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Four selected high molecular weight heterocyclic aromatic hydrocarbons: Ecotoxicological hazard assessment, environmental relevance and regulatory needs under REACH

Stephan Brendel[a,*], Christian Polleichtner[a], Andreas Behnke[b], Sönke Jessel[b], Enken Hassold[a], Christian Jennemann[c], Doreen Einhenkel-Arle[a], Albrecht Seidel[b]

[a] German Environment Agency, Dessau-Rosslau, Germany
[b] Biochemical Institute for Environmental Carcinogens Prof. Dr. Gernot Grimmer Foundation, Grosshansdorf, Germany
[c] Berlin Office for Occupational Safety, Protection of Health and Technical Safety (LAGetSi), Berlin, Germany

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ABSTRACT

Little is known about the ecotoxicity of heterocyclic aromatic hydrocarbons (NSO-HETs) to aquatic organisms. In the environment, NSO-HETs have been shown to occur in a strong association with their unsubstituted carbocyclic analogues, the polycyclic aromatic hydrocarbons (PAH), for which much more information is available. The present study addressed this issue by investigating the toxicity of four selected NSO-HETs in green algae (*Desmodesmus subspicatus*), daphnids (*Daphnia magna)* and fish embryos (*Danio rerio*). The four high molecular weight NSO-HETs dibenz[*a,j*]acridine (DBA), 7*H*-dibenzo[*c,g*]carbazole (DBC), benzo[*b*]naphtho[2,1-*d*]thiophene (BNT) and benzo[*b*]naphtho[1,2-*d*]furan (BNF) were selected, based on the results of a previous research project, indicating a lack of toxicity data and a high potential for persistence and bioaccumulation. The solubilities of the NSO-HETs in the test media were determined and turned out to be comparatively low (2.7–317 µg/L) increasing in the following order: DBA < BNT « DBC « BNF. Exposure concentrations during the toxicity tests were quantified with GC-MS and decreased strongly possibly due to sorption or metabolising during the test periods (48–96 h). Therefore, the estimated effect concentrations were related to the mean measured concentrations, as endpoints related to nominal concentrations would have underestimated the toxicity many times over. Within the range of the substance solubilities, BNF affected all test organisms with fish embryos being the most sensitive (fish: EC_{50} 6.7 µg/L, algae: EC_{10} 17.8 µg/L, daphnids: EC_{50} 55.8 µg/L). DBC affected daphnids (EC_{50} 2.5 µg/L,) and algae (EC_{10} 3.1 µg/L), but not fish embryos. The lowest toxicity endpoint was observed for BNT affecting only algae (NOEC 0.556 µg/L) and neither daphnids nor fish embryos. DBA did not show any effects on the tested organisms in the range of the water solubility. However, we would expect effects in long-term toxicity studies to fish and aquatic invertebrates for all substances at lower concentrations, which needs further investigation. All four NSO-HETs were identified in mussels (*Mytilus edulis*) from the German coasts, in green kale (*Brassica oleracea var. acephala*) and in freshwater harbor sediment in concentrations between 0.07 and 2 µg/kg, highlighting their relevance as environmental contaminants. There is a need to regulate the four NSO-HETs within the REACH regulation due to their intrinsic properties and their environmental relevance. However, acquisition of additional experimental data appears to be pivotal for a regulation under REACH.

1. Introduction

Polycyclic aromatic compounds (PACs) are a heterogenic group of thousands of individual substances with diverse chemical structures (Achten and Andersson, 2015). During the last 40 years the focus regarding hazard assessment has been made upon homocyclic polycyclic aromatic hydrocarbons (PAHs), mainly based on the 16 EPA PAHs priority list (Achten and Andersson, 2015; Andersson and Achten, 2015) established by the US environmental protection agency (EPA) (Keith, 2015). Little attention has so far been paid to the heterocyclic

* Corresponding author.
E-mail addresses: stephan.brendel@uba.de (S. Brendel), christian.polleichtner@uba.de (C. Polleichtner), andreas.behnke@biu-grimmer.de (A. Behnke), s.jessel@biu-grimmer.de (S. Jessel), enken.hassold@uba.de (E. Hassold), christian.jennemann@lagetsi.berlin.de (C. Jennemann), doreen.einhenkel-arle@uba.de (D. Einhenkel-Arle), albrecht.seidel@biu-grimmer.de (A. Seidel).

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PAHs and in particular the NSO-heterocycles (NSO-HETs), containing the heteroatoms nitrogen, sulfur or oxygen in the aromatic ring. There is limited data about their occurrence in the environment and about their adverse effects on aquatic organisms.

NSO-HETs occur mainly in substances of unknown or variable composition, complex reaction products, or biological materials (so-called UVCBs) derived from fossil fuels such as coal and crude oil (Schwarz et al., 2014). Even though the composition of such UVCBs is often largely unknown, it has been shown that coal tar consists approximately to 3–13% of NSO-HETs, which contribute to up to 40% of the water-soluble fraction (Licht et al., 1997; Muller et al., 1989; WHO, 2004). Further emissions of NSO-HETs are observed for textile materials (Luongo et al., 2016), dyestuff (Cripps et al., 1990), pesticides (Broughton and Watson, 2004) and natural sources (alkaloids or mycotoxins). NSO-HETs possess a higher water solubility in comparison with homocyclic PAHs (Feldmannova et al., 2006; Pearlman et al., 1984), resulting in an increased availability in aquatic ecosystems and an elevated risk for pollution of drinking water resources. NSO-HETs have been identified at contaminated sites, as for example sites related to former wood impregnation plants, gas plants or coke manufacturing sites (Blum et al., 2011; Pereira et al., 1987) and have been detected in various environments such as groundwater, river basins, coastal areas and sediments (Brack et al., 2007, 2005; De Voogt and Laane, 2009; Hartnik et al., 2007; Reineke et al., 2007; Siemers et al., 2017). They often appear in combination with homocyclic PAHs (Witter and Nguyen, 2016).

Even though NSO-HETs seem to be quite prominent in the environment little is known about their adverse effects on aquatic organisms. However, available studies show that NSO-HETs exhibit similar toxicities to aquatic organisms compared to their unsubstituted carbocyclic analogues: Ecotoxicological effects for algae, daphnids and fish embryos are observed for several mono- to tricyclic NSO-HETs in concentrations in the low mg/L range (Eisentraeger et al., 2008; Peddinghaus et al., 2012). Although so far poorly explored, it is shown for some NSO-HETs that (i) they have genotoxic and mutagenic potentials (Brinkmann et al., 2014; Eisentraeger et al., 2008), (ii) metabolites show an estrogenic activity comparable to homocyclic PAHs (Brinkmann et al., 2014) and (iii) degradation products can also cause adverse effects on organisms (Bleeker et al., 1999; Oberoi and Philip, 2017).

In a previous project the hazardous potential of NSO-HETs with respect to the environment and the need for potential regulatory activities were evaluated (Schwarz et al., 2014; see also Umweltbundesamt, 2012). Within this project four NSO-HETs have been identified which are very likely persistent (P), bioaccumulative (B) and toxic (T) and therefore, probably fulfill the PBT-criteria. However, data allowing a conclusion on their ecotoxicity are missing. Substances fulfilling the PBT-criteria can be identified as “Substances of Very High Concern” (SVHC) and their production, import and use in the European Union may be further regulated within the REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) regulation (EC No. 1907/2006), resulting in reduced emissions.

The present study aimed to investigate the ecotoxicity of the selected four NSO-HETs, namely benzo[*b*]naphtho[1,2-*d*]furan (BNF), 7*H*-dibenzo[*c,g*]carbazol (DBC), dibenz[*a,j*]acridine (DBA) and benzo[*b*]naphtho[2,1-*d*]thiophene (BNT). The substances contain one heteroatom in the ring system and are selected as representatives of the groups of furanes, carbazoles, acridines, and thiophenes (Table 1). Effects on green algae (*Desmodesmus subspicatus*), daphnids (*Daphnia magna*), and fish embryos (*Danio rerio*) have been assessed in standard toxicity studies according to OECD test guidelines (TG). To our knowledge, this is the first study to investigate the ecotoxicological effects of such high molecular weight NSO-HETs, relating the endpoints to measured (GC-MS) exposure concentrations. This has been concluded mandatory (Eisentraeger et al., 2008; Peddinghaus et al., 2012), as nominal concentrations overestimate the exposure concentrations and consequently underestimate the toxicity manifold, even when testing lower molecular weight NSO-HETs. Additionally to the ecotoxicological hazard assessment, the concentrations of the respective NSO-HETs were quantified in blue mussels (*Mytilus edulis*) from the Baltic Sea and North Sea, in a freshwater harbor sediment (certified reference material) and in market-fresh green kale (*Brassica oleracea var. acephala*) to investigate the environmental relevance of the four NSO-HETs. Finally it is discussed whether the test results allow to classify the four tested NSO-HETs as SVHCs and which further steps for a regulation are necessary.

2. Methods

2.1. Chemicals

The four NSO-HETs (for chemical structures see Table 1) were purchased from a commercial supplier (Sigma-Aldrich, Taufkirchen) and supplemented by own synthesis at the Biochemical Institute for Environmental Carcinogens Prof. Dr. Gernot Grimmer Foundation. To achieve a uniform quality of the compounds, the total amount was either crystallized directly from cyclohexane or previously purified by flash chromatography on silica gel 60 (particle size 0.040–0.063 nm, Merck, Germany). All substances were obtained in high purity (> 99% measured with GC-FID (gaschromatography with flame ionization detector)). The melting points of all four compounds were determined using a conventional melting point apparatus (Buchi model 510) and are uncorrected (see Table 1). Water solubility in artificial freshwater and solubility in the other test media were determined according to the ASTM method E1148-02 (ASTM, 2008) (*vide infra*).

2.2. Preparation of stock solutions and determination of NSO-HETs in the test media

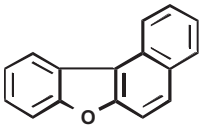
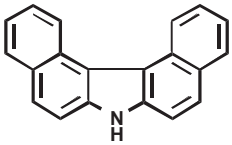
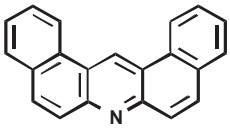
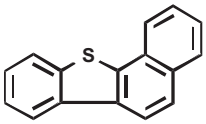
Stock solutions were already prepared in the respective test media (Elendt M4 Medium for daphnids, OECD medium for green algae and artificial freshwater for fish embryos). Solubilities of the test substances in artificial freshwater are given in Table 1 (see Table S1 for the solubilities in all test media). To obtain respective test concentrations, the stock solutions were further diluted.

The experimental procedure to determine the solubility and to prepare the stock solutions of the four test compounds was developed and further adjusted on the basis of the ASTM method E1148-02 (ASTM, 2008): The test substance dissolved in tetrahydrofuran is poured into an Erlenmeyer flask and subsequently the solvent is blown off in a stream of nitrogen, while the flask is slightly moved. Under these conditions the glass wall of the flask is coated with very fine particles of the test substance. Subsequently, the test medium is added and the mixture is incubated at the test temperature with agitation of the Erlenmeyer flask in a shaking water bath, a combined orbital/linear Shaking Bath OLS 200 obtained from Grant Instruments Ltd. (Cambridge, UK).

When applying the standard procedure, the concentrations of the stock solutions did not show a sufficient repeatability. Therefore, it was adjusted as follows: (i) use of silanized Erlenmeyer flasks, (ii) incubation of the test substance at 60 °C for 24 h to achieve a fast saturation of the solution followed by (iii) an incubation for 24 h at the test temperature, and finally (iv) the filtration using syringe filters with alumina as filter material (pore size 0.02 µm) to achieve a sufficient removal of fine undissolved particles and to avoid almost adsorption effects on the filter material.

The determination of the NSO-HETs was carried out using the established analytical method applying the stable isotope dilution GC-MS technique (Schwarz et al., 2014).

Table 1
Names, chemical structural formulae, CAS numbers, melting points (experimental), octanol-water partition coefficients (calculated) and water solubility (experimental) of the four NSO-HETs.

Name	Structural formula	CAS	MP[a] [°C]	log Kow[b] (QSAR)	WS[c] in Elendt M4 Medium (20 °C) [µg/L]
Benzo [*b*] naphtho [1,2-*d*] furan (BNF)		205-39-0	43	4.89	254.49
7*H*-Dibenzo [*c,g*] carbazol (DBC)		194-59-2	157	5.58	49.43
Dibenz [*a,j*] acridine (DBA)		224-42-0	215–217	5.67	2.72
Benzo [*b*] naphtha [2,1-*d*] thiophene (2,1-BNT)		239-35-0	183–184	5.34	4.08

[a] MP: melting point.
[b] QSAR: KOWWIN v1.68 EPIWEB.
[c] WS: water solubility.

2.3. Ecotoxicological methods

Preliminary limit-tests were conducted for each test substance with two treatments, the saturated stock solutions and a control. Apart from that limit-tests were carried out according to the procedures and OECD TG described later in this section. Only if the limit-tests indicated significant ($p < 0.05$) adverse effects, the respective substance and test organism were further tested in toxicity tests, to determine the actual effect concentrations. All ecotoxicological tests were performed following the respective OECD TG and met the defined validity criteria (for further information see the corresponding TG). Each test system consisted of seven treatments, including the control. The lowest, the middle and highest test concentration of each test substance in the three test systems given in Table 2.

The growth inhibition test with *Desmodesmus subspicatus* according to the OECD TG 201 (OECD, 2011) was performed as a static test for 72 h. Each test concentration was tested in triplicates and for the controls 6 replicates were tested. Endpoint assessed was inhibition of growth, calculated on the basis of cell counts (see OECD TG 201). The culturing and testing conditions in the control treatment allowed unrestricted exponential growth under nutrient sufficient conditions and continuous light.

The acute immobilization test with *Daphnia magna* was conducted in compliance with the OECD TG 202 (OECD, 2004) with a static test design using five replicates for treatments with the test substances and 10 for the control treatments. Young daphnids, aged less than 24 h at the start of the exposure, were exposed for a period of 48 h. Immobilization was recorded at 24 h and 48 h and compared with control values.

The acute fish embryo toxicity test (FET) with *Danio rerio* was performed according to the OECD TG 236 (OECD, 2013b) for 96 h in a semi-static test design (daily exchange of all test solutions). Tests started within 90 min post-fertilization. For each substance treatment 20 replicates and for the controls 24 replicates were exposed. Mortality as well as hatching rate (sublethal endpoint) were recorded.

2.4. Determination of the NSO-HETs in biota and sediment samples

A quantitative analysis of the four NSO-HETs in blue mussels (*M. edulis*) from the Baltic Sea (fjord of Kiel, Germany, August 2014) and North Sea (harbor entrance Neuharlingersiel, Germany, May 2014) was carried out in order to demonstrate the relevance of the four NSO-HETs for aquatic ecosystems. The two sampling points allowed for a comparison of mussels from a region with a relative high burden of environmental contaminants (fjord of Kiel) and a rather natural region (Neuharlingersiel: Lower Saxony Wadden Sea National Park). The GEOMAR, Helmholtz Centre for Ocean Research in Kiel, Germany as well as the Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research (AWI) in Bremerhaven, Germany collected and provided the samples (about 500 g fresh weight each). Additionally, the concentration of the four NSO-HETs was determined in a freshwater harbor sediment (certified reference material BCR-535, IRMM, Geel, Belgium). Market-fresh kale (*Brassica oleracea* var. *acephala*); cultivated in Bardowick (Lueneburg district, Northern Germany), another species commonly used as biological indicator for environmental pollution with PAHs (Srogi, 2007), was also analyzed for the contents of the NSO-HETs. All samples have been stored at − 20 °C until used for analysis. The determination of the NSO-HETs was carried out using the well-established analytical method applying the stable isotope dilution GC-MS technique (Schwarz et al., 2014).

2.5. Statistical analysis

All experimental results presented and statistical analysis are based on the geometric mean of measured concentrations at the start and end of the exposure, as concentrations during the ecotoxicological tests decreased strongly (see Table 2). As a conservative assumption, concentrations below the limit of quantification (LOQ) were not considered LOQ/2, but left as unchanged values.

NOEC (no observed effect concentration) and LOEC (lowest observed effect concentration) values were calculated for each ecotoxicity test with the statistics program ToxRat Professional XT, version 3.2.1 (ToxRat Solutions GmbH, Alsdorf, Germany). The FET (hatching rate

Table 2
Nominal and measured concentrations of the four NSO-HETs in the lowest, the middle and highest nominal test concentration of the three test systems given in µg/L. The decrease among the exposure concentrations at the start and end of exposure is given in percent.

Test substance	Test organism	Nominal concentration	Measured concentration		Decrease during exposure	Geometric mean
			t_{start}	t_{end}		
BNF	*D. subspicatus*	1.0	0.68	< 0.013[a]	> 98.1	0.094
		15.0	11.24	2.78	75.3	5.59
		200.0	142.2	134.4	5.5	138.25
	D. magna	1.0	0.53	0.25	52.8	0.36
		125.0	67.76	58.59	13.5	63.01
		225.0	113.85	82.60	27.4	96.97
	D. rerio	0.902	0.5	0.054	89.2	0.16
		90.24	43.90	1.59	96.4	8.35
		288.77	136.74	5.21	96.2	26.69
DBC	*D. subspicatus*	1.0	2.1	< 0.056[a]	> 97.3	0.34
		8.0	8.76	1.69	80.7	3.85
		32.0	28.19	11.21	60.2	17.78
	D. magna	0.095	< 0.056[a]	< 0.056[a]	–	0.06
		14.25	5.82	2.31	60.3	3.67
		95.00	38.02	22.67	40.4	29.36
DBA	*D. subspicatus*	1.0	0.32	< 0.039[a]	*> 87.8*	0.11
		2.0	0.71	< 0.039	*> 94.5*	0.17
		3.0	1.34	< 0.039	*> 97.1*	0.23
BNT	*D. subspicatus*	0.1	0.03	< 0.015[a]	> 50	0.02
		0.8	0.29	0.10	34.5	0.17
		4.4	1.85	0.83	44.9	1.24

[a] Value less than the LOQ.

and mortality) and the test with *D. magna* were evaluated with a step-down Cochran-Armitage test procedure. William´s test, Bonferroni median test or Bonferroni-Welch test were performed to analyse the results of the test with green algae. The testing procedure was dependent on whether the assumptions for normal distribution and homogeneity of variance, examined with Levene´s and Shapiro-Wilk´s test respectively, were met (significance level 0.01). The ecotoxicological test results were considered significant if $p < 0.05$.

Effect concentrations (EC_x) (10% and 50%) were calculated for each ecotoxicity test with dose-response models using the drc extension package (Knezevic et al., 2007) for the statistics program R Version 3.3.0, if the results show a dose-response relationship. Weibull, Log-normal and log-logistic 3-parameter (green algae) and 2-parameter (fish embryos and daphnids) models were fitted to the data. The model selection was based on the Akaike information criterion (AIC) and expert judgment.

The tests and selection of test concentrations were primarily designed to calculate effect concentrations. Therefore, the drawn conclusions should be based on the effect concentrations instead of the NOEC or LOEC values. Effect concentrations should generally be preferred over NOEC/LOEC values, as the NOEC/LOEC concept is criticized and has in comparison major disadvantages (Laskowski, 1995). However, if the results of a test do not allow fitting a dose-response model, the NOEC/LOEC concept was assumed appropriate in this study, as it also finds application in the regulatory context (OECD, 2014).

3. Results

3.1. Preliminary limit-tests

All four NSO-HETs, inhibited the growth of *D. subspicatus* significantly in the limit-tests. A significant amount of immobilized *D. magna* were observed in the limit-tests with DBC and BNF, but not with BNT and DBA. *D. rerio* showed a significant suppression of hatch in the limit-test with BNF, but not with DBA, DBC or BNT. The suppression of hatch is regarded as a sublethal endpoint and should not be used for the calculation of lethal concentrations (OECD, 2013b). Table 3 gives mean measured concentrations of the limit-tests showing no significant adverse effects on the test organisms.

3.2. Test concentrations

The solubility of the four NSO-HETs in the three test media increased in the following order: DBA < BNT « DBC « BNF (see Table 1, S1). The test concentrations in the toxicity tests were measured at the start and end of the respective exposure time. During the toxicity tests, the test concentrations decreased strongly (Table 2), resulting in a low reproducibility of the test results. However, nominal and mean measured concentrations correlate linearly in all tests (pearson correlation coefficient 0.88–0.99), showing a concentration dependent exposure of the test organisms. Due to the low solubilities and high adsorption potentials of the four tested NSO-HETs, the achievement of higher recovery rates was not possible by means of the used static or semi-static (FET) test designs.

3.3. Effects on green algae

Of the four NSO-HETs tested, BNF, DBC and BNT significantly inhibited the growth rate (Fig. 1) and yield (Fig. S1) of green algae. The effects on yield and growth rate showed comparable results. Due to mathematical basis of the respective approaches, the endpoint yield is in general more sensitive. Table 3 provides the estimated effect concentrations (EC_{10}, EC_{50}) with confidence intervals, as well as the respective NOECs and LOECs. DBC and BNT showed effects in the lower µg/L range, whereas BNF was around fivefold less toxic. DBA did not inhibit the yield or growth rate of the algae in the tested concentrations, which is very likely associated with the low solubility and resulting low test concentrations (highest tested concentration: 0.23 µg/L). Dose-response relationships can be established with narrow confidence intervals for the effects on growth rate (Fig. 1, Table 3) and yield (Fig. S1) in the toxicity tests with BNF and DBC, but not for BNT. Despite an obvious inhibition of the growth rate in the test with DBC, effects are not significant with the selected test for non-parametric data (Bonferroni mediantest). The results of DBA and BNT do not allow determining a dose-response relationship due to the low inhibition of yield and growth rate in the treatments. However, BNT caused a significant inhibition of yield and growth rate in the highest treatment concentration (1.24 µg/L; William's test: $p < 0.001$; inhibition yield: 10.7%, inhibition growth rate: 2.5%). For DBA, no significant effects are

Table 3
Effects concentrations, NOECs and LOECs for green algae, daphnids and fish embryos after 72 h, 48 h and 96 h of exposure, respectively. All endpoints are related to mean measured concentrations. If there were no significant effects in the limit-tests, effects are denoted as greater than the measured concentration in the respective limit-test. Concentrations are given in µg/L, including 95% confidence intervals.

Substance	Effect concen-tration	Algae		Daphnids	Fish	
		Growth rate	Yield	Immobility	Mortality	Hatch
BNF	EC_{10}	17.83 (17.1–18.6)	10.94 (9.5–12.4)	36.10 (28–44.2)	- [a]	0.84 (0.22–1.45)
	EC_{50}	51.15 (49.8–52.5)	21.92 (20.5–23.4)	55.75 (49.2 − 62.3)	- [a]	6.73 (3.76–9.7)
	NOEC	5.59	5.59	37.57	- [b]	3.6
	LOEC	12.79	12.79	63.11		8.35
DBC	EC_{10}	3.06 (2.4–3.8)	1.71 (1.2–2.2)	2.3690	> 10.78 [c]	> 10.78 [c]
	EC_{50}	11.28 (10.4–12.1)	4.21 (3.8–4.7)	2.5455		
	NOEC	- [b]	3.85	1.051		
	LOEC		8.7	3.669		
DBA	EC_x	- [a]	- [a]	> 3.45 [c]	-	-[c,dc,d]
	NOEC/LOEC	- [b]	- [b]			
BNT	EC_x	- [a]	- [a]	> 1.62 [c]	> 0.46 [c]	> 0.46 [c]
	NOEC	0.566	0.566			
	LOEC	1.241	1.241			

[a] No dose-response relationship.
[b] Not significant based on dose-response studies.
[c] Not significant based on the limit-test.
[d] Mean measured concentration in the limit-test not determined due to analytical errors

determinable.

3.4. Effects on daphnids

Ecotoxicity tests were conducted with BNF and DBC as only these significantly affected the mobility of *D. magna* in the preliminary limit-tests. Both test substances show a dose-response relationship regarding the relevant endpoint immobility (Fig. 2A). Effect concentrations (EC_{10} and EC_{50}) as well as NOECs and LOECs are given in Table 3. DBC affected *D. magna* in the lower µg/L range whereas for BNF effects were first seen in higher concentrations. The dose-response relationship of DBC is very steep, increasing from 0% to 100% from one treatment

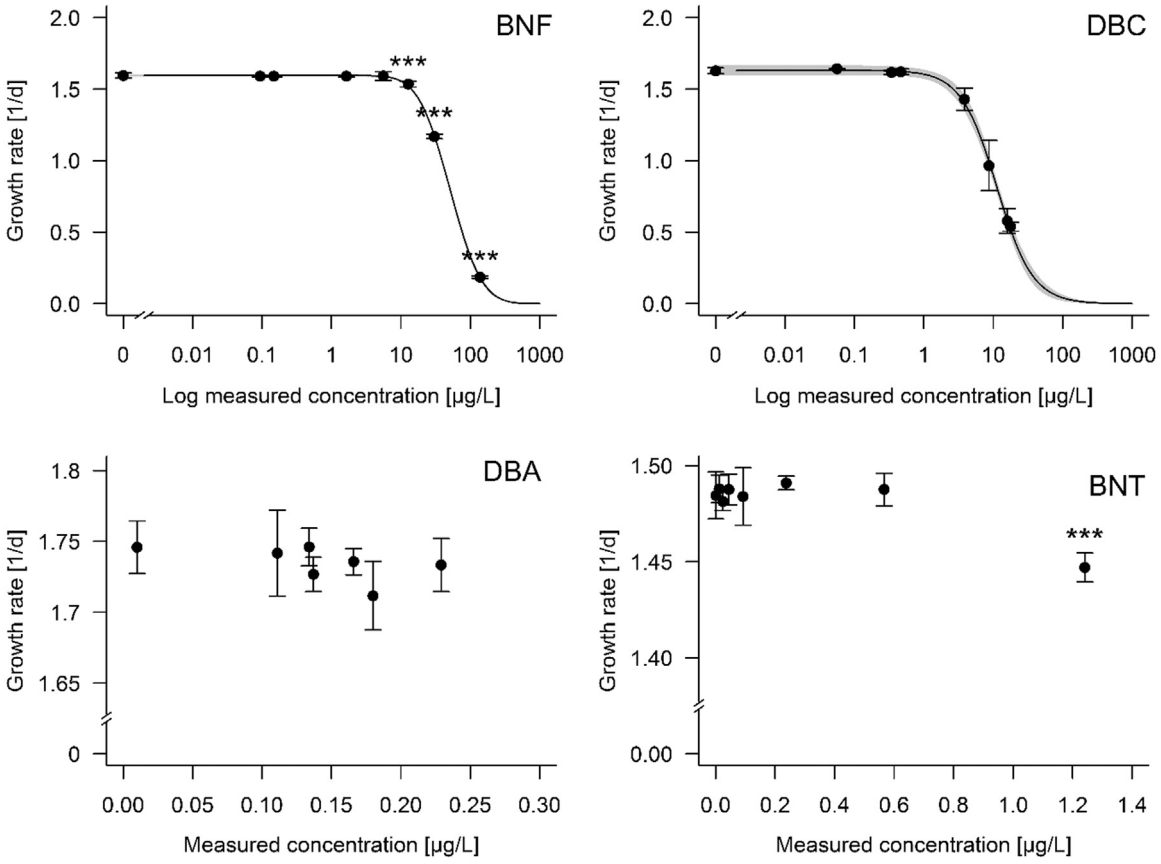


Fig. 1. Effects of the test substances BNF, DBC and BNT on the growth rate of the green algae *D. subspicatus* after 72 h of exposure. The figures show mean values (control: n = 6, treatments n = 3) with related standard deviations and dose-response relationships with 95% confidence intervals when possible. Dose-response relationships are determined with a 3-parameter log normal or 3-parameter log logistic model. Asterisks denote significant differences to the control (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Note: The logarithmic x-scale is just applied for BNF and DBC.

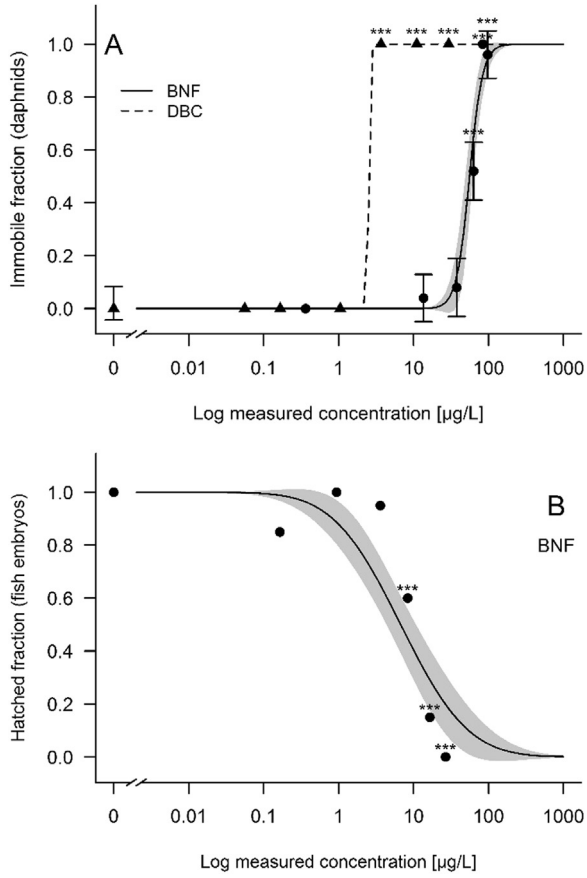


Fig. 2. A: Effects of the test substances BNF and DBC on the mobility of *D. magna.* after 48 h of exposure (control: n = 10, treatments n = 5). **B**: Effects of the test substances BNF on the hatching rate of *D. rerio* embryos after 96 h of exposure (control: n = 24, treatments n = 20). The figures show (geometric) mean values with standard deviations and dose-response relationships with 95% confidence intervals. Dose-response relationships are determined with 2-parameter lognormal and 2-parameter log logistic models. Asterisks denote significant differences to the control (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

concentration to another (1.05–3.7 µg/L) without any variation among the replicates. Therefore, no standard deviations and no confidence intervals regarding the model fit can be determined, but the range in which the effect concentrations are located is clearly localized.

3.5. Effects on fish embryos

The FET was just conducted with BNF, as in the preliminary limit-tests the other tested NSO-HETs revealed no significant toxicities towards fish embryos. The sublethal endpoint hatching rate shows a clear dose-response relationship, displayed in Fig. 2B. In addition, significant differences of the treatments to the control could be observed (Table 3). Compared to the EC_{50} of BNF in the tests with daphnids and green algae, the EC_{50} for hatching rate of zebrafish are more than one order of magnitude lower. No dose-response relationship can be established for the endpoint mortality, as there were no significant toxic effects.

3.6. Determination in sediment and biota

The four NSO-HETs were detected above their LOQ (0.02–0.04 µg/kg) in all samples, namely mussels (*M. edulis,* North and Baltic sea*)*, freshwater harbor sediment (certified reference material BCR-535) and green kale (*Brassica oleracea var. acephala*) (Table 4). The highest concentrations were observed in the freshwater harbor sediment, in accordance with the high adsorption potential of the substances. In

Table 4
Levels of the NSO-HETs in blue mussels (*Mytilus edulis*) from the North Sea and Baltic Sea, in fresh green kale (*Brassica oleracea var. acephala*) and freshwater harbor sediment. Concentrations are given in µg/kg.

NSO-HET	Concentration			
	M. edulis North Sea	*M. edulis* Baltic Sea	Green kale	Freshwater harbor sediment
BNF	0.068	0.811	0.803	171.284
DBC	1.051	1.968	0.756	36.919
DBA	0.741	1.726	1.114	31.290
BNT	0.072	1.523	0.185	477.100

comparison with the blue mussels collected from a rather natural site in the North Sea, the blue mussels collected in the Baltic Sea show, as expected, higher concentrations of the NSO-HETs reflecting the contamination caused by the commercial shipping and the surrounding industrial area. In case of BNT and BNF the difference in concentrations is very pronounced (21-fold BNT, 12-fold BNF), whereas the difference for DBC and DBA is much lower (2-fold), but still considerable. Interestingly, the BNF content in green kale is very similar to that found in the more contaminated blue mussels from the Baltic Sea, whereas the concentrations of DBA, DBC, and BNT are closer to the lower levels found in the blue mussels from the North Sea.

4. Discussion

4.1. Ecotoxicological hazard assessment

The present study examined the ecotoxicological potential of four high molecular weight NSO-HETs. Except DBA, the tested NSO-HETs showed toxic effects at very low concentrations in one or more test organisms (Table 3, Figs. 1 and 2). The EC_{50} of BNF are around 50 µg/L for daphnids and green algae and the hatching rate of zebrafish embryos is suppressed at even lower concentrations. DBC showed high toxicities to *D. subspicatus* and *D. magna* in concentrations of only a few µg/L, but not in fish. BNT already exhibited significant effects on green algae at 1 µg/L. In case of DBA and BNT higher concentrations would have been needed to establish dose-response relationships, but this was technically not feasible. Long-term toxicity studies would very likely result in effects at lower concentrations.

Even though the present study shows that three out of four investigated NSO-HETs are highly toxic to aquatic organisms and are present in the environment, there is limited data on their ecotoxicity found in the literature. Warshawsky et al. (1995) examined the toxicity of DBC and DBA under the influence of cool white light on the survival of the green algae *Selanestrum capricornutum* at nominal concentrations of 0.4 mg/L during six days. Green algae in the DBA treatment showed at the beginning of the experiment a slight inhibition of growth, followed by a recovery. DBC caused a strong inhibition of growth during the whole experiment. The author explained the toxicity of DBC to be caused either by the production of reactive metabolites or by the occurrence of highly toxic photoproducts. Those results are in line with the results of the present study, which examined even higher toxicities of DBC, attributed to the analytical monitoring conducted. However, the potential phototoxicity of the tested NSO-HETs was not addressed in the present study. Ecotoxicological data for BNF is available in the US-EPA ecotoxicity database (Maas, 1990), giving an LC_{50} for the immobility of *D. magna* after 48 h of exposure as nominal concentration of 4 mg/L. Results of the present study also show effects on the mobility of *D. magna* but at much lower concentrations, which is as in case of DBC attributed to the relation to measured instead of nominal concentrations. Eastmond et al. (1984) examined the toxicity of BNT to *D. magna* and examined no effect on immobility after 48 h of exposure. This is in accordance with the preliminary limit-tests.

Several effects may be responsible for the decline of test concentrations during exposure such as adsorption, partial precipitation or metabolism of the test compounds. However, no precipitation has been observed and the test vessels were saturated with the respective NSO-HET to minimize a loss of the test substances due to adsorption. The decrease of the test concentrations is most pronounced in the lower concentrations, which possibly indicates a saturation of the metabolic turnover and therefore, a high relevance of the metabolic capacity of the test organisms. In general PAHs and NSO-HETs are expected to be metabolically converted to hydroxylated derivatives by cytochrome P450 enzymes found to be expressed in all three test organisms (Akkanen and Kukkonen, 2003; Andreasen et al., 2002; De Voogt et al., 1999). However, the oxidative metabolism of the four studied NSO-HETs in the aquatic test organisms and the possible contribution of the formed metabolites to their toxicity is unknown.

When comparing the ecotoxicological results of the present study with the results from the literature regarding mono- to tricyclic NSO-HETs (Eisentraeger et al., 2008; Peddinghaus et al., 2012), it can be concluded that in general an increase in the log Kow results also in a higher toxicity of NSO-HETs. This was also found by Bleeker et al. (1999), showing that the toxicity of N-HETs increases with an increasing number of aromatic rings. One explanation is a higher bioavailability with an increasing amount of aromatic rings, until steric effects (Dimitrov et al., 2002) reduce the uptake of the molecule. This is supported by studies examining the bioaccumulation potential of NSO-HETs, indicating high bioconcentration factors (BCFs) for the here tested NSO-HETs. For BNT Eastmond et al. (1984) found a BCF of 8000 in *D. magna*. Furthermore, De Voogt et al. (1991) examined a BCFs of 14900 for benzo[*b*]naphtha[2,3-d]thiophene (an isomer of BNT) and of 7060 for 13*H*-Dibenzo[*a,i*]carbazole (an isomer of DBC) in guppys (*Poecilia reticulata*).

4.2. Environmental relevance

The environmental relevance of the four NSO-HETs investigated in the present study is exemplary highlighted by determining their concentrations in selected sediment and biota samples. Considering the observed ecotoxicity of three out of the four NSO-HETs in the low µg/L range, the observed concentrations in blue mussels, green kale and harbor sediment are of concern. Even at an almost natural sampling site (Neuharlingersiel, North Sea), DBA and DBC were found in the µg/kg range in blue mussels. Other studies confirm that NSO-HETs are present contaminants in sediments of coastal areas and rivers (Brulik et al., 2013; De Voogt and Laane, 2009; Tolosa et al., 1996). Compared to homocyclic PAHs, NSO-HETs seem to occur in lower concentrations in sediments, but are shown to prevail in the water phase (Siemers et al., 2015). Reliable data on the occurrence of tetra- or pentacyclic NSO-HETs are poorly available in the literature. However, BNT is shown to occur in sediments and crude oils (Li et al., 2012; Witter and Nguyen, 2016). Blue mussels are well established as bioindicators of seawater pollution with PAHs (Beyer et al., 2017). The German Specimen Bank provides time histories over the last few decades on BNT concentrations in *M. edulis* from national parks of the North Sea and Baltic Sea. In these natural areas concentrations of BNT show a decline in the last 30 years, but remained constant within the last decade (for example around 2 ng/g at Eckwarderhörne, see Fig. S2). Concentrations measured are far below the concentrations observed in the present study.

4.3. Regulatory options to reduce environmental exposure to NSO-HETs

Identification of “Substances of Very High Concern” (SVHCs) is one of the key components of the REACH regulation of the European Union (EC No. 1907/2006). Amongst other characteristics, substances that fulfill the criteria to be persistent (P), bioaccumulative (B) and toxic (T) (PBT criteria) or very persistent (vP) and very bioaccumulative (vB) (vPvB criteria) are considered of very high concern for the environment and human health. Annex XIII of REACH defines the characteristics of SVHCs. Emissions of SVHCs into the environment should be as far as possible reduced and therefore, they need to be further regulated. UVCBs (substances of unknown or variable composition, complex reaction products and biological materials) containing a constituent, impurity or additive above 0.1% (w/w) being SVHC are considered themselves as SVHC. NSO-HETs are often constituents of under REACH registered UVCBs with a high production volume, as for example BNT in diesel (CAS 68334-30-5), asphalt (CAS 232-490-9) and fuel oil (CAS 68476-33-5).

The main perspective of this study is to obtain a better knowledge on the toxicity and environmental occurrence of NSO-HETs and to fill data gaps for possible regulatory actions. Albeit the present study is focusing on the European chemicals legislation, results are also useful for the risk assessment in other chemical legislations (Van Leeuwen and Vermeire, 2007). In a preliminary study four NSO-HETs have been identified of meeting probably the persistence and bioaccumulation criteria and having an environmental relevance, but data on the ecotoxicity was so far missing. In case a substance shows toxic effects in long-term toxicity tests to aquatic organisms below a concentration threshold of 0.01 mg/L, the toxicity criteria for the respective substance is fulfilled. This study shows that DBC clearly fulfills the T criteria, as already the tests with green algae and daphnids show toxicities below the threshold value. In the test with green algae BNT shows a NOEC below 0.01 mg/L and therefore also meets the T criteria. The FET is currently only accepted together with other information in a weight of evidence approach in the REACH regulation and not fully accepted as alternative to the acute toxicity test, which is often criticized (for example Braunbeck et al., 2015). Furthermore, hatching is not a final endpoint given in the respective OECD TG 236, stating that if delayed hatch occurs, another toxicity tests might be more appropriate (OECD, 2013b). Therefore, the T criteria cannot be clearly addressed to BNF, as the toxicity to green algae barely misses the T criteria. DBA did not show effects at the maximum level of solubility. However, long-term tests with daphnids (OECD, 2012) or fish (e.g. OECD, 2013a), not conducted in the course of the present study, might result in higher toxicities and effects within the solubility range (Ahlers et al., 2006; May et al., 2016).

There are strong indications that DBC and BNT have a high bioaccumulation potential. The B or vB criteria are fulfilled if the BCF in fish is above 2000 or 5000 respectively, which is in a weight of evidence approach indicated by the studies of Eastmond et al. (1984) and De Voogt et al. (1991). The BCF of BNT in *D. magna* is 8000 and the BCFs of two isomers of BNT and DBC are 7060 and 14900, respectively. QSAR calculations confirm the high bioaccumulation potential of DBC and BNT and indicate that both NSO-HETs are persistent in the environment (Schwarz et al., 2014). For DBC the assumption of persistence is additionally supported by mineralization experiments in soils finding no mineralization within 64 days (Grosser et al., 1995). Observations in field studies indicate that NSO-HETs are in general more persistent in the environment than their unsubstituted carbocyclic analogues (Blum et al., 2011).

5. Conclusions

The present study provides reliable ecotoxicity data for the investigated tetra- and pentacyclic NSO-HETs and highlights their environmental relevance. This is the first study evaluating the ecotoxicity of such high molecular weight NSO-HETs and relating the observed effects to measured concentrations. The high observed toxicities to *D. magna* and *D. subspicatus* and the occurrence in all analyzed environmental samples (freshwater harbor sediment, blue mussels from contaminated and nearly natural coastal areas and green kale) is of concern. To fully understand the molecular mechanisms underlying the observed toxicities, elucidation of their metabolism in these aquatic test organisms warrants further investigations.

The release of NSO-HETs into the environment should be minimized. DBC und BNT fulfill the T criteria of the REACH regulation (Annex XIII) and are on a weight of evidence approach most likely "Substances of Very High Concern" requiring regulation. It is very probable that also BNF and DBA would have effects at lower concentrations in long-term toxicity studies to fish and daphnids and hence could fulfill the T criteria for PBT substances. Further standardized data according to respective guidelines regarding the PBT properties and information on the uses of NSO-HETs are mandatory to establish appropriate regulatory measures.

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The authors declare that they have no competing interests.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2018.07.035.

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