

SIXTH FRAMEWORK PROGRAMME



Project contract no. 003933

THRESHOLDS
Thresholds of Environmental Sustainability
INTEGRATED PROJECT

Priority 1.1.6 "Sustainable Development, Global Change and Ecosystems"
Sub-Priority 1.1.6.3 "Global Change and Ecosystems"

Stream 4 – D4.1.1
Identification of molecular biomarkers in the marine diatom
Thalassiosira pseudonana

Due date of delivery: June 2006
Actual submission date: June 2006

Start date of project: 1st of January 2005

Duration: 48 months

Lead authors for this deliverable: S. Bopp & T. Lettieri (European Commission – DG Joint Research Centre, Institute for Environment and Sustainability)

Project co-funded by the European Commission within the Sixth Framework Programme (2002-2006)		
Dissemination Level		
PU	Public	X
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Executive Summary

The classical way to investigate (eco)toxicological effects of chemicals or environmental pollutants is to look for acutely toxic effects. Commonly used endpoints are often not sensitive enough to detect effects at low level of contamination and thus cannot be used as early-warning tools for adverse effects. Looking at effects on subcellular level, i.e. at gene expression and protein level alterations, offers a promising alternative as every (even long-term) response elicited by a chemical will also affect cellular and molecular level processes.

It was therefore the aim of the current study to identify biomarkers at gene expression level for detection of organisms' exposure to chemicals. The marine diatom, *Thalassiosira pseudonana*, was chosen as model organism because of its ecological relevance and the fact that its genome was recently completely sequenced. Three polycyclic aromatic hydrocarbons (PAHs) were chosen as model compounds to identify molecular biomarkers. Diatoms were exposed to three PAHs: pyrene, fluoranthene, and benzo[a]pyrene as single compounds and to a mixture of all three. Firstly, dose-response curves for growth inhibition were determined and secondly, alterations at gene expression level were investigated using Real-time Polymerase Chain Reaction (RT PCR). Potentially suitable genes were selected upon an extensive literature study. All chosen genes are involved in important cellular processes and metabolic pathways, e.g. in silica shell formation, photosynthesis and signal transduction.

Growth inhibitory effects due to PAHs occurred only at relatively high concentrations, i.e. EC₅₀ values were above aqueous solubility. The effects elicited by the mixture of the three compounds, were in good agreement with predictions from calculations based on the concentration addition method.

Among the chosen target genes, one (*lacsA*) could be identified as suitable biomarker gene. For the others, a general conclusion is difficult due to high variability between results from individual biological experiments.

1. Introduction

In recent years, there has been a growing awareness of the need to improve the ability to detect and assess the water quality as well as adverse effects of environmental pollutants in water. Progressing methodologies in chemical environmental analysis enable a fast and sensitive detection of a broad variety of compounds. However, in chemical analysis, the focus is generally on pollutants known or suspected to be present at a certain site. Thus, unexpectedly occurring compounds, which could be of toxicological relevance, might be overlooked. Therefore, (eco)toxicological analyses can be an important complement.

In classical ecotoxicological studies, effects of chemicals or environmental pollutants are mostly investigated concerning their acutely toxic effects. Within the past years, also the use of biomarkers in ecotoxicology arose (e.g. Van der Oost *et al*, 2003). Biomarkers are defined as changes in biological responses (on molecular, cellular or physiological level) caused by environmental contaminants. Commonly used endpoints and biomarkers are often not sensitive enough to detect effects at low level of contamination and thus cannot be used as early-warning tools for adverse effects. The currently emerging field of molecular ecotoxicology offers new possibilities for sensitive detection of deleterious effects of environmental pollutants at an early stage, on gene regulation and at protein level. Assessment of gene expression alterations offers a promising alternative, as every (even long-term and sub-chronic) response elicited by a chemical will also affect molecular level processes.

In the present study, biomarkers on gene expression level in the marine, centric diatom *Thalassiosira pseudonana* were identified. Diatoms are eukaryotic algae, which can be found worldwide in oceans and freshwater. They play an important role in the global carbon cycle as they are thought to contribute at about 25 % to the global primary production (Falciatore *et al*, 2002). Diatoms are rarely utilized as test organisms in ecotoxicological investigations, but their use can be a meaningful, promising approach due to their ecological relevance. Furthermore, the sequencing of the whole genome of *Thalassiosira pseudonana* as the first diatom species (Armbrust *et al*, 2004) paved the way for the use of diatoms in molecular ecotoxicology.

In the current study, we investigated effects elicited by polycyclic aromatic hydrocarbons (PAHs) in *Thalassiosira pseudonana* on the entire organism and at molecular level. Three polycyclic aromatic hydrocarbons (PAHs) were chosen as model compounds to identify molecular biomarkers. Diatoms were firstly exposed to three PAHs (pyrene, fluoranthene, and benzo[a]pyrene) as single compounds

and secondly to a mixture of all three. Dose-response curves for growth inhibition were determined and four concentrations eliciting from “no effect” up to a severe growth inhibition were chosen for further investigation to detect alterations at gene expression level. Gene expression from exposed diatoms was investigated using Real-time Polymerase Chain Reaction (RT PCR). Potentially suitable genes were selected upon an extensive literature study. All chosen genes are involved in important cellular processes and metabolic pathways, e.g. in silica shell formation, photosynthesis and signal transduction.

2. Material and Methods

2.1. Culture conditions

Thalassiosira pseudonana (strain CCMP 1335) was obtained as axenic culture from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (West Boothbay Harbour, Maine, USA). Diatoms were maintained at 6-8°C under a diurnal light cycle of 13 h light and 11 h darkness. The culture medium was adapted from f/2-medium (Guillard R.R.L., 1975) prepared in filtered Boothbay Harbor sea water to a preparation in artificial sea water in order to achieve reproducible conditions. For this purpose, an artificial sea salt mixture (Sigma-Aldrich, Steinheim, Germany) was used to prepare a 3.2 % salt solution in distilled water. After autoclaving the artificial sea water (ASW) the f/2 medium components were added aseptically using an f/2 medium kit (CCMP, Maine, USA). *T. pseudonana* was cultured in Erlenmeyer flasks closed with metal caps at a density between $0.5 \cdot 10^6$ - $5 \cdot 10^6$ cells/mL. Doubling times under these conditions were ca. 24 h.

2.2. Exposure of *T. pseudonana* to PAHs

Effects of three polycyclic aromatic hydrocarbons (PAHs) on *T. pseudonana* were tested as single compounds and as a mixture of the three. The chosen PAHs were pyrene (Pyr, purity >99%, Fluka, Buchs, Switzerland), fluoranthene (Flu, purity >99%, Aldrich, Buchs, Switzerland) and benzo[a]pyrene (BaP, purity >97%, Fluka, Buchs, Switzerland).

In a first step, growth inhibition due to the chemicals was investigated in batch cultures. 20 mL of test cultures were inoculated from seven day old cultures to a start cell density of $5 \cdot 10^5$ cells/mL. To avoid lag phases in growth during the exposure times, PAHs were added after a pre-incubation of the cultures

for 24 h. After that pre-incubation phase cell density at the start of the exposure was measured as described below. PAHs were dissolved in methanol and added to the culture leading to a concentration of methanol of 0.05 % in the culture medium. Methanol was investigated before to have no effect on the diatom growth up to the highest tested concentration of 0.5 %. Additionally, a solvent blank was always added in each experiment. Dosing of methanol and PAH solutions was always performed within 30 min before the light cycle started, so that the first part of the exposure took place in the light and the second in the dark. After 24 h of exposure, growth inhibition (I_μ) with respect to the untreated control cultures was determined using the growth rates (μ). For this purpose, cell density was determined after transferring each 200 μ L of diatom suspension to 96 well plates (Falcon, BD Biosciences, Milano, Italy) and measuring the absorption at 450 nm using a microplate spectrophotometer (Biorad, Segrate, Milan, Italy) (Figure 2.1).

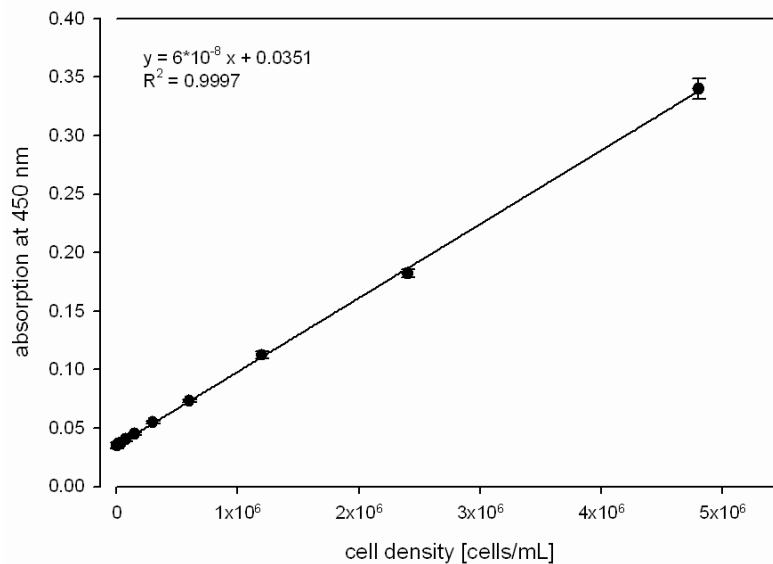


Figure 2.1: Correlation between cell density and absorption measurements at 450 nm. Even at low cell densities, absorption can be used as a measure of cell number. Cell densities used in the experiments were between $5 \cdot 10^5$ and $3 \cdot 10^6$ cells/mL.

Growth rates and growth inhibition were calculated as follows:

$$\mu = \frac{\ln N_t - \ln N_0}{t - t_0}$$

μ = growth rate
 N = cell density
 $t - t_0$ = exposure time [days]

$$I_{\mu} = \frac{\mu_C - \mu_T}{\mu_0} \cdot 100$$

I_{μ} = inhibition of the growth rate with respect to the untreated control [%]
 μ_C = growth rate of the control culture
 μ_T = growth rate of the treated culture

After testing the single compounds at least in four independent experiments, a mixture of the three compounds was tested. EC_{50} values for the three single compounds were calculated and the mixture was prepared according to the ratio of their EC_{50} values. I.e. in the highest concentration of the mixture, each single PAH was present at its single compound $EC_{50,i}$ concentration (concentration named as 1x $EC_{50,i}$). Additionally, seven dilutions of this PAH mixture, with a dilution factor of three, were tested.

2.3. Calculation of expected mixture toxicity

In order to evaluate the effects elicited by the mixture in comparison to the effects found for single compounds, an expected toxicity of the mixture was calculated from the single compound exposure results. As the three used compounds belong all to the same class of contaminants and their mode of action is supposed to be similar, the use of the concentration addition concept seems adequate (e.g. Altenburger *et al*, 2004; Maciel & Zaldivar, 2005).

For this purpose, the proportion p_i of each single compound in the mixture is calculated and divided by its own $EC_{50,i}$ value. The reciprocal of the sum of these values results in the predicted EC_{50} value for the mixture ($EC_{50,mix}$).

$$p_i = \frac{C_i}{C_{mix}}$$

C_i = concentration of the single compound in the mixture [nM]

C_{mix} = sum of all single compound concentrations [nM]

$$EC_{50,mix} = \frac{1}{\sum_{i=1}^n \frac{p_i}{EC_{50,i}}}$$

p_i = proportion of single compound in the mixture

$EC_{50,i}$ = EC_{50} for the single compound [nM]

2.4. Sample preparation for RNA extraction

Diatoms exposed to PAH were harvested at the end of the experiment. For this purpose, 20 mL of diatom suspension were centrifuged at 1500 rcf for 10 min. The supernatant was removed and the cell pellet resuspended in 0.5 mL PBS buffer (Gibco, Invitrogen, San Giuliano Milanese, Milan, Italy). The

suspension was transferred to 1.5 mL Eppendorf tubes and centrifuged at 10,000 rcf for 10 min. Afterwards, the supernatant was removed and the remaining cell pellets were frozen at -80 °C.

2.5. RNA extraction

RNA extraction was performed using the Trizol LS (Invitrogen, San Giuliano Milanese (MI), Italy) method according to the proposed protocol. In order to improve the yield and quality of the extracted RNA, small deviations from the provided protocol were adapted: for cell homogenization the pellets were lysed in Trizol LS for 15 min instead of 5 min; after the addition of chloroform, samples were centrifuged for 20 min and all centrifugation steps were performed at 16,000 rpm.

2.6. DNase treatment of the RNA samples and cDNA synthesis

For removing potential DNA contaminations from the RNA samples, they were treated with DNase (DNase I, 1000 U/ μ L, Roche Diagnostics, Monza (MI), Italy). Each 2 μ g of RNA were treated according to the manufacturer's protocol, using a 10x Reverse Transcription buffer (Applied Biosystems, Monza, Milan, Italy). Samples were incubated with the DNase for 15 min at room temperature. Afterwards, 1 μ L of 25 mM EDTA was added and the DNase in the samples was inactivated by a 10 min incubation at 65 °C.

The whole sample of DNase treated RNA was transcribed to cDNA using oligodT primers and SuperScript II Reverse Transcriptase Kit as recommended (Invitrogen, San Giuliano Milanese , Milan, Italy).

2.7. Choice of housekeeping and target genes

Eight genes involved in different cellular processes were chosen as potential biomarker genes upon literature review. Transcript sequences were taken from either the NCBI Nucleotide database or the Joint Genome Institute (JGI) *Thalassiosira pseudonana* genome project database. The potential target genes were involved in crucial cellular processes, such as e.g. photosynthesis, silica shell formation, and fatty acid metabolism (Table 2.1).

Table 2.1: Investigated housekeeping and target genes

Name of gene	Abbreviation	Cellular processes involved in
-tubulin	<i>tub2</i>	formation of microtubules
actin	<i>act</i>	formation of microfilaments / cytoskeleton
Glyceraldehyde-3-phosphate dehydrogenase	<i>GAPDH</i>	glycolysis
Fucoxanthin-chlorophyll a/c light harvesting protein	<i>3HfcpA</i>	photosynthesis
Fucoxanthin-chlorophyll a/c light harvesting protein	<i>3HfcpB</i>	photosynthesis
Long chain acyl coA synthetase	<i>lacsA</i>	fatty acid metabolism
RBJ like protein	<i>RBJ</i>	Ras-related GTP binding protein, involved in the development and maintenance of nervous system and/or reproductive apparatus
Silaffin precursor 1	<i>Sil1</i>	silica shell formation
Silaffin precursor 2	<i>Sil2</i>	silica shell formation
Silaffin precursor 3	<i>Sil3</i>	silica shell formation
Sphingolipid delta-8 desaturase	<i>desB</i>	fatty acid metabolism, signal transduction and cell recognition

In order to exclude differences occurring due to different yields in cDNA synthesis, expression values from target genes are normalized to the expression of so-called housekeeping or reference genes, which are consistently expressed. Actin and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are often reported housekeeping genes in several organisms and were therefore investigated also for *T.*

pseudonana. -tubulin seems to be a specially promising candidate as it was used as housekeeping gene in the diatom *Thalassiosira weissflogii* (Armbrust & Galindo, 2001).

2.8. Conventional PCR

Conventional PCR was performed in order to check whether the chosen gene sequences were adequate for investigation in real-time PCR. Twelve genes were investigated using the Taq DNA Polymerase Kit (Qiagen, Milan, Italy) and a RoboCycler Gradient 96 (Stratagene, M-Medical srl, Cornaredo, Milan, Italy). The PCR program was: 5 min at 95°C, then 33 cycles of 45 s at 94 °C, 45 s at 52 °C, and 1.5 min at 72 °C, followed by a last cycle of 5 min at 72°C. Afterwards, PCR products were investigated on a 2% agarose gel, and stored at -20°C. For optimization of annealing, a temperature gradient from 48 to 60 °C was investigated and 52 °C was found to be the optimum temperature for all primer pairs.

2.9. Gel electrophoresis for RNA and PCR products

RNA quality and size of PCR products were checked using gel electrophoresis. Agarose gels (1% for RNA and 2% for PCR products), containing 50 µg ethidium bromide, were prepared in 1x TAE buffer, which was also used for electrophoresis. Electrophoresis was performed at 100 V for 20 to 50 min (Wide Mini Sub Cell GT with Power Pac 300, Bio-Rad). Visualization of band was performed in a Gel Doc Station (XR Systems, Bio-Rad).

2.10. Real-time PCR

For Real-time PCR, primers and MGB-FAM-TaqMan probes were designed using Primer Express Software (Applied Biosystems). The gene regions chosen for the primer design were validated using conventional PCR beforehand. Primers and probes were synthesized by Applied Biosystems. For validation of the housekeeping genes, SYBR Green was used instead of the TaqMan probes. All reactions were performed in 96 well plates using a volume of 25 µL. Master Mix for SYBR Green as well as for TAqMan experiments were obtained from Applied Biosystems. All real-time PCR analyses were performed using the ABIPrism 7900 Sequence Detection System and the SDS.

2.11. Data evaluation

Growth inhibition curves were fitted using a nonlinear regression sigmoidal dose response curve model provided in the GraphPad Prism software, which is also used for EC₅₀ value calculation with a 95% confidence interval.

For evaluation of gene expression data from real-time PCR, the Q-Gene module, which is freely available via internet and is used as a macro for Microsoft Excel, was used (Muller *et al*, 2002). Q-gene allows that amplification efficiencies of target and reference gene, which may differ slightly, can be included in the calculation, whereas for the often used Ct-method, identical / very similar amplification efficiencies are a prerequisite for its application. For all real-time PCR results, GAPDH was used as reference / housekeeping gene and the calibrator sample was the control sample containing only the carrier solvent methanol.

3. Results

3.1. Assessment of growth inhibition elicited by PAHs

In order to analyze complete dose-response curves, a wide range of concentrations was tested for all compounds. Significant growth inhibition was found at rather high concentrations, with EC₅₀ values determined at 1031 µg/L (5.1 µM) for fluoranthene, 260.3 µg/L (1.29 µM) for pyrene, and 55.24 µg/L (0.22 µM) for benzo[a]pyrene (Figure 1).

The mixture, which contained the three compounds at a concentration ratio corresponding to their EC_{50,i} values, showed a parallel dose response curve. The concentration scale indicates at the highest tested concentration (1x EC_{50,i}) the presence of all three compounds at their single compound EC_{50,i} value. The EC_{50,mix} value determined as a dilution factor of the EC_{50,i} scale was 0.3497xEC_{50,i}. Thus, the EC_{50,mix} value of the mixture is determined at one third of the sum of the three compounds EC_{50,i} values (Figure 3.1).

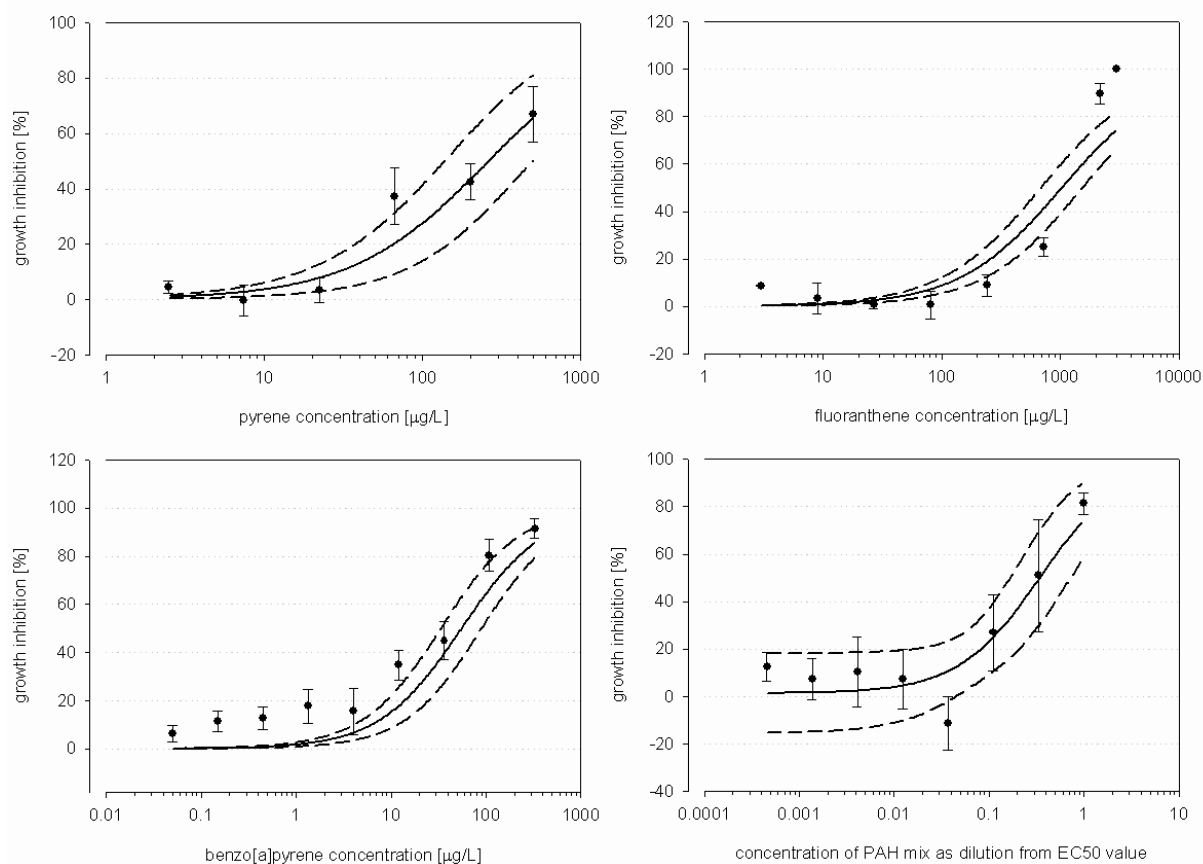


Figure 3.1: Dose-response curves for growth inhibition in *T. pseudonana* exposed for 24 h to either single PAHs (pyrene, fluoranthene, benzo[a]pyrene) or a mixture of all three compounds. The mixture contained the three PAHs at a ratio according to their EC_{50} value, i.e. at the highest tested concentration ($1 \times EC_{50}$) each single PAH was present at its single compound $EC_{50,i}$. Circles represent average measured values from at least 4 experiments, vertical lines indicate the standard error. The continuous line represents the fitted dose response-curve (using GraphPad Prism software) with dashed lines indicating the 95% confidence interval.

Furthermore, growth inhibitory effects were predicted using the concentration addition method as described in part 2.3. Using this method, a very close agreement between measured values and those predicted by the concentration addition method was observed (Table 3.1).

Table 3.1: Comparison of presence of single PAHs in the mixture at the predicted and measured $EC_{50,mix}$. Concentrations were predicted using the concentration addition method. Measured concentrations were calculated from the compounds' proportion at the experimentally determined $EC_{50,mix}$.

	single compound concentrations in the mixture at $EC_{50,mix}$ [$\mu\text{g/L}$]	
	according to prediction	according to measured data
Pyrene	87	91
Fluoranthene	344	361
Benzo[a]pyrene	18	19

3.2. Preparation of samples for gene expression analysis

In the beginning, RNA was extracted from the samples using the Trizol method directly according to the protocol of Invitrogen. Concentration and quality of RNA was checked on a NanoDrop instrument (Peqlab Biotechnologie). Yield and quality of RNA could be improved by small modifications to the manufacturer's protocol as described in Materials and Methods. The quality of RNA checked by the ratio of absorption at 260/280nm should be close to 2. For the original protocol, some DNA contaminations were indicated by a ratio below 1.7. Using the modified protocol a ratio of 1.9-2.05 was achieved.

Additionally, RNA quality was checked on 1% agarose gel (Figure 3.2).

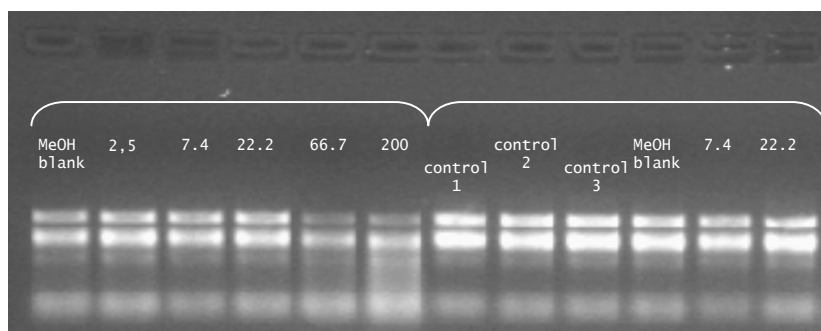


Figure 3.2: Example of RNA extracted from diatom samples according to the optimized protocol. RNA extracted from samples of two independent biological experiments was loaded on a 1% agarose gel and run for 30 min. Numbers above the bands indicate the concentration of pyrene [$\mu\text{g/L}$], these diatom samples were treated with.

3.3. Housekeeping genes

All three potential housekeeping genes, which are needed for normalization of gene expression in the target genes, were firstly investigated in conventional PCR. Amplified products had the expected size for β -actin and GAPDH and seemed to be consistently expressed in pyrene treated compared to control samples. In contrast, for β -tubulin no bands or only weak bands were observed, even when different primer pairs and annealing temperatures were tested to optimize PCR conditions. Thus, giving evidence for a very low expression level compared to the other two housekeeping genes and also compared to the target genes, β -tubulin was not further taken into account (Figure 3.3).

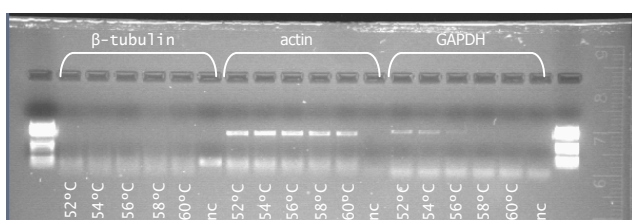


Figure 3.3: PCR products for β -tubulin, actin, and GAPDH using different annealing temperatures (within each set from 52 °C to 60 °C, and the last well for each set being the negative control (nc)). No bands were found for β -tubulin which seemed to be very low expressed. GAPDH and actin can be detected well, with the restriction that lower annealing temperatures should be used for GAPDH.

GAPDH and actin were further validated using RT PCR as a very sensitive method. Both showed a consistent expression (for GAPDH see Figure 3.4). GAPDH was chosen as housekeeping gene for all following experiments for normalization of target gene expression.

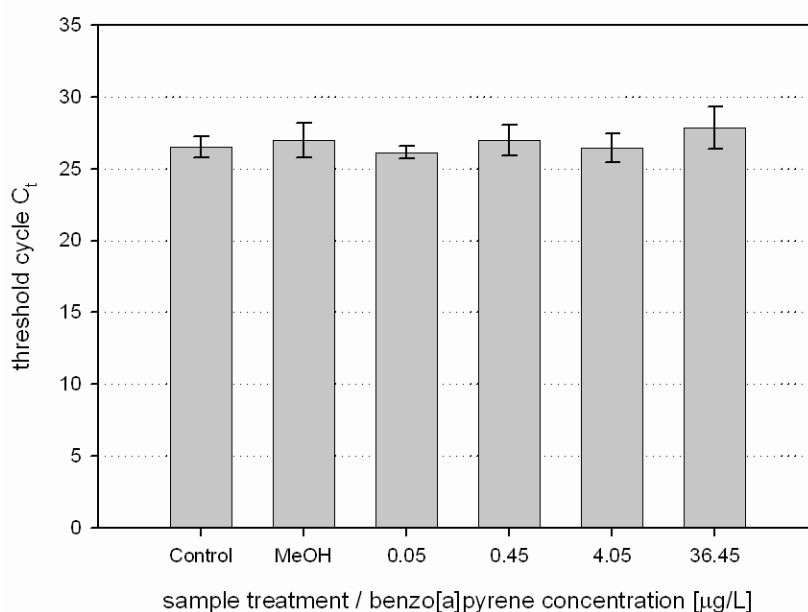


Figure 3.4: Threshold cycle values determined for GAPDH using diatom samples from different treatments (Control: untreated control, MeOH: control containing only the carrier solvent methanol, 0.05-36.45: samples treated with benzo[a]pyrene). No significant differences ($p > 0.05$) were detected using samples from three individual biological experiments tested on eight individual real-time PCR plates. Error bars indicate the standard deviation.

3.4. Investigation of target genes using real-time PCR

Samples from each three individual biological experiments, where diatoms were exposed either to benzo[a]pyrene or to pyrene, were analyzed for alterations in gene expression. Eight potential biomarker genes involved in crucial cellular processes were investigated as target genes. Differences between the individual biological experiments were relatively high in some cases. Thus, no general conclusions can be drawn for some of the genes (e.g. *Sil1* and *Sil2*, Figure 3.5) or only a trend can be ascertained. In other cases, a clear trend was confirmed in both treatments either with BaP or with Pyr (e.g. *lacsA*, Figure 3.6).

A summary of the derived results can be found in table 3.2.

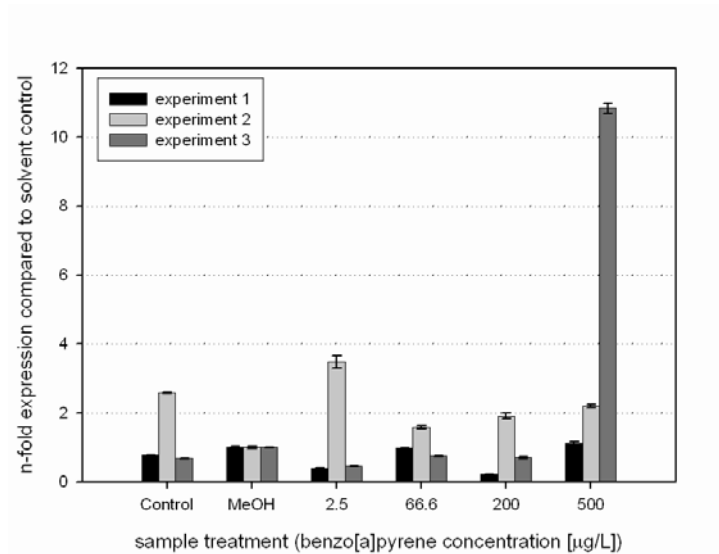


Figure 3.5: Example for high variability of results for *Sll* gene expression between the three biological experiments using benzo[a]pyrene treatment.

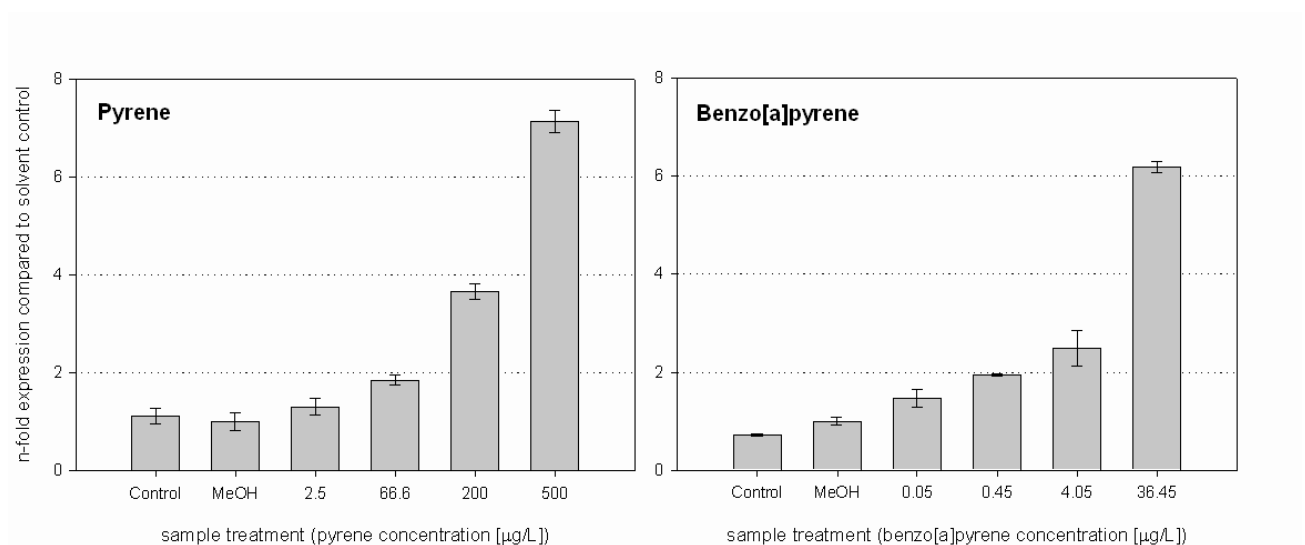























Figure 3.6: Example for enhanced gene expression in *lacsA* due to pyrene and benzo[a]pyrene treatment in each one biological experiment.

Table 3.2: Summary of the results for the regulation of eight potential biomarker genes from Real-time PCR analysis.

target gene	benzo[a]pyrene treatment*		pyrene treatment*	
	trend	details	trend	details
<i>3HfcpA</i>		Trend to down-regulation which is not consistent for lower concentrations in 3 biological experiments. Clear regulation down to 30% of the control for 4.05 and 36.45 µg/L.		Dose-dependent down-regulation, which is significant at concentrations of 66.7-500 µg/L (20-50% of control).
<i>3HfcpB</i>				
<i>lacsA</i>		Dose-dependent up-regulation starting with slight increase at 0.45 (1.5-2 fold over control), and significant up-regulation at 4.05 and 36.45 µg/L (5-10x)		Dose-dependent up-regulation at concentrations of 66.7-500 µg/L. (5-7 fold over control).
<i>RBJ</i>		Clear up-regulation at 36.45 µg/L (2-4x). At lower concentrations only in 1 of 3 biological experiments.		No clear trend identified.
<i>Sil1</i>		No clear trend identified.		Up-regulation confirmed at 200 and 500 µg/L (4x) in 2 of three experiments
<i>Sil2</i>		No clear trend identified.		Up-regulation confirmed at 200 and 500 µg/L (2x).
<i>Sil3</i>		Trend to down-regulation, which is significant at 36.45 µg/L (down to 40%).		Trend to down-regulation, which is significant at 200 and 500 µg/L (down to 30%).
<i>desB</i>		No clear trend identified.		Clear up-regulation at 500 µg/L (2-5x), in general a trend for up-regulation also at lower concentrations, but not consistent for all concentrations in all 3 biological experiments.
 no clear trend in regulation  trend of up-regulation  clear up-regulation  trend of down-regulation  clear down-regulation				

*Samples were diatoms, exposed for 24 h to four concentrations of benzo[a]pyrene (0.05, 0.45, 4.05, 36.45 µg/L) or of pyrene (2.5, 66.7, 200, 500 µg/L). Gene expression was compared to a control sample containing only the carrier solvent (methanol).

4. Discussion

4.1. *T. pseudonana* growth inhibition by PAHs

Clear growth inhibition was detected for all three singly tested PAHs as well as for the mixture. Results for the mixture were in agreement with expected effects based on the concentration addition concept.

As reported in the literature also for other algal species, benzo[a]pyrene showed the highest toxicity to *T. pseudonana*. Nevertheless, the growth inhibiting concentrations were relatively high, resulting in EC₅₀ values even above the aqueous solubility of the compounds (factor of 2 for pyrene, factor of 4 for fluoranthene and a factor of 15 for benzo[a]pyrene). As well, when comparing the effect concentrations to results reported in the literature, other algal species seem to react more sensitive to PAHs than *T. pseudonana* under the used test conditions (Table 4.1).

Table 4.1: Comparison of EC₅₀ values for algal growth/reproduction inhibition from literature and the current study. All species were exposed to the compound for 24 h.

Substance	Test organism	EC ₅₀ value [µg/L]	source
pyrene	<i>S. vacuolatus</i>	48	Altenburger <i>et al.</i> , 2004
	<i>S. vacuolatus</i>	50	Grote <i>et al.</i> , 2005
	<i>S. vacuolatus</i>	19	Djomo <i>et al.</i> , 2004
	<i>T. pseudonana</i>	260	current study
fluoranthene	<i>S. vacuolatus</i>	34	Altenburger <i>et al.</i> , 2004
	<i>S. vacuolatus</i>	34	Grote <i>et al.</i> , 2005
	<i>T. pseudonana</i>	1031	current study
benzo[a]pyrene	<i>S. vacuolatus</i>	1.77	Grote <i>et al.</i> , 2005
	<i>S. vacuolatus</i>	1.48	Djomo <i>et al.</i> , 2004
	<i>T. pseudonana</i>	55	current study

The observed lower sensitivity may have several reasons. One factor might be the low culture temperature, which leads to a reduced or decelerated metabolism. A major factor is probably the used light:dark cycle. Many other studies use longer light periods, which can enhance the production of phototoxic compounds, thus leading to phototoxic effects especially for compounds like the

investigated PAHs at lower concentrations (e.g. Grote *et al.*, 2005). Examples for diatoms used in ecotoxicological studies for assessment of effects caused by PAHs are very rare in the literature. A reason for a lower sensitivity of diatoms compared to green algae could be that contaminants have to pass the silica shell of the diatoms. However, pores in the silica shell are in the size range of 100 nm up to several μm ; thus, compounds like the PAH should not be hindered in their passage through the silica shell. Okay *et al.* (2002) used the diatom *Phaeodactylum tricorutum* to assess the (photo)toxicity of pyrene in an acute and a chronic test system. Pyrene was found to be acutely toxic to this diatom. Using ^{14}C assimilation rate as an endpoint after 4 h of exposure, EC_{50} values of 68-70 $\mu\text{g/L}$ were determined. In long-term experiments of 17 d, lag-phases in growth were prolonged in diatoms treated with 40 and 80 $\mu\text{g/L}$ of pyrene, but after 7 days growth rates between control and pyrene treated cultures were again similar. Therefore, it was concluded that pyrene has an effect only on actively growing cells. This would explain the higher EC_{50} values which were detected in the current study under the used culture conditions (low temperature, short light period).

4.2. Gene expression analysis

Housekeeping genes are required for gene expression normalization. Among the three tested genes, two (actin and GAPDH) were found to be suitable, as they were consistently expressed. However, α -tubulin, which was reported as housekeeping gene for the diatom *Thalassiosira weissflogii*, was expressed at very low level in *T. pseudonana* and thus not taken further into account. GAPDH was used for normalization of target gene expression.

Eight potential biomarker genes were investigated in pyrene and benzo[a]pyrene treated samples. For each single PAH treatment, samples from three individual biological experiments were investigated using real-time quantitative PCR. Due to the high variability between the different biological experiments, which was also observed in the detection of growth inhibition, it is difficult to draw general conclusions concerning gene expression alterations due to PAH exposure.

Among the tested genes, four (*3HfcpA*, *3HfcpB*, *lacsA*, *Sil3*) showed a similar trend in benzo[a]pyrene treatment as well as in pyrene treatment. For others, no clear trend could be observed for one of the treatments. The most promising candidate seemed to be *lacsA* which was strongly up-regulated in both (BaP and Pyr) treatments. This gene is involved in the fatty acid metabolism of the cell and thus important for the energy budget. Up-regulation of *lacsA* upon benzo[a]pyrene treatment was already

evident for concentrations, which did not show effects on diatom growth. For other genes, effects are mainly found at higher concentrations, where also growth was inhibited.

The high variability especially in gene expression analysis might be overcome, using synchronized cultures of *T. pseudonana*. In our experiments, *T. pseudonana* was already strictly adapted to a light:dark cycle, which was probably not sufficient. The cell cycle stage plays a crucial role in gene expression. Thus, in order to exclude differences between experiments due to diatoms being in different cell cycle phases, diatoms should be more adequately synchronized. A suitable method for use in future experiments will be a synchronization using silica starvation before experiments are started (Frigeri *et al.*, 2005).

5. Conclusions

T. pseudonana represents an ecologically relevant test organism which can be easily cultured in the laboratory. As its genome is completely sequenced, it is well suitable for gene expression studies. In the current study, only one of eight genes could be confirmed as potential sensitive biomarker gene. In general, adjustment to synchronized cultures could enable the detection of other biomarker genes, as it will probably reduce the variability. Thus, genes among the ones investigated here, and/or other selected genes, involved in different cellular pathways, might be identified as biomarkers.

6. References

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