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T. Baussant*, F. Provan*, M. O. Sydnes*, E. Lyng*, E. Larssen*, C. Bjørkblom*, K. Kaster*, C. Boccadoro*, A. Nævdal* & Anette C. Elde #

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Add-on studies to the current NFR Project "Impact of water- based drilling mud: a study using the epibenthic coral species *Lophelia pertusa*

(Photo Oljedirektoratet/Emile Ashley)

Report IRIS - 2010/034

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Summary

This study was made in the frame of an ongoing NFR project aiming at assessing the impact of drilling mud (DM) discharges from offshore O&G operations on the cold-water coral species *Lophelia pertusa*. Here, we have initiated developments and use of methodologies that could have a potential for application, at a later stage of their optimization, in field monitoring of coral health and hence for documenting the effects of drilling mud discharges.

Three different methodologies were used and applied on mucus samples and coral samples collected during two laboratory experiments in 2009. Concerning the mucus, a first method applied was based on mass spectrometry using both Surface Enhanced Laser Desorption Ionisation Time of Flight Mass Spectrometry (SELDI-TOF MS) to recognize protein expression signatures, and the LTQ ("linear trap quadrupole") orbitrap for protein identification; Another work on freshly collected mucus consisted in examination of microbial community through the extraction of total DNA from the coral mucus, then amplification using PCR and primers specific for the bacterial 16S rRNA gene. Denaturing Gradient Gel Electrophoresis (DGGE) was then used to identify changes in the microbial populations in the samples in relation with exposure to drilling mud. Whole coral samples were also used to extract the total pigment using a relatively simple method based on that implemented at NTNU, then the pigment signature was analyzed by visible (VIS) spectrophotometry; In addition, HPLC-NP (Normal Phase HPLC) and TLC (Thin Layer Chromatography) methods were employed to detail the main pigment observed by the VIS analysis.

Mucus has been demonstrated as a potentially sensitive diagnostic material for coral. This material is also non-destructive, a great value for a protected species. Our preliminary results showed a clear difference in the protein content of white and orange polyps and an indication that DM related effects can be revealed from the protein content in polyps and mucus. Contrary to proteins, little differences were seen in coral-associated microbial communities between orange and white corals, possibly because the actual field communities were modified following the maintenance period in the laboratory before use of the corals. However, DM exposure led to changes in coral-associated microbial communities as indicated by the different banding patterns seen on the DGGE gels between the exposed and non exposed corals. No clear changes in pigment signatures related to drilling mud could be revealed in this study but colour-related differences were observed in whole coral sample extract (shift in wavelength max absorbance). The main VIS absorbance appears to be related to esterified astaxanthin rather than actual free astaxanthin as revealed by HPLC-NP analyses.

This work needs further research to confirm the first observations reported herein. Protein quantification to confirm identification of DM sensitive markers, sequencing and identification of the dominant microorganisms in the different communities from the DGGE bands and a better separation of the different esterified compounds by reverse HPLC, combined with esterase treatment to release astaxanthin, are suggestions for follow up that could strengthen the data of this preliminary study.

1 Background and objectives

IRIS-Biomiljø is currently running a project to study the effects of drilling mud particles (DM) from O&G drilling activities to the cold-water coral *Lophelia pertusa*. One objective of this project is first to develop and implement methods that can enable to assess the health condition of *L. pertusa*. A combination of biotools is used based on behaviour, metabolism, internal damages/changes (histology) and changes in protein pattern in mucus/whole individual. Another type of methods envisioned in the project is based on changes in pigmentation in the deep-water corals. Some of these methodologies are foreseen as promising for the development of cost-effective field monitoring although optimization for potential field application is beyond the scope and the economical frame of the current NFR project.

In the add-on study financed by Total, the main objective is to contribute to the development of monitoring biotools based on mucus samples and pigment profiles obtained from corals under control and exposed conditions. The key challenges are (i) to identify, test and optimize the potential "tools" that they can help us in this inquiry and (ii) evaluate their applicability for cost-effective field screening.

Critical questions raised through the present study were:

- ➔ Can we use coral mucus as a diagnostic tool for assessing coral health by finding specific protein signatures related to DM exposure and use that as a diagnostic tool;
- ➔ Can changes in pigmentation be related to environmental stress following DM particles discharges?
- ➔ What is the role of coral-associated microbes and can changes in the microbial community be used as "early warnings".

2 Scope

In the project, two main sub-activites were conducted:

a) *Add-on study with mucus* – two types of developments were carried out:

1. Characterize specific protein patterns for *Lophelia* and use these to assess if there are significant changes related to the exposure of DM particles. Questions that should be raised are "Can we use changes in this pattern as a diagnostic tool"; "Can we identify more specifically some target proteins"; "Is there a potential to develop that into a quick non-invasive diagnostic tool e.g. biosensor for quick field diagnose"

2. Is the sensitivity of *Lophelia* to DM related to bacterial community in mucus? Can we identify changes in the microbial community based on for

example 16S rRNA gene probing techniques, and use that as "early warnings". If the microbial community in the mucus is responsive enough to the coral's metabolism, we may use that to assess indirectly the health status of the coral.

b) *Add-on study with pigment* – The role of pigmentation in *Lophelia* is not well understood. As this species lives in a deep dark environment, pigments may serve another purpose than that of tropical corals living closer to the surface. Pigments may play an important role as antioxidant, for example strengthening the immune function of polyps. A main carotenoid found in coral is astaxanthin but white corals contain less. We have examined whether pigment profiles can be used to indicate a change in the health of coral following DM exposure. Could changes in pigment by simple spectro-photometric techniques be used as a diagnostic tool reflecting crucial changes in coral physiology and to assess the impact of stressors like DM particles? Characterization by HPLC techniques have also been performed additionally to the spectro-photometric method.

These activities have been carried out using state-of-the-art analytical techniques available at IRIS-biomiljø. The SELDI-TOF instrument was used to provide a protein profile where we can look for biomarkers related to stress and the LC-MS (LTQ-ORBITRAP) instrument to identify more specifically proteins involved in stress responses. For pigment, we have based our data on measurements performed by a Perkin-Elmer UV/VIS Lambda 2S spectrophotometer and pigments were additionally characterized by HPLC-NP method. The bacterial community changes was screened by semi-quantitative screening technique using a denaturation gradient gel electrophoresis (DGGE) analysis.

3 Experimental arrangement & methods

Following collection and transport to the Akvamiljø facility, all corals used in this study were maintained in temperature-regulated running seawater at 7°C prior to their transfer to the experimental setup. They were regularly fed newly hatched *Artemia* cultured the day before feeding.

Setup in Experiment A. Photo: Anette C. Elde

The coral samples used during this study were collected during two different laboratory experiments run within the frame of the NFR project.

Experiment A was conducted in the period August/September 2009 during 7 weeks: 6 coral individuals (3 white and 3 orange) collected in the Trondheimsfjord in February 2009 (Johanna Järnegren, Norwegian Institute for Nature Research-NINA) were used. They were distributed in two different 60 L aquariums (A1&A2) supplied with seawater at a flow rate of 400 ml/min. The two aquariums had an average temperature of respectively

6°C and 11°C. During the first 3 weeks, corals were maintained in clean water only at these temperatures while the last 4 weeks were conducted with DM supplied at an average concentration of 1 to 2 mg/l.

Setup in Experiment B.

Experiment B was conducted in the period December 2009/January 2010: 3 coral individuals (2 white and 1 orange) collected off Finmark in the Lopphavet in April 2009 (r/v SARS, Havforskninginstitutet, "Hermione" cruise) were used. At the onset of the experiment, branches of the 3 corals were split and placed in four cylindrical experimental chambers supplied with seawater (7.5°C) at a flow rate of 100 ml/min. After one week in clean seawater, DM was added in 3 chambers at average concentrations of 4, 14 and 45 mg/l. The exposure lasted ca. 2 weeks (13 days) and was then followed by a clean water period of ca. 5 weeks.

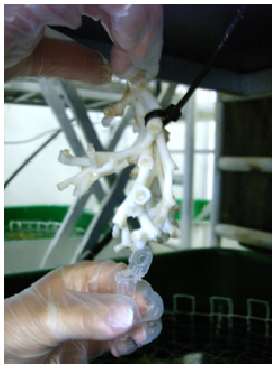
DM particle concentrations were measured with a Hach turbidimeter throughout the experimental work. A standard curve (linear regression) was first prepared to relate the concentrations of DM to the NTU measurements obtained with the turbidimeter. From this curve, the amount of particles measured from seawater samples was calculated.

During each experiment, corals were fed newly hatched *Artemia* nauplii twice a week.

3.1 Add-on study with mucus

3.1.1 Collection of mucus in coral

During the experiment A, *Lophelia pertusa* individuals were collected from the aquarium using tweezers, and held upside down in the air to produce mucus. The first few drops were discarded to prevent the mixture of seawater into the mucus sample. Mucus was collected in small containers and stored in freezer (-80). Individuals were not kept in air for more than 2 minutes to try reducing stress (Pauling 2009). The samples obtained from this experiment were used in **3.1.2**



("air stress" collection of mucus)

In experiment B, we used a new and gentler manner of collecting mucus using sample collection swabs (Catch-All, Epicentre Biotechnologies, USA). Mucus was gently smeared on the swabs underwater, then collected into an Ependorf tube before processing. This way, we avoid the possible bias by coral producing "air stress" mucus not reflecting the actual stress from exposure. The mucus samples from experiment B were used in **3.1.3.**

(mucus collection with Catch-All swab – black arrow, left picture, and transfer of mucus to Ependorf tube)

3.1.2 Characterizing changes in protein pattern

The use of mass spectrometry for proteomics involves the use of different technologies each suited for particular analysis. Complimentary mass spectrometric instruments are in daily use at IRIS Biomiljø to identify and characterise diagnostic markers in fish and other marine organisms, mainly in response to environmental impact.

In an application of mass spectrometry known as Surface Enhanced Laser Desorption Ionisation Time of Flight Mass Spectrometry (SELDI-TOF MS), entire proteomes will be studied. Here Laser Desorption Ionisation –Time of Flight technology is combined with surface enhanced protein chips, with a chromatographic surface. A protein/peptide profile of the biological sample is generated. In SELDI-TOF MS analysis a specific subset of the proteins/peptides present in a sample is specifically immobilised to the protein chip through the chromatographic surface of the chip. Following separation and detection of the ionised peptides/proteins, protein profiles are generated. These profiles can potentially be related to environmental impact/stress with some degree of specificity and are informative anyhow because they illustrate the degree of impact that the treatment has on the expression level of the peptides/proteins (Figure 1).

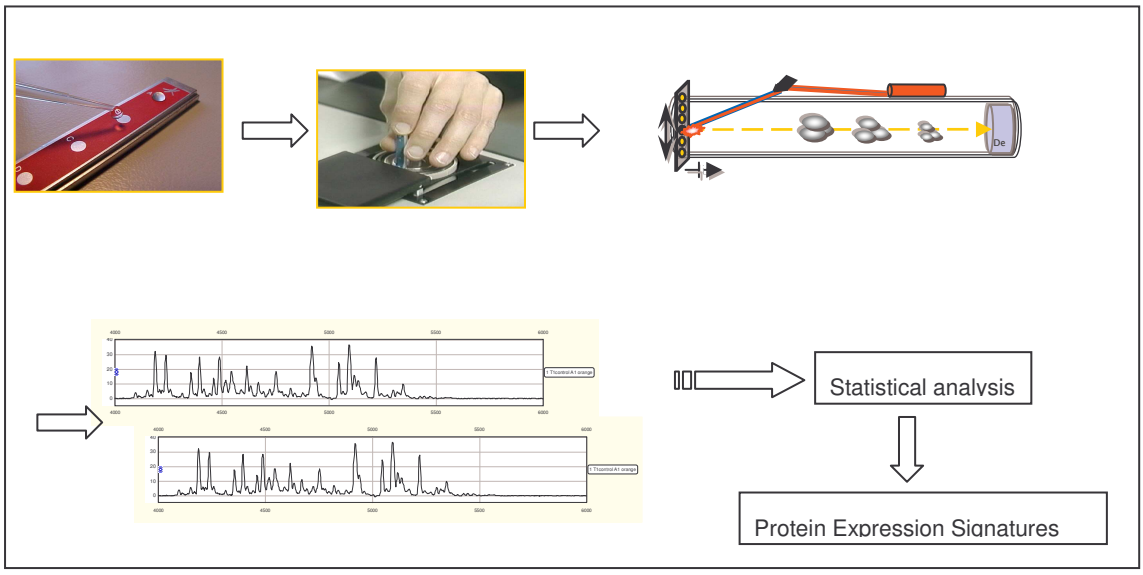


Figure 1 (previous page). SELDI-TOF analysis. Sample extracts are bound to chip. Following matrix application, the samples are ionised by laser collision, separated according to *m/z* ratio and detected. The generated spectra are analysed with bioinformatics software available with the instrument and multivariate analysis using the statistical package Primer.

A second mass spectrometer the LTQ orbitrap has been used to perform protein and peptide identification. This has been done by peptide identification by mean of bioinformatic database searching. This will let us know which peptides or proteins are present in the coral polyps (Figure 2).

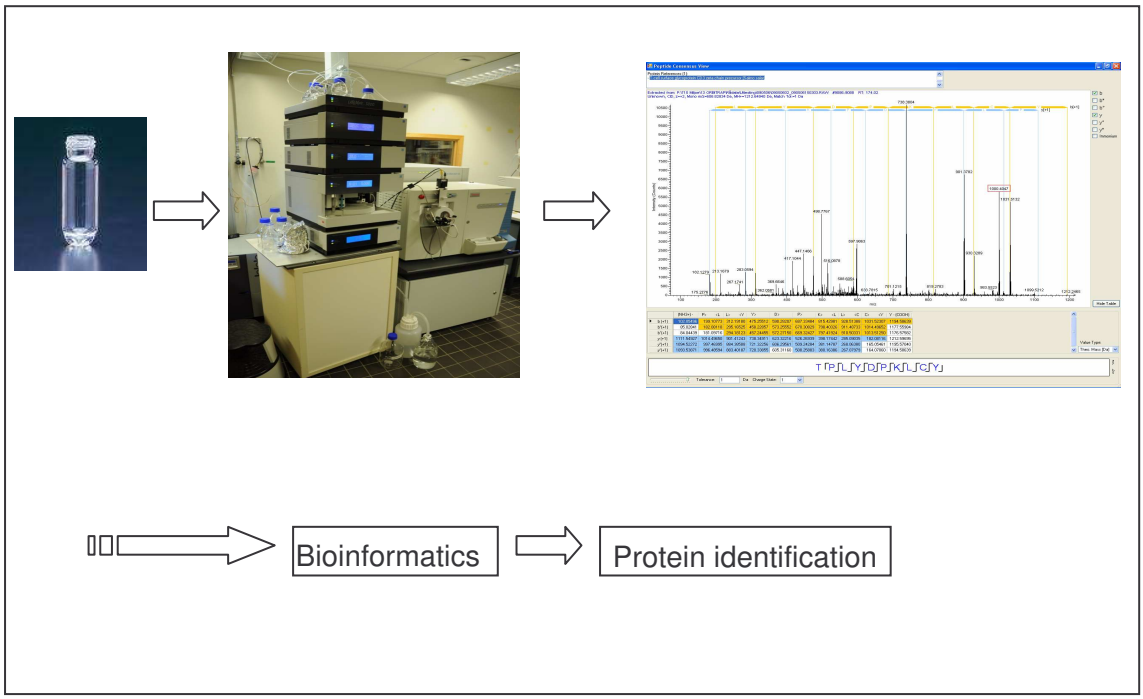


Figure 2. Illustration of workflow for LTQ Orbitrap analysis of data.

An overview of the two proteomics analysis strategies is illustrated in Figure 3 below. SELDI_TOF mass spectrometer is a good screening tool, for rapid analysis of many samples, it gives us information regarding overall changes in protein expression in the samples, however no identification of the proteins involved is given using this analysis. The information is nicely complimented by the LTQ orbitap mass spectrometer information which gives the identification of proteins in the samples.

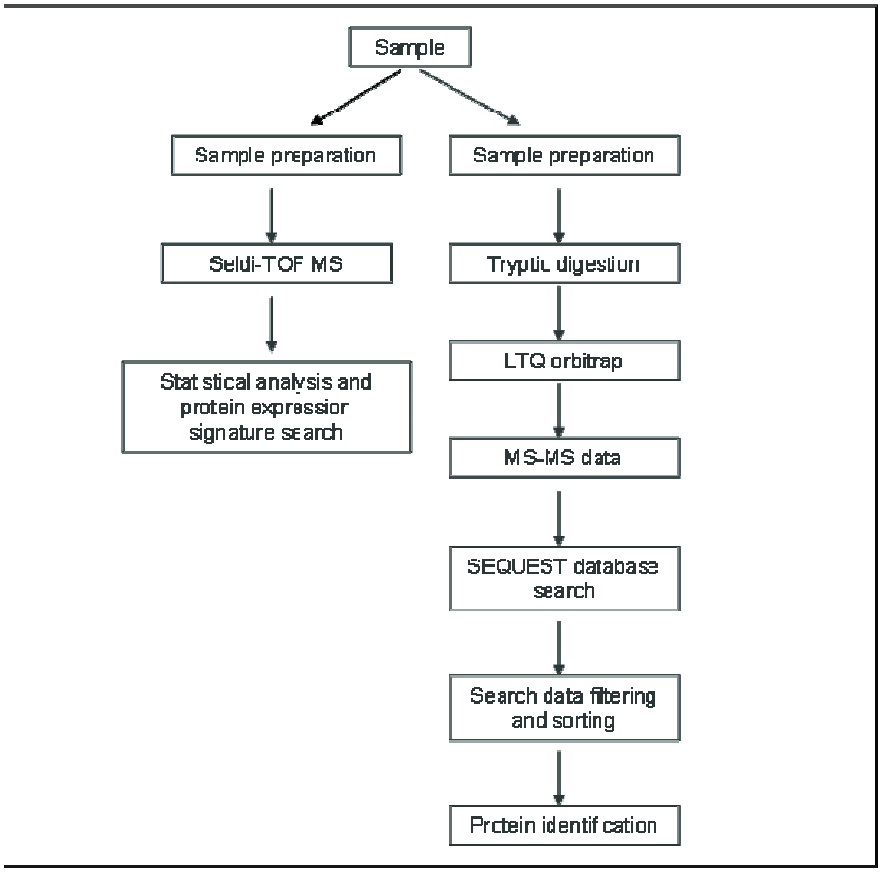


Figure 3. Proteomic analysis strategies (adapted from Wilmes P. and Bond P.L. 2006). Two analysis strategies are used; Seldi TOF analysis giving Protein Expression Signatures and LTQ orbitrap based analysis giving the identification of proteins present in the sample

3.1.2.1 Method optimisation

Considerable effort has been put into developing sample preparation methods to achieve optimal results in the analysis. These efforts involved developing procedures for generating samples from the polyps and mucus suitable for analysis on the orbitrap instrument. Finally a two step procedure involving the FastPrep® and sample clean up with ZipTips was developed for the polyp samples.

For the SELDI-TOF analysis the optimization was focused on testing various chip types with various chip surface chemistries to decide upon which chip type yielded spectra of the highest quality.

3.1.2.2 Sample extraction polyps

One polyp from each coral was added to a Lysing Matrix E tube (two samples were also prepared in Lysing Matrix D tube) together with PBS buffer (150 µl). The samples were lysed at a speed of 5.5 for 30 s utilizing FastPrep®. The samples were then centrifuged

(2.5 min at 10000 g) and aliquoted. The aliquoted samples were again centrifuged (5 min at 10000 g) and aliquoted.

Zip Tip concentration polyps - Zip Tip C_4 pipettes were conditioned by first pipeting acetonitrile (5 x 10 µl) with ejection to waste followed by washing with 0.1% TFA solution (5 x 10 µl) with ejection to waste. The sample (aliquotes from the previous step) were then applied from the top of the Zip Tip and forced through the C_4 packing material. The packing material was then washed with 0.1% TFA solution (5 x 10 µl) prior to elution with 60% acetonitrile solution (10 µl) (repeated 10 times). The aliquots were then divided into two samples (one for SELDI and one for Orbitrap analysis) and concentrated to dryness (spinvac, 5 min).

Sample preparation of polyps for Orbitrap - The dry samples from the Zip Tip step was dissolved in AMBIC (15 µl of a 50 mM solution) and dithiothreitol (DDT) (1 µl of a 1 M solution) was added. The resulting solution was left at room temperature for 45 min before iodoacetamid (IAA) (5 µl of a 1 M solution) was added and the resulting mixture was left in the dark at room temperature for 45 min. DDT (5 µl of a 1 M solution) was then added and the sample was left at room temperature for 45 min before trypsin (2 µl of a 0.1 µg/ µl in 0.01% TFA) was added and the resulting solution was incubated for 18 h at 37 ^{o}C. The samples were then cooled to 0 ^{o}C and centrifuged through a cut-off filter (30000 Da). Samples were then directly put on the Orbitrap for analysis.

3.1.2.3 Sample preparation of mucus for Orbitrap

Development of procedures facilitating the analysis of mucus by Orbitrap is currently ongoing at the laboratory of IRIS-Biomiljø in a parallel analytical activity. The heavily glycosylated mucus proteins requires a deglycosylation step prior to trypsin digestion. Work is now focusing on optimizing that step in order to provide optimum samples for Orbitrap. Treatment of mucus proteins with trifluoromethanesulphinic acid (TFMS)[1] are providing samples that are looking promising, however, further optimization of this step is required in order provide optimum samples.

3.1.2.4 Sample preparation of mucus for SELDI –TOF

Due to the low protein concentrations in the samples, the mucus samples were bound to Normal Phase ProteinChip® arrays NP20 (Bio-Rad) chips which require no dilution of samples binding buffer. The chip surface contains silicon oxide groups on the spots, where proteins with hydrophilic and charged residues are able to bind (Bio-Rad Laboratories, Inc., 2006). The binding is based on either electrostatic attraction, dipole - dipole interactions or hydrogen bonds.

To each spot on the chip array 2 µl of sample was added. The spots reserved for standards were left blank. The chip array was then dried in a heating cabinet at 37°C for 50 minutes. After drying, the spots were washed by addition and aspiration of 5 µl distilled H_2O with a pipette and subsequently dried again in the heating cabinet at 37 °C for 10 minutes. 1 µl matrix solution was added to each sample spot, and 1µl of the standard/matrix solution to the spots left blank. After the chip array was dried in the heating cabinet at 37 °C for 15 minutes, the matrix solution and the standard/matrix solution were added again, and the chips were incubated at room temperature for 30 to

60 minutes. After incubation chips were placed into the Ciphergen ProteinChip® AutoLoader (Ciphergen Biosystems, Inc. Fremont, CA) for analysis.

3.1.3 Identifying changes in bacterial community

The microbial community was examined through the extraction of total DNA from the coral mucus, which was then amplified using PCR and primers specific for the bacterial 16S rRNA gene. The 16S rRNA gene is a section of prokaryotic DNA which is found in all bacteria and archaea (as opposed to 18S rRNA gene fragments of eukaryotes). It is highly conserved and is therefore a commonly used tool for identifying bacteria. Denaturing Gradient Gel Electrophoresis (DGGE) was then used to investigate the microbial populations in the samples. DGGE is a powerful molecular based method, which is a commonly used technique in environmental microbiology. This method allows for the rapid characterisation of the microbial community composition, diversity and dynamics, and is a strong tool for looking at microbial communities and changes in a given population in response to pollutants.

DGGE banding patterns can be used to visualize variations in microbial genetic diversity and provide a rough estimate of the richness and abundance of predominant microbial community members. It allows for the separation of identical or nearly identical length PCR products that have different DNA sequences. DGGE separates the PCR products based on their different motilities as they migrate through the gel. Sequence differences are resolved based on the different melting temperatures of the DNA sequences as they migrate through a polyacrylamide gel with increasing concentrations of denaturants (urea and formamide). As a result, each DGGE band on the gel represents a different microbial species and the intensity of each band is a qualitative measure of the proportion of that species in the DNA sample. Bands appearing in different samples at the same height suggest that this species is present in both samples. Comparing band patterns from different samples provides information on the microbial diversity and differences between samples. Identification of the different species present can be provided by DNA purification and sequencing of bands of interest.

3.1.3.1 DNA extraction

Coral mucus was sampled using Catch-All swabs (EPICENTRE Biotechnologies, Madison, WI). DNA was extracted from the coral mucus samples immediately after sampling. Sterile forceps were then used to remove the swab into a PowerBead tube (Mio Bio Laboratories Inc, Solana Beach, CA) and cells were lysed using the FastPrep-24 instrument (Santa Ana, CA) at 6.0 m/s for 60 s. DNA was extracted using the PowerSoil DNA isolation kit (Mio Bio Laboratories Inc, Solana Beach, CA) with the following changes to the manufacturer's instructions: the centrifugation times in the pelleting steps were all increased to 2 min and the DNA was eluted in 50 µl of C6.

3.1.3.2 PCR amplification

The rDNA bacterial primers GM5F (5'-CCTACGGGAGGCAGCAG-3') and DS 907r (5'-CCCCGTCAATTCCTTTGAGTT-3') (Teske et al., 1996) were used to PCR amplify the 16S rDNA gene. The GM5F primer included a 40-mer GC-clamp (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3') on the 5'end. To control for any potential inhibitors in the samples, undiluted DNA template was used in the PCR reactions along with 1:10 and 1:100 dilutions of the DNA template in sterile MilliQ water. The PCR was run in 50µl mixtures, containing 2 µl DNA template, 200 µM of each d'NTP, 0.5 µM of each primer, PCR buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl) 2.5 µM $MgCl_2$) and 2.5 U Taq DNA polymerase Gold (Applied Biosystem, Foster City, CA). The PCR mixtures were heated (95^{o}C; 2 min), followed by 40 cycles, 1 each consisting of denaturation (95^{o}C, 1 min), annealing (55^{o}C; 1 min) and DNA synthesis (72 ^{o}C; 1 min). The reaction was terminated with a final extension (72^{o}C; 7 min).

3.1.3.3 Denaturing Gradient Gel Electrophoresis (DGGE)

The coral samples were compared using DGGE. PCR products were resolved on an IngenyphorU DGGE system (Ingeny International, Goes, Netherlands). A continuous gradient of 20 – 70 % of the denaturant agents urea and formamide (100% denaturant corresponds to 7 M urea and 40 % deionised formamide) was used for DGGE. PCR products were run on a 10 % polyarylamide gel at a constant 40 mA for 17 h. The gel was stained using Gel Red (Biotum, Hayward, CA) for 1 hour.

3.2 Characterizing changes in main pigment profile

The samples used in this study were collected during experiment B.

3.2.1 Spectrophotometric method

The method used was based on that implemented at NTNU (Trondheim) by Elde (2009). At each sampling event, between 1 to 1.5 grams of each coral individual was cut off. *In vitro* extracts were made by crushing coral pieces thoroughly in a chilled mortar (mortar placed on ice), adding 10 ml acetone. Both calcareous skeleton and polyps were used. Extracts were left in freezer (-20^{o}C) for 24 hours, then sampled using a pipette and filtered through 0.2 µm filter to avoid particles. Extracts were filtered once more before analysing in spectrophotometer. To measure *in vitro* absorbance, filtered extracts were run in spectrophotometer in a quartz cuvette, from 300 nm to 850 nm. Maximum VIS absorbance for astaxanthin is about 470 nm depending on solvent.

3.2.2 HPLC-NP method for identification of astaxanthin

In normal phase (NP) a polar column is used in combination with a nonpolar mobile phase. A normal phase silica column, Kromasil 100 – 3.5 SIL, 150 x 4.6 mm, was used for these analyses. The column was first conditioned with 1% ortho-phosphoric acid in methanol. Analytical conditions: heptane/ acetone 86/14 at 1.2 ml/min. Detectors: PDA

360 – 800 nm and UV/VIS detector with a fixed wavelength at 470 nm. Sample volume injected: 20 µl.

The method used for HPLC analysis is based on a standard method used and described in FHL (Fiskeri og havbruksnæringens landsforening, Østerlie, 2005) for analysing astaxanthin in fish feed. This method can detect the total amount of astaxanthin, and the isomer distribution between all-*E*-, 9*Z*-, 13*Z*- and other *Z*-isomers of astaxanthin. Most natural carotenoids are found in all-*E* form, but recently *Z*-isomers have been isolated from nature that probably has a biological function (based on Skretting Norway standard method; www.skretting.com). All-*E*-isomers easily changes into *Z*-isomers in organic solvent, exposed to light and heat.

The samples used for VIS_{470} measurements were concentrated about 8 times (using a stream of N_2 and gentle heating) for better detection in the HPLC analyses. The retention time for free astaxanthin was determined by running the neat substance before the analysis of samples from the corals.

3.2.3 TLC method for identification of mono- and diester of astaxanthin

The thin-Layer Chromatography method used in this study is based on that from NatuRose™ Technical Bulletin #003; www.cyanotech.com.

The analytical material needed was:

- ⇨ Silica Gel Plates, Merck (TLC aluminium sheets 5x10 cm, Silica gel 60).
- ⇨ Glass jar with lid (TLC chamber). Solvent: 25% acetone in hexane.

Elution time was carried out for 30 min. The original samples were concentrated as much as possible and approximately 5 µl is applied.

This method was used for its simplicity, and to get a visual confirmation of what is seen from other methods. This method can also be used for purification or separation of compounds of interest, because it is non destructive. Unfortunately the colours fade quickly when exposed to air and light. Separation of astaxanthin diesters and monoesters from free astaxanthin (as well as canthaxanthin and some other carotenoides) is possible.

4 Results

4.1.1 Characterizing changes in protein pattern in exp. A

4.1.1.1 Differentiation between mucus from white and orange polyps with SELDI-TOF

The spectra were analysed using the biomarker wizard software available with the SELDI-TOF instrument. A total of 21 peaks were identified (with relatively low stringency settings) that were significantly different in peak heights between orange and

white corals. It may be inferred that these peaks each represent a peptide or protein that is significantly altered in expression levels between the two coral types (Table 1).

Table 1 List of peaks with significantly different peak intensities in mucus from white (w) and orange corals (o). [with search criteria set at S/N3, S/N 1, minimum peak threshold 25%, cluster mass window 0.5] ($p<0.05$)

	p	Mean - o	SD - o	Mean - w	SD - w
4676,960	0,000	8,441	4,207	2,671	2,382
5047,238	0,000	13,136	8,525	4,270	4,702
4489,803	0,000	11,379	7,851	4,621	5,426
4940,375	0,000	7,571	3,963	3,627	4,012
4823,721	0,000	18,365	17,651	4,664	6,654
4752,151	0,000	5,505	3,706	3,609	3,955
5227,052	0,000	11,792	8,826	5,833	6,791
4618,217	0,000	9,935	8,707	4,304	4,351
5347,272	0,001	7,125	6,901	2,555	2,893
4464,918	0,001	5,195	4,928	2,510	2,525
4551,782	0,001	14,067	8,607	9,252	8,062
4239,946	0,002	16,889	7,902	12,012	8,261
4397,500	0,002	8,551	6,116	5,726	4,640
5096,128	0,002	16,072	12,744	8,624	9,077
4916,079	0,004	16,973	12,518	9,685	9,524
2994,978	0,004	5,059	4,413	10,236	10,186
3107,415	0,005	4,560	3,249	7,818	7,206
4725,712	0,008	4,875	2,614	4,705	4,801
4889,772	0,027	6,736	5,101	16,340	14,720
2969,158	0,031	5,103	4,116	7,709	6,819
3035,823	0,037	2,850	2,373	4,525	4,656

The peaks listed in Table 2 were identified as segregating peaks under particularly stringent settings, suggesting that these peaks are particularly robust and hence putative markers for segregating orange and white corals.

Table 2 Summary of “robust peaks” with significantly different peak intensities in mucus from white (w) and orange (o) corals

m/z	Analysis settings
4239,6	sn5, sn3 60% 0.5
4671,7	sn5, sn3 25% 0.5
5047	sn5,sn3 50% 0.5
5226	sn5sn3 50%0.5

The spectra were also analysed using multivariate statistical analysis with Primer (v 6.1.9). The peak clusters generated in the Ciphergen software are used as variables in the analysis. After square root transformation a resemblance matrix based on Bray Curtis similarity was calculated. From this matrix Multi-Dimensional Scaling (MDS) plots were created. This type of plot gives a spatial illustration of the similarity or difference between samples. The stress value indicates how well the illustration fits with the data, a value of <0.1 shows that the map corresponds well, and <0.2 indicates a potentially useful representation. The MDS plot indicating differences in profile by colour can be seen in Figure 4, each data point represents one spectrum. The stress value of 0.12 is quite low and the diagram shows relatively good separation by colour with some overlap between the groups.

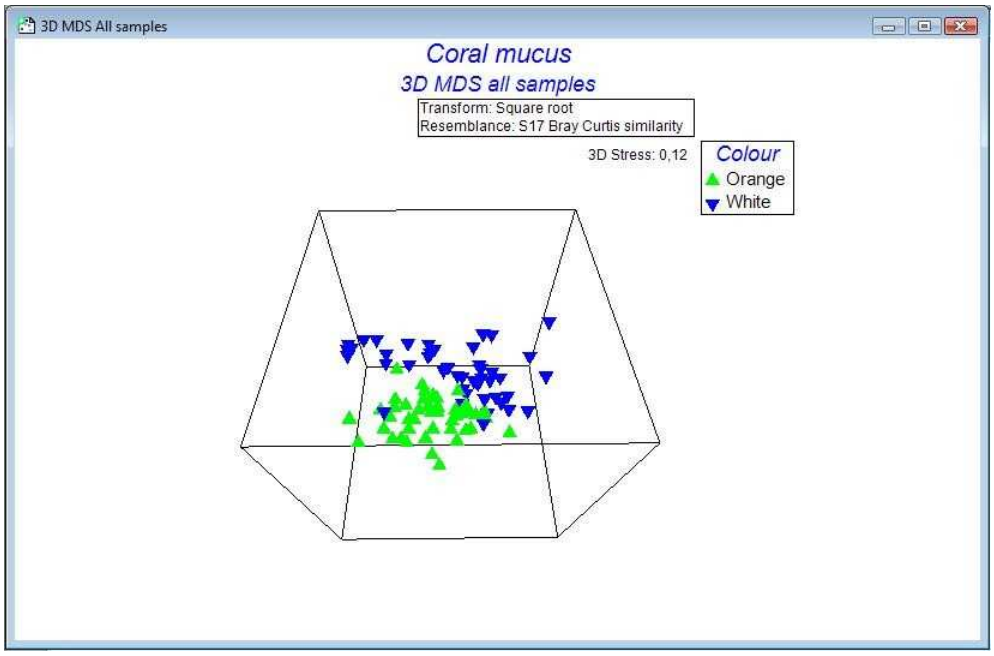


Figure 4 -Dimensional MDS plot of peak data from SELDI spectra from corals, with points labelled by coral colour (orange and white). All samples (control and exposed to drilling mud) are included.

4.1.1.2 Exposure effects on mucus with SELDI-TOF

The multivariate analysis of the SELDI TOF spectra from all data (independently of coral colour or temperature difference in experimental system) showed that the control and exposed groups did not segregate well in the 3D MDS plots (Figure 5). This suggests that overall the spectra of the polyps are similar between the two treatments.

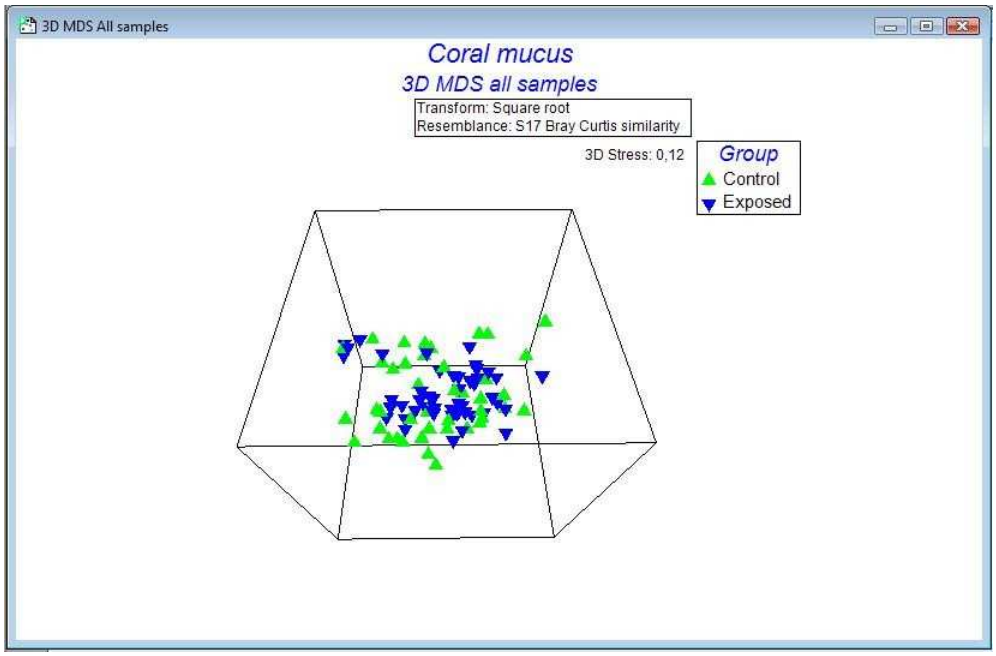


Figure 5.-Dimensional MDS plot of peak data from SELDI spectra form corals, with points labelled as control or exposed. All samples (orange and white) are included.

The analysis was repeated but this time each coral colour (orange or white) and aquarium temperature (6 or 11°C) was analysed separately. The multivariate statistical analyses are visualised in the form of 3-Dimensional MDS plots (Figure 6-9).

A tendency for segregation according to DM exposure can now be seen, especially for the orange polyps kept in aquarium 1 (Figure 6). This may suggest that the orange polyps are in some way more affected by the exposure than the white polyps. The tendency for segregation between the control and exposed groups in orange polyps in aquarium one can also be confirmed by the univariate analysis of the spectral data (Table 3). In the univariate analysis of the spectral data of orange polyps from corals in A1, 13 peaks were identified with significantly different peak intensities when comparing exposed and control (Table 3). By comparison, 5 peaks were identified to have significantly different peak intensities when comparing control and exposed groups for white polyps of corals in A 1 aquarium (Table 4).

Table 3. List of peaks with significantly different peak intensities between control and exposed groups in orange polyps, in A1 aquarium.

M/Z	p	Mean - control	SD - control	Mean - exposed	SD - exposed
1670.738	0.001	10.395	4.675	17.704	3.983
4360.219	0.002	7.310	5.562	2.143	1.354
1530.919	0.004	14.790	5.984	22.746	3.530
1554.244	0.007	15.526	5.256	22.477	4.440
1572.944	0.007	16.360	5.612	23.090	4.532
4489.803	0.018	16.271	11.186	6.617	3.296
1647.796	0.024	32.886	15.523	48.208	11.753
5138.157	0.024	8.181	4.036	4.825	2.121
4239.946	0.028	20.837	7.819	12.961	4.548
4153.789	0.033	12.936	15.276	4.762	5.781
4676.960	0.038	9.020	4.071	5.756	2.677
4190.680	0.043	19.014	9.926	11.042	3.566
5047.238	0.050	16.223	8.877	9.579	5.956

Table 4. List of peaks with significantly different peak intensities between control and treatment groups in white polyps, in A1 aquarium.

M/Z	p	Mean - control	SD - control	Mean - exposed	SD - exposed
4823.721	0.001	1.662	0.762	5.441	5.459
1530.919	0.015	15.714	5.810	22.338	7.000
1554.244	0.018	15.741	5.115	22.173	7.356
1572.944	0.024	17.075	5.063	23.213	7.653
4153.789	0.028	9.105	8.276	3.069	1.898

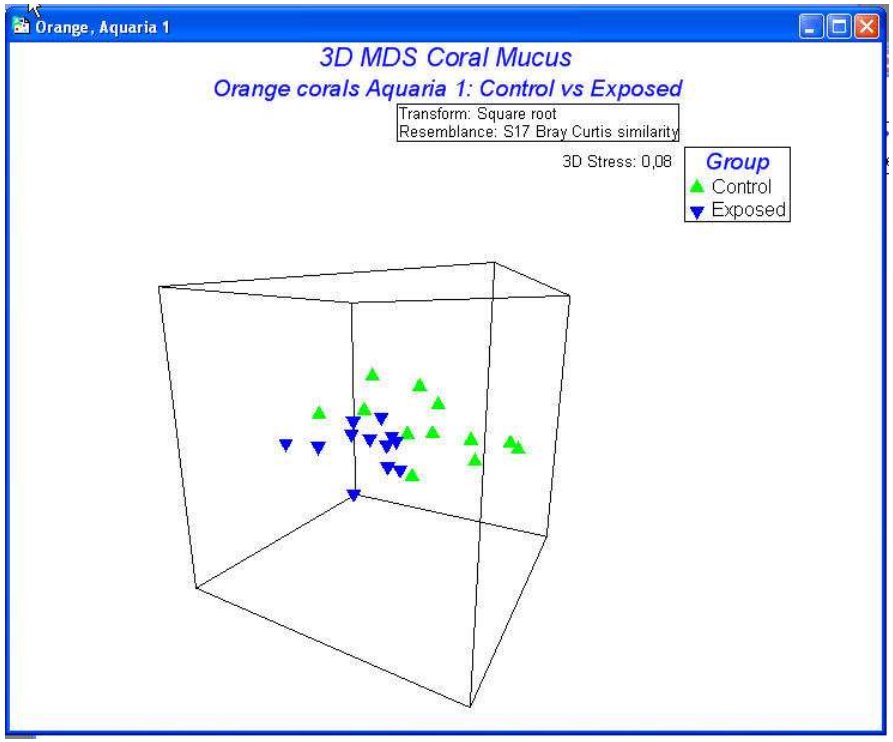


Figure 6- Dimensional MDS plot of peak data from SELDI TOF spectra from orange polyps, with points labelled by treatment (▲control ▼exposed). Corals kept in A1 aquarium at 6°C

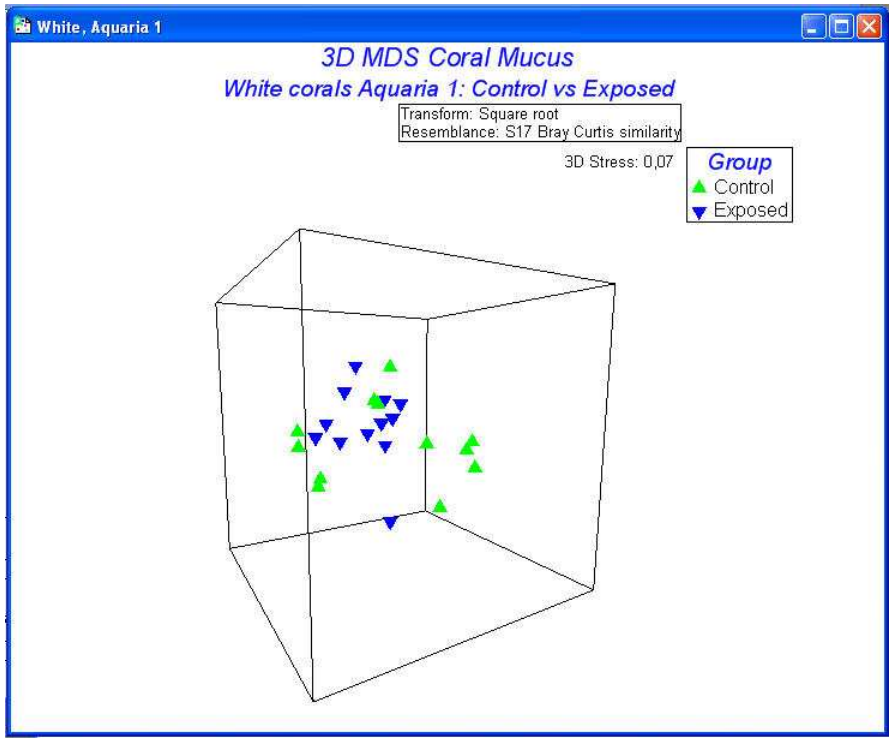


Figure 7- Dimensional MDS plot of peak data from SELDI TOF spectra from white polyps, with points labelled by treatment (▲control ▼exposed). Corals kept in A1 aquarium at 6°C

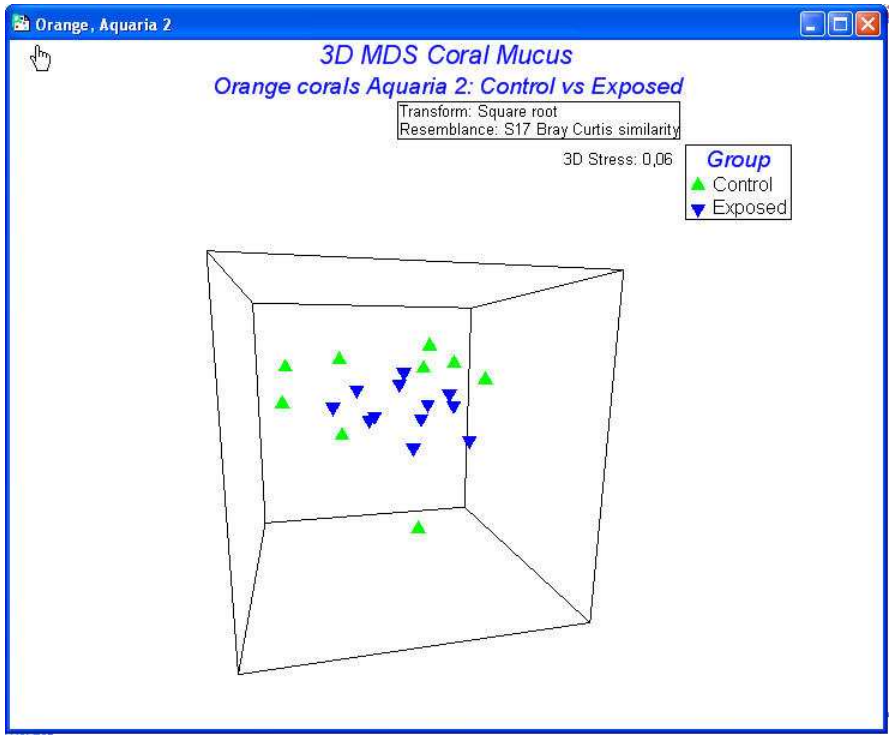


Figure 8- Dimensional MDS plot of peak data from SELDI TOF spectra from orange polyps, with points labelled by treatment (▲control ▼exposed). Corals kept in A2 aquarium at 11°C.

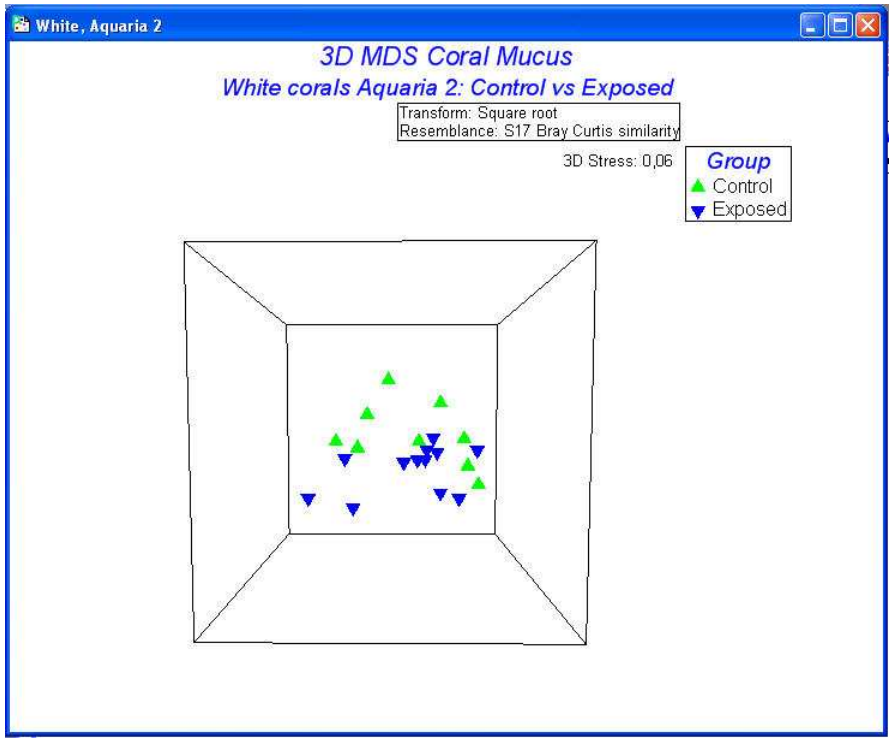


Figure 9- Dimensional MDS plot of peak data from SELDI TOF spectra from white polyps, with points labelled by treatment (▲control ▼exposed). Corals kept in A2 aquarium at 11°C.

4.1.1.3 Protein identification in whole polyps, ORBITRAP

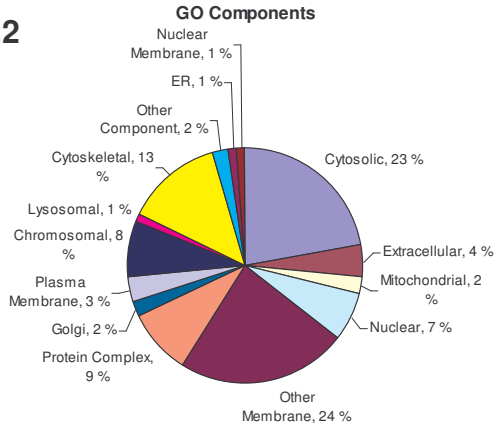
Both orange and white polyps were analysed together using the LTQ orbitrap instrument to identify proteins present in these samples. Only three samples were analyzed as a preliminary study of the potential for using this instrumentation in further analysis. Much effort was put into generating high quality sample preparations suitable for analysis. This work included sample clean ups using C4 ccontaining columns. Once this methodology was established the analysis was efficient and yielded data of good quality. The analysis revealed that 1102 peptides were identified to be present in the polyp samples, corresponding to 247 proteins (see appendix). These proteins are proposed to be associated with various functions, processes and cellular components as illustrated in Figure 10.

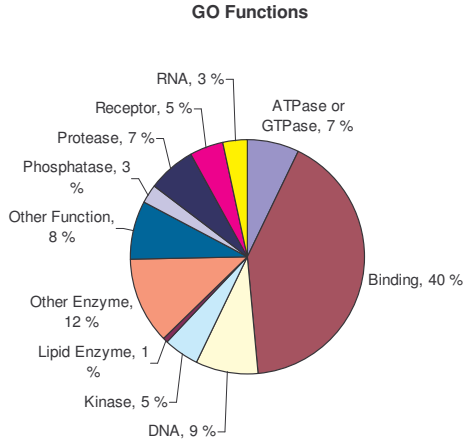
4.1.1.4 Protein identification in coral mucus, ORBITRAP

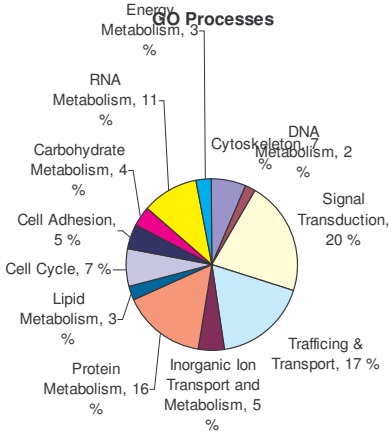
A sample preparation method was also initiated for the mucus samples which involved a deglycosylation step. Many of the proteins in mucus are glycosylated and these carbohydrate groups are proposed to reduce the accessibility of the proteins to the trypsin enzyme which digests the proteins into segments suitable for separation and analysis in the LTQ orbitrap. The method will require further steps before being fully established.

Figure 10. Gene ontology diagrams illustrating proposed functions, processes and cellular component location for the identified proteins from the three coral individuals.

4.1.2







4.1.2 Identifying changes in bacterial community in exp. B

The coral were sampled on December 6th and December 15th, 2009 (corresponding respectively to 4 and 13 days exposure to drilling mud). On January 13th, 2010 after approximately 1 month of allowing the coral to recover from drilling mud exposure, the coral mucus was sampled again. DNA was extracted from coral mucus from all individuals in both the control and exposure conditions to examine if there were any bacterial community changes in the corals exposed to drilling mud compared to the control specimens. DNA and PCR products were successfully obtained from all samples.

Generally, the DGGE results show a low diversity in the coral mucus (Figure 11 and 12). Only a few bands are seen on the DGGE gels: 3 different bands seen in Figure 11 and 6 different bands seen on the gel in Figure 12 (each band theoretically represents a different microbial species).

This study indicates that drilling mud exposure may lead to a change in the microbial community. This can be seen by the appearance of band b in individual B exposed to 3 mg particles/l of drilling mud after 4 days exposure and individual A exposed to 14 mg particles/l of drilling mud after 13 days exposure (Figure 11). Therefore, drilling mud exposure led to change in the microbial population with the presence of a new microbial species in these individuals after drilling mud exposure.

The effect of drilling mud exposure appears to result in a lasting change of coral mucus microbial community. One month after the end of exposure, differences between the control coral samples and the exposed coral samples still exist (Figure 12). Distinct differences were seen in the banding patterns in the different drilling mud exposure conditions with only band e present in the control samples and additional bands seen in the exposed conditions; 3 bands were present in individual A (bands a, d and e) and 5 bands were observed in individuals B and C (bands a, b, c, d and e) at 45 mg/l; 4 bands (bands a, c, e and f) were present in all individuals in both the 4 mg particles/l and 14 mg particles/l (Figure 12; all the bands mentioned above may not be seen in the gel due to the quality of the image). The additional bands present in the exposed samples indicated a significant increase in the diversity of the coral-associated microbial communities compared to the control individuals.

From this study, it appears that coral pigmentation had little or no effect on the coral mucus microbial community as comparable banding patterns were seen between the coral individuals independently of their colour.

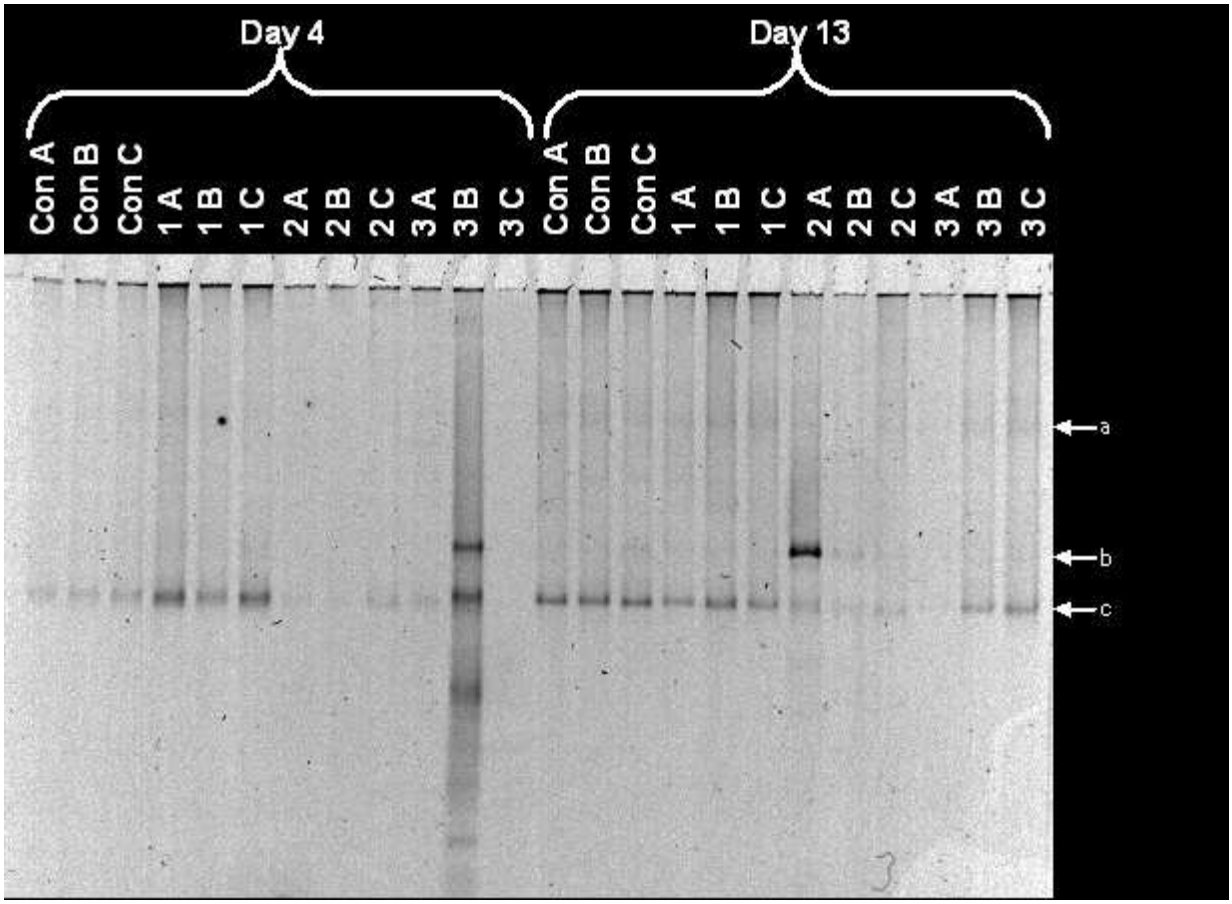


Figure 11. The DGGE profiles of 16S rRNA gene fragments amplified from coral mucus samples that were taken on days 4 and 13 of the drilling mud exposure experiment. The coral were exposed to four different concentrations of drilling mud 0 mg particles/l (Con), 45 mg particles /l (1), 14 mg particles/l (2) and 4 mg particles/l (3). Each exposure condition contained three different corals (individuals A, B and C) containing three different coral individual where individuals A and B where white *Lophelia pertusa* and individual C was an orange *Lophelia pertusa*. The location of visible bands (bands a, b, and c) on the gel is marked with arrows on the side.

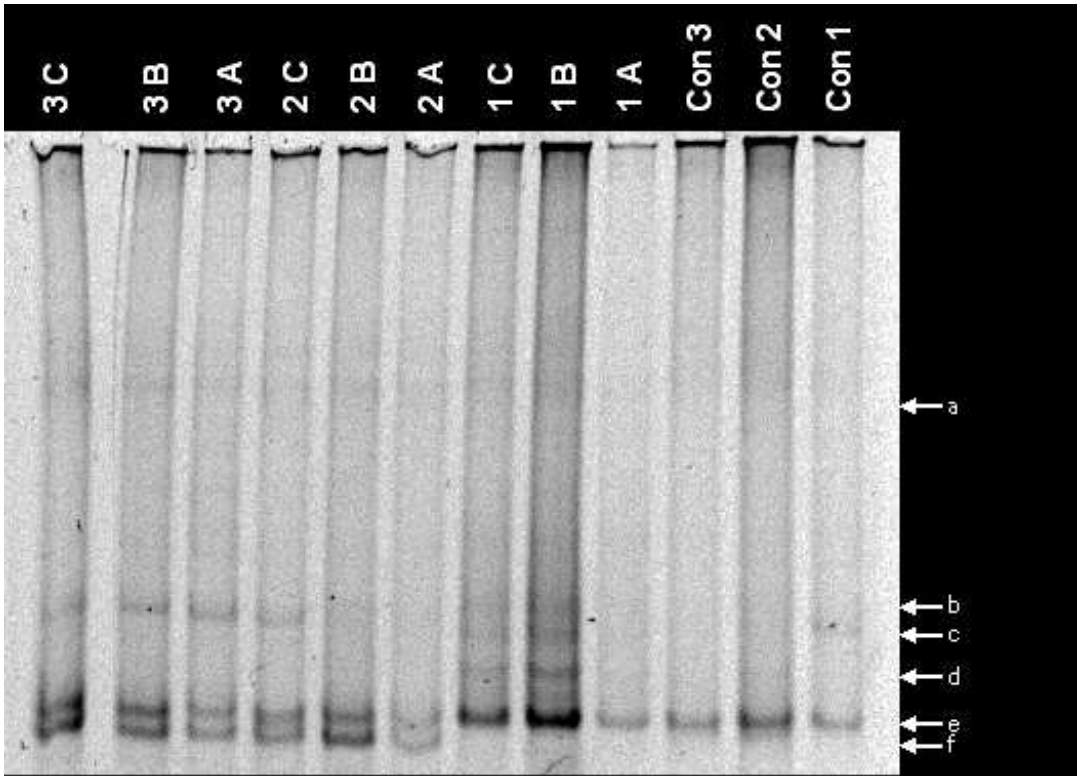


Figure 12. The DGGE profiles of 16S rRNA gene fragments amplified from coral mucus samples that were taken after an approximately month recovery after drilling mud exposure. The coral were exposed to four different concentrations of drilling mud 0 mg particles/l (Con), 45 mg particles /l (1), 14 mg particles/l (2) and 4 mg particles/l (3). Each exposure condition contained three different corals (individuals A, B and C) containing three different coral individual where individuals A and B where white *Lophelia pertusa* and individual C was an orange *Lophelia pertusa*. The locations of visible bands (bands a, b, c, d, e and f) on the gel are marked with arrows on the side.

4.1.3 Characterizing changes in main pigment profile in exp. B

4.1.3.1 Spectrophotometric method

The different *L. pertusa* individuals contained different shades and degree of colouration. This natural colour variation will contribute to variation in absorbance both between and within individuals (Table 5, Figure 13). The biological variation is greater for white *L. pertusa*, with a coefficient of variation (CV) above 30% for absorption per wet weight.

Table 5: Mean λ_{max} and mean absorption per wet weight portraying the biological variation between and within three orange and three white *L. pertusa* individuals.

Species	**n**	**Mean λ_{max}**	**CV %**	**Mean abs /wet weight at λ_{max}**	**CV %**
Orange *L. pertusa*	3	475	0.42 %	29.45	8 %
White *L. pertusa*	3	434	1.28 %	6.8	31.8 %

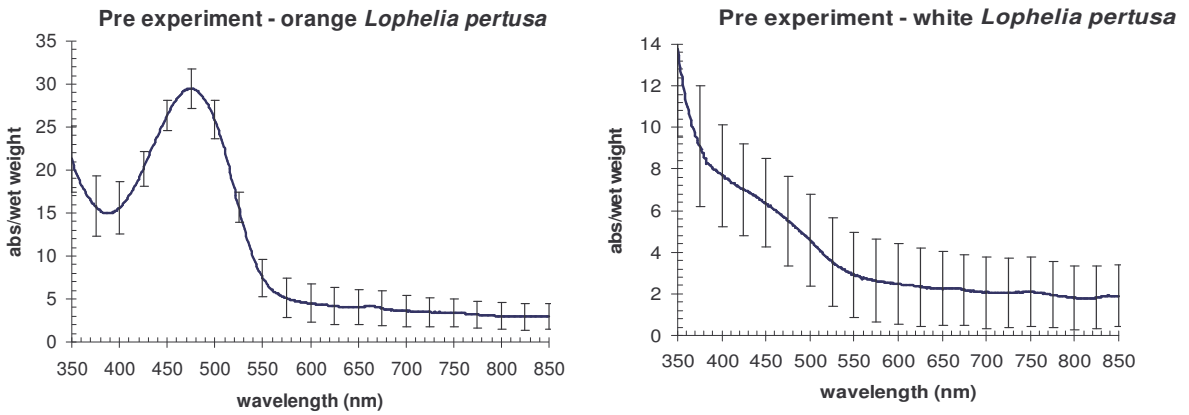


Figure 13: Pre experiment showing *in vitro* absorption per wet weight of orange and white *L. pertusa*. Bars represent standard deviation (n = 3).

The normalized absorbance (abs/wet weight) measured in coral individuals from the different treatment of exp. B are represented in Figure 14. Generally, absorbance decreased with experimental time in white corals while it appeared to increase in orange corals. This pattern was observed both in control and exposed corals.

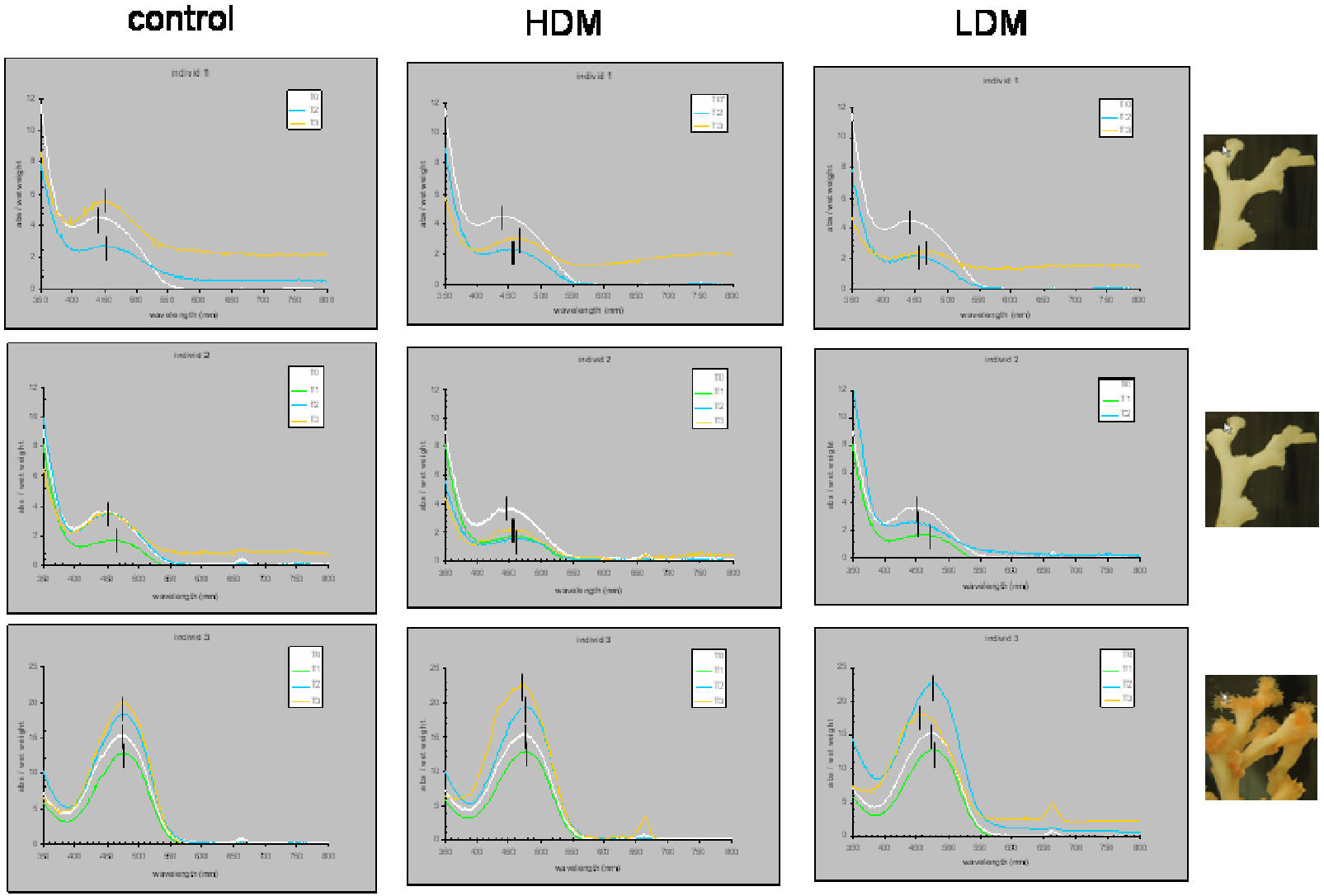


Figure 14 – Visible (VIS) spectral absorption (wet weight normalized) measured in white (top, middle) and orange (bottom) individuals in control and DM exposed treatments at start (T1), after 7 days (T2) and at end of exposure (T3) in exp. B. T0 is a pre-exposure sampling event, 10 days before T1. HDM: high (45 mg/l) drilling mud exposure; LDM: low (4 mg/l) drilling mud exposure. The symbol | is placed on each curve to visualize the wavelength at which the max VIS absorbance is detected.

Also, a shift towards higher wavelength in max absorbance was measured in white corals but not in orange corals. Although the significance of that is not yet understood, similar observations were made with samples collected and analyzed during exp. A (data not presented) in the NFR project. Fouling from micro-algae or other epibiotic species needs to be considered. No differences between control and exposed white corals were clearly demonstrated at the stage of this analysis in the present study.

4.1.3.2 HPLC-NP method for identification of astaxanthin

Very little free astaxanthin was detected (retention time about 7.8 min.), and there seems to be at least two isomers present. Two or more other peaks were detected that are less polar than astaxanthin (retention times about 2.1 min. and 4 min; the first eluting peak is the larges one). These are probably fatty acid mono- and diesters of astaxanthin or carotenoid-protein complexes.

As an example, Figures 15&16 show the pigment spectra obtained from HPLC analyses performed at the end of the exposure in a white (ind.1) and orange (ind.3) individual. The HPLC chromatogram has a main peak that is believed to be esterified carotenoids/astaxanthin, and some smaller peaks. Very little free astaxanthin is observed. Individual 3 (the orange corals) have approximately the same peak responses in the control tank (not exposed to mud) as the exposed corals in tank with 4, 14 and 45 mg/l DM. The orange corals (Individual 3) show 10 times higher (or more) response for the largest peak than the other two coral individuals (Individual 1 and 2).

The analyses are not quantitative, but still give a good picture of the differences between the three individuals.

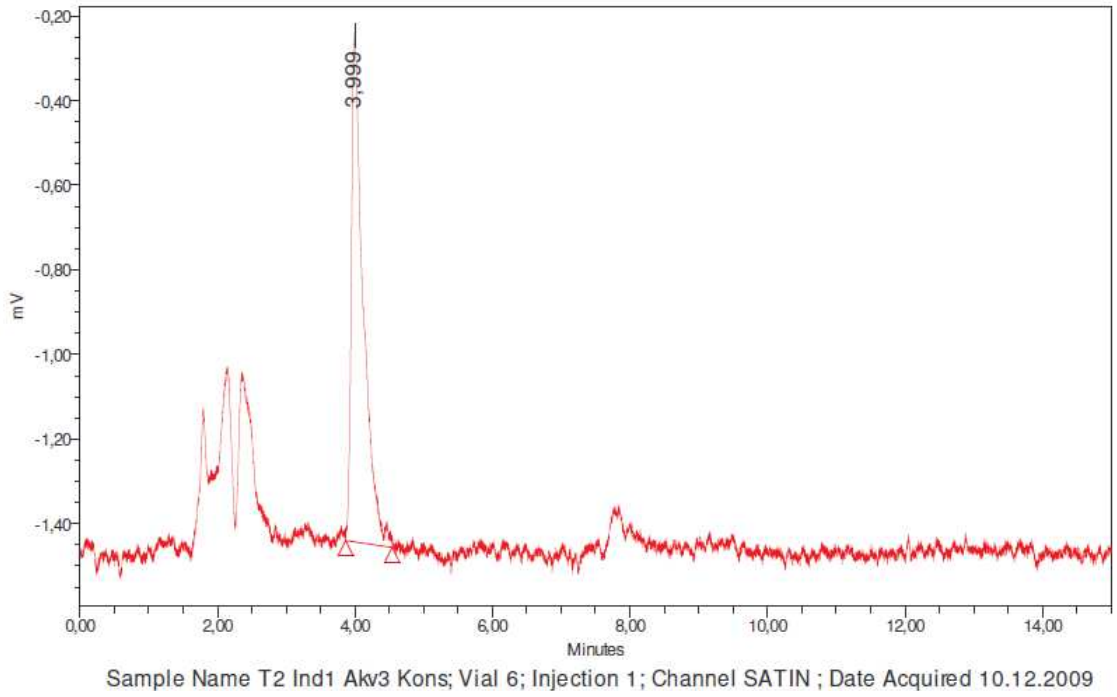


Figure 15 – HPLC-NP analysis performed on white coral (ind.1) during exp.B.

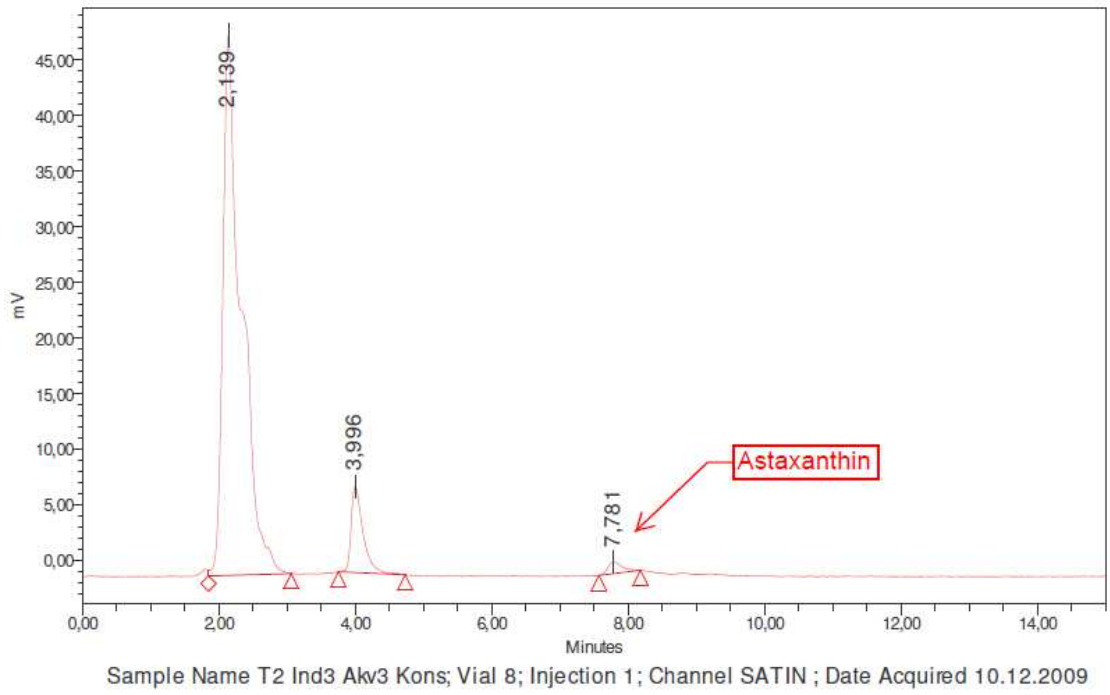


Figure 16 – HPLC-NP analysis performed on orange coral (ind.3) during exp.B.

4.1.3.3 TLC method for identification of mono- and diester of astaxanthin

The most coloured extract, applied after concentration of the sample, showed three distinct coloured lines, one of which has the same retention time as free astaxanthin. The other two lines are believed to be mono- and diesters of astaxanthin (not yet confirmed). These two lines probably contribute the most to the measured VIS_{470} absorption. Based on visually observed colour intensity, free (unesterified) astaxanthin is present at a very low concentration in the coral extracts.

5 Discussion

The effects of drilling activities related to oil and gas search in regions where reefs of the deep-sea coral *Lophelia pertusa* need to be addressed. In order to properly address the effects these activities have on *L. pertusa* tools need to be developed which can be used to asses the health of these corals. This study was made to assess the potential of some innovative and state-of-the art techniques for their use as biomonitoring techniques applied to cold water *Lophelia pertusa* in the context of drilling operations by O&G industries.

5.1.1 Characterizing changes in protein pattern

Mucus has been shown to be a powerful diagnostic material in a variety of different species. It can be sampled non-invasively and can be envisaged to be particularly sensitive to environmental effects due to its exposed location and constant contact with the surroundings. With this work we have obtained preliminary results indicating that coral mucus is also a promising diagnostic material in environmental impact analysis.

In this work we have taken a proteomic approach to the analysis, meaning we have studied overall changes in protein expression between control and treatment groups. Toxicoproteomics has been proposed to be the next step in the evolution of environmental biomarkers and this work can be said to be state of the art. No similar work has to our knowledge been published earlier. In particular the work using the LTQ orbitrap is promising with regards to identifying proteins sensitive to exposure and/or reconstitution.

Methodology has been established for analysis of mucus and polyps using mass spectrometric instrumentation. The sample optimisation procedure was relatively extensive however once established the methodology allows rapid analysis of further samples.

Continuing this work with a greater number of samples and with focus on protein identification and quantification in the samples could allow us to identify proteins sensitive to drilling mud exposure. The data quality from the SELDI-TOF analysis may be affected by low protein concentrations in the samples, the LTQ orbitrap analysis are not affected by this limitation suggesting that focus in further studies could be kept on the LTQ orbitrap analysis.

One limitation regarding this work is that the genome of the *Lophelia pertusa* coral is only partially sequenced limiting the amount of genomic information available. However searching databases of related species and de novo sequencing lets us overcome these limitations.

5.1.2 Identifying changes in bacterial community

Compared to the original field location of corals, bacterial communities in our experiment were likely to be different due to individuals held in the laboratory for several months since field collection. However, this investigation was made to assess whether there were changes of coral-associated bacterial communities present in mucus following drilling mud exposure and compare that with a control situation in the laboratory.

In this preliminary study we attempted to address if there is any effect of drilling mud exposure on coral related microbial communities and the potential of using change in the coral- associated microbial communities as "early warnings" for potential deleterious effects of drilling mud on corals.

This study indicated that drilling mud exposure leads to changes in coral-associated microbial communities as indicated by the different banding patterns seen on the DGGE gels between the exposed and non exposed corals (Figure 1 and 2). For most of the exposed samples, no changes were seen on the DGGE gel (Figure 1). This is possibly due to the low concentration of DNA in the PCR products for some of the samples, which would render differences in the microbial communities impossible to visualize on the gel. As this is a preliminary study, further optimization of the method is required to increase the DNA concentration of the PCR products to be able to visualise less dominant species within the samples. In future studies, information on the effects of drilling mud on coral-associated microbial communities can be increased by sequencing

the individual bands of the DGGE gel. In particular, identifying the organisms which are present specifically during or after drilling mud exposure would be interesting. This would allow us to determine what microbial species are present in the different exposure conditions, and if drilling mud exposure leads to an increase in potentially pathogenic bacteria on the coral.

The use of DNA/DGGE based methods to study the effect of drilling mud on coral-associated microbial communities is therefore a potentially very effective and rapid tool for assessing coral health and drilling mud effects.

5.1.3 Characterizing changes in main pigment profile

The spectrophotometric method is relying on a quick protocol based on the extraction of total pigments in corals. Using that method, the idea was to recognize possible changes in absorbance profile related to drilling mud exposure. The hypothesis was that changes in absorbance amplitude or shift in wavelength in max absorbance could reflect changes in the physiological state of corals. From this study, we were not able to distinguish clear changes related to drilling mud exposure. An interesting observation is though related to the shift of wavelength in max absorbance in white corals not observed in orange corals. The same type of observation was done in exp. A run earlier in the NFR project. At this stage, we have no clear explanation to what causes this shift.

From the analyses performed with the HPLC, no differences in control and exposed corals were seen confirming the observation made by the VIS method. However, the HPLC analyses revealed the main extracted pigment using acetone as solvent was apparently esterified astaxanthin rather than actual free astaxanthin. Hence, from that, it appears that the main absorbance measured in VIS spectra in white and orange colour corals is related to esterified astaxanthin.

Reversed phase HPLC may separate different esterified compounds in a more detailed way than the normal phase method used here.

Esterase treatment may release astaxanthin and thus confirm the assumption of esterified astaxanthin being the compound that can explain most of the colour difference in orange and white corals (Wade, N; Degnan, B.M. et.al. comparative Biochemistry 2005). Carotene-protein complexes (if present) may be split by using proteolytic enzymes (trypsin, papain, pepsin).

6 Conclusion

6.1 Changes in mucus protein patterns

- Methods have been established for sample preparation for both polyps and mucus.
- Mucus has been demonstrated as a potentially sensitive diagnostic material.

- Preliminary results indicate that it is possible to see difference in the protein content of white and orange polyps and to see treatment specific effects on the protein content in polyps and mucus.
- Proteins have been identified in the polyp samples and one can see the potential of developing this methodology to look for treatment specific protein markers.

⇨ **Protein quantification to confirm identification of treatment sensitive markers is recommended as a follow-up study**

⇨ **Confirm the present observations using mucus collected in exp. B with All-catch swab**

6.2 Bacterial community changes

- Drilling mud exposure appears to cause lasting changes in the coral-associated microbial community
- Little differences seen in coral-associated microbial communities between coral types
- DNA/DGGE based methods to study the effect of drilling mud on coral-associated microbial communities could potentially be a good tool for assessing coral health and drilling mud effects.

⇨ **Further optimization of the methods is required**

⇨ **Examination of the sea water and drilling mud associated microbial communities and comparison with coral-associated bacteria**

⇨ **Sequencing and identification of the dominant organisms in the different microbial communities from the DGGE bands allowing to recognize possible pathogenic organisms**

6.3 Changes in pigment spectral signature

- The VIS method is easy and allow for a rapid screening of the main pigment signature in white and orange corals.
- Colour-related differences were observed in the experimental work (shift in wavelength max absorbance) but no clear effect of DM was demonstrated
- The main VIS absorbance appears to be related to esterified astaxanthin rather than actual free astaxanthin.

⇨ **Longer-term experiment to confirm trends observed in experimental work**

⇨ **Test other dissolvent than acetone**

⇨ **Reversed phase HPLC may separate different esterified compounds in a more detailed way than the normal phase method used here.**

⇨ **Esterase treatment may release astaxanthin and thus confirm the assumption of esterified astaxanthin being the compound that can explain most of the colour difference in orange and white corals (Wade, N; Degnan, B.M. et.al. comparative Biochemistry 2005). Carotene-protein complexes (if present) may be split by using proteolytic enzymes (trypsin, papain, pepsin).**

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