Ecotoxicity of NSO-heterocycles (NSO-HET) and short-chained alkyl phenols (SCAP) commonly detected in contaminated groundwater

Running head (60 characters incl. spaces): Toxicity of NSO-heterocycles and short-chained alkyl phenols

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Data are available upon request from the corresponding authors.

Competing Interests

The authors have no competing interests to declare/

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Abstract

Heterocyclic aromatic hydrocarbons (NSO-HET) and short-chained alkyl phenols (SCAP) are commonly detected in groundwater at contaminated sites and in the surrounding environment. It is now scientific consensus that these chemicals pose a risk to human and ecosystem health. However, toxicity data are comparably fragmentary and only few studies have addressed the ecotoxicity of NSO-HET and SCAP in a systematic and comparative fashion. To overcome this shortcoming, we tested 18 SCAP, 16 NSO-HET, as well as the homocyclic hydrocarbons indane and indene in the Microtox® assay with *Aliivibrio fischeri*, the growth inhibition test with *Desmodesmus subspicatus*, the acute immobilization assay with *Daphnia magna*, as well as the fish embryo toxicity (FET) test with embryos of the zebrafish (*Danio rerio*). Because of the physicochemical properties of the tested chemicals (limited water solubility, volatility and sorption to test vessels), actual exposure concentrations in test media and their dissipation over time were analytically quantified by means of gas chromatography with mass spectrometry (GC/MS). Analytically corrected effect levels (EC/LC_{50}s) ranged from 0.017 to 180 mg L^{-1}, underlining the environmental relevance of some NSO-HET and SCAP. Here, we provide, for the first time, a complete high-quality dataset in support of better environmental risk assessments of these chemicals.

Keywords: SCAP, NSO-HET, Hetero-PAH, ecotoxicology, biotest

1. Introduction

A plethora of aromatic hydrocarbons are produced intentionally or released as unwanted by-products since the onset of industrialization. In addition to substituted and unsubstituted polyaromatic hydrocarbons (PAHs) and chlorinated aromatic hydrocarbons, such as polychlorinated biphenyls and polychlorinated dibenzodioxins and -furans, this group also comprises the less known NSO-heterocyclic hydrocarbons (NSO-HET). In these chemicals, one or more carbon atoms are substituted with nitrogen, sulphur or oxygen heteroatoms. This substitution makes NSO-HET more water soluble and thus mobile compared to their homocyclic analogues (Blotevogel et al., 2008), resulting in long plumes of contamination in groundwater at contaminated sites (Zamfirescu and Grathwohl, 2001) and a substantial risk of contaminating drinking water resources (Kuhn and Suflita, 1989; Meyer, 1999). Particularly high concentrations are found at sites contaminated with tar-oil (Blum et al., 2011; Brack and Schirmer, 2003; Rasmussen and Olsena, 2004; Tiehm et al., 2008), a complex mixture of roughly 10,000 chemicals (Collin and Höke, 1995), approx. 85% of which are PAHs and 5-13% NSO-HET (Meyer, 1999). NSO-HET have also been detected at sites contaminated with crude oil and petro-chemical products (Mundt and Hollender, 2005), coke manufacturing sites, gasworks and wood impregnation plants (Kohler et al., 2000). In Germany alone, there are more than 1,300 of such contaminated sites (Blotevogel et al., 2008). NSO-HET contamination may also originate indirectly from heterocylic dyes (Cripps et al., 1990; Gustavsson et al., 2007; Gustavsson et al., 2004), pesticides (Broughton and Watson, 2004; Fernández-Alba et al., 2002) and runoff from road pavement surfaces (Mahler et al., 2014).

Although NSO-HET are pollutants of increasing concern in different compartments and have been recently addressed as emerging pollutants by the NORMAN network (Dulio et al., 2018), only few representative chemicals out of the overwhelming number of NSO-heterocyclic substances have been characterized regarding their human and environmental toxicology (Blotevogel et al., 2008). There is a particularly striking data gap regarding the long-term consequences of exposure to these compounds. While selected PAHs, BTEX (benzene, toluene, ethylbenzene and xylene) and phenols are often routinely monitored in groundwater at contaminated sites (Wolz et al., 2011), the highly relevant NSO-HET are

frequently neglected; potentially because of the missing human and ecotoxicological effect data that would be required for adequate risk assessments. A basic dataset providing aquatic ecotoxicity data for algae and daphnids, as well as mutagenicity in the Ames assay, has been provided by Eisentraeger et al. (2008). Many NSO-HET (e.g. benzofuran, quinoline) have been shown to or are suspected to exhibit embryotoxic (Peddinghaus et al., 2012) or cancerogenic effects (Blotevogel et al., 2008; Robbiano et al., 2004; Tada et al., 1980). Additionally, several NSO-HET trigger aryl hydrocarbon receptor (AhR)- and estrogen receptor (ER)-mediated effects *in vitro* (Brinkmann et al., 2014; Hinger et al., 2011).

Short-chained alkylphenols (SCAP) are frequently detected alongside with NSO-HET at tar oil-contaminated sites (Sauter and Licha, 2002). SCAP are phenol derivatives which contain at least one alkyl group with a chain length of C1 to C3. These chemicals are highly soluble in water (Varhanickova et al., 1995) and rarely adsorb to solid particles (Sauter and Licha, 2002), resulting in a substantial risk of drinking water contamination. Especially at contaminated sites which had produced, stored or processed SCAP, high concentrations (up to several mg L^{-1}) are found in groundwater (Fischer and Licha, 2013). Aquatic ecotoxicity data are only available for some SCAP and related chemicals (phenol, dimethylphenols and propylphenols) (Choi et al., 2004; Kahru et al., 2000). For the group of trimethylphenols and ethylphenols ecotoxicological effect data hardly exist to date.

The objective of the present study was to provide a comprehensive dataset on the ecotoxicity of those SCAPs and NSO-HET most commonly detected in the environment to achieve more profound environmental risk assessments of contaminated sites. To this end, we conducted the Microtox® assay with *Aliivibrio fischeri*, the growth inhibition test with the green alga *Desmodesmus subspicatus*, the acute immobilization assay with the water flea *Daphnia magna*, as well as the fish embryo toxicity (FET) test with embryos of the zebrafish (*Danio rerio*). Because of the physicochemical properties of the tested chemicals (limited water solubility, volatility and sorption to test vessels), it has previously been shown that analytical verification of actual exposure concentrations in test media is a prerequisite for the generation of accurate data (Eisentraeger et al., 2008; Peddinghaus et al., 2012). To this end, analytical procedures for detection of the test chemicals by means of gas chromatography with mass spectrometry (GC/MS) were

scaled down to achieve the required analytical performance and used to analytically determine the behavior of the test chemicals during the applied bioassays.

2. Materials and methods

2.1 Chemicals

NSO-HET and SCAP were obtained from abcr chemicals (Karlsruhe, Germany), ACROS organics (Geel, Belgium), Alfa Aesar (Karlsruhe, Germany), Fluka (Seelze, Germany), Merck (Darmstadt, Germany), or Sigma-Aldrich (Steinheim, Germany) and analytical purities ranged from 95.0 to 99.5 % (Table 1). Stock solutions of SCAP were prepared directly in water or test media. Depending on water solubility, NSO-HET were either dissolved directly in water or test media, or first dissolved in dimethyl sulfoxide (DMSO) and further diluted in water or test media. The maximum DMSO concentration in the Microtox® toxicity assay was 1 %, and 0.1 % in all other assays.

2.2 Microtox® toxicity assay with Aliivibrio fischeri

The Microtox® assay with the marine bacterium *Aliivibrio fischeri* was conducted according to the international guideline EN ISO 11348-1 (EN ISO, 2008) using a LUMIStox photometer and a LUMIS-therm incubation unit (Dr. Lange, Düsseldorf, Germany). Briefly, reduction of the natural luminescence emitted by the bacteria after exposure to the investigated NSO-HET and SCAP was monitored in duplicate at 490 nm following 30 min exposure and expressed relative to unexposed control treatments. To address potential volatilization of test chemicals, exposure vessels were sealed with PTFE-lined caps during incubation. Each chemical was investigated in three independent valid tests according to EN ISO 11348-1. Stock solutions of SCAP and most NSO-heterocycles were prepared in water. DMSO was used as a solvent carrier to make stock solutions of poorly water-soluble compounds, which were further diluted in water. Concentrations of working stock solutions were adjusted according to the ongoing experiments. During

each run, chemicals were tested at eight 2-fold serial dilutions. For each dilution series, 2 % (w/v) sodium chloride were added to 5 mL of each aqueous working stock solution and serially diluted in 2 % (w/v) sodium chloride in water. Zinc sulfate, 3,5-dichlorophenol und potassium dichromate were used as reference chemicals, and 2 % (w/v) sodium chloride in water as negative control.

HACH-LANGE LUMISsoft IV software (HACH-LANGE GmbH, Düsseldorf, Germany) was used to interpolate half-maximal effect concentrations (EC_{50}s) from fitted concentration-response data. Mean values and standard deviations of interpolated EC_{50}s were calculated from the three independent replicates, and experiments were accepted if the standard deviation did not exceed 20 %.

2.3 Growth inhibition test with Desmodesmus subspicatus

The growth inhibition test with *Desmodesmus subspicatus* was conducted according to DIN 8692 (DIN, 2005) and OECD technical guideline no. 201 (OECD, 2011), and as detailed in (Di Paolo et al., 2016). A culture of the test organism (SAG 86.81) was obtained from the Culture Collection of Algae at Göttingen University ('Sammlung von Algenkulturen der Universität Göttingen', SAG) and maintained in continuous culture at the Institute for Environmental Research, RWTH Aachen University (Aachen, Germany). Three to four days prior to initiation of experiments, a pre-culture was inoculated to ensure exponential growth.

Algae were exposed in triplicate to seven serial dilutions of each chemical in a volume of 2 mL in individual wells of 24-well microplates. A starting density of 5,000 cells per mL was chosen for all experiments. Negative controls and blanks were included for each chemical. Algae were incubated at 23±2°C while being constantly shaken at 120 rpm. Algal density was measured fluorometrically (excitation: 485 nm; emission: 685 nm) after 24, 48 and 72 h using an Infinite M200 spectrofluorometer (Tecan Deutschland GmbH, Crailsheim, Germany). Growth rates were calculated and statistically evaluated using ToxRat software (ToxRat Solutions GmbH, Alsdorf, Germany). EC_{50}s for growth inhibition were calculated by probit analysis using maximum likelihood regression. Validity of the tests was assessed based on the criteria defined in OECD technical guideline no. 201 (OECD, 2011).

2.4 Daphnia magna acute immobilization test

The *Daphnia magna* acute immobilization test was conducted according to DIN EN ISO 6341:2013-01 (DIN, 2013) and as detailed in Johann et al. (2016). Test organisms (*Daphnia magna*, Clone 5) were obtained from a continuous culture at the Institute for Environmental Research, RWTH Aachen University (Aachen, Germany). Toxicities of each SCAP and NSO-HET were tested in triplicate at seven graded concentrations. In addition, four negative control replicates were treated as the exposed animals, without addition of test chemical. Each replicate received five neonate daphnids in a total volume of 10 mL. Daphnids were exposed in the dark at 20±1°C and received no food. The number of immobile animals was scored after 48 h and statistically evaluated using ToxRat software (ToxRat Solutions). EC_{50} values for percent immobilization were calculated by probit analysis using maximum likelihood regression. Validity of the tests was assessed based on the criteria defined in DIN EN ISO 6341:2013-01.

2.5 Fish embryo toxicity (FET) test with zebrafish (Danio rerio) embryos

The fish embryo toxicity (FET) test with embryos of the zebrafish (*Danio rerio*) was conducted according to the guideline DIN EN ISO 15088:2009 (DIN, 2009) and as detailed in Peddinghaus et al. (2012). Fish embryos were exposed to graded concentrations of the test chemicals in 24-well microplates. Fish were cultured, and eggs obtained and selected according to previously published protocols (Peddinghaus et al., 2012). Ten eggs per replicate were exposed to each chemical per replicate. Eggs were exposed for 48 h at 26±1°C and subsequently scored microscopically at 40 to 100× magnification for mortality criteria (coagulation, no heartbeat, lack of detachment of the tail-bud from the yolk sac) and sub-lethal effects on development. LC_{50} values were interpolated by means of sigmoidal regression using GraphPad Prism 6 software (GraphPad, San Diego, USA) of data from at least five graded concentrations of each chemical which were tested in three independent experiments (except for *m*-cresol, for which only two valid tests were obtained). For each experimental day, negative controls (40 eggs), as well as positive (3.75 mg L^{-1} 3,4-dichloroanilin) and solvent controls (0.1% DMSO, 20 eggs each) were tested simultaneously. A test

was considered valid if mortality in the negative control was ≤ 10% and mortality was >10% in the positive control.

2.6. Chemical analysis

The initial concentrations of the tested SCAP and NSO-HET in the exposure solution, as well as the residual concentrations in the media after incubation in each of the toxicity tests were analyzed following liquid – liquid extraction (Blum et al., 2011). To this end, 45 mL sample were spiked with 10 µL internal standard solution (toluene-d8 and naphthalene-d8) and then extracted with 5 mL methyl tert-butyl ether (MTBE) for 20 min. After phase separation, the extract was dried with sodium sulphate and subsequently analyzed using gas chromatography (GC; Agilent technologies GC 6890 N). The GC was equipped with an auto sampler (Agilent technologies) and mass-selective detector (MS; Agilent technologies MS 5973 Network) operated in SIM (Single Ion Monitoring) mode. Separation of the substances was achieved using a ZB-5 Inferno column (60 m × 0.25 mm × 0.25 µm; Phenomenex, Aschaffenburg, Germany). Details regarding the measurement conditions are listed in Table 1.

Nominal concentrations of test chemicals in the ecotoxicity tests were compared to real exposure concentrations as measured by GC-MS analysis. Two different approaches were used to determine real exposure concentrations for comparison to initial test concentrations: median measured concentrations were calculated and subsequently the loss between initial concentration (t_0) and the median measured concentration determined (cf. Eisentraeger et al., 2008). This value (t_{median}) presents an integrative value that averages over the test duration. Furthermore, losses over the entire duration of the tests were determined. This value (t_{30} for bacteria, t_{72} for algae, t_{48} for daphnia and zebrafish) presents the maximum possible loss over the test duration ("worst-case-scenario").

2.7 ECOSAR modelling

Experimental data were compared with predictions of the US-EPA ECOSAR model for neutral organics (for NSO-HET) and phenols (for SCAPs), respectively. Toxicity ratios (TRs), i.e. the fold-difference

between measured and modeled values, were calculated for each chemical according to Verhaar et al. (1992). A chemical is defined as a baseline toxicant according to this classification if the TR is <5, as a polar narcotic at TR≥5 and ≤10, and as specifically acting at TR > 10. Since the structure-activity relationship (SAR) for phenols already accounts for the excess toxicity of phenols, the TR classification according to Verhaar is not directly applicable. A TR of 10 was thus only assumed to be indicative of a mechanism of toxic action that differs from that of the other phenols.

3. Results and discussion

3.1 Microtox® assay with Aliivibrio fischeri

Results of the Microtox® assay with *Aliivibrio fischeri* are provided in Table 2. EC_{50} values determined for SCAP spanned approx. four orders of magnitude and ranged from 0.01 to 100 mg L^{-1}. All SCAP exhibited greater toxicities compared to the non-alkylated phenol (EC_{50} > 100 mg L^{-1}). While there was no clear pattern regarding the influence of number, position and nature of alkyl substitutions along the phenol ring on toxicity, the greatest nominal toxicities were observed for three propyl- and ethyl-substituted phenols with alkyl residues in para-position: 4-isopropylphenol (0.02 mg L^{-1}), 4-ethylphenol (0.09 mg L^{-1}) and 4-n-propylphenol (0.25 mg L^{-1}). EC_{50}s of all other ethylated, propylated and methylated phenols were greater than 1 mg L^{-1}. Our data were generally comparable with previously published data. Aruoja et al. (2011) report EC_{50}s of 39.5 mg L^{-1} and 0.43 mg L^{-1} for 2-ethylphenol and 4-ethylphenol, respectively. Similar to the values measured in the present study, the EC_{50} of 4-isopropylphenol in the Microtox® assay was 0.01 mg L^{-1} (Choi et al., 2004). An exception to the general agreement with literature values were *o*- and *m*-cresol, as well as 2,5- und 2,6-dimethylphenol and 2,4,6-trimethylphenol, for which Kaiser and Palabrica (1991) have reported considerably lower EC_{50}s. However, Kaiser and Palabrica (1991) used a different bacteria species (*Photobacterium phosphoreum*) for the Microtox® assay, and the test conditions were not completely comparable to the present study.

In addition to the above-described SCAP, EC_{50}s in the Microtox® assay were determined for 16 NSO-HET, as well as the PAHs indane and indene (Table 2). Because of their low toxicity, pyridines were only investigated in this and none of the other toxicity tests. EC_{50}s of NSO-HET, indane and indene spanned approx. five orders of magnitude and ranged from 0.62 to 1,100 mg L^{-1}. The overall lowest EC_{50}s were observed for the S-heterocycles dibenzothiophene, as well as 3- and 5-methylbenzothiophene, with values between 0.62 and 0.77 mg L^{-1}. The investigated N-heterocycles (except for the three-ring phenanthridine) almost exclusively exhibited lesser toxicity, with EC_{50}s $\geq$ 10 mg L^{-1}. The lowest toxicity was observed for the N-heterocycle pyridine (1,100 mg L^{-1}). In agreement with the results obtained for the investigated SCAP, the para-substituted 4-methylpyridine was the most toxic chemical within the group of methylpyridines. It was not possible to determine EC_{50}s within the limits of water solubility for the O-HET xanthene and xanthenone. EC_{50}s below 10 mg L^{-1} were determined for the PAHs indane and indene. Based on the average toxicities of the different classes of NSO-HET, the investigated substances can be ranked according to EC_{50}s as follows: S-HET < O-HET < N-HET. Furthermore, there appeared to be an increasing trend in toxicity with increasing number of rings and methyl residues. EC_{50}s observed in the present study were generally in good agreement with literature values (Hartnik et al., 2007; Kaiser and Palabrica, 1991; Seymour et al., 1997). Kaiser and Palabrica (1991) and Seymour et al. (1997)report slightly lesser EC_{50} values for dibenzothiophene (0.12 mg L^{-1} and 0.16 mg L^{-1}, respectively), and the value reported for xanthenone by Kaiser and Palabrica (1991) exceeds water solubility.

3.2 Growth inhibition test with Desmodesmus subspicatus

Results of the growth inhibition test with *Desmodesmus subspicatus* are provided in Table 2. All investigated SCAP exhibited toxicity in the assay. The greatest nominal EC_{50} values were observed for phenol (168 mg L^{-1}) and *o*-cresol (176 mg L^{-1}). Both *m*- and *p*-cresol decreased algae growth at approx. 10-fold lower concentration compared to *o*-cresol, with nominal EC_{50}s of 18.6 and 15.2 mg L^{-1}, respectively. Nominal toxicities of dimethylphenols in the assay were more heterogeneous

(2,6-dimethylphenol: 64.5 mg L^{-1}; 2,4-dimethylphenol: 15.8 mg L^{-1}) and nominal EC_{50} values greater compared to 2,3,5-trimethylphenol (19.6 mg/L), 3,4,5-trimethylphenol (16.8 mg L^{-1}), 2-isopropylphenol (19.5 mg L^{-1}) und 4-isopropylphenol (20.0 mg L^{-1}). The lowest EC_{50} (10.7 mg L^{-1}, based on nominal concentrations) was observed for 4-*n*-propylphenol. Based on the average nominal toxicities of the different classes of SCAP, the EC_{50}s of the investigated substances can be ranked as follows: propylphenols < ethylphenols < trimethylphenols < dimethylphenols < monosubstituted phenols < phenol. Similar to the effects observed in the Microtox® assay, the greatest toxicities based on nominal concentrations were observed for those chemicals alkylated in the para-position. Our results are in good agreement with data derived from growth inhibition assays with the alga *Pseudokirchneriella subcapitata* by Aruoja et al. (2011). The EC_{50} of phenol reported by the authors was 197 mg L^{-1}, and 168 mg L^{-1} (nominal) in the present study (Table 2). EC_{50}s of the dimethylphenols reported by Aruoja et al. (2011) ranged from 32.5 mg L^{-1} to 48.1 mg L^{-1}, while the average EC_{50} of all seven dimethylphenols investigated in the present study was 39.8 mg L^{-1}.

Test results for 11 NSO-HET, in addition to those of the PAHs indane and indene are provided in Table 2. Xanthenone, indane, and thiophene did not cause significant inhibition of growth in *D. subspicatus* within their limits of water solubility or, as in the case of thiophene, because corrosion of test vessels occurred at concentration exceeding 400 mg L^{-1}. The greatest observed nominal EC_{50} was that of indene (140 mg L^{-1}), while the lowest EC_{50}s were observed for dibenzothiophene, phenanthridine and xanthene (9.0, 4.6 and 3.5 mg L^{-1}, respectively; nominal concentrations). All other nominal EC_{50} values ranged between nominal concentrations of 14.7 mg L^{-1} (3-methylbenzothiophene) and 42.0 mg L^{-1} (2,4-dimethylquinoline). Generally, there was no clear trend or tendency regarding the influence of heteroatoms on toxicity. However, methyl-substituted N- and O-heterocycles appeared to be less toxic compared to their non-methylated analogues. Because of the general scarcity of toxicity data for NSO-HET, only few values were available for comparison with data generated in the present study. Nominal EC_{50}s determined by Eisentraeger et al. (2008) were much greater compared to our data, with nominal values of 65 mg L^{-1} and 99 mg L^{-1} for dibenzothiophene and xanthene. When corrected for substance losses, however,

values reported by Eisentraeger et al. (2008) were more similar to our results, with EC_{50}s of 4.0 mg L^{-1} (dibenzothiophene) and 38 mg L^{-1} (xanthene). These drastic differences between nominal and real concentration are further discussed in section 3.5.

3.3 Daphnia magna acute immobilization test

Results of the acute immobilization test with *Daphnia magna* are provided in Table 2. All investigated SCAP exhibited toxicity in the test after 48 h, with 2,4,6-trimethylphenol (2.6 mg L^{-1}) and 4-*n*-propylphenol (2.8 mg L^{-1}) showing the lowest nominal EC_{50} values. Apart from 3,5-dimethylphenol (14.9 mg L^{-1}), 2-ethylphenol (10.7 mg L^{-1}) und 2-isopropylphenol (11.5 mg L^{-1}), all other tested SCAP showed nominal EC_{50}s < 10 mg L^{-1}. In agreement with a previous study (Devillers, 1988), there was no clear influence of the number of alkyl substituents on toxicity in the acute immobilization test. Again, the greatest toxicity within each group based on nominal concentrations was generally found for para-substituted phenols within the mono-substituted cresols, dimethyl- and trimethyl-substituted phenols, as well as ethyl- and propylphenols, with the length of the alkyl residue being of minor importance. Keen and Baillod (1985) reported an EC_{50} of 6.6 mg L^{-1} for phenol, which is identical to the value obtained in the present study. Kühn et al. (1989) determined 24-h EC_{50}s of 10.0, 7.7, and 5.7 mg L^{-1} for phenol, 4-methylphenol (*p*-cresol) und 4-ethylphenol, while 48-h values in the present study were systematically lower, with nominal EC_{50}s of 6.6, 4.4, and 3.7 mg L^{-1}, respectively. The relative ranking of EC_{50}s of the three chemicals in the acute immobilization test, however, was identical (4-ethylphenol < 4-methylphenol < phenol).

In addition to the above-described SCAP, the toxicities of 11 NSO-HET and the PAHs indane and indene were determined in the acute immobilization test with *D. magna* (Table 2). Exposure to all 13 compounds resulted in acute toxicity, with 2,4- and 2,6-dimethylquinoline being the least toxic (nominal EC_{50}s of 55 and 21 mg L^{-1}), and phenanthridine, xanthene and dibenzothiophene being the most toxic, with nominal EC_{50}s of 4.1, 3.0 and 0.45 mg L^{-1}, respectively. Nominal EC_{50}s of all other tested substances fell within a relatively narrow range between 5.1 mg L^{-1} (3-methylbenzothiophene) and 18 mg L^{-1} (indane). Although the behavior within the groups of nitrogen-, sulfur- and oxygen-substituted heterocycles was

relatively heterogeneous, they can be ranked as follows regarding their nominal EC_{50}s in the acute immobilization test with *D. magna*: S-HET < O-HET << N-HET. Eisentraeger et al. (2008) have assessed the toxicities of 2-methylbenzofuran, dibenzothiophene and xanthene in the acute immobilization test with *D. magna*, with analytically corrected EC_{50}s being in the same range as those obtained in the present study (7.4, 0.2, and 2.3 mg L^{-1}, respectively).

3.4 Fish embryo toxicity (FET) test with zebrafish (Danio rerio) embryos

Results of the FET test with embryos of the zebrafish (*Danio rerio*) are provided in Table 2. All 18 investigated SCAP caused negative effects on development and survival of zebrafish embryos. Phenol (90.1 mg L^{-1}), *o*- (53.8 mg L^{-1}) and *m*-cresol (53.7 mg L^{-1}), as well as 2-ethyl- (39.5 mg L^{-1}) and 4-ethylphenol (65.3 mg L^{-1}) exhibited the greatest nominal LC_{50} values compared to the other investigated compounds. With a nominal LC_{50} of 19.8 mg L^{-1}, *p*-cresol was the most toxic of the three cresols, and comparable in toxicity with the investigated dimethylphenols, with LC_{50}s between nominal concentrations of 20.2 mg L^{-1} (3,4-dimethylphenol) to 32.6 mg L^{-1} (2,5-dimethylphenol). Trimethylphenols were similar or slightly greater in toxicity, with LC_{50}s of 28.1 mg L^{-1} (2,4,6-trimethylphenol), 15.1 mg L^{-1} (2,3,5-trimethylphenol) and 9.5 mg L^{-1} (3,4,5-trimethylphenol). The para-substituted propylphenols 4-isopropylphenol (14.5 mg L^{-1}) and 4-*n*-propylphenol (8.8 mg L^{-1}) showed the overall greatest toxicity and lowest nominal LC_{50}s, respectively, in the FET test. The relative ranking of nominal LC_{50}s in the FET test was: propylphenols < trimethylphenols < *p*-cresol < dimethylphenols < cresols < phenol. The most commonly observed apical endpoint that was considered as lethality criterion was coagulation. The earliest observable sub-lethal effects were changes in somatic and ocular pigmentation. To our knowledge, this is the first assessment of SCAP in the FET test with embryos of the zebrafish.

Test results for 11 NSO-HET, in addition to those of the PAHs indane and indene in the FET test are provided in Table 2. It was not possible to derive LC_{50}s for dibenzothiophene, xanthene and xanthenone within the limits of their water solubility, although all three substances caused some toxicity below 50% mortality. LC_{50}s were derived for all other tested chemicals, with thiophene exhibiting the greatest nominal

338 LC_{50} (153 mg L^{-1}), and phenanthridine the lowest (3.1 mg L^{-1}). Nominal LC_{50}s for the other tested chemicals
339 ranged from 5.0 mg L^{-1} (2,6-dimethylquinoline) to 53 mg L^{-1} (2-methylbenzofuran). Generally, the greatest
340 toxicity was observed for N-heterocycles, followed by S- and O-heterocycles. Peddinghaus et al. (2012)
341 have previously reported 48-h LC_{50}s of 14 mg L^{-1} and 3.8 mg L^{-1} for 2-methylbenzofuran and xanthene,
342 respectively. While no toxicity was observed for xanthene in the present study, we determined an approx.
343 4-fold greater nominal LC_{50} for 2-methylbenzufuran (53 mg L^{-1}) compared to Peddinghaus et al. (2012).
344
345 *3.5 Comparisons among toxicity tests and chemical losses during incubation*
346 Comparing the EC_{50}s/LC_{50}s values of the different toxicity tests for the investigated SCAP, the resulting
347 analytically corrected (nominal) values ranged between 0.017 (0.017) mg L^{-1} and 160 (180) mg L^{-1}. The
348 lowest EC_{50} value was detected for 4-iso-propylphenol in the Microtox® assay with *Aliivibrio fischeri*
349 (0.017 mg L^{-1}). For all investigated SCAP, the acute immobilization test with *Daphnia magna* with EC_{50}s
350 values below 10 mg L^{-1} for 15 SCAP, and the Microtox® assay with EC_{50}s values below 10 mg L^{-1} for 8
351 SCAP exhibited the greatest sensitivities. Para-substituted phenols (4n-propylphenol, 4-ethylphenol and
352 4-iso-propylphenol) showed the greatest toxicities in all four toxicity tests based on nominal concentrations.
353 Nominal concentrations of the tested substances in the toxicity tests were compared to real exposure
354 concentrations as measured by GC-MS analysis, and the subsequent dissipation over the test duration
355 quantified. Figure 1 illustrates the effect of dissipation of selected SCAP during the test duration on the
356 resulting EC_{50}/LC_{50}s in all four toxicity tests. Results were based on the concentrations of chemicals in test
357 media at the beginning and the end of exposure, as well as the median value of these two concentrations.
358 For the Microtox® assay with *Aliivibrio fischeri,* only phenol, *m*-cresol and 2,4,6-trimethylphenol exhibited
359 chemical losses during the incubation period ranging between 3 and 25% (Figure 1A). For the other
360 investigated SCAP, no concentration decrease was observed during the incubation period of 30 min. Similar
361 results were observed for the acute immobilization test with *Daphnia magna.* Except of 4-ethylphenol, all
362 investigated SCAP exhibited chemical losses during the test period of 48 h. However, the concentration
363 decreases were below 20% for all investigated substances (Figure 1C). During the growth inhibition test

with *Desmodesmus subspicatus* phenol, *o*-cresol and 4-n-propylphenol showed no chemical losses during the incubation time of 72 h. For all other investigated SCAP, the losses exhibited great variability and usually exceeded 20% (Figure 1B). Concentrations measured by means of GC-MS analysis differed strongly from nominal concentrations, especially for dimethylphenols and trimethylphenols. The dissipation determined for 2,4,6-trimethylphenol was greater than 50% (median measured concentration, t_{Median}) and 90% (maximum loss over test duration, t_{72h}), respectively. During the FET test with zebrafish embryos, all investigated SCAP exhibited chemical losses during the incubation time of 48 h (Figure 1D). Phenol and 2,4,6-trimethylphenol showed the greatest dissipation after 48 h in exceedance of 75%. For the other investigated substances, the determined dissipation ranged from 15 % to 30%. Similar to the algal growth inhibition test, the concentration decreases were highly variable. In summary, chemical losses in the Microtox® assay (30 min) and the acute immobilization test with *Daphnia magna* (48 h) were less than 25% for all investigated SCAP. Therefore, these chemical losses have a minor impact on the risk assessment of these chemicals. During the algal growth inhibition test and the FET test, some SCAP showed significant concentration decreases during the test duration. The greatest losses were observed for dimethylphenols (approx. 70%) and trimethylphenols (approx. 90 %) in the growth inhibition test. In this case, an assessment based only on nominal concentration would drastically underestimate the toxicity potential of these substances. No literature data were available regarding chemical losses during incubation with SCAP. To our knowledge, this is the first study comparing nominal and real exposure concentrations of phenolic compounds during incubation among different toxicity tests.

Comparing the EC_{50}s/LC_{50}s values among the toxicity tests for the investigated NSO-HET and the PAHs indane and indene, the resulting analytically corrected values ranged from 0.19 to 62 mg L^{-1} (excluding pyridines). The acute immobilization test with *Daphnia magna* and the Microtox® assay exhibited the highest sensitivities, with 11 and 9 EC_{50}s below 10 mg L^{-1}, respectively. Comparing compounds with the same heteroatoms, greater toxicities could be determined with increasing ring number and degree of methylation. However, due to the limited water solubility and high volatility of some tested

389 compounds, the determination of EC_{50}s/LC_{50}s values was not possible in all test systems (i.e. xanthene,
390 xanthenone, dibenzothiophene, 2-methylbenzofuran, thiophene).

391 As in the case of SCAP, nominal concentrations of selected NSO-HET in the toxicity tests were
392 compared to real exposure concentrations as measured by means of GC-MS analysis, and chemical losses
393 quantified during the exposure duration. Figure 2 illustrates the effects of the analytically determined
394 dissipation of selected NSO-HET and the PAHs indane and indene during the test duration on EC_{50}/LC_{50}
395 in all four toxicity tests. In the Microtox® assay with *Aliivibrio fischeri*, the investigated NSO-HET and
396 indane exhibited chemical losses during the test duration (less than 25 %, Figure 2A). However, for some
397 of the tested chemicals (thiophene, 5-methylbenzothiophene, dibenzothiophene), initial concentrations
398 were significantly lower as nominal concentrations, in some cases less than 50% (as illustrated through the
399 difference between nominal and measured t_0 concentrations). This might be due to the low water solubility
400 and the high adsorption potential of these compounds, causing chemical losses already during preparation
401 of the test media. Similar effects were observed for xanthene and xanthenone in the Microtox® assay.
402 During the acute immobilization test with *Daphnia magna*, all investigated NSO-HET and the PAHs indane
403 and indene exhibited chemical losses during the test duration (Figure 2C). As in the Microtox® assay, initial
404 concentrations of thiophene, xanthenone, indane, indene, 5-methylbenzothiophene and xanthene were
405 approx. 50% of the nominal concentrations before the test start. Furthermore, the analytically determined
406 dissipation of thiophene, 5-methylbenzothiophene, indane, indene and 2-methylbenzofurane during test
407 duration was extremely high, leading to some concentrations being below the limit of quantification after
408 48 h incubation. In the growth inhibition test with *Desmodesmus subspicatus*, phenanthridine exhibited no
409 chemical losses during test duration and the loss of 2,4-dimethylquinoline was less than 3%. In contrast, 5-
410 methylbenzothiophene, dibenzothiophene, xanthene and indene exhibited significant concentration
411 decreases during the test duration, with 5-methylbenzothiophene and indene concentrations below the limit
412 of quantification after 72 h. All selected NSO-HET and the PAHs indane and indene that caused effects in
413 the FET test exhibited chemical losses during incubation (Figure 2D). The concentration decreases of 2,4-

414 dimethylchinoline and phenanthridine were less than 20%, while that of all other investigated compounds
415 was greater than 80% over 48 h.

416 In summary, the investigated N-HET showed the lowest concentration decrease during incubation
417 (< 20%). The determined dissipation depended strongly on factors defining the test system, including
418 incubation temperature and time, where the Microtox® assay generally showed the lowest concentration
419 decrease. In the other toxicity tests, especially the sulfur-containing S-HET exhibited high chemical losses
420 during the test duration, partly resulting in concentrations below the limit of quantification. Furthermore,
421 in all tests, chemical losses occurred during preparation of the test media for thiophene, dibenzothiophene
422 and xanthene. Therefore, consideration of only nominal concentrations of this substance class may results
423 in an underestimation of the toxic potential, and the analytical verification of actual exposure concentrations
424 in test media is a prerequisite for the generation of accurate data.

425 Literature data for comparison with the results of the present study are scarce. Chemical losses have
426 been investigated for selected NSO-HET in previous studies (Eisentraeger et al., 2008; Peddinghaus et al.,
427 2012). In these studies, dissipation of different compounds was observed during incubation, e.g. for
428 dibenzothiophene, 2-methylbenzofurane, and xanthene in the FET test (Peddinghaus et al., 2012), for
429 dibenzothiophene and xanthene in the algae growth inhibition test (Eisentraeger et al., 2008) and for
430 dibenzothiophene, 2-methylbenzofurane and xanthene in the *Daphnia magna* immobilization test
431 (Eisentraeger et al., 2008). Both studies are in good agreement with the present study and recommended
432 analytical verification of actual exposure concentrations in test media.

433

434 *3.6 ECOSAR modeling*

435 The US-EPA model ECOSAR was used to estimate if any of the investigated SCAP showed excess toxicity
436 exceeding that predicted by the linear regression model for phenols in fish, daphnids and algae (Figure 3).
437 A model for bacterial toxicity does not currently exist. For all investigated test organisms, toxicity was
438 slightly over-estimated by the model. The deviation from equality, however, was less thank 10-fold for all
439 investigated chemicals.

The ECOSAR model was also used to compare measured and modeled toxicity data for the investigated NSO-HET (Figure 4). In contrast to the SCAP, we used the neutral organic SAR to predict toxicity of NSO-HET from *log* K_{ow}. Predictions were relative accurate compared to experimental results in the algal growth inhibition test, while the model slightly under-estimated the toxicity in daphnids and fish. Of the compounds investigated here, only thiophene exerted excess toxicity in daphnids (TR > 10). For comparison, we also included experimental data from Eisentraeger et al. (2008) and Peddinghaus et al. (2012) (project „KORA“) in Figure 4. In tests with fish embryos, a TR of 10 was exceeded for the N-HET carbazole and acridine, in the *D. magna* acute immobilization test for thiophene, indole and its O-HET analogue benzofuran (Figure 4). Based on these findings, it appears warranted to study the specific mechanisms of toxicity of thiophene, indole and benzofuran in daphnids, as well as carbazole and acridine in fish embryos.

4. Conclusions

In this study, we provided a comprehensive and comparative analysis of the ecotoxicity of SCAP and NSO-HET spanning four trophic levels, namely primary producers (green algae), primary consumers (daphnids), secondary consumers (zebrafish embryos) and decomposers (bacteria). Because of the dissipative losses of SCAP and NSO-HET during the test duration, resulting EC/LC_{50} values were expressed based on real concentrations determined by means of GC-MS analysis, in addition to nominal concentrations. Our study emphasizes the toxicological relevance of SCAP and NSO-HET, and will enable scientists and risk assessors to adequately assess the risks of these chemicals at contaminated sites.

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578 **Table 1**: Details of the measurement conditions for SCAP and NSO-HET during GC-MS analysis.

Instrument	Gas chromatograph Agilent 6890N	
Detector	Mass selective detector MSD Agilent 5973 Network	
Carrier gas flow	1.1 mL min^{-1}	
Carrier gas	Helium	
Injection	Split for solvent vapor exit	
Injection volume	5 µL	
Injector temperature	10°C, 1 min	
Column	ZB-5HT Inferno 60 m × 0.25 mm × 0.25 µm	
Oven temperature program	**For NSO-HET**	**For SCAP**
	40°C, 5 min	50°C, 5 min
	40-130°C with 6°C min^{-1}	50-90°C with 5°C min^{-1}
	130°C, 7 min	90-300°C with 15°C min^{-1}
	130-250°C with 5°C min^{-1}	
	350°C, 15 min	
Transfer line temperature	300°C	300°C
Total measurement time	76 min	61 min

579

580 **Table 2:** Results of the four toxicity tests (mean ± standard deviation; *n*=2 for Daphnia, *n*=3 for all other tests) with short-chained alkyl phenols
581 (SCAP) and NSO-heterocycles (NSO-HET), along with CAS numbers, suppliers and purity information. EC/LC_{50} values are reported based on
582 nominal concentrations, as well as median real exposure concentrations which account for analytically determined losses of the test chemicals.

Chemical name	**CAS no.**	**Supplier**	**Purity (%)**	**EC_{50} bacteria (mg L^{-1})**	**EC_{50} algae (mg L^{-1})**	**EC_{50} daphnia (mg L^{-1})**	**LC_{50} zebrafish (mg L^{-1})**
				nominal / real	nominal / real	nominal / real	nominal / real
Short-chained alkyl phenols (SCAP)							
Phenol	108-95-2	Sigma-Aldrich	99.5	130 ± 8.2 / 120	170 / 160	6.6 / 7.1	90 ± 16 / 95
o-Cresol	95-48-7	Sigma-Aldrich	99.0	50 ± 3.7 / 44	180 / 110	5.8 / 2.8	54 ± 11 / 41
m-Cresol	108-39-4	Acros Organics	99.0	24 ± 2.5 / 21	19 ± 9.3 / 19	8.8 / 8.1	54 ± 23 / 44
p-Cresol	106-44-5	Sigma-Aldrich	99.0	3.8 ± 0.14 / 3.3	15 ± 5.4 / 15	4.4 / 4.1	20 ± 3.7 / 16
2-Ethylphenol	90-00-6	Fluka	98.5	37 ± 6.3 / 37	36 ± 21 / 11	11 / 11	40 ± 9.2 / 34
4-Ethylphenol	123-07-9	Fluka	99.2	0.086 ± 0.0041 / 0.090	17 ± 4.4 / 32	3.7 / 3.2	65 ± 32 / 66
2,3-Dimethylphenol	526-75-0	Sigma-Aldrich	99.0	8.5 ± 0.78 / 8.5	35 ± 20 / 21	3.7 / 3.5	26 ± 6.1 / 21
2,4-Dimethylphenol	105-67-9	Fluka	99.0	5.4 ± 0.82 / 5.3	16 ± 8.1 / 32	3.7 / 2.7	29 ± 9.8 / 16
2,5-Dimethylphenol	95-87-4	Sigma-Aldrich	99.0	20 ± 1.7 / 20	57 ± 6.2 / 34	5.3 / 4.9	33 ± 4.4 / 27
2,6-Dimethylphenol	576-26-1	Sigma-Aldrich	99.5	67 ± 4.1 / 67	65 ± 4.6 / 39	7.9 / 7.4	31 ± 5.9 / 25
3,4-Dimethylphenol	95-65-8	Acros Organics	99.0	1.3 ± 0.17 / 1.3	31 ± 2.4 / 19	4.3 / 4.0	20 ± 4.1 / 17
3,5-Dimethylphenol	108-68-9	Sigma-Aldrich	99.0	41 ± 3.7 / 41	37 ± 4.3 / 22	15 / 14	25 ± 7.8 / 21
2-Isopropylphenol	88-69-7	Sigma-Aldrich	98.0	7.5 ± 1.0 / 7.6	20 ± 9.8 / 14	12 / 11	19 ± 5.6 / 14
4-Isopropylphenol	99-89-8	Sigma-Aldrich	98.0	0.017 ± 0.0023 / 0.017	20 ± 0.9 / 38	4.1 / 6.2	15 ± 3.3 / 6.9
4-*n*-Propylphenol	645-56-7	Sigma-Aldrich	99.0	0.25 ± 0.012 / 0.2	11 ± 3.4 / 30	2.8 / 3.5	8.9 ± 3.7 / 4.9
2,3,5-Trimethylphenol	697-82-5	Acros Organics	98.0	16 ± 1.2 / 15	20 ± 5.6 / 8.3	6.8 / 6.1	15 ± 6.5 / 7.8

2,4,6-Trimethylphenol	527-60-6	Acros Organics	99.0	26 ± 1.9 / 25	29 ± 0.63 / 16	2.6 / 2.6	28 ± 7.1 / 17
3,4,5-Trimethylphenol	527-54-8	Alfa Aesar	97.0	10 ± 1.2 / 9.9	17 ± 1.5 / 7.1	4.0 / 3.6	9.5 ± 2.7 / 4.9
NSO-heterocyclic hydrocarbons							
2,4-Dimethylquinoline	1198-37-4	Alfa Aesar	95.0	30 ± 1.2 / 35	42 ± 2.7 / 39	55 / 43	16 ± 6.4 / 10
2,6-Dimethylquinoline	887-43-0	abcr	98.0	9.6 ± 1.2 / 9.5	18 ± 3.4 / 18	21 / 19	5.0 ± 1.3 / 4.7
Phenanthridine	229-87-8	abcr	98.0	2.0 ± 0.18 / 1.2	4.6 ± 1.2 / 6.3	4.1 / 2.9	3.1 ± 0.51 / 1.1
Thiophene	110-02-1	Sigma-Aldrich	99.0	290 ± 21 / 62	–	15 / 2.4	150 ± 32 / 49
3-Methylbenzothiophene	1455-18-1	abcr	98.0	0.62 ± 0.079 / 0.6	15 ± 11 / 5.1	5.1 / 1.9	14 ± 3.7 / 5.1
5-Methylbenzothiophene	14315-14-1	abcr	98.0	0.77 ± 0.15 / 0.3	19 ± 22 / 0.82	6.7 / 1.0	23 ± 3.7 / 3.1
Dibenzothiophene	132-65-0	abcr	98.0	0.71 ± 0.025 / 0.2	9.0 ± 7.9 / 1.5	0.45 / 0.19	–
2-Methylbenzofuran	4265-25-2	Sigma-Aldrich	96.0	2.9 ± 0.25 / 2.4	200 ± 64* / 54	8.0 / 1.7	53 ± 17 / 13
3-Methylbenzofuran	21535-97-7	Sigma-Aldrich	97.0	4.3 ± 0.12 / 3.9	12.0 ± 0.7 / 4.1	10 / 3.5	30 ± 0.78 / 8.0
Xanthene	92-83-1	Sigma-Aldrich	99.0	–	3.5 ± 1.2 / 0.40	3.0 / 0.68	–
Xanthenone	90-47-1	abcr	99.0	–	–	13 / 3.2	–
Indane	496-11-7	Sigma-Aldrich	95.0	5.8 ± 1.0 / 5.4	–	18 / 3.0	20 ± 9.2 / 1.8
Indene	95-13-6	Sigma-Aldrich	99.0	5.1 ± 0.76 / 4.1	140 ± 35 / 21	15 / 1.7	49 ± 7.4 / 5.7
Pyridine	110-86-1	Merck	99.5	1,100 ± 0.0	n.a.	n.a.	n.a.
2-Methylpyridine	109-06-8	Merck	98.0	160 ± 14	n.a.	n.a.	n.a.
3-Methylpyridine	108-99-6	Merck	98.0	110 ± 11	n.a.	n.a.	n.a.
4-Methylpyridine	108-89-4	Merck	98.0	53 ± 3.7	n.a.	n.a.	n.a.
2,4,6-Trimethylpyridine	108-75-8	Merck	98.0	460 ± 16	n.a.	n.a.	n.a.

583 *n.a.: not analysed, – : no observable toxicity, * : This EC_{50} value was extrapolated slightly beyond the range of tested concentrations.*

Figures

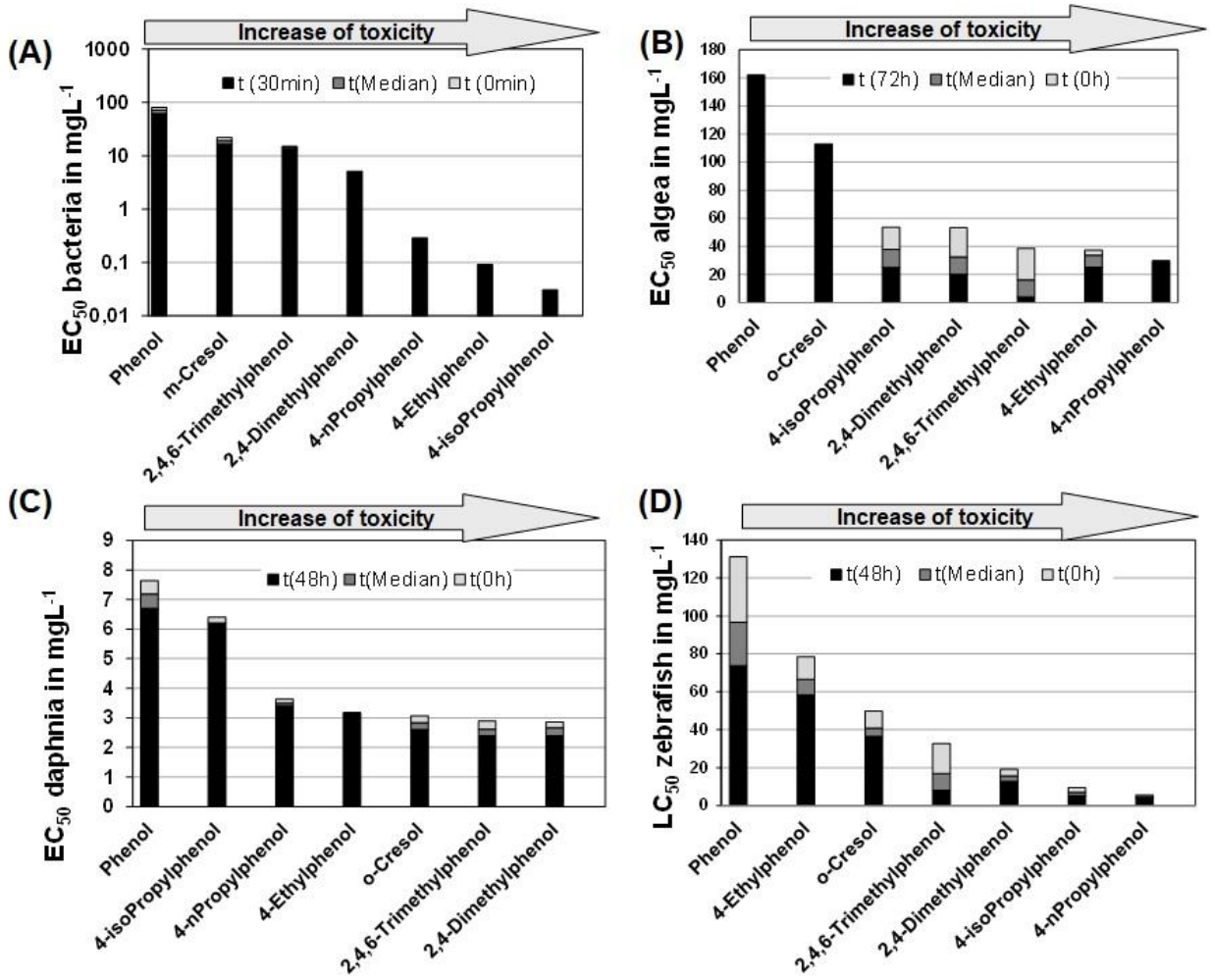
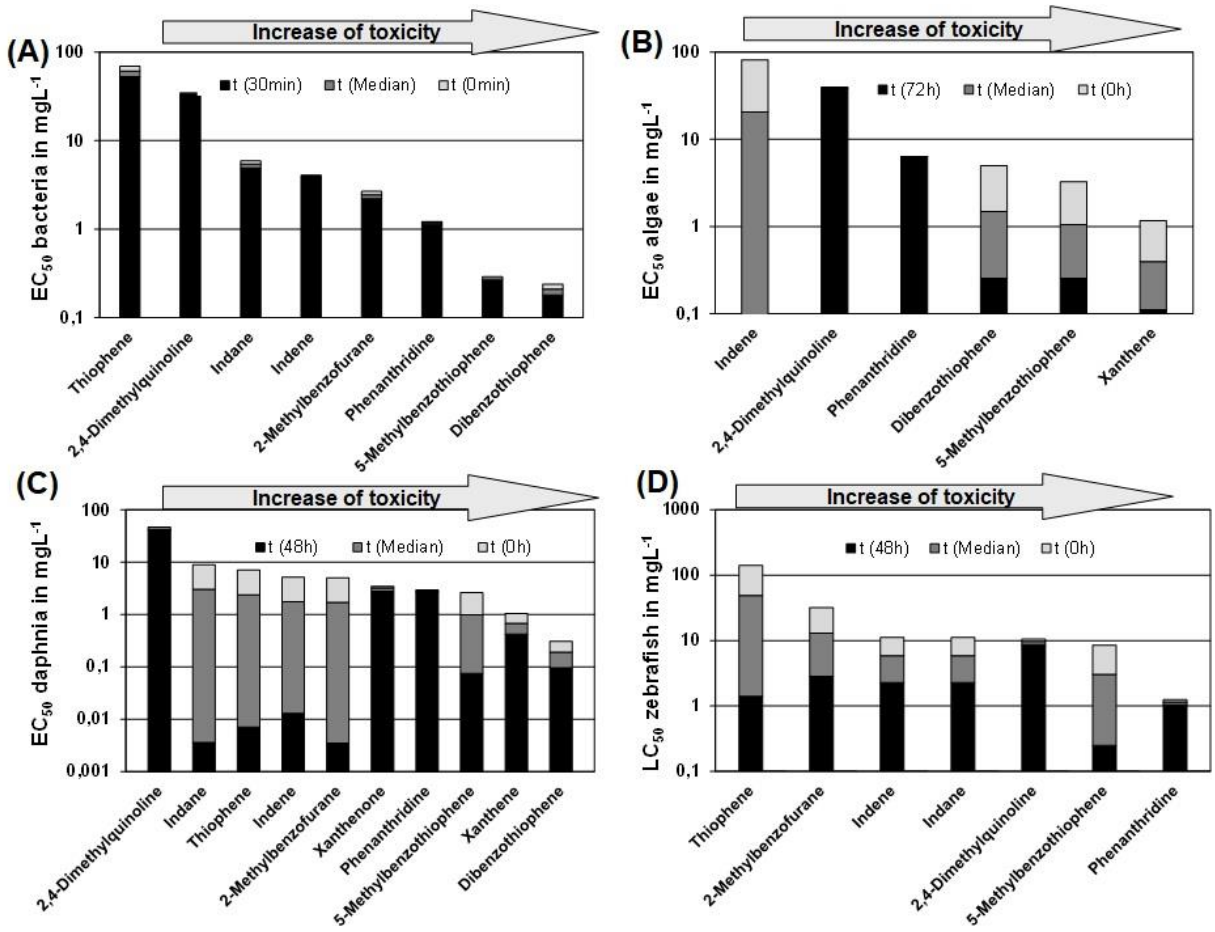


Figure 1: Effects of the analytically determined dissipation of selected short-chained alkylphenols (SCAP) during the test duration on EC_{50}/LC_{50} in the four toxicity tests: (A) bacteria, (B) algae, (C) *Daphnia*, and (D) zebrafish embryos. Results were based on the measured concentrations of chemicals in test media at the beginning (0 h) and the end of exposure (30 min/ 48 h/ 72 h), as well as the median value of these two concentrations. The increase of toxicity relates to the concentration at the beginning of the test.



591

592 **Figure 2:** Effects of the analytically determined dissipation of selected NSO-heterocycles (NSO-HET)
593 during the test duration on EC_{50}/LC_{50} in toxicity tests with four test organisms: (A) bacteria, (B) algae, (C)
594 *Daphnia*, and (D) zebrafish embryos. Results were based on the measured concentrations of chemicals in
595 test media at the beginning (0 h) and the end of exposure (30 min/ 48 h/ 72 h), as well as the median value
596 of these two concentrations. The increase of toxicity relates to the concentration at the beginning of the test.

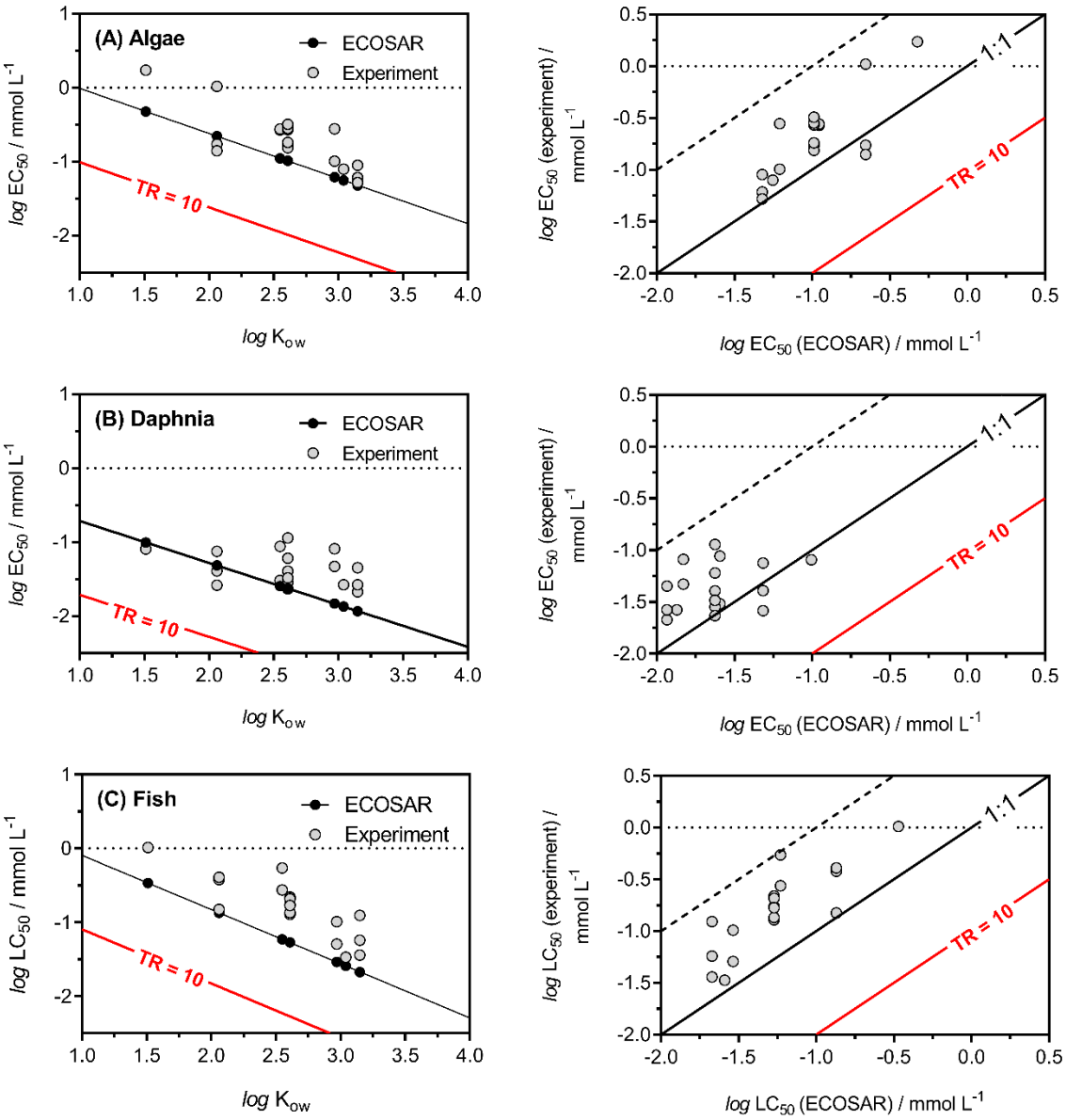
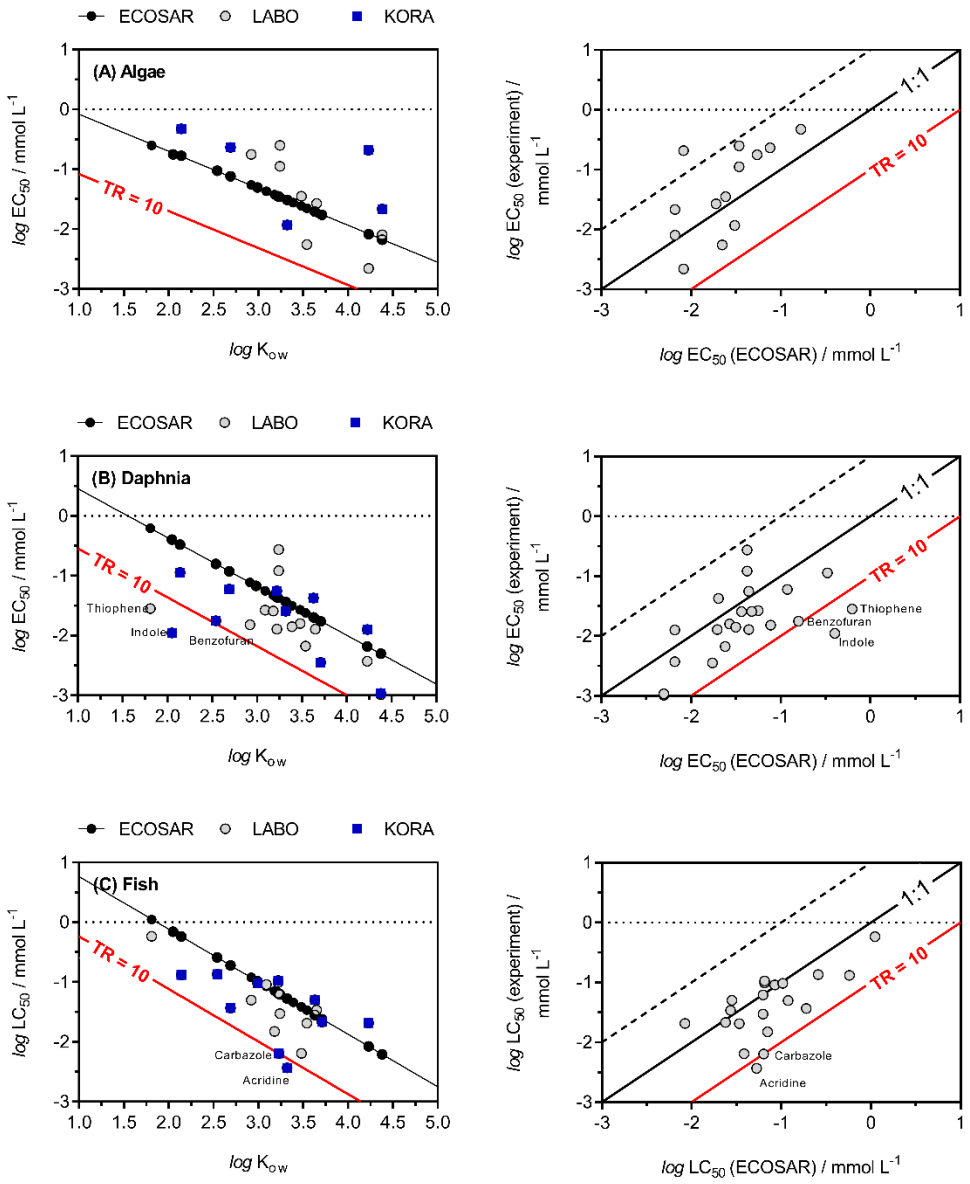


Figure 3: Comparison of experimentally determined (open circles) and modelled (filled circles) toxicity of short-chained alkyl phenols (SCAP) in (A) algae, (B) *Daphnia* and (C) fish embryos depending on the *n*-octanol/water partitioning coefficient (*log* K_{ow}). Model predictions were made using the "phenols" sub-model of the US-EPA ECOSAR model. Experimental EC/LC_{50}s are based on median real concentrations. The solid red line marks a toxic ratio (TR) of 10, i.e. a 10-fold greater experimental toxicity compared to predicted values.



604

605 **Figure 4:** Comparison of experimentally determined (this study, LABO: open circles, previous study,
606 KORA: blue squares) and modelled (filled circles) toxicity of NSO-heterocycles (NSO-HET) in (A) algae,
607 (B) *Daphnia* and (C) fish embryos depending on the *n*-octanol/water partitioning coefficient (*log* K_{ow}).
608 Model predictions were made using the US-EPA ECOSAR model. Experimental EC/LC_{50}s are based on
609 median real concentrations. The solid red line marks a toxic ratio (TR) of 10, i.e. a 10-fold greater
610 experimental toxicity compared to predicted values.