

# Diplomarbeit

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Biogeochemische Prozesse an marinen  
Walkadavern und Methanquellen

# Diploma thesis

Biogeochemical processes at marine  
whale falls and methane seeps

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## Abstract

To test the "everything is everywhere" hypothesis, four marine sediment types of different origins were enriched with either whale biomass or methane. Sulphide and methane concentrations were measured for almost 200 days. After 35 days, sediments that were from sites of high organic matter input showed constant or even increasing rates of sulphate reduction. Increasing rates of methanogenesis were detected with a lag of about 100 days from onset of sulphate reduction. The sediment that had been exposed to methane in situ exhibited a low rate of anaerobic oxidation of methane (AOM) when enriched with methane. Nitrogen and organic carbon content of the whale biomass enriched sediments showed a two- to threefold increase compared to pristine sediment. No change in elemental composition was observed in methane enriched sediments.

A whale vertebra sampled at an inactive whale fall site was examined for rates of sulphate reduction. Pockets of lipids were shown to be hotspots of sulphate reduction in deep layers of the bone. By measurement of citrate content, age determination was attempted.

In a related experiment, fresh whale and bird vertebrae were incubated on two marine sediments in flow-through aquaria and microbial mat development and sediment appearance were monitored for six months. Bones were sampled every month and light, fluorescence and scanning electron microscopy was done on surfaces and cross-sections to determine the nature and extent of bioerosion. Microbial bioerosion was found to start rapidly after incubation and no differences were observed between bones or sediments. No bioerosion was detected in bone parts incubated below sediment.

The results obtained shed an ambivalent light on the hypothesis of "everything is everywhere". Whereas some metabolic reactions in sediments were similar, others did not exhibit the same pattern. In bone incubations, mat formation and bioerosion were very similar between sediments, but identification of the actual organisms is necessary to unravel the succession and mechanism of bioerosion.

## Zusammenfassung

Um die Gültigkeit der Hypothese "alles ist überall, aber die Umwelt selektiert" zu überprüfen, wurden vier marine Sedimente unterschiedlicher Herkunft und Vorgeschichte mit Walfleisch oder Methan angereichert. Über fast 200 Tage wurde die Sulfid- und Methanentwicklung verfolgt. Die Sedimente, die an ihrem Ursprungsort hohen Organikeintrag erfahren haben, zeigten bei Anreicherung mit Walbiomasse nach 35 Tagen steigende Raten von Sulfatreduktion. Steigende Methanogeneseraten folgten mit einer Verzögerung von 100 Tagen. Das Sediment, welches ursprünglich hohen Methankonzentrationen ausgesetzt war, zeigte eine schwache Rate von anaerober Methanoxidation, als es mit Methan angereichert wurde. Nach Beendigung der Versuche zeigten Biomasse-angereicherte Sedimente einen zwei- bis dreifach erhöhten Gehalt an Stickstoff und organischem Kohlenstoff gegenüber den Anfangsgehalten. Bei Methan-angereicherten Sedimenten wurden keine Veränderungen der Elementgehalte gefunden.

An einem Wirbel eines inaktiven Tiefsee-Walkadavers wurden Messungen der Sulfatreduktion ausgeführt. Die Zentren der Sulfatreduktion waren in vom Meerwasser geschützten Lipidtaschen in tieferen Knochenschichten zu finden. Eine Altersbestimmung des Knochens wurde mittels Messung des Citratgehalts versucht.

Ein ähnliches Experiment beinhaltete die Inkubation von Wal- und Vogelknochen auf zwei verschiedenen Sedimenten in Durchflussequarien. Die Bildung bakterieller Matten und die Veränderungen im Sediment wurden dokumentiert und jeden Monat wurde ein Knochen zwecks verschiedener mikroskopischer Analysen genommen. Aufsichten und Querschnitte wurden auf Art und Intensität von biologischer Knochenerosion untersucht. Erosion setzte in allen Ansätzen schnell ein und keine Unterschiede zwischen den Sedimenten waren erkennbar. Knochenabschnitte, die unter der Sedimentoberfläche inkubiert waren, zeigten keine Erosionsspuren.

Die Ergebnisse lassen an der Gültigkeit der Hypothese "alles ist überall, aber die Umwelt selektiert" zweifeln. Während die metabolischen Reaktionen einiger Sedimente ähnlich waren, zeigten andere abweichende Muster. Die Mattenbildung und Erosion in den Knocheninkubationen war bei beiden Sedimenten sehr ähnlich, allerdings ist eine Identifizierung der beteiligten Organismen notwendig, um Aussagen über Ablauf und Mechanismen von biologischer Knochenerosion treffen zu können.

# 1. Introduction

*"alles is overal: maar het milieu selecteert"* (Baas Becking 1934)

(Everything is everywhere, but the environment selects)

Ever since the establishment of the discipline of microbiology the question of microbial biogeography has aroused discussion, leading Dutch microbiologist Lourens Baas Becking to formulate this concise statement based on realisations of his compatriot and inspirator, Martinus Beijerinck (Baas Becking 1934; Beijerinck 1913).

The lack of distinguishing morphological features only allowed a rough taxonomical identification and thus, led to the belief that many microorganisms were distributed globally (Fenchel & Finlay 2004; Finlay et al. 1999). It was hypothesised that geographical barriers do not hinder microbial dispersal due to their small body size and easy distribution by currents, wind, animals etc. (O'Malley 2008). Neither contemporary environmental conditions nor historical separation were thought to influence microbial dispersal. This seemed to be in contrast to Darwin's dispersalist theory of speciation introduced only decades before, but was nonetheless widely accepted. Generally, body size of an organism seems to be inversely related to its geographical distribution and population density. For microbes, this implies a vast geographical distribution also due to possibly high population densities (Fenchel & Finlay 2004). In addition, microbial propagation has long been thought to be clonal only, leading to low rates of divergence and speciation due to little or no genetic recombination.

As techniques in microbiology are becoming more sophisticated, Baas Becking's hypothesis is put to the test. The influences of environment and history have recently been elucidated (Martiny et al. 2006). Over large spatial scales, history has been shown to have most influence on microbiotic composition, while over very small geographic scales, environmental cues are the main factors. Only at intermediate distances, i.e., 10 - 3000 km, have studies not revealed a clear dominance of influence (ibd.). With the use of modern microscopy and molecular methods, identification of organisms has reached a much higher resolution and what has been considered one species can now be easily distinguished into several species (Katz et al. 2005; Telford et al. 2006). In addition, progress concerning microbial ecology has been made. The assumption that microbes can disperse freely and settle anywhere where favourable conditions are met has been found to be true only in special cases (O'Malley 2008). Rather, many microbes do not possess the ability to survive adverse conditions as cysts or dormant stages and the resilience of these stages varies greatly among organisms.

Obligate or even facultative anaerobes, e.g., are likely to experience high mortality when removed from anoxic conditions and exposed to oxygen (Cypionka et al. 1985; Loesche 1969). Furthermore, competition of newly arrived microbes with already established communities is likely to aggravate settlement problems. The discovery of lateral gene transfer adds a hitherto unknown possibility of genetic diversity to microbial genomes (Ochman et al. 2000). Even a case of lateral gene transfer between thermophilic archaea and eubacteria has been reported (K. E. Nelson et al. 1999).

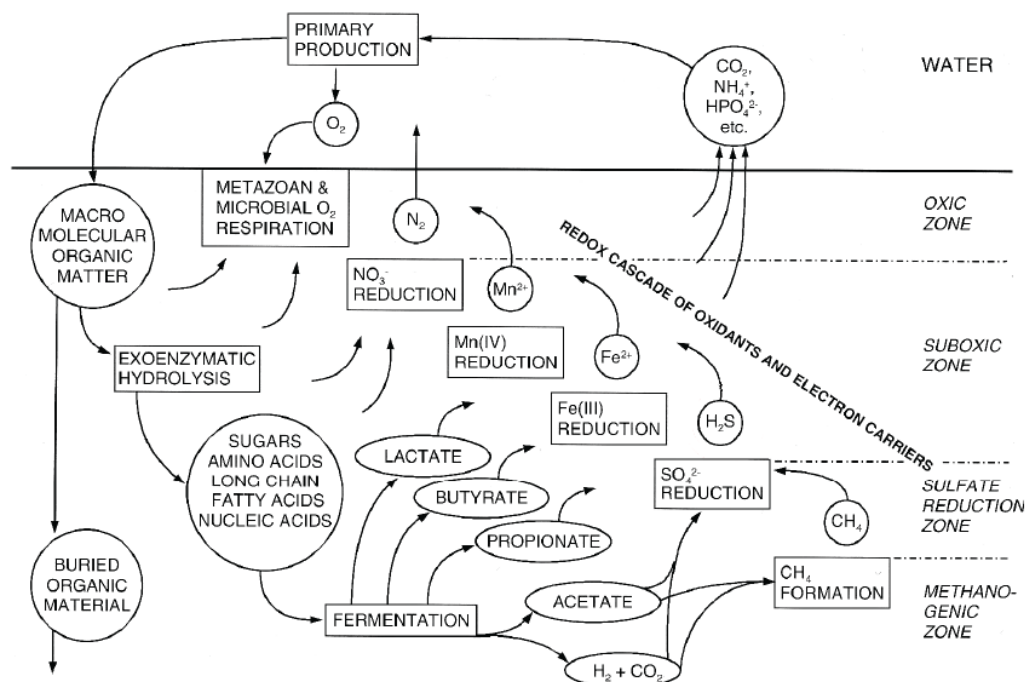
All these factors make the term "species" hard to grasp when applied to microorganisms. In contrast to researchers of macro-organisms, microbiologists define a species as a group of cells having a genome that exhibits at least 70 % DNA-DNA hybridisation. This is a very low threshold for species definition and alternative ways of assessing microbial distribution are employed. Metagenomics, which can encompass the genomic sets of habitats, are suitable for setting single organisms in a larger context. Nesbø and coworkers suggest that microbial biogeography should be based on the occurrence of genomes instead of species (Nesbø et al. 2006).

The exploration of one of the least accessible and highly under-studied realms, the deep sea, has led to the discovery of several kinds of extreme habitats. Although the deep sea itself constitutes an extreme habitat (in terms of pressure, organic matter input and light availability), hydrothermal vents, cold seeps and whale falls all exhibit extremes with regard to biogeochemical characteristics (C. R. Smith & Demopoulos 2003). This leads to unique and highly adapted microbial communities, whose functioning and dispersal strategies are still poorly understood. The isolated nature of these hot-spots for biodiversity challenges Baas Becking's hypothesis of "everything is everywhere" and stirs discussion until today.

Sediment microbiology in the marine realm is heavily influenced by organic matter input. Depending on the location and seasonality of the overlying water body, microbial processes in the sediment can be very different. Biomass in the marine environment is produced mainly in the upper, euphotic zone. Dead phytoplanktonic cells and faecal pellets of zooplankton comprise the largest part of organic material that becomes available for degradation and remineralisation. The modes of degradation in the microbial world are diverse. Because most of the water column is rich in oxygen (exceptions are the highly stratified Black Sea and seasonal suboxia in the Gotland Basin of the Baltic Sea, for instance) aerobic degradation dominates. It is estimated that in oceanic regions distant from land, 95 % of organic matter are consumed within the first several hundred meters and only about 1 % reaches the deep

sea floor below 1000 m. This has implications for the bacterial biomass in the deep sea: It has been found to decrease linearly with organic matter flux and exponentially with depth (Jørgensen & Boetius 2007).

In the sediments, replenishment of oxygen is limited by porewater exchange and diffusion. In shallow sediments exposed to sunlight, benthic photosynthesis can supersaturate oxygen in the sediment, but this oxygen source is not given in deeper waters, naturally. Once oxygen is no longer available as an electron acceptor for microbial metabolisms, alternative electron acceptors are utilised. The order of utilisation mirrors the ease with which a substrate accepts electrons and is thereby reduced. The affinity of a substrate to accept electrons is measured in the reduction (redox) potential: The more readily a substrate accepts electrons, the easier an electron donor (organic matter) can be oxidised and the more negative its redox potential is. The chemical energy generated by transferring electrons from the organic donor to the acceptor can be used to fuel metabolism and cell growth. Oxygen has the most negative redox potential, followed by nitrate ( $\text{NO}_3^-$ ), manganese ( $\text{Mn(IV)}$ ) and iron ( $\text{Fe(III)}$ ). These substrates are usually rapidly reduced within the upper layer of sediment. Further down, sulphate becomes a very important oxidising agent. Once sulphate becomes depleted, methanogenesis sets in, using mainly acetate, carbon dioxide and hydrogen as electron acceptors. The energy yield from the latter redox reaction is very low.

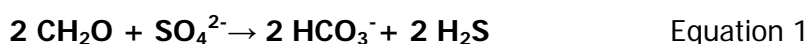


**Figure 1.** Overview of the microbial degradation processes in marine sediments (after Jørgensen, 2000).

Microbial degradation of organic matter is step-wise (Figure 1), meaning that no single organism can perform the degradation from fresh biomass to the end products such as hydrogen or carbon dioxide. Usually, aerobic bacteria and metazoans convert the food parcels to sugars, amino acids and fatty acids at the sediment-water interface. As oxygen availability decreases, these organic compounds are used as electron donors by fermenting bacteria, which thereby produce smaller molecules such as lactate, acetate, hydrogen and carbon dioxide. The sequence of substrates that are used as electron donors also exhibits an order, with C1- and C2-bodies being respired before smaller substrates such as hydrogen and carbon dioxide. A variety of microbial metabolisms depends on these substrates. Denitrification ( $\text{NO}_3^-$  reduction) and manganese ( $\text{Mn(IV)}$ ) and iron ( $\text{Fe(III)}$ ) reduction take place in the suboxic ( $< 10 \mu\text{M O}_2$ ) sediment layer and will not be discussed here any further. Especially manganese and iron reduction do not play a major role in sediment biogeochemistry (Henrichs & Reeburgh 1987).

The next most energy-yielding step is sulphate reduction, which is performed by bacteria and few archaea (Equation 1). Some sulphate reducing organisms are also capable of reducing other electron acceptors, e.g. nitrate, iron or even oxygen (Muyzer & Stams 2008). The most prominent oxidising agent used is sulphate, however. Compared to freshwater environments, sea water contains sulphate in high concentrations and it only becomes depleted very far down in marine sediments (Reeburgh 1983). This abundance of available sulphate makes sulphate reduction the most important mode of organic matter oxidation in organic-rich marine sediments, accounting for 10 - 90 % of total oxidation (Canfield & Thamdrup 1996). Because oxygen is quickly depleted at the sediment surface, sulphate reduction can occur from close to the sediment - water interface to far down since sulphate is constantly replenished from the overlying water column.

Energy is gained by transferring electrons from a variety of substrates (e.g. formate, acetate, hydrogen) to sulphate to form hydrogen sulphide. Sulphate reducers depend on fermenting bacteria to break down large organic molecules into smaller substrates.

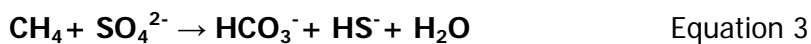


The last step in organic matter degradation is methanogenesis (Equation 2). The same substrates as in sulphate reduction and additionally methanol, trimethylamine or methionine are oxidised and methane is produced. This step is the least energy yielding and is only performed by archaea in highly reducing milieus, using the last products of fermentation,

mainly carbon dioxide, as electron acceptors. Because the reduction of sulphate is energetically more efficient than reduction of carbon dioxide, methanogens are inferior in the competition for substrates (Oremland & Polcin 1982). Methanogenesis can thus only take place at sediment depths where sulphate is depleted.



The sediment layer where sulphate becomes depleted and methane becomes enriched is called the sulphate-methane transition zone (SMTZ). Here, anaerobic oxidation of methane can take place (Boetius et al. 2000, Equation 3). This oxidation reaction is performed by archaea known to belong to three groups of ANME (anaerobic methanotrophs). This oxidation is coupled to sulphate reduction by sulphate reducing proteobacteria (Knittel & Boetius 2009) in a syntrophic consortium: electrons are transferred to sulphate through an intermediate that is hitherto unknown.



Sulphate reduction, methanogenesis and anaerobic oxidation of methane are distributed differently in sediments.

**Coastal sediments** are heavily influenced by land and river runoff by which they receive organic matter. For coastal waters in higher latitudes, stratification of the water body in summer is often interrupted by mixing events in colder months. Thus, remineralised nutrients are replenished in the upper layers. The resulting high biomass production in combination with the shallow depth leads to organically enriched sediments with diverse microbial activity in the several top centimetres of sediment. Oxygen regularly becomes depleted, and in cases of very high organic matter flux, the zone of oxygen depletion can extend upwards into the water column. Aerobic oxidation of organic matter plays only a minor role, with the most important microbial degradation process being sulphate reduction. Anaerobic oxidation of methane can occur in sediment layers as low as 5 cm (Treude, Krüger, et al. 2005) and methanogenesis takes place in deeper sediment. In Eckernförde Bay (Kiel, Germany), Treude and coworkers found anaerobic methane oxidation to be mediated by ANME-2 archaea not associated with bacterial sulphate reducers (ibid.). One of the sediments used in this study are the top 5 cm from an Eckernförde Bay core.

**Sediments in upwelling regions**, such as the Concepción Methane Seep Area (CMSA) off Chile, are characterized by seasonal upwelling (Sobarzo et al. 2007). Due to increased primary production, oxygen becomes scarce in the water column and may reach very low levels of less than 11  $\mu\text{M}$  (Roa et al. 1995). Shelf sediments down to great depth may thus become anoxic (Glud et al. 1999). Sediments become reduced as sulphate reduction becomes the most dominant metabolic pathway and extensive mats of sulphur oxidising bacteria of the genus *Thioploca* have been described from this region (Gallardo 1977). Three of the sediments that were used in this study originate from the Chilean upwelling area: Whale fall, cold seep and reference sediment were collected in the deep sea off Chile.

**Cold seeps.** Apart from production by microbial methanogenesis, methane in sub-surface sediment can also be produced abiogenically at hydrothermal vents (Sherwood Lollar et al. 2002) or thermogenically by transformation of buried organic matter (Chapman et al. 2004). Methane production over long periods of time can lead to the development of methane hydrates in outer continental margins and in permafrost soil under favourable conditions (Kvenvolden 1988). Geological faults that lead to instability of methane hydrates can cause methane to migrate upwards in the sediment and sustain a long-lasting supply of methane, often in combination with other hydrocarbons. Because methane in this environment is not limiting, it fuels constant anaerobic oxidation of methane, coupled with sulphate reduction. This "microbial filter" oxidises methane (depending on the flow rate) before it can reach the sediment-water interface and thereby diminishes the amount of this highly potent greenhouse gas that escapes to the atmosphere. The hydrogen sulphide that is produced in AOM rises in the sediment and a highly adapted community of chemoautotrophic sulphide-oxidisers can thrive. Depending on the flux intensity of methane from below, different zones have been identified (Treude et al. 2003; Torres et al. 2002). In sediment patches with very high methane flow, mats of the sulphur oxidising bacteria *Beggiatoa* are dominant. Where methane flow is intermediate (0 - 1  $\text{mmol/m}^2/\text{d}$ ), clams of the genus *Calyptogena* dominate, gaining energy by harbouring sulphide oxidising bacteria in their guts and supplying them with oxygen from the overlying water. Where methane flux is only diffusive, clams of the family Solemyidae with the genus *Acharax* are most common.

The CS (cold seep) sediment used in this study is taken from the top 5 cm of an active cold seep site, collected off Chile.

**Whale falls.** Whereas in common deep sea sediment, an organic carbon flux of less than 10 g organic carbon/m<sup>2</sup>/y is encountered (C. R. Smith & Demopoulos 2003), the arrival of large parcels of organic matter (wood, kelp, fish, marine mammals) has a huge impact on the sediment biogeochemistry. In shallow waters, gases produced during decomposition of a carcass hinder it from sinking to the sea floor. In deeper waters, however, the increase in hydrostatic pressure compresses the gases and, e.g., a dead whale becomes negatively buoyant (Allison et al. 1991). It is estimated that the biomass of a grown whale is equal to 2000 years of constant background supply of organic carbon to the underlying sediment (C. R. Smith & Baco 2003). A whale fall can sustain a succession of unique environments over decades to possibly a century (ibid.). Four faunal successions have been identified in whale falls, but the transition from one to the next is smooth:

In the mobile scavenger state, large chunks of biomass are removed mainly by sleeper sharks (*Somniosus pacificus*) and hagfish (*Eptatretus deani*). Smaller crabs and amphipods are also drawn to the carcass. This stage is estimated to last around 10 years.

As scavengers remove the soft tissue, pieces of flesh are distributed in the vicinity of the whale fall and are buried by bioturbation, especially by large scavengers and crustaceans (Solan et al. 2004). This is the onset of the so-called enrichment opportunist stage. It is characterized by a rapid recruitment of vast numbers of macrobenthic organisms, mainly polychaetes (*Vigtorniella* and dorvilleids, e.g., Wiklund et al. 2009). The number of individuals quickly drops with distance from the whale fall, but sediments are still enriched in organic carbon at least 9 m away (Treude et al. 2009). Sulphate reduction and possibly methanogenesis are observed near the carcass. The enrichment opportunist stage lasts for about one year.

When most of the soft tissue is removed from the carcass and the bones lay bare, the sulphophilic stage begins. Dense mats of sulphide oxidising bacteria, mostly *Beggiatoa*, cover the whale fall. Vesicomid clams, mussels and vestimentiferan polychaetes, all harbouring endosymbiotic sulphide oxidising bacteria, can be found around the skeleton (Deming et al. 1997). Sulphide is produced by sulphate reducing bacteria in the bones. Depending on the type of bone, whale bones can contain up to 60 % lipids (Higgs et al. 2010), a substrate that contains a lot of energy and is easily degradable. At this stage of the whale fall, the whole community depends on the sulphide produced in the bones. It is assumed that the bone lipids can sustain the sulphophilic community for a long time, because lipids are degraded slowly and sulphate reducers cannot penetrate a bone instantly. It is hypothesised that this is due to the hydrophobic nature of lipids that prevent sea water from quickly entering the

bone cavities (Treude et al. 2009). This stage of a whale fall is assumed to last up to several decades.

The whale bones themselves undergo changes when the lipids within are consumed by sulphate reducing bacteria. Not much attention has been paid to the traces of bioerosion that can be found in recent whale remains. Allison and coworkers examined vertebrae and other bones of a whale fall for the occurrence of pyrite and bioerosion patterns (Allison et al. 1991). The identities of bone-boring organisms and the interrelations of microbes and other bioeroders, such as fungi, are still largely unknown.

The WF (whale fall) sediment that was used in this study was collected in the vicinity of an old whale fall, off Chile.

The questions this study attempts to answer are: to what extent does the previous enrichment history of sediments determine the sediment's reaction to further enrichments? How quickly can sediment react to the addition of enrichment stimuli? What are the differences in reaction between organic enrichment in the form of whale biomass, and enrichment with methane?

The second part of this study extends this concept further and examines the interactions between sediments and bones incubated on them. What successional stages are observed in the colonisation of a bone? What are the morphological changes caused by bacterial bioerosion and how quickly is a bone eroded? What bacterial metabolisms are present and how long is a bone able to sustain them?

## 2. Material and Methods

### 2.1. Sediment origins

Five sediment types from different regions/ecosystems were utilised for this study. Four types (whale fall, cold seep, reference and Eckernförde Bay sediment) were used for enrichment experiments with whale biomass and methane (see 2.2). Eckernförde Bay and North Alex mud volcano sediments were used in bone incubation experiments with whale and ostrich vertebrae (see 2.3).

- Whale fall (WF): The top 5 cm of the sediment were scooped up by an ROV on the RV Sonne cruise SO210 off Chile in the Southeast Pacific at Station 21 (36° 42.31339' S, 73° 70.13855' W) from a depth of 665 m. This site was characterized by scattered whale bones of a seemingly old whale fall on top of a massive carbonate plate of an old cold seep. Appearance of the sediment was brownish-green and no sulphide smell could be detected.
- Cold Seep (CS): The top 5 cm of black sediment from an active cold seep, covered by bacterial mats, were taken with a push core by an ROV on the RV Sonne cruise SO210 off Chile. Station 42 (36° 28.241' S, 73° 40.727' W, depth of 707 m) was in the same region as the old whale fall. Large gas bubbles were visible in the core.
- Reference sediment (Ref): Light brown to green sediment from 2-9 cm depth was taken outside any cold seep or whale fall area by a multicorer on the RV Sonne cruise SO210 off Chile at station 68 (36° 28.242' S 37° 44.071' W) from a depth of 1006 m.
- Eckernförde Bay (Ebay): Located just outside a narrow bay in the western Baltic Sea (54° 30' N, 10° 02' E), this sediment receives much more organic input than deep sea sediments, owing to organic matter runoff from the nearby land combined with the shallow depth (ca. 25 m, Orsi et al. 1996). The sediment was largely reduced and black in colour from right below the surface, smelling of sulphide. The top 5 cm of a sediment core, which were retrieved on April 27, 2010 with a multicorer onboard RC Littorina, were used for the enrichment experiments. The core was kept cool and the 0 - 5 cm horizon was transferred into a 100 ml Duran glass bottle and closed with a butyl rubber stopper to avoid trapping air inside. For the bone incubations, one whole core (0 - 30 cm) taken on February 17, 2011 was used.
- North Alex mud volcano (NAMV): Situated 50 nautical miles off the Egyptian coast (31° 41' N, 29° 46' E), this large mound lies in the sediment fan created by runoff from the Nile delta. The active emission zone of the volcano features high methane and sulphide concentrations, but the sediment used in this study is reference sediment taken several hundred meters away from the centre, thus being of a light brown appearance. It was taken with a multicorer on the RV Poseidon cruise P262/2 from a depth of about 700 m.

## 2.2. Enrichment experiments

To test for and monitor the reaction of sediments to enrichment with methane or whale biomass, four different sediments were used: whale fall (WF), cold seep (CS), reference (Ref) and Eckernförde Bay (Ebay). Sediment slurries were prepared and were, together with sulphate reducer medium and whale biomass or methane, incubated over several months.

### 2.2.1. Preparation of sulphate reducer medium

The medium used for the slurries was designed to meet the needs of sulphate reducing bacteria (Widdel & Bak 1992). Under conditions of excess organic material being available, minimizing substrate competition, this medium can also be used for growing sulfate reducers together with methanogenic archaea (Treude et al. 2009). It was prepared in a 2l Widdel flask under sterile anaerobic conditions.

Several salts (for details on all amounts and solutions see Appendix I) were weighed in, solved in ultra pure water Type I ( $H_2O_{UP}$ ) and 0.5 ml/l of Resazurin (oxygen indicator) was added. After autoclaving (at 121°C for 1:05 h), the warm medium was gassed several minutes with  $N_2$ , and finally with  $N_2/CO_2$  (90:10) under constant stirring for the following procedures.

Thirty ml/l of  $NaHCO_3$  and 50 ml/l of  $NH_4Cl/KH_2PO_4$ , prepared in autoclaved vials and under anoxic conditions, were added. One ml/l of the following solutions were added, for details see Appendix I:

6 Vitamin solution

thiamine solution

cyanocobalamin (B12) solution

riboflavin solution

selenite/tungstate solution

trace elements solution (SL-10 KN-2, sulphate salts added)

$Na_2S$  solution (0.5mM, more if required to turn the medium yellowish/colourless)

pH was adjusted to 7.5 with  $Na_2CO_3$  (1 M) or HCl (6.5%). The medium was finally transferred into sterile Duran bottles flushed with  $N_2$ .

### 2.2.2. Set-up of enrichment experiment vials

In a glove box with an atmosphere of 90 % N<sub>2</sub> / 10 % CO<sub>2</sub>, 100 ml of each sediment were added to 300 ml of sulphate reducer medium with 25 ml plastic pipettes and a bulb filler to create the stock slurry in 1 l Duran glass bottles closed with butyl rubber stoppers. This slurry was stored in the dark on a shaker (70 rpm) at 10 °C until further processing.

Whale biomass was acquired from a stranded juvenile harbour porpoise, which was frozen at -25 °C before usage. Subcutaneous fatty tissue with some skin and red meat was available for this experiment. Tissue was cut into pieces of about 0.5 cm<sup>3</sup> and oil, which formed during cutting, was collected.

Volumes of 250 ml glass vials were determined by measuring the weight of water when vials were completely filled and sealed. Exact volumes of the vials are given in Appendix II. Before usage, all vials were autoclaved (at 121°C for 25min). Great care had to be taken to protect the sediments from warming, so all steps in the glove box were done on ice. To create the final mixture in the reaction vials, 50 ml of stock slurry of the respective sediment were added to 100 ml medium inside the glove box. Final dilution was thus 1 : 6 (1 part sediment in 6 parts medium). Each sediment was enriched with 16 g of whale meat and 4 g of whale oil in three replicates, yielding 3x4=12 vials. Furthermore, three replicates only contained 100 ml medium and whale tissue and served as controls. Sediment controls were set up to contain only 50 ml stock slurry and 100 ml medium. For each sediment type, only one control was prepared.

The methane enrichment was performed by filling the vials with sediments and medium and gassing with N<sub>2</sub> as before, but adding methane to the closed vials to a pressure of 1.1 bar. Similarly to above, medium controls for this setup contained only methane and medium. For a list of the experimental setup, see Table 1.

All vials were closed with butyl rubber stoppers and crimped before being taken out of the glove box and gassed with N<sub>2</sub> to ensure anoxic conditions. Both, methane and whale biomass were added to the respective treatments at the same time to be able to follow developments simultaneously. Vials containing Ebay sediment were started 40 days later than the other sediments; however, all procedures applied were exactly the same. All vials were placed in a dark 3 °C incubator and allowed to settle for 2 days prior to the first measurements.

**Table 1:** Overview of the enrichment experiment vials. WF=whale fall sediment, CS=cold seep sediment, Ref=reference sediment, Ebay=Eckernförde Bay sediment. Notice that vial 25 contained 150 instead of 100 ml medium.

Vial no.	Sediment	Enrichment	Replicate
1	WF	methane	1
2	WF	methane	2
3	WF	methane	3
4	CS	methane	1
5	CS	methane	2
6	CS	methane	3
7	Ref	methane	1
8	Ref	methane	2
9	Ref	methane	3
10	-	methane	1
11	-	methane	2
12	-	methane	3
16	WF	flesh+oil	1
17	WF	flesh+oil	2
18	WF	flesh+oil	3
19	CS	flesh+oil	1
20	CS	flesh+oil	2
21	CS	flesh+oil	3
22	Ref	flesh+oil	1
23	Ref	flesh+oil	2
24	Ref	flesh+oil	3
25*	-	flesh+oil	1
26	-	flesh+oil	2
27	-	flesh+oil	3
28	Ebay	flesh+oil	1
29	Ebay	flesh+oil	2
30	Ebay	flesh+oil	3
13	WF	-	1
14	CS	-	1
15	Ref	-	1
31	Ebay	-	1

During the course of the experiments, medium was exchanged collectively in all vials of the respective treatment as soon as any of the respective vials reached a concentration of 15 mM sulphide (only the case in whale biomass enriched treatment in this study). Medium in vials containing Ebay sediment was not exchanged at all, because of the later starting point of this experiment (non-synchronous with the other experiments), and because sulphide levels never exceeded 15 mM.

Medium for exchange was freshly prepared as described above and stored at 4 °C until use. Three medium changes were performed in total, each time in a different way:

The first medium change on May 31, 2011 (after 81 days) was done on ice in the glove box. The vial containing least supernatant was opened and as much liquid as possible was withdrawn (65 ml) without removing any sediment. The same volume was then taken from all other whale enriched vials as well. Special care had to be taken not to remove any whale flesh. It was not possible to withdraw all liquid from the supernatant, because that would have removed oil floating on top. To account for the 100 µl medium that was removed for each of the 30 sulphide measurement (see 2.2.3) before the first medium exchange, 68 ml (instead of 65 ml) of new medium were refilled. Vials were closed with new butyl rubber stoppers to avoid gas loss through needle punch holes. Finally vials were gassed with N<sub>2</sub> outside the glove box.

The next medium exchange (June 24, after 105 days) was conducted outside of the glove box to ease handling of the vials and lessen disturbance of the sediments. Medium was extracted with a 50 ml N<sub>2</sub>-flushed syringe (build-up of a vacuum) and fresh medium was filled in the same way. Since only five measuring days had passed since the last change, no additional medium was filled in to account for sulphide measurements. Vials were not flushed afterwards.

The last medium exchange (July 11, after 122 days) was done in the same way as the second one; 93 ml medium were exchanged and vials were flushed with N<sub>2</sub> afterwards. Due to the handling outside the glove box, rubber stoppers could not be renewed after the last two changes, increasing the chance of gas loss in the later stages of the experiment. To ensure equal mixing of sediment, medium and headspace among all vials of the experiment, vials that were not subject to medium change were shaken thoroughly on these three days.

To test for competition for substrates between sulphate reducers and methanogens, an alternative substrate was added to the sediment control vials after 187 days. The sediment

controls containing only sediment and medium were enriched with methanol to a final concentration of 10 mM (Oremland & Polcin 1982).

One WF-sediment vial enriched with whale biomass, Whale+WF3, was also kept for long-term monitoring.

### 2.2.3. Sulphide and methane development in enrichment experiments

Sulphide and methane levels in vials enriched with whale or methane were monitored over 191 days (except for the five vials still under observation, see above).

Sulphide and methane were measured every other day for the first three months, then every 4 - 7 days. Vials were kept in a cooled water basin inside the incubator to avoid warming during measurements. Sulphide was measured first to get a liquid sample from the clear supernatant. After this measurement, the vial was shaken thoroughly to equilibrate methane between the liquid and the gaseous phase and a gas sample was taken from the headspace.

Sulphide was measured following the method described by Cord-Ruwisch (Cord-Ruwisch 1985). Hundred  $\mu\text{l}$  of clear supernatant were sampled using a long hollow needle (0.9 x 120 mm, Rose GmbH Trier) and a 1 ml syringe (Terumo) flushed with  $\text{N}_2$  beforehand. Long needles were used because the whale biomass and oil floating on the surface had to be avoided. The sample was quickly transferred into a glass vial containing 4 ml of the Cord-Ruwisch acidified copper sulphate reagent, decanted into a 4.5 ml plastic cuvette and measured immediately photometrically at 480 nm wave length (UVmini-1240 Shimadzu spectrophotometer). The linear calibration curve was acquired by measuring sulphide standards ranging from 0.5 - 20 mM. For details on the sulphide standards, see Appendix III. Sulphide concentration was calculated using the resulting formula:

$$\text{Concentration} \left[ \frac{\text{mmol}}{\text{l}} \right] = \frac{\text{Absorption}}{0.0357} + 0.4958$$

For methane measurements, a gas-tight 250  $\mu\text{l}$  SGE syringe with a 0.5 x 40 mm needle (Braun Sterican) was flushed with  $\text{N}_2$  and several times with headspace gas before the actual gas sampling. 100  $\mu\text{l}$  of headspace were sampled. The sample was quickly injected into the gas chromatograph (Hewlett Packard 5890 Series II) equipped with a Haye SepT column (oven temperature 75 °C, injection temperature 160 °C). Carrier gas was helium, combustion gases were hydrogen and synthetic air. Before measurements, 200  $\mu\text{l}$  and 100  $\mu\text{l}$  of a

100 ppm methane standard (Scotty gases, Supelco) were injected. The peak areas detected could thus be converted to ppm methane by the linear relation.

For sulphate reduction and methanogenesis rate calculations (sulphate reduction and methanogenesis), regression lines of four or more data points (sulphide and methane, respectively) forming a straight line were calculated. After considering dilution factors of the stock and final slurry, a final rate in  $\mu\text{mol}$  of sulphide or methane produced per cubic centimetre of original (i.e., undiluted) sediment per day [ $\mu\text{mol}/\text{cm}^3/\text{d}$ ] was yielded.

#### 2.2.4. Analyses of enrichment experiment sediments

At the end of the enrichment experiment, vials were opened and sediment was taken from the bottom with a 10 ml plastic pipette and a bulb filler. Due to the high load of particulate whale biomass and the oil floating in the medium, the vials could not be shaken thoroughly before sampling, so exact dilutions sampled are not known. Half a ml sediment was taken for CARD-FISH analysis, several ml sediment were frozen at  $-20\text{ }^{\circ}\text{C}$  for CNS / organic carbon ( $\text{C}_{\text{org}}$ ) analyses and dry weight determination, and 4 ml sediment were frozen at  $-80\text{ }^{\circ}\text{C}$  for DNA / RNA analysis.

#### Quantification of cell numbers by SybrGreen staining and CAtalysed Reporter Deposition Fluorescence In-Situ Hybridisation (CARD-FISH)

For detailed information on buffers, solutions and microscope filters see Appendix IV.

CARD-FISH is a molecular method used for detecting and counting certain labelled cells. In addition to FISH, CARD facilitates the detection of rare targets by enhancing the signal.

The sample containing the cells (sediment from enrichment experiments in this study) is fixed and the cells are permeabilised. Then, cells are hybridised with an rRNA-binding, biotin-labelled probe. In this study, one archaeal probe (Arch915) and three eubacterial probes (EUB338 I-III) were employed. Horseradish peroxidase (HRP) is coupled to the probe and fluorescently labelled tyramide is added. HRP catalyses the tyramide molecules to reactive intermediates that bind to nearby proteins and can be detected by their fluorescence. By this tyramide signal amplification, few copies of rRNA can be made visible.

For fixation of the sediment, 0.5 ml of sediment were subsampled and kept in 1.5 ml of a 4 % formalin / Phosphate Buffered Saline (PBS) solution for 2 - 4 h at  $4\text{ }^{\circ}\text{C}$ . The sample was then centrifuged (10 min at 1400 g), the formalin solution replaced by PBS and the pellet resuspended with a clean spatula. Centrifugation, replacement by 1.5 ml PBS and

resuspension was done once more. After the third centrifugation step and decanting of the PBS, 1.5 ml of a 1:1 solution PBS / EtOH were added and the sample was stored at -20 °C in 2 ml cryovials.

To compare cell numbers and archaeal and eubacterial numbers in the sediments before and after the enrichment experiment, sediment samples of the initial sediment and the final sediment were taken.

The initial sample, i.e., before the enrichment, were 0.5 ml of a 1:2 mixture of base slurry and medium, the same dilution as in the experiment vials.

Sampling of the final sediment was performed using a pipette and bulb filler and sampling sediment from the bottom of the vials to avoid including whale biomass or oil from the surface. Due to differences in sediment volume acquisition using the pipette method, the height of sediment and total liquid level in the 2 ml cryovials used for storage had to be measured after fixation to get a dilution factor. The same was done with samples taken in the beginning of the experiment, which then allowed comparing cell numbers obtained with CARD-FISH. This pre-dilution is the reason why dilutions on the filters were so low (1:100 or 1:200).

Stock solutions of 1:10 were prepared with PBS / EtOH and sonicated for 20 sec on ice (Bandelin sonopuls GM200 at 20 % intensity and cycle 20). Subsequent final dilutions were 1:100 or 1:200 with PBS. 1 ml was filtered on a 2 µm Nucleopore filter (Whatman) supported by a GF / F carrier filter (Whatman), applying a weak vacuum. A solution of 0.1 % low-melt agarose in 50 ml H<sub>2</sub>O<sub>UP</sub> was sprayed on the filters to ensure adherence of the sediment and to avoid sticking of filters to the lid of the petri dish. The given concentration of agarose is sufficiently low to allow solutions and chemicals to pass.

Total cell counts: The filter was dried on Whatman filter paper one section (about 1/8) was cut with a sterile scalpel and stained. For that, the piece was placed face-up on 2.5 µl SybrGreen I (1:10) in 97.5 µl H<sub>2</sub>O<sub>UP</sub> and incubated in the dark for 15 min. After 10 min washing in H<sub>2</sub>O<sub>UP</sub> and drying, the filter was embedded in a drop of the antifadent Citifluor™ AF87 on a glass slide and covered with a cover slip. Cell counts were done at 100x magnification using the L5 filter cube (Appendix IV). Cells were counted either immediately or after 1-2 days. In the case of non-immediate counts, slides were stored in the dark at - 20 °C to avoid rapid bleaching of the dye.

Archaeal and eubacterial cell counts: The CAlyzed Reporter Deposition Fluorescence In-Situ Hybridisation was performed following a modified protocol of Pernthaler (A. Pernthaler, J. Pernthaler & Amann, 2002).

Probes used for Eubacteria were biotin-labelled Eub338 I-III oligonucleotides (R. I. Amann et al. 1990; H. Daims et al. 1999). To cover all Eubacteria, the three probes were mixed 1:1:1. Probes used for Archaea were biotin-labelled Arch915 oligonucleotides (Ishii et al. 2004).

The filter was immersed in Methanol / 0.015 %  $H_2O_2$  (30 %) for 30 min to inactivate endogenous peroxidases, then washed in  $H_2O_{UP}$  and EtOH and dried. Permeabilisation of the cells was chosen depending on the probe used later on: For the Eub338 probes, filters were kept rotating in a 5 mg/ml lysozyme solution in lysozyme buffer at 37 °C for 60 min. For Arch probes, additional 30 min incubation in 60 U/ml achromopeptidase solution in achromopeptidase buffer was done at the same temperature. Arch filters had to be washed thoroughly with  $H_2O_{UP}$  afterwards. Hybridisation buffer containing the appropriate percentage of formamide (35 % for Eub338 and Arch915 probes, (Raskin et al. 1994; Glöckner et al. 1999)) was prepared and mixed 300:1 with probe. Hybridisation time was 2 h at 46 °C. After washing in 2x SSC buffer, filters were put face-down on a drop of blocking reagent in PBS for 30 min, then in streptavidin-HRP in blocking reagent (3:297) for another 30 min at room temperature. After washing in PBS or cooled storage in PBS over night, the fluorescently-labelled tyramide (Alexa 546, 488 or 555) was applied: filters were incubated for 15 min at 46 °C in 300 parts of amplification buffer containing 1 part 30 %  $H_2O_2$  and 3 parts of the respective dye, then washed in PBS and shortly in  $H_2O_{UP}$  and EtOH before being stained with SybrGreen or 4',6-diamidino-2-phenylindole (DAPI).

A special test was conducted to compare the hybridisation success of cells in sediment to that of pure cultures. For this test, three filters were prepared: On the first filter 0.5 ml of a milky-white liquid of a pure sulphate reducer culture (*Desulfobulbus mediterraneus*) were filtered. The second filter contained a mixture of pure culture and whale enriched sediment (Whale+WF2, 1:100) taken at the end of the experiment. The third filter contained only the whale enriched sediment (1:100). These three filters were treated for CARD-FISH as described above, using the probes Eub338 I-III. Dyeing was done with the tyramide Alexa 555 instead of Alexa 546, but the chromatic filter used to view both was the same (Y3).

DAPI (Porter & Feig 1980) or SybrGreen I (Noble & Fuhrman 1998) was used to discriminate between unspecific and specific binding of the CARD-FISH probes. Both stainings were done in the dark to avoid bleaching. Samples with an Alexa 488 stain had to be counterstained with DAPI since SybrGreen I and Alexa 488 signals were viewed using the same microscope filter. DAPI filters were kept face down in a drop of 1mg/l DAPI for 15 min, then washed in  $H_2O_{UP}$  and for 1 min in EtOH, dried and embedded in Citifluor. For SybrGreen staining details, see above ("total cell counts").

Microscopic analysis and counting were performed on an epifluorescence microscope (Leitz Aristoplan) equipped with the adequate filter sets for the dyes (Y3 for Alexa 546 and 555, L5 for Alexa 488 and Sybr Green I, A4 for DAPI; see Appendix IV). Pictures were taken with a Leica DFC 420 camera and processed using Image Access easyLab (Version 8, Leica). The area counted per grid at 100x magnification was 0.0121 mm<sup>2</sup>. For total cell numbers, at least 1000 cells were counted. For eubacterial and archaeal counts, 20 grids were enumerated.

Statistical analyses to determine normal distribution, to visualise the counts in boxplots, and to compare means of counts were performed using the software "R" (version 2.14.0., The R Foundation for Statistical Computing).

#### Elemental analysis of sediments

Sediments samples that were frozen at the end of the enrichment experiment at -20 °C in 15 ml Falcon tubes, were used for CNS/C<sub>org</sub> analysis. Elemental analysis of initial sediments (i.e., WF, CS, Ref and Ebay before enrichment) was done on 4ml of each frozen in cryovials at - 80°C. Two ml of each sediment were weighed in medium-size weighing dishes. The dishes were dried for 16 h at 58 °C and weighed again. Sediments were then ground with an agate mortar and pestle.

For CNS measurement, 7-10 mg of ground sediment were weighed into a tin cartridge (HEKAtech) using a Sartorius micro scale. Standards were 0.3-1.2 mg 2,5-Bis(5-tert-butyl-2-benzo-oxal-2-yl)thiophen (BBOT) for sulphur and 1-5 mg Bodenstandard Nr.1 (3.500 %C, 0.216 %N) for carbon and nitrogen (both HEKAtech). Vanadium pentoxide was added for complete oxidation of sulphur compounds and samples were measured in a NA1500 Series 2 Elemental Analyzer (Carlo Erba Instruments). The column used for CNS measurements contained chromium oxide and, to ensure complete oxidation, silver coated cobalt oxide was placed in the combustion tube and the temperature was 1050 °C. A second tube contained metallic copper at 650 °C.

For C<sub>org</sub> measurement, silver cartridges (IVA Analysetechnik, Meerbusch) were used and ca. 8 mg of sample was weighed out. Standards were 0.2 - 1.5 mg acetanilide (71.09 %C) and 1 - 5 mg of Bodenstandard. To remove carbonic carbon, first 60 µl H<sub>2</sub>O<sub>UP</sub> and then 60 µl of 25N HCl were added. Only one combustion tube containing Al<sub>2</sub>O<sub>3</sub> and WO<sub>3</sub> as oxidation catalyst was employed and copper wire was placed in the tube.

Data were plotted in boxplots using the software "R".

## 2.3. Incubation of whale and ostrich bones on sediments

### 2.3.1. Aquaria set-up

Vertebra bones of Harbor Porpoise (*Phocoena phocoena*) and vertebrae and one large femur of Southern Ostrich (*Struthio camelus australis*) were incubated on two different sediments.

The sediments used were North Alex mud volcano from the Mediterranean (NAMV) and Eckernförde Bay from the western Baltic Sea (Ebay) (see 2.1).

Three ca. 24 l aquaria (2 Ebay, 1 NAMV) were filled with sediment to a height of about 10 cm and covered to the outlet with unfiltered water from Kiel fjord from a depth of about 3 m. To prevent the bones from getting covered by sediment over time, a fourth tank was installed as sediment trap. From there a steady supply of about 250 ml/min was maintained to each aquarium throughout the experiment. Large black plastic bags kept the installation in the dark. The room was kept at a constant temperature of 5 °C.

The juvenile harbour porpoise had recently been stranded and frozen, the ostrich recently slaughtered, so both types of bone were considered fresh. The bones were defleshed as thoroughly as possible with a knife but the periosteum could not be removed completely. Ostrich vertebrae were sawed in half because of the much larger size and smaller available number.

Parallel rows of five vertebrae of both types were incubated in two aquaria (Ebay and NAMV). The vertebrae were placed about halfway in the sediment, with at least one long projection protruding into the water. The ostrich femur was sawed in two below the head and incubated on Ebay sediment in the third aquarium.

### 2.3.2. Sampling

One whale vertebra and half an ostrich vertebra were taken every month for the duration of the incubation. The parts that had been above and under the sediment were marked and a small sample of flat and undamaged bone was taken from either and fixed further for CARD-FISH analysis: The bone pieces were incubated in 4 % formaldehyde/sterile phosphate buffer saline (PBS) at 4 °C for 16 h to ensure complete fixation. The liquid was replaced twice by PBS for 1-2 h and finally stored at -20°C in 1:1 PBS/Ethanol. The remnants of the vertebrae were frozen at -80 °C for microscopic analysis of bioerosion.

At the end of the experiment three sediment samples were taken from depths of 0-0.5, 3-4 and 6-7 cm from the femur tank with a cut-off 3 ml Braun syringe. The sediment was diluted 1:200 in PBS, filtered and stained with DAPI like CARD-FISH samples (see 2.2.4).

### 2.3.3. Thin sections of bones

A Leica CM1100 cryomicrotome was used to produce thin sections of the bones taken from the incubation experiment. After fixation for CARD-FISH analysis as described above, bones were embedded in SCAM (Super cryoembedding medium, Japan) and fixed on the sample plate. To ensure complete hardening of the embedding fluid, samples were either stored at -20 °C inside the cryomicrotome or kept at -80 °C for several hours. If the final height of the sample was too large for the first cut to go through, the top section of the bone was cut with a utility knife in order not to exert so much pressure on the sample as to loosen it from the plate. Thickness of sections was set to 5 µm. Sections were fixed on tape and stored face-down on an object slide at -80 °C until further processing.

Transmission light microscopy was done on a Zeiss Axioskop equipped with a Zeiss AxioCam ICc1 and the software AxioVision (V.4.8.0.0.).

SybrGreen staining was done as described in section 2.2.4.

One thin-section of Ebay incubated whale vertebra (five months incubation time) was used for CARD-FISH. Eub338 I-III probe and DAPI were applied. The same protocol as for sediment samples was used and the whole procedure was performed on the object slide directly. Due to the high permeabilisation and hybridisation temperatures and the small amounts of liquids used, special care had to be taken to avoid rapid evaporation. The slide was processed in a small plastic slide storage box with wet paper towels on the bottom to saturate the air inside. The same amounts of chemicals were used as with sediment samples but the slide was checked frequently during incubation and solutions were replenished if necessary. Washing steps were done with the respective washing liquid in excess to ensure that no previous solution was trapped under the tape.

### 2.3.4. Scanning electron microscopy sample preparation

Pieces of incubated bone frozen at -80 °C were cut to a size that fit the sample plate. Care was taken that mainly flat, not artificially damaged, exposed bone surface be visible afterwards. Samples were fixed in 2 % formalin / 0,04 % glutaraldehyd / PBS for 2 h. An ascending ethanol series with ethanol percentages of 15, 30, 50, 70, 90, 99 and 100 was prepared in 2 ml Eppendorf tubes and the samples were immersed in each for at least 20 min. Denatured as well as undenatured ethanol was used and the 99 %- and 100 %-steps were done twice. Afterwards the sample was immersed in a silazane for 20 min, the liquid was taken off and the caps were left in a fume hood to dry overnight.

Cryosections of 5 µm thickness were glued from the tape directly to the stronger SEM sample plate adhesive. They were treated in the same fashion but due to the smaller volume of the sample, the steps were about 5 min each.

## **2.4. Analyses of the whale vertebra from the Chilean whale fall**

One large whale vertebra was retrieved by a remotely operated vehicle (ROV) at the same station as the whale fall sediment used in the enrichment experiments and frozen at -80 °C

### **2.4.1. Sulphate reduction rates in whale vertebrae**

The vertebra was subsampled for sulphate reduction determination on board: With an electric hand-held saw blocks of about 2x1x1 cm<sup>3</sup> were sawed out covering depth horizons of 0-2 and 2-4 cm below bone surface. Five replicates of each depth were incubated in 10 ml crimped glass vials with anoxic, sterile filtered seawater. 15 µl of the radio-tracer <sup>35</sup>SO<sub>4</sub><sup>2-</sup> (activity 200 kBq) were injected into the vial and incubation time was 206 hours. Three blanks contained only seawater and tracer. After incubation the bone pieces were removed and cold chromium distillation was performed using the method described by Kallmeyer et al (2004). Rates in nmol / g wet weight bone / day were calculated by including the bones' wet weight and volume.

### **2.4.2. Elemental analysis of the vertebra**

The bone pieces used in the sulphate reduction determination (2.4.1) were saved and later on ground in an agate mortar for C/N/S and C<sub>org</sub> determination. The bone powder was treated in the same way as the ground enrichment sediments for both analyses (see section 2.2.4).

### 2.4.3. Age determination

Bone pieces from the vertebra surface were prepared for SEM microscopy (see 2.3.4) to attempt age determination by the degree of bioerosion.

A novel method of post mortem interval (PMI) determination in bones was applied first by Henry Schwarcz and coworkers (Schwarcz, Agur & Jantz 2010). By measuring the citrate content of ground bone, the PMI could be calculated with an accuracy of about 1 % of the age for time spans below 100 years in their study.

Four pieces of approximately 1x1x2 cm<sup>3</sup> were sawed off the whale vertebra stored at -80 °C. To determine the initial citrate value of a whale bone, one vertebra of the same harbour porpoise used for the incubation experiments was defleshed as thoroughly as possible and two samples were sawed out of it. To defat the samples they were incubated in a 1:1 mixture of Chloroform and Ethanol in 20 ml Scintivials for approximately 1.5 h. The liquid was discarded and the samples dried in a fume hood over the weekend. They were thoroughly ground with an agate mortar and pestle and weighed in 50 mg or 100 mg aliquots into 50 ml Falcon tubes. 2 ml of 1 M HCl was added and the tubes were placed in a waterbath held at 60 °C for 1 h to liberate the citrate into the liquid phase. Then, pH was adjusted to 5 by dropwise addition of 0.5 M KOH (approximately 100 drops necessary per sample) and the tubes were centrifuged at 1200 g for 5 min in order to form a collagen pellet. The supernatant containing the citrate was decanted into 15 ml Falcon tubes and stored in the fridge at 4 °C overnight. The porpoise samples had to be centrifuged twice because the remains of meat did not form a sufficiently solid pellet.

An EnzyPlus Kit (Xygen Diagnostics) was employed for citrate determination according to the instructions.

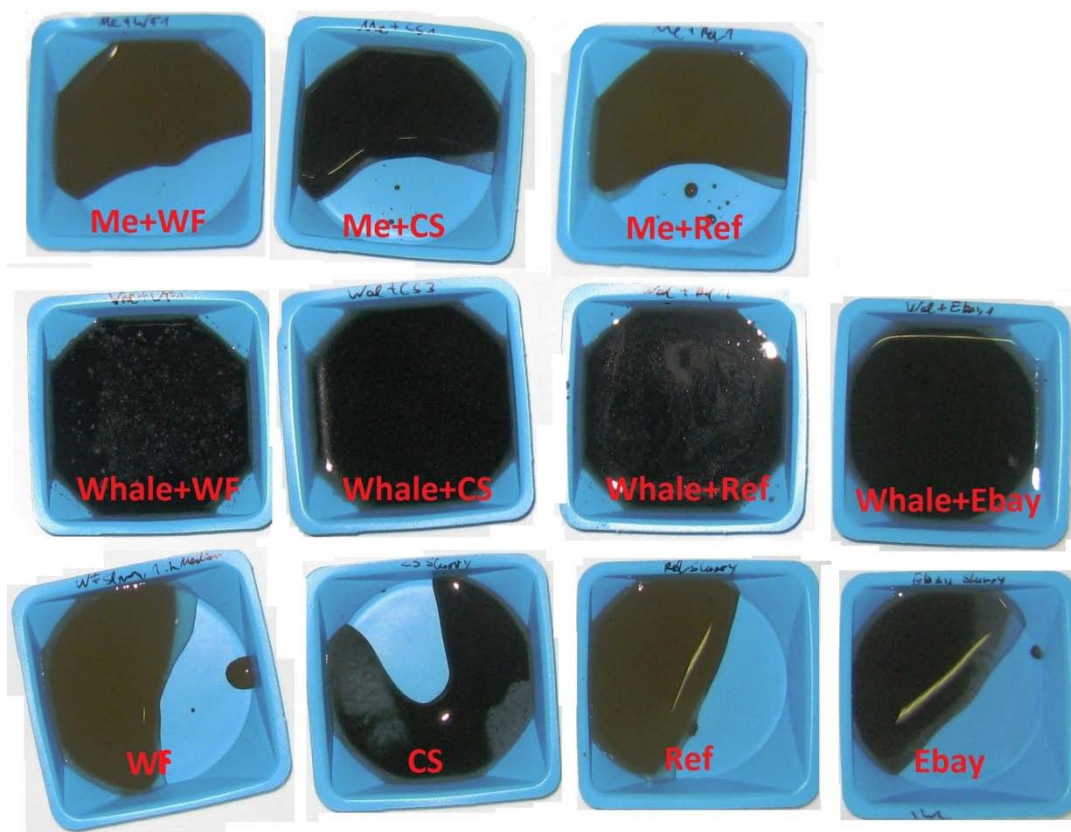
Post mortem intervals were calculated using Schwarcz' equation:

$$C(t) = -0.673 \log t + 3.021$$

where C is citrate content in weight % and t is time in days.

### 3. Results

#### 3.1. Enrichment experiments



**Figure 2.** 2 ml of a representative replicate of the enriched sediments after 191 days (top two rows) and before incubation (bottom row). Me= methane enriched, Whale=whale biomass enriched, WF=whale fall, CS=cold seep, Ref= reference, Ebay=Eckernförde Bay sediment. Notice the change in appearance in whale enriched WF and Ref sediments and the oily film covering all whale enriched sediments.

Sediments that were methane enriched did not differ in appearance from the initial ones (Figure 2). CS sediment was black from the beginning and WF and Ref sediments did not turn to black during the incubation. Whale enriched sediments, however, were all black in the end and the 2 ml poured into the weighing dishes spread over the whole bottom of the dish due to the oil, contrary to methane enriched and initial ones. A distinct film of oil is visible on all whale enriched sediments, which will become important later on (see Figure 11).

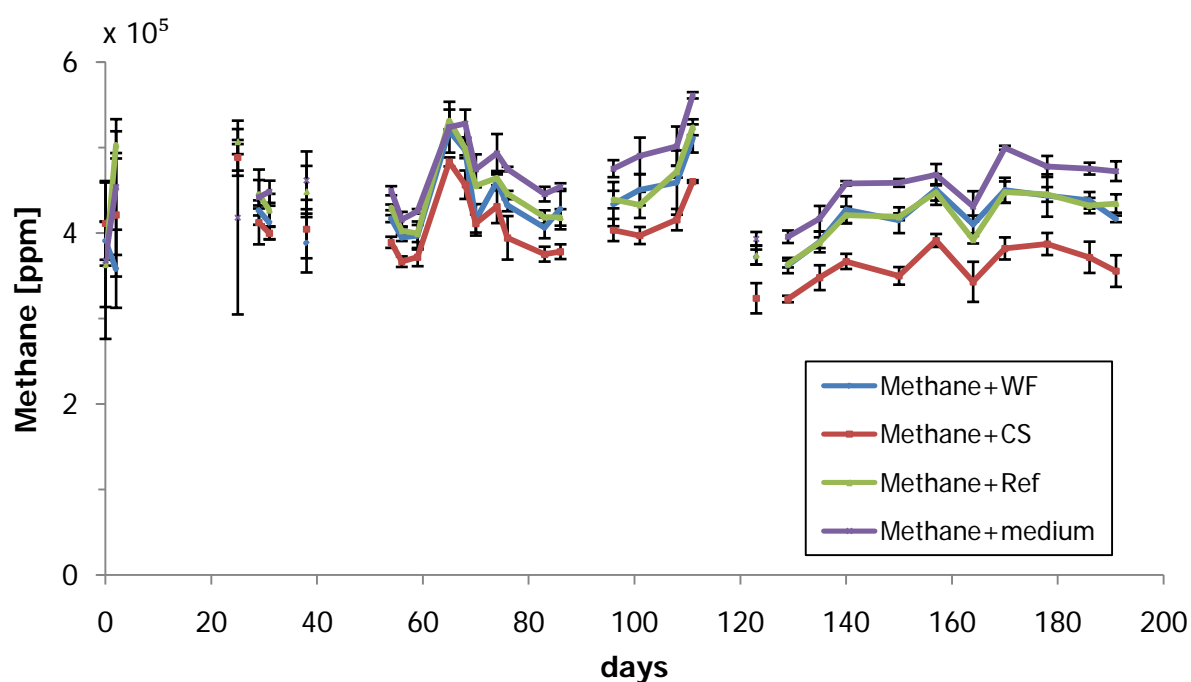
### 3.1.1. Sulphide and methane development

#### Methane enrichments

##### Methane development

Methane measurements yielded very unstable values in the first ca. 50 days. The values paralleled, however, regardless of the sediment type. Technical or measurement issues are therefore likely to have caused this inconsistency. Far outlying data points were removed from analysis.

Although the order of the four sediment types remains the same over the whole experiment with cold seep sediment (CS) featuring the lowest concentrations of methane and the medium controls the highest, no clear upward or downward trend can be observed. Whale fall (WF) and reference (Ref) sediment roughly show equal methane concentrations (Figure 3).

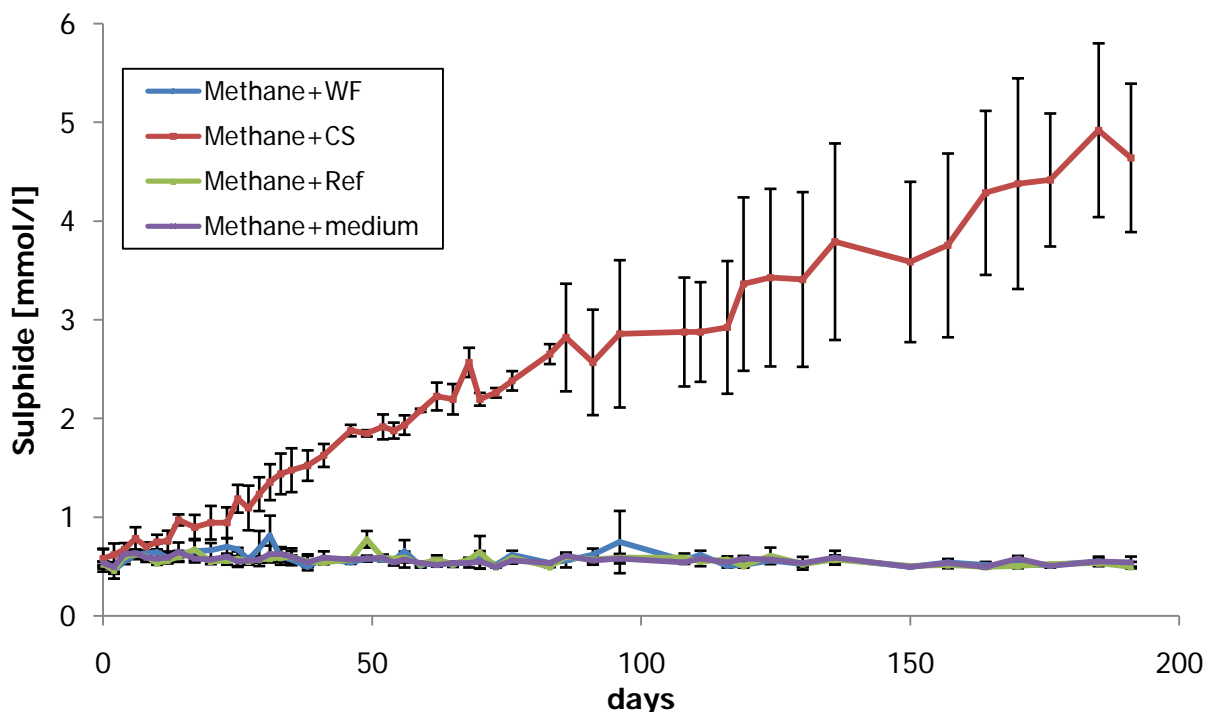


**Figure 3.** Methane concentrations in methane enriched sediments. WF = whale fall, CS = cold seep, Ref = reference, Ebay = Eckernförde Bay sediment. Outlying measurements were removed from the plot.

##### Sulphide development

Sulphide in the methane enrichments was roughly at the initial concentration of 0.5 mM throughout the experiment in WF and Ref vials and in medium controls. 0.5 mM is the lower detection limit of this method. In addition, sulfide was added to the medium to ensure

anoxic conditions. On CS sediment, however, a slow but steady increase in sulphide concentration at  $0.3 \pm 0.007 \mu\text{mol}/\text{cm}^3/\text{d}$  was observed, peaking at 5 mM after 185 days (Figure 4).



**Figure 4.** Sulphide concentrations in methane enriched sediments. WF = whale fall, CS = cold seep, Ref = reference, Ebay = Eckernförde Bay sediment.

### Whale enrichments

Methane and sulphide developed very strongly in the whale biomass enriched vials.

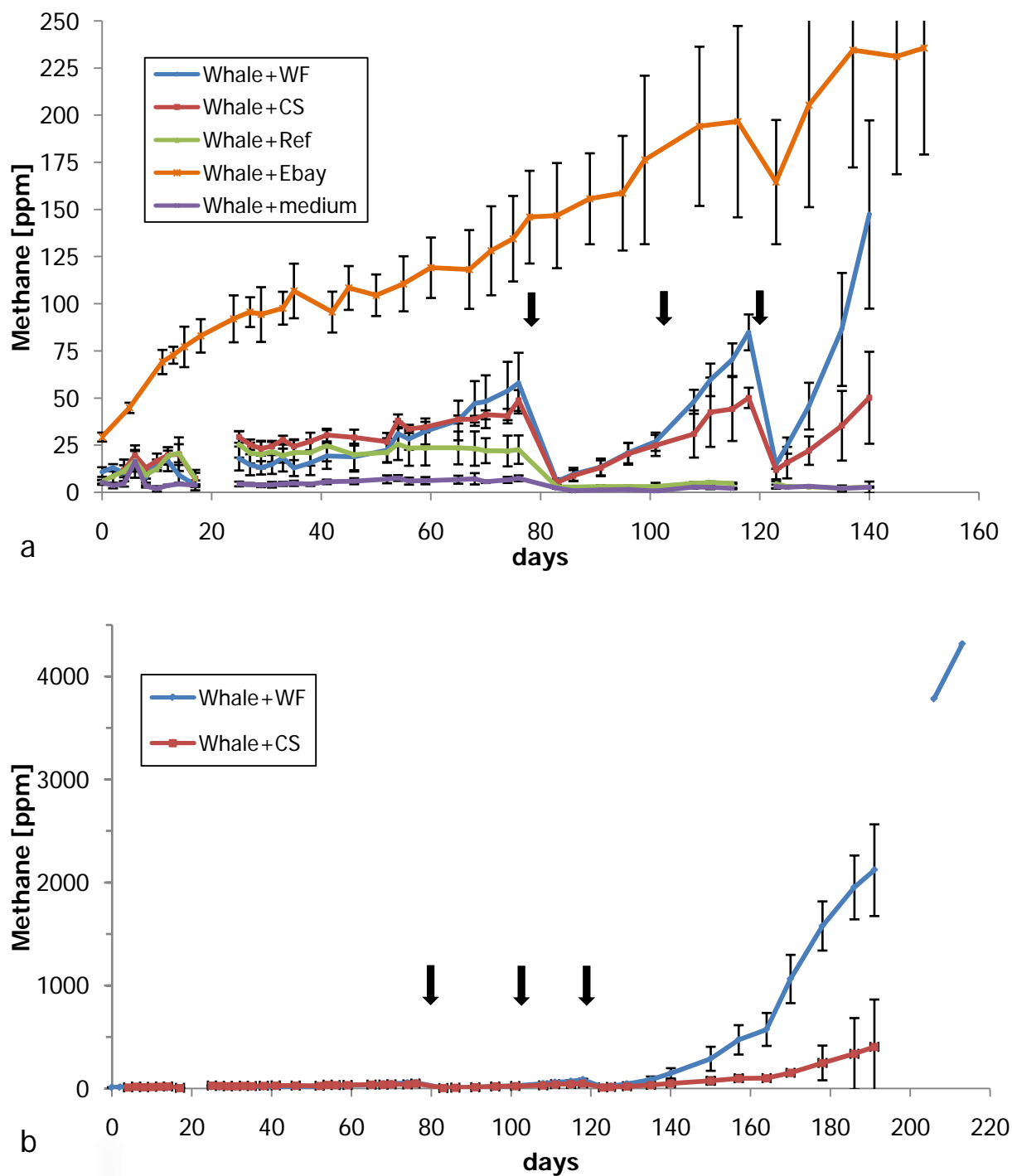
#### Methane development

Ebay sediment showed an increase of methane of  $0.9 \pm 0.2 \mu\text{mol}/\text{cm}^3/\text{d}$  right from the beginning and sloped off after approximately 24 days to only  $0.4 \pm 0.1 \mu\text{mol}/\text{cm}^3/\text{d}$  (Figure 5a).

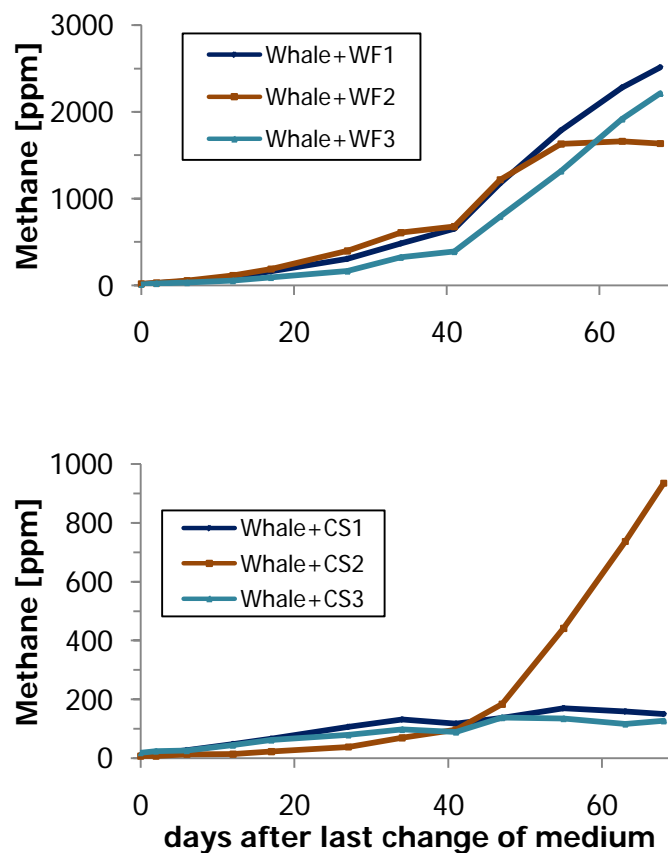
After 35 days methane concentrations rose at  $0.2 \pm 0.05 \mu\text{mol}/\text{cm}^3/\text{d}$  to 25 ppm in vials with Ref sediment and roughly twice that in WF and CS vials ( $0.5 \pm 0.1$  and  $0.3 \pm 0.1 \mu\text{mol}/\text{cm}^3/\text{d}$ , respectively) with WF showing a steeper increase and slightly higher end concentration. In Ref concentrations stagnated at the final value of ca. 25 ppm from day 25 on. After 65 days WF sediment outperformed CS sediment and featured the steepest increase in methane production. Medium controls showed a very slight but steady increase in concentration up to about 7 ppm.

After the first change of medium, Ref vials and medium controls remained at very low methane concentrations (below 4 ppm) for the remaining duration of the experiment. WF and CS vials, however, exhibited steep increases in methane concentrations with almost identical values between days 83 and 101. Rates were  $0.8 \pm 0.1 \mu\text{mol}/\text{cm}^3/\text{d}$  for WF and  $0.4 \pm 0.1 \mu\text{mol}/\text{cm}^3/\text{d}$  for CS. The second change of medium remained invisible in the methane measurements since vials were not flushed with  $\text{N}_2/\text{CO}_2$ , so headspace methane concentration remained the same. After that, WF sediment clearly showed a stronger increase ( $4.2 \pm 1.4 \mu\text{mol}/\text{cm}^3/\text{d}$ ) than CS sediment ( $0.9 \pm 0.2 \mu\text{mol}/\text{cm}^3/\text{d}$ ); it has to be noted, however, that the diagrams depict the average of the three respective vials and the standard deviation of the replicates. Looking at methane concentrations after the first change of medium, Whale+CS vials 1 and 3 produced more methane than Whale+WF vial 2 but the average of the Whale+WF values is still higher. Until the next change of medium after 120 days methane concentration of WF vials end up at ca. 85 ppm compared to CS vials at 50 ppm. After that the differences were even more pronounced: WF sediment produced methane at an average rate of  $19.8 \mu\text{mol}/\text{cm}^3/\text{d}$  and continued to do so for the remainder of the experiment. The replicate Whale+WF3 that was running for another 22 days even featured a final rate of  $27.5 \mu\text{mol}/\text{cm}^3/\text{d}$ .

CS however only display an average rate of  $4.1 \mu\text{mol}/\text{cm}^3/\text{day}$ . Thus, WF sediments reached an end concentration of almost 2200 ppm (4300 ppm in Whale+WF3) whereas CS reached just above 400 ppm (Figure 5b). Notably, discrepancy between the three replicates of each sediment type was very high (Figure 6): In WF the concentrations of each vial were similar to each other, with only WF 3 stagnating 55 days after the last medium change and later even declining, possibly due to a perforated rubber stopper. In CS the two replicates CS 1 and CS 3 showed very similar, albeit low methane concentrations between 89 and 169 ppm with hardly an increase over time ( $0.4\text{-}0.2 \mu\text{mol}/\text{cm}^3/\text{d}$ ). Replicate CS 2 exhibited a very steep increase in methane concentration from day 164 on (41 days after last medium change) and production took place at  $11.6 \mu\text{mol}/\text{cm}^3/\text{d}$  until it reached a final concentration of 935 ppm by the end of the experiment.

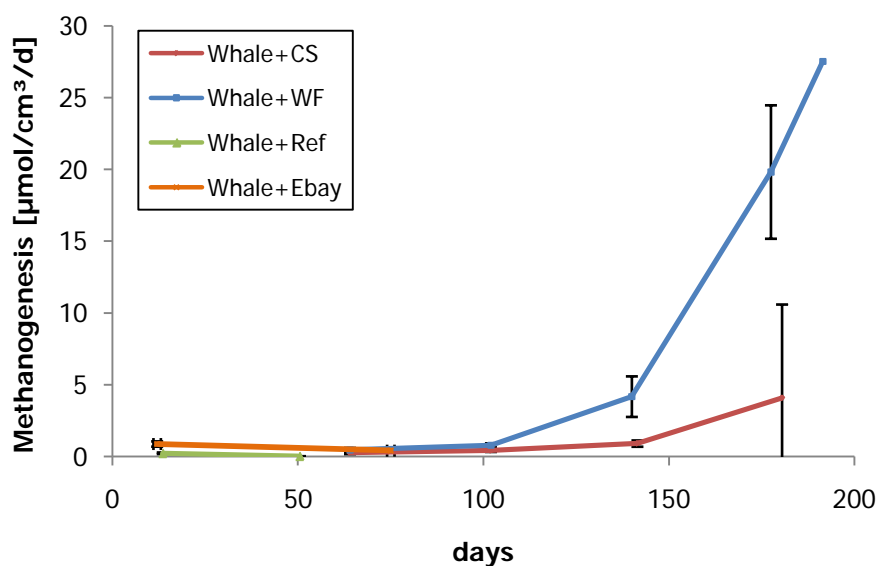


**Figure 5.** Methane concentrations in whale biomass enriched sediments. WF = whale fall, CS = cold seep, Ref = reference, Ebay = Eckernförde Bay sediment. Black arrows indicate medium changes (do not apply for Ebay sediment vials). a) First 150 days of incubation b) WF and CS plotted on a larger y-axis beyond day 150. Notice that measurements for WF after day 200 are only of one replicate.



**Figure 6.** Discrepancies between methane concentrations in whale biomass enriched whale fall (WF) and cold seep (CS) replicates during the last 68 days of the experiment. Notice the different scaling on the y-axes.

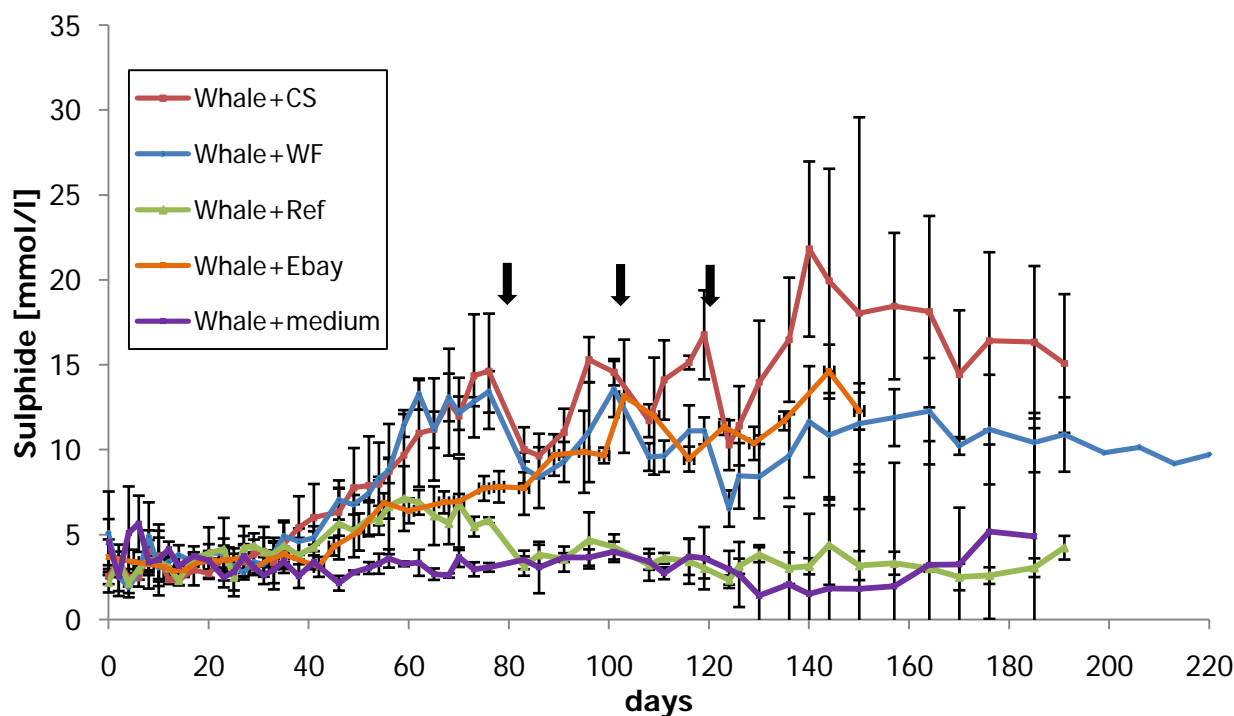
For WF and CS sediments enriched with whale biomass clear upward trends of methanogenesis were observed over time. WF methane production increases from 0.5 to 27.5  $\mu\text{mol}/\text{cm}^3/\text{d}$ . CS increases from 0.3 to 4.1  $\mu\text{mol}/\text{cm}^3/\text{d}$ . Ref sediment rates even decrease from 0.2 to virtually 0  $\mu\text{mol}/\text{cm}^3/\text{day}$  (Figure 7).



**Figure 7.** Rates of methanogenesis in whale biomass enriched sediments. WF = whale fall, CS = cold seep, Ref = reference, Ebay= Eckernförde Bay sediment. Last rate of WF calculated with only one replicate.

### Sulphide development

In the beginning of the experiment the medium in the whale enriched vials was darkened by organic particles and the measured absorptions were at times variable and unreliable. After about 14 days the minor particles floating in the medium were mostly gone and reliable measurements were possible, although occasional outliers occurred. One possibility to deal with this distortion would be to subtract a fixed, average value estimated from the first measurements from all absorption values. However, that would mask the fact that later on in the experiment the supernatant was quite clear and absorptions measured accurately mirror sulphide concentrations at the time. Subtracting this value from only the first measurements does not take into account the fact that the disappearance of organic particles in the supernatant was a continuous process and no sharp change was observed. This method would then overestimate the suddenness of sulphide production onset. Thus, values were plotted as measured: Sulphide concentrations depicted in the diagrams in the beginning are higher than expected but calculated sulphide generation rates are unaffected by this inaccuracy.



**Figure 8.** Sulphide concentrations in whale biomass enriched sediments. WF = whale fall, CS = cold seep, Ref = reference, Ebay = Eckernförde Bay sediment. Black arrows indicate medium changes (do not apply for Ebay sediment vials).

In sulphide concentrations the three changes of medium in the sediments WF, CS and Ref are clearly visible (arrows in Figure 8). Medium was not changed in Ebay vials. As vials had to rest for two days after a medium renewal and the sediments continued to produce sulphide during that time, it was impossible to get sulphide concentrations below 10 mmol/l on the next measuring day. Adding to that, not all medium could be exchanged in order to avoid sucking out sediment or whale biomass, so there was always a remainder of sulphide enriched medium.

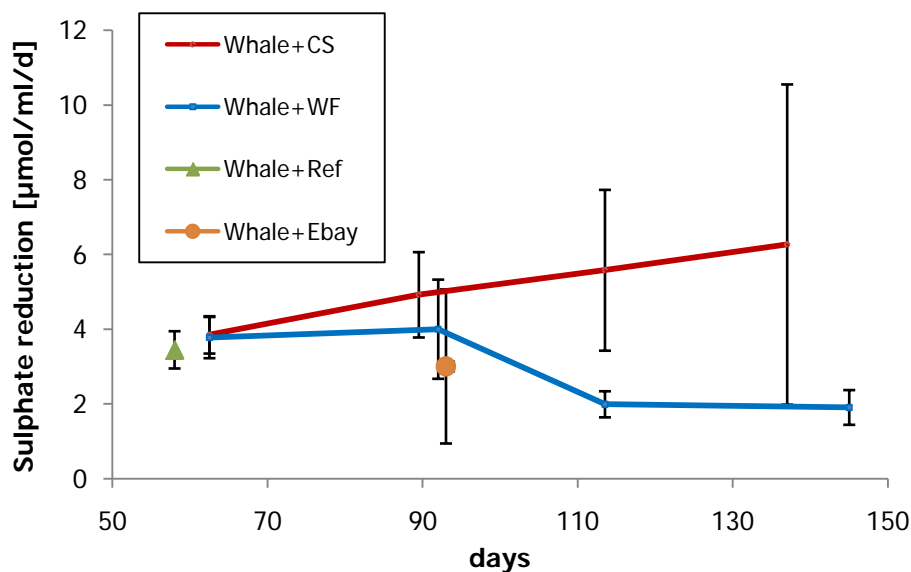
Sulphide concentrations started to rise constantly after a lag phase of about 35 days in WF and CS sediments and about one week later in Ebay and Ref sediment (Figure 8). WF and CS sediments showed very similar rates of sulphide production ( $3.8 \pm 0.6$  and  $3.9 \pm 0.5 \mu\text{mol}/\text{cm}^3/\text{d}$ , respectively) and reached levels of between 10 and 17 mmol/l right before the first change of medium with the CS average being slightly higher. Ebay sediment showed a constant but weaker increase of  $3.0 \pm 2.1 \mu\text{mol}/\text{cm}^3/\text{d}$ .

Ref sediment sulphide increase was shorter than in WF and CS sediment, with occasional peaks but an overall rate of  $3.5 \pm 0.5 \mu\text{mol}/\text{cm}^3/\text{d}$ . The peak of the average concentrations (6.7 mmol/l) was reached 15 days before the first medium change, a slight decline was observed afterwards.

CS sediment continued to produce sulphide at a higher rate than WF sediment in between the following medium changes (Figure 9). WF production even decreased from  $4.0 \pm 1.3$  to  $1.9 \pm 0.5 \mu\text{mol}/\text{cm}^3/\text{d}$ , whereas CS production increased from  $4.9 \pm 1.1$  to  $6.3 \pm 4.3 \mu\text{mol}/\text{cm}^3/\text{d}$  after medium changes. From day 126 on, no fresh medium was added. Peak average sulphide concentrations of 22 mmol/l in CS with single vial peaks as high as 27 mmol/l were observed. In WF average peak concentration was 12 mmol/l with single vials at 15 mmol/l. From around day 150 on a decrease in concentrations in WF and CS vials was observed, very likely due to perforated rubber stoppers. Although over-pressure was still noticeable during sampling it is likely that after 22 measuring days (26 for Whale+WF3 vial) some sulphide escaped.

Ref sediment did not seem to continue sulphide production after the first medium renewal. Concentrations stayed low between 2.3 and 4.7 mmol/l and no significant increases were visible.

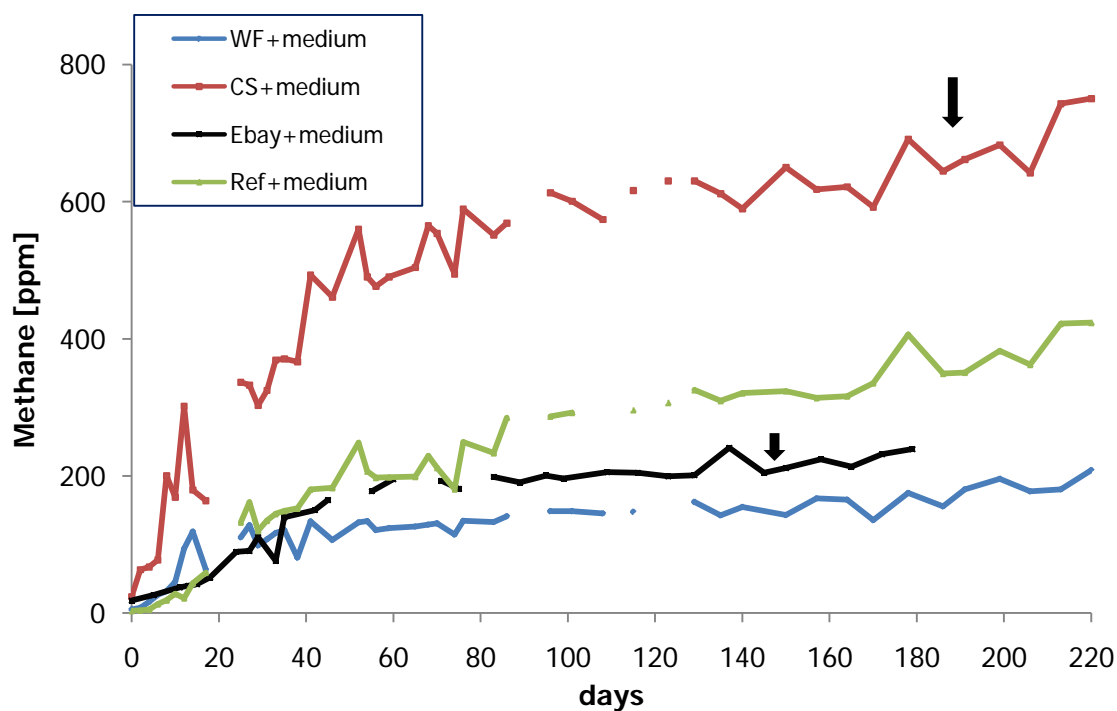
Medium controls stayed at a level of 1.5 to 4 mmol/l for the entire duration of the experiment. As mentioned above, photometric measurements might be skewed by floating particles and oil droplets at these low concentrations. However, contamination seems to have taken place around day 160, possibly by a needle used on one of the other sediment types before. From this time on, sulphide concentrations in the medium control vials rose constantly, first in replicate 1 (vial 25) and then in the other two replicates, which followed with a lag. This is again possibly due to cross-contamination as the same needle was used to sample all three replicates of a kind and the order of measuring was always the same. Towards the end, sulphide concentration in vial 25 reached up to 16.5 mmol/l and a rate that would be equivalent to  $1.9 \mu\text{mol}/\text{cm}^3/\text{d}$  if the vial had contained sediment as the others did. Any sulphide concentrations in medium controls higher than 5 mmol/l were excluded from the plotted data.



**Figure 9.** Rates of sulphate reduction in whale biomass enriched sediments. WF = whale fall, CS = cold seep, Ref = reference, Ebay= Eckernförde Bay sediment.

### Sediment controls

All four sediment types (WF, CS, Ebay and Ref) exhibited steady methane production (Figure 10) but no sulphide production at all (data not shown), so medium was never changed. On September 15 (after 188 days for WF, CS and Ref and after 147 days for Ebay) 1.5 ml of methanol was added to the vials to achieve a final concentration of 10 mM.



**Figure 10.** Methane concentrations in sediment control vials (sediment+medium only). WF = whale fall, CS = cold seep, Ref = reference, Ebay = Eckernförde Bay sediment. The small black arrow indicates time of methanol addition for Ebay, the large black arrow for the remaining sediments.

Unlike the whale enriched treatments, these sediment controls started producing methane right from the beginning at the highest production rates, then sloped off. CS showed the highest rate ( $3.2 \mu\text{mol}/\text{cm}^3/\text{d}$ ) until day 35, rate afterwards was only  $0.2 \mu\text{mol}/\text{cm}^3/\text{d}$ . The same final production rate could be observed in Ref vials with an initial rate of  $1.5 \mu\text{mol}/\text{cm}^3/\text{d}$ . WF and Ebay rates of  $1.1 \mu\text{mol}/\text{cm}^3/\text{d}$  seemed to slope off 3 - 5 days earlier.

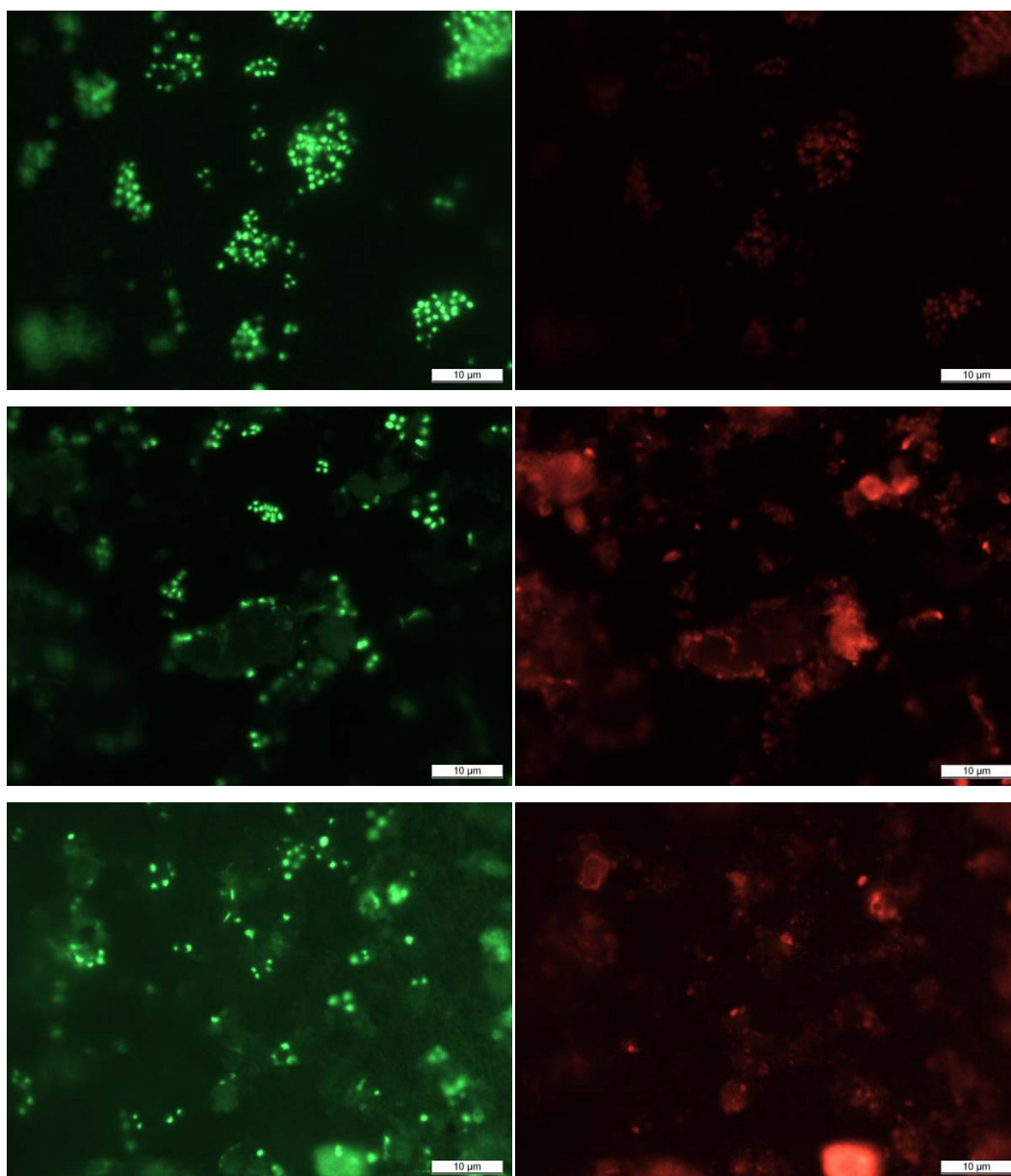
Much lower rates were observed in all four sediments after the initial phase and they did not change until the end of the experiment. Addition of methanol (black arrows in Figure 10) did not lead to a visible change of methane production rate.

### 3.1.2. CARD-FISH

Both DAPI and SybrGreen staining were tested to determine total cell numbers and to check whether a signal of a potential archaeal or eubacterial cell was really a cell or not. DAPI was found to be much more unspecific in its binding. Especially in samples with much and coarse sediment, the cell numbers counted were arbitrary. Sediment grains showed a very strong fluorescence and smaller particles that were about the size of cells were often stained as well. The contrast between background and particles was often not sufficient to distinguish single small cells. SybrGreen staining produced a much more reliable overall picture. Background fluorescence was much lower and sediment grains were rarely stained. Smaller, possibly organic, particles differed clearly from actual cells in the intensity of fluorescence and could hence easily be identified. The SybrGreen stain, however, vanished much faster than DAPI stains and counting had to be done quickly to avoid bleaching to the point where cells became indistinguishable from the background. SybrGreen was favoured and used to determine total cell numbers and check against archaeal and eubacterial signals.

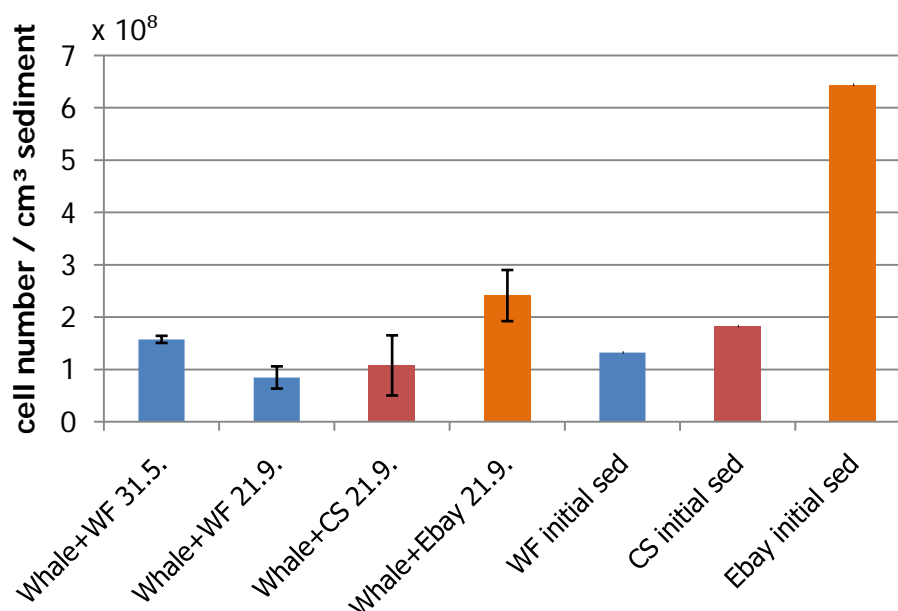
CARD-FISH of whale enriched sediments was unsuccessful despite various modifications of the protocol. Different methods for inactivation of endogenous peroxidases were tested (pure MeOH or MeOH with 0.015 %  $\text{H}_2\text{O}_2$ ), the amounts of streptavidin-HRP, probe and dye were increased and almost all buffers were prepared freshly. Signals were always very vague, if present at all, and often an archeal or eubacterial signal that seemed clearly a cell did not have a SybrGreen counterpart and could not be counted. Similar issues have been reported from the attempts of doing CARD-FISH on *Fucus*-associated bacteria. Probably due to the algae's mucopolysaccharides, no reliable fluorescent signals could be obtained (S. Neulinger, pers. communication).

The test of using a pure liquid culture of sulphate reducing Eubacteria for CARD-FISH revealed a possible reason for this problem. When the culture alone was hybridized with Eub I-III probes clear signals with corresponding SybrGreen signals were visible. When a mixture of culture and whale enriched sediment was used, signals became feeble and many false signals from particles (inorganic or organic) were observed (Figure 11). The direct comparison to the filter containing only sediment supports the assumption that whale enriched sediment impairs some process of the CARD-FISH procedure. Possible reasons are presented in the discussion.



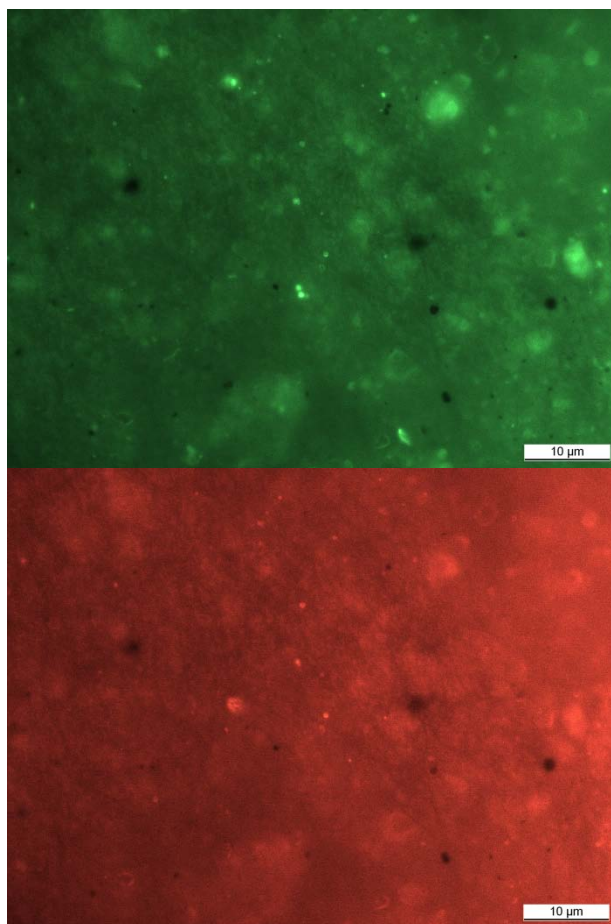
**Figure 11.** Test with SybrGreen stain (left) and Alexa546 dye (Eub338 probe CARD-FISH, (right)). The top row shows a liquid eubacterial culture, middle row a 50/50 mixture of this culture and whale biomass enriched sediment (whale fall, 194 days incubated, 1:100) and the bottom row whale enriched sediment only (1:100). Magnification 100x.

Only total cell numbers obtained by SybrGreen staining could thus be counted for whale enriched sediments and initial sediments (Figure 12). The figures obtained did not mirror the increasing sulphate reducing and methanogenic activity of the respective sediments. Cell numbers in the initial sediments seem to be higher than numbers at the first change of medium (incubation time 81 days) and in the end (191 days) regardless of the sediment origin. Furthermore cell numbers are higher after 81 days than after 194 days in WF sediments although at least methanogenic activity in the respective vials increased strongly. The very high cell count of  $6.5 \times 10^8$  cells/cm<sup>3</sup> in initial Ebay sediment also is a striking contrast to  $2.4 \times 10^8$  cells/cm<sup>3</sup> in Ebay sediment after 153 days of incubation. The only consistent pattern observed is the order of sediments: WF sediment, initial and enriched for 194 days, always features the lowest cell numbers, followed by CS and Ebay.



**Figure 12.** Total cell numbers per cm<sup>3</sup> sediment calculated by SybrGreen counts. 31.5. marks the date of the first change of medium, 21.9. the end of the experiment. WF = whale fall, CS = cold seep, Ebay = Eckernförde Bay sediment.

Due to a lack of time caused by the problems with the whale enriched sediment, only one methane enriched sediment sample could be prepared with CARD-FISH. CS sediment showed sulphide production and possibly a decrease of methane in the methane enrichment experiments. Sediment from the vial Methane+CS1 as well as initial CS sediment were thus filtered for hybridisation. Arch915 and Eub I-III probes were both applied to determine whether the percentages of Archaea or Eubacteria changed during the experiment (Figure 13).



**Figure 13.** Methane enriched cold seep sediment (1:100) stained with SybrGreen (left) and with Alexa 546 (Eub338 I-III probe, right). Magnification 100x.

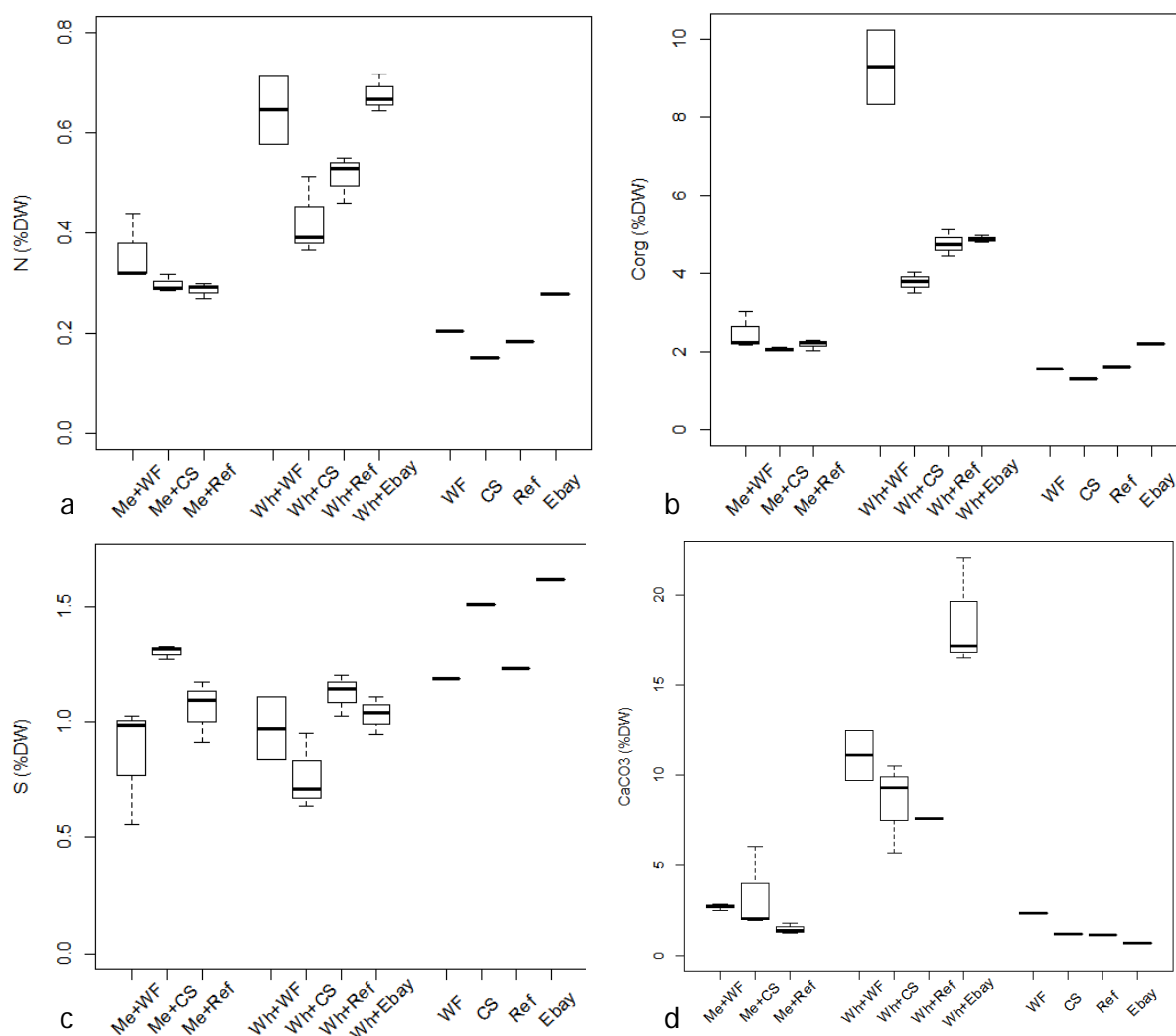
In original CS sediment, a total of  $3.79 \times 10^8$  cells/cm<sup>3</sup> sediment was calculated. Eubacteria made up  $2.59 \times 10^8$  cells/cm<sup>3</sup> and Archaea  $1.77 \times 10^8$  cells/cm<sup>3</sup>. As cell numbers of Archaea and Eubacteria combined make up more cells than calculated from the SybrGreen counts, statistical analysis was employed to test for normal distribution and comparison of means. Both the sum of archaeal and eubacterial counts and SybrGreen counts were normally distributed (tested using Shapiro-Wilk test). A two-sided Student's t-test could therefore be applied and showed that the mean of archaeal and eubacterial counts combined was indeed ( $p=0.96$ ) larger than the mean of SybrGreen counts. From the combined figures, percentages were 40.6 % for Archaea and 59.4 % for Eubacteria.

Methane+CS1 sediment showed a large number of consortia that ranged from cell numbers of 30 up to 180, calculated from the circumference cell number and assuming a spherical shape that seemed to fit for most. These cells were clearly stained with SybrGreen but it was difficult to determine whether they were also stained with Alexa dye. Total cell numbers including the consortia were  $6.47 \times 10^8$  cells/ml original sediment. Archaea made up

$9.15 \times 10^7$  and Eubacteria  $1.92 \times 10^8$  cells/ml. From Archaea and Eubacteria combined, Archaea made up 32.3 % while Eubacteria contributed 67.7 %.

### 3.1.3. Elemental analysis of sediments

The sediments of the enrichment experiment were dried and weighed for elemental analysis. Two measurements of each replicate were done. Since one vial (Whale+WF3) is still being monitored, only two replicates were measured of whale enriched WF sediment (Whale+WF).



**Figure 14.** Nitrogen (a), sulphur (b), organic carbon (c) and calcium carbonate (d) contents (% dry weight) of the sediments used for enrichment experiments (WF=whale fall, CS=cold seep, Ref=reference, Ebay=Eckernförde Bay sediment; Me=methane enriched, Wh= whale biomass enriched)

Nitrogen made up significantly higher percentages in whale enriched sediments (0.37 - 0.72 %) compared to methane enriched ones (0.27 - 0.44 %) for the respective sediment (Figure 14a). Initial sediments featured the lowest N-values (0.15 - 0.28 %). The relative order of the sediments remained the same except for a minor deviation in methane enriched reference sediment.

No consistent pattern was observed for sulphur contents (Figure 14b). Percentages were mostly not significantly different between treatments except for CS sediment where the methane enriched vials contained approximately 0.5 % more sulphur. The original sediments (1.2 - 1.6 %) contained more sulphur than the enriched ones (0.55 - 1.33 %). There was no consistent relative order within the sediments.

Whale enriched sediments contained significantly more organic carbon than original and methane enriched ones (Figure 14c). Sediments were 2- to 4-fold enriched in organic carbon after incubation. Methane treatments were only very slightly enriched. With percentages between 8.3 and 10.2 % whale enriched WF sediment issued the highest values by far. The patterns of nitrogen and organic carbon contents resembled each other closely.

Sediments incubated with whale biomass were significantly enriched in calcium carbonate in the end (Figure 14d). Percentages varied from 5.7 to 12 %, as opposed to the much lower contents in methane enriched (1.3 - 6.5 %) and original sediments (0.7 - 2.4 %). Ebay showed the lowest initial but the highest final content in the whale enrichment. The  $\text{CaCO}_3$  values of the other three sediments in the whale treatment were much closer together.

Except for the diffuse picture in sulphur contents, methane treatment percentages were much more similar between sediment types than those of the whale treatments. Here, either WF, Ebay or both tended to show very high values. Initial sediment percentages mostly lay close together.

## **3.2. Incubation of whale and ostrich bones on sediments**

### **3.2.1. Visual monitoring of the incubations**

Eckernförde Bay (Ebay) and North Alex mud volcano (NAMV) sediments reacted differently to the addition of whale and ostrich vertebrae and the large ostrich femur. One bone each was sampled once a month from both sediment types for five months.

Ebay sediment exhibited white-bluish bacterial mats around the protruding vertebrae already after two weeks. After four weeks the mats were solely white in colour and covered even large open spaces between bones. No obvious differences between mat development around whale and ostrich vertebrae was observed. Very strong sulphidic smell was detected when

the bones were taken out of the sediment. At this time, slimy thread-like webs between the bones and jellyfish-like structures were observed. Although the setup had accidentally been exposed to light for some time these did not seem to have been algae. The whitish-yellow colour of the threads and their adhesion to bones were rather indicative of fungi. The potential fungi mostly disappeared later on, but some threads remained until the end.

By the second month the mats reached their largest expansion but also showed signs of disintegration as mat thickness decreased and single radially connected colonies became visible. This development became evident after three months when the whole sediment was covered but sediment was mostly visible and interconnected colonies prevailed. Sulphide smell was not as pungent during sampling as before. Gradually, mats disappeared until, by the fifth month, only few spiky, crystalline colonies on the remaining bones were present. Sediment appeared black throughout the experiment and never changed to a brown colour.

The Ebay sediment incubated with the large ostrich femur exhibited very similar mats as Ebay incubated with vertebrae. After two weeks bluish mats were well established and the appearance changed to a slimy white coverage soon. This dense mat cover thinned to the radially connected pattern after two months first on the sediment and later on the head of the femur. Towards the end of the incubation large parts of the bone lay bare while mats still lined the sides and covered the head. The sediment surface was of brown appearance where mats had vanished, but still appeared black under the mats and at depth. Until the last visual check at the end of August the head of the femur was completely covered and mats lining the bone were present. On the plastic aquarium walls a white substance stretching from 0.5 cm below the surface about 8 cm down was observed in the vicinity of the bone. The upper margin followed the line of sediment closely and the substance was fringed at the borders. To test for potential mats having wandered inside the sediment, three depth horizons (0-0.5 cm, 3-4 cm, 6-7 cm) were sampled and checked for filaments with DAPI staining. Quick microscopic analysis showed that filaments were by far most abundant in the surface sample and less abundant in the two deeper samples. A similar substance was observed under whale falls before and identified as being palmitate (Allison 1988). Possibly, a reaction of the bones' lipids with the plastic wall of the aquaria took place.

NAMV sediment did not show any mats after two weeks and only featured mats in the vicinity of protruding bones after one month. Sediment colour remained brown until the end of the incubation where no mats were present but black sediment was visible under thinner mats. The voids created when taking out bones were black directly under the surface as well. Mats never reached the same abundance as in Ebay sediment and did not cover areas farther away from the bones than about 5 cm. During the last month mats only occurred

around whale vertebrae. Although mats appeared later and disappeared earlier in NAMV than in Ebay sediment the sulphide smell emanating when sampling bones seemed to be stronger. When taking out the last vertebra, gas bubbles that had been trapped under the bone even rose from the sediment.

For pictures of the first three month of bone incubation, see Appendix V.

### 3.2.2. Microscopic analyses: Light, fluorescence and scanning electron microscopy

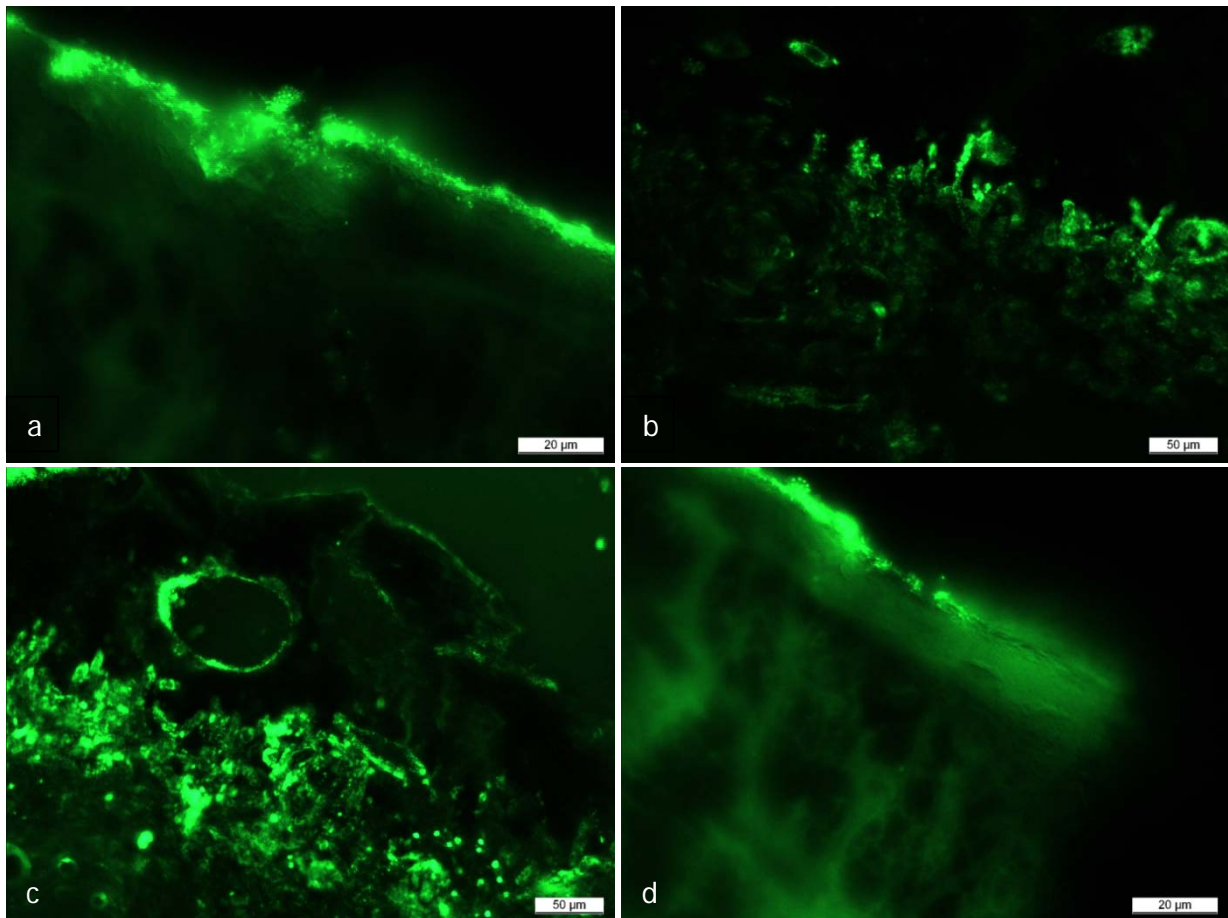
Bone pieces from above and below the sediment were fixed for CARD-FISH and thin-sections were prepared. Only whale bones were examined microscopically because ostrich bones became very soft and crumbly towards the end. The cryotome's blade was not able to cut properly but rather crushed the ostrich bones and no proper thin sections could be produced. Whale vertebrae incubated on Ebay sediment for one and for five months and on NAMV sediment for five months were examined microscopically. Cryosections were made of the dorsal or lateral processes of the vertebrae as these were thin enough to be cut without damaging the structure of the bone.

Light microscopy allowed for a rough analysis of effects of incubation time and sediment type. Cryosections of the reference bone frozen prior to incubation showed no signs of bioerosion at all, cortical and trabecular layers were easily distinguishable and undamaged. No organic matter covering the bone surface was visible. Scanning electron microscopy (SEM) analysis showed the intact periosteum and remains of flesh that could not be removed (Figure 16a).

The bone sampled one month later showed a dense cover of organic material in the part above sediment. Under the light microscope identification of this matter was impossible. Some filaments were visible and unlike sediment, the matter was translucent. With SybrGreen it could be identified as dense mats with occasional tufts of filaments. No major boreholes were seen at this stage but irregularities at the outer bone layer seemed to be occupied by notably many cells (Figure 15a).

After four to five months of incubation, however, excessive bioerosion had taken place (Figure 15b and c). The cortical bone layer was deteriorated severely by boreholes and was at times completely removed. With lengths of around 100  $\mu\text{m}$  some boreholes almost permeated the bone completely. SybrGreen staining revealed single cells in the boreholes. Staining also affected the bone itself, especially inside the holes, so not everything that appeared green were cells. At places where the cortical bone had been removed to a large extent, the disintegrated layers could still be seen as a grey shade, but cells were mostly

located at the active boreholes. Organisms seemed not to have been able to access the bone from within since no erosion and hardly any cells were observed at the inner bone surface.

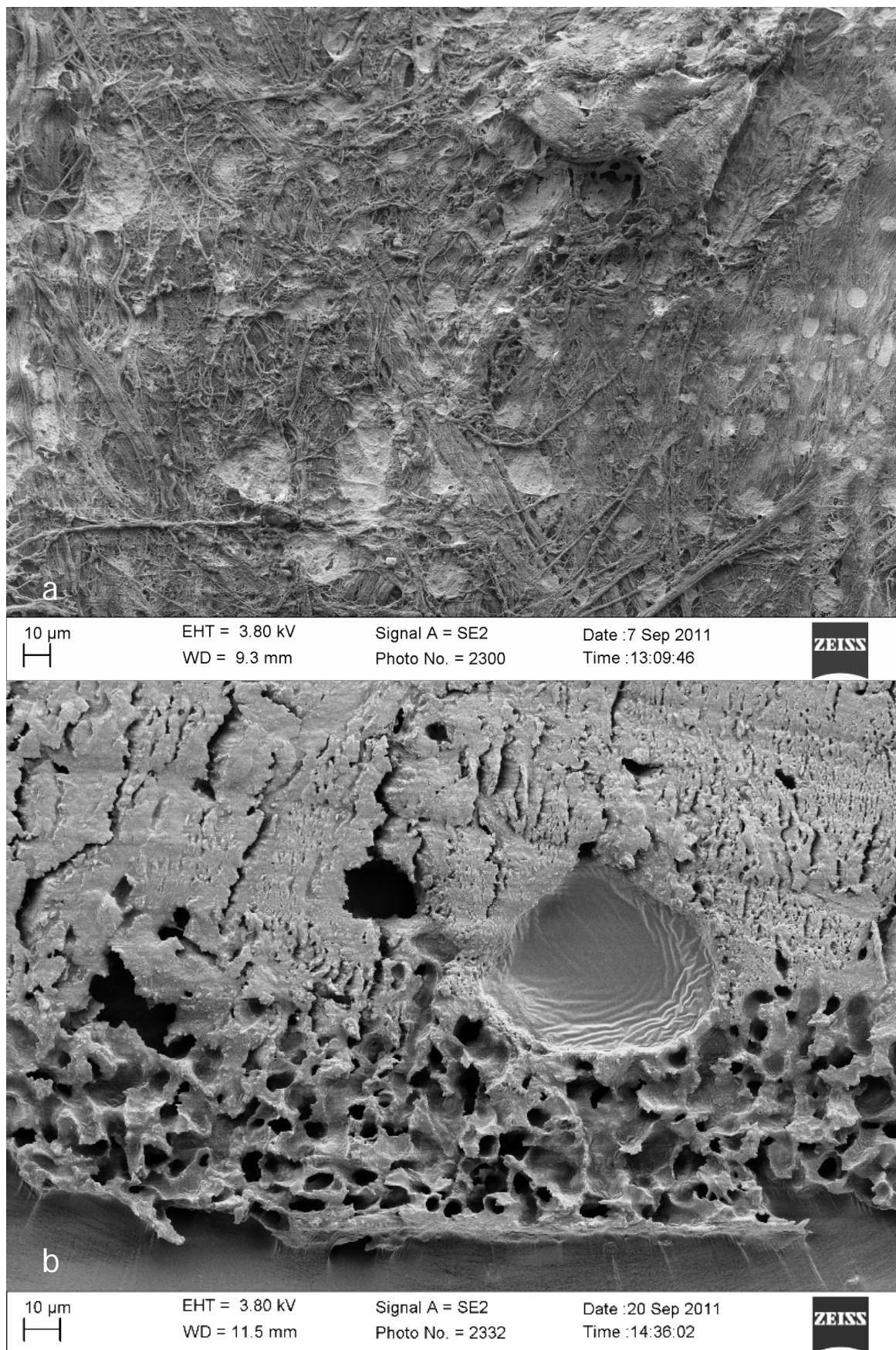


**Figure 15.** SybrGreen-stained cryosections of incubated vertebrae. a) whale vertebra above Ebay sediment after one month, b-c) whale vertebra above NAMV sediment after five months, d) ostrich vertebra below Ebay sediment after five months.

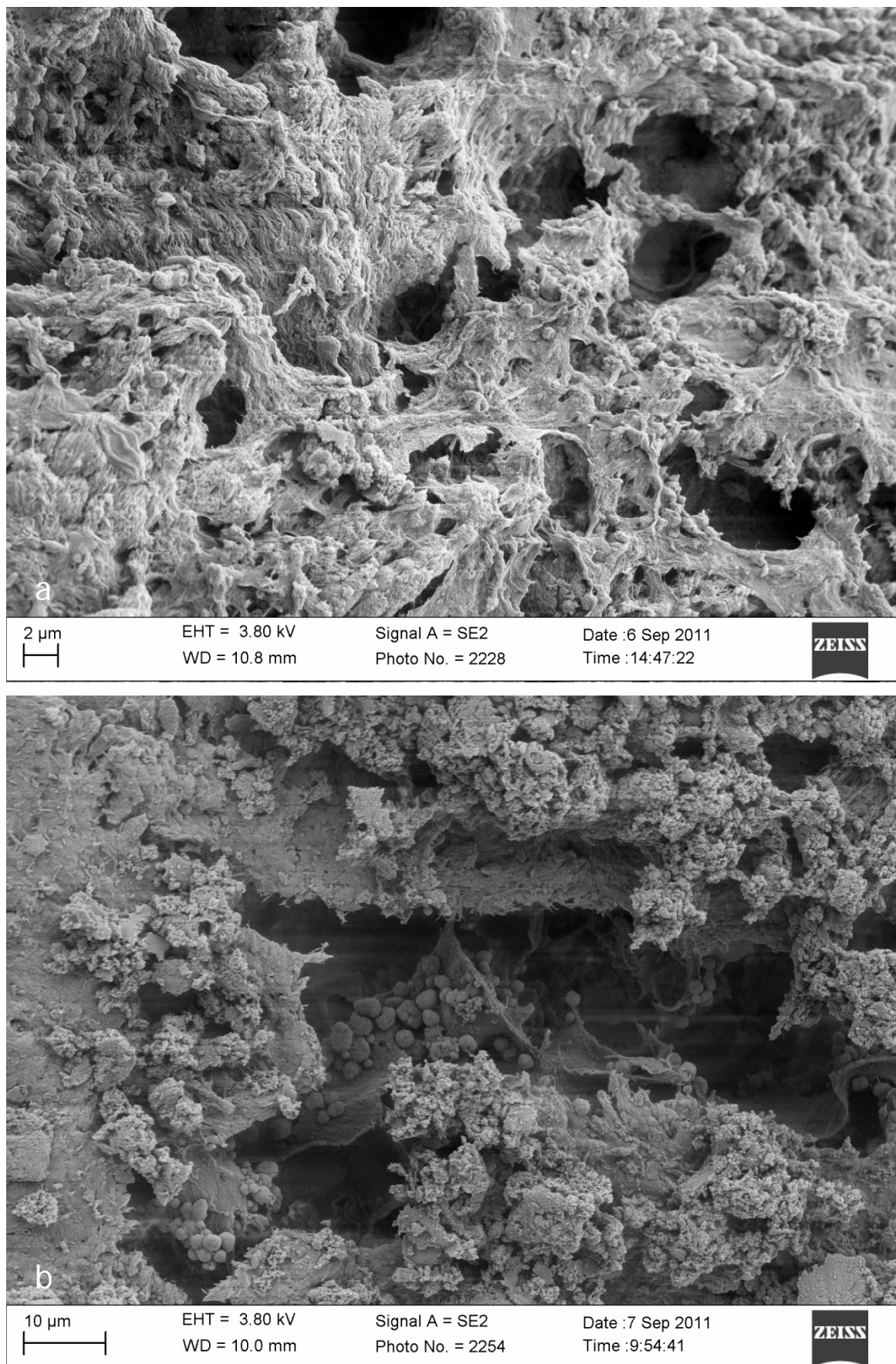
In cavities naturally occurring in the bones and close enough to the outer surface, cells were often found in high densities, but no boreholes originated from these cavities. SEM imaging revealed groups of cells located in deteriorated cortical layers (Figure 16b, Figure 17, Figure 18a) and, in cases where the outer layers had been completely removed, even in between the sheets of trabecular bone tissue (Figure 18b). The cryosection (Figure 16b) shows the morphology of the boreholes at depth. No cells are visible possibly due to problems with the ethanol dehydration executed directly on the SEM sample plate. Cells may have been washed away by frequent refilling of the ethanol solutions.

Samples from under the sediment showed no signs of bioerosion. Some organic matter was attached to the surface but no clearly identifiable boreholes were observed and bone surface was as smooth as in the non-incubated control bone (Figure 15d).

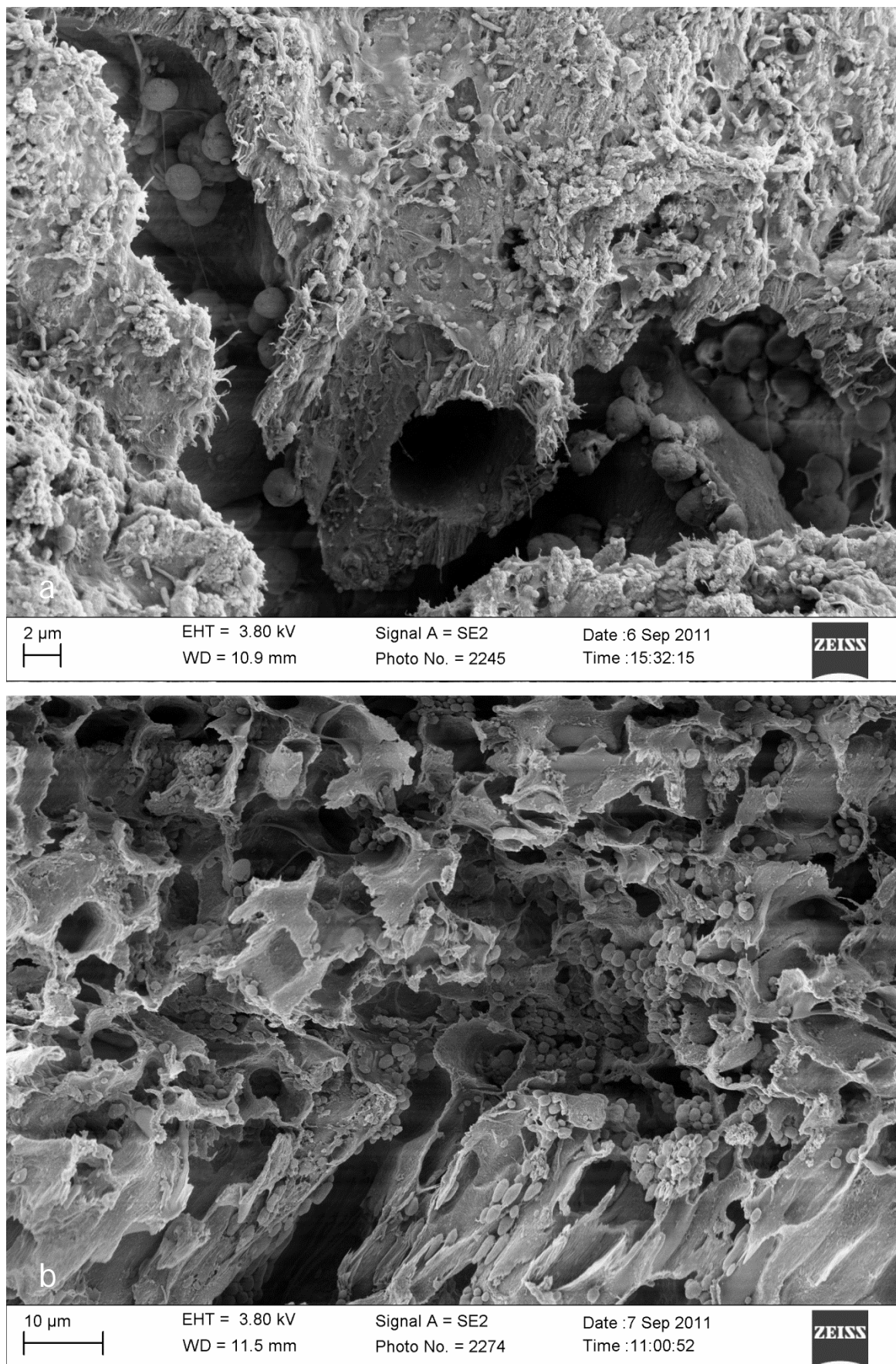
As in ostrich bones, but not to the same extent, whale bones became soft and broke more often during sectioning after longer incubation. Cracked open bone surfaces and fragile appearance were observed in the SEM.



**Figure 16.** SEM pictures of incubated whale vertebrae. a) Bone surface before incubation b) 5  $\mu$ m cross-section of whale vertebra incubated over Ebay sediment for five months.



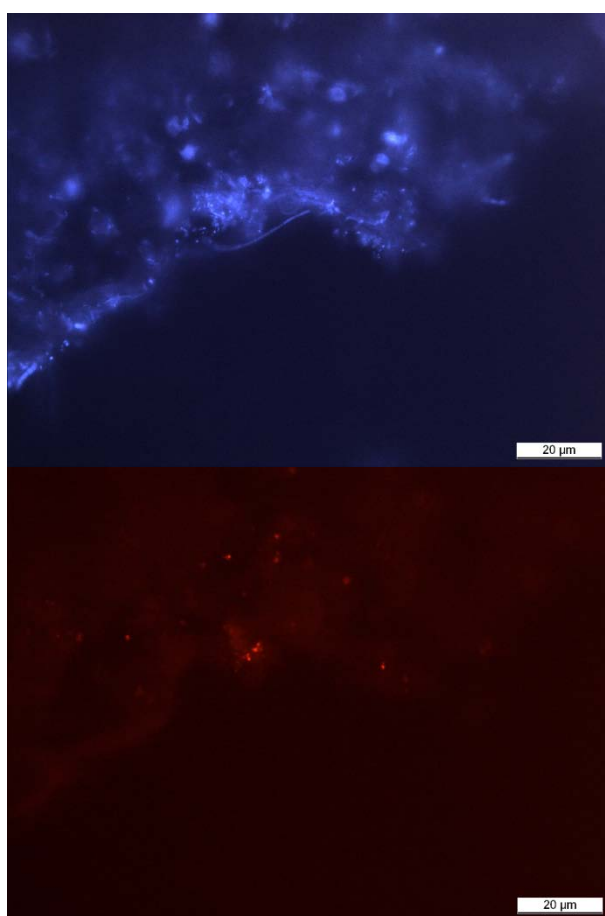
**Figure 17.** SEM pictures of incubated whale vertebrae. a) bone surface after five months incubation above Eckernförde Bay sediment. b) bone surface after five months incubation above North Alex mud volcano sediment.



**Figure 18.** SEM pictures of incubated whale vertebrae. a) bone surface after five months incubation above North Alex mud volcano sediment, b) bone surface after four months incubation above North Alex mud volcano sediment.

### 3.2.3. CARD-FISH on a cryosection

CARD-FISH was attempted on an above-sediment cryosection of an Ebay incubated whale vertebra (five months incubation time). The signals obtained from Alexa546 and DAPI were strong but the bone material itself was at times dyed as well (Figure 19). Brightly fluorescing spots the approximate shape and size of cells were often visible and not always validated by a DAPI signal. DAPI as well as SybrGreen staining of bones showed high rates of random staining and thus made an unmistakable identification of cells difficult. Adding CARD-FISH technique to that, this method can be convenient to view particular parts of the bone and check for presence or absence of cells, but should not be used quantitatively.

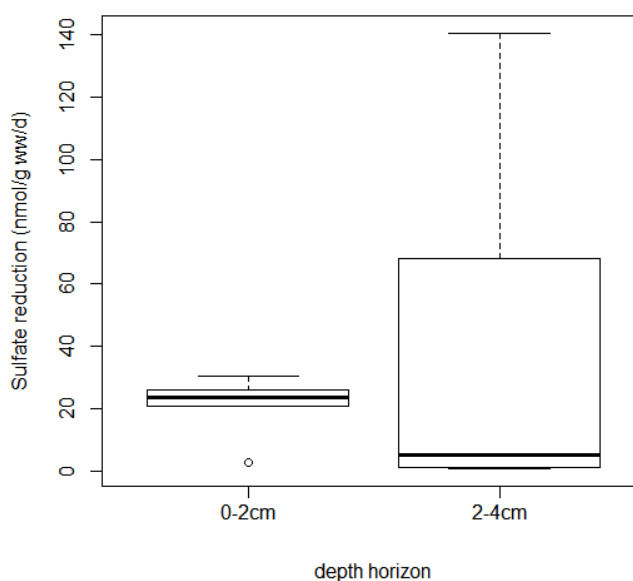


**Figure 19.** DAPI (left) and Alexa546 (Eub338 I-III probe, right) stained pictures of a 5 µm cryosection of whale vertebra incubated on Eckernförde Bay sediment for five months. Magnification 50x.

### 3.3. Analyses of the whale vertebra from the Chilean whale fall

#### 3.3.1. Sulphate reduction

Sulphate reduction rates (nmol per gram wet weight per day, nmol/g ww/d) were determined in one whale vertebra recovered off Chile. The five replicates of each depth horizon (0 - 2 and 2 - 4 cm) were treated equally. Average sulphate reduction rates of  $20.7 \pm 10.7$  nmol/g ww/day for the surface pieces and  $43.1 \pm 61.6$  nmol/g ww/d for the deeper horizon were calculated (Figure 20). The Shapiro-Wilk-test confirmed a normal distribution of the data for both samples and Student's t-test showed that the sample averages were equal. The much higher standard deviation in the deeper samples depicts the very patchy distribution of sulphate reducing activity. While three values were below 5 nmol/g ww/day, the remaining two (68.3 and 140.5 nmol/g ww/day) revealed strong activity. The five surface samples, except for one outlier (2.6 nmol/g ww/day), were much closer together (20.5 - 30.4 nmol/g ww/day).



**Figure 20.** Sulphate reduction rates [nmol per gram wet weight per day] in two depth horizons of the Chile whale vertebra.

#### 3.3.2. Elemental analysis

Elemental analysis of the same bone pieces used for sulphate reduction (see 3.3.1) resulted in a much less varied result. For sulphur (0.1 - 0.2 % DW) and organic carbon (11.3 - 12 % DW) there were no differences between replicates of one depth layer or between depth layers. Student's t-test showed a significant difference in means of nitrogen content between the two depth layers, 3.99 and 3.83 % DW for 0 - 2 and 2 - 4 cm,

respectively. This is such a minor difference that a sample size of five replicates is not enough to make a general statement.

### 3.3.3. Post mortem interval (PMI) determination

No information on the age of the whale vertebra could be drawn from SEM microscopic imaging. The bone surface was so deteriorated by bioerosion that no boreholes or any other biologically relevant traces could be distinguished (pictures not shown).

Determining the age using the citrate measurement method yielded unexpected results. After bringing the citrate from the ground bones into solution, photometric measurements resulted in roughly the same absorptions for all samples. Calculated citrate contents by percent weight were very low (0.116 - 0.391 wt%, Table 2). The resulting ages (time after death, PMI) did not show a consistent pattern: Where 50 mg had been weighed in, calculated PMI was between 22.2 and 26.5 years. However, the two fresh harbour porpoise samples that were supposed to be the baseline ( $t_0$ ) samples embraced this whole time span. One outlier (Chile 0 - 2 cm) featured a PMI of 46.2 years. Where 100 mg had been weighed in PMI determined were much higher (56.7 and 41.4 years) than with 50 mg.

**Table 2.** List of the Chilean whale vertebra pieces from two depth horizons with weight of samples, citrate content (% weight) and resulting calculated post mortem interval (PMI).

bone sample	bone weighed [mg]	citrate content [wt%]	PMI [years]
Chile 0-2 cm	50	0.348	25.6
Chile 0-2 cm	50	0.176	46.2
Chile 0-2 cm	100	0,116	56.7
Chile 2-4 cm	50	0.357	24.9
Chile 2-4 cm	50	0.355	25.1
Chile 2-4 cm	100	0.209	41.4
fresh	50	0.338	26.5
fresh	50	0.391	22.2

These PMI calculated scatter so much that no statement about the true age of the Chile whale vertebra can be made. Hardly any difference can be seen between the obviously old Chile samples and fresh bones.

## 4. Discussion

In this study several questions of marine sediment biogeochemistry were addressed.

On the one hand the effects of methane and whale biomass enrichment on different kinds of sediment were monitored. An attempt was made to answer the questions of how quickly sediments react to certain enrichments, how they are changed and what metabolisms dominate. Cell enumerations using different stains and probes were used and outcomes as well as problems with these techniques are discussed.

On the other hand, the study focuses on the effect of prolonged exposure of bones to different sediments. The course of microbial community development and bioerosion was monitored visually by different microscopic techniques and by elemental analyses and sulphate reduction measurements.

### 4.1. Sediment enrichment experiments

In the set-up of this part of the study, the whale biomass, which floated on top, separated from the sediment, which, albeit shaken thoroughly at every measurement, settled on the bottom.

It took several weeks in all four sediments used in this study (whale fall/WF, cold seep/CS, Eckernförde Bay/Ebay and reference/Ref) until increases in sulphide concentration could be observed. Three explanations might be viable for this pattern:

Either there was no suitable substrate for sulphate reducing bacteria and methanogenic archaea in the beginning and some time had to pass until anaerobic fermenters had degraded the whale biomass sufficiently. Because no scavengers were present, whale biomass remained chunky, hence the flesh cubes had a very small area : volume ratio. Microbial activity could increase visibly only after partial decomposition and, thus, surface area increase, of the flesh. The increase in exposed area might have enhanced fermentation activity and resulted in increased availability of small substrates such as acetate, hydrogen or carbon dioxide. It is possible that it took the sulphate reducers and methanogens longer to become active as they are not adapted to degrade large molecules (Oremland & Polcin 1982). With oils being easier to degrade (Wakelin & Forster 1997) than meat, the added whale oil could have straddled the initial phase but the oil was also floating on top of the medium in the vials and was not in permanent close contact with the sediment.

A second possible explanation is that traces of oxygen introduced by the biomass itself hindered anaerobes from active metabolism. The role of oxygen introduced into the enrichment vials by whale biomass might be important to consider, but is hard to quantify. Resazurin is a very sensitive oxygen indicator and turns pink at very low amounts of oxygen. Medium controls containing no sediment showed a pinkish hue after a while due to resazurin response. This was not observed in any vials containing sediment. In one vial it was seen for several days, but later quickly disappeared again. The apparent absence of oxygen from sediment vials can be explained by binding of free oxygen in partly or fully reduced sediments, e.g., by oxidation of pyrite (Lowson 1982). The oxygen in the whale flesh might nonetheless have had a slight effect on the onset of anaerobic metabolic processes. Many factors influence the oxygen content of muscle and fat tissue, and to empirically determine it in a juvenile dead whale is impossible. A considerable concentration of oxygen in the vials seems unlikely, however, as oxygen would have been used up quickly by aerobic microbes from the whale biomass itself or from the sediments.

From the fact that, when whale biomass was added, the four sediments used (WF, CS, Ebay and Ref) started to produce sulphide after roughly the same incubation period, the conclusion that all possessed similar communities of microbes in the beginning could be drawn. However, the sediments from the deep sea should not have been well adapted to deal with a large organic input quickly, as WF and Ref were most likely not fully reduced and had not received large amounts of organic matter in situ shortly. CS, on the other hand, probably contained a standing stock of active sulphate reducers, because consortia of anaerobic methane oxidizing archaea are commonly found in syntrophy with those (Boetius et al. 2000). The fact that CS sediment still reacted so slowly to the enrichment might be due to the sulphate reducers being associated in consortia. They are probably adapted to oxidising the unknown substrate delivered by the archaea. These three sediments were initially all low in nitrogen and organic carbon content. This fits into the picture as WF and Ref can be considered common deep sea sediments (maybe slightly enriched due to the Chilean upwelling). Cold seeps are not primarily driven by organic matter input but by methane flux. N and C<sub>org</sub> contents should be well above regular deep sea sediment contents in CS (Levin 2005).

The Ebay sediment used in this part of the study should have contained active sulphate reducers as it was sampled during or right after the spring bloom and seemed to have been reduced. Nevertheless, it did not produce any sulphide until after a lag phase of the same

length as in the other sediments. This may be attributable to one of the delaying factors discussed above.

All sediments enriched with whale biomass show reasonably high rates of sulphate reduction after approximately 35 days, while methanogenesis in WF, CS and Ref sediments took longer to reach measurable rates, around 50 days. This phenomenon mirrors the natural order in which organic material is consumed under anoxic conditions. Sulphate reduction takes place in anoxic sediment layers, given that sufficient sulphate from the seawater and suitable substrates such as H<sub>2</sub> and acetate are available. Methanogenesis, on the other hand, is restricted to deeper layers as sulphate becomes depleted (Whiticar 2002). At the onset of sulphate reducer activity in the experiment, methanogenesis might have been inhibited by substrate competition. Over time, more or different substrates became more readily available through the activity of fermenters and methanogens either started using one that sulphate reducers could not, or there was such an abundance of substrates that both were able to thrive on the same ones. The fact that rates of methanogenesis in WF and CS sediments increased over time, while rates of sulphate reduction increased only very slightly or even decreased might also reflect this fact. Substrates such as methanol, trimethylamine or methionine only usable by methanogens (Oremland & Polcin 1982) might have been present in excess for them to exploit, while substrates preferred by sulphate reducers yielding more energy (formate, acetate) might have been depleted already. Sulphate reducers in CS sediment were probably other species or strains with different adaptations or preferences than the ones in WF, which could explain why in CS, rates increased slightly while they decreased in WF towards the end. The preferences for and availabilities of diverse substrates might well have played a role in regulating the rates of sulphate reduction and methanogenesis. Holmer and Kristensen in a similar experiment found that sulphate reducing bacteria use acetate initially, but seem to switch to another substrate after longer incubation (180 hours), probably propionate (Holmer & Kristensen 1994).

Another reason for sulphate reduction rates to decrease could have been the high sulphide concentrations, at least in some vials. Concentrations of 16 mM have been found to inhibit growth in pure sulphate reducer cultures (Reis et al. 1992). Concentrations of 26 mM have been reached in this study; although sediments bind free sulphide by formation of pyrite (Whiticar et al. 1995), concentrations this high are likely to slow down or even inhibit sulphate reducer growth.

Final methanogenesis rates in whale enriched WF and CS sediment increased quickly. Average rates in WF sediment were higher than those in CS sediment by the end. Upon

closer examination, the replicates of WF and CS showed very large differences in methane concentrations. While all three replicates of WF show an approximately equal increase in methane concentrations, the replicates of CS vary much more among each other. One replicate exhibits rates of methanogenesis similar to the average of the WF vials, but the other two hardly show any increase in methane concentration at all. This underlines the importance of replication in this kind of experiment, because patchy distribution or possibly lumping together of cells can result in large differences between seemingly equal aliquots of sediment.

Ebay sediment was the only one that produced methane from the beginning and continued to do so at a rather low, but constant rate throughout the experiment. One possibility is that Ebay sediment commonly possesses a set of microbes apt to produce substrates suitable for methanogens. From former enrichments by algal blooms etc., Ebay may have contained residual low-energy substrates such as carbon dioxide in large quantities for methanogenic archaea to exploit.

When looking at initial sulphide increases it is important to consider the native state of the respective sediment. Only from appearance, the sediments differed before the whale biomass enrichment: While WF and Ref were brown and did not emit a sulphidic smell; CS was black and malodorous, as was Ebay. The results of the sediment enrichments have to be analysed according to their different initial reduction states. The progression of the whale enriched Ref sediment depicts this nicely: After a fairly steep increase in sulphide the concentration drops again and remains low until the end. The possibility that sulphide oxidising bacteria quickly become active in fully oxidised sediment is unrealistic. Given the rubber stoppers were airtight in the beginning and no oxygen was present, the only reasonable explanation for the disappearance of the sulphide is a reaction with and subsequent reduction of the sediment. Berner showed a linear relationship between organic matter content and pyrite formation in marine sediments (Berner 1970). In the rapid reaction of hydrogen sulphide and iron, iron monosulphides are formed. A constant resupply of hydrogen sulphide leads on to formation of pyrite (iron disulphide).

Indeed, Ref sediment was black after the enrichment experiment. It seems to have become reduced and at the very last measurement day, a slight increase in sulphide concentration was visible. Maybe reduction in the end was complete and sulphide concentrations would have risen from that point onwards.

However, WF sediment, light brown in the beginning, showed high levels of sulphide throughout the experiment and ended up completely reduced as well. Looking at the colour might not reveal the oxidation status in detail, as formation of pyrite can take months to years (Berner 1970). WF sediments might have been reduced to a large extent due to the possibly recent activity of whale fall microbial processes. It remains unclear how much of the produced sulphide reacted with the sediment and how much was actually measured.

In CS sediment, which was black from the beginning, it can be assumed that most sulphide produced remained in the medium and was measured. The same was the case with initial Ebay sediment, but surprisingly, Ebay exhibited sulphate reduction rates only about half those of WF and CS.

In a related previous study, sediment from an active whale fall (6-7 years old) was enriched with whale biomass *in vitro* (Treude et al. 2009). The authors used sediments from zero to nine meters distance from the whale fall. Comparing elemental composition of the most distant sediment to the values of the four sediments enriched with whale biomass used in the present study reveals the extent to which a sunken whale affects its surroundings; sulphur content was approximately equal while nitrogen and organic carbon contents were two- to three-fold higher in the sediment nine meters away from the active whale fall. Organic carbon contents of around 2 % (DW) in all four sediments investigated in this study were very similar to contents reported for the same region and depth off Chile in surface sediment not influenced by excessive input of organic matter (Treude, Niggemann, et al. 2005). This indicates that the WF sediment used in this study resembles typical deep sea sediment from the upwelling region off Chile much more closely than it resembles whale fall impacted sediment, even nine meters away from the whale. It is very similar in elemental composition to CS, Ebay and Ref sediments.

The fact that sediments around a sunken whale are so highly enriched in organic matter can be explained by extensive bioturbational activity by large scavenging fish first and crustaceans later on (C. R. Smith et al. 1998). Shreds of whale biomass are thereby distributed and buried in the sediment (Solan et al. 2004). The authors argue that, due to this enrichment, the sediment cannot be regarded as control sediment. In the present study this form of organic pre-load of the sediment was not present and major organic input originated solely from the added whale flesh and oil.

The rates of sulphate reduction and methanogenesis measured at an active whale fall site (Treude et al. 2009) from 0 and 3 m distance are in the same order of magnitude as those

found in the present enrichment experiment. However, sulphate reduction and methanogenesis are inversely related between the studies. At the active whale fall site, sulphate reduction rates between 4 and 30  $\mu\text{mol}/\text{cm}^3/\text{d}$  and methanogenesis rates between 4 and 8  $\mu\text{mol}/\text{cm}^3/\text{d}$  were reported. In the present study, observed sulphate reduction rates (between all sediments and replicates) were between 3 and 10  $\mu\text{mol}/\text{cm}^3/\text{d}$  and methanogenesis rates between 0.2 and 27  $\mu\text{mol}/\text{cm}^3/\text{d}$ .

In the same study by Treude and coworkers, rates were only measured for 40 days, while, in the present study, rates were measured for more than 6 months. However, sulphate reduction rates remained comparatively low in this study and methanogenesis rates did not increase until after about 100 days. It can thus be concluded that methanogens were constantly competing with sulphate reducers for substrates and rates of methanogenesis only started to increase strongly after sulphate reduction started to decline. This has implications for the conduction of long-term enrichment experiments: When enriching previously pristine sediments, it has to be taken into consideration that certain substrates have to be produced in significant quantities to induce the activation of certain metabolisms. Conducting such an experiment for only several weeks does not yield any information on microbial metabolism succession or long-term importance or dominance. Because fermenting bacteria are respiring organic matter constantly, substrate supply and demand are dynamic and a slight difference in concentration of a specific substrate could lead to a different microbial pattern.

At the end of the whale biomass enrichment experiment, elemental composition of the sediments had changed considerably.

Nitrogen contents were higher at the end than they had been in the beginning. As nitrogen-rich molecules such as amino acids are consumed rapidly (Chalupa 1976), a high N value can be a sign for fresh organic matter and thus, easily degradable microbial food. The sediments seemed to have been a very fecund culture medium in the end, making possible the high rates of methanogenesis.

An inconsistent picture was given by the  $\text{CaCO}_3$  percentages, with a very high content in Ebay and low contents in the other sediments. This could be explained by patchy distribution of calcified particles in the sediment: Ebay being of shallow origin, it is possible that tiny shell pieces were present and were accidentally ground with the sediment for elemental analysis. Alternatively, in anaerobic oxidation of methane (AOM), bicarbonate is produced, which results in formation of  $\text{CaCO}_3$  (Valentine 2002). AOM was not clearly observed in Ebay sediment, however. At least, methanogenesis was pronounced enough to mask a possible

decline in methane and sulphate reduction rates were not unusually high, either. However, with the high organic input and sediment being even gassy with methane at depth (Treude, Krüger, et al. 2005), the occurrence of AOM would not be surprising.

To determine whether the increase in microbial metabolic activity was accompanied by an increase in cell numbers, total cell counts were done. The determination of total cell numbers for whale enriched sediments was done by SybrGreen staining, while the distinction between archaea and eubacteria was performed by catalysed reporter deposition fluorescence in-situ hybridisation (CARD-FISH).

Cell number determinations in the sediments were difficult. It was not possible to obtain sediment samples in the end exactly the way it was sampled before whale and methane enrichment since the sediment on the bottom of the vials was inaccessible with the whale flesh that was floating on top. Shaking out the flesh would have led to mixing of the sediment with the remaining oil and that would have biased the elemental analyses. The way sediments were sampled yielded a much higher sediment : pore water ratio than samples from the beginning showed, so the dilution factor of liquid level : sediment level was utilised. However, this approach seems to have been quite inaccurate because cell numbers calculated in this fashion did not increase during the experiment, but rather decreased. Given the increasing rates of sulphate reduction and methanogenesis, this is a result that is unlikely to reflect the true situation. Although cells are known to increase their metabolic rate somewhat, an increase in cell numbers has to accompany the observed rates of sulphate reduction and, especially towards the end of the experiment, methanogenesis. One possibility to overcome this problem would have been to take pure sediment out of the fixed sediment samples and dilute it in a known amount of liquid so both samples would contain the same sediment : liquid ratio again.

Overall initial cell numbers determined by SybrGreen staining were fairly low. Compared to  $6.5 \times 10^8$  per  $\text{cm}^3$  found in this study in initial Ebay sediment, total cell numbers of about  $2.5 \times 10^9$  per  $\text{cm}^3$  were found in a shallow, organic matter rich coastal sediment in a comparable environment and climate (Sahm et al. 1999). Cell numbers of CS and WF sediment were even lower by factors of three and four, respectively. Cell numbers commonly found in the deep sea sediment influenced slightly by upwelling are around  $10^8$  per  $\text{cm}^3$  (Mauclaire et al. 2004). This difference could again have been due to the dilution and sampling issue. The fact that there are more cells per  $\text{cm}^3$  at the first medium change in WF sediment than in the end, however, corresponds to decreasing sulphate reduction rates in this sediment. Sulphate reducing bacteria might have experienced shortage of suitable

substrates or overly high sulphide rates and that might have led to reduction of metabolic activity and cell death.

Cell enumeration was further complicated by the multi-step process of CARD-FISH. The initial sample of CS, e.g., was counted for SybrGreen to have roughly similar numbers in SybrGreen-only and in CARD-FISH treated filters, so a negligible amount of cells was lost during filter incubations, washing and handling. However, often during the filter preparation, cells were lost. Depending on the number of filters incubated in one Eppendorf tube cells can be removed in larger quantities. When handling filters improperly with the forceps, streaks devoid of cells could be seen under the microscope. Furthermore, signals of the fluorescently labelled tyramide can be hard to see, especially when sediment is dyed as well or occludes cells. These problems led to numbers of archaea and eubacteria combined being higher than the SybrGreen based total cell number.

Compared to the whale biomass enriched vials, most methane enriched vials did not exhibit measurable rates of sulphate reduction or methanogenesis. Only in CS sediment enriched with methane a reaction was observed. Apart from the large fluctuations in the beginning, they showed only a minor downward trend, if any. All vials were gassed with roughly 1.1 bar methane in the beginning but slight deviations were likely to occur. So the fact that the medium control values were always above the sediments and CS was always lowest could be due to different methane loading in the first place. This would be a coincidence as previous studies have found the 0 - 5 cm depth horizon to be a zone of active AOM in seep areas (Nauhaus et al. 2002). The authors found very similar rates of anaerobic methane oxidation and sulphate reduction and concluded the coupled metabolisms of consortia to be at work. Pronounced differences were found at different depths within the top 5 cm, however, with surface AOM rates being much lower than at 5 cm due to the oxygen sensitivity of the involved organisms. *Beggiatoa* mats were found to be an indicator for high upward methane flow in underlying sediment (Treude et al. 2003).

In the present study, CS sediment (in situ having been covered by *Beggiatoa* mats as well) from 0 - 5 cm depth exhibited a slow, but steady increase in sulphide over the 191 days up to a final concentration of 5 mM ( $0.32 \pm 0.01 \mu\text{mol}/\text{cm}^3/\text{d}$ ). A correlative decrease in methane could not be observed in the headspace. However, it is likely that the slow consumption of methane was masked by the measurements' inaccuracies and AOM took place at fairly low rates.

The total cell numbers of methane enriched CS sediment increased by a factor of about two during the experiment and reached  $6.5 \times 10^8/\text{cm}^3$ , while the ratio of archaea to eubacteria decreased from 41 to 32 %. Assuming that AOM took place, the percentage of archaeal cells should have increased. Knittel and coworkers reported from sediments at Hydrate Ridge that over 90 % of cells were found in consortia. In the present study, the number of consortia greatly increased during the experiment. The observed consortia became much larger and about 65 % of the cells were located within consortia. Their cell numbers were roughly calculated, but they were neither counted as eubacteria nor as archaea since no staining except for SybrGreen could be detected safely. It is very likely that these consortia consisted of sulphate reducing eubacteria enclosing a core of methane oxidising archaea as they are commonly found at cold seeps (Treude, Niggemann, et al. 2005; Knittel et al. 2005). The exact cell numbers and proportions could not be determined in this study.

Assuming the sulphate reduction (and subsequent AOM) rate of  $0.32 \mu\text{mol}/\text{cm}^3/\text{d}$  was attributable to AOM consortia only, this rate would still be one order of magnitude smaller than, e.g., the rates found at a cold seep at Hydrate Ridge (Treude et al. 2003). Given that methane was delivered at much lower pressures than in the deep sea, this seems to be a reasonable rate. Nauhaus and coworkers report a four- to fivefold increase in sulphate reduction rate when methane is applied at deep sea ambient pressure (11 atm, Nauhaus et al. 2002).

The fact that no other sediment produced any sulphide when enriched with methane supports the hypothesis that AOM consortia are not commonly found in sediments that are not exposed to methane over longer periods of time. Since AOM rates at cold seeps can vary greatly within the top 5 cm of sediment, the methane enriched vials in the present study represent an average AOM rate of this sediment horizon. The modest rate of AOM and sulphate reduction could be attributed to the slow growth rate and thus, long doubling time of anaerobic methanotrophs (Nauhaus et al. 2002).

To test for potential sulphate reduction or methanogenesis that might occur without addition of methane or whale enrichment, sediment controls, containing only the respective sediment and medium, were done. Surprisingly, these controls of all four sediment types showed considerable rates of methanogenesis. The methane increase started right at the beginning, without a lag phase, but sloped off after only about one month. Finally methane concentration in Ref sediment was about half of the one in CS sediment, and in WF and Ebay sediments were even lower than that. WF (Goffredi et al. 2008) and Ebay sediments are likely to have contained methanogens due to the large organic input they had received

before sampling: Ebay might have experienced an algal bloom event several months before sampling (autumn bloom) and WF sediment was exposed to a dead whale before. However, it is unknown how long after the disappearance of most of this organic matter (keeping in mind the low initial organic carbon contents of the sediments) the archaeal community is still active and how quickly these microbes can be re-activated. Ref sediment was the only one that included a depth horizon down to 9 cm. Because all other sediments contained only the 0 - 5 cm horizon, these additional 4 cm in the Ref sediment might have contained active methanogenic archaea. This is an unusually shallow depth for methanogenesis for deep sea sediment that does not receive major organic input. Usually, methanogenesis is only found in anaerobic sediment layers where sulphate is depleted, but some methanogenic archaea can use alternative substrates to avoid competition with sulphate reducers (Vetriani et al. 1999). The high rate of methanogenesis in CS sediment is surprising because AOM, the counter-reaction of methanogenesis, is usually found to be dominant at cold seeps. Recent findings show, however, that archaeal metabolisms at cold seeps are more versatile than previously thought (Orcutt et al. 2005). One or more groups of anaerobic methanotrophic (ANME) archaea that are commonly found in association with sulphate reducers in consortia at cold seeps, seem to be capable of methanogenesis under certain conditions (ibd.). The authors hypothesize that the possession of genes for methanogenesis-related enzymes enables ANME archaea to oxidise methane by reversing the enzymatic machinery. With these enzymes, a back-reaction (equilibrium enzyme effect), i.e., methanogenesis, under altered geochemical conditions might be possible. They state that several ANME or different syntrophies are involved in this process since gain of energy by reverse processes within one organism seems unlikely. While these findings could explain methane production in the CS sediment control, they are not applicable to the other sediments as no ANME should have been present there in large numbers.

In the sediment controls during the initial phase, rates of methanogenesis were higher than initial rates of the whale biomass enriched sediments. In the CS sediment control, rates were about twice as high as in whale biomass enriched WF and CS after 100 days and equal to the rates in CS at the end. This finding is unexpected, since organic carbon contents of the initial sediments are fairly low and no sulphide development was observed. No enrichment was added in any way, so methanogenesis must have relied solely on the substrates already present in the native sediments.

The second phase of methanogenesis showed much lower rates in all sediment controls. If methanogenesis relied on residual substrates to fuel the high rates in the beginning, these substrates were depleted quickly.

The addition of methanol to the sediment control vials did not have an impact on sulphate reduction or methanogenesis rates. It was expected that methanogenesis would be stimulated by the addition of this substrate only usable by methanogens. Another possibility would have been that the already active methanogens could have switched to the newly available substrate methanol, and sulphate reducing bacteria would have started to use the other substrates. Usually, sulphate reducers dominate over methanogens in competition for substrates usable by both, so this outcome was unlikely.

Oremland incubated sediments with additional substrates such as methanol, and kept the flasks at 20 °C. He reported a steep methane increase after 17 days (Oremland & Polcin 1982). The reason why, in the present study, no increase in methane production was observed might have been the incubation temperature, which was much lower (3 °C). The vials are still being monitored, so long-term measurements might still show an impact of the methanol addition.

## 4.2. Bone incubation experiments

The incubation of whale and ostrich vertebrae and of a large ostrich femur showed an immediate response of incubation sediments. Eckernförde Bay (Ebay) and North Alex mud volcano (NAMV) sediment both reacted vigorously to the addition of bones. Whale and ostrich vertebrae as well as the large ostrich femur all induced similar reactions. However, Ebay showed bacterial mat coverage earlier than NAMV. This is most likely due to the more versatile microbial community present in Ebay sediment. Organic matter reaches shallow sea floors in much higher amounts than deep sea floors (Suess 1980). As the Ebay sediment used for the bone incubation experiments was sampled in February, it was very likely not affected by the remains of an algal bloom (Wasmund et al. 1998), but given strong winter mixing, microbial activity should have been high. Due to the strong and frequent organic matter input, sediments in the Baltic Sea can often encounter suboxic or even anoxic conditions, so aerobic as well as anaerobic metabolisms occur and degrade organic matter. Especially sulphide oxidising *Beggiatoa* (although lacking identification, the mats seen on incubations are assumed to be *Beggiatoa* mats) are commonly present at the transition of aerobic and anaerobic (sulphidic) sediment layers (Jørgensen 1977; D. C. Nelson & Jannasch

1983). Addition of the vertebrae constituted a rich input of organic matter and hence, promoted anoxia by aerobic organic matter degradation. Ebay sediment, having been in a reduced state at the time of sampling, was therefore likely better adapted to quickly respond to this enrichment with strong sulphate reduction and the development of extensive mats of these sulphide oxidising bacteria.

Typical deep sea sediments such as NAMV sediment, on the other hand, do not receive as much organic matter as shallow sediments (Suess 1980). From its light brown appearance it can be concluded that the sediment was fully oxidised and anaerobic metabolic processes did not occur at the time of sampling at the relevant sediment depth. Mat forming organisms such as *Beggiatoa* were likely absent or in dormant stages due to the lack of sulphide. The microbial community was probably much less adapted to dealing with a large parcel of organic matter than in Ebay sediment. Anaerobic processes only took place after the periosteum and remaining flesh particles had been removed by aerobic degradation, turning the sediment below the surface black with iron monosulphides and possibly pyrite after about one month.

Because the aquaria were filled with water from Kiel Fjord, salinity might have had an influence on the sediments. Strong summer stratification is observed in Kiel Bight and, combined with a continuous freshwater influx from the river Schwentine, a surface salinity of about 13 during the incubation experiment can be assumed (Smetacek 1981). A more or less stable salinity of about 23 is found in the bottom water of Eckernförde Bay. Compared to the average deep water salinity of 35 encountered at NAMV, this difference could likely contribute to the delay in sediment reaction by complicating the re-activation of dormant stages in NAMV sediment.

The possibility that *Beggiatoa* was present in the incubation water cannot be ruled out and the contribution to the formation of mats by water and sediment, respectively, cannot be determined. A control basin containing a bone and autoclaved sediment or no sediment at all could have clarified the presence or magnitude of *Beggiatoa* abundance in Kiel Fjord water.

Despite the possible complications for NAMV sediment, the quick appearance of bacterial mats could be accounted for by different processes. Treude and coworkers showed that sulphate reduction in bones leads to a seepage of sulphide across the bone-water interface (Treude et al. 2009). Since the incubated vertebrae had been defleshed with sharp utensils which might have scratched the outer bone surface, lipids inside the bones might have become readily available for sulphate reducing bacteria. Subsequent sulphide seepage from the bones could then have promoted rapid growth of sulphide oxidising mats on the bone surface. The temperature of Kiel Bight water (5 - 20 °C, Jochem 1988) taken from 3 m depth

was higher over the duration of the incubations than at the sample sites of the sediments (ca. 4 °C for Ebay (Dale et al. 2011) and NAMV. As higher temperature speeds up metabolic processes (within limits, Gillooly et al. 2001) mat growth was probably accelerated.

In order to estimate the lipid content of a bone, several things have to be considered. Whale vertebrae show a range of lipid contents depending on post mortem interval, species, age and position of the vertebra within the spine (Higgs et al. 2010). A 53 % decrease in fat in lumbar and thoracic vertebrae within 52 hours after death has been found previously. The fat had been converted to fatty acids quickly. The striped dolphin (*Stenella coeruleoalba*), comparable in size to the juvenile harbour porpoise used in the present study, has a vertebra lipid content of about 5 %, while the larger whales exhibit values of up to 60 % in their caudal vertebrae. Furthermore, juvenile whales have a higher proportion of red bone marrow and thus, less lipids in bones (ibid., and references therein). The actual fat content of the incubated bones remains unknown, as does the fat content of ostrich vertebrae.

Regardless of the initial lipid contents, both types of vertebra were able to support sulphate reduction and mats of sulphide oxidisers for several months. Slightly earlier mat disappearance in NAMV than in Ebay sediment was probably due to residual organic matter and a more reduced initial milieu in Ebay sediment.

Unfortunately, microscopic analyses could only be performed on whale vertebrae due to the fragile nature of the bioeroded ostrich vertebrae. However, it can be assumed that microbial erosion took place to the same degree in both bone types since mat cover and appearance were very similar over a long time. No bioerosion at all was observed in bone parts buried in the sediment. This finding is consistent with the much lower cell numbers found in buried bone than in exposed bone of a whale fall (Deming et al. 1997). In the present study, the whole bone surface was markedly rougher and the periosteum seemed to have been completely removed after one month. This is very likely due to initial abiotic dissolution of the mineral outer bone phase. Due to aerobic biomass consumption on the bone, evolving CO<sub>2</sub> led to a decrease in pH and destabilisation of the mineral structure (Collins et al. 2002). In a different study, Davis reported bioerosion in sunken birds after four days (Davis 1997). His incubations were performed in shallow water and under the natural light cycle, however, and the involved organisms were cyanobacteria and algae. Nonetheless, his and the present study demonstrate how quickly bone material is degraded in an aquatic environment.

The hydrophobic nature of the bones' lipids might have been a barrier for sulphate reducing bacteria in the beginning. As seawater cannot penetrate fatty bones quickly through natural crevices and holes, it cannot transport sulphate reducing bacteria inside (Treude et al. 2009).

Sulphide production could thus only start when bone boring organisms had unlocked the lipid storage and facilitated seawater permeation. In the present study, extensive bioerosion was seen after four months of incubation, with boreholes penetrating more than 100  $\mu\text{m}$  into the bone. The most likely candidates to have caused the boreholes are bacteria of the order *Oceanospirillales*, a heterotrophic group of  $\gamma$ -proteobacteria that have been found to aerobically degrade complex organic compounds in bones and are also associated with the bone-eating polychaete worm *Osedax* (Goffredi et al. 2005). Decreasing oxygen availability might also be the reason for the microbes not to dig in any deeper. It is likely that sufficient amounts of lipids for the sulphate reducers were still available at the end of the incubation but replenishment of oxygen and evacuation of carbon dioxide probably set the limit for boring depth of the aerobic *Oceanospirillales*. This seems to be the reason why bioerosion was completely absent from bones below the sediment-water interface. The anoxic conditions caused by organic matter degradation hinder these microbes from settling. However, it is not known which organisms exactly can contribute to bioerosion of bones. Based on histomorphological alterations, bioerosion has been ascribed to different organisms. Following Hackett, the tunnel-shaped erosion patterns found in this study may have been caused by bacteria following the intrusions of bone-degrading fungi (Hackett 1981). Involvement of several organisms at the same time or in a succession might have occurred.

Fossilised bones from whale fall sites were found to feature microbial bioerosion as well. Whale bones from the Eocene to the late Miocene (ca. 55 - 23 million years ago) showed boreholes that closely resemble the ones found in the present study (Kiel 2008). The author observed that in the fossil bones, boreholes were almost exclusively found in the parts in contact with seawater and concludes that bioerosion probably begins as soon as the bone surface is exposed. The findings in the present study confirm these hypotheses. Bone surface below the sediment was untouched and bioerosion above the surface started quickly after incubation. Most of the bioerosion seems to take place relatively shortly after exposure and possibly little changes in the long time after burial and during fossilisation.

Interestingly, a negative relationship between lipid content of bone and degree of microbial bioerosion has been found in both whale bones (Deming et al. 1997) and bird bones (Davis 1997). This implies that the bones with the highest lipid content last longest in marine settings. As stated earlier, lipid contents can vary depending on different factors. The whale vertebra retrieved at the inactive whale fall site off Chile was found among several shattered

vertebrae, rib pieces and a skull. Most of the whale skeleton seemed to have been completely degraded or buried. The fact that the vertebra sampled was still there might give a clue about its former position on the spine: Caudal vertebrae were found to have the highest lipid content, so degradation in these would be expected to be slowest. The vertebra analysed in this study might thus be a caudal one. Again, no conclusions should be drawn concerning the age of the whale fall, because neither species nor age of the whale was known.

There was still sufficient fat in the bone to facilitate sulphate reduction. Five replicates of two depth horizons (0 - 2 and 2 - 4 cm) showed very different distributions of sulphate reducer activity: At the bone-water interface, rates of sulphate reduction were generally low with a small spread. Deep samples showed patchy distribution with a very low background and high peaks. This supports the hypothesis that lipids in bones keep out water and impede fast colonisation by sulphate reducers. While in the shallow bone layers almost all fat seems to be used up, pockets of lipids still exist in deeper layers and bone persistence is increased by the resulting stepwise consumption. This phenomenon could prolong the sulphophilic stage of a whale fall, support growth of bacterial mats and provide for bacterial mat grazers and specialised species with chemoautotrophic endosymbionts (C. R. Smith & Baco 2003). X-ray diffraction of the same pieces that were used for determination of sulphate reduction revealed that no pyrite was present in the two depth horizons of the vertebra (Steffen Kiel, personal communication). This could lead to the conclusion that the whale fall was rather young because pyrite formation can take up to several years (see above). On the other hand, sulphate reduction was very scarce in the top 4 cm of bone (except for the lipid pockets), a young age thus seems unlikely. As sulphate reduction progresses towards the core of a bone, a concentric layer of formed iron monosulphides and pyrite is usually seen in whale fall vertebrae (Allison et al. 1991). As the pyrite layer is dissolved by aerobic seawater, it migrates towards the centre of the vertebra as it follows the border of sulphate reduction. The fact that no pyrite was found down to 4 cm into the vertebra in this study reveals that the pyrite layer must be deeper and a young age can be excluded. The lipid pockets where sulphate reduction still takes place seem to be protected from the penetrating oxygenated seawater, possibly by the hydrophobic lipids themselves.

Elemental analysis performed on ground bone pieces of the same two depth horizons (0 - 2 and 2 - 4 cm) revealed no difference in bone composition regarding sulphur and organic carbon. Only nitrogen percentage was a bit lower in the deeper part. As collagen contains over 95 % of the bones' nitrogen (Collins et al. 2002), this could show the consumption of

collagen by bone boring microorganisms. However, more and also deeper samples of the vertebrae would be necessary to find a trend in elemental contents.

Determination of the post mortem interval of the Chile whale fall was impeded by several problems. The fresh harbour porpoise bones were calculated to be about as old as the whale fall and outliers were detected where 100 mg instead of 50 mg of sample were used. Schwarcz and coworkers concluded from their work that burial conditions did not have an effect on the citrate content of bone over time (Schwarcz et al. 2010). However, no samples used in their study were incubated in water or on marine or freshwater sediment. Although the authors gave a correctional function for bones that had been incubated below 0 °C for periods of time, a permanent temperature of 4 °C on the deep sea floor might result in different citrate degradation. Less accurate than Schwarcz' method but proven to work for whale fall bones, the  $^{210}\text{Pb}/^{226}\text{Ra}$  ageing method (Schuller et al. 2004) could alternatively be applied in future studies. This method might be more time-consuming, but can be used for whale falls aged between 10 and 85 years.

### 4.3. Conclusions

This is the first comprehensive study to test microbial reactions to the addition of whale biomass and methane on various sediment types. The fundamental idea of these enrichment experiments was to determine the validity of Baas Becking's theory that "everything is everywhere, but the environment selects".

**If this theory was valid**, all sediments should have reacted similarly to the biomass and methane enrichments. Due to the recent history of each sediment, the initial lag phases would probably have differed, but the rates at the end of the experiment would have been similar. The impact of a certain enrichment would have been to stimulate the dormant stages of those microbes whose metabolism would have been apt to deal with the new situation.

The fact that the lag phases before sulphide increase in all sediments were roughly equally long points to a universal microbial community in all sediment types. It has been shown in this experiment that any sediment, regardless of its previous history, can be "transformed" into one that closely resembles active whale fall sediment. Although Ref sediment exhibited low sulphide levels throughout, the concentration might still have risen after complete reduction of the sediment. With sufficient time allowed, all sediment types might have arrived at similar rates of sulphate reduction and methanogenesis.

**In case this theory was invalid**, the each sediment's recent history would have determined the nature and extent of the reaction: WF and Ebay sediment would have reacted first with high sulphide and methane production. CS sediment would have shown lower rates of both since it was expected to possess a reasonably suitable set of microbial metabolisms, but would not have been prepared for a large parcel of organic matter. Ref sediment would not have shown any reaction.

AOM was only observed in CS sediment, where it occurred in situ. Despite the addition of methane, AOM was not inducible in the other sediments, pointing towards a non-ubiquitous distribution of the ANME/sulphate reducer-consortia. However, AOM is usually only found in deeper sediment layers and sediments in this experiment encompassed only the top 5 cm. In CS, AOM consortia can be found due to the high methane flux from below and the anoxic conditions.

The results from this experiment do not verify or falsify the "everything is everywhere" hypothesis. The results rather suggest that similar microbial communities are present in different sediments, and similar metabolic processes can be observed, but nothing is known about the exact identity of the microbes. Several different species or strains might have been present, but the metabolic waste products that were monitored were the same.

Furthermore, the experiments of this study did not encompass the whole diversity of microbial habitats, but rather marine sediments that are all interconnected, albeit by huge distances. The possibility of dispersal by currents, wind or animal vectors cannot be denied. Habitats like hot springs, aquifers or hydrothermal vents that all require cellular and metabolic adaptations, might give a completely different picture of ubiquity of microbes. These extreme environments are much more prone to harbour species that are not distributed globally.

If applying Baas Becking's theory to the bone incubation experiment, the answer is even less straightforward. The signs of bioerosion seem to be identical regardless of the sediment the bones were incubated on. But whether the microbes originated from within the sediment or from the incubation water is unknown. Even more, the boreholes found in fossil bones of whale falls, active millions of years ago in distant oceans, resemble recent ones. To answer the question of ubiquity, much more has to be learned about the organisms creating these boreholes.

## 5. References

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## Appendix

### Appendix I: Sulfate reducer medium components:

All components should be autoclaved or filtered

Salts (g/l medium)

KBr 0.09

KCl 0.6

CaCl<sub>2</sub>\*2H<sub>2</sub>O 1.47

MgCl<sub>2</sub>\*6H<sub>2</sub>O 5.67

MgSO<sub>4</sub>\*7H<sub>2</sub>O 13.6

NaCl 26.37

Resazurin: 100 mg resazurin in 100 ml H<sub>2</sub>O<sub>UP</sub>

Bicarbonate solution 30 mM NaHCO<sub>3</sub>, 30 ml/l medium

Nitrogen-/phosphate solution 4.67 mM NH<sub>4</sub>Cl and 1.47mM KH<sub>2</sub>PO<sub>4</sub>, 50 ml/lmedium

6-vitamin solution (for 100 ml in 20 mM NaP buffer)

4-aminobenzoic acid	4mg
D-(+)-biotin	1mg
nicotinic acid	10mg
calcium-D-(+)-panthothenate	5mg
pyridoxaminedihydrochloride	15mg
liponic acid	1.5mg
folic acid	4mg

Thiamin solution 10 mg thiamin in 100 ml 25 mM NaP buffer

Cyanocobalamin solution 5 mg cyanocobalamin in 100 ml H<sub>2</sub>O<sub>UP</sub>

Riboflavin solution 5 mg riboflavin in 100 ml acetic acid (20 mM)

Selenite/tungstate solution 400 mg NaOH, 8 mg Na<sub>2</sub>WO<sub>2</sub> and 6 mg Na<sub>2</sub>SeO<sub>3</sub> in 1000 ml H<sub>2</sub>O<sub>UP</sub>

Trace elements solution (in 1000 ml H<sub>2</sub>O<sub>UP</sub>)

HCl 37%	12.5 ml
FeSO <sub>4</sub> *7H <sub>2</sub> O	2100 mg
H <sub>3</sub> BO <sub>3</sub>	30 mg
I <sub>2</sub> *4H <sub>2</sub> O	100 mg
CoCl <sub>2</sub> *6H <sub>2</sub> O	190 mg
NiCl <sub>2</sub> *6H <sub>2</sub> O	24 mg
CuCl <sub>2</sub> *2H <sub>2</sub> O	2 mg
ZnSO <sub>4</sub> *7H <sub>2</sub> O	144 mg
Na <sub>2</sub> MoO <sub>2</sub> *2H <sub>2</sub> O	36 mg
MnCl <sub>2</sub> *4H <sub>2</sub> O	100 mg

**Appendix II: Volumes of the enrichment experiment glass vials**

Vial no.	Vial volume [ml]
1	244
2	244.22
3	241.23
4	243.9
5	243.53
6	244.26
7	244.94
8	244.11
9	243.59
10	244.76
11	244.02
12	243.67
13	243.44
14	243.59
15	244.31
16	244.81
17	243.95
18	244.17
19	244,65
20	243.45
21	244.26
22	244.4
23	243.74
24	245.28
25	243.9
26	244.11
27	243.26
28	244.83
29	243.65
30	243.62
31	243.76

**Appendix III: Sulphide standard preparation for calibration of photometric sulphide measurements**

Vials with anoxic ultrapure water were used for the sulphide calibration. Sodium sulphide with a known amount of water ( $\text{Na}_2\text{S} \times 9\text{H}_2\text{O}$ ) was added to final concentrations of 0.5, 1, 3, 5, 10, 20 and 30 mM. Absorption was measured the same way as in the samples, according to Cord-Ruwisch (Cord-Ruwisch 1985).

**Appendix IV: CARD-FISH solutions and buffers**

1x PBS	8 g NaCl 0.2 g 2.7 mM KCl 1.41 g 10 mM Na <sub>2</sub> HPO <sub>4</sub> *7H <sub>2</sub> O 0.27 g 2 mM KH <sub>2</sub> PO <sub>4</sub> adjust to pH 7.3 and 1 l, autoclave
Lysozyme solution	0.05 M EDTA pH 7.3 0.1 M Tris-HCl pH 7.3 5 mg/ml Lysozyme (20000 U/μl)
Achromopeptidase solution	0.01 M Tris-HCl pH 8 0.01 M NaCl 60 U/ml Achromopeptidase
Hybridization buffer 35 % (20 ml)	7 ml Formamide 7 ml H <sub>2</sub> O <sub>UP</sub> 3.6 ml 5 M NaCl 0.4 ml 1 M Tris-HCl 2 ml blocking reagent 40 μl 10 % SDS 2 g dextran sulfate disodium salt, Sigma
2x SSC	300 mM NaCl 34 mM Na-Citrate*2H <sub>2</sub> O adjust to pH 7.0

**Microscope filters**

Filtercube	Excitation Filter [nm]	Dichromatic Mirror	Suppression Filter [nm]
L5	BP 480/40	505	BP 527/30
Y5	BP 620/60	660	BP 700/75
A4	BP 360/40	400	BP 470/40

All other solutions were prepared according to instructions (Invitrogen TSA Kit#23, Eugene, Oregon).

**Appendix V:** Bone incubation experiments monitored over six months (first three months shown below). First two rows are vertebrae incubated in Eckernförde Bay (Ebay) and North Alex mud volcano (NAMV) sediments, respectively. Bottom row depicts the ostrich femur incubated on Ebay. Notice the time lag between first bacterial mat appearance between Ebay and NAMV.

