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Research Paper

New compounds, old problems. The case of C6O4 - a substitute of PFOA - and its effects to the clam *Ruditapes philippinarum*

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ABSTRACT

C6O4 (difluoro{[2,2,4,5-tetrafluoro-5-(trifluoromethoxy)-1,3-dioxolan-4-yl]oxy}acetic acid) is a new surfactant and emulsifier used as a substitute of perfluorooctanoic acid (PFOA). Recently, C6O4 has been detected in aquatic environments, but, at present, no information concerning the effects of C6O4 on aquatic species, such as bivalves, are available in the literature. Therefore, in this study we evaluated for the first time the effects of C6O4 (0.1 and 1 μg/L) and PFOA (1 μg/L) to the clam *Ruditapes philippinarum*. Short-term (7 days) and long-term (21 days) exposures of clams to the two compounds were carried out and numerous biomarkers were measured in haemocytes/haemolymph, as well as in gills and digestive gland. The MANOVA analysis demonstrated statistically significant effects of the independent variables "treatment", "time" and "treatment-time interaction" on the whole dataset of biomarker responses. The two-way ANOVA analysis performed for each biomarker response indicated that the two compounds affected most of the cellular and tissue parameters measured. Despite preliminary, the results obtained suggested that C6O4 - similarly to PFOA - can affect both cellular and biochemical parameters of clams.

1. Introduction

Per- and poly-fluoroalkyl substances (PFASs) are used since 1950s and include more than 7000 compounds characterised by fluorine-carbon bonds (Buck et al., 2011; Johnson et al., 2021). PFASs are mainly used as surfactants or repellents of both oil and water in a wide spectrum of applications, such as fluoropolymer industry, textile and food packaging industry, in aqueous film forming foam and in metal-plating industries (Lindstrom et al., 2011; Sunderland et al., 2019). Due to the highly resistant chemical bonds, such compounds are classified as persistent organic pollutants (POPs). Indeed, PFASs are highly resistant to photolysis, hydrolysis, chemical and thermal degradation (Sznajder-Katarzyńska et al., 2019). As for biodegradation of PFASs, there is evidence for biodegradation of some compounds, even if such process is incomplete and may not result in mineralisation (Parsons et al., 2008).

The most used long chain PFASs are perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) that have been respectively included in Annex A (elimination) and in Annex B (reduction of its production) of Stockholm Convention (UNEP, 2009, 2019). In addition, the International Agency for Research on Cancer (IARC) classified PFOA as a possible carcinogen to humans (IARC, 2017). PFOA, which is mainly used as wetting and dispersion agent in polytetrafluoroethylene (PTFE) production (Pistocchi and Loos, 2009), has been phased out by the USA chemical industry since 2015 (Mueller and Yingling, 2017). PFOA direct emissions into the environment have been estimated in 30 tons/year (1995–2024), even if PFOA can also originate from fluorotelomer alcohols (FTOHs) degradation (Van Zelm et al., 2008; Wallington et al., 2006). PFOA has been detected worldwide in many water bodies. It has been recorded not only downstream of fluoropolymer manufactories (up to 6500 ng/L and 0.97 mg/L in Italy and China, respectively) (Rusconi et al., 2015; Wang et al., 2016) and in Waste Water Treatment Plants

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(WWTPs) (up to 12.7 μg/l) (Vo et al., 2020), but also in rivers of South Africa (390 ng/L), Italy (303 ng/L), China (260 ng/L) (So et al., 2007; Mudumbi et al., 2014; Castiglioni et al., 2015) and in lakes of China (10.6–36.7 ng/L) (Yang et al., 2011) and USA (10–64) ng/L (Sinclair et al., 2006). PFOA can reach the sea through the water flux, with an estimated river discharge to coastal areas in Europe of about 30 tons/-year in 2007 (Pistocchi and Loos, 2009). In coastal marine ecosystems, PFOA reached levels up to 2.15 ng/L in Orbetello lagoon (Renzi et al., 2013), 19.4 ng/L and 26 ng/L in the Lagoon of Venice and Po estuary (Polesello et al., 2011), 68.6 ng/L along Korean coasts (Naile et al., 2010), 320 ng/L in Korean seawaters (So et al., 2004), 15.3 ng/L in China coastal areas and 192 ng/L in Tokyo bay (Yamashita et al., 2005). Furthermore, PFOA has been detected in the North Sea (up to 4 ng/L) (Ahrens et al., 2009a, 2009b), in Western Pacific Ocean (up to 500 pg/L) (Yamashita et al., 2008), in North Atlantic Ocean (229 pg/L) (Ahrens et al., 2009a, 2009b) and in Antarctic seawaters (up to 15 ng/L) (Cai et al., 2012). As regards the effects of PFOA, data on the molecular mechanisms and signalling pathways of PFOA-induced toxicity in animals and humans have been summarised in the review of Li et al. (2017).

Perfluoroalkyl ether carboxylic acids (PFECAs) have been recently introduced in replacement to PFOA as polymerisation aids in production of perfluoropolymers (Wang et al., 2020a, 2020b). These compounds contain oxygens in their carbon chain (ether group), but maintain similar chemical properties to those of PFOA (Wang et al., 2015; Gomis et al., 2015). Although PFECAs have been poorly researched in aquatic ecosystems and investigated from an ecotoxicological point of view, they are considered as emerging environmental contaminants (Wang et al., 2017; Gebreab et al., 2020). Strynar et al. (2015) demonstrated the presence of a series of perfluorinated ether carboxylic and sulphonic acids, along with traditional PFASs, in surface water in North Carolina (USA). The compounds consist of a homologous series of per- and polyfluorinated compounds with repeating units of CF_2 or CF_2O subunits.

A recently used PFECASs is C6O4 (difluoro{[2,2,4,5-tetrafluoro-5-(trifluoromethoxy)-1,3-dioxolan-4-yl]oxy}acetic acid) (Fig. 1). C6O4 is classified as perfluoroether non-polymer with saturated bonds available in four different forms: *i*) acid form (CAS: 1190931-41-9), *ii*) potassium salt form (CAS: 1190931-39-5), *iii*) ammonium salt form (CAS:1190931-27-1) and *iv*) reduced alcohol form (CAS: 1190931-34-0) (ECHA, 2021; Wang et al., 2020a, 2020b). C6O4 has been registered by MITENI S.p.A. (now inactive) and by Solvay (active registrant) (ECHA, 2021). ECHA registered dossier reports a total tonnage band of 10–100 ton per year (ECHA, 2021).

As ammonium salt, C6O4 is used as a polymer production aid during the manufacture of fluoropolymers as substitute of PFOA (EFSA, 2014). C6O4 has been detected in industrial discharge, ground and surface water in Italy. A range of 50–100 μg/L was measured in the discharge of the perfluoropolymer plant of Solvay, located in the Western sector of the Po River valley (Morganti et al., 2021). C604 was also found in the river Bormida, a Po tributary, downstream from the Solvay factory (Wang et al., 2020a, 2020b), and it was detected by Environmental Agency of the Veneto Region in the Po River waters close at its mouth with ranging concentration from <LOD to 1190 ng/L (ARPAV, 2020). The same Agency found C6O4 in aquifer underneath the MITENI production site with a maximum concentration of 3265 ng/L (ARPAV, 2020).

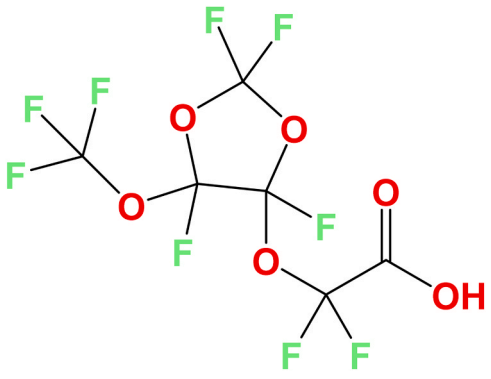

Fig. 1. Structure of C6O4 ((difluoro{[2,2,4,5-tetrafluoro-5-(trifluoromethoxy)-1,3-dioxolan-4-yl]oxy}acetic acid).

The recent replacement of PFOA with C6O4 and the limited information on the effects of C6O4 to aquatic species (ECHA, 2021) pose several questions on C6O4 ecotoxicological profile. Consequently, in this study - that is a continuation of our previous one (Bernardini et al., 2021) - we evaluated for the first time the effects of C6O4 and PFOA on cellular and biochemical parameters of the clam *Ruditapes philippinarum,* a bivalve species widely used in ecotoxicological investigations. The hypotheses we tested were *i*) whether a short-term (7 days) or a long-term (21 days) exposure to C6O4 could pose a potential risk to marine bivalves and *ii*) whether the effects induced by C6O4 are comparable to those caused by PFOA.

2. Materials and methods

2.1. Clam acclimation and exposure

R. philippinarum specimens (3.64 ± 0.32 cm shell length) were sampled in a licensed area for bivalve culture in the southern basin of the Lagoon of Venice (Italy) and acclimated for 7 days in large aquaria filled with a sandy bottom and aerated seawater (salinity of 35 ± 1, temperature of 12 ± 0.5 °C). As food supply, a suspension of the microalgae *Isochrysis galbana* was provided ad libitum.

Two stock solutions (50 mg/L) of C6O4 (CAS no. 1190931-41-9) (Wellington Laboratories, Canada; purity: > 98%) and PFOA (CAS no. 3825-26-1) (Sigma-Aldrich, Milano, Italy; purity: > 98%) were prepared in methanol. Clams (90 in total per each concentration tested) were exposed for 7 and 21 days to 0.1 and 1 μg/L of C6O4 in 30 L glass tanks (two tanks per each concentration, with 45 clams per tank) without a sandy bottom. To compare the potential toxicity of C6O4 with that of PFOA, clams were also exposed to PFOA at the highest concentration tested in C6O4-exposed group, namely 1 μg/L. A control was running in parallel by adding methanol in seawater at a concentration of 20 μL/L. Based on our preliminary observations (data not published), such concentration of methanol was unable to produce significant alterations in biomarker responses of clams with respect to a control condition without solvent. In addition, a previous study demonstrated that methanol could cause lethal or sub-lethal effects in aquatic species, such as fish and invertebrates, at relatively high concentrations, in the order of grams or milligrams per litres (Kaviraj et al., 2004). That is why we decided to prepare only one control (seawater + methanol). Seawater, working solutions of C6O4 and PFOA and food supply (microalgae suspension at an initial concentration of about 9×10^8 cells/L) in exposure tanks were renewed every 48 h.

Actual concentrations of C6O4 and PFOA in seawater samples from experimental tanks, as well as bioaccumulation of the two compounds in clams, were also evaluated and the results obtained are reported in Bernardini et al. (2021). A synopsis of the used chemicals, analytical methods and results is reported in supplementary material (S1).

2.2. Tissue collection

Haemolymph was collected from the anterior adductor muscle by a 1-mL plastic syringe and stored in Eppendorf tubes at 4 °C. At each sampling time, 5 pools of haemolymph (from four clams each) from each experimental condition were prepared. A volume of pooled haemolymph was immediately used to measure total haemocyte count (THC), haemocyte diameter and volume, haemocyte proliferation (XTT) and lactate dehydrogenase (LDH) activity. The remaining part of pooled haemolymph was then centrifuged at 780 × *g* for 10 min, the

supernatant was discharged, while the pellets (=haemocytes) were resuspended in distilled water to obtain haemocyte lysate (HL) samples. To this aim, samples of haemocytes resuspended in distilled water were vortexed for 30 s and centrifuged at 780 × *g* for 10 min at room temperature. The supernatant, corresponding to HL, was then collected, frozen in liquid nitrogen and stored at − 80 °C until analyses (lysozyme activity assay).

Gills and digestive gland from clams were then excised, pooled to obtain five different pools of four clams each, divided in aliquots, frozen in liquid nitrogen, and stored at − 80 °C until analyses.

2.3. Haemocyte parameters

THC, as well as haemocyte diameter and volume, were determined using a Scepter™ 2.0 Automated Cell Counter (Millipore, FL, USA). Briefly, 20 μL of haemolymph were added to 2 mL of Coulter Isoton II diluent. The THC was expressed as the number of haemocytes (10^7)/mL of haemolymph, while haemocyte diameter and volume were expressed in μm and picolitres (pL), respectively.

Haemocyte proliferation was evaluated using the *Cell proliferation* Kit II, Roche (a commercial kit), as described in our previous study (Matozzo et al., 2012). In brief, a volume of 200 μL of the mixture provided by the kit was added to 400 μL of pooled haemolymph and incubated for 4 h in a dark humidified chamber. The absorbance at 450 nm was then recorded using a Beckman 730 spectrophotometer. The results were normalised to THC values of each experimental groups and expressed as optical density (OD) at 450 nm.

The commercial *Cytotoxicity Detection* Kit, (Roche) was used to measure lactate dehydrogenase activity (LDH) in cell-free haemolymph (CFH). Pooled haemolymph (500 μL) from each experimental condition was centrifuged at 780 x g for 10 min, and the supernatant (=CFH) was then collected for the assay following the manufacturer's instructions. The results were expressed as optical density (OD) at 490 nm.

The lysozyme activity was measured in haemocyte lysate (HL) from pooled haemolymph (500 μL). HL was obtained as described above. Briefly, 50 μL of HL was added to 950 μL of a 0.15% suspension of *Micrococcus lysodeikticus* (Sigma) in 66 mM phosphate buffer (pH 6.2), and the decrease in absorbance (ΔA/min) was continuously recorded at 450 nm for 3 min at room temperature. The results were expressed as μg lysozyme/mg of protein. Protein concentrations in the HL were quantified according to Bradford (1976).

2.4. Gill and digestive gland enzyme activity assays

Gills and digestive gland samples were homogenised at 4 °C with an Ultra-Turrax homogeniser (model T8 basic, IKA) in four volumes of 10 mM Tris-HCl buffer, pH 7.5, containing 0.15 M KCl, 0.5 M sucrose, 1 mM EDTA and protease inhibitor cocktail (1:10 v/v) (Sigma-Aldrich), and centrifuged at 12,000*g* for 30 min at 4 °C. Supernatants (SN) were collected for analyses. The protein concentration in SN samples was quantified according to Bradford (1976).

Total superoxide dismutase (SOD) activity was measured in both gills and digestive gland in triplicate using the xanthine oxidase/cytochrome c method proposed by Crapo et al. (1978). Enzyme activity was expressed as U/mg protein, one unit of SOD has been defined as the amount of sample causing 50% inhibition under the assay conditions.

Catalase (CAT) activity was measured in gills and digestive gland SN in triplicate following the method proposed by Aebi (1984). The enzyme activity in a volume of 30 μL of tissue SN were measured at 240 nm and expressed as U/mg protein; one unit of CAT was defined as the amount of enzyme that catalysed the dismutation of 1 μmol of H_2O_2/min.

The method of Ellman et al. (1961) was used to measure acetylcholinesterase (AChE) activity in gill SN (25 μL), following the colorimetric reaction between acetylthiocholine and the reagent dithiobisnitrobenzoate. Changes in absorbance were then recorded at 405 nm for 5 min on a microplate reader at room temperature. The results were expressed as nmol/min/mg of protein.

Glutathione S-transferase (GST) activity was measured in digestive gland SN according to the method described in Habig et al. (1974) using 1-chloro-2,4-dinitrobenzene (CDNB) and reduced glutathione (GSH) as substrates. GST activity was expressed as nmol/min/mg protein.

Glutathione reductase (GR) activity was evaluated according to Smith et al. (1988), by measuring the (5-thio (2-nitrobenzoic acid)) TNB production at 412 nm. The enzyme activity was expressed as U/mg protein.

Oxidative damage in gills and digestive gland was measured through both protein carbonyl content (PCC) and the DNA strand breaks assays. Briefly, PCC was measured in duplicate using the method of Mecocci et al. (1999) following the reaction with 2,4-dinitrophenylhydrazide (DNPH). Results were expressed as nmol carbonyl group/mg protein. The DNA strand breaks were measured using a fluorescence method adapted from an alkaline precipitation assay (Olive, 1988). Fluorescence was measured using a Varian Cary Eclipse fluorescence spectrophotometer at 360 nm (excitation) and 450 nm (emission) against blanks. Salmon sperm genomic DNA standards were used for DNA calibration, and the results were expressed as μg/mg protein.

2.5. Statistical analysis

The normal distribution of data (Shapiro-Wilk's test) and the homogeneity of the variances (Bartlett's test) were assessed. The MANOVA analysis was performed to highlight the effects of the factors "treatment", "time" and "treatment-time interaction" on the whole dataset of the biomarker responses. For each biomarker, results were compared using a two-way ANOVA (with treatment and time as predictors), followed by Tukey's HSD post-hoc test for pairwise comparisons. All results are expressed as means ± standard error (n = 5).

A canonical correlation analysis (CCA) was also performed. The factors "treatment", "time" and their interaction have been included in the first set, while the measured cellular and biochemical parameters are used in the second one.

The software packages Statistica 13.4 (TIBCO Software Inc.) and R (R Core Team, 2020, Austria) with the CCA package (González and Déjean, 2012) and the r41sqrt10 package (Finos, 2020) were used for the statistical analyses.

3. Results

No clam mortality was recorded during experiments. Interestingly, during exposure at least five clams treated with 0.1 μg/L of C6O4 showed foot cut-off, clams being unable to withdraw it before shell closure (Fig. 2).

A statistically significant effect of the independent variables "treatment" (MANOVA: $F_{(54,51)} = 3.206$, $p < 0.001$) "time" (MANOVA: $F_{(18,15)} = 48.87$, $p < 0.001$) and "treatment-time interaction" (MANOVA: $F_{(54,51)} = 3.789$, $p < 0.001$) on the whole dataset of dependent variables (biomarker responses) were recorded.

As for graphs, asterisks denoting statistically significant variations in biomarker responses between control and treated groups at each sampling time were reported only when a significant effect of the factors

Fig. 2. Clams exposed to C6O4 0.1 μg/L. Two images showing clams with almost cut foot (black circle) that remained out of the shell after its closure.

"treatment" and/or "treatment-time interaction" was revealed by the two-way ANOVA analysis.

3.1. Haemocyte parameters

The two-way ANOVA analysis demonstrated that the THC was altered significantly by the factors time ($F = 13.07$, $p = 0.001$), treatment ($F = 7.32$, $p = 0.000$) and treatment-time interaction ($F = 7.59$, $p = 0.000$). As showed in Fig. 3A, the pairwise comparison test revealed a significant decrease of THC values after 7 days of exposure to 1 μg/L of C6O4 or PFOA ($p < 0.001$). Both volume and diameter of haemocytes were affected by time ($F = 7.32$, $p = 0.010$ and $F = 7.59$, $p = 0.009$, respectively) and treatment ($F = 8.27$, $p = 0.000$ and $F = 7.06$; $p = 0.000$, respectively). In details, a significant increase in both volume and diameter of haemocytes was recorded after 7 days of exposure to 1 μg/L of C6O4 ($p < 0.001$) or 1 μg/L of PFOA ($p < 0.05$ for volume and $p < 0.01$ for diameter) (Fig. 3B, C).

Exposure to the compounds affected significantly ($F = 3.48$, $p = 0.027$) LDH activity. In detail, a significant decrease in enzyme activity was recorded after 21 days of exposure of clams to 1 μg/L of PFOA ($p < 0.01$) (Fig. 3D).

The factors time ($F = 11.02$, $p = 0.002$), treatment ($F = 7.75$, $p = 0.000$) and time-treatment interaction ($F = 6.82$, $p = 0.001$) significantly influenced haemocyte proliferation, and the pairwise comparison test revealed a significantly ($p < 0.01$) higher cell proliferation in bivalves exposed for 7 days at 1 μg/L of C6O4 or PFOA (Fig. 3E). All the factors considered did not affect lysozyme activity (Fig. 3F).

3.2. Gill and digestive gland parameters

In gills, SOD activity was affected significantly by treatment ($F = 3.02$, $p = 0.044$) and time-treatment interaction ($F = 3.60$, $p = 0.023$), with a significant increase after 7 days of exposure at the two C6O4 concentrations tested ($p < 0.05$) and at 1 μg/L of PFOA ($p < 0.001$) (Fig. 4A). Conversely, digestive gland SOD activity was affected by time only ($F = 9.99$, $p = 0.003$) (Fig. 4B).

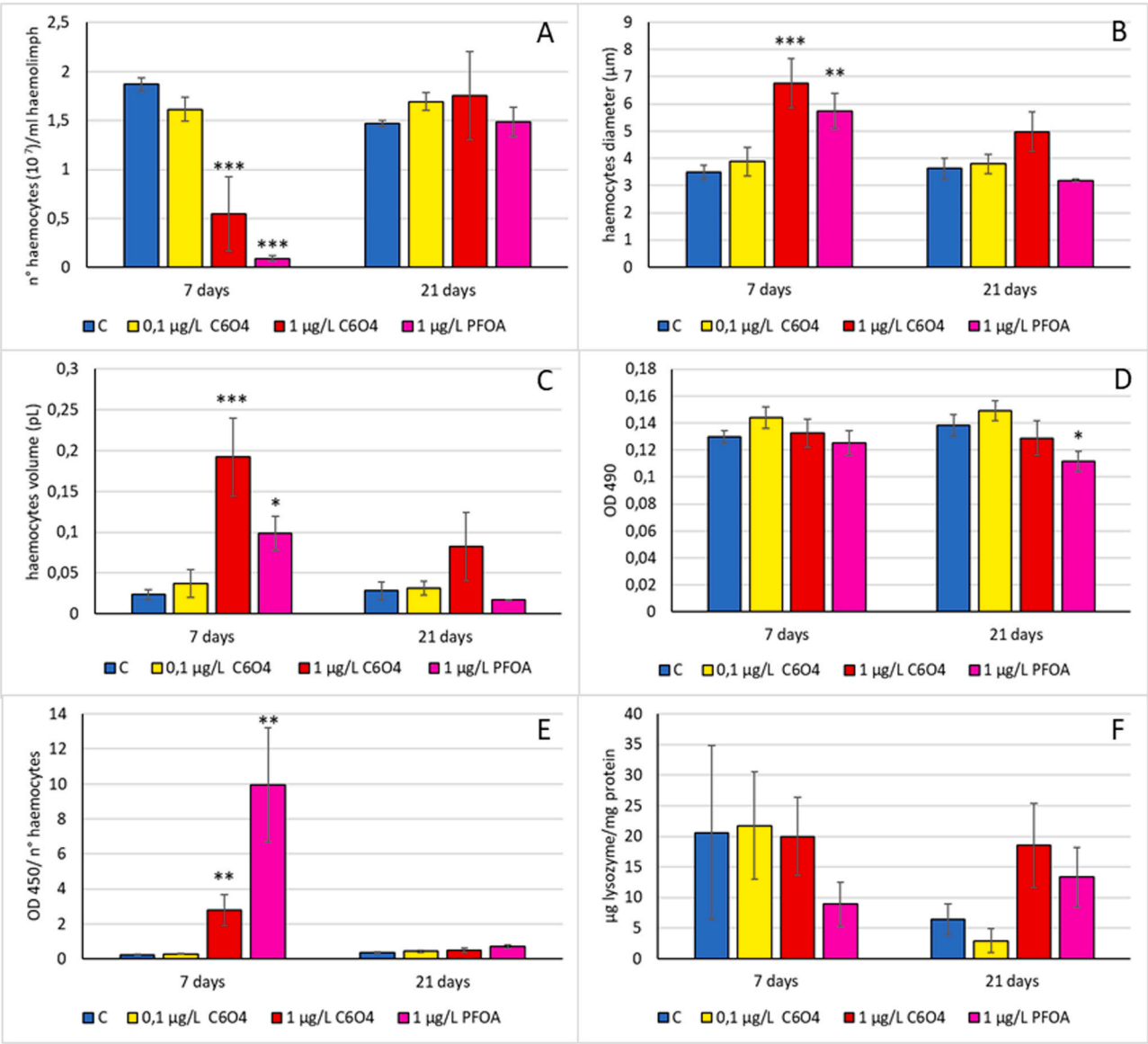

Fig. 3. Total haemocyte count (THC), expressed as n° haemocytes (10^7)/mL haemolymph (A), haemocyte diameter, expressed in μm (B), haemocyte volume, expressed in pL (C), cell-free haemolymph lactate dehydrogenase activity, expressed as OD_{490} (D), haemocyte proliferation, expressed as OD_{450}/n° haemocytes (E), haemocyte lysate lysozyme activity, expressed as μg lysozyme/mg protein (F). The values are mean ± SE (n = 5). The asterisks indicate significant differences in comparison with control: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

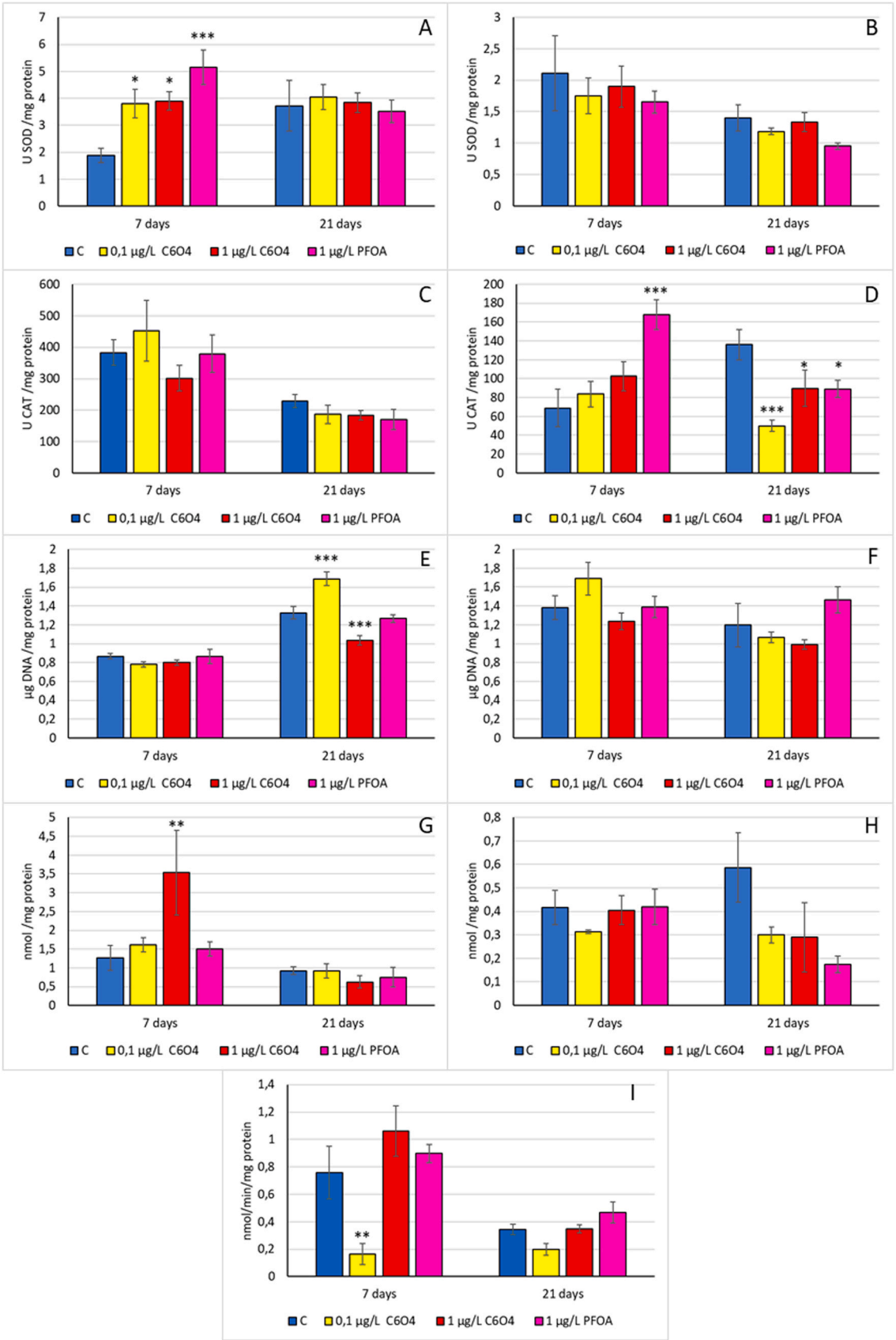

Fig. 4. SOD activity, expressed as U SOD/mg protein, in gills (A) and digestive gland (B), CAT activity, expressed as U CAT/mg protein, in gills (C) and digestive gland (D), DNA strand breaks expressed as µg DNA/mg protein, in gills (E) and digestive glands (F), protein carbonyl content, expressed as nmol/mg protein, in gills (G) and digestive gland (H), AChE activity in gills, expressed as nmol/min/mg protein (I). The values are mean ± SE (n = 5). The asterisks indicate significant differences in comparison with control: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Contrary to what was observed for SOD activity, gill CAT activity was influenced significantly only by time ($F = 29.68$, $p = 0.000$), whereas treatment ($F = 5.68$, $p = 0.003$) and treatment-time interaction ($F = 8.19$, $p = 0.000$) significantly affected CAT activity in digestive gland. In that tissue, the pairwise comparison test revealed a significant increase in CAT activity after 7 days of exposure to 1 μg/L of PFOA ($p < 0.001$), while a significant decrease was recorded after 21 days of exposure to 0.1 μg/L of C6O4 ($p < 0.001$), 1 μg/L of C6O4 ($p < 0.05$) or 1 μg/L of PFOA ($p < 0.05$) (Fig. 4C, D).

GR gills activity was affected significantly only by time ($F = 377.87$; $p = 0.000$), while digestive gland GST and GR activities were not influenced by any considered factors (supplementary material, S2).

As for oxidative damage biomarkers, gill DNA strand breaks were affected significantly by the factors time ($F = 189.95$, $p = 0.000$), treatment ($F = 12,84$, $p = 0.000$) and treatment-time interaction ($F = 15.66$, $p = 0.000$), with a significant ($p < 0.001$) increase in animals exposed to 0.1 μg/L of C6O4 followed by a significant decrease at 1 μg/L of C6O4 ($p < 0.001$) after 3 weeks of exposure (Fig. 4E). In digestive gland, the DNA strand breaks resulted influenced significantly by time ($F = 4.69$, $p = 0.038$) and treatment-time interaction ($F = 3.53$, $p = 0.025$), with a significant increase in bivalves exposed for 21 days to PFOA, when compared to the related control (Fig. 4F).

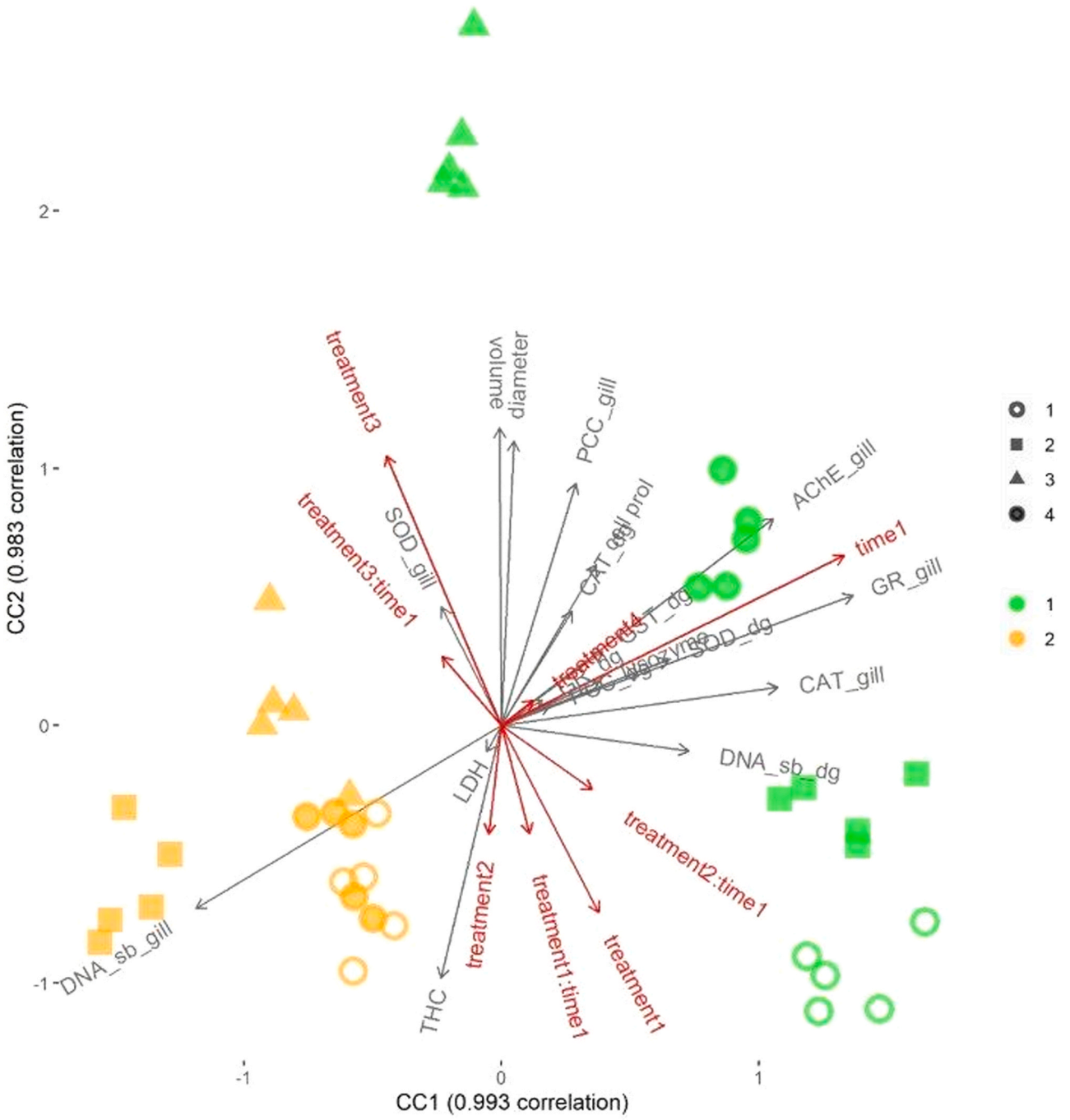

Fig. 5. CCA biplot. Cellular and biochemical parameters measured in clams, as well as experimental concentrations were used for CCA analysis. Black symbols indicate experimental concentrations (1 =0 μg/L; 2 =0.1 μg/L C6O4; 3 =1 μg/L; 4 =1 μg/L PFOA), green symbol indicates time 1 (=7 days of exposure) and yellow symbol indicates time 2 (=21 days of exposure).

PCC values in gills were affected significantly by time ($F = 13.82$, $p = 0.000$) and treatment-time interaction ($F = 3.430$, $p = 0.029$), with a significant ($p < 0.01$) increase at 1 μg/L C6O4 after 7 days of exposure, when compared to the related control (Fig. 4G). On the contrary, all the factors considered did not affect PCC values in digestive glands (Fig. 4H).

Lastly, gill AChE was significantly affected by time ($F = 25.65$, $p = 0.000$), treatment ($F = 10.37$, $p = 0.000$) and time-treatment interaction ($F = 4.24$, $p < 0.05$). Pairwise comparisons revealed a significant ($p < 0.01$) reduction of AChE in gills of clams exposed for 7 days to 0.1 μg/L of C6O4 (Fig. 4I). No significant alterations in enzyme activity were recorded after 21 days of exposure.

3.3. CCA analysis

A plot of the first two components of the CCA analysis are shown in the biplot in Fig. 5. The explained correlations are 99% and 98% for the first and the second canonical correlation, respectively. All the patterns are well separated, highlighting a strong effect of the experimental design on the measured parameters. At time 1 (= 7 days of exposure) the treatment 3 (= animals exposed to the highest concentration of C6O4) is very separated (different) from the other ones. Treatment 3 showed higher values of haemocyte diameter and volume and gill PCC, but lower THC values. A similar pattern can be seen at time 2 (= 21 days of exposure), with treatment 3 being separated from the other treatments, while the overall effects seem reduced. Furthermore, a clear time-dependent pattern of variation can be observed (time 1 on the right, time 2 on the left), with decreasing values of gill AChE, GR and CAT activities and increasing values of gill DNA strand breaks over time.

4. Discussion

4.1. Haemocyte parameters

It is well known that exposure to toxic substances can cause alterations of haemocyte parameters in molluscs (Matozzo and Gagné, 2016). In the present study, the effects of C6O4 and PFOA were investigated for the first time in haemocytes of *R. philippinarum*. Results demonstrated that the exposure to PFOA and C6O4 (at 1 μg/L) influenced haemocyte parameters, mostly after 7 days of exposure. We observed a reduction in THC values in haemolymph of clams exposed for 7 days, along with a significant increase of haemocyte volume and diameter. THC is one of the most used cell parameters to assess the effects of contaminants in bivalves at the cellular level (Matozzo and Gagné, 2016). The increase in THC values can be due to increased cell proliferation or movement of haemocytes from tissues to haemolymph, whereas a decrease in the THC values can be a consequence of cell death or an increased movement of haemocytes from haemolymph to peripheral tissues (Pipe and Coles, 1995). In this study, the reduced number of circulating haemocytes in clams exposed for 7 days to C6O4 and PFOA could be due to either substances-mediated stressful condition or increased movement of haemocytes towards peripheral tissues to increase immunosurveillance. At 21 days, THC values restored in treated clams, suggesting a recovery capability of bivalves. Regarding previous studies, it has been demonstrated that exposure for 21 days of the Chinese mitten-handed crab *Eriocheir sinensis* to PFOS (0.01–10 mg/L) caused a significant reduction in THC values, starting from the concentration of 0.1 mg/L (Zhang et al., 2015).

Overall, C6O4 and PFOA at a concentration of 1 μg/L seemed to act in a very similar way on clam haemocytes, determining a decrease in THC and an increase in size after 7 days of exposure. The increase in haemocyte size can be a consequence of alteration in cell membrane permeability, which leads to increased cell volume. Unfortunately, no studies are available in the literature concerning the effects of PFASs on cell volume and diameter. Consequently, it is difficult for us to propose a plausible explanation for these results. In our previous study, we demonstrated that haemocytes from *R. philippinarum* can divide in haemolymph (Matozzo et al., 2008). In this study, the XTT assay revealed a significant increase of haemocyte proliferation in clams exposed for 7 days to PFOA and C6O4. In a similar way for the two compounds, cell proliferation returned to control levels after 21 days of exposure. We hypothesised that increased cell proliferation in C6O4- and PFOA-treated animals was an attempt of clams to re-establish cell number that dropped after 7 days of exposure. An increase in cell proliferation was also observed in carp (*Cyprinus carpio*) hepatocytes following in vivo exposure for 56 days to PFOA (200 ng/L to 2 mg/L), even if at the highest concentration tested only (Giari et al., 2016).

In the present study, no significant reductions in haemocyte membrane stability (as indicated by the results of LDH assay) was observed in clams exposed to PFOA and C6O4. Similarly, in a recent study, Coperchini et al. (2021) demonstrated that in vitro exposure of human thyroid cells to six C6O4 concentrations (0, 0.01, 0.1, 1, 10 and 100 ng/mL) did not cause any alteration of cell viability, ROS production and cell proliferation. On the contrary, Tang et al. (2018) observed a cytotoxic effect after 12 h of in vitro exposure of lymphocytes from the freshwater *Carassius auratus* to PFOA (10 and 100 μg/L), followed by an induction of autophagy pathway genes.

Lysozyme is an important hydrolytic enzyme that can be released by haemocytes into haemolymph during phagocytosis to counteract non-self materials. In the present study, no significant alterations of lysozyme activity were observed in haemocyte lysate of clams exposed to C6O4 and PFOA, suggesting that lysozyme is not a target for the two compounds. Conversely, the enzymatic activity decreased in the liver of the of zebrafish *Danio rerio* following a 21-day exposure to 0.04 mg/L of PFOS (Guo et al., 2019) and in cell-free haemolymph of *E. sinensis* following exposure for 21 days to PFOS (0.01–10 mg/L) (Zhang et al., 2015).

4.2. Gill and digestive gland parameters

One of the possible consequences of exposure of marine organisms to environmental contaminants is oxidative stress, a condition provoked by and imbalance between the production of reactive oxygen species (ROS) and antioxidant defences. Recent studies have shown the effects of different PFASs on oxidative stress biomarkers in marine organisms (Wu et al., 2019; Miranda et al., 2020). To assess the capability of C6O4 and PFOA to induce oxidative stress in clams, as well as oxidative damage to proteins and DNA, several biomarkers were evaluated in this study. As it is well known, SOD catalyses the dismutation of superoxide anion ($O_2^{\cdot-}$) into hydrogen peroxide (H_2O_2). In this study, gill SOD activity was affected by exposure of clams to contaminants. Indeed, after 7 days of exposure enzyme activity increased significantly in C6O4- and PFOA-treated groups, compared to control ones. After 21 days of exposure, SOD activity values resulted to be like those of controls, with no significant differences between the experimental conditions. Unlike in gills, SOD activity in the digestive gland was significantly affected by exposure duration only. In our opinion, the different response of gills and digestive gland to contaminant exposure could be due to their location in animals. Indeed, gills are the first barrier between the external and internal environment of bivalves and as such must activate antioxidant defence mechanisms against oxidative stress. On the other hand, digestive gland has a different position and role in bivalves. Therefore, it is plausible that defences against oxidative stress activate later than gills. Previous studies have shown a different pattern of variation of antioxidant enzymes following exposure of organisms to various concentrations of contaminants. For example, SOD activity increased at the lower concentrations (0–100 μg/L), while it decreased at the highest concentrations (100–10,000 μg/L) in the green mussel *Perna viridis* exposed for 7 days to various perfluorinated compounds, such as PFOS, PFOA, PFNA, PFDA (Liu et al., 2014a). Similarly, in the freshwater mollusc *Unio ravoisieri* exposed for 7 days to PFOS (2–10 mg/L) gill and digestive gland SOD activity increased in animals

treated with 2 and 6 mg/L, while it decreased in molluscs exposed to 10 mg/L (Amraoui et al., 2018). Likewise, an increase in SOD activity was observed in the amphipod *Gammarus insensitives* treated for 4 days with 1 mg/L of PFOS, followed by a decrease in those exposed to 1.6 and 3.1 mg/L (Touaylia et al., 2019). Conversely, in the liver of *D. rerio* SOD activity increased following exposure for 96 h to 200 μg/L of PFOS, whereas no alterations were observed in gills (Li et al., 2017). Similarly, in *C. auratus*, no SOD activity alterations have been detected in liver exposed to 1.21 and 12.1 μmol/L of PFOA for 4 days (Feng et al., 2015). In other studies, a reduction in SOD activity has been demonstrated following exposure to PFASs. For example, SOD activity decreased in *E. sinensis* exposed for 21 days to highest concentrations of PFOS (till to 10 mg/L) (Zhang et al., 2015) and in the freshwater cladoceran *Daphnia magna* exposed for 7 days to PFOS or PFNA (0.008–5 mg/L), just at the concentration of 0.2 mg/L for both the compounds (Lu et al., 2015).

CAT catalyses the conversion of hydrogen peroxide (H_2O_2) to water and molecular oxygen and is generally found within peroxisomes. In this study, differences in CAT activity were observed between gills and digestive gland. Gill CAT activity was significantly affected by exposure duration only, whereas digestive gland CAT was influenced significantly by both treatment and time-treatment interaction. After 7 days of exposure, a significant increase in enzymatic activity was detected in digestive gland from animals treated with PFOA. After 21 days of exposure, a significant decrease in CAT activity was observed in digestive gland of animals exposed to all the concentrations tested. In previous studies, a reduction of CAT activity was detected in the liver of *Oryzias latipes* exposed for 7 days to PFOA (10, 50, 100 mg/L) (Yang, 2010) and in *D. magna* exposed for 7 days to PFOS or PFNA (0.008–5 mg/L), just at a concentration of 0.04 mg/L for both the compounds (Lu et al., 2015). Conversely, increased CAT activity was recorded both in digestive gland and in gills from *U. ravoisieri* exposed to PFOS (2–10 mg / L) (Amraoui et al., 2018). In the Australian rainbowfish *Melanotaenia fluviatilis* exposed for 24 days to PFOA (0.01, 0.1, 1 and 10 mg/L), an increase in CAT activity was recorded in the gills of animals exposed to 0.1 mg/L, whereas a reduction was found in the liver of fish treated with the two highest concentrations tested (Miranda et al., 2020). Increases in CAT activity were also observed in *P. viridis* following exposure for 7 days to PFOS, PFOA, PFNA, PFDA (0–10, 000 μg/L), showing an increase in enzyme activity up to a concentration of 100 μg/L (Liu et al., 2014b). In addition, a significant increase in CAT activity was observed in hepatocytes of *O. niloticus* exposed for 24 h to PFOS or PFOA (1, 5, 15 and 30 mg/L), but at the highest concentration tested only (Liu et al., 2007).

GR is an important enzyme that plays a key role in antioxidant defences, as it converts oxidised glutathione (GSSG) to reduced glutathione (GSH), thus replenishing functionally active GSH reserves. Although no significant effects of exposure to C6O4 and PFOA were observed in our study, a different pattern of variation of GR activity was recorded between gills and digestive gland of *R. philippinarum*. GR activity significantly decreased over time in gills of clams exposed to all the experimental conditions (including controls), whereas it did not change in digestive gland throughout the experiments. Overall, at the end of exposure (21 days), GR activity in gills resulted about five times lower than in digestive gland. An increase in GR activity was also observed in hepatocytes from *O. niloticus* exposed for 24 h to PFOS or PFOA (1, 5, 15 and 30 mg/L), particularly at the 5 mg/L of PFOA and at 30 mg/L of PFOS (Liu et al., 2007).

GST is a detoxifying enzyme of phase II of biotransformation that catalyses the conjugation of glutathione with toxic substances, to make them less reactive and more easily eliminated by organisms. Therefore, GST is indirectly involved in the antioxidant defence since it affects the availability of glutathione. In this study, digestive gland GST activity was not significantly influenced by either the treatment or the exposure time. On the contrary, alterations in GST activity were observed in the gills of *M. fluviatilis* exposed for 24 days to PFOA (0.01, 0.1, 1 and 10 mg/L), in which an increased enzyme activity was recorded at the two highest concentrations tested, while a decreased activity was found in the liver of animals treated with the highest concentration tested (Miranda et al., 2020). Increased GST activity was also observed in *Dugesia japonica* exposed for 10 days to PFOA (0.5, 5, 10, 20 mg/L) (Yuan et al., 2017), and in digestive gland of *M. galloprovincialis* exposed for 21 days to PFOS (2–10 mg/L) (Gülsever and Parlak, 2018). Conversely, inhibition of GST activity was detected in hepatocytes of *O. niloticus* exposed for 24 h to PFOS or PFOA (1, 5, 15 and 30 mg/L), even if only at the highest concentrations tested of both the compounds (Liu et al., 2007).

Protein carbonylation is one of the possible consequences of oxidative stress and PCC determination is a useful biomarker of oxidative damage to proteins (Dalle Donne et al., 2003). In our study, gill PCC was significantly affected by exposure time and time-treatment interaction. In particular, a significant increase in PCC was observed in animals treated for 7 days with 1 μg/L C6O4, with respect to the related control. Conversely, PCC in digestive gland was affected by exposure time only. Results obtained suggested that gills are more vulnerable than digestive gland to oxidative damage, at least after short-term exposure of clams to C6O4. To the best of our knowledge, no literature data on the effects of PFASs on PCC in aquatic organisms are available. Just for a comparison on the effects of other contaminants to mollusc proteins, PCC was shown to increase in the gills and digestive gland of *R. philippinarum* following exposure to titanium dioxide nanoparticles (Marisa et al., 2018), in the scallop *Chlamys farreri* exposed to chrysene (Xiu et al., 2016) and in the freshwater mussel *Dreissena polymorpha* exposed to amphetamine (Parolini et al., 2016).

Oxidative damage to DNA can occur because of oxidative stress following exposure of organisms to contaminants. In our study, DNA strand breaks in gills was significantly affected by both exposure time and treatment, with a significant increase after 21 days of exposure of clams to C6O4 (0.1 μg/L) and a decrease in PFOA-exposed animals. Conversely, no significant variations were recorded in clam digestive gland, highlighting once again the greater vulnerability of gills than digestive gland. Although a significant variation in DNA strand breaks was observed after 21 days in gills from clams exposed to C6O4, two different patterns of variation were observed, as the values increased at the lowest concentration and decreased at the highest concentration of C6O4. In this regard, we speculated that exposure to the highest concentration of C6O4 was able to activate DNA repair enzymatic systems. Increases in DNA strand breaks was observed in the planarians *Dugesia japonica* exposed for 4 days to 15 mg/L of PFOA (Zhang et al., 2020), in *P. viridis* exposed for 7 days to PFOS or PFOA (0.1–1000 μg/L) (Liu et al., 2014b), and in *C. carpio* after exposure for 4 days to PFOS (50–50, 000 μg/L) starting from the nominal concentration of 5000 μg/L, while no genotoxic effects were observed in PFOA-treated fish (Kim et al., 2010).

AChE converts acetylcholine to choline and acetic acid at the synaptic junctions, playing a key role in the transmission of the nerve signals. In our study, AChE activity was significantly influenced by treatment, exposure time and treatment-time interaction, with a significant decrease in animals exposed for 7 days to the lowest concentration of C6O4. Results obtained suggested a neurotoxic potential of C6O4 after short-term exposure and a recovery capability of clams after more prolonged exposure. Previous studies demonstrated that PFASs can reduce markedly AChE activity in various species, as observed in *D. japonica* exposed for 10 days to 15 mg/L of PFOA (Zhang et al., 2020), in *D. magna* exposed for 7 days to PFOS or PFNA (0.008–5 mg/L) (Lu et al., 2015), and in the brain of *C. auratus* after exposure for 7 days to PFOA (0.2–25,000 μg/L) (Dong et al., 2019). Conversely, no significant alterations in AChE activity were found in *C. carpio* brain samples after exposure for 4 days to PFOS or PFOA (50–50,000 μg/L) (Kim et al., 2010), in *U. ravoisieri* exposed for 7 days to 2 and 10 mg/L of PFOS (Amraoui et al., 2018) and in *G. insensibilis* exposed for 4 days to PFOS (1–3.1 mg/L) (Touaylia et al., 2019).

Interestingly, we observed that the foot of at least five clams exposed

to the lowest concentration of C6O4 (0.1 μg/L) was cut off, clams being unable to withdraw it before shell closure. Such observation, along with the reduction of AChE in clams from the same experimental group, supports the hypothesis of a possible neurotoxic effect of the contaminant. Likewise, both syphons and foot of clams were cut-off following exposure to 4-nonylphenol (Matozzo et al., 2003). In any case, narcotic effects of C6O4 to clams cannot be excluded. In this regard, further investigations are needed to evaluate deeply the narcotic and/or neurotoxic effects of C6O4 to aquatic species.

5. Conclusions

Results of the present study demonstrated that C6O4 and PFOA can affect biomarker responses in *R. philippinarum*. Of the biomarkers measured, haemocyte parameters resulted influenced by exposure to the two compounds similarly to biochemical ones. Exposure to contaminants affected THC, haemocyte volume and diameter, and cell proliferation. However, values of these parameters generally returned to control values after 21 days of exposure, indicating a transient effect of the contaminants. Interestingly, biomarkers measured in clam gills were more affected by contaminant exposure than those evaluated in digestive gland. In particular, C6O4 induce more DNA alterations than PFOA, with an increase of DNA damage at the lower concentration and a reduction of DNA damage at the higher concentration tested. Lastly, evidence of reduction in AChE activity in gills of clams exposed to 0.1 μg/L of C6O4, along with the observation of some cases of clams with foot cut off in the same experimental group, are noteworthy and suggest that other studies are necessary to investigate more fully the effects of such compounds in clams, as well as in other aquatic invertebrate species.

CRediT authorship contribution statement

Jacopo Fabrello: Methodology, Investigation, Data curation, Writing – Original draft preparation, Writing – Reviewing and Editing. **Maria Ciscato**: Methodology, Investigation, Data curation. **Luciano Masiero**: Methodology, Investigation. **Livio Finos**: Formal analysis. **Sara Valsecchi**: Data curation, Validation, Writing – Reviewing and Editing. **Stefano Polesello**: Data curation, Validation, Writing – Reviewing and Editing. **Ilaria Bernardini**: Methodology, Investigation. **Giulia Dalla Rovere**: Methodology, Investigation. **Luca Bargelloni**: Conceptualization. **Massimo Milan**: Methodology, Conceptualization, Data curation. **Tomaso Patarnello**: Conceptualization. **Maria Gabriella Marin**: Conceptualization, Methodology, Data curation. **Valerio Matozzo:** Methodology, Data curation, Writing – Original draft, Writing – Reviewing and Editing, Project administration, Funding acquisition, Supervision.

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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.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2021.126689.

References

Aebi, H., 1984. Catalase in vitro. Methods Enzymol. 105, 121–126.
Ahrens, L., Barber, J.L., Xie, Z., Ebinghaus, R., 2009a. Longitudinal and latitudinal distribution of perfluoroalkyl compounds in the surface wayer of the atlantic ocean. Environ. Sci. Technol. 43, 3122–3127.
Ahrens, L., Plassmann, M., Xie, Z., Ebinghaus, R., 2009b. Determination of polyfluoroalkyl compounds in water and suspended particulate matter in the river Elbe and North Sea, Germany. Front. Environ. Sci. Eng. China 3, 152–170.
Amraoui, I., Khalloufi, N., Touaylia, S., 2018. Effects to perfluorooctane sulfonate (PFOS) on the mollusk Unio ravoisieri under laboratory exposure. Chem. Ecol. 34, 324–339.
ARPAV, 2020. Concentrazione di sostanze perfluoroalchiliche (PFAS) nelle acque prelevate da ARPAV. Anni 2013–2020. Available online at: ⟨https://www.arpa.veneto.it/dati-ambientali/open-data/idrosfera/concentrazione-di-sostanze-perfluoroalchiliche-pfas-nelle-acque-prelevate-da-arpav⟩. Accessed on 19 December 2020.
Bernardini, I., Matozzo, V., Valsecchi, S., Peruzza, L., Dalla Rovere, G., Polesello, S., Iori, S., Marin, M.G., Fabrello, J., Ciscato, M., Masiero, L., Bonato, M., Santovito, G., Boffo, L., Bargelloni, L., Milan, M., Patarnello, T., 2021. The new PFAS C6O4 and its effects on marine invertebrates: first evidence of transcriptional and microbiota changes in the Manila clam Ruditapes philippinarum. Environ. Int. 152, 106484.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
Buck, R.C., Franklin, J., Berger, U., Conder, J.M., Cousins, I.T., de Voogt, P., Jensen, A.A., Kannan, K., Mabury, S.A., van Leeuwen, S.P., 2011. Perfluoroalkyl and polyfluoroalkyl substances in the environment: terminology, classification, and origins. Integr. Environ. Assess. Manag. 7, 513–541.
Cai, M., Yang, H., Xie, Z., Zhao, Z., Wang, F., Lu, Z., Sturm, R., Ebinghaus, R., 2012. Per- and polyfluoroalkyl substances in snow, lake, surface runoff water and coastal seawater in Fildes Peninsula, King George Island, Antarctica. J. Hazard. Mater. 209–210, 335–342.
Castiglioni, S., Valsecchi, S., Polesello, S., Rusconi, M., Melis, M., Palmiotto, M., Manenti, A., Davoli, E., Zuccato, E., 2015. Sources and fate of perfluorinated compounds in the aqueous environment and in drinking water of a highly urbanized and industrialized area in Italy. J. Hazard. Mater. 282, 51–60.
Coperchini, F., Croce, L., Pignatti, P., Ricci, G., Gangemi, D., Magri, F., Imbriani, M., Rotondi, M., Chiovato, L., 2021. The new generation PFAS C6O4 does not produce adverse effects on thyroid cells in vitro. J. Endocrinol. Investig. 44, 1625–1635.
Crapo, J.D., McCord, J.M., Fridovich, I., 1978. Preparation and assay of superioxide dismutases. Methods Enzymol. 53, 382–393.
Dalle Donne, I., Rossi, R., Giustarini, D., Milzani, A., Colombo, R., 2003. Protein carbonyl groups as biomarkers of oxidative stress. Clin. Chim. Acta 329, 23–38.
Dong, H., Lu, G., Yan, Z., Liu, J., Ji, Y., 2019. Molecular and phenotypic responses of male crucian carp (Carassius auratus) exposed to perfluorooctanoic acid. Sci. Total Environ. 653, 1395–1406.
ECHA, 2021. ECHA Information on chemicals registered substances (EC number: 682–239-6; CAS number: 1190931–41-9) ⟨https://echa.europa.eu/it/registration-dossier/-/registered-dossier/5331⟩, Accessed on 27 December 2020.
EFSA CEF Panel (EFSA Panel on food contact materials, enzymes, flavourings and processing aids), 2014. Scientific Opinion on the safety assessment of the substance, Perfluoro {acetic acid, 2-[(5-methoxy-1,3-dioxolan-4-yl) oxy]}, ammonium salt, CAS No 1190931-27-1, for use in food contact materials. EFSA J. 12 (6), 3718. https://doi.org/10.2903/j.efsa.2014.3718.
Ellman, G.L., Courtney, K.D., Andres Jr., V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95.
Feng, M., He, Q., Meng, L., Zhang, X., Sun, P., Wang, Z., 2015. Evaluation of single and joint toxicity of perfluorooctane sulfonate, perfluorooctanoic acid, and copper to Carassius auratus using oxidative stress biomarkers. Aquat. Toxicol. 161, 108–116.
Finos, L., 2020. r41sqrt10: livio's sandbox. R package version 0.1. Available online at: ⟨https://github.com/livioivil/r41sqrt10/⟩.
Gebreab, K.Y., Eeza, M., Bai, T., Zuberi, Z., Matysik, J., O'Shea, K.E., Alia, A., Berry, J.P., 2020. Comparative toxicometabolomics of perfluorooctanoic acid (PFOA) and next-generation perfluoroalkyl substances. Environ. Pollut. 265, 114928.
Giari, L., Vincenzi, F., Badini, S., Guerranti, C., Dezfuli, B.S., Fano, E.A., Castaldelli, G., 2016. Common carp Cyprinus carpio responses to sub-chronic exposure to perfluorooctanoic acid. Environ. Sci. Pollut. Res. 23, 15321–15330.
Gomis, M.I., Wang, Z., Scheringer, M., Cousins, I.T., 2015. A modeling assessment of the physicochemical properties and environmental fate of emerging and novel per-and polyfluoroalkyl substances. Sci. Total Environ. 505, 981–991.
González, I., Déjean, S., 2012. CCA: Canonical correlation analysis. R package version 1.2.
Gülsever, G., Parlak, H., 2018. Effects of perfluorooctane sulfonate compounds on the biochemical activities in mussels (Mytilus galloprovincialis). Ege JFAS 35, 417–422.
Guo, J., Wu, P., Cao, J., Luo, Y., Chen, J., Wang, G., Guo, W., Wang, T., He, X., 2019. The PFOS disturbed immunomodulatory functions via nuclear Factor-κB signaling in liver of zebrafish (Danio rerio). Fish Shellfish Immunol. 91, 87–98.
Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases: the first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7139.
IARC, Working Group on the Evaluation of Carcinogenic Risks to Humans, 2017. Some chemicals used as solvents and in polymer manufacture. IARC Monographs on the Carcinogenic Risk to Humans. Vol. 110. Lyon, France.
Johnson, M.S., Buck, R.C., Cousins, I.T., Weis, C.P., Fenton, S.E., 2021. Estimating environmental hazard and risks from exposure to per- and polyfluoroalkyl substances (PFASs): outcome of a SETAC focused topic meeting. Environ. Toxicol. Chem. 40, 543–549.

Kaviraj, A., Bhunia, F., Saha, N.C., 2004. Toxicity of methanol to fish, Crustacean, oligochaete worm, and aquatic ecosystem. Int. J. Toxicol. 23, 55–63.
Kim, W.-K., Lee, S.-K., Jung, J., 2010. Integrated assessment of biomarker responses in common carp (Cyprinus carpio) exposed to perfluorinated organic compounds. J. Hazard. Mater. 180, 395–400.
Li, K., Gao, P., Xiang, P., Zhang, X., Cui, X., Ma, L.Q., 2017. Molecular mechanisms of PFOA-induced toxicity in animals and humans: Implications for health risks. Environ. Int. 99, 43–54.
Li, Y., Men, B., He, Y., Xu, H., Liu, M., Wang, D., 2017. Effect of single-wall carbon nanotubes on bioconcentration and toxicity of perfluorooctane sulfonate in zebrafish (Danio rerio). Sci. Total Environ. 607–608, 509–518.
Lindstrom, A.B., Strynar, M.J., Libelo, E.L., 2011. Polyfluorinated compounds: past, present, and future. Environ. Sci. Technol. 45, 7954–7961.
Liu, C., Chang, V.W.C., Gin, K.Y.H., 2014a. Oxidative toxicity of perfluorinated chemicals in green mussel and bioaccumulation factor dependent quantitative structure–activity relationship. Environ. Toxicol. Chem. 33, 2323–2332.
Liu, C., Gin, K.Y.H., Chang, V.W.C., 2014b. Multi-biomarker responses in green mussels exposed to PFCs: effects at molecular, cellular, and physiological levels. Environ. Sci. Pollut. Res. 21, 2785–2794.
Liu, C., Yu, K., Shi, X., Wang, J., Lam, P.K.S., Wu, R.S.S., Zhou, B., 2007. Induction of oxidative stress and apoptosis by PFOS and PFOA in primary cultured hepatocytes of freshwater tilapia (Oreochromis niloticus). Aquat. Toxicol. 82, 135–143.
Lu, G.-H., Liu, J.-C., Sun, L.-S., Yuan, L.-J., 2015. Toxicity of perfluorononanoic acid and perfluorooctane sulfonate to Daphnia magna. Water Sci. Eng. 8, 40–48.
Marisa, I., Matozzo, V., Martucci, A., Franceschinis, E., Brianese, N., Marin, M.G., 2018. Bioaccumulation and effects of titanium dioxide nanoparticles and bulk in the clam Ruditapes philippinarum. Mar. Environ. Res. 136, 179–189.
Matozzo, V., Costa Devoti, A., Marin, M.G., 2012. Immunotoxic effects of Triclosan in the clam Ruditapes philippinarum. Ecotoxicology 21, 66–74.
Matozzo, V., Deppieri, M., Moschino, V., Marin, M.G., 2003. Evaluation of 4-nonylphenol toxicity in the clam Tapes philippinarum. Environ. Res. 91, 179–185.
Matozzo, V., Marin, M.G., Cima, F., Ballarin, L., 2008. First evidence of cell division in circulating haemocytes from the Manila clam Tapes philippinarum. Cell Biol. Int. 32, 865–868.
Matozzo, V., Gagné, F., 2016. Immunotoxicology Approaches in Ecotoxicology: Lessons from Mollusks. In: Ballarin, L., Cammarata, M. (Eds.), Lessons in Immunity: From Single-cell Organisms to Mammals, 1st edition. Academic Press-Elsevier, London (UK), pp. 29–51.
Mecocci, P., Fano, G., Fulle, S., MacGarvey, U., Shinobu, L., Polidori, M.C., Cherubini, A., Vecchiet, J., Senin, U., Beal, M.F., 1999. Age-dependent increases in oxidative damage to DNA, lipids, and proteins in human skeletal muscle. Free Radic. Biol. Med. 26, 303–308.
Miranda, A.F., Trestrail, C., Lekamge, S., Nugegoda, D., 2020. Effects of perfluorooctanoic acid (PFOA) on the thyroid status, vitellogenin, and oxidant–antioxidant balance in the Murray River rainbowfish. Ecotoxicology 29, 163–174.
Morganti, M., Polesello, S., Pascariello, S., Ferrario, C., Rubolini, D., Valsecchi, S., Parolini, M., 2021. Exposure assessment of PFAS-contaminated sites using avian eggs as a biomonitoring tool: a frame of reference and a case study in the Po River valley (Northern Italy). Integr. Environ. Assess. Manag. 17, 733–745. https://doi.org/10.1002/ieam.4417.
Mudumbi, J.B., Ntwampe, S.K., Muganza, F.M., Okonkwo, J.O., 2014. Perfluorooctanoate and perfluorooctane sulfonate in South African river water. Water Sci. Technol. 69, 185–194.
Mueller, R., Yingling, V., 2017. History and Use of Per-and Polyfluoroalkyl Substances (PFAS). Interstate Technology & Regulatory Council, USA.
Naile, J.E., Khim, J.S., Wang, T., Chen, C., Luo, W., Kwon, B.-O., Park, J., Koh, C.-H., Jones, P.D., Lu, Y., Giesy, J.P., 2010. Perfluorinated compounds in water, sediment, soil and biota from estuarine and coastal areas of Korea. Environ. Pollut. 158, 1237–1244.
Olive, P.L., 1988. DNA precipitation assay: a rapid and simple method for detecting DNA damage in mammalian cells. Environ. Mol. Mutagen. 11, 487–495.
Parolini, M., Magni, S., Castiglioni, S., Binelli, A., 2016. Amphetamine exposure imbalanced antioxidant activity in the bivalve Dreissena polymorpha causing oxidative and genetic damage. Chemosphere 144, 207–213.
Parsons, J.R., Sáez, M., Dolfing, J., de Voogt, P., 2008. Biodegradation of perfluorinated compounds. Biodegradation of perfluorinated compounds. Rev. Environ. Contam. Toxicol. 196, 53–71.
Pipe, R.K., Coles, J.A., 1995. Environmental contaminants influencing immune function in marine bivalve molluscs. Fish Shellfish Immunol. 5, 581–595.
Pistocchi, A., Loos, R., 2009. A map of European emissions and concentrations of PFOS and PFOA. Environ. Sci. Technol. 43, 9237–9244.
Polesello, S., Pagnotta, R., Rusconi, M., Valsecchi, S., 2011. Realizzazione di uno studio di valutazione del Rischio Ambientale e Sanitario associato alla contaminazione da sostanze perfluoro-alchiliche (PFAS) nel Bacino del Po e nei principali bacini fluviali italiani. Relazione finale. Istituto di Ricerca Sulle Acque del Consiglio Nazionale delle Ricerche,.
R Core Team, 2020. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. Available online at: ⟨http://www.r-project.org/index.html⟩.
Renzi, M., Guerranti, C., Giovani, A., Perra, G., Focardi, S.E., 2013. Perfluorinated compounds: levels, trophic web enrichments and human dietary intakes in transitional water ecosystems. Mar. Pollut. Bull. 76, 146–157.
Rusconi, M., Marziali, L., Stefani, F., Valsecchi, S., Bettinetti, R., Mazzoni, M., Rosignoli, F., Polesello, S., 2015. Evaluating the impact of a fluoropolymer plant on a river macrobenthic community by a combined chemical, ecological and genetic approach. Sci. Total Environ. 538, 654–663.
Sinclair, E., Mayack, D.T., Roblee, K., Yamashita, N., Kannan, K., 2006. Occurrence of perfluoroalkyl surfactants in water, fish, and birds from New York State. Arch. Environ. Contam. Toxicol. 50, 398–410.
Smith, I.K., Vierheller, T.L., Thorne, C.A., 1988. Assay of glutathione reductase in crude tissue homogenates using 5, 5′-dithiobis (2-nitrobenzoic acid). Anal. Biochem. 175, 408–413.
So, M.K., Miyake, Y., Yeung, W.Y., Ho, Y.M., Taniyasu, S., Rostkowski, P., Yamashita, N., Zhou, B.S., Shi, X.J., Wang, J.X., Giesy, J.P., Yu, H., Lam, P.K., 2007. Perfluorinated compounds in the Pearl River and Yangtze River of China. Chemosphere 68, 2085–2095.
So, M.K., Taniyasu, S., Yamashita, N., Giesy, J.P., Zheng, J., Fang, Z., Im, S.H., Lam, P.K., 2004. Perfluorinated compounds in coastal waters of Hong Kong, South China, and Korea. Environ. Sci. Technol. 38, 4056–4063.
Strynar, M., Dagnino, S., McMahen, R., Liang, S., Lindstrom, A., Andersen, E., McMillan, L., Thurman, M., Ferrer, I., Ball, C., 2015. Identification of novel perfluoroalkyl ether carboxylic acids (PFECAs) and sulfonic acids (PFESAs) in natural waters using accurate mass time-of-flight mass spectrometry (TOFMS). Environ. Sci. Technol. 49, 11622–11630.
Sunderland, E.M., Hu, X.C., Dassuncao, C., Tokranov, A.K., Wagner, C.C., Allen, J.G., 2019. A review of the pathways of human exposure to poly- and perfluoroalkyl substances (PFASs) and present understanding of health effects. J. Expo. Sci. Environ. Epidemiol. 29, 131–147.
Sznajder-Katarzyńska, K., Surma, M., Cieślik, I., 2019. A review of perfluoroalkyl acids (PFAAs) in terms of sources, applications, human exposure, dietary intake, toxicity, legal regulation, and methods of determination. J. Chem. 2019 (6), 1–20, 2717528.
Tang, J., Lu, X., Chen, F., Ye, X., Zhou, D., Yuan, J., He, J., Chen, B., Shan, X., Jiang, J., Liu, W., Zhang, H., 2018. Effects of perfluorooctanoic acid on the associated genes expression of autophagy signaling pathway of Carassius auratus lymphocytes in vitro. Front. Physiol. 9, 1748.
Touaylia, S., Khazri, A., Mezni, A., Bejaoui, M., 2019. Effects of emerging persistent organic pollutant perfluorooctane sulfonate (PFOS) on the Crustacean Gammarus insensibilis. Hum. Ecol. Risk. Assess. 25, 2133–2141.
UNEP, 2009a. Decision SC-4/17. Listing of Perfluorooctane Sulfonic Acid, Its Salts and Perfluorooctane Sulfonyl Fluoride. United Nations Environment Programme (UNEP), Secretariat of the Basel, Rotterdam and Stockholm Conventions, Geneva, Switzerland. SC-4/17.
UNEP, 2019b. Decision SC-9/12: Listing of Perfluorooctanoic Acid (2019), its Salts and PFOA-Related Compounds. UN Environment (UNEP), Secretariat of the Basel, Rotterdam and Stockholm Conventions, Geneva, Switzerland.
Van Zelm, R., Huijbregts, M.A., Russell, M.H., Jager, T., Van De Meent, D., 2008. Modeling the environmental fate of perfluorooctanoate and its precursors from global fluorotelomer acrylate polymer use. Environ. Toxicol. Chem. 27, 2216–2223.
Vo, H.N.P., Ngo, H.H., Guo, W., Nguyen, T.M.H., Li, J., Liang, H., Deng, L., Chen, Z., Nguyen, T.A.H., 2020. Poly-and perfluoroalkyl substances in water and wastewater: a comprehensive review from sources to remediation. J. Water Process Eng. 36, 101393.
Wallington, T.J., Hurley, M.D., Xia, J., Wuebbles, D.J., Sillman, S., Ito, A., Penner, J.E., Ellis, D.A., Martin, J., Mabury, S.A., Nielsen, O.J., Sulbaek Andersen, M.P., 2006. Formation of C7F15COOH (PFOA) and other perfluorocarboxylic acids during the atmospheric oxidation of 8:2 fluorotelomer alcohol. Environ. Sci. Technol. 40, 924–930.
Wang, Z., Cousins, I.T., Scheringer, M., Hungerbuehler, K., 2015. Hazard assessment of fluorinated alternatives to long-chain perfluoroalkyl acids (PFAAs) and their precursors: status quo, ongoing challenges and possible solutions. Environ. Int. 75, 172–179.
Wang, Z., DeWitt, J.C., Higgins, C.P., Cousins, I.T., 2017. A never-ending story of per-and polyfluoroalkyl substances (PFASs)? Environ. Sci. Technol. 51, 2508–2518.
Wang, D., Goldenman, G., Tugran, T., McNeil, A., Jones, M., 2020a. Nordic Working Paper, Per-and Polyfluoroalkylether Substances: Identity, Production and Use. Nordic Council of Ministers, Copenhagen.
Wang, J., Shi, G., Yao, J., Sheng, N., Cui, R., Su, Z., Guo, Y., Dai, J., 2020b. Perfluoropolyether carboxylic acids (novel alternatives to PFOA) impair zebrafish posterior swim bladder development via thyroid hormone disruption. Environ. Int. 134, 105317.
Wang, P., Yonglong, L., Tieyu, W., Meng, J., Qifeng, L., Zhaoyun, Z., Yajun, S., Wang, R., Giesy, J.P., 2016. Shifts in production of perfluoroalkyl acids affect emissions and concentrations in the environment of the Xiaoqing River Basin, China. J. Hazard. Mater. 307, 55–63.
Wu, Y., Deng, M., Jin, Y., Mu, X., He, X., Luu, N.-T., Yang, C., Tu, W., 2019. Uptake and elimination of emerging polyfluoroalkyl substance F-53B in zebrafish larvae: response of oxidative stress biomarkers. Chemosphere 215, 182–188.
Xiu, M., Pan, L., Jin, Q., 2016. Toxic effects upon exposure to polycyclic aromatic hydrocarbon (chrysene) in scallop Chlamys farreri during the reproduction period. Environ. Toxicol. Pharmacol. 44, 75–83.
Yamashita, N., Kannan, K., Taniyasu, S., Horii, Y., Petrick, G., Gamo, T., 2005. A global survey of perfluorinated acids in oceans. Mar. Pollut. Bull. 51, 658–668.
Yamashita, N., Taniyasu, S., Petrick, G., Wei, S., Gamo, T., Lam, P.K., Kannan, K., 2008. Perfluorinated acids as novel chemical tracers of global circulation of ocean waters. Chemosphere 70, 1247–1255.
Yang, J.-H., 2010. Perfluorooctanoic acid induces peroxisomal fatty acid oxidation and cytokine expression in the liver of male Japanese medaka (Oryzias latipes). Chemosphere 81, 548–552.

Yang, L., Zhu, L., Liu, Z., 2011. Occurrence and partition of perfluorinated compounds in water and sediment from Liao River and Taihu Lake, China. Chemosphere 83, 806–814.

Yuan, Z., Miao, Z., Gong, X., Zhao, B., Zhang, Y., Ma, H., Zhang, J., Zhao, B., 2017. Changes on lipid peroxidation,enzymatic activities and gene expression in planarian (Dugesia japonica) following exposure to perfluorooctanoic acid. Ecotoxicol. Environ. Saf. 145, 564–568.

Zhang, F., Wei, J., Li, Q., Jiang, R., Yu, N., Qin, J., Chen, L., 2015. Effects of perfluorooctane sulfonate on the immune responses and expression of immune-related genes in Chinese mitten-handed crab Eriocheir sinensis. Comp. Biochem. Physiol. Part C 172–173, 13–18.

Zhang, J., Shao, X., Zhao, B., Zhai, L., Liu, N., Gong, F., Ma, X., Pan, X., Zhao, B., Yuan, Z., Zhang, X., 2020. Neurotoxicity of perfluorooctanoic acid and post-exposure recovery due to blueberry anthocyanins in the planarians Dugesia japonica. Environ. Pollut. 263, 114471.