Research paper

Toxicological effects and bioaccumulation of fullerene C_{60} (FC_{60}) in the marine bivalve *Ruditapes philippinarum*

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ABSTRACT

Fullerene C_{60} (FC_{60}), with its unique physical properties, has been used in many applications in recent decades. The increased likelihood of direct release into the environment has raised interest in understanding the biological effects of FC_{60} to aquatic organisms. Nowadays, only few studies have analysed FC_{60} effects and bioaccumulation in marine organisms following *in vivo* exposure. To provide new data about FC_{60} toxicity, *Ruditapes philippinarum* was selected as target species to assess potential adverse effects of the contaminant. Clams were exposed for 1, 3 and 7 days to predicted environmental concentrations of FC_{60} (1 and 10 μg/L) and cellular and biochemical responses were evaluated in clams' gills, digestive gland and haemolymph. The FC_{60} content in gills and digestive gland was determined in all experimental conditions after 7 days of exposure. Results showed an increase in oxidative stress. In particular, a significant modulation in antioxidant enzyme activities, and changes in glutathione S-transferase activity were observed in gills. Moreover, damage to lipids and proteins was detected in FC_{60}-treated (10 μg/L) clams. In digestive gland, slighter variations in antioxidant enzyme activities and damage to molecules were detected. CAT activity was significantly affected throughout the exposure, whereas damage to lipids was evident only at the end of exposure. FC_{60} accumulation was revealed in both gills and digestive gland, with values up to twelve-fold higher in the latter. Interestingly, haemolymph parameters were slightly affected by FC_{60} compared to the other tissues investigated. Indeed, only Single Cell Gel Electrophoresis and Neutral Red uptake assays showed increased values in FC_{60}-exposed clams. Moreover, volume and diameter of haemocytes, haemocyte proliferation, and micronucleus assay highlighted significant variations in treated clams, but only in the first phases of exposure, and no changes were detected after 7 days. Our results suggested clam gills as the target tissue for FC_{60} toxicity under the exposure conditions tested: the high damage detected to lipids and proteins could contribute to long-term problems for the organism.

1. Introduction

Among carbon nanoparticles (NPs), fullerene C_{60} (FC_{60}) is a hollow sphere molecule with 60 carbon atoms. Due to its three-dimensional shape, its unsaturated bonds and its electronic structure, fullerene has unique chemical and physical characteristics (Costa et al., 2012). Since its discovery (Kroto et al., 1985), FC_{60} has received considerable attention in various fields (Kim et al., 2010), and nowadays it has many important commercial and industrial applications, including drug delivery, electronics, superconductors, and cosmetics (Langa and Nierengarten, 2007; Pulicharla et al., 2016). It is one of the most ubiquitous

and produced carbon NPs (Sanchís et al., 2015), and it is present in polluted air as a result of fuel combustion (Nielsen et al., 2008). Moreover, in view of its large production and applications, FC_{60} is inevitably released into the aquatic environments (Gottschalk et al., 2009). Once released, FC_{60} tends to take the form of colloid via several mechanisms (Andrievsky et al., 1995) and be associated with biomolecules or natural organic matter (Hyung et al., 2007). FC_{60} possesses strong hydrophobic characteristics, and, consequently, in molecular form has the tendency to associate with hydrophobic substances in the environment (e.g., natural organic matter, lipids, and living organisms).

Despite this information, very few data on the levels and the

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behaviour of FC_{60} in the aquatic environment have been reported, mostly in marine coastal waters. Predicted environmental concentrations (PECs) of FC_{60} in wastewater treatment plants were estimated to be in the range of few ng/L to about 100 μg/L (Gottschalk et al., 2009). In addition, Ferreira da Silva et al. (2011) reported FC_{60} PECs in water in the range of 0.31–5 μg/L. In water, FC_{60} forms stable aggregates and concentration and nature of salt, as well as the pH value of the water, can strongly influence the strength of the aggregates (Fortner et al., 2005). A range of ionic strengths (I) from approximately seawater (0.7 I) to groundwater (0.01 I) was tested to study particle stability. The results obtained showed that FC_{60} aggregates did not remain in solutions simulating seawater or even brackish waters with ionic strengths at or above 0.1 I (Fortner et al., 2005). Another study demonstrated that natural organic matter, ionic strength and Ca^{2+} can affect physico-chemical properties of FC_{60} in the aqueous phase, influencing both fate and transport of FC_{60} in the natural aqueous environment (Li et al., 2009).

Coastal environments tend to be major depositional areas for many pollutants, as well as for NPs. NPs can enter marine systems via different sources, either directly or indirectly (Corsi et al., 2014). These environments are commonly inhabited by various species of organisms, among which bivalve molluscs are considered target species for NP exposure. Therefore, it became important to investigate NP toxicity (Canesi et al., 2012; Moore, 2006), alone or in combination with other chemical compounds, since the effects of co-presence of NPs on organisms are still not clear (Canesi et al., 2015). Understanding the behaviour and toxicity of a single NP in an organism may represent an essential premise for future studies aimed at assessing interaction with other contaminants or environmental factors.

As filter-feeding organisms, bivalves spend their lives filtering large volumes of water, processing microalgae, bacteria, sediments, particulates, and even pollutants. In addition, the NP characteristics, together with evidence from studies comparing their presence in different types of water, suggest that NPs will partition in the sea bottom at the sediment/water interface, thus representing a major threat to benthic organisms (Rocha et al., 2015). In this regard, the use of an infaunal filter-feeder could be particularly suitable to assess NP effects in coastal habitats. Recently, in the Manila clam *Ruditapes philippinarum*, the effects of NPs have been investigated in *in vitro* (Marisa et al., 2015) and *in vivo* exposures (Aouini et al., 2019; Britto et al., 2020; De Marchi et al., 2019; García-Negrete et al., 2013; Marisa et al., 2016, 2018).

Ecotoxicological studies on FC_{60} were carried out in various organisms, including bacteria (Letts et al., 2011), algae (Baun et al., 2008), crustaceans (Tao et al., 2011), fishes (Kuznetsova et al., 2014; Oberdörster et al., 2004), insects (Waissi-Leinonen et al., 2015), worms (Wang et al., 2014), and bivalve molluscs (Al-Subiai et al., 2012; Barranger et al., 2019; Ringwood et al., 2009; Sanchís et al., 2018). FC_{60} has been shown to cause DNA damage (Al-Subiai et al., 2012; Barranger et al., 2019), to have bactericidal action (Trpkovic et al., 2012), to promote oxidative stress (Zhu et al., 2008), to alter metabolism (Sanchís et al., 2018) and to inhibit growth, development and reproduction (Tao et al., 2009; Waissi-Leinonen et al., 2015). Moreover, FC_{60} can enter living organisms via several routes and accumulate in tissues (Oberdörster et al., 2005, 2006). The primary mechanism of FC_{60} toxicity has generally been attributed to its ability to generate reactive oxygen species (ROS), although other studies have reported contradictory results, indicating the need for further investigations to understand the potential oxidizing action of this NP (Al-Subiai et al., 2012; Costa et al., 2012; Shinohara et al., 2009).

The information on FC_{60} toxicity in marine species is still scarce, even though the increasing use and inevitable release of this NP into aquatic environments highlight the need to assess better its potential impact to coastal environments. To fill this gap, the present study was aimed at evaluating potential sub-lethal effects of FC_{60} on *R. philippinarum*, by analysing a battery of biomarkers (antioxidant enzyme activities, levels of damage to molecules, and haemocyte

parameters) in three tissues (gills, digestive gland and haemolymph) throughout a 7-days exposure to 1 and 10 μg/L of FC_{60} that were in the PECs range (Gottschalk et al., 2009). The biochemical and cellular parameters measured were selected based on both physico-chemical characteristics of FC_{60} and information on FC_{60} toxicity in other species. To confirm the FC_{60} ability to penetrate and accumulate in different tissues, the FC_{60} content in gills and digestive gland was measured. Physico-chemical characterisation of FC_{60} was also performed by various techniques to validate the manufacturer information on particle features.

2. Materials and methods

2.1. FC_{60} characterisation

Nano-sized powder of FC_{60} (purity > 97.5%) was purchased from Sigma-Aldrich (Milano, Italy). The images of FC_{60} were acquired using a transmission electron microscope (TEM, FEI Tecnai G12) operated at 100 kV, with a TVIPS F114 camera. X-ray diffraction (XRD) characterisation was performed using a Bruker D8 Advance diffractometer. The analyses were performed on pristine material before clam exposure in Bragg–Brentano configuration at 30 kV and 30 mA. The mean crystallite size was evaluated using the Scherrer equation.

2.2. Clams, FC_{60} exposure and tissue collection

Specimens of *R. philippinarum* (approximately 4 cm shell length) were collected from a reference site located within a licensed clam culture area in the southern part of the Lagoon of Venice (Italy). Specimens were then acclimatised in the laboratory for 5 days before exposure to the contaminant. Clams were maintained in large aquaria, which contained a sandy bottom and aerated natural seawater (salinity of 35 ± 1, temperature of 16 ± 0.5 °C), and were fed daily with microalgae (*Isochrysis galbana*). Stock solution of FC_{60} (0.1 g/L) was prepared in Milli-Q water and sonicated at 4 °C using a Braun Labsonic U sonifier at 50% duty cycles for 30 min. Clams were exposed for 1, 3 and 7 days to nominal concentrations of 0 (control), 1, and 10 μg FC_{60}/L. The experimental setup had been used and validated in previous works (Marisa et al., 2016, 2018). In detail, for each experimental condition tested, two replicate glass aquaria (35 clams each) were prepared. During exposure, clams were maintained in aerated seawater (1 L per animal) without sediment and in the same thermo-haline conditions used during the acclimatisation period. A movement pump (Hydor, Koralia nano 900, USA) was positioned in every aquarium (both for control and treated clams) to facilitate the water circulation and to prevent FC_{60} particle sedimentation (Marisa et al., 2016). The seawater was renewed daily, and FC_{60} and microalgae (at an initial concentration of approximately 150,000 cells/L) were supplied in the experimental tanks. Before adding the contaminant, the stock solution was sonicated, as reported above.

Throughout the exposure, clam haemolymph, gills and digestive gland were collected after first (T1), third (T3), and last (T7) day of exposure. For each tissue, six pools (5 animals per pool, 2 or 3 from each replicate tank) from each experimental condition were prepared. Aliquots of each pooled tissue were either snap frozen in liquid nitrogen and stored at −80 °C until analyses or immediately processed, depending on the biological responses measured. All assays performed in this study had previously been validated (Marisa et al., 2016, 2018; Matozzo et al., 2012a, 2012b, 2013; Parolini et al., 2010, 2013).

2.3. Gill and digestive gland preparation and biochemical assays

Aliquots of pooled gills and digestive glands were homogenised at 4 °C using an Ultra-Turrax homogeniser (model T8 basic, IKA) in four volumes of 50 mM Tris-HCl buffer, pH 7.4, containing 0.15 M KCl, 0.5 M sucrose, and Protease Inhibitor Cocktail (P2714, Sigma–Aldrich) and then centrifuged at 12,000 x g for 40 min at 4 °C. Supernatants (SN) were

collected for the analyses. SN protein concentrations were quantified according to Bradford (1976) using bovine serum albumin (BSA) as standard.

Total superoxide dismutase (SOD) activity was measured in the SN of both tissues using the xanthine oxidase/cytochrome c method proposed by Crapo et al. (1978). Enzyme activity is expressed as U SOD/mg protein, where one unit of SOD was defined as the amount of sample producing 50% inhibition in the assay conditions.

Gill and digestive gland catalase (CAT) activity was measured according to the method of Aebi (1984). The results are expressed in U CAT/mg protein, where one unit of CAT was defined as the amount of enzyme that catalysed the dismutation of 1 μmol of H_2O_2/min.

Glutathione S-transferase (GST) activity was measured 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 is expressed as nmol/min/mg protein.

Lipid peroxidation (LPO) was quantified in both tissues' SN using the malondialdehyde (MDA) assay, according to the method of Buege and Aust (1978). The results are expressed as nmoles of thiobarbituric reactive substances (TBARS)/mg protein. TBARS, considered as "MDA-like peroxide products", were quantified by reference to MDA absorbance (Damiens et al., 2007). The results were not expressed as MDA levels because TBA can react with a range of chemical compounds (Csallany et al., 1984).

Protein carbonyl content (PCC) was measured via the formation of labelled protein hydrazone derivatives, after 2,4-dinitrophenylhydrazide (DNPH) reaction, which were then quantified spectrophotometrically (Dalle Donne et al., 2003; Mecocci et al., 1999). The carbonyl content was calculated from the SN absorbance via the molar absorption coefficient of 22,000 mol/cm and expressed as nmol/mg protein.

DNA strand breaks were quantified using a fluorescence technique adapted from the alkaline precipitation assay (Olive, 1988). Samples of both gills and digestive gland were weighed (Mettler Toledo, XS105 Dual Range analytical balance, 0.01 mg readability) before tissue preparation (see above), and the wet weight was recorded. Salmon sperm genomic DNA standards were added for DNA calibration, and the results are expressed as μg/g wet weight.

2.4. FC_{60} bioaccumulation in gills and digestive gland

At the end of the exposure (T7), 4 pools of gills and digestive glands per experimental condition (6 animals each) were collected to quantify FC_{60} bioaccumulation. Tissue samples were carefully washed with pure toluene to remove surface adsorbed FC_{60} particles. Then, tissues were extracted into toluene (1:6, w:v ratio) using ultra-sonication for 30 min (35 kHz). Extracts were centrifuged (9000 x g) and the SN was used in HPLC (High Performance Liquid Chromatography) analysis (Al-Subiai et al., 2012). FC_{60} was separated using Eclipse XDB-C18 4.6 mm ID x 250 mm 5 μm 80 Å column. The mobile phase for columns was toluene (Sigma–Aldrich HPLC grade), at a flow-rate of 1.0 mL/min. Sample injections were performed manually with volumes of 100 μL. The eluent was monitored at 335 nm using a HPLC Agilent 1100, Chemstation rev. A10.02. For external calibration, a standard curve was generated for FC_{60} concentrations ranging from 0.001 to 0.8 mg/mL. The FC_{60} concentration of each sample was calculated by comparison to the standard curve, and the results were expressed as μg FC_{60}/g wet weight.

2.5. Haemolymph parameters

Total haemocyte count (THC) and haemocyte diameter and volume were determined using a Model Z2 Coulter Counter electronic particle counter/size analyser (Coulter Corporation, FL, USA). THC is expressed as the number of haemocytes (x10^6)/mL of haemolymph. Haemocyte diameter and volume are expressed in μm and in femtolitres (fL), respectively.

Haemocyte proliferation was evaluated using a commercial kit (Cell proliferation Kit II, Roche). This assay has been validated in previous studies (Matozzo et al., 2012a, 2012b) according to the evidence of cell division in circulating haemocytes of Manila clams (Matozzo et al., 2008). The data were normalised to the THC values recorded for the clams from each experimental condition and expressed as the optical density (OD) at 450 nm.

Cytotoxicity was evaluated using a colorimetric assay based on the measurement of lactate dehydrogenase (LDH) activity in cell-free haemolymph. A commercial kit (Cytotoxicity Detection Kit, Roche) was used to assess cell damage. The results, normalised to THC values, are expressed as the optical density (OD) at 490 nm.

The Neutral Red uptake (NRU) assay provides a quantitative estimation of viable cells, and it was performed according to the modified method of Cajaraville et al. (1996). This test is based on the capability of cells to incorporate and bind the vital dye neutral red; thus, it was used to evaluate the capability of haemocytes to perform pinocytosis. The results, normalised to THC values, are expressed as the optical density (OD) at 550 nm.

Lysozyme activity was quantified in both cell-free haemolymph (CFH) and haemocyte lysate (HL) (Matozzo et al., 2012b). The results were expressed as μg lysozyme/mg protein. Protein concentrations in CFH and HL were quantified according to Bradford (1976).

The SCGE assay (Single Cell Gel Electrophoresis) was performed using the alkaline (pH > 13) version of the assay developed by Singh et al. (1988) with some modifications (for more details, see Marisa et al., 2016). After haemocyte electrophoretic treatment, imaging was performed using a fluorescence microscope (Leica 5000B, Germany) equipped with an FITC filter (I3, excitation BP 450–490, emission LP 515) at 10× magnification. One hundred cells per slide for a total of 600 cells per condition were analysed using an image analysis system (Comet Score®). The ratio between the migration length and the diameter of the comet head (LDR), as well as the percentage of tail DNA were chosen as indicators of DNA damage.

The micronucleus (MN) test was performed according to the method of Pavlica et al. (2000). After adhesion onto slides, haemocytes were kept in the dark at 4 °C prior to examination under the microscope. Four hundred cells were counted for each slide for a total of 2400 cells/treatment: cells were examined under the fluorescent microscope Leica 5000B equipped with a submerged lens at 100× magnification. Only intact and non-overlapping haemocytes were considered. Micronuclei were identified according to the criteria proposed by Kirsch-Volders et al. (2000), and the MN frequency (MN‰) was calculated.

2.6. Statistical analysis

The normal distribution (Shapiro–Wilk test) and the homogeneity of the variance (Bartlett test) of the data were assessed. The data were statistically compared using a two-way ANOVA test, with treatment and time of exposure as variables, and biomarkers as cases. The ANOVA was followed by a Fischer LSD post-hoc test to evaluate significant differences between treated samples and related controls at each tissue sampling time. The STATISTICA 10 software package (StatSoft, Tulsa, OK) was used for statistical analyses. Lastly, a canonical correlation analysis (CCA) was performed. The set of variables included treatment, exposure duration, and the measured cellular and biochemical parameters. The software package R (R Core Team, 2019) with the CCA package (González and Déjean, 2012) and r41sqr10 package (Finos, 2020) were used.

3. Results

3.1. Nanoparticle characterisation

In Fig. 1A and B, a TEM image of FC_{60} and the XRD pattern of FC_{60} powder are shown. The FC_{60} mean crystallite diameter was 49 nm and the crystalline phase was cubic.
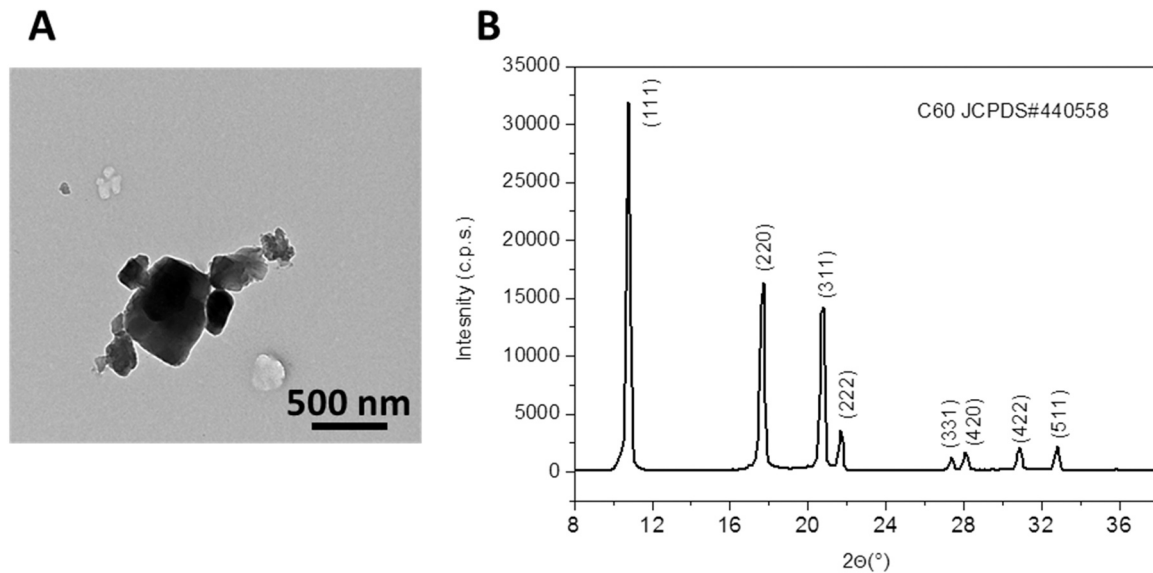
Fig. 1. A TEM image of FC_{60}. B: XRD pattern of the FC_{60} powder. The crystalline phase is C_{60} (JCPDS#440558).

3.2. Clam conditions throughout the experiment

No mortality and no visible morphological changes have been detected throughout the 7 days exposure of *R. philippinarum* to the experimental conditions.

3.3. Gill and digestive gland parameters

Gill SOD activity was significantly affected by treatment ($p < 0.001$) and time of exposure ($p < 0.001$) (Table 1). At T1 and T3, FC_{60} (1 and 10 µg/L) treated clams exhibited significantly higher values of SOD activity with respect to control, whereas at T7 a significant increase was detected only at 10 µg/L (Fig. 2A). A significant time-dependent ($p < 0.001$) and treatment*time interaction-dependent ($p = 0.009$) variation in the activity of SOD was found in the digestive gland (Table 1); a significant increase was found at T3 in clams exposed to both concentrations of FC_{60}, compared to control (Fig. 2B).

In both tissues, CAT activity was affected significantly by the exposure to FC_{60} ($p = 0.002$ in gills, $p < 0.001$ in digestive gland) and exposure time ($p < 0.001$ in gills, $p < 0.001$ in digestive gland)

(Table 1). In gills, CAT activity decreased significantly in clams exposed for 3 and 7 days to both the concentrations of FC_{60}, compared to controls (Fig. 2C). In digestive gland, a significant increase in CAT activity was found in clams exposed for 1, 3 and 7 days to 1 µg/L FC_{60}, compared to controls (Fig. 2D).

Gill GST activity exhibited a significant modulation determined by treatment ($p < 0.001$), time of exposure ($p < 0.001$) and treatment*time interaction ($p < 0.001$) (Table 1). In particular, GST activity significantly increased at T1 and T3 in clams exposed to the lower FC_{60} concentration, with respect to controls. At T3, the activity was statistically ($p < 0.001$) higher in clams exposed to 1 µg/L respect to those at 10 µg/L of FC_{60}. At T7, no significant variations were detected among the experimental conditions (Fig. 2E). In digestive gland, the GST activity was affected by time of exposure ($p = 0.002$) and treatment*time interaction ($p < 0.001$) (Table 1). Pair-wise comparisons highlighted significant difference between controls and clams exposed to the higher FC_{60} concentration only at T3. In addition, enzyme activity was statistically higher ($p < 0.001$) in clams exposed to 10 µg/L respect to 1 µg/L of FC_{60} (Fig. 2F).

Gill LPO was significantly influenced by treatment ($p < 0.001$), time

Table 1

Two-way ANOVA results for the biochemical responses measured in gills and digestive gland of *R. philippinarum* throughout the exposure to FC_{60} (1 and 10 µg/L). Statistically significant effects of the variables ''Treatment'', ''Time'' and ''Treatment*Time'' interaction are indicated in bold.

	Source of variation	Gills			Digestive gland		
		F	*df*	*p*-value	F	*df*	*p*-value
SOD activity	Treatment	13.37	2	**0.000**	1.91	2	0.159
	Time	8.79	2	**0.001**	24.45	2	**0.000**
	Treatment*Time	1.00	4	0.417	3.78	4	**0.010**
CAT activity	Treatment	6.73	2	**0.003**	10.81	2	**0.000**
	Time	29.82	2	**0.000**	100.94	2	**0.000**
	Treatment*Time	2.46	4	0.059	0.21	4	0.931
GST activity	Treatment	9.64	2	**0.000**	2.14	2	0.130
	Time	11.53	2	**0.000**	6.73	2	**0.003**
	Treatment*Time	5.65	4	**0.001**	5.71	4	**0.001**
LPO	Treatment	10.20	2	**0.000**	1.71	2	0.193
	Time	6.89	2	**0.002**	44.37	2	**0.000**
	Treatment*Time	2.64	4	**0.046**	2.03	4	0.106
PCC	Treatment	1.65	2	0.203	0.19	2	0.824
	Time	43.37	2	**0.000**	1.31	2	0.281
	Treatment*Time	4.03	4	**0.007**	0.06	4	0.994
DNA damage	Treatment	1.35	2	0.270	2.65	2	0.081
	Time	6.19	2	**0.004**	3.28	2	0.047
	Treatment*Time	1.24	4	0.307	2.16	4	0.089
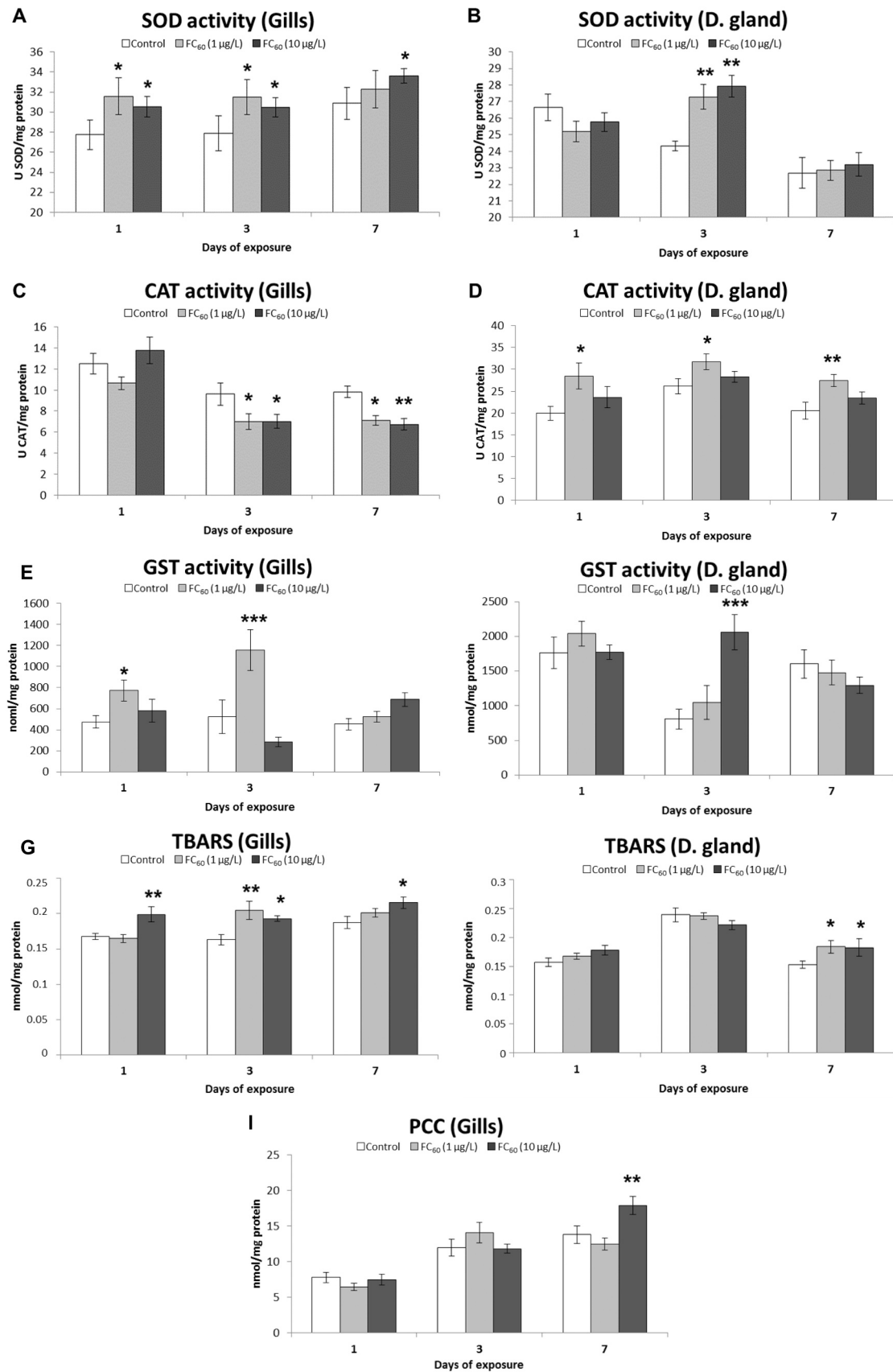
Fig. 2. Biochemical parameters measured in gills and digestive gland of *R. philippinarum* after 1, 3 and 7 days of exposure to FC_{60} (1, 10 µg/L): SOD activity (A, B) expressed as U SOD/mg protein; CAT activity (C, D) expressed as U CAT/mg protein; GST activity (E, F) expressed as nmol/min/mg protein; TBARS (G, H) and PCC (I) levels expressed as nmol/mg protein. Values are reported as mean ±SD; n = 6. Asterisks denote significant differences compared to controls at the same time of exposure: *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

of exposure ($p = 0.002$), and treatment*time interaction ($p = 0.045$) (Table 1). The TBARS levels increased significantly at the higher FC_{60} concentration tested at T1, T3 and T7. In clams exposed to the lower concentration of FC_{60}, an increase of lipid peroxidation respect to control was also shown at T3 (Fig. 2G). In digestive gland, TBARS levels were affected by time of exposure only ($p < 0.001$) (Table 1). A significant time-dependent increase in lipid damage was detected in both FC_{60}-treated clams compared to control only at the end of exposure (Fig. 2H).

PCC values were significantly affected only in gills, in particular by time of exposure ($p < 0.001$) and treatment* /time interaction ($p = 0.007$) (Table 1). The only significant difference in the pair-wise comparisons was found at T7 between clams treated with 10 µg/L FC_{60} and the related control (Fig. 2I).

DNA damage was not significantly affected by concentration, time of exposure and their interaction in both gills and digestive gland (Table 1; data not shown).

3.4. FC_{60} bioaccumulation in gills and digestive gland

Results demonstrated accumulation of FC_{60} in both gills and digestive gland. In gills, FC_{60} content showed a dose-dependent trend, with values of 1.007 µg/g_{ww} (± 0.248 sd.) and 1.638 µg/g_{ww} (± 0.157 sd.) measured in clams exposed at 1 and 10 µg/L of FC_{60}, respectively. In digestive gland, bioaccumulation was higher than in gills, but an opposite pattern was observed, with 12.377 µg /g_{ww} (± 1.153 sd.) and 6.652 (± 0.845 sd.) µg /g_{ww} detected in 1 µg/L- and 10 µg/L-treated clams, respectively.

3.5. Haemolymph parameters

No evidence of THC modulation was detected during the exposure; instead, the diameter and volume of haemocytes were significantly influenced. The diameter was affected by FC_{60} treatment ($p < 0.001$), time of exposure ($p = 0.038$) and treatment*time interaction ($p < 0.001$). The volume was affected by treatment ($p < 0.001$) and treatment*time interaction ($p < 0.001$) (Table 2). As for both diameter and volume, significant increases with respect to controls were observed at T1 in clams exposed to the two FC_{60} doses, and at T3 in FC_{60} 10 µg/L-treated clams. No significant differences were detected at T7 among the experimental conditions (Fig. 3A, B).

Haemocyte proliferation was statistically influenced by treatment ($p < 0.001$), time of exposure ($p = 0.011$) and treatment*time interaction ($p < 0.001$) (Table 2), with a pattern of variation similar to that of haemocyte diameter and volume. Indeed, haemocyte proliferation significantly increased in clams exposed to 1 and 10 µg/L at T1, and to 10 µg/L FC_{60} at T3 (Fig. 3C).

Significant variations in NR uptake due to FC_{60} exposure were observed in clam haemocytes ($p = 0.032$) (Table 2), with a significant increase in clams exposed for 7 days to the higher FC_{60} concentration, compared to controls (Fig. 3D).

LDH and lysozyme activity were not significantly affected by exposure to FC_{60}, time of exposure and the interaction between the two variables (Table 2; data not shown).

For both SCGE assay endpoints (LDR value and the percentage of DNA in the comet tail), a significant effect of FC_{60} ($p < 0.001$), time of exposure ($p < 0.001$) and treatment*time interaction ($p = 0.004$ and $p < 0.001$, respectively) was recorded in clam haemocytes (Table 2). In particular, LDR values significantly ($p < 0.01$) increased after 3 and 7 days of exposure to both FC_{60} concentrations (Fig. 3E). A statistically significant increase in the percentage of tail DNA was observed just after the first day of exposure at the highest FC_{60} concentration, and after 3 and 7 days of exposure to all FC_{60} concentrations tested (Fig. 3F).

The frequency of micronucleus was affected only by treatment*time interaction ($p = 0.013$) factor (Table 2). Difference with respect to control was significant at T3 only in the clams exposed to the lowest FC_{60}

Table 2

Two-way ANOVA results for the biochemical and cellular responses measured in the haemolymph of *R. philippinarum* throughout the exposure to FC60 (1 and 10 µg/L). Statistically significant effects of the variables "Treatment", "Time" and "Treatment*Time" interaction are indicated in bold.

|	Source of variation	Haemolymph		
		F	df	p-value
THC	Treatment	1.65	2	0.204
	Time	0.98	2	0.384
	Treatment*Time	0.92	4	0.461
Haemocyte diameter	Treatment	9.65	2	**0.000**
	Time	3.51	2	**0.038**
	Treatment*Time	6.39	4	**0.000**
Haemocyte volume	Treatment	13.42	2	**0.000**
	Time	2.26	2	0.116
	Treatment*Time	8.67	4	**0.000**
Cytotoxicity (LDH)	Treatment	0.48	2	0.621
	Time	0.22	2	0.801
	Treatment*Time	1.10	4	0.367
NR Uptake	Treatment	3.72	2	**0.032**
	Time	1.79	2	0.179
	Treatment*Time	1.45	4	0.234
Lysozyme activity (haemocyte lysate)	Treatment	0.23	2	0.793
	Time	0.78	2	0.466
	Treatment*Time	0.73	4	0.578
Lysozyme activity (cell-free haemolymph)	Treatment	0.98	2	0.382
	Time	1.25	2	0.298
	Treatment*Time	0.49	4	0.740
Haemocyte proliferation	Treatment	17.07	2	**0.000**
	Time	4.97	2	**0.011**
	Treatment*Time	5.78	4	**0.001**
MN frequency	Treatment	1.79	2	0.178
	Time	2.99	2	0.060
	Treatment*Time	3.56	4	**0.013**
LDR value (SCGE)	Treatment	23.86	2	**0.000**
	Time	16.90	2	**0.000**
	Treatment*Time	4.43	4	**0.004**
% DNA in tail (SCGE)	Treatment	34.29	2	**0.000**
	Time	27.82	2	**0.000**
	Treatment*Time	6.59	4	**0.000**

concentration (Fig. 3G).

3.6. CCA analysis

A plot of the first two components of the CCA analysis is shown in Fig. 4. The first principal correlations are about 99% and 97% for the first and the second canonical correlation, respectively. The samples highlighted a strong effect of the experimental design on the measured parameters. Furthermore, a clear time-dependent pattern of variation can be observed: day 1–7 from left to right. The treatment level increases from bottom to top. Dose 1 µg/L and 10 µg/L show an overall similar pattern, with the exception of day 3, where 1 µg/L appears more similar to control than dose 10 µg/L. Samples at day 7 are characterized by increased values of Gill_SOD, Gill_LPO, LDR and % DNA.

4. Discussion

The widespread use of FC_{60} in a variety of consumer products inevitably causes their release into aquatic environments (Sanchís et al., 2015). Filter-feeders, such as bivalves, can come into contact with FC_{60} NPs directly through the waterflow of the gills or indirectly through the diet (Sanchís et al., 2018). Manila clams, as well as Mediterranean mussels, have been extensively used in laboratory studies to understand the effects of various pollutants (Jiang et al., 2019; Matozzo et al., 2004, 2012a; Santovito et al., 2015; Trombini et al., 2019; Stara et al., 2020; Freitas et al., 2020a, 2020b), including NPs (Rocha et al., 2015). The potential detrimental effects of FC_{60} on marine species are largely unknown and therefore information is quite inconclusive in literature (Al-
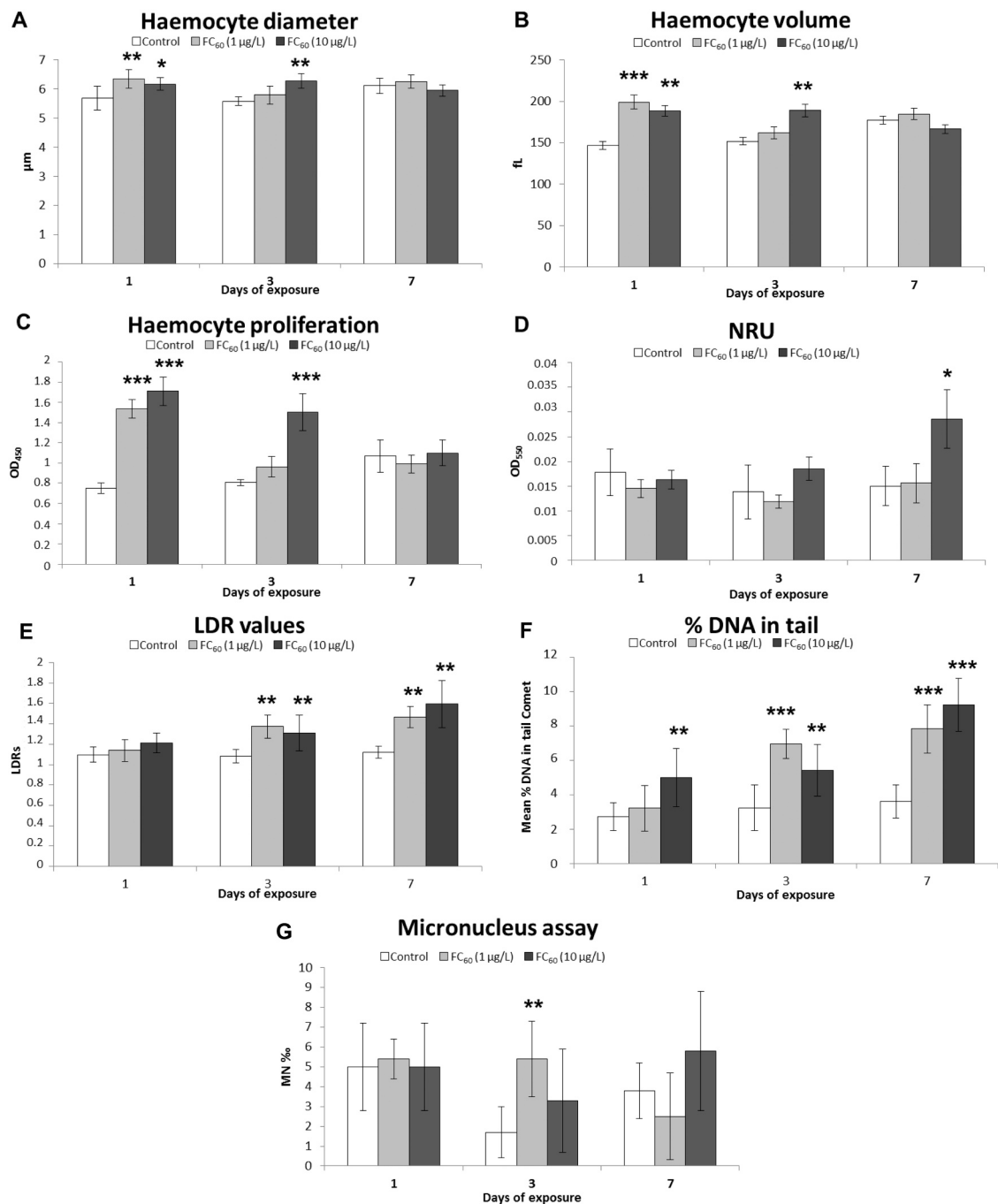
Fig. 3. Haemocyte parameters measured in *R. philippinarum* after 1, 3 and 7 days of exposure to FC_{60} (1, 10 μg/L): diameter of haemocytes (A) expressed in μm; volume of haemocytes (B) expressed in femtolitres (fL); haemocyte proliferation (C) expressed as OD_{450}; NRU (D) expressed as OD_{550}; SCGE results expressed as length/diameter ratio, LDR (E) and the mean percentage of tail DNA (F); micronucleus results (G) expressed as frequency (MN‰). Values are reported as mean ±SD; n = 6. Asterisks denote significant differences compared to controls at the same time of exposure: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Subiai et al., 2012; Canesi et al., 2010, 2015; Marques et al., 2013; Ringwood et al., 2009). Moreover, data about the real concentrations in coastal areas are missing, and only PEC values are available (Ferreira da Silva et al., 2011; Gottschalk et al., 2009).

In this study, we chose concentrations (1 and 10 μg/L) of FC_{60} that were in the PECs range. Differently, most of the published studies on NP effects were performed with high concentrations. However, high concentrations tested in previous studies, as well as acute toxicity assays and *in vitro* approaches do not provide complete information about the potential interaction of NPs with aquatic organisms. It has been noted that the behaviour of NPs can vary depending on their concentrations in

aqueous media (Al-Subiai et al., 2012; Barmo et al., 2013; Ringwood et al., 2009). In addition, bioavailability, uptake, accumulation and toxicity of NPs in aquatic organisms depend on several physico-chemical properties, such as particle size/shape, surface charge and structure, particle chemistry, solubility, and aggregation state (Baker et al., 2014; Pakarinen et al., 2014; Yang et al., 2013).

It is generally accepted that the toxic potential of NPs is not only dependent upon their features, but also on target cell types and exposure conditions. In the present study, prior to evaluate the potential detrimental biological effects of FC_{60}, its physico-chemical properties were thoroughly investigated, as recommended by Handy et al. (2008).
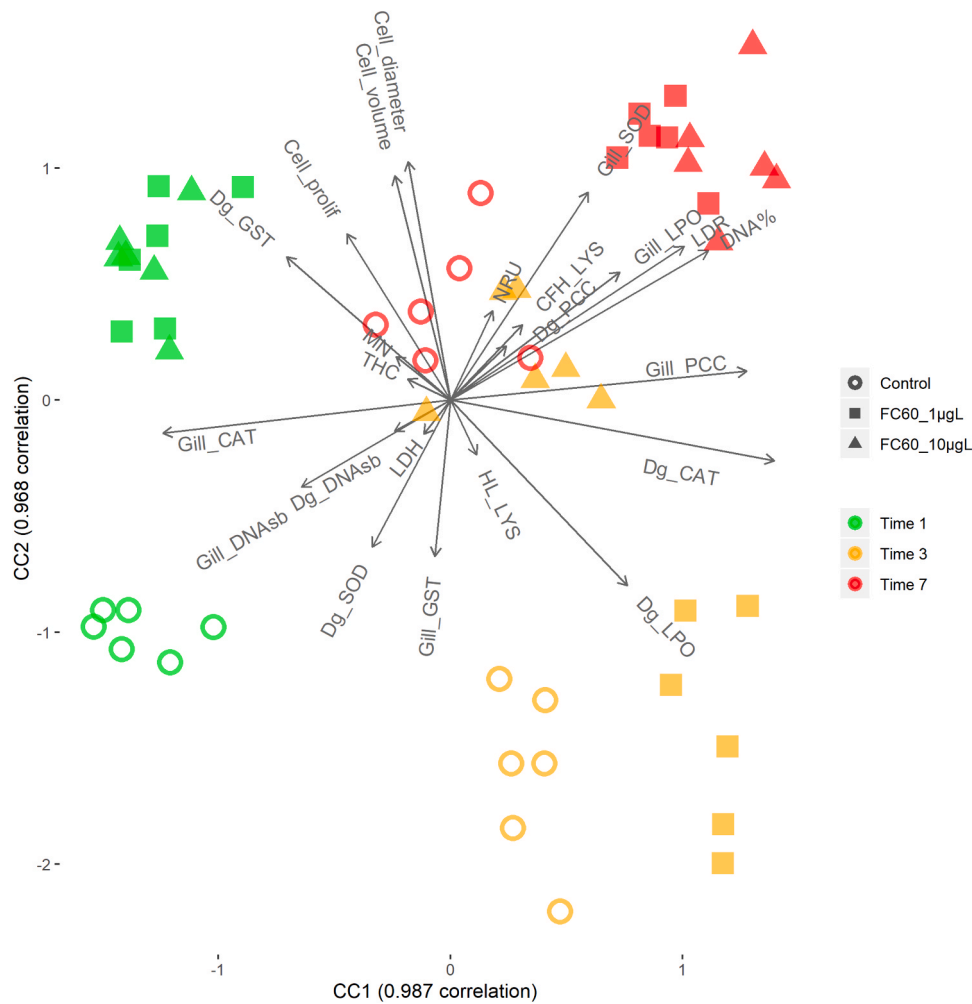
Fig. 4. CCA analysis results. Both the biomarkers measured in clams and the experimental conditions (treatment and exposure duration) are considered. Time 1, Time 3 and Time 7 correspond to tissue sampling times, i.e., after 1, 3 and 7 days of exposure, in control, 0.1 μg/L and 1 μg/L fullerene C_{60} treated clams.

Analyses performed confirmed the information available in the literature, highlighting the high hydrophobic properties of FC_{60}. Despite lacking information about FC_{60} toxicity (mainly *in vivo*), some useful indications about FC_{60} impact in bivalves can be provided by data on general action and fate of NPs in these organisms. Indeed, it is known that NPs are filtered by gills, accumulated in the digestive gland, and transferred to the haemolymph through the epithelium of the digestive gland tubules (Moore et al., 2009; Rocha et al., 2015). For this reason, in our study we investigated the effects of FC_{60} in gills, digestive gland and haemolymph of *R. philippinarum*. A battery of specific biomarkers - widely measured in previous ecotoxicological studies - was selected to assess FC_{60} toxicity in clams. The use of a multi-biomarker approach can provide an indication of the sub-lethal impacts of stressors, as well as of the biochemical mechanisms that may be affected by NPs, thus providing an "early warning" at the organism level (Handy and Depledge, 1999).

Fullerene toxicity is a controversial issue. Kahru and Dubourguier (2010) compiled fullerene toxicological data for fourteen organisms and classified this nanomaterial as very toxic, taking into account the lowest median lethal concentration values for all test organisms. However, some studies indicated the absence of fullerene toxicity (Xia et al., 2010). Nonetheless, as previously mentioned, there is evidence that carbon NPs in aquatic environments are biologically active, promoting oxidative stress (Nel et al., 2006; Oberdörster et al., 2004). The toxicity mechanism of FC_{60} has generally been attributed to its potential ability to generate ROS (Al-Subiai et al., 2012; Marques et al., 2013), even if

other studies considered the ROS generation by aqueous suspension of fullerene as a minimal effect of the contaminant (Henry et al., 2011). In an *in vivo* study of Usenko et al. (2008), FC_{60}- induced oxidative stress was evaluated in the embryonic stage of zebrafish. The authors suggested that this NP acts as a pro-oxidant and enhance toxic response by interacting with biomolecules such as DNA, proteins and lipids.

Considering all the above information, the potential induction of oxidative stress determined by FC_{60} action in clam tissues was investigated in this study, by analysing variations in i) antioxidant enzyme activity ii) detoxification enzyme activity and iii) lipid, protein and DNA damage. FC_{60} determined various effects in clam gills. Clams exposed to FC_{60} showed a modulation of gill antioxidant enzymes, with an increase in SOD activity, suggesting an increased defence against oxidative stress in such tissue. Likewise, increased SOD activity was found in gills from *R. philippinarum* exposed for 3 and 7 days to zinc oxide nanoparticles (Marisa et al., 2016). Instead, in this study CAT activity showed a decrease in treated clams compared to controls. In this scenario, considering the mechanisms of action of both SOD and CAT, increased levels of H_2O_2 can be hypothesised in gills, promoting oxidative stress (Lesser, 2006; Livingstone, 2001). The antioxidants CAT and SOD are important components of intracellular defences, and they could work together to convert O^{2-} and H_2O_2 into harmless H_2O and O_2 (Hu et al., 2010; Jamil, 2001). Overall, results obtained in this study suggested a potential H_2O_2-mediated oxidative stress following exposure of clams to FC_{60}.

The enzyme GST participates in the detoxification process due to

conjugation reaction between its substrate glutathione (GSH) and xenobiotics, assisting in their elimination (Cummins et al., 2011). Interestingly, a significant increase in GST activity was observed at 1 µg FC_{60}/L after 1 and 3 days of exposure, suggesting a contribution of the enzyme in the antioxidant defence. These findings evidenced the high potential FC_{60} toxic action and oxidative stress as the main mechanism, in spite of the short time of exposure in our experiment. In a long-term exposure, chronic problems to gill functions and to individual health could be hypothesized. Indeed, in mussels (*Mytilus* sp.) exposed to FC_{60} (0.1–1 mg/L) for 3 days hypoplasia in gills and necrosis in digestive gland were detected, compared to controls (Al-Subiai et al., 2012). High oxidative stress and LPO were revealed also in gills of fish *Cyprinus carpio* after an *in vitro* exposure to FC_{60} (1 mg/L) (Britto et al., 2012).

In our experiment, the FC_{60} content in gills of treated clams showed a dose-dependent increase, with a higher value in clams exposed to 10 µg FC_{60}/L respect to the lower concentration (1 µg/L), and bioaccumulation matched the biomarker results. In another study (Wang et al., 2014), increased CAT activity was reported in the worm *Lumbriculus variegatus* exposed for 14 days to FC_{60} (5 mg/kg d.w. sediment), but a low bioaccumulation of FC_{60} was detected in worm specimens. Nonetheless, a linear relationship between FC_{60} body residue and CAT activity was revealed. Based on their results, Wang et al. (2014) suggested the need for a more in-depth study of FC_{60} agglomerate accumulation and corresponding oxidative stress in living organisms.

In clam digestive gland, slight variations in antioxidant enzyme activities and damage to molecules were detected when compared to those observed in gills. During the exposure, an increase in CAT activity and LPO levels (only at T7) were shown in treated clams, compared to controls. Similarly to what was observed by Al-Subiai et al. (2012) in *Mytilus* sp., accumulation of FC_{60} in clams was more pronounced in digestive gland than in gills. Unlike gills, FC_{60} content did not show a dose-dependent pattern in digestive gland, and values in clams exposed to 1 µg FC_{60}/L were approximately twice higher than those in clams at 10 µg FC_{60}/L. We postulated that at lower FC_{60} concentration the formation of smaller NP agglomerates could enhance their bioaccumulation (Wang et al., 2014). Instead, at higher FC_{60} concentrations, larger NP aggregates could be retained in the gills, thus allowing the observed pattern of accumulation, but they did not reach digestive gland. Disaggregation of large size NP aggregates might occur (Rocha et al., 2015), even though it probably requires time span longer than 7 days, as in our study. On the other hand, it is generally recognized that small size NP aggregates are more likely to undergo a process of disaggregation and infiltrate biological systems compared to the larger aggregates that cannot infiltrate (Moore, 2006) and diffuse through cell membranes (Lin et al., 2010; Rocha et al., 2015). In this case, lower concentrations in short exposure could be considered more toxic than higher concentrations. In the oyster *Crassostrea virginica* exposed to FC_{60} (1, 10, 100, 500 µg/L) for 4 days, there was no significant increase in LPO in the hepatopancreas. Moreover, confocal microscopy analysis indicated the presence of FC_{60} in oyster hepatopancreas cells within 4 h. In particular, FC_{60} aggregates tended to be localized and concentrated into lysosomes (Ringwood et al., 2009). Marques et al. (2013) analysed the polychaete *Laeonereis acuta* and the bacteria that colonized the worm mucus after FC_{60} (0.01, 0.10 and 1 mg/L) exposure for 24 h. FC_{60} reduced total antioxidant capacity of bacteria from worms exposed to 0.1 mg/L. Lower antioxidant capacity and LPO reduction were observed in worms after exposure to 1 mg/L (Marques et al., 2013).

In our study, no DNA damage was detected in gills and digestive gland of treated clams. This response could be due to low concentrations of the contaminant and short *in vivo* exposure. The haemocytes of the clam *R. philippinarum* play a key role in internal defence and have various functions (Cima et al., 2000; Donaghy et al., 2009). Several studies have demonstrated the adverse effects of contaminants, including NPs (Renault, 2015), on haemocyte functionality in bivalves. In this experiment, transient increases in haemocyte diameter, volume and proliferation were observed in FC_{60}–exposed clams. After 7 days of

exposure, only a slight variation in NRU was shown in haemocytes, while a low haemocyte DNA damage, which had persisted throughout the exposure, was revealed. In particular, a significant increase in NRU values was observed at the higher FC_{60} concentration at the end of the exposure, but this finding was not confirmed by an increased cytotoxicity assessed by LDH assay. Uptake of NR cationic dye by haemocytes occurs by pinocytosis or passive diffusion across cell membranes (Coles et al., 1995). Therefore, alterations in dye uptake may reflect damage to cell membranes (including lysosomal membranes) and/or weakening of haemocyte pinocytotic capability. Our results demonstrated that FC_{60} significantly increased haemocyte NR uptake, suggesting that exposure of clams to FC_{60} induced changes in the membrane permeability of haemocytes and increased the number and/or the volume of lysosomes, which probably contained higher levels of NR than those of haemocytes from control clams. Although the percentage of tail DNA significantly increased in haemocytes of FC_{60} exposed clams with respect to controls, the level of DNA damage revealed by SCGE assay was very low (Mitchelmore et al., 1998; Riva et al., 2007). This finding, possibly related to both short time of exposure and low exposure concentrations, suggested a potential action of the contaminant directly through interaction with DNA inside the nucleus or indirectly through various mechanisms, for example the generation of ROS (Handy et al., 2008; Moore, 2006). Interestingly, in the studies of Al-Subiai et al. (2012) and Barranger et al. (2019), where the concentrations tested were higher than in the present study, FC_{60} determined high genotoxicity in mussel tissues (haemocytes and digestive gland, respectively).

Overall, various biomarkers in gills (GST activity), digestive gland (SOD and GST activity) and haemolymph (volume and diameter of haemocytes, haemocyte proliferation and MN frequency) resulted to be affected only in the first phases of exposure. In particular, increased GST activity in gills and digestive gland indicated a potential reaction of tissues to FC_{60}. In digestive gland of FC_{60} treated clams, increased activity of both SOD (at T3) and CAT (at all tissue sampling times) suggested a good capability of the tissue to respond to oxidative stress. In haemolymph, the modulation of some haemocyte parameters at T1 and T3 suggested a transient reaction to FC_{60} and overall increased stress conditions possibly responsible for other more persistent effects, such as the increasing DNA damage observed throughout the exposure. It can be hypothesized that clams take advantage from differing pathways, according to differing tissues or organs, to cope with NP impact. This may lead to dissimilar patterns of bioaccumulation and effects depending on the tissue or organ considered.

5. Conclusion

Our results indicated a different modulation of the biological parameters measured in the tissues from R. *philippinarum* following 7-days exposure to FC_{60}. Although digestive gland is generally considered to be the target tissue of NP toxicity in bivalves (Rocha et al., 2015), we demonstrated that gills can be more affected by FC_{60}, at least under the experimental conditions tested in this study. Overall, oxidative stress can be recognized as one of the primary effects of FC_{60} in clam tissues, under an *in vivo* short-term exposure at low concentrations similar to PECs. Instead, FC_{60} scarcely affected haemocytes in treated clams. In this study, the bioaccumulation of FC_{60} in gills and digestive gland was also observed, in agreement with results of previous studies. To our knowledge, this is the first study that investigated the effects of FC_{60} in Manila clams, thus providing a new topic for discussion about the impact of NPs on marine species. As recently highlighted by Yi et al. (2019), there is an increasing need for longer exposures to study effects of environmental contaminants on organisms, since they could reveal different results compared to acute approaches. Therefore, in future studies, the toxicity of FC_{60} shall be investigated after longer exposure, in order to ascertain whether NPs can more markedly affect biomarker responses in clams.

CRediT authorship contribution statement

Ilaria Marisa: Conceptualization, Methodology, Investigation, Data curation, Writing - original draft preparation. **Davide Asnicar:** Investigation, Writing- original draft preparation. **Valerio Matozzo:** Conceptualization, Methodology, Writing - reviewing & editing. **Alessandro Martucci:** Investigation. **Livio Finos:** Formal analysis. **Maria Gabriella Marin:** Conceptualization, Methodology, Investigation, Data curation, Writing- original draft preparation, Supervision, Writing-reviewing & editing.

Declaration of Competing Interest

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

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