Ecotoxicity studies of the levulinate ester series

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Abstract: The increasing interest in the development of novel green solvents has led to the synthesis of benign alternative products with minimized environmental impacts. However, most of published studies on green solvents focus primarily on their physicochemical properties, with limited emphasis on absence of ecotoxicological assessment. In this study, we evaluated the acute ecotoxicity of four levulinates (levulinic acid, methyl levulinate, ethyl levulinate and butyl levulinate) on freshwater algae (*Chlamydomonas reinhardtii*), bacteria (*Vibrio fischeri*), daphnids (*Daphnia* magna) and earthworms (*Eisenia foetida*) using various dose-response tests. As a general trend, the toxicity of levulinate esters in aquatic exposure (assessed as the EC_{50}) increased as a function of increasing alkyl chain length; accordingly, the most toxic compound for the aquatic organisms was butyl levulinate, followed by ethyl levulinate and methyl levulinate. The most toxic compound for *Eisenia foetida* (terrestrial exposure) was methyl levulinate, followed by ethyl levulinate, butyl levulinate and levulinic acid; in this case, we observed an inverse relationship between toxicity and alkyl chain length. Based on both the lowest EC_{50} found in the aquatic media and the ratio between predicted environmental concentration (PEC) and the predicted no-effect concentration (PNEC), we have estimated the maximum allowable values in the

environment for these chemicals to be 1.093 mg L^{-1} for levulinic acid, 2.761 mg L^{-1} for methyl levulinate, 0.982 mg L^{-1} for ethyl levulinate and 0.151 mg L^{-1} for butyl levulinate.

Keywords: levulinic acid, methyl levulinate, ethyl levulinate, butyl levulinate, lethal concentration, acute toxicity.

Introduction

Green Chemistry is a philosophy aimed at designing alternative products and processes that minimize the use and generation of hazardous substances (Anastas and Warner 1998), creating safer products, reducing waste, eliminating costly end-of-pipe treatments and reducing the use of energy and resources. However, it is difficult to quantify the "greenness" of chemical products or processes although several attempts have been proposed to achieve this goal; Life Cycle Assessment (Wernet et al. 2010) or the Persistence and Spatial Range methods (Scheringer 2002) are examples of this. Nonetheless, it is possible to estimate the environmental "goodness" of a chemical process based on the "Twelve Principles of Green Chemistry" (Anastas and Warner 1998). According to these Principles, solvents should exhibit low vapor pressure, high boiling point, and high biodegradability under environmental conditions; be odorless and easy to recycle after use; and be able to dissolve as many chemical compounds as possible. In addition, green solvents should be non-toxic and non-harmful to the environment and to health, inexpensive and come from renewable resources. (Ahluwalia and Varma 2009).

Although no perfect green solvent exists that could be applied in all situations, several alternatives have recently been developed (i.e., solvents from biomass, ionic liquids, supercritical fluids, etc.) (Diaz-Alvarez et al. 2011). Solvents from biomass can be obtained by enzymatic or esterification and fermentation processes. Among these solvents are levulinic acid and its esters, terpenes, lactates, furfural, glycols, etc (Timokhin et al. 1999).

Concretely, levulinate esters and levulinic acid are used as flavors, in the fragrance industry, in cigarette manufacturing (Keithly et al. 2005) or as substrates for various types of condensation and addition reactions (Khusnutdinov et al. 2007). Although

some data exist regarding their molecular behavior (Guerrero et al. 2011; Lomba et al. 2013; Lomba et al. 2011), information regarding their toxicity and ecotoxicity is scarce. Several reports deem levulinic acid (LA) to be non-toxic (Tischer et al. 1942). However, we hypothesize that the toxicity of levulinates will increase as a function of the length of the alkyl chain based on toxicity data available for analogous compounds containing alkyl chains with different lengths (Haibo X. 2013; Xiaohua Lu 2009).

We have carried out a detailed study of the environmental toxicity of levulinic acid (LA), methyl levulinate (ML), ethyl levulinate (EL) and butyl levulinate (BL). Three standard ecotoxicological tests were used: the inhibition of bioluminescence of *Vibrio fischeri* bacteria, the *Daphnia magna* (freshwater crustacean) acute immobilization test, and the acute toxicity test in *Eisenia foetida*. In addition, we have evaluated the short-term toxicity of levulinates to the photosynthesis of *Chlamydomonas reinhardtii* using fluorimetry. The results were analyzed and related to the physicochemical properties previously reported for these compounds (Guerrero et al. 2011; Lomba et al. 2013). We have also provided the first approaches for risk assessment of these compounds in aquatic and terrestrial environments.

Materials and methods

Chemicals

All solvents derived from biomass used in this study were provided by Sigma–Aldrich: LA, ML and BL with a purity of 98% and EL with a purity of 99%. These test chemicals were special-grade reagents and used without purification. The chemical structures and molecular weight formula weights of the compounds studied are shown in Fig. 1. Trend analysis and quantitative structure –activity relationship (QSAR) models were applied with the software QSAR Toolbox 2.3 (QSAR Toolbox) which

helped to determine the levulinate concentrations to be tested. QSAR is based on the correlation between structural characteristics of series of molecules and their biological activity or chemical reactivity. Furthermore, for each of the tests carried out, a previous study was performed to refine the range of concentrations and ensure concentrations within EC_{50}.

Positive and negative controls were used for all the bioassays and the results were acceptable in all the cases according to the criteria established in each of the standardized used method. Details of controls used in each test are provided below.

Assessing the impacts of levulinates on algal photosynthesis

Culture of Chlamydomonas reinhardtii

Exponential-phase unicellular green algae (*Chlamydomonas reinhardtii* CC125) were used for the experiments. The algae were grown for 72 hours on an orbital shaker at 90 rpm under a continuous illumination of 130 µE $PAR \cdot m^{-2} \cdot s^{-1}$ from four fluorescent tubes (Blau aquaristic T5HO, 39 $w \cdot 10000 \cdot K^{-1}$) in an incubator at 25ºC. The culture medium was "Talaquil", prepared as described in Szivák *et al*. (Szivak et al. 2009) except that $CuCl_2 \cdot 2H_2O$ and $ZnCl_2$ were used instead of the corresponding sulfates. Furthermore, the pH of the culture was adjusted to 7.5 using MOPS.

Exposure to levulinates

The exposure medium consisted on a MOPS solution (10 mM) with double distilled water (Milli-Q), adjusted to a pH of 7.5 using KOH. Different concentrations of these chemicals have been tested in order to obtain dose-response curves, with two replicates (flasks) for each one. In case of levulinic acid concentrations ranged between 40-1800 mg L^{-1}, while methyl, ethyl and butyl levulinates concentrations ranged between

0-33000 mg L^{-1}, 0-22000 mg L^{-1} and 0-5500 mg L^{-1} respectively. Negative controls have been also tested. Dose-response curves have been repeated 3 times. The 72-hour-old algae have been centrifuged (10 min, 3000 r.p.m.) and the concentrate have been used to obtain an optical density (OD_{685}) of 0.3, equivalent to 1150000 cells mL^{-1}.

Measuring of fluorescence parameters

A Mini-PAM fluorometer (from WALZ, Effeltrich, DE) was used to measure photochemical quantum yield, Y (II), in algal suspensions contained in an optical glass cuvette. Suspensions were maintained under constant agitation using a micro stirrer (Spinette Cel Stirrer from STARNA, Essex, UK). The settings used were as follows: an ML level of 0.15 µmol m^{-2} s^{-1}, SP of 1577 µmol m^{-2} s^{-1}, and pulses 0.8 s in duration. Fluorescence parameters were measured on 2 mL algal suspension after 1 and 2 hours of exposure. After 30 s of acclimatization to the measuring light conditions (≈ 50-60 µmol $m^{-2}\cdot s^{-1}$), between 4 and 5 consecutive Y (II) measurements were made at 10-second intervals.

The Inhibition of Bioluminescence of *Vibrio fischeri*

Vibrio fischeri culture

The lyophilized *Vibrio fischeri* (strain NRRL-B-11177) used for the bioluminescence inhibition assays were purchased from Macharey-Nagel (ref. 945 006). These experiments were carried out in accordance with the test conditions and the operating protocol of the *Vibrio fischeri* acute toxicity test (UNE-EN-ISO 11348-3 2007). Prior to testing, bacteria were rehydrated using the corresponding reactivation solution provided by the manufacturer. The reactivated bacteria were stored for 5 min in the refrigerator at a temperature between 2-8ºC.

Exposure to levulinates

Several dilutions for each of the four studied chemicals were prepared using a 2% NaCl stock solution. The different concentrations ranges assessed for this bioassay for these compounds were 0-20000 mg L^{-1} for LA, 0-2000 mg L^{-1} for ML, 0-900 mg L^{-1} for EL and 0-500 mg L^{-1} for BL. Additionally, positive controls with phenol, 42.5 mg L^{-1} and zinc sulfate, 2.2 mg L^{-1} and negative controls were tested (Jennings et al. 2001). The pH of the solutions were adjusted to 7-7.5 using either 0.1 M NaOH or 0.1 M HCl solutions.

Next, 0.5 mL of the reactivated bacterial suspension was transferred to cuvettes. The solution equilibrated for 10 min at 15ºC, and subsequently, the first measurements were taken to obtain the initial luminescence; then 0.5 mL of each dilution to be tested was added to the cuvette. The inhibition of luminescence was measured after 30 min. The test was repeated in triplicate.

Measuring the inhibition of bioluminescence

Luminescence measurements were taken with a Biofix® Lumi-10 luminometer (Macharey-Nagel) using the acute mode (Biotox B) equipped with an ultra-fast single-photon counter detector covering the 3806-660 nm spectral range. The toxic effect values reflect the ratio of the decrease in bacterial light production to the remaining light. The sensitivity is 10 fmol ATP when using ATP bioluminescence assays CLS II (Roche Diagnostics GmbH, Mannheim Germany). The ratio between the light intensity lost by the bacterial solution and that remaining after exposure to the toxic sample can be obtained using the following expression:

$$\Gamma = \frac{\overline{\overline{H}}_t}{100 - \overline{\overline{H}}_t} \quad (1)$$

where H_t is the inhibition percent.

From the results, a linear relationship can be deduced between the function Γ and the concentration of the chemicals used, in the following form:

$$\log \Gamma = \log a + \log C \quad (2)$$

The resulting function was normalized, and the EC_{50} (chemical concentration resulting in a 50% reduction of bioluminescence) was calculated.

Daphnia magna Acute Immobilization Test

Daphnia magna culture

The *Daphnia magna* vials used in the acute immobilization assays were purchased from Vidrafoc (ref. DM090812) and were stored at 4ºC. The experiment was carried out in accordance with the OECD 202 test conditions and operating protocol (OC SE TG 202 2004).

First, the medium for the eggs was prepared according to the specifications of the supplier. Next, the eggs were incubated for 72 hours at 20-22 °C with 6000 lux in a TOXKIT model CH-0120D-AC/DC incubator (supplied by ECOTEST). When a sufficient number of neonates was obtained, a vial of *Spirulina* was added to feed them 2 hours prior to starting the bioassay.

Exposure to levulinates

Several dilutions for the four studied chemicals were prepared in aqueous medium solution. In this case, the different concentrations range for these compounds have been between 2200-10000 mg L^{-1} for LA, 2000-4500 mg L^{-1} for ML, 400-1800 mg L^{-1} for EL and 0-360 mg L^{-1} for BL. Additionally, negative and positive controls with $K_2Cr_2O_7$ (0.6-2.1 mg L^{-1}) were also tested. (OECD 202 1984) Furthermore, the pH of the solutions was adjusted to be between 7-7.5 using 0.1 M NaOH or 0.1 M HCl solutions.

After 2 hours of feeding, a total of 20 organisms (aged < 24 h) were used. The organisms were divided into four groups of five organisms per group. All concentrations with the daphnids were incubated in complete darkness for 24 hours at 20-22°C. The test was repeated in triplicate.

Measuring of acute immobilization

After 24 hours in darkness, the immobilization of the daphnids was measured. The daphnids that were unable to swim for 15 seconds after gentle stirring were considered immobile. The probit method (Bliss 1934) was used to calculate the 50% effect concentration (EC_{50}).

Earthworm, acute toxicity test

Eisenia foetida culture

Adult *Eisenia foetida* earthworms (2 months old with clitellum) with individual weights in the range of 300 mg to 600 mg were used in accordance with the test conditions and the operating protocol of the Guidelines for the testing of chemicals. (OECD 207 1984)

Exposure to levulinates

The artificial soil was composed of 10% *sphagnum* peat, 20% kaolin clay and 70% industrial fine sand by dry weight. *Sphagnum* peat was purchased from Verdecora vivarium (Spain) whereas kaolin and industrial sand were obtained from Imerys Ceramics España, S.A. The water content of the mixture was determined by taking an initial sample weight, drying it to a constant mass at 105ºC for 24 hours, and taking a subsequent measurement. Deionized water was added to adjust the overall moisture content to approximately 35% of the dry weight, and the medium was thoroughly mixed. The pH measurements were taken with a Crison Basic 20 pH meter using a 1 M KCl solution.

A total of 0.5 kg of dry artificial soil was placed in a 500 mL plastic jar, and 10 adult earthworms were added to each jar. Additionally, we prepared several test concentrations in a geometric series and both negative and positive controls (chloracetamide; 24 mg kg^{-1}) (OECD 207 1984). The different concentrations prepared for each compound were 1900-6900 mg kg^{-1} for LA; 400-900 mg kg^{-1} for ML 400-1600 mg kg^{-1} for EL and 1400-2500 mg kg^{-1} for BL. Three jars, each containing 10 adult earthworms, were used for each concentration.

The jars were loosely covered with a film and some air-exchange holes were made. These jars were stored at 20-22ºC with 80–85% relative humidity under 400–800 lux of constant light. Mortality was assessed at 7 and 14 days after treatment. During the bioassay, all organisms were fed with a flavor/water mixture. The test was repeated in triplicate.

Measuring of death of earthworms

After 14 days, the number of dead earthworms was measured. All organisms that were immobile upon digital stimulation were characterized as dead. The probit method (Bliss 1934) was used to calculate the 50% effect concentration (EC_{50}).

Statistics and graphical representation

Dose-response curves for algal photosynthesis were fitted to a four-parameter logistic curve using R and the *drc* package to obtain the corresponding EC_{50} values and standard errors (SE). The same package was also used to perform comparison tests, using the R "compPAR" function; the null hypothesis was that the ratio equals 1. The ratio was obtained by dividing EC_{50} values; if it significantly differed from 1 (i.e., $p<0.05$), the null hypothesis was to be rejected. All statistical analysis for daphnids, earthworms and

bacteria was performed using the SPSS 18.00 software (IBM® SPSS software), in which a threshold of p=0.05 has been set to accept or reject the null hypothesis

Results and discussion

We found that all of the levulinates studied were toxic to the test organisms in a concentration-dependent manner. Similarly, in agreement with our hypothesis, the aquatic toxicity of levulinate series increased as a function of the alkyl chain length; accordingly the toxicity of the series of compounds for the aquatic organisms was BL, followed by EL and ML. LA was the most toxic compound in the freshwater algae assay (see Tab. 1), that was due to its high acidity (buffer capacity of MOPS was surpassed). In terrestrial exposure (tests with *Esenia foetida)*, the most toxic compound was ML followed by EL, BL and LA; in this case, the toxicity of levulinates decreased as the length of alkyl chain increased (Tab. 1).

Levulinate toxicity in algae

The toxicity followed the trend of BL, EL and ML (the values of EC_{50} decreased as the length of alkyl chain in the levulinate series increased (Fig. 2). All values were significantly different. The toxicity, measured as the decrease of in the yield of Photosystem II, Y (II), indicates that levulinates affect the electron flow. This effect is commonly induced by a wide range of chemicals such as Zn (Corcoll 2011, Corcoll et al. 2011), Hg (Juneau et al. 2001) and Cu (Juneau et al. 2002), volatile compounds (Brack et al. 1998), herbicides (Brack and Frank 1998, Conrad et al. 1993, Dorigo and Leboulanger 2001) and ultraviolet radiation (UVR) (Navarro et al. 2008). Even if the underlying processes explaining the levulinate toxicity are unknown, the behavior of minimal fluorescence (F) after 2 hours of exposure (data not shown) seems to point to

an attenuation of photosynthetic pigments, and particularly a decrease in chlorophyll *a*. The value of F was related to the stability of the light-harvesting complexes and thus to the stability of its pigments (Yamada et al. 1996). Moreover, LA has been described as an inhibitor of chlorophyll synthesis in algae and higher plants (Johanningmeier and Howell 1984). Another possible explanation may be linked to the loss of integrity of the photosynthetic membranes (Brack et al. 1998); the hydrophobicity of the levulinates would facilitate their interaction with lipid membranes (Lomize et al. 2007). The subsequent disruption of the photosynthetic electron chain might also lead to ROS production (Stoiber et al. 2011), increasing the oxidative stress (Guasch and Sabater 1998) and the concomitant toxic effects on the photosynthetic yield.

Levulinate toxicity in bacteria

The most toxic compounds as measured using the bioluminescence assay followed the decreasing ranked order series of BL followed by EL, ML and LA. All values were significantly different from each other and followed trends similar to those of the algae study, *i.e.,* the values of EC_{50} decrease as the length of the alkyl chain increases (Fig. 3). Bacterial bioluminescence reactions are coupled to the electron transport system in cellular respiration and are indicative of cellular metabolism; *i.e.,* lower bioluminescence implies decreased cellular respiration. Moreover, the addition of a variety of chemicals are known to affect the cellular respiration by the modification of both protein and lipid biosynthetic pathways, altering the luminescence emission; *i.e.*, in the presence of pollutants, the natural bioluminescence of *Vibrio fischeri* decreases (Onorati and Mecozzi 2004).

Levulinate toxicity in daphnids

Results obtained in the *Daphnia magna* bioassay are shown in Fig. 4. The toxicity rank of the levulinate series decreases as follows: BL followed by EL, ML and LA. Additionally, the trend observed for *Daphnia magna* is the same as the rest of the aquatic bioassays tested; for levulinate esters, the values of EC_{50} decreases as the length of alkyl chain increases.

The different toxicity of the studied chemicals can be due to the structural variability. We hypothesize that if the toxicants are absorbed by daphnids, the more hydrophobic compounds have a greater potential to cross the plasma membrane, thus eliciting greater ecotoxicity.

Levulinate toxicity in earthworms

Although the previous studies we performed show a direct relationship between toxicity and alkyl chain length, our results show opposite trends between aquatic and terrestrial compartments. In the *Eisenia foetida*, the toxicity of the series follows the rank order of ML followed by EL, BL and LA; *i.e.,* for levulinate esters, the values of EC_{50} increase with the length of the alkyl chain (Fig. 5). There are no studies focusing on levulinate toxicity in earthworms; however, there is a report by Roberts and Dorough in which several other chemicals were analyzed and classified according to their toxicity to *E. foetida* (Roberts and Dorough 1984). Interestingly, this study also revealed that *Eisenia* was unexpectedly tolerant to many solvents and fuels, even at very high concentrations (i.e., cyclohexane, dichloromethane or methanol). However, other solvents such as acetone or benzene were moderately toxic while benzaldehyde and dimethylacetamide were very toxic (Roberts and Dorough 1984).

According to the classification presented in this study, LA and BL can be considered as non-toxic, while ML is moderately toxic (Roberts and Dorough 1984).

Considering the differences in toxicity shown by very similar compounds it is difficult for us to correlate the structure and properties of the chemicals tested (i.e., the levulinate series) and their effect in *Eisenia*. Typically, chemicals that are extremely toxic in mammals are found to be the same in both fish and earthworms. However, some fungicides are known to show the opposite trend (non-toxic for mammals and extremely toxic to fish and earthworms). Many other examples exist that demonstrate little to no correlation between non-target species, and consequently, interspecies extrapolations of chemical hazard from one animal species to another based on toxicity data are not easy to make (Roberts and Dorough 1984). We find that the mechanisms of action for levulinate toxicity are both exposure- and species- dependent.

Environmental Risk Assessment for levulinates

Physico-chemical properties of levulinates related to toxicity

Log P can be used to estimate the lipophilicity of molecules, which is related to their ability to cross through biological membranes and influences their fate after uptake. In a previous paper (Lomba et al. 2013; Lomba et al. 2011) values of the logarithm of 1-octanol/water partition coefficient, *log P*, were computed in silico using the ALOGP method (Ghose and Crippen 1986; Ghose et al. 1988; Viswanadhan et al. 1989) with values of -0.14 for LA, -0.17 for ML, 0.38 for EL and 1.36 for BL. We have compared the computed *log P* values to those predicted with the KowWin method (available on http://esc.syrres.com/esc/est_kowdemo.htm) (Meylan and Howard 1995). Using this second approach, the predicted *log P* values for the levulinate series are -0.49 for LA

(empirical value), -0.13 for ML (empirical value), 0.29 for EL and 1.27 for BL. The values obtained from two different methods are different; nonetheless values obtained from KowWin method have been used to correlate our log EC_{50} values against the predicted *log P* values as shown in Fig. 6.

The levulinate series elicited a direct relationship between the hydrophobicity and ecotoxicity; *i.e.,* the less hydrophilic the chemicals had lower EC_{50} values in aquatic exposure. The exceptions included LA, which has the lowest EC_{50} values compared to its *log P* in the case of *Chlamydomonas reinhardtii* (EC_{50} values lower than expected), and BL, which exhibits higher EC_{50} values in the case of the *Vibrio fischeri* assay.

Differently from water exposure, it is important to note that there is no direct relationship between the toxicity in soil and the *log P*. This lack may exist because the partition coefficient is a specific parameter between water and octanol and does not take into account other factors governing the mobility of the pollutants in soils (i.e. cation exchange, chemical solubility equilibrium, biochemical reactions and sorption to soil organic matter).

Environmental toxicity

Publications related to the environmental toxicity of levulinates are scarce. Only LA has been found to inhibit microorganism growth and ethanol production during fermentation (Almeida et al. 2007). Similarly, Tischer *et al*. tested LA on rats, guinea pigs, chicks and human beings and stated its innocuousness (Tischer et al. 1942). The aquatic US-EPA ECOTOX database provides a LC_{50} lethal value of 10 mg L^{-1} during 24 h for three different fish species (MacPhee and Ruelle 1969). On the other hand, there are a few predicted aquatic LC_{50} and EC_{50} values for our series

(http://webnet.oecd.org/CCRWEB/Search.aspx) (OECD 2006) which are presented in Table 2.

Several EC_{50} values from our bioindicator assays were used as a basis for comparing our studied "green solvents" with traditional solvents; for instance, some traditional solvents, such as dimethylformamide or 2-propanol, have been tested in *Selenastrum capricornutum* with EC_{50} values of 1714 mg L^{-1} and 11.719 mg L^{-1}, respectively. (Cho et al. 2008) The algae assays for the levulinate series show lower values than 2-propanolol, with the exception of ML. However, all the studied chemicals show higher EC_{50} values than dimethylformamide except for LA.

For the *Vibrio fischeri* assay, the EC_{50} values of three levulinate esters (ML, EL and BL) are similar to the values of solvents classified as toxic for the environment, such as benzene with 435 mg L^{-1} or chloroform with 632 mg L^{-1}; however, LA can be considered as nontoxic for the environment, similarly to acetone with EC_{50}=19900 mg L^{-1}, ethanol with EC_{50}=45700 mg L^{-1} or 2-propanol with EC_{50}=28100 mg L^{-1} (Jennings et al. 2001).

Comparison of the levulinate series to other solvents based on the *Daphnia magna* assay revealed that the EC_{50} values for LA and ML are similar to those of traditional solvents such as acetone with 7600 mg L^{-1} (Pawlisz and Peters 1995), ethanol with 12340 mg L^{-1} or dimethylformamide with 14500 mg L^{-1} (Hutchinson et al. 2006) which are all considered non-toxic.

Environmental Risk of levulinates

The general estimation of environmental risk makes use of the ratio between predicted environmental concentration (PEC) and the predicted no-effect concentration (PNEC). Compounds presenting PEC/PNEC ratios lower than 1 are considered to be substances of low environmental risk. Here, PNECs have been calculated from a dataset which includes both our experimental data (table 1) and previous predicted information (table 2). We selected the most sensitive ecotoxicity experimental value in aquatic bioindicators required from the REACH directive (algae, daphnids and fish) (COM (2003) 644 final29.10.2003. Euroepan Comission). For this reason, we did not take into account bacteria, earthworms and fish results. Therefore, the EC_{50} of *Daphnia magna* has been used for the levulinate esters, while for LA, the EC_{50} of *Chlamydomonas reinhardtii* was used. According to the REACH directives a factor of 1000 was applied to the EC_{50} values. The values for PEC that would result in a PEC/PNEC ratio equal or lower than 1 would be1.093 mg L^{-1} for LA, 2.761 mg L^{-1} for ML, 0.982 mg L^{-1} for EL and 0.151 mg L^{-1} for BL.

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Table 1.

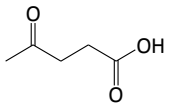
Effective concentration EC_{50} for levulinate family compounds

	Chlamydomonas reinhardtii		*Vibrio fischeri*	*Daphnia magna*	*Eisenia foetida*
	mg L^{-1}		mg L^{-1}	mg L^{-1}	mg kg^{-1}
	EC_{50} *1h*	EC_{50} *2h*			
Levulinic acid	1092.5 ± 26.7	1098.1 ± 13.7	5687±1325	6234±466	3615±68
Methyl levulinate	20720.9 ± 1083.6	18655.8 ± 986.1	883±5	2761±512	620±14
Ethyl levulinate	6889.0 ± 405.3	6065.9 ± 757.8	182±5	982±66	848±24
Butyl levulinate	2151.8 ± 190.1	1640.0 ± 127.0	83±3	151±13	1899±154

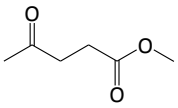
Table 2.

Predicted concentration in mg L^{-1} for levulinate family compounds. (OECD 2006)

	Levulinic acid	Methyl levulinate	Ethyl levulinate	Butyl levulinate
Feather minnow (LC_{50}) as predicted by Topkat v6.1	1500	352	117.3	24.6
Fish (LC_{50}) as predicted by Ecosar v0.99g	189000	296092	179369	64069
Fish (LC_{50}) as predicted by Aster	13613	387.3	180.2	38
Daphnia (EC_{50}) as predicted by Topkat v6.1	330.3			



Levulinic acid



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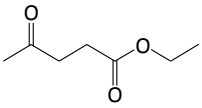

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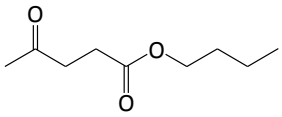
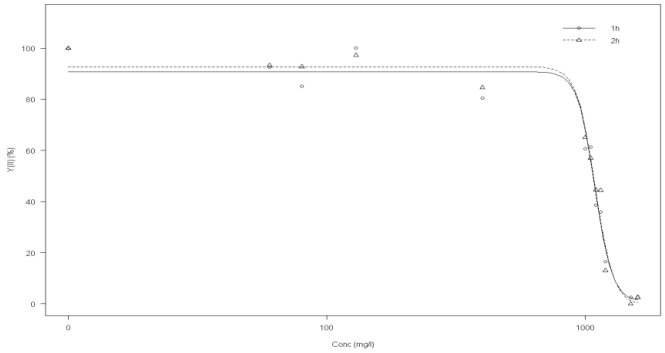
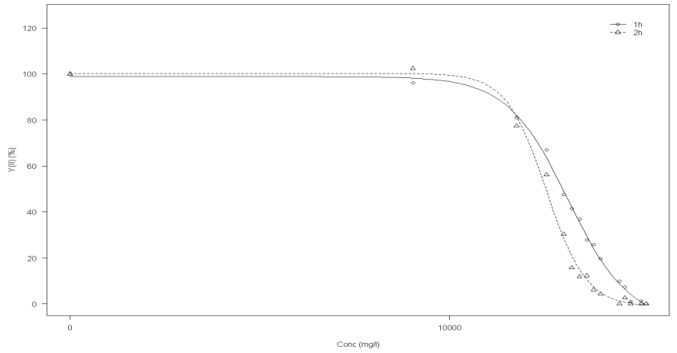


Butyl levulinate

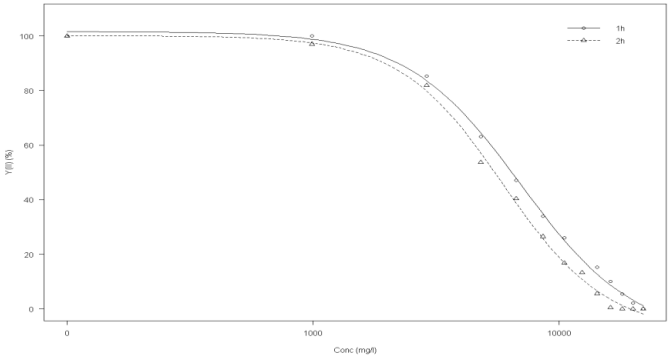
Fig.1. Chemical structures of the compounds studied



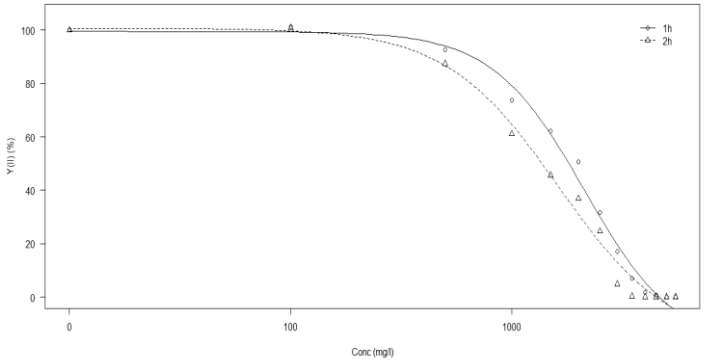
Levulinic acid



Methyl levulinate



Ethyl levulinate



Butyl levulinate

Fig.2. Dose- response curves after 1 and 2 h of exposure for compounds studied for *Chlamydomonas reinhardtii*.

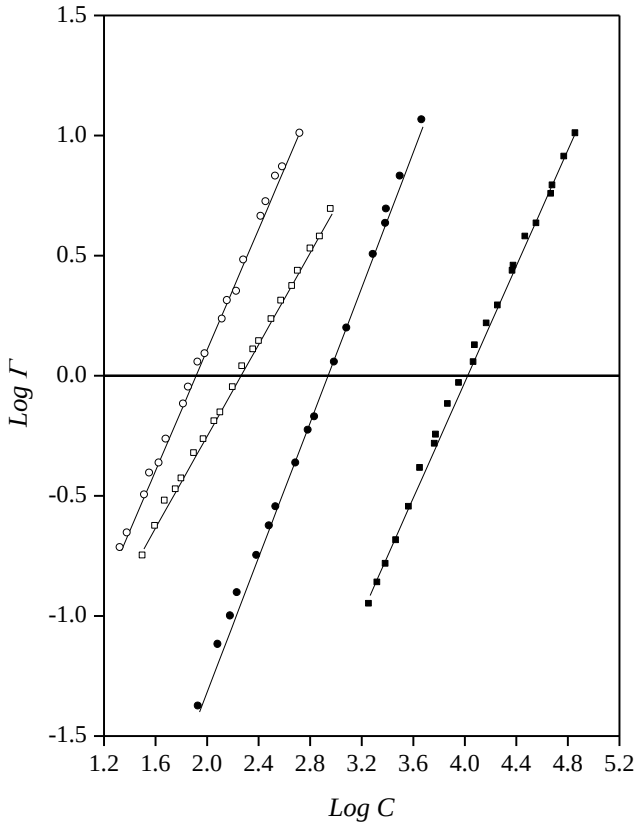


Fig. 3. Dose- response curves for compounds studied for *Vibrio fischeri:* ■ *levulinic acid,* ● *methyl levulinate,* □ *ethyl levulinate,* ○ *butyl levulinate.*

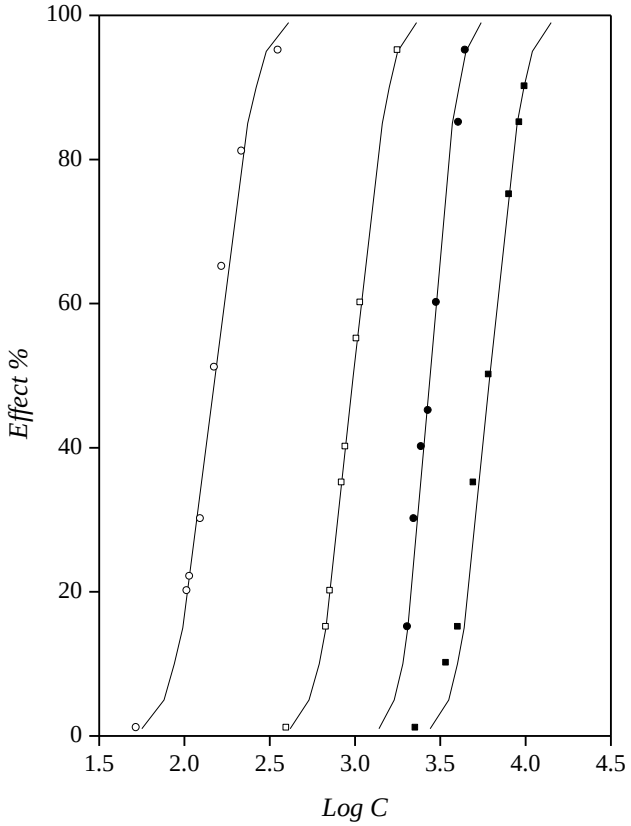


Fig. 4. Dose- response curves for compounds studied for *Daphnia magna:* ■ *levulinic acid,* ● *methyl levulinate,* □ *ethyl levulinate,* ○ *butyl levulinate.*

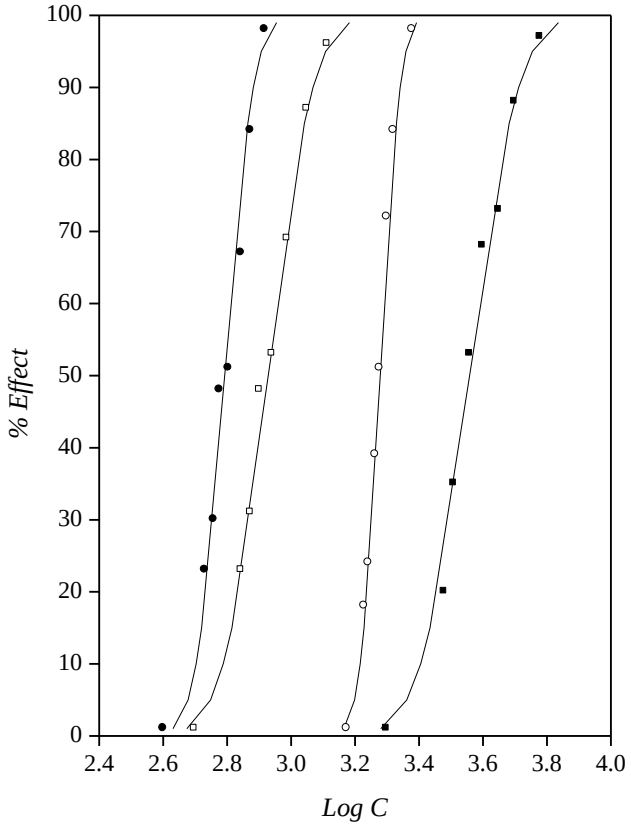


Fig. 5. Dose- response curves for compounds studied for *Eisenia foetida:* ■ *levulinic acid,* ● *methyl levulinate,* □ *ethyl levulinate,* ○ *butyl levulinate*

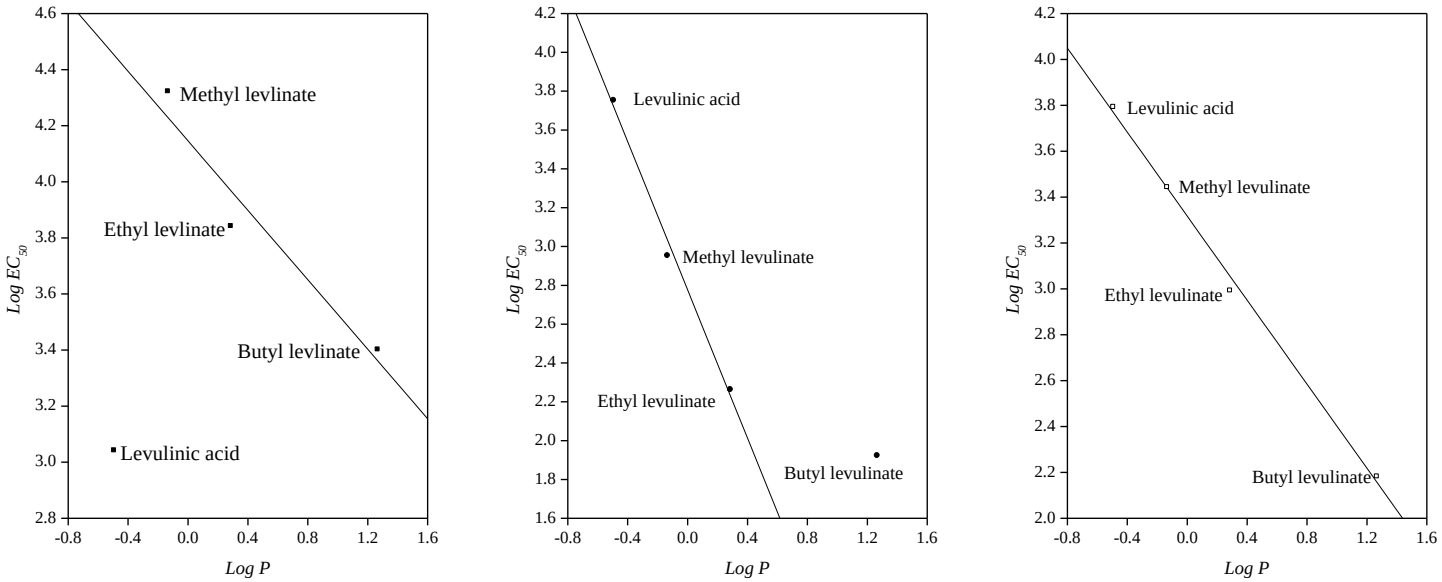


Fig. 6. Plots of acute (Log EC_{50}) toxicity for *Chlamydomonas reinhardtii*, *Vibrio fischeri* and *Daphnia magna* against the logarithm of the octanol-water partition coefficients (*Log P*): ■ *Chlamydomonas reinhardtii*, ● *Vibrio fischeri*, □ *Daphnia magna*.Position of LA in the first plot is mostly due to its high acidity.