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Acute embryo toxicity and teratogenicity of three potential biofuels also used as flavor or solvent

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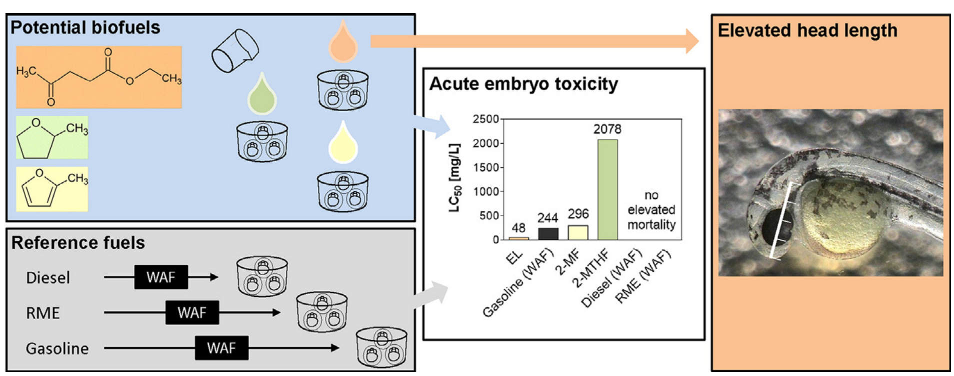
HIGHLIGHTS

- The demand for biofuels increases but their (eco)toxicological effects are unknown.
- Acute fish embryo toxicity and teratogenicity of potential biofuels were evaluated.
- Ethyl levulinate induced a higher acute toxicity compared to WAFs of gasoline.
- Ethyl levulinate caused elevated head lengths in zebrafish embryos.
- Investigations at an early stage of the R&D process of biofuels should be performed.

GRAPHICAL ABSTRACT



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ABSTRACT

The demand for biofuels increases due to concerns regarding greenhouse gas emissions and depletion of fossil oil reserves. Many substances identified as potential biofuels are solvents or already used as flavors or fragrances. Although humans and the environment may be readily exposed little is known regarding their (eco)toxicological effects. In this study, the three potential biofuels ethyl levulinate (EL), 2-methyltetrahydrofuran (2-MTHF) and 2-methylfuran (2-MF) were investigated for their acute embryo toxicity and teratogenicity using the fish embryo toxicity (FET) test to identify unknown hazard potentials and to allow focusing further research on substances with low toxic potentials. In addition, two fossil fuels (diesel and gasoline) and an established biofuel (rapeseed oil methyl ester) were investigated as references. The FET test is widely accepted and used in (eco)toxicology. It was performed using the zebrafish *Danio rerio*, a model organism useful for the prediction of human teratogenicity. Testing revealed a higher acute toxicity for EL (LC_{50}: 83 mg/L) compared to 2-MTHF (LC_{50}: 2980 mg/L), 2-MF (LC_{50}: 405 mg/L) and water accommodated fractions of the reference fuels including gasoline (LC_{50}: 244 mg DOC/L). In addition, EL caused a statistically significant effect on head development resulting in elevated head lengths in zebrafish embryos.

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2-Methylfuran
Green toxicology

Results for EL reduce its likelihood of use as a biofuel since other substances with a lower toxic potential are available. The FET test applied at an early stage of development might be a useful tool to avoid further time and money requiring steps regarding research on unfavorable biofuels.

1. Introduction

The interest in biofuels for the transport sector increased within the last years. Biofuels are considered renewable alternatives with the benefits of a reduced dependence on fossil fuels and a potential to attenuate the effects of global climate change due to decreased greenhouse gas emissions. Consequently, biofuel production as well as research on fuel production processes from biomass increased dramatically in recent years. Growing production capacities and the associated rise in biofuel consumption also increase the risk of a release into the environment due to increasing cargo turnover. However, comparatively little knowledge on (eco)toxicological effects of biofuels is available (Bluhm et al., 2012) even though useful tools for (eco)toxicological investigations of biofuel fermentation samples were identified (Heger et al., 2012) and could also be used for investigations of biofuels. The usage of such tools is advisable already within the research and developmental process of new biofuels according to principles of Green Toxicology (Maertens et al., 2014). For example, investigating the (eco)toxicological potential of biofuels at an early stage of development helps to avoid high research costs for a substance that would not commercially viable and enables researchers to focus on substances with promising properties but a low impact on the environment and human health.

The three substances ethyl levulinate (EL), 2-methyltetrahydrofuran (2-MTHF) and 2-methylfuran (2-MF) are promising biofuels currently evaluated within the interdisciplinary Cluster of Excellence "*Tailor made fuels from Biomass*" at RWTH Aachen University, Germany. These compounds can be effectively obtained from (ligno)cellulosic biomass via the platform chemicals levulinic acid, furfural and 5-hydroxymethylfurfural (Huber et al., 2006; Roman-Leshkov et al., 2007; Geilen et al., 2010; Lange et al., 2012; Climent et al., 2014) and show promising fuel properties and performances in combustion engines (U.S. Department of Energy, 2002; Janssen et al., 2011; Thewes et al., 2011; Wang et al., 2012, 2013).

Beside their promising potential as biofuels, they can also be used as (co-)solvents (Shanmuganathan et al., 2010; Winter, 2010; Geilen et al., 2011; Yang et al., 2014) and EL also as flavor in food (The Good Scents Company, 2015). EL is used as a solvent for cellulose acetate, starch and flavorings (Winter, 2010) and 2-MTHF is a promising candidate to substitute petrochemical solvents in industrial processes (Shanmuganathan et al., 2010; Geilen et al., 2011).

Even though there is a rather wide potential for applications of EL, 2-MTHF and 2-MF, information on their (eco)toxicological potential is scarce. This is especially true for biofuels (Bluhm et al., 2012) but even for EL, that is used in the food industry, only one study on its (eco)toxicological effects was found in the available literature (Lomba et al., 2014) while some toxicological data is available (e.g. acute oral and dermal toxicity, as well as information regarding skin irritation; Fragrance raw material monographs; Anonymous, 1982). For 2-MTHF and 2-MF information on, e.g., genotoxicity or impacts on histopathology, morphology, clinical biochemistry and hematology respectively, were recently published (Antonucci et al., 2011; Gill et al., 2014). However, for an intensified application of EL, 2-MTHF or 2-MF in case of the usage as a biofuel, additional knowledge about their effects on the environment and human health is essential. The number of spillages and leakages would increase with the volume used due to an increase in the quantities transported and stored. In addition, the probability of human exposure to high concentrations would increase, e.g. during the refueling process. Therefore, substances with the lowest toxic potential or with at least lower environmental and human health impacts compared to reference fuels or solvents they are supposed to replace should be preferred to minimize negative consequences of usage. Lethal and sub-lethal effects are relevant endpoints. Among others it is important to assess carcinogenicity, mutagenicity but also teratogenicity since these effects are difficult to predict e.g. via quantitative structure-activity relationship (QSAR) models but they are important endpoints regarding competitive advantages of a substance (Markiewicz et al., 2015).

For an environmentally and human health-relevant investigation we performed the fish embryo toxicity (FET) test using the zebrafish *Danio rerio* to obtain information on the acute fish embryo toxicity and the teratogenic potential of the three substances EL, 2-MTHF and 2-MF as well as of water accommodated fractions (WAFs) of the reference fuels diesel, gasoline and rapeseed oil methyl ester (RME). The test is widely used for (eco)toxicological investigations and biomedical research (Strähle et al., 2012). Transparency of the embryos allows a microscopic investigation during development, and thus enables to observe sensitive sub-lethal effects. The test offers a high throughput capability at low costs compared to animal experiments, e.g. with mice, and only small amounts of a sample are needed to reveal acute toxicity as well as teratogenic effects. Furthermore, early life-stages of zebrafish (until 5 days post-fertilization) are not protected as animals according to European legislation (Directive 2010/63/EU). Therefore, it can be easily applied as a screening test at an early stage of development of new products even in accordance with Green Toxicology, i.e., to predict toxicity at an early stage of the chemical developmental process to improve the design for hazard reduction. Within biomedical research, the zebrafish is an attractive model to study human diseases (Guyon et al., 2007; Feitsma and Cuppen, 2008; Bakkers, 2011) due to a high number of homologous gene sequences compared with the human genome (Howe et al., 2013). In addition, zebrafish embryos are promising models for drug toxicity screening (Eimon and Rubinstein, 2009) and were found to be useful as a screening model for the teratogenic potential of antiepilepsy drugs (Lee et al., 2013). Thus, the FET test is well suited for the investigation of fuels with respect to an early (eco)toxicological investigation during the developmental process of potential biofuels and to reveal potential adverse effects on human development.

2. Materials and methods

2.1. Chemicals

EL (99%) 2-MTHF (≥99%) and 2-MF (99%) were purchased from Sigma-Aldrich (Munich, Germany). Ultra-low sulfur diesel fuel and gasoline were purchased from Shell (Deutsche Shell Holding GmbH, Hamburg, Germany) and RME was kindly provided by Archer Daniels Midland Company (ADM Hamburg AG, Germany).

2.2. Preparation of water accommodated fractions (WAFs)

Fossil diesel fuel and gasoline are complex mixtures which are, like RME, only partially soluble in water. To allow an investigation in aqueous media, WAFs were prepared. WAFs only contain the fraction of a partially soluble substance that dissolves in the aqueous phase but in contrast to a water-soluble fraction (WSF) might also contain small oil droplets. WAFs were solely prepared and stored in or handled with glass vessels and glass pipettes. The partitioning of compounds into the aqueous phase is not directly correlated to the ratio of oil to water and in WAFs the relative volatile hydrocarbon concentration does not