mass ratios in bone, muscle, liver, and gonadal tissue to determine the fluxes and fates of selected minerals and metals in the eels' bodies. We find that bone loss is more pronounced in females than in males and eventually may reach a point at which the mechanical stability of the skeleton is challenged. P and Ca are released and translocated from skeletal tissues to muscle and gonads, leaving both elements in constant proportion in remaining bone structures. The depletion of internal stores from hard and soft tissues during maturation-induced body reorganization is accompanied by the recirculation, translocation, and maternal transfer of potentially toxic metals from bone and muscle to the ovaries in gravid females, which may have direct deleterious effects on health and hinder the reproductive success of individuals of this critically endangered species.

eel | maternal transfer | bone loss | metals | spawning migration

Stocks of several eel species of the genus *Anguilla* have diminished severely in recent decades worldwide, putting them at risk for conservation. The European eel (*Anguilla anguilla*) is now rated as critically endangered on the red list of the International Union for Conservation of Nature, and the American eel (*Anguilla rostrata*) and Japanese eel (*Anguilla japonica*) are rated as endangered. The reason for these declines is not fully understood, however. Anguillid eels undergo a long oceanic larval development before inhabiting inland and coastal waters for their premature growth phase. With the onset of sexual maturation, they change their appearance from resident yellow eel to migratory silver eel to meet the physiological requirements for an up to 6,000-km, once-in-a-lifetime migration back to their oceanic spawning areas. This peculiar changeover, termed "silvering," involves morphological adaptations, such as increase in eye diameter and fin length, and physiological changes, including the cessation of feeding, degeneration of the gut, and initiation of gonadogenesis (1–3). Consequently, eels rely on the breakdown of their lipid-rich muscle tissue to fuel gonadogenesis and locomotion during their migration (4–6).

No European or American eel in an advanced maturation state has yet been found in the wild, limiting observations to natural initial maturation stages and artificially matured eels. Previous research has shown that the depletion of soft tissues during fasting and maturation is accompanied by the resorption of phosphorus and calcium from the bone, which acts as a mineral reservoir (7, 8). The endoskeleton (plus scales in most bony fish) forms the largest depot for minerals in vertebrates, storing the majority of Ca (~99%) and P (85–90%) (9–11). It has been postulated that this feature could have promoted the evolution of the early dermal skeleton millions of years ago (12, 13).

The use of the maternal body as a reservoir of nutrients during migration in eels illustrates how bone loss, lipid metabolism, and reproduction can be directly physiologically connected. Among teleosts, diadromous salmonids also use energy reserves and lose bone and scale mass during spawning migration (13, 14). While Pacific salmon die after spawning, Atlantic salmon can restore their

Significance

Body reorganization in eels during gametogenesis can induce undesired side effects with possible pathological significance. This study provides analytical evidence for the maternal transfer of toxic metals from soft and hard tissues to the ovaries of mature females. By illustrating the metabolic fluxes and fate of mobilized minerals and metals in the fishes' bodies during sexual development, we have identified a previously unreported aspect of anthropogenic impact on endangered anguillid eels. Furthermore, our findings suggest a physiologically connected interplay of energy metabolism and bone resorption in the reproductive strategy of eels. Consequently, we propose the eel as an interesting model organism for investigating the physiological pathways connecting lipid metabolism and mineral retention, known also to affect the health state of humans.

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skeletons and survive. Depletion and restoration of bone mineral reserves connected to reproduction is also known from mammals, which lose bone during pregnancy and lactation and restore it afterward (10). Not only for this reason, the connection of bone and energy metabolism has received attention in human health sciences (15). Bone metabolism is hormonally linked to fat metabolism (15–20), which can affect several diseases and pathologies, including type 2 diabetes, osteopenia, and osteoporosis (11, 17).

In summary, bone loss in connection with reproduction can be a designated and physiologically purposeful feature, in contrast to the bone loss caused by pathological conditions. Nonetheless, maturation-related and disease-related bone loss may involve similar endocrine mechanisms. In short, appetite, reproduction and bone remodeling are regulated by a complex hormonal system in which leptin and other adipokines control energy homeostasis and modulate bone cells through direct and indirect actions (18–20). At the same time, bone-forming cells (osteoblasts) secrete osteocalcin, which affects lipid storage by regulating insulin (17). The involvement of leptin and its actions in sexual reproduction and energy metabolism seems to be a conserved evolutionary feature found in basal teleosts, such as freshwater eels, as well as in basal osteichthyans, such as gars (20, 21). Interactions between bone and adipose tissue are influenced by an array of hormones, including peripheral hormones and prehormones (18, 19, 22, 23), that have been described in eels (21, 24–26).

Body reorganization during maturation in eels also carries risks. Eels are long-lived, semelparous predators with a high body fat content that are especially prone to the uptake and accumulation of toxic substances during their growth phases (27–29). While many organic compounds are lipophilic and thus concentrate in the eels' lipid-rich soft tissues, the mineral phase of bone can also act as a sink for metals (23, 30). Some of these elements can exert toxic effects by interfering with nutritionally essential metals, by competing for binding sites, or by inhibiting metabolic pathways (31–33). During migration and sexual maturation, similar to organic xenobiotics, these metals can be further concentrated due to catabolic processes, remobilized, and maternally transferred to the gonads, posing a threat to the reproductive fitness of this endangered species (6, 34–36). However, little is known about the physiological effects and the fate of remobilized substances during the phase of fasting and sexual development.

In this study, we investigated the body reconstructions that may occur during the migration of maturing European eels. Our analyses illustrate details of bone resorption at anatomic, microanatomic, and cellular levels and at the same time draw a comprehensive picture of the remobilization and fate of relevant minerals and potentially toxic metals through different relevant somatic body matrices of eels in several maturation stages.

Results

Female Eels Lose More Bone Than Males During Sexual Maturation. Biological data of individual fish are provided in *SI Appendix*, *Text* and Table S1. Demineralization of skeletal structures in premature and artificially matured eels was confirmed by noninvasive whole-body computed tomography (CT) imaging (Fig. 1*A*, *Top*). Yellow female eels (yf) exhibited less total bone volume than silver females (sf) and maturing females (gf), and sf had greater skeletal bone volume than the group of fully mature females (mf). No significant differences in skeletal bone volume were found between silver males (sm) and mature males (mm) (Fig. 1*A*, *Bottom*). Color-coded Ca maps show differences in bone mineral density (BMD) among the groups (Fig. 1*B*, *Top*). Quantitative analyses showed a trend toward decreasing BMD during maturation in both sexes (*SI Appendix*, *Text* and Table S2), with significant differences among the groups (Fig. 1*B*, *Bottom*). Distinct differences across the progressing maturation stages were also evident in Ca maps of the skull structures (*SI Appendix*, Fig. S1*A*). Along with the decrease in BMD, Ca maps of the CT scans also showed signals of elevated mineral density in the body cavities around the developing ovaries, marking the onset of gonadal maturation in sf (*SI Appendix*, Fig. S1*B*).

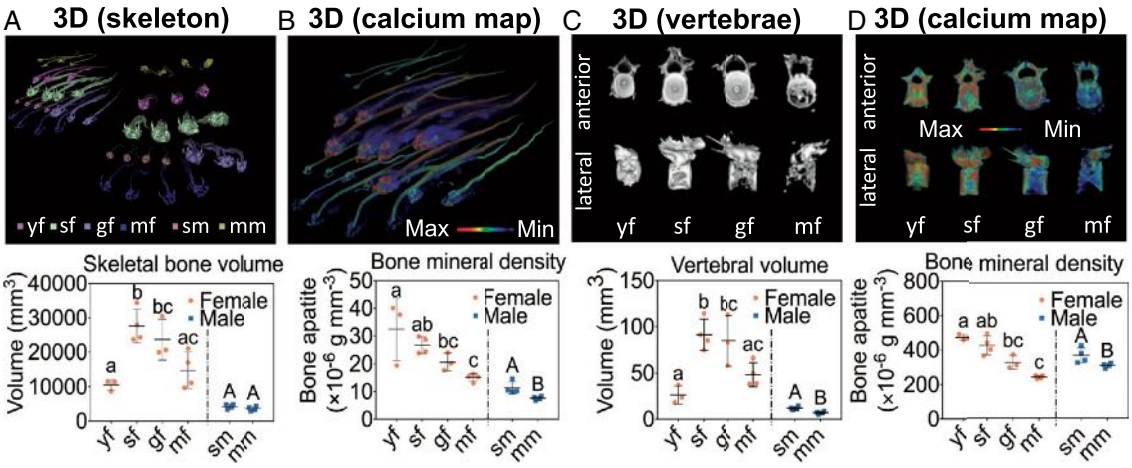

Fig. 1. Bone loss in skeletons and skeletal elements from eels in different maturation stages. (*A*, *Top*) CT-derived 3D renderings of whole eel skeletons depict the total skeletal volumes of female and male eels in different maturation stages. (*A*, *Bottom*) Comparative analyses of total bone volume in eel skeletons revealed significant differences among female maturation stages. Sf and gf females showed the greatest skeletal volumes. (*B*, *Top*) Ca maps (Imalytics Preclinical software) depict BMD in female and male eels of different maturation stages. (*B*, *Bottom*) BMD in skeletal bone from eels of both sexes differed significantly, with an overall trend toward a decrease with progressing maturation. (*C*, *Top*) 3D reconstructions of C2 cervical vertebrae bodies of female eels in different maturation stages show the successive decline in bone volume. (*C*, *Bottom*) Vertebral volumes in female eels increased during growth phase from yellow stage to silver stage and decreased in both sexes in fully mature stages. (*D*, *Top*) Color-coded volume renderings of single skeletal elements of female eels show the decline in BMD during maturation. (*D*, *Bottom*) BMD in vertebral bodies from female and male eels decreased from silver stage to fully mature stage. In *A*–*D*, different letters indicate statistically significant differences among maturation stages per sex ($P < 0.05$). yf, $n = 3$; sf, $n = 4$; gf, $n = 3$; mf, $n = 4$; sm, $n = 4$; mm, $n = 4$.

Micro-CT scans and Ca maps of isolated female eel vertebrae of different maturation stages provided insight into spatial differences in bone resorption (Fig. 1 *C* and *D*, *Top*). Bone loss in all areas intensified with progressing maturation. While trabecular bone structures in cervical vertebrae from yf and sf were mostly intact and stable, hormone-treated groups showed signs of progressive bone loss. Vertebrae from hormone-treated groups (gf and mf) had resorbed structures in all parts of the vertebral body (Fig. 1*C*, *Top*). Quantitative analyses revealed lower vertebral volumes in yf compared with sf and gf, but no difference between yf and mf (Fig. 1*C*, *Bottom* and *SI Appendix*, *Text* and Table S2). In contrast, sm had larger vertebral volumes than mm (Fig. 1*C*, *Bottom*). In female eels, BMD in vertebrae showed a decreasing trend from yf to mf, in line with maturation (Fig. 1*D*, *Top* and *Bottom*). In male eels, BMD in vertebrae was greater in sf compared with mf (Fig. 1*D*, *Bottom*).

Structural Examination of Vertebral Bodies Indicates a Conserved Functionality of the Notochord. Scanning electron microscopy (SEM) images of vertebral bodies provided details of bone loss in individual bone compartments. While overview images of cut vertebrae (Fig. 2*A*) depicted a uniform degradation of bone trabeculae, magnified views (Fig. 2*B*) illustrated microstructural changes in the trabecular bone. Fenestration and increased widths in bone marrow-like structures, lined by exposed collagen fiber bundles, were present in sf and further progressed in the two hormone-treated groups (gf and mf). The loss of trabecular bone structure in male eels was less pronounced compared with that in females. Histological examination of vertebrae (Fig. 2*C*) revealed abundant bone remodeling. Osteons were clearly visible in yf, sf, and sm. Compared with the other groups, yf had denser bone structures and showed only minor signs of bone resorption. Sf showed a regular amount of bone at the vertebral body end plates with their bone trabeculae connected and the intervertebral space and the notochord still intact. In contrast, both artificially matured female groups (gf and mf) showed progressive bone loss with disconnected bone trabeculae and indications of substantial bone resorption (asterisk) at the vertebral body end plates. In mf, most of the bone was resorbed down to the notochord sheath with no open fenestration, leaving the notochord functional. Traces of previous notochord fenestration with scarring tissue indicated repair of this damage. In male eels (*SI Appendix*, Fig. S2), maturation-related bone resorption was less pronounced than in females, yet mm showed greater bone resorption compared with untreated sm.

Eels Use Bone as a Mineral Reservoir to Build up Gonads During Maturation. Energy-dispersive X-ray spectroscopy (EDXS) was used to determine emission signals for Ca and P in cervical vertebrae of animals from all the experimental groups. Emission spectra revealed similar Ca:P ratios and thus stable elemental composition (Fig. 3 *A* and *B*). In contrast, emission strength and peak areas differed considerably among the groups, indicating decreasing BMD along progressing maturation in both sexes. Integrals of both targeted elements ranged from 0.1 to 0.6 normalized counts, with yf displaying the highest counts for both elements, ranging from 0.55 to 0.59 (Fig. 3*A*). Ca and P signals in vertebrae from sf showed smaller peaks, and the lowest signals were detected in vertebrae from gf and mf, with counts ranging from 0.10 to 0.20. Vertebrae from male eels also showed differences in emission intensity according to stage (Fig. 3*B*); however, compared with females, the differences by life stage were not as pronounced.

Elemental inductively coupled plasma mass spectrometry (ICP-MS) mass-balance analyses of P and Ca in different tissues revealed differences in body composition across the maturation stages (Fig. 3 *C* and *D*). P was found mainly in bone (between 82% in mm and 93% in yf), with some (between 2% and 16%) allocated in muscle tissue. With advancing maturation, the soft tissue/bone mass ratio rose, with 9–13% of total allocated P found in gonads of gf and mf. The relative amount of P bound in muscle tissue decreased compared with earlier maturation

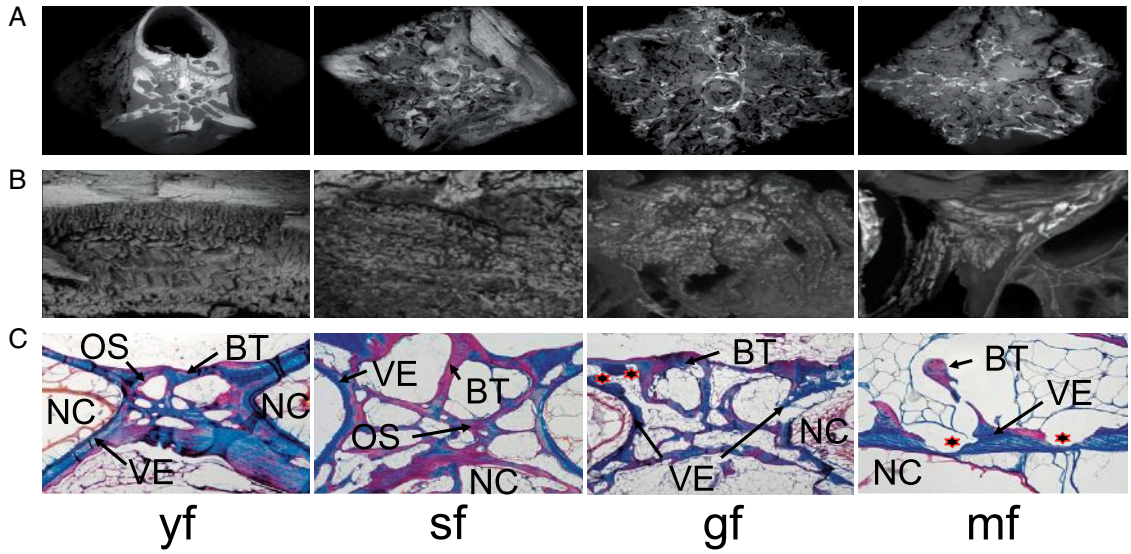

Fig. 2. Structural aspects of bone loss during sexual maturation in eels. Superior view (*A*) and 500× magnified (*B*) SEM images of entire vertebral body end plates of female eels in different maturation stages depict the successive bone loss on a supracellular level. (*C*) Bone histology based on azan-dyed, parasagittal sections of vertebral bodies illustrates changes in bone structures during the maturation process on a cellular level. Defined structures are marked and labeled: BT, bone trabeculae; NC, notochord; OS, osteon; VE, vertebral body end plate. *Indication of bone resorption.

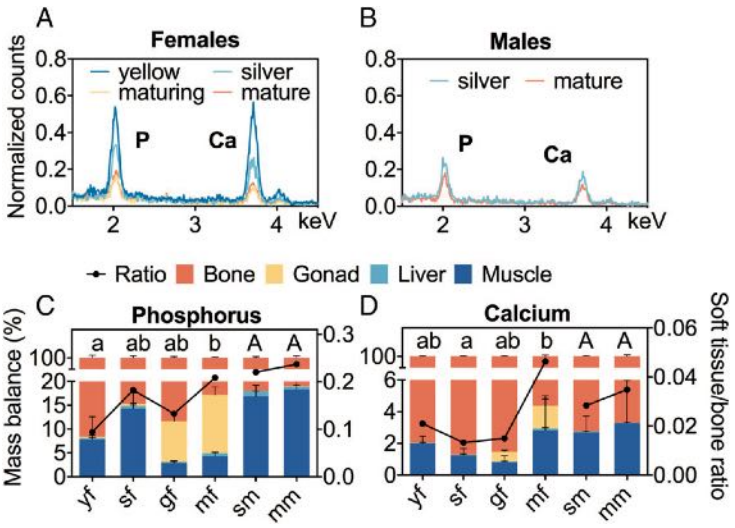

Fig. 3. Ca and P fluxes in eels of different maturation stages. SEM-EDXS spectra revealed decreasing bone mineralization in vertebral bones of representative female (*A*) and male (*B*) individuals according to maturation stage (colors). Relative mass balances (%) of P (*C*) and Ca (*D*) differed among various somatic tissues (colors) as obtained by quantitative ICP-MS. Trend lines illustrate the ratio of total bone-bound analytes in relation to total soft tissue-bound analytes. Different letters indicate significant differences in this ratio across maturation stages by sex ($P < 0.05$). yf, $n = 3$; sf, $n = 4$; gf, $n = 3$; mf, $n = 4$; sm, $n = 4$; mm, $n = 4$.

stages. The ratio of bound P in total soft tissue to bone tissue was significantly greater in mf than in yf (Fig. 3*C*).

By far the largest share of total Ca mass balance was detected in bones, accounting for ∼95% to >99% of the total estimated mass (Fig. 3*D*). While Ca was not found in substantial amounts in liver, the amount bound in soft tissue of females rose slightly with onset of hormone treatment, with the highest soft tissue-to-bone mass ratio seen in mf.

Body Reorganization During Maturation Leads to Maternal Transfer of Toxic Metals. Mass balance analyses (Fig. 4*A*) and dry weight concentrations (Fig. 4*B*) were also obtained to analyze the redistribution of different metals in body compartments of relevance in female eels from sf stage to mf stage (*SI Appendix*, *Text* and Tables S2 and S4). Cd and Cu exhibited quite similar characteristics. While in sf, both metals accumulated in muscle tissue (60–80%), with low amounts found in bone (5–20%), in gf and mf, 50%–65% of Cd was found in the gonads. Concentrations of Cd were <0.1 mg kg^{-1} dry weight (dw) in most matrices of all stages, elevated only in livers (>1 mg kg^{-1} dw) and gonads (>0.2 mg kg^{-1} dw). Cu in gonads was found in similar ranges but at significantly lower concentrations (<3 mg kg^{-1} dw) in both gf and mf compared with sf.

For Mn, the greatest shares were bound in skeletons of sf (90%), with the proportion decreasing with advancing maturation (∼70% in gf and 60% in mf). While the relative mass balance in muscle tissue of all stages remained low (<10%), amounts of Mn in gonadal tissue rose to around 20–30% of total mass balance in the gf and mf. The highest concentrations of Mn were found in bone tissue (20–37 mg kg^{-1} dw) and revealed a slight decreasing trend during maturation, with no significant differences across the groups. Concentrations in liver and gonads increased during maturation, exceeding 10 mg kg^{-1} dw in livers of mf and remaining below 5 mg kg^{-1} dw in gonads of all groups.

For Hg, in untreated sf, almost the entire body burden (∼97%) was found in muscle tissue, while in gf and mf, substantial Hg levels were detected in gonads (Fig. 4*A*). Total concentrations of Hg in all organs were significantly greater in mf than in females at earlier maturation stages (>0.1 to >3.0 mg kg^{-1} dw vs. <0.1 to <1.0 mg kg^{-1} dw) (Fig. 4*B*).

Discussion

This study demonstrates that body reconstruction in European eels during sexual development can initiate the mobilization and maternal transfer of toxic metals, with possible detrimental

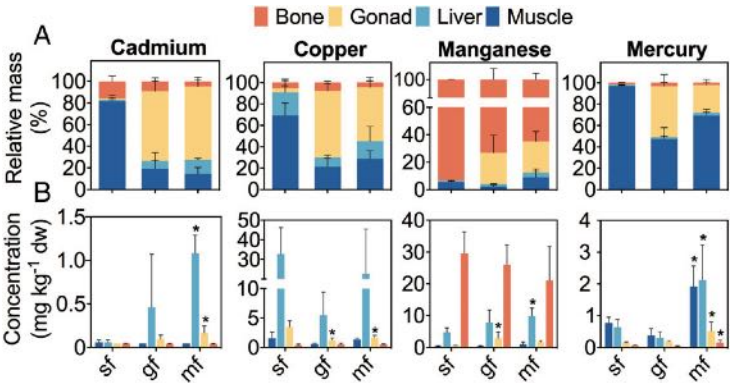

Fig. 4. Mass balance ratios and tissue concentrations of toxic metals in female eels. (*A*) Relative mass balances of Cd, Cu, and Mn were obtained by quantitative ICP-MS, and Hg was analyzed using TDA-AAS. Metabolic fluxes led to notable relocation of metals from muscle (Cd, Cu, and Hg) and bone (Mn) tissue in sf to gonadal tissue in gf and mf. (*B*) Metal dry weight concentrations differed among maturation stages and sampled tissues. Metabolic reorganization during fasting and maturation affected dry weight concentrations in several organs. Asterisks indicate significant differences compared with sf. yf, $n = 3$; sf, n = 4; gf, $n = 3$; mf, $n = 4$.

consequences on health and reproductive success. By analyzing the changes in relative mass balances of selected elements within different body compartments, we have depicted the dispersal and fate of remobilized minerals and metals during maturation. Our results suggest a reciprocal interrelation between the storage and depletion processes in bone and soft tissues of European eels during their spawning migration. Since the speciation of the genus *Anguilla* 20–40 Mya (37), the reproductive strategy of these fishes has evolved to include a "programmed death" resulting from use of the parental body as a storehouse to supply the requirements of locomotion, maturation, and successful spawning. The migrating eel's body provides energy resources, such as lipids and proteins, from muscles (5), as well as minerals from the bones (7, 8).

Eels provide an example of the tight interaction between bone turnover and lipid metabolism in a vertebrate species. As this connection can also be found in extant mammals, the eel may pose an interesting model for generating a better understanding of the physiological pathways involved in associated human pathological conditions and diseases.

In the current study, hormone-treated eels did not constantly swim before being killed. This is important, since with sufficient nutrition, exercise can be beneficial for bone retention and formation in humans and other vertebrates (11). Constant locomotion displayed by nonfeeding, migrating eels however, could amplify bone resorption since muscle mass is continuously depleted during their journey. It has been shown in simulated migration trials that active swimming initiates lipid mobilization and hormonal changes which lead to gonadal maturation in female and male silver eels (5, 38, 39). Due to the lack of feeding during the time of gonadogenesis, especially increasing P requirements would conflict with exercise-triggered bone formation and mineralization. Since the maturing gonads in our sampled fish were supplied with minerals from the bones, we conclude that under natural conditions, the swimming performance necessary for transoceanic migration adds an additional effect to the observed processes.

Ca maps of skeletal structures in this study reveal a corresponding picture. Beginning with initial silvering and thus the start of migration, average mineral content and skeletal mass successively declined in both sexes. This decreasing volume is characterized by the loss of bone tissue in all areas of the vertebrae (Fig. 1 *C* and *D*, *Top*). In fact, single vertebrae in large mf shrunk to the size of those in much smaller, immature yf. This finding is remarkable, since the size of vertebrae in premature eels usually correlates well with their full body size (40). The observed severe bone loss at the vertebral body end plates combined with the disconnection of bone trabeculae until final maturation strongly suggests the loss of mechanical support by vertebral bodies. At the same time, the notochord remained intact and likely functional as axial skeleton (14).

In line with the decline in BMD and bone volume throughout maturation, structural bone loss was more accentuated in female eels compared with males. This was not surprising, since anguillids are sexually dimorphic and females grow larger than males. During oogenesis, female eels also use a greater percentage of their body mass for egg production than male eels do for spermatogenesis (5). To provide sufficient P (and partly Ca) for early embryonic development, egg yolk must be enriched from maternal resources that are stored before migration. Accordingly, CT-derived Ca maps revealed elevated mineral signals located around the gonads of sf (*SI Appendix*, Fig. S1*B*, red arrows). At this stage, female gonads are still at an initial maturation stage without further dilution effects initiated by protein metabolism or hydration (41).

EDXS spectra revealed that gross mineral composition in bone remained unchanged during maturation of both sexes. P and Ca persist at the same ratio, which is in line with the observed bone resorption, a process that liberates all bone minerals equally. Yamada et al. (7) found only small amounts of Ca from the bones transferred to the gonads of eels, suggesting discharge of excess Ca from the body. Unlike P, Ca is generally not limited in aqueous marine environments, since fish are usually able to take up Ca^{2+} from sea water via gill and stomach epithelia (42). At times of extreme Ca demand (e.g., during gonadal maturation), water-derived Ca may be insufficient, and food can provide additional Ca for fish (42, 43). This does not apply to nonfeeding migrating eels (2, 3) and suggests that along with seawater-derived Ca taken up via gills, Ca liberated from the skeleton can be used for oogenesis, muscle function, and other physiological processes.

Elemental analyses also indicated that maturation in eels can induce the redistribution of toxic metals from the animals' bone and soft tissues. Metals such as Cd, Cu, Mn, and Hg are inducers of oxidative stress and are known to cause toxicity in aquatic organisms. Their toxicity mostly originates from their capacity to bind and interfere with proteins and nucleic acids, and the generation of reactive oxygen and nitrogen species may result in oxidative damage of these biomolecules (44). As reviewed by Jezierska et al. (45), teleost early-life stages are particularly sensitive, and the influence of toxic metals may lead to impaired embryonic development. It was further concluded that exposure of spawning animals to metal might result in contamination of eggs and sperm, with potential adverse effects on fertility and embryogenesis.

How the quality of sperm in eels is affected by paternal metal levels and the extent to which male gametes contribute to the overall transfer of metals into fertilized eggs are unclear at present. In female eels, the distribution of these elements changes substantially with ongoing body restructuring, leading to the incorporation of substantial amounts into the gonads, with possible implications for individual reproductive success. Recirculated and relocated metals are of concern, since their concentration in liver, muscle, or gonads, in line with the metabolic reconstructions, may exceed critical values and produce toxic effects.

Cd has also been shown to interfere with bone turnover (46). Pierron et al. (47) showed that Cd is a strong endocrine disruptor in eels, with the potential to interfere with hormone synthesis, to alter egg quality and quantity, and to lead to exhaustion during migration. Since it can negatively affect the lipid storage capacities of European eels, Cd may impact the interaction of lipid metabolism and bone remodeling once the element is released from muscle during migration (48).

Our results for Cu suggest that this element redistributes similar to Cd. Even though Cu is essential to fish nutrition (49), it can exert genotoxic effects (45) and is known to become toxic to aquatic life at concentrations slightly greater than essentially required concentrations (50).

Knowledge of Mn toxicity in fish is scarce, yet it is of relevance here, as it has been shown to accumulate in vertebrate skeletons (32) and to interfere with bone metabolism (51).

Due to its distinct physiochemistry, body distribution, and mass ratio, Hg differed substantially from Cd, Cu, and Mn. This element is known to produce a range of deleterious effects in vertebrates, including microtubule destruction, mitochondrial damage, and lipid peroxidation (52). It also has been shown to negatively affect reproduction of teleost fish (53) and has specifically been suggested to pose a health risk to eels (36). Although mostly associated with the brain and nervous system, Hg can impair any organ and lead to severe organ malfunction and interference with Ca homeostasis (33). Our results indicate a limited but proportional transfer of Hg from muscle tissue into the ovaries, with elevated concentrations in livers of mature fish, indicating a possible metabolic pathway via the liver. Our results are generally in line with Hg levels in somatic tissues of artificially matured female European eels reported by Nowosad et al. (36).

In conclusion, our findings on the resorption and mineral release from the bones of eels contribute to a better understanding of physiological processes involved in the sexual maturation of European eels. Moreover, this study shows that the metabolic turnover of somatic tissue into gametic tissue can induce mobilization

of potentially toxic metals. Proof of maternal transfer of these elements into the ovaries of female eels demonstrates the fairly incalculable challenges that eels encounter in the course of reproduction. For future studies, a desirable focus would be an assessment of specific toxicity biomarkers to generate effect data, to better support the hypothesis that maternal transfer of metals to the gonads represents a specific risk to offspring in eels. Due to the involvement of apparently ancient, congeneric physiological pathways, new knowledge of the pertinent mechanisms in eels may be helpful to better understand the interconnections of lipid metabolism and mineral retention that are also involved in conditions and diseases seen in humans. However, additional investigations are needed to further elucidate the involved endocrine interactions that regulate energy metabolism, storage depletion, and bone remodeling in eels during migration and sexual maturation.

Methods

Female and male European eels in different natural and artificially induced maturation stages were analyzed postmortem for BMD and maturation-related structural changes in skeletal elements using clinical CT and micro-CT. Structural changes on a microanatomic level in single skeletal elements were investigated using histology and SEM. Elemental composition of eel soft and hard tissues was analyzed using EDXS, ICP-MS, and thermal decomposition, amalgamation, and atomic absorption spectrometry (TDA-AAS). Individual elemental tissue burdens were calculated using measured concentrations in the respective tissue multiplied with extrapolated, stage-specific total dry weight of the respective organ, derived from a reference database (*SI Appendix*, *Text* and Tables S2 and S4). Further details on biological variables, hormone treatment, sampling and handling, methodological procedures and instrumental settings, and test statistics, as well as the reference database for mass balance calculations, are provided in *SI Appendix*.

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Supplementary Information for

Bone resorption and body reorganization during maturation induce maternal transfer of toxic metals in anguillid eels

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This file includes

Supplementary Text

Table S1 to S4

Figures S1 and S2

References for SI reference citations

Supplementary Text

Sampling of animals:

A total of 22 live European eels (*Anguilla anguilla*) were originally obtained from German commercial fisheries and eventually divided into six groups according to their sex, life history and maturation stages: Yellow, silver, (hormone treated) maturing female, (hormone treated) fully matured female, silver males and (hormone treated) fully mature male developmental stages. Due to low availability, no male yellow eels were included in the analyses (*See Table S1 for detailed biological data of all used individuals*). In accordance with German animal welfare law (1), eels used for this study were killed by an overdose of 2-phenoxyethanol (ROTH, Karlsruhe, Germany) until final stop of the heart and subsequently stored at -20°C before further data collection.

Hormone treatment:

All hormone-treated groups consisted of silver eels that were artificially matured by regular hormone injections prior to analyses. While the maturing female group consisted of fish that were incompletely stimulated due to shorter hormonal treatment, individuals in the mature female and mature male groups consisted of ready-to-spawn individuals with fully matured gonads. During the time of hormone treatment, these eels were held under a gentle but constant water flow in a round recirculation system equipped with aeration and a trickle filter for mechanical filtration and denitrification.

To induce maturation in female eels, individuals were treated with weekly intramuscular injections of aqueous salmon pituitary extract (SPE; Argent Aquaculture, Redmond, USA; 20 mg/kg). Injections were administered close to the dorsal fin into the dorsal muscle. Full maturation was indicated by a steep increase in weight caused by the hydration of oocytes. To reach this stage, between 26 to 32 weekly SPE injections per individual were required. Individuals in the group of maturing females did not reach the full maturation status and received between 13 and 27 SPE-injections before they died during the maturation process. They were immediately stored at 20°C when death was confirmed. Male eels were treated with weekly intraperitoneal injections of 150 IU human chorionic gonadotropin (hCG; Sigma-Aldrich, Schnelldorf, Germany). These fish were regarded mature when they produced active spermatozoa, which was regularly monitored and visually confirmed using a stereo microscope. It took ≥8 weeks of hCG-treatment to reach this stage.

Sampling of skeletal elements:

The first spinal, postcranial vertebrae of each individual per group were excised for µCT scans. The same vertebrae of each, one representative individual per group was then also used for SEM scans and the third to fifth postcranial vertebrae taken from the same individuals were excised for bone histology. For body burden calculations and for inductively coupled plasma mass spectrometry (ICP-MS) measurements, all fish were carefully further dissected and body compartments of interest (muscle, liver,

gonads, selected vertebrae and remaining spinal cord) were separated, weighed and subsequently freeze-dried and weighed again to record the water contents.

Whole-body computed tomography (CT) imaging:

Non-invasive whole-body scans from eels were performed at a clinical CT system (Somatom Definition Flash, Siemens Medical Solutions, Forchheim, Germany). All animals were scanned within one single CT scan (voltage 120 kV, current 54 mA, exposure time 285 ms, focal spot 1.2, filter type wedge 3) and CT slices were reconstructed using the Syngo software (Syngo CT 2012B, Siemens Medical Solutions) at voxel size 0.77 mm with a medium kernel (B35f). (*For further instrumental settings, see Table S3*) Individual whole-body volumes and skeletal structures were semi-automatically rendered based on contrast thresholding using Imalytics Preclinical Software, as previously described (2,3). Each individual animal was assigned a distinct class and color to facilitate visualization. BMD was estimated by comparing bone density with a hydroxyapatite calibration phantom (Osteo Phantom, Siemens Medical Solutions, Erlangen, Germany) of known BMD (200 mg / cm^3) scanned simultaneously with the eels**.**

Vertebrae imaging (µCT):

Making use of the same individuals from the whole-body CT scans, detailed imaging of the *second* postcranial spinal vertebrae was performed in a preclinical µCT system (TomoScope DUO 30s, CT-Imaging, Erlangen, Germany). Following the standard scanning protocol HQD-6565-360-90 as described by Gremse *et al.* (4), the vertebrae from individuals within the same sampling groups were scanned. Volumetric images were reconstructed at voxel size 70µm using a Feldkamp-type reconstruction with ring artifact correction. Vertebrae were segmented using a region-growing algorithm based on their increased image intensity, to determine the vertebrae volume per animal.

Calcium maps:

Calcium maps were derived from the clinical-CT (whole-body) and micro-CT (vertebrae) data, making use of proportionality between calcium-content and x-ray density (5). Those were employed to assess the mean and total calcium concentration (values normalized to g/10^6 mm^3) per animal, and for providing a color-coded volumetric depiction of the calcium distribution within the scanned specimen or body compartment.

Scanning electron microscopy (SEM) of selected vertebrae:

Ex vivo validation of bone demineralization was performed through high-resolution scanning electron microscopy (ESEM XL 30 FEG, FEI, Eindhoven, sputter coater EM SCD500, Leica, Wetzlar, Germany) of harvested vertebrae. Of each group, one representative, of the first three *postcranial* vertebra per developmental stage was scanned for overview, 50x, 500x and 2500x magnifications (BSE scan, 10 kV,

VD: 10, Spot 3.0, Blender 3.0). Energy dispersive X-ray spectroscopy (EDXS) analyses were performed at 50x magnifications (Preset 50, Amp 35 µs), providing the semi-quantitative assessment of average Ca and P content within each sample Results were compared to the ICP-MS analytical evaluation.

Bone histology:

For histological analyses, frozen abdominal (type II) vertebrae of one representative individual (with median bone density) per group were thawed in 10% neutral-buffered formalin at room temperature and subsequently fixated in formalin for additional three days. After formalin fixation, these samples were rinsed in running tap water overnight and then transferred stepwise into 70% ethanol (storage solution). Then, prior to decalcification, the vertebrae were again transferred stepwise to 100% ethanol to remove tissue fat overnight on a shaker and after this, retransferred stepwise to tap water again. Decalcification was carried out with Decal™ Decalcifier (Statlab Medical, McKinney, USA) twice for 24 h; with each step performed on a shaker at room temperature. After decalcification, samples were rinsed overnight in tap water followed by dehydration in a series of ethanol solutions with increasing concentration (30-100% ethanol in water). In the paraffin embedding process, the bones were treated with HistoChoice (Sigma-Aldrich, St. Louis, USA), two times overnight on a shaker, followed by graded HistoChoice - paraffin solutions (30-100% paraffin at 60°C). Serial sections of 5 µm thickness were prepared in the sagittal plane of the vertebral column, starting at the lateral periphery of vertebral bodies and ending in the mediosagittal plane. Sections were mounted on treated microscope slides (Superfrost Plus Micro, VWR International, Radnor, USA) and stained with Heidenhain's Azan following the protocol of Presnell & Schreibman (6). DPX (Sigma-Aldrich, St. Louis, USA) was used to mount the sections on slides. All sections were analyzed and photographed at magnifications between 40x and 400x with a Leitz Dialux 22EB microscope (Leica, Wetzlar, Germany) equipped with a 5MP color CCD camera.

Analytical validation by ICP-MS & TDA AAS

Before further utilization, muscle, liver, bone and gonad samples were weighed, lyophilized (Lyovac GT 2; GEA Pharma Systems, Wommelgem, Belgium) and then weighed again before being homogenised using a laboratory mill (IKA A11; IKA, Staufen, Germany).

Concentrations of most minerals and metals were determined by means of ICP-MS. Briefly, samples were digested in Polytetrafluoroethylene (PTFE) high-pressure vessels (HPR-1000/10S, MLS GmBH, Leutkirch, Germany) using an MLS Ethos plus microwave oven with temperature and pressure control (MLS GmbH, Leutkirch, Germany). To this end, approx. 50-100 mg was weighed into a microwave vessel containing 5 mL nitric acid (65%, SupraPure, Sigma Aldrich), 7 mL hydrogen peroxide (SupraPure, Sigma Aldrich), and 1 mL internal rhodium standard (1µg mL^{-1}). The digestion temperature was ramped from room temperature to 210°C for 45 min, and held for 15 min. After digestion, samples were rehydrated 2- and 10-fold with ultrapure water. Instrumental ICP-MS analyses were performed on

an ELAN DRC II system (PerkinElmer SCIEX™, PerkinElmer, Waltham, MA, USA). The technical parameters and operating conditions are summarized in supplementary material (S2). Calibration was performed using a commercial multi-element standard (CertiPUR®, Merck, Darmstadt, Germany) with element concentrations ranging from 0.1 – 1000 mg L^{-1}. Limits of detection (LODs) were calculated by applying the dilution factor to LODs based on the signal obtained from the analysis of 10 replicates of a solution containing a digested unexposed filter using the three standard deviation criteria.

Mercury concentrations in the body compartments of interest were determined using thermal decomposition, amalgamation, and atomic absorption spectrometry (TDA AAS) (Direct Mercury Analyzer, Milestone Inc, Shelton, USA) with integrated autosampler, control system lab-terminal 1024 and laboratory weighing scale (Precisa XT220, Milestone, Leutkirch, Germany). Dry samples were weighed to approximately 1,5 - 60 mg and quadruplicate measurements were conducted for each sample. If values varied more than 20 %, another 4-fold measurement was performed.
Analytically derived dry weight-based concentrations of metals and minerals in homogenized samples from individual organs were multiplied with the total dry-weight of the respective organ, which was estimated based on life-history stage-dependent percentages of the body wet-weight of each fish. For this, a reference database consisting of whole-body dry-weight distribution data from 120 European eels of different stages was used (*SI Table S4*). Results were expressed as relative percentages in the individual compartments, and as soft tissue to bone mass ratios.

Statistical Analyses

Statistical testing was carried out using GraphPad Prism 6.0h (GraphPad software Inc, California, USA). Analysis of variances (ANOVA) followed by Tukey's or Dunnett's post hoc test was used to test for differences in volume and calcium map- derived calcium content of whole-body and single vertebrae sampling groups of female eels. For the two groups of male eels, student's t-test was used to test for differences in volume and calcium content of whole bodies and single vertebrae sampling groups. A significance level of $P < 0.05$ was used for all tests. (*Details on test results available in Table S2*)

Table S1.

Detailed biological parameters of eels used in this study

ID	System / Origin	Sex	Stage (Durif)	Length (cm)	Mass (g)	Liver mass (g)	Gonad mass (g)	Muscle sample (g)	Stomach & Gut (g)	Total muscle lipid *(fatmeter device)*	Swim-bladder (g)	Skeletal bone volume (mm^3)	Bone mineral density in whole skeleton (apatite / mm3)	Vertebral bone volume (mm^3)	Bone mineral density in Vertebrae (apatite / mm3)
yf1	Warnow / Peene	f	2	51	177	1.25	0.28	17.39	6.12	14.5	1.13	11444.30	37.62	37.16	457.29
yf2	Warnow / Peene	f	1	45	118	1.41	0.1	10.25	3.73	12.9	0.18	8675.78	40.09	17.99	491.39
yf4	Warnow / Peene	f	3	55	302	4.0	3.04	21.53	7.44	33.1	0.77	11556.70	19.43	23.66	469.92
sf1	Warnow / Peene	f	5	66	632	7.8	11.34	38.4	12.48	26.3	2.98	23699.70	24.36	85.03	411.02
sf2	Warnow / Peene	f	5	73	818	8.4	11.45	41.45	19.05	30.2	2.96	27983.50	28.68	114.36	374.80
sf3	Warnow / Peene	f	5	73	782	0.94	10.58	36.92	18.38	29.5	2.24	24316.50	23.76	74.28	450.60
sf4	Warnow / Peene	f	5	76	877	10.53	12.59	34.52	21.48	38.2	4.89	34374.70	29.77	91.07	503.94
gf1	Ems	f	5	71	528	4.8	74.07	18.95	2.84	20.9	1.35	19986.00	17.66	82.82	305.01
gf2	Arresö (Dk)	f	5	73	709	10.64	10.9	33.58	6.95	31.5	3.72	30512.10	23.92	113.07	380.54
gf4	Ems	f	5	73	675	8.52	124.1	29	4.78	20.7	1.47	20626.70	20.25	58.34	320.64
mf1	Weser	f	5	69	634	4.66	155	13.39	1.66	NA	3.8	11152.30	13.04	38.06	244.46
mf2	Weser	f	5	66	532	5.14	152	15.5	4.1	NA	0.5	9757.93	15.49	38.89	258.49
mf3	Weser	f	5	77	826	14.21	295.6	12.46	1.92	NA	1.05	17118.30	1534	49.47	258.69
mf4	Weser	f	4	81	1219	17.08	471.87	25.6	9.1	NA	2.81	21026.90	16.31	66.08	238.66
sm1	Warnow / Peene	m	6	44	125	1.25	0.1	14.44	2.24	29.6	0.58	4071.39	10.50	14.49	348.71
sm2	Warnow / Peene	m	6	39	107	1.38	<LOQ	10.82	2.6	27.3	1.09	3390.63	10.19	10.92	334.14
sm3	Warnow / Peene	m	6	42	122	1.39	<LOQ	14.69	1.87	37.4	0.69	4799.40	9.95	12.00	388.27
sm4	Warnow / Peene	m	6	43	141	1.25	<LOQ	10.39	1.53	33.4	0.58	4750.95	14.62	10.20	432.65
mm1	Raised in farm	m	6	43	136	1.63	8.77	8.82	1.33	32.8	0.21	3926.61	7.41	8.70	330.84
mm2	Raised in farm	m	6	41	155	1.96	14.94	9.68	0.94	34.2	0.11	3357.13	7.81	5.19	300.31
mm3	Raised in farm	m	6	39	106	1.1	10.65	9.7	1.69	36.3	0.05	2929.22	8.30	6.75	323.04
mm4	Raised in farm	m	6	43	88	0.85	2.32	8.65	0.78	37.6	0.05	4398.01	6.75	7.43	316.86

Table S2.

Details of statistical tests between tested groups. Bold letters indicate significant results

Skeletal Bone Volume							
FEMALES (one-way ANOVA and Tukey's multiple comparisons test)							
	Mean 1	Mean 2	Mean Diff.	Summary	95.00% CI of diff.	Adjusted P value	
Yellow vs. Silver	**10559**	**27594**	**-17035**	**	**-28238 to -5832**	**0.0042**	
Yellow vs. Maturing	**10559**	**23708**	**-13149**	*	**-25126 to -1173**	**0.0310**	
Yellow vs. Mature	10559	14764	-4205	ns	-15408 to 6998	0.6701	
Silver vs. Maturing	27594	23708	3885	ns	-7318 to 15088	0.7194	
Silver vs. Mature	**27594**	**14764**	**12830**	*	**-2458 to 23202**	**0.0158**	
Maturing vs. Mature	23708	14764	8944	ns	-2259 to 20147	0.1312	
Males (unpaired t-test)							
	Mean 1	Mean 2	Mean Diff	Summary	95.00% CI of diff.	P value	R^2
Silver vs. Mature	4253	3653	-600	ns	-1732 to 532	0.2423	0.2189

Skeletal Bone Mineral Density							
FEMALES (one-way ANOVA and Tukey's multiple comparisons test)							
	Mean 1	Mean 2	Mean Diff.	Summary	95.00% CI of diff.	Adjusted P value	
Yellow vs. Silver	32.4	26.64	5.757	ns	-7.215 to 18.73	0.5504	
Yellow vs. Maturing	**32.4**	20.61	11.79	ns	-2.082 to 25.66	0.1030	
Yellow vs. Mature	**32.4**	**15.04**	**17.35**	**	**4.382 to 30.33**	**0.0098**	
Silver vs. Maturing	26.64	20.61	6.029	ns	-6.943 to 19	0.5147	
Silver vs. Mature	26.64	15.04	11.6	ns	-0.4131 to 23.61	0.0591	
Maturing vs. Mature	20.61	15.04	5.568	ns	-7.405 to 18.54	0.575	
Males (unpaired t-test)							
	Mean 1	Mean 2	Mean Diff	Summary	95.00% CI of diff.	P value	R^2
Silver vs. Mature	11.31	7.564	-3.75	ns	-6.577 to -0.9225	0.0176	0.6371

Vertebral Volume							
FEMALES (one-way ANOVA and Tukey's multiple comparisons test)							
	Mean 1	Mean 2	Mean Diff.	Summary	95.00% CI of diff.	Adjusted P value	
Yellow vs. Silver	**26.27**	**91.19**	**-64.92**	**	**-105.9 to -23.98**	**0.0031**	
Yellow vs. Maturing	**26.27**	**84.74**	**-58.47**	**	**-102.2 to -14.71**	**0.0099**	
Yellow vs. Mature	26.27	48.2	-21.93	ns	-62.87 to 19.01	0.4017	
Silver vs. Maturing	91.19	84.74	6.442	ns	-34.50 to 47.38	0.9615	
Silver vs. Mature	**91.19**	**48.2**	**42.99**	*	**5.083 to 80.89**	**0.0260**	
Maturing vs. Mature	84.74	48.2	36.54	ns	-4.395 to 77.48	0.0840	
Males (unpaired t-test)							
	Mean 1	Mean 2	Mean Diff	Summary	95.00% CI of diff.	P value	R^2
Silver vs. Mature	**11.9**	**7.018**	**-4.89**	**	**-7.796 to -1.974**	**0.0063**	**0.7376**

Vertebral Bone Mineral Density

FEMALES (one-way ANOVA and Tukey's multiple comparisons test)

	Mean 1	Mean 2	Mean Diff.	Summary	95.00% CI of diff.	Adjusted P value
Yellow vs. Silver	0.04442	0.04351	0.0009096	ns	-0.008852 to 0.01067	0.9919
Yellow vs. Maturing	**0.04442**	**0.03354**	**0.01088**	*	**0.0003348 to 0.02142**	**0.0426**
Yellow vs. Mature	**0.04442**	**0.02501**	**0.01941**	***	**0.009649 to 0.02917**	**0.0005**
Silver vs. Maturing	0.04351	0.03354	0.009969	ns	-0.0005748 to 0.02826	0.0656
Silver vs. Mature	**0.04351**	**0.02501**	**0.0185**	***	**0.008739 to 0.02826**	**0.0007**
Maturing vs. Mature	0.03354	0.02501	0.008532	ns	0.0002012 to 0.02908	0.1273

Males (unpaired t-test)

	Mean 1	Mean 2	Mean Diff	Summary	95.00% CI of diff.	P value	R^2
Silver vs. Mature	**0.03759**	**0.03178**	**-0.005818**	*	**-0.01145 to -0.0001844**	**0.0449**	**0.5156**

Concentration in Bones From ADULT FEMALES

(one-way ANOVA with Dunnett's multiple comparisons test)

	Mean 1	**Mean 2**	**Mean Diff.**	**Summary**	**95.00% CI of diff.**	**Adjusted P value**
Cadmium						
Silver vs. Maturing	43.75	40	3.75	ns	-4.116 to 11.62	0.3810
Silver vs. Mature	43.75	40	3.75	ns	-3.532 to 11.03	0.3337
Copper						
Silver vs. Maturing	397.5	543	-145.5	ns	-451.1 to 160.1	0.3819
Silver vs. Mature	397.5	534	-136.5	ns	-419.4 to 146.4	0.3737
Manganese						
Silver vs. Maturing	29625	26033	3592	ns	-13428 to 20611	0.8013
Silver vs. Mature	29625	21025	8600	ns	-7157 to 24357	0.2989
Mercury						
Silver vs. Maturing	56	43.33	12.67	ns	-85.56 to 110.9	0.9181
Silver vs. Mature	**56**	**157.8**	**-101.8**	*	**-192 to -10.81**	**0.0310**

Concentration In Gonads From Adult Females

(one-way ANOVA with Dunnett's multiple comparisons test)

	Mean 1	**Mean 2**	**Mean Diff.**	**Summary**	**95.00% CI of diff.**	**Adjusted P value**
Cadmium						
Silver vs. Maturing	**45**	**96**	**-51**	**ns**	-160.2 to 58.25	0.3941
Silver vs. Mature	**45**	**168.4**	**-123.3**	*	**-224.4 to -22.11**	**0.0208**
Copper						
Silver vs. Maturing	**3481**	**1261**	**2220**	**	**789.1 to 3651**	**0.0059**
Silver vs. Mature	**3481**	**1690**	**1791**	*	**465.9 to 3115**	**0.0124**
Manganese						
Silver vs. Maturing	**612.8**	**2845**	**-2233**	*	**-4324 to -141.4**	**0.0381**
Silver vs. Mature	612.8	1765	-1153	ns	-3089 to 783.6	0.2492
Mercury						
Silver vs. Maturing	139	186	-47	ns	-408 to 314	0.9166

Silver vs. Mature	**139**	**520.3**	**-381.3**	*	**-715.5 to -47.01**	**0.0284**

Concentration In Livers From Adult Females (one-way ANOVA with Dunnett's multiple comparisons test)						
	Mean 1	**Mean 2**	**Mean Diff.**	**Summary**	**95.00% CI of diff.**	**Adjusted P value**
Cadmium						
Silver vs. Maturing	58.75		-400.3	ns	-1077 to 276.8	0.2530
Silver vs. Mature	**58.75**		**-1024**	**	**-1651 to -397.2**	**0.0044**
Copper						
Silver vs. Maturing	32700	5510	27190	ns	-1457 to 55838	0.0619
Silver vs. Mature	32700	22617	10083	ns	-18565 to 38730	0.5760
Manganese						
Silver vs. Maturing	4711	7795	-3083	ns	-8471 to 2304	0.2713
Silver vs. Mature	**4711**	**9956**	**-5245**	*	**-10233 to -257.1**	**0.0406**
Mercury						
Silver vs. Maturing	629.3	300.7	328.6	ns	-1098 to 1755	0.7700
Silver vs. Mature	**629.3**	**2123**	**-1494**	*	**-2815 to -172.8**	**0.0295**

Concentration In Muscle From Adult Females (one-way ANOVA with Dunnett's multiple comparisons test)						
	Mean 1	**Mean 2**	**Mean Diff.**	**Summary**	**95.00% CI of diff.**	**Adjusted P value**
Cadmium						
Silver vs. Maturing	1532	584.5	947.5	ns	-751.2 to 2646	0.2764
Silver vs. Mature	1532	1352	180	ns	-1207 to 1567	0.9126
Copper						
Silver vs. Maturing	58.75	45	13.75	ns	-20.63 to 48.13	0.4897
Silver vs. Mature	58.75	45	13.75	ns	-18.08 to 45.58	0.4423
Manganese						
Silver vs. Maturing	414.3	450.3	-36.08	ns	-890.4 to 818.3	0.9907
Silver vs. Mature	414.3	1049	-635	ns	-1426 to 156	0.1110
Mercury						
Silver vs. Maturing	767	383.3	383.7	ns	-479.8 to 1247	0.4248
Silver vs. Mature	**767**	**1922**	**-1155**	**	**-1954 to 355.3**	**0.0088**

Table S3.

Instrumental parameters for the analysis with ICP-MS

ICP-MS Operating Conditions and Parameters.	
ICP-MS	Elan-DRCII (Perkin-Elmer)
Nebulizer	Meinhard Type A quartz Part No.: WE02-4372
Spray Chamber	Quartz Cyclonic Part No.: WE02-5222
RF Power	1100 Watt
Plasma Ar Flow	15 L/min
Nebulizer Ar Flow	0.93 L/min
Aux. Ar Flow	1.1 L/min
Injector	2.0 mm i.d. Quartz Part No.: WE02-3916
CeO+/Ce+	<3%

Table S4.

Reference dataset with biological values for mass balance calculations

Origin	Yellow /Silver	Sex	Stage (Durif)	Length (cm)	Weight (g)	Fatmeter % in Filet	Fat (%ww) (analyt.)	Kidney (g)	Kidney (%)	Liver (g)	Liver (%)	Gonads (g)	Gonads (%)	Gut (g)	Gut (%)	Total muscle (g)	Muscle (%)	Skin (g)	Skin (%)	Carcass (g)	Carcass (%)	SB (g)	SB (%)	Gills (g)	Gills (%)	Splee n (g)	Splee n (%)	Brain(g)	Brain (%)
Aquaculture	y	f	1	34	57.75	NA	7.35	0.72	1.24	0.80	1.38	NA	NA	2.25	3.90	26.31	45.56	6.36	11.01	12.87	22.28	NA	NA	0.97	1.68	0.09	0.16	0.05	0.08
Aquaculture	y	f	1	35	52.81	NA	7.35	0.86	1.62	0.80	1.51	NA	NA	2.15	4.06	23.25	44.02	5.79	10.96	13.93	26.37	NA	NA	0.52	0.98	0.12	0.23	0.03	0.06
Aquaculture	y	f	1	40	94.05	NA	8.72	1.26	1.34	1.29	1.37	0.33	0.35	3.78	4.02	40.24	42.79	9.77	10.38	28.75	30.57	NA	NA	1.73	1.84	0.20	0.21	0.05	0.05
Aquaculture	y	f	1	39	108.65	NA	20.99	0.90	0.83	1.37	1.26	0.37	0.34	5.67	5.22	56.53	52.03	9.65	8.89	23.58	21.70	NA	NA	1.29	1.19	0.14	0.13	0.05	0.05
Aquaculture	y	m	1	35	83.7	NA	23.24	0.61	0.73	1.28	1.53	NA	NA	4.85	5.79	39.90	47.67	9.07	10.84	19.19	22.93	NA	NA	1.18	1.41	0.11	0.13	0.04	0.05
Aquaculture	y	m	1	34	76.12	NA	23.50	0.46	0.60	1.04	1.37	NA	NA	3.61	4.75	38.77	50.93	8.38	11.01	18.58	24.41	NA	NA	0.80	1.06	0.05	0.06	0.03	0.04
Aquaculture	y	f	1	36	78.2	NA	11.32	0.82	1.04	0.93	1.18	0.10	0.13	2.11	2.69	44.91	57.43	8.55	10.94	13.69	17.51	NA	NA	0.93	1.19	0.07	0.10	0.05	0.06
Aquaculture	y	f	1	35	61.39	NA	9.91	0.45	0.73	0.86	1.41	0.16	0.27	3.40	5.53	27.34	44.54	5.49	8.95	17.71	28.85	NA	NA	0.65	1.06	0.07	0.12	0.04	0.06
Aquaculture	y	f	1	35	73.47	NA	21.35	0.84	1.14	0.98	1.34	0.31	0.42	4.14	5.64	41.36	56.30	6.35	8.65	12.86	17.50	NA	NA	1.27	1.73	0.10	0.14	0.07	0.09
Aquaculture	y	f	1	35	65.9	NA	15.10	0.45	0.68	0.90	1.36	0.42	0.64	2.31	3.51	38.40	58.27	4.57	6.93	9.97	15.13	NA	NA	1.47	2.23	0.05	0.08	0.06	0.09
Aquaculture	y	f	1	34	66.84	NA	13.75	0.73	1.09	0.91	1.36	0.10	0.14	2.63	3.93	32.93	49.27	7.73	11.56	15.61	23.36	NA	NA	0.89	1.33	0.14	0.21	0.05	0.07
Aquaculture	y	f	1	35	63.7	NA	17.02	0.72	1.13	0.84	1.32	NA	NA	1.63	2.56	34.81	54.65	6.48	10.18	11.91	18.70	NA	NA	0.91	1.42	0.08	0.13	0.04	0.06
Aquaculture	y	f	1	31	57.33	NA	26.49	0.48	0.84	0.61	1.06	NA	NA	2.48	4.32	29.68	51.78	5.85	10.20	12.77	22.28	NA	NA	0.42	0.73	0.06	0.10	0.03	0.05
Aquaculture	y	m	1	31	56.38	NA	25.35	0.54	0.96	0.92	1.63	NA	NA	1.54	2.74	35.77	63.44	4.65	8.25	6.52	11.56	NA	NA	0.54	0.95	0.07	0.13	0.04	0.07
Aquaculture	y	m	1	34	85.45	NA	27.37	0.61	0.71	1.01	1.18	NA	NA	3.29	3.85	47.30	55.35	9.49	11.11	17.29	20.23	NA	NA	0.81	0.94	0.04	0.05	0.04	0.04
Aquaculture	y	f	1	35	66.65	NA	20.91	0.60	0.89	0.82	1.23	0.04	0.06	2.73	4.09	39.37	59.07	5.89	8.83	10.27	15.41	NA	NA	0.79	1.18	0.04	0.06	0.05	0.08
Eider	y	f	1	41	115	11.1	NA	NA	NA	1.78	1.55	0.20	0.17	7.18	6.24	60.89	52.95	17.17	14.93	16.86	14.66	1.0 5	0.9 1	2.45	2.13	NA	NA	NA	NA
Elbe	y	f	2	52	265	34.9	NA	NA	NA	2.88	1.09	2.52	0.95	11.19	4.22	160.30	60.49	32.50	12.26	36.47	13.76	1.0 3	0.3 9	1.71	0.65	NA	NA	NA	NA
Elbe	y	f	2	56	295	35.2	NA	NA	NA	3.53	1.20	1.48	0.50	16.10	5.46	174.31	59.09	32.62	11.06	51.47	17.45	0.8 8	0.3 0	2.83	0.96	NA	NA	NA	NA
Weser	y	f	2	55	320	32.7	NA	NA	NA	4.28	1.34	1.60	0.50	23.19	7.25	191.16	59.74	34.41	10.75	46.48	14.53	2.6 0	0.8 1	3.59	1.12	NA	NA	NA	NA
Weser	y	f	2	61	406	32.8	NA	NA	NA	5.10	1.26	1.86	0.46	21.88	5.39	247.64	61.00	38.11	9.39	60.03	14.79	0.1 8	0.0 4	5.75	1.42	NA	NA	NA	NA
Weser	y	f	2	60	395	29.9	NA	NA	NA	9.08	2.30	2.44	0.62	18.51	4.69	247.90	62.76	36.94	9.35	56.37	14.27	1.6 6	0.4 2	5.71	1.45	NA	NA	NA	NA
Weser	y	f	2	52	285	31.4	NA	NA	NA	4.82	1.69	1.67	0.59	16.40	5.75	151.91	53.30	29.76	10.44	52.00	18.25	1.0 3	0.3 6	4.15	1.46	NA	NA	NA	NA
Weser	y	f	2	57	306	20.4	NA	NA	NA	4.79	1.57	2.18	0.71	17.45	5.70	183.99	60.13	32.56	10.64	43.61	14.25	2.0 4	0.6 7	3.95	1.29	NA	NA	NA	NA
Weser	y	f	2	55	288	30.7	NA	NA	NA	3.90	1.35	1.25	0.43	14.28	4.96	182.94	63.52	25.57	8.88	43.71	15.18	0.7 9	0.2 7	3.69	1.28	NA	NA	NA	NA

Weser	y	f	2	58	508	33.3	NA	NA	NA	8.80	1.73	4.03	0.79	29.10	5.73	323.47	63.68	48.60	9.57	62.87	12.38	0.95	0.19	6.31	1.24	NA	NA	NA	NA
Weser	y	f	2	53	314	28	NA	NA	NA	5.85	1.86	1.30	0.41	12.23	3.89	203.29	64.74	27.85	8.87	38.90	12.39	0.61	0.19	2.99	0.95	NA	NA	NA	NA
Weser	y	f	2	51	218	15.3	NA	NA	NA	2.48	1.14	0.91	0.42	12.84	5.89	127.34	58.41	19.88	9.12	34.73	15.93	1.00	0.46	3.51	1.61	NA	NA	NA	NA
Weser	y	f	2	53	290	21.6	NA	NA	NA	5.66	1.95	1.02	0.35	19.83	6.84	164.31	56.66	28.79	9.93	46.29	15.96	1.00	0.34	4.96	1.71	NA	NA	NA	NA
Eider	y	f	2	52	201	10.8	NA	NA	NA	2.95	1.47	0.51	0.25	9.58	4.77	85.87	42.72	28.89	14.37	60.77	30.23	0.81	0.40	4.65	2.31	NA	NA	NA	NA
Eider	y	f	2	55	355	7.9	NA	NA	NA	4.53	1.28	0.82	0.23	11.73	3.30	122.46	34.50	37.20	10.48	58.21	16.40	1.36	0.38	5.52	1.55	NA	NA	NA	NA
Schlei	y	f	2	55	323	11.2	NA	NA	NA	6.23	1.93	1.35	0.42	15.16	4.69	172.46	53.39	36.64	11.34	70.25	21.75	2.68	0.83	3.85	1.19	NA	NA	NA	NA
Rhein	y	f	2	56	281	19.6	NA	NA	NA	5.91	2.10	1.22	0.43	12.92	4.60	174.00	61.92	23.21	8.26	43.48	15.47	0.93	0.33	2.73	0.97	NA	NA	NA	NA
Rhein	y	f	2	51	206	21.2	NA	NA	NA	4.75	2.31	0.67	0.33	9.70	4.71	123.00	59.71	22.09	10.72	34.71	16.85	0.46	0.22	2.12	1.03	NA	NA	NA	NA
Rhein	y	f	2	52	206	18.3	NA	NA	NA	2.97	1.44	0.54	0.26	10.80	5.24	116.95	56.77	24.08	11.69	37.48	18.19	0.74	0.36	2.67	1.30	NA	NA	NA	NA
Rhein	y	f	2	55	276	29.8	NA	NA	NA	3.77	1.37	0.90	0.33	14.84	5.38	162.80	58.99	32.22	11.67	44.79	16.23	1.53	0.55	3.09	1.12	NA	NA	NA	NA
Rhein	y	f	2	53	272	30.2	NA	NA	NA	4.57	1.68	2.33	0.86	12.48	4.59	164.82	60.60	38.54	14.17	36.12	13.28	1.58	0.58	2.68	0.99	NA	NA	NA	NA
Schlei/ Trave	y	f	3	79	995	35.4	NA	NA	NA	11.93	1.20	13.61	1.37	30.95	3.11	668.23	67.16	110.57	11.11	121.43	12.20	2.58	0.26	8.42	0.85	NA	NA	NA	NA
Elbe	y	f	3	52	238	13.3	NA	NA	NA	2.68	1.13	0.89	0.37	15.99	6.72	127.18	53.44	26.86	11.29	47.09	19.79	2.53	1.06	2.86	1.20	NA	NA	NA	NA
Weser	y	f	3	59	354	21.3	NA	NA	NA	5.12	1.45	1.65	0.47	19.96	5.64	223.36	63.10	32.99	9.32	52.78	14.91	1.04	0.29	4.63	1.31	NA	NA	NA	NA
Eider	y	f	3	71	660	21.8	NA	NA	NA	8.15	1.23	3.10	0.47	25.05	3.80	403.63	61.16	76.32	11.56	106.66	16.16	3.04	0.46	9.35	1.42	NA	NA	NA	NA
Eider	y	f	3	63	442	15.3	NA	NA	NA	7.62	1.72	1.99	0.45	24.64	5.57	248.82	56.29	44.71	10.12	77.15	17.45	2.99	0.68	7.76	1.76	NA	NA	NA	NA
Eider	y	f	3	54	302	7	NA	NA	NA	3.97	1.31	0.72	0.24	18.73	6.20	167.84	55.58	37.04	12.26	47.98	15.89	1.57	0.52	5.16	1.71	NA	NA	NA	NA
Eider	y	f	3	54	276	10.1	NA	NA	NA	4.59	1.66	0.96	0.35	12.88	4.67	151.70	54.96	40.49	14.67	45.44	16.46	2.12	0.77	4.39	1.59	NA	NA	NA	NA
Eider	y	f	3	54	290	17.4	NA	NA	NA	4.34	1.50	0.80	0.28	13.97	4.82	163.55	56.40	36.80	12.69	47.79	16.48	1.72	0.59	2.72	0.94	NA	NA	NA	NA
Eider	y	f	3	56	303	9.3	NA	NA	NA	3.65	1.20	0.94	0.31	11.65	3.84	179.44	59.22	40.78	13.46	48.88	16.13	1.15	0.38	4.85	1.60	NA	NA	NA	NA
Eider	y	f	3	61	357	9.6	NA	NA	NA	5.37	1.50	1.00	0.28	16.64	4.66	183.65	51.44	46.63	13.06	82.70	23.17	3.30	0.92	7.22	2.02	NA	NA	NA	NA
Eider	y	f	3	60	374	18.2	NA	NA	NA	5.46	1.46	1.59	0.43	16.86	4.51	194.18	51.92	52.62	14.07	80.52	21.53	2.44	0.65	5.63	1.51	NA	NA	NA	NA
Schlei/ Trave	y	f	3	71	564	30	NA	NA	NA	7.63	1.35	6.28	1.11	22.28	3.95	310.54	55.06	73.09	12.96	113.52	20.13	1.39	0.25	6.18	1.10	NA	NA	NA	NA
Schlei/ Trave	y	f	3	84	1047	23.9	NA	NA	NA	12.80	1.22	14.51	1.39	22.33	2.13	590.01	56.35	141.73	13.54	217.14	20.74	3.41	0.33	10.95	1.05	NA	NA	NA	NA
Rhein	y	f	3	67	544	40	NA	NA	NA	6.76	1.24	3.65	0.67	19.98	3.67	333.08	61.23	47.84	8.79	87.15	16.02	1.51	0.28	5.85	1.08	NA	NA	NA	NA
Rhein	y	f	3	53	280	20.2	NA	NA	NA	4.42	1.58	1.16	0.41	11.01	3.93	184.20	65.79	21.87	7.81	44.84	16.01	1.29	0.46	2.82	1.01	NA	NA	NA	NA
Eider	y	f	3	63	544	22.5	NA	NA	NA	5.08	0.93	3.55	0.65	19.11	3.51	323.07	59.39	78.10	14.36	79.52	14.62	1.68	0.31	6.75	1.24	NA	NA	NA	NA
Eider	y	f	3	67	484	18	NA	NA	NA	5.90	1.22	3.12	0.64	21.10	4.36	293.55	60.65	49.87	10.30	86.78	17.93	1.66	0.34	8.39	1.73	NA	NA	NA	NA

Eider	y	f	3	61	450	24.1	NA	NA	NA	5.64	1.25	2.39	0.53	21.94	4.88	274.19	60.93	52.00	11.56	68.45	15.21	1.50	0.33	6.31	1.40	NA	NA	NA	NA
Eider	y	f	3	64	424	26.1	NA	NA	NA	6.14	1.45	2.16	0.51	20.17	4.76	256.11	60.40	51.95	12.25	64.84	15.29	1.57	0.37	4.80	1.13	NA	NA	NA	NA
Eider	y	f	3	62	376	19.2	NA	NA	NA	4.90	1.30	1.33	0.35	20.24	5.38	202.96	53.98	49.05	13.05	77.45	20.60	1.58	0.42	6.95	1.85	NA	NA	NA	NA
Eider	y	f	3	60	330	24.9	NA	NA	NA	4.98	1.51	1.23	0.37	15.64	4.74	180.08	54.57	44.78	13.57	67.26	20.38	1.68	0.51	6.10	1.85	NA	NA	NA	NA
Eider	y	f	3	57	351	22.3	NA	NA	NA	4.70	1.34	1.62	0.46	16.96	4.83	196.91	56.10	42.72	12.17	69.23	19.72	2.28	0.65	4.38	1.25	NA	NA	NA	NA
Schlei/ Trave	s	f	4	91	1829	20.1	NA	NA	NA	20.68	1.13	25.81	1.41	48.78	2.67	1027.89	56.20	239.36	13.09	347.43	19.00	5.45	0.30	16.50	0.90	NA	NA	NA	NA
Schlei/ Trave	s	f	4	97	2110	25.2	NA	NA	NA	19.86	0.94	27.80	1.32	61.18	2.90	1265.15	59.96	249.03	11.80	397.06	18.82	5.31	0.25	22.06	1.05	NA	NA	NA	NA
Schlei/ Trave	s	f	4	91	1412	24.6	NA	NA	NA	14.49	1.03	16.68	1.18	45.96	3.25	797.11	56.45	178.38	12.63	282.62	20.02	3.84	0.27	14.25	1.01	NA	NA	NA	NA
Schlei/ Trave	s	f	4	80	1283	18.5	NA	NA	NA	18.23	1.42	12.13	0.95	39.94	3.11	614.18	47.87	179.49	13.99	316.20	24.65	20.06	1.56	16.23	1.27	NA	NA	NA	NA
Schlei/ Trave	s	f	4	90	1318	25.3	NA	NA	NA	15.02	1.14	16.56	1.26	43.37	3.29	738.89	56.06	177.96	13.50	258.80	19.64	4.11	0.31	11.46	0.87	NA	NA	NA	NA
Schlei/ Trave	s	f	4	80	1153	24.5	NA	NA	NA	15.77	1.37	15.81	1.37	35.98	3.12	646.87	56.10	146.62	12.72	237.13	20.57	3.39	0.29	13.67	1.19	NA	NA	NA	NA
Schlei/ Trave	s	f	4	91	1625	26.9	NA	NA	NA	19.82	1.22	15.13	0.93	52.94	3.26	706.67	43.49	208.03	12.80	253.61	15.61	5.65	0.35	14.69	0.90	NA	NA	NA	NA
Schlei/ Trave	s	f	4	86	1283	20.9	NA	NA	NA	12.91	1.01	16.54	1.29	35.43	2.76	715.71	55.78	189.25	14.75	245.41	19.13	3.86	0.30	12.61	0.98	NA	NA	NA	NA
Schlei/ Trave	s	f	5	66	620	24.7	NA	NA	NA	6.97	1.12	8.72	1.41	26.69	4.30	367.78	59.32	80.91	13.05	98.65	15.91	2.92	0.47	7.09	1.14	NA	NA	NA	NA
Eider	s	f	5	69	611	22.5	NA	NA	NA	7.96	1.30	8.07	1.32	21.29	3.48	350.80	57.41	87.56	14.33	95.78	15.68	3.07	0.50	8.28	1.36	NA	NA	NA	NA
Eider	s	f	5	70	681	24.7	NA	NA	NA	9.04	1.33	8.52	1.25	20.47	3.01	411.36	60.41	102.97	15.12	92.79	13.63	3.89	0.57	7.47	1.10	NA	NA	NA	NA
Eider	s	f	5	62	528	27.9	NA	NA	NA	7.19	1.36	4.07	0.77	26.02	4.93	300.07	56.83	62.78	11.89	94.47	17.89	4.47	0.85	5.91	1.12	NA	NA	NA	NA
Eider	s	f	5	62	602	21.9	NA	NA	NA	6.42	1.07	7.57	1.26	17.57	2.92	375.28	62.34	88.35	14.68	71.52	11.88	3.11	0.52	5.62	0.93	NA	NA	NA	NA
Eider	s	f	5	67	579	28.7	NA	NA	NA	5.13	0.89	6.52	1.13	19.89	3.44	360.54	62.27	75.03	12.96	78.58	13.57	3.21	0.55	7.88	1.36	NA	NA	NA	NA
Eider	s	f	5	62	405	6	NA	NA	NA	8.20	2.02	0.94	0.23	21.03	5.19	190.43	47.02	47.57	11.75	107.10	26.44	1.63	0.40	9.56	2.36	NA	NA	NA	NA
Weser	s	f	5	70	683	19.8	NA	NA	NA	9.73	1.42	10.17	1.49	31.42	4.60	326.87	47.86	117.24	17.17	8.09	1.18	2.67	0.39	152.49	22.33	NA	NA	NA	NA
Weser	s	f	5	63	519	18.7	NA	NA	NA	8.12	1.56	9.56	1.84	22.35	4.31	252.25	48.60	102.92	19.83	96.47	18.59	1.90	0.37	4.77	0.92	NA	NA	NA	NA
Schlei/ Trave	s	f	5	72	736	23.7	NA	NA	NA	11.83	1.61	8.03	1.09	23.80	3.23	366.53	49.80	97.07	13.19	194.97	26.49	3.69	0.50	6.11	0.83	NA	NA	NA	NA
Schlei/ Trave	s	f	5	84	988	22.1	NA	NA	NA	10.84	1.10	13.99	1.42	32.05	3.24	540.07	54.66	147.61	14.94	195.55	19.79	5.07	0.51	12.47	1.26	NA	NA	NA	NA
Schlei/ Trave	s	f	5	74	709	25	NA	NA	NA	7.44	1.05	6.87	0.97	27.09	3.82	401.27	56.60	100.49	14.17	128.73	18.16	2.71	0.38	8.44	1.19	NA	NA	NA	NA
Schlei/ Trave	s	f	5	78	807	27.1	NA	NA	NA	8.96	1.11	8.75	1.08	22.10	2.74	464.95	57.61	116.61	14.45	147.42	18.27	2.80	0.35	10.10	1.25	NA	NA	NA	NA
Schlei/ Trave	s	f	5	64	459	25.6	NA	NA	NA	6.55	1.43	7.07	1.54	16.53	3.60	234.38	51.06	80.69	17.58	82.45	17.96	4.00	0.87	10.38	2.26	NA	NA	NA	NA
Eider	s	f	5	63	484	11.1	NA	NA	NA	7.05	1.46	2.19	0.45	20.34	4.20	263.19	54.38	57.18	11.81	103.64	21.41	2.14	0.44	8.93	1.85	NA	NA	NA	NA
Eider	s	f	5	64	427	17.4	NA	NA	NA	4.90	1.15	2.06	0.48	20.32	4.76	245.93	57.59	47.48	11.12	78.23	18.32	1.73	0.41	7.91	1.85	NA	NA	NA	NA

Eider	s	f	5	60	521	24.5	NA	NA	NA	7.32	1.40	5.92	1.14	22.86	4.39	315.51	60.56	71.24	13.67	81.64	15.67	1.60	0.31	6.74	1.29	NA	NA	NA	NA
Weser	s	m	6	43	143	23.2	NA	1.01	NA	1.99	1.39	0.01	0.01	4.06	2.84	75.78	52.99	27.27	19.07	19.36	13.54	0.66	0.46	1.67	1.17	NA	NA	NA	NA
Weser	s	m	6	39	120	23.8	NA	1.09	NA	1.72	1.43	0.07	0.06	3.25	2.71	66.51	55.43	19.55	16.29	14.92	12.43	0.53	0.44	1.37	1.14	NA	NA	NA	NA
Weser	s	m	6	40	117	20.9	NA	1.39	NA	1.60	1.37	0.09	0.08	4.54	3.88	61.82	52.84	15.66	13.38	16.66	14.24	0.22	0.19	1.23	1.05	NA	NA	NA	NA
Weser	s	m	6	38	106	20.4	NA	1.18	NA	2.06	1.94	0.08	0.08	2.66	2.51	45.98	43.38	11.99	11.31	13.44	12.68	1.23	1.16	1.21	1.14	NA	NA	NA	NA
Weser	s	m	6	40	130	25.3	NA	1.83	NA	3.20	2.46	0.21	0.16	4.18	3.22	74.87	57.59	16.89	12.99	15.29	11.76	1.07	0.82	1.30	1.00	NA	NA	NA	NA
Weser	s	m	6	39	92	24.6	NA	0.55	NA	1.41	1.53	0.03	0.03	2.88	3.13	49.89	54.23	11.05	12.01	14.74	16.02	0.29	0.32	0.86	0.93	NA	NA	NA	NA
Weser	s	m	6	43	120	25.5	NA	0.94	NA	1.83	1.53	0.09	0.08	3.03	2.53	67.66	56.38	16.55	13.79	15.43	12.86	0.58	0.48	1.09	0.91	NA	NA	NA	NA
Weser	s	m	6	36	81	25.4	NA	0.81	NA	1.33	1.64	0.01	0.01	3.22	3.98	46.66	57.60	9.63	11.89	10.56	13.04	0.31	0.38	0.62	0.77	NA	NA	NA	NA
Weser	s	m	6	43	129	21.5	NA	0.81	NA	1.95	1.51	0.11	0.09	2.97	2.30	72.04	55.84	18.08	14.02	16.49	12.78	0.42	0.33	1.26	0.98	NA	NA	NA	NA
Weser	s	m	6	36	90	27.9	NA	0.57	NA	1.72	1.91	0.01	0.01	3.92	4.36	45.99	51.10	11.64	12.93	14.63	16.26	0.31	0.34	0.69	0.77	NA	NA	NA	NA
Ems	**mature**	f	M	81	1385	NA	34.39	NA	NA	18.38	1.33	743	53.65	13.20	0.95	249.66	18.03	131.20	9.47	82.50	5.96	4.20	0.30	11.24	0.81	NA	NA	NA	NA
Ems	**mature**	f	M	65	584	NA	22.32	NA	NA	6.39	1.09	282	48.34	7.60	1.30	123.67	21.18	69.34	11.87	35.34	6.05	2.63	0.45	5.43	0.93	NA	NA	NA	NA
Ems	**mature**	f	M	79	1073	NA	21.85	NA	NA	16.61	1.55	457	42.61	11.91	1.11	263.56	24.56	107.35	10.00	55.63	5.18	3.40	0.32	9.15	0.85	NA	NA	NA	NA
Schlei	**mature**	f	M	80	1177	NA	35.03	NA	NA	14.28	1.21	659	55.99	10.38	0.88	176.53	15.00	112.22	9.53	69.24	5.88	4.20	0.36	9.43	0.80	NA	NA	NA	NA
Schlei	**mature**	f	M	63	567	NA	25.00	NA	NA	6.38	1.13	325	57.32	5.40	0.95	93.88	16.56	48.25	8.51	23.63	4.17	2.03	0.36	7.82	1.38	NA	NA	NA	NA
Arresö	**mature**	f	M	76	808	NA	NA	NA	NA	7.82	0.97	397.3	49.17	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Elbe	**mature**	f	M	70	609	NA	NA	NA	NA	6.65	1.09	302.1	49.61	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Arresö	**mature**	f	M	81	992	NA	NA	NA	NA	NA	NA	342.0	34.48	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Arresö	**mature**	f	M	81	934	NA	NA	NA	NA	11.23	1.20	473.9	50.74	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Arresö	**mature**	f	M	78	912	NA	NA	NA	NA	14.22	1.56	432.3	47.40	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Elbe	**mature**	f	M	64	601	NA	NA	NA	NA	7.86	1.31	238.0	39.60	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Elbe	**mature**	f	M	79	1049	NA	NA	NA	NA	13.77	1.31	569.2	54.26	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Arresö	**mature**	f	M	73	730	NA	NA	NA	NA	13.30	1.82	366.0	50.14	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Arresö	**mature**	f	M	75	1013	NA	NA	NA	NA	18.51	1.83	403.0	39.78	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Weser	**mature**	f	M	82	966	NA	NA	NA	NA	13.50	1.40	428.2	44.33	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Weser	**mature**	f	M	69	628	NA	NA	NA	NA	9.60	1.53	230.1	36.64	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Ploen lake	**mature**	f	M	91	1484	NA	NA	NA	NA	NA	NA	393	26.48	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Weser	**mature**	f	M	71	654	NA	NA	NA	NA	11.30	1.73	309.8	47.37	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

Weser	**mature**	f	M	76	791	NA	NA	NA	NA	NA	NA	305.3	38.60	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Ploen lake	**mature**	f	M	94	1941	NA	NA	NA	NA	23.60	1.22	871.2	44.89	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Ploen lake	**mature**	f	M	84	1153	NA	NA	NA	NA	20.70	1.80	322.5	27.97	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Weser	**mature**	f	M	72	777	NA	NA	NA	NA	12.70	1.63	368.2	47.39	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Ploen lake	**mature**	f	M	85	1104	NA	NA	NA	NA	13.29	1.20	307.8	27.88	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Weser	**mature**	f	M	77	967	NA	NA	NA	NA	14.21	1.47	471.4	48.75	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Weser	**mature**	f	M	67	633	NA	NA	NA	NA	10.00	1.58	269.7	42.61	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Weser	**mature**	f	M	75	891	NA	NA	NA	NA	13.20	1.48	298.5	33.50	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Weser	**mature**	f	M	66	786	NA	NA	NA	NA	9.01	1.15	321.4	40.89	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Ploen lake	**mature**	f	M	78	1159	NA	NA	NA	NA	16.10	1.39	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

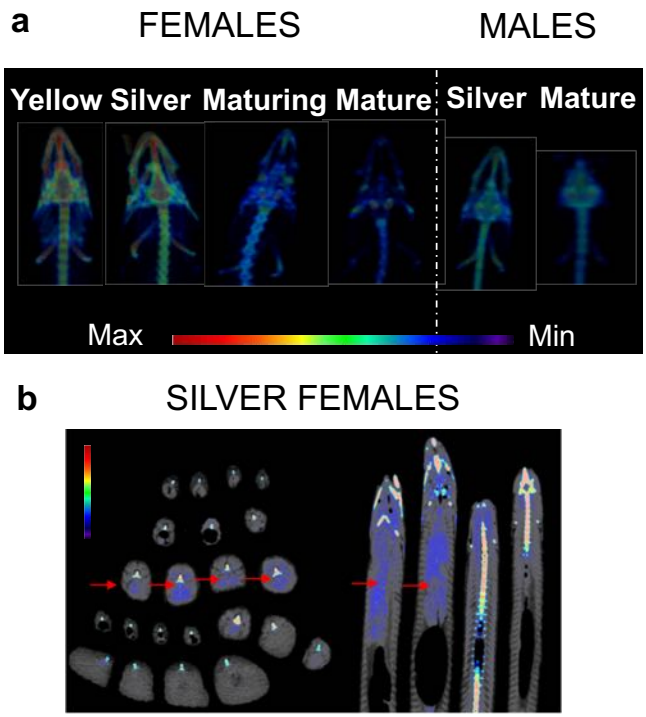

Figure S1: Bone loss in skeletal elements from eels in different maturation stages and mineral accumulation in female silver eels.
(a) Clinical tomography calcium maps pf skull and skeletal elements of eels showed declining bone density along progressing maturation. (b) Note accumulated mineral signals around gonadal tissue in silver females at onset of maturation. Images obtained by clinical computed tomography.

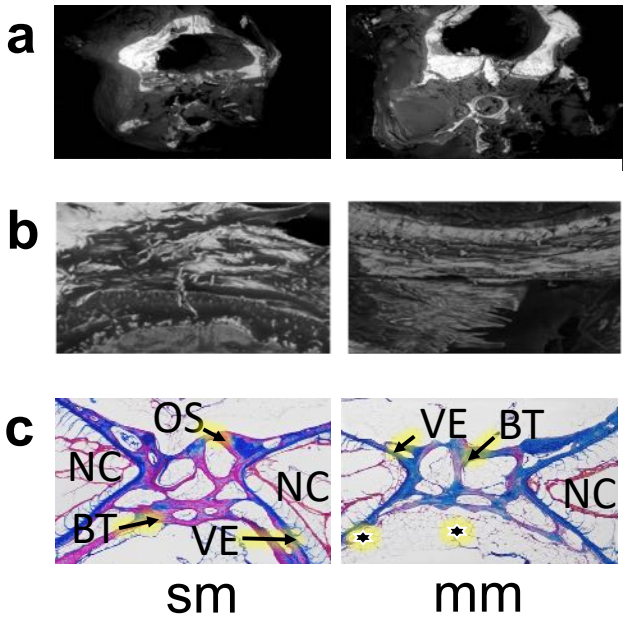

Figure S2: Structural aspects of bone loss during sexual maturation in male eels.
Superior view (a) and (500x) magnified (b) SEM images of entire vertebral body endplates of male eels in different maturation stages depict the successive bone loss on a supracellular level. (c) Bone histology is based on azan-dyed, para-sagittal sections of vertebral bodies and illustrates changes of bone structures along the maturation process on a cellular level. Defined structures are marked and labeled by abbreviations: (NC = Notochord, BT = Bone Trabeculae, VE = Vertebral Body Endplate, OS = Osteon, * = indication of bone resorption). (sm= silver male; mm= mature male).

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CHAPTER V

A physiologically based toxicokinetic (PBTK) model for moderately hydrophobic organic chemicals in the European eel (*Anguilla anguilla*)

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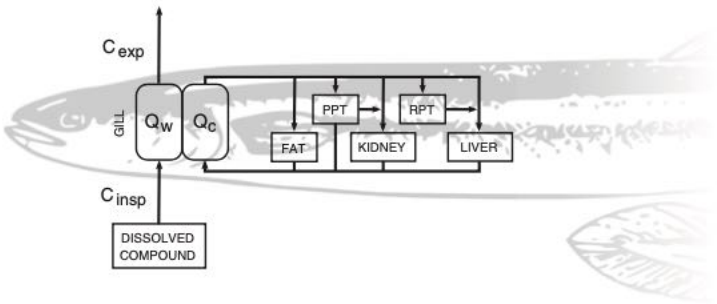

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A physiologically based toxicokinetic (PBTK) model for moderately hydrophobic organic chemicals in the European eel (*Anguilla anguilla*)

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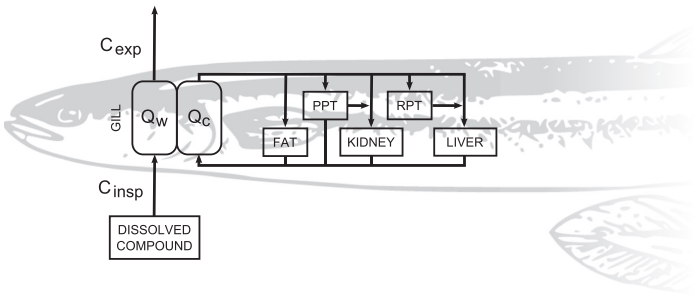
HIGHLIGHTS

- A PBTK model was developed for European eel (*Anguilla anguilla*).
- Own experimental data and data from the literature were used for parameterization.
- The predictive power of the model was excellent, with RMSE of 0.28 *log* units.
- The developed model can be amended with sub-models for dietary and dermal exposure.

GRAPHICAL ABSTRACT



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ABSTRACT

The European eel (*Anguilla anguilla*) is a facultatively catadromous fish species with a complex life cycle. Its current population status is alarming: recruitment has decreased drastically since the 1980s and its stock is still considered to be outside safe biological limits. Although there is no consensus on the reasons for this situation, it is currently thought to have resulted from a combination of different stressors, including anthropogenic contaminants. To deepen our understanding of the processes leading to the accumulation of lipophilic organic contaminants in yellow eels (*i.e.* the feeding, continental growth stage), we developed a physiologically based toxicokinetic model using our own data and values from the literature. Such models can predict the uptake and distribution of water-borne organic chemicals in the whole fish and in different tissues at any time during exposure. The predictive power of the model was tested against experimental data for six chemicals with *n*-octanol-water partitioning coefficient (*log* K_{ow}) values ranging from 2.13–4.29. Model performance was

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Bioconcentration
Organic pollutants
PBTK

excellent, with a root mean squared error of 0.28 *log* units. This model has the potential to help identify suitable habitats for restocking under eel management plans.

1. Introduction

The European eel (*Anguilla anguilla* L.) is a facultatively catadromous fish species that practices one of the longest spawning migrations known in nature. With the onset of maturation, silver eels migrate up to 7500 km from their brackish and freshwater habitats in Europe and North Africa to their spawning grounds in the Sargasso Sea. Eel larvae subsequently make their way back to Europe's continental shelves, travelling with oceanic currents for an estimated 2–3 years, while metamorphosing into the transparent glass eel stage. Once they reach European and North African coastal waters and river outlets, glass eels start to become pigmented and partly ascend up European rivers and freshwater habitats. As yellow eels, they feed and grow for several years in order to store the energy reserves required for migration. The cycle is completed by the transformation of yellow eels into silver eels, which marks the beginning of their migration to the spawning grounds and the beginning of their gonadal maturation (van den Thillart et al., 2008).

Recruitment of the European eel has decreased since the 1980s, when its panmictic stock started to decline (ICES, 2012). Today, it is considered 'critically endangered' by the International Union for Conservation of Nature (Stone, 2003), and is also listed as a vulnerable species by the Convention on International Trade in Endangered Species of Wild Fauna and Flora. The causes of this alarming situation are unclear but they are thought to reflect a combination of different stressors, including reduced habitat quality and habitat loss (Castonguay et al., 1994), over-fishing (van Ginneken and Maes, 2005), climatic changes (Friedland et al., 2007), and introduced parasites, which may affect eel fitness during migration (Kirk, 2003; Wysujack et al., 2014). Anthropogenic contaminants may also reduce fitness and fat reserves, thereby reducing the escapement success of this species (Buet et al., 2006; Kammann et al., 2013; Marohn et al., 2008; Palstra et al., 2006; van Ginneken and Maes, 2005).

A prerequisite of successful measures for the recovery of European eel stocks is their need to increase silver eel escapement, a measure of the number of eels with the potential to migrate to their spawning grounds in the Sargasso Sea. Thus, the European Commission mandates its member states to guarantee 40% escapement of silver eels, relative to the estimated escapement under pristine conditions (EU, 2007). To meet this target in a particular river basin system, it is necessary either to reduce the mortality of naturally recruited eels or to use restocking to artificially increase the number of recruits (Marohn et al., 2013). In the absence of clear steps by fisheries managers in many European countries to significantly reduce anthropogenic mortality, restocking is still the most common measure used to fulfil the 40% escapement target (Kammann et al., 2013).

The success of restocking is generally highly dependent on the quality of the stocked habitat. Suitable stocking habitats need to be identified based on a number of different criteria. Apart from the habitat's ecological quality, anthropogenic pollution needs to be recognised as an important criterion. Due to their catadromous and semelparous lifecycle, eels form a unique fish taxon. Their lengthy migration and their semelparity rely on an extraordinarily high total lipid content; this makes them vulnerable to the accumulation of high concentrations of lipophilic pollutants, especially during their continental growth phase (Belpaire and Goemans, 2007). A detailed knowledge of the bioaccumulation and distribution processes acting on lipophilic pollutants within the fish body is required to understand the effects of these pollutants on escapement, fitness and ultimately, reproductive success. When analytical data relating to the internal chemical concentrations in laboratory- or field-exposed fish are unavailable, the use of physiologically based toxicokinetic (PBTK) models can provide a powerful tool (Groh et al., 2015). Organs and tissues are explicitly represented as individual compartments within PBTK models, where each compartment is characterised by its volume (as a fraction of total body weight), its total lipid and water contents (as a fraction of tissue wet weight [w.w.]), and by the blood flow to the compartment (as a fraction of cardiac output). PBTK models are capable of predicting the concentration of neutral organic pollutants in the whole fish and in different tissues at any time during exposure (Louisse et al., 2012; Yoon et al., 2012). Furthermore, they facilitate application of the results of *in vitro* bioassays, which have a higher throughput and reduce the requirement for experimental animals, to predict the effects *in vivo*; these models thus have the potential to make a valuable contribution to predictive toxicology (Brinkmann et al., 2014a; Stadnicka-Michalak et al., 2014).

PBTK models have been developed for a number of fish species (Bungay et al., 1976; Liao et al., 2005; Lien and McKim, 1993; Lien et al., 2001; Nichols et al., 1998, 1990, 1993), but to our knowledge, none exist for members of the *Anguilla* genus. We have successfully used PBTK models to test hypotheses on the physiology of bioconcentration and the distribution of neutral organic contaminants in rainbow trout (Brinkmann et al., 2014b). A PBTK model for the European eel would provide a powerful research tool, facilitating the prediction and understanding of bioconcentration in this species. The main aims of the present study were to: (a) develop a PBTK model for the bioconcentration of neutral organic chemicals in European eels as a basis for further developments using our own data and published parameter values; (b) evaluate the predictive power of this model using published experimental bioconcentration data; and (c) discuss future research needs for applications of the PBTK model.

2. Materials and methods

2.1. Study design

In the present study, we determined model parameters (total lipid and water contents, as well as tissue/organ volumes) and combined them with physiological data from the literature to re-parameterise the PBTK model for rainbow trout (*Oncorhynchus mykiss*) published by Nichols et al. (1990) for the European eel (*A. anguilla*), as conceptually presented in Fig. 1. This model was then used to predict bioconcentration factors (BCFs), as well as accumulation (k_1) and elimination (k_2) rate constants for a number of chemicals. To assess the model performance, these predictions were compared with published experimental values.

2.2. Experimental fish

Live European eels were obtained from the French Atlantic coast as glass eels and reared to the early yellow eel stage at the Thünen-Institute of Fisheries Ecology research station at Ahrensburg, Germany. The fish were held in an aerated 1500-L tank with recirculated water from a 57 m^3 system (approx. 18 °C; pH 6.7 ± 0.3; $NH_3 < 0.1$ mg L^{-1}). The water was continuously exchanged at a rate of 10–12 m^3 d^{-1}. Fish were subject to a natural day/night rhythm. The eels were fed daily *ad libitum* with a diet of commercial pellets (C-3 Pro Aqua K18, Skretting, Stavanger, Norway). All animals were treated in accordance with the animal welfare act and with the permission of the German Federal authorities.

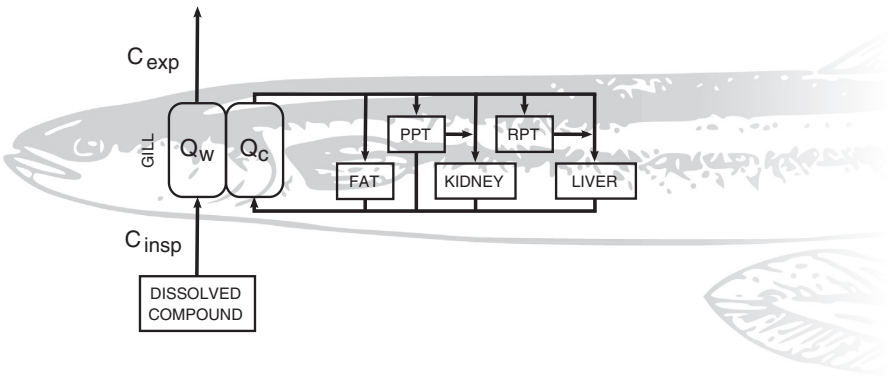

Fig. 1. Conceptual representation of the physiologically based toxicokinetic (PBTK) model for the European eel (*Anguilla anguilla*). Q_w: effective respiratory volume, Q_c: cardiac output, C_{insp}: inspired chemical concentration, C_{exp}: expired chemical concentration, PPT: poorly perfused tissues, and RPT: richly perfused tissues. Modified from Brinkmann et al. (2014b).

2.3. Model parameterisation

The parameterisation of PBTK models requires a number of experimental values to describe the underlying physiological processes, as described in Nichols et al. (1990); these are summarized in Table 1 and Appendix A.

2.3.1. Volumes and total lipid/water contents in organs and tissue groups

A total of 18 eels (twelve females, four males, two undetermined) were anaesthetised with 2-phenoxyethanol (Carl Roth, Karlsruhe, Germany) and killed by severing the spinal cord. Blood samples were taken from the caudal vein, using 0.55 × 25 mm stainless steel needles (Braun, Melsungen, Germany) and 1 mL syringes (Braun).

The following tissues/organs were then dissected from the fish: liver, kidney, spleen, gonads, muscle, brain, gills, viscera (including stomach, pyloric caeca, intestines and the associated visceral fat), skin and carcass (the tissue that remained after the excision of all other fractions). The excised tissues/organs from each individual were weighed (0.0001 g resolution) using a precision scale (Sartorius, Göttingen, Germany), and stored at −20 °C. The tissue/organ compartment volumes were calculated from these weights, assuming that all tissues had a density of 1.0 g mL^{-1}.

Total lipid and water levels were determined in all muscle samples individually ($n = 18$). The remaining tissue compartments were pooled, depending on the amount of available tissue. Carcass, skin, gills, kidney, liver and the viscera were grouped in four thoroughly homogenised pools ($n = 4$ animals in each; three females, one male), whereas spleen, brain and blood were each grouped to one single pool of 16 fish (twelve females, four males). Gonad tissue was also analysed in a single pool of 7 eels (all female), since too little tissue was available to allow for clean separation in the remaining fish. The total lipid content of viscera-associated adipose tissue was determined in duplicates using tissues from three wild-caught yellow eels. The water content was determined gravimetrically after freeze-drying (Lyovac GT 2; GEA Pharma Systems, Wommelgem, Belgium).

The total lipid levels were determined as described by Smedes (1999), with the modifications introduced by Schlechtriem et al. (2012). Briefly, freeze-dried samples were re-suspended in water and homogenised using a laboratory mill (IKA A11; IKA, Staufen, Germany). Subsequently, these homogenates were freeze-dried and briefly ground in the laboratory mill in order to achieve a good degree of homogenisation and to avoid separation of the fat from the rest of the sample; this was especially important in fatty tissues. Homogenates were dried at 105 °C until they reached a constant weight, indicating the removal of residual moisture. Approximately 100 mg of each sample was used for lipid extraction using a mixture of cyclohexane (2.50 mL), propan-2-ol (2.00 mL) and water (2.75 mL), followed by a second extraction using cyclohexane (2.175 mL) and propan-2-ol (0.325 mL). The organic phase was collected after each extraction and the solvents were evaporated prior to gravimetric determination of the fat content. All samples were analysed in duplicate. If the relative deviation exceeded 5%, the samples were re-analysed.

Apart from those present in the muscle, most of the lipid deposits in European (yellow) eels were associated with visceral organs and with the carcass (Table 2). Most of the lipids measured in the carcass seemed to have resulted from incomplete dissection of muscle tissue from the carcass. The total volume of the adipose tissue compartment, *i.e.* the sum of adipose tissue in the viscera and carcass, was estimated using the total lipid levels and the volumes of the viscera (Eq. (1.1)) and

Table 1
Physiological parameters (and corresponding symbols) used to re-parameterise a physiologically based toxicokinetic (PBTK) model (Nichols et al., 1990) for European eels (*Anguilla anguilla*).

Physiological parameter	Symbol	Unit	Value
Body wet weight	W	kg	Model input
Cardiac output	Q_c	L kg^{-1} h^{-1}	Eq. (2)
Oxygen consumption rate	VO_2	mg kg^{-1} h^{-1}	Eq. (3)
Effective respiratory volume	Q_w	L kg^{-1} h^{-1}	Eq. (4)
Arterial blood flow to different tissues			
Liver	Q_l	L h^{-1}	1.9% of Q_c
Fat	Q_f	L h^{-1}	12.2% of Q_c
Poorly perfused tissues[1]	Q_m	L h^{-1}	64.3% of Q_c
Richly perfused tissues[2]	Q_r	L h^{-1}	20.2% of Q_c
Kidney	Q_k	L h^{-1}	1.4% of Q_c
Organ/tissue group volumes (fraction of W)[3]			
Liver	V_l	L	1.5% of W
Fat	V_f	L	4.2% of W
Poorly perfused tissues[1]	V_m	L	87.0% of W
Richly perfused tissues[2]	V_r	L	6.3% of W
Kidney	V_k	L	1.0% of W
Organ/tissue total lipid content (fraction w.w.)			
Liver	α_l	–	3.9%
Fat	α_f	–	68.1%
Poorly perfused tissues	α_m	–	18.2%
Kidney	α_k	–	5.3%

[1] Mainly white muscle.
[2] Viscera, spleen, gonads, and gills.
[3] All tissues were assumed to have a specific gravity of 1.0 w.w., wet weight.

Table 2
Relative tissue wet weight (w.w.), total lipid and water levels in European eels (*Anguilla anguilla*). Total lipid contents of spleen, brain, blood, and gonads were determined in a single pooled sample; those of muscle were determined in individual fish samples. All other lipid and water levels were determined in five pools, with 2–4 animals per pool. All values are expressed as mean ± standard deviation.

	Tissue w.w. (%)	Lipid content (%)	Water content (%)
Liver	1.49 ± 0.16	3.89 ± 0.88	74.88 ± 0.03
Kidney	1.04 ± 0.30	5.29 ± 0.68	75.75 ± 2.80
Muscle	57.27 ± 6.28	18.24 ± 7.15	64.04 ± 5.80
Skin	10.85 ± 1.41	5.20 ± 1.46	72.32 ± 2.33
Viscera[1]	4.61 ± 1.01	10.79 ± 2.57	71.65 ± 5.44
Gill	1.42 ± 0.41	4.86 ± 0.77	73.86 ± 5.12
Carcass	22.97 ± 5.08	14.19 ± 2.23	62.18 ± 3.82
Spleen	0.13 ± 0.06	2.88	75.63 ± 1.79
Gonad	0.14 ± 0.21	7.24	80.23 ± 5.26
Brain	0.07 ± 0.02	4.87	19.90 ± 1.58
Adipose tissue[2]	–	68.14 ± 11.67	25.52 ± 10.80
Blood	–	1.43	82.71 ± 4.39

[1] Includes stomach, pyloric caeca, intestines, and viscera-associated adipose tissue.
[2] Values for viscera-associated adipose tissue of three female yellow eels.

carcass (Eq. (1.2)), as well as using the total lipid content of viscera-associated adipose tissue according to Nichols et al. (1993). Since eel muscle had a high lipid content, we used the average total lipid level in lean tissues (α_{lean}; *i.e.* spleen, liver and gills; 3.87%), instead of the white muscle lipid level.

$$V_v \cdot \alpha_v = V_f(viscera) \cdot \alpha_f + (V_v - V_f(viscera)) \cdot \alpha_{lean}$$
$$4.61\% \cdot 10.79\% = V_f(viscera) \cdot 68.14\% + (4.61\% - V_f(viscera)) \cdot 3.87\%$$
$$V_f(viscera) = 0.50\% \quad (1.1)$$

$$V_c \cdot \alpha_c = V_f(carcass) \cdot \alpha_f + (V_c - V_f(carcass)) \cdot \alpha_{lean}$$
$$22.97\% \cdot 14.19\% = V_f(carcass) \cdot 68.14\% + (22.97\% - V_f(carcass)) \cdot 3.87\%$$
$$V_f(carcass) = 3.69\% \quad (1.2)$$

Where V_v, V_c, and V_f are the volumes of viscera, carcass and fat (in % of the total body weight), respectively, and α_v, α_c, α_f and α_l are the total lipid levels in viscera, carcass, fat and liver (in % w.w.), respectively; this determined a total fat compartment volume, V_f (sum of fat from viscera and carcass), of 4.19%.

The volumes deduced from the weights of the liver and kidneys were directly used in the model. The volume of the richly perfused tissue compartment was estimated by adding the experimentally determined weights of the viscera, spleen, gonads, and gill. The volume of the poorly perfused tissue compartment (mainly white muscle) was assumed to be the difference between the total body volume and the volumes of all other compartments. Compartment volumes were expressed relative to the total body volume, while all compartments were assumed to have a specific gravity of 1.0.

2.3.2. Distribution of arterial blood flow and cardiac output

Data on the distribution of arterial blood flow to the different organs was obtained from a study on American eels (*Anguilla rostrata*) that applied the radiolabelled microsphere method (Butler and Oudit, 1994). Table 3 shows some of the cardiovascular parameters, such as heart rate and cardiac output (Q_c), determined by various studies. Imbrogno et al. (2001) investigated 292 isolated hearts from European eels (*A. anguilla*) that weighed 98 g on average and found that the Q_c was 10.9 mL min^{-1} kg^{-1}, which corresponds to 0.654 L h^{-1} kg^{-1} (Table 3). Other European eel studies have reported comparable Q_c values. The results from Imbrogno et al. were used for the PBTK model in the present study because this study provided a broader dataset than the other available publications. Q_c (in L h^{-1} kg^{-1}) was scaled to body weight using allometric scaling (Eq. (2)), according to Adolph (1949) and Nichols et al. (1993), where W is the total body w.w. (kg). The coefficient of 0.366 results in a Q_c value of 0.654 L h^{-1} kg^{-1} for an eel weighing 98 g.

$$Q_c = 0.366 \cdot W^{-0.25} \quad (2)$$

2.3.3. Oxygen consumption rate and effective respiratory volume

Values for the oxygen consumption rate (VO_2, in mg kg^{-1} h^{-1}) of European eels with different weights and at different water temperatures (T, in °C) were found in the literature (Degani et al., 1989). These data were then used for three-dimensional regression by TableCurve 3D 4.0 (Systat Software, Erkrath, Germany), which resulted in Eq. (3) ($R^2 = 0.95$). According to Berg and Steen (1965), European eels receive between 10 and 15% of the consumed oxygen *via* their skin, rather than *via* the gills. Oxygen taken up *via* the skin does not contribute to the effective respiratory volume (Q_w, in L h^{-1}), *i.e.* the volume of water that equilibrates with blood in the gill lamellae. VO_2 was thus reduced by 12.5% (resulting from subtraction of the average of 10 and 15%) to account for cutaneous respiration. The amount of chemical available for uptake into fish *via* the gills is assumed to be limited to that present in the Q_w, which was calculated and scaled to body weight as proposed by Stadnicka et al. (2012) and shown in Eq. (4), where C_{ox} is the dissolved oxygen concentration (in mg L^{-1}).

$$\frac{1}{VO_2} = -9.11 \cdot 10^{-3} + 1.95 \cdot 10^{-2} \cdot W^{0.5} + \frac{3.45 \cdot 10^{-1}}{T} \quad (3)$$

$$Q_w = \frac{0.875 \cdot VO_2}{0.8 \cdot C_{ox}} \cdot W^{0.75} \quad (4)$$

To verify the accuracy of the interpolated values for VO_2 calculated using Eq. (3), we conducted a simple respiration experiment. Briefly, 13 eels were fasted and their VO_2 was measured for 96 h. Prior to the experiment, the eels were fed *ad libitum* and kept in individual 15-L tanks (in the recirculation system described above) to assure sufficient food intake by each individual. The experiment was conducted in a respiratory system similar to the one described by Focken et al. (1994), but

Table 3
Previously published experimental data on the cardiac output of the indicated species within the *Anguilla* genus.

No	Species	*n*	Cardiac output (ml min^{-1} kg^{-1})	Stroke volume (ml kg^{-1})	Heart rate (bpm)	Body weight (g)	Temp. (°C)	Method	Reference
1	*A. rostrata*	12	15.9 ± 0.5	0.54 ± 0.01	29.7 ± 1.0	980–1590	12.0 ± 0.5	*In situ*	Butler and Oudit (1995)
2	*A. australis*	5	10.2 ± 1.1	0.21 ± 0.02	50.0 ± 3.1	900–1450	16.0–20.0	*In situ*	Hipkins and Smith (1983)
3	*A. anguilla*	10	12.2 ± 1.7	0.33	36.6 ± 0.8	600–1000	9.5 ± 1.0	*Ex situ*	Hughes et al. (1982)
4	*A. anguilla*	10	11.5 ± 0.6	0.29 ± 0.01	37.1 ± 1.2	510 ± 33	15.0 ± 0.5	*Ex situ*	Peyraud-Waitzenegger and Soulier (1988)
5	*A. rostrata*	6	28.6 ± 4.2	–	–	980 ± 1590	12.0 ± 1.0	*In situ*	Butler and Oudit (1994)
6	*A. dieffenbachii*	9	9.6 ± 0.3	0.26 ± 0.01	37.1 ± 1.6	2700–6300	15.0	*Ex situ*	Davie et al. (1992)
7	*A. anguilla*	292	10.9 ± 1.6	0.21 ± 0.09	51.4 ± 12.2	98 ± 3	18.0–21.0	*Ex situ*	Imbrogno et al. (2001)
8	*A. australis*	5	10.4 ± 0.6	0.21 ± 0.02	50 ± 3.4	900–1100	16.0–20.0	*In situ*	Hipkins (1985)

equipped with a WTW Oxi 730 oxygen probe (WTW, Weilheim, Germany), with 13 experimental units and using washout times of 102 s. The eels were weighed, transferred to the experimental units and acclimatised at a water temperature of 11.5 ± 0.6 °C for 8 days prior to data recording. Light and dark phases were 12 h throughout the acclimatisation and experimentation periods. The water temperature was stable at 11.6 ± 0.6 °C during the experiment. A total of 170 measurements were conducted during the experiment and VO_2 was calculated as mg O_2 kg^{-1} h^{-1}. Since no significant differences were found between VO_2 in the light and dark phases (Student's t-test, $p = 0.53$), all VO_2 measurements were averaged.

2.3.4. Description of chemical flux at the eel gill

Chemical flux at the eel gill was calculated using the approach described by Erickson and McKim (1990) and Nichols et al. (1993); this corresponds to counter-current blood and water flows that are separated by a diffusion barrier that comprises the gill epithelium, as well as the stagnant boundary layers of both blood and water (Eq. (5)).

$$F_g = k_x \cdot \left(C_{insp} - \frac{C_{ven}}{P_{bw}}\right) \tag{5}$$

where F_g is the total flux across the gill epithelium (in μg h^{-1}), k_x is the exchange coefficient, P_{bw} is the blood:water partitioning coefficient for the relevant compound (Appendix A, Equation A.1), C_{insp} is its concentration in water, and C_{ven} is its concentration in venous blood.

According to Erickson and McKim (1990), the exchange coefficient k_x can be calculated from the chemical capacities of water and blood (k_w and k_b), and the average resistance to chemical diffusion, k_d (Eq. (6)).

$$k_x = \frac{e^{-\frac{k_d}{k_b}} - e^{-\frac{k_d}{k_w}}}{\frac{e^{-\frac{k_d}{k_b}}}{k_w} - \frac{e^{-\frac{k_d}{k_w}}}{k_w}} \tag{6}$$

The chemical capacity of water flowing to the gill lamellae is equal to Q_w, while that of blood equals $k_b = Q_c \cdot P_{bw}$. The diffusional resistance k_d can be calculated as $k_d = D \cdot \frac{S}{d}$, where D is the molecular diffusivity (in units m^2 h^{-1}), S is the total lamellar surface area of the gill (in m^2), and d is the thickness of the diffusion barrier (in m). S can be determined using the allometric equation (Eq. (7), recalculated to the units used in this publication) provided by Bennett (1988).

$$S = 0.1703 \cdot W^{0.715} \tag{7}$$

Assuming an average lamellar frequency of 18 lamellae per mm gill filament (Bennett, 1988), the combined thickness of water, blood and epithelial layers amounts to approximately 28 μm (Nichols et al., 1993). Assuming that d is the sum of the thickness of the epithelial layer (6.4 μm; Lorin-Nebel et al., 2013) and one-third of the collective thickness of the water and blood layers $d = 6.4\ \mu m + \frac{28\ \mu m - 6.4\ \mu m}{3}$ (Erickson and McKim, 1990), d is approximately 14 μm. D was calculated at 20 °C (m^2 h^{-1}) using the equation (Eq. (5) in the original publication) published by Wilke and Chang (1955). The resulting values were multiplied by a factor of 0.75 to obtain the combined diffusivity value that reflected the reduced permeability of the gill epithelium (Erickson and McKim, 1990).

Previously published PBTK models, *e.g.* for rainbow trout, assume constant movement of the water surrounding the gill lamellae. However, *Anguilla* species show intermittent ventilation separated by periods of apnoea (Smith et al., 1983). Eels may transition between eupneic and apneic ventilation or hold each breath for several minutes (Berg and Steen, 1965). Interestingly, VO_2 is roughly equal in eels ventilating continuously or intermittently, because a higher oxygen extraction efficiency (up to 77%; Smith et al., 1983) compensates for the reduced ventilation volume. Since eels are more likely to show intermittent ventilation patterns at rest, with an eupnoeic fraction of only 15% (Smith et al., 1983), the amount of chemicals available for uptake *via* the gills was further limited to 0.15 · Q_w.

2.4. Calculation of BCFs and rate constants

Kinetic BCFs (BCF_k, in L kg^{-1}), k_1 and k_2 were calculated using the internal concentrations predicted by the PBTK model, in accordance with OECD 305 (2012), using Eqs. (8) and (9).

$$BCF_k = \frac{k_1}{k_2} \tag{8}$$

$$C_m(t) = C_{insp}(t) \cdot \frac{k_1}{k_2} \cdot \left(1 - e^{-k_2 \cdot t}\right) \tag{9}$$

Where $C_m(t)$ (in mg kg^{-1}) and $C_{insp}(t)$ (in mg L^{-1}) are the predicted chemical concentrations in fish muscle and the reported dissolved concentration in water, respectively, at time t. k_2 (in d^{-1}) was determined prior to k_1 (in L kg^{-1} d^{-1}) as the slope of a straight line fitted to *ln*-transformed $C_m(t)$ plotted against t.

2.5. Estimation of model performance

The model performance was verified against several bioconcentration experiments that were available in the literature. One European eel dataset was generated under flow-through conditions (Sancho et al., 1998). In this study, eels (20–30 g) were exposed to the organophosphate pesticide fenitrothion (CAS 122-14-5; *n*-octanol-water partitioning coefficient [*log* K_{ow}] = 3.30) at 20 °C. The concentration of this compound, which was frequently used in the past but is no longer used in the European Union, in water was 40 μg L^{-1} during a 72-h accumulation phase and fish were subsequently subjected to an additional 72-h depuration phase in clean water. The internal concentration of this chemical in muscle tissue was reported. Moreover, changes in the total lipid content of muscle tissue were observed as a toxicological effect of fenitrothion that was relevant to its bioconcentration in muscle; this lipid level decreased to approximately 25% of the initial value after 48-h exposure to 40 μg L^{-1} fenitrothion. This decrease was also included in our model. In addition, a dataset relating to the elimination of benzene (CAS 71-43-2; *log* K_{ow} = 2.13), toluene (CAS 108-88-3; *log* K_{ow} = 2.73), *m*-xylene (CAS 108-38-3; *log* K_{ow} = 3.20) and *o*-xylene (CAS 95-47-6; *log* K_{ow} = 3.12), which are collectively referred to as BTX and are a widely distributed class of environmental contaminants, from the muscle of Japanese eels (*Anguilla japonica;* 130–180 g) that had been exposed to crude oil suspensions at 20 °C was available (Ogata and Miyake, 1978). Furthermore, Ogata et al. (1980) published a dataset on the toxicokinetics of dibenzothiophene (CAS 132-65-0; *log* Kow = 4.29) and other organosulphur compounds in Japanese eels of the same weight and under the same exposure conditions. Such compounds are released during contamination with crude or heavy oils and are frequently found in the vicinity of industrial sites or harbours. In this study, data were only reported as BCFs and no internal concentration information was available. The PBTK model was used to predict the time-course of internal chemical concentrations; in addition, values for BCF_k, k_1 and k_2 were predicted where applicable, assuming the exposure conditions reported in these publications. These predicted values were then compared with the experimentally derived values. For all studies, the water was assumed to be saturated with oxygen and the dissolved oxygen concentration was calculated as described by Weiss (1970). *Log* K_{ow} values were taken from the US-EPA EPI Suite software. Experimental database values were preferred over predicted values. As a quantitative measure of model performance, we calculated the root mean squared error (RMSE) of the residuals. Unless indicated, all values are reported as mean values ± standard deviations.

3. Results and discussion

3.1. Model parameterisation

As detailed in the following paragraphs, we successfully developed a PBTK model for the bioconcentration of neutral organic chemicals in European eels. Physiological parameters were compiled from the literature or determined experimentally (Tables 1–3). Furthermore, the accuracy of interpolated VO_2 rates (Eq. (3)) was experimentally verified using our own data. The measured respiration rates were consistent with the predicted values. In the present study, the young yellow eels used in the respiratory experiment showed a mean VO_2 of 33.9 ± 6.73 mg O_2 kg^{-1} h^{-1} (Table 4), which did not differ significantly from the modelled value (37.8 ± 0.57 mg O_2 kg^{-1} h^{-1}, Mann–Whitney U-test, $p = 0.09$). The data available in the scientific literature were sufficient to parameterise the PBTK model with a satisfactory level of confidence. One major physiological irregularity of the *Anguilla* genus, as compared with other fish species, is their intermittent ventilation pattern (Berg and Steen, 1965; Smith et al., 1983). This reduces their Q_w, which considerably reduces chemical flux across the gills in eels.

3.2. Evaluation of the model performance

The predictive power of the eel PBTK model was evaluated using published toxicokinetic data. Data of sufficient quality were available for six different chemicals. Full accumulation and elimination data were available for the pesticide fenitrothion (Sancho et al., 1998), and the organosulphur compound dibenzothiophene (Ogata et al., 1980). For *m*-xylene, *o*-xylene, toluene and benzene, only the elimination phase was considered because the exposure concentration was not reported by the authors (Ogata and Miyake, 1978). Out of these compounds, only fenitrothion had been studied in European eels; the other values were derived from the Japanese eel, which is physiologically very similar to the European eel.

3.2.1. Predicted and measured internal concentrations

Internal concentrations in eel muscle tissues were predicted using the PBTK model with the exposure conditions reported in the corresponding publications as the model inputs; a comparison of predicted *versus* modelled values for all tested concentrations of all six chemicals is shown in Fig. 2. The predicted internal concentrations were accurate: the RMSE was 0.28 *log* units. All predicted values deviated from the measured values by less than 5-fold, and 71% of these deviated by less than 2-fold.

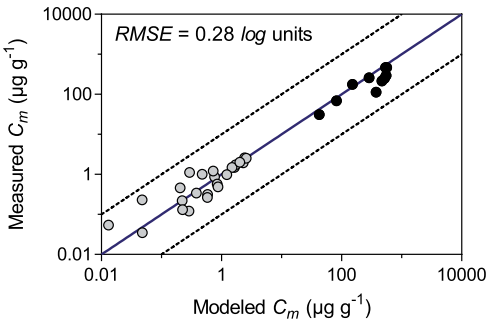

Fig. 2. Relationship between measured (muscle) and modelled (poorly perfused tissues) internal concentrations (values represent all available measured values for the chemicals) in eels. Open circles represent the actual measured values (Ogata and Miyake, 1978) (Sancho et al., 1998), closed circles are based on the bioconcentration factors (BCFs) reported by Ogata et al. (1980). The solid line represents the equality line and dashed lines indicate a 10-fold deviation from equality. RMSE, root mean squared error.

Table 4
Measured and modelled respiratory data (170 measuring cycles in 96 h) from 13 individual European eels (*Anguilla anguilla*) at a mean temperature of 11.6 ± 0.6 °C.

Chamber/fish (No)	Body mass (g)	Oxygen consumption (mg O_2 h^{-1})	Measured VO_2 (mg O_2 kg^{-1} h^{-1})	Predicted VO_2 (mg O_2 kg^{-1} h^{-1})
1	98.40	2.19 ± 0.78	22.30	37.39
2	89.80	2.16 ± 0.84	24.02	37.78
3	95.60	3.01 ± 0.99	31.48	37.51
4	85.40	2.67 ± 1.14	31.31	37.99
5	99.20	2.92 ± 1.21	29.43	37.36
6	86.40	3.76 ± 2.6	43.53	37.94
7	102.40	3.07 ± 1.31	29.95	37.22
8	74.20	3.00 ± 1.39	40.41	38.55
9	101.20	3.59 ± 1.54	35.44	37.27
10	104.80	3.48 ± 1.49	33.17	37.12
11	66.60	2.89 ± 1.49	43.41	38.97
12	83.00	3.22 ± 1.59	38.83	38.10
13	79.20	2.94 ± 1.56	37.07	38.29
Mean	89.71	2.99	33.87	37.81
SD	11.84	0.47	6.73	0.57

3.2.2. Accumulation and elimination rates

In addition to the internal concentrations at different time points, k_1 and k_2 were calculated using the predicted and published experimental toxicokinetic data. In general, the elimination kinetics of *m*-xylene, *o*-xylene, toluene, benzene and fenitrothion were predicted accurately (Figs. 3 and 4, Table 5). On average, the predicted values for k_2 deviated from the measured values by 39%. The errors for dibenzothiophene and benzene were much greater than those observed for the other compounds, which might reflect hepatic metabolism. Predictions of k_1 (and consequently for BCF_k) were only possible for fenitrothion and dibenzothiophene, since no accumulation kinetics were reported for the other four compounds. The predictions for k_1 were reasonably accurate, deviating an average of 18% from the measured values, while the weak fit of k_2 for dibenzothiophene resulted in a 2-fold overestimation of BCF_k. The calculated BCF_k for fenitrothion deviated only 9% from the measured value (Table 5).

3.3. Conclusions and further directions

In the present study, we developed and tested a PBTK model of the uptake and disposition of neutral organic chemicals in European yellow eels. We successfully tested the predictive power of the model for six different chemicals with *log* K_{ow} values ranging from 2.13–4.29.

Because of the slow rate of accumulation of very lipophilic compounds (*e.g.* polychlorinated biphenyls or dioxins/furans), growth of the organism cannot be neglected when modelling their bioconcentration. In particular, essential parameters of the PBTK model, such as the volumes and lipid levels of tissues and organs, are not constant and can change significantly before the organism, *i.e.* the growing yellow eel, achieves equilibrium with the surrounding medium. Incorporation of a scientifically sound sub-model for growth (including changes in volume, lipid levels and perfusion rates in growing individuals) will therefore represent an important addition to the proposed model. These data are not currently available. Furthermore, the PBTK model would need to be amended with sub-models for dietary and dermal exposure. Studies encompassing depuration periods of several years (*e.g.* de Boer et al., 1994) could be modelled confidently with such extensions.

One particular advantage of models that contain explicit sub-models for growth, as well as dietary and dermal exposure is that they have the potential to model time-variable exposures. European eels have a continental growth phase that lasts for up to 15 years and have been

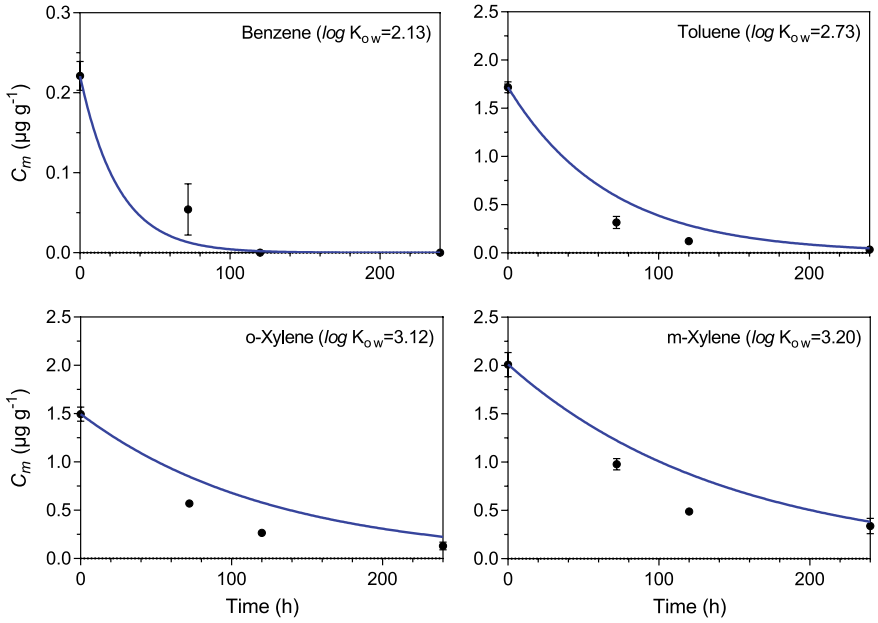

Fig. 3. Measured (muscle) and modelled (poorly perfused tissues) elimination kinetics of *m*-xylene, *o*-xylene, toluene and benzene in Japanese eels (*Anguilla japonica;* 130–180 g) that had been exposed to crude oil suspensions (Ogata and Miyake, 1978). Dots represent experimental data from the literature (mean values with standard errors); the solid line represents the model prediction.

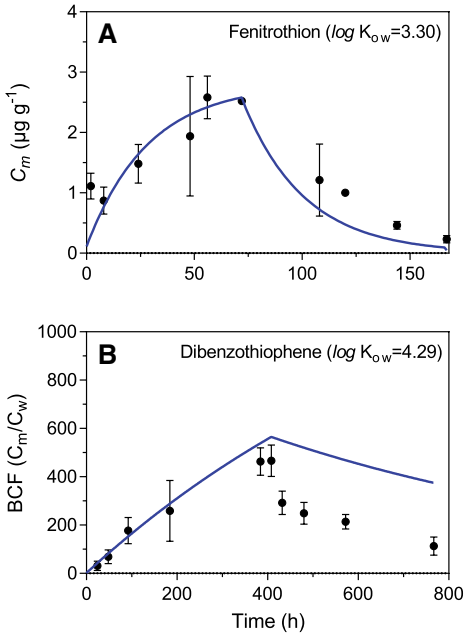

Fig. 4. Experimental (muscle) and modelled (poorly perfused tissues) kinetics of uptake and elimination of (A) the organophosphate pesticide fenitrothion, and (B) the organosulphur compound dibenzothiophene in European eels (*Anguilla anguilla*) and Japanese eels (*Anguilla japonica*), respectively. Dots represent experimental data (mean values with standard deviations) from the literature (Ogata et al., 1980; Sancho et al., 1998) and the solid line represents the predictions of the physiologically based toxicokinetic (PBTK) model. Data for dibenzothiophene were reported as BCF values only, *i.e.* ratios between concentrations in muscle (C_m) and water (C_{insp}).

demonstrated to be fairly mobile in inland waters, sometimes even transitioning between freshwater, brackish water and the ocean (Marohn et al., 2013). PBTK models have been used successfully in marine mammals to model the accumulation and tissue distribution of lipophilic organic contaminants over time-spans of more than 20 years (Weijs et al., 2010). Our PBTK model lays the groundwork for successful modelling of contaminant uptake and distribution in European yellow eels during their continental growth phase. It should be emphasised that, in its present form, the model performance has only been verified for neutral organic substances with moderate lipophilicity. Such compounds could include endocrine disrupting chemicals, such as the birth control agent, ethinyl estradiol, as well as other pharmaceuticals, personal care products, biocides and plant protection products.

In order to realise the full potential of the proposed methodology, the European eel PBTK model can be extended by the inclusion of toxicodynamic (TD) models, resulting in a PBTK/TD model. PBTK models have been used successfully to link results of *in vitro* bioassays with *in vivo* effects (Brinkmann et al., 2014a; Stadnicka-Michalak et al., 2014). With the present model, toxicological data from European eels can thus be retrospectively linked to internal concentrations at the target site. Combined PBTK/TD models have the potential to predict a large variety of toxicological effects semi-quantitatively, without the need to perform additional animal experiments; these include acute toxicity and receptor-mediated effects, but also hepatotoxicity, aneugenic or clastogenic effects, hepatic lesions and carcinogenesis, and can be used to derive threshold values for aqueous exposure concentrations. In this way, the ultimate goal of developing a tool to identify suitable habitats for stocking measures could be achieved. Considering the lack of relevant threshold values for most lipophilic contaminants and the clear knowledge gaps in relation to their physiological consequences for eel gonadal development and bioenergetics, it is important that future studies adapt and re-parameterise this model to silver eels and their oceanic migration.

Table 5
Measured (muscle) and modelled (poorly perfused tissues) accumulation and elimination rate constants (k_1 and k_2), as well as kinetic bioconcentration factors (BCF_k) of fenitrothion, dibenzothiophene, *m*-xylene, *o*-xylene, toluene and benzene in eels. The accumulation rate (and consequently also BCF_k) could only be calculated for fenitrothion and dibenzothiophene because only elimination phase data were available for the other four compounds.

Chemical	k_1 ($L\,kg^{-1}\,d^{-1}$)		k_2 (d^{-1})		BCF_k ($L\,kg^{-1}$)	
	Measured	Modelled	Measured	Modelled	Measured	Modelled
Fenitrothion	47.69	59.74	0.610	0.843	78.18	70.87
Dibenzothiophene	46.13	41.64	0.067	0.027	688.5	1542
m-Xylene	–	–	0.178	0.166	–	–
o-Xylene	–	–	0.242	0.189	–	–
Toluene	–	–	0.381	0.358	–	–
Benzene	–	–	0.470	0.942	–	–

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2015.07.046.

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CHAPTER V

Appendix A

A physiologically based toxicokinetic (PBTK) model for moderately hydrophobic organic chemicals in the European eel (*Anguilla anguilla*)

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Total page number: 3, including

10 equations and one table

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Table A.1 Model inputs and parameters of the PBTK models for European eel (*Anguilla anguilla*). Based on Stadnicka *et al.* (2012).

Symbol	Units	Description	Value
W	kg	Body wet weight	– Model input –
log K_{ow}	-	Octanol-water partitioning coefficient	– Model input –
C_w	µg L^{-1}	Chemical concentration in inspired water	– Model input –
T	°C	Water temperature	– Model input –
C_{ox}	mg L^{-1}	Dissolved oxygen concentration in inspired water	– Model input –
lipid	-	Total lipid content (fraction of body weight)	– Model input –
$lipid_l$	-	Lipid content of lean tissue (fraction of wet weight)	Eq. A.1
P_{bw}	-	Blood:water partitioning coefficient	Eq. A.2
P_l,P_f,P_m	-	Liver/fat/muscle:blood partitioning coefficient	Eq. A.3
P_k	-	Kidney:blood partitioning coefficient	Eq. A.4
P_r	-	Richly perfused tissue:blood partitioning coefficient	P_l
A_i	µg	Chemical amount in fat, poorly and richly perfused tissues	Eq. A.5
A_l	µg	Chemical amount in the liver compartment	Eq. A.6
A_k	µg	Chemical amount in the kidney compartment	Eq. A.7
C_{int}	µg g^{-1}	Internal concentration in the whole fish	Eq. A.8
C_{art}	µg L^{-1}	Chemical concentration in arterial blood	Eq. A.9
C_{ven}	µg L^{-1}	Chemical concentration in venous blood	Eq. A.10

Model equations, based on Stadnicka *et al.* (2012)

Volume of the lean tissue compartments

$$lipid_l = \frac{V_l \cdot a_l + V_r \cdot a_r + V_m \cdot a_m + V_k \cdot a_k}{V_l + V_r + V_m + V_k} \quad \textbf{(Eq. A.1)}$$

Blood:water partitioning coefficient

$$P_{bw} = 10^{0.72 \cdot \log Kow + 1.04 \cdot log(\alpha_b) + 0.86} + \gamma_b \quad \textbf{(Eq. A.2)}$$

Liver/fat/muscle:blood partitioning coefficient

$$P_{l,f,m} = \frac{10^{0.72 \cdot \log Kow + 1.04 \cdot log(\alpha_{l,f,m}) + 0.86} + \gamma_{l,f,m}}{P_{bw}} \quad \textbf{(Eq. A.3)}$$

Kidney:blood partitioning coefficient

$$P_k = \frac{10^{0.72 \cdot \log Kow + 1.04 \cdot log(\alpha_k) + 0.86} + \gamma_k}{P_{bw}} \quad \textbf{(Eq. A.4)}$$

Chemical amount in fat, poorly and richly perfused tissues

$$\frac{dA_i(t)}{dt} = Q_i \cdot \left(C_{art}(t) - \frac{A_i(t)}{V_i \cdot P_i}\right) \quad \textbf{(Eq. A.5)}$$

Chemical amount in the liver compartment

$$\frac{dA_l(t)}{dt} = Q_r \cdot \frac{A_r(t)}{V_r \cdot P_r} + Q_l \cdot C_{art}(t) - (Q_r + Q_l) \cdot \frac{A_l(t)}{V_l \cdot P_l} \quad \textbf{(Eq. A.6)}$$

Chemical amount in the kidney compartment

$$\frac{dA_k(t)}{dt} = 0.6 \cdot Q_m \cdot \frac{A_m(t)}{V_m \cdot P_m} + Q_k \cdot C_{art}(t) - (0.6 \cdot Q_m + Q_k) \cdot \frac{A_k(t)}{V_k \cdot P_k} \quad \textbf{(Eq. A.7)}$$

Internal chemical concentration in the whole fish

$$C_{int}(t) = \frac{A_f(t) + A_m(t) + A_r(t) + A_l(t) + A_k(t)}{1000 \cdot w\_w} \quad \textbf{(Eq. A.8)}$$

Chemical concentration in arterial blood

$$C_{art}(t) = \min\left(Q_w, Q_c \cdot P_{bw}\right) \cdot C_w - \frac{C_{ven}(t)}{P_{bw}} \cdot \frac{1}{Q_c} + C_{ven}(t) \quad \textbf{(Eq. A.9)}$$

Chemical concentration in venous blood

$$C_{ven}(t) = \left(Q_f \cdot \frac{A_f(t)}{V_f \cdot P_f} + 0.4 \cdot Q_m \cdot \frac{A_m(t)}{V_m \cdot P_m} + (0.6 \cdot Q_m + Q_k) \cdot \frac{A_k(t)}{V_k \cdot P_k} + (Q_r + Q_l) \cdot \frac{A_l(t)}{V_l \cdot P_l}\right) \cdot \frac{1}{Q_c} \quad \textbf{(Eq. A.10)}$$

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Chapter VI

Fipronil and two of its transformation products in water and European eel from the river Elbe

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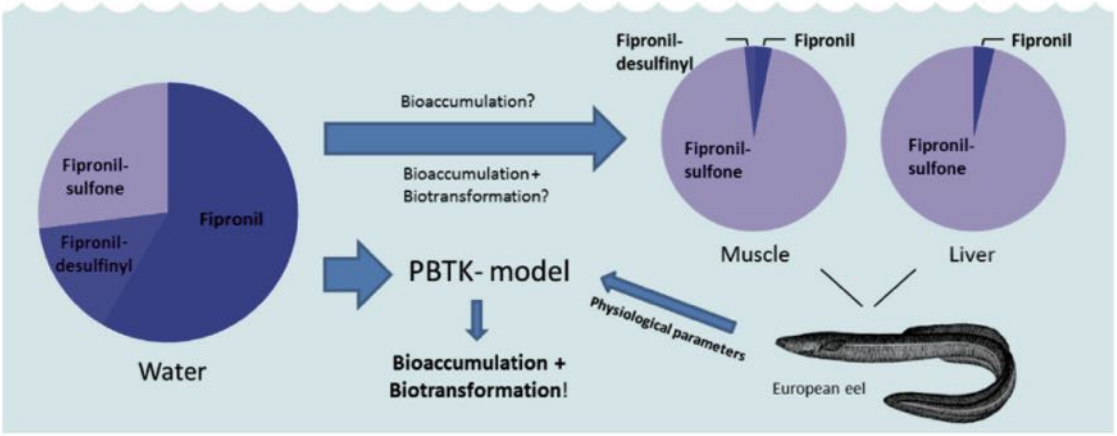

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Fipronil and two of its transformation products in water and European eel from the river Elbe

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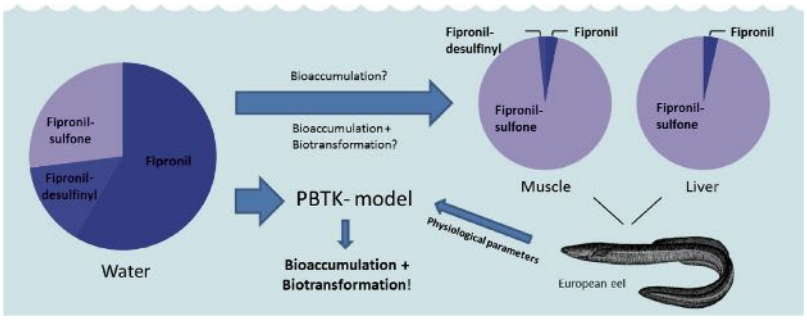
HIGHLIGHTS

- Fipronil, Fipronil-desulfinyl and Fipronil-sulfone were detected in water samples and eels.
- In water, Fipronil was predominant over its metabolites.
- Analytes concentrations in water did not reflect seasonal application of Fipronil.
- In muscle and liver tissue of eels, Fipronil-sulfone was the predominant compound.
- Using a PBTK model, distributions in eels could be attributed to metabolic processes.

GRAPHICAL ABSTRACT



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ABSTRACT

Fipronil is an insecticide which, based on its mode of action, is intended to be predominantly toxic towards insects. Fipronil bioaccumulates and some of its transformation products were reported to be similar or even more stable in the environment and to show an enhanced toxicity against non-target organisms compared to the parent compound. The current study investigated the occurrence of Fipronil and two of its transformation products, Fipronil-desulfinyl and Fipronil-sulfone, in water as well as muscle and liver samples of eels from the river Elbe (Germany). In water samples total concentrations of FIP, FIP-d and FIP-s ranged between 0.5–1.6 ng L^{-1} with FIP being the main component in all water samples followed by FIP-s and FIP-d. In contrast, FIP-s was the main component in muscle and liver tissues of eels with concentrations of 4.05 ± 3.73 ng g^{-1} ww and 19.91 ± 9.96 ng g^{-1} ww, respectively. Using a physiologically based toxicokinetic (PBTK) model for moderately hydrophobic organic chemicals, the different distributions of FIP, FIP-d and FIP-s in water and related tissue samples could be attributed to metabolic processes of eels. The measured concentrations in water of all analytes and their fractional distribution did not reflect the assumed seasonal application of FIP and it seems that the water was constantly contaminated with FIP, FIP-d and FIP-s.

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1. Introduction

Fipronil (FIP) is an insecticide belonging to the group of phenylpyrazoles (Cole et al., 1993). It came to broad use as a pesticide for crop protection and pest control but also in households as an insecticide, e.g. against tick and flea infestations on pets.

Its mode of action is based on blocking the γ-aminobutyric acid (GABA)-gated chloride channel (Cole et al., 1993). Similar effects are known from older-generations pesticides such as Lindane (γ-HCH), Dieldrin and Endosulfan (Wafford et al., 1989; Cole et al., 1993; Hainzl et al., 1998). In comparison, FIP seems to show higher selective toxicity in terms of a higher binding affinity for the GABA-receptors of insects compared to those of vertebrates (Hainzl et al., 1998; Zhao et al., 2003). For this reason, the application of FIP is considered to have a low toxic impact on non-target organisms. However, concerns were raised over FIP being a threat to several non-target organisms such as bees (APENET, 2011) or freshwater crustaceans (Schlenk et al., 2001) through direct toxicity or, as for most pesticides, indirectly by influencing food webs within the application area (Peveling et al., 2003).

FIP is not stable in the environment and forms several transformation products of which Fipronil-desulfinyl (FIP-d) and Fipronil-sulfone (FIP-s) are two of the most common ones (USEPA, 1996; Gunasekara et al., 2007). While FIP-d seems to be exclusively formed during abiotic photolysis, FIP-s is supposed to be produced via biotic transformation processes, either by vertebrates, invertebrates and plants (Caboni et al., 2003; Durham et al., 2002; Hainzl and Casida, 1996; Lu et al., 2010; Raveton et al., 2006; Scharf et al., 2000) or through microbiological degradation of FIP in soils (Raveton et al., 2007; Tan et al., 2008) and additionally also through photooxidation (Hainzl and Casida, 1996; Raveton et al., 2006) (Fig. 1).

The degradation of FIP highly depends on environmental conditions of the application area. Thus, reported half-life values for FIP in soils vary widely, e.g. from 36 h (Bobé et al., 1998) to approx. 1.5 months (Mandal and Singh, 2013; USEPA, 1996). Similarly, reported half-life values for FIP in aqueous solutions exposed to UV light vary from, e.g. several minutes (Walse et al., 2004a) to approx.56 h (Raveton et al., 2006). However, due to the apparent instability of FIP, it degrades readily and therefore, every application of FIP will most likely lead to the presence of FIP-d and FIP-s in the environment due to the aforementioned processes, as was confirmed by many studies focusing on FIP and its transformation products in environmental field samples from soils, urban influenced water bodies and streams as well as residential runoffs (Bobé et al., 1998; Gan et al., 2012; Jiang and Gan, 2016; Mandal and Singh, 2013; Raveton et al., 2007; Weston and Lydy, 2014).

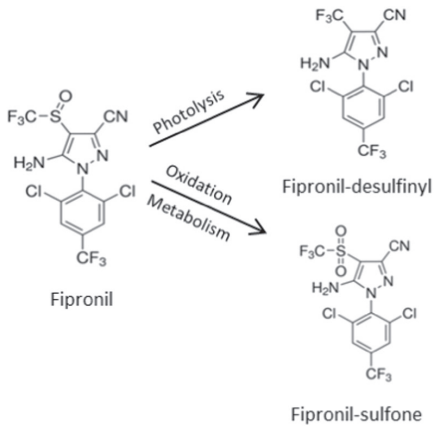

Fig. 1. Chemical structures of Fipronil, Fipronil-desulfinyl and Fipronil-sulfone.

Compared to the parent compound, FIP-d and FIP-s were found to be similar or even more persistent, e.g. spiked to urban sediments FIP showed maximum half-lives of 18.5 d and 91.2 d for anaerobic and facultative conditions, respectively, while half-lives of FIP-d and FIP-s ranged between 217 d–712 d (Brennan et al., 2009; Lin et al., 2009; Mandal and Singh, 2013; Ngim and Crosby, 2001). Furthermore, FIP-d and FIP-s seem to be less selective in binding at GABA-receptors of insects than FIP. In a comparative study in 1998, Hainzl et al. showed FIP to have the most selective binding affinity, followed by Lindane and FIP-d, while FIP-s and α-Endosulfan were the least selective ones. Confirming these results, Zhao et al. (2005) found a 20-fold higher affinity in blocking GABA-receptors in mammals for FIP-s compared to FIP. These results may explain why FIP-d and FIP-s, compared to the parent compound, were often found to be similar or even more toxic towards aquatic organisms (Iwafune et al., 2011; USEPA, 1996, 2005; Weston and Lydy, 2014) and they question the theoretical benefit of FIP, which is supposed to be predominantly toxic towards insects.

Under laboratory conditions FIP bioaccumulates in fish and is then metabolized to FIP-s (Konwick et al., 2006; USEPA, 1996). Although the *n*-octanol-water partitioning coefficient (log K_{ow}) for FIP-s (3.68) is reported to be slightly lower than that of FIP (4.01) (Walse et al., 2004b), FIP-s was found to be three times more persistent in rainbow trout (*Oncorhynchus mykiss*) than the parent compound (Konwick et al., 2006). No indications about a potential of FIP-d to bioconcentrate in fish were found. However, based on its lipophilicity (log K_{ow} of 4.63 (Walse et al., 2004b)) and the observation that FIP-d was found to bioaccumulate in mice (Hainzl and Casida, 1996), bioaccumulation in fish has to be considered. Still, only little is known about the abundance and behavior of these compounds in the aquatic environment and studies focusing on the determination of FIP in biota samples taken from the field are rare. However, FIP was detected in samples of tiger fish (*Hoplias malabaricus*) from South Brazil, variety of other aquatic species from different trophic levels as well as in muscle and liver samples of European eel (*Anguilla anguilla*) from southern France (Miranda et al., 2008; Ribeiro et al., 2005; Roche et al., 2009). Unfortunately, FIP-d and FIP-s were not determined in any of these studies.

Suitable organisms for studying the bioaccumulation potential of chemicals found in the aquatic environment are diadromous eels of the genus *Anguilla.* Due to their high body fat content and semelparous lifestyle, they are extremely predisposed towards chemical contaminations (Belpaire et al., 2009; Freese et al., 2016; Sühring et al., 2014). However, the majority of available studies on contaminants in eels focus on classic lipophilic chemicals such as trace metals or halogenated pollutants and so far, only little attention was paid to modern compounds with potentially harmful effects on aquatic species, as was e.g. done by Belpaire et al. (2015) and Sühring et al. (2013).

To the best of our knowledge, we provide for the first time data about the occurrence of FIP, FIP-d and FIP-s in water samples of the river Elbe (Hamburg, Germany) and we discuss whether or not the measured concentrations reflect the seasonal application of FIP. Furthermore, we measured FIP in muscle and liver samples of silver eels caught in the river Elbe and included for the first time FIP-d and FIP-s into the investigation of fish taken from field. A recently developed physiologically based toxicokinetic (PBTK) model for European eel (Brinkmann et al., 2015) was used to link the measured concentrations of FIP, FIP-d and FIP-s in water samples to those concentrations measured in eel samples.

2. Materials and methods

2.1. Water

2.1.1. Sample collection

FIP and its two transformation products FIP-d and FIP-s were at first discovered and then quantified in water samples of the river Elbe in April 2014. Two water samples were additionally taken the following

two weeks (May 14a; May 14b) confirming the presence of these substances over a period of at least three weeks. Further two water samples were analyzed in December 2014 and January 2015 expecting the absence of FIP as the application of insecticides is more common during the spring and summer season. Unexpectedly, even in the samples taken in winter FIP, FIP-d and FIP-s could be detected. For analyzing the potential accumulation of FIP in biota, eels caught between 2013 and 2014 were made available from the Thünen-Institute, Institute of Fisheries Ecology (Hamburg, Germany). For that reason, disposable reference water samples from 2013 obtained by the Federal Maritime and Hydrographic Agency were additionally analyzed. All water samples were taken off a pier located in Wittenbergen (Hamburg, Germany). As the lower river Elbe is influenced by tidal phase, all samples were collected during low tides approximately 1 m below surface using brown glass bottles (2 L). Filtration and extraction were done immediately after sampling. Information about sample storage is displayed in Supplementary Information S1. All samples were taken as duplicates. Data of river flow rates at times related to water sampling were obtained from the Federal Waterways and Shipping Administration-Hamburg.

2.1.2. Sample extraction

Prior to their extraction water samples were filtered with glass fiber filters (GF/F, pore size 0.7 μm, Whatman®) and subsequently an internal standard solution mix (details see S2) was added. The filtered samples were extracted using an automated SPE device (Quicksampler Q-3000 Aqua, Biontis) equipped with 3 mL cartridges filled with 200 mg polymer-based adsorber Strata-X (Phenomenex). Prior to their use, cartridges were washed with eluent (Acetonitrile/Methanol, see below) and then Methanol (LC-MS CHROMASOLV®, Sigma-Aldrich) and conditioned with high-purity water (LC-MS CHROMASOLV®, Sigma Aldrich). The water sample volume used for extraction was 200 mL. Pure water treated equivalent to samples was used for blank value determinations. Elution from the SPE-column was performed with 2 × 3 mL eluent mixture, consisting of methanol (LC-MS CHROMASOLV®, Sigma-Aldrich) and acetonitrile (HPLC grade, J.T. Baker®) (v/v, 70:30) and added buffer giving 1.25 mM HAc and 2.5 mM NH_4Ac. From the eluate, 1.5 mL were taken and concentrated to 0.5 mL by a gentle nitrogen stream. The final extract was then analyzed by HPLC-MS/MS.

2.2. Eel samples

2.2.1. Sample collection and age determination

A total of 13 silver eels of migrating stages IV and V (according to Durif et al., 2005) were caught with stow nets in lower stretches of the river Elbe near Hamburg (Hoopte and Winsen; Germany) in November 2013 and 2014 in line with the EU Data Collection Framework (European Council, 2008; European Commission, 2010). Eels were killed by decapitation after being anaesthetized in a water bath of 25 L containing approximately 10 mL 2-Phenoxyethanol (ROTH, Germany). Together with the assessment of biometric parameters (length, weight, sex and life history stage) eels were aged based on otolith readings following the cutting and burning method (Graynoth, 1999) as recommended by the International Council for the Exploration of the Sea (ICES, 2009, 2011). Biological data and additional sampling details of eel samples are displayed in Supplementary Information S3.

2.2.2. Sample extraction

For chemical analyses, whole livers ($n = 13$) were excised from the sampled animals. To investigate possible physiological transformation mechanisms of FIP in eels, we also analyzed muscle samples from 6 of the sampled eels. For each muscle sample, between 10 and 25 g skeletal muscle tissue were excised just behind the level of the anus. Samples were kept frozen at −20 °C until further analyses. To prevent possible sources of cross-contamination, samples were strictly handled with clean equipment made of glass, aluminum or steel. After storage, biota samples were thawed and homogenized using an analytical mill (IKA-A11 basic, IKA-Werke GmbH & Co. KG) until evenly homogenous. Tissue-homogenates were then freeze-dried (Lyov GT2 Typ 8, SRK Sytem Technik GmbH) and subsequently weighed with a precision scale (VWR-124, Sartorius). Total lipid content in dry weight (dw) and wet weight (ww) was then quantified gravimetrically after extraction of total lipids, following the protocol published by Smedes (1999) and modified by Schlechtriem et al. (2012).

Analyte extraction for LC-MS/MS analyses was done based on the methods described in Theobald et al. (2011a, 2011b): 2 g of muscle tissue or 1 g of liver tissue were extracted three times in a 35 mL centrifuge tube with 9 mL acetonitrile (HPLC Ultra Gradient Grade, J.T. Baker ®) for 30 min each run, using a shaker device. Previous to extraction, an internal standard solution mix (details see S2) was added. After each extraction step, the sample was centrifuged at 3000 rpm for 10 min and the supernatant liquid was collected in a pear-shaped flask. Extracts were then split and transferred into 15 mL centrifuge tubes and were stored at −18 °C over night. Afterwards, the frozen samples were again centrifuged at 3000 rpm for 1 min to remove the fatty matrix. Extracts were then reduced in volume to 3 mL with a vaporizer (Syncore®Analyst, BÜCHI) and the freeze out step was repeated, followed by another centrifugation for 1 min. 1 mL of the final extract was transferred into 1.5 mL vials for LC-MS/MS analysis.

2.3. Sample analysis

Analyses of water and eel extracts were performed using a HPLC system (Ultimate 3000, DIONEX) coupled with tandem mass spectrometer (5500QTrap, AB Sciex) with an electrospray interface (ESI). For chromatographic separation, a C-18 type column (Kinetex 100 × 2.1 mm, 2.6 μm particle size, Phenomenex) was used, equipped with a security-guard column AQ C18 (4x2.1 mm, Phenomenex). The flow was 0.220 mL/min and column oven temperature was set to 28 °C. Eluent A was ultrapure water (LC-MS CHROMASOLV®, SIGMA-ALDRICH) and Eluent B was methanol (LC-MS CHROMASOLV®, SIGMA-ALDRICH). To both eluents an acetate buffer was added, resulting in 5.6 mM acetic acid and 5 mM ammonium acetate. Separation started with eluent A 90%, followed by a gradient of 4 min to 30% A and then to 5% A within 18 min followed by an isocratic step for the next 10 min. Scheduled multiple reaction monitoring (sMRM) mode with negative ESI was used for the detection of all analytes.

2.4. Quality assurance

All analyses were performed in an ISO 17025 accredited laboratory for the LC-MS/MS analysis. Analytical standards for FIP, FIP-d and FIP-s were purchased from Dr. Ehrenstorfer (LGC, Augsburg, Germany). Analytical standards for ^{13}C-labeled PFOA and ^{13}C-labeled PFOS, which were used as internal standards in water and eel samples, were obtained from Wellington Laboratories Inc. (ON, Canada) (see S2).

The limit of quantification (LOQ) was determined by taking into account a signal to noise ratio (S/N) of ten for the quantifier ion (Q1) and a minimum S/N of three for the qualifier ion (Q2) as well as the ratio of their peak areas (Q2/Q1). In water samples a LOQ of 0.08 ng L^{-1} for FIP as well as FIP-d and 0.04 ng L^{-1} for FIP-s was determined. LOQ for FIP and FIP-s in liver samples were 0.03 ng g^{-1} ww and 0.015 ng g^{-1} ww, respectively. LOQ for FIP, FIP-d and FIP-s in muscle samples were 0.015 ng g^{-1} ww for FIP and FIP-d and 0.008 ng g^{-1} ww for FIP-s.

The limit of detection (LOD) was determined based on a required minimum S/N of three for both Q1 and Q2 transitions, which resulted in LOD values for FIP and FIP-d of 0.04 ng L^{-1} and 0.02 ng L^{-1} for FIP-s in water samples. The LOD for FIP and FIP-d in muscle tissue was 0.008 ng g^{-1} ww and in liver tissue 0.02 ng g^{-1} ww. For FIP-s, LOD was determined in muscle tissue to be 0.003 ng g^{-1} ww and in liver samples 0.006 ng g^{-1} ww.

Relative recoveries (internal standard corrected; $n = 7$) for FIP, FIP-d and FIP-s in spiked Elbe-water samples were 97.8 ± 2.4%, 89.3 ± 2.2%

and 85.5 ± 5.1%, respectively. Ultrapure water treated as sample was used for blank determination with every water sampling ($n = 9$). In all blanks, FIP-d could not be detected. Traces of FIP could be detected in one blank sample (Oct. 13) with a value slightly above LOD. In the same blank sample FIP-s was detected with a value two times above LOQ (0.08 ng L^{-1}).

Relative recoveries ($n = 9$) for FIP and FIP-s in spiked muscle and spiked liver samples were 103.2 ± 6.1% and 99.9 ± 5.0%, respectively. Relative recoveries for FIP-d in spiked muscle samples ($n = 3$) achieved a value of 103.7 ± 0.6%. With every sample batch, depending on the sample batch size, one or two blanks (acetonitrile used for the extraction and treated as a sample) were determined. In all blank values ($n = 9$) FIP, FIP-d and FIP-s could not be detected.

2.5. PBTK model

Physiologically based toxicokinetic (PBTK) models are capable of predicting the lipid-based absorption, disposition and elimination of neutral organic chemicals in the whole fish and in different tissues at any time during aqueous exposure (Nichols et al., 1990; Yoon et al., 2012). Within PBTK models, organs and tissues are explicitly represented as individual compartments, each of which is characterised by its volume (as a fraction of total body weight), its total lipid and water contents (as a fraction of tissue ww), and by the perfusion of the compartment (as a fraction of cardiac output).

We used a recently developed PBTK model for the European eel (Brinkmann et al., 2015) to investigate if the measured concentrations of all three analytes in muscle and liver samples of eels may be explained by their uptake solely via the water phase. Therefore, predictions were calculated by assuming bioconcentration of FIP, FIP-d and FIP-s with and without simulated metabolism.

To conform to the conditions of the present study, the model parameters body ww, as well as the total lipid contents of liver and muscle needed to be adjusted. The log K_{ow} values of FIP, FIP-s and FIP-d (4.01, 3.68 and 4.63, respectively) were taken from the literature (Walse et al., 2004b). As silver eels stop feeding, exposure due to dietary uptake was not included in the current model.

Metabolic processes were simulated based on the results reported in Konwick et al. (2006) who investigated the bioaccumulation and biotransformation of FIP in rainbow trout (*Oncorhynchus mykiss*). In rainbow trout, FIP is quickly metabolized, mostly to FIP-s. The experimental whole-body biotransformation rate constant of FIP in rainbow trout (12.2 ± 0.5 g) at 12 °C was 1.006 d^{-1}, while the biotransformation of FIP-s was negligible (Konwick et al., 2006). The biotransformation rate of FIP in European eel was assumed to be equal to that of rainbow trout. In the model, internal concentrations of FIP-s resulted from both uptake through the water phase and biotransformation of FIP. No data on the biotransformation of FIP-d in fish was available; thus, its biotransformation rate was assumed to be equal to that of FIP. Biotransformation rates were allometrically scaled and temperature-corrected according to (Arnot et al., 2008). All predictions were continued until equilibrium conditions were reached. Uncertainties of model parameters and input variables were addressed by generating sets of parameters and variables that were randomly drawn from the statistical (Gaussian) distributions defined by the measured data (mean ± standard deviation, i.e. body ww [0.83 ± 0.32 kg], total lipid contents of liver [9.01 ± 3.15% ww] and muscle [26.40 ± 4.65% ww], as well as water temperature of the sampling area [14.3 ± 7.7 °C] and the aqueous concentrations of FIP [0.66 ± 0.23 ng L^{-1}], FIP-s [0.29 ± 0.08 ng L^{-1}] and FIP-d [0.18 ± 0.14 ng L^{-1}]), during each model run (Monte Carlo simulation). A total number of 1000 Monte Carlo simulations were performed for each condition and the mean value and its standard deviation were calculated and compared to the experimental data (Manly, 1991). Detailed information about parameters and equations used for the PBTK model can be found in Appendix B of the Supplementary Material.

3. Results and discussion

3.1. Concentrations of FIP, FIP-d and FIP-s in water samples

Total concentrations of targeted analytes (sum of FIP, FIP-d and FIP-s) ranged between 0.5–1.6 ng L^{-1}. Negative Spearman correlation was found between stream flow and sum concentrations of FIP, FIP-d and FIP-s ($r_s = -0.87$; $p < 0.005$), with highest total concentrations measured in water samples taken during periods with the lowest stream flow rates (April 14, May 14a, May 14b), while those samples taken at times with high stream flow rates (June 2013, January 2015) showed the lowest total concentrations (Fig. 2). The water sample taken in June 2013 showed a total concentration of FIP, FIP-d and FIP-s two to three times lower compared to the rest of the samples which could be explained by a strong flood event during this period and a resulting dilution of FIP, FIP-d and FIP-s within the water phase.

FIP was the main component in all samples with concentrations ranging between 0.24–0.92 ng L^{-1}, followed by FIP-s (0.16–0.39 ng L^{-1}) and FIP-d (<LOQ – 0.37 ng L^{-1}). Seasonal trends could be detected for FIP and FIP-d. Fractional distributions of FIP decreased from an average of 71.3% in water samples taken during seasons with low sunshine exposure (Dec. 2013; Dec. 2014; Jan. 2015) to an average of 52.2% in those samples taken during periods of higher sunshine exposure and higher water temperatures (June, July 2013; April, May-a, May-b 2014). At the same time the fraction of FIP-d increased by nearly the same amount (18.6%), which is a plausible result, as FIP-d is formed due to photolysis. Except for the sample influenced by the flood event in June 2013, FIP-d showed similar concentrations (0.28 ± 0.08 ng L^{-1}) during the spring and summer seasons compared to those of FIP-s (0.3 ± 0.07 ng L^{-1}; average of all water samples except sample June 2013), for which no seasonal effect was detected (Figs. 2 and 3, Table S2).

A direct comparison of the measured concentrations of FIP, FIP-d and FIP-s in water with studies from different regions is difficult as the concentrations in water depend on regional conditions such as climate conditions, regional pests and legal regulations which in turn determines the amount of FIP used. However, many water bodies within the United States were monitored for FIP and its transformation products and the reported maximum concentrations of FIP, FIP-d and FIP-s (lower μg L^{-1}–higher ng L^{-1} level) were much higher compared to those of the current study (Gunasekara et al., 2007; Weston and Lydy, 2014; Wu et al., 2015). Different pest pressures (e.g. fire ants and termites) may be one reason for the higher concentrations of FIP, FIP-d and FIP-s in water as in California FIP is not used in agriculture and the found concentrations of FIP, FIP-d and FIP-s in urban streams were related to

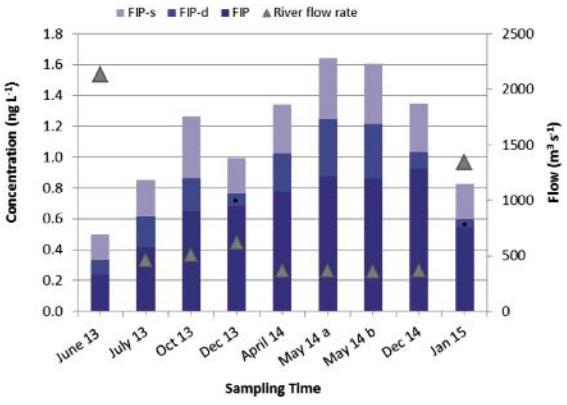

Fig. 2. Absolute concentrations of FIP, FIP-d and FIP-s (ng L^{-1}) in water samples from the river Elbe and related river flow rates (m^3 s^{-1}); •Concentration < LOQ.

Table 1
Toxicity data of FIP, FIP-d and FIP-s for different aquatic organisms.

Species tested	FIP	FIP-d	FIP-s	Toxicity endpoint	Study
					USEPA (1996, 2005)
Daphnia magna	EC_{50} = 190 μg L^{-1}		EC_{50} ~ 28.8 μg L^{-1} (factor 6.6 reported)	Not specified	
Mysid shrimp (estuarine) [a]	EC_{50} = 0.14 μg L^{-1}		LOEC = 5 ng L^{-1}	Survival, reproduction, growth	
Bluegill sunfish (*Lepomis macrochirus*)	LC_{50} (96 h) = 83 μg L^{-1}		LC_{50} (96 h) ~ 83 μg L^{-1} (factor 3.3 reported)	Death	
Rainbow trout (*Oncorhynchus mykis*)	LC_{50} (96 h) = 246 μg L^{-1}		LC_{50} (96 h) ~ 39 μg L^{-1} (factor 6.3 reported)	Death	
					Key et al. (2003)
Adult Grass shrimp (*P. pugio*)	LC_{50} (96 h) = 0.32 μg L^{-1}			Death	
Larvae Grass shrimp (*P.pugio*)	LC_{50} (96 h) = 0.68 μg L^{-1}			Death	
					Konwick et al. (2005)
Ceriodaphnia dubia	LC_{50} (48 h) = 17.7 μg L^{-1}	LC_{50} (48 h) = 355 μg L^{-1}		Death	
					Stark and Vargas (2005)
Daphnia pulex	LC_{50} (48 h) = 15.6 μg L^{-1}			Death	
					Iwafune et al. (2011)
Daphnia magna	EC_{50}(48 h) = 42.9 μg L^{-1}	EC_{50}(48 h) > 9 μg L^{-1}	EC_{50}(48 h) = 5.17 μg L^{-1}	Mobility	
Cheumatopsyche brevilineata	EC_{50}(48 h) = 0.133 μg L^{-1}	EC_{50}(48 h) = 0.1377 μg L^{-1}	EC_{50}(48 h) = 0.066 μg L^{-1}	Mobility	
					Clasen et al. (2012)
Carp (*Cyprinus carpio*)	EC_{tested} (90 d) = 0.65 μg L^{-1} – < LOD within 60 d			Enzyme activity of superoxide dismutase (SOD) and catalase (CAT); Lipid peroxidation	
					Baird et al. (2013)
Fathead minnow (*Pimephales promelas*)	LC_{50} (96 h) = 448.5 μg L^{-1}			Death	
	LC_{50} (7 d) = 208 μg L^{-1}			Death	
					Weston and Lydy (2014)
Chironomus dilutes (most sensitive out of 14 tested freshwater invertebrates)	EC_{50} (96 h) = 0.03–0.035 μg L^{-1}		EC_{50} (96 h) = 0.0075–0.0079 μg L^{-1}	Ability to thrash when prodded	
					Wu et al. (2014)
Juvenil Zebrafish (*Danio rerio*)	LC_{50} (24 h) = 220.4 μg L^{-1}			Death	
	$EC_{min, tested}$ (24 h) = 2 μg L^{-1}			Cytochrome P450 activity	

[a] Not specified.

landscape maintenance and structural pest control (Weston and Lydy, 2014).

The application of FIP as pesticide in agriculture is not permitted in Germany. However, the Federal Office of Consumer Protection and Food Safety (BVL-Germany) is authorized to permit the application of FIP in potato cultivation as curative treatment against wireworms for 120 days (European Parliament and the Council of the European Union, 2009). The permission was granted since 2009. While the sale of FIP used as pesticide within Germany for the years 2013 and 2014 is reported to be less than one ton per year (Federal Office of Consumer Protection and Food Safety, 2014, 2015), no data about the amount of FIP applied or the sites of application are available from the responsible authorities. However, as the river Elbe runs through the Federal State of Lower Saxony (connecting to Hamburg), which is the federal state with the highest harvested quantities of potatoes (>40%) in Germany (Federal Statistical Office of Germany, 2015), soil leaching processes may be one possible source for the detected contaminations of FIP, FIP-d and FIP-s in water.

Furthermore, FIP is registered as a biocide in Germany for domestic use against insects such as ants and moths (e.g. NEXA LOTTE ®) as well as parasites of domestic animals (e.g. FRONTLINE®). A contribution to the found water contaminations (e.g. through residential runoffs) due to these pest-control agents has to be considered as well.

However, in both cases FIP is used mainly during the spring and summer seasons. Due to this seasonal use and its chemical instability the absence of FIP and its transformation products in samples taken in the winter months or at least different patterns of distribution compared to samples taken during the spring and summer seasons would have been expected. Different patterns of distributions were e.g. found for FIP-s in urban residential runoffs (California, USA) with an increasing trend from ~21% in April to ~41% in October (Gan et al., 2012). However, in the current study, the fractions of FIP-s stayed nearly constant in all samples (26.6% ± 3.3%) while the fractions of FIP were always high and ranged between 48.5%–74.8%.

Even if the here presented data do not provide a comprehensive concentration profile of FIP, FIP-d and FIP-s during the whole years, it appears that the water was constantly contaminated with FIP and its two transformation products.

The measured water concentrations of FIP, FIP-d and FIP-s never exceeded the reported effect concentrations (EC) and lethal concentrations (LC) of toxicity studies (Table 1). However, most studies were conducted over a short amount of time and long-time exposure of aquatic

organisms to FIP, FIP-d and FIP-s (as it is assumed for the sampling site) may result in lower EC and/or LC values. Such effect was demonstrated by Baird et al. (2013) who investigated an approx. 2-fold lower LC_{50} due to a prolonged exposure time from 96 h to 7 d of fathead minnow to FIP (Table 1). At sublethal concentrations of 2 μg L^{-1}, FIP increased the amount of the detoxification enzymes cytochrome P450 in gill, liver and muscle of juvenile zebrafish (Wu et al., 2014). Under field conditions, long time exposure (90 d) of carp to decreasing FIP concentrations from 0.65 μg L^{-1} to below LOD (FIP-d and FIP-s were not measured) within 60 d induced significant changes in certain enzymes activities, that are related to oxidative stress, in liver tissue. Furthermore, an increased lipid peroxidation in brain, muscle and liver was detected (Clasen et al., 2012) (Table 1). Based on these results and especially due to the bioaccumulation potential of FIP, FIP-d and FIP-s, similar effects in eels (and other aquatic organisms) may be assumed with the consequence of a higher energy demand due to detoxification processes. These effects would have a special importance for eels, since eels are a migratory species, that need to migrate back several thousand kilometers to their spawning grounds, without feeding, solely relying on their lipid reserves as an energy source.

3.2. Concentrations of FIP, FIP-d and FIP-s in muscle and liver tissue of eels

FIP, FIP-d and FIP-s were measured in muscle and liver tissues of six eels from the river Elbe. Additionally, FIP, FIP-d and FIP-s were determined in further seven eel liver samples. Concentrations in muscle tissue ranged from 0.04–0.32 ng g^{-1} ww for FIP, 0.02–0.13 ng g^{-1} ww for FIP-d and 0.52–11.24 ng g^{-1} ww for FIP-s. In liver samples concentrations of FIP ranged between 0.09–1.96 ng g^{-1} ww and 6.83–44.29 ng g^{-1} ww for FIP-s. FIP-d was not detected in any of the analyzed liver samples. In all cases FIP-s was found to be the dominant species within a sample with ratios of average concentrations for FIP-s and FIP ($c_{FIP\text{-}s}/c_{FIP}$) of 30.6 for muscle tissue and 26.4 for liver samples. Regarding the six muscle-liver pairs, ratios of average concentrations of liver and muscle tissue (c_{liver}/c_{muscle}) were 4.6 for Fipronil and Fipronil-sulfone, respectively, showing that the liver is the major accumulation organ for these substances (Table 2, Fig. 3).

Even though lipid content plays an important role for bioaccumulative pollutants, no correlations between concentrations of FIP, FIP-d and FIP-s and lipid contents (Tables S3 and S4) of muscle and liver samples were found. These results stand in contrast to those of Miranda et al. (2008), who reported a positive correlation between the lipid contents and FIP concentrations of livers in tiger fishes (*Hoplias malabaricus*) from South Brazil. Regarding the six muscle-liver pairs a strong negative Pearson correlation ($R^2 = 0.95$, $p = 0.001$) between the lipid-weight concentrations of FIP-s in liver and the lipid content in muscle tissue were found. This may indicate that the accumulation of FIP-s is linked to the fat metabolism of eels. However, due to the limited sample number further investigations are needed to verify this hypothesis.

In comparison to the current study, FIP concentrations in muscle and liver samples of eels as reported by Ribeiro et al. (2005) (only dry weight concentrations were provided), were much higher (243–335-fold and 23–67-fold, respectively). As the sampling area (Camargue Nature Reserve, France) in Ribeiros study was influenced by rice cultivation in which FIP was an authorized pesticide at that time (Mesléard et al., 2005) it may explain the high concentrations of this compound. Ratios of average FIP concentrations of liver and muscle tissue (c_{liver}/c_{muscle}) were lower compared to those of the current investigation (0.6–1.4 and 7, respectively).

Maximum residue levels (MRLs) for FIP, FIP-d and FIP-s in fish (edible tissue) are not regulated in the European Union. However, MRLs are defined by the European Commission for more than 300 food products as the sum of FIP and FIP-s, and the reported MRLs for products of animal origin range between 0.005–0.09 mg kg^{-1} (European Commission, 2014). In the current study, the sum concentrations of FIP and FIP-s in edible muscle tissues ranged between 0.002–0.01 mg kg^{-1} and may therefore be relevant for food safety assessments.

3.3. PBTK model- linking concentrations of FIP, FIP-d and FIP-s in water to those in eel tissue

The PBTK model (Brinkmann et al., 2015) was used to asses if the measured concentrations of FIP, FIP-d and FIP-s in water samples may explain the measured concentrations of all three analytes in muscle and liver samples of eels. Therefore, we considered two scenarios. In the first scenario we assumed only bioconcentration of FIP, FIP-d and FIP-s due to their uptake via the water phase. The second scenario additionally implied their biotransformation due to metabolic processes. In this context we choose to investigate the pollution of silver eels with FIP, FIP-d and FIP-s as silver eels stop feeding and therefore the influence of contaminated diet is minimized while the uptake of contaminants via the water phase becomes more relevant. Furthermore, the results of Konwick et al. (2006) indicate that FIP and FIP-s are eliminated fast, with half lives of 0.6 d for FIP (mainly due to biotransformation) and 2 d for FIP-s. Eels were caught near the water sampling sight, meaning those concentrations measured in water most likely contributed to the pollution with all three analytes in eel tissues.

In general, total lipid contents in eels were larger in muscle compared to liver tissue. As reflected by the PBTK model, thus chemical concentrations would be expected to be higher in muscle than in liver due to the lipid-based nature of the partitioning process; instead, the contrary was observed in the experimental dataset.

Assuming bioconcentration alone average concentrations of FIP, FIP-d and FIP-s in muscle predicted by the PBTK model deviated 7-, 11- and 17-fold compared to measured values while predicted concentrations for liver tissue deviated 3-, 13- and 253-fold, respectively. FIP and FIP-d would be predicted to be at similar concentrations in muscle and liver samples, and with concentrations exceeding those of FIP-s by factor approx. 4–5 in muscle and approx. 2.5–4 in liver samples. Again, the contrary was observed in field data with FIP-s being the analyte with the highest concentrations in both, muscle and liver samples exceeding those concentrations of FIP and FIP-d by a factor of ~30 and ~57, respectively, in muscle samples and by a factor of ~261 in liver samples compared to FIP concentrations (FIP-d was not detected in liver samples) (Fig. 3).

Table 2
Average concentrations (ng g^{-1} ww, ng g^{-1} lw) of FIP, FIP-d and FIP-s in liver and muscle samples.

	Unit	Liver $n = 13$	Muscle $n = 6$
Fipronil	(ng g^{-1} ww)	0.76 ± 0.54	0.13 ± 0.10
Fipronil	(ng g^{-1} lw)	9.41 ± 9.15	0.49 ± 0.38
Fipronil-desulfinyl	(ng g^{-1} ww)	n.d.	0.07 ± 0.04
Fipronil-desulfinyl	(ng g^{-1} lw)	n.d.	0.25 ± 0.14
Fipronil-sulfone	(ng g^{-1} ww)	19.91 ± 9.96	4.05 ± 3.73
Fipronil-sulfone	(ng g^{-1} lw)	238.66 ± 137.73	14.17 ± 11.83

n.d., not detected; lw, lipid weight.

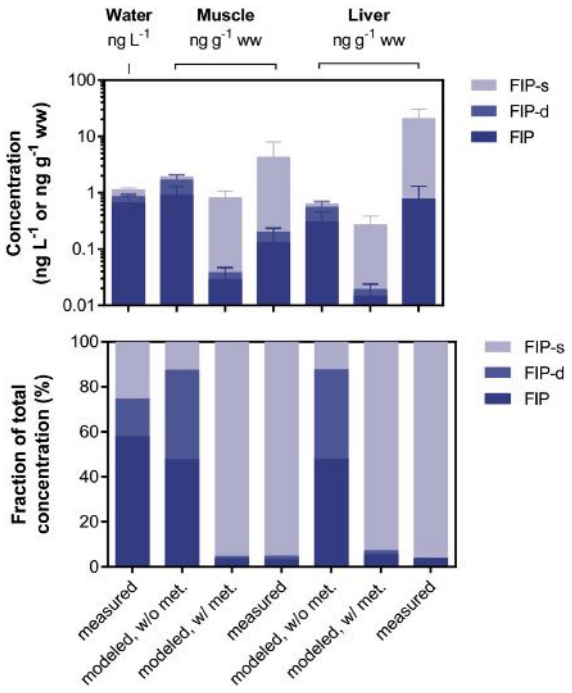

Fig. 3. Absolute (top, ng L^{-1}/ng g^{-1} ww) and relative (bottom, fraction of total, %) concentrations of Fipronil (FIP), Fipronil-desulfinyl (FIP-d) Fipronil-sulfone (FIP-s) in water, as well as eel muscle and liver (both measured and modeled with and without metabolism, respectively). Bars represent mean values of $n = 9$ water samples, $n = 6$ muscle and $n = 13$ liver samples (experimental dataset), as well as 1000 Monte Carlo simulations for the model predictions. Error bars represent the standard deviation.

Assuming bioconcentration together with metabolism, which can be considered to be more realistic, concentrations of FIP, FIP-d and FIP-s in muscle tissue predicted by the PBTK model were slightly underestimated (4-, 8- and 5-fold, respectively) and were comparable to the measured concentrations. The concentrations of FIP and FIP-s in liver tissue were underestimated (52-,and 80-fold, respectively) and their high concentrations in liver tissue could not be explained by the PBTK model. While FIP-d was not detected in liver samples, the PBTK model predicted a value of 0.005 ng g^{-1} ww (value below LOD). Yet, the predicted FIP concentrations in liver tissue were more precise for assuming bioconcentration alone. However, in contrast to the results of the PBTK model without considering metabolism, the relative contributions of FIP, FIP-d and FIP-s to the total concentrations of all FIP analogues was accurately reflected by the model predictions for both, muscle and liver tissue (Fig. 3).

Regarding the average concentrations of FIP, FIP-d and FIP-s in muscle and liver tissue predicted by the PBTK model including metabolism, their measured concentrations in water can explain for the most part the average concentrations measured in muscle samples as well as the concentrations below LOD of FIP-d in liver tissue. Furthermore, predominance of FIP-s over FIP and FIP-d in muscle and liver tissue can be explained by the rapid biotransformation of FIP to FIP-s rather than by accumulation of FIP-s from the water alone.

However, different parameters such as exposure via contaminated food (prior silver stage), exposure to higher concentrations of FIP, FIP-d and FIP-s in water from different sites of the river Elbe or sediment-bound FIP and its transformation products were not included into the PBTK model and it may explain why the measured concentrations of FIP, FIP-d and FIP-s in eels varied widely among each other and why the PBTK model including metabolism underestimated the concentrations of FIP, FIP-d and FIP-s in muscle and liver tissues. Moreover, the assumed elimination rates of FIP, FIP-d and FIP-s may differ from those assumed, especially for FIP and FIP-s in liver tissue as elimination rates may be reduced, e.g. due to the compounds binding-affinity to plasma proteins (Lacroix et al., 2010).

4. Conclusions

The results of this study show that the presence of FIP, FIP-d and FIP-s may be of concern for the aquatic environment especially due to their bioaccumulation potential and the observed predominance of the transformation product FIP-s in muscle and liver tissues of eels, which is supposed to be more toxic than the parent compound. The distribution of FIP, FIP-d and FIP-s in muscle and liver samples could be explained by the used PBTK model but not their absolute concentrations in liver tissue of eels. Thus, further investigations regarding the behavior of FIP in the aquatic environment are needed. The measured concentrations of FIP and FIP-s in muscle tissues were in the range of MRLs, defined by the European Commission for products of animal origin, and may therefore be relevant for food safety assessments.

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Appendices A and B

Appendices A and B to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2016.05.210.

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Supplementary Material

Appendix A

Fipronil and two of its transformation products in water and European eel from the river Elbe

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S1. Water samples -storage

All water samples were filtered and extracted (SPE) immediately after sampling.

Water samples taken in 2014/2015 (April 14, May a 14, May b 14, Dec 14, Jan 15):

The dried SPE columns were stored in the dark at 3°C in an air-tight container to a maximum of three days. After their elution, the resulting extracts were immediately analyzed. Measured concentrations for FIP, FIP-d and FIP-s never differed more than 15 % within one set of duplicate samples.

Water samples taken in 2013 (June 13, July 13, Oct 13, Dec 13):

The samples were disposable reference water samples obtained by the Federal Maritime and Hydrographic Agency. Duplicate samples were available in terms of one extract that was stored in the dark at 3°C in a crimp-sealed vial, while the respective duplicate sample were stored at 3°C in the dark in an air-tight container as dried SPE column. SPE columns were eluted as described in Material and Methods and afterwards both extracts were analyzed. Measured concentrations for FIP, FIP-d and FIP-s never differed more than 15 % within one set of duplicate samples. Measured concentrations of FIP, FIP-d and FIP-s and their fractional distributions were in accordance with those concentrations and fractional distributions measured in samples taken in 2014/2015, showing that the influence of storage was negligible.

S2. LC-MS/MS analysis

The internal standard mix used for quantification contained 27 deuterated or ^{13}C-labeled substances. In general, this standard mix was used within the monitoring program of the Federal Maritime and Hydrographic Agency (BSH) for water samples of the river Elbe, North Sea and Baltic Sea. Based on this standard mix we developed our method for the quantification of FIP, FIP-d and FIP-s to obtain the possibility to analyse even retained monitoring samples. While the component ^{13}C-labeld PFOS was well suited as internal standard in analyses of three analytes in eel tissues in terms of recovery, relative recovery and retention time, its use in Elbe water analyses resulted in an overestimation of FIP, FIP-d and FIP-s concentrations, mainly due to its poor recovery (< 75 %). Therefore we chose ^{13}C-PFOA as internal standard for the quantification of all three analytes in Elbe water.

S3. Sampling and biometric parameters of eels

Table S1 Biological data and additional sampling details of eel samples used in this study. Life history stages were determined according to the method described by Durif (2005). Sex was macroscopically confirmed.

Sample number	River	Location	Time	Mass (g)	Length (cm)	Liver (g)	Stage (s.i.)	Sex
2401	Elbe	Hoopte	November 2013	1182	82	19.10	4.00	f
2402	Elbe	Hoopte	November 2013	1428	88	21.05	4.00	f
2403	Elbe	Hoopte	November 2013	1128	84	21.90	4.00	f
2406	Elbe	Hoopte	November 2013	884	84	14.09	5.00	f
2409	Elbe	Hoopte	November 2013	531	67	9.82	5.00	f
2410	Elbe	Hoopte	November 2013	460	65	8.78	5.00	f
2411	Elbe	Hoopte	November 2013	1210	90	24.54	4.00	f
2412	Elbe	Hoopte	November 2013	695	74	10.32	5.00	f
2413	Elbe	Hoopte	November 2013	990	82	18.69	4.00	f
2414	Elbe	Hoopte	November 2013	595	71	8.32	5.00	f
3470	Elbe	Winsen	Nov 2014	644	64	6.84	5	f
3471	Elbe	Winsen	Nov 2014	571	64	5.57	5	f
3472	Elbe	Winsen	Nov 2014	482	63	5.06	5	f

S4. Concentrations of FiP, FIP-d and FIP-s in water samples and eel tissue/Lipid content of eels

Table S2 Concentrations of FIP, FIP-d and FIP-s in water samples of the river Elbe; n.d. = not detected

Date	FIP [ng/L]	Blind FIP [ng/L]	FIP-d [ng/L]	Blind FIP-d [ng/L]	FIP-s [ng/L]	Blind FIP-s [ng/L]
June 13	0.239	n.d.	0.095	n.d.	0.164	n.d.
July 13	0.414	n.d.	0.200	n.d.	0.240	n.d.
Oct 13	0.650	0.052	0.213	n.d.	0.400	0.078
Dec 13	0.685	n.d.	0.077	n.d.	0.231	n.d.
April 14	0.771	n.d.	0.255	n.d.	0.316	n.d.
May 14 a	0.878	n.d.	0.373	n.d.	0.391	n.d.
May 14 b	0.861	n.d.	0.353	n.d.	0.395	n.d.
Dec 14	0.925	n.d.	0.108	n.d.	0.317	n.d.
Jan 15	0.550	n.d.	0.045	n.d.	0.231	n.d.

Table S3 Concentrations of FIP, FIP-d and FIP-s in liver (L) and muscle (M) samples; dw=dry weight; ww=wet weight; lw=lipid weight; n.d. = not detected

	Fipronil			Fipronil-sulfone			Fipronil-desulfinyl		
Sample	Conc. [ng/g] dw	Conc. [ng/g] ww	Conc. [ng/g] lw	Conc. [ng/g] dw	Conc. [ng/g] ww	Conc. [ng/g] lw	Conc. [ng/g] dw	Conc. [ng/g] ww	Conc. [ng/g] lw
L2401	3.715	1.058	12.089	53.430	15.214	173.858	n.d.	n.d.	n.d.
L2402	0.467	0.163	1.127	49.471	17.301	119.390	n.d.	n.d.	n.d.
L2403	3.649	1.193	8.002	57.605	18.832	126.345	n.d.	n.d.	n.d.
L2406	8.294	1.961	36.706	75.294	17.801	333.215	n.d.	n.d.	n.d.
L2409	1.420	0.376	5.874	49.527	13.111	204.850	n.d.	n.d.	n.d.
L2410	4.603	1.368	14.585	73.563	21.859	233.072	n.d.	n.d.	n.d.
L2411	2.958	0.900	9.201	77.087	23.457	239.768	n.d.	n.d.	n.d.
L2412	1.760	0.465	6.273	76.000	20.087	270.864	n.d.	n.d.	n.d.
L2413	0.376	0.093	1.538	39.332	9.737	160.999	n.d.	n.d.	n.d.
L2414	1.217	0.433	3.541	96.620	34.369	281.222	n.d.	n.d.	n.d.
L3470	0.840	0.221	3.563	60.600	15.948	257.027	n.d.	n.d.	n.d.
L3471	2.533	0.742	10.501	151.154	44.286	626.712	n.d.	n.d.	n.d.
L3472	2.874	0.844	9.309	23.245	6.828	75.289	n.d.	n.d.	n.d.
M2409	0.279	0.129	0.437	9.202	4.251	14.427	0.287	0.132	0.449
M2410	0.258	0.113	0.414	7.643	3.347	12.294	0.201	0.088	0.323
M2413	0.088	0.038	0.139	5.473	2.369	8.644	0.204	0.088	0.322
M3470	0.747	0.323	1.219	5.912	2.554	9.639	0.081	0.035	0.133
M3471	0.144	0.049	0.280	1.551	0.523	3.009	0.050	0.017	0.096
M3472	0.268	0.142	0.466	21.281	11.238	36.977	0.089	0.047	0.154

Table S4 Lipid content of liver and muscle samples; n.m. =not measured

Sample number	Lipid % (liver)	Lipid % (muscle)
2401	8.75	n.m.
2402	14.49	n.m.
2403	14.91	n.m.
2406	5.34	n.m.
2409	6.40	29.5
2410	9.38	27.2
2411	9.78	n.m.
2412	7.42	n.m.
2413	6.05	27.4
2414	12.22	26.5
3470	6.205	17.4
3471	7.066	30.4
3472	9.069	n.m.

Appendix B

PBTK model

Fipronil and two of its transformation products in water and European eel from the river Elbe

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Total page number: 5, including

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Table B.1 Model inputs and parameters of the PBTK models for European eel (*Anguilla anguilla*). Based on Stadnicka et al. (2012). Adapted from Brinkmann et al. (2015). Asterisks indicate values that were used for random generation of parameter/ variable sets from the statistical (Gaussian) distributions defined by the measured data during each model run (Monte Carlo simulation).

Symbol	Units	Description	Value
W	kg	Body wet weight	0.83 ± 0.32*
log K_{ow}	-	Octanol-water partitioning coefficient	FIP: 4.01 FIP-s: 3.68 FIP-d: 4.63
C_{insp}	ng L^{-1}	Chemical concentration in inspired water	FIP: 0.66 ± 0.23* FIP-s: 0.29 ± 0.08* FIP-d: 0.18 ± 0.14*
T	°C	Water temperature	14.3 ± 7.7*
C_{ox}	mg L^{-1}	Dissolved oxygen concentration in inspired water	Saturation assumed
P_{bw}	-	Blood:water partitioning coefficient	Eq. B.1
P_l,P_f,P_m	-	Liver/fat/muscle:blood partitioning coefficient	Eq. B.2
P_k	-	Kidney:blood partitioning coefficient	Eq. B.3
P_r	-	Richly perfused tissue:blood partitioning coefficient	P_l
A_i	µg	Chemical amount in fat, poorly and richly perfused tissues	Eq. B.4
A_l	µg	Chemical amount in the liver compartment	Eq. B.5
A_k	µg	Chemical amount in the kidney compartment	Eq. B.6
C_{int}	µg g^{-1}	Internal concentration in the whole fish	Eq. B.7
C_{art}	µg L^{-1}	Chemical concentration in arterial blood	Eq. B.8
C_{ven}	µg L^{-1}	Chemical concentration in venous blood	Eq. B.9

Table B.2: Physiological parameters (and corresponding symbols) used in the physiologically based toxicokinetic (PBTK) model for European eels (*Anguilla anguilla*). Adapted from Brinkmann et al. (2015). Asterisks indicate values that were used for random generation of parameter/ variable sets from the statistical (Gaussian) distributions defined by the measured data during each model run (Monte Carlo simulation).

Physiological parameter	**Symbol**	**Unit**	**Value**
Cardiac output	Q_c	L kg^{-1} h^{-1}	Eq. B.10
Oxygen consumption rate	VO_2	mg kg^{-1} h^{-1}	Eq. B.11
Effective respiratory volume	Q_w	L kg^{-1} h^{-1}	Eq. B.12
Arterial blood flow to different tissues			
Liver	Q_l	L h^{-1}	1.90% of Q_c
Fat	Q_f	L h^{-1}	12.20% of Q_c
Poorly perfused tissues[1]	Q_m	L h^{-1}	64.30% of Q_c
Richly perfused tissues[2]	Q_r	L h^{-1}	20.20% of Q_c
Kidney	Q_k	L h^{-1}	1.40% of Q_c
Organ/ tissue group volumes (fraction of W)[3]			
Liver	V_l	L	1.50% of W
Fat	V_f	L	4.20% of W
Poorly perfused tissues[1]	V_m	L	87.00% of W
Richly perfused tissues[2]	V_r	L	6.30% of W
Kidney	V_k	L	1.00% of W
Organ/ tissue total lipid content (fraction w.w.)			
Liver	α_l	-	9.01 ± 3.15%*
Fat	α_f	-	68.10%
Poorly perfused tissues	α_m	-	26.40 ± 4.65%*
Kidney	α_k	-	5.30%

[1] *mainly white muscle*

[2] *viscera, spleen, gonads, and gills*

[3] *all tissues were assumed to have a specific gravity of 1.0 w.w., wet weight*

Model equations, based on Stadnicka et al. (2012)

Blood:water partitioning coefficient

$$P_{bw} = 10^{0.72 \cdot \log Kow + 1.04 \cdot log(\alpha_b) + 0.86} + \gamma_b \quad \textbf{(Eq. B.1)}$$

Liver/fat/muscle:blood partitioning coefficient

$$P_{l,f,m} = \frac{10^{0.72 \cdot \log Kow + 1.04 \cdot log(\alpha_{l,f,m}) + 0.86} + \gamma_{l,f,m}}{P_{bw}} \quad \textbf{(Eq. B.2)}$$

Kidney:blood partitioning coefficient

$$P_k = \frac{10^{0.72 \cdot \log Kow + 1.04 \cdot log(\alpha_k) + 0.86} + \gamma_k}{P_{bw}} \quad \textbf{(Eq. B.3)}$$

Chemical amount in fat, poorly and richly perfused tissues

$$\frac{dA_i(t)}{dt} = Q_i \cdot \left(C_{art}(t) - \frac{A_i(t)}{V_i \cdot P_i}\right) \quad \textbf{(Eq. B.4)}$$

Chemical amount in the liver compartment

$$\frac{dA_l(t)}{dt} = Q_r \cdot \frac{A_r(t)}{V_r \cdot P_r} + Q_l \cdot C_{art}(t) - (Q_r + Q_l) \cdot \frac{A_l(t)}{V_l \cdot P_l} \quad \textbf{(Eq. B.5)}$$

Chemical amount in the kidney compartment

$$\frac{dA_k(t)}{dt} = 0.6 \cdot Q_m \cdot \frac{A_m(t)}{V_m \cdot P_m} + Q_k \cdot C_{art}(t) - (0.6 \cdot Q_m + Q_k) \cdot \frac{A_k(t)}{V_k \cdot P_k} \quad \textbf{(Eq. B.6)}$$

Internal chemical concentration in the whole fish

$$C_{int}(t) = \frac{A_f(t) + A_m(t) + A_r(t) + A_l(t) + A_k(t)}{1000 \cdot W} \quad \textbf{(Eq. B.7)}$$

Chemical concentration in arterial blood

$$C_{art}(t) = \min(Q_w, Q_c \cdot P_{bw}) \cdot C_w - \frac{C_{ven}(t)}{P_{bw}} \cdot \frac{1}{Q_c} + C_{ven}(t) \quad \textbf{(Eq. B.8)}$$

Chemical concentration in venous blood

$$C_{ven}(t) = \left(Q_f \cdot \frac{A_f(t)}{V_f \cdot P_f} + 0.4 \cdot Q_m \cdot \frac{A_m(t)}{V_m \cdot P_m} + (0.6 \cdot Q_m + Q_k) \cdot \frac{A_k(t)}{V_k \cdot P_k} + (Q_r + Q_i) \cdot \frac{A_l(t)}{V_l \cdot P_l}\right) \cdot \frac{1}{Q_c}$$

(Eq. B.9)

Cardiac output

$$Q_c = 0.366 \cdot W^{-0.25}$$ **(Eq. B.10)**

Oxygen consumption rate

$$\frac{1}{VO_2} = -9.11 \cdot 10^{-3} + 1.95 \cdot 10^{-2} \cdot W^{0.6} + \frac{3.45 \cdot 10^{-1}}{T}$$ **(Eq. B.11)**

Effective respiratory volume

$$Q_W = \frac{0.875 \cdot VO_2}{0.8 \cdot C_{OC}} \cdot W^{0.75}$$ **(Eq. B.12)**

References

Brinkmann, Markus; Freese, Marko; Pohlmann, Jan-Dag; Kammann, Ulrike; Preuss, Thomas G.; Buchinger, Sebastian et al. (2015): A physiologically based toxicokinetic (PBTK) model for moderately hydrophobic organic chemicals in the European eel (*Anguilla anguilla*). *Sci Tot Environ* 536, 279–287.

Stadnicka, J., Schirmer, K., Ashauer, R., 2012. Predicting concentrations of organic chemicals in fish by using toxicokinetic models. *Environ Sci Technol* 46, 3273-3280.

GENERAL DISCUSSION

The present thesis addresses the role of chemical pollution during the continental growth phase of freshwater eels as a detrimental factor for the health and reproductive success of affected individuals in light of current management goals and recovery approaches. In a first chapter (CHAPTER I) it was investigated how different continental life history stages of *Anguilla anguilla* are affected by dioxin-like compounds and whether exposure in separate aquatic habitats determines the contaminant load of eels. Two further chapters focus on if and to what extent incorporated amounts of selected POPs, namely DLCs (CHAPTER II) and halogenated flame retardants (CHAPTER III), are being passed on to the offspring of contaminated fishes during sexual maturation with the goal of assessing how this may impact their reproductive success. Another chapter formed by an interdisciplinary study on physiological processes involved in the body changes that eels undergo during maturation, revealed how these transformations can induce adverse side effects caused by pollution after onset of migration (CHAPTER IV).

In order to gain a better understanding of the bioaccumulation and distribution processes of organic substances within the body of affected eels, a first PBTK model for organic chemicals in Anguillids was created in another chapter (CHAPTER V). These models facilitate powerful in-vitro research tools to predict and understand bioconcentrations of organic chemicals at any time of exposure. In a follow-up study (CHAPTER VI), this model was successfully used to resolve whether water concentrations of the insecticide Fipronil and its metabolites in a German river were causative for their fractional concentrations found in muscle and liver samples of silver eels caught in the same area. With still many remaining uncertainties and unanswered questions, the studies included in this thesis contribute largely to the current state of knowledge on how eels incorporate pollutants during their continental life. This work further provides empirical evidence and estimates on possible risks for the reproduction success of eel populations. As a result, the new knowledge resulting from these findings can finally help to include chemical pollution as a recognized threat for the species and contribute to attempts developing better strategies for stock management and recovery approaches of this endangered species.

GENERAL DISCUSSION

The specialized biology of anguillid eels as benthic opportunistic predators with large amounts of body fat makes them explicitly susceptible to contamination by environmental pollution (Belpaire & Goemans 2007; ANNEX II; CHAPTER I; Pannetier *et al.* 2016). Accumulated contaminant concentrations may vary substantially depending on location and life stage of an individual, as incorporated types and amounts of chemicals depend on the local sources for contaminants (ANNEX V). As shown in Chapter I and Appendix I, young life history stages such as glass eels and elvers show still comparably low body burdens of lipophilic POPs. Yet, as suggested before by Belpaire *et al.* (2011a, 2011b; ANNEX V) yellow eels can be suitable bio-indicators for monitoring sources, composition and distribution of certain metals and chemical pollutants (de Boer & Hagel 1994; ANNEX I; Byer *et al.* 2013a; 2013b; Pannetier *et al.* 2016). This is due to the fact, that yellow eels during their feeding and growth phase continuously accumulate these compounds over time until they reach a final stage of contamination at the silver eel stage, as they stop feeding and leave the polluted continental habitats. This clarified how the growth habitat affects the fishes' final body burden before their onset to spawning migration (CHAPTER I).

It is known from several other studies that this is not only the case for lipophilic POPs but also for metals, insecticides and other contaminants. Different from most other fishes though, semelparous eels do not regularly reduce incorporated contaminants by releasing gametes during repeated spawning. As a result and adding to their high body lipid levels, their accumulated body burdens of different lipophilic contaminants are usually much higher than those of other species in the same habitats (Bodin *et al.* 2014). While these stored contaminants are inactive and inert as long as the lipid reserves remain unused, the chemicals are thought to be released into the bloodstream during migration. This is when they potentially enfold detrimental effects on the lipid metabolism or when they are started being transferred to the reproductive organs (Geeraerts & Belpaire 2010; Belpaire *et al.* 2016; ANNEX V). The study in Chapter II on the maternal transfer of dioxin-like compounds clarified how the body burden of a mature silver eel is decisive for the amount of chemicals transferred to the ovaries during migration. This release of lipophilic contaminants from the muscular lipid stores via the bloodstream, as well as the conveyance of metals to oocytes by vitellogenin, represent a risk and may affect gametogenesis and eventually larval development and survival (van den Thillart *et al.* 2007; Pierron *et al.* 2008; Belpaire *et al.* 2009; Chapter II; Chapter IV).

In order to utilize the gained knowledge from maternal transfer rates of artificially matured eels, chapter II eventually presented a tentative approach in order to estimate expected concentrations in eggs of a silver eel by using its body concentration of DLCs. However, results from this modelled approach haved to be viewed at with caution since the complex biology of eels makes it very difficult to simulate the natural reproductive cycle in an experimental design. Eels in this study did not swim the entire migration distance as they would have under natural conditions. It has to be expected that the addition of energy metabolism (and thus lipid consumption) during migration would result in further concentration of lipophilic contaminants in the body and ultimately to higher amounts found in eggs of the respective individuals.

The physiological changes that eels are expected to undergo during migration include the consumption of their own body mass, which apparently happens in a reciprocal interaction between lipid consumption and bone resorption (Chapter IV). The break-down of their lipid-rich muscle tissue is necessary in order to fuel the energetic demands of their distant spawning migration while the resorption of their skeletons is thought to provide the necessary amounts of phosphorus and calcium needed for gonadogenesis (Chapter IV). Results from this study underlined the importance of learning more about the still little understood last parts in the life history of eels (migration and spawning). New knowledge about what exactly happens in the fish's body during this phase helps to better understand how life long contamination really enfolds its detrimental impact on the physiology and spawning success of contaminated eels. In Brinkmann, Freese & Pohlmann et *al.* 2015 (Chapter V) we demonstrated how the kinetics of hydrophobic chemicals in different body compartments of eels can be modelled based on simple factors such as lipid content, time and blood flow / heart rate. Models like these are of great value in order to create a better understanding of the origin and fate of pollutants with distinct chemical properties in the body of eels. In Michel *et al.* 2016 (Chapter VI) we used our own PBTK model and managed to indicate that found amounts of (Fipronil), a metabolizable insecticide in eels from a German waterbody, did not correspond with found water concentrations, and thus most likely must have originated from food sources.

Contributing factors to the decline

The dramatic stock declines of the European, American and the Japanese eel began between the early 1970s and the 1980s, and still challenge the minds of researchers in the field. A number of different causes have been identified since, yet it is still not clear in which proportion these different impacts have contributed to the situation. Chemical

pollution of our environment has been an issue for longer than a century even though it has massively expanded and accelerated after the onset of industrialization and even more after the second world war. Today, pollution in its various facets is considered as one of the world's greatest problems (Rockström *et al.* 2009b). Global production and application of various chemicals, including chlorinated persistent organic chemicals such as PCBs, had their production peak in the late 1960s and were still industrially produced until 1993 (Breivik *et al.* 2002). Today, sediment concentrations as well as tissue concentrations of these legacy chemicals (e.g. lead, HCB, DDT or PCBs) found in biota and more specifically in eels have declined and slowly continue to decrease (de Boer *et al.* 2010; Geeraerts *et al.* 2011; ANNEX IV; Byer *et al.* 2015). However, to a certain extent some of these now forbidden halogenated compounds (Chapter I; Appendix X) have simply been replaced by emerging chemicals with similar physico-chemical features and thus applicability (Chapter III). Some of these substitutes (e.g. brominated and fluorinated compounds) are still unregulated, poorly understood and environmental concentrations and effect data are insufficient or simply unavailable, even though some of them have already been shown to also exhibit toxic and endocrine-disrupting characteristics (ANNEX II).

Almost four decades ago, Brian Knights reviewed contamination levels of anguillid eels by organochlorine pesticides and PCBs and conducted what is assumingly the first comprehensive inter-regional risk assessment approach (Knights 1997). In this work, even though he stated that cause-effect relationships and critical loadings are unclear, Knights concluded that contamination has not been a major cause of recent declines in eel recruitment. He argued that there was a temporal mismatch between the timing of major pollution and declines, as massive organochlorine pollution occurred in St Lawrence and Lake Ontario in the 1950 and recruitment declines did not occur until the late 1970s. The rationale of this conclusion can be seen as a bit incomprehensible, as he did not further consider the prolonged generation times of eels nor the non-existent interrelation of local escapement and recruitment due to the panmictic reproduction strategy. The reproduction biology of freshwater eels is comparably long in duration, with adults needing 8 to more than 16 years in order to fulfill their continental growth phase in addition to a several months long spawning migration and the assumed 1-3-year duration of larval drift before recruitment (Westerberg *et al.* 2018). As a result, the time of global production peaks of PCBs correspond fairly well with the timing of the global eel decline as a decreased reproduction of a population would only be visible 10-20 years

after the exposure of the animals to the toxic pollution (Figure 4). In summary, the peak production period along with the introduction of halogenated POPs and emerging contaminants displays a time-frame that would largely correspond to the timing of the steep recruitment declines of anguillid eel stocks around the globe.

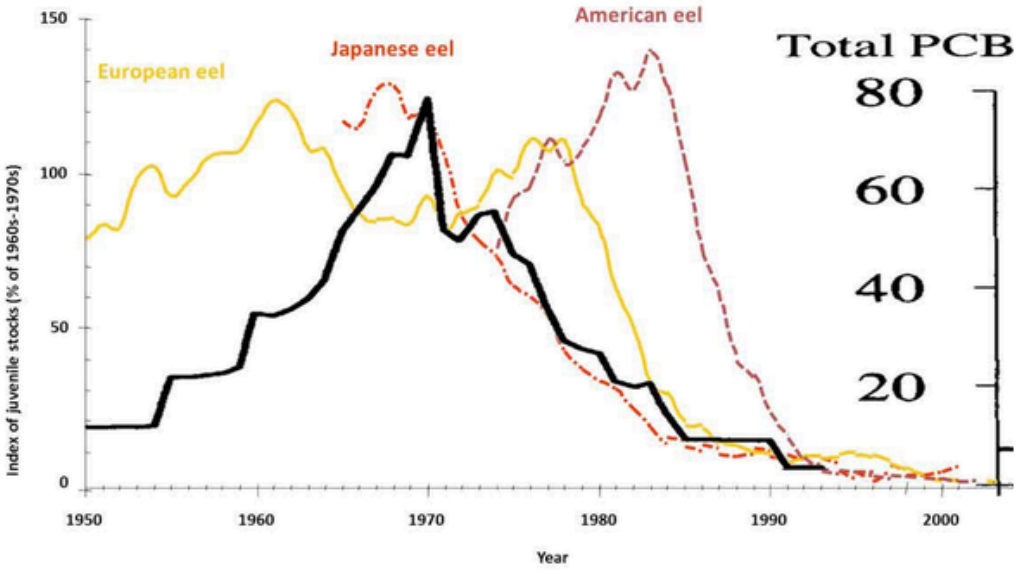

Fig. 4 Recruitment declines of *Anguilla anguilla* (yellow), *Anguilla japonica* (orange) and *Anguilla rostrata* (red) (y-axis) as well as an estimated temporal trend (black) in the global production of total PCBs (z-axis). (Modified after Dekker, 2004, PCB data from Breivik *et al.* 2002).

It has to be kept in mind though, that this timing (1970-1990s) was concurrent with the presumed peak effectiveness of many other contributing factors that were recognized as detrimental to the stock situation. The European eel is regarded as a fish species with exceptional important commercial value in many regions of the world (Violi *et al.* 2015). Besides targeted fisheries and utilization of all continental life stages from glass eels to migrating silver eels as fresh or processed fish, the industry surrounding eel fisheries also includes the trade of live eels as seedstock for eelfarms or for restocking measures in waters with low natural recruitment. This trade of live seedstock became a globally connected problem, when the stock situation also for Japanese eels showed a massive shortage in supply of wild-caught juvenile Japanese eels in the 1990s. Asian eel farms then also shifted to European and American eels (Crook 2010; Crook and Nakamura 2013), which led to an exceptional increase of the market price of European caught glass eels (Anonymous 2007; Stein *et al.* 2016). As a result, the eel was listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) and export out of Europe has been forbidden since 2009 (Jacoby & Gollock 2014).

Due to the prevailing high consumer demand and the comparably high prices that can be achieved with glass eel trade, these measures have never put an end to the glass eel trade with Asia, as export from non-EU countries is allegedly ongoing and a black market for extensive illegal trade out of Europe has emerged (Crook *et al.* 2010; Stein *et al.* 2016).

Another factor that has negatively affected many fish species and the aquatic fauna in river systems was the upgrowth and development of alternative energy sources worldwide. The best proxy for the growth of the hydropower industry assumingly is the total installed capacity of dams, that has increased steadily since the beginning of last century. The increase of installed hydropower capacity and thus numbers of hydropower plants in total did also intensely increase in the first half of the twentieth century, which would also correspond well with the timing of observed decline in eel recruitment (Figure 5). Hydropower plants pose an obstacle in the continental migration of eels and without appropriate implementation of fish passes or fish ladders cause high mortalities in eels, that try to pass the respective damn through the turbines (McCleave 2001; Pedersen *et al.* 2012; Piper *et al.* 2013).

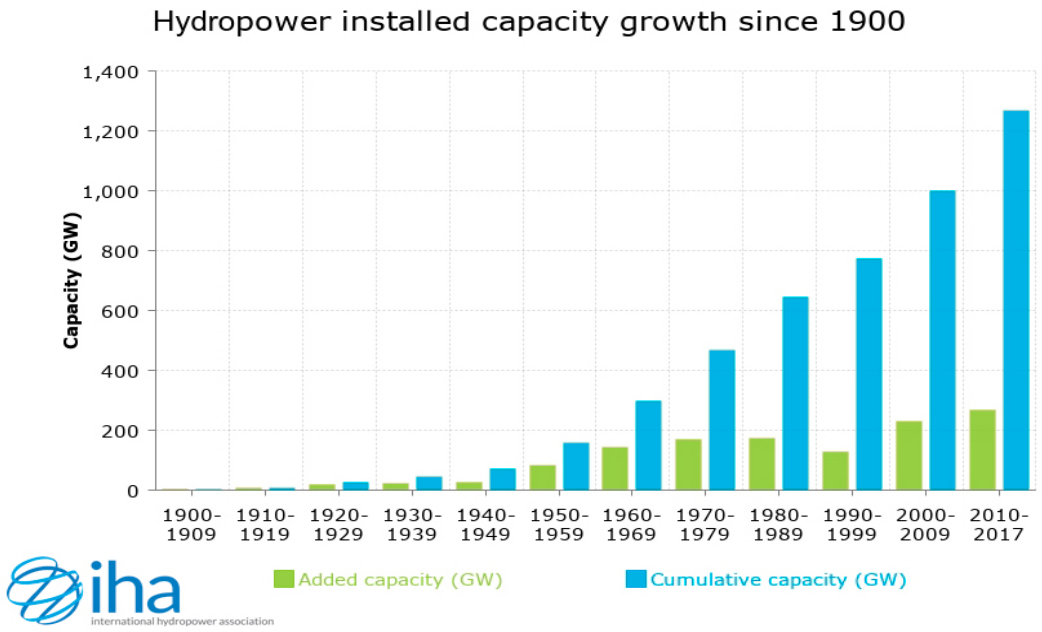

Fig. 5 Historical worldwide increase of hydropower installed capacity growth since 1900. (Source: IHA international hydropower association).

Some of the pioneering research on the effects of hydropower on mortality rates in European eel populations were done by Von Raben (1955, 1957) and Berg (1968). In their papers, the authors estimated that the average mortality caused by turbines in hydropower plants in a local eel population could range between 15 and 38% depending

on number and design of plants. Estimates reported by the expert working group on eels (ICES 2017) suggest that hydropower mortality accounts for more than 50% of all quantifiable anthropogenic mortality in more than half of 62 Eel management units, where data for fisheries and hydropower were reported.

The accidental introduction of the invasive swim bladder parasite *Anguillicola crassus* to Europe in the early 1980s is another stressor that made its first appearance in Atlantic eel stocks during the time of steep decline. The presence of this alien parasite has led to high infection rates of yellow and silver eels throughout the natural distribution range of the European eel (Kennedy & Fitch, 1990; Maamouri *et al.* 1999; Lefebvre *et al.* 2002; Kirk 2003; Drouineau *et al.* 2018). Even though reported in lesser magnitude, the parasite has also established populations in North America affecting *Anguilla rostrata* shortly after its introduction into Europe (Kirk 2003; Machut & Limburg 2008; Drouineau *et al.* 2018). In *A. anguilla* and in *A. rostrata* it was shown, that *A. crassus* infection can severely damage the swim bladder (Würtz *et al.* 1996; Würtz & Taraschewski 2000) and induce a number of physiological stress responses that may lead to increased metabolic rate (Sures *et al.* 2001, Gollock *et al.* 2005). Given the fact that maturation in anguillid eels leads to a significant increase in rate of gas deposition and thus improvement in swim bladder function (Kleckner, 1980, Righton *et al.* 2012), it seems self-evident that the swim bladder function is vital for the spawning migration. As a result, high infestation rates that result in damage and impaired functioning of the organ will have restricting impact on the migratory capability and thus potentially the reproductive success of strongly affected individuals. However, even though infestations rates in the distribution range of the European and the American eel can reach very high rates today, this stressor is unlikely most responsible for the steep recruitment decline of all three aforementioned anguillid species during the 1970s. Reasons for this unlikeliness are, that the stock decline in *Anguilla anguilla* was recognizable already before the introduction of *A.* crassus, and that the parasite has its origin in the distribution range of *A. japonica*. As a result, the Japanese eel has co-evolved with this parasite for a long time and thus is more resistant and shows less severe immune reactions to swim bladder damage caused by *A. crassus* infection.

Climate change scenarios such as changing ocean currents that could inhibit successful larval transport to the growth areas as well as match/mismatch hypotheses on how changing sea temperatures may spatially and/or seasonally change the food webs and thus impact the food availability of eel larvae in the nursery areas could have also had impact on the stock (Friedland *et al.* 2007; Bonhommeau *et al.* 2008). As these impacts were also

most likely effective during the same period of time even these hypotheses seem appropriate and comprehensible. These hypotheses, however, were explicitly postulated for the Sargasso Sea and not for the Marianna Sea, the spawning area of the Japanese eel. Consequently, it is not entirely clear how these hypotheses can be accounted for the simultaneous decline of all three species. Also, these changes in the spawning grounds and their impact on larval survival are complicated and thus difficult to verify and they do not rule out any of other presented influential effects for the declining stock. As a result, all of the direct anthropogenic pressures must be considered. It even seems likely that the declines of the eel stocks have suffered and collapsed under addition of synchronistic pressures that could even have interactively affected one another and in the end, led to the phenomenon in temperate eel species in different parts of the world's ocean.

Current management and actions

Alarmed by the vast decline in recruitment of the European eel, the European Council (EC) issued a recovery strategy under Regulation No. 110/2007 in September 2007. This regulation is broadly known as the Eel Management Plans (EMPs), and obligates member states to install measures to increase and stabilize the number of escaping silver eels out of their water bodies in order to secure reproduction and recruitment of the species. After now 8 years of applied actions of the management plans, glass eel recruitment numbers have stabilized at a low level and show slight increase since 2011 (ICES WGEEL 2019). Nevertheless, the abundance of eels at all life stages remains very low and a distinct recovery of the stock has not been achieved to date. It has to be kept in mind that the long and complicated life cycle and semelparity of anguillid eels prevent applied management measures to show clear effects between 10-20 years after first being installed anyways (Anonymous 2012).

Albeit, efforts and measures for an improved management need to be maintained and developed further until achievement of a sustainable stock status inside safe biological limits. Among the officially proposed measures for EU member states to achieve targeted numbers of escapees are the reduction and further regulation of commercial and recreational fisheries, assisted migration and structural actions to increase the passability of water bodies as well as restocking programs to areas with low natural recruitment.

Even though stocking measures should be conducted only in suitable habitats, this usually refers to the passability of the respective water body in order to avoid stocking young eels into rivers with many unpassable obstacles or landlocked inland waters. No requirements and no definitions yet exist to exclude waters from stocking measures that, depending on the degree of its contamination, could negatively impact the reproductive capacity of the fishes in these areas. Obviously, lowering the reproductive capacity of a population by translocating (already severely diminished) wild-caught seedstock from coastal to contaminated areas is strongly inconsistent with the conservational intent of stocking measures for stock recovery purposes. Additionally, the fact that in many countries stocking of young eels takes place especially in water bodies in which fisheries for eels is still active only intensifies the problem, as stocking into (fishing) mortality in order to prevent natural mortality due to an exceedance of a habitat's carrying capacity leaves open the question for a net benefit for the stock.

Yet, given the variety and large number of potentially harmful substances and different sources of contamination, this admittedly makes it difficult to formulate an applicable and specific assessment tool to distinguish suitable from unsuitable habitats with regard to pollution as a general stressor. Furthermore, the unique biology of anguillid eels hinders the assessment of pollution effects on a stock level. Frankly, it is very complex to establish the link between polluted freshwater habitats, embryo-larval toxicity caused by maternally-transferred contaminants and reduced recruitment since clear evidence for this connection in the eel stock decline has yet to be provided. Although plenty of evidence exists that show the negative effects of several contaminants on vertebrate health, the reproductive capacity of fishes and even on the specific physiology of eels, most of these data have been produced in laboratory experiments that focus on certain life stages and endpoints with unrealistic pathways, exposure times or intensities (ANNEX V). Lifetime exposure and "cocktail-effects" of various, simultaneously interacting compounds are virtually unknown (ANNEX V). Many other challenges in the assessment of pollution-associated effects are rooted in uncertainties connected to the spawning migration and larval development of eels and make interpretation of laboratory data fairly speculative and difficult. To generalize effects or large-scale impacts from most pollutants is controversial as drawn conclusions often only cover part of the whole story and do not cover all influencing aspects. This is one reason why some voices in the scientific community consciously relativize or even downplay interpretations on field data deduced from experiments and models (Maceina and Sammons 2019).

Hamilton *et al.* (2016) acknowledged this controversy and phrased the statement: "Integrating data on biological effects between laboratory-based studies and wild populations, and building an understanding on adaptive responses to sublethal exposure are some of the priority research areas for more effective evaluation of population risks and resilience to contaminant exposure". Therefore, increased caution is suggested, when applying laboratory-derived perceptions on population levels. Knowledge about a present degree of contamination does not automatically allow for a detailed interpretation of the consequences. Nevertheless, tissue concentrations and body burdens of a respective compound still constitute the most crucial benchmarks to assess the quality of spawners in order to predict their overall reproductive success (Pierron *et al.* 2008; Geeraerts *et al.* 2011; Chapter I, Chapter II; Chapter III; ANNEX III). With all given uncertainties, basic laboratory-based research remains crucial to gain knowledge about the effects of contaminants in the environment and specifically on biota. While modern analytical chemistry continues to develop rapidly and modern instruments and methods allow to identify and quantify more and more substances often even in trace amounts, a bottle neck remains the production of valid effect data on wildlife, particularly on a species level.

Conclusions and outlook

In sum, the results in this thesis generally support the hypothesis that chemical pollution had been capable of affecting the European eel on a stock level. Due to the multiple and diverse stressors that were concurrently present and may have acted synergistically before and during the time of the steep recruitment decline, it remains difficult to quantify their partial share of the overall impact. Regardless of these fractions, however, the need for further actions to initiate recovery of the stock remains of utmost importance.

In order to reach the goal of a long-term stock recovery, eel management and conservation should continue, review and improve the already implemented actions for the stock, and hold on to the precautionary approach of limiting the anthropogenic mortality to close as zero as possible. As suggested in Chapter 1; ANNEX V as well as by De Meyer *et al.* (2018), management for eels connected with stocking and translocation of glass eels and young recruits, must consider the habitat quality in respect of chemical pollution and provide the best possible conditions for the growth phase of the eels in order to maintain the conservational intent of the management measure. The EU Water Framework Directive is a tool for integrated river basin management and has been

implemented to create an overview of the current situation of pollution and its effects and is meant in the long term, to constantly improve the situation to an extent to eventually reach a good environmental standard. Even though data derived from this framework could be a helpful tool in order to select suitable habitats for stocking measures, the restoration of habitats in general needs to be retained and even furthered as a goal. This implies a reduction of chemical discharges, clean-up of contaminated sediments, frequent monitoring and controls by a legislative power. The proposed intentions to build up a pan-European monitoring of Eel quality by Belpaire *et al.* (2011b) seem reasonable, as this would provide a tool to receive information about the spawner-quality related habitat traits covering a large area of the species' distribution range. In addition to this, the number of spawners must be maximized by restricting or banning mortality caused by fisheries, by reducing obstacles to migration and by generally improving habitat quality. Stocking can only provide a net-benefit for the stock if it eventually contributes to a higher number of healthy and fecund (high quality) spawners compared to a scenario without stocking.

For improved stock management, it would also be helpful to define standards including benchmark values of average fat content and contaminant burden derived from species-specific dose-response curves in order to evaluate and assess the average parental condition (spawner quality) that silver eels from a certain watershed may reach before the respective water can be defined suitable for stocking. With regards to this, Byer *et al.* stated (2013a): "*The method of mortality evaluation could have a dramatic impact on the outcome of recruitment risk assessment. Therefore, it is essential for future research to focus on the development of relative potencies for eels and resultant eel specific thresholds for dioxin-like compound mortality*."

Indeed, improvement and further development of PBTK models in combination with physiological data for energy efficiency of maturing fish derived from swimming tunnel experiments would help to facilitate tools to assess potential pollution impacts. Combined with species-distinct dose-response curves derived from experiments with larvae after improved laboratory rearing of anguillid eels, or at least from in-vitro experiments with specific receptor genes, could further establish a population-based assessment of spawner quality. For this, monitoring contamination status and contaminant burdens in relevant body compartments over their distribution range could then translate into precisely anticipated effect concentrations and thus survival probability in the spawning area.

In order to improve our understanding about the realistic effects of contaminants for both, individual fish and on population level, further development and implementation of

new available tools and technologies including artificial reproduction, swimming tunnels experiments, data from analytical chemistry, biomarkers and biomolecular methods (ANNEX V) are crucial. Also, most publications dealing with detrimental effects of pollution on eel reproduction had their focus on how chemicals may impact female maturation in terms of egg quality or egg and embryo development. However, it is understudied how contaminants could affect the reproductive capacity of male eels (Annex V). Only few studies have dealt with this, even though indications have been presented that for example metals can impair male eel endocrine pathways and maturation (Pierron *et al.* 2008). Also studies on other fish species have already shown or suggested effects on the reproductive capacity of males by endocrine disruption such as feminization, reduced fertility, as occurs for other fish species (Matthiessen *et al.* 2018).

Pollution has been shown to induce transcriptomic responses in eels (Maes *et al.* 2013; Pujolar *et al.* 2012; Pujolar *et al. 2013b*, Baillon *et al*. 2015). However, these changes in gene transcription are yet not fully usable to really assess the reproductive capacity of eels. Especially variability in individual life history and also several abiotic influences can alter gene transcription, which makes the interpretation of such data extremely challenging. For the future, a number of new insights have to evolve in order to completely clarify the remaining uncertainties regarding the contributing role of contaminants to the stock decline in anguillid eels. The catch of mature, spawning eels in the breeding grounds would help to solve many questions including knowledge about the levels and fingerprints of contaminants in muscle and eggs of actual spawners, as tissue samples from these fish may help develop thresholds and even allow to identify the fishes' origins. Closing the lifecycle in captivity in line with proceedings in the controlled reproduction and upbringing of eels in closed aquaculture systems, including gaining the ability to feed and maintain eel larvae in a stable laboratory or aquaculture environment, would make it possible to conduct embryo toxicity tests to further clarify critical concentration values in spawning fish. And last but not least, due to the production ban and (slowly but steady) sinking environmental concentrations of DLCs, it is crucial to not only focus on dioxin-like or halogenated compounds, but also keep an eye on emerging contaminants and other chemicals that may potentially interfere with the health and physiology of these mysterious and iconic species.

Annex I

Brominated flame retardants and dechloranes in eels from German Rivers

Roxana Sühring, Axel Möller, **Marko Freese**, Jan-Dag Pohlmann, Hendrik Wolschke, Renate Sturm, Zhiyong Xie, Reinhold Hanel, Ralf Ebinghaus

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Review

Brominated flame retardants and dechloranes in eels from German Rivers

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HIGHLIGHTS

- Elvers had low PBDE contamination, alternate BFRs and dechloranes could be detected.
- PBDEs were main contaminants in yellow and silver eels with BDE-47 as main congener.
- The isomer ratio of syn- and antiDP changes with the life cycle stage.
- First detection of Dec-602 and Dec-603 in aquatic organisms from Europe.
- First detection of DPTE, BEHTBP and PBEB in European Eels.

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ABSTRACT

The levels of PBDEs, alternate BFRs and dechloranes in European Eel (*Anguilla anguilla*) samples (elvers, yellow and silver eels) were investigated to compare the contamination of eels from the rivers Elbe and Rhine and to estimate the BFR contamination throughout the eel's life cycle.

PBDEs were the dominating flame retardants (FRs) in muscle tissues of yellow and silver eels, while the alternate BFR 2,3-dibromopropyl-2,4,6-tribromophenyl ether (DPTE) and the Dechlorane 602 were the dominating FRs in elvers (juvenile eels). Concentrations of FRs in silver eels from river Rhine were generally higher than concentrations in other eels analysed with up to 46 ng g^{-1} wet weight (ww) ∑PBDEs. The concentrations in yellow and silver eels from river Elbe were similar with an average of 9.0 ± 5.1 ng g^{-1} ww and 8.1 ± 3.7 ng g^{-1} ww respectively. PBDE concentrations in elvers were comparably low (0.02 (BDE-100) to 0.1 (BDE-183) ng g^{-1} ww), which lead to the conclusion that these contaminants were mostly ingested within the rivers.

Among the alternate BFRs and dechloranes, DPTE as well as the Dechlorane 602 and Dechlorane Plus (DP) were found in all life cycle stages and rivers with concentrations between 0.01 ng g^{-1} ww and 0.7 ng g^{-1} ww. Dechlorane 603 could only be detected in silver eels from river Rhine. Pentabromoethylbenzene (PBEB) was only found in yellow and silver eels and bis(2-ethylhexyl)tetrabromophthalate (BEHTBP) could only be detected in elvers.

These are the first reports of Dec-602 and 603 in aquatic organisms from Europe. The results of this study show the lasting relevance of PBDEs as contaminants in rivers and river-dwelling species but also the growing relevance of emerging contaminants such as alternate BFRs and dechloranes.

Contents

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1. Introduction

The European Eel (*Anguilla anguilla*) is a catadromous, carnivorous fish. It is widely distributed over Europe and has a high economic value for the fishing industry.

Its overall population has been declining rapidly since the 1980s and has by now dropped to 1% of the average population during the 1970s (Fisheries Forum, 2003; ICES, 2008). Therefore the European Eel was added to the UN CITES Appendix II list, implying trading restrictions, as well as to the Red List of species by the International Union for Conservation of Nature (IUCN), rating it as "critically endangered". Several natural as well as anthropogenic causes, such as overfishing, destruction of habitats, parasites, hydropower plants, predation and chemical pollution have been discussed (Dekker, 2004). Chemical pollution has become one of the main focuses as eels are predestined to take up large quantities of lipophilic organic pollutants due to their high lipid contents (Robinet and Feunteun, 2002; Palstra et al. 2006; Belpaire and Goemans 2007). This is especially problematic as eels are a possible way of human exposure to hazardous chemicals.

One group of organic pollutants possibly threatening to the European Eel are halogenated flame retardants (HFRs) and especially brominated flame retardants (BFRs). For several decades polybrominated diphenyl ethers (PBDEs) have been applied as BFRs. Some PBDEs are known to be bioaccumulative, persistent and to undergo long-range transport (LRT) (Darnerud 2003; Wania and Dugani, 2003). Many of them are toxic for aquatic organisms, some induce endocrine effects or are carcinogenic (de Wit, 2002). Due to these adverse effects to the environment and human health PBDEs have been banned for production and usage in the European Union (EU) (European Court of Justice, 2008). As a further banishment step congeners used in the technical penta- and octa-BDE mixtures have been officially classified as Persistent Organic Pollutants (POPs) under the Stockholm Convention (SCOP, 2009).

Due to the restriction of PBDEs and the increasing demand of flame retardants (FRs) the usage of alternate (non-PBDE) BFRs have increased. There is little knowledge concerning POP potential of these substitutes for PBDEs yet many alternate BFRs are suspected to at least partially fulfil the criteria (Harju et al. 2009).

Another HFR used and recommended by the EU as substitute for Deca-BDE is the highly chlorinated Dechlorane Plus (DP) (Pakalin et al., 2007). It was originally developed as a substitute for the banned pesticide Mirex but has mostly been applied as FR (Hoh et al., 2006). Even though it has been produced and used for more than 40 years there is little data available on behaviour and possible adverse effects in the environment. Since its first detection in 2006 (Hoh et al., 2006) reports on DP in the environment have increased rapidly and it has even been reported from remote areas such as the Arctic and Antarctic (Möller et al., 2010). For other used dechloranes, namely the twelvefold chlorinated cycloaliphatic ether Dec-602, the twelvefold chlorinated cycloaliphatic Dec-603 and the both brominated and chlorinated cycloaliphatic Dec-604 there are even less data available even though they are suspected to be bioaccumulative and have been reported in biota far away from production sites (Sverko et al., 2011).

This paper presents the analysis of PBDEs, alternate BFRs and Decs in elvers (juvenile eels) from river Vidå, yellow eels (stationary, river dwelling adult eels) from six sampling sides along the river Elbe and silver eels (adult eels migrating back to the spawning grounds in the Sargasso Sea) from the rivers Elbe and Rhine. The aim of this research project was to compare the contamination level of silver eels from Elbe and Rhine as well as estimate the BFR and Dec contamination during the eel's freshwater phase.

2. Materials and methods

2.1. Samples

All adult eels were caught as part of the EU Data Collection Regulation (DCR) (Stransky et al., 2008). All eels were taken in the German part of the rivers. 30 elvers with a mean length of 12 cm were taken from the river Vidå and combined into ten samples of three fish each. From six sampling sites along the river Elbe five yellow eels per sampling site were taken. All yellow eels used were between 8 and 12 years old and in the silvering stage II or III (growth phase) (Durif et al., 2005). Ten silver eels were taken each from the estuary mouth of the river Elbe and the upper river Rhine. All silver eels were in the silvering stage V (migrating phase) (Durif et al., 2005). Contact with materials containing brominated flame retardants was avoided at all sampling sites. Muscle tissue was excised from the skeletal muscle behind the level of the anus from yellow and silver eels and as much muscle tissue as possible from elvers. A detailed list of the analysed samples can be found in Table S1.

2.2. Extraction and clean-up

The frozen yellow and silver eel samples were homogenised with anhydrous Na_2SO_4 (Merck) (2:1; w/w) for approximately 20 min. using a stainless steel/glass 1 L laboratory blender (neoLab Rotorblender). For each extraction 11 mL stainless steel extraction cells were filled with 3 g Na_2SO_4 and 3 g of the Na_2SO_4–eel -mixture (equal to 1 g eel tissue) or one of the pooled elver samples. The samples were spiked with mass labelled (internal) standards (IS) ^{13}C-HBB, ^{13}C-BDE-77, ^{13}C-BDE-138 and ^{13}C-synDP. The remaining volume was filled with anhydrous Na_2SO_4.

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The samples were extracted via accelerated solvent extraction (Dionex ASE-200) using dichloromethane (DCM) at 100 °C and 120 bar. The lipid content of the samples was determined gravimetrically from separate sample aliquots.

After extraction the samples were reduced in volume to approx. 2 mL using rotary evaporators. Gel permeation chromatography (GPC) was used as a first clean up step, using a glass column (height: 500 mm, i.d.: 30 mm) filled with 35 g Bio-Beads S-X3 (pre swollen with 200 mL DCM:hexane (1:1 v/v) for 12 h) (Bio-Rad Laboratories). Analytes were eluted with 110 mL DCM:hexane (1:1; v/v).

The eluates were again reduced to about 2 mL and the solvent changed to hexane. The samples were further purified by 10% deactivated silica gel (2.5 g, 0.063–0.200 mm) (Merck) and eluted with 20 mL hexane.

The eluates were reduced to 150 μL under a gentle stream of nitrogen and transferred to measurement vials. Finally, 50 μL PCB-207 (10 ng mL^{-1}) were added as an injection standard. For further specifications regarding the used method and standards see Tables S2 and S3.

2.3. Instrumental analysis

For instrumental analysis a method developed and published by Möller et al. (2010) (Möller et al., 2010) was used. Briefly, analyses were done by a GC/MS-system (6890 GC/5973 MSD) in negative chemical ionisation mode (NCI) with methane as ionisation gas fitted with a HP-5MS column (30 m × 0.25 mm i.d. × 0.25 μm film thickness, J&W Scientific). The instrument was operated in selected ion monitoring mode. Samples were analysed for nine PBDEs, 11 alternate (non-PBDE) BFRs, DP, the one- and two-fold dechlorinated DP species (aCl_{11}DP [−1Cl + 1H], aCl_{10}DP [−2Cl + 2H]), DPMA and Dechlorane 602, 603 and 604 (see Table S4 for chemical structures and properties).

2.4. QA/QC

Extraction and clean-up were conducted in a clean lab (class 10000). BFR containing material was avoided during preparation and analysis.

Recovery rates of IS were determined for every sample (for a detailed list see Table S5). Mean IS recoveries ranged from 45 ± 19% for ^{13}C-HBB to 86 ± 19% for ^{13}C-DP in elvers; 68 ± 24% to ^{13}C-BDE-138 and 82 ± 20% for ^{13}C-BDE-77 in yellow eels and 66 ± 31% to ^{13}C-BDE-138 and 83 ± 24% for ^{13}C-BDE-77 in silver eels. All concentrations were recovery corrected.

Relative recoveries of the analytes (corrected by recovery rates of the IS) were determined during method development and ranged from 67% for BDE-66% to 159% for DPTE. The recovery for BEHTBP was low (5%). Results for BEHTBP were therefore treated as semi-quantitative.

A blank test, using Na_2SO_4 treated similar to real samples, was conducted with every extraction batch (11 samples). DPTE and Dec-602 could each be detected in one blank sample with absolute concentrations of 98 pg and 22 pg respectively. BDE-183 was found in five of eleven blank samples in absolute concentrations between 320 pg and 860 pg. The blank concentrations were considered in the calculation of the sample concentrations of the appropriate batch. For a detailed list of the measured blanks see Table S6.

The limit of detection (LOD) was calculated from a signal to noise ratio of three, the limit of quantification from a signal to noise ratio of ten. The LOD ranged from 0.004 ng g^{-1} wet weight (ww) for Dec-602 to 0.073 ng g^{-1} ww for BDE-183 in elvers; 0.008 ng g^{-1} ww for Dec-602 to 0.14 ng g^{-1} ww for BDE-183 in yellow eels and 0.004 ng g^{-1} ww for Dec-603 to 0.14 ng g^{-1} ww for BDE-100 for silver eels. The LOQ ranged from 0.013 ng g^{-1} ww for Dec-602 to 0.24 ng g^{-1} ww for BDE-183 in elvers; 0.026 ng g^{-1} ww for Dec-602 to 0.46 ng g^{-1} ww for BDE-183 in yellow eels and 0.014 ng g^{-1} ww for Dec-603 to 0.46 ng g^{-1} ww for BDE-100 in silver eels. For a detailed list of LODs, LOQs see Tables S7 and S8.

A twofold measurement was done for every sample. The standard deviation between measurements of five aliquots of one eel sample was 12%.

3. Results and discussion

3.1. BFRs and dechloranes throughout the eels lifecycle

The average results for PBDEs, alternate BFRs and dechloranes from this study in comparison to recent studies are displayed in Table 1.

For a complete list of the results of this study see Tables S9 and S10.

3.1.1. PBDEs

The elvers analysed in this study have been in fresh water between a few months and 1 year. Their journey from the Sargasso Sea to Europe has taken up to 3 years (Tesch et al., 1990; Bonhommeau et al., 2010). It is therefore likely that most of the contaminations found were ingested during their stay in the ocean and estuary or passed on by spawners.

Elvers had low PBDE concentrations compared to the PBDE levels in eels from other life cycle stages and the contribution of PBDEs to the sum contamination in elvers was similar or lower than the contribution of alternate BFRs and dechloranes. Three of the nine analysed PBDE congeners could be detected in elvers, with concentrations ranging from 0.02 (BDE-100) to 0.1 (BDE-183) ng g^{-1} ww. In all other eels analysed PBDEs were the major group of contaminants. Six and seven different congeners could be detected in yellow eels from river Elbe and silver eels from river Rhine, respectively. ∑PBDEs concentrations ranged from 9.0 ± 5.1 ng g^{-1} ww in yellow eels from river Elbe to 21.3 ± 13.8 ng g^{-1} ww in silver eels from river Rhine.

The congener distribution of the PBDEs differed in elver samples and samples from other life cycle stages. In elvers BDE-183 was the main congener, indicating a contamination through the technical octa-BDE mixture. In yellow and silver eels BDE-47 was the main congener with concentrations between 6.2 ± 3.6 ng g^{-1} ww in yellow eels from river Elbe and 14.3 ± 9.05 ng g^{-1} ww in silver eels from river Rhine. The congener distribution in adult eels matched the distribution reported in other studies analysing PBDEs in eels (Belpaire, 2008) with BDE-47 > BDE-100 > BDE-153 > BDE-99 > BDE-154 > BDE-183.

The low concentrations in elver samples indicated that PBDEs have mostly been ingested in the rivers. The strong contribution of lower brominated PBDEs yellow and silver eels suggests the technical penta-BDE mixture as main source of the contamination. The high contribution of BDE-47 is typical for all fish due to the higher uptake rate and biomagnifications of BDE-47 within the aquatic food web (Eljarrat and Barceló, 2011). BDE-47 has also been proven to be formed via enzymatic debromination of higher brominated diphenyl ethers during the metabolism in fish (Eljarrat and Barceló, 2011).

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3.1.2. Alternate BFRs

DPTE could be detected in eels of all life cycle stages analysed with mean concentrations between 0.2 ± 0.1 ng g^{-1} ww in elvers, 0.22 ± 0.35 ng g^{-1} ww in yellow eels from river Elbe and 0.89 ± 0.64 ng g^{-1} ww in silver eels from river Rhine.

The detection of DPTE within the elver samples could be an indication that the eels ingested DPTE during their time in the ocean or estuary as well as the river. There are no data on current DPTE production, however, DPTE has frequently been detected in various matrices most recently by Möller et al. (2012) who detected DPTE in water samples from the North Sea, river Elbe and river Weser. DPTE is suspected to be persistent in sediments making them a possible source of DPTE contamination (Fisk et al., 2003).

BEHTBP could only be detected in elvers, with a medium concentration of about 0.1 ng g^{-1} ww and does therefore seem to not be ingested within the rivers. In recent studies BEHTBP has as well mostly been detected in ocean dwelling species such as dolphins and porpoise (Lam et al., 2009) while it could not be detected in sources typically discharging into fresh water such as sewage sludge (Moskeland, 2010). The concentrations found in this study were higher than the average PBDE concentration in elvers which again indicated, that the main contamination with PBDEs occurred within the rivers.

The second alternate BFR detected in yellow and silver eels was PBEB. The detected concentrations were similar for all adult eels analysed with 0.025 ± 0.007 ng g^{-1} ww in yellow eels from river Elbe, 0.027 ± 0.009 ng g^{-1} ww in silver eels from river Elbe and 0.027 ± 0.015 ng g^{-1} ww in silver eels from river Rhine. It could not be detected in elver samples and has therefore probably only been ingested in the rivers. These results accorded with results from recent studies that reported PBEB in samples from industrialised areas rather than oceanic samples (Harju et al. 2009). Recently the German Environment Agency also detected low amounts of PBEB in bream samples from German rivers such as Elbe and Mulde (Sawal et al., 2011).

3.1.3. Dechloranes

Dechlorane Plus and Dec-602 could be detected in all life cycle stages analysed with up to 0.67 ng g^{-1} ww (in elvers). Dec-603 could only be detected in silver eels from river Rhine with concentrations between < LOD (0.0042 ng g^{-1} ww) and 0.076 ng g^{-1} ww.

In elvers, yellow eels and silver eels from river Rhine the syn-isomer of the two technical stereoisomers syn- and antiDP could be detected in slightly higher concentrations and more individual samples. The synDP/$\sum$DP ratio (f_{syn}) was highest in yellow eels with an average of 0.96 ± 0.12, followed by f_{syn} in elvers with an average of 0.80 ± 0.14. In silver eels from river Rhine syn- and antiDP concentrations were almost equal (0.040 ± 0.030 ng g^{-1} ww and 0.033 ± 0.022 ng g^{-1} ww respectively, f_{syn} = 0.52 ± 0.084), yet synDP could be detected in more individual samples. In silver eels from river Elbe the detected synDP and antiDP concentrations were similar as well (n.d. – 0.030 ng g^{-1} ww and n.d. – 0.021 ng g^{-1} ww respectively) yet antiDP could be detected in 70% of the samples, while synDP was detectable in only 30% of the samples. The resulting f_{syn} was therefore low with only 0.24 ± 0.30.

The significant change in the isomer ratio from the technical mixture (75% antiDP) to the isomer ratio found in the majority of the eel samples (between 50% and 90% synDP) matched observations from previous studies indicating that synDP bioaccumulates and biomagnifies stronger than antiDP in fish (Wu et al., 2010; Shen et al., 2011b). The high contribution of antiDP in silver eels however suggests that without further uptake synDP is eliminated quicker than antiDP. For the eels analysed in this study the isomer ratio of syn- and antiDP seems to have mostly been driven by uptake rate and/or metabolism and not by location, as the significant changes were between life cycle stages (yellow and silver eels) and not between rivers (silver eels from Elbe and Rhine).

Dec-602 has not yet been reported in aquatic organisms in Europe. It has however been found in sea bird eggs from Spain and various matrices from the US and Canada (Guerra et al., 2011). The detection in eels from all life cycle stages was surprising as there is no reported producer or importer within the EU. Dec-602

Table 1
BFRs and dechloranes in elvers, yellow and silver eels from this and recent studies (ng g^{-1} ww, ng g^{-1} lw) Results are displayed in ng g^{-1} wet weight (ww) and ng g^{-1} lipid weight (lw) values below the limit of detection are labelled "not detected" (n.d.) substances that were not analysed in the study are labelled "not applicable" (n.a.).

Location	Water system	Sample	Unit	∑PBDEs	BDE-47	BEHTBP	DPTE	PBEB	∑DP	Dec-602	Dec-603	Reference
Germany	Vidå	Elvers (*n* = 30)	ng g^{-1} ww	0.22 ± 0.08	n.d. – 0.088	0.10 ± 0.032	0.22 ± 0.08	n.d.	n.d.- 0.46	n.d. – 0.66	n.d	This study
			ng g^{-1} lw	10.2 ± 1.3	n.d. – 6.5	7.4 ± 2.4	16.06 ± 5.7	n.d.	n.d.- 33.8	n.d. – 48.8	n.d	
Germany	Elbe	Yellow eels (*n* = 30)	ng g^{-1} ww	8.9 ± 3.4	6.0 ± 2.2	n.d.	0.19 ± 0.18	0.020 ± 0.010	0.041 ± 0.027	n.d. – 0.25	n.d	This study
			ng g^{-1} lw	33.5 ± 13.0	22.5 ± 8.3	n.d.	0.67 ± 0.30	0.28 ± 0.19	0.14 ± 0.085	n.d. – 0.73	n.d	
Germany	Elbe	Silver eels (*n* = 10)	ng g^{-1} ww	8.3 ± 3.7	5.9 ± 2.9	n.d.	0.62 ± 0.72	0.027 ± 0.009	0.028 ± 0.015	0.017 ± 0.009	n.d	This study
			ng g^{-1} lw	30.2 ± 13.5	21.5 ± 10.4	n.d.	2.3 ± 2.8	0.10 ± 0.030	0.38 ± 0.067	0.060 ± 0.033	n.d	
Germany	Rhine	Silver eels (*n* = 10)	ng g^{-1} ww	21.3 ± 13.8	14.3 ± 9.05	n.d.	0.89 ± 0.64	0.027 ± 0.015	0.073 ± 0.051	0.073 ± 0.055	n.d. – 0.076	This study
			ng g^{-1} lw	88.7 ± 65.9	59.2 ± 42.5	n.d.	3.6 ± 2.8	0.13 ± 0.060	0.34 ± 0.26	0.30 ± 0.26	n.d – 0.37	
Czech Republic	Elbe	Eels (*n* = 2)	ng g^{-1} ww	n.a.	4.3	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	Santillo et al. (2005)
England	Thames	Eels (*n* = 5)	ng g^{-1} ww	n.a.	46	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	Santillo et al. (2005)
Scandinavia	Various	Fish (*n* = 14)	ng g^{-1} ww	n.a.	n.a.	0.46	0.026–0.049	0.0001–0.004	0.030–0.042	n.a.	n.a.	Schlabach et al. (2011)
Netherlands	Rhine	Eels (*n* = 25)	ng g^{-1} lw	n.a.	259	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	de Boer et al. (2010)
Canada/USA	Lake Ontario	Lake Trout (*n* = 29)	ng g^{-1} lw	n.a.	n.a.	n.a.	n.a.	n.a.	0.2–1.9	8–180	0.03–0.40	Shen et al. (2011a)
Spain		Falcon eggs (*n* = 13)	ng g^{-1} lw	n.a.	n.a.	n.a.	n.a.	n.a.	1.78	8.36	3.98	Guerra et al. (2011)

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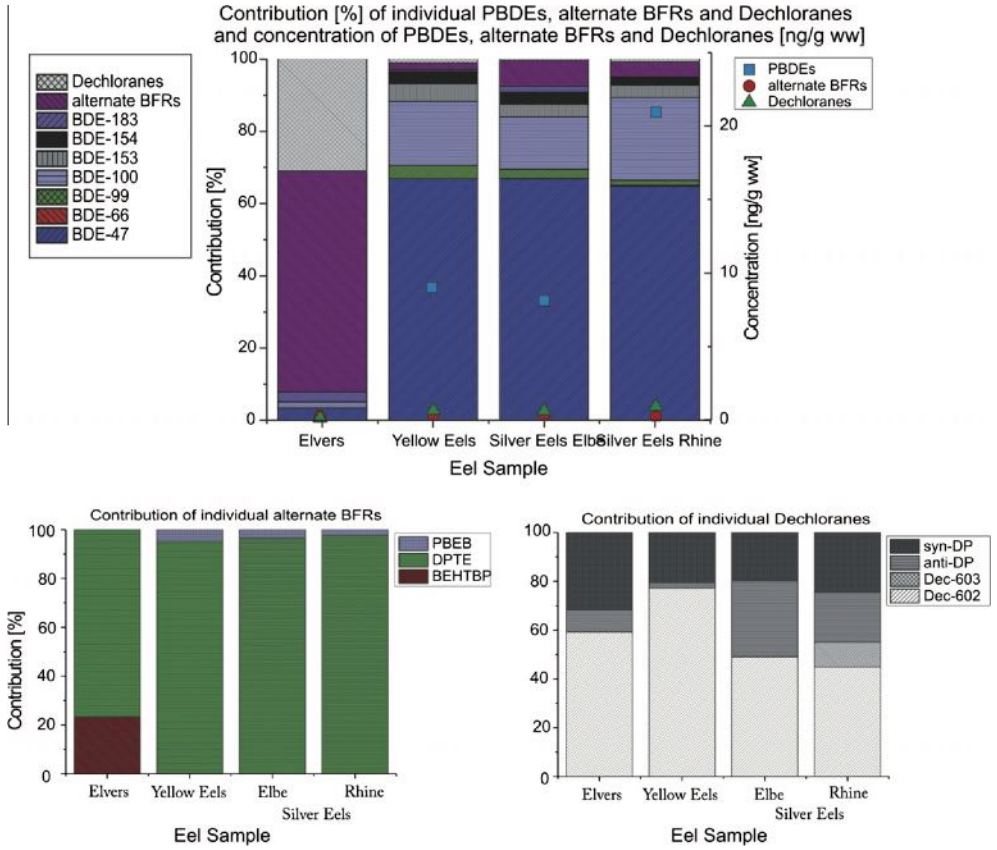

Fig. 1. Contribution of individual PBDEs, alternate BFRs and Dechloranes (%), displayed as columns and concentrations of sum PBDEs, alternate BFRs and Dechloranes (ng g^{-1} ww) displayed as symbols.

has however been reported to have a high bioaccumulation potential (the biota- sediment accumulation factor (BSAF) is about 500 times higher than the BSAF of DP) and to be very bio available (Shen et al., 2011b).

There is no reported source for Dec-603 in Europe yet it has as well been detected in sea bird eggs from Spain (Guerra et al., 2011). Dec-603 has also been detected in the banned organochlorine pesticides formulations of aldrin and dieldrin (Shen et al., 2011a). As the reported half-life for Dec-603 in sediments is 11 years (Sverko et al., 2011) residues of these pesticides leaking from sediments could be a possible source. The fact that it could only be detected in silver eels from the river Rhine indicates that it, so far, mainly occurs in highly industrialised areas (in this case the Rhine–Ruhr metropolitan region) close to sources. Both Dec-602 and Dec-603 could also enter the EU incorporated in products. Dec-602 for example is used in fibreglass- reinforced nylon (Shen et al. 2011) which is a common component in consumer products.

In the group of dechloranes Dec-602 was the main contaminant in yellow eels while in silver eels from river Rhine ∑DP and Dec-602 had similar concentrations and ∑DP concentrations in silver eels from river Elbe slightly exceeded Dec-602 concentrations (see Fig. 1). This change of the contamination pattern could indicate that Dec-602 is easier metabolised and/or eliminated than DP. An increase of the DP/Dec-602 ratio could not have been caused by a change of diet, as silver eels stop feeding. The increase is therefore likely to have been caused by different metabolism strategies or different ways of uptake between Dec-602 and DP, such as a higher uptake of DP via gills or skin. Another reason could be that the highly migratory silver eels ingested the high DP concentration at a different part of the river and have not ingested any new contaminants as silver eels stop feeding due to their physiological changes from yellow to silver eels.

3.2. Concentration profile of BFRs and dechloranes in eels along the Elbe

PBDEs showed increasing concentrations (significance: 99.9% confidence level; Neumann-test) towards inland sampling sites, again supporting the thesis that eels were primarily exposed to these contaminants in the rivers.

The trend was mainly driven by the BDE-47 congener but most PBDEs measured apart from BDE-183 and BDE-154 showed a similar trend. Highest PBDE concentrations were measured in eels from the Dessau sampling site (km 261) (12.6 ± 5.7 ng g^{-1} ww) close to where the river Mulde flows into the Elbe. The Mulde is known to be contaminated by a variety of chemicals (e.g. hexachlorocyclohexane) due to leakage of landfills containing chemical waste from the former German Democratic Republic (Ministerium für Landwirtschaft und Umwelt, 2005). A study done by the German Federal Environmental Agency, analysing PBDEs as well as some alternate BFRs in bream from rivers Mulde and Elbe also re-

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ported higher concentrations in the Mulde than in any of the samples from river Elbe (Sawal et al., 2011).

The Mulde as main source for PBDEs in the Elbe would explain the decrease in the concentration upstream the Dessau sampling site as well as the gradually decreasing trend towards the estuary mouth as the contamination is bound to decrease with distance to the source. As yellow eels are relatively residential the decreasing trend of contamination along the river can be expected to be reflected in the contamination of the eels at different sampling sites.

The concentrations of alternate BFRs were relatively constant throughout the Elbe with two exceptions for DPTE. One exception was the low concentrations at the Hohengöhren sampling site (km 378). The second exception was one very high contaminated eel from Jork sampling site (km 643). The lack of a trend in the contamination indicated continuous contamination throughout the river via e.g. diffuse emission and/or deposition. Remobilisation from contaminated sediments could also be a possible reason for this lack of a clear contamination pattern. The high DPTE concentration at Jork sampling site (km 643) however indicated that this specific eel was exposed to a large dose of DPTE probably by a point source. PBEB concentrations were found in low concentrations in samples from most sampling sites again indicating diffuse emissions and/or immission via deposition or discharge from contaminated sediments.

At Gorleben sampling site (km 492) highest individual Dec-602 concentrations were measured (0.25 ± 0.24 ng g^{-1} ww). Towards the estuary mouth Dec-602 could however be detected in more individual samples. Upstream Gorleben some fish still had high Dec-602 concentrations (at Hohengöhren (km 378)) yet overall synDP was the main contaminant of the dechloranes. The high concentrations of Dec-602 at Gorleben sampling site might indicate a point source in that area. The contamination found in fish from Hohengöhren sampling site could be due to the movement of the fish along the river even though yellow eels are supposed to be relatively stationary. The overall DP concentration was highest at the Dessau sampling site (km 261) (0.038 ± 0.013 ng g^{-1} ww) and gradually decreased towards the estuary mouth (significance: 99.9% confidence level; Neumann-test) apart from one high contaminated sample from Jork sampling site (km 643). The trend indicated that the primary DP source was near the Dessau sampling site and therefore probably influenced by the river Mulde. The high contaminated sample from Jork was the same sample that also showed alternate BFR concentrations above average, again indicating a contamination of this individual fish by a point source.

3.3. Comparison of silver eels from Elbe and Rhine

The concentrations of PBDEs and dechloranes in silver eels from river Rhine were up to three times higher than the concentrations found in silver eels from river Elbe. This was to be expected as the samples from river Rhine were taken in a highly industrialised area (close to potential sources) and fish from river Rhine are known to be contaminated with up to several 100 ng g^{-1} lw PBDEs (Sawal et al., 2011). The congener distribution of the PBDEs in samples from Elbe and Rhine were similar, yet in addition to the PBDEs found in silver eels from river Elbe BDE-66 could be detected in silver eels from river Rhine.

The contribution of the individual dechloranes to the sum dechlorane contamination differed for silver eels from Rhine and Elbe. Again there were more individual substances detectable in the river Rhine (DP, Dec-602, Dec-603). The concentrations of alternate BFRs found in silver eels from river Rhine and Elbe were similar, indicating a contamination through diffuse sources.

The comparably high concentrations of FRs and detection of additional components like Dec-603 and BDE-66 display the overall higher contamination of the river Rhine in comparison to river Elbe and might be an indication for sources in this area.

4. Conclusions

The results of this study show the lasting relevance of PBDEs as contaminants in rivers and river-dwelling species but also the growing relevance of emerging contaminants such as alternate BFRs and dechloranes. There are in many cases not enough data to evaluate the risk of the emerging contaminants yet many BFRs are expected to be toxic for aquatic organisms and are therefore likely to affect the eel's health and ability to reach its spawning ground.

Further tests concerning adverse effects and properties of the analysed substances and their metabolites should be conducted. Sources and ways of environmental release and distribution, especially for substances without a known source such as DPTE and the dechloranes have to be identified and monitored.

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Appendix A. Supplementary material

Tables on the samples, method, used standards, recovery rates, blank values, detection and quantification limits as well as a detailed list of the results is available in the supporting information. Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2012.08.016.

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Annex II

Brominated flame retardants and Dechloranes in European and American eels from glass to silver life stages

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Brominated flame retardants and Dechloranes in European and American eels from glass to silver life stages

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HIGHLIGHTS

- Bioaccumulation of PBDEs over the life cycles of European and American eels.
- Bans on PBDEs are effectively reducing the contamination of juvenile eels in Europe.
- Rapid uptake of Dechlorane 602 as soon as juvenile eel enter the freshwater phase.
- Increasing relevance of alternative brominated flame retardants and Dechloranes in juvenile eels.

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ABSTRACT

The populations of American (*Anguilla rostrata*) and European eels (*Anguilla anguilla*) have been declining rapidly in the last decades. Organic contaminants are suspected to be one of the possible causes for the decline; however, so far there have been few investigations of the uptake of specific compounds by different life cycle stages (e.g. freshwater or marine stage) and how the contamination patterns develop throughout the eel's life cycle. In the present study we measured concentrations of polybrominated diphenylethers (PBDEs), alternate brominated flame retardants (alternate BFRs) and Dechloranes (Decs) in different life stages of European and American eels to compare the contamination patterns and their development throughout the eel's life cycle.

In general, concentrations of flame retardants (FRs) were similar to or higher in American than in European eels, and a greater number of FRs were detected. PBDE congeners that are characteristic of the Penta-PBDE formulation were the most abundant FRs in all adult eels as well as American glass eels. In European glass eels the alternate BFR 2,3-dibromopropyl-2,4,6-tribromophenylether (DPTE) and Dechlorane Plus were the dominating FRs, with average concentrations of 1.1 ± 0.31 ng g^{-1} ww and up to 0.32 ng g^{-1} ww respectively. Of the PBDEs BDE-183 was the most abundant congener in European glass eels. Low concentrations (less than 10% of the total contamination) of Tetra and Penta-PBDEs in juvenile European eels indicated that bans of technical Penta-PBDE in the European Union are effective. Enrichment of PBDEs was observed over the life stages of both European and American eels. However, a greater relative contribution of PBDEs to the sum FR contamination in American eels indicated an ongoing exposure to these substances. High contributions of alternate BFRs in juvenile eels indicated an increased use of these substances in recent years. Concentrations seemed to be driven primarily by location, rather than life stage or age.

1. Introduction

European eel (*Anguilla anguilla*) and American eel (*Anguilla rostrata*) are facultatively catadromous, carnivorous, and, during their continental phase, benthic species with unusual life cycles (Dekker, 2000; van Ginneken and Maes, 2005; Ministry of Natural

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Resources, 2007). Both spawn in the Sargasso Sea, hatch, and are transported as larvae by oceanic currents to the North African, European and American coastal waters (Dekker, 2000; Ministry of Natural Resources, 2007). There they first metamorphose into glass eels and develop further to elvers and yellow eels. During their continental growth phase, eels build up large energy resources (Belpaire and Goemans, 2007; Belpaire et al., 2009). Prior to maturation and migration back to their spawning grounds, eels undergo a silvering process accompanied by drastic changes in physiology including the degeneration of the alimentary tract (Durif et al., 2005). Stored fat is used to develop gonads and as energy reserves for their migration back to the Sargasso Sea to reproduce once and die (Dekker, 2000).

The European eel is of high economic value. However, its population has been declining rapidly since the 1980s (Fisheries Forum, 2003; ICES, 2008) leading to its listing under Appendix II of CITES (the Convention on International Trade in Endangered Species of Wild Fauna and Flora) as well as on the Red List of species (IUCN), rating it as "critically endangered". A similar downward trend in American eel has led to the closure of commercial yellow eel fishery in Lake Ontario in 2004 and the rating "threatened" in Canada by COSEWIC (Committee on the Status of Endangered Wildlife in Canada) in 2012.

Chemical contaminants are postulated as one of the possible causes for the decline of freshwater eel populations because, due to their high lipid contents (Palstra et al., 2006; Belpaire and Goemans, 2007; Belpaire et al., 2009), eels are predestined to accumulate potentially harmful lipophilic organic pollutants.

Halogenated flame retardants (HFRs) are a group of possibly harmful and accumulating organic contaminants. They are used in a variety of consumer products such as textiles, electronic equipment, plastics, and furniture (De Wit, 2002). The largest group among the currently used HFRs are brominated flame retardants (BFRs). For several decades polybrominated diphenyl ethers (PBDEs) were the most widely used additive BFRs (De Wit, 2002). However, due to their adverse effects on the environment and human health, PBDEs have been banned for production and usage in the European Union (EU) (European Court of Justice, 2008), and are being voluntarily withdrawn or phased out in North America (US EPA 2009). Congeners used in the technical penta- and Octa-PBDE mixtures have been classified as Persistent Organic Pollutants (POPs) under the Stockholm Convention (SCOP, 2009).

Government regulations require consumer products to meet certain standards for flame retardancy, which has encouraged the use of substitutes such as alternate (non-PBDE) flame retardants both brominated or chlorinated such as Dechloranes (Decs) (Covaci et al., 2011). There is little knowledge concerning production, usage, or the persistence potential of these substitutes for PBDEs, yet many are suspected to at least partially fulfil the criteria for POPs (Harju et al., 2009; Covaci et al., 2009; Sverko et al., 2011).

This paper presents a comparison of concentrations and contamination patterns of PBDEs, alternate BFRs, and Decs throughout the life cycle of European and American eels. The aim was to identify the decisive factors for spatial and life cycle dependent distribution of halogenated flame retardants.

2. Materials and methods

2.1. Samples

The life stages examined were glass eels, elvers, yellow and silver eels for European eels, and glass eels, young yellow eels, yellow eels, silver eels for American eels.

One hundred European glass eels, originally caught at the French Atlantic coast, were purchased from a glass eel distributer and combined into ten samples. Data for elvers and adult European eels from the Elbe and Rhine River in Germany were previously published in Sühring et al. (2013). Thirty-seven American glass eels from Baie des Sables, Matane, Quebec, Canada were pooled into three samples. Ten young American yellow eel samples were taken from the Saint Lawrence River, Canada at each of the Beauharnois Dam, Quebec and the Moses-Saunders Dam, Ontario. Fifteen muscle tissue samples were taken from older yellow eels sampled from Lake Ontario and the upper Saint Lawrence River; dorsal muscle tissue was excised posterior to the anus. Data for American silver eels from Lake Ontario were previously published in Byer et al. (2013).

The primary sampling areas (Lake Ontario/Saint Lawrence River, Canada and Elbe River, Germany) are both major waterways in industrialised areas with major urban areas such as Toronto and Hamilton (Lake Ontario), and Dresden and Hamburg (River Elbe). Including the estuary both the Elbe and the Saint Lawrence River are over 1000 km in length (Netzband et al., 2002; Canadian Geographic, 2008). However, the Saint Lawrence River is downstream the Laurentian Great Lakes, and therefore, potentially receives contaminants from a large geographic area, while the river Elbe originates from a spring in the Riesengebirge. Another major difference is the average discharge of the rivers with over 16,000 $m^3 s^{-1}$ for the Saint Lawrence River and ~860 $m^3 s^{-1}$ for the Elbe River (Netzband et al., 2002; Environment Canada, 2009).

A detailed list of the analysed samples can be found in Table S1.

2.2. Extraction and clean-up

The frozen yellow eel samples were homogenised with anhydrous Na_2SO_4 (Merck) (2:1; w/w) for approximately 20 min. using a stainless steel/glass 1 L laboratory blender (neoLab Rotorblender). For glass eel samples, 28 × 60 mm glass–fibre extraction thimbles for Soxhlet extraction were filled with Na_2SO_4–eel -mixture (equal to 3 g eel tissue). All samples were spiked with mass labelled surrogate standards ^{13}C–HBB, ^{13}C–BDE-77, ^{13}C–BDE-138, and ^{13}C–synDP.

Glass eel samples were Soxhlet-extracted using DCM at 55 °C for 24 h. Adult eels were extracted with DCM by accelerated solvent extraction, using the method described in Sühring et al. (2013). The lipid content of samples was determined gravimetrically from separate sample aliquots. Extracts were purified as described by Sühring et al. (2013). Briefly, a gel permeation chromatography (GPC) was used as first clean-up step, using 30 g Bio Beads SX-3 and DCM:hexane (1:1; v:v) as eluent. The first fraction (75 mL) was used to determine the lipid content of the sample; the second fraction (110 mL) contained the target substances and was reduced in volume to about 2 mL. 2.5 g 10% H_2O deactivated silica gel was used as a second clean-up step. Analytes were eluted with 20 mL hexane and the volume reduced to 150 μL under a gentle stream of nitrogen. Finally, 500 pg (absolute) ^{13}C PCB-208 was added as an injection standard to each sample.

2.3. Instrumental analysis

Extracts were analysed by gas chromatography/mass spectrometry (GC/MS; 6890 GC/5973 MSD) in negative chemical ionisation mode (NCI) with a method developed by Möller et al. (2010). Eels were analysed for nine PBDEs (BDE-28, -47, -66, -85, -99, -100, -153, -154, -183), 10 alternate BFRs (PBBz, PBT, DPTE, HBB, PBEB, TBB, BTBPE, TBPH, OBIND, HCDBCO), DP, aCl11DP, aCl10DP, 1,5-DPMA and Dechlorane 602, 603 and 604. A detailed list of standards can be found in Table S2.

Peak areas of the obtained chromatograms were integrated using Agilent Technologies MassHunter Workstation Software Quantitative Analysis B.05.02 for GCMS. Further data analysis was performed with Microsoft Office Excel 2010 and Origin Lab 9.0 SR1.

Table 1
Comparison of the mean (±SD) flame retardant concentrations [ng g^{-1} ww], [ng g^{-1} lw] and contribution of synDP to sum DP (fsyn) found in European glass eels from France (FR), American glass eels from Canada (CA), young American yellow eels and yellow eels from Lake Ontario (LO) and the Saint Lawrence River (SLR) in Canada from this study with concentrations [ng g^{-1} ww], [ng g^{-1} lw], [pg g^{-1} lw] reported in recent studies on European elvers from the river Vidå at the German- Danish border (GER), yellow eels from the river Elbe in Germany, as well as European and American silver eels from the river Rhine, Elbe (Germany) and from Lake Ontario and the Saint Lawrence River, respectively.

		Glass eels (Estuary, FR)	Elvers (Vidå, GER)	Yellow Eels (Elbe, GER)	Silver Eels(Elbe, Rhine, GER)	Glass eels (Estuary, CA)	Young Yellow Eels (LO, SLR, CA)	Yellow eels (LO, CA)	Yellow eels (SLR, CA)	Silver Eels (LO, CA)	Silver Eels (LO, CA)
∑PBDEs	ng g^{-1} ww	1.8 ± 0.89	0.22 ± 0.08	8.9 ± 3.4	14.9 ± 11.9	1.7 ± 0.85	4.4 ± 2.7	16*	5*	26.7 ± 21.4	n.a.
	ng g^{-1} lw	176.0 ± 98.1	10.2 ± 1.3	33.5 ± 13.0	59.7 ± 47.7	168.8 ± 85	44 ± 27	77*	23*	n.a.	
BDE-47	ng g^{-1} ww	<LOD	<LOD–0.088	6.0 ± 2.2	10.06 ± 7.8	1.1 ± 0.55	2.1 ± 1.8	11*	4*	15.3 ± 14.3	n.a.
	ng g^{-1} lw		<LOD–6.5	22.5 ± 8.3	40.2 ± 31.3	114.2 ± 55	21 ± 18	53*	18*	n.a.	
ATE	ng g^{-1} ww	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	ng g^{-1} lw										226 ± 223 pg g^{-1} lw
BEHTBP	ng g^{-1} ww	<LOD	0.10 ± 0.032	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	n.a.	n.a.
	ng g^{-1} lw		7.4 ± 2.4								25.7 ± 26.3 pg g^{-1} lw
DPTE	ng g^{-1} ww	2.0 ± 0.31	0.22 ± 0.08	0.19 ± 0.18	0.74 ± 0.68	<LOD–0.76	<LOD–0.76	2.0 ± 0.78	1.4 ± 0.54	n.a.	n.a.
	ng g^{-1} lw	199 ± 31	16.06 ± 5.7	0.67 ± 0.30	3.0 ± 2.7	<LOD - 76	<LOD - 7.6	9.5 ± 3.7	6.7 ± 2.6		
HBB	ng g^{-1} ww	<LOD	<LOD	<LOD	<LOD	<LOD	LOD	LOD	<LOD	n.a.	n.a.
	ng g^{-1} lw										3.72 ± 4.06 pg g^{-1} lw
PBEB	ng g^{-1} ww	<LOD	<LOD	0.020 ± 0.010	0.022 ± 0.014	<LOD–0.027	<LOD–0.020	<LOD	<LOD	n.a.	n.a.
	ng g^{-1} lw			0.28 ± 0.19	0.086 ± 0.057	<LOD - 2.7	<LOD - 0.20				
PBT	ng g^{-1} ww	0.012 ± 0.0013	<LOD	<LOD	<LOD	0.023–0.19	0.027 ± 0.014	< LOD	<LOD– 0.12	n.a.	n.a.
	ng g^{-1} lw	1.2 ± 0.13				2.3–19	0.27 ± 0.14		<LOD– 0.57		0.91 ± 1.09 pg g^{-1} lw
∑DP	ng g^{-1} ww	<LOD - 0.32	<LOD - 0.46	0.041 ± 0.027	0.043 ± 0.048	< LOD	0.17 ± 0.092	0.19 ± 0.086	0.29 ± 0.20	n.a.	n.a.
	ng g^{-1} lw	<LOD - 31.8	<LOD–33.8	0.14 ± 0.085	0.17 ± 0.19		1.7 ± 0.92	0.90 ± 0.41	1.4 ± 0.95		66.9 ± 48.1 pg g^{-1} lw
DPMA	ng g^{-1} ww	< LOD	< LOD	< LOD	< LOD	< LOD	<LOD–0.037	0.070 ± 0.019	0.10 ± 0.016	n.a.	n.a.
	ng g^{-1} lw						<LOD–0.37	0.33 ± 0.090	0.48 ± 0.076		0.37 ± 0.57 pg g^{-1} lw
Dec-602	ng g^{-1} ww	< LOD	<LOD–0.66	<LOD–0.25	0.044 ± 0.048	<LOD	0.0070–0.29	2.7–1.2	0.26–2.4	n.a.	n.a.
	ng g^{-1} lw		<LOD–48.8	<LOD–0.73	0.18 ± 0.19		0.070–2.9	12.9–5.7	1.2–11.4		882 ± 515 pg g^{-1} lw
Dec-603	ng g^{-1} ww	<LOD	<LOD	<LOD	<LOD–0.076	< LOD	<LOD– 0.020	0.14 ± 0.015	0.12 ± 0.067	n.a.	n.a.

Table 1 (*continued*)

		Glass eels (Estuary, FR)	Elvers (Vidå, GER)	Yellow Eels (Elbe, GER)	Silver Eels(Elbe, Rhine, GER)	Glass eels (Estuary, CA)	Young Yellow Eels (LO, SLR, CA)	Yellow eels (LO, CA)	Yellow eels (SLR, CA)	Silver Eels (LO, CA)	Silver Eels (LO, CA)
	ng g^{-1} lw				<LOD–0.37		<LOD–0.20	0.67 ± 0.071	0.57 ± 0.32		12.4 ± 5.08 pg g^{-1} lw
f(syn)		0.94 ± 0.08	0.80 ± 0.14	0.97 ± 0.11	0.40 ± 0.09	n.a.	0.71 ± 0.34	0.64 ± 0.21	0.89 ± 0.17	n.a.	0.44
Average lipid content	%			1	1.4	27	25	1	10	21	23
20	20										
Ref.		this study	Sühring et al 2013	Sühring et al 2013	Sühring et al. 2013	this study	this study	this study	this study	Byer et al. 2013	Byer et al. 2013

* data on BDE-47 in American yellow eels is semi-quantitative.

2.4. Qa/Qc

Extraction and clean-up of juvenile European and American eels (glass eels and young yellow eels) were conducted in a clean lab (class 10,000). Adult American eels (yellow eels) were extracted in a regular laboratory. Materials containing FR were avoided during sample preparation and analysis.

Surrogate recoveries were determined for every sample. Mean recoveries were 58 ± 18% for ^{13}C–HBB, 130 ± 20% for ^{13}C–BDE-77, 117 ± 22% for ^{13}C–BDE-138, and 78 ± 23% for ^{13}C–DP. 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 processed in the clean lab were in general low; PBT, BDE-99 and BDE-183 were measured in one blank samples each at concentrations of 0.002 ng g^{-1} wet weight (ww), 0.0016 ng g^{-1} ww and 0.078 ng g^{-1} ww respectively. BDE-47 was detected in two blank samples at 0.088 ng g^{-1} ww and 0.24 ng g^{-1} ww. DPTE was detected in the majority of blank samples with average concentrations of 0.19 ± 0.036 ng g^{-1} ww. Samples processed at the regular laboratory showed greater contamination by technical Penta-PBDE and Octa-PBDE, with average concentrations between 0.12 ± 0.011 ng g^{-1} ww for BDE-66 and 1.75 ± 0.76 ng g^{-1} ww for BDE-47. Of the alternate BFRs, PBT, PBEB, and HBB were detected at average concentrations of 0.45 ± 0.12 ng g^{-1} ww, 0.075 ± 0.014 ng g^{-1} ww and 0.12 ± 0.0069 ng g^{-1} ww, respectively. DPTE was found in one blank sample at 0.12 ng g^{-1} ww. SynDP and antiDP were found in two and three blank samples with concentrations up to 0.14 ng g^{-1} ww and 0.21 ng g^{-1} ww, respectively. Blank concentrations were considered in the calculation of the sample concentrations and limit of detection (LOD) of the appropriate batch. In case of high blank values and detection frequencies, as e.g. in the case of DPTE, only samples with concentrations at least one order of magnitude higher than the average blank were considered in order to ascertain that concentrations found in the samples were environmental concentrations and not caused by contamination in the lab. The average blank value was then subtracted from the concentration found in the samples (see supplement information Tables S3 and S4 for a detailed list of blank values, LOD and LOQ).

The limit of detection (LOD) was calculated from a signal to noise ratio of three or by using the blank standard deviation method (where applicable). The limit of quantification (LOQ) was calculated from a signal-to-noise ratio of ten or using the blank standard deviation method (where applicable). For juvenile eels, LODs ranged from 0.0022 ng g^{-1} ww for BDE-66 to 0.45 ng g^{-1} ww for BDE-47. For adult American eels, LODs ranged from 0.005 ng g^{-1} - ww for BDE-153 to 4.03 ng g^{-1} ww for BDE-47 due to the higher average blank levels. The LOQ for juvenile European and American eels (glass eels and young yellow eels) ranged from 0.0073 ng g^{-1} - ww for BDE-66 to 1.51 ng g^{-1} ww for BDE-47. The LOQ for large American yellow eels ranged from 0.017 ng g^{-1} ww for BDE-153 to 13.45 ng g^{-1} ww for BDE-47. Due to the high blank levels, BDE-47 results for American yellow eels were considered semi-quantitative.

3. Results and discussion

Results for European yellow eels and silver eels were previously published in Sühring et al. (2013). Results for American silver eels were published by Byer et al. (2013). The average results for PBDEs, alternate BFRs, and Dechloranes from this study are compared to

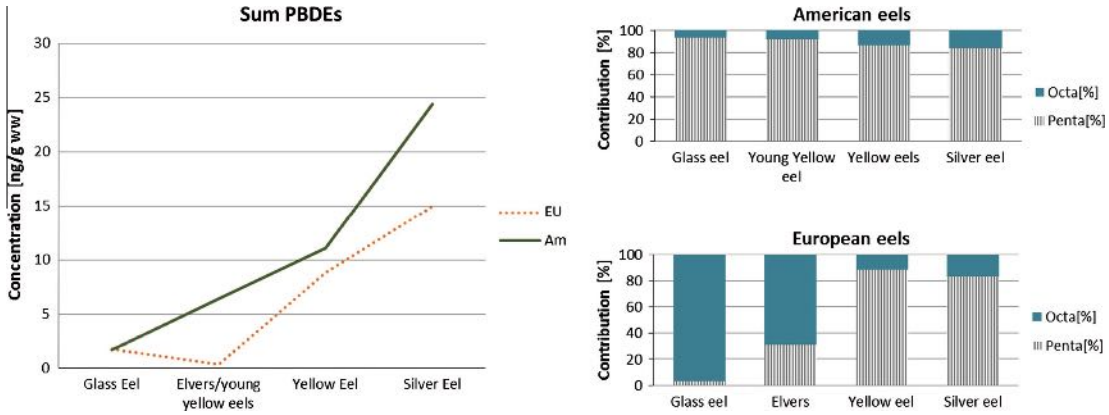

Fig. 1. Concentration [ng g^{-1} ww] of Sum PBDEs in American and European eels throughout their life cycle stages (left) and contribution [%] of technical Penta- and OctaBDE (right).

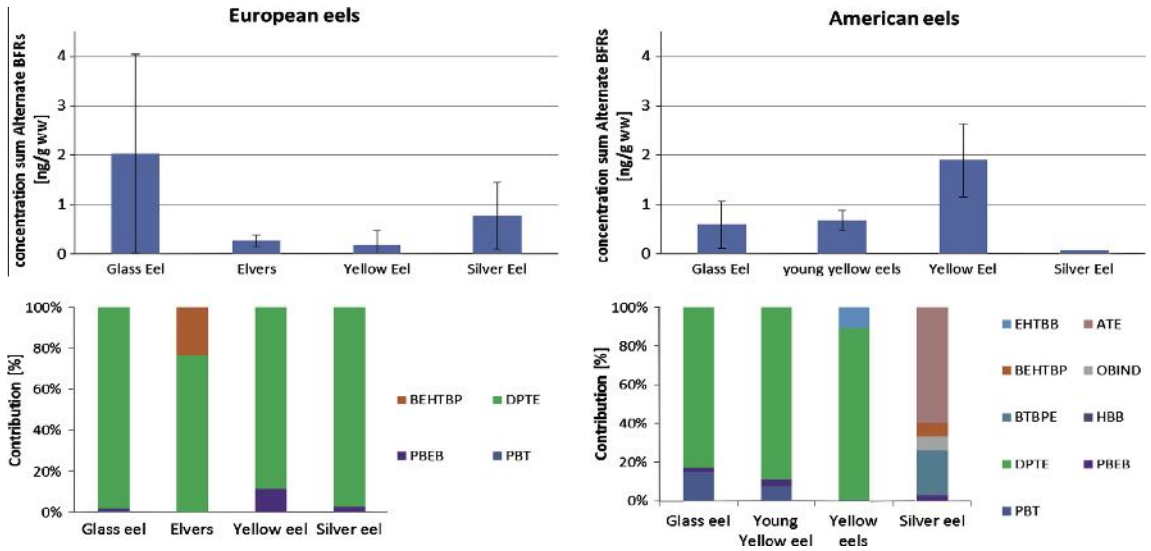

Fig. 2. Concentration [ng g^{-1} ww] of Sum Alternate BFRs (top) and contribution [%] of individual substances (bottom) to the different groups throughout the life cycle of European (left) and American (right) eels.

recent studies in Table 1. A detailed list of all results is provided in supplement information Tables S4 and S5.

3.1. PBDEs

The sum concentrations of PBDEs were similar in European and American glass eels (1.8 ± 0.89 ng g^{-1} ww and 1.7 ± 0.84 ng g^{-1} ww, respectively) yet more congeners were detected in American eels (Table 1).

The concentrations of congeners attributed to the technical Penta-PBDE mixture (BDE-47, BDE-99, BDE-100 and low amounts of BDE-153 and-154) were noticeably lower in European compared to American glass eels. More than 90% of PBDE in American glass eels was comprised of a technical Penta-PBDE mixture (Fig. 1). In contrast 97% of the PBDE contamination in European glass eels

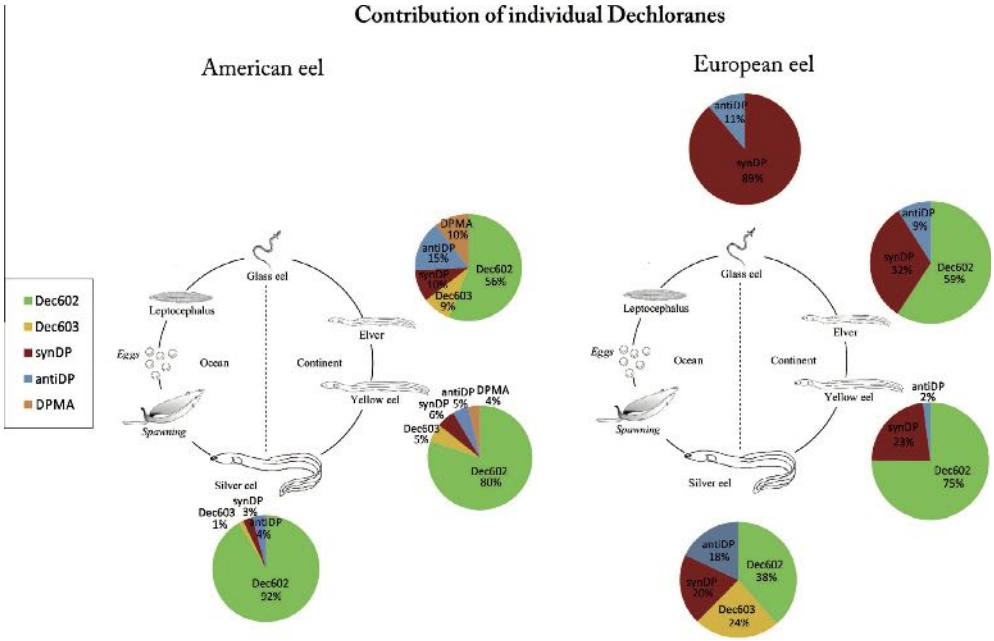

Fig. 3. Contribution [%] of individual Dechloranes to the Sum Dechlorane contamination in American (left) and European (right) eels throughout their life cycle stages (picture life cycle: Dekker, 2000).

consisted of BDE-183 and BDE-153, which are congeners of the technical Octa-PBDE mixture. The presence of technical Octa-PBDE in European glass eels has two possible explanations: The detected concentrations could indicate an on-going exposure to technical Octa-PBDE despite the restrictions. It could, however, also indicate an exposure to technical Deca-PBDE and subsequent debromination to lower brominated PBDE congeners as described by Eljarrat et al., 2011.

The difference in the congener pattern between American and European glass eels exhibits a fundamental difference between the contamination glass eels are exposed to in the European and American coastal environments. The low Penta-PBDE concentrations in European glass eels might indicate that restrictions on importation and use of technical Penta-PBDE in the European Union are having an effect on environmental inputs. The continued application of technical Deca-PBDE, on the other hand, could be the reason for the high contribution of its debromination product BDE-183 The high contribution of technical Penta-PBDE in American glass eels reflects its historically higher use in North America compared to the EU (7100 T/a in North American vs 150 T/a in the EU in 2001 (BSEF 2013)), but can also be an indication for continued emissions. This would be congruent with the findings of Csiszar et al. (2013), who estimated Penta- and Octa-PBDE (BDE-28, -47, -100, -154, -183) emissions into the air of Toronto in 2008 to be 18 kg y^{-1}. They concluded, that, despite the restrictions, many buildings, homes and vehicles were still equipped with Penta- and Octa-PBDE containing materials, making them possible contamination sources (Csiszar et al., 2013). Higher current as well as historical emissions along with the persistence of PBDEs lead to generally higher concentrations in the aquatic environment in North America (US EPA, 2010). However, the up to 16× lower PBDE concentrations in both European and American glass eels compared to the other life stages indicate that the primary uptake of PBDEs occurs in the later life stages. The uptake of PBDEs is therefore probably driven by ingestion or dermal uptake due to contact with sediments, because eels become more predatory with size (before they stop feeding in their silver stage) and become benthic during their yellow eel stage (Tesch and Thorpe, 2003, p. 152). The primarily pelagic glass eels (Tesch and Thorpe, 2003, p. 122) are therefore mostly exposed to contamination through water, plankton, suspended matter or maternal transfer.

Technical Penta-PBDE was the predominant analysed flame retardant in European and American yellow and silver eels, contributing 89–92% and 86–91% of PBDEs, respectively (Fig. 1), reflecting its persistence in the environment and biota. In general, the congener profile followed distributions reported in previous studies (Belpaire, 2008) with an order of abundance of BDE-47 > BDE-100 > BDE-153 > BDE-99 > BDE-154 > BDE-183. High concentrations of BDE-47 were expected due to its high uptake rate and biomagnification within the aquatic food web (Domínguez et al., 2011), as well as its formation via enzymatic debromination of higher PBDEs during metabolism in fish (Eljarrat et al., 2011). However, in young American yellow eels, BDE-100 and BDE-47 were found in similar concentrations (2.9 ± 0.93 ng g^{-1} ww and 2.8 ± 1.8 ng g^{-1} ww respectively) indicating a continued exposure of juvenile eels to congeners from the technical Penta- and Octa-PBDE mixtures. PBDE concentrations increased significantly (significant trend at 99% confidence level according to Neumann trend test) over the life cycle, consistent with the bioaccumulation of PBDEs (Fig. 1).

3.2. Alternate BFRs

DPTE was detected in European eels of all life cycle stages analysed with average concentrations of 1.1 ± 0.31 ng g^{-1} ww in glass eels, n.d.–1.7 ng g^{-1} ww in yellow eels and 0.12–2.4 ng g^{-1} ww in silver eels. In American eels, DPTE was detected in the majority of the glass eel samples with up to 0.76 ng g^{-1} ww, and all yellow eel samples (Saint Lawrence River and Lake Ontario) with a mean of 1.68 ± 0.73 ng g^{-1} ww. However, DPTE was only detected in two of the young American yellow eel samples indicating that the contamination was not driven by life stage or age of the eel, but rather by local contamination sources such as e.g. contaminated sediments. European silver eels showed similar concentrations of DPTE (0.12–2.4 ng g^{-1} ww) to yellow eels indicating that this substance does not accumulate strongly throughout the life cycle, has been reintroduced recently, or is metabolised and excreted as soon as the eels stop feeding in their silver eel stage. The high concentration and abundance in American and European glass eels supports the hypothesis enunciated in our previous study that the uptake of DPTE happens in estuaries as well as rivers and is mostly driven by local contamination sources and not by age or life stage of individual eels (Sühring et al., 2013). It could, however, also be an indication for maternal transfer of DPTE. There are no data on current DPTE production (Vetter et al., 2010). However, it is thought to be persistent in sediments, a possible source of DPTE contamination for aquatic species (Fisk et al., 2003). The higher concentrations and abundance in European eels can be explained by its former production and application in Germany (Vetter et al., 2010).

PBEB was also detected in European eels with similar average concentrations in different life stages, yet the frequency of detection increased with life stage. A variety of alternate BFRs were detected in American eels of all life stages, in lower concentrations than DPTE (Fig. 2). In American glass and young yellow eels, the pattern of alternate BFRs concentrations was similar, with DPTE > PBT > PBEB. Byer (2013) reported a different distribution and more substances by high-resolution mass spectrometry in electron ionisation mode, but lower concentrations in American silver eels; the order of concentrations was ATE > BTBPE > O-BIND > TBPH > PBEB > HBB > PBT. The difference in patterns might be due to differences in the analytical process especially because most alternate BFRs were detected in concentrations close to the limit of detection. In yellow eels from the upper Saint Lawrence River, TBB was detected in the majority of the samples, suggesting proximity to a point source (Table 1).

In general, it was concluded that the contamination patterns of alternate BFRs were induced by local contamination sources. The high frequencies of specific compounds at specific locations indicated that American eels were exposed to point sources. A possible source close to the American eel sampling sites is the OxyChem manufacturing facility at Niagara Falls, NY, which is known to produce flame retardants such as Dechlorane Plus (Sverko et al., 2011). Other sources at Lake Ontario could be wastewater treatment plants of the major urban centres Toronto and Hamilton. In European eels there was no characteristic contamination pattern at specific sampling sites, indicating an exposure to diffuse sources (Sühring et al., 2013). Possible sources could be e.g. diffuse emissions from waste incineration plants or leaching from consumer products. The high contributions of alternate BFRs to the sum contamination in both American and European glass eels compared to the older life stages emphasise the increasing relevance of these compounds since the phase-out and restriction of PBDEs.

3.3. Dechloranes

In general, Dechlorane concentrations were highest in American yellow eels from Lake Ontario (1.7–5.0 ng g^{-1} ww), mostly driven by Dec-602 concentrations. Along the Saint Lawrence River, Dec-602 concentrations decreased towards the Atlantic Ocean, suggesting a source close to or at Lake Ontario (possibly OxyChem in Niagara Falls, NY, who are a known producer of DP (Sverko et al., 2011)).

DP concentrations were highest in yellow eels from the upper Saint Lawrence River (0.10–0.69 ng g^{-1} ww). In European eels, Dechlorane concentrations were similar in yellow and silver eels (0.013–0.50 ng g^{-1} ww in yellow eels and 0.017–0.38 ng g^{-1} ww in silver eels), suggesting that these eels were exposed to diffuse sources rather than to a specific point source. The overall contamination pattern was similar in European and American yellow and silver eels, with Dec-602 > DP > Dec-603 > DPMA (DPMA was only detected in American eels). This concurred with distributions reported in previous studies (Shen et al., 2010). The variability among samples, on the other hand, was higher for European eels, whereas a greater number of Dechloranes were detected in American eels (Fig. 3).

The high contribution of Dec-602 in European eels was unexpected, because it is not produced or imported to the EU. Even in North America (close to production facilities), it is only listed in the Non-domestic Substances List published by Environment Canada (http://www.ec.gc.ca/CEPARegistry/subs_list/NonDomestic.cfm). This indicated that Dec-602 is used internationally, but to date is not considered a substance of high priority or concern. However, Dec-602 has been reported to have a high bioaccumulation potential (higher for example than DP) and to be very bioavailable (Shen et al., 2011). Glass eels did not contain detectable concentrations of Dec-602, but it was the predominant Dechlorane in all other life cycle stages, suggesting little uptake during the oceanic phase of the eel. To determine how quickly Dec-602 becomes the major Dechlorane contaminant, the results of glass and adult eels were compared with the concentration in young American yellow eels and concentrations previously found in European elvers (Sühring et al., 2013). Elvers that had been in freshwater for less than a year already showed a predominance of Dec-602 (59% of total Dechlorane contamination). In American eels a similar progression was observed with no Dec-602 in glass eels and a relative contribution of 56% Dec-602 to total Dechlorane contamination in young yellow eels. This indicated a rapid uptake when juvenile eels enter their freshwater phase (Fig. 3).

DP was detected in all analysed life stages of the European eel and all adult American eels (Fig. 3). Of the two stereoisomers (syn- and antiDP), synDP was predominant in glass and yellow eels, with 96% relative contribution in European and 72% relative contribution in American yellow eels, respectively. These findings matched observations from previous studies indicating that synDP bioaccumulates and biomagnifies in fish to a greater extent than antiDP (Wu et al., 2010; Shen et al., 2011). However, the two isomers had a similar relative contribution to sum DP in European and American silver eels (60% and 56% respectively). This significant change in the isomer ratio over the life cycle of eels and from the technical product (75% antiDP; Sverko et al., 2011) has several implications. It confirms the assumption that synDP is the more bioaccumulative isomer in yellow eels. In contrast, when eels have stopped feeding in their silver phase, there seems to be either an uptake of antiDP via gills and skin, or a faster elimination of synDP from muscle tissue. Elimination could be induced by metabolism, excretion or redistribution of synDP to other fatty tissues such as gonads (Peng et al., 2012).

DPMA was detected in American yellow eels only, but was reported in American silver eels from a similar area (Byer et al. (2013)).

4. Conclusions

This study described the bioaccumulation of PBDEs over the life cycle of both American and European eels. Additionally, it was concluded that concentrations of alternate BFRs and Dechloranes were mostly driven by location and not by life stage. Contamination of American eels was likely caused by point sources in Lake Ontario or the upper Saint Lawrence River. In contrast, European eels seemed to be exposed primarily to diffuse sources, with no specific trend in the contamination pattern. In both American and European eels DPTE was a major contaminant, indicating existing sources and a continued release to the environment. Bans on the use of Penta-PBDE in the EU are effectively reducing PBDE contamination of juvenile eels. A significant increase of Dec-602 concentrations in the eel's freshwater phase was observed consistent with its high bioavailability and bioaccumulation potential.

In general, this study showed the relevance of continued monitoring of PBDE contamination in eels, and the emerging importance of contamination by alternate BFRs and Dechloranes. Further research is needed to identify the sources of contamination of compounds with no official record on production or application such as DPTE and Dec-602. It should also be investigated if the contaminations found in juvenile eels were caused by maternal transfer, as the transfer of BFRs to offspring could be a critical reason for concern.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2013.10.096.

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Annex III

Evidence for High Concentrations and Maternal Transfer of Substituted Diphenylamines in European eels Analyzed by Two-Dimensional Gas Chromatography–Time-of-Flight Mass Spectrometry and Gas Chromatography–Fourier Transform Ion Cyclotron Resonance Mass Spectrometry

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Evidence for High Concentrations and Maternal Transfer of Substituted Diphenylamines in European Eels Analyzed by Two-Dimensional Gas Chromatography−Time-of-Flight Mass Spectrometry and Gas Chromatography−Fourier Transform Ion Cyclotron Resonance Mass Spectrometry

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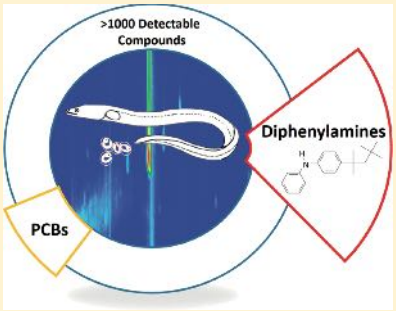
Supporting Information

ABSTRACT: Chemical pollution is hypothesized to be one of the factors driving the strong decline of the critically endangered European eel population. Specifically, the impact of contaminants on the quality of spawning eels and subsequent embryo survival and development has been discussed as crucial investigation point. However, so far, only very limited information on potential negative effects of contaminants on the reproduction of eels is available. Through the combination of nontargeted ultrahigh-resolution mass spectrometry and multidimensional gas chromatography, combined with more-conventional targeted analytical approaches and multimedia mass-balance modeling, compounds of particular relevance, and their maternal transfer in artificially matured European eels from the German river Ems have been identified. Substituted diphenylamines were, unexpectedly, found to be the primary organic contaminants in the eel samples, with concentrations in the $\mu g\ g^{-1}$ wet weight range. Furthermore, it could be shown that these contaminants, as well as polychlorinated biphenyls (PCBs), organochlorine pesticides, and polyaromatic hydrocarbons (PAHs), are not merely stored in lipid rich tissue of eels but maternally transferred into gonads and eggs. The results of this study provide unique information on both the fate and behavior of substituted diphenylamines in the environment as well as their relevance as contaminants in European eels.



1. INTRODUCTION

The European eel (*Anguilla anguilla*) is regarded as a critically endangered species.[1,2] Scientists agree that the "quality of spawners" is a vital factor for the survival of the species[1] and that its impairment might be one of the reasons for the strong decline of juvenile eel (glass eel) recruitment during the last three decades. "Quality of spawners" is, in this context, defined as the health status of mature silver eels migrating back to their spawning ground in the Sargasso Sea and their ability to produce healthy offspring.[1]

Halogenated contaminants have been postulated as potential compounds of high concern for the quality of spawning eels.[3] They are suspected to affect the eel's lipid metabolism, decrease its ability to reproduce, or affect the viability of offspring.[3]

Our recent study[4] reported that the majority of brominated and chlorinated flame retardants were maternally transferred into gonads and eggs of artificially matured silver eels.

The targeted analysis of the maternal transfer of potentially hazardous compounds in European eels has been a unique opportunity to gather more information on the potential impact of contaminants on the quality of spawning eels and their developing gonads and eggs. However, the approach of targeted analysis only provides information on a selection of compounds, which means that important, not-targeted, compounds might be missed.

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Table 1. Analyzed Substituted Diphenylamines[a]

compound	formula	MW	log K_{OW}	log K_{AW}	MRM transitions	RT (min)
diphenylamine (DPA)	$C_{12}H_{11}N$	169	3.5	−3.9	169→167	16.8
monostyrenated DPA 1	$C_{20}H_{19}N$	273	5.5	−5.3	273→180	23.34
monooctyl-DPA	$C_{20}H_{27}N$	281	7.3	−2.2	281→210	23.42
monostyrenated DPA 2	$C_{20}H_{19}N$	273	5.5	−5.3	273→258	25.46
monooctyl-monostyrenated -DPA 1	$C_{28}H_{35}N$	385	9.2	−4.4	385→314	27.21
monooctyl-monostyrenated -DPA 2	$C_{28}H_{35}N$	385	9.2	−4.4	385→314	28.20
dioctyl-DPA	$C_{28}H_{43}N$	393	11		393→322	28.47
monooctyl-monostyrenated -DPA 3	$C_{28}H_{35}N$	385	9.2	−4.4	385→314	30.25
dioctyl-monostyrenated -DPA	$C_{36}H_{51}N$	497	13		497→496	31.15

[a]The summary includes compound name, structural formula, molecular weight (MW) octanol−water partitioning coefficient (log K_{OW}), air−water partitioning coefficient (log K_{AW}), and MRM transitions and retention time (RT) [min]. Partitioning coefficients were estimated using EPISuite KOWWIN v1.67 and HENRYWIN v3.10.[13].

To gather the necessary understanding on the importance of different organic contaminants in the contamination and maternal transfer of European eels, selected samples of artificially matured eels through hormonal treatment and a nontreated comparison group from the same habitat were analyzed by two-dimensional gas chromatography coupled with time-of-flight mass spectrometry (GC×GC−ToF-MS) and gas chromatography Fourier transform ion cyclotron resonance mass spectrometry (GC−FTICR-MS). Selected identified compounds were subsequently analyzed by gas chromatography tandem mass spectrometry (GC−MS/MS) to quantify levels in samples.

The focus of all analysis steps was on nonpolar compounds because eels have been reported to accumulate lipophilic contaminants especially during their continental life phase[5,6] due to their high body fat content (up to 40% of total body weight)[7] and their longevity.

2. MATERIALS AND METHODS

2.1. Sampling and Sample Preparation. Sampling and sample preparation as well as the artificial maturation process have been described in Sühring et al.[4] For the nontarget analysis, three female eels from the German river Ems, caught at the onset of migration and treated with salmon pituitary extract (SPE) to induce maturation, were used. Additionally, two female eels without hormone treatment from the same habitat were sampled to assess the initial contamination situation in the habitat, control for potential contamination through the hormone treatment process, and gather information on the potential impact of maturation on the contaminant distribution within the eel. Muscle and gonad tissue (3 g each) as well as 5 g eggs (in the case of artificially matured eels) were sampled in duplicate and stored at −20 °C until analysis. For targeted analysis, three additional female eels were sampled to increase the comparison group.

Extraction and cleanup methods are described in Sühring et al.[8] The frozen samples were homogenized with anhydrous sodium sulfate and extracted by pressurized liquid extraction (ASE-200, Dionex, Sunnyvale, CA) using dichloromethane (DCM, ROTH, Karlsruhe, Germany) at 100 °C and 120 bar. All samples were spiked with ^{13}C isotope labeled polychlorinated biphenyl standards (PCB-77, -81, -105, -114, -118, -126, -156, -157, -167, -169, -189, -170, and -180 cleanup standards ($^{13}C_{12}$, 99%), Cambridge Isotope Laboratories (Tewksbury, MA)) prior to extraction. Clean-up consisted of a gel permeation chromatography with hexane−DCM (1:1, v/v) and a 10% deactivated silica gel column cleaned with hexane. The lipid content of samples was determined gravimetrically from separate aliquots following a method described in Sühring et al.[8]

2.2. Instrumentation. *GC×GC−ToF-MS.* Samples and blanks were analyzed in accordance with the method reported in Pena-Abaurrea et al.[9] using a Pegasus 4D (Leco Corp., St. Joseph, MI) consisting of a modified GC×GC Agilent 6890 chromatograph and a ToF-MS with electron ionization (EI) mode, fitted with a Rtx-5MS × BPX-50 column set (30 m × 0.25 mm internal diameter (i.d.) × 0.25 μm film thickness and 1.6 m × 0.15 mm i.d. × 0.15 μm film thickness, respectively). A nitrogen quad-jet dual-stage modulator was used for sample focusing and reinjection in the secondary column.

FTICR-MS. Samples and blanks were analyzed in accordance with the method reported in Ortiz et al.,[10] using a Varian gas chromatography−triple-quadrupole−Fourier transform ion cyclotron resonance mass spectrometer (GC−QQQ−FTICR-MS) (Varian Inc., Walnut Creek, CA). The GC was fitted with a DB5-MS capillary column (40 m × 0.18 mm i.d. × 0.18 μm film thickness) from Agilent (Santa Clara, CA). Samples were injected (1 μL) in splitless mode with helium as carrier gas. The mass spectrometer was operated in the EI mode (70 eV) and was set to pass all ions. The FTICR-MS was operated at a resolving power of 100 000 to 150 000 (fwhm). Mass spectra were obtained using arbitrary waveform excitation and broadband detection from m/z 75 to 650. Detection and cycle times were set at 524 ms and 1.5 s, respectively. External mass calibration was performed using perfluorotributylamine, and internal mass calibration was performed using protonated diisononyl phthalate (background ion) at m/z 419.315 60.

GC−MS/MS. Samples and blanks were analyzed by gas chromatography with tandem mass spectrometry, GC−MS/MS (Agilent QQQ 7010) in EI mode. The instrument was fitted with a HP-5MS column (30 m × 0.25 mm i.d. × 0.25 μm film thickness; J&W Scientific) with helium (purity 99.999%) as carrier gas and nitrogen as collision gas. The instrument was operated in multiple reactions monitoring mode (MRM) at 70 eV ionization energy. A technical benzamine, *n*-phenyl-, reaction products with styrene, and a 2,4,4-trimethylpentene (BNST) standard (AK Scientific Inc., Union City, CA; lot TC36296) were used to quantify substituted diphenylamines in the eel samples with ^{13}C isotope labeled PCB standards as an indication how much analyte was lost during extraction and cleanup. A total of nine diphenylamine reaction products with styrene and 2,4,4-trimethylpentene were analyzed (Table 1).

2.3. Quality Assurance and Quality Control. Extraction and cleanup were conducted in a clean lab (class 10 000). A

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Environ. Sci. Technol. 2016, 50, 12678−12685

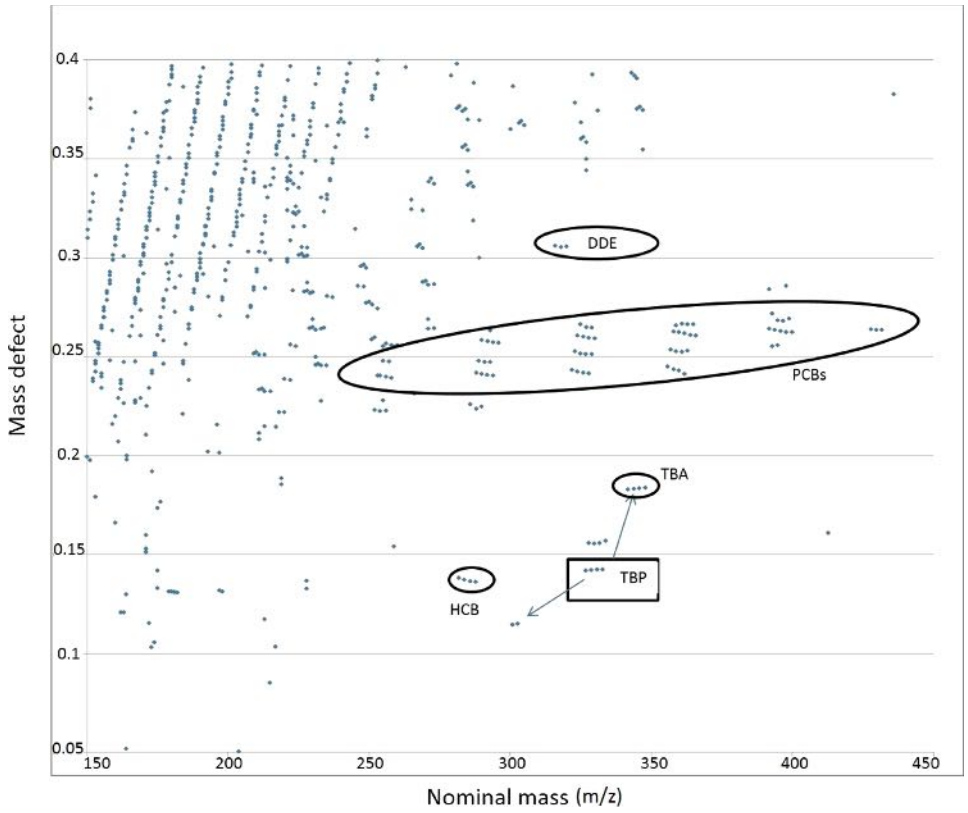

Figure 1. Kendrick plot displaying results of a muscle sample of a hormone treated eel from river Ems. The mass defect (*y*-axis) is plotted as a function of nominal mass (*x*-axis) in a mass scale based on the exact and IUPAC mass of Cl, as defined in Taguchi et al.[12]

blank test using Na_2SO_4 treated similar to real samples was conducted with every extraction batch (five samples). Solvent blanks were run between experimental runs to ensure no carryover between samples. Recoveries for the ^{13}C PCB standards ranged from 57 ± 26% for PCB 81 to 96 ± 34% for PCB 169. However, due to the impurity of the technical standard and the lack of accurate isotope labeled reference standards, only the order of magnitude (i.e., pg g^{-1}, ng g^{-1}, or μg g^{-1}) could be determined.

2.4. Data Analysis. Analysis of GC×GC−ToF-MS results was performed using ChromaToF software (version 4.50). FTICR spectra were analyzed using Varian Omega (version 9.1.21). Elemental compositions were assigned through a combination of custom macros for Excel (GC×GC−ToF-MS) described in Pena-Abaurrea et al.,[9] analysis through Kendrick mass defect plots[11] as well as the Elemental Composition Calculator (Varian Inc.) and subsequent library matching (similarity).

Kendrick mass defect plots utilize the convention by the International Union of Pure and Applied Chemistry (IUPAC) who defined a mass scale based on the carbon exact mass (C = 12.000 00 Da). By the use of a mass scale based on CH_2 (i.e., the Kendrick mass scale, where CH_2 = 14.000 00 Da), a homologous series of hydrocarbons can be grouped on the basis of their difference between exact mass and nominal mass (mass defect).[11] This principle can be applied similarly to chlorinated analogue series. The resulting Kendrick mass defect can then be plotted against the nominal mass, resulting in simplified visualizations of large data sets in which compounds containing halogens are aligned horizontally.[12]

GC−MS/MS data were analyzed using Agilent Technologies MassHunter Workstation Software Quantitative Analysis B.06.00. Further data analysis was performed with Microsoft Office Excel 2010.

Physical and chemical properties of substituted diphenylamines, namely octanol−water and air−water partitioning coefficients (log K_{OW} and log K_{AW}, respectively) were estimated using EPISuite KOWWIN v1.67 and HENRYWIN v3.10.[13] Distribution of substituted diphenylamines into different environmental media was estimated using the Level III multimedia mass balance model (fugacity model) by Mackay et al.[14]

3. RESULTS AND DISCUSSION

3.1. From "Usual Suspects" to Unexpected Findings. An analysis of the GC×GC−ToF-MS results and Kendrick plots of the FTICR-MS data first returned a compound spectrum of what could be called "usual suspects" in the analysis of organic contaminants in eels. Both Kendrick Plots and GC×GC−ToF-MS chromatograms were dominated by polychlorinated biphenyls (PCBs) (Figure 1).

Furthermore, the dichlorodiphenyltrichloroethane (DDT) transformation product dichlorodiphenyldichloroethylene (DDE) and the fungicide hexachlorobenzene (HCB) could

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Environ. Sci. Technol. 2016, 50, 12678−12685

Table 2. Summary of Commonly Detected Contaminants in Muscle, Gonads, and Eggs (Number of Samples) Of Hormone-Treated Eels Analyzed with GC×GC−ToF-MS, Including Retention Time (s) in 1D and 2D Dimensions

compounds		retention time (s)		detection frequency (%)		
	m/*z*	1D	2D	muscle (5)	gonads (5)	eggs (3)
phenylethene	178.15	852	2.34	100	100	100
1,8-dichloronaphthalene	197.06	750	1.86	100	100	83
pyrene	202.25	1014	3.37	100	100	100
fluorenthene	202.26	1062	3.78	100	100	100
hexachlorobenzene	284.80	822	1.75	100	100	100
dichlorodiphenyldichloroethylene	318.02	1092	2.86	100	100	100

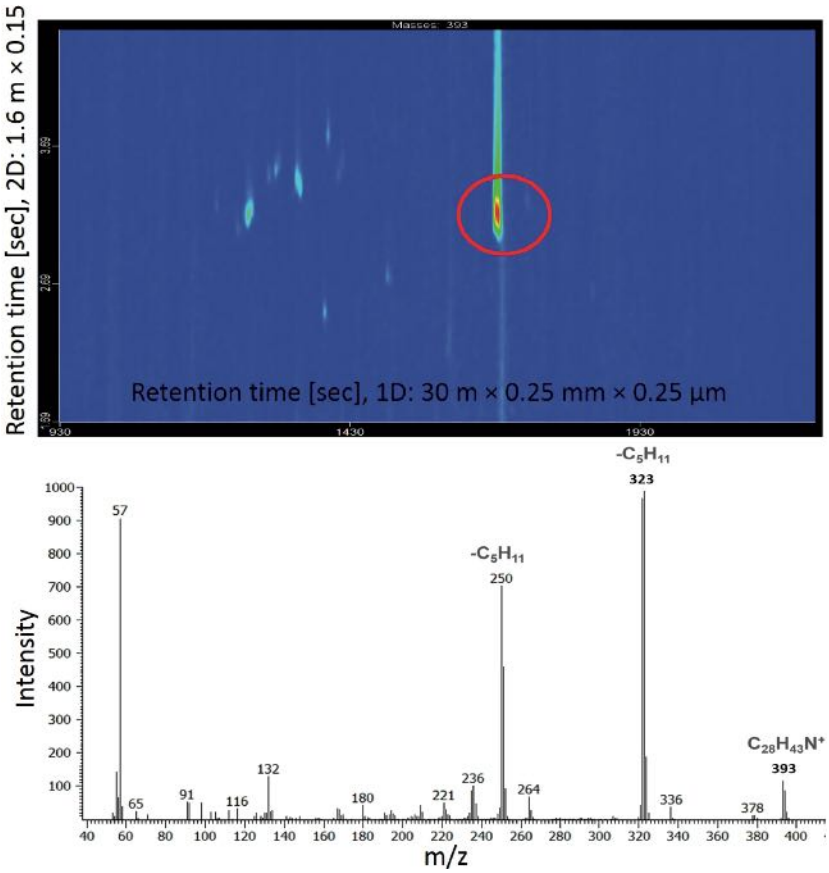

Figure 2. GCxGC chromatogram (top) and mass-spectrum for the peak marked by the red circle (bottom) of nonhalogenated compound (*m*/*z* 393) with high abundance in GC×GC−ToF-MS analysis of gonads of artificially matured eels.

be detected in every sample (Table 2). The polyaromatic hydrocarbons (PAH) pyrene and fluoranthene, as well as phenylethene (styrene) and 1,8-dichloronaphtalene (PCN-9) were detected in the majority of samples using the custom macros for Excel (GC×GC−ToF-MS) described in Pena-Abaurrea et al.[9] (Table 2). The brominated flame retardant tribromophenole (TBP) as well as its transformation product tribromoanisole (TBA) were identified during FTICR-MS analysis and could primarily be detected in muscle tissue (Figure 1). The presence of these compounds in eels from Europe as well as North America has been reported previously.[3,5,6,15−19] However, the detection of all of these contaminants in gonads and eggs of eels further underlined the necessity of controlling and monitoring these hazardous chemicals. A closer analysis of the compound pattern found in gonads of artificially matured eels revealed a new picture of chemicals of potential concern for the quality of spawning eels.

3.2. Detection and Characterization of Substituted Diphenylamines. Unexpectedly, high abundance compared to other detected compounds of substituted diphenylamines was found in GC×GC−ToF-MS measurements of gonads of artificially matured eels for nonhalogenated compounds with primary fragments at *m*/*z* 210 and 393, respectively, using the

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Environ. Sci. Technol. 2016, 50, 12678−12685

Table 3. Overall Summary of Relative Contribution (%; Average ± Standard Deviation) of Compounds of the BNST Mixture in the Technical Mix[a]

		hormone-treated eel			comparison group	
	technical mixture	muscle (n = 9)	gonad (n = 9)	egg (n = 9)	muscle (n = 10)	gonad (n = 10)
DPA	n.a.	1.3 ± 0.096	0.24 ± 0.67	8.8 ± 6.0	8.1 ± 24	33 ± 25
monostyrenated DPA 1,2	9	1.2 ± 21	0.76 ± 17	48 ± 28	71 ± 59	15 ± 6
monooctyl-DPA	30	0.075 ± 1.4	16 ± 8.9	7.7 ± 0.022	2.2 ± 2.1	7.6 ± 4
monooctyl-monostyrenated DPA 1−3	8−10	0.019 ± 0.34	0.039 ± 0.020	13 ± 14	1.8 ± 1.4	n.d.
dioctyl-DPA	19	97 ± 23	83 ± 15	19 ± 11	17 ± 34	45 ± 15
dioctyl-monostyrenated DPA	15	n.d.	0.0090 ± 0.0052	2.4 ± 2.2	n.d.	n.d.

[a]Each three samples of muscle, gonads, and eggs of from three hormone-treated eels as well as each two samples of muscle and gonads of the five comparison-group eels. The total number of analyzed samples (including replicates) is depicted as n. n.a.: not applicable; n.d.: not determined.

elemental composition calculator and library matching (similarity) (Figures 2 and S1).

GC−FTICR-MS measurements confirmed a high abundance of nonhalogenated compounds at the respective m/z fragments. Through elemental composition assignment, using a combination of custom macros for Excel, the Elemental Composition Calculator (Varian Inc.), and library matching (Figure S1), it was possible to identify the two compounds as monooctyl-DPA (m/z 281 and 210) and dioctyl-DPA (m/z 393 and 323) with $\Delta m/z$ < 2 ppm for the elemental composition search (Figure S1). Both of these compounds are integral parts of benzamine, n-phenyl-, reaction products with styrene, and 2,4,4-trimethylpentene (BNST). BNST (CAS 68921-45-9) is a high-volume production chemical with production and import of over 10 000 tons yearly in 2006 in Canada[20] and 100−1000 tons per year in the European Union.[21] It is used as additive antioxidant in vehicle engine oil and in some commercial and industrial lubricants.[22]

A final screening assessment report by Environment Canada[23] concluded that BNST is highly persistent and bioaccumulative and "...is entering or may be entering the environment in a quantity or a concentration or under conditions that have or may have an immediate or long-term harmful effect on the environment or its biological diversity" (ref 22, section 1.2, first paragraph). This assessment led to the addition of BNST to the Prohibition of Certain Toxic Substances Regulations,[24] leading to prohibitions of its use, sale and offer for sale in Canada by March 2013.[25] Exemptions include the use in vehicle engine oils and commercial and industrial lubricants until March 2015 and the manufacture, sale, use, and import as additive in rubber (except tires) as well as in products such as vehicle engine oil intended for personal use only.[25] In the European Union, BNST has been registered under REACH, classifying it as "very persistent" (vP) but lacking evidence for bioaccumulation or toxicity.[21] One of the key missing factors, at present, is the limited experimental data on BNST in biota. The detection in European eels could therefore significantly increase and impact the information regarding environmental fate and behavior of BNST.

To verify the presence of BNST and related substituted diphenylamines in the eel samples, minimize potential errors due to blank contamination or carry-over, and get information on the magnitude of substituted diphenylamines present in different tissue types of eels, all samples and laboratory blanks were analyzed with GC−MS/MS using the technical substituted diphenylamines mixture for identification and calibration. Considering the impurity of the standard and the lack of isotope-labeled reference standards, it was only possible to determine the relative order of magnitude (i.e., pg g^{-1}, ng g^{-1}, or $\mu g\ g^{-1}$) of substituted diphenylamines in the analyzed samples. Highest levels were detected in gonads of the artificially matured eels with concentrations in the $\mu g\ g^{-1}$ wet weight range for dioctyl-DPA and monooctyl-DPA. In the gonads and muscle tissue of nonhormone treated eels, as well as eggs, dioctyl-DPA and monooctyl-DPA concentrations were in the ng g^{-1} wet weight range, with the highest concentrations in gonads followed by muscle tissue and eggs. Other BNST components were in the >10 ng g^{-1} wet weight range for most tissue types. Exemptions were monooctyl-monostyrenated DPA 3 and dioctyl-monostyrenated DPA that were detected in concentrations below 1 ng g^{-1} wet weight for the majority of tissue types.

Even with the limitations regarding quantitative analysis using a technical mixture as standard, the magnitude of substituted diphenylamines in the European eel samples exceeded concentrations reported for halogenated contaminants such as flame retardants (HFRs) and polychlorinated biphenyls (PCBs).[19] PCB concentrations measured in the same samples were in the ng g^{-1} wet weight range. This implies that concentrations of substituted diphenylamines in eels from the comparably small and remote river Ems not only exceeded the PCB concentrations in the same samples but also are in the same range or even higher than concentrations of PCBs and organochlorine pesticides reported in eels form the known highly polluted river Scheldt in Belgium[14] or Lake Ontario in Canada.[16]

3.3. Patterns and Tissue Distribution of the Detected Substituted Diphenylamines. The pattern of substituted diphenylamines differed strongly between the composition of the technical mixture analyzed via GC−MS/MS, the hormone-treated eels, and the comparison group, as well as among the analyzed tissue types.

The technical mixture was found to primarily contain monooctyl-DPA (approximately 30%), dioctyl-DPA (approximately 19%), dioctyl-monostyrenated DPA (approximately 15%), and monooctyl-monostyrenated DPA (approximately 10%), as well as smaller amounts (5−10% each) of monostyrenated DPA and the monooctyl-monostyrenated DPA isomers (Table 3). Observed patterns in eel tissue, however, did not resemble the composition of the technical mixture but rather showed strong accumulation of individual compounds (Table 3).

In the muscle and gonads of hormone-treated eels, dioctyl-DPA was predominant with relative contributions of 97 ± 23% and 83 ± 15%, respectively. In eggs, however, monostyrenated DPA was predominant with 48 ± 28% contribution, followed by dioctyl-DPA (19 ± 11%) and monooctyl-monostyrenated DPA (13 ± 14%). Patterns in muscle samples from the

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Environ. Sci. Technol. 2016, 50, 12678−12685

comparison group also primarily displayed contamination with monostyrenated DPA (71 ± 59%), followed by dioctyl-DPA (17 ± 34%). Gonad tissue of comparison group eels was the only tissue type with a significant contribution of DPA (33 ± 25%). Apart from DPA, it displayed patterns close to muscle and gonads of hormone treated eels, with 45 ± 15% dioctyl-DPA, followed by monostyrenated DPA (15 ± 6%) (Table 3).

These differences in patterns could indicate different persistence of the substituted diphenylamines in the environment, transformation processes during uptake and distribution in the eel's body, and transformation or redistribution processes during the maturation process as well as different uptake and distribution processes, depending on the properties of the compound.

Table 4. Overview of the Fugacity III Model Predictions of Partitioning (%) of Substituted Diphenylamines into Air, Water, Soil, and Sediment, Assuming 100% Emission into Water

compound	% air	% water	% soil	% sediment
DPA	0	96	0	4
monostyrenated DPA 1,2	0	95	0	5
monooctyl-DPA	0	4	0	96
monooctyl-monostyrenated DPA 1−3	0	2	0	98
dioctyl-DPA	0	94	0	6
dioctyl-monostyrenated DPA	0	97	0	3

Physical and chemical properties of the analyzed substituted diphenylamines varied strongly with estimated octanol−water partitioning coefficients (log K_{OW}) between 3.5 (DPA) and 13 (dioctyl-monostyrenated DPA) and estimated air−water partitioning coefficient (log K_{AW}) between −5.3 (monostyrenated DPA) and −2.2 (monooctyl-DPA) (Table 1).

This work used the Fugacity III model developed by Mackay et al.[14] to estimate distribution of substituted diphenylamines in air, water, soil, or sediments assuming emissions into air (scenario 1) or water (scenario 2). This distribution into different environmental media could provide indications of potential sources of substituted diphenylamines for eels and could furthermore give indications on partitioning behavior of the tested compounds into nonpolar matrices (e.g., sediment and lipid-rich tissue) or primarily aqueous matrices (e.g., water and blood).

The first scenario (emission into air) showed that most substituted diphenylamines would partition into soil if emitted into air and would not, or only in limited amounts, reach the water phase (Table S1). It could therefore be concluded that emission into air and subsequent deposition was not likely the primary source of BNST contamination in the analyzed eels. The second scenario (emission into water), however, showed strong differences in the predicted partitioning of different substituted diphenylamines (Table 4 and Figure S2). Monooctyl-DPA and monooctyl-monostyrenated DPA were predicted to primarily partition into sediments, while all other substituted diphenylamines were predicted to primarily partition into the water phase.

Correlating the contamination pattern found in different tissue types of hormone-treated eels and the comparison group with patterns of the technical mixture and this predicted partitioning into sediments and water provided indications on potential tissue distribution and sources of substituted diphenylamines (Table 5). Predicted partitioning into sediments could indicate an affinity of the substances to partition into nonpolar matrices such as lipids in biota, whereas a predicted partitioning into water would be an indication of the substances' presence in the blood of the eels.

The results of the correlation could only provide a first indication, mainly due to the limited available samples. The apparent correlations with a regression coefficient (r) above 0.5 have been marked with "+" (see Table 5). Nevertheless, this analysis only contains three tissue types from three hormone-treated fish and two tissue types from five reference fish. Therefore, these results should be treated with caution as they have limited statistical validity.

Patterns of substituted diphenylamines in muscle and gonad tissue of hormone-treated eels as well as the comparison group indicated correlations with the patterns for substituted diphenylamines that were predicted to partition into sediments (r = 0.7−0.99). This could be an indication that uptake from sediments might be a relevant source for substituted diphenylamine contamination in these eels. It, furthermore, indicated that uptake and tissue distribution into muscle and gonads could be related to the lipid distribution and lipid redistribution during the maturation of eels, as previously reported for halogenated flame retardants (HFRs) in eels[4] and zebrafish[26] as well as organochlorines in oviparous organisms[27] and walleye.[28] Contamination patterns in eel eggs, however, were dominated by compounds predicted to partition into the water phase,

Table 5. Summary of Pearson Correlation of Substituted Diphenylamine Patterns in the Technical Mixture, Sediment, Water, Muscle, and Eel Samples[a]

					hormone treated eels			comparison group	
		technical mixture	sediment	water	muscle (n = 3)	gonads (n = 3)	eggs (n = 3)	muscle (n = 5)	gonads (n = 5)
	technical mixture	–	0.35	−0.39	0.09	0.45	−0.28	0.02	0.29
	sediment	0.35	–	−0.24	0.86+	0.99+	0.15	0.67+	0.99+
	water	−0.39	−0.24	–	0.23	−0.25	0.81+	0.50	−0.24
hormone-treated eels	muscle	0.09	0.86+	0.23	–	0.86+	0.63+	0.70+	0.88+
	gonads	0.45	0.99+	−0.25	0.86+	–	0.21	0.49	0.98+
	eggs	−0.28	0.15	0.81+	0.63+	0.21	–	0.65+	0.21
comparison group	muscle	0.02	0.67+	0.50	0.70+	0.49	0.65+	–	0.52+
	gonads	0.29	0.99+	−0.24	0.88+	0.98+	0.21	0.52+	–

[a]All apparent correlations with r > 0.5 were marked with +; it was not possible to determine statistical significance of the correlations due to the limited sample size (n).

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Environ. Sci. Technol. 2016, 50, 12678−12685

indicating that the maternal transfer into eggs might not solely be related to the lipid transfer, as described for HFRs but by transfer via primarily aqueous media such as e.g. blood and water (Table 5). The comparably high contribution of DPA in gonads of eels from the comparison group could be an indication for transformation or metabolism processes. Previous research on the maternal transfer of halogenated flame retardants suggests a significant increase of metabolites in gonad tissue compared to muscle tissue.[4]

3.4. Implications. The results of this study have shown the benefits and necessity of combining targeted and nontargeted analytical approaches. These mechanisms can help to comprehensively assess chemicals with potential negative impact on the quality of spawning eels. Without the nontarget analysis, the high contribution and potential relevance of substituted diphenylamines as contaminants in eels would not have come to our attention.

Our results showed that substituted diphenylamines were taken up and accumulated by eels to concentrations similar or even exceeding those of pesticides and PCBs and were several orders of magnitude higher than concentrations of halogenated flame retardants in the same samples.[4] Despite the limited number of samples and analyzed habitats, the results of this study, clearly require further research, particularly on the environmental fate, behavior and, especially, uptake and impacts on biota of substituted diphenylamines.

Furthermore, it could be observed that the majority of detected organic contaminants, including legacy POPs such as PCBs, organochlorine pesticides, and DDT transformation products as well as PAHs and substituted diphenylamines, are not merely stored in the lipid-rich tissue of adult eels but maternally transferred into gonads and eggs, making them a potential threat to the quality of spawners and recovery of the European eel stock. Model predictions by, e.g., recently developed physiologically based toxic−kinetic model for the European eel[29] could provide valuable information on the kinetics and tissue distribution of contaminants, especially considering the limited availability of samples.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b04382.

A comparison of measured mass spectra and fugacity model predictions. (PDF)

■ AUTHOR INFORMATION

Notes

The authors declare no competing financial interest.

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Annex IV

PAH metabolites, GST and EROD in European eel (*Anguilla anguilla*) as possible indicators for eel habitat quality in German rivers

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RESEARCH ARTICLE

PAH metabolites, GST and EROD in European eel (*Anguilla anguilla*) as possible indicators for eel habitat quality in German rivers

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Abstract The stock of the European eel (*Anguilla anguilla* L.) continues to decline and has reached a new minimum in 2011. Poor health status of the spawners due to organic contaminants is one of the possible causes for this dramatic situation. Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants, which are rapidly metabolized in vertebrates. EROD (ethoxyresorufin-*O*-deethylase) and GST (glutathione-*S*-transferase) are two enzymes involved in PAH detoxification in fish. In this study, PAH metabolites as well as EROD and GST activity in a large, comprising dataset of more than 260 migratory and pre-migratory eels from five large German river basin districts were used to describe PAH exposure and its metabolism as possible indicators for the habitat quality for eels. Eel from the river Elbe appear to be moderately contaminated with PAH. Highest mean values of PAH metabolites were analysed in fish from the river Rhine. However, the results suggest that contaminants such as PAH are metabolized in the fish and may have contributed to EROD activity in eels caught from the Elbe estuary to 600 km upstream. Since the eel's onset of cessation of feeding is closely linked to maturation and migration, we propose bile pigments as new indicators contributing to identify the proportion of migratory eel, which is crucial information for eel management plans. We showed that PAH metabolites normalized to bile pigments as well as EROD could be used to describe the habitat quality and might be suitable parameters in search for suitable stocking habitats.

Keywords PAH metabolite · 1-Hydroxypyrene · EROD · GST · Silver eel · Maturation · Elbe · Rhine

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Introduction

The European eel (*Anguilla anguilla L.*) has its supposed spawning area in the Sargasso Sea which is 5,000–7,500 km away from its fresh water habitats in Europe. At the onset of gonadal maturation, eels start their migration back to their spawning grounds (van den Thillart et al. 2008). The process of maturation goes along with morphological and physiological changes for the fish: the transformation from so-called yellow eel into silver eel, including an increasing eye diameter and prolonged fins. Durif et al. (2005) used these and other externally visible characteristics to create a silvering index (SI), describing the maturation stages of the eel. Another important physiological change for maturing eels is that, while they migrate, they stop feeding and become reliant on their body energy reserves, mostly muscle lipid. It is assumed that larger females can use their lipid reserves more effectively and therefore have a better chance of successful migration and spawning (Clevestam et al. 2011). This is also supported by the observation that *Anguilla rostrata* tend to become larger and older at higher latitudes (Jessop 2010). Age and lipid have been reported to influence proposed spawning success (Palstra and van den Thillart 2010). Belpaire et al. (2009) reported decreasing lipid contents in yellow eels caught in Belgium, which the authors regarded as a crucial element for reproductive success.

The stock of the European eel continues to decline, reached a new minimum in 2011 and is considered to be "outside safe biological limits" (ICES 2011a). One hypothesis for the cause

of this dramatic situation is low spawner quality due to contaminant effects (Palstra et al. 2006). Polycyclic aromatic hydrocarbons (PAHs) belong to the group of organic contaminants, are known to accumulate in sediments and suspended particulate matter (Keiter et al. 2008, Woelz et al. 2008), and have significant impact on the habitat of yellow and silver eels. In German rivers, the environmental quality standards were more often exceeded by chemicals belonging to the PAH group, than by other organic chemicals (Federal Environment Agency 2012). This underlines the importance of PAH for environmental quality assessment. PAH are rapidly metabolized and their metabolites are detectable in the bile. Because of this fast metabolization, chemical quantification of PAH in fish tissues provides only limited information. In marine monitoring, PAH metabolites in the bile of fish have been applied as indirect indicators of PAH exposure. Numerous studies concerning PAH metabolites in different fish species have been published before (Brinkmann et al. 2010, 2013; Tairova et al. 2012; Kammann 2007) as well as in the European eel (Ruddock et al., 2003; Nagel et al. 2012a). The main metabolite in fish bile is known to be 1-hydroxypyrene (1-OHPyr) which contributes up to 76 % of the sum of PAH metabolites. Other metabolites, detected in considerably lower levels in fish bile are 1-hxdroxyphenanthrene (1-OHPhen), 1-hydroxychrysene, and three metabolites of benzo(a)pyrene (Ruddock et al. 2003). PAH metabolites are prominent parameters in marine monitoring. They have recently been proposed in a suite of 13 core indicators to monitor hazardous substances and their effects in the Baltic Sea (HELCOM, 2012) and are part of marine monitoring programs (OSPAR 1998; Kammann et al. 2012). This background lets PAH metabolites become candidates do describe PAH exposure as possible indicators for the habitat quality for eels.

PAH are metabolized by enzymes belonging to the group of cytochrome P450 monooxygenases (CYPs). Especially enzymes from the CYP1A sub-family are involved in phase I biotransformation of xenobiotics in many vertebrates. Substrates for CYP1A enzymes have to be of planar conformation: Hahn et al. (2005) showed that the expression of CYP1A mRNA is mainly regulated by binding of planar aromatic hydrocarbons to the cytosolic aryl hydrocarbon receptor (AhR). The enzymatic activity of CYP1A is typically assessed indirectly by measuring the *O*-deethylation of the substrate 7-ethoxyresorufin to the fluorescent product resorufin by ethoxyresorufin-*O*-deethylase (EROD). The second step of PAH metabolism is conducted by phase II biotransformation enzymes such as glutathione-*S*-transferases (GST), also controlled by the AhR. Both enzyme families, as well as their activities or corresponding mRNAs and proteins have been extensively studied in various fish species as biomarker of exposure to planar aromatic compounds, e.g., polychlorinated biphenyls (PCB), dioxins, and furans, as well as PAH (Schlenk et al. 2008) and also in European eel (Agradi et al. 2000; Bonacci et al. 2003; Buet et al. 2006; Fenet et al. 1996; Hewitt et al. 1998; Marohn et al. 2008; Pujolar et al. 2013, Teles et al. 2004; van der Oost et al. 1996). However, there are many confounding factors that influence the signal-to-noise ratio of the biomarkers (for review, see Whyte et al. 2000). Although the link between elevated enzyme activities and adverse effects in organisms is well established, induction of EROD or GST cannot be directly equated with toxicity. Therefore, it is important to compare the enzymatic activities with contaminant data.

The nutrition status of the fish is one important confounding factor for PAH metabolites in eel because cessation of feeding is a natural process for the eel, occurring during the silvering process. Starvation may mark the eels' start of migration accompanied by regression of the digestive tract (Tesch 2003). It is known that, during periods of starvation, the amount of glucuronidated and sulphated PAH metabolites in bile increase (Beyer et al. 1997). Consequently, an increase in concentration of other bile contents such as bile pigments can be expected during starvation periods of fish (Richardson et al. 2004). In previous studies, we described the relation of PAH metabolite concentration in eel bile with maturation (Nagel et al. 2012a). We showed that this bias could be overcome when PAH metabolites were put into relation to bile pigments (Nagel et al. 2012b). Because of the fact that the concentration of PAH-metabolites and bile pigments in eel bile are influenced by cessation as part of the silvering process, bile pigments might be used to identify migratory status of eel. The condition of these fish is of special importance since healthy and well conditioned specimens are possibly favored for a successful reproduction. The eel management plans of the European Union allot that, for a successful restoration of the panmictic stock of the European eel, it has to be permitted that at least 40 % of the silver eel biomass can escape to the sea, relative to the best estimate of escapement that would have existed if no anthropogenic influences had impact on the stock (Council Regulation (EC) 1100/2007). For these reasons, it is of special importance to identify migrating eel to archive a better understanding of the mechanisms involved in silvering of eels and to determine their proportion in the local populations. Also, stocking of glass eel and elvers is a prevalent practice to support and sustain local fisheries. Nevertheless, no significant restoration of the population has been observed so far, suggesting that restoration plans are inefficient. Identification of migrating eel in the fresh water population might help to shed light on this problem.

The aim of the present study was (1) to prove PAH metabolites as well as GST and EROD activity as possible criteria for habitat quality in eels from German rivers and (2) to investigate the relation of bile pigment concentration and the migratory status of silver eels.

Materials and methods

Sampling of fish

All eels used in this study derived from commercial fishermen and were originally bought alive for stock assessment purposes within the EU Data Collection Framework, as defined by the European Commission (2008) and further specified for the presented time frame (European Commission 2010). Out of animal welfare considerations, it is of importance to the authors to mention that no additional eel had to be killed for the present study. In sum, 262 fish were caught between June and October 2011 in the German rivers Eider, Elbe, Rhine, Schlei/Trave, and Weser (Fig. 1). This time frame was intentionally chosen to lie in the expectable main feeding season of European eels in Germany (Tesch 2003).In the river Elbe, eel were sampled at five different locations from the estuary to 600 km upstream (Fig. 2). Fish were held in flow-through holding tanks for a maximum of 10 days until dissected for the collection of biological data. Eels were individually anesthesized by using diluted clove oil, weighed, measured, staged according to Durif et al. (2005), and then killed by decapitation. After decapitation, livers were excised, and the left distal lobe of each liver was directly transferred into liquid nitrogen for enzymatic analysis. Bile was directly extracted from the gallbladder by using a 1-mL disposable syringe with a hypodermic cannula, transferred to 1.5-mL cryovials, and stored at −20 °C until examination.

Lipid and age analysis

Muscle fat content was derived using the Distill Fish Fatmeter (Model FM-692, Distell.com), with the "EEL-2" preset (whole carcass including skin, head, tail, fins, and intestines). Measurements were done according to the manufacturer's instructions with two exceptions: Fat content was determined on the left side of the fish only using a mean of four measurements, independent of fish length. In order to determine the accuracy of this method, lipid values of 51 eel were measured with both Fatmeter and a gravimetric method (Sühring et al. 2013) as reference. The average deviation of Fatmeter results to the reference values was −1.73 (standard deviation 4.44) percentage points, indicating that the Fatmeter rather produces estimates, which are, however, sufficient for the present study.

For ageing of individual fish, otoliths were cut out and prepared using the cutting and burning method (Graynoth 1999; Richards 1989; Todd 1980). Age readings were done according to a manual established by international experts (ICES 2009, 2011b).

PAH metabolites and bile pigments

PAH metabolites in bile samples were determined by a modified version of the method described by Kammann (2007) based on Krahn et al. (1984) but using slightly divergent high-performance liquid chromatography (HPLC) conditions: A volume of 25 μl bile was mixed with 95 μl water to which

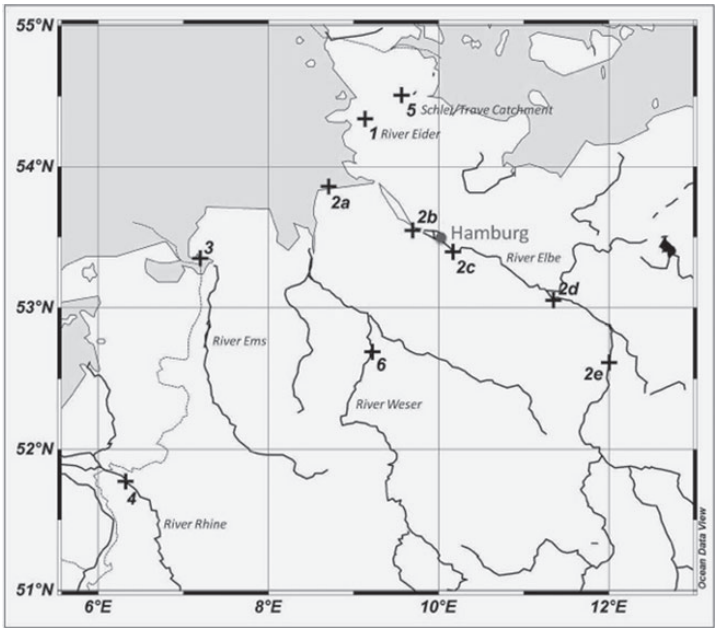

Fig 1 Sampling positions of 262 female eels caught in 2011 in the German rivers Eider (*1*), Elbe (*2*, *a*: Cuxhaven, *b*: Jork, *c*: Winsen, *d*: Gorleben, *e*: Hohengöhren), Ems (*3*), Rhine (*4*), Schlei/Trave (*5*), and Weser (*6*)

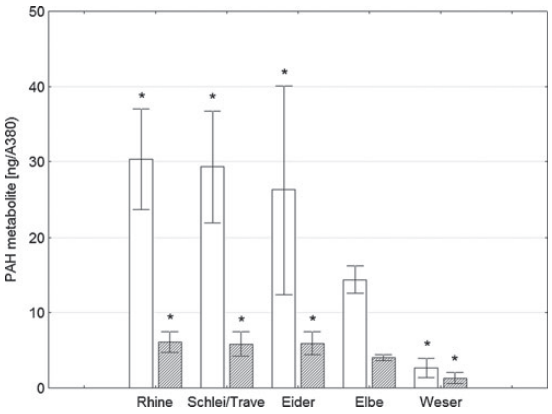

Fig 2 PAH metabolites 1-hydroxypyrene (*light*) and 1-hydroxyphenanthrene (*dark*) related to bile pigments (A380) in female eel caught in 2011 in German rivers; means and 95 % confidence intervals. *Asterisks* denote significant differences compared with animals from Elbe (Kruskal–Wallis ANOVA on ranks, Dunn's post hoc test $p \leq 0.05$)

5 μl of ß-glucuronidase/arylsulfatase solution (30/60 U/ml) were added and incubated for 2 h at 37°C on a heated shaker. The addition of 125 μl ethanol containing 5 mg/ml ascorbic acid stopped the reaction. The final solution represents a tenfold dilution of the bile sample and was centrifuged (5 min, 700×*g*). The clear supernatant was used for HPLC analysis immediately. The concentrations of PAH metabolites were determined using a LaChrom HPLC system (Merck Hitachi) comprising a quaternary pump (L-7100), an auto sampler (L-7200), and a fluorescence detector (L-7480). Standard solutions were diluted in acetonitrile with 5 mg/ml ascorbic acid. The column was a Nucleosil 100–3 C18 (3×125 mm) reverse phase and run at a flow of 0.55 ml/min. The initial mobile phase was acetonitrile/0.1% trifluoroacetic acid 50/50 (*v/v*). After 10 min, the solvent composition progressively changed to 60 % acetonitrile over 4 min and reached afterwards 100 % acetonitrile within 2 min. The excitation/emission wavelength pairs for 1-OHPyr and 1-OHPhen were 346/384 and 256/380 nm, respectively.

PAH metabolite concentrations were related to bile volume as well as to bile pigments measured as total absorbance at 380 nm (A380). For quality assurance of PAH metabolite analysis, each sample was processed twice. The limit of detection (LD) and the limit of quantification (LQ) were calculated according to DIN 32645 (DIN 1994) with a confidence level of 99 %. Considering the dilution during sample preparation, a LD of 3.4 (0.5) and a LQ of 22.5 (1.7)ng/ml bile were determined for 1-OHPyr (1-OHPhen). A fish bile sample as laboratory reference material was included in every sample batch to monitor the stability of the method (variation coefficient 15 % for 1-OHPyr). An intercalibration exercise of the method leads to z scores below +/−2 (Kammann et al. 2013).

For bile pigment determination, a volume of 25 μl bile was added to 475 μl water, and absorbance of 300 μl was recorded at 380 and 660 nm, respectively, using 96-well microplates and a UV/VIS microplate reader (Fluostar Optima, BMG Labtech, Offenburg, Germany). The concentration of biliverdin (nanograms per milliliter) was calculated from the absorbance at 660 nm using its molar extinction coefficient of 10,800 cm^{-1} mM^{-1} (Grossbard et al. 1987). Bile pigments are expressed as absorption units per milliliter (a.u./mL) in case of A380.

EROD and GST activity

Liver subcellular fractions were prepared according to the methods described by Bonacci et al. (2003). Briefly, pieces of liver samples were excised and added to 50 mM potassium phosphate buffer (pH 7.5) containing 0.5 mM dithiothreitol and 0.4 mM phenylmethylsulfonyl fluoride at a ratio of 1:10 (*w/v*) and homogenized using an electric disperser. Homogenates were centrifuged at 9,000×*g* and 4 °C for 20 min. The resulting supernatant (S9) was collected in fresh tubes. Samples were kept on ice during the whole procedure. Concentration of total proteins in S9 was measured following a dilution step in homogenization buffer using the bicinchoninic acid assay provided as kit (Sigma-Aldrich, Deisenhofen, Germany).

EROD activity was then measured according to Maria et al. (2005). In a quartz cuvette, 100 μl S9 were mixed with 1 ml 0.5 μM 7-ethoxyresorufin and 10 μl 10 mM reduced nicotinamide adenine dinucleotide phosphate solutions in 100 mM TRIS buffer (pH 7.4) containing 150 mM potassium chloride. Fluorescence of the reaction product resorufin was measured in 10-s intervals for 5 min in a spectrofluorometer (Jasco FP-750, Gross-Umstadt, Germany) with excitation and emission wavelengths of 530 and 585 nm, respectively. All samples were measured in duplicates. Blank measurements were performed to correct for spontaneous substrate conversion. A serial dilution reference curve for resorufin was recorded and used for interpolation of changes in product concentrations. Specific EROD activities were calculated and expressed as picomoles resorufin generated per minute reaction time and milligrams total protein.

The activity of GSTs in S9 was measured using to the method of Habig et al. (1974) according to the protocol recently published by Brinkmann et al. (2010). Briefly, 750 μl 100 mM sodium phosphate buffer (pH 6.5) was mixed with 30 μl 25 mM 1-chloro-2,4-dinitrobenzene (CDNB) solution in ethanol and dilutions of the S9 fraction in homogenization buffer in a cuvette. The reaction was initialized by addition of 75 μl 11 mM solution of reduced glutathione, and absorbance was recorded at 340 nm and 25 °C for 5-min in intervals of 5 s. All samples were

measured in duplicates. Blank measurements were performed to correct for spontaneous substrate conversion, and the specific GST activity was expressed as nanomoles CDNB converted per minute reaction time and milligrams total protein.

Statistical methods and PCA

Since all datasets did not pass either the Barlett's test for equal variances ($p<0.05$) or the Kolmogorov–Smirnov test on Gaussian distribution ($p<0.05$), they were analyzed by use of nonparametric Kruskal–Wallis ANOVA on ranks ($p\leq0.001$) or Spearman correlation test ($p<0.05$). Dunn's method or Mann–Whitney U test ($p\leq0.05$) was used to identify significant differences between sampling locations. Unless indicated, values are expressed as mean value 95 % confidence intervals. The principal component analysis (PCA) with Varimax rotation was performed with STATISTICA 6.0 (Stat. Soft, USA).

Calculation for the use of bile pigments as indicator for pre-migratory stage of eel

To elucidate the hypothesis that bile pigments could be an indicator for pre-migratory stage, the eel with higher bile pigment concentrations were distinguished from those individuals which were clearly not migrating and most probably still feeding. Therefore, in a first step, the 119 eels in stages 1 and 2 (yellow eels) shown in Table 1 were chosen as a group of non-migrating eels. Three individuals with SI of 2 and lipid contents below 11 % were excluded from the whole dataset because they were obviously not feeding due to a fishing hook in the intestine or due to injury. The remaining fish comprised the group of non-migrating eels with A380<106 a.u./mL and biliverdin<1554 ng/mL. These two threshold values were used in a second step to separate migrating and non-migrating eels in the whole data set to check the hypothesis described above. The ranges of biological characteristics of the resulting groups are presented and compared with other studies in Table 2.

Results

PAH metabolites in all rivers

Mean values and standard deviations of the PAH metabolites 1-OHPyr and 1-OHPhen in bile, concentration of bile pigments, and biological parameters of 262 individually analyzed eels are given in Table 1. Data are grouped for rivers and SI, respectively. The mean values of 1-OHPyr (1-OHPhen) cover a broad range from 323 to 3,806 (110 to 699)ng/mL or 2.5 to 38.8 (0.9 to 8.8)ng/A380, respectively. Highest mean values of 1-OHPyr and 1OHPhen were analyzed in fish from the river Rhine, which is the case for both: volume-related [nanograms per milliliter] and bile pigment-related concentrations [nanograms per A380]. Mean bile pigment concentration varied from 35.1 to 205.3 a.u./mL. Highest concentrations of bile pigments were found in fish with an SI of 3 or higher in all rivers. Concentrations of 1-OHPyr in fish bile differed significantly ($p\leq0.05$) between most rivers. Only samples from Schlei/Trave and Rhine showed no significant difference from each other. Even when the maturation stages are regarded separately (data in Table 1), fish from Schlei/Trave, Eider, and Rhine appear to be more highly contaminated. Regarding PAH metabolite concentration related to bile pigments in eel, regional differences are visible (Fig. 2): While eel from Rhine, Schlei/Trave, and Eider showed the highest means in 1-OHPyr, fish from Elbe and Weser tend to provide lower concentrations. The influence of maturation and the linked nutrition status are predominantly ruled out with the relation to bile pigments (A380). The second metabolite under investigation, 1-OHPhen, was found in lower concentrations than 1-OHPyr in all samples. Only in the relatively low contaminated fish from the Weser were the concentrations of the two metabolites close (Fig. 2, Table 1). The lower concentration of 1-OHPhen compared with 1-OHPyr is typical for fish bile (Ruddock et al. 2003; Kammann 2007; Kammann and Gercken 2010).

PAH metabolites EROD and GST in the River Elbe

Mean values and standard deviations of the activities of EROD and GST and biological parameters of individually analyzed eel ($n=232$) are given in Table 1. The maximum mean activity of EROD (GST) of 7.3 pmol/mg*min (236 nmol/mg*min) were determined in samples from the River Elbe. Both PAH metabolites relative to bile pigments, as well as EROD activity in eels sampled at different sites along the lower Elbe River showed a similar spatial pattern (Fig. 3). The lowest values for both biomarkers from the Elbe catchment were found in eels from Cuxhaven, located directly at the estuary. Compared with this location, EROD activities were significantly higher in eels from Gorleben, Winsen, and Jork. Animals from Jork showed both the highest EROD activities (5.4-fold higher compared with Cuxhaven) and biliary PAH metabolite concentrations (2.8-fold higher, Kruskal-Wallis ANOVA on ranks, Dunn's post hoc test, $p\leq0.05$). A significant correlation between EROD activity and SI could be detected regarding eel from all rivers ($n=232$, $r=-0.135$, $p=0.039$, Spearman rank correlation) but was not present in the data subset from the river Elbe ($n=153$, $r=-0.085$, $p=0.293$).

Table 1 PAH metabolites 1-hydroxypyrene (1-OHPyr) and 1-hydroxyphenanthrene (1-OHPhen) related to bile volume or bile pigments (A380) respectively; ethoxyresorufin-*O*-deethylase (EROD), glutathione-*S*-transferases (GST), age, lipid, and length

	SI	N1	1-OHPyr [ng/mL]		1-OHPhen [ng/mL]		1-OHPyr [ng/A380]		1-OHPhen [ng/ A380]		Bile pigments A380[a.u./mL]		Age [y]		Lipid [ww%]		Length[cm]		N2	EROD [pmol/(mg *min)]		GST [nmol/(mg *min)]	
River			M	SD	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD	M	*SD*		M	SD	M	SD
Eider	1	7	1324	1043	299	213	38.7	32.6	8.8	6.6	35.1	16.9	5.0	1.0	16.6	6.4	39.0	6.1	6	2.4	1.5	187.5	31.3
	2	20	791	661	195	96	16.7	14.0	4.3	2.6	62.2	60.7	7.0	1.6	18.4	7.0	52.3	4.4	11	2.9	1.3	210.2	61.5
	3	9	1578	2500	447	329	38.5	79.8	7.1	6.1	71.2	33.7	10.6	2.6	23.1	5.7	60.9	6.4	5	2.8	1.3	208.8	24.2
	5	2	1118	63	367	28	22.9	11.4	7.6	3.9	55.1	24.8	10.0	2.8	25.3	2.3	68.5	6.4	–	n.a.		n.a.	
	all	38	1093	1367	283	216	26.3	42.0	6.0	4.7	59.0	48.5	7.6	2.6	19.5	6.8	52.7	9.6	22	2.7	1.3	203.7	47.4
Elbe	1	22	466	260	110	64	13.7	8.8	3.2	1.8	38.3	24.7	5.2	1.2	20.3	6.7	41.8	4.4	33	7.3	5.9	217.4	53.2
	2	71	666	841	170	110	15.6	15.2	4.3	3.1	49.2	29.5	8.2	2.1	23.3	7.2	55.3	6.1	60	6.7	4.9	236.2	64.3
	3	56	723	632	229	162	13.6	9.1	4.3	2.4	73.2	101.5	10.3	2.7	26.2	5.1	65.7	7.9	49	6.9	5.6	197.6	40.3
	4	2	2872	972	358	134	18.1	4.9	2.3	0.7	156.8	10.8	12.5	0.7	22.6	1.8	86.5	3.5	3	6.2	2.4	214.4	23.0
	5	13	1407	979	312	168	11.9	5.0	3.5	2.4	118.8	56.5	10.5	3.5	26.0	3.1	66.2	8.4	8	3.3	1.4	196.0	33.3
	all	164	744	792	196	141	14.4	11.9	4.0	2.7	62.4	68.4	8.8	2.9	24.1	6.4	58.3	10.9	153	6.7	5.2	217.3	55.3
Rhine	3	9	3285	1662	699	538	38.7	36.2	7.9	7.6	108.6	57.9	10.3	1.7	25.0	1.4	76.6	5.3	13	5.4	3.3	205.7	45.1
	4	27	2998	1307	583	193	28.2	14.1	5.8	2.7	126.5	101.9	10.4	2.2	23.5	1.5	82.4	4.9	27	4.6	2.2	184.6	39.4
	5	5	3806	1084	606	204	25.8	11.6	4.3	2.5	167.6	78.9	12.4	2.9	26.0	4.9	67.0	7.0	7	3.4	1.9	217.5	47.4
	all	41	3160	1361	612	298	30.2	20.6	6.0	4.2	127.6	91.5	10.6	2.3	24.1	2.2	79.2	7.3	47	4.6	2.5	195.3	43.3
Schlei/Trave	3	4	2082	911	391	80	38.8	10.2	8.0	3.0	61.0	42.7	9.5	2.4	24.5	1.4	67.8	8.2	–	n.a.		n.a.	
	4	4	2778	1099	544	190	30.7	6.5	6.1	1.1	90.5	33.9	13.3	3.0	24.1	0.5	85.3	12.6	1	2.1	0.0	139.3	0.0
	5	6	2009	1906	379	347	22.1	14.3	4.3	2.7	88.1	38.3	13.2	2.3	24.5	1.7	64.2	5.2	4	2.9	1.1	208.3	31.2
	all	14	2250	1410	430	248	29.3	12.8	5.9	2.8	81.1	37.7	12.1	2.9	24.4	1.3	71.2	12.2	5	2.5	0.5	173.8	15.6
Weser	3	3	547	448	176	88	2.5	1.4	0.9	0.3	205.3	114.0	7.7	1.5	24.4	1.2	73.3	2.5	3	3.0	0.9	232.8	38.5
	4	2	323	62	210	60	3.0	0.5	1.9	0.1	111.8	39.0	9.0	4.2	23.8	1.2	80.0	5.7	2	3.1	0.7	174.2	23.8
	all	5	457	341	190	71	2.7	1.0	1.3	0.6	75.2	74.2	8.2	2.5	24.2	1.1	76.0	4.9	5	3.0	0.8	203.5	31.1
All		262	1248	1358	286	242	19.2	21.5	4.7	3.4			9.1	3.0	23.4	6.0	61.8	13.4	232	5.7	4.7	210.9	52.3

Results are given in mean values (M) and standard deviation (SD) grouped for rivers and silvering index (SI, Durif et al. 2005). The number of single fish analyzed is given for PAH metabolites (N1) and enzyme activity (N2) separately

n.a. not analyzed

Table 2 Biological indicators of female silver eel in pre-migratory stage from German rivers selected by either A380 or biliverdin thresholds respectively and compared with published data for migrating and mature eel

Indicator	Present study		Published data	
	A380 (n=46)	Biliverdin (n=10)		Reference
Age [years]	4–17	5–15	5–28	Clevestam et al. 2011
Length [mm]	520–950	640–890	492–973	Clevestam et al. 2011
Weight [g]	302–1,689	463–1,256	189–1,609	Clevestam et al. 2011
Condition factor	0.15–0.29	0.18–0.22	0.14–0.28	Clevestam et al. 2011
Lipid [%]	21.2–32	21.2–31.5	20.2–37.9	Clevestam et al. 2011
Eye index	5.9–12.9	6.6–10.2	6.5–11.8	Pankhurst 1982
Silvering index	2–5	3–5	3–5	Durif et al. 2005
A380 [a.u./ml]	>106		None	
Biliverdin [ng/ml]		>1,554	None	

PCA

The PCA in Fig. 4a explains 54.7 % of the variance with the first two factors. Factor 1 explains 31.1 % of the variance and refers mainly to PAH metabolites 1-OHPyr and 1-OHPhen related to bile pigments (factor loadings (FL)<=−0.93). Factor 2 explains 23.6 % of the total variance and is dominated by SI (FL-0.71) and the inversely related variables GST and lipid (FL=0.60 and −0.56) as well as EROD (FL=0.44). In Fig. 4b, the first two factors explain 52.9 % of the variance. Factor 1 represents 32.5 % of the variance and is dominated by the two PAH metabolites (FL<=0.78) and by EROD (FL=0.56). Factor 2 stands for 20.3 % of the variance and explains mainly the variables SI and lipid (FL<=0.61). GST, however, shows weaker relations to the first two factors. An overview on all factor loadings is given in Table 3.

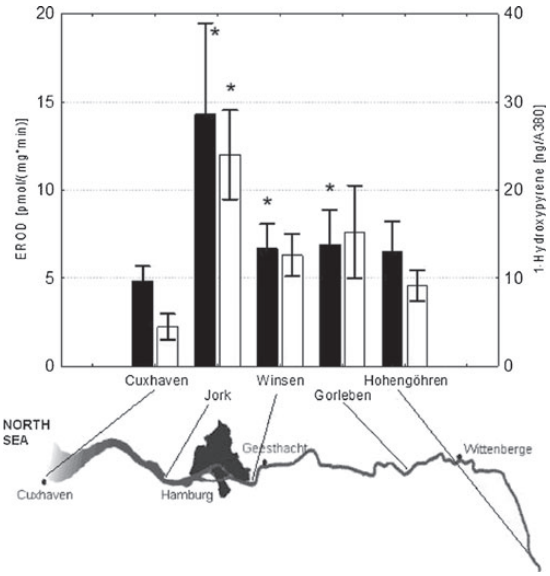

Fig. 3 EROD (ethoxyresorufin-*O*-deethylase) activity (*white bars*) and concentration of 1-hydroxypyrene relative to A380 (*black bars*) at different sampling locations along the river Elbe given as means and their 95 % confidence intervals (n>=13). *Asterisks* denote significant differences compared with animals from Cuxhaven (Kruskal–Wallis ANOVA on ranks, Dunn's post hoc test $p \leq 0.05$)

Discussion

Spatial differences

The concentrations of PAH metabolites in fish bile exhibited spatial differences between the rivers: Eel from Schlei/Trave, Eider, and Rhine exhibited the highest contamination with PAH metabolites (Fig. 2).These findings are in accordance with Nagel et al. (2012a) who detected elevated concentrations of PAH metabolites in eel caught in the river Trave and described eels from the Elbe as "medium contaminated". However, Nagel et al. (2012b) did not relate their results to bile pigments. Investigations on PAH metabolites in European eel caught in UK estuaries revealed 1-OHPyr concentrations in bile ranging from 120 to 7,600 ng/ml (Ruddock et al. 2003), which is twice as high as the mean values presented in Table 1 but quite close of the range of individual results (97–6,609 ng/ml). Sühring et al. (2013) analyzed up to three times higher concentrations of brominated flame retardants in eel from the Rhine compared with eel from the Elbe. This is in accordance with the findings of the present study: a twofold higher value of 1-OHPyr in eels from the Rhine compared with the Elbe (Fig. 2). It is also in accordance with the poorer chemical status of the Rhine compared with the river Elbe (Federal Environment Ministry 2010). However, the high 1-OHPyr levels in Schlei/Trave and Eider, as found in the present study, are not reflected in the assessments cited above. The results suggest that PAH metabolites can contribute to an assessment of habitat quality for eel in German rivers. On the other hand, it has to be mentioned that

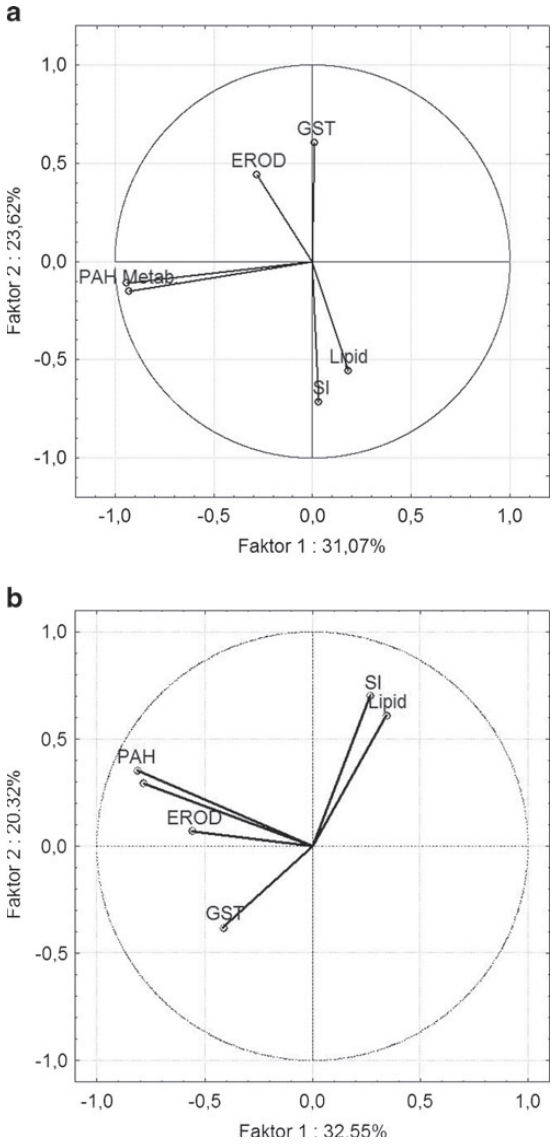

Fig 4 Principal component analysis of female European eel from German rivers considering fish from all rivers (**a**) or from the river Elbe exclusively (**b**): PAH=PAH metabolites 1-hydroxypyrene and 1-hydroxyphenanthrene [nanograms per A380]; *SI*=Silvering Index according to Durif et al. (2005); Lipid [%], *EROD*=ethoxyresorufin-*O*-deethylase activity [pmol/(mg protein*min)]; *GST*=glutathione-*S*-transferase activity [nmol/(mg protein*min)]

EROD activity in eel is at least partly influenced by the maturation because of the significant correlation between EROD and SI regarding fish from all rivers which is in accordance with Whyte et al. (2000). However, in a subset of the data, comprising fish from the river Elbe, no such correlation could be detected. It can thus be assumed that

Table 3 Factor loadings for the first two factors (F1, F2 with variance levels) of a principal component analysis of female European eel from German rivers considering fish from all rivers or from the river Elbe exclusively: PAH metabolites=1-hydroxypyrene and 1-hydroxyphenanthrene [nanograms per A380]

	All rivers		Elbe only	
	F1 (54.7%)	F2 (31.1%)	F1 (32.5%)	F2 (20.3%)
PAH metabolite	**−0.93**	−0.15	**−0.78**	0.29
PAH metabolite	**−0.94**	−0.11	**−0.82**	0.35
SI	0.03	**−0.72**	0.27	**0.70**
Lipid	0.18	**−0.56**	0.34	**0.61**
EROD	−0.28	0.44	**−0.56**	0.07
GST	0.01	**0.60**	−0.42	−0.38

Factor loadings above 0.5 are marked in bold

SI Silvering Index according to Durif et al. (2005); Lipid [%]; *EROD*=ethoxyresorufin-*O*-deethylase activity [pmol/(mg protein*min)]; *GST*=glutathione-*S*-transferase activity [nmol/(mg protein*min)]

EROD activity in fish from the river Elbe was not strongly influenced by sexual maturation, and the observed spatial differences can be related to a possible influence of pollutants. Therefore, this data subset has been chosen for a closer look on the spatial EROD pattern: PAH metabolites relative to bile pigments and EROD activity in eels sampled at different sites along the lower Elbe River in shown in Fig. 3. The sampling site Jork at the Elbe River with tidal influence is situated in only approximately 20 km distance downstream from the harbor of the city Hamburg. The sampling site Winsen is located approximately 20 km upstream of Hamburg. An influence of migrating eels from this heavily polluted industrial area on PAH metabolites and EROD activities is very likely. Many studies have demonstrated that PAH and PCB concentrations decreased in suspended particulate matter with increasing stream kilometer, with the highest concentrations close to the Czech border (BFG 2008; Heise et al. 2005). Dioxins and furans in freshly deposited sediments also show such spatial trend, although the highest concentrations are typically found close to the tributary Mulde. In the vicinity of the sampling site Gorleben, elevated dioxin/furan concentrations of about 50 pg WHO-TEQ/g dw sediment (compared with 20 pg WHO-TEQ/g dw at Cuxhaven) have been measured (Stachel et al. 2011), being one potential explanation for the elevated EROD activities. The results suggest that PAH among other contaminants may contribute to the enhanced EROD activities in eels from the river Elbe. However, the high EROD activities in eel from the river Elbe compared with the other rivers under investigation are not reflected in concentrations of PAH metabolites (Table 1). This fact suggests that PAHs are not likely to be always the major cause for EROD activities in eel.

PAH metabolites, EROD, and GST linked to eel physiology

Besides contaminants, the physiology of the fish may influence enzyme activities. Two PCAs were performed to elucidate the main linkages between six selected variables from Table 1: Two PAH metabolites (contaminants), silvering index and lipid content (physiology) as well as the enzyme activities EROD and GST. These variables were chosen to obtain a balanced PCA approach with equal numbers of variables from the three groups: contaminants, physiology, and enzymatic effects. The known linkage of GST and lipid-related processes in fish (Leaver and George 1998) is reflected in Fig. 4a. It has been shown in laboratory experiments before that EROD or CYP1A levels respectively are influenced by the hormonal status and sexual maturity (here represented by SI) of the fish (Whyte et al. 2000), which is in accordance with Fig. 4a. There is no evidence from Fig. 4a that EROD is influenced by the exposure of the eel to PAHs because of the fact that EROD and PAH metabolites are related to two different factors (nearly 90° angle between the variables). Therefore, we doubt that EROD and GST generally reflect effects related to PAH exposure in the samples. However, in fish from the river Elbe, PAH metabolites and EROD showed very similar spatial patterns (Fig. 3). In addition, the PCA of eel from the river Elbe (Fig. 4b) shows a close relation of EROD and the two PAH metabolites. Thus, we cannot exclude that EROD reflects pollution with organic contaminants at least in some samples. On the other hand, GST activities did not differ significantly between the rivers or the investigated sampling sites (results not shown). Van der Oost et al. (2003) reported that GST activity in fish does not respond always to xenobiotics: In only one third of laboratory studies, a significant GST activity increase was reported after exposure to organic pollutants.

Both enzyme activities, GST and EROD, appear to be strongly linked to physiological status of the eel (SI and lipid, respectively). But, for EROD, there is evidence that in some cases a pollution effect, e.g., of PAH or other AhR binding contaminants, might be reflected by this enzyme activity in eel. When EROD and GST are used as biological effect markers, eel should represent one maturation stage only (e.g., yellow eel) to avoid interference with physiology as described above.

Bile pigments and pre-migratory stage of eel

As mentioned before, concentrations of PAH metabolites and bile pigments increase when fish stop feeding. For bile pigments, this is a well known fact for decades (McCormick and Podoliak 1984). Consequently, bile pigment concentration may indicate the duration of starvation process in eel, which coincides with maturation. We hypothesize that the concentration of bile pigments measured as A380(a.u. per milliliter) or biliverdin (nanograms per milliliter) in bile indicates starvation and may therefore act as an additional indicator for the migratory status of silver eels. Figure 5 shows that high A380 values coincide in most cases with a small range in lipid content and with the SI stages 3, 4, and 5, which are typical

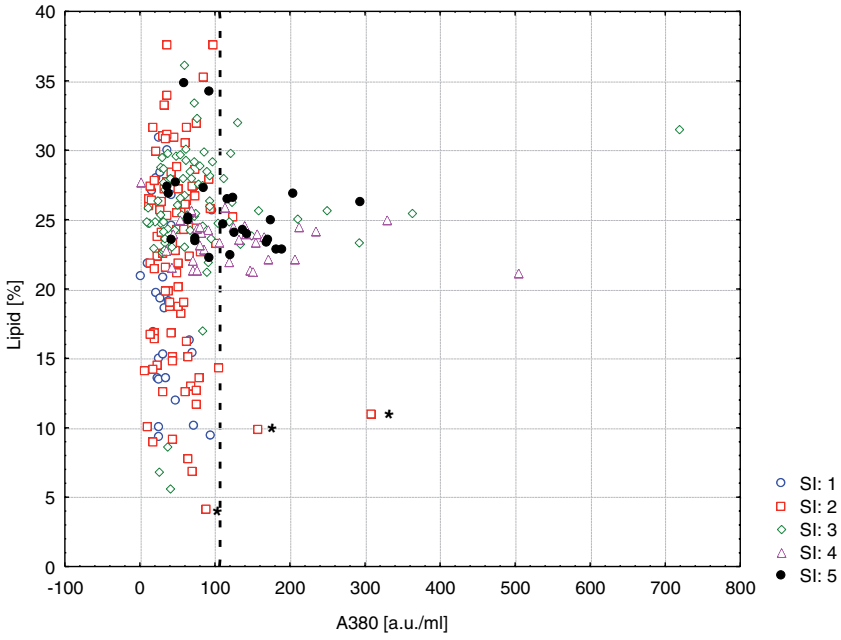

Fig 5 A380 (absorption at 380 nm) related to lipid content and maturation expressed as SI (Silvering Index, Durif et al. 2005). *Dashed line*: threshold of potential migrating eel characterized by A380 >106 a.u./ml. *Asterisks*: fish starving due to fishing hooks or injury

for migrating eel. However, some fish with lower SI also exhibit higher concentrations of bile pigments, indicating that maturation is not the only cause for cessation of feeding. On the other hand, not all eels staged as SI 3, 4, or 5 are starving (Fig. 5). This observation indicates that not all mature eel (according to SI) in German rivers have stopped feeding or at least not doing so at once. To elucidate the hypothesis described above that bile pigments could be an indicator for pre-migratory stage, potential migratory eel were selected by higher bile pigment concentrations (for details compare the "Materials and methods" section) and compared with migratory eel selected by other criteria in Table 2. Clevestam et al. (2011) investigated a larger number of female silver eel (n = 387) caught during autumn in Danish waters near the Öresund Strait. Those eels are considered to be migrating because they were caught while leaving the Baltic Sea towards their spawning regions in the Sargasso Sea. Pankhurst (1982) used the eye diameter related to the body length to distinguish non-migration yellow eel from migrating silver eel. Durif et al. (2005) used external characteristic of eel including length, weight, eye diameter, and fin length to stage female eel from 1 to 5 with increasing maturity. Most biological values from the present study, especially age, SI, and length show good accordance to the cited studies (Table 2). However, the lipid contents of migrating eel are lower in the present study than described by Clevestam et al. (2011). Although eels with higher lipid content up to 37.6 % have been detected in the present study (Fig. 5), they were apparently not starving. The large number and different origins of eels in the study of Clevestam et al. (2011) are causes for the broader-ranging biological parameters compared with the present study.

This is a first attempt to show that the cessation of feeding indirectly measured by bile pigments (either A380 or biliverdin) could be an additional indicator to enlighten the physiological processes during eel maturation in fresh water habitats. However, the threshold values might differ in other regions because they are influenced by the source or the overall level of nutrition. Eel identified as migratory due to analysis of bile pigments have been caught in all investigated rivers at different dates and by different fishermen together with non-migratory yellow eel. Therefore, it is not likely that artificial starvation during sampling has influenced the results. However, the proof of this assumption is still missing, and we can therefore not exclude a possible bias in the data caused by the sampling process. A380 or biliverdin could be new indicators, preferably applied in combination with other parameters, like those listed in Table 2, to identify migrating silver eel and to give additional insight in maturation. This hypothesis has to be confirmed in the future by controlled laboratory experiments and by enhancing the number of data and comparing the findings presented in this study to fish from other regions.

Conclusion

We conclude that the PAH metabolites 1-OHPyr and 1-OHPhen in the bile as well as EROD activity in the liver of European eel can be used to describe the habitat quality in German rivers. Bile pigments can be new indicators contributing to identify the proportion of migratory eel, which is crucial for a fresh water habitat in the light of the European eel management. In search for suitable stocking areas for glass eels, PAH metabolites and EROD can provide valuable information, even if the general benefit of stocking can be discussed (Marohn et al. 2013). Healthy and well conditioned silver eel growing in suitable habitats have the best prepositions for successful reproduction, which is in accordance with the goals of the eel management plans of the European Union.

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Annex V

Impact of chemical pollution on Atlantic eels: facts, research needs and implications for management

Claude Belpaire, Peter Hodson, Fabien Pierron, **Marko Freese**

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Impact of chemical pollution on Atlantic eels: Facts, research needs, and implications for management

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Abstract

Multiple eel species of the genus Anguillidae are under anthropogenic pressure. This review presents strong evidence that chemical pollution is a driving force behind the catastrophic decline in recruitment and abundance of both the European (*Anguilla anguilla)* and the American eel (*Anguilla rostrata*). In response to this crisis, stock and habitat management policies have blindly focused on increasing the areas available for the recruitment and rearing of yellow eels, and increasing the numbers of silver eels escaping to spawn in the Sargasso Sea. No specific policies or regulations have been adopted to foster recruitment of yellow eels to uncontaminated watersheds, to monitor the quality and condition of silver eels, or to protect silver eels from contaminated environments. Research is needed to identify existing and emerging contaminant problems, to understand their potential impacts on eel reproduction, and to develop indicators of spawner quality and management actions that would increase the likelihood of successful eel reproduction and recruitment.

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European eel, American eel, Contaminants, Stock decline.

Introduction

Multiple Anguillid eel species are threatened or near-threatened due to continuous and persistent declines in recruitment and abundance over past decades. The most affected is the European eel *Anguilla anguilla*, for which recruitment has decreased to 2.1% of the 1960–1979 average in the North Sea data series [1]. Despite measures taken at national levels, there is no clear recovery, and in most (84%) eel management units, stock indicators remain far below management targets [1]. At the same time, there have been alarming declines in stocks of two other temperate eel species of high commercial value, the American eel *Anguilla rostrata* and the Japanese eel *Anguilla japonica*. These dramatic developments prompted global interest in anthropogenic causes, including overfishing, habitat degradation, barriers to migration, diseases, pollution, and climate change. While the causes may interact synergistically, only pollution and climate change affect every single life stage [2].

Anguillid species are semelparous (once-in-a-lifetime spawners) and panmictic (random mating), reproducing far from their continental habitats (e.g., the Sargasso Sea for *A. anguilla* and *A. rostrata*). The oceanic larvae drift and develop for 0.5 to >2.5 years before they metamorphose into glass eels at the continental slopes and enter estuaries and rivers [3]. After pigmentation, they begin to feed and grow for 6 to >20 years as yellow eels. In their final life stage, they cease feeding, transform to silver eels and mature sexually while migrating back to the Sargasso to spawn and die. Silver eels rely on lipid stores to fuel gonadal maturation and migrations up to 7000 km. This review summarizes the current knowledge and critical research needed to understand how chemical pollution impairs the survival, growth, and reproduction of Atlantic eels.

Unique sensitivity of anguillid species to chemical contamination

Eels are benthic and opportunistic predators that accumulate extraordinarily high amounts of body fat during their continental lives in coastal and freshwater habitats. Thus, they are particularly prone to accumulating and biomagnifying lipophilic and persistent organic pollutants and other chemicals of concern [4–9].

Semelparous eels cannot reduce contaminant burdens by releasing gametes during repeated spawning, so their body burdens of contaminants clearly exceed those of

other fish species from the same habitat [10]. Fat stores catabolized during migration release these stored contaminants to the bloodstream, where they can contaminate and affect reproductive organs and gametogenesis. Concentrations of tissue contaminants provide a crucial benchmark for the quality of spawners and their overall reproductive success [11–16].

Pioneering work in analyzing and monitoring eels and developing standard methods for assessing bioaccumulating chemicals was done in the Netherlands and Belgium for *A. anguilla* [4,17] and in Canada for *A. rostrata* [18,19]. Larsson et al. [20] were probably the first to suggest that declining stocks of *A. anguilla* might be explained by chemical contamination. Groundbreaking research in the Netherlands on the toxicity to European eel embryos of maternally derived dioxin-like compounds (DLCs) [21] and comparisons of the swimming performance of adult eels to their chemical burdens [22] suggested realistic mechanisms linking contamination to impaired reproduction.

Bioaccumulation in eels—spatially and physiologically driven

Pollutant concentrations in both Atlantic eel species are characterized by extreme variability [7,23–26], and body burdens reflect atmospheric transport and the proximity of rearing habitats to urban, agricultural, and industrial development (Table 1). There are clear correlations between local contamination pressure and the pollution fingerprint of wild yellow eels. Yellow eels are efficient bioindicators for monitoring the sources and distribution of metals and lipophilic compounds [8,23,27,28]. For example, concentrations of mirex (i.e. organochloride insecticide) in *A. rostrata* provided a clear chemical marker of eels migrating from L. Ontario, which is uniquely contaminated by a single point source [18,19]. For both species, tissue concentrations of legacy chemicals (e.g., lead; polychlorinated biphenyls (PCBs)) that first attracted attention in the 1970s have since declined [12,19,29–31], to be replaced by emerging chemicals (e.g. brominated and fluorinated compounds). Many of those new chemicals are ubiquitous in eel (Table 2), at concentrations that reflect the extent of habitat degradation. In general, the effects on eels of these newly recognized compounds are poorly understood, yet some are known for their toxic and endocrine-disrupting properties.

In eels, lipophilic contaminants are usually measured in muscle where most lipids are found as stored energy. However, contaminants are not distributed evenly among eel tissues. This makes impact studies more challenging because each contaminant can exert specific damage in the target organ where it accumulates. For example, the eel brain is an important target for DDT, a neurotoxic pollutant [32]. Similarly, mercury is typically measured in muscle due to concerns for human safety, but it accumulates mainly in the liver, kidneys, and brain [33].

Physiologically based toxicokinetic models estimate uptake and distribution of chemicals in distinct body compartments during exposure. Brinkmann et al. [34] developed the first physiologically based toxicokinetic model for European eels with excellent predictive precision for moderately hydrophobic chemicals. The same model described the metabolic pathways of the pesticide fipronil and two of its metabolites in muscle and liver of eels from a German river [9]. Further model development may help in future quantification and assessment of potential pollution impacts.

Pollution impairs the health of eels: spawner energetics, embryo-larval survival, and endocrine disruption

Research on contaminant effects on eels has focused on traits affecting their fitness to complete their life cycle, including their ability to swim, accumulate energy reserves, develop healthy oocytes, and reproduce. Lipid stores are crucial for eel reproduction. It has been estimated that a minimum of 20% in muscle is needed for normal migration and reproduction [35]. Lipid concentrations in female silver European eels vary considerably over their distribution range, suggesting large differences in their capacity to complete spawning migrations and in reproductive potential (number of eggs produced) [55], particularly because pollutants impair lipid metabolism [36]. Significant declines in lipid levels in European and American eels from polluted watersheds [30,31,37] suggest that eel stocks might well be governed by pollution-impaired lipid storage, spawning migrations, and/or fecundity [24].

The release of organic contaminants from lipid stores mobilized to support eel migration and gonadal development represents a risk of toxicity to migrating adults, developing oocytes, and early developmental stages of fertilized eggs [7,21,36,37]. Similarly, stored metals can be conveyed to oocytes by vitellogenin [11]. For *A. rostrata*, DLCs extracted from muscle lipids of Lake Ontario yellow eels captured between 1988 and 1998 were toxic to mummichog (*Fundulus heteroclitus)* embryos, extracts from eels captured in 2008 were not [39]. The decline in embryotoxicity corresponded to parallel declines in tissue concentrations of DLCs [31].

The rates of survival and deformities of European eel embryos were correlated to concentrations of DLCs in ovaries of contaminated females induced to spawn in the laboratory [21]. However, this landmark study was limited by low sample numbers. More recent studies demonstrate that substituted diphenylamines, flame retardants, DLCs, and metals can be transferred from artificially matured females to eggs [13–16,40].

While most studies focus on chemical effects on female reproduction and embryo development, contaminants also impair the reproductive capacity of males by endocrine disruption, either by feminization or reduced fertility, as occurs for other fish species (reviewed in Matthiessen et al. [41]). Even though metals such as cadmium may disrupt eel endocrine pathways and gonadal maturation [11], this field is understudied.

The role of pollution in eel decline: confounding factors and evidence from other species

Although there is substantial evidence of contaminant effects on eel physiology (reviewed in the study by Geeraerts and Belpaire [36]), most is derived from experiments and is limited to specific life stages and endpoints with unrealistic exposure times and pathways. The effects on eels of lifetime exposures to complex mixtures of chemicals are essentially unknown. Some promising *in situ* approaches to produce valid effects data include the measurement of molecular biomarkers (reviewed by the International Council for the Exploration of the Sea (ICES) [42]). Although transcriptomic responses demonstrated pollution impacts on Atlantic eels (e.g. Refs. [43–46]), changes in gene transcription are not yet reliable indicators of the potential for eels to successfully migrate and reproduce [42]. Moreover, because many environmental factors unrelated to pollution also affect these indices, simple comparisons of individuals between clean and contaminated sites could be misleading. Interpretation of transcriptomics is especially challenging and should consider the interindividual variability and diversity of life history traits of eels [46,47]. Nonetheless, high-throughput sequencing technologies hold promise for further progress. Laporte et al. [48] recently applied restriction site–associated DNA sequencing to demonstrate within-generation polygenic selection of wild Atlantic eels exposed to PCB153, *p,p*' DDE and selenium. The evidence suggests nonrandom mortality of Atlantic eels by human-driven environmental selection with potential long-term impacts on genetic diversity and evolutionary potential.

Compared to other fish species, assessing the comprehensive effects of pollution on the stock of Atlantic eels is extremely challenging due to their eurytopic behavior and specialized biology. Oceanic mating and subsequent distribution of larvae to freshwater rearing habitats are considered totally random (see Ref. [49]), so there are no clear links between reduced recruitment to polluted freshwater habitats and embryo-larval toxicity caused by maternally transferred contaminants [50]. Chemical effects on other species may improve the understanding of the effects of maternally derived contaminants on larval development, condition, and survival and on subsequent stock recruitment. Well-known examples include the population collapse of several birds of prey due to DDT (e.g. Ref. [51]), the total elimination of natural reproduction of Lake Ontario lake trout (*Salvelinus namaycush*) by DLCs [52], reductions in abundance of Atlantic salmon (*Salmo salar*) after large-scale forest treatment with an insecticide containing nonylphenol [53], and reproductive disturbances and lower fecundity in populations of brown bullhead *Ameiurus nebulosus* from agricultural watersheds [54]. Based on toxicity thresholds for PCB effects on reproduction of other fish species, ICES [55] estimated that >60% of European eels from eight countries were at risk of reproductive impairment (e.g. compared to North Sea whiting *Merlangius merlangus*). Similar conclusions were drawn for American and European eels when tissue concentrations of DLCs were compared to threshold concentrations affecting lake trout reproduction [15,31].

Declines in fish reproduction and abundance followed the release of a panoply of new chemicals from the 1940s onwards (e.g. Ref. [52]). However, the decline in eel stocks occurred later, in the early eighties, corresponding to the longer generation times of eels. PCBs likely attained their highest concentrations in eel by the late seventies, contributing to lower recruitment during the early eighties [24,31]. Finally, the concurrent timing of recruitment decline in *A. anguilla*, *A. rostrata*, and *A. japonica* suggests that a common global pressure was involved, including the global distribution of one or more legacy or emerging contaminants of concern, combined with other stressors such as climate change.

Research needed to understand the impact of chemicals on eel stocks

Apart from monitoring to assess the status of contaminants and the quality of eels over their range [42], collaborative international research on pollution impacts is urgently required [42,56] (Table 3), taking advantage of new tools and technologies (e.g. artificial reproduction, swimming tunnels, analytical chemistry, biomarkers, genetic work). As detailed in Table 3, research is needed on the effects of specific contaminants on eel reproduction, lipid metabolism, epigenetics during metamorphosis, and toxicogenomics; contaminant distributions among tissues; and development of methods to support reproduction of eels in the laboratory and to assess the capacity of wild eels to migrate and reproduce.

Do eel management policies account for the effects of pollution?

For the European eel, current stock management is focused on regulating fisheries, assisting migration, or translocating and stocking wild-caught recruits to areas with low natural recruitment [57]. These policies will allow more spawners to escape and reproduce in the short term. However, they do not recognize, integrate, or implement measures that would reduce pollution as a

Table 1

Contaminant concentrations (ranges) in eels from different watersheds, sorted by species and country.

Species	Contaminant and concentration range	Matrix	Site	Reference
A.a.	DDT 4.9–392.3 ng/g PCBs 1.7–288.5 ng/g DLCs(PCDD/F/dl-PCBs) 1.42–14.59 pg TEQ/g PBDEs 0.07–8.19 ng/g HBCDD 0.16–17.52 ng/g	Muscle (all), liver (ww) (PBDEs; PCBs; pesticides)	5 sites in Poland, 2010–2012	Szlinder-Richert et al., 2014 [62]
A.a.	PAH metabolites 1-OHPyr 323–3806 ng/mL 1-OHPhen 110–699 ng/mL	Bile	5 river systems, 10 sampling sites in Germany, 2011–2012	Kammann et al., 2014 [28]
A.a.	Σdl-PCBs 2.3–266.0 ng/g	Muscle (ww)	6 river systems, 13 sampling sites in Germany, 2011–2012	Freese et al., 2016 [7]
A.a.	Sum 7 PCBs 3.5–12455 ng/g Sum DDTs 1.5–3995 ng/g Hg 5–1185 ng/g Cd 1–2474 ng/g Pb 1–3453 ng/g	Muscle (ww)	365 sites in Belgium 1994–2005	Maes et al., 2008 [29]
A.a.	Sum 6 PCBs 5–2600 ng/g ww Sum DDTs 110–7000 ng/g lw PBDEs 12–1400 ng/g lw HBCD 7–9500 ng/g lw	Muscle (ww); muscle (lw)	60 sites in Belgium 2000–2009	Malarvannan et al., 2014 [61]
A.a.	Hexachlorobenzene 2.1–3.2 ng/g Lindane 0.47–9.87 ng/g Sum DDTs 4.6–149.1 ng/g Sum 7 PCBs 53–1220 ng/g	Muscle (dw)	4 sites in France, 2011–2012	Laporte et al., 2016 [48]
A.a.	Metals Cu 70–125 µg/g muscle Se 22–52 µ/g liver Zn 250–290 µg/g liver Ag 0.65–2.0 µg/g liver As 1.5–15 µg/g muscle Cd 0.5–37 µg/g kidney Cr 1.5–4.2 µg/g liver Hg 0.2–0.9 µg/g liver Ni 0.5–0.8 µg/g kidney Pb 0.2–1.8 µg/g kidney	Muscle; liver, kidney (dw)	4 sites in France, 2011–2012	Pannetier et al., 2016 [8]

(*continued on next page*)

Table 1. (*continued*)

Species	Contaminant and concentration range	Matrix	Site	Reference
A.r.	Hexachlorobenzene 0.8–2.3 ng/g Lindane 0.16–0.21 ng/g Sum DDTs 8.1–63.8 ng/g Sum 7 PCBs 21–120 ng/g	Muscle (dw)	4 sites in Canada, 2011–2012	Laporte et al., 2016 [48]
A.r.	Metals Cu 60–270 µg/g muscle Se 22–80 µ/g liver Zn 240–490 µg/g liver Ag 1.1–2.1 µg/g liver As 0.5–3.5 µg/g muscle Cd 0.5–14 µg/g kidney Cr 1.9–5.8 µg/g liver Hg 0.3–1.8 µg/g liver Ni 0.8–1.1 µg/g kidney Pb 0.1–0.6 µg/g kidney	Muscle; liver, kidney (dw)	4 sites in Canada, 2011–2012	Pannetier et al., 2016 [8]
A.r.	Various pesticides 87–1480 ng/g Mirex 1–474 ng/g Hg 50–990 ng/g PCBs 142–5391 ng/g	Gutted carcass w/o head (muscle, skeleton, skin)	Migrating silver eels in the St. Lawrence R. estuary, 1990 (includes eels from Lake Ontario, the St. Lawrence R. and tributaries) North America	Hodson et al., 1994 [19]
A.r.	Sum DDTs 11–250 ng/g ww Sum chlordanes 1.1–10.5 ng/g ww Sum HCH 0.10–0.83 ng/g ww Sum Nonachlor 1.89–17.9 ng/g ww Mirex 0.037–19.6 ng/g ww Sum PBDE 2.1–39.4 ng/g ww Sum PCBs 12.5–2345 ng/g ww	Whole-fish homogenates minus the liver, a few grams of ovary, and otoliths;	Large yellow eels in L. Ontario, the St. Lawrence R. (ON), R. Sud Ouest (Qc), Miramichi R., NB, Margaree R., NS, Hudson R. NY; Silver eels – St. Lawrence River estuary. N = 3-17	Byer et al., 2013b, Table SI-2 [26]
A.r.	Various pesticides 0.4–209 ng/g Mirex 5.2–39 ng/g Hg ND PCBs 163–719 ng/g PBDE 5.9–63 ng/g PCDD/PCDF 3.8–13 ng/g	Whole body minus the liver and small samples of gonad and muscle	Lake Ontario North America, 2008	Byer et al., 2015 [31]

Table 2

Examples of new emerging chemicals as reported from eel studies.

Anguillid Species	Chemical	Country	Reference
A.a.	Brominated flame retardants (PBDEs) and dechloranes	Belgium; Germany; Poland; France	Sühring et al., 2013a,b; 2015 [5,6,13]; Malarvannan et al., 2014 [61]; Szlinder-Richert et al., 2014 [62]; Laporte et al., 2016 [48]
A.a.	(per)Fluorinated substances	Belgium and Germany	Sühring et al., 2013b [6]; Roland et al., 2014 [63]
A.a.	Organophosphorus flame retardants and plasticizers	Belgium	Malarvannan et al., 2015 [64]
A.a.	Fipronil (insecticide)	Germany	Michel et al., 2016 [9]
A.a.	Toxic textile dyes (such as malachite green)	Belgium	Belpaire et al., 2015 [65]
A.a. - *A.r.*	Thallium	France; Canada	Rosabal et al., 2015 [66]
A.r.	Brominated flame retardants (PBDEs) and dechloranes	USA; Canada	Ashley et al., 2007 [67]; Byer et al., 2013b [26]; Sühring et al., 2013b [6]; Laporte et al., 2016 [48].

A.a., Anguilla anguilla; A.r., A. rostrata.

factor contributing to stock decline. The regulations target a defined quantity of silver eels to leave continental catchments but fail to consider their quality, even though pollution effects on quality have been identified as a crucial cause of recruitment failure.

The situation is little different for the American eel but aggravated by eel habitats that are distributed among numerous watersheds of the Caribbean, the Gulf of Mexico, eastern United States, and eastern Canada. Unlike the EU, there are no consistent approaches to habitat or fisheries management. Some jurisdictions such as the Province of Ontario, Canada, have detailed scientifically sound eel recovery strategies [58], but this is the exception not the rule. And even in Ontario, the impacts on eels of chemical pollution are given only the briefest of nods. Although 'historic' problems are acknowledged (e.g., DLC toxicity to fish embryos), there are no recommendations to mitigate widespread problems of pollution and habitat quality, and none at all for assessing the reproductive quality of silver eels.

As recently suggested by Freese et al. [7] and De Meyer et al. [59], stock management of anguillids must integrate the condition and quality of rearing habitats and of the eels leaving continental waters. Effective eel stock management must be redefined to include standards for judging the success of eel and watershed management. These standards must ensure that recruits have access to unpolluted watersheds, that productive but contaminated watersheds are rehabilitated as suitable habitat for healthy eels that are safe to eat, and that targets are set for spawner quality (e.g, lipid and contaminant content, parasites and viruses). Given the complex life cycle of anguillids and their wide range of habitats and sensitivity to multiple stressors, effective management requires a multifaceted approach.

Habitat remediation requires a reduction of chemical discharges and removal of contaminated sediments. However, these are long-term solutions. Given the precarious status of eel stocks, relying solely on existing regulations to decrease pollutant pressure (e.g., EU WFD, REACH) is not sufficient. Specific new measures are needed to further document and understand the impact of pollutants on eels, and to recognize current knowledge in management actions. ICES [42,60] initiated work to harmonize monitoring strategies and to understand contaminant effects on the stock. However, pollution-related monitoring and management of eels is not yet coordinated and there is no clear indication of its effectiveness in improving the spawning stock and recruitment. Possible management measures may include refraining from stocking glass eel in heavily polluted catchments and maximizing protection of less-polluted catchments that produce well conditioned females. These valuable habitats must be identified and used as 'reserves' to foster appropriate stocking. The

Table 3

Summary of research needed to understand the role of contaminants in eel decline.

Objective	Tools	Relevant references
1. Improving the controlled reproduction of eels is crucial for research on reproductive impacts of contaminants (see 2). One major obstacle to understanding the contaminant effects on eel reproduction is the lack of tools and understanding needed to experimentally reproduce Atlantic eels in the laboratory. While the production of fertilized eggs and early-stage larvae (<20 days) is feasible, rearing of larvae beyond 20 days remains a major bottleneck.	Aquaculture zootechnical tools. Artificial reproduction in *A. anguilla* and *A. rostrata*, benefitting from experiences with *A. japonica*. Development of early-stage food.	Butts et al., 2014 [68]; Masuda et al., 2012 [69]
2. Assessing the effects of specific contaminants on eel reproduction. Taking advantage of progress under 1, assess the effects of specific legacy and emerging contaminants on eel reproduction, gamete viability, and development of larvae and juveniles.	Classical ecotoxicological approaches tailored to eel. Challenge experiments at different doses combined with artificial reproduction.	Palstra et al., 2006 [21]; Pierron et al., 2008 [11]
3. Assessing partitioning and distribution of contaminants among eel tissues. Contaminant concentrations are usually measured in muscle tissue but may not be evenly distributed among other organs. Tissue distributions will help predict toxic concentrations in target organs of wild eels.	Experimental work and development of physiologically based toxicokinetic (PBTK) models; identification of critical body burdens for specific contaminants	Brinkmann et al. (2015) [34]; Michel et al. (2016) [9]; Sühring et al., 2015; 2016 [13,14]; Freese et al., 2017 [15]; Nowosad et al., 2018 [40]; Freese et al., 2019 [16]

4. Contaminant effects on lipid metabolism. In other species, contaminants alter lipid physiology, but there are few studies of eel, despite the crucial role of lipids in migration and reproduction.	Chemical exposures combined with swim tunnels and physiological responses and energetical studies	See for an overview Geeraerts and Belpaire, 2010 [36](e.g. Palstra and van den Thillart [38])
5. Tools to assess the quality of eels over its distribution area in relation to their capacity to migrate and reproduce. Biomarkers are needed to assess survival, migration, and reproduction capacity.	Monitoring of chemicals in silver eels over their distribution area. Development of biomarkers	Couillard et al., 2011 [70]; see for an overview ICES, 2015 [42]
6. Epigenetic mechanisms of pollutant impacts on eels. Role of epigenetic marks and their potential pollutant-induced changes during the critical windows of metamorphoses, the early stages of eels, and their subsequent consequences on the completion of the eel's life cycle.	Use of molecular methods to investigate the impacts of contaminants on epigenetic marks. Correlate changes in DNA methylation, histone marks, or miRNAs expression with contaminant burden in critical life phases	Trautner et al., 2017 [71]; Pierron et al., 2014 a,b; 2019 [47,72,73];
7. Toxicogenomic studies of pollutant effects on eel stocks. Assessments of eels from contaminated environments to establish links between pollution and genetic biomarkers	Toxicogenomics, biomarkers	Maes et al., 2013 [43]; Pujolar et al., 2012; 2013 [44,45]; Baillon et al., 2015 [46]

production of escaping high-quality spawners must be also maximized by removing obstacles to migration, restricting development that affects habitat, and banning fisheries.

Conclusion

Currently, a clear and quantitative assessment of pollution impacts on eel stocks is not available. While new chemical and bioanalytical tools have enabled significant progress, research, monitoring, and management are inadequate to understand and mitigate stock-wide impacts of contaminants. A reliance solely on fisheries measures to restore declining stocks risks losing the species if contaminant issues crucial for eel restoration are overlooked.

Conflict of interest statement

Nothing declared.

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LIST OF PUBLICATIONS

All chapters (as well as annexes) of this thesis have already been published in peer-reviewed scientific journals.

Chapter I
A question of origin: dioxin-like PCBs and their relevance in stock management of European eels
Chapter II
Maternal transfer of dioxin-like compounds in artificially matured European eels
Chapter III
Maternal transfer of emerging brominated and chlorinated flame retardants in European eels
Chapter IV
Bone resorption and body reorganization during maturation induce maternal transfer of toxic metals in anguillid eels
Chapter V
A physiologically based toxicokinetic (PBTK) model for moderately hydrophobic organic chemicals in the European eel (*Anguilla anguilla*)
Chapter VI
Fipronil and two of its transformation products in water and European eel from the river Elbe

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Annex I
Brominated flame retardants and dechloranes in eels from German Rivers
Annex II
Brominated flame retardants and Dechloranes in European and American eels from glass to silver life stages
Annex III
Evidence for High Concentrations and Maternal Transfer of Substituted Diphenylamines in European eels Analyzed by Two-Dimensional Gas Chromatography–Time-of-Flight Mass Spectrometry and Gas Chromatography–Fourier Transform Ion Cyclotron Resonance Mass Spectrometry
Annex IV
PAH metabolites, GST and EROD in European eel (*Anguilla anguilla*) as possible indicators for eel habitat quality in German rivers
Annex V
Impact of chemical pollution on Atlantic eels: facts, research needs and implications for management

CONTRIBUTIONS OF AUTHORS

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

Chapter I -
Marko Freese wrote the majority of the manuscript, designed and coordinated the study, performed sampling, was responsible for sample selection as well as preparation, participated in all laboratory analyses and conducted data evaluation. Roxana Sühring developed the methods for PCB analyses and lead the analyses in the laboratory. Victoria Magath assisted with statistical testing. Reinhold Hanel helped to develop the study and supervised throughout its development. All co-authors contributed to the writing process and finalization of the manuscript prior to submission.

Chapter II -
Marko Freese wrote the majority of the manuscript, designed and coordinated the study, performed sampling, sample preparation and selection, participated in laboratory analyses and conducted data evaluation and statistical testing. Roxana Sühring developed the used methods for contaminant analyses and lead the analyses in the laboratory. Lasse Marohn and Klaus Wysujack performed artificial maturation procedures and wrote the corresponding section in the manuscript. Reinhold Hanel helped to develop the study and supervised throughout its development. All co-authors substantially contributed to the writing process and thus finalization of the manuscript prior to submission.

Chapter III -
Roxana Sühring wrote the majority of the manuscript. The study was mainly designed by Roxana Sühring and Marko Freese. Marko Freese designed, coordinated and performed sampling of the fishes. Roxana Sühring coordinated the compilation of data for the study, lead all laboratory analyses and conducted data evaluation and statistical testing. All co-authors substantially contributed to the writing process and thus finalization of the manuscript prior to submission.

Chapter IV -
Marko Freese wrote the majority of the manuscript, yet designed and coordinated the study together with Larissa Yokota Rizzo and Markus Brinkmann. Marko Freese and Jan-Dag Pohlmann selected the samples. Lasse Marohn and Klaus Wysujack performed artificial maturation procedures and wrote the corresponding section in the manuscript. Larissa Yokota Rizzo, Fabian Kiessling, Nihan Guenever and Twan Lammers conducted computer tomography analyses. Felix Gremse calculated calcium maps. Eckhard Witten conducted histology and respective analyses of eel bones for the study and wrote the corresponding section in the manuscript. Reinhold Hanel helped to develop the study and supervised throughout its development. All co-authors substantially contributed to the writing process and thus finalization of the paper prior to submission.

Chapter V –

This study was designed and the manuscript was written by Markus Brinkmann, Marko Freese and Jan-Dag Pohlmann, who contributed equally to the study and share the first authorship. Marko Freese and Jan-Dag Pohlmann conducted sampling, sample selection and the respirometric experiments. Markus Brinkmann was responsible for model adaptations and model calculations. Reinhold Hanel helped to develop the study and supervised throughout its development. All co-authors substantially contributed to the writing process and thus finalization of the manuscript prior to submission.

Chapter VI –

Natascha Michel and Marko Freese designed and coordinated this study. Marko Freese and Jan-Dag Pohlmann performed sample selection, sampling and sample preparation. Natascha Michel planned and conducted all laboratory analyses as well as data evaluation. Markus Brinkmann performed the modeling by adjusting the previously developed PBTK model with concentration data from the study. Reinhold Hanel helped to develop the study and supervised throughout its development. All co-authors substantially contributed to the writing process and thus finalization of the manuscript prior to submission.

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ERKLÄRUNG

Hiermit erkläre ich, dass die vorliegende Dissertation selbständig von mir angefertigt wurde. Die Dissertation ist nach Form und Inhalt meine eigene Arbeit und es wurden keine anderen als die angegebenen Hilfsmittel verwendet. Die in dieser Dissertationsschrift enthaltene Veröffentlichung aus Kapitel III mit dem Titel „Maternal transfer of emerging brominated and chlorinated flame retardants in European eels“ wurde von der Erstautorin Dr. Roxana Sühring auch im Rahmen ihrer kumulativen Promotionsarbeit an der Fakultät Nachhaltigkeit der Leuphana Universität Lüneburg zur Prüfung vorgelegt. Die Veröffentlichung aus Kapitel V mit dem Titel „A physiologically based toxicokinetic (PBTK) model for moderately hydrophobic organic chemicals in the European eel (*Anguilla anguilla*)“ wurde von dem Co-Erstautoren Prof. Dr. Markus Brinkmann auch im Rahmen seiner kumulativen Promotionsarbeit an der Fakultät für Mathematik, Informatik und Naturwissenschaften der RWTH Aachen Universität zu Erlangung des Doktorgrades vorgelegt. Abgesehen davon wurde die hier vorliegende Arbeit nicht einer anderen Stelle im Rahmen eines Prüfungsverfahrens eingereicht. Die Arbeit ist unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft entstanden. Dies ist mein einziges und bisher erstes Promotionsverfahren. Mir wurde noch kein akademischer Grad entzogen. Die Promotion soll im Fach Biologische Meereskunde erfolgen.

Kiel, den 30.04.2020

Marko Freese