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1 **Co-exposure to polystyrene plastic beads and polycyclic aromatic**
2 **hydrocarbon contaminants in fish gill (RTgill-W1) and intestinal (RTgutGC)**
3 **epithelial cells derived from rainbow trout (*Oncorhynchus mykiss*)**

4
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25
26

27 **ABSTRACT**

28 Microscopic plastic (MP) particles are a ubiquitous contaminant in aquatic environments,
29 which may bind hydrophobic chemicals, such as polycyclic aromatic hydrocarbons (PAHs),
30 altering their environmental fate and interactions with biota. Using rainbow trout gill (RTgill-
31 W1) and intestinal (RTgutGC) epithelial cells we investigated the effects of polystyrene
32 microbeads (PS-MBs; 220 nm) on the cyto- and genotoxicity of the environmental pollutants
33 benzo[*a*]pyrene (BaP) and 3-nitrobenzanthrone (3-NBA) over 48 h (0, 0.1, 1 and 10 μ M). The
34 Alamar Blue bioassay, used to assess cytotoxicity, showed that both pollutants significantly
35 decreased cell viability by 10-20% at 10 μ M in both cell lines after 48 h whereas PS-MBs (5
36 or 50 μ g mL⁻¹) were non-toxic. Cytotoxicity in cells treated with PS-MBs together with BaP
37 or 3-NBA were similar to those observed after exposure to BaP or 3-NBA alone. Using the
38 formamidopyrimidine-DNA glycosylase (FPG)-modified comet assay 3-NBA, but not BaP,
39 induced DNA damage in RTgutGC cells at 10 μ M (~10% tail DNA in the absence and ~15%
40 tail DNA in the presence of FPG versus ~1% in controls), whereas PS-MBs alone showed no
41 detrimental effects. Interestingly, comet formation was substantially increased (~4-fold) when
42 RTgutGC cells were exposed to PS-MBs (50 μ g mL⁻¹) and 10 μ M 3-NBA compared to cells
43 treated with 3-NBA alone. Further, using ³²P-postlabelling we observed strong DNA adduct
44 formation in 3-NBA-exposed RTgutGC cells (~900 adducts/10⁸ nucleotides). 3-NBA-derived
45 DNA adduct formation was significantly decreased (~20%) when RTgutGC cells were exposed
46 to MB and 3-NBA compared to cells treated with 3-NBA alone. Our results show that PS-MBs
47 impact on the genotoxicity of 3-NBA, causing a significant increase in DNA damage as
48 measured by the comet assay in the intestinal cell line, providing proof of principle that MPs
49 may alter the genotoxic potential of PAHs in fish cells.

50

51 **Keywords:** Microplastic beads, polycyclic aromatic hydrocarbons, fish cell lines, RTgill-W1,
52 RTgutGC, genotoxicity

53 **Capsule:** Polystyrene microbeads (~220 nm) in conjunction with the polycyclic aromatic
54 hydrocarbon 3-nitrobenzanthrone (3-NBA) increased DNA damage, as measured by the comet
55 assay, by 4-fold in the fish gut cell line RTgutGC.

56

57 INTRODUCTION

58 The contamination of the aquatic environment with plastic debris is now a globally
59 recognised problem (Moore 2008; Jambeck et al. 2015). This coincides with its increased
60 prevalence in society – global production has risen from ~2 million tons in 1950 to >330 million
61 tons in 2016 (Duis and Coors 2016; PlasticsEurope 2017), and reflects the high durability and
62 persistence of these polymers.

63 In recent years microplastics (MPs; defined as 0.1– 5000 μm in diameter) have received
64 increased attention because there is an urgent need to assess the risk they pose to the
65 environment and human health (Wright et al. 2013a; Wright and Kelly 2017). These primarily
66 originate from the breakdown of larger plastic items (Thompson et al. 2004; Roy et al. 2011),
67 potentially down to the nano-scale (Lambert and Wagner 2016). Further, environmental inputs
68 come from the use of MPs in cosmetics (Napper et al. 2015), detergents and the construction
69 sector. In addition, MP fibers used in clothing may enter the waste effluence system following
70 washing (Browne et al. 2011; Napper and Thompson 2016). These particles consist of many
71 different types such as polyethylene terephthalate (PET), polyvinylchloride (PVC), polystyrene
72 (PS), and polypropylene (PP) amongst others (Kanhai et al. 2017).

73 The ingestion of MPs has been reported for a wide range of organisms including
74 bivalves and crustaceans, fish and larger mammals and seabirds. Following ingestion, MPs can
75 compromise energy reserves (Wright et al. 2013b), reproductive success and cause
76 inflammation (Sussarellu et al. 2016) as well as impede feeding activity. Furthermore, they
77 may pose a health risk due to the leaching of chemicals used in plastic production (e.g.
78 plasticisers) and they are a proposed conduit for the transfer of other chemicals (i.e.
79 hydrophobic organic chemicals [HOCs]) adhered to the surface or absorbed into the polymer
80 matrix of the MP (Teuten et al. 2009; Browne et al. 2011; Rochman et al. 2013a,b,c; Koelmans
81 et al. 2016). Of the priority substances listed in the EU more than ~80% are classified as HOCs
82 and thus are expected to be able to bind to MPs (Rochman et al. 2014). However, sorption
83 characteristics will depend on physiochemical properties of the HOC and microbead surface
84 chemistry and Gouin et al (2011) predicted only a limited amount (<1%) of environmental
85 HOCs with $\text{LogK}_{\text{ow}} < 5$ partitioning to polystyrene microbeads (PS-MBs) in aquatic
86 environments. As smaller MP particles have a larger surface area per unit of volume, it could
87 be hypothesised that they could facilitate a larger mass transfer of HOCs to a level capable of
88 inducing toxicity. However, the evidence available either does not support that microplastics
89 can act as vector of HOCs into organisms or is inconclusive (Burns and Boxall, 2018). What

90 may be of concern is the potential for additive or synergistic effects to arise following co-
91 exposure, but enhanced detrimental effects of combined MP and HOC exposures on biota are
92 not always observed (Lohmann et al 2017; Guven et al. 2018), highlighting the need for more
93 research to fully understand this.

94 These HOCs include polycyclic aromatic hydrocarbons (PAHs) which are widespread
95 environmental and aquatic pollutants (Amaeze et al. 2015; Sogbanmu et al. 2016; Banni et al.
96 2017). PAHs like benzo[*a*]pyrene (BaP; Fig. S1A) are formed by incomplete combustion of
97 organic matter. BaP is carcinogenic acting via a genotoxic mechanism. Metabolic activation is
98 catalysed by cytochrome P450 (CYP) enzymes, particularly CYP1A1, resulting in highly
99 reactive diol-epoxides (i.e. BaP-7,8-diol-9,10-epoxide [BPDE]; Fig. S1A) capable of forming
100 covalent DNA adducts (Wohak et al. 2016; Reed et al. 2018). Diesel exhaust is composed of a
101 complex mixture of PAHs, nitro-PAHs and particulates (Jarvis et al. 2018). One such nitro-
102 PAH is 3-nitrobenzanthrone (3-NBA; Fig. S1B) which is highly mutagenic and a potent
103 carcinogen (Arlt 2005). As a likely consequence of atmospheric washout, 3-NBA was
104 detectable in rainwater, surface soil and river sediments (Lubcke-von Varel et al. 2012) and
105 thus may co-contaminant MPs. Metabolic activation is required for 3-NBA to form DNA
106 adducts (White et al. 2017). Formation of the DNA-reactive metabolite *N*-hydroxy-3-
107 aminobenzanthrone (*N*-OH-3-ABA; Fig. S1B) is primarily catalysed by nitroreductases such
108 as NAD(P)H:quinone oxidoreductase 1 (NQO1). The combined effects of MPs and absorbed
109 PAH co-contaminants on genotoxicity is poorly understood.

110 As the amount of plastic entering our environment is increasing each year (Jambeck et
111 al. 2015; Geyer et al. 2017), it is important to assess the risk it poses and to develop effective
112 policies and management. Although the possible effects of MPs on the environment are not
113 covered by current environmental risk assessment procedures, current risk assessments for
114 testing HOCs require the use of fish. However, to date only a few *in vivo* studies have been
115 carried out regarding the issue of MPs functioning as vectors of HOCs (Rochman et al. 2013a;
116 Batel et al. 2016), and little information is yet available on the effects of MPs and associated
117 HOCs using *in vitro* assays. *In vitro* assays play a key role in ecotoxicology as they allow
118 studying the effect of a chemical on the cell surface or inside a cell; the initial place of
119 interaction. Bearing in mind the 3R's principle which focuses on the replacement, refinement
120 and reduction of animals used for *in vivo* experimentation, *in vitro* assays are valuable tools
121 (Schnell et al. 2016). The fish gill RTgill-W1 cell line assay is currently being considered as a
122 new possible standard method within the International Organization for Standardisation
123 (Lillicrap et al. 2016). It has been recently subjected to an international round robin test which

124 demonstrated to be robust and to show inter-laboratory reproducibility (Lillicrap et al. 2016).
125 More recently the fish intestinal RTgutGC cell line (Kawano et al. 2011) has been used to
126 evaluate the risk posed by novel pollutants (Langan et al. 2018; Stadnicka-Michalak et al.
127 2018). Thus, this cell line offers another *in vitro* model with the opportunity to reduce the
128 numbers of fish used in regulatory procedures.

129 In the present study we have evaluated the effects of co-exposure to MP beads and
130 HOCs. For proof-of-principle we tested PS-MBs (~200nm). PS is the 4th highest polymer type
131 in the global primary production and primary waste generation (Geyer et al. 2017) and is
132 commercially available in defined size classes. We evaluated cellular responses towards two
133 HOCs, namely BaP and 3-NBA, alone or in combination with PS-MBs in fish gill (RTgill-W1)
134 and intestinal (RTgutGC) epithelial cells derived from rainbow trout (*Oncorhynchus mykiss*).
135 Cytotoxicity was assessed using the Alamar Blue assay. DNA damage and oxidative damage
136 to DNA was determined by the single cell gel electrophoresis (comet assay). DNA adduct
137 formation was measured by ³²P-postlabelling.

138

139 MATERIAL AND METHODS

140

141 Carcinogens

142 Benzo[*a*]pyrene (BaP, CAS number 50-32-8; purity ≥96 %) was obtained from Sigma
143 Aldrich (UK). 3-Nitrobenzanthrone (3-NBA, CAS number 17117-34-9) was prepared as
144 previously reported (Arlt et al. 2005).

145

146 Microplastics

147 Polystyrene microbeads (PS-MB; 220 nm; PP-025-10) were obtained from Spherotech
148 (USA). Beads were sonicated (Soniprep 150 Plus, amplitude level 10) for 3-5 seconds
149 immediately before use.

150

151 Cell culture and treatment

152 Fish gill (RTgill-W1) (Bols et al. 1994) and intestinal (RTgutGC) (Minghetti and
153 Schirmer 2016) epithelial cells derived from rainbow trout (*Oncorhynchus mykiss*) were
154 routinely cultured in 75-cm² culture flasks at 18 °C in DMEM medium (Gibco) supplemented
155 with 5% fetal bovine (FBS) serum (Invitrogen, UK), penicillin (100 units mL⁻¹) and
156 streptomycin (0.1 mg mL⁻¹) (Invitrogen, UK). Cells were seeded at a density of 1.5 × 10⁵ cells
157 mL⁻¹ and incubated at 18 °C for 48 h prior to exposure. BaP and 3-NBA were dissolved in

158 dimethyl sulfoxide (DMSO) and cells were treated as indicated (0, 1 or 10 μM). Controls were
159 treated with solvent, DMSO, only; the final concentration of DMSO was always kept at 0.5%.
160 The commercial PS-MBs stock solution was diluted in fresh medium to 5 and 50 $\mu\text{g mL}^{-1}$; the
161 PS-MB stock contains sodium azide and the final concentration in the culture media was 0.3
162 and 3 μM , respectively. For controls the addition of PS-MBs was omitted. In co-exposure
163 experiments fish cells were treated with 10 μM BaP or 3-NBA together with PS-MBs (50 μg
164 mL^{-1}). Test compounds were diluted in fresh medium to final concentrations and then added to
165 the cells (seeding medium was aspirated immediately prior). Cells were exposed up to 48 h.

166 Culture conditions (L15 + 5% FBS) may influence the size of PS-MBs. To evaluate the
167 actual size of PS-MBs our cell cultures were exposed to PS-MBs at 5 and 50 $\mu\text{g mL}^{-1}$ were
168 incubated for 0, 24 and 48 h in culture media, each experiment was performed in triplicate and
169 for each replicate 3 subsamples were taken for bead size analysis using a Malvern Zetasizer
170 Nano ZS. Results from the nanosizer were adjusted to consider refractive index and viscosity
171 according to the methods of Fröhlich et al [2013].

172

173 **Analysis of cell viability, DNA damage by comet assay and DNA adduct formation by ^{32}P -** 174 **postlabelling.**

175 Cell viability was assessed using the Alamar Blue assay, DNA damage via the alkaline
176 comet assay as described previously [Amaeze et al. 2015] and DNA adduct formation by ^{32}P -
177 postlabelling as reported [Arlt et al., 2008]. Further methodological details are described in the
178 Supplementary Information.

179

180 **Statistical Analysis**

181 All biological data are presented as mean \pm standard deviation (SD) and derived from
182 three or four independent experiments with cells from different passage numbers. For
183 cytotoxicity (viable cells as % of control [untreated]), 4 technical replicates (e.g. wells) were
184 measured for each sample (i.e. each treatment condition) in each independent experiment
185 ($n=3$). For the comet assays 50 nuclei per sample were scored in each independent experiment
186 ($n=3$, i.e. 3 independent replicates). For DNA adduct analysis each DNA sample obtained in
187 independent experiments ($n=4$) was analyzed once in separate ^{32}P -postlabelling analyses. For
188 statistical analysis, cytotoxicity data was normalised to control (untreated) which was set to
189 1.0, then log₂ transformed and analysed using a single sample *t*-test with Bonferroni correction
190 against the population control mean of 0. Similarly, comparisons were made to cells treated
191 with MBs alone to account for potential effects to sodium azide. For the effects of MBs on

192 DNA adduct formation, adduct data was normalized to carcinogen treatments without MBs
193 which was set to 1.0, then log₂ transformed and analysed using a single sample *t*-test with
194 Bonferroni correction against the population control mean of 0. For the comet assay data, two-
195 way ANOVA followed by Tukey post-hoc test was performed. For assessment of the effects
196 of culture conditions on PS-MB size data was initially tested for homogenous variance
197 (Levene's Test of variance; *p*=0.064), followed by a two-way ANOVA with Bonferroni
198 correction where time and concentrations were independent variables. Significance difference
199 were identified via a Tukey's HSD post-hoc test. All statistical analyses were performed using
200 GraphPad Prism 7.

201

202 **RESULTS**

203

204 **Cytotoxicity of BaP and 3-NBA in RTgill-W1 and RTgutGC cells**

205 No cytotoxic effects were observed for both BaP and 3-NBA in both RTgill-W1 (Fig.
206 S2) and RTgutGC (Fig. S3) cells after 24 h exposure. In contrast, BaP and 3-NBA did induce
207 significant cytotoxicity in both RTgill-W1 (Fig. S2) and RTgutGC (Fig. S3) cells after 48 h
208 exposure, with cell viability decreasing by 10-20% compared to controls.

209

210 **Genotoxicity of BaP and 3-NBA in RTgill-W1 and RTgutGC cells**

211 No significant DNA damage (measured as % tail DNA) was found for both BaP and 3-
212 NBA in RTgill-W1 cells neither in the absence nor presence of FPG (Fig. 1A). In RTgutGC
213 cells DNA damage was significantly increased at the highest 3-NBA concentration tested (i.e.
214 10 μM; ~20% tail DNA in 3-NBA-treated cells *versus* ~2% in controls) using the FPG-
215 modified comet assay (Fig 1B). No significant DNA damage was induced in BaP-exposed
216 RTgutGC cells in the absence or presence of FPG (Fig 1B).

217 RTgill-W1 and RTgutGC cells were both capable of generating BaP-induced DNA
218 adducts (Fig. 2), with the major DNA adduct detected (assigned spot B1, Fig. 2 *upper panels*)
219 previously identified as 10-(deoxyguanosin-*N*²-yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydro-BaP
220 (dG-*N*²-BPDE) (Arlt et al. 2008). In RTgill-W1 cells BaP induced ~2.5 adducts per 10⁸
221 nucleotides, with adduct levels being ~4-fold higher in BaP-exposed RTgutGC cells (Fig. 2).
222 3-NBA exposure also resulted in DNA adduct formation in both RTgill-W1 and RTgutGC
223 cells, with 4 major DNA adducts (assigned spots N1-N4, Fig. 2 *lower panels*) in both cell lines.
224 Three of these adducts were previously identified as 2'(2'-deoxyadenosine-*N*⁶-yl)-3-
225 aminobenzanthrone (dA-*N*⁶-3-ABA; spot N1), *N*-(2'-deoxyguanosine-*N*²-yl)-3-

226 aminobenzanthrone (dG-*N*²-3-ABA; spot N3), and *N*-(2'-deoxyguanosin-8-yl)-3-
227 aminobenzanthrone (dG-C8-*N*-3-ABA; spot N4) (Arlt et al. 2006; Gamboa da Costa et al.
228 2009). Spot N2 was previously identified as deoxyadenosine adduct but its structure has not
229 yet been elucidated. 3-NBA induced extremely high DNA adduct levels in RTgutGC cells
230 (~900 adducts per 10⁸ nucleotides) which were 25 times higher than in RTgill-W1 cells under
231 the same experimental conditions (Fig. 2). No DNA adduct spots were detected in control
232 (untreated) cells (Fig. 2).

233

234 **The effect of cell culture media on PS-MB size**

235 The mean-processed Zeta-average particle size (d.nm) data appears to show all overall
236 increase in particle size, from 279.1 ± 17.5 nm at 0 h to 415.9 ± 54.7 nm at 24 h and 407.4 ±
237 60.0 nm at 48 h (Supplementary Information Fig. S4 and Table S1). PS-MBs also appeared
238 larger in the higher concentration of 50 µg mL⁻¹, with a mean diameter of 407.3 ± 84.5 nm
239 compared to 327.6 ± 49.2 nm at 5 µg mL⁻¹ at 48 h. However, when looking at the frequency
240 distributions of particle size, PS-MBs at the higher concentration had a consistent modal size
241 of 295.30 nm over time (Supplementary Information Table S1). This slightly increased in the
242 initial 24 h for the lower concentration (to 342.0 nm) before decreasing again at 48 h. It is
243 important to note that suspensions at both concentrations gained a multi-modal distribution at
244 24 h (Supplementary Information Fig. S4). This can be observed between 43.82 nm and 58.77
245 nm and <10 nm for the lower concentration and at 91.28 nm for the higher concentration

246

247 **The impact of PS-MB co-exposure on BaP- and 3-NBA-induced cytotoxicity in RTgill- 248 W1 and RTgutGC cells**

249 Since treatment with 10 µM BaP or 3-NBA showed genotoxic effects in both RTgill-
250 W1 and RTgutGC cells after 48 h of exposure, these concentrations and exposure times were
251 used in further experiments to explore the effects of co-exposure to PS-MBs and BaP or 3-
252 NBA in RTgill-W1 and RTgutGC cells. Both PS-MB concentrations were observed to be non-
253 toxic in RTgill-W1 and RTgutGC cells (Fig. 3). In subsequent experiments both cell lines were
254 treated with 10 µM BaP or 3-NBA, in the presence or absence of PS-MBs (5 and 50 µg mL⁻¹),
255 with cells also treated with either DMSO or MBs control treatments for 48 h. Overall, exposure
256 to MBs together with BaP or 3-NBA had marginal influence on cell viability in RTgill-W1 and
257 RTgutGC cells [i.e. between 5 - 20% loss in viability (Fig. 3)]. However, the loss of cell

258 viability was significant in all treatments where cells are co-exposed with MBs and PAHs if
259 compared to the respective MB concentrations (Fig. 3).

260

261 **The impact of PS-MB co-exposure on BaP- and 3-NBA-induced genotoxicity in RTgill- 262 W1 and RTgutGC cells**

263 Exposure to PS-MBs did not enhance DNA damage (measured as % tail DNA) in both
264 cell lines (Fig. 4). Furthermore, no significant changes in DNA damage in RTgill-W1 were
265 observed in any treatments (e.g. PS-MB + PAH) in the absence or presence of FPG (Fig. 4A).
266 In contrast, in RTgutGC cells the presence of PS-MBs substantially increased 3-NBA-induced
267 DNA damage in both the unmodified and FPG-modified comet assay (Fig. 4B). This effect
268 was not observed in BaP-treated RTgutGC cells (Fig 4B).

269 PS-MB co-exposure did not alter the characteristic DNA adduct pattern induced by BaP
270 and 3-NBA in RTgutGC cells (Fig. 5 *insert panel*), however, DNA adduct formation was
271 significantly decreased (~20%) in RTgutGC cells co-exposed to PS-MB and 3-NBA relative
272 to cells treated with 3-NBA alone (Fig. 5).

273

274 **DISCUSSION**

275 The present study aimed to evaluate the influence of microplastics (220nm PS-MBs)
276 on the toxicity of BaP and 3NBA in two epithelial cell lines derived from rainbow trout gill
277 and gut tissue, RTgill-W1 and RTgutGC,. These two cell lines were chosen because they
278 represent the epithelia of fish that are exposed to pollutants via the water or diet. Notably, the
279 culture conditions impacted the size of the PS-MBs. The presence of particles approximately
280 400-500 nm in size suggests that the protein-supplemented and electrolyte-rich media resulted
281 in potential adsorption of biomacromolecules to the surface of the PS-MBs. This could
282 influence hydrodynamic behaviour, alter stability and modify functionality, resulting in the
283 formation of aggregates. Moreover, the presence of smaller modes in the lower concentration
284 of PS-MBs may be due to agglomerated serum proteins, however, testing this was not in the
285 scope of the present study. However, the key findings were that co-exposure to PS-MBs
286 enhanced the genotoxicity of 3-NBA, as measured by the normal and FPG-modified comet
287 assay in RTgutGC, but not RTgill-W1 cells (Fig. 4), and that both BaP and 3-NBA induced
288 DNA adduct formation in RTgutGC and RTgill-W1 cells (Fig. 2), with a significantly higher
289 levels of adducts seen in the RTgutGC cells. The formation of 3-NBA-derived DNA adducts
290 indicated that RTgutGC and RTgill-W1 cells have active nitroreductases (e.g. NQO1), because

291 nitroreduction is the primary pathway of metabolic activation for 3-NBA (Arlt et al. 2005;
292 Stiborova et al. 2014). However, it should be noted that this is a proof-of-principle study and
293 the concentrations of both MBs and PAHs may exceed those that would be found in fresh or
294 marine waters or inside prey items.

295

296 **Cytotoxicity of BaP and 3-NBA in RTgill-W1 and RTgutGC cells**

297 The Alamar Blue assay, a measure of cellular metabolic activity, indicated that PS-MBs
298 at 5 or 50 $\mu\text{g mL}^{-1}$ were not cytotoxic to RTgill-W1 or RTgutGC cells over a 48 h exposure
299 period (Fig. S3). This corroborates findings by Schirinzi et al. (2017) who found no significant
300 effect of PS (10 nm and 40–250 nm) and polyethylene (3–16 μm and 100–600 nm) microplastic
301 exposure on cerebral (T98G) and epithelial (HeLa) human cells up to 10 mg L^{-1} (Schirinzi et
302 al. 2017). Dose response assays showed moderate (10–20%), but significant, reductions in cell
303 viability when RTgill-W1 cells are exposed to 10 μM BaP and 3-NBA and when RTGutGC
304 cells were exposed to 10 μM BaP and 1 μM 3-NBA (Fig. S2 and S3). Stadnicka-Michalak et
305 al (2018) observed similar moderate (~10%) effects on cytotoxicity (which was not significant
306 in their study), in both these cell lines after BaP exposure for 48 h. Cytotoxicity of these
307 pollutants is often associated with biotransformation which produces reactive metabolites
308 and/or free-radicals (Imanikia et al. 2016; Banni et al. 2017; White et al. 2017; Jarvis et al.
309 2018; Reed et al. 2018). CYP1A1 enzyme activity in the RTgill-W1 cell line, as measured by
310 ethoxyresorufin O-deethylase (EROD) activity, has been reported to be low and is not
311 significantly induced by BaP (Stadnicka-Michalak et al. 2018), which may explain the low
312 cytotoxicity to this PAH (present study) and extracts from oil contaminated sediment (Amaeze
313 et al. 2015). To our knowledge, the cytotoxicity of 3-NBA has not been ascertained in RTgill-
314 W1 and RTgutGC cell lines previously.

315

316 **Genotoxicity of BaP and 3-NBA in RTgill-W1 and RTgutGC cells**

317 The significant increase in DNA damage (% tail DNA), as measured by the comet
318 assay, in RTgutGC cells compared to RTgill-W1 cells indicates that they have a greater ability
319 to metabolise PAHs/nitro-PAHs (Fig. 1 and 4). The FPG-modified comet assay reflects
320 oxidative damage to DNA (Landvik et al. 2010) and in most instances the DNA damage
321 induced was greater in assays with FPG indicating ROS generation in these cells. These
322 findings are in line with other studies which assessed 3-NBA genotoxicity using the comet
323 assay in a variety of human cells lines including lung A549 epithelial cells (Nagy et al. 2005;
324 Nagy et al. 2007; Oya et al. 2011). Metabolic activation of 3-NBA leads to the formation of *N*-

325 OH-3-ABA (see Fig. S1B), which in turn can generate DNA adducts (Fig. 2 and 5). *N*-OH-3-
326 ABA can further be reduced to 3-aminobenzanthrone (3-ABA) by nitroreductases which in
327 turn can be activated by CYP1A1 and CYP1A2 leading again to the formation of *N*-OH-3-
328 ABA (Arlt et al. 2004). The involvement of CYP1A1 in 3-NBA metabolism has been suggested
329 to explain the pro-oxidative properties of 3-NBA (Hansen et al. 2007). Indeed, generation of
330 ROS, measured as 2',7'-dechlorofluorescein-diacetate fluorescence, has been observed in
331 human lung A549 and bladder RT4 cells (Hansen et al. 2007; Reshetnikova et al. 2016).
332 However, another study showed that the extent of production of these free radicals was
333 dependent on dose and duration of exposure with only marginal increase in ROS production
334 seen in 3-NBA-exposed RT4 cells (Pink et al. 2017). It is also noteworthy that no significant
335 formation of 8-oxo-2'-deoxyguanosine (8-oxodG), which is commonly used as biomarker for
336 oxidative stress, has been observed in 3-NBA-treated A549 cells using high-performance liquid
337 chromatography with electrochemical detection or liquid chromatography-tandem mass
338 spectrometry (LC/MS-MS) (Nagy et al. 2005; Rossner et al. 2016). In future LC/MS-MS could
339 be a sensitive method to assess 8-oxodG formation in 3-NBA-exposed RTgutGC cells which
340 was beyond the scope of the present study.

341 A number of PAHs including BaP have been shown to induce CYP1A activity in
342 another rainbow trout cell line derived from the liver (RTL-W1) with EC50 for inducible
343 EROD activity in the low to high nM range, unfortunately no nitro-PAHs were tested in this
344 study (Bols et al. 1999). RTL-W1 cells are able to metabolise BaP forming BaP-7,8-
345 dihydrodiol and to a lesser extent BaP-6,12-quinone (Schirmer et al. 2000). BaP-7,8-
346 dihydrodiol is the precursor of BPDE which is capable of forming covalent DNA adducts (i.e.
347 dG-*N*²-BPDE). Although BaP metabolite formation was not determined in RTgill-W1 and
348 RTgutGC cells after BaP exposure in the present study, RTgutGC cells have previously been
349 shown to metabolise BaP (Stadnicka-Michalak et al. 2018), and the detection of BaP-derived
350 DNA adducts (i.e. dG-*N*²-BPDE) (Fig. 2) is indirect proof that both cell lines are capable of
351 forming BaP-7,8-dihydrodiol/BPDE. The presence of BaP-DNA adducts in RTgill-W1 cells
352 after exposure to BaP, albeit, at considerably low levels, may indicate some but minimal
353 CYP1A activity. Although BaP has not been seen to induce EROD activity in RTgill-W1 cells
354 over 72 h exposure to 1 µM BaP (Stadnicka-Michalak et al. 2018), it is possible that induction
355 did occur at 10 µM in the current study. Alternatively, other CYP enzymes in this piscine model
356 maybe capable of metabolise BaP to form adduct biotransformation products. However, the
357 differences (~4-fold) in BaP-DNA adduct levels (Fig. 2) between RTgill-W1 and RTgutGC

358 cells again indicates a greater CYP1A activity which is in accordance with differences in
359 EROD activity seen between both cell lines (Stadnicka-Michalak et al. 2018). It is also
360 noteworthy that induction of EROD activity in BaP-treated RTgutGC cells has previously
361 shown to follow a bell-shaped concentration curve with inhibition at higher ($>0.5 \mu\text{M}$) BaP
362 concentrations (Langan et al. 2018; Stadnicka-Michalak et al. 2018) which may have impacted
363 on the degree of BaP-DNA adduct formation (present study). The formation of 3-NBA-derived
364 DNA adducts in both RTgill-W1 and RTgutGC cells indicates active NQO1 (Fig. 2), however,
365 again the large difference (~ 25 -fold) in DNA adduct levels between cell lines provide
366 additional evidence that RTgutGC cells are more biotransformationally active. It is also
367 noteworthy for our study that BaP-derivatives can also be metabolised by NQO1 (Luch and
368 Baird 2005). This indicates that potentially both CYP1A and NQO1 enzymes are active in
369 RTgill-W1 and RTgutGC cells and are critical determinants of BaP and/or 3-NBA
370 genotoxicity.

371

372 **The impact of PS-MB co-exposure on BaP- and 3-NBA-induced cytotoxicity and** 373 **genotoxicity in RTgill-W1 and RTgutGC cells**

374 The observed genotoxicity indicates that PAHs/nitro-PAHs have entered the RTgill-
375 W1 and RTgutGC cells, and the enhancement of DNA damage in the presence of PS-MBs (Fig.
376 4) suggests that the MBs may have been taken up by the cells, at least in the RTgutGC cells.
377 The mechanisms by which these particles can be taken up by epithelial cells is via two main
378 endocytotic pathways, clathrin-mediated endocytosis ($\sim 120 \text{ nm}$) and caveolae-mediated
379 endocytosis ($50\text{--}100 \text{ nm}$), or macropinocytosis which is a non-specific pathway that facilitates
380 the uptake of particles of $>1 \mu\text{m}$ (Gratton et al. 2008; Petros and DeSimone 2010; Reinholz et
381 al. 2018). He et al (2013b) demonstrated the uptake of 100 nm carboxyl-functionalised PS-
382 MBs by the human intestinal Caco-2 cells via macropinocytosis where the majority of these
383 MBs entered the endolysosomal pathway and degradation in the lysosome. A smaller proportion
384 of the MBs underwent transcytosis via unknown processes with the rate of excretion being
385 limited by basal exocytosis (Reinholz et al. 2018) indicating a potential route for HOC-bound
386 MBs of entering the circulation. Clathrin- and caveolae-mediated endocytosis have been
387 implicated in Madin-Darby canine kidney (MDCK) cell uptake of polymer nanoparticles (NP)
388 of $\sim 80 \text{ nm}$ in size and all three processes in Caco-2 cells formed the majority of uptake
389 pathways of the NPs accumulated in lysosomes (He et al 2013a,b). The uptake processes of
390 spherical PS-MPs of 200 nm in the RTgut-GC cells still must be ascertained. However,

391 (Minghetti and Schirmer 2016) have observed that lysosomal membrane integrity was
392 significantly affected in RTgut-GC cells exposed to citrate-Ag nanoparticles of ~30 nm in size,
393 indicating internalisation of these particles. Whether the increase in DNA damage (i.e. comet
394 formation) seen in RTgutGC cells co-exposed to 3-NBA and PS-MBs (Fig. 4) is due to
395 increased intracellular 3-NBA concentrations is unclear. Should a PS-MB gain entry into a cell,
396 it's chemical burden may be released; PAH desorption may be facilitated by intracellular
397 conditions (Bakir et al. 2014). Alternatively, the PS-MBs may have sedimented out of
398 suspension over the duration of exposure leading to direct contact exposure with cell surfaces
399 (Hartmann et al. 2017). Desorption and subsequent diffusion of PAH molecules across the cell
400 membranes could lead to increased intracellular concentrations and therefore DNA damage.
401 Sorption/desorption characteristics in the media or within cells was not the focus of this study
402 and further work is necessary to elucidate the mechanism(s) that caused an induction of comet
403 formation during co-exposure.

404 Since exposure to PS-MBs alone did not increase DNA damage in RTgutGC cells as
405 measured by the comet assay, it can be speculated that any 3-NBA bound to PS-MBs may have
406 altered the surface characteristics of the particles and subsequently their potency to induce
407 particle-related genotoxicity. However, we observed a decrease in DNA adduct formation in
408 these cells in the presence of 3-NBA and PS-MBs, which may suggest a decrease in 3-NBA
409 metabolism, the reason for which is currently unclear, and further research is required to
410 determine the temporal dynamics of 3-NBA metabolism in the presence of PS-MBs.
411 Nevertheless, it is important to point out that the ³²P-postlabelling and comet assay are
412 assessing different types of DNA lesions. While the ³²P-postlabelling assay determines
413 covalent DNA binding of 3-NBA (i.e. bulky 3-NBA-DNA adducts), the comet assay measures
414 DNA strand break formation linked to ROS production and/or DNA repair (e.g. removal of 3-
415 NBA-DNA adducts by nucleotide excision repair or 8-oxodG by base excision repair) in these
416 cells. Thus, the underlying mechanisms for an increase in 3-NBA-induced DNA damage (i.e.
417 comet formation) and inhibition of 3-NBA-DNA adduct formation in the presence of PS-MBs
418 in RTgutGC cells can be different.

419

420 **Conclusion**

421 The greater induction of genotoxicity in RTgutGC cells by BaP and 3-NBA (Fig. 2 and 4),
422 supports the hypothesis that these cells possess a greater metabolic activity as seen in the study
423 of Stadnicka-Michalak et al (2018). The high DNA adduct levels observed with 3-NBA suggest
424 it is a potent mutagen to fish cells. 3-NBA is mainly a product of diesel combustion and is

425 present in ambient air (Feilberg et al. 2002), rainwater (Murahashi et al. 2003a) and soil
426 (Murahashi et al. 2003b). Lubcke-von Varel et al. (2012) is the only study to date to identify
427 3-NBA in the aquatic environment as part of an effect-directed analysis of the polar fractions
428 of sediments extracts from Bitterfeld on the German Elbe, and attributed the genotoxicity,
429 based on the Ames test, of these sediment extracts to 3-NBA. The aquatic environmental fate
430 of 3-NBA is unclear. The polarity of PAHs, suggest that they may bind to MPs (Teuten et al.
431 2009), however, others predict that only small amounts of environmental HOCs with LogK_{ow}
432 < 5 may bind to PS-MBs in aquatic environments (Gouin et. 2011). In our study 220 nm PS-
433 MBs enhanced 3-NBA-induced DNA damage (i.e. comet formation), suggesting that in the
434 laboratory fish gut epithelia are able to take up plastic of ≥ 200 nm, as has been observed for
435 other vertebrate epithelium (He et al 2013a,b; Reinholz et al 2018). However, our proof-of-
436 principle study has some limitations, for example, the concentrations of both MBs and PAHs
437 exceed those that would be found in fresh or marine waters or inside prey items, and we did
438 not focus on the sorption/desorption characteristics of the PAHs and MPs. On the other hand,
439 chronic low-level exposure scenarios as found in the environment are difficult to mimic in any
440 cell culture models. Thus, testing acute exposures at higher concentrations still has value to
441 predict cellular responses and toxic mechanisms that might establish with chronic exposures at
442 low concentrations. In summary, our study demonstrates that MPs can alter the genotoxicity of
443 PAH contaminants in fish epithelial cells and more work is required to elucidate the underlying
444 mechanisms.

445

446

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457

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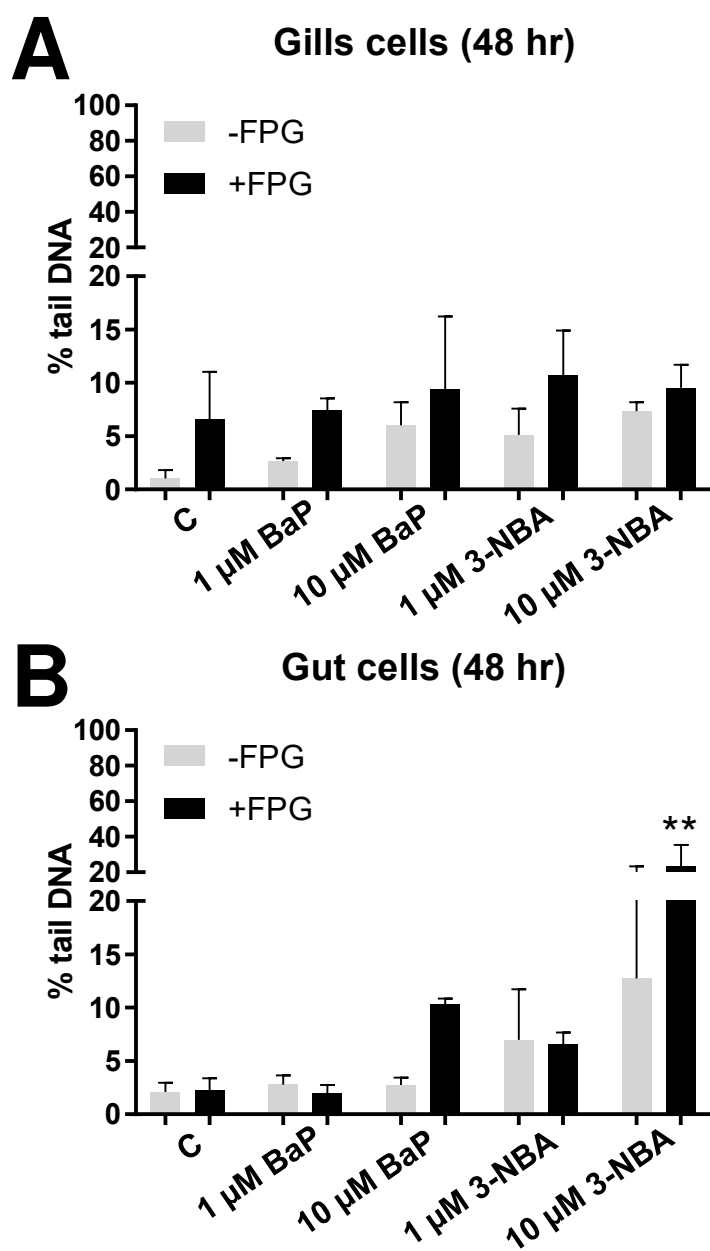
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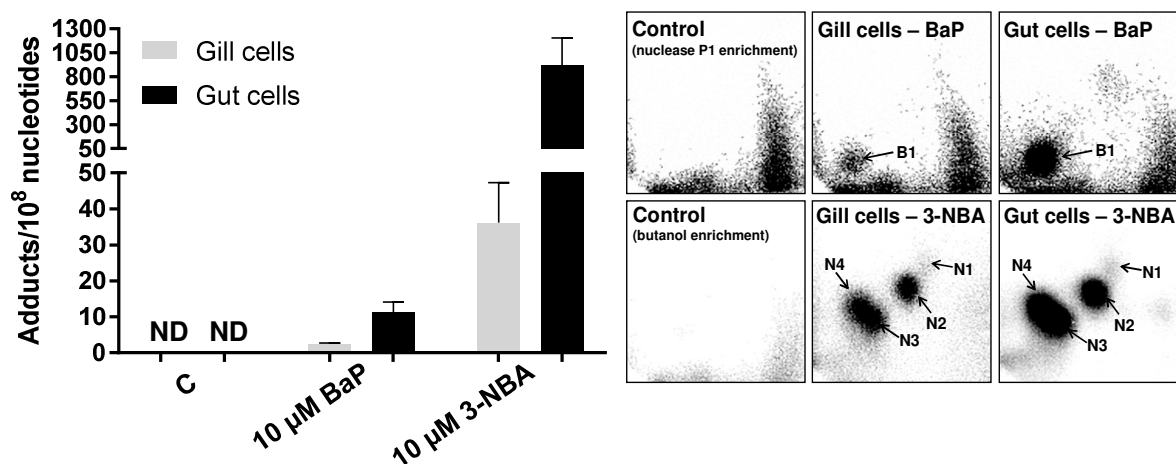
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- 694

695

696
697**Figure 1**

698 Effect of BaP and 3-NBA exposure on DNA damage (% tail DNA) in fish gill RTgill-W1 (A)
 699 and intestinal RTgutGC cells (B) at 48 h as assessed by the alkaline comet assay. The comet
 700 assay was used to detect alkali-labile lesions. Formamidopyrimidine glycosylase (FPG) which
 701 detects oxidative damage to DNA including 8-oxo-dG was added in additional experiments.
 702 Values represent mean \pm SD ($n=3$) derived from three independent experiments with cells from
 703 different passage numbers; 50 nuclei per sample were scored. Statistical analysis was
 704 performed by two-way ANOVA followed by Tukey post-hoc test (** $p<0.01$, different from
 705 control).

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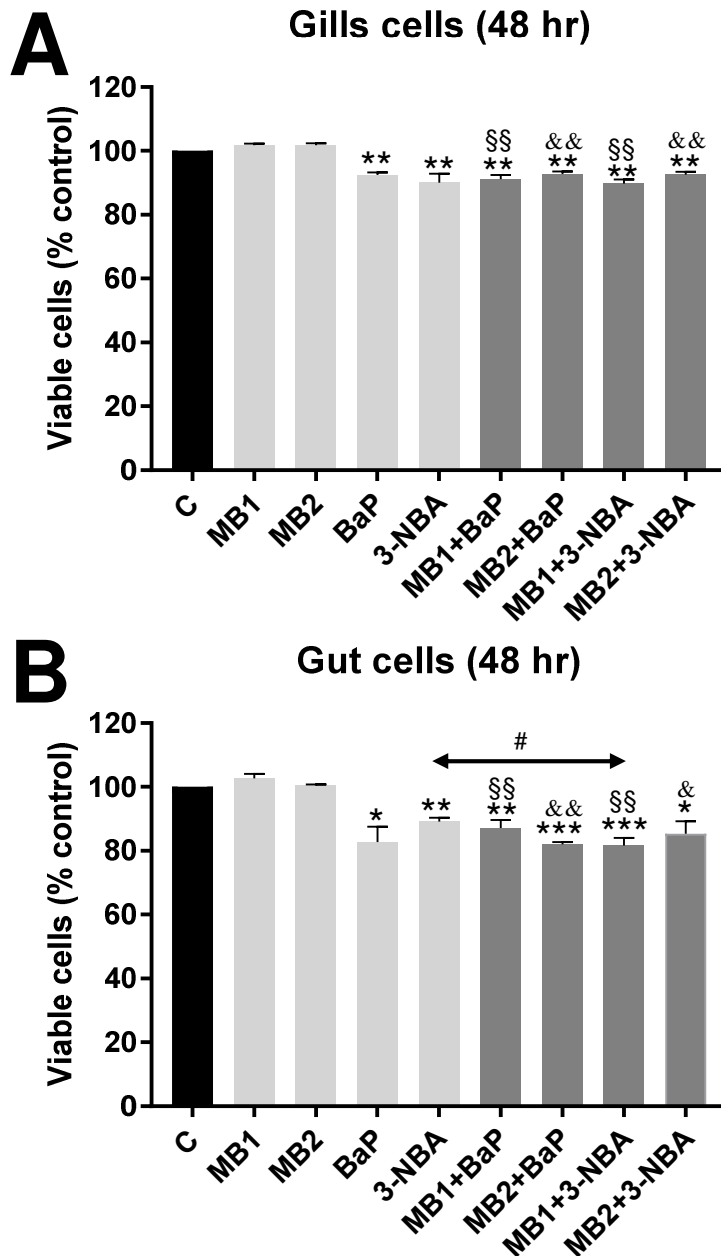


707

708 **Figure 2**

709 DNA adduct levels measured by ³²P-postlabelling in fish gill RTgill-W1 and intestinal
 710 RTgutGC cells exposed BaP and 3-NBA for 48 h as assessed. For BaP-derived DNA adducts
 711 nuclease P1 digestion, for 3-NBA-derived DNA adducts butanol extraction was used as
 712 enrichment procedure. Values represent mean ± SD (*n*=4) derived from four independent
 713 experiments with cells from different passage numbers. Inserts: Autoradiographic profiles of
 714 DNA adducts formed in fish gill RTgill-W1 and intestinal RTgutGC cells after exposure; the
 715 origin, at the bottom left-hand corner, was cut off before exposure. B1, 10-(deoxyguanosin-*N*²-
 716 yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydro-BaP (dG-*N*²-BPDE); N1, 2'(2'-deoxyadenosine-*N*⁶-
 717 yl)-3-aminobenzanthrone (dA-*N*⁶-3-ABA); N2, as-yet unidentified adenine adduct derived
 718 from nitroreduction; N3, *N*-(2'-deoxyguanosine-*N*²-yl)-3-aminobenzanthrone (dG-*N*²-3-
 719 ABA); N4, *N*-(2'-deoxyguanosin-8-yl)-3-aminobenzanthrone (dG-C8-*N*-3-ABA).

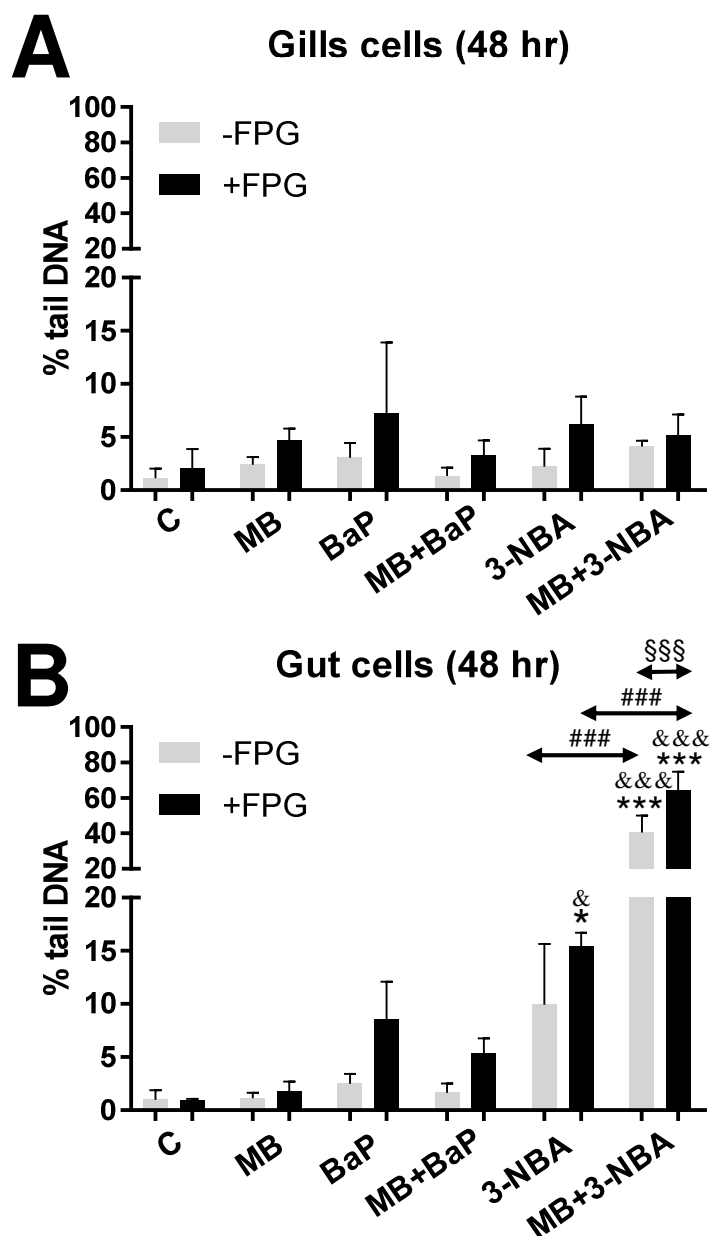
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722 **Figure 3**

723 Effect of PS-MB alone or in combination with BaP and 3-NBA exposure on cell viability (%
 724 control) of fish gill RTgill-W1 cells (A) and intestinal RTgutGC cells (B) at 48 h. PS-MBs
 725 were used at $5 \mu\text{g mL}^{-1}$ (MB1) or $50 \mu\text{g mL}^{-1}$ (MB2). Values represent mean \pm SD ($n=3$)
 726 derived from three independent experiments with cells from different passage numbers; 4
 727 technical replicates per sample were scored. For statistical analysis the cell viability data was
 728 normalised to 1.0, data then log₂ transformed and analysed using a single sample *t*-test with
 729 Bonferroni correction against the population control mean of 0 ($*p<0.05$, $**p<0.01$,
 730 $***p<0.001$, different from control; $§§p<0.01$, different from cells treated with MB1 alone;
 731 $&p<0.05$, $&&p<0.01$, different from cells treated with MB2 alone; $\#p<0.05$, different from cells
 732 treated with 3-NBA alone).

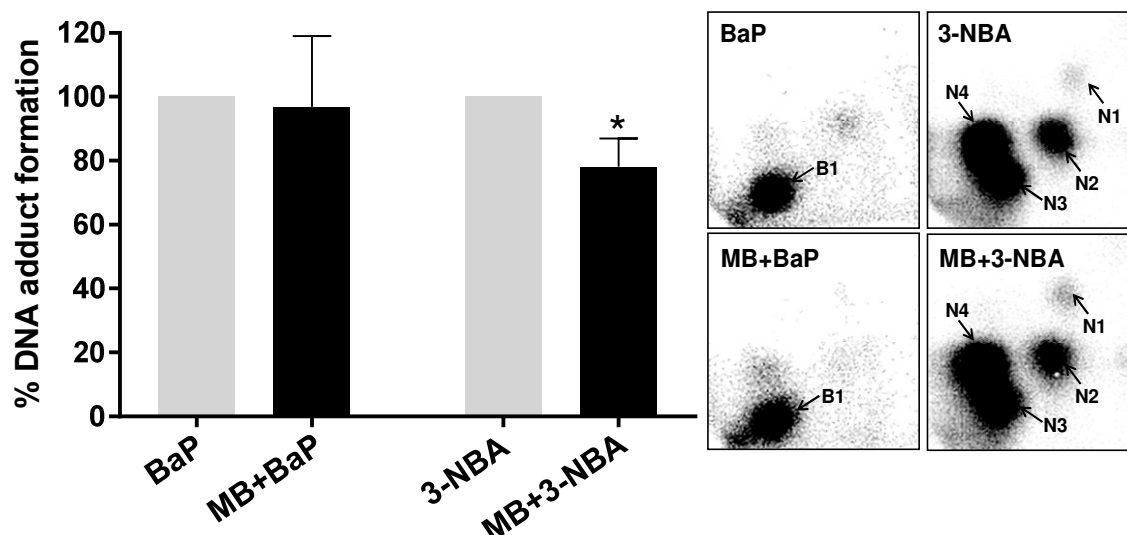


733

734 **Figure 4**

735 Effect of PS-MB alone (MB; 50 $\mu\text{g mL}^{-1}$) or in combination with BaP and 3-NBA exposure on
 736 DNA damage (% tail DNA) in fish gill RTgill-W1 (A) and intestinal RTgutGC cells (B) at 48
 737 h as assessed by the alkaline comet assay. The comet assay was used to detect alkali-labile
 738 lesions. Formamidopyrimidine glycosylase (FPG) which detects oxidative damage to DNA
 739 including 8-oxo-dG was added in additional experiments. Values represent mean \pm SD ($n=3$)
 740 derived from three independent experiments with cells from different passage numbers; 50
 741 nuclei per sample were scored. Statistical analysis was performed by two-way ANOVA
 742 followed by Tukey post-hoc test (* $p<0.05$, *** $p<0.001$, different from control; & $p<0.05$,

743 &&& p <0.001, different from cells treated with MB alone; ### p <0.001, different from cells treated
744 with 3-NBA alone; §§§ p <0.001, different from -FPG).



745
 746 **Figure 5**
 747 Effect of PS-MB (MB; 50 $\mu\text{g mL}^{-1}$) on BaP- and 3-NBA-derived DNA adduct formation (%)
 748 in fish intestinal RTgutGC cells exposed for 48 h. ^{32}P -postlabelling was used to assess DNA
 749 adduct formation; for BaP-derived DNA adducts nuclease P1 digestion, for 3-NBA-derived
 750 DNA adducts butanol extraction was used as enrichment procedure. Values represent mean \pm
 751 SD ($n=4$) derived from four independent experiments with cells from different passage
 752 numbers. For statistical analysis the adduct data was normalised to 1.0, data then log2
 753 transformed and analysed using a single sample t -test with Bonferroni correction against the
 754 population control mean of 0 ($*p<0.05$, different from cells treated with 3-NBA alone). Inserts:
 755 Autoradiographic profiles of DNA adducts formed in fish gill RTgill-W1 and intestinal
 756 RTgutGC cells after exposure; the origin, at the bottom left-hand corner, was cut off before
 757 exposure. B1, 10-(deoxyguanosin- N^2 -yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydro-BaP (dG- N^2 -
 758 BPDE); N1, 2'(2'-deoxyadenosine- N^6 -yl)-3-aminobenzanthrone (dA- N^6 -3-ABA); N2, as-yet
 759 unidentified adenine adduct derived from nitroreduction; N3, N -(2'-deoxyguanosine- N^2 -yl)-3-
 760 aminobenzanthrone (dG- N^2 -3-ABA); N4, N -(2'-deoxyguanosin-8-yl)-3-aminobenzanthrone
 761 (dG-C8- N -3-ABA).
 762

763 **Supplementary Information:**
764
765 **Co-exposure to polystyrene plastic beads and polycyclic aromatic**
766 **hydrocarbon contaminants in fish gill (RTgill-W1) and intestinal (RTgutGC)**
767 **epithelial cells derived from rainbow trout (*Oncorhynchus mykiss*)**
768
769 **Daniel Bussolaro, Stephanie L. Wright, Sabine Schnell, Kristin Schirmer, Nicolas R.**
770 **Bury, and Volker M. Arlt**
771

772 **Materials and Methods:**

773

774 **Cell viability assay.**

775 Both cell lines were cultured in 96 well plates and, after exposure, cell viability was assessed
776 using the Alamar Blue assay which is based on the reduction of resazurin to resorufin in
777 metabolically active cells. After exposure, cells were incubated with a 10% solution of Alamar
778 Blue reagent (Invitrogen, UK) in culture medium at 18°C for 3 h. Subsequently, fluorescence
779 was measured at excitation/emission wavelengths 530/590 nm using a Synergy HT plate reader
780 (Biotek, UK).

781

782 **DNA damage assessed via the comet assay.**

783 The DNA damage detected by the alkaline version of the comet assay includes single- and
784 double-strand breaks and alkali-labile (e.g. apurinic) sites [Arlt et al. 2004]. The lesion-specific
785 repair enzyme Formamidopyrimidine-DNA glycosylase (FPG) was employed to characterise
786 oxidative damage to DNA. The comet assay was performed essentially as described previously
787 [Ersson et al., 2013; Amaeze et al. 2015]. Briefly, three-window diagnostic slides
788 (X2XER203B, Thermo Fisher Scientific; UK) were coated with 1.5% (w/v) agarose (#16500,
789 Invitrogen, UK) per window and left to dry overnight at room temperature. Cells were collected
790 following exposure, resuspended in 0.65% (w/v) low melting agarose (A9414, Sigma-Aldrich,
791 UK) and applied per window of the diagnostic slide. Slides were placed in cold lysis buffer
792 (2.5 M sodium chloride, 10 mM Tris, and 100 mM EDTA, 250 mM NaOH, pH 10 with 1%
793 Triton X-100 and 10% DMSO) for 120 minutes on ice. For FPG treatment, 10 µL of FPG
794 enzyme (NorGenoTech AS, Norway; 10,000× diluted in buffer) in enzyme buffer (100 mM
795 KCl, 40 mM HEPES, 0.5 mM EDTA, pH 8 with 0.2 mg/mL bovine serum albumin) or enzyme
796 buffer alone as control were added and incubated for 30 minutes at 37°C in a humidity chamber.
797 The activity of the FPG enzyme was tested using H₂O₂-treated cells as positive control.
798 Alkaline unwinding was performed in 0.3 M sodium hydroxide, 1 mM EDTA for 30 minutes
799 at 4°C. Electrophoresis was performed in the same buffer and temperature for 24 minutes at 22
800 V, 300 mA, 1.4 V/cm² as reported previously (Ersson et al., 2013) using a horizontal
801 electrophoresis tank. Subsequently, slides were neutralized in 0.4 M Tris-HCl (pH 7.4) and
802 then fixed in 100% methanol for 10 minutes before air-drying overnight in the dark. Nuclei
803 were stained with ethidium bromide (10 µg/mL in water) and washed in deionized water. A
804 total of 50 nucleoids/sample were scored using a Leica DMLB fluorescent microscope and
805 Comet IV capture system (Perceptive Instruments, UK). Results were derived from three
806 independent experiments with cells from different passage numbers. All samples were measured
807 blind. Tail intensity (% tail DNA), defined as the percentage of DNA migrated from the head
808 of the comet into the tail, was used as a measure of DNA damage.

809 **Analysis of DNA adduct formation by ³²P-postlabelling.**

810 Genomic DNA was isolated from cells using a standard phenol/chloroform extraction
811 method. DNA adduct formation was analysed by ³²P-postlabelling as reported [Arlt et al.,
812 2014]. For BaP, adducts were enriched using nuclease P1 digestion, whereas for 3-NBA,
813 adducts were enriched using butanol extraction. For separation by thin-layer chromatography

814 (TLC) on polyethylenimine (PEI)-cellulose sheets (Macherey-Nagel, Düren, Germany) the
815 following solvents were used: for all experiments – D1, 1 M sodium phosphate, pH 6.5; D5,
816 1.7 M sodium phosphate, pH 6.0; for BaP – D3, 3.5 M lithium formate, 8.5 M urea, pH 3.5;
817 D4, 0.8 M lithium chloride, 0.5 M Tris, 8.5 M urea, pH 8.0; for 3-NBA – D3, 4 M lithium
818 formate, 7.0 M urea, pH 3.5; D4, 0.8 M lithium chloride, 0.5 M Tris, 8.5 M urea, pH 8.0. After
819 chromatography, TLC sheets were scanned using a Packard Instant Imager (Dowers Grove, IL,
820 USA) and DNA adduct levels (RAL, relative adduct labelling) were calculated from the adduct
821 cpm, the specific activity of [γ -³²P]ATP (Hartmann-Analytic, Braunschweig, Germany) and
822 the amount of DNA (pmol of DNA-P) used. No DNA adduct spots were observed in control
823 (untreated) cells. Results were derived from 4 independent experiments with cells from
824 different passage numbers.

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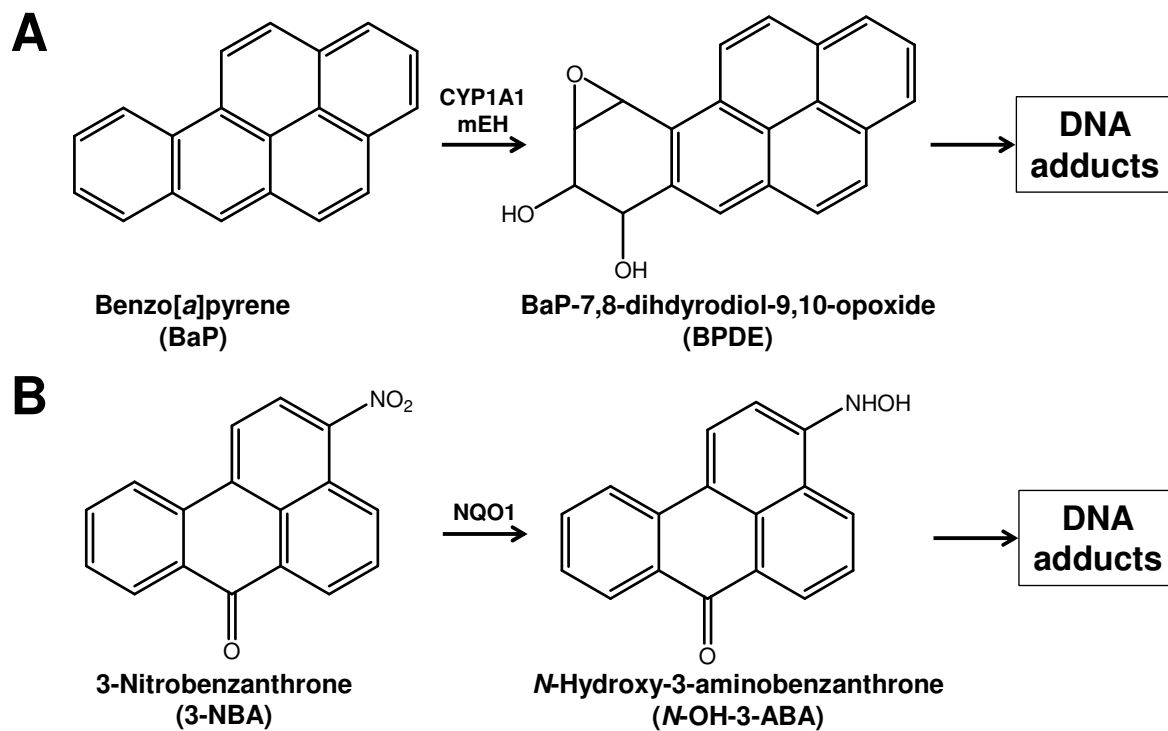
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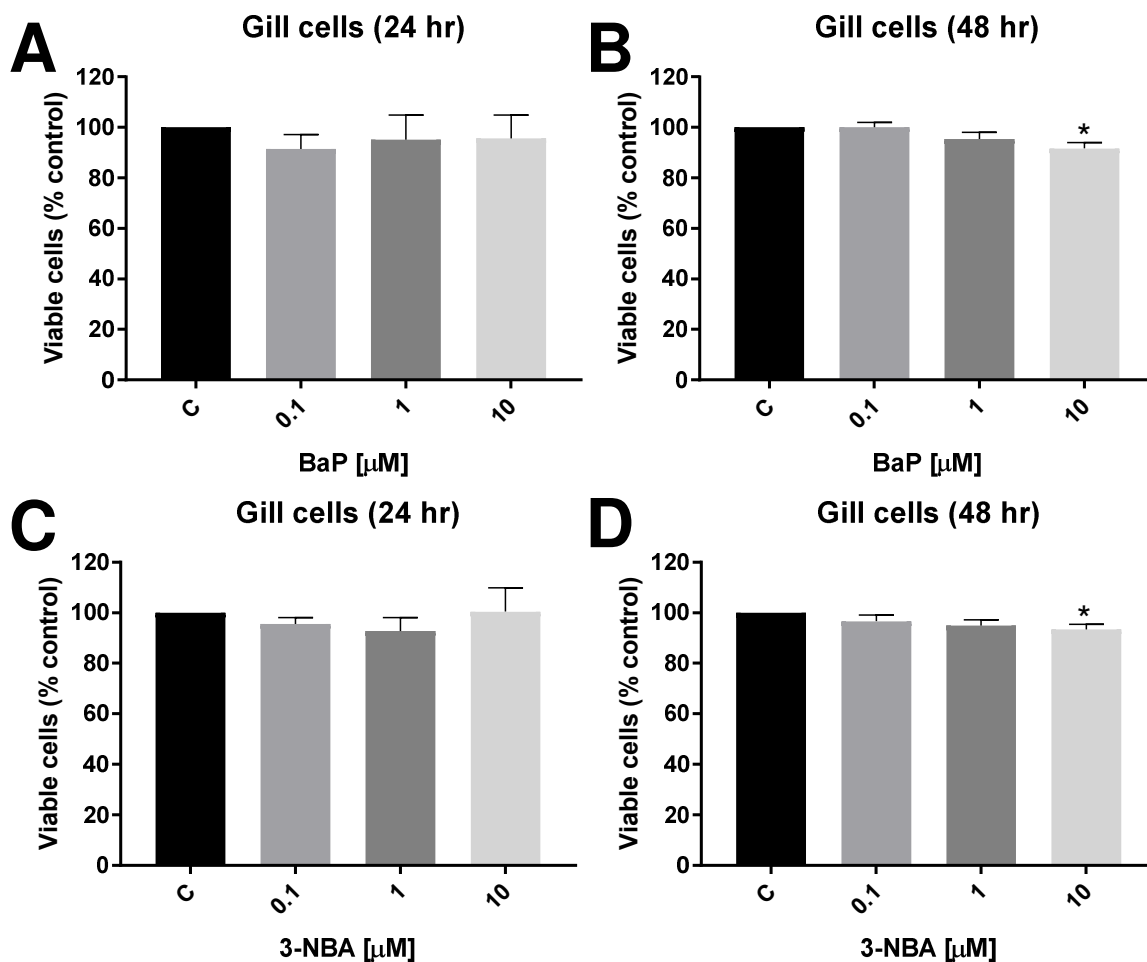
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841 **Figure S1.** Metabolic activation and DNA adduct formation of (A) BaP and (B) 3-NBA.

842



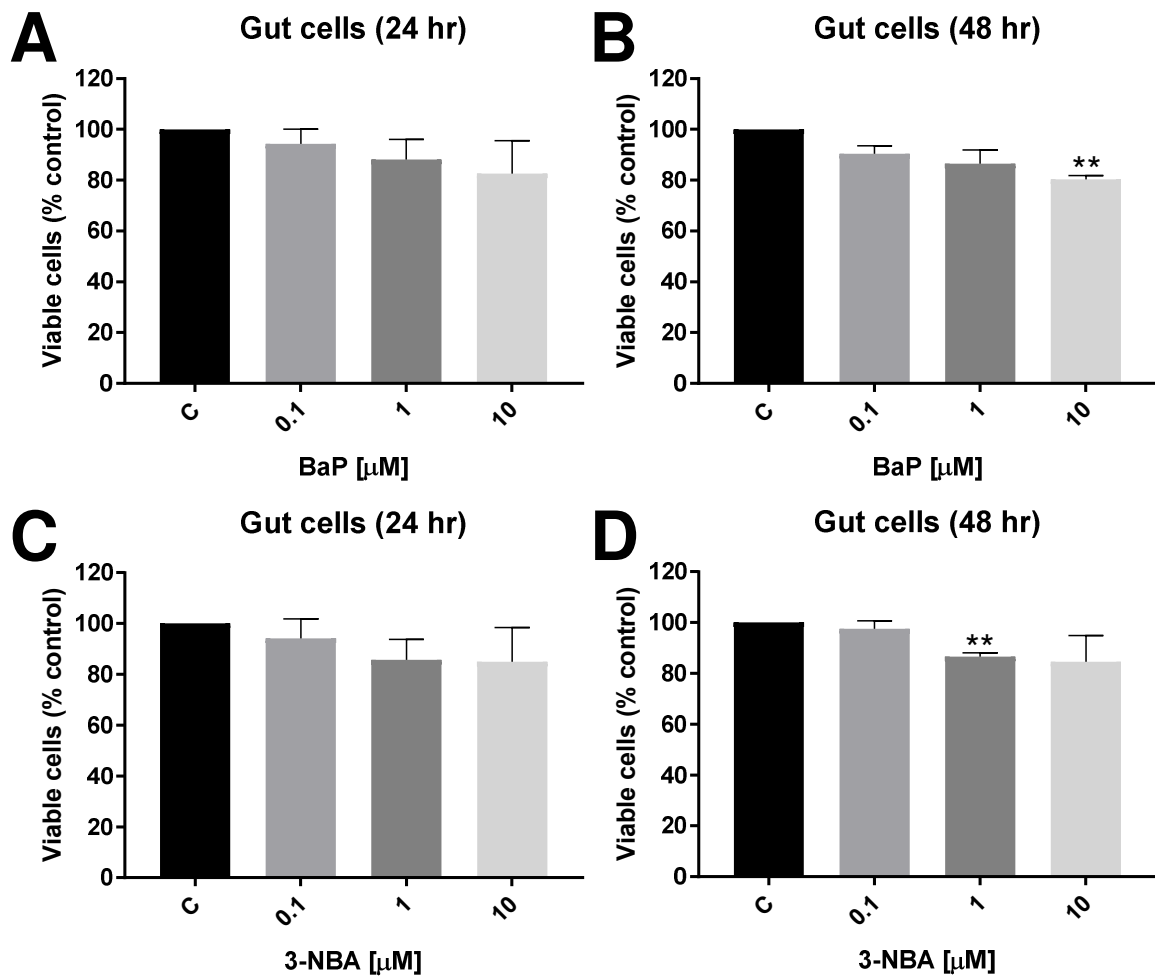
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844 **Figure S2**

845 Effect of BaP (A and B) and 3-NBA (C and D) on cell viability (% control) of fish gill RTgill-
 846 W1 cells at 24 (A and C) and 48 h (B and D). Values represent mean \pm SD ($n=3$) derived from
 847 three independent experiments with cells from different passage numbers; 4 technical
 848 replicates/sample were scored. For statistical analysis the cell viability data was normalised to
 849 1.0, data then the log₂ transformed and analysed using a single sample *t*-test with Bonferroni
 850 correction against the population control mean of 0 (* $p < 0.05$, different from control).

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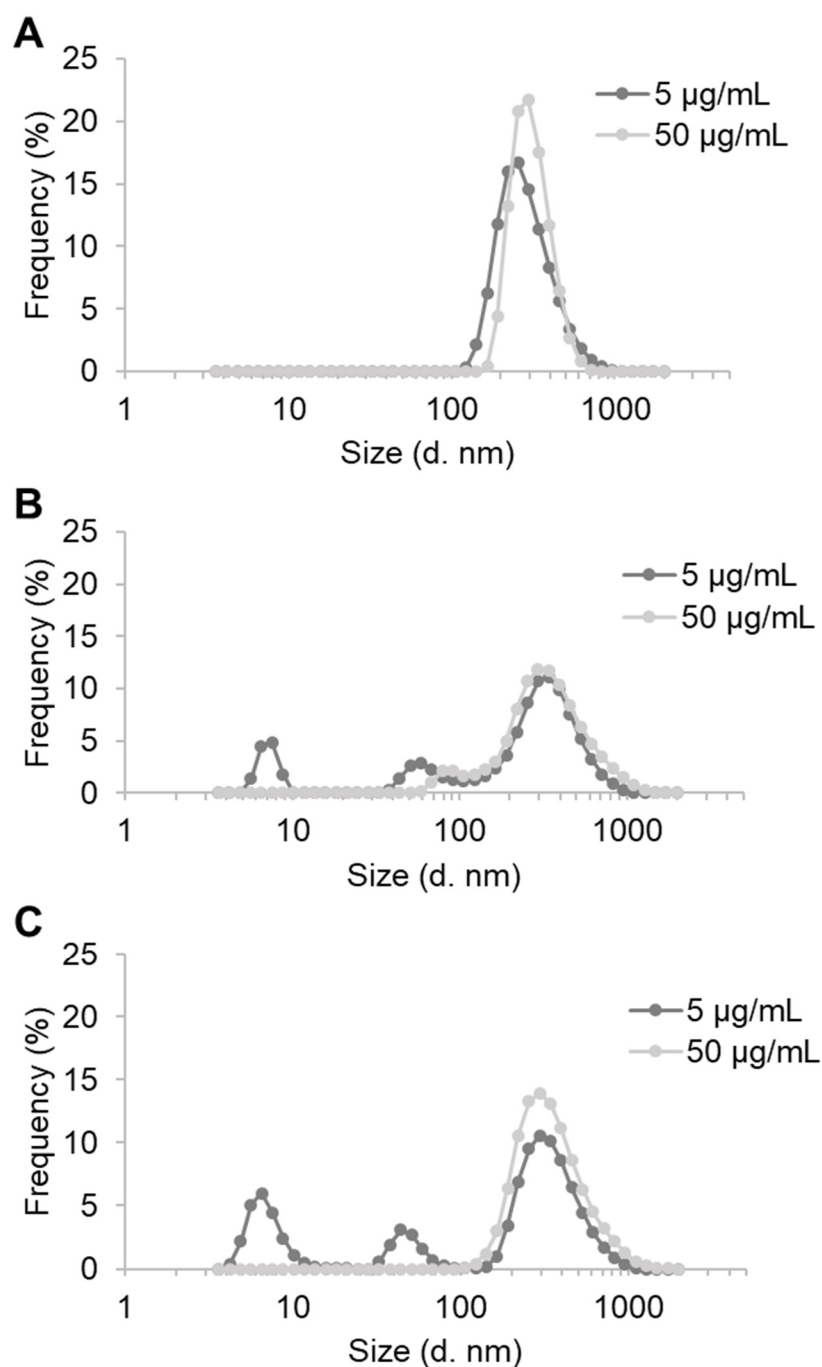
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854 **Figure S3**

855 Effect of BaP (A and B) and 3-NBA (C and D) on cell viability (% control) of fish intestinal
 856 RTgutGC cells at 24 (A and C) and 48 h (B and D). Values represent mean \pm SD ($n=3$) derived
 857 from three independent experiments with cells from different passage numbers; 4 technical
 858 replicates/sample were scored. For statistical analysis the cell viability data was normalised to
 859 1.0, data then log₂ transformed and analysed using a single sample *t*-test with Bonferroni
 860 correction against the population control mean of 0 (** $p<0.01$, different from control).

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863

864 **Figure S4.** The size distribution of PS MB suspensions (5 and 50 $\mu\text{g/mL}$) in DMEM medium
865 with 5% fetal bovine serum following A) 0 h; B) 24 h; and C) 48 h incubation at 18°C. '0 h'
866 was measured immediately following sonication of the stock PS MBs and subsequent
867 preparation of suspensions. Values show the mean of three independent replicates, resulting
868 from the mean of three technical replicates.

869

870 **Table S1.** Zeta-sizer data for PS-MBs (5 and 50 $\mu\text{g mL}^{-1}$) incubated in culture medium (+ 5%
 871 FBS) up to 48 h. Z-average is derived from 3 replicates in triplicate; mean and median are for
 872 the Z-averages; mode is derived from the size distribution data for Fig. S4.
 873

Time (h)	0		24		48	
Concentration ($\mu\text{g mL}^{-1}$)	5	50	5	50	5	50
Z-average (d.nm)	259.57	295.00	376.87	460.93	357.87	469.33
	262.00	294.63	368.00	466.30	341.93	459.10
	268.47	294.63	354.40	468.63	359.20	456.87
Mean	263.34	294.76	366.42	465.29	461.77	353.00
SD	4.60	0.21	11.32	3.95	6.65	9.61
Overall Mean (time)	279.05		415.86		407.38	
SD	17.45		54.68		60.03	
Median	262.00	294.63	368.00	466.30	459.10	357.87
Interquartile range	4.45	0.18	11.23	3.85	6.23	8.63
Mode	295.30	295.30	342.00	295.30	255.00	295.30

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