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Aquatic toxicity of biofuel candidates on *Daphnia magna*

Sebastian Heger[a], Kerstin Bluhm[a], Miaomiao Du[a], Gunnar Lehmann[a], Nico Anders[b], Dominique Dechambre[c], André Bardow[c], Andreas Schäffer[d,e,f], Henner Hollert[a,e,f,g,*]

[a] RWTH Aachen University, Institute for Environmental Research, Department of Ecosystem Analysis, Aachen, Germany
[b] RWTH Aachen University, Aachener Verfahrenstechnik – Enzyme Process Technology, Aachen, Germany
[c] RWTH Aachen University, Institute of Technical Thermodynamics, Aachen, Germany
[d] RWTH Aachen University, Institute for Environmental Research, Chair of Environmental Biology and Chemodynamics, Aachen, Germany
[e] Chongqing University, College of Resources and Environmental Science, Chongqing, China
[f] Nanjing University, State Key Laboratory of Pollution Control and Resource Reuse, School of Environment, Nanjing, China
[g] Tongji University, College of Environmental Science and Engineering and State Key Laboratory of Pollution Control and Resource Reuse, Shanghai, China

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ABSTRACT

The increasing need for carbon-neutral, low-emission transportation sector has led to the development of advanced biofuels with tailor-made production and combustion processes. Even though the large-scale deployment of these advanced biofuels also increases the risk for their release into the environment, their toxic potency remains largely unknown. To identify hazardous biofuel candidates as early as possible, the fuel development process can be expanded by "Green Toxicology". To demonstrate such early Green Toxicology testing, this study investigates the aquatic toxicity for the two biofuel candidates 2-methyltetrahydrofuran (2-MTHF) and 2-methylfuran (2-MF) on *Daphnia magna*. We performed the prolonged acute immobilisation assay (96 h) and the *D. magna* reproduction test. 2-MF induced acute effects on *D. magna* that were two orders of magnitude stronger than those of 2-MTHF. Furthermore, both substances affected the growth and reproductive output of *D. magna* in a 21 d reproduction test, with 2-MF already inducing effects with concentrations one to two orders of magnitude lower than those of 2-MTHF. Thus, our assessment of the aquatic toxicity suggests that further biofuel development should focus on 2-MTHF. Furthermore, the acute immobilisation test with *D. magna* was identified as a promising tool for a rapid and sensitive "Green Toxicology" screening of further biofuel candidates.

1. Introduction

The consumption of petroleum-based fuels is expected to rise rapidly due to an increase in the global population and emerging economies (US Energy Information Administration, 2016). This leads to concerns regarding energy security and global climate change caused by the emission of greenhouse gases (Cubasch et al., 2013; Lefèvre, 2010). In response to these concerns, established fossil fuels could be substituted by biofuels as promising and renewable alternatives (Lefèvre, 2010; Ragauskas et al., 2006; Soyez and Graßl, 2008). Consequently, the European Union (EU) plans to replace up to 10% of the final energy consumption in the transport sector with energy derived from renewable sources, such as biomass-derived fuels by 2020 (Commission of the European Communities 2009/28/EC) and to establish a mandatory target of 20% for renewable sources in the overall EU energy consumption. Research and development of sustainable and economically feasible biofuels is therefore increasing all over the world. At the same time, the development of novel tailor-made biofuels could allow to overcome drawbacks of fossil fuels beyond greenhouse gas emissions such as soot and NOx emissions (Leitner et al., 2017). However, novel biofuels may also have potential disadvantages. Many negative aspects of the use of biomass for fuel production have already been discussed extensively, such as competition with food production, cost efficiency or land-use (Petrou and Pappis, 2009; Ramachandran and Stimming, 2015; Tilman et al., 2009; Young, 2009). However, potential (eco)toxicological effects of biofuels are a relatively new subject of consideration. Until now, most of the available (eco)toxicological studies focus on the exhaust emissions of biodiesel or diesel/biodiesel blends and reveal often contradictory results (Bluhm et al., 2012b). Information on the toxic potency of novel synthetic fuel molecules are rare (Bluhm et al., 2012a), even though this information should be a critical parameter in the fuel design process. Since biofuels

* Correspondence to: RWTH Aachen University, Institute for Environmental Research, Department of Ecosystem Analysis, Worringerweg 1, 52074 Aachen, Germany.
E-mail address: Henner.Hollert@bio5.rwth-aachen.de (H. Hollert).

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will be introduced in the same infrastructure and used in the same way as the petroleum-derived fuels, an environmental contamination as a consequence of fuel transport, leakages from underground storage tanks and pipelines, or accidental spills can be expected in similar concentrations as for petroleum-derived fuels. Fuels are considered one of the main potential sources of environmental contamination due to their large-scale use (Gallego et al., 2001; Panagos et al., 2013) and were projected to make up 23.8% of the contaminants affecting soil and 21.9% of the contaminants affecting groundwater in Europe (Panagos et al., 2013). In the United States, 69,000 barrels of oil (~10,970,123.35 litres) and other hazardous liquids, on average every year, were spilled from pipelines from 2012 to 2014 (US Department of Transportation and Statistics, 2016). Therefore, safety considerations should be moved to the earliest stage in a chemicals life cycle according to the emerging discipline of "Green Toxicology" for the benign design of safer chemicals (Maertens et al., 2014). Identifying hazardous biofuel candidates already in early development process could reduce research costs by excluding these substances from further research. A prominent example highlighting the need for a prospective (eco)toxicological screening of particular harmful molecules to prevent unintended environmental consequences is the gasoline octane booster methyl *tert*-butyl ether (MTBE) (Davis and Thomas, 2006). MTBE was introduced as an oxygenate additive in the existing infrastructure as a substitute for tetraethyl lead in leaded gasoline. However, the MTBE application resulted in health issues of exposed workers as well as widespread contamination of aquatic systems (Davis and Thomas, 2006). An early and prospective (eco)toxicological testing of MTBE could have identified its relative hazard potential and, thus, prevented harmful consequences towards human health and environment.

A fuel design process considering simultaneous fuel production, combustion as well as toxicity is currently developed in the Cluster of Excellence "Tailor-Made Fuels from Biomass" (TMFB) at RWTH Aachen University. Research on both biofuel production and combustion has identified 2-methyltetrahydrofuran (2-MTHF) and 2-methylfuran (2-MF) as promising biofuel candidates for Diesel and spark-ignition engines, respectively (Kerschgens et al., 2015; Ragauskas et al., 2006; Thewes et al., 2011; Tripathi et al., 2017). However, the oxygenate nature of these biofuels, which is beneficial in combustion, has been shown to also strongly increase both the water solubility in the biofuels as well as solubility of the biofuels in water compared to fossil fuels (Dechambre et al., 2017).

In this work, we therefore investigate the aquatic toxicity of the promising biofuels 2-MTHF and 2-MF. Since aquatic systems are very vulnerable to the contamination by fuels and biofuels (Davis and Thomas, 2006; Khan et al., 2007; Zhang et al., 1998), testing of biofuel candidates focuses primarily on the investigation of aquatic toxicity. In previous publications, we demonstrated the advantage of using (eco) toxicological bioassays to gain a better understanding of the (eco)toxicological effects of potential biofuels (Bluhm et al., 2016; Heger et al., 2016). In this study, we focus on a deeper investigation of these two biofuel candidates using the waterflea *Daphnia magna*. Daphnids are widely recognized and standardised test animals for assessing the aquatic toxicity for aquatic invertebrates due to their sensitivity towards water quality, their reproductivity and their availability (Khan et al., 2007; Wagner et al., 2017). We investigated 96 h acute toxicity for neonate and adult daphnids in a prolonged acute immobilisation test with *D. magna* that was based on the OECD guideline 202:2004. Furthermore, effects of the two biofuel candidates on reproduction and length growth of *D. magna* were also investigated by means of the 21 d *D. magna* reproduction test (OECD 211:2012).

2. Material and methods

2.1. Chemicals

2-MTHF (CAS# 96–47-9; > 99%) and 2-MF (CAS# 534–22-5; 99%) were purchased from Sigma-Aldrich (Munich, Germany).

2.2. Maintenance of Daphnia magna culture

Individuals of *Daphnia magna* STRAUS (clone 5) were kept in ~80 mL of aerated Elendt M4 medium (OECD 202:2004) at 20 ± 1 °C and a photoperiod of 16 h light: 8 h darkness. Daphnids were fed three times a week with *Desmodesmus subspicatus* and once a week with yeast (1 mg/L). Medium was renewed once a week. Neonate daphnids to be used for testing at the age of 4–5 weeks were kept in 1000 mL M4 medium (20 individuals each) under identical conditions as described above.

2.3. Prolonged acute immobilisation test with Daphnia magna (OECD 202:2004)

Acute immobilisation of *D. magna* was investigated according to OECD 2024 with modifications stated below. Neonate (< 24 h) and adult (4–5 weeks) daphnids were exposed to different nominal concentrations of 2-MTHF (0.269, 0.538, 1.075, 2.150, 4.300 g/L) and 2-MF (0.018, 0.023, 0.027, 0.032, 0.036, 0.041 g/L) in M4 medium for 96 h. Four test bottles of each concentration were prepared for each substance. Daphnids were fed 24 h before testing, but no food was provided during the experiment.

For testing of 2-MTHF and 2-MF, four and five individuals were transferred in one test bottle, respectively. Neonate daphnids were placed in 20 mL glass bottles (CS-Chromatographie Service GmbH, Langerwehe, Germany), whereas adult daphnids were tested in 100 mL glass bottles (Rixius AG, Mannheim, Germany). Headspace in all bottles was reduced to a minimum to minimize evaporation, thus resulting in volumes of medium up to 22 mL in neonate bottles and 110 mL in adult bottles. As a negative control, M4 medium and five individuals per bottle were used. All 20 mL bottles and the 100 mL bottles used for testing of 2-MF were sealed using screw caps with a PTFE-coated silicon seal (CS-Chromatographie Service GmbH; Rixius AG). 100 mL bottles used for testing of 2-MTHF were sealed with aluminium foil. To remain within 80% of the nominal 2-MTHF concentration, the medium was renewed once after 48 h. Sealing 100 mL bottles with screw caps maintained the 2-MF concentrations within 80% of the nominal 2-MF concentration, therefore, no medium change was required. All tests were conducted in three independent replicates.

The test duration of 48 h as required by the OCED 202:2004 (OECD/OCDE, 2004) was extended to a 96 h test duration. Immobility was recorded after 24 h, 48 h, 72 h and 96 h. Daphnids were considered immobile if no directed movement was observed within 15 s after gentle agitation of the test bottles (OECD 202:2004). O_2-concentration and pH were measured after 96 h by means of a fiber-optic micro sensor (PreSens OXY-4 micro NTH-PSt1-L5-TF-NS40/0.8-OIW, PreSens Precision Sensing GmbH, Regensburg, Germany) and a pH electrode (S20 - SevenEasy™ pH, Mettler Toledo, Erftstadt, Germany).

2.4. Reproduction test with D. magna (OECD 211:2012)

Effects on reproduction of *D. magna* were investigated according to OECD 211:2012 (OECD/OCDE, 2012) with some modifications. Neonate daphnids (< 24 h) were exposed to five concentrations of 2-MTHF (0.134, 0.269, 0.538, 0.806, 1.075 g/L) and 2-MF (0.005, 0.009, 0.014, 0.027, 0.036, g/L) and 10 bottles per concentration in M4 medium for 21 d. Single neonate daphnid was transferred in one 100 mL bottle covered with aluminium foil and a Teflon coated screw cap, respectively, and fed three times per week with 0.1 mg carbon (*D. subspicatus*) per day for the first week and 0.2 mg Carbon per day for the last two weeks.

Medium was renewed every 48 h with the exemption of the first medium change for 2-MF, which was performed after 72 h in order to reduce stress on the neonate daphnids. Medium with 2-MF was