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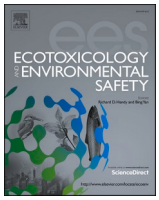

Effect of waterborne exposure to perfluorooctanoic acid on nephron and renal hemopoietic tissue of common carp *Cyprinus carpio*

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ABSTRACT

Per- and poly-fluoroalkyl substances (PFAS) are synthetic contaminants of global concern for environmental and public health. Perfluorooctanoic acid (PFOA) is an important PFAS, and considerable attention has been paid to its hepatotoxicity and reproductive and developmental impact, while potential nephrotoxic effects are largely ignored, especially in fish. This study documents the structural and ultrastructural effects on kidney of common carp *Cyprinus carpio* exposed to waterborne PFOA at an environmentally relevant concentration of 200 ng L^{-1} and at 2 mg L^{-1}. Dilation of the glomeruli capillary bed, increased vesiculation in the proximal tubular segment, compromised mitochondria, apical blebbing, and sloughing of collecting duct cells occurred in exposed fish, primarily at 2 mg L^{-1}. Perfluorooctanoic acid exposure resulted in higher numbers of rodlet cells (RC), putative immune cells exclusive to fish, mainly in the renal interstitium, than seen in controls, increased association with cells of myeloid lineage and modifications to ultrastructure. No differences in other cells of innate immunity were observed.

Despite the absence of severe histological lesions, PFOA was shown to affect both nephron and hemopoietic interstitium at high concentration, raising concern of the impact on renal and immune function in fish. The response of RCs to PFOA concentration of 200 ng L^{-1} suggests a potential role as a biomarker of PFOA exposure.

1. Introduction

Perfluorooctanoic acid (PFOA) belongs to a large and heterogeneous class of chemicals (> 4700), called per- and poly-fluoroalkyl substances (PFAS), that have been released into the environment since the late 1940s (Wang et al., 2017). Per- and poly-fluoroalkyl substances, called 'forever chemicals' were developed to be resistant to degradation and, because of properties such as water and oil repellence and high surface activity, have been employed in a wide range of consumer and industrial products (Wang et al., 2017). The common feature of PFAS is a partially or fully fluorinated carbon chain with the extremely strong C-F bonds conferring high stability and biological, chemical and physical inertness (Alexander et al., 2008; Cousins et al., 2020). Due to their amphiphilic nature and proteinophilia, PFAS have mobility in aquatic environments and a high affinity to liver and plasma proteins (Alexander et al., 2008). Some PFAS, especially the long-chain ones, can bioaccumulate along the food webs (Cousins et al., 2020). Only recently have PFOA and several other PFAS been subject to regulatory measures (inclusion in the Stockholm Convention on Persistent Organic Pollutants and restriction under REACH), based on their ubiquity and growing evidence of the ecological and health risks (Ankley et al., 2021).

Perfluorooctanoic acid, one of the most representative PFAS, is a perfluoroalkyl carboxylic acid [$CF_3(CF_2)_6COOH$] having a linear chain of eight carbon (long-chain). It is generally completely dissociate in water and shows a high water solubility, very low vapour pressure, and moderate sorption to solids (Alexander et al., 2008). Of primary concern is PFOA persistence in the environment and ability to accumulate in biota and alter endocrine, reproductive, and immune systems (Lee et al., 2020; Vierke et al., 2012; Zhang et al., 2021). Recently the European Food Safety Authority (EFSA) underlined that PFAS effects on the immune system are the most critical for risk assessment (Schrenk et al., 2020). Perfluorooctanoic acid can affect innate and adaptive immune responses, cytokine secretion, and inflammatory reactions in different animals (Stein et al., 2016) and its immunotoxicity mechanisms have recently been reviewed (Liang et al., 2022). Per-poly-fluoroalkyl substance pollution shows particular impact on aquatic ecosystems, with

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exposure of aquatic organisms and the terrestrial animals and humans that consume contaminated water and prey (Falk et al., 2015). Data for PFAS, especially PFOA and perfluorooctane sulfonate (PFOS), toxicity to aquatic organisms is considerable. A review of the potential effects in both invertebrates and vertebrates is provided by Lee et al. (2020) and Ankley et al. (2021). Most fish studies have been conducted in cyprinids, especially zebrafish *Danio rerio* (Ankley et al., 2021). *Cyprinus carpio*, widespread in freshwater ecosystems worldwide, could be a better model of PFOA impact on natural fish populations. This species is easy to obtain from fish farms and to maintain in captivity, represents an important food source, is abundant and plays a crucial role in aquatic environments as 'ecological engineer' (Rahman, 2015). Common carp is recommended for evaluation of emerging pollutants and has already been used successfully both for field and experimental studies on PFAS (Kim et al., 2010; Ye et al., 2008).

The liver is a major target of PFOA and much information is available on hepatic accumulation and responses at biochemical, genetic, cellular, and histological levels in aquatic organisms (Lee et al., 2020). In contrast, renal effects are poorly documented, although the kidney can accumulate high concentrations of PFOA and plays a primary role in its elimination in fish as in other vertebrates (Consoer et al., 2014; Falk et al., 2015). In rainbow trout, 24 h after injection of PFOA at 1.0 mg kg^{-1}, at steady-state concentration, PFOA tissue distribution was reported to be blood plasma > liver > kidney > muscle (Consoer et al., 2014). In rainbow trout exposed to PFAS in feed for 28 days, liver, blood, and kidney were identified as the primary tissues for PFOA storage (Goeritz et al., 2013). Chen et al. (2021) studied PFAS tissue distribution in fish, including common carp from a Chinese lake, and reported liver, followed by kidney, gill, intestine, and muscle as sites of accumulation.

The mesonephros, as the adult fish kidney, contains nephrons (each composed of a glomerulus and different tubular segments) and hemopoietic (erythropoietic, leukopoietic, thrombopoietic) tissue thus having both excretory and immunological functions (Zapata et al., 2006). As a consequence, it offers the opportunity to explore both nephrotoxicity and immunotoxicity (Zapata et al., 2006).

In vitro studies of mammalian cells suggest how PFAS can impact renal function (Gong et al., 2019; Qian et al., 2010). Perfluorooctane sulfonate causes oxidant stress and increased permeability of human kidney endothelial cells that are essential for structure and function of the glomeruli (Qian et al., 2010). Perfluorooctanoic acid impairs amino acid and purine metabolism in rat mesangial cells, inducing inflammation and enhancing kidney injury under diabetic conditions (Gong et al., 2019). Epigenetic alterations of various markers of fibroblast activation were reported in kidney tissues of mice dosed with PFOA (Rashid et al., 2020). In animal models, the structural renal damage associated with PFAS is largely ignored, with only a single published report dealing with histological observations of rat exposed to PFOS and PFOA (Cui et al., 2009). To the best of our knowledge, there have been no reports of kidney structure and ultrastructure in fish exposed to PFAS.

Given this gap and the crucial importance of electron microscopy in assessing toxicity (Grover et al., 2021), we aimed to determine the effect of waterborne exposure to PFOA on nephron and renal hemopoietic tissue of common carp, especially with regard to rodlet cell (RC) response. Rodlet cells are putative immune cells unique to fish (Manera and Dezfuli, 2004; Mazon et al., 2007; Reite, 2005) and are known to respond to exposure of a wide range of pollutants, making them a candidate for biomarker of exposure (Manera and Dezfuli, 2004).

2. Materials and methods

2.1. Experimental design

Kidney samples were obtained from a previously conducted experiment described in Giari et al. (2016). Thirty-one common carp (stage/age: adults two-year-old; total length ± SD 19.32 ± 2.49 cm; weight ± SD 104.84 ± 27.80 g) were obtained from a local fish farm (Azienda Ittica Ferioli, Cento, Ferrara). During both the four-week acclimation period and the exposure test fish were maintained at a photoperiod of 14: 10 h light: dark and fed manually with a commercial pellet food (Tetra Pond Pellets Mini, Tetra, Melle, Germany) three times per week.

Fish were divided into three groups: an unexposed control (n = 10) and groups exposed to 200 ng L^{-1} PFOA (n = 10), and to 2 mg L^{-1} (n = 11). Each group was placed into a 120 L glass aquarium filled with tap water and received a continuous supply of fresh water (flow-through rate of 500 mL min^{-1}). Stock solutions of PFOA were continuously dispensed (open system) through a peristaltic pump in the two treatment aquaria to deliver the tested concentrations. The dose of 200 ng L^{-1} was chosen based on PFOA reports in surface water (Loos et al., 2008) to represent an environmentally relevant concentration. The dose of 2 mg L^{-1} was chosen based on data of experimental exposures that lead to a histological response in cyprinid fish (Wei et al., 2007). In each aquarium water temperature, pH, and oxygen saturation were measured three times weekly and resulted 10–15 °C, 6.70–8.00, and > 80% respectively.

After a sub-chronic exposure of 56 days, carp were killed by anaesthesia with MS-222 followed by severing the spinal cord and necropsied.

2.2. Chemical analysis

PFOA quantification in kidney samples was conducted following an analytical method detailed in (Giari et al., 2015, 2016) and summarised below. PFOA was extracted using an ion-pairing extraction procedure and measured using high performance liquid chromatography with electrospray ionisation tandem mass spectrometry. About 1 g of sample was homogenised with 5 mL of ultrapure water supplied by a Milli-Q system from Millipore (Watford, UK). One mL of 0.5 M tetrabutylammonium (TBA) hydrogen sulphate (Sigma Aldrich, St. Louis, MO) solution and 2 mL of sodium carbonate buffer (Sigma Aldrich, St. Louis, MO) (0.25 M, pH 10) were added to 1 mL of the homogenate samples in a polypropylene tube and thoroughly mixed for extraction. Five mL of methyl tert-butyl ether (MTBE) (Sigma Aldrich, St. Louis, MO) were added to the above mixture and shaken for 20 min. The organic and aqueous layers were separated by centrifugation, and an exact volume of MTBE (4 mL) was removed from the solution. The aqueous mixture was rinsed with MTBE and separated twice; both the rinses were combined in a second polypropylene tube. The solvent was evaporated under nitrogen and replaced with 0.5 mL of HPLC grade methanol (Sigma Aldrich, St. Louis, MO). This extract was passed through a nylon filter (0.2 lm, Supelco, Bellefonte, PA) into an HPLC vial. Extraction blanks were prepared using Milli-Q water. Analytes separation was performed using a Finnigan Surveyor Plus HPLC System, consisting of a quaternary pump, vacuum degasser, and autosampler. Chromatographic separation was achieved using a Betasil C18 column supplied by Thermo Electron Corporation, San Jose, CA. For quantitative determination, the HPLC system was interfaced to a Finnigan LTQ linear ion trap mass spectrometer (Thermo Electron Corporation, San Jose, CA) operating in negative electrospray mode. Instrumental parameters were optimised to transmit the [M–H]- ions for the analytes. Primary and product ions monitored for PFOA determinations were 498.8 > 368.9. Ten mL of each extract were injected in the LC–MS with 2 mM LC–MS grade ammonium acetate (> 99%), from CNW, Dusseldorf, Germany/HPLC grade methanol (Sigma Aldrich, St. Louis, MO) as the mobile phase, starting at 10% methanol, increasing to 95% methanol at 10 min before reverting to original conditions at 15 min. Standards for the five-point calibration curve were prepared by progressive dilution with methanol from a neat standard purchased from Dr. Ehrenstorfer (Augsburg, Germany) and concentrations were evaluated in comparison to this unextracted standard curve and were not corrected for the recoveries or for the purity of the standard (more than 98%). Stock solution of the analyte was prepared in methanol and stored in polypropylene bottles.

Teflon coated labware were avoided during the whole process of sampling, pre-treatment and analysis to minimise contamination of the

samples. Data quality assurance and quality control protocols included matrix spikes, laboratory blanks, and continuing calibration verification. Blanks were analysed with each set of five tissue samples as a check for possible laboratory contamination and interferences; recoveries, assessed using spiked matrix with a concentration of 5 ng g^{-1}, were over 89%. The reproducibility was also determined and expressed as the relative standard deviation (RSD) for each concentration level (PFOA 1.9%). The level of detection (LOD), determined as three times the signal-to-noise (S/N) ratio, was 0.4 ng g^{-1} wet weight.

2.3. Light and transmission electron microscopy

For morphological analysis kidney pieces were fixed in 2.5% glutaraldehyde buffered with sodium cacodylate (pH 7.3) at 4 °C for 3 h, post-fixed in 1% osmium tetroxide for 2 h, dehydrated in a graded series of acetone, and embedded in epoxy resin (Durcupan™ ACM, Fluka, Sigma-Aldrich, USA).

Semithin sections (1.5 μm) of embedded kidney fragments were cut, stained with toluidine blue, and examined and photographed using a Nikon Eclipse 80i light microscope (Nikon, Tokyo, Japan) equipped with a digital camera.

Ultrathin sections (90 nm) were contrasted with uranyl acetate and lead citrate and examined under a Hitachi H-800 transmission electron microscope (Hitachi Ltd, Tokyo, Japan).

2.4. Rodlet cell biometry

Five semithin sections from five carp of each exposure group were screened for rodlet cells (RCs) via light microscopy at 400 × magnification using image analysis software (Nis Elements AR 3.0). In each screened microscopic field (64,000 μm^2; mean of 29 fields in each screened section; mean of 1.86 mm^2 of renal tissue screened), the total number of RCs and the number of RCs in hemopoietic tissue, lining sinusoids, in renal tubules, and in collecting ducts were counted. Each tissue section was examined without knowledge of the exposure group and screened in a zig-zag trajectory, avoiding duplicate RC counts, empty spaces, and non-homogeneous tissue, to ensure replicability.

2.5. Statistical analysis

The differences among experimental groups in total number of RCs and the number of RCs in each tissue class were quantified. Because data were not normally distributed, a nonparametric test (Kruskal-Wallis and Dunn as pairwise test) was adopted. Data distribution was tested according to discrete distribution models. JAMOVI (JAMOVI 2.2.2, JAMOVI project) and JASP (JASP 0.15; JASP Team) were used as statistical package for analysing differences and data distribution, respectively.

3. Results

3.1. PFOA tissue concentrations

Concentrations of PFOA in carp organs have been reported previously in Giari et al. (2016). Both in unexposed control fish and fish exposed to 200 ng L^{-1}, PFOA tissue concentrations were under the LOD (0.4 ng g^{-1}). In kidney of carp exposed to the highest dose (2 mg L^{-1}) PFOA was detected at a mean concentration ± standard deviation of 1.08 ± 0.54 ng g^{-1} wet weight.

3.2. Light microscopy

The histoarchitecture of kidney of unexposed carp was typical of piscine mesonephros, with a complete excretory nephron (renal corpuscle, proximal and distal tubular segments, and collecting ducts) and hemopoietic interstitium with erythroid and cells of myeloid lineage prominent (Fig. 1A–D). Typical mature rodlet cells were detected, mainly within hemopoietic tissue (Fig. 1B, D). Perfluorooctanoic acid exposure affected both excretory and hemopoietic kidney compartments. In exposed fish, the glomerular capillary bed was dilated and did not display the normal folding pattern seen in controls (Fig. 2A–B). Morphological evidence of efferent glomerular arteriole contraction was detected at 2 mg L^{-1} (Fig. 2B). Cells from the first proximal tubular segment showed increased apical cytoplasm vesiculation, mainly at 2 mg L^{-1}. At 2 mg L^{-1}, cells of the second proximal tubular segment were less affected compared to the first proximal tubular segment. The distal tubular segment was unaffected by PFOA exposure. Slight vesiculation was observed in main cells of the collecting ducts in fish exposed to 2 mg L^{-1}, along with apical blebbing and sloughing in the tubular lumen (Fig. 2C–D). Increased occurrence of RCs in microscope fields of PFOA-exposed fish was the most notable observation, with up to 32 cells in a single field at 2 mg L^{-1}. In exposed fish, RCs occurred in clusters, mainly in proximity to cells of myeloid lineage (Fig. 2A–C).

3.3. Rodlet cell biometry

The group exposed to PFOA at 2 mg L^{-1} showed significantly higher numbers of rodlet cells than observed in the environmentally relevant dose group, and significant higher numbers were seen in both exposures compared to controls (Table 1; Fig. 3). The rodlet cell tissue distribution fits a negative binomial model, in particular the zero-inflated variant, for all experimental groups, implying that RCs are not randomly distributed within the tissue as would be the case with a Poisson distribution (Fig. 4). Effectively, the pattern reflects the distribution of RCs in hemopoietic tissue, which was the most frequent RC-occurrence site in the screened fields.

3.4. Transmission electron microscopy

Cells from first proximal tubular segment showed an increase in both number and volume of vesiculations with content appearing more flocculent and electron-dense than in unexposed fish, to a greater extent at 2 mg L^{-1}. Mitochondria exhibited no (200 ng L^{-1}) or incipient vesiculation (at 2 mg L^{-1}), reflecting enhanced pinocytosis of glomerular ultrafiltrate leading to accumulation of vesiculations in cytoplasm (athrocytosis) rather than an energy/metabolic disturbance (Fig. 5A–B). Cells of the second proximal tubular segment showed a mild increase in cytoplasm vesiculation in terms of occurrence and diameter at the highest PFOA dose, with some mitochondria showing scattered focal vesiculation (Fig. 5C). Cells of the distal tubular segment in PFOA-exposed groups did not differ from controls; whereas cells, particularly the main/clear cells, from collecting ducts showed focal vesiculation, spotted matrix rarefaction of mitochondria, and sloughing (Figs. 5D, 6A), at the higher dose. Rodlet cells displayed the typical key ultrastructure features of their mature stage: a polar, pear-shaped form with a large circular to oval nucleus at the expanded base; a fibrillar subplasmalemma layer; markedly vesiculated cytoplasm; typical rod-shaped inclusions displaying an amorphous outer rodlet sac, and a more electron-dense core converging at the cell apex where the fibrillar layer became thinner facilitating cell content expulsion. They were in close proximity to thin, elongated stromal reticular cells and myeloid lineage cells, particularly neutrophil granulocytes at various differentiation stages and, less likely, a putative basophil granulocyte precursor. Macrophages and erythroid lineage cells were also observed in proximity. Perfluorooctanoic acid exposure caused an apparent increased association of RCs with myeloid lineage cells (Fig. 6B, C). Rodlet cells of PFOA-exposed fish showed increased cytoplasm vesiculation, rodlet sac dissolution, and evidence of discharge of their contents into the interstitium (Figs. 5D, 6A, B, D). All the above reported PFOA-induced RC modifications were dose-dependent.

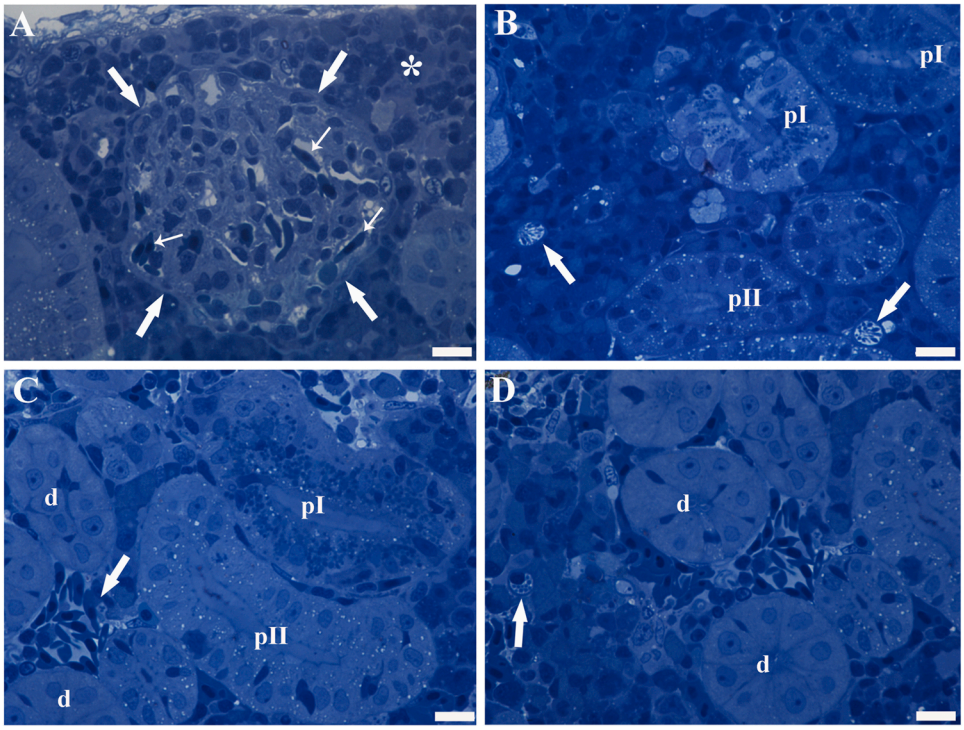

Fig. 1. Semithin sections of unexposed carp kidney. Toluidine blue. Scale bar = 10 μm. (A) Normal renal corpuscle (thick arrow) encircled by hemopoietic tissue (asterisk). Erythrocytes (thin arrows) within the lumen of glomerular capillary bed are visible. (B) First and second proximal tubular segments with prominent brush border and small cytoplasm vesiculations visible. Lysosomes (small, intensively blue-stained granules) are present only in the first proximal segments. Two RCs (thick arrows) are visible within the hemopoietic tissue. (C) First (pI) and second (pII) proximal tubular segments, distal tubular segments (d), and sinusoid (thick arrow) lined by tubules. (D) The absence of cytoplasm vesiculations and of a prominent brush border is a distinctive feature of the distal tubular segments (d) from proximal tubular segments. A rodlet cell (thick arrow) in closed proximity to erythroid lineage cells is present.

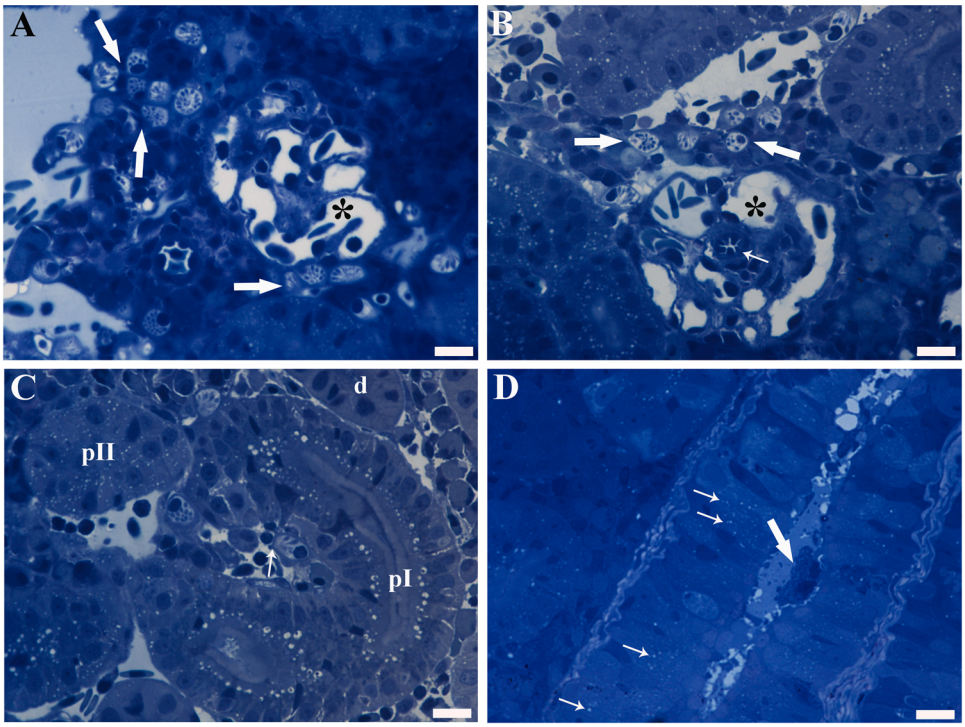

Fig. 2. Semithin kidney sections of carp exposed to 2.0 mg L^{-1} PFOA. Toluidine blue. Scale bar = 10 μm. (A) and (B) Glomerular capillary bed is considerably dilated (asterisks), showing loss of folding pattern. Efferent glomerular arteriole (thin arrow) contraction is evident in (B). Higher number of RCs than in controls, mainly in the hemopoietic tissue (A), also lining sinusoids (A, B). Clusters of RCs (thick arrows) visible (B). (C) Enhanced cytoplasm vesiculation mainly in the first (pI), and slightly in the second (pII), proximal segments. In the first proximal segment, vesiculations are larger than in controls and toluidine-positive spots may occur. Cells of distal tubular segments (d) show no evident alterations. Two RCs lining a sinusoid are visible, one displaying apical protrusion (thin arrow) as a possible sign of incipient discharging activity. (D) Vesiculations (thin arrows) of main/clear cells of collecting ducts, cell sloughing (thick arrow) in tubular lumen are visible.

4. Discussion

4.1. Nephron pathophysiology

Kidneys are the main route of PFOA excretion (Butenhoff et al., 2004). Similar to mammals, elimination of PFOA in trout was shown to occur mainly via the renal route, possibly by means of transporters facilitating PFOA movement from plasma to urine (Consoer et al., 2014). In experimentally exposed rats, the organ displaying the highest PFOA concentration was the kidney, in which interstitium proliferation and microvascular alterations were observed (Cui et al., 2009). The present study revealed a dose-dependent PFOA effect on nephron morphology. The observed glomerular capillary bed dilation and the loss of its folding pattern is indicative of high intraglomerular capillary pressure (Kriz and Endlich, 2005). Because filtration across the glomerular capillary is mainly driven by hydrostatic pressure, this results in hyperfiltration

Table 1
Number of rodlet cells per microscopic field in common carp exposed to perfluorooctanoic acid (PFOA) for 56 days, according to experimental group and tissue location.

Experimental group (microscopic field n)	Variable	Total n	Hemopoietic tissue n	Sinusoids n	Tubules n	Collecting ducts n
Controls (177)	Mean ± SE	0.89 ± 0.10	0.81 ± 0.10	0.07 ± 0.02	0.01 ± 0.01	0.00 ± 0.00
	Median	0	0	0	0	0
	Range	0–8	0–8	0–2	0–1	0
PFOA 200 ng L^{-1} (132)	Mean ± SE	4.44 ± 0.40	4.02 ± 0.37	0.39 ± 0.08	0.02 ± 0.01	0.01 ± 0.01
	Median	3	3	0	0	0
	Range	0–23	0–23	0–4	0–1	0–1
PFOA 2 mg L^{-1} (122)	Mean ± SE	7.75 ± 0.66	6.47 ± 0.55	0.93 ± 0.20	0.00 ± 0.00	0.35 ± 0.18
	Median	6	5	0	0	0
	Range	0–32	0–26	0–16	0	0–15

(Benzing and Salant, 2021). The absence of histological signs of glomerulonephritis and tubular protein (hyaline) casts rules out massive qualitative variation in glomerular filtration, although subtle morpho-functional changes such as mild proteinuria cannot be rejected and may be detected by urinalysis, clearly not practical in fish, with the requirement of labour-intensive cannulation. In effect, the greater vesiculation observed in the first proximal tubular segment is compatible with increased glomerular protein ultrafiltrate pinocytosis. Cells of the proximal tubular segment are known to reabsorb most ultrafiltrate molecules (Stockham and Scott, 2008). Protein uptake is mediated by megalin and cubilin, a nonselective multireceptor complex, leading to clathrin-mediated endocytosis. Endocytosed proteins undergo transcytosis and/or intracellular sorting, also including lysosomal digestion depending on molecular integrity (Dickson et al., 2014; Nielsen et al., 2016). Excess protein reabsorption by proximal tubular segment cells, as a consequence of glomerulonephrosis, may lead to cell overload and engulfment known as hyaline droplet degeneration (Schwaiger et al., 2004; Uwagawa et al., 1992). This degenerative pattern was not observed in the present research, strengthening the conclusion of only mild protein glomerular leakage.

With regard to possible pathophysiology and toxicity mechanism, perfluorooctane sulfonate, another PFAS, was shown to alter in vitro human microvascular endothelial permeability by reactive oxygen species (ROS) promotion and consequent actin filament remodelling (Qian et al., 2010). Reactive oxygen species were supposed to be involved in PFOA-induced ultrastructure modifications in hepatocytes of the same fish sampled for the present research, with mitochondria showing reduced or absent cristae; matrix lysis and vesiculation, swelling, and ballooning, autophagosomes; and myelin figures/myeloid bodies (Manera et al., 2021). This involvement of ROS was supported by increased expression of the glutathione S-transferase (GST) gene in the liver tissue of these same fish as a consequence of increased ROS production (Rotondo et al., 2018). Exposure to PFOA was suggested to inhibit mitochondrial β-oxidation and/or uncouple oxidative phosphorylation via its chemical similarity to fatty acids and by alteration in fluidity of mitochondrial membranes, respectively (Manera et al., 2021). The more pronounced ultrastructure changes observed in liver of the studied carp are in line with its significantly higher PFOA concentration than in kidney (Giari et al., 2016). With respect to kidney mitochondria ultrastructure changes, a higher degree of alteration than in other kidney tubular tracts was observed in the main/clear cells of the collecting ducts. Moreover, although neither apoptosis nor necrosis was greater in the 2 mg L^{-1} group compared to the low-dose group and controls, evidence of cell sloughing was documented in collecting ducts in fish of both PFOA-exposed groups. There was no sign of neutrophil or macrophage recruitment, although the 2 mg L^{-1} group showed significantly greater RC numbers than seen in other groups. Fish inhabiting freshwater do not need to reabsorb water from ultrafiltrate, therefore distal and collecting ducts are mainly deputed to the reabsorption of NaCl by a dedicated transporter, relying on related ATP-ase (Katoh et al., 2008). Per- and poly-fluoroalkyl substances are excreted by the action of organic anion transporters (OAT) in kidney (Hagenbuch and Meier, 2004) that are also expressed in proximal tubules and collecting ducts (Burckhardt and Burckhardt, 2011) and appear to be phylogenetically conserved and present in zebrafish (Dragojević et al., 2019; Hagenbuch and Meier, 2004). A study of the solute carrier 22 protein family in zebrafish, comprising OATs, showed marked variation with sex and tissue. The nephron segments in humans are known to be differentially affected by toxicants, depending on the implied transporter and its possible sex- and genetic-based modifications. Accordingly, a nephron topographic toxicologic pathology is known for human beings (Barnett and Cummings, 2018), but not for fish.

4.2. *Hemopoietic tissue pathophysiology*

The interstitium of fish mesonephros, the adult kidney, serves as erythropoietic, myelopoietic, and lymphopoietic tissue, along with other hemopoietic sites including pronephros, spleen, and thymus (Zapata et al., 2006). Fish, notably zebrafish, have become a reference model for study of hemopoiesis and immune function, particularly innate immunity (Renshaw and Trede, 2012). Recently, an experimental model roughly comparable to the present study in dose (0.05, 0.1, 0.5, 1 mg L^{-1}) and duration (7, 14, and 21 days) demonstrated PFOA effect on zebrafish kidney immune cells (Zhang et al., 2021) with hydropic endoplasmic reticulum, swelling of mitochondria, and vacuolisation. With regard to toxicity mechanism, low concentrations of PFOA were reported to disturb cytokine and antibody secretion, altering the TLR/myd88/NF-κB pathway (Zhang et al., 2021). Perfluorooctanoic acid, like other toxicants, activates TLRs primarily devoted to promoting inflammation as a response to microbial pathogens (Hu et al., 2019; Zhang et al., 2021). Evidence of decreased neutrophil migration in response to injury in zebrafish embryos exposed to PFOA was also reported (Pecquet et al., 2020), suggesting a potential negative impact of PFOA on both innate and adaptive immunity. In the present study, although no histological signs of innate cellular immunity activation or inflammation were observed, an increased association of RCs with myeloid cells (mainly neutrophil lineage) was noted.

Rodlet cells are unique to teleost fishes (Manera and Dezfuli, 2004). Widely accepted key features of the mature stage of RCs include a polarised, pear-shaped form with a large spherical to ovoid nucleus at the expanded base, a fibrillar sub-plasmalemmar layer, markedly vesiculated cytoplasm, typical rod-shaped inclusions displaying an amorphous outer rodlet sac, and an electron-dense core converging at the cell apex where the fibrillar layer becomes thinner, favouring cell content expulsion (Depasquale, 2020; Manera and Dezfuli, 2004). Rodlet cells are considered to be involved in innate cellular immunity (Manera and Dezfuli, 2004; Mazon et al., 2007; Reite, 2005) based on increased numbers and degranulation in infected tissues, immunoreactivity to lysozyme, inducible nitric oxide synthase, and to tumour necrosis factor alpha (Bosi et al., 2018; Ronza et al., 2015; Sayyaf Dezfuli et al., 2022). Possible influence of stress hormones and stress-related changes on RCs has been reported (Manera et al., 2001; Manera and Dezfuli, 2004; Mazon et al., 2007). The unique association of RCs with stromal reticular cells and leucocytes observed in the present study has

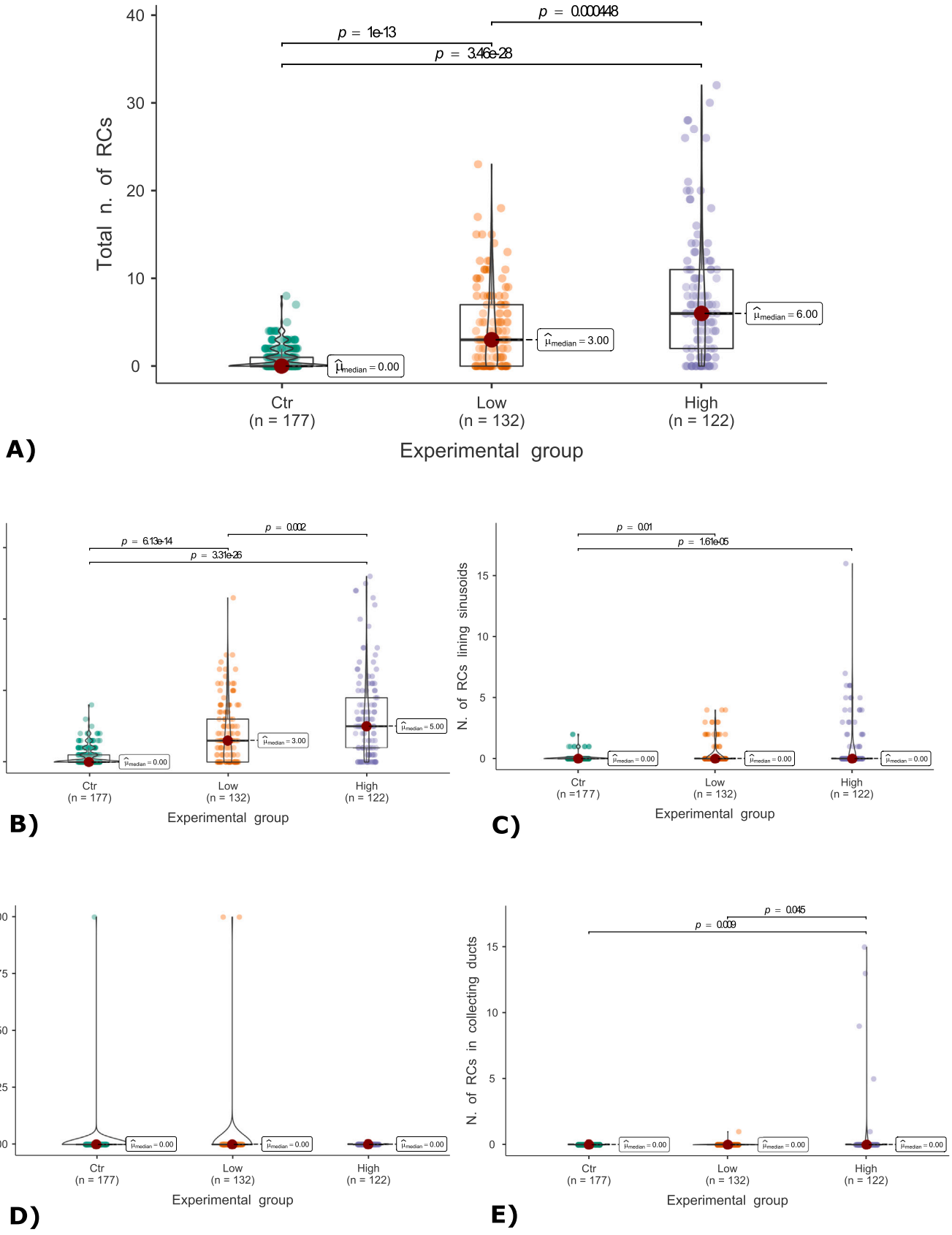

Fig. 3. Violin-box plot of the number of RCs per microscopic field, according to experimental group and tissue. (A) Total RCs; (B) RCs in hemopoietic tissue; (C) RCs lining sinusoids; (D) RCs in tubules; (E) RCs in collecting ducts. Significant differences among groups (Kruskal-Wallis and Dunn test as pairwise test) and related p-values are shown as annotated, pairwise connecting lines. The primary source of variation in the number of RCs is the hemopoietic tissue, the only tissue group showing significant difference in each pairwise comparison (control vs. 200 ng L^{-1}; control vs. 2 mg L^{-1}; 200 ng L^{-1} vs. 2 mg L^{-1}).

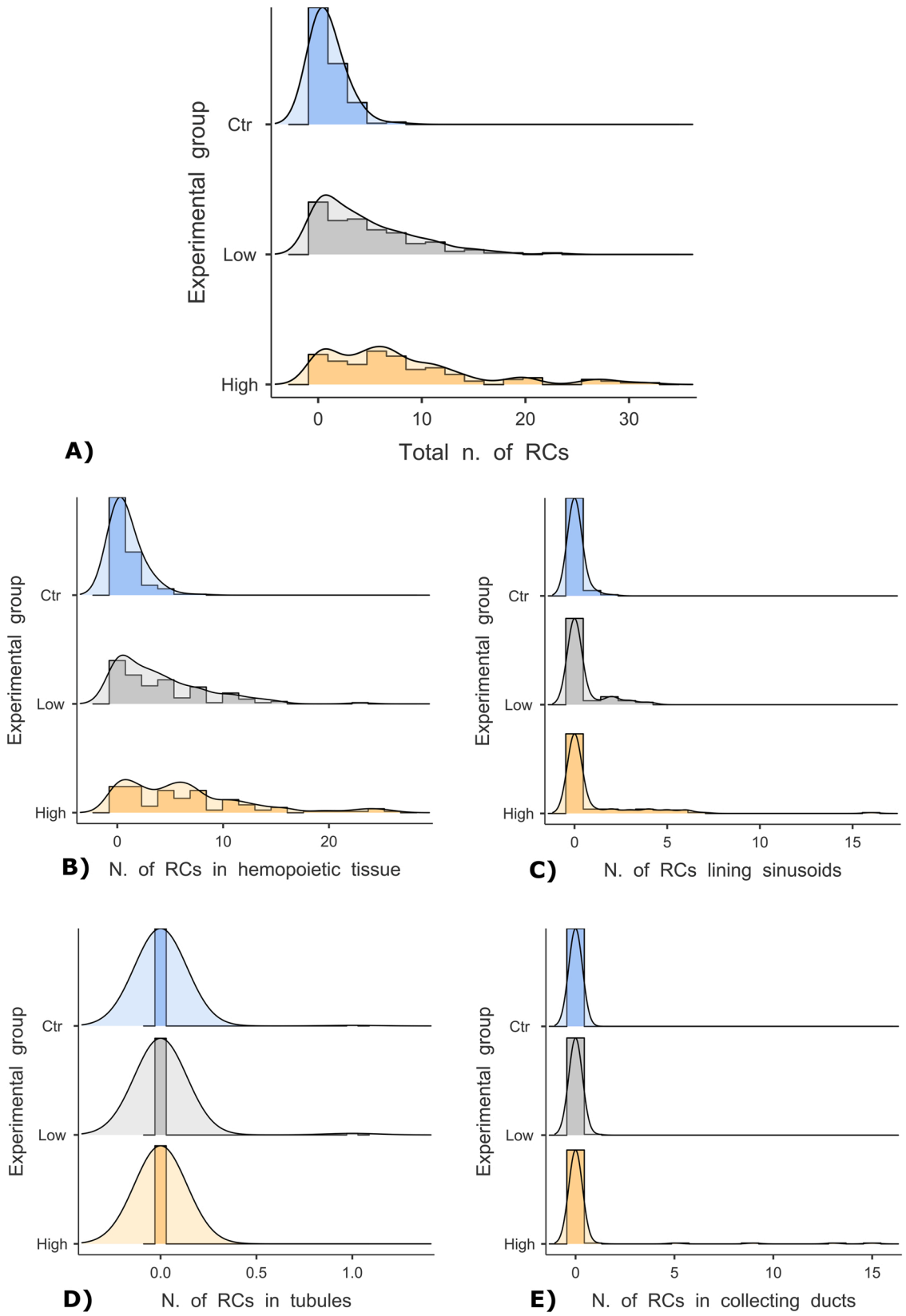

Fig. 4. Frequency distribution of RCs per microscopic field. (A) Total; (B) in hemopoietic tissue; (C) lining sinusoids; (D) in tubules; (E) in collecting ducts. Distribution pattern of RCs reflects distribution in hemopoietic tissue, confirming it as the most frequent RC occurrence site.

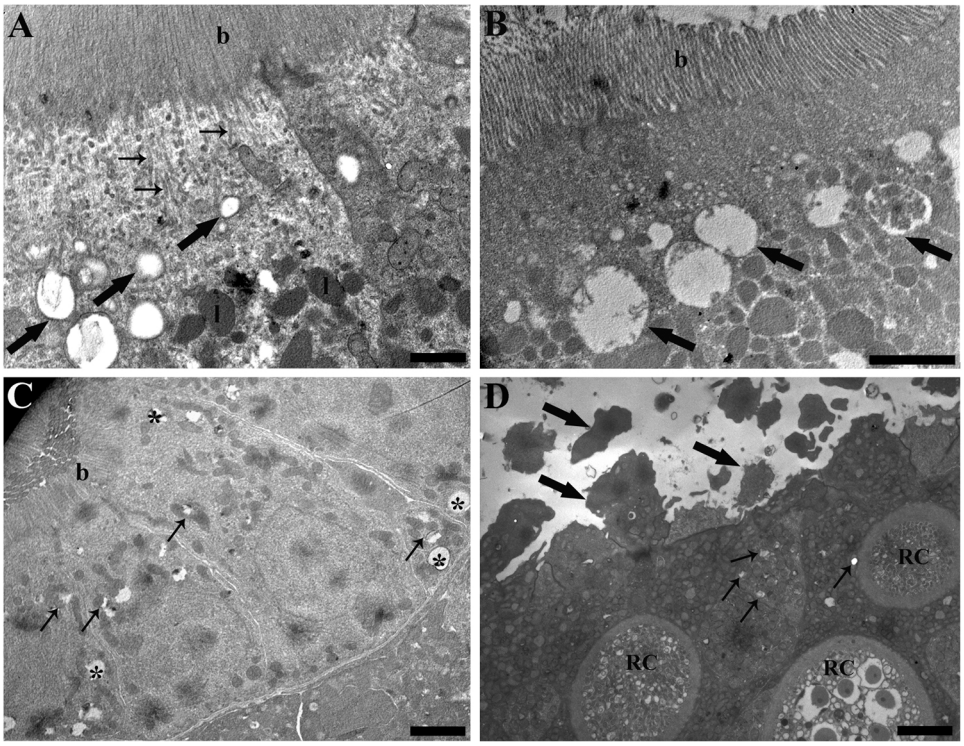

Fig. 5. Transmission electron micrographs of ultrathin sections of common carp kidney. (A) Cells from the first proximal segment of a control fish show a prominent brush border (b), typical apical endocytic apparatus formed by endocytic vesicles (thick arrows) and dense tubules (thin arrows), and large lysosomes (l). Scale bar = 1 μm. (B) Cells from the first proximal segment of fish exposed to 2 mg L^{-1} PFOA show higher numbers of endocytic vesicles than in controls (thick arrows), which appear larger, with electron dense, foamy to compact content. Scale bar = 2 μm. (C) Cells from the second proximal segment of fish exposed to 2 mg L^{-1} PFOA show some focal mitochondria vesiculation (thin arrows). A less prominent brush border (b) compared to cells from the first proximal segment is a distinctive feature of the segment. Scattered cytoplasm vesicles (asterisks) are present. Scale bar = 2 μm. (D) Focal mitochondria vesiculation (thin arrows) and sloughing (thick arrows) of tubular lumen in cells of the collecting ducts of fish exposed to 2 mg L^{-1} PFOA. Three rodlet cells (RC) are visible within the epithelium, one displaying enlargement and vacuolisation of the rodlet sac. Scale bar = 2.5 μm.

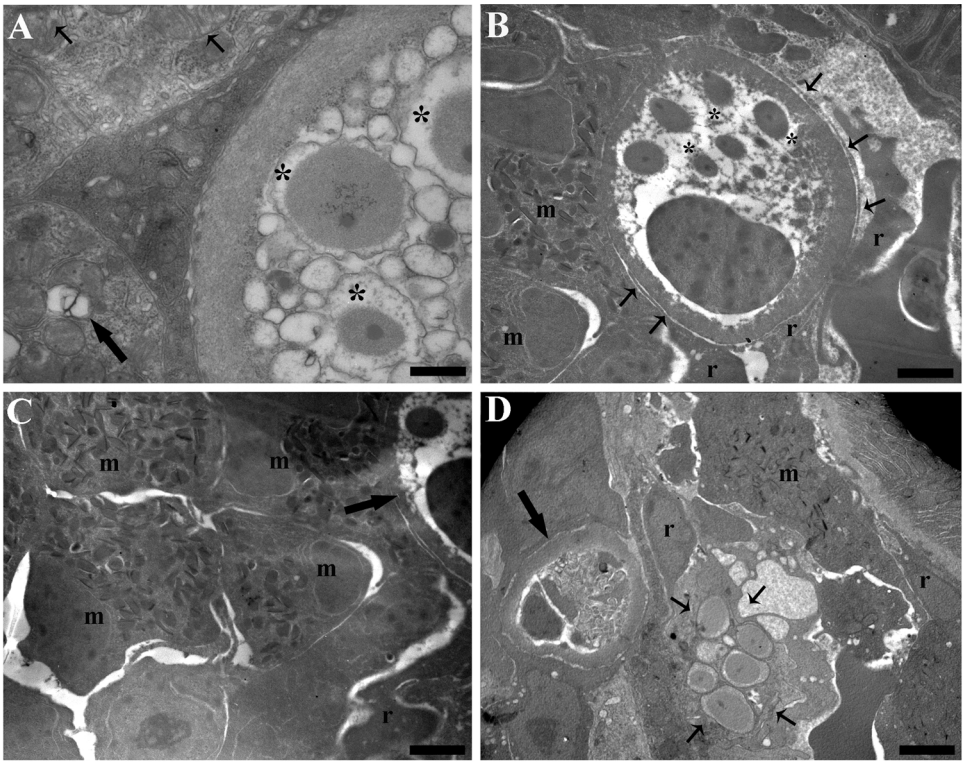

Fig. 6. Transmission electron micrographs of ultrathin sections of common carp kidney. (A) Spotted mitochondrial matrix rarefaction (thin arrows) and focal vesiculation (thick arrow) in main clear cells of the collecting duct from fish exposed to 2 mg L^{-1} PFOA. Rodlet cell present, showing enhanced cytoplasm vesiculation and enlargement, vacuolation, and rarefaction of the rodlet sac asterisks). Scale bar = 0.5 μm. (B) Rodlet cell of fish exposed to 2 mg L^{-1} PFOA displaying cytoplasm vacuolation and rodlet sac rarefaction (asterisks) is surrounded by myeloid neutrophilic lineage cells (m). Cytoplasmic projections (thin arrows) of a stromal reticular cell are visible, partially encircling the RC. Endothelial cells are also visible. Scale bar = 1.5 μm. (C) Myeloid neutrophilic lineage cells (m) near an RC (thick arrow) in the hemopoietic tissue of fish exposed to 2 mg L^{-1} PFOA. A stromal reticular cell (r) is present. Scale bar = 1.5 μm. (D) Discharged RC cytoplasm (thin arrows) is visible within the hemopoietic interstitium of fish exposed to 200 ng L^{-1} PFOA, near a partially depleted RC (thick arrow), a myeloid neutrophilic lineage cell (m), and stromal reticular cells (r). Scale bar = 2 μm.

been previously reported in the *bulbus arteriosus* of goldfish *Carassius auratus*, a carp-related species, at the subendothelial level, as a potential micro-environment for RC full development (Manera et al., 2001). In the present research, RC quantitative and qualitative difference was the only immune response observed in PFOA exposure. Significantly greater RC numbers, compared to unexposed fish, was documented in kidney even in fish exposed to the environmental relevant PFOA concentration of 200 ng L^{-1}, where tissue concentrations were below the level of detection, representing a possible reliable biomarker of exposure. This apparent discrepancy between chemical analytical results and morphopathological results has been previously reported in the liver tissue of the same fish of this study, even with regard to the expression level of

the glutathione S-transferase (GST) gene (Manera et al., 2017; Rotondo et al., 2018). There is currently no available data of biomolecular signalling related to RC recruitment and activation. It is not known whether RCs may act as sentinel cells, able to independently mount an innate immune cellular response, or if they need to be activated by macrophages or neutrophils as sentinel cells. The absence of leucocyte recruitment and activation may lead to speculation of direct involvement of RCs as an alternative sentinel cell in teleost fish (Manera et al., 2001; Reite, 2005). Referring to the possible pathophysiology, further investigation is needed to clarify whether this is due to direct (TLR) or indirect (tissue damage, stress-induced) PFOA mediation. Degranulating/degranulated RCs have been previously reported in kidney hemopoietic interstitium (Mazon et al., 2007), and evidence of increased degranulation was documented in the present study as a consequence of PFOA exposure. Because no evident pathogen, tissue damage, and/or leucocyte activation were detected near discharging/discharged RCs, it may be argued that degranulation exerts other biological effects, possibly directed toward the surrounding hemopoietic tissue.

5. Conclusion

Perfluorooctanoic acid affects kidney architecture, tubular cells, and RC ultrastructure and occurrence in experimentally exposed carp. Nephron-associated changes are primarily observed in fish exposed to 2 mg L^{-1} and are related to glomerular hyperfiltration, consequent protein readsorbance by the proximal tubular segment, and collecting duct mitochondria impairment. As reported in the liver of the same fish (Manera et al., 2021), ROS are presumed to be involved in PFOA-induced mitochondria changes. Rodlet cell qualitative-quantitative response occurs at the environmentally relevant dose of 200 ng L^{-1}. Further research is needed to clarify whether RCs react directly to PFOA stimulation or indirectly to tissue damage signalling or stress. This study was based on morphological evidence, and it is premature to speculate on either a possible proliferation-inducing function on myeloid cells or direct recruitment of RCs as a consequence of degranulation. Further biomolecular study should investigate possible molecular signalling. Nevertheless, regarding RC-induced recruitment of RCs, renal tissue from PFOA-exposed fish showed greater probability of occurrence of more than one RC per microscopic field and more frequent occurrence of RC clusters.

The implication of RCs in innate fish immunity makes them a candidate for a reliable, though generalist, biomarker of PFOA exposure at environmentally relevant concentrations.

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CRediT authorship contribution statement

Maurizio Manera Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing, Supervision. **Giuseppe Castaldelli** Project administration, Funding acquisition, Supervision. **Cristiana Guerranti** Methodology, Investigation. **Luisa Giari** Designed the investigation, Resources, Writing – original draft, Writing – review & editing, Funding acquisition, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Alexander, J., Auðunsson, G.A., Benford, D., Cockburn, A., Dogliotti, E., Domenico, A., Di, Fernández-cruz, M.L., Fürst, P., Galli, C., Grandjean, P., Gzyl, J., Johansson, N., Mutti, A., Schlatter, J., Leeuwen, R., Van, Peteghem, C., Van, Verger, P., 2008. Perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA) and their salts – scientific opinion of the Panel on Contaminants in the Food chain. EFSA J. 653, 1–131. https://doi.org/10.2903/j.efsa.2008.653.

Ankley, G.T., Cureton, P., Hoke, R.A., Houde, M., Kumar, A., Kurias, J., Lanno, R., McCarthy, C., Newsted, J., Salice, C.J., Sample, B.E., Sepúlveda, M.S., Steevens, J., Valsecchi, S., 2021. Assessing the ecological risks of per- and polyfluoroalkyl substances: current state-of-the science and a proposed path forward. Environ. Toxicol. Chem. 40, 564–605. https://doi.org/10.1002/etc.4869.

Barnett, L.M.A., Cummings, B.S., 2018. Nephrotoxicity and renal pathophysiology: a contemporary perspective. Toxicol. Sci. 164, 379–390. https://doi.org/10.1093/toxsci/kfy159.

Benzing, T., Salant, D., 2021. Insights into glomerular filtration and albuminuria. N. Engl. J. Med. 384, 1437–1446. https://doi.org/10.1056/nejmra1808786.

Bosi, G., DePasquale, J.A., Manera, M., Castaldelli, G., Giari, L., Sayyaf Dezfuli, B., 2018. Histochemical and immunohistochemical characterization of rodlet cells in the intestine of two teleosts, *Anguilla anguilla* and *Cyprinus carpio*. J. Fish Dis. 41, 475–485. https://doi.org/10.1111/jfd.12751.

Burckhardt, G., Burckhardt, B.C., 2011. In vitro and in vivo evidence of the importance of organic anion transporters (OATs) in drug therapy. Handb. Exp. Pharm. 201, 29–104. https://doi.org/10.1007/978-3-642-14541-4_2.

Butenhoff, J.L., Kennedy, G.L., Hinderliter, P.M., Lieder, P.H., Jung, R., Hansen, K.J., Gorma, G.S., Nokers, P.E., Thomford, P.J., 2004. Pharmacokinetics of perfluorooctanoate in cynomolgus monkeys. Toxicol. Sci. 82, 394–406. https://doi.org/10.1093/toxsci/kfh302.

Chen, M., Zhu, L., Wang, Q., Shan, G., 2021. Tissue distribution and bioaccumulation of legacy and emerging per-and polyfluoroalkyl substances (PFASs) in edible fishes from Taihu Lake, China. Environ. Pollut. 268, 115887 https://doi.org/10.1016/j.envpol.2020.115887.

Consoer, D.M., Hoffman, A.D., Fitzsimmons, P.N., Kosian, P.A., Nichols, J.W., 2014. Toxicokinetics of perfluorooctanoate (PFOA) in rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 156, 65–73. https://doi.org/10.1016/j.aquatox.2014.07.022.

Cousins, I.T., Dewitt, J.C., Glüge, J., Goldenman, G., Herzke, D., Lohmann, R., Miller, M., Ng, C.A., Scheringer, M., Vierke, L., Wang, Z., 2020. Strategies for grouping per-and polyfluoroalkyl substances (PFAS) to protect human and environmental health. Environ. Sci. Process. Impacts 22, 1444–1460. https://doi.org/10.1039/d0em00147c.

Cui, L., Zhou, Q.F., Liao, C.Y., Fu, J.J., Jiang, G.Bin, 2009. Studies on the toxicological effects of PFOA and PFOS on rats using histological observation and chemical analysis. Arch. Environ. Contam. Toxicol. 56, 338–349. https://doi.org/10.1007/s00244-008-9194-6.

Depasquale, J.A., 2020. Tropomyosin and alpha-actinin in teleost rodlet cells. Acta Zool. 102, 323–332. https://doi.org/10.1111/azo.12344.

Dickson, L.E., Wagner, M.C., Sandoval, R.M., Molitoris, B.A., 2014. The proximal tubule and albuminuria: really! J. Am. Soc. Nephrol. 25, 443–453. https://doi.org/10.1681/ASN.2013090950.

Dragojević, J., Mihaljević, I., Popović, M., Smital, T., 2019. Zebrafish (*Danio rerio*) Oat1 and Oat3 transporters and their interaction with physiological compounds. Comp. Biochem. Physiol. Part - B Biochem. Mol. Biol. 236, 110309 https://doi.org/10.1016/j.cbpb.2019.110309.

Falk, S., Failing, K., Georgii, S., Brunn, H., Stahl, T., 2015. Tissue specific uptake and elimination of perfluoroalkyl acids (PFAAs) in adult rainbow trout (*Oncorhynchus mykiss*) after dietary exposure. Chemosphere 129, 150–156. https://doi.org/10.1016/j.chemosphere.2014.06.061.

Giari, L., Guerranti, C., Perra, G., Lanzoni, M., Fano, E.A., Castaldelli, G., 2015. Occurrence of perfluorooctanesulfonate and perfluorooctanoic acid and histopathology in eels from north Italian waters. Chemosphere 118, 117–123. https://doi.org/10.1016/j.chemosphere.2014.06.066.

Giari, L., Vincenzi, F., Badini, S., Guerranti, C., Dezfuli, B.S., Fano, E.A., Castaldelli, G., 2016. Common carp *Cyprinus carpio* responses to sub-chronic exposure to perfluorooctanoic acid. Environ. Sci. Pollut. Res. 23, 15321–15330. https://doi.org/10.1007/s11356-016-6706-1.

Goeritz, I., Falk, S., Stahl, T., Schäfers, C., Schlechtriem, C., 2013. Biomagnification and tissue distribution of perfluoroalkyl substances (PFASs) in market-size rainbow trout (*Oncorhynchus mykiss*). Environ. Toxicol. Chem. 32, 2078–2088. https://doi.org/10.1002/etc.2279.

Gong, X., Yang, C., Hong, Y., Chung, A.C.K., Cai, Z., 2019. PFOA and PFOS promote diabetic renal injury in vitro by impairing the metabolisms of amino acids and purines. Sci. Total Environ. 676, 72–86. https://doi.org/10.1016/J.SCITOTENV.2019.04.208.

Grover, A., Sinha, R., Jyoti, D., Faggio, C., 2021. Imperative role of electron microscopy in toxicity assessment: a review. Microsc. Res. Tech. https://doi.org/10.1002/jemt.24029.

Hagenbuch, B., Meier, P.J., 2004. Organic anion transporting polypeptides of the OATP/SLC21 family: phylogenetic classification as OATP/SLCO superfamily, new nomenclature and molecular/functional properties. Pflug. Arch.-Eur. J. Physiol. 447, 653–665. https://doi.org/10.1007/s00424-003-1168-y.

Hu, X., Chi, Q., Liu, Q., Wang, D., Zhang, Y., Li, S., 2019. Atmospheric H2S triggers immune damage by activating the TLR-7/MyD88/NF-KB pathway and NLRP3 inflammasome in broiler thymus. Chemosphere 237, 124427. https://doi.org/10.1016/j.chemosphere.2019.124427.

Katoh, F., Cozzi, R.R.F., Marshall, W.S., Goss, G.G., 2008. Distinct $Na^+/K^+/2Cl^-$ cotransporter localization in kidneys and gills of two euryhaline species, rainbow trout and killifish. Cell Tissue Res. 334, 265–281. https://doi.org/10.1007/s00441-008-0679-4.

Kim, W.-K., Lee, S.-K., Jung, J., 2010. Integrated assessment of biomarker responses in common carp (*Cyprinus carpio*) exposed to perfluorinated organic compounds. J. Hazard. Mater. 180, 395–400. https://doi.org/10.1016/j.jhazmat.2010.04.044.

Kriz, W., Endlich, K., 2005. Hypertrophy of podocytes: a mechanism to cope with increased glomerular capillary pressures? Kidney Int. 67, 373–374. https://doi.org/10.1111/j.1523-1755.2005.00141.x.

Lee, J.W., Choi, K., Park, K., Sung, C., Yu, S., Do, Kim, P., Seong, C., Yu, S., Do, Kim, P., 2020. Adverse effects of perfluoroalkyl acids on fish and other aquatic organisms: a review. Sci. Total Environ. 707, 135334 https://doi.org/10.1016/j.scitotenv.2019.135334.

Liang, L., Pan, Y., Bin, L., Liu, Y., Huang, W., Li, R., Lai, K.P., 2022. Immunotoxicity mechanisms of perfluorinated compounds PFOA and PFOS. Chemosphere 291, 132892. https://doi.org/10.1016/j.chemosphere.2021.132892.

Loos, R., Locoro, G., Huber, T., Wollgast, J., Christoph, E.H., de Jager, A., Manfred Gawlik, B., Hanke, G., Umlauf, G., Zaldívar, J.M., 2008. Analysis of perfluorooctanoate (PFOA) and other perfluorinated compounds (PFCs) in the River Po watershed in N-Italy. Chemosphere 71, 306–313. https://doi.org/10.1016/j.chemosphere.2007.09.022.

Manera, M., Castaldelli, G., Fano, E.A., Giari, L., 2021. Perfluorooctanoic acid-induced cellular and subcellular alterations in fish hepatocytes. Environ. Toxicol. Pharmacol. 81, 103548 https://doi.org/10.1016/j.etap.2020.103548.

Manera, M., Dezfuli, B.S., 2004. Rodlet cells in teleosts: a new insight into their nature and functions. J. Fish Biol. 65, 597–619. https://doi.org/10.1111/j.1095-8649.2004.00511.x.

Manera, M., Giari, L., Vincenzi, F., Guerranti, C., Depasquale, J.A., Castaldelli, G., 2017. Texture analysis in liver of common carp (*Cyprinus carpio*) sub-chronically exposed to perfluorooctanoic acid. Ecol. Indic. 81, 54–64. https://doi.org/10.1016/j.ecolind.2017.05.001.

Manera, M., Simoni, E., Dezfuli, B.S.S., 2001. The effect of dexamethasone on the occurrence and ultrastructure of rodlet cells in goldfish. J. Fish Biol. 59, 1239–1248. https://doi.org/10.1006/jfbi.2001.1733.

Mazon, A.F., Huising, M.O., Taverne-Thiele, A.J., Bastiaans, J., Verburg-van Kemenade, B.M.L., 2007. The first appearance of Rodlet cells in carp (*Cyprinus carpio* L.) ontogeny and their possible roles during stress and parasite infection. Fish Shellfish Immunol. 22, 27–37. https://doi.org/10.1016/j.fsi.2006.03.012.

Nielsen, R., Christensen, E.I., Birn, H., 2016. Megalin and cubilin in proximal tubule protein reabsorption: from experimental models to human disease. Kidney Int. 89, 58–67. https://doi.org/10.1016/j.kint.2015.11.007.

Pecquet, A.M., Maier, A., Kasper, S., Sumanas, S., Yadav, J., 2020. Exposure to perfluorooctanoic acid (PFOA) decreases neutrophil migration response to injury in zebrafish embryos. BMC Res. Notes 13, 408. https://doi.org/10.1186/s13104-020-05255-3.

Qian, Y., Ducatman, A., Ward, R., Leonard, S., Bukowski, V., Guo, N.L., Shi, X., Vallyathan, V., Castranova, V., 2010. Perfluorooctane sulfonate (PFOS) induces reactive oxygen species (ROS) production in human microvascular endothelial cells: role in endothelial permeability. J. Toxicol. Environ. Heal. - Part A Curr. Issues 73, 819–836. https://doi.org/10.1080/15287391003689317.

Rahman, M.M., 2015. Role of common carp (*Cyprinus carpio*) in aquaculture production systems. Front. Life Sci. 8, 399–410. https://doi.org/10.1080/21553769.2015.1045629.

Rashid, F., Ramakrishnan, A., Fields, C., Irudayaraj, J., 2020. Acute PFOA exposure promotes epigenomic alterations in mouse kidney tissues. Toxicol. Rep. 7, 125–132. https://doi.org/10.1016/J.TOXREP.2019.12.010.

Reite, O.B., 2005. The rodlet cells of teleostean fish: their potential role in host defence in relation to the role of mast cells/eosinophilic granule cells. Fish Shellfish Immunol. 19, 253–267. https://doi.org/10.1016/j.fsi.2005.01.002.

Renshaw, S.A., Trede, N.S., 2012. A model 450 million years in the making: zebrafish and vertebrate immunity. Dis. Model. Mech. 5, 38–47. https://doi.org/10.1242/dmm.007138.

Ronza, P., Losada, A.P., Villamarín, A., Bermúdez, R., Quiroga, M.I., 2015. Immunolocalization of tumor necrosis factor alpha in turbot (*Scophthalmus maximus*, L.) tissues. Fish Shellfish Immunol. 45, 470–476. https://doi.org/10.1016/j.fsi.2015.04.032.

Rotondo, J.C., Giari, L., Guerranti, C., Tognon, M., Castaldelli, G., Fano, E.A., Martini, F., 2018. Environmental doses of perfluorooctanoic acid change the expression of genes in target tissues of common carp. Environ. Toxicol. Chem. 37, 942–948. https://doi.org/10.1002/etc.4029.

Sayyaf Dezfuli, B., Pironi, F., Maynard, B., Simoni, E., Bosi, G., 2022. Rodlet cells, fish immune cells and a sentinel of parasitic harm in teleost organs. Fish Shellfish Immunol. 121, 516–534. https://doi.org/10.1016/j.fsi.2021.09.045.

Schrenk, D., Bignami, M., Bodin, L., Chipman, J.K., del Mazo, J., Grasl-Kraupp, B., Hogstrand, C., Hoogenboom, L., Leblanc, J.C., Nebbia, C.S., Nielsen, E., Ntzani, E., Petersen, A., Sand, S., Vleminckx, C., Wallace, H., Barregård, L., Ceccatelli, S., Cravedi, J.P., Halldorsson, T.I., Haug, L.S., Johansson, N., Knutsen, H.K., Rose, M., Roudot, A.C., Van Loveren, H., Vollmer, G., Mackay, K., Riolo, F., Schwerdtle, T., 2020. Risk to human health related to the presence of perfluoroalkyl substances in food. EFSA J. 18, e06223 https://doi.org/10.2903/J.EFSA.2020.6223.

Schwaiger, J., Ferling, H., Mallow, U., Wintermayr, H., Negele, R.D., 2004. Toxic effects of the non-steroidal anti-inflammatory drug diclofenac. Part I: Histopathological alterations and bioaccumulation in rainbow trout. Aquat. Toxicol. 68, 141–150. https://doi.org/10.1016/j.aquatox.2004.03.014.

Stein, C.R., Ge, Y., Wolff, M.S., Ye, X., Calafat, A.M., Kraus, T., Moran, T.M., 2016. Perfluoroalkyl substance serum concentrations and immune response to FluMist vaccination among healthy adults. Environ. Res. 149, 171–178. https://doi.org/10.1016/j.envres.2016.05.020.

Stockham, S.L., Scott, M.A., 2008. Fundamentals of Veterinary Clinical Pathology, 2nd ed. Blackwell Publishing, Oxford, UK.

Uwagawa, S., Saito, K., Nakayama, A., Umihira, M., Okuno, Y., 1992. Comparison of hyaline droplets in rats with chronic progressive nephropathy and chemical-induced ALPHA2u-globulin nephropathy. J. Toxicol. Pathol. 5, 195–203. https://doi.org/10.1293/tox.5.195.

Vierke, L., Staude, C., Biegel-Engler, A., Drost, W., Schulte, C., 2012. Perfluorooctanoic acid (PFOA) — main concerns and regulatory developments in Europe from an environmental point of view. Environ. Sci. Eur. 24, 16. https://doi.org/10.1186/2190-4715-24-16.

Wang, Z., Dewitt, J.C., Higgins, C.P., Cousins, I.T., 2017. A never-ending story of per- and polyfluoroalkyl substances (PFASs)? Environ. Sci. Technol. 51, 2508–2518. https://doi.org/10.1021/acs.est.6b04806.

Wei, Y., Dai, J., Liu, M., Wang, J., Xu, M., Zha, J., Wang, Z., 2007. Estrogen-like properties of perfluorooctanoic acid as revealed by expressing hepatic estrogen-responsive genes in rare minnows (*Gobiocypris rarus*). Environ. Toxicol. Chem. 26, 2440–2447. https://doi.org/10.1897/07-008R1.1.

Ye, X., Schoenfuss, H.L., Jahns, N.D., Delinsky, A.D., Strynar, M.J., Varns, J., Nakayama, S.F., Helfant, L., Lindstrom, A.B., 2008. Perfluorinated compounds in common carp (*Cyprinus carpio*) fillets from the Upper Mississippi River. Environ. Int. 34, 932–938. https://doi.org/10.1016/j.envint.2008.02.003.

Zapata, A., Diez, B., Cejalvo, T., Gutiérrez-De Frías, C., Cortés, A., 2006. Ontogeny of the immune system of fish. Fish Shellfish Immunol. 20, 126–136. https://doi.org/10.1016/j.fsi.2004.09.005.

Zhang, H., Shen, L., Fang, W., Zhang, X., Zhong, Y., 2021. Perfluorooctanoic acid-induced immunotoxicity via NF-kappa B pathway in zebrafish (*Danio rerio*) kidney. Fish Shellfish Immunol. 113, 9–19. https://doi.org/10.1016/j.fsi.2021.03.004.