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OCSPP, OPPT, ECRAD, RAB6 **Date:** August 26, 2026

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OCSPP, OMCO, RASD, HECSB **Date:** August 26, 2026

EPA Publication Number: EPA 747/R-26-002

DATA EVALUATION RECORD

STUDY TYPE: *In vitro* assessment of point-of-contact respiratory toxicity of surfactants in human cells following a single dose exposure (Non-Guideline)

PC CODE: Not available **DP BARCODE:** Not available

TEST MATERIAL (CONCENTRATION): Triton X-100 (Laboratory Grade)

SYNONYMS: Polyethylene glycol p-(1,1,3,3-tetramethylbutyl)phenyl ether

CITATIONS: Stucki, A. O., Sharma, M., Murray, J. R., Verstraelen, S., Frijns, E., Hollanders, K., Jacobs, A., Luz, A., Remy, S., Roldan, N., Laer, J. V., Yoon, M., Salazar, K., Vinas, N. G. R., Lowit, A., & Clippinger, A. J. (2026). Human in vitro airway models to assess point-of-contact respiratory toxicity of surfactants and derive human equivalent concentrations after single dose exposure. *[Submitted, Under Review]*.

SPONSOR: PETA Science Consortium International

EXECUTIVE SUMMARY:

The purpose of this study was to evaluate the response of BEAS-2B cells and MucilAir tissue to Triton X-100. BEAS-2B is a human bronchial epithelial cell line and MucilAir is an *in vitro* model reconstructed from human respiratory epithelial. These *in vitro* assays represent new approach methodologies (NAMs) for assessing airway irritation. Three MucilAir donor tissues were exposed to 5 concentrations of Triton X-100 ranging from 5 to 80 µg/cm^2 for 24 hours (hrs), while BEAS-2B cells were exposed to 5 concentrations of Triton X-100 ranging from 3 to 15 µg/cm^2 for 24 hrs. Experiments with both *in vitro* models included incubator controls, solvent controls, and positive controls (i.e., lipopolysaccharides [LPS] and isoproterenol hydrochloride [ISO]). Immediately after the 24-hr exposure, BEAS-2B cells and MucilAir tissues were evaluated for cell viability (i.e., resazurin metabolism via PrestoBlue Cell Viability Reagent), cytotoxicity (i.e., lactate dehydrogenase (LDH) release via the LDH Assay Kit-WST), and cytokine secretion (i.e., interleukin-6 [IL-6] and chemokine (CXC) motif ligand 8 [CXCL-8] secretion via the Meso Scale Discovery [MSD] V-PLEX Assay). MucilAir tissues, but not BEAS-2B cells, were also evaluated for barrier integrity using transepithelial electrical resistance (TEER), cilia average active area (AAA) and cilia beating frequency (CBF) via microscopy, and morphological changes via histology. MucilAir tissues, but not BEAS-2B cells, were also

assessed seven days post-exposure to Triton X-100 to assess the reversibility of observed effects following a single chemical application.

BEAS-2B Results: Treatment with Triton X-100 for 24 hrs reduced cell viability (resazurin metabolism) at concentrations of 9 μg/cm^2 and above, and increased cytotoxicity (LDH release) at concentrations of 12 μg/cm^2 and above. IL-6 and CXCL-8 secretion was significantly increased at 9 μg/cm^2 Triton X-100. IL-6 and CXCL-8 secretion was not reported at the highest two concentrations of Triton X-100 (12 and 15 μg/cm^2) due to low cell viability (<10% of control). All observed effects in BEAS-2B cells occurred in a concentration-dependent manner. Based on benchmark dose/concentration (BMD/BMC) modeling, the most sensitive endpoint was IL-6 secretion ($BMC_{1SD}/BMCL_{1SD}$ = 5.77/3.88 μg/cm^2), followed by CXCL-8 secretion ($BMC_{1SD}/BMCL_{1SD}$ = 6.90/5.16 μg/cm^2), LDH release ($BMC_{1SD}/BMCL_{1SD}$ = 7.12/5.49 μg/cm^2), and cell viability ($BMC_{1SD}/BMCL_{1SD}$ = 6.53/6.24 μg/cm^2).

MucilAir Results: A biphasic response to Triton X-100 was observed for resazurin metabolism, a biomarker of cell viability, with resazurin metabolism increasing approximately 28% at 10 μg/cm^2 Triton X-100, before returning to control levels at 20 μg/cm^2, and then significantly declining to 28 and 11% of control values at the highest two concentrations (40 and 80 μg/cm^2). Cytotoxicity (LDH release) was significantly increased in a concentration-dependent manner at the highest three concentrations of Triton X-100 (20, 40 and 80 μg/cm^2). IL-6 and CXCL-8 secretion increased starting at the lowest concentration of Triton X-100 (5 μg/cm^2). Cytokine secretion was not reported in MucilAir at the highest two concentrations of Triton X-100 (40 and 80 μg/cm^2) due to low cell viability (11–28% of control). IL-6 secretion increased in a concentration dependent manner with the largest response (107-fold increase) occuring at the highest concentration of Triton X-100 evaluated (20 μg/cm^2), while CXCL-8 secretion increased from 5 to 10 μg/cm^2, before plateauing at 20 μg/cm^2 Triton X-100. TEER measurements decreased in a concentration-dependent manner starting at 10 μg/cm^2 Triton X-100. CBF and cilia AAA were both significantly reduced starting at 20 μg/cm^2 Triton X-100. However, CBF and AAA responses were highly variable. Variability in cilia AAA and CBF responses are likely related to the high levels of cytotoxicity and reduced cell viability observed at the two highest concentrations of Triton X-100 (40 and 80 μg/cm^2), and as noted by study authors cilia AAA and CBF measurements reported at these concentrations should be interpreted with caution.

Based on BMC modeling, the two most sensitive endpoints in MucilAir were IL-6 secretion ($BMC_{1SD}/BMCL_{1SD}$ = 1.79/1.15 μg/cm^2) and CXCL-8 secretion ($BMC_{1SD}/BMCL_{1SD}$ = 3.59/2.94 μg/cm^2), followed by TEER ($BMC_{1SD}/BMCL_{1SD}$ = 5.87/4.31 μg/cm^2), CBF ($BMC_{1SD}/BMCL_{1SD}$ = 9.11/4.98 μg/cm^2), LDH release ($BMC_{1SD}/BMCL_{1SD}$ = 11.7/7.81μg/cm^2), and cilia AAA ($BMC_{1SD}/BMCL_{1SD}$ = 15.3/9.33 μg/cm^2). No models adequately fit the MucilAir cell viability (resazurin metabolism) dataset, which may be due to the observed biphasic dose-response for this endpoint.

This *in vitro* study evaluating airway irritation is Acceptable/Non-Guideline. It was developed in support of a NAM for refining inhalation risk assessment for contact irritants.

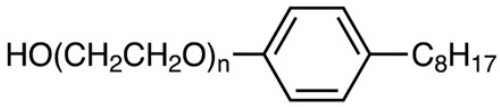
COMPLIANCE: Good Laboratory Practice (GLP) regulations are not applicable for this non-guideline study.

I. MATERIALS AND METHODS

A. MATERIALS:

1. Test Material:

Identification: Triton X-100
Physical Description: Colorless viscous liquid
Supplier: Sigma-Aldrich, Saint Louis, Missouri, USA
Batch #: WXBD9346V
Retest Date: Per manufacturer certificate of analysis: 10/14/2022 (quality release date); 10/12/2024 (date retested); 10/2026 (recommended retest date)
Triton X-100 Concentration: Laboratory Grade
Storage Conditions: Room Temperature
CAS #: 9002-93-1



2. Other Reagents:

2.1. Positive Control:
Identification: Lipopolysaccharides (LPS) from Escherichia coli O111:B4
Physical Description: White lyophilized powder
Product #: L4391
Batch #: 0000252542
Supplier: Sigma Aldrich (Saint Louis, Missouri, USA)
Expiration Date: July 2029
CAS # 93572-42-0

2.2. Positive Control:
Identification: Isoproterenol hydrochloride
Physical Description: White solid
Product #: PHR2722
Batch #: LRAD5082
Supplier: Merck KGaA (Darmstadt, Germany)
Expiration Date: February 27, 2027
Concentration: Neat
CAS # 51-30-9

2.3. Vehicle Control:
Identification: BEBM™ Bronchial Epithelial Cell Growth Medium
Catalogue #: CC-3171
Lot #: Not available
Supplier: Lonza (Basel, Switzerland)
Expiration Date: Not available

2.4. Vehicle Control:
Identification: Saline Solution (0.9% sodium chloride (NaCl), 1.25 mM calcium chloride ($CaCl_2$), 10 mM HEPES)
Catalogue #: $CaCl_2$: 1.42002
NaCl: S7653
HEPES: 15630080
Batch #: Self-made/mixed
Supplier: $CaCl_2$ and NaCl: Sigma-Aldrich, Saint Louis, Missouri, USA
HEPES: Thermo Fisher Scientific, Waltham, Massachusetts, USA
Expiration Date: 4 weeks after preparation

3. **Test System:** BEAS-2B cells (Catalog # CRL-9609) were obtained from the American Type Culture Collection (ATCC) (Manassas, Virginia, USA). Culture medium, including BEBM™ Bronchial Epithelia Cell Growth Basal Medium (Catalog # CC3171) and BEGM™ Bronchial Epithelial Cell Growth Medium BulletKit™ (Catalog # CC3170) was obtained from Lonza (Basel, Switzerland).

4. **Test System:** MucilAir primary human bronchial epithelial cells (Catalog # EP01MD) from three non-smoking donors (Table 1) and MucilAir culture medium (Catalog # EP05MM) was obtained from Epithelix Sàrl (Genevea, Switzerland).

Table 1. Donor Information Provided for MucilAir Tissues by the Manufacturer

Experimental Run	Sex	Age	Pathology Reported	Smoker	Morphology
22/05/2024	Male	42	None	No	Normal with some mucus vesicles
18/6/2024	Male	17	None	No	Normal
02/07/2024	Female	64	None	No	Normal
Table from supplementary document associated with Stucki et al.					

5. **Cell Viability Assay:** PrestoBlue™ Cell Viability Reagent (Catalog # A13261) was obtained from Thermo Fisher Scientific (Waltham, Massachusetts, USA).

6. **Cytotoxicity Assay:** The Cytotoxicity LDH Assay Kit-WST (Catalog # Ck12-05) was obtained from Tebu-bio (Le Perray-en-Yvelines, France).

7. **Interleukin (IL)-6 and CXCL-8 Assay:** The Meso Scale Discover (MSD) V-PLEX Custom Human Biomarkers Proinflammatory Panel (human): Human IL-2, Human IL-6, Human CXCL-8, and Human TNF-α assay (Catalog # K151A9H-2) was obtained from Meso Scale Diagnostics (Rockville, Maryland, USA).

8. **Transepithelial Electrical Resistance (TEER) Assay:** The EVOM Manual voltohmmeter (Catalog # EVM-MT-03-01) and EVOM STX4 electrodes (Catalog # STX4) were obtained from World Precision Instruments Germany, GmbH, Friedberg, Germany.

9. **Cilia Beating Frequency (CBF) and Average Active Area (AAA):** The Zeiss Axiovert 10 microscope was obtained from Zeiss (Oberkochen, Germany), the Basler acA1300-200µm USB3 video camera was obtained from Ammons Engineering (Clio, Michigan, USA), and Cilia-X software (CiliaX V1.0) was obtained from Epithelix Sàrl, (Geneva, Switzerland).

10. **Fixation for Histology:** 4% Formaldehyde solution (Catalog # F2006) was obtained from Avantor (Radnor, Pennsylvania, USA).

B. METHODS

1. **Test System (BEAS-2B):** BEAS-2B cells are normal, immortalized human bronchial epithelial cells. The cell line was established in 1988 by immortalizing non-tumorigenic human bronchial cells with an adenovirus 12-SV40 virus. Cells were cultured in Bronchial Epithelial Cell Growth Medium (BEGM), which consists of Bronchial Epithelial Growth Basal Medium

(BEBM) supplemented with BEGM BulletKit. Although bovine pituitary extract is a component of the BEGM BulletKit, it was not used in this study because it is not necessary to culture BEAS-2B cells. Cells were cultured in T-75 culture flasks (Greiner Bio-One nv, Kremsmunster, Austria; Catalog # 658170) coated with 4.5 mL (0.06 mL/cm^2) of 10 µg/mL human plasma fibronectin (Sigma-Aldrich, Saint Louis, Missouri, USA; Catalog # F2006), 30 µg/mL VitroCol (Sigma-Aldrich, Saint Louis, Missouri, USA; Catalog # 5008), and 10 µg/mL human serum albumin (Sigma-Aldrich, Saint Louis, Missouri, USA; Catalog #A9731) dissolved in BEBM. Cells were cultured in a humidified incubator at 37°C and 5% atmospheric carbon dioxide (CO_2) and were sub-cultured before reaching 80% confluence using 1× TrypLE Express Enzyme (Thermo Fisher Scientific, Waltham, Massachusetts, USA; Catalog # 12604013). Culture medium was refreshed every 2-3 days and cells were sub-cultured every 4-5 days (1,500-3,000 cells/cm^2). Cells were used until passage 20. Prior to exposure to Triton X-100 and controls, cells were seeded on a 24-well PET clear insert with pore size of 0.4 µm (SABEU GmbH & Co Kg, Northeim, Germany; Catalog # 932 04 12) at a density of 72,725 cells/cm^2. Inserts were coated with the same coating solution and concentration as used for coating of T-75 culture flasks. After seeding, inserts were placed in sterile 24-well plates (Geiner Bio-One nv, Kremsmunster, Austria; Catalog # 662160) with 200 µL and 600 µL of BEGM in the apical and basolateral compartments, respectively. Right before exposure, cells were airlifted by removing apical medium and adding fresh medium basolaterally.

2. **Test System (MucilAir):** MucilAir is derived from human airway cells collected from healthy donors. The culture process reconstructs a functional model of the human airway epithelium, exhibiting a pseudostratified, ciliated epithelium which secretes mucus. The MucilAir tissues used in this study were derived from three healthy donors (Table 1). Upon delivery, MucilAir tissues were incubated (37°C, 5% CO_2) for one hour (hr). Cells were then removed from the nutritive gel used during transport and transferred to new 24-well plates (Geiner Bio-One nv, Kremsmunster, Austria; Catalog # 662160) containing 700 µL of prewarmed MucilAir Medium at 37°C. MucilAir cells were then incubated (37°C, 5% CO_2) for 48–72 hrs before basal medium was changed with 700 µL of fresh basal medium and mucus was removed from each MucilAir insert via apical washing with MucilAir medium. To remove mucus, 200 µL of warm (37°C) medium was added to the apical side of each insert and then incubated at 37°C for 10-20 minutes. Following incubation, 100 µL of apical medium was gently pipetted up and down to remove mucus from the cells surface, before all apical medium was removed by aspiration. Cells were incubated for an additional 72 h (i.e., 24 h before exposure to Triton X-100), and then cilia beat frequency (CBF) and cilia average active area (AAA) were evaluated before mucus washing. After mucus washing, TEER was evaluated. Basal medium (700 µL) was then changed and cells were incubated (37°C, 5% CO_2) for an additional 24 hrs before exposure to Triton X-100.

3. **Preparation of Triton X-100 Dosing Solutions:** For experiments with BEAS-2B cells, five concentrations of Triton X-100 (i.e., 51.0, 102.0, 153.1, 204.1, 255.2 µM; equivalent to 3, 6, 9, 12, 15 µg/cm^2) were prepared from a stock solution (255 µM or 0.165 mg/mL) (Table 2). For experiments with MucilAir cells, five concentrations of Triton X-100 (i.e., 85.0, 170.1, 340.1, 680.2, 1,360.4 µM; equivalent to 5, 10, 20, 40, 80 µg/cm^2) were prepared from a stock solution (1.36 mM or 0.879 mg/mL) (Table 2). From the stock solutions, serial dilutions were prepared in BEBM (for BEAS-2B exposures) or saline (for MucilAir exposures) and mixed by vortexing. BEBM (BEAS-2B) and saline (MucilAir) were used as the solvent controls for all Triton X-100 exposures. The exposure concentrations were selected based on a range-finding experiment. Data from the range-finding experiment were not presented by Stucki et al., 2026.

4. **Analysis of Triton X-100 Dosing Solutions:** All dilutions of Triton X-100 were analytically verified using ultra performance liquid chromatography (UPLC)-tandem mass spectrometer (UPLC-MS) using a Waters H-class Acquity UPLC system (Waters, Milford, Massachusetts, USA). 100 µl of all dilutions were sampled, diluted 10× in ultrapure water/methanol and vortexed before analysis. Two microliters of each sample were used for injection and each sample was analyzed in duplicate. Triton X-100 was separated on an Acquity UPLC BEH C18 column (100 mm × 2.1 mm; 1.7 µm) at 50 °C. The mobile phase consisted of ultrapure water (solvent A) and methanol / acetonitrile with 2 mM ammonium acetate (1/1 v/v; solvent B), and both solvents A and B were acidified with 0.1% acetic acid. The gradient was programmed from 10% to 100% of solvent B (v/v) in 6 minutes (min) at a 0.4 mL/min flow rate. For chemical identification, the UPLC system was coupled to a Waters Xevo tandem mass spectrometer (Waters, Milford, Massachusetts, USA), which was operated in the positive electrospray ionization mode (ESI+). Triton X-100 was identified using a selected ion recording (SIR) mode and its LC retention time. Quantification was conducted with an external calibration method. The limit of quantification (LOQ) was 0.25 µg/mL. Samples were analyzed in batches of 10. Every analysis batch contained the necessary quality control samples, including procedure blank, spiked water sample, spiked sample (trueness), and sample duplicate analysis (intra-day precision). Nominal and analytically measured concentrations of Triton X-100 dosing solutions are provided in Table 2.

Table 2. Test Concentrations Used in BEAS-2B Cells and MucilAir for Liquid Exposures

BEAS-2B			MucilAir		
Dilution Conc. (µM)	**Nominal Conc. (µg/cm²)**	**Measured Conc. (µg/cm²)**	**Dilution Conc. (µM)**	**Nominal Conc. (µg/cm²)**	**Measured Conc. (µg/cm²)**
51.0	3	2.2 ± 0.38	85.0	5	4.2 ± 0.67
102.0	6	5.5 ± 0.76	170.1	10	9.1 ± 0.32
153.1	9	7.9 ± 0.78	340.1	20	18.6 ± 2.72
204.1	12	13.9 ± 2.21	680.2	40	37.8 ± 2.57
255.1	15	16.4 ± 1.18	1360.4	80	77.9 ± 4.61
Measured Concentration Displayed as Mean ± Standard Deviation. Data from Table 2 in Stucki et al.					

5. **Treatment/Exposure (BEAS-2B):** Immediately before exposure, cells were checked for confluency using a conventional inverted microscope. BEGM was then completely removed from the apical and basolateral sides of all the inserts, and 600 µL of fresh BEBM (without growth factors) was added basolaterally. 30 µL of test solution was pipetted on the apical surface of the cells and the tissues were then placed in the incubator for 24 hrs. After 24 hrs of exposure, 200 µL basolateral medium was collected for assessment of cytotoxicity (LDH assay). The remaining volume was frozen and stored at -80°C for later assessment of cytokine (IL-6 and CXCL-8) secretion. Apical wash solutions were not stored for analysis. The inserts were then transferred to a new, sterile 24-well plate prefilled with warm 600 µL fresh BEBM. Three biologically independent experimental runs, with three replicates per treatment, using different cell passages, were performed for each exposure condition.

6. **Treatment/Exposure (MucilAir):** 24 hrs before exposure, CBF, AAA, and TEER were measured for quality control purposes and mucus was removed. Immediately before exposure, each tissue insert was inspected under a conventional inverted microscope to ensure the quality of the epithelia. Subsequently, tissues were exposed by pipetting 30 µL of test solution on the apical surface of MucilAir and then placed in the incubator for 24 hrs. After 24 hrs of exposure, 200 µL basolateral medium was collected for cytotoxicity assessment (LDH assay). The

remaining medium was stored at -80°C for later assessment of cytokine (IL-6 and CXCL-8) secretion. The inserts were then transferred to a new, sterile 24-well plate prefilled with 700 µL MucilAir medium. Next, CBF and AAA were measured, before the inserts were washed apically 3 times with 37°C warm saline solution. Apical wash solutions were not stored for analysis. TEER was measured after apical washing. Two out of four tissues were used for assessing cell viability (PrestoBlue assay) and afterwards fixed for histology. The other two remaining tissues were kept for an additional 6 days (i.e., 7 days post-exposure). Medium was changed at days 3 and 6 post-exposure and inserts were apically washed with saline solution to remove mucus. At 7 days post-exposure, basolateral medium was collected and stored at -80°C for cytokine (IL-6 and CXCL-8) measurements. CBF, AAA, and TEER were then measured. The tissues were then used for assessing cell viability (PrestoBlue assay) and then fixed for histology. Three biologically independent runs, using a different single donor in each run (see Table 1 for donor information), were performed for each exposure condition. Each run included 4 replicates of tissues from a single donor per treatment group.

7. **Cell Viability Assay:** Cell viability was assessed via resazurin metabolism (i.e., CYP1A1-1A2 activity) by measuring the ability of BEAS-2B and MucilAir cells to reduce resazurin to resorufin and dihydroresorufin using the PrestoBlue Cell Viability Reagent according to the manufacturer's instructions with some modifications for the air-liquid interface (ALI) cell culture format. Cell viability was assessed 24 hrs and 7 days (MucilAir only) post-exposure. After removing the basolateral medium, 200 µL of a 10% v/v PrestoBlue solution in BEGM (for BEAS-2B) or in MucilAir Medium (MucilAir) was added on the apical side of the inserts. After 1 hr in the incubator (at 37°C, 5% CO_2) in the dark, 2 samples/well (90 µL each) were taken from the apical solution of each insert, transferred to a 96-well plate, and fluorescence was measured using a multi-mode microplate reader in fluorescence mode (excitation: 552-22 nm, emission: 590-20 nm). 10% v/v PrestoBlue diluted in the corresponding cell culture media was also incubated for 1 hr at 37°C in the dark and used as a blank.

8. **LDH Release Cytotoxicity Assay:** Cytotoxicity was assessed in BEAS-2B and MucilAir cells after 24 hrs and 7 days (MucilAir only) of exposure. Cytotoxicity was assessed using the LDH Assay Kit-WST. After 24 hrs of exposure, 2 × 100 µL of the basolateral medium (two technical replicates) were transferred to a 96-well plate and used for the LDH assay. Basolateral samples from the insert(s) treated with the positive control [lysis solution included in the LDH assay kit; 50 µL incubated for 18 hrs (BEAS-2B) or 3 hrs (MucilAir)] and blanks (BEBM for BEAS-2B and MucilAir Medium) were also transferred to the 96-well plate. Subsequently, 100 µL LDH substrate mix were added to each sample well and incubated for 30 minutes at room temperature in the dark. The reaction was stopped by the addition of 50 µL stop solution. Absorbance measurements were conducted using a multi-mode microplate reader (wavelength: 490 nm).

9. **IL-6 and CXCL-8 Secretion:** Secretion of pro-inflammatory markers (IL-6 and CXCL-8) was evaluated 24 hrs and 7 days (MucilAir only) post-exposure. IL-6 and CXCL-8 secretion was evaluated using the Meso Scale Discovery (MSD) V-PLEX Assay according to manufacturer instructions. The assay uses an electrochemiluminescent detection method and is a highly sensitive multiplex enzyme-linked immunosorbent assay (ELISA). Calibration curves were employed to determine the cytokine concentrations, which are expressed in pg/mL. 24 hrs or 7 days post-exposure (MucilAir only), basolateral medium was collected and stored at -80°C until analysis using the MSD assay. LPS was included as a positive control for cytokine secretion in each experimental run. Briefly, BEAS-2B cells were exposed to 20 µg/mL LPS

and MucilAir tissues were exposed to 200 µg/mL LPS apically for 24 hrs. The basolateral medium was collected 24 hrs post-exposure and stored at -80°C until analysis using the MSD assay.

The pre-coated MSD plate was washed three times. Calibrators and samples were diluted 4-fold (standard curve) and 10-fold (samples) in assay diluent, then added to the MSD plate. The plate was sealed and incubated overnight at 4°C. After another three washes, the detection antibody solution was added to each well. The plate was incubated at room temperature for 2 hrs on an orbital shaker, followed by another three washes. Finally, the read buffer was added, and the plate was analyzed on the MSD instrument. For both IL-6 and CXCL-8, the MSD software calculated the protein concentrations in each sample based on the calibrators that were incubated alongside the samples and applying a weighted, four parameter logistic fit per default. Reported concentrations were then processed further using R. Concentrations below the lower limit of detection (LLOD) were removed. Significant changes in protein concentration were analyzed relative to vehicle control and were assessed by mixed models upon logarithmic transformation (ln) while considering experiment ID (biological replicate) as random factor. R-packages for mixed model analyses "lme4" (Bates et al. 2015)[1] and "lmerTest" (Kuznetsova et al. 2017)[2] were applied. P-value less than 0.05 was used as cut-off for statistical significance.

10. **TEER Measurement (MucilAir only):** TEER measurement was evaluated before exposure, as well as 24 hrs and 7 days post-exposure. The EVOM Manual voltohmmeter was used with STX4 EVOM electrode. The voltohmmeter was calibrated using the 99673-test resistor and the electrodes were calibrated using KCl (40 mM, 80 mM, and 160 mM) solutions at room temperature. The electrode was washed with 70% ethanol before use and wiped. For measurement, MucilAir Medium was used as electrolyte solution and equilibrated at 37°C for 30 minutes. The electrodes were equilibrated in MucilAir Medium for 5 minutes before measurement. The MucilAir tissues were removed from the incubator or, if measured after exposure, they were processed for TEER measurement after CBF/AAA measurement and apical washing steps. The medium was exchanged (200 µL MucilAir Medium apical and 700 µL basolateral) and inserts were placed back in the incubator for 5 minutes. After the incubation time, the inserts were transferred to the laminar flow hood, a timer was set to record the duration for which the test system was out of the incubator, and the TEER was measured. The electrodes were rinsed with MucilAir Medium between measurements for each experimental group. After measurement, the electrolyte solution was removed via pipetting, air liquid interface was restored apically and 700 µL fresh MucilAir Medium was added to the basolateral side of the insert before putting it back in the incubator. The inserts were outside the incubator for 3 to 4 minutes.

11. **Cilia Beating Frequency (CBF) and Average Active Area (AAA) (MucilAir only):** Cilia AAA and CBF within active areas only (>1.5 Hz) was measured 24 hrs and 7 days post-exposure. On each MucilAir insert, two microscopic fields were measured with a 10× objective on a Zeiss Axiovert microscope (Zeiss, Jena, Germany) equipped with a stage-top incubator system (Chamlide WP-S-10F with air mixer CU-501, Microsystem, Sweden) and a Basler

[1] Bates, D., Mächler, M., Bolker, B. M., and Walker, S. C. (2015). Fitting Linear Mixed-Effects Models Using lme4. *J. Stat. Softw.* 67, 1–48. doi:10.18637/JSS.V067.I01.

[2] Kuznetsova, A., Brockhoff, P. B., and Christensen, R. H. B. (2017). lmerTest Package: Tests in Linear Mixed Effects Models. *J. Stat. Softw.* 82, 1–26. doi:10.18637/JSS.V082.I13.

acA1300-200 µm USB3 video camera. A 5-second video, 100 frames per second, 512 frames was taken and analyzed with the CiliaX software (CiliaX V1.0). All measurements were recorded at 37ºC. Results are provided as percentage cilia AAA and CBF (Hz).

Isoproterenol (ISO) was included as a positive control for CBF (i.e., ISO is known to increase CBF) in each experimental run for the 24 hr and 7 day time points. ISO was administered as a liquid exposure. MucilAir tissues were exposed to 50 µM ISO apically for 2 hrs before CBF was measured.

Study authors reported that a data handling error caused some data loss for the AAA and CBF endpoints, resulting in a single technical replicate (n = 1) for the 5 µg/cm^2 Triton X-100 exposed tissues of Donor 2 and resulting in loss of the ISO positive control data for Donor 2 at the 24 hr time point. No other samples were reported to be affected by the data handling error.

12. **Histology (MucilAir only):** Two inserts per treatment were processed for histology. Inserts were washed 3 times for 5 minutes in PBS+ (PBS Ca^{+2}, Mg^{+2}) and fixed for 30 minutes at room temperature using 4% formaldehyde solution (0.4 mL apical and 0.4 mL basolateral). After fixation, the inserts were washed 3 times for 5 minutes each in PBS+ in a new 24-well plate. After washing, the inserts were transferred to a 25 mL tube filled with 25 mL PBS+ at 4°C and shipped to Cerba Research (France) for processing. After embedding, the samples were sectioned at 3µm ±1 thickness and dried for at least 2 hrs in a ventilated oven at 45°C (±5°C) before proceeding to staining (Alcian Blue/ Periodic Acid Schiff/ Hematoxylin). The slides were digitized using the NANOZOOMER-S360 scanner in bright field mode with a 20× objective. No z-stacks were acquired.

C. DATA EVALUATION

A. Endpoint Calculations

1. **PrestoBlue Cell Viability Measurement:** Measured fluorescence readings (RFI) were corrected by subtracting the mean value of background controls that were included on each plate (resazurin solution in cell-free environment to determine spontaneous resorufin reduction). Resazurin metabolism was expressed relative to the vehicle control after correcting for background. Significant changes in percent cell viability compared to vehicle control were assessed by mixed models considering experiment ID (biological replicate) as the random factor. The R-packages for mixed model analyses "lme4" (Bates et al., 2015) and "lmerTest" (Kuznetsova et al., 2017) were applied. P-value less than 0.05 was used as cut-off for statistical significance.

$$Resazurin\ Reduction = 100\%\ x\ \frac{RFI_{Sample} - RFI_{Blank}}{RFI_{Average\ Vehicle\ Control\ per\ Experimental\ Day} - RFI_{Blank}}$$

2. **LDH Release:** Measured absorbance values (OD) were corrected for the background absorbance of cell culture media by subtracting the mean absorption of cell culture media (i.e., BEBM for BEAS-2B cells and MucilAir Medium for MucilAir cells) controls included on each plate. LDH release by treated cells was expressed as a fold-change relative to the solvent control:

$$LDH\ Release\ (fold\ change\ rel.to\ vehicle) = \frac{OD_{Sample} - OD_{Blank}}{OD_{Average\ Vehicle\ Control\ per\ Experimental\ Day} - OD_{Blank}}$$

Fold change in LDH release levels relative to vehicle control exposure was calculated prior to statistical analysis assessed by mixed models upon logarithmic transformation (ln) while considering experiment ID (biological replicate) as the random factor. R-packages for mixed model analyses "lme4" (Bates et al., 2015) and "lmerTest" (Kuznetsova et al., 2017) were applied. P-value less than 0.05 was used as cut-off for statistical significance.

3. **TEER Measurement (MucilAir Only):** Resistance of the cells was (R_{cells}) was calculated by subtracting blank resistance (R_{blank}) [100 $\Omega\ cm^2$] from total measured resistance (R_{total}).

$$R_{cells} = R_{total} - R_{blank}$$

TEER was then calculated by multiplying R_{cells} by the cell culture insert surface area (SA; 0.33 cm^2).

$$Corrected\ TEER\ (\Omega) = R_{cells}\ (\Omega)\ x\ SA\ (cm^2)$$

Significant changes in TEER compared to the vehicle control exposure for a particular time point (i.e., pre-exposure, 24 h post-exposure, 7 days post-exposure) were assessed by mixed models while considering experiment ID (biological replicate) as random factor. R-packages for mixed model analyses "lme4" (Bates et al., 2015) and "lmerTest" (Kuznetsova et al., 2017) were applied. P-value less than 0.05 was used as cut-off for statistical significance.

B. Benchmark Dose (BMD) Modeling

Benchmark dose (BMD) analysis was performed on TEER, viability (resazurin metabolism), cytotoxicity (LDH release), IL-6 and CXCL-8 secretion, ciliary beat frequency (CBF) and average active area (AAA) using BMDS Online (version 25.1) developed by the U.S. EPA (https://bmdsonline.epa.gov/). Fits were performed with normal constant and non-constant variance versions of a linear (unrestricted), power (restricted), polynomial degree 2–6 (restricted), exponential 3 and 5 (restricted), and Hill (restricted) models using default settings for continuous models. A benchmark response (BMR) of 1 control standard deviation from the control mean (BMD_{1SD}) was utilized. Lower 95% confidence intervals of the BMD ($BMDL_{1SD}$) were also calculated and reported. For BMD analysis, technical replicates were averaged (n=2–4 per MucilAir donor or BEAS2B exposure group) and the mean and standard deviation were entered as datapoints into BMDS. Both absolute values and normalized values (% or fold-change normalized to vehicle controls) were analysed. In all cases, data normalization improved BMD_{1SD} model fits and therefore all selected $BMDL_{1SD}$ values were based on normalized data.

The BMDS recommended model was selected as the best model whenever possible, which reflects the best-fitting model consistent with EPA's Benchmark Dose Technical Guidance (https://www.epa.gov/risk/benchmark-dose-technical-guidance). If a constant variance model was deemed appropriate based on the statistical test provided in BMDS (i.e., Test 2 p-value >0.05), the final selected BMD results were estimated from a constant variance model. If the test for homogeneity of variance was rejected (i.e., Test 2 p-value < 0.05), then non-constant variance models, which model the variance as a power function of the mean to account for non-constant variance, were considered. For three endpoints (IL-6 secretion in BEAS-2B

cells, TEER and viability measurements in MucilAir), no model was recommended by BMDS. In the absence of a BMDS recommendation, the best model was selected based on consideration of p-value (goodness of fit, only p-values >0.1 considered), model with lowest Akaike's Information Criterion (AIC) value from models with viable fits (i.e., goodness of fit p-value >0.1), BMDL values, visual fit, and the absolute value of scaled residuals near the control and BMD. Due to the biphasic nature of viability measurements in MucilAir, the data were deemed unsuitable for BMD modeling and NOAEC/LOAEC values were reported instead.

II. RESULTS

Note: Percent change and fold-change values reported in Stucki et al. and this data evaluation record for BEAS-2B cells and MucilAir tissues differ slightly because values calculated by Stucki et al. are based on median values, while calculations in this data evaluation record are based on mean values.

1. Results for BEAS-2B Cells After 24 hrs of Triton X-100 Exposure

1.1 Cell Viability (PrestoBlue – Resazurin Metabolism)

Mean and standard deviation of percentage resazurin metabolism in BEAS-2B cells following 24 hrs of treatment with Triton X-100 are shown in Table_Apx 1 (Appendix A). Resazurin metabolism was significantly decreased in a concentration-dependent manner at the three highest concentrations of Triton X-100 (i.e., 78%, 5.8%, and 0% of control at 9, 12, and 15 µg/cm^2, respectively) (Figure 1). Resazurin metabolism was comparable to the solvent control at the lowest two concentrations of Triton X-100 (3 and 6 µg/cm^2).

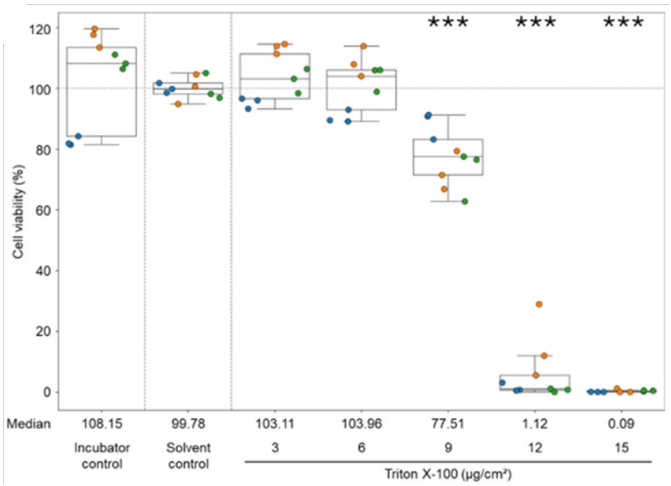


Figure 1. Effect of Triton X-100 Exposure (24 hrs) on Cell Viability (Resazurin Metabolism) in BEAS-2B Cells

From Figure 2 in Stucki et al. The graph shows results from three separate experiments (N = 3) represented in different colors, with three technical replicates (N = 3) included in each experiment. Asterisks (*) show statistically significant differences compared to the solvent control; ***, P < 0.001.

1.2 Cytotoxicity (LDH Release)

Mean and standard deviation of fold-change in LDH release in BEAS-2B cells following 24 hrs of treatment with Triton X-100 are shown in Table_Apx 2 (Appendix A). Compared to the

solvent control, LDH release was slightly decreased (0.75-fold) at the lowest concentration of Triton X-100 (3 µg/cm^2), and increased at the highest two concentrations of Triton X-100 (2.6-fold and 3.2-fold at 12 and 15 µg/cm^2, respectively) (Figure 2). LDH release was comparable to the solvent control at concentrations of 6 and 9 µg/cm^2 Triton X-100. The lysis solution positive control gave expected results, increasing LDH release compared to the solvent control.

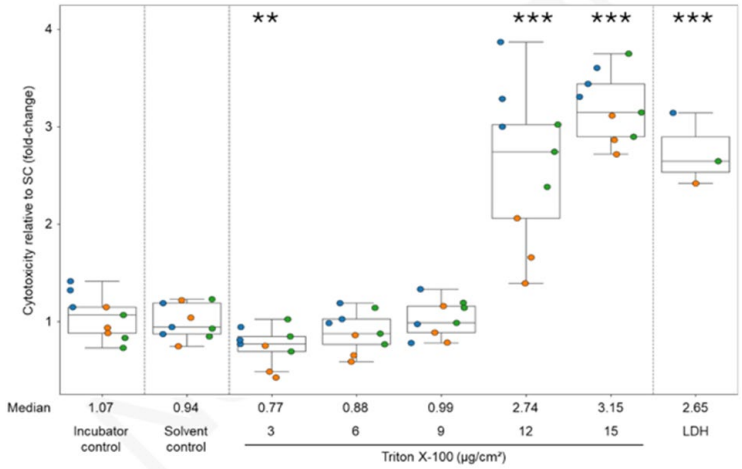


Figure 2. Effect of Triton X-100 Exposure (24 hrs) on Cytotoxicity (LDH Release) in BEAS-2B Cells

From Figure 2 in Stucki et al. The graph shows results from three separate experiments (N = 3) represented in different colors, with three technical replicates (N = 3) included in each experiment. Asterisks (*) show statistically significant differences compared to the solvent control; **, P < 0.01; ***, P < 0.001.

1.3 IL-6 and CXCL-8 Secretion

Mean and standard deviation of fold-change in IL-6 and CXCL-8 secretion in BEAS-2B cells following 24 hrs of treatment with Triton X-100 are shown in Table_Apx 3 and Table_Apx 4, respectively (Appendix A). IL-6 and CXCL-8 secretion was not included in the analysis at the highest two concentrations of Triton X-100 (12 and 15 µg/cm^2) due to low cell viability (< 10% of the solvent control at both concentrations). Compared to the solvent control, IL-6 and CXCL-8 secretion increased 7.8-fold and 4.8-fold, respectively, at 9 µg/cm^2 Triton X-100 (Figure 3 and Figure 4). IL-6 and CXCL-8 secretion at the lowest two concentrations of Triton X-100 (3 and 6 µg/cm^2) were comparable to the solvent controls. The positive control (LPS) gave expected results, significantly increasing IL-6 and CXCL-8 secretion compared the solvent control.

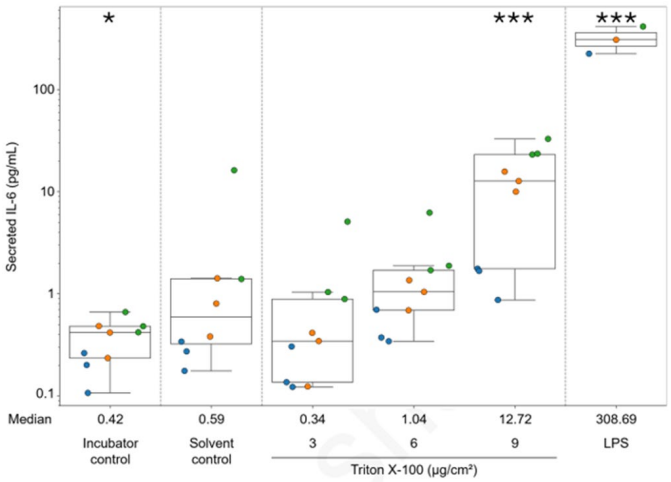


Figure 3. Effect of Triton X-100 Exposure (24 hrs) on IL-6 Secretion in BEAS-2B Cells
From Figure 3 in Stucki et al. The graph shows results from three separate experiments (N = 3) represented in different colors, with three technical replicates (N = 3) included in each experiment. Asterisks (*) show statistically significant differences compared to the solvent control; *, P < 0.05; ***, P < 0.001.

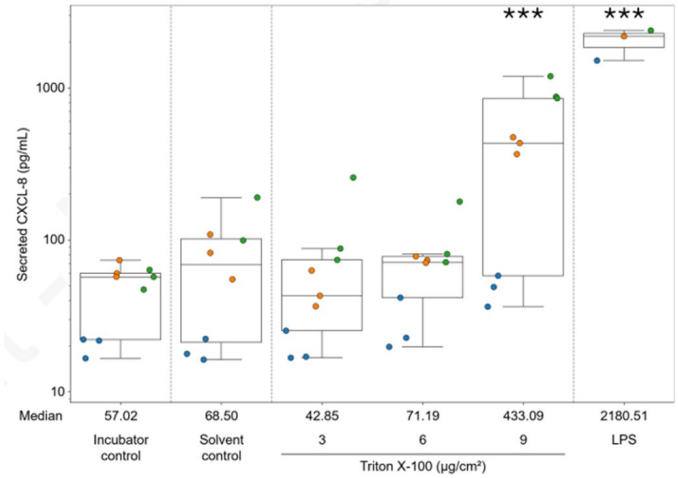


Figure 4. Effect of Triton X-100 Exposure (24 hrs) on CXCL-8 Secretion in BEAS-2B Cells
From Figure 3 in Stucki et al. The graph shows results from three separate experiments (N = 3) represented in different colors, with three technical replicates (N = 3) included in each experiment. Asterisks (*) show statistically significant differences compared to the solvent control; ***, P < 0.001.

2. Results for MucilAir Tissues After 24 hrs of Triton X-100 Exposure

2.1 Cell Viability (PrestoBlue – Resazurin Metabolism)

Mean and standard deviation of percentage resazurin metabolism in MucilAir tissues following 24 hrs of treatment with Triton X-100 are shown in Table_Apx *5* (Appendix A). Resazurin metabolism in MucilAir tissues showed a biphasic response to Triton X-100 exposure (Figure 5). Compared to the solvent control, resazurin metabolism significantly increased (128% of control) at 10 µg/cm^2 and then decreased at the highest two concentrations of Triton X-100 (28% and 11% of control at 40 and 80 µg/cm^2, respectively). Resazurin metabolism was slightly increased (117% of control) at the lowest concentration of Triton X-100 (5 µg/cm^2); however, the increase was not statistically significant. Resazurin metabolism was comparable to the solvent control at 20 µg/cm^2 Triton X-100. Study authors hypothesized that the increase in resazurin metabolism

at 10 µg/cm^2 likely reflects a transient increase in cellular metabolic activity, which can be elevated under stress conditions due to adaptive metabolic responses and induction of redox enzymes such as NQO1 via NRF2 signaling.

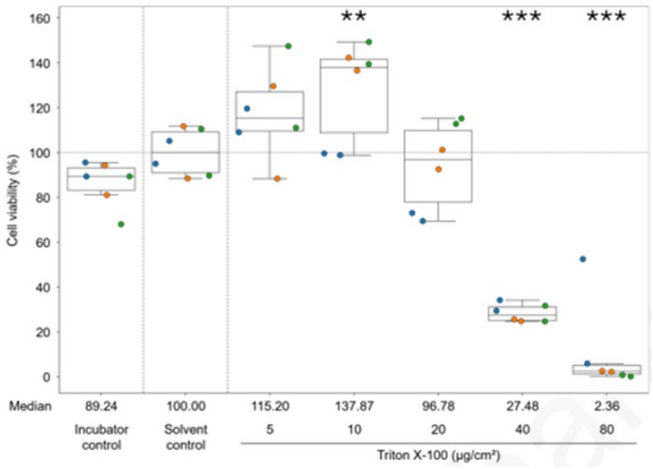


Figure 5. Effect of Triton X-100 Exposure (24 hrs) on Cell Viability (Resazurin Metabolism) in MucilAir

From Figure 4 in Stucki et al. The graph shows results from three separate experiments (N = 3) represented in different colors, with two technical replicates (N = 2) included in each experiment. Asterisks (*) show statistically significant differences compared to the solvent control; **, P < 0.01; ***, P < 0.001.

2.2 Cytotoxicity (LDH Release)

Mean and standard deviation of fold-change in LDH release in MucilAir tissues following 24 hrs of treatment with Triton X-100 are shown in Table_Apx 6 (Appendix A). Compared to the solvent control, LDH release was slightly decreased (0.8-fold) at the lowest concentration of Triton X-100 (5 µg/cm^2), and increased in a concentration-dependent manner at the highest three concentrations of Triton X-100 (i.e., 2.3-, 15-, and 60-fold at 20, 40, and 80 µg/cm^2, respectively) (Figure 6). LDH release was comparable to the solvent control at 10 µg/cm^2 Triton X-100. The lysis solution positive control gave expected results, increasing LDH release compared to the solvent control.

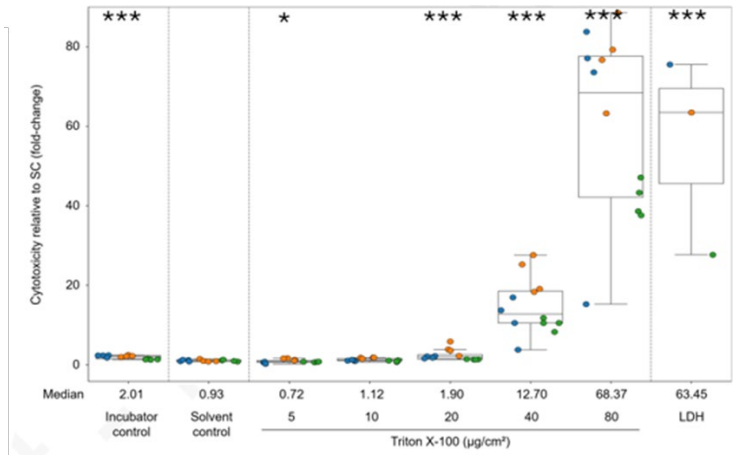


Figure 6. Effect of Triton X-100 Exposure (24 hrs) on Cytotoxicity (LDH Release) in

MucilAir
From Figure 4 in Stucki et al. The graph shows results from three separate experiments (N = 3) represented in different colors, with four technical replicates (N = 4) included in each experiment. Asterisks (*) show statistically significant differences compared to the solvent control; *, P < 0.05; ***, P < 0.001.

2.3 IL-6 and CXCL-8 Secretion

Mean and standard deviation of fold-change in IL-6 and CXCL-8 secretion in MucilAir tissues following 24 hrs of treatment with Triton X-100 are shown in Table_Apx 7 and Table_Apx 8, respectively (Appendix A). IL-6 and CXCL-8 secretion was not included in the analysis at the highest two concentrations of Triton X-100 (40 and 80 µg/cm^2) due to low cell viability (11–28% of the solvent control). Compared to the solvent control, IL-6 secretion increased significantly in a concentration-dependent manner at the three lowest concentrations of Triton X-100 (i.e., 10-, 69-, and 107-fold at 5, 10, and 20 µg/cm^2, respectively) (Figure 7). Similarly, CXCL-8 secretion also increased significantly in a concentration-dependent manner at the three lowest concentrations of Triton X-100 (i.e., 2.1-, 2.4-, and 2.4-fold at 5, 10, and 20 µg/cm^2, respectively) (Figure 8). The positive control (LPS) gave the expected result, significantly increasing IL-6 and CXCL-8 secretion compared the solvent control.

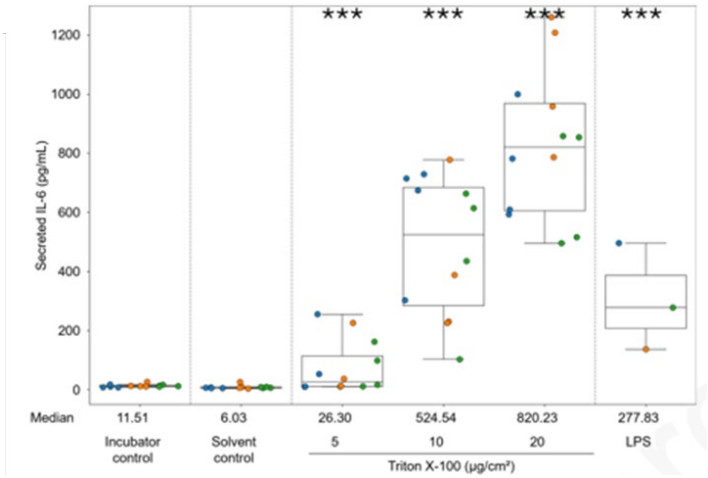


Figure 7. Effect of Triton X-100 Exposure (24 hrs) on IL-6 Secretion in MucilAir
From Figure 5 in Stucki et al. The graph shows results from three separate experiments (N = 3) represented in different colors, with four technical replicates (N = 4) included in each experiment. Asterisks (*) show statistically significant differences compared to the solvent control; ***, P < 0.001.

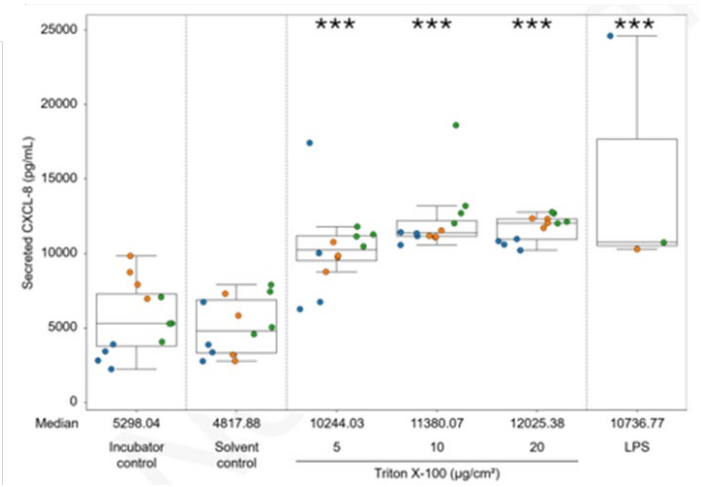


Figure 8. Effect of Triton X-100 Exposure (24 hrs) on CXCL-8 Secretion in MucilAir
From Figure 5 in Stucki et al. The graph shows results from three separate experiments (N = 3) represented in different colors, with four technical replicates (N = 4) included in each experiment. Asterisks (*) show statistically significant differences compared to the solvent control; ***, $P < 0.001$.

2.4 TEER Measurements

Mean and standard deviation of all TEER measurements in MucilAir tissues taken pre-treatment and following 24 hrs of treatment with Triton X-100 are shown in Table_Apx 9 (Appendix A). A narrow range of TEER measurements in MucilAir tissue was observed pre-treatment, ranging from 505 to 540 $\Omega \times cm^2$ (Table_Apx 9). Post-exposure TEER measurements for the solvent control appeared elevated (approximately 20%) compared to pre-exposure TEER measurements (i.e., mean ± standard deviation pre- and post-exposure TEER = 506 ± 51 and 606 ±77 $\Omega \times cm^2$, respectively, for the solvent control; Table_Apx 9).

Per manufacturer recommendations, TEER values greater than 200 $\Omega \times cm^2$ are generally accepted to indicate a tight barrier for MucilAir. Compared to the solvent control, a concentration-dependent decrease in TEER measurements was observed following 24 hrs of exposure to Triton X-100 at concentrations of 10 $\mu g/cm^2$ and above (i.e., 76%, 18%, 12%, and 7.9% of control at 10, 20, 40, and 80 $\mu g/cm^2$, respectively; Figure 9). TEER measurements were below the 200 $\Omega \times cm^2$ threshold indicative of a tight barrier at the three highest concentrations of Triton X-100. TEER measurements were comparable to the solvent control at the lowest concentration of Triton X-100 (5 $\mu g/cm^2$).

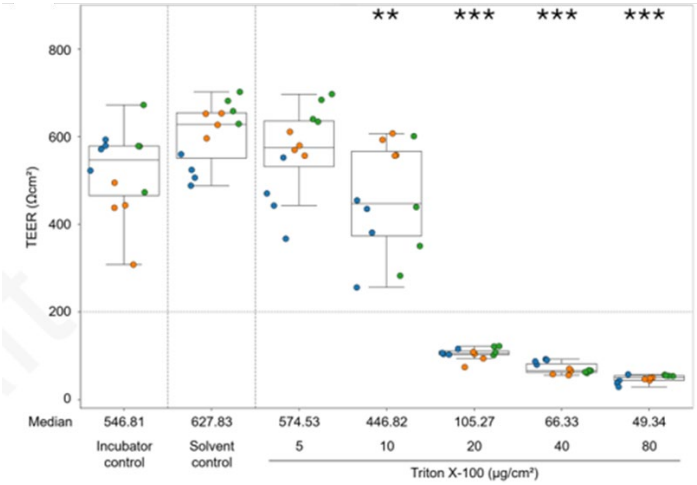


Figure 9. Effect of Triton X-100 Exposure (24 hrs) on Barrier Integrity (TEER) in MucilAir

From Figure 6 in Stucki et al. The graph shows results from three separate experiments (N = 3) represented in different colors, with four technical replicates (N = 4) included in each experiment. Asterisks (*) show statistically significant differences compared to the solvent control; **, $P < 0.01$; ***, $P < 0.001$.

2.5 Cilia Beating Frequency (CBF) and Average Active Area (AAA)

Mean and standard deviation of percent control CBF and AAA in MucilAir tissues pre-treatment and following 24 hrs of treatment with Triton X-100 are shown in Table_Apx 10 and Table_Apx 11, respectively (Appendix A). Study authors did not discuss the results of pre-treatment CBF and AAA values. Using the raw data provided in the supplementary document associated with Stucki et al., EPA calculated mean and standard deviation pre-treatment CBF and AAA values (Table_Apx 10 and Table_Apx 11). Little variability was observed in pretreatment CBF (ranged from 7.5–8.4 Hz) and AAA values (% AAA varied from 99.6% to 100%). However, some variability between pre-exposure and post-exposure CBF and AAA values was observed for the solvent control. Cilia AAA declined from 99.8% ± 0.30% (mean ± SD) in pre-treatment solvent controls to 81.6% ± 19.4% in post-treatment controls (Table_Apx 11), while CBF increased approximately 22% in post-exposure solvent control MucilAir tissue (i.e., mean ± SD pre- and post-exposure CBF = 7.794 ± 1.61 and 9.530 ± 1.30 Hz, respectively; Table_Apx 10).

Compared to the solvent control, CBF was significantly reduced 27% and 16% at concentrations of 20 and 40 µg/cm^2 Triton X-100, respectively, and increased 36% at the highest concentration (80 µg/cm^2) (Figure 10). Cilia AAA significantly decreased 83%, 56%, and 92% at 20, 40, and 80 µg/cm^2 Triton X-100, respectively (Figure 11). CBF and AAA were comparable to the solvent control at the lowest two concentrations of Triton X-100 (5 and 10 µg/cm^2). As noted by study authors, substantial cell death was observed at the two highest concentrations in several assays (cell viability, cytotoxicity, TEER, as well as histology), therefore cilia AAA and CBF measurements reported for these concentrations should be interpreted with caution. Although all datapoints are shown below in Figure 10, the high-dose group was removed for purposes of BMD modeling in Section 4 due to the biphasic nature of the response.

Study authors included isoproterenol hydrochloride (ISO) as a positive control for CBF. As expected, treatment with ISO significantly increased CBF compared to the solvent control (Figure 10).

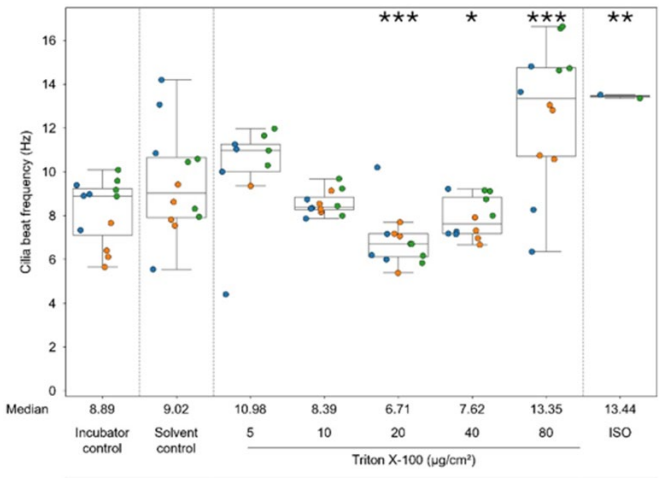


Figure 10. Effect of Triton X-100 Exposure (24 hrs) on Cilia Beating Frequency (CBF) in MucilAir

From Figure 7 in Stucki et al. The graph shows results from three separate experiments (N = 3) represented in different colors, with four technical replicates (N = 4) included in each experiment. Note: due to a data handling error, three datapoints at 5 µg/cm^2 and the ISO positive control are missing at 24 hours for Donor 2 (orange). Asterisks (*) show statistically significant differences compared to the solvent control; *, P < 0.05; ***, P < 0.001.

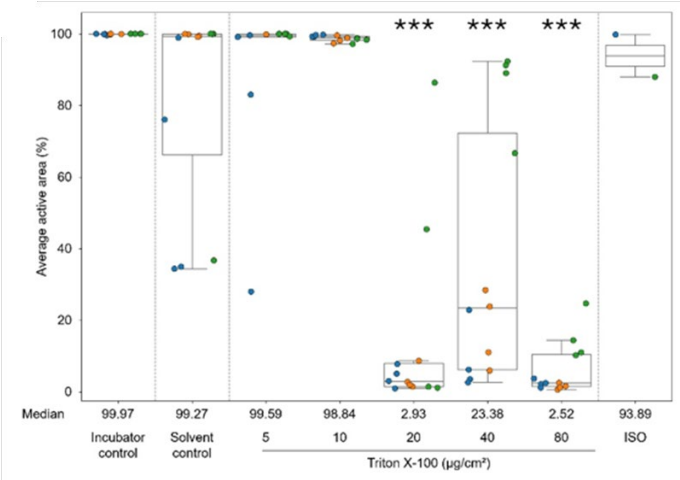


Figure 11. Effect of Triton X-100 Exposure (24 hrs) on Average Active Area (AAA) in MucilAir

From Figure 7 in Stucki et al. The graph shows results from three separate experiments (N = 3) represented in different colors, with four technical replicates (N = 4) included in each experiment. Note: due to a data handling error, three datapoints at 5 µg/cm^2 and the ISO positive control are missing at 24 hours for Donor 2 (orange). Asterisks (*) show statistically significant differences compared to the solvent control; ***, P < 0.001.

2.6 Histology

Incubator control and solvent control cells showed the typical pseudostratified structure of airway epithelium comprising basal cells, ciliated cells, and mucus-secreting goblet cells (Figure 12). No changes in tissue structure were observed at the lowest two concentrations of Triton X-100 (5 and 10 µg/cm^2). MucilAir tissues treated with 20 µg/cm^2 Triton X-100 for 24 hrs showed damage to the cell layer with loss of stratification, while tissues treated with 40 µg/cm^2 Triton X-100 showed substantial changes, characterized by loss of stratification and loss of cilia. No

histological images are available for tissues exposed to 80 µg/cm^2 Triton X-100 due to high cytotoxicity and subsequent cell loss.

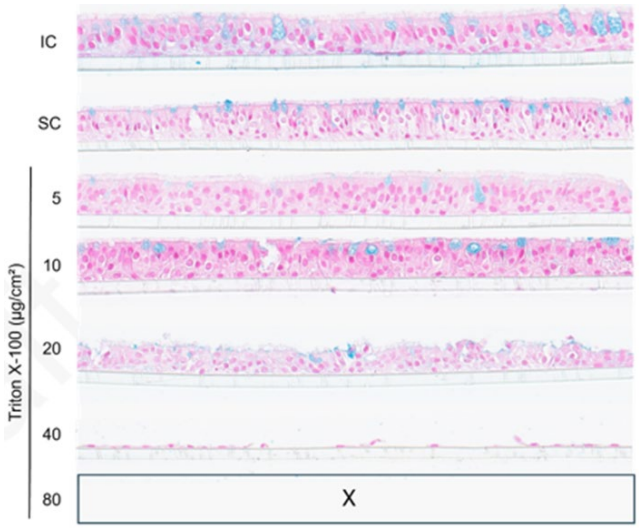


Figure 12. Effect of Triton X-100 Exposure (24 hrs) on Bronchial MucilAir Tissue
From Figure 8 in Stucki et al. Representative images from one experimental run. Abbreviations: IC = incubator control; SC = solvent control.

3. Results for MucilAir Tissues 7 Days Post-exposure to Triton X-100

Cell viability, cytokine secretion (IL-6, CXCL-8), TEER, CBF, cilia AAA, and histology was also evaluated in MucilAir tissues 7 days post-exposure. Cytotoxicity (LDH release) was not measured at this timepoint. Overall, study authors concluded that for this study the 7 day timepoint did not provide additional useful information compared to the 24 hr exposure and did not provide any additional value to understanding respiratory irritation or benchmark dose modeling. Therefore, results at the 7 day post-exposure timepoint are summarized briefly below, but are not further discussed herein.

- **Cell viability (Resazurin metabolism)**: No recovery in cell viability was observed 7 days post-exposure to Triton X-100. Compared to the solvent control, cell viability was significantly decreased at the two highest concentrations of Triton X-100 (i.e., 31% and 20% of control at 40 and 80 µg/cm^2, respectively).
- **IL-6 and CXCL-8 Section**: IL-6 and CXCL-8 secretion partially recovered to solvent control levels. Although not statistically significant, IL-6 secretion appeared increased by 32 to 50% at 10 and 20 µg/cm^2 Triton X-100, while CXCL-8 secretion appeared increased by 46 to 54% at 5, 10, and 20 µg/cm^2 Triton X-100.
- **TEER**: Partial recovery in barrier integrity was observed 7 days post-exposure to Triton X-100. TEER measurements recovered to control levels at 10 and 20 TEER µg/cm^2 Triton X-100, increased by approximately 32% at 40 µg/cm^2 Triton X-100, and showed no recovery at 80 µg/cm^2 Triton X-100 (i.e., TEER measurements were 11% of control).
- **Cilia AAA:** No recovery in cilia AAA was observed 7 day post-exposure to Triton X-100. Cilia AAA was significantly reduced to 64, 1.4, and 1.4% of control at 20, 40, and 80 µg/cm^2, respectively.

- **CBF:** CBF in active areas recovered to control levels at 20 and 40 µg/cm^2 Triton X-100. Similar to the 24 hour timepoint where CBF increased 36%, CBF remained increased by 38% at 80 µg/cm^2 Triton X-100 7 days post-exposure.
- **Tissue Histology:** Although no histology images were available at the 24 hour timepoint at the highest concentration of Triton X-100 (80 µg/cm^2) due to high cytotoxicity, remaining cells proliferated and formed a monolayer on the membrane by 7 days post-exposure. Consistent with the 24 hr timepoint, no obvious changes in histology were observed at the two lowest concentrations (5 and 10 µg/cm^2). MucilAir tissues treated with 20 µg/cm^2 showed slight damage to the cell layer with loss of stratification after 24 hrs of exposure and loss of cilia after 7 days of exposure.

4. Benchmark Concentration (BMC) Analysis

EPA confirmed that the BMC analysis conducted by Stucki et al. using all standard continuous models in EPA's Benchmark Dose Software Online were performed appropriately. The analysis was conducted using a normal distribution with both constant and non-constant variance models. All endpoints were modeled using normalized (i.e., as % vehicle control or fold-change relative to the control) and raw data. However, data normalization improved model fit and all results reported in Table 3 are for normalized data.

Using the BEAS-2B data reported in Table_Apx 1 through Table_Apx 4, and the MucilAir data reported in Table_Apx 5 through Table_Apx 11, EPA was able to replicate the BMC analysis reported in Stucki et al. BMC modeling results are reported in Table 3. For BEAS-2B cells, the most sensitive endpoint was IL-6 secretion ($BMC_{1SD}/BMCL_{1SD}$ = 5.77/3.88 µg/cm^2), followed by CXCL-8 secretion ($BMC_{1SD}/BMCL_{1SD}$ = 6.90/5.16 µg/cm^2), LDH release ($BMC_{1SD}/BMCL_{1SD}$ = 7.12/5.49µg/cm^2), and cell viability ($BMC_{1SD}/BMCL_{1SD}$ = 6.53/6.24 µg/cm^2). Similarly, for MucilAir tissues, the two most sensitive endpoints were IL-6 secretion ($BMC_{1SD}/BMCL_{1SD}$ = 1.79/1.15 µg/cm^2) and CXCL-8 secretion ($BMC_{1SD}/BMCL_{1SD}$ = 3.59/2.94 µg/cm^2), followed by TEER ($BMC_{1SD}/BMCL_{1SD}$ = 5.87/4.31 µg/cm^2), CBF ($BMC_{1SD}/BMCL_{1SD}$ = 9.11/4.98 µg/cm^2), LDH release ($BMC_{1SD}/BMCL_{1SD}$ = 11.7/7.81 µg/cm^2), and cilia AAA ($BMC_{1SD}/BMCL_{1SD}$ = 15.3/9.33 µg/cm^2). No models adequately fit the MucilAir cell viability (resazurin metabolism) dataset. This may be due to the observed biphasic dose-response for this endpoint (i.e., cell viability [measured via P4501A1/1A2 enzymatic activity] increased 17–28% at the lowest two concentrations of Triton X-100 [5 and 10 µg/cm^2] before decreasing at the highest two concentrations [40 and 80 µg/cm^2]).

Table 3. BMC Modeling Results for BEAS-2B Cells and MucilAir Tissues Exposed to Triton X-100 (24 Hrs)

Model	Endpoint	NOEC/ LOEC (µg/cm^2)	Selected Model	Distribution + Variance	BMC_{1SD}/ $BMCL_{1SD}$ (µg/cm^2)	BMD Notes
BEAS-2B	Cell viability	5.5 / 7.9	Hill	Normal + Non-constant	6.53 / 6.24	
	LDH release	7.9 / 13.9	Polynomial 3	Normal + Non-constant	7.12 / 5.49	
	IL-6 secretion	5.5 / 7.9	Polynomial 3	Normal + Constant	5.77 / 3.88	See footnotes a IL-6 not reported at 12 and 15 µg/cm^2
	CXCL-8 secretion	5.5 / 7.9	Power	Normal + Non-constant	6.90 / 5.16	CXCL-8 not reported at 12 and 15 µg/cm^2

MucilAir	Cell viability	4.2 / 9.1	No models adequately fit the dataset.			
	LDH release	9.1 / 18.6	Exponential 5	Normal + Non-constant	11.7/ 7.81	
	IL-6 secretion	None / 4.2	Hill	Normal + Non-constant	1.79 / 1.15	IL-6 not reported at 40 and 80 µg/cm^2 Lowest dose/BMDL ratio = 3.7
	CXCL-8 secretion	None / 4.2	Hill	Normal + Constant	3.57 / 2.94	CXCL-8 not reported at 40 and 80 µg/cm^2
	TEER	4.2 / 9.1	Exponential 5	Normal + Constant	5.87 / 4.31	See footnote b
	CBF	9.1 / 18.6	Exponential 5	Normal + Constant	9.11 / 4.98	High dose group removed to improve model fit due to high cytotoxicity.
	Cilia AAA	9.1 / 18.6	Exponential 5	Normal + Constant	15.26 / 9.33	

[a] Constant variance models failed Test 2 for homogenous variance. However, non-constant variance models failed to improve model fit, with goodness of fit P-values <0.1 in all cases. The Polynomial 3 model (constant variance) was selected because of the models providing a viable fit (goodness of fit P-value >0.1) and it had the lowest AIC. The Polynomial 3 model had scaled residuals less than 2 and provided a good visual fit. Further, all other viable models (i.e., those with goodness of fit P-value >0.1) supported similar BMCL estimates ranging from 3.26 to 5.15 µg/cm^2.

[b] Constant variance models failed Test 2 for homogenous variance. However, non-constant variance models failed to improve model fit, with goodness of fit P-values <0.1 in all cases. The Exponential 5 model (constant variance) was selected because of the two models providing a viable fit (goodness of fit P-value >0.1) and it had the lowest AIC. The Exponential 5 model had scaled residuals less than 2 and provided a good visual fit. Further, the Hill model also provided a viable fit (goodness of fit P-value >0.1), and supported a similar BMCL estimate of 5.52 µg/cm^2.

III. DISCUSSION AND CONCLUSIONS:

A. INVESTIGATORS' CONCLUSIONS

In general, concentration-dependent responses were observed for all assessed endpoints in both BEAS-2B and MucilAir *in vitro* test systems. BMC modeling was applied using a BMR of one control standard deviation. Based on BMC modeling results, IL-6 secretion was the most sensitive outcome for both *in vitro* cell models, yielding a BMC/BMCL of 5.77/3.88 µg/cm^2 Triton X-100 for BEAS-2B cells and a BMC/BMCL of 1.79/1.15 µg/cm^2 Triton X-100 for MucilAir.

In the MucilAir cell model, cellular effects were assessed after 24 hrs of exposure to Triton X-100, and after 7 days. Generally, at 7 days, partial recovery of cellular health effects was observed at lower exposure concentrations, except when cells died. Overall, the 7 day timepoint produced highly variable data and did not provide additional useful information compared to the 24 hr timepoint.

B. REVIEWER CONCLUSIONS

BEAS-2B Cells: Treatment with Triton X-100 for 24 hrs reduced cell viability (resazurin metabolism) at concentrations of 9 µg/cm^2 and above, and increased cytotoxicity (LDH release) at concentrations of 12 µg/cm^2 and above. IL-6 and CXCL-8 secretion was significantly increased at 9 µg/cm^2 Triton X-100. IL-6 and CXCL-8 secretion was not reported at the highest two concentrations of Triton X-100 (12 and 15 µg/cm^2) due to low cell viability (< 10% of control). All observed effects in BEAS-2B cells occurred in a concentration-dependent manner.

Based on BMC analysis, the most sensitive endpoint in BEAS-2B cells was IL-6 secretion ($BMC_{1SD}/BMCL_{1SD}$ = 5.77/3.88 μg/cm^2), followed by CXCL-8 secretion ($BMC_{1SD}/BMCL_{1SD}$ = 6.90/5.16 μg/cm^2), LDH release ($BMC_{1SD}/BMCL_{1SD}$ = 7.12/5.49 μg/cm^2), and cell viability ($BMC_{1SD}/BMCL_{1SD}$ = 6.53/6.24 μg/cm^2).

MucilAir: A biphasic response to Triton X-100 was observed for resazurin metabolism, a biomarker of cell viability, with resazurin metabolism increasing approximately 28% at 10 μg/cm^2 Triton X-100, before returning to control levels at 20 μg/cm^2, and then significantly declining to 28 and 11% of control values at the highest two concentrations (40 and 80 μg/cm^2).Study authors hypothesized that the increase in resazurin metabolism at 10 μg/cm^2 likely reflects a transient increase in cellular metabolic activity, which can be elevated under stress conditions due to adaptive metabolic responses and induction of redox enzymes such as NQO1 via NRF2 signaling. Cytotoxicity (LDH release) was significantly increased in a concentration-dependent manner at the highest three concentrations of Triton X-100 (20, 40 and 80 μg/cm^2). IL-6 and CXCL-8 secretion increased starting at the lowest concentration of Triton X-100 (5 μg/cm^2). Cytokine secretion was not reported in MucilAir at the highest two concentrations of Triton X-100 (40 and 80 μg/cm^2) due to low cell viability (11–28% of control).

There is some uncertainty associated with TEER measurements, as well as CBF and AAA responses. TEER measurements were significantly reduced at concentrations of 10 μg/cm^2 Triton X-100 and above. Although there was little variability in pre-exposure TEER measurements, TEER measurements were elevated approximately 20% in post-exposure solvent controls compared to pre-exposure solvent controls (i.e., pre- and post-exposure TEER = 506 and 606 Ω × cm^2, respectively, for the solvent control). A similar 18% increase in TEER measurements was also observed for pre- and post-exposure solvent controls in the oleoyl sarcosine experiments reported by Stucki et al. Study authors do not provide a reason for the increase in post-exposure TEER measurements in the solvent control; however, it may be due to the liquid application and/or the choice of vehicle (0.9% NaCl). The impact on TEER measurements in each of the Triton X-100 treatment groups and on the dose-response for this outcome is unknown and is a source of uncertainty. CBF and cilia AAA were both significantly reduced starting at 20 μg/cm^2 Triton X-100. However, CBF and AAA responses were highly variable. Variability in CBF and AAA measurements were likely related to the observed increase in cytotoxicity and decrease in cell viability at the highest two concentrations (40 and 80 μg/cm^2). This is further corroborated by histological findings at 40 and 80 μg/cm^2 Triton X-100 that showed substantial changes, including loss of most cells by 40 μg/cm^2, while histology at 80 μg/cm^2 could not be evaluated due to excessive cytotoxicity and cell loss. As noted by study authors, cilia AAA and CBF measurements at 40 and 80 μg/cm^2 should be interpreted with caution. Consistently, removing the high-dose group for CBF measurements improved BMD model fits.

BMC analysis indicated that the two most sensitive endpoints in MucilAir following 24 hrs of exposure to Triton X-100 were IL-6 secretion ($BMC_{1SD}/BMCL_{1SD}$ = 1.79/1.15 μg/cm^2) and CXCL-8 secretion ($BMC_{1SD}/BMCL_{1SD}$ = 3.59/2.94 μg/cm^2), followed by TEER ($BMC_{1SD}/BMCL_{1SD}$ = 5.87/4.31 μg/cm^2), CBF ($BMC_{1SD}/BMCL_{1SD}$ = 9.11/4.98 μg/cm^2), LDH release ($BMC_{1SD}/BMCL_{1SD}$ = 11.7/7.81 μg/cm^2), and cilia AAA ($BMC_{1SD}/BMCL_{1SD}$ = 15.3/9.33 μg/cm^2). No models adequately fit the cell viability (resazurin metabolism) dataset. This may be a reflection of the observed biphasic dose-response for this endpoint (i.e., resazurin metabolism increased 17–28% at the lowest two concentrations of Triton X-100 [5 and 10 μg/cm^2] before decreasing at the highest two concentrations [40 and 80 μg/cm^2]). As discussed above, there is

some uncertainty associated with the TEER, CBF, and cilia AAA measurements. IL-6 and CXCL-8 secretion were more sensitive endpoints than cell viability (resazurin metabolism) and cytotoxicity (LDH release). For IL-6 and CXCL-8 secretion, 10-fold and 2.1-fold increases in secretion were observed at the lowest concentration of Triton X-100 tested, respectively, and resulting BMC/BMCL estimates for both endpoints were below the lowest concentration (4.2 $\mu g/cm^2$) evaluated. The lowest concentration-to-BMCL ratio was 1.4 for CXCL-8 and 3.7 for IL-6. Because BMC/BMCL estimates for IL-6 and CXCL-8 are being extrapolated below the lowest concentration tested, there is some uncertainty in the BMC/BMCL estimates.

As discussed above, there is some uncertainty associated with the TEER, CBF, and cilia AAA measurements. Based on BMD analysis, cell viability (resazurin metabolism) and cytotoxicity (LDH release) were not found to be as sensitive as other endpoints in either BEAS-2B or MucilAir *in vitro* models. IL-6 and CXCL-8 secretion were consistently the most sensitive outcomes in both BEAS-2B and MucilAir *in vitro* models. Further, pro-inflammatory cytokine production and inflammatory responses are key events at the cellular and tissue levels in the respiratory tract following acute exposures to inhaled material (Clippinger et al., 2018)[3], and good BMD model fits were observed for these measurements. Therefore, the BMD values for IL-6 and/or CXCL-8 secretion are recommended for use in the NAM approach to calculate human equivalent concentrations.

This *in vitro* study evaluating airway irritation is Acceptable/Non-Guideline. It was developed in support of a NAM for refining inhalation risk assessment for contact irritants.

[3] Clippinger, A. J., Allen, D., Behrsing, H., BéruBé, K. A., Bolger, M. B., Casey, W., DeLorme, M., Gaça, M., Gehen, S. C., Glover, K., Hayden, P., Hinderliter, P., Hotchkiss, J. A., Iskandar, A., Keyser, B., Luettich, K., Ma-Hock, L., Maione, A. G., Makena, P., . . . Jarabek, A. M. (2018). Pathway-based predictive approaches for non-animal assessment of acute inhalation toxicity. *Toxicology in Vitro*, *52*, 131-145. https://doi.org/https://doi.org/10.1016/j.tiv.2018.06.009

APPENDIX A

Note: All mean and SD values shown in Table_Apx 1 through Table_Apx 11 were calculated by EPA using the raw data provided in the supplementary document associated with Stucki et al.

Table_Apx 1. Effect of Triton X-100 Treatment on Cell Viability (PrestoBlue) in BEAS-2B Cells

Dose (µg/cm²)	N	PrestoBlue (RFI)		PrestoBlue (% Control)	
		Mean	SD	Mean	SD
0	3	77,535.111	12,072.480	100.0	15.6
2.2	3	80,862.167	17,148.673	103.7	9.0
5.5	3	78,857.111	18,098.446	100.9	9.3
7.9	3	59,537.333	3,341.215	77.7	9.2
13.9	3	4,650.333	6,820.590	5.8	8.4
16.4	3	-28.444	328.987	0	0.4

Table_Apx 2. Effect of Triton X-100 Treatment on Cytotoxicity (LDH Release) in BEAS-2B Cells

Dose (µg/cm²)	N	LDH (OD)		LDH (Fold-Change)	
		Mean	SD	Mean	SD
0	3	0.070	0.022	1.000	0.311
2.2	3	0.050	0.009	0.748	0.170
5.5	3	0.060	0.007	0.897	0.185
7.9	3	0.071	0.019	1.025	0.082
13.9	3	0.169	0.014	2.601	0.847
16.4	3	0.219	0.049	3.205	0.281

Table_Apx 3. Effect of Triton X-100 Treatment on IL-6 Secretion in BEAS-2B Cells

Dose (µg/cm²)	N	IL-6 (pg/mL)		IL-6 (Fold-Change)	
		Mean	SD	Mean	SD
0	3	3.315	4.773	1.000	1.440
2.2	3	0.941	1.214	0.440	0.241
5.5	3	1.592	1.483	1.118	0.713
7.9	3	13.605	12.573	7.754	6.213

Table_Apx 4. Effect of Triton X-100 Treatment on CXCL-8 Secretion in BEAS-2B Cells

Dose (µg/cm²)	N	CXCL-8 (pg/mL)		CXCL-8 (Fold-Change)	
		Mean	SD	Mean	SD
0	3	81.677	62.844	1.000	0.769
2.2	3	68.806	62.657	0.864	0.250
5.5	3	70.652	41.102	1.052	0.387
7.9	3	481.300	464.923	4.818	2.118

Table_Apx 5. Effect of Triton X-100 Treatment on Cell Viability (PrestoBlue) in MucilAir

Dose (µg/cm²)	N	PrestoBlue (RFI)		PrestoBlue (% Control)	
		Mean	SD	Mean	SD
0	3	105,937.417	10,938.403	100.0	10.3
4.2	3	123,892.167	10,346.065	117.4	10.5
9.1	3	133,385.000	15,000.374	127.5	24.7
18.6	3	97,952.333	13,015.769	93.9	21.5
37.8	3	30,212.167	6,197.624	28.4	3.3
77.9	3	12,401.000	18,986.737	10.7	16.1

Table_Apx 6. Effect of Triton X-100 Treatment on Cytotoxicity (LDH Release) in MucilAir

Dose (µg/cm²)	N	LDH (OD)		LDH (Fold-Change)	
		Mean	SD	Mean	SD
0	3	0.025	0.009	1.000	0.373
4.2	3	0.019	0.009	0.817	0.472
9.1	3	0.028	0.005	1.213	0.429
18.6	3	0.052	0.016	2.347	1.325
37.8	3	0.334	0.093	14.647	6.842
77.9	3	1.386	0.091	60.300	17.737

Table_Apx 7. Effect of Triton X-100 Treatment on IL-6 Secretion in MucilAir

Dose (µg/cm²)	N	IL-6 (pg/mL)		IL-6 (Fold-Change)	
		Mean	SD	Mean	SD
0	3	7.971	2.720	1.000	0.341
4.2	3	74.904	5.994	10.251	3.949
9.1	3	487.971	104.190	68.601	35.017
18.6	3	826.749	199.030	106.988	20.748

Table_Apx 8. Effect of Triton X-100 Treatment on CXCL-8 Secretion in MucilAir

Dose (µg/cm²)	N	CXCL-8 (pg/mL)		CXCL-8 (Fold-Change)	
		Mean	SD	Mean	SD
0	3	5,070.950	1,057.032	1.000	0.208
4.2	3	10,352.088	725.297	2.082	0.314
9.1	3	12,159.511	1,702.436	2.422	0.206
18.6	3	11,714.839	936.242	2.353	0.318

Table_Apx 9. Effect of Triton X-100 Treatment on TEER in MucilAir

Dose (µg/cm²)	N	TEER (Ω × cm²)				TEER (% Control)			
		Pre-Exposure		24 hr Exposure		Pre-Exposure		24 hr Exposure	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
0	3	505.670	51.017	606.348	77.292	1.000	0.101	1.000	0.127
4.2	3	505.285	79.078	566.885	103.376	0.997	0.075	0.931	0.058
9.1	3	517.495	108.831	459.415	104.750	1.024	0.184	0.759	0.146
18.6	3	506.440	74.777	105.078	9.119	1.002	0.104	0.175	0.028
37.8	3	539.660	82.490	71.115	13.959	1.064	0.080	0.121	0.041
77.9	3	506.523	70.355	47.889	6.488	1.002	0.090	0.079	0.004

Table_Apx 10. Effect of Triton X-100 Treatment on Cilia Beating Frequency (CBF) in MucilAir

Dose (µg/cm²)	N	CBF (Hz)				CBF (% Control)			
		Pre-Exposure		24 hr Exposure		Pre-Exposure		24 hr Exposure	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
0	3	7.794	1.610	9.530	1.295	1.000	0.207	1.000	0.136
4.2	3	7.984	0.939	9.919	1.134	1.040	0.111	1.055	0.190
9.1	3	7.539	1.691	8.564	0.261	0.965	0.042	0.911	0.134
18.6	3	7.455	1.788	6.858	0.517	0.953	0.063	0.725	0.080
37.8	3	7.851	1.389	7.893	0.785	1.013	0.055	0.836	0.119
77.9	3	7.796	1.209	12.736	2.564	1.009	0.065	1.359	0.348
ISO	2	9.173	0.619	13.443	0.115	–	–	–	–

Table_Apx 11. Effect of Triton X-100 Treatment on Cilia Average Active Area (AAA) in MucilAir

Dose (µg/cm²)	N	Cilia AAA (% Active Area)				Cilia AAA (% Control)			
		Pre-Exposure		24 hr Exposure		Pre-Exposure		24 hr Exposure	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
0	3	99.813	0.298	81.611	19.377	1.000	0.003	1.000	0.237
4.2	3	98.846	1.453	92.367	12.937	0.990	0.017	1.152	0.136
9.1	3	99.837	0.209	98.729	0.652	1.000	0.001	1.261	0.330
18.6	3	99.633	0.361	13.859	17.106	0.998	0.002	0.169	0.200
37.8	3	99.852	0.169	36.996	41.627	1.000	0.001	0.442	0.490
77.9	3	99.966	0.033	6.344	7.604	1.002	0.003	0.078	0.089
ISO	2	99.977	0.033	93.891	8.374	–	–	–	–