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Eco- and genotoxicity profiling of a rapeseed biodiesel using a battery of bioassays

Bettina Eck-Varanka[a], Nora Kováts[a,*], Eszter Horváth[a], Árpád Ferincz[b], Balázs Kakasi[c], Szabolcs Tamás Nagy[d], Kornélia Imre[e], Gábor Paulovits[f]

[a] University of Pannonia, Institute of Environmental Sciences, Egyetem str. 10, 8200 Veszprém, Hungary
[b] Department of Aquaculture, Szent István University, Páter K. str. 1, 2100 Gödöllő, Hungary
[c] University of Pannonia, Research Institute of Biomolecular and Chemical Engineering, Egyetem str. 10, 8200 Veszprém, Hungary
[d] University of Pannonia, Georgikon Faculty, Department of Animal Sciences, Deák Ferenc str. 16, 8360 Keszthely, Hungary
[e] MTA-PE Air Chemistry Research Group, Egyetem str. 10, 8200 Veszprém, Hungary
[f] Balaton Limnological Institute, Centre for Ecological Research, Hungarian Academy of Sciences, Klebelsberg Kunó str. 3, 8237 Tihany, Hungary

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ABSTRACT

Biodiesel is considered an important renewable energy source but still there is some controversy about its environmental toxicity, especially to aquatic life. In our study, the toxicity of water soluble fraction of biodiesel was evaluated in relatively low concentrations using a battery of bioassays: *Vibrio fischeri* bioluminescence inhibition, *Sinapis alba* root growth inhibition, *Daphnia magna* immobilization, boar semen live/dead ratio and DNA fragmentation and *Unio pictorum* micronucleus test. While the *S. alba* test indicated nutritive (stimulating) effect of the sample, the biodiesel exerted toxic effect in the aquatic tests. *D. magna* was the most sensitive with EC_{50} value of 0.0226%. For genotoxicity assessment, the mussel micronucleus test (MNT) was applied, detecting considerable genotoxic potential of the biodiesel sample: it elucidated micronuclei formation already at low concentration of 3.3%. Although this test has never been employed in biodiesel eco/genotoxicity assessments, it seems a promising tool, based on its appropriate sensitivity, and representativity.

1. Introduction

Biofuel is regarded as a renewable energy source and considered a clean, economically efficient possibility to substitute fossil fuels (Ji, 2016). The European Directive 2009/28/CE sets a target to establish a 10% biofuel share in the motor fuel market by 2020 (Escobar et al., 2014).

However, the environmental hazard of biodiesel in comparison to fossil fuels has not been assessed unambiguously. Most studies address the toxicity (either cyto- or genotoxicity) of diesel exhaust produced by combustion of biodiesel. Steiner et al. (2013) compared the in vitro toxicity of diesel exhaust produced by bio- and fossil diesel combustion in human lung cells and found that exhaust from pure rapeseed methyl ester decreased oxidative stress but increased pro-inflammatory responses, while the blend of 20% rapeseed-methyl ester (RME) and 80% fossil diesel decreased both oxidative stress and pro-inflammatory responses. Some studies revealed quite similar behavior of fossil fuels and biodiesel blends: for example, in the test series of Turrio-Baldassarri et al. (2004), diesel and biodiesel blend emissions showed similar mutagenic potency and genotoxic profile assessed by the *Salmonella typhimurium* and mammalian microsome assays. On the other hand, Kooter et al. (2011) assessed the environmental performance of biodiesel and pure plant oil after combustion in comparison to conventional fuels and reported that biofuels resulted in lower PM mass, but also concluded that they should be treated with caution due to potentially increased toxicity. Liu et al. (2009) evaluated the extracts of gaseous emissions of a biodiesel blend (B10, 10% palm fatty acid methyl ester) and a diesel. Samples were collected at different loading modes (idling, 10%, 33%, and 55%) and it was concluded that the addition of biodiesel increased the toxicity for all operation modes.

In order to get a better view about the environmental fate and potential hazard of biodiesels in aquatic environments, several ecotoxicological studies have been conducted on different biodiesel samples. Rosen et al. (2014) compared the ecotoxicity of two biofuels (one derived from *Camelina sativa* (wild flax) seeds and the other derived from algae) to that of a jet fuel and a ship diesel. Both acute and chronic/sublethal tests were conducted on four standard marine species: topsmelt larvae (*Atherinops affinis)*, mysid shrimp (*Americamysis bahia*), purple sea urchin (*Strongylocentrotus purpuratus*) and Mediterranean mussel (*Mytilus galloprovincialis*). Alternative fuels proved significantly

* Corresponding author.
E-mail address: kovats@almos.uni-pannon.hu (N. Kováts).

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less toxic to marine organisms. In order to assess potential risk of fuel spills in aquatic ecosystems, Khan et al. (2007) compared ecotoxicity of diesel, neat biodiesel (B100) and biodiesel blends (B50, B20, and B5) on two freshwater organisms, *Daphnia magna* (water flea) juveniles and *Oncorhynchus mykiss* (rainbow trout) fry. Diesel was found to have the highest toxicity both expressed as mortality rate and EC_{50} while B100 exerted the lowest toxicity. In general, the more diesel fraction was added, the higher toxicity was experienced. Bluhm et al. (2012) give a comprehensive review on aquatic toxicity testing of different biodiesel blends.

Though all studies which assess the environmental risk of biodiesels on aquatic ecosystems agree that biodiesels exert lower toxicity than fossil fuels, there is some indication that the risk of biodiesels is far from negligible. In the study of Khan et al. (2007), *Daphnia* LC_{50} of neat biodiesel was 4.65 ppm, while that of fossil fuel was 1.78; the two EC_{50}s were in the same order of magnitude. Nogueira et al. (2011) found that pure biodiesel and biodiesel blends triggered biochemical responses in Nile tilapia (*Oreochromis niloticus*) after short-term exposure. Another study conducted on armored catfish (*Pterygoplichthys anisitsi*) gave similar results (Nogueira et al., 2013).

The main aim of our study was to provide a comprehensive eco- and genotoxicological profile for a Hungarian blend biodiesel, including a wide range of available test organisms and end-points:

Method	Test organism	End point
ISO 21338:2010	*Vibrio fischeri*	bioluminescence inhibition
ISO 11269–1:2012	*Sinapis alba*	root growth inhibition
OECD Guideline No. 202.	*Daphnia magna*	immobilization
Flow cytometry	Boar semen	live/dead ratio and DNA fragmentation
Micronucleus test	*Unio pictorum*	micronuclei number

Of the selected bioassays, the *Daphnia* immobility test and the *Vibrio fischeri* bioluminescence inhibition test have already been used for assessing the toxicity of different biodiesels (e.g. Khan et al., 2007; Hollebone et al., 2008). Also, the *V. fischeri* bioassay has been found sensitive to characterize traffic-related emissions (Lin and Chao, 2002; Liu et al., 2009; Vouitsis et al., 2009; Kováts et al., 2013).

The *Sinapis alba* root growth inhibition assay was selected to represent the toxic effect of biodiesel to terrestrial plants. Though this bioassay has not been directly used in biodiesel toxicity assessment, it has been proven to be an appropriate test organism for assessing PAH (Polycyclic Aromatic Hydrocarbons) contaminated soils (Sverdrup et al., 2003).

In addition to characterization of this biodiesel blend by the given bioassays, the study was aimed at assessing the applicability and sensitivity of two additional tests which have not been used in previous biodiesel toxicity evaluations.

The boar sperm bioassay was developed by Andersson et al. (1998, 2004) as a mammalian cell model. Boar sperm can be obtained non-invasively therefore it does not require the sacrifice of laboratory animals and represents multiple modes of action of different chemicals which interfere with mitochondrial activity (Vicente-Carrillo et al., 2015). It has been mostly used for detecting the toxicity of bacterial and fungal toxins (e.g. Andersson et al., 2010; Rasimus et al., 2012; Mikkola et al., 2015) and was recently adapted to flow cytometry to measure different end points like plasma membrane integrity or mitochondrial transmembrane potential changes (Ajao et al., 2015).

The mussel micronucleus test is also a non-invasive and relatively easy-to-perform tool to detect the effect of any kind of genotoxic compounds in aquatic environments. Micronuclei formation indicates chromosomal DNA damage occurring as a result of either chromosome breakage or mitotic chromosome mis-segregation (Bolognesi et al., 2012). It can be used for metal pollution (Guidi et al., 2010; Falfushynska et al., 2013), to determine the genotoxic effect of PAH compounds (Wozniczki et al., 2004; Michel et al., 2013) or in in situ environmental status assessments (Kolarević et al., 2009, Štambuc et al., 2009).

2. Materials and methods

2.1. Biodiesel

Sample used was a rapeseed-based biodiesel, kindly provided by Rossi Biofuel Co., Komárom, Hungary. According to the safety data sheet, the composition of the biodiesel was 99.7% FAME (Fatty Acid Methyl Ester) and 0.3% methanol, pH = 7 and its density was 0.875–09 g/cm^3.

Because the main goal was to investigate the biodiesel effect on the aquatic environment, a stock solution was made by adding water to the sample in 1:1 ratio. The solution was shaken at 130 rpm at 20 °C for 24 h, then it was allowed to settle for 30 min. The aqueous phase was separated from the oily phase in a separatory funnel.

2.2. Vibrio fischeri bioluminescence inhibition test

The test was made according to ISO 21338:2010: Water quality - Kinetic determination of the inhibitory effects of sediment, other solids and colored samples on the light emission of *Vibrio fischeri* (kinetic luminescent bacteria test). The kinetic reading allows the measurement of highly turbid or colored samples (Lappalainen et al., 1999, 2001).

The freeze-dried photobacteria were rehydrated with the reconstitution solution and stabilized at 15 °C for 15 min before the measurement. For the assay the Ascent Luminometer (marketed by ABOATOX Co.) was used. After the sample was added to the bacterial suspension, bioluminescence intensity was continuously recorded for the first 30 s. After the pre-set exposure time, 30 min in our case, luminescence intensity was read again. The light output of the unstressed bacteria (the first 30 s) was used as a reference in calculating the results.

EC_{50} and EC_{20} values were calculated from the light inhibition percentages by the Aboatox software provided with the Ascent Luminometer. The light inhibition (INH%) was calculated based on the following equations:

$$KF = \frac{IC_{30}}{IC_0}$$

$$INH\% = 100 - \frac{IT_{30}}{KF \times IT_0} \times 100$$

where KF is the correction factor, IC_0 and IC_{30} are the luminescence intensities of the control at the beginning and after 30 min, IT_0 and IT_{30} are the luminescence intensities of the sample at the beginning and after the 30 min contact time.

From the inhibition data of each concentration the software calculates Gamma using the equation below:

$$Gamma = \frac{INH\%}{100 - INH\%}$$

and the inhibition that belongs to the Gamma = 1 value gives the EC_{50}.

2.3. Sinapis alba root growth inhibition test

The root growth inhibition test was performed according to ISO 11269–1:2012 Soil quality - Determination of the effects of pollutants on soil flora - Part 1: Method for the measurement of inhibition of root