Marine Habitat Committee **ICES CM 2001/E:01**
Ref.: ACME

REPORT OF THE

STEERING GROUP FOR A SEA-GOING WORKSHOP ON PELAGIC BIOLOGICAL EFFECTS METHODS

ICES Headquarters
18–19 January 2001

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International Council for the Exploration of the Sea

Conseil International pour l'Exploration de la Mer

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1 OPENING OF THE MEETING

The Chair, Dr Ketil Hylland, opened the meeting of the Study Group for a Sea-going Workshop on Pelagic Biological Effects Methods (SGSEA) and welcomed participants to the meeting. He noted that only a few of the research groups involved in the practical workshop were not represented. The meeting participants comprised both members of the Steering Group and representatives from participating institutions.

The full list of participants and their affiliations is shown in Annex 1. It was noted that some research groups were represented by more than one scientist due to the range of different methods to be used by that group within the framework of the workshop.

2 APPOINTMENT OF RAPPORTEURS

It was agreed that the rapporteuring would be split among members of the group. Each presenter would prepare the necessary text concerning his or her presentation.

3 PROGRAMME FOR THE MEETING

The programme for the meeting was presented to the participants (Annex 2). It was noted that a tight schedule would be required to have time for both presentations and discussions. The list of meeting papers and working documents is attached as Annex 3.

4 AN OVERVIEW OF THE WORKSHOP

Dr K. Hylland gave a brief overview of the workshop, as presented at the ICES Annual Science Conference in Brügge, September 2000 (Annex 4).

5 AREAS TO BE INVESTIGATED DURING THE WORKSHOP

Dr T. Utvik presented the Statfjord oil production area. The geographic position of the Statfjord B platform was given. Estimated discharge volumes of produced water in 2001 from Statfjord A, B and C are 36 000 tonnes/day, 117 600 tonnes/day and 43 800 tonnes/day, respectively. An overview of studies performed in the water column by the oil companies was given, i.e., Statfjord area 1995 (Semi-permeable Membrane Devices (SPMDs)/Blue mussels (*Mytilus edulis*)), Tampen area 1997 (Semi-permeable Membrane Devices (SPMDs)/Blue mussels (*Mytilus edulis*)/*In situ* large volume water sampling/"At-line" solid phase extraction/Direct water sampling), Ekofisk area 1999 (Environmental monitoring programme (mussels and SPMDs), Sleipner area 2000 (Environmental monitoring programme (mussels and SPMDs)).

The DREAM model (dose-related risk and effect assessment model) has been used to predict dispersion of produced water from Statfjord B. The model is based on field specific discharge data, and 3D current fields calculated from meteorological data. Some results from runs with the DREAM model are shown in Annex 5.

Dr Gerd Becker presented the hydrography and environmental status of the German Bight.

6 THE CRUISE PLAN

Dr T. Lang presented information on the cruises that will be carried out in the framework of the BECPELAG workshop and highlighted the major tasks to be fulfilled during each cruise.

In total, seven cruises with RVs "Walther Herwig III" (Germany) (two cruises), "Scotia" (UK Scotland), "Johan Hjort" (Norway), "Belgica" (Belgium), "Cirolana" (UK England) and "G.M. Dannevig" (Norway) will be performed in the period from late February to early September 2001.

The first workshop cruise to the German Bight and the Statfjord area stations will be on-board RV "Walther Herwig III" (23.2–10.3.2001) and will cover field sampling of fish embryos/larvae and adult/juvenile herring for biomarker measurements and chemical analyses. In addition, ripe fish will be collected for on-board stripping and artificial fertilisation experiments. Further tasks include the sampling of water for studies on bacterial diversity/degradation and the sampling of the sea surface microlayer for subsequent use in various bioassays.

The second workshop cruise will be carried out by RV "Scotia" (17–30.3.2001), again in both main workshop areas. Priority tasks will be the collection of herring, other adult fish, zooplankton and, in addition, water samples for studies on microzoo-/phytoplankton.

The main task of the third cruise with RV "Johan Hjort" (23.4–1.5.2001) is to transport the live organisms (cod, stickleback, blue mussels) for the exposure experiments and to deploy the cages and the attached equipment (SPMDs and DGTs) at the four sites each in the Statfjord area and the German Bight. In addition, water samples will be taken for chemical analyses and studies on water bacteria.

After an exposure period of 5–6 weeks, the cages and the other equipment will be collected and the exposed organisms will be processed. In the German Bight, this will be done during two consecutive cruises with RV "Belgica" (11–15.6. 2001) and RV "Cirolana" (16.–23.6.2001). RV "G.M. Dannevig" (1–10.6.2001) will do the same in the Statfjord area.

The final workshop cruise will again be carried out with RV "Walther Herwig III" (24.8.–9.9.2001). The major objectives are to collect sea surface microlayer and other water samples for bioassay, chemical analyses and bacteriological studies.

It was mentioned that the dates of the cruises given above still have to be considered somewhat preliminary and that more minor changes may be necessary due to the workload expected or the availability of the vessels.

In the discussion of the cruise plan, it was pointed out that it will be crucial that the workshop participants attend the cruises whenever possible in order to ensure that samples are being collected and processed in an appropriate way. Particularly for some biomarker methods requiring sophisticated preparation techniques, it will be inevitable that trained experts are on-board. Although some of the above cruises are part of national monitoring programmes and can, therefore, not fully be dedicated to the purposes of the workshop, there will be sufficient space on-board (berths) all vessels for workshop participants.

Since some of the techniques applied on caged organisms require an addition incubation phase subsequent to the exposure in cages (e.g., scope-for-growth in blue mussels), it was suggested to explore ways to conduct the incubation and some of the tissue preparation techniques in land-based laboratories. Suitable places could be located on the island of Helgoland (Alfred-Wegener-Institute for Polar Research, Biological Station Helgoland) in the German Bight and in Bergen, Norway (Institute of Marine Research). The transport of samples and live organisms on shore could be carried out either by vessels or helicopters. T. Lang agreed to take the responsibility for making contact with the Helgoland laboratory, and J. Klungsøyr will check the availability of laboratory space in the IMR, Bergen.

An overview of the cruises is given in Annex 6.

7 STATISTICAL DESIGN AND DATA COLLECTION

Dr W. Wosniok could not be present at the meeting so his contribution was given by Dr K. Hylland.

7.1 Guidelines for Sampling Design

The aim of the workshop is to assess the ability of various methods to detect biological effects of contaminants in pelagic systems. The size of these effects is expected to vary according to the distance from a contamination source. It is also expected to differ between areas with different contamination profiles. Moreover, some biological effects can only be investigated during a certain period within a year. Hence, the cruise plan includes sampling in various positions along the estimated contamination gradient, sampling in different areas, and sampling during different times over the year.

Within this framework, the sampling must allow answers to be obtained for the following:

- Does the method under study show a gradient at all?
- If yes, is this gradient in line with the contamination gradient?
- Can detection or determination limits be estimated?
- Do gradients derived by various methods share a common tendency?
- Are there groups of methods that can mutually backup one another?
- Which methods should be combined to generate an overall picture of the situation?

Sampling design considerations differ between methods with results on a continuous scale, and other methods producing counts or percentages that are based on counts. Typical examples for continuous quantities are salinity, temperature, or concentrations of chemical compounds given as mg/l or ppm. Examples of count-based data are the number of species found, the number of individual animals found, the proportion of diseased fish with respect to the number examined, or the prevalence of embryonic malformations. However, the distinction between continuous and count data is not always that obvious. Mixed forms and rank data require specific treatment. In cases of doubt or when dealing with situations not covered here, W. Wosniok[1] should be contacted.

A common recommendation for both continuous and count methods is that samples are to be taken at least at three different points along a gradient and at a reference site. This set-up is already incorporated into the cruise plan. If for any reason not all of these samples can be taken, attempts should be made to take those samples which are likely to show the largest differences. This means:

- if only two sample positions along a gradient are possible, omit the middle one;
- If only two sample positions at all are possible, take the reference sample and the one with the highest expected effect.

Given that the full number of samples cannot be taken, this strategy maximizes the chance for a meaningful statement about the method's ability to detect differences in biological effects. Nevertheless, every reduction in the number of samples reduces this chance.

For the determination of sample sizes, two sources of variation must be considered separately:

- the *analytical variation,* which leads to different measurement results even if the sample analysed is the same;
- the *biological variation*, which is a true variation in the sense that members of the same population do not necessarily have the same biological properties (in fact, in most cases they do not).

Ideally the analytical variation of a method is already known and can simply be reported, e.g., as a precision profile. If this variation is not known, it must be determined from the data to be collected (see below). The biological variation must be quantified from replicates, which means that at each sampling position and for each point in time, several replicates must be taken and each of these must be analysed individually. The term "replicates" refers to the specimens that are taken under identical conditions (location, time). As an example, if a method is applied to cod liver, then a sample might consist of ten cod caught at one location and one time, and if the method is applied to each individual cod liver, then these constitute ten replicates in the above sense.

Pooling of specimens (livers in the example above) might be technically necessary in order to obtain sufficiently large amounts of material for analysis. Pooled replicates tend to "average out" biological variation, a basically desirable feature. However, if a response within a population is not uniform over the population, but only exhibited by some individuals, then pooling reduces the chance to detect their deviating response, with the consequence that the method under study appears to be less sensitive. On the other hand, individual measurements (no pooling) are prone to have high biological variation, which might also obscure the chance to detect existing differences, if the number of measurements is small. A recommendation concerning the amount of pooling cannot be given at the present instance, as the calculation of an optimal design requires knowledge of the analytical and biological variance involved, that is not yet available. Researchers who use methods with known analytical and biological variance should contact W. Wosniok for advice.

Required sample sizes, i.e., the number of replicates per location and time point, depend on:

- the sizes of analytical and biological variation;
- the size of the change along a gradient that should be detected; and
- the safety with which the size change should be detected.

The last two quantities are fixed deliberately by the researcher. However, the analytical and biological variation (here: variance) must be supplied by the researcher. As this is not yet available, a general sample size calculation is not possible at the present time. If for particular methods these variances are already known or can reasonably be estimated from previous experience, please contact W. Wosniok.

[1] see end of section for ways of contact

Summarizing, the following design is recommended as a minimal requirement:

- take three replicates at each sampling position and for each point in time and apply your method to each replicate;
- if the analytical variance is not known, determine it from multiple (> 2) measurements of the same replicate at at least one sampling location and sampling time.

Larger sample sizes will increase the chance to detect slight gradients, which might not be detected using only the minimal requirements. It is therefore desirable to obtain and analyse more replicates than implied by the above design. Also, the use of additional replicates to determine a precision profile for the analytical variance should be considered. Using fewer samples/replicates than recommended will make a method appear as less effective and could also prevent a meaningful analysis. If the recommended design does not seem achievable, an alternative should be discussed with W. Wosniok.

As a result of the project, *a posteriori* power calculations for each method will be undertaken where possible. These calculations can serve for the planning of further sample sizes, and in the case of no trend detection they will show what size of trend could have been detected by the sample size used.

For methods that generate proportions, the required sample size does not depend on an analytical error, but instead on the size of the unknown proportion. The closer the true proportions are to 50 %, the larger is the sample size required to detect an existing difference/gradient. As an example, in order to detect a prevalence gradient from 50 % to 30 %, about 100 individuals must be considered.

For questions on sampling and statistical analysis within the project, Dr W. Wosniok can be contacted by e-mail wwosniok@math.uni-bremen.de or by phone +49 421 218 3471 (after 10 May 2001).

7.2 Guidelines for Data Reporting

Workshop participants are requested to report their data to the SGSEA Steering Group for a uniform analysis of all methods in the project and for a joint analysis of several methods simultaneously, as indicated in the Guidelines for Sampling Design.

All information relevant for statistical analysis must be reported. The basic record in each report refers to:

- in the case of continuous measurements: one measurement (the value obtained from one replicate, or if the replicate has been split up into several sub-replicates to determine analytical variation, then one observation refers to one sub-replicate);
- in the case of observed numbers or proportions: the absolute counts of number examined and number affected obtained at one sample location at one point in time.

Aggregated quantities such as means, standard deviations, and percentages can only be accepted as additional information for plausibility checks. They cannot replace the basic records described above. Each basic record must contain a unique identification and a description of what the record refers to. This requires, among others, the following information:

Information	**Example**
project identification	BE 015
date (containing year, month, day)	270301 or 27Mar01
location (in geographical coordinates, i.e., latitude and longitude down to seconds)	60° 35.75’ N for 60° 35’ 45” N (three separate fields for degree, min, sec)
station identification (to be given by the cruise leader)	XY35
sample number (one sample comprises all measurements stemming from one location, one time point)	same number for all cod taken at 27Mar01 at 60° 35.75’ N, 02° 15.07’ E
replicate number (identifies sampled object) (could also be pool number)	5 (for cod number 5)

Information	Example
subreplicate number (if replicate was split up to determine analytical variance	2 (for liver part no. 2 from cod no. 5)
number in pool	1 (for non-pooled measurement)
matrix description (may contain several components. If so, use one field in the record for each component)	cod (field 1) liver (field 2)
description of measured quantity	Cd
unit	µg/kg
reference	liver fat
additional information	measurement technique, fish size, weight, age, …
measurement (the result of the method)	3.47

The methods involved in BECPELAG require diverse quantities to be reported, hence it is not feasible to provide a comprehensive presentation here.

Accepted technical formats are:

- plain ASCII files, fields separated by tabs or delimiter characters (, ; etc), or with fixed format fields without particular delimiters;
- Excel files up to Excel 2000, dbase files (upon request);
- other formats must be discussed in advance with W. Wosniok.

Files must be organized with horizontal rows referring to records/measurements and vertical columns referring to fields containing all entries for one parameter/quantity. All files must be accompanied by a description of their contents. This can happen by descriptive heads at the top of each column. Files should be sent as early as possible. In particular, if in one project there are data from spring and autumn cruises, do not wait until all data are complete, but send the data from the spring campaign when they are available.

Send your files as e-mail attachments to

pelagic@math.uni-bremen.de

or on disk to

Werner Wosniok
Institute of Statistics
Universität Bremen, FB3
D 28334 Bremen
Germany

This is also the contact address for any technical questions concerning data submission.

On submission of your data, you will get a statement of receipt (immediately). Your data will be transformed into a standard form for statistical analysis, and a safety copy will be made. You will get a copy of the transformed data, with the request to check the transformed structure, to make sure your data description has been correctly understood. Together with your data you will receive the basic oceanographic and chemical data that will be supplied by SGSEA to all participants.

Please consult http://www.statistik.uni-bremen.de/BECP-guidelines.html for more details.

8 THE CHEMISTRY PROGRAMME

An overview of the sample matrices for the chemical programme was given by Dr T. Utvik. The calculated concentrations from the DREAM model and experimental bioaccumulation factors in zooplankton (*Calanus finnmarchicus*) gives the following expected concentrations of naphthalene, phenanthrene, and alkylated phenols in zooplankton in the Statfjord area 500 m from the platform: 7–17 ng g^{-1}, 16–36 ng g^{-1}, 20–40 ng g^{-1} wet weight.

These results show that the detection limits are of importance when the methods for chemical analysis of such samples are to be chosen. Other important criteria are cost, quality assurance system, experience, and strategy. The oil companies that are financing the analytical part of the Workshop have to fulfill quality criteria set by the Norwegian State Pollution Control Authorities in their field monitoring programmes, and this will lead to some guidance to the Steering Committee in the final choice of laboratories. The Steering Committee will work further on the decision of which laboratories to choose to perform the different analyses.

9 METHODS USING FIELD-COLLECTED ORGANISMS

The session on methods using field-collected organisms was chaired by Dr T. Lang.

9.1 CEA and antioxidant enzymes in fish and fish embryos (P. Roose/K. Cooreman)

This project was presented by Dr P. Roose. The project involves two Belgian institutes: MUMM and DvZ. The Management Unit of the North Sea Mathematical Models (MUMM) is a department of the Royal Belgian Institute of Natural Sciences, Belgian Federal Office for Scientific, Technical and Cultural Affairs. MUMM is responsible for marine environmental protection and resource assessment. The main task of the Sea Fisheries Department (DvZ) is to provide the scientific basis for the rational and sustainable exploitation of living marine resources, from a biological, technical and socio-economic point of view, the protection of the marine environment as a habitat for these resources, and the quality control and assurance of fishery products.

The contribution of MUMM/DvZ to the workshop is threefold: 1) to provide ship time aboard the RV "Belgica", 2) to contribute to the basic chemistry, and 3) to evaluate the use of two biological effects techniques.

The first biological effect technique measures the Cellular Energy Allocation (CEA) in organisms or tissues of organisms. The concept is based on the measurement of the energy consumption at the cellular level. CEA is thus a general indicator of stress. The technique has the potential to be a rapid and cost-effective method for detecting long-term effects, which emerge at higher levels of organization. All measurements are colorimetric measurements. So far there is only one drawback: the measurement of the electron transport activity might need to be performed on fresh samples. This needs to be examined as soon as possible. The technique has been studied in a few laboratory experiments, but was, to our knowledge, never evaluated or used in field surveys. Therefore, this Workshop provides an excellent opportunity for testing the effectiveness of the technique. It is also applicable to any organism or tissue, e.g., algae, zooplankton (performed by Wim de Coen, University of Antwerp), fish larvae, etc.

CEA has been developed to quantify the cellular energy budget of the organism or part of the organism. The method is a biochemical assessment, using spectrophotometric methods, of the organism's energy consumption (E_a) and energy reserves available for metabolism (E_c). The energy consumption (E_a) is estimated by measuring the electron transport (ETS) activity in the homogenates. Measuring the total lipid, protein and sugar contents of the test organism assesses the energy reserves available for metabolism (E_c). The ETS activity is determined by reduction of p-IodoNitroTetrazolium Violet or INT-reduction. The quantity of oxygen consumed is proportional to the quantity of formed INT-formazan. 2 µmol INT-formazan is equivalent to 0.5 µmol oxygen. The difference between the energy factors E_a and E_c represents the energy available for growth and reproduction of the test organism. CEA is expressed in enthalpic equivalents (Joule/(organism or tissue)/hour. CEA is considered to be complementary to Scope for Growth (SfG). The main similarities are the facts that both techniques determine the energy budget of the animal and they are both general indicators of stress. However, there are some important differences. SfG is clearly a more complex approach. Moreover, CEA is measured at the cellular level, using homogenates, while SfG is performed on living "whole organisms". This Workshop provides an opportunity to compare the field assessments obtained in both assays.

The second proposed biological effect method focuses on oxidative stress. Potential mechanisms of contaminant-mediated toxicity in aquatic organisms include the enhanced generation of reactive oxygen species (oxyradicals), such as the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), which might lead to changes in antioxidant enzyme levels, changes in oxidant scavenger levels, and oxidative damage to biomolecules (proteins, DNA membranes, etc.). Techniques to measure oxidative toxicity can be subdivided into measurements of indicators of oxidative stress and indicators of oxidative damage.

The first set of indicators comprises the measurement of antioxidant enzymes and/or oxidant scavengers. The role of these parameters is to protect against oxyradicals. Examples of indicators of oxidative stress are the antioxidant enzymes superoxide dismutase, catalase and glutathione-peroxidase. Examples of oxidant scavengers are glutathione, vitamins E and C, and carotenoids.

An indicator of oxidative damage is the reaction product malondialdehyde, produced during lipid peroxidation. The tissue concentration of malondialdehyde is reasonably indicative of the lipid peroxidation process.

The proposal is therefore to compare CEA and SfG in mussels because this animal has been proposed in the SfG proposal. CEA would then be determined in whole mussel homogenates. Five replicate samples of one mussel each would be necessary. The determination of the Electron Transport Activity in fresh homogenates on-board the research vessel should not pose any obstacle. All necessary handling can be performed during the cruise.

The use of indicators of oxidative stress and damage is included in two other proposals. Furthermore, there are several proposals on determinations in digestive glands of mussel. This might pose practical problems because approximately 10 digestive glands per replicate are needed to perform the analysis of antioxidant enzymes, oxidant scavengers and lipid peroxidation. Therefore, we propose to measure these parameters in tissues other than mussel digestive glands, e.g., fish liver tissues in which EROD was measured. Or, the part on oxidative stress could be omitted from the proposal and, for instance, the application of the CEA technique could be expanded to other organisms or tissues.

9.2 Larval Abnormalities (V. Dethlefsen/H. van Westernhagen/T. Lang)

This project was presented by Dr T. Lang. Early developmental stages of fish are extremely sensitive towards a variety of factors. Small deviations from optimum conditions can result in malfunctions during the embryo development which become manifest in elevated prevalence of malformed embryos. This has been proven in a vast number of experiments. The elevated prevalence of malformed embryos *in situ* indicates that some of the factors that are requisite for normal embryo development deviate from the optimum. Causes for malfunctions of embryos may already be set during the development of the gonads of parental fish. Possible reasons at this stage are malnutrition of parent, chronic stress during development of gonads, but also the accumulation of pollutants.

After spawning further factors can act in the water column. These may include unfavourable temperature or salinity but also elevated concentrations of various pollutants. Therefore, the eventual occurrence of malformed embryos in the water column may be the result of pre-exposure during parental gonad development plus exposure in the water column. Given the high sensitivity of developing fish embryos, it can be assumed that low prevalences of malformed embryos are also occurring under near to optimum conditions. Elevated prevalences always indicate a deviation from normal.

Since 1984 areas off the Danish, Dutch and German coasts have been visited once a year in spring (February/March), and embryos of pelagic spawning fish species have been monitored for the occurrence of morphological aberrations. Highest malformation rates were always found for embryos of whiting (*Merlangius merlangus*), followed by those of flounder (*Platichthys flesus*), dab (*Limanda limanda*), cod (*Gadus morhua*) and plaice (*Pleuronectes platessa*). At times of maximum malformation rates in March 1987, 60 % of the early embryos of whiting in the German Bight were malformed, 28 % of dab embryos, 17 % of plaice embryos, and 18 % of flounder embryos. The pattern of distribution of malformed embryos was typical throughout the period of investigation, with significantly elevated percentages of malformed embryos in near-coastal areas, in an area located northwest of the island of Helgoland, off the East Frisian Islands (shipping lane) and off the mouth of the river Rhine.

The fluctuation of malformation rates in dab embryos from 1984 to 1998 can be taken as typical. From 1984 to 1987 malformation rates increased. Beginning in 1990 until 2000 there was a trend towards decreasing prevalences, with the exception of 1996. The lowest value ever was found in 1998 (2.7 %). Fluctuation patterns for malformation rates were similar for embryos of the other species studied during this monitoring programme. Prevalence rates were negatively correlated with water temperatures related to medium temperatures at Helgoland Roads measured during the first quarter of each year in a long-term monitoring programme of the Biologische Anstalt Helgoland. Highest malformation rates were found at temperatures in a range around or lower than 2 °C. This applied for the years 1985, 1986, 1987 and 1996. In temperature ranges between 3.5 °C and 6.5 °C lower malformation rates were encountered.

Comparing fluctuations of malformation rates of dab over time with residues of organochlorines in livers of dab from the German Bight it was only for DDE that a significant positive correlation existed. Low water temperatures (< 2 °C) exert a pronounced negative influence on malformation rates of pelagic fish embryos. With decreasing water temperatures in the German Bight, also malformation rates decreased over time. In addition, there existed correlations between certain pollutants accumulated in tissues of parental fish (dab, flounder) and the occurrence of malformed embryos.

Multivariate statistical evaluations are presently under way to elucidate in more detail associations between a variety of potential causative factors and fluctuations of malformations of fish embryos over time and space.

During SGSEA/BECPELAG, malformation rates in fish embryos will be studied at multiple sites in the two areas (German Bight, Statfjord).

9.3 Reproductive Impairment in Fish, Developmental Success of Eggs and Larvae (G. Petersen)

Dr G. Petersen presented a study in which ripe fish in the sampled areas will be stripped and the fertilization success and development of larvae determined. During the past two decades, different types of reproductive disturbances, including inadequate ovary maturation, low fecundity and early life stage mortality, have been demonstrated for a number of fish species in Europe. It has been suggested that larval deformities and increased mortality in pelagic eggs of plaice, flounder, and whiting and demersal eggs of herring are caused by persistent bioaccumulative compounds. The present study aims at assessing links between the viability of eggs and larvae from North Sea whiting and the concentrations of PAH-metabolites in bile and of organochlorines in the ovaries of the females. Eggs from running ripe female whiting will be stripped, artificially inseminated and incubated in sea water. If fish, or their eggs, are pre-exposed to toxicants, only a low extra dose is likely to induce a toxic effect. This approach, known as a "challenge test", was used as a tool for examining whether the individual fertilized cod larvae from the Bornholm Basin have different intrinsic sensitivities towards a single extra toxicant. A higher intrinsic sensitivity in the different larvae batches may be a result of the mother-to-egg transfer of toxicants causing elevated burdens of toxicants in the eggs and larvae.

9.4 Histopathological and Biochemical Markers in Fish and Mussels (I. Cancio; M. Soto; M. P. Cajaraville; I. Marigómez)

This project was presented by Dr I. Cancio. The aim of this contribution is to compare biomarkers and histological approaches (applied in both fish hepatocytes and mussel digestive gland cells) with those used by other research groups involved in the detection and assessment of the biological effects of contaminants in marine ecosystems and, particularly, in pelagic ecosystems. Biomarkers to be employed within the framework of the BECPELAG workshop can be subdivided into those that can be employed to assess heavy metal pollution, those specific for organic pollution, and those that are nonspecific biomarkers of stress. Concerning the biomarkers of heavy metal pollution we propose to study heavy metal accumulation applying autometallographic techniques on paraffin sections and metallothionein induction using immunochemical techniques (western blot and immunohistochemistry).

Autometallographed Deposits Screening—Autometallography (AMG), a technique introduced by Danscher (1981), is a cost-effective technique that allows an accurate determination of the metal levels in biological tissues (Marigómez *et al.,* 1995, 1998; Soto *et al.,* 1996b, 1998; Soto and Marigómez, 1997a, 1997b). AMG has been applied in various investigations to demonstrate the association of metals in cells of fish and molluscs with environmental levels of the metals (Marigómez *et al*., 1996, 1998; Soto *et al*., 1996a, 1996b, 1999; Soto and Marigómez, 1997a, 1997b). A close parallel has been found between AMG deposits (Black Silver Deposit-extent or BSD-extent) in target cell compartments and the metal concentrations determined by AAS in the soft tissues of molluscs (Soto and Marigómez, 1997a, 1997b; Soto *et al*., 1998). AMG deposits, that appear specifically confined to the molluscan digestive cell and to the fish hepatocyte lysosomal system, will be measured by image analysis on paraffin sections (Soto and Marigómez, 1997a, 1997b).

The volume density of BSD will be compared in animals (wild herring liver, caged cod liver and caged mussel digestive gland) coming from sites with different pollutant bioavailability. In the case of molluscan tissues, due to the fact that metals are only accumulated in the digestive cells, the relative digestive to basophilic cell ratio will be measured, since a replacement of digestive cells by basophilic cells is a common non-specific response of molluscs exposed to xenobiotics (Marigómez *et al*., 1998). An increase in the number of basophilic cells results in a reduced capacity to accumulate metals leading to misinterpretations of the bioavailable fraction of metals in the environment (Soto and Marigómez, 1997b). The determination of possible basophilic cell increases will be performed by stereological procedures on paraffin sections.

Metallothionein Induction—Metallothioneins are low molecular weight proteins, rich in cysteine residues, that specifically bind toxic and essential metals (Aspholm and Hylland, 1998). Induction of metallothionein synthesis represents a specific response to pollution by heavy metals such as Cu, Zn, Cd and Hg. Binding of metal cations by *de novo* synthesized apothioneins produces non-toxic forms, thus reducing the deleterious effects of metals. Differential pulse polarography is currently used in fish and molluscs to evaluate the concentration of metallothioneins in tissues (Aspholm and Hylland, 1998). Alternatively, competitive ELISA (enzyme-linked immunosorbent assay) is being applied too. Our task in the project will be to localize metallothioneins by western blotting and immunohistochemistry in wild juvenile herring and caged cod hepatocytes. Positive labelling will be quantified using a specific computer

program for the quantitation of immunolabelling intensity on western blots, and semiquantified in the case of the immunohistochemical demonstration. Interestingly, the presence of metallothioneins in fish liver could be related to the presence of metals using serial sections stained with AMG.

As a biomarker of general stress, we propose to employ lysosomal membrane destabilization and/or lysosomal enlargement. These parameters of worldwide use have been employed in our laboratory for the assessment of pollution in Basque and Mediterranean estuaries for over 12 years. For the specific biomonitoring of organic xenobiotics, we propose to measure peroxisome proliferation and neutral lipid accumulation.

Peroxisome Proliferation—Peroxisomes are membrane-bound cytoplasmic organelles appearing in most eukaryotic cells (Cancio and Cajaraville, 2000). One of the unique features of peroxisomes is their ability to proliferate and to enhance their metabolic activity, a phenomenon termed "peroxisome proliferation", which is induced by a number of endogenous compounds and xenobiotics (Fahimi and Cajaraville, 1995). Peroxisome proliferation consists of an increase in peroxisome number (Nv) and volume (Vv) densities, which is usually accompanied by the induction of some peroxisomal enzyme activities, particularly those of the fatty acid β-oxidation system (Cajaraville *et al*., 1997; Cancio *et al*., 1998; Cancio and Cajaraville, 2000). In molluscs, peroxisome proliferation is a potential alternative as a biomarker of exposure to organic contaminants since cytochrome P450 induction (measured as EROD induction) does not give consistent results (Cajaraville *et al*., 1989).

Peroxisome proliferation has been typically described in hepatic tissue of rodents but has also been reported in piscine liver (Cancio and Cajaraville, 2000). We will look for peroxisome proliferation onto catalase-stained cryostat and/or resin sections of caged mussel digestive gland and cod liver and on liver of wild juvenile herring. These studies will be complemented by biochemical studies of peroxisomal acyl-CoA oxidase (AOX) activity and, when necessary, by western blot studies of both catalase and AOX.

Lysosomal Membrane Stability (LMS) and Lysosomal Structural Changes (LSC)—In both piscine liver and molluscan digestive gland, destabilization of the lysosomal membrane and lysosomal enlargement are significant alterations resulting from pollution insult (Cajaraville *et al*., 1989, 1991, 1995a, 1995b; Etxeberria *et al*., 1994; Marigómez *et al*., 1996; Lekube *et al*., 2000) and may be quantified by the LMS test (UNEP, 1997) and the LSC test (Cajaraville *et al*., 1995b). Liver and digestive gland samples will be processed for enzyme cytochemistry of β-glucuronidase or hexosaminidase after cryopreservation, freezing and cryosectioning (Cajaraville *et al*., 1995b) to be further quantified by grading (lability period) and by image analysis (Vv, surface density (Sv), surface to volume ratio (S/V) and Nv). Eventually, an immunochemical approach (Lekube *et al*., 2000), based on the use of specific antibodies against lysosomal enzymes, will be also applied at particular sites of interest with comparative purposes.

Neutral lipid accumulation—Pathological accumulation of neutral lipids in liver cells is a well-known phenomenon, induced by, e.g., chlorinated hydrocarbons. In an extensive review of the effects of pesticides on livers of fish, Couch and Harshbarger (1985) reported that the most commonly encountered liver lesion was abnormal fatty accumulation. In particular, exposure to organic contaminants such as PAHs and PCBs is often linked to an increased accumulation of neutral lipids leading to "fatty change". This phenomenon has been reported repeatedly in mussels (Lowe, 1988; Moore, 1990) and is an established biomarker of exposure to organic contaminants. Neutral lipid accumulation will be measured by image analysis in cryostat sections stained with oil red 0 (OR0; Cancio *et al*., 1999).

9.5 Biomarkers in Field-collected Fish (O. Aspholm)

The objective is to investigate whether biomarkers in herring, *Clupea harengus*, caught at Statfjord and in the German Bight are affected by contaminants in the pelagic system. Juvenile or adult herring will be collected by pelagic trawl. After catching the fish must be kept alive on-board prior to sample collection. The project will analyse vitellogenin concentrations and *zona radiata* protein concentrations in plasma from 25 fish. For this 2 vials are needed of at least 30 µl plasma from each fish. Hepatic CYP1A protein concentrations and mRNA expression will be analysed and 2 samples are needed of approximately 1 g liver from each fish. Both liver and plasma samples must be stored in liquid nitrogen and be distributed on dry ice.

9.6 Bacterial Production and Grazing (A. Tobiesen)

In the open ocean it has been found that there is a tight coupling between bacterial growth rate and bacterial grazing, resulting in nearly constant amounts of bacterial biomass over time within a biotope. It is hypothesized that processed water may uncouple this link either by increasing bacterial growth or by reducing grazer efficiency.

Use of ^{3}H-thymidine is a well-established method for measuring bacteria-specific growth, although there are difficulties with respect to what conversion factor to use on the amount of thymidine incorporated when calculating bacterial biomass. However, this problem is cancelled out when looking at relative changes within the same area, using the same method and performing it in the same way (same scientist).

This property has been exploited to measure grazing of bacteria by microzooplankton in a modified version of the Landry and Hasset (1982) Dilution Experiment. This method was developed to measure grazing of phytoplankton. By diluting out grazers and incubating for 24 h it is possible measure bacterial gross growth rate while at the same time estimating grazing on bacteria. An assumption must be fulfilled, i.e., that the growth rate of bacteria is constant in both diluted and undiluted samples.

9.7 Effect on Photosynthesis (A. Tobiesen)

Algal production is the basis for production in the sea. Because of the heterogeneity and patchiness of algae in the sea, it is not possible to make direct comparisons of effects on photosynthesis *in situ*. The next best approach is then to use algal cultures and see if it is possible to observe any relative difference in photosynthesis between different water samples. It is well known that the sensitivity to pollutants differs for different algae, therefore at least 5 different algal species will be tested.

The test will look at gross production rates (2-hour incubation) and net production rates covering incubation for 24 h. The gross production rate is expected to yield information with respect to the need for adaption to exposed pollutants; net production rates are expected to yield the most sensetive indicator as this measurement will incorporate energy used to detoxify pollutants and cover a whole life cycle. Therefore, effects due to inability to divide (reproduction) would also be included.

9.8 Biomarker Responses in Fish (E. Aas, S. Sanni, M. Depledge, L. Balk, B. E. Grøsvik)

There is currently a need for improved biological effects monitoring of pelagic ecosystems with regard to oil activity. Fish are both ecologically important as well as a resource for human exploitation, and for these reasons highly relevant to include in environmental monitoring programmes. This proposal emphasizes studies on three aspects of biological effects in fish: early biochemical responses, genotoxic and endocrine effects. Several compounds have been shown to exert genotoxic effects on organisms. Among the best documented are the polyaromatic hydrocarbons (PAHs). PAHs are important contaminants regarding oil production offshore, e.g., in operational discharges of produced water. Endocrine disruption in marine organisms, e.g., fish, is another concern which has been addressed lately. Alkyl phenols are also of particular concern regarding discharges of produced water from the oil industry.

Several different methods for evaluating genotoxicity exist, more or less well established and suited for monitoring. Detection of DNA adducts by the ^{32}P-postlabelling method is possibly one of the most sensitive and well established methods available today for monitoring genotoxic effects at the molecular level in fish (Reichert *et al*., 1998). Due to the use of radioactivity in the DNA ^{32}P-postlabelling methodology and it being a rather time-consuming method, there is a need for simpler methods, which also could be more suited for field monitoring.

Interpretation possibilities regarding biomarker responses are increased by prior laboratory experiments. Various laboratory experiments have been and will be carried out at Akvamiljø as part of the research programme for developing the DREAM model (Statoil, Hydro, Elf and ENI-Agip). The organism used in the experiments is the "model" fish sheepshead minnow, *Cyprinodon variegatus*, which is exposed to mixtures of PAH components and alkylated phenols. End-point parameters of reproductive effects applied are egg production, hatching success and larval survival. The biomarkers included are PAH metabolite detection in bile, cytochrome P4501A/ EROD, DNA adducts (^{32}P-postlabelling), COMET assay, vitellogenin and *zona radiata* protein.

The objective is to evaluate different molecular biomarkers in fish with regard to their suitability for monitoring. The main source of contamination is hydrocarbons discharged from oil-producing installations in the North Sea. The biomarkers suggested for analysis are PAH metabolites in bile, cytochrome P4501A/ EROD, vitellogenin, *zona radiata* protein and COMET (Single Cell Gel) assay. Additionally, we will consider to include alkaline unwinding assay (plate reader technique) and micronuclei assay. These two methods are presently not included as established methods at Akvamiljø, but will be evaluated, and implemented if they are found suitable Cooperating laboratories will supplement with other methods. In order to compare sensitivity and suitability for monitoring, the selected methodologies should be performed on samples taken from the same fish, either egg, larvae or adult fish. Eventually, independent proposals, in particular on genotoxicity studies of fish, should be coordinated.

Since the focus of this programme is water column monitoring, priority will be given to the pelagic species herring and mackerel. However, in order to reveal site-specific contamination, including a more stationary species like plaice would be recommended. Samples from 15–25 individuals from each species at each site are desired. Liver is the required tissue for analysis by all methods, with blood, additionally, for the COMET assay. Processing of samples for the COMET assay should normally be conducted on fresh material, which means on-board. For the other methods, approximately one gram of liver frozen on liquid nitrogen is sufficient.

9.9 Biological Effects of Contaminants in Pelagic Marine Ecosystems using Invertebrates as Bioindicator Species (B.E. Grøsvik, F. Regoli, M. Depledge, J.F. Børseth)

Please refer to Section 10.6, below.

9.10 Biodegradation of Oil in Sea Water. Genetic Diversity and Catabolic Genes (O.G.Brakstad)

Microbial degradation is recognized as a major process contributing to the fate and effects of oil after discharge to sea water, either through accidental discharges or after regular releases (e.g., as produced water). After release to the water column, the oil will disperse and oil compounds distribute between soluble and oil-droplet phases. Biodegradation of oil components in the two phases will differ significantly, both due to the differences in bioavailability and in component characteristics. In addition, the microbial activities will influence the oil droplet characteristics by microbial production of surfactants or by changing the droplet surface characteristics by cellular adherence.

Several studies have shown that specific microbial traits are associated with defined pollution conditions, e.g., the prevalence of catabolic genes for BTEX or PAH degradation in oil-contaminated soils and sediments. PCR-based studies have also demonstrated increasing microbial genetic diversity during active biodegradation conditions. Changes in microbial community structures or catabolic gene expression during biodegradation indicate microbial adaptation to a pollution situation, and will have subsequent influences on the degradation potentials.

In this project, seawater samples will be collected from the Statfjord field and the German Bight according to defined transects. Samples will be collected through different cruises either as seawater samples for laboratory biodegradation studies, or by onboard filtering of sea water through filters (0.2 μm pore limit) for trapping seawater bacteria. Filtered bacteria will be lysed immediately. Correspondingly treated sea water from our laboratory (Trondheimsfjorden, 90 m depth) will be used as reference water.

After arrival to the laboratory, the microbial diversity will be analysed both in lysed samples and sea water by PCR amlification of extracted DNA. PCR primers defining eubacterial and archaeal rDNA, and selected BTEX and PAH oxygenase genes, will be included. PCR products will be detected by standard agarose gel electrophoresis and Southern blot/dot blot methods. The eubacterial and archaeal PCR products will be further processed by denaturing gradient gel electrophoresis (DGGE) for studies of community structures. Dominating genotypes may be excised from the DGGE gels, re-amplified and sequenced for the species determination.

Seawater samples will be subjected to biodegradation experiments with a crude Statfjord oil immobilized on hydrophobic Teflon fabrics. Biodegradation will be performed during a period of 2 months in selected seawater samples, and at temperatures corresponding to the original seawater temperatures. Biodegradation will be measured by respiratory analysis (biological oxygen demand) and by measurements of oil component depletion by GC-FID and GCMS analysis. Eubacterial and archaeal community changes will be analysed by DGGE of PCR-products. Changes in catabolic genes will be analysed by PCR, but reverse transcriptase (RT) PCR will also be conducted on some samples to investigate the expression of these genes.

The expected outcome of this project will be expanded information concerning the microbial diversity and hydrocarbon catabolic genes both in sea water with considerable organic input, and during marine oil biodegradation. The project results will also be used for verification of data from previous experiments conducted in our laboratories.

9.11 Histopathological Studies of Gonads from Pelagic Fish Caught in the German Bight and Statfjord Area (L. Norrgren)

A variety of fish species has been shown to be affected by different reproduction disorders including inadequate gonad maturation, low fecundity, and early life stage mortality. Habitat destruction, eutrophication, toxic algae, halogenated hydrocarbons and heavy metal contamination are among the most well-known threats to fish reproduction and recruitment. Furthermore, other potential environmental threats like leaking canisters of war gasses dumped after the First World War, recently introduced industrial chemicals, e.g., brominated flame retardants, plasticizers, antifouling

agents, must be considered as possible factors which may affect fish reproduction. Histopathological studies of fish gonads are the most important methodology to describe whether gonadogenesis is normal or not. Numerous studies based on a variety of fish species show that the maturation and development of germ cells is an important biomarker that reflects delayed and absent reproduction. The objective of the present study is to investigate gonad maturation in pelagic fish caught in the German Bight and in the Statfjord Area. The description will be based on a light microscopic morphometric quantification of different maturation stages.

9.12 EROD and PAH-metabolites in Bile (A. McIntosh)

In complement to other methods, FRS Marine Laboratory will investigate the relationship between polycyclic aromatic hydrocarbon (PAH) concentrations and P4501A monooxygenase activity in liver tissue from juvenile herring. This activity will be measured by the kinetic fluorescent assay as 7-ethoxyresorufin *O*-deethylase (EROD). PAH metabolites will be estimated from bile samples taken from the same fish using an HPLC method. In addition to the investigations on juvenile herring, an adult pelagic fish such as herring, or another species, will also be investigated. Fifteen replicate samples will be taken from each of the 4 stations identified at the Statfjord and the German Bight transects. All samples will be snap frozen in liquid nitrogen and stored at – 70 ºC aboard the research vessel for subsequent analyses at the Aberdeen Marine Laboratory.

9.13 Virus Isolation from Fish (P. Dixon, D. Stone, K. Way)

Until recently, viral haemorrhagic septicaemia (VHS) was considered to be a disease that primarily affected cultivated rainbow trout in Europe, and severe losses occur in both freshwater and marine cage culture. However, the causative virus (VHSV) has been isolated from an increasing number of marine or anadromous fish species, and the isolations from marine fish in Europe have all been from the North Sea, or waters linked to the North Sea such as the Baltic Sea. Rather than the virus being transferred from the freshwater environment to the marine environment, there is evidence that the reverse may have occurred and that there is a reservoir for VHSV in marine fish; the herring, *Clupea harengus*, is one species that may be a major reservoir. The reason for the increase in the number of isolations of VHSV from marine fish is unknown, but it cannot be explained entirely by an increase in surveillance for the virus. VHSV has also been isolated from marine fish from the Pacific Coast of North America, and experimental exposure of Pacific herring, *C. harengus pallasi*, to PAHs caused expression of VHSV. It has also been suggested that immunosuppression of fish by pollutants may reactivate a sub-clinical infection or make them more susceptible to the virus. We propose to take samples of liver, kidney and spleen from herring (50 juveniles/adults from each station), and test both for infectious virus by inoculation of an extract onto established fish cell cultures, and for the presence of viral RNA by the polymerase chain reaction (PCR). Liver, kidney and spleen will be dissected from fish, using clean (disinfected/sterilized) instruments for each fish. Approximately 1g total tissue made up of equal proportions of each organ will be put into virus transport medium which will be frozen at –20 °C. Up to 200 mg (total) of approximately equal portions of the same organs will be put into "RNA later" to prevent degradation of viral RNA prior to PCR. All analyses will be conducted at the laboratory.

9.14 Pathology and Molecular Biomarkers in Marine Fish Larvae (B. Lyons, M. Kirby, S. Feist)

This study was presented by Dr P. Dixon. The majority of studies evaluating the causal relationships between contaminant exposure and observable biological effects in aquatic organisms have so far focused on utilizing multiple biomarker approaches (analytical chemistry, cellular biomarkers and histopathology) in adult life stages of aquatic organisms. However, it is increasingly becoming acknowledged that larval stages may represent critical life stages that are sensitive to the effects of pollutants. We propose to employ a suite of pathological biomarkers along with the determination of EROD activity by standard methods to determine whether pollutant exposure is having a biologically detectable effect on exposed larvae. The pathological study will include assessment of deformities, histopathological changes and assessment of pathogen involvement. Targeted scanning electron microscopy will be used for surface morphological assessment of deformed fish. Our target species is the dab, *Limanda limanda*, although the plaice, *Pleuronectes platessa*, or whiting, *Merlangius merlangus*, are acceptable alternatives. For histopathology, a minimum of 50 larvae per site must be immediately placed into fixative (10 % neutral buffered formalin); sorting of larvae into species can take place later. For EROD analysis, the larvae must be sorted as quickly as possible, then 100–200 mg need to be stored in liquid nitrogen.

9.15 CYP1A and MT in Herring (J. Wedderburn, J. W. Langston, I.R.B. McFadzen, L. Peters, P. Pipe)

The PML work package was presented by Dr J. Wedderburn and consists of six techniques across a range of species, metallothionein (MT), CYP1A, genotoxic assays, immunological assays, histopathology, and early life stage assays. MT will be measured in collected wild fish, *Clupea harengus*. MT is an important intracellular component involved in

buffering metal ions. Techniques will also focus on caged mussels and fish. CYPIA, a biochemical marker of organic pollution, and MT are being used on caged cod, *Gadus morhua*.

9.16 References on Field-Collected Organisms

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10 METHODS USING CAGED ORGANISMS

The session on methods using caged organisms was chaired by Dr K. Hylland.

10.1 BPH, CYP1A-Like Protein and AChE in Mussels (T. Burgeot , G. Bocquené, J. Faucet, L.D. Peters)

Two biomarkers of exposure will be studied in the blue mussel, *Mytilus edulis,* in two areas, the German Bight and Statfjord. The main objective is to evaluate the environmental stress of the pelagic ecosystem. AChE is the first biomarker of neurotoxic effects recommended for biomonitoring in invertebrates by ICES. Levels of cytochrome P4501A-like protein and BPH activity constitute the second biomarker of detoxification identified as a promising biomarker for biomonitoring in mussels by ICES.

Evaluation of the environmental stress is complex and must be based on a structured approach. A classical tryptich based on the development of a suitable analytical technique, an appropriate sampling design, and an integrated method for biomarker interpretation constitute the main basis of this approach.

We will apply the standardized protocol for AChE analysis described by Bocquené and Galagni (1998) and two analytical methods published by, respectively, Akcha *et al*., (2000) for BPH activity and Peters *et al*. (1999) for CYP1A-like protein.

Sampling design: From each station in the two areas studied (German Bight and Statfjord), 30 pairs of gills and 30 digestive glands will be collected, placed in eppendorf tubes in 6 replicate pools containing 5 tissues each, snap frozen and stored in liquid nitrogen until their transport in dry ice to the IFREMER laboratory. Prior to dissection, one mussel from each pool will be bled from the adductor muscle sinus, and the samples smeared on glass slides for hemocyte microsatellite analysis. In addition, 6 individual mussel digestive glands will be collected to determine levels of CYP1A-like mRNA. All samples will be maintained in dry ice during the transport and will be sent to the IFREMER laboratory. Appropriate samples will be forwarded on to PML from the IFREMER laboratory.

Spatial and temporal design: Each biomarker will be studied in individuals and in pools (6 pools of 5 individuals). Biomarkers analysis will be done on "point zero" before transplantation. The other series of biomarker analyses will be done on the different stations of the two areas.

Replicates: It is important to have several replicates for statistical analysis. One or two replicates could be done the same day (morning and afternoon) and the same week (two samples on two different days) on one selected station in the two different areas. Inter-individual variations will be analysed in each area.

Integrated methods should be developed in order to improve biomarker interpretation and more generally the environmental stress. We suggest using the simple graphic tools developed by Belieaff and Burgeot (1999) in order to obtain a biomarker index on each station and on each area. The star plot method can be applied with other parameters analysed by other participants (PAH components or indicators of physiology; condition index).

10.2 CEA and Antioxidant Enzymes in Blue Mussel (P. Roose/K. Cooreman)

Please refer to Section 9.1, above.

10.3 AChE and GST in Atlantic Cod (D. Danischewski)

Two biomarkers will be measured in caged cod, *Gadus morhua*, from 4 locations in the Statfjord area and 4 locations in the German Bight. A number of 25 animals will be analysed from each location.

Acetylcholinesterase (AChE) (project DE-2a) is a well-established biomarker for the exposure to neurotoxic contaminants. The enzyme is responsible for the cleavage of the neurotransmitter acetylcholine at neuronal and neuromuscular junctions, thereby regenerating the acetylcholine receptor at the postsynaptical membrane. This process is vital to the transmission of nerve impulses, because it restores the excitability of the postsynaptical membrane. Inhibition of AChE leads to an accumulation of acetylcholine at the synapse, causing overstimulation of the receptor. A number of sublethal effects of AChE inhibition have been observed, ranging from impairment of locomotor activity to behavioural changes and neuronal malformations of developing embryos. These effects occur at concentrations considerably lower than those required to cause mortality.

AChE has traditionally been regarded as a highly specific biomarker for organophosphorus and carbamate pesticides. In recent years, however, a growing body of evidence has indicated that substances other than pesticides may play a significant role in the AChE inhibitions observed in various marine organisms. In particular, oil and combustion-type hydrocarbons have been suggested as possible inhibitors.

The enzyme activity will be measured in muscle tissue and according to the ICES TIMES protocol (Bocquené and Galgani, 1998).

Glutathione *S*-transferase (GST) (project DE-2b) is an enzyme of the phase II (conjugative) xenobiotic metabolism. It conjugates electrophilic substances with glutathione, rendering them more water-soluble and/or making them susceptible to further transformation and excretion via the mercapturic acid pathway.

As the enzyme is inducible by electrophilic compounds or phase I metabolites, it can serve as a biomarker for these substances. GST induction rates observed in contaminated aquatic environments are often lower than those measured for cytochrome P450 activities. In some cases, however, GST induction has been observed, while P450 activities remained unaffected. This has been considered as an indication that, unlike in mammals, GST induction may be regulated by a different receptor.

In most organisms multiple forms of GST are present, each of them possessing slightly different substrate specificities and induction characteristics. In marine organisms, however, CDNB (1-chloro-2,4-dinitrobenzene) has proven to be a universal substrate for all GST isoforms. The enzyme activity will therefore be measured spectrophotometrically, using

CDNB as a substrate. The assay is based on the "classic" method of Habig *et al*. (1974), modified for use in a microplate reader. As GST activity is predominantly located in the cytosol, the cytosolic fraction of liver tissue will be used for this investigation.

10.4 Histopathological and Biochemical Markers in Fish and Mussels (I. Cancio; M. Soto; M. P. Cajaraville; I. Marigómez)

Please refer to Section 9.4, above.

10.5 Biomarkers in Caged Cod (Ole Aspholm)

The objective is to study physiological effects in caged Atlantic cod, *Gadus morhua*, exposed to produced water or coastal pollution by the analysis of biomarkers. Aquacultured cod at approximately 700 g will be held in cages at four stations in the German Bight and four stations at the Statfjord field. The caging will be for approximately 6 weeks. The project will analyse vitellogenin concentrations and *zona radiata* protein concentrations in plasma from 25 fish. For this, 2 vials are needed of at least 30 µl plasma from each fish. Hepatic CYP1A protein concentrations and mRNA expression will be analysed and 2 samples of approximately 1 g liver are needed from each fish. In addition to the caged fish, the same parameters will be analysed in 25 samples collected from cod from the same aquaculture batch that not has been caged. Both liver and plasma samples must be stored in liquid nitrogen and be distributed on dry ice.

10.6 Biological Effects of Contaminants in Pelagic Marine Ecosystems using Invertebrates as Bioindicator Species (B.E. Grøsvik, F. Regoli, M. Depledge, J.F. Børseth)

Blue mussels, *Mytilus edulis,* are used extensively in biomonitoring programmes. They are sessile, filter large amounts of water, and are easy to use in caging studies, and in many respects very interesting as a model species in pelagic monitoring surveys. Elevated levels of PAHs in mussels deployed in cages are reported from the North Sea Tampen region (OLF, 1999). Research on some biomarkers has been done in relation to these levels (Børseth *et al*., in press), but a wider range of biomarkers is interesting for further development of the potential of mussels deployed in cages for biomonitoring. *Calanus* and krill (*Euphausiacea* sp.) are important organisms in the food chain of the North Sea and thus highly interesting for studies of environmental impact. This proposal emphasizes studies with these species on three aspects of biological effects: cellular defence systems/oxidative stress, genetic damage and endocrine disruption.

Cellular defence systems/oxidative stress: Haemocytes are important for immunological function and effects on these cells could be measured by a decrease in lysosomal membrane stability (neutral red retention time (NRRT) and plate reader) or decreased immunocompetence measured by the sheep red blood cell assay. A decrease in NRRT in mussels has been demonstrated to be correlated to contamination in several field studies (e.g., Lowe *et al*., 1995; Grøsvik *et al*., 1999).

The TOSC-assay (Total Oxyradical Scavenging Capacity) is a method for measuring the protective antioxidant potential of biological tissues and has been shown to be repressed after exposure to environmental contaminants (Regoli, 2000). We suggest measurements on scavenging capacity toward peroxyl radicals and hydroxyl radicals, in addition to measurements of glutathione reductase, glutathione-*S*-transferases and catalase activities. Effects on levels of glutathione-*S*-transferase and catalase have been demonstrated in blue mussels caged in the North Sea (Børseth *et al*., in press).

Genetic damage: Whether chemicals released from offshore activities in the North Sea may lead to genetic damage in organisms may be assessed from mussels deployed in cages, using either gill or digestive gland tissue. The most sensitive method for assessing such effects is the ^{32}P-postlabelling technique, but we also propose to sample material to study whether other, less time-consuming and expensive methods may be used for this material. There are several techniques that may be alternatives, like the COMET assay or increased occurrence of micronuclei. Recently, a new method using DNA unwinding read by a fluorescent plate reader has been published, measuring single-strand breaks and alkaline labile sites. The method requires only 30 ng DNA per single well and can be done on frozen material (Batel *et al*., 1999).

Endocrine disruption: Laboratory experiments with nonylphenol in flow-through water have demonstrated that several invertebrates, including blue mussels, express significantly higher levels of vitellogenin (Vtg)- and eggshell (Zrp)-cross reacting proteins in haemolymph. It could therefore be interesting to study whether mussels deployed in cages and *Calanus*/krill in the North Sea demonstrate induced expression of these proteins.

Material needed: *Caged mussels:* We would be interested in samples collected from all stations after 3–4 weeks of exposure, preferably mussels originating from Trøndelag (mid-Norway) as with previous cage monitoring studies in the North Sea (OLF, 1999) and sampled no later than the end of May. The arguments for this are to use individuals from a homogeneous aquaculture population and to be able to collect mussels before spawning, as this has been shown for several biomarkers to influence the results. Lysosomal membrane stability, immunocompetence, and COMET assay have to be performed on live cells. The mussels should be transported alive (i.e., dry and cold) to the laboratory or, preferably, trained personnel should be on-board to sample and to perform the analyses.

Some of the methods mentioned above could also be used for *Calanus* or krill. Samples of *Calanus* need to be pooled and will not allow for analyses of tissues. For measurements of oxidative stress, 0.5–1 g of whole zooplankton tissue is needed per sample. TOSC will be used as the test biomarker. If successful, it can be extended with other antioxidant defence parameters on the same material. Measurements of DNA damage could also be assessed on these organisms. With regard to Vtg- and Zrp-cross reacting proteins in krill, only a small amount of haemolymph per animal would be needed for each measurement.

10.7 DNA Adducts Analysed in Fish Early Life Stages by the ^{32}P-Postlabelling Technique to Screen for Genotoxic Exposure to Contaminants (L. Balk)

One of the most significant criteria for a healthy, well-functioning ecosystem is that the different species living in it have the capacity to reproduce in a non-disturbed manner. Disturbances of the reproductive outcome of one or several species is therefore probably the most serious threat to an ecosystem.

The early life stages of fish are potentially exposed to organic pollutants in the environment in at least three different ways. The earliest route for exposure is via the female (maternal exposure). This is followed by uptake directly from the water phase over the skin and gill membranes. The third route of exposure is via food particles. The relative importance of the different routes is dependent on, among other factors, the chemical structure of the pollutant.

Compounds most likely of great importance to be biologically active in these matters are organic hydrophobic structures. These substances are not recognised in the first case as having highly acute toxic effects. Instead they are known to express their effects at a time long after exposure or during a prolonged exposure period. Stable organic pollutants also show the ability to bioaccumulate both by bioconcentration directly from the water phase and by biomagnification via the food web, whereby an elevated risk for maternal exposure occurs. Further, the parent compounds themselves are not always toxic, but instead the ultimate toxic substances are often formed during the metabolism of these compounds. In connection with this biotransformation process, the reactive intermediates formed may attack different biomolecules within the cell. The nuclear genetic material is a special critical site for these attacks, since it give rise to genotoxicity. This damage to the genetic material, in the form of DNA adducts, can in itself be a very significant observation with respect to disorders described in pelagic embryos and larvae in polluted areas (Grauman and Sukhorukova, 1982; Westerhagen *et al*., 1988; Åkerman *et al*., 1996; Åkerman and Balk, 1998).

A recently performed study has found very high levels of DNA adducts in cod eggs and larvae in the polluted Baltic Sea compared with the Lofoten area in Norway, as the control site (Ericson *et al*., 1996). The elevated levels of DNA adducts were in agreement with a number of developing disorders among the embryos and larvae (Åkerman *et al*., 1996; Åkerman and Balk, 1998). This strongly indicates that the measurement of DNA adducts could be a valuable tool in evaluating exposure to genotoxic compounds in pelagic ecosystems.

For the investigations suggested for this pelagic project, we will use the same technique as above for the DNA adduct measurements, which is the ^{32}P-postlabelling with the nuclease P1 version (Randerath *et al*., 1982; Reddy and Randerath, 1986) as well as recent adjustments and improvements in our laboratory, as presented in Ericson *et al*. (1999a, 1999b), and Aas *et al*. (2000). Depending on sample success, we are most interested in newly hatched larvae from a species that could be collected at all sampling points. According to "Table 1. Organisms expected to.....", mackerel might fulfil this criterion. However, if other species like, e.g., cod are available in a material that gives the opportunity for good scientific comparison between sites, it could be an additional option to use. Artificially stripped and fertilised eggs, examined on the boat deck where the parent animal is subject to additional investigation, are, of course, a very interesting material to investigate in these matters. If the offspring are additionally investigated for developmental disorders and other biomarkers, it might be a perfect material to use.

10.8 The Three-spined Stickleback: A Universal Indicator Species For Endocrine Disruption (I. Mayer, I. Katsiadaki, S. Scott)

An increasing number of studies have linked the appearance of reproductive disorders in wildlife species to exposure to environmental contaminants that are capable of eliciting responses typically induced by sex steroids. These compounds have been termed endocrine-disrupting chemicals (EDCs). The three-spined stickleback, *Gasterosteus aculeatus,* is presently being promoted as an indicator species for endocrine disruption in the aquatic environment. It is an ideal model species for the following reasons:

- It is an ubiquitous species found in all aquatic habitats from full sea water to fresh water; it has a circumpolar distribution in the Northern Hemisphere and can readily be sampled in the field in large numbers; this latter characteristic is especially important for *in situ* monitoring.
- It is easy to maintain in small laboratory aquaria, has a relatively short life cycle, and is readily stimulated into reproductive condition by manipulation of photothermal regimes, making it a particularly good species for "partial life-history" tests.
- The female has a relatively low fecundity, the eggs being laid in a nest built by the male; in this respect, the suitability of the stickleback is enhanced by the fact that very high egg/fry survival rates can routinely be obtained, which is an important factor for comparisons between test and control groups.
- The male shows pronounced androgen-dependent secondary sexual characters, notably kidney hypertrophy and breeding colours; the production of the glue protein "spiggin" is probably the most clearly defined male secondary sexual character of any fish.
- An ELISA for spiggin has been developed and validated; we are presently doing the same for vitellogenin with the intention of developing a test which will allow the simultaneous assessment of androgens and oestrogens. This has potential for considerably reducing testing costs for the European chemical industry.

In meeting the above criteria, the stickleback not only represents an ideal sentinel species for laboratory testing, but also for field studies. Indeed, preliminary studies involving both laboratory animals exposed to suspected effluent and cage deployments near sites of high contamination have yielded evidence of androgenic disruption. BECPELAG will offer a unique opportunity to evaluate the stickleback as a valid biomarker of androgenic contamination. Evidence of androgenic EDCs will be evaluated in caged female sticklebacks using the spiggin ELISA, which has proved to be a sensitive and robust assay for androgenic compounds.

At each of the proposed localities, 50 adult female sticklebacks will be placed in a cage. After about six weeks' exposure, the fish will be retrieved and immediately frozen in liquid nitrogen. Following transportation to the CEFAS Weymouth Laboratory, individual fish will be weighed and the kidney dissected out and weighed. Half the kidney will be used for the spiggin assay, while the other half will be processed for histology for the determination of kidney epithelium height (KEH). Initial controls (taken at the time of cage deployment) will be sampled in the same way.

10.9 Biomarkers, Genotoxicity, Condition and Spawning Success in Mussels (J. Wedderburn, J. W. Langston, I.R.B. McFadzen, L. Peters, P. Pipe)

Mussels, *Mytilus edulis*, will be used for six techniques, the intention being to link a number of responses that operate at different levels of biological organisation. An assessment will be made of genotoxic damage to mussel blood cells as well as an examination of the immunological response. Mussel digestive gland will also be examined for CYP1A induction in conjunction with further genotoxic assessment. Mussel tissues will be prepared for histopathological examination, which will allow their health and condition to be determined. Stereology will also be used to quantitatively assess reproductive status. Caged mussels will be induced to spawn and resultant offspring will be examined for their ability to develop successfully. An assessment will be made of larval chromosomal aberrations and growth to determine the extent of any genetic damage that has occurred. The use of these techniques, as part of the same package, will potentially allow a greater understanding of the way in which different biological responses interrelate. By not only combining the data obtained from these techniques with the available chemistry, but also with biological data obtained by the other participants, valuable information should be obtained as to the applicability of these methods for marine risk assessment.

10.10 Caged Mussel and Stickleback Assays (J. Thain)

In the early stages of planning the workshop, CEFAS agreed to assist with the deployment of *in situ* caged blue mussels, *Mytilus edulis,* and sticklebacks, *Gasterosteus aculeatus*. CEFAS has experience in deploying these two species over periods of up to three months. The methods of caging were presented.

At each study site, two cages of sticklebacks will be deployed and they will be placed inside the cod cage. This will provide good protection for the small cages and maximise the provision of food sources as provided for the cod. One hundred fish will be exposed at each site, fifty males and fifty females. The fish will be greater than 50 mm in length and will be provided fully acclimated to sea water. Ian Mayer (University of Stockholm) has agreed to provide the sticklebacks from a clean source and transport the fish to Bergen prior to the cruise. The cages are being provided by CEFAS. The main objective in using sticklebacks in this study was to use the spiggin assay (see Section 10.8, above) and to measure EROD induction. Material required for both of these assays will be taken on-board the ship at the time of recovery of the cages and stored in liquid nitrogen for subsequent analysis at the respective laboratory. In addition, there is a requirement by other participants for stickleback material for other assays and these were presented at the workshop. It is anticipated that there will be a sufficient number of fish deployed to meet these needs.

At each study site, cages of mussels will be deployed attached to the outside of the cod cage. Each mussel cage will contain fifty mussels. The mussels will be obtained from two sources, the west coast of Norway and southern Ireland. This is necessary because some of the assays require mussels post-spawned and one assay needs to use mussels close to spawning. CEFAS agreed to carry out two techniques, Mt analysis and Scope For Growth (SFG). Data on SFG studies in the UK were presented which included two offshore studies. In both these cases, the measurement of SFG was conducted ashore and it was emphasised that this was the only practical and realistic approach to ensure success in conducting this work. Other workshop participants also emphasised the need to conduct some of the mussel assays ashore, e.g., lysosomal latency. It was agreed that for some of the techniques, the mussels will be transported ashore to Bergen (Statfjord) and Helgoland or a laboratory in the Netherlands (German Bight). The number of mussels to be deployed is still to be determined since there is a large requirement for mussel tissue. An important aspect of the mussel work will be the potential to integrate physiological measurements with biomarker measurements and concentrations of contaminants in mussel tissue.

10.11 References for Caging Studies

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11 BIOASSAY METHODS

The session on bioassay methods was chaired by Dr D. Vethaak.

11.1 Neurotoxicity, Genotoxicity, Cytotoxicity and Embryotoxicity (D. Danischewski, U. Kammann, M. Vobach)

BFA-Fi will perform four different bioassays with extracts from SPMDs (8 samples) and produced water (one sample). These bioassays will cover different modes of toxic action as well as different levels of biological organisation, ranging from the biochemical to the cellular and organism levels:

Neurotoxicity—Acetylcholinesterase (AChE) inhibition test (*in vitro* – purified enzyme);

Genotoxicity—COMET assay (*in vitro* – fish cell line);

Cytotoxicity—Apoptosis (*in vitro* – fish cell line);

Embryotoxicity—Early-life stage test with fertilised eggs of *Danio rerio* (mortality, malformations);

The AChE inhibition test (project DE-1) is an *in vitro* assay for the detection of potentially neurotoxic contaminants, using a purified freeze-dried enzyme preparation. AChE is an enzyme involved in the transmission of nerve impulses at neural and neuromuscular junctions. Inhibition of the enzyme can lead to lethal and sublethal effects including behavioural changes and impairment of locomotor behaviour. The assay is based on a German standard method (DIN 38415-1). The enzyme is incubated with the sample, allowing inhibitors to react with the enzyme. A specific AChE inhibitor (ethyl paraoxon, an organophosphorus pesticide) is used as a positive control. Enzyme activity is measured spectrophotometrically. Comparison with the positive control allows the inhibition caused by contaminants in the sample to be expressed as paraoxon equivalent values.

The COMET assay and the measurement of apoptotis (project DE-1b) will be done with the EPC cell line, an adherent epithelial cell line from carp, derived from a virus-induced epithelioma.

The COMET assay is a single cell gel electrophoresis assay for the detection of DNA strand breaks. After incubation with the sample, cells are immobilised in agarose gel, gently lysed, and subjected to electrophoresis. If DNA fragmentation has occurred due to the action of xenobiotics, these fragments will migrate out of the area of the nucleus. After staining with a fluorescent dye, DNA fragments that have migrated in the gel appear as comet-like structures, their tail size corresponding to the degree of DNA fragmentation. Cells are assigned to one of four classes of DNA fragmentation under a fluorescence microscope. Observation of 100 cells (two replicates) and recording of class frequencies yields a "comet score" as a measure of DNA damage.

Apoptosis, also termed "programmed cell death", is an active process allowing an organism to dispose of cells that have suffered irreparable damage or have otherwise become obsolete (e.g., in the course of embryo development). Apoptosis can be triggered by a number of pathways, including the action of chemicals disrupting membrane integrity. A recent research project has given evidence that environmental contaminants may induce apoptosis in dab, *Limanda limanda*. BFA-Fi therefore has started investigations on the ability of contaminants to induce apoptosis in an *in vitro* system using fish cell lines. This test system is still under development within the framework of an on-going research project. Apoptosis will be measured using two different techniques. One characteristic of apoptosis is the occurrence of histone-complexed DNA fragments (multiples of 180 bp) in the cytoplasma of otherwise intact cells. These fragments can be detected by a commercially available one-step colorimetric sandwich ELISA (Cell Death Detection ELISA, Boehringer Mannheim). The second technique used to measure apoptosis is the caspase-3 activity assay. Caspases (cystein-dependent, aspartate specific proteases) are a family of proteases acting as the executioners of apoptosis. The different caspases work together in a cascade reaction, with the "upstream" (initiator) caspases activating the "downstream" (effector) enzymes. Caspase-3 is one of the key effector caspases, responsible for a number of structural and biochemical changes typical of cells undergoing apoptosis. Caspase-3 activity assay kits are available from several suppliers.

The zebra fish early life stage test (project DE-1c) is a test for embryotoxicity using freshly fertilised eggs of the zebra fish, *Danio rerio*. The test has proven to be sensitive to a wide range of compounds with differing modes of toxic action. After successful fertilisation, eggs are collected and exposed to samples in 24-well microplates. A total number of 48 eggs is used for every sample. The development of the embryo is monitored over 48 hours. After 24 hours and 48 hours, the following toxicological endpoints are observed: coagulation of the embryo, formation of somites, spontaneous movements, development of the eye, extension of the tail, development of melanocytes, and rate of heartbeat. The test is well-established in ecotoxicological research and is currently being standardised nationally as well as internationally.

11.2 Tests with the Copepod *Acartia tonsa (*K. O. Kusk)

Acartia tonsa is a small planktonic copepod about 1 mm long at the reproductive stage. It is abundant in coastal areas of the Atlantic Ocean. The genus *Acartia* often dominates the zooplankton, thus making it an important ecological link between the primary producers (algae) and fish larvae and bigger zooplankton species. During its development, it goes through 12 stages from egg to adult. There are six nauplia stages, which have a body shape clearly distinct from that of the six later copepodite stages, the last of which is the reproductive stage.

It is planned to perform 48 hr lethality tests and 5–6 day larval development tests on extracts. The last test is finished when about 50 % of the larvae have reached the copepodite stages. The fraction of larvae that has reached the copepodite stages in exposed samples is also identified and compared to the same fraction of the control animals. On this basis, an inhibition percentage is calculated and used to find EC_{10} and EC_{50} (EC = Effect Concentrations).

11.3 Microinjection of SPMD Extracts in Atlantic Salmon Embryos (L. Norrgren)

Fish are potentially exposed to organic pollutants in the environment in at least three different ways. The earliest route for exposure is via the female through maternal transfer. This is followed by uptake directly from the water phase over skin and gills. The third route of exposure is via food particles. Toxicity studies of lipophilic substances in fish are complicated due to the low water solubility of these compounds. Methods currently in use include injection of juveniles/adults, feeding with contaminated food or exposure to contaminated water or sediments. Exposure of early life stages of fish using various microinjection techniques comprise alternative methods which are useful when studying the toxicity of lipophilic extracts from environmental samples, i.e., sediment, water, and tissue extracts. Microinjection has been used in studies of carcinogenic properties of chemicals and sediment extracts. Another approach to assess the integrated toxicity of a sediment extract is to measure the activity of P450-dependent enzymes in whole-body homogenates or in liver tissue. EROD activity has been shown to be induced after microinjection of several technical mixtures of PCBs and polychlorinated naphthalenes (PCNs) as well as specific congeners, i.e., PCB126. The strategy to bring SPMD samples to the laboratory and extract lipophilic compounds for testing is one significant method to estimate the biological impact of extracts and thereby reflect the environmental situation. Major advantages include that the amount of extract needed is small, and the dose administered is easily controlled so that an environmentally realistic concentration can be achieved. Disruption of normal biological functions can be observed at different levels of biological organisation including subcellular processes (enzyme activities) and on the individual level (pathology, mortality). The major objective of the proposed study is to inject different concentrations of SPMD extracts originating from the Statfjord area and the German Bight. The study will be performed on early life stages of Atlantic salmon.

11.4 Fish Bile as a Biomarker of Water Quality (H. Ek, G. Dave)

A wide variety of xenobiotics are concentrated in the bile of the fish. Fish bile can be used as a biomarker of water quality in two ways. The first, and most widely used, is chemical analysis. The chemical composition of the bile is expected to reflect the concentrations that the fish has been exposed to recently. Chemical analysis of fish bile has, therefore, been recommended as a tool in water quality monitoring. This method has been used in several studies, and substances that have been detected in fish bile are, for example, PAH metabolites and phenols. There are, however, disadvantages of only relying on chemical analysis. Apart from being expensive and time consuming, chemical analysis cannot possibly detect all substances that the bile contains. Some toxicants are probably still unknown and are, therefore, not detected and identified. Furthermore, since the fish is exposed to a mixture of substances, it is impossible to know if synergistic effects arise.

We, therefore, suggest a complementary method in addition to chemical analysis. By measuring the toxicity of fish bile to the test organism *Daphnia magna*, we estimate the toxicity of all chemicals, known as well as unknown. A study with pentachlorophenol (PCP) showed that the toxicity of bile, as determined with a *Daphnia magna* bioassay, could be used to measure sublethal exposure in fish (Andréasson and Dave, 1994). The major limitation of fish bile as a biomarker of water quality is that not all chemicals are stored in the bile to the same extent. The concentration in the bile depends not only upon the concentration in the surrounding medium but also on the capability of the fish to metabolise and excrete various substances. However, a large number of chemicals of environmental concern are concentrated in the bile of fish, and toxicity tests of bile can, therefore, be useful as a general indicator of toxic exposure.

11.5 Water Column Bioassays (K. Thomas)

Many biological assays are insensitive to the levels of contamination found in seawater samples collected offshore. One way to overcome this problem is to test concentrated seawater extracts. It is proposed that within this study solid phase extraction (SPE) and semi-permeable membrane devices (SPMD) be used as a means of pre-concentrating samples prior to assay. SPE will provide extracts that contain organic compounds at 10^3 times their concentrations in sea water.

The SPE and SPMD extracts will then be tested using the oyster, *Crassostrea gigas,* embryo bioassay, a marine copepod, *Tisbe battagliai*, marine algae, *Skeletonema costatum*, and fish larvae. These assays will provide LC50/EC50 data expressed as a concentration factor. This in turn can be used to rank the effects observed at each station along a contaminant gradient. Using the yeast oestrogen screen (YES) and the yeast androgen screen (YAS), the *in vitro* oestrogenic and androgenic activity of each sample will be determined. These data will be compared with the results of other bioassays (H. Klamer, RIKZ, NL) and where significant activity is determined, a toxicity identification evaluation (TIE) study will be performed to identify the cause of the effect.

11.6 Potential for UV-light to Increase the Toxicity and/or Genotoxicity of Seawater Samples to Invertebrate Bioassay Organisms (B. Lyons, I. McFadzen)

This study was presented by Dr P. Dixon. Traditionally, the study of PAH toxicology in the aquatic environment has focused on the ability of organisms to metabolise PAHs *in vivo*, via components of the mixed function oxygenase system (MFO), to derivatives with increased toxicity over the parent PAH compound. In general, PAHs do not show extremely high acute toxicity to aquatic life, with the majority of intermediate to high molecular weight PAHs not exhibiting acute toxicity within their water solubility limits. Lower molecular weight PAHs may be acutely toxic to aquatic organisms, although the toxic concentrations (> 10 $\mu g\ l^{-1}$) tend to be several orders of magnitude above those found in North Sea waters. Therefore, studies concerning PAH contamination of the aquatic environment have primarily focused on chronic effects such as carcinogenicity. However, there is a growing body of evidence to suggest that certain PAHs may pose a greater hazard to aquatic organisms than previously thought, due to their potential to cause photo-induced toxicity (phototoxicity) when exposed to environmental levels of ultraviolet (UV) light. Such phototoxic effects can be up to 1000-fold greater than in the absence of UV light. We will undertake a series of studies to investigate the potential for various intensities of UV light to enhance or mitigate the toxicity of environmental samples (bulk water and sea surface microlayer (SSML) samples). In order to achieve this, we will employ a number of routine invertebrate toxicity tests (e.g., oyster embryo bioassay, *Tisbe* bioassay). Environmental samples (SSML and SPMD extracts) will be tested both under traditional testing protocols omitting UV light and under conditions simulating natural sunlight.

11.7 SPMD Water Extract Injection in Atlantic Salmon (O. Aspholm)

The aim of the study is to investigate if intraperitoneal injection of SPMD water extracts into Atlantic salmon, *Salmo salar,* with subsequent biomarker analyses is a suitable test system for contaminants in pelagic systems.

Smoltified juvenile Atlantic salmon, *Salmo salar,* will be injected intraperitoneally (i.p.) with extracts from SPMDs. Two SPMDs placed at cages deployed at four stations in the Statfjord field and four stations in the German Bight will be dialysed (e.g., by hexane). Co-extracted constituents will be separated with gel permeation chromatography (GPC). Following volume reduction by evaporation, the extract will be mixed with peanut oil and injected intraperitoneally into the salmon. Nine fish distributed in three aquaria will be exposed for each station. As a control, nine fish (in three aquaria) will be exposed to extracts from clean SPMDs. In addition, three different concentrations of water extracts sampled directly from the produced water will be i.p. injected into salmon. Nine fish distributed in three tanks will be used for each concentration. All fish will be individually coded. Before exposure, weight and length will be measured and blood plasma sampled. The exposure will last for 14 days, then the fish will be sacrificed and blood plasma, liver, bile and gonad will be sampled. In addition, biological parameters such as length, weight, and liver and gonad weight will be measured. Analysis endpoints will be plasma vitellogenin concentration, plasma *zona radiata* protein concentration, hepatic CYP1A protein concentration, and mRNA expression.

11.8 *In vitro* Screening of Pelagic Water and Biota Extracts (H. Klamer, K.C.H.M. Legierse, J.H.F. Bakker, D.H. Vethaak, P. den Besten and C. Tuk)

In an on-going research effort concerning the adverse effects of contaminants in the marine and estuarine environment, RIKZ and RIZA are involved in the development and validation of an instrument for integrated biological and chemical assessment of ecological risks of sediment, water and biota samples. The instrument consists of three building blocks: *in vivo* bioassays, *in vitro* screening assays and, at RIKZ, a TIE (Toxicity Identification and Evaluation) tool.

The *in vitro* screening assays concern either broad-spectrum or so-called "toxic mechanism based" assays. Examples are Microtox (broad-spectrum toxicity), Mutatox and *umu*-C and COMET (genotoxicity), DR-CALUX (dioxin-like or Ah-mediated toxicity), ER-CALUX (oestrogenic toxicity), and AR-CALUX (androgenic toxicity). These assays have in common that they require the use of solvent extracts. Research activities involve the analytical, chemical and ecological validation of these assays, the achievement of controlled chemical exposure, and standardisation and protocolisation. At present, the screening assays have already been partly validated, standardised and protocolised and/or have been

successfully applied in several field studies. However, until now, the assays have been predominantly applied to sediment extracts, while only limited experience has been gained with their application to aqueous and biota extracts.

The TIE tool under development focuses primarily on the evaluation of the toxicity as observed in *in vitro* screening assays. The tool consists of a package of fractionation techniques combined with a Quality Peak Identification and Database system (QPID). QPID aims at the identification of organic compounds responsible for effects in screening assays and features a database with GC-MS performing characteristics, and physico-chemical and toxicological data for an extended range of environmental chemicals. The performance of the system is currently being evaluated in a case study on Dutch harbour sediment toxicity.

The goals of the proposed project are:

1) to assess the applicability (e.g., sensitivity) of Microtox, Mutatox, *umu*-C, ER-CALUX, DR-CALUX, and COMET assays to extracts of aqueous samples from pelagic marine ecosystems;

2) to assess food chain effects of mixtures of dioxin-like, oestrogenic, and genotoxic chemicals by application of the screening assays DR-CALUX, ER-CALUX and *umu-C* to solvent extracts of phytoplankton, microzooplankton, copepods, fish larvae, fish embryos, fish ovaries and fish liver;

3) to identify chemical(s) responsible for responses in ER-CALUX and Microtox by applying QPID to a few hot samples, and to investigate to what extent the total molar concentration of organic chemicals in hot samples explains toxicity in Microtox.

11.9 *In vitro* Monitoring of Endocrine Disruptors and "Dioxin"-like Chemicals in Pelagic Marine Ecosystems (K.E. Tollefsen, A. Kelly, A. Goksøyr)

This study was presented by Dr K. Hylland. Produced water (PW) emissions have been pointed out as a substantial contributor to the discharge of pollutants from offshore oil production platforms. In 1998 the discharge of PW in the Norwegian sector of the North Sea amounted to 340 million m^3 and total emissions are expected to reach 120 million m^3 this year (E&P Forum, 1994; OLF, 1997). With the aim to adopt "zero effect" emissions by the year 2005 (OLF, 1999), the need to monitor pollutant impact on pelagic marine species is highly warranted. In this context, the use of biomarkers for detection of toxicological effects of chemicals has a large potential for use.

Previously, it has been shown that effluents from land-based oil production activity contain chemicals with oestrogenic (oestrogen mimics) and cytochrome P450 (CYP)-inducing properties using piscine biomarkers *in vivo* (Goksøyr, 1995; Arukwe, 1997 #1168; Knudsen, 1997 #1169). In this proposal, environmental monitoring of PW from oil production platforms in the Norwegian sector of the North Sea is proposed using biomarkers for oestrogen mimics and CYP-inducers. The aim of the project is to combine chemical extraction of PW with tests of the extracts in piscine *in vitro* bioassays for detection and future identification of chemicals with oestrogenic and CYP-inducing properties.

Samples of produced water and sea water are concentrated using solid phase extraction (SPE), Empore-disks or semi-permeable membrane devices (SPMD). The extracts are purified and toxic (active) extracts further separated in different fractions according to their water solubility for a Toxicity Identification and Evaluation testing set-up. Detection of oestrogen mimics (EMs) and CYP-inducing chemicals in the extracts/fractions is performed by a fish *in vitro* bioassay utilising antibody technology established at Biosense Laboratories. In short, liver cells from fish (rainbow trout or Atlantic salmon) are exposed to the extracts/fractions individually. The cell culture medium is analysed for the presence of the oestrogenic biomarker vitellogenin (Vtg) by quantitative ELISA, whereas liver microsomes are analysed for the presence of CYP1A induction by semi-quantitative ELISA. As a continuation of the project, active fractions may be analysed by GC-MS with the purpose to identify components responsible for the observed toxic activity (additional funding necessary).

11.10 Effects of SPMD Extracts on Stickleback (I. Mayer)

The caged experiments will be complemented by laboratory studies. The extent of these important laboratory studies will depend on the quantity of SPMD extracts available.

Spiggin induction in adult female sticklebacks will be investigated following i.p. injections of SPMD extracts. Androgenic properties of the extracts will again be determined using the spiggin assay and histological evaluation of the kidney.

A number of international committees on endocrine disruption have stressed that valid partial life-history tests should be given a high priority. Laboratory testing, based on partial life-cycle tests, will be carried out on adult sticklebacks to evaluate the effects of the collected extracts on fertility and reproductive success. Test animals will be exposed at a number of critical time points in the life cycle corresponding to key developmental phases (e.g., gonadal development). Following exposure to environmentally relevant concentrations of suspect chemicals, possible reproductive disturbances will be determined by assessing sperm and egg quality.

Sperm quality: since sperm quality is a major factor contributing to successful reproduction in fish, measurement of its motility will provide a sensitive and accurate bioindicator of reproductive disturbance. Computer-assisted sperm analysis (CASA) has already been successfully employed in monitoring the deleterious effects of heavy metals on sperm quality in fish (Kime *et al*., 1996). This CASA technique has recently been successfully applied to the stickleback in our laboratory.

Fecundity and egg quality: probably the most valid reproductive endpoint for the accurate assessment of endocrine disruption is that of fecundity and egg viability. Following exposure to the test compound, these reproductive endpoints can be accurately quantified, enabling an assessment of possible reproductive disturbance. In this respect, the suitability of the stickleback is enhanced by the fact that very high egg/fry survival rates can routinely be obtained, this being an important factor for comparisons between test and control groups.

11.11 References

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12 MARINE POLLUTION TRACKING SYSTEM

T. Nissen-Lie presented MARPOT, Marine Pollution Tracking System. MARPOT is a project initiated by Det Norske Veritas (DNV) with the support from ChemTAG AS. The aim of the project is to physically trace the produced water from Statfjord B, and thereby:

- evaluate the dilution processes and dispersion of produced water downstream of the release;
- verify exposure of test organisms;
- verify model results against measurements.

The tracing of produced water will be performed with C-tag^{TM}, which is an "intelligent tracer" developed by ChemTAG AS (see http://www.chemtag.com/indexie.html). C-tag^{TM} is a synthetically produced DNA molecule. Information can be encoded in the tracer so that it is specific for the release, and the technique is quantitative and very sensitive. The tracer is not harmful to the environment.

The project consists of six main tasks:

1) modelling the dispersion of the produced water that is discharged into the sea, in order to calculate C-TAG (concentrations needed and to optimise sampling stations);
2) adding C-TAG to the produced water discharge system;
3) water sampling;
4) analysis of the water samples and interpretation of results;
5) verifying the DNA dispersion models against the obtained results;
6) documenting procedures for tracing of produced water with C-tag™.

Adding C-tag to the produced water discharge system and the water sampling will be the most challenging tasks in the project. Diluted C-tag will be added to the discharge system of Statfjord B which carries produced water, cooling water, and formation water. The water samples will be collected by a CTD with water bottles that can be electronically "fired" from the ship. Water will be sampled at approximately 20 stations, at several depths at each station, out to approximately 2000 m downstream of the release. The final station grid will cover both the centre and the outer edge of the plume. Other parameters such as salinity, temperature and density profiles and current speed and direction will be recorded at the stations. The main part of the sampling will be conducted from the "stand-by" ship, which belongs to Statfjord B. In order to conduct the sampling while the concentrations in the plume remain at a "steady state" level, the samples must be collected within a limited time frame. The collected samples (three parallels) will be frozen down at −20 °C before they are transported onshore for C-tag analysis.

13 RECOMMENDATIONS

SGSEA recommends that results from the workshop be presented to WGBEC in time for its meeting in 2002. A special session should be arranged at the 2002 ICES ASC for presentations covering results from the workshop.

14 CLOSURE OF THE MEETING

The meeting was adjourned at 1700 hrs 19 January 2001.

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ANNEX 2: PROGRAMME FOR THE MEETING

18.1.2001

0900-0915	welcome and introductions
0915-0945	overview of the workshop
0945-1000	the Statfjord area
1000-1015	the German Bight
1015-1045	sampling design
1045-1100	coffee break
1100-1130	reporting and data storage
1130-1200	overview of cruises and caging
1200-1300	lunch
1300-1330	the chemistry programme
1330-1500	methods based on field-collected samples, chair: Thomas Lang
1500-1515	coffee break
1515-1715	methods based on field-collected samples continued
1715-1730	summing-up field-collected samples
1900	joint dinner

19.1.2001

0900-0920	Special presentation: marine pollution tracking
0920-1100	bioassay methods; chair: Dick Vethaak
1100-1115	coffee break
1115-1230	methods based on caged organisms; chair: Ketil Hylland
1230-1330	lunch
1330-1500	methods based on caged organisms continued
1500-1515	coffee break
1515-1530	summing-up caged organisms
1530-1600	logistics, practical details
1600-1700	protocols, tasks
1700	Meeting adjourned

ANNEX 3: LIST OF MEETING PAPERS - WORKING DOCUMENTS

SGSEA 1	Draft programme
SGSEA 2	Produced water discharges to the North Sea: fate and effects in the water column. Summary report. OLF, 1998.
SGSEA 3	Becker et al., 1992. Mar Ecol Prog Ser
SGSEA 4	ICES ASC 2000 CM 2000/S:05

ANNEX 4: ICES ASC 2000 CM 2000/S:05

International Council for the Exploration of the Sea

2000 Annual Science Conference – Bruges, Belgium

Theme Session on Temporal and Spatial Trends in the Distribution of Contaminants and their Biological Effects in the ICES Area

Biological effects of contaminants in pelagic ecosystems a practical workshop

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Abstract

There is a lack of agreed methods to assess the impact of contaminants in pelagic ecosystems. Earlier workshops arranged under the auspices of ICES and IOC have successfully stimulated research into the use of biological effects methods to monitor contaminant impacts in benthic ecosystems. Many of the techniques developed have now been incorporated in national and international monitoring programmes. Since benthos constitutes only a part of the marine ecosystem potentially exposed to contaminants, however, there has been increasing interest to initiate co-ordinated studies on effects in pelagic ecosystems as a basis for future monitoring programmes.

The objective of the workshop is to bring together scientists involved in relevant work in a practical workshop in order to assess the ability of selected methods to detect biological effects of contaminants in pelagic ecosystems under uniform and standardised conditions. The methods will be assessed for their applicability in future monitoring programmes.

During February-September 2001, research vessels from England, Germany, Norway and Scotland will collect water samples and selected pelagic organisms at eight sampling stations in two areas of the North Sea. Samples will be obtained on a contaminant gradient in the German Bight (at three stations of the 1991 IOC/ICES Bremerhaven Workshop on Biological Effects of Contaminants), at three stations in the vicinity of an oil field in the central/northern North Sea plus a reference location in each area. In addition, cages with fish and mussels will be positioned at each site for 4-5 weeks. Facilities will be made available following some of the cruises to perform exposure and challenge studies under controlled conditions at shore laboratories.

Introduction and objectives

There is a widely recognised need for methods to detect and quantify effects of contaminants in pelagic ecosystems, not least in relation to oil-production activities. In the past few decades, the main focus of biological effect methods development has been on benthic organisms and processes, to some extent fuelled through three practical workshops. One was held in Oslo/Langesund/Solbergstrand (Bayne et al., 1988), the second at Bermuda (Addison & Clarke, 1990) and the third in Bremerhaven (Stebbing & Dethlefsen, 1992). The three workshops were held under the auspices of UN (GEEP, IOC) and/or ICES. The workshops stimulated research into the use of biological effects methods to monitor pollution impacts in marine ecosystems and contributed towards a framework for general and contaminant-specific monitoring (JAMP, 1998a,b).

Whereas there has been progress in developing methods to assess pollution effects in benthic systems, there is still a lack of agreed methods to evaluate biological effects in pelagic systems. There are obvious reasons for this situation. Firstly, the quality of the water at any location may vary substantially with time. Secondly, organisms in the pelagial either move with the water (plankton) or move through large volumes of water (nekton). For many of the methods in question, plankton represent very small samples and it is difficult to find sufficient material for analyses. The nekton will integrate over large areas and may not represent the water quality at any given location very well.

It is probable that pelagic ecosystems along European coasts are affected by contaminants to a greater or lesser extent. There are many sources of contaminants to pelagic ecosystems including coastal inputs, dumping and long-range transport by ocean currents and the atmosphere. In areas of oil-production, there will be inputs of produced water. The composition of produced water varies considerably from well to well, but generally contains mono- and polycyclic aromatic hydrocarbons, other organic contaminants and trace metals.

The objective of the workshop is to bring together scientists involved in relevant work in a practical workshop in order to assess the ability of selected methods to detect biological effects of contaminants in pelagic ecosystems under uniform and standardised conditions. The methods will be assessed for their applicability in future monitoring programmes.

Organisation

The initiative for this workshop stemmed from the ICES working group on biological effects of contaminants (WGBEC). Earlier workshops (Oslo, Bermuda, Bremerhaven; see above) had a main focus on benthic systems, although the Bremerhaven workshop also contained pelagic elements (see e.g. Karbe, 1992). The proposed workshop is co-ordinated through a scientific steering committee with the following members: Gerd Becker (FMHA, Germany), Ian Davies (FRS, UK), Ketil Hylland (NIVA, Norway), Peter Matthiessen (CEFAS, UK), Thomas Lang (BfF, Germany), Bjørn Serigstad (IMR, Norway), Kurt Tande (University of Tromsø, Norway), Toril Inga Røe Utvik (Norsk Hydro, Norway), Dick Vethaak (RIKZ, Netherlands) and Werner Wosniok (University of Bremen, Germany). The steering group has been chaired by Ketil Hylland. At the first meeting agreement was reached on the wording of a Prospectus (invitation to submit research proposals), subsequently distributed to most relevant European and North American laboratories. This call for proposals resulted in 37 submitted projects involving 20 institutions in a number of European countries. Following the initial assessment of the projects by the steering group, an appropriate selection of methods has now been included in the workshop.

Structure of the workshop

The practical work will focus on two areas with inputs of contaminants into the pelagic ecosystem: a coastal area (German Bight) and an area in the vicinity of an oil rig (Statfjord). In both areas, four locations will be identified, three within a contaminantion gradient and a fourth outside the affected area (reference). At each of the eight sites, sampling for water and pelagic organisms will take place on multiple occasions in 2001. In addition, buoys with SPMDs[2], DGTs[3], cages with blue mussels, fish (Atlantic cod, 3-spined stickleback) and fish eggs (herring) will be deployed at each of the eight sites. The cruise plan is indicated in Table 1. There will be a need for one more vessel in June to collect buoys from the Statfjord locations.

Table 1. Overview of cruises, areas and special tasks. GB – German Bight.

Period	Vessel	Areas sampled	Remarks
Feb-Mar (14d)	Walther Herwig III	GB, Statfjord	embryos/larvae
March (10 d)	Scotia	Statfjord, GB	zooplankton
Apr-May (14 d)	Johan Hjort	Statfjord, GB	deploy cages
June (7 d)	Cirolana	GB	collect cages
June (7 d)	Mikael Sars	Statfjord	collect cages
June (7 d)	Belgica	GB	collect cages, SSML
Aug-Sep (14 d)	Walther Herwig III	GB, Statfjord	fish

The workshop will provide vessels, basic chemistry, statistical guidance and analysis of data. In addition, specially trained crew and laboratory staff will do pre-treatment and/or extraction of materials. Each participant is expected to cover the costs of their own analyses and, for the few methods where it is required, provide personnel to perform special tasks on board the vessels.

[2] SPMD – semipermeable membrane device; used to estimate integrated accumulation of hydrophobic contaminants from water

[3] DGT – diffusive gradient in thin films; used to estimate integrated accumulation of metals from water

Overview of methods

The methods that will be used in the workshop can be divided into three categories: (i) methods that use *in vitro* techniques on water samples, extracts or concentrates from water, (ii) methods applied on caged organisms, (iii) methods applied on field collected organisms. Within each category there is a broad range of projects.

***In vitro* techniques**

During all five cruises, water will be sampled. Projects will use extracts of water, extracts of the sea surface microlayer (SSML) and extracts from SPMDs deployed at each site. There will be a main focus on the use of extracts from SPMDs in laboratory-based tests. In addition, one project will use suspended particulate matter (SPM), sampled by filtering seawater. The projects are summarised in Table 2.

Caged organisms

At each of the eight sites, cages will be deployed. Caging structures and the cages themselves will be provided by the Institute for Marine Research (IMR), Bergen, Norway. Cages will be used for Atlantic cod (*Gadus morhua*), blue mussel (*Mytilus edulis*), 3-spined stickleback (*Gasterosteus aculeatus*) and herring (*Clupea harengus*) eggs. Buoys with SPMDs and DGTs will be deployed at each site during the first cruise. SPMDs and DGTs will be sampled and redeployed at each subsequent cruise in the relevant area.

Blue mussels will be collected from the Norwegian west coast and deployed in cages from the end of April until June. Hatchery-reared Atlantic cod will be deployed in cages from the end of April until June. Stickleback will be collected on the UK east coast and adapted to full-strength seawater at CEFAS, Burnham-on-Crouch. Specially made cages with stickleback will be deployed at the same time as cod and blue mussels.

Table 2. Overview of proposed *in vitro* techniques to be performed with water extracts.

Test system	**Endpoint**
pure enzyme	AChE inhibition
primary fish hepatocytes	apoptosis, DNA damage, viability, mitochondrial function, vg, CYP induction
modified cell lines with reporter genes	dioxin, estrogen, androgen receptor
bacteria	microtox, mutatox
modified yeast with reporter gene	estrogen receptor
juv. salmon, i.p. injection	vg, zrp, CYP induction
egg microinjection, salmon	embryonal development
early life stage Danio rerio	embryonal development
Corophium volutator	toxicity
oyster embryo, Tisbe sp, algae	toxicity
Arbacia punctulata	fertilisation, embryonal development
invertebrate larvae	toxicity; UV-exposure*
Neomysis integer	sublethal effects
Acartia tonsa	survival, reproduction
Nitocra spinipes	survival, life-cycle

Vg – vitellogenin; zrp – zona radiata protein; CYP – cytochrome P450, AChE – acetylcholinesterase.

Table 3. Techniques with caged organisms. MT – metallothionein, BPH – benzo(a)pyrene hydroxylase, TOSC – total oxyradical scavenging capacity, EROD – ethoxyresorufin *O*-deethylase, GST – glutathione *S*-transferase, other abbreviations see Table 2.

Organism	Tissue	Endpoint
blue mussel (*Mytilus edulis*)	gills	MT induction
	hepatopancreas	MT induction, histochemistry, AChE, BPH, CYP, oxidative damage, antioxidant enzymes, TOSC, DNA damage
	haemolymph	immunotoxicity, lysosomal stability (platereader), immunocompetence
	whole mussels, haemolymph	lysosomal stability genotoxicity pathology
	whole mussel	scope for growth
Atlantic cod (*Gadus morhua*)	liver	MT, EROD, DNA adducts, CYP (protein, mRNA), GST, histopathology
	bile	PAH-metabolites
	plasma	vg, zrp (protein, mRNA)
	muscle	AChE
3-spined stickleback (*Gasterosteus aculeatus*)	kidney	spiggin
	liver	EROD, MT
	bile	PAH-metabolites

Herring eggs and larvae will be collected in the Norwegian Sea immediately prior to deployment of cages with cod and mussels. The eggs will be attached to a glass surface within a net cage for exposure at the eight sites, alongside the other caged organisms. The endpoint for the method is embryonal development. Larvae will be kept in specially constructed cages. The endpoints for this study are development, EROD, RNA/DNA ratios and chromosome aberrations.

Field collected organisms

In each of the two main areas (Statfjord and German Bight), samples will be taken at four sites. Three sites will be in a gradient from the contaminant source, the fourth a reference site outside this region. The projects range from studies on bacterial diversity to biomarkers in pelagic fish species (Table 4).

Table 4. Techniques with field collected samples, abbreviations see Tables 2 and 3.

Organism(s), endpoint(s)	Comment
bacteria; genetic diversity, degradation	whole water sample
phytoplankton, photosynthesis	whole water sample
microzooplankton; grazing	whole water sample
zooplankton; biomarkers	whole Calanus
zooplankton; TOSC, DNA damage	whole Calanus
krill; TOSC, DNA damage, vg, zrp	haemolymph, whole krill
fish embryos; aberrations	embryos
fish embryos and larvae; EROD, histopathology	larvae
fish larvae; DNA damage	
juvenile herring; EROD, MT	liver
juvenile herring; vg, zrp, CYP (protein and mRNA)	plasma, liver
juvenile herring; histopathology	liver
ripe dab, whiting; stripping, *in vitro* fertilisation	ripe male and female fish

Discussion

There is a clearly identified need for methods to monitor effects of contaminants in pelagic ecosystems. In previous studies there has been some indications of effects on fish embryos and larvae in contaminated parts of the North Sea (Dethlefsen et al. 1996; von Westernhagen et al. 1980; von Westernhagen et al. 1988; von Westernhagen et al. 1989) and some projects within the Bremerhaven workshop also indicated toxic effects of water from the German Bight on test organisms (Thain, 1992; Williams, 1992). Similarly, studies have indicated that outputs from the oil fields in the North Sea have affected fish and fish larvae (Stagg et al., 1995, 1996). In addition to studies indicating effects, there is a substantial database of transport and bioavailability of the components in produced water from North Sea oil fields (see e.g. Utvik & Johnsen, 1999; Utvik et al., 1999).

The development of methods to assess the effects of contaminants in pelagic systems will be important for monitoring in coastal waters and to understand impacts of oil production in the North Sea. One objective of the workshop is to assess the methods used during the workshop and propose methods suitable for monitoring and management. Considering the wide range of methods to be implemented during the workshop, it should provide the tools required to monitor contaminant impacts on pelagic systems in the North Sea region.

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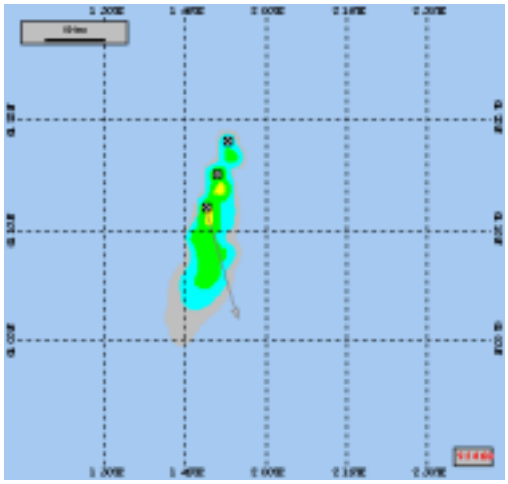
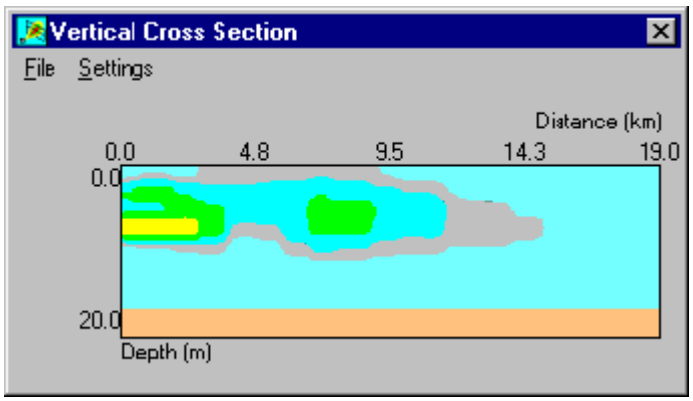
ANNEX 5: DATA FOR THE STATFJORD STUDY AREA

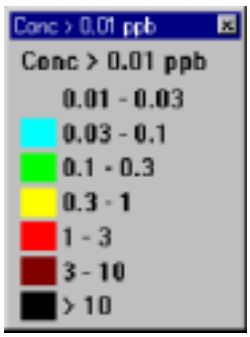
Composition of produced water from the Statfjord oil fields.

Compound	Concentration (mg/l)
Naphthalenes	0,4400
PAH (2-3 rings)	0,0870
PAH (4 ring +)	0,0004
Dispersed oil	10,8000
Alkylatd phenols (C0-C3)	2,0570
Alkylated phenols (C4+)	0,0320
Organic acids	368,000
Zn	0,0020
Triethylene glycol	2,3300
Flocculant	0,1000
Defoamer	0,0003
Emulsion breaker	0,6500
Corrosion inhibitor	0,1200
H2S scavanger	0,0050
Scale inhibitor	4,7600

Simulation of spread and potential environmental effects of discharge from Statfjord B (using the DREAM model)







ANNEX 6: PRELIMINARY CRUISE PLAN

ICES/IOC WORKSHOP ON BIOLOGICAL EFFECTS OF CONTAMINANTS IN PELAGIC ECOSYSTEMS (BECPELAG)

CRUISE	AREA	PRIORITY TASKS
1 23.2.-10.3.2001 RV Walther Herwig III (DE)	German Bight Statfjord	BIOTA FIELD SAMPLES - Fish embryos/larvae - Adult/juvenile herring - Ripe fish (dab, whiting etc.) WATER SAMPLES - Bacteria - Chemical analyses
2 17.-28.3.2001 RV Scotia (UK)	Statfjord German Bight	BIOTA FIELD SAMPLES - Adult/juvenile herring - Adult fish - Zooplankton (incl. Krill) WATER SAMPLES - Microzoo-/Phytoplankton - Chemical analyses
3 23.4.-1.5.2001 RV Johan Hjort (NO)	Statfjord German Bight	TRANSPORT OF LIVE FISH/MUSSELS DEPLOY CAGES/SPMDs/DGTs BIOTA FIELD SAMPLES WATER SAMPLES - Bacteria - Chemical analyses
4 11.-15.6.2001 RV Belgica (BE)	German Bight	COLLECT CAGES/SPMDs/DGTs PROCESSING OF CAGED FISH/MUSSELS PROCESSING OF SPMDS/DGTs WATER SAMPLES - Chemical analyses
5 16.-22.6.2001 RV Cirolana (UK)	German Bight	COLLECT CAGES/SPMDs/DGTs PROCESSING OF CAGED FISH/MUSSELS PROCESSING OF SPMDS/DGTs WATER SAMPLES - Chemical analyses
6 1.6.-10.6.2001 RV G.M. Dannevig (NO)	Statfjord	COLLECT CAGES/SPMDs/DGTs PROCESSING OF CAGED FISH/MUSSELS PROCESSING OF SPMDS/DGTs WATER SAMPLES - Chemical analyses
7 24.8.-9.9.2001 RV Walther Herwig III (DE)	German Bight Statfjord	COLLECT SEA SURFACE MICROLAYER WATER SAMPLES - Bacteria - Chemical analyses