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

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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.

Keywords: biodiesel; aquatic toxicity; bioluminescence inhibition; flow cytometry; *Daphnia* immobilization test; micronucleus test

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. In most cases, toxicity hazards are evaluated within the framework of Life Cycle Assessment, that is, toxic impact generated during production of either biofuels or fossil fuels are quantified (e.g. Yang, 2013). When the environmental hazard of the product is addressed, most studies report on 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 compared to exhausts from fossil diesel, 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. On the other hand, Turrio-Baldassarri et al. (2004) found that diesel and biodiesel blend emissions showed similar mutagenic potency and genotoxic profile assessed by the *Salmonella typhimurium* and mammalian microsome assays. 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 aquatic environments, 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. For ecotoxicity assessments, 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 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), though diesel exerted higher toxicity than biodiesel, *Daphnia* LC_{50} of neat biodiesel was 4.65 ppm, while that of fossil fuel was 1.78. 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 the 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 studies.

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 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., 2012), to determine the genotoxic effect of PAH compounds (Woznicki et al., 2004, Michel et al., 2013) or in *in situ* environmental status assessments (Kolarevic et al., 2009, Stambuc et al., 2009).

Materials and methods

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 hours, then it was allowed to settle for 30 min. The aqueous phase was separated from the oily phase in a separatory funnel.

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 minutes 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 sec. 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 sec) 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}.

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 growth. The test assesses toxic effects on seedlings and early growth of higher plants following exposure to the test substance in the soil or aqueous solution.

The test was run in two replicates, in 4 concentrations. Filters were put in petri dishes then 5-5 cm^3 sample/control were poured on each filter. When the filters got completely wet, 25-25 seeds were placed at equal distance from each other in every petri dish and the dishes were covered. The samples were stored in a dark place at 20-22°C for 72 hours. After the exposure time, root length of each plant was measured. Root length inhibition was calculated using the following equation:

$$X = \frac{K - M}{K} \times 100$$

where X is the root length inhibition (%) for each concentration, K is the root length of the control plants (mm), and M is the root length of the plants in each concentration (mm).

Daphnia magna immobilization test

This is an acute immobilization test that was carried out by the OECD Guideline No. 202. For the 48 hour immobilization test not more than 24 hour old daphnids were used, bred under accredited GLP conditions. The stock solution was made from the biofuel sample with aerated, stale tap water then it was ultrasonicated (Branson Sonifier; 3x1 min, 30% amplitude). After a range finding test we adjusted a dilution series of bisecting dilution from 0.1% to 0.0008% biofuel concentration. The test was made in 3 replicates, each with 10 animals per dilution. After 48 hours the immobile animals were counted and a log-logistic model was fitted on the concentration-immobility data from which the EC_{50} value was calculated (R software, drc package).

Flow cytometry (FC)

Boar semen was obtained from a local pig farm. The sperm was transferred to the lab immediately after collection and extended with a commercial semen extender (BTS - Minitube) to approximately 30 million spermatozoa per ml. Cell concentrations were measured with a Minitube SDM-1 photometer, calibrated for porcine sperm. The sperm samples were used for testing within a few days after collection.

For the flow cytometric boar sperm assay, 200 µl extended boar semen was exposed to 5 µl of test substance (biodiesel sample) for 30 minutes at room temperature in the dark to monitor short term cellular effects (Andersson et al., 2004). For long term effects, 20 µl biodiesel was added into 2 ml extended boar sperm and incubated for 1 day (Hoornstra et al., 2003). Methanol was used as control the same way according to the applied exposure time.

When the incubation time expired each sample was extended further with PBS (phosphate buffered saline, P4417-Sigma) to one million sperm cells per ml, the optimal cell concentration for the applied Beckman Coulter FC500 flow cytometer (Beckman Coulter, Inc., Brea, CA, USA). The cytometer was equipped with a 488 nm 20 mW Ar ion laser. The proper alignment of the flow cytometer was monitored daily with FlowCheck fluorospheres (6605359, Beckman Coulter). Acquisitions were automatically stopped after 300 sec or 20 000 events. Data files were stored as list mode (LMD) files and were analyzed with Flowing Software (Version 2.5.1, http://www.flowingsoftware.com).

LIVE/DEAD® Sperm Viability Kit (L-7011, Life Technologies) was used to determine the live/dead cell ratio. The labelling protocol followed the manual of the kit, supplied by the manufacturer. Briefly, 1 µl SYBR14 (0.1 mM solution in DMSO) and 5 µl of PI (2.4 mM solution in distilled water) were added to each sperm suspension, then incubated in the dark at room temperature for 10 minutes.

The DNA fragmentation was measured as the quick method described in Riccardi and Nicoletti (2006). Sperm suspensions were washed once with PBS (400 × g, 10 min). After that 1 ml of propidium iodide (PI) fluorochrome solution was added to the samples and incubated at 4 °C for an hour in the dark then measured directly. PI histograms were used to determine cellular DNA content. In case of DNA fragmentation, DNA fragments may leak out of the cells hence the remaining DNA content represent lower intensity peaks below the main PI peak (Figure 1.)

Results were compared to controls using Yates corrected Chi-square test. The statistical analysis was performed using GraphPad QuickCalcs software.

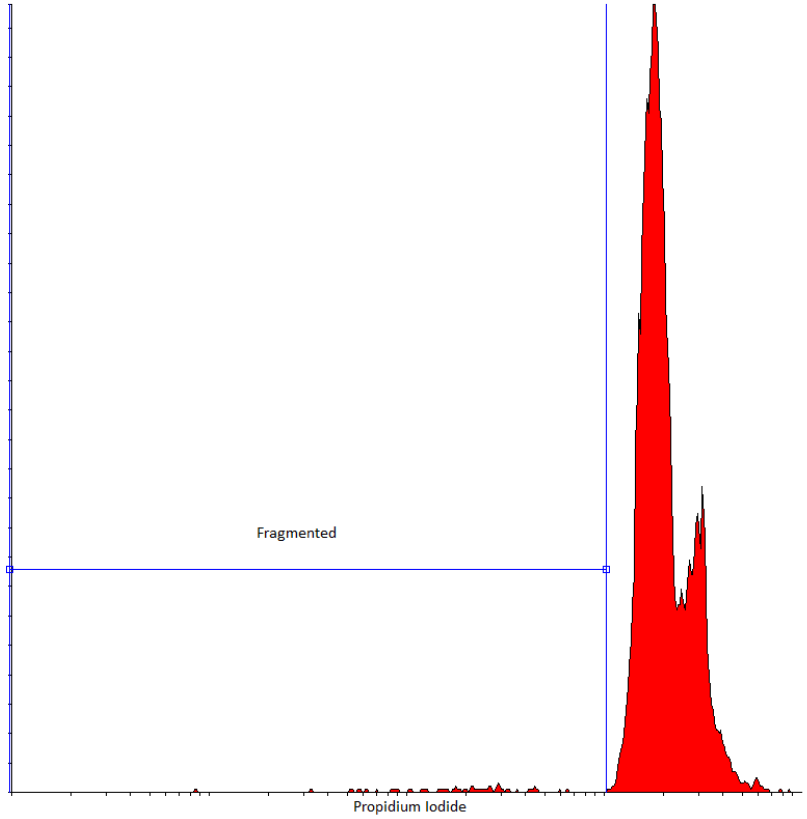

Figure 1. DNA fragmentation based on propidium iodide fluorescence intensities

Micronucleus (MN) test

Although no standardized test method is available for the mussel MN test, there are well described, step-by-step test protocols published. Our assay was performed based on the protocol given by Wozniczki et al. (2004), with some modifications. Treatments were performed in 3 replicates for each concentration and for the control. 10 individuals were kept in aquaria of 3 L volume. In the aquaria Lake Balaton water was used. The mussels were not fed during the experiment, aquaria were constantly aerated, and the temperature was set at 22°C. Organisms were exposed for 4 days, and the sample was renewed after 2 days. As test organism, the freshwater bivalve *Unio pictorum* was selected as it already proved to have high sensitivity for a wide range of environmental contaminants (Vuković-Gačić et al., 2014).

After 4 days, hemolymph was taken from the posterior adductor muscle using an improved non-lethal technique based on the method described by Gustafson et al (2005). 1 ml hemolymph sample was mixed with 0.3 ml 10% acetic acid in methanol as a fixative and centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded and the rest was fixed in 1 ml 80% ethanol, thus the sample can be kept refrigerated for a few weeks. For processing the

samples, refrigerated samples were centrifuged again at 1000 rpm for 5 minutes and the supernatant was discarded. The pellet which contained the hemolymph cells in a more concentrated form, was smeared onto a microscope slide and allowed to dry. After that the slides were fixed in 80% methanol, air dried again and stained with 5% Giemsa in distilled water for 20 minutes.

Photos of the cells were taken by a Zeiss AxioScope A1 microscope with an AxioCam ICC1 camera and Zen 2011 program at 400x magnification. For each animal 1000 cells were counted, micronuclei frequency was identified according to Fenech (1992).

One-way ANOVA with Tukey post hoc test was used to compare the mean MN numbers between the treatments. To use the ANOVA test the following assumptions were met: each group has approximately normal distribution (Shapiro-Wilk normality test: W = 0.9732, p = 0.3099), all groups have a common variance (Bartlett's test: Bartlett's K-squared = 3.1215, df = 4, p = 0.5377), independence of observations and all groups has equal sample number. In each group there were 15 individuals but for the statistical analysis the 10 most undoubtable were used (where the color and the quality of the pictures were the best). No transformations were applied on the data.

Results

Vibrio fischeri bioluminescence inhibition test

Figure 2 shows the bioluminescence reading for the first 30 sec. An immediate decrease in the light output after adding the bacterial suspension to the sample already gives an indication on the toxicity of the sample (Mortimer et al., 2008). After 30 minutes of exposure, calculated EC_{50} was 12.52% and EC_{20} was 1.90%.

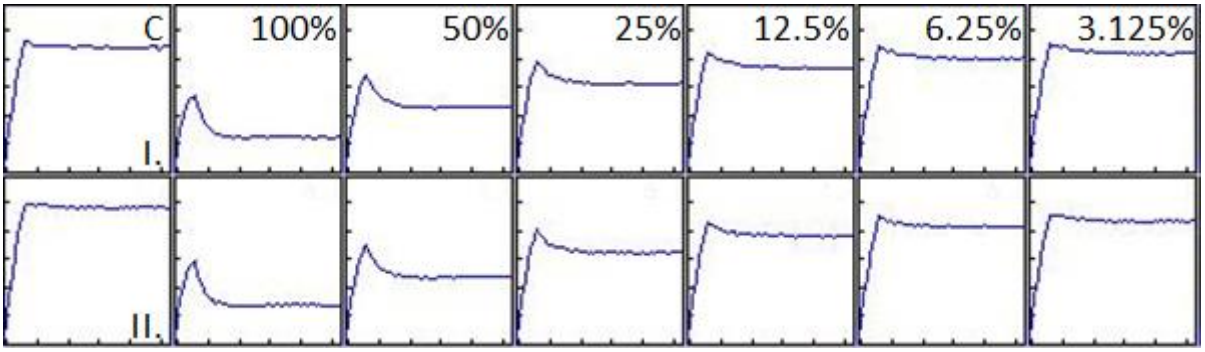

Figure 2. Light output during the first 30 secs of the 30 minutes exposure. I and II depict the two replicates. C: Control. The peak shows the maximum light output of the bacteria, which immediately starts to diminish after the test bacteria get in contact with the sample.

Sinapis alba growth inhibition test

The measured root length of the treated seeds was greater than in the control in every concentration but no clear trend could be noticed as Figure 3 shows below. Due to the stimulating effect on the seeds neither EC_{50} values nor inhibition was calculated. This pattern can be experienced for samples which contain plant nutrients: in this case nutrients might mask the toxic effect in low concentrations (USEPA, 2000).

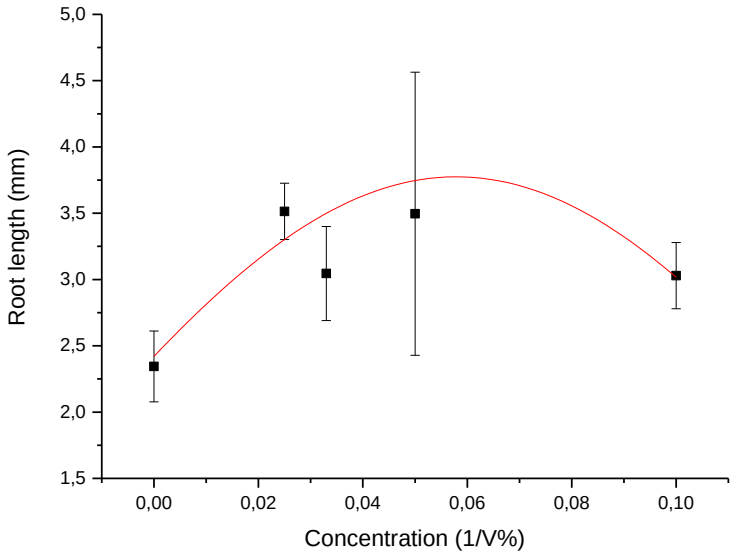

Figure 3. Concentration-response curve for the *Sinapis alba* test.

Daphnia magna immobilization test

Of the conducted tests, the *D. magna* immobilization test appeared to be the most sensitive. After a few range finding test the adjusted concentration was between 0.001% and 0.1%, calculated EC_{50} value was 0.0226%.

Flow cytometry

A well distinguishable sperm region was established according to forward scatter versus side scatter properties (Figure 4.). This sperm region was gated to SYBR14 vs. PI dot plots,

where distinct living, dead and moribund populations were discriminated. Moribund cells were included in the dead category during data analysis (Figure 5.).

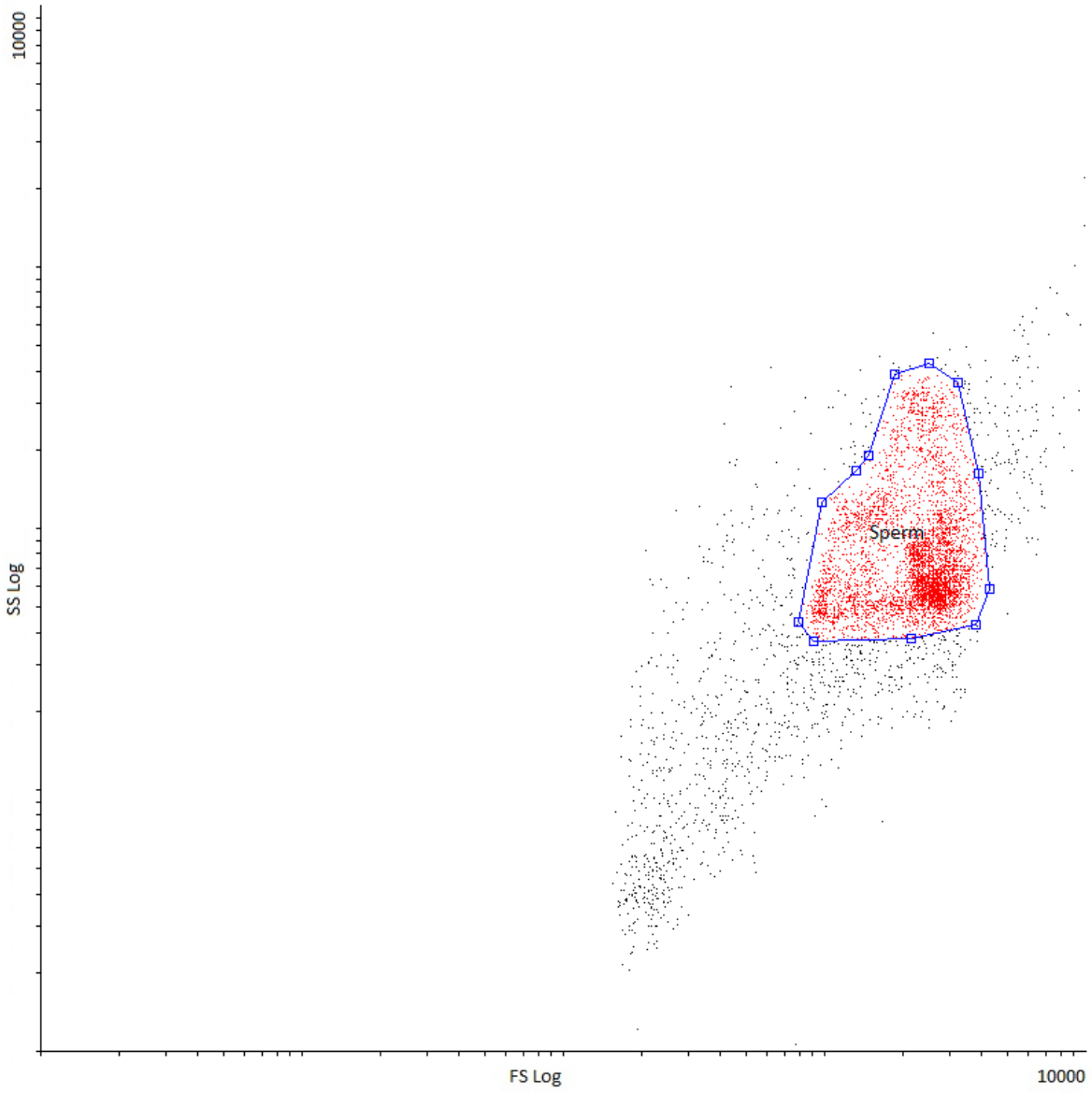

Figure 4. Differentiation of sperm population based on forward scatter versus side scatter properties

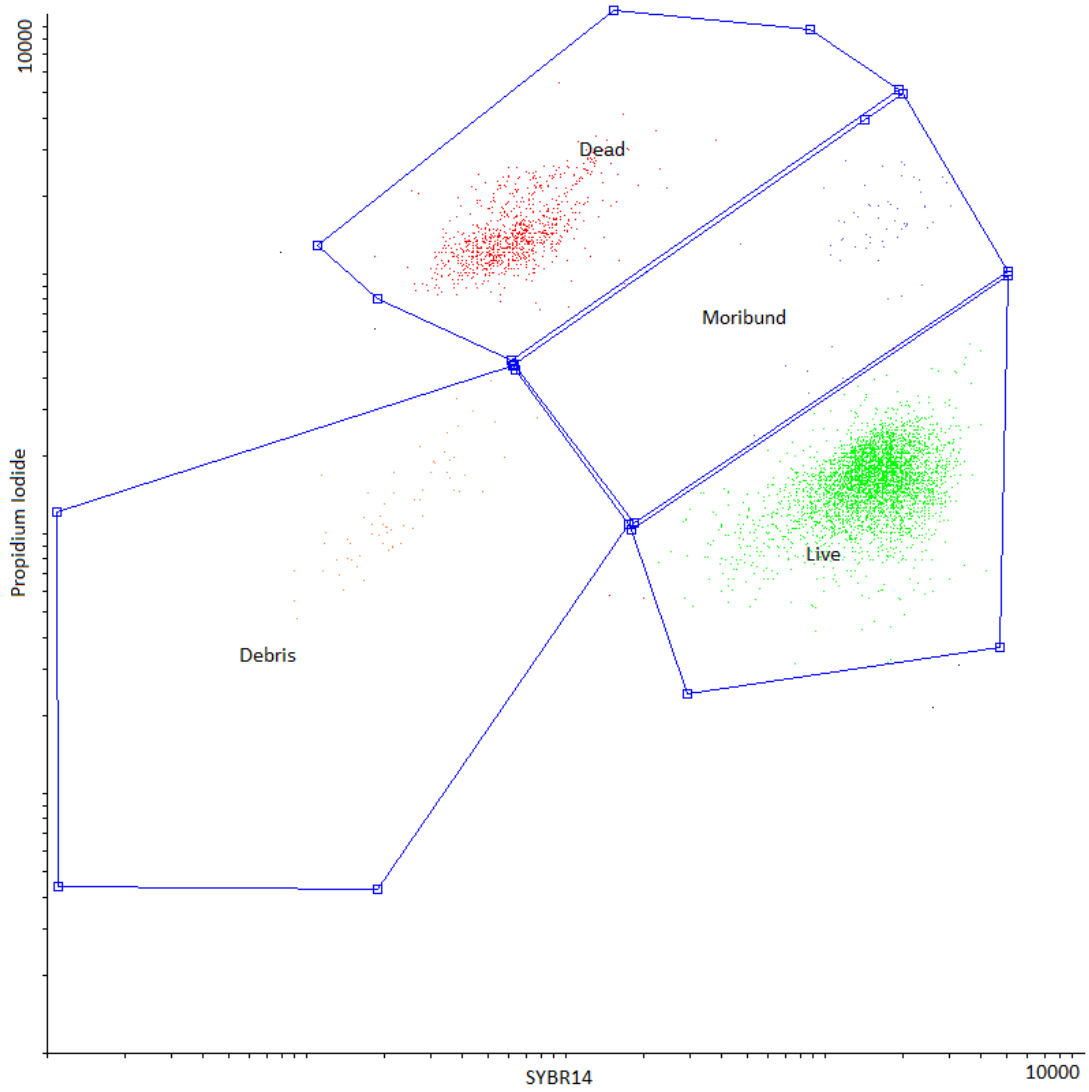

Figure 5. Live-, moribund- and dead regions according to SYBR14 and PI fluorescence

The results show that live cell ratio was around 82-83% after 30 minutes exposition and the samples did not differ from controls significantly ($p= 0.0547$). After one day exposure, the biodiesel treated samples showed statistically significant (from 83% to 77%; $p<0.0001$) decrease in live cell ratio.

After 30 minutes, the biodiesel samples indicated only a few percent (less, than 2%) of spermatozoa with DNA fragmentation similarly to the control and the percentage of cells with fragmented DNA did not change after one day exposition.

Micronucleus test

The genotoxic response was expressed as the number of micronuclei/1000 cells. Figure 6 shows typical micronucleus formation, a concentration-effect curve is given in Figure 7. The data of the test were not suitable for calculating EC_{50} values so statistical analysis was performed. The result of the one way ANOVA was p=0.00025 (F=6.7152, df=4) so the effect of each concentration could be separated from each other. To determine the difference between the control and the treatments, a two sample t-test was carried out. The results show that the control and the most diluted concentration do not differ significantly (p=0.882), but for the other concentrations (3.3%, 5% and 10%) statistically significant difference could be established (p=0.009; p=0.019 and p=0.0003, respectively).

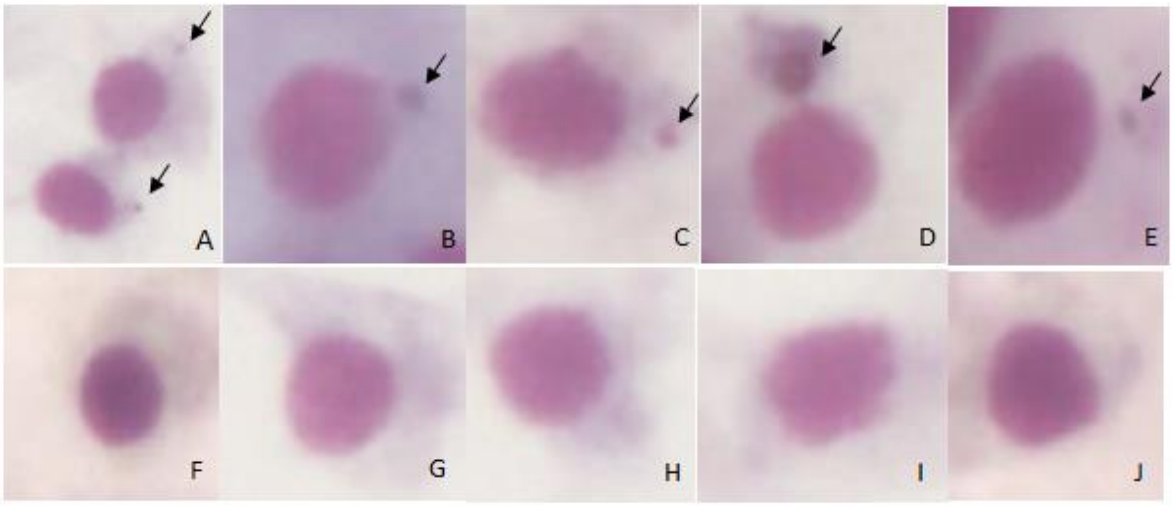

Figure 6. Typical micronucleus formation (A-E) and normal agranular hemolymph cells (F-J) from *Unio pictorum* Giemsa painted hemolymph.

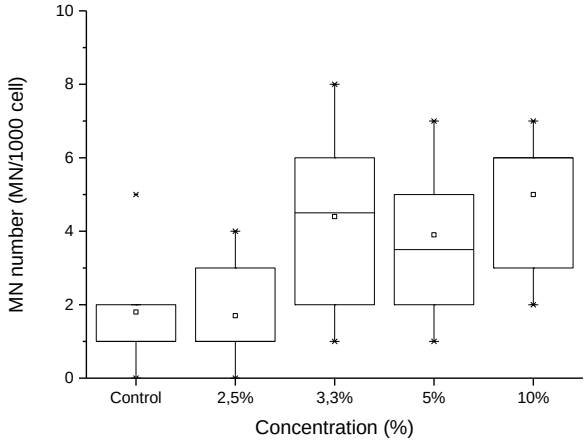

Figure 7. Concentration-effect relationship for the micronucleus test.

Discussion

The biodiesel impact on water resources is composed of several factors. Biofuel production demands a great volume of water that can be replaced with seawater or wastewater in a certain amount (Wu et al. 2009). The spilled biofuel (as well as any other type of fuel) forms a non-aqueous phase layer on the water surface damaging sea birds and other animals that try to pass through. Biodiesel has a low solubility in water but the intensive waving and water flow cause some degree of mixing. We used a similar mix for the tests that appeared to exert highly toxic and genotoxic effect on the aquatic life.

Terrestrial ecosystems were represented by the standardized *S. alba* seedling emergence and seedling growth test. The results of this test showed no sign of toxicity. Moreover, in this test lower concentrations seemed to exert stimulating effect. This is a typical concentration-response relationship in cases where the sample contains plant nutrients (USEPA, 2000).

However, these negative results do not necessarily imply that biodiesel should be completely safe for terrestrial ecosystems. On one hand, several studies have been targeted to assess biodegradability of biodiesel or different biodiesel-fossil fuel blends. These studies support that biodiesel can be biodegraded considerably faster than diesel both under aerobic (e.g. Lapinskienė et al., 2006, Yassine et al., 2013) and anaerobic conditions (e.g. Wu et al., 2015).

On the other hand, seed germination tests showed that biodegradation products might pose actual risk. Tamada et al. (2012) followed biodegradation of biodiesel and vegetable oils for a period of 180 days. Seed germination tests revealed an increasing toxicity of biodiesel metabolites as bacterial decomposition went by. In the same study, using the earthworm (*Eisenia foetida*) test, biodiesel was the only contaminant that proved to be toxic. A similar study was conducted by Cruz et al. (2013a, b). *Cucumis sativus* and *Brassica oleracea* seed germination inhibition showed that after two months of biodegradation, biodiesel was the most toxic contaminant in comparison to diesel and waste lubricant oil. Phytotoxicity of metabolites was also demonstrated in the study of Hawrot–Paw and Izwikow (2015) using garden cress (*Lepidium sativum*) and spring barley (*Hordeum vulgare*).

In order to assess the potential ecotoxicity of different biodiesel blends on aquatic life, two standard and widely used assays were used in our study, the *Daphnia magna* immobility assay and the *Vibrio fischeri* bioluminescence inhibition bioassay. The *V. fischeri* bioluminescence inhibition bioassay detected considerable toxicity with the EC_{50} of 12.52%. This assay was used in a study of Yassine et al. (2012) to assess the toxicity of the water

accommodated fraction (WAF) of six commercial soybean biodiesel/petrodiesel blends at different oil loads. These results can provide a good basis for comparison with our results, as in the preparation of WAF, oils were introduced to water with the highest load of 1:1. *V. fischeri* EC_{50}s for WAFs of B20, B40, B60, B80 and B100 blends fall very close to each other, app. 5%. In our test, only neat biodiesel was assessed, test results showed slightly lower ecotoxicity. Differences might have been caused by different biodiesel types: while in the study of Yassine et al. soybean-methyl ester biodiesel was used, our sample was a rapeseed methyl ester. In a comparative study of Hollebone et al. (2008) three different biodiesels (two based on vegetable oils of canola and soy, and one animal-source waste fry oil) were assessed using Microtox. The soy-based biodiesel exerted the highest toxicity on the test bacterium.

In addition, although the same test organism, *V. fischeri* was used in both assessments, test protocols differed. In our study a kinetic protocol was followed, which was developed especially for the assessment of turbid and/or colored samples. As light output in the sample is assessed independently from the control, false toxicity readings caused by turbidity and/or color of the sample can be avoided (Lappalainen et al., 2001).

D. magna showed extreme sensitivity with EC_{50} value of 0.0226%. Though the *Daphnia* bioassay is the most frequently used test in biodiesel ecotoxicity assessments, results given by different studies are rather difficult to compare with each other due to different sample preparation protocols (oil in water dispersion, OWD vs. water accommodated fraction, WAF) or differences in test protocols (e.g. different exposure regimes) (Bluhm et al., 2012). Khan et al. (2007) in a comparative study used OWD of biodiesels derived from recycled cooking oils and fats, employing daphnids and rainbow trouts (*Oncorhynchus mykiss*) as test organisms. Daphnids were found more sensitive: EC_{50} in the *D. magna* assay was 4.65 ppm, while *O. mykiss* EC_{50} was 455.28 ppm (after 24 hour exposure in the *D. magna* test and 96 hour exposure in the *O. mykiss* test). Acute *Daphnia* EC_{50} value determined by Tjarinto et al. (2014) fall very close, 3.157 ppm.

Hollebone et al. (2008) suggest that OWD sample might not be representative when ecotoxicity on daphnids is to be evaluated. When OWD of different biodiesels were investigated, higher toxicity was detected than in WAFs of the same biodiesels. The possible explanation was that in OWDs oil layers formed which might have caused either physical smothering or trapping of daphnids, enhancing mortality rate. Based on these findings, the WAF of our biodiesel was further ultrasonicated to avoid such possible physical effects, therefore the experienced low EC_{50} must have reflected actual toxicity.

Literature studies reveal that apart from standard bioassays, tests conducted using other test organisms also support the potential risk of biodiesels on different elements of aquatic ecosystems, both freshwater and marine. A study of Leite et al. (2011) determined the toxicity of the water-soluble fractions (WSF) of three different biodiesel fuels to two marine organisms, the sea urchin *Echinometra lucunter* and the microalga *Tetraselmis chuii*. A non-lethal bioassay was conducted by Gauthier (2012) using behavioral alterations of the crayfish *Orconectes rusticus* and found that biodiesel and crude oil had equal negative effects on chemosensory behavior of the crayfish. Gorcharoenwat et al. (2017) evaluated the effects of the water soluble fraction of palm biodiesel on *Macrobrachium rosenbergii*, the giant freshwater prawn, which is an economically important native aquatic organism in Southeast Asia living in freshwater to brackish water. It was found that histologically abnormal alterations appeared in the gills of tested larvae. Some freshwater plant species such as duckweed (*Lemna minor*) or water milfoil (*Myriophyllum spicatum*) were seriously affected by biodiesel in a comprehensive study of Birchall et al. (1995).

Considering genotoxicity/mutagenicity, in most cases biodiesel exhaust emission has been evaluated (reviewed by Bluhm et al., 2012, Claxton, 2015). Direct genotoxicological assessments of biodiesel samples have been carried out much less often, these literature studies, however, indicate the genotoxic nature of biodiesel samples.

Leme et al. (2012) carried out spill simulations with neat diesel and biodiesel. In their study, water soluble fraction of the biodiesel exerted mutagenic and genotoxic effects in the *Salmonella*/microsome preincubation assay and the in vitro Chinese hamster ovary cell MN test. The authors attributed these effects to the presence of potentially toxic compounds in the biodiesel derived from the raw material source used in the production chain.

Cavalcante et al. (2014) found that biodiesel can cause cytotoxic, biochemical and genotoxic alterations in the hepatocyte cell line of *Danio rerio* (ZFL), depending on the production route: methylic (BdMt) route producing biodiesel with more intense effect than ethylic (BdEt) route.

In our study, 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%. This test has never been employed in biodiesel eco/genotoxicity assessments, however, it seems promising. It shows appropriate sensitivity, and moreover, mussels are a representative group when ecological risk to aquatic ecosystems is to be addressed. Different mussel biomarkers have been used for example to assess or monitor ecological impacts of oil spills (e.g. Pérez-Cadahía et al., 2004, Laffon et al., 2006).

The mechanism of micronuclei formation is relatively well discussed. Acentric chromosome or chromatid fragments from misrepaired or unrepaired DNA double-strand breaks can lead to MN formation (Savage, 1988). MN can also originate from broken nucleoplasmic bridges during telophase when chromosome fragments fail to be included in the daughter nuclei (Dianov et al., 1991). Lagging whole chromosomes at anaphase also can create MN. This can happen in the centromeric and pericentromeric DNA repeat sequence by the hypomethylation of cytosine or by the defects of the kinetochore protein or the mitosis check point (Pironon et al., 2010). Abnormal centrosome amplification and some spindle dysfunction could also be the cause of MN formation (Gisselsson, 2008).

As the results of the flow cytometric boar sperm test indicated, biodiesel has a slight cytotoxic effect.

Conclusions

A battery of bioassays was employed to provide complex information on the eco- and genotoxicity of a rapeseed biodiesel, including the *Sinapis alba* root growth inhibition, the *Daphnia magna* immobilization, *Vibrio fischeri* bioluminescence inhibition, boar semen live/dead ratio as well as DNA fragmentation and the *Unio pictorum* micronucleus tests.

The sample exerted significant effect on aquatic test organisms, *D. magna* being far the most sensitive with EC_{50} value of 0.0226%. The *V. fischeri* bioluminescence inhibition bioassay also detected considerable toxicity with the EC_{50} of 12.52%. These results raise environmental concern about biodiesel, especially in case of accidental oil spills.

On the other hand, no acute toxicity was shown by the terrestrial *S. alba* test.

The mussel micronucleus test, using the freshwater *Unio pictorum* detected considerable genotoxic potential of the biodiesel sample: it elucidated micronuclei formation already at low concentration of 3.3%. It was the first time this bioassay has been employed in biodiesel genotoxicity assessment and one of the aims of the study was to evaluate its applicability for such samples. It seems to be an appropriate tool, based on its representativity, sensitivity and cost effectiveness.

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