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**Expression of heat shock protein 70 in a permanent cell line (EPC) exposed to
sediment extracts from the North Sea and the Baltic Sea**

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ABSTRACT

Heat shock proteins 70 (hsp70) are known to be induced by a great variety of chemical stressors. The effects of different environmental contaminants, which were identified in sediments of the North Sea and the Baltic Sea as main contaminants or as contaminants of special toxicological interest, on the extent of expression of hsp70 in a permanent cell line *Epithelioma papulosum cyprini* (EPC) were investigated. Concentrations of the single contaminants leading to elevated hsp70 levels were compared to the respective concentrations of these contaminants encountered in the sediment extracts thus allowing an assessment of their contribution to the toxicity of these sediments. Analysis of hsp70 was performed using western blotting techniques with subsequent comparative quantification by densitometry. Eleven of thirteen contaminants have shown a dose-dependent increase in hsp70 levels. An expression of hsp70 was observed in most cases between 0,1-10 µg/mL test solution. Some of the investigated stressors led to extremely high hsp70 contents even at low concentrations. However, in most cases contaminant concentrations in sediments were too low to cause an effect. For this reason, effects of the tested sediments are attributed to other contaminants or rather to mixtures of stressors.

Keywords: EPC, marine sediment, Heat shock proteins, bioassay, sediment toxicity, sublethal stress

INTRODUCTION

A multitude of anthropogenic substances reaches the North Sea and the Baltic Sea and leads to a considerable burden on the marine environment. Sediments function as both a sink and, in the case of remobilisation, as a source. Many national and international monitoring programmes include the analysis of contaminants that are known to be toxic and / or persistent. Chemical analysis alone is insufficient for the assessment of sediment toxicity. It is impossible to determine every anthropogenic contaminant and, furthermore, the effects of most contaminants on the aquatic organisms are still unknown (Ankley *et al.*, 1996). For this reason, the combination of chemical analysis with biological tests becomes important (Fernández *et al.*, 1992; Brack *et al.*, 1999).

Cellular stress protein response, in particular the induction of hsp70, has received increasing attention as a potential marker for sublethal stress, because of its sensitivity to a wide variety of environmental contaminants (Williams *et al.*, 1996; Werner *et al.*, 1998; Marino *et al.*, 1999). Members of the hsp70 family are involved in the cellular protein repair system. They stabilise or 'repair' damaged or aberrant proteins and dissolve protein aggregates present in cells (Ellis, 1990; Gething & Sambrook, 1992;

Rutherford & Zuker, 1994; Hartl, 1996). Increasing occurrence of damaged proteins due to xenobiotics or other forms of stress leads to elevated stress protein content. Numerous inorganic and organic xenobiotics are well known for their ability to induce the synthesis of hsp70 (Salminen *et al.*, 1996; Bierkens *et al.*, 1998; Schröder *et al.*, 1999; Kilemade & Mothersill, 2001).

The aim of this study was to determine the induction of hsp70 as the result of incubation with two extracts of one North Sea and one Baltic Sea sediment. These sediments differed much from each other regarding to their anthropogenic contamination. While a wide range of contaminants existed in the North Sea sediment, the sediment from the Baltic Sea was mainly contaminated by polycyclic aromatic hydrocarbons (Biselli *et al.*, 2005). In addition to the sediment extracts, several quantitatively dominating contaminants (e.g. polycyclic aromatic hydrocarbons) or substances with structural characteristics of toxicological potential (e.g. quinones, halogenated compounds, sulphides) were selected for individual testing. Single contaminants will be discussed as potential causes for the toxicity observed in the sediment extracts.

MATERIALS AND METHODS

Chemicals

All chemicals were of analytical grade and purchased from Sigma-Aldrich (Germany) or Merck (Germany) if not otherwise noted.

Sediment collection and sediment extraction

The North Sea sediment was collected from the German Bight (53°50'N, 8°10'E) in 2000. The Baltic Sea sediment was collected from the 'Kieler Förde' (54°20'N, 10°10'E) in 2001. The German Bight is influenced by the rivers 'Elbe' and 'Weser' and a known sink for contaminants coming from these both rivers. The 'Kieler Förde' is in a coastal region and known to be relatively highly contaminated. Sediments were sampled by a box corer with the research vessel 'Gauss' of the German Federal Maritime and Hydrographic Agency. The upper 2 cm of sediment were collected and stored until extraction at -20 °C in the dark. In the first place pore water was separated by centrifugation (25 min, 3500 x g). With the pore water even inorganic compounds were separated, so the experiments were focused on sediment bound organic contaminants. Afterwards the wet sediment was sequentially extracted with acetone and *n*-hexane by ultraturrax-treatment. Solvents were combined and the water phase was removed by

refrigeration. Extracts were reduced by rotary evaporation with dimethyl sulfoxide (DMSO) as keeper. Solvent residues were removed under a nitrogen stream. The resulting extracts contained equivalents of about 4 to 5 g sediment (dry weight) per mL DMSO.

Identification and quantification of single substances

Identification and quantification of single substances were performed by different methods of GC/MS-analysis. For detailed information see Biselli et al. (2005). The entire procedure (extraction, clean-up, and quantification) was accompanied by suitable methods of quality assurance. All relevant steps of analysis were checked for recovery rates and contaminations. The average recoveries of most PAHs and chlorinated hydrocarbons ranged from 60-100 %. The accuracy of GC/MS quantification was ascertained by measuring certified standard solutions.

Cell line and cell treatment

The applied fish cell line 'Epithelioma papulosum cyprini' derived from a skin tumour of carp (*Cyprinus carpio* L.) was provided by the Collection of Cell Lines in Veterinary Medicine of the Federal Research Centre for Virus Diseases of Animals (Riems, Germany) and was kindly provided by the Federal Research Centre for Fisheries, Institute of Fisheries Ecology. Cells were cultured as described by Kammann *et al.* (2001). After developing a complete monolayer, cells were trypsinised using Trypsin/EDTA, washed twice with phosphate buffered saline (PBS; 0.1 M K₂HPO₄, 0.15 M NaCl, pH 7.2), resuspended in PBS and distributed to several incubation vessels. By that procedure results proved to be more reproducible as experiments were performed with an homogenous cell suspension. For exposure to sediment extracts or single contaminant test solutions (sediment extracts or solutions of contaminants at concentrations of 0.1 to 10 µg/mL DMSO) were added to resuspended cells in duplicate. Since DMSO, under the described conditions, led to an hsp70-expression at concentrations higher than 2 % (data not shown), a DMSO concentration of 1 % was chosen for all experiments. Incubation time was 2 h at room temperature. In previous investigations this incubation time prove to be sufficient for an hsp70-expression. Longer incubation times yielded a slight increase of hsp70-levels until a decrease due to cytotoxic effects (data not shown). In addition, hsp70-levels of four to six control groups (depending on the size of the experiment) were determined using untreated cells without incubation. In every experiment an incubation with cadmium chloride (100 µM) was performed as a positive control to verify the success of the experiment. After incubation cells were washed twice with PBS, resuspended in PBS supplemented

with 1 mM phenylmethylsulfonyl fluoride, and homogenised via ultra sonic treatment. A reducing sample buffer (Vieths *et al.*, 1992) was immediately added to each sample at a 1:1 dilution, and samples were boiled for three minutes.

Gel electrophoresis and Western blotting

The reduced cell suspension containing about 25 µg total protein was subjected to a 10 % polyacrylamide gel with a 5 % stacking gel (Invitrogen) using the buffer system described by Laemmli (1970). Gels were run at 120 mA for 35 minutes (Xcell SureLock Mini Cell, Invitrogen).

Proteins were electroblotted onto nitrocellulose membranes (Schleicher&Schuell, Germany) at a constant current of 180 mA for 1 h using a semi-dry blotting apparatus (Invitrogen) in blotting buffer (25 mM Tris, 20 % ethanol; pH 10.4). Membranes were twice blocked with PBS containing 0.1 % Tween20 and 5 % skimmed milk powder for 15 minutes under permanent shaking and washed twice with double distilled water. Blots were incubated overnight with monoclonal anti-hsp70 directed against bovine hsp70 (BRM-22, Sigma). The primary antibody was diluted 1:10,000 with PBS containing 0.1 % bovine serum albumin and 0.05 % Tween20. Blots were then washed three times with PBS containing 0.05 % Tween20. An biotinylated secondary antibody (goat-anti-mouse IgG, Dako; dilution 1:5,000 in PBS containing 0.1 % bovine serum albumin and 0.05 % Tween20; incubation time 3 h) was used to detect primary anti-hsp70. After three washing steps blots were incubated with horseradish-peroxidase conjugated streptavidine solution (Medac) diluted 1:20,000 with PBS containing 0.1 % bovine serum albumin and 0.05 % Tween20 for 20 minutes. Hsp70-bands were visualized with the substrate 3,3',5,5'-tetramethyl benzidine (Fluka; incubation time approx. 5 minutes). Developed blots were scanned and quantification was performed by densitometric evaluation (Biometra, Scan Pack 3.0) by comparing band areas of samples and an hsp70 standard of bovine brain (Sigma) on the same membrane. For checking transfer efficiency each membrane contained a sample of the control group as well. Variation in bands of equal amounts of hsp70 was approx. 15 %, derived from a multiple determination of one sample.

Cytotoxicity

In preliminary experiments cytotoxicity was detected by the lactate dehydrogenase leakage assay (Sigma). The lactate dehydrogenase activity was determined after incubation and centrifugation in the supernatant with *p*-iodonitrotetrazolium violet as substrate according to Decker & Lohmann-Matthes

(1988). Simultaneously, the total protein content was measured in cell suspension after homogenisation by the method of Bradford (1976) and expressed as percentage of control. A good correlation between protein loss and the lactate dehydrogenase leakage assay was observed (data not shown). Cytotoxic effects resulted in a protein loss of more than 20 % referring to the control group. In further experiments the protein content was used as an indicator for cytotoxic effects.

Statistical analysis

In order to compare toxic effects a threshold concentration of the investigated sediment extracts or the investigated contaminants was determined. The threshold concentration was defined as the concentration of sediment extracts or contaminants calculated from the dose-effect-curves leading to an induction of hsp70 of 150 % with reference to the control group (100%).

RESULTS

Induction of hsp70 by incubation with sediment extracts

The results of the relative induction of hsp70 produced by incubation of EPC-cells with sediment extracts in a range of 0.1 to 50 mg dry weight/mL test solution are shown in Fig. 1. In both cases incubation with higher sediment concentrations (4-50 mg dry weight/mL test solution) did not result in significantly elevated hsp70-contents or to cytotoxic effects (data not shown). However, a significant expression of hsp70 was observed after incubation with sediment extracts in the range between 0.2 and 1-4 mg dry weight/mL test solution. In this range hsp70-contents up to 175 % relative to the control group were measured. Threshold concentrations of approx. 0.25 mg dry weight/mL test solution (North Sea) and approx. 0.7 mg dry weight/mL test solution (Baltic Sea) were determined from the dose effect relationships.

Induction of hsp70 by selected contaminants found in the North Sea and the Baltic Sea sediments

The results of the application of selected contaminants to EPC-cells are summarized in Table 1. Presented results show that the tested substances exerted different effects on the induction of hsp70.

Hsp70 was induced by nearly all compounds tested except for *N,N'*-diethyl-*N,N'*-diphenyl urea and 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethyl-cyclopenta[g]-2-benzopyran (HHCB, galaxolide®).

Cytotoxic effects were caused by benzantrone and HHCB in the highest concentrations applied to EPC-cells.

Remarkable effects were measured after incubation of EPC-cells with tris(2-chloroethyl)phosphate and 2-methylthiobenzothiazole. These compounds, especially 2-methylthiobenzothiazole, led to a strong expression of hsp70, up to approximately 20-fold increase in content compared to the controls. In all other experiments in this study, the expression of hsp70 did not exceed 250 % compared to the control. Furthermore, significantly elevated hsp70 levels were observed for these compounds as well as for fluoranthene over nearly the whole range tested, whereas in contrast after incubation with the other compounds significantly increased hsp70 levels were determined at one to three concentrations. Also noteworthy is the fact, that in some cases the maximum induction was not associated with the highest concentrations of contaminants. Only in case of the compounds phenanthrene, 9,10-phenanthrene quinone, 4-bromo phenol, tris(2-chloroethyl)phosphate, and carbazole the concentration of 10 µg/mL test solution induced maximal elevated hsp70 levels of about 1.5 to 5-fold compared to controls. During exposure to 3-methyl quinoline and benzantrone highest expression of hsp70 was observed at a concentration of 5 µg/mL test solution. A test solution of 2 µg/mL led to the maxima of the dose-effect-relationship in the case of pyrene, dieldrine, and 2-methylthiobenzothiazole, while after exposure to fluoranthene the maximum of hsp70 induction was observed at 1 µg/mL test solution.

DISCUSSION

The dose-effect relationships resulting from exposure to crude sediment extracts as well as from incubation with single contaminants showed a similar development. After having reached the threshold concentration a dose dependent increase in expression of hsp70 up to a maximum of induction was observed. Further increasing concentrations led to a decrease in hsp70 expression down to contents of the control group or even lower contents. However, in the latter case this can be attributed to cytotoxic effects. The curve described above was observed after exposure to both sediment crude extracts and to the single contaminants 3-methyl quinoline, pyrene, fluoranthene, 2-methylthiobenzothiazole, dieldrine, and benzantrone. In the case of phenanthrene, 9,10-phenanthrene quinone, 4-bromo phenol, tris(2-chloroethyl)phosphate, and carbazole the maximum of induction was reached with the highest concentrations, so an equivalent cytotoxic response is still possible if higher concentrations of these substances were applied to the test.

The observed dose relationship for hsp70 expression was described for numerous single substances such as heavy metals (Eckwert *et al.*, 1997), paracetamol (Neuhaus-Steinmetz & Rensing, 1997), sodium dodecyl sulfate (Vijayan *et al.*, 1998), oxyfluorfen (Hassanein *et al.*, 1999), and sodium arsenite (Edwards *et al.*, 1990) in other investigations. To date, a dose-effect relationship resulting from incubation with complex mixtures like sediment extracts have not been noted by the authors.

Eckwert *et al.* (1997) subdivided the curve of hsp70 expression into three zones. The first zone, at low concentrations of contaminants, shows no increase in hsp70 contents. Any small damage is compensated by existing levels of hsp70 or just marginal induction of hsp70. In the second zone hsp70 levels increase drastically due to the cellular damage caused by higher concentrations of stressors. The maximum of hsp70 expression is reached at the end of this zone. In the third zone hsp70 expression decreases down to control or even lower levels. The decrease in hsp70 expression is partly due to strong damage of the cells' protein synthesis apparatus required for hsp70 synthesis (Meyer *et al.*, 1995). Marked pathological damage is also partly responsible for the reduction in hsp70 (Pawert *et al.*, 1996; Triebkorn & Köhler, 1996). Cytotoxic effects like membrane damage lead to decreasing hsp70 contents compared to control groups (Köhler *et al.*, 1996).

In this study no decrease in hsp70 levels compared to control groups was observed in the case of sediment extract exposure. After having returned to the control levels of hsp70 at a distinct sediment concentration, this level stayed the same even at higher sediment concentrations. Another kind of effect, an inhibition of hsp70 synthesis, may be responsible for this result. An inhibition of the hsp70 synthesis by substances like glycerol, copper and some flavonoids was described by several research groups (Edington *et al.*, 1989; Hosokawa *et al.*, 1990; Sanders & Martin, 1994). Werner *et al.* (1998) observed a negative correlation between hsp60 induction and some organotin compounds between expression of hsp70 and benzo[*b,k*]fluoranthene and benzo[*ghi*]perylene after exposure of 23 sediments to amphipods (*Ampelisca abdita*), respectively. There was a suggestion of an inhibition by some polycyclic aromatic hydrocarbons in the course of a bioassay-directed fractionation (data not shown). Specific substances from complex mixtures leading to an inhibition of hsp70 syntheses still need to be determined.

In conclusion, the observed shape of the dose-effect-curve makes it clear, that measured effects depend on the concentration tested. To avoid false conclusions, the analysis at different concentrations is essential, regardless whether testing single contaminants or complex mixtures. The procedure of determining threshold concentrations for complex mixtures requires that substances, which inhibit the hsp70-

synthesis, are not included – but this is not practicable for samples of unknown composition. Altogether, the mentioned aspects show that hsp70-induction is not a practical marker for analysis of complex mixtures.

Comparison of the concentrations of contaminants leading to hsp70 inductions and concentrations in sediments

In Table 2 the minimal concentrations of the tested contaminants leading to a significant expression of hsp70 are compared to the concentrations existing in the test solution during exposure to sediment extracts. Obviously, no single contaminant is responsible for the observed effects. On the other hand, effects in sediment crude extracts were even determined at dilutions of 1:100 and more. Only for the contaminants fluoranthene, pyrene and tris(2-chloroethyl)phosphate is a contribution to an effect produced by the undiluted crude extract feasible (Table 2). The results suggest that the effects observed after exposure to the two sediment extracts can probably be traced back to a mixture of various contaminants present in the sediment, rather than to a single substance.

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Fig. 1. Dose effect relationships of hsp70-induction and dry weight of the investigated sediment extracts applied to the bioassay. Cells were exposed for two hours to several concentrations of dry weight/mL test solution. A -Sediment of the German Bight (North Sea); B - Sediment of the Kieler Förde (Baltic Sea). Represented is the range of Hsp70 levels determined by two individual experiments.

TABLE 1

Expression of hsp70 after a two hours exposure to various contaminants of the North Sea and the Baltic Sea.

Contaminants ($\mu\text{g/mL}$ test solution)	0.1	0.2	0.5	1	2	5	10
Phenanthrene	108	103	99	113	122	129	154
9,10-Phenanthrene quinone	111	97	103	103	112	150	180
3-Methyl quinoline	101	109	107	103	147	231	168
Pyrene	75	104	117	120	179	130	89
Fluoranthene	100	113	128	254	200	188	149
4-Bromophenol	98	90	107	100	126	138	153
Tris(2-chloroethyl)phosphate	99	132	135	170	420	486	523
<i>N,N'</i> -diethyl- <i>N,N'</i> -diphenyl urea	105	94	108	103	90	97	99
HHCB (Galaxolide [®])	107	95	100	113	107	113	<u>103</u>
2-Methylthiobenzothiazole	132	183	197	225	2183	683	245
Dieldrine	104	108	108	150	183	137	93
Benzanthrone	109	108	104	97	99	147	<u>76</u>
Carbazole	87	99	92	103	111	123	145

Hsp70 values are shown as mean relative contents according to the control group (n=2). Bold numbers show hsp70-contents significantly elevated to control ($p<0.05$). Underlined numbers show cytotoxic effects.

Table 2

Comparison of concentrations of contaminants leading to an expression of hsp70 with the concentrations in the sediment extract applied to EPC-cells.

Contaminants	c ^a [ng/g d.w.]		c ^b [ng/mL test solution]		c ^c [ng/mL test solution]	f	
	NS	BS	NS	BS		NS	BS
Phenanthrene	18	654	1	26	5000	5x10 ³	190
9,10-Phenanthrene quinone	-	x	-	x	5000	-	> 1x10 ⁵
3-Methyl quinoline	x	x	x	x	2000	> 1x10 ⁵	> 1x10 ⁵
Pyrene	27	2225	1.5	89	1000	670	11
Fluoranthene	30	1924	1.6	77	200	125	2.6
4-Bromophenol	360	-	19	-	10000	530	-
Tris(2-chloroethyle)phosphate	160	x	8.6	x	200	23	> 1x10 ⁵
2-Methylthiobenzothiazole	20 ^d	-	1 ^d	-	100	100	-
Dieldrine	x	x	x	x	1000	> 1x10 ⁵	> 1x10 ⁵
Benzanthrone	x	x	x	x	5000	> 1x10 ⁵	> 1x10 ⁵
Carbazole	x	20 ^d	x	1 ^d	5000	> 1x10 ⁵	0.02

^a = concentration in sediments [ng/g dry weight], ^b = concentrations in test solution (considering 5.4 (4.9) g d.w./mL DMSO and 1 % DMSO/test solution), ^c = minimal concentration leading to an significant expression of hsp70, f = factor between c^c and c^b, NS = North Sea, BS = Baltic Sea, - = not detected, ^d = estimated concentration, x = just present in traces (< 1 ng/g d.w. respectively < 0.05 ng/mL test solution)