SUPPORTING INFORMATION

Effects of solvents and dosing procedure on chemical toxicity in cell-based *in vitro* assays

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SUMMARY OF SUPPORTING INFORMATION

EXPERIMENTAL SECTION

Cell culture. The cells were routinely cultured at 19°C in 75 cm^2 cell culture flasks (TPP, Trasadingen, Switzerland) with Leibovitz's L15 culture media (Invitrogen, Basel, Switzerland; *1*), supplemented with 5% fetal bovine serum (Sigma-Aldrich, Buchs, Switzerland) and 1% penicillin/streptomycin solution (Sigma-Aldrich, Buchs, Switzerland; 10 000 U/ml penicillin, 10 mg/ml streptomycin).

The RTgill-W1 cell line appears to have low metabolizing activity. For example, to our knowledge, it does not express inducible cytochrome CYP1A catalytic activity. Together with the fact that RTgill-W1 cells are exposed in our study to chemicals in a very simple medium, L15/ex, which does not favor metabolism, we assume metabolism to play a minor or no role.

For exposure to test chemicals, confluent flasks were used to seed 24-well microtiter plates (TPP, Trasadingen, Switzerland) at an initial cell density of 150 000 cells in each well in 1000 µL of L15 culture media. After 24 h the seeded cells were used for toxicity analyses.

Sampling of exposure medium for chemical analysis. Samples taken for chemical analysis were stored in 1.5 mL sampling vials (VWR, Darmstadt, Germany). In the case of 1,2-DCB and 3,4-DCA, 500 µL of the solvent cyclohexane (Pestiscan, LabScan Analytical Sciences, Dublin, Ireland) were added to each sample vial prior to sampling. Thereafter, 500 µL of the exposure mixtures (at the start of exposure (C_{0h}) and after 24 h (C_{24h}) of exposure) were taken out of the respective wells and were transferred into the sampling vial containing the cyclohexane. The vials were closed and vortexed for 1 min and then stored at -20°C until chemical analysis. In the case of indirect dosing, a sample of the dosing mixture (C_{DM}) was taken directly from the glass vial containing the dosing mixture, right after the 15 min of shaking. The total handling time for sampling was kept as short as possible, especially between pipetting the solvent and adding the dosing mixture, to avoid evaporation of the solvent and the

chemical. In case of SDS, sampling was conducted without the addition of any solvent. Just 500 µL of the respective exposure mixtures were added into auto-sampling vials and stored at -20°C.

Chemical extraction procedure. Vials containing the cyclohexane/medium sample from the 3,4-DCA and 1,2-DCB experiments were kept frozen at -20°C. Before analysis, vials were thawed and shaken for 30 min on a horizontal two dimensional shaker (IKA, Stauffer, Germany). Afterwards, samples were left in static conditions for 15 min, and 100 µL from each vial was transferred to a new vial with an insert for gas chromatography with electron capture detection (GC-ECD) analysis.

For the preparation of the SDS samples, a clean-up procedure was performed before liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Samples were first diluted (1:10) in Millipore water. The resulting solution was cleaned-up by Solid Phase Extraction (SPE) using octadecyl reversed-phase silica (C18) columns (Supelclean™ ENVI™-18, 0.5 g, Supelco, Bellefonte, PA, USA) following a similar procedure as developed for the anionic surfactant linear alkylbenzene sulfonate (*2, 3*). Briefly, the columns were preconditioned with 5 mL of analytical MeOH, followed by 5 mL of Millipore water. Next, the whole sample (10 mL) was transferred and the column was then rinsed with 4 mL of Millipore water. 4 mL of MeOH were used in two steps of 2 mL to elude the SDS from the column. Depending on their nominal concentration, final SPE extracts were further diluted in MeOH-water solution or, in case of no dilution needed, 1 mL of Millipore water was added to get a final eluent composition as the LC-MS eluents.

Chemical analysis of the extracts. Cyclohexane extracts for 3,4-DCA and 1,2-DCB samples were analyzed using GC-ECD. The system consisted of a TriPlus autosampler, a Fisons HRGC 8000 GC, and a Carlo Erba ECD 40 electron capture detector with an ECD 400 controller (all instruments from Thermo Scientific, Rodano, Italy). Helium (150 kPa) was used as a carrier gas. 1.3 µL (for 3,4-DCA samples) or 1 µL (for 1,2-DCB samples) of cyclohexane extract was split injected on a deactivated

uncoated precolumn (1.5 m x 0.53 mm) connected to the fused-silica Restek RTX 5Sil MS separation column of 30 m x 0.25 mm and 0.25 µm coating thickness (Restek, Bellefonte, PA, USA). Isothermal separation was performed at 190 and 115°C for 3,4-DCA and 1,2-DCB samples, respectively. The GC-ECD performance was tested every 10 samples by injection of an external standard with a known solution concentration. Chromatograms were checked by hand after automatic integration (Chromcard 2.2.3. software, Thermo Scientific, Rodano, Italy). Sample concentrations for medium extracts were corrected for recovery (cyclohexane extraction recovery from medium) of 82% ± 1.6% and 87% ± 1.5% for 3,4-DCA and 1,2-DCB, respectively. Limit of quantification for both compounds was 0.04 mg/L.

SDS samples were analyzed on an LC-MS/MS system, API 3000 triple quadruple analyzer (MDS Sciex-Applied Biosystems, Foster City, CA, USA) and with a RP-C18 column (3 µm Econosphere, 50 x 3 mm, Alltech, Deerfield, IL). The interface was a Turbo ion Spray source used in the negative mode, the source temperature was 450°C, the ion-spray voltage was set to –1200 V, and the source gas flow was 7 to 8 L/min. Injection volume of the samples was 10 µL and the flow rate was 0.4 mL/min. SDS was measured by screening of the molecular ion $[M-H]^-$ 265 *m/z* and the daughter ion 97 *m/z*. Eluent and final sample solution composition consisted on 80:20 (v:v) MeOH-water. Quantification was done using the software Analyst 1.4.1 (AB/MDS Sciex Instruments). Calculated sample concentration was corrected by the SPE method recovery (92% ± 4%). Limit of quantification (peak/noise signal≥10) was set to 10 pg/ injection.

Cell viability assay. After removing the exposure solution and washing the cells with 1000 uL PBS (Invitrogen, Basel, Switzerland), 400 µL of the AlamarBlue dye solution (5% (v/v) AlamarBlue in PBS) was added to each well and the plate was incubated for 30 min at 19°C in the dark. The fluorescence was then quantified using the Infinite M200 plate reader (TECAN, Männedorf, Switzerland). The excitation and emission wavelengths used were respectively 530 and 595 nm. The fluorescence readings were recorded as relative fluorescent units (FU) for each well. For the elimination of background

AlamarBlue fluorescence, the FUs from wells without cells (no-cell controls) were subtracted from the FUs from wells with cells. For the determination of the dose-response curves and the corresponding EC_{50} values, the FUs were expressed as a percentage of the FUs in the solvent control wells. The means were calculated and plotted with their standard deviations using GraphPad Prism version 4.03 (GraphPad Software, San Diego, California, USA; http://www.graphpad.com). The concentrations effecting 50% of the cells (EC_{50}) were likewise calculated with GraphPad Prism (see statistical evaluation).

SUPPORTING TABLES

Table S1. Physico-chemical properties of the test chemicals.

test chemical	1,2-DCB	3,4-DCA	SDS
CAS number	95-50-1	95-76-1	151-21-3
log K_{ow}[a]	3.28	2.37	1.60[b]
log H[a] [atm-m^3/mol]	-2.53	-5.98	-6.74
water solubility [mg/L][a]	104.13	1350.9	$1*10^{5}$[(c)]

a - calculated with EPI Suite (version 4.0): http://www.epa.gov/oppt/exposure/pubs/episuite.htm

b - the actual meaning of K_{ow} estimates for surfactants is unclear and hydrophobicity of surfactants will also depend on the medium composition

c - data taken from PhysProp database: http://www.syrres.com/esc/physdemo.htm (experimental value included because of poor predictability of water solubility for the amphiphilic molecule SDS by the EPI Suite algorithm [0.013 mg/L])

SUPPORTING FIGURES

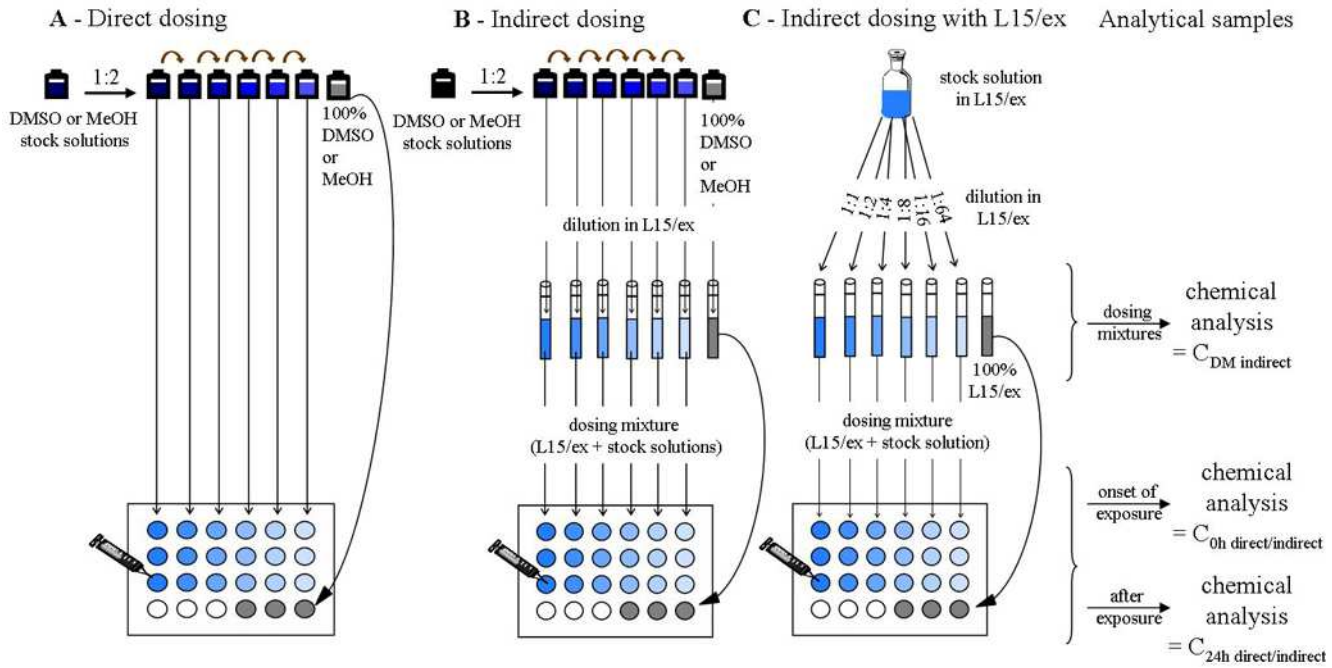

Figure S1. Scheme of chemical dosing procedures and sampling. (A) Direct dosing was conducted when DMSO and MeOH were used as solvents. Stock solutions were added directly into the exposure medium and onto the cells. (B) For indirect dosing with DMSO and MeOH stock solutions, the respective stock solutions were diluted in L15/ex. This dosing mixture was than added onto the cells. (C) In case where the chemicals were dissolved in L15/ex, only one stock solution was prepared. The other dilutions were prepared from this stock solution. The resulting dosing mixtures were added afterwards onto the cells. Samples for chemical analysis were taken as indicated from the dosing mixtures (C_{DM}) and out of the well at 0 h (C_{0h}) and 24 h (C_{24h}).

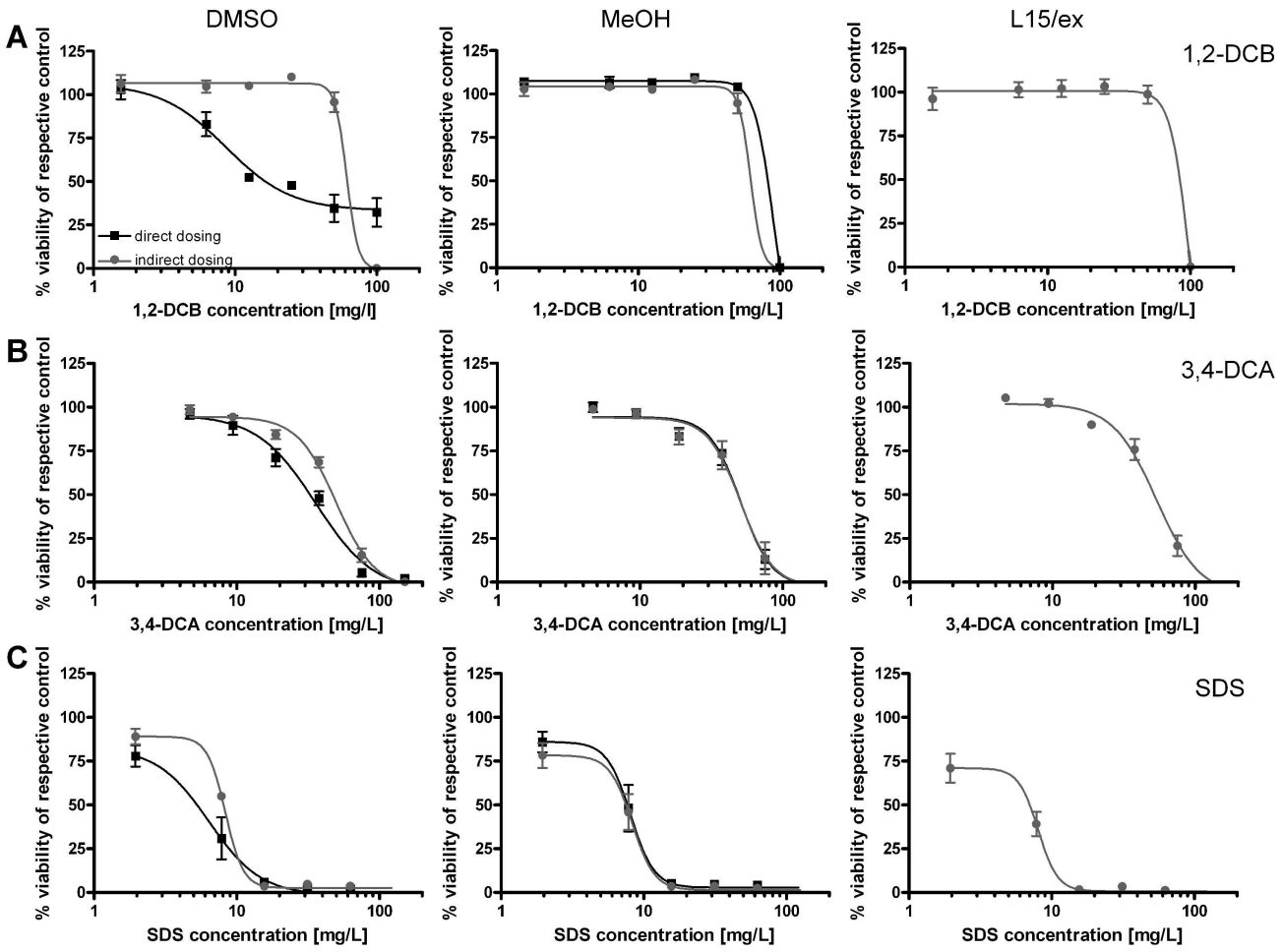

Figure S2. Cell viability of RTgill-W1 cells after 24 h of exposure to 1,2-DCB (A), 3,4-DCA (B) and SDS (C) declines in a concentration-dependent manner. Test chemicals were dissolved either in DMSO (left), MeOH (middle) or L15/ex (right) and dosed directly (closed black squares) or indirectly (closed grey circles) onto the cells. The cell viability as percentage of the control cells are plotted against the nominal chemical concentration. Data points are means of three independent experiments with the vertical lines showing the standard deviation (SD) for cell viability.

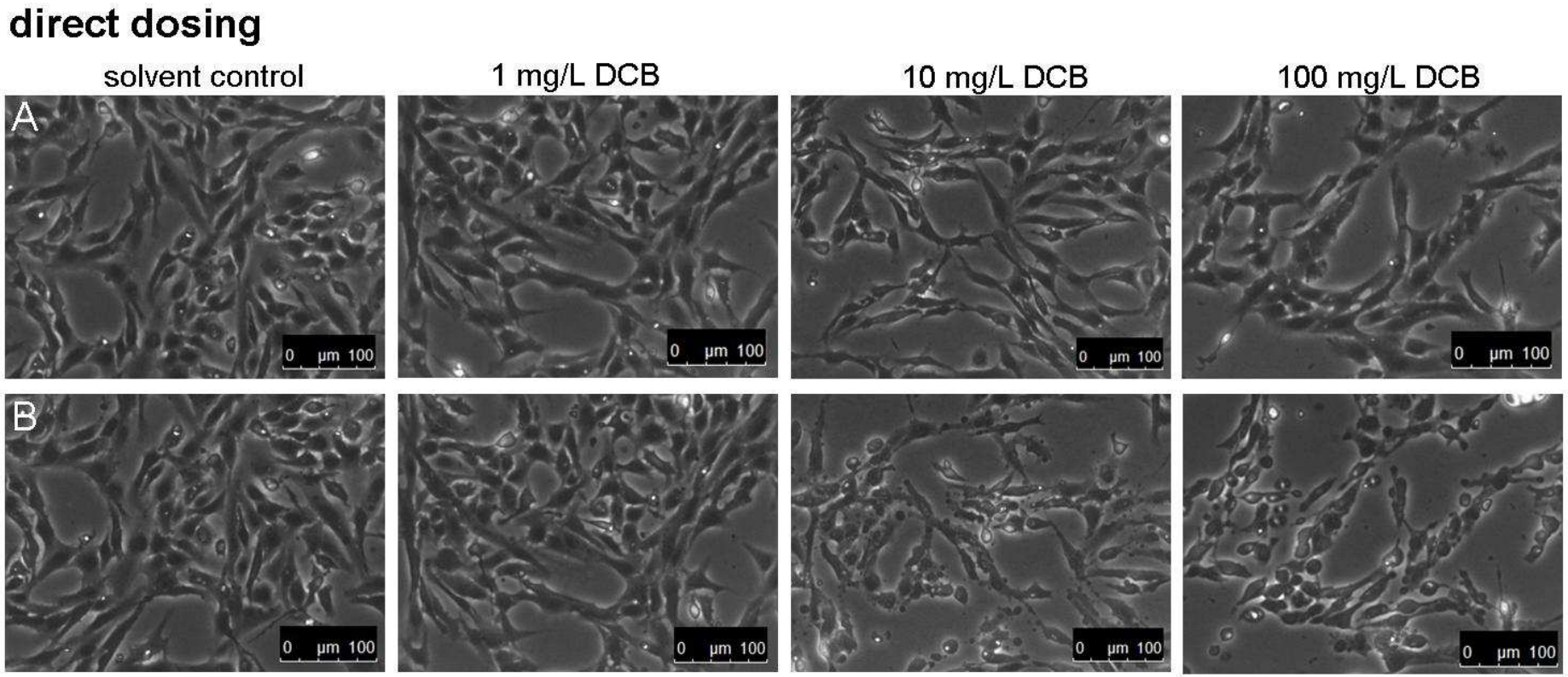

Figure S3. Appearance of RTgill-W1 cells after direct dosing of 1,2-DCB. Cells were exposed to 0.5 % solvent (DMSO), 1, 10 and 100 mg/L 1,2-DCB (in DMSO). Pictures were taken at t = 0 min (A) and t = 5 min (B). After 5 min of exposure, cell damage was clearly visible at 10 mg/L 1,2-DCB, a concentration close to the EC_{50} value, and at 100 mg/L DCB (the highest used test concentration). At these concentrations, cells retracted and/or rounded up, indicating cell damage and rapid cell death. Pictures were taken with the Leica DMI 6000B microscope and at a 100x magnification.

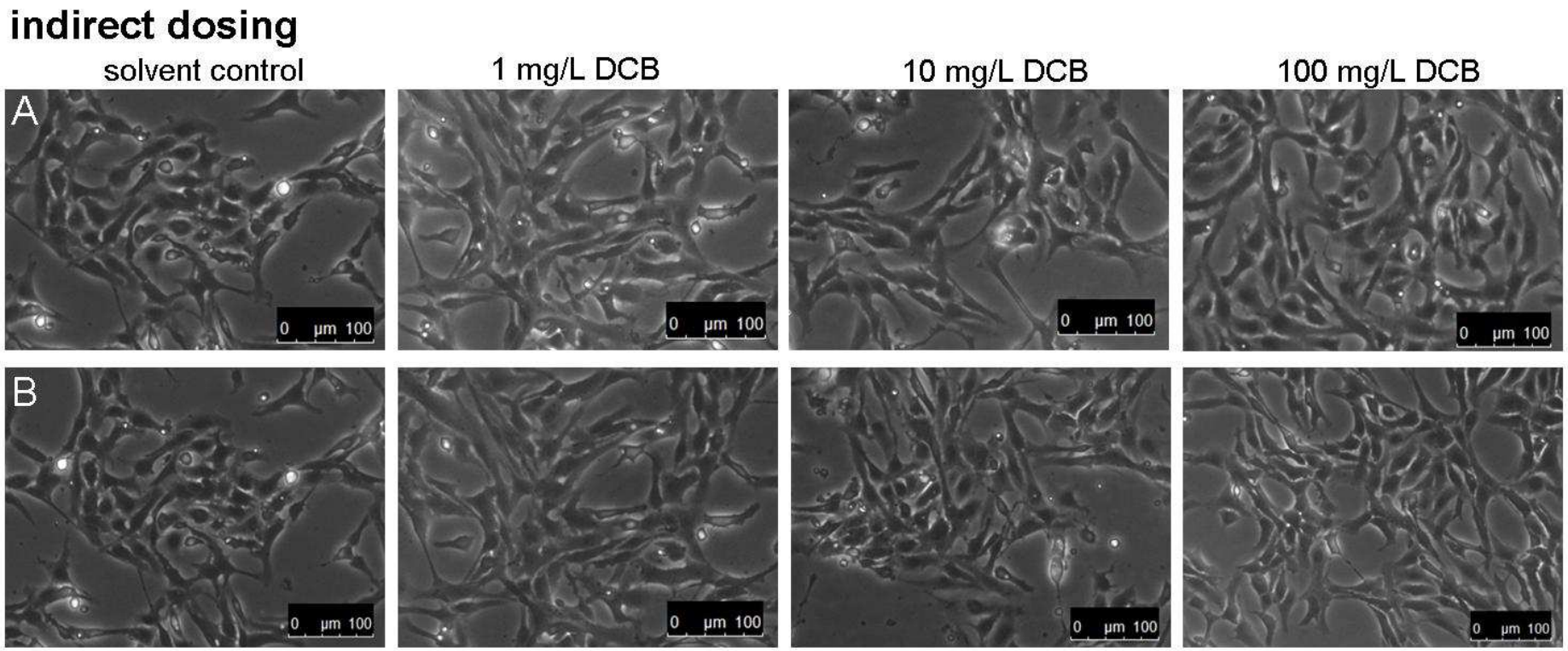

Figure S4. Appearance of RTgill-W1 cells after indirect dosing of 1,2-DCB. Cells were exposed to 0.5 % solvent (DMSO), 1, 10 and 100 mg/L 1,2-DCB (in DMSO). Pictures were taken at t = 0 min (A) and t = 5 min (B). In contrast to direct dosing, no change in appearance of cells was observed for any of the chosen concentrations at either t = 0 or t = 5 min. Pictures were taken with the Leica DMI 6000B microscope and at a 100x magnification.

SUPPORTING REFERENCES

(1) Leibovitz, A. Growth and maintenance of tissue-cell cultures in free gas exchange with atmosphere. *Am. J. Hyg.* **1963**, *78* (2), 173-180.

(2) Rico-Rico, A.; Temara, A.; Behrends, T.; Hermens, J. L. M. Effect of sediment properties on the sorption of C_{12}-2-LAS in marine and estuarine sediments. *Environ. Pollut.* **2009**, *157* (2), 377-383.

(3) Rubio, J. A.; GonzalezMazo, E.; GomezParra, A. Sorption of linear alkylbenzenesulfonates (LAS) on marine sediment. *Mar. Chem.* **1996**, *54* (1-2), 171-177.