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Molecular responses of European flounder (Platichthys flesus) chronically exposed to contaminated estuarine sediments

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ABSTRACT
Molecular responses to acute toxicant exposure can be effective biomarkers, however responses to chronic exposure are less well characterised. The aim of this study was to determine chronic molecular responses to environmental mixtures in a controlled laboratory setting, free from the additional variability encountered with environmental sampling of wild organisms. Flounder fish were exposed in mesocosms for seven months to a contaminated estuarine sediment made by mixing material from the Forth (high organics) and Tyne (high metals and tributyltin) estuaries (FT) or a reference sediment from the Ythan estuary (Y). Chemical analyses demonstrated that FT sediment contained significantly higher concentrations of key environmental pollutants (including polycyclic aromatic hydrocarbons (PAHs), chlorinated biphenyls and heavy metals) than Y sediment, but that chronically exposed flounder showed a lack of differential accumulation of contaminants, including heavy metals. Biliary 1-hydroxypyrene concentration and erythrocyte DNA damage increased in exposed fish. Transcriptomic and metabolomic analyses of liver tissues detected small but statistically significant alterations between fish exposed to different sediments. These highlighted perturbance of immune response and apoptotic pathways, but there was a lack of response from traditional biomarker genes. Gene-chemical association annotation enrichment analyses suggested that polycyclic aromatic hydrocarbons were a major class of toxicants affecting the molecular responses of the exposed fish. This demonstrated that molecular responses of sentinel organisms can be detected after chronic mixed toxicant exposure and that these can be informative of key components of the mixture.

1. Introduction
The majority of terrestrial and aquatic toxicology is conducted using acute exposures to individual chemicals, usually at concentrations higher than those detected in the environment. In contrast, wild animals are typically chronically exposed to complex mixtures of chemical contaminants where each constituent is present at a relatively low concentration. Acute toxicity studies are...
useful for determining toxicant modes of action, but relating these results to field studies is not straightforward, due to the differences outlined above and the non-pollutant variation inevitably encountered in the environment. Certain biomarker genes, such as cytochrome P450 1A (CYP1A) and vitellogenins (VTG), have been found to be useful in revealing the classes of contaminants to which wild animals have been exposed (van der Oost et al., 2003). In aquatic research, transcriptomics techniques have been applied both to acute toxicological studies (Van Aggelen et al., 2010) and, to a lesser extent, to field studies (Williams et al., 2003, 2011; Meyer et al., 2005; Falciani et al., 2008; Sellin Jeffries et al., 2012; Asker et al., 2013). The European flounder fish (Platichthys flesus) is a species employed for biomonitoring in North-Western European coastal areas (Kirby et al., 2004), where it is exposed to marine and estuarine sediments due to its demersal habitat. Using flounder we have demonstrated several important concepts which strongly support the use of omics based measurements in the analysis of chemical exposures. Flounders sampled from differently-contaminated environments display different gene expression profiles (Williams et al., 2003). Molecular responses to individual toxics can provide information on their mechanisms of action (Sheader et al., 2006; Williams et al., 2006, 2007) and different toxics result in different hepatic gene expression profiles (Williams et al., 2008). Statistical models based on gene expression responses to single chemical exposures can discriminate between fish sampled from differently contaminated sites (Falciani et al., 2008) and network integration of multiple biomarkers, transcriptomics and metabolomics can partially predict both exposure and effects of aquatic pollutants in field-sampled animals (Williams et al., 2011).

In a study described previously (Leaver et al., 2010) we reported that hepatocytes isolated from flounders that had been exposed in mesocosms for 7 months to relatively clean, or multiply-polluted, sediments did show differences in gene expression. However these differences were indicative of chronic inflammation, immune response and apoptosis, rather than corresponding to the classic biomarkers of environmental pollution (e.g. CYP1A, VTG). Subsequent acute treatment of the hepatocytes with model toxics benzo(a)-pyrene, copper or estradiol did indeed elicit the expected biomarker gene expression responses (Leaver et al., 2010), and these were modulated by the different prior chronic exposure conditions. Here, we instead focus on liver tissue from the individual fishes that were maintained in the mesocosms, rather than pooled hepatocytes. We aimed to determine if the different sediment types elicited different transcriptional and metabolic responses from the flounders, whether there was evidence of DNA damage and whether there was accumulation of contaminants and their metabolites in flounder tissue and bile. This would not only allow further validation of the results from the previous hepatocyte study, but also contribute to understanding the differences between chronic and acute chemical exposures.

2. Materials and methods

2.1. Experimental animals and mesocosms

Details of experimental animals and the mesocosms employed were described previously by Robinson et al. (2009) and Leaver et al. (2010). Briefly, four 1.5 m tanks were established, containing 4–5 cm depth of test sediment, supplied with ~100 L h$^{-1}$ carbon-filtered seawater. Two tanks contained sediment from the Ythan estuary (Y) (North East Scotland, UK) and two tanks contained a mixture of sediments (FT) from the Forth estuary (Grangemouth, UK) and the Tyne estuary (Riverside Quay, Jarrow, UK). The Ythan sediment was chosen as a control as discrimination between sediments containing low levels of pollutants and those containing high levels is key in environmental monitoring. The Forth and Tyne sediments were mixed to include high levels of both metal and organic pollutants. Sediment chemical analyses have been described previously (Leaver et al., 2010). All mesocosms were stocked with benthic invertebrates sampled from the Ythan and from the Firth of Tay (Balmerino, NW Scotland, UK). Juvenile flounders (Platichthys flesus) were caught from the Ythan estuary and 60 fish (3.1 ± 1.1 g, 56 ± 6 mm) were added to each mesocosm. Fish were additionally fed with mysid shrimp and commercial pellets (Bio-Optimal START 1.5 mm; Biomar, Grangemouth, UK). Tanks were regularly inspected and any dead fish removed. After 7 months the majority of the fish were sampled for transcriptomic and metabolomic analyses, while the remaining fish were maintained in the mesocosms for a further 7 months before sampling. A number of morphometric parameters were measured to assess the general health and stage of sexual development, including the gonadosomatic index (GSI, gonad wt/body wt × 100), the hepatosomatic index (HSI, liver wt/body wt × 100), and the condition factor (body wt/length$^3$ × 100). Blood was taken from a subset of individuals into heparinised micro-haematocrit tubes and immediately transported on ice. For gene expression and metabolomic analysis, samples of liver and muscle tissue and bile were snap frozen and stored at −80°C; tissue samples for chemical analysis were frozen at −20°C.

2.2. Tissue chemistry

Chemical analyses were carried out for trace metals and for biliary 1-hydroxyxyprene (a PAHs metabolite) on flounder muscle tissue at the 7 month sampling point (n = 55 per sediment). At the 14 month sampling point, trace metals were analysed in pooled muscle and in pooled liver samples (n = 2–4) and chlorinated biphenyls were also measured in pooled muscle and pooled liver tissue. Metal and chlorinated biphenyl concentrations were determined as shown previously (Robinson et al., 2009: Leaver et al., 2010). The concentration of conjugated 1-hydroxyxyprene in flounder bile was determined following the fixed wavelength fluorescence method (Aas et al., 2000) as recommended by the International Council for the Exploration of the Sea (ICES) for use as a routine screening tool to assess recent PAHs exposure (Ariese et al., 2005). Bile samples were initially diluted 1600-fold in methanol and the concentration of 1-hydroxyxyprene determined by comparison with reference calibration standards.

2.3. Comet assay

The alkaline single cell gel electrophoresis Comet assay allows assessment of DNA damage by detecting migration of DNA from the immobilised nucleus. The method described by Emmanouil et al. (2006) was employed. Briefly, fresh blood cells (mainly erythrocytes) were immobilised in agarose, layered onto microscope slides, lysed, subjected to electrophoresis (25 V, 300 mA, 20 min) and stained with ethidium bromide. The Comets were examined using a fluorescent microscope (×20 magnification, Zeiss Axiosvert 10 inverted fluorescent microscope; Carl-Zeiss, Jena, Germany). The cells were scored using an image analysis package (Comet 3.0 European Kinetic Imaging, Liverpool, UK). 100 cells were scored per individual animal (Tice et al., 2000). Median percentage of DNA in tail (% tail DNA) was compared for 25 fish from Ythan mesocosms and 25 fish from Forth/Tyne mesocosms.
distributed by the Shapiro–Wilks test and therefore were compared using the Mann–Whitney U test.

2.4. Microarray experiments

Samples of livers from flounders exposed to Ythan sediment (12 male, 16 female) and Forth/Tyne sediment (15 male, 22 female) were analysed by transcriptomics and metabolomics. Liver tissues from individual flounders were homogenised in 8 ml g⁻¹ (v/w wet weight) methanol and 2.5 ml g⁻¹ (v/w) water using a bead-based homogenizer (Precellys 24; Stretton Scientific, Stretton, UK) (Wu et al., 2008). Aliquots were then taken for both metabolomics and transcriptomics (Katsiadaki et al., 2010). The GENIPOL European flounder microarray (Williams et al., 2006) was used for determination of differential gene expression. This microarray consisted of 13 824 flounder clones and controls spotted in duplicate onto Corning UltraGAPS slides (Corning Inc., Corning, NY, USA) with an MGII robot (Biorobotics, Cambridge, UK) at Birmingham University Functional Genomics Laboratory, representing approximately 3336 unique genes. We have previously shown details of clones incorporated into the microarray (Williams et al., 2003, 2006, 2007; Diab et al., 2008). Microarray experiments were carried out as described previously (Diab et al., 2008). Briefly, total RNA samples were purified from liver tissue homogenates from individual fish by RNeasy (Qiagen, Crawley, Oxford), with DNA contamination removed by DNA-free (Ambion, Austin, TX, USA) followed by spectrophotometry for quality control. RNA samples were reverse transcribed to cDNA (Superscript II; Invitrogen, Paisley, UK) then labelled with Cy5-dCTP (GE Lifesciences, Amersham, UK) using Klenow polymerase (Invitrogen). Each array consisted of Cy5-labelled cDNA from one individual fish hybridised versus a Cy3-labeled artificial reference sample (Diab et al., 2008). Hybridisations were carried out for 18 h, before stringent washing and scanning with an Axon 4000B scanner (Molecular Devices, Wokingham, UK). Data were captured using Genepix software (Molecular Devices), and each slide was checked in detail, with spots showing poor morphology or arrays showing experimental artefacts being repeated. MiAME compliant data have been deposited to ArrayExpress and assigned the accession E-MTAB-1847. The data used in analyses consisted of local background-subtracted median intensities. Microarray data were quantile normalized using geWorkbench software version 2.0.1 (Floratos et al., 2010). Only data from spots designated as ‘present’ were used. Data for low intensity, highly variable spots were removed.

2.5. Metabolomics

For metabolomics, liver homogenate aliquots were further extracted using methanol/chloroform/water (2:2:1.8 final volumes) (Bligh and Dyer, 1959; Wu et al., 2008). One-dimensional ¹H NMR spectroscopy was performed upon the hydrophilic fraction as previously described (Katsiadaki et al., 2010). Briefly, NMR spectra were measured at 500.11 MHz using an Avance DRX-500 spectrometer and cryogenic probe (Bruker, Coventry, UK), with 200 transients collected into 32 k data points. NMR data sets were zero-filled to 64 k points, exponential line-broadenings of 0.5 Hz were applied before Fourier transformation, and spectra were phase and baseline corrected, then calibrated (TMSP, 0.0 ppm) using TopSpin software (version 1.3; Bruker). The subsequent processing and statistical analyses of the NMR data were similar to those described in a previous study (Katsiadaki et al., 2010). Residual water was removed, each spectrum was segmented into 0.005 ppm bins, and the total area of each binned spectrum was normalized to subjectivity as so to facilitate comparison between the samples and subjected to a generalized log transformation.

2.6. Omics data analyses

Metabolomic and transcriptomic datasets were normalised, log transformed and scaled, then combined for analysis. Lists of transcripts and metabolites that changed in abundance between Ythan and Tyne/Forth sediment-exposed fish were generated using TMEV software (Saeed et al., 2006) using the Rank Products (RP) test, employing a multiple-test-corrected q-value cut-off of 0.05, calculated by permutation. Data from male and female fish were considered together as both sets of samples had similar sex ratios (Y = 43% male, TF = 41% male). Enrichment analyses were carried out at the DAVID website (Huang et al., 2009a,b) for genes with identifiable human orthologs, employing a false discovery rate (FDR) cut-off of 0.05 or 0.1. All detected identifiable genes were used as a background set for comparison. Enrichment analysis of gene-chemical annotation, derived from the Comparative Toxicology Database (CTD) (Davis et al., 2009) was performed using the EASE software package within TMEV using a FDR cut-off of 0.1 (Williams et al., 2011). Briefly, for presumed orthologs of all genes detected by microarray, chemical to gene expression relationships for any species were downloaded from CTD, segregated into induction and repression and used to annotate each gene with its ‘chemical inducers’ and ‘chemical repressors’. Gene-lists were interrogated for enrichment of annotation by EASE in comparison with all genes detected. False-discovery rates were multiplied if the same chemical matched to induced gene lists as an inducer and to repressed gene lists as a repressor.

3. Results

3.1. Chemical analyses and morphological parameters

Sediment chemistry data have been reported previously (Leaver et al., 2010). Briefly, chemical analyses showed that, in comparison to Ythan sediment, Forth/Tyne sediments contained significantly (P < 0.05) higher concentrations of hexachlorobenzene (HCB), total organochlorine pesticides (OCP), chlorinated biphenyls (CB), naphthalenes, total polycyclic aromatic hydrocarbons (PAHs), and B[a]P equivalents of PAHs, tributyltin, dibutytin, As, Cr, Cu, Hg, Ni, Pb and Zn. Dichlorodiphenyltrichloroethane (DDT) and its metabolites were not significantly different. Cd was detected in Forth/Tyne but was below the limit of detection in Ythan sediment. In contrast, the flounder muscle tissues showed no significant differences in As, Cd, Cr, Cu, Mn, Hg or Zn concentrations between Forth/Tyne and Ythan sediment-exposed fish after 7 months or in liver after 14 months. There was a significantly higher concentration of Pb in FT fish muscle after 7 months (FT = 0.038 ± 0.092 mg/kg; Y = 0.014 ± 0.016 mg/kg; P < 0.001). Chlorinated biphenyl concentrations were no different in muscle but appeared elevated in liver after 14 months, though not statistically significantly (sum of liver PCBs: FT = 31 µg/kg; Y = 19.8 µg/kg). Liver chlorinated biphenyl concentrations were an order of magnitude higher than those found in muscle (P < 1.4E–6). Biliary 1-hydroxyxypyrrene appeared elevated in Forth/Tyne sediment exposed fish (1.7 ± 1.3 µg/ml) compared with Ythan sediment exposed fish (0.9 ± 0.5 µg/ml) after 7 months (P = 0.065, Mann–Whitney U test). No measured morphological parameters significantly differed between sexes or sediment-exposed groups, apart from gonad weights and GSI that were higher for female fish than males.

3.2. Comet assay

The flounders exposed to Ythan sediments showed 3.8% mean tail DNA while those exposed to Forth/Tyne sediments showed a mean 7.8% tail DNA (Fig. 1A). These data were variable for both
groups (Fig. 1B) but were found to be statistically significant by the Mann–Whitney test with $P$-value of 0.015.

### 3.3. Transcriptional and metabolic changes

The changes in metabolite and transcript abundance between flounders exposed to the different sediments for 7 months were not extensive. Indeed, hierarchical clustering and principal components analysis of the data set failed to identify any relationships between overall gene expression or metabolic profiles and sediment type. Rank Products analysis identified 259 transcripts and metabolites significantly ($q < 0.05$) altered in abundance in Forth/Tyne sediment exposed fish compared with Ythan sediment exposed fish in both males and females. Data are shown in full in Supplementary Table 1. Of the transcripts induced with FT sediment, 51 were identified and 45 were not identified. Of those repressed with FT sediment, 76 transcripts were identified and 69 were not identified (Table 1). Six metabolites significantly increased in concentration with FT sediment and 12 significantly decreased, but none of these could be identified. A number of metabolites similarly expressed in both groups were identified; choline, glucose, glutamine, glycine, lactate, malonate, maltose or taurine, N,N-dimethylglycine, o-phosphocholine, taurine, tyrosine and valine. The statistically significant changes in transcript expression and metabolite concentration were relatively modest in magnitude between FT and Y samples, with only 17% of those identified exceeding a 1.5-fold change in males and 6% in females.

### 3.4. Annotation analyses

Gene Ontology annotation enrichment analysis, carried out using DAVID, identified significant (FDR < 0.05) enrichment of the GO term ‘iron ion binding’ (GO:0005506) among transcripts up-regulated with FT sediment and significant enrichment (FDR < 0.05) of the GO term ‘calcium ion binding’ (GO:0005509) among transcripts down-regulated with FT sediment, as well as a less significant (FDR < 0.1) enrichment of ‘microsome’ (GO:0005792).

Gene-chemical annotation enrichment analysis highlighted chemical pollutants whose associated gene expression changes were consistent with those detected in response to TF versus Y sediment in this experiment (Table 2). These included fungicides (pro-cymidone, vinclozolin and trichostatin A), the polycyclic aromatic hydrocarbon benzo(a)pyrene, a brominated flame retardant (BDE-47), a fluoro-surfactant (PFOS) and a model genotoxic hepatocarcinogen (N-nitrosomorpholine). The highest number of matches between gene expression changes in these flounders and those in CTD were for benzo(a)pyrene (56 matches).

### 4. Discussion

Despite FT sediment containing significantly higher concentrations of a range of organic and inorganic contaminants than Y sediment, there was little evidence of differential accumulation of these contaminants in flounder tissue. It is therefore possible that the majority of contaminants assayed were not bioavailable to the flounders, despite their demersal foraging behaviour. The only exceptions were a higher, but variable, concentration of lead in FT fish muscle and an increase in biliary 1-hydroxypyrene. The biliary 1-hydroxypyrene elevation was consistent with exposure to and metabolism of pyrenes (Ariese et al., 2005), members of the group of polycyclic aromatic hydrocarbons (PAHs). PAHs are well-known genotoxins, and may have contributed to the significantly elevated DNA damage detected in FT flounder blood cells by the Comet assay. ICES recommend a Background Assessment Criteria (BAC) value for the Comet assay of 5% DNA in tail to assess whether exposure to genotoxins has occurred in fish (dab and cod; Davies et al., 2012); the values here of 3.8% in the Y fish and 7.8% in the FT fish are consistent with the latter being exposed to genotoxins and the former not being highly exposed. PAHs tend not to accumulate chronically in fish tissue (Du-Lacoste et al., 2013).
Transcripts differentially expressed between TF and Y flounders fell into a number of functional categories. The category with the greatest number of differentially expressed transcripts was ‘immune response’ (Table 1), as was found in hepatocytes (Leaver et al., 2010), with a similar induction of hepcidin, encoding an antibacterial peptide. Additionally DIABLO expression was induced in FT fish similarly to responses of isolated hepatocytes. This transcript has recently emerged as a possible biomarker of chronic chemical contamination (Zacchino et al., 2012), with the DIABLO protein playing a key role in promoting apoptosis. Therefore the transcriptomic responses detected in fish livers were broadly consistent with those found in isolated hepatocytes, indicative of alteration of immune response and apoptosis. Indeed, these classes of responses have been detected in other fish species directly sampled from polluted environments (Asker et al., 2013).

Similarly to Leaver et al. (2010) we found that typical biomarker genes were not induced in FT fish. Indeed, the transcription of CYP1A was repressed, as was that of UDP-glucuronosyltransferase (UGT), despite the evidence for increased biliary PAHs metabolites. As many PAHs are effective inducers of CYP1A expression and activity via activation of the aromatic hydrocarbon receptor, this was unexpected. There are a number of possible reasons for a decline in CYP1A transcription. As outlined previously there may have been insufficient exposure or a lack of responsiveness after long term exposure, with transcription returning to equilibrium (Reynolds et al., 2003; Leaver et al., 2010). If so, this demonstrates one of the main differences between acute and chronic responses. Alternatively, there may be mixture effects interfering with CYP1A transcription, such as the repression of PAHs-induced CYP1A transcription in flounder when co-exposed with heavy metals such as cadmium (Lewis et al., 2006). This is not necessarily an effect mediated solely by heavy metals, as estrogens (Maradonna et al., 2004) and polybrominated diphenyl ethers (Wahl et al., 2010), amongst many other compounds, have been found to repress CYP1A in fish. It is possible that such repression is mediated by oxidative stress (Morel and Barouki, 1998). Both time-dependent and mixture-dependent factors may contribute to CYP1A repression in this scenario. Although transcription of CYP1A, UGT and aldehyde dehydrogenases ALDH and ALDH2 decreased in FT fish, there was induction of glutathione-S-transferases (GST) of the omega and theta classes and of alcohol dehydrogenase. While GSTs are key components of phase II metabolism of xenobiotics such as PAHs, GST induction is also associated with oxidative stress (Leaver et al., 1997), and this is not inconsistent with chronic inflammation and immune response.

While the transcriptional changes detected were modest and initially appeared non-specific, interrogation of previous literature via CTD gene-chemical annotation enrichment analysis did identify significant (FDR < 0.1) similarities between gene expression responses recorded in response to individual chemical exposures and the gene expression responses found to this chronic complex mixture exposure (Table 2). Most notably, there was significant similarity between the genes differentially expressed between FT and Y fish and the genes previously found to respond to benzo(a)-pyrene treatment. This implies that, while the individual biomarker approach can be very useful, especially in acute studies, a multi-biomarker readout is more effective at identifying stressors affecting chronically exposed animals. Additional similarities predicted included fungicides (procyomide, vinclozolin and trichostatin A), a brominated flame retardant (BDE-47), a fluoro-surfactant (perfluorooctane sulfonic acid, PFOS) and a model genotoxic hepatocarcinogen (N-nitrosomorpholine). BDE-47 was indeed detected in the FT sediment (Robinson et al., 2009) and the presence of fungicides and PFOS would not be unexpected in contaminated estuarine sediment. N-nitrosomorpholine is unlikely to occur in detectable concentrations in the environment, but due to their metabolism by cytochromes P450 and subsequent conjugation and excretion via the bile.

Alterations in gene expression and metabolite concentrations were detectable, but not of great magnitude. Again this was consistent with low bioavailability leading to modest hepatic exposure. While none of the metabolites that altered in concentration were identifiable, the detection of metabolic change in these chronically exposed animals adds weight to the finding that chronic exposure does elicit functional phenotypic alterations in the long term. Although the magnitude of gene expression changes was small, it was evident that there were similarities with the responses of isolated hepatocytes and qPCR data (CYP1A, hepcidin, DIABLO) on additional fish from the same mesocosms described by Leaver et al. (2010). Table 1

### Table 1

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Table 2

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effectively illustrates detection of the DNA damage that was also confirmed by Comet assay.

5. Conclusions

Chemical analyses of flounder chronically exposed to more highly contaminated Tyne/Forth estuarine sediment demonstrated a lack of contaminant accumulation in fish tissues. Transcriptomic and metabolomic analyses of liver tissue detected small but statistically significant alterations between the groups of fish exposed to different sediments. These broadly confirmed the results of a previous study on isolated hepatocytes, highlighting alterations in immune response and apoptosis, but a lack of response from traditional biomarker genes. Together with analyses of fluorescent bile metabolites and DNA damage, the data were consistent with uptake, metabolism and excretion of genotoxic polycyclic aromatic hydrocarbons. Gene-chemical association annotation analyses also suggested that PAHs were a major class of toxicants affecting the molecular responses of contaminant exposed fish. The study highlighted the importance of bioavailability and the benefits of a multi-biomarker approach in determining responses to chronic chemical mixture exposure.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2014.01.028.

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