1 # Supplementary Information (SI)

2 # Hexachlorobenzene exerts genotoxic effects in a

3 # humpback whale cell line under stable exposure

4 # conditions

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18 **Table of contents**

49

1. Establishment of the passive dosing setup for HCB

1.a. Table S1

Table S1. Equations characterising the partitioning between different phases in the passive dosing setup

Efficiency of silicone O-ring loading	$$Efficiency = \frac{m_{HCB,sil}^{*}}{m_{HCB,LB,t0}}$$
Mass balance of silicone O-ring loading	$$Recovery = \frac{m_{HCB,LB}^{*} + m_{HCB,sil}^{*}}{m_{HCB,LB,t0}}$$
Partitioning coefficient between LB and silicone O-ring, based on the volume of silicone	$$K_{LB:sil} = \frac{C_{LB}^{*}}{C_{sil}^{*}} = \frac{m_{HCB,LB}^{*}/V_{LB}}{m_{HCB,sil}^{*}/V_{sil}} \; [\frac{L}{L}]$$
Predicted equilibrium concentration on silicone O-ring as a function of starting concentration in LB	$$C_{sil}^{*} = \frac{C_{LB,t0}}{K_{LB:sil} + \dfrac{V_{sil}}{V_{LB}}}$$
Starting concentration in LB required to achieve a specified equilibrium concentration on silicone O-ring	$$C_{LB,t0} = C_{sil}^{*}\frac{V_{sil}}{V_{LB}} + C_{sil}^{*}K_{LB:sil}$$
Partitioning coefficient between silicone O-ring and cell culture medium DMEM/F12, based on the volume of silicone	$$K_{sil:DMEM/F12} = \frac{C_{sil}}{C_{DMEM/F12}^{*}} = \frac{m_{HCB,sil}/V_{sil}}{C_{DMEM/F12}^{*}} \; [\frac{L}{L}]$$
Predicted equilibrium concentration in cell culture medium DMEM/F12 as a function of concentration on silicone O-ring	$$C_{DMEM/F12}^{*} = \frac{C_{sil}}{K_{sil:DMEM/F12}}$$
Theoretical partitioning coefficient between LB and in cell culture medium DMEM/F12	$$K_{LB:DMEM/F12} = K_{LB:sil} \cdot K_{sil:DMEM/F12}$$
Partitioning coefficient between LB and silicone O-ring, based on the mass of silicone	$$K'_{LB:sil} = \frac{C_{LB}^{*}}{C'_{sil}^{*}} = \frac{m_{HCB,LB}^{*}/V_{LB}}{m_{HCB,sil}^{*}/m_{sil}} \; [\frac{kg}{L}]$$
Partitioning coefficient between silicone O-ring and cell culture medium DMEM/F12, based on the mass of silicone	$$K'_{sil:DMEM/F12} = \frac{C'_{sil}}{C_{DMEM/F12}^{*}} = \frac{m_{HCB,sil}/m_{sil}}{C_{DMEM/F12}^{*}} \; [\frac{L}{kg}]$$

$m^{*}_{HCB,sil}$ - mass of HCB on silicone O-rings at equilibrium; $m_{HCB,LB,t0}$ - mass of HCB in loading buffer (LB) at the beginning; $m^{*}_{HCB,LB}$ - mass of HCB in the LB at equilibrium; $K_{LB:sil}$ - partitioning coefficient between LB and silicone;

55   C^*_{LB} - equilibrium concentration in the LB; C^*_{sil} - equilibrium concentration in silicone; V_{LB} - volume of LB; V_{sil} – volume
56   of silicone; $C_{LB,t0}$ - starting concentration in LB; $K_{sil:DMEM/F12}$ - partitioning coefficient between silicone and DMEM/F12;
57   C_{sil} - concentration in silicone[‡]; $m_{HCB,sil}$ - mass of HCB in silicone[‡]; $C^*_{DMEM/F12}$ - equilibrium concentration of HCB in
58   DMEM/F12. [‡]Due to the low solubility of HCB in aqueous solution the loss of HCB to the medium can be considered
59   negligible in comparison to the amount loaded onto the O-ring, thus C_{sil} and m_{sil} can be considered as constant and are
60   therefore not denoted as equilibrium concentrations[1].

61 **1.b. Figure S1**
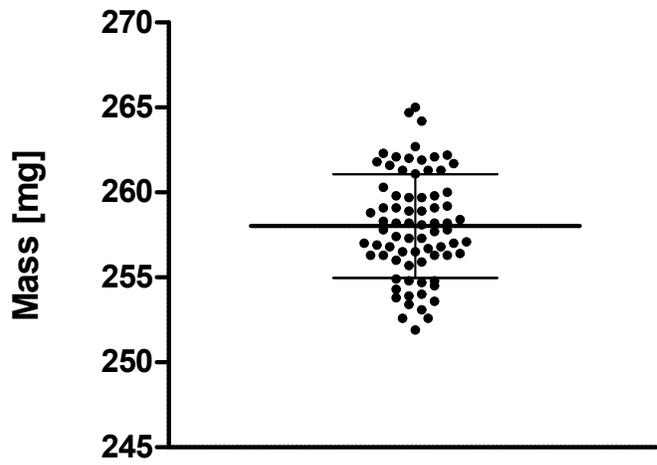
62

63 **Figure S1. Mass of silicone O-rings.** O-rings from Hutchinson Suisse (Langnau am Albis, Switzerland). Error bars
64 represent mean with standard deviation (SD) (n = 75). Size of O-rings is highly standardised with a relative SD of only
65 1.2%.

66

67 **1.c. Comparison of loading techniques**

68 Two different methods of loading were compared: partitioning loading and push loading.

69 Partitioning loading is the most common approach in passive dosing assays. The working principle

70 of this technique is equilibrium partitioning of the chemical from a loading buffer (LB) — usually

71 methanol[2–4] or a mixture of methanol and water[1] — to silicone. Hydrophobic chemicals

72 preferentially partition from the solution into the silicone. Partitioning loading was carried out as

73 described in section 2.2 of the main manuscript.

74 In push loading, a chemical is dissolved in methanol, followed by a stepwise addition of water,

75 thus decreasing the chemical's solubility in the LB and 'pushing' it into the silicone. Birch et al.

76 (2010) reported complete mass transfer of fluoranthene to silicone using this technique[5]. Push

77 loading was carried out as described in Birch et al. (2010)[5] with modified volumes of LB. Briefly,

78 pre-cleaned O-rings were placed in 100 µL per O-ring of a solution of HCB in methanol. Then,

79 increasing volumes of dH_2O were added in 10 minute intervals as follows: 100 µL, 100 µL, 100

80 µL, 200 µL, 400 µL, 600 µL, to a final volume of 1.6 mL. O-rings were equilibrated in the LB for

81 24 hours with constant shaking at 250 rpm, then 10 mL of dH_2O were added, and O-rings were

82 equilibrated for another 24 hours. This technique was used with the following concentrations of

83 HCB in methanol: 100, 50, 10, and 0 (control) µg/mL.

84 To compare the two techniques the loading efficiency was determined (Table S2). Both push and

85 partitioning loading were efficient for loading considerable amounts of HCB onto O-rings.

86 However, partitioning loading was slightly more efficient, while at the same time more convenient

87 in terms of handling. The solubility of HCB in the LB of push loading would be predicted to be

88 lower than with partitioning loading where methanol content is 60% compared to 0.9% in push

89 loading after the addition of water. It can be reasoned that the rapidly decreasing amount of

90 methanol during push loading, instead of leading to partitioning into the silicone, might cause HCB

91 losses through evaporation or crystallisation. As a consequence, partitioning loading was chosen

92 for our passive dosing setup. A full mass balance calculation determined a recovery of $104 \pm 9\%$

93 $(n = 6)$ for this technique, indicating no major losses in the process.

94

95 **1.d. Table S2**

96 **Table S2. Comparison of loading efficiencies (mean ± SD) of partitioning loading and push loading**

	Partitioning loading	Push loading
Loading efficiency	84% ± 10% (n=6)	74% ± 10% (n=9)

97

98

99 **1.e. Estimation of HCB concentration in blood plasma of humpback whales**

100 <u>i. Estimation based on partitioning coefficients for HCB between blubber and plasma developed</u>

101 <u>for bottlenose dolphins[6]</u>

102 a) Based on wet-weight partitioning coefficient:

103 $\quad$ $C_{blubber} = 60 \rightarrow 200$ ng/$g_{lipid(blubber)}$ [7]

104 $\quad$ $Lipid_{blubber} = {\sim}50\%$ [7]

105 $\quad$ $C_{blubber} = 60 \rightarrow 200$ ng/$g_{lipid} \cdot 0.5$ $g_{lipid}/g_{blubber} = 30 \rightarrow 100$ ng/$g_{blubber}$

106 $\quad$ $K_{blubber:plasma} = 155$ (juveniles) / <u>94</u> (adult males) / 144 (adult females) (values for bottlenose
107 $\quad$ dolphins)[6]; partitioning coefficient for adult males was selected to avoid confounding factors
108 $\quad$ such as contaminant offload to offspring during reproduction

109 $\quad$ <u>C_{plasma}</u> = 30 $\rightarrow$ 100 ng/$g_{blubber}$ / 94 = 0.32 $\rightarrow$ 1.06 ng/g_{plasma} <u>= ${\sim}0.32 \rightarrow 1.06$ ng/mL$_{plasma}$</u>

110 b) Based on lipid-normalised partitioning coefficient:

111 $\quad$ $C_{blubber} = 60 \rightarrow 200$ ng/$g_{lipid(blubber)}$ [7]

112 $\quad$ $K_{blubber:plasma} = 1.54$ (juveniles) / <u>1.55</u> (adult males) / 1.17 (adult females) (values for bottlenose
113 $\quad$ dolphins)[6]; partitioning coefficient for adult males was selected to avoid confounding factors
114 $\quad$ such as contaminant offload to offspring during reproduction

115 $\quad$ $C_{plasma} = 60 \rightarrow 200$ ng/$g_{lipid(blubber)}$ / 1.55 = 38.7 $\rightarrow$ 129.0 ng/$g_{lipid(plasma)}$

116 $\quad$ $Lipid_{plasma} = {\sim}0.5\%$ (values from bottlenose dolphins)[6]

117 $\quad$ <u>C_{plasma}</u> = 38.7 $\rightarrow$ 129.0 ng/$g_{lipid(plasma)} \cdot 0.005$ $g_{lipid}/g_{plasma} = 0.19 \rightarrow 0.65$ ng/g_{plasma} <u>= ${\sim} 0.19 \rightarrow$
118 $\quad$ 0.65 ng/mL$_{plasma}$</u>

119

120 ii. Estimation based on physiologically based pharmacokinetic model of HCB distribution in a

121 humpback whale[8]

122 a) At the beginning of migration

123      $C_{blubber} = \sim 60$ ng/g_{lipid} [7]

124      $C_{blubber} \sim 10\%$ higher than C_{plasma} [8]

125      $C_{blubber} = \sim 60$ ng/g_{lipid} → $C_{plasma} = \sim 54$ ng/g_{lipid}

126      $Lipid_{plasma} = \sim 0.5\%$ (values from bottlenose dolphins)[6]

127      $\underline{C_{plasma}} = \sim 54$ ng/g_{lipid} · 0.005 g_{lipid}/g_{plasma} = 0.27 ng/g_{plasma} $\underline{= \sim 0.27}$ ng/mL$_{plasma}$

128 b) At the end of migration

129      $C_{blubber} = \sim 200$ ng/g_{lipid} [7]

130      $C_{blubber} \sim 35\%$ lower than C_{plasma} [8]

131      $C_{blubber} = \sim 200$ ng/g_{lipid} → $C_{plasma} = \sim 270$ ng/g_{lipid}

132      $Lipid_{plasma} = \sim 0.5\%$ (values from bottlenose dolphins)[6]

133      $\underline{C_{plasma}} = \sim 270$ ng/g_{lipid} · 0.005 g_{lipid}/g_{plasma} = 1.35 ng/g_{plasma} $\underline{= \sim 1.35}$ ng/mL$_{plasma}$

134

135 iii. Average

136 a) At the beginning of migration

137      $\underline{C_{plasma}} = \sim (0.32+0.19+0.27)/3 = \underline{0.3}$ ng/mL$_{plasma}$

138 b) At the end of migration

139      $\underline{C_{plasma}} = \sim (1.06+0.65+1.35)/3 = \underline{1}$ ng/mL$_{plasma}$

140

141 **1.f. Figure S2**
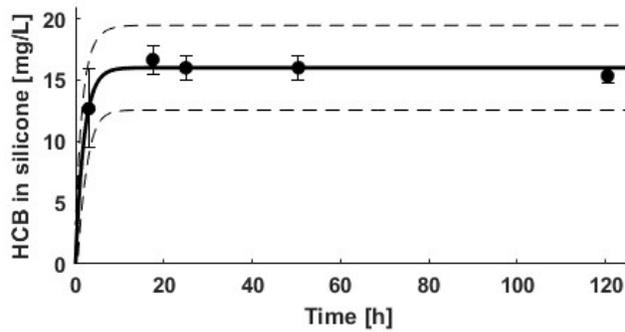
142

143 **Figure S2. Time to steady-state and concentration stability during loading**. Loading of silicone O-rings by
144 partitioning from a loading buffer of methanol/water 60:40 (v/v) at room temperature and 250 rpm. Each point represents
145 the mean of three technical replicates (i.e. three O-rings), error bars represent SD. Equilibrium was reached within 6 h
146 (see Fig. 1a), and remained stable for at least 120 h.

147

148 **1.g. Figure S3**
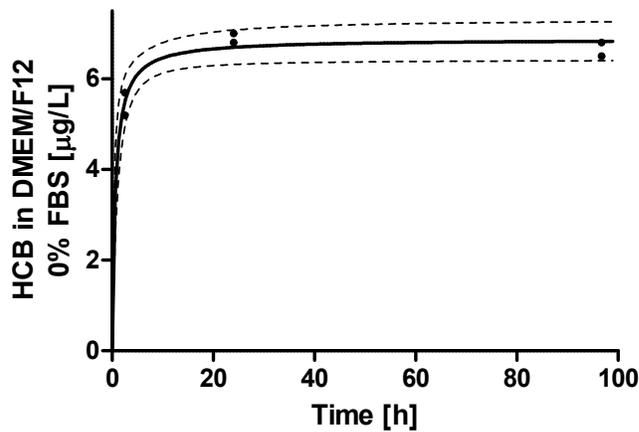
149

150 **Figure S3. Pre-equilibration of serum-free DMEM/F12 exposure medium.** Time to steady-state in DMEM/F12 (0%
151 FBS) with submerged HCB-loaded silicone O-rings in amber glass vials at 37°C and 250 rpm. Each point represents
152 one technical replicate.

153

154

1.h. Figure S4

156 Plastic foil, with which microtiter plates are commonly covered, has a high capacity for sorption

157 of hydrophobic chemicals, leading to lower exposure concentrations. In 24 well plates covered

158 with plastic foil (Figure S3) equilibrium was established at 20% lower medium concentrations as

159 compared to aluminium foil (see Figure 1c of the main manuscript).
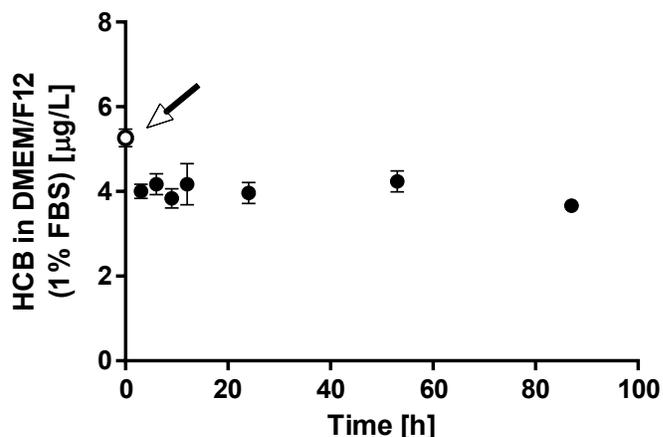
160

161 **Figure S4. Stability of HCB concentration in a 24 well-plate in the presence of cells.** Concentration of HCB in
162 exposure medium DMEM/F12 (1% FBS) immediately after pre-equilibration (at 0 hours, open symbol ○ indicated by
163 arrow), and after transfer to a 24-well plate containing a confluent monolayer of cells (1×10^4 cells/cm^2 seeded two days
164 prior to exposure), with a loaded O-ring placed afloat and the plate covered with plastic foil, sealed with parafilm and
165 incubated at 37°C and 30 rpm (solid symbols ●). Each point represents the mean of three technical replicates, error
166 bars represent SD. After transfer of pre-equilibrated medium to the cell-containing 24-well plate there was a drop in
167 aqueous HCB concentration; equilibrium re-established at a lower concentration equal to 75% of the pre-equilibrated
168 concentration. This concentration remained stable for at least 87 hours.

169

170 **1.i. Table S3**

171 The partitioning coefficients for HCB between the three phases LB, silicone O-ring, and exposure

172 medium DMEM/F12 (with 1% FBS or without) can be determined in terms of volume (K) or mass

173 (K') of silicone. For the purpose of comparison with literature values both have been determined.

174 Partitioning coefficients K are given in Figure 1c (main manuscript), partitioning coefficients K'

175 are given in Table S3.

176

177 **Table S3. Partitioning coefficients for HCB between LB, silicone, and exposure medium DMEM/F12**

$logK'_{LB:sil}$	$logK'_{sil:DMEM/F12}$ (1%FBS)	$logK'_{sil:DMEM/F12}$
-2.10	3.57	4.17

178

179

1.j. Comparison of partitioning coefficients with published literature

The HCB partitioning coefficients found here are in good agreement with previously published partitioning coefficients for HCB (Table S4) and for other chlorobenzenes and benzo[a]pyrene (B[a]P) (Table S5). There is a negative linear relationship between $\log K_{ow}$ and partitioning into serum-free medium. The addition of serum increases partitioning to the medium of substances with high $\log K_{ow}$ values, but has little influence on substances with low $\log K_{ow}$ values. HCB is more hydrophobic than 1,2-dichlorobenzene (1,2-DCB) and 1,2,4-trichlorobenzene (1,2,4-TCB), but less so than B[a]P. Consequently, it partitions less readily from silicone into medium, especially serum-free medium, than the two chlorobenzenes, but more so than B[a]P.

1.k. Table S4

Table S4. Comparison of published partitioning coefficients for HCB between reservoir and water

Description	Partitioning Coefficient	Source
Partitioning coefficient for HCB between silicone and water	$\log K'_{sil:w} = 5.05$ [L/kg]	Gilbert et al. (2015)[9]
Partitioning coefficient for HCB between octadecyl discs and water	$\log K_{disc:w} \approx 6.3$ [L/L]	Mayer et al. (1999)[10]
Linear relationship of partitioning coefficients for PAHs from silicone to water based on their hydrophobicity: $\log K_{sil:w} = 0.799 \cdot \log K_{ow} + 0.234$; applied to HCB	$\log K_{sil:w} = 4.81$ [L/L]	Smith et al. (2010)[4]
Partitioning coefficient for HCB between silicone and DMEM/F12 (without FBS)	$\log K_{sil:DMEM/F12} = 4.25$ [L/L] $\log K'_{sil:DMEM/F12} = 4.17$ [L/kg]	This study

194 **1.l. Table S5**

195 **Table S5. Comparison of partitioning properties of HCB with 1,2-dichlorobenzene (1,2-DCB), 1,2,4-**
196 **trichlorobenzene (1,2,4-TCB),and benzo[a]pyrene (B[a]P)**

	$logK_{ow}$	Henry's law constant [Pa·m³/mol]	$K_{LB:sil}$[c]	$logK_{sil:medium}$ (-)serum[d]	$logK_{sil:medium}$ (+)serum[e]
HCB[a]	5.73	35[f]	$6.56 \cdot 10^{-3}$	4.25	3.66
1,2-Dichlorobenzene[b]	3.43	195	$8.05 \cdot 10^{-2}$	3.53	3.54
1,2,4-Trichlorobenzene[b]	4.05	101	$3.8 \cdot 10^{-2}$	3.96	3.83
Benzo[a]pyrene[b]	6.13	0.034	$1.17 \cdot 10^{-2}$	5.08	2.39

197 [a]This study; [b]Data from Kramer et al. (2010)[1]; [c]LB = MeOH/H_2O 60:40% (v/v); [d]Data for HCB in DMEM/F12 medium,
198 data for 1,2-DCB, 1,2,4-TCB and B[a]P in L15 medium; [e]Data for HCB in DMEM/F12 medium with 1% FBS, data for
199 1,2-DCB, 1,2,4-TCB and B[a]P in L15 medium with 5% FBS; [f]Data from Jantunen and Bidleman (2006)[11]

200

1.m. Figure S5

202   Relative to the medium concentration aimed for, quite large quantities of highly concentrated HCB

203   stock solutions are required to load O-rings. Thus, an experiment was carried out to assess the

204   possibility of re-using loaded O-rings. To this end, nine O-rings were loaded to the same

205   concentration and then used between 0 and 5 times to pre-equilibrate DMEM/F12 medium with

206   1% FBS for 24 hours, followed by 24 hours exposure in 24-well plates covered with aluminium

207   foil, sealed and incubated at 37°C and 30 rpm. After exposure the O-rings were extracted with

208   cyclohexane to analyse remaining HCB in silicone. After each cycle of pre-equilibration and

209   exposure there was a slight reduction in the concentration of HCB in silicone. Thus, used O-rings

210   cannot be used to achieve the same concentration. Yet, if the lower concentrations are taken into

211   account re-use is possible. Nevertheless, with the aim of our study being to perform exposures at

212   the same starting concentrations, we used freshly prepared O-rings in our experiments.
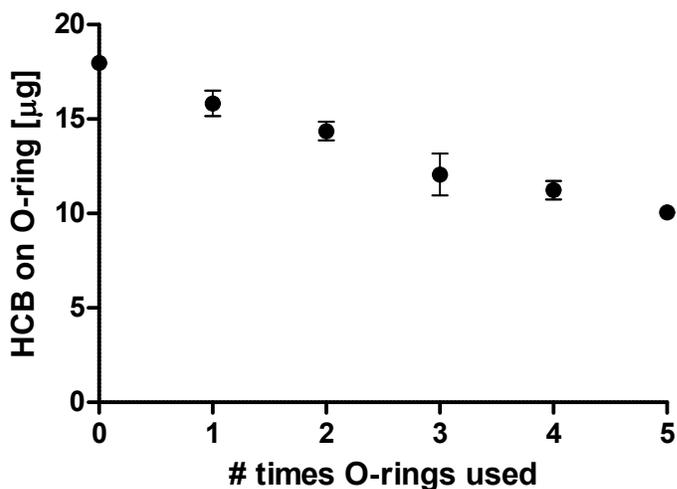
213

214   **Figure S5. Re-use of O-rings.** Loaded O-rings were extracted after using them up to 5 times in a simulated exposure
215   including 24 hours pre-equilibration of DMEM/F12 medium (1% FBS) and 24 hours exposure in 24-well plates. Each
216   point represents the mean of three technical replicates, error bars represent SD. After each cycle of pre-equilibration
217   and exposure in 24-well plates there is a slight reduction in the concentration of HCB in silicone.

218

219 **2.** __Effect of HCB on viability of HuWa$_{TERT}$ cells__

220 **2.a. Figure S6**

221
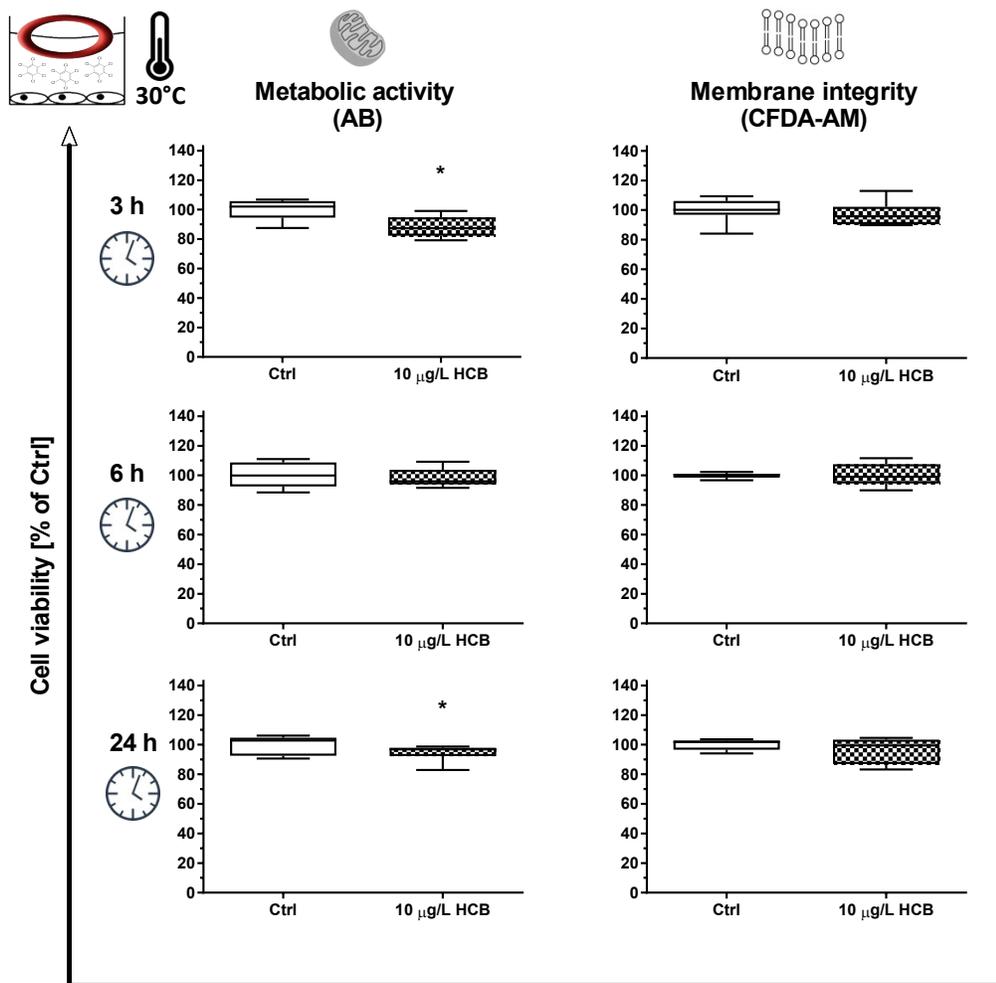
222

223 **Figure S6. Impact of HCB exposure on viability of cells in monolayer under temperature stress.** Attached
224 monolayers of HuWa$_{TERT}$ were exposed to 10 µg/L HCB using the pre-equilibrated DMEM/F12 (1% FBS) exposure
225 medium and silicone O-rings for passive dosing at a reduced temperature of 30°C. Metabolic activity and membrane
226 integrity were assessed relative to control after 3, 6, and 24 hours. Plots represent the median, 1st and 3rd quartile, and
227 whiskers the 5th and 95th percentile of three biological replicates.

228

229 **2.b. Figure S7**
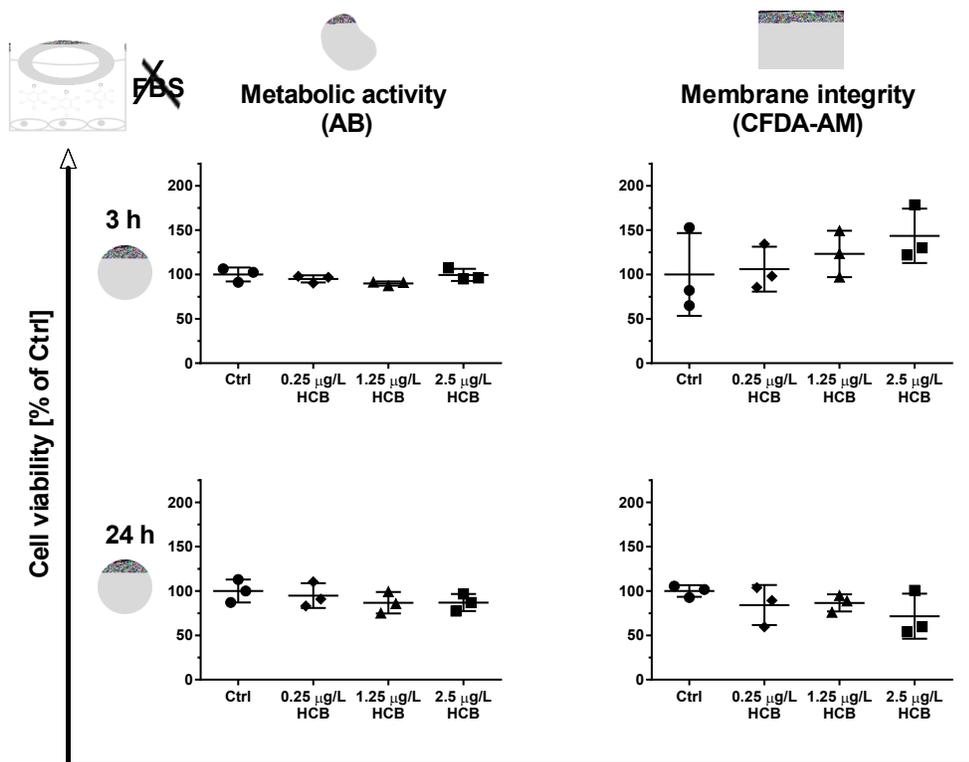
Figure S7. Impact of HCB exposure on viability of cells in monolayer with serum-free exposure medium.
Attached monolayers of HuWa$_{TERT}$ were exposed to 0.25, 1.25, and 2.5 µg/L HCB using the serum-free pre-equilibrated DMEM/F12 exposure medium and silicone O-rings for passive dosing. Metabolic activity and membrane integrity were assessed relative to control after 3 and 24 hours. Data represents the mean and error bars the SD of three technical replicates.

237 **3.** **Genotoxic effects of HCB in HuWa_{TERT} cells**

238 **3.a. Figure S8**
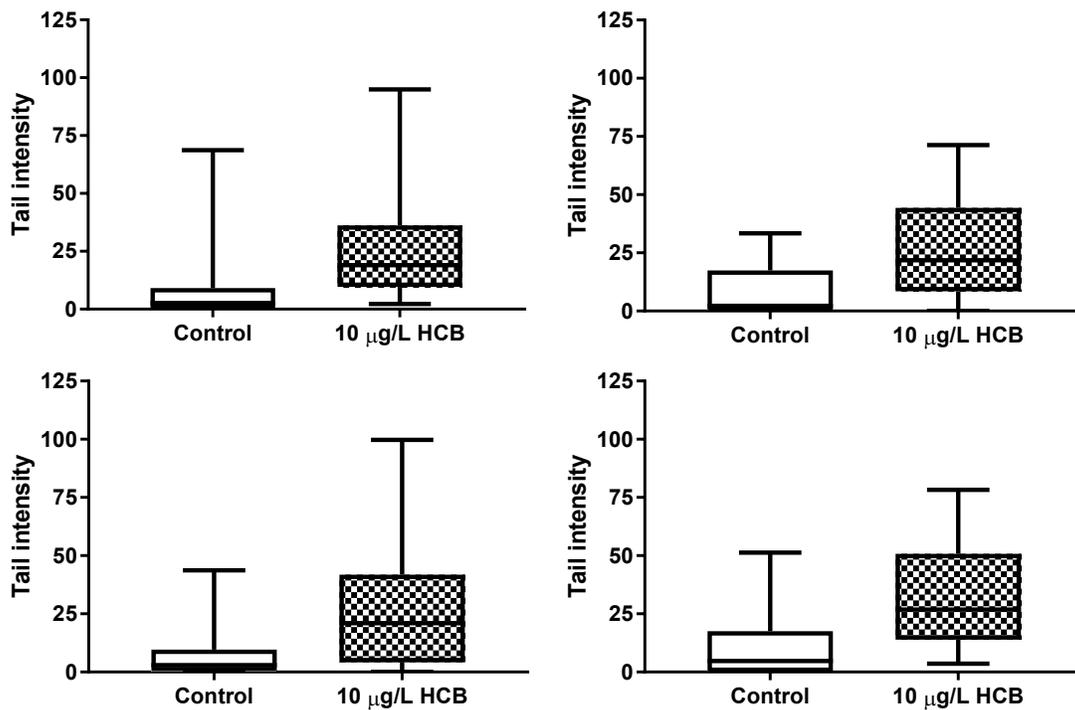
239

240 **Figure S8. Visualisation of data obtained in four replicates of the comet assay.** Four biological replicates of the
241 comet assay were performed with HuWa$_{TERT}$ cells after exposure to 10 µg/L HCB for three hours during the phase of
242 attachment to well bottoms using pre-equilibrated DMEM/F12 (1% FBS) exposure medium with passive dosing. Each
243 plot represents the median, 1st and 3rd quartile, and whiskers the 5th and 95th percentile, of one biological replicate with
244 100 analysed cells each in the control and the HCB-treated group.

245

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