

# 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 – D4.3.1**

Report on variation of toxicity of two model contaminants  
*Revision [1]*

Due date of delivery: M17  
Actual submission date: M17

Start date of project: 1<sup>st</sup> of January 2005

Duration: 48 months

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<b>Project co-funded by the European Commission within the Sixth Framework Programme (2002-2006)</b>		
<b>Dissemination Level</b>		
<b>PU</b>	Public	X
<b>PP</b>	Restricted to other programme participants (including the Commission Services)	
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## Table of Contents

<b>EXECUTIVE SUMMARY</b> .....	<b>2</b>
<b>1. INTRODUCTION</b> .....	<b>3</b>
1.1. CONTEXT AND OBJECTIVES OF 4.3.1 .....	3
1.2. EFFECTIVE WORK REPORTED .....	3
<b>2. METHODOLOGY</b> .....	<b>4</b>
2.1. GENERAL APPROACH .....	4
2.2. SHORT-TERM TEST ON ALGAE .....	4
2.2.1. <i>Sampling and analysis</i> .....	4
2.3. SHORT-TERM TEST ON BACTERIA .....	5
2.3.1. <i>Sampling and analysis</i> .....	5
2.4. SHORT-TERM TEST ON ZOOPLANKTON .....	5
2.4.1. <i>Algal labelling</i> .....	5
2.4.2. <i>Sampling and incubation of zooplankton communities</i> .....	6
2.5. COMMUNITY ANALYSIS .....	6
2.5.1. <i>Algae community analysis</i> .....	6
2.5.2. <i>Bacterial community composition</i> .....	6
2.5.3. <i>Zooplankton community analysis</i> .....	7
<b>3. PRELIMINARY RESULTS</b> .....	<b>8</b>
3.1. VARIATION IN SENSITIVITY OF PYRENE .....	8
3.2. VARIATION IN SENSITIVITY OF CADMIUM.....	8
<b>4. CONCLUSIONS</b> .....	<b>8</b>
<b>5. REFERENCES</b> .....	<b>9</b>

## **Executive Summary**

This task has been postponed in part as Task 3.3 was moved forward. The aim is to analysis the cause of variation in sensitivity of algal, bacterial and zooplankton communities for two of the model compounds within Thresholds Project: Pyrene and Cadmium. Initial experiments have been run, but the results so far are not sufficient to enable the sought analysis of causes of variations. However, the preliminary data suggests that there is indeed a variation in sensitivity displayed as ten-fold variations in Lowest Observed Effect Concentrations. This report contains methods used and some preliminary data is provided. This task will continue in 2006 and 2007 to ensure that sufficient data are available for more conclusive analyses.

# 1. Introduction

The overall objective of Stream 4 is to analyse, compare and assess the effects of contaminants in coastal ecosystems from the threshold perspective. WP 3 is the work package where uncertainties in thresholds for contaminants due to combined effects with nutrients are investigated. Therefore WP3 is concentrated on tests performed with algal, bacterial and zooplankton communities under varying environmentally realistic conditions as a complement to WP1, where mainly thresholds for laboratory species and conditions are reported.

## 1.1. Context and objectives of 4.3.1

The aim was to study the variation in toxicity of two of the model substances (Pyrene and Cadmium) over a productive season. Response parameters include productivity of alga and bacteria and to a lesser extent zooplankton. Environmental factors such as nutrient concentrations, algal, bacterial and zooplankton community composition and total organic carbon will be linked to variation observed. For the metal chosen, influence of speciation on toxicity is included in selected experiments, as speciation has to be analysed without storage of the samples.

## 1.2. Effective work reported

Due to changes in the order of tasks within this work package, where Task 3.3 was put forward to 2005 only part of Task 3.1 has been performed (see First Year Report). The prolonged ice coverage in spring 2006 has further delayed this task. However, work on this task has been performed, but the data set is so far too small in order to analyse variations in toxicity and to assign these variations to environmental factors. The full report will therefore first be submitted in 2007.

## 2. Methodology

### 2.1. General approach

The experiments on algae, bacterial and zooplankton communities are performed on the communities present on the day of the individual experiments. At the same time samples for temperature, salinity, chlorophyll a, nutrients, DOC and community composition are collected to be used as explanatory variables in the analysis of the causes for variation in toxicity. The experiments are run immediately to avoid too much confounding influences of laboratory conditions; however, the experiments are all run at 18 degrees in order to keep exposure- and  $^{14}\text{C}$ -incorporation time the same. This is a compromise between keeping strictly to the environmental conditions present at the day and having comparable experimental conditions. However, since the experiments are performed at once, the impact of succession due to acclimatisation should be avoided given the short time span from collection to end of experiment, which is maximum three hours.

### 2.2. Short-term test on algae

The functional endpoint for the algae test is  $\text{H}^{14}\text{CO}_3^-$  incorporation as an estimate of primary production (Maraldo et al. 2004a).

#### 2.2.1. Sampling and analysis

Samples of natural algal communities are collected from surface water in Roskilde Fjord, DK. The algae are separated from larger zooplankton by gentle sieving through a 45- $\mu\text{m}$  mesh. Four replicates of 10 ml are taken with a 10 ml automatic kip dispenser pipette (Schott Duran, Mainz Germany) and transferred to 20 ml glass vials (BN Instruments, Denmark). 2  $\mu\text{Ci}$   $\text{H}^{14}\text{CO}_3$  (1 mCi  $\text{ml}^{-1}$ ,  $^{14}\text{C}$  Agency, Hørsholm, Denmark) is added to each sample, and the samples are incubated for 2 hours under cool white light (2x Pope FTD 18W/33, Holland). To test for abiotic  $^{14}\text{C}$  adhesion and bacterial incorporation of  $^{14}\text{C}$ , two dark samples are run in parallel with each experiment. Immediately after the incubation, 200  $\mu\text{l}$  1 mol  $\text{L}^{-1}$  HCl is added to remove non-incorporated  $^{14}\text{C}$  in the samples. After 24 h, 10 ml of Insta-gel Plus (Perkin Elmer Life and Analytical Sciences, Inc., Boston USA) is added, and the samples are stored for at least 24 h and at most for one week at room temperature. Finally, the samples are radioassayed in a Beckman LS 1801 scintillation counter. The total incorporation is measured as the amount of radiolabelled carbon, and all the samples are corrected for the amount of abiotic  $^{14}\text{C}$  in the dark sample. Specific primary production is estimated as the total incorporation divided by the amount of chlorophyll a.

### 2.3. Short-term test on bacteria

The functional endpoint for bacteria is  $^{14}\text{C}$ -leucine incorporation as an estimate of bacterial growth (Maraldo 2004b).

#### 2.3.1. Sampling and analysis

From each of the algal incubations, sub samples of 1 ml are transferred after one-hour pre-incubation with the contaminant, to 2 ml Eppendorf tubes. 50  $\mu\text{l}$  of 4  $\text{mmol l}^{-1}$  [ $^{14}\text{C}$ ]-l-leucine (295  $\text{mCi mmol}^{-1}$ , Amersham, Life Science) are added to achieve a final concentration of 190  $\text{nmol l}^{-1}$  in each tube. Blind samples are prepared as 100  $\mu\text{l}$  100% trichloroacetic acid (TCA) in 1 ml of water to estimate any abiotic leucine adhesion and contamination of the leucine solution. All samples are incubated for 60 min and terminated with 100  $\mu\text{l}$  cold 100% TCA. 15  $\mu\text{l}$  of skim milk are added to enhance protein precipitation. The samples are stored at 5  $^{\circ}\text{C}$  until centrifugation and washing steps. The samples are centrifuged twice for 10 minutes (13 000  $\times$  g, 4  $^{\circ}\text{C}$ ) where the supernatant is discarded and the pellet washed with 1 ml ice cold 100% TCA between centrifugation steps. Finally, the supernatant is removed, 1 ml of Ecoscint A (National Diagnostics, Atlanta USA) is added and the tubes are vortexed. After 24 h of storage at room temperature (18  $^{\circ}\text{C}$ ), the samples are radioassayed in a Beckman LS 1801 scintillation counter.

### 2.4. Short-term test on zooplankton

The zooplankton short-term test is a method for assessing impact on grazing through up-take of  $^{14}\text{C}$ -labelled algae (Hjorth et al 2006).

#### 2.4.1. Algal labelling

For grazing studies of natural zooplankton communities, algal communities are collected at same site as zooplankton communities, by filtering approximately 5 L of surface water through a 45  $\mu\text{m}$  filter to remove large particles and zooplankton. Alternatively, *Rhodomonas baltica* can be used. When labelling, *R. baltica* or phytoplankton communities are incubated with  $\text{NaH}^{14}\text{CO}_3$  (0.1  $\mu\text{Ci/ml}$ , 1.77  $\mu\text{M}$  inorganic carbon,  $^{14}\text{C}$ -central, DHI, DK) in nutrient enriched water for 24 hours in an aerated glass container under cool white light. The duration of the incubation ensures a uniform labelling of the algal cells (Nielsen & Olsen, 1989). After the incubation, the algae are filtered onto a 2  $\mu\text{m}$  filter, thoroughly rinsed to remove unincorporated  $^{14}\text{C}$ , after which the filtrate is resuspended in approximately 100 ml 0.2  $\mu\text{m}$  filtered seawater and diluted to a final volume of 600 ml with an activity of approximately 20,000 dpm/ml.

### ***2.4.2. Sampling and incubation of zooplankton communities***

Samples of natural zooplankton communities are collected from surface water in Roskilde Fjord, DK. The organisms are carefully collected on a 45 µm mesh, rinsed with 0.2 µm filtered seawater and resuspended in a 300 ml round flask. Within one hour after sampling, two 5 ml sub-samples are transferred to a 20 ml plastic vial with a 5 ml automatic kip dispenser pipette mounted on a 250 ml glass flask (Schott Duran, Mainz Germany), which collects the chosen volume of water in a reservoir during sampling. Sub-sampling with this pipette allows a representative sampling of the organisms without eliciting escape responses since any suction or drag effect is present, as long as the flask is shaken and not swirled (personal observations). Four replicate samples were taken for each treatment and placed in random order for the subsequent toxicant exposure and incubation. After one hour preincubation with the contaminant, the zooplankton samples are fed <sup>14</sup>C-labelled algae (1 ml added) for 1 hour under dim light at ambient temperature. Grazing of the labelled algae by zooplankton is terminated by collecting the zooplankton on 45 µm filters (3x3 cm) mounted on small homemade funnels. The filters are carefully rinsed three times with approximately 5 ml of 0.2 µm-filtered seawater to avoid adhesion of labelled algae. Each filter is then transferred to a 6 ml scintillation vial, and 3 ml scintillation cocktail (Ecoscint, National Diagnostics) is added. The activity of the samples is measured on a Beckman LS 1801 scintillation counter within a month from the experiment.

## **2.5. Community analysis**

### ***2.5.1. Algae community analysis***

The phytoplankton biomass is determined as the concentration of chlorophyll *a*. Each sample is vacuum pressure filtered through a 0.2µm filter (GF25, Adventec), which, afterwards, is extracted in 5 ml ethanol for 24 hours and, thereafter fluorometrically analysed (Turner, Denmark). Phytoplankton speciation and abundance is determined in Lugol-preserved samples using an inverted microscope to group level. Algae less than 2 µm are not identified or counted in any of the samples.

### ***2.5.2. Bacterial community composition***

Bacterial community analysis is based on community fingerprinting using PCR-DGGE methodology according to Dahllöf et al. 2000 and Petersen et al. 2005. In short 50 ml of water is sampled on a 0.2µm filter and is extracted by bead beating according to Petersen et al. (2005). In short, the filters are put in a 2 ml cryo-tube and extracted with one ml of extraction buffer consisting of 400 µl 6.25 M ammonium acetate, 100 µl 1 M TRIS (pH 8), 40 µl 0.5 M EDTA (pH 8), and 460 µl Milli-Q. To each

tube 80  $\mu\text{l}$  CTAB/NaCl and 15  $\mu\text{l}$  fluorescent Extr<sub>IS</sub> ( $\sim 25 \text{ ng } \mu\text{l}^{-1}$ ) were added together with 200  $\mu\text{l}$  of silica beads (Biospec Products, Inc., Bartlesville, USA), and 300  $\mu\text{l}$  of chloroform: isoamylalcohol (24:1) (Lab Scan and Merck). The samples are beaten (30 s at  $5.5 \text{ m s}^{-1}$ ) in a BIO-101 bead beater (Savant, Holbrook, NY, USA) followed by centrifugation of the supernatants for 20 min at  $15000 \times g$  to precipitate proteins. The new supernatants are transferred to 2-ml Eppendorf tubes; 100  $\mu\text{l}$  3 M NaAC (0.1  $\times$  volume) is added, and the tubes are topped up with isopropanol (Sigma) (minimum 0.6  $\times$  volume), vortexed, and precipitated overnight ( $4^\circ\text{C}$ ). The samples are centrifuged (30 min,  $15000 \times g$ ,  $15^\circ\text{C}$ ) and the pellets are washed with ice-cold 75% ethanol and then the DNA is dissolved in 100  $\mu\text{l}$  TE.

A fragment of the RNA polymerase beta subunit gene, *rpoB*, is amplified for the subsequent DGGE analysis using the following primers, *rpoB*1698F (5'- AACATCGGTTTGATCAAC -3') and *rpoB*2041R (5'- CGTTGCATGTTGGTACCCAT - 3'). A GC-clamp is added to the forward primer at the 5'-end (5'- CGCCCCCGCGCCCCGCGCCCCGGCCCGCCCGCCCCGCCCC -3'). The PCR<sub>IS</sub> mix including PCR<sub>IS</sub> template (*D. melanogaster* DNA) and PCR<sub>IS</sub> primers is added to the PCR master mixture. The PCR master mixture for one PCR tube contained 2  $\mu\text{l}$  PCR buffer; 0.7  $\mu\text{l}$  dNTP (20 pmol/ $\mu\text{l}$  of each); 0.5  $\mu\text{l}$  of each *rpoB* primer (25 pmol/ $\mu\text{l}$ ); 0.2  $\mu\text{l}$  of each PCR<sub>IS</sub> primer (25 pmol/ $\mu\text{l}$ ); 1  $\mu\text{l}$  of PCR<sub>IS</sub> template ( $\sim 3 \text{ ng}/\mu\text{l}$ ); 0.5  $\mu\text{l}$  bovine serum albumin (10 mg/ml); 2  $\mu\text{l}$  JumpStart™ RedTaq™ polymerase and 10.4  $\mu\text{l}$  Milli-Q. To 18  $\mu\text{l}$  of reaction mixture, 2  $\mu\text{l}$  sample DNA (50 ng/ $\mu\text{l}$ ) is added, giving a final volume of 20  $\mu\text{l}$ . The PCR is performed on a Hybaid PCR Express Thermal Cycling machine (Franklin, MA, USA). The thermal cycling conditions are as follows: initial denaturing and activation of polymerase for 5 min at  $94^\circ\text{C}$ , followed by 25 cycles consisting of denaturing for 30 s at  $94^\circ\text{C}$ , annealing for 1 min at  $50^\circ\text{C}$  and extension for 1.5 min at  $72^\circ\text{C}$ , with a final 10 min  $72^\circ\text{C}$  extension period at the end of the PCR. Primers are supplied by DNA Technology A/S (Aarhus C, Denmark), nucleotides from Invitrogen (Carlsbad, CA, USA) and chemicals are supplied by Sigma unless otherwise stated.

### 2.5.3. Zooplankton community analysis

Zooplankton abundance and composition is determined through inverted microscope analysis to group and/or species levels. Number of naupili and larvae are also noted.

## **3. Preliminary results**

### **3.1. Variation in sensitivity of Pyrene**

So far, only a few short-term experiments on algae and bacteria have been performed, mainly in early spring and late autumn (due to changes in the order of tasks) when the productivity of the communities is low. However, it is still evident that there are differences in sensitivity which for algae is expressed as variation in Lowest Observed Effect Concentration (LOEC) from 3.2-250 nM. However, hormesis effects are observed at lower concentrations. In order to evaluate if this apparent stimulation is an initial stress response resulting in adverse effects at a later stage, time series experiments have to be performed. For bacteria LOECs vary between 2.5 and >250 nM. Also here hormesis effects are observed. LOEC for zooplankton is only determined once as the time of sampling has been seasonal mismatch to the presence of zooplankton in a large enough abundances. At this occasion LOEC was determined to be 25 nM.

### **3.2. Variation in sensitivity of Cadmium**

A preliminary experiment with Cadmium has been performed in order to establish the concentration range to be used. This range was found to be 0.01-100 ppb Cd.

## **4. Conclusions**

Since this task has been postponed in favour of task 3.3 no final conclusion can be drawn so far. This task continues in 2006 and 2007 in order to collect a sufficient amount of data for a more stringent analysis.

## 5. References

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