Report

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EDCAT 5. A catchment-based study of endocrine disruption in surface waters: multivariate evaluation of the health of a sentinel fish species exposed to sewage treatment works effluent.

Annexe to EDCAT Final report – CYP1A gene expression results

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5.2 Methods

5.2.12 CYP1A gene expression: RNA was extracted from stickleback livers using the Qiagen RNeasy mini kit (Qiagen) and was converted to cDNA using a high capacity cDNA reverse transcriptase kit (Applied Biosystems). The yield and purity of RNA extracts was assessed at 260 nm using the Nanodrop ND-1000 spectrophotometer (Labtech). Relative expression of CYP1A was determined using a StepOne real-time PCR machine (Applied Biosystems). The sequences for amplification primers and a minor groove binding (MGB) Taqman fluorogenic probe were obtained from the Defra Project report A1149 "A multi-biomarker approach using the three-spined stickleback as a sentinel species: Implementation of genome and proteome analysis". Primers CYP1AFP (5'-GGAATTGTCAATGACCTGTTTGG–3') and CYP1ARP (5'-CGGATGAGCCACCATGTACA-3') and the MGB Taqman probe CYP1ATP (5'-6FAM-ACACCGTCAGCACGACATTGTCATGG-3') were checked for specificity by using BLASTn (Altschul *et al*., 1997) within the NCBI suite of facilities (www.ncbi.nlm.nih.gov). All reagents and kits used in amplifications were obtained from Applied Biosystems. Optimum primer (300 nM) and MGB probe concentration (250 nM) were determined empirically using cDNA pooled from fish liver RNA extractions as template. Duplex relative gene expression real-time PCR assays were performed in 20 µl reaction volumes. All reactions received the following: 3 µl cDNA; 10 µl Taqman Gene Expression Master Mix (2x concentration); 2 µl of each primer (300 nM); 2 µl (250 nM) MGB and 1 µl Human 18S rRNA endogenous control mixture (containing limited concentration primers and VIC-labelled MGB Taqman probe specific to 18S rRNA). The cycling parameters of 1 cycle of 50^{o}C for 2 min (activation of uracyl glycosylase) followed by 1 cycle of 95^{o}C for 10 min (activation of Amplitaq gold) and 45 cycles of 95^{o}C for 15 secs and 60oC for 1 min were maintained in all cases. In accordance with accepted guidelines for carrying out the comparative cycle threshold (C_t) method the relative amplification efficiencies of template and endogenous control was tested and confirmed. Baselines and cycle threshold values were automatically calculated by the StepOne software. Expression of CYP1A in each sample was normalised to that of 18s rRNA using the equation $R = 1000*[(2^{Ct18s}) / (2^{CtCYP1A})]$ where R is relative expression level, and Ct is the cycle threshold for target and control genes. Amplification efficiencies were not adjusted for each sample. The overall mean C_t value of 9.0 obtained for the internal control gene (18s rRNA) was similar to the value previously reported for 18s in zebra fish liver (11.7; Filby and Tyler, 2007). The overall mean C_t value for CYP1A was approximately 22.5.

It was not found to be possible to consistently transform the relative expression data to provide normality and homogeneity of variances. Instead, where data did not conform to parametric assumptions, a nonparametric Kruskall–Wallis ANOVA on ranks was conducted followed by a pairwise multiple comparison procedure (Dunn's Test).

5.3 Results and Discussion

5.3.13 Relative CYP1A expression levels

Measurement of the activity of cytochrome P450 isoform CYP1A is widely employed to evaluate the exposure of fish to organic contaminants (Whyte *et al*, 2000). This isoform is induced by dioxins, dibenzofurans, polychlorinated biphenyls (PCBs),

polycyclic aromatic hydrocarbons (PAHs), and other compounds that bind to the aryl hydrocarbon receptor (AhR). In this study, CYP1A induction was measured indirectly, as EROD activity (see 5.3.13 in the EDCAT Final Report), and directly, as the abundance of CYP1A transcripts relative to the abundance of transcripts coding for an internal normalising control gene, 18s rRNA.

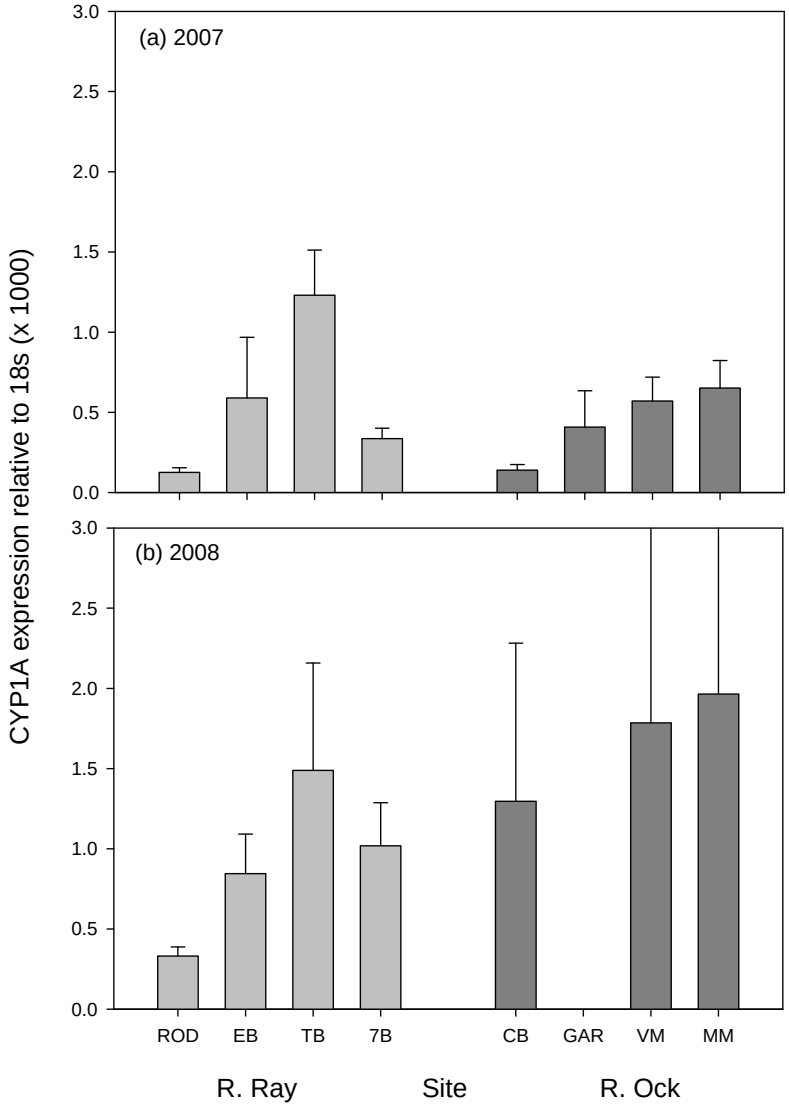

Figure 5.28. Relative expression of Cytochrome P4501A in liver tissue of sticklebacks collected during (a) September and November 2007, (b) September and November 2008. Each bar is the mean ± SEM. Sample sites are indicated along the x-axis: R. Ray (light grey bars): ROD – Rodbourne STW (site 3); EB - Elborough Bridge (site 4); TB - Tadpole Bridge (site 6); 7B - Seven Bridges (site 7); R. Ock (dark grey bars): CB - Charney Basset (site 10); GAR – Garford (site 11); VM - Venn Mill (site 12); MM - Marcham Mill (site 13). For details of significant differences between rivers, years and sites, see text.

Mean values for the expression of CYP1A relative to 18s rRNA are presented in Figure 5.28a (combined September and November 2007 data) and 5.28b (combined September and November 2008 data). In 2007, there was no significant difference in

relative expression level (REL) of CYP1A overall between rivers. However, the REL in fish from Tadpole Bridge (site 6) on the Ray was significantly greater than the REL in fish from Charney Bassett (site 10) and Garford (site 11) on the Ock. Within rivers there were also significant differences in REL between sites. On the R. Ray a clear and significant trend was evident for REL to increase between Rodbourne (site 3) and Tadpole Bridge ($P<0.05$). On the R. Ock, CYP1A expression was lowest in fish from Charney Bassett and highest in fish from Venn Mill and Marcham Mill ($P<0.05$).

Overall, relative expression of CYP1A was significantly higher in 2008 than 2007 ($P<0.001$) in both rivers. On the R. Ray, as was the case for 2007, a trend for increasing levels of CYP1A downstream of Rodbourne was evident ($P<0.05$). However, in 2008, expression of CYP1A overall was significantly greater in fish from the Ock than in fish from the Ray.

There are several notable features of these data. The EROD data for January and March 2008 (see 5.3.13, Figure 5.27, in the EDCAT Final Report) indicated that there was no marked alteration in EROD activity between the pre- and post-remediation periods on the Ray. That finding is mirrored here, with slightly higher CYP1A expression levels in fish from the Ray during September/November 2008, post-remediation than in fish collected during September/November 2007. The EROD results for March 2008 also indicated surprisingly high levels of activity in fish collected from sites on the Ock, at least as high as was evident in fish collected from the Ray. That anomalous observation is supported by the CYP1A expression data which indicate that the REL of CYP1A in fish from the Ock was higher during 2008 than 2007 and significantly higher overall than that of CYP1A in fish from the Ray during 2008.

If, as is reasonable to assume, the variation apparent in CYP1A expression levels reflects exposure of the fish to chemical contaminants then the trend for increasing CYP1A levels downstream of Rodbourne suggests that the primary route of exposure is not to water-borne chemicals. Contaminants introduced into the Ray at Rodbourne would be expected to show higher concentrations at the discharge point than at sites downstream. See, for example, the data for steroid estrogens reported in 4.3.2.1 (Figure 4.1) and that for PPCPs and phenols (Table 4.6). The pattern of expression of CYP1A in fish from the Ray instead matches more closely the pattern of change in concentrations of contaminant chemicals within sediments downstream of Rodbourne (see Table 4.7) where concentrations tend to be highest at Elborough Bridge (site 4). It is possible that the ingestion by the resident fish of invertebrates that have been in contact with the sediment accounts for this pattern of CYP1A expression. This interpretation of the results may also account for the complete absence of any apparent effect of remediation on CYP1A expression levels between 2007 and 2008 despite the likelihood that the GAC plant effectively removed most if not all organic contaminants. It is probable that sediment-associated contamination will take some time to depurate after the removal of the source of contamination and an immediate response would not be expected. It would be informative to take annual samples of both sediment and fish at each of the EDCAT sites on the Ray to assess the rate at which recovery proceeds.

As a caveat to these conclusions, it should also be considered that fish captured at Rodbourne may have spent time upstream of the STW discharge and thus experienced

a discontinuous exposure to the effluent. Interpretation of the Ray data may also be complicated by the fact that the Tadpole Bridge site is downstream of a steam railway depot and on several occasions clear signs of oil or diesel contamination of the water course were evident during fish sampling visits.

The CYP1A data for fish taken from the Ock in 2008 indicate that the elevated EROD activity detected in fish from the Ock during March 2008 is likely to have been a real response to a new source of contamination. The fact that there is a marked increase in both EROD activity and CYP1A expression in fish from the Ock between 2006/2007 and mid to late 2008 suggests that the river received a contaminant load during this period. We have no information as to the source or nature of this but, as noted in the Final Report, although the Charney Bassett, Garford and Marcham Mill sites are on the Ock, the Venn Mill site is on Childrey Brook and it is therefore unlikely that the source of contamination was agricultural activity. It may be the case that sufficient road run-off entered both rivers during periods of high rainfall to raise the levels of EROD-inducing chemicals. If so, this effect was not evident in the Ray, or in the Ock during 2007. Effects on the Ock were evident in March, September and November 2008 indicating either a prolonged episode of contamination, or that more than one episode occurred.

In summary, the levels of CYP1A expression vary significantly between sites on the Ray, exhibiting a similar pattern in 2007 and 2008, and these trends seem to be linked with sediment contaminant loading, as reported by EDCAT 3/4, rather than water-borne contamination. If the primary route of exposure to organic chemicals for the fish in the Ray is indeed linked to sediment concentrations (e.g. via sediment-dwelling invertebrates) this may explain the complete absence of effect of remediation on CYP1A expression in fish in the Ray. *It also suggests that exposure of the resident fish populations to contaminating chemicals may continue for some time after remediation of the STW effluent*. Further chemical and fish monitoring effort would establish how long is required for the system to depurate fully.

Relatively high levels of CYP1A expression were detected in fish from sites on the Ock during 2008. This is consistent with data obtained from EROD analyses of fish collected on a different occasion during 2008 and is strongly suggestive of a contaminant episode. The source and nature of this contamination remains unknown.

References

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