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1 Marine Environmental Research

2

3 **Biomarker induction in tropical fish species on the Northwest Shelf of**  
4 **Australia by produced formation water**

5

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41 **ABSTRACT**

42

43 Normal operation of oil well platforms results in the discharge of produced formation  
44 water (PFW). The expression of CYP1A, CYP2M1- and 2K1-like proteins was  
45 examined for use as possible biomarkers of PFW exposure. A pilot study on the  
46 Northwest Shelf of Australia had indicated that PFW contamination possibly  
47 contributes to induction of CYP1A-like proteins in Gold-Spotted Trevally  
48 (*Carangoides fulvoguttatus*). The pilot study samples were re-examined for CYP1A,  
49 and, in addition, CYP2K1/2M1-like proteins. In a subsequent caged-fish study in the  
50 same location a second species, Stripey seaperch (*Lutjanus carponotatus*), caught at a  
51 clean site, were distributed to three caging sites in a PFW gradient from the Harriet A  
52 production platform: A (near-field), B (far-field) and C (a non-impacted reference  
53 site). Fish were sampled at time (T) T=0, T=3 and T=10 days. Significant increases of  
54 CYP1A, one CYP2K1- and two CYP2M1-like proteins were noted at Site A at T=10  
55 d. For another CYP2K1-like protein, a significant increase was observed at Site A  
56 only at T=3 d. These results support a previous study indicating that CYP1A protein is  
57 sensitive to PFW exposure. Importantly, statistically significant environmental  
58 induction of both CYP2M1- and CYP2K1-like proteins in tropical fish due to PFW  
59 exposure had not previously been described and induction of enzymes in the CYP2  
60 family suggest new biomarkers for PFW. In addition, the novel response of one  
61 CYP2K-like protein requires further verification, but offers promise for improved  
62 monitoring of sub-lethal responses in marine organisms.

63

64 *Keywords:* CYP1A, CYP2K1, CYP2M1, produced formation water, tropical fish,  
65 Australia, biomarkers

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69 **1. Introduction**

70 Produced formation water (PFW), the oily water usually discharged from a platform  
71 after separation from the oil, is made up from formation water (water associated with  
72 the oil in the reservoir) and potentially includes water which was injected into the  
73 reservoir to maintain pressure for oil production (Holdway, 2002). As oil fields age,  
74 the amount of PFW increases as the reservoir fills with injected water (Henderson et  
75 al., 1999).

76 When discharged, the PFW contains a large variety of naturally occurring materials  
77 from the formation, which include residual volatile and non-volatile hydrocarbons not  
78 removed by the separation regime, and also contains chemicals added to the  
79 production stream (Burns et al., 1999). Therefore, the composition of the discharged  
80 PFW is very complex. Almost all offshore oilfields produce formation water that can  
81 have significant environmental effects if not handled properly. Australia's oil and gas  
82 production activity is predominantly on the Northwest Shelf of Western Australia, off

83 the continental margins between the North West Cape and Dampier (Swan et al.,  
84 1994). There has been excellent work on the toxicology of PFW (Holdway, 2002;  
85 Neff, 2002; Neff et al., 2006). Unfortunately, little is known of the effects of PFW on  
86 the tropical marine environment, especially for tropical reef fish. One study  
87 demonstrated minor toxicity of PFW from Harriet A oil platform to corals and their  
88 symbiotic algae (Jones and Heyward, 2003). Another study illustrated a preliminary  
89 toxicological assessment of PFW including analytical detection and acute toxicity  
90 bioassay using European sea bass larvae (Mariani et al., 2004). An independent  
91 scientific review commissioned by the Australian Petroleum Production and  
92 Exploration Association (APPEA) and the Energy Resource Development  
93 Corporation (ERDC) highlighted the need to conduct environmental assessments of  
94 industry practices in shallow coastal ecosystems around Australia.

95 In 1998, the Australian Institute of Marine Science (AIMS) conducted a pilot study to  
96 evaluate the use of sub-lethal stress indicators in fish for assessing exposure to  
97 organic contaminants associated with PFW discharged from an oil and gas production  
98 platform on the Northwest Shelf of Australia. The pilot study results confirmed that  
99 within 1000 m from the Harriet A platform there was potential for biological effects in  
100 fish populations exposed to PFW effluent and that further investigation was warranted  
101 at this site (Codi King et al., 2005a). For this study, these samples were re-examined  
102 for CYP1A using more universally repeatable conditions, and, in addition, the  
103 response of CYP2K1/2M1-like proteins was determined. In May 2003, AIMS  
104 conducted a comprehensive biological and chemical assessment of PFW effluent at  
105 the Harriet A platform. This more comprehensive study utilized a controlled system in  
106 which fish from a reference site were collected and placed in cages, and the cages  
107 were located in a gradient line moving away from the point source of PFW being  
108 discharged at Harriet A. The work described here is a subset of the suite of biomarkers  
109 conducted for the 2003 study and presents in detail the cytochrome protein  
110 biomarkers in an ecologically relevant reef species, Stripey seapearch (*Lutjanus*  
111 *carponotatus*) for the evaluation of the potential effects of PFW discharge at the  
112 Harriet A Platform on the Northwest Shelf of Australia.

## 113 **2. Materials and Methods**

### 114 **2.1 Pilot study: study site and fish collection**

115 Harriet A, an oil and gas production platform located 135 km west of Karratha on the  
116 Northwest Shelf of Australia, was the main focus of this study (Figure 1). The  
117 hydrocarbon concentration of PFW effluent in the sea-surface micro-layer, water  
118 column, sediments and biota, at this location, have been well documented (Burns and  
119 Codi, 1999) The site selections for the pilot study have previously been described in  
120 Codi King et al. (2005a). Gold-spotted Trevally was chosen for the pilot study  
121 because of its importance both commercially and recreationally to the NW Shelf of  
122 Australia. A total of 41 Gold-spotted Trevally were collected from each of three sites:  
123 Harriet A (n=23, Mean  $\pm$  SD; 3817  $\pm$  1532 g total weight, 654  $\pm$  103 mm total length,  
124 age 1-5 yr); Harriet C (n=6, Mean  $\pm$  SD; 3608  $\pm$  1503 g total weight, 654  $\pm$  103 mm

125 total length, age 2-6 yr); and reference site off the Montebello Islands (n=12, Mean  $\pm$   
126 SD; 3992  $\pm$  1122 g total weight, 660  $\pm$  75 mm total length, age 2-5 yr). The data  
127 demonstrated there were no significant differences in total weight, total length and  
128 age of fish between the three study sites ( $P < 0.05$ ). All fish were processed as  
129 previously described in Codi King et al. (2005a). Briefly, livers were removed, frozen  
130 in liquid nitrogen and transported to AIMS via a liquid nitrogen dry shipper remaining  
131 stored in liquid nitrogen until thawed for microsomal preparation (see Section 2.3).

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## 132 2.2 Caged fish study: Study site and fish collection

133 The PFW plume from Harriet A forms a fine surface slick that moves in a NNW  
134 direction on the ebb tides and SSE direction on the flood tide. The PFW hydrocarbons  
135 in the sea-surface microlayer were detectable to a distance of 1 km. The lighter MW  
136 components (naphthalene and homologs) were in the dissolved phase while the  
137 heavier MW hydrocarbons (phenanthrene and homologs) were associated with the  
138 particulate phase. The particulate phase was removed from the water column within a  
139 distance of 0.9 – 2 km of Harriet A (Burns and Codi, 1999; Holdway and Heggie,  
140 1998). Therefore for the caged fish study, the cages were situated along the gradient  
141 of PFW formed as the plume moves away from the discharge site. Site A (within 200  
142 m NNW of Harriet A) was the near-field site; Site B (~ 1000 m NNW of Harriet A)  
143 was the far-field site and Site C (~20 km NNW of Harriet A; in the Montebello  
144 Islands) was chosen as the reference site due to the lack of known contamination  
145 (Figure 2). The field component of this project was conducted aboard the AIMS RV  
146 Cape Ferguson from May 16-31, 2003. Initially, the target species was Red Emperor  
147 (*Lutjanus sebae*), since it could be caged successfully (Mike Cappo, pers. comm.). On  
148 the first day of collection; however, only 5 juvenile *L. sebae* were caught and another  
149 species, Stripey seaperch (*Lutjanus carponotatus*) were being trapped at a much  
150 higher proportion (10:1). *L. carponotatus* is an important recreational fishery in  
151 Dampier Harbor and preliminary biomarker research with this species from the  
152 Harriet A pilot study (Codi et al., 2001) suggested that it would suffice as a substitute  
153 species for *L. sebae*. From May 16-19 2003, an estimated 130 *L. carponotatus* were  
154 collected by fish traps along the Dampier Harbor LNG pipeline and maintained in a  
155 1000-L holding tank with running seawater and air. On May 21, all fish were placed  
156 in cages with 2 cages per station, 15 fish per cage at each of 3 sites (Figure 2). Fish  
157 were fed individually quick frozen (IQF) pilchards of the highest quality (Western  
158 Australia Bait Supply, Fremantle), collected from clean local waters off of Albany,  
159 WA. Food canisters were replenished every two days during the field exposure. Fish  
160 were sampled at T=0, 3 and 10 d.

161 A total of 50 Stripey seaperch were analyzed for CYP1A, CYP2M- and CYP2K-like  
162 proteins from the holding tank (T=0), and T=3 and T=10 for cage Sites A, B, and C.  
163 There were no significant differences for total weight, total length and age between  
164 the study sites ( $P < 0.05$ ; Table 1). All fish were handled and processed in a similar  
165 manner as stated for the pilot study.

## 166 2.3. Microsomal preparation and Western blot

167 Hepatic microsomal samples were prepared at the Australian Institute of Marine  
168 Science, Townsville, Qld (AIMS) following methods previously described (Haasch,  
169 2002) and shipped on dry ice to The University of Mississippi, University, MS.  
170 Hepatic microsomal samples were stored at  $-80^{\circ}\text{C}$  until analyzed. Protein content of  
171 the microsomal fraction was determined by Bio-Rad DC Protein Assay (Hercules,  
172 CA) using bovine serum albumin (BSA) as a standard. Microsomal proteins (10  $\mu\text{g}$

173 per well) were separated using pre-cast Criterion SDS-PAGE gels (10%T, 2.7%C  
174 resolving gel; 4%T, 2.7%C stacking gel; Bio-Rad) at 200V for 1 h with the Bio-Rad  
175 Criterion apparatus. Proteins were then electrotransferred (100V for 0.75 h) to a  
176 PVDF membrane (FluoroTrans W, 0.2  $\mu$ m; VWR) together with one lane containing  
177 MagicMark protein standards (contain proteins with IgG binding site; Invitrogen,  
178 Carlsbad, CA) detectable by chemiluminescence, for molecular weight determination  
179 and for evaluation of transfer efficiency. Sample loading buffer also contained  
180 Pyronin Y which is visible on the membrane and marks the dye front. Detection of  
181 proteins of interest was accomplished by using one of the primary polyclonal  
182 antibodies; rabbit anti-fish CYP1A (two synthetic peptides used as immunogens;  
183 BioSense, Bergen, NO distributed by Cayman Chemical, Ann Arbor, MI), rabbit  
184 anti-trout CYP2K1, rabbit anti-trout CYP2M1 (both gifts from Donald R. Buhler,  
185 PhD, Oregon State University, Corvallis, OR). Proteins of interest were visualized  
186 with goat anti-rabbit alkaline phosphatase conjugate (Bio-Rad) and the Western  
187 Breeze Novex Western Blot Immunodetection Kit (Invitrogen) according to the  
188 manufacturer's directions using Western Lightning CDP-Star Chemiluminescent  
189 reagent (Perkin Elmer Life Sciences, Boston, MA). Image analysis was accomplished  
190 using the VersaDoc 3000 Imaging System and QuantityOne analysis software  
191 (Bio-Rad). A summary of CYP1A results of the pilot study samples was previously  
192 presented (Codi King et al., 2005a) in which the samples were analyzed with a  
193 different primary antibody (anti-trout CYP1A1<sub>277-294/KLH</sub>) under different SDS-PAGE  
194 conditions (mini-Protean II, individually poured gels in the previous study) and with  
195 different methods for quantification (film, scanning densitometry and NIH Image<sup>®</sup>  
196 in the previous study). For this study, a commercially available antibody was used and  
197 the number of samples that could be compared on one gel was increased improving  
198 the statistical analyses.

#### 199 **2.4 Densitometry and Statistical Analysis**

200 The optical density of the samples is given as a Trace Quantity (QTY; Bio-Rad  
201 QuantityOne) or the quantity of a band as measured by the area under its intensity  
202 profile curve. Trace QTY units are intensity  $\times$  area. For standardization, one sample in  
203 triplicate was loaded on each gel. The mean Trace QTY of this sample was used as a  
204 gel factor for standardization between gels. Trace QTY results are presented as an  
205 Arbitrary Optical Density (without gray-scale calibration). Statistical analysis was  
206 performed using GraphPad Prism. For the pilot study, a two-way ANOVA was  
207 performed using both gender and site as covariates. The results indicated that for each  
208 individual isozyme site was a significant factor, but not gender, and there were no  
209 interactions between these two factors. Therefore, for the one-way ANOVA the males  
210 and females were pooled, and the ANOVA results were analyzed by Turkey's multiple  
211 comparison test ( $P < 0.05$ , 0.01 and 0.001). Similar analysis of covariates allowed the  
212 use of the same statistical approach for the caged fish study.

### 213 **3. Results**

#### 214 **3.1 Pilot study**



215 The results of the immunodetection of the CYP1A-, CYP2M1-, and CYP2K1-like  
216 proteins indicated that proteins of the appropriate molecular weight range for each  
217 isozyme were detectable in Gold-Spotted Trevally. Most Gold-Spotted Trevally  
218 showed one immunodetectable band of CYP1A-, CYP2M1- and CYP2K1- like  
219 proteins; some samples had a second CYP2M1- like protein. Because this second  
220 protein was not present in each sample, only the first band of lower molecular weight  
221 was quantified (Figure 3). Similar to the previous study (Codi King et al., 2005a),  
222 CYP1A-like protein levels in Gold-Spotted Trevally from the near-field (Harriet A)  
223 site were significantly higher than those from the far-field (Harriet C) site and the  
224 reference site. Although not significant, Gold-Spotted Trevally also exhibited a trend  
225 of increased CYP2M1-like protein levels at Harriet A as compared to Harriet C and  
226 the reference site (Figure 3).

227 For CYP2K1-like protein, a significant increase was observed at the Harriet C site  
228 compared to the reference site (Figure 3). The induction of CYP2K1-like proteins at  
229 the Harriet C site in Trevally may be due to possible exposure to hydrocarbons from  
230 natural oil seepages found in this region, or due to the movement of fish between  
231 platforms. The elimination of these variables was one of the main reasons for  
232 undertaking the subsequent controlled field study using caged fish.

### 233 **3.2. Caged fish study**

234 Immunodetection of CYP1A-like proteins indicated that proteins of the appropriate  
235 molecular weight range were detectable in Stripecy seaperch at all three sites and over  
236 the time of the exposure (Figure 4a). The results for the CYP1A-like proteins (Figure  
237 4b) demonstrated that by Day 3 of exposure both Site A and Site B had higher mean  
238 values than Site C, albeit not significant. By Day 10, fish collected at Site A had  
239 significantly higher induction of CYP1A-like proteins as compared to the fish at Site  
240 B and Site C. Comparisons also demonstrated that Site A fish after 10 days of  
241 exposure had significantly induced levels of CYP1A-like proteins as compared to the  
242 holding tank fish (T=0).

243 Figure 5a presents the optical densities for the species-specific pattern of  
244 CYP2M1-like proteins determined in Stripecy seaperch, with two distinct appropriate  
245 molecular weight proteins in most samples; CYP2M1-like Protein 1 (52.8 kDa) and  
246 CYP2M1-like Protein 2 (49.0 kDa). Immunodetection of CYP2M1-like proteins  
247 demonstrated a similar pattern of expression for both Protein 1 and Protein 2 in  
248 Stripecy seaperch with significant induction occurring at Day 10 for both proteins  
249 (Figures 5b and 5c) and at Day 3 the same increasing but not significant trend. For  
250 Site A both Day 3 and Day 10 exposed Stripecy seaperch had significantly higher  
251 levels of CYP2M1-like proteins than the holding tank fish. It is interesting that Site C  
252 Day 3 exposed fish also demonstrated site-specific differences from the holding tank  
253 fish.

254 Figure 6a presents the optical densities for the species-specific pattern of  
255 CYP2K1-like proteins determined in Stripecy seaperch, again showing two distinct  
256 appropriate molecular weight proteins; CYP2K1-like Protein 1 (52.5 kDa) and

257 CYP2K1-like Protein 2 (48.2 kDa). Unlike the two CYP2M1-like proteins, the  
258 induction patterns for the two CYP2K1-like proteins were dissimilar. For  
259 CYP2K1-like Protein 1, levels (Figure 4b) were significantly elevated for Site A Day  
260 3 as compared to fish collected on Day 3 from Site B and Site C. At Day 10 there  
261 were no site-specific differences in expression, although Site A Day 10 and Site A  
262 Day 3 Stripey seaperch had significantly higher CYP2K1-like Protein 1 levels than  
263 the holding tank fish. For CYP2K1-like Protein 2 levels (Figure 4c) the induction  
264 trend was similar to the pattern observed for CYP1A- and both CYP2M1-like Protein  
265 1 and Protein 2 in that by Day 10, Site A fish demonstrated the highest mean level of  
266 induction. The same site-specific differences were also observed with Site A Day 10  
267 fish and Day 3 fish having significantly higher CYP2K1-like Protein 2 induction  
268 levels than the holding tank fish.

#### 269 4. Discussion

270 Fish are important organisms for monitoring environmental contaminants because of  
271 their position in the food web, their lifestyle, their relative abundance and their  
272 adaptable physiology. Fish CYP1A induction is an extremely useful biomarker of  
273 chemical exposure in the environment (Flammarion et al., 2002), particularly for  
274 persistent pesticides (Levine et al., 1999), polychlorinated biphenyls (Burton et al.,  
275 2002; Haasch et al., 1993), dioxins (Zodrow et al., 2004; Smith, 2003), pulp mill  
276 effluents (Oikari et al., 2002), xenoestrogens (Katchamart et al., 2002), spilled crude  
277 oil (Jewett et al., 2002), and polycyclic aromatic hydrocarbons (PAHs) (Stagg et al.,  
278 2000). The induction of CYP1A has implications for human fish consumption, as well  
279 as for the health status of aquatic organisms (Arinc et al., 2000).

280 The induction of CYP2 family proteins in bluegill and catfish due to exposure to  
281 peroxisome proliferating agents (PPAs; Haasch, 1996) was the first description of an  
282 inducible P450 in fish other than CYP1A. The CYP2M1- and 2K1-like proteins in  
283 bluegill and catfish were later shown to be lauric acid hydroxylases (Haasch et al.,  
284 1998). Bivalves exposed to petroleum water-accommodated fraction (WAFs) produce  
285 peroxisome proliferation (Cajaraville et al., 1997). Therefore, petroleum hydrocarbon,  
286 as the major contaminant in PFW, may be considered a PPA, even though the  
287 mechanism is unknown. These findings provided an indication that a petroleum  
288 hydrocarbon-initiated peroxisome proliferation response may occur in lower  
289 vertebrates similar to the model PPA-mediated induction of CYP2M1/2K1 (Ackers et  
290 al., 2000; Johnson et al., 1996).

291 These two CYP2 family P450 isozymes have been most thoroughly characterized in  
292 rainbow trout, (*Oncorhynchus mykiss*; Buhler and Wang-Buhler, 1998). The molecular  
293 weight of CYP2K1 in rainbow trout is about 54 kDa, and has been shown to catalyze  
294 the ( $\omega$ -1) and ( $\omega$ -2) hydroxylation of lauric acid and some longer chain fatty acids; the  
295 molecular weight of CYP2M1 in rainbow trout is about 50 kDa, and this isozyme is  
296 the only known P450 responsible for laurate hydroxylation at the ( $\omega$ -6) position. Both  
297 isozymes also catalyze the hydroxylation of estradiol, testosterone and progesterone  
298 (Buhler and Wang-Buhler, 1998). It follows that induction of CYP2K1- and

299 CYP2M1-like proteins in fish may have effects on fatty acid and steroid homeostasis,  
300 with possible links to developmental and reproductive toxicity as well as  
301 carcinogenesis (Biegel et al., 1995; Vanden Heuvel, 1996; Vanden Heuvel et al., 1991).  
302 A variety of structurally diverse xenobiotics and pharmaceuticals are known to  
303 produce peroxisome proliferation and concordant fatty acid metabolizing enzyme  
304 induction in mammals, therefore, the monitoring of CYP2 family isozymes in fish  
305 may have implications for human health and has the potential to become an important  
306 indicator of environmental health.

307 The immunodetection results indicated one highly immunoreactive protein for  
308 CYP1A and multiple immunoreactive proteins for the CYP2-like proteins. Some  
309 cross-reactivity between CYP2-like proteins is expected with the polyclonal  
310 anti-CYP2K1 and anti-CYP2M1 antibodies (some affinity for the same protein  
311 epitopes) as these antibodies have cross-reactivity to both rainbow trout CYP2K1 and  
312 CYP2M1 (Buhler and Wang-Buhler, 1998). Further evidence for cross-reactivity was  
313 evident in samples that also included one or more minor proteins of intermediate size  
314 that were specific to individuals and not consistently represented across all  
315 individuals. The CYP2 family is known to be fairly complex in number of isozymes  
316 and expression levels (Buhler and Wang-Buhler, 1998). Multiple cross-reactivity was  
317 not unexpected and did not detract from the relatively clear induction of two proteins  
318 using the anti-CYP2 antibodies. The proteins were designated CYP2M1- and  
319 CYP2K1-like proteins because appropriate standards for the tropical fish species do  
320 not yet exist and were beyond the scope of this study. Nevertheless, it is evident that  
321 the CYP2 family antibodies recognize constitutively present and inducible proteins  
322 that could potentially be used as biomarkers. Furthermore, it is possible, with  
323 appropriate sequence information, for commercially-available anti-CYP2 family  
324 antibodies to be developed.

325 The pilot study CYP1A results supported the findings of an earlier analysis of those  
326 samples using a different methodology indicating induction of CYP1A (Codi King et  
327 al., 2005a). Unlike the previous analysis, the current study did not indicate  
328 gender-specific responses. Multiple differences between the two analyses could  
329 contribute to the discrepancy including the use of a different primary antibody  
330 preparation. Similar to the previous analysis, site-specific responses were detected.  
331 Analysis of the pilot study samples for CYP2M1- and CYP2K1-like proteins  
332 indicated possible site-specific induction and prompted the further examination of  
333 these proteins in the caged fish study.

334 Studies of environmental contaminant-mediated induction of the cytochromes P450 in  
335 tropical fish are lacking (Codi King et al., 2005a; Vrolijk et al., 1994). Both the pilot  
336 and caged fish field studies indicated significant results pertaining to the induction of  
337 cytochromes P450 in tropical fish species by PFW. First, the induction of CYP1A in  
338 Gold-Spotted Trevally and Stripey seaperch at the near-field site indicated that even  
339 though PFW discharge limits are being met (Codi King et al., 2005b), exposure to  
340 CYP1A inducing chemicals within the diluted PFW produced a significant induction

341 effect. Numerous studies have demonstrated that chronic low level exposure of fish  
342 and especially larvae to PAHs can have a significant impact and can produce  
343 long-term, adverse health and reproductive effects (Incardona et al., 2004; Stegeman  
344 et al., 2001). These adverse effects are most likely to be more serious to those  
345 organisms attached to the platform or living under or near the platform in a restricted  
346 range but could also be detrimental to organisms cyclically exposed to pools of PFW.  
347 Second, the significant induction of the CYP2K1/2M1-like proteins in both the  
348 Gold-Spotted Trevally and the Stripey seaperch is the first documented significant  
349 environmental induction in fish of any cytochrome P450 isozyme other than  
350 CYP1A1. The similar induction pattern of CYP1A1 and CYP2K1-like Protein 2 and  
351 CYP2M1-like Protein 1 and 2 may indicate that all of these isozymes are induced by  
352 the same chemical class contained within the PFW. While this finding alone is a  
353 reason for further study, it is perhaps even more interesting that there is an apparent  
354 response difference for induction of particular CYP2K1/2M1-like proteins. Only the  
355 CYP2K1-like Protein 1 was induced at the near-field site (Site A) on Day 3, but not  
356 on Day 10. The apparently different induction of the CYP2K1 Protein 1 compared to  
357 the other CYP isozymes potentially represents a unique chance to develop another  
358 bioindicator of PFW exposure. Although the metabolic activities of the CYP2M1 and  
359 CYP2K1 isozymes in Stripey seaperch are not known, it is reasonable to assume that  
360 the fatty acid and reproductive steroid hormone substrates would differ among the  
361 different proteins. Induction of different isozymes may therefore have different short-  
362 and long-term effects on the exposed organisms.

363 In support of the previous findings, the results indicate that CYP1A protein is  
364 sensitive to PFW exposure. Importantly, statistically significant environmental  
365 induction of both CYP2M1- and CYP2K1-like proteins in tropical fish due to PFW  
366 exposure had not previously been described and CYP2 family induction may  
367 represent possible new biomarkers (other than CYP1A) associated with PFW. In  
368 addition, the novel response of one CYP2K-like protein requires further verification  
369 but offers promise for improved monitoring of sub-lethal responses in marine  
370 organisms.

371 **References**

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373

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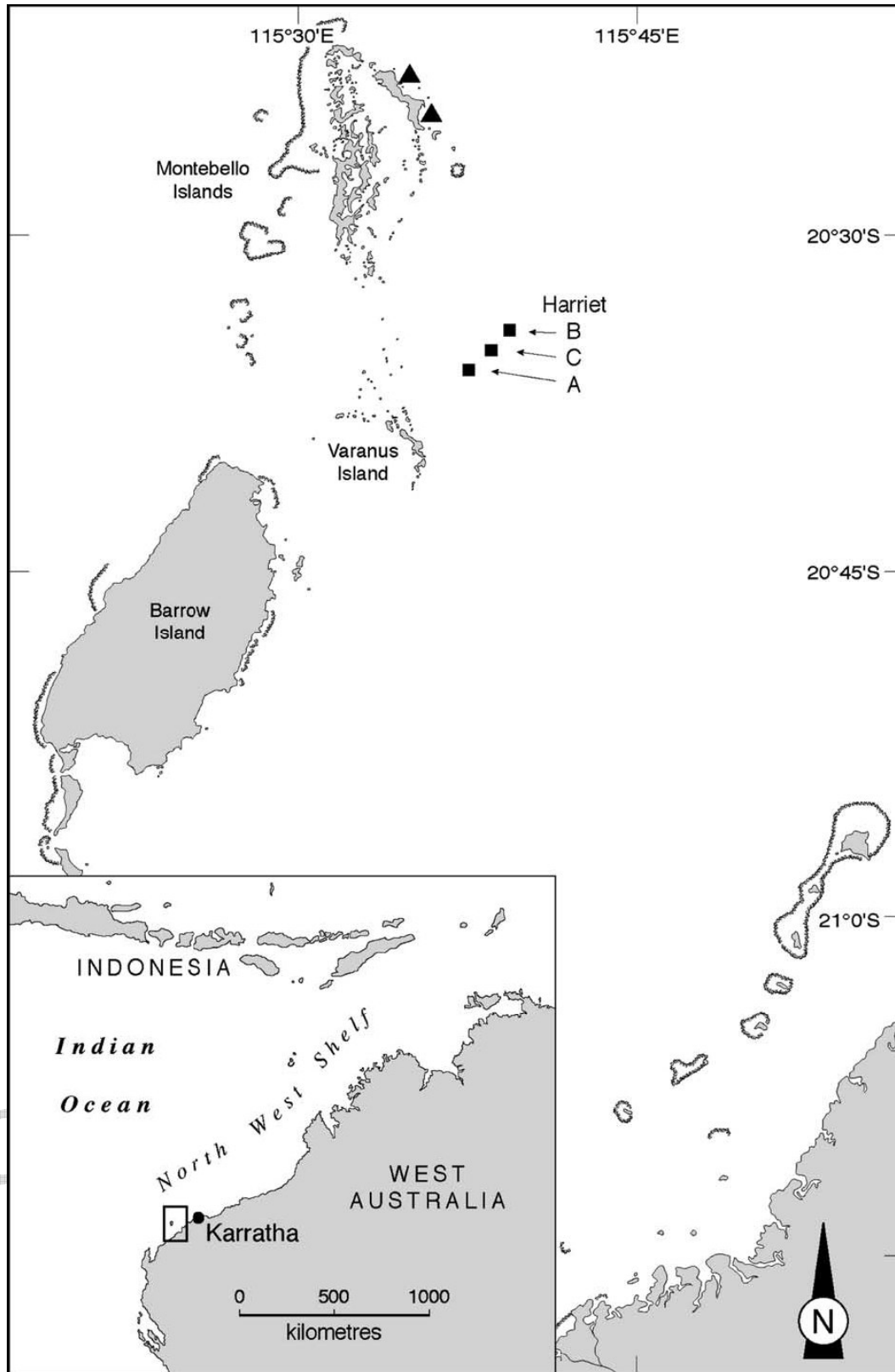
383 Table 1: Characteristics of Stripey seaperch used in the caged fish study. All of the  
 384 fish were of intermediate reproductive status (sex-stage) and had plasma vitellogenin  
 385 levels that were below the detection limit.

| Holding Tank or Cage Site | Time (days) | Sex Ratio (Female/Male) | Body Weight* (g) | Body Length* (mm) | Age* (yr) |
|---------------------------|-------------|-------------------------|------------------|-------------------|-----------|
| <b>Holding Tank</b>       | <b>0</b>    | (4/7)                   | 271 ± 85         | 261 ± 22          | 5 ± 2     |
| <b>Reference Site C</b>   | <b>3</b>    | (4/4)                   | 292 ± 83         | 265 ± 30          | 6 ± 4     |
|                           | <b>10</b>   | (4/4)                   | 284 ± 52         | 257 ± 19          | 4 ± 2     |
| <b>Far-Field Site B</b>   | <b>3</b>    | (0/5)                   | 273 ± 123        | 260 ± 34          | 5 ± 3     |
|                           | <b>10</b>   | (0/5)                   | 324 ± 92         | 269 ± 21          | 3 ± 2     |
| <b>Near-Field Site A</b>  | <b>3</b>    | (3/5)                   | 371 ± 88         | 285 ± 20          | 5 ± 3     |
|                           | <b>10</b>   | (1/4)                   | 293 ± 79         | 262 ± 28          | 5 ± 2     |

386 \*Values are mean ± SD for male and female combined.

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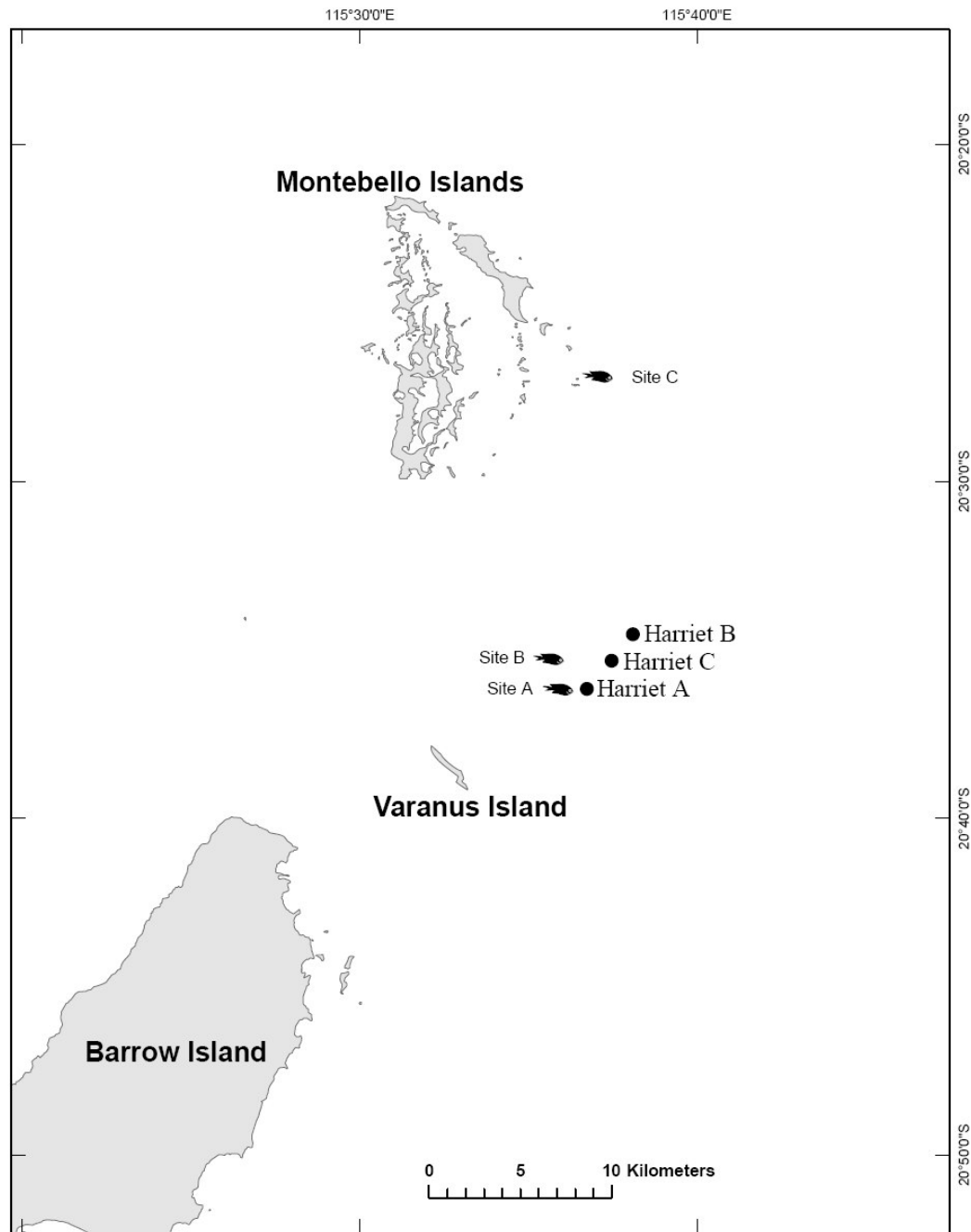




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389 Figure 1: Pilot study site locations: Harriet A, Harriet B, Harriet C, and the reference sites, indicated by  
 390 triangles, off the Montebello Islands.

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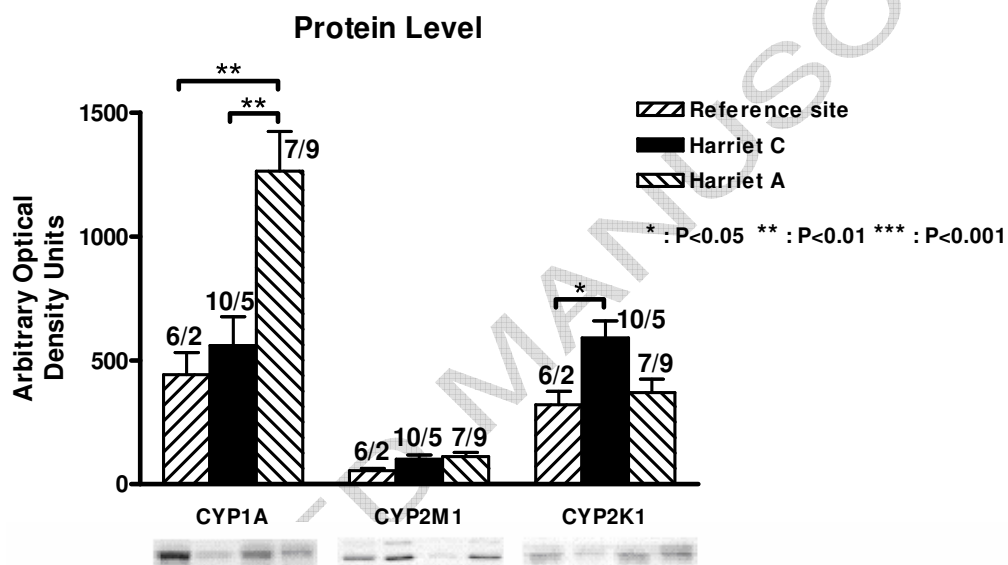
393 Figure 2. Site locations of the caged fish study: (Site A ~200 m, Site B ~1000 m and Site C ~20 km)

394 relative to the Harriet A PFW discharge.

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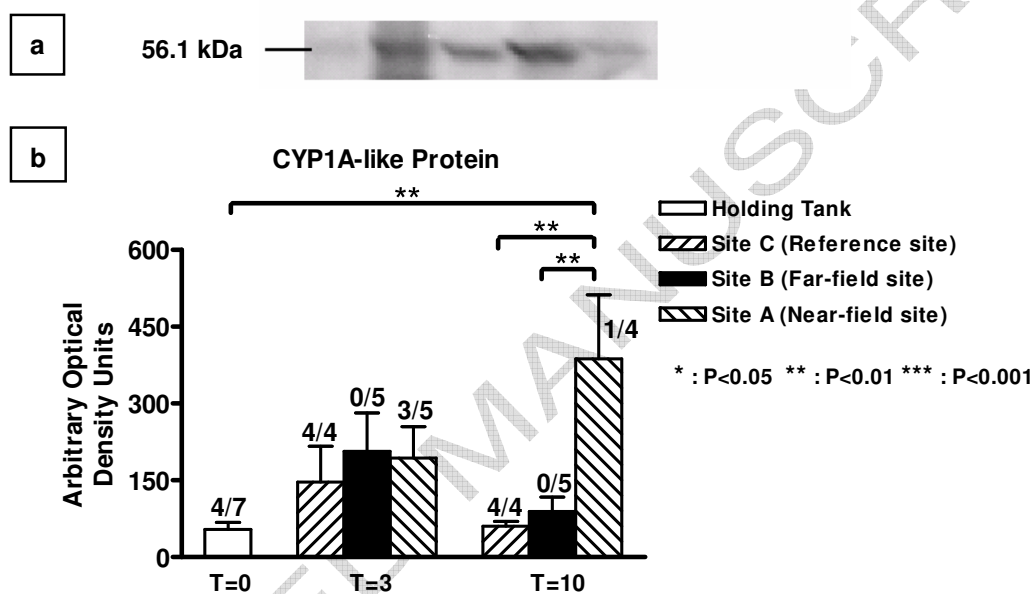
396 Figure 3. Pilot study: the species specific pattern of CYP1A-, CYP2M1-, and CYP2K1- like proteins  
 397 determined in Gold-Spotted Trevally (shown for comparison of isozyme expression not for illustration  
 398 of site effects), and graphical representation of the optical densities (Mean  $\pm$  SD) determined for each  
 399 site. The molecular weight range of the proteins is typical of cytochromes P450 and is between 45 and  
 400 60 kDa for all three isozymes. Numbers above the bars indicate the number of female and male fish  
 401 (e.g., 6/2, female/male) represented in the average densitometry values. No statistical difference was  
 402 observed between genders.

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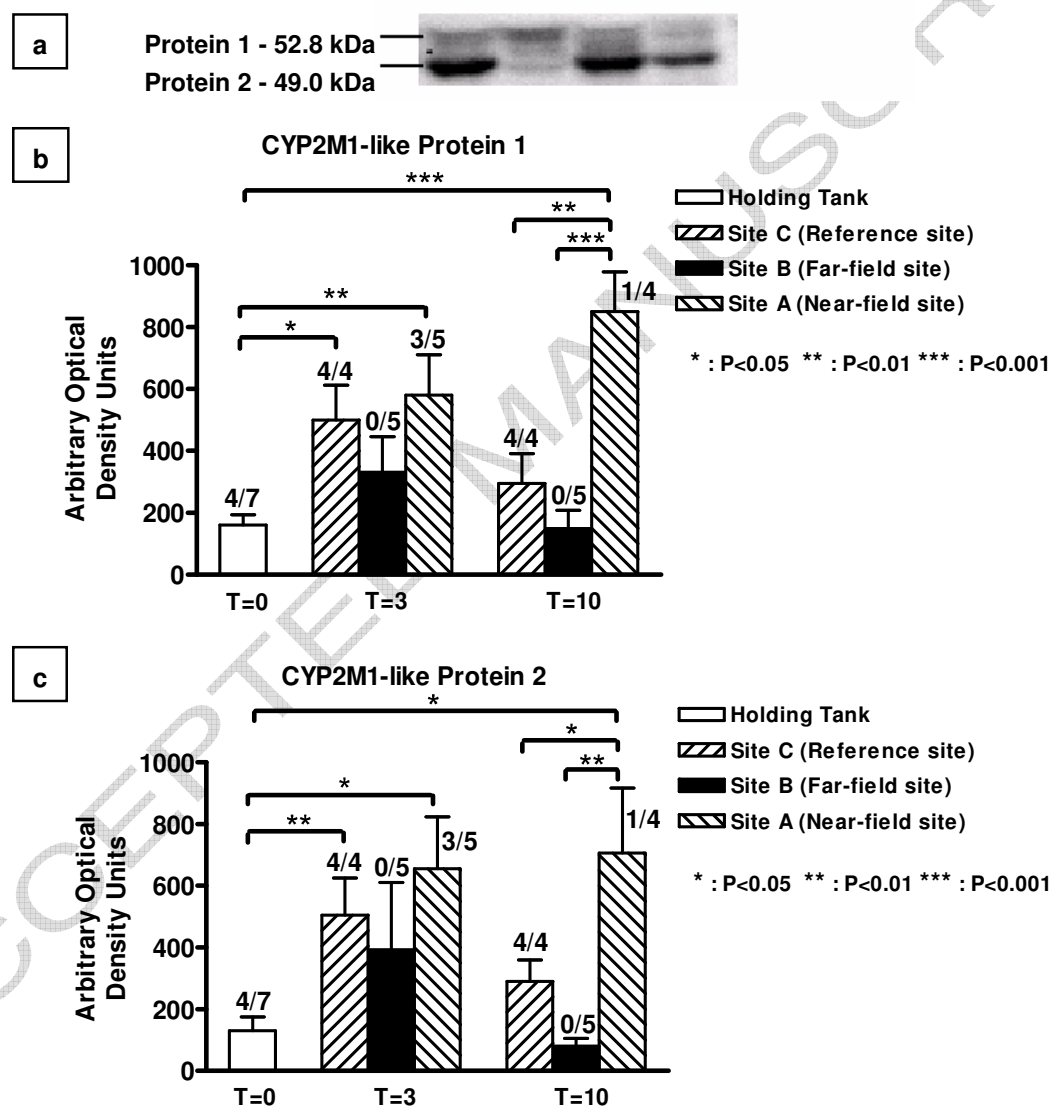
404

405 Figure 4 a-b. Caged fish study: the species-specific pattern of CYP1A-like proteins determined in  
 406 Stripey seaperch. (a) Image of the chemiluminescence detection of CYP1A-like proteins with a  
 407 single distinct band (56.1 kDa) in the appropriate molecular weight range (shown for comparison  
 408 of isozyme expression not for illustration of site effects), and (b) graphical representation of the  
 409 optical densities (Mean  $\pm$  SD) determined in Stripey seaperch at each site over time. Numbers  
 410 above the bars indicate the number of female and male fish (e.g., 4/7, female/male) represented in  
 411 the average densitometry values. No statistical difference was observed between genders.



412

413 Figure 5 a-c. Caged fish study: the species specific pattern of CYP2M1-like proteins determined in  
 414 Striped seaperch. (a) Image of the chemiluminescence detection of CYP2M1-like proteins with two  
 415 distinct bands of protein (shown for comparison of isozyme expression not for illustration of site  
 416 effects), (b) the graphical representation of optical densities (Mean  $\pm$  SD) determined for Protein 1 and  
 417 (c) for Protein 2. Numbers above the bars indicate the number of female and male fish (e.g., 4/7,  
 418 female/male) represented in the average densitometry values. No statistical difference was observed  
 419 between genders.



420

421 Figure 6 a-c. Caged fish study: the species specific pattern of CYP2K1-like proteins determined in  
 422 Stripecy seaperch. (a) Image of the chemiluminescence detection of CYP2K1-like proteins with two  
 423 distinct bands of protein (shown for comparison of isozyme expression not for illustration of site  
 424 effects), (b) the graphical representation of optical densities (Mean  $\pm$  SD) determined for Protein 1 and  
 425 (c) for Protein 2. Numbers above the bars indicate the number of female and male fish (e.g., 4/7,  
 426 female/male) represented in the average densitometry values. No statistical difference was observed  
 427 between genders.

