Comparative rapid toxicity screening of commercial and potential 'green' plasticizers using bioluminescent bacteria

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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 toxicity of plasticizers. Comparison of EC_{50} of similar compounds having a different alkyl chain length indicated a decrease in toxicity of dibenzoate plasticizers with respect to an increasing alkyl chain size. The Microtox technique that we adapted to testing of compounds having low solubility was proven to be useful to evaluate the toxicity of potential plasticizers relative to

commercial products. However, these results cannot be used alone to single out the best candidate. The Microtox technique is complementary to biodegradation experiments and plasticizing properties tests and allow, at the development stage, the screening of a large number of potential plasticizers based on their relative toxicity.

Keywords: Microtox, *Vibrio fisheri*, 'green' plasticizer, dibenzoate, glycol dibenzoate, fumarate, maleate, succinate, phthalate

1. Introduction

Plasticizers are additives used to improve polymer workability and flexibility [1]. About 50 to 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 to 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], 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 replacement 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 based solely on 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 have 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 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 co-solvent 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 an organic co-solvent 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 eleven commercial and thirteen 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 with 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. 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 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 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 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. In order to increase the solubility of the plasticizers, an in-house diluent solution was prepared containing 2% v/v DMSO. This solvent has shown to provide sufficient solubilization of the substances of interest while having low toxicity towards the bacteria (Table S2, Supporting Information) and 2% w/v NaCl in H_2O (necessary to avoid cell lysis due to osmotic pressure [26]). Briefly, tests were performed using a Model 500 Analyzer manufactured by SDIX (Newark, DE) using 4 controls, which were 3 blanks (2% v/v DMSO and 2% w/v NaCl in H_2O) and 1 positive control (33.6 mg L^{-1} phenol and 2% w/v NaCl in

H_2O), and 6 concentrations of each plasticizer, which were prepared by two-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 homogenous composition throughout the assay is of major importance since the DMSO content must be equal in all test solutions. In this way, the small bioluminescence inhibition due to the toxicity of the DMSO cancels out and only the inhibition due to the plasticizer is observed. 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 reagent. As a result, the measured EC_{50} values obtained when using a co-solvent 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 since all experiments were carried out using test solutions below their turbidimetric solubility reported in Table 1. For the evaluation of the potential 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} 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 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 show that PrDDB, BDDB and DHM (EC_{50} < 1 mg L^{-1}) are about 1 to 4 orders of magnitude more potent than the commercial plasticizers to inhibit 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 towards *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 EC_{50} < 1 mg L^{-1} is DBF. Thus, strictly from a toxicity point of view and the arbitrary scale defined in section 2.4, all the potential plasticizers tested, except PrDDB and BDDB, 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 type compounds also 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 compared 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

discriminate the best candidate plasticizers but rather as a complementary technique to biodegradation studies and plasticizing properties.

3.1. 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 similar toxicities to those observed for DEGDB and DPGDB. Therefore, toxicity data suggests 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) than the one 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 non-toxic (*i.e.* no EC_{50} could be measured) towards *V. fischeri*.

178 It has been widely observed that the toxicity of organic compounds towards aquatic species is directly
179 proportional to the octanol-water partition coefficient (K_{ow}), which is a measure of hydrophobicity.
180 Therefore, compounds with more C atoms in their alkyl chains chains should be more toxic because
181 hydrophobicity increases with the number of saturated C atoms on the chain [32]. However, results in
182 which the biological activity of a homologous series of substances increases and then decreases or
183 disappears with increasing number of C atoms have also been previously reported. This phenomenon,
184 which is called the "cutoff effect", was observed for diverse compounds such as *n*-alkanols in rat spinal
185 ganglion neurons[33] and in both *V. fischeri* and submitochondrial particles [34], *n*-alkanes in squid giant
186 axons and frog sciatic nerves [35], *N,N*-dimethylalkylamine oxides in *Staphylococcus aureus* and
187 *Escherichia coli* [36] and ester derivatives of gallic acid in L-1210 leukemia cells [37]. Several hypothesis
188 have been put forward to explain the cutoff effect as a function of number of C atoms in the alkyl chain
189 [38]. Although there is no agreement on the specific molecular mechanism behind this phenomenon,
190 current theories point towards direct [39, 40] or indirect [41] disruption of membrane proteins. Nonetheless,
191 these theories rely on water solubility, which limits the amount of a substance that can interact with the
192 target sites.

193

194 While results presented here do not suggest which particular molecular mechanism is responsible for the
195 observed cut-off, experimental data could not be interpreted only as the consequence of low solubility.
196 According to the turbidimetric solubility measurements, some of the most toxic dialkyl esters such as
197 DHM and DHS also have low turbidimetric solubilities (< 20-50 μM), which are at the same order of
198 magnitude as all the plasticizers for which no effect was observed (DOF, DEHF, DOM, DEHM, DEHS,
199 DEHP, DINCH and DEHA). Therefore, an insufficient amount of plasticizer in solution to interact with
200 the bacterial membrane cannot explain completely the observed results. Another factor, which may be
201 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. Based only on its toxicity towards *V. fischeri*, all the potential plasticizers considered, except the dibenzoates PrDDB and BDDB and the dialky ester DHM, seem to 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 section 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 non-specific 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 non-toxic 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.

4. Acknowledgments

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5. Associated content

Supporting Information

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

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Tables

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* (2.66-6.12)	12.9* (8.5-19.6)
HDDB	< 11.8	0.276 (0.12-0.358)	0.845 (0.651-1.10)	2.14* (1.30-3.51)	6.54* (3.98-10.8)
DHF	< 20.2-33.6	3.15 (1.92-5.17)	11.1 (6.74-18.2)	149* (33.2-664)	523* (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* (19.0-161)	194* (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* (8.19-13.7)	31.0* (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* (146-198)	599* (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* (270-500)	1290* (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 3 times higher than the interquartile range of the absorbance of the blank (2% v/v DMSO + 2% w/v NaCl) (n = 12).* Extrapolated value.

Comparative rapid toxicity screening of commercial and potential 'green' plasticizers using bioluminescent bacteria

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Supporting Information

1. Supporting Information

1.1. Determination of EC_{50} values

Gamma values (Γ) were calculated for each dose-response solution according to equation A.1:

$$\Gamma = \left(\frac{R_5 I_0}{I_5}\right) - 1 \quad \text{(A.1)}$$

where R_5, the blank correction factor, is equal to the ratio of average blank emission after and before 5 min of exposure, I_0 is the test solution light emission before exposure and I_5 the light emission after 5 min of plasticizer exposure. Linear least squares regression of the logarithm of the concentration as a function of the logarithm of Γ was used to calculate the median effective concentrations (EC_{50}). The lower and upper 95% confidence intervals (LCI95% and UCI95%, respectively) were calculated using the fitted values equation [1] according to the formulas used by the manufacturer:

$$\mathrm{LCI}_{95\%} = 10^{(logEC50 - t \cdot PF)} \quad \text{(A.2)}$$

$$\mathrm{UCI}_{95\%} = 10^{(logEC50 + t \cdot PF)} \quad \text{(A.3)}$$

where t is Student's t value with α=0.05 with n-2 degrees of freedom, and PF is the confidence pre-factor, which is given by:

$$\mathrm{PF} = se_{reg}\sqrt{\frac{1}{n} + \frac{(log\Gamma_{EC50} - log\bar{\Gamma})^2}{\sum(log\Gamma_i - log\bar{\Gamma})^2}}$$

where se_{reg} is the standard error of the regression, n is the number of data pairs, $log\ \Gamma_{EC50}$ is the logarithm of gamma corresponding to the EC_{50} and $log\ \bar{\Gamma}$ is the mean of $log\ \Gamma_i$ values.

1.2. Turbidimetric solubility

Turbidimetric solubility of each plasticizer in 2% v/v DMSO and 2% w/v NaCl in H_2O was determined in order to the set the maximum concentration to be tested in the Microtox assay. Turbidity is the result of light scattering caused by suspended particles in solution [2]. Therefore, light absorbance measurements of samples containing increasing amounts of a substance can be used to indicate the concentration at which the studied compound is no longer soluble. This solubility threshold is indicated by the increase in absorbance caused by light scattering, since the solubilized compounds absorb light weakly in the 600-800 nm range. This approach has been previously developed for the rapid solubility screening of new compounds in drug discovery and development settings [3, 4]. The solubility determined by this method is also called "kinetic solubility" because it is not obtained at equilibrium conditions (thermodynamic solubility), and the formation of a precipitate is kinetically driven [5].

Since it was observed that some plasticizers formed turbid solutions in 2% w/v NaCl in H_2O; a method for the measurement of the turbidimetric solubility based on procedures reported in literature was applied [3, 4]. The absorbance of a series of aqueous solutions containing 2% v/v DMSO and 2% w/v NaCl was measured at different concentrations of the target plasticizer using a Benchmark Plus microplate spectrophotometer (Bio-Rad, Hercules, CA). Seven working solutions of each plasticizer, typically between 50 mM and 0.1 µM, were prepared in DMSO by serial dilution from stock solutions. The test solutions were prepared in Corning Costar 3596 polystyrene 96-well microplates (Corning, NY) first by adding 245 µL of 2% w/v NaCl in H_2O to each well and then a volume of 5 µL of each working solution in triplicate. Before measuring the absorbance at 800 nm and 620 nm, microplates were mixed for 60 s at room temperature (23 ± 2 °C). Turbidimetric solubility was defined as the concentration at

which the median absorbance (n = 3) was 3 times higher than the interquartile range of the absorbance of the blank (2% v/v DMSO and 2% w/v NaCl in H_2O) (n = 12).

1.3. Results of the turbidimetric analysis of the solubility

Turbidimetric solubility measurements were essential to determine the maximum concentration that could be used in the bioassay given that the preparation of dose-response solutions above the solubility limit would lead to heterogenous solutions and EC_{50} values would not be accurate. Furthermore, the main advantage of using kinetic over thermodynamic solubility for the Microtox assay is that equilibrium conditions are not necessarily reached in the time scale of the bioassay and thus kinetic solubility should be a better measure of the actual amount of substance that is readily available to the bacteria.

Results of the experiments determined at 800 nm are shown in Table 2. This wavelength was chosen to determine the turbidimetric solubility instead of 620 nm, which is commonly used to measure turbidity, because it provided more consistent results. These data demonstrate that, as expected, solubility decreases with increasing number of C atoms in the alkyl chains for the dibenzoates, the glycol dibenzoates and the dialkyl esters.This relationship is observed because plasticizers with longer or bulkier hydrocarbon chains are less hydrated in solution and more likely to aggregate in order to reduce unfavorable solvent-solute interactions [6].

For the dibenzoates, turbidimetric solubility varied only slightly as the number of C atoms in the alkyl chain increased. For example, PrDDB, which contains 3 C atoms in its alkyl chain, and HDDB (6 C

atoms), had turbidimetric solubility values of < 52-105 and < 11 μM, respectively. The two studied glycol dibenzoates, DEGDB and DPGDB, were more soluble than PDDB and HDDB (dibenzoates of similar molecular size), which could be explained by the presence of the ether functional group in their structures. As for the dialkyl esters (fumarates, maleates and succinates), diethyl compounds were very soluble (> 1000 μM), but solubility rapidly decreased to about < 10-50 μM for the alkyl chains with higher number of C atoms. Therefore the differences observed on the relationship between the number of C atoms in the alkyl chains and the solubility of dibenzoates and alkyl esters could be explained by the location and the number of the alkyl chains in the molecular structure of these compounds: for the former there is a single alkyl chain between the benzoate groups as opposed to two side chains in the case of the latter.

1.4. Additional tables

Table S1. Plasticizers selected for this study.

Plasticizer	CAS RN	Purity [a] (%)	Provenance
Potential 'green' plasticizers			
PrDDB	2451-86-7	≥99	Synthesized in-house
BDDB	19224-27-2	≥99	Synthesized in-house
PDDB	6624-73-3	≥99	Synthesized in-house
HDDB	22915-73-7	≥99	Synthesized in-house
DHF	19139-31-2	≥99	Synthesized in-house
DOF	2997-85-5	≥99	Synthesized in-house
DEHF	141-02-6	≥99	Synthesized in-house
DHM	16064-83-8	≥99	Synthesized in-house
DOM	2915-53-9	≥99	Synthesized in-house
DEHM	142-16-5	≥99	Synthesized in-house
DBS	141-03-7	≥99	Synthesized in-house
DHS	15805-75-1	≥99	Synthesized in-house
DOS	14491-66-8	≥99	Synthesized in-house
DEHS	2915-57-3	≥99	Synthesized in-house
Commercial plasticizers			
DEGDB	120-55-8	96.0	Sigma-Aldrich
DPGDB	27138-31-4	99.6 [b]	Sigma-Aldrich
DEF	623-91-6	99.6	Sigma-Aldrich
DBF	105-75-9	100.0	Scientific Polymer Products

DEM	141-05-9	98.2	Sigma-Aldrich
DBM	105-76-0	96.0	Acros Canada
DES	123-25-1	99.9	Sigma-Aldrich
DEHP	117-81-7	99.7	Sigma-Aldrich
DINCH	166412-78-8	≥99.5 [b]	BASF Canada
DEHA	103-23-1	99.6	Sigma-Aldrich

[a] Purity was determined by NMR. The impurities would be mostly a mix of the reagents that were used in the synthesis (small acids, aliphatic alcohols). However these substances would be in trace amounts that together amount for less than 1% of the total mass of the plasticizer.

[b] Determined as ester content by the manufacturer.

Table S2. Toxicity of the co-solvent and the control in different conditions

Substance	EC_{20}[a] (CI 95%)	EC_{50}[a] (CI 95%)
DMSO	3.02 (2.41-3.79)	8.34 (6.78-10.24)
Phenol (2% NaCl)	8.02 (5.90-10.90)	34.6 (28.9-41.4)
Phenol (2% NaCl + 2% DMSO)	7.08 (6.67-7.50)	29.9 (28.7-31.1)

[a] Units for DMSO are in % (v/v) and for phenol in mg L^{-1}

1.5. Additional figures

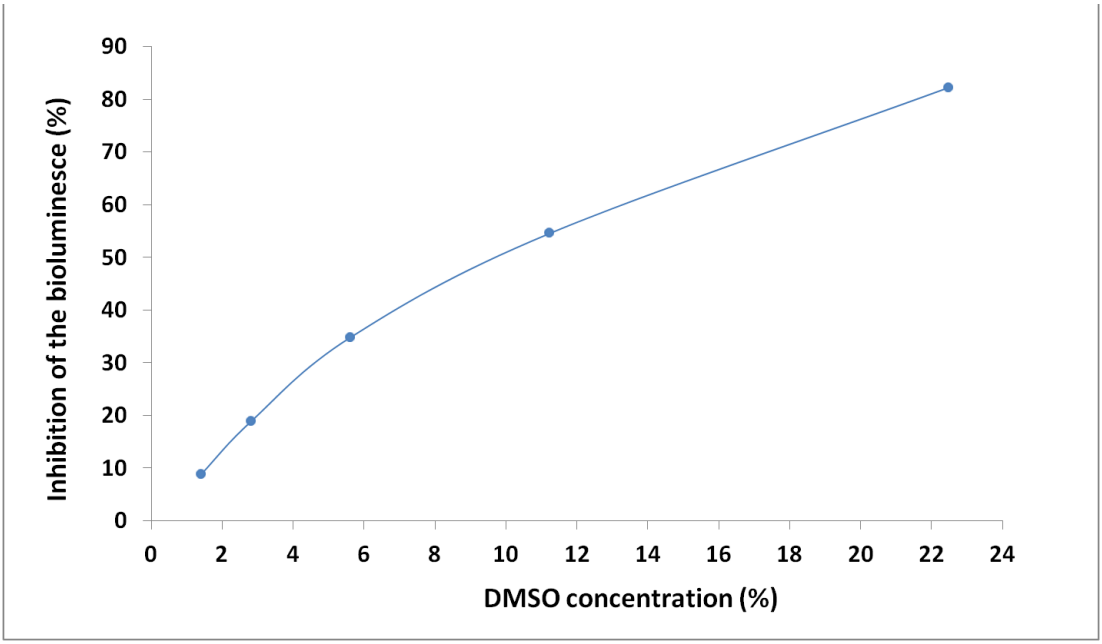

Figure S1. Dose-response curve of DMSO in the 5 min Microtox test.

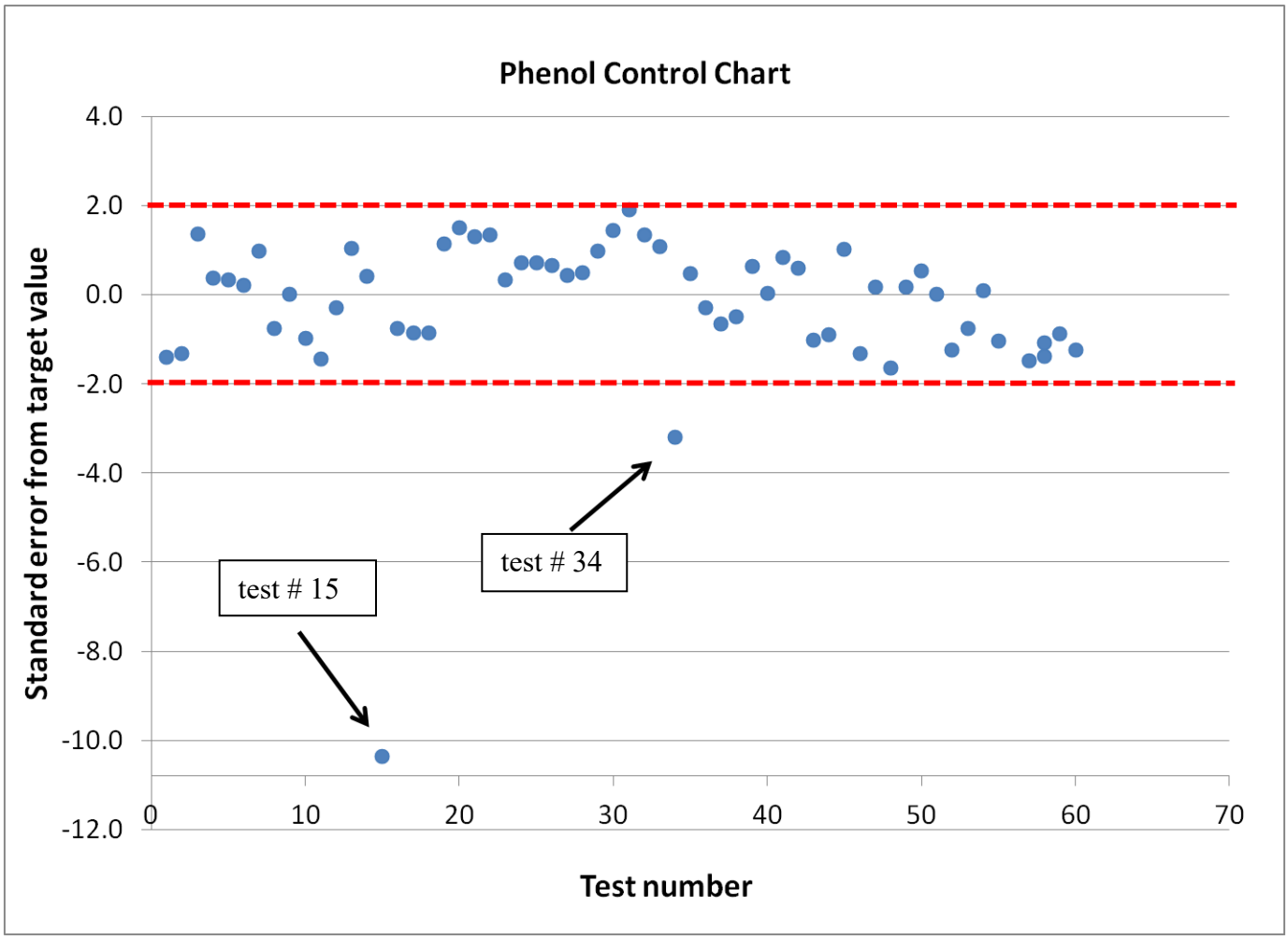

Figure S2. Control chart of phenol used to evaluate the viability bacteria throughout the experiments. Tests number 15 and 34 were repeated since the phenol value was outside of the variation range (± 2 standard errors).

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