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"Microbial Diversity and Metal Fluxes in Contaminated North Sea Sediments"

«MICROMET»

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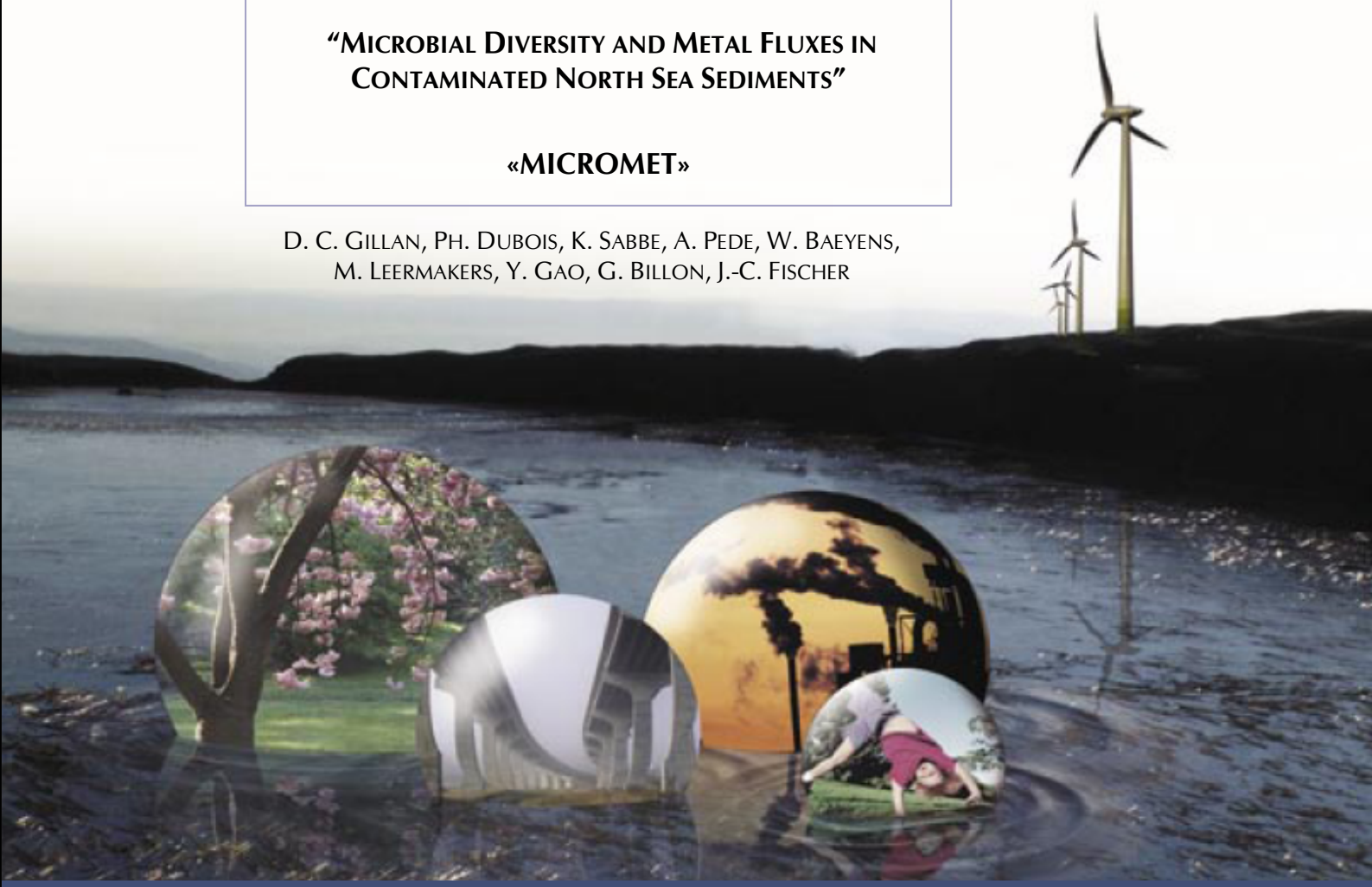

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North Sea

FINAL REPORT PHASE I

MICROBIAL DIVERSITY AND METAL FLUXES IN CONTAMINATED NORTH SEA SEDIMENTS

"MICROMET"

SD/NS/04A

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ACRONYMS, ABBREVIATIONS AND UNITS

ATP	adenosine triphosphate
AVS	acid volatile sulfides
BCCM	belgian co-ordinated collections of microorganisms
BCP	belgian continental plate
BET theory	Brunauer, Emmet and Teller theory
BLAST	basic local alignment search tool (http://www.ncbi.nlm.nih.gov/blast)
bp	base pair
BMDC	Belgian Marine Data Center
CFB	Cytophaga-Flavobacter-Bacteroides
CL	clone library
CLPP	community level physiological profiling
cm	centimeter
CRS	chromium reducible sulfur
DAPI	4', 6-diamidino-2-phenylindole
DET	diffusive equilibrium in thin films
DGGE	denaturing gradient gel electrophoresis
DGT	diffusive gradients in thin films
DNA	desoxyribonucleic acid
dw	dry weight
Eh	redox potential
FF	fine fraction
FISH	fluorescent *in situ* hybridization
FTIR	Fourier transformed infra red
mg	milligram
MGS	mean grain size
mV	millivolt
Nb	number
nmile	nautical mile
OM	organic matter
PCA analysis	principal component analysis
PCR	polymerase chain reaction
POC	Particulate Organic Carbon
RDA	redundancy analysis
rDNA	ribosomal desoxyribonuceic acid
rRNA	ribosomal ribonucleic acid
SEM	simultaneously extracted metals
SSU	small sub unit
SWI	sediment-water interface
TI	toxicity index
TRFLP	terminal restriction fragment length polymorphism
WP	work package
ww	wet weight

1. SUMMARY

As many toxic metals are complexed by organic matter in contaminated marine sediments, metals might be released in the water column when organic matter is degraded by microorganisms. This might affect microbial biodiversity and lead to metal bioaccumulation in higher trophic levels. To date, microbial communities in marine sediments of the Belgian Continental Plate (BCP) remain poorly studied, particularly in heavy metal contaminated zones. The MICROMET project fits within two priority research domains of the Science for a Sustainable Development programme (Biodiversity and Marine Ecosystems) and is directly connected with the aims of the Water Framework Directive (2000/60/EC).

The aim of the MICROMET project is to understand the link between microbial activity and metal fluxes in marine sediments. To this end, the whole microbial communitiy will be studied in the BCP area using an interdisciplinary approach in which geochemical and microbiological methods will be closely integrated. The research is subdivided in three Work Packages (WP 1-3). The aim of WP 1 is to determine the metallic contaminants and the microbial diversity in sediments. The aim of WP 2 is to assess the importance of microorganisms in the leaching of metal contaminants from the sediments into the water column. The aim of WP 3 will be to run numerical models with the data collected during the project. This will allow predictions and lead to a better understanding of the benthic ecosystems.

Phase I of the MICROMET project was entirely devoted to WP 1. During phase I, nine sampling stations on the BCP have been examined during the first year (2007). These stations were stations 120, 130, 140, 230, 330, 435, 700, DCG and ZG03 (for the coordinates, see the MICROMET website : http://ulb.ac.be/sciences/micromet). Sediments have been sampled before (February) and after (July) the major phytoplankton blooms. Microbial diversity has been determined using the DGGE approach. Biomass was determined using DAPI counts. Pure cultures of microorganisms have been characterized and geochemical properties of the sediment have been determined (Eh, pH, DGT-sulfides, AVS & CRS, granulometry, simultaneously extracted metals – SEM). Pore-water metal concentrations have been determined *in situ* using the DET technique (Diffusive Equilibrium in Thin films) and the DGT technique (Diffusive Gradients in Thin films). In the second year (2008), sediments of two selected, silty stations (130 and 700) have been sampled monthly to take different organic matter sedimentation patterns into account. In addition to DGGE, fragments of the SSU rRNA gene have been sequenced in order to have a better understanding of the microbial diversity and physiologies in the sediments.

Results have first indicated that the BCP sampling stations followed in the study may be classified in three groups: sandy stations with a mean grain size (MGS) of 400 µm (group I: DCG, 330 and 435), sandy stations with a MGS of 200 µm (group II: 120, 230 [except 0-1 cm], ZG03), and muddy stations with a MGS of 12.5 µm (group III: 130, 140 and 700).

In group I sediments oxygen penetration depth is important, probably due to the low quantity and bioavailability of organic matter at the water-sediment interface. Oxygen and sulphates are not significantly reduced and only small quantities of CRS were detected. Low amounts of simultaneously extracted metals (SEM) have been measured.

In group III sediments, oxygen is completely consumed within the first mm and the Eh values drop to about -200 mV within the first cm. Production of dissolved and solid sulfides confirm sulfate-reducing activities, mostly in the first cm. Concentrations of Pb, Cu and Zn in SEM are generally more than 10 x lower compared to the AVS, which means that sulfides might act as a sink for the metals present. However, as demonstrated by the DET/DGT analyses, metallic pollutants are nevertheless present in the pore waters of these stations and

may therefore be released in the water column. In group II sediments, oxygen is consumed in the first cm and Eh values decrease more slowly than in the muddy stations.

The February 2007 data show that eubacterial taxon richness increased from the coast (group III sediments) to the open sea (group I sediments), when DGGE results were expressed per unit of biomass. Even when normalization was not performed it was observed that the raw DGGE diversity values were never low in offshore stations DCG and 435 (always >16 DGGE bands) and that the lowest values (6 DGGE bands) were always observed in coastal stations. In addition, no archaebacterial 16S rRNA sequences could be obtained by PCR in coastal stations; on the contrary, many archaebacterial sequences were obtained offshore, in sediments of stations DCG and 435. Reduced diversity in sediments from coastal areas on the BCP was also observed in other studies for other groups, such as nematods and harpacticoid copepods (e.g., Vincx 1990). It should be noted that bacterial diversity values may nevertheless be elevated in coastal stations (e.g., the sandy station 120 in February 2007 and the metal contaminated station 130 in July 2007). This means that the situation is much more complex than initially thought and that the observed trend may not be valid for all sediment types and/or all periods of the year.

Although phase II of the project is absolutely necessary to conclude, the reduction of biodiversity observed in February 2007 might be related to elevated concentrations of metallic pollutants and metalloids in the pore waters, particularly arsenic, as demonstrated by DET/DGT analyses. Multivariate analyses of the eukaryotic DGGE data of 2007 also show pronounced changes in community (phylotype) composition with sediment type (group I-III). Although the eukaryotic data was not normalized to biomass, there appears to be no strong shift in diversity between the group I and group III sediments.

From the 2007 and 2008 data we can conclude that microbial biodiversity, as measured with DGGE, is not a variable that can be easily related to the environmental variables considered in this study. This might be due to the long exposition time of the microbial communities to metals in the coastal area. The present-day communities are probably adapted to the elevated metal concentrations in that zone. This may explain the high biodiversity values that were observed in contaminated sediments of station 130 in July 2007. Such a situation was also observed in the bacterial communities living in marine sediments of the Sørfjord in Norway, exposed for more than 80 years to high levels of Cd and Zn (Gillan et al. 2005).

On the contrary, bacterial biomass is a variable that displayed elevated and significant correlations to some environmental variables, particularly to dissolved Mn, Fe and As. This is not surprising as these metals may serve as electron donors or acceptors depending on their oxidation state. Bacterial biomass was also significantly correlated to chlorophyll a levels in sediments. This may be explained by the proliferation of bacteria on decaying phototrophic micro-eukaryotes such as diatoms and *Phaeocystis*.

Eubacterial rRNA sequencing (February 2007 samples) has shown that 5 to 10 major eubacterial groups are present in the BCP sediments examined (DCG, 435, 130, 700). Three major groups were present in all the four stations examined (γ-Proteobacteria, δ-Proteobacteria and CFB bacteria). Acidobacteria represent 2.6 - 14.6% of the clones in most of the stations. For micro-eukaryotes, 18S rDNA based DGGE analyses of February 2007 samples revealed a surprisingly high diversity of microbial eukaryotes, mainly comprising stramenopiles (diatoms), as yet unidentified (or ambiguously identified) marine eukaryotes and Fungi, but also protozoa and microalgae belonging to other groups. The DGGE procedure also picked up many metazoan sequences. DGGE and clone library analyses on the 2008 samples confirmed the 2007 results and in addition, due to the use of group-specific primers sets (for Cercozoa and ciliates) for the clone libraries, allowed a more detailed identification of the protozoan communities present.

With the DET/DGT approaches, high resolution profiles of trace metals in the sediments were obtained. Trace elements presented a variable geochemical behavior in the sediments, confirming that remobilization is occurring at specific depths. Seasonal variations of trace elements (Mn, Fe, As) have been observed during the cruises in 2007 and 2008. Although variations in the oxygen concentration and redox potential may explain most of the patterns obtained, the importance of microorganisms in this seasonal phenomenon has still to be determined (phase II). There is apparently no depletion of trace elements in the sediment porewaters at station 130 and 700. The flux calculations based on DGT profiles show that elements such as Mn, Fe, Co, As and Ni will diffuse out of the sediment into the overlying water column, at least for station 130. Flux calculations based on the DGT piston experiments confirm that metallic toxicants may reach the SWI and be released in the seawater. This might be detrimental for the benthic ecosystem. Other metallic toxicants such as Cu, Zn, and Cd will diffuse towards the sediments in station 130.

2. INTRODUCTION

2.1 Context

Many coastal areas worldwide are contaminated by metallic toxicants such as Cd, Ag, Pb, Hg and Ni, and these contaminants usually accumulate in sediments. On the Belgian Continental Plate (BCP) the concentration of metallic pollutants in sediments is above, or just at the level of, the Ecotoxicological Assessment Criteria (EAC), which are defined by the OSPAR Commission as concentration levels above which concern is needed. In addition, metals are on the European Union's list of priority substances for the Water Framework Directive (2000/60/EC).

As the most abundant organisms in the sediments, microorganisms are key players in the biogeochemistry of benthic ecosystems, including a variety of processes which may affect metal mobility and bioavailability. For example, benthic microbial communities are responsible for a substantial part of the remineralization of organic matter produced by local phytoplankton blooms and/or imported from adjacent estuaries. As most metals are usually complexed by organic matter, metals are mobilized when organic matter is degraded. This may lead to both the alteration of microbial biodiversity and to metal bioaccumulation in higher trophic levels through increased leaching of metals from the sediments. To date, the composition, structure and physiology of microbial communities in marine sediments remain poorly studied, and there is virtually no information on microbial assemblages and their functioning in metal contaminated zones. Nothing is known about the impact of metallic toxicants on microbial diversity in sediments of the BCP or about the importance of microbial communities in the leaching of metallic pollutants from the sediments in this area. This is problematic as the BCP is characterized by massive algal blooms in spring (mainly *Phaeocystis globosa*) and receives large amounts of nutrients and organic matter from the Scheldt estuary. In addition, future changes in nutrient inputs into the BCP may affect the nature and quantity of organic matter inputs into the sediments, with unknown consequences on the fate of trapped metals.

2.2 Objectives and expected outcomes

The aim of this project is to study the interactions between metallic contaminants and the microbial communities (archaebacteria, eubacteria, and eukaryotes) living in marine sediments of the BCP area. The research comprises three main parts in which microbiological and geochemical approaches are closely integrated. The first part (WP 1) is devoted to the measurement of the metallic contaminants and the microbial diversity of the sediments using a combination of state-of-the-art molecular and geochemical tools. The first phase of MICROMET (i.e., this report) is entirely devoted to WP1. The aim of the second part (WP 2) will be to assess the importance of microorganisms in the leaching of metallic contaminants from the sediments into the water column. In the third part (WP 3), results obtained during the research project will be used to run numerical models. These models will be used for the prediction and, ultimately, for the remediation of metal remobilization events.

Special attention will be paid to the influence of oxygen and organic matter on the relation between microbes and metals. These factors have been identified as the main forces affecting the diversity and activity of microbial communities (Giller et al. 1998, Bak et al. 1995), and can be expected to change in the near future as nutrient and organic matter inputs from the Scheldt estuary will be further reduced through the activation of the Brussels North

wastewater treatment plant in March 2007 (www.aquiris.be) and the continuing implementation of various EU directives on surface water quality (such as the Water Framework Directive).

The research project aims at gathering new data on environmental variables and microbiological processes not previously investigated in the BCP such as taxon richnesses (number of DGGE bands) and diversity indexes of microbial diversity, 16S and 18S rRNA sequences, pure cultures of microorganisms, DAPI counts, oxygen, metals in porewaters, metal fluxes, pH, Eh, S(-II) profiles within sediment cores, granulometry, organic C and N, etc.

3. METHODOLOGY

The first and second year of the MICROMET project (first phase, from the 15th December 2006 to the 31st January 2009) were entirely devoted to WP 1, i.e. the measurement of metallic contaminants and the determination of microbial diversity in the sediments.

Task 1.1. Sampling and microelectrode analysis.

In 2007, nine sampling stations were selected on the BCP on the basis of previous research (Danis et al. 2004, Gillan & Pernet 2007) and SPSD 2 programs (Fig. 1) : stations 120, 130, 140, 230, 330, 435, 700, DCG, ZG03. The coordinates of the stations were 51°11.10 N - 02°42.07 E (120), 51°16.25 N - 02°54.30 E (130), 51°19.57 N - 03°02.93 E (140), 51°18.50 N - 02°51.00 E (230), 51°26.00 N - 02°48.50 E (330), 51°34.84 N - 02°47.42 E (435), 51°22.60 N - 03°13.20 E (700), 51°45.00 N - 02°42.00 E (DCG), and 51°15.70 N - 02°40.00 E (ZG03). Sediments encompassed a wide range of metal loads and granulometries. In 2007, sediments were sampled in two periods to take different organic matter sedimentation patterns into account [before (7-9th February) and after (4-6th July) the spring bloom]. In 2008, the two stations with the highest metal loads were sampled monthly between February and July (St. 130 & 700). Sandy stations were not selected as porewater metal concentrations (see DET/DGT analyses) were very low. Each month, the 10 cm cores were completely subdivided into 1cm slices which were further analysed using the same methods as 2007. Although this was originally only planned for Phase II, this more detailed approach allowed a much better assessment of the seasonal and spatial (vertically within the cores) occurrence of microbial organisms (phylotypes) in relation to the presence of metals (see 4, 1.5). This will speed up the selection of suitable phylotypes for FISH / real time PCR study and the experiments planned for Phase II.

Sediments were sampled using a Reineck corer (ø 15 cm) onboard the RV Zeeleeuw (n=4 in 2007; n=2 in 2008). Subcores were obtained using 11 cm plastic corers (ø 3 cm and ø 7-10 cm). From each Reineck core, one or two narrow subcores were used for molecular analyses (DGGE, cloning) and another one was used for determination of microbial biomass. The largest plastic subcores (ø 7-10 cm) were used for DET/DGT and microelectrode analyses. For microbiology, only two sediment horizons (0-1 and 9-10 cm) were considered in 2007. In 2008, ten depths (every 1 cm up to 10 cm) were sampled. In 2007, subsamples for molecular analyses were taken on board, stored in cryovials and transported in liquid nitrogen to the lab, where they were stored at -80°C. In 2008, whole cores were frozen on board in liquid nitrogen and subdivided in the lab. The subsamples for biomass determination (DAPI counts) were fixed with an equal volume 2.5 % gluteraldehyde (micro-eukaryotes) or 4% paraformaldehyde (bacteria), and stored in darkness at 4°C until processing. At each station, replicate pigment samples were also collected for all depths considered.

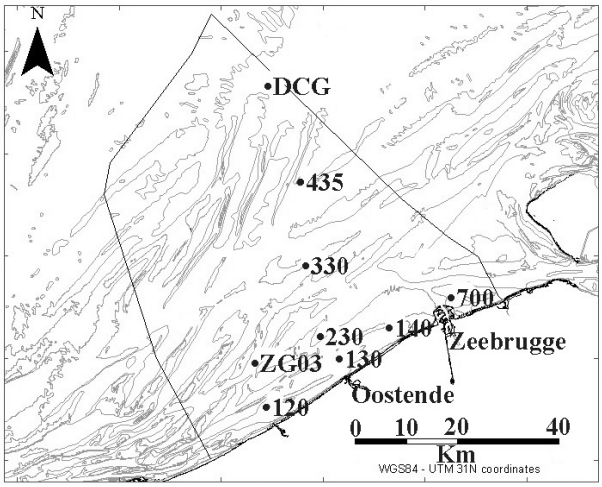


Figure 1. Sampling stations of the MICROMET project in 2007 and 2008.

Oxygen, pH and Eh were determined in sediments using microelectrodes. These parameters are important because they directly influence microbiology as well as the type of metals released in the pore-waters and consequently in the water column (Petersen et al. 1996). Oxygen profiles were obtained with a microelectrode of 500 µm in diameter fixed on a micromanipulator (Unisense company). pH was estimated directly in the field by means of a glass micro-electrode (Ingold). It was combined with an Ag/AgCl reference electrode with a potential equal to +0.22 V *vs* a hydrogen normal electrode (HNE). Redox potential (Eh) profiles were measured with a home-made platinium micro-electrode associated with a reference electrode Ag/AgCl, [KCl] = 3 M.

Task 1.2. Determination of the microbial diversity.

Task 1.2.1. DGGE analysis (Denaturing Gradient Gel Electrophoresis).

In 2007 and 2008, the *in situ* genetic diversity of microbial communities was determined using DGGE analysis of the 16S (for eubacteria and archaebacteria) and 18S (for eukaryotes) rRNA gene. The DGGE approach will be complemented in phase II of the project with other methods in order to obtain more detailed and quantitative data on the microbial communities (real-time PCR and/or FISH for prokaryotes and eukaryotes, epifluorescence microscopy counts for eukaryotes).

For bacteria, DNA extraction was performed as described in Gillan & Danis (2007). The general eubacterial primers GM5F and 518r were used (Muyzer et al. 1993). They amplify a fragment of the 16S rDNA, approximately 195 bp long. For Archaebacteria, we designed two new primers: ARC-349-F and ARC-853-R (see below). A 40 bp GC clamp was attached to the 5' end of all the forward primers. DGGE was performed in 10% polyacrylamide gels submerged in 1X TAE buffer at 60°C. Electrophoresis conditions were: 16h at 75V in a linear 25 to 75% denaturant gradient. The gels were stained for 60 min in 1X TAE buffer with ethidium bromide and visualized with UV radiation.

For micro-eukaryotes, DNA extraction was performed using zirconium beads (Zwart et al. 1998). Elimination of extracellular DNA was performed as described by Corinaldesi et al. (2005). Extracted DNA was amplified for DGGE analysis using the PCR procedure described by Muyzer et al (1993). We used the general eukaryotic primers 1427f-GC and 1637r, designed by Van Hannen et al. (1998), which amplifies a ± 180 bp fragment of the 18S rDNA. In addition, we tested the general eukaryotic primers Euk1A and Euk516r-GC

designed by Diez et al. (2001), as these yield a larger DNA fragment (± 560bp). We also adapted 3 specific primer sets for important benthic protozoan groups (Alveolata:ciliates, Excavata:kinetoplastids and Rhizaria:cercozoans) for use in DGGE (nested PCR approach) to check whether the diversity of these groups may be underestimated due to the presence of high amounts of diatom DNA (see task 1.2.1) [ciliate-specific primers (Cil-315f; Cil-959r(I-II-III): 600-670bp, Lara *et al*. (2007), Kinetoplastida-specific primers (Kineto14F; Kineto2026R: 1900-2200bp, Von der Heyden and Cavalier-Smith (2005) and Cercozoa-specific primers (25F; 1256R: ±1260bp, Bass and Cavalier-Smith (2004)), all in combination (second step in nested PCR) with the general eukaryotic primers (1427f-GC and/or Euk516r-GC)]. DGGE was performed with 7% polyacrylamide gels submerged in 1X TAE buffer at 60°C. Electrophoresis conditions were: 16h at 100V in a linear 30 to 55% denaturant gradient. The gels were stained for 30 min in 1X TAE buffer with SybrGold and visualized with UV radiation. DGGE bands were excised from the gel, resuspended in 30µl 1X TE buffer, reamplified, and sequenced.

Task 1.2.2. SSU rRNA clone libraries (CL) and sequencing.

Originally planned for 2008, we decided to start this task in 2007 in order to obtain clones for DGGE marker selection (micro-eukaryotes) and to also obtain information on the eubacterial molecular diversity of the sandy stations (e.g. DCG and 435), which were not sampled in 2008. In addition, the CL's were also used for the development of new DGGE primers for Archaebacteria, as the originally used primers proved to be unsuccessful. Complete 16S and partial 18S rRNA genes were amplified from environmental DNA using universal archaeal, bacterial or eukaryotic primers (Gillan et al. 2005, Van Hannen et al. 1998, Wilms et al. 2006) and sequenced.

For micro-eukaryotes, three 18S clone libraries were constructed for stations 130 and 700 for 2008, using group-specific eukaryotic primers (first tested using PCR-DGGE-see 2.1.2) for three major protozoan groups, viz. ciliates, Cercozoa and Kinetoplastida (cf. above). The 18S rRNA genes were PCR amplified; PCR products from several reactions (2008 samples) were pooled to provide adequate amounts of product for the preparation of the clone library, positive (white and light blue) colonies were picked out; 250 colonies for ciliates, 100 c for Cercozoa, 250 c for Kinetoplastida. Presence of the 18S rRNA gene insert was checked by PCR using T7 and SP6 primers and agarose gel electroforese. No insert was found for clones of Kinetoplastida. Clones with the correct insert size were PCR amplified for fast screening by DGGE using the nested PCR approach described above. Clones which showed different DGGE banding patterns, were selected to be sequenced. Sequencing was performed with forward primer cil-315f and reverse primers cil-959r(I-II-III) for ciliates, and with forward primer 25F, reverse primer 1256R, and internal primers 528 and Euk516r for Cercozoa (Bass and Cavalier-Smith 2004, Lara *et al*. 2007, Diez *et al.* 2001, Huss *et al*. 1999). Forward, reverse and internal sequences were aligned in Bionumerics 5.10.

Task 1.2.3. Isolation of microorganisms.

For bacteria, five types of medium were used in February 2007. These are described in Köpke et al. (2005). Briefly, medium I was used for oxic incubations and media II to V were used for anoxic incubations. Medium II is the standard anoxic medium for sulfate reducers (with sulfate); medium III is the standard anoxic medium without sulfate (for fermenters); and medium IV & V are the standard anoxic medium with manganese or iron oxides (5 mM). The MPN plates were incubated 3 months at 15°C in the dark. After that, MPN counts were obtained and subcultures were then incubated for 3 months. Isolations of pure cultures were

then performed on agar plates. For protozoa, isolations from stations 130 and 700 will largely be restricted to the second half of 2009. This way, the cultures can be used for experiments planned in phase 2 of the project.

Isolates are characterized by DGGE, ARDRA and/or partial sequencing (a short SSU rRNA fragment is used). All selected colonies are characterized using conventional phenotypic tests. After characterization, strains are deposited in the BCCM collections.

Task 1.3. Determination of microbial biomass.

For bacteria, total counts were determined in the two selected sediment sections for the February and July 2007 samples (0-1 and 9-10 cm), and in all sediment sections (from 0-10 cm) for the 2008 samples. Due to initial problems in methodology in February 2007 eukaryotic DAPI counts could not be obtained. For July 2007, eukaryotic DAPI counts were obtained for all samples except those of stations 330, 435 and DCG.

Total bacterial biomass was obtained using DAPI counts as described in Gillan et al. (2005) and MPN counts as described in Köpke et al. (2005). Bacteria were counted manually and by using the Image J software (14 pictures were counted for each filter, as explained in Gillan et al. (2005).

For isolation of micro-eukaryotes from the fixed sediment samples we used the density gradient centrifugation technique described by Starink et al. (1994, see also Hamels et al. 2004). This involves creating a 50 % Percoll density gradient (ultracentrifugation during 30 min at 38.800 g) and loading of the fixed sediment samples onto Percoll density gradients and centrifugation during 15min at 4.300 g. After centrifugation, the supernatans containing the extracted micro-eukaryotes is filtered onto 0.8 µm polycarbonate filters and stained with DAPI (10µg/ml final concentration). The filters are then immersed in immersion oil, covered with a cover slip and kept frozen and dark until epifluorescence microscopic analysis.

Task 1.4. Geochemical properties of the sediment

Task 1.4.1. General properties of the sediment

Granulometry was determined by laser analysis and the specific area of the sediments was determined by nitrogen adsorption and the BET theory. Specific area of the sediments was used to normalize the prokaryotic DGGE data. Acid Volatile Sulfides (AVS) and Chromium Reducible Sulfur (CRS) have been determined in the sediments because they are closely linked to the behaviour of heavy metals. AVS are mainly amorphous FeS, but also crystallized iron sulfides precipitates, mostly Fe_3S_4 (greigite) and FeS_{1-x} (mackinawite). Other metal sulfides such as PbS, CdS and ZnS are also included in the AVS but generally at significant lower amount. CRS are mainly composed of elemental S and FeS_2 (pyrite). The sequential extraction of AVS and CRS has been previously described in detail and reviewed elsewhere (Canfield et al. 1986, Billon et al. 2001). Dilute HCl-extractable metals (SEM - Simultaneously Extracted Metals) have also been obtained as they reveal the easily exchangeable metals (Gillan et al. 2005). A solution of HCl (1M) was used. To determine the concentration of dissolved sulfides in the sediments with high resolution AgI DGT probes were used (see Task 1.4.2. for a description of the DGT approach). Briefly, in presence of sulfides, the white AgI compound present in the probe is replaced by a dark Ag_2S precipitate. The colour intensity is then digitised and calibrated to achieve the concentrations initially present in the porewaters (Teasdale et al. 1999). For pigment analyses, sediment samples were extracted in acetone using sonication. Pigments were analyzed using HPLC with standard protocols (cf. Wright et al. 1991). Chlorophyll a concentrations (µg/g DW) were determined.

Task 1.4.2. DET/DGT analysis.

In this study, high-resolution vertical profiles of metals in sediment porewaters were obtained by the DET (Diffusive Equilibrium in Thin films) and the DGT (Diffusive Gradients in Thin films) approaches (Davison et al. 1991; Davison & Zhang 1994; Fones et al. 2004; Gao et al. 2006; Zhang et al. 2002; Leermakers et al. 2005). Porewaters were also analysed by conventional sampling (centrifugation technique).

For the DET approach the preparation procedure was similar to that of Docekalova et al. (2002). A gel containing 1.5% agarose was prepared by dissolving it in an appropriate volume of 80°C warm Milli-Q water. The mixture was placed in a boiling water bath, covered and gently stirred until all the agarose was dissolved and the solution was immediately pipetted into a preheated gel-casting probe and left to cool down to its gelling temperature (36°C or below). The constrained DET probe's material was obtained from DGT Research Ltd. The size of the DET probes was 180 mm * 40 mm, with a window of 150 mm * 18 mm open to the aquatic system. After the gels were set, they were covered with a 0.45 µm cellulose acetate filter (Millipore). Finally the window plate was put on top of the probe and all the elements gently pressed together.

For the DGT technique, diffusive and resin gels were prepared as described by DGT Research Ltd (www.dgtresearch.com). The probes were 180 mm*40 mm in size, with a window of 150 mm*18 mm open to the aquatic system. The resin gel was covered by diffusive gel (polyacrylamide) and a 0.45 µm pore size cellulose acetate filter. The front window plate gently pressed the various layers together.

Before deployment, the entire gel assemblies (DET and DGT) were de-oxygenated by immersing them for 24 hours in a container filled with 0.40 M NaCl trace metal free solution and by bubbling with nitrogen. A pair of DET and DGT probes, arranged back to back, was inserted vertically into one core. The water-sediment interface was marked when the probes were retrieved from the sediment core. In the laboratory, all manipulations of the gels were carried out in a laminar flow hood located in a clean room. The DET gels (typically 20 µl) were transferred into pre-weighed 2 ml tubes, weighed and eluted in 1 ml 1 N HNO_3. They were generally not further diluted for analysis. The DGT probes were opened, the filter and diffusive gel were removed and the resin gel was cut into 5 mm intervals using a Plexiglass gel cutter. Each gel slice was eluted in 1 ml 1 N HNO_3 for 24 hours (elution factors of 0.8) and further diluted to 10 ml for analysis by Inductively Coupled Mass Spectrometry (ICPMS).

Blank DET and DGT probes went together with the sample probes through all previous described steps including casting, probe construction, and deoxygenation except for the last step, the deployment. After the deployment, blank and sample probes were again treated in the same way. The resin gel of DGT was sliced into 32 intervals of 5 mm; 10 slices were randomly chosen for analysis. For the DET probe 10 out of 75 blank slices were randomly chosen for analysis.

Conventional sampling and centrifugation. In 2007, in addition to the DET/DGT approaches, porewaters were also analysed using a conventional approach. All handling, including sample sectioning and filtration, was carried out inside a nitrogen flushed glove-bag. The sediment samples collected with conventional presectioned core samplers were cut after removing each time the plastic cover, and split into two parts: one part was put in the centrifuge vessels and another small part about 2 grams was kept inside a small container used for AVS analysis (USTL, Lille). The samples were then all sealed in order to prevent oxidation. The samples in the centrifuge vessels were centrifuged for 30 minutes at 2500 rpm. To eliminate residual small size particles, the obtained pore waters were further filtered through a 0.45 µm cellulose acetate disposable filter, collected in a clean polyethylene tube

and acidified with 1% HNO_3. HR-ICPMS (Thermo Finnigan Element II) was used to determine the concentrations of the elements As, Cd, Cr, Co, Cu, Fe, Mn, Ni, Pb and Zn.

Task 1.5. Data analysis.

Microbial diversity, composition and biomass data were analyzed and related to the measured geochemical variables using standard correlation and (multiple) regression statistical analyses. DGGE gels were digitized and analyzed using Bionumerics 1.5 (Applied Maths BVBA, Kortrijk, Belgium) and Quantity One v. 4.2 (Bio Rad). The DGGE data on microbial community structure were analysed using multivariate statistical tools using Statistica 7.0 and the Canoco 4.5 software package. A toxicity index (TI) was calculated for the simultaneously Extracted Metals (Di Toro et al. 1992): $TI = [Cd + Co + Cu + Ni + Pb + Zn]_{SEM} / [S]_{AVS}$. In this formula, all concentrations are expressed in $\mu mol.kg^{-1}$. Biodiversity (i.e., the Shannon index) was calculated as described in Gillan (2004).

4. RESULTS

Task 1.1. Micro-electrode analysis.

Oxygen and redox profiles for the 2007 sampling campaigns are shown in Figs 2-3. For sandy sediments (stations 330, 435 and DCG), no significant decreases in dissolved oxygen were measured within the upper cm, indicating either a poor bacterial activity or a quick resupply of oxygen because of the high porosity of the sediment (Fig. 2, 330 and DCG not shown). For the other, muddier stations, the drop of the oxygen concentrations is sharp, immediately below the water-sediment interface. In these sediments, oxygen is completely consumed in the upper 2-4 mm's (Fig. 2).

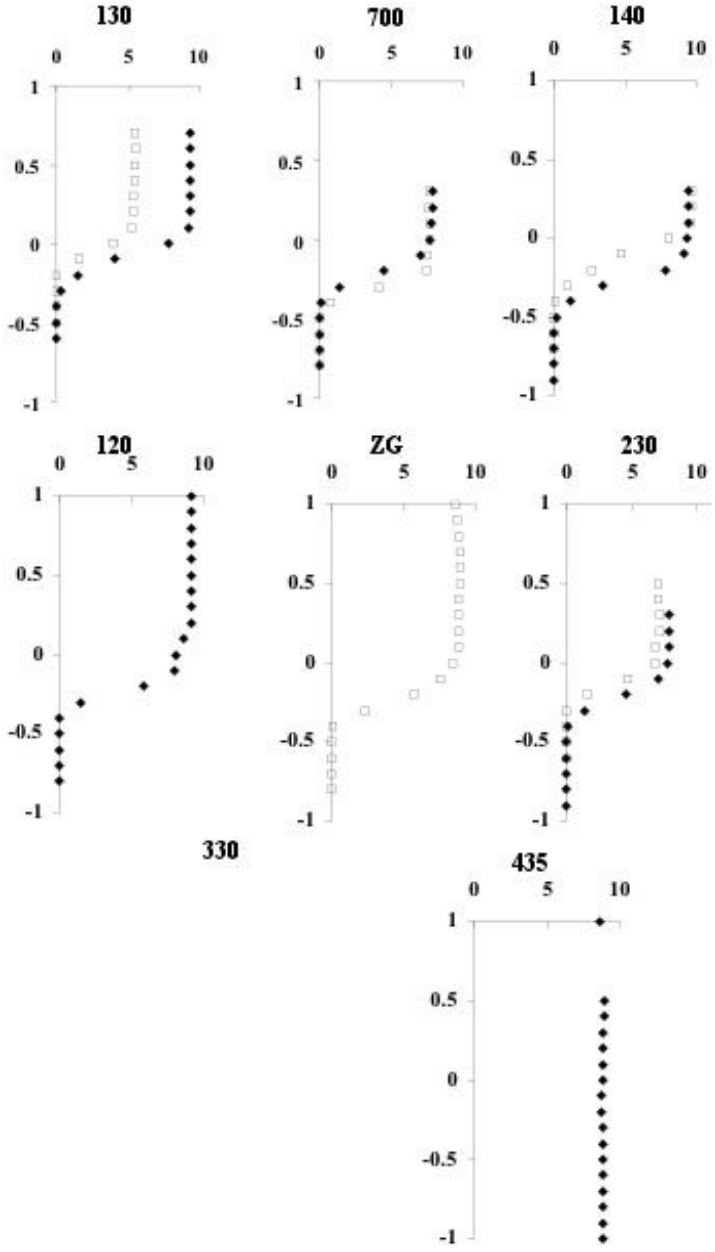


Figure 2. Oxygen profiles (mg.L^{-1}) in function of depth (cm). ◆: February 2007; □: July 2007.

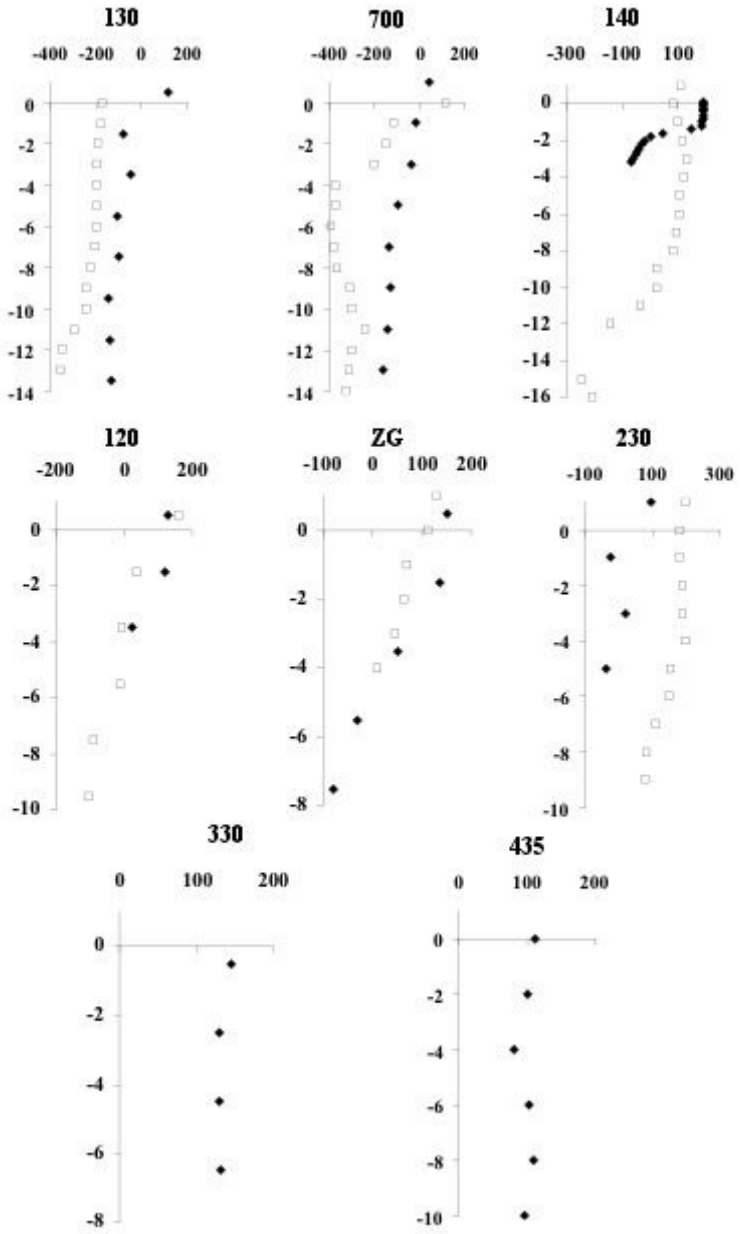


Figure 3. Eh profiles (mV) in function of depth (cm). ◆: February 2007; □: July 2007.

For the two muddy stations sampled in 2008 (130 and 700, not shown), we also noticed that oxygen decreased sharply at the water-sediment interface down to 0 at around 2-3 mm. Oxygen concentrations just above the interface are usually lower in summer than in winter, which could be explained by higher bacterial activity during the warmest periods.

Redox potential profiles are displayed in Fig. 3 and corroborate the differences between sandy (330, 435, DCG) and muddy sediments (130, 140, 700). Oxic conditions remained stable in function of depth in the sandy stations with redox potential values higher than 100 mV (*vs* reference electrode). In contrast, a drop of 200 mV occurs in the 2-4 cm of the muddy sediments (stations 130 and 700), indicating that other oxidants (than O_2) are thereby consumed by microorganisms. For the other stations (especially stations 120, 140 and ZG03), the evolution of the redox potential is more slow, suggesting lower microbial activities. Measurements of pH profiles give information on early diagenetic processes since

degradation of organic matter produces alkalinity but also hydronium ions. Our data (not shown here) show a distinct drop of ± 0.5 pH units in the muddy stations between the interface and -10 cm (stations 130 and 700), down to pH 6.9. In sandy sediments (DCG, 435 & 330), most of the pH values remain higher than 8, while in station 120, 140 and ZG03, values are between 7.1 and 8.5.

In 2008, Eh and pH values were recorded every month in stations 130 & 700. Values of pH did not indicate any clear seasonal trend. A decrease of ± 0.5-1.0 pH unit generally occurred at the interface and pH stabilized between 7.0 and 7.5 below 2 cm of depth. Strongly reduced sediments have been observed each time through Eh measurements. Eh values (given *vs* the Ag/AgCl reference electrode) were always negative below 1-2 cm depth and could reach -400 mV at -10 cm depth. Thus, sulphide compounds (and especially AVS, which are very sensitive to oxygen), which are known to trap several metallic pollutants, will never easily oxidize whatever the season.

Task 1.2. Determination of the microbial diversity.

Task 1.2.1. DGGE analysis.

DGGE of Eubacteria.

In 2007, the total number of DGGE bands (i.e., biodiversity) observed for 500 mg (ww) of sediments varied between 6 and 27 (Table 1). For the surface sediments (0-1 cm) in February, bacterial biodiversity values were significantly higher in sandy stations 120/DCG than in coastal station 700; in July, it was significantly higher in stations 130/DCG than in station 120. For the 9-10 cm layer of the sediments in February, biodiversity was significantly higher in station DCG than in stations 330/140; in July, it was at the same level in all the stations (Table 2). Significant seasonal differences were observed between February and July : for the 0-1 cm sediments, diversity increased in stations 130/700 and decreased in stations 120/230; for the 9-10 cm sediments diversity increased in station 330 (Table 1).

TABLE 1. Number of DGGE bands (range; n=4) observed in marine sediments of 9 stations on the Belgian Continental Plate in February 2007 (FEB) and July (JUL) 2007. Two sediment sections were considered (0-1 cm and 9-10 cm). Values in boldface refer to significant seasonal differences, within the same station and sediment layer (Mann Whitney U-test, $P < 0.05$). Letters (a, b) refer to comparisons between stations (within one sediment layer); stations are not significantly different if at least one letter is shared (Dunn's test, $P < 0.05$).

	120	130	140	230	330	435	700	DCG	ZG03
FEB 0-1 cm	**20 - 27** a	**9 - 19** ab	13 - 17 ab	**17 - 19** ab	15 - 15 ab	18 - 21 ab	**7 - 13** b	22 - 23 a	16 - 19 ab
FEB 9-10 cm	14 - 20 ab	16 - 18 ab	10 - 12 b	12 - 14 ab	**10 - 13** b	16 - 19 ab	11 - 15 ab	20 - 22 a	15 - 18 ab
JUL 0-1 cm	**10 - 13** b	**20 - 21** a	14 - 14 ab	**11 - 16** ab	15 - 19 ab	17 - 21 ab	**14 - 14** ab	19 - 22 a	11- 17 ab
JUL 9-10 cm	10 - 16 a	17 - 22 a	15 - 18 a	6 - 15 a	**13 - 18** a	17 - 21 a	12 - 14 a	16 - 21 a	15 - 21 a

The number of DGGE bands normalized to the DAPI counts, i.e. normalized to the number of bacteria counted in the initial sediment sample of 500 mg (ww), is shown in Table 2. Values are expressed as DGGE bands for 10^6 bacteria. The normalized values ranged between 0.01 and 13.5 bands / 10^6 bacteria. For surface sediments (0-1 cm) in February, values were significantly higher in sandy stations 435/DCG than in coastal stations 130/140/700; in July, it was significantly higher in stations 435/DCG than in station 700. For the 9-10 cm layer of the sediments in February, biodiversity was significantly higher in station 435/DCG than in stations 130/700; in July, it was at the same level in all the stations (Table 2). Using the normalized values, significant seasonal differences were also observed between February and

July : for the 0-1 cm sediments, diversity increased in station 130 and decreased in stations 120/230/330/DCG/ZG03; for the 9-10 cm sediments diversity increased in stations 130/700 and decreased in station ZG03 (Table 2).

TABLE 2. Number of DGGE bands for 10^6 bacteria (range; n=4) observed in marine sediments of 9 stations on the Belgian Continental Plate in February 2007 (FEB) and July (JUL) 2007. Two sediment sections were considered (0-1 cm and 9-10 cm). Values in boldface refer to significant seasonal differences, within the same station and sediment layer (Mann Whitney U-test, $P < 0.05$). Letters (a, b) refer to comparisons between stations (within one sediment layer); stations are not significantly different if at least one letter is shared (Dunn's test, $P < 0.05$).

	120	130	140	230	330	435	700	DCG	ZG03
FEB 0-1 cm	**0.18 – 0.65** ab	**0.02 – 0.05** b	0.02 – 0.15 b	**0.18 – 2.03** ab	**1.44 – 4.24** ab	2.65 – 9.63 a	0.01 – 0.03 b	**8.81 – 13.5** a	**0.29 – 2.27** ab
FEB 9-10 cm	0.11 – 0.29 ab	**0.02 – 0.04** b	0.02 – 0.04 ab	0.06 – 1.65 ab	0.08 – 0.57 ab	2.07 – 4.27 a	**0.02 – 0.03** b	0.47 – 5.78 a	**0.38 – 4.20** ab
JUL 0-1 cm	**0.04 – 0.06** ab	**0.07 – 0.07** ab	0.23 – 0.31 ab	**0.05 – 0.19** ab	**0.12 – 0.28** ab	6.12 – 15.5 a	0.03 – 0.09 b	**1.31 – 3.79** a	**0.06 – 0.20** ab
JUL 9-10 cm	0.07 – 0.26 a	**0.05 – 0.10** a	0.05 – 0.06 a	0.07 – 0.09 a	0.17 – 0.63 a	1.19 – 1.54 a	**0.05 – 0.17** a	0.34 – 2.11 a	**0.09 – 0.21** a

For eubacterial biodiversity no clear trend was observed with the raw DGGE data. Elevated biodiversities were found in sandy offshore sediments (e.g., station DCG in February and July) but also in coastal sediments (e.g., the sandy station 120 in February) even in metal contaminated areas (station 130 in July). A clear trend was nevertheless observed when DGGE results were expressed per unit of biomass : coastal stations (stations 130, 140, 700) displayed the lowest bacterial diversities per unit of biomass, in the 0-1 cm layer of the sediments, and the offshore stations (stations DCG, 435) located at more than 20 km from the coast the highest. However, the validity of such a normalization may be questioned. Indeed, as biomass was more elevated in coastal muddy sediments than in sandy sediments (up to two orders of magnitude), the result of normalization will automatically result in lowered values in coastal areas. In fact, the same result may be obtained with any variable displaying high values in coastal areas such as contamination or surface area of the sediments (data not shown). Although the reality of the observed trend may be questioned, it should be noted that raw diversity DGGE values were never low in offshore stations DCG and 435 (always >16 DGGE bands) and that the lowest values (6 DGGE bands) were always observed in coastal stations. Similar results were found for other taxa such as nematodes in the same area (Vincx 1990). It's thus possible that the trend observed in the present study, i.e. an increasing eubacterial biodiversity offshore, is not an artefact. This observation is corroborated by the fact that archaebacterial 16S rRNA sequences could not be obtained by PCR in coastal stations; on the contrary, many archaebacterial sequences were obtained offshore, in sediments of stations DCG and 435 (data not shown).

In 2008, vertical profiles of DGGE taxon richness were obtained for stations 130 and 700. About 15 band positions were followed each month in both stations between 0 and 10 cm. An example of a profile, (DGGE band 2/2) is shown in Fig. 4. In that figure, it can be seen that reproducibility is good and that the relative intensity of the band varies with sediment depth, with a peak in the 8-9 cm section of the sediments. The profiles of the relative intensities for all DGGE bands considered up to date have been used in ordination analyses and good correlations were found between some band profiles and some environmental parameters (see below). The DGGE bands showing the best correlations to environmental parameters that are the most relevant for the MICROMET project (such as metals with upward fluxes) will be identified and followed in phase II of the project.

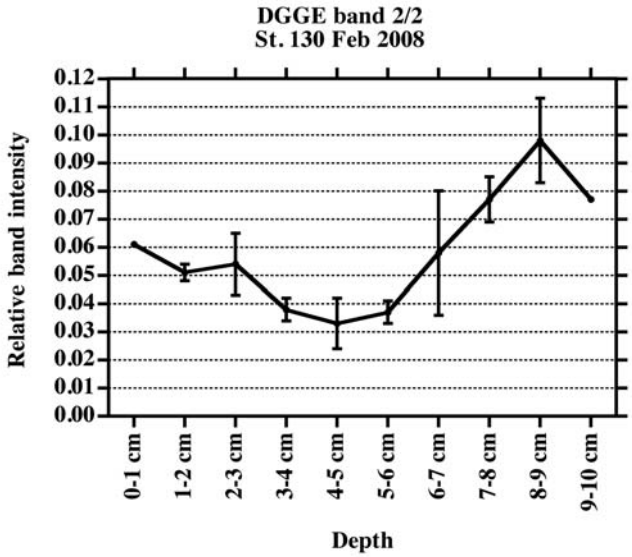


Figure 4. Relative band intensity according to depth for DGGE band 2/2 in February 2008 (station 130); n=2.

DGGE of Archaebacteria.

On the basis of the 16S rRNA sequences obtained in the cloning approach (see Task 1.2.2.) the existing PCR primers for studying archaebacterial communities by DGGE were first adapted to the BCP communities. The adapted primers were called ARC-349-F and ARC-853-R. These primers are homologous to primers S-D-Arch-0344-a-S-20 and 907r (Wilms et al. 2006), but contrary to them they amplify the rDNA genes from both Euryarchaeota and Crenarchaeota of the BCP (the two major groups of Archaebacteria). A 40 bp GC clamp was attached to the 5' end of primer ARC-349-F. These new primers amplify a 544 bp region of the archaeal 16S rRNA gene over the V3 and V5 regions (bp 330-874, *Methanococcus jannaschii* M59126 numbering).

As the quantity of archaeal DNA in the BCP sediments was very low, a nested PCR approach was used with a first amplification of the complete 16S rDNA with primers ARC21F and U1492R (Gillan & Danis 2007) followed by a nested PCR with primers ARC-349-F-GC-clamp and ARC-853-R. This approach was tested in stations 700 and DCG (sediments of February 2007). No PCR amplifications were observed in station 700, which can be explained by a total absence of Archaebacteria in that station (various PCR conditions were tested). On the contrary, PCR amplifications were successful in station DCG and the DGGE profiles featured only one band. The diversity of Archaebacteria is thus very low in that station. No PCR amplifications have been obtained in the other stations. This might be due to the absence, or very low numbers, of archaebacteria on the BCP.

DGGE of microeukaryotes

DNA extraction and DGGE profiling of the February and July 2007 samples, and the 2008 samples, has been completed using the Van Hannen (1998) primer set. All DGGE gels were digitized and aligned using the program Bionumerics. These data were then used to analyze variation patterns in micro-eukaryotic community structure and relations with environmental parameters using multivariate techniques (see below).

For 2007, the DGGE analyses revealed a surprisingly high number of eukaryotic phylotypes (DGGE bands), with values ranging between ± 20 – 50 (data not shown). There

were no clear trends in diversity between stations, depths or seasons, but the data need to be further analyzed (normalized to biomass). In order to get an idea of the identity of these bands, a total of about 404 bands was excised, of which 114 have been sequenced. BLAST analyses and alignments revealed 63 different sequences, most of which belonged to the Stramenopiles (mainly diatoms), metazoans (annelids, nematodes, arthropods, Cnidaria, …) and Fungi (Table 3 and Fig. 5). Truly protozoan sequences (Alveolata, Cercozoa, Foraminifera) accounted for 13 respectively 14 % of all sequences in February and July. However, many sequences could not be assigned to a known eukaryotic group ('uncultured') or gave ambiguous BLAST results ('various'); these groups may well comprise further Protozoan diversity (see 1.2.2). In addition, we also found sequences matching with Chlorophyta and dinoflagellates. No pronounced compositional differences were observed between February and July (not shown).

TABLE 3. Phylogenetic affiliation of DGGE bands of micro-eukaryotes (primers Van Hannen 1998) for 2007 (63 seq) and 2008 (44 seq).

	2007		2008	
	Nb	%	Nb	%
Stramenopila	24	38,1	14	31,8
various eukaryote	8	12,7	0	0,0
uncultured eukaryote	2	3,2	0	0,0
Alveolates	4	6,3	1	2,3
Ciliates	0	0,0	3	6,8
Cercozoa	3	4,8	7	15,9
Metazoa	9	14,3	5	11,4
Dinophyceae	2	3,2	4	9,1
Fungi	7	11,1	3	6,8
Foraminifera	2	3,2	2	4,5
Chlorophyta	2	3,2	2	4,5
Acantharea	0	0,0	2	4,5
Amoebozoa	0	0,0	1	2,3
	63		**44**	

Figure 5. Phylogenetic affiliation of DGGE bands of micro-eukaryotes (primers Van Hannen 1998) for 2007 (63seq) and 2008 (44seq) expressed in percentages.

For the 2 silty stations sampled in 2008 (130 and 700), DGGE analyses again revealed a high eukaryotic species richness (between 35 and 70 bands). 240 bands have been excised, of which so far 77 have been sequenced. BLAST analyses and alignments revealed 44 unique sequences. Stramenopila (mainly diatoms), Metazoa (Nematoda, Nemertea, Gastrotricha) and Fungi accounted for 50% of these sequences. Protozoan sequences (Alveolata, Ciliata, Cercozoa, Foraminifera, Acantharea and Amoebozoa) were relatively more abundant (36%) than in 2007. In addition sequences matching Dinophyceae and Chlorophyta were found (Table 3). Some of the sequences (13) found in 2008, corresponded with the sequences found in 2007.

The predominance of DGGE bands affiliating with diatoms in the dark subtidal sediments is surprising, especially in the 2007 samples, since the sediments were anoxic and aphotic. The molecular data however are confirmed by the microscopic analyses which reveal the presence of live diatom cells in almost all samples.

Distinct changes in seasonal and depth distribution have been detected for the relative abundance of selected phylotypes (see e.g. Fig. 6). An example of a profile of a ciliate (*Acineta* sp-DGGE band 47.6) is given for two depths (0-1 cm and 1-2 cm) for station 130 and of a cercozoan species (*Protaspis* sp – DGGE band 58.4) for two depths for station 700. The relative abundance of *Protaspis* increases from February onwards with a peak in June in the top sediment layer, while a decrease is seen in a deeper layer (8-9 cm). The June peak in the top layer is also present in station 130 (not shown) and could be related to an increase in organic matter deposition (< algal blooms) in early summer.

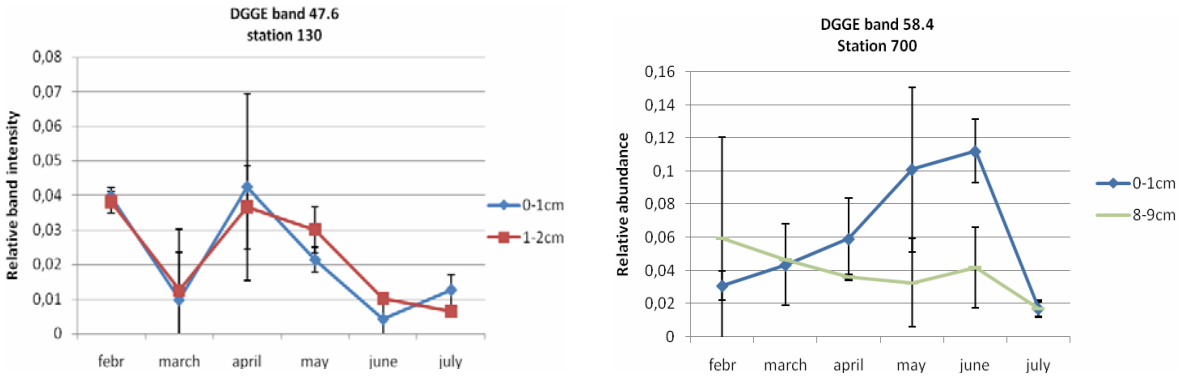


Figure 6. Relative band intensities of a Ciliate (DGGE band 47.6-left) and a Cercozoa (DGGE band 58.4-right) according to months for 2 sediment horizons in station 130 (left) and station 700 (right). N=2; ±stdev.

In addition to the DGGE analyses with the Van Hannen primers, we also tested the Diez et al. (2001) DGGE primer set. These primers amplify a larger DNA fragment and thus harbour more phylogenetic information. After fine-tuning of the protocol, preliminary DGGE runs revealed a molecular diversity which is even higher than the one observed with the Van Hannen primers. The high amount of band classes however made both band excision and alignment problematic. For our analyses we therefore opted for a combination of DGGE using the Van Hannen primer set combined with a clone library approach.

To exclude the possibility that the diversity of certain important protozoan groups is underestimated due to the predominance of diatom DNA in the samples (or PCR bias for this DNA) (cf. Shimeta et al. 2007), we also developed and tested a nested PCR – DGGE approach for three primer-sets which are specific for important benthic protozoan groups, viz. ciliates, Cercozoa and Kinetoplastida (cf. 2, task 1.2.1). The primers were tested on 16 samples from 4 stations (435, ZG, 130 and 700, both February and July). About 40 bands of each DGGE were excised, of which so far respectively 11 'ciliate', 14 'kinetoplastid' and 8 'cercozoan' bands have been sequenced and analyzed using BLAST. The Cercozoa-specific

primers proved to be 100% Cercozoa-specific, whereas ciliate- and kinetoplastida-specific primers revealed a majority of sequences belonging to various other groups of microbial eukaryotes (Stramenopila, Fungi, Dinophyceae,…). A possible explanation is that PCR conditions are not optimal (annealing temperature too low). Comparison of the primers with the sequences showed that one to two mismatches could be found between the 'aspecific' sequences and the primers. Due to this aspecific amplification we did not further use the group-specific DGGE approach. The group-specific primers sets were however used for construction of the clone libraries (see below, task 1.2.2).

A PCA ordination analysis of the fully aligned micro-eukaryote DGGE data set of 2007 is shown in Fig. 7a. Sandy and mixed sediments are composed of different eukaryotic communities, with mixed sediments taking an intermediate position. Grain size, either directly or through modification of the chemical and physical environment (including the presence of pollutants, cf. above and below) thus strongly influences eukaryotic community structure. No clear differentiation could be seen between top and bottom sediment layers, or between seasons (not shown). The envelope in Fig. 7a (right) shows that stramenopile sequences (mainly diatoms) are largely restricted to silty and mixed sediments, which may be related to deposition and preservation of sedimented diatom blooms in these sediment types.

PCA ordinations were also performed on the fully aligned 2008 seasonal DGGE data for stations 130 and 700 (see Fig. 7b). The 2008 show that within a single sediment type (silty) there are pronounced changes in eukaryotic community composition with season and depth. Seasonal changes are most pronounced in the surface layers, variation with depth is most pronounced in the period March to May.

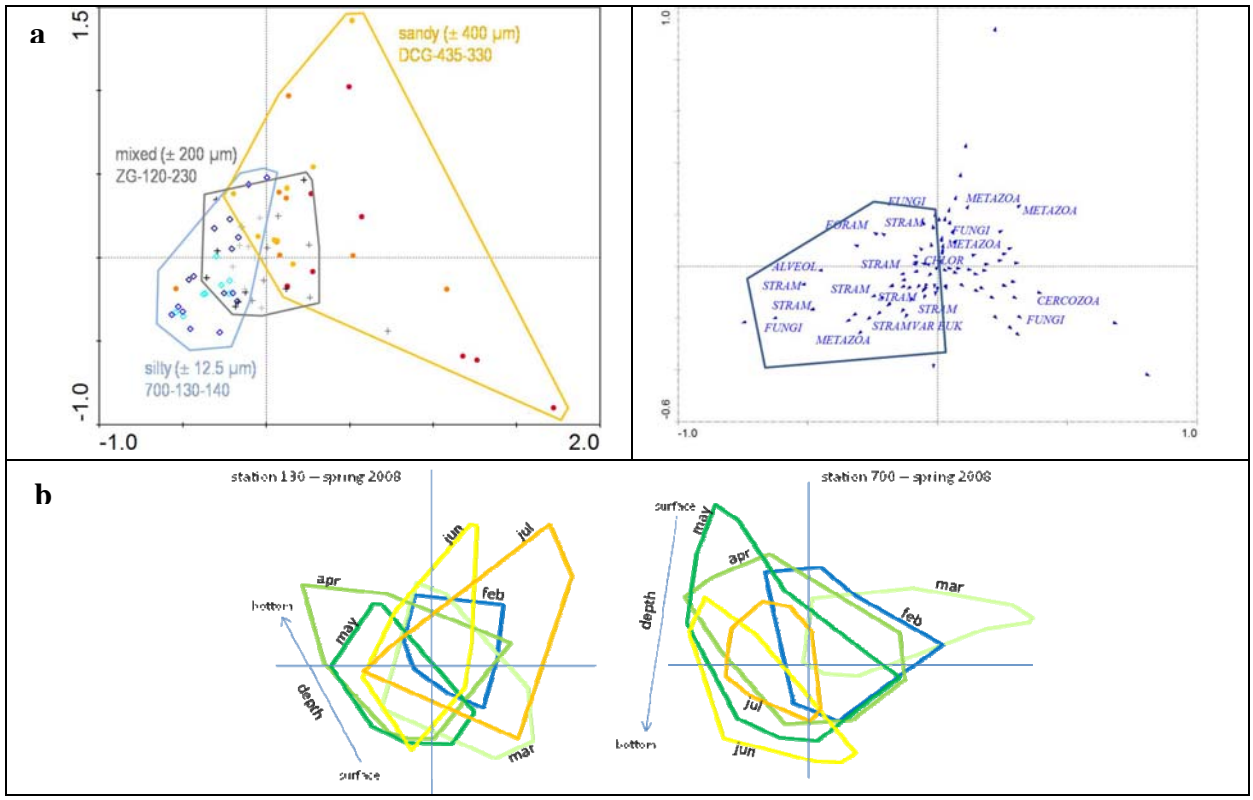


Figure 7. PCA analysis of the full 2007 (a-above) and 2008 (b-below) data sets. (a) The sample ordination is shown on the left; the species ordination (with labels given for identified bands) is on the right. Envelopes show the sediment group affiliation (see below 1.4.1) of the stations (left) and the position of the so far identified stramenopile (diatom) phylotypes (right). (b) Sample ordinations for stations 130 and 700 – only monthly envelopes are shown. The 'depth' arrow shows the direction in which depth varies.

Task 1.2.2. SSU rRNA clone libraries and sequencing.

Eubacteria

In 2007, eight DNA libraries were obtained (4 stations, 2 sediment horizons). The stations were DCG, 435, 700 & 130. The universal primers 8F and 1492R were used. A total of 288 sequences of about 600 bp were obtained (Table 4). Sequences were identified using BLAST.

TABLE 4. Number (Nb) and percentage (%) of clones obtained in each of the 4 stations analysed. H, 0-1 cm sediments; B, 9-10 cm sediments.

	DCG-H		DCG-B		435-H		435-B		130-H		130-B		700-H		700-B	
	Nb	%	Nb	%	Nb	%	Nb	%	Nb	%	Nb	%	Nb	%	Nb	%
α-Proteobacteria	1	2.4					2	4.9							3	7.9
β-Proteobacteria	1	2.4			2	5.3										
γ-Proteobacteria	18	43.9	13	34.2	12	31.6	18	43.9	10	37	10	30.3	13	40.6	12	31.6
δ-Proteobacteria	5	12.2	6	15.8	11	28.9	3	7.3	9	33.3	10	30.3	5	15.6	11	28.9
ε-Proteobacteria																
Actinobacteria	6	14.6	3	7.9	4	10.5	2	4.9			1	3.03	1	3.13		
Acidobacteria	3	7.3	5	13.2	1	2.6	6	14.6	1	3.7	3	9.09			3	7.9
CFB bacteria	2	4.9	4	10.5	4	10.5	1	2.4	5	18.5	6	18.2	8	25	7	18.4
Planctomycetes	3	7.3	3	7.9			3	7.3			1	3.03	1	3.13		
Nitrospirales	1	2.4	1	2.6			4	9.8								
Verrucomicrobia			2	5.3			1	2.4								
Chlorobi													1	3.13	1	2.6
Chloroplastes													1	3.13	1	2.6
OD1							1	2.4								
WS3											1	3.03				
TG3											1	3.03				
OP8													1	3.13		
Spirochaetes			1	2.6												
Unknown	1	2.4			4	10.5			2	7.41			1	3.13		
Nb of clones	41		38		38		41		27		33		32		38	
Nb of groups	10		9		7		10		5		8		9		7	

As shown in Table 4, the number of bacterial groups varied between 5 and 10 in the sediments analysed [500 mg of sediments (ww) were used in the analyses; this corresponds to a surface of 0.4 m^2 for stations DCG & 435 and 2.9-3.2 m^2 for stations 130 & 700]. Gamma- and delta-Proteobacteria, as well as CFB bacteria, were observed in all the DNA libraries.

Archaebacteria

A total of 17 16S rDNA sequences were obtained in station DCG (in the two sediment horizons considered; sediments of February 2007). 10 of these sequences are complete (± 1500 bp). The majority of these sequences cluster in the Marine Group 1 Crenarchaeota (Gillan & Danis 2007). These new BCP archaeal sequences were aligned to other similar sequences in the GenBank database and were then used to adapt the existing PCR primers for DGGE (see Task 1.2.1.). The PCR amplifications were not successful in the other BCP stations considered.

Micro-eukaryotes

We first made two clone libraries (250 clones each), one for the upper sediment horizon (combination of top cm's of stations DCG and 700) and one for the lower sediment horizon (combination of bottom cm's of stations 130 and 700) using the general eukaryotic primers 1427f and 1637r (Van Hannen et al., 1998). These clones were used for the construction of a set of markers that were used in the DGGE analyses.

We then made 3 group-specific clone libraries with protozoan primers for ciliates, Cercozoa and Kinetoplastida (cf. above); the kinetoplastid-specific clone library however was not successful (no positive clones). CL were made with lumped PCR products of PCR reactions performed with template DNA of both stations 130 and 700, different months and different depths. Eighty-seven positive ciliate-clones and 37 Cercozoa-clones (positive clones after screening) were found. After sequencing (69 clones for Ciliates, 27 clones for Cercozoa) and BLAST analysis, 52% and 93% respectively of the sequences could be assigned to the correct taxonomic group. The remaining sequences were related to Bacillariophyta (Diatoms) and uncultured eukaryotes, despite the specificity of the primers. For ciliates, 14 unique sequences were found; sequences related to *Acineta* sp. (class Phyllopharyngea) and representatives of the class Spirotrichea (*Strombidium, Trachelostyla, Holosticha, Tintinnopsis*) dominated the clone library. In addition representatives related to other important ciliate groups were found (Oligohymenophorea, Litostomatea, Karyorelictea). For Cercozoa, 5 unique sequences were found; BLAST analyses of obtained sequences were all most closely related to *Protaspis grandis* (Thaumatomonadida). Maximum Likelihood phylogenetic analyses (not shown) reveal that our sequences fall within a diverse cluster of as yet largely unidentified cercozoan representatives.

When comparing the diversity observed with the clone libraries with that observed in the DGGE sequence analyses (cf. above), we note that 3 ciliate sequences (one Strombidium and two Acineta) are identical. Rarer clones were not observed amongst the sequences obtained with the DGGE approach. For Cercozoa on the other hand, a higher diversity is found using the DGGE approach with the general eukaryotic primers (4 Thaumatomonadida, 1 Cercomonadida, 1 Vampyrellidae and 1 uncultured Cercozoa) than in the CL approach with the group-specific primers. Our presumption that by using the general eukaryotic primers, protozoan diversity is underestimated, is thus correct for ciliates (and probably other protozoan), but not for Cercozoa.

Task 1.2.3. Isolation of microorganisms

This task is in progress for bacteria and will be started in fall 2009 for the micro-eukaryotes. To date, a total of 11 pure cultures have been obtained in the aerobic medium (medium I) and 23 cultures in the anaerobic media (media II-IV). No cultures were obtained in medium V. The pure cultures will be identified using 16S rRNA sequencing and will be transferred to the BCCM culture collection.

Task 1.3. Determination of microbial biomass.

Bacteria

MPN counts were obtained with 4 types of media in BCP sediments collected in February 2007 (medium V was unsuccessful). An example for medium I (oxic conditions) is given in Fig. 8; the MPN counts varied between 4.9 x 10^2 to 7.0 x 10^7 per gram of sediments (ww). Counts were very similar in the two sediment horizons considered. In anaerobic medium II, counts varied between 4.6 x 10^2 to 2.0 x 10^6 per gram of sediments (ww). Counts were always significantly higher in the 9-10 cm horizon for sediments of station 130, 140, 230 & 700. In anaerobic medium III, counts varied between 2.3 x 10^2 to 1.0 x 10^6 per gram of sediments (ww). Except in stations 120, 330 & DCG, counts were always significantly higher in the 9-10 cm horizon. Finally, in medium IV, counts varied between 4.5 x 10^1 to 1.7 x 10^6 per gram of sediments (ww). No MPN counts were obtained for station 435.

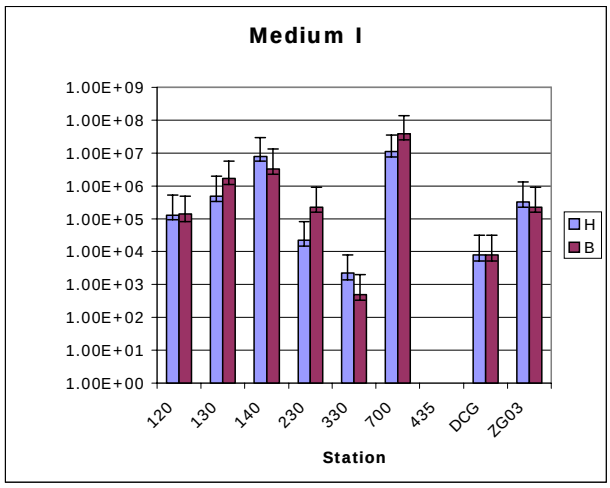


Figure 8. MPN counts in BCP sediments (February 2007). H, 0-1 cm horizon; B, 9-10 cm horizon.

DAPI counts were obtained in all stations in February and July 2007 (n=4) (Table 5). These counts were used to normalize the DGGE data.

TABLE 5. DAPI-counts (mean ± SD) in 500 mg (ww) of sediments (n=4); Feb, February; Jul, July. Two sediment sections were considered (0-1 cm and 9-10 cm). Letters (a, b) refer to comparisons between stations (within one sediment layer); stations are not significantly different if at least one letter is shared (Dunn's test, $P < 0.05$).

	120	130	140	230	330	435	700	DCG	ZG03
Feb 0-1 cm	4.2E+07 – 1.2E+08 ab	3.7E+08 – 5.2E+08 a	1.1E+08 – 7.5E+08 ab	9.4E+06 – 9.5E+07 ab	3.5E+06 – 1.0E+07 ab	2.1E+06 – 7.9E+06 b	2.6E+08 – 8.9E+08 a	1.6E+06 – 2.5E+06 b	7.1E+06 – 5.8E+07 ab
Feb 9-10 cm	6.9E+07 – 1.6E+08 ab	4.9E+08 – 7.1E+08 a	1.9E+07 – 2.7E+08 ab	7.9E+06 – 2.4E+08 ab	2.3E+07 – 1.6E+08 ab	4.5E+06 – 7.9E+06 b	3.9E+08 – 6.8E+08 a	3.6E+06 – 4.4E+07 ab	3.6E+06 – 4.8E+07 ab
Jul 0-1 cm	1.6E+08 – 3.2E+08 a	2.8E+08 – 3.2E+08 a	4.6E+07 – 8.1E+07 ab	7.7E+07 – 2.1E+08 ab	5.8E+07 – 1.3E+08 ab	5.3E+05 – 3.7E+06 b	1.68E+08 – 5.2E+08 a	5.8E+06 – 1.5E+07 ab	8.4E+07 – 2.0E+08 ab
Jul 9-10 cm	5.8E+07 – 1.6E+08 ab	1.8E+08 – 3.4E+08 a	2.9E+08 – 6.3E+08 a	8.3E+07 – 1.9E+08 ab	2.8E+07 – 9.2E+07 ab	8.2E+06 – 1.4E+07 b	7.4E+07 – 2.8E+08 ab	9.6E+06 – 4.5E+07 b	8.0E+07 – 2.3E+08 ab

The DAPI-counts varied between 5.3 10^5 and 7.5 10^8 bacteria in 500 mg of sediments (ww) (Table 5). For the surface sediments (0-1 cm) in February, DAPI-counts were significantly higher in stations 130/700 than in stations DCG/435 (up to 2 orders of magnitude); the same situation was observed in July although the difference was not significant for station DCG. For the 9-10 cm layer of the sediments in February, DAPI-counts were significantly higher in stations 130/700 than in stations 435 (not significant for DCG); in July, the DAPI-counts were significantly higher in stations 130 than in stations 435 and DCG (not significant for 700) (Table 5).

Significant differences of bacterial biomass were thus found among the BCP stations considered in this study. Muddy stations of Group III featured the highest numbers of bacteria, and sandy stations of Group I the lowest. As aquatic bacteria are almost inevitably associated to surfaces (Cooksey & Wigglesworth-Cooksey 1995), DAPI-counts may also be expressed per unit of fine fraction. After such a normalization it was found that coastal stations (stations 130, 140, 700) always featured the lowest biomass per unit of fine fraction when compared to the open sea stations (data not shown). Such a relationship was also found

in another study conducted on the BCP area and might be explained by several factors such as the toxicity of the available surfaces (Gillan & Pernet 2007).

In 2008, DAPI-counts were obtained for sediments of stations 130 & 700 between February and July. An example of profile is shown in Fig. 9. As for the DGGE profiles, reproducibility was good and DAPI-count profiles were used in an ordination approach (see below).

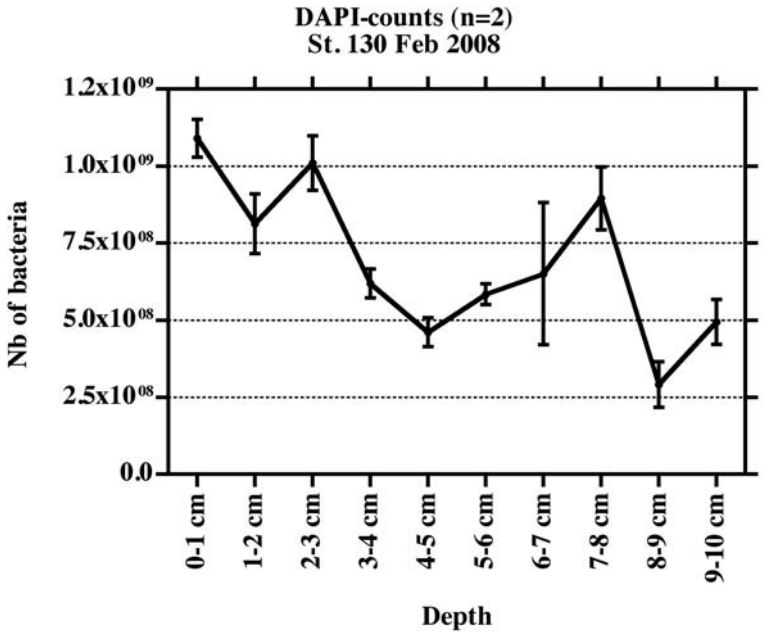


Figure 9. Example of a DAPI-count profile obtained in February 2008 in station 130 (n=2).

Micro-eukaryotes

Due to breakdown of the ultracentrifuge used for the density centrifugation method (cf. Starink et al. 1994) during the February 2007 sampling campaign, no preparations for biomass counts could be made for this period. Preparations have been successfully made for the July 2007 (except stations DCG, 330 and 435) and all 2008 samples. The July 2007 counts (n=3-4) yielded values ranging between 8,25 x 10^5 to 5,19 x 10^6 cells per ml sediment (Fig. 10). These values are in the same order of magnitude as those reported in the literature for subtidal sediments (e.g. Shimeta et al. 2007). Cell numbers tended to be on average higher in the deeper sediment layers. Error bars however are large and further tests need to be carried out. Unfortunately, no data are available for the sandiest stations, which have the lowest prokaryote counts. Heterotrophic nanoflagellates were most abundant in all samples (> 90 % of cell count; not shown).

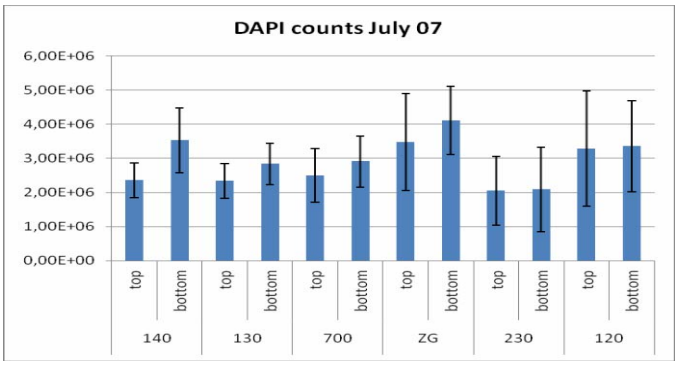


Figure 10. DAPI-counts (mean ± SD) of protozoa per ml of sediments (n=3-4); July 2007. Top= 0-2cm; bottom= 8-10cm.

Task 1.4. Geochemical properties of the sediments.

Task 1.4.1. General properties of the sediment.

Laser granulometry.

Sediments sampled in 2007 in the 9 stations were studied using a Malvern Mastersizer 2000 laser granulometer. They have been classified in three groups. In Group I (stations DCG, 435 and 330), sediments have a MGS of 400 µm (Fig. 11). In Group II (stations 120, 230 0-1 cm, ZG03) sediments have a MGS of 200 µm. In Group III (130, 140, 700, 230 9-10 cm), sediments are very muddy, with a MGS of 12.5 µm (Fig. 11). The shape of curves appear very similar whatever the depth or the season, except in station 230 which features sand at the surface and mud at 9-10 cm. Stations 130, 140 and 700, located close to the coast, are dominated by clays and silt particles. Particle size distribution from stations 120, 230 and ZG03 display variable contents of clays (0-76%) mixed with sandy particles smaller than those present in the open sea. Station 140 showed very heterogeneous sediments and reproducibility between different cores was bad.

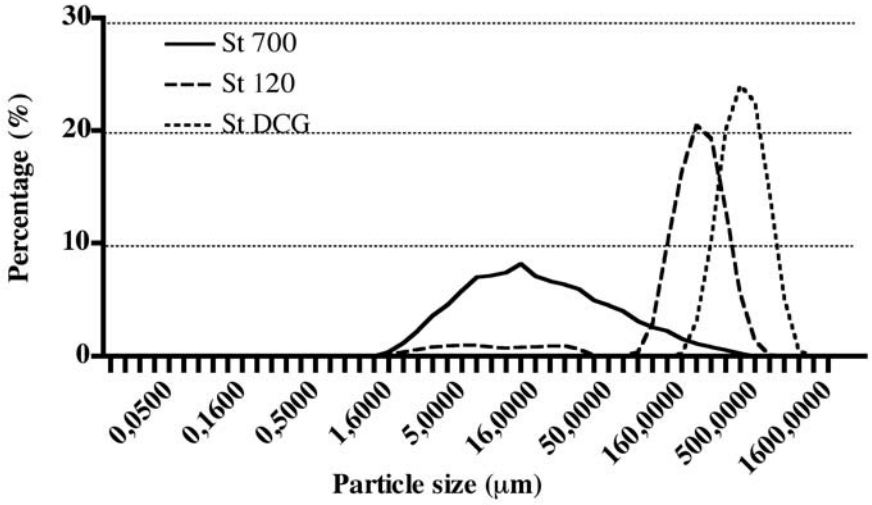


Figure 11. Particles size distribution as measured with a laser granulometer.

Specific area measurements, porosity and core pictures.

Granulometric data have been completed by core pictures and specific surface area (SSA) measurements, which are of paramount importance to normalize bacterial activities in function of the available adsorption sites (see Task 1.2.1.). Sediments from stations 130, 140 & 700 (fine fraction < 63 µm higher than 80 %), displayed SSA values of 13.6 (130), 10 (140), and 11 (700) $m^2.g^{-1}$. A mesoporosity characterizes also the surface of these particles, with a main size at 30 nm. Sediments from stations 120 and ZG03 displayed lower SSA values, between 1.2 and 1.5 $m^2.g^{-1}$. All the other stations displayed SSA values inferior to 1 $m^2.g^{-1}$, which is the detection limit of the technique.

Dissolved sulfide as determined by the DGT approach.

For stations 140 and 700 in 2007, results indicate clearly a sulfate-reducing bacterial activity just below the water-interface characterized by a strong S(-II) gradient of about 40 $\mu mol.L^{-1}$ in the first cm (Fig. 12). Under this level, sulfides concentration did not evolve quantitatively, which means that organic matter and/or sulfates are the limiting factor of the bacterial activities. In addition, the high contents of sulfides in the porewaters suggest, that all the labile iron and other heavy metals should be trapped at least in the solid phase, and probably in AVS compounds. Increase of dissolved sulfides in the station 230 is more regular in the first 8 cm, then the same concentration level as for the two previous stations is reached. It seems that microbial activity in this station occurs with a lower kinetic. Station 130, with very muddy sediment, should be however considered as a heterogeneous site, since the presence of dissolved sulfides is quantitatively detected between 6-12 cm depth. In that station, sulfate-reducing microbial activity takes place in well defined zones, suggesting for instance a discontinuous organic supply in these sediments. This behaviour has already been observed and partly explained using lipid compounds as a biomarker (Billon et al., 2007). In sandy sediments (DCG, 435 & 330, ZG03), the concentration of dissolved sulfides is under the detection limit, probably due to the fact that sediments remain oxic all along the core. This could be explained by a low supply of organic matter whatever the season.

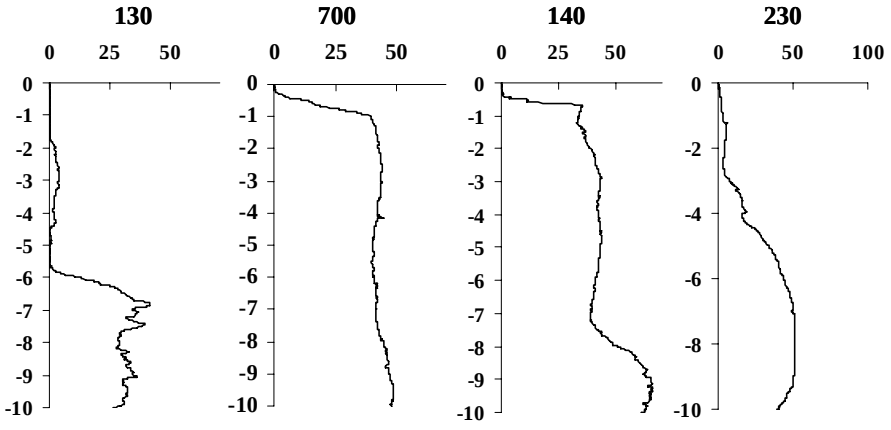


Figure 12. Dissolved sulfide concentration profiles (in $\mu mol.L^{-1}$) in function of depth (cm) determined by AgI DGT in 4 sediment cores from stations 130, 700, 140 and 230 sampled in February 2007.

In 2008, several other profiles have been measured only for the stations 130 and 700. In station 130 (see Fig. 13), the concentrations of dissolved sulfides in the winter and spring are low in the 4 first cm (< 1$\mu mol.L^{-1}$). Below 5 cm, sulfides contents increased to reach

variable maxima, that range from 15 to 80 µmol.L^{-1}. In June and July, the supply of organic matter due to the organism degradation at the surface of the sediments did increase the activity of sulfate reducing bacteria, close to the water-sediment interface. Profiles exhibits therefore high maxima at around 3-4 cm depth with dissolved sulfide values at around 400 µmol.L^{-1} in the porewaters. According to these profiles, the bioturbation seems not sufficient enough to get also high concentrations of sulphides below 8 cm and below this depth the concentrations are comparable to those obtained in the other seasons. Thus, the active production of dissolved S(-II) in the sediment porewaters in summer should trap efficiently the trace metals in the solid sulfide pool. In the other periods studied, metal availability in the surface sediments should be higher.

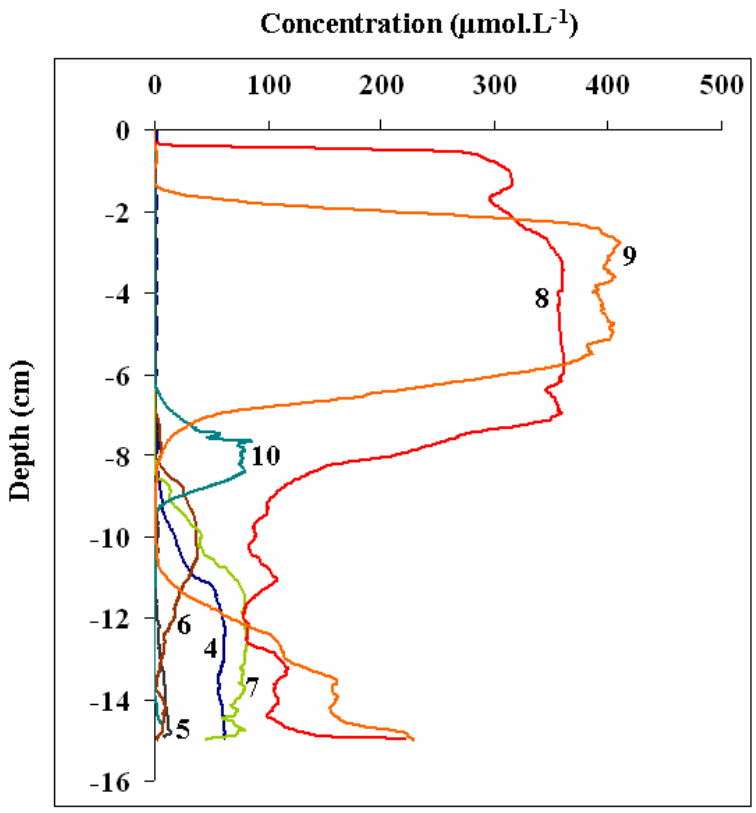


Figure 13. Dissolved sulfide concentration profiles (in µmol.L^{-1}) in function of depth (cm) determined by AgI DGT in station 130 for the following sampling dates: Feb-08 (4); Mar-08 (5); April-08 (6); May-08 (7), June-08 (8), July-08 (9) and November-08 (10).

Acid Volatile Sulfides (AVS) and Chromium Reducible Sulfur (CRS).

Results presented in Fig. 14 indicate the absence of AVS in sandy sediments (DCG, 435), because they are either not produced or quickly reoxidized, for producing CRS for instance. Low contents of CRS were also found in these stations. For the other sites, AVS are generally present at lower concentrations than the CRS. One surprising feature is the higher concentrations of AVS in the surface sediments than in the bottom, suggesting that a transformation of AVS occurs in function of the burial of the particles. However, this trend is not necessarily bound to an increase of CRS with depth, suggesting a more complex pathway of production of CRS, not only based on the partial reoxidation of the AVS.

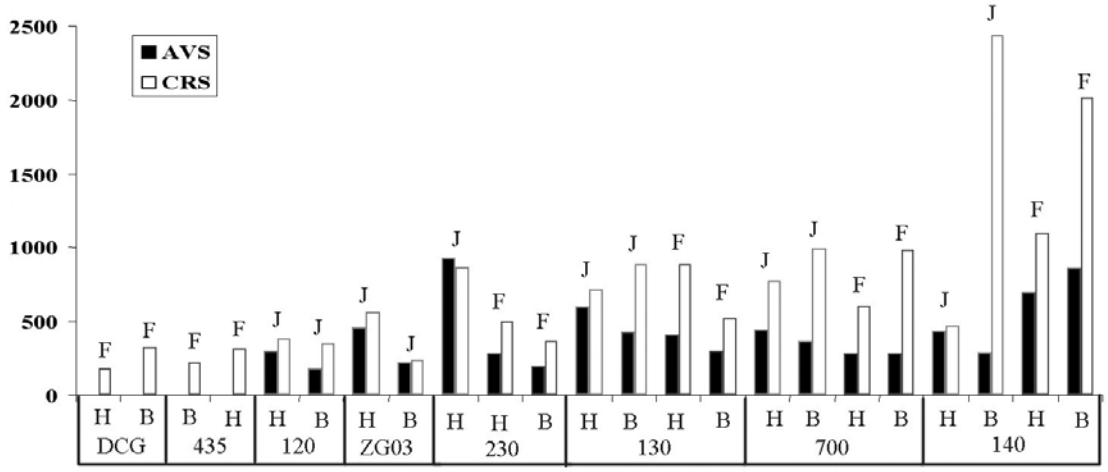


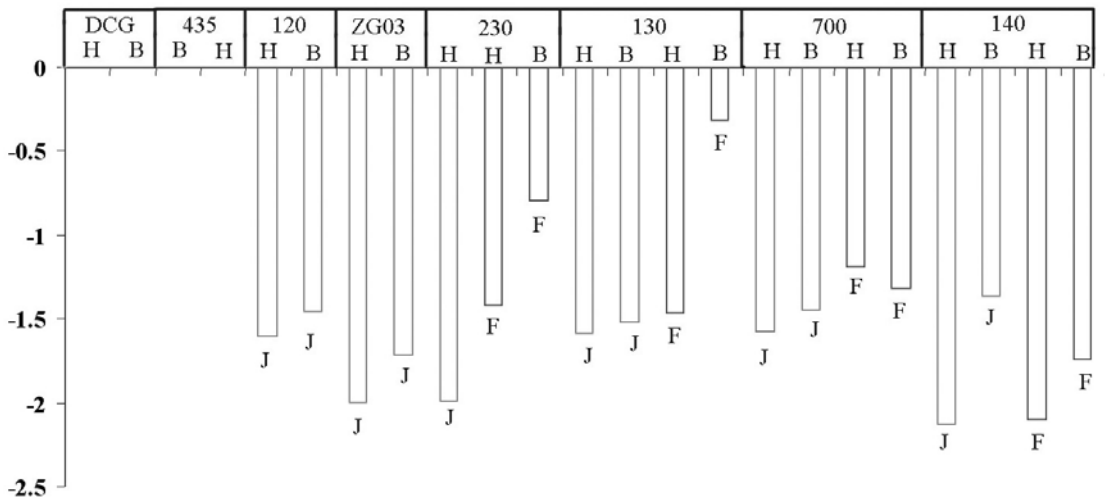
Figure 14. AVS and CRS contents in the sediments sampled from the stations DCG, 435, 120, ZG03, 230, 130, 700 and 140 in February 2007 (F) and July 2007 (J). H, surface sediments (0-2 cm); B, bottom sediments (8-10 cm).

More analyses of AVS and CRS contents have been performed in 2008 in the stations 130 and 700. For station 130, concentrations indicate large scattering of the data. For instance, in the surface sediments [S-AVS] = 660 ± 1700 ppm (for 8 campaigns between Feb-07 and July-08). Differences in bacterial activity, partial reoxidation of surface sediments and heterogeneity of the sediments are probably the main reasons of such a high standard deviation. Profiles of AVS and CRS have also been measured in station 700 and indicated slightly lower concentrations than in the station 130. In both cases, a slow increase of CRS contents occur in the sediments as a function of depth, whereas no such tendency has been pointed out for AVS, indicating that most of the sulfidization process takes place in the first centimeter of the sedimentary column. Generally, the AVS/CRS ratio is lower than 1. That means the transformation of AVS into CRS is effective in our sediments. The results pointed also out that the sulfidization process in sediment is fully achieved and labile iron is still in excess. It is therefore surprising to notice no relevant production of AVS and/or CRS in surface sediments of the station 130 in June and July 08. Several explanations may be proposed: (i) the solubility product of FeS is not achieved in these porewaters; (ii) although concentrations of sulfides are high, reoxidation processes occurs and do not permit a quantitative production of inorganic sulfide precipitates and; (iii) the remaining labile iron fraction is not quickly reactive to a kinetically point of view. To sum up, sulfidization processes occur mainly in the surface of the sediments. In deeper depths, the lack of biodegradable organic matter is a limiting factor to subsequent production of AVS and CRS.

Simultaneously Extracted Metals (SEM) and Toxicity Index (TI).

According to the results obtained in 2007 and 2008 (Fig. 15), we can assume that AVS are in excess compared to the labile metals Cu, Zn and Pb (generally more than 10 time higher), which are thereby trapped in sulfides under anoxic conditions. Consequently, sediments should not be toxic towards Cu, Zn and Pb. For the sites DCG and 435 TI has not been calculated because AVS were not detected. However, concentrations of SEM in these samples remain low (about ten times lower than in muddy sediments), mainly due to the very low quantity of fine fraction. In addition, for the station 130, no seasonal effect has been observed on the TI; the values remain very negative whatever the sampling date, with always a large excess of AVS.

(A)



(B)

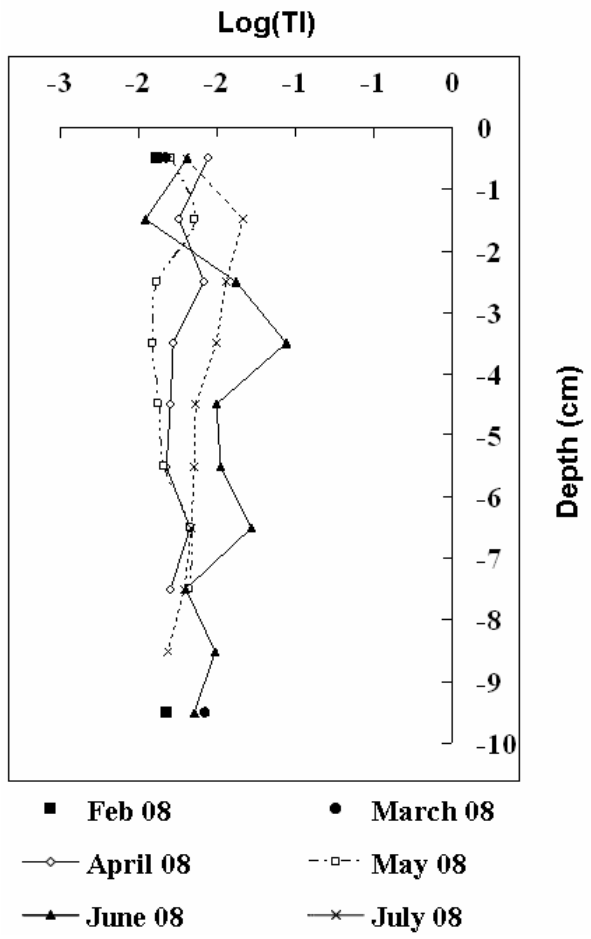


Figure 15. (A) Logarithm of TI calculated for the sediments sampled in February 2007 (F) and July 2007 (J), in stations DCG, 435, 120, ZG03, 230, 130, 700 and 140; **(B)** Logarithm of TI calculated for sediments of station 130 sampled in February (Feb), March, April, May, June and July 2008.

Pigment analyses

The 2007 chlorophyll a concentrations in sediments are shown in Fig. 16 (left). Chl a is very low to zero in the sandy (DCG, 435 and 330) and mixed (230, 120 and ZG) sediments, both in February and in July. The silty stations (700, 130 and 140) however show much higher values. For the siltiest stations 700 and 130, chl a concentrations in winter are on average higher at depth than at the sediment surface; the opposite appears in summer.

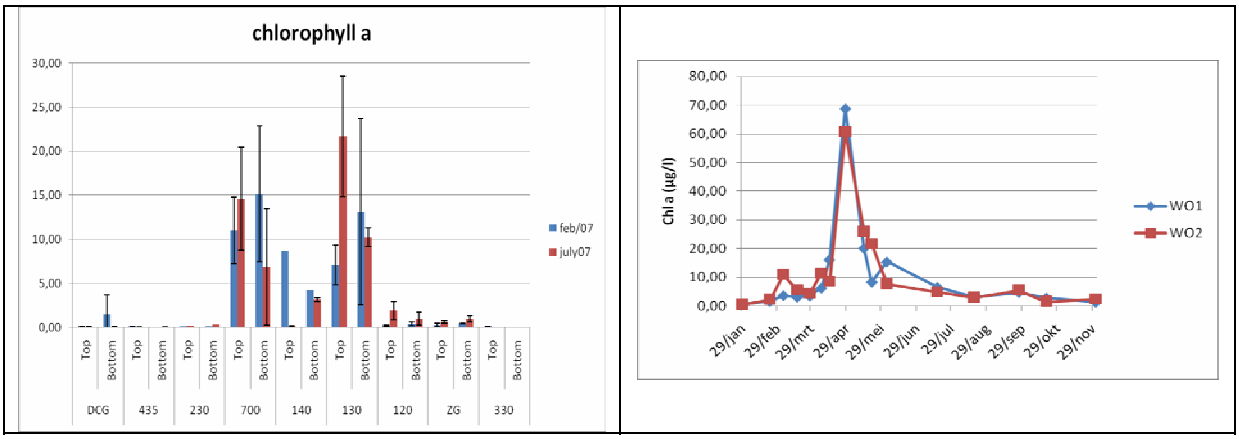


Figure 16. Left: Chl a concentrations (µg/g dw) (mean ± SD) at the 2 depths at the 9 sampling stations for February and July 2007 (n=2-3). Top= 0-2cm; bottom= 8-10cm. Right: Chla a concentrations (µg/L) in the water column at two coastal stations (WO1 and WO2, closely situated to stations 130 and 700 respectively) in spring 2008.

In 2008, the main spring bloom occurred in late April-early May (Fig. 16, right). Pigment analyses were done for all depths (0-10cm) for the silty stations 130 and 700, and are shown per month (Fig. 17). Again (cfr 2007), February shows higher chl a concentrations at higher depth than in the top sediment (not clear for station 700). Chl a concentrations in the surface layers, derived from the spring phytoplankton bloom, mainly increase from May onwards. Note that in all months chl a concentrations can be high in deeper layers.

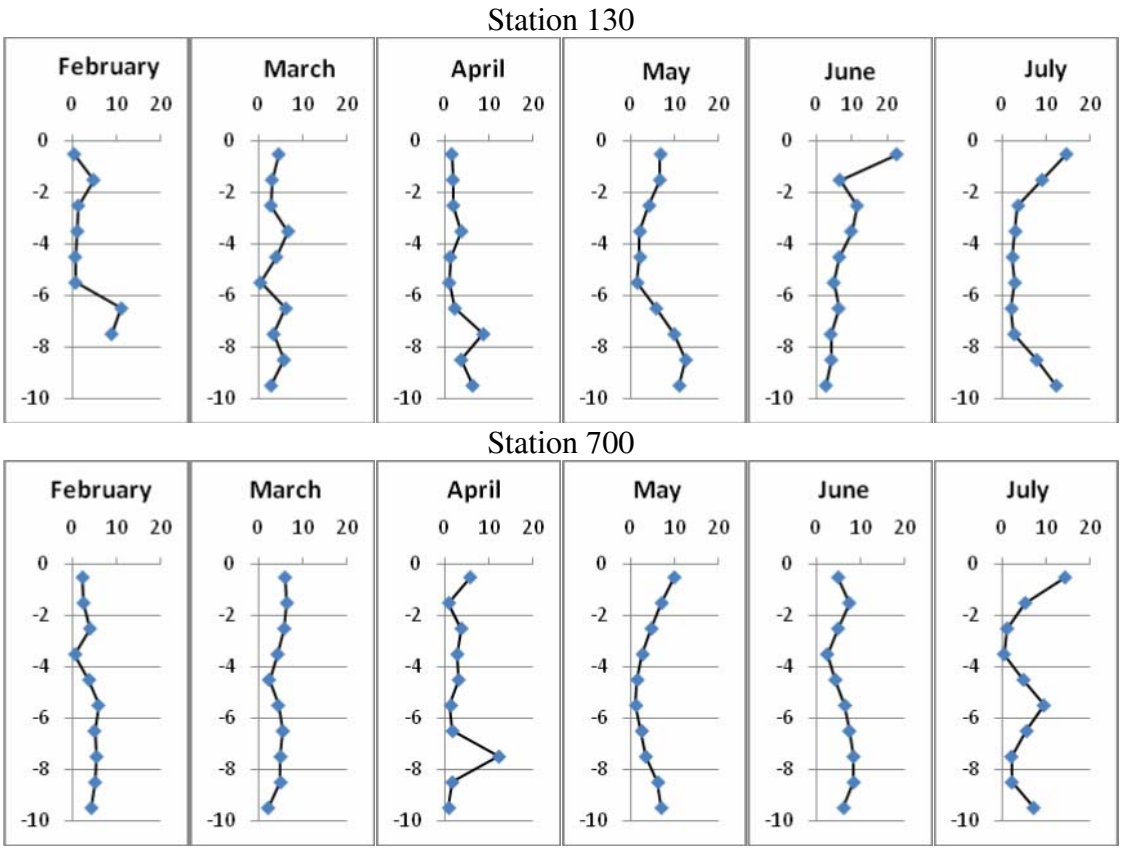


Figure 17. Vertical profiles of Chl a concentrations for the silty stations 130 and 700 for 2008. X-axis: chl a (µg/g DW); Y-axis: depth (cm)

Task 1.4.2. DET/DGT analysis

1. Spatial variability.

Sediments of the sandy stations 435, ZG03 and DCG contain low amounts of organic matter (OM) and consequently associated trace metals. Dissolved oxygen and other oxidants are abundantly present. As a result, the vertical profiles of trace elements Mn, Fe, Co and As assessed by DET and DGT did not present major concentration variations with the depth (Fig. 18).

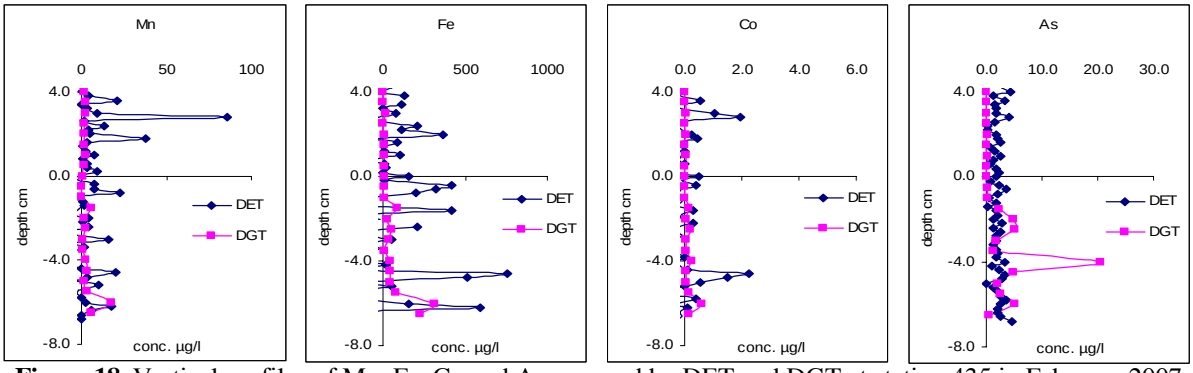


Figure 18. Vertical profiles of Mn, Fe, Co and As assessed by DET and DGT at station 435 in February 2007.

High resolution profiles of trace elements were assessed by DET and DGT in muddy sediments of stations 130, 140 and 700. Generally good correspondence between DET and centrifuge data were observed at the 3 stations. The trace element profiles at those three

stations show more pronounced differences (Figs 19 & 20). The remobilization zones of the trace elements appear at specific depths for the different stations. The trace elements at station 700 show much higher concentrations than at the two other ones. High concentrations have been observed for As (at the 3 stations) and further research should be carried out on this element.

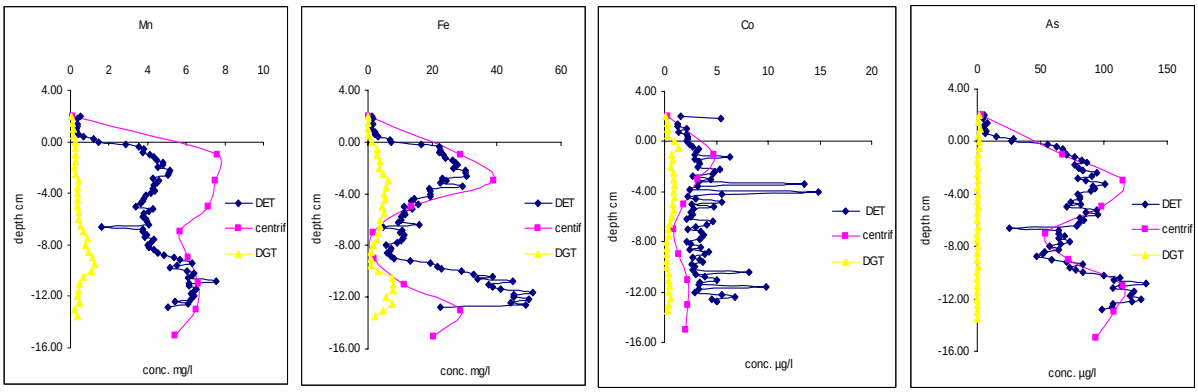


Figure 19. Vertical profiles of Mn, Fe, Co and As assessed by DET, DGT and centrifuge at station 130 in February 2007.

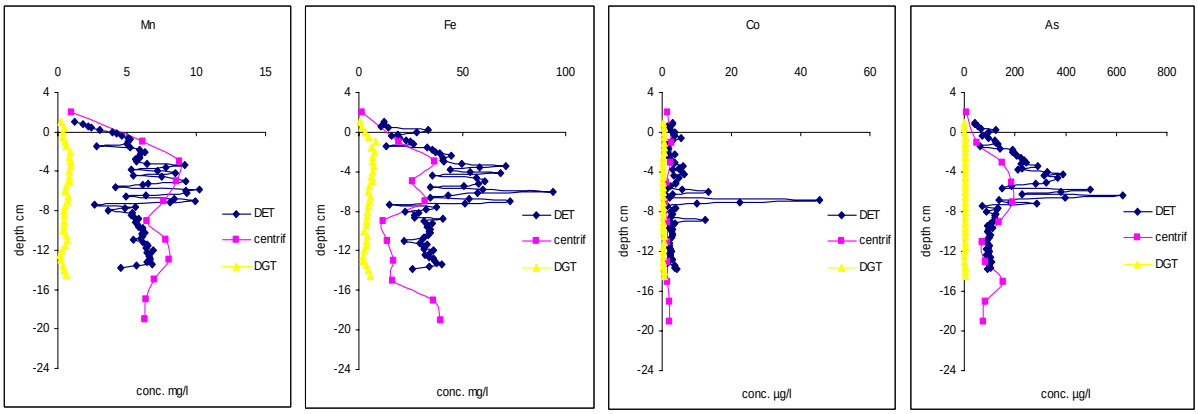


Figure 20. Vertical profiles of Mn, Fe, Co and As assessed by DET, DGT and centrifuge at station 700 in February 2007

2. Seasonal variability.

2.1 Seasonal variation of POC, $\delta^{13}C$ and $\delta^{15}N$ at station 130

Dissolved oxygen profiles indicate highest concentration during the winter months of February and March 2008 in overlying bottom waters and sediment pore waters (Fig. 21). At 0.3 cm of depth the sediment becomes anoxic. This depth rises to 0.1 cm in the spring months of April and May (Fig. 21). These results suggest an increased microbial activity in the sediment surface layer in the spring months, probably due to a supply of freshly produced organic matter.

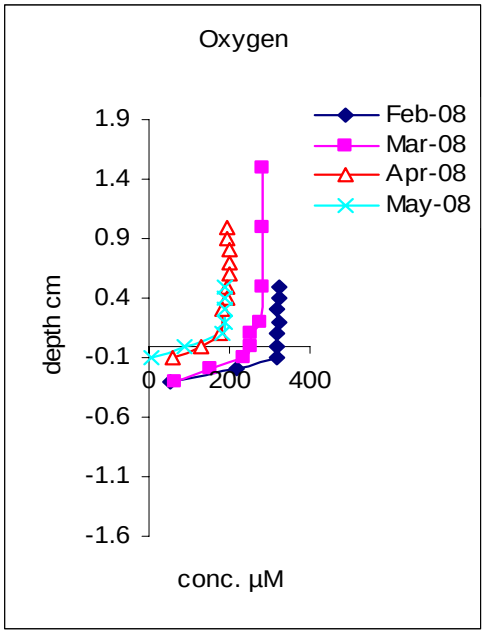


Figure 21. Oxygen concentration in sediments of station 130 (February to May 2008).

The POC varied between 1.4 and 2.3 % between February and May 2008 (Table 6). This can be explained by a variable input of organic matter from the Scheldt estuary and by the spatial heterogeneity of the sediments. The lower percentage of POC during February to March is not a result of bacterial degradation because bacteria preferentially use the lighter isotopes, which would result in more positive $\delta^{13}C$ values of POC instead of lower ones as observed for March. On the contrary, the isotope ratios of organic carbon suggest an input of freshly produced POC in March because the $\delta^{13}C$ values decreased, in agreement with the lower $\delta^{13}C$ values of marine phytoplankton (Michener and Schell, 1994). The $\delta^{15}N$ values in sedimentary POC also decreased, but the interpretation of the values is more difficult. The degradation of the organic matter in the following months is corroborated by a decrease in dissolved oxygen levels in the surface sediment layer (Fig. 21) and by a progressive increase in $\delta^{13}C$ and $\delta^{15}N$ because the lighter isotopes are preferentially assimilated by the microbial community (Blair et al. 1985). This progressive increase in the isotopic ratios is in fact a balance between POC degradation and input of freshly produced organic matter, with the former process dominating the latter.

The redox profiles corroborate the results obtained on dissolved oxygen, with lowest values in spring (- 135 mV at the SWI and – 372 mV at 10 cm of depth). During March 2008, pH values were at the lowest and most variable and dissolved sulfide levels remained always very low compared to other months. In February, sulfide concentrations rapidly increased from 5 µM at 8 cm to 63 µM at 12.3 cm; values remained stable untill the bottom of the sediment cores (Fig. 14). Measurable amounts of S^{2-} were not detected during April and May in the upper 6.5 cm of the sediments (Fig. 14). In April, sulfides increased to 40 µM between 8 and 11 cm of depth and dropped again below 5µM below 14 cm. In May, sulfide levels increased to a stable concentration of 40-80 µM between 8 and 12 cm of depth (Fig. 13). Seasonal variations of these parameters induce temporal variation in some of the trace metals as we will see further in this report.

TABLE 6. $\delta^{15}N$, $\delta^{13}C$ and POC values in the sediment top layers.

Month	*$\delta^{15}N$ ‰*	*$\delta^{13}C$ ‰*	*POC %*
Feb	9.93	- 23.00	2.3
Mar	7.95	- 23.41	1.4
Apr	8.46	- 23.03	1.6
May	8.22	- 22.62	1.7

2.2 Seasonal variation of trace elements assessed by DET and DGT probes.

Subsurface DET profiles (Fig. 22) displayed a range of concentrations from detection limits in deep layers to a maximum of 94 µM of Mn, 720 µM of Fe and 3.8 µM of As indicating a typical redox pattern for coastal marine sediments. Iron presented a typical pattern in relationship to the redox conditions (oxic, sub-oxic and anoxic). In the suboxic pore water layers (between 2 - 6 cm of depth), Fe oxides were reduced and total dissolved Fe levels (DET) reached concentrations of about 700 µM during all surveys, except in April 2008 where the maximum was 160 µM (Fig. 22B).

In February and May 2008, when sulfide levels were important in the sediments below 8 cm of depth, low total dissolved Fe levels (DET) were observed, while during the other months Fe levels reached 340 µM. The seasonal variability of the Mn profiles (DET) is much less pronounced (Fig. 22A). In the deepest sediment layers, even with dissolved sulfide levels above 5 µM, dissolved Mn levels were still relatively high. This is not surprising as the affinity of Mn for sulfides is relatively low. Several studies have indeed shown that MnS is not observed in most pore waters (Canavan et al., 2007; Huert-Diaz et al., 1998; Morse and Luther, 1999; Billon et al., 2001). For arsenic, an important suboxic maximum was observed in February 2008 (DET) (Fig. 22C).

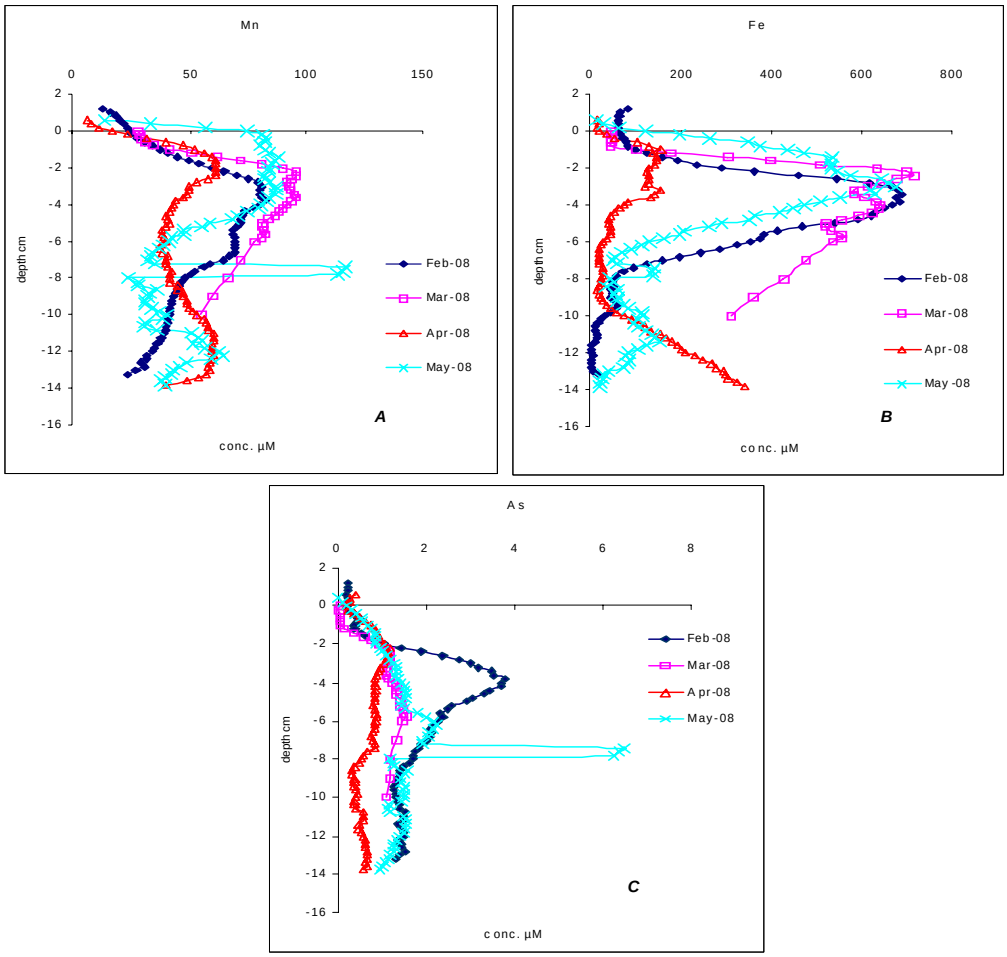


Figure 22. Vertical profiles of Mn (*A*), Fe (*B*) and As (*C*), assessed by DET from February to May 2008.

The DGT approach gives a direct measurement of time averaged fluxes (mass of metal (M)/cm^2.s) rather than concentrations. Concentrations can however, be estimated when (1) a constant pore water flux is maintained and (2) all compounds binding onto the resin have the same diffusion coefficient in the gel layer. If the flux is constant (no depletion in concentration or rapid re-supply from the solid phase), the concentration (C_b) can be calculated by the following formula:

$$M = \frac{C_b D A t}{\Delta g} \qquad (1)$$

If, for different gel thicknesses (Δg), the calculated C_b is constant then the flux is constant during the exposure period.

In 2008, a couple of DGT probes (thickness of the diffusive gels : 0.80 and 0.12 mm) arranged back to back was inserted into one large sediment core (7 cm) at stations 130 and 700. Another couple of probes (one DET and one DGT-AgI probe), arranged back to back, was inserted into the same core. No systematic differences were observed between the two DGT probes with different thicknesses of diffusive gel (see Annex1, Figure A1), neither in sediment porewaters at station 130 or at station 700 (data not shown). The random differences between the two profiles corresponding to the thinner and the thicker gel layers can be explained by sample heterogeneity. In order to validate this hypothesis of heterogeneity, twin

DGT probes (with the same thickness of diffusive gel and arranged back to back), were inserted into one sediment core at station 130. Since two similar DGT probes were inserted into exactly the same sediment area, vertical profiles of trace elements should almost be identical. Actually good comparable results were obtained for the twin DGT probes. No systematic differences were observed between the concentrations found in the two DGT probes. The random differences between the two profiles were within the error due to sample heterogeneity (Annex1, Figure A2).

The second condition mentioned above (i.e., the same diffusion coefficient for all compounds within the gel) is much more difficult to prove *in situ*. In fact, free ions diffuse faster than complexes (Zhang, 2004) but the percentage of free ions, inorganic and organic complexes is difficult to determine in the sediments as well as their diffusion rate. Since the overall diffusion coefficient used in our pore water concentration calculations (equation 1) is overestimated, the pore water concentration and hence the exchange fluxes are underestimated causing our reported fluxes to be minimum.

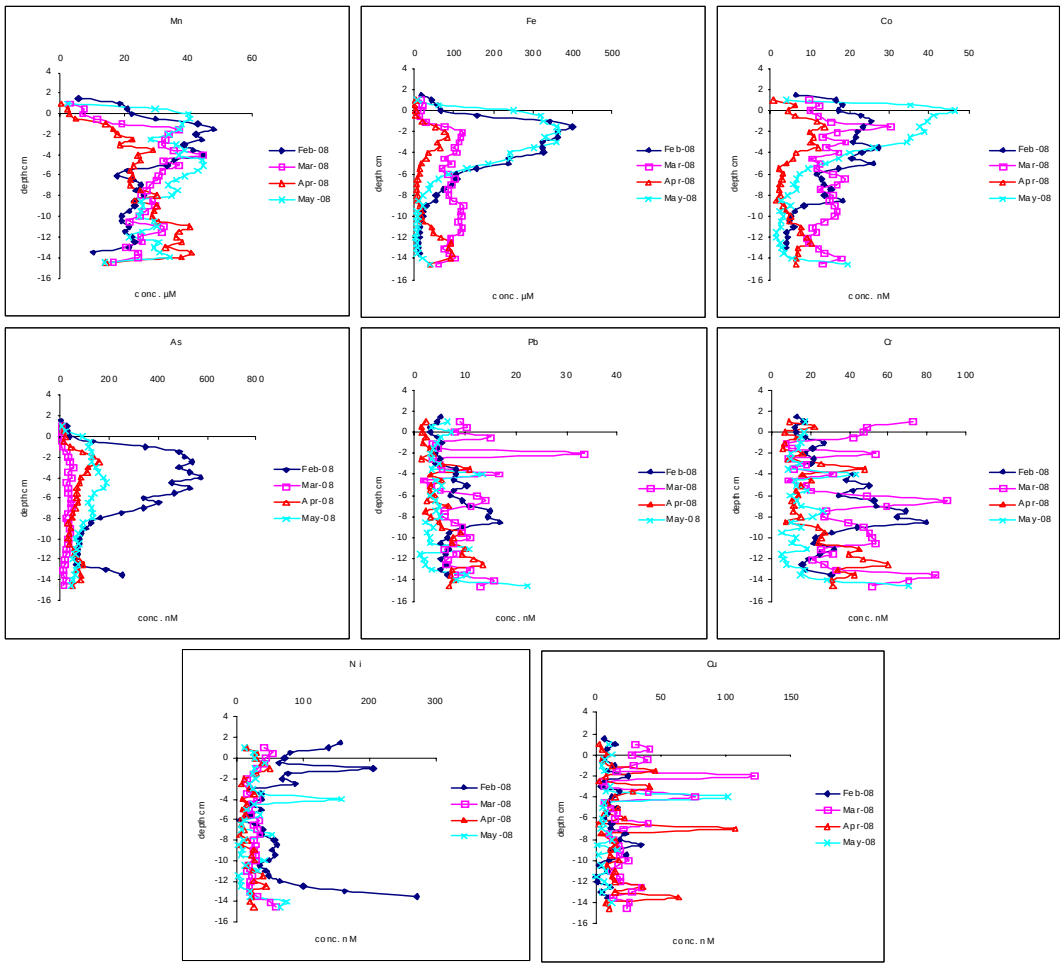


Figure 23. Vertical profiles of Mn (*A*), Fe (*B*), Co (*C*), As (*D*), Pb (*E*), Cr (*F*), Ni (*G*) and Cu (*H*) assessed by DGT from February to May 2008.

The labile Fe, Mn and As levels (DGT approach) displayed profiles similar to those observed with the DET approach except for Fe in March (Fig. 23). For Co and Ni suboxic

maxima were observed, while the Pb and Cr profiles presented several high peaks at various depths in March (for Cu this was observed for all sampling periods). These peaks may be explained by local heterogeneities and/or analytical artifacts (Fig. 23). In the <63 µm fraction of the solid phase in the surface sediment the following concentrations were observed (all concentrations are in µg g^{-1} dw) : 20,000-23,000 (Fe), 620-780 (Mn), 55-65 (Cr), 26-33 (Ni), 13-16 (Cu), 25-32 (Pb), 7-10 (As), 0.36-0.48 (Cd) and 28,000-31,000 (Al). This means that all elements are enriched versus Fe, the enrichment factor ranging from 1.5 to 2.5 for Cr, Cu, Mn, Ni and Pb, and up to 6 for As and Co. Trace elements are known to be associated with Fe oxides (adsorption or coprecipitation) and can thus be released in the pore water together with Fe, during suboxic conditions. The vertical profiles of Mn, Fe and As assessed by DET and DGT in this study indicate that the remobilization zone and subsurface maximum of these elements shifts upwards, closer to the sediment-water interface (SWI), from winter to spring, corresponding with a decrease in oxygen concentrations and redox potential (Figs 22 & 23).

It is worthwhile to investigate seasonal effects of phytoplankton blooms (in surface sediment) and sulfide in the deepest sediment layer, on the trace metal profiles in the pore waters. The phytoplankton biomass at station 130 is mainly composed of diatoms (De Bock & Sabbe, unpubl. data) and was < 1.5 µg chl-a L^{-1} until March, then gradually increased to approximately 16 µg chl-a L^{-1} by mid April. Based on the chl-a in the water column and the $\delta^{13}C$ - POC levels in sediments, we can assume that pore waters became enriched in newly supplied DOC (dissolved organic carbon) / POC (particulate organic carbon) in March – April potentially forming large Fe organic complexes which are incapable of diffusing into the small pores of the DET and DGT gels (Davison and Zhang, 1994 & Zhang and Davison, 1999), explaining the lower subsurface concentrations of Fe in April. The behavior of Mn and As is similar to Fe but less pronounced. For labile dissolved Fe (DGT), this is also valid in March (Fig. 23B). Another possible reason for low Fe could be the introduction of oxygen during DET and DGT deployment in April (the oxygen concentration at the SWI is then very low), but this should normally result in small colloidal Fe and Mn oxhydroxides. Such small colloids are measured with the DET probe, but not with the DGT unless these colloids are very unstable. However, for Fe both the subsurface DET and DGT profiles are strongly reduced in April, suggesting that oxygen is not the key parameter causing the subsurface suppression in April.

In the deep sediment layers dissolved sulfide clearly controls the level of dissolved trace metals. In March, the elevated concentrations of Fe, Mn and several other trace metals may be explained by the absence of dissolved sulfide. On the contrary, DET and DGT analyses in February and May have revealed low levels of trace metals with high levels of dissolved sulfide. It is interesting to compare the solubility product of iron-sulfide compounds (FeS) reported in literature (Davison, 1991, Rickard and Morse, 2005) with our results. Iron sulfide solubility (pK_s) decreases as follows in marine systems : amorphous ferrous sulfide (2.95 ± 0.1), mackinawite (3.6 ± 0.2), greigite (4.4 ± 0.1), pyrrhotite (5.25 ± 0.2) and pyrite (16.4 ± 1.2) (Davison, 1991). The concentration levels of Fe^{2+}, dissolved sulfide and pH observed in our study are comparable to those reported by Davison (1980 & 1991). The solubility of FeS in a marine waters (pH>7) is not well defined (e.g., Rickard and Morse, 2005). Using the equations reported by Davison (1991), which define the ionic activity product (IAP),

$$IAP = [Fe^{2+}]\,[HS^-]\,\gamma Fe^{2+}\,\gamma HS^-\,(H^+)^{-1} \qquad (2)$$

$$K_1 = a\,[HS^-]\,a\,[H^+] / a\,[H_2S] \qquad (3)$$

we attempted to predict the presence and the nature of the FeS compounds. Equation (3) was used to derive the concentration of HS^- from the total soluble sulfide, i.e. $H_2S + HS^-$ (S^{2-} being negligible) levels. Values of γFe^{2+} and γHS^- in seawater were taken from Davison (1980). In the case of high Fe^{2+} (about 700 µM for DET, 100 µM for DGT) and low dissolved sulfide concentrations (about 1.5 µM) (observed at 3 - 4 cm of depth in the month of February), the –log(IAP) values was 2.83, using DET values for Fe^{2+}, and 3.67, using DGT values for Fe^{2+}, which are in the range of amorphous ferrous sulfide and mackinawite. In the case of low Fe^{2+} (about 70 µM for DET and 5 µM for DGT) and high dissolved sulfide concentrations (about 80 µM) (deeper sediment layers in the month of May), similar –log(IAP) values were obtained. The calculated –log(IAP) value based on the DET Fe concentrations is perhaps overestimated because not all DET-Fe is labile.

In order to corroborate the above findings, the Visual MINTEQ ver. 2.40b software was used to calculate the saturation index (SI) (equation 4) of iron, manganese, arsenic, cobalt, copper and lead precipitates (Gustafsson, 1999).

$$SI = log\ (IAP / K_s) \qquad (4)$$

For these calculations the following compounds have been taken into account: (1) cations, H^+, Ca^{2+}, K^+, Mg^{2+}, Na^+, Fe^{2+}, Mn^{2+}, As^{3+}, Co^{2+}, Cu^{2+}, Pb^{2+}; (2) anions, HCO_3^-, SO_4^{2-}, Cl^-, HS^- and (3) DOC, 50% are fulvic and 50% are humic acids. According to these calculations, FeS (amorphous) should not exist since SI values are always below 0. Conversely, troillite and pyrrhotite are oversaturated, implying that these compounds do not precipitate quickly in our sediments. Both iron sulfide minerals mackinawite and greigite are probably present in our sediments. By using DGT data, that are the best for this kind of calculations because only the labile fraction of iron is taken into account, greigite is slightly oversaturated whereas mackinawite is at equilibrium or slightly undersaturated. Finally, siderite ($FeCO_3$) may exist at some depths. MnS does not precipitate as a pure mineral; hence Mn should be incorporated into other labile sulfides like greigite and / or pyrite compounds. Conversely, $MnCO_3$ may exist as a pure mineral depending upon the season and the depths.

Task 1.5. Data integration.

The proposed data analyses are aimed at describing the observed variation patterns in biomass, biodiversity and (molecular) community composition of the microbial assemblages present in BCP sediments, but are also crucial for integrating the results of both the microbiological and geochemical analyses. They allow to evaluate the importance of the main biogeochemical drivers of microbial diversity and biomass in these sediments (including the metal pollutants), and more specifically to identify microbial phylotypes which are strongly related to the presence of metals. This information will form the basis for the development of specific FISH and real time PCR protocols to monitor the occurrence of these phylotypes in both *in situ* and experimental conditions, and to select isolates for use in the experiments (see phase II).

In 2007, 47 variables were analyzed in 9 stations of the BCP taking two sediment sections into account (0-1 cm and 9-10 cm) and two different periods (February and July). As most of the data was non-parametric, a Spearman's rank correlation global analysis was first used to identify main trends in our data (February and July were grouped in the same analysis). Surprisingly, the biomass of bacteria, as determined with DAPI counts, was the only microbial variable which was significantly correlated to the biogeochemical variables considered in this study. The biomass of bacteria was significantly correlated to Cu (0.87) and Zn (0.89) extracted during AVS analysis (SEM). It was also significantly correlated to Mn

(0.87), Fe (0.76), and As (0.73) as determined with DET analysis. For DGT, the metal showing the highest correlation coefficient to bacterial biomass was Fe (0.74). Bacterial biomass was also significantly correlated to chlorophyll a (0.88), pH (-0.79), QFF (0.89) and SSA (0.80). The biomass of heterotrophic nanoflagellates could only be studied in 6 stations in July 2007. It was significantly correlated to Fe (0.83) as determined with DET analysis, and also to pH (-0.90) and SSA (0.88).

Bacterial biodiversity, as measured with the maximum number of DGGE bands, was not correlated to most of the variables measured in this study. Globally, the maximum number of bacterial DGGE bands was only negatively correlated to Co (-0.68) as determined with DET analysis. For micro-eukaryotes, the maximum number of DGGE bands was only correlated to Ag (0.63). Biodiversity can also be estimated using the Shannon index. For bacteria the best correlations were again for Co, as determined with DGT analysis (-0.72) and DET analysis (-0.67). No good correlations were observed for micro-eukaryotes using the Shannon index.

Various biogeochemical variables were, as expected, correlated to each other. For instance when DET results are considered, levels of Fe were significantly correlated to Mn (0.94) and As (0.93) levels. Similarly, levels of Pb were significantly correlated to levels of V (0.73) and Ag (0.71). We observed that DET and DGT values can be correlated for some metals. For instance, DET values for Ag were negatively correlated to Ni values determined by DGT (-0.80), and DET values for Fe were always positively correlated to DGT values (0.84).

In 2008, 43 variables were analyzed in 2 stations of the BCP (130 and 700) taking 10 sediment sections into account (0-10 cm) and 6 different periods (February to July). As for 2007, most of the data was non-parametric and a Spearman's rank correlation global analysis was first used to identify main trends in the data. These analyses were performed separately in station 130 and 700. In station 130 the situation for bacterial biomass and biodiversity was different from the trend observed in 2007. Indeed, bacterial biomass in 2008 did not correlate well to any variable in station 130. However, bacterial biodiversity as determined by the maximum number of DGGE bands was significantly correlated to Zn levels as determined by DGT (0.70). For micro-eukaryotes no good correlations to any geochemical variable was observed in station 130. In station 700 the situation for bacterial biomass and biodiversity was also different from the trend observed in 2007. No good correlations were observed for biomass and the only good correlations observed for DGGE was for DET-Fe (0.63).

Results may also be analyzed on a monthly basis. As an example, we here show the results of a redundancy analysis (RDA) of the eubacterial DGGE data of station 130 for February 2008, using forward selection of environmental variables and significance testing using Monte Carlo permutation tests (Fig. 24).

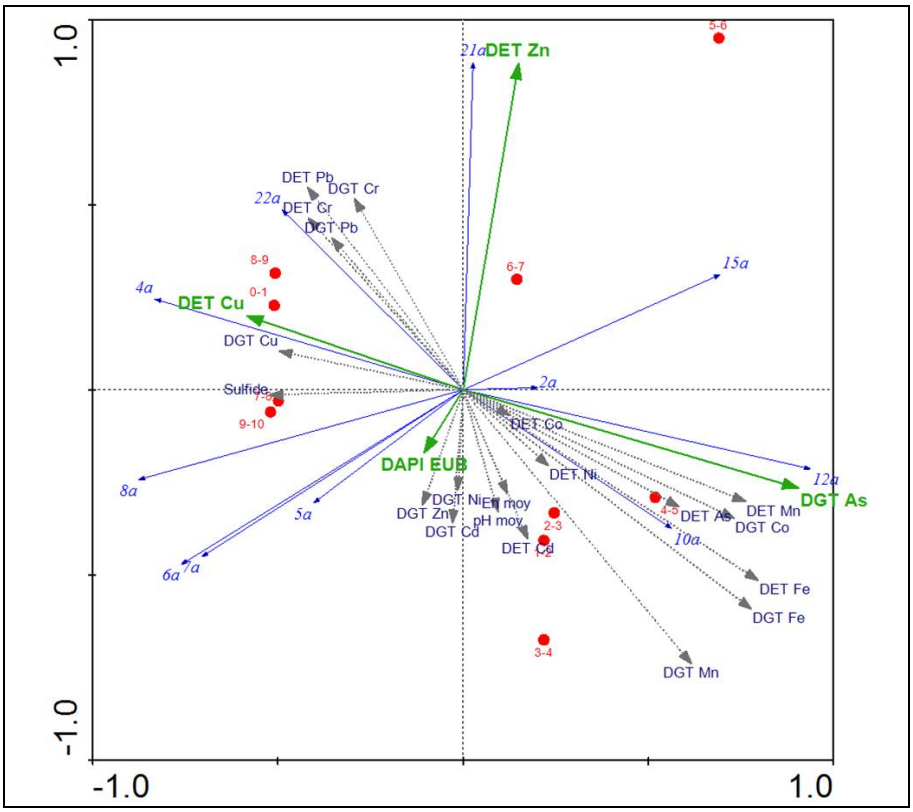


Figure 24. RDA analyses of eubacterial DGGE data (Feb. 2008, core A, st. 130), with significant environmental variables (green), supplementary environmental variables (dotted arrows), phylotype numbers (blue arrows) and samples (depth horizons, red).

This analysis shows that there are pronounced changes in phylotype composition with depth (cf. also Figs 4 & 6), and that these changes can be significantly related to specific geochemical (i.c. DET Cu, DGT As, DET Zn) and biological variables (DAPI Eub). Bearing in mind that these relations are purely regressions between the biological and geochemical data, they do allow to identify specific phylotypes whose relation to the metal variables (e.g. note the strong correlation between phylotype 12a and As, or phylotype 21a and Zn) which can be further studied using FISH and real time PCR, and in the experimental manipulations in Phase II.

The DGT profiles for Mn, Fe, Co, As and Cu at station 130 in February and April (Cr only in February) displayed increasing concentrations with depth (Fig. 23). Ni presented a reversed trend in February, while Pb (in February and April) and Cr (in April) had more or less constant concentrations in the water column and porewater layers adjacent to the SWI (Fig. 23). We calculated concentration gradients and exchange fluxes over 2 cm of depth and, as mentioned above, for each element at the SWI using a diffusion coefficient of 10^{-5} cm^2 s^{-1} (Baeyens et al., 1986), which was corrected for a porosity value of 0.9 (the ratio of the fluid volume to the total volume of sediment) (Table 2). Exchange fluxes of trace metals in February can be slightly higher due to diagenetic processes, than in April. Oxygen and redox values, as well as Fe and Mn concentrations, were lower in April than in February. As suggested earlier, freshly produced organic matter (POM) entering the sediment porewaters can bind dissolved Fe and Mn compounds, reducing their porewater concentrations. Fe and Mn out-fluxes (from sediments to overlying water) are higher (0.063-1.6 $mmol \cdot m^{-2} \cdot d^{-1}$) compared to the other elements (Co, As and Cu : $4.2 \cdot 10^{-5}$ - $1.6 \cdot 10^{-3}$ $mmol \cdot m^{-2} \cdot d^{-1}$). Also the Ni influx in February is of the order of the outfluxes of the latter elements (Table 7).

TABLE 7. Exchange fluxes of trace elements at the Sediment Water Interface (SWI) in station 130 (calculated from vertical pore water profiles).

Element	*Calculated flux in February (mmol·m^{-2}·d^{-1})*	*Calculated flux in April (mmol·m^{-2}·d^{-1})*
Mn	0.10	0.063
Fe	1.6	0.26
Co	5.7E-05	4.2E-05
As	1.6E-03	4.4E-04
Cr	6.2E-05	0
Ni	-1.2E-03	1.6E-04
Cu	8.6E-05	5.4E-05
Pb	0	0

Remobilization fluxes of trace metals at the SWI, estimated by "DGT pistons" placed at the SWI, were used to calculate fluxes based on the element concentrations in the DGT resin and on the DGT equation (equation 1). We estimated minimal fluxes with the assumption that all solutes moved with the speed of free ions through the DGT gel layer, while metal complexes move slower (Zhang, 2004). The fluxes correspond to the trace elements that can be mobilized in the sediment (including the labile fraction in the solid sediment phase) and that is vertically transported to the probe (Table 8). The "DGT piston" fluxes and those calculated via the concentration gradients for Mn, Fe, Co As are within the same order of magnitude. For Cr, Ni, Cu and Pb fluxes are at least 10 times more elevated. In fact, the DGT piston acts as a "pump" at the SWI and trace element concentrations may be directly measured. As the trace element concentrations at the SWI and the overlying water are zero (the "DGT piston" is placed at the SWI), the concentration gradient is maximized which is not always accomplished when exchange fluxes are calculated based on the vertical concentration profiles. For Mn, Fe, Co and As both fluxes are rather similar (Table 8) which can be explained by the fact that their dissolved phase concentrations are very low in the overlying water as a result of rapid oxidation and precipitation.

TABLE 8. Remobilization fluxes of trace elements at the Sediment Water Interface (SWI) in April 2008, calculated from pore water profiles and estimated by DGT pistons.

Element	*Calculated flux (mmol·m^{-2}·d^{-1})*	*DGT piston (mmol·m^{-2}·d^{-1})**
Mn	0.063	0.10
Fe	0.26	0.095
Co	4.2E-05	4.4E-05
As	4.4E-04	3.7E-04
Cr	0	2.8E-04
Ni	1.6E-04	3.0E-03
Cu	5.4E-05	9.5E-04
Pb	0	7.0E-05

**These values are average values obtained from three different deployment times: 2, 4 and 6 hours in different sediment cores. For most elements, Relative Standard Deviation is about 100% due to sample heterogeneity.*

5. CONCLUSIONS.

The BCP sampling stations followed in this study may be classified in three groups: sandy stations with a mean grain size (MGS) of 400 µm (group I : DCG, 330 and 435), sandy stations with a MGS of 200 µm (group II: 120, 230 [except 0-1 cm], ZG03), and muddy stations with a MGS of 12.5 µm (group III: 130, 140 and 700).

In group I sediments the consumption of oxidants does not occur efficiently (i.e., the oxygen penetration depth is important), probably due to the low quantity and bioavailability of organic matter at the water-sediment interface. In addition, the low specific area of the sediments does not encourage adsorption of bacteria (that are mostly restricted to the fine fraction in all stations). Oxygen and sulphates are not significantly reduced and only small quantities of CRS are detected. Low amounts of SEM have been measured (about 10 times lower than in the other sites) but the low contents of AVS may result in a positive toxicity index.

In group III sediments, oxygen is completely consumed within the first mm and the Eh values drop to about -200 mV within the first cm. Production of dissolved and solid sulfides confirms sulfate-reducing activities, mostly in the first cm. Concentrations of Pb, Cu and Zn in SEM are generally more than 10 times lower compared to the AVS, which means that sulfides might act as a sink for the metals present. However, as demonstrated by the DET/DGT analyses, metallic pollutants are nevertheless present in the pore waters of these stations and may therefore be released in the water column. In group II sediments, oxygen is consumed in the first cm and Eh values decrease more slowly than in the muddy stations.

The February 2007 data show that eubacterial taxon richness increased from the coast (group III sediments) to the open sea (group I sediments), when DGGE results were expressed per unit of biomass. Even when normalization was not performed it was observed that the raw diversity DGGE values were never low in offshore stations DCG and 435 (always >16 DGGE bands) and that the lowest values (6 DGGE bands) were always observed in coastal stations. In addition, no archaebacterial 16S rRNA sequences could be obtained by PCR in coastal stations; on the contrary, many archaebacterial sequences were obtained offshore, in sediments of stations DCG and 435. Reduced diversity in sediments from coastal areas on the BCP was also observed in other studies for other groups, such as nematods and harpacticoid copepods (e.g., Vincx 1990). It should be noted that bacterial diversity values may nevertheless be elevated in coastal stations (e.g., the sandy station 120 in February 2007 and the metal contaminated station 130 in July 2007). This means that the situation is much more complex than initially thought and that the observed trend is not valid for all sediment types and/or only valid for some periods of the year.

It appears that the reduction of biodiversity observed in February 2007 is not simply related to anoxia because stations such as 120 and ZG03 were anoxic below 0.5 cm and still featured high biodiversity. Other factors than granulometry or oxygen must therefore be considered in order to explain the low bacterial biodiversities in some of the group III muddy stations. Although sediment mineralogy, predation, the abundance of viruses, and the quality and quantity of nutrients and organic matter are important factors to consider, one of the main striking differences between group III stations and stations in groups I & II is the presence of high concentrations of metallic pollutants and metalloids in the pore waters, particularly arsenic, as demonstrated by DET/DGT analyses. Arsenic is probably detrimental for many microorganisms as the arsenate oxyanion is an analog of phosphate. This might also explain the low DAPI counts of bacteria in group III stations (when expressed per gram of fine fraction).

Multivariate analyses of the eukaryotic DGGE data of 2007 also show pronounced changes in community (phylotype) composition with sediment type (group I-III). Although the eukaryotic data was not normalized to biomass, there appears to be no strong shift in diversity

between the group I and group III sediments. This may be due to the fact that deposited diatom cells are preserved in the group III sediments [see the PCA diagram (Fig. 8) but also the high chlorophyll a values (Fig. 17)], which increases diversity.

In 2008, eubacterial and eukaryotic taxon richness and relative DGGE band intensities were followed monthly in two stations (130 & 700) from the sediment/water interface up to -10 cm of depth, from February to July. Results show that the relative DGGE band intensities change with sediment depth and that reproducibility was good between the replicate cores. Ordination analyses reveal pronounced changes in both bacterial and eukaryotic community composition with season and depth, which will allow to identify the DGGE bands/phylotypes that show the best correlations to environmental parameters. These DGGE bands may then be sequenced (excision and16S/18S rRNA sequencing) and compared to the clone libraries. This will permit to design specific probes to follow the corresponding bacteria and micro-eukaryotes in the sediments during the in situ analyses and laboratory incubations in phase II.

From the 2007 and 2008 data we can conclude that microbial biodiversity, as measured with DGGE, is not a variable that can be easily related to the environmental variables considered in this study. This might be due to the long exposition time of the microbial communities to metals in the coastal area. The present-day communities are probably adapted to the elevated metal concentrations in that zone. This may explain the high biodiversity values that were observed in contaminated sediments of station 130 in July. Such a situation was also observed in the bacterial communities living in marine sediments of the Sørfjord, exposed for more than 80 years to high levels of Cd and Zn (Gillan et al. 2005). In other words, despite elevated metal levels, bacterial biodiversity cannot be related to metal contamination in the field, as shown by the multivariate analyses performed here. To complicate things, other contaminants such as PCBs are abundant on the BCP, and their effect on the diversity of microbial communities is poorly known. Indeed, sediments on the BCP have $\sum_6$PCB levels ranging from 0.23 to 10.6 μg g^{-1} (Danis et al. 2004).

On the contrary, bacterial biomass is a variable that displayed elevated and significant correlations to some environmental variables, particularly to dissolved Mn, Fe and As. This is not surprising as these metals may serve as electron donnors or acceptors depending on their oxidation state (Canfield et al. 2005). With the exception of arsenic, these metals are also essential components of metalloenzymes and electron transport systems (Beinert et al. 1997, Kendrick et al. 1992). Bacterial biomass was also significantly correlated to chlorophyll a. This may be explained by the proliferation of bacteria on decaying phototrophic micro-eucaryotes such as diatoms and *Phaeocystis*.

Eubacterial rRNA sequencing (February 2007 samples) has shown that 5 to 10 major eubacterial groups are present in the BCP sediments examined (DCG, 435, 130, 700). Three major groups were present in all the four stations examined (g-Proteobacteria, d-Proteobacteria and CFB bacteria). Acidobacteria represent 2.6 - 14.6% of the clones in most of the stations. For micro-eukaryotes, 18S rDNA based DGGE of February 2007 samples revealed a surprisingly high diversity of microbial eukaryotes, mainly comprising stramenopiles (diatoms), as yet unidentified (or ambiguously identified) marine eukaryotes and Fungi, but also protozoa and microalgae belonging to other groups. The DGGE procedure also picked up many metazoan sequences. In 2008, the use of Protozoa-specific primer sets (for Cercozoa and ciliates) in combination with full length 18S rDNA CL allowed a more detailed identification of the protozoan communities present.

With the DET/DGT approaches, high resolution profiles of trace metals in the sediments were obtained. The general good correspondence between the DET and centrifuge technique proved that reliable data are produced with our sampling approach. Trace elements present a variable geochemical behavior in the sediments, confirming that remobilization is occurring at specific depths. Seasonal variations of elements (Mn, Fe, As) have been observed

during the cruises in 2007 and 2008. Although variations in the oxygen concentration and redox potential may explain most of the patterns obtained, the importance of microorganisms in this seasonal phenomenon has still to be determined. There is apparently no depletion of trace elements in the sediment porewaters at station 130 and 700. The flux calculations based on DGT profiles show that elements such as Mn, Fe, Co, As and Ni will diffuse out of the sediment into the overlying water column at least for station 130. Flux calculations based on the DGT piston experiments confirm that metallic toxicants may reach the SWI and be released in the seawater. This might be detrimental for the benthic ecosystem. Other metallic toxicants such as Cu, Zn, and Cd will diffuse inside the sediments in station 130.

6. SUPPORT TO THE DECISION.

Although definitive conclusions will only be reached at the end of phase II we can already present important findings of the MICROMET network that can be of great use to support decision processes :

- The contamination of the coastal zone by transition metals and metalloids is significant. Eubacterial diversity expressed per unit of biomass is reduced (at least during the winter months) in the BCP zone extending up to 3-4 km in the open sea, when compared to sediment stations far from the coast. Similarly, archaebacteria were absent from this zone. These observations are corroborated by previous reports on meiobenthos biodiversity (nematods, copepods) that was also reduced in the same zone. Although various factors may be invoked to explain these lowered bacterial biodiversities, and that the relationship between bacterial diversity and metal levels is not straightforward, elevated anthropic contamination in the Belgian coastal areas is striking and efforts should be made to reduce the human impact on that zone, especially in the area extending up to 3-4 km in the open sea between Oostende and Zeebrugge. Sediments are able to accumulate large quantities of contaminants that may affect benthic biodiversity and ecology of many invertebrates and vertebrates.

- In itself, eubacterial diversity as measured with DGGE is not a variable which can be considered as a good indicator of marine sediment quality. The same conclusion may be applied to micro-eukaryotes. This is probably the result of adaptation to elevated concentrations of toxic metals, especially in areas with a long history of contamination. The result of this is that elevated microbial biodiversities may be observed in highly contaminated areas such as stations 130 and 700. Highly contaminated stations have probably to be studied separately from the other stations.

- Bacterial biomass is a variable which is a better indicator than biodiversity for marine sediment quality, especially for As in the porewaters. Future studies should concentrate on the quantification of resistance genes in bacteria and on the time needed for microbial communities to adapt.

- Benthic upward fluxes of toxic metals (such as cobalt and arsenic) have been calculated for the BCP coastal zone extending up to 3-4 km in the open sea (especially near stations 130, 140 and 700 on the eastern part of the belgian coast). Products from fisheries (especially benthic and demersal fishes) and mussel-farming from this zone should be carefully monitored for metals. The effect of microbial communities on these upward fluxes will be determined in Phase II of Micromet.

- In October 2000 the EU Water Framework Directive (WFD) entered into force. The implementation of this WFD is currently raising a number of technical challenges for the Member States, particularly for Belgium, which should meet the WFD environmental objectives in 2015. Clearly, measurements performed during the Micromet project in 2007 and 2008 suggest that a "good ecological and chemical status" will not be met in 2015 for the Belgian coastal ecosystem extending up to 3-4 km in the open sea between Oostende and Zeebrugge. A less stringent goal should be considered for this area.

7. PERSPECTIVES FOR PHASE II

As planned in Annex I of the MICROMET contract, the 3rd and 4th years of the project will be devoted to Work Packages 2 and 3 (WP2 & WP3). There are no major deviations from the original work plan. Experiments will mainly be conducted with the heavily polluted sediments of the site 130.

WP 2. Importance of microorganisms in the leaching of metallic contaminants from the sediments.

Task 2.1. Sampling and micro-electrode analysis (ULB, UGent, VUB, USTL).
Intact sediment cores will be collected mainly in the sampling site 130. As in phase I (year 2), several sediment horizons (0-1 cm, 1-2 cm, etc...) will be considered (tasks 2.2 to 2.5). The sediment cores will mainly be used in laboratory simulation experiments. Additional cores can be taken when necessary in collaboration with VLIZ (Flanders Marine Institute).

Task 2.2. Determination of microbial biodiversity (UGent, ULB).
Task 2.2.1. DGGE analysis. DGGE analyses will be performed on all sediment horizons in the sediment samples and the same sediment samples will be analyzed by the two partners.

Task 2.3. Determination of microbial biomass (UGent, ULB).
Task 2.3.1. Total DAPI counts. As in phase I, all the sediment horizons in the sediment samples will be considered.
Task 2.3.2. FISH and Real-Time PCR. Key organisms have been identified in phase I of the project (WP 1). FISH will be carried out as described in Gillan et al. 2005 and Stoeck et al. (2003). Real-Time PCR will be carried out as described in Bowman et al. (2005) and Créach et al. (2006). These analyses will be conducted during the laboratory simulation experiments.

Task 2.4. Microbial productivity measurements (VUB, ULB, UGent).
Task 2.4.1. ATP & CLPP approaches. ATP measurements will be carried out according to Nakamura & Takaya (2003); CLPP according to Lehman et al. (1997) and Boivin et al. (2006); and the chitinase activity according to Gillan (2004).
Task 2.4.2. Sulfate reduction rate (SRR). The SRR will be determined with the whole-core $^{35}SO_4^{2-}$ injection method (Jørgensen 1978); ^{35}S incorporation into total reducible inorganic sulfur will be determined by the one-step acidic Cr-II method (Fossing & Jørgensen 1989, de Beer et al. 2005).

Task 2.5. Geochemical properties of the sediment (VUB, ULB, UGent).
Task 2.5.1. General properties of the sediments. Total organic carbon and nitrogen will be determined on freeze-dried sediment slices with an elemental analyzer. For pigment analyses, sediment samples will be freeze-dried to remove water and pigments will be extracted in acetone using sonication. Pigments will be analyzed using HPLC with standard protocols (cf. Wright et al. 1991). Mineral phases will be determined using FTIR spectroscopy and X-ray diffraction (Böttcher et al. 2000). Dilute HCl-extractable metals will be determined using dilute 0.5M HCl treatments (Sutherland 2002, Gillan et al. 2005).

Task 2.5.2. DET/DGT analysis. DET and DGT measurements will be done as described in Gao et al. (2006) and Leermakers et al. (2005). Fluxes of heavy metals will be calculated using Fick's law of diffusion.

Task 2.6. Laboratory simulation approach (VUB, UGent, USTL, ULB).
Laboratory simulation experiments will be set up in order to assess the impact of benthic microbes on leaching of metals from the sediments according to the type of organic matter deposited onto the sediments. Experiments will be designed according to standard principles (e.g. Kuehl 2000). A static set-up will mainly be used (i.e., no water flow over the sediments). Intact sediment cores will be placed in Plexiglass tubes (Ø 15 cm, h: 20 cm) and incubated in the laboratory (at field temperature in the dark) for several weeks. Changes of the microbiological characteristics will mainly be studied at the seawater interface (SWI) but also by investigating depth profiles in parallel incubated cores at different times. The sediment surface of all cores will be exposed to a small volume of overlying seawater. The main treatment will consist in an exposition to organic matter from unicellular algae. Two types of algae will be used : diatoms and *Phaeocystis*. These two organisms are forming blooms each year on the BCP and might influence the type of metals released by the sediments. Pure cultures of these algae will be used. The cells will then be deposited on the sediments. Sediments and overlying water will then be followed over time (Eh, pH, metals, sulfides, etc.).

Task 2.7. Data analysis (ULB, UGent, VUB, USTL).
Microbial biomass and diversity for the experiments will be related to the measured geochemical variables using standard correlation and (multiple) regression statistical analyses. DGGE gels will be digitized and analyzed using the software package Bionumerics 1.5 (Applied Maths BVBA, Kortrijk, Belgium) and the Quantity One software (BioRad). These softwares perform a density profile through each lane, detect the bands and calculate the relative contribution of each band to the total band signal in the lane after applying a rolling disk as background subtraction. The DGGE data on microbial community structure will then be analysed using multivariate statistical tools as described in e.g. Jongman et al. (1987) and McCune & Grace (2002) using various software packages (e.g. Canoco 4.5 and Primer 5). Choice of the most appropriate methods will depend on the nature of the data and will include clustering and ordination techniques (such as principal components and correspondence analysis and their canonical variants, and multidimensional scaling) (e.g. Coteur et al. 2003, Van der Gucht et al. 2005).

Work Package 3. Numerical modelisation (VUB, ULB, Ugent and USTL).

Modelling will largely be restricted to the end of phase II. The main reason is that models will be run with data obtained during phase II, which will be entirely devoted to the laboratory simulation approach (Task 2.6.). The MICROMET research project will use the MEDIA environment (Meysman et al. 2003b). This improved software is a powerful tool for reactive transport modelling. The MEDIA environment consists of a toolbox of building blocks (element, species and process objects), which can be combined freely by the user to construct new models (without the need for recompilation). An object-oriented database stores current objects and accommodates new user-defined building blocks (Meysman et al. 2003a,b). It is to be expected that the modeling part of the project will greatly benefit from the fact that Dr. F. Meysman recently joined the ANCH Department at VUB.

8. PUBLICATIONS AND VALORIZATION

8.1. Peer reviewed publications of the team

Yue GAO, Ludovic LESVEN, David GILLAN, Koen SABBE, Gabriel BILLON, Sandra DE GALAN, Marc ELSKENS, Willy BAEYENS, Martine LEERMAKERS. 2009. Geochemical behavior of trace elements in sub-tidal marine sediments of the Belgian coast. Marine Chemistry. In press.

David C. GILLAN, Willy BAEYENS, Rafeh BECHARA, Gabriel BILLON, Kevin DENIS, Philippe DUBOIS, Jean-Claude FISCHER, Yue GAO, Philippe GROSJEAN, Martine LEERMAKERS, Ludovic LESVEN, Annelies PEDE, Stéphanie ROOSA, Koen SABBE. Links between Microbial Community Composition, Biomass and Dissolved Metals in Porewaters of Marine Sediments. Submitted to *Applied and Environmental Microbiology*.

8.2. PhD thesis including Micromet data

Ludovic LESVEN. 2008. Devenir des éléments traces métalliques au sein du sédiment, un compartiment clé de l'environnement aquatique. USTL, Lille, France. 181 pp.

Yue GAO. 2009. Trace metal behavior in sedimentary environments. VUB, Brussels, Belgium. 310 pp.

8.3. Poster presentations and oral communications

- Gillan DC (2007) The MICROMET Project : Microbial Diversity and Metal Fluxes in Contaminated North Sea Sediments. Programme Science for Sustainable Development. Kick-off Meeting, Belgian Science Policy, Brussels, 26-27 Mars (oral communication).

- Pede A., Verstraete T., Vyverman W., Sabbe K. (2007) Eukaryotic Microbial Diversity and Metal Fluxes in Contaminated North Sea Sediments (MICROMET) : preliminary results. NecoV working group on Ecology of Aquatic Microorganisms, University of Amsterdam, 7th December (oral communication).

- Baeyens W, Bechara R, Billon G, Dubois P, Fischer JC, Gao Y, Gillan DC, Leermakers M, Lesven L, Pede A, Sabbe K (2008) Preliminary results of potential correlations between geochemical parameters, metals contamination and microbial diversity in North Sea sediments. 35th International Symposium on Environmental Analytical Chemistry, ISEAC 35, Gdansk, Poland, June, 22-26 (poster).

- Gao Y, Lesven L, Gillan D, Sabbe K, Billon G, Baeyens W (2008) Geochemical behavior of trace elements in sediments inside the Scheldt estuary plume. 10th International Estuarine Biogeochemistry Symposium, 18th May, Xiamen, China (poster).

- Gillan DC. (2008) The structure of microbial communities in North Sea sediments contaminated by metallic pollutants. Symposium ECODIM V: Interaction between microbes and metals. University of Concepcion, Chile, 18th January (oral communication).

- Pede A., Gillan D., Gao Y., Billon G., Lesven L., Leermakers M., Baeyens W., Vyverman W. & Sabbe K. (2009) Patterns in microbial diversity in North Sea sediments; correlations with specific sediment characteristics and heavy metal pollution. VLIZ jongerencontactdag, Brugge, 6th March (poster).

- Pede A., Gillan D., Gao Y., Billon G., Lesven L., Leermakers M., Baeyens W., Vyverman W. & Sabbe K. (2009) Spatial And Seasonal Variation In Microbial Diversity In Marine Subtidal Sediments In Relation To Sediment Geochemistry And Heavy Metal Pollution. ASLO Aquatic Sciences Meeting 2009, Nice (France), January, 25-30 (poster).

- Pede A., Gillan D., Gao Y., Billon G., Lesven L., Leermakers M., Baeyens W., Vyverman W. & Sabbe K. (2009) Spatial And Temporal Microbial Dynamics In Metal Contaminated North Sea Sediments. 44th EMBS 2009 (Liverpool), September, 7-11 (oral communication)

8.4. Other activities

Design and implementation of the MICROMET website
(http://www.ulb.ac.be/sciences/micromet) [1962 connections since January 2007]

9. REFERENCES

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