TransFER: a new device to measure the transfer of volatile and hydrophobic organic chemicals across an *in vitro* intestinal fish cell barrier

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Abstract

Transfer of compounds across cellular barriers is a critical step of compound uptake into organisms. Using *in vitro* barrier systems to evaluate such transfer is attractive because of the higher throughput and reduced resource needs compared to animal studies. Thus far, however, studying the transfer of hydrophobic and volatile compounds was hampered by the unavailability of *in vitro* exposure systems that allow for stable and predictable chemical exposure concentrations. To overcome this limitation, we constructed a novel exposure chamber, TransFER, and tested it with an *in vitro* epithelial barrier model using the rainbow trout (*Oncorhynchus mykiss*) intestinal cell line, RTgutGC. Key features of the chamber are its closed design and rotatable silicon segments, which can serve for chemical dosing and sampling. Using the fragrance Damascone beta ($\log K_{ow}$:3.7, $\log HLC$:-3.9) as a pilot chemical, we were able to demonstrate that our exposure chamber provides for stable chemical exposure concentrations and full mass balance. The RTgutGC epithelium served as barrier for Damascone beta transfer, which we attribute to chemical retention and biotransformation in the intestinal cells. Nevertheless, substantial transfer of the chemical across the epithelium occurred. When a chemical sink, i.e. a silicon segment, was included in the basolateral chamber to mimic blood constituents binding *in vivo*, transfer was about three-fold enhanced. We suggest that the presented methodology can help to obtain insights into chemical uptake mechanisms via the intestinal or other epithelia of fish and other animals for hydrophobic and volatile chemicals.

Keywords: Chemical transfer, Exposure chamber, *in vitro*, fish intestine

55 Introduction

56 This paper describes the development of the TransFER exposure chamber which was designed to
57 measure the transfer of difficult to test chemicals across *in vitro* epithelial cellular barrier models.
58 Cellular epithelia, as in the respiratory and digestive organs, are important gate keepers that regulate
59 the interaction of the organism with its surrounding environment while providing protection from
60 environmental influences. Transfer across cellular barriers is the first critical step for compounds to
61 enter an organism; it is thus a matter of great interest not only in pharmacological research¹ but as
62 well in human and environmental toxicology². Importantly, cell- rather than animal-based test
63 systems are increasingly used. Here, the cells are seeded on a permeable membrane in well-plate
64 inserts creating two compartments: an apical (upper) compartment, representing the environmental-
65 facing side, and a basolateral (lower) compartment, representing the blood-facing side.

66 The advantage of cell-based transfer assays are, among others, that they require small amounts of
67 compound, can be performed in large numbers, have controlled physical conditions, such as
68 temperature and pH. In this way, such systems are suitable for compound screening and also permit
69 mechanistic studies of compound transport and biotransformation. In pharmacology, the application
70 of human intestinal cell lines for drug transport studies has become a standard both in academic and
71 industrial research³⁻⁴, in particular using the human colon adenocarcinoma epithelial cell line, Caco-
72 2⁵. In environmental toxicology, a fish gill and intestinal barrier model⁷ have been recently
73 developed. The fish gill model has been successfully applied to study the uptake of pharmaceuticals
74 into the organism with good correlation of *in vitro* transfer rates with *in vivo* measured plasma
75 concentrations⁶, presenting a promising tool for environmental risk assessment of chemicals. Thus
76 far, the fish intestinal model has been effectively used to study the transport of nanoparticles⁸ and
77 fluorescent model compounds⁷ across the fish intestine. Both *in vitro* barrier setups have been shown
78 to be applicable for compounds which are well soluble and not volatile. While these chemicals are
79 relatively easy to dose and test, they only represent a fraction of existing industrial chemicals. Approx.
80 a third of the registered chemicals within the ECHA-REACH database are rather hydrophobic
81 chemicals ($\log K_{ow} \geq 3$)⁹ and require more thorough assessments because they are prone to
82 accumulate in biota. Moreover, if combined with volatility, such chemicals could even bioaccumulate
83 in organisms in remote regions.

84 Testing of hydrophobic and volatile compounds poses well-known difficulties. The most challenging
85 short-comings are unstable exposure conditions and poor mass balance due to sorption to test
86 vessels of hydrophobic compounds and evaporation of volatile ones¹⁰⁻¹². It is, moreover, difficult to

87 achieve defined exposure concentrations without the use of co-solvents such as DMSO or methanol
88 in the first place due to the low aqueous solubility¹³. To overcome these difficulties, we developed
89 and constructed a new exposure chamber. The aim was to provide a flexible exposure device, which
90 can easily be adapted to the specificities of the chemicals to test and the scientific questions to
91 answer. By choosing an appropriate material, loss via sorption to surfaces is reduced. By having an
92 air-tight lid, loss due to evaporation is avoided. Further, the chamber dimensions allow applying
93 sufficiently large volumes of solutions, which are necessary to quantify chemicals when tested at non-
94 toxic concentrations. Finally, it should allow for a tunable exposure and sampling regime established
95 via movable trapping and release segments, i.e. segments coated with silicone, to mimic a chemical
96 source and/or a chemical sink while ensuring mixing in the apical and basolateral compartment,
97 respectively.

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103 **Materials and Methods**

104 The TransFER chamber was constructed in the internal workshops at Eawag and Firmenich according
105 to optimized specifications as described in the results and discussion. Afterwards, the chamber was
106 tested following the study design and the workflow presented in SI Figure 1.

107 **Study design**

108 We use the *in vitro* fish intestinal barrier model, which comprises the intestinal epithelial cell line
109 from rainbow trout (*Oncorhynchus mykiss*), RTgutGC¹⁴, cultured on a permeable support.⁷⁻⁸ Among
110 other features, the barrier model has been found to well reflect the leaky nature of the intestinal
111 epithelium of salmonids⁷, important for transport studies. For the transfer experiments, the highest
112 non-cytotoxic but well quantifiable concentration was chosen. We first evaluate the exposure
113 conditions assuring cell viability and barrier integrity. Transfer was measured over permeable
114 supports without (cell-free) and with RTgutGC cells and concentrations of our pilot test chemical (see
115 below) were quantified over time in all compartments in order to establish a mass balance. Transfer
116 coefficients, often also referred to as permeation coefficients, were calculated based on the mass
117 quantified for the test chemical in the apical and the basolateral compartment over time. To better
118 understand the underlying processes, we applied different exposure setups. The first focused on the
119 transfer of the test chemical from the apical to the basolateral compartment at 19°C, which reflects
120 the physiological temperature of the RTgutGC cells. In the second setup, transfer experiments were
121 conducted at 4°C. Exposure at such low temperatures is commonly used to explore the role of energy
122 requiring processes, such as transport by ATP-dependent carriers or biotransformation. In the third
123 setup, a chemical sink (= Polydimethylsiloxane [PDMS] coated stir bar) was introduced in the
124 basolateral compartment to present the blood components in the fish. This stir bar would be
125 expected to bind the chemical and therefore maximize the concentration gradient. A schematic
126 presentation of the workflow can be found in the supplemental information (SI Figure 1).

127 **RTgutGC cell culture**

128 **Routine cell culture** The rainbow trout intestinal epithelial cell line, RTgutGC, was originally isolated
129 from the intestine of a female rainbow trout (*Oncorhynchus mykiss*).¹⁴ Cells are routinely cultured
130 with Leibovitz L-15 medium (Invitrogen, Basel, Switzerland) supplemented with 5 % fetal bovine
131 serum (FBS; PAA, Basel, Switzerland) and 1 % gentamycin (PAA, Basel, Switzerland) (= complete
132 medium, L-15/FBS). Cells are cultured in 75 cm² flasks (TPP, Transadingen, Switzerland) at 19°C (all
133 temperatures given are within a range of $\pm 1^\circ\text{C}$) in absence of light in ambient atmosphere and sub-
134 cultured every 7 to 10 days by washing the cells twice with Versene followed by trypsinization

135 (Biowest, Nuaille, France). Routinely, rainbow trout cell lines are physiologically adapted to a
136 temperature range of 18-22°C¹⁵.

137 **Cultivation on permeable supports** For the transfer experiments, the RTgutGC cells were cultured in
138 6-well plates according to optimized protocols that yield a polarized epithelial monolayer on
139 permeable supports within three weeks of culture.⁷ Prior to cell seeding, permeable membrane
140 supports with PET-membranes (pore size = 0.4 µm, pore density = 2 x 10⁶, culture surface = 4.5 cm²,
141 Greiner-bio-one, Frickenhausen, Germany) were coated with 300 µl of 30 µg/ml fibronectin (Roche,
142 Mannheim, Germany) in sterile PBS (Invitrogen, Basel, Switzerland) for 1 h at room temperature and
143 100 rpm horizontal shaking. Fibronectin solution was discarded and supports were washed twice with
144 sterile PBS. The cells were seeded at a density of 5.8 x 10⁴ cells per cm² in 3.5 ml of complete medium
145 (L-15/FBS). The basolateral compartment was filled with 4 ml L-15/FBS. The cells were cultured for
146 three weeks at 19°C in an incubator at ambient atmosphere with weekly medium changes of the
147 apical and basolateral solution. As a control, cell free permeable supports were filled with 3.5 ml
148 apically and 4 ml L-15/FBS basolaterally and treated identically.

149 **Pilot chemical: Damascone beta**

150 Damascone beta ((2E)-1-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2-buten-1-one, CAS 23726-91-2, MW =
151 192 g/mol) was chosen as pilot test chemical to proof the concept because it has challenging
152 properties though yet not extreme. On the one hand, it combines significant hydrophobicity (logK_{ow}:
153 3.65¹⁶, experimentally determined) and volatility (logHLC:-3.9, from EPIWin model) – adsorption to
154 container walls when inappropriate materials are used can be expected as well as significant losses
155 by volatilization. On the other hand, its solubility is relatively high (solubility 221 mg/L¹⁷,
156 experimentally determined), which allowed to directly dissolve Damascone beta in the exposure
157 medium without the need of a co-solvent or a passive dosing system. The relatively high water
158 solubility moreover allowed us to quantify the test chemical with a high accuracy, ensuring a robust
159 evaluation of the mass balance.

160 In the apical and basolateral compartment, L-15/ex, a medium more closely reflecting the fish
161 intestinal lumen, was used.¹⁸ This exposure medium is a simplified version of the L-15 culture medium
162 and contains only the salts, galactose and pyruvate.¹⁸⁻¹⁹ Damascone beta was first dissolved in
163 deionized water to a final concentration of 25 mg/L (= 130.2 µM) as stock solution. The exposure
164 solution was prepared freshly on the day of the experiment by diluting the stock solution in sterile L-
165 15/ex to a concentration of 1 mg/L (= 5.2 µM). With the detection limit of Damascone beta being 3

166 µg/L, the exposure concentration of 1 mg/L was expected to be quantifiable in all different
167 compartments.

168 **Exposure of RTgutGC cells to Damascone beta**

169 As a pre-requisite for the transfer experiments, the exposure conditions were confirmed as non-toxic
170 by assessing the cell viability and monolayer integrity, the latter being measured as transepithelial
171 electrical resistance (TEER) and transfer of fluorescent model molecules. These experiments were
172 conducted identically to the transfer study, but instead of sampling for chemical quantification (see
173 below), the different endpoints were measured. Three weeks old RTgutGC monolayers cultured on
174 permeable supports were used as starting material (see SI Figure 1).

175 To initiate each experiment, TEER was assessed as a quality measure of monolayer formation
176 according to Minghetti et al.¹⁸ and monolayers with a TEER $\geq 20 \Omega \cdot \text{cm}^2$ were used for viability and
177 transfer studies. TEER was measured using an epithelial tissue EVOM (Epithelial Volt/Ohm Meter)
178 Voltohmmeter equipped with an Endohm-24SNAP chamber (World Precision Instruments, Berlin,
179 Germany). To adjust the cells to the exposure conditions, the apical medium was replaced with 3.5
180 ml L-15/ex and the basolateral medium with 4 ml L-15/FBS and incubated at 19°C for 30 min.
181 Thereafter, TEER measurement was repeated. Then, the basolateral compartment was washed twice
182 with L-15/ex to remove FBS-containing medium, potentially remaining at the bottom side of the
183 permeable supports. Next, 3.5 ml of Damascone beta exposure solution was added to the apical side
184 and the permeable supports were transferred to the experimental chambers, containing 12 ml L-
185 15/ex. The lid was closed and a 9 V block battery was attached to power the motor. Chambers were
186 incubated at 19 °C in the incubator or at 4 °C in the refrigerator for the desired time period. In the
187 presence of a basolateral PLMS-coated stir bar (Gerstel, Sursee, Switzerland) (see Figure 1), the
188 chambers were incubated on magnetic stir plates (100 rpm).

189 **Cell viability and monolayer integrity of RTgutGC cells grown on permeable supports**

190 Cell viability was assessed via a combination of two non-invasive dyes: AlamarBlue as a measure of
191 metabolic activity and 5-carboxyfluorescein diacetate acetoxy-methyl ester (CFDA-AM) as a measure
192 of cell membrane integrity (Invitrogen, Basel, Switzerland)¹⁹. After 0.5, 1.5, 3, 5, 10 and 24 h of
193 exposure to Damascone beta, permeable supports were washed with PBS and TEER was measured.
194 Then, 800 µl of AlamarBlue/CFDA-AM solution in sterile PBS was incubated in the apical compartment
195 at 19°C in the dark for 30 min. 400 µl of AlamarBlue/CFDA-AM solution were transferred to a 24-well
196 plate and the fluorescence was recorded at excitation and emission wavelengths of $\lambda_{\text{ex}}/\lambda_{\text{em}}$ 530/590

197 for AlamarBlue and $\lambda_{\text{ex}}/\lambda_{\text{em}}$ 493/541 for CFDA-AM, using a multi-well plate reader (Tecan Infinite
 198 2000, Männedorf, Switzerland).

199 In order to assess the monolayer integrity, TEER and the transfer of two integrity markers was
 200 measured in the same set of samples. As integrity markers, the hydrophilic molecule Lucifer Yellow
 201 (LY, MW = 520 Da, hydrodynamic diameter = 1 nm) and the larger molecular Rhodamine-Dextran
 202 (Rho, MW = 10 kDa, hydrodynamic diameter = 4 nm) were used. Lucifer Yellow is postulated to diffuse
 203 between the cells. Rhodamine-Dextran, due to its larger hydrodynamic diameter, either suggests the
 204 presence of a fraction of wide inter-cellular spaces²⁰ for the molecule to be able to cross the cell layer
 205 or transcellular transfer.¹⁸ Lucifer Yellow (0.14 μM) and Rhodamine-Dextran (0.19 μM) were added
 206 in 1.75 ml L-15/ex to the apical compartment (Sigma Aldrich, Buchs, Switzerland) and 6.55 ml L-
 207 15/FBS was added to the basolateral compartment. Over a period of 4 h, 3 x 50 μl were transferred
 208 from the basolateral compartment to a 96-well plate and replaced with 150 μl L-15/FBS. The
 209 fluorescence was measured at $\lambda_{\text{ex}}/\lambda_{\text{em}}$ 420/520 nm for Lucifer Yellow and at $\lambda_{\text{ex}}/\lambda_{\text{em}}$ 520/580 nm for
 210 Rhodamine-Dextran using the multi-well plate reader.

211 **Sampling of Damascone beta for transfer assessment**

212 Cell-free and cell containing permeable supports were prepared and exposed to Damascone beta as
 213 described above. After 0.5, 1.5, 3, 5 and 10 h of exposure, the apical and basolateral medium, the
 214 fraction containing the cells, the membrane and the plastic parts of the permeable support were
 215 sampled. Cells were removed by scraping and two consecutive rinses with water. The apical,
 216 basolateral and cell fraction containing solutions were transferred to amber glass vials with teflon
 217 coated lids to minimize the risk of photodegradation or loss due to sorption (Supelco, Sigma Aldrich,
 218 Buchs, Switzerland). For quantification via LC-MS MS (LC-20AD, Shimadzu, Germany, Qtrap ABSciex
 219 4000, AbSciex, Germany), the medium solutions were diluted 1:1 with acetonitrile containing 19.4
 220 μM of Tonalide as internal standard. Plastic parts and the membrane were cut and placed into sample
 221 vials. Membrane and plastic were extracted with a soxhlet (pentane) operated overnight
 222 (corresponding to roughly 10 cycles) or with acetonitrile. GC-MS (Agilent 5975, Agilent, Germany)
 223 was used to analyse pentane extracts, and 1,4-di-bromobenzene (212 μM in heptane) was added as
 224 internal standard. PDMS-coated twistors were extracted three times using 500 μl heptane and
 225 quantified by GC-FID (Agilent 6890, Agilent, Germany). Samples were stored at -20°C before chemical
 226 quantification. A more detailed procedure of the chemical quantification can be found in SI, Table 1.

227 **Data treatment and statistical evaluation**

228 **Cell viability (AlamarBlue, CFDA-AM)** Background fluorescence was subtracted from absolute
229 fluorescent values and the three biological replicates were plotted as mean ± SD.

230 **Monolayer integrity (TEER)** TEER values were corrected for the resistance of cell-free permeable
231 supports and normalized to the surface area by multiplication with the area of the membrane
232 supports (4.52 cm²). TEER values from each permeable support after the exposure were normalized
233 to the respective TEER values before the exposure. TEER data are shown as individual replicates and
234 as mean ± SD of three biological replicates.

235 **Monolayer integrity (integrity markers)** Fluorescence values were converted to mass transported
236 over time based on linear regression from calibration curves. The transfer or permeability coefficient
237 in cm/s for integrity markers was calculated assuming sink conditions:

238
$$P \left[\frac{\text{cm}}{\text{s}} \right] = \left(\frac{dQ}{dt} \right) * \left(\frac{1}{A \times C_0} \right) \quad \text{Eq.1}$$

239 where P is the transfer coefficient [cm/s] across the cell-containing permeable support (= P_{total}) or
240 across the cell-free membrane (= P_{membrane}), Q is the quantity of the chemical [nmol], t is the time [s],
241 $\frac{dQ}{dt}$ is the steady state flux [nmol/s]; A the filter surface area [cm²] and C₀ the initial donor
242 concentration [μmol/L].¹ To obtain only the transfer across the cells, the total transfer measured
243 across the membrane in the presence of cells was corrected for the transfer across the membrane
244 by the following equation²¹:

245
$$\frac{1}{P_{total}} = \frac{1}{P_{cells}} + \frac{1}{P_{membrane}} \quad \text{Eq.2}$$

246 where P_{total} is the transfer or permeability across the cell-containing permeable supports, P_{cells} the
247 transfer or permeability across the cell layer only and P_{membrane} the transfer across the cell-free
248 membrane. The resulting transfer across the cell layer is shown as individual biological replicates and
249 as mean ± SD thereof.

250 **Transfer studies** Damascone beta mass extracted from the compartments was normalized to the
251 introduced mass in each experiment. Calculated values are presented as the mean ± SD of three
252 (experiments at 19°C) or two (experiments at 4°C) biological replicates. The mass balance was
253 calculated as sum of all analysed compartments.

254 Calculations based on sink conditions can only be applied if the basolateral concentration remains
255 below 10 % of the apical concentration. For Damascone beta, these conditions were not granted for

256 all time points; the permeability coefficients were therefore calculated assuming non-sink
 257 conditions^{1, 22}:

$$258 \quad C_R(t) = \left(\frac{M_{tot}}{V_R + V_D} \right) + \left(C_{R0} - \frac{M_{tot}}{V_R + V_D} \right) * e^{-P * A * \left(\frac{1}{V_R} + \frac{1}{V_D} \right) * t} \quad \text{Eq.3}$$

259 where $C_R(t)$ is the concentration in the receiver (=basolateral) compartment over the time, M_{tot} is the
 260 total mass in the system, V_R and V_D are the volume of the receiver and donor (=apical) compartment,
 261 C_{R0} is the concentration in the receiver compartment at the start of the experiment, A is the surface
 262 area of the permeable support, t is the time and P is the transfer coefficient [cm/s] across the cell-
 263 containing permeable support (= P_{total}) or across the cell-free membrane (= $P_{membrane}$). The transfer
 264 coefficient across the cell layer (without the membrane) was calculated applying equation 2.

265 **Fittings and statistics** Graphs and fittings of Damascene beta transfer over time were made using
 266 GraphPad Prism version 5.02 (Graphpad Software Inc., San Diego, CA). For permeability calculations,
 267 R (version R-3.3.0) and R studio (version 0.99.902) were used. Cell viability and monolayer integrity
 268 data were analysed via ANOVA and Dunnett's post-hoc test.

269

Results & Discussion

Novel exposure chamber TransFER

In order to reduce chemical loss via sorption to plastic, the TransFER exposure chamber was constructed of mirror polished stainless steel as material with minimal adsorption capacity, which is as well recommended by the OECD for the testing of hydrophobic chemicals.²³ Chemical loss via evaporation was minimized by having a gas-tight lid sealed with Teflon, where only a small headspace with a defined volume remains. Each TransFER chamber fits one 6-well permeable support to provide sufficient amount of material for chemical quantification. The apical solution can be stirred at 1 rpm with a stainless steel agitator operated by a mini-motor, installed in the lid of each chamber to assure even apical distribution of the compound. Generally, stirring ensures a faster homogenisation of the concentration and allows to control the stagnant boundary layer close to the silicon tube, the cell layer or just the membrane in the cell free system.²⁴⁻²⁵ A PDMS tube can be connected to the agitator. It can be kept apically and serve as partition controlled sampling and dosing system. The latter compensates any remaining loss in the system due to evaporation or absorption and avoids the use co-solvents, which are otherwise often required when working with hydrophobic chemicals. The basolateral compartment provides sufficient distance between the permeable support and bottom to include a (silicone coated) stir bar to agitate the basolateral solution and serve as a passive dosing or sampling (= chemical sink) system as well. Such a sink has been reported to represent blood proteins and blood flow and aims to maintain the concentration gradient as driving force of transfer.²⁴⁻²⁵

291 Establishing exposure conditions for Damascone beta

292 To study the transfer of Damascone beta, the medium L-15/ex was selected for both the apical and
293 basolateral side. This medium reflects the milieu of the fish gut lumen⁸ and offered a stable
294 Damascone beta concentration, whereas some losses were observed in complete L-15 medium with
295 or without FBS (SI Figure 2). The cell viability and monolayer integrity of RTgutGC cells was used to
296 set the maximal exposure time for the mass balance and transfer experiments.

297 In the selected conditions, RTgutGC cells were viable for at least 10 h of exposure, but viability was
298 strongly impacted at 24 h in the presence of Damascone beta (Figure 2, A). We observed an initial
299 tightening of the monolayer, indicated by higher TEER values, both in the presence and absence of
300 Damascone beta at the early time of exposure (Figure 2 B). This change in TEER values can be
301 attributed to a tightening of cell-cell contacts as a reaction to the exposure medium L-15/ex alone. A
302 similar reaction has been shown with freshly isolated gill cells upon exposure to freshwater
303 samples.²⁶ In the presence of Damascone beta, the tightness of the monolayer is slightly decreasing
304 over time. However, up to 10 h the TEER decrease was not significant compared to the control. The
305 hydrophilic molecule Lucifer Yellow and Rhodamine-Dextran were used as an integrity marker, where
306 higher transfer coefficients would indicate loosened cell-cell contacts and potentially impacted
307 monolayer integrity. Even though both integrity markers show a slightly increased transfer across the
308 monolayer after longer exposure times (Figure 2 C), the differences compared to the control were
309 not significant up to 10 h (all p-values > 0.53). Taken together, the final setup for Damascone beta
310 mass balance and transfer experiments comprised exposure for up to 10 h in L-15/ex.

311

312 Mass balance and indication of biotransformation

313 Despite a sensitive limit of quantification ($= 3 \mu\text{g/L}$ or 15.6 nM in solvent), Damascone beta was not
314 quantifiable in membrane or plastic parts of the permeable support, i.e. $< 0.3 \%$ of total added mass.
315 It appears that the physico-chemical properties of Damascone beta do not favour plastic binding.
316 Also, the Damascone beta mass associated with the RTgutGC cells was lower than expected: less than
317 0.5% of total dosed Damascone beta was measured within the cell fractions (SI Figure 3). By
318 comparing to a chemical with similar size and $\log K_{OW}$, Propiconazole, from an *in vitro* study with the
319 rainbow trout gill cell line, RTgill-W1, we expected to find about $2\text{--}3 \%$ of total Damascone beta mass
320 intracellularly.²⁷ Because, to achieve a fast and sensitive detection, the developed analytical methods
321 focussed on the parent compound only, we cannot preclude structural modifications of Damascone
322 beta in the presence of cells, which might explain the low intracellular Damascone beta mass. A
323 significant Damascone beta loss in the presence of cells becomes indetectable when evaluating the
324 recovery of Damascone beta from the entire system. The recovery was calculated by adding up the
325 medium compartments and, where applicable, Damascone beta mass absorbed to the PDMS-coated
326 stir bar. In the absence of cells, almost all mass ($87.0 \pm 3.8 \%$) was recoverable from the system (Figure
327 3). However, in the presence of cells, substantial Damascone beta loss was measured ($49.3 \pm 12.0 \%$).
328 We postulated two potential causes for this.

329 The first postulated cause is covalent binding to intracellular nucleophilic groups, such as thiol groups
330 present in the amino acid cysteine, which we suspected based on the alpha-beta unsaturated
331 carbonyl moieties of Damascone beta that could easily lead to Michael addition (SI Figure 4). Indeed,
332 if cysteine was added in 5-fold excess ($1310 \mu\text{M}$ cysteine with $260 \mu\text{M}$ Damascone beta), a rapid and
333 significant loss of Damascone beta was observed (SI Figure 5); at the same time, the corresponding
334 cysteine-Damascone beta adduct was detected by LC-MS/MS. A cysteine-containing tripeptide,
335 glutathione, was added in 4-fold excess, which relates to an approx. 1:1 ratio with regard to cysteine.
336 Again, the amount of quantifiable Damascone beta was reduced but to a much lower extent (SI Figure
337 5). Despite these findings, we do not expect thiol-binding to be a dominant process explaining the
338 Damascone beta loss in the RTgutGC cells because much less chemical was lost when cysteine was
339 present in a more complex solution as demonstrated with the L-15 cell culture medium (SI Figure 2).
340 L-15 contains a cysteine concentration of $990 \mu\text{M}$, resulting in a much higher Damascone
341 beta:cysteine ratio of 1:190 compared to the cysteine/glutathione experiments. This indicates
342 different binding kinetics of Damascone beta in the presence of other amino acids, vitamins and salts
343 which likely reflects the conditions in a cell more closely than a single amino acid or peptide solution.

344 As a second cause for loss, we therefore postulate intracellular biotransformation of Damascone
345 beta. In support of this second proposal, Laue et al. found biotransformation of the isomer
346 Damascone delta in isolated rainbow trout liver S9 fractions²⁸. Further, Damascone beta loss is
347 reduced in experiments at 4°C (Figure 3, white circles) where active processes, such as
348 biotransformation, can be expected to be strongly reduced.²² This finding is in line with previous
349 reports on the ability of intestinal cells ²⁹ and specifically the RTgutGC cells³⁰, to be capable of
350 biotransforming chemicals.

351 In the context of the fish intestinal barrier model, this implies that the determined transfer rate is a
352 combined measure of intestinal biotransformation and permeation across the cell layer. This
353 observation reflects the *in vivo* situation, where the intestine of fish was suggested to be a crucial
354 site for first pass biotransformation of chemicals taken up via the diet.³¹ These processes might
355 impact the bioavailability and the toxicity of the chemical before reaching the blood.³¹⁻³² In addition
356 to hepatic biotransformation, the intestinal biotransformation processes may be important to
357 consider for reducing uncertainty in bioaccumulation prediction.²

358 **Transfer of Damascone beta across the intestinal cell barrier**

359 ***Transfer of Damascone beta at 19°C***

360 The RTgutGC monolayer presents a barrier for Damascone beta transfer: compared to cell-free
361 controls, only about 50 % of Damascone beta is transferred from the apical to the basolateral side,
362 significantly reducing the amount of Damascone beta in the apical compartment (Figure 4 A, B). We
363 assume that Damascone beta partly permeates between (paracellular) and partly through
364 (transcellular) the cells along its concentration gradient. It has been reported that for hydrophilic
365 chemicals, the paracellular pathway dominates, whereas for hydrophobic chemicals, the transcellular
366 pathway is of more importance.³³⁻³⁵ One observation pointing towards a transcellular pathway is the
367 hydrophobicity of Damascone beta. The logK_{OW} of Damascone beta was measured to be 3.65, which,
368 according to Nichols et al.^{2, 36}, is characterized as hydrophobic. Also, since Damascone beta appeared
369 to be biotransformed, we expected at least a fraction of chemical to be absorbed in the cells which
370 was confirmed by, albeit low, concentrations quantified in the cells (SI Figure 3). At the same time,
371 however, the RTgutGC monolayer, like the fish intestinal epithelium^{2, 38}, is characterized as leaky^{8, 18},
372 which indicates a high ability of chemicals to passively permeate between cells.³⁹ The integrity marker
373 Lucifer Yellow (MW = 520 Da) permeates across the RTgutGC cell layer with a P_{cells} of 6.0 * 10⁻⁶ cm/s.
374 Lucifer Yellow has a hydrodynamic diameter of 1 nm, thus, requiring at least 1 nm functional pore
375 size between the cells to allow its transfer. Damascone beta, with a molecular weight of 192 Da is
376 about half the size of Lucifer Yellow and should therefore permeate between the cells without size
377 restriction. Transfer of Damascone beta across the cells, P_{cell}, was calculated to be 2.5 * 10⁻⁵ cm/s
378 (Figure 4 B, SI Table 2), which is 4-times faster than Lucifer Yellow. P_{cell} of Damascone beta fitted well
379 with the permeation coefficients of four fluorescent compounds, where P_{cell} decreased exponentially
380 with increasing molecular weight of the fluorescent compounds (SI Figure 6). The transfer across the
381 RTgutGC monolayer was about 10-fold faster compared to pharmaceuticals across a primary gill
382 epithelium⁶, which highlights potentially important differences between the barrier properties of the
383 gill and intestine for the transfer of chemicals.

384 ***Adding a chemical sink to the basolateral compartment***

385 The addition of a chemical sink increased the total amount of Damascone beta that reached the
386 basolateral compartment by approx. 20-30 % (Figure 4 C/D compared to Figure 4 A/B). Freely
387 available Damascone beta was absorbed in the PDMS of the stir bar and was therefore removed from
388 the basolateral solution (SI Figure 7). The concentration gradient as driving force of transfer was
389 maximized, which is also reflected in an increased permeation coefficient of P_{cells} = 6.1*10⁻⁵ cm/s

across the cell layer (Figure 4 D, SI Table 2). The chemical sink was introduced to mimic potential binding of Damascone beta to blood components and the distribution via the blood flow in the *in vivo* situation. *In vivo*, these processes would remove Damascone beta from the intestinal side and could, as shown in the *in vitro* system, increase the total mass taken up into the organism. The incorporation of a sink has also been suggested as a refinement for cell culture models. Specifically, the addition of bovine serum albumin (BSA) has been suggested to increase solubility of highly hydrophobic chemicals ($\log K_{ow} > 5$)¹ and for experiments with chemicals potentially displaying different protein binding affinity.⁴⁰ In contrast to BSA, the PDMS coated stir bar presents a well-defined alternative, which can be easily extracted to quantify specifically the chemical fraction which is bound to the PDMS compared to the chemical fraction which is freely dissolved in the basolateral solution. However, in case of the PDMS the removal is predominantly dependent on the hydrophobicity ($\log K_{ow}$) of the chemical.

Transfer at 4°C

Decreasing the exposure temperature from 19°C to 4°C did evidently influence the mass of Damascone beta present in the apical side in the presence of cells (Figure 4 E), but only slightly affected the mass of Damascone beta that permeated to the basolateral side over time (Figure 4 F). After 10 h and in the presence of cells, more Damascone beta was recovered from the apical compartment at 4°C (62 % of dosing mixture) compared to 19°C (38.0 ± 5.0 % of dosing mixture). This difference in recovery was discussed before as indication for biotransformation in the RTgutGC cells. In the absence of cells, apical mass was comparable between experiments at 4°C (64 % of dosing mixture) and 19°C (59 ± 7.5 % of dosing mixture) (Figure 4 E compared to Figure 4 A). In contrast to the apical mass, the amount of Damascone beta that permeated to the basolateral was comparable between experiments conducted at 4°C (RTgutGC: 14.7 and cell-free: 23.3 % of dosing mixture) and at 19°C (RTgutGC: 12.7 ± 3.8 and cell-free: 27.4 ± 5.2 % of dosing mixture). It has been shown that temperature and permeability are related exponentially and that a change in temperature in a lower temperature range has only little influence on the transfer rates.⁴¹ Based on the Wilke-Chang equation, a temperature decrease of 15°C should theoretically decrease the diffusion rate by approx. 5.1 %⁴²⁻⁴³, which is expected to only slightly affect the permeation rate. The calculation of the permeation rate across the cells, however, depends on the total mass in the system. Since the total mass was higher in experiments conducted at 4°C compared to 19°C, the calculated P_{cell} at 4°C was with $1.5 \cdot 10^{-5}$ cm/s approx. 1.7 times lower than the rates at 19°C (see Figure 4 F, SI Table 2).

Conclusion

The aim of this study was to design an exposure device, which enables stable *in vitro* exposure concentrations for hydrophobic and volatile chemicals. The exposure system TransFER was developed and tested with a focus on fish, using the recently presented fish intestinal barrier model. The exposure chamber, however, can easily be transferred to other cell based barrier systems, such as the primary fish gill cell model⁶ or the human intestinal Caco-2 model.¹ In the intestinal fish system, we successfully studied the transfer of the fragrance Damascone beta and calculated an *in vitro* permeability coefficient, which combines biotransformation and transfer across the intestinal epithelium. The well-defined exposure scenario established in our system comprises a prerequisite for studying mechanisms underlying chemical transfer, as for example active transport or biotransformation. With this study, we provided a proof of concept, which can be further evaluated by testing more chemicals with a wider range of physico-chemical properties, especially logK_{OW} and logHLC. Such efforts will also help to determine the exact applicability range of the novel TransFER device.

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Supporting Information

Contains details on the work flow, protocols for chemical quantification, as well as additional results (stability of Damascone beta in different solutions, Damascone beta mass in cells and on PDMS in basolateral compartment, potential reaction mechanisms, permeability coefficients).

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Accepted version

Figure 1 Composition of the TransFER exposure chamber

The TransFER chamber is built of a holder, accommodating one 6-well permeable membrane (insert) and a motor agitating a passive dosing/sampling system in the apical compartment. The basolateral chamber provides sufficient space to include a PDMS coated stir bar to serve as a passive sampling/dosing system and to agitate the basolateral solution (A, B). The entire system can be closed air-tight and the motor is powered by a 9 V block battery (C).

A

B

C

Figure 2 Cell viability and monolayer integrity of RTgutGC cells on permeable supports exposed to Damascone beta.

The cell viability (A) and monolayer integrity, measured as TEER (B) and as transfer of integrity markers (C), were assessed after different times of exposure with 1 mg/L Damascone beta and after 24 h exposure of L-15/ex serving as a control (Ctrl, grey consigned). Cell viability values are shown as background-corrected absolute fluorescence values (A). Background corrected TEER values from each permeable support after the exposure were normalized to the respective TEER values before the exposure (dashed line = 100 %). Values above the dashed line indicate an increase and values below this line a decrease of TEER as a consequence of the exposure (B). The transfer coefficient [cm/s] of Lucifer Yellow and of Rhodamine Dextran across the cell layer, P_{cell} , was calculated and is plotted on a log-scale (C). All data are shown as biological replicates and mean $\pm$ SD (n=3), except for the control (n=2) and the 24 h time point (n=1). Up to 10 h of exposure, no significant differences compared to the control were detected by ANOVA followed by a Dunnett's post-hoc test.

580 **Figure 3 Mass balance of Damascone beta in the TransFER exposure chamber.**

581 The mass balance was calculated as sum of the apical and basolateral compartment for experiments at 19°C, with (grey)
 582 and without (black) PDMS-coated stir bar (= sink) and at 4°C (white). The analysed masses for cell-free membranes
 583 (squares) and membranes seeded with RTgutGC cells (circles) were normalized to the introduced mass for each
 584 experiment. The dashed line indicates the 100% recovery. All 19°C data are shown as mean ± SD of three and 4°C data as
 585 the mean ± SD of two biological replicates and the line fitted against the mean.

586

Time of exposure [h] with 1 mg/L Damascone beta

Figure 4 Transfer of Damascone beta in different exposure scenarios.

Change of Damascone beta mass in the apical (A, C, E) and basolateral (B, D, F) compartment in experiments conducted at 19°C and 4°C in the presence and absence of RTgutGC cells and at 19°C in the presence and absence of a chemical sink. The analysed mass for cell-free (grey-filled circles) and permeable supports seeded with RTgutGC (white circles) was normalized to the introduced mass for each experiment. All experiments at 19°C are shown as individual replicates with the fitted line $\pm$ 95 % confidence intervals in dashed lines. Experiments at 19°C consist of 4 biological replicates, experiments at 19°C in the presence of a chemical sink of 3 biological replicates and experiments at 4°C represent two biological replicates. The transfer of Damascone beta across the cell layer P_{cells} with 95 % confidence intervals was calculated as indicated in the experimental section; values are shown in Panel B, D and F.