Environmental Science & Technology

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Inflammation of Gill Epithelia in Fish Causes Increased Permeation of Petrogenic Polar Organic Chemicals via Disruption of Tight Junctions

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Cite This: https://doi.org/10.1021/acs.est.1c05839

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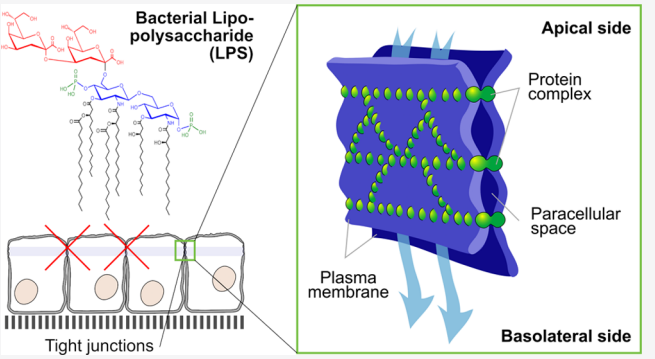
ABSTRACT: The epithelial cell layer that lines the gills of fish controls paracellular permeation of chemicals through tight junctions. The integrity of tight junctions can be affected by inflammation, which likely affects the bioavailability of chemicals. Here, the inflammation of the rainbow trout gill cell line RTgill-W1 was induced *via* exposure to bacterial lipopolysaccharides (LPS). Cells were then coexposed to extracts of oil sands process-affected water (OSPW), which contain complex mixtures of chemicals. After 24 h of exposure, cells exposed to LPS showed a reduction in transepithelial electrical resistance, an indicator of tight junction integrity. Quantitative reverse-transcription polymerase chain reaction (RT-PCR) analysis determined that abundances of transcripts of genes coding for tight junction proteins were significantly less in cells exposed to 20, 50, or 100 mg L^{-1} LPS. Chemical analysis revealed increased permeation of constituents of OSPW across epithelia at all studied LPS concentrations. These in vitro findings were confirmed in vivo in rainbow trout exposed to LPS and OSPW for 48 h, which resulted in greater accumulation of chemicals relative to that for fish exposed to OSPW alone. Our results demonstrated that inflammation and disruption of tight junctions could lead to greater uptake of potentially harmful chemicals from the environment, which has implications for mixture risk assessment.



KEYWORDS: *tight junctions, inflammation, pathogen, ionizable organic chemicals, OSPW*

1. INTRODUCTION

Some physiological processes of central importance to homeostasis take place at the fish gill, including gas exchange and osmoregulation.[1] Exchange of gasses at the gill follows a countercurrent process, during which deoxygenated blood flows in the opposite direction of oxygen-rich water. This feature makes the gill not only highly effective in oxygen transfer but also results in rapid uptake of other compounds such as waterborne contaminants. In fish, the gill also plays a vital role in osmoregulation in that it maintains the appropriate balance of water and ions between the internal body and its surrounding environment.[2] The osmoregulatory function of gill epithelia arises from the reduction and control of paracellular permeation of ions through cellular structures called tight junctions. Tight junctions are composed of claudins, a family of proteins that are located between cells and act to create a selectively permeable barrier between the apical and basolateral sides of the epithelium.[3,4] Tight junctions are not only effective in controlling paracellular permeation of major inorganic ions but also in controlling that of ionizable organic chemicals (IOCs). The permeability of IOCs in their ionized form is often orders of magnitude lesser compared to that of their neutral form but can show complex dynamics in bioavailability and interactions with environmental factors.[5,6] Thus, IOCs have received increasing attention among researchers and regulators likewise.

The integrity of tight junctions can be affected by various endogenous and exogenous stressors,[4,7] including pathogen-induced inflammation.[8] Inflammation of gill epithelium is not only expected to disrupt its osmoregulatory and respiratory roles but might also impair its barrier function against IOCs. This might be of significant toxicological relevance because several pesticides, household chemicals, and pharmaceuticals

Received: August 30, 2021
Revised: December 3, 2021
Accepted: December 27, 2021

are IOCs. In addition to individual chemicals, there are also complex mixtures of concern that contain large numbers and quantities of IOCs, for example, oil sands process-affected water (OSPW).

OSPW is created as a waste product of bitumen extraction from oil sands and because of its toxicity, it cannot be released into the environment but is stored in large on-site tailing ponds. There are concerns over the risks of potential spills and seepage into soils, from where OSPWs may eventually reach ground and river water.[9] OSPW is a complex mixture of chemicals, many of which have significant toxic potencies. In a recent review by Li et al.,[10] some of the effects of OSPW on aquatic invertebrates and fish included adverse effects on reproductive endpoints, such as the rate of emergence in insects, fertilization and embryo deformities in fish, and an increase in oxidative stress. A main constituent of OSPW and a major driver of toxicity are naphthenic acids (NAs),[11] which are weak acids, and thus IOCs. At the alkaline pH of OSPW, most NAs are ionized and unlikely to cross gills of fishes through passive diffusion and transcellular transport, while, at the same time, tight junctions prevent their uptake by blocking their paracellular passage. Impairment of tight junctions, which might result from pathogen-induced inflammation, can thus be expected to result in an increase in permeation of NAs across the gill epithelium and result in an increased toxicological risk. While a rich body of research has studied the effects of inflammation on tight junction integrity in mammals, little work has been done addressing this question in fish, especially at the gill. However, additional work in this area is needed to adequately characterize the environmental risks associated with IOC exposure in this taxonomic group.

The work presented here informs the assessment of risk associated with multiple stressors, namely, pathogens and chemicals. While the vast majority of studies have addressed how chemical exposure can impair the immune response of organisms, here, we focus on the reverse, that is, how pathogen-induced inflammation can modulate bioavailability. Specifically, we aimed at establishing a test system that enables the assessment of effects of inflammation on the integrity of tight junctions and associated contaminant uptake and to apply this system to assess its utility for characterizing the risk of uptake of IOCs from an environmental matrix of concern, OSPW. Specifically, bacterial lipopolysaccharide (LPS) was used to induce inflammation in the permanent rainbow trout gill cell line RTgill-W1. LPS has been used successfully in previous studies to experimentally induce inflammation in many model species. LPS is thought to function through the activation of cytokine signaling.[12,13] RTgill-W1 cells have been used previously for whole-effluent toxicity testing, as well as for evaluating toxic potencies of OSPW.[14,15] Acute cytotoxicity tests with this cell line have furthermore been standardized recently.[16] To determine if the induced inflammation and concurrent decrease in tight junction integrity led to an increase in the movement of chemicals from OSPW across the epithelia, cells were then exposed to reconstituted extracts of OSPW. In vitro results were further verified in an in vivo coexposure experiment using fingerling rainbow trout.

2. MATERIALS AND METHODS

2.1. Sources of LPS and OSPW. LPS from the O111:B4 strain of *Escherichia coli*, which has been successfully used in prior studies to induce inflammation in a variety of model species, was obtained from Sigma Aldrich (St. Louis, USA).[13] OSPW was collected from Base Mine Lake, Fort McMurray, Alberta, on the site of Syncrude Canada Ltd. in September 2012. Whole OSPW was used for in vivo exposure. For in vitro experiments, the OSPW sample was extracted and fractionated using mixed-mode sorbents according to a recently published method.[17] A reconstituted sample at 1000-fold concentration was prepared by combining equal volumes of neutral, acidic, and basic fractions of the OSPW extracts. This reconstituted fraction was then used for dosing of cells during in vitro experiments.

2.2. In Vitro Experiments. *2.2.1. Cell Culture.* The rainbow trout gill cell line RTgill-W1 (ATCC CRL-2523) was obtained from the American Type Culture Collection (ATCC, Manassas, USA) and cultured as described previously,[18,4] with slight modifications. Briefly, cells were cultured in 75 cm^2 tissue culture flasks at 20 °C. Leibowitz's L-15 medium was used as the culture medium and supplemented with 10% fetal bovine serum (FBS), 100 units mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin (all obtained from Thermo Fisher Scientific, Waltham, USA). The cells were harvested at ∼95% confluency and either subcultured or directly used in bioassays.

2.2.2. Cytotoxicity of LPS and Extracts of OSPW. Cytotoxicity of LPS and reconstituted OSPW fractions was determined by the use of the neutral red retention assay, with modifications of previously described methods.[19] Briefly, cells were seeded into 96-well microplates, allowed to adhere for 24 h, and then exposed to seven concentrations of samples in L-15/ex at pH 7.4 for 48 h, with twofold serial dilutions starting at maximum concentrations of 100% reconstituted OSPW and 100 mg LPS L^{-1}, respectively. L-15/ex only contains the salts, sodium pyruvate, and galactose of the original L-15 medium to avoid interactions of test chemicals with medium constituents.[20] Subsequently, the dosing medium was removed and exchanged with a neutral red solution (500 mg L^{-1} in unsupplemented L-15). Following 3 h incubation, cells were washed twice with phosphate-buffered saline (PBS, Fisher Scientific), and neutral red was extracted with a mixture of 1% v/v acetic acid and 50% v/v ethanol in PBS). After 30 min extraction on an orbital shaker, the absorbance at 540 nm was recorded photometrically using a POLARstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany). Viability was expressed as the percentage of the negative control and concentration-response curves fitted to the experimental data as described below.

2.2.3. Effect of LPS on the Abundance of Transcripts of Tight Junction Proteins and Inflammation Markers. To determine the molecular effects of LPS on the abundance of transcripts of tight junction proteins and inflammatory markers, cells were seeded into 24-well microplates (1 mL $well^{-1}$, 500,000 cells mL^{-1}) and allowed to adhere for 24 h. Subsequently, cells were exposed in quadruplicate to graded concentrations of LPS (0, 5, 10, 20, 50, or 100 mg L^{-1} in L-15/ex media at pH 7.4), then incubated for 4 and 24 h for subsequent assessment of the expression of genes encoding for inflammation markers and of tight junction proteins, respectively. Exposure times were chosen based on the time point of maximum induction observed in previous experiments.[21] Subsequently, cells were washed with PBS, and the plates were stored at −80 °C until reverse transcription-quantitative real-time PCR (RT-qPCR) analysis.

RNA was extracted using RNeasy Mini kits (QIAGEN, Hilden, Germany), following the manufacturer's instructions.

https://doi.org/10.1021/acs.est.1c05839
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Table 1. Genes and Primer Sequences Used for Quantitative Real-Time PCR of Rainbow Trout Transcript Targets

target	primer sequence	reference
β-actin	F: 5′-TCC TCG GTA TGG AGT CTT GC-3′	Trubitt et al. (2015)[4]
	R: 5′-AGC ACT GTG TTG GCG TAC AG-3′	
TNF-α2	F: 5′-GGT GTG GCG TTC TCT TAA TAG CAG C-3′	Alvarez et al. (2017)[21]
	R: 5′-ATT CCG TCC TGC ATC GTT GC-3′	
interleukin- β1	F: 5′-GTC ACA TTG CCA ACC TCA TCG-3′	Alvarez et al. (2017)[21]
	R: 5′-GTT GAG CAG GTC CTT GTC CTT GAA-3′	
claudin-10e	F: 5′-ATC AAG GTG GCC TGG TAC TG-3′	Trubitt et al. (2015)[4]
	R: 5′-GAC CAG AGC ACA GGG AAG TC-3′	
claudin-28b	F: 5′-CGA CTC CCT GGC CTT AC-3′	Trubitt et al. (2015)[4]
	R: 5′-GCA ATT ATG GCG ATT ATG ATC AGA-3′	
claudin-30	F: 5′-TGA TCA TTG GAG GGT TC-3′	Trubitt et al. (2015)[4]
	R: 5′-AAC ATA GTC CCT GGG TGC TG-3′	

Recommendations for cell lines rich in RNases were followed, that is, β-mercaptoethanol was added to the lysis buffer, and the cell suspension was repeatedly passed through a fine cannula to facilitate extraction. RNA concentrations were determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific) and 75−500 ng of RNA reverse-transcribed into cDNA using the QuantiTect reverse transcription kit (QIAGEN). The abundance of transcripts of several genes related to inflammation and tight junction integrity was determined in these samples by means of qPCR using gene-specific primers (Thermo Fisher Scientific, Table 1). Genes were chosen based on the previous literature, showing their dysregulation during inflammation.

Analysis by qPCR was performed using a QuantStudio 6 Flex RealTime PCR System (Applied Biosystems). Each cDNA sample was analyzed in duplicate. A 10 μL reaction mixture was setup in PCR plates by means of a QIAgility pipetting robot (QIAGEN), combining 9 μL gene-specific reaction mixture containing appropriate amounts of Power SYBR Green PCR Master Mix (Thermo Fisher Scientific) and gene-specific primers (0.4 μM final concentration) with 1 μL of each cDNA sample. The reaction mixture for qPCR was denatured at 95 °C for 10 min, followed by a thermal cycle profile consisting of denaturation at 95 °C for 15 s and a combined annealing and extension step at 60 °C for 1 min for a total of 40 PCR cycles. A subsequent melt curve analysis was performed to ensure single product amplification. The ΔΔCT method was used to calculate fold changes in gene expressions relative to controls. Transcript abundance was normalized against the reference gene β-actin.

2.2.4. Effect of LPS on the Global Transcriptome Response. To determine more globally the suitability of RTgill-W1 cells as a gill inflammation model, we have studied the transcriptome-wide ability of LPS to mirror some of the hallmarks of inflammation established in animal models. RTgill-W1 cells were exposed as described above (Section 2.2.3), the only differences being that only control and 100 mg L^{-1} LPS treatment groups were used, and cells were only exposed for 4 h. Subsequently, cells were washed with PBS, and the plates were stored at −80 °C. Upon thawing, total RNA was extracted from each replicate well, as described above (Section 2.2.3). Only samples with an RNA integrity number (RIN) > 8 were used for dsDNA library preparation. Libraries were prepared using the TruSeq Stranded mRNA Library Prep kit (Illumina Inc., San Diego CA, USA) and were subsequently run for paired-end sequencing (75 bp × 2150 cycles) using the NextSeq 500/550 High Output Kit v2.5 (Illumina Inc) on an Illumina NextSeq 500 System (Illumina Inc). Raw reads are available through the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO; accession number GSE182953). Sequencing was conducted on four control replicates and four 100 mg L^{-1} LPS treatment group replicates (one of which failed QA/QC criteria and was subsequently excluded).

The quality of raw reads was evaluated using FastQC v0.11.81,[22] and reads were trimmed to a minimum Phred score of 20 and a minimum length of 35 bases per reading using Trimmomatic v0.38.1.[23] The abundance of transcripts was quantified by pseudoalignment to the reference rainbow trout transcriptome (GenBank Acc # GCF_013265735.2) using Kallisto v0.46.0.4.[24] The overall pseudoalignment rates of all control and treated samples were in the range 85−87%. Feature counts were filtered to a minimum of 5 counts per million in at least 3 samples and normalized using the trimmed mean of the M (TMM) approach.[25] Differential expression analysis was performed using edgeR v3.26.5 with a significance threshold false discovery rate (Benjamini-Hochberg) ≤ 0.05.[26]

Over-representation analyses were conducted in a g:Profiler using the g:GOSt functional profiling module with g:SCS threshold ≤0.05 and only the annotated transcripts of *O. mykiss* as the statistical domain.[27] The term size was limited to 7−1000 using Gene Ontology databases for cellular components (CC), biological processes (BP), and molecular function (MF). Overrepresentation mapping was built using EnrichmentMap in Cytoscape.[28,29]

2.2.5. Effect of LPS on Tight Junction Integrity and Permeation of Chemicals from OSPW. To assess the effects of LPS on the permeability of simulated gill epithelia, RTgill-W1 cells were seeded to full confluency into 24-well plates with permeable ThinCert cell culture inserts (Greiner Bio-One, Frickenhausen, Germany) using previously established methods.[30] Briefly, cells were grown in these inserts under symmetric conditions (supplemented L-15 culture media in both apical and basolateral chamber) for 2 weeks, with one exchange of media after 7 days of incubation. Thereafter, cells were adapted to L-15/ex media in the apical chamber. Experiments were initiated following a 7 days acclimation period to these conditions, that is, after 21 days of the total culture. Media were decanted from inserts rather than aspirated or pipetted to avoid damaging epithelia. The maturity of epithelia was verified by determining transepithelial electrical resistance (TEER) using a volt-ohm-meter with chopstick-type electrodes (World Precision Instruments Inc., Sarasota, USA).

https://doi.org/10.1021/acs.est.1c05839
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Exposure was initiated by dosing inserts, in duplicate, with a noncytotoxic concentration of the reconstituted OSPW fraction, either alone or in combination with noncytotoxic concentrations of LPS (see Section 2.2.2), and incubated for 24 h. Cells received 0, 5, 10, 20, or 50 mg L^{-1} LPS in L-15/ex and either no or 3.125% OSPW equivalent.

TEER measurements were taken according to the manufacturer's instructions prior to media changes occurring on days 14 and 21, and after 24 h of exposure on day 22. TEER readings were normalized to the surface area of the inserts the cells were growing in (0.336 cm^2) and blank-corrected. After TEER measurements were taken, media from apical and basal chambers of permeable inserts were collected and stored at −80 °C for subsequent chemical analysis.

2.3. In Vivo Confirmation Experiment. Fingerling rainbow trout (30−60 days of age, 0.54 ± 0.16 g) were exposed to 300 mg kg^{-1} dietary LPS and 10% v/v waterborne OSPW. Dietary exposure was chosen over waterborne exposure to LPS because of similar efficacies to induce inflammation, while at the same time posing reduced biohazards to research staff. Fish were obtained from an in-house culture at the Aquatic Toxicology Research Facility (University of Saskatchewan, Canada). This study was conducted in accordance with Canadian Council on Animal Care regulations (University of Saskatchewan Animal Use Protocol 20170103), following previously published protocols.[30] LPS-spiked fish food was prepared by thoroughly mixing 10 mL of a 1.5-mg mL^{-1} solution of LPS in ultrapure water with 50 g of dry fish food (size #0 Biovita starter food, Bio-Oregon, Vancouver, Canada). The mixture was dried at 45 °C for 6 h and subsequently stored at 4 °C.

Briefly, 10 fish were exposed in individual aerated 8 L tanks, with three replicate tanks per treatment under static renewal conditions (50% of exposure solutions exchanged daily). Fish were acclimatized to laboratory conditions at 14 °C with an 8:16 h light:dark cycle for 14 days and the pH gradually adjusted to 7.4 using diluted hydrochloric acid, respectively. The treatments consisted of a negative control (facility water at pH 7.4), an LPS-only control (300 mg kg^{-1} dietary LPS at pH 7.4), a 10% v/v OSPW-only (at pH 7.4), and a coexposure to both 300 mg kg^{-1} dietary LPS and 10% v/v OSPW (at pH 7.4). Fish were fed *ad libitum* every morning with either clean fish food (non-LPS treatments) or LPS-spiked fish food. Temperature, ammonia, conductivity, and dissolved oxygen concentration were determined before the initiation of exposure, and pH and temperature were recorded over the entire duration of the experiment.

At 24 and 48 h, two fish from each tank were euthanized by immersion in buffered MS-222. Fish were rinsed in clean water, blotted with tissue paper, individually weighed and measured, and stored at −20 °C for chemical analysis. At 96 h, all remaining fish were euthanized in buffered MS-222. Two fish from each tank were rinsed with clean water, weighed and measured, their livers and gills were excised and weighed, flash-frozen in liquid nitrogen, and transferred to −80 °C for final storage. Remaining fish were rinsed with clean water, weighed and measured, and stored for chemical analysis.

2.4. Extraction and Ultrahigh-Performance Liquid Chromatography High-Resolution Accurate Mass Spectrometry. All samples were extracted prior to analyses by ultrahigh-performance liquid chromatography and high-resolution accurate mass spectrometry (UPLC-HRAM MS). Media samples from in vitro exposures were extracted twice with equal volumes of a 1:4 (v/v) mixture of ethyl acetate and hexane containing 0.03% (v/v) formic acid. Extracts were left to evaporate overnight and reconstituted in 100 μL of methanol. The resulting MeOH solution was collected into liquid chromatography vials for chemical analysis. Whole fish samples were homogenized using the Omni Bead Ruptor 24, with 2 mm stainless steel balls (Omni International). Homogenates were extracted with acidified dichloromethane (2% v/v formic acid), reduced, and reconstituted in 100 μL of methanol.

UPLC-HRAM MS analysis was carried out, as described by Sun et al.,[31] with slight modifications. Briefly, extracts were analyzed on a Q Exactive HF (Thermo Fisher Scientific, Waltham, USA) equipped with a Dionex UltiMate 3000 UPLC system (Thermo Fisher Scientific). Chemicals were separated on a XTerra MS C18 (3.5 μm; 2.1 mm × 100 mm, Waters, Mississauga, Canada) using a 5 μL injection volume. Ultrapure water (A) and methanol (B) were used as the mobile phase at a flow rate of 0.25 mL min^{-1} and a temperature of 30 °C. Data were acquired in full scan mode with parameters for full MS1 scan (100−1000 m/z) set to be recorded at resolution R = 70,000 (at m/z 200) with a maximum of 3×10^6 ions collected within 100 ms. The general MS settings for negative electrospray ionization (ESI) mode were as follows: spray voltage of 2.8 kV; capillary temperature of 350 °C; sheath gas at 35 L/h; auxiliary gas at 8 L/h; and probe heater temperature of 350 °C.

Prediction of chemical formulas was carried out using the formula prediction feature in MZmine 2.29, where possible formulas were set to contain up to 100 C, 200 H, 6 N, 10 O, and 3 S per molecule, as described previously.[30] Final peak lists were used for suspect and nontarget screening. Specifically, exact masses of $[M\text{-}H]^-$ ions with an m/z tolerance of 5 ppm were used in negative ion mode to search for suspect compounds.

2.5. Statistical Analyses. Data were expressed as mean ± standard deviation. Statistical differences among treatment groups were assessed using GraphPad Prism 7 software (GraphPad, San Diego, USA). Grubb's outlier test was used to eliminate statistically significant outliers. Data were assessed for homoscedasticity and normality using Bartlett's test and Shapiro−Wilk's normality test, respectively. Whenever assumptions were met, significant differences among treatment groups were characterized using one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test. If assumptions of ANOVA were not met, data were assessed using the nonparametric ANOVA analogue, Kruskal−Wallis test, followed by Dunnett's multiple comparisons post hoc test. Differences were considered statistically significant at $p \leq 0.05$. Detailed information on the statistical analyses conducted for each data set is included in the Results section.

3. RESULTS AND DISCUSSION

3.1. Effects of LPS on Tight Junction Integrity. Membrane integrity was adversely affected by LPS, as expected. Measurements of TEER in unexposed mature epithelial cultures, that is, after 14 days growth in inserts, stabilized at 24.4 ± 2.22 Ω·cm^2. After exposure of cells to graded concentrations of LPS for 24 h, cells exhibited significantly reduced TEER than the control (29.3 ± 1.43 Ω·cm^2), with readings of 20.0 ± 3.09, 20.3 ± 5.47, and 13.1 ± 2.38 Ω·cm^2 in the 10, 20, and 50 mg L^{-1} LPS treatments, respectively ($p \leq 0.05$; Figure 1A). The significantly reduced

https://doi.org/10.1021/acs.est.1c05839
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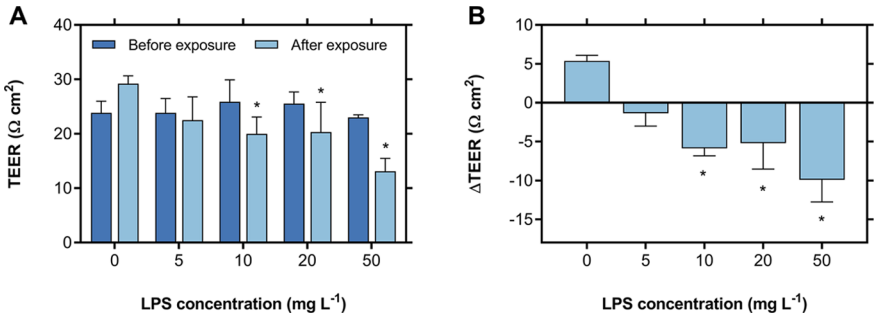

Figure 1. TEER in RTgill-W1 cells before and after 24 h exposure to LPS (A) and change in TEER during the 24 h exposure period (B). Bars represent the mean ± standard deviation of $n = 2$ replicates. (A) Asterisks indicate significant differences in TEER between the measurement before and after exposure (repeated measures two-way ANOVA with Dunnett's multiple comparisons test, $p \leq 0.05$). (B) Asterisks indicate significant differences in the change in TEER between treatments and control (one-way ANOVA with Dunnett's multiple comparisons test, $p \leq 0.05$).

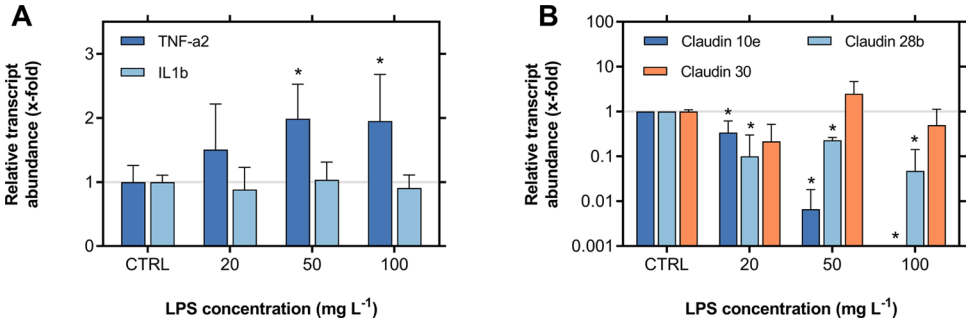

Figure 2. Relative abundances of transcripts of genes coding for inflammation-related proteins in RTgill-W1 cells exposed to various concentrations of LPS for 4 h (A) and abundance of transcripts of genes coding for tight junction proteins in RTgill-W1 cells exposed to various concentrations of LPS for 24 h (B). Bars represent mean values ± standard deviation of $n = 4$ replicates and are expressed relative to transcript abundance in control treatments. Grubb's outlier test was used to eliminate statistically significant outliers. Asterisks indicate significant differences compared to controls (one-way ANOVA and Dunnett's multiple comparisons test $p \leq 0.05$).

TEER at the three greatest concentration ($p \leq 0.05$; Figure 1B) indicates that exposure to LPS resulted in decreased integrity of epithelia cultured on the permeable inserts. Other studies have also shown that TEER can be modulated by exposure to physical or chemical stressors that ultimately results in less integrity of tight junctions.[4,7] Because tight junctions are an integral element in maintaining the barrier function of the gill to unwanted molecules, a disrupted epithelial barrier increases the risk of unwanted molecules to potentially enter the organism *via* paracellular transport. This means that contaminants in water that are not normally taken up into the organism, because of their charge or molecular mass, could potentially pose a threat to organisms with inflamed gills (e.g., as a result of chemical exposure or disease) because of increased paracellular uptake, causing toxicity that would not otherwise occur.

3.2. Effects of LPS on Abundances of Transcripts of Inflammation Markers and Tight Junction Proteins. The observed decreased TEER might have been due to inflammatory responses of cells to LPS, potentially leading to a decrease in the abundance of tight junction proteins. This link has been thoroughly established in mammalian models,[33] but is as of yet not studied in rainbow trout. This interpretation and hypothesis were supported by the concentration-dependent increase in the abundance of transcripts of the pro-inflammatory cytokine tumor necrosis factor $\alpha 2$ (TNF-$\alpha 2$) that was observed in RTgill-W1 cells exposed to LPS for 4 h (Figure 2). This increase was significantly greater in cells exposed to 50 and 100 mg LPS L^{-1} relative to controls ($p \leq 0.05$; Figure 2A). This result confirmed that exposure to LPS resulted in inflammatory responses, which are in good agreement with previously published findings. In a study investigating the effects of exposure of LPS on the rainbow trout gut cell line RTgutGC, abundances of transcripts of both TNF-$\alpha 1$ and TNF-$\alpha 2$ were increased, with TNF-$\alpha 2$ showing greater induction.[32] However, the abundance of transcripts of TNF-$\alpha 1$ was not significantly altered after exposure to any LPS concentration (data not shown) in this study. Similarly, the administration of LPS to dogs induced both TNF-like and interleukin (IL)-like activity, indicating that LPS stimulates both TNF and IL cytokines.[8]

In contrast to the induction of immune response genes, cells sampled after 24 h of exposure showed a significant decrease in the relative abundance of transcripts coding for tight junction proteins, namely, claudin 10e and 28b in cells exposed to 20, 50, or 100 mg LPS L^{-1} ($p \leq 0.05$; Figure 2B). Relative abundances of transcripts of claudin 10e decreased to 0.34-fold of control levels at 20 mg LPS L^{-1} to nondetectable levels at 100 mg LPS L^{-1}. The abundance of transcripts of claudin 28b significantly decreased to 0.1-fold of control levels at 20 mg LPS L^{-1} and to 0.05-fold at 100 mg LPS L^{-1}. Claudin 30 did not show any significant changes with LPS exposure at any of the tested concentrations (Figure 2B). Together, these results indicate that the reduction in tight junction integrity that was seen in the TEER results was likely the result of a decrease in the abundance of transcripts coding for tight junction proteins, leading to a consequential decrease in the abundance of the tight junction proteins themselves and subsequently a functional impairment of tight junctions.

https://doi.org/10.1021/acs.est.1c05839
Environ. Sci. Technol. XXXX, XXX, XXX−XXX

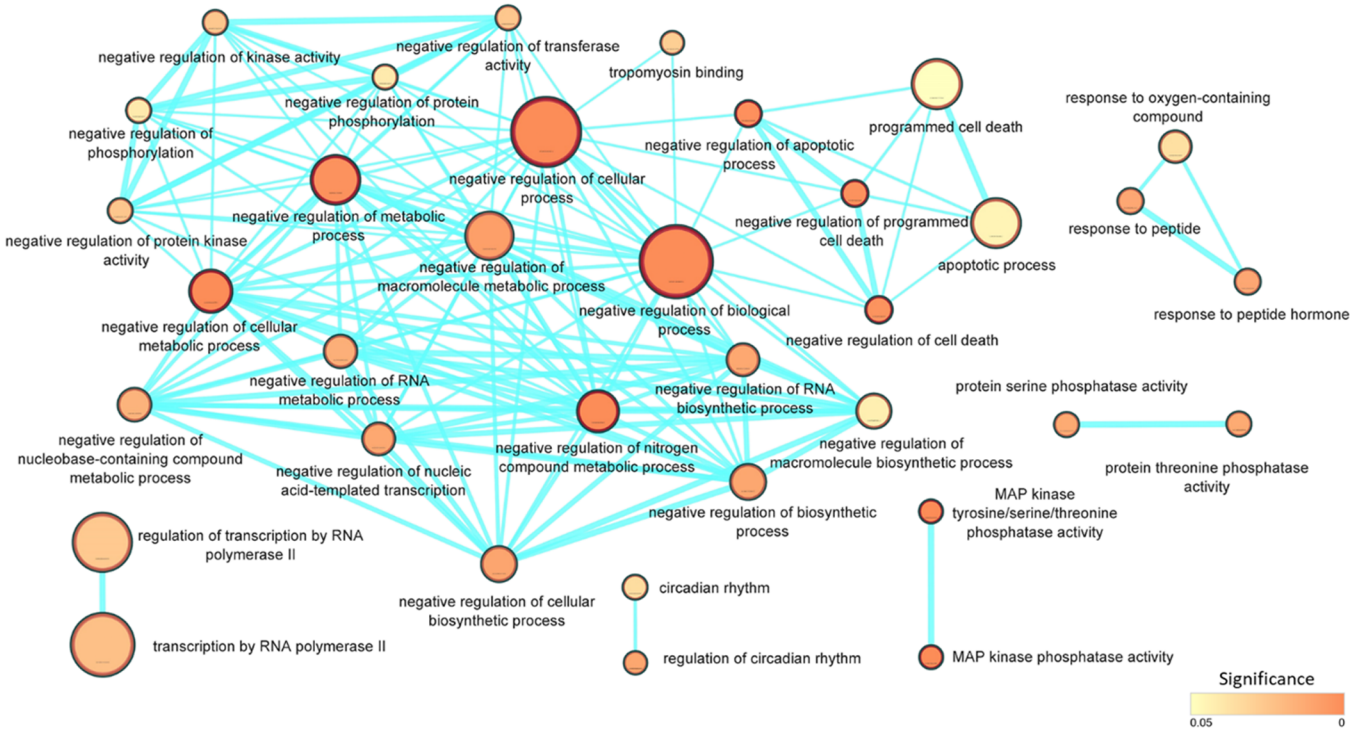

Figure 3. Gene ontology overrepresentation network (FDR ≤ 0.05) from significantly dysregulated genes (FDR ≤ 0.05; |FC| ≥ 1.5). Node size corresponds to the number of input genes within the gene set. Color of the node corresponds to the significance of the gene set. Edge size corresponds to the number of overlapping genes between two connected gene sets.

The relationship between inflammation and integrity of tight junctions is well established. For example, exposure of Sertoli cells to greater concentrations of TNF-α resulted in less mRNA for another tight junction protein, claudin 11.[33] Furthermore, viral infections of the intestinal barrier resulted in significant dysregulation of several claudins and inflammatory genes in carp.[34] A study looking at the diet in young grass carp found that the expression of tight junction proteins was negatively correlated with the expression of proinflammatory cytokines IL and TNF.[35]

3.3. Effects of LPS on Global Transcriptome Response. The results of gene expression studies here are in good agreement with various other model systems, in which exposure to pathogens or pathogen-derived immune-modulants (i.e., LPS) caused inflammatory responses, which in turn resulted in perturbations in the expression of genes coding for tight junction proteins, leading to increased epithelial permeability. The comparison of transcriptomes of control and LPS-treated cells after exposure to 100 mg L^{-1} LPS for 4 h found 473 significantly dysregulated transcripts (FDR ≤ 0.05; |FC| ≥ 1.5), among which 336 were upregulated and 137 were downregulated. Upregulated transcripts included those coding for a member of the TNF ligand superfamily, interferon regulatory proteins, transforming growth factor beta 1a, and IL-11, indicating proinflammatory responses at the molecular level. These results are consistent with the results of the targeted gene expression at 100 mg LPS L^{-1} for 4 h, as discussed above. None of the claudin isoforms were significantly dysregulated after 4 h, which is consistent with 4 h qPCR results (data not shown).

Results of transcriptomic analyses showed significant dysregulation of multiple transcripts coding for the transcription factors early growth response (Egr) protein, insulin-like growth factor (IGF) binding proteins, immediate early response (IER) proteins, nuclear factor interleukin-3-regulated (NFIL3) protein, and nuclear factor kappa-B (NF-κB) protein, all of which are classic proteins associated with the mediation of inflammatory responses in higher mammals. Furthermore, the most highly dysregulated transcripts were those coding for E3 ubiquitin-protein ligase Mdm2, protein LYRIC, transcriptional activator GLI3, rho GTPase-activating protein, voltage-dependent calcium channel subunit alpha-2/delta-1, ferritin H-3, and endophilin-A3(−20 > FC > 20). These proteins are associated with the modulation of epithelial tight junction barriers. For instance, E3 ubiquitin-protein ligase Mdm2 plays a role in the regulation of cell junction stability via post-translational ubiquitination-deubiquitination processes; the p80 isoform of ubiquitin ligase promotes the removal of claudins in tight junctions, which may result in the reduction of tight junction strands.[36,37] Similarly, the downregulation of the iron-storage protein ferritin and the upregulation of the LYRIC protein suggest disruptions in the normal structure and functions of the tight junctions.[38] Ferritin is involved in iron trafficking in the epithelial barrier cells, while LYRIC colocalizes with tight junction proteins in polarized epithelial cells.[39]

Over-representation analyses showed 35 over-represented GO terms (Figure 3). Most terms pertained to the negative regulation of multiple biological processes; however, the terms were too general to infer specific effects. In summary, however, it can be concluded that RTgill-W1 cells appear to be a suitable experimental model for studying inflammation of the fish gill.

3.4. Effects of LPS on Relative Permeability of Chemicals from OSPW through Gill Epithelia. The observed inflammation of epithelia, along with the reduced abundance of transcripts of tight junction proteins and

https://doi.org/10.1021/acs.est.1c05839
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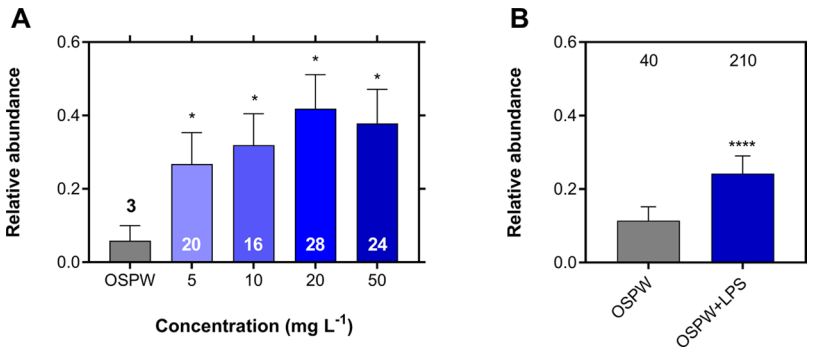

Figure 4. Relative abundances of chemicals from OSPW that permeated through gill epithelia in vitro in the absence and presence of graded concentrations of LPS, following 24 h exposure (A) and in whole-body extracts of fingerling rainbow trout exposed to 10% waterborne OSPW in the absence and presence of dietary LPS (300 mg kg^{-1}) for 48 h (B). (A) Bars represent mean values ± standard deviation of $n = 2$ replicates. Asterisks indicate significant differences compared to the control (one-way ANOVA with Dunnett's multiple comparisons test, $p \leq 0.05$). (B) Bars represent mean values ± standard deviation of $n = 3$ replicates. Asterisks indicate significant differences among treatment groups (nonparametric Mann−Whitney test, $p < 0.0001$). Numbers within/above the bars represent the number of different chemicals that were detected in the basal chamber or fish extracts in each treatment.

decreased epithelial integrity, as shown through a decrease in TEER, might have increased the paracellular permeability of chemicals, potentially allowing chemicals to move across epithelia that would have otherwise not been able to pass this barrier. There was a concentration-dependent and statistically significant increase in the relative abundance of chemicals originating from OSPW that was able to cross the epithelia from apical to basal compartments within 24 h when cells were coexposed to reconstituted OSPW and LPS as opposed to reconstituted OSPW alone ($p \leq 0.05$; Figure 4A). In addition, the number of different chemicals from OSPW permeating across the insert in either treatment increased from three in the OSPW-only treatment to 28 and 24 chemicals in the OSPW +20 mg L^{-1} LPS and OSPW +50 mg L^{-1} LPS treatments, respectively. These findings confirmed in vitro that the disruption of epithelial integrity through LPS exposure also resulted in increased permeation of chemicals from reconstituted OSPW that would have not otherwise been able to permeate the epithelium. These findings suggest that there might be similarly stark differences in the toxico-kinetics of IOCs originating from OSPW in fish in vivo, which could affect toxicity. This conclusion is consistent with the results of several studies that have shown that inflammation of other epithelia such as those present in the gastrointestinal tract can affect their permeability or uptake dynamics for chemicals including nutrients and contaminants.[40] This study, for the first time, demonstrated that inflammation of gill tissues in vitro can similarly result in the differential bioavailability of waterborne contaminants.

3.5. In Vivo Verification of In Vitro Responses. Exposure of fingerling rainbow trout to diluted OSPW in the presence and absence of dietary LPS showed that there was a significant increase in the abundance of chemicals from OSPW in fish exposed to OSPW + LPS compared to those exposed to OSPW alone (Figure 4B). As well, the total number of different chemicals from OSPW that was detected in fish increased when coexposed to LPS. Chemical species detected in fish increased from 40 in the OSPW alone treatment to 210 in the OSPW + LPS treatment (Figure 4B). These results confirmed the in vitro findings and suggested that the effects of inflammation on tight junction integrity and subsequent permeation of IOCs from OSPW might have also occurred in vivo, suggesting that inflammation can impact the extent, and potentially also the rate, of contaminant uptake. The observation that there was a greater variety of chemicals from OSPW taken up into the fish that were exposed to OSPW + LPS, as opposed to those exposed to OSPW alone, indicates that chemicals can permeate across inflamed epithelia that would typically not be bioavailable in healthy fish. As discussed for the results of the in vitro assays mentioned above, these findings are in line with previous studies that investigated the impact of inflammation on the gastrointestinal uptake of chemicals.[40] Previous studies have highlighted that chemical-induced immunomodulation could alter disease susceptibility in fish, but to our knowledge, the effect of inflammation on the uptake of contaminants has not been demonstrated before.

Another factor that might be involved in the observed greater paracellular permeability is the ambient pH of exposure water, which can have a marked impact on the fraction of ionized versus neutral fraction of IOCs, depending on the chemical properties of the contaminant in question. The present study did not investigate this aspect systematically but maintained the pH stable at 7.4 during exposure. It should, however, be the subject of dedicated future research as it has been shown that the uptake of IOCs is a function of the varying permeabilities of neutral and ionized organic chemicals across gill epithelia.[5,6] For example, weak acids would show increased uptake at lower pHs because of an increased fraction of neutral versus ionized chemicals, and this pattern might differ under inflammation conditions. These processes gain particular importance in the context of global climate and land use changes, where anthropogenic changes in pH, salinity, and flow patterns in estuarine ecosystems might further contribute to varying bioavailability to exposed fish.

3.6. Future Directions. Overall, the results of this study showed that inflammation affects the integrity of tight junctions through a decrease in the transcript abundance of tight junction proteins, leading to increased paracellular transport of IOCs (here, weak acids at basic pHs) across gill epithelia. The investigation of these processes in various OSPW fractions (i.e., separated according to the physicochemical properties of chemicals into neutral, acidic, and basic fractions) at different pHs should be the subject of future dedicated research, as the observed behavior can be hypothesized to be specific to weak acids. While we examined the permeation of the reconstituted fraction of OSPW in our in

https://doi.org/10.1021/acs.est.1c05839
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vitro exposures, it would be interesting to see if other fractions would show the same increased permeation upon inflammation of cells. Furthermore, other IOCs and mixtures thereof should be studied, for example, in municipal wastewater effluents (MWWEs). Additionally, MWWEs can contain high concentrations of LPS and other endotoxins, rendering them the ideal medium to study the interactions of inflammation and contaminant uptake.[41] The question of how long after an infection the impairment of tight junction integrity would be sustained is another important future research direction and could be supported with studies to further anchor the effects of LPS exposure in a more solid description of the paracellular transport phenotype.

The findings of this study have important implications for the consideration of combined stressors in environmental risk assessment.[42] Chemical toxicity data to be used in risk assessments are typically generated using practically disease-free laboratory animal cultures. These data might be of limited value to understanding the uptake or effects of a chemical in infected animals. Infections and resulting inflammation can be quite common in wild populations, especially during vulnerable periods of a fish's life cycle, for example, during and after spawning. Several studies have investigated the impacts of environmental contaminants on the susceptibility of organisms to fight infections, and the effects of pathogens on the effects of environmental contaminants; in most cases, these interactions were synergistic.[42] Our results suggest a potential positive feedback mechanism, in addition to other molecular and biochemical processes, that might explain these synergistic effects: contaminant exposure might result in greater susceptibility of aquatic organisms to pathogen infections, which increases the uptake of contaminants into the organisms, in turn leading to a yet greater susceptibility to pathogens. This might create a situation in which the organism is unable to simultaneously fight infection and repair damage from toxicity, and which may occur at far lower concentrations of an environmental contaminant than suggested by toxicity tests performed as part of chemical risk assessments.

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Notes

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ACKNOWLEDGMENTS

The research was supported by a Discovery Grant from the Natural Science and Engineering Research Council of Canada (Project # 326415-07) and a grant from the Western Economic Diversification Canada (Project # 6578, 6807, and 000012711). The authors wish to acknowledge the support of an instrumentation grant from the Canada Foundation for Innovation. The research published in this paper is part of the project titled "Next generation solutions to ensure healthy water resources for future generations" funded by the Global Water Futures program, Canada First Research Excellence Fund. Additional information is available at www.globalwaterfutures.ca. Dr. Brinkmann was supported through the Canada First Research Excellence Funds (CFREF) Global Water Futures (GWF) program led by the University of Saskatchewan. Drs. Giesy and Hecker were supported through the Canada Research Chairs (CRC) program. Dr. Giesy was also supported by a Distinguished Visiting Professorship in the Department of Environmental Sciences, Baylor University in Waco, TX, USA. Dr. Alharbi was supported through the research project number RSP-2021/128, King Saud University, Riyadh, Saudi Arabia. The authors would like to acknowledge M. Ruiz Villarreal (LadyofHats) for releasing an original drawing that formed the basis for the abstract/TOC artwork of this article into the public domain, as well as Tim Vickers for providing the LPS molecular structure under a CreativeCommons license.

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