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Pathology working group review of histopathologic specimens from three laboratory studies of diclofenac in trout☆

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ABSTRACT

While the pathology peer review/pathology working group (PWG) model has long been used in mammalian toxicologic pathology to ensure the accuracy, consistency, and objectivity of histopathology data, application of this paradigm to ecotoxicological studies has thus far been limited. In the current project, the PWG approach was used to evaluate histopathologic sections of gills, liver, kidney, and/or intestines from three previously published studies of diclofenac in trout, among which there was substantial variation in the reported histopathologic findings. The main objectives of this review process were to investigate and potentially reconcile these interstudy differences, and based on the results, to establish an appropriate no observed effect concentration (NOEC). Following a complete examination of all histologic sections and original diagnoses by a single experienced fish pathologist (pathology peer review), a two-day PWG session was conducted to allow members of a four-person expert panel to determine the extent of treatment-related findings in each of the three trout studies. The PWG was performed according to the United States Environmental Protection Agency (US EPA) Pesticide Regulation (PR) 94-5 (EPA Pesticide Regulation, 1994). In accordance with standard procedures, the PWG review was conducted by the non-voting chairperson in a manner intended to minimize bias, and thus during the evaluation, the four voting panelists were unaware of the treatment group status of individual fish and the original diagnoses associated with the histologic sections. Based on the results of this review, findings related to diclofenac exposure included minimal to slightly increased thickening of the gill filament tips in fish exposed to the highest concentration tested (1000 μg/L), plus a previously undiagnosed finding, decreased hepatic glycogen, which also occurred at the 1000 μg/L dose level. The panel found little evidence to support other reported effects of diclofenac in trout, and thus the overall NOEC was determined to be >320 μg/L. By consensus, the PWG panel was able to identify diagnostic inconsistencies among and within the three prior studies; therefore this exercise demonstrated the value of the pathology peer review/PWG approach for assessing the reliability of histopathology results that may be used by regulatory agencies for risk assessment.

1. Introduction

Historically, there have been few toxicological studies in which organisms were exposed to human or veterinary pharmaceuticals in surface waters, ground water, and/or sewage sludge (Boxall et al., 2012; Corcoran et al., 2010). This is particularly true for investigations of potential chronic toxicity effects. Consequently, it can be challenging to understand the toxicological and ecological importance of drug concentrations measured in the environment. Although only limited data are available relevant to the ecotoxicological impact of non-endocrine pharmaceutical ingredients released into the environment, some reports have suggested that

Abbreviations: DCF, diclofenac; PWG, pathology working group; SP, study pathologist; RP, reviewing pathologist; NOEC, no observed effect concentration; LOEC, lowest observed effect concentration.

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Table 1
Summary of experimental designs and reported outcomes from four laboratory studies of diclofenac in trout.

Study	Design	Diclofenac conc. tested (μg/L)	*n* per dose group (total n)	Fixation/staining	Tissues examined	Tissues with exposure related findings	No observed effect concentration (NOEC)	Organ with lowest observed effect concentration (LOEC)
Schwaiger et al. (2004)	1.8 y rainbow trout; 28 d exposure; no replicate tanks	0, 1, 5, 20, 100, 500	10 (60)	Formalin/H&E, PAS (kidney)	Gills, liver, kidney, spleen, intestine	Gills, kidney	1 μg/L	Gills, kidney (5 μg/L)
Hoeger et al. (2005)	18 m brown trout; 21 d exposure; no replicate tanks	0, 0.5, 5, 50	6 (24)	Formalin/H&E; IHC (granulocytes, Thrombocytes, MHCII)	Gills, liver, head and trunk kidney, spleen, intestine	Gills, liver, trunk kidney	0.5 μg/L	Liver (5 μg/L)
Mehinto et al. (2010)	Juvenile (6 w+) female rainbow trout; 21 d exposure; 2 replicate tanks	0, 0.5, 1, 5, 25	10 (50)	Bouin's/H&E	Gills, liver, kidney, intestine	Kidney, intestine	0.5 μg/L	Kidney, intestine (1 μg/L)
Memmert et al. (2013)	Juvenile rainbow trout; 95 d exposure (33 d prehatch; 62 d posthatch); 4 replicate tanks	0, 3.2, 10, 32, 100, 320, 1000	20 (140)	Davidson's/H&E; Azan Heidenhains (gills)	Gills, liver, kidney (whole fish sections)	Gills	320 μg/L	Gills (1000 μg/L)

their presence may be related to adverse effects in wildlife (Boxall et al., 2012).

Diclofenac (DCF) is a widely available non-steroidal anti-inflammatory drug (NSAID) that is used to treat a diverse spectrum of pain and inflammatory disorders. Concerns pertaining to possible environmental effects of DCF arose approximately a decade ago when initial reports linked the veterinary use of this compound to vulture population declines in the Indian Subcontinent (Oaks et al., 2004; Shultz et al., 2004). According to these and subsequent articles, a major cause of vulture deaths involved renal damage and visceral gout that were induced by the birds' consumption of DCF-treated livestock carcasses (Oaks et al., 2004; Shultz et al., 2004; Meteyer et al., 2005; Swan et al., 2006).

Diclofenac is regularly detected in sewage treatment plant effluents and surface waters across the globe (Zhang et al., 2008; Johnson et al., 2013); consequently, a number of studies have investigated the potential for DCF to exert toxicological effects in aquatic organisms. Included among these are several laboratory investigations in which salmonid fishes were exposed to DCF by water bath (Schwaiger et al., 2004; Hoeger et al., 2005; Mehinto et al., 2010; Memmert et al., 2013). Although each of these four studies described effects related to diclofenac exposure, histopathologic findings among the accounts varied in terms of the organs that were affected, the types of lesions that were observed, and the no observed effect concentrations (NOEC) that were reported (Table 1). For example, exposure related findings were reported for the gills in three of the four studies (Schwaiger et al., 2004; Hoeger et al., 2005; Memmert et al., 2013) and for the kidneys in three of the four studies (Schwaiger et al., 2004; Hoeger et al., 2005; Mehinto et al., 2010). In addition to different findings with respect to the target organs, the studies also differed with respect to the specific organ lesions ascribed to DCF exposure in each case (Table 2). Exposure related findings were also reported for the liver in one study (Hoeger et al., 2005) and for the intestines of another study (Mehinto et al., 2010). The NOEC and lowest observed effect concentration (LOEC) values varied widely among the three studies and were based on histopathologic findings reported in different tissues.

It is reasonable to presume that some of this interstudy variability could be attributed to experimental design differences such as disparities in fish species and age, test concentrations, test compound bioavailability, exposure duration, and/or specific technical procedures employed for autopsy and histologic slide preparation. However, because the histopathological assessments were conducted at different laboratories by different individuals, it is also possible that variation in the results may have been caused by differences in diagnostic interpretation. In order to explore this latter possibility, a Pathology Working Group (PWG) was convened to review the histologic sections and compare findings among these salmonid DCF studies. This independent PWG panel consisted of several expert anatomic pathologists who have extensive experience in the microscopic evaluation of tissues from chemically exposed fish.

The pathology peer review and PWG paradigm (Fig. 1) is employed routinely to enhance overall quality of histopathological results produced by toxicological bioassays conducted in mammals; however, such reviews have had relatively limited application in ecotoxicological investigations. Briefly, pathology peer review involves the examination by a second reviewing pathologist (RP) of histologic slides that were previously evaluated by the initial study pathologist (SP). The primary goal of this type of review is to improve the accuracy of the histopathologic evaluation, by ensuring that treatment-related findings have been identified properly, and the severity grading of such findings has been scored consistently (Mann and Hardisty, 2013). Peer review is not the same as a de novo re-evaluation of the study, because all original diagnoses for each evaluated animal are available to the RP, who can thereby choose to agree or disagree with each individual finding made by the study pathologist; thus, the outcome of a peer review is intended to be a single data set rather than two completely independent sets of data. A peer review may be conducted prospectively, i.e., prior to finalization of a study and/or publication of the results. In such instances, the SP and the RP may meet following the peer review to jointly reconcile diagnostic differences, which often occurs. Alternatively, persisting interpretive differences between the SP and RP may be resolved subsequently by the consensus decisions of a multi-pathologist PWG panel. Retrospective peer reviews are generally performed to address issues involving diagnostic criteria and interpretation for changes reported in certain target tissues. The results may be

Table 2
Specific histopathologic findings as reported in four laboratory studies of diclofenac in trout.

	Gills	Liver	Kidney	Intestine
Schwaiger et al. (2004)	**1. Pillar cell necrosis** **2. Dilation of capillary walls/telangiectasia** **3. Respiratory epithelial cell necrosis**[a] 4. Focal interlamellar cell proliferation (NS[b]) 5. Inflammation (NS) 6. Epithelial lifting (NS)	No findings	**1. Severe tubular hyaline droplet degeneration** **Interstitial nephritis**	No findings
Hoeger et al. (2005) (Study 1)	1. Lamellar clubbing 2. Telangiectasis (NS) 3. Hyperplasia (NS) 4. Thickened tips (NS) 5. Mucus cell hyperplasia (NS) 6. Secondary lamellar fusion (NS)	**1. Monocyte infiltration (5 μg/L only)** 2. Sinusoid distension (NS) 3. Diffuse cytoplasm (NS) 4. Focal necrosis (NS) 5. Basophilic foci (NS) 6. Interstitial proteinaceous fluid (NS) 7. Scanty cytoplasm (NS)	**1. Interstitial proteinaceous fluid** **2. Tubular necrosis** 3. Interstitial hyaline droplets (NS) 4. Proteinaceous casts (NS) 5. Interstitial and intravascular proteinaceous fluid (NS)	No results reported
Mehinto et al., 2010 (Study 2)	Not evaluated	No findings	**1. Increased developing nephrons** **2. Tubular necrosis** **3. Loss of Bowman's space**	**1. Fusion of villi** **2. Hyperplasia of villi** 3. Increased numbers and size of goblet cells (NS)
Memmert et al. (2013) (Study 3)	**1. Focal interlamellar cell proliferation** **2. Increased chloride cells** **3. Thickened lamellar tips** 4. Mononuclear cell foci (NS) 5. Inflammation (NS) 6. Angiectasis (NS) 7. Lamellar fusion (NS) 8. Single cell necrosis of interlamellar cells (NS)	1. Inflammatory foci (NS) 2. Enhanced basophilia (NS)	1. Hyaline inclusions (NS) 2. Single cell necrosis (NS)	Not evaluated

[a] Bolded findings were statistically significant.
[b] NS = no significant difference between diclofenac exposed trout and controls.

used for the determination of appropriate NOEC levels, and/or the use of the results in risk assessment. The usual purpose of a retrospective peer review is to identify potential areas of diagnostic concern for further evaluation by a PWG panel (Mann and Hardisty, 2013). In the specific case of diclofenac, existing trout histopathology data has been cited as evidence of a relationship between the environmental presence of that pharmaceutical and fish population declines (European Commission, 2011). Therefore, the reliability of such data must be substantiated if regulatory agencies are to make informed decisions.

Selection criteria for the voting PWG panel members, who are invited to participate by the non-voting PWG chairperson,

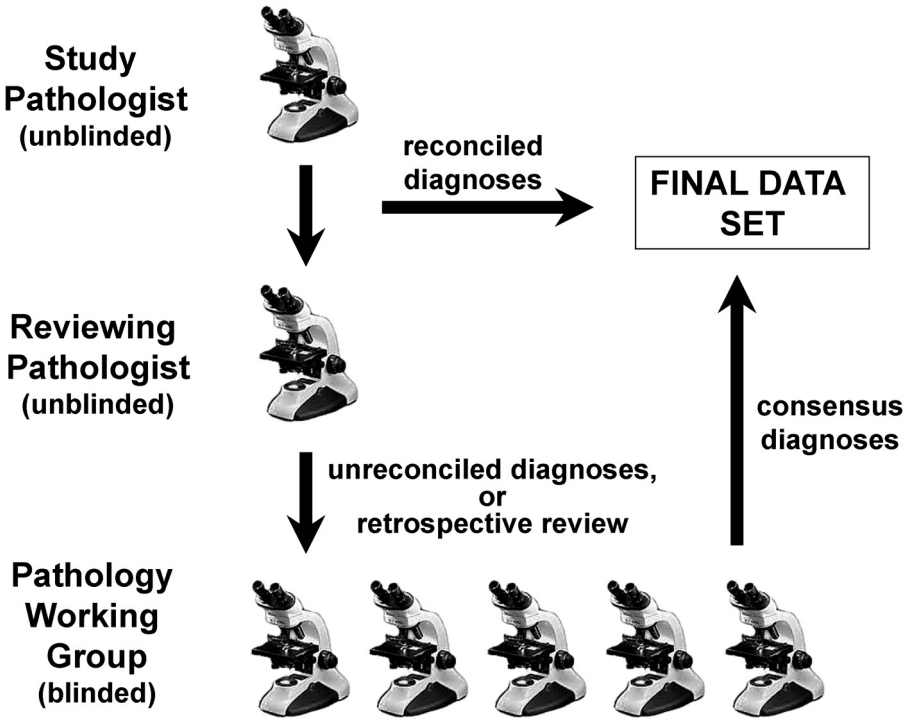

Fig. 1. The peer review/pathology working group (PWG) paradigm.

generally include advanced experience in toxicologic pathology, special expertise in the area of pathology that is relevant to the review, and a lack of conflicting interests (Mann and Hardisty, 2013). Because they have the most familiarity with the specific lesion types to be addressed, it is also customary to include SPs and RPs in the panel. During their evaluations, the members of this expert group are kept unaware of ("blinded" to), the original diagnoses and treatment group status of individual animals. Further information on the theory and conduct of pathology peer review and PWG can be found in a number of review and best practices documents (Hardisty and Boorman, 1986; The Society of Toxicologic Pathologists, 1991; Ward et al., 1995; The Society of Toxicologic Pathologists, 1997; Crissman et al., 2004; Boorman et al., 2010; Mann, 1996; Morton et al., 2010).

The purpose of the present paper is to describe the procedures used for this particular DCF trout PWG, report the PWG findings, and illustrate the importance of microscopic slide review for ensuring the reliability of histopathology data that may be utilized by regulatory bodies as criteria for ecotoxicological risk assessment.

2. Materials and methods

The original intent of this project was to review material from all four of the DCF trout studies that were described in the introduction; however, materials from the (Schwaiger et al., 2004) study were not available. Consequently, reviews were limited to slides and accompanying data from Hoeger et al. (2005) (designated here as Study 1), Mehinto et al. (2010) (designated as Study 2), and Memmert et al. (2013) (Study 3). Table 1 provides an overview of the experimental designs and reported outcomes from the four DCF studies, whereas Table 2 compares the specific histopathologic findings from those studies.

2.1. Study 1 materials

All slides and a spreadsheet containing transcribed individual animal data were available for Study 1 (the original data sheets from the pathology evaluation were no longer retrievable). The spreadsheet contained scores for the DCF-exposed fish but no findings for any control animals. The Study 1 slide set consisted of 24 histologic slides (1 per fish) that had been stained with hematoxylin and eosin (H&E). Each slide contained two sections of excised liver, two sections of excised posterior kidney, and a single section of excised gill arch with filaments (primary lamellae).

2.2. Study 2 materials

All slides and the published summary data were furnished for Study 2, but the individual animal data upon which the publication was based were no longer available. A total of 297 H&E-stained slides were on hand from this study. There were three slides (A, B, and C) of excised kidney for each of 50 fish, and three slides (A, B, and C) of excised intestine for each of 50 fish. The animal numbers for the kidney slide labels did not correlate to those of intestine slides, so the total number of animals represented could not be determined. Each kidney or intestine slide contained two or three ribbons of serial sections (approximately 10 to 30 sections total per slide), and sections from all three slides per fish (A, B, and C) had been cut in series from the same block (thus for each fish, all sections of kidney and all sections of intestine were virtually identical). The kidney sections appeared to have been microtomed in the longitudinal (axial) plane, and comprised varying proportions of anterior (hematopoietic) and/or posterior (urinary) kidney. The intestine sections had been microtomed transversely, i.e., perpendicular to the longitudinal axis of the gut.

2.3. Study 3 materials

Because the conduct of Study 3 followed OECD Test Guideline 210 (OECD, 1992), all slides and the entire final toxicology report including individual animal data were provided. A total of 140 H&E-stained slides were available (1 per fish). Each slide contained two parasagittal sections of decalcified whole fish. Following an initial assessment, duplicate slides that had been stained with Heidenhain's Azan stain did not appear to provide additional information and were thus not further examined.

2.4. Pathology peer review of Studies 1–3

Prior to the re-evaluations, the original individual animal data for Studies 1 and 3 were entered electronically into proprietary dedicated peer review software (PQA). For Study 2, only the animal numbers were entered into the PQA software. Peer review of the initial histopathology findings from three studies was performed by Jeffrey C. Wolf, DVM, DACVP. Dr. Wolf (the RP) examined by light microscopy all slides that contained one or more of the target tissues (i.e., gills, liver, kidney, and/or intestine), in concert with each of the original diagnoses recorded by the SP. A combined total of 461 slides were examined for the three studies. These slides contained >6000 sections, which were associated with 37 different types of diagnoses. For Study 2, in which the individual animal results were not available, the RP examined the tissues for the types of changes that were reported in the published article (Mehinto et al., 2010). For each of the three studies, the peer review findings were recorded into the PQA software. This software allowed the RP to either agree or disagree with each individual original diagnosis that had been recorded by the SP. The RP also had the option of providing an alternate diagnosis for each disagreement. Diagnoses made by both the SP and RP were retained so that comparisons could be made in software-generated reports. During the peer review, findings were graded progressively for severity according to the scoring systems that were used in the original studies:

Study 1: no findings, mild, mild to moderate, moderate, or severe
Study 2: no findings, mild, moderate, or severe
Study 3: no findings, minimal, mild, moderate, marked, or severe.

Ultimately, the results of the peer review assessment helped the PWG chairperson to identify the types of diagnostic differences that the PWG panel would need to resolve.

2.5. PWG review of studies 1–3

The PWG was chaired by Jerry F. Hardisty, DVM, DACVP, FIATP, Experimental Pathology Laboratories, Inc. (EPL®), who organized and presented the material to the panel, and served as the author of the PWG report following the PWG review. Voting members of the independent PWG panel consisted of four pathologists who have expertise in the diagnosis of toxicologic effects in fish tissues, and these included Helmut Segner, PhD, Christine Ruehl-Fehlert, DVM, Klaus Weber, DVM, PhD, and Dr. Wolf (the RP). Dr. Weber was also the SP for Study 3. The two-day PWG was conducted at AnaPath GmbH, located in Oberbuchsiten, Switzerland on May 1–2, 2013.

The purpose of the two-day PWG was to review H&E-stained sections of kidney, liver, gills, and/or intestine from each of the three laboratory studies in trout to characterize spontaneous and potential exposure-related changes, if any, as they relate to findings that were reported by the SP and RP, and to confirm the no observed effect concentration (NOEC) of diclofenac based on histopathologic findings. The PWG was performed according to the United

Table 3
Slide selection criteria for each of the three studies evaluated in the pathology working group (PWG) review.

Study 1
Liver, kidney and gills from the first five fish in the control group
Liver, kidney and gills from the first five fish in the LOEC (5 μg/L) dose group
Liver, kidney and gills from the first five fish in the NOEC (1 μg/L) dose group
The subset above included slides representing differences of opinion in diagnosis between the reviewing pathologist's findings and the original findings reported by the study pathologist
Study 2
Kidney and intestine from the first five fish in the control group
Kidney and intestine from the first five fish in the LOEC (1 μg/L) dose group
Kidney and intestine from the first five fish in the NOEC (0.5 μg/L) dose group
Kidney was examined from additional animals when urinary kidney was not available from the first five fish
Because each of the three slides (A–C) for each organ consisted of 10–30 serial sections that were derived from a single paraffin block, only slide B was examined by the PWG
In order to confirm the incidence of goblet cell proliferation in the highest dose group (25 μg/L), sections of intestine were examined from all control and exposed fish
Study 3
Gills, kidney and liver from the first five fish in the control group replicate B and D
Gills, kidney and liver from the first five fish in the LOEC (1000 μg/L) dose group from replicates B and D
Gills, kidney and liver from the first five fish in the NOEC (320 μg/L) dose group from replicates B and D
This included slides representing differences of opinion in diagnosis between the reviewing pathologist's findings and the original findings reported by the study pathologist
Following the initial review of the above fish, all livers from all fish in each replicate for the controls, 320 μg/L dose group, and 1000 μg/L dose group were examined to determine the distribution of decreased glycogen among those groups

States Environmental Protection Agency (US EPA) Pesticide Regulation (PR) 94-5 (EPA Pesticide Regulation, 1994). At the onset of the PWG, Dr. Wolf presented an overview of the three studies to be reviewed, including photomicrographic figures from the original publications. Dr. Hardisty then presented the PWG approach and posed specific questions that were to be addressed by the panelists following microscopic examination of the slides.

The slides selected for evaluation included representative sections that were deemed necessary to determine the NOEC for each study and to resolve diagnostic differences between findings of the RP and the original diagnoses reported by the SPs. It was the responsibility of the PWG Chairperson to select slides for review by methods that strive to minimize potential bias, and slide selection criteria for each of the three studies are presented in Table 3. Label identifications on the selected slides were masked and coded by the non-voting Chairperson. Thus, when these materials were examined by the panelists, they had no knowledge of the diclofenac exposure status or previous findings associated with individual slides. This allowed the PWG to determine the nature of the changes without being influenced by awareness of the original diagnoses. Each participant recorded his/her diagnoses and comments on worksheets that were prepared by the PWG Chairperson.

At the end of the slide evaluation period, the PWG data were locked (i.e., no further changes to the data were permitted) and tabulated, and the results were revealed to the PWG participants. The participants were then asked to characterize the findings in each of the examined tissues, and to distinguish between spontaneous and exposure related changes. The PWG panelists were also asked to determine the NOEC for diclofenac in each of the three studies based on their findings. After the slides had been examined, the final PWG consensus diagnoses were recorded on the Chairperson's worksheets. Consensus diagnoses of the PWG were reached when the majority of the PWG participants were in agreement.

After the data were locked (i.e., could no longer be changed) group-wise differences in lesion prevalence between diclofenac treated fish and controls were confirmed using a 2-sided Fisher's exact test (Piegorsch and Bailer, 1997), with findings tested for significance at $p \leq 0.05$.

3. Results

The Pathology Working Group (PWG) is considered to be the arbiter of diagnostic differences between the study and reviewing pathologist; therefore, for simplicity only the results of the PWG will be described. These results represent the consensus opinions of the panel. By the conclusion of the two-day workshop, initial differences among the PWG panelists were resolved to the satisfaction of all four panel members.

3.1. PWG results for Study 1

The study pathologist's (SP's) original diagnoses for the gills included lamellar clubbing, hyperplasia (lamellar epithelial hyperplasia), mucous cell hyperplasia, secondary lamellar fusion, telangiectasia (angiectasis), and thickened (filament) tips; original diagnoses for the liver included diffuse cytoplasm (decreased glycogen), focal necrosis, foci of enhanced basophilia, interstitial proteinaceous fluid, monocyte accumulation (mononuclear cell infiltrates), scanty cytoplasm (increased glycogen), and sinusoidal distension; and original diagnoses for the posterior kidney included interstitial hyaline droplets, interstitial proteinaceous fluid, intraluminal/intratubular proteinaceous fluid, intravascular proteinaceous fluid, proteinaceous casts, and tubular necrosis. The overall quality of the histologic sections for Study 1 was judged by the PWG participants to vary from good (liver) to fair (kidney and gill). Kidney sections were often considered to be excessively thick.

Results of the PWG evaluation for Study 1 are compared to the original findings in Table 4. The most prevalent types of findings identified by the PWG occurred in the gills and included lamellar clubbing, lamellar epithelial hyperplasia, secondary lamellar fusion, telangiectasis, and thickened lamellar tips. The most frequent finding in the liver was the presence of mononuclear cell infiltrates, and in the kidney, interstitial hyaline droplets and interstitial proteinaceous fluid were recorded at the highest frequency. Regarding these latter two findings, the PWG panelists believed the interstitial hyaline droplets were probably an artifact of the histologic staining process, whereas the dark pink appearance of interstitial proteinaceous fluid (most of which actually appeared to be intravascular) was likely a function of excessive section thickness. The overall severity of these findings varied from mild to moderate, and none of these findings was substantially more prevalent or severe in diclofenac exposed fish when compared to unexposed controls.

There were several findings reported by the SP that were not observed to any degree by the PWG. For the liver, these included foci of enhanced basophilia, interstitial proteinaceous fluid, and sinusoidal distension. For the kidney, types of findings that were not confirmed by the PWG included proteinaceous casts and tubular necrosis.

The SP's original data did not report any diagnoses for any of the control group animals. However, there were many instances in which the PWG panel recorded findings for that group. Examples for the gill included lamellar clubbing, lamellar epithelial hyperplasia, secondary lamellar fusion, telangiectasis, and

Table 4
Prevalence and severity of Study 1 findings as determined by the study pathologist and the pathology working group (PWG).

		Study pathologist				PWG		
	Diclofenac concentration (μg/L)	0	0.5 (NOEC)	5 (LOEC)	50	0	0.5	5
	n[a]	*6*	*6*	*6*	*6*	*6*	*6*	*6*
Gills	Lamellar clubbing	0[b]	0	0	4	4	5	2
	Mild	–[c]	–	–	3	4	5	2
	Mild–moderate	–	–	–	1	–	–	–
	Lamellar epithelial hyperplasia	0	0	2	2	5	6	6
	Mild	–	–	2	2	4	3	2
	Mild–moderate	–	–	–	–	1	3	3
	Moderate	–	–	–	–	–	–	1
	Mucous cell hyperplasia	0	0	1	0	0	1	2
	Mild	–	–	1	–	–	1	1
	Mild–moderate	–	–	–	–	–	–	1
	Secondary lamellar fusion	0	0	1	2	4	4	4
	Mild	–	–	–	1	3	4	4
	Mild–moderate	–	–	1	1	1	–	–
	Telangiectasis	0	5	2	4	2	6	2
	Mild	–	2	–	–	2	3	–
	Mild–moderate	–	–	–	3	–	–	–
	Moderate	–	2	2	1	–	3	2
	Thickened filament tips	0	0	1	0	5	3	4
	Mild	–	–	1	–	3	2	1
	Mild–moderate	–	–	–	–	2	1	2
	Moderate	–	–	–	–	–	–	1
Liver	Decreased glycogen	0	1	2	1	4	2	2
	Mild	–	1	1	–	1	1	1
	Mild–moderate	–	–	1	1	3	1	1
	Focal necrosis	0	0	2	3	0	0	0
	Mild	–	–	1	3	–	–	–
	Mild–moderate	–	–	1	–	–	–	–
	Foci of enhanced basophilia	0	0	1	1	0	0	0
	Mild	–	–	1	1	–	–	–
	Increased glycogen	0	2	1	0	1	2	1
	Mild	–	2	1	–	1	2	1
	Interstitial proteinaceous fluid	0	0	1	1	0	0	0
	Mild	–	–	1	1	–	–	–
	Mononuclear cell infiltrates	0	2	5	4	5	6	5
	Mild	–	2	2	3	5	5	4
	Mild–moderate	–	–	3	1	–	1	1
	Sinusoidal distension	0	1	2	3	0	0	0
	Mild	–	–	2	2	–	–	–
	Mild–moderate	–	1	–	1	–	–	–
Trunk kidney	Interstitial hyaline droplets	0	5	2	3	3	5	2
	Mild	–	5	2	1	3	4	2
	Mild–moderate	–	–	–	2	–	1	–
	Interstitial proteinaceous fluid	0	0	3	5	2	3	3
	Mild	–	–	3	2	2	3	3
	Mild–moderate	–	–	–	2	–	–	–
	Moderate	–	–	–	1	–	–	–
	Intratubular proteinaceous fluid	0	0	1	1	0	0	1
	Mild	–	–	1	1	–	–	1
	Intravascular proteinaceous fluid	0	0	0	1	1	0	0
	Mild	–	–	–	1	1	–	–
	Proteinaceous casts	0	0	0	1	0	0	0
	Mild	–	–	–	1	–	–	–
	Tubular necrosis	0	0	0	3	0	0	0
	Mild–moderate	–	–	–	1	–	–	–
	Moderate	–	–	–	2	–	–	–

[a] Number of sections evaluated.
[b] Number of animals affected.
[c] A dash indicates that no animals were affected at that severity grade.

thickened lamellar tips. For the liver, these included increased glycogen and mononuclear cell infiltrates. And for the posterior kidney, diagnoses recorded by the PWG in control fish included interstitial hyaline droplets, interstitial proteinaceous fluid, and intravascular proteinaceous fluid.

Based on the results of the PWG evaluation, there were no findings for which the prevalence and/or severity was significantly increased in diclofenac-treated fish relative to controls. Thus, there were no exposure related findings for any of the three tissue types examined in Study 1.

3.2. PWG results for Study 2

According to the published results of Study 2, findings identified in the kidney included increased developing nephrons, loss of glomerular Bowman's space, and tubular necrosis. Findings in the intestines included fusion of villi (mucosal folds), hyperplasia of villi, and increased numbers and sizes of goblet cells.

The overall quality of the histologic sections for Study 2 was judged by the PWG participants to vary from fair (intestine) to poor (kidney). The H&E staining had faded substantially in both the

Table 5
Prevalence and severity of Study 2 findings as determined by the peer review pathologist and the Pathology Working Group (PWG).

Diclofenac concentration	0 μg/L	0.5 μg/L (NOEC)	1 μg/L (LOEC)	5 μg/L	25 μg/L	
Kidney	*n*[a]	*5*	*5*	*3*	*7*	*7*
	Developing nephrons	4[b]	4	3	nd[c]	nd[c]
	Mild	4	4	3		
	Loss of Bowman's space	5	5	3	7[d]	7[d]
	Mild	5	4	3	5	5
	Moderate	–[e]	1	–	2	2
Intestine	*n*	*9*	*10*	*10*	*8*	*13*
	Increased goblet cell size and/or number	3	3	2	2	7
	Mild	3	3	2	2	5
	Moderate	–	–	–	–	2

[a] Number of renal sections that contained urinary kidney.
[b] Number of animals affected.
[c] No diagnoses of increased developing nephrons were recorded by the reviewing pathologist for any fish, and fish in this dose group were not evaluated by the PWG.
[d] Findings in this dose group are based on results of the reviewing pathologist, as the PWG did not evaluate this group.
[e] A dash indicates that no animals were affected at that severity grade.

kidney and intestine sections, and the kidney sections were excessively thick and had microtomy chatter artifacts. Another issue relevant to the kidney sections involved the amount of urinary (mesonephric, posterior) kidney that was available for analysis. Slides from 20 of the 50 evaluated fish contained insufficient amounts of urinary kidney for diagnostic purposes. This included 10 fish for which no urinary kidney was available (i.e., sections consisted entirely of hematopoietic [anterior] kidney), and 10 fish for which only minimal amounts of urinary tissue (e.g., only a few tubules) were available. Because the original findings pertained only to the urinary elements of the kidney (i.e., glomeruli and tubules), these 20 fish were effectively excluded from further evaluation. There were eight additional animals for which the amount of urinary kidney present in the sections was considered suboptimal; however, diagnoses were recorded by the PWG for fish of this subset.

Results of the PWG evaluation for Study 2 are presented in Table 5. The prevalence and severity results recorded by the PWG cannot be compared directly to those of the SP, because the latter data were reported as mean severity scores per dose group (Mehinto et al., 2010).

During the peer review, the reviewing pathologist (RP) had not recorded any diagnoses of increased developing nephrons, because a low number (usually 1–2) of developing nephrons were present in most of the sections that contained sufficient amounts of urinary kidney, and these structures were not observed to be increased in any diclofenac-treated fish when compared to controls. However, the PWG opted to record the presence of developing nephrons as an absolute (versus relative) finding, and confirmed that, compared to controls, there were no increases in the prevalence and severity of developing nephrons in fish exposed at the NOEC and LOEC concentrations (i.e., 0.5 and 1 μg/L diclofenac). The PWG also did not find the loss of Bowman's space to be an exposure related finding. Throughout their evaluation, panelists observed that the size of this microanatomic cavity tended to vary considerably among glomeruli within the same kidney section, and that glomeruli with little or no apparent Bowman's space could be visualized in virtually every examined section of urinary kidney. However, the PWG did endeavor to evaluate the prevalence and severity of this finding, and the results of that blinded scoring did not provide evidence that diclofenac exposure was associated with a substantially greater prevalence or severity of collapsed Bowman's space. Finally, the PWG did not identify any instances of tubular necrosis in any of the sections that contained urinary kidney (nor was tubular necrosis observed by the RP during the peer review phase).

Concerning the intestines, the PWG did not find any morphologic evidence of fused villi or hyperplasia of villi during the microscopic review of intestine sections. Based on depictions in photomicrographic figures from the associated publication (Mehinto et al., 2010), the panelists concluded that these findings were plane-of-section artifacts. The mucosal surface of the trout intestine actually consists of short folds rather than true finger-like villi (Dale et al., 2009). Evidence from the photomicrographs suggested that tangential sectioning across the edges of folds was responsible for the appearance of mucosal epithelial hypercellularity, whereas oblique sectioning through the broad base of a single fold created the appearance of two villi that were fused at the tips. However, the PWG did observe a tendency for greater prevalence and severity of increased goblet cell size and/or number in the intestines of fish in the 25 μg/L dose group as compared to controls, although the difference in prevalence was not statistically significant ($p = 0.3050$). Panelists also questioned whether this slight variation in goblet cell size/number may have been a function of sample collection, as goblet cell numbers are known to vary progressively along the length of the trout intestine (Anderson and Mitchum, 1974); Yasutake and Wales, 1983; Khojasteh et al., 2009).

Based on the results of the PWG evaluation, there were no findings for which the prevalence and/or severity was significantly increased in diclofenac-treated fish relative to controls. Thus, there were no exposure related findings for any of the three tissue types examined in Study 2.

3.3. PWG results for Study 3

The SP's original diagnoses for the gills included angiectasis, focal proliferation of interlamellar cells, focal proliferation of chloride cells, thickened lamellar tips, lamellar fusion, single cell necrosis of interlamellar cells, inflammation, and focal mononuclear cells. Original diagnoses for the liver included inflammatory cell foci and enhanced basophilia, whereas original diagnoses for the kidney included (single cell) necrosis and the presence of hyaline deposits/droplets.

The overall quality of the histologic sections for Study 3 was judged by the PWG participants to be very good.

Results of the PWG evaluation for Study 3 are presented in Table 6. Two findings that the PWG identified as being more prevalent in the exposure groups than in the control group were thickened lamellar tips in the gills and decreased glycogen in the liver. Thickened lamellar tips of the gills were characterized by broadening of the distal end of the filaments secondary to focal epithelial hyperplasia. The severity of thickened lamellar tips was graded as minimal to mild, and the prevalence of this finding was only significantly increased at the highest diclofenac concentration (1000 μg/L; $p = 0.0457$). Decreased glycogen was diagnosed when there was a generalized reduction in the amount of translucent space within the cytoplasm of hepatocytes, and both

Table 6
Prevalence and severity of Study 3 findings as determined by the study pathologist and the pathology working group (PWG).

	Diclofenac concentration (μg/L)	Study pathologist							PWG		
		0	3.2	10	32	100	320 (NOEC)	1000 (LOEC)	0	320	1000
Gills	n[a]	*20*	*20*	*20*	*20*	*20*	*20*	*20*	*20*	*20*	*20*
	Angiectasis[b]	3[c]	9	9	9	10	5	11	0	0	0
	Minimal	3	5	1	–[d]	1	–	4	–	–	–
	Slight	–	4	8	5	5	4	4	–	–	–
	Moderate	–	–	–	4	4	1	3	–	–	–
	Focal proliferation of interlamellar cells/interlamellar cell hyperplasia	10	6	5	9	4	7	13	6	6	6
	Minimal	10	5	5	9	4	7	11	6	6	5
	Slight	–	1	–	–	–	–	2	–	–	1
	Focal proliferation of chloride cells	0	0	0	0	1	0	4	0	1	3
	Minimal	–	–	–	–	1	–	3	–	1	3
	Slight	–	–	–	–	–	–	1	–	–	–
	Inflammation	1	0	0	1	0	0	1	0	0	0
	Minimal	1	–	–	1	–	–	1	–	–	–
	Lamellar epithelial lifting	0	0	0	0	0	0	0	14	16	15
	Minimal	–	–	–	–	–	–	–	7	10	7
	Slight	–	–	–	–	–	–	–	6	5	5
	Moderate	–	–	–	–	–	–	–	1	1	3
	Lamellar fusion	0	0	1	0	0	0	0	0	0	0
	Minimal	–	–	1	–	–	–	–	–	–	–
	Mononuclear cells, focal	5	2	6	6	1	2	7	1	0	3
	Mild	5	2	6	6	1	2	6	1	–	2
	Mild–moderate	–	–	–	–	–	–	1	–	–	1
	Single cell necrosis of interlamellar cells	1	0	0	0	0	0	0	1	0	0
	Mild	1	–	–	–	–	–	–	1	–	–
	Thickened lamellar tips	0	0	0	3	1	0	4	1	1	6*
	Minimal	–	–	–	3	1	–	3	1	1	2
	Slight	–	–	–	–	–	–	1	–	–	1
Liver	*n*	*20*	*20*	*20*	*20*	*19*	*20*	*20*	*20*	*20*	*20*
	Enhanced Basophilia	0	0	0	0	0	0	1	0	0	0
	Minimal	–	–	–	–	–	–	1	–	–	–
	Decreased glycogen	0	0	0	0	0	0	0	2	4	10**
	Minimal	–	–	–	–	–	–	–	2	4	3
	Slight	–	–	–	–	–	–	–	–	–	1
	Marked	–	–	–	–	–	–	–	–	–	5
	Severe	–	–	–	–	–	–	–	–	–	1
	Inflammatory cell foci	1	1	2	0	2	3	1	2	3	1
	Minimal	1	1	2	–	2	3	1	2	3	1
Kidney	*n*	*17*	*16*	*20*	*20*	*20*	*19*	*19*	*17*	*19*	*19*
	Granular casts	0	0	0	0	0	0	0	0	0	1
	Minimal	–	–	–	–	–	–	–	–	–	1
	Hyaline inclusions/droplets	2	4	6	2	2	3	6	7	6	8
	Minimal	2	4	6	2	2	3	6	7	6	8
	Single cell necrosis	0	1	2	2	1	0	2	1	1	4
	Minimal	–	1	2	2	1	–	2	1	1	4

* $p \leq 0.05$.
** $p \leq 0.01$.
[a] Number of sections evaluated.
[b] Original diagnoses of angiectasis were revised to lamellar epithelial lifting by the PWG.
[c] Number of animals affected.
[d] A dash indicates that no animals were affected at that severity grade.

the prevalence and severity of this finding were increased relative to controls in the 1000 μg/L diclofenac dose group ($p = 0.0069$). Other findings were not substantially more prevalent or severe in diclofenac exposed fish relative to controls.

The PWG observed that initial diagnoses of angiectasis were more appropriately categorized as lamellar epithelial lifting, because these changes were characterized by expansion of the interstitial space between the lamellar capillary and pavement cell epithelium, rather than a dilation of the vascular compartment of the capillaries themselves. The panelists determined that this finding was unrelated to diclofenac exposure.

Based on the results of the PWG evaluation, there were two findings for which the prevalence and/or severity was significantly increased in diclofenac-treated fish relative to controls. These findings, which occurred in the 1000 μg/L diclofenac dose group, were thickened lamellar tips in the gills, and decreased glycogen in the liver.

4. Discussion

There are a number of reasons why histopathology is an invaluable tool for the identification of potential adverse effects in toxicological bioassays. First, a wide variety of homeostatic disturbances can be observed at the cellular, organ system, and whole organism levels. Second, unlike many other types of assays, histopathologic studies can be designed to incorporate a full range of toxicologic, toxicokinetic, and biotransformative effects. Finally, histopathology has the ability to furnish mechanistic clues that are unavailable from other endpoints. However, it is well understood that the interpretation of morphologic changes in tissue sections is an inherently subjective exercise. Consequently, pathology peer review and PWG procedures were developed to enhance the objectivity of the histopathology endpoint for non-clinical toxicologic bioassays. Over the past 30 years, this process has been implemented and refined by the National Toxicology Program of the

National Institute of Environmental Health Sciences, U.S. Department of Health and Human Services (Boorman et al., 2002), and it has gradually become the industry-wide standard for assuring the quality of histopathology data (Mann and Hardisty, 2013). Although application of the pathology peer review/PWG model to environmental toxicological studies has thus far been limited, this is not entirely without precedence. For example, pathology peer review was used to confirm histopathologic findings in an experiment involving the effects of 17 beta-estradiol on *Xenopus laevis* gonads (Wolf et al., 2010), and the PWG format was employed to develop consensus diagnostic criteria for assessing toxicologically induced liver lesions in medaka fish (Boorman et al., 1997).

It is important to distinguish pathology peer review from journal peer review. While it is accepted that journal peer review is an essential element for improving the overall quality of scientific articles, the ability of journal peer review to confirm the accuracy of histopathology data is limited, because the actual tissue sections are never examined as part of that process. Instead, journal peer review relies on evaluation of printed text descriptions and published photomicrographic figures, which may or may not represent the complete spectrum of the histopathologic results, and on the expertise of the journal reviewers, who may or may not have extensive histopathology experience.

The current project involved an independent blinded review by several highly experienced toxicologic pathologists of the original histologic slides from three studies of diclofenac in trout, among which there were substantive differences in the original histopathologic findings. This PWG review identified key areas of concern for each of those three studies. For Study 1, the most toxicologically significant issue in the published paper was the complete absence of diagnoses for each of the six control group fish. This outcome by itself was considered unusual, given the fact that many types of findings diagnosed by the SP (such as lamellar epithelial hyperplasia) would be anticipated to occur to at least some degree in untreated fish. Because the original data recording sheets were no longer retrievable for Study 1, the lack of recorded findings in control fish could not be verified via documentation. Once the PWG panel established that findings such as lamellar clubbing in the gills and mononuclear cell infiltrates in the liver actually occurred to a similar extent in both control and diclofenac-exposed fish, it became clear that the presence of these lesions was entirely unrelated to diclofenac exposure. The situation for Study 2 was somewhat different from that of Study 1. In Study 2, the PWG disagreed with several of the SP's original diagnostic interpretations. For example, diagnoses of renal tubular necrosis, fusion of intestinal villi, and hyperplasia of intestinal villi were not observed to any degree by the PWG panel, whereas the prevalences of other findings, such as increased developing nephrons, loss of Bowman's space, and goblet cell size/numbers, were found to be comparable in diclofenac exposed fish and controls. The results of Study 3 were further different from those of Studies 1 and 2. In Study 3, the PWG identified decreased hepatic glycogen as a treatment-related finding that was not previously reported, while confirming the existence of another treatment-related finding: thickened lamellar tips of the gills. These examples demonstrate the value of the peer review/PWG process, as it is unlikely that any of the aforementioned issues could have been discovered and assessed by means other than through direct slide review.

As a result of the PWG evaluation, findings that were found to be significantly more prevalent in diclofenac exposed trout relative to controls included increased thickened lamellar tips in the gills and decreased glycogen in the liver in Study 3. Both of these findings were only significant at the highest concentration tested (1000 μg/L). The gill lesions, which were graded as minimal to slight, would not be expected to impact the functionality of the gills under normal circumstances, because only the distal segments of the filaments were affected (Speare and Ferguson, 2006). Due to the overlapping conformation of the gill arches, the distal tip of the filament is the area of the gill that is most exposed to the environment, and in salmonids, thickening of the filament tip is thought to be a non-specific long-term response to chronic irritation or injury (Speare and Ferguson, 2006). Similarly, decreased liver glycogen storage, which is presumed to be reversible, would not necessarily impair survival at the individual or population level. Decreased hepatic glycogen can be a direct response to toxic insult in some cases, but often this change is a consequence of negative energy balance due to treatment-induced inanition or physiological stress (Wolf and Wolfe, 2005). However, the published account of Study 3 (Memmert et al., 2013) reported no behavioral abnormalities or significant differences in growth among diclofenac-treated trout as compared to negative control fish.

Diagnoses of thickened lamellar tips in the gills and decreased glycogen in the liver were not reported in the study conducted by Schwaiger et al., 2004 (see Table 2). Conversely, findings from Schwaiger et al. (2004) that were not at all evident in the three trout studies evaluated by the PWG included pillar cell necrosis and respiratory epithelial cell necrosis in the gill, and interstitial nephritis in the kidney. Telangiectasis (angiectasis) of the gills, which refers to aneurysmal dilation of one or more lamellar capillaries, was reportedly related to diclofenac administration in Schwaiger et al. (2004); however, this finding was observed but was not associated with diclofenac exposure in Study 1 (Hoeger et al., 2005), and was ultimately shown to not be present at all in Study 3 (Memmert at al., 2013), despite the thousand-fold higher top concentration of diclofenac used in that study (i.e., 1000 μg/L versus 1 μg/L). Because telangiectatic lesions tend to resolve via lamellar thrombosis (Speare and Ferguson, 2006), observations of telangiectasis without thrombosis suggest that such changes probably occurred at some point in the perimortem period, possibly as a consequence of handling during the euthanasia process (Speare and Ferguson, 2006). Another reported effect of diclofenac administration in Schwaiger et al. (2004) was severe tubular hyaline droplet degeneration. Even though hyaline inclusions were observed occasionally as an incidental finding in renal tubular epithelial cells of both control and diclofenac-exposed trout Study 3, the severity of this alteration was never graded higher than minimal by either the SP or the PWG panel. Furthermore, while it is understood that hyaline droplets in the kidney can be toxicologically induced, Reimschuessel and Ferguson (2006) noted that it is also possible to observe numerous hyaline droplets in the proximal renal tubules of normal fish as a function of varying husbandry conditions, and thus they urged caution in the interpretation of that particular finding.

5. Conclusions

This outcome of this project serves to illustrate the utility of the peer review/PWG process. Although it was discovered that some findings among the three studies were truly different (e.g., the presence of telangiectasis in Study 1 but not Study 3), potentially as a function of differing experimental designs, the PWG revealed that much of the interstudy variation was related to issues of diagnostic interpretation. The results of the PWG review were also instrumental for determining appropriate NOEC levels. Based on the PWG findings, the NOEC for each of the three studies was as follows: Study 1: 50 μg/L (the highest concentration tested); Study 2: 25 μg/L (the highest concentration tested); and Study 3: 320 μg/L (the next to highest concentration tested). Consequently, the effective overall NOEC for diclofenac based on the three studies analyzed by the PWG was 320 μg/L.

The potential toxicological and ecological impacts of a test substance cannot be determined by the results of a single endpoint. Histopathology is most effective when integrated

with other in-vivo and in-vitro methodological approaches (e.g., ultrastructural, morphometric, molecular, chemical, biochemical, physiological, and/or epidemiological investigations). However, whichever approach is chosen to address a specific scientific question, it is vital to demonstrate that the results produced by each individual assay are accurate and reliable.

The peer review/PWG paradigm provides a transparent, efficient, and effective method for assuring the accuracy, consistency, and objectivity of histopathology findings. While this model has become standard practice in mammalian toxicologic pathology, to date pathology peer review has been underutilized in ecotoxicological research. However, this situation is anticipated to change as concern about environmental issues continues to increase, and the need for objective scientific data becomes more apparent. Once environmental scientists become more familiar with the peer review/PWG approach, it will be recognized that investigators who are willing to subject their results to this degree of scrutiny display the highest levels of professionalism and scientific integrity. More importantly, it will become evident that confidence in reliability of the data, as inspired by the pathology peer review/PWG process, is of vital consequence to regulatory agencies as they endeavor to incorporate study results in a weight-of-evidence approach to risk assessment.

Conflict of interest statement

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