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Bioavailability and Kidney Responses to Diclofenac in the Fathead Minnow (*Pimephales promelas*)

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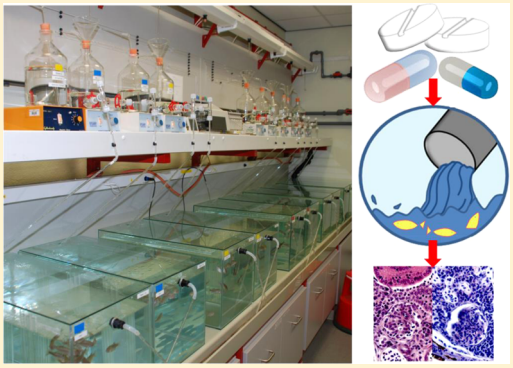
Supporting Information

ABSTRACT: Diclofenac is one of the most widely prescribed nonsteroidal anti-inflammatory drugs worldwide. It is frequently detected in surface waters; however, whether this pharmaceutical poses a risk to aquatic organisms is debated. Here we quantified the uptake of diclofenac by the fathead minnow (*Pimephales promelas*) following aqueous exposure (0.2−25.0 μg L^{-1}) for 21 days, and evaluated the tissue and biomolecular responses in the kidney. Diclofenac accumulated in a concentration- and time-dependent manner in the plasma of exposed fish. The highest plasma concentration observed (for fish exposed to 25 μg L^{-1} diclofenac) was within the therapeutic range for humans. There was a strong positive correlation between exposure concentration and the number of developing nephrons observed in the posterior kidney. Diclofenac was not found to modulate the expression of genes in the kidney associated with its primary mode of action in mammals (prostaglandin-endoperoxide synthases) but modulated genes associated with kidney repair and regeneration. There were no significant adverse effects following 21 days exposure to concentrations typical of surface waters. The combination of diclofenac's uptake potential, effects on kidney nephrons and relatively small safety margin for some surface waters may warrant a longer term chronic health effects analysis for diclofenac in fish.



INTRODUCTION

Diclofenac is one of the most widely prescribed nonsteroidal anti-inflammatory drugs (NSAID) worldwide.[1] It is used to treat a wide variety of pain and inflammatory disorders. Globally, approximately 1000 tons of diclofenac are consumed annually.[2] Diclofenac has a favorable safety profile in humans, but has nevertheless been associated with occasional hepatotoxicity, gastrointestinal and adverse renal effects.[3] Concerns regarding possible adverse environmental effects of diclofenac first arose more than a decade ago when reports implicated the veterinary use of diclofenac in the poisoning and decline of Asian vulture populations.[4] Consumption of diclofenac-treated livestock carcasses by the vultures has subsequently been proven to induce renal damage (arising as a consequence of altered renal blood flow and a buildup of uric acid) and visceral gout. This has resulted in *Gyps* vulture population declines of >95%, including localized extinctions, and at least one *Gyps* species is now critically endangered as a consequence.[5−8] The adverse effects of diclofenac on *Gyps* vultures are still a major international concern.[9,10]

Diclofenac is present in wastewater treatment plant (WwTP) effluents (where it has been measured to reach up to 5.45 μg L^{-1})[11−16] and in the wider aquatic environment, including rivers and estuaries (up to 1.2 μg L^{-1}).[13,17,18] Diclofenac has also been measured in groundwater (9.7−477 ng L^{-1})[19−21] and in some drinking water sources (2.5−35 ng L^{-1}).[22,23] The removal of diclofenac at WwTPs is variable and depends on the treatment system. Poor adsorption of diclofenac to activated sludge and resistance to biodegradation means its removal rate is generally low (measured at between 21 and 40%)[2,24−26] and this, together with evidence for adverse effects on renal function

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in vultures, highlights the potential of this pharmaceutical for impacting aquatic life.

Standardized ecotoxicity tests report acute EC_{50} values for diclofenac at between 15 and 68 mg L^{-1} and between 23 and 72 mg L^{-1} for algae and daphnia, respectively.[27−29] In juvenile medaka (*Oryzias latipes*) the 96 h lethal concentration $(LC)_{10}$ is reported at 8 mg L^{-1}.[30] No observed effect concentrations (NOECs) have been reported at 10 mg L^{-1} in algae, 1 mg L^{-1} in daphnia, 12.5 mg L^{-1} in rotifers, and 4 mg L^{-1} in fish embryos (for growth, reproduction, embryo and larvae mortality, respectively)[31] suggesting diclofenac is unlikely to adversely affect these aquatic organisms at concentrations with environmental relevance.

The end points of standardized toxicity tests, however, do not necessarily capture the mode of action for diclofenac and possible effects on the kidney. Some studies in fish have suggested that chronic diclofenac exposure can cause effects at lower concentrations than those established as safe by acute or subchronic toxicity testing. Histopathological effects have been reported in the kidney, liver and gill of diclofenac exposed fish, including in rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*) at the relatively low concentrations of 1 to 5 μg L^{-1}.[32−35] In contrast, in a study by Memmert et al.[36] in rainbow trout, an effect on the gills, specifically on the presence and incidence of interlamellar chloride cells, thickened lamellar tips, and mononuclear cell foci, was seen only at 1000 μg L^{-1} diclofenac. These different reported effect concentration thresholds prompted a review of selected histopathological findings[37] and based on the authors' interpretive assessments, they suggested an overall effective NOEC for histopathological effects of 320 μg L^{-1}. Diclofenac exposure in fish has also been shown to induce changes in the expression of a variety of genes; related to prostaglandin synthesis (its primary pharmacological mode of action)[32] as well as genotoxicity,[30] inflammation, and the immune response,[38,39] at exposure concentrations in the low μg L^{-1} range.

The question of whether diclofenac, at concentrations detected in European surface waters, has the potential to exert adverse effects in fish is still unclear. This is an especially important question given that diclofenac is now included by the European Commission on a watch-list of pharmaceutical substances for targeted EU-wide monitoring.[40] The possible regulation of diclofenac and associated potential restrictions would have wide ranging and economic implications for a number of stakeholders, including for the human and environmental health, and water treatment service sectors. It is important therefore, that there are sufficient high quality data available on the effects of exposure of aquatic organisms to diclofenac to allow for an accurate environmental risk assessment to be made.

Here we investigated uptake following waterborne exposure to diclofenac and responses in the kidney of fathead minnow (*Pimephales promelas*), a fish species widely used in international regulatory guidelines for chemical testing. We hypothesized that diclofenac exposure would result in altered pathology in the kidney and we investigated this through assessing molecular and histological changes in the kidney of fish exposed to a range of diclofenac concentrations, including concentrations that have been measured in the environment.

MATERIALS AND METHODS

Chemicals. All chemicals and reagents were obtained from Sigma-Aldrich (Dorset, UK) unless otherwise stated.

Fish Maintenance. Fathead minnows were obtained from breeding populations sourced from U.S. EPA, Duluth, Minnesota and maintained at Brixham Environmental Laboratory, UK. Their age at the start of the experiment was 11 months. Fish were fed twice daily, with frozen brine shrimp (Gamma, Tropic Marine Centre, UK) and commercial pellet (Special Diet Services, Essex, UK) at each feeding. All husbandry conditions, including photoperiod, light intensity, water temperature, aeration, aquarium size and source of aquarium water are detailed in Supporting Information (SI) Table S1.

Experimental Protocol. Male fathead minnows were exposed to water containing diclofenac (0, 0.2, 1.0, 5.0, and 25.0 μg L^{-1}; CAS number 15307−79−6) for 21 days via a continual flow-through system in duplicate tanks. These concentrations were selected to cover environmentally and pharmacologically relevant exposures. Water quality parameters (temperature, dissolved oxygen, pH, conductivity, hardness, alkalinity, chlorine, and ammonia levels) were monitored throughout the study, and exposure conditions including number of animals per aquarium are all detailed in SI Table S1. All animal procedures were carried out according to UK Home Office guidelines.

Biological effects assessments were carried out after 48 hours (h) and 21 days (d) of exposure to diclofenac as described in SI 1.1. Fish were euthanised by immersion in benzocaine, followed by destruction of the brain, and then sampled for molecular and histological analysis according to details described in SI 1.1.

Determination of Diclofenac Concentrations in Water and Plasma. Concentrations of diclofenac in the exposure water were quantified on exposure days −3, 0, 2, 7, 14, and 21 in all tanks. Diclofenac concentrations in plasma samples were measured in terminated fish at 48 h and 21 d of exposure. Detailed methods on the chromatographic separation and mass spectrometry analysis of diclofenac in the water and plasma are provided in SI 1.2 and 1.3, respectively. The limit of quantitation was 0.05 μg L^{-1} for water and 0.5 μg L^{-1} for plasma. In this manuscript we use these measures to calculate bioconcentration factors (BCF_{plasma}), defined here as the ratio of the concentration of diclofenac in the fish plasma to the concentration of diclofenac in the aquarium water at the times of the sample collections.

Histological Analysis of the Kidney. Fish were fixed in Bouin's solution and subsequently decalcified (using 10% formic acid and 5% formalin) and cut in to 4 mm transverse sections. Sections were embedded in paraffin wax and processed for histology according to Paull et al.[41] Serial sections were cut at 4 μm and stained with hematoxylin and eosin. A detailed description of the histology methods is provided in SI 1.4.

An average of six slides (minimally three), were assessed per fish, incorporating sections taken at multiple positions between the anterior kidney and midway along the longitudinal axis of the posterior kidney. Numbers of glomeruli, developing nephrons (DNs) and basophilic cell clusters (BCs) were counted in the posterior kidney sections, alternating between the right and left side of the organ. Numbers of DNs and BCs have been validated as useful biomarkers of nephrotoxicity.[42] All measurements were standardized relative to the area of the transverse kidney cross section analyzed (the number of glomeruli, DN and BC were expressed per square millimeter, and criteria for the identification of DN and BC were followed according to Cormier et al.[42]). Analysis of the cell types and

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numbers were conducted on slides that were blinded to avoid possible bias in the assessments. Measurements were taken using image analysis software (analySIS, version 3.2; Soft Imaging System, GmHB, Germany).

Statistical Analysis. Data are presented as mean ± SEM. All statistical analyses were carried out using SigmaStat 3.1 (Systat Software Inc.) unless otherwise stated. Measured concentrations of diclofenac below the limit of quantitation in control water were assigned a value of 0 (as no diclofenac was added to the controls). For plasma samples where measured concentrations of diclofenac were below the limit of quantitation, these were assigned a value half the limit of quantitation for the data analyses, as they could in fact contain a very low level of diclofenac. Data were assessed for normality and homogeneity of variances using the Kolmogorov−Smirnov and Levene's median tests, respectively. When parametric assumptions were met, data were analyzed using analysis of variance (ANOVA) with post hoc assessment of differences made using the Holm-Sidak multiple comparison method. Kruskal−Wallis one-way ANOVA was used to compare medians where normality assumptions were not met and post hoc Dunn's multiple comparison procedure applied as appropriate. A two factor nested ANOVA, with tank as a random factor, was applied to assess for tank effects in data analyses. We tested whether the number of basophilic cell clusters (BCs) and developing nephrons (DNs), and the number of glomeruli, measured in posterior kidney tissue increased with the exposure concentration of diclofenac using Spearman's rank correlation (using SPSS Statistics, version 22, IBM). We characterized the statistically significant relationship between the number of BCs and DNs, and exposure concentration by fitting a three parameter asymptotic exponential function by least-squares regression. Data were considered statistically significant at $p < 0.05$.

RNA Extraction, Library Preparation, and Sequencing. RNA was isolated from the anterior kidney of adult male fathead minnows using Tri Reagent, according to the manufacturer's instructions and further purified using RNeasy MinElute Cleanup with on column DNase treatment (Qiagen Ltd. West Sussex, UK). RNA quality was assessed on the Agilent BioAnalyser with RNA 6000 Nano Kit (Agilent Technologies Ltd. Berkshire, UK) according to the manufacturer's instructions. All RNA used for library construction had A_{260}/A_{280} and A_{260}/A_{230} ratios >1.8 and RIN scores in the range 7.0−9.2. cDNA libraries were prepared using Illumina TruSeq RNA Sample Preparation Kit and subsequent cluster generation was conducted using TruSeq Paired-End Cluster Generation kit (Illumina, San Diego CA).

In total 24 samples were prepared for sequencing: six replicate samples from each of four treatment groups (0 $\mu g\ L^{-1}$ diclofenac (control) and 25.0 $\mu g\ L^{-1}$ diclofenac, at 48 h and 21 days of exposure). Eight samples were sequenced per lane using an Illumina GAII platform (76bp paired-end).

Transcriptomic Analysis. Raw reads were quality-trimmed, clipped and filtered using the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Overlapping reads were separated from orphan reads and combined into single extended reads using FLASH.[43] A de novo fathead minnow transcriptome was created from all orphan reads, overlapping (extended) reads and nonoverlapping reads (forward reads only) using the Trinity platform (trinityrnaseq_r2013_08_14).[44]

Annotation of the de novo assembled transcripts was carried out using Trinotate (http://trinotate.sourceforge.net/) and Blast+.[45] Abundance was estimated for all annotated transcripts using RSEM[46] and differential gene expression was analyzed using EdgeR[47] and R/Bioconductor (version 2.2.0)[48] using a selection of scripts provided by Trinity. For each time point (48 h and 21 d), differences in gene expression were determined between the diclofenac exposed fish and their time-matched controls. Genes were considered differentially expressed when the false discovery rate (FDR) < 0.1 (Benjamini-Hochberg multiple testing correction). Word clouds were generated using the fold-change values for up- and down-regulated genes using www.wordle.net. Hierarchical clustering (Ward's method and Euclidean distance) was performed on TMM-normalized FPKM expression values of the differentially expressed genes (FDR < 0.1) and visualized by creating heatmaps. Functional annotation analysis was conducted on lists of differentially expressed genes to identify enriched Gene Ontologies (GO terms) and/or KEGG pathways using DAVID (v6.7).[49] GO terms and pathways were considered to be enriched when P values adjusted for multiple-testing <0.1 (Benjamini-Hochberg). Functional enrichments between control (48 h vs 21 d) and diclofenac (48 h vs 21 d) were visualized using the Enrichment Map plugin[50] for Cytoscape 2.8.3[51] and the Functional Annotation Charts produced by DAVID (cutoff values used for *P* value, *Q* value, and overlap coefficient were 0.001, 0.05, and 0.6, respectively). The same methods were used to perform comparisons between control fish at 48 h and 21 d, and between exposed fish at 48 h and 21 d, to determine the effect of time on the transcript profile of anterior kidneys within our experimental system. Full details on the transcriptomic analyses are provided in SI 1.5. All raw sequence data are accessible at the NCBI Sequence Read Archive through accession number SRP076715.

RESULTS

Water Chemistry. The mean (arithmetic) measured concentrations of diclofenac were 0.19 ± 0.005 $\mu g\ L^{-1}$ (93.8% of the corresponding nominal concentration), 0.95 ± 0.02 (94.9%), 5.15 ± 0.13 (103.0%), and 23.92 ± 0.31 (95.7%), for the 0.2, 1.0, 5.0, and 25.0 $\mu g\ L^{-1}$ diclofenac treatment groups, respectively (SI Table S2). Diclofenac was not detected in the control treatment group (limit of detection = 0.05 $\mu g\ L^{-1}$).

Concentrations of Plasma Diclofenac. There were no differences between replicate tanks within treatment groups (two factor nested ANOVA, $F_{1,30} = 0.094$, $p = 0.993$). Following 48 h of exposure, plasma concentrations of diclofenac were 6.4 ± 1.2, 35.3 ± 7.1, and 205.0 ± 30.5 $\mu g\ L^{-1}$ for the 1, 5, and 25 $\mu g\ L^{-1}$ treatment groups, respectively. Diclofenac concentrations in the plasma of the control and 0.2 $\mu g\ L^{-1}$ treatment groups were below the limit of detection (0.5 $\mu g\ L^{-1}$) in all the samples measured (Figure 1). Fish exposed to diclofenac at 25 $\mu g\ L^{-1}$ for 48 h had significantly higher plasma concentrations compared with other treatment groups, including control (two-way ANOVA with Holm-Sidak multiple comparison procedure, $F_{4,99} = 54.3$, $p < 0.001$).

The calculated ratios between water and plasma concentrations (BCF_{plasma}) after 48 h exposure to diclofenac were 6.7 ± 1.3, 7.0 ± 1.4, and 8.49 ± 1.3 for the 1, 5, and 25 $\mu g\ L^{-1}$ treatment groups, respectively. There were no significant differences in the calculated ratios between these treatment

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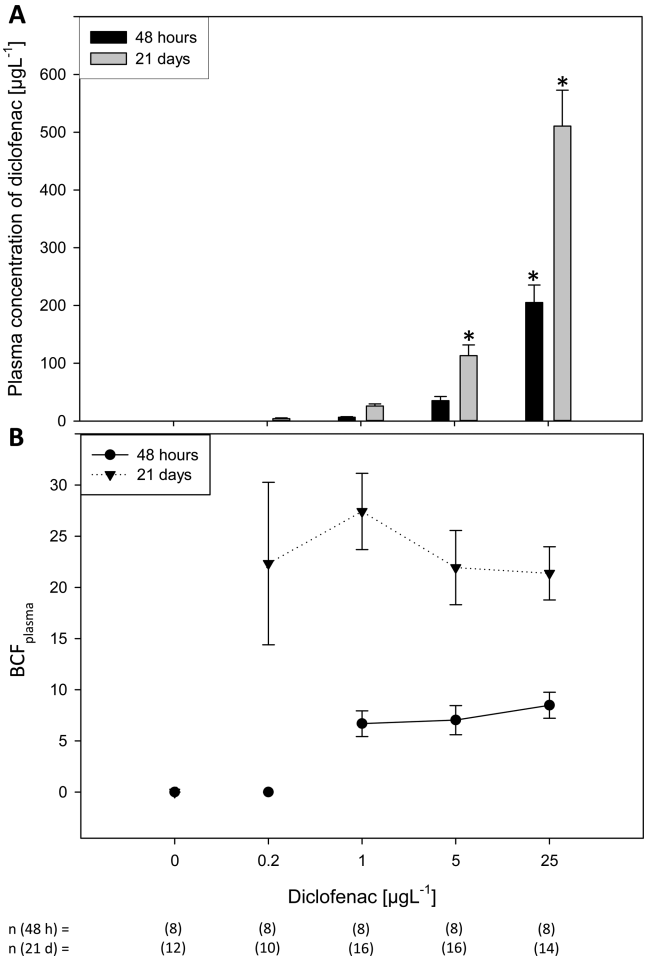

Figure 1. (A) Plasma concentrations of diclofenac in fish at 48 hours (h) and 21 days (d) exposure. * denotes significantly different to control treatment group (Two Way ANOVA with Holm-Sidak multiple comparison procedure, $F_{4,99}$ = 54.3, P < 0.001). (B) Bioconcentration factors (BCF) of diclofenac for blood plasma after 48 h and 21 d exposure. There were no significant differences between treatment groups measuring above 0 at 48 h (Kruskal–Wallis one-way ANOVA, H_2 = 1.582, p = 0.453) or 21 d exposure (Kruskal–Wallis one-way ANOVA, H_3 = 1.98, p = 0.576). BCF values were 0 for both the 0 (control) and 0.2 μg L^{-1} diclofenac treatment groups at 48 h, and 0 μg L^{-1} diclofenac (control) treatment group at 21 d.

groups (Kruskal–Wallis one-way ANOVA, H_2 = 1.582, p = 0.453; Figure 1).

At 21 d exposure, there were no differences between replicate tanks within treatment groups (two factor nested ANOVA, $F_{1,59}$ = 1.475, p = 0.212) and plasma concentrations of diclofenac had increased significantly to 26.1 ± 3.5, 113.1 ± 18.7, and 510.7 ± 62.1 μg L^{-1} for the 1, 5, and 25 μg L^{-1} treatment groups respectively (two-way ANOVA with Holm-Sidak multiple comparison procedure, $F_{1,99}$ = 19.2, p < 0.001; Figure 1). In the lowest diclofenac treatment group (0.2 μg L^{-1}), concentrations were above the limit of detection in 50% of samples measured (4.1 ± 1.4 μg L^{-1}). All samples were measured below the limit of detection in the control treatment group. There was a statistically significant interaction between exposure concentration and the day of analysis ($F_{4,99}$ = 9.7, p < 0.001). At the highest exposure concentration (25 μg L^{-1}), the average plasma concentration of diclofenac (510.7 μg L^{-1}) had reached human plasma therapeutic levels (C_{max} >420 μg L^{-1}).[52,53]

The calculated ratios between water and plasma concentrations of diclofenac (BCF_{plasma}) after 21 d exposure were 22.3 ± 7.9, 27.4 ± 3.7, 21.9 ± 3.6, and 21.4 ± 2.6 for the 0.2, 1, 5, and 25 μg L^{-1} treatment groups, respectively. There was no significant difference in these ratios between treatment groups (Kruskal–Wallis one-way ANOVA, H_3 = 1.98, p = 0.576; Figure 1).

Fish Morphometrics. There were no significant differences in either condition factor (K) or hepatosomatic index (HSI) between any of the treatment groups after 48 h or 21 d exposure (K: Kruskal–Wallis one-way ANOVA, H_4 = 3.939, p = 0.414, and H_4 = 4.255, p = 0.373, for 48 h and 21 days, respectively; HSI: one-way ANOVA, $F_{4,78}$ = 0.466, P = 0.760 for 48 h and Kruskal–Wallis one-way ANOVA, H_4 = 1.749, p = 0.782 for 21 d). All associated data (including length and weight) are presented in SI Table S3.

Histology. Typical histological sections and the structural features of the posterior kidney in control and diclofenac exposed fish are shown in Figure 2. There was no evidence of differences in the morphology of glomeruli, BCs or DNs in any diclofenac treatment groups compared with controls. However, the number of BCs and DNs observed in the posterior kidneys increased with diclofenac concentrations (Spearman rank correlation, r_s = 0.76, n = 10 tanks, p = 0.01; Figure 3). The relationship was described by number of BCs and DNs = 2.63 ($1.97e^{-1.6\ \text{diclofenac}\ [\mu gL\text{-}1]}$), R^2 = 0.86. We observed increased cellularity and inflammation of the glomeruli (glomerulitis) and inflammation of the tubules (nephritis) in the kidneys of fish exposed to 25 μg L^{-1} diclofenac (SI Figure S1). Inflammatory cell infiltrates were predominantly chronic-active in composition. Associated cells were comprised mainly of mononuclear lymphocytes and histiocytes, together with a lesser number of neutrophils and eosinophils. Glomeruli and associated Bowman's capsules were observed along the posterior kidney but not in the anterior kidney. There was no significant correlation between the number of glomeruli and diclofenac exposure concentration (Spearman rank correlation, r_s = 0.172, n = 10 tanks, p = 0.634). No abnormal pathologies were observed in the anterior kidney. This tissue predominantly consisted of hematopoietic cells, with some steroidogenic and chromaffin cells located around the blood vessels. Furthermore, no notable changes were observed in the sinusoidal or vascular profile structure of either control or diclofenac exposed fish.

Transcriptome Assembly and Annotation. In total, 233 824 042 raw Illumina sequence reads were obtained from the 24 sample libraries. After preprocessing, 108 659 244 sequence reads were retained and assembled de novo to create a reference anterior kidney transcriptome. SI Table S4 shows the number of sequence reads for each sample library before and after the various processing steps. A total of 144 701 contigs were constructed with sizes ranging from 200 to 14 002 bp (mean contig size of 934 bp) and the transcriptome had an N50 (the contig length where half of the assembly is represented by contigs of this size or longer) of 1600. A total of 66 329 contigs were annotated using a variety of public sequence databases and peptide/protein predictions (e.g., signal peptides and protein motifs; see SI 1.5 for further details and SI Table S5 for the full annotation table, including transcript sequences).

Effect of Diclofenac Exposure on Gene Expression in the Anterior Kidney. Analysis of the MDS plot (SI Figure

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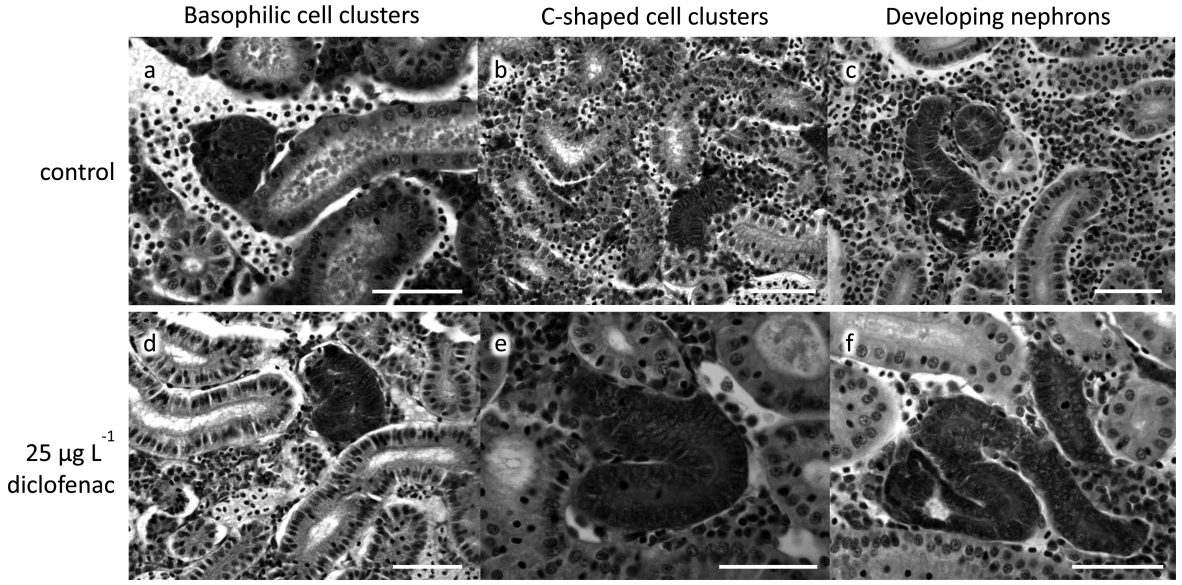

Figure 2. Histopathology of male fathead minnow kidney from control (a–c) and 25 μg L^{-1} diclofenac exposed fish (d–f) at 21 days, showing basophilic cell clusters (clusters of small, basophilic, crescent shaped cells) (a and d), further proliferation of basophilic cell clusters to form c-shaped clusters (b and e) and formation of s-shaped developing nephrons (organized groups of basophilic epithelial cells usually forming a narrow lumen) (c and f)). Sections are taken from various locations longitudinally along the posterior kidney. Hematoxylin & Eosin stain. Scale bar = 50 μm.

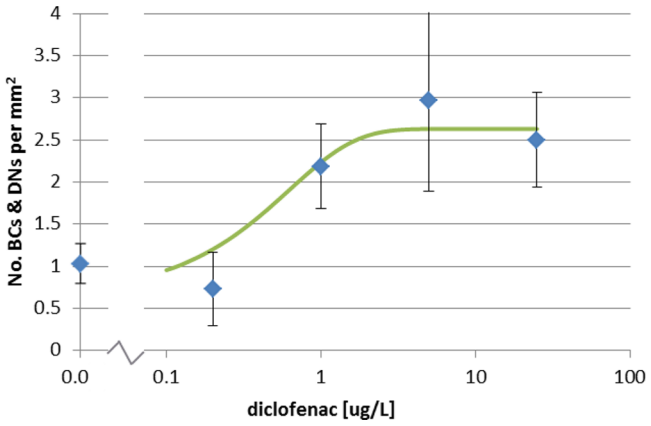

Figure 3. Relationship between diclofenac and the number of basophilic cell clusters (BCs) and developing nephrons (DNs) per mm^2 in the posterior kidney. The number of BCs and DNs observed in the posterior kidneys increased with increased diclofenac concentrations (Spearman rank correlation, r_s = 0.76, n = 10 tanks, p = 0.01). The relationship is described by y = 2.63 ($1.97e^{-1.6x}$) (R^2 = 0.86), where y = number of BCs and DNs and x = diclofenac [μg L^{-1}]. Values shown are mean ± standard error.

S2) showed two control samples (one from 48 h and 21 d treatment groups respectively) were outliers and these were subsequently removed from the data set prior to differential gene expression analysis.

Exposure of fathead minnow to diclofenac for 48 h resulted in the differential regulation of 74 genes compared to controls, 23 of which were down-regulated and 51 were up-regulated (FDR < 0.1; Figure 4, SI Table S6). Some of these genes have previously been shown to be affected by diclofenac and they include; interleukins,[54,55] *nitric oxide synthase 2b* (*nos2b*),[56] *suppressor of cytokine signaling 3b* (*socs3b*),[57] c–c motif chemokines,[57] *clusterin*,[58] desmin a (*desma*), solute carrier family genes,[59] *jun B*,[60] *nuclear factor, erythroid 2*, and *ectonucleoside triphosphate diphosphohydrolases*.[61]

Functional annotation analysis did not identify enriched gene ontologies and/or KEGG pathways in the lists of genes differentially expressed at 48 h diclofenac exposure. There was no effect of diclofenac treatment on the expression of prostaglandin-endoperoxide synthase (ptgs) genes in the anterior kidney. Previously known as cyclooxygenase, these genes code for enzymes through which the therapeutic effects of NSAIDs are exerted in mammals. There was however a group of 13 differentially expressed genes associated with processes and pathways linked with inflammation (SI Table S7). A further group of differentially expressed genes were associated with extracellular matrix and muscle related proteins, including some genes previously associated with kidney fibrosis. Most of these 18 differentially expressed genes were up-regulated (SI Table S8). Other genes differentially expressed at this time point included some associated with the immune system (e.g., *h2-k1 and* Ig mu chain C region membrane bound), as well as calcium binding and ion transport genes (e.g., *cacna2d4b*, *efcab1*, *scn1b*, *lin7a*, *atp1b1*, and *cadpsb*).

After 21 d exposure, only 14 genes were found to be differentially expressed compared with controls; six being down-regulated and eight up-regulated (Figure 4, SI Table S6). Of these, cytochrome p450 family 2[57,62,63] and *bax* (an apoptosis regulator)[64] have been identified previously to be affected by diclofenac. Functional annotation analysis did not identify enriched gene ontologies and/or KEGG pathways in these differentially expressed genes. Furthermore, there was no overlap between differentially expressed genes at 48 h and 21 d.

Differential gene expression was also observed for each treatment group over time: in control samples, comparisons between the expression profiles of samples analyzed at 48 h compared to 21 d identified 93 genes differentially expressed (71 up- and 22 down-regulated), and in the samples from diclofenac exposed fish, 258 genes were differentially expressed over time (145 up- and 113 down-regulated) (SI Table S6). Functional enrichment was found within the groups of genes that were altered over time within each treatment (SI Table S9). Some molecular processes were significantly over-

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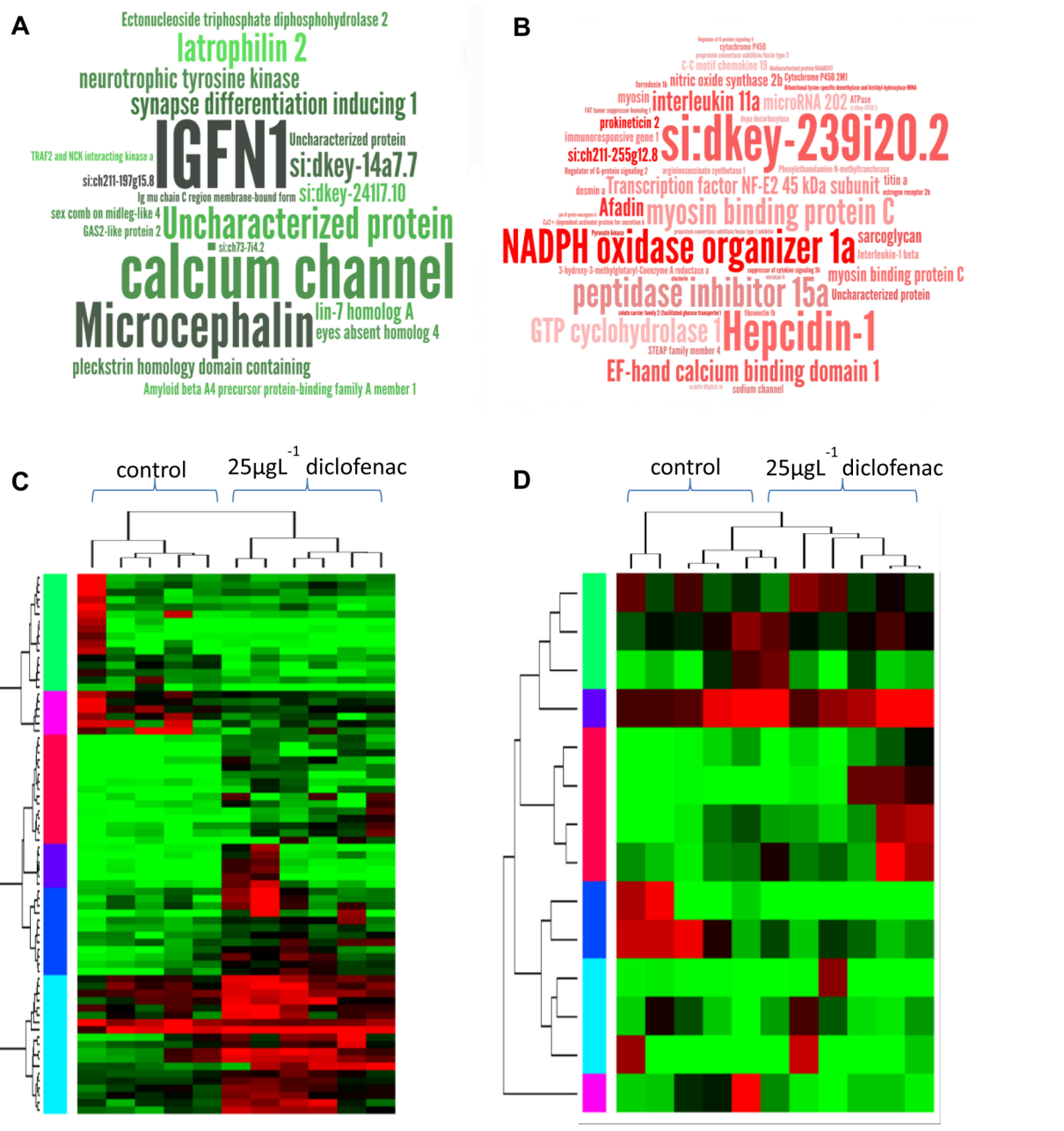

Figure 4. (A and B) Differentially expressed genes in the anterior kidneys of fathead minnow exposed to diclofenac for 48 h, represented as a word cloud. Up- and down-regulated genes are represented in green (A) and red (B) respectively, and letter height and shade are proportional to the fold-change values. (C and D) Heatmap shows differential gene expression in control and diclofenac libraries after 48 h (C) and 21 days (D) of exposure. The cluster diagrams were created using all differentially expressed genes (adjusted *P* values <0.01). The level of gene expression is visualized by using a color gradient from dark green (low expression) to dark red (high expression).

represented (adjusted *P* values < 0.1) in both control and diclofenac-exposed fish, including oxygen binding and oxygen transporter activity. In addition, enriched processes specific for each of the treatment groups were also observed, including iron ion binding (controls) and structural constituent of ribosome and structural molecule activity (diclofenac) (SI Table S9).

DISCUSSION

In this study we demonstrated concentration and time dependent uptake of diclofenac into the plasma of fish following aqueous exposures. Differentially expressed genes in the anterior kidney did not include the known primary targets (e.g., ptgs) through which the therapeutic effects of NSAIDs are exerted (in mammals) but did include other genes related to the mode of action of diclofenac at plasma concentrations equivalent to human therapeutic levels. There was a strong positive correlation between increasing concentrations of diclofenac and the number of BCs and DNs observed in the posterior kidneys, which may be indicative of recovery from recent kidney damage. The significance of these effects on kidney function, however, is not known.

Current environmental concentrations of diclofenac (spanning between 0.003 and 5 $\mu g\ L^{-1}$) are low compared with human therapeutic plasma concentrations, which range between 500 and 3000 $\mu g\ L^{-1}$.[52,53] At the highest exposure concentration in our study (25 $\mu g\ L^{-1}$), the plasma

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concentration of diclofenac reached human therapeutic plasma concentrations and at more environmentally relevant exposure concentrations (1 and 5 $\mu g\ L^{-1}$; the 95th percentile for diclofenac in UK WwTP effluents is 0.85 $\mu g\ L^{-1}$[16] and measured up to 1.2 $\mu g\ L^{-1}$ for rivers and estuaries globally[13,17,18]), plasma diclofenac was 6 and 27% of the human therapeutic dose, respectively. Fish and other aquatic organisms may bioconcentrate diclofenac leading to internal concentrations higher than those in the water. Furthermore, in fish, as occurs in humans, diclofenac undergoes enterohepatic cycling, which potentially prolongs its availability within the organism.[65] The average calculated ratios between water and plasma concentrations (BCF_{plasma}) across treatment groups after 48 h and 21 d exposure were 7.4 (±0.56) and 23.3 (±1.4), respectively. The difference between these values is likely because steady state diclofenac plasma concentrations were not reached at 48 h exposure. Brown et al.[11] report steady state to occur in rainbow trout plasma after 2 d exposure to diclofenac, however, Memmert et al.[36] suggest that between 10 and 14 d exposure are required (measured in whole bodies of rainbow trout). The calculated ratios between water and plasma concentrations of diclofenac (BCF_{plasma}) in the present study in fathead minnow are in most cases slightly higher than those previously reported for diclofenac in rainbow trout (from 2.5 to 29),[11,12,38,66] which may indicate species or environmental (e.g., temperature) differences in the bioconcentration potential of diclofenac and/or differences in the metabolism and excretion dynamics of diclofenac between species. Diclofenac has also been shown to accumulate in other tissues, including muscle, kidney, liver, and gill, in the bile, and also in whole bodies.[32,33,36,67]

Our global gene expression analysis in the fathead minnow did not identify any diclofenac induced differential expression of ptgs (cyclooxygenase) genes in the anterior kidney. The most widely recognized pharmacological activity of diclofenac in humans is inhibition of the ptgs enzymes.[1] ptgs1 is constitutively expressed in most tissues and regulates the synthesis of prostanoids with functions that include cytoprotection of the gastric mucosa, regulation of renal blood flow, and platelet aggregation.[68] ptgs2 is mostly inducible, although it is also constitutively present in the kidney,[69] and expression is greatly increased at inflammatory sites in response to cytokines, hormones, growth factors and hypoxia. These drug targets are conserved across vertebrates[70] and both ptgs genes have been characterized in a number of fish species.[71−73] Diclofenac (at concentrations as low as 1 $\mu g\ L^{-1}$) has previously been shown to reduce the transcription of *ptgs1* and *ptgs2* in the liver, gills and kidney of rainbow trout,[32] and reduce the synthesis of prostaglandin E2 in brown trout anterior kidney macrophages in vitro.[35] However, microarray analysis of hepatic gene expression in rainbow trout found exposure to diclofenac at concentrations between 11.5 and 81.5 $\mu g\ L^{-1}$ did not alter ptgs transcription, although a down-regulation was seen at the lower concentration of 1.6 $\mu g\ L^{-1}$.[38] Similarly, in a recent study in three-spined sticklebacks, exposure to diclofenac at 1 $\mu g\ L^{-1}$ had no effect on the expression of ptgs genes in the gill.[74] The regulation of ptgs is complex, and little is known with respect to differences in fish species. Furthermore, the prostanoids produced via the cyclooxygenase pathway have a diverse array of effects on a variety of cellular processes, and they do not always act in a similar and coordinated manner.[75,76] The expression of ptgs genes may therefore depend on the net effects of prostaglandins, their balance or timing of expression within a tissue or during a physiological response, and this may account for the variability in expression seen across different studies.

Over 20% of the genes differentially expressed in response to diclofenac exposure (at a concentration equivalent to a human therapeutic level) were associated with inflammation. These genes included: two members of the interleukin cytokine family (*il11a* and *il1b*, up regulated by 66.5- and 14-fold, respectively), the latter an important mediator of the inflammatory response and inducer of *ptgs2* expression; hepcidin (*hepc1*, up-regulated by 147-fold), important in the maintenance of iron homeostasis and induced by cytokines during inflammation; *nos2b* (up-regulated by 34-fold), induced by pro-inflammatory or mechanical stresses including tissue injury, and catalyzes the production of nitric oxide, which is important for cytokine signaling; GTP cyclohydrolase 1 (*gch1*, up-regulated by 76-fold), which transcribes a rate-limiting enzyme in tetrahydrobiopterin biosynthesis, an essential cofactor required by nitric oxide synthases, and also a modulator of inflammatory pain; and *socs3b* (up-regulated by 4.7-fold), an inflammatory regulator that mediates a negative feedback system regulating cytokine signal transduction via the JAK/STAT pathway. Although these responding genes appear to be associated with the established and putative mechanisms of action of diclofenac, a commonly prescribed anti-inflammatory drug,[1] the lack of significant effects seen on the ptgs genes make it difficult to draw any specific pathway associations.

In the present study, we observed a concentration-dependent increase in the number of developing nephrons in the kidney, and at the highest exposure concentration we found some evidence of increased cellularity and inflammation of both the glomeruli and tubules. As for mammalian kidneys, fish kidneys have the ability to repair after injury, including following exposure to a variety of toxicants.[42,77,78] Additionally, fish are also able to regenerate kidney nephrons de novo after renal damage.[79−81] Intensely basophilic, compact developing tubules are occasionally seen in normal adult fish kidneys, but are increased significantly after nephrotoxicant induced injury, allowing fish to rapidly regenerate lost nephrons.[82] The presence of increased numbers of new nephrons in adult fish therefore implies recently induced toxicant damage.[42] Studies in zebrafish suggest this process is reminiscent of nephrogenesis in mammals and that similar mechanisms govern nephron formation in both species.[83] However, the extent to which the fish kidney compares with that of higher vertebrates, including mammals, in terms of toxicant exposure is less well understood. Evidence from this investigation of nephron neogenesis in fish following exposure to diclofenac suggests exposure (to 25 $\mu g\ L^{-1}$ diclofenac) may cause damage to fish kidneys. We did not see evidence for any obvious kidney damage, such as tubular necrosis, however such an analysis would require a more comprehensive, and a time-course analysis, to effectively capture this information. We also found that a relatively high proportion (>20%) of differentially expressed genes following exposure to 25 $\mu g\ L^{-1}$ diclofenac were associated with extracellular matrix proteins. Extra cellular matrix has been recognized as having a key influence on organ formation and repair,[84] and many kidney diseases, including inflammatory processes, are associated with changes in the expression and distribution of components of extracellular matrix.[85,86] Most of these genes were up-regulated and include: *FAT atypical cadherin 1* (*fat1*, up-regulated by 4.8-fold), involved in controlling cell proliferation during development

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and also a regulator of actin cytoskeletal organization, playing an important role in actin dynamics and filament rearrangement characteristic of the kidney's response to podocyte injury;[87] *clusterin* (*clu*, up-regulated by 4.6-fold), an extracellular chaperone that prevents aggregation of non-native proteins and a regulator of cell proliferation, it has also been shown to be associated with renal repair and cell proliferation in kidney tubules[88] and its up-regulation during renal injury may act as a protective response against the development of renal fibrosis;[89] two regulators of G protein signaling, including *rgs2* (up-regulated by 8.6-fold), previously shown to negatively regulate progression of fibrosis in kidneys;[90] *fibronectin 1b* (*fn1b*, up-regulated by 6.5-fold), a large adhesive glycoprotein involved in the regulation of cell adhesion, proliferation and differentiation, and associated with the development of fibrosis;[91] and *socs3b*, not only a major regulator of inflammation but may also play an important role in the renal fibrosis repair process, controlling cellular proliferation and regeneration through HNF-1β regulation.[92] Renal fibrosis is the consequence of an excessive accumulation of extracellular matrix that occurs in many types of chronic kidney disease.[93] However, we found no evidence of fibrosis at the tissue level in the histopathology of the anterior or posterior kidneys. Furthermore, many of these transcriptomic responses were transient and not maintained over the whole exposure period. The possible health impacts of the molecular and cellular responses we see in the kidney tissues are unknown and will also depend on the wider biological costs of kidney tissue injury and regeneration where it occurs.

We also observed significant changes in the transcriptome over time, both in the control and to a greater extent in the diclofenac treatment group. To our knowledge, temporal changes in transcriptomic data across all treatment groups are uncommonly reported but important for understanding the biology of the organism under investigation. The present study was designed to avoid confounding variability arising from factors including sex and stage of development, and to maintain animals in a highly controlled environment. However, fish were moved from large mixed sex stock tanks into smaller tanks with males only. There was also a reduction in fish density over the exposure period due to terminal sampling of a proportion of fish at 48 h. Such environmental perturbations have the potential to change the social behaviors of fish, particularly promoting territoriality and the formation of dominance hierarchies within groups. These behaviors have been associated with stress[94] and this may have altered levels of activity within tanks, which in turn may explain the temporal changes in genes associated with oxygen transport. There were many more genes differentially expressed over time in the diclofenac exposed fish compared to control fish, possibly reflecting differences in the way these fish adjusted to their tank environment.

Our study has demonstrated that diclofenac can be taken up by fish and can cause transcriptomic and morphological alterations in the kidney at plasma concentrations equivalent to human therapeutic levels. Of particular note is the finding that exposure to diclofenac can result in abnormal kidney pathology that might suggest kidney damage as indicated by nephron neogenesis. However, the consequences of these alterations on individual health are unknown and further work is needed to inform on this. Furthermore, these effects were seen at aqueous exposure concentrations that exceed current reported concentrations in the environment, although in some cases by 5-fold only.[95,96] Overall, considering current exposure levels in most ambient aquatic environments, the accumulative potential, and exposure effects threshold for diclofenac, our study indicates a low likelihood for significant health impacts to fish for exposures over a period of a few weeks. However, the combination of diclofenac's uptake potential, effects on kidney nephrons and relatively small safety margin for some surface waters may warrant a longer term chronic health effects analysis for diclofenac in fish.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b05079.

Additional information as noted in the text (PDF)
(XLSX)

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The authors declare no competing financial interest.

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■ REFERENCES

(1) Gan, T. J. Diclofenac: an update on its mechanism of action and safety profile. *Curr. Med. Res. Opin.* **2010**, *26* (7), 1715−1731.

(2) Zhang, Y. J.; Geissen, S. U.; Gal, C. Carbamazepine and diclofenac: Removal in wastewater treatment plants and occurrence in water bodies. *Chemosphere* **2008**, *73* (8), 1151−1161.

(3) Donati, M.; Conforti, A.; Lenti, M. C.; Capuano, A.; Bortolami, O.; Motola, D.; Moretti, U.; Vannacci, A.; Rafaniello, C.; Vaccheri, A.; Arzenton, E.; Bonaiuti, R.; Sportiello, L.; Leone, R. on behalf of, D.-I. T. S. G., Risk of acute and serious liver injury associated to nimesulide and other NSAIDs: data from drug-induced liver injury case−control study in Italy. *Br. J. Clin. Pharmacol.* **2016**, *82* (1), 238−248.

(4) Oaks, J. L.; Gilbert, M.; Virani, M. Z.; Watson, R. T.; Meteyer, C. U.; Rideout, B. A.; Shivaprasad, H. L.; Ahmed, S.; Chaudhry, M. J. I.; Arshad, M.; Mahmood, S.; Ali, A.; Khan, A. A. Diclofenac residues as the cause of vulture population decline in Pakistan. *Nature* **2004**, *427* (6975), 630−633.

(5) Shultz, S.; Baral, H. S.; Charman, S.; Cunningham, A. A.; Das, D.; Ghalsasi, G. R.; Goudar, M. S.; Green, R. E.; Jones, A.; Nighot, P.; Pain, D. J.; Prakash, V. Diclofenac poisoning is widespread in declining vulture populations across the Indian subcontinent. *Proc. R. Soc. London, Ser. B* **2004**, *271*, S458−S460.

(6) Meteyer, C. U.; Rideout, B. A.; Gilbert, M.; Shivaprasad, H. L.; Oaks, J. L. Pathology and proposed pathophysiology of diclofenac poisoning in free-living and experimentally exposed oriental white-

backed vultures (*Gyps bengalensis*). *J. Wildl. Dis.* **2005**, *41* (4), 707−716.
(7) Swan, G. E.; Cuthbert, R.; Queved, M.; Green, R. E.; Pain, D. J.; Bartels, P.; Cunningham, A. A.; Duncan, N.; Meharg, A. A.; Oaks, J. L.; Parry-Jones, J.; Shultz, S.; Taggart, M. A.; Verdoorn, G.; Wolter, K. Toxicity of diclofenac to Gyps vultures. *Biol. Lett.* **2006**, *2* (2), 279−282.
(8) The IUCN Red List of Threatened Species, 2015−4 Website; http://www.iucnredlist.org/ (accessed June 2016).
(9) Cuthbert, R. J.; Taggart, M. A.; Saini, M.; Sharma, A.; Das, A.; Kulkarni, M. D.; Deori, P.; Ranade, S.; Shringarpure, R. N.; Galligan, T. H.; Green, R. E. Continuing mortality of vultures in India associated with illegal veterinary use of diclofenac and a potential threat from nimesulide. *Oryx* **2016**, *50* (1), 104−112.
(10) Becker, R. Cattle drug threatens thousands of vultures. *Nature News* **2016**, *532* (7600), 413−536.
(11) Brown, J. N.; Paxeus, N.; Forlin, L.; Larsson, D. G. J. Variations in bioconcentration of human pharmaceuticals from sewage effluents into fish blood plasma. *Environ. Toxicol. Pharmacol.* **2007**, *24* (3), 267−274.
(12) Fick, J.; Lindberg, R. H.; Parkkonen, J.; Arvidsson, B.; Tysklind, M.; Larsson, D. G. J. Therapeutic levels of levonorgestrel detected in blood plasma of fish: results from screening rainbow trout exposed to treated sewage effluents. *Environ. Sci. Technol.* **2010**, *44* (7), 2661−2666.
(13) Ternes, T. A. Occurrence of drugs in German sewage treatment plants and rivers. *Water Res.* **1998**, *32* (11), 3245−3260.
(14) Lishman, L.; Smyth, S. A.; Sarafin, K.; Kleywegt, S.; Toito, J.; Peart, T.; Lee, B.; Servos, M.; Beland, M.; Seto, P. Occurrence and reductions of pharmaceuticals and personal care products and estrogens by municipal wastewater treatment plants in Ontario, Canada. *Sci. Total Environ.* **2006**, *367* (2−3), 544−558.
(15) Yu, J. T.; Bouwer, E. J.; Coelhan, M. Occurrence and biodegradability studies of selected pharmaceuticals and personal care products in sewage effluent. *Agricultural Water Management* **2006**, *86* (1−2), 72−80.
(16) Gardner, M.; Comber, S.; Scrimshaw, M. D.; Cartmell, E.; Lester, J.; Ellor, B. The significance of hazardous chemicals in wastewater treatment works effluents. *Sci. Total Environ.* **2012**, *437*, 363−372.
(17) Metcalfe, C. D.; Miao, X. S.; Koenig, B. G.; Struger, J. Distribution of acidic and neutral drugs in surface waters near sewage treatment plants in the lower Great Lakes, Canada. *Environ. Toxicol. Chem.* **2003**, *22* (12), 2881−2889.
(18) Moder, M.; Braun, P.; Lange, F.; Schrader, S.; Lorenz, W. Determination of endocrine disrupting compounds and acidic drugs in water by coupling of derivatization, gas chromatography and Negative Chemical ionization mass Spectrometry. *Clean: Soil, Air, Water* **2007**, *35* (5), 444−451.
(19) Teijon, G.; Candela, L.; Tamoh, K.; Molina-Diaz, A.; Fernandez-Alba, A. R. Occurrence of emerging contaminants, priority substances (2008/105/CE) and heavy metals in treated wastewater and groundwater at Depurbaix facility (Barcelona, Spain). *Sci. Total Environ.* **2010**, *408* (17), 3584−3595.
(20) Vulliet, E.; Cren-Olive, C. Screening of pharmaceuticals and hormones at the regional scale, in surface and groundwaters intended to human consumption. *Environ. Pollut.* **2011**, *159* (10), 2929−2934.
(21) Lopez-Serna, R.; Jurado, A.; Vazquez-Sune, E.; Carrera, J.; Petrovic, M.; Barcelo, D. Occurrence of 95 pharmaceuticals and transformation products in urban groundwaters underlying the metropolis of Barcelona, Spain. *Environ. Pollut.* **2013**, *174*, 305−315.
(22) Heberer, T.; Mechlinski, A.; Fanck, B.; Knappe, A.; Massmann, G.; Pekdeger, A.; Fritz, B. Field studies on the fate and transport of pharmaceutical residues in bank filtration. *Groundwater Monit. Rem.* **2004**, *24* (2), 70−77.
(23) Togola, A.; Budzinski, H. Multi-residue analysis of pharmaceutical compounds in aqueous samples. *J. Chromatogr. A* **2008**, *1177* (1), 150−158.
(24) Kimura, K.; Hara, H.; Watanabe, Y. Elimination of selected acidic pharmaceuticals from municipal wastewater by an activated sludge system and membrane bioreactors. *Environ. Sci. Technol.* **2007**, *41* (10), 3708−3714.
(25) Johnson, A. C.; Dumont, E.; Williams, R. J.; Oldenkamp, R.; Cisowska, I.; Sumpter, J. P. Do concentrations of ethinylestradiol, estradiol, and diclofenac in European rivers exceed proposed EU environmental quality standards? *Environ. Sci. Technol.* **2013**, *47* (21), 12297−12304.
(26) Gardner, M.; Jones, V.; Comber, S.; Scrimshaw, M. D.; Coello-Garcia, T.; Cartmell, E.; Lester, J.; Ellor, B. Performance of UK wastewater treatment works with respect to trace contaminants. *Sci. Total Environ.* **2013**, *456−457*, 359−369.
(27) Cleuvers, M. Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects. *Toxicol. Lett.* **2003**, *142* (3), 185−194.
(28) Ferrari, B.; Paxeus, N.; Lo Giudice, R.; Pollio, A.; Garric, J. Ecotoxicological impact of pharmaceuticals found in treated wastewaters: study of carbamazepine, clofibric acid, and diclofenac. *Ecotoxicol. Environ. Saf.* **2003**, *55* (3), 359−370.
(29) Haap, T.; Triebskorn, R.; Kohler, H. R. Acute effects of diclofenac and DMSO to *Daphnia magna*: Immobilisation and hsp70-induction. *Chemosphere* **2008**, *73* (3), 353−359.
(30) Hong, H. N.; Kim, H. N.; Park, K. S.; Lee, S. K.; Gu, M. B. Analysis of the effects diclofenac has on Japanese medaka (*Oryzias latipes*) using real-time PCR. *Chemosphere* **2007**, *67* (11), 2115−2121.
(31) Ferrari, B.; Mons, R.; Vollat, B.; Fraysse, B.; Paxeus, N.; Lo Giudice, R.; Pollio, A.; Garric, J. Environmental risk assessment of six human pharmaceuticals: Are the current environmental risk assessment procedures sufficient for the protection of the aquatic environment? *Environ. Toxicol. Chem.* **2004**, *23* (5), 1344−1354.
(32) Mehinto, A. C.; Hill, E. M.; Tyler, C. R. Uptake and biological effects of environmentally relevant concentrations of the nonsteroidal anti-inflammatory pharmaceutical diclofenac in rainbow trout (*Oncorhynchus mykiss*). *Environ. Sci. Technol.* **2010**, *44* (6), 2176−2182.
(33) Schwaiger, J.; Ferling, H.; Mallow, U.; Wintermayr, H.; Negele, R. D. Toxic effects of the non-steroidal anti-inflammatory drug diclofenac Part 1: histopathological alterations and bioaccumulation in rainbow trout. *Aquat. Toxicol.* **2004**, *68* (2), 141−150.
(34) Triebskorn, R.; Casper, H.; Heyd, A.; Eikemper, R.; Kohler, H. R.; Schwaiger, J. Toxic effects of the non-steroidal anti-inflammatory drug diclofenac Part II. Cytological effects in liver, kidney, gills and intestine of rainbow trout (*Oncorhynchus mykiss*). *Aquat. Toxicol.* **2004**, *68* (2), 151−166.
(35) Hoeger, B.; Kollner, B.; Dietrich, D. R.; Hitzfeld, B. Water-borne diclofenac affects kidney and gill integrity and selected immune parameters in brown trout (*Salmo trutta f. fario*). *Aquat. Toxicol.* **2005**, *75* (1), 53−64.
(36) Memmert, U.; Peither, A.; Burri, R.; Weber, K.; Schmidt, T.; Sumpter, J. P.; Hartmann, A. Diclofenac: New data on chronic toxicity and bioconcentration in fish. *Environ. Toxicol. Chem.* **2013**, *32* (2), 442−452.
(37) Wolf, J. C.; Ruehl-Fehlert, C.; Segner, H. E.; Weber, K.; Hardisty, J. F. Pathology working group review of histopathologic specimens from three laboratory studies of diclofenac in trout. *Aquat. Toxicol.* **2014**, *146*, 127−136.
(38) Cuklev, F.; Kristiansson, E.; Fick, J.; Asker, N.; Forlin, L.; Larsson, D. G. J. Diclofenac in fish: blood plasma levels similar to human therapeutic levels affect global hepatic gene expression. *Environ. Toxicol. Chem.* **2011**, *30* (9), 2126−2134.
(39) De Felice, B.; Copia, L.; Guida, M. Gene expression profiling in zebrafish embryos exposed to diclofenac, an environmental toxicant. *Mol. Biol. Rep.* **2012**, *39* (3), 2119−2128.
(40) *Report from the Commission to the European Parliament and the Council on the outcome of the review of Annex X to Directive 2000/60/EC of the European Parliament and of the Council on priority substances in the field of water policy*, 31.1.2012 COM (2011) 875 final; European Commission: Brussels. Website; https://ec.europa.eu/transparency/

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Environ. Sci. Technol. 2017, 51, 1764−1774

regdoc/rep/1/2011/EN/1-2011-875-EN-F1-1.Pdf (accessed June 2016).
(41) Paull, G. C.; Lange, A.; Henshaw, A. C.; Tyler, C. R. Ontogeny of sexual development in the roach (*Rutilus rutilus*) and its interrelationships with growth and age. *J. Morphol.* **2008**, *269* (7), 884−895.
(42) Cormier, S. M.; Neiheisel, T. W.; Wernsing, P.; Racine, R. N.; Reimschuessel, R. New nephron development in fish from polluted waters a possible biomarker. *Ecotoxicology* **1995**, *4* (3), 157−168.
(43) Magoc, T.; Salzberg, S. L. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* **2011**, *27* (21), 2957−2963.
(44) Haas, B. J.; Papanicolaou, A.; Yassour, M.; Grabherr, M.; Blood, P. D.; Bowden, J.; Couger, M. B.; Eccles, D.; Li, B.; Lieber, M.; MacManes, M. D.; Ott, M.; Orvis, J.; Pochet, N.; Strozzi, F.; Weeks, N.; Westerman, R.; William, T.; Dewey, C. N.; Henschel, R.; Leduc, R. D.; Friedman, N.; Regev, A. De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. *Nat. Protoc.* **2013**, *8* (8), 1494−1512.
(45) Altschul, S. F.; Gish, W.; Miller, W.; Myers, E. W.; Lipman, D. J. Basic Local Alignment Search Tool. *J. Mol. Biol.* **1990**, *215* (3), 403−410.
(46) Li, B.; Dewey, C. N. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinf.* **2011**, *12*, 323.
(47) Robinson, M. D.; McCarthy, D. J.; Smyth, G. K. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **2010**, *26* (1), 139−140.
(48) Gentleman, R. C.; Carey, V. J.; Bates, D. M.; Bolstad, B.; Dettling, M.; Dudoit, S.; Ellis, B.; Gautier, L.; Ge, Y. C.; Gentry, J.; Hornik, K.; Hothorn, T.; Huber, W.; Iacus, S.; Irizarry, R.; Leisch, F.; Li, C.; Maechler, M.; Rossini, A. J.; Sawitzki, G.; Smith, C.; Smyth, G.; Tierney, L.; Yang, J. Y. H.; Zhang, J. H. Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol.* **2004**, *5* (10), R80.
(49) Huang, D. W.; Sherman, B. T.; Lempicki, R. A. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* **2009**, *4* (1), 44−57.
(50) Merico, D.; Isserlin, R.; Stueker, O.; Emili, A.; Bader, G. D. Enrichment Map: A Network-Based Method for Gene-Set Enrichment Visualization and Interpretation. *PLoS One* **2010**, *5* (11), e13984.
(51) Smoot, M. E.; Ono, K.; Ruscheinski, J.; Wang, P. L.; Ideker, T. Cytoscape 2.8: new features for data integration and network visualization. *Bioinformatics* **2011**, *27* (3), 431−432.
(52) Regenthal, R.; Krueger, M.; Koeppel, C.; Preiss, R. Drug levels: Therapeutic and toxic serum/plasma concentrations of common drugs. *J. Clin. Monit. Comput.* **1999**, *15* (7−8), 529−544.
(53) Schulz, M.; Iwersen-Bergmann, S.; Andresen, H.; Schmoldt, A. Therapeutic and toxic blood concentrations of nearly 1,000 drugs and other xenobiotics. *Critical Care* **2012**, *16* (4), R136.
(54) Mahdy, A. M.; Galley, H. F.; Abdel-Wahed, M. A.; El-Korny, K. F.; Sheta, S. A.; Webster, N. R. Differential modulation of interleukin-6 and interleukin-10 by diclofenac in patients undergoing major surgery. *Br. J. Anaesth.* **2002**, *88* (6), 797−802.
(55) Yano, A.; Higuchi, S.; Tsuneyama, K.; Fukami, T.; Nakajima, M.; Yokoi, T. Involvement of immune-related factors in diclofenac-induced acute liver injury in mice. *Toxicology* **2012**, *293* (1−3), 107−114.
(56) Kakita, H.; Aoyama, M.; Hussein, M. H.; Kato, S.; Suzuki, S.; Ito, T.; Togari, H.; Asai, K. Diclofenac enhances proinflammatory cytokine-induced nitric oxide production through NF-kappa B signaling in cultured astrocytes. *Toxicol. Appl. Pharmacol.* **2009**, *238* (1), 56−63.
(57) Lee, E. H.; Oh, J. H.; Selvaraj, S.; Park, S. M.; Choi, M. S.; Spanel, R.; Yoon, S.; Borlak, J. Immunogenomics reveal molecular circuits of diclofenac induced liver injury in mice. *Oncotarget* **2016**, *7* (12), 14983−15017.
(58) van Swelm, R. P. L.; Laarakkers, C. M. M.; Pertijs, J.; Verweij, V.; Masereeuw, R.; Russel, F. G. M. Urinary proteomic profiling reveals diclofenac-induced renal injury and hepatic regeneration in mice. *Toxicol. Appl. Pharmacol.* **2013**, *269* (2), 141−149.
(59) Lauer, B.; Tuschl, G.; Kling, M.; Mueller, S. O. Species-specific toxicity of diclofenac and troglitazone in primary human and rat hepatocytes. *Chem.-Biol. Interact.* **2009**, *179* (1), 17−24.
(60) Singh, R.; Cadeddu, R. P.; Frobel, J.; Wilk, C. M.; Bruns, I.; Zerbini, L. F.; Prenzel, T.; Hartwig, S.; Brunnert, D.; Schroeder, T.; Lehr, S.; Haas, R.; Czibere, A. The non-steroidal anti-inflammatory drugs Sulindac sulfide and Diclofenac induce apoptosis and differentiation in human acute myeloid leukemia cells through an AP-1 dependent pathway. *Apoptosis* **2011**, *16* (9), 889−901.
(61) Fredriksson, L.; Wink, S.; Herpers, B.; Benedetti, G.; Hadi, M.; de Bont, H.; Groothuis, G.; Luijten, M.; Danen, E.; de Graauw, M.; Meerman, J.; van de Water, B. Drug-induced endoplasmic reticulum and oxidative stress responses independently sensitize toward TNF alpha-mediated hepatotoxicity. *Toxicol. Sci.* **2014**, *140* (1), 144−159.
(62) Masubuchi, Y.; Ose, A.; Horie, T. Mechanism-based inactivation of CYP2C11 by diclofenac. *Drug Metab. Dispos.* **2001**, *29* (9), 1190−1195.
(63) Kirchheiner, J.; Meineke, I.; Steinbach, N.; Meisel, C.; Roots, I.; Brockmoller, J. Pharmacokinetics of diclofenac and inhibition of cyclooxygenases 1 and 2: no relationship to the CYP2C9 genetic polymorphism in humans. *Br. J. Clin. Pharmacol.* **2003**, *55* (1), 51−61.
(64) Siu, W. P.; Pun, P. B. L.; Latchoumycandane, C.; Boelsterli, U. A. Bax-mediated mitochondrial outer membrane permeabilization (MOMP), distinct from the mitochondrial permeability transition, is a key mechanism in diclofenac-induced hepatocyte injury: Multiple protective roles of cyclosporin A. *Toxicol. Appl. Pharmacol.* **2008**, *227* (3), 451−461.
(65) Hoeger, B.; Dietrich, D. R.; Schmid, D.; Hartmann, A.; Hitzfeld, B. Distribution of intraperitoneally injected diclofenac in brown trout (*Salmo trutta f. fario*). *Ecotoxicol. Environ. Saf.* **2008**, *71* (2), 412−418.
(66) Lahti, M.; Brozinski, J. M.; Jylha, A.; Kronberg, L.; Oikari, A. Uptake from water, biotransformation, and biliary excretion of pharmaceuticals by rainbow trout. *Environ. Toxicol. Chem.* **2011**, *30* (6), 1403−1411.
(67) Kallio, J.-M.; Lahti, M.; Oikari, A.; Kronberg, L. Metabolites of the aquatic pollutant diclofenac in fish bile. *Environ. Sci. Technol.* **2010**, *44* (19), 7213−7219.
(68) Vane, J. R.; Botting, R. M. Mechanism of action of nonsteroidal anti-inflammatory drugs. *Am. J. Med.* **1998**, *104*, 2S−S8.
(69) Kirkby, N. S.; Chan, M. V.; Zaiss, A. K.; Garcia-Vaz, E.; Jiao, J.; Berglund, L. M.; Verdu, E. F.; Ahmetaj-Shala, B.; Wallace, J. L.; Herschman, H. R.; Gomez, M. F.; Mitchell, J. A. Systematic study of constitutive cyclooxygenase-2 expression: Role of NF-kappa B and NFAT transcriptional pathways. *Proc. Natl. Acad. Sci. U. S. A.* **2016**, *113* (2), 434−439.
(70) Chandrasekharan, N. V.; Simmons, D. L. The cyclooxygenases. *Genome Biol.* **2004**, *5* (9), 241.
(71) Zou, J.; Neumann, N. F.; Holland, J. W.; Belosevic, M.; Cunningham, C.; Secombes, C. J.; Rowley, A. F. Fish macrophages express a cyclo-oxygenase-2 homologue after activation. *Biochem. J.* **1999**, *340*, 153−159.
(72) Roberts, S. B.; Langenau, D. M.; Goetz, F. W. Cloning and characterization of prostaglandin endoperoxide synthase-1 and-2 from the brook trout ovary. *Mol. Cell. Endocrinol.* **2000**, *160* (1−2), 89−97.
(73) Grosser, T.; Yusuff, S.; Cheskis, E.; Pack, M. A.; FitzGerald, G. A. Developmental expression of functional cyclooxygenases in zebrafish. *Proc. Natl. Acad. Sci. U. S. A.* **2002**, *99* (12), 8418−8423.
(74) Lubiana, P.; Prokkola, J. M.; Nikinmaa, M.; Burmester, T.; Kanerva, M.; Gotting, M. The effects of the painkiller diclofenac and hypoxia on gene transcription and antioxidant system in the gills of three-spined stickleback. *Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol.* **2016**, *185−186*, 147−54.
(75) Poligone, B.; Baldwin, A. S. Positive and negative regulation of NF-kappa B by COX-2-Roles of different prostaglandins. *J. Biol. Chem.* **2001**, *276* (42), 38658−38664.

DOI: 10.1021/acs.est.6b05079
Environ. Sci. Technol. 2017, 51, 1764−1774

(76) Vichai, V.; Suyarnsesthakorn, C.; Pittayakhajonwut, D.; Sriklung, K.; Kirtikara, K. Positive feedback regulation of COX-2 expression by prostaglandin metabolites. *Inflammation Res.* **2005**, *54* (4), 163−172.
(77) Cengiz, E. I. Gill and kidney histopathology in the freshwater fish *Cyprinus carpio* after acute exposure to deltamethrin. *Environ. Toxicol. Pharmacol.* **2006**, *22* (2), 200−204.
(78) Liney, K. E.; Hagger, J. A.; Tyler, C. R.; Depledge, M. H.; Galloway, T. S.; Jobling, S. Health effects in fish of long-term exposure to effluents from wastewater treatment works. *Environ. Health Perspect.* **2006**, *114*, 81−89.
(79) Reimschuessel, R.; Bennett, R. O.; May, E. B.; Lipsky, M. M. Development of newly formed nephrons in the goldfish kidney following hexachlorobutadiene-induced nephrotoxicity. *Toxicol. Pathol.* **1990**, *18* (1), 32−38.
(80) Salice, C. J.; Rokous, J. S.; Kane, A. S.; Reimschuessel, R. New nephron development in goldfish (*Carassius auratus*) kidneys following repeated gentamicin-induced nephrotoxicosis. *Comp. Med.* **2001**, *51* (1), 56−59.
(81) Diep, C. Q.; Ma, D. D.; Deo, R. C.; Holm, T. M.; Naylor, R. W.; Arora, N.; Wingert, R. A.; Bollig, F.; Djordjevic, G.; Lichman, B.; Zhu, H.; Ikenaga, T.; Ono, F.; Englert, C.; Cowan, C. A.; Hukriede, N. A.; Handin, R. I.; Davidson, A. J. Identification of adult nephron progenitors capable of kidney regeneration in zebrafish. *Nature* **2011**, *470* (7332), 95−U108.
(82) Reimschuessel, R. A fish model of renal regeneration and development. *ILAR J.* **2001**, *42* (4), 285−91.
(83) Diep, C. Q.; Ma, D. D.; Deo, R. C.; Holm, T. M.; Naylor, R. W.; Arora, N.; Wingert, R. A.; Bollig, F.; Djordjevic, G.; Lichman, B.; Zhu, H.; Ikenaga, T.; Ono, F.; Englert, C.; Cowan, C. A.; Hukriede, N. A.; Handin, R. I.; Davidson, A. J. Identification of adult nephron progenitors capable of kidney regeneration in zebrafish. *Nature* **2011**, *470* (7332), 95−U108.
(84) Wang, J.; Karra, R.; Dickson, A. L.; Poss, K. D. Fibronectin is deposited by injury-activated epicardial cells and is necessary for zebrafish heart regeneration. *Dev. Biol.* **2013**, *382* (2), 427−435.
(85) Saball, E.; Salvarrey, M.; Serra, E.; Pico, G.; Elias, M. M. Potential mechanism of fibronectin deposits in acute renal failure induced by mercuric chloride. *Mol. Cell. Biochem.* **2001**, *226* (1−2), 67−75.
(86) Sorokin, L. The impact of the extracellular matrix on inflammation. *Nat. Rev. Immunol.* **2010**, *10* (10), 712−723.
(87) Patari-Sampo, A.; Ihalmo, P.; Holthofer, H. Molecular basis of the glomerular filtration: Nephrin and the emerging protein complex at the podocyte slit diaphragm. *Ann. Med.* **2006**, *38* (7), 483−492.
(88) Nguan, C. Y. C.; Guan, Q.; Gleave, M. E.; Du, C. Promotion of cell proliferation by clusterin in the renal tissue repair phase after ischemia-reperfusion injury. *Am. J. Physiol. Renal Physiol.* **2014**, *306* (7), F724−F733.
(89) Jung, G. S.; Kim, M. K.; Jung, Y. A.; Kim, H. S.; Park, I. S.; Min, B. H.; Lee, K. U.; Kim, J. G.; Park, K. G.; Lee, I. K. Clusterin attenuates the development of renal fibrosis. *J. Am. Soc. Nephrol.* **2012**, *23* (1), 73−85.
(90) Jang, H. S.; Kim, J. I.; Noh, M.; Rhee, M. H.; Park, K. M. Regulator of G protein signaling 2 (RGS2) deficiency accelerates the progression of kidney fibrosis. *Biochim. Biophys. Acta, Mol. Basis Dis.* **2014**, *1842* (9), 1733−1741.
(91) To, W. S.; Midwood, K. S. Plasma and cellular fibronectin: distinct and independent functions during tissue repair. *Fibrog. Tissue Repair* **2011**, *4* (1), 21.
(92) Ogata, K.; Shimamura, Y.; Hamada, K.; Hisa, M.; Bun, M.; Okada, N.; Inoue, K.; Taniguchi, Y.; Ishihara, M.; Kagawa, T.; Horino, T.; Fujimoto, S.; Terada, Y. Upregulation of HNF-1 beta during experimental acute kidney injury plays a crucial role in renal tubule regeneration. *Am. J. Physiol. Renal Physiol.* **2012**, *303* (5), F689−F699.
(93) Liu, Y. H. Renal fibrosis: New insights into the pathogenesis and therapeutics. *Kidney Int.* **2006**, *69* (2), 213−217.
(94) Larson, E. T.; O'Malley, D. M.; Melloni, R. H. Aggression and vasotocin are associated with dominant-subordinate relationships in zebrafish. *Behav. Brain Res.* **2006**, *167* (1), 94−102.
(95) Heberer, T. Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. *Toxicol. Lett.* **2002**, *131* (1−2), 5−17.
(96) Andreozzi, R.; Marotta, R.; Paxeus, N. Pharmaceuticals in STP effluents and their solar photodegradation in aquatic environment. *Chemosphere* **2003**, *50* (10), 1319−1330.

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