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Integrated biological responses and tissue-specific expression of *p53* and *ras* genes in marine mussels following exposure to benzo(α)pyrene and C₆₀ fullerenes, either alone or in combination

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Abstract

1 We used the marine bivalve (*Mytilus galloprovincialis*) to assess a range of biological or
2 biomarker responses following exposure to a model engineered nanoparticle (ENP), C₆₀
3 fullerene, either alone or in combination with a model polycyclic aromatic hydrocarbon (PAH),
4 benzo(α)pyrene [B(α)P]. An integrated biomarker approach was used that included: (a)
5 determination of ‘clearance rates’ (a physiological indicator at individual level), (b)
6 histopathological alterations (at tissue level), (c) DNA strand breaks using the comet assay (at
7 cellular level) and (d) transcriptional alterations of *p53* (anti-oncogene) and *ras* (oncogene)
8 determined by real-time qPCR (at the molecular / genetic level). In addition, total glutathione
9 (tGSH) in the digestive gland was measured as a proxy for oxidative stress. Here we report that
10 mussels showed no significant changes in ‘clearance rates’ after 1 day exposure, however
11 significant increases in ‘clearance rates’ were found following exposure for 3 days.
12 Histopathology on selected organs (i.e. gills, digestive glands, adductor muscles and mantles)
13 showed increased occurrence of abnormalities in all tissues types, although not all the exposed
14 organisms showed these abnormalities. Significantly, increased levels of DNA strand breaks
15 were found after 3-day exposures in most individuals tested. In addition, a significant induction
16 for *p53* and *ras* expression was observed in a tissue and chemical-specific pattern, although
17 large amounts of inter-individual variability, compared to other biomarkers, were clearly
18 apparent. Overall, biological responses at different levels showed variable sensitivity, with
19 DNA strand breaks and gene expression alterations exhibiting higher sensitivities. Furthermore,
20 the observed genotoxic responses were reversible after a recovery period, suggesting the ability
21 of mussels to cope with the toxicants C₆₀ and/or B(α)P under our experimental conditions.
22 Overall, in this comprehensive study, we have demonstrated mussels as a suitable model marine
23 invertebrate species to study potential detrimental effects induced by possible genotoxicants and
24 toxicants, either alone or in combinations at different levels of biological organisation (i.e.
25 molecular to individual levels).

26

1 **Introduction**

2 The aquatic environment is often the ultimate recipient of an increasing range of anthropogenic
3 contaminants, many of which are potentially genotoxic and carcinogenic [1, 2]. Furthermore,
4 contaminants in the environment are present in all probable combinations. In recent years
5 therefore, environmental policies have recognised ‘mixture effects’ as a major issue in risk
6 assessment [3]. For example, the European Union (EU) is reviewing approaches for
7 environmental risk assessment which could take into account systematic mixture considerations
8 [4]. In this context, there has been considerable regulatory concern with respect to the presence
9 of contaminants, which are known to be carcinogenic, mutagenic and reproductive toxicants, so
10 called ‘CMR’ under the Water Framework Directives (WFD) of the EU [5]. Such sub-lethal
11 biological responses, which are inherently linked, could also have long-term effects on
12 environmental sustainability [1,2]. Apart from ubiquitous pollutants such as polycyclic aromatic
13 hydrocarbons (PAHs), other contaminants (e.g., metals, organometallics or other legacy and
14 emerging organic pollutants) are also known to induce a range of negative biological responses
15 in aquatic organisms [6]. Organisms exposed to complex mixtures of different substances can
16 interact in many ways (e.g. additively, synergistically or antagonistically) to induce biological
17 responses. The interactions between compounds can potentially change the responses compared
18 to single compound exposures [7, 8]. In this context, for total carcinogenic/mutagenic risk,
19 several researchers have simply added the risk contributions (potency \times dose) from the most
20 important carcinogens (e.g. PAHs) present in inhaled air. In some cases this additive model
21 could be justified, but chemical-chemical multiplicative synergistic action reveals that the
22 research is conspicuously incomplete [3]. This needs further elaboration to elucidate more
23 realistic exposure scenarios applicable to the environment. An integrated approach is therefore
24 required to assess the biological response at different levels of biological organisation.

25 It is well established that various environmental contaminants including the PAH
26 benzo(α)pyrene [or B(α)P], nanoparticles and metals can induce a series of responses in marine
27 mussels, *Mytilus sp.*, at different levels of biological organisations [5,9–11]. Emerging new
28 molecular technologies have raised our expectations to elucidate the potential interactive effects

1 of environmental contaminants under different exposure scenarios (e.g. chronic and acute). In
2 this context, our previous study has suggested that gene expression patterns of *p53* and *ras* can
3 present tissue-specific changes after exposure to B(α)P [9]. Limited available information on
4 aquatic organisms also suggests that these tissue expression patterns could also be influenced as
5 a function of seasonal variation [12]. Whilst these molecular approaches provide the opportunity
6 to investigate responses to mixed chemical exposures, including engineered nanoparticles
7 (ENPs), which are being increasingly manufactured and released in the environment [13,14],
8 these need further validation and elaboration, before they can be successfully employed for
9 environmental hazard and risk assessments.

10 Fullerenes, a family of carbon allotropes in the shape of a hollow spheres are one of the most
11 ubiquitous ENPs with C₆₀ being the most prevalent. C₆₀ fullerenes are released into the
12 environment through wastewater discharges [15, 16] or to the atmosphere through combustion
13 of common fuels [17]. In common with PAHs, they have been detected in river water, surface
14 sediments and soils as well as on aerosols from the sea atmosphere [18–20]. A number of
15 studies have indicated that C₆₀ can potentially cause cellular damage by inducing oxidative
16 stress [21]. They have been shown to be able to cross cellular membranes and could be
17 preferentially localized to organelles [22 - 24]. Another consideration concerning C₆₀ is the
18 potential interactive effects between suspended C₆₀ and other aquatic pollutants. This potential
19 vector-function of C₆₀ may be a significant factor, when considering their environmental effects
20 due to possible interaction with other anthropogenic contaminants. In this context, apolar
21 contaminants (e.g. PAHs) as well as polar contaminants (e.g. pesticides) have demonstrated a
22 strong sorption to suspended fullerenes, suggesting that their combined presence in the
23 environment might affect their fate, availability, exposures and consequently the biological
24 effects [25–29]. The limited information available on the environmental levels of C₆₀ ranging
25 from pg/L to low ng/L [15, 16, 19], however, makes it difficult to estimate ecologically relevant
26 concentrations of C₆₀.

27 In the backdrop of the above information, to determine an holistic assessment, an integrated
28 approach was employed in this study to evaluate the biological responses at different levels of

1 biological organisation in *Mytilus* sp. following exposure to B(α)P and C₆₀ either alone or in
2 combination. This assessment included biochemical, molecular, cellular as well as physiological
3 evaluation at the whole animal level. Whilst the genotoxic effects were evaluated using single
4 cell gel electrophoresis or the comet assay (cellular level), histopathology of specific organs (i.e.
5 tissue level effects) and ‘clearance rate’ as a measure of physiological effects (individual or
6 organism level). Tissue specific transcriptomics expression of key tumour-related genes (i.e *p53*
7 and *ras*) as genetic or molecular responses and total glutathione (tGSH) content level in
8 adductor muscle (at biochemical level) were also selected to indicate the potential oxidative
9 stress. To complement the biological responses, organ specific accumulation of C₆₀ fullerenes
10 and B(α)P in water samples were also determined.

11 **Materials and methods**

12 *Experiment design*

13 The overall experimental design has been presented in Figure.1. Briefly, mussels were collected
14 at Trebarwith Strand, North Cornwall, a pristine / reference site. Mussel collection and
15 maintenance (15 °C) procedures have been described in detail in previous publications from our
16 laboratory [5, 9, 24, 30]. Prior to exposure, haemocyte viabilities from all the experimental
17 scenarios mussels were checked using the Trypan Blue assay to ensure that the cells are in
18 healthy conditions. The exposure vessels were 12 L glass tanks, each containing 10 L of
19 seawater (filtered to 10 μ m) and containing ten mussels. The tanks were aerated to maintain the
20 water quality (i.e. pH, salinity, oxygen, ammonia) which was checked daily during the
21 experimental period and was found to be within the expected range.

22 Appropriate volumes of B(α)P dissolved in acetone were added to the seawater to yield a
23 nominal concentration of 56 μ g/L with a final acetone concentration of 0.01% (v/v). This
24 selected B(α)P concentration (56 μ g/L) has been previously found to induce biological
25 responses in mussels by us [9] and by other workers [31]. The stock suspension of C₆₀ in
26 seawater was prepared in an ultra-sonication bath (35 kHz frequency, Fisherbrand FB 11010)
27 for 2 h prior to the start of the experiment to ensure the C₆₀ was thoroughly suspended and was
28 added to seawater to yield a final concentration of 1 mg/L with minimal ageing. This

1 concentration of C₆₀ was adopted from our previous study [24] which was found to induce a
2 series of responses in this species of mussels. The combined dosing of both compounds was 56
3 µg/L B(α)P and 1 mg/L C₆₀ (Fig 1). The same concentrations of chemicals were re-dosed 1 h
4 after the seawater was changed on daily basis and mussels were fed (2 h) every day prior to
5 water change during 3 days of exposure [30]. After exposure, the seawater was changed and
6 mussels were fed daily (20 min before the complete water change) for another 3 days without
7 any addition of chemicals to allow the mussels to recover. In addition to water quality
8 parameters, the tanks were checked for mortalities on a daily basis during the entire experiment.

9 *Characterisation of C₆₀ nanoparticles*

10 The aqueous fullerene aggregates came from a batch already characterised in our previous work
11 [24]. As there have been very limited information with respect to characterisation of commercial
12 C₆₀, a broad range of analytical approaches were adopted to the concentrated stock suspension
13 (10 mg/L in filtered seawater). Samples (in triplicates) were analysed for hydrodynamic
14 diameters, polydispersity index, zeta potential along with the purity of the samples in terms of
15 element composition of discrete C₆₀ particles. In addition, shapes and sizes of the particles using
16 transmission electron microscopy were also determined as described in detail elsewhere [24].

17 *Determination of B(α)P concentration in water samples by GC-MS*

18 The B(α)P analysis is based on a protocol previously developed and validated by us [24].
19 Briefly, water samples (9 cm³) were collected into glass vials and dichloromethane (1 cm³,
20 HPLC grade, Rathburn Chemicals Ltd., UK) was added. Phenanthrene d10 (1.1 µg in 10 µL
21 dichloromethane) was then added as an internal standard. Following thorough shaking, the
22 mixtures were stored in the dark at 4 °C. Immediately prior to analyses, the dichloromethane
23 layers were removed into glass micro-vials. 2 µL aliquots of the sample extracts were analysed
24 using an Agilent Technologies 6890 N Network GC system interfaced with an Agilent 5973
25 series Mass Selective detector. A DB-5MS (crosslinked 5% phenyl methyl siloxane) capillary
26 column (30 m) with a film thickness of 0.25 µm and internal diameter 0.25 mm was used for
27 separation, with helium as a carrier gas (maintained at a constant flow rate of 1 mL/min).
28 Extracts were injected splitless, with the injector maintained at 280 °C. The oven temperature

1 programme was 40 °C for 2 min and then increased at 6 °C/min to a final temperature of 300 °C,
2 where it was held for 4 min. The mass spectrometer was operated in electron impact mode (at
3 70 eV) with the ion source and quadrupole analyser temperatures fixed at 230 °C and 150 °C,
4 respectively. Samples were screened for B(α)P and phenanthrene d10 using selected ion
5 monitoring, in which the target ions were 252 and 188 respectively. Full scan GC-MS was
6 performed for confirmational purposes. Prior to sample extract analyses, the system was
7 calibrated using authentic standards. With each batch of samples, a solvent blank, a standard
8 mixture and a procedural blank were run in sequence for quality assurance purposes. B(α)P
9 concentrations were calculated based on the internal standard.

10 ***Determination of C₆₀ concentrations in tissue samples by Liquid Chromatography (LC-UV)***

11 The analysis of C₆₀ concentrations in tissues was thoroughly validated and has been previously
12 reported [24]. Adductor muscle, digestive gland and gill tissues were dissected from individual
13 mussels exposed to C₆₀ only and were carefully washed with pure toluene (HPLC grade,
14 Rathburn Chemicals Ltd., UK) to remove C₆₀ particles adsorbed to the surfaces of the organs.
15 Tissues were then treated by ultrasonic assisted extraction in toluene (1 cm³) for 15 min and
16 centrifuged at 9000 rpm. The HPLC method was developed for C₆₀ analysis using a Hypersil
17 Elite C18 (250×4.6 mm I.D., 5 μm) column. The mobile phase was toluene (HPLC grade) at a
18 flow-rate of 1.0 mL/min. Sample injections were performed manually with volumes of 100 μL.
19 The UV detector was set at a 330 nm wavelength (Shimadzu SPD-6 AV, Shimadzu, Germany).
20 Integration was performed using a Shimadzu-C-R3A Chromatopac data processor (Shimadzu,
21 Germany). The C₆₀ response was externally calibrated.

22 ***Determination of clearance rate, histopathological effects and DNA strand breaks***

23 A total of 6 mussels were collected from each treatment at each sampling day. Clearance rate,
24 histopathological effects and DNA strand break were analysed as described by us in previous
25 publications [9,10, 24].

26 For clearance rate, briefly, mussels were allowed to acclimatise until their valves opened
27 (approximately 10 min) prior to the addition of 500 μl of *Isochrysis* algal suspension (supplied
28 by Cellpharm Ltd., Malvern, UK). The algae were mixed manually with a glass rod and then 20

1 ml of water sample was removed using a glass syringe. This procedure was repeated again after
2 20 min. Samples from both time zero and 20 min were analysed using a Beckman Coulter
3 Particle Size and Count Analyser (Z2) adjusted to count particles between 4.0-10.0 μm in
4 diameter. Clearance rate of the mussels were calculated as described elsewhere in detail [10,32].

5 For histopathological analyses, tissues dissected from exposed animals (i.e. adductor muscle,
6 digestive gland, gills and mantle) were examined by normal histological methods [9, 10, 24].
7 Each organ was initially fixed in 10% buffered formal saline for at least 48 h. Specimens were
8 then processed in ascending grades of alcohol. Tissue samples were embedded into paraffin and
9 cut with a microtome at 5-7 μm thickness and mounted on slides. Slides were stained with
10 haematoxylin and eosin (H and E) following Mayer's standard protocols. It is to be mentioned
11 that due to a shortage of tissue samples no histopathological analysis could be applied to
12 mussels exposed to C_{60} only in this study.

13 For the determination of DNA Strand breaks, alkaline single cell gel electrophoresis or comet
14 assay was used. Single strand breaks in the haemocytes were determined using a standard assay
15 as described elsewhere [9,10,24,32]. Briefly, haemolymph (200 μL) samples were obtained
16 from the posterior adductor muscle from individual mussels and centrifuged at $9600 \times g$ for 2
17 min. The supernatant was discarded and replaced with 200 μL 0.75% (w/v) low melting point
18 agarose. The mixture was then applied as two gel-drops (100 μL) to the slides which were pre-
19 coated with 1.5% normal melting agarose 24 hours in advance. Coverslips were placed over
20 each gel-drop and gels were allowed to solidify at 4 $^{\circ}\text{C}$ for 1 h. The slides were then immersed
21 in cold lysis solution [2.5 M NaCl, 100 mM EDTA, 10 mM Tris base, 1% N-Lauroyl-sarcosine,
22 1% Triton X 100, 10% DMSO, pH=10] for 1 h to remove membranes and histones from DNA.
23 After the lysis period, slides were placed in a horizontal electrophoresis unit (TS-COMET-RB,
24 Thistle Scientific, Norway) containing freshly prepared electrophoresis buffer [0.3 M NaOH, 1
25 mM EDTA, pH>13]. The DNA was allowed to unwind for 30 min to denature before
26 electrophoresis proceeded at 25 V for 30 min. The slides were then removed from the
27 electrophoresis tank and gently immersed in neutralization buffer [0.4 M Tris base, pH=7.5] to
28 rinse (3 times) before drying overnight for visualization. The level of DNA damage in 100 cells

1 sample⁻¹ was measured by Komet 5.0 Image Analysis System (Kinetic Imaging, Liverpool, UK)
2 using an epifluorescence microscope (Leica, DMR). Data for % tail DNA are presented as a
3 reliable measure of single-strand DNA breaks/alkali labile sites [33].

4 ***Determination of tGSH level in adductor muscle extractions***

5 The posterior adductor muscles from three mussels (0.2 g wet weight), collected both after 3
6 days exposure and 3 days recovery, were dissected and were homogenized using the method as
7 described by Al-Subiai et al. [34]. Briefly, the tissues were ground with acid-washed sand (0.5 g)
8 using ice-cold extraction buffer [20 mM Tris-chloride, pH=7.6, containing 0.15 M KCl, 0.5 M
9 sucrose and 1 mM EDTA, freshly supplemented with 1 mM DTT and 100 μ L protease inhibitor
10 cocktail (Sigma-P2714; reconstituted according to the manufacturer's instructions)] using a
11 ratio of 1:3 (w/v). The crude homogenate was centrifuged for 35 min ($10,500 \times g$ at 4 °C) and
12 then the supernatant was separated and stored at -80 °C until use.

13 The tGSH [i.e. reduced: GSH, and oxidised: glutathione disulphide (GSSG)] content in adductor
14 muscle extract was determined as described by Al Subiai et al. [34]. Samples were treated with
15 5-5'-Dithio-bis (2-nitrobenzoic acid) (DTNB) by mixing at a 1:1 ratio with buffered DTNB (10
16 mM DTNB in 100 mM potassium phosphate, pH=7.5, containing 5 mM EDTA). Potassium
17 phosphate (100 mM, 235 μ L, pH=7.5, containing 5 mM EDTA) and glutathione reductase (0.6
18 U, Sigma G-3664 from *Saccharomyces cerevisiae*) was mixed with DTNB-treated samples (40
19 μ L). After equilibration for 1 min, the reaction was started by the addition of 60 μ L 1 mM
20 NADPH. The rate of absorbance decrease at 412 nm was measured over 5 min. A 20 μ M GSH
21 standard and a blank were used to calibrate the results. tGSH contents were measured in
22 triplicate in 96 well plates using a microplate reader (Optimax, Molecular Devices, Sunnyvale,
23 CA).

24 ***Gene expression analyses***

25 Haemolymph and tissues, including digestive gland, adductor muscle, mantle and gill, were
26 collected from a total of 6 mussels from each treatment at each sampling day. Total RNA was
27 extracted, cleaned by DNase and reverse transcribed (10 ng of RNA) to cDNA as described in

1 details elsewhere [9]. Real-time qPCR for target genes (*p53*, *ras* and 18S rRNA) was performed
2 in triplicate for each sample as described in previous studies [35, 36]. Details of primers used
3 for each gene and their PCR reaction conditions are provided in Supplementary Table 1. It is
4 important to note that the reference gene (18S rRNA) was chosen on the outcome of the analysis
5 of stability by geNorm qbase^{PLUS} software (geNorm biology, USA) following manufacturer's
6 instruction. The software has been written to automatically calculate the gene-stability (M value,
7 the average pairwise variation of a particular gene) which relies on the principle that the
8 expression ratio of two ideal internal control genes is identical in all samples, regardless of the
9 experimental condition or cell type. Genes with the lowest *M* values have the most stable
10 expression [37]. In our case the reference gene 18S rRNA performed the best over *actin* as
11 previously used elsewhere [9], Relative *p53* and *ras* gene expression was compared to the
12 reference 18S rRNA gene expression. The comparative Ct method based on the comparison of
13 distinct cycle differences was used [9, 38].

14 ***Statistical analyses***

15 Statistical analyses were carried out with the aid of Minitab V15 statistical package (Minitab
16 Inc., USA). Significant differences between untreated control and treated exposed mussels were
17 studied using the Student's t-test and one-way analysis of variance (ANOVA) after testing for
18 normality of the data and homogeneity of variance. All values were provided as means \pm SEM
19 (standard error of mean). Significance was established at $P < 0.05$.

20 **Results**

21 ***C₆₀ nanoparticle characterisation***

22 A summary of the C₆₀ characterisation results is presented in Table 1. Briefly, Dynamic Light
23 Scattering measurements indicated the formation of large and highly polydispersed aggregates
24 (Z-average hydrodynamic diameter of 680 ± 19 nm). Micrographs obtained by transmission
25 electron microscopy showed that the aggregates were composed of distinct particles. Size
26 measurements of discrete particles within the agglomerates on the TEM micrographs and AFM
27 images showed oval particles with diameters in the 100-200 nm range (Table 1). The difference

1 in mean diameter given by TEM (160 nm) and AFM (122 nm) can be explained by the fact that
2 the TEM measures the height along the surface of the grid whilst AFM height measures the
3 particle sizes above the muscovite surface. No significant differences were observed between
4 EDX-spectra acquired on C₆₀ particles and on the background carbon-coated Cu-grid indicating
5 that elemental impurities were below the detection limit of EDX [24].

6 *Chemical analyses using GC-MS and HPLC-UV*

7 Seawater B(α)P concentrations in the exposure tanks were measured at 47±15 µg/L immediately
8 after spiking for 56 µg/L nominal concentrations. The calibration curve for C₆₀ showed a good
9 linear fit for the selected range (R²=0.996). Adductor muscle, digestive gland and gill tissue
10 were dissected from mussels sampled after 3 days C₆₀ (1 mg/L) exposure and after another 3
11 days recovery from C₆₀ exposure in fresh seawater. The analysis of all mussel tissues unexposed
12 to C₆₀ exhibited a very small and repeatable positive signal at the retention time of C₆₀s which
13 was attributed to an interfering co-extractant. The results were subsequently corrected for blanks
14 and showed significant C₆₀ concentrations in all three tissues after 3 days of exposure,
15 confirming the ability of mussels to accumulate C₆₀ in organs (Figure 2). Significantly higher
16 amounts of C₆₀ (14.2 ± 7.2 µg C₆₀/gww) was bioaccumulated in the digestive gland, followed by
17 gill and adductor muscle. After 3 days recovery in fresh seawater, C₆₀ concentrations in the
18 three tissues dropped below the method detection limit (<1.5 µg C₆₀/gww), indicating the C₆₀
19 that had accumulated in each tissue had been bio-transformed and/or excreted from the tissues
20 after this time.

21 *Clearance rate*

22 There was no significant difference in clearance rate after 1 day exposure to any
23 chemical/particles compared to the fresh seawater control (Figure 3). Significant increases in
24 clearance rate were found following exposure to the chemical/particles for 3 days. Mussels
25 showed the most activated feeding behaviour after B(α)P exposure only (about 2-fold increase
26 compared to the fresh seawater control samples) followed by fresh C₆₀. The combination of
27 B(α)P and C₆₀ did not change the physical response of mussels in terms of feeding behaviour.
28 After 3 days recovery from exposure, all mussels showed a further increase in clearance rate

1 including controls (but not significantly) compared to samples collected after exposure. The
2 increase was significant compared to 1 day exposure but not significant in comparison to 3 days
3 exposure, except for samples recovering from the B(α)P and C₆₀ combined exposure.

4 *Histopathological analysis*

5 Histopathological analysis of the adductor muscle, digestive gland, gill and mantle tissues
6 showed no pathological signs in control specimens such as haemocyte infiltration, necrosis or
7 other injuries. However, there were pathological alterations in treated mussel tissues (Figures
8 4.1 and 4.2).

9 *Posterior adductor muscle:* Transverse section of the posterior adductor muscle showed normal
10 histology consisting of muscle blocks, each made of distinct bundles of muscle fibres. The
11 bundles of muscle were surrounded with connective tissue. There was no evidence of
12 haemocyte infiltration, necrosis or other injuries in the controls (Figure 4.1A2). The adductor
13 muscle showed histological abnormalities after B(α)P and B(α)P in combination with C₆₀
14 exposures, i.e. loss of muscle bundle structure, increase in intracellular spaces and decrease in
15 extracellular spaces of connective tissue with an extreme example of complete breakdown of
16 bundles of muscle fibres (Figure 4.1A3).

17 *Digestive gland:* Transverse sections of the digestive gland in controls showed normal
18 structures (several round/oval) lined by columnar epithelia. All the digestive tubules were
19 connected to each other by connective tissue. There was no evidence of haemocyte infiltration,
20 necrosis or other injuries in the digestive gland of control mussels (Figure 4.1B1). Most of the
21 digestive tubules collected after B(α)P exposures showed reduced epithelial cell height and
22 haemocyte infiltration inside the tubules and in surrounding connective tissue. The histological
23 abnormalities after combined exposure showed different features, such as no clear distinction in
24 some epithelial cells and destroyed architecture of digestive tubules. In some extreme cases, the
25 complete breakdown of the epithelium was observed (Figure 4.1B3).

26 *Gill:* The histopathological analysis showed several abnormalities in gills of mussels in
27 comparison between control and exposed conditions. Gills from the control group showed well

1 preserved structures including gill filaments covered with a ciliated epithelium on their external
2 surface, simple frontal cilia, and lateral cilia. The frontal cilia are emerging from the front
3 epithelia, while the lateral cilia are emerging from lateral cells (Figure 4.1C1). Most of the gills
4 from B(α)P treated mussels exhibited injuries featured as swollen gill filaments filled with
5 haemocytes, inflammation and filament necrosis. Most of gills from mussels collected after
6 combined exposure showed abnormalities such as absence of the front epithelial border,
7 hyperplasia in the frontal and lateral cilia, and hypoplasia in the lateral cilia. In addition, pore
8 structures were only found in frontal epithelial of gills dissected from mussels after combined
9 exposure (Figure 4.1C2 and C3).

10 *Mantle:* The histopathological analysis showed normal mantle tissues to contain gonads (testis
11 for male and ovary for female) and connective tissues. Gonads consist of an organized network
12 of branching tubules and appear as follicles. The tubules terminate into a short gonado-duct that
13 opens into mantle cavity (Figure 4.2). There were no significant histological abnormalities in
14 mantle tissue after B(α)P exposure, either alone or in combination with C₆₀.

15 Although histopathological alterations were observed in some tissue samples after exposures to
16 the chemicals, it is worthy to note that not all treated samples exhibited abnormalities. Figure
17 presented here only show the examples of histopathological profiles of tissues in unexposed
18 (control) and exposed groups. The summary of percentage of tissues that showed abnormalities
19 is summarised in Table 2. Increased occurrence of abnormalities was found in all tissues after
20 exposure and a slightly decreased occurrence was also found after recovery in fresh seawater for
21 3 days compared to the 3 days exposure period. There was no difference in percentage of
22 abnormalities induced by the 2 types of exposure. Qualitatively, no tissue showed increased
23 sensitivity to a particular exposure type.

24 **DNA strand break analysis by comet assay**

25 In our previous studies using a range of concentrations of B(a)P, C₆₀ fullerenes and fluoranthene
26 either alone or in combinations, no significant loss of cell viability (as determined by Trypan
27 Blue exclusion assay) were observed [9, 24]. These observations gave us the required
28 information for further experiments. As the amount of haemocytes to be procured from mussels

1 poses restrictions (it is to be noted that in this study we also used haemocytes for gene
2 expression analyses), cellular viability was detected in haemocytes from individual mussels
3 before the exposure to ensure their health status. The results showed no cytotoxicity presented
4 (cell viability > 90%, supplementary Table 2). Results of tail DNA (%) showed no significant
5 increase in DNA strand break after 1 day exposure to B(α)P and/or C₆₀ (Figure 5). Significantly
6 increased DNA strand breaks ($p < 0.05$) were found after 3 days exposure where the highest
7 DNA damage (70% tail DNA) was induced by B(α)P exposure only, followed by C₆₀ only (62%)
8 and surprisingly only a 56% induction of DNA strand breaks for exposure to B(α)P in
9 combination with C₆₀. However, differences in these numerical values are not statistically
10 significant. After 3 days recovery, DNA damage was significantly decreased compared to the 3
11 days exposure samples. However, there was still a significantly increased DNA damage induced
12 by chemicals compared to control conditions.

13 **tGSH analysis**

14 The tGSH level in adductor muscle tissue was measured in mussels sampled after 3 days of
15 exposure to B(α)P and/or C₆₀ (Figure 6). There was an increased level of total glutathione after
16 exposure to chemical treatments. The increase was significant for individual C₆₀ or B(α)P
17 exposed samples but not significant after combined exposure compared to control samples.

18 **Gene expression analyses**

19 *Relative quantification of p53 and ras expression in different tissues:* The relative
20 quantification of *p53* and *ras* expression was normalized in different tissues by 2^{- $\Delta\Delta$ Ct} method [9]
21 using 18S rRNA as the housekeeping gene. Relative expression of 1 was defined as the control
22 level after normalization with housekeeping gene and control. High inter-individual variation
23 was found in all the gene expression results, including *p53* and *ras* expressions in various
24 tissues (Supplementary Figure 1).

25 *Relative expression of p53 gene in different tissues:* In haemocytes, induced *p53* expression
26 was only found after 3 days exposure to B(α)P alone (1.8 \pm 0.3- fold; Fig. 7a). After exposure to
27 C₆₀ alone, significantly increased *p53* relative expression was detected after 1 day of exposure

1 and kept increasing after 3 days exposure, however, the increase was not significant compared
2 to the 1 day exposure. After 3 days recovery from exposure, *p53* relative expression decreased
3 dramatically, but was still significantly higher than control levels (17.2 ± 6.6 - fold; Fig. 7a).
4 After the exposure to combined B(α)P and C₆₀, *p53* expression was significantly induced by
5 17.8 ± 5.6 - fold. The induction increased to 98.4 ± 8.5 - fold after 3 days exposure (Fig. 7a). The
6 induction of *p53* expression after both exposure times was lower compared to C₆₀ exposure
7 alone. The recovery from combined chemicals exposure showed a decline in *p53* relative
8 expression, but was the same as the C₆₀ exposure; the level was still significantly higher than the
9 control (28.4 ± 14.7 - fold; Fig. 7a).

10 Relative expression of *p53* in the digestive gland showed a similar pattern as haemocytes but
11 with a quicker response to C₆₀ exposure (Figure 7b). B(α)P only induced *p53* expression after 3
12 days exposure. The combination of B(α)P and C₆₀ intended to induce more *p53* expression
13 compared to B(α)P alone, about 4.5 ± 0.5 -fold of *p53* expression induced after 1 day exposure
14 and 6.3 ± 1.7 - fold after 3 days exposure. Relative expression of *p53* dropped to control levels
15 after exposure to both B(α)P alone and in combination with C₆₀. *p53* expression responding to
16 C₆₀ exposure showed a different pattern. Significantly increased *p53* expression (over a
17 thousand-fold) was found after 1 day exposure. This level dramatically decreased to $32.0 \pm$
18 15.0 - fold after 3 days exposure. After recovery, the level continued decreasing but was still
19 higher than control level.

20 Relatively higher *p53* expression was induced in mantle compared to the other tissues (Figure
21 7c). After B(α)P exposure alone, 6.3 ± 5.2 - fold of *p53* expression was induced after 1 day
22 exposure. The induction increased to 283 ± 157 - fold after 3 days exposure. No induction of *p53*
23 expression was detected after 3 days recovery. After C₆₀ exposure alone, significantly increased
24 *p53* expression was detected after 1 day exposure. The level was similar after a longer exposure
25 time (3 days) but returned to control levels after recovery. Unlike haemocytes and the digestive
26 gland, the combined exposure showed the ability to induce more *p53* expression in mantle tissue.
27 A significant induction about 3515 ± 2491 - fold of *p53* was detected after 1 day exposure.

1 Further increased *p53* expression was induced after 3 days exposure. After recovery, induced
2 *p53* expression decreased but was still significantly higher than the control level.

3 In the adductor muscle, a similar *p53* expression pattern was found (Figure 7d) compared to the
4 mantle. After B(α)P exposure alone, significant induction of *p53* was found after 1 day of
5 exposure. No induction of *p53* expression was found after both 3 days exposure and 3 days
6 recovery. For C₆₀ exposure alone, highest induction of *p53* expression was shown after 1 day
7 exposure, less but still significantly induced *p53* expression was found after 3 days exposure.
8 The level was similar after recovery compared to 3 days exposure. The combined exposure of
9 B(α)P and C₆₀ induced significantly *p53* expression after 1 day exposure. Decreased expression
10 was found after longer exposure and no induction of *p53* expression after 3 days recovery.
11 Similar to mantle tissue, combined chemical exposure induced more *p53* expression compared
12 to single chemical exposure in adductor muscle (Figure 7d).

13 The relative expression pattern of *p53* in gill tissue after exposure was similar to digestive gland
14 and haemocytes (Figure 7e). There was no induction of *p53* relative expression after B(α)P
15 exposure alone at any sampling time. Induced expression was found after 1 day exposure to C₆₀
16 alone and the level increased after 3 days exposure. *p53* expression decreased after recovery.
17 However, there was no significant difference in *p53* expression when comparing different time
18 points after C₆₀ exposure due to high variability between replicates. The combined exposure
19 induced *p53* expression after 1 day exposure. The level dropped after 3 days exposure but
20 increased slightly after recovery. The differences in *p53* expression for different time points
21 were still not significant (Figure 7e).

22 ***Relative expression of ras gene in different tissues:*** The relative expression of *ras* in
23 haemocytes (Figure 8a) showed a different trend and pattern compared to *p53* expression. There
24 was no up-regulation of *ras* expression at any sampling time after any treatment. Similar results
25 were found in gill (Figure 8e), where *ras* relative expression remained at control levels after all
26 the treatments. In the digestive gland (Figure 8b), no significant induction of *ras* expression was
27 found after B(α)P exposure alone. After C₆₀ exposure alone, *ras* relative expression was
28 significantly induced after 1 day of exposure (108 \pm 19- fold). The level dramatically decreased

1 after 3 days exposure but returned back to the control level after 3 days recovery. The combined
2 exposure also induced *ras* expression after 1 day exposure; however, the level (3.1 ± 0.2 - fold)
3 was significantly lower than C_{60} exposure alone. A decline of *ras* expression was found after 3
4 days exposure and remained at a similar level after 3 days recovery (Figure 8b).

5 Relative expression of *ras* in mantle (Figure 8c) showed a different pattern compared to
6 digestive gland, but similar to *p53* expression in the same tissue as mentioned earlier. There was
7 no significant induction of expression after 1 day exposure to B(α)P exposure alone, but the
8 level increased dramatically (73.2 ± 40.2 - fold) after exposure to B(α)P for 3 days. After 3 days
9 recovery, no induction of *ras* expression was found in mantle. There was no induction of *ras*
10 expression after C_{60} exposure only, after all treatments in this tissue. The combined exposure of
11 B(α)P and C_{60} showed the ability to induce significant *ras* expression. After 1 day exposure,
12 over a 200- fold increase in *ras* expression was found. The level increased to over 4000- fold
13 after 3 days exposure to the combined chemicals. Unlike other tissues, *ras* expression in mantle
14 remained at a relatively higher level (221 ± 220 -fold) after recovery compared to control.

15 Expression of *ras* gene in the adductor muscle showed a similar expression pattern to mantle
16 tissue but with quicker response times (Figure 8d). Expression was induced after 1 day exposure
17 to B(α)P alone and then the level dropped to the control level after 3 days exposure and
18 remained at a similar level after 3 days recovery. There was no induction of *ras* expression after
19 C_{60} exposure alone at any sampling time in this tissue (Figure 8d). The expression level
20 remained slightly below the control level. The combined exposure significantly induced *ras*
21 expression after 1 day exposure, approximately 647 ± 424 - fold. After 3 days exposure, the
22 induction decreased to 30 ± 28 - fold higher than the control and decreased to similar to the
23 control level after 3 days recovery in fresh seawater (Figure 8d).

24

25 **Discussion**

26 ***Determination of B(α)P concentration by GC-MS and C_{60} concentration by LC-UV***

27 The measured concentrations of B(α)P in the water samples were on average 16 % lower than

1 nominal, in agreement with B(α)P's low solubility in seawater [9]. Regarding C₆₀ analyses, the
2 digestive gland was found to accumulate more C₆₀ after exposure compared to the other two
3 tissues. This was not surprising given that its main function is to digest absorbed compounds.
4 C₆₀ tissue concentrations after the 3 days recovery period in fresh sea water suggest that all three
5 tissues are able to metabolise or excrete the C₆₀ back to control levels. This ability appears to
6 exhibit a tissue-specific pattern which is consistent with previous studies e.g. different
7 concentrations of C₆₀ were recorded in rat tissues after tail vein administration [39]. However,
8 the exact mechanism of how each tissue at whole organism level functions to metabolise,
9 excrete or eliminate C₆₀ remains unknown. Several studies aimed to determine the interactions
10 between nanoparticles and tissues have been performed using transmission electron microscopy
11 (TEM) or confocal laser scanning microscopy (CLSM). These studies have shown diffusion and
12 localization of selected nanoparticles [e.g.: TiO₂, poly (D, L-lactide-co-glycolide) nanoparticles]
13 into cells at different sites [40–42]. However, measuring the interaction between absorbed C₆₀
14 and *Mytilus sp.* tissues following exposures using microscopic techniques is technically very
15 challenging because it is not possible to distinguish between C₆₀ and naturally occurring carbon
16 present in the cells/tissues or with cell structure within the same size range as C₆₀/C₆₀ aggregates
17 [43].

18 **Clearance rates**

19 In general, the largest increase of clearance rate was for B(α)P alone followed by C₆₀ alone.
20 Surprisingly, the lowest stimulation of feeding was from the combined treatment. Variability in
21 the measurements is, however, quite substantial. The explanation for enhanced feeding activities
22 in treated groups might relate to higher energy demands and metabolic activities required to deal
23 with accumulated chemicals in mussels meaning that the mussels feed more to get the energy to
24 maintain metabolic activities [44]. Following the recovery period, clearance rate / feeding
25 activity increased further and whilst quite variable, were quite similar, although was highest for
26 the combined mixture. It is to be noted, however, that after the 6 days, clearance rates also
27 marginally increased in the control as well, possibly indicating that an unknown environmental
28 variable may have contributed to the increase in feeding.

1 **Histopathological alterations**

2 Histopathology indicated physiological changes in the mussel tissues following exposure.
3 Mussels exposed to B(α)P and B(α)P with C₆₀ tended to exhibit more tissue damage compared
4 to unexposed mussels. The observations are in accord with previous studies [9]. Overall, the
5 histopathological observations provide evidence for the toxic effects of B(α)P and C₆₀ which
6 can cause tissue abnormalities even at these exposure scenarios. These tissue abnormalities
7 could then lead to suppression of immune function over time and subsequently development of
8 pathophysiological conditions such as neoplasia in the natural environment [45]. It is important
9 to note that no samples after C₆₀ exposure alone were examined for histopathological analysis
10 due to the tissues being specifically preserved for C₆₀ concentration analyses rendering them
11 unsuitable for histopathology. It has been reported previously by our group that C₆₀ is able to
12 cause tissue abnormalities following exposure to the concentration used in the present study
13 [10]. In addition, after exposure to both B(α)P and C₆₀, gill tissues showed different
14 abnormalities in comparison to B(α)P alone with pore structures observed in frontal cilia of gill.
15 This observation has also been reported previously with the suggestion that it could be either the
16 structure of nanoparticles themselves after their accumulation in the tissues or due to their
17 accumulation in the tissues [46].

18 **DNA strand breaks**

19 DNA strand breaks measured by comet assay reflect the degree of DNA damage and also can be
20 influenced by factors such as cellular viability [47]. This suggested that haemocytes collected
21 from the experimental mussels were in the healthy condition. Significant increases in DNA
22 strand breaks were observed after 3 days exposure to the chemicals. The highest level of DNA
23 damage was induced by B(α)P alone followed by C₆₀ alone and then by the combined exposure
24 of chemicals. There was no significant DNA damage after 1 day of exposure. This probably
25 suggests that organisms take time to switch on the essential machinery to manifest the
26 detrimental effect, i.e. the induction of DNA damage, after exposure to xenobiotics. It is
27 interesting that the mixture actually induces slightly less damage than the individual exposures.
28 After 3 days recovery, DNA damage was significantly less compared to 3 days exposure. This

1 suggests the involvement of DNA repair processes. It is also likely that the damaged cells are
2 replaced by cellular proliferation or through apoptosis [48]. The replacement of damaged cells
3 by newly generated cells could therefore also dilute the observed responses. The results
4 confirmed that, similar to B(α)P, C₆₀ can induce DNA strand breaks, which is consistent with
5 previous studies [49].

6 **Total glutathione levels**

7 It has been reported that the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent
8 metabolism of B(α)P in the digestive gland of marine mussels results in the production of
9 hydroxyl and superoxide anion radicals, which are extremely potent oxidants and capable of
10 reacting with critical cellular macromolecules, including DNA and proteins [50,51]. C₆₀, itself,
11 has also been reported to generate oxidative stress in cells [52]. Therefore, biomarkers which
12 can indicate the ability of cells to cope with oxidative stress are required for analysis of C₆₀
13 induced responses in organisms. It is well documented that total glutathione (tGSH), including
14 both reduced and oxidized forms, is widely distributed among living cells and participates in
15 essential aspects of cellular homeostasis [53]. Cell injury induced by electrophiles was long
16 believed to be the mere result of alkylation of cellular macromolecules by their reactive
17 metabolites [54]. Several studies, however, highlight that, in some instances, most of the cell
18 injury occurs after total GSH depletion and may actually depend on the onset of extensive,
19 uncontrolled oxidative processes [55–57]. Our results show that C₆₀ or B(α)P alone can increase
20 the glutathione level, suggesting that antioxidant defences have been switched on in response to
21 pollutant exposure by generating more glutathione. This result is consistent with previous
22 studies where up-regulation of glutathione has been correlated with increased burden in the
23 bivalve *Perna viridis* [50]. Although both C₆₀ and B(α)P are able to induce oxidative stress in
24 mussels, the interaction between these two chemicals did not lead to higher glutathione levels
25 compared to the single chemical treatments.

26 In this study we measured total glutathione levels to detect oxidative stress induction. It is being
27 also suggested that total glutathione concentration is not sufficient to provide a holistic picture
28 of oxidative stress and should be accompanied by measurement of oxidized glutathione. It is to

1 be noted that glutathione is widely distributed in living cells and composed of reduced and
2 oxidized forms. In healthy cells, oxidized GSH (GSSG) can be produced when the oxidative
3 stress increases, and it can be further catalyzed into reduced GSH by glutathione reductase.
4 Therefore, by measuring the total glutathione and GSSG concentration and calculating the ratio
5 of reduced/oxidized GSH, can directly indicate the oxidative stress. However, total GSH dose
6 not only circulate between its two forms, it can also be the substrate in xenobiotics metabolism
7 (both phase I and phase II reactions) catalyzed by GSH-S-transferase. Glutathione is consumed
8 to maintain cells in a reduced condition. Consequently, glutathione levels are expected to be
9 changed in cells at different health stages.

10 In this study, there were two reasons for looking solely at total glutathione rather than
11 measuring both the reduced and oxidised forms, and looking at the ratio. The first is the
12 accurate measurement of GSSG requires relatively large amount of cell samples, usually pooled
13 haemolymph samples from several mussels to reduce statistical errors. In this study,
14 haemolymph collected has been used to assess DNA strand breaks, tGSH concentration and
15 gene expression. Insufficient sample was available for additional oxidative GSH concentration
16 analysis. The second reason relates to the level of oxidative stress that is expected. If the level of
17 oxidative stress is low then the expectation is that there will be a response by the organism to
18 up-regulate antioxidant defences, one possibility being to increase the synthesis of glutathione
19 (as shown here, and elsewhere, in mussels). Under these circumstances, where the organism is
20 able to deal with the oxidative stress, it would be expected that there would be little change in
21 the GSH:GSSG ratio, and hence little to be gained from separate measurements of GSH and
22 GSSG, but the increase in total glutathione is clear evidence in itself of oxidative stress. At
23 higher levels of oxidative stress then, depression of the GSH:GSSG ratio may occur, followed
24 by transport of GSSG out of cells in an attempt to maintain the ratio, in which case there will be
25 a decline in total intracellular glutathione. If total glutathione levels are below control levels
26 (which is not the case here) then it might be worth looking at the GSH:GSSG ratio to confirm
27 that it is depressed.

1 **Gene expression**

2 *Expression of p53 and ras genes in different tissues in untreated organisms*

3 The expression abundance of *p53* and *ras* in different tissues under control conditions were
4 analysed before the relative expression analysis was carried out to take into account the
5 expression of the housekeeping genes (i.e. 18S rRNA). As expected, the expression abundance
6 trend of results for *p53* and *ras* after normalization with 18S was the same as the *actin*
7 normalized results [9]. *p53* tends to be more expressed in the digestive gland, followed by gill,
8 mantle and adductor muscle tissues. *ras* was expressed at similar levels in the digestive gland,
9 gill and adductor muscle and less in mantle tissue. These results confirm that the change in
10 housekeeping gene for normalisation does not affect the normalization results and make the
11 results comparable to the previous study.

12 *Relative expression of p53 and ras in haemocytes following exposure to B(α)P and/or C₆₀*

13 In bivalves, the haemocytes are responsible for cell mediated immunity through phagocytosis
14 and various cytotoxic reactions [58]. In the marine mussels, haemocytes have been shown to
15 represent a sensitive target for a number of environmental contaminants, including heavy metals
16 and organic xenobiotics, with consequent immunotoxic effects or stimulation of immune
17 parameters, leading to inflammation, depending on the compound and on the conditions of
18 exposure [59,60]. In particular, changes in lysosomal membrane stability and phagocytosis, and
19 stimulation of lysosomal enzyme release and oxyradical production have been observed in
20 response to different contaminants. Many of these effects are known to be due to interference
21 with components of the signalling pathways involved in activation of the immune response [61,
22 62]. Therefore, analysis of the expression of key genes in haemocytes will represent the generic
23 genetic response of mussels to environmental contaminants.

24 The increased expression of *p53* in haemocytes after exposure to B(α)P and/or C₆₀ confirmed its
25 function in DNA repair and cell cycle related process. The reduced level of expression after
26 recovery in all the treatment suggests that mussels are able to cope with the applied exposure
27 concentration because there is no need for more *p53* to be expressed. The damaged DNA has

1 either been repaired and cells are allowed to pass through the cell cycle checkpoint, or the
2 damage cannot be repaired and has led the cell to the apoptosis pathway. These results are
3 closely related to the DNA strand break results, where less DNA damage has been found after
4 recovery. However, higher *p53* expression after exposure to B(α)P in combination with C₆₀ was
5 found with no significantly induced DNA damage compared to B(α)P exposure alone,
6 suggesting that potentially more B(α)P was delivered through the combination[23]. It is also
7 possible that DNA strand breaks detected by comet assay cannot cover all types of DNA
8 damage induced by exposure as reported by Canesi et al [63], or *p53* is involved in a common
9 signalling pathway which can sense a wide range of stress, apart from DNA damage [64].
10 Therefore, *p53* expression was induced in response to DNA repair rather than DNA strand
11 breaks. A higher *p53* was induced after C₆₀ exposure alone compared to the other two exposures.
12 This could be attributed to haemocytes being either more sensitive to C₆₀ or to the combination
13 with B(α)P which can protect cells from the toxic effects of C₆₀ alone. This might occur by
14 changing the structure or acting as radical scavengers [39, 46]. Information about the
15 mechanisms of how organisms process nanoparticles after absorption is however limited. *p53*
16 expression in haemocytes collected from combined and C₆₀ exposure showed very high level of
17 expression even after recovery, indicating that haemocytes are probably still under stress and
18 either need more time for recovery or cannot cope with the stress completely. This could
19 potentially impair immune function of the individuals and could lead to other
20 pathophysiological conditions.

21 Expression of *ras* gene did not show any changes in haemocytes after all the treatments. Down-
22 regulation was found after 3 days exposure to chemicals suggesting *ras* is still kept in the proto-
23 oncogene form which is not as closely involved in the DNA repair process as *p53*. Ruiz et al.
24 [65] found no mutation in *ras* gene at the traditional hotspots, i.e. codons 12, 13 and 61 in
25 mussels after exposure to heavy fuel and styrene, suggesting *ras* was still in proto-oncogene
26 (inactive) form following exposure to these contaminants. Whilst *ras* has been proven to
27 function in cell differentiation and proliferation in mammalian cells [66], its function in
28 invertebrates is still to be well established. Our results indicate that *ras*, as a proto-oncogene, is

1 involved in cell growth by pathways other than directly involved in DNA repair as no
2 significant difference in expression between treated and recovered group was detected. Non-
3 induction of *ras* was not surprising as over expression of *ras* has only been reported in tumour
4 cells [67].

5 When comparing the three different exposure scenarios, B(α)P was found to induce more DNA
6 strand breaks compared to the other two treatments, *p53* expression was higher in C₆₀ alone
7 exposures, and total glutathione analysis showed highest GSH induction after C₆₀ exposure
8 alone. Taken together, this suggests antagonistic effects of combined exposure of B(α)P and C₆₀
9 with reduced oxidative stress. This observation is consistent with previously reported alteration
10 of phagocytic activity after exposure to TCDD and n-TiO₂ either alone or in combination[63].
11 In our study, using the same target cells (i.e. haemocytes), we have shown a direct comparison
12 of levels of induced DNA damage, anti-oxidative ability (tGSH) and expression of a key gene
13 (i.e. *p53*) involved in processing the damage. Due to technical limitations (e.g. amount of
14 haemocytes available for analyses etc.), we could not perform modified comet assay to
15 determine oxidative DNA damage or oxidative GSH in the present study.

16 ***Relative expression of p53 and ras genes in different tissues following exposures***

17 Even though the expression of *p53* and *ras* genes in haemocytes following different treatments
18 showed concomitant induction of DNA damage, the expression patterns of these two genes in
19 different tissues are of interest. In the previous study [9], mussels exposed to B(α)P at the same
20 concentration (i.e. 56 μ g/L) for 6 and 12 days showed significantly increased expression for
21 both genes in adductor muscle and mantle tissues. However, no recovery analysis was included
22 and no B(α)P was re-dosed on a daily basis in the previous study. After improving the
23 limitations of the previous experimental design, both *p53* and *ras* gene expression showed a
24 dramatic increase after the combined exposure in the mantle and to a lesser extent in the
25 adductor muscle. The interaction between B(α)P and C₆₀ has an increased effect in these two
26 tissues, possibly as a result of ‘Trojan Horse’ effects. Interestingly, the gene expression levels
27 recovered from combined exposure were close to control in adductor muscle, whereas *p53* and
28 *ras* remain at a high level of expression in mantle. This suggests that cells in the mantle cannot

1 cope with the combined exposure and this could potentially leads to the development of
2 pathophysiological conditions. This theory is supported by research on mussels collected from
3 contaminated sites, where only leukaemia (haemocytes) and gonadal (mantle) neoplasia have
4 been found. No neoplasia has been found in other tissues of mussels [68]. Mantle is the main
5 tissue to produce germ cells and requires rapid development compared to other cells. Therefore,
6 DNA abnormalities are under higher risk to be passed to next generation and un-repaired
7 damage could initiate neoplastic development.

8 In contrast to the mantle and adductor muscle, the combination of B(α)P and C₆₀ induced an
9 antagonistic rather than an additive effect in the digestive gland. C₆₀ concentrations measured in
10 different tissues after exposure showed that digestive gland accumulated more C₆₀ than the
11 adductor muscle and gill which might explain the higher response level of gene expression in
12 this tissue. Although, a high level of *p53* and *ras* expression was induced after exposure, the
13 level dropped to control levels after recovery, suggesting the digestive gland is capable to cope
14 with the chemical concentrations applied in this study or it is more resistant to induced stress.

15 **Conclusions**

16 B(α)P and/or C₆₀ induce tissue and DNA damage in exposed mussels, confirming their function
17 as genotoxicants. The effects of individual or combined exposures to B(α)P and C₆₀ compounds
18 at the same concentrations are diverse. For example, concerning genotoxicity (Comet assay), the
19 mixture actually shows marginally less damage than the individual exposures. The same is true
20 for total glutathione level. Explanations for these observations require further investigation.

21 The experimental exposures also induced expression of tumour-regulating genes (i.e. *p53* and
22 *ras*) with high inter-individual variation. B(α)P and/or C₆₀ induced *p53* and *ras* expression in a
23 tissue specific manner with the mantle and adductor muscle being more sensitive to the
24 combined exposure and the digestive gland being more sensitive to C₆₀ exposure alone. The
25 adductor muscle and digestive gland were found to respond more quickly compared to the
26 mantle and haemocytes. Gill was found to be more tolerant to the chemical exposures and did
27 not exhibit dramatic change for the expression of *p53* and *ras* genes.

1 Direct measurement of DNA damage in the haemocytes as the target cell type correlated with
2 expression of tumour-regulating genes. In addition, it has been suggested that both *p53* and *ras*
3 function is closely related to post-transcriptional modification in response to DNA damage
4 [69,70]. With each stress, the responses may show some levels of similarities, but there will also
5 be differences essential for eliciting a unique molecular signalling outcome. It appears, therefore,
6 that multiple sites targeted by an integrated network of signalling pathways highly sensitive to
7 genotoxic stresses must be modified to yield functional *p53* and *ras* responses.

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14

15

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2 **Figure and Table Legends**

3 **Figures:**

4 **Figure 1.** Overall experimental design to determine the biological impacts of B(α)P and C₆₀ *in*
5 *vivo* exposure in mussels.

6 **Figure 2.** C₆₀ concentration in tissues after 3 days C₆₀ exposure. Star indicates significantly
7 increased concentration in exposed mussel tissues in comparison to control maintained in fresh
8 seawater only.

9 **Figure 3.** Clearance rate in mussels sampled after 1 ,3 days exposure and 3 days recovery (n=6).
10 * indicates significant difference between treated and control group at same sampling day. #
11 indicates significant difference between samples collected after 6 days incubation compared to 1
12 and 3 days incubation within control group.

13 **Figure 4.1.** Light micrographs of sections through digestive gland, gill, adductor muscle of *M.*
14 *edulis* showing histological structures of control and treated mussels stained with H & E at 5-
15 8 μ m thickness. A1-C1: control; A2-C2: exposed to B(α)P; A3-C3: exposed to B(α)P with C₆₀. A:
16 adductor muscle (\times 200 times); B: digestive gland (\times 400 times); C: gill (\times 400 times). dt =
17 digestive tubule; ct=connective tissues; fc=frontal cilia; lc=lateral cilia; gf= gill filaments;
18 amb=adductor muscle block. Black triangle indicates abnormalities. Scale bar = 20 μ m.

19 **Figure 4.2.** Light micrographs of sections through mantle of *M. edulis* showing histological
20 structure of control mussels stained with H & E at 5-8 μ m thickness. MF: female mantle; MM:
21 male mantle; MC: mantle connective tissue. mgt= male gonad tubule; fgt= female gonad tubule;
22 ct= connective tissue. Scale bar = 20 μ m.

23 **Figure 5.** Induction of DNA strand break (represented as % Tail DNA) in *Mytilus sp.*
24 haemocytes following 1 & 3 days *in vivo* exposure to B(α)P and/or C₆₀. * indicates significant
25 increase of % Tail DNA in exposed groups compared with control group ($p < 0.05$). # indicates
26 significant differences of % Tail DNA among different time treated samples ($p < 0.05$).

1 **Figure 6.** Total glutathione level in adductor muscle after 3 days exposure to chemicals. *
2 indicate significant increase compared to control ($p < 0.05$).

3 **Figure 7.** Relative quantitative p53 expression pattern in haemocytes (a.), digestive gland (b.),
4 mantle (c.), adductor muscle (d.) and gill (e.) exposed to B(α)P at 56 $\mu\text{g/L}$ and/or C₆₀ 1 mg/L
5 for 1 and 3 days followed by 3 days recovery. Each Histogram represents the means of 6
6 replicates ($n=6$) and S.E.M are indicated by error-bars. Histogram marked with the letter (a to e)
7 indicate no significant difference when one mean value compared to another, based on the
8 statistical analysis. * indicated significant up-regulated genes expression compared to control
9 only. # indicated significant down-regulated genes expression compared to control only.

10 **Figure 8.** Relative quantitative *ras* expression pattern in haemocytes (a.), digestive gland (b.),
11 mantle (c.), adductor muscle (d.) and gill (e.) exposed to B(α)P at 56 $\mu\text{g/L}$ and/or C₆₀ 1 mg/L for
12 1 and 3 days followed by 3 days recovery. Each histogram represents the means of 6 replicates
13 ($n=6$) and S.E.M are indicated by error-bars. Histogram marked with the same letter (a to f)
14 indicate no significant difference when one mean value compared to another, based on the
15 statistical analysis.. * indicated significant up-regulated genes expression compared to control
16 only. # indicated significant down-regulated genes expression compared to control only.

17 **Tables**

18 **Table 1.** Characterisation measurements of C₆₀ fullerenes particles.

19 **Table 2.** Percentage of histopathological abnormalities in different tissues following in vivo
20 B(α)P exposure alone or in combination with C₆₀.

21 **Supplementary Information**

22 **Supplementary Figure 1.** Constitutive *p53* (a.) and *ras* (b.) genes expression pattern in various
23 tissues. The gene transcript levels were semi-quantified in tissues using real-time qPCR and are
24 expressed relative to *18S* level. Each histogram represents the mean of 6 replicates examination
25 ($n=6$) and S.E.M were indicated by T-bars. Histograms marked with different letters (a, b or c)

1 indicate significant difference when one mean value compared to another, based on the
2 statistical analysis.

3 **Supplementary Table 1.** Primers designed for each gene and their PCR reaction conditions.

4 **Supplementary Table 2.** Cell viability of haemocytes collected from posterior adductor muscle
5 of experimental mussels prior to exposure (n=16).

6