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

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A B S T R A C T

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

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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).