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Evidence of variation in cholinesterase activity in fish along a pollution gradient in the North Sea

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ABSTRACT: During the Bremerhaven Workshop cholinesterase measurements in dab *Limanda limanda* muscle were evaluated as a monitoring tool to assess the effect of pollutants along a 360 km transect in the North Sea, and around a drilling site. The basic properties of cholinesterases, together with their natural variability related to sex and size, were investigated. The results show the presence of at least 2 different enzymes, acetylcholinesterase and butyrylcholinesterase, with high activities in brain, muscle and liver. No variation was observed in relation to sex or size. The activity of both acetylcholinesterase and butyrylcholinesterase was depressed in nearshore stations along the transect and no variation was observed around the drilling site. The K_m of acetylcholinesterase from muscle of dab varied along the transect. The results lead us to interpret enzyme variation as the result of effects of neurotoxic compounds coming from the Elbe and Weser rivers into the German Bight, and validate cholinesterase activity as a tool for biological monitoring at sea.

INTRODUCTION

Considerable efforts have been made in recent years to rationalize the techniques of research and monitoring of biological effects of pollutants in marine waters, exemplified by the IOC and ICES practical workshops in temperate and tropical waters, and the Bremerhaven Workshop. A number of physiological techniques have been sufficiently developed to provide for the implementation of a monitoring programme (Payne et al. 1987). However, the search continues for new monitoring tools which may be used as specific markers for contaminant effects.

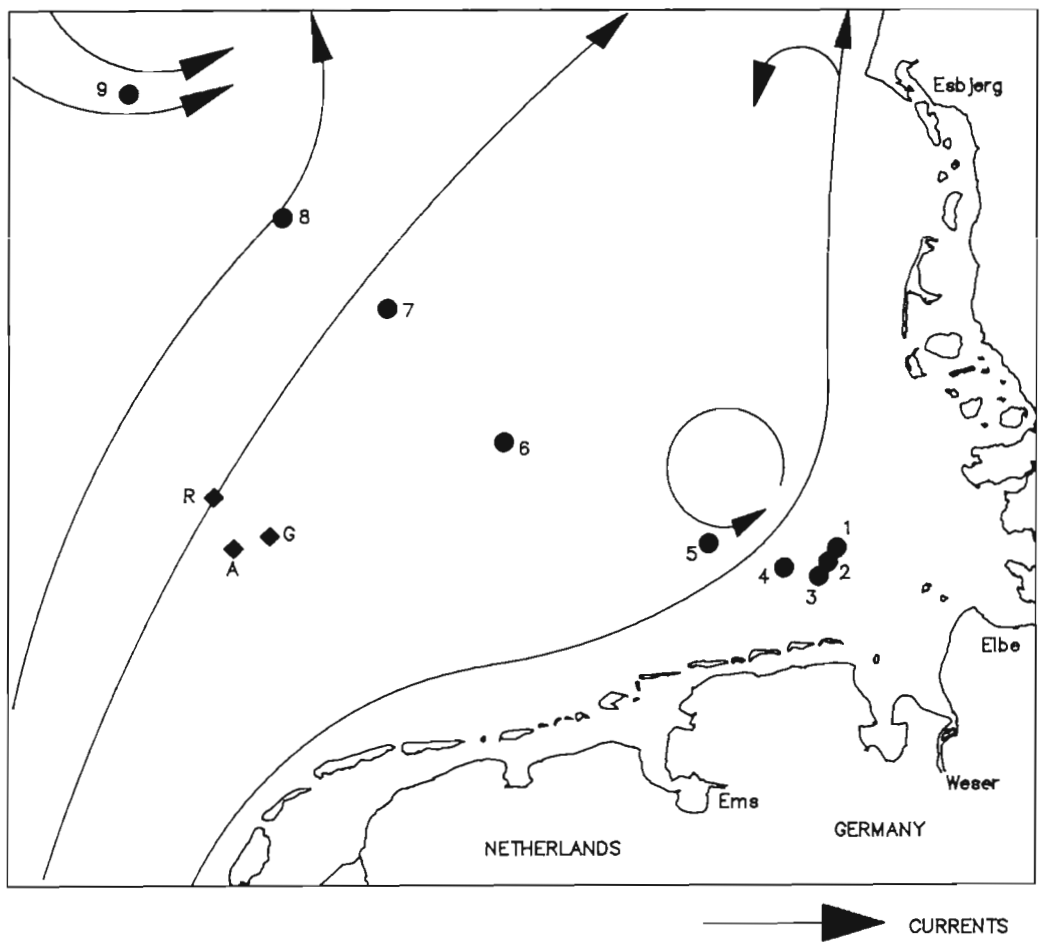
The principal biological role of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) is the regulation of nerve impulse transmission by hydrolysis of the neurotransmitter acetylcholine. This paper deals with cholinesterase inhibition, a marker that has been used for years in monitoring effects of pesticides such as organophosphorus and carbamate compounds in both terrestrial and freshwater organisms (Holland et al. 1967, WHO 1986a, b). The study of cholinesterase in marine fishes has focused on biochemical characterization (Zinkl et al. 1987, Habig et al. 1988, Bocquené et al. 1990, Galgani & Bocquené 1990) or toxicity tests (Coppage & Matthews 1974, Habig et al. 1986, Van der Wel & Welling 1989, Bocquené & Galgani 1991). In this paper, we evaluate the use of enzyme measurements at sea and as a tool for monitoring pollution effects in marine animals.

MATERIALS AND METHODS

Animals. *Limanda limanda* were obtained during the Bremerhaven Workshop (10 to 28 March 1990). Samples (5 males and 5 females per site) were trawled during the cruise of RV 'Victor Hensen' at 7 stations along a 360 km transect in the German Bight, and on a drilling site transect during the cruise of RV 'Aurelia' (Fig. 1) as described by Stebbing & Dethlefsen (1992).

Enzymatic assays. Cholinesterase measurements were performed on tissues sampled from live fish and stored at −20 °C. Tissues were suspended in 0.1 M TRIS buffer pH 8.0 (2/1 v/w) and homogenized for 1 min using an Ultra Turrax. Extracts were then centrifuged at 10 000 × *g* for 10 min and the supernatants were analysed for cholinesterase activity. Proteins were determined by the method of Bradford (1976)

using bovine serum albumin (BSA) as a standard. All assays were done in quadruplicate. Acetylcholinesterase and butyrylcholinesterase were determined spectrophotometrically using, respectively, acetylthiocholine and butyrylthiocholine as substrates. The principle of Ellman et al. (1961) was used as modified for microtitration plate reading (Galgani & Bocquené 1991). For each microplate well, 300 µl of TRIS buffer 0.1 M, pH 8.0, 20 µl of dithio*bis*nitrobenzoic acid (DTNB, 0.01 M) and 10 µl of enzyme suspension were added successively. Substrate (10 µl, 0.1 M) was added before the enzymatic reaction was started and absorbance (A) was monitored on a microplate reader (Titertek MCC 340) at 405 mm. One unit of AChE activity is the variation of 0.001 A. Results are given as units min^{-1} or units min^{-1} mg^{-1} protein for specific activities.



LOCATIONS (GERMAN BIGHT TRANSECT)

	No.	Sediment	Coordinates		Water depth	
	1	mud	54°04′N	08°07.5′E	19 m	
	2	mud	54 02	08 03	27 m	(no fishing)
	3	muddy sand	54 00	08 00	29 m	
	4	–	54 01	07 49	36 m	(no fishing)
	5	–	54 06.5	07 24	35 m	
	6	–	54 25	06 15	40 m	
	7	–	54 50	05 35	43 m	
	8	–	55 06	05 00	42 m	
	9	sand	55 30	04 10	30 m	
Oil platform	A	mud	54 06.2	04 45.5		
	R	–	15 Km from A			
	G	–	5 Km from A			

Fig. 1. Position of sampling sites and currents along the German Bight transect and at the drilling site

Enzyme kinetics. Michaelis-Menten kinetics were assumed in determining enzymatic characteristics:

$$E + S \longrightarrow ES \longrightarrow E + P;$$

$$\text{and } V = V_{max} \frac{[S]}{[S] + K_m}$$

where V = velocity of the reaction; V_{max} = maximum velocity; [S] = substrate concentration; K_m = Michaelis constant.

Computational procedures. Measurements of enzyme kinetics were done on the plate reader connected through an RS232 C serial port to a computer. Routine measurements were performed as described by Galgani et al. (1991).

RESULTS

The specific activity of acetylcholinesterase and butyrylcholinesterase was determined for each organ of the dab to eliminate tissues without sufficient activity. The highest activity (Table 1) was found for acetylcholinesterase with the highest values occurring in brain (7449 units mg^{-1} protein) and muscle (2654 units mg^{-1} protein). For BChE, the highest value was found in muscle (1487 units mg^{-1} protein) and liver (336 units mg^{-1} protein). The activity of both AChE and BChE was very low in gonads.

The optimum pH of the assay was established using dab muscle. Results are given in Fig. 2. For AChE, a sharp peak was observed with a maximum pH value of around 8.45. For butyrylcholinesterase, no variation of enzyme activity was observed in the range pH 7.5 to 9.5.

The specific activity of muscle acetylcholinesterase (Fig. 3) showed a gradient along the transect, with values increasing from 2296 ± 743 units mg^{-1} of protein near the shore (Stn 3) to 6392 ± 852 units mg^{-1} protein in the centre of the North Sea (Stn 8). However, significantly lower values ($p < 0.05$) were obtained for Stn 9 (4952 ± 1091 units mg^{-1} of protein) compared to Stn 8. Results with butyrylcholinesterase show an identical

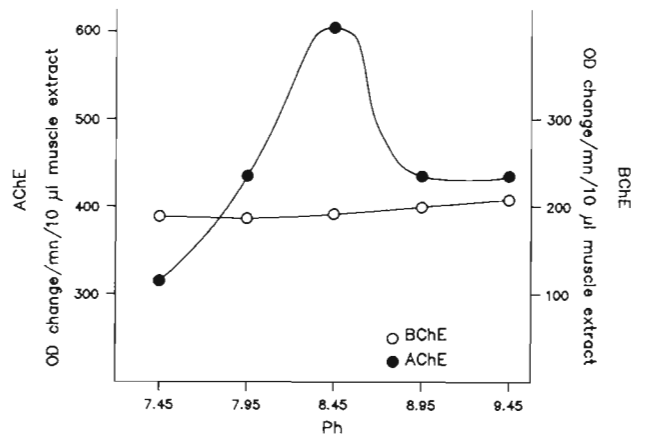


Fig. 2. *Limanda limanda*. Activity of AChE and BChE from the muscle of dab from fish collected at Stn 5 measured under different conditions of pH (mean value of 3 assays)

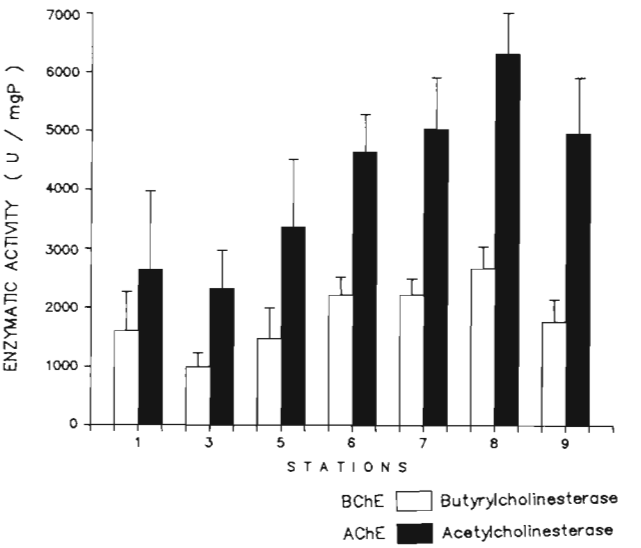


Fig. 3. *Limanda limanda*. Specific activity of acetylcholinesterase and butyrylcholinesterase in muscle of dab caught along the German Bight transect. Results (mean ± SD) are expressed in absorbance units per mg of protein. Ten fish were sampled per station and 4 measurements were made per sample

Table 1. *Limanda limanda* acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activity in organs from dab collected at Stn 5. Four measurements were made per sample. Results [mean ± SD (no. of samples)] are expressed in absorbance units per mg protein

Organ	Protein (mg ml^{-1})	AChE activity (U mg^{-1} protein)	BChE activity (U mg^{-1} protein)
Brain	4.40	7449 ± 1647 (5)	234 ± 144 (6)
Gonad	11.6	98.7 ± 110 (6)	127 ± 823 (5)
Gills	5.04	480 ± 176 (5)	138 ± 173 (6)
Testis	13.4	26.8 ± 21.0 (5)	25 ± 18.3 (6)
Liver	14.8	1583 ± 398 (6)	556 ± 141 (6)
Muscle	8.4	2654 ± 710 (4)	1487 ± 229 (4)

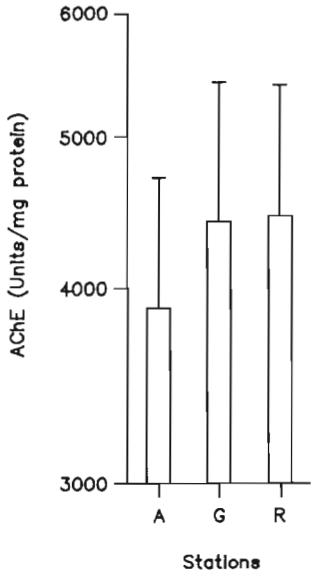


Fig. 4. *Limanda limanda*. Specific activity of acetylcholinesterase activity in muscle from dab collected at different sampling sites around the drilling site

profile, with specific activity values ranging from 984 ± 225 units mg^{-1} (Stn 3) to 2729 ± 422 units mg^{-1} (Stn 8). In this case, however, values for Stn 1 were significantly different from those for Stn 3.

There was no significant variation in AChE or BChE relation to the size of the fish (Table 2). No significant sex-related differences were observed for AChE for BChE (Table 3), except for a slight variation in BChE of samples collected at Stn 8.

The link between oil contamination and cholinesterase activity was investigated by measuring the activity of the enzyme in samples collected near the drilling site. Three points were sampled along a 23 km transect. No significant variation of the enzyme activity was noted as shown in Fig. 4.

To determine whether the system could be used directly on board ship, assays were performed on board and compared to laboratory measurements on frozen tissue samples. Comparisons were made during the cruises of RV 'Victor Hensen' (German Bight, 2 stations) and RV 'Aurelia' (drilling site, 2 stations). Results are shown in Fig. 5. All measurements at sea were obtained in less than 30 min. The correlation between the 2 procedures was 0.86.

Table 2. *Limanda limanda*. Size variability in acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities in male dab muscle from Stn 5. Results (mean ± SD, n = 4) are expressed in absorbance units per mg protein. There were no significant differences between the size groups tested (ANOVA, p = 0.05)

Size (cm)	AChE	BChE
< 12	3298 ± 1449	2001 ± 631
12–20	2654 ± 710	1487 ± 229
20–25	2689 ± 1700	1398 ± 1024

Measurements of the K_m of acetylcholinesterase of dab were made along the German Bight transect. Results are shown in Fig. 6. K_m values for the enzyme ranged from 192.3 ± 30 µM at Stn 3 to 367.9 ± 120 µM at Stn 8. A slight decrease in enzyme affinity occurred over the Dogger Bank (Stn 9).

DISCUSSION

The high activity of both AChE and BChE in muscle and brain leads us to consider those tissues useful for monitoring purposes, as is the case in other species (Bocquené et al. 1990). This enzyme activity must be related to the importance of nerve impulse transmission in tissues with high neuromuscular activity. Dissection of the brain is difficult to perform, especially at sea, and the amount of tissue collected was small in

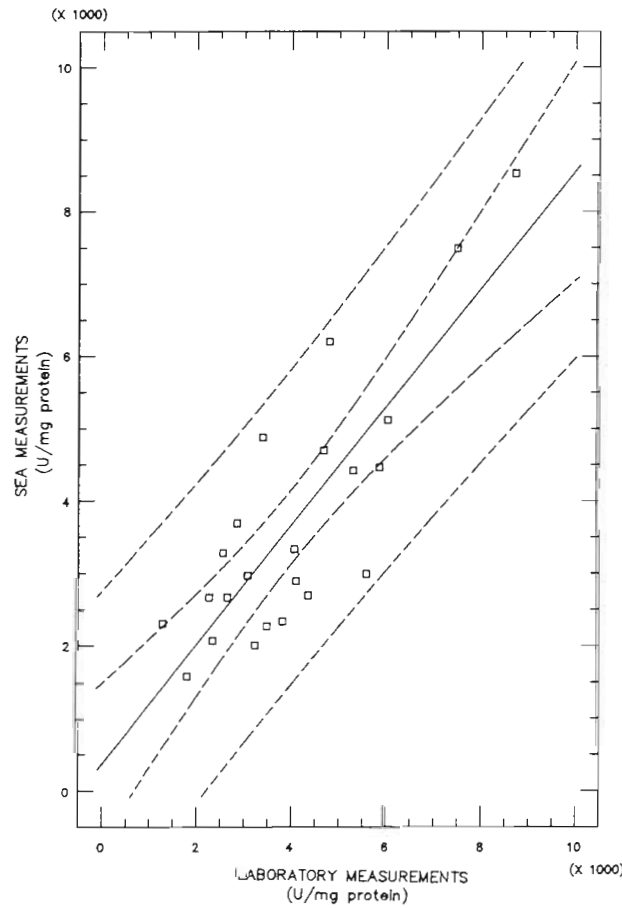


Fig. 5. *Limanda limanda*. Correlation of cholinesterase measurements performed by microplate reader at sea on fresh dab muscle (4 fish per site and 4 analyses per fish) with results obtained on muscle from the same fish stored at −20 °C and analyzed in the laboratory (r = 0.86)

comparison with that of muscle. Therefore, for the experiments at sea, the activity of muscle extracts towards acetylthiocholine and butyrylthiocholine was used as the enzymatic marker. The differences in tissue enzyme activities and pH profiles for both AChE and BChE show the existence of at least 2 different enzymes acting on synthetic substrates. Evidence for different enzymes, including a true acetylcholinesterase and a pseudocholinesterase, has been reported previously (Leibel 1988). The poor BChE activity observed in the dab brain, together with its high activity in muscle and liver, support the hypothesis that butyrylthiocholine is hydrolysed by an atypical cholinesterase (Toutant et al. 1989) and acetylthiocholine by both a true cholinesterase and a pseudocholinesterase.

The activities of both enzymes varied according to the expected pollution gradient. We hypothesize that, overall, the results indicate a gradient of effects related to Weser and Elbe river inputs and to an accumulation of pollutants on the Dogger Bank (Stn 9). The most marked effects were noted at Stn 3 (as compared to Stns 1 & 5) and at Stn 9, as compared to Stn 8, because of the general conditions of water movement in the North Sea which produced considerable roughness at Stns 6, 7 & 8 relative to inputs from the English Channel, or to eddying around Stns 1, 3 & 5 and on the Dogger Bank. The accumulation of various pollutants in this area has been attributed to the currents parallel to the coast of the British Isles entering the central part of the southern North Sea exactly over the Dogger Bank where their speed decreases (Dethlefsen 1988).

The effects observed may indicate the presence of organo-phosphorus or carbamate molecules known to be cholinesterase inhibitors (Olson & Christensen 1980, WHO 1986 a, b, Galgani & Bocquené 1991) or of molecules with a neurotoxic action, although no information is available about the presence of these pesticides in the North Sea. These compounds remain to be identified, and their presence in the German Bight and on Dogger Bank must be explained.

K_m values varied according to the expected dilution gradient of the 'plume' of the Rivers Elbe and Weser. Since an increase in K_m corresponds to a decrease in enzyme affinity, our results probably suggest a modification of the enzyme properties related to the pollution gradient. The most probable explanation might be that the cholinesterases measured are mixtures of isoenzymes of which one or more forms might be inhibited selectively, leaving others of different catalytic properties to predominate in the assay mixture. In that case, the inhibition of cholinesterase activity along the transect could be related to specific inhibition of one or more isozymes. This could only be tested by examining preparations of higher purity and demonstrating changes in isoenzyme patterns. This phenomenon is not uncommon when crude enzyme preparations are used (Low & Somero 1976, Galgani 1985).

Recent enzyme studies have demonstrated the use of the 96-well microplate reader in the routine determination of enzyme activities (Ashour et al. 1987, Bocquené & Galgani 1991). Such assays give results that are sensitive, accurate, adequately reproducible, widely applicable and amenable to extensive data

Table 3. *Limanda limanda*. Variability of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities in dab muscle with sex. Five individuals of each sex of length 12 to 25 cm were sampled at each station; data are expressed in absorbance units per mg protein (mean ± SD). There were no significant differences between different sizes of fish tested (ANOVA, $p = 0.05$). There was no significant difference between males and females at each station except for butyrylcholinesterase activity from samples at Stn 8 (*t*-test, $p < 0.05$)

Stn	Sex	AChE	BChE
6	Male	4549 ± 912	1880 ± 267
	Female	5448 ± 605	2637 ± 463
7	Male	5632 ± 949	2664 ± 687
	Female	4539 ± 293	1862 ± 379
8	Male	5848 ± 468	3093 ± 498
	Female	6954 ± 801	2274 ± 321
9	Male	5097 ± 1080	1728 ± 430
	Female	4807 ± 1083	1989 ± 463

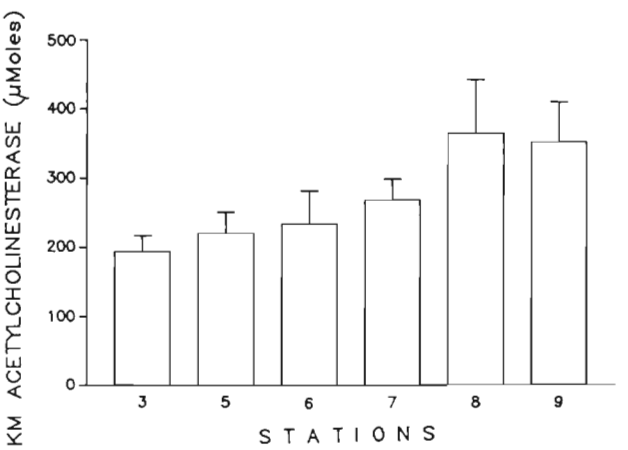


Fig. 6. *Limanda limanda*. K_m values of acetylcholinesterase from dab muscle (mean ± SD of 6, 6, 4, 6, 7 and 6 analyses from Stns 3, 5, 6, 7, 8 & 9, respectively) along the German Bight transect. Michaelis constants were determined in pools of 10 muscles per station. Each determination was performed in duplicate using 4 substrate concentrations. Results are mean values of K_m for which the measurements give a regression line with a correlation coefficient greater than 0.85

analysis. The present work provides an application of the routine determination of kinetic parameters from data collected from plate reader. When using a classical spectrophotometer under the best conditions, an investigator can run no more than one K_m and V_{max} determination simultaneously. Using the plate reader, the same investigator can easily complete 12 determinations in about 10 min with no additional automation. In the context of the workshop, more than 60 determinations were performed in 3 h, indicating the potential of enzyme kinetic measurements made on such a system to be used as an oceanographic monitoring tool.

To our knowledge, this is the first time that cholinesterases have been used at sea as a marker of contaminant effects. The link between enzyme inhibition and fish pathology remains to be established. However, the opportunity to obtain measurements rapidly on board ship makes this technique quite useful for sea-going monitoring, as well as for detecting the effects on marine organisms of chemicals whose analysis is difficult, and whose presence and stability in the marine environment (WHO 1986 a, b, Tully & Morissey 1989) constitute basic questions.

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LITERATURE CITED

Ashour, M. B. A., Gee, S. J., Hammock, B. D (1987). Use of a 96-well microplate reader for measuring routine enzyme activities. Anal. Biochem. 166: 353–360

Bocquené, G., Galgani, F. (1991). Acetylcholinesterase activity in the common prawn (*Palaemon serratus*) contaminated by carbaryl and phosalone. Ecotox. Environ. Safety 22: 337–345

Bocquené, G., Galgani, F., Truquet, P. (1990). Characterisation and assay conditions for use of AChE activity from several marine species in pollution monitoring. Marine environ. Res. 30: 75 -89

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248–254

Coppage, D. L., Matthews, E. (1974). Short-term effects of organophosphate insecticides on cholinesterases of estuarine fishes and pink shrimp. Bull. environ. Contam. Toxicol. 11: 483–488

Dethlefsen, V. (1988). Status report on aquatic pollution problems in Europe. Aquat. Toxicol. 11: 259–286

Ellman, G. L., Courtney, K. O., Andrres, V., Featherstone, R. M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7: 88–95

Galgani, F. (1985). Régulation de l'activité des protéases digestives de *Penaeus japonicus* Bate en relation avec la température. J. exp. mar. Biol. Ecol. 94: 11–18

Galgani, F., Bocquené, G. (1990). *In vitro* inhibition of acetylcholinesterase from four marine species by organophosphate and carbamates. Bull. environ. Contam. Toxicol. 45: 243–249

Galgani, F., Bocquené, G. (1991). Semi-automated colorimetric and enzymatic assays for aquatic organisms using plate readers. Water Res. 25: 147–150

Galgani, F., Cadiou, Y., Bocquené, G. (1991). Routine determination of enzyme kinetics. Biotech. Bioeng. 38: 434 -437

Habig, C., Di Giulio, R. T., Abou-Donia, M. B. (1988). Comparative properties of channel catfish (*Ictalurus punctatus*) and blue crab (*Callinectes sapidus*) acetylcholinesterases. Comp. biochem. Physiol. 91C: 293–300

Habig, C., Di Giulio, R. T., Nomeir, A. A., Abou-Donia, M. B. (1986). Comparative toxicity, cholinergic effects, and tissue levels of S,S,S-Tri-*n*-Butyl Phosphorotrithioate (DEF) to channel catfish (*Ictalurus punctatus*) and blue crabs (*Callinectes sapidus*). Aquatic Toxicol. 9: 193–206

Holland, H. T., Coppage, D. L., Butler, P. A. (1967). Use of fish brain acetylcholinesterase to monitor pollution by organophosphorus pesticides. Bull. environ. Contam. Toxicol. 2: 156–162

Leibel, W. S. (1988). An analysis of esterase activities from surgeonfish tissues yields evidence of an atypical pseudocholinesterase. Comp. biochem. Physiol 91B: 437–447

Low, P. S., Somero, G. N. (1976). Adaptation of muscle pyruvate kinases to environmental temperatures and pressures. J. exp. Zool. 198: 1–12

Olson, D. L., Christensen, G. M. (1980). Effects of water pollutants and other chemicals on fish acetylcholinesterase in vitro. Environ. Res. 21. 327–335

Payne, J. F., Fancey, L., Rahimtula, A., Porter, E. (1987). Review and perspective on the use of mixed function oxygenase enzymes in biological monitoring. Comp. biochem. Physiol. 86C: 233–245

Stebbing, A. R. D., Dethlefsen, V (1992). Introduction to the Bremerhaven Workshop on Biological Effects of Contaminants. Mar. Ecol. Prog. Ser. 91: 1–8

Toutant, J. P., Massoulie, J., Bon, S. (1989). Polymorphism of pseudocholinesterase in *Torpedo marmorata* tissues. J Neurochem. 44: 580–582

Tully, O., Morrissey, D. (1989). Concentrations of dichlorvos in Beitreach Bui Bay, Ireland. Mar. Pollut. Bull. 20: 190–191

van der Wel, H., Welling, W. (1989). Inhibition of acetylcholinesterase in guppies (*Poecilia reticulata*) by Chlorpyrifos at sublethal concentrations: methodological aspects. Ecotoxicol. Environ. Safety 17: 205–219

WHO (1986a). IPCS International Programme on Chemical Safety. Organophosphorus insecticides: a general introduction. Environmental Health Criteria 63. OMS, Genève

WHO (1986b) IPCS International Programme on Chemical Safety. Carbamate pesticides: a general introduction. Environmental Health Criteria 64. OMS, Genève

Zinkl, G. J., Shea, P. J., Nakamoto, R. J., Callman, J. (1987). Brain cholinesterase activity of rainbow trout poisoned by carbaryl. Bull. environ. Contam. Toxicol. 38: 29–35

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Detection of cytochrome P450 1A1 in North Sea dab liver and kidney

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ABSTRACT: Liver and kidney tissue of dab *Limanda limanda* were analyzed during the Bremerhaven Workshop by immunochemical and catalytic assays for cytochrome P450 1A1 induction. Antibodies to cod P450 1A1 cross-reacted with a protein of M_r = 58 000 daltons in the dab liver samples, suggested to be the dab P450 1A1 protein. By employing a recently developed indirect ELISA with these antibodies, we observed elevated levels of P450 1A1 protein in the innermost stations of the German Bight transect in both liver and kidney (head and trunk kidney) samples. Ethoxyresorufin O-deethylase (EROD) activities in trunk kidney were also elevated in the innermost stations. Along the drilling station gradient no induction could be detected. Immuno-detection of the cytochrome P450 1A1 induction response should provide a convenient indicator for biological effects of several classes of organic contaminants.

INTRODUCTION

Among various biological indicators studied to detect effects of marine pollutants on organisms, the cytochrome P450 (CYP or P450) monooxygenase induction response is considered to be of great promise (see e.g. Payne et al. 1987, Goksøyr & Förlin 1992). The response of this system in fish to aromatic and chlorinated hydrocarbons has been studied in a number of laboratory and field studies, traditionally by catalytic assays such as 7-ethoxyresorufin O-deethylase (EROD) or aryl hydrocarbon hydroxylase (AHH). In fish, these activities are believed to represent the catalytic activity of a single CYP form, CYP 1A1 or P450 1A1, a member of the CYP 1A subfamily, being responsive to organic contaminants by gene activation. The induction response can also be studied at other levels, such as with nucleotide probes for measuring mRNA levels (Renton & Addison 1992), or with antibody probes for measuring the translated protein product (reviewed in Goksøyr & Förlin 1992).

Catalytic measurements have certain disadvantages compared to immunodetection of protein levels, including their critical dependence on fresh samples and time and cost effectiveness. Sampling for catalytic determinations demands fresh processing and/or cryopreservation, conditions that may not be easily met in remote areas, far off-shore, or in developing regions of the world. Simple immunoassays can overcome these problems. By measuring the presence of immunoreacting protein with appropriate antibodies, biological integrity and freshness is not critical in this system, and the assay can be automated so that large numbers of samples can be processed and analyzed simultaneously. Such systems are also more easily adapted to low-cost laboratories.

Using polyclonal antibodies against cod *Gadus morhua* P450 1A1 (= cod P-450c; Goksøyr 1985), we have developed a simple, indirect enzyme-linked immunosorbent assay (ELISA) for detecting the P450 induction response in fish samples (Goksøyr 1991). The method has been applied to a large number of samples from both laboratory and field studies, generally showing good correlation with contaminant exposure in different species of fish (Goksøyr et al. 1991a, 1991b, Boon et al. 1992).

Although the induction response is generally studied in liver tissue, other organs are also affected by inducing agents. In some cases, studies of induction in extrahepatic tissue may give additional information to that from liver samples (Payne et al. 1984, Pesonen et al. 1987). The fish kidney is divided into the pronephros (head kidney) and mesonephros (trunk kidney). These organs are important in steroidogenesis,