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Annual cycles of apoptosis, DNA strand breaks, heat shock proteins, and metallothionen isoforms in dab (*Limanda limanda*): influences of natural factors and consequences for biological effect monitoring

MARKUS LACORN[1], GUNDULA PIECHOTTA[1,2], THOMAS J. SIMAT[1], ULRIKE KAMMANN[2], WERNER WOSNIOK[3], THOMAS LANG[2], WERNER E. G. MÜLLER[4], HEINZ C. SCHRÖDER[4], HANS-STEPHAN JENKE[2] AND HANS STEINHART[1]*

[1] Institute of Biochemistry and Food Science, University of Hamburg, Grindellallee 117, 20146 Hamburg, Germany.

[2] Federal Research Centre for Fisheries, Institute of Fisheries Ecology, Wüstland 2, 22589 Hamburg, Germany

[3] Institute of Statistics, University of Bremen, Postfach 330440, 28334 Bremen, Germany

[4] Institute of Physiological Chemistry, University of Mainz, Duesbergwet 6, 55099 Mainz, Germany

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The present study was undertaken to investigate the influence of natural and anthropogenic stressors on the induction of apoptosis, metallothionein (MT) isoforms, heat shock proteins and DNA strand breaks in the marine flatfish dab (*Limbanda limanda*) Seasonal changes and possible physiological influences were evaluated over a 1-year period at a fixed location northwest of Helgoland in the German Bight. These results were compared with data from sampling sites in the North Sea and the Baltic Sea. Annual cycles could be observed for all parameters except for Cd. The data revealed that changes in biomarker are not only linked to physiological processes related to reproduction but also to factors like water temperature changes, lipid content and zinc content. Cd and organochlorines had no influence on biomarkers whereas an influence of Cd on MT levels revealed in the regional observations was possibly masked by the major changes in Zn content during the annual cycle. Due to different abiotic factors we supposed that the annual cycles at each sampling site in the North and the Baltic Sea might be shifted temporally and therefore measurements at different locations during a small time window of a few weeks may lead to misinterpretation in biomarker research.

Keywords: biomarker, apoptosis, metallothionein, heat shock protein, DNA strand breaks, dab, annual cycle.

Introduction

In the last few years biomarker research has been accompanied by increased information about influences of abiotic and biotic factors such as reproductive cycle, changes in water temperature or nutritional status on biomarker levels. For example, Saborowski (1995) found seasonal changes in ethoxyresorufin-*O*-deethylase (EROD) activity in dab (*Limanda limanda*) liver due to water temperature variation and Lange *et al*. (1998) identified temperature as the key

* Corresponding author: Hans Steinhart, Institute of Biochemistry and Food Science, University of Hamburg, Grindelalle 117, 20146 Hamburg, Germany.

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factor for regional variations of EROD in dab liver. Olsson *et al*. (1987) observed the highest metallothionein (MT) values in rainbow trout (*Oncorhynchus mykiss*) during spawning, parallel to Zn contents of the liver. The application of biomarkers in the field requires exact knowledge of these influencing factors. Without this information differences in biomarker levels in organisms from distinct locations may erronously be attributed to anthropogenic influences.

Apoptosis, also known as programmed cell death, is a physiological and irreversible process of cells to maintain tissue homeostasis (Kerr *et al*. 1972). During apoptosis nuclear chromatin condenses and a calcium-dependent endonuclease is activated. The result is a DNA fragmentation into multiples of 180–200 bp (Wyllie *et al*. 1980). Electrophoretic separation of apoptotic DNA fragments reveals a typical 'ladder' pattern (Arends *et al*. 1990). Apoptosis is induced by a variety of chemical substances, e.g. tributyltin (Batel *et al*. 1993), Cd (Xu *et al*. 1996) and polychlorinated biphenyls (Piechotta *et al*. 1999). As for DNA strand breaks, a possible influence of natural stressors on apoptosis is not known at the present time.

Two major types of damage in cellular DNA caused by chemical, physical and biological agents are known, single- and double-stranded breaks. These strand breaks are induced by several chemicals such as PAH, PCB and Cd (Steinert 1996, Schröder *et al*. 1999a,b). These strand breaks can be measured directly in cell or tissue lysates by a method using a fluorescence dye which specifically binds to double-stranded DNA (Müller *et al*. 1997). A possible influence of natural stressors on DNA strand breaks is not known at the present time.

Heat shock proteins (HSP) are assumed to contribute to the survival of cells after exposure to diverse environmental stresses (Lindquist 1986). The expression of HSP 70, a protein of M_r 70 kDa, can be affected by a variety of stressors (Sanders 1990), including temperature shock and exposure to heavy metals, e.g. organotin and methyl mercury (Batel *et al*. 1993). HSPs have been shown to be involved in stabilizing unfolded proteins (chaperone function). HSP70 is considered as a biomarker of exposure and has been used as a probe for monitoring stress in aquatic environments (Koziol *et al*. 1996). Because of HSP induction by temperature changes (Koziol *et al*. 1997), a possible influence of natural factors is supposed.

Metallothioneins (MT) are regarded as the most suitable heavy metal-specific biomarkers and are an integral part of a current international intercalibration programme (BEQUALM 1999). They are characterized by their low molecular weight, the ability to bind metals in metallothiolate clusters and the presence of distinct isoforms due to certain amino acid substitutions, with the exception of highly conserved cysteine residues (George and Olsson 1994). The main function of MT is to maintain a trace metal homeostasis of Zn and Cu (Bremner and Beattie 1990). MTs in fish normally consist of two major isoforms (Olsson and Haux 1985, Duquesne and Richard 1994), which play different physiological roles. For example, Kammann *et al*. (1997) showed differences in inducibility and mercury binding properties of isoforms isolated from bream (*Abramis brama*). Kuroshima (1995) found differences in inducibility of both isoforms by Cd and Zn in Red Sea bream (*Pargus major*). As described above, there is information about the induction of MTs by 'natural' stressors (Olsson *et al*. 1987) and about seasonal variations of MT levels in fish (Hylland *et al*. 1998).

The marine flatfish dab was selected for our investigations because of its use as a

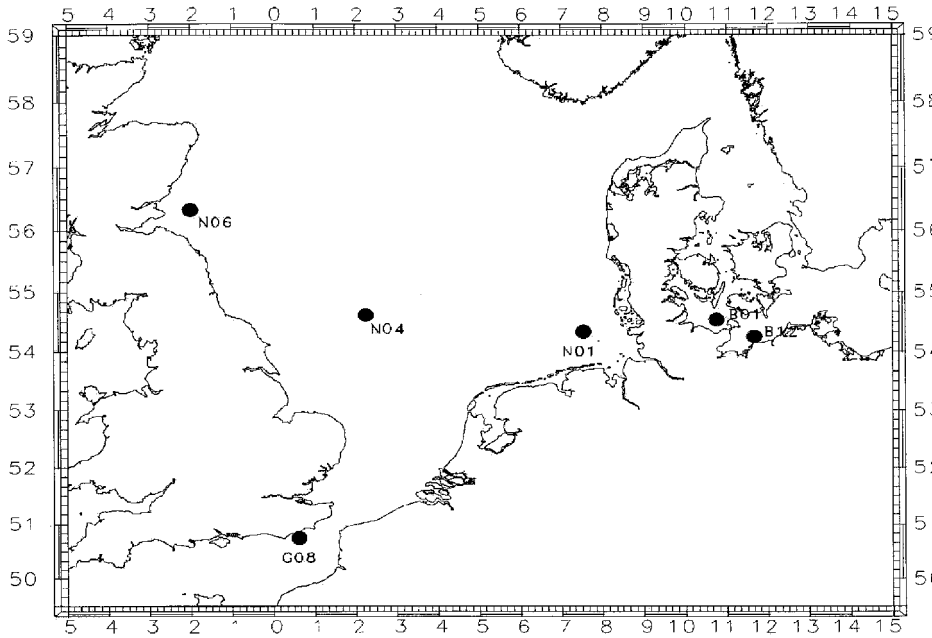


Figure 1. Sampling locations in the North Sea and the Baltic Sea.

monitoring organism, e.g. Joint Assessment and Monitoring Programme (JAMP 1997), and due to its widespread occurrence, frequency and known data about contaminants (ICES 1989, Lang and Dethlefsen 1996).

The aim of this study was to assess the influence of the seasonally variable factors water temperature, hepatic lipid content, Zn content and also the Cd content on changes in the possible biomarkers apoptosis, heat shock proteins, DNA strand breaks, and metallothionein isoforms. The time course of these variables were observed in fish caught at one location in the German Bight during 1 year. These results are compared with data from other locations in the North Sea and Baltic Sea and consequences for biomonitoring strategies are discussed.

Materials and methods

Chemicals

All chemicals were of analytical grade or better and obtained from Merck (Darmstadt, Germany), except for the following substances: agarose was purchased from Biozym (Hess. Oldendorf, Germany). DNA basepair marker, Tris and monoclonal antibody against heat shock protein-70 [anti-hsp70 antibody (bovine); H 5147] were from Sigma (St Louis, MO, USA). The cod liver oil was a gift from Dr K.-O. Henkel (Lamotte, Bremen, Germany).

Sampling

Annual cycle. Female dab (> 25 cm; $n = 10$) were collected monthly from February 1998 to January 1999 by bottom trawling with the research vessels '*Walter Herwig III*' (Federal Research Centre for Fisheries, Germany) or '*Uthörn*' (Biological Station Helgoland, Germany). The sampling site was located northwest of Helgoland (see figure 1; N 01) in the German Bight (North Sea). Data from measurements of bottom water temperatures during the year were made available by the Federal Maritime and Hydrographic Agency of Germany.

Regional discrimination. Additionally dab were collected by bottom trawling during a sampling with

the '*Walther Herwig III*' in December 1997 at the following locations (see figure 1): B 01 (Kiel Bight; 54°30´N–54°35´N, 10°40´E–10°50´E), B 12 (54°10´N–54°20´N, 11°35´E–11°45´E), N 01 (Helgoland; 54°15´N–54°25´N, 07°26´E–07°39´E), N 04 (Doggerbank; 54°25´N–54°50´N, 02°00´E–02°31´E), N 06 (Firth of Forth; 56°15´N–56°25´N, 01°50´W–02°10´W), G 08 (English Channel; 50°40´N–50°50´N, 00°30´E–00°50´E).

Parameters. The following hepatic parameters were determined (for further description, see below): apoptosis, DNA strand breaks, heat shock protein, metallothionein isoforms, Zn and Cd. Furthermore age (Maier 1908), organochlorines (Schröder *et al*. 1999a) and gonado somatic index (GSI; quotient of gonad weight and total weight without gonads) were determined in samples from the various regions. Lipid contents in samples from the annual cycle were measured according to Zöllner and Kirsch (1962) and in samples from different regions according to Schröder *et al*. (1999a).

Dab were killed by a cut through the spinal cord, livers were removed and cut into pieces for each determination. Samples for biochemical analysis were stored at –80 °C and samples for chemical analysis at –20 °C.

Cadmium and zinc determination

Metal contents were determined as described by Piechotta *et al*. (1999). Briefly, dab liver samples were digested with conc. HNO_3 and subsequently analysed by graphite furnace atomic absorption spectrophotometry (GF-AAS) with deuterium background correction for Cd and by flame atomic absorption spectrophotometry for Zn. Quantification was performed according to the External Standard method with multi-point calibration. Detection limits in the digested sample were 0.005 μg g^{-1} for Cd and 2.5 μg g^{-1} for Zn. All metal concentrations were expressed on a wet weight basis.

Quantification of apoptotic DNA fragments

The quantification of apoptotic DNA fragments (hereafter called 'apoptosis') is described in detail by Piechotta *et al*. (1999). Briefly, DNA was extracted by using the QIAamp Tissue Kit (Qiagen GmbH, Hilden, Germany). Gel electrophoresis was carried out in 1.5 % agarose and run for approximately 1 h at 6 V cm^{-1}. The gels were stained with ethidium bromide solution, visualized on a 302 nm UV transilluminator (Intas, Göttingen, Germany) and analysed densitrometrically. For quantification of apoptotic DNA, fragments between 180 and 1500 bp were chosen. Their integrated optical density was referred to a DNA basepair marker standard curve. Quantified amounts of apoptotic fragments were related to the total amount of DNA.

A second method to determine apoptosis was the TUNEL assay (hereafter called 'TUNEL') with frozen liver sections (Piechotta 1999). Briefly, the tissue was lysed, DNA strand breaks were marked enzymatically (terminal transferase) with digoxygenin-labelled nucleotides, coupled with peroxidase labelled anti-digoxygenin and stained by reaction with diaminobenzidine. TUNEL-labelled nuclei were counted by Image Analysis.

Determination of heat shock protein

The determination was performed as described previously (Müller *et al*. 1998). Briefly, proteins were extracted, subjected to electrophoresis (Laemmli 1970) and electrotransferred to PVDF-membranes (Kyhse-Andersen 1984). After blocking, the membranes were processed as described by Bachmann *et al*. (1986), incubated with mouse anti-hsp70 antibody, visualized by incubation with peroxidase-conjugated anti-mouse IgG and disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2-5-chloro)tricyclo[3.3.1.1]decan}-4-yl)phenyl phosphate (CPSD; Boehringer Mannheim, Germany), and evaluated using a Model GS 525 Molecular Imager (Bio-Rad, München, Germany). The values were related to one randomly chosen sample.

DNA strand breaks

DNA single strand-breaks and/or alkali-labile sites were determined as described by Müller *et al*. (1997) using Fast Micromethod. Briefly, frozen liver tissue samples were homogenized under liquid nitrogen and applied to a microplate. Lysis of tissue was performed by addition of lysing solution containing the fluorescent dye PicoGreen. DNA unwinding at pH 12.4 was started and was followed by measuring the fluorescence at 480 nm excitation and 520 nm emission. Six replicate assays were performed. Human lymphocytes were used as reference cells (100% dsDNA). Effects are expressed as strand scission factors (SSF) and were calculated after 20 min of unwinding, as follows: SSF = log (% dsDNA in sample/% dsDNA in control). Negative values for SSF are indicative of increased frequencies of strand breaks/alkali-labile sites. For a better representation values were multiplied by (–1).

Metallothionein isoform analysis

MT isoform analysis was performed according to Lacorn *et al.* (2000). Briefly, samples were homogenized and Cd saturated. After centrifugation the resulting cytosols were divided into subsamples for Bradford protein determination (Bradford 1976) and MT-determination. For MT-determination the cytosol was partially purified by a two-step acetone precipitation, and the 50–80% acetone precipitate was reconstituted and subjected to anion exchange chromatography.

For elution a NaCl gradient was used and 2 ml-fractions were collected for Cd determination. MT-containing fractions were diluted, acidified and analysed by GF-AAS as described above. MT-content was calculated assuming that one MT molecule binds seven Cd atoms (Kuroshima 1995) and referred to total protein content.

Quality control/assurance

Accuracy and precision of the Cd and Zn determination were tested by analysing certified reference materials (CEC, Community bureau of reference, CRM No. 185) during the analysis of dab liver. The mean was within the certified 0.95 confidence interval and the coefficient of variation was 5% ($n = 20$), respectively.

For MT analysis a reference material is not available and calculation of recovery by addition of MT isofrom from rabbit liver was estimated as not promising. The coefficient of variation was less than 15% for both isoforms ($n = 6$). The detection limit calculated from GF-AAS detection limit was about 0.002 $\mu g\ mg^{-1}$ protein or 0.04 $\mu g\ g^{-1}$ liver.

The detection limit for apoptosis measurement is 0.9% DNA fragments (180–1500 bp) related to total DNA. Values below this limit are represented as 0%. Repeatability of the described method was measured with homogenized material and resulted in a coefficient of variation of 15% ($n = 10$).

Lipid determination

The determination of the hepatic lipid content was performed according to Zöllner and Kirsch (1962) in samples from the annual examination. Briefly, lipids were extracted, mixed with concentrated sulphuric acid, stained with a phosphoric acid–vanillin mixture and the extinction was measured at 526 nm. Quantification was performed according to the external standard method with multi-point calibration using cod liver oil as a standard. The lipid content was expressed in per cent of the liver wet weight.

Statistical analysis

Data are presented as box–whisker-plots (median, box: 25–75%, whisker: non-outlier, Min.–Max.). Comparisons in pairs were carried out by using the Mann–Whitney rank order test (U-Test). To examine and characterize the correlation between two parameters, Spearman rank correlations were calculated. The correlation structure between all parameters was investigated by factor analysis on the correlation matrix of the logarithms of the variables. In the case of apoptosis, a fourth-order root was used because of observed zero values.

For the comparison of annual cycles, the measured data were standardized to a mean of zero and a standard deviation of one. Logarithmic transforms were used, except for apoptosis where a fourth order root was used, to reduce the impact of extreme values and to obtain an approximately symmetrical error distribution. The average annual cycle of each parameter was estimated by a smooth function (locally weighted second order regression, Wand and Jones 1995). Partial copies of the observed data were used as additional input for a period of 3 months before and after the actually-observed data. This ensures that the estimated function is cyclic. However, all tests and displays use only data from the actually-observed period. Cross-validation was used to determine the smoothing parameter involved (Hastie and Tibshirani 1990). A 90% confidence region for the estimated cycle was calculated by bootstrap simulation with 1000 replicates (Efron and Tibshirani 1993), employing a kernel density estimate (Silverman 1990) of the empirical measurement distributions to generate the replicates. Distances between bootstrap replications and the basic annual cycle estimate were calculated as Kolmogorov–Smirnov distances, weighted by the local error standard deviation. Pointwise bounds of the confidence regions are displayed graphically, so that a test on the existence of a non-constant cycle can easily be performed: if a horizontal line fits completely inside the bounds, the hypothesis of a non-constant cycle has to be rejected. The pairwise comparison of the estimated cycles for two different parameters is based on the 90% confidence regions for the standardized estimated cycles: if these regions overlap over the whole time span, then the cycles are considered as not significantly different. From the estimated cycles, ranges of maximal parameter values during the annual cycle are derived as those intervals in which the parameter is at most 10% smaller than the maximum (see dark coloured fields in table 3). Values between 10 and 40% of the maximum value representing increasing or decreasing values are coloured in grey (see table 3).

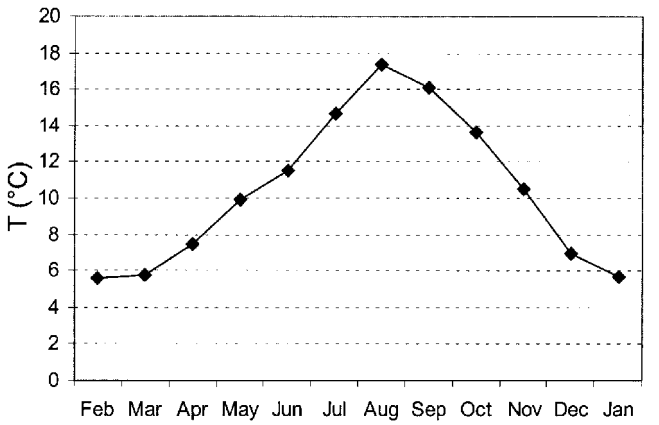


Figure 2. Water temperature curve during the annual cycle at station N 01.

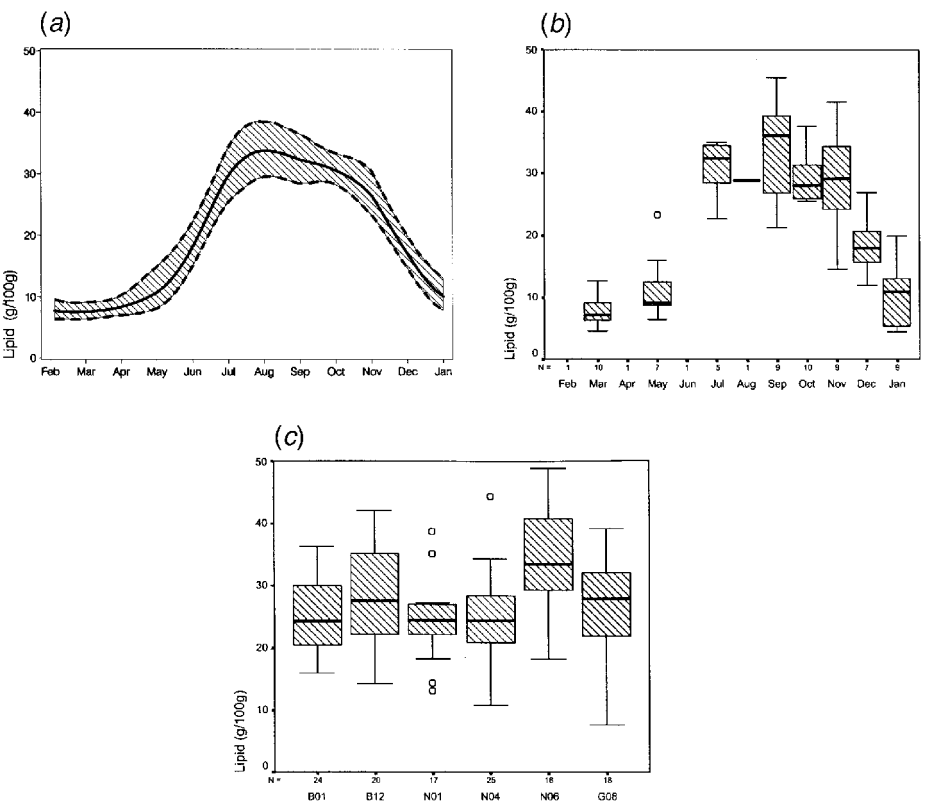


Figure 3. Lipid content (%). (a) Calculated annual cycle. (b) Data of the annual cycle presented as box–whisker-plot. (c) Data of the regional discrimination presented as box–whisker-plot. For detailed information see Materials and Methods.

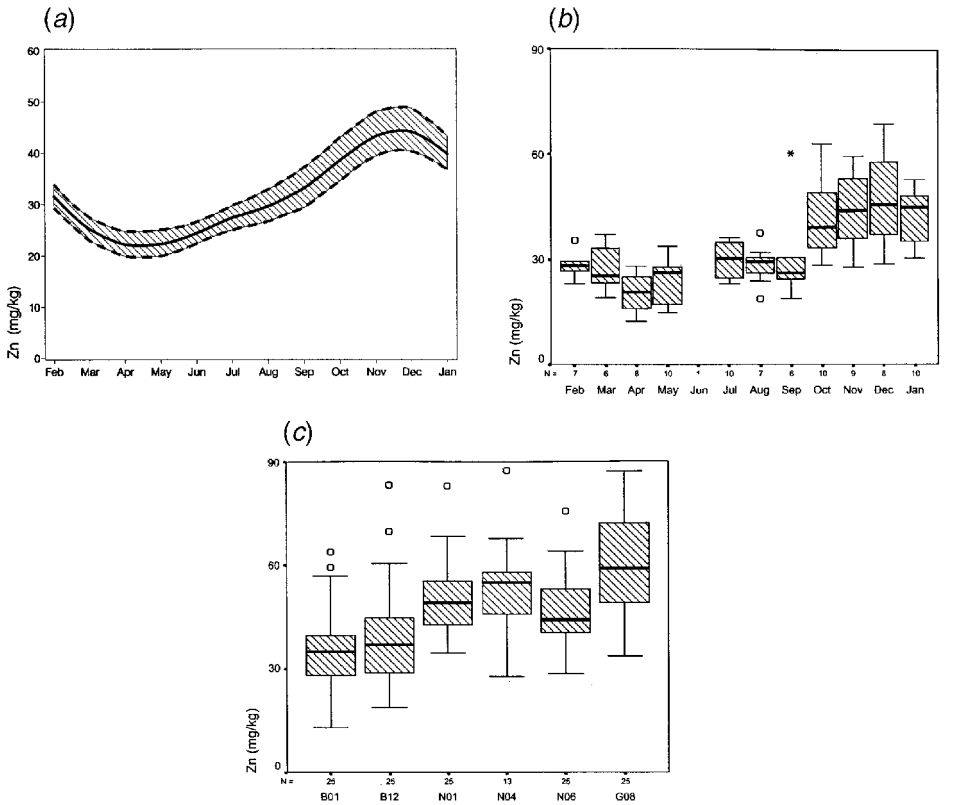


Figure 4. Zinc content (mg kg^{-1} liver). (a) Calculated annual cycle. (b) Data of the annual cycle presented as box–whisker-plot. (c) Data of the regional discrimination presented as box–whisker-plot. For detailed information see Materials and Methods.

Results

Annual cycles

The changes in water temperature during the annual cycle at station N 01 are illustrated in figure 2. The water temperature varied from about 5.6 °C in February to 17.4 °C in August. The maximum temperature increase was in July (3.2 °C per month) and the maximum decrease in November (3.4 °C per month).

Maximum hepatic lipid contents (figure 3(a,b)) were observed between July and September (28.9–36.2 %). The content in August is calculated from a single value. There was a strong correlation between lipid content and water temperature ($p < 0.001$; $r_s = 0.762$).

The annual changes in Zn contents in dab liver are shown in figure 4(a,b). The values ranged from 20.8 mg kg^{-1} in April to 45.6 mg kg^{-1} in December with a marked increase in October from 26.3 to 39.1 mg kg^{-1}. A correlation analysis between Zn contents and water temperature changes (monthly) resulted in a strong negative correlation ($p < 0.001$; $r_s = -0.596$).

Figure 5(a,b) shows the apoptotic DNA fragmentation measured in dab liver during an annual cycle. The rate of hepatic apoptosis has obvious seasonal variations. Lowest values were reached in late winter/spring during January to April and highest values were measured in late summer/autumn from July to

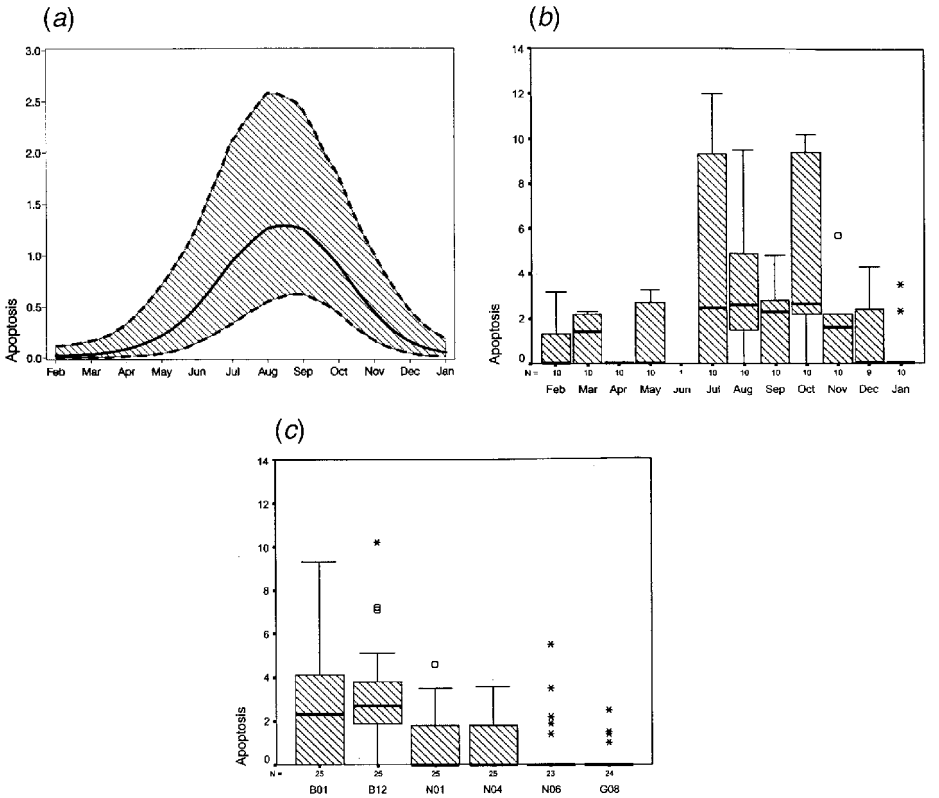


Figure 5. Apoptosis. (a) Calculated annual cycle. (b) Data of the annual cycle presented as box–whisker-plot. (c) Data of the regional discrimination presented as box–whisker-plot. For detailed information see Materials and Methods.

October (up to 2.7% in October). The apoptotic rates in July and October are significantly elevated in comparison to the period from January to May ($p \leq 0.05$). Apoptosis is strongly correlated with water temperature ($p < 0.001$; $r_s = 0.430$) and lipid content ($p < 0.01$; $r_s = 0.355$).

The determination of SSF in dab liver samples during 1 year shows a significant annual cycle with a broad maximum peak in October with values of about 0.4 figure 6(a,b). Minimal SSF ratios are reached from February to April with values of about 0.15. SSF is strongly correlated with water temperature ($p < 0.001$; $r_s = 0.546$) and lipid content ($p < 0.001$; $r_s = 0.570$).

The annual changes in relative HSP contents are shown in figure 7(a,b). The values ranged from about 50% in March to 150% in October with a marked increase in September from 90% to 140%. HSP contents and water temperature ($p < 0.001$; $r_s = 0.563$) were strongly correlated as were HSP and lipid contents ($p < 0.001$; $r_s = 0.477$).

MT-I contents ranged from 0.14 μg mg^{-1} to 0.51 μg mg^{-1} with a moderate distinct maximum in September (figure 8(a,b)).

MT-II contents showed highest values from October to December (5.16–6.11 μg mg^{-1}) with a distinct increase from September to October (figure 9(a,b)). There was a second significant increase in July (from 1.87 μg mg^{-1} in May to 4.27 μg mg^{-1}). Lowest values were observed in March (1.24 μg mg^{-1}).

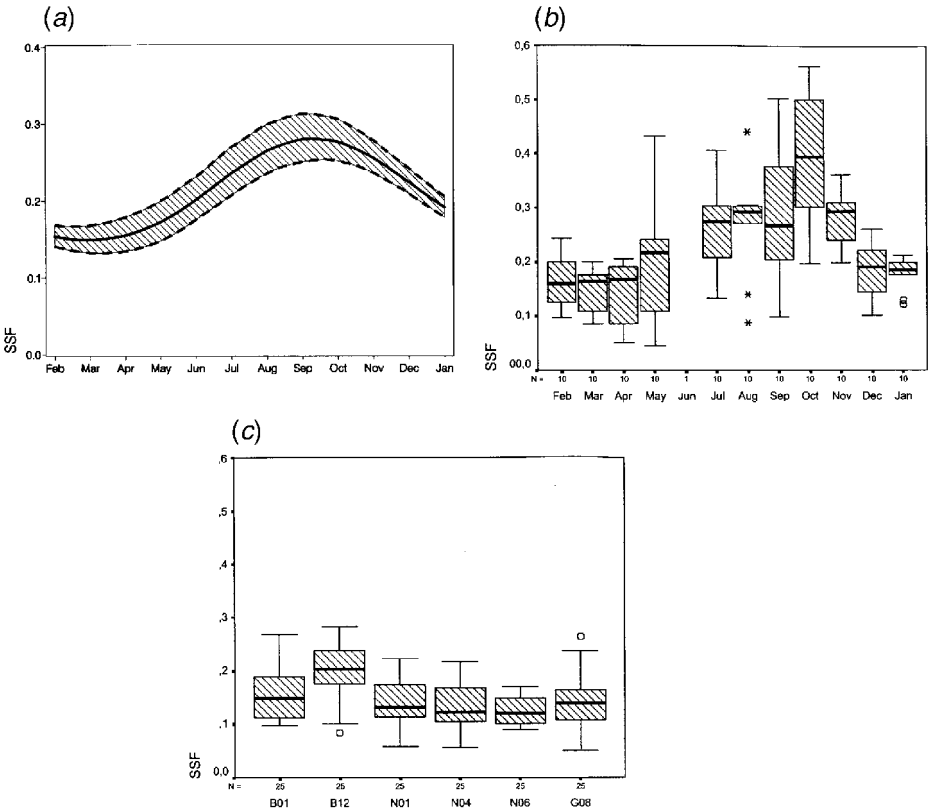


Figure 6. SSF. (a) Calculated annual cycle. (b) Data of the annual cycle presented as box–whisker-plot. (c) Data of the regional discrimination presented as box–whisker-plot. For detailed information see Materials and Methods.

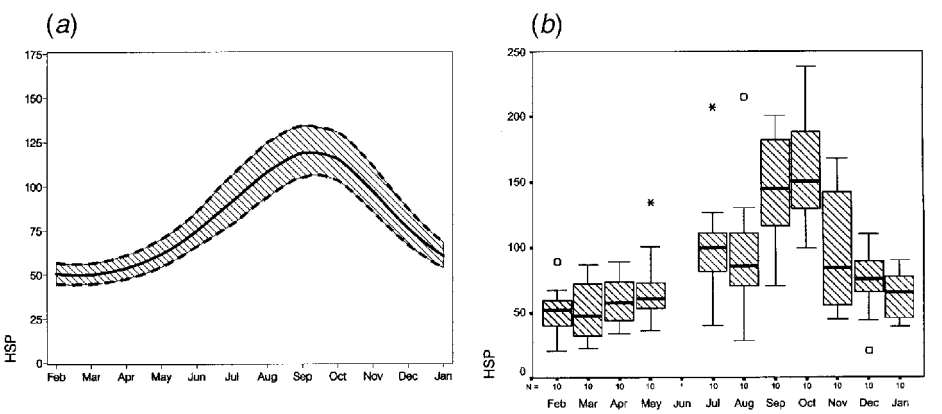


Figure 7. HSP. (a) Calculated annual cycle. (b) Data of the annual cycle presented as box–whisker-plot. For detailed information see Materials and Methods.

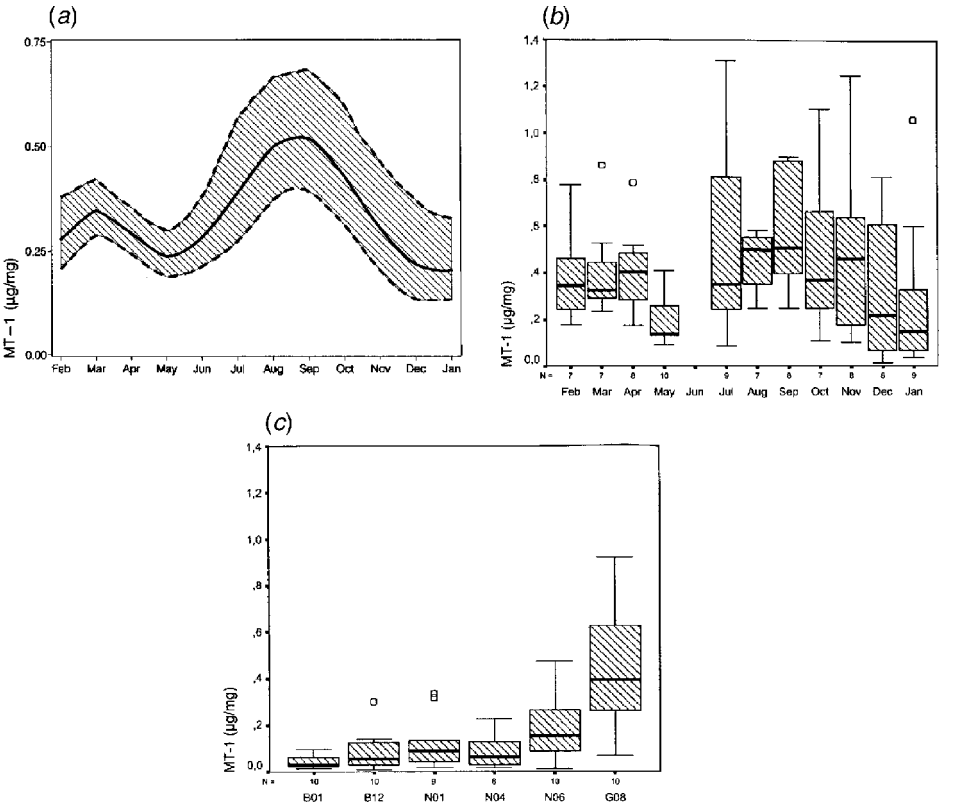


Figure 8. MT-I (µg kg^{-1} protein). (a) Calculated annual cycle. (b) Data of the annual cycle presented as box–whisker-plot. (c) Data of the regional discrimination presented as box–whisker-plot. For detailed information see Materials and Methods.

There was a strong correlation between MT-II values and Zn contents ($p < 0.001$; $r_s = 0.684$). Furthermore we observed a strong correlation between MT-II and water temperature, as well as with lipid content ($p < 0.001$). MT-I showed correlations with both temperature and lipid content ($p < 0.01$).

The Cd contents did not show any noticeable changes during the year (figure 10(a,b). The lowest values were in October (70.7 µg kg^{-1}) and the highest values in December (132.8 µg kg^{-1}). There was no significant correlation between Cd and any of the other parameters.

Figures 3(a)–10(a) show the calculated annual cycles with 90% confidence regions. All parameters except the one for Cd are significantly non-constant.

The pairwise comparison of standardized cycles revealed different cycles in 18 cases, while in 10 cases no difference was found (table 1). Zn turned out to change differently from all other parameters over the year. The fact that Cd was found to vary differently from all other parameters is a consequence of Cd not having a significant cycle, while all other parameters do have. The remaining parameters do not show a uniform pattern. It should be noted that different scales are irrelevant in the comparison, while the shape of the cycles as well as the width of the confidence regions are relevant. A difference in a single month only is sufficient to consider two

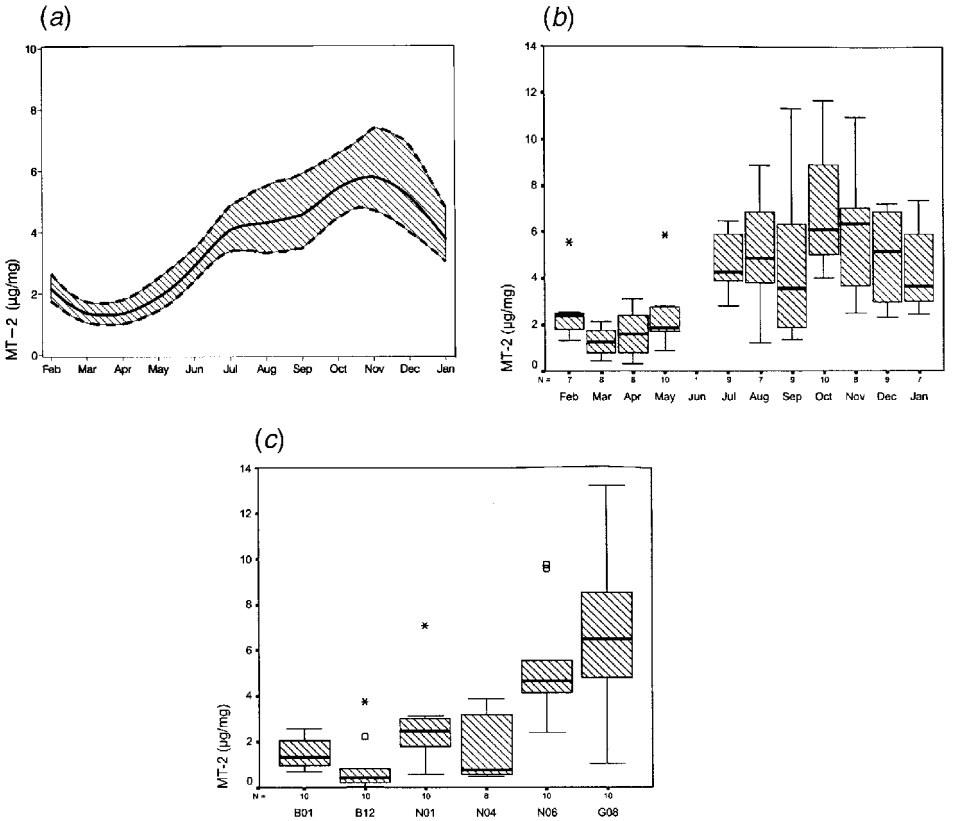


Figure 9. MT-II (µg kg^{-1} protein). (a) Calculated annual cycle. (b) Data of the annual cycle presented as box–whisker-plot. (c) Data of the regional discrimination presented as box–whisker-plot. For detailed information see Materials and Methods.

parameters as having different cycles. This is the case in the pairwise comparison of Zn and MT-II (month of July).

The factor analysis allows a comprehensive representation of the correlation matrix of all measured parameters. Small distances between parameters in the graphic display indicate high correlation. Parameters on the same axis but at opposite ends are negatively correlated, whereas parameters in perpendicular directions are not correlated. Figure 11 shows that HSP, SSF, lipid and temperature, are closely clustered, whilst apoptosis and MT-I are less correlated than the other four parameters. In contrast Zn is located on the other axis and therefore not related to the above mentioned group. MT-II is located between both axes and therefore explained by both factors. Cd and MT-I are only extracted to a slight extent.

Regional variation

Results from dab sampled at different locations in the North Sea and the western Baltic Sea are shown in figures 3(c)–10(c). The water temperatures were 6.3 °C (B 01), 6.6 °C (B 12), 6.8 °C (N 01), 8.2 °C (N 04), 9.0 °C (N 06) and 11.6 °C (G 08). The value at station N 01 is therefore comparable to the value from the

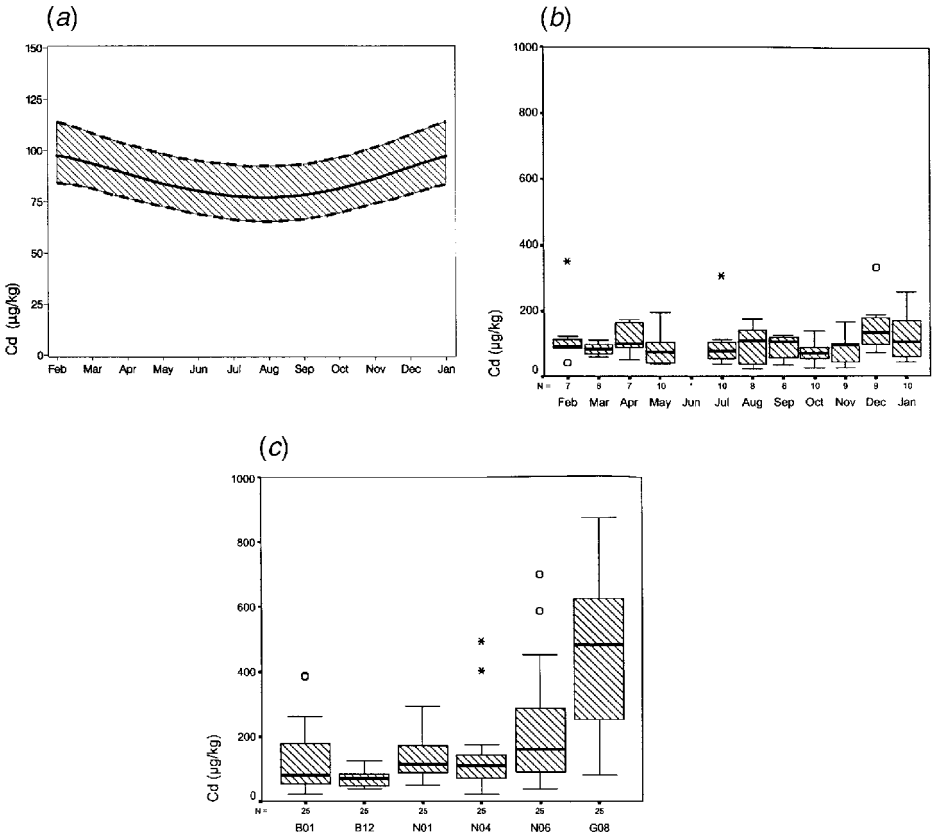


Figure 10. Cadmium content (µg kg^{-1} liver). (a) Calculated annual cycle. (b) Data of the annual cycle presented as box–whisker-plot. (c) Data of the regional discrimination presented as box–whisker-plot. For detailed information see Materials and Methods.

annual cycle examination in December 1998. The hepatic lipid content varied between 24.3 % (Baltic Sea; B 01) and 33.5 % (Firth of Forth; N 06). All values are slightly above the value from the seasonal examination in December. The highest GSI values were measured at locations N 01 and N 04 whereas the lowest values occurred at the Baltic Sea stations, reflecting the different times of vitellogenesis and spawning. With the exception of B 12 all dab were of comparable age. Dab from B12 were significantly younger. Concentrations of Zn were significantly lower in dab from the Baltic Sea compared with dab from the North Sea. The highest values were found in dab from location G 08 (59.3 mg kg^{-1}) and the lowest values at location B 01 (35.0 mg kg^{-1}).

The determination of apoptotic DNA-fragmentation resulted in significantly higher values in dab from the Baltic Sea (B 01: 2.3 %; B 12: 2.7 %) compared with dab from the North Sea (median of 0 % for all locations). There were strong correlations ($p < 0.001$) between apoptosis and water temperature ($r_s = -0.438$) and the age of the dabs ($r_s = -0.284$) as well as with zinc content ($r_s = -0.296$).

The extent of DNA strand breaks ranged from 0.120 (N 06) to 0,230 (B 12). Dab from station B 12 showed significantly higher levels of DNA strand breaks compared with dab from the other stations. A correlation analysis revealed

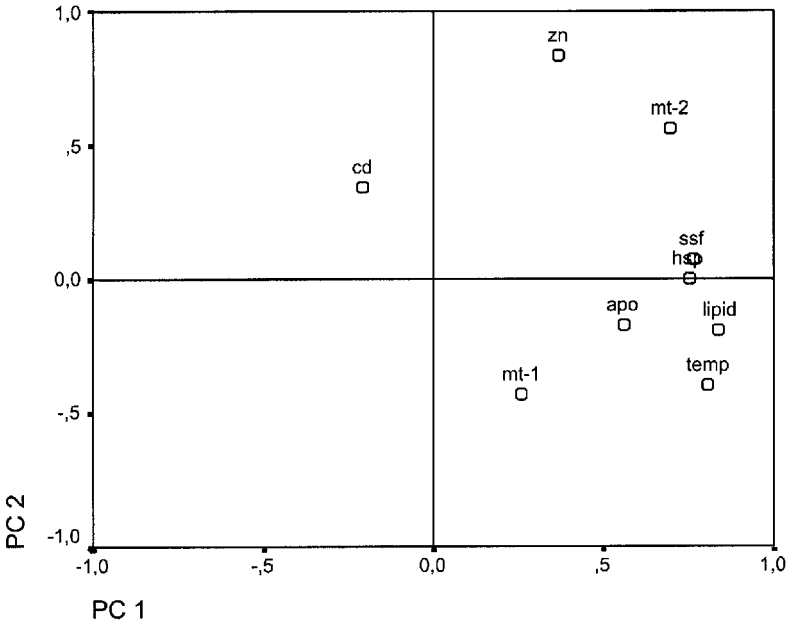


Figure 11. Factor analysis of the annual cycle data. For detailed information see Materials and Methods (Statistical Analysis).

Table 1. Results of the tests on significantly non-zero average annual cycles (see figures 3(a)–10(a) and of the pairwise comparisons of all standardized cycles (not displayed).

		Significant difference between parameter 1 and…						
Parameter 1	Significant cycle?	Cd	Lipid	HSP	MT1	MT2	SSF	Zn
Apoptosis	Yes	Yes	No	No	No	Yes	No	Yes
Cd	No	—	Yes	Yes	Yes	Yes	Yes	Yes
Lipid	Yes		—	No	Yes	Yes	No	Yes
HSP	Yes			—	Yes	No	No	Yes
MT1	Yes				—	Yes	No	Yes
MT2	Yes					—	No	Yes
SSF	Yes						—	Yes
Zn	Yes							—

significance between SSF and either water temperature ($p < 0.001$; $r_s = -0.279$), age ($p < 0.001$; $r_s = -0.320$) or zinc content ($p < 0.05$; $r_s = -0.199$). HSP were not determined in this examination.

MT-II contents showed the highest values at station G 08 (6.49 μg mg^{-1}). The lowest values were observed at station B 12 (0.42 μg mg^{-1}). There was a significant correlation between MT-II contents and either Zn ($p < 0.01$; $r_s = 0.371$) or water temperature ($p < 0.001$; $r_s = 0.663$) or Cd ($p < 0.001$; $r_s = 0.423$). MT-I ranged from 0.03 μg mg^{-1} (B 01) up to 0.39 μg mg^{-1} (G 08). Correlations of MT-I and the factors mentioned for MT-II were comparable.

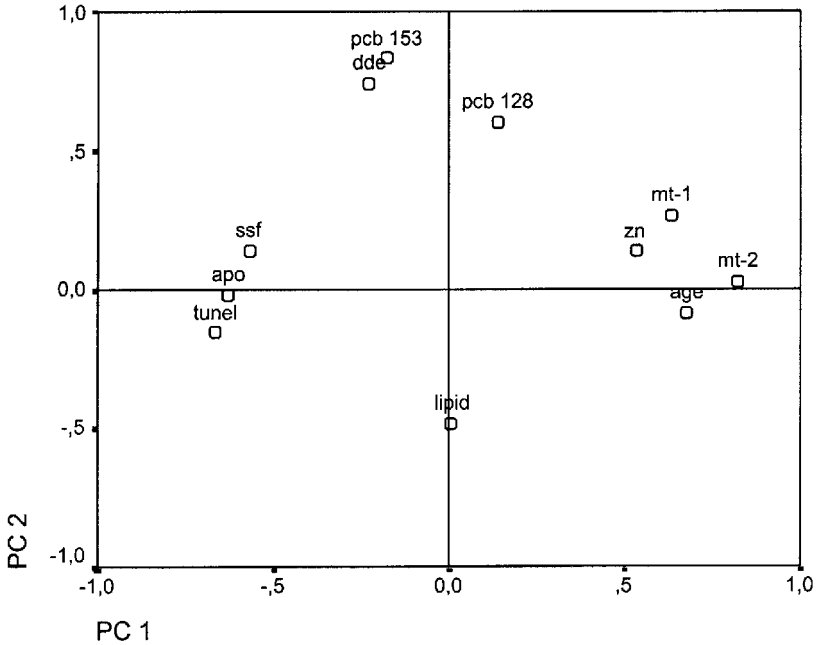


Figure 12. Factor analysis of the regional discrimination data. For detailed information see Materials and Methods (Statistical Analysis).

Table 2. Regional discrimination. Measured xenobiotics and the GSI at the sampling stations in the North Sea and Baltic Sea. Values are given as medians. Levels indicated by identical superscript letters are not significantly different ($p < 0.05$).

	B 01	B 12	N 01	N 04	N 06	G 08
Cd (μg kg^{-1})	80.1^{a}	71.0^{a}	114.6^{a}	110.0^{a}	161.2^{b}	482.6^{c}
DDE (μg kg^{-1})	64.9^{a}	207.8^{b}	52.4^{a}	71.0^{a}	77.0^{a}	82.0^{a}
CB 28 (μg kg^{-1})	11.3^{a,b}	20.2^{c}	17.9^{a}	10.0^{a,b}	12.8^{a,b}	20.2^{c}
CB 153 (μg kg^{-1})	54.7^{a}	136.0^{b}	110.6^{a}	44.1^{a}	53.0^{a}	163.7^{b}
GSI	8.3	7.1	1.3	3.8	6.5	1.9

Table 2 shows the results of the Cd and organochlorine analysis; the high values of Cd and PCBs at station G 08 are noteworthy. Furthermore high concentrations were observed at station B 12.

The factor analysis of the regional discrimination data (figure 12) revealed four distinct clusters. The first component describes correlations between apopotosis, SSF and TUNEL. Negatively correlated to these are the clustered parameters Cd, Zn, MT-I, MT-II and age. Clusters on the vertical axis are formed by the organochlorines and, in the opposite direction, by lipids. This indicates that the level of contamination with the determined organochlorines does not induce a noticeable response of the biomarkers of the sampled dabs.

Discussion

Correlations and their biological reasons

Ideally biomarkers are biological/biochemical parameters which respond to anthropogenic stressors. Basic investigations with regard to EROD activity and MT induction additionally revealed strong influences of physiological and abiotic factors (Goksøyr *et al*. 1996). At present, no detailed knowledge exists about the extent to which these physiological factors contribute to the changes in biomarkers as compared with anthropogenic factors. Therefore our examination focussed on variations in biomarkers under changing physiological conditions.

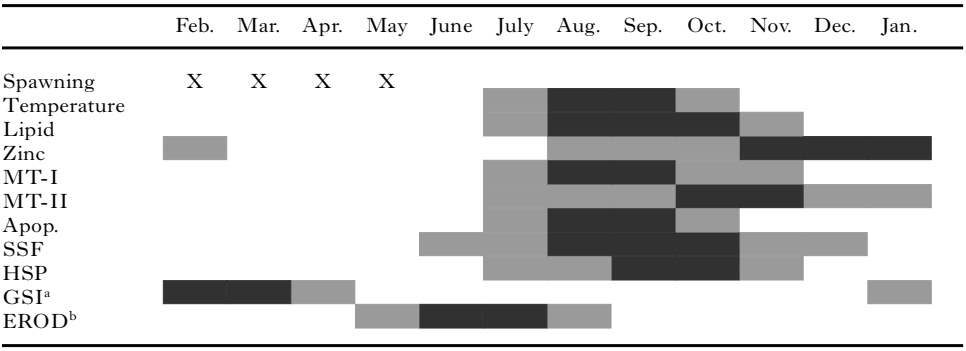
The physiology of the female dab is predominantly influenced by the water temperature and the reproductive cycle. The development of the ovaries represented by GSI starts in October during a strong temperature decrease and is initiated by increasing steroid hormone levels. In parallel, the exogenic vitellogenesis in the liver is initiated, accompanied by elevated metabolic activity reflected by raised mRNA and Zn levels. This process demands hepatic lipids which are accumulated during the summer. In spring the spawning season is characterized by minimal lipid and Zn contents in the liver. At this time water temperature increases and dab built up hepatic lipids as new energy reserves (Bohemen *et al*. 1981, Olsson *et al*. 1987, Saborowski and Buchholz 1996).

The results of the present study indicate a relationship between the factors water temperature and lipid metabolism to apoptosis, DNA strand breaks and HSP. It can be concluded that an elevated metabolic activity seems to be the key factor for the regulation of these indicators. Our observations do not allow a more detailed statement about the nature of the influencing factors. In contrast to MT induction, gonad development and spawning do not coincide with these biomarkers. MT isoforms show different cycles throughout the year, with MT-I peaking in a similar way to the above mentioned indicators. Therefore, it can be concluded that the regulation of MT-I is influenced by the temperature-dependent metabolic activity. As described in the literature, MT-II peaked at the same time as Zn (Olsson *et al*. 1987), namely during the gonad development. This result provides evidence for a possible role of MT-II in the Zn homeostasis. As for all other examined biomarkers no correlation with Cd nor with MT-I nor MT-II could be shown. A possible influence of Cd on MT induction during our annual observation could be masked by the major changes in Zn content.

The assessment of the contamination status at different locations showed differences in Cd and organochlorine contents. As demanded by the biomarker concept, these differences should be reflected by the selected indicators. This was found for MT isoforms and CD only, whereas all other biomarkers did not correlate with the measured contaminants. Parallel to our examinations, measurement of EROD activity and PCB values showed no correlation at the same stations at the same time (unpublished results).

Corresponding to results from the annual cycle, regional patterns of apoptosis, DNA strand breaks, HSP and MT isoforms were mainly influenced by the biological/abiotic factors Zn, GSI, and water temperature. Interestingly, induction of MT isoforms is correlated to Cd as well as to biological/abiotic factors, which can be explained by a far greater range of Cd values at the six stations compared with the Cd data during the annual cycle.

Table 3. Schematized presentation of the maxima in each annual cycle. Maxima are represented with dark coloured fields and increasing or decreasing values with grey coloured fields. For calculation see Materials and Methods.



[a] Sabrowski (1996).
[b] Vobach and Kellermann (1999).

Consequences for biological monitoring

For assessing the status of the marine environment of the North Sea and the Baltic Sea, international monitoring programmes are performed in the framework of the international conventions for the protection of the marine environment of the North Atlantic Ocean and the Baltic Sea.

Biological effect monitoring is part of international programmes like the 'Joint Assessment and Monitoring Programme' (JAMP) and the 'Cooperative Monitoring in the Baltic Marine Environment' (COMBINE). Therein guidelines are presented which are the basis for monitoring activities in the addressed regions such as the North Sea and the Baltic Sea, respectively. These guidelines include so called contaminant specific monitoring of biological effects and mention well known biological effect markers like EROD and MT (JAMP 1998a,b).

However, quality assurance is an important precondition for all monitoring activities and must be performed before monitoring activities start. With the EU funded project BEQUALM the installation of international intercalibration of biological effects methods had just begun. Therefore biological effect monitoring is desired but not mandatory under JAMP and COMBINE.

The intention of the cited guidelines is the measurement of biological effects in fish once a year outside the spawning season preferably simultaneously with measurements of relevant contaminants. These guidelines are based on the conviction that only physiological processes directly (cor)related to reproduction influence biomarker responses.

Our investigation suggests that annual cycles beyond those due to spawning behaviour exist. If it is understood that measurements should be made out of the spawning season during periods of low marker values, then a suitable period for each single marker can be taken from table 3. Analogously, in order to use a battery of biomarkers simultaneously (Goksøyr *et al*. 1996, Van der Oost *et al*. 1996), a period containing only marker minima is required. However, table 3 shows that such a common period for EROD, MT, HSP, SSF and apoptosis jointly does not exist. Moreover, the observed cycles seem to be associated with water temperature, lipid and zinc content. The cycles of these quantities can be assumed to vary to

some extent from year to year. Hence a corresponding variation in the biomarkers of interest must be expected simply as a consequence of variation in natural factors. Anthropogenic influences would induce additional variation in biomarker values. In order to derive a proper assessment of biomarker values, particularly to identify anthropogenic effects, the natural annual cycle variation of a marker must be accounted for.

Our measurements from different locations showed clear differences in parameter values, even though taken during a small time window. This could be explained by generally location-specific levels, but also as a consequence of location-specific annual cycles, or, most plausibly, as a mixture of both. Since there is no information about annual cycles at locations different from N 01, we are not able to identify the source of these observed differences. At least it seems unjustified to explain differences in biomarker response exclusively as caused by anthropogenic influences. Instead, a more elaborate assessment of biomarker values which includes the assessment of annual cycle effects is necessary. This needs location-specific information about the cyclic behaviour of the markers involved, and also the appropriate statistical methodology to separate natural and anthropogenic effects.

It should be noted that our conclusion about the existence of annual cycles in biomarker values is based upon the observation of a 1 year cycle at one location. Because of the explanations why such cycles might exist and the parallels to some well investigated markers, we assume that such cycles are general, although the shapes of the cycles actually found cannot simply be carried over to other times or locations. A confirmation of this assumption requires the analysis of additional data in this respect.

We conclude that the observed annual cycles are the major problem in biomarker evaluation. From this point of view the current guidelines for biological effect monitoring should be reconsidered.

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