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Comparative Rapid Toxicity Screening of Commercial and Potential "Green" Plasticizers Using Bioluminescent Bacteria

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Supporting Information

ABSTRACT: The *Vibrio fischeri* bioluminescence inhibition assay (Microtox) was slightly modified to use an in-house diluent containing 2% DMSO and was successfully applied to perform a rapid toxicity screening of 24 compounds including commercial plasticizers and chemicals currently studied as potential "green" plasticizers. Comparison of the EC_{50} values obtained indicated that 1,3-propanediol dibenzoate (PrDDB), 1,4-butanediol dibenzoate (BDDB), and dihexyl maleate (DHM) might not be good candidates as "green" plasticizers because of their higher toxicity (EC_{50} < 1 mg L^{-1}). Results also indicated that Microtox is a useful technique to better understand the effect of key structural features on the toxicity of plasticizers. Comparison of EC_{50} values of similar compounds having a different alkyl chain lengths indicated a decrease in toxicity of dibenzoate plasticizers with respect to increasing alkyl chain size. The Microtox technique that we adapted to test compounds having low solubility was proven to be useful to evaluate the toxicity of potential "green" plasticizers relative to commercial products. However, these results cannot be used alone to select the best candidates. The Microtox technique is complementary to biodegradation experiments and plasticizing properties tests and allows, at the development stage, the screening of a large number of potential "green" plasticizers on the basis of their relative toxicity.

1. INTRODUCTION

Plasticizers are additives used to improve polymer workability and flexibility.[1] About 50−100 different types of plasticizers are used nowadays commercially, which include phthalates, aliphatic dibasic esters, benzoate esters, and citrates, among many others.[2] In 2006, about 5.8×10^9 kg of plasticizers were used globally for polyvinyl chloride (PVC), and approximately 75% were phthalate plasticizers.[3] Studies have shown that phthalate plasticizers such as diethyl phthalate (DEP), di-*n*-buytl phthalate (DBP), and di(2-ethlylhexyl) phthalate (DEHP) can negatively affect the development of fish ovaries,[4] damage testicular tissue of amphibians,[5] and delay the growth of insects[6] at environmentally relevant concentrations (0.3−100 μg L^{-1}). In humans, exposure to phthalates has been linked to decreased anogenital distance in male infants, lower semen quality, and younger gestational age in newborns.[7] Consequently, the European Union,[8] the United States,[9] and Canada[10] have regulated the concentration of phthalates such as DEHP, dibutyl phthalate (DBP), benzyl butylphthalate (BBP), diisonomyl phthalate (DINP), diisodecyl phthalate (DIDP), and di(*n*-octyl) phthalate (DNOP) in consumer products, especially children toys. For these reasons, there has been increased research activity to develop new "green" plasticizers based on the principles of green chemistry, which aims to design safer chemicals by reducing potential hazards of new substances.[11−13] Our research team has proposed several new substances as replacements for toxic plasticizers.[12,14−16] Important characteristics of these compounds such as plasticizing properties and biodegradation have been evaluated; however, not much is known about their toxicity.

Bioassays are required to measure the toxicity of a compound on a given species given that a biological response is not easily predicted solely on the basis of molecular structure. Since its introduction in 1979, the *Vibrio fischeri* bioluminescence inhibition assay (also known as Microtox) has become a fast and simple approach to assess acute toxicity effects of organic and inorganic compounds.[17] In this bioassay, toxicity is determined by measuring the reduction of light emitted by bacteria exposed to increasing concentrations of the substance of interest. After short exposure times (≤30 min) to a toxicant, reduction of the bioluminescence in the bacterium *V. fischeri* occurs as a result of disruption of cell proteins and/or membrane damage.[18] Several comparative studies have highlighted the benefits of this test such as sensitivity for a broad range of substances, rapidity, low cost, and minimal training and equipment required in comparison to other bioassays.[19,20] Although it is well-known that a multitrophic battery of tests has to be used to evaluate more accurately the toxicity of a substance, it has been shown that results obtained using this bacterial assay correlate well with traditional bioassays based on species such as trout, minnows, and daphnids[17] and can be used to screen a large number of compounds.

However, the Microtox assay is limited by the water solubility of the test substance.[21] For this reason, addition of a cosolvent is necessary to broaden the application of this bioassay to include hydrophobic substances. Water-miscible organic solvents such as methanol, ethanol, and DMSO have been previously used to increase the test substance solubility. The Microtox manufacturer recommends using organic solvent at levels no higher than 1%;[22] however, higher amounts up to 10% have been reported.[23] Calleja and Persoone[24] showed that

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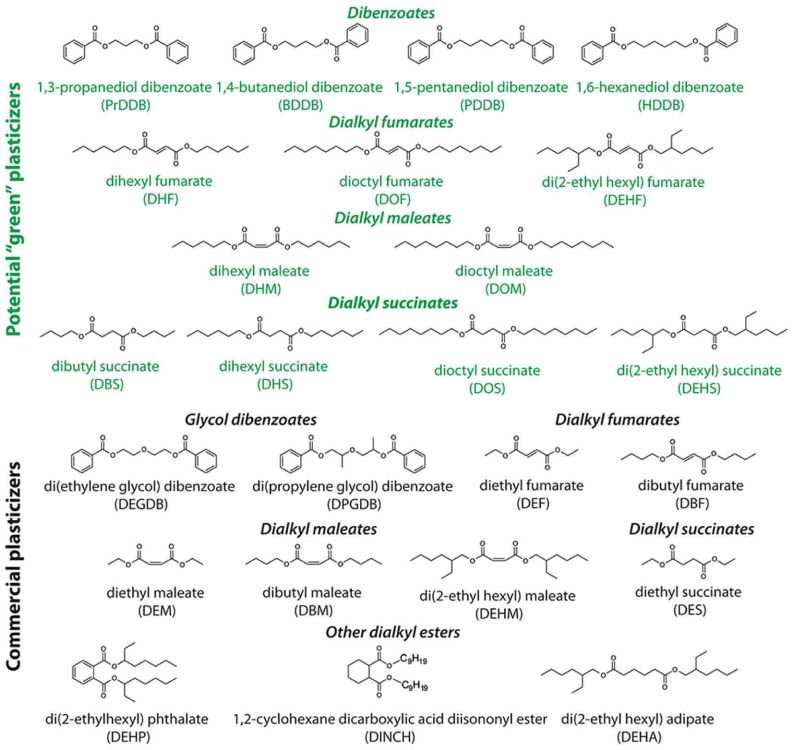

Figure 1. Molecular structure, name, and acronym of the 24 plasticizers selected for this study.

an organic cosolvent can significantly modify the effect of a test substance on the exposed organism. According to these authors, this may be due to an enhanced bioavailability, which allows the test substance to more easily reach the biological membrane.

The goal of this study was to perform a rapid toxicity screening of 11 commercial and 13 potential "green" plasticizers with a modified *V. fischeri* bioluminescence inhibition test using a diluent containing 2% v/v DMSO. The working hypothesis is that the Microtox assay will provide valuable information about the relationship that exists between the molecular structural features of the target plasticizers and the toxic response observed in *V. fischeri*, which in turn will lead to better designed plasticizers.

2. MATERIALS AND METHODS

2.1. Reagents and Materials. Solvents used for both standard and sample preparation were of high purity (>99%). Dimethyl sulfoxide (DMSO) and phenol (99.9% pure) were purchased from Sigma-Aldrich, ethyl acetate (EtOAc) from Fisher Canada, and LCMS grade water from J.T. Baker. In total, 24 plasticizers (purity >99%) were chosen for this study. Structures of these plasticizers are presented in Figure 1, while CAS registry number and provenance of these plasticizers are presented in Table S1 (Supporting Information). Lyophilized luminescent bacteria *V. fischeri* strain NRRL-B-11177 (Microtox Reagent), Microtox Osmotic Adjustment Solution (MOAS), and 12 × 50 mm glass cuvettes were purchased from Polycontrols (Brossard, QC).

2.2. Sample Preparation. Stock solutions at 50 mM of each plasticizer were prepared in 100% DMSO with the exception of DBF, DOF, DEHF, DINCH, and DEHA, which required EtOAc for proper dissolution (80%, 10%, 10%, 30%, and 10% v/v, respectively). Working solutions were prepared for all of the plasticizers (except DEF, DEM, and DES for which the stock solution was used undiluted) by diluting the stock solutions in 100% DMSO. The dilution factor was chosen according to both the turbidimetric solubility and the toxicity (determined by preliminary Microtox assays) of each plasticizer. Therefore, concentrations of the working solutions ranged from 50 to 5000 μM. A solution of phenol (3500 mg L^{-1}) in 100% DMSO was prepared for use as the positive control.

2.3. Toxicity Assay. The bioluminescence inhibition assay used in the experiment was based on a protocol obtained from Environment Canada.[25] This procedure was followed without modification for both the bacteria reconstitution and the luminescence reading. However, the method was slightly modified for the preparation of the dose–response solutions to account for the low solubility of the compounds tested. To increase the solubility of the plasticizers, an in-house diluent solution was prepared containing 2% v/v DMSO and 2% w/v NaCl in H_2O (necessary to avoid cell lysis due to osmotic pressure[26]). DMSO has been shown to provide sufficient solubilization of the substances of interest while having low toxicity toward the bacteria (Table S2, Supporting Information). Briefly, tests were performed using a model 500 analyzer manufactured by SDIX (Newark, DE) using four controls, which were three blanks (2% v/v DMSO and 2% w/v NaCl in H_2O) and one positive control (33.6 mg L^{-1} phenol and 2% v/v DMSO and 2% w/v NaCl in H_2O), and six concentrations of each plasticizer, which were prepared by 2-fold serial dilutions of the working solutions. This preparation procedure ensured that all dose–response solutions contained 2% v/v DMSO and 2% w/v NaCl. A homogeneous composition throughout the assay is of major importance because the DMSO content must be equal in all test solutions. In this way, the small bioluminescence inhibition due to the toxicity of the DMSO

Table 1. Plasticizers Selected for This Study

plasticizer	turbidimetric solubility[b] (μM)	EC_{20} (mg L^{-1}) (CI 95%)	EC_{20} (μM) (CI 95%)	EC_{50} (mg L^{-1}) (CI 95%)	EC_{50} (μM) (CI 95%)
		Potential "Green" Plasticizers			
PrDDB	<54.7−109	0.00936 (0.00837−0.0105)	0.0329 (0.0294−0.0368)	0.031 (0.029−0.034)	0.109 (0.100−0.119)
BDDB	<54.7	0.151 (0.144−0.158)	0.506 (0.483−0.530)	0.546 (0.523−0.569)	1.83 (1.75−1.91)
PDDB	<10.3−51.4	0.698 (0.568−0.857)	2.23 (1.82−2.74)	4.04[c] (2.66−6.12)	12.9[c] (8.5−19.6)
HDDB	<11.8	0.276 (0.12−0.358)	0.845 (0.651−1.10)	2.14[c] (1.30−3.51)	6.54[c] (3.98−10.8)
DHF	<20.2−33.6	3.15 (1.92−5.17)	11.1 (6.74−18.2)	149[c] (33.2−664)	523[c] (117−2340)
DOF	<12.2	>2.08	>6.12	>2.08	>6.12
DEHF	<62.0	>10.5	>31.0	>10.5	>31.0
DHM	<20.9−34.9	0.137 (0.102−0.185)	0.483 (0.360−0.649)	0.657 (0.539−0.801)	2.31 (1.90−2.82)
DOM	<51.2	>8.72	>25.6	>8.72	>25.6
DBS	<558	0.471 (0.435−0.511)	1.66 (1.53−1.80)	1.89 (1.69−2.12)	6.65 (5.94−7.45)
DHS	<34.3−51.8	0.294 (0.250−0.347)	1.04 (0.879−1.22)	1.24 (1.03−1.50)	4.36 (3.61−5.27)
DOS	<53.8	7.05 (4.55−10.9)	24.8 (16.0−38.4)	55.3[c] (19.0−161)	194[c] (66.9−565)
DEHS	<11.9−59.7	>10.2	>29.9	>10.2	>29.9
		Commercial Plasticizers			
DEGDB	<103−514	1.07 (0.974−1.17)	3.39 (3.10−3.72)	4.39 (4.06−4.74)	14.0 (12.9−15.1)
DPGDB[a]	<53.6	2.46 (2.13−2.84)	7.19 (6.23−8.29)	10.6[c] (8.19−13.7)	31.0[c] (23.9−40.1)
DEF	>1170	9.94 (9.44−10.5)	34.9 (33.2−36.8)	37.1 (35.6−38.5)	130 (125−136)
DBF	<54.0−107.9	0.149 (0.141−0.159)	0.53 (0.49−0.56)	0.585 (0.561−0.610)	2.06 (1.97−2.140
DEM	>1060	46.1 (42.4−50.1)	162 (149−176)	170[c] (146−198)	599[c] (514−697)
DBM	<500.0	0.388 (0.310−0.368)	1.19 (1.09−1.29)	1.39 (1.26−1.53)	4.90 (4.45−5.39)
DEHM	<50.8	>8.64	>25.4	>8.64	>25.4
DES	>1230	0.137 (0.102−0.185)	267 (232−307)	367[c] (270−500)	1290[c] (949−1760)
DEHP	<10.4−52.1	>10.2	>26.0	>10.2	>26.0
DINCH[a]	<10.8−53.9	>11.4	>27.0	>11.4	>27.0
DEHA	<11.0	>2.03	>5.49	>2.03	>5.49

[a]Mixture of isomers. [b]Turbidimetric solubility was defined as the concentration at which the median absorbance (n = 3) was three times higher than the interquartile range of the absorbance of the blank (2% v/v DMSO + 2% w/v NaCl) (n = 12). [c]Extrapolated value.

cancels out, and only the inhibition due to the plasticizer is calculated. Light output readings at 5 min of exposure were preferred over readings at 15 or 30 min of exposure because of the advantage of being able to run more samples per Microtox Reagent vial. As a result, the measured EC_{50} values obtained when using a cosolvent might not represent the real environmental toxicity of these compounds. Nonetheless, this approach is useful as a comparative tool considering substances are tested using the same experimental parameters, in our case, 2% v/v DMSO and 5 min of exposure. All tests were performed in triplicate except for the substances for which no bioluminescence inhibition could be observed; in that case, duplicate measurements were considered sufficient.

2.4. Data Analysis and Quality Control. Equations used to calculate EC_{50} values and their confidence intervals are described in the Supporting Information. Phenol was used as the positive control (reference toxicant) to evaluate the viability of the bacteria during each test. A target value was calculated using the effect of the positive control for the first 20 Microtox tests. Subsequent tests in which the effect of the positive control was higher or lower than the calculated target value ±2 standard error were repeated. The phenol control results can be found in the Supporting Information (Figure S1). Data correction for turbidity was not necessary because all experiments were carried out using test solutions below their turbidimetric solubility reported in Table 1. For the evaluation of the potential "green" plasticizers, compounds with EC_{50} values <1 mg L^{-1} were considered not apt as replacement plasticizers, those with EC_{50} values >1−10 mg L^{-1} were considered as good replacements, and those with EC_{50} > 10 mg L^{-1} were considered as excellent replacements. This arbitrary scale is based on European legislation (Directive 93/21/EEC).[27]

3. RESULTS AND DISCUSSION

3.1. Comparison of Potential "Green" and Commercial Plasticizers. Results of bioluminescence inhibition assays are shown in Table 1. Comparison of the EC_{50} values of the commercial and the potential "green" plasticizers shows that PrDDB, BDDB, and DHM (EC_{50} < 1 mg L^{-1}) are about 1−4 orders of magnitude more potent than the commercial plasticizers in the inhibition of the bioluminiscence of *V. fischeri*. Interestingly, none of the di(2-ethyl hexyl) compounds (DEHF, DEHM, DEHS, DEHP, and DEHA) inhibited the luminescence of the bacteria. The lack of toxicity of DEHP toward *V. fischeri* is in agreement with previous Microtox results for that compound.[28] Studies performed on the toxicity of DEHP in other species such as fish and invertebrates showed that this plasticizer was unable to elicit acute toxic effects[29] and its toxicity toward aquatic organisms was observed rather in chronic tests, usually acting as an endocrine disruptor.[30] The only commercial plasticizer with an EC_{50} < 1 mg L^{-1} is DBF. Thus, strictly from a toxicity point of view and according to the arbitrary scale defined in section 2.4, all of the potential "green" plasticizers tested, except PrDDB, BDDB and DHM, should be considered as good or excellent replacements. However, studies on the commercial di(2-ethyl hexyl) plasticizers DEHP and DEHA have shown that these types of compounds form stable metabolites when biodegraded.[16,31] In addition, plasticizing properties studies have demonstrated that DEHF[16] and short

dialkyl succinates such as DES and DBS[12] are not efficient plasticizers in comparison to commercial compounds on a mass percent basis (these properties have yet to be determined for the other candidates). These findings indicate that the 2% DMSO Microtox assay should not be used alone to determine the best candidate plasticizers, but rather as a complementary technique to biodegradation studies and plasticizing properties tests.

3.2. Relationship between Structure and Toxicity. Data shown in Table 1 indicate an inverse relationship between the number of C atoms in the alkyl chain and the bioluminescence inhibition for the dibenzoate plasticizers (PrDDB, BDDB, PDDB, and HDDB). That is, toxicity decreases (i.e., higher EC_{50} values) as a function of increasing number of C atoms in the alkyl chain. Table 1 also shows that PDDB and HDDB elicit toxicities similar to those observed for DEGDB and DPGDB. Therefore, toxicity data suggest that the presence of the ether functional group in these glycol dibenzoates does not have a significant impact on the luminescence inhibition of *V. fischeri*. A different trend for the dialkyl esters (fumarates, maleates, and succinates) from that observed for the dibenzoates is shown in Table 1. Toxicity appears to reach a maximum value at a specific number of C atoms in the alkyl chains (which number depends on the dialkyl ester class), and for either lower or higher number of C atoms in the alkyl chains a decrease or disappearance in toxicity is observed. To summarize the results, for the fumarates, maximum toxicity is observed for DBF (4 C atoms in alkyl chain, EC_{50} = 0.585 mg L^{-1}); while for the maleates, DHM is the most toxic compound (6 C atoms, EC_{50} = 0.657 mg L^{-1}); and for the succinates, DHS is the most toxic compound (6 C atoms, EC_{50} = 1.24 mg L^{-1}). Compounds with 8 C atoms in the alkyl chains [dioctyl and di(2-ethyl hexyl)] are either weakly (DOS EC_{50} = 55.3 mg L^{-1}) or nontoxic (i.e., no EC_{50} could be measured) toward *V. fischeri*.

It has been widely observed that the toxicity of organic compounds toward aquatic species is directly proportional to the octanol–water partition coefficient (K_{ow}), which is a measure of hydrophobicity. Therefore, compounds with more C atoms in their alkyl chains chains should be more toxic because hydrophobicity increases with the number of saturated C atoms on the chain.[32] However, results in which the biological activity of a homologous series of substances increases and then decreases or disappears with increasing number of C atoms have also been previously reported. This phenomenon, which is called the "cutoff effect", was observed for diverse compounds such as *n*-alkanols in rat spinal ganglion neurons[33] and in both *V. fischeri* and submitochondrial particles,[34] *n*-alkanes in squid giant axons and frog sciatic nerves,[35] *N*,*N*-dimethylalkylamine oxides in *Staphylococcus aureus* and *Escherichia coli*,[36] and ester derivatives of gallic acid in L-1210 leukemia cells.[37] Several hypotheses have been put forward to explain the cutoff effect as a function of number of C atoms in the alkyl chain.[38] Although there is no agreement on the specific molecular mechanism behind this phenomenon, current theories point toward direct[39,40] or indirect[41] disruption of membrane proteins. Nonetheless, these theories rely on water solubility, which limits the amount of a substance that can interact with the target sites.

While the results presented here do not suggest which particular molecular mechanism is responsible for the observed cutoff, experimental data could not be interpreted only as the consequence of low solubility. According to the turbidimetric solubility measurements, some of the most toxic dialkyl esters such as DHM and DHS also have low turbidimetric solubilities (<20–50 μM), which are at the same order of magnitude as all of the plasticizers for which no effect was observed (DOF, DEHF, DOM, DEHM, DEHS, DEHP, DINCH, and DEHA). Therefore, an insufficient amount of plasticizer in solution to interact with the bacterial membrane cannot explain completely the observed results. Another factor, which may be either a direct or an indirect interaction with a membrane protein as predicted by current protein and lipid theories, could be responsible for the drastic diminution of toxicity observed such as in the case of DHM and DOM. Further study of this interaction is, however, outside the scope of the present work.

4. CONCLUSIONS

The main goal of the application of the Microtox test to the design of new plasticizers is to rapidly screen which compounds are better suited for further development and studies. Only on the basis of toxicity toward *V. fischeri*, all of the potential "green" plasticizers considered, except the dibenzoates PrDDB and BDDB and the dialky ester DHM, seem to be good or excellent candidates. However, previous studies on biodegradation have shown that di(2-ethyl hexyl) compounds degrade into toxic metabolites. Also, investigation on the plasticizing properties of succinate dialkyl esters has shown that short alkyl chain analogues are not efficient plasticizers in a mass percent basis. Therefore, the Microtox assay results are complementary to studies on biodegradation and plasticizing properties to help in choosing the best potential "green" plasticizers at the development stage.

The working hypothesis formulated in the Introduction suggesting that the Microtox assay will provide valuable information about the relationship that exists between structure and the toxic response observed in *V. fischeri* was partially validated. Experimental data did not suggest a clear trend due to structural differences in the central group between the different plasticizers. This could be explained by the nonspecific nature of the mode of toxic action of these compounds to *V. fischeri*. However, the Microtox assay was successful in identifying significant differences due to another key structural feature in plasticizer development, the alkyl chain size. Maximum toxicity among the classes of plasticizers tested was observed for the compounds having 3 C atoms (dibenzoates), 4 C atoms (fumarates), and 6 atoms (maleates and succinates). Compounds with smaller or larger chains were less toxic or nontoxic to the bacteria.

Finally, it is essential to measure the chronic effect of these new substances on higher organisms and perform assays using cell cultures and biomarkers to investigate more specific effects such as endocrine disruption and mutagenicity to fully assess their potential environmental and health impact prior to commercialization.

■ ASSOCIATED CONTENT

Supporting Information

Determination of EC_{50} values. Turbidimetric solubility. Results of the turbidimetric analysis of the solubility. Tables S1 and S2. Figures S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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