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Article

Mechanisms of pH-Dependent Uptake of Ionizable Organic Chemicals by Fish from Oil Sands Process-Affected Water (OSPW)

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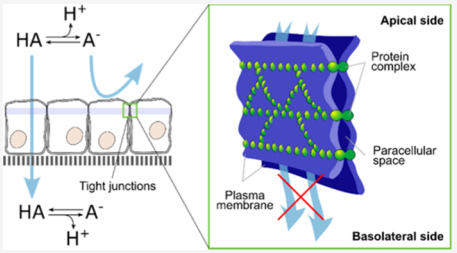
ABSTRACT: Uptake and effects of ionizable organic chemicals (IOCs) that are weak acids in aqueous solution by fish can differ as a function of pH. While the pH-dependent behavior of select IOCs is well-understood, complex mixtures of IOCs, e.g., from oil sands process-affected water (OSPW), have not yet been studied systematically. Here, we established an in vitro screening method using the rainbow trout gill cell line, RTgill-W1, to investigate pH-dependent cytotoxicity and permeation of IOCs across cultured epithelia using ultra-high-performance liquid chromatography with high-resolution mass spectrometry (UPLC-HRMS). The assay was benchmarked using model chemicals and technical mixtures, and then used to characterize fractions and reconstituted extracts of field-collected OSPW. Significant pH-dependent cytotoxicity of individual IOCs, acidic fractions, and reconstituted extracts of OSPW was observed. In vitro data were in good agreement with data from a 96 h in vivo exposure experiment with juvenile rainbow trout. Permeation of some IOCs from OSPW was mediated by active transport, as revealed by studies in which inhibitors of these active transport mechanisms were applied. We conclude that the RTgill-W1 in vitro assay is useful for the screening of pH-dependent uptake of IOCs in fish, and has applications for in vitro−in vivo extrapolation, and prioritization of chemicals in nontarget screenings.



INTRODUCTION

It is widely accepted that internal concentrations of organic xenobiotics in aquatic organisms[1,2] or in specific target tissues[3] are critical determinants of their biological activity, and knowledge of the processes that affect internal concentrations is often vital to understanding interspecies differences in sensitivity to pollutants.[1,4] Assessments of internal concentrations often rely on measured whole-body or tissue-specific bioconcentration factors (BCFs) to relate median effect concentrations (EC_{50}s) based on exposure concentrations to corresponding internal concentrations.[3] This critical body burden concept demonstrates that for neutral organic molecules, toxic effects are a function of the molecular volume attained in a tissue and that this is proportional to the BCF. Many organic molecules, when corrected for their potential to be accumulated into lipids, in particular phospholipid membranes, have the same critical body burdens of approximately 3−5 mM. Therefore, computational models include predictions of internal concentrations and BCFs in the absence of experimental data on accumulation.[5−9] The conceptual basis for most of these models is that partitioning between the lipid-fraction of tissues of aquatic organisms and water is similar to that between bulk *n*-octanol and water.[5,10,11] This assumption works sufficiently well for neutral organic substances, particularly if elimination through hepatic biotransformation is explicitly accounted for (e.g., through in vitro biotransformation experiments, even though uncertainties remain).[12] Polar organic chemicals such as various pesticides, plasticizers, pharmaceuticals, and personal care products, however, do not meet these assumptions. This is especially true for ionizable organic chemicals (IOCs), i.e., chemicals that can be present in partly or fully dissociated forms under environmental conditions, for which predictions can be inaccurate.[11,13,14]

This shortcoming is particularly concerning because half of all industrial chemicals, and the majority of all human and veterinary pharmaceuticals, are IOCs.[15] Predictions of their bioaccumulation potential and toxicity often over- or underestimate measured values by orders of magnitude. The reasons for these discrepancies are manifold: the ability of charged and neutral species to permeate across epithelia, to partition between compartments, and to be actively transported or transformed by specific proteins varies greatly among chemicals and among species.[14] Furthermore, pH, ionic strength, and sorbent fractions can differ significantly between environment and organism and even between microenvironments within organisms, thereby further complicating assess-

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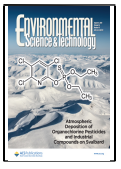

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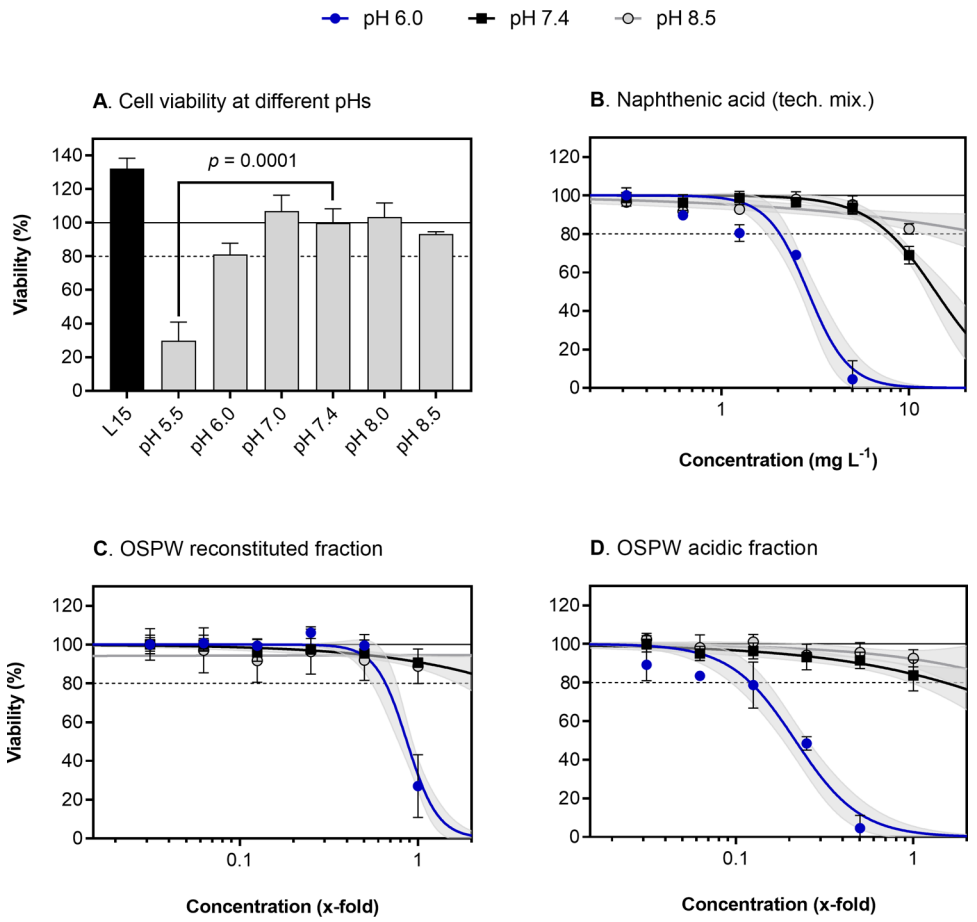

Figure 1. Viability of RTgill-W1 cells at several pHs (A), as well as cytotoxicity of a technical mixture of naphthenic acids (B) and two fractions of the aqueous phase of oil sand process-affected water (OSPW) in the cell line RTgill-W1 (C, D). (A) Viabilities of cells were determined in L15/ex at pHs ranging from 5.5 to 8.5 and normalized to that at the physiological pH of 7.4; viability in L15 culture medium is provided for comparison. Bars represent mean values, error bars the standard deviation of $n = 3$ technical replicates. Viability was significantly less in the pH 5.5 treatment compared to that in pH 7.4 (one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test). (B–D) Cytotoxicity was determined at pH 6.0, 7.4, and 8.5. Data points represent mean values, error bars the standard deviation of $n = 6$ technical replicates. A four-parameter logistic model was used to fit experimental data (solid lines) and 95% confidence interval computed (shaded areas).

ment of BCFs of IOCs.[16] To date, few studies have addressed these processes systematically,[17] and even fewer have focused on environmentally relevant conditions and complex environmental mixtures, such as municipal wastewater or mining effluents.

One such example of a complex sample matrix is oil sands process-affected water (OSPW), which is produced during the extraction of bitumen from oil sands.[18,19] The aqueous phase of OSPW is a complex mixture of neutral and ionizable organic chemicals, including "classic" naphthenic acids (NAs; $C_nH_{2n+2}O_2$), as well as mono- and polyoxygenated compounds, and multiple sulfur- and nitrogen-containing chemicals, the structures of which are unknown.[20−24] Previous studies have characterized OSPW and have found chemicals with log D_{OW} (pH-dependent *n*-octanol−water distribution ratio) ranging between −4.2 and 2.0[25] and dissociation constants (pK_a) between 3.7 and 7.0.[26] OSPW can exert biological effects in exposed organisms through various mechanisms of toxicity. Acute toxicity, however, is thought to be mostly associated with NAs present in the acidic fraction of some OSPWs.[27,28] Hence, it is reasonable to expect a strong influence of environmental pH on bioavailabilities and toxic potencies of constituents of OSPW. While the differential partitioning of IOCs from OSPW to neutral and polar lipids on toxicity has been comprehensively studied previously by employing the target lipid model,[29,30] effects of pH on bioavailabilities of these chemicals have not been systematically addressed to date.[5]

To overcome the limitations of assessments of bioaccumulation of IOCs in general, and dissolved organics present in OSPW specifically, in the research presented here, a rapid, in vitro screening method using the rainbow trout gill cell line RTgill-W1 was applied to study pH-dependent permeation of IOCs across epithelia of gills of fish. Fish are important sentinel species of aquatic ecosystem quality, and rainbow trout specifically are an important species in the context of bioaccumulation assessments. Specifically, the objectives were to determine (1) pH tolerance of RTgill-W1 cells; (2) pH-dependent cytotoxicity of single IOCs, technical mixtures of IOCs, and extracts of a sample of OSPW in adherent cultures; (3) permeation of IOCs across cells cultured in transwell inserts by means of ultra-high-performance liquid chromatography with high-resolution mass spectrometry (UPLC-HRMS); and (4) to verify the in vitro findings in a 96 h in vivo experiment with juvenile rainbow trout.

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Environ. Sci. Technol. 2020, 54, 9547−9555

MATERIAL AND METHODS

Oil Sands Process-Affected Water (OSPW). OSPW used during the present study was collected from the West-In-Pit tailings pond (Syncrude Canada Ltd.). This tailings pond was commissioned as a permanent storage basin, "Base-Mine Lake", in December 2012. For in vitro experiments, an aliquot of filtered OSPW (0.45 μm glass-fiber filter, Whatman) was extracted and fractionated using mixed-mode sorbents (MMSs) for in vitro experiments, as previously described.[31] During this process, neutral, acidic, and basic organic fractions were obtained at 1000× concentration in ethanol. Also, a reconstituted total extract that contained equal amounts of each of these three fractions (1000× final concentration) was prepared. Diluted native OSPW was used for in vivo experiments with rainbow trout. Furthermore, a technical mixture of naphthenic acids (NAs) as well as six individual weak acids (4-chlorophenol, 2,4-dichlorophenol, and 2,4,5-trichlorophenol as reference chemicals with known cytotoxicity, as well as a 4-nonylphenol technical mixture, cyclohexyl carboxylic acid, and dicyclohexylacetic acid) were used for in vitro experiments (Sigma Aldrich, St. Louis). Stock solutions of these chemicals were prepared in dimethyl sulfoxide (DMSO).

Cell Culture. The permanent rainbow trout gill cell line RTgill-W1 was obtained from the American Type Culture Collection (CRL-2523, ATCC, Manassas). Cells were cultured in Leibowitz's L15 medium (Gibco, Thermo Fisher Scientific, Waltham) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific), 100 units/mL penicillin, and 100 μg mL^{-1} streptomycin (Gibco). Cells were cultured with modifications to previously described methods.[32,33] Briefly, cells were grown in 75 cm^2 tissue culture flasks (Greiner BioOne, Monroe) at 20 °C and subcultured or harvested for bioassays at approximately 95% confluency using trypsin solution (Gibco, Thermo Fisher Scientific).

Neutral Red Retention Assay. Cytotoxicities of single chemicals, complex mixtures of IOCs, and fractions of OSPW extracts were determined in L15/ex medium at different pHs using the neutral red retention assay as described previously.[34] The readout of this assay provides a direct proxy for cell count rather than metabolic activity and was thus chosen over other cytotoxicity assays. L15/ex is a reduced-complexity L15 medium that, in addition to the L15 salts and buffer constituents, only contains sodium pyruvate and galactose.[35] We chose this medium to avoid interactions between tested samples, medium constituents, and pH of the media. The pH of L15/ex was adjusted with dilute hydrochloric acid or sodium hydroxide. Before exposure, 100 μL of cell suspension in complete L15 culture medium, which had been adjusted to 5×10^5 cells mL^{-1}, was seeded into 96-well microplates (Greiner BioOne) and allowed to adhere for 24 h. Next, cells were exposed in quadruplicate wells to six graded concentrations of test samples based on 2-fold serial dilutions of test compounds and mixtures in L15/ex medium of pH 6.0, 7.4, and 8.5. Individual chemicals and technical mixtures were tested at concentrations ranging from 0.3125 to 10 mg L^{-1} and OSPW extracts at concentrations ranging from 0.03125- to 1-fold. To avoid the influence of interplate differences, all three pH treatments were included on one microplate. A range-finding assay (pHs from 5.5 to 8.5) was conducted to assess the pH range acceptable for cell viability assays (Figure 1A). Negative controls (L15/ex only) or solvent controls (L15/ex with 0.1% DMSO or ethanol), where appropriate, as well as positive controls (methanol treatment after exposure) at the appropriate pH, were included on each plate. After 48 h of exposure, dosing media were removed and exchanged with a 0.005% (w/v) neutral red solution in L15 medium. After 3 h of incubation, cells were washed twice with phosphate-buffered saline (PBS), and neutral red was extracted with a solution of 1% (v/v) glacial acetic acid in 50% ethanol (v/v). After 30 min of extraction on a shaker, absorbance at 540 nm was read using a POLARstar Optima microplate reader (BMG LABTECH, Cary). Results were expressed as percent viability observed in the negative control relative to the positive control. Data were fitted using four-parameter logistic regression using Prism 7 software (GraphPad, San Diego), and half-maximal effect concentration (EC_{50}s) was interpolated. Concentrations of samples that resulted in less than 20% reduction in viability were considered adequate for permeation experiments.

Permeable Insert Experiments. To assess the effects of pH on permeabilities of IOCs across gill epithelium, RTgill-W1 cells were seeded to full confluence into permeable, 0.4 μm pore diameter, translucent cell culture inserts in 24-well plates (Greiner ThinCert). Briefly, cells were initially grown for 14 days under symmetric conditions in supplemented L15 culture medium in the apical chamber, i.e., within the insert, and the basolateral chamber, i.e., in the well below the insert, with one medium change after 7 days. To avoid any damage to epithelia, media were decanted rather than aspirated. To verify the maturity of the epithelium, transepithelial electrical resistance (TEER) of each insert was determined using an epithelial volt-ohm meter with chopstick-type electrodes (World Precision Instruments Inc., Sarasota). TEER readings were corrected for blank measurements and normalized to the surface area of the insert. Subsequently, cells were cultured under asymmetric conditions and acclimatized to different pHs, with supplemented culture medium (pH 7.8) in the basolateral and L15/ex of the appropriate pH in the apical chamber. After an additional 7 days, TEER was measured one more time. Inserts were dosed in duplicate with noncytotoxic concentrations of single chemicals, complex mixtures, as well as fractions and reconstituted total extracts of OSPW. Control inserts were treated as dosed inserts but contained only L15/ex of the appropriate pH with 0.1% (v/v) solvent only, rather than samples. Following 24 h of exposure on a rocking shaker (1 rpm), media from apical and basolateral chambers as well as inserts including cells were collected and stored at −80 °C for chemical analysis.

Active Transport Experiments. To assess the effects of active transport on permeation of chemical species from OSPW, experiments with the active transport inhibitor cyclosporin A (CsA), which inhibits both solute carrier (SLC) and ATP-binding cassette (ABC) transporter families,[40] were performed. First, the effective concentration in RTgill-W1 cells was verified using a previously described calcein AM efflux assay.[36] A co-exposure experiment with CsA and reconstituted total extracts of OSPW was then performed as described above. Reference samples with CsA only and OSPW only and a control sample were included during incubation. In line with previous observations,[36] a CsA concentration of 20 μM, which caused maximum inhibition of calcein AM efflux, was used.

In Vivo Exposure Experiment with Juvenile Rainbow Trout. In vitro findings were compared with results of a 96 h semistatic in vivo exposure of fingerling rainbow trout. Fish were obtained as fertilized eggs from the Troutlodge hatchery (Benney Lake) and reared for approximately 60 days (final

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weight: 611 ± 188 mg, final length 35.4 ± 4.2 mm) in the Toxicology Centre's Aquatic Toxicology Research Facility at the University of Saskatchewan, Saskatoon, Canada. After swim-up, fish were fed size 0 BioVita starter food (Bio-Oregon, Vancouver, Canada). A total of 10 fish were exposed per 8 L tank (n = 3 replicates per treatment) in a temperature-controlled chamber (14.1 ± 0.2 C). Fish were exposed to either uncontaminated facility water (ammonia: <0.25 mg L^{-1}, dissolved oxygen: 89% saturation, conductivity: 266 ± 7.8 μS cm^{-1}) or 10% (v/v) OSPW diluted in facility water at pHs 6.0, 7.4, and 8.5. The pH was adjusted to the desired value using concentrated hydrochloric acid or sodium hydroxide. Because pH drifted over time due to carbonate buffering, pH was measured before and after each 24 h period, and time-weighted average (TWA) pHs were calculated. TWAs were 6.74 ± 0.03, 7.63 ± 0.03, and 8.21 ± 0.03 for pH 6.0, 7.4, and 8.5 controls and 6.80 ± 0.02, 7.75 ± 0.02, and 8.32 ± 0.01 for pH 6.0, 7.4, and 8.5 OSPW treatments, respectively. Fish were fed ad libitum every morning, size 0, BioVita starter food, before 50% changes of exposure solutions for each tank in the afternoon. Two fish were subsampled from each tank at 24 and 48 h, before termination at 96 h, at which time the remaining four fish in each tank were sampled. Fish were euthanized by immersion in buffered Tricaine methanesulfonate (MS-222). Each fish was weighed, measured, and frozen at −20 °C for future chemical extractions. Two fish per timepoint were dissected and their gills collected, weighed, snap-frozen in liquid nitrogen, and transferred to −80 °C for final storage. This study was approved by the institutional animal welfare committee of the University of Saskatchewan, Canada (Animal Use Protocol number 20170103), and was conducted following the Canadian Council on Animal Care (CCAC) regulations.

Chemical Analyses. Before analysis by ultra-high-performance liquid chromatography and high-resolution (accurate mass) spectrometry (UPLC-HRMS), samples were thawed and extracted. RTgill-W1 cells were extracted with a 1:4 (v/v) ethyl acetate/hexane mixture containing 0.03% (v/v) formic acid. Samples of media were liquid−liquid extracted twice with equal volumes of the same solvent mixture. Samples were vortex-mixed, and the top layer was collected and allowed to evaporate overnight at room temperature. Samples were reconstituted in 100 μL of methanol (with 500 ng L^{-1} myristic acid as the internal standard) and transferred into chromatography vials for chemical analysis. Whole fish and gill samples from in vivo experiments were first homogenized in a Bead Ruptor 24 bead mill homogenizer using 2 mm stainless steel (Omni International, Kennesaw). Homogenates were extracted twice with 3 mL of 2% formic acid in dichloromethane, and then reduced and reconstituted in methanol as described above.

All UPLC-HRMS analyses were performed as described previously,[37] with slight modifications. Briefly, analyses of extracts were performed on a Q Exactive hybrid, quadrupole-orbitrap mass spectrometer (Thermo Fisher Scientific) interfaced to a Dionex UltiMate 3000 UPLC system (Thermo Fisher Scientific) or a Q Exactive HF hybrid quadrupole-orbitrap mass spectrometer (Thermo Fisher Scientific) coupled to a Vanquish Flex UPLC system (Thermo Fisher Scientific). Chemicals were separated on a Waters (Milford) XTerra MS C18 column (3.5 μm particle size; 2.1 mm diameter × 100 mm length; injection volume 5 μL). Ultrapure water (A) and methanol (B) were used as mobile phases. Initially, 5% B was increased to 60% over 7 min, then increased to 100% in 14 min, and held static at 100% for 10 min, followed by a decrease to initial conditions of 5% B and a 3 min equilibration period. The flow rate was set to 0.25 mL min^{-1}, and the column and sample compartments were temperature-controlled at 30 and 10 °C, respectively. Data were acquired in full scan mode (100−1000 m/z) at a resolution of 70 000 (FWHM at m/z 200) with a maximum of 3×10^6 ions collected (maximum collection time: 100 ms). The mass spectrometer was operated in negative electrospray ionization (ESI) mode, with a spray voltage of 2.8 kV, a capillary temperature of 350 °C, sheath gas flow rate of 35 L h^{-1}, an auxiliary gas flow rate of 8 L h^{-1}, and a probe heater temperature of 350 °C.

Prediction of chemical formulas was carried out using the formula prediction feature in MZmine 2.29, wherein possible formulas were set to contain up to 100 carbon, 200 hydrogen, 6 nitrogen, 10 oxygen, and 3 sulfur atoms per molecule. To generate peak lists for each sample, the exact mass chromatograms of the samples were used. A noise cutoff of 200, followed by an FTMS shoulder peak detection with a mass resolution of 100 000, was used for detecting masses in individual scans. To build chromatograms, a minimum peak width of 0.2 min, a minimum height of 5 000, and a mass tolerance of 0.002 m/z were used. Peaks were smoothed with a filter width of 6 and deconvoluted by the local minimum search (chromatographic threshold 35%, a search minimum in the retention time range of 0.5 min, a minimum absolute height of 50 000, a minimum ratio of peak top/edge 2.3, peak duration range 0.2−10 min). Retention times were normalized by the use of the retention normalization option with a mass tolerance of 0.002 m/z, a retention time tolerance of 0.3 min, and a minimum intensity of standards for normalization of 500 000. The normalized peak lists were aligned (m/z tolerance of 0.0009, retention time tolerance of 0.4 m, retention time tolerance after correction at 0.2 min, the minimum number of points at 60%, a threshold value of 10), gaps in aligned peak lists were filled, and peak lists were filtered for duplicates. Final peak lists were used for the suspect and nontarget screening. Specifically, exact masses of $[M - H]^-$ ions with an m/z tolerance of 5 ppm were used in negative ion mode to search for suspect compounds. In the absence of calibration standards, all concentrations were expressed as relative abundances, i.e., the peak area of a given feature measured at a specific pH divided by the respective maximum peak area detected at any of the studied pHs.

Statistical Analysis. All statistical analyses were performed using Prism 7 software (GraphPad, San Diego). Since all data passed tests for deviation from normality (Shapiro−Wilk's test, $p \leq 0.05$) and homoscedasticity (Bartlett's test, $p \leq 0.05$), data were analyzed using parametric t-tests (two groups) or one-way analysis of variance (ANOVA), followed by Dunnett's or Tukey's multiple comparison post hoc tests (three and more groups). Differences were considered statistically significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

RTgill-W1 Cells Can Be Acclimatized to a Wide Range of pHs. To identify the range of acceptable pHs for subsequent cytotoxicity and permeation experiments, the viabilities of RTgill-W1 cells at different pHs were determined (Figure 1A). Although viabilities of cells were slightly less in cells exposed to L15/ex at pH 7.4 compared to those exposed

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Environ. Sci. Technol. 2020, 54, 9547−9555

to the complete L15 medium of the same pH (Figure 1A, black bar), this difference was not statistically significant and was likely related to differences between the minimal exposure medium and the richer culture medium.[38] Relative to L15/ex at pH 7.4, all tested pHs resulted in viabilities ≥80% of that at pH 7.4. The only exception was the pH 5.5 treatment, which resulted in viability of only 30% of that at pH 7.4 (one-way ANOVA with Dunnett's multiple comparison test, $p = 0.0001$).

Although rainbow trout can live in acidic waters, previous studies of pH tolerance of rainbow trout have reported that long-term exposure to water of pH 5.5 and less resulted in significant embryo lethality and poor survival of adult fish.[39] Based on these findings, it can be concluded that permeation experiments with RTgill-W1 cells, similar to their live donor organisms, should not be conducted at pHs less than 6. Previous experiments with primary cultures of gill cells from rainbow trout have been conducted at pHs ranging from 6 to 9, and therefore are consistent with our findings.[40] Although more acidic waters are occasionally found, including acid mining effluents, industrial effluents, and acid deposition in poorly buffered waters, the pH range of the present study sufficiently represents the conditions naturally found in most rivers, lakes, and wetlands in the Northern Hemisphere.[41]

Cytotoxicity of Single Chemicals and the Mixture Is Strongly pH-Dependent. Cytotoxicity of a technical mixture of NAs as well as fractions of OSPW was dependent on pH (Figure 1B–D). While no cytotoxicity was observed for any of the samples at pH 8.5, a marked increase in toxicity was observed with decreasing pH for the technical mixture of NAs (Figure 1B), as well as reconstituted OSPW and the acidic fraction of OSPW (Figure 1C,D). It should be noted that the chemical composition of the technical mixture of NAs does not reflect the organic or inorganic chemical composition of OSPW and that toxicities of technical mixtures are poor surrogates for the toxicity of OSPW.[42−44] Neither the neutral nor basic fraction of OSPW (data not shown) caused cytotoxicity at any tested combination of concentration and pH. The half-maximal effect concentration (EC_{50}) of the technical naphthenic acid mixture at pH 7.4 was 13.9 mg L^{-1} and decreased almost by 5-fold to 2.90 mg L^{-1} at pH 6.0. The acidic fraction of OSPW and reconstituted OSPW did not cause cytotoxicity at pH 7.4, while their EC_{50} was 0.22× and 0.87× OSPW equivalents at pH 6.0, respectively. To ensure that subsequent permeation experiments would not be impacted by cytotoxicity, a concentration of 0.03× OSPW equivalents was chosen because this concentration did not cause cytotoxicity for any sample at any pH. Additionally, the performance of the assay was verified with six individual weak acids (Supporting Information Figure S1), where cytotoxicity showed an inverse relationship with pH.

Based on their physicochemical properties, pH-dependent cytotoxicities of the technical naphthenic acid mixture and samples of OSPW and weak acids were expected. The relative proportion of neutral (protonated) species of weak acids compared to their charged (deprotonated) counterparts increased with decreasing pH, thereby allowing for greater apparent uptake and effects in cells. These findings are consistent with results of experiments with Chinese hamster ovary (CHO) cells, in which the cytotoxicity of the chemotherapeutic, weak acid chlorambucil was inversely proportional to pH.[45] Similar observations were made related to the toxicity of different chloro-, bromo-, and nitro-substituted phenols to the guppy (*Poecilia reticulata*).[46] In this study, toxic potencies of primary ionized phenols were inversely proportional to pH. Last, Rendal et al.[47] critically reviewed existing literature and found that both toxicity and BCFs were greater for acids at lower pH values, whereas the opposite effect was observed for bases. The authors observed differences across the studied pH range up to a factor of 100, and the changes were most pronounced approximately three pH units around the pK_a.[47] Based on the observed pK_a range of IOCs from OSPW between 3.7 and 7.0,[26] significant differences in bioaccumulation and/or toxicity of OSPW are expected across the natural pH range of aquatic ecosystems.

Permeability of Weak Acids across Epithelia Is Inversely Proportional to pH. Because manipulation of apical pH might have resulted in altered integrity of epithelia, transepithelial electric resistance (TEER) of all inserts was quantified before initiation of exposure,[33] and it was confirmed that apical pH alone did not result in a reduction of epithelial integrity (Supporting Information Figure S2). Also, performance of the epithelial permeation assay was verified by observing transepithelial permeation of two weak acids, dicyclohexylacetic acid and a technical mixture of isomers of nonylphenol, at several pHs of exposure media in the apical chamber. For both chemicals, permeability was inversely proportional to pH (one-way ANOVA with Dunnett's multiple comparison test, $p \leq 0.05$; Supporting Information Figure S3).

The pH of exposure solutions might have influenced the transepithelial permeabilities of technical NAs and IOCs from fractions of OSPW. In the epithelial permeation assays, both the number and relative abundance of individual chemical species that were able to cross the epithelium, or that accumulated within the cell, were affected by pHs of apical exposure media (Figure 2). At pHs 6.0 and 7.4, a total of 186

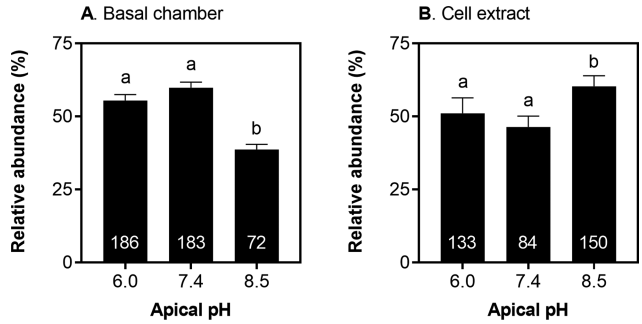

Figure 2. Relative abundances of naphthenic acids from a technical mixture of naphthenic acids in the basal chamber (A) and in extracts of cells (B) at different pHs of exposure media in the apical chamber. Data represent the mean ± standard error of the mean and were normalized to the mean of the group with the greatest relative abundance. The respective number of chemicals with the greatest relative abundance in each group is provided within the bars. Differences were tested for statistical significance using repeated-measure one-way ANOVA with Tukey's multiple comparison test ($p \leq 0.05$).

and 183 chemical species, respectively, from the technical NA mixture were detected in extracts of basal media (Figure 2A). In contrast, only 72 species were detected at pH 8.5 (one-way ANOVA with Tukey's test, $p \leq 0.05$). The average relative abundances of all chemical species detected were 55.4 ± 2.06, 59.9 ± 1.91, and 37.7 ± 1.80% for pH 6.0, 7.4, and 8.5, respectively (Figure 2A). A similar tendency toward a decreased average relative abundance of chemical species in

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Environ. Sci. Technol. 2020, 54, 9547−9555

basal extracts at pH 8.5 was observed for reconstituted extracts of OSPW (Figure 3A; one-way ANOVA with Tukey's multiple

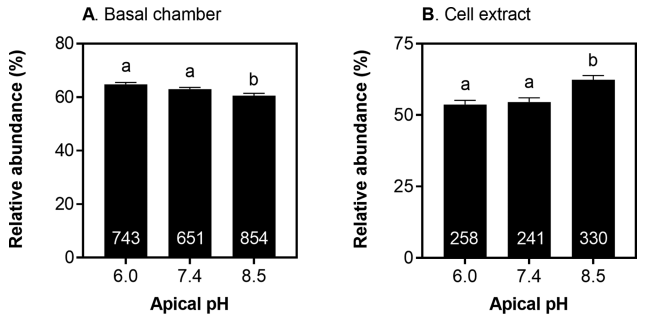

Figure 3. Relative abundances of chemical species from reconstituted extracts of the aqueous phase of oil sand process-affected water (OSPW) in the basal chamber (A) and extracts of cells (B) at different pHs of exposure media in the apical chamber. Data represent the mean ± standard error of the mean and were normalized to the mean of the group with the greatest relative abundance. The respective number of chemicals with the greatest relative abundance in each group is provided within the bars. Differences were tested for statistical significance using repeated-measure one-way ANOVA with Tukey's multiple comparison test ($p \leq 0.05$).

comparison test, $p \leq 0.05$). Contrary to the observations for technical NAs, however, the total numbers of chemical species in basal extracts with 854 individual chemicals were greatest in treatments with an apical pH of 8.5.

Lesser permeation of chemical species across the cells of the epithelium from apical to basal chambers at pH 8.5 might have been caused by retention of these chemicals within the cell or cell membranes. Average relative abundances of chemical species in extracts of cells, after exposure to technical naphthenic acid or reconstituted OSPW, were significantly greater at pH 8.5 compared to pH 6.0 and 7.4, respectively (Figures 2B and 3B; one-way ANOVA with Tukey's multiple comparison test, $p \leq 0.05$). Also, total numbers of these chemicals in cells, with 150 and 330 total chemicals in the technical NA and reconstituted OSPW treatments, respectively, were greater at pH 8.5 compared to all other pHs.

Although the chemical compositions of the technical NA and reconstituted OSPW are different,[42−44] results were generally in good agreement, as greater permeabilities from apical to basal chambers were observed with decreasing pH. This is likely because, despite their different compositions, the physicochemical properties of the two classes of chemicals were similar. However, it was surprising to find that some chemicals of potentially ionized speciation were taken up by cells or cell membranes from the apical exposure media to a great extent at pH 8.5, some of which were not found at lower pH treatments. These findings might be explained by the physicochemical properties of the charged organic acids found in the technical mixture of NAs and OSPW. These often show amphiphilic characteristics and tend to partition into phospholipids, as has been demonstrated recently for OSPW at its native pH of approximately 8.5.[25,48] Furthermore, these observations might suggest accumulation through active transport by energy-requiring transporter proteins present in the membranes of cells.[40] These might further complicate assessments of the bioavailability of IOCs beyond simple physicochemical considerations. Last, there might be an impact of pH on biotransformation among the treatments, which should be the subject of dedicated future research.

Permeability of Charged Species Across Epithelia Appeared to Be Facilitated by Active Transport. Absorption of ionized chemical species into cells and potentially their permeation into basal media at pH 8.5 might have been the result of active transport processes. To test this hypothesis, the commonly used inhibitor of active transport proteins, cyclosporin A (CsA), was used, which inhibits both solute carrier (SLC) and ATP-binding cassette (ABC) transporter families.[40] The half-maximal inhibitory concentration (IC_{50}) of CsA in the calcein AM efflux assay with RTgill-W1 cells was 4.15 ± 1.24 μM (Figure 4A), which is in close agreement with a previously published value of 3.12 ± 1.24 μM.[36] To achieve maximum inhibition of transporter activity, subsequent permeation experiments were conducted using CsA at a concentration of 20 μM.

Co-exposure with CsA and OSPW led to a decrease in the relative abundance of almost all chemical species from OSPW in basal media compared to treatment in the absence of CsA

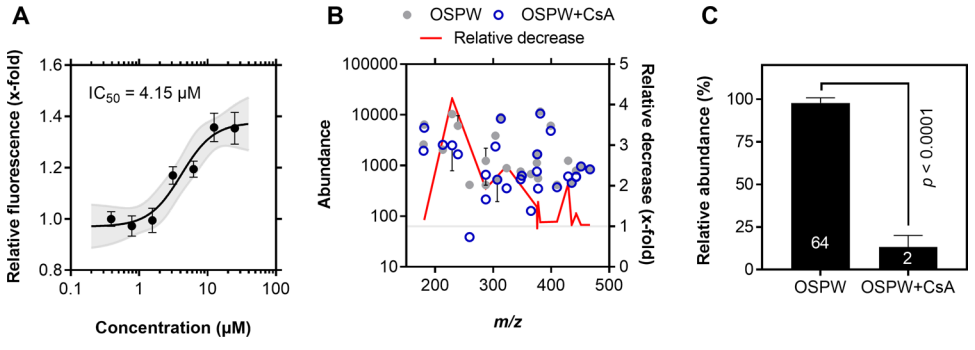

Figure 4. Inhibition of calcein AM efflux in RTgill-W1 cells (A) and reduction of the abundance of various chemical species from OSPW in basal media (B, C) following exposure to the active transport inhibitor cyclosporin A (CsA) at pH 8.5. (A) Data points represent mean values, error bars the standard deviation of $n = 6$ technical replicates. A four-parameter logistic model was used to fit experimental data (solid lines) and 95% confidence intervals computed (shaded areas). (B) Data represent the mean ± standard deviation of raw abundances (areas) of $n = 2$ replicate inserts. Only those chemicals present in both treatments are shown. The solid red line indicates the x-fold reduction in chemical abundance following CsA treatment compared to OSPW alone. (C) Data represent the mean ± standard error of the mean and were normalized to the mean of the group with the greatest relative abundance. The respective number of chemicals with the greatest permeability in each group is provided within the bars. Relative abundance in basal media was significantly lesser following CsA treatment compared to OSPW alone (paired Student's t-test).

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Environ. Sci. Technol. 2020, 54, 9547−9555

(Figure 4B). This change was most pronounced in the m/z range between 200 and 380 (Figure 4B), and the difference in mean relative abundance between both treatments was statistically significant (paired Student's t-test, p <0.0001; Figure 4C). These findings could help explain the results of a previous study that showed inhibitory effects of OSPW, in particular, the neutral and basic fractions of the aqueous phase of OSPW, on active transport in early-life stages of Japanese medaka.[31] Given the observations of the present study, these effects might well be explained by a competitive inhibition process. Future research is needed to further identify the exact transporters involved in these processes and to further narrow down the chemical identity of the substrates.

Permeability of Weak Acids In Vivo Is Impacted by pH. Results of the 96 h exposure of rainbow trout fingerlings to a 10% concentration (v/v) of raw OSPW revealed a pattern similar to the effects of reconstituted OSPW on the movement of IOCs observed in vitro. Specifically, relative abundances and numbers of permeated chemicals in extracts of gill tissue were directly proportional to pH (two-way ANOVA with Tukey's multiple comparison test, $p \leq 0.05$; Figure 5). Results of

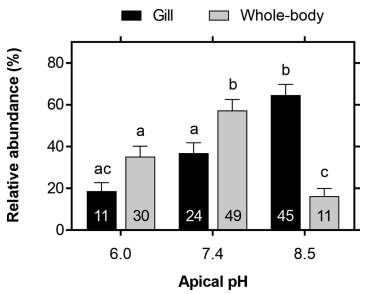

Figure 5. Relative abundances of chemical species from oil sand process-affected water (OSPW) in gill and whole-body of juvenile rainbow trout following 96 h exposure to 10% OSPW at pHs 6.0, 7.4, and 8.5. Bars represent mean values, error bars the standard deviation of relative abundances across 80 (gill) and 90 (whole-body) chemicals, respectively. Numbers of chemicals with the greatest relative abundance in each group are provided within the bars. Differences were tested for statistical significance using two-way ANOVA with Tukey's multiple comparison test ($p \leq 0.05$).

whole-body extracts of the same fish, however, did not follow the same pattern as those obtained from the basal chamber of cell culture inserts. The greatest permeation and/or partitioning of chemicals, as indicated by absolute numbers and relative abundances, was observed at pH 7.4, followed by pHs 6.0 and 8.5 (two-way ANOVA with Tukey's multiple comparison test, $p \leq 0.05$; Figure 5). These differences might be due to physiological factors between in vivo experiments and the in vitro model. In the intact gills of live fish, there are an acidic microenvironment, various cell types and epithelial thicknesses present, and diffusion and flow limitations. These might have influenced dispositions of chemicals from OSPW in vivo but not in the more simple in vitro model.[13,14] These findings might also suggest that charged organic acids from OSPW are preferentially retained in cell membranes of gill epithelia at alkaline pHs due to their amphiphilic character and resulting partitioning into phospholipids.[25,48] Additionally, similar active transport processes to the ones studied here in gill epithelia might be of relevance in other compartments as well, and these might have had a confounding effect on the resulting whole-body concentrations.

Potential Implications for the Environmental Risk Assessment of IOCs. In this study, it was demonstrated that in vitro transepithelial permeation assays with RTgill-W1 cells are useful tools to characterize uptake and bioavailability of IOCs. Results for single chemicals were in good agreement with expectations based on the physicochemical properties of these chemicals. Findings for organic chemicals from the aqueous phase of OSPW in vitro and intact OSPW in vivo showed that pH affected permeability and uptake of weak acids. However, our findings conflicted with the expectation that weak acids from OSPW would generally show much greater permeability at lower pH based on whole-body concentrations. Also, the results supported the hypothesis that at least a fraction of potentially charged or polar chemical species present in OSPW might be actively transported across biological membranes by active transport proteins. These findings could help explain the inhibitory effects of OSPW on active transport proteins in fish, as previously demonstrated.[31]

The results of our in vitro and in vivo experiments provide insights that suggest that studies on the bioconcentration of chemicals from OSPW need to consider pH as a significant contributing factor. In this light, previous studies characterizing the partitioning of chemicals from OSPW to neutral and polar lipids[5] should be repeated with varying pH to verify if this relationship also pertains to lipid partitioning. Additionally, there is the potential to refine current approaches for the prediction of OSPW toxicity based on the target lipid model and passive samplers also to include pH-dependent bioavailability and active transport-related processes.[11] The results presented herein indicate that the in vitro assay with RTgill-W1 cells could hold great potential to contribute to chemical risk assessment if appropriate in vitro−in vivo extrapolation methods can be developed.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.0c02522.

Cytotoxicity and epithelial permeability of other ionizable organic chemicals, as well as changes in transepithelial electric resistance over time; cytotoxicities of six ionizable organic chemicals in the cell line RTgill-W1 (Figure S1); changes in transepithelial electrical resistance (TEER) after changing the pH of exposure media in the apical chamber of culture inserts containing established RTgill-W1 epithelia (Figure S2); relative permeabilities of dicyclohexylacetic acid (A) and nonylphenol technical mixture of isomers (B) at different pHs of exposure media in the apical chamber (Figure S3) (PDF)

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Notes

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SUPPLEMENTARY INFORMATION

Mechanisms of pH-dependent uptake of ionizable organic chemicals by fish from oil sands process-affected water (OSPW)

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Total page number: four, including
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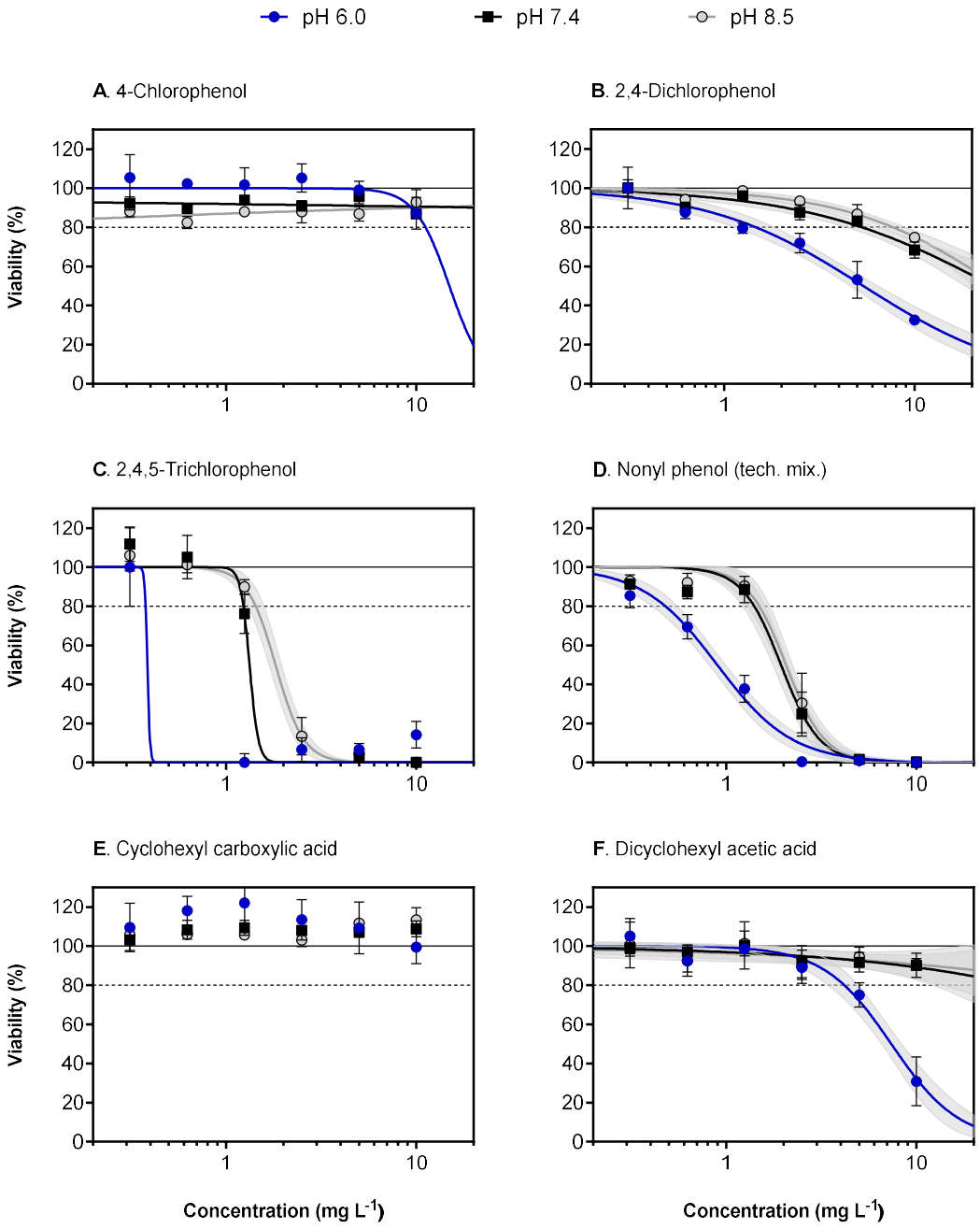

Figure S1. Cytotoxicities of six ionizable organic chemicals in the cell line RTgill-W1. Cell viability was determined at pH 6.0, 7.4, and 8.5. Data points represent mean values, error bars the standard deviation of *n*=6 technical replicates. A four-parameter logistic model was used to fit experimental data (solid lines) and 95% confidence intervals computed (shaded areas).

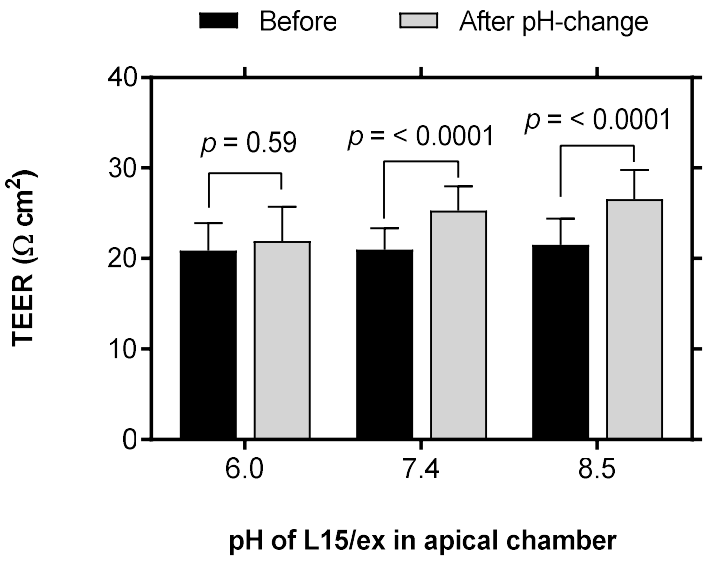

Figure S2. Changes in trans-epithelial electrical resistance (TEER) after changing the pH of exposure media in the apical chamber of culture inserts containing established RTgill-W1 epithelia. Data represent the mean ± standard deviation of *n*=22 technical replicates, i.e., all inserts used in subsequent permeation experiments. Differences were tested for statistical significance using two-way repeated-measures ANOVA with Bonferroni's multiple comparisons test.

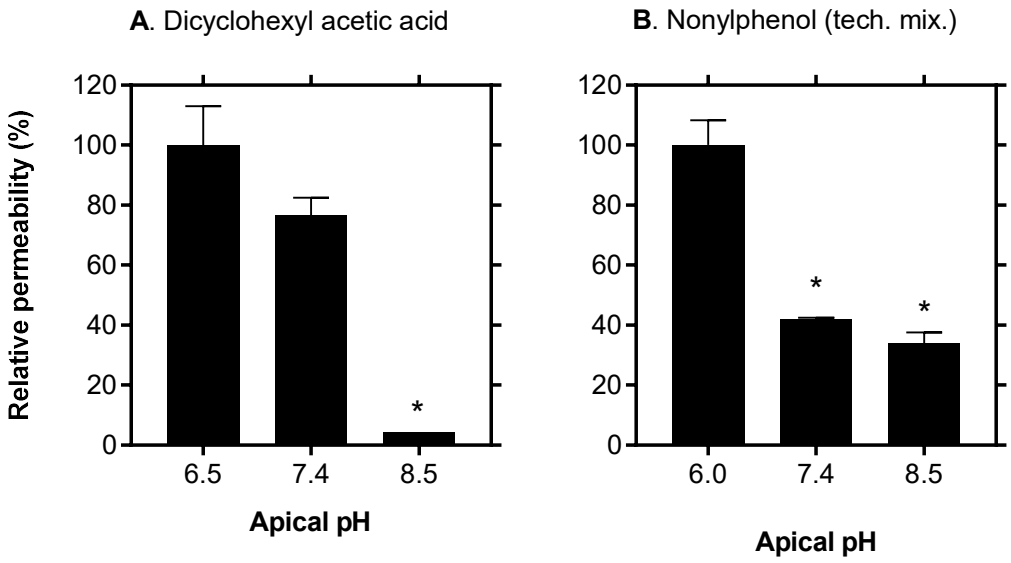

Figure S3. Relative permeabilities of dicyclohexylacetic acid (A) and nonylphenol (technical mixture of isomers; B) at different pHs of exposure media in the apical chamber. Data represent the mean ± standard deviation of n=2 replicate inserts and were normalized to the mean of the group with the greatest permeability (pH 6.0). Differences were tested for statistical significance using one-way ANOVA with Dunnett's multiple comparisons test ($p \leq 0.05$).