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Contaminant-induced oxidative stress underlies biochemical, molecular and fatty acid profile changes, in gilthead seabream (*Sparus aurata L.*)

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ABSTRACT

Chemical contaminants such as heavy metals, polybrominated diphenyl ethers (PBDEs) and drugs, are constantly found in the marine environment determining the interest of the scientific community for their side effects on animal welfare, food safety and security. Few studies have analyzed the effects of mix of contaminants in fish, in terms of molecular and nutritional composition response, beside it is indispensable to think more and more on effect of contaminants along the food web system. In this study, *Sparus aurata* specimens were exposed for 15 days, by diet, to a mixture of carbamazepine (Cbz), polybrominated diphenyl ether-47 (PBDE-47) and cadmium chloride ($CdCl_2$), at two doses (0.375 μg g-1 D1; 37.5 μg g-1 D2) (T15). After, fish were fed with a control diet, without contaminants mix, for other 15 days (T30). The study explored the effect on oxidative stress in the liver, analyzing specific molecular markers and effects on quality, by fatty acid profile and lipid peroxidation. Molecular markers involved in ROS scavenging, such as superoxide dismutase (sod), catalase (cat) and glutathione peroxidase (gpx) were evaluated by gene expression; as markers of quality and lipid peroxidation, the fatty acids (FAs) profile and the level of malondyaldeide (MDA) were assessed. *Sod* and *cat* genes underwent to up-regulation after 15 days of diet containing contaminants and showed down-regulation after the next 2 weeks of detoxification (T30). At T15, the FAs profile showed an increase of the saturated fatty acids (SFA), and a decrease of the polyunsatured fatty acids (PUFA). The MDA levels increased over time, indicating an ongoing radical damage. These results suggest that the effects of the contaminants can be perceived not only at molecular but also at nutritional level and that the molecular and biochemical markers adopted could be differently used to monitor the health of aquatic organisms in the marine environment.

1. Introduction

It is well known that the release of pollutants and xenobiotics into the environment, by a variety of anthropogenic activities, could represent a serious threat for human and ecosystems health (Goretti et al., 2020). Among contaminants the non-biodegradables, as heavy metals, bioaccumulate through the food web, in the organisms tissues, with a lot of negative side effects (Morcillo et al., 2016). In particular, Cadmium (Cd) is one of the most relevant, representing an important risk for human health (Mensi et al., 2008), included by the USA Environmental Protection Agency in the "Priority List of Chemicals" and classified as a human carcinogen by the International Agency for Research on Cancer (Nordberg et al., 1992). In the marine environments, Cd has been found at concentration ranged approximately 0.01–42 μg L^{-1} and, additionally, has been demonstrated to induce, both *in vivo* and *in vitro*, cytotoxicity, oxidative stress and immune system impairment (Bonsignore et al., 2022; Espinosa-Ruiz et al., 2021; Morcillo et al., 2016). Regarding xenobiotics, pharmaceuticals are considered a class of emerging environmental contaminants and for that reason can represent a potential risk for the living organisms (Palacios-Rosas and Castro-Pastrana, 2019).

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In fact, once in the environment, pharmaceuticals could be bioavailable, threatening aquatic organisms. Within this category, carbamazepine (Cbz) is widely distributed and abundant in the aquatic environment; it can be found in wastewater treatment plants effluents, surface waters, and soils (Oliveira et al., 2015). Actually, only 10% of Cbz is removed in wastewater treatment plants (Chen et al., 2014), persisting in the aquatic environment around 82 days (Brandão et al., 2013). Although, some studies performed in last years, highlighted possible lethal and sublethal effects of Cbz on a variety of marine organisms such as algae, cladocerans and fish, its chronic effects on marine communities still remains uncertain (Deblonde et al., 2011). Furthermore, another class of persistent contaminants in the marine environment, is represented by the polybrominated diphenyl ethers (PBDEs), widely used as flame-retardants in a lot of products as building and electronic materials, textiles, paints and foams. Due to their ubiquities in the environment and toxicities, PBDEs are a threat to both human health and ecosystems (Wu et al., 2020). The 2, 2′, 4, 4′ -tetrabromodiphenyl ether (BDE-47) and the 2, 2′, 4, 4′, 5-pentabromodiphenyl ether (BDE-99), are the most abundant congeners of PBDEs in aquatic environments. Nowadays the majority of studies about the biological impacts of PBDEs in fish, investigated on their ability to act as thyroid hormone disruptors leading to alterations in thyroid related gene expression and growth (Lema et al., 2008). However, recently researches highlighted that PBDEs exposure can also lead to alterations in immune function in fish (Arkoosh et al., 2015; Espinosa-Ruiz et al., 2021).

In marine environments, organisms are simultaneously exposed to a variety of contaminants resulting in complex mixtures. These, depending on the nature of the toxicant and the action mechanisms, produce abnormal effects compared to exposures of single compounds such as cumulative, synergistic and antagonistic effects (Espinosa-Ruiz et al., 2020). The accumulation of contaminants has an impact not only on fish but also on fish consumers. Thus, for the fishery value-chains, could be important to understand the mechanisms of contaminant's bio-accumulation, biomagnification and the side effects of their transfer to humans, through the diet (Bonsignore et al., 2022; Di Bella et al., 2020).

Among the negative effects induced by chemical pollutants on marine organisms, the phenomenon of oxidative stress is widely recognized as a common event, detrimental for all cellular components (Bonsignore et al., 2022; Espinosa-Ruiz et al., 2021; Messina et al., 2020). This event occurs when the reactive oxygen species (ROS) are produced in excess in respect to the antioxidant systems, represented also by the scavenger enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) (Pan et al., 2018), with negative effects such as DNA damage, protein degradation, enzyme inactivation and lipid peroxidation. In particular, oxidative stress can adversely impact poly-unsaturated fatty acids (PUFA) (Filimonova et al., 2016). It has been demonstrated that PUFA, and hence membranes with a higher unsaturation degree, are more prone to lipid peroxidation (Bonsignore et al., 2022). PUFA, including omega-3, play a key role in the health and function of all marine organisms through trophic web. In fish, they cannot be synthesized *de novo*, or at least not in sufficient amounts so are accumulated along the food chain (starting from primary producers) (Filimonova et al., 2016). Although, marine organisms are among the most rich in omega-3, the relationship between single chemical contaminants or combined, and fatty acids profile alteration is still unclear (Filimonova et al., 2016). Fatty acids are transferred across the aquatic food webs and are considered good bioindicator for ecosystem health and stress for their sensibility to environmental changes (Filimonova et al., 2016). Malondialdehyde (MDA), the final product of tissues lipid peroxidation, is indicator of oxidative damage (Pan et al., 2018).

Our previous studies demonstrated that prolonged exposure to mixture of contaminates, commonly spreaded in sea water at sub-lethal concentrations, influenced immunity and inflammatory response in different marine organisms both *in vitro* and *in vivo* (Espinosa-Ruiz et al., 2021, 2020, 2019b, 2019c, 2017; Messina et al., 2020). Recently, the interest of the scientific community focused also on the effects of contaminants in compromising of the nutritional values of fish, that represent food safety and security issues (Bonsignore et al., 2022). Up to now, few studies have analyzed the effects of mix of contaminants in fish, in terms of molecular and nutritional composition response (Bonsignore et al., 2022), beside it is indispensable to think more and more on effect of contaminants along the food web system, from sea to human, according to the "one health" approach.

This study aimed to investigate the effects of different concentrations of BDE-47, Cbz and $CdCl_2$ mixture, on biomarkers involved in oxidative stress and lipid peroxidation in *S. aurata*, used as marine model system, in order to understand the effects of contaminants not only in fish health but also on its quality, focusing on side effects of contaminants on one of the emblematic components of fish quality, represented by n-3 PUFA.

2. Materials and methods

2.1. Sea bream maintenance

Forty specimens (25 ± 3 g mean body weight) of gilthead sea bream (*Sparus aurata* L.), obtained from a farm in Murcia (Spain), were randomly assigned and kept in three running seawater aquaria (250 L, flow rate 900 L h^{-1}) in the Marine Fish Facilities at the University of Murcia at 28‰ salinity, 20 °C, and a photoperiod (12 L:12 D) and kept in quarantine for four weeks, according to the experimental design described in Espinosa-Ruiz et al. (2021). Fish were fed with a commercial pellet diet (Skretting Italia, Vr, Italy) at a rate of 1.5% body weight day^{-1}.

2.2. Experimental design, treatment with contaminants mix and sampling

After this period, fish were then randomly located in six running seawater aquaria (6 specimens per aquarium) at the same conditions described above, while four specimens were separated for time zero, as described below. Animals in each aquarium received one of the following experimental diets made with a commercial pellet diet (Skretting Italia, Vr, Italy) at a rate of 1.5% body weight day^{-1}: commercial diet containing vehicle (control) or containing a mixture of 0.375 μg g^{-1} of BDE-47, Cbz and $CdCl_2$ (low dose - D1) or a mixture of 37.5 μg g^{-1} of BDE-47, Cbz and $CdCl_2$ (high dose - D2) according to the experimental design described in Espinosa-Ruiz et al. (2021).

Six specimens of each diet were sampled after 15 days of treatment with mixture (T15) and after the next 15 days of detoxification period (T30), while four fish (for economy and ethics matter) were sampled as the beginning of the experiment (considered time zero). Specimens were sacrificed by an overdose of MS222 (100 mg L^{-1}; Sandoz) and samples of liver were obtained from each specimen.

The fragments of liver were stored in PUREzol Reagent (Bio-Rad, Hercules, CA, USA) at −80 °C for gene expression analysis. Other fragments of the same organ were freeze-dried then stored at −80 °C for analyses of lipid peroxidation and fatty acids profile.

2.3. Analysis of mRNA expression

Total RNA was extracted from the liver using PUREzol Reagent (Bio-Rad, Hercules, CA, USA) and the concentration was assessed spectrophotometrically at 260 nm (Chomczynski, 1993). The absorbance ratios A260/A280 and A260/A230 were evaluated as indicators of RNA purity. Then, 1 μg of RNA was reverse-transcribed for each sample, in a volume of 20 μL, by the 5× iScript Reaction Mix Kit (Bio-Rad, USA) according to manufacturer's instructions. The amplification was performed in a total volume of 20 μL, which contained: 0.4 μmol L^{-1} of each primer, cDNA diluted 1:10 of the final reaction volume, 1× IQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and nuclease-free water. Conditions for real-time PCRs were optimized in a gradient cycler using a C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA, USA) and using the primers shown in Table 1 and the following run protocol. An initial

Table 1
Sparus aurata primer sequences used for real-time PCR.

Gene	Accession number	F/R Primer sequence (5′–3′)
sod		CCATGGTAAGAATCATGGCGG CGTGGATCACCATGGTTCTG
cat		TTCCCGTCCTTCATTCACTC CTCCAGAAGTCCCACACCAT
gpx		CTTTGAGCCAAAGATCCAGC CTGACGGGACTCCAAATGAT
ef1a	AF184170	CTTCAACGCTCAGGTCATCAT GCACAGCGAAACGACCAAGGGGA
18S	AM490061	CTTCAACGCTCAGGTCATCAT AGTTGGCACCGTTTATGGTC

activation step at 95 °C for 3 min, followed by 39 cycles of 95 °C for 10s and 60 °C for 30s, with a single fluorescence measurement. Melting curve program was achieved at 65–95 °C with heating rate of 0.5 °C/cycle and a continuous fluorescence measurement. All reactions were performed in triplicate. For each PCR, we checked linear range of a standard curve of serial dilutions. The relative quantification of superoxide dismutase (*sod*), catalase (*cat*) and glutathione peroxidase (*gpx*) gene expression was evaluated after normalization with the reference genes. Data processing and statistical analysis were performed using CFX Manager Software (Bio-Rad, Hercules, CA, USA). The relative expression of all genes was calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), using *S. aurata ef1α* and *18S* as the endogenous reference.

2.4. Fatty acid determination

The total lipids were determined according to Folch et al. (1957). The fatty acid methyl esters (FAME) were determined by the method of Lepage and Roy (1984). The gas chromatography was carried out under the conditions described by Messina et al. (2013) employing a Perkin Elmer (Waltham, MA, USA) Clarus 580 instrument equipped using a silica capillary column (30 m × 0.32 mm, d_f 0.25 μm, Omegawax 320, Supelco, Bellefonte, PA, USA). Individual FAME were measured by comparison of known standards (mix of PUFA 1, PUFA 2 and PUFA 3 mixed oil, Supelco) while individual fatty acids were expressed as percentage of total fatty acids.

2.5. Lipid peroxidation

Lipid peroxidation was determined by measuring thiobarbituric acid reactive substances (TBARS) according to Botsoglou et al. (1994) and analyzed as described by Messina et al. (2021). Freeze-dried liver samples (30 mg) were homogenized in 800 μL of 5% trichloroacetic acid and 500 μL of 0.8% butylhydroxytoluene. Homogenates were centrifuged at 3000 rpm for 10 min at 4 °C for the extraction. The MDA in the sample reacted with thiobarbituric acid (TBA) under high temperature (70 °C) to generate the MDA-TBA adduct. MDA was measured by colorimetric determination at 532 nm. Its concentration was expressed as mg/kg of sample.

2.6. Statistical analysis

Statistical differences among the groups were assessed by one-way ANOVA analyses, followed by the Bonferroni or Games Howell test, depending on the homogeneity of the variables. The normality of the variables was confirmed by the Shapiro–Wilk test and homogeneity of variance by the Levene test. The significance level was 95% in all cases ($P < 0.05$). All the data were analyzed by the computer application SPSS for Windows® (version 15.0, SPSS Inc., Chicago, USA).

3. Results

3.1. Gene expression analyses

The results of gene expression of biomarkers related to the antioxidant activity in liver of *S. aurata* are shown in Fig. 1. The expression of *sod* resulted significantly ($P < 0.05$) decreased in fish fed both doses of contaminants (D1 and D2) at 15 days (T15) compared to control group ($P < 0.05$), while increased at T30, after 2 weeks of detoxification with both doses of contaminants (D1 and D2). The expression of *cat* showed no significant difference in fish fed with lower dose of mixture (D1) at T15 and T30 compared to control. Instead, a significant ($P < 0.05$) decrease in animals exposed to the D2 at T15 and an increase at T30, after two weeks of detoxification compared to control group was observed. The expression of *gpx* showed no significant difference in liver of *S. aurata* exposed to the lowest dose (D1) at both T15 and T30 compared to control. While a marked decrease after 15 days of mixture

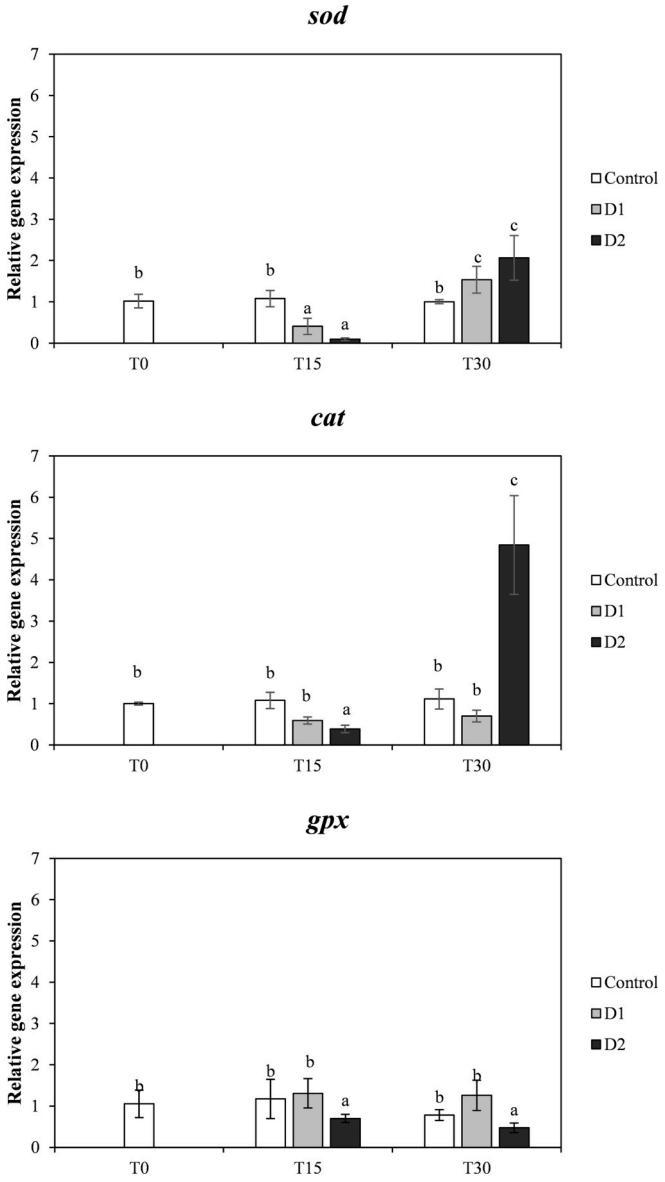

Fig. 1. Relative gene expression of molecular markers related to oxidative stress (*cat, sod and gpx*) determined in liver of *S. aurata* at T0, T15 and T30 of treatment, fed with Control diet and experimental diets (D1 and D2). Values are means ± SEM (n = 6). Statistical differences ($P < 0.05$) among groups are indicated by different superscript letters.

administration at the highest dose (D2), and did not recover at 30 days, beside the 15 days of detoxification was observed.

3.2. Fatty acid and MDA profile

The fatty acids composition of the liver of mix exposed fish, presented some alterations with respect to control specimens (Table 2), more specifically: the lot fed the highest dose of mix (D2), showed a significant increase of saturated fatty acids (SFA) at T15 (in particular for 16:0 and 18:0) ($P < 0$ 0.05), followed by a remarkable decrease after 2 weeks of detoxification (T30) ($P < 0.05$) with respect to control. While no significant differences were observed in the liver of the specimens fed the lower dose of mixture (D1) at T15 and T30 compared to control (Table 2).

Monounsaturated fatty acids (MUFA), which represent the most abundant class of fatty acids, were analyzed in liver of *S. aurata* (Table 2). In the specimens fed the lower dose of mixture (D1) at T15 and T30 no significant differences were observed compared to control. An increase at T30 in D2 lot was observed, specifically, higher concentrations of 24:1n9, and a significant ($P < 0.05$) reduction of 18:1n9 were observed (Table 2).

Among the polyunsaturated fatty acids (PUFA), the class of Tot n-3 in specimens exposed to the lowest mix dose (D1) both at T15 and T30 showed no significant differences compared to control (Table 2). Concerning specimens exposed to the highest mix dose (D2) after 15 days of experimental feeding, a significant decrease ($P < 0.05$) was observed, while Tot n-3 content was comparable to control at T30 (Table 2). Interestingly, the most affected fatty acids resulted the eicosapentaenoic acid (EPA) (20:5n-3) and the docosahexaenoic acid (DHA) (22:6n-3). Even the class of Tot n-6 in D1 lot, showed no significant differences at T15 and T30 compared to control. Instead the specimens fed the highest dose (D2) after 15 days exhibited the highest content Tot n-6. A significant increase ($P < 0.05$) of 18:2n-6 was observed, while in D2 lot at T30 no significant differences were observed compared to control (Table 2).

The evaluation of the peroxidation status in liver, by MDA quantification, was showed in Fig. 2. The obtained results highlighted a significant production of MDA after 15 days of exposure at the highest dose (D2) of contaminants in the diet, with respect to control and D1 lot ($P < 0.05$). At T30, after 2 weeks of detoxification, MDA levels continued to increase in lot D2, attesting the radical damage (Fig. 2).

4. Discussion

The adverse effects of different stressors, including chemicals contaminants, were extensively investigated in marine organisms, in term of cytotoxicity, immune and biochemical response, especially with regard to induction of oxidative stress (Bonsignore et al., 2022; Guardiola et al., 2015). It is well known that the interactions between chemicals and organisms can lead diverse biochemical pathways with different toxicological responses, depending on the natures of the chemicals and on the target organism (Prato and Biandolino, 2007). On the contrary, few papers have considered the implication of biochemical responses to contaminants on modification of quality traits of the organisms that become components of the food system (Bonsignore et al., 2022; Polak-Juszczak, 2018).

Itis well known that the bioaccumulation of the contaminants depends largely on the time of exposure and tissues (Bowen et al., 2006). The liver plays a key role also in fish physiology and detoxification patterns (Chen et al., 2012) and changes in its structure and enzymatic activities can provide information on fish welfare (Espinosa-Ruiz et al., 2021). The liver is also considered as the most vulnerable organ to oxidative damages (Bonsignore et al., 2022). Metals are important inducers of oxidative stress also in aquatic organisms, inducing reactive oxygen species (ROS) formation through two different mechanisms: redox active metals generate ROS through redox cycling, while metals without redox potential impair antioxidant defenses, especially those of

Table 2
Fatty acid profile determined at T0, T15 and T30 of treatment in liver of *S. aurata*, fed with control and experimental diet (D1 and D2). Values are the mean ± SEM (n = 6). Statistical differences ($P < 0.05$) among groups are indicated by different superscript letters.

	Control	D1		D2	
		T15	T30	T15	T30
14:0	1.71 ± 0.02^{b}	1.42 ± 0.31^{b}	1.59 ± 0.20^{b}	1.34 ± 0.34^{ab}	1.14 ± 0.15^{a}
16:0	12.60 ± 0.17^{b}	12.38 ± 0.48^{b}	12.90 ± 0.57^{b}	14.75 ± 0.95^{c}	10.48 ± 0.59^{a}
16:1n-7	4.23 ± 0.06^{a}	4.23 ± 0.04^{a}	4.44 ± 0.13^{a}	4.13 ± 0.16^{a}	4.91 ± 0.54^{b}
16:2n-4	0.46 ± 0.01	0.57 ± 0.01	0.50 ± 0.22	0.64 ± 0.06	0.48 ± 0.18
16:3n-4	0.54 ± 0.01^{a}	0.79 ± 0.06^{ab}	0.92 ± 0.34^{ab}	1.10 ± 0.14^{b}	0.63 ± 0.01^{a}
18:0	4.55 ± 0.06^{a}	4.91 ± 0.15^{a}	4.78 ± 0.39^{a}	5.36 ± 0.26^{b}	4.36 ± 0.12^{a}
18:1n-9	20.12 ± 0.26^{b}	20.37 ± 0.81^{b}	19.85 ± 0.86^{b}	16.73 ± 0.41^{a}	15.79 ± 1.06^{a}
18:1n-7	3.10 ± 0.04^{a}	3.80 ± 0.27^{a}	3.45 ± 0.21^{a}	7.22 ± 0.84^{b}	4.03 ± 1.92^{a}
18:2n-6	11.52 ± 0.15^{b}	12.03 ± 0.19^{b}	12.25 ± 1.46^{b}	14.04 ± 0.51^{c}	10.19 ± 0.17^{a}
18:3n-6	0.89 ± 0.01^{a}	0.99 ± 0.07^{a}	0.90 ± 0.36^{a}	0.61 ± 0.07^{a}	1.43 ± 0.20^{b}
18:3n-3	3.46 ± 0.05^{c}	2.58 ± 0.17^{b}	2.56 ± 0.44^{b}	1.99 ± 0.00^{a}	2.82 ± 0.09^{b}
18:4n-3	0.71 ± 0.01^{bc}	0.59 ± 0.07^{b}	0.63 ± 0.28^{b}	0.28 ± 0.05^{a}	0.94 ± 0.07^{c}
18:4n-1	1.22 ± 0.02^{ab}	1.76 ± 0.21^{c}	1.49 ± 0.34^{bc}	2.66 ± 0.01^{d}	0.95 ± 0.07^{a}
20:1n-9	1.73 ± 0.02^{c}	1.36 ± 0.13^{bc}	1.07 ± 0.23^{ab}	0.78 ± 0.02^{a}	1.66 ± 0.47^{c}
20:2n-6	0.54 ± 0.01^{a}	0.15 ± 0.14^{a}	0.46 ± 0.19^{ab}	0.38 ± 0.21^{ab}	0.08 ± 0.14^{a}
20:3n-6	0.40 ± 0.01^{c}	0.25 ± 0.03^{b}	0.24 ± 0.06^{b}	0.17 ± 0.00^{a}	0.26 ± 0.01^{b}
20:4n-6	0.64 ± 0.01	0.59 ± 0.01	0.54 ± 0.04	0.57 ± 0.02	0.56 ± 0.08
20:5n-3	4.17 ± 0.05^{c}	3.54 ± 0.13^{bc}	3.21 ± 0.37^{ab}	2.75 ± 0.04^{a}	3.85 ± 0.52^{bc}
22:1n-11	3.73 ± 0.05^{b}	3.99 ± 0.10^{c}	4.02 ± 0.10^{c}	3.45 ± 0.01^{a}	4.96 ± 0.02^{d}
22:1n-9	0.47 ± 0.01	0.25 ± 0.04	0.61 ± 0.16	0.25 ± 0.18	0.43 ± 0.27
22:5n-3	2.53 ± 0.03^{b}	1.91 ± 0.12^{a}	1.85 ± 0.30^{a}	1.57 ± 0.10^{a}	1.95 ± 0.01^{a}
22:6n-3	11.05 ± 0.14^{a}	11.71 ± 0.12^{a}	11.64 ± 1.03^{a}	10.52 ± 0.75^{a}	13.47 ± 0.16^{b}
24:1n-9	7.40 ± 0.10^{a}	8.04 ± 0.38^{a}	8.15 ± 1.01^{a}	8.46 ± 0.01^{a}	11.59 ± 1.08^{b}
NI	2.23 ± 0.03	1.82 ± 0.40	1.96 ± 0.14	0.26 ± 0.04	3.04 ± 0.10
SFA	18.87 ± 0.25^{b}	18.71 ± 0.60^{b}	19.27 ± 1.09^{b}	21.45 ± 1.03^{c}	15.97 ± 0.61^{a}
MUFA	40.77 ± 0.53^{a}	42.03 ± 1.16^{a}	41.59 ± 0.72^{a}	41.02 ± 0.05^{a}	43.38 ± 0.11^{b}
PUFA	38.14 ± 0.50	37.44 ± 0.51	37.18 ± 0.37	37.28 ± 1.03	37.61 ± 0.62
Tot n-3	21.92 ± 1.29^{b}	20.32 ± 0.55^{b}	19.89 ± 2.36^{b}	17.11 ± 0.84^{a}	23.02 ± 0.52^{b}
Tot n-6	14.00 ± 0.57^{ab}	14.01 ± 0.24^{b}	14.39 ± 1.27^{b}	15.77 ± 0.60^{c}	12.52 ± 0.10^{a}
Other PUFA	2.22 ± 0.13^{a}	3.11 ± 0.28^{b}	2.91 ± 0.85^{ab}	4.40 ± 0.28^{c}	2.07 ± 0.13^{a}

Different superscript letters in the same row indicate significant differences $P < 0.05$.
SFA Saturated fatty acids; MUFA Monounsaturated fatty acids; PUFA Polyunsaturated fatty acids; NI Not identified

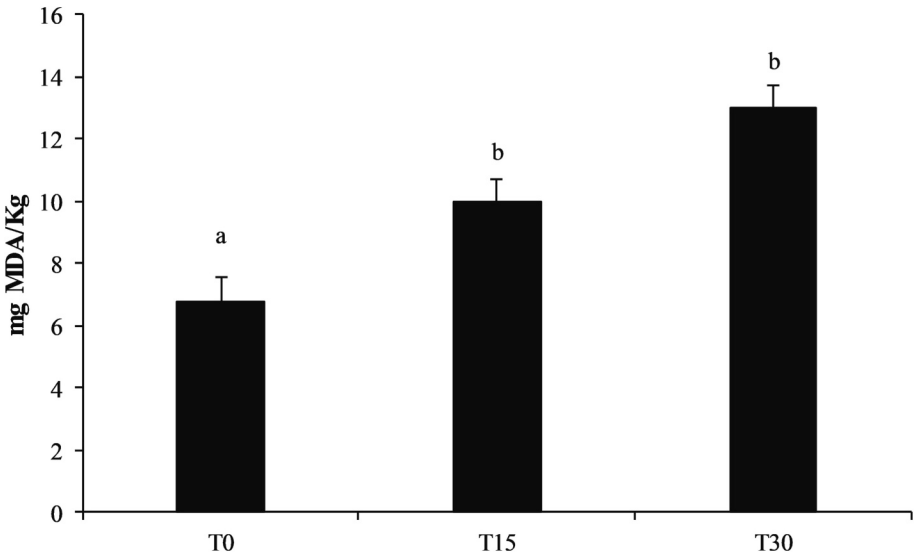

Fig. 2. MDA levels in liver of *S. aurata* fed with different diets containing 0.375 μg g^{-1} (D1) and 37.5 μg g^{-1} contaminants mix (D2) at 0, 15 and 30 days of treatment (T0, T15 and T30). Values are the mean ± SEM (n = 6). Statistical differences ($P < 0.05$) among groups are indicated by different letters.

thiol-containing antioxidants and enzymes. Elevated levels of reactive oxygen species lead to oxidative damage including lipid peroxidation, protein and DNA oxidation, and enzyme inactivation (Sevcikova et al., 2011).

Different studies reported the relationship between the exposition to chemical contaminants and the induction of oxidative stress, with related modification of lipid and fatty acid profile, in different marine organisms (Fokina et al., 2013); on the contrary few studies have considered at the same time the effects of the exposure to contaminants at molecular and fatty acids profile level (Bonsignore et al., 2022). It is known that in all organisms and in marine too, a situation of stress induces a metabolic adaptation necessary to counteract the energetic restriction imposed by the stress its-self and that this situation is attested by biochemical cascades that promotes physiological and molecular changes, in charge for the adaptation response (Bonsignore et al., 2018; Espinosa-Ruiz et al., 2021; Manuguerra et al., 2019; Messina et al., 2020). These adaptive responses for metabolic adaptations, if prolonged, could lead negative effects in terms of growth and reproductive performances in marine organisms at levels that overcome the organisms and could involve also the ecosystem level, with severe repercussions in the equilibrium of the oceans (Bonsignore et al., 2022; Messina et al., 2020).

Our study was carried out at realistic concentrations of chemical stressors (Popova et al., 2008), reported also by others authors: Cbz concentrations ranged from 0.03 to 11.6 μg L^{-1} (Bahlmann et al., 2009), for PBDEs concentrations ranged from ng g^{-1} to μg g^{-1} in the marine organisms (Parolini and Binelli, 2012; Vidal-Liñán et al., 2016) and finally, for $CdCl_2$ concentrations ranged from 200 μg g^{-1} in dry tissue in carp and of 1.3 μg g^{-1} of dry tissue in *S. aurata*. In addition, the concentrations of Cbz (Brandão et al., 2013), BDE-47 (Espinosa-Ruiz et al., 2019a, 2019c, 2019b) and $CdCl_2$ (Avallone et al., 2017) used in our experiment could be considered as sub-lethal doses as no mortality was recorded during the trial.

In the present study we evaluated the combined effects of a mixture of the contaminants $CdCl_2$, BDE-47 and Cbz using multi-levels biochemical and molecular approach based on the gene expression involved in oxidative stress, fatty acids profile and lipid peroxidation in liver of *S. aurata* used as marine model organism.

Fish utilize enzymatic and non-enzymatic defense against oxidative stress due to xenobiotics exposure (Cao et al., 2012). Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and lipid peroxidation were extensively used as biomarkers of oxidative stress (Shahjahan et al., 2022). Overall, biomarkers are considered as signals reflecting the adverse biological responses due the environmental contaminants and are commonly employed in environmental quality and/or risk assessment. Thus, the biomarkers monitoring in fish is a validated approach that can provide information about changes and damages resulting from stressors exposure (Van der Oost et al., 2003).

Our results showed that the exposure of *S. aurata* to mixture of contaminants modulated some key biomarkers related to oxidative stress. The expression of *sod* was significantly down-regulated after 15 days of exposure at both concentrations, showing a dose-dependent expression's reduction (Fig. 1), indicating impairment of the antioxidant mechanism in fish (Yang et al., 2020) subsequent to the exposition (Fig. 1). A similar trend was observed also for *cat* gene (Fig. 1) at the highest dose of exposition. CAT activity is often correlated to SOD activity (Cao et al., 2012). These enzymes constitute the first line of defense against oxidative stress. CAT activity is considered as a sensitive biomarker of oxidative stress in fish. However, the reduction of CAT activity leads to the accumulation of hydrogen peroxide causing toxicity in fish and increasing lipid peroxidation (Yang et al., 2020). In this study, the upregulation of both genes after 2 weeks of detoxification at T30 attested the ability of the antioxidants scavenging enzyme system to counteract a transitory sub-lethal condition of oxidative stress (Shahjahan et al., 2022), as reported by Messina et al. (2020) in *Mytilus galloprovincialis*, exposed to BDE-47. This ability is not always guaranteed as it depends on a combination of factors such as animal species and chemical characteristics and concentration: Nkoom et al. (2020) showed in *Carassius carassius* exposed to diclofenac, carbamazepine, and their mixture for 7 days exposure followed by 10 days recovery period, an inefficiency in the antioxidant defense system enzymes. Qiu et al. (2018) reported that chronic exposure to bisphenol F increased the levels of lipid peroxidation and inhibited CAT, and SOD activities due to excess ROS formation, causing oxidative damage in the liver of juvenile common carp (*Cyprinus carpio*). In our study, the expression of *gpx*, which has a direct role in body defense mechanism against the formation of hydrogen peroxide, showed significant decrease at T15 and after 2 weeks of detoxification with the highest dose (D2) (Fig. 1). The low expression of *gpx* during the 2 weeks of detoxification could be due to the increased CAT activity which eliminates excess peroxides generated by the action of SOD. This result suggests that the inactivation of *gpx*, due to the exposure to mixture of contaminants, represents a defense cellular mechanism against oxidative injury (Miyamoto et al., 2003).,

The induction of antioxidant defenses can be interpreted as organism

capacity to counteract an oxidative challenge, while the inhibition could reflect the overwhelming of such defenses (Regoli and Giuliani, 2014). Our results highlighted a specific and rapid response of liver to counteract oxidative stress due to long-term mixture exposure by scavenging ROS.

Oxidative stress induced by intake of chemical contaminants for 2 weeks (T15), induced a radical damage, responsible for modification of PUFAs, that are particularly sensible to this event (Bonsignore et al., 2022; Filimonova et al., 2016). In particular, a decrease in Tot n-3 in specimens fed D2 for 15 days (T15) was observed (Table 2). The variation in PUFAs and SFAs content during exposure to chemical contaminant could be attributed to PUFAs oxidation (Bonsignore et al., 2022). Similar observations were previously reported in the same species (Bonsignore et al., 2022; Sánchez-Muros et al., 2013) and in other species such as *Salmo trutta* (Bayir et al., 2013), *Pseudosciaena crocea, Pampus argenteus, Anguilla japonica, Oreochromis mossambicus, Ctenopharyngodon idellus* (Geng et al., 2015) and *Sebastiscus marmoratus* (Sun et al., 2013) exposed to different pollutants, that showed a reduction in eicosapentaenoic (EPA; 20:5n − 3) and docosahexaenoic (DHA; 22:6n − 3) acids. After 2 weeks of detoxification (T30), the fatty acids profile returned to levels comparable to control. Lipid peroxidation mechanism leads to free radicals production, as MDA, one of the main oxidation product of PUFA (Roméo et al., 2000). Despite the activation of antioxidant systems during the detoxification period, the oxidative stress induced a radical damage that continued to express its negative effects also during the detoxification phase, as confirmed by the high levels of MDA at T30 (Fig. 2). This result indicated that antioxidant defense system was not enough to counteract oxidative stress induction in *S. aurata* liver, suggesting ongoing radical damage (Nkoom et al., 2020). Similar results were reported by Solomando et al. (2020) in gut of *S. aurata* after chronic exposure to microplastics.

It is important to consider that, apart the environmental issues related to the presence of the contaminants in the marine environments, the modification of the biochemical composition of fish, *i.e.* the reduction of the n-3 PUFA contents, could represent also a negative aspect for the value of fish as prominent components of the food system. It is well known, in fact, that fish are the most important source of n-3 PUFA, having beneficial effects for human health, but it is not neglectable the role that these biochemical components have, as essential nutrients, also for fish (Swanson et al., 2012). The lacking of these compounds in marine organisms is deleterious for their health, welfare, immunological responses, stress resistance, so the effects of contaminants in marine organisms not only compromise their health but also their nutritional value as food commodities (Tocher, 2015).

5. Conclusions

Our results showed that realistic concentrations of chemical stressors used in this study, altered oxidative balance, affecting antioxidant defense system, modifying the fatty acid contents and increasing lipid peroxidation levels. The observed alterations at molecular and biochemical levels suggest, that chronic exposure to chemical contaminants, could lead to possible long-term or permanent effects on the single organism until population level.

Overall, the obtained results suggest that some biomarkers are good to attest oxidative stress and radical damage in progress, while other biomarkers are able to attest the damage also when the stress input is removed: in the first group we can include the scavenging enzymes and the fatty acids, that are able to recover more easily its original status and performances; in the second group of biomarkers we can include the free radicals as MDA, that continued to increase its levels, attesting that the marine organisms were previously exposed to a source of oxidative stress. The different sensibility and behavior of the biomarkers could be considered in environmental biomonitoring.

Author contributions

Author Contributions: Conceptualization C.M.M and A.S.; Data curation C.M.M; Formal analysis C.E., S.M., E.C., and R.A. M.A.E.; Funding acquisition C.M.M. and A.S.; Investigation C.M.M.; Methodology C.M.M; Project administration C.M.M. and A.S.; Resources C.M.M. and A.S.; Supervision C.M.M. and A.S., Validation C.M.M.; Visualization C.M.M.; Roles/Writing - original draft S.M.,R.A. and C.M.M.; Writing - review & editing C.M.M, M.A.E. and A.S.

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Declaration of Competing Interest

None.

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