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Synergistic embryotoxicity of polycyclic
aromatic hydrocarbon aryl hydrocarbon receptor
agonists with cytochrome P4501A inhibitors in
Fundulus heteroclitus

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19 **Running title: Synergy of PAHs and CYP1A inhibitors**

20 **Key Words:**

21 Polycyclic aromatic hydrocarbons, aryl hydrocarbon receptor, *Fundulus heteroclitus*,
22 cytochrome P4501A, polychlorinated biphenyls, deformity, benzo(a)pyrene, β -
23 naphthoflavone, α -naphthoflavone, fluoranthene

24

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31

32

33 **Abbreviations:**

34 ATSDR – U.S. Agency for Toxic Substances and Disease Registry, AHR – aryl
35 hydrocarbon receptor, CYP1A – cytochrome P4501A, PAHs – polycyclic aromatic
36 hydrocarbons, pHAHs – planar halogenated aromatic hydrocarbons, PCB –
37 polychlorinated biphenyl, PCB126 –3,3',4,4',5-pentachlorobiphenyl, ANF – α -
38 naphthoflavone, BNF – β -naphthoflavone, BaP – benzo(a)pyrene, AA – 2-
39 aminoanthracene, PBO – piperonyl butoxide, FL – fluoranthene, DMSO – dimethyl
40 sulfoxide

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58 **Abstract:**

59 Widespread contamination of aquatic systems with polycyclic aromatic hydrocarbons
60 (PAHs) has led to concern about effects of PAHs on aquatic life. Some PAHs have been
61 shown to cause deformities in early life stages of fish that resemble those elicited by
62 planar halogenated aromatic hydrocarbons (pHAHs) that are agonists for the aryl
63 hydrocarbon receptor (AHR). Previous studies have suggested that activity of
64 cytochrome P4501A, a member of the AHR gene battery, is important to the toxicity of
65 pHAHs, and inhibition of CYP1A can reduce the early life stage toxicity of pHAHs. In
66 light of the effects of CYP1A inhibition on pHAH-derived toxicity, we explored the
67 impact of both model and environmentally relevant CYP1A inhibitors on PAH-derived
68 embryotoxicity. We exposed *Fundulus heteroclitus* embryos to two PAH-type AHR
69 agonists, β -naphthoflavone and benzo(a)pyrene, and one pHAH-type AHR agonist,
70 PCB126, alone and in combination with several CYP1A inhibitors. In agreement with
71 previous studies, co-exposure of embryos to PCB126 with the AHR antagonist and
72 CYP1A inhibitor α -naphthoflavone decreased frequency and severity of deformities over
73 PCB126-alone exposed embryos. In contrast, embryos co-exposed to the PAHs with
74 each of the CYP1A inhibitors tested were deformed with increased severity and
75 frequency than PAH-alone dosed embryos. The mechanism by which inhibition of
76 CYP1A increased embryotoxicity of the PAHs tested is not understood, but these results
77 may be helpful in elucidating mechanisms by which PAHs are embryotoxic.
78 Additionally, these results call into question additive models of PAH embryotoxicity for
79 environmental PAH mixtures that contain both AHR agonists and CYP1A inhibitors.

80 **Introduction:**

81 Polycyclic aromatic hydrocarbons (PAHs) are important environmental contaminants that
82 are generated by the incomplete combustion of organic compounds. PAHs enter the
83 environment through natural sources such as forest fires and seeps in ocean floors, and
84 through anthropogenic activities including combustion of fossil fuels and wood and
85 petroleum refining (Douben 2003; Latimer and Zheng 2003). PAH contamination in
86 estuarine settings originates from point sources such as municipal wastewater discharges,
87 industrial outfalls, and oil shipping and refinery operations, and from non-point sources
88 such as urban runoff and dry and wet depositions of atmospheric PAHs (Latimer and
89 Zheng 2003). The ubiquity of PAH contamination at U.S. national priority sites
90 (Superfund sites), along with their known and suspected human toxicity has led to the
91 listing of PAHs as number 8 on the U.S. Agency for Toxic Substances and Disease
92 Registry's (ATSDR) priority list; 15 individual PAHs are also listed throughout the
93 priority list of 275 entries (ATSDR 2003). Furthermore, environmental contamination
94 by PAHs has steadily increased in recent years (Van Metre et al. 2000).

95

96 Some PAHs have impacts on early life stages of fish including reduced growth, cranial-
97 facial malformations, yolk sac and pericardial edema, and subcutaneous hemorrhaging
98 (Billiard et al. 1999; Carls et al. 1999; Hawkins et al. 2002). These deformities closely
99 resemble the "blue sac syndrome" that has been described in several fish species
100 including rainbow trout (*Oncorhynchus mykiss*), zebrafish (*Danio rerio*), medaka
101 (*Oryzias latipes*), and killifish (*Fundulus heteroclitus*) exposed to certain halogenated
102 aromatic compounds that are agonists for the aryl hydrocarbon receptor (AHR) (Chen

103 and Cooper 1999; Elonen et al. 1998; Helder 1981; Toomey et al. 2001; Walker and
104 Peterson 1991; Wannemacher et al. 1992). These compounds include co-planar PCBs
105 and 2,3,7,8 tetrachlorodibenzo-*p*-dioxin (TCDD), collectively referred to here as planar
106 halogenated hydrocarbons, (pHAHs). Some of the PAHs that induce these deformities
107 are, like TCDD and co-planar PCBs, agonists for the AHR (Billiard et al. 2002).

108

109 The AHR is a cytoplasmic receptor whose activation initiates the transcription of a
110 battery of genes including the monooxygenase, cytochrome P4501A (here, generally
111 referred to as CYP1A, although two CYP1As exist in mammals as well as in rainbow
112 trout; mammalian CYP1As are referred to as CYP1A1 and CYP1A2; Hankinson 1995).

113 The AHR pathway is similar between mammals and non-mammalian vertebrates,
114 including fish, reptiles and birds (Hahn 1998), however two AHRs (AHR1 and AHR2)
115 have been identified and characterized in several fish species including *Fundulus* and
116 zebrafish (Andreasen et al. 2002; Hahn et al. 1997; Karchner et al. 1999). The
117 mechanism for the toxicity of pHAHs has been widely studied and there are well-
118 established positive relationships among compounds' affinity for the AHR, their potency
119 for CYP1A induction and their toxicity (Guiney et al. 1997; Heid et al. 2001; Safe
120 1990,1993).

121

122 The AHR's critical role in pHAH toxicity has been demonstrated by AHR knockout
123 studies in which AHR knockout mice do not show typical dioxin induced toxicity
124 compared to their AHR expressing littermates (Fernandez-Salguero et al. 1996). There is
125 evidence that some of the toxicity of these pHAHs may be directly due to CYP1A

126 activity; for example, CYP1A2 knockout mice are resistant to liver damage and
127 uroporphyrin when exposed to TCDD (Smith et al. 2001). And CYP1A1 knock out male
128 mice are protected against TCDD-mediated lethality and wasting syndrome (Uno et al.
129 2004b). Furthermore, Cantrell et al. (1996) were able to reduce TCDD-induced DNA
130 degradation and damage to the medial yolk vein in medaka by co-treating the embryos
131 with the P450 inhibitor piperonyl butoxide (PBO). Dong et al. (2002) found that co-
132 treatment of zebrafish embryos with the partial AHR antagonist and CYP1A inhibitor
133 alpha-naphthoflavone (ANF) or the P450 inhibitors SKF525A or miconazole reversed the
134 reduction of blood flow in the mesencephalic vein and midbrain apoptosis caused by
135 TCDD. Another study by Teraoka et al. (2003) showed that a morpholino knockdown of
136 CYP1A and AHR2 in zebrafish prevented the pericardial edema and trunk circulation
137 failure caused by TCDD.

138

139 Although there is a strong, positive relationship between PAHs' ability to bind the AHR
140 and their induction of CYP1A (Billiard et al. 2002), conclusions regarding the role of the
141 AHR pathway and CYP1A activity in the toxicity of PAHs have been less clear. In a
142 mammalian study, homozygous CYP1A1 knock out mice showed less liver damage and
143 survived the acute effects of injection of the PAH, benzo(a)pyrene (BaP) for three days
144 longer than those that were heterozygous for CYP1A1. However these CYP1A1 knock
145 out mice also showed 4-fold higher levels of BaP-DNA adducts than those heterozygous
146 for CYP1A1 (Uno et al. 2001). This study suggests that acute lethality of BaP was
147 reduced by lack of CYP1A1, but genotoxicity was actually increased by the lack of
148 CYP1A1. In a recent study by this group, BaP administered in the diet caused lethality in

149 CYP1A1 knock out mice at a dose that was not lethal to CYP1A1 expressing mice (Uno
150 et al. 2004a). The authors of this study suggested that rather than CYP1A1 activity
151 enhancing the toxicity of BaP, as has been previously suggested, CYP1A1 is critical for
152 the detoxication of orally administered BaP in mice.

153

154 Billiard (2002) compared a variety of PAHs with various affinities for the AHR and
155 potencies for CYP1A induction in juvenile rainbow trout; chemicals ranged from the
156 strong CYP1A inducer benzo[k]fluoranthene, to the relatively weak, alkylated inducer
157 retene and the non-inducer phenanthrene. She found that the rank order for CYP1A
158 induction in these fish did not predict the rank order for the induction of blue-sac-like
159 symptoms; in fact the only PAHs that caused blue-sac like symptoms were retene and
160 phenanthrene, the low- and non-inducing PAHs used in this study. Hawkins et al. (2002)
161 observed apparent additive toxicity in juvenile and larval rainbow trout co-exposed to one
162 of two PAHs, the alkylated AHR agonist retene, or the non-AHR agonist phenanthrene,
163 with the P450 inhibitor piperonyl butoxide (PBO). In contrast, another study found that
164 co-treatment with the partial AHR antagonist and CYP1A inhibitor ANF prevented the
165 reduction of circulation in the dorsal midbrain of zebrafish caused by the PAH-type AHR
166 agonist β -naphthoflavone (BNF; Dong et al. 2002). From these studies, it is clear that the
167 relationship between CYP1A activity and PAH toxicity is complex, and reduced CYP1A
168 activity is sometimes, but not always, protective of PAH toxicity.

169

170 In an attempt to: 1) clarify the role of CYP1A activity in the toxicity of PAHs, and 2)
171 explore the possible effects of co-occurring PAH-type CYP1A inducers and inhibitors,

172 we co-treated *Fundulus heteroclitus* (killifish) embryos with three different AHR agonists
173 (the pHAH, 3,3',4,4',5-pentachlorobiphenyl; PCB126; and the PAHs, BNF and BaP) and
174 four CYP1A inhibitors that work by various mechanisms (Figure 1). The compounds
175 here collectively referred to as CYP1A inhibitors have all been shown to inhibit CYP1A
176 activity (see references in Figure 1), however, the inhibitory compounds' specificities for
177 CYP1A over other P450s in our system are not known. These inhibitors included the
178 aforementioned model compounds, ANF and PBO, and the environmentally relevant
179 hydrocarbons, fluoranthene (FL) and 2-aminoanthracene (AA) (Watson et al. 1995;
180 Willett et al. 1998; Willett et al. 2001). Embryos were then observed for *in ovo* CYP1A
181 activity, as measured by ethoxyresorufin-O-deethylase (EROD) activity and for
182 deformities, including pericardial edema, heart elongation, cranial facial malformations,
183 and tail abnormalities. In these experiments a wide range of concentrations of AHR
184 agonists were used to elicit a range of EROD inductions with and without inducing
185 deformities; concentrations of inhibitors were selected with the goal of eliciting the
186 maximal inhibition of EROD without inducing deformities. Our results indicate that co-
187 exposure to PAH-type AHR agonists and CYP1A inhibitors consistently enhanced
188 embryotoxicity beyond levels predicted by an additive toxicity model.

189

190 **Materials and Methods:**

191 **Reagents**

192 BaP, BNF, ANF, FL, AA, PBO, and ethoxyresorufin were purchased from Sigma Aldrich
193 (Saint Louis, MO). PCB126 was purchased from Chem Service (West Chester, PA).

194 Dimethyl sulfoxide (DMSO) and acetone were purchased from Mallinckrodt Baker
195 (Phillipsburg, NJ).

196

197 **Fish care**

198 Adult killifish were captured with minnow traps from King's Creek, VA (a well
199 characterized reference with low sediment PAH levels; Mulvey et al. 2002) and
200 transported to the Ecotoxicology Laboratory of Duke University. Fish were maintained
201 in 70 or 100 liter aquaria at 24° C with a 16 h. light 8 h. dark cycle and were fed
202 TetraMin flakes (Tetra Sales, Blacksburg, VA) *ad libitum*. Fish were held in laboratory
203 conditions for at least 3 weeks prior to embryo acquisition. Embryos were obtained from
204 *in vitro* fertilization of pooled oocytes stripped from 9-12 females with pooled milt from
205 4-5 males.

206

207 ***In ovo* EROD**

208 An *in ovo* ethoxyresorufin-O-deethylase (EROD) method, modified slightly from the
209 method described in Nacci et al. (1998; accepted), was used to measure the CYP1A
210 activity of embryos. Several hours after fertilization, embryos with dividing cells were
211 selected and placed individually in 20 mL scintillation vials with 10 mL artificial sea
212 water (Instant Ocean 20 ppt) containing 21 µg/L ethoxyresorufin with or without an
213 EROD inducer, (BNF, BaP or PCB126) and/or an EROD inhibitor, (ANF, AA, FL or
214 PBO). Acetone or DMSO were used as solvents and solvent concentrations were less
215 than 0.015% for all treatments except the high doses in the ANF-alone dose response
216 (Figure 2) in which solvent concentrations were 0.1% or less. Embryos were in dosing

217 solution for 7 days during which resorufin, the fluorescent product of CYP1A
218 metabolism of ethoxyresorufin, accumulated in the embryos' bi-lobed urinary bladders.
219 On day 7 of development, embryos were placed in clean artificial seawater and embryo
220 bladders were visualized by fluorescent microscopy (Zeiss Axioskop, 50x magnification
221 using rhodamine red filter set). EROD activity was measured as intensity of the bladder
222 fluorescence and was quantified digitally by IP lab software (Scanalytics, Inc., Fairfax,
223 VA). *In ovo* EROD values were expressed as a percent of control intensity. Individuals
224 with deformed bladders or with fluorescence in areas other than the bladder (such as the
225 pericardial sac in some embryos with severe pericardial edema) were excluded from *in*
226 *ovo* EROD measurement. Although ethoxyresorufin has been shown to be non-
227 detrimental to embryos (Nacci et al. 1998), co-exposures of ANF and BNF were done
228 with and without ethoxyresorufin to rule out a possible interactive effect of the
229 ethoxyresorufin. No differences were observed between the deformities of embryos with
230 or without ethoxyresorufin (data not shown).

231

232 **Deformity assessment**

233 Embryos were scored blind for heart elongation (tube heart), pericardial edema, tail
234 shortening and hemorrhaging on day 10 of development. Heart deformities were found
235 to be the most sensitive endpoint scored, so this endpoint was used for further analysis.
236 Heart elongation severity was ranked between 0 and 5, and a deformity index for each
237 treatment was calculated as sum of scores for individuals in that treatment group divided
238 by the maximum score possible (the number of individuals multiplied by 5). This
239 quotient was then multiplied by 100.

240

241 **Experimental approach**

242 Embryos were exposed to nominal concentrations of one of three AHR agonists alone
243 and in combination with nominal concentrations of one of four CYP1A inhibitors. The
244 AHR agonists used are listed in Figure 1. BNF and BaP were chosen as model PAH-type
245 AHR agonists. BNF is a synthetic compound, commonly used as a model AHR agonist
246 in studies, while BaP is a naturally occurring PAH, commonly found in environmental
247 mixtures. PCB126 was chosen as a model pHAH-type AHR agonist.

248

249 Inhibitors used in this study and their mechanisms of actions are listed in Figure 1. ANF
250 was chosen because it is well characterized for its activities as both a partial AHR
251 antagonist (Merchant et al. 1990; Merchant et al. 1992), and a competitive CYP1A
252 inhibitor (Goujon et al. 1972; Testa and Jenner 1981). BNF and ANF dose response
253 curves were first established using a range of concentrations and scoring for deformities
254 and *in ovo* EROD (Figure 2). Subsequently, co-exposures were performed using a range
255 of BNF concentrations that spanned concentrations found to induce EROD, but not
256 deformities, to concentrations that caused both EROD induction and deformities, with a
257 concentration of ANF (100 µg/L) that dramatically lowered *in ovo* EROD measurements,
258 but did not by itself cause deformities (Figure 3).

259

260 In order to distinguish between the effects of AHR antagonism and CYP1A inhibitory
261 effects, both of which occur with ANF exposure, we also used the P450 inhibitor PBO.
262 PBO is a quasi-irreversible P450 inhibitor that acts by forming a metabolic intermediate

263 complex with the heme group of P450 enzymes, thereby preventing the redox cycling of
264 the enzyme (Hodgson and Philpot 1974; Testa and Jenner 1981). We co-treated embryos
265 with a range of BNF concentrations (1-100 µg/L) and either 1 or 9 mg/L PBO (Figure 4).

266

267 To test the effects of EROD inhibition on embryos co-exposed to an environmentally
268 relevant AHR agonist, BaP and ANF co-exposures were conducted. In this experiment
269 the ANF concentration was 100 µg/L, a concentration previously established as effective
270 at lowering *in ovo* EROD without inducing deformities. BaP concentrations ranged from
271 1-100 µg/L (Figure 5).

272

273 To test the effectiveness of environmentally relevant PAHs at inhibition of *in ovo* EROD,
274 and to determine how inhibition by these compounds affected deformities, embryos were
275 exposed to a range of FL and AA concentrations alone and with 1 µg/L BNF (Figure 6).

276

277 In order to assess interactions between a representative pHAH and a CYP1A inhibitor in
278 *Fundulus*, embryos were exposed to concentrations of PCB126 that spanned from
279 concentrations known to induce EROD that cause low deformity indices, to
280 concentrations that induce severe deformities, with and without 100 µg/L ANF (Figure
281 7).

282

283 **Data analysis and representation**

284 Data were analyzed using Statview[®] for windows (Version 5.0.1, SAS Institute Inc.,
285 Cary, NC). EROD values were analyzed by one- and two-way analysis of variance

286 (ANOVA). When ANOVA yielded significance ($p < 0.05$) Fisher's Protected Least-
287 Significant Differences was used as a post-hoc test. Deformity data were ordinal in
288 nature and were therefore assessed using rank order tests, the Mann Whitney U test for
289 analyses with 2 variables and the Kruskal-Wallis test for analyses with 3 or more
290 variables. P values corrected for ties in rank are reported for these analyses as "tied p
291 values." Each graph represents a separate experiment. While deformities were analyzed
292 statistically using individual severity rankings, deformity data are shown as a deformity
293 index for clarity. Interactions were characterized as synergistic based on significance of a
294 one group chi square analysis comparing the observed frequencies of deformities to
295 frequencies predicted by an additive interaction (calculated as a sum of the deformity
296 frequency for each treatment; predicted frequency had minimum value of 1 for this
297 analysis as chi square calculation requires predicted frequency in the denominator of an
298 equation).

299

300 **Results:**

301 Embryos dosed with BNF alone showed *in ovo* EROD induction at all concentrations
302 tested ($p \leq 0.0002$) that was maximal at the 10 $\mu\text{g/L}$ concentration (Figure 2). At 50 and
303 100 $\mu\text{g/L}$, EROD activities declined to below the maximal level ($p = 0.0001$ and 0.0003 ,
304 respectively). Coincident with this decline, embryos exposed to 50 and 100 $\mu\text{g/L}$ BNF
305 exhibited elevated deformity indices (effect of BNF on deformities, tied $p < 0.0001$).
306 Embryos exposed to ANF alone displayed lower EROD activities than controls at the
307 lowest exposure concentrations (10, 100 and 500 $\mu\text{g/L}$; $p < 0.0001$). Embryos exposed to
308 ANF levels higher than 500 $\mu\text{g/L}$ were too deformed to allow for measurement of *in ovo*

309 EROD. Embryos exposed to 10 µg/L ANF and 100 µg/L ANF exhibited no deformities,
310 while those exposed to 500 µg/L ANF and higher exhibited high deformity indices (effect
311 of ANF on deformities tied $p < 0.0001$).

312

313 In a separate experiment designed to explore the interaction between ANF and BNF co-
314 exposures, embryos were dosed with a range of BNF concentrations with or without 100
315 µg/L ANF (Figure 3), using the dose of ANF shown (Figure 2) to be most effective in
316 inhibiting EROD without causing deformities by itself. Embryos exposed to BNF alone
317 exhibited significant EROD induction at all concentrations ($p < 0.0001$). Co-treatment
318 with ANF significantly inhibited *in ovo* EROD activities ($p < 0.0001$). Embryos co-
319 treated with ANF and 110 µg/L BNF were too deformed for *in ovo* EROD measurements.

320 In BNF-alone treated embryos, deformities were noted only at the 110 µg/L
321 concentration (effect of BNF-alone on deformities, tied p value = 0.0011). However,
322 ANF co-treated embryos were deformed at all BNF concentrations. That is, embryos
323 were deformed at BNF concentrations three orders of magnitude lower when BNF
324 treatment was combined with 100 µg/L ANF than when treated with BNF alone (overall
325 effect of BNF and ANF on deformities tied $p < 0.0001$ for each).

326

327 In an experiment exploring the effect of co-treatment of embryos with BNF and PBO
328 (Figure 4), all BNF concentrations significantly induced *in ovo* EROD activities ($p <$
329 0.0001). Co-treatment with both concentrations of PBO (1 and 9 mg/L) lowered *in ovo*
330 EROD across all BNF concentrations ($p < 0.0001$). Embryos exposed to PBO at the low
331 concentration had very low deformities that were not statistically different from controls

332 (tied $p = 0.3173$). Embryos exposed to the high concentration of PBO had an elevated
333 deformity index (effect of PBO-alone on deformities, tied $p = 0.0448$). Co-exposures to
334 BNF and PBO caused increased deformity indices over those seen in BNF-alone or PBO-
335 alone dosed embryos at all BNF concentrations (overall effect of BNF and PBO on
336 deformities, tied $p < 0.0001$ and $= 0.0021$ respectively).

337

338 We also examined a range of concentrations (1-100 $\mu\text{g/L}$) of the environmentally
339 relevant PAH, BaP, with and without co-exposure to 100 $\mu\text{g/L}$ ANF (Figure 5). BaP
340 alone significantly induced EROD at all doses tested ($p < 0.0001$), and ANF co-treatment
341 lowered the *in ovo* EROD activity ($p < 0.0001$). Embryos dosed with BaP alone
342 exhibited low deformity indices that were not statistically different from controls (effect
343 of BaP alone on deformities, $p = 0.1856$), while those dosed with BaP in combination
344 with 100 $\mu\text{g/L}$ ANF had elevated deformity indices at all BaP concentrations tested
345 (overall effect of ANF on deformities, tied $p < 0.0001$).

346

347 Exposure to environmentally relevant CYP1A inhibitor FL by itself caused *in ovo* EROD
348 activities below control levels ($p < 0.0001$; Figure 6); however when embryos were co-
349 exposed to FL with 1 $\mu\text{g/L}$ of the inducer BNF, EROD activities were induced ($p <$
350 0.0001) and there was FL-dose dependant decrease in *in ovo* EROD activities ($p <$
351 0.0001). Embryos exposed to FL by itself did not exhibit elevated deformity indices
352 (effect of FL alone on deformities, tied $p = 0.3764$), BNF at this concentration also did
353 not cause an elevated deformity index (effect of BNF alone on deformities tied $p =$
354 0.1681), however, when FL exposure was combined with 1 $\mu\text{g/L}$ BNF high deformity

355 indices were observed at FL levels of 50 µg/L and higher (overall effect of FL on
356 deformities tied $p = 0.0002$, overall effect of BNF on deformities tied $p < 0.0001$).

357

358 Exposure to AA by itself elicited slight EROD induction at the 10 and 50 µg/L
359 concentrations ($p < 0.0001$ and $p = 0.0163$ respectively; Figure 6), however when
360 embryos were co-exposed to 1 µg/L BNF, the BNF-mediated EROD induction in EROD
361 was inhibited in a dose-dependent fashion by increasing AA concentrations ($p < 0.0001$).
362 AA-alone dosed embryos exhibited low deformity indices (the effect of AA alone on
363 deformities was not significant, tied $p = 0.6609$), but when embryos were co-exposed to
364 AA with 1 µg/L BNF, deformity indices were elevated in co-treatments of BNF with AA
365 concentrations of 50 µg/L and greater (overall effect of AA and BNF on deformities, tied
366 $p < 0.0001$ for each).

367

368 The pHAH, PCB126 significantly induced *in ovo* EROD over controls at all doses tested
369 ($p < 0.0001$; Figure 7). Concentrations of 300 and 600 ng/L induced EROD levels less
370 than the maximal levels achieved by 30 and 100 ng/L ($p < 0.0001$ for each). Deformity
371 indices were elevated in embryos exposed to PCB concentrations of 100 ng/L and higher
372 (effect of PCB alone on deformities, tied $p > 0.0001$). In the case of PCB126, however,
373 co-exposure with 100 µg/L ANF dramatically decreased the deformity indices of PCB
374 treatment groups (overall effect of PCB and ANF on deformities, tied $p < 0.0001$ and =
375 0.0003 respectively).

376

377 Synergistic interactions, determined by one group chi square analyses, yielded deformity
378 frequencies greater than predicted additive values for BNF+ANF, BaP+ANF, BNF+FL
379 and BNF+AA co-treatments ($p < 0.001$ for each; Figures 3, 5 and 6, respectively). The
380 interaction for BNF+1 mg/L PBO co-treatment approached significance ($p = 0.051$),
381 however the BNF+9 mg/L PBO co-treatment was not synergistic (Figure 4).

382

383 **Discussion:**

384 The results of this study demonstrate that the embryotoxicity of the pHAH PCB126 was
385 decreased with co-exposure to the CYP1A inhibitor and AHR antagonist ANF. This
386 result is in general agreement with other studies showing the reduction of early life stage
387 toxicity of pHAHs when CYP1A activity or AHR-mediated signaling was decreased
388 (Cantrell et al. 1996; Dong et al. 2002; Teraoka et al. 2003). In contrast, in this study the
389 embryotoxicities of two PAH-type AHR agonists were increased when CYP1A was
390 inhibited by chemicals that act by various modes of action. The data for the interactions
391 between the PAH-type inducers and inhibitors clearly indicate a synergistic effect on
392 embryotoxicity for co-exposures to BNF+ANF, BaP+ANF, BNF+FL and BNF+AA. The
393 BNF+1 mg/L PBO dose was nearly significant for synergism ($p = 0.051$).

394

395 The various inhibitors used in this study caused similar increases in PAH toxicity, though
396 these inhibitors varied in structure and mechanism of inhibition. This suggests that the
397 increased toxicity of PAHs by CYP1A inhibitors is due to the shared characteristic of
398 CYP1A inhibition, and is not specific for a particular structure or mechanism of
399 inhibition. The PAH interaction with CYP1A inhibitors observed in this study are in

400 general agreement with those of a previous study in which we showed that the toxicity of
401 an extract from a highly PAH-contaminated site were more toxic when co-exposed with
402 several CYP1A inhibitors (Wassenberg and Di Giulio 2004).

403

404 While the pHAH PCB126 and the PAHs BNF and BaP share the characteristic of being
405 AHR agonists, the difference between the effect of CYP1A inhibition in the pHAH
406 versus the PAH dosed embryos is striking. This difference may be due to the
407 fundamentally different chemistries and somewhat different toxicities of these two
408 classes of compounds. PCBs and other halogenated compounds are relatively stable,
409 long lived compounds. Although pHAHs induce monooxygenases such as CYP1A,
410 metabolism of these compounds is relatively slow (White et al. 1997). The half life of
411 PCB126 administered to juvenile rainbow trout in their diet was found to be between 82-
412 180 days (Brown et al. 2002). In contrast, PAHs are rapidly metabolized. Half lives of
413 nine PAHs orally administered to adult rainbow trout were estimated to be 9 days or less
414 (Niimi and Palazzo 1986). *In vitro* metabolism of BaP was found to be 2000-4000 times
415 faster than metabolism of the co-planar pHAH PCB77 in induced scup (*Stenotomus*
416 *chrysops*) microsomes (Stegeman et al. 1981; White et al. 1997). This rapid metabolism
417 of PAHs allows for more rapid excretion of the compound, but can also activate PAHs
418 into more reactive intermediates that can bind to and damage cellular constituents.
419 Studies of PAH metabolism by fish embryos are very limited. However, Fong et al.
420 (1993) demonstrated extensive phase 1 and phase 2 metabolism of 7,12—
421 dimethylbenz[a]anthracene by rainbow trout embryos. Additionally, the presence and
422 inducibility of CYP1A in killifish embryos observed in this and previous studies (Nacci

423 et al., 1998; Toomey et al., 2001; Meyer et al.) support the supposition that PAH
424 metabolism is occurring in embryos in this study. Therefore, it is possible that inhibition
425 of CYP1A in the PAH-treated embryos extended the half-life of the PAH, causing
426 prolonged AHR agonism, similar to AHR agonism in pHAH-treated animals.

427

428 Some PAHs act through a narcotic mechanism in which the compounds accumulate in
429 tissues to a level at which they physically interfere with membranes (McCarty and
430 Mackay 1993). The inclusion of a CYP1A inhibitor with PAHs would be likely to slow
431 metabolism of the PAHs. However, it is not likely that narcosis is responsible for the
432 synergy observed in these experiments. First, even if the total amount of compound to
433 which the embryos were exposed in deformed treatment groups accumulated within the
434 embryo, the concentration of PAH would not reach the 2-8 mMol/kg threshold for acute
435 narcosis (McCarty and Mackay 1993). Second, narcotic modes of action are, by
436 definition additive, and an additive model of toxicity does not fit our data.

437

438 It has been suggested that the toxicity of pHAHs is at least in part tied to an oxidative
439 stress mode of damage (Nebert et al. 2000; Stohs 1990). The pHAHs fit into the active
440 site for CYP1A, but are poor substrates for CYP1A metabolism, causing an uncoupling
441 of electron flow between the enzyme and the substrate. This uncoupling, together with
442 increased expression of CYP1A via the AHR, are believed to lead to the production of
443 reactive oxygen and oxidative damage (Schlezinger and Stegeman 2001; Schlezinger et
444 al. 1999; Shertzer et al. 2004). We included PBO as an inhibitor in our studies because
445 PBO binds to the heme group of P450s, thereby inhibiting electron flow from the enzyme

446 and preventing this uncoupling. Since PBO enhanced toxicity in PAH co-treated
447 embryos, P450 uncoupling is not supported as the mechanism underlying the interactive
448 toxicity of PAHs and CYP1A inhibitors observed in this study.

449

450 However, other mechanisms of oxidative stress may play a role in PAH-driven toxicity.

451 An oxidative stress mechanism for the toxicity of the alkylated PAH retene was proposed
452 based on reduced glutathione to glutathione disulfide ratios (GSH:GSSG) in rainbow
453 trout larvae at retene exposures that exhibited blue-sac like symptoms (Billiard 2002).

454 Many PAHs (including BaP) can be metabolized to quinones (Bolton et al. 2000). These
455 reactive metabolic intermediates are capable of further AHR agonism, redox cycling and
456 generation of reactive oxygen species, which can then perturb cellular redox status,

457 damage macromolecules and are cytotoxic and mutagenic (Bolton et al. 2000; Burezynski
458 and Penning 2000). The metabolism of PAHs to reactive compounds is clearly

459 associated with their genotoxicity and carcinogenicity (Levin et al. 1982; Sjögren et al.
460 1996). Inhibition of CYP1A would likely alter the metabolism of PAHs, possibly

461 generating more embryotoxic intermediates. However, the extent to which altered

462 metabolism impacted the PAH toxicity observed in this study is not known. Current

463 studies are addressing mechanisms underlying the interactive toxicities reported herein.

464

465 **Importance of findings**

466 PAH contamination levels are increasing in aquatic systems across the United States

467 (Van Metre et al. 2000). Sites with PAH mixtures generally contain agonists for the AHR

468 that can induce CYP1A activity such as BaP, chrysene and benzo(k)fluoranthene. These

469 mixtures may also contain compounds that can act as CYP1A inhibitors. The non-
470 competitive CYP1A inhibitor fluoranthene, for example, is one of the more prevalent
471 PAHs found in marine sediments, lakes and rain water (Latimer and Zheng 2003; Van
472 Metre et al. 2000). Aminoanthracenes are components in coal liquefaction products
473 (Pelroy and Wilson 1981; Wilson 1980) and may also be found in environmental
474 mixtures. It is possible that other compounds found in environmental mixtures may also
475 be, as yet, uncharacterized CYP1A inhibitors. The synergisms found in this study
476 indicate that compounds such as BaP, FL, AA that can be commonly found in
477 environmental mixtures may be substantially more toxic in their mixtures than an
478 additive approach to PAH toxicity would predict, and that additive models currently used
479 to estimate PAH toxicity (for examples, Barron et al. 2004; Di Toro et al. 2000) may
480 underestimate the toxicity of PAH mixtures. Additionally, the observed endpoint for this
481 synergy was cardiovascular development during early development, a sensitive life stage
482 for vertebrates in general.

483

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797
798

799 **Figure legends:**

800 Figure 1

801 AHR agonists and CYP1A inhibitors used in this study.

802

803 Figure 2

804 Dose response curves showing percent control *in ovo* EROD induction and deformity
805 index in embryos exposed to BNF (A) or ANF (B). Missing EROD values at the 1000,
806 5000, and 10,000 µg/L concentrations were because embryos from these treatment
807 groups were too deformed to score for *in ovo* EROD. For BNF control group, n = 20, for
808 all other BNF treatments n = 9 or 10. For each ANF treatment group, n = 8-10. EROD
809 values are means ± S.E.M. Statistical differences are explained in results section.

810

811 Figure 3

812 Effects of BNF with and without 100 µg/L ANF co-treatment on *in ovo* EROD and
813 deformity index. The missing EROD value from 110 µg/L BNF + ANF treatment group
814 was because embryos in this treatment group were too deformed to score for *in ovo*
815 EROD. N = 8 or 9 for each treatment group except for EROD measurement in the 1.1
816 µg/L BNF + ANF (n = 6) and 11 µg/L BNF + ANF (n = 2) treatments because the
817 remainder of embryos were too deformed to score for *in ovo* EROD. EROD values are
818 means ± S.E.M. Statistical differences are explained in results section.

819

820 Figure 4

821 Effects of BNF with and without 1 or 9 mg/L PBO co-treatment on *in ovo* EROD and
822 deformity index. N = 7-10 for each treatment group except for EROD measurements in
823 the 50 µg/L BNF + 9 mg/L PBO (n = 5) and 100 µg/L BNF + PBO (n = 6) treatments
824 because the remainder of embryos were too deformed to score for *in ovo* EROD. EROD
825 values are means ± S.E.M. Statistical differences are explained in results section.

826

827 Figure 5

828 Effects of BaP with and without 100 µg/L ANF co-treatment on *in ovo* EROD and
829 deformity index. N = 9 or 10 for each treatment group except for EROD measurement in
830 the 5 µg/L BaP + ANF (n = 7), 10 µg/L BaP + ANF (n = 7) and the 100 µg/L BaP + ANF
831 (n = 3) treatments because the remainder of embryos were too deformed to score for *in*
832 *ovo* EROD. EROD values are means ± S.E.M. Statistical differences are explained in
833 results section.

834

835

836 Figure 6

837 Effects of FL (A) and AA (B) with and without 1 µg/L BNF co-treatment on *in ovo*
838 EROD and deformity index. For experiment in graph A, n = 9 or 10 for each treatment
839 group except for EROD measurement in the 1 µg/L BNF + 1000 µg/L FL, treatment in
840 which n = 4, because the remainder of embryos were too deformed to score for *in ovo*
841 EROD. For experiment in graph B control and BNF-alone treatment groups n = 16 and
842 19 respectively, other treatment groups n = 6-10. EROD values are means ± S.E.M.
843 Statistical differences are explained in results section.

844

845 Figure 7

846 Effects of PCB126 with and without 100 µg/L ANF co-treatment on *in ovo* EROD and
847 deformity index. N = 9 or 10 for all treatment groups. EROD values are means ± S.E.M.

848 Statistical differences are explained in results section.

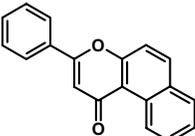
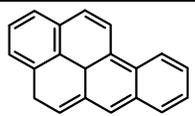
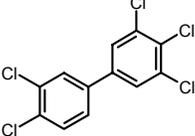
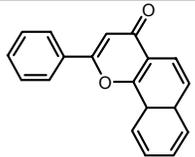
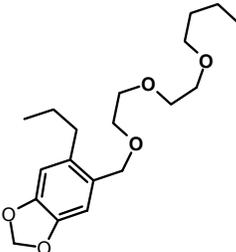
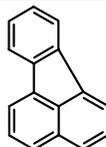
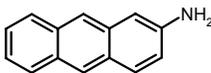
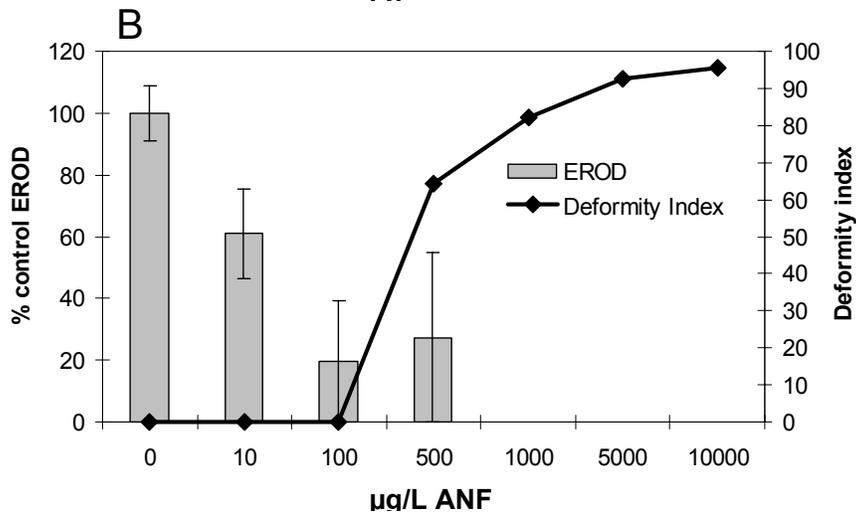
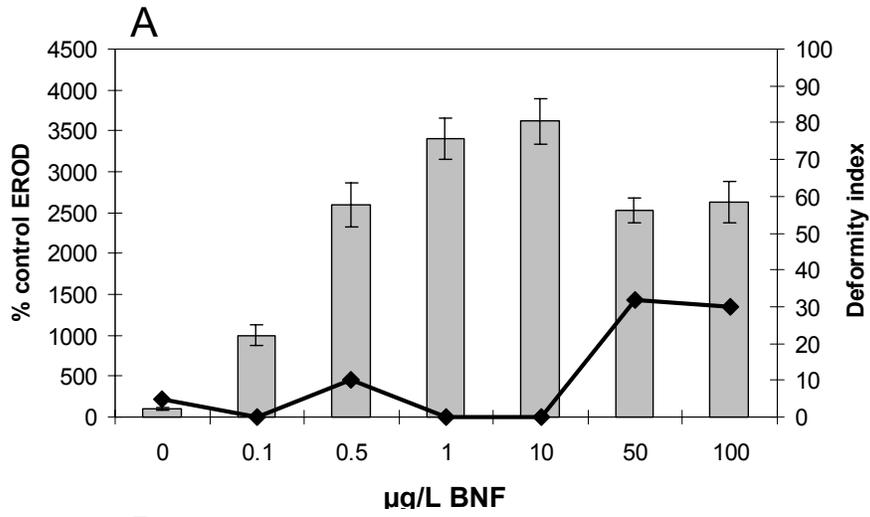
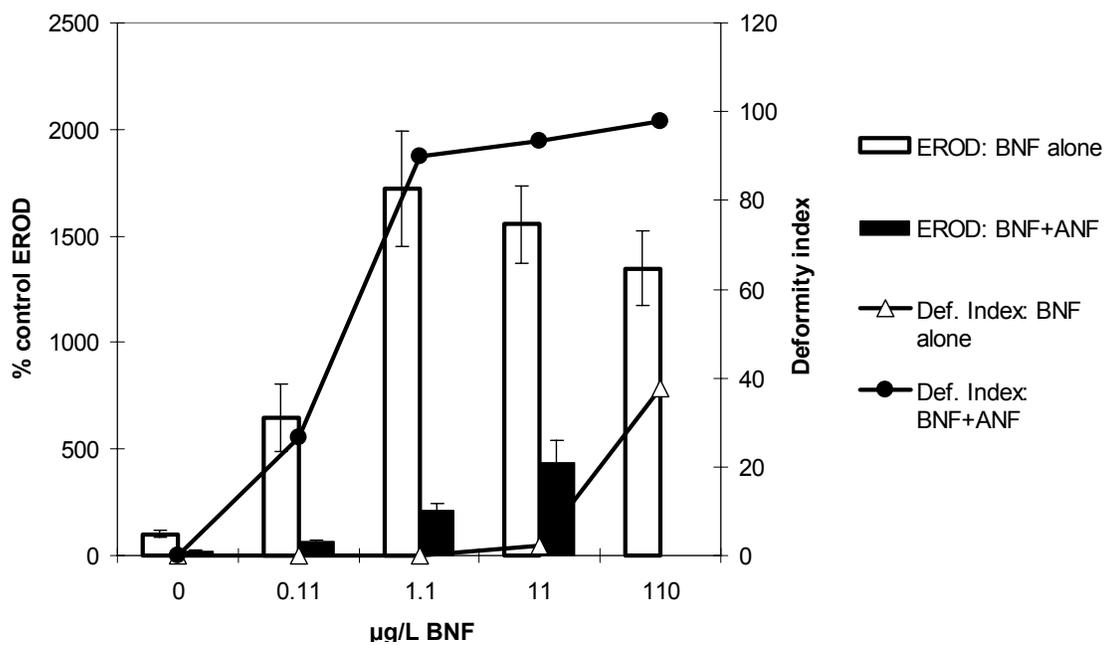
AHR agonists				
Compound	Type	Structure	Sample References for AHR agonism	
β -naphthoflavone (BNF)	Synthetically derived model PAH		(Matsuda et al. 1995; Ronisz and Förlin 1998)	
Benzo(a)pyrene	Environmentally relevant PAH		(Chaloupka et al. 1993; Fent and Bätischer 2000; Van Veld et al. 1997)	
3,3',4,4'-pentachlorobiphenyl (PCB126)	Environmentally relevant pHAH		(Abnet et al. 1999; Dabrowska et al. 2000)	
CYP1A inhibitors				
Compound	Type	Structure	Mechanism of action	Sample References
α -naphthoflavone (ANF)	Synthetically derived model PAH		Partial AHR antagonist and competitive inhibitor of CYP1A	(Goujon et al. 1972; Lu et al. 1996; Merchant et al. 1990; 1993; 1992; Merchant and Safe 1995; Miranda et al. 1998; Testa and Jenner 1981)
Piperonyl butoxide (PBO)	Methylenedioxybenzene derivative		P450 inhibitor. Forms a metabolic intermediate with heme group of P450	(Hodgson and Philpot 1974; Miranda et al. 1998; Murray and Reidy 1990; Testa and Jenner 1981)
Fluoranthene (FL)	Environmentally relevant PAH		Competitive inhibitor of CYP1A <i>in vitro</i> . Modestly lowers CYP1A protein expression <i>in vivo</i>	(Willett et al. 1998; 2001)
2-Aminoanthracene (AA)	Environmentally relevant aromatic amine		Mechanism-based CYP1A inhibitor. Binds to CYP1A and causes its degradation	(Watson et al. 1995)

Figure 1



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Figure 2



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Figure 3

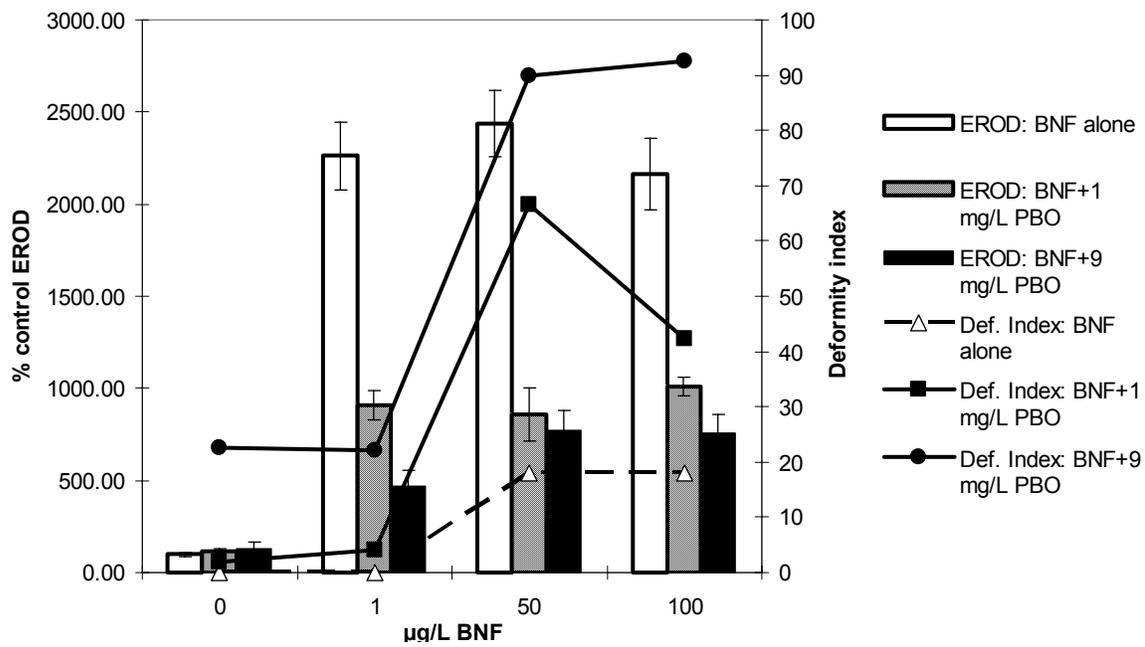


Figure 4

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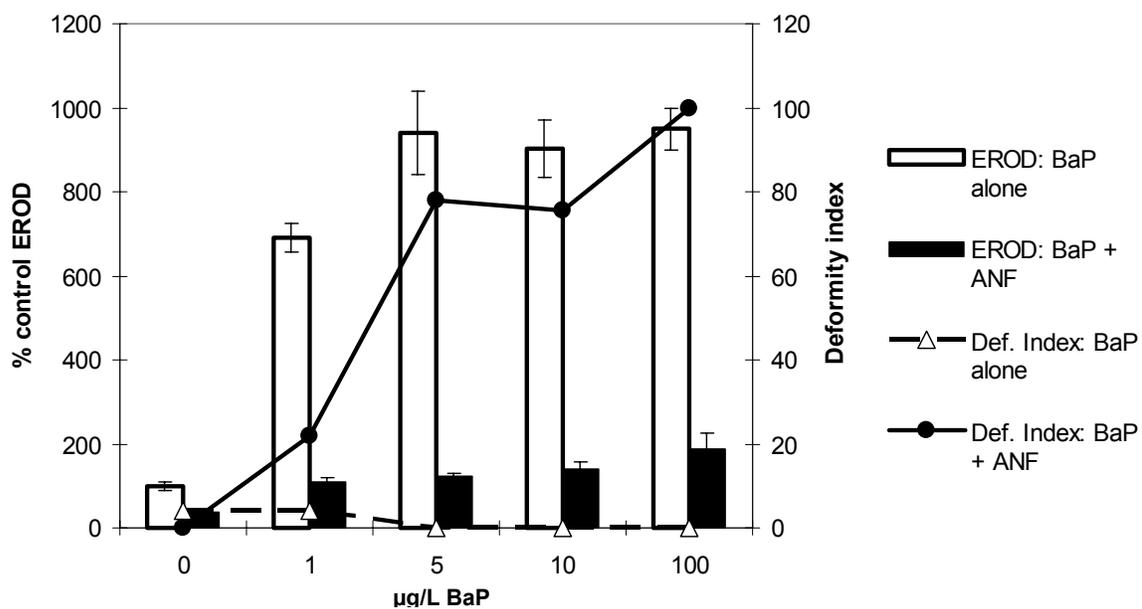
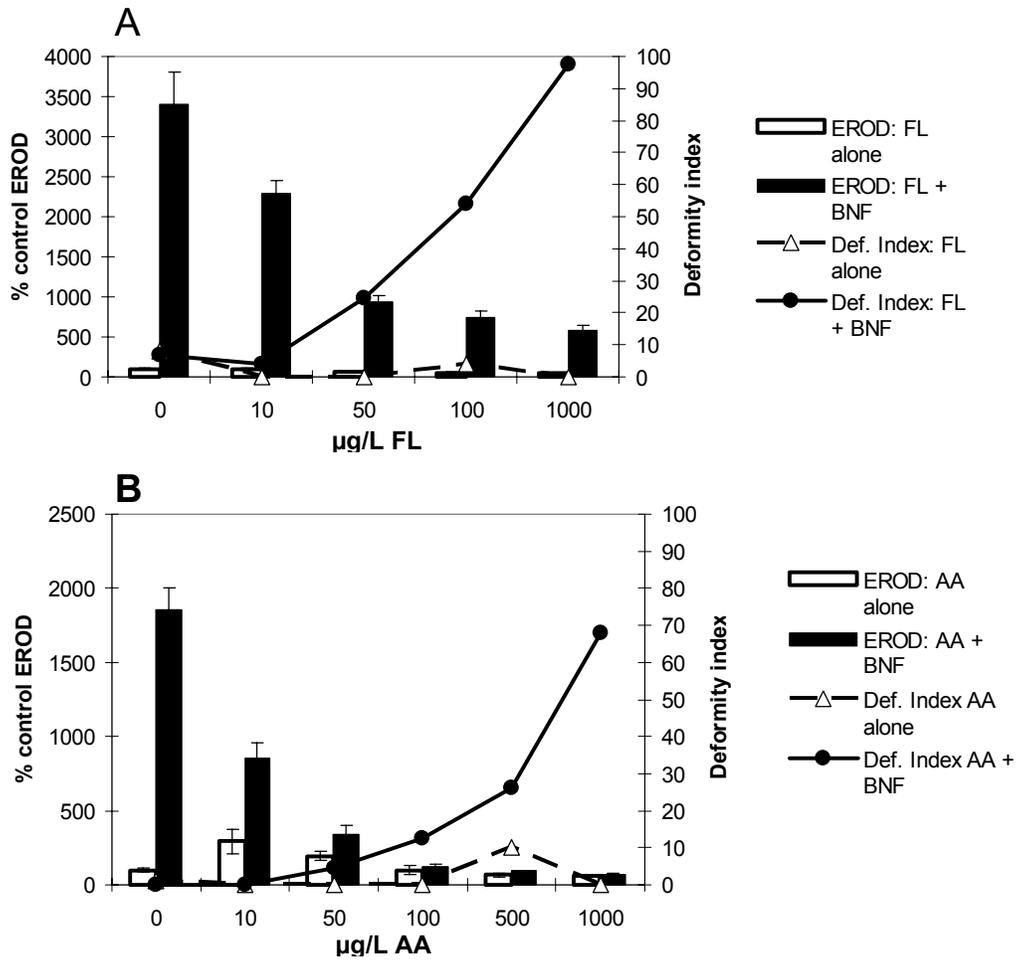


Figure 5

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Figure 6

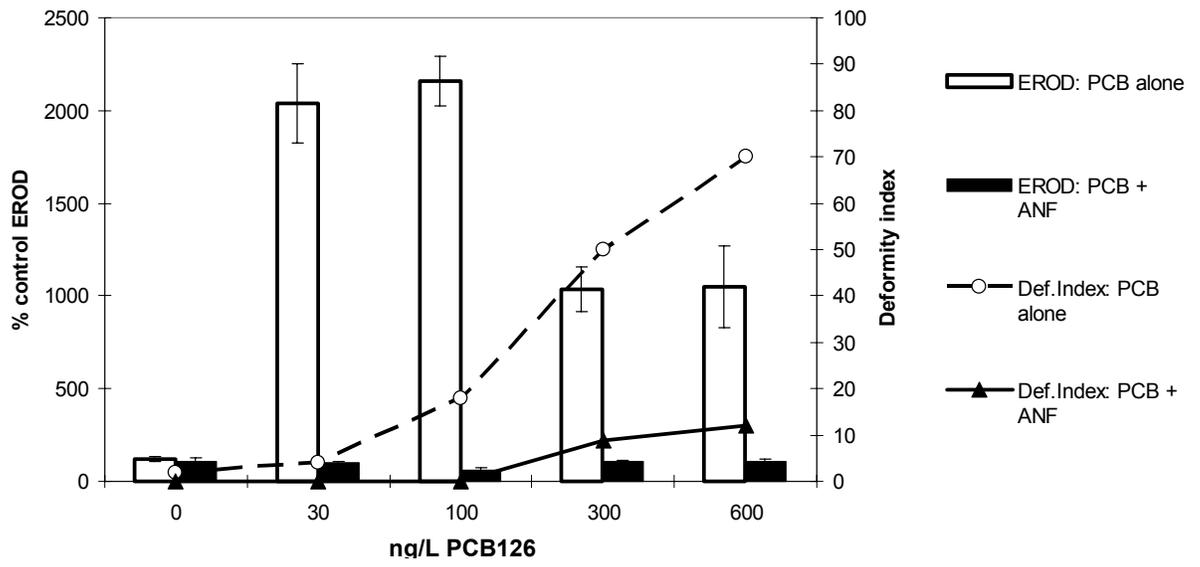


Figure 7

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