International Council for the Exploration of the Sea

Temporal and Spatial Trends in the Distribution of Contaminants and their Biological Effects in the ICES Area
CM 2000/S:06

EROD and ChE measurements in flounder (*Platichthys flesus*) as monitoring tools in English estuaries

Mark F. Kirby, Mark R. Hurst, Carole A. Kelly, Sonia J. Kirby, Paula Neall, Tina A. Tylor, Steven Morris and Peter Matthiessen.

Centre for Environment, Fisheries and Aquaculture Science, Burnham-on-Crouch, Essex CM0 8HA, U.K. [tel:+44 1621 787200, fax: +44 1621 784989, e-mail: m.f.kirby@cefas.co.uk]

ABSTRACT

Recent studies have made it clear that fish populations in UK estuaries are being exposed to an extensive mixture of contaminants at the sub-lethal level. Amongst the tools used by CEFAS to investigate the extent of these effects are a range of enzyme biomarkers. This paper outlines measurements of ethoxyresorufin O-deethylase (EROD) activity, a marker of induction of the mixed function oxygenase (MFO) detoxication system, and cholinesterase (ChE) inhibition, a marker of neurotoxicity, in tissues of the flounder (*Platichthys flesus*) from a number of key estuaries.

Hepatic EROD activities were found to be significantly ($p<0.05$) induced at 8 out of 15 sites whilst muscle ChE activities were significantly ($p<0.05$) lower in fish from 12 of 15 sites when compared to specimens from a reference site. Specimens from industrialised estuaries such as the Mersey, Tyne and Tees routinely showing the greatest effects in both assays.

It is clear that flounder populations in certain English estuaries are experiencing sub-lethal effects, probably as a result of exposure to a number of contaminant classes. Potential consequences of these and other, non-contaminant, influences are also discussed. Chemical analytical data are presented that provides partial evidence that polycyclic aromatic hydrocarbons (PAH)/ poly chlorinated biphenyls (PCB) and organophosphate/ carbamate pesticides are contributors to the observed EROD and ChE effects respectively.

Keywords: pollution monitoring, flounder (*Platichthys flesus*), EROD, cholinesterase, biomarker, estuary.

INTRODUCTION

A wide variety of contaminants enter the estuarine and coastal waters of the UK. Conventional bioassay methods and chemical analysis techniques have allowed scientists to assess the concentrations of specific contaminants in water and sediment and the potential biological effects of these on variables such as survival, growth and reproduction in fish and other organisms. However, the need to detect and assess the effects of contamination at lower concentrations has led to the development of a wide range of sub-lethal indicators of exposure to, and effects of, contaminants and other environmental stressors (Livingstone *et al*., 1997). These indicators are collectively referred to as 'biomarkers' and can be either specific (monitoring the presence/effects of specific chemical classes) or general. This paper describes the use of two specific enzyme biomarkers (EROD and ChE) by CEFAS to monitor the exposure and effects of particular contaminants in populations of the estuarine flounder (*Platichthys flesus*).

EROD

The mixed function oxygenase (MFO) enzyme system is the primary detoxification pathway for a number of planar, organic contaminants, specifically polycyclic aromatic hydrocarbons (PAH) and some polychlorinated biphenyls (PCB) and is induced in fish by exposure to such compounds. The degree of induction of one of its components, the cytochrome P450s, can be readily assessed through the *in vitro* measurement of 7-ethoxyresorufin-O-deethylase (EROD) activity. The specific nature of the EROD response has resulted in its frequent use to measure MFO activity in the environment (Stegeman *et al*., 1988; Burgeot *et al*., 1996) but its application to UK estuaries appears to have been limited to the Forth Estuary in Scotland (Sulaiman *et al*., 1991). Furthermore, the flounder (*Platichthys flesus*) has been a common choice as the sentinel species (Beyer *et al*., 1996; Eggens *et al*., 1996; Draganik *et al*., 1996; Kirby *et al.*1999b) for use in EROD applications.

ChE

Acetylcholine is the primary neurotransmitter in the sensory and neuromuscular systems of fish. The activity of this system is vital to normal behaviour and muscular function (Payne *et al* 1996) and it represents a prime target on which some toxicants can realise a detrimental effect. The levels of ACh at a neuro-junction must be carefully regulated, and this role is fulfilled by the enzyme acetylcholinesterase (AChE), which degrades the ACh into the inactive products choline and acetic acid. These are reabsorbed and used as raw materials for the continued production of ACh. Inhibition of the AChE enzyme will result in a build up of ACh causing a continuous and excessive stimulation of the nerve/muscle fibres leading to tetany, paralysis and eventual death.

Pesticides have been highlighted as important estuarine contaminants (Matthiessen, 1988). Organophosphate and carbamate insecticides are potent neurotoxins and exert their toxicity by blocking the breakdown of acetylcholine (ACh) by the enzyme, acetylcholinesterase (AChE). It is primarily with the environmental monitoring of these chemicals in mind that an *in vitro* measurement technique to determine ChE inhibition in the muscle tissue of flounder has been applied. Although ChE activity has previously been measured in the European

flounder (Stieger *et al.* 1989; Sturm *et al.* 1999), this appears to be the first time that this species has been used for the environmental monitoring of ChE inhibition.

Chemical Analysis

Wherever possible appropriate tissue and/or water samples were taken for chemical analysis in an effort to link contaminant levels with biomarker responses. Polycyclic aromatic hydrocarbons (PAH) and poly chlorinated biphenyls (PCB) were targeted in order to investigate their possible link with MFO induction. A selection of organophosphate (OP) and carbamate (C) pesticides were analysed in water in an attempt to link the ChE levels observed to the presence of these potent neurotoxic contaminants.

MATERIALS AND METHODS

Sampling Strategy

The positions of the estuaries sampled are shown in Fig. 1. Eight estuaries were sampled using 2-3 metre beam trawls between September and December 1997. Trawls were towed at 3-4 knots for 20-30 minutes so as to minimise stress to the catch. The estuary of the River Alde on the Suffolk coast was chosen as the control or reference site as, unlike the other estuaries sampled, it is essentially free from industrial inputs, and only has two small (<1000 m^3d^{-1}) sewage treatment works discharging to the estuary.

Once on deck, flounder were separated into tanks containing flowing seawater. Fish of approximately 20 cm were taken unless samples were scarce, when those over 10 cm were taken. Specimens were killed within 1 h of capture via cranial strike and dissections were then performed. Pieces of liver were excised and a strip of muscle (approx. 2 cm^3) was removed from the dorsal surface near to the spine. Tissue samples were placed in a cryovial which was immediately transferred to liquid nitrogen for storage. Whole fish carcasses were frozen (-20^{o}C) and total, liver and gonad weights were measured on return to the laboratory. Condition factor (CF) was determined as (total weight (g)/length $(cm)^3$) x 100. Somatic indices for liver (HSI) and gonad (GSI) were determined as (liver weight/fish weight) x 100 and (gonad weight/fish weight) x 100 respectively.

In estuaries where excess fish were available (Alde, Tees, Humber and Mersey) livers were pooled and frozen for chemical analysis of PAH/PCB. In six of the estuaries samples of estuarine waters were acquired from 1m below the surface at low water for analysis of OP and C pesticide residues. These were stored in clean, amber, borosilicate bottles and extracted immediately. Separate samples were taken for the determination of OPs and Cs.

EROD Determination

Liver samples remained in liquid nitrogen until analysis, a maximum of 2-3 months after collection. A 200mg (± 10) slice of liver was homogenised with 1ml of ice cold homogenising buffer (50mM TRIS pH 7.5, 1mM EDTA, 1mM dithiothrietol, 150 mM NaCl) using six strokes of a Potter-Elvehjem automatic homogeniser set at 4000 rpm. The homogenate was then transferred to a lidded polyethylene eppendorf tube and stored on ice. The homogenates were then centrifuged at 10,000g for 20 minutes in a refrigerated unit set at 4 °C. After centrifugation, supernatants were removed, kept on ice and assayed within 30 minutes.

EROD measurement was performed using a modification of the method described in Stagg *et al.,* (1995). A Perkin Elmer LS50B fluorescence spectrometer set at 535nm excitation and 580nm emission with a cuvette stirring function was used. All assay reagents were kept at 20°C (±1) in a water bath so as to control the assay temperature. The reaction mixture, final volume 2ml, contained 1.96ml assay buffer (100 mM pH 7.5 TRIS, 100 mM NaCl), 20µl liver homogenate, 10µl ethoxyresorufin substrate (0.4 mM in dimethyl sulphoxide, DMSO) and 10µl of resorufin internal standard (25 µM in DMSO). The standard equates to an addition of 250 pM of resorufin against which the assay was calibrated. To ensure quality control on the substrate and standard, a batch was made and then 1-2 ml aliquots were frozen and defrosted prior to use in the assay. The reaction was initiated by the addition of 10 µl NADPH (0.25mM) and emission readings were recorded at 0, 15, 30, 45 and 60 seconds post addition.

EROD activity was normalised to protein content and expressed as pM resorufin/min/mg protein. Protein analyses were carried out on the same liver homogenate as the EROD activity measurements using a plate reader modification of the Bradford method (1976) with a bovine serum albumin standard.

ChE Determination

Samples were kept at -80°C for no longer than 4 months before the assays were performed. Approximately 1g of muscle tissue was placed in 10 mls of homogenising buffer (0.1M pH7 Tris/HCl containing 0.1%Triton X100) in a suitably sized, clean glass beaker. The muscle was processed using an Ultra-Turrax homogeniser for 15-20 seconds whilst keeping the beaker on ice to minimize the temperature increase. The crude homogenate was then decanted into an eppendorf tube and centrifuged at 10,000g for 20 minutes. The resultant supernatants were used as the cholinesterase source.

ChE activity determinations were performed using a modification of the technique described by Bocquené and Galgani (1996). Briefly, using a 96-well microplate assays were performed in quadruplicate with each test well initially containing 10µl of supernatant, 340µl of assay buffer (0.1M pH 7 Tris/HCl – no triton X100) and 20µl of 0.01M dithiobisnitrobenzoate (DTNB). Blanks contained no supernatant and 350µl of buffer. Assay reactions were initiated by quick addition, via stepping pipette, of 10µl of 0.1M acetylthiocholine (ACTC) which acted as the substrate. The plate was then placed in a microplate reader set to read absorbance at 412nm and optical density (OD) readings were taken every 15 seconds for 1 minute. The assay was temperature controlled at 25°C and all reagents were brought to this temperature prior to use.

ChE activity was normalised to protein content and expressed as mU/min/mg protein (1U = 1mOD unit). Protein analyses were carried out on the same muscle homogenate as the AChE activity measurements using a plate reader modification of the Bradford method (1976) with a bovine serum albumin standard.

Chemical Analysis

PAH and PCB concentrations in samples of flounder liver were determined from sites in the Rivers Mersey, Tees, Humber and Alde. Homogenised samples of flounder liver were pooled by site in groups of 5 for PAH and PCB analysis. Total PAH was reported as the sum of naphthalene, C_1-N, C_2-N and C_3-N alkyl naphthalenes, phenanthrene and anthracene concentrations. In all, nineteen individual

PAH and groups of isomers or alkylated derivatives were determined (up to a molecular weight of 276Da - benzo[*ghi*]perylene being the last eluting compound) by gas chromatography/mass spectrometry, but several were found to be below the limit of detection of the method used (0.1 μg kg^{-1} wet weight) due to the high metabolic capacity of teleost fish for these compounds (Kelly and Law, 1998; Law *et al*., 1998). Total PCB concentration was reported as the sum of 25 individual congeners and was analysed using gas chromatography with electron-capture detection (Allchin *et al*.1989).

For OP analysis the water sample pH was reduced to 6 and deuterated Parathion-methyl added as a surrogate standard. Enrichment of the OPs was achieved *in situ* by solid phase extraction using pre-solvent rinsed and conditioned octadecylsilane (C18) bonded cartridges (International Sorbent Technology, Mid-Glamorgan, UK). All OP analyses were performed using a high resolution gas chromatography mass spectrometry (GC-MS) system that consisted of a Varian GC connected via a capillary transfer line (250°C) to a Finnigan MAT ion trap detector (Finnigan MAT Ltd., Hemel Hempstead, UK). Helium was used as the carrier gas at a flow rate of 1 ml min^{-1} and a head pressure of 15psi. Detection of OPs in sample extracts was confirmed by matching the retention times with authentic standards as well as operating a computerised library search. For positive confirmation of an OP analyte, three designated ions were required to be evident with the correct relative abundances.

For the determination of carbamate residues, water samples were extracted at the native pH of the sample using a styrene divinylbenzene sorbent and subsequently stored at -10°C. Extracts were analysed by high performance liquid chromatography - atmospheric pressure chemical ionisation mass spectrometry. Chromatographic separation of carbamate analytes was achieved using a Hewlett Packard 1050 and an Envirosep-CM analytical column (175 x 3.2 mm; 5μm, Phenomenex, Cheshire, UK) with a guard column (10 x 3.2 mm) and an in-line filter (0.25μm), all maintained at 45°C. The mobile phase flow rate was 1ml min^{-1}. Optimum tuning parameters were investigated for each compound and a compromised set of conditions was established.

Further details of the OP and C analysis techniques are described in Kirby *et al.* 2000.

Statistical Analysis

Means of EROD activity, ChE activity, size, GSI, HSI and CF were calculated and analysed for significant ($p<0.05$) inter-site differences using single factor ANOVA. Inter-gender differences in the activities of both enzymes were also determined using single factor ANOVA. Correlation coefficients (r) were determined between fish size, GSI, HSI and CF and enzyme activities to determine the possible influences of these variables.

RESULTS

EROD

Mean EROD activities from each estuarine site are displayed in Table 1. Significantly ($p<0.05$) elevated levels, compared to the Alde reference site, were evident in 8 of the 15 stations sampled. The samples from the Mersey and Tees showed the greatest induction, reaching a maximum of 161.6 $pmol.min^{-1}.mg\ pro^{-1}$, at the Bromborough site on the Mersey, a four fold increase in induction over the

reference level. The span of EROD activities from all the sites sampled is represented in Fig. 2.

The mean length, weight, gonado-somatic index , hepato-somatic index and condition factor for each site are shown in Table 1. No significant inter-gender differences were demonstrated for any variable at any site suggesting that sex was not a significant factor in intra- or inter-site differences.

Linear correlation coefficients were calculated between mean EROD activity and mean length, weight, GSI, HSI and CF for pooled gender data, males and females across all sampled sites. Also, due to the extensive range of sites sampled on the Tyne a comparable intra-estuary correlation analysis for EROD against the same variables from this estuary was carried out. These analyses are summarised in Table 2.

When all sites were included in the correlation analysis only weak correlations were demonstrated between mean EROD activity and any other measured variable for pooled or single gender datasets. However, when the correlation analysis was performed on the dataset from the Tyne sites alone, some stronger relationships begin to emerge, which are more apparent in the females than in the males. In particular female mean EROD activity was quite strongly negatively correlated to both GSI ($r = -0.84$) and CF ($r = -0.80$).

Total PAH and PCB concentrations in the fish livers for each of the four estuaries analysed are shown in Table 3. The reported figures are means of all the pooled samples from the river and therefore represent an integrated value for the estuary.

ChE

Mean ChE activities at each of the estuarine sites are shown in Fig. 3 and Table1. Mean activities at each site were statistically compared to the mean for the River Alde using single factor ANOVA.

In total, 12 out of the 15 sites showed significantly ($p<0.05$) depressed ChE activities compared with the Alde. The only sites that showed 'near normal' levels were from the Wear (but this is a mean of only four samples) and the two outer sites within the Tyne, at Jarrow and Lloyds Hailing Station. Whilst the Alde showed a mean activity of 16573 mU min^{-1} mg $protein^{-1}$, Bromborough Port on the Mersey showed a mean of only 5206 mU min^{-1} mg $protein^{-1}$ and Scotswood Bridge on the Tyne only showed 5894 mU min^{-1} mg $protein^{-1}$ which are the equivalent of 68% and 64% inhibition of ChE activity respectively. Other low activities were observed at sites on the Tees and Humber which showed >50% inhibition.

The mean length, weight, GSI, HSI and CF for each site are shown in Table 1. Linear correlation coefficients were explored between mean ChE activity and mean length, weight, GSI, HSI and CF for pooled gender data, males and females across all sampled sites. Also, due to the extensive range of sites sampled on the Tyne a comparable intra-estuary correlation analysis for ChE against the same variables from this estuary was carried out. These analyses are summarised in Table 4 and commented upon in the discussion.

In the representative estuarine water samples analysed for the presence of OP and C pesticides, a total of 8 OP and 6 C compounds were identified. These were present at concentrations of 13-57 ng l^{-1} and 8-32 ng l^{-1} for a range of OPs and Cs respectively. The data are presented in Table 5. A further 6 OP and 3 C compounds were found at other estuarine and coastal sites not covered by this study (S. Morris,

unpublished data). All OP and C determinands were found to be below the limit of quantitation in water samples from the River Alde reference site.

DISCUSSION

EROD

It is clear from the estuary survey that the mixed function oxygenase (MFO) enzyme system in flounder is significantly induced in a number of English estuaries (Table 1; Fig. 2) compared to the clean control estuary (River Alde). These results reflect the anthropogenic contamination of estuaries such as the Mersey, Tees and Tyne, and suggest that the flounder (and probably other fish) populations in these areas are subject to sub-lethal contaminant stress. However, it should also be borne in mind that the endocrine system in the most EROD-induced fish was highly disturbed (Allen *et al*. 1997,1999; Matthiessen *et al.* 1998b), with high levels of plasma vitellogenin occurring in male fish from the Mersey, Tees and Tyne. It is also possible that the MFO system was responding to oestrogenic contaminants such as ethynyl estradiol or nonylphenol (which have been shown to inhibit EROD activity in the Atlantic salmon, *Salmo salar* (Arukwe *et al.* 1997)) in addition to planar PCBs or PAH (some of which are themselves oestrogenic or anti-oestrogenic). The survey nevertheless allowed a tentative ranking of contamination in the estuaries in terms of their EROD inducing capabilities in flounder : Mersey, Tees > Tyne, Humber, Wear, Southampton Water > Alde. Kirby *et al*. (1998) ranked a number of English estuarine waters on the basis of the toxicity of hexane extracts to the harpacticoid copepod, *Tisbe battagliai* and the estuaries sampled, common to both studies, were ranked: Tees > Wear > Mersey > Tyne > Southampton Water. It can be seen that the ranking of estuaries was not dissimilar in the two studies. This approximate agreement between these relatively disparate studies is not surprising bearing in mind the correlation found by Kirby *et al*. (1998) between biological water quality, as revealed by the *T. battagliai* assay, and the PAH concentrations in water, as PAH are known to be potent inducers of EROD activity.

If the EROD results obtained from the River Tyne are observed in isolation it is evident that in this estuary there are inter-site differences in EROD activity with the mean activity at Hebburn (54.3 $pmol.min^{-1}.mg\ pro^{-1}$) being significantly less than mean EROD activity at three other Tyne stations: Redheugh, Newcastle and Jarrow. There are no obvious up or downstream trends in measured effects, which was also the case for EROD activity in flounder from the Forth Estuary (Sulaiman *et al*. 1991). A recently published survey of sediment toxicity in the River Tyne (Matthiessen *et al*. 1998a) suggested that sediments taken from the Jarrow area were relatively non-toxic compared to other areas in the estuary, and that the most toxic sediments were sampled from the Redheugh/Newcastle area. The same study also recorded the highest sedimentary PCB and hydrocarbon concentrations at Redheugh and Newcastle, which reflects the elevated EROD activity expressed in samples from these sites. The relatively low levels of contaminants measured at Jarrow are at variance with the EROD activities from this study which were comparable to those seen at Newcastle and Redheugh. This is further evidence that no one form of water or sediment quality assay is representative of the entire ecosystem, and supports Matthiessen *et al.'s* (1998a) statement that it should not be assumed that samples giving non-toxic results in acute bioassays will not exhibit significant chronic toxicity. Furthermore, Lye *et al*., (1997) demonstrated high serum vitellogenin levels and

testicular abnormalities in male flounder at a sampling station close to the Jarrow site sampled in this study. These effects were tentatively linked to the presence of a sewage treatment works outfall, some of the constituents of which could potentially also affect the MFO system. Of course this type of intra-estuary site comparison must be treated with caution when dealing with a mobile species such as flounder and although we have been unable to quantify the magnitude of intra-estuary migration in this study it is recognised that this could significantly contribute to the inter-site differences.

Previous surveys of MFO activity in flatfish have shown significant differences between genders. This has been apparent in several species including winter flounder (*Pleuronectes americanus*) (Vandermeulen and Mossman, 1996), plaice (*Pleuronectes platessa*) (George *et al*., 1990), dab (*Limanda limanda*) (Kirby *et al*., 1999a) and also flounder (*Platichthys flesus*) (Eggens *et al.* 1996). However, in each of the cases above, this inter-gender difference has occurred at times associated with spawning or gonadal development. In this study no significant inter-gender difference was demonstrated in mean EROD activities from any of the sites where sufficient numbers of each sex were collected. In fact the EROD results for individual sexes, with respect to their difference from the reference site, closely matched those of the pooled data, with the mean activities from Newcastle and Jarrow being significantly elevated over the Alde for both males and females, and also at Redheugh for females only. These results suggest that, as expected, the fish in this study were in their resting phase (despite severe endocrine disturbances in Mersey, Tees and Tyne fish) and therefore the amalgamation of results from both sexes in the overall analysis was justified. In fact no significant inter-gender difference was demonstrated for any of the measured variables at any of the sites.

Several variables including length, weight, gonado-somatic index (GSI), hepato-somatic index (HSI) and condition factor (CF) were also correlated against EROD activity for each gender. The resultant correlation coefficients for these analyses across all estuaries and more specifically in the Tyne estuary are also shown in Table 2. Further scrutiny of Table 2 reveals that whilst there is some indication of a relationship between size (length and weight) and EROD activity in male flounder by far the most consistently correlated parameter is GSI. EROD activity in flounder has already been shown to exhibit high levels of seasonal fluctuation but these influences appear to be at a minimum during the period June to October (Eggens *et al*., 1996). The influence of reproduction may significantly obscure the desired biomarker response (Goksøyr *et al*., 1996) and therefore the samples taken in this study were timed to coincide with a period when reproductive influences were thought to be minimal. In general this was indeed the case, and so we are confident that the inter-estuary differences shown are contaminant induced, with oestrogens and their mimics possibly contributing somewhat to the overall effect. However, correlation analysis (Table 2) of mean GSI with mean EROD does suggest that even in the optimal sampling period there may be a residual effect of the reproductive cycle on the MFO system in some estuaries. For example, the female samples from the Tyne sites showed a high negative correlation ($r = -0.84$) of EROD with GSI which reflects a trend that is more evident during the reproductive season of several flatfish species (Eggens *et al*., 1996; Kirby *et al*., 1999a; Elskus *et al*., 1992).

Livingstone *et al*. (1997) mention several instances where increased EROD activity in fish has been linked to higher order effects on processes such as disease and reproduction. This study has shown that the MFO system of flounder is significantly elevated above baseline levels in many English estuaries. Whilst the

MFO system is essential for detoxification of certain xenobiotics and the metabolism of some endogenous compounds (for example steroid hormones and vitamins), its induction may also produce deleterious side effects. First, whilst many xenobiotics are rendered less harmful, others form carcinogenic or genotoxic compounds after transformation by the MFO system (eg formation of benzo[*a*]pyrene diol-epoxide from the parent PAH compound). Secondly, since essential endogenous substances such as steroid hormones are metabolised by the MFO system, the latter's elevated activity could have serious repercussions for an organism's normal reproductive development. However, it is also possible that heavy exposure to exogenous oestrogenic hormones and their mimics is actually causing some of the differences in EROD induction observed. On balance, it seems possible that flounder populations (and probably other species) in industrialised English estuaries are facing a significant threat to their long-term health and viability from anthropogenic contamination.

ChE

These surveys have shown that significantly ($p<0.05$) different levels of muscle ChE activity (Table 1, Fig. 3) are apparent in populations of flounder both between and within estuaries. On the assumption that the fish sampled from the reference estuary exhibited 'normal' levels of activity then the majority of those from other estuarine sites were showing high levels (>40%) of muscle ChE inhibition. In particular, samples from Bromborough Port and Scotswood Bridge in the industrialised estuaries of the rivers Mersey and Tyne, respectively, showed mean muscle ChE inhibition levels of 68.6 and 64.4 % in comparison to the Alde reference site. In ranking the estuaries with respect to levels of potential neurotoxicity as exhibited in flounder muscle the following was found; (in descending order) Mersey> Tyne, Tees, Tamar, Humber >Alde > Wear.

When the results from the river Tyne are observed in isolation, a clear downstream trend is apparent. The furthest upstream site, Scotswood Bridge, shows significantly ($p<0.05$) depressed ChE activity levels compared to all other sites in the river. Furthermore, those 2 sites furthest downstream, Jarrow and Lloyds Hailing Station, exhibited ChE activity levels which are comparable to those in the Alde and significantly higher ($p<0.05$) than those at all the other upstream Tyne sites. The spatial distribution of ChE inhibition in flounder muscle from the Tyne strongly indicated that a neurotoxic gradient exists in this estuary, presumably caused by upstream sources of ChE inhibitors.

Organophosphate (OP) and carbamate (C) insecticides are considered to be the most potent cholinesterase inhibitors among the likely environmental contaminants (Burgeot, *et al.*, 1996). Although OPs and Cs are relatively non-persistent in the aquatic environment, their potency is such that their use remains a concern, and it is for the detection of these chemicals that most ChE monitoring programmes appear to have been designed. The ChE results from this study appear to indicate that these insecticides could be present at chronically toxic levels in certain UK estuaries and chemical analysis of water samples from these estuaries confirms the presence of a number of OPs and Cs (Table 5). Weiss (1961) showed that exposures of 100 $\mu g\ l^{-1}$ diazinon for 6 h and 10 $\mu g\ l^{-1}$ azinphos-methyl for 8 h resulted in 95% and 65% inhibition of brain AChE activity in a fish, bluegill, respectively, and Zinkl *et al.*, 1987, found that a 24 h exposure at 250 $\mu g\ l^{-1}$ of the carbamate carbaryl caused up to 85% inhibition of brain AChE in rainbow trout. In this study we found concentrations of the OPs diazinon and azinphos-methyl up to 57 and 46 $ng\ l^{-1}$

respectively, the former at its highest concentrations in the Humber and Tyne and the latter in the Tees. Carbaryl was also detected at up to 18 ng l^{-1} in the Tees. Whilst the measured concentrations of OPs and Cs in water of UK estuaries appear to be below those reported in the literature to have major effects on fish, there are two reasons why they could nevertheless be the prime causative agents of the observed suppression of neural activity. Firstly, several different OPs and Cs will be present in the estuaries at any one time. Analyses undertaken in this study revealed six and five separate OP and C compounds in the Tyne and Tees respectively (Table 5). Not only may the sum of all these compounds reach levels of significance in terms of anti-cholinesterase effect, but, moreover, combinations of OPs and Cs have been shown to be highly synergistic in their ability to inhibit ChE activity (Bocquené *et al.* 1995). Secondly, these insecticides were only determined in water, but several are relatively non-polar and may be associated with the sediment, or alternatively, bioaccumulate in the dietary organisms of the flounder. Exposure via the sediment or dietary path could, therefore, be a contributory factor in the total exposure of flounder to neurotoxic compounds. However, a number of other important contaminants have been shown to have anti-ChE properties, including heavy metals (Zinkl *et al.* 1991), hydrocarbons and detergents (Payne *et al.* 1996). It seems more likely that the reductions in ChE activity observed in this study, if caused by pollution, could be attributed to the integrated effect of several classes of contaminant.

The neurotoxicity gradient observed in the Tyne would suggest that the primary neurotoxins responsible for this trend have their main source upstream of the Scotswood Bridge site, and that the increased flushing by marine waters downstream acts to remediate its effects. This supports the hypothesis that agricultural/urban run-off from the upper watershed may be a significant factor, and suggests that agricultural contaminants, such as OP and C insecticides, as opposed to the industrial contaminants (e.g. metals, hydrocarbon etc.) associated with downstream industrialisation, are more likely to be the primary neurotoxins responsible. However, this requires much more investigation and needs to be verified by the sampling of upstream transects in other estuaries.

In this study muscle tissue was used exclusively. Other tissues have been used for the determination of cholinesterase activity in fish including liver, heart (Bocquené *et al.,* 1990), plasma and eye (Ceron *et al.,* 1996). However, brain tissue has been most commonly used in studies with fish (Zinkl *et al.,*1987;Sancho *et al.,*1997; Inbaraj and Haider, 1988). Muscle offers some logistical advantages over brain tissue, including ease of collection and the availability of a larger quantity of material. Moreover, it offers a different perspective on neurotoxicity than the inhibition of brain tissue neural activity. Whilst brain cholinesterase consists entirely of acetylcholinesterase (AChE) muscle not only contains AChE but also butyrylcholinesterase (BChE). In fact, BChE activity in flounder (*Platichthys flesus*) muscle has been shown to account for up to 46% of the total cholinesterase activity (Sturm *et al.*, 1999). Inhibition of BChE activity has been demonstrated to be more sensitive to certain OPs and Cs (Sturm *et al.*, 1999) and therefore the measurement of ChE activity in muscle could prove to be a more sensitive monitoring tool of neurotoxicity in certain circumstances. Whilst the essential role of AChE in cholinergic neurotransmission is well documented, the physiological significance of BChE is poorly understood. It is generally accepted that 60-70% inhibition of brain AChE in fish will result in death (Zinkl, *et al.* 1991). In this study, however, muscle ChE inhibition of >60% (compared to a reference site) was apparent in flounder populations that were not manifesting any obvious signs of neurotoxic stress. It seems

likely that much of this effect was due to inhibition of ChE forms other than AChE. This, however, does not detract from the fact that significant total ChE inhibition was occurring, and that this indicates the presence of bioavailable neurotoxins in UK estuaries.

Comparison of the means by ANOVA for each gender at each site showed that no significant ($p<0.05$) differences existed. This observation is in agreement with those previously reported for dab (Galgani *et al.,* 1992) and bluegills (Hogan, 1970) and it was therefore justified to amalgamate data from each sex for all other statistical analyses. Correlation analysis of mean ChE activity levels with the mean values of GSI, HSI and CF for all the sites together, and also for the six Tyne sites in isolation, revealed no relationship between these measurements. Several studies have shown that AChE activity is lower in larger (older) fish than in smaller (younger) fish (Zinkl *et al,* 1991). This is possibly the case over wide size/age differences and whilst there is some evidence, although weak, to support it in this study (correlation coefficients for ChE:length, $r = -0.43$ and $r = -0.64$ for all sites and Tyne sites respectively) the results presented here are similar to those reported by Galgani *et al* (1992) for dab from the North Sea. It is interesting to note that the length associated effect on ChE activity was much more pronounced in males (with correlations of $r=-0.87$ and $r=-0.97$ for all estuaries and Tyne respectively) but due to the narrow range of fish length and the small number of fish sampled the data must be treated with some caution. Nevertheless, it is recommended that in monitoring surveys a specific size range should be sampled at each site where possible.

With the possible exception of size, there was a lack of evidence to suggest that any of the above biotic factors has significantly influenced the ChE activity levels observed in this study. It can therefore be concluded that the observed intra- and inter-estuarine differences were, at least in part, neurotoxin-mediated.

GENERAL CONCLUSION

The simultaneous use of two enzyme biomarkers to study sub-lethal contaminant effects in UK estuaries has clearly demonstrated their value as important tools in environmental monitoring. Furthermore, the results indicate that flounder populations in a number of UK estuaries are experiencing exposure to different classes of contaminant at levels high enough to elicit sub-lethal responses. Work is on-going at CEFAS to investigate what the biological significance of biomarker responses are with respect to higher order effects (growth, disease and reproductive capacity).

ACKNOWLEDGEMENTS

The authors are indebted to many people for assistance with these studies including Sarah Haworth, Tony Fagg and Yvonne Allen and the crews of the vessels we used. The work was funded by the UK Ministry of Agriculture, Fisheries and Food (MAFF) and the UK Department of Environment, Transport and the Regions (DETR).

REFERENCES

Allchin, C.R., Kelly C.A., Portmann J.E. (1989). Methods of analysis for chlorinated hydrocarbons in marine and other samples. *Aquatic Environment Protection: Analytical Methods*, MAFF Directorate of Fisheries Research, Lowestoft, **6**: 25pp.

Allen Y., Thain, J., Matthiessen, P., Scott, A. P., Haworth, S. and Feist, S. (1997). A survey of oestrogenic activity in UK estuaries and its effects on gonadal development of the flounder *Platichthys flesus*. International Council for the Exploration of the Sea, Copenhagen, ICES report C.M. 1997/U:01, 13 pp + figs.

Allen, Y., Matthiessen, P., Scott, A.P., Haworth, S., Feist, S.W. and Thain, J.E.

(1999). The extent of oestrogenic contamination in the UK marine environment. *Environmental Toxicology and Chemistry*, **18(8),** 1791-1800.

Arukwe, A., Forlin, L. and Goksøyr, A. (1997). Xenobiotic and steroid biotransformation enzymes in Atlantic salmon (*Salmo salar*) liver treated with an estrogenic compound, 4-nonylphenol. *Environmental Toxicology and Chemistry*, **16(12),** 2576-2583.

Beyer, J., Sandvik, M., Hylland, K., Fjeld, E., Egaas, E., Aas, E., Skåre, U. and Goksøyr, A. (1996). Contaminant accumulation and biomarker responses in flounder (*Platichthys flesus* L.) and Atlantic cod (*Gadus morhua* L.) exposed by caging to polluted sediments in Sørfjorden, Norway. *Aquatic Toxicology*, **36**, 75-98.

Bocquené, G., Galgani, F., and Truquet, P. (1990). Characterization and assay conditions for use of AChE activity from several marine species in pollution monitoring. *Marine Environmental Research*, **30**, 75-89.

Bocquené, G., Bellanger, C., Cadiou, Y. and Galgani, F. (1995). Joint action of combinations of pollutants on the acetylcholinesterase activity of several marine species. *Ecotoxicology*, **4**, 266-279.

Bocquené, G. and Galgani, F. (1996). Cholinesterase inhibition by organophosphorus and carbamate compounds. Techniques in Marine Environmental Sciences: IFREMER internal report.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. *Analytical Biochemistry*, **72**, 248-254.

Burgeot, T., Bocquené, G., Porte, C., Dimeet, J., Santella, R. M., Garcia de la Parra, L. M., Pfohl-Leszkowicz, A., Raoux, C. and Galgani, F. (1996). Bioindicators of pollutant exposure in the northwestern Mediterranean Sea. *Marine Ecology Progress Series*, **131**, 125-141.

Ceron, J.J., Ferrando, M.D., Sancho, E., Gutierrez-Panzio, C. and Andreu-Moliner, E. (1996). Effects of diazinon exposure on cholinesterase activity in different tissues of European eel (*Anguilla anguilla*). *Ecotoxicology and Environmental Safety*, **35**, 222-225.

Draganik, B., Bykowski, P., Krawczak-Krogulecka, W. and Kuczynski, J. (1996). Seasonal and spatial variation in EROD activity in flounder along the Polish coast. *Bulletin of Sea Fisheries Institute*, **2(138),** 3-13.

Eggens, M. L., Opperhuizen, A. and Boon, J. P. (1996). Temporal variations of CYP1A indices, PCB and 1-OH pyrene concentration in flounder, *Platichthys flesus*, from the Dutch Wadden Sea. *Chemosphere*, **33**, 1579-1596.

Elskus, A. A., Pruell, R. J. and Stegeman, J. J. (1992). Endogenously-mediated, pretranslational suppression of cytochrome P4501A in PCB-contaminated flounder. *Marine Environmental Research*, **34**, 97-101.

Galgani, F., Bocquené and Cadiou, Y. (1992) Evidence of variation in cholinesterase activity in fish along a pollution gradient in the North Sea. *Marine Ecology Progress Series*, **91**, 77-82.

George, S., Young, P., Leaver, M. and Clarke, D. (1990). Activities of pollutant metabolising and detoxication systems in the liver of the plaice, *Pleuronectes platessa*: Sex and seasonal variations in non-induced fish. *Comparative Biochemistry and Physiology*, **96C**, 185-192

Goksøyr, A., Beyer, J., Egaas, E., Grøsvik, B. E., Hylland, K., Sandvik, M. and Skaare, J. U. (1996). Biomarker responses in flounder (*Platichthys flesus*) and their use in pollution monitoring. *Marine Pollution Bulletin*, **33**, 36-45.

Hogan, J.W. (1970). Water temperature as a source of variation in specific activity of brain acetylcholinesterase of bluegills. *Bulletin of Environmental Contamination and Toxicology*, **5 (4)**, 347-352.

Inbaraj, R. M. and Haider, S. (1988). Effect on brain acetylcholinesterase and ovarian steroidogenesis of *Channa punctatus* (Bloch). *Ecotoxicology and Environmental Safety*, **16**, 123-128.

Kelly, C.A. and Law, R.J. (1998). Monitoring of PAH in fish and shellfish following the *Sea Empress* incident. The *Sea Empress* Oil Spill: Proceedings of the International Conference held in Cardiff, 11-13 February 1998. R. Edwards and H. Sime (Editors). Chartered Institute of Water and Environmental Management, London. pp. 467-473.

Kirby, M. F., Blackburn, M. A., Thain, J. E. and Waldock, M. J. (1998a). Assessment of water quality in estuarine and coastal waters of England and Wales using a contaminant concentration technique. *Marine Pollution Bulletin*. **36(8),** 631-642.

Kirby, M. F., Neall, P. and Tylor, T. (1999a). EROD activity measured in flatfish from the area of the *Sea Empress* oil spill. *Chemosphere*, **38 (12)**, 2929-2949.

Kirby, M. F., Matthiessen, P., Neall, P., Tylor, T., Allchin, C. R., Kelly, C. A., Maxwell, D. L. and Thain, J. E. (1999b). Hepatic EROD activity in flounder (*Platichthys flesus*) as an indicator of contaminant exposure in English estuaries. *Marine Pollution Bulletin.* **38(8)**, 676-686.

Kirby, M. F., Morris, S., Hurst, M., Kirby, S. J., Neall, P., Tylor, T. and Fagg, A. (2000). The use of cholinesterase activity in flounder (*Platichthys flesus*) muscle tissue as a biomarker of neurotoxic contamination in UK estuaries. *Marine Pollution Bulletin*, **40(9).**

Law, R.J., Thain, J.E., Kirby, M.F., Allen, Y.T., Lyons, B.P., Kelly, C.A., Haworth, S., Dyrynda, E.A., Dyrynda, P.E.J., Harvey, J.S., Page, S., Nicholson, M.D. and Leonard, D.R.P. (1998). The impact of the *Sea Empress* oil spill on fish and shellfish. The *Sea Empress* Oil Spill: Proceedings of the International Conference held in Cardiff, 11-13 February 1998. R. Edwards and H. Sime (Editors). Chartered Institute of Water and Environmental Management, London. pp. 109-136.

Livingstone, D. R., Förlin, L. and George, S. G. (1997). Molecular biomarkers and toxic consequences of impact by organic pollution in aquatic organisms. In: Water quality and stress indicators in marine and freshwater ecosystems. Linking levels of organisation (individuals, populations, communities). Ed: Sutcliffe, D. W., ISBN 0-980386-54-1, 154-171.

Lye, C. M., Frid, C. L. J., Gill, M. E. and McCormick, D. (1997). Abnormalities in the reproductive health of flounder *Platichthys flesus* exposed to effluent from a sewage treatment works. *Marine Pollution Bulletin*, **34**, 34-41.

Matthiessen, P. (1988). Recent attempts to measure the behaviour and impact of pesticides in aquatic environments. In: Field methods for the study of environmental effects of pesticides, BCPC monograph No. 40, Eds. Greaves, M. P., Smith, B. D., and Grieg-Smith, P.W., ISBN 0-948404-23-X, 203-212.

Matthiessen, P., Bifield, S., Jarrett, F., Kirby, M. F., Law, R. J., McMinn, W. R., Sheahan, D. A., Thain, J. E. and Whale, G. F. (1998a). An assessment of sediment toxicity in the River Tyne estuary, UK by means of bioassays. *Marine Environmental Research*, **45**, 1-15.

Matthiessen, P., Allen, Y. T., Allchin, C. R., Feist, S. W., Kirby, M. F., Law, R. J., Scott, A. P., Thain, J. E. and Thomas, K. V. (1998b). Oestrogenic endocrine disruption in flounder (*Platichthys flesus*) from United Kingdom estuarine and marine waters. Centre for Environment, Fisheries and Aquaculture Science, Lowestoft, UK, Science Series Technical Report **107**, 48pp.

Payne, J.F., Mathieu, A., Melvin, W. and Fancey, L.L. (1996). Acetylcholinesterase, and old biomarker with a new future? Field trials in association with two urban rivers and a paper mill in Newfoundland. *Marine Pollution Bulletin*, **32 (2)**, 225-231.

Sancho, E., Ferrando, M.D. and Andreu, E. (1997). Response and recovery of brain acetylcholinesterase activity in the European eel, *Anguilla anguilla*, exposed to fenitrothion. *Ecotoxicology and Environmental Safety*, **38**, 205-209.

Stagg, R. M., McIntosh, A. and Mackie, P. (1995). Elevation of hepatic monooxygenase activity in the dab (*Limanda limanda* L.) in relation to environmental contamination with petroleum hydrocarbons in the northern North Sea. *Aquatic Toxicology*, **33**, 245-264.

Stegeman, J. J., Woodin, B. R. and Goksoyr, A. (1988). Apparent cytochrome P-450 induction as an indication of exposure to environmental chemicals in the flounder *Platichthys flesus*. *Marine Ecology Progress Series*, **46**, 55-60.

Stieger, S., Gentinetta, R. and Brodbeck, U. (1989). Cholinesterases from flounder muscle. *European Journal of Biochemistry*, **181**, 633-642.

Sturm, A., da Silva de Assis, H.C. and Hansen, P.-D. (1999). Cholinesterases of marine teleost fish: enzymological characterization and potential use in the monitoring of neurotoxic contamination. *Marine Environmental Research*, **47**, 389-398.

Sulaiman, N., George, S. and Burke, M. D. (1991). Assessment of sublethal pollutant impact on flounders in an industrialised estuary using hepatic biochemical indices. *Marine Ecology Progress Series*, **68**, 207-212.

Vandermeulen, J. H. and Mossman, D. (1996). Sources of variability in seasonal

hepatic microsomal oxygenase activity in water flounder (*Pleuronectes americanus*) from a coal tar contaminated estuary. *Canadian Journal of Fisheries and Aquatic Sciences*, **53**, 1741-1753.

Weiss, C.M. (1961). Physiological effect of organic phosphorus insecticides on several species of fish. *Transactions of the American Fisheries Society*, **90**, 143-152

Zinkl, J.G., Shea, P.J., Nakamoto, R.J. and Callman, J. (1987). Technical and biological considerations for the analysis of brain cholinesterase of rainbow trout. *Transactions of the American Fisheries Society*, **116**, 570-573.

Zinkl, J. G., Lockhart, W. L., Kenny, S. A. and Ward, F. J. (1991). The effects of cholinesterase inhibiting insecticides on fish. In: *Cholinesterase inhibiting insecticides* (Ed: Mineau, P.), Elsevier, Amsterdam, Chap.10, p. 233-254.

Table 1. Mean EROD activity, ChE activity and other variables in Flounder from English estuaries.

Location	EROD (n) (pmol.min^{-1}.mg pro^{-1})	ChE (n) (mU.min^{-1}.mg pro^{-1})	Length (cm)	Weight (g)	GSI Female	GSI Male	HSI	CF
Alde (Reference)								
North of Orford	38.7 (24)	16573 (22)	25.4	156.8	0.72	0.76	1.03	0.95
Mersey								
Speke	117.9 (16)*	9402 (38)*	25.0	185.3	0.82	-	1.35	0.96
Bromborough	161.6 (11)*	5206 (27)*	28.1	238.2 *	0.98	-	1.58 *	0.98
Tees								
Tees Dock	99.8 (4)*	7852 (5)*	23.9	171.4	1.47	0.17	1.77 *	1.26 *
Redcar Terminal	95.6 (4)	10847 (7)*	22.0	125.3	1.04	0.08	1.66 *	1.17 *
Humber								
Whitton	17.44 (3)	7699 (17)*	22.4	106.0	1.40	-	1.27	0.94
Foul Holme Channel	77 (9)	9498 (19)*	21.9	117.8	1.11	-	1.79 *	0.98
Spurn Head	84.6 (13)*	10191 (29)*	25.4	181.6	1.27	0.86	0.92	1.07 *
Southampton Water								
Bird Pile Beacon	62.1(12)	-	27.2	218.5	2.52 *	1.36	1.42 *	0.95
Wear								
Wearmouth Bridge	84.61(4)	20888 (4)	20.4	99.6	0.69	0.18	1.44	1.08 *
Tyne								
Scotswood Bridge	70.4 (26)*	5894 (23)*	29.7 *	288.1 *	2.36 *	1.35	1.70 *	1.05 *
Redheugh	90.0 (41)*	9901 (40)*	27.2	237.2 *	1.72 *	1.37	1.78 *	1.05 *
Newcastle	95.8 (22)*	9704 (20)*	25.4	183.7	1.53	0.56	1.68 *	1.02
Hebburn	54.3 (28)	10918 (24)*	24.2	176.3	1.94 *	0.80	1.59 *	1.08 *
Jarrow	94.8 (30)*	18478 (31)	20.9 *	105.5	1.51	0.16	1.74 *	1.04 *
Lloyds Hailing Station	57.6 (3)	15984(8)	28.2	271.6	2.54	0.27	2.03 *	1.19 *
Tamar								
Warren Point	-	9717 (17)*						

* Significantly different ($p<0.05$) from the Alde reference site.

Table 2. Linear correlation coefficients (r) for mean EROD with other variables

All Estuaries

	Number of Stations	Length	Weight	GSI	HSI	CF
All	16	-0.09	0.05	-	0.21	0.22
Female	13	0.09	0.14	-0.33	0.31	0.24
Male	7	-0.54	-0.37	-0.44	0.11	0.52

Tyne Only

	Number of Stations	Length	Weight	GSI	HSI	CF
All	6	-0.55	-0.60	-	-0.17	-0.67
Female	5	0.01	-0.24	-0.84	-0.07	-0.80
Male	5	-0.51	-0.51	-0.43	0.09	-0.63

Table 3. Mean total PAH and PCB in bulked samples of flounder liver from selected estuaries

Location	PAH (µg kg-1 wet wgt.)	PCB (µg kg-1 wet wgt.)
River Mersey	363	1082
River Tees	365	n/a
River Humber	71.2-100.6	424
River Alde	109.7	32

n/a = not analysed

Table 4. Linear correlation coefficients (r) for mean ChE with other variables

	No. of stations	Length	Weight	GSI	HSI	CF
All estuaries						
All	16	-0.43	-0.36	-	-0.06	0.05
Female	13	-0.31	-0.33	-0.17	-0.11	0.23
Male	7	-0.87	-0.84	-0.63	-0.14	-0.37
Tyne only						
All	6	-0.64	-0.55	-	0.46	0.39
Female	5	-0.82	-0.86	-0.54	-0.26	0.06
Male	5	-0.97	-0.96	-0.91	0.39	-0.40

Table 5. Concentrations (ng l^{-1}) of organophosphates and carbamates from selected estuaries

Pesticide	LOQ (ng/l)	Estuary					
		Alde	Humber	Tamar	Mersey	Tees	Tyne
Organophosphates							
Heptenophos	30	<30	<30	<30	<30	39	<30
Diazinon	20	<20	39-57	<20	36	<20	28-43
Disulfoton	30	<30	32	<30	<30	<30	<30
Etrimfos	10	<10	<10	26	<10	<10	<10
Fenthion	10	<10	<10	<10	13-29	<10	<10
Chlorfenvinphos	10	<10	25	<10	18-31	<10	31-50
Triazophos	20	<20	48	<20	<20	37	<20
Azinphos-methyl	30	<30	<30	<30	<30	46	<30
Carbamates							
Carbofuran-3-OH	10	<10	<10	<10	<10	26	<10
Carbofuran	2	<2	<2	<2	<2	<2	13-21
Bendiocarb	2	<2	<2	<2	<2	<2	18
Carbaryl	2	<2	<2	<2	<2	18	<2
Ethiofencarb	5	<5	<5	<5	8	<5	32
Pirimicarb	2	<2	<2	<2	<2	<2	12

LOQ = Limit of quantitation

Figure 1. Estuarine sampling locations.

Figure 2. Mean EROD activities in English estuaries.Error bars = 95% confidence limits.

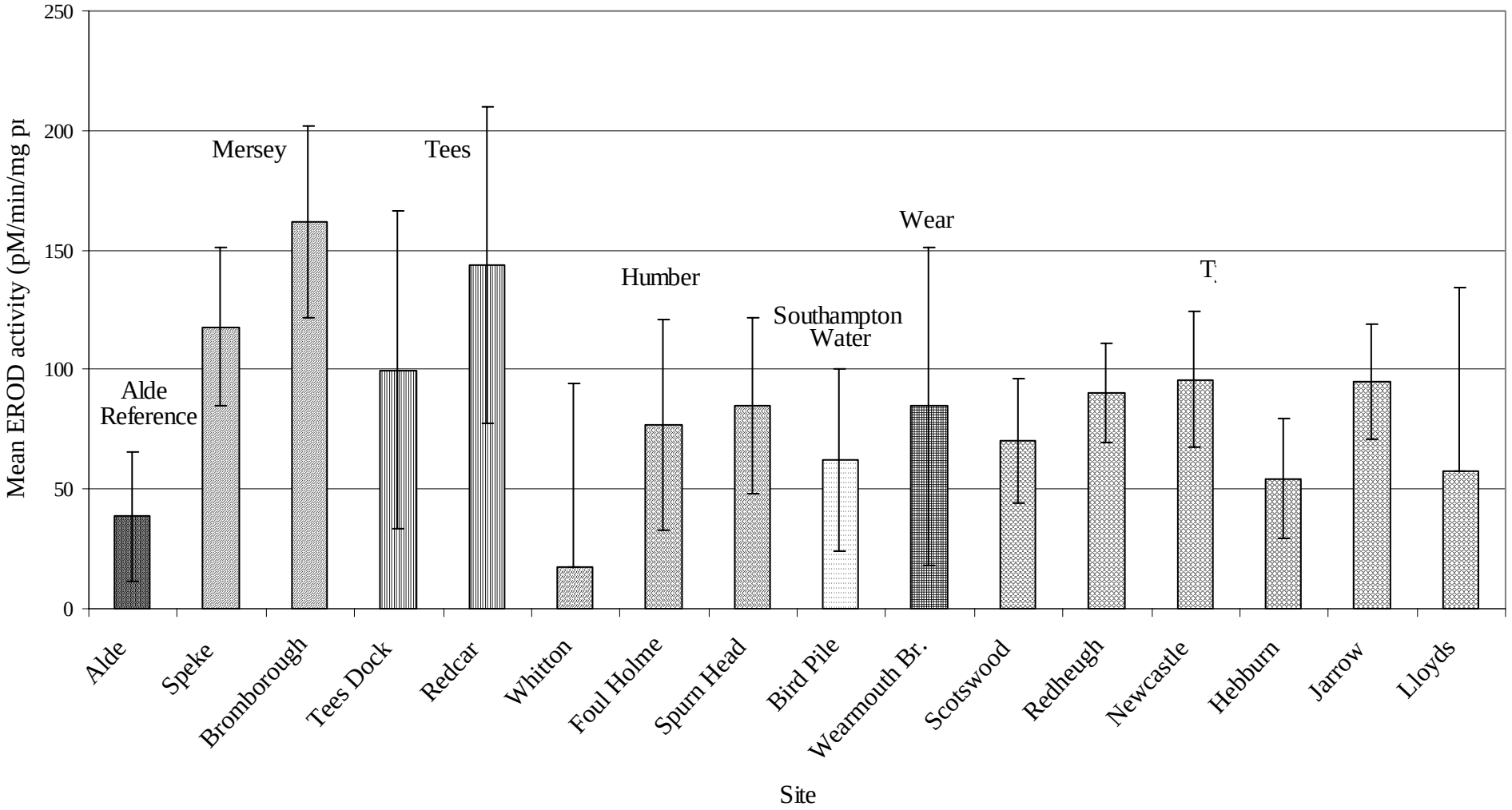

Figure 3. Cholinesterase activity in flounder muscle from English estuaries. Error bars = 95% confidence limits.