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1 **Cytochrome P4501A mRNA and protein induction in the striped bass**

2 **(*Morone saxatilis*)**

3

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6

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23

24 **Abstract**

25 The striped bass (*Morone saxatilis*) support a valuable recreational fishery, and are among the most  
26 important piscivorous fish of the San Francisco Estuary. This species has suffered a significant  
27 decline in numbers over the past decades and there is indication that contaminants are important  
28 contributors. Polycyclic Aromatic Hydrocarbons (PAHs) and Polyhalogenated aromatic  
29 hydrocarbons (PHAHs) including PCBs and dioxins are widespread in the Estuary, they typically  
30 bioaccumulate through trophic levels, reaching highest levels in top predators, and are known to  
31 affect fish health and development. The aim of this study was to investigate the dynamics of  
32 cytochrome P4501A (*Cyp1a*) induction simultaneously at different levels of biological  
33 organization (RNA transcription and protein synthesis) as a biomarker of exposure to PAHs and  
34 PHAHs. We utilized  $\beta$ -naphthoflavone (BNF) as a model PAH to induce *Cyp1a* responses in  
35 juvenile striped bass in both dose-response, and time-response assessments and determined *Cyp1a*  
36 mRNA and protein levels. Significant responses were measured in both systems at 10 mg BNF.Kg<sup>-1</sup>,  
37 a concentration used for time-response studies. Messenger RNA levels peaked at 6 h post-  
38 injection, while protein levels increased progressively with time, significantly peaking at 96 h post-  
39 injection; both remaining elevated throughout the duration of the test (8 days). Our data suggest  
40 that rapid induction of gene transcription following exposure and subsequent cumulative protein  
41 synthesis could provide a useful means of identifying temporal variants in exposure to *Cyp1a*  
42 inducers in *Morone saxatilis*. The potential application of this combined *Cyp1a* gene and protein  
43 biomarker in this species for field studies is discussed.

44

45 **Keywords:** *Cyp1a*, biomarker, fish, *Morone saxatilis*, “beta-naphthoflavone”, “Polycyclic  
46 Aromatic Hydrocarbon”

47

## 48 **1. Introduction**

49 The San Francisco Estuary is subject to diverse anthropogenic pressures, including the release of  
50 numerous contaminants that have greatly altered the functioning of this ecosystem (Bennett and  
51 Moyle 1996; Sommer et al. 2007; Strange 2008). Among contaminants, polycyclic aromatic  
52 hydrocarbons (PAHs) and polyhalogenated aromatic hydrocarbons (PHAHs) including  
53 polychlorinated biphenyls (PCBs) and dioxins are widespread in the Estuary (Davis et al. 2007;  
54 Oros et al. 2007). These chemicals generally occur as complex mixtures originating from a variety  
55 of sources: storm water runoff, wastewater treatment plant effluent, atmospheric deposition and  
56 dredged material disposal. PAHs and especially PHAHs typically bioaccumulate through the food  
57 web, reaching highest levels in top predators (Hylland 2006; Beyer and Biziuk 2009). In fish, water  
58 or food exposure to PAHs and PHAHs has been reported to result in numerous biological effects,  
59 such as weight loss, immunotoxicity, hepatotoxicity, cardiotoxicity, edema formation, genotoxicity  
60 and tumor promotion (e.g. Tanguay et al. 2005; Reynaud and Deschaux 2006, Pinkney et al. 2010;  
61 Nahrgang et al. 2010; Wills et al. 2010; Hiken et al. 2011). Such compounds are known to affect  
62 early life stages development, i.e. malformations of embryo and larvae (Barron et al. 2004;  
63 Sundberg et al. 2006, Billiard et al. 2008) and cause alteration of endocrine system function and  
64 reproduction (Monosson 1999/2000). These deleterious effects may reduce survival leading to  
65 population level effects.

66  
67 The striped bass (*Morone saxatilis*) were introduced from the East Coast of USA in the late 1880s,  
68 now support a valuable recreational fishery, and are among the most important piscivorous fish in  
69 the San Francisco Estuary. Along with a number of other pelagic fish species in the estuary, this  
70 species has suffered a significant decline in numbers (Bennett and Moyle 1996; Feyrer et al. 2007).

71 Extensive research on this and other pelagic fish species have also indicated that invasive species,  
72 food limitation, intra- and inter-specific competition and predation, increased entrainment, habitat  
73 losses, disease and impaired offspring (Ostrach et al. 2008; Sommer et al. 2007) likely contribute  
74 to population declines, and there is indication that contaminants are important contributors  
75 (Stevens et al. 1985; Bailey et al. 1994; Bennett et al. 1995; Ostrach et al. 2008). Monitoring and  
76 assessment of the striped bass population in the San Francisco Estuary has been extensive over the  
77 past 50 years for various reasons. Striped bass exploit the entire estuary ecosystem including the  
78 rivers, Delta region, outer bays and nearshore Pacific Ocean (Stevens et al. 1985, Sommer et al.  
79 2007). Striped bass are an apex predator in the system and as such are important sentinels for this  
80 ecosystem (Ostrach et al. 2009). The majority of fish species declining in the San Francisco  
81 Estuary are listed as either threatened or endangered and cannot be studied in the laboratory due to  
82 their status as well as the difficulty of handling and lack of appropriate methods for  
83 experimentation. Unlike these other species, striped bass are not a listed species and are amenable  
84 to breeding and experimentation in captivity (Ostrach et al. 2008).

85  
86 To understand the effect of contaminants on fish we must use systems that measure the extent of  
87 sublethal biological effects resulting from chemical exposure. Molecular and biochemical  
88 biomarkers, in particular, are valuable tools for monitoring contaminant exposure (Tom and  
89 Austlander 2005; Sarkar et al. 2006; Fedorenkova et al. 2010), however, most require validation  
90 for better interpretation of field data (e.g. Connon et al. 2010). Toxic responses are directly linked  
91 to a contaminant's mode of action, and are generally preceded by modifications in gene  
92 transcription. RNA is not the final product of a gene, as the gene function is carried out by the  
93 resulting protein. The level of protein may be regulated through transcriptional, post-

94 transcriptional, and post-translational mechanisms. Thus measurements of mRNA transcription  
95 may or may not be indicative of subsequent protein levels and activity.  
96  
97 Induction of cytochrome P4501A (*Cyp1a*) is a powerful biomarker of exposure to PAHs and  
98 PHAHs (i.e. PCBs and dioxins) and has been used for biomonitoring assessments in a number of  
99 aquatic organisms (Anderson et al. 1994; Stegeman and Hahn 1994; van der Oost et al. 2003;  
100 Hylland 2006). Cytochrome P4501A is a monooxygenase involved in xenobiotic biotransformation  
101 and detoxification of many PAHs, PCBs and dioxins, and is induced by the binding of these  
102 compounds with Aryl hydrocarbon receptor (AhR), i.e. a cytosolic ligand-activated transcription  
103 (Stegeman and Hahn 1994; Hahn 2001). The induction of *Cyp1a* may then be regulated at various  
104 post-transcriptional steps (Abdelhamid et al. 2010; Amara et al. 2010). Cytochrome P4501A can  
105 also be activated by other pathways and plays a strong role in steroid hormone biosynthesis,  
106 tryptophan and retinol metabolism (review in Monostory et al. 2009). Induction of *Cyp1a* varies  
107 between fish species (Wirgin et al. 1996, Willet et al. 1997, Oris and Roberts 2007), so that a  
108 specific evaluation needs to be performed before using this biomarker on a sentinel species in  
109 environmental monitoring programs. In this study we thus focus on both *Cyp1a* gene expression  
110 and protein content in order to provide a combined biomarker specific for the striped bass *Morone*  
111 *saxatilis*.

112  
113 The aims of this study are two-fold, investigating the dynamics of *Cyp1a* induction at different  
114 biological levels of organization (RNA transcription and protein synthesis), and to explore the  
115 relationship between the measured parameters. By conducting sublethal exposure response studies  
116 that integrate molecular and biochemical assessments, we are better able to understand the  
117 relationship between gene expression and effects at higher levels of biological organization. In the

118 present study, we utilize the PAH model,  $\beta$ -naphthoflavone (BNF), to induce *Cyp1a* responses in  
119 juvenile striped bass in both i) dose response and ii) time response experiments, in order to identify  
120 and utilize transcriptional and translational differences as measures of temporal variants of  
121 xenobiotic exposure.

122

## 123 **2. Methods**

124 **2.1. Fish handling:** One-year old striped bass juveniles (fork length:  $19.9 \pm 2.5$  cm; body mass:  
125  $114 \pm 40$  g) used in this study were produced from a domestic brood stock maintained at the Center  
126 for Aquatic Biology and Aquaculture at UC Davis. Fish were moved to the experimental facility  
127 two days prior to the start of the experiment. They were maintained in aerated circular (diameter:  
128 60 cm; height: 35 cm) flow-through freshwater tanks (10 fish per tank) at 19 – 20 °C water  
129 temperature and under natural photoperiod. Fish were fed daily at 2.5% body weight with #3  
130 Crumbled Salmon/Trout chow (Silver Cup, Murray, UT 84157), but were fasted for 24 h prior to  
131 test initiation to avoid any post-anesthesia risk (e.g. regurgitation of food and/or fecal  
132 contamination of recuperation water)

133 **2.2. BNF exposures and sample preparation:** Fish were exposed to BNF through intra-peritoneal  
134 injections in two experiments: (i) a 24h dose response to BNF concentrations (Sigma-Aldrich  
135 Corporation) of 0.1, 1, 10, and 25 mg.kg<sup>-1</sup>, and (ii) a time response study, commencing at time 0 h  
136 with either a 10.0 mg  $\beta$ -naphthoflavone.kg<sup>-1</sup> or solvent control (SC), followed by a metabolic  
137 detoxification time of 6 h, 12 h, 24 h, 2 d, 4 d and 8 d. Each group of fish (N=10) for both dose and  
138 time response was placed in an individual circular tank (see above). Corn oil (Sunny Select, Super  
139 store industries, Lathrop, CA 95330) was used for BNF dilution and as solvent control. Fish

140 sampled at time zero received no injection. Prior to injection, fish were anesthetized with MS-222  
141 (tricaine methanesulfonate, Sigma, St. Louis, MO, USA) at 100 mg.L<sup>-1</sup>. Upon termination of the  
142 experiment, fish were euthanized using an overdose of MS-222, their livers dissected and  
143 immediately snap frozen in liquid nitrogen. Liver tissue was then ground in liquid nitrogen to a  
144 fine powder using mortar and pestle, aliquots were placed into two separate microcentrifuge tubes,  
145 to ensure a homogeneous sample, and stored at -80°C for subsequent molecular and biochemical  
146 analyzes.

### 147 **2.3. *Cyp1a* Transcription assessment:**

148 RNA extraction and cDNA synthesis: Total RNA was extracted from powdered liver tissue using  
149 RNeasy Mini Kit (Qiagen Inc., Valencia, CA), with on-column DNase digestion, as per  
150 manufactures protocol. Samples were quantified using a NanoDrop spectrophotometer and their  
151 quality verified by electrophoresis on 1% agarose gel. Complementary DNA (cDNA) was  
152 synthesized using 100 units of SuperScript III (Invitrogen, Carlsbad, CA, USA), 600 ng random  
153 hexadeoxyribonucleotide (pd(N)<sub>6</sub>) primers (random hexamer primer), 10U RNaseOut (RNase  
154 inhibitor), and 1mM dNTPs (all Invitrogen, Carlsbad, CA, USA).

155 Quantitative PCR: We measured *Cyp1a* gene (precisely CYP1A1) expression in striped bass using  
156 specific probes and primers designed for quantitative PCR (qPCR), as detailed in Table 1. TaqMan  
157 Universal PCR Mastermix (Applied Biosystems) was used in cDNA amplifications using an  
158 automated fluorometer (ABI PRISM 7900 Sequence Detection System, Applied Biosystems).  
159 Quantitative PCR data was analyzed using the relative quantification 2<sup>(-Delta Delta CT)</sup> method  
160 (Livak and Schmittgen, 2001), using *β-actin* expression as a reference to which *Cyp1a* expression  
161 was normalized. Statistical assessments were conducted on Log<sub>2</sub> normalized data (see below).



162

163 **2.4. *Cyp1a* Protein quantification:** A *Cyp1a* competitive-ELISA for striped bass was developed  
164 following Tom et al. (2002). A brief method description is provided below.

165 *2.4.1. Cyp1a protein production:* A strain of *Escherichia coli* that contained an antigenic fragment  
166 of *Lithognathus mormyrus Cyp1a* gene was obtained from Dr. Moshe Tom, Israel Oceanographic  
167 and Limnological Research, Haifa, Israel. Colonies were grown over night in Lysogeny Broth  
168 medium with 100 µg/mL ampicillin at 37 °C to produce a starter culture. This was used to  
169 inoculate one liter of Terrific Broth medium with 50 µg/mL ampicillin and 1.0 mM thiamine at 37  
170 °C. Four hours later heme precursor δ-aminolevulinic acid and isopropyl β-D-1-  
171 thiogalactopyranoside (IPTG) inducer were added to a final concentration of 0.5 mM, and the  
172 culture was incubated at 28 °C for 24 hours. After centrifugation at 5,000 x g for 15 minutes in  
173 250ml flasks, bacteria were transferred into 50 mL centrifuge tubes and spun again. Cells were  
174 resuspended into 30 mL of 0.1 M potassium phosphate buffer, 20% glycerol, 0.1 mM  
175 Dithiothreitol (DTT) and 0.1 mM Ethylene Diamine Tetraacetic Acid (EDTA) (pH 7.4). Egg white  
176 lysozyme (0.5 mg/ml) was added to the buffer and incubated for one hour at 4 °C. A 1/10,000  
177 dilution of nonselective protease inhibitor (Sigma P8340) and 1 µg/ml DNAase I were added. The  
178 resulting spheroplast suspension was then sonicated with a high powered probe sonicator at 25  
179 Watts, 8 times for 30 seconds on ice and then centrifuged at 30,000 x g for 2 hours. The *Cyp1a*  
180 containing cytosol was saved and the pellet was discarded.

181 *2.4.2. Cyp1a protein purification:* The *Cyp1a* containing cytosol was purified on a Ni<sup>+</sup>  
182 Nitrilotriacetic acid Agarose (Qiagen) column equilibrated with running buffer 50 mM potassium  
183 phosphate buffer containing 0.2 M NaCl, 20 mM glycine, 20% glycerol, 0.1 mM EDTA and 0.1

184 mM DTT. The poly-His tail that was added on the recombinant protein fragment has a high affinity  
185 for the Ni<sup>+</sup> ion and allows for the crude extract to pass through and enrich the solution with the  
186 protein of interest. The protein was eluted from the column with running buffer containing 50 mM  
187 histidine. Fractions were analyzed for total protein with the BioRad DC protein assay, and further  
188 analyzed with Sodium Dodecyl Sulfate (SDS) polyacrylamide gel (Invitrogen NuPAGE) to  
189 determine the fractions containing high concentrations of *Cyp1a*. These fractions were pooled, and  
190 analyzed with the BioRad DC protein assay to determine total protein content and by SDS gel  
191 electrophoresis to determine the *Cyp1a* protein concentration. SDS gels showed a prominent band  
192 at 55kDa matching the molecular weight of the *Cyp1a* protein fragment with a concentration of  
193 0.18 µg/µL in the resulting stock.

194  
195 2.4.3. *Cyp1a* competitive-ELISA optimization and measurement: For the competitive-ELISA, we  
196 used the monoclonal mouse anti-fish *Cyp1a* antibody (C10-7, Biosense Laboratories) that has been  
197 shown to cross-react with a variety of fish species including rainbow trout (*Oncorhynchus mykiss*),  
198 Atlantic salmon (*Salmo salar*), carp (*Cyprinus carpio*), turbot (*Scophthalmus maximus*) and  
199 Atlantic cod (*Gadus morhua*). The produced *Cyp1a* protein fraction was coated on high binding 96  
200 well plates (Costar 3925, Black) at 100 or 200 ng/mL *Cyp1a* protein fragment in 0.05 M Sodium  
201 Bicarbonate, pH 9.6. Plates were then blocked with PBS buffer pH 7.5 containing 1%  
202 Hammerstein Casein and 1.0 mM EDTA. Plates were then co-incubated with increasing  
203 concentrations of *Cyp1a* protein fragment with 1/5,000 or 1/10,000 diluted Mouse anti-fish *Cyp1a*  
204 antibody in PBS buffer pH 7.5 containing 1% Hammerstein Casein, 1.0 mM EDTA, and 0.05%  
205 Tween 20, and then rinsed with PBS and 0.05% Tween 20. Following incubation with 1/10,000  
206 diluted Goat Anti Mouse Horse Radish Peroxidase (HRP) conjugate (Jackson Immuno Research)  
207 and rinsing with PBS and 0.05% Tween 20, HRP substrate ( 35.4 µM ADHP (Anaspec, Inc.), 1.76

208 mM Urea Hydrogen Peroxide in 0.05 M Sodium Phosphate buffer, pH 7.4) was added. After  
209 approximately 30 minutes, fluorescence was measured at 544 nm excitation with 590 nm emission  
210 on a Wallac Victor 2 Fluorescence Plate Reader. The results confirmed that the antigenic *Cyp1a*  
211 protein fragment was present. The competitive *Cyp1a* ELISA described above was shown to be  
212 optimized by coating plates overnight with 200 ng/ml *Cyp1a* at 4°C, co-incubating S9 fraction  
213 sample with 1/10,000 dilution of Mouse anti-fish *Cyp1a* in the competitive binding step, and  
214 incubating with 1/10,000 dilution of Goat anti Mouse-HRP (Ostrach et al., 2009; Spearow et al.,  
215 2011).

216  
217 S9 fraction sample was prepared: powdered liver tissues of each sample was homogenized in 10  
218 volumes by weight of ice-cold extraction buffer consisting 0.02M 4-(2-hydroxyethyl)piperazine-1-  
219 ethanesulfonic acid (HEPES) 0.15M KCl, (pH 7.5) 0.22mM 4-(2-aminoethyl) benzenesulfonyl  
220 fluoride and 1/200 protease inhibitor cocktail (Sigma). After centrifugation at 10,000 g for 15  
221 minutes at 4C, the S9 supernatant fractions of *Morone saxatilis* liver samples were obtained for  
222 *Cyp1a* protein measurement through competitive-ELISA as mentioned above.

223  
224 **2.5. Statistical Analyses:** Data-sets were not normally distributed; therefore assessments were  
225 tested for significance using non-parametric tests. Time responses were assessed using Kruskal-  
226 Wallis analysis of variance, with Siegel-Castellan post-hoc multiple correction tests. Differences  
227 between data-points and respective solvent controls were assessed using Mann Whitney U tests.  
228 Dose response data were compared to controls using Mann Whitney U tests. Analyses were  
229 performed using the R software (R Development Core Team, 2008).

230

## 231 **3. Results**

### 232 **3.1. Dose Response to BNF exposure:**

233 Striped bass hepatic *Cyp1a* mRNA and protein responses to BNF exposure were dose dependent at  
234 24 h post injection (Fig. 1), with both q-PCR and competitive ELISA results correlating positively  
235 with increased BNF exposure ( $p < 0.001$ ). *Cyp1a* mRNA abundance was significantly increased  
236 3.83-fold by 1 mg  $\beta$ -naphthoflavone.Kg<sup>-1</sup> relative to solvent controls ( $p < 0.05$ ), and almost 13-fold  
237 by 10.0 mg  $\beta$ -naphthoflavone.Kg<sup>-1</sup> ( $p < 0.05$ ). *Cyp1a* Protein content was significantly upregulated  
238 by 10 mg  $\beta$ -naphthoflavone.Kg<sup>-1</sup>; ( $p < 0.01$ ).

### 239 *3.2. Timed Response to BNF exposure:*

240 There was a significant time-dependent response in hepatic *Cyp1a* mRNA abundance and protein  
241 content in juvenile striped bass exposed to 10.0 mg  $\beta$ -naphthoflavone.Kg<sup>-1</sup> (Fig. 2). *Cyp1a* mRNA  
242 abundance peaked at 6 h with a 40.80 fold-increase relative to time zero ( $p < 0.001$ ), then decreased  
243 with metabolic detoxification time to 8 d, though remaining significantly up-regulated at 12 h, 24 h  
244 and 48 h relative to time zero ( $p < 0.01$ ). The quantity of *Cyp1a* protein, however, increased with  
245 time, being significantly higher relative to time zero from 48 h onwards ( $p < 0.001$ ), peaking at 96 h  
246 post-injection (9.3  $\mu\text{g} \cdot \text{mg}^{-1}$  total protein) and remained elevated at 8 d post-injection (6.33  $\mu\text{g} \cdot \text{mg}^{-1}$   
247 total protein). We note that control fish (SC) also show some increase in mRNA and especially  
248 protein levels during the first 24 hours after the beginning of the experiment, which may be due to  
249 the effects of handling stress. However, *Cyp1a* mRNA and protein levels were significantly higher  
250 with respect to solvent controls throughout the 6 h to 8d post-injection period (Fig. 2).

## 251 **4. Discussion**

252

253 The cytochrome P4501A is considered an excellent indicator of PAHs and PHAHs exposure, with  
254 a number of reviews highlighting its use in monitoring (Anderson et al. 1994; Stegeman and Hahn  
255 1994; van der Oost et al. 2003; Hylland 2006). Liver microsomal 7 ethoxyresorufin-O-deethylase  
256 (EROD) activity is commonly used as a proxy of *Cyp1a* induction. Although *Cyp1a* is the main  
257 enzyme catalyzing the de-ethylation of ethoxyresorufin, other CYP enzymes also can contribute to  
258 EROD activity (Spearow et al. 2011). It has also repeatedly been reported that EROD can  
259 substantially be inhibited by elevated levels of PCBs, fluoranthene (a prevalent PAH), dioxins and  
260 heavy metals (e.g., Viarengo et al. 1997; Gooch et al. 1989; Willett et al. 2001; Ortiz-Delgado et al.  
261 2008). EROD activity is technically easy to measure and it is still considered as a robust biomarker  
262 (Oris and Roberts 2007) however, this enzymatic activity biomarker shows some limitations that  
263 can make interpretation sometimes difficult in field studies (Spearow et al. 2011). Although,  
264 several studies have focused separately on *Cyp1a* induction at different organizational levels: gene  
265 expression (e.g. Rees 2003; Chung-Davidson 2004; Aluru 2008) and protein content or EROD  
266 activity (e.g. Gravato and Santos 2002; Miller et al. 2004; Ortiz-Delgado et al. 2008), relatively  
267 few have compared gene expression and protein level (see: Kloepper-Sams and Stegeman 1989;  
268 Hahn and Stegeman 1994). In this study we focus on both *Cyp1a* induction through gene  
269 expression and protein content with state of the art techniques in order to provide a combined  
270 biomarker applicable for *Morone saxatilis*. Molecular and biochemical biomarkers are promising  
271 tools to assess the effects of chemicals on exposed organisms; however, data interpretation is  
272 sometimes the limiting factor hindering their use. Nevertheless, through assessing the combined  
273 responses at different levels of biological organization, it is possible to gather more extensive

274 information towards a more reliable interpretation of data, thus substantiating the use of  
275 biomarkers in monitoring efforts. Although, ongoing validation is required in order to apply these  
276 biomarkers in field situations, the current study strongly supports the use of *Cyp1a* in striped bass  
277 as a monitoring tool of sublethal exposure, particularly when two or more assessment methods can  
278 be applied synchronously.

279  
280 This study has been successful in refining and validating a *Cyp1a* protein assay technique for  
281 striped bass developed by Ostrach et al. (2009), based on methods by Tom et al. (2002). Results  
282 were comparable to responses reported by Kloepper-Sams and Stegeman in *Fundulus heteroclitus*  
283 (1989; 1994), though the newly developed approach, and current techniques permit the  
284 presentation of these responses in a more specific manner for *Morone saxatilis*. The present study  
285 applied a competitive-ELISA technique that uses a recombinant *Cyp1a* and that allows for a  
286 higher-throughput quantitative evaluation of fish *Cyp1a* (Tom et al. 2002), than the semi-  
287 quantitative western blot method used in the reference study by Kloepper-Sams and Stegeman  
288 (1989). In the present study, hepatic *Cyp1a* mRNA abundance in juvenile striped bass responded  
289 rapidly to BNF exposure peaking within six hours post-exposure. The corresponding  
290 biotransformation and detoxification protein was induced progressively peaking at four days post-  
291 exposure and remaining elevated throughout the duration of the experiment. Thus the rapid  
292 increase in mRNA abundance and subsequent cumulative protein synthesis can provide a useful  
293 mean of identifying temporal variants in exposure, where mRNA levels are used to detect recent  
294 exposures and protein levels are indicative of past exposures. The time course of *Cyp1a* induction  
295 may also vary following single or continuous exposure (e.g. Levine and Oris 1999) and to different  
296 kind of contaminants; for instance poorly metabolizable AhR inducers such as PCBs or dioxins  
297 show prolonged induction even at gene expression level (e.g. Hahn and Stegeman 1994). In field

298 conditions, chronic exposure is the common environmental situation and fluctuations in pollution  
299 levels occur. Time course of biomarker are important in evaluating changes in exposure due to  
300 pollution sources or prevention actions. Therefore further studies are evidently required with  
301 different modes of exposure and using different *Cyp1a* substrates, inhibitors and mixtures before  
302 application to field conditions. The difference between these conditions would therefore need to be  
303 established based on statistical differences in expression from negative control samples, or  
304 previously determined basal expression levels (Tom and Auslander 2005). Nevertheless models  
305 that make use of the shorter half life of *Cyp1a* mRNA, relative to that of *Cyp1a* protein, could  
306 potentially detect temporal variants in exposure. This is likely to hold true, not only for *Cyp1a*, but  
307 for a number of other biomarkers of toxicant exposure and effect.

308  
309 Increasing the informative power of biomarkers, by measuring multiple levels of organization  
310 along with establishing suites of biomarkers, indicative of exposure and effect of multiple groups  
311 of chemicals, will allow a more comprehensive evaluation of contaminant impacts in exposed  
312 organisms. This is especially needed for field monitoring. Current monitoring efforts in the San  
313 Francisco Estuary have highlighted the presence of a number of contaminants, including PAHs and  
314 PHAHs (SFEI 2008). The newly developed *Cyp1a* ELISA described here, along with knowledge  
315 based applications of numerous other molecular and biochemical biomarkers, and numerous other  
316 molecular and biochemical biomarkers (Burge et al. 2004; Geist et al. 2007; Picha et al. 2008;  
317 Washburn et al. 1996; Durieux et al. 2010; Spearow et al. 2011) will permit the assessment and  
318 understanding of the sublethal effects of complex mixture of chemical contaminants on striped  
319 bass. In addition, as an apex predator, striped bass is a sentinel species in this threatened ecosystem  
320 such that the approach developed and described here can be an extremely useful and effective  
321 mean of assessing, understanding and potentially mitigating sublethal contamination in estuarine

322 habitats where striped bass reside. These promising results show the need for continuing research  
323 towards establishing links between cellular responses to toxic contaminants with effects on growth,  
324 development, behavior, reproduction and survival.

325

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338



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507



508 **Table 1.** Primer and Probes for *Cyp1a* (precisely CYP1A1) and  $\beta$ -*actin* used for quantitative PCR  
 509 assessments of gene expression in striped bass.

510

<b>Gene</b>	<b>Species for starting sequence</b>	<b>NCBI GenBank accession number</b>	<b>Primer</b>	<b>Roche Probe</b>
<i>Cyp1a</i>	<i>Dicentrarchus labrax</i>	AJ251913	Right: GCGGCACAACCCAGAGTA	No. 65; CTGGAGGA
			Left: CAGCTTTCATGACGGTGTTGAG	
$\beta$ - <i>actin</i> <sup>a</sup>	<i>Dicentrarchus labrax</i>	AJ493428	Right: GCAATGAGAGGTTCCGTTGC	No. 11; CTTCCAGC
			Left: GCAGGACTCCATACCGAGGAA	

511

512 <sup>a</sup> reference gene

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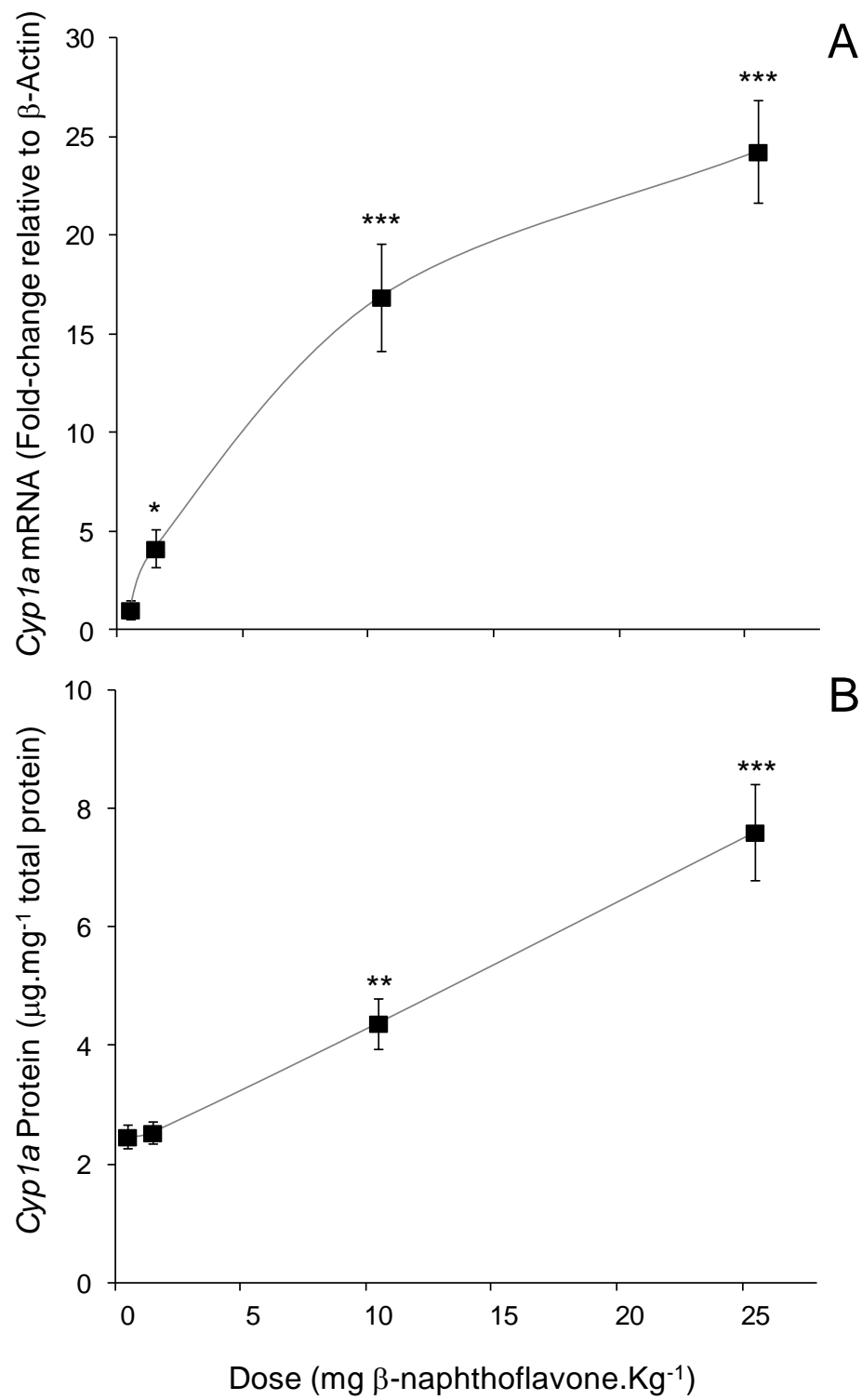
518 **Figures captions**

519

520 Figure 1. Hepatic *Cyp1a* mRNA levels (A) and *Cyp1a* protein levels (B) in juvenile *Morone*  
521 *saxatilis*, at 24 h following the injection of 0.1, 1, 10 and 25 mg  $\beta$ -naphthoflavone.Kg<sup>-1</sup>. Values  
522 represent means  $\pm$ SE. With sample size for the respective concentrations: N = 9, 10, 10, 8 and 9  
523 (A); 10, 10, 10 and 10 (B). Significant differences from solvent control are indicated: \*  $p < 0.05$ ;  
524 \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$

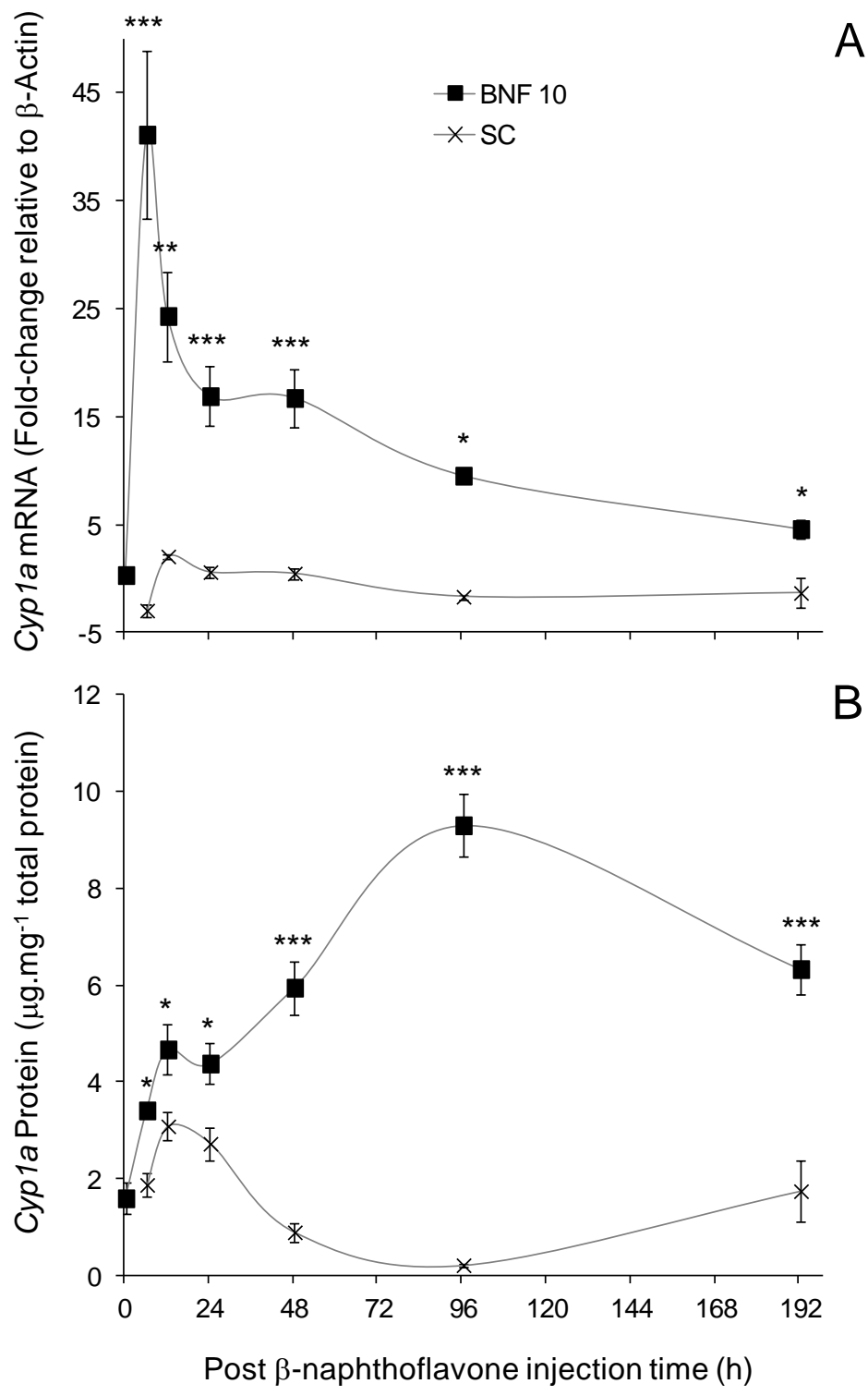
525

526 Figure 2. Timed responses in hepatic *Cyp1a* mRNA (A) and protein levels (B) in juvenile *Morone*  
527 *saxatilis*, following the injection with 0 (solvent control: SC) and 10 mg  $\beta$ -naphthoflavone.Kg<sup>-1</sup>  
528 (BNF 10) at 0, 6, 12, 24, 48, 96 and 192 h. Values represent means  $\pm$ SE. With sample size for the  
529 respective timed responses: N = 9, 8, 8, 8, 10, 4 and 10 (A: BNF 10); 10, 8, 9, 10, 4 and 10 (A:  
530 SC); 9, 8, 10, 10, 10, 4 and 10 10 (B: BNF 10); 10, 10, 9, 10, 4 and 10 (B: SC). Significant  
531 differences from solvent control are indicated: \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$



532

533 Fig. 1



534

535 Fig. 2