A retrospective analysis to explore the applicability of fish biomarkers and sediment bioassays along contaminated salinity transects

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Biological-effects monitoring in estuarine environments is complex as a result of strong gradients and fluctuations in salinity and other environmental conditions, which may influence contaminant bioavailability and the physiology and metabolism of the organisms. To select the most robust and reliable biological-effect methods for monitoring and assessment programmes, a large-scale field study was conducted in two estuarine transects in the Netherlands. The locations ranged from heavily polluted harbour areas (the ports of Rotterdam and Amsterdam) to cleaner coastal and freshwater sites. Assessment methods used included a variety of biomarkers in flounder (*Platichthys flesus*) and a range of *in vitro* (sediment extracts) and *in vivo* bioassays. Multivariate statistical analysis was applied to investigate correlations and relationships between various biological effects and contaminant levels in flounder liver or sediments. Several biological methods seemed to be too much affected by salinity differences for routine use in estuaries. The most discriminative biomarkers in the study were hepatic metallothionein content and biliary 1-OH pyrene in fish. Mechanism-based *in vitro* assays DR-CALUX and ER-CALUX applied to sediment extracts for screening of potential toxicity were much more responsive than *in vivo* bioassays with macro-invertebrates using survival as an endpoint.

Keywords: bioassays, biomarkers, estuaries, field study, flounder, multivariate analysis.

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Introduction

Biological-effects monitoring is an important element of programmes that aim to assess the quality of the environment, because it can demonstrate links between bioavailable contaminants and organism health. It can be used not only to identify particular sites and geographic areas where environmental quality is poor, but also to identify substances, or mixtures of substances, that have not been identified previously as harmful. Over

the past few decades, biological-effects research and monitoring programmes have focused mainly on the marine environment. This has resulted in the development and validation of a broad range of biological-effect techniques (e.g. *in vitro* screening tests, *in vivo* bioassays, and biomarkers) to assess marine and coastal contamination (Stebbing *et al.*, 1992a, b; Hylland *et al.*, 2002; Diamant and von Westerhagen, 2003; Thain *et al.*, 2008). Following appropriate quality assurance and control (QA/QC) procedures, several of these techniques have now been adopted in international monitoring programmes, such as the OSPAR Joint Assessment Monitoring Programme (JAMP; Hill *et al.*, 1993; ICES, 2004; Thain *et al.*, 2008). Recently, however, there has been increasing effort to apply these techniques to biota from contaminated estuarine environments (Chapman and Wang, 2001). Estuaries are a notoriously complex environment for biological-effects monitoring and risk assessment, however. Salinity can interfere with the interpretation of the contamination and biological results, either as a factor in its own right or as a confounding factor, obscuring correlations. There are also strong fluctuations and gradients in temperature, pH, dissolved oxygen, redox potential, and particle composition (Chapman and Wang, 2001). The applicability and sensitivity of conventional biomarkers and bioassays in complex estuarine environments have not yet been assessed fully.

The principal objective of this study was to test and compare the available biological-effects monitoring techniques to select the most robust and reliable methods for further use in monitoring programmes for complex estuarine environments. The study area consisted of pollution gradients associated with two east–west regional transects in the Dutch delta, extending from freshwater upstream to saltwater closer to the North Sea. The transects were situated in the Amsterdam and Rotterdam port areas, with major influence from the River Rhine. The study included a wide range of biological responses including fish biomarkers, *in vitro* bioassays, *in vivo* bioassays, and accompanying chemical analysis. Fish biomarkers and gross health indices were measured in flounder, *Platichthys flesus*. This flatfish was used as a sentinel species because (i) it is found in environments ranging from entirely saline to freshwater (it is euryhaline), (ii) it is a bottom-dwelling fish that lives on soft substrata in intimate contact with sediments (Vethaak and Jol, 1996; Matthiessen and Law, 2001), and (iii) it has been selected as a suitable species for use in national and international monitoring of estuarine and marine environments (JAMP, 1998a, b). The biomarkers chosen in flounder were cytochrome P450 1A content (CYP1A), metallothioneins (MT), glutathione-*S*-transferase (GST) activity, superoxide dismutase (SOD) activity, catalase activity (CAT), and several other biomarkers for oxidative stress, vitellogenin (VTG) in plasma of male fish, 1-OH pyrene in bile, acetyl cholinesterase (AChE) activity, and RNA/DNA ratio. *In vivo* sediment bioassays were conducted with invertebrate species such as the amphipod *Corophium volutator*, the burrowing heart urchin *Echinocardium cordatum*, and the polychaete *Arenicola marina*. In addition, a number of *in vitro* screening assays was applied for toxicity characterization of sediment extracts. These included the Microtox Solid Phase® bioluminescence inhibition assay, the reporter gene assays DR-CALUX and ER-CALUX for dioxin-like and oestrogenic effects, respectively, and the recombinant Yeast (O)Estrogen Screen (YES) assay.

A detailed account of the chemical analysis of sediment and flounder liver along the two transects has previously been published by de Boer *et al.* (2001). Results of oestrogenic activity in sediment extracts using the ER-CALUX assay (Legler *et al.*, 2002), and with NADP(H)-dependent radical oxygen species (ROS) biomarkers in flounder (Livingstone *et al.*, 2000), have been published separately, but are included in our overall analysis. Although the large-scale field study was conducted more than a decade ago (1996), we believe that the findings are worth publishing because test protocols are still valid and few data seem to be available in the area.

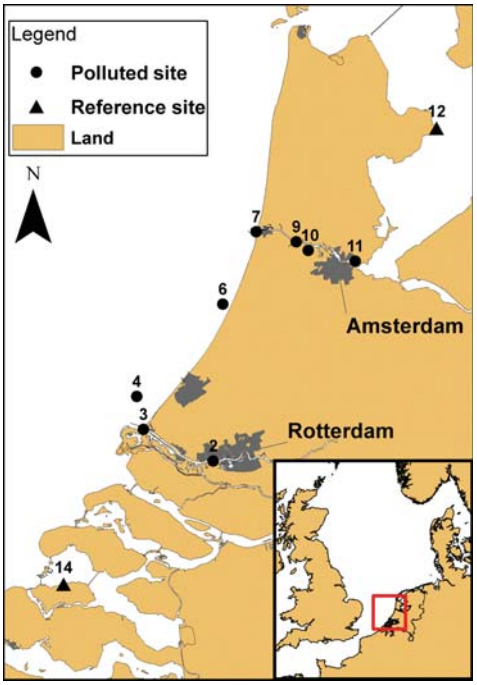

Figure 1. Distribution of sampling sites along two major estuarine areas in the Netherlands; sample locations at Port of Rotterdam transect (Euromonding including the New Waterway shipping lane: site 2 Benelux tunnel; site 3 Splitsingsdam; site 4 Loswal Noord; site 6 Noordwijk) and the Port of Amsterdam (IJmonding including the North Sea Canal: sites 7 IJmuiden Haven; 9 Buitenhuizen; 10 Amerikahaven; 11 Oranjesluis). The two reference sites are 12 Enkhuizen near IJsselmeer (brackish) and 14 Oesterput near Eastern Scheldt (saline).

Material and methods

Study area

The sample locations are shown on Figure 1. Euromonding (Port of Rotterdam) and the North Sea Canal (Port of Amsterdam) are well-known areas of pollution. The sites in the North Sea are situated at the end of the estuarine harbour areas, except for Noordwijk (site 6), which is a typical coastal location. Two relatively clean sites, the Oesterput in the eastern Scheldt estuary (site 14) and IJsselmeer near Enkhuizen (site 12), were also sampled and used as salt- and freshwater reference sites, respectively. In the Port of Rotterdam transect, the salinity gradient is continuous, whereas in the Port of Amsterdam transect, it is interrupted and more complex owing to canalization. The numbers of the sampling sites correspond to the site numbers reported previously for the results of the chemical sediment and flounder liver analyses (de Boer *et al.*, 2001). The transects were sampled from 19 to 26 September 1996, the time of year when flounder

are considered to be indicative of the contamination status of the capture location because they have been there continuously for several months (Vethaak and Jol, 1996).

Sampling

At each site, water, sediment, and flounder (*P. flesus*) tissue were sampled, stored, and transported to various laboratories for analysis. Environmental abiotic factors (e.g. depth, temperature, salinity) were recorded. Sediment samples were collected using a van Veen grab operated from a ship. At each location, 40 l of sediment was sampled and stored in PVC barrels at 4°C. After 2 weeks, they were homogenized for 20 min in an IKA-WERK RW-20 stirrer. From locations presented in Figure 1, all sediments were homogenized, and subsamples were taken and used for chemical and biological analyses. Subsamples were taken and sent by mail to the various laboratories for chemical analysis and testing in bioassays, where they were kept at 4°C until analysis within 2 weeks. Pore-water extracts were prepared as described by Murk *et al.* (1996). Flounder were captured with a fykenet at the same location at which the sediments were collected. Only flounder measuring 20 cm or more were kept and placed in tanks containing water from the sampling sites. After anaesthetization, 30 specimens per site were sacrificed with a blow to the head. This was done within 12 h of capture. The sex, length, body weight, liver weight, and gonad weight of the fish were recorded, and tissue samples were subdivided for chemical and biomarker analyses. Body weight was measured after removal of the viscera. The otoliths of each fish were removed for age determination. The whole brain, blood serum, gonads, bile, muscle tissue, and part of the liver were taken from each fish and stored in liquid nitrogen at −70°C before distribution for biomarker analysis. Two pooled samples of flounder liver from both sexes (numbers 1–15 and 16–30) were used for a few additional biomarkers. The same two pools and one pooled sample of female flounder livers only (numbers 31–45) were analysed chemically for organic contaminants and trace metals, as described by de Boer *et al.* (2001). All samples for biomarker analysis were sent to laboratories inside and outside the Netherlands by express mail. They were stored on dry ice during transportation, except for the formalin-fixed liver samples used for histopathology.

The sediment from Amerikahaven (site 10) contained unexpectedly low contaminant levels during sampling in 1996 (de Boer *et al.*, 2001). This was attributed to repeated dredging activity. The sediment was therefore sampled a second time in September 1997 at a non-dredged site. Analysis of that sediment showed considerably higher levels of contaminants. These results were considered more representative of the location and were therefore used instead of the 1996 data in the multivariate statistical analysis of biomarker data. However, sediment bioassays were conducted with the material collected in 1996, and these data for location number 10 were used for multivariate analysis when sediment chemistry was included.

As part of the field study, relevant QA/QC criteria and guidelines (SETAC, 1993; JAMP, 1998a, b) have to be set to ensure the quality of the data generated during the assessments. The development of QA/QC criteria for this study involved conducting a series of replicate bioassays with each method. Samples tested included a control sediment, contaminated sediments, and reference toxicants. Based on the results of the bioassay replicates, the variability associated with the tests was quantified and we were able to determine what we considered to be acceptable QA/QC criteria for the methods.

Gross health indices, biomarkers, and bioassays

The fish biomarkers and other indices measured during the study are listed in Table 1 along with the acronyms used, the type of biomarker, the substances that induce the biomarker (van der Oost *et al.*, 2003), and the ranges of the number of fish samples analysed per biomarker. Several biomarkers, namely hepatic GST, SOD, and AChE activities in muscle tissue, were analysed by two laboratories. The utility of these biomarkers appeared to be method- rather than laboratory-specific. The units used to present the biomarker results sometimes varied from one laboratory to the other.

The condition factor (CF) and the hepatosomatic and gonadosomatic indices (HSI and GSI) were calculated as follows: $CF = 100 \times \text{body weight (g)}/\text{length}^3$ (cm); $HSI = 100 \times \text{liver weight (g)}/\text{body weight (g)}$; $GSI = 100 \times \text{gonad weight (g)}/\text{body weight (g)}$. References to the methods used for biomarker analysis are summarized in Table 2. Methods with references and expression of units for the *in vivo* bioassays with invertebrate species, *in vitro* bioluminescence, and reporter gene assays used in this study are also presented in Table 2.

Statistical analysis

The univariate response data on all standard biomarker data were analysed, including analysis of variance (ANOVA) for unbalanced design, using Genstat v7.1 statistical software (VSN, 2003). In addition, *a priori* pairwise *t*-tests were performed with the mean reference value, using the pooled variance estimate from the ANOVA. The real-value data were not transformed. The average values for the KMBA and WOP biomarkers were not based on different flounder captured at the sites, but on replicate measurements of pooled liver tissue. The nominal response data of the immunohistochemical biomarkers (classification of effects) were analysed by a Monte Carlo test (Manly, 1997) using the Chi-squared statistic (Snedecor and Cochran, 1967) because of the many low-number (or zero) observations. In the simulations, the number of observations per location was kept fixed. For each test, 10 000 simulations were performed and pairwise comparisons made using this method.

Multivariate techniques are increasingly used to link field concentrations of chemicals with bioassay responses (Ter Braak, 1995; Shaw and Manning, 1996; Del Valls *et al.*, 1997; van den Brink *et al.*, 2003; van den Brink and Kater, 2006). Here, principal component analysis (PCA), a frequently used ordination technique (Ter Braak and Smilauer, 2002), was used to correlate concentrations in sediment and fish with each other and with the responses of biomarkers and bioassays. A PCA triplot was used for exploratory data analysis. As these data were collected in a field-monitoring programme, no conclusions can be drawn as to causality. PCA provides a correlation plot that serves as a graphic summary of the dataset, showing the correlations between all variables. In PCA, imaginary, latent explanatory variables are calculated from the dataset that best explains the variation in concentrations of chemicals in the sediments from the different sampling sites. The first two latent variables are normally used to construct an ordination diagram which provides an overview of mutual correlations between "species" (here, the concentrations of chemicals in sediments or fish) on the one hand and passive "environmental" variables (here, concentrations in fish, bioassay, or biomarker responses) on the other (van den Brink *et al.*, 2003). For a more elaborate description of PCA plot interpretation, the reader is referred to Ter Braak (1995). For further explanation of the use of PCA and related methods to analyse ecotoxicological

Table 1. Gross indices for general health and pollution biomarkers in flounder (*P. flesus*) measured during the estuarine field study in the Netherlands.

Biomarker	Acronym	Type of biomarker	Indicative of	Organ/tissue used in the study	Fish analysed per site
Gross indices					
Condition factor	CF	General condition	Nutritional status and environmental pollutants	Whole animal	17–30
Gonadosomatic index	GSI	Reproduction	Reproductive activity	Gonad (females only)	0–13
Hepatosomatic index	HSI	Liver disease and nutritional status	PCBs, OCPs, BKME, PCDDs, PAHs[a]	Liver	4–30
Liver protein content	LivProt	General condition	Nutritional status and environmental pollutants	Liver	6–15
Biochemical and enzyme markers					
Cytochrome P450 1A content	CYP1A	Phase I biotransformation enzyme	PAHs, PCBs, PCDDs, PCDFs[a]	Liver tissue	6–15
Glutathione-*S*-transferase activity	GST	Phase II biotransformation enzyme	PAHs, PCBs, OCPs, PCDDs[a]	Cytosol liver tissue	6–15
Superoxide dismutase activity	SOD	Oxidative stress parameter	Paraquat, PCDFs, HCB, BKME[a,b]	Liver tissue	1–15
Catalase activity	CAT	Oxidative stress parameter	PCBs, BKME, PAHs, DNOC[b], TCB[a,b]	Liver tissue	0–11
Malondialdehyde content	MDA	Oxidative stress parameter	Lipid peroxidation inducers	Liver tissue	6–15
2-Keto-4-methiolbutyric acid	$KMBA_{cyt}$	Oxidative stress parameter	OCPs, nitroaromatics, PAHs[c]	Cytosol liver tissue	Pooled
2-Keto-4-methiolbutyric acid	$KMBA_{mit}$	Oxidative stress parameter	OCPs, nitroaromatics, PAHs[c]	Mitochondrial fraction liver tissue	Pooled
2-Keto-4-methiolbutyric acid	$KMBA_{micr}$	Oxidative stress parameter	OCPs, nitroaromatics, PAHs[c]	Microsomal fraction liver tissue	Pooled
Western blot of oxidized proteins	WOP	Oxidative stress parameter	Pro-oxidant contaminants	Microsomal fraction liver tissue	Pooled
Metallothionein content	MT	Stress protein	Cd, Cu, Zn, Hg, oxidative stress[a]	Liver tissue	6–15
1-Hydroxy pyrene content	1-OHpyr	Biotransformation product	PAHs[a]	Bile fluid	6–15
Acetyl cholinesterase activity	$AChE_{brain}$	Neurotoxic parameter	Organophosphate and carbamate pesticides[a]	Brain tissue	13–15
	$AChE_{muscle}$		Organophosphate and carbamate pesticides[a]	Muscle tissue	10–15
Vitellogenin content	VTG	Reproductive and endocrine parameter	(Xeno-)oestrogenic compounds	Blood plasma of male fish	0–13
RNA and DNA	RNA/DNA	Growth, metabolic state	Environmental pollutants	Liver tissue	2–12
DNA adduct number	DNAadd	Genotoxic parameter	PAHs and other environmental mutagens[a]	Liver tissue	5–6
Histological markers					
Vacuolation (presence)	Vac	Metabolism, general condition	Environmental pollutants	Liver tissue	16–32
Glycogen content	Glyc	Nutritional status	Environmental pollutants	Liver tissue	16–32
Fibrillar structures (presence)	Fibr	Possible genotoxic parameter	Genotoxic compounds	Liver tissue	16–32
Cytochrome P450 1A (presence)	CYP1Ahis	Phase I biotransformation enzyme	PAHs, PCBs, PCDDs, PCDFs[a]	Liver tissue	16–32

BKME, bleach kraft pulp mill effluent; DNOC, 4,6-dinitro-*o*-cresol.
[a]van der Oost *et al*. (2003).
[b]Inhibition rather than induction by marked compound(s) reported.
[c]Livingstone *et al*. (2000).

Table 2. References for the methods used to measure biomarkers in flounder (*P. flesus*) and to conduct bioassays.

Biomarker	Gross indices	References
Condition factor	CF	Text
Gonadosomatic index	GSI	Text
Hepatosomatic index	HSI	Text
Liver protein content	LivProt	Text
Biochemical and enzyme markers		
Cytochrome P450 1A content	CYP1A	Celander and Forlin (1992)
Glutathione-*S*-transferase activity	GST lab1	Habig *et al.* (1974) and Wilbrink *et al.* (1991)
Glutathione-*S*-transferase activity	GST lab2	Leaver *et al.* (1993)
Superoxide dismutase activity	SODlab3	Sazuka *et al.* (1989)
Superoxide dismutase activity	SODlab4	McCord and Fridovich (1988)
Catalase activity	CAT	Clairbone (1985)
Malondialdehyde content	MDA	Esterbauer and Cheeseman (1990)
2-Keto-4-methiolbutyric acid	$KMBA_{cyt,mit,micr}$	Lemaire and Livingstone (1997) and Livingstone *et al.* (2000)
Western blot of oxidized proteins	WOP_{micr}	Fessard and Livingstone (1998) and Keller *et al.* (1993)
Metallothionein content	MT	Eaton and Toal (1982)
1-Hydroxy pyrene content	1-OHpyr	Ariese *et al.* (2005)
Acetyl cholinesterase activity	$AChE_{brain}$	Ellman *et al.* (1961) and Sturm *et al.* (1999)
Acetyl cholinesterase activity	$AChE_{muscle}$ lab 5	Ellman *et al.* (1961) and Sturm *et al.* (1999)
Acetyl cholinesterase activity	$AChE_{muscle}$ lab 6	Bocquené *et al.* (1990)
Vitellogenin content	VTG	Allen *et al.* (1999a, b)
DNA adduct number	DNAadd	Reddy and Randerath (1986)
Ribonucleic acid	RNA	Munro and Fleck (1966)
RNA/DNA ratio	RNA/DNA	Bradford (1976), Munro and Fleck (1966), Cesarone *et al.* (1979), and Labarca and Paigen (1980)
Histological markers		
Vacuolation (presence)	Vac	Grinwis *et al.* (2000, 2001)
Glycogen content	Glyc	Grinwis *et al.* (2000, 2001)
Fibrillar structures (presence)	Fibr	Grinwis *et al.* (2000, 2001)
Cytochrome P450 1A (presence)	CYP1Ahis	Grinwis *et al.* (2000, 2001)
In vivo bioassays		
C. volutator (10 d)		ASTM (1988) and Schipper and Stronkhorst (1999)
E. cordatum (14 d)		Bowmer (1993) and Schipper and Stronkhorst (1999)
A. marina (10 d)		Thain and Bifield (2001)
C. elegans		Donkin and Williams (1995)
In vitro bioassays		
Microtox SP®		Schipper and Stronkhorst (1999) and Ringwood *et al.* (1997)
Mutatox		SDI (1996) and Schipper and Stronkhorst (1999)
DR-CALUX		Murk *et al.* (1996), Schipper and Stronkhorst (1999), and Stronkhorst *et al.* (2002)
ER-CALUX		Schipper and Stronkhorst (1999) and Legler *et al.* (2002)
YES		Routledge and Sumpter (1996) and Legler *et al.* (2002)

and biomonitoring data, see van den Brink *et al.* (2003). Four PCA analyses were performed to correlate the following:

(i) the sediment concentrations of chemicals at the different sampling sites with the response of biomarkers;

(ii) the sediment concentrations of chemicals at the different sampling sites with the concentrations of chemicals in fish;

(iii) the concentrations of chemicals in fish at the different sampling sites with the response of biomarkers and gross health indices;

(iv) the sediment concentrations of chemicals at the different sampling sites with the response of *in vitro* and *in vivo* bioassays.

In all analyses, chemical concentrations were transformed using a natural logarithm. Concentrations below the limit of detection were replaced by concentrations at half the limit of detection. Parameters with more than two missing data were not included in the analysis (i.e. GSI, Mutatox). As salinity may interfere with the response of biomarkers, it was decided to add the salinity measurements (Table 3) to the chemical data used in the multivariate analysis of biomarker results. This was deemed pointless for the analysis of the bioassay data because either seawater was added during all tests or the animals were exposed to extracts (nematodes). The input for the histopathology and immuno-histochemical parameters was the weighted average effect class per location. The PCA triplot analyses were performed using the software program Canoco for Windows 4.5 (Ter Braak and Smilauer, 2002), by centring and standardizing the concentrations of the chemicals. The results were presented in correlation diagrams (van den Brink *et al.*, 2003). The significance of the correlation between the separate biomarkers and bioassays with the concentrations of the chemicals in the two matrices was tested using Monte Carlo permutation tests applying the redundancy analysis (RDA, a constrained form of PCA) option (van den Brink and Kater, 2006).

Table 3. Average values (± pooled s.e. of the mean) for different health parameters and pollution biomarkers in flounder (*P. flesus*) and salinity at the time of sampling.

Site	Salinity (psu)	Average age (years)	Average weight (g)	Average length (cm)	CF	GSI	HSI	SG	LivProt (mg g^{-1} wet weight)	CYP1A (units mg^{-1} pms protein)	GST (μmol min^{-1} mg^{-1} protein; laboratory 1)	GST (μmol min^{-1} mg^{-1} pms protein; laboratory 2)
2	2.6	2.43	210	27.3	1.03 ± 0.02	1.21 ± 0.22^{ff}	1.56 ± 0.11^{ff}	19.6	121 ± 7^{ssf}	47.7 ± 3.10^{ssff}	0.293 ± 0.043^{f}	0.561 ± 0.031^{f}
3	25.1	2.26	177	25.9	0.97 ± 0.02^{f}	–	1.56 ± 0.25^{f}	11.5	118 ± 7^{ssff}	32.5 ± 3.33^{ss}	0.230 ± 0.054^{ff}	0.399 ± 0.036
4	31.0	2.48	250	29.4	1.01 ± 0.03	–	–	33.9	128 ± 7^{ss}	35.1 ± 3.21^{ss}	0.375 ± 0.054	0.452 ± 0.032
6	31.6	2.37	267	29.1	1.04 ± 0.02	–	1.93 ± 0.14^{s}	45.8	176 ± 7^{ff}	40.3 ± 3.10^{ssf}	0.443 ± 0.042^{s}	0.493 ± 0.031
7	12.0	3.37	229	27.8	1.03 ± 0.02	1.24 ± 0.20^{ff}	1.71 ± 0.09^{ff}	1.1	166 ± 7^{sf}	37.7 ± 3.10^{ss}	0.301 ± 0.042^{f}	0.373 ± 0.031^{f}
9	20.0	3.60	271	29.6	1.04 ± 0.02	1.61 ± 0.23	1.41 ± 0.11^{ff}	8.4	136 ± 11^{ss}	35.0 ± 4.90^{ss}	0.298 ± 0.061^{f}	0.388 ± 0.049
10	20.0	4.26	216	27.9	0.98 ± 0.02^{f}	1.28 ± 0.51	1.24 ± 0.10^{sff}	−16.2	138 ± 7^{ss}	51.3 ± 3.10^{ssff}	0.327 ± 0.049	0.415 ± 0.031
11	5.0	4.17	218	27.2	1.02 ± 0.02	1.91 ± 0.27	1.32 ± 0.11^{ff}	−14.6	155 ± 7^{ss}	34.8 ± 3.10^{ss}	0.374 ± 0.057	0.322 ± 0.031^{ff}
12Fref	<2.0	3.57	209	27.0	1.03 ± 0.02	2.03 ± 0.24	2.04 ± 0.09^{ss}	–	143 ± 7^{ss}	32.4 ± 3.33^{ss}	0.438 ± 0.047^{s}	0.472 ± 0.033
14Sref	30.8	3.22	245	28.7	1.01 ± 0.02	1.58 ± 0.27	1.57 ± 0.11^{ff}	–	182 ± 7^{ff}	16.9 ± 3.10^{ff}	0.316 ± 0.042^{f}	–

Site	SOD mean (U mg^{-1} protein; lab 3)	Total SOD (U mg^{-1} pms protein; lab 4)	CAT (ng min^{-1} mg protein)	MDA (nmol TMA mg^{-1} protein)	$KMBA^{a}_{cyt}$ (pmol ethylene min^{-1} m^{-1} protein)	$KMBA^{a}_{mit}$ (pmol ethylene min^{-1} mg^{-1} protein)	$KMBA^{a}_{micr}$ (pmol ethylene min^{-1} mg^{-1} protein)	WOP^{a}_{micr} (AU)
2	132 ± 12^{s}	40.1 ± 6.5	–	5.67 ± 0.88^{ssf}	95.9 ± 9.90^{s}	983 ± 126	46.7 ± 9.91^{ss}	169 ± 6.99
3	136 ± 15^{s}	57.8 ± 7.2^{s}	85 ± 13	5.61 ± 0.92^{ssf}	123.3 ± 9.90^{ssff}	1856 ± 126^{ssff}	55.2 ± 9.91^{ss}	174 ± 6.99
4	125 ± 15	66.7 ± 6.9^{ssf}	100 ± 12	4.34 ± 0.96	95.1 ± 9.90^{s}	1843 ± 126^{ssff}	112.2 ± 9.91^{ff}	165 ± 6.99
6	123 ± 12	39.6 ± 6.5	–	2.72 ± 0.92	113.9 ± 9.90^{ssff}	2425 ± 126^{ssff}	135.0 ± 9.91^{ssff}	160 ± 6.99
7	115 ± 12^{f}	79.0 ± 25.0	109 ± 11	2.14 ± 0.82	71.7 ± 9.90	1190 ± 126	55.4 ± 9.91^{ss}	156 ± 6.99
9	121 ± 18	49.4 ± 11.2	99 ± 14	6.23 ± 1.30^{ssf}	77.8 ± 9.90	1264 ± 126^{sf}	31.7 ± 9.91^{ss}	193 ± 6.99^{ssff}
10	159 ± 14^{ss}	64.8 ± 7.2^{ssf}	98 ± 12	7.25 ± 0.82^{ssff}	91.8 ± 9.90	1990 ± 126^{ssff}	80.2 ± 9.91^{f}	165 ± 6.99
11	127 ± 16	62.6 ± 6.5^{s}	138 ± 13^{ssf}	2.10 ± 0.82	85.8 ± 9.90	1121 ± 126	127.0 ± 9.91^{sff}	168 ± 6.99
12Fref	148 ± 14^{ss}	48.2 ± 6.9	109 ± 11	3.28 ± 0.88	74.5 ± 9.90	934 ± 126	52.9 ± 9.91^{ss}	165 ± 6.99
14Sref	100 ± 12^{ff}	41.7 ± 6.5	87 ± 11	2.41 ± 0.82	70.8 ± 9.90	928 ± 126	96.0 ± 9.91^{ff}	157 ± 6.99

Site	MT (μg mg^{-1} pms protein)	1-OHpyr (ng ml^{-1})	$AChE_{brain}$ (ng min^{-1} mg^{-1} protein)	$AChE_{muscle}$ (ng min^{-1} mg^{-1} protein)	$AChE_{muscle}$ (U min^{-1} mg^{-1} protein)	VTG in males (μg ml^{-1})	RNA/protein (μg ng^{-1})	RNA/DNA ratio (μg $μg^{-1}$)	DNAadd (nr. 10^{-8} nucleotides)
2	1.55 ± 0.18^{f}	50 ± 29	128 ± 5^{ssff}	54 ± 5^{ssff}	8347 ± 941	0.1 ± 426^{f}	0.116 ± 0.077	9.19 ± 1.67	13.0 ± 3.2
3	1.76 ± 0.19^{sff}	70 ± 37	105 ± 5	91 ± 5	9458 ± 941	0.1 ± 426^{f}	0.140 ± 0.071	6.86 ± 0.83^{ss}	14.0 ± 3.2
4	1.99 ± 0.17^{ssff}	37 ± 45	105 ± 5	60 ± 5^{ssff}	6949 ± 941^{sf}	–	0.118 ± 0.032	7.96 ± 0.89	20.1 ± 3.5^{s}
6	1.40 ± 0.17	32 ± 33	127 ± 5^{ssff}	65 ± 5^{ssff}	7738 ± 974	551 ± 337	0.138 ± 0.167	7.52 ± 0.71^{s}	15.2 ± 3.2
7	2.14 ± 0.17^{ssff}	365 ± 31^{ssff}	83 ± 5^{sf}	61 ± 5^{ssff}	7537 ± 941	1.8 ± 337^{f}	0.078 ± 0.054	5.83 ± 0.75^{ss}	15.4 ± 3.2
9	2.27 ± 0.26^{ssff}	79 ± 35	71 ± 5^{ssff}	60 ± 5^{ssff}	10 558 ± 1214	0.9 ± 476^{f}	0.094 ± 0.047	6.62 ± 0.79^{ss}	14.4 ± 3.2
10	1.37 ± 0.17	117 ± 39^{sf}	87 ± 5^{ff}	72 ± 5^{ssff}	9295 ± 974	2.2 ± 476^{f}	0.088 ± 0.035	6.22 ± 0.75^{ss}	15.8 ± 3.2
11	2.61 ± 0.17^{ssff}	103 ± 45^{f}	75 ± 5^{ssff}	86 ± 5	9800 ± 941	0.4 ± 476^{f}	0.098 ± 0.030	7.73 ± 0.96	16.3 ± 3.5
12Fref	1.07 ± 0.18	7.9 ± 31	109 ± 5^{s}	91 ± 5	9290 ± 1010	962 ± 318^{s}	0.050 ± 0.021	6.64 ± 0.83^{ss}	17.3 ± 3.5
14Sref	1.18 ± 0.17	21 ± 29	97 ± 5^{f}	90 ± 5	9393 ± 974	0.2 ± 264^{f}	0.069 ± 0.023	9.51 ± 0.68^{ff}	11.0 ± 3.2

Fref, freshwater reference site; Sref, saltwater reference site.
[a]Pairwise comparison based on replicate measurements rather than replicate fish.
[s]Significantly different from saltwater reference site (Sref) at $p < 0.05$; [ss]significantly different from Sref at $p < 0.01$.
[f]Significantly different from freshwater reference site (Fref) at $p < 0.05$; [ff]significantly different from Fref at $p < 0.01$.

Results

Salinity and contaminants in sediments and flounder

The salinity of the water above the sediments during sampling at each site is shown in Table 3. As expected, the salinity gradient was less distinct in the Port of Amsterdam transect than in the Port of Rotterdam transect. The concentrations of contaminants in sediments and flounder liver as previously published by de Boer *et al.* (2001) are summarized in Table 4. The highest levels of polychlorinated biphenyls (PCBs), several organochlorine pesticides (OCPs), polybrominated diphenylethers (PBDEs), and organotins (e.g. TBT and TPhT) were found in the industrialized harbour areas in the low-salinity segment of each transect. The contamination level generally decreased and salinity increased towards the marine end of the estuaries, but there are specific differences between the two transects. For example, fairly high concentrations of PBDE and cadmium were found in flounder liver from the Port of Rotterdam, and elevated levels of PAH were found in sediments at most sites from the Port of Amsterdam transect (Table 5).

Gross health indices in flounder

Table 3 lists the results of the biological parameters and gross health indices of flounder captured at the different sites. There were considerable differences in the age compositions of the flounder >20 cm. Flounder from the sites in the Port of Amsterdam transect were older than those from the Port of Rotterdam transect (average age range 3.6–4.4 and 2.3–2.5 years, respectively). The Splitsingsdam location (site 3) yielded not only the youngest specimens but, compared with the reference sites, the flounder with the lowest weight and lowest average length. Compared with the freshwater reference site (IJsselmeer near Enkhuizen; site 12), average CFs were slightly but significantly reduced at two polluted sites, sites 3 (Splitsingsdam) and 10 (Amerikahaven). In general, the flounder seemed to have a fairly similar average CF and none of the locations deviated from the saltwater reference site in the eastern Scheldt estuary.

The average GSI of female flounder and HSI of both sexes at the saltwater and freshwater reference locations differed significantly from each other ($p < 0.01$). Average GSI was significantly lower at sites 2 (Benelux tunnel) and 7 (IJmuiden), but only compared with the freshwater reference site where flounder had the greatest average GSI of all locations where gonad weights were measured (no samples at sites 3, 4, and 6). The difference in average HSI between the two reference locations was also significant ($p < 0.01$). Average HSI values at all other sites also differed significantly from one or both references, although those index values were all within the range between the two reference sites.

Biomarkers in flounder

The average values of the biomarker measurements in flounder are also listed in Table 3. For several of the biomarkers, there was a distinct and significant difference between the average values at the saltwater and freshwater reference sites. This was the case for CYP1A, protein content in the liver, GST measured by lab 1 (GST at the saltwater reference site was not measured by lab 2), SOD analysed by lab 3, microsomal 2-Keto-4-methiolbutyric acid ($KMBA_{mic}$), RNA/DNA ratio (differences with $p < 0.01$), $AChE_{brain}$, and VTG in male fish (all with $p < 0.05$). For those biomarkers, parameter values at non-reference sites were rarely significantly different from both freshwater and saltwater reference sites, although some differences were expected. The first exception was CYP1A in the liver, which still showed a significant increase at three other sites (site 2, Benelux tunnel; site 6, Noordwijk; site 10, Amerikahaven) compared with both (significantly different) reference sites. The second exception was $AChE_{brain}$, but both significant increases and significant decreases compared with the two references were observed for this biomarker.

With only one freshwater and one saltwater reference site, biomarkers that did not seem to be affected by the differences in salinity and showed significant and unambiguous responses at several non-reference sites were GST2, SOD3 (one of two laboratories), MDA, cytosolic and mitochondrial KMBA, western blot of microsomal oxidized proteins (WOP), MT, 1-OH pyrene, AChE in muscle tissue (two laboratories), and DNA adducts. The average VTG content of flounder varied considerably from site to site, but the difference between the reference sites was large and significant. However, for VTG, the variation was three orders of magnitude greater than the mean. Some biomarkers showed limited power to discriminate between polluted and less polluted sites (i.e. a significant response at just one polluted location). These were DNA adducts, $AChE_{muscle}$, CAT, and WOP. However, for the latter two, the increases are highly significant ($p < 0.01$) and occurred at two of the most polluted sites (11 and 9, respectively; see Table 4).

The results of the histopathological and immunohistochemical biomarker analyses are shown in Table 5. Vacuolation of the liver tissues differed significantly between the saltwater and freshwater reference sites. The average degree of vacuolation was highest at the saltwater site. Compared with this location, vacuolation was significantly lower ($p < 0.05$) at sites 4 (Loswal Noord), 9 (Buitenhuizen), and 11 (Oranjesluis). Glycogen content barely differed between sites. Only flounder from site 10, Amerikahaven, had a somewhat greater quantity of PAS positive material in their hepatocytes. Fibrillar structures in hepatocytes did not differ significantly between sampling locations. Average immunoreactivity against CYP1A was low in flounder from all sites, except at North Sea Canal sites 10 (Amerikahaven) and 11 (Oranjesluis), where the frequency of higher classes was significantly higher than at either reference site. Site 10 was also the site with the greatest average CYP1A content in flounder liver homogenate.

In vivo bioassays

Most of the *in vivo* bioassays conducted with the whole sediments from the locations in the study showed barely any differences between sites (Table 6). Endpoints and tests that appeared particularly indiscriminative were survival of *C. volutator* (in tests performed by two laboratories), survival of *A. marina*, and survival and reburrowing behaviour of field heart urchin *E. cordatum*. The total number of casts produced by *A. marina* during the bioassays varied greatly between sediments from different locations, and also between the two reference sites. The same was found for the average dry weight of *C. volutator* at the end of the 10-d experiments. Most *in vivo* whole sediment bioassays were not replicated, so could not be tested statistically. Significant reproductive effects with the nematode *C. elegans* were observed at sites 2, 3, 6, 7, 9, and 11 for female nematodes exposed to sediment extracts for 24 h before egg-laying. The magnitude of the effects followed the degree of contamination on the different gradients. Interestingly, although the total number of offspring was not significantly less at location 11 (Oranjesluis in the Port of Amsterdam), their development typically halted at stage J3, and was not completed.

Table 4. Summary of chemicals measured in the sediments and flounder (*P. flesus*) liver (pooled) from the Port of Rotterdam and Port of Amsterdam sites (according to de Boer *et al*., 2001).

		Site										
Chemical	**Unit**	**2**	**3**	**4**	**6**	**7**	**9**	**10**	**10[a]**	**11**	**12**	**14**
Sum organotins (MBT, DBT, TBT, MPT, DPT, TPhT)	μg Sn kg^{-1} dry weight	106	110	52	43	237	170	14	73	206	8	12
Cu	mg kg^{-1} dry weight	47	38	21	20	77	40	18	140	129	41	10
As	mg kg^{-1} dry weight	16	15	14	17	29	17	11	29	41	14	13
Cd	mg kg^{-1} dry weight	2.34	1.46	0.88	0.81	1.92	0.90	0.24	3.02	3.16	2.90	0.38
Hg	mg kg^{-1} dry weight	0.69	0.53	0.34	0.30	2.43	0.54	0.08	–	1.24	0.65	0.16
Cr	mg kg^{-1} dry weight	110	93	92	100	142	76	78	109	121	75	61
Zn	mg kg^{-1} dry weight	295	231	162	238	587	269	88	648	719	422	90
Ni	mg kg^{-1} dry weight	28	27	21	20	41	37	35	41	50	25	15
Pb	mg kg^{-1} dry weight	72	69	57	88	272	85	34	255	234	72	30
Sum 13 PAHs (Ant, BaA, Bap, BbF, BeP, BghiPe, BkF, Chr, dBahA, Fen, Flu, Pyr, InP)	mg kg^{-1} dry weight	6 132	4 343	4 774	1 313	11 463	7 726	673	11 269	11 660	2 013	2 265
Sum 7 PCBs (28, 101, 118, 138+163, 153, 180)	μg kg^{-1} dry weight	89.5	54.5	38.2	23.7	57.6	33.4	6.8	82.1	84.6	41.4	23.7
Sum 28 PCBs (28, 31, 44, 47, 49, 52, 56, 66+95, 87, 97, 99, 101, 105, 110, 118, 128, 137, 138,+163, 141, 149 151, 153, 156, 170, 180, 187, 194, 202, 206)	μg kg^{-1} lipid weight	11 545	6 596	3 115	1 635	2 927	6 587	5 455	n.a.	6 173	1 578	2 081
Sum OCPs (Aldrin, Endrin, Dieldrin, Lindane gamma Heptachlor epoxide, Hexachlorobenzene)	μg kg^{-1} lipid weight	392.4	242.6	184	148.8	152.2	218.3	159	n.a.	111	92.1	136.5
Cd	μg kg^{-1} lipid weight	0133	0.054	0.107	0.065	0034	0.031	0.028	n.a.	n.a.	0.006	0.075
Cr	μg kg^{-1} lipid weight	0.005	0.02	0.005	0.005	0.07	0.005	n.a.	n.a.	n.a.	0.005	0.002
Hg	μg kg^{-1} lipid weight	0.18	n.a.	0.06	0.03	0.05	0.05	n.a.	n.a.	n.a.	0.015	0.16
Zn	μg kg^{-1} lipid weight	50	n.a.	66	43	40	41	n.a.	n.a.	n.a.	29	51
Sum of brominated flame retardants (2,4,2,4′-TBDE, 2,4,5,2,4′-PeBDE)	μg kg^{-1} lipid weight	304	149	<62	<36	<44	81	<62	n.a.	123	<40	<17

n.a., not analysed.
[a]Sediment was sampled a second time at a non-dredged site in this harbour.

Table 5. Frequency distribution of numbers of flounder livers in histological-effect classes.

	Vacuolation					Glycogen content					Fibrillar structures				CYP1A				
Site	**0**	**1**	**2**	**3**		**0**	**1**	**2**	**3**		**0**	**1**	**2**	**3**	**0**	**1**	**2**	**3**	
2	13	1	7	6		15	4	6	2		23	1	2	1	27	0	0	0	
3	9	1	4	10		15	1	5	3		23	1	0	0	24	0	0	0	
4	15	0	2	13	s	16	2	6	6		29	0	1	0	27	2	1	0	
6	15	1	6	8		16	4	3	7		29	0	0	1	30	0	0	0	
7	9	0	5	10		11	1	6	6		23	0	1	0	23	1	0	0	
9	12	0	2	11	s	13	7	4	1		24	0	1	0	25	0	0	0	
10	14	0	8	7		15	0	4	10	s	28	0	1	0	18	4	7	0	f
11	15	3	4	5	s	18	5	3	1		26	0	1	0	20	7	0	0	f
12Fref	17	0	1	14	ss	19	0	11	2		32	0	0	0	32	0	0	0	
14Sref	1	0	6	9	ff	7	5	4	0		15	0	1	0	16	0	0	0	

An explanation of the classes is given in text. s, significantly different from saltwater reference site (Sref) at $p < 0.05$; ss, significantly different from Sref at $p < 0.01$; f, significantly different from freshwater reference site (Fref) at $p < 0.05$; ff, significantly different from Fref at $p < 0.01$.

In vitro bioassays

The bacterial Microtox tests and the other *in vitro* bioassays clearly indicated differences in sediment toxicity between locations (Table 7). In the Microtox SP® assay, inhibitory effects were found in sediment extracts from the Port of Amsterdam transect (i.e. TU values >20 at sites 7 and 11). The highest response in the Mutatox® assay was at Oranjesluis (site 11) in the Port of Amsterdam. The reference values from the Mutatox® assay at that site were below the detection limit. A high response was also found at Oranjesluis (site 11) and Buitenhuizen (site 9), with the 6 h (indicating toxic PAHs) and 48 h (indicating dioxin-like toxicity) results of the DR-CALUX. Other locations where toxic equivalent (TEQ) values in the 6, 24, and 48 h DR-CALUX were greater than the saltwater references were at Splitsingsdam (site 3) and Buitenhuizen (site 9). EEQs in the sediment extracts measured with the ER-CALUX varied by almost one order of magnitude. The greatest oestrogenic activity (EEQ >10 pmol g dry weight^{-1}) was found in sediments from the interregional locations at the Port of Rotterdam (sites 2 and 3), Noordwijk (site 6), and IJmuiden Harbour (site 7). EEQs in the sediment extracts measured using the YES were an order of magnitude higher than the references at locations Splitsingsdam (site 3) and IJmuiden Harbour (site 7), but lower at Loswal Noord (site 4), Buitenhuizen (site 9), and Amerikahaven (site 10).

Multivariate analysis

The results of the PCA of internal pollutant concentrations in flounder liver at the sampling locations plotted against the concentrations of the same substances in the sediments are shown in Figure 2. There is a clear distinction between the more polluted sites on the right site of the triplot and the cleaner locations on the left. Figure 2 also reveals that the pattern of most individual PCB congeners in flounder liver is strongly correlated with the distribution of the PCBs in the sediment. The only exception to this is PCB105. There is a weaker correlation between the concentrations of HCB in flounder liver and measured concentrations of these contaminants in the sediments, and none at all for metals (Zn, Cd, Cr, and Hg).

A number of biomarkers correlated to some extent with the chemical compounds that were measured in flounder liver tissue. Those explaining >10% of the variance are shown in Figure 3. Biomarkers and health parameters that correlated significantly ($p < 0.10$)—either positively or negatively—with contamination were HSI ($p = 0.015$), LivProt ($p = 0.027$), GST (measured by laboratory 1; $p = 0.058$), MDA ($p = 0.076$), WOP_{micr} ($p = 0.079$), and VTG ($p = 0.080$). There was no correlation between VTG values in male fish and the sediment oestrogenic activity. HSI, LivProt, and GST decreased with higher concentrations of many higher PCBs. MDA and WOP_{micr} were positively correlated with high body burdens of lower PCBs and several other organochlorine contaminants.

Figure 4 shows a similar triplot of biomarkers and health indices at the various sites against sediment concentrations of measured contaminants. Biomarkers and health indices that correlated significantly ($p < 0.10$) with pollutants in the sediments were 1-OH pyrene ($p = 0.022$), MT ($p = 0.045$), HSI ($p = 0.053$), SOD (laboratory 4; $p = 0.066$), CYP1A ($p = 0.070$), and CYP1Ahis ($p = 0.098$). These parameters also correlated with the degree of contamination in the sediments. CYP1A and CYP1Ahis corresponded well with higher levels of PCBs (and with each other) and 1-OH pyrene with high levels of PAHs. MT correlated with high levels of organotin compounds but not with metals such as Zn and Cd.

The flounder health indices and biomarkers in multivariate analysis that explained <10% of the observed variance were CF (Figures 3 and 4), GST (laboratory 2; Figure 3), SOD (measured by laboratories 3 and 4; Figure 3), CAT (Figure 3), MDA (Figure 4), KMBA (mitochondrial, microsomal, and in cytosol; Figures 3 and 4), WOP_{micr} (Figure 4), 1-OH pyrene (Figure 3), $AChE_{muscle}$ (laboratories 5 and 6; Figure 4), $AChE_{brain}$ (Figure 3), RNA/DNA (Figure 3), DNAadd (Figures 3 and 4), Glyc (Figures 3 and 4), Fibr (Figure 4), and CYP1Ahis (Figure 3).

Figure 5 displays the PCA triplot for the sediment bioassay responses against the measured concentrations of pollutants in sediments. Most *in vivo* bioassays correlate negatively with pollutants in a general sense, i.e. survival (*C. volutator*), number of casts (*A. marina*), and reburrowing behaviour (*E. cordatum*) increase with decreasing contamination, although not significantly. The bioassay *C. volutator* showed a slightly decreased survival rate at site 11, associated with sediment containing high concentrations of organotin, PAH, and PCB. The survival of *E. cordatum* correlates significantly ($p = 0.031$) with increasing contamination, although survival at all sites was barely affected, if at all (Table 6). The heart urchin showed only a slightly decreased survival rate at site 10, which is associated with sediment contamination by PCBs (Figure 5).

Table 6. Results of *in vivo* bioassays with sediment or sediment pore water from estuarine and coastal locations in the Netherlands and at fresh- and saltwater reference sites.

	C. volutator			*A. marina*		*E. cordatum*		*C. elegans*	
Site	Average survival (%) lab 7	Average dry weight (µg ind.$^{-1}$) lab 7	Average survival (%) lab 8	Average survival (%)	Average total cast number	Average14-d survival (%)	Average dug in after 30 min (%)	Average number of offspring per female (s.d.)	*n*
2	–	–	100	100	20	100	100	43.0** (13.9)	12
3	93	514	100	90	12	100	100	50.8** (6.7)	11
4	82	513	100	100	35	100	100	57.5 (10.2)	12
6	85	522	100	90	32	100	100	51.5* (12.5)	12
7	94	437	100	100	37	100	100	31.7*** (11.0)	11
9	94	337	97	100	10	100	100	36.9*** (11.2)	12
10	94	394	97	100	28	80	100	40.9** (11.9)	12
11	80	365	93	80	10	100	95	54.4 (13.7)	11
12Fref	83	399	100	80	2	–	–	66.3 (20.2)	12
14Sref	97	512	100	100	36	100	100	62.0 (10.5)	12

*Significant effect ($p < 0.05$) compared with the control.
**Significant effect ($p < 0.01$) compared with the control.
***Significant effect ($p < 0.001$) compared with the control.

Table 7. Results of Microtox tests and *in vitro* bioassays.

			DR-CALUX (with clean-up dry sediment)			Pore water	ER-CALUX on polar fraction sediment	YES on 10× diluted sediment
Site	MSP (TU)	Mutatox [average LOEC (mg ml^{-1})]	6 h TEQ (pg g^{-1})	24 h TEQ (pg g^{-1})	48 h TEQ (pg g^{-1})	24 h TEQ (pg ml^{-1})	Average 24 h-EEQ ± s.d. (pmol g^{-1} dry weight)	Average EEQ ± s.d. (pmol g^{-1} dry weight)
2	<20	31.8	2.5	16	23	3.6	38.4 ± 9.54	0.213 ± 0.001
3	<20	10.5	16	78	79	2	22.0 ± 4.54	0.533 ± 0.012
4	<20	47.8	2.6	10	dl	3.1	6.42 ± 1.51	0.160 ± 0.002
6	<20	191	0.24	3	dl	2.4	15.0 ± 5.68	0.284 ± 0.006
7	12	65.4	7	23	15	5.7	27.1 ± 10.1	0.593 ± 0.007
9	<20	dl	32	87	65	6.7	5.44 ± 1.46	0.213 ± 0.002
10	<20	dl	2.7	12	9	1.9	7.14 ± 2.94	0.160 ± 0.002
11	24	2.6	119	31	135	5.9	6.02 ± 1.20	0.309 ± 0.007
12Fref	44	dl	8.1	36	37	1.7	7.46 ± 0.56	0.267 ± 0.003
14Sref	<20	dl	3.9	29	20	1.2	4.86 ± 0.8	0.309 ± 0.006

dl, detection limit.

The results of the Microtox SP® assay did not correlate significantly with sediment contamination. However, individual sediment response results at sites 7 and 11, showed a moderate response. The results for DR-CALUX correlated significantly with the sediment concentrations ($p = 0.011$), especially with PAHs, organotins, and some metals (Figure 5). There was a difference between the correlations of the responses in the DR-CALUX after 6, 24, and 48 h of incubation with sediment extract at sites 3, 9, and 11, or pore-water extracts at all sites. This suggests that PAHs are dominant in the sediment extract and that the cells were not able to metabolize them within 24 h. The ER-CALUX response increased with rising levels of the more chlorinated PCB congeners in the sediment, but this was not significant ($p = 0.205$). The YES assay did not correlate with PCB congeners in the sediment.

Discussion

Contaminants in sediments and flounder

The Port of Amsterdam transect yielded four locations in the estuarine system with highly polluted sediment (sites 7, 9, 10, and 11). The chemical concentrations there were higher than at the sites in the Port of Rotterdam transect (Table 4). PCB and trace metal concentrations in Rotterdam harbour sediments and TBT, TPhT, and PAH concentrations in all sediments exceeded the Dutch maximum permissible concentration environmental quality standard [the concentration that theoretically protects 95% of the species in ecosystems (de Boer *et al.*, 2001)]. In Euromonding, including the New Waterway shipping lane (Figure 1), the lower concentrations of contaminants found may have been the consequence of intensive dredging, because nearly 20 million cubic metres of sludge is disposed of in the open sea each year. Moreover, tides appeared to transport sediment-associated contaminants westwards (Stronkhorst *et al.*, 2003a).

The contamination ranking from high to low sediment concentrations of metals (copper, zinc, mercury, chromium, nickel, and lead), sum PCB, and sum PAH in the Port of Amsterdam locations was as follows: Oranjesluis (site 11), IJmuiden (site 7), Amerikahaven (site 10; 1997), and Buitenhuizen (site 9). De Boer *et al.* (2001) concluded that in flounder liver, the highest concentrations of PCBs, several OCPs, and PBDEs were found at the inland side of the Port of Amsterdam and Port of Rotterdam transects (Table 4). Although the relative concentrations may be

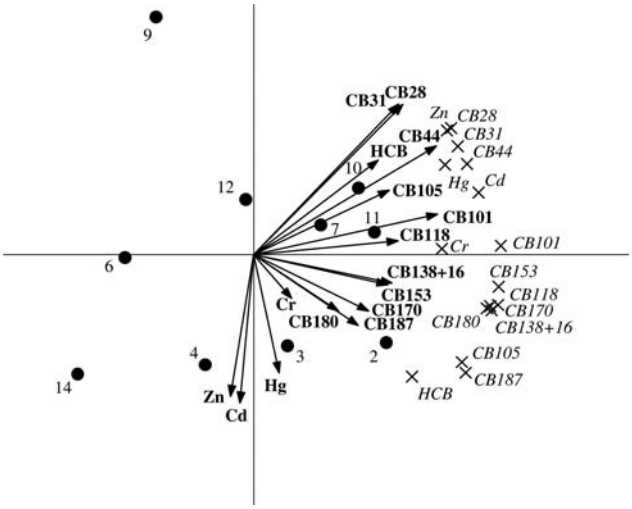

Figure 2. PCA triplot showing the correlation between chemical contaminants in estuarine sediments and their levels in flounder ("environmental" variables). The horizontal first axis displays 74% of the variation in contaminants in the sediment, the vertical second axis another 12%. Contaminants in flounder, arrows; contaminants in sediments, crosses; sampling locations, filled circles. Abbreviations of substances are provided in the Appendix.

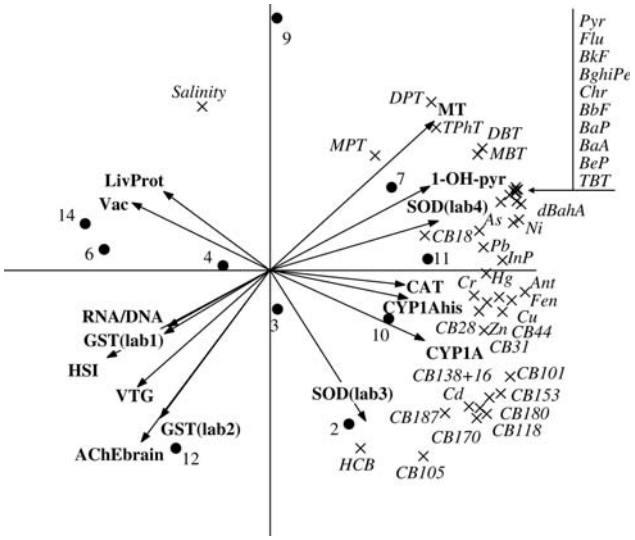

Figure 4. PCA triplot showing the correlation between chemical contaminants in sediments (and salinity) and the response of the biomarkers. 12% of the total variance was captured by the covariable age. The horizontal first axis displays 65% of the remaining variation in contaminants in the fish, the vertical second axis another 14%. Only biomarkers that explained 10% or more of the total variance are shown. Biomarkers, arrows; contaminants in flounder, crosses; sampling sites, filled circles. Abbreviations of substances are provided in the Appendix.

inferred from the location on the PCA plots, PCA triplot calculations express the relative covariation between variables, not their absolute value. Overall, the PCA triplot revealed a positive correlation between sediment contamination and the body burden in flounder at the sites (Figure 2). The PCB concentrations in most sediments correlate weakly with body burden in pooled flounder livers (correlation coefficient, $r^2 = 0.404$). Interestingly, the highest levels of PCB contamination in sediments were at the Amsterdam transect locations IJmuiden (site 7), Buitenhuizen (site 9), Amerikahaven (site 10), and Oranjesluis (site 11), whereas the body burdens from Buitenhuizen and IJmuiden were higher and lower, respectively, than the matched sediments. The PCA triplot (Figure 2) clearly shows that flounder caught at Buitenhuizen (site 9) showed no correlation between sediment contamination and body burden, indicating that flounder may be locally migratory and that those sampled at Buitenhuizen (site 9) are probably not representative of the location, but possibly migrants from the more highly polluted locations IJmuiden (site 7) or Oranjesluis (site 11). PCBs and trace metal concentrations in flounder liver were not higher than the literature data (Kopecka *et al.*, 2006). PCBs accumulate in fatty tissues such as the liver. As the levels of most individual PCB congeners closely reflected the pattern in the sediments, equilibrium between flounder and their surroundings may be readily achieved for these substances. PCB105, the only congener measured whose levels in sediment and flounder corresponded less well, is readily metabolized in fish (Boon and Duinker, 1985). In our study, organic contaminants and metal concentrations were measured in liver tissue. The correlation of liver contamination with sediment contamination was much better for PCBs than for metals and HCB. For metals, this may be caused by the fact that contaminants were only measured in flounder livers; certain metals may accumulate more efficiently in other tissues, such as the kidneys and gills (Camusso *et al.*, 1995; Storelli and Marcotrigiano, 2001).

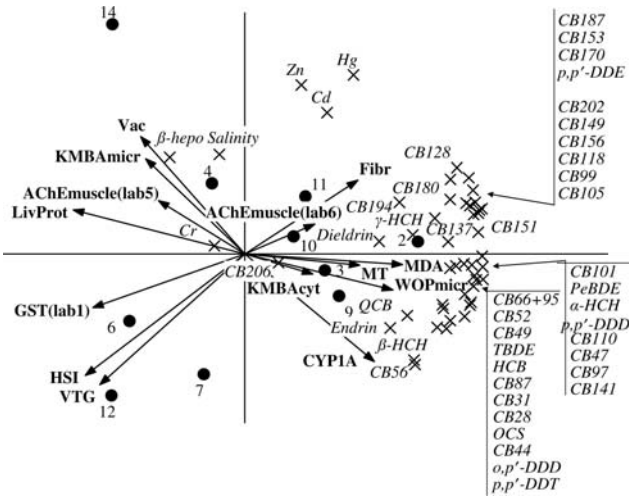

Figure 3. PCA triplot showing the correlation between chemical contaminants in flounder liver (and salinity) and the response of the biomarkers. 12% of the total variance was captured by the covariable age. The horizontal first axis displays 74% of the remaining variation in contaminants in the fish, the vertical second axis another 9%. Only biomarkers that explained 10% or more of the total variance are shown. Biomarkers, arrows; contaminants in flounder, crosses; sampling locations, filled circles. Abbreviations of substances are provided in the Appendix.

Gross indices in flounder

Gross indices of health, such as GSI (high values indicate increased reproductive activity) and HSI (high values indicate good nutritional status, but this may also be caused by increased liver activity caused by exposure to organic pollutants), and CF (high values

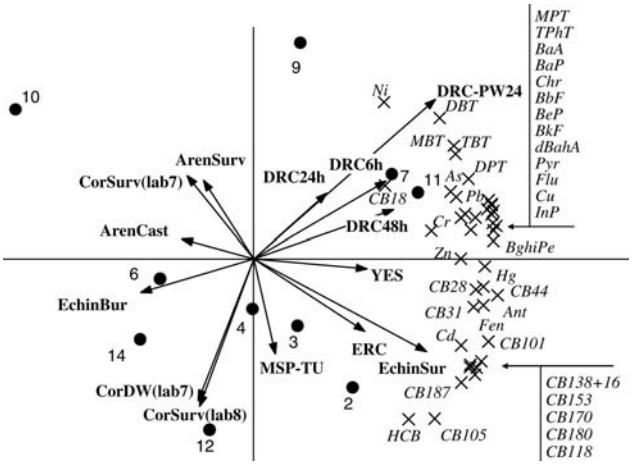

Figure 5. PCA triplot showing the correlation between the chemical contaminants in estuarine sediments and the response of bioassays. The horizontal first axis displays 74% of the variation in contaminants in the sediment, the second vertical axis another 10%. Bioassays, arrows; contaminants in sediments, crosses; sampling sites, filled circles. Abbreviations of substances are provided in the Appendix.

indicate good nutritional status) in flounder can be used to underpin evaluations of more specific biomarker measurements. Although small differences in these indices between sites are generally difficult to relate to a specific cause, when many minor differences are taken into account they can together provide clues to contaminant effects on populations (Kleinkauf *et al.*, 2004a). The indices measured in our study (CF, GSI, and HSI) could not, however, distinguish well between more polluted sites and seemed unrelated to the concentrations of PCBs, PAHs, and trace metals.

It is well known that the growth rate of flounder depends on environmental conditions, particularly temperature, salinity, and diet (Fonds *et al.*, 1992; Drevs *et al.*, 1999), and that females grow faster than males. Growth rates are higher in Dutch freshwater and brackish water than in marine water, and differences occur between areas and estuaries (van Leeuwen and Vethaak, 1988). The observed differences in age-at-length data of flounder between the two transects may be caused by the possibly less favourable environmental conditions in the semi-closed Port of Amsterdam area. Differences in somatic growth rate may confound the biomarker responses that are linked to somatic growth, such as RNA/DNA ratios in flounder liver tissue in the present study or RNA in crustaceans (Korsloot *et al.*, 2004). The potential confounding effects of factors such as age, growth, gender, and diet on biomarker responses should therefore be investigated (Kleinkauf *et al.*, 2004a; Mayeux, 2004).

Certain skin diseases of flounder are known to be general indicators of stress, including chemical contaminants in estuarine environments (Vethaak and Jol, 1996). However, skin lesions such as ulcers were not monitored in our study, because such indices require larger samples. However, a parallel study conducted in the Port of Amsterdam transect in September 1996 showed that up to 6.5% of flounder were afflicted with skin ulcers and that there was a significant correlation between the odds for ulcers and the concentration of PCBs and cadmium in flounder liver (Pieters *et al.*, 2000).

Biomarkers in flounder

Average parameter values per biomarker often varied from one site to another. In general, biomarker responses were less strong in flounder captured in relatively clean coastal areas (sites 4, 6, and 14) and at the freshwater reference site 12 (Figures 3 and 4). Consequently, combinations of biomarkers in flounder can be used to distinguish between cleaner and more polluted estuarine areas. Several biomarkers such as MDA, $KMBA_{mit}$, $KMBA_{cyt}$, $KMBA_{mic}$, WOP, Glyc, and Fibr were not able to distinguish more polluted sites and lost track of the distribution in sediment of PCBs, PAHs, and metals. Although the biomarkers CYP1A, GST (lab 1), SOD (lab3), $KMBA_{mic}$, $AChE_{brain}$, VTG, and RNA/DNA responses differed between the two freshwater and saltwater reference sites, the differences were not necessarily attributable to salinity. This implies that these particular biomarkers may be less suitable for use in estuarine environments where salinity may fluctuate on a daily or seasonal basis, or where fish may migrate between areas. The biomarker responses at the IJmuiden location (site 7) do not show a clear correlation with PCB sediment contamination (Figure 3) because, compared with sediment at that site, they showed relatively low PCB body burdens. CYP1A content in flounder liver varied between locations, but the largest difference was between the salt- and freshwater reference sites (Table 3). CYP1A content in liver tissue seemed to be influenced by (extreme) salinity differences, but still showed significant increases at some polluted locations compared with both the fresh- and saltwater reference sites. In this study, the CYPT1A results showed no correlation with contaminant levels of PCBs and PAHs in sediments. It is unfortunate that the results for CYP1A content cannot be compared with its activity, because EROD activity was not determined in this study. However, most studies (Eggens *et al.*, 1995; Rotchell *et al.*, 2001; Kirby *et al.*, 2004) were not conducted over an entire transect, from fresh- to saltwater, as in the present study.

The biotransformation enzyme GST showed significant and unambiguous decreased responses compared with references, but it did not correlate with contamination levels for PCBs and PAHs. As it appeared to be influenced by salinity differences, it would seem to have little value as an estuarine biomarker.

Oxidative enzymes CAT, SODlab4, MDA in liver tissue, KMBA in liver cytosol/mitochondria, and WOP appeared to be unaffected by salinity (Table 3), and showed significant differences between locations in univariate statistical analysis. The responses of oxidative enzymes WOP and MDA in multivariate analysis (Figure 3) were positively correlated with high body burdens of lower PCBs and several organochlorine contaminants. The concentrations of MDA obscured the biological significance in relation to chemical exposure at sites 2, 3, 9, and 10. It may be that multistress status influences malondialdehyde production (Huggett *et al.*, 2002). SOD and CAT are both biomarkers for oxidative stress, though for a different part of the chain of reactions that such compounds provoke (van der Oost *et al.*, 2003). For SOD, the two laboratories used different methods. Univariate analysis proved that a significant difference for CAT was found only at Oranjesluis (site 11); compared with the reference it correlated with high contamination levels for PCBs and PAHs in the Port of Amsterdam transect. The biomarkers KMBA (mitochondrial, microsomal, and in cytosol) did not correlate with the chemical compounds measured in flounder liver tissue and explained $<10\%$ of the observed variance of contaminants in sediments.

The MT biomarkers in flounder liver correlate with the gradient of contamination in both sediment and fish (Figures 3 and 4). This may be an indicator of specific stress by organotins, although the expected correlation was not found for metals such as Zn and Cd. Surprisingly, there was a good correlation between the concentration of MT and chemical exposure to total organotin in sediment ($r^2 = 0.7304$). In a few studies, it has been shown that in fish, detoxification of TBT is induced by metalloproteins (Kawano *et al.*, 1996; Padros *et al.*, 2000).

The OH-pyrene biochemical marker varied greatly among individual flounder. Averages for several biomarkers differed only marginally, if at all, between locations (Table 3). The OH-pyrene biomarker did not show correlation with the biota PAH levels (Figure 3). However, in the PCA triplot (Figure 4), the relationship between OH-pyrene and high concentrations of PAHs in sediment correlated ($r^2 = 0.4949$) at sites 7, 10, and 11. This is clearly not a significant result, although the 1-OH-pyrene value of site 7 (IJmuiden) seemed to be an outlier, because the 1-OH-pyrene value measured was extremely high compared with flounder liver, which showed low body burden. Moreover, pairwise univariate comparison of individual biomarker data showed that for several biomarkers, there were considerable and often significant differences in the average response between flounder captured at the fresh- and saltwater reference sites (Table 3). The positive experiences with the biomarkers MT in the liver and OH pyrene during the present study with some of the more established biomarkers for specific groups of compounds were in agreement with the findings of other studies in which they were measured in flounder: MT in liver (Goksøyr *et al.*, 1996; Padros *et al.*, 2000; Rotchell *et al.*, 2001; George *et al.*, 2004) and 1-OH pyrene in bile (Vethaak *et al.*, 1996; Richardson *et al.*, 2001; Ruddock *et al.*, 2002).

$AChE_{brain}$ induction correlated with the body burden of total pesticides in 15 pooled flounder livers at the Benelux tunnel (site 2) and Noordwijk (site 6) locations. The highest concentrations of several OCPs (total 150–400 $\mu g\,kg^{-1}$ lipid weight) were found in flounder from the Rotterdam transect (de Boer *et al.*, 2001). The Rotterdam transect (sites 2–6) is characterized by acetylcholinesterase inhibition (mainly brain), which may reflect contamination by pesticides in this transect. The concentrations of total OCPs in SPM varied in the Rotterdam and Amsterdam transects from below the detection limit to 1.8 and 1.6 $\mu g\,kg^{-1}$, respectively. Rotterdam showed relatively higher levels of OCPs from upstream, caused by one of the major sources of OCP residues in the rivers Meuse and Rhine. The contamination might be classified as historical, or industrial centres might still be emitting these compounds (Voorspoels *et al.*, 2004).

One biomarker for which the results proved difficult to interpret during the study was VTG in male flounder. The reason for this phenomenon is still unknown. In the UK, VTG in male flounder has proved to be an excellent and highly specific biomarker for the effects of oestrogenic contamination in estuaries (Allen *et al.*, 1999a; Kleinkauf *et al.*, 2004b).

In the present study, RNA/DNA ratios in flounder liver tissue did not correlate with contaminants in the flounder or sediments. Nucleic acid contents can rise over a season, influenced by biotic and abiotic factors such as fish size, somatic growth, temperature effects, and salinity. This is in contrast to studies in which the ratio of RNA to DNA in fish has been positively correlated with long-term growth and is therefore used in environmental sciences as a parameter for growth rate (Bulow, 1987). This biomarker was found to be suitable for assessing the general condition of fish (Rooker and Holt, 1996; Suthers *et al.*, 1996), and growth rates were negatively correlated with sublethal contamination levels.

DNA adducts in flounder liver tissue differed slightly, but not significantly, between sampling locations, and these effects were not PAH-exposure-related. This is not surprising, because it is consistent with the finding that this parameter does not discriminate for locations based on an ANOVA calculated on the values for individual fish. However, in a study in two UK estuaries, the average number of DNA adducts at the four UK sampling sites was positively correlated with average biliary 1-OH-pyrene concentrations (Lyons *et al.*, 1999).

Earlier analysis showed that CYP1A immunoreactivity in flounder could be induced in laboratory experiments by exposure to substances such as TCDD and PCB126 (Grinwis *et al.*, 2000). The present study demonstrated that this induction of CYP1A immunoreactivity can be found in flounder liver. The other histopathological biomarkers in the present study proved either difficult to interpret (vacuolation) or relatively insensitive to contaminants at the locations in this study (glycogen content, fibrillar structures).

In vivo bioassays

The finding that the *in vivo* sediment bioassays *C. volutator*, *E. cordatum*, *A. marina*, and *C. elegans* showed little response to the contaminated sediments from sites 2, 7, 9, 10, and 11 (Table 6) seems remarkable in retrospect (van den Brink and Kater, 2006). The bioassay with the amphipod *C. volutator* has since been included in the set of standard bioassays used for routine assessment of polluted harbour sediments using the chemical toxicity test (CTT) in the Netherlands (Schipper and Schout, 2004), and the burrowing heart urchin *E. cordatum* has long been under consideration for the purpose. Both showed only significant effects upon exposure to heavily polluted harbour sediments at the Oranjesluis site (site 11). However, the relation between bioavailability of the contaminants (Reichenberg and Mayer, 2006) and measured contaminants in those sediments is not always evident (Stronkhorst *et al.*, 2003b).

The *A. marina* bioassay is routinely used in the UK, but only in more saline conditions (Thain and Bifield, 2001). The survival of this polychaete in our study was hardly affected by the sediments tested during the study, but the number of casts produced varied considerably (Table 6). Significant effects with the above sediment *in vivo* bioassays used here have been demonstrated in other studies, not only in Dutch harbour sediments, but also in marine and estuarine environments (Matthiessen *et al.*, 1998; Kater *et al.*, 2001; Stronkhorst *et al.*, 2003c).

The nematode *C. elegans* assay did show a location- and gradient-related response for reproduction and development (at sites 2, 3, 7, 9, and 10). The assay seems independent of the salinity of a sample, and even of the source of the sample (biotic or abiotic), as long as extracts are used. The results obtained with the *C. elegans* assay are, however, difficult to extrapolate to field conditions, partly because this is not a native benthic marine or estuarine species.

In vitro bioassays

The Microtox SP®, Mutatox, DR-CALUX, ER-CALUX, and YES *in vitro* assays all revealed some differences between the sediments tested in this study (Table 7). The PCA overall analysis showed that the levels of PAHs, PCBs, trace metals, and organotins in the

sediment correlate strongly with the *in vitro* bioassays. The most discriminant *in vitro* bioassays in the present study were DR-CALUX, ER-CALUX, and Microtox SP®. Microtox SP® did not, however, find the expected correlations for PAHs. The DR-CALUX response was sometimes higher after 48 h of incubation, suggesting that the effects are greater after longer exposure times, and indicating that the response attributable to PCBs or dioxins/furans and the concentrations were too high for the test cells to metabolize them all within 24 h (Hamers *et al.*, 2000).

There is no significant correlation between the ER-CALUX bioassay and the VTG biomarker, given the doubtful statistical validity of the VTG data. The ER-CALUX was more sensitive than the YES assay. All the above *in vitro* tests have been applied successfully to field sediments from marine, estuarine, and freshwater environments (Johnson and Long, 1998; Stronkhorst *et al.*, 2002; Legler *et al.*, 2003; Houtman *et al.*, 2004; Thomas *et al.*, 2004). The Mutatox® assay for genotoxicity showed differences between locations, but was not applied to sediments from either the salt- or freshwater reference sites. Suitable sediment bioassays are the Microtox SP® bioluminescence assay and the *in vitro* reporter gene assays DR-CALUX and ER-CALUX. The Microtox SP® and DR-CALUX have been included in the battery of standard bioassays used to assess Dutch harbour sediments with the CTT (Schipper and Schout, 2004). In general, the Microtox SP® and DR-CALUX results hinted that the most toxic sediment collected during the study came from site 11 (Oranjesluis; see Tables 5 and 6). This is in line with the results of the chemical analysis (de Boer *et al.*, 2001).

Conclusions

The results of this study show that body burden in benthic fish at least partly fingerprints the chemical characteristics of the sediment from the sites along the salinity transects where they were sampled. The most discriminant biomarkers and bioassays were those based on types of biological effects for specific groups of compounds, which gave more straightforward results than gross indices and biomarkers of general damage or condition. Mechanism-based *in vitro* assays DR-CALUX and ER-CALUX applied to sediment extracts for screening of potential toxicity were more responsive than *in vivo* bioassays with macro-invertebrates using survival as an endpoint. However, no clear responses were found between induction of Microtox SP® by PAHs, metallothioneins by metals such as Zn and Cd, and 24 and 48 h DR-CALUX by PCBs or HCB. The utility of some biotransformation and oxidative stress biomarkers in detecting differences between sites or transects was also limited. These comparisons may, however, have been blurred by the effects of salinity on the bioavailability of contaminants such as metals, shortcomings in the extraction and clean-up methodology, and for some biomarkers, by the migratory behaviour of flounder. Based on this study, a tentative list of appropriate biomarkers in flounder for monitoring and assessing chemical contaminants in estuarine environments would include the induction of hepatic metallothionein content (indicating exposure to organotins) and concentration of 1-OH pyrene in bile (an indicator of exposure to PAHs). Mechanism-based *in vitro* assays DR-CALUX and ER-CALUX applied to sediment extracts for screening of potential toxicity were much more responsive than *in vivo* bioassays with macro-invertebrates using survival as an endpoint.

This is undoubtedly an area requiring further investigation. In the past, the ICES Working Group on Biological Effects of Contaminants (ICES, 2008) and field surveys such as the EU BEEP project in the Baltic Sea (Lethonen *et al.*, 2006) have been calling for more research on the effects of interfering factors such as salinity affecting biomarker responses in marine and estuarine fish and bioassays to help fill this knowledge gap.

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Appendix

Abbreviations of substances used in PCA triplots (Figures 2–5). For abbreviation of biomarkers and bioassays, see Tables 1 and 2.

HCH	hexachlorocyclohexane
DDD	dichlorodiphenyldichloroethane
DDE	dichlorodiphenyldichloroethylene
DDT	dichlorodiphenyltrichloroethane
QCB	pentachlorobenzene
HCB	hexachlorobenzene
HCBD	hexachlorobutadiene
OCS	octachlorostyrene
β-hepo	heptachloroepoxide
TBDE	2,4,2,4′-tetrabromodiphenylether
PeBDE	2,4,5,2,4′-pentabromodiphenylether
TBT	tributyltin
DBT	dibutyltin
MBT	monobutyltin
TPhT	triphenyltin
DPT	diphenyltin
MPT	monophenyltin
Ant	anthracene
BaA	benzo[*a*]anthracene
BaP	benzo[*a*]pyrene
BbF	benzo[*b*]fluoranthene
BeP	benzo[*e*]pyrene
BghiPe	benzo[*ghi*]perylene
BkF	benzo[*k*]fluoranthene
Chr	chrysene
dBahA	dibenzo[*ah*]anthracene
Fen	phenanthracene
Flu	fluoranthene
Pyr	pyrene
InP	indeno[1,2,3-*cd*]pyrene
CB	polychlorinated biphenyl.

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