1 **Depurated fish as an alternative reference for field-based biomarker monitoring**

2

3

4

5 Diane Webb[1]*, Marthe Monique Gagnon[1]

6

7 [1] Curtin University of Technology, Department of Environmental Biology, Kent Street,

8 Bentley WA 6102, Australia

9

10 *Corresponding author and address

11 Diane Webb

12 Department of Environment and Agriculture

13 Curtin University of Technology

14 GPO Box U1987

15 Perth, Western Australia 6845

16 Email: d.webb@curtin.edu.au

17 Voice: +61 8 9266 2513

18 Fax: +61 8 9266 2495

19

20 Running head: Depurated fish as reference for field based biomarker studies

21

22 **ABSTRACT**

23 The entire Swan-Canning Estuary, south-western Australia, is impacted by human activity,

24 and the selection of a reference site to assess the impact of contamination on the health of

25 biota is not possible. To determine whether the use of fish depurated under laboratory

26 conditions is a suitable substitute, adult *Acanthopagrus butcheri* were collected from the

27 estuary and maintained in clean water (24 ppk) for 3 months. A suite of biomarkers were

28 assessed, namely; mixed-function oxygenase enzymes [ethoxycoumarin-*O*-deethylase

29 (ECOD) and ethoxyresorufin-*O*-deethylase (EROD)] activities, serum sorbitol

30 dehydrogenase (s-SDH), naphthalene-, pyrene-, and benzo[*a*]pyrene-type biliary

31 metabolites, DNA strand breaks, and heat shock protein (HSP70) levels. The results were

32 compared to field captured black bream from three sites within the estuary (Ascot,

33 Claisebrook, and Riverton), and to hatchery-bred juvenile fish. Biomarker levels were

34 lower (up to 3.8 times) in depurated fish compared to field captured fish, while DNA

35 integrity was higher. EROD activity was marginally lower in the hatchery-bred black

36 bream than the depurated fish while s-SDH levels were 2 times higher in the hatchery fish,

37 comparable to levels measured in the field. From the results obtained, field captured fish

38 depurated for 3 months are suitable to determine reference/baseline levels for biomarker of

39 health studies in estuarine environments.

40

41

42

45

46

47 **INTRODUCTION**

48 Defining the reference condition is considered to be an essential feature of ecosystem risk

49 assessment to determine a baseline against which all experimental evidence is compared

50 (Baird and Burton Jr 2001; Suter *et al.* 2007; Schmidt *et al.* 2009). However, this assumes

51 that a reference baseline exists in each ecosystem that is constant, except when influenced

52 by human activity (Kapustka 2008). Suter *et al.* (2007) defines different types of reference

53 conditions, such as historical, self-reference, local, regional natural, regional acceptable, or

54 no reference. None of these conditions can be applied to biochemical marker of fish health

55 studies in estuaries such as the Swan-Canning Estuary (SCE) in south-western Australia,

56 where anthropogenic inputs affect the whole system, with the exception of the no reference

57 definition. This is in accord with Foran and Ferenc (1999) who state that a priori definition

58 of a reference sites is not always possible especially where all sites within a study area are

59 considered impaired.

60

61 The SCE is a highly modified, wave-dominated salt wedge estuary (NLWRA 2002) with

62 an open (via training walls and dredging) connection to the ocean at Fremantle (Fig. 1).

63 Like all estuarine environments this estuary is a complex ecosystem undergoing high levels

64 of natural variability (Elliott and Quintino 2007; Schulte 2007). Physico-chemical

65 conditions are extremely variable with daily and seasonal movements of the salt wedge

66 within the estuary playing an important role in the distribution of soluble hydrocarbons at

67 the sediment/surface water interface (Thomson *et al.* 2001; Twomey and John 2001;

68 Westbrook *et al.* 2005).

69

70 The SCE is the focal point around which the city of Perth, with a population approaching

71 1.6 million people, has developed. There are no areas within its catchment that have not

72   been impacted by some form of human activity, either through urban, light industrial or

73   agricultural development. Direct discharge, surface runoff and groundwater all have the

74   potential to contribute nutrients, pesticides, fertilisers, heavy metals, and polycyclic

75   aromatic hydrocarbons (PAHs) in the form of fuel and oil into the estuary (Foulsham *et al.*

76   2009; Nice 2009; Nice *et al.* 2009).

77

78   Fish biomarker of health studies in the Swan-Canning Estuary commenced in 2000 at a

79   limited number of sites within the system (Webb and Gagnon 2002) after 170 years of

80   expanding human development throughout the catchment. A historical baseline for

81   biomarker studies in fish from this estuary is therefore not available. Estuaries similar to

82   the SCE in south-western Australia are all influenced by anthropogenic inputs from

83   agriculture, urbanisation and other human activity. Foran and Ferenc (1999) suggest

84   assessing site results based on a gradient of biochemical response from low to extremely

85   stressed. However studies to date show the proximity of major roads and stormwater

86   drains, former landfill sites and industrial precincts, significant weather events, are the

87   most significant factors affecting biomarker levels in biota. There is no evidence of

88   gradients of biomarker responses up- or downstream in the SCE (Webb and Gagnon 2002;

89   Webb *et al.* 2005a; b; c).

90

91   Depuration is a process designed to cleanse or remove contaminants from an animals gut

92   or tissue. This process is often used in purging shellfish of pathogens (Barile *et al.* 2009),

93   or to remove detritus from the gut of crustaceans (McClain 2000), to improve hygiene or

94   palatability for human consumption. Other studies use depuration techniques to determine

95   the bioaccumulation potential of lipophilic compounds in aquatic organisms (Ellgehausen

96   *et al.* 1980).

97

98   In this study black bream (*Acanthopagrus butcheri*), a fish species endemic to estuaries in

99   south-western Australia, were depurated in order to determine a baseline (reference)

100   condition for the assessment of health of fish in the estuary using biochemical markers.

101   Black bream were captured from the estuary and maintained under laboratory conditions

102   using clean water replicating the physico-chemical conditions within the estuary for a

103   period of three months. At the end of 3 months the depurated fish were sacrificed and a

104   selection of biomarkers were compared with fish, of a similar size and age, collected

105   directly from the estuary at the same time the laboratory depuration was terminated.

106   Further comparison was undertaken for several biomarkers measured in juvenile fish

107   sourced from a fish hatchery. It was hypothesised that laboratory depurated fish would be

108   suitable to use to determine a reference state for fish health biomarker studies in estuarine

109   environments.

110

111   **METHODS**

112   *Depuration of Fish*

113   Ten (10) black bream (*Acanthopagrus butcheri*) were collected from the Swan River in

114   January 2006 by a commercial fisherman using a 120 m, 100 mm monofilament haul net

115   then transferred to a 1000 litre tank at the Curtin Aquatic Research Laboratory with UV

116   sterilised recirculating water filtration. The fish were maintained at 24ppk and 20°C for 92

117   days, and fed human food quality mussels (*Mytilus* sp.). On the 92nd day the fish were

118   sacrificed and tissue and fluid biopsies taken

119

120   *Field collection*

121   In April 2006, 56 black bream were collected from two sites in the Swan River

122   (Claisebrook and Ascot; $N = 20$ respectively) and one site in the Canning River (Riverton;

123    $N = 16$; Fig 1). The fish were sacrificed within 2 hour of capture and biopsies taken.

124

125   *Hatchery-bred Fish*

126   Twenty (20) juvenile black bream, purchased from the Challenger Institute of Technology

127   hatchery Fremantle, Western Australia, had been maintained as control fish for an

128   unrelated laboratory experiment. These fish were kept in 4 x 100 litre aquariums under

129   similar conditions to the hatchery (37ppk, 20°C) in semi-static water conditions with 50%

130   daily water changes. These fish were sacrificed on day 21.

131

132   *Sample Collection*

133   Total weight, gutted weight and standard lengths were recorded for each fish. An external

134   examination was conducted for abnormalities and any sign of parasite infestation. A blood

135   sample was taken from the caudal vein using a vaccutainer, which was allowed to clot on

136   ice for 15 minutes then centrifuged for 10 minutes at 3000 x g. Each fish was killed by the

137   method of ike jime, bile collected from the gall bladder, and gill and liver biopsies taken.

138   All samples were immediately placed in liquid nitrogen then later transferred to a freezer

139   and held at –80 ^{o}C until analysis. Total protein content in the bile, and liver and gill

140   supernatants, was determined using the method of Lowry *et al.* (1951).

141

142   *Biomarker Analyses*

143   Ethoxyresorufin-*O*-deethylase (EROD) activity was measured in liver supernatant using

144   the methods of Hodson *et al.* (1991) adapted to black bream as detailed in Webb (2005).

145   The fluorescence of the supernatant was read on a Perkin-Elmer LS-45 Luminescence

146   Spectrometer at excitation/emission wavelengths of 535/585 nm (slit 10 ex/10 em). EROD

147   activity was expressed as picomoles of resorufin produced, per mg of total protein, per

148   minute (pmol R mg Pr^{-1} min^{-1}).

149

150   Bile Metabolites - Three biliary metabolite-types were measured, naphthalene, pyrene and

151   benzo(*a*)pyrene (B[*a*]P) by fixed wavelength fluorescence (FF) using the methods of

152   Krahn *et al.* (1986) for pyrene-type metabolites and Lin *et al.* (1996) for both naphthalene-

153   type and B(*a*)P-type metabolites. Biliary PAHs are standardised to biliary protein

154   (metabolite mg $protein^{-1}$).

155

156   Serum sorbitol dehydrogenase (s-SDH) activity - The s-SDH assay was modified from the

157   Sigma Diagnostics (St Louis, USA) Sorbitol Dehydrogenase Procedure No. 50-UV as

158   described by Webb and Gagnon (2007). The linear decrease in the rate of absorbance (ΔA)

159   over one minute was read on a Pharmacia UV-Visible Spectrophotometer at 340 nm. The

160   s-SDH activity is expressed as milli-International Units (mU) mL^{-1} serum.

161

162   The following biomarkers were measured in the depurated and field captured fish only.

163   Ethoxycoumarin-O-deethylase (ECOD) activity was measured in liver supernatant using

164   methods previously detailed by Webb *et al.* (2005a). The fluorescence of the supernatant

165   was read on a Perkin-Elmer LS-45 Luminescence Spectrometer at excitation/emission

166   wavelengths of 380/452 nm. ECOD activity was expressed as picomoles of 7-

167   hydroxycoumarin produced, per mg of total protein, per minute (pmol H mg Pr^{-1} min^{-1}).

168

169   DNA strand breakage - DNA strand breaks in liver supernatant was determined using the

170   alkaline unwinding assay method of Shugart (1996). Incubation times and temperatures to

171   obtain partially unwound DNA (DSS) and single stranded DNA (SS) in each sample were

172   optimized for black bream. Hoechst dye 33258 is used to bind with the isolated DNA in

173   solution. When DNA is in the single-stranded form, the intensity of fluorescence of the

174   bound dye is reduced to approximately one half of that observed for double-stranded DNA.

175   This development constitutes the basis for determining the amount of double- and single-

176   stranded DNA present in a sample of DNA during the alkaline unwinding assay (Shugart

177   1996). The fluorescence of the double stranded DNA (DS), DSS (incubated at $35^{o}C$ for 5

178   mins), and SS (incubated $85^{o}C$, 30 mins) present in each sample was read on a Perkin-

179   Elmer LS-5 Luminescence Spectrometer at excitation/emission wavelengths of

180   350ex/453em nm. The ratio of double-stranded DNA in the sample (F value) was

181   calculated using the equation, F = (DSS - SS)/(DS - SS). The F value is a measure of DNA

182   integrity with a high value corresponding to high DNA integrity.

183

184   Stress protein (HSP70) - Stress protein response was measured in gill supernatant using the

185   methods of Martin et al. (1996) optimized for black bream as outlined in Webb and

186   Gagnon (2009). HSP70 levels in the black bream are expressed as pixel density per μg of

187   total protein (pixels μg pr^{-1}).

188

189   *Statistical analysis*

190   Data are presented as mean ± standard error (SE) and analysis was done using the SPSS

191   statistical package (Version 17; SPSS GmbH, Germany). Where necessary, Log_{10}

192   transformations were done for each biomarker to achieve normality and homoscedasticity.

193   Student *t*-tests were carried out to determine whether any sexual differences were present

194   for each biomarker ($\alpha = 0.05$). As no interactions were found in the data sets main effects

195   were analysed using one-way ANOVAs. Where significant differences were found ($p <$

196   0.05), a Dunnett's (2 sided) test was run to compare the field and hatchery-bred fish with

197   the depurated fish group.

198

199   **RESULTS**

200   No significant differences were detected between male and female black bream for any

201   morphological measure, physiological indices, or biomarker analysed in this study ($p \geq$

202   0.05) so the results for both sex were pooled for each variable.

203

204   The length and weights of the black bream purchased from the hatchery were significantly

205   smaller than either the depurated fish or the freshly captured fish from the estuary (field

206   captured fish; $p \leq 0.001$; Table 1). The liver somatic index (LSI) of the hatchery-bred fish

207   was also much smaller compared to the other fish in the study ($p \leq 0.001$). However, the

208   hatchery-bred fish had a significantly higher condition factor (CF) compared to both the

209   depurated fish and the field captured fish ($p \leq 0.001$; Table 1). The depurated fish and the

210   field captured fish had similar morphology and physiological indices ($p \geq 0.05$; Table 1).

211

212   No significant differences were found in EROD activity ($p = 0.21$; Fig 2a), naphthalene-

213   type biliary metabolites ($p = 0.43$; Fig 3a), pyrene-type biliary metabolites ($p = 0.52$; Fig

214   3b) and B[a]p-type biliary metabolites (p=0.99; Fig 3c) measured in the hatchery-bred

215   black bream when compared with the depurated fish. Compared to the depurated fish, the

216   hatchery-bred black bream measured significantly higher s-SDH activity (p = 0.006; Fig 4)

217   which was at similar levels to the field collected fish.

218

219   *Field-captured vs. Depurated black bream*

220   EROD activity in the black bream was lower in the depurated fish than the field captured

221   fish ($p \leq 0.001$). This difference was significant between the depurated fish and fish

222   captured from Ascot ($p = 0.01$) and Claisebrook ($p \leq 0.001$) but not Riverton ($p = 0.81$; Fig

223   2a).

224

225   All biliary metabolites measured in the depurated black bream were lower than the field

226   captured fish in this study. This difference was significant at Claisebrook for all

227   metabolites (naphthalene and pyrene-type $p \leq 0.001$; B[a] P-type $p = 0.01$; Figs 3a, b, c).

228   The fish collected from Ascot had significantly higher pyrene-type metabolites ($p = 0.016$;

229   Fig 3b) while the fish from Riverton had higher B[a] P-type ($p = 0.02$; Figs 3c) when

230   compared to the depurated fish.

231

232   The activity of s-SDH was lower in the depurated black bream compared to all field

233   captured fish in this study but this difference was only significant when compared to the

234   fish from Ascot ($p = 0.003$) and Claisebrook ($p = 0.004$; Fig 4).

235

236   ECOD activity was significantly lower in the depurated fish ($p = 0.003$) than the field

237   captured black bream. This difference was statistically significant between the depurated

238   fish and fish captured from Claisebrook ($p = 0.04$) but not Ascot ($p = 0.99$) nor Riverton (p

239     $= 0.30$; Fig 2b).

240

241   The depurated black bream displayed higher DNA integrity than the field captured fish (p

242     $= 0.01$), which was only significant compared to the fish from Riverton ($p = 0.004$). Ascot

243   ($p = 0.06$) and Claisebrook ($p = 0.07$) also had lower DNA integrity but this was not

244   statistically lower (Figure 5a).

245

246 HSP70 levels were lower in the depurated black bream compared to the field captured fish

247 ($p = 0.01$) but this difference was only significant compared to Riverton ($p = 0.02$) and not

248 Claisebrook ($p = 0.06$) or Ascot ($p = 0.93$; Fig 5b).

249

250 **DISCUSSION**

251 This study has clearly validated the use of depurated fish to establish baseline levels for a

252 suite of biochemical markers of fish health in the black bream from the Swan-Canning

253 Estuary.

254

255 The biomarkers measured in the depurated black bream are all significantly lower that

256 those measured in the fish collected directly from the field. There was an almost 50%

257 reduction in the Cytochrome (CYP) detoxification enzymes as measured by both EROD

258 and ECOD activity induction levels. Serum SDH levels in the depurated were also up to

259 50% below the levels measured in the field fish demonstrating that the lower levels of

260 EROD and ECOD levels were not due to liver damage. There was a 100 to 275%

261 improvement in the levels of biliary metabolites measured. DNA integrity was higher in

262 the depurated fish although this was only significant when compared to the fish captured

263 from the Riverton site. Finally, HSP70 expression was between 119% lower that the black

264 bream measured from Claisebrook and 155% lower than the fish analysed from Ascot

265 indicating a reduction in oxidative stress levels. These results clearly show that the

266 biomarker levels had come close to, or attained, baseline levels in the black bream which

267 compares well to the results of Ferreira *et al.* 2007 using sea mullet (*Mugil cephalus*).

268

269   Ferreira *et al.* 2007 studied long term depuration on the levels of oxidative stress

270   biomarkers in *M. cephalus*). The fish, chronically exposed to contaminants in the Douro

271   estuary, were transferred to and maintained in unpolluted seawater for periods of 1, 4, and

272   8 months. The researchers found that the mullets had the capacity to recover oxidative

273   damage following long term depuration. Mixed results were observed after 1 month

274   depuration whereas the activities of antioxidant enzymes, as well as stress induced

275   oxidative damaged proteins, had returned to normal values at 4 months.

276

277   Depurated fish also provided an improved benchmark when compared to hatchery-bred

278   fish. The fish collected for depuration were adults of comparable size/age and with similar

279   life histories as the fish sampled directly from the field whereas as the hatchery-bred fish

280   were appreciably smaller juvenile fish (Table 1) that had been bred and reared under

281   artificial conditions. Although EROD activity induction was lower in hatchery-bred fish,

282   measured s-SDH levels were comparable to the highest readings measured in the field

283   captured fish. This result suggests the possibility that some hepatocellular injury had

284   occurred in the juvenile black bream impacting the CYP1A detoxification enzymes.

285

286   3. Implications for ERM

287   • According to Baird and Burton Jr (2001) the establishment of an appropriate benchmark

288   or reference condition for comparison is required to assess biochemical responses of

289   fish to contaminant exposure under field conditions.

290   • Preston 2002 – Reference sites in ecological risk assessment used to compare an

291   impacted to a 'pristine' or non-impacted site. Often located in close proximity.

292   Reference may be compromised by indirect results. E.g. periodic foraging of a fish in a

293   relatively small contaminated patch may have adverse effects over a much larger

294   geographic area with adjacent areas. Adjacent non-contaminated ecosystems may still

295   be affected at a distance by contamination and therefore not suitable as reference.

296 • Elliott and Quintino 2007 – Estuarine Quality Paradox – the difficulty in separating

297   natural and anthropogenic stress in estuaries – repercussions for implementation of all

298   environmental management systems reliant on ability to detect changes based on a

299   defined reference condition.

300 • Lobry *et al.* 2006 - particularly difficult in estuarine situations to define a reference

301   condition as estuaries are complex ecosystems and fluctuate with time (seasonally).

302 • This is the case in many estuarine environments, such as the Swan-Canning Estuary

303   (SCE), where anthropogenic inputs affect the whole system.

304

305 4. Conclusions

306 From the results obtained, field captured fish depurated for 3 months are suitable to

307 determine baseline levels and therefore the reference state for biomarker of health studies

308 in estuarine environments.

309

316

317   **REFERENCES**

318   Baird D.J., Burton Jr G.A., editors. 2001. Ecological variability: separating natural from

319   anthropogenic causes of ecosystem impairment. Pensacola, FL, USA: SETAC

320   Press. 336 p.

321   Barile N.B., Scopa M., Nerone E., Mascilongo G., Recchi S., Cappabianca S., Antonetti L.

322   2009. Study of the efficacy of a closed cycle depuration system on bivalve

323   molluscs. Vet. Ital. 45:555-566.

324   Ellgehausen H., Guth J.A., Esser H.O. 1980. Factors determining the bioaccumulation

325   potential of pesticides in the individual compartments of aquatic food chains.

326   Ecotoxicol. Environ. Saf. 4:134-157.

327   Elliott M., Quintino V. 2007. The estuarine quality paradox, environmental homeostasis

328   and the difficulty of detecting anthropogenic stress in naturally stressed areas. Mar.

329   Pollut. Bull. 54:640-645.

330   Ferreira M., Moradas-Ferreira P., Reis-Henriques M.A. 2007. The effect of long-term

331   depuration on levels of oxidative stress biomarkers in mullets (*Mugil cephalus*)

332   chronically exposed to contaminants. Mar. Environ. Res. 64:181-190.

333   Foran J.A., Ferenc S.A., editors. 1999. Multiple stressors in ecological risk and impact

334   assessment: proceedings from the Pellston Workshop on Multiple Stressors in

335   Ecological Risk and Impact Assessment; 13-18 September 1997. Pensacola, FL,

336   USA: SETAC Press. 100 p.

337   Foulsham G., Nice H.E., Fisher S., Mueller J., Bartkow M., Komorova T. 2009. A baseline

338   study of organic contaminants in the Swan and Canning catchment drainage system

339   using passive sampling devices. Perth, Western Australia: Department of Water.

340   Water Science Technical Series Report No. 5.

341 Hodson P.V., Klopper-Sams P.J., Munkittrick K.R., Lockhart W.L., Metner D.A., Luxon

342   P.I., Smith I.R., Gagnon M.M., Servos M., Payne J.F. 1991. Protocols for

343   measuring mixed function oxygenases of fish liver. Canadian Technical Report of

344   Fisheries and Aquatic Sciences 1829, Department of Fisheries and Oceans, Quebec.

345   51 p.

346 Kapustka L. 2008. Limitations of the current practices used to perform ecological risk

347   assessment. Integr. Environ. Assess. Manag. 4:290-298.

348 Krahn M.M., Rhodes L.D., Myers M.S., Macleod Jr W.D., Malins D.C. 1986. Association

349   between metabolites of aromatic compounds in bile and the occurrence of hepatic

350   lesions in English sole (*Parophrys vetulus*) from Puget Sound, Washington. Arch.

351   Environ. Contam. Toxicol. 15:61-67.

352 Lin E.L.C., Cormier S.M., Torsella J.A. 1996. Fish biliary polycyclic aromatic

353   hydrocarbon metabolites estimated by fixed-wavelength fluorescence: comparison

354   with HPLC-fluorescent detection. Ecotoxicol. Environ. Saf. 35:16-23.

355 Lobry J., Lepage M., Rochard E. 2006. From seasonal patterns to a reference situation in

356   an estuarine environment: Example of the small fish and shrimp fauna of the

357   Gironde estuary (SW France). Estuar. Coast. Shelf Sci. 70:239-250.

358 Lowry O.H., Rosebrough N.J., Farr A.L., Randall R.J. 1951. Protein measurement with the

359   Folin Phenol reagent. J. Biol. Chem. 193:265-275.

360 Martin L.S., Nieto S.R., Sanders B.M. 1996. Characterization of the cellular stress

361   response in aquatic organisms. In: Ostrander G., editor. Techniques in Aquatic

362   Toxicology. Boca Raton, Florida: CRC Press Inc. p 341-370.

363 McClain W.R. 2000. Assessment of depuration system and duration on gut evacuation rate

364   and mortality of red swamp crawfish Aquaculture 186:267-278.

365   Nice H.E. 2009. A baseline study of contaminants in the sediments of the Swan and

366   Canning estuaries. Perth, Western Australia: Department of Water. Water Science

367   Technical Series Report No. 6. 54 p.

368   Nice H.E., Grassi M., Foulsham G., Morgan B., Evans S.J., Robb M. 2009. A baseline

369   study of contaminants in the Swan and Canning catchment drainage system. Perth,

370   Western Australia: Department of Water. Water Science Technical Series Report

371   No. 3. 150 p.

372   NLWRA. 2002. Australian Catchment, River and Estuary Assessment 2002. Turner, ACT,

373   Australia: National Land and Water Resources Audit. 192 p.

374   Preston B.L. 2002. Indirect effects in aquatic ecotoxicology: implications for ecological

375   risk assessment. Environ. Manag. 29:311-323.

376   Schmidt S.I., Konig-Rinke M., Kornek K., Winkelmann C., Wetzel M.A., Koop J.H.,

377   Benndorf J. 2009. Finding appropriate reference sites in large-scale aquatic field

378   experiments. Aquatic Ecology 43:169-179.

379   Schulte P.M. 2007. Responses to environmental stressors in an estuarine fish: interacting

380   stressors and the impacts of local adaptation. J. Therm. Biol. 32:152-161.

381   Shugart L.R. 1996. Application of the alkaline unwinding assay to detect DNA strand

382   breaks in aquatic species. In: Ostrander G., editor. Techniques in Aquatic

383   Toxicology. Boca Raton, Florida: CRC Press Inc. p 205-218.

384   Suter G., Cormier S., Norton S. 2007. Ecological epidemiology and causal analysis. In:

385   Suter G.W., editor. Ecological Risk Assessment 2nd ed. Boca Raton, FL: CRC

386   Press. p 39-68.

387   Thomson C., Rose T., Robb M. 2001. Seasonal water quality patterns in the Swan River

388   Estuary, 1994-1998, Technical Report. Perth, Western Australia: Swan River Trust.

389   29 p.

390   Twomey L., John J. 2001. Effects of rainfall and salt-wedge movement on phytoplankton

391   succession in the Swan-Canning Estuary, Western Australia. Hydrolog. Proc.

392   15:2655-2669.

393   Webb D. 2005. Assessment of the health of the Swan-Canning River system using

394   biochemical markers of exposure in fish. PhD Thesis [Online]. Perth, Western

395   Australia: Curtin University of Technology. 253 p. Available at

396   http://adt.curtin.edu.au/theses/available/adt-WCU20061204.135553/.

397   Webb D., Gagnon M.M. 2002. Biomarkers of exposure in fish inhabiting the Swan-

398   Canning Estuary, Western Australia - a preliminary study. J. Aquat. Ecosys. Stress

399   Rec. 9:259-269.

400   Webb D., Gagnon M.M. 2007. Serum sorbitol dehydrogenase activity as an indicator of

401   chemically induced liver damage in black bream (*Acanthopagrus butcheri*).

402   Environ. Bioind. 2:172-182.

403   Webb D., Gagnon M.M. 2009. The value of stress protein 70 as an environmental

404   biomarker of fish health under field conditions. Environ. Toxicol. 24:287-295.

405   Webb D., Gagnon M.M., Rose T. 2005a. Interannual variability in fish biomarkers in a

406   contaminated temperate urban estuary. Ecotoxicol. Environ. Saf. 62:53-65.

407   Webb D., Gagnon M.M., Rose T. 2005b. Interseasonal variability in biomarkers of

408   exposure in fish inhabiting a southwestern Australian estuary. Environ. Toxicol.

409   20:522-532.

410   Webb D., Gagnon M.M., Rose T. 2005c. Metabolic enzyme activities in black bream

411   (*Acanthopagrus butcheri*) from the Swan-Canning Estuary, Western Australia.

412   Comp. Biochem. Physiol. C 141:356-365.

413   Westbrook S.J., Rayner J.L., Davis G.B., Clement T.P., Bjerg P.L., Fisher S.J. 2005.

414   Interaction between shallow groundwater, saline surface water and contaminant

415   discharge at a seasonally and tidally forced estuarine boundary. J. Hydrol. 302:255-

416   269.

417

418

420 TABLE 1: Mean (± SEM) measurements and physiological indices of black bream

421 collected during autumn in the Swan-Canning Estuary. Within columns, values marked

422 with an asterisk are significantly different to the depurated fish ($p \geq 0.05$).

Site	N	Standard length (cm)	Gutted weight (g)	Condition Factor[1]	Liver Somatic Index[2]
Depurated	7	26.1 ± 1.2	534 ± 59	2.98 ± 0.14	1.62 ± 0.27
Ascot	20	25.3 ± 0.4	460 ± 25	2.81 ± 0.03	1.24 ± 0.08
Claisebrook	10	24.8 ± 0.3	439 ± 12	2.87 ± 0.03	1.17 ± 0.04
Riverton	16	25.8 ± 0.8	517 ± 58	2.89 ± 0.03	1.56 ± 0.09
Hatchery bred	17	13.8* ± 0.03	91* ± 6	3.37* ± 0.07	1.02* ± 0.07
p-value		≤0.001	≤0.001	≤0.001	0.008

423 [1] Condition Factor = (gutted weight/standard lenght^3) x 100. [2] Liver Somatic Index = (Liver

424 weight/gutted weight) x 100.

425

426 **List of Figures**

427 **Figure 1.** Field collection sites within the Swan-Canning Estuary (adapted from Swan

428 River Trust, 1999).

429

430 **Figure 2**. Mixed function oxygenase activities (mean ± SEM) in black bream (A) EROD

431 activity (pmol R mg Pr^{-1} min^{-1}); (B) ECOD activity (pmol H mg Pr^{-1} min^{-1}). Bars marked

432 with an asterisk are significantly different to the depurated fish ($p \geq 0.05$).

433

434 **Figure 3**. Biliary metabolites (mean ± SEM) in black bream; (A) naphthalene-type (mg

435 metabolite mg protein^{-1}); (B) pyrene-type (µg metabolite mg protein^{-1}); (C) B[a]p-type (µg

436 metabolite mg protein^{-1}). Bars marked with an asterisk are significantly different to the

437 depurated fish ($p \geq 0.05$).

438

439 **Figure 4**. s-SDH activity (mU; mean ± SEM) in the serum of black bream. Bars marked

440 with an asterisk are significantly different to the depurated fish ($p \geq 0.05$).

441

442 **Figure 5**. Biomarkers of effect (mean ± SEM) in black bream. (A) DNA integrity (F

443 value); (B) HSP70 levels (pixels µg pr-1). Bars marked with an asterisk are significantly

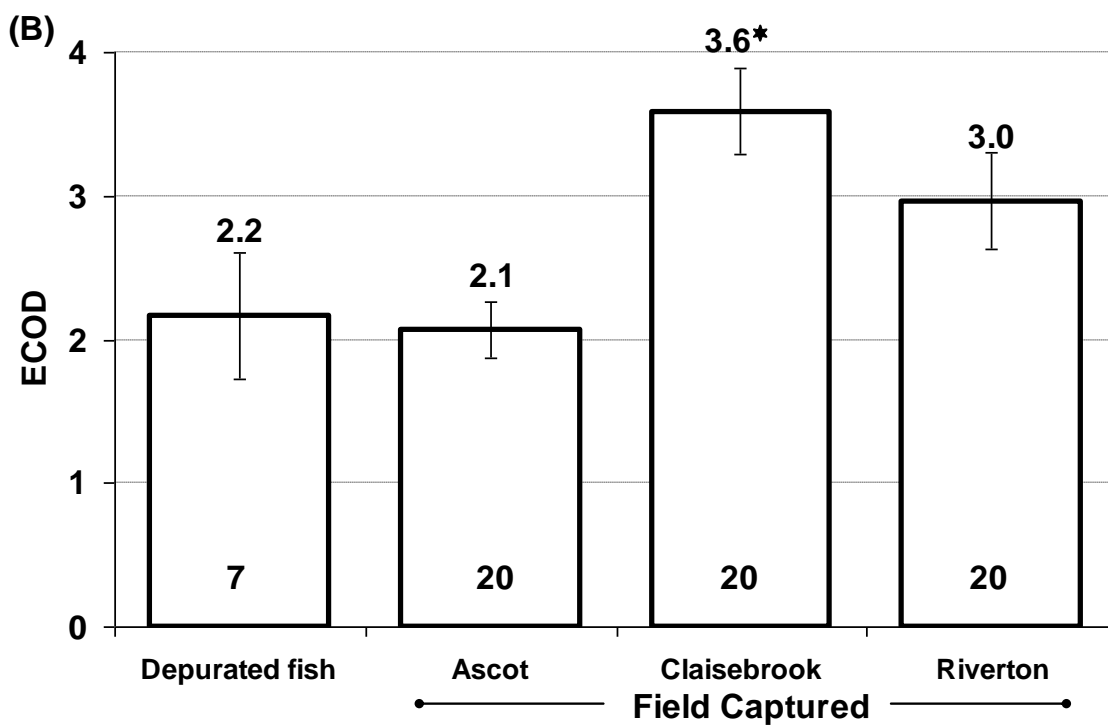444 different to the depurated fish ($p \geq 0.05$).
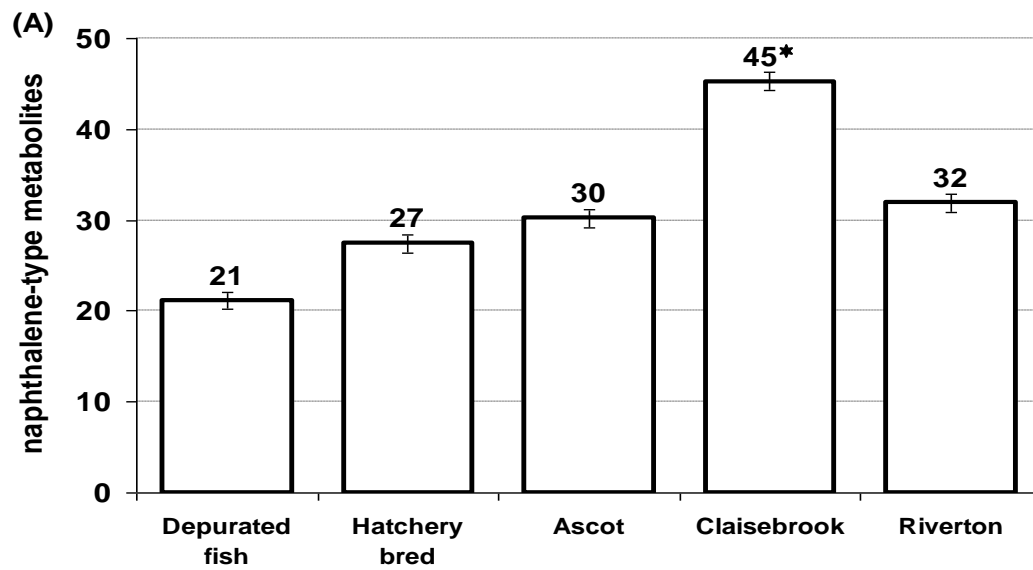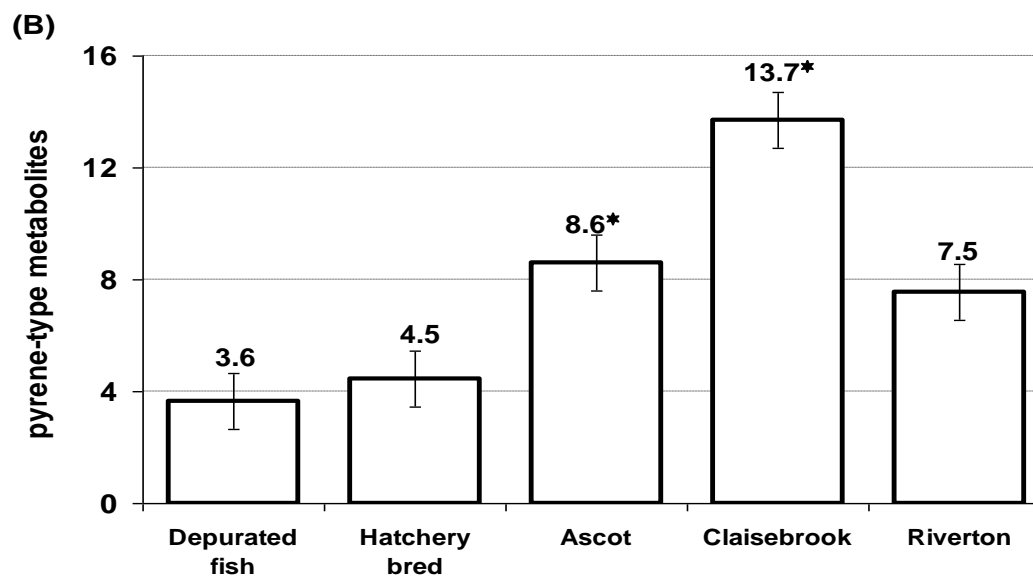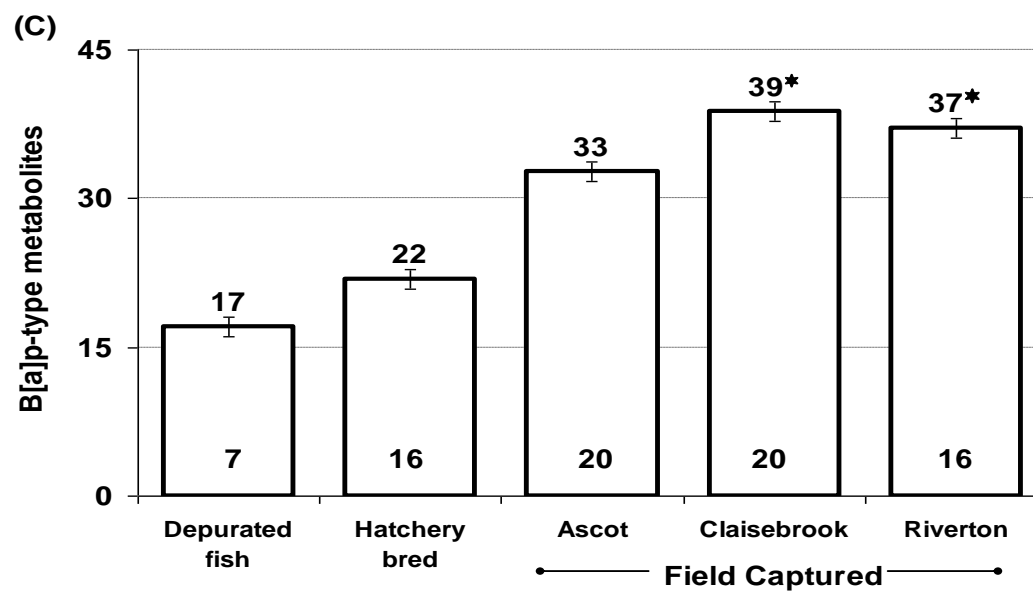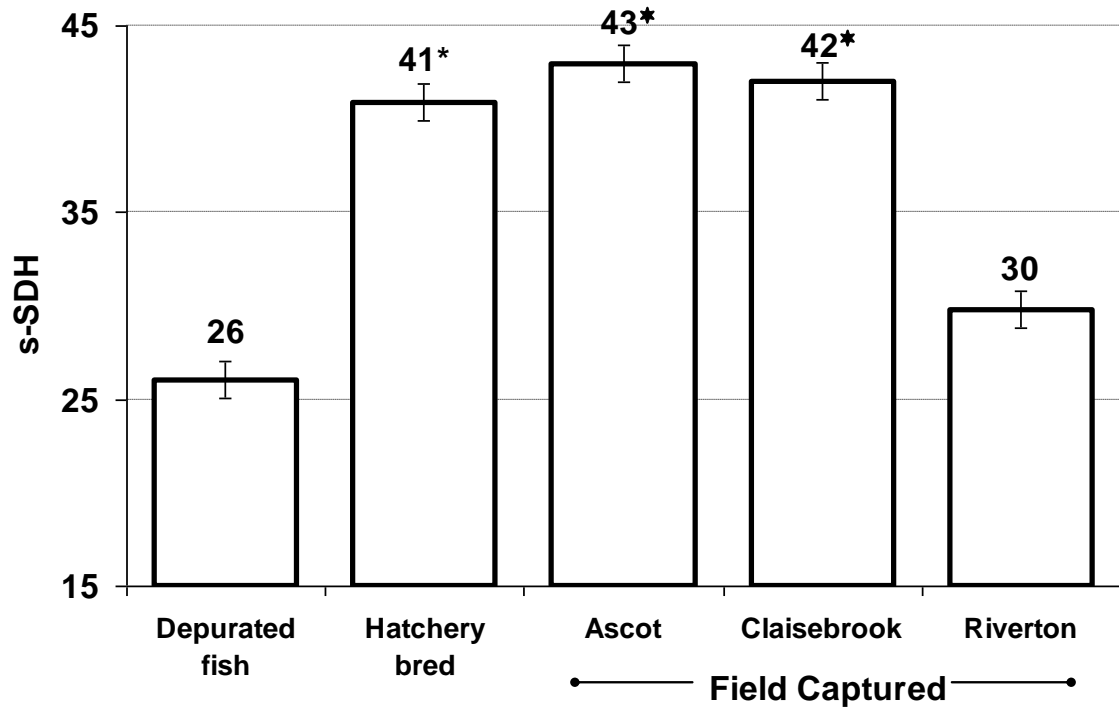
445
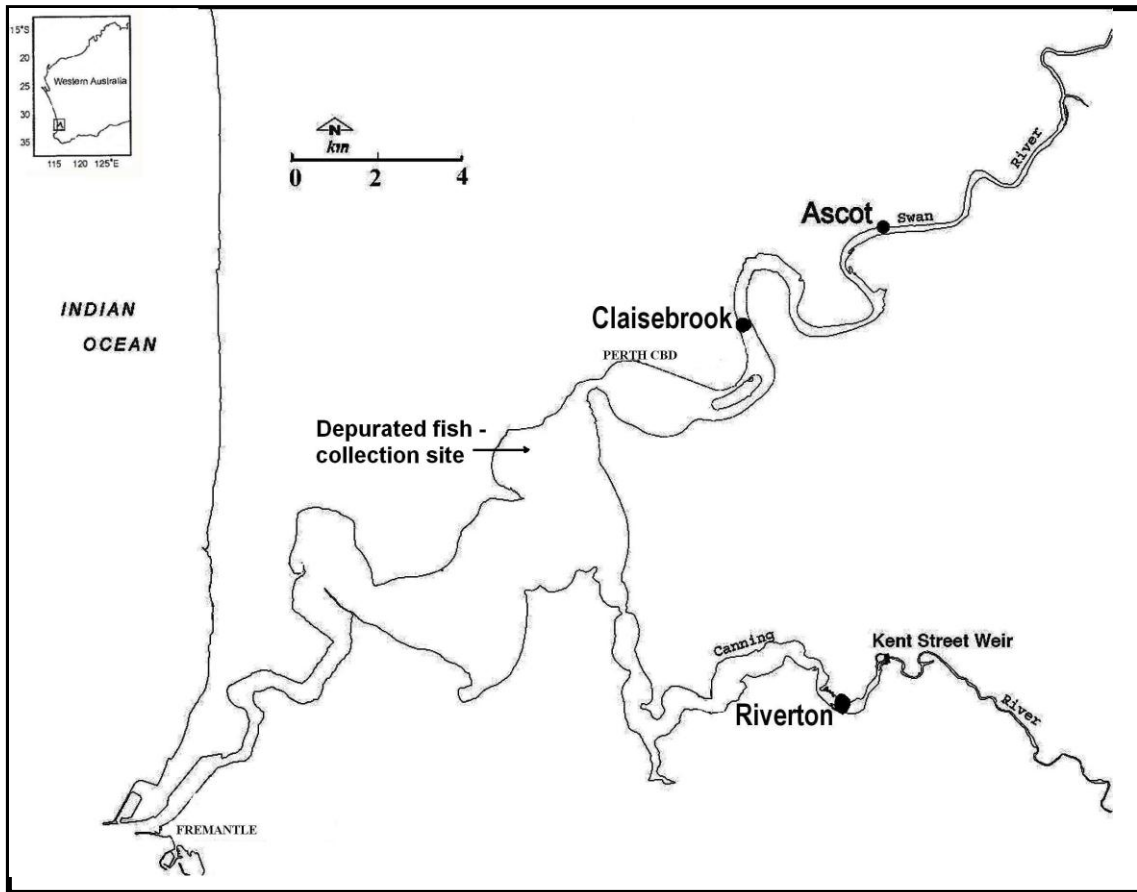
446

447

448
449

450
451

(A)

(B)

(C)

452

453

454

455

456
457

(A)

458

(B)

459