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1 Marine Environmental Research

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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°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 μg

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 μ 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_{277-294/KLH}) 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[®]
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 Strikey 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 Strikey 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 Strikey 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 Strikey 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 Strikey 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 (ω -1) and (ω -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 (ω -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.

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372

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373

374 **ACKNOWLEDGMENTS**

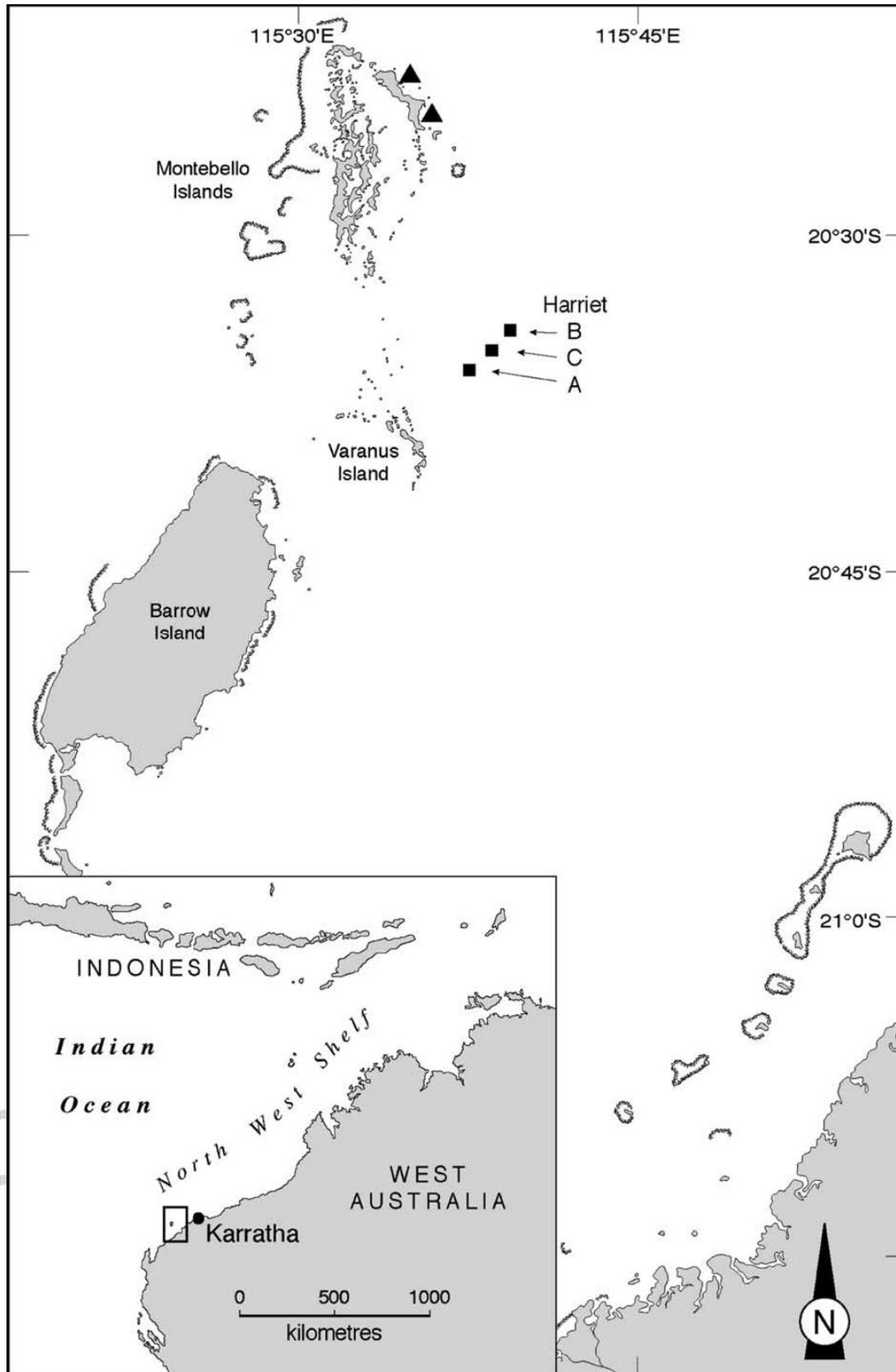
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383 Table 1: Characteristics of Stripey seaperch used in the caged fish study. All of the
 384 fish were of intermediate reproductive status (sex-stage) and had plasma vitellogenin
 385 levels that were below the detection limit.

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

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

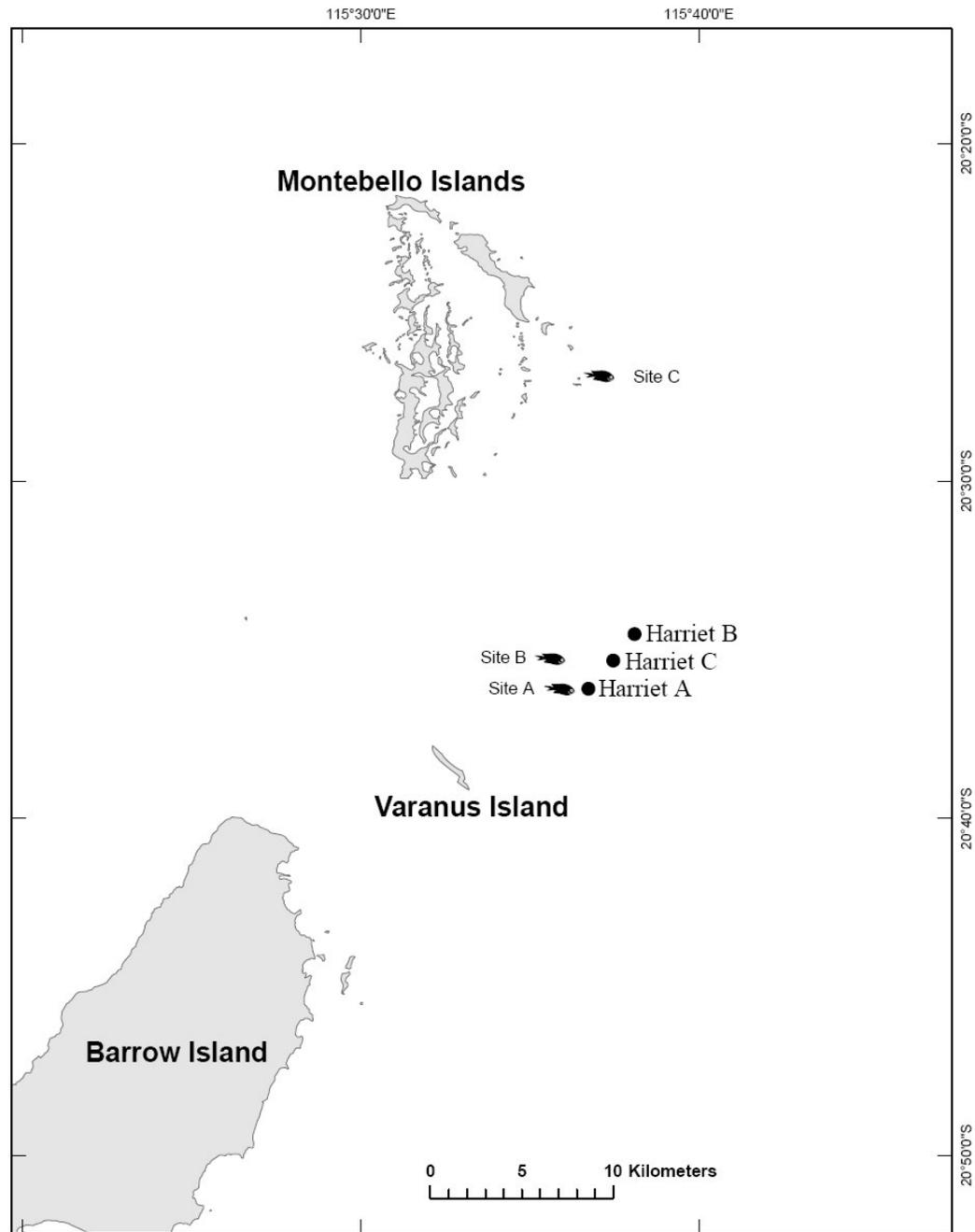
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389 Figure 1: Pilot study site locations: Harriet A, Harriet B, Harriet C, and the reference sites, indicated by
 390 triangles, off the Montebello Islands.

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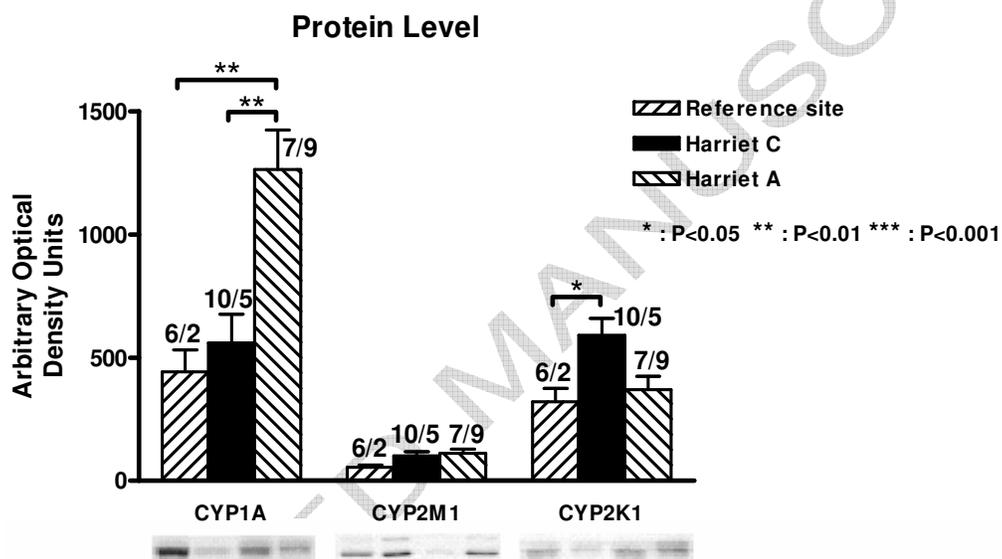
393 Figure 2. Site locations of the caged fish study: (Site A ~200 m, Site B ~1000 m and Site C ~20 km)

394 relative to the Harriet A PFW discharge.

395

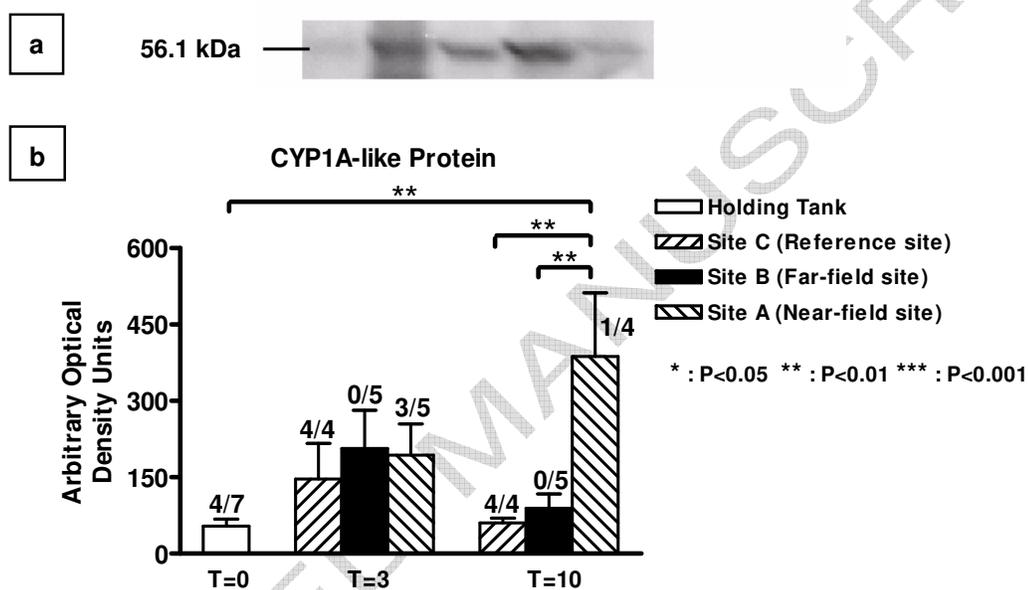
396 Figure 3. Pilot study: the species specific pattern of CYP1A-, CYP2M1-, and CYP2K1- like proteins
 397 determined in Gold-Spotted Trevally (shown for comparison of isozyme expression not for illustration
 398 of site effects), and graphical representation of the optical densities (Mean \pm SD) determined 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.

403



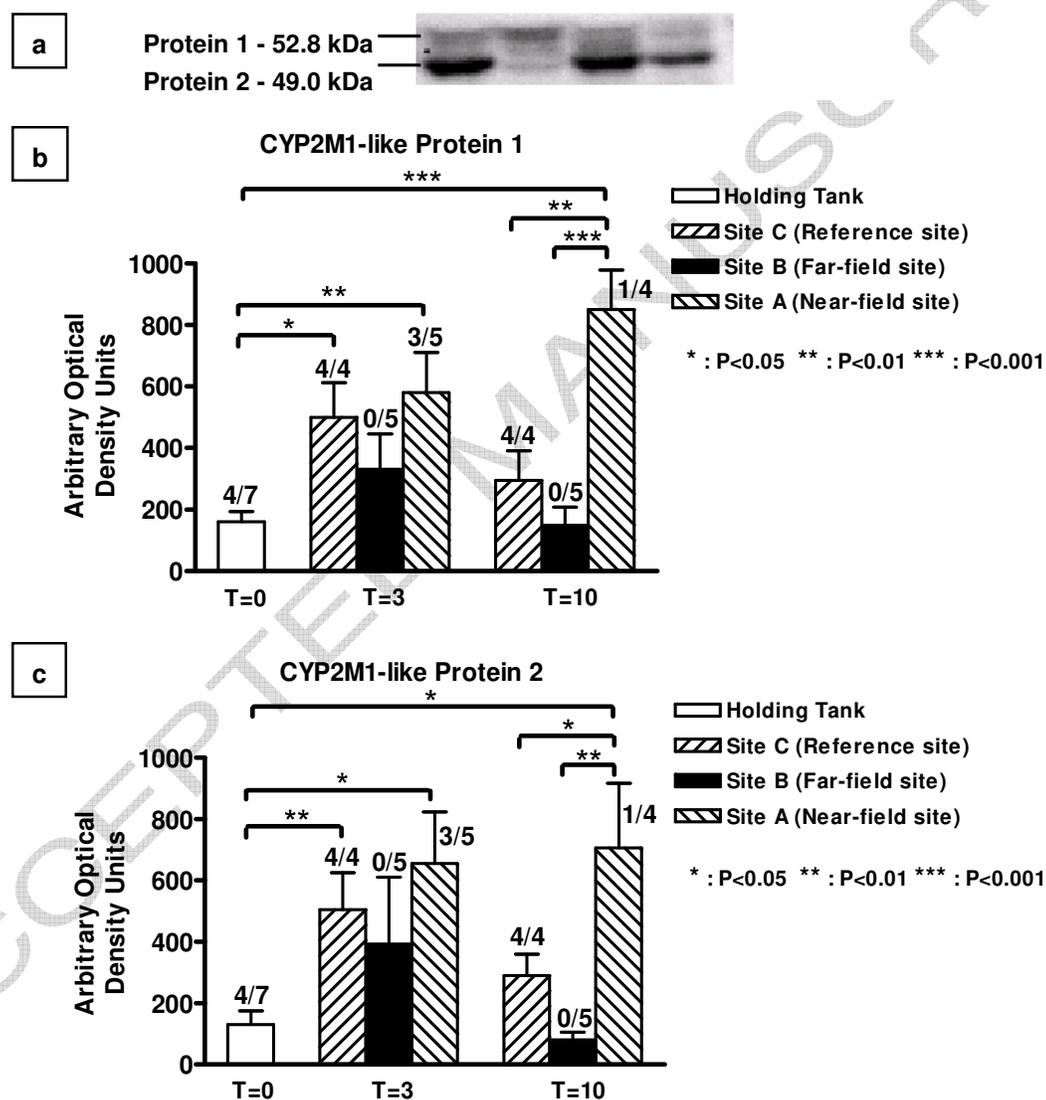
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405 Figure 4 a-b. Caged fish study: the species-specific pattern of CYP1A-like proteins determined in
 406 Strikey seaperch. (a) Image of the chemiluminescence detection of CYP1A-like proteins with a
 407 single distinct band (56.1 kDa) in the appropriate 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 Strikey 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.

