EFFECT OF BACTERIAL MINERALISATION OF PHYTOPLANKTON-DERIVED PHYTODETRITUS ON THE RELEASE OF ARSENIC, COBALT AND MANGANESE FROM MUDDY SEDIMENTS IN THE SOUTHERN NORTH SEA

A MICROCOSM STUDY

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I contributed to this chapter by cultivation of Phaeocystis and Skeletonema, performing analyses during the experiment, delivering the data for chlorophyll a in the sediment and heterotrophic flagellate biomass, and by revision of the manuscript.
Chapter 4

Abstract

Muddy sediments of the Belgian Continental Zone (BCZ) are contaminated by metals such as Co, As, Cd, Pb, and Ni. Previous studies have suggested that mineralization of phytodetritus accumulating each year on sediments might cause secondary contaminations of the overlying seawater (metal effluxes). The aim of the present research was to investigate these effluxes using a microcosm approach. Muddy sediments were placed in microcosms (diameter: 15 cm) and overlaid by phytodetritus (a mix of Phaeocystis globosa with the diatom Skeletonema costatum). The final suspension was 130.6 mg L\(^{-1}\) (dw) and the final chlorophyll \(a\) content was 750 ± 35 \(\mu g\) L\(^{-1}\) (mean ± SD). Natural seawater was used for controls. Microcosms were then incubated in the dark at 15°C during 7 days. Metals were monitored in overlying waters and microbial communities were followed using bacterial and nanoflagellate DAPI counts, thymidine incorporation, community level physiological profiling (CLPP) and fluorescein diacetate analysis (FDA). Benthic effluxes observed in sediments exposed to phytodetritus were always more elevated than those observed in controls. Large effluxes were observed for Mn, Co and As, reaching 1084 nmol m\(^{-2}\) d\(^{-1}\) (As), 512 nmol m\(^{-2}\) d\(^{-1}\) (Co), and 755 \(\mu mol\) m\(^{-2}\) d\(^{-1}\) (Mn). A clear link was established between heterotrophic microbial activity and metal effluxes. The onset of mineralization was very fast and started within 2h of deposition as revealed by CLPP. An increased bacterial production was observed after two days (8.7 mg C m\(^{-2}\) d\(^{-1}\)). The bacterial biomass was stable and controlled by heterotrophic nanoflagellates. Calculations suggest that during phytoplankton blooms the microbial activity alone may release substantial amounts of dissolved arsenic in areas of the BCZ covered by muddy sediments.

Introduction

The Belgian Continental Zone (BCZ) is located in the Southern part of the North Sea. As in many coastal areas the delivery of continental nutrients into the BCZ results in large phytoplankton blooms characterized by high biomass levels (Lancelot et al., 1987; Lancelot et al., 2007; Gypens et al., 2007). Strong seasonal patterns are observed in the BCZ with diatom blooms initiating the succession in February-March followed by the main spring bloom composed of diatoms and Phaeocystis globosa in April-May (Rousseau et al., 2002; 2008). During the blooms, the biomass of Phaeocystis may reach 10 mg C L\(^{-1}\) which corresponds to
35 µg L\(^{-1}\) of chlorophyll \(a\) (Schoemann et al., 2005). Phytoplankton is usually a major source of organic matter for coastal sediments and it was estimated for the BCZ that 24% of the phytoplankton production is deposited onto the sediments (Lancelot et al., 2005). After deposition, phytodetritus are actively mineralized by various benthic organisms including microbial communities (Van Duyl et al., 1992).

As a consequence of human activities and the presence of the nearby Scheldt estuary, many areas of the BCZ are contaminated by metals (Danis et al., 2004; Gillan and Pernet, 2007; Gao et al., 2009). Metal concentrations in sediments are frequently above the Ecotoxicological Assessment Criteria (EAC), defined by the OSPAR Commission as concentration levels below which no harm to the environment or biota is expected (OSPAR, 2000). Metals are much more abundant in muddy sediments where concentrations in porewaters can exceed those in overlying waters by several orders of magnitude (Gao et al., 2009). As a consequence, the Fick’s first law predicts that benthic effluxes of metals may occur. Such effluxes are well-known phenomena and have been observed in many parts of the world using benthic chambers (Elderfield et al., 1981; Westerlund et al., 1986; Skrabal et al., 1997; Pakhomova et al., 2007). Among the factors that influence metal effluxes we find metal speciation, the presence of Mn and Fe oxide phases, sulfides, organic matter content, salinity gradients, temperature, water advection, bottom currents and thickness of the diffusive boundary layer (DBL), oxygen, and finally microbial communities and bioturbation (Jørgensen and Revsbech, 1985; Ciceri et al., 1992; Skrabal et al., 2000; Iskrenova-Tchoukova et al., 2010; Liu and Cai, 2010).

The influence of microorganisms on metal effluxes, through mineralization of freshly deposited phytodetritus, has been poorly investigated. Organic matter and phytodetritus may influence metal effluxes because they are able to complex metals (Wells et al., 1998; Iskrenova-Tchoukova et al., 2010; Liu and Cai, 2010). The subsequent degradation of phytodetritus by microorganisms may then release complexed metals in bottom waters. Increased oxygen consumption at the water–sediment interface (WSI) may also affect metal speciation in porewaters and promote effluxes. Previous studies have already suggested that mineralization of phytodetritus may cause increased metal effluxes such as for Mn (Aller, 1994; Fones et al., 2004). However, for highly contaminated sediments such as those of the BCZ (containing high levels of Co, As, Cd, Pb, and Ni; Gao et al., 2009; Gillan & Pernet, 2007) and phytodetritus containing *Phaeocystis*, the type of metal released and the importance of the effluxes have, to our best knowledge, never been measured. In addition, on the
microbiological point of view many questions remain to be answered: is there a delay in the onset of mineralization? Will the biomass of bacteria and protozoa increase at the same time? At what moment will change the activity of microorganisms?

The aim of the present research was to investigate dissolved trace metal effluxes during phytodetritus mineralization by microorganisms at the WSI. For that purpose, metal contaminated muddy sediments from the BCZ were placed in microcosms, with or without phytodetritus, and followed during one week in the laboratory. Contrary to the use of benthic chambers deployed in situ, our microcosm approach has the advantage to permit easier sediment manipulations like phytodetritus addition, sampling of microorganisms at the interface, Eh/pH measurements and control of temperature. In previous experiments, microcosm approaches have already been used with success for the study of phytodetritus degradation by microorganisms on sandy sediments (Van Duyl et al., 1992).

**Materials and methods**

**Sediment collection**

Sediments were collected by a Reineck corer (diameter 15 cm) at station 130 (51°16.25 N - 02°54.30 E; depth: ± 11 m) in March 2010 onboard the "Zeeleeuw" research vessel. Station 130 is one of the most metal-contaminated subtidal stations of the BCZ (Gillan and Pernet, 2007; Gao et al., 2009). Sediments are muddy and have a mean grain size of 12.5 μm. Undisturbed cores were immediately transferred in cylindrical plexiglass microcosms of the same diameter (15 cm) together with 4 cm of overlying seawater. All cores were transported to the laboratory in insulated boxes and stabilized during 10 days in the dark at 15.0 ± 1°C. This stabilization period was necessary because cultivated algae were not fully developed at the time of sediment sampling and it was also necessary to ensure the absence of leaks that would introduce biases in flux calculations. Aerobic conditions were maintained in the overlying water during the stabilization period (oxygen concentration was regularly monitored).
Algal cultures and algal suspension

Two independent 20 liter cultures of unicellular algae were prepared: *Phaeocystis globosa* (a Prymnesiophyte) and *Skeletonema costatum* (an early spring diatom). These algae are naturally present on the BCZ, are dominant members of the phytoplankton and may co-occur in spring (Rousseau et al., 2002). Inocula were obtained from the ESA Lab, ULB, Brussels (both strains were initially isolated from the BCZ). Algae were cultivated in a medium that is composed of natural seawater in which major nutrients, trace metals and vitamins were added. The medium is derived from the medium used by Veldhuis and Admiraal (1987) except for the saline matrix which was composed of natural instead of artificial seawater (*Phaeocystis* cultured in artificial seawater does not form colonies). The natural seawater used was obtained on the BCZ and was filtered through 0.2 μm Sartorius membrane and autoclaved for 20 min at 121 °C before use. Streptomycin (25 and 50 μg mL⁻¹) and Penicillin (75 and 100 μg m L⁻¹) were then added to prevent the development of bacteria (final concentrations, for *Phaeocystis* and *Skeletonema*, respectively). These antibiotics were completely eliminated by the subsequent treatments (centrifugations) and had consequently no effects on sediment bacteria (as demonstrated further by bacterial activity measurements). Cultures were performed at 9-10 °C with an irradiance of 100 μE m⁻² s⁻¹ and a gentle agitation of the culture flasks. After a few days of development cultures reached the stationary phase. At that moment, they were decanted and centrifuged. Cell pellets were kept at -20°C until the start of the experiment. At the start of the experiment (Day-0) cell pellets were thawed and placed into 8 liters of natural BCZ seawater at 15°C (that seawater was not filtered and not autoclaved). This algal suspension was then immediately used for the microcosm experiment. The dry matter content of the final algal suspension was 130.6 mg L⁻¹ (dry weight, measured after centrifugation and lyophilisation of three 50 mL samples) and the salinity was 30 %o. The proportion of the two algae in the algal suspension was 50:50 (w/w), and the final chlorophyll a content was 750 ± 35 μg L⁻¹ (mean ± SD). Metal concentrations of the algal biomass were as follows (in μg g⁻¹ dry weight): Cd, 0.53; Pb 38.6; Cr, 5.1; Mn, 64.5; Fe, 7533; Co, 0.54; Ni, 5.1; Cu, 246; Zn, 381; As, 4.4. Dissolved metal concentrations of the algal suspension (after filtration on 0.45 μm) were as follows (values in μg L⁻¹): Cd, 0.71; Pb, 0.26; Cr, 0.94; Mn, 12.4; Fe, 6.9; Co, 0.28; Ni, 2.1; Cu, 9.9; Zn, 12.0; As, 2.8).
Experimental design

The experimental design featured a total of 18 large microcosms (15 cm ø). These microcosms were used to test two conditions [with and without (controls) phytodetritus]. Samples were taken on three different sampling occasions during the experiment (Day-0, Day-2, Day-7) in triplicate. At the start of the experiment the overlying seawater was slowly removed from all the microcosms and cores without disturbing the sediment-seawater interface. A volume of 710 mL o² algal suspension was then slowly deposited on half of the microcosms, the other half were considered as controls and received 710 mL of natural seawater (that seawater was not filtered and not autoclaved). The height of the overlying seawater in all microcosms was 4 cm. After two hours of sedimentation 6 microcosms (3 experimental and 3 controls) were used for the Day-0 analyses. These analyses included microbial and geochemical parameters (see below). After two days (Day-2) and 7 days (Day-7) the same procedure was repeated. Microcosms were kept in the dark throughout the experiment. Salinity variations due to evaporation were avoided by regular MilliQ additions and monitoring by a salinometer. The temperature of the water was maintained at 15.0 ± 1°C during the whole experiment which is the water temperature that may be reached at the end of spring (Hondeveld et al., 1994; Vanaverbeke et al., 2008; Sir Jacobs et al., 2011). To allow phytodetritus accumulation at the WSI and prevent their resuspension the overlying water was not agitated. In such a system the DBL is thicker than in the field (Jørgensen and Revsbech, 1985). As a consequence, molecular diffusion completely sustains contaminant transport, as long as chemical activities of the contaminants in the sediment porewater are higher than in the overlying water. For this reason, only a short-term experiment was conducted (7 days). To prevent the development of anaerobic conditions the height of the water was limited to 4 cm and oxygen concentration as well as redox potential were regularly monitored.

Geochemical parameters

Geochemical parameters included Eh and pH profiles in the sediments, oxygen levels at the sediment-water interface, salinity of the overlying seawater, dissolved organic carbon (DOC) in porewaters, chlorophyll a in surface sediments, and metals in overlying seawater. Eh and pH measurements were performed at various depths by potentiometry. For that purpose, a glass electrode (specifically designed for abrasive and hard media, Ingold), and a home made platinum electrode (diameter about 1-2 mm) were used as indicator electrodes to detect pH and Eh, respectively. Both electrodes were combined with an Ag/AgCl, [KCl] =
3M reference electrode with a potential equal to 0.22 V versus a hydrogen normal electrode (HNE). All profiles were determined every 5 mm up to 3 cm into the sediments. Electrodes were fixed on a micromanipulator. All potential values further in this paper, refer to the Ag/AgCl electrode. The pH electrode was calibrated with Merck buffers, type NBS (National Bureau of Standards). In addition, since measurements were carried out in seawater, a correction of the values was made according to Aminot and Kerouel (2004). The salinity and temperature of the overlying water were monitored using a WTW Cond 340i conductivity meter. The dissolved oxygen in the overlying water was measured at ± 5 mm above the sediments using a WTW CellOx 325 galvanic oxygen sensor adapted for measurements in seawater.

For chlorophyll a, samples of surface sediments were frozen in liquid nitrogen (immediately after sampling), and kept at -80°C until further analysis. After freeze-drying of the sediment (0.4-2.1 g of sediment dry weight - SDW), pigments were extracted in 90% acetone and analyzed using HPLC with standard protocols (Wright et al., 1991). Chlorophyll a concentrations were expressed as µg g⁻¹ SDW.

For dissolved organic carbon (DOC) in porewaters, surface sediments (ca. 20 g of the 0-5 mm layer) were centrifuged and the porewater was collected using a glass syringe. The porewater was then filtered using Whatman GF/F glass fiber filters. Porewaters were preserved with phosphoric acid (5 µL of H₃PO₄ per mL of sample) and stored at 4°C. All glassware and filters were previously treated at 500°C for 4 hours. Before analysis samples were diluted 9 times with MilliQ water. DOC concentrations were measured by a Dohrman Apollo 9000 total organic carbon analyzer in which inorganic carbon is eliminated by bubbling in the presence of phosphoric acid and organic carbon is oxidized at high temperature (680°C). The produced CO₂ was then detected by non-dispersive infra-red (NDIR) analysis. The instrument response was calibrated by the method of standard additions.

For flux calculations, metals in the overlying seawater of the microcosms were determined by HR-ICP-MS as described elsewhere (Gao et al. 2009). For that purpose, a 10 mL water sample was filtered on 0.45 µm and acidified using Suprapur HNO₃. Benthic fluxes were then calculated using dissolved metal concentrations in the overlying waters after 2 and 7 days of exposure (concentrations at Day-0 were subtracted from those at Day-2, and concentrations at Day-2 were subtracted from those at Day-7, respectively). The volume of the overlying water was 710 mL (V) and the surface area (A) of the microcosms was 0.0176
The efflux can be calculated as \( F = C \times V / (A \times T) \). Effluxes were expressed in mol m\(^{-2}\) d\(^{-1}\).

**Microbial parameters**

Microbial parameters included bacterial and nanoflagellate biomass (DAPI counts) as well as three bacterial activity measurements: (i) bacterial production estimated by the tritiated thymidine incorporation, (ii) fluorescein diacetate analysis (FDA), and (iii) community level physiological profiling (CLPP). All analyses were performed on the 0-5 mm layer of the sediments (on the day the analyses were performed, the overlying water was carefully removed and sediment samples were taken with a spatula).

Bacterial DAPI counts were used to evaluate bacterial biomass. Sediment samples (1 mL) were preserved in 8 mL of 4% paraformaldehyde at 4°C. Bacteria were then extracted by sonication and placed on filters as described elsewhere (Gillan and Pernet, 2007). Bacteria in each microscopic field (14 fields per filter) were counted automatically using the ZooImage software v.1.2.2 (http://www.sciviews.org) based on Image J v.1.38r. Image background was first subtracted using a rolling ball radius of 40, and contrast was automatically adjusted. Images were then transformed in 16-bit and the threshold function was used with a lower threshold level of 180, and an upper threshold level of 255. The lower threshold level had to be adapted for each station to avoid the count of mineral particles. This value varied between 140 and 180, and the best value was found by comparing manual counts with automatic counts and outlines generated by ZooImage/Image J. Particles of the size of bacteria were counted automatically with the "Analyse particle" function (size pixel\(^2\): 100-2500; circularity: 0). A specific plugin was then developed in ZooImage/ImageJ to process automatically the 2016 pictures generated in this study. For each sediment sample, bacteria counted on the 14 pictures (total area observed = 5.39 \(10^{-8}\) m\(^2\)) were summed and the number obtained was compared to the effective filtration area (1.77 \(10^{-4}\) m\(^2\)). Four replicate filters were counted for each type of sediments (n=4) and the mean number of bacteria per field varied between 30 and 60 (the coefficient of variation was 10-30% of the mean). Such a counting scheme guarantees the lowest error in environments with great spatial heterogeneity (Kirchman et al., 1982; Montagna, 1982).

For nanoflagellate DAPI counts, sediment samples (2 mL) were fixed in 4 ml of 2.5% gluteraldehyde at 4°C. Heterotrophic nanoflagellates were isolated within 24h of sampling,
using the Percoll-gradient centrifugation technique of Starink et al. (1994). Upon centrifugation, the supernatant containing the nanoflagellates was decanted from the centrifuge tubes. A subsample was filtered on 0.6 μm pore-size black polycarbonate filters (Whatman) and stained with 4,6-diamino-2-phenylindole (DAPI; 10 μg ml⁻¹ final concentration). Filters were mounted on a slide with low fluorescence immersion oil and kept frozen in dark until enumeration. Heterotrophic nanoflagellates were counted manually using epifluorescence microscopy with UV radiation (Zeiss Axioplan2) as described in Hamels et al. (2005). Absence of chlorophyll was checked by switching to blue light excitation. Per filter, at least 20 randomly selected fields were observed (magnification 1000x).

For bacterial production, the tritiated thymidine incorporation approach was used with 1.0 g (ww) of living surface sediments that was suspended in 6 mL of autoclaved sterile seawater. These sediments were collected over a surface of ca 1 cm². The sediment suspension was then sonicated for 30 sec to detach bacteria from particles (Gillan and Pernet, 2007). The suspension was then centrifuged 5 min. at 180 g (4°C) to precipitate mineral particles. A volume of 5 mL of supernatant (containing bacteria) was placed in a clean tube with 6.4 μL of a ³H-thymidine stock solution (specific activity, SA : 64 Ci mmol⁻¹; 1.0 mCi mL⁻¹) (MP Biomedicals). The final concentration of tritiated thymidine was 0.1 nmol in 5 mL. Tubes were then incubated for 90 min in the dark at 15°C under orbital agitation. The incorporation was stopped using 1.5 mL of 25% cold trichloracetic acid. For the incorporation blanks (controls) the trichloracetic acid was added before the 90 min incubation. All samples were then filtered using Sartorius cellulose acetate filters (0.2 μm). Filters were placed in scintillation vials with 5 mL of scintillation fluid (Filter Count). Radioactivity of the samples was determined using a Packard Tri-Carb scintillation counter. Counts per minute (CPM) were automatically converted to disintegrations per minute (DPM) using a quench curve stored in the counter. Incorporation blanks were then subtracted from the experimental tubes and the rate of incorporation of thymidine was obtained in mg C m⁻² d⁻¹ using the following formulae:

\[
\frac{[DPM/SA \times 2.22 \times 10^{12} \times 1000]}{[2 \times 10^{18} \times 60/T] \times 24 \times 10^4 \times 2.44 \times 10^{-11}}
\]

where SA is the specific activity of the added ³H-thymidine (in Ci mmol⁻¹), and T is the time.
of incubation (min). We considered that $2 \times 10^{18}$ cells were produced per mol of thymidine incorporated (Moriarty et al., 1985) and that one cell of 0.1 $\mu$m$^3$ contained $2.44 \times 10^{-11}$ mg C (Servais, 1990).

Community level physiological profiling (CLPP) was determined using living sediments and the Biolog EcoPlate system (Garland and Mills, 1991). Bacteria were first extracted from the sediments. Fresh sediments (2.0 g, wet weight) were placed in 5 mL of autoclaved artificial seawater (Sigma, 40 g L$^{-1}$). The sediment suspension was sonicated (same protocol as above). The suspension was then centrifuged 5 min. at 180 g (4°C) to precipitate mineral particles. A volume of 150 $\mu$L of supernatant (containing bacteria) was placed in each well of an EcoPlate and the microplates were incubated 48 h at 15°C in the dark. The optical density of each well was recorded at 590 nm using a FLUOstar Optima microplate reader (BMG Labtech). The absorbance value of the least utilized substrate (among the 31 substrates) was subtracted from the absorbance value of the remaining wells (Hitzl et al., 1997; Stefanowicz, 2006). Absorbance values obtained with control and experimental microcosms were then compared to each other using the Mann-Whitney U test.

For fluorescein diacetate analysis (FDA), which estimates the total esterase activity of microbial communities (Battin, 1997), sediments (500 mg ww) were placed in sterile 15 mL polypropylene tubes and diluted with 2750 $\mu$L of sterile artificial seawater (Sigma, 40 g L$^{-1}$; autoclaved). Samples were sonicated 30 seconds as explained above. A volume of 250 $\mu$L of a FDA working solution (Sigma) in 100% acetone (analytical grade) was then added to the samples to a final concentration of 200 $\mu$M. Control tubes were immediately inactivated with acetone 100% to a final concentration of 50% (v/v). Samples were incubated for 60 min in the dark at 15°C. Incubation was stopped with 3 mL of acetone 100%. Absorbance of the supernatant at 490 nm was then measured spectrophotometrically against a water/acetone (50% v/v) blank. A standard curve was prepared with a fluorescein disodium salt (Sigma). FDA hydrolysis was expressed per g of wet weight.

**Results**

The algal suspension was added on Day-0 in half of the microcosms and samples were taken after 2h (Day-0), 2 days (Day-2) and 7 days (Day-7). A slight but significant acidification was observed at the sediment-seawater interface on Day-2 and Day-7 in the microcosms that received algae: the initial pH values were 7.9 at the beginning of the incubation and dropped
down to 7.7-7.8 after one week (Figure 1). No effects of algae addition were noticed on the redox potential or on the oxygen levels of the overlying waters: Eh values around 200 mV were observed up to -0.5 cm into the sediments for all microcosms, and values dropped to -130 mV at -1.5 cm of depth (not shown). Oxygen levels, measured at 5 mm above the sediments, were maintained at an average of 5 mg L⁻¹ throughout the experiment with no significant differences between experimental and control microcosms. Similarly, salinity was stable, with values of 30.0 ± 0.5‰ in all microcosms.

Figure 1. pH profiles measured in sediments of the microcosms on Day-2 and Day-7 (mean ± SD, n=3). Asterisks (*) indicate significant differences (Student T test, α = 0.05).

Metals released into the overlying waters were monitored by HR-ICP-MS throughout the experiment (Figure 2). After two hours of exposure to the algal suspension a significant increase of the metal concentration was observed for Cd, Co and As. After two days of exposure (Day-2) the concentrations of four metals were significantly higher in the overlying water of the experimental microcosms when compared to the controls: Cd (x 2.7), Co (x 5.6), Mn (x 13.8), and As (x 1.8) (Figure 2). After 7 days of exposure no differences were observed in the overlying waters between controls and experimental microcosms.
Figure 2. Metals in the overlaying water as measured by ICP-MS (mean ± SD). Significant differences are indicated by an asterisk (Student t-test, α = 0.05). Day-0 represents the overlying water at the end of the 10-days stabilization period, i.e. before the start of the experiment; this overlying water was then eliminated and replaced by the algal suspension or fresh seawater for controls.
Exchange fluxes (in mol m\(^{-2}\) d\(^{-1}\)) were calculated and are presented in Table 1. During the first 48 hours, 4 measured metals in experimental microcosms (As, Cd, Co and Mn) presented fluxes that differed significantly from those observed in the control microcosms. For As, Co and Mn, large negative fluxes (i.e., effluxes, from sediments to overlying water) were observed in the experimental microcosms. These negative fluxes reached \(-1084\) nmol m\(^{-2}\) d\(^{-1}\) for As, \(-512\) nmol m\(^{-2}\) d\(^{-1}\) for Co, and \(-755\) mmol m\(^{-2}\) d\(^{-1}\) for Mn. A positive flux was noticed for Cd (i.e., towards the sediments). For the other metals (Pb, Fe, Ni, Cu and Zn) no differences were observed between control and experimental microcosms and fluxes were either negative or positive (Table 1A). During the last 5 days (Day-2 to Day-7) only 2 measured metals in experimental microcosms (As and Cd) presented fluxes that differed significantly from those measured in the control microcosms (Table 1B). For As in the experimental microcosms, fluxes were less negative than during the first 48 hours and some microcosms displayed large positive fluxes (+262 nmol m\(^{-2}\) d\(^{-1}\)). For Cd, fluxes were still positive but less elevated than during the first 48 hours.

The chlorophyll \(a\) content of the sediments was measured at the seawater-sediment interface (Table 2). All sediment cores presented a background chlorophyll \(a\) concentration around 20 \(\mu\)g g\(^{-1}\) (dw). The introduction of phytodetritus into the experimental microcosms was clearly visible; after 2h values of chlorophyll \(a\) significantly increased to 30 \(\mu\)g g\(^{-1}\). After 2 and 7 days, chlorophyll \(a\) values were respectively 1.8 and 1.6 more elevated in the experimental microcosms than in the controls. Values of DOC in the 0-5 mm porewaters are shown in Table 3. Although mean DOC values of the experimental microcosms clearly increased with the incubation time, which is indicative of a mineralization process, no significant differences were found (variability between replicate microcosms was particularly high on Day-7). Bacterial DAPI counts are listed in Table 4. Bacterial counts did not evolve during 48h (the differences between controls and experimental microcosms were not significant). At Day-7 bacterial counts were significantly higher (1.3 times) in the experimental microcosms. DAPI counts of nanoflagellates are shown in Table 5. Only on Day-2 these counts were significantly higher (1.4 times) in the experimental microcosms.
Table 1. Exchange fluxes (negative values are from sediments to overlying water) in mol m\(^{-2}\) d\(^{-1}\) calculated using metal concentrations in overlying waters. Minimum and maximum values obtained from 6 microcosms (A) or 3 microcosms (B) are indicated. Significant differences between algae and controls are indicated in boldface (Mann-Whitney U test, two-tailed, \(\alpha=0.05\)).

<table>
<thead>
<tr>
<th></th>
<th>A. Between Day-0 and Day-2</th>
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<th>B. Between Day-2 and Day-7</th>
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<tbody>
<tr>
<td></td>
<td>Algae</td>
<td>Controls</td>
<td>Algae</td>
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<tr>
<td></td>
<td>Min</td>
<td>Max</td>
<td>Min</td>
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<tr>
<td>As</td>
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<td>(-349 \times 10^{-9})</td>
<td>(-266 \times 10^{-9})</td>
</tr>
<tr>
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<tr>
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<td>(-34 \times 10^{-9})</td>
<td>(-47 \times 10^{-9})</td>
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<tr>
<td>Mn</td>
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<td>(-77.4 \times 10^{-6})</td>
</tr>
<tr>
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</tr>
<tr>
<td>Fe</td>
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<tr>
<td>Ni</td>
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</tr>
<tr>
<td>Cu</td>
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<td>(+848 \times 10^{-9})</td>
<td>(-225 \times 10^{-9})</td>
</tr>
<tr>
<td>Zn</td>
<td>(-19 \times 10^{-6})</td>
<td>(+1.9 \times 10^{-6})</td>
<td>(-10 \times 10^{-6})</td>
</tr>
</tbody>
</table>

Table 2. Chlorophyll a in 0-5 mm sediments (mean ± SD; µg g\(^{-1}\) dw).

<table>
<thead>
<tr>
<th></th>
<th>Day-0 (2h)</th>
<th>Day-2</th>
<th>Day-7</th>
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<tbody>
<tr>
<td></td>
<td>Algae</td>
<td>Controls</td>
<td>Algae</td>
</tr>
<tr>
<td></td>
<td>19.7 ± 1.4 a</td>
<td>22.4 ± 3.6 a</td>
<td>21.9 ± 2.2 a</td>
</tr>
<tr>
<td>Algae</td>
<td>30.3 ± 3.1 b</td>
<td>41.6 ± 1.3 b</td>
<td>35.2 ± 4.0 b</td>
</tr>
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</table>

Different letters (a, b) indicate significant differences between controls and treatment (Student t-test, \(\alpha=0.05\)).
Table 3. DOC in 0-5 mm sediments (mean ± SD; mg L⁻¹).

<table>
<thead>
<tr>
<th></th>
<th>Day-0 (2h)</th>
<th>Day-2</th>
<th>Day-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>17.3 ± 5.2 a</td>
<td>14.6 ± 2.8 a</td>
<td>17.2 ± 1.3 a</td>
</tr>
<tr>
<td>Algae</td>
<td>16.8 ± 0.5 a</td>
<td>18.1 ± 1.5 a</td>
<td>24.1 ± 5.4 a</td>
</tr>
</tbody>
</table>

Different letters (a, b) indicate significant differences between controls and treatment (Student t-test, α = 0.05).

Table 4. Bacterial DAPI counts (x 10⁶) in 0-5 mm sediments (mean ± SD; cells g⁻¹ dw).

<table>
<thead>
<tr>
<th></th>
<th>Day-0 (2h)</th>
<th>Day-2</th>
<th>Day-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>4.0 ± 0.73 a</td>
<td>3.0 ± 0.27 a</td>
<td>2.9 ± 0.31 a</td>
</tr>
<tr>
<td>Algae</td>
<td>5.1 ± 1.6 a</td>
<td>4.1 ± 0.85 a</td>
<td>3.8 ± 0.44 b</td>
</tr>
</tbody>
</table>

Different letters (a, b) indicate significant differences between controls and treatment (Student t-test, α = 0.05).

Table 5. DAPI counts of nanoflagellates (x 10⁶) in 0-5 mm sediments (mean ± SD; cells ml⁻¹).

<table>
<thead>
<tr>
<th></th>
<th>Day-0 (2h)</th>
<th>Day-2</th>
<th>Day-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>8.4 ± 2.7 a</td>
<td>4.5 ± 0.2 a</td>
<td>6.1 ± 1.1 a</td>
</tr>
<tr>
<td>Algae</td>
<td>5.6 ± 2.4 a</td>
<td>6.2 ± 0.7 b</td>
<td>5.7 ± 1.4 a</td>
</tr>
</tbody>
</table>

Different letters (a, b) indicate significant differences between controls and treatment (Student t-test, α = 0.05).

The mineralization activity of the microbial community was first assessed using the Biolog EcoPlate system (CLPP approach). Mineralization was clearly visible, with a total of 15 enzymatic activities that differed significantly between control and experimental microcosms (Figure 3). The majority of these activities (13 over 15) were more elevated in the experimental microcosms by a factor 1.3 to 15.1 and occurred on Day-2 and Day-7. Only two activities were significantly reduced (on Day-2, by a factor 0.6-0.8). Microbial mineralization already started after 2h of incubation as indicated by the significant increase of three enzymatic activities (Figure 3). The mineralization activity of the microbial community was also studied using the FDA approach. The FDA approach indicated that the esterase activity...
progressively increased from Day-0 to Day-7 (Figure 4) with an esterase activity that was 1.9 times more elevated at Day-7 in the experimental microcosms than in the controls.

Figure 3. Bacterial activity as measured using Community Level Physiological Profiling (CLPP). The EcoPlate™ system of Biolog was used, which measures the use of 31 carbon sources. Only the carbon sources which produced significant differences between controls and experimental microcosms (Algae) are indicated (Mann-Whitney U test). E2 to E31 refer to the type of carbon source degraded by the microbial community. E2, pyruvic acid methyl ester; E3, Tween 40; E4, Tween 80; E5, α-cyclodextrin; E9, β-methyl-D-glucoside; E14, D-glucosaminic acid; E20, 4-hydroxy-benzoic acid; E21, γ-hydroxybutyric acid; E22, itaconic acid; E26, L-asparagine; E27, L-phenylalanine; E28, L-serine; E30, glycyl-L-glutamic acid; E31, phyllethylamine; E32, putrescine. D2, Day-2; D7, Day-7.

Finally, bacterial production during the incubation of the microcosms was evaluated by the incorporation of tritiated thymidine. Values were stable in the control microcosms and ranged between 0.2 and 0.8 mg C m⁻² d⁻¹ (Figure 5). In the experimental microcosms values were significantly more elevated, especially on Day-2 when a maximum was observed at 8.7 mg C m⁻² d⁻¹. This value was at least 10 times more elevated than in the controls.
Figure 4. Fluorescein Diacetate Analysis (FDA) which estimates the total esterase activity of the microbial community as the quantity of released fluorescein according to time (mean ± SD). Significant differences are indicated by an asterisk (Student T test, \( \alpha = 0.05 \)).

Figure 5. Bacterial production in surface sediments (at the interface) during the incubation of the microcosms as measured by incorporation of tritiated thymidine (mean ± SD). Significant differences are indicated by an asterisk (Student T-test, \( \alpha = 0.05 \)).
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Discussion

The experiments performed in this study clearly demonstrate that microbial mineralization of phytoplankton-derived phytodetritus accumulated at the surface of contaminated muddy sediments may lead to an increased efflux of trace elements from these sediments. Several important points must be considered. First, the quantity of phytodetritus used in this experiment was in the range of what can be observed in the field. Although the algal suspension used here was 21.4 times more concentrated than the maximum values observed in the North Sea during a *Phaeocystis* bloom (chlorophyll $a$: 750 $\mu$g L$^{-1}$ versus 35 $\mu$g L$^{-1}$) (Schoemann et al., 2005), it should be noted that the height of the water column over the microcosms was only 4 cm, and that station 130 is located at ca. 10 m of depth. Consequently, considering that all phytodetritus may reach sediments, the present experiment was equivalent to an 85 cm column of seawater containing 35 $\mu$g L$^{-1}$ of chlorophyll $a$ (4 cm x 21.4), or to a 10 m column containing 3 $\mu$g L$^{-1}$ of chlorophyll $a$ ($750 \times 0.04 \times 10$). Such a chlorophyll $a$ concentration (3 $\mu$g L$^{-1}$) is exactly the mean concentration observed in spring in coastal areas such as the Bay of Biscay (France), with peaks at 10 $\mu$g L$^{-1}$ (Gohin et al. 2003). On the coastal BCZ and the English Channel, mean values are commonly higher than 30 $\mu$g L$^{-1}$ (Denis and Desroy 2008). On the BCZ, values of ca. 20 $\mu$g L$^{-1}$ at station 130 were observed throughout March and April 2008, with values reaching 100 $\mu$g L$^{-1}$ in May (unpublished observations). Lancelot et al., 2005 estimated that 24% of the production may reach sediments, the rest being exported or degraded in the water column. As a result, for the period between March and April on the BCZ, a quantity of 4.8 $\mu$g L$^{-1}$ may potentially reach the sediments (24% of 20 $\mu$g L$^{-1}$). Although the actual input of phytodetritus in North Sea sediments is hard to determine, as it depends on currents and wind (Van Duyl et al., 1992; Denis and Desroy, 2008), we may consider that the quantity of phytodetritus used in the present experiment (3 $\mu$g L$^{-1}$) is in the lower range of values that may potentially be observed on sediments during a real phytoplankton bloom.

Another point to consider is the origin of metals appearing in the overlying waters during the experiment. Metals measured after 2h originate mostly from the added algal suspension in itself (clearly our algal suspension was contaminated with Cd) but also from the disturbance of the WSI. Indeed, it can be seen that the increases observed after 2h are of the same range as the dissolved metal concentrations of the algal suspension (see materials and methods). As a consequence, no conclusions can be drawn about effluxes of metals from sediments after 2h.
of exposure to phytodetritus. However, this is not the case for Day-2: concentrations of Mn, Co and As in the experimental microcosms were respectively 60 times, 3.6 times and 2.1 times more elevated than the values observed in the same microcosms after 2h. These increases can only be explained by (i) the mineralization of phytodetritus that releases metals directly into the overlying water, and (ii) by an efflux of metals from the sediments. It has been calculated that the complete mineralization of the phytodetritus on Day-2 would only introduce 8.4 (Mn), 0.07 (Co) and 0.58 (As) μg of metals per liter in the overlying water (Appendix A). This means that the complete mineralization of phytodetritus at the interface only represents a maximum of 0.9% (Mn), 12.4% (Co) and 23.3% (As) of the increases observed on Day-2 in the overlying waters. As a result, 99.1% (Mn), 87.6% (Co) and 76.7% (As) of the metals are coming from the sediments.

A third point is the physico-chemical conditions during the experiment. Like with many approaches, the microcosm experiment presented here is not free from biases: (1) Because stagnating water was used oxygen levels were lower than in the field. Low oxygen levels may promote or impede the flux of metals like Fe^{2+}; (2) In the field, hydrodynamics is known to play an important role in the rate of diffusion as it directly influences the thickness of the DBL, which in turn acts as a diffusion barrier; (3) In the sea, currents and waves may cause water advection and sediment resuspension which may influence metal fluxes. For the first bias (1) it must be noted that microcosms were far from anoxia: oxygen levels at 5 mm above the sediments were about 5 mg L^{-1} and increased Fe fluxes were not observed during the experiment (Table 1). Iron effluxes are frequently observed when anoxia is developing (Petersen et al. 1996). For some metals like Mn^{2+} it was shown in other reports that the concentration in porewaters of the top sediment layer (0-5 mm) is not dependent on dissolved oxygen levels of the bottom water, at least on the short term (Pakhomova et al. 2007). Manganese fluxes are therefore not influenced by the redox conditions in the near-bottom water. For the second bias (2) it must be noted that high water flow velocities near the bottom will tend to decrease the DBL thickness and thus increase benthic effluxes (Jørgensen and Des Marais, 1990). Consequently, we may view our measurements in stagnating water as lower estimates of what can be released under high flow velocities. Finally, for the third biases (3), sediment resuspension and water advection are also expected to release more metals into the bottom waters. To sum up, metal fluxes determined in our microcosm approach must be considered as an approximation, probably underestimated, of the real benthic fluxes in muddy sediments of the BCZ (they cannot be applied to the whole BCZ...
which also feature coarse and sandy sediments).

Although fluxes determined here are approximations, the most important point of the present research is that mineralization of phytodetritus at the WSI increases effluxes of Mn, Co and As. Flux values obtained in this study are in the range of those observed in situ using benthic chambers deployed on other shallow coastal oxic sediments which may be exposed to phytodetritus (Hunt, 1983; Ciceri et al., 1992; Aller, 1994; Thamdrup et al., 1994; Warnken et al., 2001; Pakhomova et al. 2007). For instance, Mn effluxes were found to vary between 70–4450 µmol m⁻² d⁻¹ in the Gulf of Finland and the Vistula Lagoon of the Baltic Sea (Pakhomova et al. 2007), or between 420–2600 µmol m⁻² d⁻¹ in Galveston Bay, Texas (Warnken et al. 2001). The maximum effluxes obtained here for Mn were only 755 µmol m⁻² d⁻¹. Similarly, for As effluxes, other investigators have observed 0.008–2.5 µmol m⁻² d⁻¹ for Amazon shelf sediments (Sullivan & Aller, 1996) and about 5.1 µmol m⁻² d⁻¹ for Chesapeake Bay sediments (Riedel et al., 1987). In the present study, As effluxes were 0.045–1.084 µmol m⁻² d⁻¹, exactly in the range of the previous observations.

Another point to consider is the role of the benthic microbial communities. It is clear that the onset of mineralization is very fast and starts within 2h of deposition as revealed by CLPP analyses. Benthic microbial communities were shown in other studies to react quickly to fresh inputs of organic carbon (Turley and Lochte, 1990; Meyer-Reil and Köster, 1992). The increased activity of the microbial heterotrophs results in the consumption of the oxygen (Franco et al., 2007; Rauch et al., 2008) which is known to be responsible for >90% of the organic carbon mineralization at the WSI (Bender and Heggie, 1984). As a consequence, the oxic-anoxic interface [usually located at 3-4 mm into the sediments of that station (Gao et al., 2009)] moves upwards and reduced elements like Fe²⁺, Mn²⁺, Co²⁺ and arsenic species, may diffuse out of the sediments. Fe and Mn oxides are important electron acceptors in anaerobic marine sediments (Thamdrup et al., 1994). Mn oxides are reductively dissolved by the action of bacteria that actively mineralize the phytodetritus thereby releasing Mn²⁺ into the porewater (Froelich et al., 1979; Burdige and Gieskes, 1983; van der Zee and van Raaphorst, 2004). This Mn²⁺ is then able to diffuse out of the sediments as it is relatively stable to chemical oxidation. In comparison, Fe²⁺ ions are more reactive and precipitate at the WSI after its re-oxidation in the contact with oxygen (Pakhomova et al., 2007). Consequently, Fe is not expected to accumulate in oxic seawater as observed in the present microcosm experiment. Increased benthic effluxes of Mn, Co and As from sediments may thus be
explained by the development of anaerobic conditions at the WSI which is here a consequence of an increased microbial heterotrophic activity. The effect of anaerobiosis on such metal effluxes was observed in previous microcosm experiments (Petersen et al., 1996) but also in situ (Sundby et al., 1986).

The deposition of fresh phytodetritus at the WSI explains the increased bacterial production observed on Day-2 (ca. 4.3 times, from 2.0 to 8.7 mg C m\(^{-2}\) d\(^{-1}\)). However, this increased bacterial production was not followed by an increased bacterial biomass because the biomass of heterotrophic nanoflagellates immediately increased \((6.2 \times 10^6\) cells mL\(^{-1}\) at Day-2). Nanoflagellates, which are known to be the main bacterial grazers (Hondeveld et al., 1995), were thus able to control the bacterial biomass in the microcosm experiment and were not impeded by high arsenic levels. The total heterotrophic activity increased (bacteria and nanoflagellates) and this resulted in a consumption of oxygen with a lowering of the pH which in turn promoted the reductive dissolution of Fe/Mn oxyhydroxides that release many metals and metalloids. In addition, large quantities of organic acids were probably generated during the mineralization process. Such organic acids are then able to complex many metallic elements and transport them into the overlying water.

During the last 5 days of mineralization (Day-2 to Day-7) effluxes of trace elements were reduced. This is another indication that effluxes are linked to the mineralization of phytodetritus, as the quantity of phytodetritus is decreasing. Although effluxes of trace elements were reduced, the microbial enzymatic activity was still intense, as indicated by the esterase activity and the CLPP profiles on Day-7. These increased bacterial activities were not followed by an increased bacterial production: values on Day-7 were at the level of those observed in the controls (ca. 2 mg C m\(^{-2}\) d\(^{-1}\)). But the bacterial biomass was now 1.3 times more elevated. This may be explained by a reduced predation by nanoflagellates as these may have been consumed by higher trophic levels (large ciliates, nematodes, etc.). Although the experiment was stopped after 7 days of incubation it is probable that enzymatic activities and bacterial biomass slowly return to pre-exposure values within a few days.

The consequences of arsenic effluxes may be huge in areas covered with contaminated muddy sediments and submitted to an intense rain of phytodetritus. In the microcosm experiment described here the quantity of added phytodetritus was only 5.24 g m\(^{-2}\) (dw) \((0.092726\) g / \(0.017670\) m\(^{2}\)). It has been calculated that this quantity of deposited phytodetritus has led to the release of 76.4 \(\mu\)g of arsenic per square meter of sediment after only 48h (see Appendix
B). The Belgian Continental Zone (BCZ) is 3600 km$^2$ and we may estimate that about 20% of that surface area (720 km$^2$) is composed of muddy contaminated sediments located close to the coast between the cities of Oostende and Zeebrugge (Van den Eynde, 2004; Ruddick and Lacroix, 2008; Gao et al., 2009). These muddy sediments have been monitored in various points and results show that the arsenic content is almost unchanged in the area [the monitored stations include contaminated stations 140 and 700 (Gillan and Pernet, 2007)]. If sediments of the area are considered as equivalent, this means that about 55 kg of arsenic may be released into the seawater of the BCZ, in only two days and as a result of the microbial activity alone, when only 5.24 g (dw) of phytodetritus are mineralized on each square meter of this area. As explained before this quantity of phytodetritus is probably a low estimation of what may be deposited on the BCZ during a real phytoplankton bloom (Schoemann et al., 2005). In addition, arsenic effluxes might even be higher because (1) the metal content of the phytodetritus was not taken into account in the above calculations; (2) hydrodynamic effects which lower the DBL and resuspension events were not taken into account.

These large quantities of arsenic released from sediments in the continental zone may then return to the sediments. Indeed positive fluxes (i.e., towards the sediments) up to +262 nmol m$^{-2}$ d$^{-1}$ have been observed in the present experiment (Table 1). However, during the stay in the overlying water, arsenic species may be accumulated in the biota. The main arsenic compound in oxic seawater is arsenate ($\text{AsO}_4^{3-}$) (Mukhopadhyay et al., 2002). Arsenate is known to be taken up by marine organisms, ranging from phytoplankton, algae, crustaceans, mollusks and fishes (Knowles and Benson, 1983). Fish and marine invertebrates retain 99% of accumulated arsenic in organic form, and crustacean and mollusk tissues are known to contain higher concentrations of arsenic than fishes (Mukhopadhyay et al., 2002). Arsenic, which is now recognized as a carcinogenic element (Pershagen, 1985; Bates et al., 1992), may thus accumulate in marine foodstuffs such as prawns at levels approaching 200 ppm (Nriagu, 1994).

To conclude, measures to limit eutrophication and phytoplankton blooms in contaminated areas are urgently needed for the Belgian Continental Zone. The reason is that upward fluxes of toxic elements like arsenic occur when contaminated muddy sediments are exposed to Phaeocystis-derived phytodetritus. A clear link was established here between heterotrophic microbial activity at the sediment-seawater interface and increased effluxes of Mn, As and Co. Calculations have suggested that this microbial activity alone may release substantial amounts of dissolved elements in only 48h when sediments are exposed to large quantities of
phytodetritus, i.e. every year between February and May.

**Acknowledgements**

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**Appendices**

**Appendix A :**

- The dry matter content of the final algal suspension used as phytodetritus was 130.6 mg L⁻¹.
- Four cm of overlying water in one microcosm of 15 cm of ø = 710 mL.
- A quantity of 92.7 mg (dw) of phytodetritus were deposited in each microcosm (130.6 mg x 710 mL / 1000 ml = 92.7). This corresponds to 0.092726 g.
- The Mn concentration of the phytodetritus was 64.5 µg g⁻¹ (dw).
- The Co concentration of the phytodetritus was 0.54 µg g⁻¹ (dw).
- The As concentration of the phytodetritus was 4.4 µg g⁻¹ (dw).
- The complete mineralization of the 92.7 mg of phytodetritus in one microcosm containing 710 mL of overlying water may thus produce a maximum of 5.98 µg of Mn (0.092726 x 64.5 = 5.98), 0.050 µg of Co (0.092726 x 0.54 = 0.050), and 0.40 µg of As (0.092726 x 4.4 = 0.40).
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- If 5.98 µg of Mn are released in 710 mL of water, this corresponds to 8.4 µg L⁻¹.

- If 0.050 µg of Co are released in 710 mL of water, this corresponds to 0.07 µg L⁻¹.

- If 0.40 µg of As are released in 710 mL of water, this corresponds to 0.56 µg L⁻¹.

Appendix B:

- In Fig. 2, the increase observed for arsenic in experimental microcosms, when 2h is compared to Day-2, is 2.49 µg L⁻¹ (4.76 – 2.27 µg L⁻¹). For 710 mL (one microcosm) this corresponds to the arrival of 1.76 µg of As (2.49 x 710 / 1000 = 1.76).

- These 1.76 µg of arsenic in one microcosm can only come from the sediments or from the phytodetritus. As 92.7 mg of phytodetritus were deposited in each microcosm (130.6 mg x 710 mL / 1000 ml = 92.7) and that 1 g of phytodetritus (dw) contains 4.44 µg of As, this means that the maximum of As released by the mineralisation of the phytodetritus in one microcosm is 0.41 µg (0.0927 g x 4.44 µg / 1 g = 0.41 µg).

- 0.41 µg is 23.4% of 1.76 µg. This means that the rest (76.6% = 1.35 µg) is coming from the sediments for one microcosm.

- One microcosm is 0.017670 m².

- We may conclude that, following the deposition of 92.7 mg of phytodetritus over a surface of 0.017670 m² (one microcosm) a quantity of 1.35 µg of As is released from the sediments.

- The quantity of As released from 1 m² is thus 76.40067 µg (1.35 / 0.017670 m²).

- One km² = 10⁶ m²

- The quantity of As released from 1 km² is thus 76.40067 g.

- The quantity of As released from 720 km² is thus 55 008.49 g = 55 kg.