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Evaluation of per- and polyfluoroalkyl substances (PFAS) toxic effects on the acute inflammatory response in the medicinal leech *Hirudo verbana*

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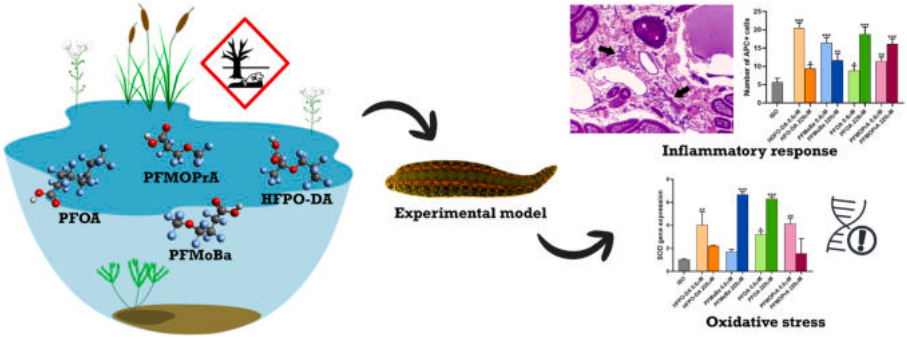
HIGHLIGHTS

- PFAS compounds cause inflammation and angiogenesis in *Hirudo verbana*, varying by type and dose.
- PFAS induces oxidative stress, marked by increased expression of GSTA4 and SOD stress markers.
- PFOA induces the strongest response, followed by PFMOPrA, HFPO-DA, and PFMoBa, respectively.
- At higher concentrations, HFPO-DA and PFMoBa suppress immune responses, unlike lower doses.
- High PFOA and PFMOPrA levels are linked to increased angiogenesis and inflammation.

GRAPHICAL ABSTRACT



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ABSTRACT

Per- and polyfluoroalkyl (PFAS) substances are a large group of chemicals with elevated water and oil-resistance properties, widely implicated in various applicative fields. Due to the extensive use and high resistance to degradative factors, these compounds pose a significant risk of environmental spreading, bioaccumulating also in living organisms. In this context, despite many researches have been performed to demonstrate "legacy" PFAS harmfulness, only few data are still available about all the emerging fluorinated molecules, industrially introduced to replace the previous ones. For this reason, we proposed the medicinal leech *Hirudo verbana* as consolidated invertebrate model to assess the effects of four different PFAS (HFPO-DA, PFMoBa, PFOA and PFMOPrA) following freshwater dispersion. Morphological, immunohistochemical and molecular analyses demonstrate that, despite all the compounds basically induce an acute inflammatory and oxidative stress response, a different cellular and molecular response has been observed. Whereas for PFOA and PFMOPrA an increase in the tested concentration leads to a corresponding rise in the immune response, HFPO-DA and PFMoBa trigger an entirely opposite effect. Indeed, the significant recruitment of both granulocytes and macrophage like

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cells, typically involved in the removal of non-self, is inhibited with increasing concentrations of these compounds. The data collected revealed a different sensitivity of the leech immune system following PFAS exposure, requiring to deepen the current knowledge on the potential toxicity of these compounds.

1. Introduction

Per- and poly-fluoroalkyl substances (PFAS) are a widely group of synthetically manufactured fluorinated compounds that are largely used for many industrial applications, including the production of fluoropolymer plastics i.e., polytetrafluoroethylene (PTFE), firefighting aqueous film forming foams (AFFF), food packaging, non-stick cookware, cosmetics, etc. The C–F bonds in their chemical structure confer unique features among which an exceptionally thermal and chemical stability (Cui et al., 2020; Parolini et al., 2022). Due to their remarkable surfactant, amphipathic and water/oil-repellent properties their production and socio-economic impact have grown exponentially during the 20th century (Liu et al., 2014; Pan et al., 2017).

Although both PFAS resistance and amphiphilic nature represent an advantage at industrial level, these molecules result practically non-degradable under natural conditions, showing elevated environmental persistence, bio-accumulative properties and toxicity in the long term, as judged by the International Agency for Research on Cancer (IARC), who has recently re-evaluated the carcinogenicity of perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS), assigning them respectively in Group 1 (carcinogen to human) and 2 (probably carcinogen) (Cui et al., 2020; Sunderland et al., 2019).

In the last decades, PFAS have been globally detected in almost all the environments, such as soil, sediments, marine and freshwaters (Crone et al., 2019; Kurwadkar et al., 2022; Mahinroosta and Senevirathna, 2020; Sima and Jaffé, 2021). PFAS can reach surface and groundwaters via sewage deriving from fluorochemical plants, electroplating operations, landfills, laundries, dry cleaners, households, directly or by means of wastewater treatment plants (WWTP). In other cases, they can be directly poured in the soil as constituents of chemicals compounds such as fire-extinguishing foams or sludges (Behnami et al., 2024; Brunn et al., 2023). As regards aquatic environments, it has been observed as some PFAS are highly water-soluble, while others tend to be differently distributed into the water-sediment fraction (Chen et al., 2017; Mussabek et al., 2019), contaminating also drinking waters (Bonato et al., 2020). To date, upward of 12,000 PFAS have been identified (EPA, 2020), of which many result to be low recoverable, persistent, non-biodegradable and toxic. In this context, the European Commission paid specific attention to the revision of the Water Framework Directive, including PFAS in the list of priority substances setting new environmental quality standards (COM, 2022).

As concern the possible their toxicity, recent experimental data performed both *in vivo* and *in vitro* have demonstrated as PFAS induce different dangerous outcomes both in plants and animals, included also human (DeWitt, 2015; Jane L Espartero et al., 2022; Melo et al., 2022; Podder et al., 2021; Wu et al., 2022). The exposition to PFAS, occurring through food or water ingestion, leads to negative effects at immunological, developmental, neurological, and endocrine level both in vertebrates (Cui et al., 2020) and in invertebrates (Liu et al., 2014; Lu et al., 2015; MacDonald et al., 2004; Marziali et al., 2019).

Among all the fluorinated compounds, PFOA is one of the most monitored and registered, whose effects have been tested in different experimental models (Wee and Aris, 2023). PFOA is a legacy PFAS with an eight-carbon backbone, well known for its strong immunotoxic and inflammatory effects (Hagenaars et al., 2013; Li et al., 2017). Given its persistence, bioaccumulation potential, and toxicity, regulatory measures have driven the development of newer PFAS compounds with shorter carbon chains as potential safer alternatives. Indeed, it has been demonstrated that PFOA can persist for many years in the soil, for then infiltrating also into ground and drinking waters (Filipovic et al., 2015), in which different concentrations have been specifically found worldwide (from 0.3 to 4300 ng/L), suggesting as the potential health risks can be induced also at low levels of exposure (Wee and Aris, 2023). In vertebrates, the contact with PFOA leads to neurodevelopmental deficits, tumor liver formation and a decrease of survival rate of following generations (Brunn et al., 2023; Brase et al., 2021). As concerns the effects on the reproductive and development rate, it has been observed as increasing concentrations negatively influence the animals gestation, revealing a dose-dependent effects on embryos survival, both in mice and in rats (Lau et al., 2006; Luebker et al., 2005; Takacs and Abbott, 2007). Similar effects were observed in bivalve mollusks, plathelminths and echinoderms, in which PFOA bioaccumulation also cause oxidative stress and DNA damage, antioxidant enzymes change and apoptosis also at low dosage (Ma et al., 2022). Therefore, not only it results important to develop new ecotoxicological models to assess the ecological risk posed by these fluorinated molecules, especially discharged into different aquatic environments (Calisi, 2023), but also to assess the hazard of PFAS in living organisms, particularly considering the alternative next generation molecules (Cui and Deng, 2022).

For these reasons, in the present work we proposed the leech *Hirudo verbana*, already used to assess the role of different pollutants (Girardello et al., 2015; Baranzini et al., 2022), as suitable freshwater experimental model. In particular, our aim was to investigate the short-term effects of less studied emerging PFAS, such as hexafluoropropylene oxide dimer acid (HFPO-DA), along with their associated by-products perfluoro-3-methoxypropanoic acid (PFMOPrA) and perfluoro (4-methoxybutanoic) acid (PFMoBa) on the leech inflammatory response, including also perfluorooctanoic acid (PFOA) as a reference legacy PFAS, whose toxicity is already well-documented, as previously described (Hagenaars et al., 2013; Li et al., 2017).

Indeed, while legacy PFAS have established health risks, emerging compounds may pose similar or new effects that are not yet fully understood, making ongoing research crucial to assess their environmental impact. Since 2013, the presence of HFPO-DA, developed as a replacement for PFOA, has been widely detected in aquatic ecosystems. As observed in various countries, such as China, the Netherlands, and the USA, recorded levels of HFPO-DA are often much higher than those of the main legacy fluorinated chemicals. Furthermore, analyses of biological matrices from the same areas reveal that HFPO-DA possesses a high bioaccumulation rate. Not only has it been identified in different fish (muscle, blood, and liver) and plant samples (grass, leaf), but also in human serum and urine (Mullin et al., 2019). Similarly, although little information is currently available about PFMOPrA and PFMoBa pollution, their accumulation in living organisms has already been determined (Song et al., 2022), highlighting the importance of studying these PFAS compounds.

However, these emerging compounds, such as HFPO-DA, PFMOPrA, and PFMoBa remain less studied in terms of their toxicological effects on immune and inflammatory responses in living organisms. These fluoropropylene oxides share similar chemical features with PFOA but differ in carbon chain length and the presence of ether groups, which may alter their biological effects. Despite these modifications, the potential immunotoxicity of these compounds is not yet fully understood, particularly in aquatic organisms like *H. verbana*. Therefore, it is crucial to investigate how these emerging PFAS compare to PFOA in terms of their biological impacts.

Our hypothesis is that the biological effects of these PFAS compounds are influenced by their molecular structure, particularly carbon chain length and the presence of oxygen atoms (ether groups). We expect that PFOA, with its longer carbon chain, will elicit stronger immune and

inflammatory responses compared to the shorter-chain fluoropropylene oxides (HFPO-DA, PFMOPrA, and PFMoBa), which are being tested as potential safer alternatives. This study aims to assess these differences in immune response and inflammation in the freshwater invertebrate *H. verbana*.

2. Materials and methods

2.1. Animals and treatments

Medicinal leeches (*H. verbana*, Annelida, Hirudinea), deriving from the ILFARM breeding (Italian Leech Farm ILFARM, Varese, Italy), were kept in lightly salted water (NaCl 1.5 g/L) at 20 °C in aerated tanks. For the experiments, adults animals (10 cm in length) were randomly divided into separate experimental groups and subjected to different treatments. For each condition, defined by the specific PFAS concentration and the time point analyzed, a total of five animals has been used, and all the experiments were performed in triplicate. Except for the more apical cephalic and posterior regions, leech presented a consistent body structure along their entire length, with identical anatomical features observable across different sections. Since PFAS exerted their effects uniformly throughout the body, small fragments, only a few millimeters in size, were collected from each individual for the various analyses. This approach allowed multiple samples to be taken from the same animal, providing sufficient biological material for all the planned assays.

After 1 week of PFAS exposure, leeches were anesthetized by immersion in 10% ethanol solution, until they appeared completely asleep and sacrificed.

- Group 1: Leeches exposed to isopropanol [$(CH_3)_2CHOH$] (0.005%), used as control. The same concentration of isopropanol has been used to diluted all the PFAS tested. For this reason, control experiments were necessary to exclude any possible side effects induced by this compound.
- Groups 2–3: Leeches treated for 1 week with 0.6 μM or 229 μM of ammonium perfluoro(2-methyl-3-oxahexanoate) (HFPO-DA), diluted in 0.005% isopropanol.
- Groups 4–5: Leeches treated for 1 week with 0.6 μM or 229 μM of perfluoro-4-methoxybutanoicacid (PFMoBa), diluted in 0.005% isopropanol.
- Groups 6–7: Leeches treated for 1 week with 0.6 μM or 229 μM of perfluorooctanoic acid (PFOA), diluted in 0.005% isopropanol.
- Groups 8–9: Leeches treated for 1 week with 0.6 μM or 229 μM of perfluoro-3-methoxypropanoic acid (PFMOPrA), diluted in 0.005% isopropanol.

The specific PFAS concentrations used in this study were selected to assess short-term ecotoxicological effects, within the framework of an EU project on PFAS (https://scenarios-project.eu). This subacute (sublethal) range spans from submicromolar concentrations, of levels found in contaminated soil and leachate at typical PFAS hotspots (Bräunig et al., 2019; Hamid et al., 2018).

2.2. Light microscope morphological analyses

Leech tissues were dissected and fixed in 4% glutaraldehyde, diluted in 0.1 M cacodylate buffer (pH 7.4), for 2h at room temperature. After several washings in the same buffer, samples were post-fixed in 2% osmium tetroxide (OsO_4) solution for 1h in the dark. Subsequently, tissues were washed in 0.1 M cacodylate buffer, and dehydrated with increasing ethanol concentrations (30%, 50%, 70%, 90%, 96%, 100%). Samples were then transferred into a solution of propylene oxide and resin (1:1 ratio) for 1h, and finally embedded in an Epon-Araldite 812 mixture epoxy resin (Sigma Aldrich, Milan, Italy). Semithin sections, prepared for light microscopy (750 nm in thickness), have been obtained with a Reichert Ultracut S ultratome (Leica, Nussloch, Germany), collected on a slide and then stained with crystal violet (1g in 100 mL of distilled water) and basic fuchsin (0.13g in 100 mL of distilled water). Samples were finally observed under a light microscope (Eclipse Nikon, Amsterdam, Netherlands), and data recorded with a DS-5M-L1 digital camera system (Nikon, Tokyo, Japan).

2.3. Immunofluorescence assays

All the collected samples were instantly included into OCT (Polyfreeze, TebuBio, Le Perray-en-Yvelines, France), frozen in liquid nitrogen, and stored at −80 °C. Cryosections (7 μm in thickness) have been obtained with a cryostat (Leica CM1850), collected on gelatinated slides, and held at −20 °C. The obtained cryosections were then rehydrated for 10min in PBS (138 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 – pH 7.4) and incubated for 30min in the BSA blocking solution (2% Bovine Serum Albumin, 0.1% Tween diluted in PBS). BSA was also used to dilute both primary and secondary antibodies. Samples were incubated for 1h at room temperature with the primary antibodies (Supplementary Table 1) and after several washes in PBS buffer, tissues were incubated for 45min with secondary antibodies conjugated with fluorescein isothiocyanate (FITC, dilution 1:250, ThermoFisher scientific, Waltham, MA, USA). The cell nuclei were then counterstained with 4,6-diamidino-2-phenylinedole (DAPI) 0.1 mg/mL diluted in PBS for 5min and slides were mounted with PBS/Glycerol Cityfluor (PBS/Glycerol, Cityfluor, London, UK). Negative control experiments were performed by omitting primary antibodies. All the samples were examined with fluorescence microscope (Nikon Digital Sight DS-SM, Tokyo, Japan), and the staining visualized using excitation/emission filters of 490/525 nm for FITC, and 340/488 nm for DAPI. The obtained images were then combined using Adobe Photoshop (Adobe System, Inc.).

2.4. RNA extraction and quantitative PCR (qPCR)

Entire leech body wall sections were instantly frozen in liquid nitrogen and homogenized with a pestle and mortar, further resuspended and lysed in 1 mL of Trizol™ reagent (ThermoFisher scientific, Waltham, MA, USA) by multiple pipetting, and total RNA extracted according to the manufacturer instructions, finally resuspended in 30 μL of RNAse free water. Samples were then quantified and the RNA purity evaluated by means of high-resolution 1.5% agarose gel electrophoresis under denaturing conditions. 2 μg of RNA were retrotranscribed into cDNA using M-MLV reverse transcriptase (ThermoFisher scientific, Waltham, MA, USA) in the presence of oligodT (Invitrogen, Thermo Fisher Scientific, length: 16bp) in a final volume of 20 μL. Quantitative real time PCR (qPCR) was carried out in triplicate in a CFX Connect Real Time PCR Detection System (Bio-Rad) using the iTaq Universal SYBR™ master mix (BioRad, Richmond, USA) and 0.2 μM each forward and reverse primer, in final a volume of 15 μL. After initial denaturation, the PCR reaction was performed at 95 °C (10s), 60 °C (5s), and 72 °C (10s) for 39 cycles. Relative gene expressions were calculated using the ΔΔCt method, and GAPDH (D-glyceraldehyde-3-phosphate dehydrogenase) as reference gene (Baranzini et al., 2020; Baranzini et al., 2020b). The primers used for qPCR amplifications are reported in Supplementary Table 2. All the primer sequences possessed an amplification efficiency of 99%.

2.5. Acid phosphatase assay (ACP)

As reported by Grimaldi (2016), cryosections were rehydrated in PBS physiological solution for 10min, incubated for 5min in 0.1 M acetic acid-sodium acetate buffer and subsequently with the reaction mixture (sodium acetate-acetic acid buffer 0.1 M, 0.01% naphthol phosphate, 2% NN-dimethylformamide, 0.06% Fast Red and $MnCl_2$ 0.5 nM) for 90min, at 37 °C. After multiple washings in PBS, slides have been

observed under light microscope after Cityfluor (Cityfluor, London, UK) mounting.

2.6. Statistical analyses

All experiments were performed in triplicate and the presented graphics showed the average values ± standard deviations. The statistical analyses were conducted using the Prism - GraphPad™ 8 software (GraphPad Software, La Jolla, CA, USA) and significant differences were calculated by means of one-way ANOVA variance analysis after verification of normality and homoscedasticity. For each statistical analysis, significant differences were calculated followed by Dunnett's *post hoc* test and values of $p < 0.05$ were considered statistically significant. In the graphs, * means that $p < 0.05$; ** means that $p < 0.01$; *** means that $p < 0.001$. For the count of blood vessels, granulocytes, CD45 fluorescent signal intensity and APC^+ macrophages, ten different slides (random fields of 45.000 μm^2 for each slide) for each experimental group were analyzed using the ImageJ software package (Bethesda, Maryland, USA). In the evaluation of statistically significant relative mRNA abundances, ΔΔCt values between a gene of interest (GOI) and GAPDH in each experimental group were compared to those of the vehicle control in the ANOVA analysis. In the graphs, the averages ± standard deviation (SD) with asterisks represent the significant differences between vehicle control animals and PFAS-treated leeches at different concentration.

3. Results

3.1. Morphological analyses of H. verbana tissues exposed to PFAS

PFAS biological effects on leeches have been first assessed by means of morphological analyses (Fig. 1), in animals treated for 1 week at two different concentrations (0.6 and 299 μM). The body wall organization of animals treated with only vehicle (0.005% isopropanol) used as reference, showed the distinctive anatomical structure of control leeches, in which few blood vessels and resident immune cells were detectable close to the epithelium and in the inner part of the musculocutaneous sac Fig. 1A and B). Moreover, the number of both Type 1 and Type 2 mucous cells was reduced in the same area, while muscle fibers resulted well-organized in groups and surrounded by a loose connective tissue. Contrariwise, although the activation of an inflammatory state was traceable in all the animals treated with the different PFAS congeners, distinct biological effects have been observed during the acute phase exposure (Fig. 1C–N). Indeed, despite the number of both new blood vessels and immune or mucous cells generally increased in all samples compared to controls, different behaviors have been observed. In leeches exposed to the lowest concentration (0.6 μM) of both HFPO-DA (Fig. 1C) and PFMoBa (Fig. 1F), a significant recruitment of macrophages and granulocytes was observable. These migrating cells were mainly localized underneath the epithelium and around muscle fibers (Fig. 1E). Instead, the administration of a higher HFPO-DA and PFMoBa concentration (229 μM) (Fig. 1D–G) mainly promoted angiogenic events, with a more evident development of blood circulation (Fig. 1 H). As concern the mucous cells, while the total amount of Type 1 cells remained comparable to that observed in the controls, the quantity of Type 2 increased at both the exposure level tested. These cells appeared elongated towards the external cuticle, indicating an active secretion. Moreover, the presence of mucous cells situated more internally, away from the commonly detected area, suggested an ongoing renewal process due to continue mucous secretion.

As regards PFOA (Fig. 1I–K) and PFMOPrA (Fig. 1L–N), a completely opposite trend has been observed. Indeed, a greater formation of new blood vessels was evident particularly at 0.6 μM (Fig. 1I–L), indicating that both these congeners promoted angiogenic processes at lower concentrations. In addition, a minor proliferation of both macrophages and granulocytes was visible in these samples, whose quantity only rose at the highest tested concentrations (229 μM) (Fig. 1J–M). Instead, a strong activation of Type 2 mucous cells was observable in these samples, especially after PFOA exposure, as well as in HFPO-DA and PFMoBa treated samples.

3.2. Activation of the angiogenetic response

Based on the conducted morphological analyses, the total amount of the blood vessels has been evaluated and the obtained results have been summarized in Fig. 2A. Compared to HFPO-DA and PFMoBa, in which a significant increase of newly formed blood vessels was mainly detectable at the highest concentration used (229 μM), both PFOA and PFMOPrA were able to significantly activate angiogenic processes already at the lowest dose (0.6 μM), confirming the different effects induced by these perfluorinated compounds. These data were also supported by immunofluorescent analyses, in which a specific antibody for the endothelial marker CD31 has been used (Fig. 2B–K). Indeed, in the vehicle treated samples (Fig. 2B), a basal CD31 signal was detectable indicating the presence of few blood vessels underneath the epithelium and surrounding muscle fibers. By contrast, the presence of $CD31^+$ cells enhanced in the PFAS congener treated samples. A greater increase was observable after HFPO-DA and PFMoBa treatment in a dose-dependent manner (Fig. 2D–G) as expected, while both PFOA and PFMOPrA largely stimulated endothelial cells proliferation at the lowest concentration (0.6 μM) (Fig. 2H–K). No signal were detected in mock experiments, in which primary antibody was omitted (Fig. 2C).

3.3. Recruitment of granulocytes

Given the previous morphological results, cell counts and immunofluorescent analyses have been performed using an anti CD11b antibody, specific for detecting leech granulocytes, to verify the recruitment of these immune cells as a response to PFAS insults. As shown in Fig. 3A, the number of these immune-related cells significantly increased due to PFAS exposure. In detail, a greater number of these immune cells could be observable at the lowest concentration (0.6 μM) after HFPO-DA and PFMoBa treatment, while PFOA and PFMOPrA mainly stimulated their proliferation at the highest level. This evidence were also confirmed by means of immunohistochemistry (Fig. 3B–K). In vehicle treated samples (Fig. 3B), a weak CD11b signal was visible. Differently, the positivity for CD11b appeared clear in both HFPO-DA and PFMoBa-treated animals (Fig. 3D–G), in which positive cells were mainly visible underneath the epithelium and in the more external area of the body wall. Similarly, the CD11b signal rose after PFOA and PFMOPrA administration (Fig. 3H–K), in which a greater signal was observed at 229 μM (Fig. 3H–J) No signals were detected in negative control experiments, in which primary antibody was omitted (Fig. 3C).

3.4. Recruitment of CD45-positive cells

Immunofluorescence experiments were also performed using the anti-CD45 antibody (Fig. 4), in order to evaluate the ability to recruit innate immune cells belonging to the monocytic-macrophage cell line, usually involved in the modulation of the leech inflammatory response.

Compared to control leeches (Fig. 4A), during the acute exposure a remarkable improvement of the fluorescent signal was clearly detectable (Fig. 4C–J). An increase in the CD45 positivity was visible in samples treated with both 0.6 μM and 229 μM concentrations of HFPO-DA, PFOA and PFMOPrA (Fig. 4C and D,G-J), revealing a significative $CD45^+$ cells recruitment. As regards PFMoBa (Fig. 4E,F), a higher positivity was mainly detected at a 229 μM concentrations (Fig. 4E and F). The graph showed the total fluorescent CD45 signal calculated in each sample (Fig. 4K). No signals were highlighted in negative controls (Fig. 4B).

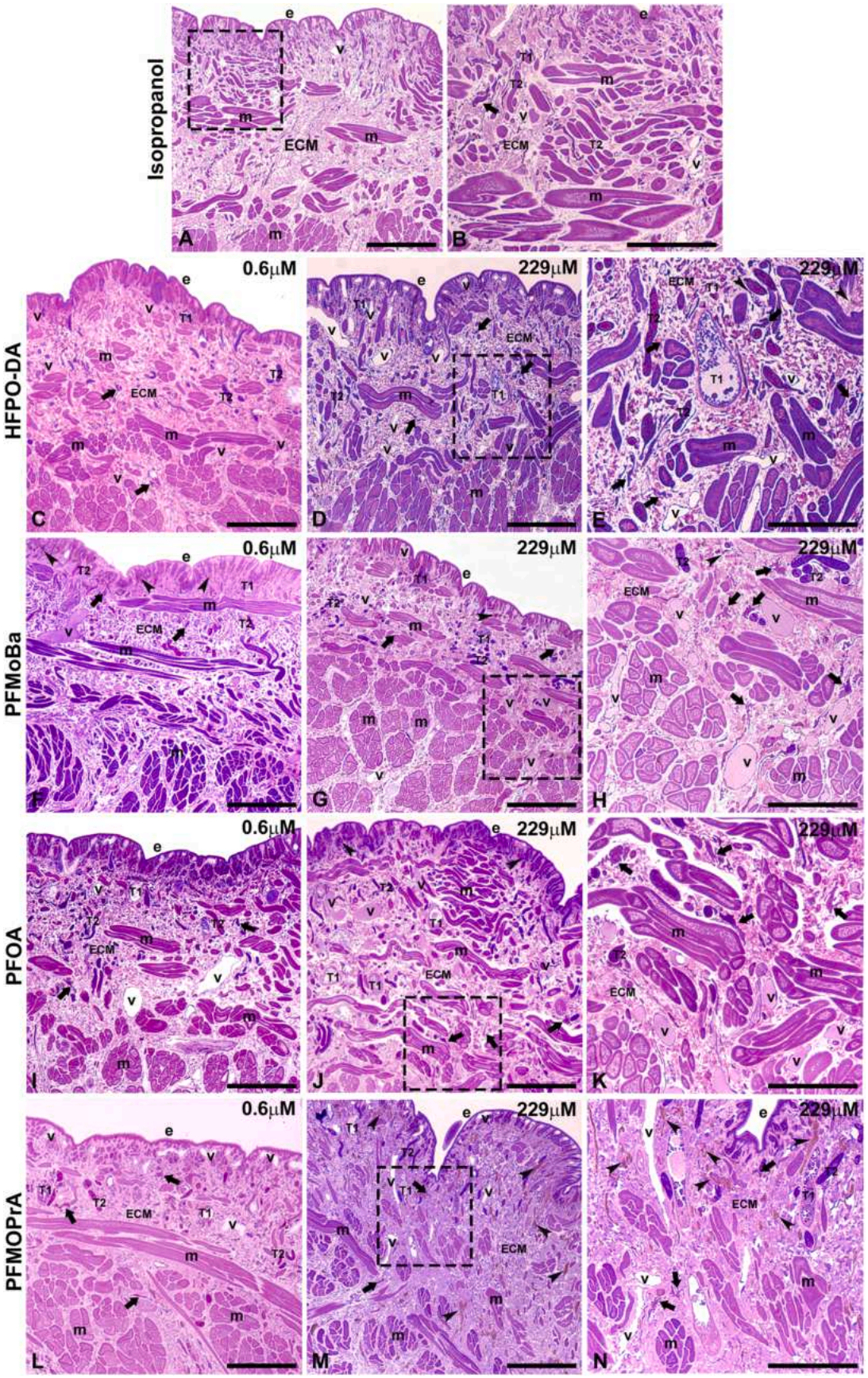


Fig. 1. Morphological analyses of isopropanol control and PFAS treated leeches following 0.6 μM and 229 μM exposure. Details at higher magnification of isopropanol control (B) and PFAS 229 μM treated (E,H,K,N) samples are referred to dotted squares. Bars in A, C-D, F-G, I-J, L–M: 100 μm; bars in B,E,H,K,N: 50 μm. ECM: extracellular matrix; e: epithelium; v: blood vessels arrows: macrophages; arrowheads: granulocytes; T1: Type 1 mucous cells; T2: Type 2 mucous cells.

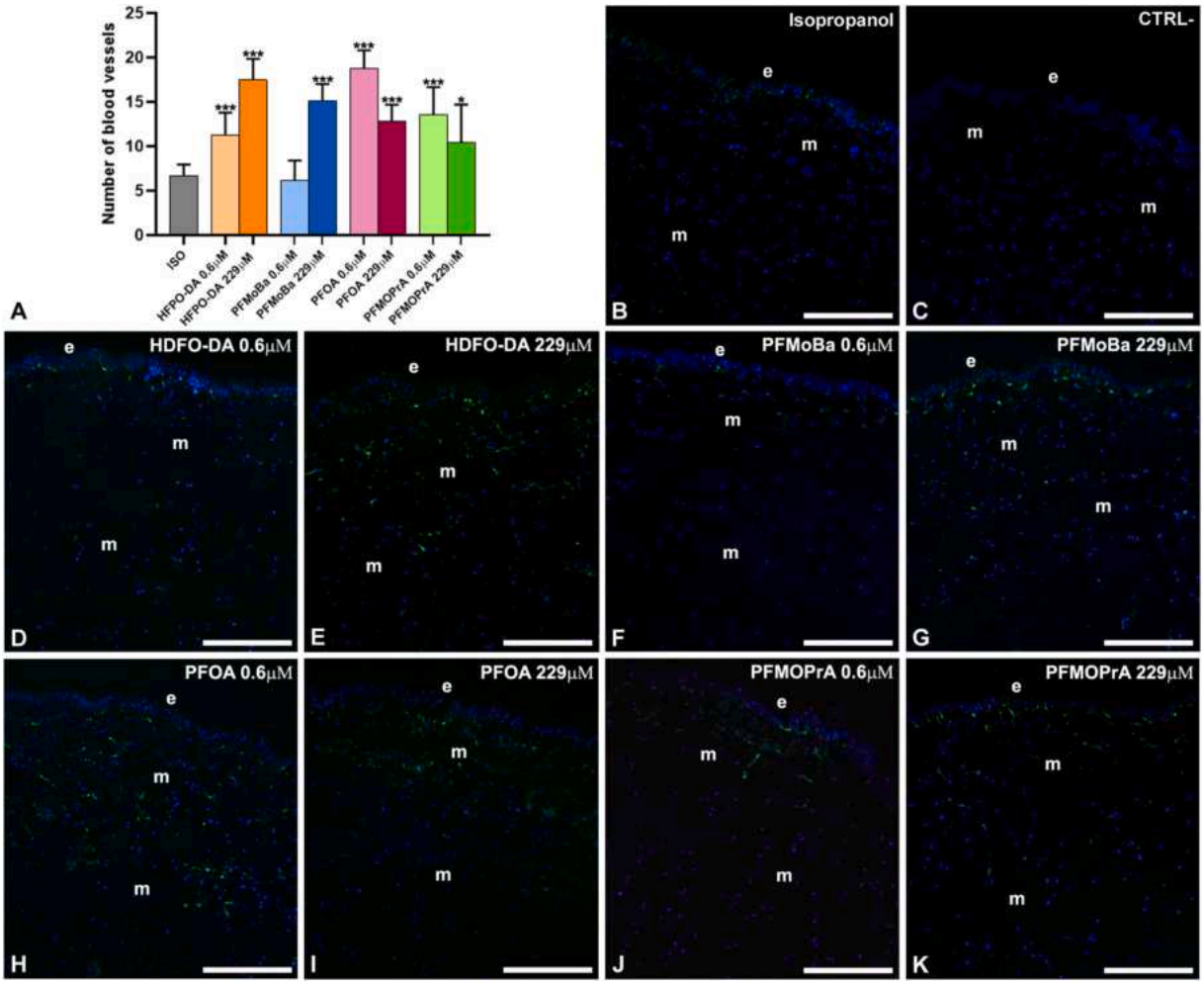


Fig. 2. Evaluation of angiogenic induction following acute PFAS exposure (A–K). The graph indicates the number of blood vessels observed by means of morphological analyses in different treated samples (A). Immunofluorescence analyses using anti-CD31 antibody (B–K). Cells nuclei are stained with DAPI and no signals are visible in negative control experiments (C), in which primary antibody is omitted. Bars in B–K: 100 μm. e: epithelium; m: muscles fibers.

3.5. Recruitment of ACP $^{+}$ macrophages

The acid phosphatase (ACP) histochemical enzymatic assay was conducted to reveal the presence of activated phagocytic cells, such as macrophages. During the acute PFAS response, in vehicle-treated leeches (Fig. 5A), only a few ACP^{+} cells were detectable underneath the epithelium and in the inner tissues. Whereas the number of ACP positive cells increased in almost all PFAS treated samples, the effects of HFPO-DA (Fig. 5B and C) and PFMoBa (Fig. 5D and E) were statistically significant only at the lower tested level. By contrast, in tissues exposed to PFOA (Fig. 5F and G) and PFMOPrA (Fig. 5H,I), a greater effect was determined at 229 μM, in which many activated cells were noticeable also in the inner part of the musculocutaneous sac, migrating from this district toward the epithelial layer. These data were summarized in the graph based on the ACP^{+} cell count, in which the number of positive cells has been determined (Fig. 5J).

3.6. Gene expression profiling

To assess PFAS modulation of inflammatory response genes, we evaluated the relative mRNA abundances of the RNASET2 and AIF-1 inflammatory factors by QPCR (Fig. 6). Higher expression levels of the RNASET2 transcript were detected across all conditions except for HFPO-DA and PFMoBa at 0.6 μM (Fig. 6A). Conversely, these two conditions exhibited higher mRNA relative abundances of AIF-1, alongside exposure to PFMOPrA and PFOA at 229 μM (Fig. 6B). Furthermore, the expression levels of both superoxide dismutase (SOD) and glutathione-*s*-transferase (GST), have been assessed (Fig. 7). The expression of SOD varied in response to PFAS treatment, showing distinct trends. SOD expression significantly increased at HFPO-DA and PFMOPrA 0.6 μM, as well as at PFMoBa 229 μM and both levels of PFOA (Fig. 7A). Conversely, GST production significantly enhanced across all concentrations analyzed, except for HFPO-DA 229 μM and both PFMOPrA conditions (Fig. 7B).

4. Discussion

Due to the anatomical and structural similarities and the elevated conservation of both biological processes and related molecules involved shared with vertebrates, the medicinal leech has recently found extensive use as innovative experimental models in diverse fields of life science. Despite this invertebrate species might not be as commonly associated with scientific research as other model organisms like fruit fly or nematode, it possesses unique characteristics that make it useful for investigating the impact of environmental factors on freshwater organisms (Grimaldi, 2016; Baranzini et al., 2020a). Indeed, given the highly sensitivity to changes in water quality and habitat conditions, leeches play an important role in aquatic ecosystems and may act as environmental monitors. As already demonstrated in recent studies, the impacts induced by hazardous substances are observable by evaluating tissues,

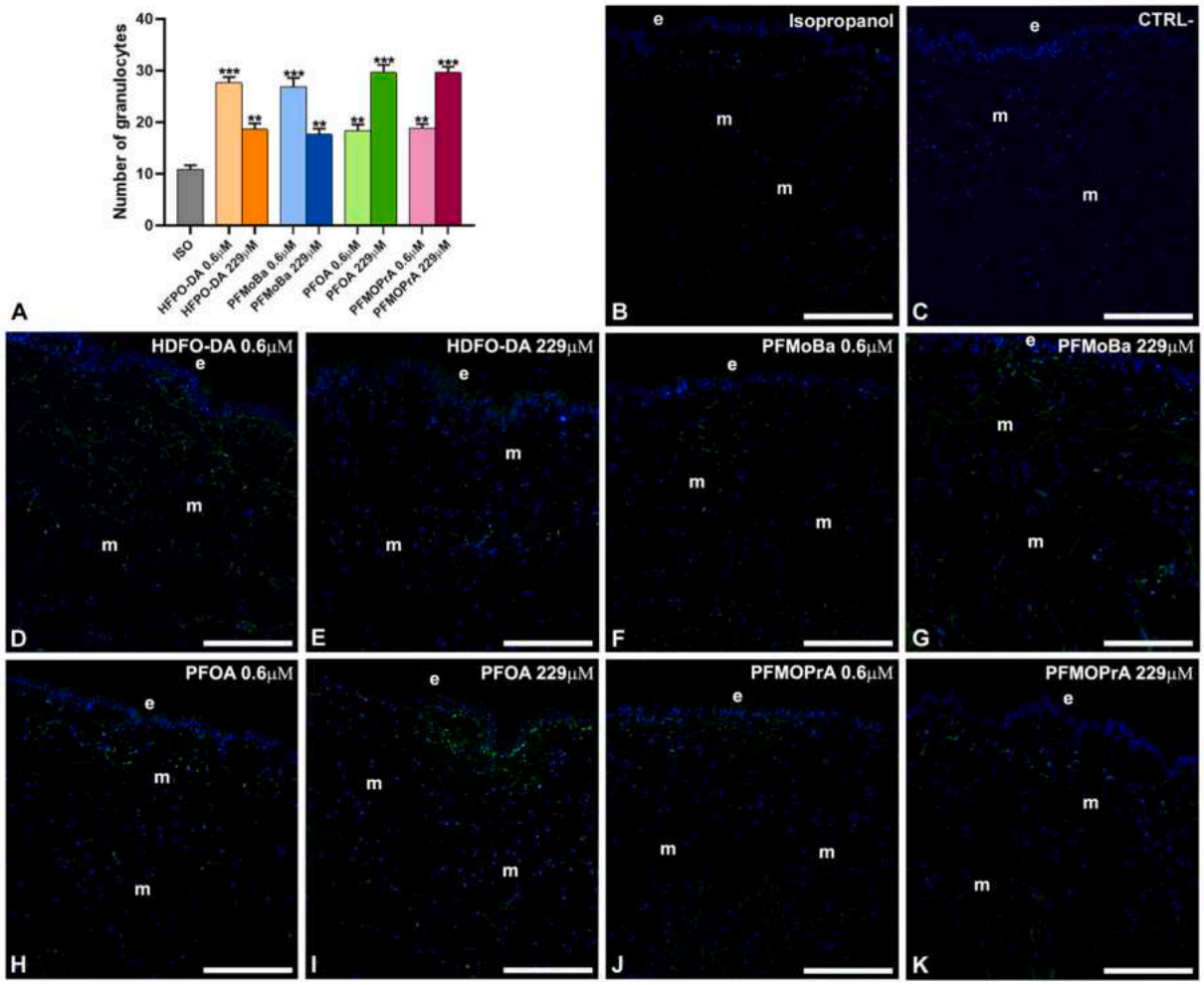


Fig. 3. Evaluation of type I granulocytes recruitment following acute PFAS exposure (A–K). The graph indicates the number of granulocytes observed by means of morphological analyses (A). Immunofluorescence analyses using anti-CD11b antibody (B–K). Cells nuclei are stained with DAPI and no signals are visible in negative control experiments (C), in which primary antibody is omitted. Bars in B–K: 100 μm. e: epithelium; m: muscles fibers.

cells, and molecular responses (Grimaldi, 2016; Baranzini et al., 2022; Girardello et al., 2015, 2017; Pirillo and Baranzini, 2022). In particular chemicals infiltrate the external cuticle and muscle fibers, leading to the formation of numerous blood vessels and recruitment of immune precursors to the affected areas. This inflammatory response can result in the proliferation and differentiation of immune cells, such as macrophages, supporting the innate immune response (Grimaldi, 2016).

In this study, the medicinal leech *H. verbana* has been chosen for assessing the potential toxic effects of PFAS to freshwater organisms, investigating the short-term effects of less studied emerging PFAS compounds, such as hexafluoropropylene oxide dimer acids such as HFPO-DA, also known as Dupont-GenX), along with their associated by-products perfluoro(4-methoxybutanoic) acid (PFMOBA) and perfluoro-3-methoxypropanoic acid (PFMOPrA). We also included perfluorooctanoic acid (PFOA) as a reference legacy PFAS, whose cancerogenic effects have been recently assessed by IARC (Zahm et al., 2024). Thus, to obtain a comprehensive overview of the generated outcomes, two broad concentrations i.e., 0.6 μM and 229 μM have been tested during leech acute response. These concentrations are consistent with previous studies that tested sub-micromolar and higher concentrations of PFAS in environmental hotspots, such as firefighting sites and manufacturing districts (Rotondo et al., 2024). Although these concentrations are relatively high compared to those found in typical freshwater environments, it is important to note that this is a short-term ecotoxicological study. Such concentrations are commonly used to derive points of departure for further classification of impact outcomes and to inform risk assessment and regulatory guidelines.

Based on these premises, here the attention has been focused on the leech inflammatory response, demonstrating as PFAS were differently able to modulate and to induce both cellular and molecular immune mechanisms, leading to an increased expression of inflammatory and oxidative stress markers (Baranzini et al., 2019, 2022; Girardello et al., 2015; Pirillo and Baranzini, 2022). In the medicinal leech, the first barrier with the external environment is represented by a thin cuticle, from which an abundant amount of mucus is produced when the organism is stressed or when it comes into contact with harmful substances. Two different typologies of mucous cells (Type 1 and the Type 2) are involved in this process, which normally present a rounded shape that indicates an inactive state. Type 1 mucous cells are more closely localized to the epidermis and they are the lightest cells stained by means of histochemical analyses, while the Type 2 are more internally organized in groups near muscle fibers (Baranzini et al., 2022; Gül and Çakıcı, 2022). At the lowest PFAS concentration tested (0.6 μM), although the number of Type 1 cells remains almost unchanged and comparable with reference control animals, numerous elongated Type 2 cells become more visible underneath the epithelium. These cells are recognizable by the dark staining and they are normally found in the inner parts of the body wall. Their elongation towards the cuticle indicates that these cells are actively involved in the mucus production (Baranzini et al., 2022; Gül and Çakıcı, 2022). A similar result is

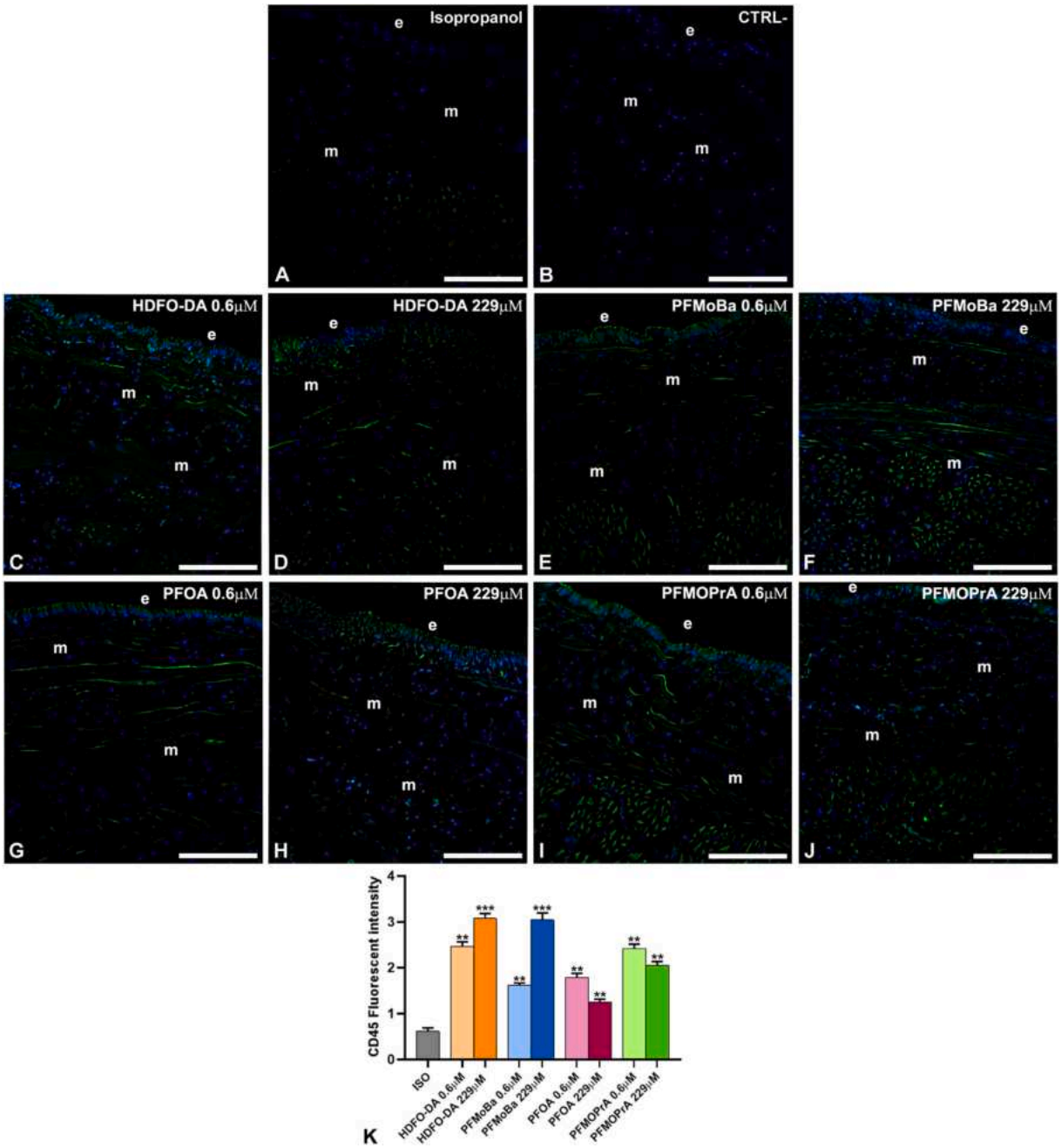


Fig. 4. Evaluation of type CD45^{+} hematopoietic precursors cells recruitment following acute PFAS exposure (A–K). Immunofluorescence analyses using anti-CD45 antibody (A–J). Cells nuclei are stained with DAPI and no signals are visible in negative control experiments (B), in which primary antibody is omitted. Graph showing the total amount of fluorescent CD45 fluorescent signal calculated in each sample Bars in A–J: 100 μm. e: epithelium; m: muscles fibers.

definitely noticeable also after testing higher concentrations (229 μM), suggesting that PFAS leads to mucous secretion and induce a continuous cells renewal. Interestingly, as already observed for other treatments such as polypropylene plastic particles (Baranzini et al., 2022), both cell types appear strongly activated, suggesting that serial waves of secretion are produced for limiting the persistent entry of the PFAS compounds and for isolating the inner microenvironment. Type 2 cells not only secrete mucous, but they seem to be also involved in the expression of important antioxidant enzymes in leeches. To this end, quantitative PCR analyses confirmed that PFAS exposure can lead to an increment in the expression of GSTA4 and SOD. The first comprises a large supergene family, whose best-known function is to catalyze the reactions involving the conjugation of the reduced form of glutathione (GSH) to various xenobiotic substrates with the purpose of detoxification of electrophilic substrates, in order to make these compounds more water-soluble (Boryslawskyj et al., 1988). In particular, GSTA4 is implicated in cell growth, oxidative stress, disease progression and oxidative damages prevention. SOD catalyzes the dismutation of superoxide ion into oxygen and hydrogen peroxide. This enzyme is ubiquitously found in living organisms and three forms are known in vertebrates, each of which relies on the metal cofactor involved in its active site (Kim et al., 2011). After PFAS acute exposure, the expression of these enzyme coding-genes were significantly higher in almost all the experimental conditions and the continuous activation of Type 2 mucous cells might be correlated. Several studies already highlighted the induction of oxidative stress by PFAS (Arukwe and Mortensen, 2011; Liu et al., 2023). The relative abundances of SOD mRNA were significantly higher in response to PFOA, reflecting an oxidative stress response. However, HFPO-DA and

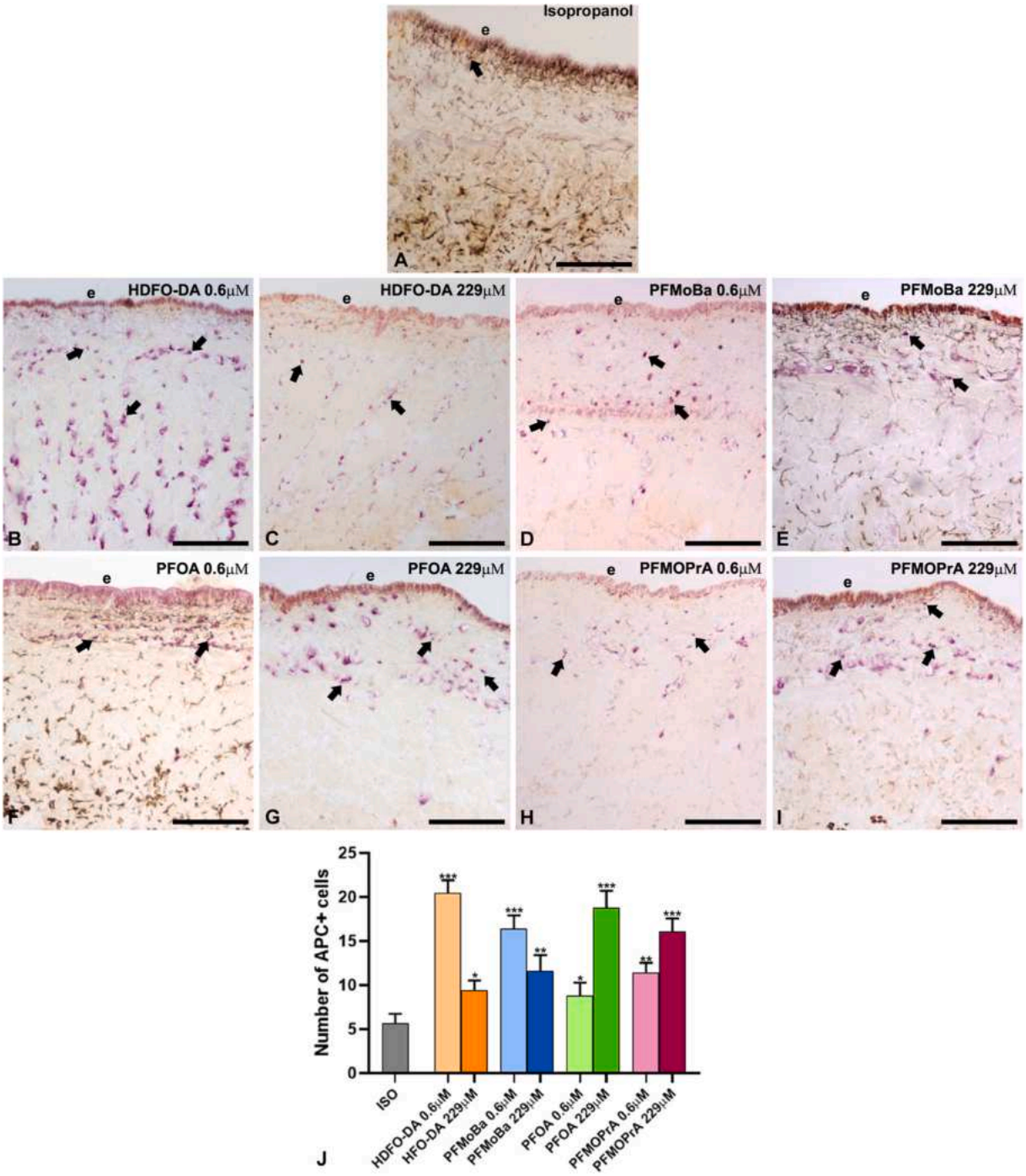
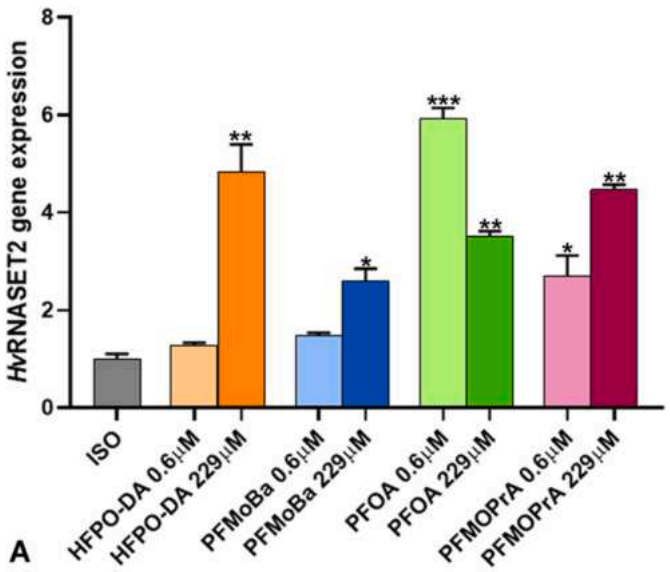
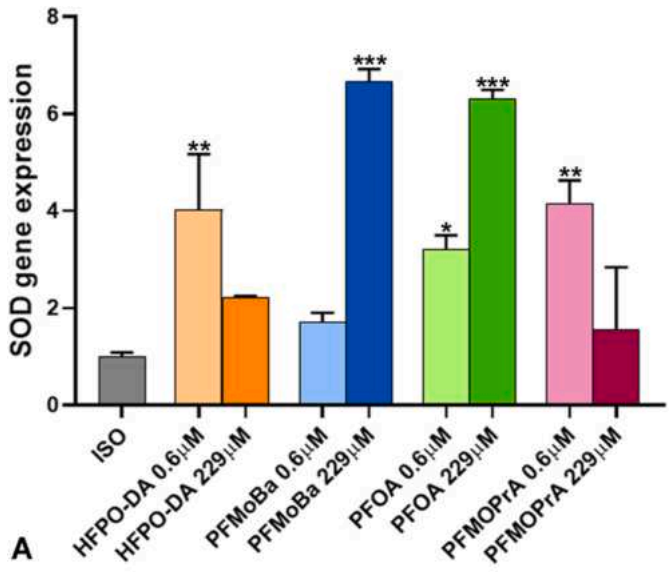


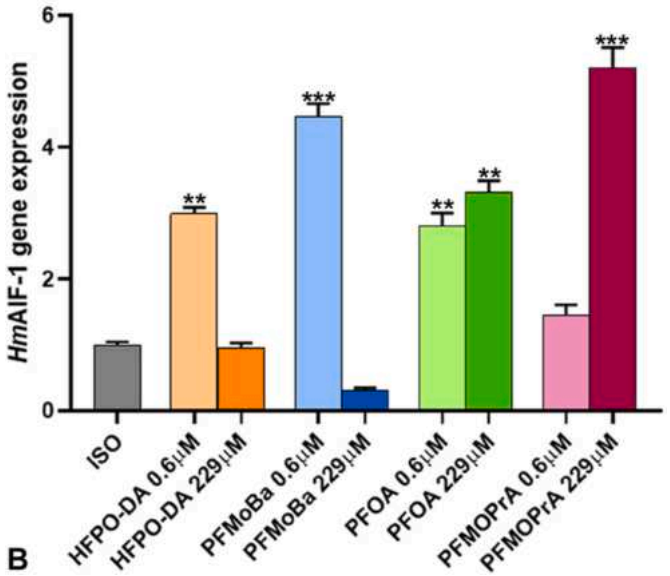
Fig. 5. Acid phosphatase assay (ACP) for the phagocytic cells recruitment evaluation following acute PFAS exposure (A–J). The total number of ACP-positive cells observed is reported in the graph (J). Bars in A–I: 100 μm. e: epithelium; arrows: ACP-positive macrophages.

PFMOPrA did not induce significant changes in SOD mRNA expression at lower concentrations. By contrast, at higher concentrations, all three compounds (PFOA, HFPO-DA, and PFMOPrA) resulted in a significant reduction in SOD mRNA levels, suggesting that excessive ROS production overwhelms the antioxidant defenses and suppresses SOD enzyme activity (Wang et al., 2021; Rotondo et al., 1826).

Morphological analyses further revealed that, although all examined PFAS compounds are able to stimulate an inflammatory response they differently promote immune cells recruitment and angiogenesis, depending on timings and concentration analyzed. During HFPO-DA and PFMoBa acute exposure at the lower concentration (0.6 μM), a more consistent enlistment of immune cells arises. This data is also confirmed by immunofluorescent and immunohistochemical analyses, in which the presence of numerous $CD11b^{+}$ granulocytes and ACP^{+} macrophages could be clearly detectable. At a concentration of 229 μM, a more significantly improve in the blood vessels number occurs. Instead, as concerns immune cells, only the number of granulocytes increases while that of macrophages appears reduced, suggesting that PFAS can differently influence the innate immune system response. Interestingly, these results validate previous experimental analyses already present in the literature, which demonstrated a dose-dependent impact of several PFAS substances on the immune activation. As beforehand noted in various organisms, elevated concentrations are often associated with statistically significant decreases in common immune







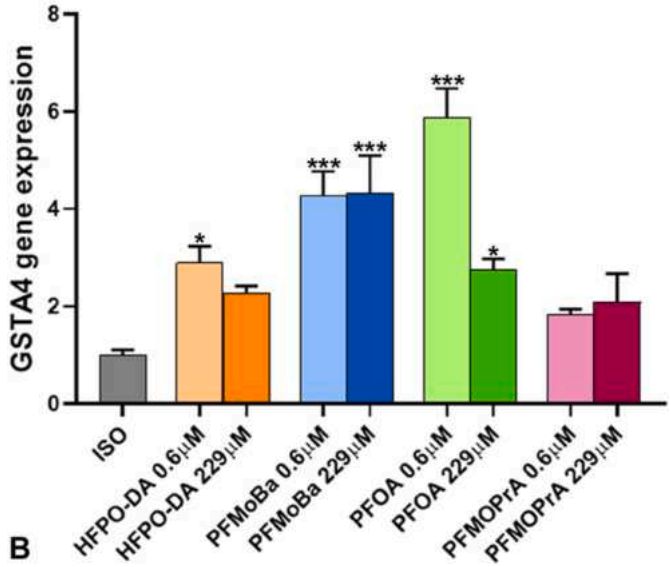


Fig. 6. *Hv*RNASET2 and *Hm*AIF-1 qPCR analyses (A–B). The graphs show the expression of both genes in isopropanol control tissues or in PFAS treated samples. D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is used as housekeeping reference gene.

Fig. 7. Superoxide dismutase (SOD) and Glutathione-S-transferase (GST) qPCR analyses (A–B). The graphs show the expression of both genes in isopropanol control tissues or in PFAS treated samples. D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is used as housekeeping reference gene.

functions, leading to a negative immunomodulation of the cells activity (Antoniou et al., 2022; Liu and Gin, 2018; Ma et al., 2023; Yang et al., 2023). It has been observed that PFMoBa is able to reduce the numbers of B and Natural Killer (NK) cells decreased in mice (Woodlief et al., 2021). Moreover, other PFAS negatively affect both innate and adaptative immune cells activation, compromising also the expression of cytokines and chemokines (Maddalon et al., 2023).

Instead, the leech treatment with PFOA and PFMOPrA induced an intense inflammatory response, whose effects are greater with the rise in concentration used. Indeed, both morphological and immunofluorescent assays demonstrate that a rapid activation of the angiogenic processes occurs, which lead to an immediate rise in the number of newly formed blood vessels, followed by an innate immunity triggering the abundant recruitment of different immune cells. Specifically, although the administration of both PFOA and PFMOPrA initially caused a significant increase of the inflammatory response at the lower concentrations (0.6 µM), a noteworthy difference is visible at 229 µM. These data could be confirmed by both histological analyses and immune-histochemical data using the specific CD31-antibody. *In vitro* three-dimensional experiments conducted on human HTR8/SVneo cell line, an embryonic trophoblast cell type that resemble endothelial cells, showing a PFAS strong ability to generate vascular-like networks in 3D matrices, revealed a similar outcome. Indeed, PFOA, and not PFOS, was able to induce morphological changes via the NOTCH pathway, reducing the sprouting and promoting the elongation of the isolated stalks of these cells in pseudo-vascular networks (Poteser et al., 2020). The ability of PFAS to positively or negatively affect the formation of new blood vessels has been already observed in vertebrates (Forsthuber et al., 2022). In leeches, the formation of these new blood vessels is necessary to recruit immune precursors belonging to the monocytes-macrophage cell line, which migrate into the bloodstream to reach all the body wall districts and differentiate into mature immune cells (Grimaldi, 2016). By

amplifying the concentrations of both PFOA and PFMOPrA, an increment in the CD45 signal could be also observed in a dose-dependent manner. In this context, a positive association between PFAS, such as PFOA or PFOS, and monocytes has been observed in the blood collected from patients exposed to contaminated water in the Mid-Ohio Valley (Emmett et al., 2006; Lopez-Espinosa et al., 2021). However, it must be considered that depending on the PFAS considered and the cell types examined, fluorinated compounds differently affect their number or viability. Also in this case, opposite results have been obtained and reported in the literature (Lopez-Espinosa et al., 2021). In this case, the ability of PFOA and PFMOPrA to modulate immune response is confirmed, revealing a strong recruitment of both $CD11b^{+}$ granulocytes and $CD45^{+}$ precursors cells, which then specifically differentiate into macrophages, as confirmed by ACP assay. Interestingly, making a comparison between PFOA and PFMOPrA, our results demonstrate that the first compound can induce a stronger response.

The induction of an inflammatory response in leeches is also supported by molecular analyses, in which the expression levels of *Hv*RNASET2 and *Hm*AIF-1 pro-inflammatory factors have been analyzed by means of qPCR analysis. These two genes play a pivotal role as alarmin or cytokine respectively, enhancing innate immune response (Baranzini et al., 2019, 2020a; Drago et al., 2014; Schorn et al., 2015). Although no significant differences are detected for the exposure to HFPO-DA and PFMOBA at 0.6μM, *Hv*RNASET2 is highly expressed in all the other samples during acute response. Instead, *Hm*AIF-1 mRNA is mainly expressed only after HFPO-DA or PFMoBa challenge at 0.6 μM or PFOA and PFMOPrA at 229 μM. These discordant results indicate that these two factors are differently involved in PFAS cellular response. Indeed, the *Hv*RNASET2 expression is closely related to granulocytes, whose number increased in a dose-dependent manner, while *Hm*AIF-1 rise followed macrophages recruitment, resulting highly expressed only in those samples in which also the number of ACP^{+} cells increased. As previously mentioned, a reduction in the cytokine expression and consequent immune cell activation has been already observed both *in vivo* and *in vitro* (Maddalon et al., 2023). Moreover, these data are in accordance with previous results obtained in leech, in which it has been observed that although these pro-inflammatory molecules are closely connected, their expression is related to different immune cell types (Baranzini et al., 2019; T. Schorn et al., 2015).

As recently observed in a *Daphnia magna* study, PFAS exposure induces oxidative stress, mucus production, inflammation, and immune responses (Villeneuve et al., 2024). *Daphnia* has long been recognized as a model organism for ecotoxicological studies. Interestingly, when integrating these findings, it becomes evident that the leech *H. verbana* can also serve as a valuable model for investigating the effects of PFAS. Indeed, in *Daphnia* as well as in leech transcriptomic analyses revealed genes involved in oxidative stress, innate immune response modulation, mucus secretion and inflammation, highlighting conserved mechanisms across species. All these findings, emphasizing the broad ecological risks posed by PFAS contamination and the importance to better understand the role of emerging fluorinated chemical compounds.

In conclusion, this study reveals that the toxicological effects of PFAS on *H. verbana* are influenced by both carbon chain length and molecular structure, particularly the presence of ether groups in emerging compounds such as HFPO-DA and PFMOPrA. Our initial hypothesis, which focused on carbon chain length as the primary determinant of toxicity, is challenged by the observed effects of these emerging PFAS. While PFOA, with its eight-carbon backbone, consistently induced the strongest inflammatory and angiogenic responses, PFMOPrA, despite being a shorter-chain compound, exhibited more potent effects than expected. This ranking suggests that additional structural factors, such as molecular polarity and the presence of ether groups, play a significant role in modulating toxicity. At lower concentrations, HFPO-DA and PFMOPrA exhibited weaker immune responses compared to PFOA, but PFMOPrA showed a more notable effect than HFPO-DA. At higher concentrations, all three compounds induced comparable toxic effects, with PFMOPrA displaying a stronger overall response than HFPO-DA in terms of immune activation and angiogenesis.

These findings lead us to a refined hypothesis: the toxicity of PFAS compounds is driven not only by carbon chain length, but also by molecular features such as ether groups and polarity. The ranking of toxic effects observed (PFOA > PFMOPrA > HFPO-DA > PFMoBa) indicates that PFMOPrA, despite its shorter chain, may have enhanced bioavailability or stronger interactions with biological membranes, likely due to its specific molecular architecture. While HFPO-DA and PFMoBa exhibited weaker responses, their effects at higher concentrations still indicate a shared toxicological pathway with PFOA and PFMOPrA.

5. Conclusion

The toxicity of PFAS should not be judged solely by chain length, but rather by the entire molecular architecture, including functional groups that affect their bioavailability, membrane interactions, and immune activation potential. This refined understanding has significant implications for the assessment of emerging PFAS, emphasizing the need for holistic evaluation in toxicity studies and regulatory frameworks. It also highlights the need for continued research to fully understand the scope of PFAS toxicity across diverse organisms and ecosystems. Future studies should prioritize long-term exposure effects, elucidating mechanistic pathways, and the development of effective remediation strategies to protect both environmental and human health.

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CRediT authorship contribution statement

A. Calisi: Writing – review & editing, Writing – original draft, Validation, Investigation, Data curation. **N. Baranzini:** Writing – review & editing, Writing – original draft, Validation, Investigation, Data curation. **G. Marcolli:** Writing – review & editing, Methodology, Formal analysis. **C. Bon:** Writing – review & editing, Methodology, Formal analysis. **D. Rotondo:** Writing – review & editing, Methodology, Formal analysis. **D. Gualandris:** Writing – review & editing, Methodology, Formal analysis. **L. Pulze:** Writing – review & editing, Methodology, Formal analysis. **A. Grimaldi:** Writing – review & editing, Writing – original draft, Validation, Conceptualization. **F. Dondero:** Writing – review & editing, Writing – original draft, Validation, Project administration, Funding acquisition, Conceptualization.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2024.143519.

Data availability

No data was used for the research described in the article.

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