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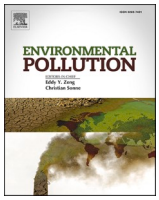

Exposure to PFAS induced differential oxidative stress-related responses in nestlings of two resident passerine birds nearby a fluoropolymer production plant

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ABSTRACT

Per- and polyfluoroalkyl substances (PFAS) are a diverse group of chemicals of growing concern due to their persistence and potential impacts on human and environmental health. While the occurrence and toxicity of several legacy PFAS are relatively well understood, knowledge of emerging PFAS, particularly in free-living organisms, remains limited. This study investigated the occurrence and biological effects of a mixture of thirty-eight legacy and emerging PFAS nearby a Fluoropolymer Production Plant (FPP) located in Northwestern Italy. Legacy and emerging PFAS were measured in eggs of two resident passerine species, the European starling (*Sturnus vulgaris*) and the great tit (*Parus major*), breeding near the FPP and in an agricultural-rural area (AA). Oxidative stress-related biomarkers, including antioxidant and detoxifying enzyme activities and lipid peroxidation, were assessed in nestling blood samples. PFAS were detected in eggs of both the species, reaching concentrations of Σ_{21}PFAS up to 1451 ng/g f.w. in starlings and 6576 ng/g f.w. in great tits. Eggs collected near the FPP showed PFAS levels up to ten-fold higher than those from the AA, with chloro-perfluoropolyether carboxylic acids (Cl-PFPECAs) dominating the contamination profile. Despite similar exposure patterns, biological responses differed between species. No significant effects were observed in great tit nestlings, whereas starling nestlings breeding near the FPP exhibited altered antioxidant defenses and increased lipid peroxidation, indicating they suffered an oxidative stress condition. These findings demonstrate that environmental exposure to PFAS mixtures near an FPP can induce oxidative stress in free-living birds and highlight the need for further field-based studies to improve understanding of PFAS toxicity and support environmental risk assessment.

1. Introduction

Per- and polyfluoroalkyl substances (PFAS) encompass a large, heterogeneous group of synthetic fluorinated chemicals widely used in industrial applications and manufactured goods because of their surfactant and water- and oil-repellent properties. Their persistence, mobility and bioaccumulation potential have led to widespread environmental contamination and increasing concern for ecosystem and human health (Buck et al., 2011; Güzel, 2025). Because of their multifaceted applications and extensive use, diverse PFAS have entered the environment, where some among the thousands of existing PFAS have emerged as a critical issue because of their environmental persistence and bioaccumulative potential (Payne et al., 2024; US EPA, 2024), so that they have nicknamed as 'forever chemicals' (Joerss and Menger, 2023). Long-chain perfluoroalkyl carboxylic acids (PFCA) and perfluoroalkyl sulfonic acids (PFSA) are the groups that have received most attention because of their environmental presence, bioaccumulation potential and toxicity (Zhang et al., 2024). Regulatory restrictions on legacy PFAS such as perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) (Stockholm Convention, 2009; ECHA, 2015) have promoted the use of alternative fluorinated compounds, including short-chain molecules, which are currently unregulated (Dosunmu et al., 2025). Although generally considered less bioaccumulative and toxic compared to their long-chain relatives, these compounds are increasingly detected in the environment and their ecological risks remain poorly understood (Jensen and Warming, 2015; Yao et al., 2018;

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Palazzolo et al., 2022).

Legacy PFAS have been measured in different environmental compartments worldwide, including air, water, sediment, soil and organisms (Zhang and Liang, 2023; Shen et al., 2024). Moreover, PFAS have been detected in many human tissues (Domingo, 2025) as a consequence of diverse exposure pathways, including contaminated drinking water and food (Domingo and Nadal, 2017; Domingo, 2025), consumer products, and occupational exposure (Brase et al., 2021), raising concerns about long-term human health effects (Dimitrakopoulou et al., 2024). However, in recent years, the increase of scientific knowledge on fluorinated compounds and the improvement of analytical techniques have allowed the identification of other PFAS never measured before in the environment, albeit they have been used for many years (Rehman et al., 2023). Short-chain fluorinated replacements and newly identified PFAS can be considered as emerging compounds, whose environmental presence, fate, and potential toxicity are totally (or largely) unknown (Ruan et al., 2022). Some recent studies have shown the occurrence of emerging PFAS in different ecosystems and environmental matrices (Brase et al., 2021; Ruan et al., 2022; Zarębska et al., 2024). In particular, as industrial emissions from fluorochemical facilities (FF) have been identified as a major source of environmental PFAS contamination (Parolini et al., 2022). A recent OECD report has indicated that fluoropolymer manufacturing is associated with complex emission profiles involving not only fluoropolymers themselves but also polymerization aids, residual monomers, impurities, transformation products and other PFAS released throughout different stages of the production process. Consequently, environmental and human exposure close to FF is expected to result from complex mixtures rather than from single compounds (OECD, 2024). The highest concentrations of legacy and emerging PFAS have been consistently reported in groundwater, surface waters, soils, and biota in the surrounding of these areas (e.g., Valsecchi et al., 2015; Morganti et al., 2021; Megson et al., 2024; Zhang et al., 2024; Valsecchi et al., 2026). Despite these findings, knowledge of the risks associated with exposure to emerging PFAS, both singularly and in complex mixture, remains very limited. Whilst the toxicity of legacy PFAS is well established (Antonoupolou et al., 2024), and the toxicity of few emerging ones (e.g., GenX and C6O4) has been documented in aquatic organisms under controlled laboratory conditions (Fabrello et al., 2021a,b; Liu et al., 2022; Rico et al., 2024), the adverse effects of environmentally relevant mixtures on free-living organisms remain poorly understood. Accordingly, comprehensive monitoring programs are urgently needed to improve exposure assessment and elucidate the biological effects of environmentally relevant mixtures of legacy and emerging PFAS in free-ranging organisms. Although field-based investigations are inherently challenged by multiple confounding factors relative to laboratory studies, they are essential to achieve a comprehensive and ecologically realistic assessment of PFAS risk (Ankley et al., 2021).

The present study aimed at monitoring the occurrence and potential adverse effects induced by environmentally relevant PFAS mixture in the surroundings of a Fluoropolymer Production Plant (FPP) located in the Western sector of the Po River valley (Northwestern Italy), compared to an agricultural-rural area (AA). Since the 1980s, this FPP has produced several fluorinated monomers and fluids, as well as fluorocarbon-based polymers (e.g., fluorinated plastomers and elastomers) and PTFE. Specifically, PFOA has been used for a long time in PTFE polymerization as an emulsifier, resulting in high concentrations in FPP discharge (Rusconi et al., 2015) and receiving river water (i.e., Bormida River; Valsecchi et al., 2015). Since 2013, PFOA has been replaced by C6O4 (CAS 1190931-27-1) and in 2020, the measured concentrations of these compounds in the water discharged were <10 μg/L and 50–100 μg/L, respectively (Morganti et al., 2021). In parallel, the processing aid ADV, a polymerization reaction mixture of chloroperfluoropolyether carboxylic acids (Cl-PFPECAs) with multiple isomers (CAS 330809-92-2), has been used in FPP for several decades (Fustinoni and Consonni, 2023) and it was still in use when this study was carried out.

PFAS occurrence was measured in the eggs of two resident passerine bird species, namely the European starling (*Sturnus vulgaris*) and the great tit (*Parus major*), while adverse effects focused on the onset of oxidative stress condition (i.e., changes in oxidative status and oxidative damage) was explored in blood of their nestlings. The European starling is a widespread and highly adaptable passerine species commonly inhabiting urban and industrialized environments, foraging primarily on the ground where feeds soil invertebrates (Cabe, 2020). The great tit is a largely resident species with a small home range living diverse habitats, from agro-forest to urban ecosystems, making it especially suitable for evaluating local anthropogenic contamination (Van den Steen et al., 2009). It predominantly has insectivorous diet based on lepidopteran larvae, and occasionally plant material (Sinkovics et al., 2021). They are both cavity-nesting birds that readily use artificial nest-boxes (Arenal et al., 2004; Lee et al., 2023) and income breeders allocating energy and lipid reserves to eggs during the egg production or at most few previous weeks. Therefore, contaminants in their eggs reflect maternal body burden experienced before egg laying, and consequently local contamination patterns (Morganti et al., 2021). Previous studies have reported PFAS accumulation in eggs and tissues of these species living near FF, supporting their use as valuable sentinels for assessing local PFAS exposure in terrestrial ecosystems (e.g., Morganti et al., 2021; Buytaert et al., 2023; Lasters et al., 2024; Chu et al., 2025). Moreover, some studies have highlighted that the environmental exposure to PFAS mixture from FF might induce diverse adverse effects in organisms belonging to different taxonomic groups, including birds (see Parolini et al., 2022 and references therein). These studies have reported variable alterations in the oxidative status and increase in oxidative damage, as well as reproductive impairments (Groffen et al., 2019), in birds living nearby FF, revealing some associations with specific or groups of PFAS (e.g., Lopez-Antia et al., 2017, 2019; Buytaert et al., 2023). Based on previous studies, we expected that PFAS levels and pattern in eggs differ between study areas, while nestlings grown nearby the FPP suffering oxidative stress condition compared to conspecifics from the AA.

2. Materials and methods

The present biomonitoring study was performed nearby a Fluoropolymer Production Plant (FPP) located in the Western sector of the Po River valley (see Valsecchi et al., 2026 for details). In late autumn 2020, 28 wooden nest-boxes (13 nest-boxes for starlings and 15 nest-boxes for tits) were placed within a 3 km buffer nearby the FPP, while 10 nest-boxes (5 for starlings and 5 for tits) were placed in a agricultural-rural area (AA) about 15 km far from the FPP (i.e., the main source of PFAS release) (Fig. 1). Nest-boxes were installed at a height of 2–3 m above ground level on trees within semi-wooded areas characterized by mixed deciduous trees interspersed with evergreen conifers and shrub vegetation, or along the margins of agricultural fields.

At the beginning of the breeding season of the focal species (i.e., April), the occupancy of nest-boxes and the progress of egg laying were monitored every two weeks. One egg, typically the first laid one, was collected from each nest-box as a proxy of PFAS contamination. Eggs were stored at −20 °C until chemical analyses (see *2.1 Analysis of PFAS in eggs*). The other eggs were left in the nest-boxes to allow parental incubation. The hatching was monitored and a blood sample was collected from each nestling of both the species when it was 8-days old. After weighing the nestling (to the nearest g), a blood sample (30-70 μl) was collected in heparinized capillary tubes after puncturing the ulnar vein. The blood sample was then centrifuged in a haematocrit centrifuge NF 048 (ESSE 3, Castelnuovo D.B, Italy) at 11,500 rpm for 10 min in order to separate red blood cells from plasma, which were both stored at − 80 °C until biochemical analyses (see 2.2 *Oxidative stress-related biomarkers*). An aliquot of red blood cells was used for molecular sexing of nestlings through the amplification of a section of the CHD gene (Lee et al., 2010). Egg and blood sampling methodology was approved by

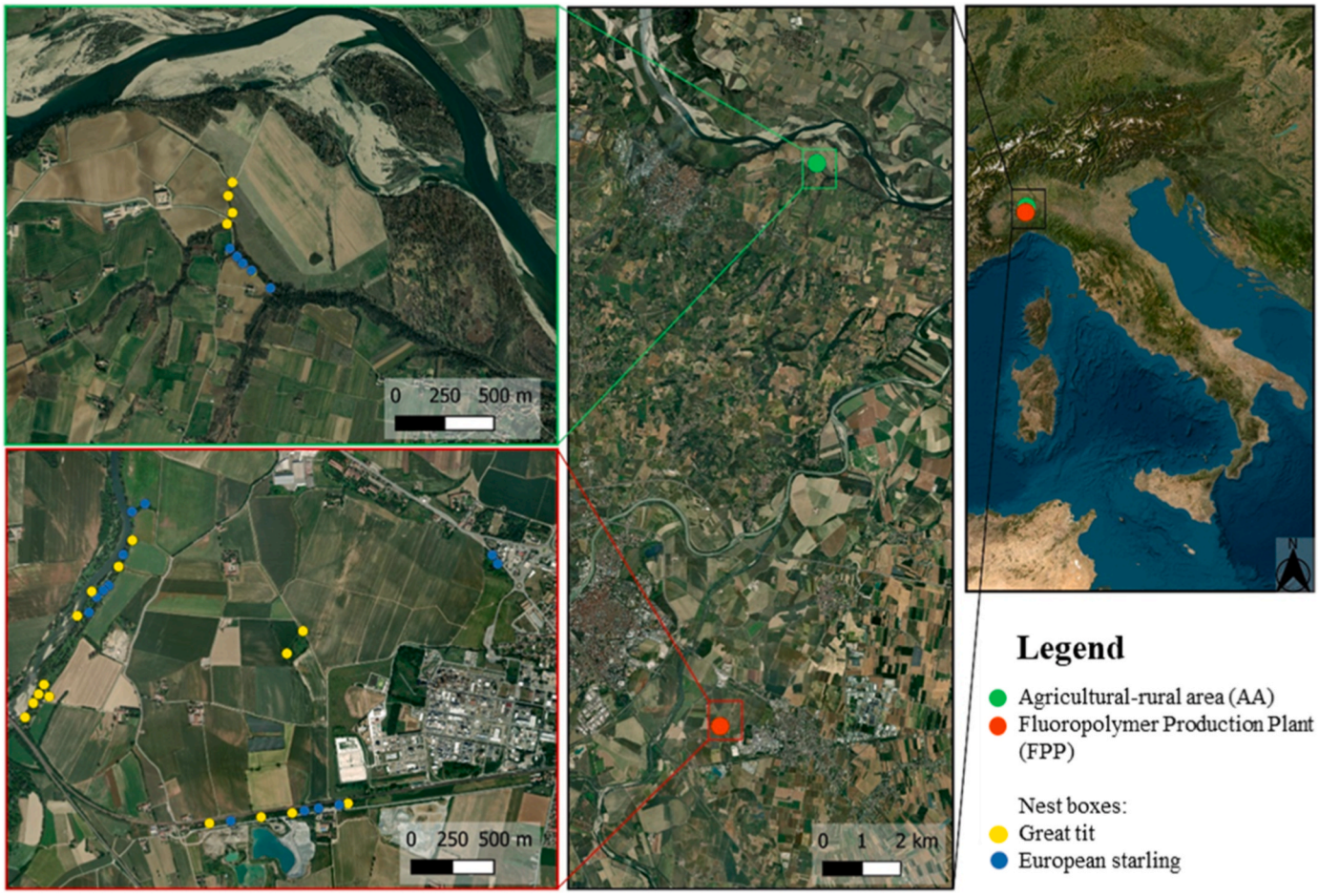

Fig. 1. Location of the nest-boxes for European starlings (blue dots) and great tits (yellow dots) placed in the agricultural-rural area (AA) and close to the Fluoropolymer Production Plant (FPP). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

ISPRA (Istituto Superiore Protezione e Ricerca Ambientale, n.p. Number 36917/2020, confirmed for samplings in the following year) and then authorized accordingly to National and Regional legislation with the permits issued by the Alessandria province with the following codes: Det. Prov. Aless. N° DDAL2-816-2020, DDAL2-12-2023, DDAL2-89-2023.

2.1. Analysis of PFAS in bird eggs

Target analytes included C6-C14 perfluoroalkyl carboxylates (PFCAs), C4-C6-C8-C10 perfluoroalkyl sulfonates (PFSAs), perfluorooctane sulfonamide (FOSA) and 6:2, 8:2 fluorotelomer sulfonates (FTSs), chloro-perfluoropolyether carboxylic acids (Cl-PFPECAs). The main components of the Cl-PFPECA mixture have their own acronyms (ADV or MFS), with ADV being used in the manuscript. Details on the analyte names and abbreviations are reported in Table S1. Egg samples were extracted by acetonitrile assisted by sonication after internal standard addition. The extraction process was repeated twice, and the combined supernatants were purified with $MgSO_4$ and NaCl, then stored overnight at −20 °C. After volume reduction to 1 mL under a nitrogen stream, phospholipids were removed by filtration of the extracts through HybridSPE® phospholipid cartridges.

PFAS were determined by liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) coupled to a turbulent flow chromatography (TFC) for the online purification of the extracts (Mazzoni et al., 2016). A full description of the standards, reagents, and methods used for PFAS analysis in eggs is provided in Supplementary Materials. Sample concentrations were corrected for analytes detected above the limit of detection (LOD) in the corresponding batch procedural blanks. Calibration curves (0.25 -200 μg/L) by isotopic dilution method were acquired for each analytical run. Limits of Detection (LOD), Limits of Quantification (LOQ) and recovery results are reported elsewhere (Valsecchi et al., 2026).

2.2. Oxidative stress-related biomarkers

Oxidative stress-related biomarkers were measured on red blood cells (RBC) from samples of both the species. About 20 μL RBC were homogenized with a pellet motor pestle (Sigma-Aldrich®) in 500 μL of 100 mM phosphate buffer (pH 7.4), containing EDTA (1 mM), KCl (100 mM), dithiothreitol (DTT; 1 mM) and 1:100 vol/vol protease inhibitors (Protease Inhibitor Cocktail containing six individual components for inhibiting serine, cysteine, and acid proteases, and aminopeptidases; P8340 - Sigma-Aldrich®). The homogenate was then centrifuged at 15,000 × g for 10 min at room temperature and the supernatant was processed to assess the protein content and the activity of antioxidant and detoxifying enzymes, specifically superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione S-transferase (GST). The pellet was used to measure the levels of lipid peroxidation. The enzyme activities were assessed through spectrophotometric methods described by Possenti et al. (2019). SOD activity (SOD Units/mg protein) was assessed measuring the inhibition of cytochrome *c* (10 μM) reduction due to the superoxide anion generated by the xanthine oxidase (1.87 mU/mL)/hypoxanthine (50 mM) reaction for 1 min at $\lambda = 550$ nm. The CAT activity (μM/min/mg protein) was assessed measuring the consumption of H_2O_2 (50 mM) in potassium phosphate

buffer for 1 min at λ = 240 nm. The GPx activity (μM/min/mg protein) was assesses measuring the consumption of NADPH (0.12 mM) using H_2O_2 (0.2 mM) in a potassium phosphate buffer added with glutathione (2 mM), sodium azide (1 mM) and glutathione reductase (2 U/mL) for 1 min at λ = 340 nm. The GST activity (μM/min/mg protein) was measured the for 1 min at λ = 340 nm assessing the conjugation of reduced glutathione (1 mM) with 1-chloro-2,4-dinitrobenzene (CDNB; 1 mM). Lipid peroxidation (LPO; nmol TBARS/mg protein) was evaluated through the thiobarbituric acid reactive substances (TBARS) method (Ohkawa et al., 1979). The activity of enzymes was normalised on the protein content, which was measured through the Bradford method (Bradford, 1976). Biomarker analyses were performed using reagents purchased from Merck (Darmstadt, Germany) and a Genova Bio spectrophotometer (Jenway).

2.3. Statistical analysis

For the calculation of ∑PFAS, levels below the limit of detection (LOD) were set to zero in order to avoid overestimating the contribution of values below the LOD. Differences in the concentrations of legacy and emerging PFAS measured in the eggs of the European starling and the great tit collected in nest-boxes located nearby the FPP or AA were assessed through the application of both univariate and multivariate analyses. First, the differences in the concentration of each single PFAS, as well as the ∑PFCA and ∑PFAS, measured in the eggs of the focal species occurring between the two monitoring sites (i.e., FPP and AA), were assessed through the application of one-way analysis of the variance (ANOVA). Preliminarily, normality and homoscedasticity of the data were checked through Shapiro–Wilk and Levene's tests, respectively. Pairwise differences of PFAS concentrations in eggs of the passerine species between FPP and AA were checked through the Tukey's post-hoc test. Significance was set at $P < 0.05$ (*) and $P < 0.01$ (**). As a large number of statistical tests were run to assess the differences in concentrations of PFAS in the same egg, P values of univariate analyses were corrected with the false discovery rate procedure.

Redundancy analysis (RDA) was used to assess differences in the pattern of PFAS contamination between the monitoring sites. The concentration of each PFAS measured in the eggs of both the passerine species was standardized before the analysis by subtracting the mean value and dividing it for the standard deviation to reduce the importance of PFAS whose concentrations showed the largest variances. The significance of RDA models was assessed through the same randomization procedure used for univariate analyses (see Parolini et al., 2021 for details).

The effects of PFAS exposure on oxidative stress-related responses were investigated through the application of Linear Mixed Models (LMMs), including the sampling site (i.e., FPP and AA), the sex of the nestlings, and their two-way interactions as predictors, while the identity of the nest-boxes was considered as random factor. The weight of the individuals was included in the models as a covariate to account for potential effects due to differences in nestling age at blood sampling. Non-significant interactions were excluded from the final models in a single step. The models were run separately for each species. Tukey's post-hoc test was applied to check for significant differences and the significance was set at $P < 0.05$ (*) and $P < 0.01$ (**). Intra-nest dependence of oxidative stress-related responses was assessed using the Intraclass Correlation Coefficient (ICC), estimated from linear mixed effects models, with nest included as a random effect. All the statistical analyses were performed with the R statistical software version 3.6.2 (R Core Team, 2019), using *lme4* (Bates et al., 2015), *vegan* (Oksanen et al., 2019), *glmmTMB* (Brooks et al., 2017), *emmeans* (Russell, 2020), *multtest* (Pollard et al., 2005) packages.

3. Results

The occupancy rate of nest-boxes was higher than 80% for the European starling (11/13 = 84% close the FPP and 4/5 = 80% in the AA) and 57% for the great tit (5/15 = 33% close the FPP and 4/5 = 80% in the AA). One egg, typically the first laid one, was collected from all the nest-boxes. Regardless the study area, the mean (± standard deviation) number of starling nestlings in the nest-boxes was 4 (±1), while for the great tit it was 6 (±2).

3.1. Levels and pattern of PFAS contamination in the eggs

Among the 38 measured PFAS, 10 were never detected above the limit of detection (LOD), while 21 were consistently quantified in the eggs of the European starling and the great tits. Overall, the sum of the concentrations of all the 21 measured compounds (i.e., ∑PFAS, expressed in ng/g fresh weight – ng/g f.w.) measured in the eggs collected in the surroundings of the FPP was higher compared to those from the AA for both the species. The mean (±SE) ∑PFAS measured in the eggs of starlings collected close the FPP (758 ± 124 ng/g f.w.; range 229 – 1452 ng/g f.w.) was 9-fold higher compared to those measured in the AA (82 ± 11 ng/g f.w.; range 76 - 104 ng/g f.w.) (Fig. 2A).

The same difference was observed for the great tit, with levels measured in the eggs from the surroundings of the FPP (1866 ± 1200 ng/g f.w.; range 221 – 6577 ng/g f.w.) higher compared to those from the AA (206 ± 29 ng/g f.w.; range 201 – 258 ng/g f.w.) (Fig. 2B). These levels measured were forced by extreme concentrations measured in the egg from a single nest-box located very close to the outlet channel of the FPP (<5 m), whose ∑PFAS was 6577 ng/g f.w. Excluding this extreme value, the mean contamination in the eggs from the surroundings of the FPP was 685 ± 296 ng/g f.w. (range 221 – 1506 ng/g f.w.).

The contamination pattern was dominated by ADV oligomers, which accounted for more than 90% of the ∑PFAS measured in eggs of both the species collected close and far from the FPP. The sum of the concentrations of all the other compounds accounted for less than the 10%, with a percentage contribution of each single compounds ranging between 1 and 8% of the total (Fig. S1). The ADV-N3 (C10) was the main compound of the pattern (accounting for 38-51% of the total). Mean (±SE) concentrations measured in starling (285 ± 43 ng/g f.w.) and great tits (776 ± 525 ng/g f.w.) eggs collected close the FPP were 9-fold higher compared to those measured in eggs of both the species from the AA (42 ± 5.7 ng/g f.w. and 85 ± 12 ng/g f.w., respectively). The longer-chain compounds ADV-M4 and ADV-N4 (C12 and C13) accounted on average for the 13% and 11% of the pattern, respectively. The mean concentrations (±SE) of ADV-M4 measured in starling (107 ± 27 ng/g f.w.) and tit (239 ± 129 ng/g f.w.) eggs from the FPP were approximatively 10-fold higher compared to those from the AA (10 ± 1.5 ng/g f.w. and 25 ± 3.8 ng/g f.w., respectively). Similarly, the concentration of ADV-N4 resulted higher in eggs collected close to the FPP in both the species (European starling: 102 ± 25 ng/g f.w. and great tit: 202 ± 100 ng/g f.w.) than in the AA (European starling: 7.2 ± 1.7 ng/g f.w. and great tit: 19 ± 3.3 ng/g f.w.).

In contrast, the concentration profiles of the perfluoroalkyl carboxylic acids (PFCA) mixture was consistent between areas, but differed markedly between the two species. In the great tit eggs, the most abundant compound was the PFDA (mean ± SE: 22 ± 20 ng/g f.w. in FPP and 2.5 ± 0.75 ng/g f.w. in AA), followed by the PFUnA (19 ± 17 ng/g f.w. in FPP and 2.3 ± 0.62 ng/g f.w. in AA) and PFNA (17 ± 16 ng/g f.w. in FPP and 1.1 ± 0.19 ng/g f.w. in AA). Overall, short-chain alkyl acids (PFHxA and PFHpA, rarely detected in the eggs) and the longest-chain compound (PFTeDA, C13) were measured at the lower concentrations. Conversely, in starling eggs, the PFCA concentrations generally increased with increasing fluorinated alkyl chain length from C5 (PFHxA) to C12 (PFTrDA), except of PFOA (17 ± 4.0 ng/g f.w. in FPP and 1.1 ± 0.24 ng/g f.w. in AA), which occurred at levels 2–10-fold higher than other PFCA. The most abundant compound were PFTrDA (12 ± 2.1 ng/g f.w. in FPP and 1.4 ± 0.66 ng/g f.w. in AA), PFDoDA (5.6 ± 0.93 ng/g f.w. in FPP and 0.82 ± 0.27 ng/g f.w. in AA) and PFUnA (5.1 ± 1.2 ng/g f.w. in FPP and 0.58 ± 0.15 ng/g f.w. in AA).

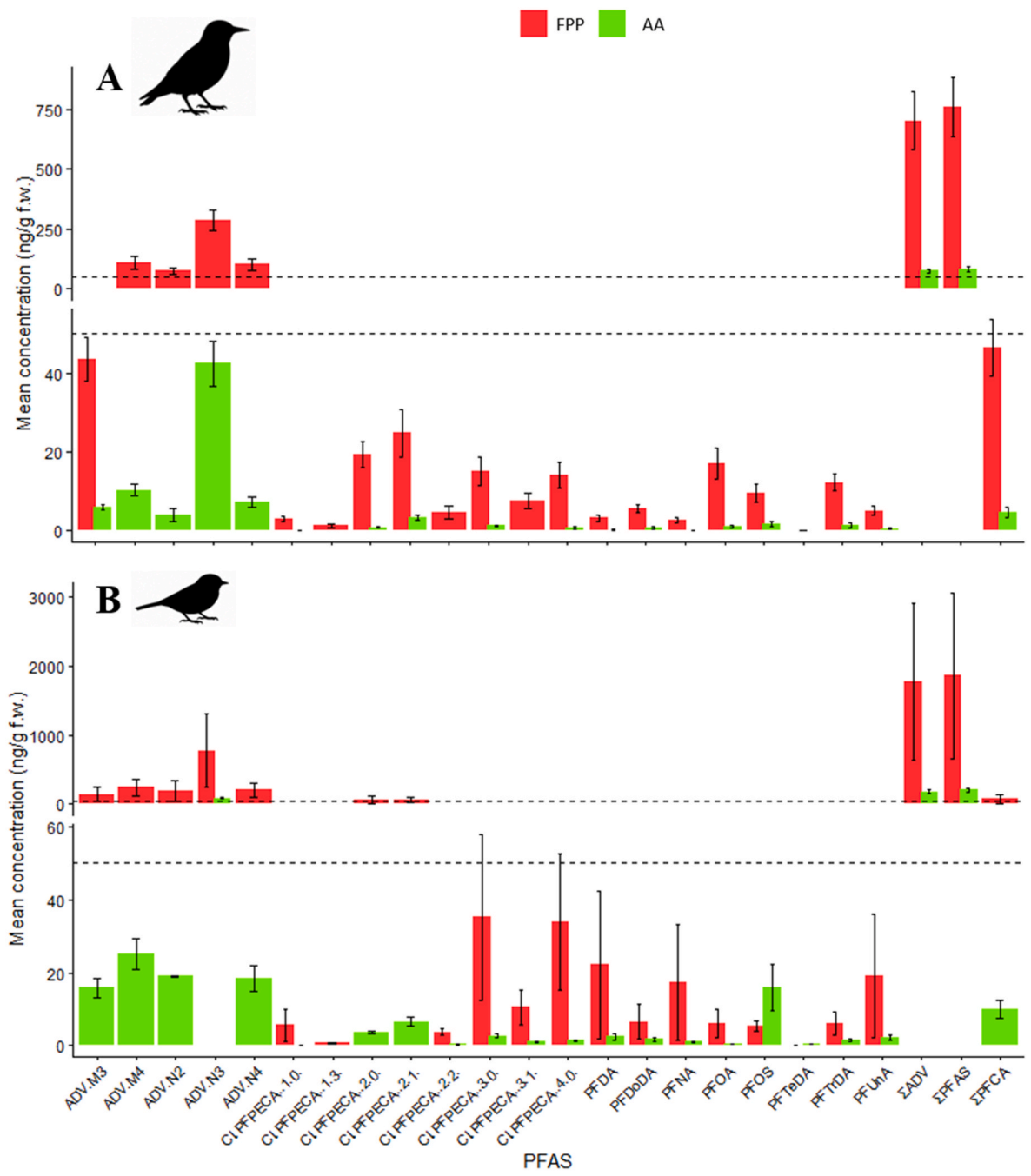

Fig. 2. Mean (±SE) concentrations of individual PFAS compounds and summed concentrations (∑ADV, ∑PFCA and ∑PFAS) measured in eggs of the European starling (A) and great tit (B) collected from nest boxes located near the Fluoropolymer Production Plant (FPP, red bars) and in the agricultural-rural area (AA, green bars). For both panels (A and B), the y-axis is split into a lower and an upper section to improve visualization of compounds occurring at markedly different concentrations. The lower section displays concentrations < 50 ng/g fresh weight, whereas the upper section displays concentrations > 50 ng/g fresh weight. The horizontal dotted line indicates the 50 ng/g fresh weight threshold used to separate the two axis sections. Bars appearing only in the lower or upper section indicate that the concentration of the corresponding compound (or summed PFAS group) was entirely below or above this threshold, respectively. When the concentration exceeded the threshold in only one study area (FPP or AA), the corresponding bars are shown separately in the lower and upper sections of the same panel. This layout may result in apparent differences in bar width, which are solely a graphical consequence of the split y-axis and does not convey any quantitative information. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Interestingly, PFOS has been measured only in great tit and its mean (±SE) concentrations in eggs collected in the AA (16 ± 5.5 ng/g f.w.) were 3-fold higher compared to those measured in eggs from the FPP (5.4 ± 1.4 ng/g f.w.).

Regardless of the bird species, the RDA showed that of PFAS contamination measured in eggs significantly differed among areas ($F_{1,21} = 2.905$, $p = 0.012$). Focusing on the single species, the pattern of PFAS contamination observed in the eggs of the European starling collected nearby the FPP significantly differed from that measured in the eggs laid by conspecifics from the rural area (AA) ($F_{1,12} = 3.902$, $p = 0.017$; Fig. 3A). In contrast, marginally non-significant differences were observed in the eggs of the great tit collected in the different areas ($F_{1,7} = 1.879$, $p = 0.061$; Fig. 3B).

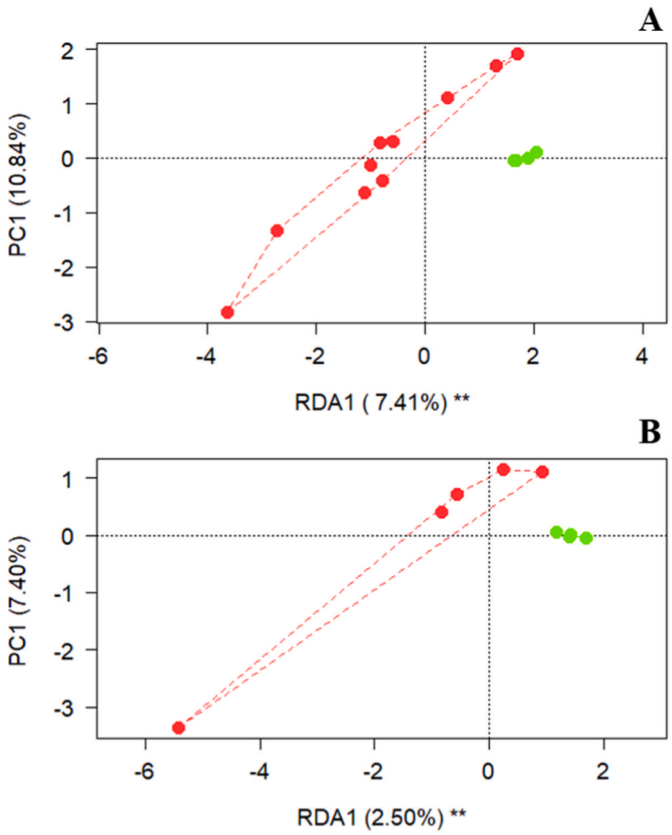

Fig. 3. Biplot from the redundancy analysis (RDA) of standardized PFAS concentrations measured in the eggs of the European starling (A) and the great tit (B) collected in nest-boxes located nearby the FPP (red dots) and in the AA (green dots). Each symbol represents an egg from a nest-box. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.2. Oxidative stress-related responses in nestlings

A modulation in the oxidative status of European starling nestlings that grew nearby the FPP was noted (Fig. 4A). Although no changes in the activity of SOD ($F_{1,8.38} = 0.758$; $P = 0.408$) and CAT ($F_{1,8.05} = 1.120$; $P = 0.320$), a significant modulation of GPx activity occurred ($F_{1,8.38} = 7.420$; $P = 0.025$), with individuals grown nearby the FPP showing higher levels compared to conspecifics from the AA ($p = 0.023$). Moreover, also the activity of the phase II detoxification enzyme GST was not significantly modulated in nestlings from the FPP compared to conspecifics from the AA ($F_{1,9.59} = 0.300$; $P = 0.596$). A significant increase of lipid peroxidation levels was noted in nestlings from the FPP ($F_{1,7.72} = 6.041$; $P = 0.040$), which resulted significantly higher compared to those measured in conspecifics from the AA ($p = 0.037$).

In contrast, neither modulations in the oxidative status nor changes in oxidative damage were noted in great tit nestlings (Fig. 4B). Indeed, no significant differences in the activity of SOD ($F_{1,6.98} = 0.472$; $P = 0.514$), CAT ($F_{1,6.30} = 0.269$; $P = 0.621$), GPx ($F_{1,6.16} = 2.581$; $P = 0.157$), GST ($F_{1,6.70} = 0.039$; $P = 0.842$) and lipid peroxidation ($F_{1,6.98} = 0.031$; $P = 0.859$) levels occurred between the study areas.

3.3. Correlations between biological responses and PFAS contamination

The ICC was > 0.3 (0.317 – 0.504) for all the responses measured in starling nestlings collected in the two areas, while it was > 0.198 (0.198 – 0.401) for responses measured in the great tit. While in the great tit no significant correlation occurred between the biological responses and the egg concentration of single PFAS or ∑ADV, ∑PFCA and ∑PFAS (Fig. S2), in starling nestling lipid peroxidation levels positively and significantly correlated with the PFAS contamination. Specifically, LPO levels were significantly correlated with the concentration of Cl-CPFPECA (1,0), Cl-CPFPECA (2,0), PFNA, PFDA, PFUnA, PFDoDA, PFOS, as well as with the ∑PFCA ($P < 0.05$ in all the cases, except $P < 0.01$ with PFNA; Fig. S2), measured in the first-laid egg from the same nest-box where the nestling grew.

4. Discussion

PFAS were detected in all the eggs collected in nest-boxes where the great tit and the European starling breed, with levels measured in eggs from the close surroundings of the FPP up to ten-fold higher compared to those collected in the agricultural-rural area (AA). Although the PFAS levels in the eggs of both species were similar, the contamination patterns showed notable species-specific differences, resulting in different biological responses. Indeed, while no changes in oxidative status and damage occurred in great tit nestlings between the study areas, European starling nestlings from the FPP clearly suffered an oxidative stress condition compared to conspecifics from the agricultural-rural area.

4.1. Levels of PFAS in bird eggs

Eggs of both resident passerines effectively reflected PFAS contamination originating from the FPP. Regardless of the bird species, PFAS concentrations were up to ten-fold higher near the FPP than at AA, confirming the usefulness of these species as local biomonitors of both legacy and emerging PFAS. Moreover, being income breeders with different trophic niches, they allows disentangling distinct exposure pathways within the same environmental scenario. Similar findings were observed in eggs of two passerine birds, the blackbird (*Turdus merula*) and the great tit, collected at varying distances from a fluoropolymer manufacturing plant located within a residential area in Kvistgaard, Denmark (Trier et al., 2026). Close to the factory, the eggs had elevated organofluorine levels (blackbirds: 670 – 2500 ng/g f.w. and great tits: 260–670 ng/g f.w.), fourfold higher for blackbirds than in the control area (Trier et al., 2026). However, the ∑PFAS measured in the eggs close to the FPP was lower compared to other studies performed nearby a fluorochemical plant located in Belgium, where the median concentrations measured in eggs of the great tits were 10,380 ng/g f.w. (extrapolated), 99.3 ng/g f.w. and 47.7 ng/g f.w. for PFOS, PFHxS and PFDS, respectively (Groffen et al., 2017). This discrepancy could be due to the differences in contamination patterns, which were dominated by PFOS.

Although total PFAS burdens were similar between species at each site, congener-specific patterns differed markedly. The Cl-PFPECA (ADV) mixture showed a consistent distribution in eggs of both species at FPP (Figs. 2 and 3), with ADV-N3 (C10) as the dominant congener, followed by ADV-M4 and ADV-N4 (C12 and C13). This consistency suggests that the relative composition of the ADV mixture in eggs largely mirrors the current emission profile of the FPP, with limited changes during environmental transfer and trophic uptake (Valsecchi et al., 2026).

In contrast, the profiles of PFCA were species-specific. In great tit eggs, PFCA concentrations followed a bell-shaped trend with carbon chain length, with low contributions from short-chain homologues and PFTeDA, and increasing concentrations from C8 to C12 (Fig. 2). In starling eggs, PFCA concentrations generally increased with increasing the chain length, with the exception of PFOA, which occurred at concentrations 2–10 fold higher than the other PFCA (Fig. 2). These contrasting patterns likely reflect the combined effects of emission history of the FPP, compound-specific environmental fate, and species-specific diet (Chu et al., 2025; Valsecchi et al., 2026). PFOA was phased out by 2013, the use and emission of its substitute C6O4 began only in 2010, while the Cl-PFPECA mixture was still in use during the sampling period (Valsecchi et al., 2026). Thus, the pattern reflects both ongoing

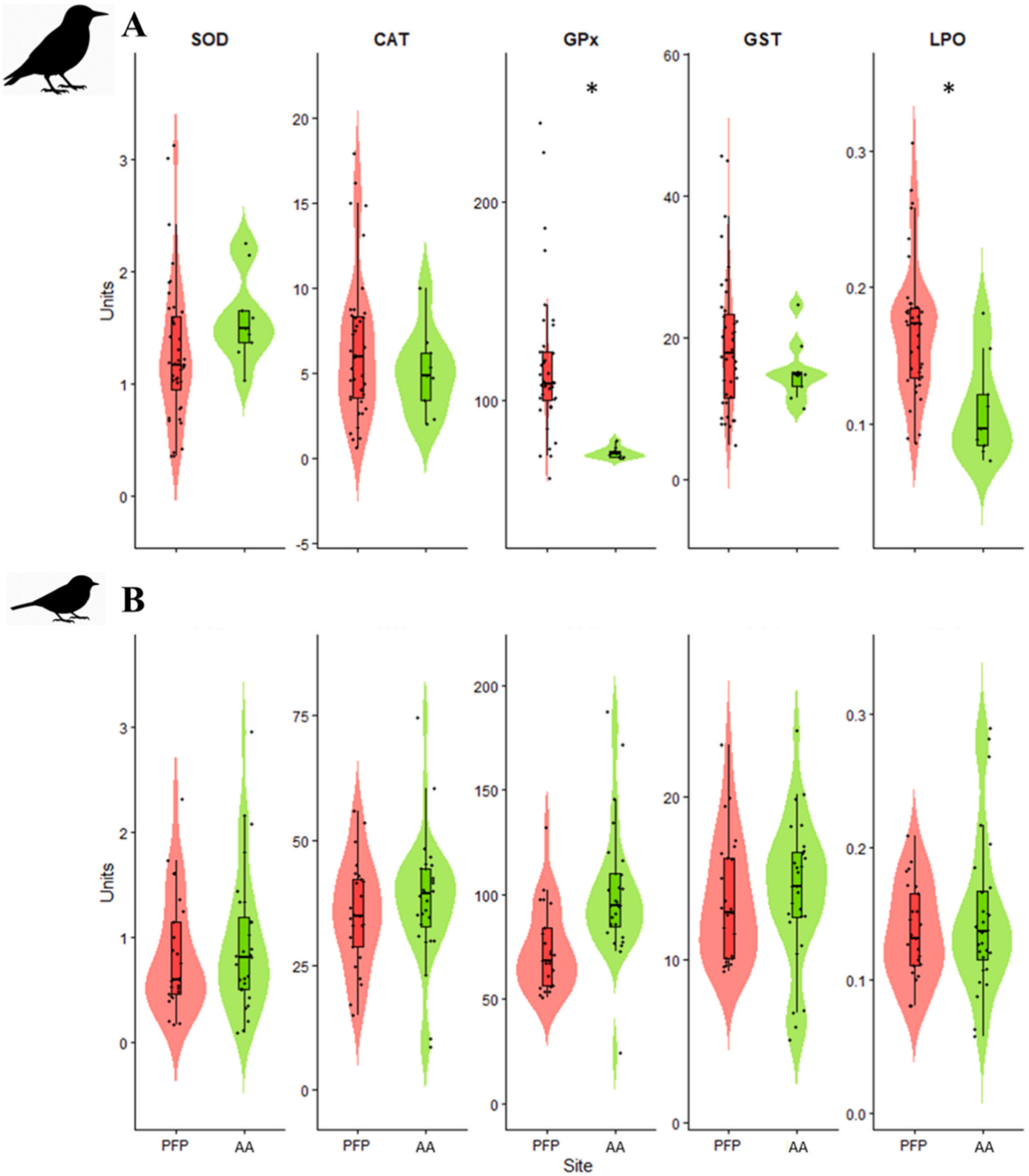

Fig. 4. Effects of PFAS exposure on European starling (A, upper panel) and great tit (B, bottom panel) nestlings. Violin plots with overlaid boxplots show the distribution of enzyme activities (superoxide dismutase, SOD; catalase, CAT; glutathione peroxidase, GPx; glutathione S-transferase, GST) and lipid peroxidation (LPO) across the two sampling sites (FPP = Fluoropolymer Production Plant and AA = agricultural-rural area). Violin shapes represent data distribution density, boxplots indicate median and interquartile range, while individual points show single observations. Each response is displayed in a separate panel with independent y-axis scaling (see the specific Units for each response in the 2.2 *Oxidative stress-related biomarkers* section). Asterisks (*P < 0.05) show statistically significant differences between groups.

emissions of replacement PFAS and residual contamination from legacy compounds. Environmental partitioning processes shaped the contamination pattern, as mid- and long-chain PFAS tend to accumulate in biota and progressively migrate downward through soils, whereas vegetables more efficiently accumulate ultrashort- and short-chain molecules (Davis et al., 2023; Wen et al., 2026). These mechanisms are expected to differently affect the transfer of PFAS in the eggs of the two species because they feed on different diet. The great tit feeds primarily on canopy-dwelling insects and vegetation (Sinkovics et al., 2021), whereas the European starling forages mainly on soil-dwelling invertebrates, particularly earthworms (Cabe, 2020). Thus, PFAS accumulation in great tit eggs was likely driven by current atmospheric emissions, as contamination of canopy-dwelling prey mainly depends on airborne deposition (Wen et al., 2026). In contrast, the PFAS profile of starling eggs characterized by enrichment increasing with fluorinated carbon number, suggests an additional exposure pathway beyond atmospheric inputs. In particular, starlings can be exposed to PFOA through the ingestion of earthworms and other soil-dwelling invertebrates inhabiting soil layers, where PFOA was accumulated over decades (Valsecchi et al., 2026). These trends are especially evident in eggs collected close to the discharge canal of the FPP, which showed the highest PFAS levels of the whole study and site-specific patterns of contamination (Valsecchi et al., 2026). On one hand, the intense aerosol deposition on the leaves of trees along the riverbanks receiving the effluent can affect canopy-dwelling prey, which constitute the primary diet of the great tits, resulting in very high PFAS levels. The large amount of PFOA and ADV released into discharge waters from the FPP over decades of fluorochemical production accumulated into the soil, as well as in earthworms and other soil-dwelling invertebrates, which are the main prey of the starlings. Consistently, the PFOA concentrations measured in the eggs of starlings breeding close to the FPP effluent were markedly higher than those measured in eggs from the close surroundings of the FPP. Overall, our findings show how species-specific ecology and compound-specific environmental fate of different PFAS create complex exposure pathways, which may cause substantially different contamination patterns and biological responses even among sympatric species.

4.2. Oxidative stress-related responses

Recent studies suggest that PFAS exposure can enhance reactive oxygen species (ROS) production. In response, organisms activate endogenous and exogenous antioxidant defence systems to mitigate oxidative stress (Beaulieu and Costantini, 2014). When ROS production exceeds antioxidant capacity, oxidative imbalance occurs, leading to cellular oxidative damage (Beaulieu and Costantini, 2014; Lopez-Antia et al., 2019). Therefore, a battery of oxidative stress biomarkers provides a comprehensive approach for assessing PFAS-induced effects. Antioxidant biomarkers reflect physiological responses to PFAS exposure, whereas oxidative damage biomarkers indicate its adverse consequences (Beaulieu and Costantini, 2014).

Our data showed a species-specific, differential response to PFAS exposure. No alteration in oxidative status nor in oxidative damage was observed in great tit nestlings breed close the FPP compared to conspecifics from the AA, suggesting that PFAS exposure did not induce an oxidative stress condition. These results suggest that the concentrations of PFAS experienced by great tits nestling due to pre- and early postnatal exposure were not able to induce modulations of the oxidative status. Alternatively, we might speculate that dietary antioxidant molecules can counteract the overproduction of ROS and the activation of enzyme antioxidants, preventing subsequent oxidative damage (Osawa et al., 1995). Our findings contrast with previous studies on 14-day-old great tit nestlings breeding near a FF in Belgium, where plasma PFAS concentrations were positively associated with antioxidant enzyme activities (CAT and GPx) and protein carbonyls (Lopez-Antia et al., 2019). Consistently, another study reported higher total antioxidant capacity and peroxidase activity in adults sampled near the FF, with plasma PFAS levels positively correlated with oxidative stress responses (Buytaert et al., 2023). These discrepancies can be due to both biological and contamination issues. In the present study, blood samples for oxidative stress analyses were collected from 8-days old nestlings, whereas previous studies performed the same analyses on 14-days old nestlings or adult individuals. The effects of PFAS exposure might become evident after prolonged exposure. Previous studies reported biological effects associated with PFAS, and mainly PFOS, probably because of the highest concentrations of this molecule ever measured in wild birds breeding in the vicinity of a fluorochemical plant (Lopez-Antia et al., 2017, 2019; 2021). For instance, highest PFOS levels were measured in the eggs of great tit (up to 80,231 $\pm$ 37,684 ng/g f.w.) or plasma from individuals (43,757 $\pm$ 16,870 pg/μL in adults and up to 16,406 $\pm$ 7113 in nestlings) breeding close the production plant (Lopez-Antia et al., 2019). Thus, PFOS levels in great tit eggs may have been too low to elicit detectable biological responses. Moreover, PFOS concentrations were higher in eggs from the AA site than in those from the FPP site, potentially masking other site-specific effects. Lastly, other contaminants maternally transferred to eggs or experienced during early postnatal period by nestlings in the AA might elicit biological responses similar to those observed in conspecifics from the FPP, confounding the effects specifically due to PFAS exposure. Indeed, many other contaminants can affect the production of ROS and the activity of antioxidants, thus PFAS might not be the only factor contributing to the modulation of oxidative status in great tits (Rijnders et al., 2021).

In contrast, a modulation of antioxidant defences and an increase in lipid peroxidation were observed in starling nestlings grown nearby the FPP compared to conspecifics from the AA. Specifically, the increased GPx activity in nestlings from the FPP indicated the activation of enzymatic antioxidant defences to counteract hydrogen peroxide overproduction. However, the concurrent increase in lipid peroxidation suggested that these defences were insufficient to prevent oxidative damage to cellular macromolecules, resulting in oxidative stress compared with nestlings from the AA. Lipid peroxidation can alter membrane structure and function, reducing fluidity and membrane potential while increasing ion permeability, membrane disruption, and leakage of cellular contents (Gaschler and Stockwell, 2017). In addition, reactive carbonyl species (RCS) generated during lipid peroxidation have longer intracellular half-lives than ROS and can diffuse within and between cells, thereby propagating oxidative damage beyond the initial sites of generation (Buttemer et al., 2010). By an eco-physiological point of view, lipid peroxidation excess may impair muscle performance, ultimately reducing flight endurance and recovery capacity (Dick and Guglielmo, 2019). In addition, elevated oxidative damage can reduced longevity and survival probability in free-living birds, suggesting that oxidative balance plays a central role in avian life-history trade-offs (Vágási et al., 2019).

Despite similar PFAS contamination levels, the different biological responses observed in starling and great tit nestlings under the same environmental conditions may reflect species-specific contamination profiles. In particular, starling eggs contained a higher proportion of long-chain PFAS than those of great tits. This difference may explain the observed variation in oxidative stress responses, as long-chain PFAS have been shown to exert stronger effects on oxidative status in free-living birds (Costantini et al., 2019) and to induce the expression of genes involved in oxidative stress regulation more effectively than short-chain congeners (Nobels et al., 2010). A correlation analysis between the levels of oxidative damage measured in the blood of the nestlings and the levels of single and groups of PFAS measured in the eggs collected in the same nest-box confirmed this hypothesis.

In income breeders, egg contaminant burdens reflect maternal exposure and accumulation immediately prior to laying, providing an accurate measure of local contamination. Because nestlings fed on the same prey consumed by parents during egg formation and laying, they are likely to experience a similar contamination profile after hatching. Consequently, high PFAS concentrations in eggs are expected to

correspond to elevated exposure in nestlings, potentially leading to stronger biological responses. Moreover, a recent study has confirmed that the concentration of PFOS and PFOA measured in eggs and plasma of great tit and blue tit (*Cyanistes caeruleus*) were positively correlated (Groffen et al., 2024). For these reasons, concentrations of PFAS measured in the eggs can be considered as robust predictors of the biological responses observed in nestlings. Significant positive correlations occurred between lipid peroxidation levels and some long-chain PFAS (≥C8), as well as ∑PFCA. These results are consistent with previous studies performed on wild birds, showing significant correlations between oxidative stress-related responses, including oxidative damage endpoints, and concentrations of PFOS and other long-chain legacy PFAS (Lopez-Antia et al., 2019; Buytaert et al., 2023).

5. Conclusions

The present study confirms that FF are PFAS contamination hotspots and that PFAS emissions can adversely affect free-living birds. Resident passerines occupying different trophic niches provide complementary indicators of PFAS exposure and effects. Bird eggs effectively reveal maternal transfer and bioaccumulation patterns, while reflecting species-specific contamination linked to diet. Great tit eggs mainly indicate current atmospheric PFAS emissions, whereas European starling eggs integrate both recent and historical contamination through trophic transfer from soil invertebrates inhabiting deeper soil layers. These differences are relevant because PFAS-induced biological responses depend not only on exposure levels but also on compound-specific properties, particularly C–F chain length. Overall, eggs of both species are useful for biomonitoring PFAS contamination near fluoropolymer manufacturing facilities and for assessing exposure to mixtures of legacy and emerging PFAS. However, the European starling appears to be a more suitable indicator of PFAS-related adverse effects. Its semi-colonial breeding behavior facilitates larger sample sizes, while its invertebrate-based diet promotes the accumulation of a broader range of PFAS, including potentially more toxic long-chain compounds. These findings highlight the need for further field studies on the ecological risks of PFAS, particularly on emerging compounds. Specifically, although the present study focused on the association between oxidative stress-related responses and some PFAS, the possible contribution of other fluorinated molecules, co-occurring contaminants and other halogenated organic pollutants associated with fluoropolymer production, cannot be excluded. For instance, a limitation of the present study concerns the lack of information on the levels of hydroperfluoroether carboxylic acids (H-PFPECAs), i.e., the dechlorinated/hydrogen-substituted analogues of the chlorinated PFPECAs associated with ADV-related emissions, in the eggs of resident birds. The presence of H-PFPECAs was documented in environmental and biological matrices collected nearby FPP and in some biota samples their levels were measured at concentrations exceeding those of their corresponding chlorinated analogues (Evich et al., 2022; Moretti et al., 2023). Consequently, their exclusion should result in an underestimation of the overall PFAS fingerprint, particularly of the ADV-related exposure. Thus, future monitoring studies conducted nearby the FPP should consider a broader chemical characterization, integrating the measurement of both chlorinated PFPECAs and their corresponding H-PFPECAs to provide a more comprehensive assessment of PFAS fingerprint and ADV-related exposure. The implementation of PFAS characterization, including transformation products and other organic contaminants, can be necessary to shed light on the relative contribution of individual chemicals and complex mixtures to the observed oxidative stress responses. Lastly, additional investigations on PFAS contamination in soils and food sources across trophic niches would improve understanding of trophic transfer and species-specific effects. Considering the European starling as a suitable ecological indicator for PFAS biomonitoring, future research should also evaluate the role of age and sex on PFAS-related sub-lethal effects and other life-history traits.

CRediT authorship contribution statement

Marco Parolini: Writing – original draft, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Michelangelo Morganti:** Writing – review & editing, Investigation. **Beatrice De Felice:** Investigation. **Marianna Rusconi:** Investigation. **Maria Teresa Palumbo:** Investigation. **Stefano Polesello:** Writing – review & editing, Conceptualization. **Sara Valsecchi:** Writing – review & editing, Conceptualization.

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Declaration of competing interests

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

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Data availability

Data will be made available on request.

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