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Impacts of drilling mud discharges on water column organism and filter feeding bivalves

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Impacts of drilling mud discharges on water column organism and filter feeding bivalves

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Content

Preface

The two main objectives of our project was to study possible effects of drilling mud discharges on water column organism (plankton and fish), and filter feeding bivalves, and to analyse metals in fish and bivalves exposed to suspended particles of drilling mud to find out if metals from the mud was taken up by the organisms.

The results from the different parts of the project have been reported in 8 manuscripts/reports. The ultimate aim is to publish as many of these manuscripts as possible. Hence this report is a collection of early versions of future publications.

Here is a list of the manuscripts/reports:

I. Exposure of fish and bivalves to suspended particles of drilling mud: A new continuous flow exposure system (R.K. Bechmann, L. P. Myhre, R. C. Sundt)

II. Metals in tissues of mussels, scallops and cod exposed to suspended particles of water based drilling mud (S. Westerlund & R. K. Bechmann*)*

III. Filtration rate, growth, histology and biomarker responses in mussels and scallops exposed to suspended particles of water based drilling mud (R. K. Bechmann, T. Baussant, A. H. Tandberg, D. Lowe).

IV. Effects of suspended particles of water based drilling mud on cod (R. K. Bechmann, K. B. Øysæd, E. Lyng, D. Lowe)

V. Effects of suspended particles of drilling mud on development, growth and feeding of the mussel *Mytilus edulis* embryos and larvae (T. Baussant, I.C. Taban, K. Alfsnes, R.K. Bechmann)

Appendix to paper V: Validation of the Analysis of Growth of the Common Mussel (*Mytilus edulis*) larvae Using AxioVision Software and Manual Measurements (K. Alfsnes & T. Baussant)

VI. Effects of suspended particles of drilling mud on survival and growth of cod larvae (R. K. Bechmann, I. C. Taban, R. C. Sundt, T. Baussant, E. Otterlei, S. Handeland)

VII. Changes in protein pattern (analysed by SELDI-TOF) in plasma from cod and haemolymphe from mussel and scallop exposed to suspended particles of water based drilling mud (D. Pampanin & R. K. Bechmann*).*

VIII. Prediction of metal bioaccumulation in organisms exposed to drilling mud (Mathijs Smit).

Acknowledgement

We are grateful to the Norwegian Research Council for financing this project through the PROOF program (project no. 159183/S40). All experiments and analyses were preformed in 2004 and 2005.

Summary

Drilling discharges spread over large areas and remain in the water column for a long time. Between 50 000 and 100 000 tons of barite is discharged to the North Sea each year. A wider spread of particles is associated with the use of water-based mud compared to oil-based mud. Discharges of oil-based mud is no longer allowed to the North Sea. Consequently, it is important to know whether negative effects can be expected in organisms exposed to suspended particles from water based drilling mud (WBM). Potential impacts from drilling discharges need to be elucidated to clearly establish what actions, if any, are needed to comply with the "no harmful environmental effects" goal stated by the Norwegian petroleum industry and the Norwegian State Pollution Authorities.

The two main objectives of our project was to study possible effects of drilling mud discharges on water column organism (plankton and fish), and filter feeding bivalves, and to analyse metals in fish and bivalves exposed to suspended particles of drilling mud to find out if metals from the mud was taken up by the organisms.

We have developed an exposure system where the smaller range of particles in the drilling mud can be tested. These particles are most relevant for animals in the water column, and bottom living animals at some distance from the discharge. Cods, scallops and mussels have been exposed to barite and to three concentrations of used water based drilling mud (WBM) with barite as the weighting material (0.5, 2 and 20 mg/L dry weight). In addition the cods were exposed to ilmenite. A mixture of metals was used as a positive control exposure for all three species (concentrations in the exposure tanks: 10.2 µg/L Cu, 34.8 µg/L Zn, 1.4 µg/L Cd and 3.2 µg/L Pb). After three weeks exposure, samples of the animals were taken for analysis of metals in the tissues, biomarker responses and histopathology.

Long term exposure of both mussels and scallops have been done to test if the used water based drilling mud affected growth and filtration rate.

Negative effects of drilling mud particles (used WBM and barite) was observed in the concentration range 0.5 – 62 mg/L. Scallops were most sensitive to the used mud, but negative effects were also observed in cod and mussels (adults and larvae). Drilling mud caused reduced filtration rate and growth for bivalves in addition to histological damage to gills and digestive gland. In addition changed protein patterns (especially for bivalves), increased oxidative stress (TOSC) in bivalves (especially for mussels), reduced lysosomal membrane stability (bivalves), and indications of DNA damage in bivalves was observed. A summary of the main effects and results from analysis of metals in the tissues is given below.

The highest concentration of used WBM (and barite and ilmenite particles) are in the range (10-50 mg/L) estimated to be found in the lower 10 meters of the water column 500 meters from the spill site following a batch release of water based drilling mud (*pers. com.* Mathijs Smit, TNO). The lowest concentration tested in our experiments is close to the concentration where Cranford *et al*. detected responses on growth of Canadian scallops *(Placopecten magellanicus*) exposed to barite.

Below is an overview of the main results from the project. Only statistically significant differences have been included.

WBM: Suspended particles of used water based drilling mud with barite as weighting material

0.5 mg/L used WBM

Metal accumulation. Significantly increased level of:
- Ba in gill and digestive gland of mussels and scallops
- Cu in digestive gland of scallops

Effects:
- Significantly reduced filtration rate (scallops)
- Changed protein pattern in plasma/haemolymph (mussels and cod and scallops)

2 mg/L used WBM

Cod exposed to 4 mg/L. The bivalves reduced the concentration to 2 mg/L.
Metal accumulation. Significantly increased level of:
- Ba in gill and digestive gland of mussels and scallops

Effects:
- Changed protein pattern in plasma/haemolymph (cod, mussels and scallops)
- Increased oxidative stress (Total Oxygen Scavenging Capacity) (mussels)
- Reduced lysosomal membrane stability (scallops)
- Histopathology: Tissue damage (gills of cod, mussels and scallops)
- Reduced filtration rate (scallops)
- 40% reduced survival time (LT50) in 'stress on stress' test (mussels)
- Reduced feeding efficiency of mussel larvae
- Reduced growth of mussel larvae

20 mg/L used WBM

Cod exposed to 39 mg/L. The bivalves reduced the concentration to 20 mg/L.
Metal accumulation. Significantly increased level of:
- Ba in gill and digestive gland of mussels and scallops and in gills of cod
- Cu in digestive gland of mussels and scallops
- Pb in digestive gland of mussels and scallops and gill of scallops
- Zn and Cd in liver of cod

Effects:
- Changed protein pattern in plasma/haemolymph (cod, mussels and scallops)
- Increased oxidative stress (TOSC) (mussels)
- Increased level of DNA strand breaks (scallops)
- Reduced lysosomal membrane stability (scallops)
- Histopathology: Tissue damage (gills of mussels and scallops)
- Reduced filtration rate (mussels and scallops)
- 30% reduced survival time (LT50) in 'stress on stress' test (mussels)
- Reduced growth or condition factor (mussels, scallops and cod)
- Increased mortality following long term exposure (scallops)

Barite particles

Metal accumulation
Cod exposed to 62 mg/L barite. Significantly increased level of: Ba in gill
Scallops and mussels exposed to 23 mg/L barite. Significantly increased level of:
- Ba in gill and digestive gland of mussels and scallops
- Cu in gill and digestive gland of mussels and scallops
- Zn in gill of mussels
- Cd in gill of scallops
- Pb in gill and digestive gland of mussels and scallops

Effects:
- Changed protein pattern in plasma/haemolymph (more response in scallops than cod and mussels)
- Increased oxidative stress (TOSC) (mussels and scallops)
- Increased level of DNA strand breaks (scallops)
- Histopathology: Tissue damage (gills of cod, mussels and scallops)
- Reduced filtration rate (scallops)
- Reduced growth or condition factor (mussels, scallops and cod)

Ilmenite particles (cod only)

Metal accumulation. Significantly increased level of:
- Zn in gill and liver
- Cd in liver

Effects:
- Histopathology: Tissue damage in cod gills
- Proteomics: Changed protein pattern in plasma
- Slightly reduced condition factor and liver somatic index (not stat. sign.)

Metal mix (Cu, Zn, Cd and Pb)

Mussels and scallops. Significantly increased level of:
- Cu in gill and digestive gland of mussels and scallops
- Zn in gill of mussels and scallops and in digestive gland of scallops
- Cd in gill and digestive gland of mussels, and in digestive gland of scallops
- Pb in gill and digestive gland of mussels and scallops

Cod. Significantly increased level of:
- Cd in gill of cod
- Pb in gill, bile and liver of cod

Effects:
- Changed protein pattern in plasma/haemolymph (scallops, cod, mussel).
- Increased oxidative stress (TOSC) (mussels and scallops)
- Reduced lysosomal membrane stability (mussels and scallops)
- Histopathology: Tissue damage (mainly on scallops gills, less on gills of cod and mussels)
- Reduced filtration rate (mussels and scallops)
- Reduced growth (scallops)
- Increased mortality following long term exposure (scallops) (not tested statistically)
- 50% reduced survival time (LT50) in 'stress on stress' test (mussels)
- Increased percentage of deformed mussel larvae; none of the embryo developed to D-shell larvae

I.

Exposure of fish and bivalves to suspended particles of drilling mud: A new continuous flow exposure system

R.K. Bechmann, L. P. Myhre, R. C. Sundt

ABSTRACT

Drilling discharges spread over large areas and remain in the water column for a long time. Between 50 000 and 100 000 tons of barite is discharged to the North Sea each year. A wider spread of particles is associated with the use of water-based mud compared to oil-based mud Consequently, it is important to know whether negative effects can be expected in organisms exposed to suspended particles from water based drilling mud. A new continuous flow exposure system has been developed to test the effects of the small particles of drilling mud present in the water column following off shore drilling operations. Both fish and filter feeding bivalves can be exposed in the new exposure system. This paper gives a description of the exposure system including details about the size and number of particles in exposure tanks with fish and bivalves.

INTRODUCTION

Drilling discharges spread over large areas and stay in the water column for a long time (Rye et al., 1998, Muschenheim and Milligan, 1996). The potential for impacts are thus considerable given the volumes and suit of components being discharged. Drilling mud with water based fluids (WBM : Water Based Muds) are the only currently being discharged to the North Sea. A wider spread of particles is associated with the use of water-based mud compared to oil-based mud. The fate of historic drilling discharges have been investigated through the UKOOA studies (Westerlund et al. 2001, Kjeilen et al., 2001, see also UKOOA web-site: www.oilandgas.org.uk). Through this work, it was established that cuttings piles may be affected by storm incidents down to depths of 100 m (Sabeur et al. 2002). Studies have also shown that erosion of cuttings piles may be a significant process (Vefsnmo and Lothe, 2001), resulting in re-suspension and spreading in the water column. Hence, both pelagic and benthic organisms can be repeatedly exposed. Consequently, it is important to know whether negative effects can be expected in organisms exposed to suspended drilling mud particles.

The drilling mud is designed to have specific properties such as cooling and lubricating the drilling bit, balancing underground hydrostatic pressure and to transport drilled cuttings away from the head of the drill bit (OLF, 2001). The weighting agent, usually barite or ilmenite, can make up more than 90% of the drilling formulation (the mud). To meet the required mud design criteria, the weighting materials are used as small particles with a diameter of 15-20 micrometer. Barite and ilmenite may contain trace impurities of an array of different metals, and if these metals are bioavailable they may cause toxicity. Moreover, the largest discharge of chemicals to the sea from oil production comes from drilling of the wells (SFT. 2000). Chemicals added to obtain wanted quality of drilling mud include compounds such as viscosifiers, emulsifiers, biocides, lubricants, wetting agents, corrosion inhibitors, surfactants, detergents, caustic

soda, salts, organic polymers and fluid loss control agents. These chemicals may remain on the discharged drilling mud particles, and may be available to organisms in the water column from ingested particles or particles trapped on the gills. Barite has the longest tradition for use as a weighting material in offshore drilling operations. The use of ilmenite as a weight material in drilling mud started quite recently in Norwegian offshore drilling operations. It was first used drilling two wells at the Ekofisk 2/4 X platform (Ekofisk field centre), and is now being used for all drilling operations by operator Statoil.

In addition to the possibility of physical effects, leakage of metals and adsorbed drilling mud chemicals may cause effects in animals exposed to suspended particles of drilling mud. It is important to use an exposure system that simulate conditions some distance away from the drilling site. The drill cuttings and the larger particles will sink rapidly, but the small particles may be transported far away from the drill site. The new exposure system was made to test whether the small particles cause effects on filter feeding bivalves and on fish. The new exposure system and the particle exposures in the different treatments will be described.

The methods used to monitor the particle exposure is described in the materials and methods section. A description of the exposure system including technical details and data on the exposure conditions for fish and bivalves in the exposure tanks is given in the results and discussion section.

MATERIALS AND METHODS

Concentration of particles

Water samples from a series of sampling times have been filtrated to determine the dry weight of the particles in the exposure tanks. A Whatman GF/F: 0.7 filter retaining particles down to 0.7 µm (also used by Cranford, 1995). The filter was weighed before filtration of the water sample and again after drying at 80°C for 24 h. The concentration of particles in the lowest used WBM exposure has been estimated from filtered water samples from the inlet to the exposure tank (from the Teflon tube delivering stock solution of particles to the tank). The flow and particle size distribution of samples from the inlets was monitored regularly to ensure that the input of particles to the tank was constant.

Particle size distribution

Laser analysis of particle size distribution was done on samples of the used WBM, the barite and the ilmenite particles (Malvern instruments Ltd, Mastersizer 2000 Ver 5.1). The Malvern instrument measures particles with diameter in the range 0.02 – 2000 µm, but this method can not be used for determination of particle size distribution of highly diluted samples of particles in sea water. The size distribution of particles in samples from the exposure system was measured by a Coulter© multisizer (model TA II, Coulter, Toronto, ON, Canada). The 70 µm aperture measures particles with diameters in the size range 1.6 – 50 µm. The quantitative data from filtering of water samples give the most correct data on the concentration of particles in each exposure. The Coulter Counter data show the relative number and volume of particles in the inlet and in the tank, and relative differences between barite, ilmenite and used mud.

Analysis of particle size distribution with the Malvern instrument showed that for used WBM 14 % of the particles were smaller than 1.6 µm and 4 % were larger than 50 µm, 6 % of the barite particles were smaller than 1.6 µm and 25 % were larger than 50 µm, 2 % of the ilmenite particles were smaller than 1.6 µm and 8 % were larger than 50 µm. Hence particle size distribution measured with the Coulter counter underestimate the volume of small particles most for the used WBM and the large particles for the barite, while a very small faction of the ilmenite particles are excluded from the Coulter measurements.

Pilot test

A pilot test was done to find out if the concentration of particles was the same at different depths in the exposure tank. A stock solution of 100 L seawater and 5 kg used WBM was made and the system was run for ten days. Water samples were taken five times at 8 cm, 23 cm and 38 cm depth (the total water depth was 48 cm). The samples were taken ca 30 cm from the tank wall.

Mud sinking test

A test was done to compare sinking times for used WBM particles, barite and ilmenite particles in a static system. Stock solutions (3 L) of diluted used WBM, barite and ilmenite particles were prepared in 5 L beakers. The number and volume of particles with diameter in the range 1.5 – 50 µm were measured by Coulter counter after 15, 30, 60 and 120 minutes.

Mud filtration test

Mussels *(Mytilus edulis)* and scallops *(Pecten maximus)* were exposed to mud in a static system (10°C; same temperature as in the exposure system) to find out how much mud they were able to filter from the water. Scallops from the control and mussels exposed to 2 mg/L mud were used in these experiments. Seven beakers (5 L) were filled with 3 L mud + seawater from the inlet to the 2 mg/L used WBM exposure. One mussel was placed in each beaker and the number of particles with diameter in the range 1.5 – 3.5 µm were measured after 8, 16, 32 and 64 minutes by Coulter counter. One hour after this experiment was finished a new experiment was started with the same mussels. New samples of 2 mg/L used WBM was taken for the inlet to the exposure tank and algae were added in addition. The size of the algae are larger than most of the mud particles. Number of particles with size between 1.5 – 3.5 µm (mud) and 3.5 – 8 µm (algae/*Isochrysis*) was calculated by the Coulter counter.

RESULTS AND DISCUSSION

Description of the exposure system

A new continuous flow exposure system has been made to simulate the conditions in the water column following off shore drilling operations. Three concentrations of used water based drilling mud with barite as the weighting material was tested in addition to one treatment with barite particles and one with ilmenite (only with cod). There was one unit (figure 1A) for each of these 5 treatments. In addition there was one control tank for cod and one for bivalves. Cod, mussels and scallops were exposed for 3 weeks to

study metal accumulation, biomarker responses and histopathology (PAPER II-IV). Scallops were also exposed for 10 weeks, and mussels for 6-8 weeks to study growth and feeding efficiency.

The principle of the exposure system is illustrated in figure 1A. For each particle treatment a stock solution of particles diluted in seawater was made in a 100 L header tank. The particles were kept in suspension by a propel. The stock solution was pumped into two exposure tanks, one for fish and one for bivalves. The flow of stock solution into each tank was 8 ml/min and this was diluted with 2 L/min sea water. Circulation pumps helped to keep the particles in suspension in the exposure tanks. The retention time was approximately 4 hours. The header tanks were refilled when the level of stock solution was reduced with approximately 50 %. When serving both fish and bivalve tanks this was done every second day, and when serving only the bivalve tank the header tanks were refilled twice each week. Sedimented mud and faeces was siphoned from the bottom of the exposure tanks twice each week, and the outlets were flushed (but the bivalves were kept submerged at all times).

The header tanks were made of polyeten LD (Polimoon Cipax). The diameter of the header tanks were 52 cm and the height 71 cm. A stirrer was mounted at the top (Eurostar-ST P DV), and a stainless steel propel was used at 300 rpm. The propel diameter was 10 cm, and the length of the rod 50 cm. A peristaltic pump (25 rpm) was used to deliver stock solution to each exposure tank. Marprene (1.6 mm i.d.) tubing was used in the pump, and teflon tubing (1.9 mm i.d.) on each side. The volume of water in the exposure tank was 500 L (1 m x 1m, 50 cm depth). The header tank for ilmenite and the propel was smaller than for the other treatments, and the exposure tank was not as tall (deep) as the other headertanks. Mussels and scallops were kept in cages (figure B,C). Seawater (34 ppt salinity, 9 ± 1°C) was directly pumped from 80 m depth (Atlantic water) and sand-filtered prior to use.

The nominal concentrations of particles (dry weight) in the used WBM exposures were 0.9 mg/L, 9 mg/L and 90 mg/L. To prepare the three concentrations of used WBM 50, 500 and 5000 g of (wet) used WBM was added to 100 L seawater while stirring. The nominal concentration of barite was 180 mg/L dry weight. The stock solution in the header tank consisted of 4500 g (dry) barite and 100 L of sea water. The nominal concentration of ilmenite was 180 mg/L dry weight. A smaller header tank (60 L) and propel was used for the ilmenite exposure. The stock solution consisted of 1800 g (dry) ilmenite to 40 L sea water.

Description of the size and number of particles in the exposure tanks

The pilot test showed that there was no statistically significant difference in dry weight of particles at different depth in the exposure tank, but there was some variation with time depending on the level of stock solution in the header tank. The header tanks were filled up more frequently in the main experiment to reduce variability in the exposure concentration. The overall mean concentration of particles in the water in the pilot test was 30 mg/L (st. dev. 6). In the main experiment all water samples were taken at mid depth (23 cm depth).

The water in the exposure tank with the lowest concentration of used WBM (0.5 mg/L) was transparent, but in the 2 mg/L tanks the water was slightly muddy. In the 20 mg/L used WBM the water was milky/brownish-grey and the visibility was very low. It was not possible to see the cod, and the bivalves were only visible in the upper compartment

of the cage. The 23 mg/L barite exposure was similar to the 20 mg/L used WBM exposure, but the water appeared to be even more muddy and more brown than grey in colour. The ilmenite particles coloured the water black and reduced the visibility.

The measured concentrations of particles in the exposure tanks were considerably lower than the nominal concentrations (table 1). The difference between nominal and measured concentration of particles in the exposure tanks for fish was largest for ilmenite (92 % reduction), less for barite (66 % reduction) and least for the used mud exposures (56 % reduction) (table 1). When calculating the nominal concentration only the dilution of the added amount of particles is taken into consideration; it is assumed that no particles are lost anywhere in the system. A fraction of the particles did, however, sink to the bottom of both header tanks and exposure tanks. Particle size distributions for stock solutions of particles in sea water and samples from the exposure tanks show that it is mainly the larger particles that are lost (figure 3).

The sinking time for used WBM particles, barite and ilmenite particles has been studied in a static system to find out if sinking of the 'larger' particles can explain the difference between nominal and measured concentration of particles. The volume of particles that had sunk after 2 hours in a static system represented 50 % of the used WBM particles and 80 % of the ilmenite and barite particles. The largest reduction of volume of particles was observed during the first 15 minutes for all treatments (ca. 40 %), indicating that sinking of larger particles explain the lower measured than nominal concentration of particles in the exposure tanks. The *number* of used WBM particles, however, was only reduced with 5 % after 2 hours, and the number of ilmenite and barite particles was reduced with 30 % and 50 %, respectively. The used WBM had more of the smaller particles and hence the difference between nominal and measured particle concentration was smaller than for barite and ilmenite. A larger part of the added particles of ilmenite than barite was lost due to sedimentation in header tank and exposure tank. This may be due to the difference in experimental design, in addition to different properties of the particles.

The dry weight of particles per litre was lower in samples from the bivalve tanks than the fish tanks (table 1). The particle concentration in the barite exposure tank with bivalves was 63 % lower than in the corresponding fish exposure tank, and the concentration in the high and medium used WBM exposure tank with bivalves was 50 % lower than in the in the corresponding fish exposure tanks. Coulter counter data confirm that the volume of particles was lower in bivalve tanks than in cod tanks, although the flow of stock solution into the tanks and the particle size distribution and volume of particles in samples from the inlet was similar (data not shown). The ability of mussels and scallops to remove suspended mud particles from the water was tested to validate that the filtering activity of the bivalves could explain the difference in concentration between cod and bivalve tanks. Each mussel and scallop was able to remove more than 50 % of the particles from 3 L of seawater with used WBM particles (2 mg/L) during one hour (table 2/figure 6). The scallops removed a similar number of mud particles regardless of whether algae were present or not, but the mussels removed mud particles more efficiently when algae were present (figure, table, Student's paired t-test, $p < 0.0001$). During one hour the mussels removed 80 % of the mud particles when algae were present. Due to sampling, the total number of mussels and scallops

varied from 120 – 240 during the experiment. The retention time for water in the exposure tanks was 4 hours. When each bivalve can remove more than 50% of the particles in 3 litres during one hour it is likely that > 120 bivalves can keep the concentration of particles in 500 L at a 50 % lower level than in the fish tanks, even though it is a continuous flow system.

Particle size distributions based on volume of particles. The mean particle size for the used WBM (13 µm) was considerably smaller than for the barite particles (33 µm) (Figure 2). The mean particle size for the ilmenite particles was 20 µm. It is the particles in the used WBM that are most relevant for the drilling mud discharge. Mean particles size (based on volume) for stock solutions measured by the Coulter counter was lower (9-10 µm for all treatments) because only particles in the range 1.6 – 50 µm were measured (Figure 3). Mean particle size for samples from the exposure tanks was smaller than in stock solutions because the larger particles had sunk to the bottom of the header tanks and exposure tanks. In the high and medium concentration of used WBM mean particle size based on volume was 4.3 µm. In the lowest used WBM concentration the mean was higher (7.6 µm). The larger particles appeared to be more easily kept in suspension when the concentration in the header tank was lower, giving a larger mean particle size. The mean particle size in the exposure tank with barite and ilmenite was 5.6 µm and 4.9 µm, respectively.

Number of particles. The number of particles per ml may be more important for the effect of particle exposure than the volume (or weight) of the particles. Many small particles may cause more damage to e.g. gills than a few large particles, and it is the small particles that will be transported away from the drill site. The mean number of particles with diameter in the range 1.6 – 50 µm in our seawater was 2700 particles per ml (n = 16). The number of particles per ml in the low, medium and high exposure to used WBM was approximately 6, 30 and 300 times higher, respectively, than in the seawater (n = 4). The number of particles in the barite and ilmenite exposures were approximately 200 and 40 times higher than in seawater, respectively (n = 4). More than 90 % of the number of particles were smaller than 5 µm both in the stock solutions and in samples from the exposure tanks, but the volume of the particles with diameter above 5 µm represented around 70 % of the volume of particles in the stock solutions, and around 40 % of the volume of particles in the exposure tank. The potential problems for animals that are exposed to suspended drilling mud particles may not be reduced as fast as the reduction in concentration (dry weight/volume) of particles if it is the physical stress of small particles that cause effects (either because of physical stress or leaking of metals or adsorbed drilling mud chemicals).

The highest concentration of used mud (and barite and ilmenite particles) are in the range (10-50 mg/L) estimated to be found in the lower 10 meters of the water column 500 meters from the spill site following a batch release of water based drilling mud (*pers. com.* Mathijs Smit, TNO). The lowest concentration is close to the concentration where Cranford detected responses on growth of Canadian scallops *(Placopecten magellanicus)* exposed to barite.

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TABLES

Table 1. Dry weight of particles pr litre in exposure tanks with cod and bivalves. Nominal concentrations are calculated based on dry weight of particles added to the headertanks. Measured concentrations are based on filtering of water samples from the exposure tanks (GF/F; 0.7 µm). Mean ± st.dev (number of samples). WBM: water based mud with barite as the weighting material.

Treatment	Nominal concentration (mg dw/L)	Measured concentration (mg dw/L)	
		Cod	Bivalves
Ilmenite	180	14 ± 13 (7)	-
Barite	180	62 ± 18 (6)	23 ±13 (12)
High conc. Used WBM	90	39 ± 8 (8)	20 ± 7 (12)
Medium conc. Used WBM	9	4 ± 1 (7)	2 ± 1 (13)
Low conc. Used WBM based on mg mud in inlet to tanks	0.9	0.5 (2)	0.5(2)

Table 2. Filtering of mud particles (range 1.5 – 3.5 µm) with or without algae present, and filtering of algae (3.5 – 8 µm) with mud present. Seven individual mussels and scallops were tested for each treatment.

Treatment	Time (min)	Scallops from control Mean ± St. dev.		Mussels from 2 mg/L used WBM Mean ± St. dev.	
		Mud particles 1.5-3.5 µm	Algae 3.5 - 8 µm	Mud particles 1.5-3.5 µm	Algae 3.5 - 8 µm
2 mg/L used WBM + algae	8	95 ± 5	91 ± 13	82 ± 8	86 ± 6
	16	87 ± 6	60 ± 13	65 ± 8	66 ± 9
	32	62 ± 8	15 ± 6	48 ± 7	46 ± 9
	64	36 ± 6	1 ± 1	20 ± 7	18 ± 8
2 mg/L used WBM	8	85 ± 6		92 ± 6	
	16	74 ± 6		81 ± 10	
	32	65 ± 11		67 ± 19	
	64	42 ± 5		48 ± 19	

Figure 1

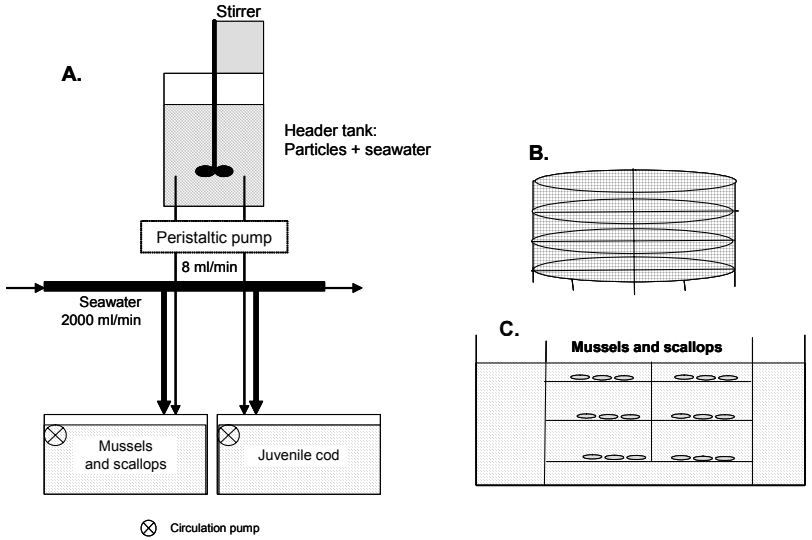

Figure 1. Principle of the exposure system; one unit like A for each particle exposure (barite, ilmenite, and 3 concentrations of used water based mud (WBM) with barite as the weighting material). B: Cage for mussels and scallops. C: Bivalve tank.

Figure 2

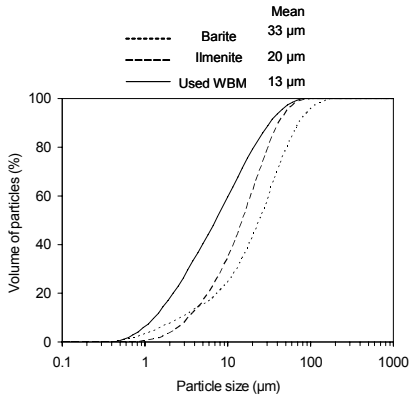

Figure 2. Particle size distribution and volume weighted means for barite, ilmenite and used water based mud (WBM) particles with diameter in the range 0.02 – 2000 µm. The graphs show accumulated volume (%) of particles with size below x µm. Laser analysis done with Malvern instruments Ltd, Mastersizer 2000 Ver 5.1.

Figure 3

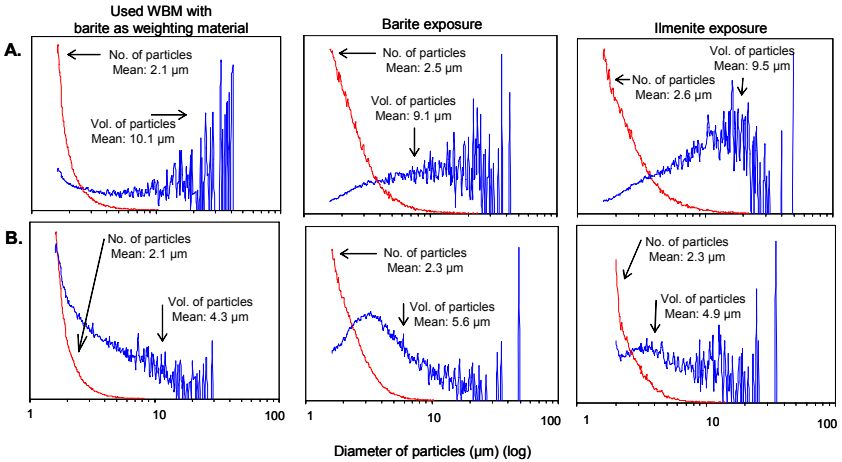

Figure 3. Particle size distributions based on volume and number of used WBM, barite and ilmenite particles with diameter in the range 1.5 – 50 µm. Top panel (A): stock solutions of particles in seawater; B): samples taken in the exposure tanks (mean for 4 representative sampling days). The mean particle size based on volume and number of particles is given in each figure.

Figure 4

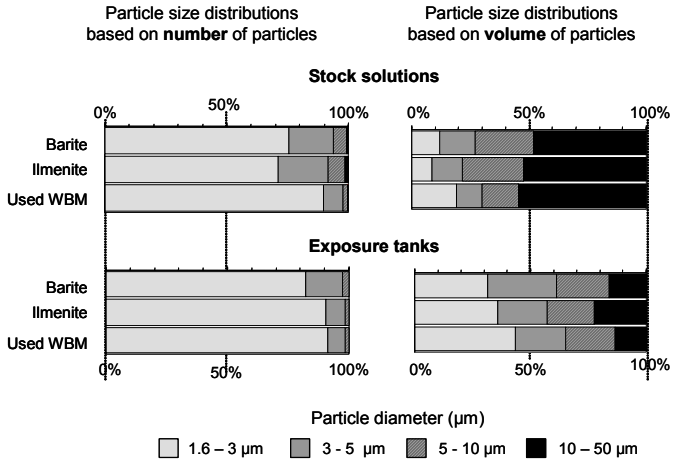

Figure 4. Differences in particle size distribution between stock solutions and exposure tanks. For each type of exposure the percentage of particles within 4 size ranges is illustrated. The figure show data based on number of particles (left) and volume (right) of particles within each size range.

Figure 5

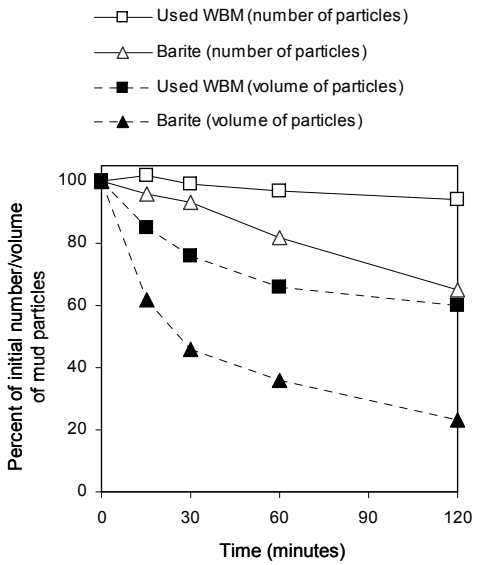

Figure 5. Sinking of drilling mud particles in a static system. The 5 L beakers used for testing feeding efficiency of mussels and scallops exposed to mud was also used to test sinking time for particles in the used WBM and the barite particles themselves. The number and volume of particles with mean size 1.5 – 50 µm was measured by coulter counter. Percent reduction of the number of particles and the volume of particles is plotted against time. The dashed lines show how the volume of particles was reduced with time for the used WBM and the barite, and the line with open markers show how the number of particles was reduced with time.

Figure 6

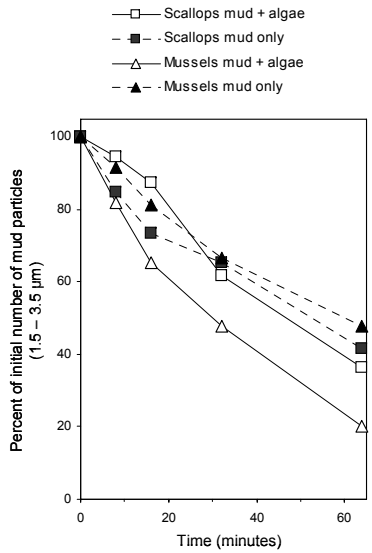

Figure 6. Filtering of mud particles (range 1.5 – 3.5 µm) with or without algae present. Seven individual mussels and scallops were tested for each treatment. Percentage of initial number of mud particles is plotted against time.

II.

Metals in tissues of mussels, scallops and cod exposed to suspended particles of water based drilling mud

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ABSTRACT

Cod (*Gadus morhua*), mussels (*Mytilus edulis*) and scallops *(Pecten maximus)* were exposed for 3 weeks to 3 concentrations of used drilling mud with barite as weighting material, barite particles and to a mixture of metals as a positive control (10.2 µg/L Cu, 34.8 µg/L Zn, 1.4 µg/L Cd and 3.2 µg/L Pb in the exposure tanks). The cod were exposed to ilmenite particles in addition to the other treatments. Concentrations of metals were analysed in gills and digestive glands of mussels and scallops, and in gills, liver and bile of the cod.

In addition to a large increase in barium in the gills and digestive glands of the bivalves, Cu, Zn, Cd and Pb increased significantly in some tissues. Significant increase in the level of Cu, Zn, Cd and Pb in cod, mussels and scallops was only detected in animals exposed to relatively high concentrations (> 10 mg/L) of drilling mud particles. The accumulated metals may be unavailable inside particles trapped in the tissues, and may not contribute to the overall effects observed in bivalves and cod exposed to drilling mud particles. Based on the partition coefficients for these metals in the water it is unlikely that they can leak out of the particles, but we can not exclude the possibility that metals are available for uptake once they are adsorbed to or absorbed in the tissues. It is also a possibility that predators on filter feeding organisms may take up metals from their prey (lower pH in the digestive system). The metal concentrations in animals exposed to drilling mud particles generally increased more in the filter feeding bivalves than in the fish. The dissolved metals in the positive control was also accumulated to a lesser extent in fish than in bivalves. The increased levels of some metals in liver and bile of cod exposed to used drilling mud with barite and to the ilmenite particles indicate that metals may be available for uptake in exposed organisms.

INTRODUCTION

One of the main objective in the project was to study bioavailability of metals from suspended drilling mud particles. Cod (*Gadus morhua*), mussels (*Mytilus edulis*) and scallops *(Pecten maximus)* were exposed for 3 weeks to 3 concentrations of used drilling mud with barite as weighting material (0.5, 2 and 20 mg/L), barite particles (23 mg/L) and to a mixture of metals as a positive control (10.2 µg/L Cu, 34.8 µg/L Zn, 1.4 µg/L Cd and 3.2 µg/L Pb in the exposure tanks). The cod were exposed to ilmenite particles (14 mg/L) in addition to the other treatments. Concentrations of metals were analysed in gills and digestive glands of mussels and scallops, and in gills, liver and bile of the cod.

Barite is collected from quarries, and is composed of $BaSO_4$ which may contain trace impurities of an array of different metals. The barite always contains Sr and Pb as these metals precipitates as sulphates together with the Ba in the formation of barite. Other trace elements like Cu, Cd and Zn is often also found in the barite. Different sources of barite are more or less

contaminated with metals, and Norwegian operators need to meet the OLF barite purity recommendations. Although this is a considerable improvement from using the more contaminated sources of barite, there are still significant metal contamination in the discharged mud. Barite has the longest tradition for use as a weighting material in offshore drilling operations. The use of ilmenite as a weight material in drilling mud started quite recently in Norwegian offshore drilling operations. It was first used drilling two wells at the Ekofisk 2/4 X platform (Ekofisk field centre), and is now being used for all drilling operations by operator Statoil. The ilmenite is a mineral used to produce Titanium (Ti), and the main constituents of ilmenite are Ti- and Fe-oxides. The ilmenite used in the North Sea is from Tellnes, Norway. The ilmenite also typically contains traces of Zn, Ni and Cr. The total concentration of these elements is found at higher levels in ilmenite compared to "normal" levels in barite. The total concentration of Pb and Cu seems however to be lower (Fjogstad et al., 2002).

Preliminary results from the UKOOA studies show that suspended barite particles proved significantly more toxic than suspended ilmenite particles. Since ilmenite contained significantly lower level of heavy metals than barite, the results may indicate effects of dissolved metals. Leaching studies of metals from historic cuttings material (Westerlund *et al.* 2001) showed that the elements Zn, Cu, Cd and Hg were found in fractions loosely bound to particles, thus indicating that these elements have a potential to accumulate in biota.

METHODS

The exposure system is described in paper I, and the effects are described in paper II and III below.

Metal analysis of tissues from mussels, scallops and cod

Metals in Bile. The bile for the metal analysis was transferred to 10 ml polypropylene test tubes. Depending on the availability of the bile in the fish 50-200 µl bile was transferred to the test-tube. The sample was then digested by adding 100 µl 15 M HNO_3 and 100 9 M H_2O_2. The digestion was performed in a water bath for 2 hours at 80 degrees. The sample was then diluted to 5 ml total volume with dilute HNO_3. This solution also contained In (indium) to give In-concentration of 5 µg/l to act as internal standard. The metals were then analysed with ICP-MS.

Metals in Tissue. The liver tissue samples were stored in a freezer in polypropylene vials after the dissection of the fish. The liver sample was then digested with a mixture of HNO_3 and H_2O_2 in a microwave oven system. The other tissue samples were digested with HNO_3 only. About 1 gram tissue sample was placed in a Teflon vessel, 5 ml 15 M HNO_3 and 1ml 9 M H_2O_2 vas added. The Teflon vessel was sealed and placed in the microwave oven. The samples were then diluted to 50 ml in 50 ml polypropylene test tubes. The samples were diluted further and In was added to act as internal standard. The final metal determination was performed using an ICP-MS (see below).

ICP-MS metal determination. For the determination of the metals a VG-PQ2+ ICP MS was used. A peak jump procedure was used with as many masses of each element to be able to evaluate possible isobaric interferences. In the procedure indium was used as internal standard to compensate for instrumental drift. In evaluating the results the mass with the highest abundance and lowest interferences was used for the quantification. The operating conditions can be found in table 1.

RESULTS

Metal concentration in barite, ilmenite and used WBM

There was less barium and more lead in the used WBM than in the barite (barite compared to 'used mud dos' in table 2). The concentrations of Cu, Zn and Cd were similar in the barite and the used WBM. The concentration of several other metals was higher in the used WBM than in the barite. The concentrations of metals in the used WBM were more similar to the metal concentrations in a typical coastal clay sediment (the right most column in table 2), indicating that the barite had been mixed with other particles and/or that particles from the bedrock affect the metal content of the used WBM compared to the barite.

Metal concentration in the positive control

The nominal concentrations of metals in the positive control (metal mix) exposure were: 2 µg/L cadmium, 15 µg/L copper, 40 µg/L zinc, and 5 µg/L lead (sum metals: 62 µg/L). The measured concentrations were slightly lower than the nominal (table 3), and the concentrations were constant during the experiment.

Background levels of metals in the tissues

The highest metal concentrations were detected in the digestive glands of the bivalves, followed by the cod gills (table 4, figure 1). There was high concentrations of Fe in most tissues, but in mussel gills and cod bile the concentrations were below the detection limit (< 100 mg/kg wet weight or pr litre of bile). The total concentration of the analysed metals decreased in the following way:

Mussel DG > Scallop DG > Cod gills > Scallop gills > mussel gills ~ cod liver and bile

The total concentration of metals excluding Fe decreased in a similar way:

Scallop DG > Mussel DG > Cod gills > mussel and scallop gills ~ cod liver gills > cod bile

The four metals selected for the positive control were copper, zinc, cadmium and lead. Table 4 show the mean concentration of these metals in the tissues of the control animals. The concentration of Zn was highest in all tissues except bile where the Zn concentration was considerably lower than in all other analysed tissues. The copper concentration was higher than the concentrations of Cd and Pb, except in scallop digestive gland where the concentration of Cd was 100 times higher than in the digestive gland of mussels.

The background level of Cu, Zn, Cd and Pb was higher in the digestive gland than in the gills of the bivalves (table 4 and 5). In bivalves exposed to the mixture of metals the concentrations in general increased more in gills than digestive gland, but the results are more variable for the particle treatments (table 5).

Positive control: Exposure to a mixture of metals

Mussels, scallops and cod exposed to dissolved metals in the positive control accumulated more Cu, Zn, Cd and Pb than in any other treatment. The highest increase of Cu, Pb and Zn was detected in gills from mussels in the positive control. The concentrations of Cu, Pb and Zn were 46, 8 and 2 times higher than the control respectively (table 7, figure 2). The highest increase of Cd was observed in gills of cod from the positive control, where the Cd concentration was 4 times higher than in the control (table 7, figure 3).

Lead (Pb). The concentration of Pb was significantly (t-test, $p<0.05$) increased (2-8 times compared to control) in all the analysed tissues of metal exposed mussels, scallops and cod.

Cadmium (Cd). The concentration of Cd was significantly increased (3-4 times) in mussel gill, mussel and scallop digestive gland and cod bile. There was a tendency to increased Cd concentration in cod liver (t test, $p = 0.055$) and bile (t-test, $p = 0.1$). In the scallop gill the concentration of Cd was 30 % lower than in the control (t-test, $p<0.05$).

Copper (Cu). Cu was significantly increased in all the bivalve tissues (3-46 times). The accumulation of Cu in mussel and scallop gills was the highest observed for any metal (except barium in mud exposed animals). The mean Cu concentration in mussel gills was 46 times higher than in the control, and the concentration in scallop gills was 18 times higher.

Zinc (Zn). Zn was significantly increased (t test, $p < 0.05$) in mussel and scallop gills and in scallop digestive gland. There was a similar increase in the mussel digestive gland (t test, $p = 0.05$). The concentration of Zn was 40 % reduced in the bile of cod from the positive control (t-test, $p<0.05$).

Based in these results the recommended tissues for studies of accumulation of dissolved metals would be mussel gills followed by scallop digestive gland. Scallop gills and mussel digestive gland are also appropriate tissues for studying Cu and Pb accumulation, and cod gills can be used to study accumulation of dissolved Pb and Cd.

Metal accumulation in cod. Lead was the only metal that increased significantly in all the analysed tissues from cod exposed to a mix of metals dissolved in seawater. Copper did not increase significantly in any of the tissues, cadmium increased significantly only in the gills and zinc was significantly *reduced* in the bile of cod exposed to dissolved metals. Hence there is no simple correlation between water and tissue concentration of these metals, even when they are dissolved in seawater. This makes it challenging to conclude about the relationship between metals in drilling mud and metals in the tissues of cod.

Barium in the tissues of cod and bivalves exposed to drilling mud

There was a dose dependent increase of barium in gills and digestive gland of mussels and scallops exposed to the three concentrations of used drilling mud (table 6). The barium concentration in cod gills was higher in the barite exposure than in the highest mud exposure. The barium concentration was not significantly changed in bile and liver of mud exposed cod. Metals have not been analysed in cod from the lower exposure concentrations.

The concentration of barium in bivalves exposed to 23 mg/L barite particles was higher than in the 20 mg/L used mud exposure and it decreased further in the 2 mg/L and 0.5 mg/L treatment.

The largest increase in barium was observed in gills of the three species exposed to barite particles. The concentration of barium in the gills of barite exposed scallops, mussels and cod were 1255, 488, and 89 times higher than in the corresponding control. The concentration of barium in the gills of scallops exposed to the two highest concentrations of used mud also increased more than in the digestive gland, but the opposite was observed for mussels. In the lowest concentration of used mud the increase in barium concentration was highest in the digestive gland for both species.

Cu, Zn, Cd and Pb in tissues of cod and bivalves exposed to drilling mud

The barite exposure

Bivalves in the barite treatment generally accumulated more metals than in the used mud treatments (table 7 and figure 2).

The concentrations of Cu, Pb, Zn and Cd in gill, liver and bile of cod exposed to barite particles did not increase significantly (t test, $p>0.05$), although the concentration of Pb and Cd increased significantly in gills of cod from the positive control and significant increases of Cd and Zn was detected in liver of cod exposed to used mud (UM3) (table 7 and figure 3).The mean Pb concentration in the cod liver was 60% higher than in the control, but generally the variability was higher in the liver and the bile of cod than in the gills and in the gills and digestive gland of the two bivalves (t-test, $p = 0.08$).

Lead (Pb). The concentration of Pb was significantly increased (2-5 times compared to control) in all analysed bivalve tissues for bivalves exposed to 23 mg/l barite particles (t test, $p<0.05$).

Cadmium (Cd). The concentration of Cd was 20% higher in gills from scallops exposed to barite than in control scallops (t test, $p<0.05$), and a 10 % increase in Cd was detected in scallop digestive gland ($p = 0.05$).

Copper (Cu). The concentration of Cu was 2 - 4 times higher in all analysed bivalve tissues compared to the control (t test, $p<0.05$).

Zinc (Zn). There was a 20-30 % increase in Zn in the bivalve tissues. The p-value for mussel gills was 0.04 while the p-values for the other bivalve tissues were in the range 0.07-0.09 (t test).

Mussel and scallop gills and digestive glands can be used to study uptake of Pb and Cu. In addition the Zn level was increased in mussel gill and Cd was increased in scallop gill.

The used mud exposure

There was a decreasing trend of metal accumulation with decreasing concentration of used mud.

Lead (Pb). The mean concentration of Pb was increased in all tissues of bivalves and cod exposed to the highest concentration of used mud (Table 7 and figure 2 and 3, treatment UM3). The difference between control and exposed was however only significant in the digestive gland of mussels and scallops and in the gills of scallops (80-90% increase, t-test, $p<0.05$). In cod liver there was high variability in the data (figure 3), but a tendency to an increase ($p = 0.07$).

Cadmium (Cd). The concentration of Cd was twice as high in the liver of cod exposed to the highest concentration of used mud as in the control (t test, $p < 0.05$). The Cd concentration was reduced relative to the control level in the digestive gland of mussels exposed to all concentrations of used mud (UM2 and UM3: $p < 0.05$, UM1: $p = 0.09$).

Copper (Cu). There was a significant increase (50 %) in the level of Cu in the digestive gland of the bivalves exposed to the highest concentration of used mud. In the digestive gland of scallops exposed to the lower concentrations of mud the Cu concentration increased 10-20 % ($p = 0.05/0.07$).

Zinc (Zn). There was a 40 % increase in Zn in the liver of cod exposed to the highest mud concentration ($p<0.05$).

Mussel and scallop digestive glands can be used for studies of accumulation of Pb and Cu from used WBM, and cod liver can be used for studies of accumulation of Cd and Zn from used WBM. Scallop gill can also be used to study accumulation of Pb from used WBM.

Zn and Cd were accumulated in the liver of UM3 exposed cod. Metals may be taken up in the gut and then reach the liver. The problem with the cod liver was that the analysis was more variable, or the levels were more variable than in the cod gills, and in the bivalve tissues.

In figure 4 and 5 PCA analyses have been done on the composition of barium, lead, copper, zinc and cadmium in the tissues of bivalves and cod. For the digestive gland and gills from the bivalves and the cod gills the tissues from animals exposed to the metal mixture clearly have a different metal composition than control tissues and tissues from other treatments. The differences between treatments that were caused by drilling mud exposure was mainly determined by the barium concentration in the tissues, but also the concentration of Pb, Cu and Zn contributed to the difference (figure 4). In liver and bile from cod no clear groupings of the data was observed, not even for the cod exposed to the metal mixture (figure 5).

Metals in cod exposed to ilmenite particles

Used water based drilling mud with ilmenite was not available at the time of the experiment. The ilmenite particles were tested on cod, but not bivalves.

Lead (Pb). The concentration of lead in the liver of cod exposed to ilmenite was 4 times higher than in the control (t-test, $p = 0.07$).

Cadmium (Cd). The concentration of Cd was twice as high in the liver of ilmenite exposed cod than in the control (t-test, $p<0.05$).

Copper (Cu). The concentration of Cu was 70 % higher in the bile of ilmenite exposed cod (t test, $p = 0.052$).

Zinc (Zn). The level of Zn was 20% higher in the gills and in the liver of cod exposed to ilmenite (t-test, $p<0.05$). There was also a 40 % increase of Zn in the bile ($p = 0.053$).

Comparison of ilmenite and barite. Zn and Cd ($p<0.05$) and Pb ($p<0.1$) increased more in the liver of ilmenite exposed cod than barite exposed cod. Zn increased more in gills of ilmenite exposed than barite exposed cods ($p<0.05$). Cu and Zn ($p<0.1$) increased more in bile of

ilmenite exposed than barite exposed. Hence our results do not indicate that less metals are accumulated from ilmenite particles than from barite particles. Filter feeding bivalves should also be exposed to ilmenite, and both cod and the bivalves should be exposed to suspended particles of used water based ilmenite mud to study accumulation of metals and effects and compare to the results from the used barite mud in the present project.

CONCLUSIONS

In addition to the very large increase in barium in the gills and digestive glands of the bivalves, Cu, Zn, Cd and Pb increased significantly in some tissues. Significant increase in the level of Cu, Zn, Cd and Pb in cod, mussels and scallops was only detected in animals exposed to relatively high concentrations (20-60 mg/L) of barite/used WBM.

The accumulated metals may be unavailable inside particles trapped in the tissues, and may not contribute to the overall effects observed in bivalves and cod exposed to drilling mud particles. Based on the partition coefficients for these metals in the water it is unlikely that they can escape the particles, but we can not exclude the possibility that metals are available for uptake once they are adsorbed to or absorbed in the tissues (Neff, 2002 and references therein). It is also a possibility that predators on filter feeding organisms may take up metals from their prey (lower pH in the digestive system). The metal concentrations in animals exposed to drilling mud particles generally increased more in the filter feeding bivalves than in the fish. The dissolved metals in the positive control was also accumulated to a lesser extent in fish than in bivalves. The increased levels of some metals in liver and bile of cod exposed to used drilling mud with barite and to the ilmenite particles indicate that metals may be available for uptake in exposed organisms. The plan was to use liver and bile of cod to conclude if metals detected in gills and digestive glands were taken up or only attached to the tissues. But only Pb increased significantly in liver and bile of cod exposed to dissolved metals (metal mix positive control), hence it is difficult to use metal concentrations in these tissues to decide whether or not the metals are available from the drilling mud particles. In future experiments we could use other tissues that are not directly in contact with the particles (e.g. gonad). But even when metals are available, the animals have varying abilities to regulate the concentrations, and different tissues have different concentrations. A combination of tissue specific metal concentrations and histology of tissues that are not in direct contact with the particles may be able to determine if metals contribute to the effects observed in cod and bivalves exposed to suspended particles of drilling mud.

Higher levels of metals were measured in tissues of bivalves exposed to barite than used WBM, this is probably due to the higher concentration of barite particles than used WBM particles added to the exposure tanks. The bivalves continuously removed a large fraction of the particles in the exposure tanks. The particle concentrations measured in the fish exposure tanks show that there was more barite particles (62 mg/L) than used WBM particles (39 mg/L). The measured concentration in the bivalve tanks was ca 20 mg/L in both tanks, indicating that the bivalves continuously removed 63% of the barite particles and 50% of the used mud particles. The bivalves removed a larger fraction of the barite particles than the used WBM particles. Hence the amount of particles passing over the gills and through the digestive gland was higher in the barite treatment. This can explain the higher levels of metals measured in tissues of bivalves

exposed to barite than used WBM. It is, however, possible that the bioavailable fraction of metals was higher in the used mud exposure because of the smaller particle size. But we can not conclude that from our results.

The metal analysis of the particles show that there was less barium and more lead in the suspended particles of used WBM than in the barite. The concentration of Cu, Zn and Cd was similar in the barite and the used WBM. Hence it is difficult to explain that the level of all these metals was higher in the barite exposed bivalves, and not only barium. The difference in actual input of particles to the exposure tanks appear to be more important for the metal levels in the tissues than the difference in metal concentration in barite and used WBM particles.

Five hypotheses were set up in the project proposal. Here are the hypotheses and a short conclusion for each:

Hypothesis 1: Higher concentrations of metals are accumulated in the tissues of mussels than cod.

> *Conclusion:* Higher concentrations of metals were accumulated in the tissues of both scallops and mussels than in the tissues of cod.

Hypothesis 2: The concentration of metals in bivalves and cod exposed to barite is higher than in animals exposed to ilmenite.

> *Conclusion:* Used ilmenite mud was not available for the experiment, hence only ilmenite particles were tested on the cod. The results from metal accumulation in cod tissues indicate more metal accumulation in the ilmenite exposed cod than in the barite exposed. More data is needed.

Hypothesis 3: The bioavailability of metals is higher from the weighting material alone than from used drilling mud.

> *Conclusion:* Higher metal levels were detected in bivalved exposed to barite than used WBM, but the results indicate that the metal level in the tissues mainly depend on the particle concentration added to the exposure tanks, and secondly on the metal concentration in these particles.

Hypothesis 4: The increase in concentration of metals in the gills of the filter feeding bivalves is higher than in the digestive gland (Table 5).

> *Conclusion:* Yes and no; it depends on the species (mussel or scallop), the metal (Ba, Cu, Cd, Zn, Pb) and the treatment (dissolved metals, barite, used mud).

Hypothesis 5. The increase in concentration of metals in the liver and bile of cod is higher than in the gills.

> *Conclusion:* The metal concentrations in bile and liver was more variable than in the gills of cod. For cod exposed to dissolved metals the metal level increased most in the gills, but for cod exposed to 39 mg/L used WBM and to ilmenite the metal levels increased most in the liver.

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TABLES

Table 1. *ICP-MS (VG PlasmaQuad 2+) operating conditions.*

ICP-system:	VG-PQ2+
Plasma RF power	1.35 kW
Reflected power	<10 watts
Torch	VG/Fassel
ID of torch injector	1.5 mm
Coolant argon flow	17 l/min
Auxiliary argon flow	0.8 l/min
Nebulizer argon flow	0.8 l/min
Double pass spray chamber temperature	5 °C
Peristaltic pump	Gilson miniplus 3
Nebulizer	Meinhard TR-30-A3
Ni sampling cone orifice	1.0 mm
Ni skimmer cone orifice	0.75 mm
Resolution	0.7 m/z at 5% of peak height
Maximum scan speed	2500 m/z s-1

Table 2. Solid samples analysed with NS 4770 digestion (7 M nitric acid at 120 degrees for 30 min in autoclave). All data based on dry weight. *Barite:* Drilling grade barite used under in the experiment. *Ilmenite:* Drilling grade ilmenite used under the experiment. *Used mud:* The used drilling mud used during the exposure. Samples were taken directly from the homogenised material in the containers we received the material in. *Used mud dos*: Sample of the used mud taken from the header tank (see PAPER I). The mud was diluted with seawater in the header tank. As an effect of this part of the mud directly sedimented. *Sediment:* Sample of a typical coastal clay sediment. The main focus in this paper is on Ba, Cu, Cd, Pb and Zn.

mg/kg	Barite		Ilmenite		Used mud		Used mud dos		Sediment
Aluminium	132	134	555	510	>1000	>1000	>1000	>1000	>1000
Vanadium	<1	<1	23.5	21.7	14.1	14.3	44.1	43.2	46.1
Chromium	15.6	16.0	19.4	18.2	31.46	50.6	72.2	71.4	31.8
Manganese	141	142	4.9	4.3	88	91	115	115	28.1
Iron	6919	6986	11306	10402	14169	14785	19746	20116	19910
Cobalt	0.30	0.34	8.38	8.15	4.70	4.92	6.79	6.75	7.23
Nickel	1.30	2.21	34.6	33.6	21.5	21.4	29.6	29.5	18.9
Copper	38.5	38.5	11.4	11.1	32.8	32.6	39.0	39.1	16.0
Zinc	45.8	43.5	1.8	0.8	48.3	48.6	63.0	64.5	153
Arsenic	<1	<1	<1	<1	4.1	4.5	6.7	5.4	6.7
Molybdenum	2.27	1.91	0.34	0.16	23.75	20.42	10.71	8.37	6.52
Cadmium	0.27	0.27	0.02	0.02	0.20	0.21	0.20	0.19	0.61
Barium	6644	5385	8.8	7.6	3785	3205	1889	1836	36.7
Lead	12.3	12.2	-	-	20.1	18.2	24.1	25.8	22.8

Table 3. Metal concentration in test tanks in the cod and bivalve exposure 2004.

	Iron	Cobalt	Nickel	Copper	Zinc	Cadmium	Lead
	Fe	Co	Ni	Cu	Zn	Cd	Pb
Exposure tank and date:	**µg/l** metals in the sea water						
Bivalves 29/3-04	14.0	0.017	0.256	9.56	28.45	1.49	3.70
Bivalves 31/3-04	6.1	0.036	0.121	10.39	34.06	1.42	3.29
Bivalves 2/4-04	10.7	0.030	0.271	9.82	34.81	1.43	3.30
Bivalves 7/4-04	19.9	0.036	0.263	9.37	33.91	1.35	2.97
Bivalves 15/4-04	10.2	0.024	0.286	9.38	34.15	1.42	3.22
Cod 29/3-04	16.5	0.031	0.296	12.12	39.56	1.44	3.23
Cod 31/3-04	9.0	0.032	0.278	10.49	38.03	1.39	3.25
Cod 2/4-04	7.0	0.031	0.255	10.55	34.90	1.36	3.23
Cod 7/4-04	12.6	0.028	0.263	10.53	36.36	1.38	3.13
Cod 15/4-04	17.6	0.031	0.268	9.98	33.31	1.31	3.04
mean (µg/l)	**12.4**	**0.03**	**0.26**	**10.2**	**34.8**	**1.4**	**3.2**
st.dev.	4.6	0.01	0.05	0.8	3.0	0.05	0.2

Table 4. Background levels (mg/kg wet weight) of Cu, Zn, Cd and Pb in the tissues of mussels, scallops and cod from the control, and concentration of metals in the seawater (background level) and in the exposure tanks of the positive control. Mean values.

	Cu	Zn	Cd	Pb	
Mussel gills (mg/kg)	0.8	7.3	0.07	0.124	Zn>Cu>Pb>Cd
Mussel digestive gland (mg/kg)	4.2	34.4	0.44	0.525	Zn>Cu>Pb>Cd
Scallop gills (mg/kg)	0.6	5.2	0.47	0.021	Zn>Cu>Cd>Pb
Scallop digestive gland (mg/kg)	9.8	53.3	42.55	0.369	Zn>Cd>Cu>Pb
Cod gills (mg/kg)	0.6	22.5	0.014	0.015	Zn>Cu>Pb~Cd
Cod liver (mg/kg)	0.9	8.3	0.018	0.005	Zn>Cu>Cd>Pb
Cod bile (mg/L)	1.2	0.8	0.001	0.002	Cu>Zn>Pb~Cd
Positive Control (ME) (µg/L)	10.2	34.8	1.4	3.2	Zn>Cu>Pb>Cd
Background: Ekofisk 70 m (µg/L)	0.29	1.1	0.03	0.10	Zn>Cu>Pb>Cd
Background: Mekjarvik 280403 (µg/L)	0.31	1.3	0.04	0.06	Zn>Cu>Pb>Cd

Table 5. Comparison of the increase in metal concentration in gills and digestive gland of scallops and mussels exposed to a mixture of metals, to barite particles and to used drilling mud with barite as weighting material. Only differences that were significant are included in the comparison (t-test, $p<0.05$).

	Metal mix		Barite particles		Used drilling mud	
Metal	Mussels	Scallops	Mussels	Scallops	Mussels	Scallops
Ba			Gill>DG	Gill>DG	DG>gill	Gill>DG
Cu	Gill>DG	Gill>DG	DG>gill	DG>gill	Only DG	Only DG
Zn	Gill>DG	DG>gill				
Cd	Gill = DG	DG>gill		Gill only		
Pb	Gill>DG	Gill>DG	DG>gill	Gill>DG	Only DG	DG ~ Gill

Table 6. Mean (± st.dev.) concentration of barium (mg/kg wet weight) in mussels, scallops and cod exposed for 3 weeks to barite, used drilling mud with barite as weighting material and a mix of metals (Cu, Cd, Zn, Pb). Three pooled samples were analysed for each treatment and tissue. The means were compared using each pair Student's t test on log 10 transformed data. Blue boxes indicate barium concentrations that were significantly higher than in the corresponding control ($p < 0.05$).

Treatment	**Mussel** *(Mytilus edulis)*		**Scallop** *(Pecten maximus)*		**Cod** *(Gadus morhua)*
	Gills	**Digestive gland**	**Gills**	**Digestive gland**	**Gills**
Control	0.7 ± 0.2	4.1 ± 1.2	0.5 ± 0.2	3.9 ± 0.3	0.9 ± 0.3
Metal mix	1.1 ± 0.7	8.2 ± 5.3	3.6 ± 3.1	7.1 ± 5.5	0.9 ± 0.1
0.5 mg/l used mud	4.4 ± 2	155 ± 140	4.6 ± 3.4	232 ± 84	-
2 mg/l used mud	32 ± 22	802 ± 56	128 ± 40	474 ± 258	-
20 mg/l used mud	62 ± 49	1233 ± 700	229 ± 56	421 ± 106	22 ± 5
23 mg/l barite	319 ± 140	1396 ± 311	637 ± 46	1468 ± 687	80 ± 25

Table 7. Relative changes in the concentration of Cu, Zn, Cd, and Pb in the tissues of mussels, scallops and cod exposed for 3 weeks to mud and metals. Mg/kg wet weight in exposed tissue/mg/kg metal in corresponding control tissue (or pr litre of bile). Blue cells: p<0.05 when compared to the corresponding control by use of t-test. Yellow cells: p<0.1 0.10 (n = 3 pooled samples for all tissues except liver where 6 samples were analysed.). ↓ reduced metal concentrations compared to control. The metal concentration in the water in the positive control (ME) was 33 (Cu), 26 (Zn), 38 (Cd) and 57 (Pb) times higher than in the inlet water in Akvamiljø (2003 data).

		Cu	Zn	Cd	Pb
Mussel gill	UM1	1.1	1.2	0.9	0.8 ↓
	UM2	1.1	1.4	1.0	1.1
	UM3	1.1	1.1	1.0	1.4
	BA	1.5	1.3	1.1	1.7
	ME	45.6	1.7	2.9	7.5
Mussel digestive gland	UM1	0.8	0.9	0.9 ↓	0.7 ↓
	UM2	0.9	0.9	0.7 ↓	1.1
	UM3	1.5	1.0	0.8 ↓	1.8
	BA	1.9	1.2	1.0	2.4
	ME	3.3	1.6	2.9	5.0
Scallop gill	UM1	0.9	2.0	0.9	1.2
	UM2	1.1	1.3	1.1	1.1
	UM3	1.1	1.1	0.9	1.8
	BA	1.6	1.2	1.2	5.3
	ME	18.3	1.2	0.7 ↓	7.5
Scallop digestive gland	UM1	1.1	1.0	1.0	1.2
	UM2	1.2	1.1	1.0	1.6
	UM3	1.5	1.1	1.0	1.9
	BA	3.9	1.3	1.1	4.1
	ME	4.7	1.6	1.2	2.5
Cod gill	BA	1.1	0.9	0.8	1.1
	UM3	1.2	1.0	1.4	1.4
	IL	1.1	1.2	0.7	0.9
	ME	1.1	1.0	4.4	6.2
Cod liver	BA	1.1	1.1	1.3	1.6
	UM3	1.3	1.4	2.1	4.7
	IL	0.9	1.2	2.3	4.1
	ME	1.3	1.1	1.4	3.1
Cod bile	BA	1.1	1.0	0.8	1.7
	UM3	1.2	0.9	0.9	2.4
	IL	1.7	1.4	0.9	5.7
	ME	1.3	0.6 ↓	1.7	2.2

FIGURES

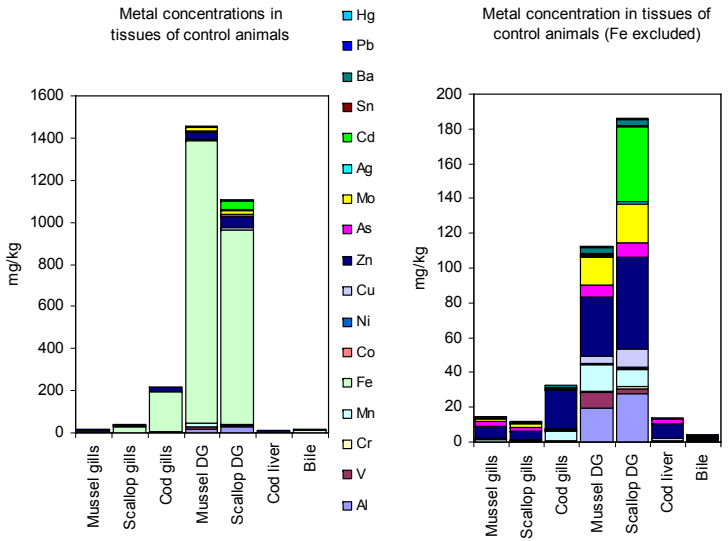


Figure 1. Mean concentration of metals in mussels, scallops and cod from the control. The left figure show the sum of all metals, and in the right figure iron (Fe) is excluded from the graph.

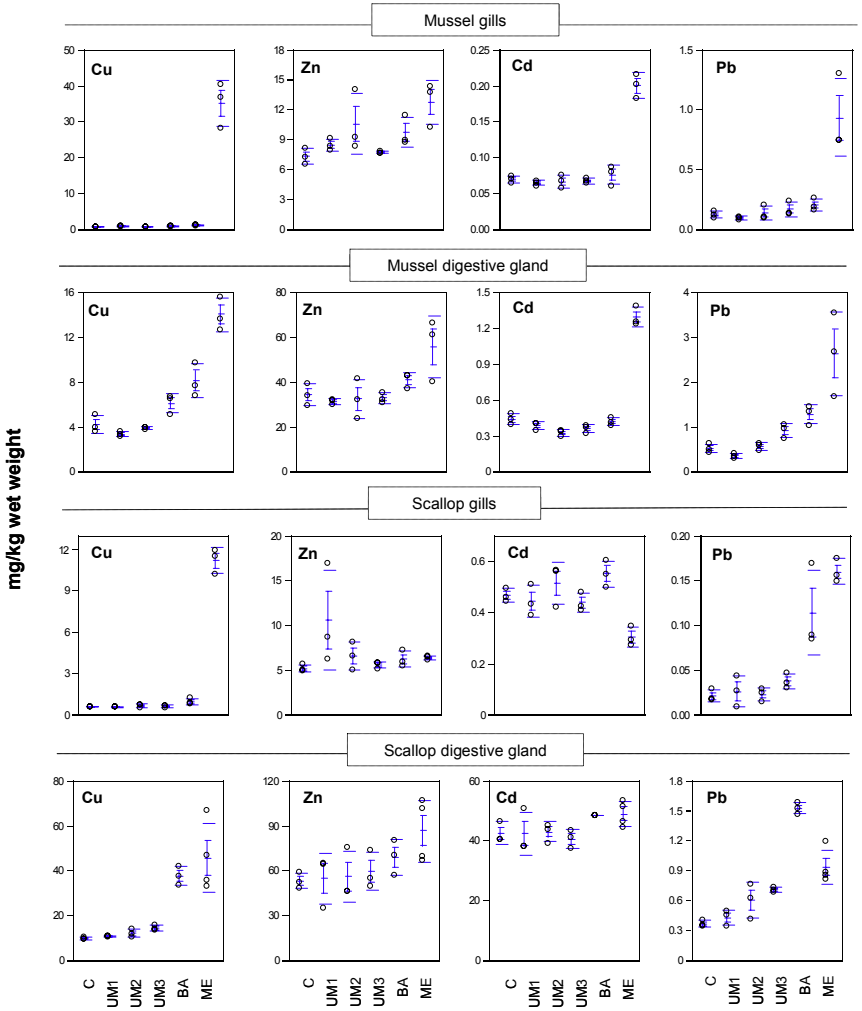


Figure 2. Concentrations of Cu, Zn, Cd and Pb in gills and digestive gland of mussels *(Mytilus edulis)* and scallops *(Pecten maximus)* exposed for 3 weeks to 3 concentrations of used drillling mud with barite as weighting material (UM1, UM2 and UM3: 0.5, 2, and 20 mg/L particles), barite particles (BA, 23 mg/L particles) and a mixture of metals (ME): 10.2 µg/L Cu, 34.8 µg/L Zn, 1.4 µg/L Cd and 3.2 µg/L Pb in the exposure tanks. The graphs show all the data and means, standard error and standard deviation.

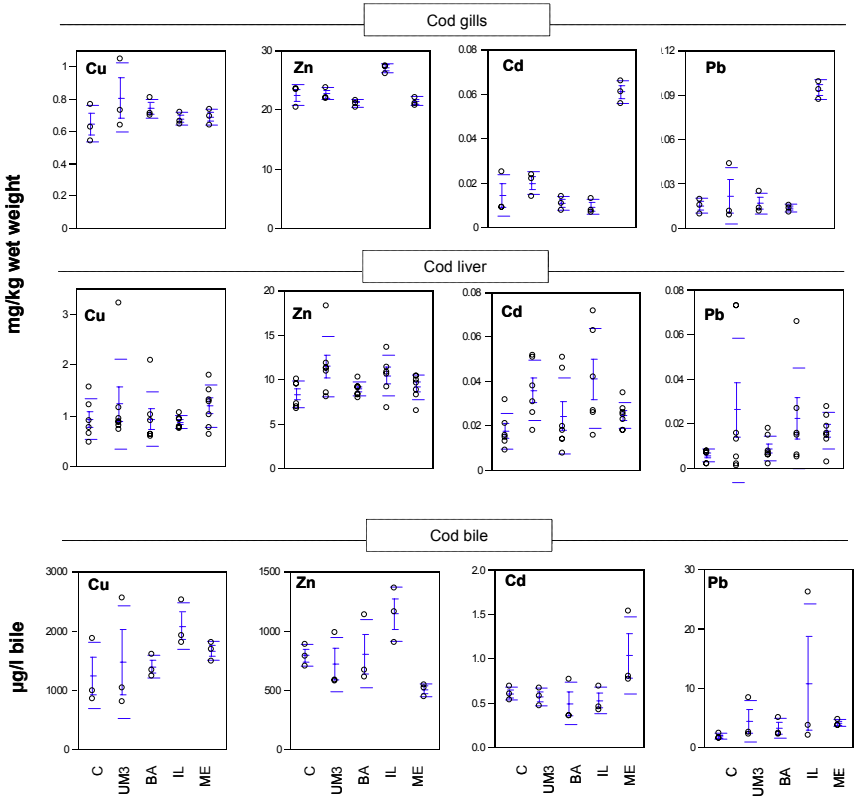
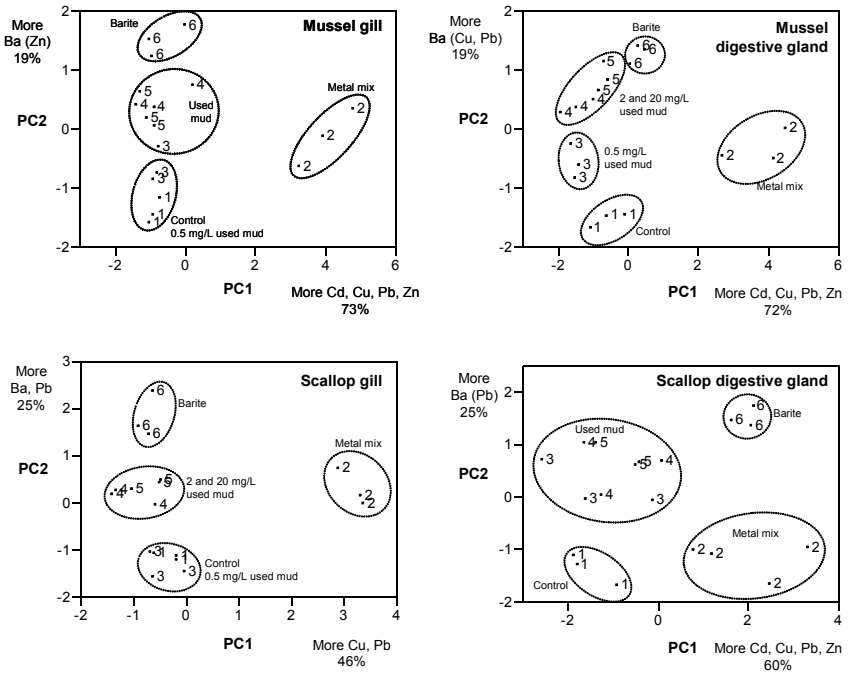


Figure 3. Concentrations of Cu, Zn, Cd and Pb in gills, liver and bile of cod *(Gadus morhua)* exposed for 3 weeks to used to used drillling mud with barite as weighting material (UM3, 20 mg/L particles), barite particles (BA, 23 mg/L particles), ilmenite particles (IL) and a mixture of metals (ME): 10.2 µg/L Cu, 34.8 µg/L Zn, 1.4 µg/L Cd and 3.2 µg/L Pb in the exposure tanks. The graphs show all the data and means, standard error and standard deviation.



Metal composition in tissues of mussels and scallops exposed to drilling mud and a mixture of metals (Cu, Zn, Cd, Pb). Principal component analysis on correlations. The groups are based on cluster analysis of PC1 and PC2 (centroid). The metals included: Ba, Cu, Zn, Cd, Pb. The rawdata was log transformed (log10 (x+1)). The numbers refer to treatments: 1: Control, 2: Metal mix, 3-4-5: 0.5 mg/L, 2 mg/L and 20 mg/L used water based mud with barite as the weighting compound, 6: 23 mg/L barite particles.

Figure 4.

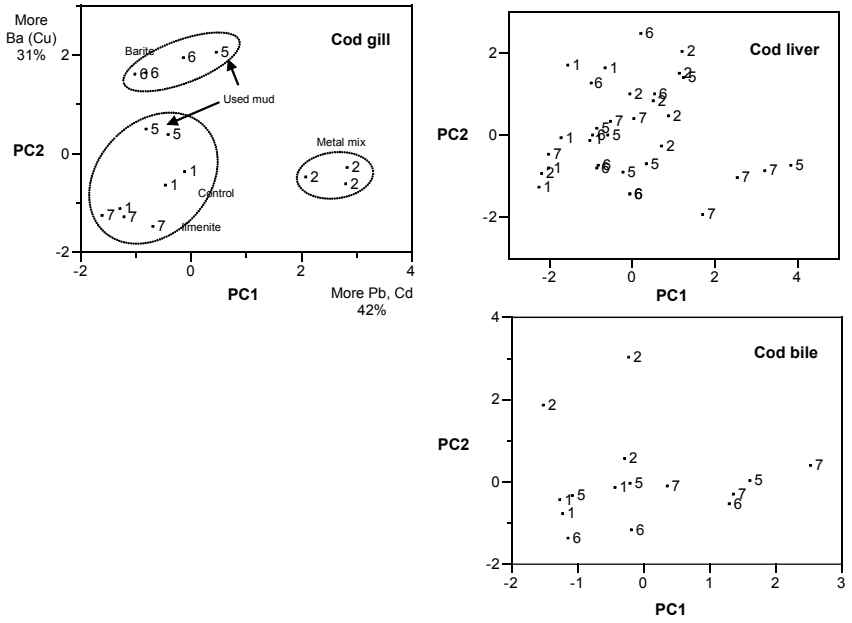


Figure 5. Metal composition in tissues of cod exposed to drilling mud and a mixture of metals (Cu, Zn, Cd, Pb). Principal component analysis on correlations. The groups are based on cluster analysis of PC1 and PC2 (centroid). The metals included: Ba, Cu, Zn, Cd, Pb. The rawdata was log transformed (log10 (x+1)). The numbers refer to treatments: 1: Control, 2: Metal mix, 5: 20 mg/L used water based mud with barite as the weighting compound, 6: 23 mg/L barite particles, 7: ilmenite particles. (one UM3 outlier with extreme Cd level excluded in the liver).

III

Clearance rate, growth, histopathology and biomarker responses in mussels and scallops exposed to suspended particles of water based drilling mud

R. K. Bechmann, T. Baussant, A. H. Tandberg, D. Lowe

ABSTRACT

Mussels (*Mytilus edulis*) and scallops *(Pecten maximus)* were exposed to suspended particles of used water based drilling mud (WBM) with barite as weighting material (0.5, 2, 20 mg/L dry weight), barite (23 mg/L) and to a mixture of metals as a positive control (10.2 µg/L Cu, 34.8 µg/L Zn, 1.4 µg/L Cd and 3.2 µg/L Pb in the exposure tanks). The level of DNA damage (strand breaks measured by Comet assay), oxidative stress (TOSC), lysosomal membrane stability (NRRT) and histopathology was analysed after three weeks exposure. The rest of the bivalves were exposed for up to 10 weeks to study effects on growth, clearance rate of bivalves transferred to clean water, and 'stress on stress' test for the mussels. The metal mix caused increased level of oxidative stress, reduced lysosomal membrane stability and clearance rate for both mussels and scallops. In addition histopathological damage was observed in the gills of scallops exposed to metals, and the growth of scallops was reduced. The 'Stress on stress test' for mussels showed that survival time was more reduced for metal exposed mussels than in any of the particle treatments. The barite exposure caused increased level of oxidative stress and reduced growth for both mussels and scallops. In addition increased level of DNA damage and reduced clearance rate was observed for scallops exposed to barite. In mussels exposed to barite histopathological damage was observed in the gills and digestive gland cells. The used WBM caused increased level of oxidative stress and reduced lysosomal membrane stability, histopathological damage and reduced clearance rate and growth for both mussels and scallops. In addition increased level of DNA damage was observed for scallops, and reduced survival time for mussels was observed in the 'stress on stress test'. Increased mortality of scallops was also observed in the last period of the 10 weeks exposure to 20 mg/L used WBM. The overall lowest effect concentration (LOEC) for used drilling mud was the lowest tested concentration (0.5 mg/L). It was the clearance rate for scallops that was the most sensitive parameter for detecting effects of used drilling mud. At the 2 mg/L used WBM exposure significant responses were detected for most parameters in both scallops and mussels.

INTRODUCTION

The effects of drilling discharges have been investigated primarily for the benthic fauna, such as through the regular Norwegian monitoring programme. Studies of the benthic communities around platforms in the North Sea (when oil-based drilling mud were used) showed that the affected area was a 3 km radius round the platforms (Gray, Bakke, Beck & Nilssen, 1999; Olsgard & Gray, 1995). Analyses linking fauna and environmental variables indicated that the effects were mainly related to THC, barium and strontium, but also to metals like zinc, copper, cadmium and lead, which are all discharged in drill-cuttings. Less is known about the potential long-term effects of suspended particles from drilling mud discharges on water-column organisms. There is a general belief that water based drilling discharges pose little environmental risk to organisms in the water column. Such believes are partly founded on the ongoing process of testing acute toxicity of single chemicals (OSPARCOM tests), but toxicity testing of drilling formulations does not always match up with field observations, probably because (assumed) chemically inert material such as barite are not routinely included in such tests (Barlow & Kingston, 2001). It is generally assumed that the dissolved

fraction of a toxic substance in surface water is mainly responsible for toxicity to aquatic organisms (Weltens, Goossens & Van Puymbroeck, 2000). Weltens *et al*, however, showed that the particle bound fraction of metals can become available within the body of filter feeding *Daphnia* (Weltens et al., 2000). Adsorbed metals might desorb in the gastrointestinal tract due to different physico-chemical conditions and exert toxic effects, lead to unexpected high tissue concentrations (Weltens et al., 2000). The digestive physiology of the animal and the behaviour of the chemical within the animal's gut influence contaminant assimilation (Wang & Fisher, 1999a). For suspension feeders such as mussels, uptake of metals from the dissolved phase and food ingestion can be equally important to metal accumulation (Wang & Fisher, 1999b). Particle bound contaminants can also be bioavailable to fish (Qiao & Farrell, 1996; Van den Belt, Van Puymbroeck & Witters, 2000).

Suspended clay either from natural sources or from drilling mud can interfere with suspension feeding and damage the gills of bivalves (Morse, 1982, Stevens 1987, Cranford & Gordon, 1991, 1992) and fish (Sprague and Logan, 1979). Chronic intermittent exposure of sea scallops *(Placopecten magellanicus)* to dilute concentrations of operational drilling wastes, characterised by acute lethal tests as practically non-toxic, can affect growth, reproductive success and survival (Cranford, Gordon, Lee, Armsworthy & Tremblay, 1999). Negative effects were observed at 0.5 mg/L barite. The gills of the suspension feeder *Cerastoderma edule* and the deposit feeder *Macoma balthica* were damaged by exposed to suspended barite particles (Barlow & Kingston, 2001). Levels of barite accumulation that can be expected 100-500 m from a point of active drill cuttings discharge, caused 100% mortality within 12 days.

Mussel have been used in numerous environmental assessment studies, chiefly because they are sessile suspension feeding organisms found in or near sites of environmental concern, and they can be easily collected, sorted and deployed at sites of interest. Mussels are useful for caging and biomonitoring of discharges from platforms. Because they are filter feeders, they can accumulate high concentrations of toxic compounds and particles. Cranfords results indicate that scallops are sensitive to drilling mud. In long-term exposure studies in the laboratory Cranford *et al.* observed effects on scallops at 0.5 mg/L barite (Cranford et al., 1999). Scallops (*Pecten maximus)* may be more sensitive to suspended drilling mud particles than mussels. The responses in mussels and scallops exposed to barite and used WBM will be compared.

Physical stress from exposure to suspended barite, and/or effects of metals that may leak from barite particles, and chemicals adsorbed to the particles may affect animals exposed to suspended particles of drilling mud. The main objective of the present project was to test if suspended particles of drilling mud affected bivalves at concentrations relevant for the field. The weighting material in drilling mud (barite or ilmenite) contain heavy metals. If these metals are bioavailable they may cause toxicity. Tissue concentrations of metals were analysed and compared to a positive control with dissolved metals. The effects of exposure to suspended particles of used water based mud with barite was compared to the effects of exposure to barite particles and to a mixture of metals using different biological effect parameters. The aim was to get increased knowledge about the possible causes of effects observed in animals exposed to used WBM.

The parameters studied in mussels and scallops exposed to suspended particles of drilling mud were survival, growth, clearance rate, protein patterns (SELDI-TOF),

oxidative stress (TOSC), genetic damage (Comet), lysosomal membrane stability (NRRT) and histopathology, in addition to a stress on stress test for mussels. Biomarker responses may be induced by contaminants within particles (metals) or adsorbed to particle surfaces (chemicals and metals). Biomarker responses can later be used as part of biomonitoring (e.g. caged bivalves). Morphological changes to the gill structure of scallops and mussels were examined as well as the impacts on digestive and reproductive processes in mussels. It is important to link possible biomarker responses to effects at higher levels of biological organisation, like the tissue and individual level. The results from the protein pattern analysis is presented in PAPER VII.

MATERIALS AND METHODS

Test organisms

Mussels *(Mytilus edulis)* were purchased from Aspøy Skjell og Produktutvikling AS, Hundvåg, Norway (week 9, 2004). The mean length of the mussels was 66 mm (st. dev.: 6). Scallops *(Pecten maximus)* were purchased from Helland Skjell AS, Manger, Norway (week 8, 2004). The mean height of the scallops was 70 mm (st. dev.: 6). The mussels and scallops were kept for 5 weeks before start of exposure.

The bivalves were fed a mixture of *Isochrysis galbana* and *Skeletonema costatum* during the whole exposure period. *Isochrysis galbana* was purchased from Stolt Sea Farm As (Øyestranda, Norway) and *Skeletonema costatum* was supplied by Scalpro As (Rong, Norway). *I. galbana* was cultivated in 200L tanks in a temperature-regulated room at 22°C and under constant illumination. The culture was maintained in constant growth using a *Rhinomonas* medium consisting of both a working solution with main nutrients and trace elements and a working solution with vitamins each dosed at 1ml.l^{-1} seawater. *S. costatum* was cultivated in 600L tanks at a temperature of ≈15°C and under constant illumination. Solutions of natrium silicate (25ml.l-1; Bim Krystal as, Drammen, Norway) and fertilizer (180g.l^{-1}; Rød Superba NPK7:4:21, Hydro, Norway) were prepared to maintain the culture. They were dosed at 0.29ml.l^{-1} and 0.25ml.l^{-1} respectively. In our cultivation system, *Isochrysis galbana* reached a maximum density of ≈ 2x10^{6} cells.ml^{-1} and *Skeletonema costatum* had a maximum density of ≈ 4.5x105 cells.ml^{-1}. Density was estimated by Coulter Counter measurements using a 70 µm glass aperture for *I. galbana* and by microscope observation using a Neubauer counting chamber for *S. costatum*. A 200L tank filled with a volume of 25% *I. galbana* and 75% *S. costatum* was used to supply the algae mixture to the bivalves for 9 hours every day. The nominal density of algae in the exposure tanks during that period was 16000±8000 cells/ml. Approximately 70% of the total number of algae was *Isochrysis*.

Experimental design

A new continuous flow exposure system has been made to expose organisms to suspended particles of drilling mud simulating the conditions in the water column following off shore drilling operations (see PAPER I).

Three concentrations of used water based drilling mud (WBM) with barite as the weighting material was tested in addition to one treatment with barite, and a positive control with a mixture of metals relevant for drilling mud. Mussels and scallops were exposed for 3 weeks to study metal accumulation (PAPER II), biomarker responses and

histopathology. In each of the 5 treatments 120 mussels and 120 scallops were exposed. After 3 weeks 60 mussels and 60 scallops were sampled, and the rest remained for an additional 3 weeks (mussels) or 7 weeks (scallops). The metal exposed scallops were sampled after 2 weeks because mortality was observed during the first part of the experiment. After 10 weeks exposure of scallps and 6 weeks exposure of mussels growth and feeding efficiency were tested. Cod were exposed for 3 weeks in parallel to the bivalves (see PAPER IV).

For logistic reasons (to be able to analyse DNA strand breaks by comet assay on samples from all treatment groups) one group of exposed bivalves (n = 21) and one group from the control (n = 9) were sampled each day. There were 5 sampling days for the mussels and 5 for the scallops. The exposure of the different treatment groups was started with a time lag to ensure the same exposure time for all treatments. The length of the mussels and height of the scallops was measured.

Haemocytes were sampled with saline in the syringe for comet and NRRT. Haemocytes from 9 control and 9 exposed bivalves were used for comet assay. Haemocytes from 9 control and 15 exposed bivalves were used for the NRRT assay.

The digestive gland was dissected out, cut in two, transferred to two eppendorf tubes, and stored at -80°C. The digestive glands from 21 bivalves from each treatment were cut in two pieces; one piece to metal analysis and one to TOSC. For analysis of TOSC samples of digestive gland samples from three individuals was pooled together to make 7 pooled samples from each treatment.

Gills were dissected out; one piece for histology and the rest for metal analysis. Gills were sampled from 15 bivalves from each treatment for metal analysis. From each treatment 3 pooled sampled was made.

Comet assay

The procedure is described in e.g. Taban *et al.* (2004) and Mamaca *et al.* (2005).

TOSC

Effects of particles on the respiratory function or toxic effect of metals or chemicals in the drilling mud may cause oxidative stress. These changes can be detected by use of the TOSC assay. TOSC was analysed in samples of digestive gland of mussels and scallops. The procedure is described in e.g. Regoli (2000) and Camus *et al.* (2004).

NRRT

Lysosomal response, measured as the retention of the neutral red probe, was measured in haemocytes of the mussels. This biomarker method is characterised as a general health parameter and is earlier shown to respond to PAH and crude oil exposed mussels (Camus, Grøsvik, Børseth, Jones & Depledge, 2000; Fernley, Moore, Lowe, Donkin & Evans, 2000).

Lysosomal response was measured with the NRRT method described by Lowe (1994). Haemolymph samples were made of 15 exposed individuals and 9 controls from each sampling day (in total 9x5 controls) 0.2 ml was sampled from a sinus in the posterior adductor muscle and mixed 1:1 with a physiological ringer (pH 7.4). 40 µl haemolymph

fluid was transferred to an object glass and incubated 20 min in a light proof and high humidity box prior to addition of 40 µl of the toxic colour pigment neutral red (NR). The neutral red solution had a concentration of 0.1 µg/µl.

NR is selectively taken up by lysosomes of the haemocytes and this adds an extra stress to the membranes. After some time, from 15 to 210 minutes, depending of the health status of the mussels, the membrane will start to burst and NR will leak out in the cytosol. This caused the form of the cells to change from irregular to round shaped. The time from NR is added the cells and until they round up and "die" is observed visually with a microscope. The cells are observed repeatedly by microscopy at 15, 30, 60, 90, 120, 150, 180 and 210 minutes of incubation with NR. For mussels the endpoint of the analysis is when 50 % of all cells are rounded up and dead. The endpoint for scallops was 80 % dead cells.

Clearance rate

Exposure to drilling mud particles and metals may affect the bivalves response to food (algae). They may remain closed for a longer period after algae is added to the water and they may filter the algae less efficiently than unexposed scallops. In the present experiment focus was on the combined response on behaviour (open/closed) and the ability to filter algae from the water. Samples were taken at regular intervals after algae had been added to the water and the reduction of algae density in the water was measured. Based on these measurements the percentage of remaining algae was calculated and plotted against feeding time (figure 4, table 5). The clearance rate (the volume of water cleared of particles per hour) can be calculated based on data from the linear part of these curves, but the clearance rate will vary depending on the selection of sampling times (which part of the curve in figure 4) is used. The tests were run for longer periods of time than usual for this type of tests. That was done to find out if the bivalves were able to filter the water given enough time. Figure 4 give a more complete picture of the effects of drilling mud exposure on the bivalves than calculation of clearance rate based on e.g. the first 30 minutes of the scallop experiment.

Tests in clean water. The clearance rate of mussels exposed for 8 weeks and scallops exposed for 10 weeks to barite, used WBM, metal mix and control was measured. The bivalves were transferred from the exposure tanks to beakers with clean seawater and algae to measure how many algae they were able to remove from the water with time. The bivalves were rinsed in a bucket with running seawater prior to transferring them to the test chambers to remove particles that could interfere with the test. The test chamber was a 5 L polypropylene beaker, with an outlet to allow quick and easy sampling of the water without disturbing the bivalves. For each treatment 7 replicate mussels/scallops were tested, one in each test chamber with 3 litres of algal solution with 40 000 cells/ml *Isochrysis galbana*. An extra test was done with scallops from the control and the 2 mg/L exposure, hence 14 scallops were tested in total from these two treatments. The tests were run at 10°C, the same temperature as in the exposure. Samples of the water was taken immediately after transferring the bivalve to each test chamber, and then after (4), 8, 16, (24), 32, 64, 95 and 136 minutes. It was observed whether the bivalve was open or closed at each sampling time. Additional samples were taken from the scallops exposed to 20 mg/L used WBM after 3 and 4 hours.

Tests with WBM particles. Two clearance tests with mud particles were done using the same test chambers as described above. Test solution was taken from the inlet to the 2 mg/L WBM exposure tank and algae (40 000 cells/ml) were added. The size of the algae are larger than most of the mud particles, hence it is possible to measure the efficiency of filtration of both mud particles and algae. Number of particles with size between 1.5 – 3.5 µm (mud particles) and 3.5 – 8 µm (algae) was measured by Coulter counter after 8, 16, 32 and 64 minutes. In the first test the clearance rate of algae from a solution of 2 mg/L mud particles and algae was measured using control scallops. The clearance rate of algae with and without mud present was compared. The efficiency of mud clearance was also tested (see paper 1). A similar test was done with mussels, but in this test mussels from the 2 mg/L mud exposure was used. This was done because no negative effect was observed when mussels from 2 mg/L WBM was transferred to clean water and the clearance efficiency was tested (see paper 1 for more details about the filtering of mud particles).

Two tests were done to check if algae and mud particles stayed in suspension for the duration of the experiment. The results of the sinking test for mud is presented in PAPER I. After 2 hours (static) 94 % of the number of used WBM particles remained in suspension. This show that when we measure the reduction in number of particles in the feeding test where used mud was present it is the mussels and scallops that remove the particles (it is not caused by sinking). In the algae 'sinking test' samples were taken after 0, 45, 120 and 180 minutes (n = 3 replicates). There was no difference in the mean number of algae in suspension after 3 hours.

Growth

At the start 40 mussels and 40 scallops were dissected to find the weight of the gonad and digestive gland for each species, and the muscle of the scallops. After 6 weeks 40 mussels from each treatment were dissected to find whether there were differences in weight of gonad and digestive gland between treatments, and to test whether they had grown since the start of the exposure. The plan was to sample at the end of the exposure, but during sampling for biomarkers it was observed that the mussels were almost ready to spawn (at least in some treatments), hence we sampled the mussels after 6 weeks, but the scallops were sampled after 10 weeks. The tissues were dried (80°C) and weighed. Mean dry weight of tissues from exposed bivalves was compared to the weight of control tissues at the end of the exposure (6 or 10 weeks).

Histopathology

A cross-section of 10 mussels from each treatment was made and fixed in Davidson solution. A piece of the gill from 10 scallops from each treatment was also fixed in Davidson solution for histology. After 48 hours the samples were transferred to 70 % ethanol. All tissues were prepared as follows for histological analysis using the same protocol on an automatic tissue processor. The scallop gill sample was cut in half transversely as it was too large to be accommodated in the wax cassettes. The mussel samples were processed as supplied, however, it should be noted that in many instances the samples were too large for the cassettes and as a result had been squashed into the cassettes at the time of dissection which may have resulted in some physical damage. Regarding the fixative used for the scallop and mussels samples, whilst Davidson's is an excellent nuclear fixative it is less effective with cytoplasmic components. Sections

were cut at 7µm and stained using the following procedures. The scallop gills and mussel tissue sections were stained in Papanicoloau to view general structure and Periodic Acid Schiff/ Alcian blue for neutral glycoproteins and acidic glycoproteins respectively.

Cellular biomarkers in mussels. In that the samples of mussels contained elements of the digestive gland, reproductive system and gills the opportunities for impact assessment was far greater and as such the following parameters were investigated.

Atrophy of the digestive tubule epithelium: Shrinkage of the epithelium cells of the digestive tubules. The no effect score for this parameter is 'absent' designated as 1.

Phasic activity of digestive tubules: The condition of the digestive system following exposure of the mussels was judged by the presence or absence of clusters of digestive tubules in different states of activity. The no effect score is 'common' designated as 2.

Digestive cell vacuolation: The condition of the digestive cells following exposure of the mussels was judged by the presence or absence of large lipid vacuoles in the digestive tubule epithelium of wax sections. The no effect score is 'absent' designated as 1.

Basophile cell vacuolation: The condition of the basophile cells following exposure of the mussels was assessed by the presence or absence of lipid vacuoles in wax sections. The no effect score is 'absent' designated as 1.

Infiltration of the digestive gland connective tissues with blood cells: The level of granular haemocytes infiltration of the connective tissues of the mussels was assessed qualitatively. The no effect score is 'absent' designated as 1.

Ratio of basophile to digestive cells in tubule epithelium: The ratio of digestive cells to basophile was assessed qualitatively and the no effect score is 'common' designated as 2.

Sex: The sex of each animal was recorded whenever possible as this is dependent on the presence of germinal cells in a suitably advanced stage of development. Female mussels were designated by the number 1, and males by number 2.

Necrotic eggs: The eggs of mussels detach from the germinal membrane prior to vitellogenesis and maturity. Sometimes in response to contaminant exposure there is a premature release of small eggs into the follicular space which round up and have altered staining characteristics. The no effect score is absent which is as 1.

Reproductive development: The reproductive state was categorised into a series of simple stages as follows, stage 1.0, no germinal tissue, stage 1.5, developing cells only, stage 2.0, more developing that ripe, stage 2.5, more ripe than developing, stage 3.0, all ripe, stage 3.5, spawning, stage 4.0, spent/regressing. Finally atretic eggs were scored as 1 (absent), 2 (present) and regressing germinal cells as 1 (absent), 2 (present).

Gill changes: The increased occurrence of acid mucin cells in the abfrontal region of the gills was assessed and scored as 1 absent, 2 present and 3 abundant.

Cellular biomarkers in scallops. For the study of the impacts on scallop gill structure, alterations indicative of pathological change in the dorsal respiratory expansion (DRE) were investigated and scored as follows: Absent 1, present 2, common 3.

Background on scallop gills. Scallops possess an euleutherorhabdicplicate gill, which means that the W-shaped left and right gills, each comprising an inner and an outer demibranch, are composed of a series of two different types of filaments, suspended from the gill axis in a corrugated or plicate fashion. The principal filaments are situated in the troughs of the plicae, separated from each other by a number of ordinary filaments. The ascending (outer) branches of the filaments are approximately two-thirds of the length of the descending branches (Fig. 2).

The individual filaments of the lamellibranch gill are thin, greatly elongated, and bend to form a V shape so that the entire ctenidium is generally shaped like a W. A ciliated ventral food groove lies between the 2 limbs of each filament, at the base of each V and the cilia within this groove pass food particles from one filament to the next, toward the mouth. A descending limb descends from the central axis, and the ascending limb bends upward from the bottom of the descending limb. When 2 V-shaped filaments unite to form a W on each side of the foot adjacent Ws in a gill are always attached to each other.

Sometimes the Ws are attached by interfilamental ciliary junctions, as in the protobranch gills (e.g. mussels). The lamellibranch gills-consisting of individual filaments linked together solely by ciliary disc are termed filibranch gills. In the most highly modified bivalve gills, called eulamellibranch gills such as occurs in *Pectin maximus*, the junction between adjacent filaments are made of tissue rather than cilia; these interfilamental tissue junctions completely attach adjacent filaments together. In both filibranch and eulamellibranch gills, the series of ascending and descending limbs form continuous sheets of tissue, or lamellae. Water passes through the gill lamellae-between adjacent gill filaments before leaving the mantle cavity.

The proximal third of the principal filament presents a complex structure which Dakin (1909) called the dorsal respiratory expansion. In spite of its name and suggestive anatomy, however, no studies have yet been performed to evaluate its contribution to gas exchange. The dorsal respiratory expansion consists of an abfrontal afferent vessel, an efferent vessel contained within the wall of the principal filament, and a number of variously-shaped interconnecting vessels. The afferent vessel is covered with numerous short cilia and occasional tufts of sensory cilia, whereas the efferent and interconnecting vessels have only tufts of sensory cilia (Beninger et al., 1988). The convoluted basal lamella of the shallow one cell thick layer of the interconnecting vessels, together with their extensive, ramified apical microvilli strongly indicate a specialized role in transport or diffusion of dissolved, as yet undefined, substances between the external medium and the gill (Le Pennec et al.,1988).

Stress on stress

The survival in air test measures how long mussels survive when removed from water. Although removal from water for several days in unlikely to occur in the natural environment, the method has been used as a way of testing the relative fitness of mussels (see references and more details in Pampanin *et al.* (2005)).

After 8 weeks continuous exposure the remaining mussels were placed on wet paper in a plastic box at a constant temperature of 10°C. There were lids on the boxes to maintain a constant humidity. Mussel mortality was checked daily, and animals were considered to be dead when their valves gaped and failed to close when the mussel was physically stimulated. Median survival time (LT_{50}) was calculated for each treatment

group and compared using the Wilcoxon signed rank test. At the start of the test there was 20 mussels from control, 13 from 0.5 mg/L WBM, 30 from 2 mg/L WBM, 22 from 20 mg/L WBM, 37 from barite and 26 exposed to metals.

Statistics

The level of DNA damage, NRRT and TOSC in each exposed group was compared to the corresponding controls using the Wilcoxon sign-rank test ($p<0.05$). Survival time in the stress on stress test with mussels was also tested statistically using Wilcoxon.

The mean percent remaining alga for each treatment group at each sampling time in the clearance rate test was compared to the control using Student's paired t-test. The growth (dry weight at start and end of exposure) of bivalve tissues was compared statistically using Student's paired t-test.

The results of the histopathological biomarkers derived from the mussel sections were tested for significance using multivariate analysis software, PRIMER V6. Non-metric MDS, derived from Bray Curtis similarity matrices was used to visualise dissimilarities between sample groups, nearby points in the plot reflecting similar biomarker values. The results were then further tested for significance using ANOSIM which is roughly analogous to a univariate ANOVA and reflects differences between treatment groups in contrast to differences among replicates within sites (the R statistic). Under the null hypothesis H_o"(no difference between treatment"), R=0 and this was tested by a non-parametric permutations approach; there should be little or no effect on the average R value if the labels identifying which replicates belong to which groups are randomly rearranged. Finally, the biomarkers that make the greatest contribution to the observed treatment differences were determined using SIMPER (similarity percentages), which partitions the tested dissimilarity between groups into contributions from each biomarker. In as much as only a single parameter was investigated for the scallops a univariate multiple range test (Statgraphics) was used to determine significant differences between treatments. Analysis of the mussel data using the ANOSIM and SIMPER tests gave the same main results shown in table 4. (ANOSIM test results: Sample statistic (Global R): 0.197. Significance level of sample statistic: 0.1%. Number of permutations: 999 (Random sample from 92378 possible). Number of permuted statistics greater than or equal to Global R: 0).

RESULTS

Oxidative stress (TOSC)

For mussels significant increase in TOSC was observed in all treatments except the lowest exposure of used mud. For scallops significant increase in TOSC was observed in the metal and barite treatment. The TOSC results from mussels indicate that used WBM and barite cause higher oxidative stress than the metal mix. In scallops barite and metal mix caused similar increase in TOSC.

DNA strand breaks (Comet assay)

Generally there were only small differences in the level of DNA damage between control and exposed mussels and scallops (table 2). Cells from mussels and scallops exposed to metal mix had the same level of DNA strand breaks as the corresponding

control cells. Cells from scallops exposed to the lowest concentration of used WBM had a lower level of DNA strand breaks than the control, but in all other treatments there were indications of increased DNA damage compared to the corresponding controls (table 2). Statistical comparisons of the level of DNA damage based on all analyzed cells from each treatment (n = 450 cells) show that scallops exposed to the highest concentration of used WBM and to barite were the only groups significantly different from the control (Wilcoxon, $p<0.01$). Statistical comparisons of the level of DNA damage based on mean level of DNA damage in each individual animal (n = 9 animals) show that scallops exposed to barite was the only group significantly different from the control (Wilcoxon, $p<0.01$). None of the exposed groups of mussels had significantly more DNA damage than the corresponding control group when comparisons were done based on means for individuals (n = 9).

NRRT

A combined control with 9 x 5 bivalves was planned, but the median retention time for control groups sampled on different days varied. Hence the median NRRT for each exposed group (n = 15) is compared to the corresponding control animals (n = 9) sampled and analysed on the same day. The median NRRT was lower in all exposed groups than in the corresponding controls (table 3). Exposure to the metal mix caused a significant reduction of median retention time for both species. There was a tendency to reduced NRRT in the barite treatment for both species. Because of high variability both between control individuals and exposed individuals, the difference was not statistically significant. The NRRT was significantly lower in the two highest concentrations of used WBM for the scallops, but only in the lowest concentration for the mussels. Overall there was less effect on NRRT from barite and used WBM than the metal mix, but the results also show that used WBM can cause significant reduction of NRRT for both mussels and scallops.

Histopathology

Main results. The metal mix did not cause significant changes in the tissues of mussels (gills and digestive gland), but the scallop gills were severely impacted. On the contrary barite caused significant damage to gills and digestive cells of mussels, but did not affect the gills of scallops. Significant damage was observed in gills and digestive cells from mussels and gills from scallops exposed to the two highest concentrations of used drilling mud. Significant effects were not detected in bivalves exposed to the lowest exposure concentration of used drilling mud.

Scallop gills. Exposure to barite had little impact on the scallop gills after 3 weeks exposure which is in sharp contrast to the metals mixture treatment group where pathologies were common after 2 weeks exposure (this group was sampled earlier because of mortality in the first part of the experiment). Exposure to drilling mud at the middle and higher concentrations also impacted on the gills as compared to the control at 3 weeks exposure. Significant differences between treatment groups were less in number after 10 weeks exposure (Table 4, figure 3).

Mussel Tissues. The mussels were impacted most severely following the barite exposure. Exposure to 2 mg/L and 20 mg/L used drilling mud impacted on the mussels with gill abnormalities contributing significantly to differences between treatment groups as well as changes in the digestive epithelium. The Simper test indicates that the

significant differences between treatment groups was due to increases in digestive cell vacuolation and the increased incidence of mucous cells in the gill. The only other contributing factor was an increase in tubule atrophy in mussels exposed to 2 mg/L used WBM as compared the control group. Whilst the 2D plot (Fig 5) suggests a close relationship between the barite and highest used WBM exposure in terms of their responses, the 3D plot (Fig 6) indicates that they are very different which is supported by the Simper results which also indicate a significant difference between those two groups.

Owing to the different numbers of male and female mussels present in the samples it was not possible to undertake a statistical comparison between the various treatment groups which included any parameters related to sex such as atresion and egg abnormalities. Therefore the data resulting from the microscope analysis was analysed with sex dependent factors removed, i.e. sex, gamete atresion and small eggs. There were also indications of an increase in egg abnormalities and atresion in mussels exposed to barite and to the 0.5 mg/L and 2 mg/L used WBM treatments.

Clearance rate

Scallops. The clearance rate was reduced with increasing concentration of used drilling mud, and there was a significant reduction even at the lowest exposure concentration (0.5 mg/L used WBM, table 5). Scallops exposed to 20 mg/L used WBM for 10 weeks appeared to have irreversible damage. Hardly any algae had been removed from the water even after 4 hours (table 5). Scallops exposed to barite and metals also showed significantly reduced clearance rate, but the effect was even more pronounced in the highest used mud exposure.

For control scallops there was a linear reduction in remaining algae in the first 30 minutes of the test (figure 4). For all exposed groups of scallops very little feeding occurred during the first 30 minutes, but continuing the experiment for up to two hours show that some of the exposed groups were able to filter algae from the water given enough time, while other groups appeared to be unable to feed. Already after 16 minutes the control scallops had removed 39 % of the algae in the beaker (mean for 14 scallops) while none of the exposed groups transferred to clean water with algae had started feeding. After 32 minutes 83 % of the algae in the control beakers were removed while ≥ 90 % of the algae in all previously exposed groups were left. With time there was a dose dependent increase in feeding for the WBM exposed groups (figure 4).

Generally the number of open scallops increased with time in the clearance test. Two tests with control scallops were preformed and in both tests all 7 scallops were open after 16 minutes. At the same time 6 of 7 scallops exposed to 0.5 mg/L WBM were open. Two tests with scallops exposed to 2 mg/L WBM were preformed; 7 of the 14 scallops were open after 16 minutes. Five of the 7 scallops exposed to 20 mg/L WBM and to barite were open, and 2 of the 7 scallops exposed to metals.

The clearance rate of scallops that remained open from 3 minutes after addition of algae until the end of the clearance tests show the same dose-dependent responses as described in figure 4; the control scallops filtered the water most efficiently followed by scallops exposed to 0.5 mg/L WBM, 2 mg/L WBM and barite, and scallops exposed to 20 mg/L WBM and metals filter least efficiently. Hence the exposed scallops remained

closed longer than control scallops, and the scallops that were open filtered less algae from the water than the control scallops.

Control scallops transferred to beakers with 2 mg/L WBM and algae managed to remove the algae from the water just as efficiently as when they were kept in clean water with algae (figure 4). After one hour 99 % of the algae were removed from the water. In addition the scallops removed approximately 60 % of the mud particles both when algae were present and not (PAPER I).

Mussels. The clearance rate of mussels were less affected by drilling mud than scallops, but significantly reduced clearance rate was observed in the 20 mg/L used WBM exposure. No negative effect was observed on the clearance rate for mussels exposed for 8 weeks to barite particles. The metal exposed mussels responded similarly to the scallops exposed to 20 mg/L used WBM: even after 136 minutes hardly any algae had been filtered out of the water indicating irreversible damage to the mussels ability to feed.

There was a linear reduction of remaining algae in the beakers with control mussels from start and during the first hour of the experiment. During the first 30 minutes the mussels that had been exposed to the highest concentration of mud filtered less algae from the water than mussels from the other particle treatments and the control, but after 136 minutes only 8% of the alga remained (table 5).

The mussels exposed to the lowest concentration of WBM filtered algae from the water more efficiently than the control mussels. The same tendency was also observed for the mussels from 2 mg/L WBM, but only the results from the 32 min sampling time showed a statistically significant change from the control in the 0.5 mg/L exposure (table 5).

Generally the number of open mussels increased with time in the clearance test. After 16 minutes 6-7 of the 7 mussels from control, 0.5 and 2 mg/L WBM were open, 5 of the 7 mussels in barite and 4 of the 7 mussels from 20 mg/L WBM were open. After 32 minutes 6-7 mussels of the 7 tested from all treatment were open.

The clearance rate of exposed mussels that remained open from 4 minutes after addition of algae until the end of the clearance tests show the same dose-dependent responses as described in figure 4; mussels exposed to 20 mg/L WBM filtered the water less efficiently than the control, and mussels exposed to metals had filtered very few algae from the water even after 136 minutes. Mussels responded less to drilling mud than scallops, but there was a significant reduction of clearance rate for mussels exposed to 20 mg/L used WBM. The mussels exposed to 20 mg/L WBM used longer time to open after transfer to clean water with algae, and mussels that opened filtered less algae from the water than the control mussels. Clearance rate for mussels exposed to metals were more reduced than for the drilling mud exposed mussels.

Mussels exposed to 2 mg/L WBM were also tested in beakers with 2 mg/L mud and algae. The results indicate that mussels filter algae more efficiently (faster) when mud is present. After 32 minutes there was significantly less algae remaining in the beakers with mud added than in the beakers with clean water (figure 4, Student paired t-test, $p<0.05$).

After one hour the mussels had cleared approximately 80 % of the algae and 80 % of the mud particles from the water. When no algae were present the mussels removed 50 % of the mud particles (PAPER I).

Growth of mussels and scallops

Tissues from 40 mussels and 40 scallops were dried and weighed at the start of the experiment and from each treatment at the end of the exposure. The mean dry weight of the digestive gland of scallops increased with 54 % during the experiment (weight at start compared to the control after 10 weeks, table 6). The gonad of scallops was very small and did not grow more than 6 % (they were not mature). The dry weight of the muscle from control scallops also remained unchanged (table 6). There was less change in the mussel tissues, possibly because they had a shorter exposure time. They were sampled earlier than scallops because some groups appeared to be ready to spawn (this was observed at the 3 weeks sampling). The gonad of mussels was 15 % larger after 6 weeks exposure than at start, but the digestive gland was reduced with 12 %.

The largest reduction of weight was observed for gonads of mussels exposed to barite and to the highest concentration of used WBM (44% and 39% lower weight than the corresponding controls). The weight of the digestive gland of exposed mussels was however similar to the control. The weight of the digestive gland, gonad and muscle of scallops exposed to the highest concentration of used WBM and to barite particles was 20 - 30 % lower than the weight of these tissues from control scallops. The weight of muscles from scallops exposed to the metal mix was 22 % lower than in the control, but the mussel digestive glands were 16% larger in this treatment.

After 6 weeks exposure, when many mussels were sampled the scallops got more food and hence had the possibility to grow more, hence more differences was observed between control and exposed scallops than mussels.

Survival of mussels and scallops

Survival was high for mussels from all treatments; 3% or less mortality was observed. Increased mortality was observed for scallops exposed to metals and to the high concentration of used WBM. During the first 4 weeks 18% of the metal exposed scallops died. Later in the experiment, however, only 9% of the surviving metal exposed scallops died. Sampling for biomarker analysis and histopathology was done after 2 weeks exposure to metals (instead of 3 weeks) because of the observed mortality. In the high used WBM exposure 27% of the scallops died during the last 2 weeks of the 10 weeks exposure, but no mortality was detected during the first 8 weeks. Maximum 5% mortality was observed in the other treatments during the 10 week experiment.

'Stress on stress' test for mussels

After 8 weeks exposure a 'stress on stress test' was done on mussels exposed to a mixture of metals, barite particles and three concentrations of used water based drilling mud with barite as the weighting material (and control). LT50 is defined as the number of days to 50 % mortality for each group. Statistical comparison of survival time for mussels from the different treatments was done using the non-parametric Wilcoxon test.

The LT50 was significantly reduced for mussels exposed to 2 and 20 mg/L used WBM and for the mussels exposed to metals. The LT50 for mussels exposed to barite and to the lowest used mud concentration was not significantly lower than the control (figure 5, $p<0.05$, Wilcoxon). The results show that the mussels were more affected by the used WBM than by barite. In the first part of the test the mussels exposed to metals were dying most rapidly, but later the mussels from the medium used WBM exposure showed higher mortality rates. In the used WBM treatments LT50 was 20%, 40% and 30% lower than in control. There were more effects in all used mud treatments than in the barite exposure, but the metal mix exposed mussels showed most effect in this test (50% reduction of LT50, $p<0.0001$, Wilcoxon).

CONCLUSIONS

Effects of the metal mix. The metal mix caused increased level of oxidative stress (TOSC), reduced lysosomal membrane stability and clearance rate for both mussels and scallops. In addition histopathological damage was observed in the gills of scallops exposed to metals, and the growth of scallops was reduced. Survival time (LT50) for mussels in the stress on stress test was significantly reduced.

Effects of barite. 23 mg/L barite caused increased level of oxidative stress and reduced growth for both mussels and scallops. In addition increased level of DNA damage (comet) and reduced clearance rate was observed for scallops. In mussels histopathological damage was observed in the gills and digestive gland cells.

Effects of used WBM. The used drilling mud caused increased level of oxidative stress and reduced lysosomal membrane stability, histopathological damage and reduced clearance rate and growth for both mussels and scallops. In addition increased level of DNA damage was observed for scallops, and reduced survival time for mussels was observed in the 'stress on stress test' for mussels. Increased mortality of scallops was also observed in the last period of the 10 weeks exposure to 20 mg/L used WBM.

The overall lowest effect concentration (LOEC) for used drilling mud was the lowest tested concentration; 0.5 mg/L. It was the clearance rate for scallops that was the most sensitive parameter for detecting effects of used drilling mud. In the 2 mg/L used WBM exposure significant responses were detected for most parameters in both scallops and mussels.

Cranford *et al.* exposed adult sea scallops *(Placopecten magellanicus)* to different types and concentrations of used operational drilling fluids and their major constituents in the laboratory under environmentally representative conditions (Cranford et al., 1999). They concluded that threshold waste concentrations causing reductions in somatic and/or reproductive tissue growth were: greater than 10 mg/L for used waterbased mud (WBM); 2 mg/L for bentonite; and less than 0.5 mg/L for barite and used oil-based mud (OBM). Cranfords results showed that chronic intermittent exposure of sea scallops to dilute concentrations of operational drilling wastes, characterized by acute lethal tests as practically non- toxic, can affect growth, reproductive success and survival. The results from our experiment with *Pecten maximus* confirm that low concentrations of WBM have negative effects on scallops.

The results from the present experiment confirm Cranford et al.'s overall conclusion that low concentration of drilling mud has negative effects on bivalves. A more detaild comparison/discussion of our results and the results from relevant papers (especially Cranford et al. (1999), Barlow & Kingston (2001) and relevant references in these papers) will be included in our coming paper.

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TABLES

Table 1. Experimental design; treatments (dry weight of particles), test species, exposure time and test parameters. WBM: Used Water Based Mud with barite as weighting material. Metal mix: 10.2 µg/L Cu, 34.8 µg/L Zn, 1.4 µg/L Cd and 3.2 µg/L Pb.

Treatments	Species	Exposure time (weeks)	Test parameters
Control Metal mix 23 mg/L barite particles 0.5 mg/L WBM 2 mg/L WBM 20 mg/L WBM	*Pecten maximus* (Scallop)	3	Lysosomal membrane stability (NRRT), DNA strand breaks (Comet assay), oxidative stress (TOSC), histopathology
		10	Growth of gonad, digestive gland and muscle Filtration rate
	Mytilus edulis (Mussel)	3	Lysosomal membrane stability (NRRT), DNA strand breaks (Comet assay), oxidative stress (TOSC), histopathology
		6	Growth of gonad and digestive gland
		8	Filtration rate, 'stress on stress test'

Table 2. Results from Comet assay on haemocytes from mussels and scallops exposed for 3 weeks to three concentrations of used WBM, barite and metal mix. The table show the mean percent tail DNA in all control groups and the mean percent tail DNA for exposed cells divided by the corresponding control value (Exposed/Control) (** p<0.01, Wilcoxon, n = 450 cells)

Treatment	Mussels		Scallops	
	Control	Exposed / control	Control	Exposed / control
0.5 mg/L WBM	7.6	1.2	11.3	0.9
2 mg/L WBM	7.2	1.2	10.2	1.1
20 mg/L WBM	7.8	1.1	9.1	1.2 **
23 mg/L Barite	10.5	1.1	9.7	1.3 **
Metal mix	8.0	1.0	12.2	1.0

Table 3. Results from the lysosomal membrane stability test on haemocytes from mussels and scallops exposed for 3 weeks to three concentrations of used WBM, barite and metal mix. The table show median NRRT and significance level from comparisons using the Wilcoxon test (n = 9 for control and n = 15 for exposed, *p<0.05, **p<0.01, ***p<0.001)

Treatment	Mussels		Scallops	
	Control	Exposed	Control	Exposed
0.5 mg/L WBM	180	120*	180	165
2 mg/L WBM	90	60	180	120*
20 mg/L WBM	150	120	150	120*
23 mg/L Barite	150	45	120	90
Metal mix	120	30***	150	30***

Table 4. Overview of the groups that were significantly different from each other: ***. Scallops: Multiple range tests. Mussels and cod: ANOSIM and SIMPER tests (both gave the same result for the treatments in the table).

	Mussels 3 weeks	Scallops 3 weeks	Scallops 10 weeks
Control *vs* Barite	***		
Control *vs* Metals		***	***
Control *vs* 0.5 mg/L WBM			
Control *vs* 2 mg/L WBM	***	***	
Control *vs* 20 mg/L WBM	***	***	
Barite *vs* Metals		***	***
Barite *vs* 0.5 mg/L WBM			
Barite *vs* 2 mg/L WBM		***	
Barite *vs* Mud 3	***	***	
Metals *vs* 0.5 mg/L WBM		***	***
Metals *vs* 2 mg/L WBM		***	***
Metals *vs* 20 mg/L WBM	***	***	***
0.5 mg/L WBM *vs* 2 mg/L WBM		***	
0.5 mg/L WBM *vs* 20 mg/L WBM	***		
2 mg/L WBM *vs* 20 mg/L WBM	***		

Table 5. Statistical comparison (Students paired t test) of feeding efficiency of mussels and scallops continuously exposed to drilling mud for 6 and 10 weeks respectively. After the long term exposure the bivalves were transferred to beakers with clean seawater and algae. The table show percentage of remaining algae with increasing time and the p-values (n = number of bivalves). *p<0.05, **p<0.01, ***p<0.001

Time (min)	Control	0.5 mg/L WBM	2 mg/L WBM	20 mg/L WBM	23 mg/L Barite	Metal-mix
Mussels (*Mytilus edulis*) exposed for 6 weeks						
4	99 ± 2 (7)	99 ±4 (7)	97 ± 2 (7)	100 ± 2 (7)	98 ± 2 (7)	101 ± 4 (7)
8	94 ± 4 (7)	93 ± 6 (7)	94 ± 5 (6)	99 ± 3 (7)	92 ± 8 (7)	100 ± 2 (7)
16	83 ± 14 (7)	79 ± 12 (7)	79 ± 12 (7)	97 ± 6 (7)	83 ± 14 (7)	99 ± 3 (7)
24	74 ± 22 (7)	62 ± 14 (7)	69 ± 18 (7)	94 ± 8 (7)*	71 ± 18 (7)	98 ± 4 (7)**
32	64 ± 29 (7)	46 ± 17 (7)*	57 ± 23 (7)	89 ± 10 (7)***	66 ± 21 (7)	96 ± 6 (7)***
64	31 ± 30 (7)	16 ± 10 (7)	24 ± 21 (7)	53 ± 14 (7)***	32 ± 33 (7)	93 ± 11 (7)***
95	12 ± 15 (7)	4 ± 4 (7)	8 ± 7 (7)	23 ± 11 (7)	21 ± 35 (7)	84 ± 15 (7)***
136	4 ± 5 (7)	1 ± 1 (7)	2 ± 2 (7)	8 ± 4 (7)	16 ± 36 (7)	71 ± 23 (7)***
Scallops (*Pecten maximus*) exposed for 10 weeks						
8	83 ± 19 (14)	100 ± 2 (7)*	99 ± 1 (14) *	104 ± 6 (2)	100 ±3 (7)	101 ± 3 (7)*
16	61 ± 33 (14)	100 ± 2 (7)***	98 ± 3 (14) ***	105 ± 6 (2)**	99 ± 3 (7)***	100 ± 2 (7)***
32	17 ± 15 (14)	91 ± 10 (7) ***	90 ±11 (14)***	101 ± 3 (7)***	97 ± 4 (7)***	99 ± 4 (7)***
64	1.4 ± 2.4 (14)	35 ± 23 (7) ***	72 ± 27 (14)***	98 ± 5 (7)***	83 ± 17 (7)***	96 ± 5 (7)***
95	0.3 ± 0.5 (14)	15 ±22 (7)	55 ± 35 (14)***	95 ± 5 (7***	63 ± 26 (7)***	65 ± 25 (7)***
136		10 ±19 (7)	39 ± 7 (14)***	93 ± 9 (7)***	58 ± 26 (6)***	43 ± 36 (7)***
180				86 ± 20 (7)***		
240				79 ± 30 (7)***		

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Table 6. Dry weight (mg) of bivalve tissues (mean (st.dev)) at the start of the exposure and after 10 weeks exposure of scallops and 6 weeks exposure of mussels (n = 40 for each treatment/species). * significantly different from the control (Student's paired t-test, p<0.05).

	Start	Control	0.5 mg/L WBM	2 mg/L WBM	20 mg/L WBM	23 mg/L barite	Metal mix
Mussels *(Mytilus edulis)* - 6 weeks exposure							
Digestive gland	89 (38)	78 (26)	70 (25)	77 (35)	77 (25)	78 (24)	90 (43)
Gonad	352 (190)	405 (244)	331 (171)	378 (227)	227 (126)*	249 (164)*	358(212)
Scallops *(Pecten maximus)* - 10 weeks exposure							
Digestive gland	90 (26)	139 (32)	133 (27)	129 (42)	99 (27)*	97 (22)*	127 (44)
Gonad	17 (12)	18 (12)	17 (6)	16 (9)	13 (5)*	14 (5)*	17 (7)
Muscle	557 (243)	565 (191)	575 (174)	529 (201)	451 (166)*	403 (125)*	440 (170)*

Table 7. Summary of statistically significant responses in *Mytilus edulis* (top) and *Pecten maximus* (bottom) exposed to drilling mud and metals (+ : significant effect p< 0.05).

Mussels	Used water based drilling mud			Barite	Metal
(Mytilus edulis)	0.5 mg/L	2 mg/L	20 mg/L	23 mg/L	mix
TOSC		+	+	+	+
Comet					
NRRT	+				+
Filtration rate			+		+
Weight of digestive gland					
Weight of gonad			+	+	
Histopathology		+	+	+	
'Stress on stress'		+	+		+

Scallops	Used water based drilling mud			Barite	Metal
(Pecten maximus)	0.5 mg/L	2 mg/L	20 mg/L	23 mg/L	mix
TOSC				+	+
Comet			+	+	
NRRT		+	+		+
Filtration rate	+	+	+	+	+
Weight of digestive gland			+	+	
Weight of gonad			+	+	
Weight of muscle			+	+	+
Histopathology		+	+		+

FIGURES

Figure 1

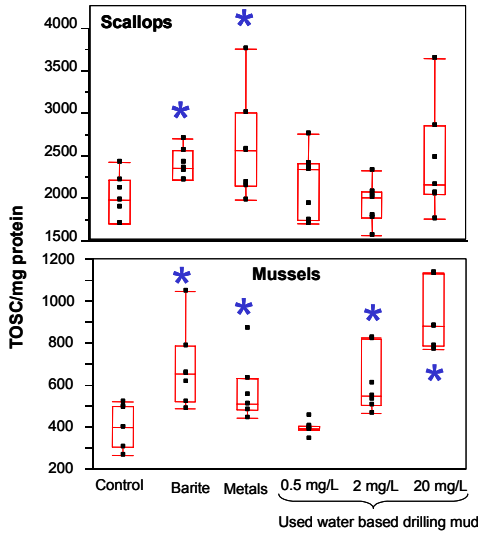

Figure 1. Total oxygen scavenging capacity (TOSC) in digestive gland of mussels and scallops exposed to a mixture of metals, barite particles and three concentrations of used drilling mud with barite as the weighting material. Each exposed group was compared to the control using the Wilcoxon sign-rank test, $p<0.05$: *

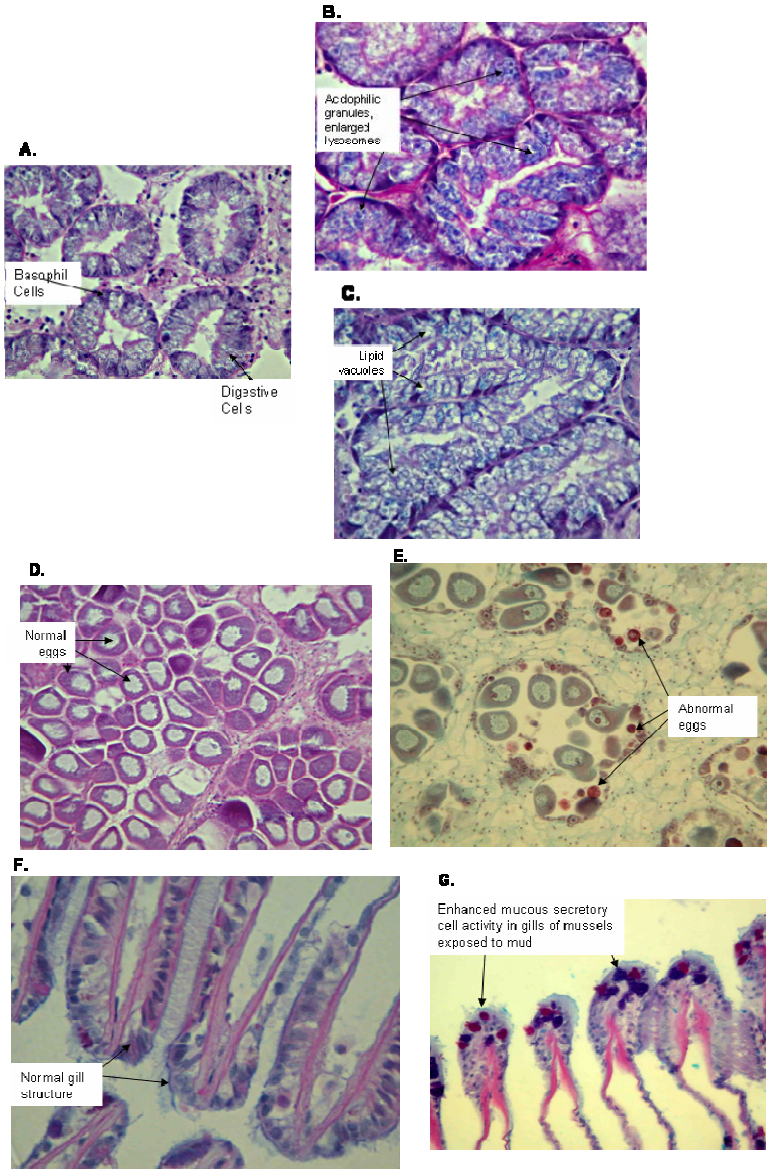

Figure 2. A: Digestive tubules in control mussels. B and C: Digestive tubules in mussels exposed to 20 mg/L WBM. D: Normal eggs in gonad from control mussel. E: Abnormal eggs in mussels exposed to 20 mg/L WBM. F: Normal gill structure from control mussel. G: Enhanced mucous secretory cell activity in gills of mussels exposed to 20 mg/L WBM (Photos: David Lowe, PLM, UK).

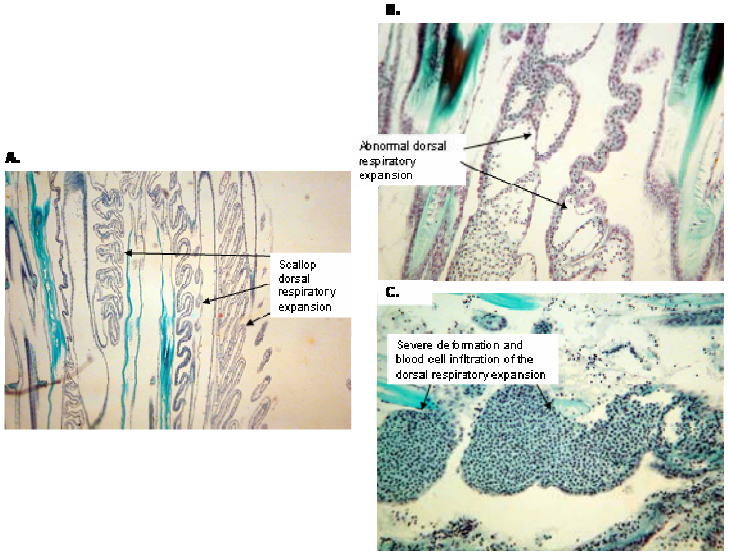

Figure 3. A: Normal gill structure from control scallops. B: deformities in scallop gills exposed to a mixture of metals.

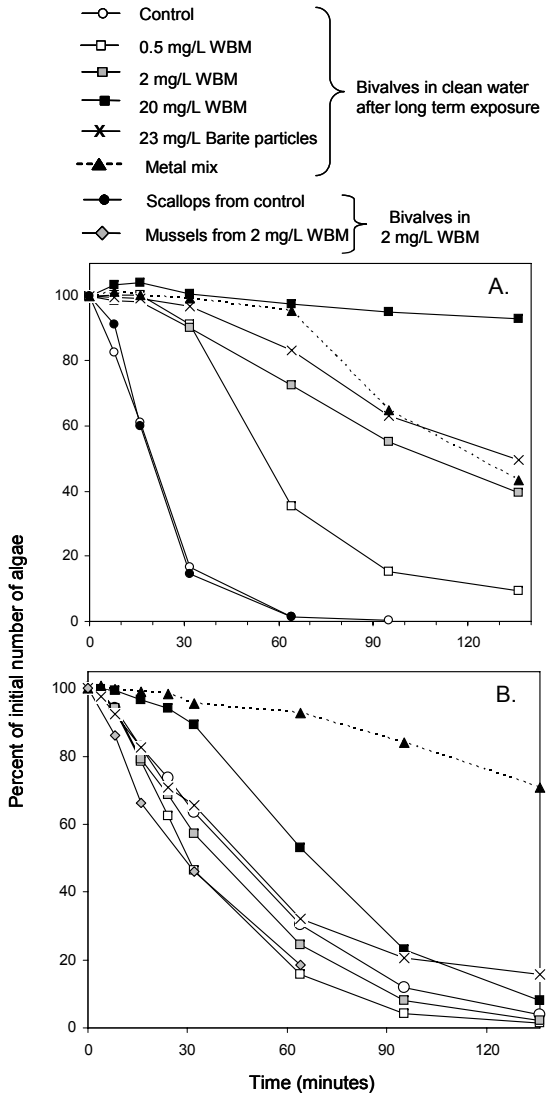

Figure 4. Feeding efficiency of scallops continuously exposed to drilling mud for 10 weeks (A) and mussels exposed for 8 weeks (B). The bivalves were transferred from the exposure tanks to beakers with clean water with alga. Filtration of algae with 2 mg/L WBM present was also measured for control scallops and mussels from the 2 mg/L WBM treatment. The graphs show percentage of remaining algae with increasing time.

A.

LT50 (days)	Control	0.5 mg/L WBM	2 mg/L WBM	20 mg/L WBM	23 mg/L Barite	Metal mix
	25	21	14	18	23	13

	Control	0.5 mg/L WBM	2 mg/L WBM	20 mg/L WBM	23 mg/L Barite	Metal mix
Control		p = 0.08	p = 0.0001	p = 0.02	p = 0.6	p = 0.002
0.5 mg/L WBM			p = 0.13	p = 0.9	p = 0.06	p = 0.15
2 mg/L WBM				p = 0.01	p < 0.0001	p = 0.4
20 mg/L WBM					p = 0.007	p = 0.02
23 mg/L Barite						p < 0.0001
Metal mix						

B.

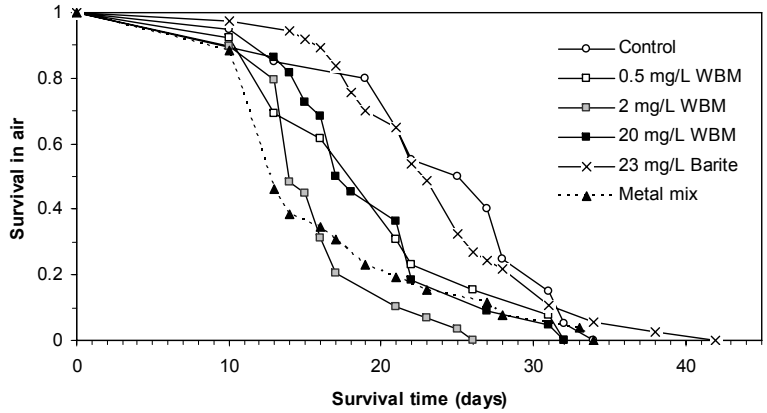

Figure 5. Survival time (in days) for mussels in air. Results from the stress on stress test with mussels exposed for 8 weeks to a mixture of metals, barite particles and three concentrations of used water based drilling mud with barite as the weighting material. **A.** LT50: days to 50% mortality for each group. The table show the results from statistical comparison of survival time for mussels from the different treatments (Wilcoxon). B. Survival in air plotted against time.

IV.

Effects of suspended particles of water based drilling mud on cod

R. K. Bechmann, K. B. Øysæd, E. Lyng, D. Lowe

ABSTRACT

Juvenile cod *(Gadus morhua)* were exposed for 3 weeks to suspended particles of used water based drilling mud with barite as the weighting material (WBM), barite particles, ilmenite particles and a mixture of metals. Metal concentrations in gills, liver and bile, protein pattern (SELDI-TOF), oxidative stress (TOSC), condition factor, liver somatic index and gill histopathology was analysed.

There was a small, but significant reduction of the condition factor of cod exposed to 39 mg/L used water based drilling mud. There was also an indication of reduced liver somatic index in this treatment. There was no significant changes in the total oxygen scavenging capacity (TOSC) in any treatment. A high incidence of aneurysms was observed in the gills of cod exposed to 62 mg/L barite particles as well as clubbing of the lamellar and epithelial lifting. Epithelial lifting and hypertrophy of the gill lamellar were also prevalent in several of the treatment groups as was thickening of the filament epithelium in the 0.5 mg/L and 4 mg/L used WBM exposure. Aneurysms were present in the gills of 50 % of the fish from the barite exposure group. The aneurysms in many instances occupied 75% or more of the length of the lamellar and as such are a most extreme abnormality. There was no evidence of aneurysms in the fish exposed to used WBM. There was a high incidence of both lamellar clubbing and fusion in the ilmenite and the 4 mg/L and 39 mg/L used WBM exposures compared to the control. All fish in the lowest used WBM exposure exhibited clubbing of the gill lamellae.

The gills of barite exposed cod appeared to be more damaged than the gills of cod exposed to used WBM, although the condition factor was more affected by the used WBM than the barite particles. The proteomics results indicated very little change in protein metabolism in the barite exposed cod compared to cod exposed to used WBM.

Based on the results we have so far, the most likely explanation to the histological damage observed in the gills of cod exposed to barite is that the particles caused physical damage. The type of histological damage observed in the gills of cod exposed to barite, dissolved metals and used WBM was not the same. Different types of histological damage was observed in these treatments also for scallops and mussels, indicating that chemicals in the used WBM may contribute to the observed effects.

INTRODUCTION

Suspended clay either from natural sources or from drilling mud can interfere with suspension feeding and damage the gills of bivalves (Morse, 1982, Stevens 1987, Cranford & Gordon, 1991, 1992) and fish (Sprague and Logan, 1979).

Waterborne metals can bind to gills of fish and disrupt the ionoregulatory and respiratory functions of the gills (Playle, 1998). Histopathological changes have been observed in gills from metal exposed fish (Muhvich, Jones, Kane, Anderson & Reimscheussel, 1995; Thophon, Kruatrachue, Upatham, Pokethitiyook, Sahaphong & Jaritkhuan, 2003) and bivalves (Clark, Teh & Hinton, 2000) (Teh, Clark, Brown, Luoma & Hinton, 1999).

Cod (*Gadus morhua)* was chosen as a representative fish species for the North Sea because of its ecological and economic importance. The parameters studied in juvenile cod were changes in protein pattern (proteomics), oxidative stress (TOSC), DNA damage (comet assay), gill histopathology, condition factor and liver somatic index.

Fish gills constitute a multifunctional organ which by virtue of its structural complexity accounts for well over 50% of the total surface area of the animal. The gill consists of three components: the gill arch, which supports the filaments which in turn supports the secondary lamellae which are the sites of gas exchanges (Fig 1). Each gill arch has two rows of filaments from which arise the lamellar which are regularly spaced along their length. Gill arches and filaments distribute the blood flow into the lamellae. Mucous cells are numerous on the gills, and are predominantly located with the filaments, principally on the efferent side; mucous cells can also be found on the gill arches themselves. Whilst there is no clear evidence suggesting the mucous cells are more or less abundant in any particular conditions that the fish may encounter they have been shown to discharge in response to extreme changes in the water such as a decrease in pH.

MATERIALS AND METHODS

Experimental design

The exposure system is described in PAPER I. Juvenile cod *(Gadus morhua)* were purchased from Sagafjord Sea Farm AS, Stord, Norway (18.02.04, week 8). They were kept in two large tanks with continuous flow of sea water, and fed pellets. The fish were acclimated for 5 weeks before start of the experiment. Three concentrations of used water based drilling mud with barite as the weighting material was tested in addition to one treatment with barite particles and one with ilmenite. The fish were exposed for 3 weeks to study metal accumulation (PAPER II), biomarker responses and histopathology. Mussels and scallops were exposed to the same treatments (except ilmenite) in separate tanks (PAPER III). Eighty cod were exposed in each of the 7 treatments (table 1 and PAPER I). At start of the experiment the mean weight of the fish was 43 grams. During the three week exposure the cod were fed approximately 30 g pellets for each tank each day (1% of the weight of the fish in the tank). In tanks with good visibility it was observed that all the food was eaten. For logistic reasons (to be able to analyse DNA strand breaks by comet assay on samples from all treatment groups) one group of exposed cod (n = 21) and one group from the control (n = 9) was sampled each day for 6 days. There were 6 sampling

days. The exposure of the different treatment groups was started with a time lag to ensure the same exposure time for each treatment.

Sampling

On each of the 6 sampling days one exposed group (n = 21) and a group of control fish (n = 9) were sampled. Length and weight of the fish, and the weight of their liver was measured, and the condition factor and the liver somatic index was calculated for fish from each treatment:

$$\text{Condition factor (CF)} = (\text{weight, gram}) \cdot 100 / (\text{length, cm})^3$$

$$\text{Liver somatic index (LSI)} = (\text{weight of liver}) / (\text{weight of fish}) \cdot 100$$

Blood from the 9 control fish and 9 exposed fish were used for comet assay, but because the controls were variable the results have been excluded. Gills, liver and bile was sampled from each cod. The livers were cut in two pieces: one part was kept at -20°C for metal analysis, and one part to TOSC analysis. For TOSC samples from three cods were pooled together to make 7 pooled samples from each treatment. The gall bladder was emptied in cryo tubes and kept at -20°C for metal analysis. Gill arch number 2 and 3 were sampled from 10 cod in each treatment. A piece of the gill arches were fixed in 4% buffered formalin for histology, and the rest was kept at -20°C for metal analysis.

TOSC

Liver samples were homogenised in phosphate buffer (pH 7.4, 0.5 g liver to 2 ml buffer) and centrifuged at 12000g for 20 min. The supernatant was further centrifuged at 100.000g for 1 hour, and the supernatant (cytosol fraction) was taken out for TOSC analyses. Samples were stored in -80°C until analysis.

Peroxyl radicals were generated by the thermal homolysis of 2-2'-azo-bis-(2 methyl-propionamidine)-dihydrochloride (ABAP) at 35°C. Peroxyl radical can oxidize the substrate α-keto-methiolbutiryc acid (KMBA) to ethylene gas, which is measured with gas chromatography. The optimal assay conditions were 0.2 mM KMBA, 20 mM ABAP in 100 mM KH_2PO_4 buffer, pH 7.4. Reactions were carried out in 10 ml rubber septa sealed vials in a final volume of 1 ml. Ethylene formation was monitored for 96 minutes with a Hewlett Packard (HP 5890 series II) gas chromatograph, equipped with a supelco SPB-1 capillary column (30 m, 0.32 mm, 0.25 µm) and a flame ionization detector (FID). The oven, injection and FID temperatures were 35, 160 and 220°C, respectively; helium was the carrier gas (1 ml/min flow rate) and a split ratio 20:1 was used. The data acquisition system was run by the software Millenium32® (Waters). Each analysis requires the measurement of a control (no antioxidants in the reaction vial) in addition to the liver samples. By plotting the absolute value of the difference between the ethylene peak areas obtained at each time point for the sample and control reactions it is possible to visualize whether the oxyradical scavenging capacity of the solution is changed. TOSC is quantified according to Equation 1, where IntSA and IntCA are the integrated areas from the curve defining the sample and control reactions, respectively.

$$\text{TOSC} = 100 - (\text{IntSA}/\text{IntCA} * 100) \qquad (1)$$

Thus, a sample that displays no oxyradical scavenging capacity would give an area equal to the control (IntSA/IntCA = 1) and a resulting TOSC = 0. On the other hand as IntSA/IntCA goes to 0 the hypothetical TOSC value approaches 100. Because the area obtained with the sample is

related to that of the control, the obtained TOSC values are not affected by small variations in instrument sensitivity, reagents or other assay conditions. The specific TOSC value is expressed per mg protein in the sample.

Histological analyses

Gill arch number 2 and 3 were sampled from 10 cod in each treatment. A piece of the gill arches were fixed in 4% buffered formalin for histology (the rest was frozen and used for metal analysis). After 48 hours the samples were transferred to ethanol. One cod gill arch with its associated filaments and lamellae was prepared for sectioning The tissues were prepared as follows for histological analysis using the same protocol on an automatic tissue processor. Sections were cut at 7µm and stained using the following procedures. The fish gills sections were all stained in Papanicoloau to view general structure and Periodic Acid Schiff/ Alcian blue for neutral glycoproteins and acidic glycoproteins respectively.

The effects of exposure on the cod gill tissue was considered in terms of the presence of the following conditions, aneurysms, lifting of the lamellar epithelium, fusion of the lamellar, clubbing of the lamellar tips and thickening of the filament walls. These pathologies were scored as 1 (absent), 2 (present) and 3 common.

Statistical analysis – histology. The results of the histopathological biomarkers derived from the cod sections were tested for significance using multivariate analysis software, PRIMER V6. Non-metric MDS, derived from Bray Curtis similarity matrices was used to visualise dissimilarities between sample groups, nearby points in the plot reflecting similar biomarker values. The results were then further tested for significance using ANOSIM which is roughly analogous to a univariate ANOVA and reflects differences between treatment groups in contrast to differences among replicates within sites (the R statistic). Under the null hypothesis H_o"(no difference between treatment"), R=0 and this was tested by a non-parametric permutations approach; there should be little or no effect on the average R value if the labels identifying which replicates belong to which groups are randomly rearranged. Finally, the biomarkers that make the greatest contribution to the observed treatment differences were determined using SIMPER (similarity percentages), which partitions the tested dissimilarity between groups into contributions from each biomarker.

RESULTS

Condition factor and liver somatic index

Juvenile cod are able to increase their bodyweight with 1% each day when fed 1-3% of their bodyweight, and kept at 14-16°C in a fish farm. In the present experiment the temperature was 9°C (below the temperature for max growth). To avoid accumulation of excess food on the bottom of the tanks the fish were fed 1 % of their bodyweight pr day. At the start of the exposure the mean weight of the cod was 43 gram. It was observed that all pellets were eaten in the exposures with good visibility, but it was difficult to see whether or not the fish were eating in the high mud and barite exposures. There was a significantly lower condition factor (CF) and liver somatic index (LSI) for cod in the highest mud exposure (figure 3). The CF for cod exposed to the high concentration of used WBM was 8 % lower than in the control (p= 0.02; Dunnett's Method, p=0.004; Student's t), and the LSI for cod exposed to high concentration of used WBM was 19 % lower than in the control (p= 0.09, Dunnett's Method, p=0.02, Student's

t). Cod exposed to ilmenite also had lower CF and LSI than control fish, but the difference was not statistically significant. The CF for cod exposed to ilmenite was 5 % lower than in the control, and the LSI was 13 % lower, but the differences were not statistically significant. The highest particle exposure was barite, but CF and LSI were slightly higher in fish exposed to barite than control fish.

TOSC

There was no significant difference in TOSC between control cod and cod exposed to drilling mud or metals (figure 2 and 3). In bivalves, however, significantly increased TOSC was observed. For mussels significant increase in TOSC was observed in all treatments except the lowest exposure of used mud. For scallops significant increase in TOSC was observed in the metal and barite treatment.

Histopathology in gills

The results from the histopathological analysis of the gills are presented in table 2-4 and figure 4–8. Whilst there was evidence of some background histopathological changes in the control treatment group there was a high incidence of aneurysms in the barite treatment group as well as clubbing of the lamellar and epithelial lifting (see table 2, and figure 7). Epithelial lifting and hypertrophy of the gill lamellar were also prevalent in several of the treatment groups as was thickening of the filament epithelium in the 0.5 mg/L and 4 mg/L used WBM exposure (UM1, UM2) (figure 4-6). There was no evidence of any residual mud particles on the gills. With the exclusion of the control group all other treatment groups exhibited a high incidence of leukocyte infiltration in the filaments and lamellae.

There was no evidence of an increase in mucous cells on the gill lamellae of fish from any treatment group. Aneurysms, were present in the gills of 50% of the fish from the barite exposure group as well as in one fish from each of the metal and ilmenite exposure groups. There was no evidence of aneurysms in the fish exposed to used mud (UM1, UM2 or UM3). The aneurysms in many instances occupied 75% or more of the length of the lamellar and as such are a most extreme abnormality. The fact that 50% of the barite exposure group exhibited aneurysms after only 3 weeks exposure is a significant finding and indicates that it is detrimental to the cod's general health status. There was a high incidence of both lamellar clubbing and fusion in the ilmenite and the4 mg/L and 39 mg/L used WBM exposures (UM2 and UM3) compared to the control. All fish in the lowest used WBM exposure (UM1) exposure exhibited clubbing of the gill lamellae.

The statistical comparisons of the different treatment groups using Primer and the MDS plot clusters showed that the barite and ilmenite together, the 3 mud exposures and the metals together and the control stands out on its own. The test results from ANOSIM indicate significant differences in the incidence of the abnormalities between control and barite, ilmenite and 2 mg/L used WBM (table 3). In addition the incidence of abnormalities in the cod exposed to barite was different from the abnormalities in the metal exposed cod and in the cod exposed to used WBM. The incidence of abnormalities in cod exposed to ilmenite was also different from the abnormalities in cod exposed to 0.5 and 2 mg/L used WBM. The ilmenite exposed group was, however, not significantly different from the other exposed groups (only from the control) when using the Simper test (table 4). The other results from the ANOSIM (table 3) was

confirmed with the Simper test. The principal driver for significant differences between treatment groups was epithelial lifting, however, the presence of aneurysms was also a contributing factor (Simper test, table 4).

In conclusion the 3 species used in these studies have responded to different degrees following exposure to mud, barite, and metals. The scallop gills were severely impacted by exposure to the metals mixture, the cod, whilst also affected by metals, were impacted most severely following barite exposure as were the mussels.

The concentration of barium was significantly increased in the gills of barite exposed cod (but not the concentration of Cu, Cd, Pb and Zn) (PAPER II). Based on the partition coefficients for $BaSO_4$ in water it is unlikely that barium can leak out of the barite particles, but we can not exclude the possibility that the availability of barium increase once particles are adsorbed to or absorbed in the gill tissue. Based on the results we have so far, the most likely explanation to the histological damage observed in the gills of cod exposed to barite is that the particles caused physical damage. The type of histological damage observed in the gills of cod exposed to barite, dissolved metals and used WBM was not the same. Different types of histological damage was observed in these treatments also for scallops and mussels, indicating that chemicals in the used WBM may contribute to the observed effects.

References

Hughs, G.M.and Grimstone, A.V. 1965. The fine structure of the secondary lamellae of the gills of *Gadus pollachius*. *Quarterly Journal of Microscopical Science*, 106, 343-353.

Table 1. Overview of treatments and endpoints/parameters for cod (*Gadus morhua)* exposed to suspended particles of drilling mud for 3 weeks. The particle concentrations are dry weight of particles in exposure tanks measured in water samples from the cod exposure tanks. WBM: water based mud with barite as the weighting material. Metal mix: 10.2 µg/L Cu, 34.8 µg/L Zn, 1.4 µg/L Cd and 3.2 µg/L Pb.

Treatment (mg dw/L)	**Parameter**
Control Metal mix 14 mg/L ilmenite 62 mg/L barite 39 mg/L used WBM 4 mg/L used WBM 0.5 mg/L used WBM	Change in protein pattern (proteomics) DNA damage (Comet assay) Oxidative stress (TOSC) Gill histology Condition factor Liver somatic index

Table 2. Percentage incidence of pathologies within treatment groups; + pathologies were exhibited (+). Percentage incidence of pathologies within treatment groups designated as category 3, i.e. common (+++).

Treatment	Aneurysms		Epithelial Lifting		Filament Thickening		Lamellar Fusion		Lamellar Clubbing	
	+	+++	+	+++	+	+++	+	+++	+	+++
Control	0	0	100%	10%	20%	0	40%	0	60%	0
ME	10%	10%	100%	0	50%	0	70%	0	90%	0
IL	10%	0	100%	40%	30%	0	90%	0	100%	0
BA	50%	0	100%	50%	20%	0	90%	0	90%	0
UM1	0	0	100%	0	60%	0	50%	0	100%	0
UM2	0	0	100%	0	70%	0	90%	10%	90%	0
UM3	0	0	90%	0	50%	0	80%	10%	80%	0

Table 3. ANOISM test results. Sample statistics (global R): 0.1, p = 0.003, no. of permutations: 999, no of permuted statistics greater than or equal to global R: 2. *** indicate significant differences between treatment groups.

	Control	Barite	Ilmenite
Metals		***	
Barite	***		
Ilmenite	***		
UM1		***	***
UM2	***	***	***
UM3		***	

Table 4. Average percentage dissimilarity between treatment groups (SIMPER). *** indicate significant differences between treatment groups.

	Control	Barite
Metals		***
Barite	***	
Ilmenite	***	
UM1		***
UM2	***	***
UM3		***

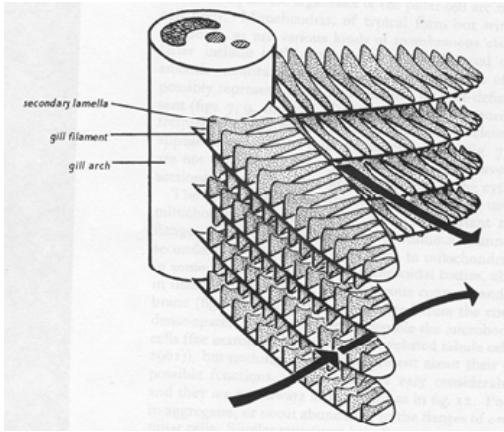

Figure 1. Diagram showing the arrangement of fish gill filaments and lamellae, arrows indicate direction of water flow (from Hughs and Grimstone, 1965).

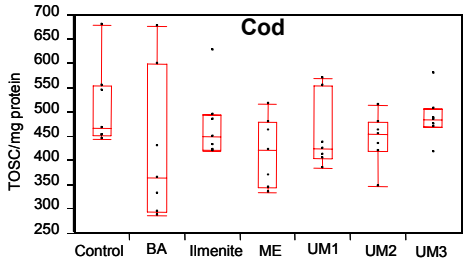

Figure 2. Total oxygen scavenging capacity (TOSC) in liver of juvenile cods exposed to a mixture of metals (ME), barite particles (BA), ilmenite particles (IL) and three concentrations of used drilling mud with barite as the weighting material (UM1, UM2, UM3). There were no significant differences between the different exposed groups and the control (Wilcoxon sign-rank test, p>0.05).

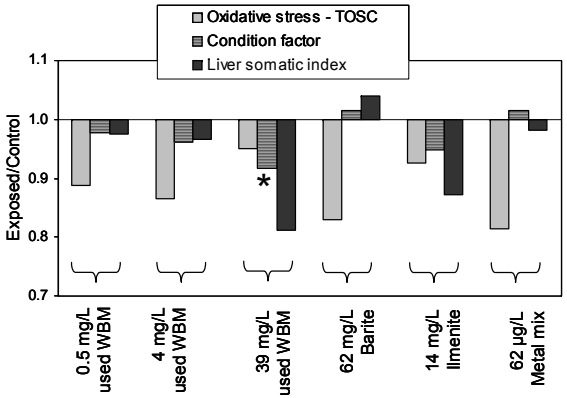

Figure 3. Overview of effects of used water based drilling mud (WBM), barite and ilmenite particles and a mixture of metals (Pb, Cu, Zn, Cd) on TOSC, condition factor and liver somatic index of cod *(Gadus morhua)*. Relative values on the y-axis: response in exposed cod/response in control cod. * statistically significant difference from control.

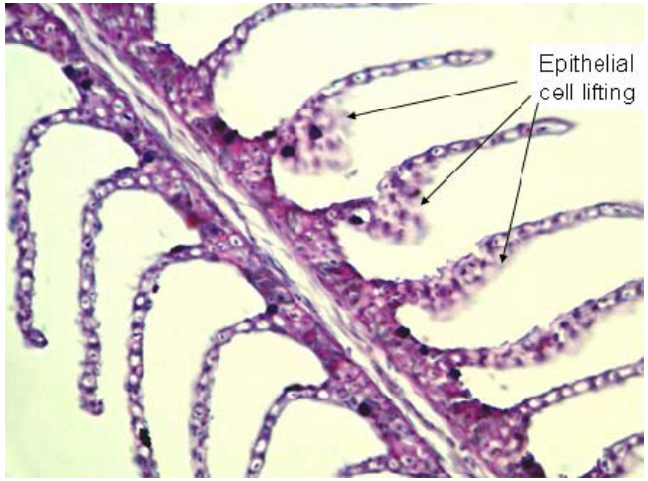

Figure 4. Abnormalities in gills of cod: Example of epithelial cell lifting (Photo: David Lowe, PLM, UK).

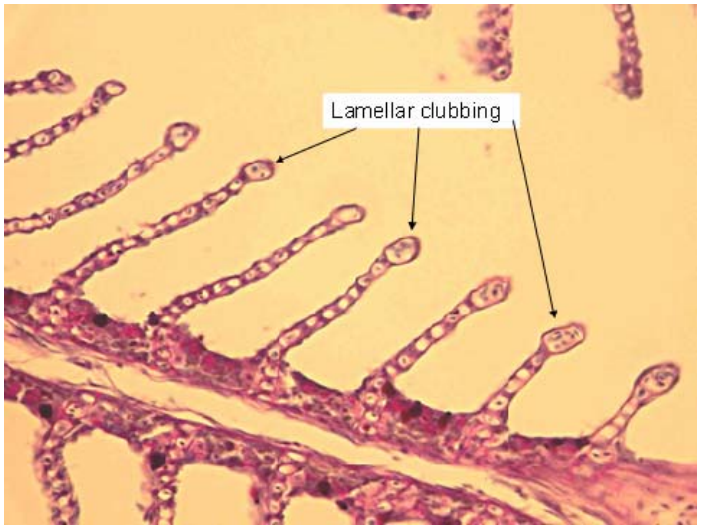

Figure 5. Abnormalities in gills of cod: Example of lamellar (Photo: David Lowe, PLM, UK).

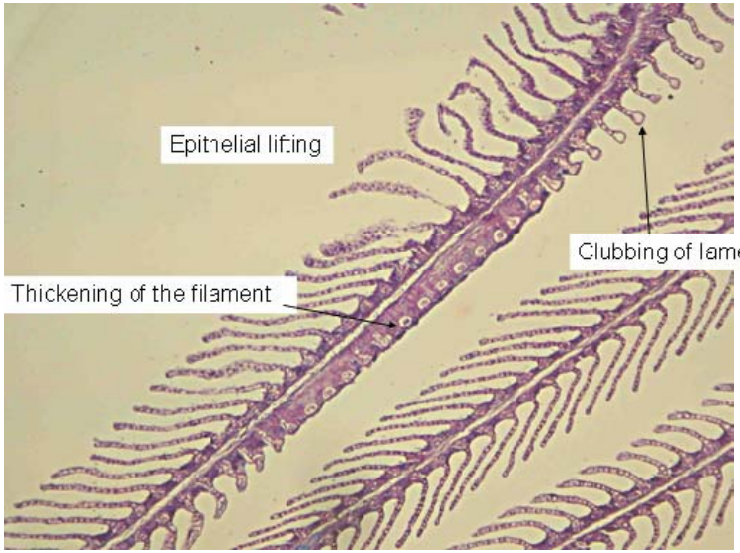

Figure 6. Abnormalities in gills of cod exposed for 3 weeks to 2 mg/L used water based drilling mud: thickening of the filament, epithelial lifting, clubbing of lamella (Photo: David Lowe, PLM, UK).

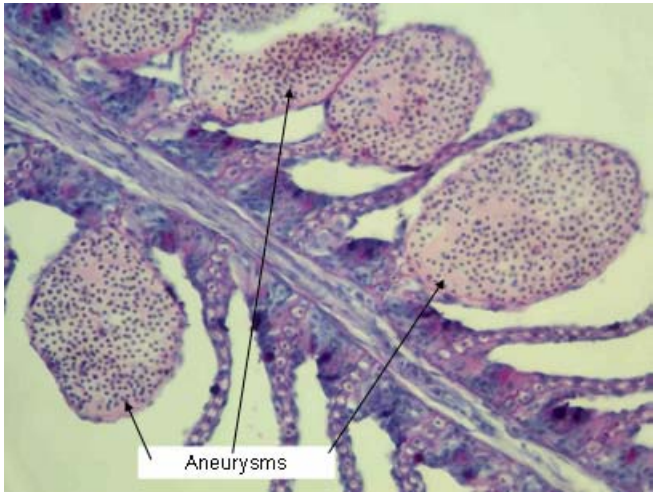

Figure 7. Abnormalities (aneurysms) in gills of cod exposed for 3 weeks to 23 mg/L barite particles (Photo: David Lowe, PLM, UK).

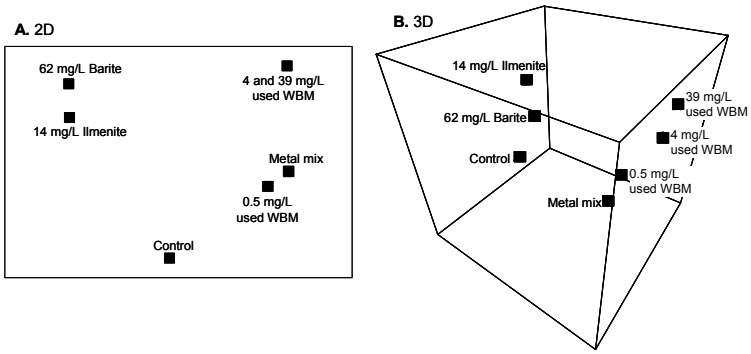

Figure 8. A: two dimensional and B: three dimensional non-metric MDS plots, derived from Bray Curtis similarity matrices, showing average dissimilarities between sample groups, nearby points in the plot reflect similar biomarker responses.

V.

Effects of suspended particles of drilling mud on development, growth and feeding of the mussel *Mytilus edulis* embryos and larvae

T. Baussant, I.C. Taban, K. Alfsnes, R.K. Bechmann

ABSTRACT

Mussel (*Mytilus edulis*) embryos and larvae were exposed continuously for a period of 11 days to used water-based drilling mud (WBM), barite particles and a solution of metals in experiments performed in 2004 and 2005. In WBM, barite was used as the weighting material and two concentrations were tested (measured concentrations: ≈4mg.l^{-1} [WBM_4] and ≈0.6mg.l^{-1} [$WBM_{0.6}$]). Barite particles concentrations were 62 and ≈ 8 mg.l^{-1} in 2004 and 2005 respectively. More than 90% of particles were below 5 μm both in barite and in WBM but a larger volume of fine particles was represented in WBM. The metal mixture concentration was composed of 10.2 μg.l^{-1} Cu, 34.8 μg.l^{-1} Zn, 1.4 μg.l^{-1} Cd and 3.2 μg.l^{-1} Pb. It was used as a positive control. The end points measured were the percentage of normal D-shell larvae 3 days post-fertilization and growth reduction at day 11. In both years, the metal mixture showed strong inhibition at an early stage of embryos development and none of the embryos reached the D-shell larvae stage 3 days post-fertilization. Barite particles exposure showed no difference in the percentage of D-shell compared to the control. In 2004, only 10% of the embryos developed normally to the D-shell in WBM_4 and 25% were still at the trochophore stage. However, these observations were not confirmed in the 2005 experiment and no difference to the control was observed in any of the WBM treatments. The measurement of morphometric parameters (area, perimeter, width and height) on 11-days old veligers using image analysis techniques show that growth was reduced in WBM_4 both in 2004 and 2005, while barite particles appeared to enhance growth (2005 experiment). Also, based on epifluorescence observations of ingestion and digestion of microalgae in the 2005 experiment, feeding efficiency was reduced in veligers exposed to WBM_4. This may explain the reduction in veligers growth in that exposure. The underlying mechanisms for these impairments remain unclear. Interference of fine particles (<3μm) with the ciliated velum, physical damage (particles) or toxicological (metals and/or possibly chemicals added to the drilling fluids) effects may explain the reported observations. Considering that any delay in growth will ultimately affect the number of offspring surviving to the settlement stage and beyond in bivalve molluscs, these results show that 4 mg.l^{-1} WBM can have important ecological consequences. This concentration may also affect the development and survival of other pelagic planktotrophic larvae.

INTRODUCTION

Large volumes of fluids are discharged during offshore drilling operation. The composition of the discharges is complex and formulation is quite variable. A major constituent is however represented by solid material like barite ($BaSO_4$) particles which are used as a weighting material. In the North Sea, the use of oil-based mud has been prohibited and the fluid component of drilling mud consists only of water. To date, there is no regulatory limit to the discharge of used water-based mud (WBM) into the sea. Concern and controversy exist

regarding the potential damaging effects of WBM to biota. In particular, there is an issue concerning the potential for chronic toxicity effects. Numerical model predictions indicate that fine particles of drilling fluids may spread over much greater distances than expected and can arise to sublethal effects which are documented in bivalve molluscs. Bivalves are filter feeding organisms that appear to be particularly sensitive to drilling fluids. Within a concentration range between 0.5 and 10 mg.l^{-1}, effects on growth, reproduction and survival have been reported in adult organisms (Cranford *et al.*, 1999; Armsworthy, 2005). These effects appear to be attributed to physical effects i.e. the ingestion of non-nutritious fine particles in the diet of these organisms. However a direct chemical toxicity of barite and other metals bound to particles may also play a significant role although it is unclear whether they are actually bioavailable for these organisms. Hence, environmental authorities are requesting investigations about the actual risk posed by discharges of WBM at sea.

An answer to that concern can be elucidated through early developmental stage bioassay. The young stages of organisms are more sensitive than adults. The use of early life stages in field monitoring is challenging but their use in laboratory-simulated exposure is highly relevant to assess the actual ecological impact of pollutant. Hence, when performed in realistic exposure conditions, the value of such bioassay is important and in addition to the ecological relevance the data can be directly used to validate risk assessment models.

The use of bivalve embryos and larvae to assess water quality and marine pollution has conducted to the development of rapid and reliable methods that can determine ecologically-relevant end points like the percentage of normal larvae after production of the first shell and , further in the development, the growth of the larvae (see for example review by His *et al.*, 2000). During the first two to three days following egg fertilization, embryos are characterised by sensitive cell divisions and both morphological and physiological changes for transformation into the next stage occur during that period (Widdows, 1991; His *et al.*, 2000). Hence, this period is particularly critical for the survival of the larvae. The sensitivity of early-life stages of bivalves and other invertebrates to metals and other pollutants has been well documented (Beiras and His, 1995; Hansen *et al.*, 1997; His *et al.*, 2000; Wedderburn *et al.*, 2000; Geffard *et al.*, 2003; Beiras and Albentosa; 2004). Since energy acquisition and utilisation are affected in adult bivalves exposed to drilling fluids, it is expected that such effects may also exist in early-life stages. Feeding of the D-shell larvae shortly after hatching is essential to their growth and ultimately survival (Bayne *et al.*, 1975; Widdows, 1991). These larvae have little storage capacity and must ingest nutritious organic material to grow. Micro-algae constitute their primary source of nutrition. Consequently, the presence of fine inorganic particles may alter feeding efficiency in feeding larvae.

The objective of this study was to investigate the chronic effects on early-life stages of the blue mussel *Mytilus edulis* of WBM. Developing embryos and larvae were exposed continuously in a flow-through system during a period of 11 days post-fertilization to WBM, to one of the major solid constituent i.e. barite and also a mixture of metals (metal-mix). The end points studied were the developmental abnormalities of embryos to the D-shell veliger stage and further growth reduction. Epifluorescence measurements were also performed using micro-algae to assess possible impairments of ingestion and digestion following WBM exposure in growing larvae.

MATERIALS AND METHODS

Early life stage assay

The bioassays were based on the recommendations given by ASTM (1989) and adapted after His *et al.* (2000) and Quiniou *et al*. (2005). Yet, in these experiments, the developing embryos and larvae were maintained in cylinders with flow-through of the exposed and control seawater. The tests were carried on in June 2004 and April 2005.

Origin of the mussels

The mussels were obtained from Aspøy Skjell og Produkutvikling as (Hundvåg, Norway) in 2004 and Vea Management as (Åkrehamn, Norway) in 2005. They were maintained in our research facility in clean running seawater for several weeks before use. During that period, mussels were fed microalgae (*Isochrysis galbanae* and *Skeletonema costatum*).

Spawning of mussels

At the onset of the test, the shell surface was brushed and byssus filaments were removed. Mussels were induced to spawn by thermal stimulation. They were placed in a dish and covered with seawater. Seawater was then rapidly changed alternating temperature between 9/10°C and 20°C for a period of maximum 2 hours. Algae was also added to induce the spawning which usually started after no longer than 1 hour. Oxygen concentration in the dish was monitored. Seawater was changed before measured oxygen reached 5 $mg.l^{-1}$.

Sperm and eggs collection

Gametes were collected individually from the spawning individuals (7/8 males and 9/10 females) and thereafter a separate solution of pooled sperm and pooled eggs was made. Sperm was first filtrated through a 30 µm nylon sieve and eggs were filtrated through a 100 µm nylon sieve to remove gross debris. Fecundation was obtained by transferring 5/6 ml of a pooled dense sperm solution to the pooled egg solution into a 2 L flask filled with filtrated seawater at 9°C (2004) or 10 °C (2005). Homogenisation was made by turning gently the sealed bottle upside down during the first hour thereafter. All glassware was pasteurised prior to their use.

Embryos and larvae exposure

Fecundity success (polar body emission), assessed on subsamples of 40 µl prior to the transfer, was over 90% in both experiments. The exposure of WBM and barite particles is described elsewhere in Bechmann *et al.*(PAPER I). Additional equipment was used to exposed embryos and larvae. The developing embryos were transferred into a continuous flow system consisting of a series of plexiglass cylinders with a volume of 250 ml, open at the top and siliconed with a 40 µm nylon mesh at the bottom (see figure 1 and 2). At start, the density of the eggs in each test cylinder was $30.ml^{-1}$. The medium inside the cylinders (exposed or control seawater) was continuously supplied with a peristaltic pump (Watson Marlow model 205S, UK) from the exposure tanks (see experimental design) at a flow of 8 ml/min. Five cylinders were used for both exposed and control groups. In 2004, two additional cylinders were used in the control group. Sampling was made 3 days, 11 days and 21 days (only 2004 but not reported in the present paper) post-fertilization. The samples were collected in scintillation glass vial and fixed in 4% formalin. Continuous feeding with a monoculture of *Isochrysis galbane* (density ≈ $30.000.ml^{-1}$) was started after the sampling at day 3 (D-shell larvae). Temperature in the exposure tank was 9±0.5°C in 2004 and 10±0.5°C in 2005.

Analysis of samples

Normal D-shell and growth assessment- The 3-days samples were transferred onto a 1 ml Sedgewick Rafter counting cell, observed under an optical Leica microscope (x400) and the percentage of trochophores, D-larvae with normal shell and D-larvae with abnormal shell was estimated after His *et al* (2000) and Quiniou *et al.* (2005). Usually a minimum of 100 larvae was observed in each sample but for samples containing a high number of particles, this number was lower. Veligers sampled at day 11 were used to assess growth. An image analysis software (Axiovision ver.4.3) connected to a Zeiss Axioplan 2 imaging epifluorescence microscope with an AxioCam MRm camera was used for the morphometric measurements of width, height, perimeter and area. A comparison of the image analysis data and visual measurements of height and width of veligers prior (n=138 observations) and subsequent (n=105 observations) to the analysis was performed using both samples from the 2004 and the 2005 experiments (see appendix to PAPER V). A one-way ANOVA test (the Dunnett's mean comparison) was used to test differences to the control group of larvae.

Ingestion and digestion assay - In the 2005 experiment, we applied epifluorescence microscopy technique to observe ingestion and digestion of the microalgae *Isochrysis galbanae* by 11-days old veligers from each of the treatment groups (except metal) and the control group. The methodology followed the description found in Babinchak and Ukeles (1979), Le Pennec and Rangel-Davalos (1985) and Martinez-Fernandez *et al*. (2004) (see figure 3). Feeding of the larvae was stopped right after the day 11 sampling but larvae were maintained in their respective cylinder. At day 13 post-fertilization, larvae were collected and added to a small dish with 60.000 cells.ml-1 *Isochrysis galbana* for 1 hour. A group of unfed control larvae was used to verify that digestive gland was empty with no fluorescence of algae (stage III) before the onset of the assay. Ingestion (stage I) was defined as the stage when whole microalgal cells were well defined in the digestive gland of the larvae i.e. observation of individual red fluorescence. Digestion (stage II) was defined as the stage when individual microalgal cells were not recognized but fluorescence was observed as a spot of pink to orange colours inside the digestive gland. After one hour, the larvae were filtered to remove the microalgae in seawater, fifty to hundred larvae were observed under the microscope and three replicates were used for each group. Thereafter, the larvae were left with no additional supply of algae for 24 hours and scored for ingestion/digestion once again.

Treatments / Exposure concentrations

The fertilized eggs were divided in five treatment groups: control, dissolved metal exposure (10.2 $\mu g.l^{-1}$ Cu, 34.8 $\mu g.l^{-1}$ Zn, 1.4 $\mu g.l^{-1}$ Cd and 3.2 $\mu g.l^{-1}$ Pb), barite particles (nominal concentrations: 180 $mg.l^{-1}$ in 2004 and 16 $mg.l^{-1}$ in 2005), two concentrations of used water-based mud (WBM) with barite as the weighting element (nominal concentrations: 9 $mg.l^{-1}$ and $0.9mg.l^{-1}$). The measured concentrations in the cylinders were lower than nominal both for WBM and barite particles. We measured respectively ≈$4mg.l^{-1}$ in the highest WBM (WBM_4) and ≈$0.6mg.l^{-1}$ in the lowest ($WBM_{0.6}$). For barite particles, we estimated 62 and ≈8 $mg.l^{-1}$ in 2004 and 2005 respectively.

RESULTS

Exposure 2004

In the 2004 collection of samples, we observed a contamination by several planktonic species – mainly copepods –together with the samples containing mussel larvae. Possibly, this problem was due to a failure in the filtration units of the inlet seawater. This contamination of species was however present in all exposure tanks.

Day 3 - We observed a strong development inhibition effect of the dissolved metal mixture to the embryos 3-days post-fertilization. None of the embryos in this group had developed to the D-shell stage. Moreover embryos were in an early trochophore stage. Many showed severe deformations and extruding cell material (see illustration figure 4). Only 10% of the larvae were normally developed in WBM4 and 64% were deformed. The larvae hinge and margin showed pronounced irregularities. In the barite group and the $WBM_{0.6}$ group, the percentage of normal D-shell was not different from that of the control (95%)(see figures 5 to 7).

Day 11 – The analyses of the larvae sampled 11-days post-fertilization revealed that veligers in WBM_4 were growing significantly less than in all the other groups. Due to the high number of particles in the barite exposure, barite particles had a tendency to accumulate on the surface of the plankton mesh siliconed at the bottom of the cylinders. This obstructed the flow and resulted in difficulty to assess properly the size of a large number of veliger with the image analyser. Based on few measurements, it appears that the size of the larvae was larger but this difference was not significant (see table 1).

Exposure 2005

In 2005, there was no planktonic species contamination of the exposure tanks as in 2004.

Day 3 – In the metal mixture group, none of the embryos developed to the D-shell and the same observations as in 2004 were made. Contrary to the 2004 experiment, there was no difference in the percentage of normal D-shell in WBM_4 or any of the other treatment groups compared to the control.

Day 11 – The size of larvae in WBM_4 was significantly reduced compared to the control. Larvae growth in $WBM_{0.6}$ was not different to that of the control. We found a significant increase in the growth of veligers exposed to barite.

Ingestion and digestion of the larvae – In the non-fed larvae, 13% of the observed larvae were at stage I and 42% to 45% were at stage II and III, respectively. Larvae fed had all ingested algae but with a different rate. Only 41% of the mussels larvae exposed to WBM_4 were in stage I (ingestion) while more than 93% of the control larvae had ingested algae. This difference was significant. In barite and in $WBM_{0.6}$, respectively 72% and 75% of the larvae were at stage I one hour after feeding. However, there was not significant difference to the control. Twenty four hours after feeding, 90% of the control larvae were in a stage II phase and only 3% in phase I. This shows that the control larvae digested efficiently the algae. In all the other treatments, the percentage of larvae remaining in stage I was higher, indicating a slower digestion rate. In $WBM_{0.6}$, there were still 25% of the larvae in phase I. However, this difference was not statistically significant (figure 7).

DISCUSSION / CONCLUSIONS

Embryogenesis in the metal mixture was strongly inhibited both in 2004 and 2005. EC_{50} (50% inhibition of normal embryogenesis) values for the metals used in this mixture are reported by several authors (see for example His *et al.*, 2000; Beiras and Albentosa, 2004). The concentration of zinc, cadmium and lead in the seawater of the metal-mix group was several orders of magnitude below the reported EC_{50} (respectively 100 to 300 $\mu g.l^{-1}$, $\approx$2000 $\mu g.l^{-1}$ and 100 to 1000 $\mu g.l^{-1}$) and consequently probably too low to have significant effects to the embryos. Only copper is reported to have an EC_{50} value in the range of concentration used in the seawater exposure of this group ($\approx$ 10 $\mu g.l^{-1}$). Hence, it is likely that copper was the major element arising for the toxicity in embryogenesis in this group. In our experiment, none of the embryo developed to the D-shell. This strong response may be attributed to the continuous flow of metals in the system while most EC_{50} measurements are obtained from static mode experiments.

The development to the D-shell larvae in the WBM_4 showed a significant number of abnormalities in 2004 but not in 2005. The same stock of WBM was used in both years and therefore we can explain this difference by a different exposure composition. However, the exposure conditions were not as good in 2004 as it was in 2005 because we observed a contamination of planktonic species inside the exposure cylinders. So it is rather possible that the results observed in WBM_4 in 2004 were in part biased by that contamination. However, a contamination of species was also observed in the other exposure tanks. It might be that the mussel stock in 2004 was more sensitive than the one used in 2005.

In both years, reduced growth of veliger larvae was observed in WBM_4. Hence, 4 $mg.l^{-1}$ water-based drilling mud with barite as the weighting element causes a reduction of the fitness in mussel larvae. The consequence of reduced growth is an extension of the period of larval development. This can be accompanied by a higher mortality and a reduction in the chance of survival to the settlement stage and beyond (Widdows, 1991). Hence, the effect of 4 $mg.l^{-1}$ of WBM can have important ecological consequences. Mussel larvae in the barite group appear to have a better growth than the control. Interestingly, a similar observation was made in a similar study with cod larvae exposed to 5$mg.l^{-1}$ barite (see PAPER VI). However in their study, Bechmann *et al.* showed that WBM containing 10 $mg.l^{-1}$ barite enhanced growth compared to the control group. Our results tend to demonstrate that particles are not impairing the development of the larvae (even though the increase might be considered a disturbance causing an actual enhancement of the development) i.e. the growth reduction is not related to a physical effect of the particles. Spangenberg and Cherr (1996) showed an EC_{50} of 189 $mg.l^{-1}$ in a study with *Mytilus californianus* exposed to barium as barium acetate in the water. They found that the embryonic stage between 16 and 32h post-fertilization was most sensitive to barium. Based on barium concentration in barite particles, we can estimate that the concentration of barium in the 2004 experiment was 360 $\mu g.l^{-1}$ and in the 2005 experiment it was only 48 $\mu g.l^{-1}$. No significant difference in the percentage of normal development to the D-shell was observed in the barite exposure in 2004 and in 2005. Barium in our exposure was most likely not bioavailable in the water and only bound to barite particles, hence reducing the risk for effect documented by Spangenberg and Cherr.

The composition of metals and the size distribution of particles differed between barite and WBM exposure groups in the stock solutions (see report by Bechmann *et al.*, PAPER I). In the

exposure tanks, there was a large proportion (>90%) of small particles with a size < 5µm both in the barite and the WBM exposures. However, the volume of fine particles was higher in WBM than in barite. For example, ≈30% of the particles was below 3 µm in WBM while ≈10% was present in barite. Early veliger larvae are able to size select particles ingested. They show a maximum ingestion rate for particles in the range 2-6 µm (Widdows, 1991; Baldwin, 1995). Hence, the size range of particles in barite and WBM exposures was in the range of those normally ingested by mussel larvae. Growth inhibition in WBM_4 is possibly related to the poor ingestion of algae observed in this group but it is not easy to elucidate whether the impairment has a physical (particles) or a toxicological (metals and/or possibly chemicals added to the drilling fluids) origin or a combination of both. A more thorough investigation using for example induction of metallothionein proteins (Geffard *et al.*, 2003; Damiens *et al.*, 2006) and histological observations of both the digestive apparatus and the ciliated filtrating organ (velum) of mussel larvae could have helped to elucidate this question.

In adult sea scallop *Placopecten magellanicus* exposed to drilling wastes (including water-, low toxicity mineral oil-, synthethic-, and ester-based muds) significant impact on growth and reproduction can occur at concentrations from 1 to < 10 $mg.l^{-1}$ (Cranford and Gordon, 1992; Cranford *et al*., 1999; Armsworthy, 2005).In adults of *Mytilus edulis*, Bechmann *et al.* (PAPER III) found reduced growth and clearance rate at ≈20$mg.l^{-1}$ WBM. Based on the present study, it appears that early-life stage of *Mytilus edulis* exposed to diluted WBM have growth impairment at concentration range 5-fold lower than that found for adults.

Typically, early life stage bioassay with *Mytilus edulis* are performed in static mode and uses the percentage of normal D-shell obtained after 48h to assess water quality or biological effects. This study shows that it is also important to consider other end point parameters like growth. Even though it is usually showed that the period between egg fertilization and hatching of larvae is critical for the survival of the next larvae stages, feeding efficiency related to the ingestion and digestion of microalgae is also critical for veliger larvae exposed to drilling fluidsand hence for their survival.

The use of image analysis for growth assessment in larvae has enabled to optimise considerably the performance of the bioassay with early-life stages. Due to the inherent important variations existing in such assay, it is necessary to have a relatively large number of observations to interpret the data properly. The choice of the measurement parameters of area, perimeter and size (both width and height) was made after their discriminatory capability following a study by Johnson *et al.* (2001). The automatic measures of these parameters gave comparable results to those obtained under visual observations. Hence, this type of automation can readily help to improve the cost-effectiveness of the mussel embryo-larval bioassay.

We used also epifluorescence technique to measure the clearance rate of microalgae in veliger larvae. This technique is often used to estimate the suitability of different algae species for the feeding and growth of cultivated species. In this study we performed the test as a mean to assess the feeding efficiency of WBM exposed larvae. Together with automatic measurement of growth parameters, this technique was very useful to give a holistic assessment of the fitness of the larvae exposed to drilling fluids. Hence, we recommend the use of these techniques to address the long term effects of contaminant exposure in the embryo-larval development test with veliger larvae of bivalves or other invertebrates.

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TABLES

Table 1 – Growth parameters measured by image analysis of 11-days old veliger larvae in the 2004 and 2005 experiments. Mean±stdev.

Parameter	2004				2005			
	control	$WBM_{0.5}$	$WBM_{4.0}$	BA_{62}	control	$WBM_{0.7}$	$WBM_{3.5}$	$BA_{7.8}$
Area (μm^2)	8169±794	8052±891	7137±792	8294±804	10160±1009	9967±944	9337±1132	10413±1109
Heigth (μm)	88±6	88±6	82±6	90±4	99±5	98±5	95±7	100±6
Width (μm)	119±5	119±6	114±5	120±6	131±7	130±6	126±7	132±7
Perimeter (μm)	352±17	352±19	335±17	354±16	388±20	385±18	375±21	394±20
n	248	229	95	7	452	470	350	258

FIGURES

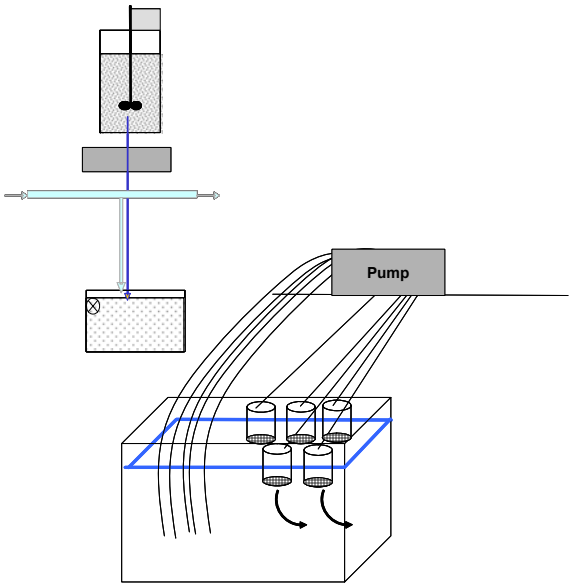

Figure 1 - Exposure system for mussel larvae. See method paper for details on particle size distributions.

Figure 2 - Experimental design and sperm and eggs produced my mussels.

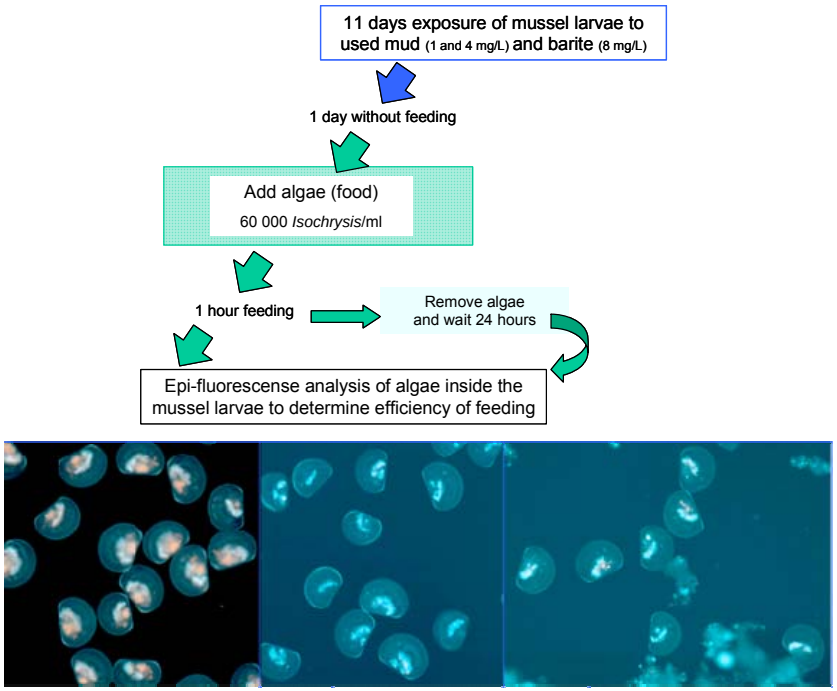

Figure 3 Experimental design for mussel larvae feeding test. Photos: Thierry Baussant. Exp. 2 (2005).

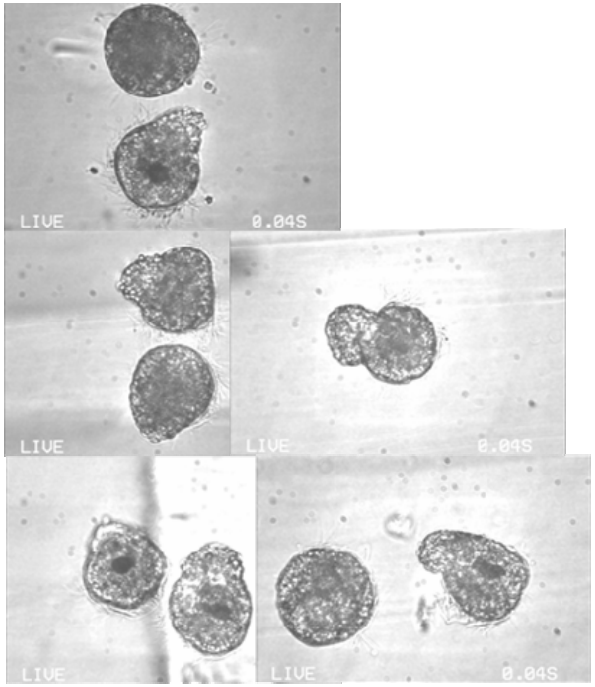

Figure 4 - Abnormal embryonic development in metal mix group after 3 days exposure in 2004. None of the embryos developed into D-shell larvae. Only early stages of trochophore larvae were observed day 3, and high occurrence of abnormal development are detected.

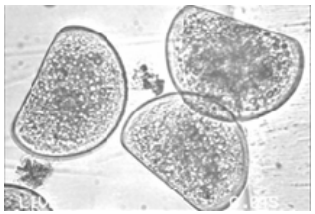

Figure 5- Normal D-shell larvae. 3 days exposure. Results from Experiment 1 (2004)

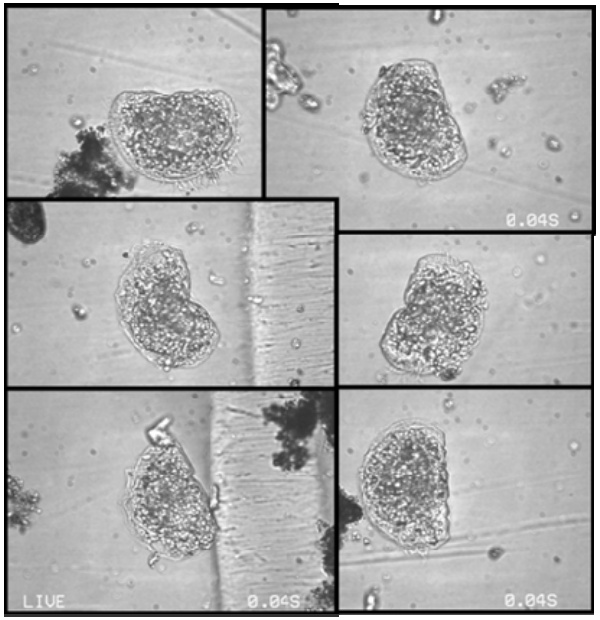

Figure 6 - Abnormal D-shell from 4 mg/L used WBM. 3 days exposure. Results from Experiment 1 (2004)

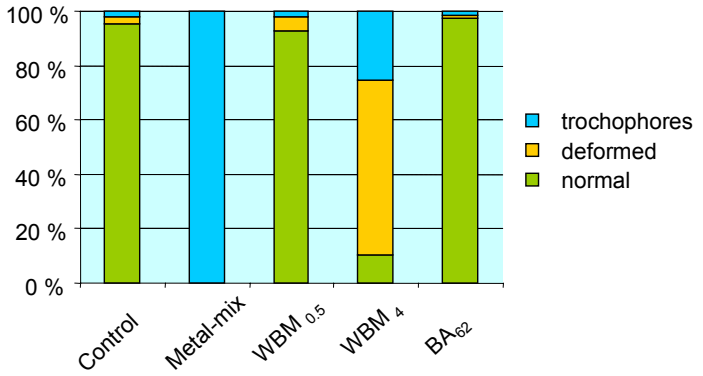

Figure 7 - Proportion of normal, abnormal veliger larvae and remaining trochophores 3 days post-fertilization in 2004. The number above the bars is the number of observations scored in each group. *

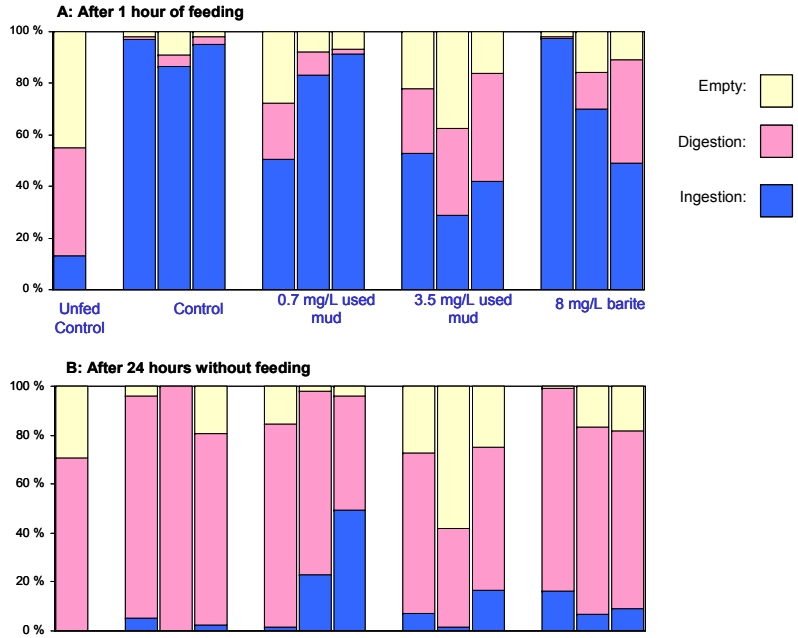

Figure 8 - Results from feeding test. Exp. 2 (2005).

APPENDIX TO PAPER V

Validation of the Analysis of Growth of the Common Mussel (*Mytilus edulis*) larvae Using AxioVision Software and Manual Measurements

Kristian Alfsnes & Thierry Baussant

INTRODUCTION

Growth analysis of the Common Mussel (*Mytilus edulis*) is a time consuming and expensive practise in laboratories, however digital photographing and software has been developed to easier execute these analysis. AxioVision is software designed to differentiate and enhancing objects on a picture and subsequently analysing the dimensions of these objects. Mussel larvae grown for 2-21 days have distinguishable margins and are easily differentiated from other objects commonly mixed with the shells in exposure experiments. Interacting with the AxioVision software, data can be obtained faster and with higher accuracy, and additional parameters hard to measure manually can be obtained.

METHODS

Batches of larvae (both from exposed groups and controls) were analysed both manually and with the aid of the software in question. Slides with shells were first taken picture of in the software supported microscope, and then manually measured using an ocular ruler on a different microscope. Two parameters were compared height and width, measured manually as the widest portion of the shell parallel to the hinge and the widest portion perpendicular to the hinge, and as FeretMin and FeretMax (1) using the software. The data were pooled in a spreadsheet and calculations were done to establish the validity of the software.

Two validation tests were done; before an analysis of approximate 50 batches (with a sample of approximate 50 from each batch) from two separate experiments and one after. A total of 245 samples were analysed for the validation, 138 samples prior to the other analyses and 107 samples after. This was done to ensure that the settings in the software were valid both before and after executing the other analysis.

During the analysis of the experiments thorough validations were executed prior to analysing new batches (e.g. from 11 to 13 days or from 2004 to 2005 batches) and at random during the period of analysis (these tests are not presented in this report).

RESULTS

The preliminary validation tests resulted in the standard settings (2) used in the experiment analyses. These settings were not changed during the analyses, except during reanalysis throughout the duration of the experiment analysis. “Shading correction” filter (3) was deactivated in most samples except those with a clearly defined margin (namely control and certain low exposure groups). Larvae grown for 2 days and high exposure groups were found to lack a defined margin; hence certain filters (such as “Shading correction” and “Sigma filter”) had to be deactivated. Light settings also varied slightly from picture to picture, as did the amount of foreign objects.

The grey values were set manually

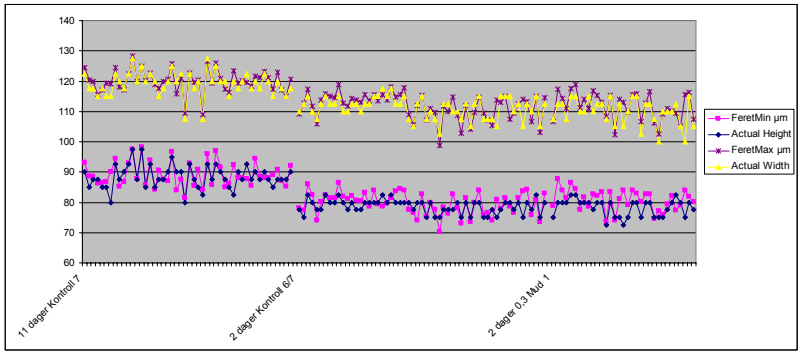

Figure 1 – Correlations between measured height and width and calculated FeretMin and FeretMax from 2004 samples using the AxioVision software (validation done prior to the analysis).

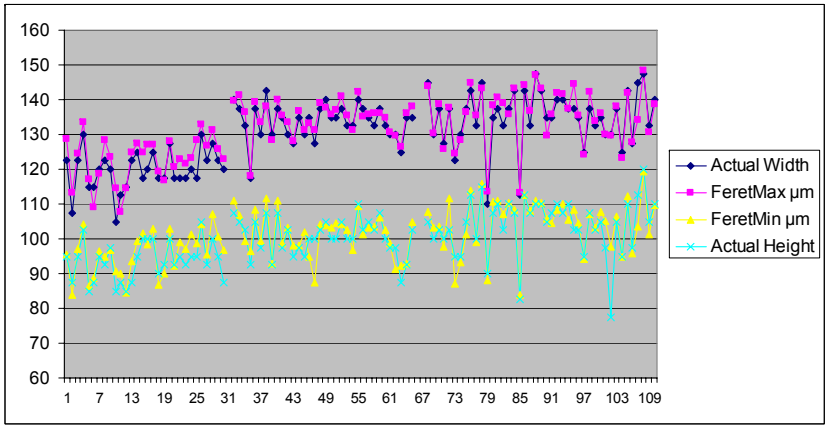

Figure 2 – Correlations between measured height and width and calculated FeretMin and FeretMax from 2005 samples using the Axiovision software (validation done subsequent to the analysis).

DISCUSSION

Certain larvae that lacked a clearly defined margin demanded certain settings to be changed, thought this was found to give a better approximation of the actual values, since the default values made the mask around the objects too small.

References/Descriptions

1. **Feret Minimum** - The minimum feret of a region is determined on the basis of distance measurements. Two straight lines are positioned on opposite sides of

the object, like a sliding caliper, at 32 angle positions. The corresponding distance is measured for each angle position. The minimum value determined is the minimum feret.

Feret Maximum - The maximum feret of a region is determined on the basis of distance measurements. Two straight lines are positioned on opposite sides of the object, like a sliding caliper, at 32 angle positions. The corresponding distance is measured for each angle position. The maximum value determined is the maximum feret.

2. "Standard for using AxioVision Release 4.3 software for analysis of D-shaped larva of the Common Mussel (*Mytilus edulis*)" – Lab manual by Kristian Alfsnes and Thierry Baussant

3. Details on the various filters can be obtained in the help entry in the AxioVision Release 4.3 software

VI.

Effects of suspended particles of drilling mud on survival and growth of cod larvae

Renée K. Bechmann, Ingrid C. Taban, Rolf Sundt, Thierry Baussant,
Erling Otterlei & Sigurd Handeland

ABSTRACT

The effects of exposure to barite particles and two concentrations of used water based drilling mud (WBM) with barite as the weighting material was tested in two experiments with cod larvae. The first experiment was started at hatching (Exp. 1), and the second experiment was started with 7 weeks old larvae from the same batch (Exp. 2). Both experiments were run for two weeks. The main objective was to find out if exposure to suspended particles of drilling mud reduce the growth and survival of cod larvae.

In Exp. 1 survival was twice as high in the two treatments with the highest concentrations of suspended particles (5 mg/L barite, 10 mg/L used WBM) as in the control, the metal mix and the 1.5 mg/L used WBM exposure. The difference was statistically significant. In Exp. 2 survival was also better in the treatments with high particle concentrations than in the control, but the difference between control and exposed was smaller than in Exp. 1. The difference in survival between control and exposed was only statistically significant for the barite treatment.

In Exp. 1 the cod larvae exposed to all particle treatments was larger than the control larvae and the larvae exposed to metals (based on myotom height). The cod larvae exposed to used WBM was longer than larvae from the control, metal mix and the barite treatment. There was less difference in growth between the treatments in Exp. 2 (7 weeks old larvae) than in Exp. 1 (larvae exposed from hatching). There was no significant difference in dry weight of larvae exposed to the different treatments in Exp. 2, but the larvae exposed to the low used WBM treatment were significantly longer.

There was twice as many empty stomachs in the control and the metal treatment as in the 3 particle treatments in Exp. 1. The difference was statistically significant. The feeding test preformed in clean water after 2 weeks exposure of the larvae in Exp. 2, showed that the particle exposed cod larvae were able to eat *Artemia* nauplii significantly faster than the control cod. The feeding test confirm the results from the analysis of stomach content in Exp. 1: The particle exposed larvae appear to eat more efficiently than the control larvae.

The indirect positive effects of suspended particles on survival and growth of cod larvae was more important than any negative effect from the drilling mud particles. The results from histological analysis of the gills of larger cod exposed to barite and used drilling mud show that three weeks exposure to 4 – 39 mg/L used WBM and 62 mg/L barite damaged the gills of cod. We can not exclude the possibility that the gills of the cod larvae also were damaged by the particles, or that longer exposure would have caused histological damage to the gills. Two weeks exposure to 1-10 mg/L drilling mud/barite did not, however, have negative effects on survival and growth of the cod larvae.

INTRODUCTION

The effects of exposure to barite particles and two concentrations of used water based drilling mud with barite as the weighting material was tested in two experiments with cod larvae. The first experiment was started at hatching, and the second experiment was started with 7 weeks old larvae from the same batch. Both experiments were run for two weeks. The main objective was to find out if exposure to suspended particles of drilling mud reduced the growth and survival of cod larvae.

Background on cod larvae. The size of the cod eggs is 1.2 – 1.9 mm. At 8°C Atlantic cod embryos hatched in 11-12 days. The larvae are 4 mm long when they hatch. One week after hatching the yolk has been absorbed (size of larvae: 4.5-5.1 mm), and the larvae need to start feeding. Rotifers *(Brachionus plicatilis)* is used for first-feeding in the lab. Later the cod larvae feed on nauplii of *Artemia salina.* The larvae metamorphose at 12 mm. Nissling *et al.* (1998) reports high egg and larval survival to day 10 post hatch (> 80%), and low variability between batches, but in the abstract of another paper by Vallin and Nissling (1998), they report large variations in hatching success, between batches, both from individual egg incubation and from incubation in batches (viable hatch varied between 14% and 97%).

MATERIALS AND METHODS

Cod larvae. Embryo and larvae (early juveniles) of cod *(Gadus morhua)* were purchased from the Sagafjord fish farm (Stord, Norway). Erling Otterlei and Sigurd Handeland gave valuable advice on how to cultivate the cod larvae. The exposure system is the same as described in PAPER I above, with some modifications (see figure 1 and 2). Preparation of stock solutions of particles for the header tanks. Barite: 900 g in 100 litre. Used water based drilling mud: 1 kg in 100 litre and 100 g in 100 litre. Metal mix: see PAPER II. The test conditions are described in table 1, and general information about the exposure system is given in PAPER I.

Experiment 1: The eggs were transferred to the test chambers 8 March 05, and the exposure was started 9 March and ended 22 March 05.

Experiment 2: 7 weeks old larvae (early juveniles) were exposed for 2 weeks from 27 April 05. Larvae from the same batch as in Exp. 1 hatched and cultivated at Sagafjord (7 weeks old).

Oxygen in the water. There was above 80% saturation of oxygen in all the test chambers (7-8 mg/L oxygen, 10°C).

Light conditions. In Exp. 2 the light conditions in the water in the exposure tanks was measured with a spektroradiometer (sum W/cm^2 400-700 nm) at the level of the bottom of the test chambers, in the middle of each exposure tank. The results from these measurements and measurements of light (lux) in the air above the tanks is presented in table 2. The light conditions (lux) above the water was measured with an Ema 1335 lightmeter. The measurements were done underneath the plastic that covered the tanks in Exp. 1 (see table 2).

Feeding of cod larvae. The cod larvae in Exp. 1 were fed rotifers once each day. Cultivation of rotifers was done in a 200 litre tank in a climate room (23°C) with continuous illumination. The rotifers were fed algae *(Isochrysis*) every day, and the culture was aerated. The culture was cooled to 10°C before they were fed to the cod larvae. Approximately 0.7 L culture containing 25 000 rotifers were added to each test chamber every day. A bottle with a thin tube leading into each exposure chamber was used to feed the larvae (see figure 2). It took ca 40 minutes before the bottle was empty.

The cod larvae in Exp. 2 were fed *Artemia* nauplii. Cultivation of *Artemia* nauplii: 7 L seawater + 3 L fresh water + 6 gram *Artemia* cysts + aeration and continuous illumination in clime room (28°C) for 24 hours. The nauplii were rinsed with sea water (115 µm sieve) before they were fed to the cod larvae (table 1). 5000 nauplii was served 2-3 times every day. We added the dense culture using a syringe. First all the replicates got 4 ml then another 4 ml. This was done 2-3 times every day.

This design (the way the food was spread around) ensured that all the replicates and all the treatments got the same amount of food. The results indicate that the cod larvae should have been feed more frequently, but at least the conditions were the same in all treatments/replicates. In both experiments we observed that the cod larvae were eating. It was easier to observed the feeding in Exp. 2 with the larger larvae. In Exp. 2 we fed 2-3 times each day to improve the feeding conditions to get increased growth and reduced mortality.

The cod larvae had eaten all the *Artemia* after ca 30 minutes; we did not see any *Artemia* in water samples from the exposure chambers taken 30 minutes after adding the nauplii. We observed that the larvae were eating rapidly immediately after *Artemia* were added to the chambers. We did not observe any obvious differences in feeding activity between treatments; they all appeared to be eating rapidly (but see the results from the feeding test).

Sampling and effect parameters

Survival. Dead larvae were counted and removed from each test chamber daily in both experiments.

Feeding. In Exp. 2 a feeding experiment was done at the end of the 2 weeks of mud exposure. The experimental design for the feeding test is presented in figure 8. Cod larvae exposed to suspended particles of WBM and barite for two weeks were transferred to beakers with 2 L clean seawater with 100 *Artemia* nauplii. Six replicate beakers were used for each treatment. The test was run in a climate room with 10˚C. The cod larvae were give 10 minutes to eat the *Artemia*. After 10 minutes the cod larvae were removed and the remaining *Artemia* were counted.

Growth. At the end of Exp. 1 photos were taken of a randomly sampled larvae from each test chamber and treatment (see figure 3). The length and myotom height of these larvae was measured using image analysis software (Leica QWin ver. 2.5). The fullness of the stomach of the same larvae was also characterized as ‘full’, ‘half-filled’ and ‘empty’ by studying the photos.

At the end of Exp. 2 five larvae were randomly sampled from 7 test chambers in each treatment (n = 35 for each treatment). The larvae were frozen individually in aluminium foil. Later the larvae were dried and weighed. One frozen larvae was placed in each

numbered and pre-weighed aluminium cup, and dried for 24 h at 80˚C. A test was first done to see if the larvae were completely dry after 24 hours. The dry weight of the larvae was the same after 24 and 48 hours drying, hence 24 hours was used for the rest of the larvae.

RESULTS AND DISCUSSION

Description of exposures

Exposure concentrations. Dry weight of particles per litre in each treatment is presented in table 3. In addition the number and volume of particles with diameter 1.6-50 µm was measured with Coulter counter.

Number of particles. The number of particles pr ml may be more important for the effect of particle exposure than the volume/weight of the particles. Many small particles may cause more damage to e.g. gills than a few large particles, and it is the small particles that will be transported away from the drill site. The mean number of particles with diameter in the range 1.6 – 50 µm in our seawater was 2700 particles per ml (n = 16) (mean for the controls in the cod larvae part: 2459, n = 3, st.dev 365). The number of particles per ml in the 0.6 mg/L and the 6 mg/L used WBM exposures were approximately 10 and 80 times higher, respectively, than in the seawater (n = 7, test chamber). The number of particles in the barite exposures was 19 times higher than in seawater (n = 6, test chamber).

The concentration of particles in the low used WBM exposure in Exp. 1 was estimated from volume of particles in water samples from the 1.8 and 18 mg/L nominal exposures measured by Coulter Counter (particle size range: 1.5 - 50 µm). In Exp. 1 the volume of particles in the low exposure was 16 % of the volume in the high exposure. In the second experiment with cod larvae the volume of particles in the low exposure was 13 % of the volume in the high exposure.

Dry weight of particles. The mean measured concentrations (dry weight) of particles in each treatment is presented in table 3. We have compared the volume of particles within the test chambers to the volume of particles in the water outside the chambers (Table 4). This was done to find out how well the exposure system worked. It is important to know how high the particle load was in the test chambers. The results from the filtered water samples from Exp. 1 showed that it was 35% less particles (based on dry weight) inside the barite test chamber than in the exposure tank (see figure 1 and 2 for drawing/photo of set up). It was 25 % less particles (based on dry weight) inside the high used WBM test chamber than in the exposure tank. The Coulter Counter measurements confirmed that the difference between tank and chamber was larger for barite than used WBM. The sinking test done in connection to the filter feeding experiment also showed that the barite fell more quickly than particles from the used WBM with barite.

The measured concentration of barite particles was lower in Exp. 1 than Exp. 2, although the nominal concentration was the same. The Coulter Counter measurements from Exp. 1 confirmed that it was a higher volume of particles in the high used WBM exposure than in the barite exposure in this experiment.

The flow into the exposure tank was 2 litre per minute and a circulation pump helped to keep the particles in suspension in the tank. The test solution was pumped from the

exposure tank and into each of the 8 test chambers. The flow into each chamber was 20-30 ml/minute. The flow was a compromise between not stressing the cod larvae with too high flow and maintaining a constant concentration of particles inside the test chamber. The lower volume/concentration inside the barite test chambers show that the flow was not high enough to maintain as high concentration inside as outside. It may be most correct to used the measured or estimated particle concentrations inside the test chambers. The particle concentrations tested were in the range 1 – 10 mg/L.

- The volume (concentration) and number of particles in the low used WBM concentration was very similar in the exposure tank and inside the test chamber.
- The number of particles in the high used WBM concentration was very similar in the exposure tank and inside the test chamber, but the concentration/volume of particles was 20-30% lower inside then test chamber than in the tank. This indicate that many small particles remained in suspension inside the test chamber, but the larger, heavier particles fell through the 200 µm plankton mesh in the bottom of the test chambers.
- The barite particles fall more quickly than the used mud particles (because of difference in size and content of other types of particles and compounds in the used mud – see PAPER III). This is also evident from the larger difference in volume inside the test chamber and outside.

Survival

Experiment 1. Mortality was low in all treatment the first five days of the experiment, but increased considerably after 12-13 days exposure. This is an indication that the first feeding may not have been successful for all the larvae, although we observed that they were eating rotifers, and we observed rotifers in the stomachs. More frequent feeding may have given higher survival. Survival was twice as high in the two treatments with the highest concentrations of suspended particles (ca 20% mortality) as in the other treatments (control, metal mix, low mud concentration: 40-50% mortality). One possible explanation is that the particles adsorb (harmful) bacteria and hence have an indirect positive effect on survival. It is also possible that the cod larvae thrive better with particles in the water because of different light conditions or maybe they are adapted to feeding better when the particle density is high (like inside a patch of plankton).

The metal mix did not affect survival and growth of cod larvae. The same concentration had a serious effect on mussel larvae development; none of the larvae developed to the D-shell stage, and some of the trochophores were deformed. Chronic exposure to this concentration also affected filtration rate of adult mussels and scallops and caused histological damage to scallop gills, reduced lysosomal membrane stability and increase oxidative stress in the bivalves.

Experiment 2. There was high survival in all treatments during the two weeks exposure of the early juveniles in exp. 2. Survival was higher than in Exp. 1, which is expected for this age group of cod larvae. The survival was, however, even better in the treatments with high particle concentration than in the control (the metal mix was not included in this experiment). This result confirms the result from Exp. 1: suspended barite particles caused reduced mortality of cod larvae. The concentration of barite in the water was higher in Exp. 2 than in Exp. 1. The highest concentration of used WBM

was slightly lower (7 compared to 10 mg/L) in Exp. 2, and the mortality in Exp. 2 was not significantly lower than in the control in this treatment. Hence although the results from Exp. 1 was confirmed, the differences in survival between the control cod and the cod in the high particle treatments was smaller than in Exp. 1. The low concentration of used WBM (ca 1 mg/L) did not have a significant effect on survival in any of the experiments.

To avoid any 'tank-effect' (placement of tank in the room could affect the results due to variation in light and noise and other factors) we switched the placement of the different groups compared to the first experiment. We also fed the cod more frequently; 2-3 times each day compared to once each day in Exp. 1.

Growth

Exp. 1.

- The myotom height (width) of cod larvae exposed to all particle treatments was larger than in the control and the metal mix. The larvae exposed to suspended particles of drilling mud was significantly bigger than the barite exposed larvae (based on myotom height).
- The cod larvae exposed to used WBM were longer than larvae from the control, metal mix and the barite treatment.
- There was approximately twice as many empty stomachs in control and metal exposed larvae than in larvae exposed to the 3 particle treatments. The particle exposed larvae had eaten significantly more (or digested the food significantly slower) than the control and metal exposed larvae. Since growth and survival was higher for the particle exposed fish, it is likely that this is a result of more efficient first feeding in these groups.

Exp. 2.

There was less difference in growth between the treatments in Exp. 2 with the 7 weeks old larvae than in Exp. 1 where larvae were exposed from hatching. There was no significant difference in dry weight of larvae to the different treatments, but the larvae exposed to the low used WBM treatment were significantly longer. The mean length of the cod larvae from the different treatments varied from 18.5 mm to 20.5 mm, and the mean dry weight varied from 4.4 mg to 5.3 mg. The feeding test, preformed in clean water after end of the exposure, showed that the particle exposed cod were able to eat *Artemia* nauplii significantly faster than the control cod (figure 8 and 9). The feeding test confirm the results from the analysis of stomach content in Exp. 1: The particle exposed larvae appear to eat more efficiently than the controls.

The indirect positive effects of suspended particles in the water was more important for the cod larvae than any negative effects from the used drilling mud/barite particles. The results from histological analysis of the gills of larger cod exposed to barite and used drilling mud show that three weeks exposure to 4 – 39 mg/L used WBM and 62 mg/L barite damaged the gills of cod. We can not exclude the possibility that the gills of the cod larvae also were damaged by the particles – or that longer exposure would have caused histological damage to the gills. Two weeks exposure to 1-10 mg/L did not, however, have negative effects on survival and growth of the cod larvae.

Acknowledgement. Thanks to Marita Sanni for technical assistance.

REFERENCES

Nissling A, Larsson R, Vallin L, Frohlund K. Assessment of egg and larval viability in cod, Gadus morhua: methods and results from an experimental study. Fisheries Research 1998;38:169-186.

Vallin L, Nissling A. Cell morphology as an indicator of viability of cod eggs - results from an experimental study. Fisheries Research 1998;38:247-255.

TABLES

Table 1. Test conditions and experimental design for two experiments with cod *(Gadus morhua)* larvae exposed to suspended particles of used water based drilling mud and barite.

	Exp. 1	**Exp. 2**
Life stage Exposure time	Larvae exposed from hatching 14 days exposure	7 weeks old larvae (early juveniles) 14 days exposure
Temperature	7°C	10°C
Treatments	Used water based drilling mud (2 concentrations), barite particles, metal mix, control	Used water based drilling mud (2 concentrations), barite particles, control
Replicates	7 replicate test chambers per treatment.	8 replicate test chambers per treatment.
Number of larvae at start	300 eggs in each test chamber at start	60 larvae in each test chamber at start
Feeding	Rotifers (*Brachionus plicatilis)* 25 000 per test chamber per day	*Artemia salina* nauplii 5000 nauplii was served 2-3 times every day (2x4 ml *Artemia* culture each chamber each time)

Table 2. In Exp. 2 the light conditions in the water in the exposure tanks was measured with a spektroradiometer (sum W/cm^2 400-700 nm)at the level of the bottom of the test chambers, in the middle of each exposure tank. Results from light measurements (lux) in the air above each tank. Light was measured below the plastic covering each tank. *(Comment: The placement of tanks were not the same in Exp. 1 and 2 to avoid possible "tank-effects". The effects on survival and growth were, however, similar) .*

Treatment	Exp. 2 Sum 400-700 nm	Lux	
		Exp. 1	Exp. 2
Control	1.6 x 10^{-5} W/cm^2	4.5 lux (without the plastic cover: 480 lux)	78-90 lux
0.6 mg/L	4.3 x 10^{-5} W/cm^2	21 lux	345-390 lux
6 mg/L	3.2 x 10^{-5} W/cm^2	1 lux	231 -249 lux
6 mg/L (no lights on, but sun outside)	62.0 x 10^{-5} W/cm^2	-	-
Barite	3.0 x 10^{-5} W/cm^2	5 lux	243-249 lux

Table 3. Dry weight of particles pr litre in 2 experiments with cod larvae. Nominal concentrations are calculated based on dry weight of particles added to the headertanks. Measured concentrations are based on filtering of water samples from the exposure tanks (GF/F; 0.7 µm). Mean ± st. dev (number of samples). WBM: water based mud with barite as the weighting material. (one control: 0.8 mg/L)

Nominal concentration (mg dw/L)		Measured concentration (mg dw/L)	
		Cod larvae - Exp. 1	Cod larvae - Exp. 2
18 mg/L used WBM	Exposure tank	9.5 ± 2.3 (5)	6.8 ± 0.8 (9)
	Test chamber	7.1 ± 1.2 (3)	-
1.8 mg/L used WBM	Exposure tank	1.5[a)]	0.9[a)]
	Test chamber	1.1[a)]	-
16 mg/L Barite	Exposure tank	4.6 ± 1.4 (5)	8.8 ± 1.7 (9)
	Test chamber	3.0 ± 0.7 (3)	-

[a)] Estimated from volume of particles in water samples from the 1.8 and 18 mg/L nominal exposures measured by Coulter Counter (particle size range: 1.5 - 50 µm)

Table 4. Percentage of particles (mg or volume or number) in the test chambers *vs* the exposure tanks in the cod larvae experiments.

	'Low' conc. used WBM	'High' conc. used WBM	Barite
	% particles in test chamber *vs*. exposure tank		
Cod larvae Exp. 1			
Particle concentration based on filtered water samples	-	75	65
Volume of particles based on Coulter counter measurements	108	67	39
Number of particles based on Coulter counter measurements	88	99	66
Cod larvae Exp. 2			
Volume of particles based on Coulter counter measurements	95	83	47
Number of particles based on Coulter counter measurements	99	94	66

Table 5. Accumulated mortality. Mean percent accumulated mortality (including eggs that did not hatch) in each test chamber. In Exp. 1 there was 300 eggs in each test chamber at start, and in Exp. 2 there was 60 larvae in each test chamber.

Exposure time (days)	Control	Metal mix	1.5 mg/L used WBM	9.5 mg/L used WBM	4.6 mg/L barite
Exp. 1: Mean percent accumulated mortality (including eggs that did not hatch)					
0	0	0	0	0	0
5	2	1	2	3	6
8	9	6	19	13	9
12	26	25	38	16	15
13	43	43	53	21	26
Exp. 2: Mean percent accumulated mortality					
	Control		1 mg/L used WBM	7 mg/L used WBM	9 mg/L barite
0	0	-	0	0	0
2	2	-	2	3	1
5	5	-	7	6	3
7	7	-	9	7	4
9	9	-	11	8	5
12	11	-	13	9	7
14	17	-	18	12	9

FIGURES

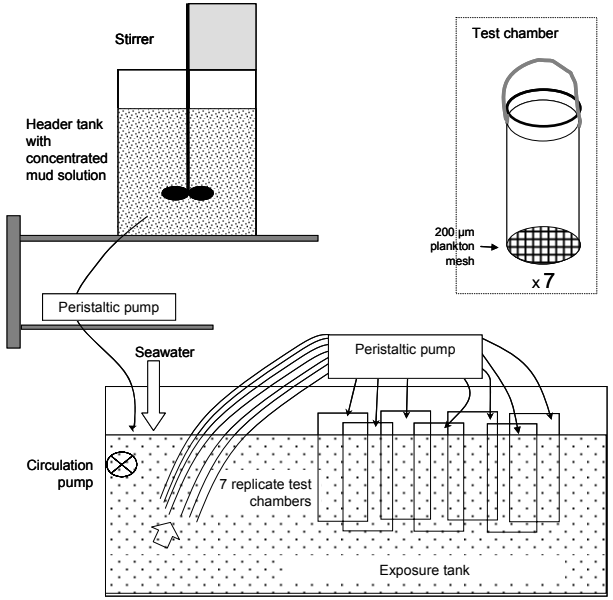

Figure 1. Experimental design in cod exposures. In addition: metal mix in Exp. 1 and controls in both experiments. Mud from the exposure tank was pumped into each test chamber using a multi channel peristaltic pump (MCP, 25 ml/min). The test chambers were plexi glass cylinders with 200 µm plankton mesh at the bottom.

Figure 2. Experimental design/test chambers for cod larvae. Note the bottles used for feeding rotifers to the cod larvae in Exp. 1.

Figure 3. Exp. 1 Cod larvae. Length and myotom height was measured on photos taken after 14 days exposure.

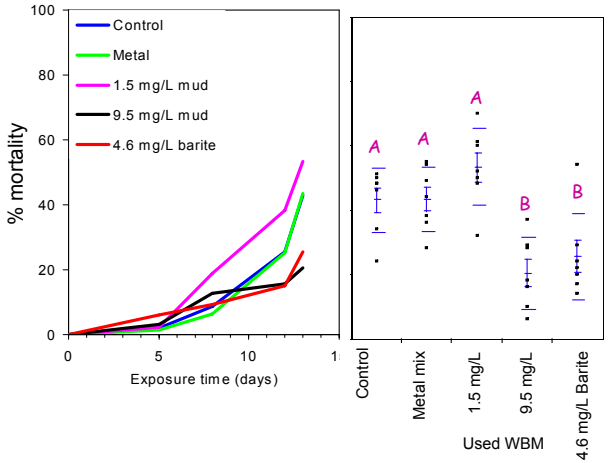

Figure 4. Exp. 1: Mortality of cod *(Gadus morhua)* larvae exposed to suspended particles of used water based drilling mud (WBM), barite and metals for two weeks from hatching. Different letters indicate groups that are significantly different from each other (Each pair Student's t-test, $p<0.05$). The figure to the left show mean percent accumulated mortality with time. The figure to the right show mean percent mortality with standard error and standard deviation lines (7 replicate chambers per treatment).

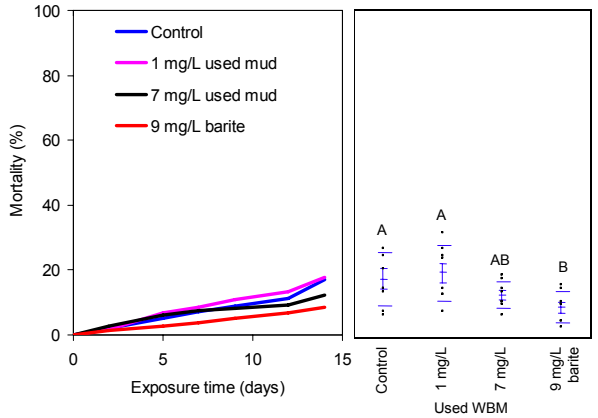

Figure 5. Exp. 2: Mortality of cod *(Gadus morhua)* larvae exposed to suspended particles of used water based drilling mud (WBM) and barite for two weeks. The cod were ca. 7 weeks old at start of the exposure. Different letters indicate groups that are significantly different from each other (Each pair Student's t-test, $p<0.05$). The figure to the left show mean percent accumulated mortality with time. The figure to the right show mean percent mortality with standard error and standard deviation lines (8 replicate chambers per treatment).

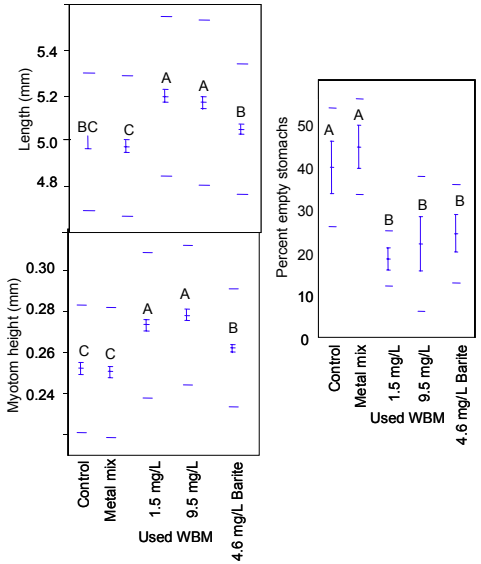

Figure 6. Exp. 1: Growth and feeding of cod *(Gadus morhua)* larvae exposed to suspended particles of used water based drilling mud (WBM), barite and metals for two weeks from hatching. The figures to the left show mean length and mean myotom height of larvae from the different treatments after 2 weeks exposure weeks (7 replicate chambers per treatment). The figure to the right show mean percent empty stomachs for cod larvae exposed to suspended drilling mud particles and metals for 2 weeks (50 larvae from 5-7 replicate test chambers was studied for each treatment). Different letters indicate groups that are significantly different from each other (Each pair Student's t-test, $p<0.05$).

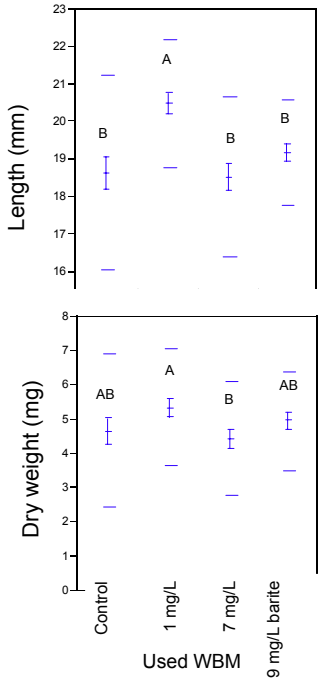

Figure 7. Exp. 2: Length (top) and dry weight (bottom) of cod larvae exposed to suspended particles of used WBM and barite for 2 weeks. The cod were ca. 7 weeks old at start of the exposure. Different letters indicate groups that are significantly different from each other (Each pair Student's t-test, $p<0.05$). Five larvae from 7 test chambers in each treatment were measured, dried and weighed (n = 35 for each treatment).

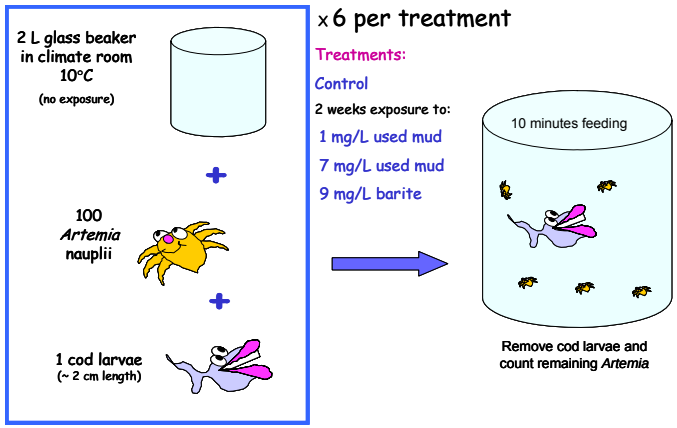

Figure 8. Experimental design for feeding test with cod *(Gadus morhua)* larvae exposed to suspended particles of used water based drilling mud (WBM) and barite for two weeks (Exp. 2). The test was run in clean water after the 2 weeks of exposure.

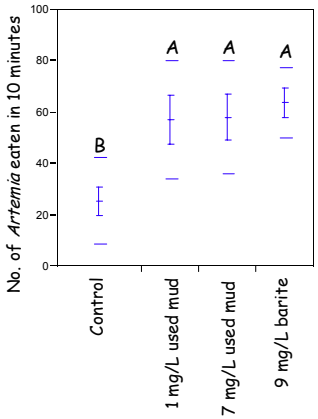

Figure 9. Results from feeding test. Exp. 2. Mean number of *Artemia* eaten in 10 minutes per cod larvae (n = 6 beakers; 100 *Artemia* and 1 cod larvae in each beaker).

VII.

Changes in protein pattern (analysed by SELDI-TOF) in plasma from cod and haemolymph from mussel and scallop exposed to suspended particles of water based drilling mud

Daniela Pampanin & R.K. Bechmann

ABSTRACT

Changes in protein patterns in plasma from cod and haemolymph from mussel and scallops exposed to suspended particles of used WBM (water based drilling mud) particles, to barite particles and to a mixture of metals were analysed by SELDI-TOF (Surface Enhanced Laser Desorption/Ionisation Time of Flight) mass spectrometry. Good separations were obtained for proteins in mussel and scallop haemolymph and cod plasma. Biomarker Wizard analysis identified statistically different proteins in control and treated samples for all three species and all treatments. Reproducibility of protein profile within the same group: the scallop data had the lowest coefficients of variation, showing the lower variability between individuals. The total number of peaks (proteins) detected between 1.5 and 200 kDa is: 345 in cod plasma, 321 in mussel haemolymph and 230 in scallop haemolymph. The resolution between 1.5 and 20 kDa (number of peaks detected in the more powerful area for SELDI-TOF) was: 237 peaks in mussel, 200 peaks in cod and 140 peaks in scallop. The largest difference between control and used WBM was detected for proteins in scallop haemolymph (45 peaks). The difference in the other two species consisted of 17 and 14 peaks for mussel and cod respectively. A dose-dependent response (increased concentration of WBM - increased number of proteins statistically different from the control) was evident in mussels exposed to the three concentrations of used WBM. Scallops showed the highest number of altered proteins in samples treated with metal mix and barite particles; this can reflect a bigger change in the scallop metabolism compared to the other two species. All 3 species showed a similar response to the used WBM, 20-40% of the proteins were significantly different from the control.

INTRODUCTION

During stress situations, such as exposure to toxicants, not only one or a few proteins/genes are up or down-regulated, but more likely hundreds of proteins show altered expression. Consequently, in order to get a more complete picture and to get a better understanding of the underlying mechanisms of toxicity, the study of the whole proteome (proteomics) needs to be emphasised. Furthermore, the analysis of the whole proteome is potentially a very powerful tool in the determination of specific diagnostic toxicological responses to various classes of pollutants (Rogowska et al 2003). The protein chip technology used at IRIS-Biomiljø is based on SELDI-TOF (**S**urface **E**nhanced **L**aser **D**esorption/**I**onisation **T**ime **o**f **F**light) mass spectrometry (Ciphergen; Fremont, USA). This approach has been used successfully and almost exclusively in medical research as a diagnostic tool (e.g. Petrecoin et al 2002). The results obtained at IRIS-Biomiljø show that it has similar potential in ecotoxicology (Knigge et al, 2004; Bjørnstad et al., 2005; Provan et al., 2005). One of the major advantages with this technique is that it is possible to analyse many samples in a relatively fast and easy way.

The objective of the present activity was to evaluate the use of the SELDI-TOF to detect stress specific protein patterns in haemolymph/plasma of marine organisms exposed to used WBM.

If the protein pattern in samples from animals exposed to the weighting material alone is different from the protein pattern in samples from animals exposed to used drilling mud and to metals, it is an indication that adsorbed chemicals on the used drilling mud is a contributing factor to the effects observed.

MATERIALS AND METHODS

SELDI-TOF mass spectrometry.

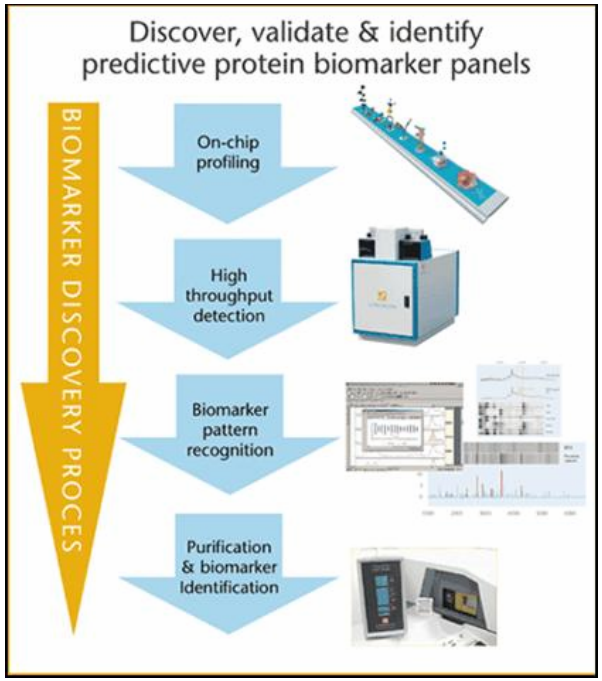

Figure 1. Proteomics approach to evaluate the effect of used water, mix of metals and barite in mussel, scallop and cod (from Ciphergen).

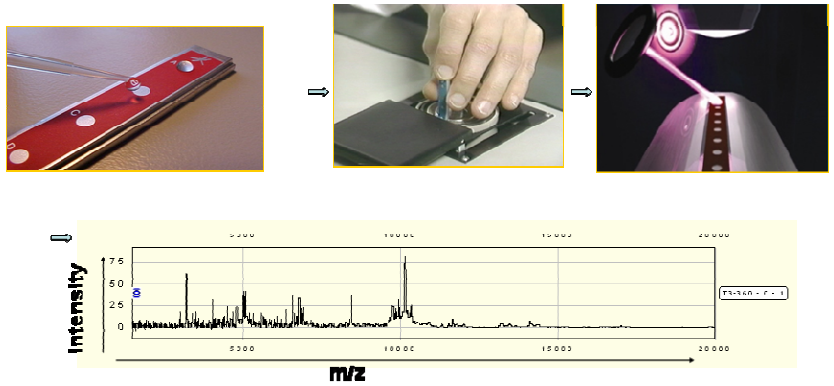

Figure 2. SELDI-TOF (Surface Enhanced Laser Desorption/Ionization – Time Of Flight) mass spectrometry. Some of the analytical procedures are reported in this figure: a) the ProteinChip Array, b) insertion of the ProteinChip Array in the mass spectrometer, c) shooting of the ProteinChip array with the laser, d) chromatogram reporting the protein profile of the samples.

ProteinChip® technology

- Retained proteins are "eluted" from the ProteinChip Array by laser desorption/ionization.
- Ionized proteins are detected and their mass accurately determined by Time-of-Flight mass spectrometry.

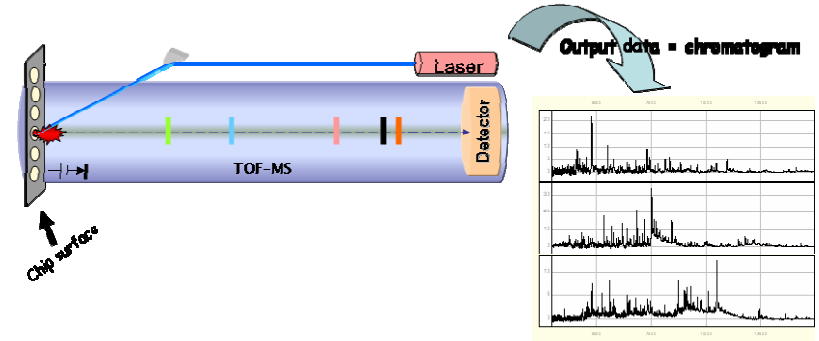

Figure 3. ProteinChip® technology: SELDI TOF mass spectrometry detection.

Various steps were necessary to develop a protocol for mussel and scallop haemolymph and cod plasma:

- ✓ Protein Chip Array selection
- ✓ Binding buffer selection
- ✓ Evaluation of dilution factor
- ✓ Protein profiling of haemolymph/plasma
- ✓ Generation of quality data from SELDI
- ✓ Analysis of data: Biomarker Wizard

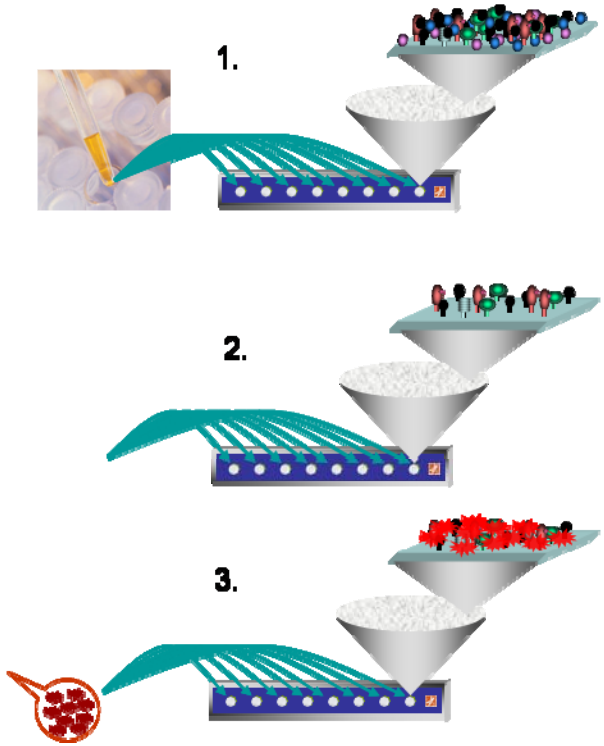

Figure 4. ProteinChip Array preparation: 1) Apply Crude Sample. Proteins within the sample bind to chemical or biological "docking sites" on the ProteinChip surface through an affinity interaction. 2) Wash ProteinChip Array. Proteins that bind non-specifically or buffer contaminants are washed away, eliminating sample "noise". 3) Add Energy Absorbing Molecules. After sample processing the chip is dried and EAM is applied to each spot to facilitate desorption and ionization in the TOF-MS (from Ciphergen).

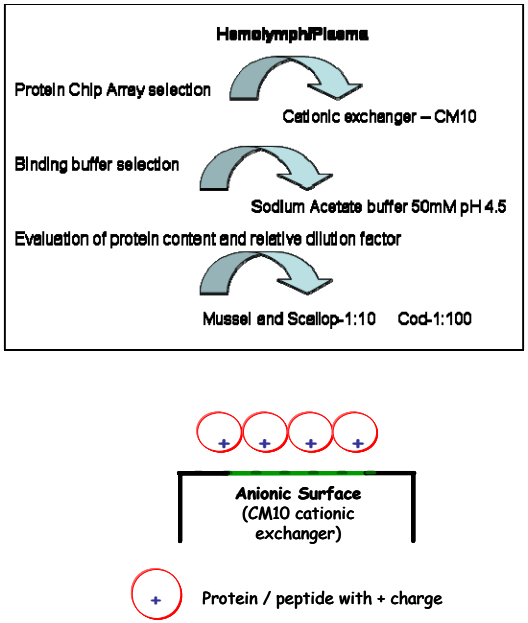

Figure 5. Selection of the Chip type is a very important step. CM10 was the selected Chip array in the developed method, proteins with a pI > pH of the binding buffer (pH 4.5) bind on the ProteinChip surface and are detected by SELDI-TOF analysis.

Protein profiling and generation of quality data:

Low mass spectrum:

- laser intensity to 170.
- sensitivity to 8.
- acquisition parameters 20. delta to 5. transients per to 5 ending position to 80.
- warming positions with 2 shots at intensity 190
- Noise setting from 1,5 kDa to 20 kDa of spectrum
- Normalization: total ion current
- No outliers were detected

High mass spectrum:

- laser intensity to 190.
- sensitivity to 8.
- acquisition parameters 21. delta to 5. transients per to 5 ending position to 81.
- warming positions with 2 shots at intensity 210
- Noise setting from 20 kDa to 200 kDa of spectrum

- Normalization: total ion current
- No outliers were detected

RESULTS

Reproducibility within a group

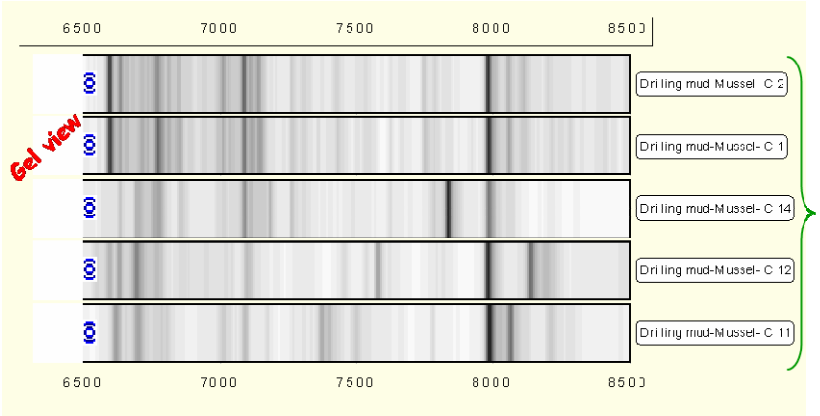

Figure 6. Reproducibility within a group in mussel samples.

Table 1. Coefficient of variance (CV= SD/mean) for mussel.

	Control	Metal Mix	Barite	Used water a	Used water b	Used water c
C.V.	0,762	0,861	0,780	0,745	0,865	0,800

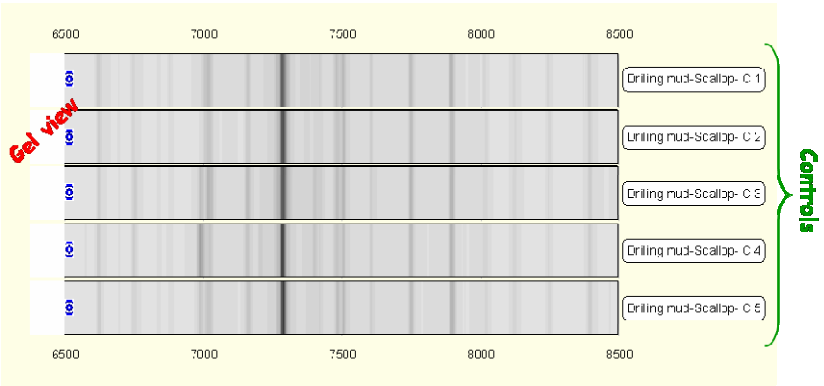

Figure 7. Reproducibility within a group in scallop samples.

Table 2. Coefficient of variance (CV= SD/mean) for scallop.

	Control	Metal Mix	Barite	Used water a	Used water b	Used water c
C.V.	0,411	0,543	0,576	0,459	0,427	0,450

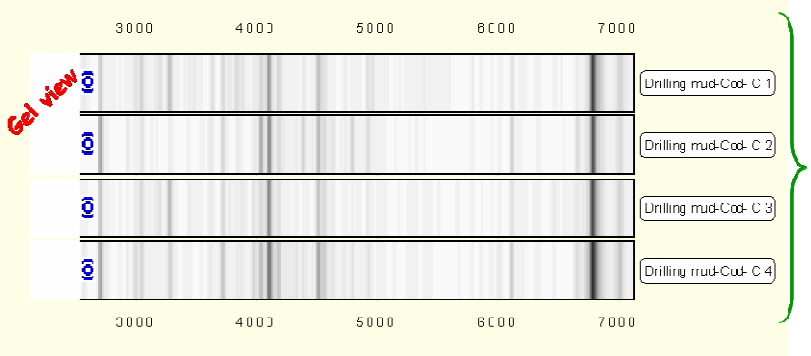

Figure 8. Reproducibility within a group in cod samples.

Table 3. Coefficient of variance (CV= SD/mean) for cod.

	Control	Metal Mix	Barite	Ilmenite	Used water a	Used water b	Used water c
C.V.	0,652	0,657	0,617	0,636	0,608	0,726	0,727

Summary of results

Table 4. Summary of results from mussel, scallop and cod exposed for 3 weeks.

	Mussels No. Of peaks	Scallops No. Of peaks	Cod No. Of peaks
1.5-20 kDa	237	140	200
20-100 kDa	78	72	113
100-200 kDa	6	18	32
	321	230	345

Table 5. Summary of results from mussel, scallop and cod exposed for 3 weeks.

	Peaks with altered intensity	Up-regulated	Down-regulated	% changed	% up	% down
Scallops *(Pecten maximus)*						
Metal mix	155	70	85	67	45	55
Barite	103	12	91	45	12	88
UM1	94	17	76	41	19	81
UM2	75	24	51	33	32	68
UM3	90	23	67	39	26	74
Mussels *(Mytilus edulis)*						
Metal mix	28	14	14	9	50	50
Barite	25	9	16	8	36	64
UM1	36	15	21	11	42	58
UM2	105	55	50	33	52	48
UM3	107	53	54	33	50	50
Cod *(Gadus morhua)*						
Metal mix	32	15	17	9	47	53
Barite	25	4	21	7	16	84
UM1	84	32	52	24	38	62
UM2	69	34	35	20	49	51
UM3	70	38	32	20	54	66
Ilmenite	37	19	18	11	51	49

Table 6. Summary of results from mussel, scallop and cod exposed for 3 weeks.

	Percent peaks with altered intensity		
	Scallops n = 230 peaks	Mussels n = 321 peaks	Cod n = 345 peaks
Metal mix	67	9	9
Barite	45	8	7
UM1	41	11	24
UM2	33	33	20
UM3	39	33	20
Ilmenite	-	-	11

Table 7. Details. Common peaks with significantly altered intensity for all used WBM (UM 1, 2, 3) concentrations compared to control and number of peaks that only were significantly different from the control in the highest mud exposure (UM3).

	No of peaks common for UM1,2,3	up - down regulated
Mussels	17 (5%)	5-12
Scallops	45 (20%)	8-37
Cod	14 (4%)	15-17
	No of peaks only in UM3	up - down regulated
Mussels	33 (10%)	2-31
Scallops	17 (7%)	8-9
Cod	14 (4%)	15-17

Table 8. Details. Number of peaks with significantly altered intensity in metal mix (ME) compared to control – common peaks with the samples from used WBM (UM 1, 2, 3) exposures.

	No of peaks common for ME and UM1,2,3	up - down regulated
	Mussels	
ME	28 (9%)	14-14
UM1,2,3	4 (1%)	2-2
UM1	11 (3%)	3-8
UM2	13 (4%)	6-7
UM3	9 (3%)	6-3
	Scallops	
ME	155 (67%)	70-85
UM1,2,3	36 (16%)	5-31
UM1	64 (28%)	13-51
UM2	59 (26%)	17-42
UM3	68 (30%)	18-50
	Cod	
ME	32 (9%)	15-17
UM1,2,3	4 (1%)	3-1
UM1	9 (3%)	7-2
UM2	14 (4%)	5-9
UM3	13 (4%)	9-4

Table 9. Details. Number of peaks with significantly altered intensity in barite (BA) compared to control – common peaks with the samples from used WBM (UM 1, 2, 3).

	No of peaks common for BA and UM1,2,3	up - down regulated
	Mussels	
BA	25 (8%)	9-16
UM1,2,3	3 (1%)	1-2
UM1	10 (3%)	3-7
UM2	11 (3%)	4-7
UM3	10 (3%)	4-6
	Scallops	
BA	103 (45%)	12-91
UM1,2,3	40 (17%)	7-33
UM1	72 (31%)	8-64
UM2	53 (23%)	12-41
UM3	64 (28%)	9-55
	Cod	
BA	25 (7%)	4-21
UM1,2,3	4 (1%)	2-2
UM1	12 (3%)	2-10
UM2	9 (3%)	6-3
UM3	7 (2%)	4-3

Conclusions

- Good separations were obtained for proteins in mussel and scallop haemolymph and cod plasma.
- Biomarker Wizard analysis identified statistically different proteins in control and treated samples.
- Reproducibility of protein profile within the same group: the scallop data showed the lowest coefficients of variation.
- Total number of peaks (proteins) detected with SELDI between 1.5 and 200 kDa: 345 in cod plasma, 321 in mussel haemolymph and 230 in scallop haemolymph.
- Resolution between 1.5 and 20 kDa (number of peaks detected in the more powerful area for SELDI-TOF analysis): 237 in mussel haemolymph, 200 in cod plasma and 140 in scallop haemolymph.
- The largest difference between control and used WBM (at all concentrations) was found in scallop haemolymph (45 peaks). The difference in the other two species consisted of 17 and 14 peaks for mussel and cod respectively.
- For mussels the number of proteins that was different in control and WBM exposed samples increased significantly with increasing concentration of WBM.

The proteomics results indicated that scallops were more sensitive to metals than mussels and cod. This was also the conclusion for the histopathological analysis, but the filtration rate of both mussel and scallop exposed to metals was negatively affected. The filtration rate was, however, tested after longer exposure time than proteomics and histology.

Scallops showed a common response for the metal mix and used WBM, and for barite and used WBM (for scallop 20% of the peaks were common for all three concentrations of used WBM, compared to 4-5% for mussel and cod). Approximately 30% of the peaks were common for the metal exposed scallop and the scallop exposed to used WBM, and a similar percentage of peaks were common for the barite exposed scallops and scallops exposed to used WBM. For mussel and cod less than 10% of the peaks were common for barite and the used mud exposed groups and for the metal exposed and the used WBM.

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Mussels

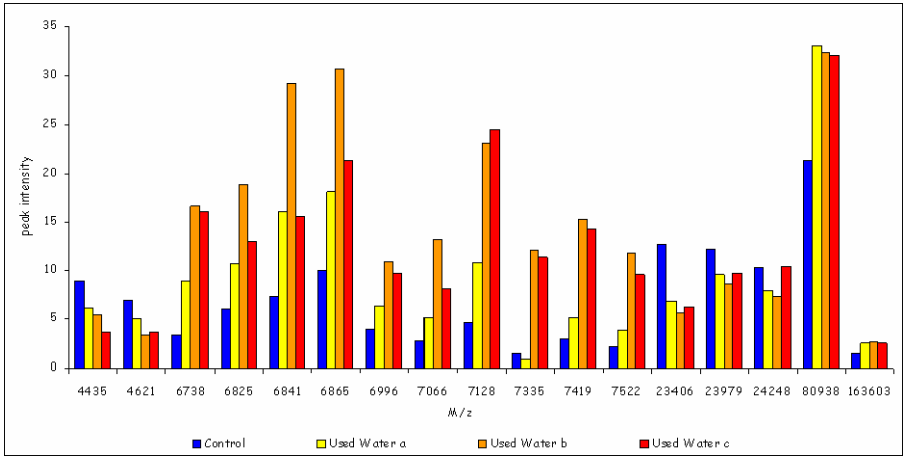

Figure 9. Mussels. Statistically different peaks in Used Water based mud (at all concentrations) compared to control = 17 peaks. Peak intensity on y-axis, and M/z on x-axis. Control values are reported in blue, 0.5 mg/L used WBM in yellow, 2 mg/L in orange, and 20 mg/L in red.

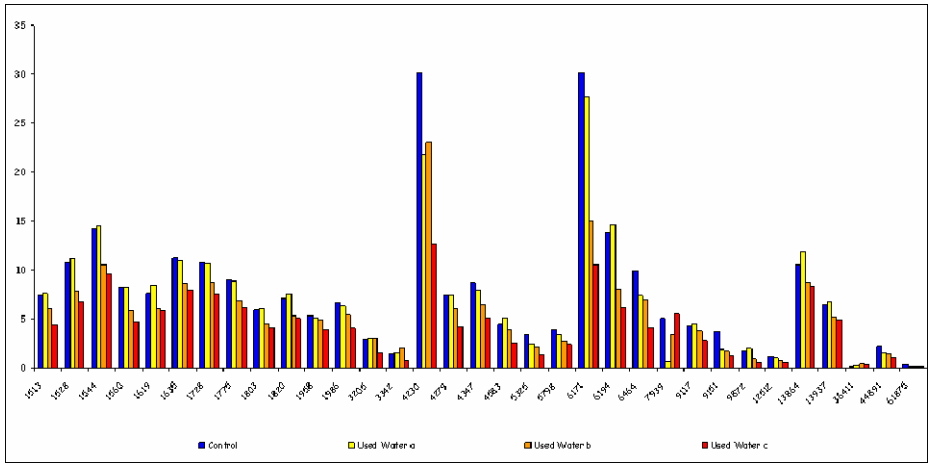

Figure 10. Mussels. Statistically different peaks in used Water Based Mud (WBM) at THE highest concentration compared to control = 33 peaks.

Scallops

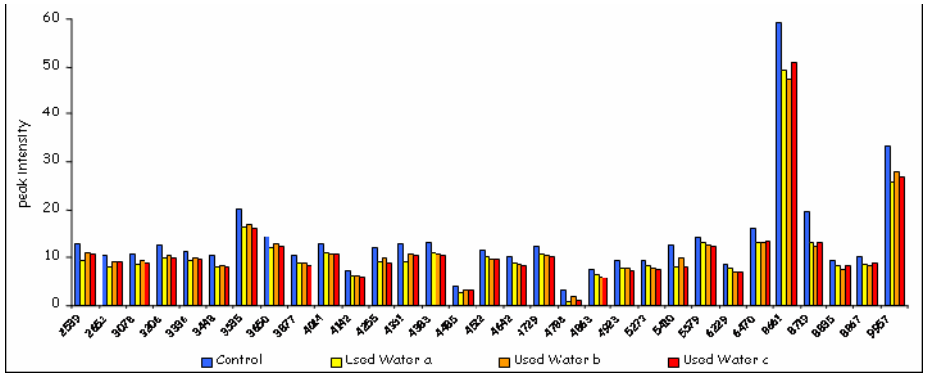

Figure 11. Scallops. Statistically different peaks in used Water Based Mud (WBM) at all concentrations compared to control = 45 peaks. Part 1-

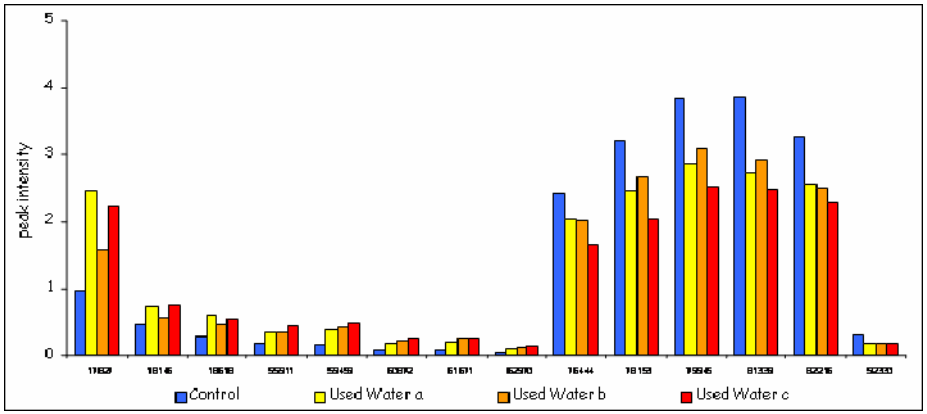

Figure 12. Scallops. Statistically different peaks in used Water Based Mud (WBM) at all concentrations compared to control = 45 peaks. Part 2-

Cod

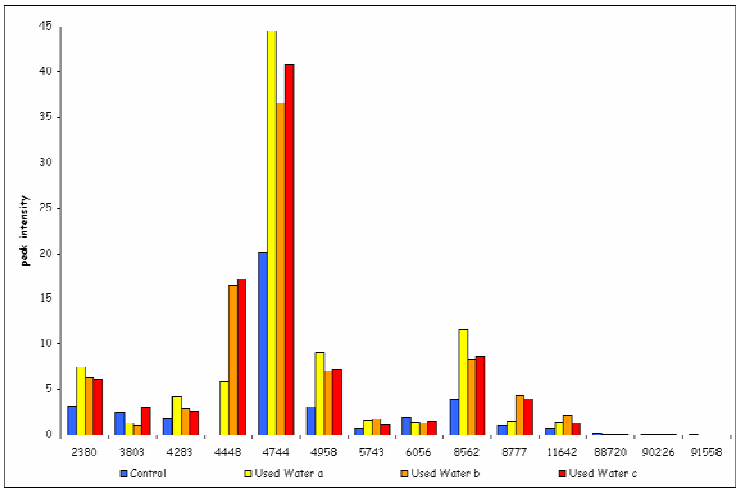

Figure 13. Cod. Statistically different peaks in used Water Based Mud (WBM) at all concentrations compared to control = 14 peaks.

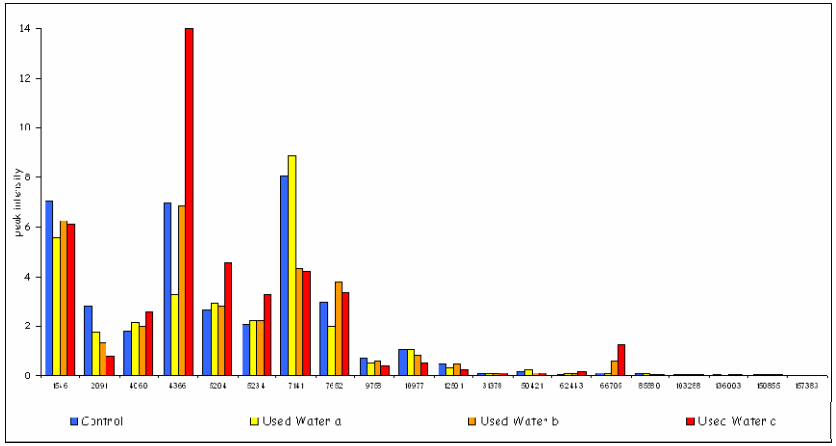

Figure 14. Cod. Statistically different peaks only in the high used Water Based Mud (WBM) compared to control = 20 peaks.

VIII.

Prediction of metal bioaccumulation in organisms exposed to drilling mud

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1. INTRODUCTION

In assessing the environmental risk of metals, including essential ones, care has to be taken on the risk assessment methodology (Karman and Jak, 1998). Both low and high concentrations can cause effects on biota. Normally the free-available fraction is considered to be bio available. This fraction is the part of the metals that is being considered in risk assessment procedures.

Drilling discharges contain both particles and metals. Most of the metals are more or less incorporated in the matrix structure of minerals and are therefore strongly attached to particles. The objective of this study is to check whether normal risk assessment procedures apply for these discharges with high (metal contaminated) particle densities. The focus will be to investigate how biota is exposed to the metals and compare this route to the assumed route in risk assessment. Finally a tool needs to be developed to assess the risk arising from combined metal and particle exposure, as a result of the release of drilling muds. On the basis of this tool risks arising from particles and metals can be predicted and used in environmental decision supporting tools, like the Environmental Impact Factor (EIF; Smit *et al*., *in prep*).

To assess the effects of suspended particles in the drilling mud on the bioavailability of metals, suspended matter can be considered as metal-binding ligands (Karman *et al*., 1999). The strength of the binding capacity, and its implications on uptake and effects in biota, can be predicted by adjusting modelling tools that are developed to predict metal speciation related to water quality parameters (Paquin *et al*., 1999).

In experiments performed in this project animals were exposed to drilling muds with metal contaminated barite as weighting component and barite and ilmenite separately. Chemical and biological responses were analysed. These test results are analysed and integrated on bioaccumulation and effects in order to apply the results in an environmental risk based decision-supporting framework (e.g. EIF). This report summarises the data analyses to describe and predict the bioaccumulation and toxic effects from metals with use of a simple partition-equilibrium method. (Also the use of a more sophisticated model was considered: the Biotic Ligand Model (BLM). However, this model was not flexible enough to be used in this study). Based on these data the validity of the current risk assessment procedures to assess the combined risks of particles and toxicants is discussed. Suggestions for further research will be defined.

The following steps are described in this document:

With use of a simple partitioning-equilibrium model the free metal concentration can be estimated from the (contaminated) barite concentration. The free metal concentration should be related to the amount of metals that can bioaccumulate in biota.

Bioconcentration factors (BCFs) and the partitioning-equilibrium model or BLM model can be applied to assess the uptake of metals by organisms from the free available fraction. The exposure route is described, based on the type of tissue that is contaminated.

Current risk assessment procedures applied to metals present in drilling mud are discussed. Results on the relation between the concentrations of free metal and metal present in mud and the metal uptake by organisms are used to assess the likelihood of occurrence of effects related to the exposure of metals.

Results are discussed in the light of existing PNEC values for barite.

2. EXPOSURE EXPERIMENTS

Cod (*Gadus morhua*), mussels (*Mytilus edulis*) and scallops *(Pecten maximus)* were exposed for 3 weeks to 3 concentrations of used drilling mud with barite as weighting material (UM1-UM3), barite particles (BA) and to a mixture of metals as a positive control (ME) (PAPER I-III). Additional, cod was exposed to ilmenite particles (IL). Concentrations of metals were analysed in gills and digestive glands of mussels and scallops, and in gills, liver and bile of cod (PAPER II). Table 1 presents the metal concentrations in barite, ilmenite and used mud. These concentrations are used together with the concentrations of suspended matter in the experiments to calculate the different exposure concentrations as shown in Table 2.

Table 1 Metal content of barite, ilmenite and used mud (mg/kg)

	Barite (mg/kg)	Ilmenite (mg/kg)	Used mud (mg/kg)
Aluminium	133.4	532.8	1000
Vanadium	1.0	22.6	14.2
Chromium	15.8	18.8	41.0
Manganese	142	4.6	89.8
Iron	6953	10855	14478
Cobalt	0.3	8.3	4.8
Nickel	1.8	34.1	21.4
Copper	38.5	11.3	32.7
Zinc	44.7	1.3	48.5
Arsenic	1.0	1.0	4.3
Molybdenum	2.1	0.3	22.1
Cadmium	0.3	0.0	0.2
Barium	6015	8.2	3496
Lead	12.2	0.0	19.1

Table 2 Total metal concentrations in the different exposures

	Used water based mud			Barite	Ilmenite	Metal mix
code:	UM1	UM2	UM3	BA	IL	ME
Particle concentration (mg/l)						
Nominal conc.	0.9	9	90	180	180	-
Measured conc.	0.5	2	20	23	14	-
Metal concentration (µg/l) based on nominal concentration						
Aluminium	0.90	9.00	90.00	24.01	96	
Vanadium	0.01	0.13	1.28	0.18	4.06	
Chromium	0.04	0.37	3.69	2.85	3.38	
Manganese	0.08	0.81	8.08	25.47	0.82	
Iron	13	130	1303	1252	1954	
Cobalt	0.00	0.04	0.43	0.06	1.49	
Nickel	0.02	0.19	1.93	0.32	6.13	
Copper	0.03	0.29	2.94	6.92	2.03	10.2
Zinc	0.04	0.44	4.36	8.04	0.23	34.8
Arsenic	0.00	0.04	0.39	0.18	0.18	
Molybdenum	0.02	0.20	1.99	0.38	0.05	
Cadmium	0.00	0.00	0.02	0.05	0.00	1.4
Barium	3.15	31	315	1083	1.47	
Lead	0.02	0.17	1.72	2.20	0.00	3.2
Metal concentration (µg/l) based on measured concentration						
Aluminium	0.500	2.00	20.00	3.07	7.46	
Vanadium	0.007	0.03	0.28	0.02	0.32	
Chromium	0.021	0.08	0.82	0.36	0.26	
Manganese	0.045	0.18	1.80	3.25	0.06	
Iron	7.24	29	290	160	152	
Cobalt	0.002	0.01	0.10	0.01	0.12	
Nickel	0.011	0.04	0.43	0.04	0.48	
Copper	0.016	0.07	0.65	0.89	0.16	10.2
Zinc	0.024	0.10	0.97	1.03	0.02	34.8
Arsenic	0.002	0.01	0.09	0.02	0.01	
Molybdenum	0.011	0.04	0.44	0.05	0.00	
Cadmium	0.000	0.00	0.00	0.01	0.00	1.4
Barium	1.748	6.99	70	138	0.11	
Lead	0.010	0.04	0.38	0.28	0.00	3.2

The concentrations in the table above are total concentrations in the different exposures. Partly these concentrations are 'attached' to the particles and partly these concentrations are free available in the water. Normally, in risk assessment, only the free-available fraction is considered. Therefore, in the following part, this part of the concentration will be assessed for the different exposures.

3. CALCULATION OF FREE METAL CONCENTRATIONS

Two different methods were considered to use for predicting the free metal concentration:

A simple partitioning-equilibrium equation;

The Biotic Ligand Model.

3.1 PARTITIONING-EQUILIBRIUM

On the basis of the total metal concentrations in the exposures (Table 2) and available

partitioning coefficients (Table 3) the concentration of free metal is calculated. The following equation is applied for this:

$$Kd = \frac{[metals\ attached\ to\ particles](\mu g / kg)}{[metals\ \ free\ in\ water\ \ phase](\mu g / l)}$$

Table 3 Partitioning coefficients for metals present in barite and mud (taken from Neff, s.a.)

Metal	Kd (SW-polymer mud)	Kd Barite
Arsenic	>106	
Cadmium	52	400 - 6000
Chromium	140	13000 - 22000
Copper	41	20000 - 80000
Lead	100	20000 - 30000

Table 4 presents the results of the calculations. The lowest Kd values have been selected, which will result in the highest concentrations in the water.. For ilmenite the same Kd value as for Barite is used as no Kd values specific for ilmenite were available. The highest free metal concentrations are calculated for copper (15- 70 ng/l).

Table 4 Predicted free metal concentrations in ng/l assuming equilibrium-partitioning

	Used water based mud			Barite	Ilmenite	Metal-mix
	UM1	UM2	UM3	BA	IL	ME
Free metal concentration (ng/l) based on nominal particle concentration						
Chromium	0.26	2.62	26.2	0.22	0.26	-
Arsenic	0.02	0.08	0.80	-	-	-
Copper	0.70	7.01	70.1	0.35	0.10	10200
Cadmium	0.00	0.03	0.34	0.13	0.00	1400
Lead	0.17	1.70	17.0	0.11	0.00	3200
Free metal concentration (ng/l) based on measured particle concentration						
Chromium	0.15	0.58	5.82	0.03	0.02	-
Arsenic	0.04	0.36	3.62	-	-	-
Copper	0.39	1.56	15.6	0.04	0.01	10200
Cadmium	0.00	0.01	0.08	0.02	0.00	1400
Lead	0.09	0.38	3.78	0.01	0.00	3200

PNECs for Cd, Cu and Pb in seawater were calculated by Holthaus *et al.* (2004) using validated chronic toxicity data for marine organisms and the assessment factors defined by the marine EU-TGD. These PNECs were 115, 2 and 100 ng/l respectively. PNECs for As and Cr were

obtained from Crementuijn *et al.* (1997), which calculated "Maximum Permissible Concentrations (MPCs)" as national risk limits for these metals. The procedures for derivation of MPCs and PNECs from validated toxicity data are quite similar, therefore the MPCs of 25000 ng As/l and 8700 ng Cr/l for fresh water can be used as PNECs. The predicted free metal concentrations listed in Table 4 are well below the PNECs, except for copper for which the concentration in water is 7 to 35 times higher than the PNEC.

3.2 BIOTIC LIGAND MODEL

The Biotic Ligand Model (BLM) has proven to be an effective tool to estimate toxicity of dissolved metals (Herbert, 2002). The BLM mathematically integrates the interaction of trace metal with solution phase ligands to predict its speciation and its subsequent interaction with receptor sites on the organism. From a chemical and mathematical perspective, the organism's receptor site is treated as a ligand, the **"**biotic ligand**."** The BLM includes two features—one chemical and one biological—that enable it to predict toxicity of dissolved metals based on the accumulation of the metal on the receptor site. In Figure 3 the different features of the BLM are presented.

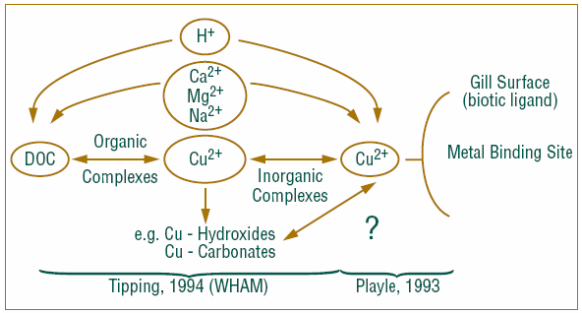

Figure 3 Conceptual diagram of the Aquatic Biotic Ligand Model (Herbert, 2002)

The added barite, ilmenite or used WBM can be regarded similar to suspended matter as both suspensions are complexes which can contain metals. The only input parameters which can be selected by the user of the BLM model are the temperature, pH, Dissolved Organic Carbon (DOC), major cations (Ca, Mg, Na and K), major anions (SO^4_2 and Cl), alkalinity and sulfide. Most of these parameters were measured in the experiments (see previous section) or can be estimated. However, the amount of particulate organic or inorganic matter and their corresponding partition coefficients cannot be selected for input in the BLM model. Therefore, the BLM model could not be used for the determination of the speciation of WBM metals and their binding to receptor sites of aquatic organisms, as the physico-chemical variables used in the model are not comparable to those of the experiments.

4. ASSESSMENT OF EXPOSURE AND UPTAKE

First, the internal concentrations in fish and molluscs exposed to metals in different test media are predicted (calculated concentrations). Then, fish and molluscs are exposed to metals in different test media after which the internal concentrations are determined (measured concentrations). The measured concentrations are then compared to the calculated concentrations in order to determine the relationship between speciation and uptake.

4.1. CALCULATED CONCENTRATIONS

The calculations are based on BCF values from literature and calculated copper concentrations with the partitioning equilibrium method.

The results of the calculations are compared to the observations from the experiments. Table 5 provides an overview of the BCF factors applied in the calculations of the internal concentrations. The predicted concentrations are presented in Table 6.

Table 5. Overview of BCFs for fish and molluscs for different metals. The highest values from a range were selected to represent a worst case scenario (Brix & DeFrost, 2000)

	Chromium	Arsenic	Copper	Cadmium	Lead
BCF-fish	17	20	1933	15471	80
BCF-molluscs	192	350	28624	12561	2568

The internal concentrations are calculated for fish and molluscs and are based on either the total metal concentration (assuming all metal is bioavailable) or on the free metal concentration (assuming only 'free' metal is bioavailable).

The calculations show that the highest concentrations of metals in tissues were calculated for molluscs, except for cadmium. This was expected since for all metals, except cadmium, the BCF values are higher for molluscs than for fish. Furthermore, as expected, the tissue concentrations based on total metal concentration are higher than those based on free metal concentration. When comparing the different types of metal exposure (barite, ilmenite, used mud and metal mix) the results show the highest tissue concentrations in organisms exposed to the metal mix. This was also expected since the exposure concentrations in the metal mix test media were (much) higher than the exposure concentration in the test media with bound metal. When comparing the bound metal exposures, the calculations based on total metal show little variation between barite, ilmenite and used mud. The calculations based on free metal show the highest concentrations for UM3 (used mud). The partitioning coefficients for used mud are lower than barite and ilmenite (see Table 3), so the fraction of free metal is higher resulting in higher tissue concentrations.

Table 6. Predicted concentrations of metals in different organisms, based on nominal exposure concentrations and BCFs

	Used water based mud			Barite	Ilmenite	Metal mix
	UM1	UM2	UM3	BA	IL	ME
Tissue concentration (mg/kg) in fish based on free metal concentration						
Chromium	4,48E-06	4,48E-05	4,48E-04	3,72E-06	4,42E-06	-
Arsenic	7,33E-07	7,33E-06	7,33E-05	n.a.	n.a.	-
Copper	1,39E-03	1,39E-02	1,39E-01	6,69E-04	1,96E-04	19,72
Cadmium	5,50E-05	5,50E-04	5,50E-03	1,88E-03	1,32E-04	21,66
Lead	1,38E-05	1,38E-04	1,38E-03	8,81E-06	-1,70E-08	0,26
Tissue concentration (mg/kg) in fish based on total metal concentration						
Chromium	6,27E-04	6,27E-03	6,27E-02	4,84E-02	5,74E-02	-
Arsenic	7,77E-05	7,77E-04	7,77E-03	3,60E-03	3,60E-03	-
Copper	5,69E-02	5,69E-01	5,69E+00	1,34E+01	3,92E+00	19,72
Cadmium	2,86E-03	2,86E-02	2,86E-01	7,50E-01	5,27E-02	21,66
Lead	1,38E-03	1,38E-02	1,38E-01	1,76E-01	-3,39E-04	0,26
Tissue concentration (mg/kg) in molluscs based on free metal concentration						
Chromium	5,06E-05	5,06E-04	5,06E-03	4,20E-05	4,99E-05	-
Arsenic	1,28E-05	1,28E-04	1,28E-03	n.a.	n.a.	-
Copper	2,05E-02	2,05E-01	2,05E+00	9,91E-03	2,90E-03	2,92E+02
Cadmium	4,46E-05	4,46E-04	4,46E-03	1,52E-03	1,07E-04	1,76E+01
Lead	4,42E-04	4,42E-03	4,42E-02	2,83E-04	-5,44E-07	8,22E+00
Tissue concentration (mg/kg) in molluscs based on total metal concentration						
Chromium	0,01	0,07	0,71	0,55	0,65	-
Arsenic	0,00	0,01	0,14	0,06	0,06	-
Copper	0,84	8,42	84,20	198,16	57,99	291,96
Cadmium	0,00	0,02	0,23	0,61	0,04	17,59
Lead	0,04	0,44	4,42	5,66	-0,01	8,22

4.2. EXPERIMENTAL RESULTS

Bivalves in the barite treatment generally accumulated more metals (Ba, Cu, Zn, Cd and Pb) than in the used mud treatments (UM3). The concentrations of Cu, Pb, Zn and Cd in gill, liver and bile of cod exposed to barite particles did not increase significantly, although the concentration of Pb and Cd increased significantly in gills of cod from the positive control and significant increases of Cd and Zn was detected in liver of cod exposed to used mud

There was an increasing trend of metal accumulation with increasing concentration of used mud. As there is a difference in exposure concentration of the barite and mud exposures the uptake of metals in both exposures cannot be directly related.

Zn and Cd were accumulated in the liver of UM3 exposed cod. Metals may be taken up in the gut and then reach the liver. Metals in particles possibly are more easily stored in liver tissue than dissolved metals while dissolved metals seem to enter cod gills easier than metals attached to particles. However, high variation is observed in the levels of the concentrations of metals in cod liver. Levels were more variable than in the cod gills, and even more than in the bivalve tissues. This makes the interpretation of the analysis results difficult.

Positive control

Mussels and scallops and cod exposed to dissolved metals in the positive control accumulated more Cu, Zn, Cd and Pb than in any other treatment. The highest increase of Cu, Pb and Zn was detected in gills from mussels in the positive control. The concentrations of Cu, Pb and Zn were 46, 8 and 2 times higher than the control respectively. The highest increase of Cd was observed in gills of cod from the positive control, where the Cd concentration was 4 times higher than in the control.

Table 7 Observed metal concentrations in different organisms and tissues in the different exposures

	Used water based mud			Barite	Ilmenite	Metal mix	Control
	UM1	UM2	UM3	BA	IL	ME	
FISH							
Cu in gills mg/kg	n.a.	n.a.	1,2	1,1	1,1	1,1	0,6
Cu in liver mg/kg	n.a.	n.a.	1,3	1,1	0,9	1,3	0,9
Cu in bile mg/L	n.a.	n.a.	1,2	1,1	1,7	1,3	1,2
Pb in gills mg/kg	n.a.	n.a.	1,4	1,1	0,9	6,2	0,015
Pb in liver mg/kg	n.a.	n.a.	4,7	1,6	4,1	3,1	0,005
Pb in bile mg/L	n.a.	n.a.	2,4	1,7	5,7	2,2	0,002
Cd in gills mg/kg	n.a.	n.a.	1,4	0,8	0,7	4,4	0,014
Cd in liver mg/kg	n.a.	n.a.	2,1	1,3	2,3	1,4	0,018
Cd in bile mg/L	n.a.	n.a.	0,9	0,8	0,9	1,7	0,0006
SCALLOPS							
Cu in gills mg/kg	0,95	1,12	1,06	1,61	n.a.	18,29	0,61
Cu in digestive gland mg/kg	1,09	1,24	1,48	3,85	n.a.	4,67	9,79
Pb in gills mg/kg	1,2	1,1	1,8	5,3	n.a.	7,5	0,02
Pb in digestive gland mg/kg	1,2	1,6	1,9	4,1	n.a.	2,5	0,4
Cd in gills mg/kg	0,9	1,1	0,9	1,2	n.a.	0,7	0,5
Cd in digestive gland mg/kg	1,0	1,0	1,0	1,1	n.a.	1,2	42,5
MUSSELS							
Cu in gills mg/kg	1,09	1,06	1,08	1,45	n.a.	45,57	0,77
Cu in digestive gland mg/kg	0,80	0,93	1,46	1,93	n.a.	3,33	4,20
Pb in gills mg/kg	0,8	1,1	1,4	1,7	n.a.	7,5	0,1
Pb in digestive gland mg/kg	0,7	1,1	1,8	2,4	n.a.	5,0	0,5
Cd in gills mg/kg	0,9	1,0	1,0	1,1	n.a.	2,9	0,07
Cd in digestive gland mg/kg	0,9	0,7	0,8	1,0	n.a.	2,9	0,4

Except for the high concentration of Cu in gills of molluscs compared to other tissues, there is little variation between measured concentrations in fish, scallops and mussel. In most cases the concentrations in organisms exposed to barite, ilmenite, used WBM and metal mix are higher than those from fish not exposed to contaminants (control). Cu concentrations in fish exposed to the metal mix were not (or not much) higher than in other fish. This indicates that the metal speciation had no significant effect on bioaccumulation of Cu in fish.

In scallops the exposure to metal mix results in higher concentrations in especially gills. In most cases the concentrations in the digestive gland are higher than in the gills except when exposed to the metal mix. This assumes that only free Cu accumulates in the gills of scallops.

In mussels the concentration of Cu in gills is also much higher when exposed to free Cu. Mussels exposed to barite and WBM showed no elevated concentrations in their tissues compared to the control.

4.3. COMPARISON OF MEASURED AND CALCULATED INTERNAL CONCENTRATIONS

In Table 8 the measured and predicted metal concentrations in organisms are presented to compare these values and therewith determine if calculated values present a realistic prediction of the actual values.

Metal mix

The calculated Cu and Cd concentrations in organisms exposed to metal mix are much higher than the measured concentrations. Therefore, it can be assumed that prediction of internal concentrations by calculation represent an overestimation of the actual concentrations measured.

Particulate matter (barite, ilmenite, mud)

The calculated concentrations based on the fraction of free metal are usually much lower than the measured concentrations, except for molluscs exposed to Cu in UM3. The calculated concentrations based on total metal are also lower than the measured concentrations except for fish and molluscs exposed to Cu and molluscs exposed to Pb. Therefore, it can be assumed that, with exposure to drilling discharges, prediction of internal concentrations usually represent an underestimation of the actual concentrations.

Table 8 . Predicted and measured concentrations in organisms exposed to different metal exposures

	Used water based mud			Barite	Ilmenite	Metal mix
	UM1	UM2	UM3	BA	IL	ME
FISH						
Cu in gills mg/kg	n.a.	n.a.	1,20	1,10	1,10	1,10
Cu in liver mg/kg	n.a.	n.a.	1,30	1,10	0,90	1,30
Cu in bile mg/L	n.a.	n.a.	1,20	1,10	1,70	1,30
calculated Cu (free)	0,00	0,01	0,14	0,00	0,00	19,72
calculated Cu (total)	0,06	0,57	5,69	13,38	3,92	19,72
Pb in gills mg/kg	n.a.	n.a.	1,40	1,10	0,90	6,20
Pb in liver mg/kg	n.a.	n.a.	4,70	1,60	4,10	3,10
Pb in bile mg/L	n.a.	n.a.	2,40	1,70	5,70	2,20
calculated Pb (free)	0,00	0,00	0,00	0,00	0,00	0,26
calculated Pb (total)	0,00	0,01	0,14	0,18	0,00	0,26
Cd in gills mg/kg	n.a.	n.a.	1,40	0,80	0,70	4,40
Cd in liver mg/kg	n.a.	n.a.	2,10	1,30	2,30	1,40
Cd in bile mg/L	n.a.	n.a.	0,90	0,80	0,90	1,70
calculated Cd (free)	0,00	0,00	0,01	0,00	0,00	21,66
calculated Cd (total)	0,00	0,03	0,29	0,75	0,05	21,66
SCALLOPS						

	Used water based mud			Barite	Ilmenite	Metal mix
	UM1	UM2	UM3	BA	IL	ME
Cu in gills mg/kg	0,95	1,12	1,06	1,61	n.a.	18,29
Cu in digestive gland mg/kg	1,09	1,24	1,48	3,85	n.a.	4,67
calculated Cu (free)	0,02	0,21	2,05	0,01	0,00	291,96
calculated Cu (total)	0,84	8,42	84,20	198,16	57,99	291,96
Pb in gills mg/kg	1,20	1,10	1,77	5,34	7,47	7,50
Pb in digestive gland mg/kg	1,20	1,64	1,93	4,13	2,54	2,50
calculated Pb (free)	0,00	0,00	0,04	0,00	0,00	8,22
calculated Pb (total)	0,04	0,44	4,42	5,66	-0,01	8,22
Cd in gills mg/kg	0,90	1,10	0,94	1,18	0,65	0,70
Cd in digestive gland mg/kg	1,00	1,01	0,95	1,14	1,15	1,20
calculated Cd (free)	0,00	0,00	0,00	0,00	0,00	17,59
calculated Cd (total)	0,00	0,02	0,23	0,61	0,04	17,59
MUSSELS						
Cu in gills mg/kg	1,09	1,06	1,08	1,45	n.a.	45,57
Cu in digestive gland mg/kg	0,80	0,93	1,46	1,93	n.a.	3,33
calculated Cu (free)	0,02	0,21	2,05	0,01	0,00	291,96
calculated Cu (total)	0,84	8,42	84,20	198,16	57,99	291,96
Pb in gills mg/kg	0,80	1,09	1,37	1,65	7,52	7,50
Pb in digestive gland mg/kg	0,70	1,07	1,75	2,44	5,02	5,00
calculated Pb (free)	0,00	0,00	0,04	0,00	0,00	8,22
calculated Pb (total)	0,04	0,44	4,42	5,66	-0,01	8,22
Cd in gills mg/kg	0,90	0,95	0,96	1,09	2,87	2,90
Cd in digestive gland mg/kg	0,90	0,74	0,82	0,96	2,94	2,90
calculated Cd (free)	0,00	0,00	0,00	0,00	0,00	17,59
calculated Cd (total)	0,00	0,02	0,23	0,61	0,04	17,59

The predicted concentrations are based on the equilibrium method. This method does not include the aspect of regulatory mechanisms. Bivalves are able to regulate internal metal concentrations by means of storage mechanisms. Fish are able to actively regulate (uptake and excretion of) Cd, Pb and Cu and store Cd and Pb. This could explain the higher measured concentrations compared to the predicted concentrations.

When dividing the measured internal concentrations by the corresponding BCFs, a pseudo-exposure concentration is obtained. In Table 9 the pseudo-exposure levels are presented. Comparing these levels with the PNEC values indicates whether there is a risk of metals in drilling discharges.

Table 9 Pseudo-exposure levels

	Used water based mud			Barite	Ilmenite
	UM1	UM2	UM3	BA	IL
FISH					
pseudo Cu conc	n.a.	n.a.	0,64	0,57	0,64
pseudo Pb conc	n.a.	n.a.	35,42	18,33	44,58
pseudo Cd conc	n.a.	n.a.	0,09	0,06	0,08
SCALLOPS					
pseudo Cu conc	0,04	0,04	0,04	0,10	n.a.
pseudo Pb conc	0,47	0,53	0,72	1,84	1,95
pseudo Cd conc	0,08	0,08	0,08	0,09	0,07
MUSSELS					
pseudo Cu conc	0,000	0,000	0,000	0,000	n.a.
pseudo Pb conc	0,29	0,42	0,61	0,80	2,44
pseudo Cd conc	0,07	0,07	0,07	0,08	0,23

The PNEC values for Cu, Pb and Cd are 0,002; 0,1 and 0,115 µg/l, respectively. When comparing the pseudo-exposure levels to the PNEC values, the following can be concluded:

For fish and scallops, the exposure level of Cu exceeds the PNEC and therewith indicates a risk for these organisms of Cu in drilling discharges.

For fish, scallops and mussels, the exposure level of Pb exceeds the PNEC and therewith indicates a risk for these organisms of Pb in drilling discharges.

For mussels the exposure level of Cd in ilmenite exceeds the PNEC and therewith indicates a risk for these organisms of Cd in ilmenite.

5. COMPARISON TO RISK LEVELS FOR WEIGHING MATERIAL

Within the ERMS project (Environmental Risk Management System) PNEC levels for different weighing agents in drilling muds were determined. These PNECs are based on literature data and constructed using Species Sensitivity Distributions (SSDs). More background information on this work can be obtained from Smit *et al.(in prep*)These SSDs were based on EC_{50} values for barite, bentonite, attapulgite and WBMs. (Figure 4).

The mean (Xm) of the SSD curves represents the position of the distribution on the x-axis and the standard deviation (Sm) determines the slope of the curve. In terms of the sensitivity of species, the Xm gives an indication of the *mean concentration for the physical effects* of suspended mud particles to marine species. The Sm represents the *interspecies variation in sensitivity* of suspended WBM particles for marine species.

Table 10 provides an overview of the data used to construct the SSDs. For attapulgite only fish data was available. Therefore the SSD cannot be considered as representative for general marine biota.

Table 10 Overview of EC_{50} data for attapulgite, barite, bentonite and WBMs to construct the Species Sensitivity Distributions (SSDs). Xm and Sm values for the SSD are presented together with the HC_5 value

Type of weighting material	barite	bentonite	attapulgite	WBMs
Number of EC_{50} values	20	12	8	63
Number of species with 1 or more EC_{50} values	15	12	7	13
Xm	8.01	7.51	9.22	8.81
Sm	3.05	3.25	2.70	1.05
HC_5	20.0	8.8	1800	79.8

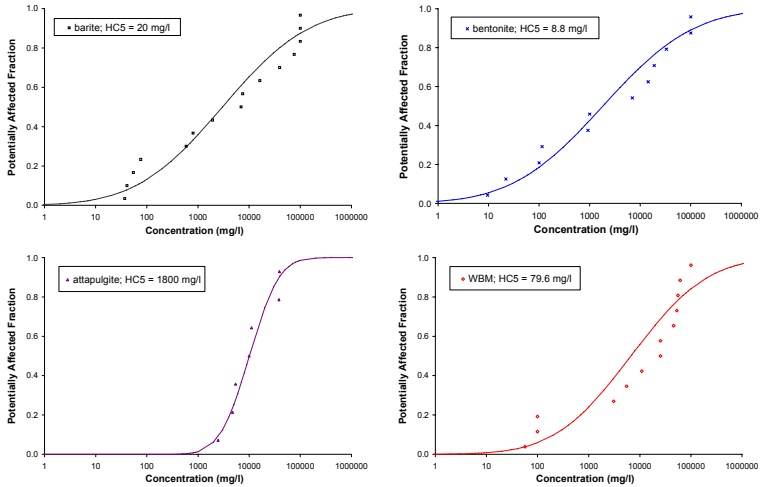

Figure 4 Effects related to risk distributions for barite, bentonite, attapulgite and WBMs at acute exposure (log-transformed).

In order to transform the HC_5 value based on acute effect data to a PNEC, assessment factors need to be applied (Posthuma *et al.*, 2002). A factor of 10 should be applied to account for the translation from EC_{50} level to no-effect level. A second value of 10 should be applied to extrapolate from acute effects to chronic effects. It can be discussed whether this factor is necessary. It is unclear if chronic physical effects of WBM are likely to occur as the increased turbidity and sedimentation of SPM from discharged WBMs might only have a temporary impact on organisms. Another factor of 10 could be applied to translate laboratory effects to field effects. This factor is also under discussion while most of the data results from non standardised test carried out under semi-field conditions.

To the derived HC_5 levels one assessment factor is applied for the translation from EC_{50} to NOEC level. Because the relevance of the acute to chronic translation and the lab to field translation can be questioned for this data, only one assessment factor of 10 is applied for these two translation steps. Because of the lack of data on more taxonomic groups an additional assessment factor of 10 is applied to the HC_5 for attapulgite. This results in PNEC levels for barite, bentonite, atapulgite and WBM of 0.2, 0.09, 1.8 and 0.8 respectively (Table 11).

The proposed PNECs for the weighting agents are all lower than the lowest observed effect levels as presented in table 4. For barite the PNEC is a factor of 2.5 lower than the 0.5 mg/l value determined by Cranford *et al*. (1999). The PNEC values for barite, bentonite and WBM derived from the HC_5 are higher than the levels determined with assessment factors. For attapulgite the values are similar.

Table 11 Overview of assessment factors applied to the HC_5 to derive the PNEC level

Type of weighting material	**barite**	**bentonite**	**attapulgite**	**WBMs**
HC_5 (mg/l)	20.0	8.8	1800	79.6
Proposed assessment factors				
EC_{50} to NOEC level	10	10	10	10
Lab to field & acute to chronic translation	10	10	10	10
Lack of data on different taxa	-	-	10	-
PNEC (mg/l)	***0.20***	***0.088***	***1.8***	***0.8***

The PNEC value for WBM derived from literature is in the range as the lowest concentration tested in experiments in this study (UM1). It is however not recommended to use one PNEC level for the whole mud, as many different compositions of mud exist and added chemicals might contribute to the overall effect of the mud. It is suggested to use the PNEC for barite together with PNEC levels for added chemicals to assess the risk of the total WBM mixture.

The PNEC for barite (0.2 mg/l) is lower than the observed effect levels in the experiments in this study and the effect value reported by Cranford et al. (1999). This level will also be protective for acute metal exposure. Additional studies to uptake and food chain transfer of metals in biota is needed to assess the risk of metals at this level.

6. CONCLUSIONS AND RECOMMENDATIONS

It can be conclude from the experimental results and this analysis that metals attached to barite might be a part of the total risk of drilling discharges. On fore hand the exposure levels of metals in the experiments cannot be regarded as irrelevant. For Cu, Pb and Cd effect levels might be exceeded by the free-available as well as the total and internal concentrations. The observed effects in the experiments can be a result of combined exposure to particles and metals. Further investigation to discriminate between these two stressors seems necessary.

Even in the lowest exposure concentration effects were observed. These effect levels were higher than the suggested PNEC level for barite of 0.2 mg/l. However long term food chain effects from exposure to these metals levels needs further investigation.

Risk assessment based on only the free-available fraction of metals underestimates the potential risk from the metals attached to the particles. Taking into account the total concentration of metals overestimates the risk.

[more recommendations are already taken up in the new project]

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