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Merging nano-genotoxicology with eco-genotoxicology: An integrated approach to determine interactive genotoxic and sub-lethal toxic effects of C_{60} 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 C_{60} fullerenes (C_{60}; 0.10–1 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 C_{60} and fluoranthene alone, in order to determine the effects on total glutathione levels (as a measure of generic oxidative stress), genotoxicity (DNA strand breaks using Comet assay in haemocytes), DNA adduct analyses (using ^{32}P-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 C_{60} plus fluoranthene (0.10 mg l^{-1} and 32 μg l^{-1}, respectively) was carried out to evaluate all endpoints mentioned above. Both fluoranthene and C_{60} 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 C_{60} 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 C_{60} was observed in all organs, with highest levels in digestive gland (24.90 ± 4.91 μg C_{60} 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 the control with exposure to C_{60}, and C_{60}/fluoranthene combined treatments, but not after fluoranthene exposure alone. This study demonstrated that at the selected concentrations, both C_{60} and fluoranthene evoke toxic responses and genetic damage. The combined exposure produced enhanced damage with additive rather than synergistic effects.

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, in paints, biocides, electronics, biomedicines, cosmetics and pharmaceuticals [1]. Given their widespread applications and intensified production in the recent years, it is expected that aquatic environments 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 (EASW) [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

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reliable data for risk assessment in order to meet European standard regulations. For the current study, a Buckminster fullerene (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 oxidising 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 $\mu g\,g^{-1}$ 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]. After ROS induction, a series of complex biological responses can be triggered by attack on DNA, proteins and lipid membranes [10]. The biological effects of C_{60} on its own however, appear to be contradictory. Whilst C_{60} has been shown to induce detrimental biological responses under in vitro and in vivo conditions, including on aquatic organisms using a range of parameters [11–18], other studies have suggested that it has no biological effects under different experimental conditions [19,20]. Furthermore, investigations of the potential interaction of C_{60} with other contaminants have been very limited. Following 2 months of stirring in water, Baun et al. [21] suggested that 85% of phenanthrene, a model environmental toxicant, sorbed to C_{60}-aggregates and increased C_{60} toxicity in algae (*Pseudokirchneriella subcapitata*) and freshwater crustaceans, *Daphnia magna*. A preliminary study by Yang et al. [22] reported the effects of suspended C_{60} 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 C_{60} structure to cross cell membranes. The authors further suggested that interactions between C_{60} and fluoranthene decrease both the uptake rate and increase the elimination rate for fluoranthene.

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

In the current study, the role of C_{60} in contaminant delivery, and the potential effects of the interaction between C_{60} and fluoranthene on the living organism, was investigated using the filter feeding bivalve mussels, *Mytilus* sp. as a model organism. Mussels are known to exhibit measurable biochemical and behavioural endpoints 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 and 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 μm) seawater. The stock C_{60} suspensions (1 and 10 mg 10 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 0.10 and $1.0\,mg\,l^{-1}$ 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 C_{60} ($100\,mg\,l^{-1}$) 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 C_{60} was controlled by determining the element composition of discrete C_{60} particles using energy-dispersive X-ray spectroscopy (EDX) and TEM (Philips Tecnai F20). Samples were prepared by drop deposition of C_{60} suspensions ($10\,mg\,l^{-1}$) onto freshly cleaved muscovite (for AFM) and 300 mesh Cu-formvar grids (for TEM). The C_{60} 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 three 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 *Mytilus edulis*, whereas those found in the Mediterranean are *Mytilus 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 [28–30].

After collection, animals were immediately transported (in a cool box) to the laboratory where they were maintained in tanks under controlled conditions to acclimatise 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 ($0.04 \pm 0.02\,mg\,l^{-1}$), temperature ($15 \pm 1$ °C) and salinity (31.5 ± 0.15‰) (Multi 340i/SET; WTW, Weilheim, Germany). A photoperiod of 12 h light: 12 h dark was maintained throughout.

2.5. Exposure conditions

The experiments were divided into three short-term (3 days) 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