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Merging nano-genotoxicology with eco-genotoxicology: An integrated approach to determine interactive genotoxic and sub-lethal toxic effects of C₆₀ fullerenes and fluoranthene in marine mussels, *Mytilus sp.*

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Abstract

Whilst there is growing concern over the potential detrimental impact of engineered nanoparticles (ENPs) on the natural environment, little is known about their interactions with other contaminants. In the present study, marine mussels (*Mytilus sp.*) were exposed for 3 days to C₆₀ fullerenes (C₆₀; 0.1-1 mg l⁻¹) and a model polycyclic aromatic hydrocarbon (PAH), fluoranthene (32-100 µg l⁻¹), either alone or in combination. The first two experiments were conducted by exposing the organisms to different concentrations of C₆₀ and fluoranthene alone, in order to determine the effects on total glutathione level (as a measure of generic oxidative stress), genotoxicity (DNA strand breaks using Comet assay in haemocytes), DNA adduct analyses (using ³²P-postlabelling method) in different organs, histopathological changes in different tissues (i.e. adductor muscle, digestive gland and gills) and physiological effects (feeding or clearance rate). Subsequently, in the third experiment, a combined exposure of C₆₀ plus fluoranthene (0.1 mg l⁻¹ and 32 µg l⁻¹, respectively) was carried out to evaluate all endpoints mentioned above. Both fluoranthene and C₆₀ on their own caused concentration-dependent increases in DNA strand breaks as determined by the Comet assay. Formation of DNA adducts however could not be detected for any exposure conditions. Combined exposure to C₆₀ and fluoranthene additively enhanced the levels of DNA strand breaks along with a 2-fold increase in the total glutathione content. In addition, significant accumulation of C₆₀ was observed in all organs, with highest levels in digestive gland (24.90 ± 4.91 µg C₆₀ g⁻¹ ww). Interestingly, clear signs of abnormalities in adductor muscle, digestive gland and gills were observed by histopathology. Clearance rates indicated significant differences compared to control with exposure to C₆₀, and C₆₀ /fluoranthene combined treatments, but not after fluoranthene exposure alone. This study demonstrated that at the selected concentrations, both C₆₀ and fluoranthene evoke toxic responses and genetic damage. The combined exposure produced enhanced damage with additive rather than synergistic effects.

Key words: Engineered nanoparticles (ENPs); C₆₀ fullerenes; Fluoranthene; DNA adducts, Comet assay, Histopathology; Clearance rate; Bivalve mollusc

1. Introduction

Manufactured or engineered nanoparticles (ENPs; size 1-100 nm) have in recent years captured the attention of scientific organisations, governments and industry worldwide. There has been much debate on the future environmental implications of ENPs as a result of their wide usage, as in paints, biocides, electronics, biomedicines, cosmetics and pharmaceuticals [1]. Given their widespread applications and intensified productions in the recent years, it is expected that aquatic environment and human(s) will be increasingly exposed to them. This warrants early evaluations of their potential environmental and health impacts [2, 3].

ENPs have different properties from their mother bulk analogues, due to the fact that they have a very large surface area to volume ratio. This feature could potentially result in (a) a high affinity for organic and metallic pollutants; (b) direct generation of reactive oxygen species (ROS); and (c) the ability to penetrate cells. A recent report by the European Agency for Safety and Health at Work [4] suggested that ENPs pose the strongest emerging risk to human health. EASW recommended that *in vivo* toxicological investigations are needed for nanomaterials to obtain more reliable data for risk assessment in order to meet European standard regulations. For the current study, Buckminster fullerenes or fullerenes (C_{60}) was chosen as it is an elementary component in many modern manufactured products. It is one of the most ubiquitous ENPs, generally present in polluted air as a result of fuel combustion [5]. There have been concerns about the potential dermal and inhalation effects of C_{60} , due to its strong oxidizing and phototoxic properties [6]. In common with other ENPs, the potential health risk of C_{60} has however not been properly evaluated.

Fluoranthene is one of the most common pyrogenic polycyclic aromatic hydrocarbons (PAHs) and is present as a ubiquitous contaminant in human foods and in environmental samples [7]. The U.S. Environmental Protection Agency (EPA) has classified fluoranthene as one of the 16 priority PAHs. Its concentration in sediment has been found to range from tens to hundreds $\mu\text{g g}^{-1}$ dry weight sediment [8]. Because fluoranthene can be metabolised by aquatic organisms it may generate reactive oxygen species (ROS) and form adducts, which can exert both acute toxic and genotoxic effects if antioxidant defences are overcome by pro-oxidant forces [9].

1 After ROS induction, a series of complex biological responses can be triggered by
2 attack on DNA, proteins and lipid membranes [10]. The biological effects of C₆₀ on its
3 own however, appear to be contradictory. Whilst C₆₀ has been shown to induce
4 detrimental biological responses under *in vitro* and *in vivo* conditions, including on
5 aquatic organisms using a range of parameters [11, 12, 13, 14, 15 16, 17, 18], other
6 studies have suggested that it has no biological effects under different experimental
7 conditions [19, 20]. Furthermore, investigations of the potential interaction of C₆₀ with
8 other contaminants have been very limited. Following two months of stirring in water,
9 Baun et al. [21] suggested that 85% of phenanthrene, a model environmental
10 toxicant, sorbed to C₆₀-aggregates and increased C₆₀ toxicity in algae
11 (*Pseudokirchneriella subcapitata*) and freshwater crustaceans, *Daphnia magna*. A
12 preliminary study by Yang et al. [22] reported the effects of suspended C₆₀ on the
13 photo-induced toxicity of fluoranthene in *D. magna*. The study suggested that
14 fluoranthene may be transported from the surface of the cage-like C₆₀ structure to
15 cross cell membranes. The authors further suggested that interactions between C₆₀
16 and fluoranthene decrease both the uptake rate and increase the elimination rate for
17 fluoranthene.

18 In the environment, organisms are generally exposed to mixtures of different
19 contaminants or pollutants. These include combinations of organics, trace metals
20 and ENPs [23, 24] which can interact in many ways (i.e. additively, synergistically, or
21 antagonistically) to induce biological responses at different levels of biological
22 organisation. However, investigations of the combined toxic effects of multiple
23 chemicals on an animal are much more challenging than of a single compound [25],
24 and are therefore sparse. For example, the number of possible combinations of
25 pollutants is extremely large, and the combination that is likely to be most important
26 is unknown. Moreover, it is more difficult to choose realistic ranges of exposure
27 concentrations and the biological parameters to be tested than for a single pollutant.

28 In the current study, the role of C₆₀ in contaminant delivery, and the potential
29 effects of the interaction between C₆₀ and fluoranthene on the living organism, was
30 investigated using the filter feeder bivalve mussels, *Mytilus sp.* as a model
31 organism. Mussels are known to exhibit measurable biochemical and behavioural
32 end-points following exposure to toxicants. An integrated approach was adopted to

examine cellular and subcellular responses, as well as specific organ accumulation and the physiology to provide a more holistic assessment of the overall biological significance of such environmental variations. In addition to genotoxic effects in terms of DNA strand breaks (in haemocytes) and DNA adduct formation using ^{32}P -postlabelling method (in different tissues), the interactive effects were determined at several levels of the biological organisation. This included determination of total glutathione content (in adductor muscle), at the biochemical level; C_{60} accumulation and histopathology in adductor muscle, gills and digestive gland at the tissue and organ levels, and 'the clearance rate' as a measure of physiological effects at the organism level.

2. Materials & Methods

2.1. Chemicals

All chemicals and reagents were of high purity analytical grade and were obtained from Sigma-Aldrich (Poole, UK) unless stated otherwise.

2.2. Solution preparation

A primary stock solution (5.0 mg ml^{-1}) of fluoranthene was prepared in acetone. The fluoranthene concentration in the experimental exposure water was measured using solvent (dichloromethane) extraction with GC-Mass Spectrometry quantification (Agilent Technologies 6890 N Network GC system interfaced with an Agilent 5973 series mass selective detector). The C_{60} (lot number 11401DB; with purity 99.5% according to the manufacturer's information) was obtained from Sigma-Aldrich. The nanomaterial was dispersed in filtered ($0.45 \mu\text{m}$) seawater. The stock C_{60} suspensions (1 and $10 \text{ mg } 10 \text{ ml}^{-1}$) were ultrasonicated (35 kHz frequency, Fisherbrand FB 11010) for 1 h to attempt uniform dispersion before adding to the exposure tanks to reach 0.1 and $1.0 \text{ mg l}^{-1} \text{ C}_{60}$.

2.3. Characterisation of C_{60} nanoparticles

With limited characterisation data available for the commercial C_{60} , a broad analytical approach was applied to the concentrated stock suspension (10 mg l^{-1}). Hydrodynamic diameters, polydispersity index (PI) and zeta potential (surface

charges) of the C₆₀ (100 mg l⁻¹) in filtered seawater were measured at 15°C using a Zetasizer Nano ZS ZEN3600 (Malvern Instruments Ltd., Malvern, UK) based on dynamic light scattering (DLS). The shapes and the sizes of the particles were also investigated using transmission electron microscopy (TEM, JEOL1200EX, 80 kV) and Atomic Force Microscopy (AFM, XE100, Park Systems) and the purity of the C₆₀ was controlled by determining the element composition of discrete C₆₀ particles using energy-dispersive X-ray diffraction and TEM (Philips Tecnai F20). Samples were prepared by drop deposition of C₆₀ suspensions (10 mg l⁻¹) onto freshly cleaved muscovite (for AFM) and 300 mesh Cu-formvar grids (for TEM). The C₆₀ particles were allowed to adsorb to muscovite for 5 min and to the TEM-grids for 30 min, after which the samples were washed by dipping into milli-Q water and air-dried overnight. Replicate samples were also prepared with fullerene dispersed in dichloromethane. Sample analyses were run in triplicate.

2.4. Animal collection and maintenance

Mussels (*Mytilus sp.*) of similar shell length (51-58 mm) were collected at low tide in April 2008 from Trebarwith Strand (Cornwall, UK), a relatively clean site (grid reference: SX048 866). These mussels were maintained under the standard laboratory conditions (mentioned below) until the end of June 2008 for 3 different sets of experiments. It should be pointed out that it had been assumed that mussels found around most of the coast of the UK, including in Cornwall, are *M. edulis*, whereas those found in the Mediterranean are *M. galloprovincialis*. Recently, however, using a molecular probe for the *Glu* gene, encoding an adhesion protein gene which demonstrates interspecies variation [26], it has been shown that the species composition of mussels at different sites in Devon and Cornwall (south west England) is quite variable and includes *M. edulis*, *M. galloprovincialis* and their hybrids [27]. At Trebarwith Strand, where our samples were collected, 97% of the organisms have been reported to be *M. galloprovincialis* and 3% to be hybrids [27]. Since the relative sensitivity of these two species to different contaminants has not been thoroughly investigated, and the species composition needs to be further confirmed using other markers with a larger sample size, it is appropriate to use the term *Mytilus sp.* in the present context, in line with other authors [28, 29, 30].

After collection, animals were immediately transported (in a cool box) to the laboratory where they were maintained in tanks under controlled conditions to acclimatize and used until the end of June 2008. During the experimental period, seawater quality was confirmed in each of the beakers by measuring % dissolved oxygen ($96.1 \pm 0.3\%$), pH (7.8 ± 0.02), total ammonia ($0.04 \pm 0.02 \text{ mg l}^{-1}$), temperature ($15 \pm 1^\circ\text{C}$) and salinity ($31.5 \pm 0.15 \text{ ‰}$) (Multi 340i/SET; WTW, Weilheim, Germany). A photoperiod of 12h light: 12h dark was maintained throughout.

2.5. Exposure conditions

The experiments were divided into 3 short-term (3 day) exposures to assess the toxicity of fluoranthene and C_{60} , both individually and in combination. This 3 day exposure period was based on earlier studies carried out in our laboratory which had shown genotoxic and physiological effects in this species following exposure to reference genotoxic agents [31, 32]. The first two experiments were conducted using differing levels of exposure to fluoranthene and C_{60} to evaluate potential dose response relationships for toxicological responses. Three animals from each beaker were taken and the tissues (i.e. adductor muscle, digestive gland and gills) were dissected and prepared for biochemical, histopathological and chemical analyses. Prior to dissection and collection of haemolymph samples, potential physiological effects were determined by 'clearance rate' assay. Animals were not fed during the experiment and all treatments were set up in triplicate.

2.5.1. Fluoranthene exposures

Initial experiments were designed to validate the sensitivity of the genotoxicity test (Comet assay) by using fluoranthene (3 day exposure) as a reference agent. The range of exposure concentrations for fluoranthene was chosen based on published results demonstrating lysosomal membrane damage in this species [33]. The concentration range used was, however, slightly modified to fit the semi-logarithmic scale widely applied in ecotoxicological studies [32, 34]. In 2 l glass beakers (3 animals beaker⁻¹), individuals were exposed to 32, 56, 100 $\mu\text{g l}^{-1}$ fluoranthene. Exposure to solvent controls was also carried out; acetone (0.05 g ml⁻¹

¹) was added instead of fluoranthene stock solution. The seawater was changed daily and re-dosed with appropriate quantities of the fluoranthene stock solutions.

2.5.2. *C₆₀* exposures

In 10 l glass tanks (10 animals per tank⁻¹) mussels were exposed to 0.1 and 1.0 mg l⁻¹ C₆₀ for 3 days. This concentration range was based on previous studies to evaluate lysosomal membrane stability as an indicator of cytotoxicity and other biological responses in adult and embryo-larval stages on bivalve and other aquatic species [12, 18]. In parallel to the exposure to C₆₀, exposure to 32 µg l⁻¹ fluoranthene was also carried out as positive control. The seawater was changed daily and re-dosed with the appropriate concentration of C₆₀.

2.5.3. Combined exposures to *C₆₀* and fluoranthene

In a separate experiment (June 2008), mussels (*n* = 120) were divided into twelve 10 l glass tanks (10 animals tank⁻¹) with sea water. Three tanks were used per exposure group and mussels were exposed to 0.1 mg l⁻¹ C₆₀, 32 µg l⁻¹ fluoranthene and a mixture of C₆₀ with fluoranthene at the same concentrations. The three remaining tanks were used as controls. In the experiment, two exposure durations were considered: three day exposure and three day post exposure in clean seawater, with removal of mussels at the end of each period for the analysis.

2.6. Collection of haemolymph samples and preparation of adductor muscle extract

From each individual mussel, approximately 0.20 ml of haemolymph was extracted from the posterior adductor muscle and was diluted with 0.20 ml physiological saline. Haemolymph-physiological saline suspension samples were centrifuged at 60 *g*_{av} in microfuge tubes for 2 min to pellet out the haemocytes which were then kept on ice for the Comet assay. Posterior adductor muscles from two mussels (approximately 0.2 g ww) were dissected and homogenized in extraction buffer (20 mM Tris-chloride, pH 7.6, containing 0.5 M sucrose and 1 mM EDTA) using a ratio of 1:3 (w/v) for total glutathione analysis as described by us in detail [35].

2.7. Determination of DNA strand breaks using the Comet assay

Haemocyte viability was checked using the Eosin Y assay. Single strand breaks in the mussels' haemocytes were determined using the alkaline Comet assay as described elsewhere [31, 32, 36]. The level of DNA damage in 100 cells sample⁻¹ was measured by Komet 5.0 Image Analysis System (Kinetic Imaging, Liverpool, UK) using an epifluorescence microscope (Leica, DMR). Data for % tail DNA are presented as a reliable measure of single-strand DNA breaks/alkali labile sites [37].

2.8. DNA adduct analysis

DNA was isolated from digestive gland, gills and adductor muscle using a standard phenol/chloroform extraction method as described elsewhere [38]. DNA concentration and purity were determined spectrophotometrically using a NanoDropTM1000 spectrophotometer (USA). DNA adduct analysis for each DNA sample (4 µg) was carried out using the nuclease P1 enrichment version of the ³²P-postlabelling method as described previously [39, 40]. Resolution of ³²P-labelled adducts was carried out by chromatography on polyethyleneimine-cellulose (PEI-cellulose) thin-layer chromatography (TLC) sheets (10 × 20 cm, Macherey-Nagel, Düren, Germany) using the following solvents: D1, 1.0 M sodium phosphate, pH 6.0; D3, 4 M lithium-formate, 7 M urea, pH 3.5; D4, 0.8 M lithium chloride, 0.5 M Tris, 8.5 M urea, pH 8.0. TLC sheets were scanned using a Packard Instant Imager (Dowers Grove, IL, USA). An external anti-(±)-*trans*-7,8-dihydrooxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE)-DNA standard [41] was employed as positive control.

2.9. Determination of total glutathione levels in adductor muscle extract

The total glutathione (i.e. reduced, GSH, and oxidised, GSSG) content of adductor muscle extract was determined essentially as described before [35]. The rate of absorbance decrease at 412 nm was measured using a microplate reader (Optimax, Molecular Devices, Sunnyvale, CA, USA) over 5 min using 96-well plates at 22 °C.

2.10. Histological preparation for adductor muscle, digestive gland and gills

Tissues dissected from exposed animals (i.e. adductor muscle, digestive gland and gills) were examined by normal histological methods [36, 42, 43]. Each

organ was initially fixed in 10% buffered formal saline for at least 48 h. Specimens were then processed in ascending grades of alcohol. Tissue samples were embedded into paraffin and cut with a microtome at 5-7 μm thickness and mounted on slides. Slides were stained with haematoxylin and eosin (H and E) following Mayer's standard protocols.

2.11. Clearance rate

Clearance or feeding rates of individual mussel were determined as described elsewhere [32, 36, 43]. Briefly, mussels were allowed to acclimatise until their valves opened (approximately 10 min) prior to the addition of 500 μl of *Isochrysis* algal suspension (supplied by Cellpharm Ltd., Malvern, UK). The algae were mixed manually with a glass rod and then 20 ml of water sample was removed using a glass syringe. This procedure was repeated again after 20 min. Samples from both time zero and 20 min were analysed using a Beckman TM Coulter Particle Size and Count Analyser (Z2) to count particles between 4.0-10.0 μm in diameter. Clearance rate of the mussels were calculated based on the Coughlan (1969) equation as described elsewhere in detail [32, 36].

2.11. Chemical analyses of fluoranthene and C₆₀

2.11.1. GC-analysis of fluoranthene in water samples

Water samples were placed into glass vials with dichloromethane (DCM) in 1:9 ratio (v/v) and stored in darkness at $-20\text{ }^{\circ}\text{C}$. Phenanthrene d₁₀ (CAS: 1571-22-2) was added as an internal standard. Quantitative analyses of 1 μl aliquots of the DCM extracts were performed using an Agilent Technologies 6890 N Network GC system interfaced with an Agilent 5973 series mass selective detector. An HP-5MS (cross-linked 5% phenyl methyl siloxane) capillary column (30 m) with a film thickness of 0.25 μm and internal diameter 0.25 mm was used for separation, with helium as a carrier gas (maintained at a constant flow rate of 1 ml min^{-1}). Extracts were injected in splitless mode. Column temperature was programmed at 40 $^{\circ}\text{C}$ for 1 min, 40-300 $^{\circ}\text{C}$ at 15 $^{\circ}\text{C per min}$ and then held at 300 $^{\circ}\text{C}$ for 5 min. Samples were screened for fluoranthene using selected ion monitoring (m/z 202) with quantification calculated relative to the phenanthrene d₁₀ internal standard (monitored using m/z 188). Prior to

sample extract analyses, the system was calibrated using authentic standards. With each batch of samples, solvent and procedural blanks together with calibration standards were run in sequence.

2.11.2. HPLC-analysis of C₆₀ in tissue samples

Adductor muscle, digestive gland and gills were carefully washed with pure toluene to remove surface adsorbed C₆₀ particles. Then, tissues were extracted into toluene (1:9 w:v ratio) using ultra-sonication for 15 min (35 kHz frequency, Fisherbrand FB 11010). Extracts were centrifuged (9000 *g*_{av}) prior to HPLC analysis. C₆₀ was separated using a Hypersil (5 µm) Elite C18 (250 × 4.6 mm I.D.) column. In the case of combined exposed samples (C₆₀ and fluoranthene), a Plgel 5 µm 50 Å (manufactured by Agilent Technologies; USA) column was used to resolve the PAHs from the C₆₀. The mobile phase for both columns was toluene at a flow-rate of 1.0 ml min⁻¹. Sample injections were performed manually with volumes of 100 µl. The eluent was monitored at 330 nm using a Shimadzu SPD-6 AV UV–Vis spectrophotometer. Integration was performed using a Shimadzu-C-R3A chromatopac. For external calibration, a standard curve was generated for C₆₀ concentrations ranging from 0.125 and 2.0 mg l⁻¹. The concentration of test sample was calculated by comparison to the standard curve.

2.12. Statistical analyses

Statistical analyses were performed using the statistical package StatGraphics plus, version 5.1 (Statistical Graphics Corp). All results are presented as mean ± S.E. Significant differences (*P* < 0.05) between groups were studied using one way analysis of variance (ANOVA) or Kruskal-Wallis test, followed by multiple range tests to differentiate between the groups of data.

3. Results

3.1. C₆₀ characterisation

The DLS measurements of C₆₀ in filtered seawater showed a large Z-average hydrodynamic diameter (680 ± 19 nm) and high polydispersity index (0.57),

1 indicating the formation of large agglomerates (Table 1). TEM micrographs of C₆₀
2 particles in seawater and dichloromethane (Fig. 1a), and AFM images C₆₀ in
3 dichloromethane (Fig. 1b) showed aggregates that were composed of distinct
4 particles. The purity of the C₆₀ was assessed by TEM-EDX on 12 different C₆₀
5 particles (example shown in Fig. 1c). No significant differences could be observed
6 between EDX-spectra acquired on C₆₀ particles and on the background carbon-
7 coated Cu-grid (Fig. 1d), implying that element impurities were below the detection
8 limit of EDX (typically 0.1 % of the total particle mass). The samples of C₆₀ in
9 dichloromethane were used for determination of particle sizes, since these gave
10 TEM-micrographs with much higher contrast, and since the seawater samples gave
11 AFM-images that were not consistent with the TEM-images, probably as a result of
12 the extensive particle agglomeration in seawater. While the dichloromethane
13 samples are not representative for the whole particle distribution in seawater
14 (including agglomerates), they should give accurate sizes of discrete non-
15 agglomerated particles. Size measurements of discrete particles within the
16 agglomerates on the TEM micrographs and AFM images showed oval particles with
17 diameters in the 100-200 nm range (Table 1). The difference in mean diameter given
18 by TEM (160 nm) and AFM (122 nm) can be explained by the fact that the AFM
19 height was measured as the height of particles above the muscovite surface, while
20 the TEM diameter was measured along the surface of the grid.

22 *3.2. Determination of DNA damage using the Comet assay*

23 No significant loss of cell viability was observed for haemocytes (cell viability >
24 90% in all samples after Eosin Y staining) after exposure to fluoranthene, C₆₀, or C₆₀
25 + fluoranthene (C₆₀ + F) (data not shown). Since the Comet assay data were not
26 normally distributed, the non-parametric Kruskal-Wallis test was applied to
27 investigate significant differences between groups. Our results showed that there
28 was a concentration-dependent response for fluoranthene (Fig. 2A). Also, in the
29 second experiment significant differences were found between the control and C₆₀
30 exposures ($P = 0.0036$) with higher DNA damage at 1.0 mg l⁻¹ C₆₀-exposed animals
31 (Fig. 2B). Multiple range tests (Fisher's LSD) showed a significant difference
32 between all exposures. An equivalent DNA damage response was also observed

1 with the positive control (fluoranthene at 32 $\mu\text{g l}^{-1}$), indicating the robustness of the
2 Comet assay.

3 *3.2.2 DNA damage following combined exposures of C₆₀ and fluoranthene*

4 Mussels showed a significant elevation in DNA damage in exposed animals in
5 comparison with the control for all the treatments alone or in combination (Fig. 2C).
6 The results of the Comet assay for single and joint effects of C₆₀ + F showed that
7 combined exposure led to the highest DNA damage among the exposure
8 experiments which was found to be additive rather than synergistic (Fig. 2C). After 3
9 day post-exposure, however, there was a significant decrease in DNA damage under
10 all treatment conditions showing equal levels of damage in all the exposure
11 conditions, but the decrease was greatest (approx. 3-fold) for the combined
12 treatment of fluoranthene and C₆₀. Using 2-way ANOVA, a significant interaction was
13 found between exposure type and time of measurement ($P = 0.0001$). Overall, these
14 results indicate that the C₆₀ interaction with PAHs contributes to the enhanced (i.e.
15 additive) genotoxic effect in haemocytes and suggest that following both exposures
16 (C₆₀ and C₆₀ + F), C₆₀-induced DNA damage recovered to the normal level after 3
17 day depuration.

18 *3.3. Formation of DNA adducts*

19 Under the chromatographic conditions used no DNA adducts were detectable
20 in DNA samples obtained from adductor muscle, digestive gland and gills either from
21 single exposure to C₆₀ and fluoranthene or the combined exposure (Fig. 3).

22 *3.4. Determination of total glutathione levels in adductor muscle*

23 As shown in Figure 4A, C₆₀ exposure resulted in a slight increase in the total
24 glutathione content (i.e. reduced, GSH and oxidised, GSSG, combined) in adductor
25 muscle in comparison to the control, but the effect was not significant. On the other
26 hand, a significant increase ($P < 0.05$, ANOVA) in total glutathione levels was
27 reported in C₆₀ + F-exposed animals. An approximately 2-fold increase in total
28 glutathione content was seen in the adductor muscle after exposure to the C₆₀ and
29 fluoranthene mixture in comparison to other groups. Multiple range tests (Fisher's
30 LSD) showed significant differences at the 95% confidence level, between the C₆₀ +

F and fluoranthene exposure alone (Fig. 4B). Total glutathione levels in control samples from the C₆₀ + F experiment were lower than the control from the C₆₀ experiment, but this effect could be attributed to laboratory acclimatisation of the mussels at higher temperature (i.e. 15° C) as the two experiments were carried out at different times (i.e. 6 weeks part) after their collection from the field.

3.5. Histopathological observations

Table 2 summarises the main histopathological abnormalities in different treatment groups. Histological examinations indicated that there were no pathological alterations in control specimens. However, different tissues showed varying degrees of abnormalities (e.g. haemocyte infiltration, necrosis or other injuries) following exposures (Fig. 5). Adductor muscle samples from the 'C₆₀ exposure' group did not show any histological abnormalities at 0.1 mg l⁻¹ C₆₀, but at 1.0 mg l⁻¹ C₆₀ caused atrophy in myocyte cells and a decrease in extracellular spaces between muscle bundles in three (i.e. 33%) out of nine examined animals (Fig. 5B). Similar effects were also observed with the adductor muscle exposed to combined exposure of C₆₀ + fluoranthene, with even more adverse effects; six (i.e. 66%) animals showed abnormalities including two (i.e. 22%) animals showing hypertrophy (swelling) and loss of muscle bundle organisation (Fig. 5C; Table 2), and another four (i.e. 44%) animals had muscle atrophy and decreased extracellular spaces between muscle bundles.

The histological observations in the digestive gland specimens showed that C₆₀ exposure caused alterations in the digestive tubules (Fig. 5E; Table 2). Low C₆₀ exposure (0.1 mg l⁻¹) caused loss of definition of the digestive tubules, probably caused by necrosis in three (i.e. 33%) out of nine specimens, whereas the other six (i.e. 66%) specimens exhibited clear signs of necrosis in the digestive tubules. These effects were even more pronounced when fluoranthene interacted with C₆₀ in the combined exposure. Histological changes were characterised by necrosis in digestive cells within the digestive tubules and were observed in all animals examined. We found atrophy of the digestive tubule epithelium in which the epithelium cells shrank and exhibited thin tubules, only basement membrane remained and cellular debris was often observed within the lumen of the digestive tubules (Fig. 5F; Table 2).

Histological examination of the gills revealed that 0.1 mg l⁻¹ C₆₀ caused abnormalities. Two (i.e. 22%) mussels at the low C₆₀ concentration showed signs of hypoplasia in frontal and lateral cilia. Progressively more abnormalities were seen with high C₆₀ exposure (1.0 mg l⁻¹); 4 (i.e. 44%) animals out of 9 had hypoplastic effects in the frontal and lateral cilia (Fig. 5H). Gills examined after C₆₀ + F exposure showed areas with erosion of cilia and filament necrosis in 3 (i.e. 33%) out of 9 specimens (Fig. 5I; Table 2).

3.6. C₆₀ accumulation

In order to understand the pattern of C₆₀ accumulation in mussel tissues, after 3 day exposure, adductor muscle, digestive gland and gills were analyzed for C₆₀ content using HPLC. Results are shown in Figure 6A. The ranking of organs according to increasing C₆₀ concentrations was identical at both exposure concentrations: adductor muscle < gills < digestive gland. No significant difference was found between C₆₀ levels in the adductor muscle in comparison to the control. Gills displayed a significant accumulation of C₆₀ in animals exposed to 0.1 mg l⁻¹ C₆₀, but no significant difference was observed with the higher exposure ($P = 0.15$, ANOVA). In contrast, significant differences in C₆₀ levels were found with 0.1 and 1.0 mg l⁻¹ exposures for digestive gland (ANOVA; $P = 0.04$ and $P = 0.03$, respectively), with the highest C₆₀ concentrations measured in the 1.0 mg l⁻¹ exposed animals ($24.90 \pm 4.91 \mu\text{g C}_{60} \text{ g}^{-1} \text{ ww}$). Thus, the digestive gland appears to be the most important target tissue/repository for C₆₀.

Results of the C₆₀ accumulation in the combined exposure experiment are summarised in Fig. 6 B, C and D. It appears that C₆₀ accumulated more in animals exposed to C₆₀ alone than those exposed to C₆₀ + F. Whilst a significant increase in C₆₀ accumulation is shown following exposure, after 3 day depuration there was a significant decrease in C₆₀ content both in the digestive gland and gills.

3.7. Clearance rate (CR)

The CR data measured in mussels exposed to C₆₀ (Fig. 7A) showed that mussels exposed to 1.0 mg l⁻¹ C₆₀ were significantly affected ($P < 0.05$, ANOVA). Whilst not significant, a reduction in CR is also indicated for animals exposed to 0.1

1 mg l⁻¹ ($P = 0.0002$, t -test). Mussels exposed to the combination of C₆₀ with
2 fluoranthene also showed a significant decrease in CR ($P = 0.017$, t -test; Fig. 7B). It
3 should be noted, however, that the clearance rates in control animals from the 'C₆₀ +
4 F' experiment was lower than in the 'C₆₀' experiment, possibly indicating a slight
5 decline in health with time, although the clearance rate for C₆₀ exposure alone in
6 both sets of exposures were comparable. After 3 days depuration, CRs of both C₆₀
7 and C₆₀ + fluoranthene increased significantly, indicating that the feeding activity of
8 the mussels had, to an extent, recovered.

10 4. Discussion

11 In common with other environmental contaminants, interactive effects of C₆₀
12 and ubiquitous organic contaminants, such as fluoranthene, represent an area of
13 research that has not yet been fully covered. As mentioned earlier, despite having
14 strong oxidizing and phototoxic properties [6], the potential detrimental effects of C₆₀
15 on its own and in combination with other agents are inadequately studied and are
16 therefore inconclusive in the literature [44]. Using mammalian systems, either no
17 cytotoxic, mutagenic effects [20, 45, 46] or positive responses for the induction of
18 DNA and cellular damage [11, 47, 48], have been reported. In this context, it is
19 generally accepted that the toxic potential of ENPs is not only dependent upon its
20 quality, but also on target cell types, quality, purity and synthesis method of the
21 ENPs, and treatment of the cells [49, 50, 51, 52]. While determining the induction of
22 DNA damage and other toxicological parameters, it is also very important that the
23 methodologies have been thoroughly optimised and validated against reference
24 genotoxic and toxic agents to ensure the assay is reliable, reproducible and sensitive
25 [34, 53, 54]. In this context, the endpoints analysed in this study were thoroughly
26 optimised and validated in this model system before evaluating the toxic potential of
27 C₆₀ and fluoranthene either alone or in combination [36]. Where appropriate,
28 concurrent positive controls were also used in the experimental design to make the
29 results robust.

30 Prior to evaluation of the potential detrimental biological responses of ENPs,
31 their physico-chemical properties were thoroughly quantified as recommended [50,

51]. The zeta potential of C₆₀ in filtered seawater was a relatively small negative charge, indicating that the fullerenes are unlikely to be fully stabilised. This colloidal instability was confirmed by DLS, TEM and AFM where large agglomerates were recorded. However, the DLS data for polydisperse agglomerate samples are only qualitative [55], since the DLS sensitivity increases rapidly with particle size, and must therefore be treated with caution. However, taken together, the characterisation data show that the C₆₀ were composed of 100-200 nm particles (indicated by AFM and TEM) that were present both as discrete particles and formed agglomerates of several hundred nm in seawater (indicated by DLS). These results are expected from un-functionalised commercial fullerenes.

Previous *in vivo* studies have evaluated C₆₀-induced oxidative stress in the embryonic stage of zebrafish, suggesting that it can act as a pro-oxidant and enhance toxic response by interacting with biomolecules such as DNA, proteins and lipids [17]. This is broadly in line with positive responses observed in our study for the Comet assay following exposure to C₆₀ on its own, but is in contrast to the study of Jacobson et al., [46], who could not find a positive response in the same assay in mouse lung epithelial cells. Whilst there is some information on potential effects of C₆₀ in mammalian systems, data is limited for aquatic organisms. For interactive effects, a preliminary study reported that the toxicity of phenanthrene on the fresh water flea (*D. magna*) increased significantly in the presence of C₆₀ [21]. It was assumed that phenanthrene is only bioavailable form [21], but that C₆₀ aggregates acted to deliver the phenanthrene to the cell membrane, where it is known to have a targeted narcotic effect [21]. If this hypothesis is accepted, the apparent induction of DNA damage in our study could be a result of oxidative stress, resulting in lipid peroxidation of the cell membrane, as lipid peroxidation has been positively linked with induction of DNA damage [56]. Relative contributions of C₆₀ and fluoranthene for the generation of free radicals however could be difficult to speculate as mentioned below, PAHs are also known to generate ROS. Similarly, Yang et al. [22] also reported the effects of suspended C₆₀ on the photo-induced toxicity of fluoranthene in *D. magna*. The study suggested that fluoranthene may be transported from the surface of the cage-like C₆₀ structure to cross the cell membranes. Once inside the cells, varieties of mechanisms could lead to generation of different qualities and quantities of free radicals leading to induction of genetic damage [57, 58]. In addition,

1 other indirect mechanisms of genotoxicity (i.e. via interaction of the contaminants
2 with non-DNA targets) could also result in observed genotoxic effects.

3 Besides the reported toxic effect of ENPs, either directly or through generation
4 of ROS [1], many studies have shown that PAHs such as fluoranthene in addition to
5 binding with the biomolecules (i.e. forming adducts) have the ability to produce ROS
6 resulting in induction of oxidative damage [9, 33, 59, 60, 61]. In the absence of
7 formation of any bulky DNA adducts in different tissues, our results indicate that C₆₀
8 and fluoranthene cause DNA damage, possibly by generating ROS. This could
9 however only be confirmed using a modified Comet assay to quantify oxidised
10 purines and pyrimidines, as carried out by us using fish cells under in vitro and in
11 vivo conditions [49, 62, 63]. In this context, however, it has also been suggested that
12 antibacterial activity of fullerene water suspensions is not due to ROS-mediated
13 damage [15]. Due to technical and logistic limitations (mainly the amount of
14 haemolymph samples required for modified Comet assay depending upon the
15 number of bacterial enzymes and animals to be used, increased number of samples
16 and sampling times), determination of oxidative DNA damage could not be carried
17 out in our study. As a measure of global oxidative stress however, as mentioned
18 earlier and discussed later, levels of total glutathione from the adductor muscle
19 extracts were determined. This assay has been thoroughly optimised and validated
20 in our laboratory conditions [35, 36]. The observed recovery of induced DNA damage
21 in the post-exposure period in the combined exposure experiments (i.e. C₆₀ +
22 fluoranthene) could be explained by either an enhanced repair process when the
23 mussels were returned to the clean seawater or as a result of enhanced turnover
24 rate of haemocytes. Similar observations have been made in other studies following
25 exposure to environmental contaminants [64].

26 At the biochemical level, combined exposure of C₆₀ plus fluoranthene caused
27 a significant increase in glutathione levels in the adductor muscle which further
28 indicates an induction of oxidative stress, and reflects the increased DNA damage in
29 the haemocytes and histological changes in different tissues. Even where there was
30 evidence of uptake of C₆₀ into adductor muscle (Fig. 6B), this was not associated
31 with elevated glutathione levels except in the combined exposure with fluoranthene
32 indicating that C₆₀ alone did not cause oxidative stress in this tissue. In a previous

study, we found that adductor muscle is a useful tissue to determine biomarker responses to oxidative stress. Elevated levels of glutathione in adductor muscle of *Mytilus edulis* have been reported by us after 5 day acute exposure to Cu ($40 \mu\text{g l}^{-1}$) [35]. A possible explanation for this induction is that under exposure conditions mussels tend to activate γ -glutamyl-cysteine synthetase (GCS) to restore glutathione levels (GSH synthesis is regulated in part by a non-allosteric competitive feedback inhibition of GCS by GSH). If this activation is maintained for several days, GSH levels could increase as suggested by Yan et al. [65]. Peña-Llopis and co-workers [66] found that a higher muscular GSH or GSH/GSSG ratio can be expected in mussels after long fenitrothion exposure. Different results on glutathione content have been obtained for organisms exposed to different types of chemical compounds. In common with other biological responses, the variability in these investigations may be due to a number of intrinsic and extrinsic factors including the C_{60} treatment regime (exposure time and nanomaterial preparation), the cell type or organisms used, as well as the metabolic/antioxidant capacity of the organism [43]. As estimation of glutathione levels in mussels in our laboratory conditions has been optimised and validated extensively [35, 36], we attribute the significant differences observed for enhanced levels of glutathione in combined exposures (i.e. F+ C_{60}) to acclimatisation of mussels at higher temperature (i.e. 15°C) in the laboratory conditions following their collection from the natural environment. Both impact of temperature and seasons on enzyme activities in mussels and other aquatic organisms is well documented, accounting for disparity in observed biomarker responses following exposure to contaminants [67-69]. This acclimatisation of mussels in our study had however no detrimental effects on the overall physiology of the mussels as in both sets of experiments (i.e. C_{60} and combined exposures), C_{60} alone treatment had comparable levels of clearance rate.

Highly reactive metabolites following exposure to PAHs can lead to covalent binding with DNA forming DNA adducts. The presence of DNA adducts following dietary exposure ($50 \text{ mg kg}^{-1} \text{ dw}$) to benzo[a]pyrene have previously been reported in mussels [70]. Another study also showed tissue specific differences in DNA adduct formation in *M. galloprovincialis* collected from PAH-contaminated sites [71]. However, in the present investigation, *in vivo* exposure to $32 \mu\text{g l}^{-1}$ fluoranthene for 3 day did not reveal any DNA adduct formation in any of the tested organs. In mussels,

1 there is a general lack of information on the potential formation of DNA adducts
2 either by fluoranthene or C₆₀. The possible explanation of our results is that either
3 fluoranthene was not able to form DNA adducts in mussel or that the experimental
4 conditions used for ³²P-postlabelling were not sensitive enough to detect them. It
5 however should be pointed out that DNA-adduct analysis using ³²P-postlabelling
6 method was used to detect bulky adducts and not oxidised bases, which would result
7 from oxidative stress. Absence of any bulky DNA adduct further supports the notion
8 that under the experimental conditions oxidative stress is mainly responsible for
9 observed biological responses.

10 After the biochemical and cellular changes, a sequence of biological
11 responses at higher order of biological organisation which included histopathological
12 and physiological alterations was observed in the exposed mussels. In addition, C₆₀
13 accumulation in different tissues was also observed. The effects of C₆₀ were
14 investigated in the tissues that are indicative of the overall health of the organism
15 (adductor muscle), metabolism (liver) or absorption (gills). Adductor muscle exhibited
16 a clear adverse atrophy in myocyte cells in C₆₀ plus fluoranthene exposed animals
17 which led to weak muscle bundle structure and therefore decreased extra-cellular
18 spaces of connective tissue. De Oliveira Ribeiro et al., [72] also found similar effects
19 in the muscles of adult male zebrafish following exposure to methyl mercury. They
20 found a decrease of the space between fibre bundles and disorganisation of
21 myofibrils. No studies have however reported potential histopathological changes of
22 the adductor muscle in mussels after exposure to ENPs. Further, gills and digestive
23 gland tissues showed marked degenerative changes. In gills of C₆₀ plus
24 fluoranthene-exposed mussels, ciliary erosion and necrosis of filaments were
25 observed. Similar toxic effects were noticed previously in mussels exposed to other
26 toxicants including heavy metals [36, 73]. As mussel gills are critical organs for their
27 respiratory and osmoregulatory functions, injuries in gill tissues may reduce the
28 oxygen consumption and impair the physiological function of the organ. The
29 apparent reductions in clearance rate observed in the current study are probably the
30 result of ciliary erosion which leads to the failure in physiological processes in the
31 whole organism [74]. Moreover, C₆₀ exposure caused reduction in digestive tubule
32 thickness (tubule atrophy) as a result of loss of digestive and basophilic secretory
33 cells within the lumen of digestive tubules. The effects of C₆₀ were enhanced when

fluoranthene was added, with even more severe changes observed in digestive tubules. The digestive gland of the exposed mussels showed necrosis (diffuse nuclei and no clear distinction in some epithelial cells), adverse atrophy of digestive tubule and stomach epithelium. Weinstein [75] found that oysters (*Crassostrea virginica*) exposed to 100 µg l⁻¹ fluoranthene in the laboratory for 21 days also exhibit a reduction in their mean digestive epithelial thickness. Digestive gland tissue of mussel is an important organ for active metabolism and detoxification and is extremely sensitive to pollutants [76]. Histological changes in the digestive tubules have been used to assess the effects of environmental contaminants [27,77, 78] and in common with other observations, it could be suggested that C₆₀-and fluoranthene-induced histopathological changes are not unique and quantitatively or qualitatively they often do not show concentration-dependent changes as observed in earlier studies [36,43]. Despite the fact that under laboratory conditions it has not been possible to induce neoplasia in invertebrates including bivalve molluscs [43, 54], neoplasia has been reported in bivalve molluscs from different parts of the world often correlated with exposures to environmental contaminants [43, 79]. The gross histopathological changes observed in our study could however be indicative of factors that promote neoplastic development in these organisms [80].

Chemical analyses of mussels exposed to C₆₀ alone showed predominant accumulation of C₆₀ in the digestive gland with more modest accumulation in the gill. In contrast, lower accumulation of C₆₀ was found in tissues from animals co-exposed to C₆₀/fluoranthene. Accumulation of C₆₀ was influenced by co-exposure to fluoranthene; animals exposed to C₆₀ alone accumulated more C₆₀ than combined exposed mussels. It is also interesting to note that whilst the uptake was higher in C₆₀ only exposures, the biological responses indicate that combined exposures were more effective in inducing detrimental effects. In line with limited information pertaining to potential interactive toxic effects of ENPs with other contaminants [21, 22], it appears that C₆₀ could act as *Trojan Horses* in facilitating the uptake of fluoranthene and enhancing the biological responses or conversely fluoranthene can enhance the toxicity of C₆₀. It is also worth mentioning that limited data are available in the literature showing differential patterns of tissue-specific accumulation, according to single or co-contaminant exposures. For example, whilst co-exposure of phenanthrene in C₆₀ suspensions showed higher bioaccumulation of phenanthrene

(no C₆₀ was measured) [21], another study using mussels showed that gold nanoparticles (GNPs) are better accumulated when used alone, whereas no GNPs were detectable in tissues when used in combination with menadione, a pro-oxidant [81]. Despite being metallic ENPs, in line with our observations, the GNPs predominantly accumulated in digestive glands and modestly in gills with no accumulation in mantle. Interestingly, no trace of gold was found in tissues from animals co-exposed to GNPs/ menadione [81]. The authors conclude that, as a consequence of the menadione toxicity, accumulation of GNPs may be impaired during feeding. Some minor peaks in the chromatograms of our study were also observed in the digestive gland and gill tissue samples, indicating that some metabolites could be produced over the exposure period. Understanding of toxicokinetics and metabolites for C₆₀ and fluoranthene would greatly help to explain the tissue/ organ- specific damage. There has however been very limited study in aquatic invertebrates as a whole to elucidate toxicokinetics for organic contaminant uptake and tissue-specific deposition to link with tissue specific damage [43]. From an ecotoxicological perspective, while the study has been carried out using adult life stages of the mussels, it is also important to evaluate the sensitivity of early life stages of this species [82] and establish relative sensitivity with other ecologically important species [54, 83, 84].

5. Conclusions

Our experimental results confirm that exposures of mussels to both fluoranthene and C₆₀ on their own result in concentration-dependant increases in DNA strand breaks but there was no evidence of DNA adduct formation under the experimental conditions. We show, for the first time, that combined exposures enhance the levels of DNA strand breaks and elevate total glutathione levels indicating oxidative stress to the exposed organisms. The effects at lower levels of biological organisation also become more pronounced at higher levels in terms of histopathological and physiological changes. Following this study involving short term exposures, longer exposure times are now required to further explore the potential effects of ENPs, either alone or in combination with other contaminants. Our integrated study adds to very limited information available on the potential impact of ENPs on aquatic biota and underlines the advantages of using filter-

feeding organisms in future investigations of environmental effects of manufactured or engineered nanomaterials at the whole organism level. This approach could be translated to other ecologically relevant species to assess the ecotoxicological impact of ENPs.

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Figure legends & Table captions

Fig. 1 Characterisation of C₆₀ fullerenes particles; (A) TEM micrograph, (B) AFM image, (C) EDX-spectrum of C₆₀-particle, (D) EDX-spectrum from the background.

Fig. 2 Induction of DNA strand breaks, represented as %Tail DNA in mussel haemocytes following 3 days *in vivo* exposure to: (A) fluoranthene (32, 56 and 100 µg l⁻¹); (B) C₆₀ (0.1 and 1.0 mg l⁻¹); and (C) C₆₀ + fluoranthene (0.1 mg l⁻¹ C₆₀ and 32 µg l⁻¹ fluoranthene). The values are mean ± S.E. Bars with the same letters are not significantly different whereas different letters show significant difference from other treatment ($P < 0.05$) according to the multiple range test (LSD). +ve control= 32 µg l⁻¹ fluoranthene; F = fluoranthene; C60 = C₆₀ fullerenes; F+C60 = fluoranthene+ C₆₀ fullerenes.

Fig. 3 ³²P-post labelling analysis of DNA from cells exposed to C₆₀ fullerenes (C₆₀), fluoranthene and C₆₀ + fluoranthene. DNA adduct profiles measured in digestive gland. Same results were obtained with gills and adductor muscle. BPDE = anti-(±)-*trans*-7, 8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene.

Fig. 4 Total glutathione levels in the adductor muscle extract following 3 days *in vivo* exposure to: (A) C₆₀; and (B) mixture of C₆₀ + fluoranthene (0.1 mg l⁻¹ C₆₀ and 32 µg l⁻¹ fluoranthene). The values are mean ± S.E. Bars with the same letters are not significantly different whereas different letters show significant difference from other treatment ($P < 0.05$) according to the multiple range test (LSD). F = fluoranthene; C60 = C₆₀ fullerenes; F+C60 = fluoranthene+ C₆₀ fullerenes.

Fig. 5 Haematoxylin and eosin (H & E) light micrographs of 5-8 µm thick sections through adductor muscle, digestive gland and gills of *M. edulis* showing the histological structure of control and treated mussels. A, D & G: control; B, E & H: exposed to C₆₀; C, F & I: exposed to C₆₀ + fluoranthene. mc = myocyte cell; si = sinus; dc = digestive cell; pbc = pyramidal basophilic secretory cell; dd = digestive diverticula; gf = gill filaments; fc = frontal cilia; lc = lateral cilia; ge = gill epithelium; dt = digestive tubules; at = atrophy; n = necrosis; hypo = hypoplasia; e = erosion. Scale bar: 100 µm.

Fig. 6 C₆₀ concentrations in tissues. (A) accumulation of C₆₀ in adductor muscle, digestive gland and gills after exposure to 0.1 & 1.0 mg l⁻¹. (B, C & D) accumulation of C₆₀ after exposure to mixture of C₆₀ + fluoranthene (adductor muscle, digestive gland and gills, respectively). Data are mean ± S.E., n = 3 mussel per treatment after 3 days of exposure. Bars with the same letters are not significantly different whereas different letters show significant difference from other treatment ($P < 0.05$) according to the multiple range test (LSD). F = fluoranthene; C60 = C₆₀ fullerenes; F+C60 = fluoranthene+ C₆₀ fullerenes.

Fig. 7 Clearance rate (represent as $l\ h^{-1}$) in *Mytilus* sp. following 3 days *in vivo* exposure to: (A) C_{60} (0.1 & 1.0 $mg\ l^{-1}$), and (B) mixture of C_{60} + fluoranthene (0.1 $mg\ l^{-1}$ C_{60} and 32 $\mu g\ l^{-1}$ fluoranthene). The values are mean $\pm$ S.E. Bars with the same letters are not significantly different whereas different letters show significant difference from other treatment ($P < 0.05$) according to the multiple range test (LSD). F = fluoranthene; C60 = C_{60} fullerenes; F+C60 = fluoranthene+ C_{60} fullerenes.

Table captions

Table 1

Results showing characterisation measurements of C_{60} fullerenes particles using different techniques. Values are mean $\pm$ S.E.

Table 2

Summary of histopathological evaluations of different organs of mussels, *Mytilus* sp. following exposure to C_{60} fullerenes (C_{60}) and fluoranthene, either alone or in combination.

Fig. 1 Characterisation of C₆₀ fullerenes particles; (A) TEM micrograph and (B) AFM image

Fig. 2

Fig 3.

Fig. 4

Fig. 5

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