

In vitro inhalation bioaccessibility of phthalate esters and alternative plasticisers present in indoor dust using artificial lung fluids

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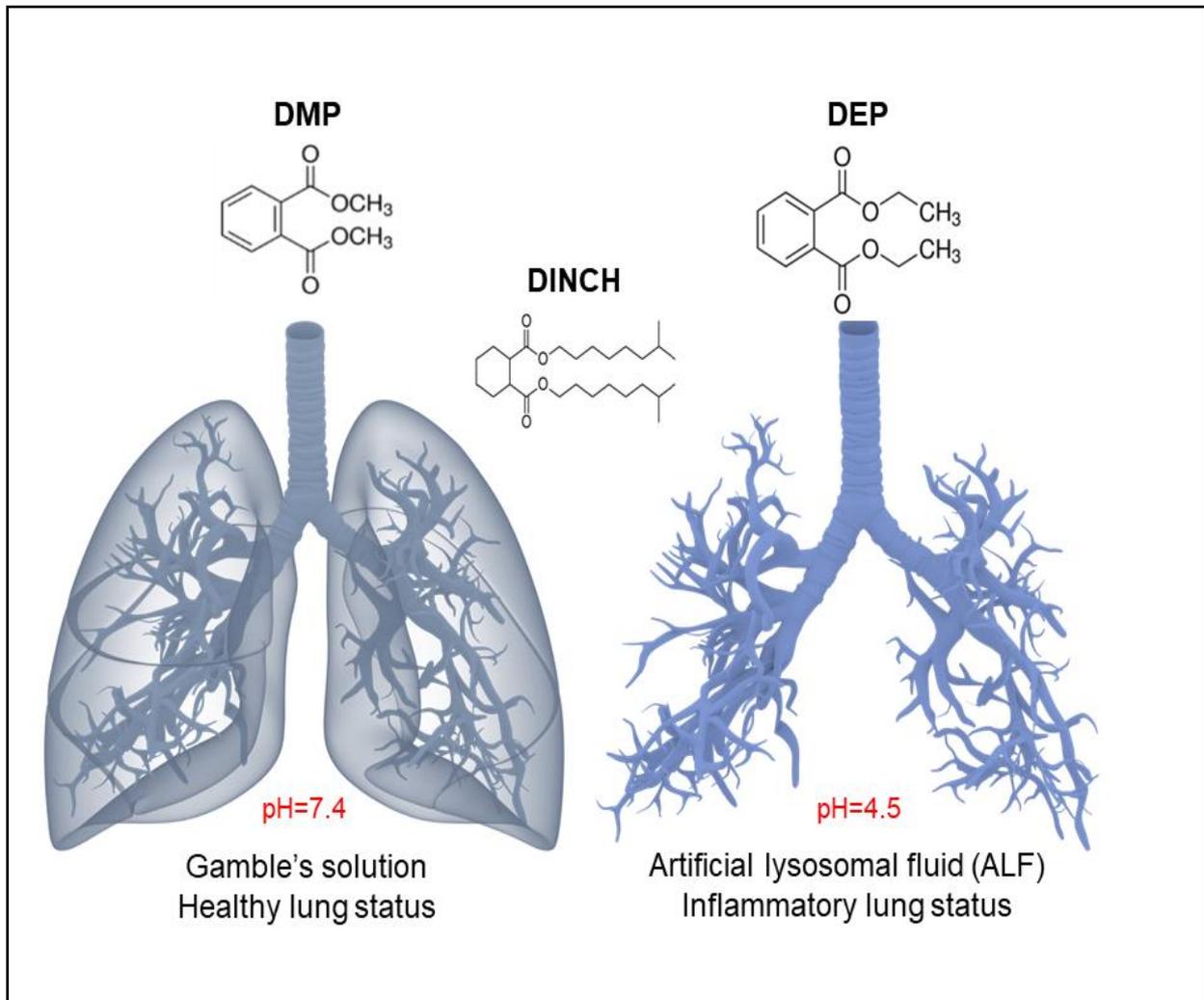
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Graphical abstract



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Highlights

4

- First study on *in vitro* inhalation bioaccessibility of organics from house dust

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- Gamble's solution and artificial lung fluid were used as pulmonary surrogate media

6

- DMP and DEP were > 75 % bioaccessible in both lung media

7

- Alternative plasticisers DINCH and DEHT were < 5% bioaccessible

8

- Inhalation bioaccessibility was highly influenced by hydrophobicity

9

10 *In vitro* inhalation bioaccessibility of phthalate esters and alternative plasticisers present in
11 indoor dust using artificial lung fluids

12 Katerina Kademoglou^{a, b*}, Georgios Giovanoulis^{c, d}, Anna Palm-Cousins^c, Cynthia de Wit^d,
13 Line Småstuen Haug^e, Adrian C. Williams^f, Jörgen Magnér^c, Chris D. Collins^{a**}

14 ^a Soil Research Centre, University of Reading, Reading, RG6 6DW, UK

15 ^b RECETOX, Masaryk University, Kamenice 753/5, pavilion A29, 62500 Brno, Czech
16 Republic

17 ^c IVL Swedish Environmental Research Institute, SE-100 31, Stockholm, Sweden

18 ^d Department of Environmental Science and Analytical Chemistry (ACES), Stockholm
19 University, SE-106 91, Stockholm, Sweden

20 ^e Norwegian Institute of Public Health (NIPH), P.O. Box 4404, Nydalen, 0403 Oslo, Norway

21 ^f School of Pharmacy, University of Reading, Whiteknights, Reading, UK

22

23 * - Corresponding authors: Katerina Kademoglou: kademoglou@recetox.muni.cz &

24 Katerina.kademoglou@gmail.com ; **Chris Collins: c.d.collins@reading.ac.uk

25

26 **Abstract**

27 Phthalate esters (PEs) are plasticiser additives imparting durability, elasticity and flexibility
28 to consumer products. The low migration stability of PEs along with their ubiquitous
29 character and adverse health effects to humans and especially children has resulted in their
30 classification as major indoor contaminants. This study assesses inhalation exposure to PEs
31 via indoor dust using an *in vitro* inhalation bioaccessibility test (*i.e.* uptake) for of dimethyl
32 phthalate (DMP), diethyl phthalate (DEP) and di-(2-ethylhexyl) phthalate (DEHP) and the
33 alternative non phthalate plasticisers bis(2-ethylhexyl) terephthalate (DEHT) and
34 cyclohexane-1,2-dicarboxylic acid diisononyl ester (DINCH), exposure. Using artificial lung
35 fluids, which mimicktwo distinctively different pulmonary environments, namely artificial
36 lysosomal fluid (ALF, pH = 4.5) representing the fluid that inhaled particles would contact
37 after phagocytosis by alveolar and interstitial macrophages within the lung and Gamble's
38 solution (pH = 7.4), the fluid for deep dust deposition within the pulmonary environment.
39 Low molecular weight (MW) PEs such as DMP and DEP were highly bioaccessible (> 75 %)
40 in both artificial pulmonary media, whereas highly hydrophobic compounds such as DEHP,
41 DINCH and DEHT were < 5 % bioaccessible via the lung. Our findings show that the *in vitro*
42 pulmonary uptake of PEs is primarily governed by their hydrophobicity and water solubility,
43 highlighting thus the need for the establishment of a unified and biologically relevant
44 inhalation bioaccessibility test format, employed within the risk assessment framework for
45 volatile and semi-volatile organic pollutants.

46

47 **Keywords:** bioaccessibility, inhalation, phthalate esters, indoor dust, artificial lysosomal
48 fluid, DINCH

49

50 Introduction

51 Phthalate esters (PEs) are plasticiser additives enhancing durability, elasticity and flexibility
52 in consumer and polymeric products ¹. Low molecular weight (LMW) PEs such as dimethyl
53 phthalate (DMP) and diethyl phthalate (DEP) are added as synthetic stabilisers to industrial
54 solvents and personal care products they are also used as colouring or fragrance additives ^{2,3}.
55 High MW (HMW) PEs such as di-(2-ethylhexyl) phthalate (DEHP) and di-iso-nonyl
56 phthalate (DiNP) are primarily used in polyvinyl chloride (PVC) products including floor
57 polishing, wall coatings, children's toys, medical products and food packaging ⁴⁻⁶. Their low
58 migration stability and vapour pressure influence PE release to the indoor environment,
59 resulting in their classification as major indoor organic contaminants ^{7,8}. Consequently,
60 considerably high levels of PEs have been found in indoor dust worldwide ^{5,9-13}.

61 Human exposure to PEs in the indoor environment is a phenomenon of growing concern due
62 to the potentially adverse health effects of PEs such as DEHP, di-n-butyl phthalate (DnBP)
63 and di-iso-butyl phthalate (DiBP) in adults, such as disrupted endocrine and thyroid
64 homeostasis, reduced fertility and reproduction ^{3,14,15}. Hence, the US and the EU have partly
65 restricted the use of DiBP, DnBP, and DEHP in toys and childcare products ^{16,17}. Such
66 actions paved the way for the introduction of less toxic, non-phthalate substitutes (*i.e.*
67 alternative plasticisers) in consumer products in the early 2000s, such as di-isononyl-
68 cyclohexane-1,2-dicarboxylate (DINCH; DEHP and DiNP replacement) and bis(2-
69 ethylhexyl) terephthalate (DEHT), a structural isomer of DEHP ¹⁸⁻²¹. However, due to their
70 dominant use and rapid substitution, considerable levels of DINCH and DEHT have been
71 reported in the indoor environment, raising concerns about their potential effects on humans
72 ²²⁻²⁵.

73 Due to their critical and vulnerable developmental status, pre and postnatal children's
74 exposure to PEs via indoor dust and PVC materials has been linked with chronic respiratory
75 problems such as allergies, asthma, bronchial hyperactivity and inflammation, as well as
76 neurodevelopmental disorders manifesting in adulthood ²⁶⁻³¹. Franken et al. (2017) reported
77 the high occurrence of asthma in Belgian teenagers (especially girls) associated with high
78 DEHP and DnBP exposure ³². DEHT and DINCH administration to rodents revealed no signs
79 of DEHP-like toxicity ³³⁻³⁵. However, DINCH *in utero* exposure has been associated with
80 signs of impaired liver metabolism and premature testicular aging such as decreased
81 testosterone secretion, physical changes in seminal glands and testicular atrophy in rats and

82 their young offspring³⁶. Thus, the debate regarding the safety of alternative plasticisers is
83 ongoing especially during early-life exposure.

84 Physiologically-based extraction tests (PBET) have been employed to assess the oral
85 bioaccessibility (*i.e.* uptake) of PEs via dust ingestion³⁷⁻³⁹. PE gut bioaccessibility decreased
86 as logK_{ow} increased; LMW PEs such as DMP and DEP were found to be 32 % and 26 %
87 bioaccessible, respectively, while DEHP was only 10 % bioaccessible via the gut³⁸. In a
88 comparative study between different dust size fractions and oral bioaccessibility, Wang et al.
89 (2013) reported the highest gut uptake for LMW PEs in < 63 µm size fraction, compared to
90 particles > 63 µm³⁹. Dermal absorption of DEP and DnBP directly from air has been
91 proposed by Weschler et al⁴⁰. Since no studies exist regarding the inhalation bioaccessibility
92 of organic pollutants, this calls for their development⁴¹.

93 This is the first study we are aware of quantifying the inhalation bioaccessibility of PEs and
94 alternative plasticisers employing two artificial lung fluids, mimicking two distinctively
95 different interstitial lung conditions. Artificial pulmonary fluids have been previously
96 employed in inhalation bioaccessibility studies of water-soluble metals and nanoparticles⁴²⁻
97⁴⁶. Artificial lysosomal fluid (ALF, pH=4.5) represents the fluid which inhaled particles come
98 into contact with after phagocytosis by alveolar and interstitial macrophages within the lung.
99 Gamble's solution (GMB, pH=7.4) is a surrogate fluid for deep dust deposition within the
100 interstitial fluid of the lung^{43,46}. The objectives of the present study are to evaluate the *in*
101 *vitro* inhalation bioaccessibility of PEs, DINCH and DEHT present in indoor dust by
102 employing two different artificial pulmonary fluids, *i.e.* Gamble's solution and ALF
103 representing the healthy and inflammatory status of the tracheobronchial environment,
104 respectively and to assess possible factors influencing inhalation bioaccessibility of PEs,
105 DINCH and DEHT.

106 **Material and methods**

107 Sampling and dust particle properties

108 Details on the A-TEAM sampling protocols are given elsewhere⁴⁷. Pre-existing vacuum
109 cleaner dust samples (N=10) were passed through a methanol-washed, metallic sieve (< 63
110 µm) with respect to the inhalable aerodynamic particle cut off convention according to the
111 International Organization for Standardization (ISO)⁴⁸. Specific surface area and dust particle
112 size were determined by laser diffraction spectroscopy (Mastersizer 3000, Malvern Ltd.,

113 UK), while total carbon (TC %) and nitrogen (TN %) contents were determined by Thermo
114 Flash 2000 and organic matter content (OMC %) was determined by loss-on-ignition (LOI)
115 as described elsewhere ⁴⁹.

116 Dust extraction and clean-up

117 Details of the indoor dust extraction have been published previously ^{24,50}. Briefly, 100 mg of
118 dust (< 63 μm) were extracted with 10 mL acetone: n-hexane (1:1 v/v) using microwave-
119 assisted extraction (MAE) under controlled pressure and temperature. Prior to extraction, 400
120 ng ISTD mix prepared in n-hexane (DMP-d₄, DnBP-d₄ and DEHP-d₄) were spiked into all
121 samples. The dust extracts were concentrated to 0.5 ml under a gentle nitrogen (N₂) stream
122 which was filtrated through a glass Pasteur pipette tip containing charcoal in order to
123 eliminate any traces of external contamination and the solvent was exchanged to n-hexane.
124 This solution was loaded onto an ENVI-Florisil cartridge (500 mg / 3 mL, Biotage Isolute,
125 Uppsala, Sweden) and 9 mL of n-hexane were added as a cleaning elution step. During the
126 second elution, all target analytes were eluted using the 9 mL acetone: n-hexane (1:1) and the
127 resulting eluate was concentrated to 1 ml with a gentle N₂ flow at room temperature, filtered
128 as described above. Finally, all extracts were transferred to GC vials and biphenyl (300 ng)
129 was added as an injection recovery standard prior to GC-MS/MS analysis (Fig SI 1). Further
130 details about instrumental analysis are available in SI.

131 Lung fluid extraction

132 All lung fluid extractions were conducted in duplicate. Both media were freshly prepared 24
133 h before the initiation of each test in ultra-pure H₂O (18.2 Ω) as described elsewhere⁴³ (Table
134 SI 3), pH-adjusted using HCl 1 M and NaOH 1 M, stored at 4°C and were checked for
135 background phthalate contamination prior use. According to Boisa et al (2014), the
136 experimental volume for simulated lung fluid extraction tests should be equal to 20 mL,
137 given the pulmonary fluid volume capacity of healthy non-smoking adults (0.3 mL / kg; 70
138 kg body mass)⁴². In order to maintain 1:100 solid-to-liquid (S/L) ratio between the incubated
139 matrix and the pulmonary fluid, 0.2 g of indoor dust (< 63 μm) were combined with 20 mL of
140 each artificial lung fluid separately, as suggested by Schaidler et al⁵¹. All samples were
141 covered on top with oven-baked aluminium foil to avoid background phthalate
142 contamination, followed by continuous incubation inside a thermostatic chamber (60 rpm; 37
143 °C) for 96 h , a time point relevant to the human alveolar clearance capacity ^{45,52}. After 96 h,
144 the samples were separated by centrifugation (1500 rpm; 3 min) and the lung supernatants

145 were subjected to liquid-liquid extraction (LLE) using 7 mL Hexane: MTBE 3:1 twice, while
146 ultrasonication-assisted extraction was employed for the residual dusts twice for 10 min using
147 7 mL of Acetone: Hexane 1:1. Prior to all extractions, all samples were spiked with 400 ng
148 ISTD mix prepared in n-hexane (DMP-d₄, DnBP-d₄ and DEHP-d₄). To avoid any water
149 residue and remove any gel-like emulsion formulated during LLE, sufficient amount of oven-
150 baked Na₂SO₄ (powder) was added to all extracts, followed by 1 min vortexing and organic
151 phase collection after centrifugation (1500 rpm; 3 min). All extracts were combined, solvent
152 was exchanged to n-hexane and concentrated to 1 ml under a gentle N₂ stream at room
153 temperature, filtered as described above. The residual dust extracts were subjected for clean-
154 up through ENVI-Florisil SPE cartridge (500 mg / 3 mL, Biotage Isolute, Uppsala, Sweden),
155 similarly to the dust extraction procedure described above. Briefly, the residual dust extracts
156 were loaded onto the Florisil[®] columns, the first hexane eluate was discarded, while the
157 second eluate was collected using 9 mL of MTBE. The resulting eluate was concentrated to 1
158 ml under a gentle N₂ flow at room temperature, filtered as described above. Finally, all
159 extracts were transferred to oven-baked GC vials and biphenyl (300 ng) was added as an
160 injection recovery standard prior to GC-MS/MS analysis (Fig SI 2).

161 Data analysis

162 Inhalation bioaccessibility (IBAF) was determined using Eq. 1, where mass phthalate (lung
163 supernatant) is set as the phthalate mass (ng) determined in the lung supernatant of the *in*
164 *vitro* pulmonary system and mass phthalate (dust residual) is the mass (ng) determined in the
165 dust residual collected after the 96 h-incubation of the *in vitro* pulmonary system which is
166 considered as the non-bioaccessible fraction.

168 IBAF%

$$169 = \frac{\text{mass phthalate} \left(\begin{array}{c} \text{lung} \\ \text{supernatant} \end{array} \right)}{\text{mass phthalate (lung supernatant)} + \text{mass phthalate (dust residual)}} \times 100 \quad (\text{Eq. 1})$$

167

170 GraphPad Prism[®] version 7.00 for Windows, (GraphPad Software, La Jolla CA, USA) was
171 used for statistical analysis. Prior to statistical analysis, all data were checked for normality
172 using the Shapiro–Wilk test and not all data passed the normality test. All data were arc-sine
173 transformed, as this mathematical transformation is necessary for statistical analysis of results
174 set in percentages in order to equalise variances among treatments⁵³. Ordinary two-way
175 ANOVA (Uncorrected Fisher’s test, p<0.05) was performed to assess statistically significant

176 differences of target analytes between both pulmonary fluids. Spearman's correlation
177 ($p < 0.05$) was employed to assess statistical dependence and correlation between artificial
178 lung fluids and the physicochemical properties of all target analytes.

179 Quality assurance and quality control

180 The methods were evaluated using SRM 2585 as QC sample during dust ($n=5$) and lung fluid
181 ($n=4$) extractions, respectively. Oven-baked, uncontaminated sand was used as a procedural
182 blank during dust extractions; four blank lung fluid samples with no added matrix (two for
183 each lung fluid) were sequentially incubated and analysed as procedural blanks. The results
184 were blank-corrected for all target analytes by subtraction of the mean blank values from the
185 raw target analytes values (expressed in ng g^{-1}) according to Abdhlah and Covaci⁵⁴.
186 Extraction efficiency for all target analytes ranged from 70 – 120% for both lung fluids
187 respectively (Table SI 6). Method limits of detection (mLOD) were calculated as three times
188 the standard deviation of the lung fluid blanks (Table SI 7).

189 **Results and discussion**

190 PEs and alternative plasticisers in indoor dust

191 Apart from DEHT, levels of PEs and DINCH from floor ($N=61$) and vacuum cleaner dust
192 ($N=58$) from the ATEAM cohort have been previously reported²³ and were of the same order
193 of magnitude as reported here ($N=10$; **SI Table xxx**). Besides the smaller dust particle size
194 used in this study compared to Giovanoulis et al.²³ ($< 63 \mu\text{m}$ and $< 500 \mu\text{m}$, respectively), the
195 median values for all target analytes were marginally different apart from DINCH (this study:
196 $17.06 \mu\text{g g}^{-1}$, Giovanoulis et al.: $32.82 \mu\text{g g}^{-1}$; **$p < 0.05$**). Substantial differences between the
197 maximum values of two studies were also found, e.g. DEP (this study: $54.2 \mu\text{g g}^{-1}$,
198 Giovanoulis et al: $240 \mu\text{g g}^{-1}$) or DiNP (this study: $2470 \mu\text{g g}^{-1}$, Giovanoulis et al: $1490 \mu\text{g g}^{-1}$).
199 These findings can be attributed to a) differences in sample size assessed and b)
200 differences in particle size cut off and specific surface area which are likely to influence a
201 pollutant's concentration in dust^{39,55}. However, the aim of the present study is primarily to
202 assess the inhalation bioaccessibility of PEs and their alternatives plasticisers, rather than
203 report on their levels in dust.

204 Inhalation bioaccessibility

205 This is the first study on the *in vitro* inhalation bioaccessibility of PEs and alternative
206 plasticisers via indoor dust. Inhalation bioaccessibility for DMP and DEP exceeded 70 % in
207 both pulmonary media (Fig. 1). Statistical comparison of IBAF between the two pulmonary
208 media did not reveal any statistically significant differences for any target analyte regarding
209 the fluids' pH (pH Gamble's = 7.4; pH ALF = 4.5) and composition, apart from DMP
210 ($p=0.017$) with 71 % and 82 % IBAF for Gamble's solution and ALF, respectively. DEP was
211 also readily absorbed with 76 % and 75 % IBAF in Gamble's solution and ALF, respectively
212 ($p>0.05$), showing thus that inhalation is an important route of exposure for LMW PEs.
213 Gamble's solution is representative of the interstitial fluid of the deep lung area and ALF is
214 representative of the more acidic environment following phagocytosis by alveolar and
215 interstitial macrophages within the lung^{42,43}. Hence, the inhaled dust particles would not have
216 to be phagocytised before a considerable uptake of plasticisers occurs, with the exception of
217 DMP.

218 Similarly to gut bioaccessibility which is partly governed by a pollutant's physico-chemical
219 properties including MW and $\log K_{ow}$ ^{56,57}, inhalation bioaccessibility of PEs decreased
220 against the increasing trend in MW and $\log Kow$ (> 4). DiBP pulmonary uptake was 15.5 %
221 and 12 %, in Gamble's solution and ALF, respectively, whereas DnBP and HMW PEs were
222 10 % and < 5 % bioaccessible in both media, including DEHP and its alternatives, DEHT and
223 DINCH (Fig 1). Such findings endorse ingestion (food or dust) and dermal uptake as the
224 predominant exposure routes for medium and HMW PEs, strongly influenced by their
225 hydrophobic character and low water solubility^{6,23,38}. However, no consensus exists
226 regarding pulmonary media composition for inhalation bioaccessibility studies of organics.
227 Employing modified media formulations with the addition of biologically relevant pulmonary
228 surfactants such as albumin, mucin and dipalmitoylphosphatidylcholine (DPCC) have been
229 proposed^{41,42,58}; the case of DPCC makes biological sense and it should be thus
230 systematically investigated along with other test parameters including S/L, incubation
231 duration and particle size cut off^{41,59}, aiming towards a unified approach similarly to gut
232 bioaccessibility⁵⁶.

233 Method performance using SRM 2585

234 Method performance was assessed using SRM 2585, since the pulmonary media used here
235 were initially designed for nanoparticle and trace element inhalation bioaccessibility

236 studies^{43,45,60}. IBAF > 75 % was found for LMW PEs, while DEHP and DiNP were the least
237 bioaccessible (IBAF < 5 %) as highly hydrophobic compounds (Table 1), following a
238 comparable pattern to the Norwegian house dust IBAF results. The SRM 2585 batch
239 purchased in our study was prepared using a pool of dust samples collected during mid to late
240 1990s. Thus, DINCH and DPHP were not detected, since they were introduced in the market
241 after 2000^{18,61}.

242 In this study we propose an *in vitro* method regarding the inhalation bioaccessibility of PEs
243 and their alternatives via indoor dust. Low MW PEs such as DMP and DEP were highly
244 bioaccessible in both artificial pulmonary media (> 75 %), regardless of the medium's pH
245 and composition. Unlike DEP which presented similar pulmonary uptake in both media,
246 DMP was more readily absorbed through ALF than Gamble's solution. HMW PEs along with
247 DEHP alternatives, DEHT and DINCH did not exceed 5 % pulmonary uptake. Therefore,
248 inhalation is a considerable route of exposure for LMW and less hydrophobic PEs. The lung
249 uptake potential for compounds with comparable physico-chemical properties, *e.g.* LMW
250 polycyclic aromatic hydrocarbons (PAHs) or organophosphates (PFRs) should be further
251 assessed. Our results show that inhalation bioaccessibility of organic pollutants is primarily
252 governed by hydrophobicity and water solubility. Future research should be targeted towards
253 a unified and biologically relevant *in vitro* pulmonary uptake test for organics relevant to dust
254 deposition in the lung, human lung function and inflammation *in vivo*. Finally, animal studies
255 are more representative of the *in vivo* situation, marking them as necessary for the validation
256 of *in vitro* inhalation bioaccessibility tests.

257 **Conflict of interest**

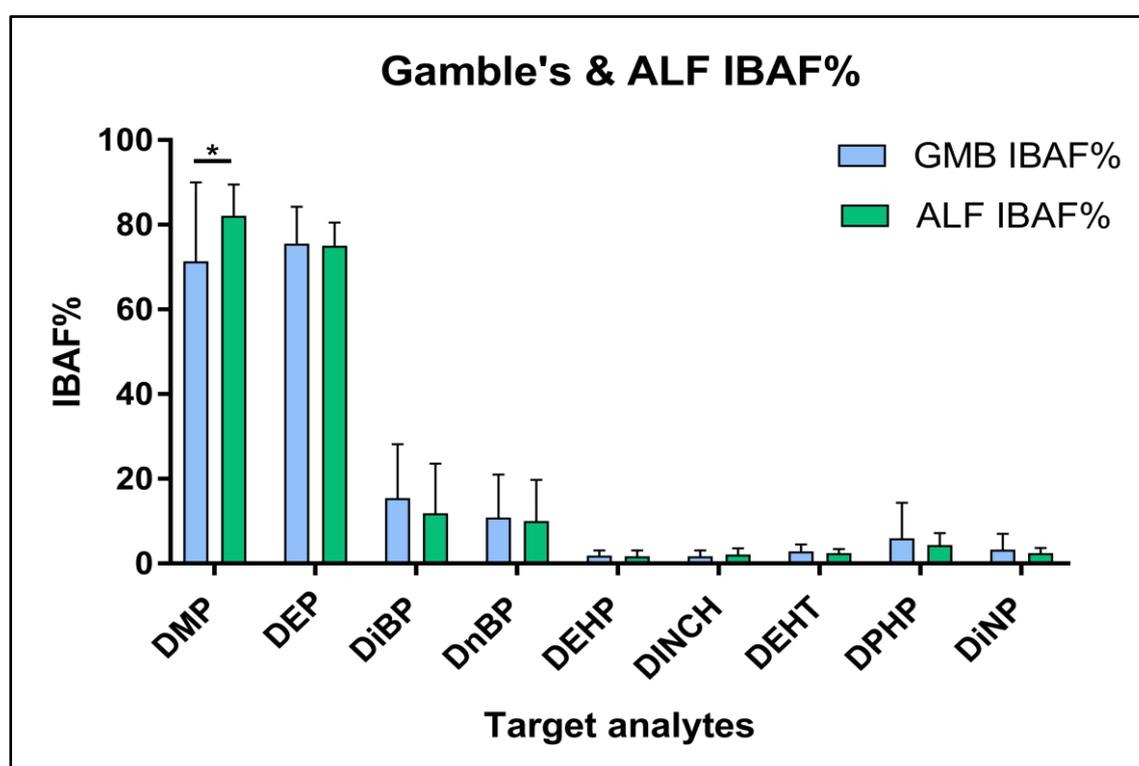
258 The authors declare no conflict of interest.

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 272 scientists.

273 Artwork and tables



274
 275 Figure 1 – *In vitro* inhalation bioaccessibility (IBAF%) of phthalate esters and alternative
 276 plasticisers present in indoor dust samples (N=10), using two different simulated lung fluids,
 277 namely Gamble’s solution (GMB) and artificial lysosomal fluid (ALF). Statistically
 278 significant differences shown here (*; $p < 0.05$). Bar charts represent average values in
 279 duplicates. Error bars represent 1 STDEV.

280 Table 1 - Lung fluid method performance using SRM 2585 (n=4) for Gamble’s solution and
 281 artificial lysosomal fluid (ALF)

Target analytes [†]	Gamble’s IBAF% (n=2)	STDEV	ALF IBAF% (n=2)	STDEV

DMP	89.9	1.8	89.5	0.3
DEP	80.7	1.2	73.7	1.0
DiBP	17.6	2.7	8.0	0.6
DnBP	9.8	1.3	6.2	0.5
BzBP	18.5	3.6	13.2	0.6
DEHP	3.1	1.6	2.0	0.2
DEHT	4.9	1.6	4.6	0.6
DiNP	3.9	1.0	3.5	0.3

282 †DINCH and DPHP not present in SRM 2585

283 Table 2 –Spearman’s correlation between inhalation bioaccessibility (IBAF) in Gamble’s
 284 solution (GMB) and artificial lysosomal fluid (ALF) and the physicochemical properties of
 285 plasticisers studied here

Physico-chemical properties†	GMB IBAF		ALF IBAF	
	Spearman's ρ	p value	Spearman's ρ	p value
MW	-0.561	0.096	-0.561	0.096
Log Kow	-0.705	0.027*	-0.705	0.027*
Log Koa	-0.588	0.081	-0.624	0.060
Vapour pressure	-0.535	0.115	-0.559	0.098
Water solubility	0.661	0.044*	0.636	0.054

286 *levels of statistical significance: p<0.05

287 † Physicochemical properties of plasticisers studied here can be found at [Table SI xxx](#)

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