ICES SGEH REPORT 2011

SCICOM STEERING GROUP ON REGIONAL SEA PROGRAMMES

ICES CM 2011/SSGRSP:04

REF. SSGHIE, SCICOM

Report of the Study Group for the Development of Integrated Monitoring and Assessment of Ecosystem Health in the Baltic Sea (SGEH)

11–15 April 2011

Riga, Latvia

ICES International Council for the Exploration of the Sea
CIEM Conseil International pour l'Exploration de la Mer

International Council for the Exploration of the Sea
Conseil International pour l'Exploration de la Mer

H. C. Andersens Boulevard 44–46
DK-1553 Copenhagen V
Denmark
Telephone (+45) 33 38 67 00
Telefax (+45) 33 93 42 15
www.ices.dk
info@ices.dk

Recommended format for purposes of citation:

ICES. 2011. Report of the Study Group for the Development of Integrated Monitoring and Assessment of Ecosystem Health in the Baltic Sea (SGEH), 11–15 April 2011, Riga, Latvia. ICES CM 2011/SSGRSP:04. 110 pp. https://doi.org/10.17895/ices.pub.9013

Contents

Executive summary **1**

1 Opening of the meeting **3**

2 Adoption of the agenda **3**

3 Appointment of rapporteurs **4**

4 Review of the progress in the BONUS+ BEAST project (ToR a) **4**

4.1 Overview of the progress on BEAST in 2010 4

4.2 BEAST WP1: Field studies and experiments in selected subregions of the Baltic Sea 4

4.3 BEAST WP2: Application and validation of methods in monitoring and assessment in the Baltic Sea 6

4.4 WP 3: Developing tools for Ecosystem Health assessment in the Baltic Sea 7

4.5 Future plans 8

5 Develop background documents for biological effects methods in the Baltic Sea (ToR b) **8**

6 Examine the status of assessment criteria for biological effects parameters in the Baltic Sea (ToR c) **9**

7 Review developments in MSFD related to the implementation of biological effects methods (ToR d) **10**

8 Propose a list of biological effect methods for integrated monitoring and assessments in the Baltic Sea (ToR e) **12**

9 Update project planning for the BONUS call in 2011 (ToR f) **13**

10 Review biological effects methods applied in ERAs, EIAs and "post-accident" studies (ToR g) **13**

11 Biological effects of perfluorinated compounds relevant to the Baltic Sea (ToR h) **17**

12 Continue compilation of effects of hazardous substances on biodiversity in the Baltic Sea (ToR i) **20**

13 Assess fish diseases as an indicator of ecosystem health (ToR j) **20**

14 Start a discussion on how to evaluate the cumulative effects of impacts from a differing human sources such as contaminants, eutrophication, noise and habitat change/impacts (ToR k) **21**

15 Any other business **22**

15.1 SSGRSP and future of SGEH 22

15.2 Progress in EUSBSR PA3 23

15.3 Specific presentations 23
15.3.1 Biomarkers in zooplankton 23
15.3.2 Development in on-line monitoring 25

Annex 1: List of participants 28

Annex 2: Draft meeting agenda and timetable of the meeting 30

Annex 3: Share of work 31

Annex 4: Draft work schedule 33

Annex 5: Supporting Information 35

Annex 6: Recommendations 36

Annex 7: CORESET Hazardous Substances - Biological Effects Indicators 37

Annex 8: Numerical Assessment Criteria (AC) for Baltic Sea organisms to assess biological effects 41

Annex 9: Overview of Core and selected Candidate Indicators to assess the effects of hazardous substances at different biological levels 44

Annex 10: ICES SGEH Biological Effects methods Background Documents for the Baltic Sea region* 47

Annex 11: ICES SGEH Biological Effects methods Background Documents for the Baltic Sea region* 55

Annex 12: ICES SGEH Biological Effects methods Background Documents for the Baltic Sea region* 65

Annex 13: ICES SGEH Biological Effects methods Background Documents for the Baltic Sea region 71

Annex 14: ICES SGEH Biological Effects methods Background Documents for the Baltic Sea region* 76

Annex 15: ICES SGEH Biological Effects methods Background Documents for the Baltic Sea region* 86

Annex 16: ICES SGEH Biological Effects methods Background Documents for the Baltic Sea region* 92

Annex 17: ICES SGEH Biological Effects methods Background Documents for the Baltic Sea region* 97

Annex 18: ICES SGEH Biological Effects methods Background Documents for the Baltic Sea region* 105

Executive summary

ICES Study Group for the Development of Integrated Monitoring and Assessment of Ecosystem Health in the Baltic Sea (SGEH) met at the Faculty of Geography and Earth Sciences, University of Latvia in Riga, Latvia on 11–15 April 2011. SGEH is targeted on (1) hazardous substances and especially their biological effects, and (2) biodiversity. The group focuses especially on linkages between hazardous substances and their effects at different biological levels, from the molecular "early warning" level via effects on individuals and populations up to the ecosystem level. The main task of SGEH is to contribute to the development of integrated chemical-biological monitoring of hazardous substances in the Baltic Sea following the requests of the Baltic Sea Action Plan. This has been done in a harmonised way with the work accomplished in OSPAR (SGIMC) and in close collaboration with HELCOM. This was the final meeting of the Study Group which is to be dissolved after three years of work.

Although the group has an extensive list of members, the SGEH meetings have mainly been attended by researchers of the Baltic Sea BONUS+ Programme project BEAST (Biological Effects of Anthropogenic Chemical Stress: Tools for the Assessment of Ecosystem Health, 2009–2011), and has inherently been very much relying on the practical work and results achieved within this project. Thus, a part of meeting time was also in 2011 allocated to examining the progress of the BEAST project, including collection of new field data in the different subregions of the Baltic Sea, laboratory experiments, progress in the development of the project database, development of guidelines and Standard Operating Procedures for the Baltic Sea as well as training workshops and intercalibration activities. In general the project is progressing as planned and following the schedule. Testing and development of integrated assessment indices for pollution and ecosystem health is still awaiting the submission of more data to the database.

All of the ToRs set for the SGEH meeting in 2010 were covered during the meeting (except for one that was considered of smaller importance). Being the last meeting of the SGEH and having some problems with reaching experts to properly cover some of the intended ToRs, the group set out to work prioritizing the following ToRs that are directly related to the implementation of biological effects methods in the Baltic Sea area: 1) develop background documents for biological effects methods in the Baltic Sea; 2) examine the status of assessment criteria for biological effects parameters in the Baltic Sea, and 3) propose a list of biological effect methods for integrated monitoring and assessments in the Baltic Sea. The establishment of methodological standards Assessment Criteria (AC) for biological effects methods is a critical issue in the development of environmental monitoring and assessment. The significant work carried out in the OSPAR area during the recent years (WKIMON and SGIMC) have been taken advantage of when developing a revision of the Baltic Sea monitoring strategy. As an important result of the work of SGEH the background documents and ACs available for the biological effects methods proposed to be taken on board have been carefully examined and modified as needed to be applied in the Baltic Sea region, and further conveyed to the use of HELCOM via the CORESET project.

SGEH is aware that interactions and collaboration especially between ICES groups dealing with integrated ecosystem assessments in different regional sea areas (mainly WGIAB, WGINOSE, WGNARS, WGEAWESS) is a key aspect to be developed, e.g. by forming a cluster of expert groups of the Regional Seas Programme; this would ensure that the basic approaches would be more-or-less consistent between the regions to achieve comparability of the assessments. Regarding the continuation of the SGEH-type activities the group suggests a formation of a new expert group/workshop (WG or WK) aimed at linking hazardous substances and their biological effects to existing integrated assessment and modelling schemes and those under development for the different regional sea areas with direct involvement and collaboration with members of these groups. The work in other relevant EGs (such as WGBEC dealing with the biological effects of contaminants) should be linked closely to the work of the new group.

1 Opening of the meeting

ICES Study Group for the Development of Integrated Monitoring and Assessment of Ecosystem Health in the Baltic Sea (SGEH) met at the Faculty of Geography and Earth Sciences, University of Latvia, in Riga, Latvia on 11–15 April 2011.

The SGEH Chair, Kari Lehtonen, welcomed the participants of the meeting. He invited the participants to introduce themselves and their affiliations and describe their area of interest and field of expertise. The list of attendees is given in Annex 1.

The Chair then expressed the warm gratitude of the group to the local co-hosting organizations, the Latvian Institute of Aquatic Ecology (LHEI) and the Institute of Biology of University of Latvia (IB UL) represented by SGEH members Maija Balode and Elmira Boikova, who introduced the practical arrangements for the meeting. A welcome address was kindly presented by the Director of the Institute of Biology, University of Latvia, Prof. Viesturs Melecis.

The Chair presented the Terms of References (ToRs) for the meeting (as adopted by SCICOM 2010): The ICES Study Group for the Development of Integrated Monitoring and Assessment of Ecosystem Health in the Baltic Sea (SGEH), chaired by K. Lehtonen, Finland, will meet in Riga, Latvia on 11–15 April 2011 to:

a) Review progress in the BEAST project;

b) Develop background documents for biological effects methods in the Baltic Sea;

c) Examine the status of assessment criteria for biological effects parameters in the Baltic Sea;

d) Review developments in MSFD related to the implementation of biological effects methods;

e) Propose a list of biological effect methods for integrated monitoring and assessments in the Baltic Sea;

f) Update project planning for the BONUS-169 call in 2011;

g) Review biological effects methods applied in ERAs, EIAs and "post-accident" studies;

h) Review biological effects of perfluorinated compounds relevant to the Baltic Sea;

i) Continue compilation of effects of hazardous substances on biodiversity in the Baltic Sea;

j) Assess fish diseases as in indicator of ecosystem health.

A late requirement from ICES had also been added to the list of ToRs for the meeting:

k) Start a discussion on how to evaluate the cumulative effects of impacts from differing human sources such as contaminants, eutrophication, noise and habitat change/impacts.

2 Adoption of the agenda

The Chair invited participants to examine the ToRs and went through the agenda recalling the backgrounds and priority of the items. A draft agenda had been circulated prior to the meeting (Annex 2) and it was agreed that the ToRs were reflected in the agenda.

Since a number of key members of the group could not attend the meeting and because of some last-minute postponements, adjustments had to be made the meeting agenda accordingly. However, some of the absentees contributed by correspondence. The agenda was adopted by the meeting and a tentative timetable and share of work was agreed upon (Annexes 3 & 4).

3 Appointment of rapporteurs

Principle contributors and rapporteurs to the agenda items were agreed upon (Annex 4) and are mentioned in connection with different contributions in this report. PowerPoint presentations, background documents, and reporting documents etc. were viewed and circulated using the ICES SharePoint.

4 Review of the progress in the BONUS+ BEAST project (ToR a)

4.1 Overview of the progress on BEAST in 2010

This section was led by Kari Lehtonen, the co-ordinator of the BEAST project (Biological Effects of Anthropogenic Chemical Stress: Tools for the Assessment of Ecosystem Health, 2009–2011). More detailed information on the project can be obtained from its website at www.environment.fi/syke/beast or from the SGEH 2010 report.

The BEAST project fulfilled all of its milestones and deliverables planned for each three WPs and tasks (subregions) for the year 2010. During 2010, many BEAST partners made significant contributions to HELCOM activities, including a key input to the HELCOM Assessment of Hazardous Substances in the Baltic Sea (HAZAS), especially concerning the writing and co-ordinating the compilation of the "Biological effects" chapter of the assessment. Related work initiated later in the HELCOM CORESET project Hazardous Substances component is also strongly supported by participation and inputs from several BEAST experts. Several abstracts describing results gained from BEAST have been submitted and will be presented at Euro SETAC meeting 2011. BEAST was nominated as one of the flagship projects of the EU Strategy for the Baltic Sea Region (EUSBSR) Priority Area 3.

Collaboration with the BONUS+ project BALCOFISH (Integration of pollutant gene responses and fish ecology in Baltic coastal fisheries and management, http://www.balcofish.science.gu.se/english). BEAST achieved close collaboration with the BALCOFISH project, and common samplings, experimental and workshop activities are planned to be continued during 2011. BEAST also attracted collaboration with a number of various non-BONUS projects related to biological effects of contaminants and ecosystem health, and collaboration (participation in cruises, sample sharing, testing of new analytical methods and approaches) with these projects will be continued and new interested parties are actively sought for.

4.2 BEAST WP1: Field studies and experiments in selected subregions of the Baltic Sea

BEAST WP1 Leader Brita Sundelin presented the progress in this component of the project. WP1 is focused on studies regarding biological effects of hazardous substances in a variety of target organisms (bioindicators), reflecting different taxa and habitats in different subregions of the Baltic Sea, i.e. Belt Sea, Gulf of Gdansk, Gulf of Riga, Gulf of Bothnia and Gulf of Finland. By using field studies (subregional sampling and caging) combined with laboratory exposure experiments, WP1 addresses specific basic research topics related to geographical locations, methods, species and chemical compound groups. The biological effects measurements are carried out at various levels of biological organisation, i.e. sub-cellular, cell, tissue, organ, whole organism, and represent lower-order and higher-order responses, reflecting different degrees of ecological relevance. The biological effects and chemical analysis studies are divided into two categories: core programme: carried out at each 5 subregion with a minimum of 2 sampling campaigns in each region and research and development (R&D) programme: carried out in 1–4 subregions according to resources and feasibility.

Four field campaigns have successfully been performed during 2010. The planned cruise in December 2010 was divided in two parts to be able to reach more coastal areas and the more shallow coastal parts were visited in January 2011.

1) Gulf of Bothnia: Amphipods (*Monoporeia affinis*) for genetic analyses and analyses of body burden of contaminants were sampled in August in the Bothnian Bay. Sediment for contaminant analyses (trace metals, PAHs, PCBs, DDT, TBT and chloroguaicols) were collected at the same sites with KVB 005 (SE). R/V *Aranda* (FI) operated in August/September in the Bothnian Sea. Hydrography (nutrients, salinity, pH, temperature and oxygen content) were measured. Sediment for bioassays and contaminant analyses and clams (*Macoma balthica*) for PAH content in biota and biomarker analyses were collected on several stations. Abundance, biomass and structure of benthos, phytoplankton and zooplankton communities were measured. *Mytilus edulis* was caged in June –August. R/V *Walther Herwig* III (DE) operated in December for fish examination (fish diseases) and sampling (eelpout and herring) in the Bothnian Sea. In addition, hydrographic measurements were done. KVB 005 visited Bothnian Sea in January 2011 for sampling of amphipods and sediment for contaminant analyses.

2) Belt Sea: Field campaigns in the fall and spring were performed in November and April to May. Sediment, mussels, snails, amphipods and eelpout were collected for contaminant analyses, bioassays, reproduction success and biomarker analyses with a national vessel or by coastal sampling. During December 2010, R/V Walther Herwig III operated in the western Baltic Sea and collected fish samples (flounder, herring) and carried out fish disease examinations and hydrographic measurements.

3) Gulf of Riga: Field campaigns were performed in June and September. Amphipods were collected and analysed for reproduction success and biomarker response in June. Body burdens of trace metals, PAHs, and PCBs were analysed. R/V *Aranda* visited the Gulf of Riga in September. Hydrography (nutrients, salinity, pH, temperature and oxygen content) were measured. Sediment for bioassays and contaminant analyses and clams (*M. balthica*) for PAH content in biota and biomarker analyses were collected. Abundance, biomass and structure of benthos, phytoplankton and zooplankton communities were measured.

4) Gulf of Gdansk: Amphipods were collected and analysed for reproduction success and biomarker response in June. Body burdens of trace metals, PAHs, and PCBs were analysed.

Bioassays

1) The amphipods *M. affinis, Gammarus zaddachi* and *Bathyporeia pilosa* were used in bioassays for acute toxicity testing of sediments from the Gulf of

Bothnia and Gulf of Riga. Sediments from the Gulf of Gdansk and Belt Sea have not been processed yet.

2) Sediment from the Bothnian Bay and Baltic proper were tested for reproductive success in *M. affinis* and biomarkers for oxidative stress, AChE and LMS were measured.

Experimental work

1) Linking biomarkers to reproduction success in amphipods and comparison between field and lab exposure were performed in the Bothnian Bay.

2) Correlation between contaminant concentrations in sediment/body burdens in amphipods to malformed embryos was assessed in the Gulf of Bothnia, Baltic proper, Gulf of Riga and Gulf of Gdansk.

3) Behavioural and electrophysiological responses were measured in crucian carp (*Carassius carassius*) to study effect of pH.

4) Injections of contaminants in eelpout to study embryo malformations and biomarker response have been performed during autumn 2010.

4.3 BEAST WP2: Application and validation of methods in monitoring and assessment in the Baltic Sea

A report on progress in BEAST WP2 had been compiled by its Leader Thomas Lang and in his absence presented to the group by Ulrike Kammann. The identification and validation of suitable methods for integrated monitoring and assessment is underway and will be finalised at the end of the project, based on the practical experiences made during the field sampling, cage exposure and bioassay work and the results of the integrated data assessment. For this activity, efforts within ICES, OSPAR, and HELCOM have been utilised and emphasis is placed on coherence of the methodologies identified to the extent possible. The field sampling program for the five Baltic Sea subregions under study was applied successfully and the final major sampling campaign took place end of 2010. Work on the Handbook with Guidelines and Standard Operating Procedures (SOPs) for integrated monitoring and assessment of contaminant and biological effects in subregions of the Baltic Sea proceeded in 2010. The goal is to publish the handbook and make it available for future national and HELCOM Baltic Sea monitoring and assessments. The handbook and the results achieved in WP 3 will form the basis for recommendation for future monitoring and assessment of contaminants and their biological effects in the Baltic Sea to be finalised at the end of the project duration.

In 2010, the following training and intercalibrations took place:

- training and intercalibration of methods for field sampling of biomarkers and fish disease studies (Lead: vTI/FOE, SYKE);
- intercalibration exercise on measurement of PAH metabolites in fish bile (Lead: vTI/FOE);
- intercalibration of measurements on histochemical biomarkers (Lead: AWI).

Plans have been made for further activities scheduled for 2011 and for possible follow-up activities after the end of BEAST: (a) a training/intercalibration workshop for studies on liver histopathology in flounder, herring and eelpout, (b) a 'Special Biomarker' workshop. Possibility to utilise external funding will be explored.

SGEH appreciated the progress made in intercalibration, harmonization of biological effects methods within the BEAST project as a prerequisite of successful application of these methods in international monitoring programmes. The meeting noticed the BEAST workshop in Askö on effects in amphipods at the Askö laboratory (Sweden) in June 2009 as well as the joint BALCOFISH/BEAST workshop on eelpout sampling and examination in Denmark in October 2009. SGEH supported the ongoing process of quality assurance for biological effects methods under BEQUALM and QUASIMEME (imposex and intersex in marine snails). Recently WGBEC announced to initiate additional intercalibration exercises on relevant methods not covered by BEAST or BEQUALM.

4.4 WP 3: Developing tools for Ecosystem Health assessment in the Baltic Sea

Doris Schiedek, the Leader of BEAST WP3 presented the progress in this part of the project. Set-up and maintenance of the BEAST database (BonusHAZ) has been continued. In close relation with all BEAST partners and discussions during the annual BEAST meeting in St. Petersburg, more parameters have been included and the report format for submission of data has been further improved. The BEAST partners have started to submit the data from the various field studies performed in 2009 and 2010 (WP1). Presently, data from about 130 stations covering all studied subareas and different biological effect measures have been included in the BonusHAZ. In addition, yearly data have been added concerning biological effects measurements in eelpout (12 stations) and blue mussels (6 stations) originating from the Danish National Monitoring (NOVANA). Most of the station information and biomarker data from the EU project BEEP have also been imported into BonusHAZ. With the present set-up of the BonusHaz, a tool has been developed allowing the use of quality-assured data for the multivariate analyses foreseen for 2011.

In applying a subset of this data (BEEP offshore, flounder), a first trial was made combining biomarker data and chemical contamination data to test and compare different multivariate statistical analyses and biomarker indices. Differences between stations were found, possibly due to differences in contamination level. In using a PCA-based approach, station differences were not so clear. Presently, work is carried out to test some other integrated biomarker indices such as the Integrated Biomarker Index (IBR) or weigh of evidence approach.

As contribution to the HELCOM Integrated Thematic Assessment of Hazardous Substances in the Baltic Sea (HAZAS), published in spring 2010, the ”traffic light approach” was applied, using different biomarkers and indicators for reproductive disorders or prevalence of certain fish diseases, respectively.

Available monitoring data from pristine and contaminated areas along the Swedish coast were used to calculate limit values for malformed embryos of the amphipod *Monoporeia affinis* in the Baltic Sea, based on station mean values of 13 stations monitored from 1994–2009. More comprehensive analyses are presently carried out as contribution to the development of specific health indices in relation to reproductive success.

A first trial of an integrated assessment of TBT pollution has been presented during the BONUS Annual Science Conference in Vilnius (2010), combining chemistry and biological effects measures, using relevant monitoring data from the Western Baltic Sea.

Based on a literature study and BEAST expertise, an overview paper is in preparation concerning "Indices for integrated assessment of Ecosystem Health" for application in Baltic Sea sub regions.

In collaboration with ICES SGEH, background documents are in preparation to develop Baltic Sea specific Assessment Criteria needed for the application of biological effects measures as monitoring tool. As part of the task to develop and apply tools for a science-based assessment and management, BEAST has used the existing knowledge to select suitable biomarkers and other measures (i.e. BEAST reproductive success or fish disease index) for the HELCOM CORESET project. It is an ongoing activity which will be continued in 2011.

4.5 Future plans

The first phase of Baltic Sea BONUS programme, BONUS+, terminates at the end of 2011. The next call of the programme opens in winter 2011–2012 and the themes to be funded will be announced in summer 2011. If hazardous substances and ecosystem health will be among the themes of the next call, the BEAST consortium, likely with additional partners, will start planning for a new project targeted at this call.

Being selected as a Flagship project in the EUSBSR may also signify improve the opportunities in receiving further funding for future BEAST activities.

The most immediate possibility to acquire continued funding after 2011 was the 4th and final call of the Baltic Sea Region Programme with a deadline in March 2011. The Steering Group of BEAST acted as a nucleus in preparing a proposal for the BSRP call. The new project proposal, called BEAST BSR consists of 20 partners from all Baltic Sea EU countries and Norway. Partners from the Russian Federation were not eligible for this call but are involved as Associated Organisations, together with several EPAs and NGOs from the Baltic Sea region. The amount of funds applied for the new project is 2.6 million € and the funding decisions are made in June 2011.

5 Develop background documents for biological effects methods in the Baltic Sea (ToR b)

Methodological background documents are one of the requirements of parameters to be considered in monitoring programmes. As agreed during the SGEH meeting in 2010, one of the main tasks of intersessional work and the work during the meeting in 2011 would consist of developing background documents for selected biological effects methods to be considered for application in monitoring in the Baltic Sea area. This was seen as a very timely process concerning the progress in the HELCOM CORESET project (http://www.helcom.fi/projects/on_going/en_GB/coreset/).

In CORESET the target is to develop a core set of indicators for biodiversity and hazardous substances with quantitative targets to allow an assessment of the status of the Baltic Sea in relation to the corresponding ecological objectives. In the hazardous substances components, in addition to providing indicators for chemicals, experts from the BONUS+ BEAST project have been invited to come up with suggestions for candidate core indicators for the assessment of biological effects such as AChE inhibition, PAH metabolites, eelpout reproductive disorders, lysosomal membrane stability, micronuclei formation, metallothionein and fish diseases. HELCOM has stressed the importance of including at least some biological effects indicators in the core set. CORESET had been informed that BEAST is currently developing specific assessment criteria for the Baltic Sea for the biological effects indicators and aims to finalise the assessment criteria proposals by the next meeting of HELCOM CORESET Hazardou Substances group in May 2011.

HELCOM CORESET has pointed out that currently monitoring for biological effects exists only in few Baltic Sea countries and that for any indicators to be included in the operational core set, the indicators will need either existing or planned monitoring activities supporting them. Hence, has CORESET emphasized the need to prioritise the biological effects indicators to be included as core indicators and invited the BEAST group to come with a proposal for up to four indicators for core indicators that should have a supporting monitoring program to operationalize them. CORESET has asked the BEAST project experts to provide a justification and an estimate of the efforts needed for laboratory analyses of the prioritised indicators.

The idea was to use existing OSPAR background documents developed by ICES SGIMC (reports 2010 and 2011) as a basis of these documents. The documents were scrutinised by named experts of SGEH examining their relevance for the Baltic Sea region, and modified versions tailored to the Baltic Sea were prepared.

The outcome of the work on background documents on selected biological effects methods is gathered in Annexes 10–18 of the present SGEH report. The same documents have been passed on to HELCOM CORESET project to be examined by the meeting of the Joint Advisory Board (JAB) of HELCOM CORESET and TARGREV projects in June 2011.

6 Examine the status of assessment criteria for biological effects parameters in the Baltic Sea (ToR c)

In addition to the background documents the development of assessment criteria (AC) is a vital step in the process of implementation of indicators. As described in the previous section (5), HELCOM CORESET requested the BEAST project to deliver AC for the proposed biological effects methods. Since the great majority of the SGEH meeting participants consist of partners of the BEAST project the document on AC was collectively prepared during the meeting. As with the background documents this work is also based on the information found in the documents of SGIMC (2010 and 2011), adjusted to fit the species monitored specifically in the Baltic Sea as well and on data measured only in organisms collected in this sea area (with some exceptions).

The document on AC for selected biological effects methods in Baltic Sea monitoring species can be found in Annex 9 of the present report.

PAH metabolites

For PAH metabolites BAC were calculated using fish from a relatively unpolluted sites like Iceland. Until BAC for flounder will be been available the BAC calculated for dab can be used also for flounder and be applied to North Sea as well as to the Baltic Sea. EAC were calculated from a laboratory exposure experiment of fish to crude oil. EAC were calculated relating PAH metabolites in bile to level of DNA adducts and general fitness indicators. The group agreed on pushing the development on assessment criteria forward.

BAC/EAC for PAH metabolites available for the North Sea are transferrable to the Baltic Sea for each species separately, because they are not population dependent. Regions for BAC calculation (e.g. Iceland) do not need to be close to the monitoring region. Since EAC are calculated from laboratory exposure experiments they are not related to single regions but to the species. Until EAC for all relevant species are developed the ones from biologically close species can be used instead.

Fish Disease index (FDI)

BAC/EAC for dab FDI are not transferrable from North Sea to Baltic Sea, because they are species and population dependent. But the FDI concept itself can be transferred and BAC/EAC will be calculated for cod and flounder from the Baltic Sea in near future. For these two species the necessary data (time-series from the Baltic Sea) are available.

General BAC for more than one fish species might be helpful for the interspecies assessment of monitoring data. For some biological effects techniques like PAH metabolites a common BAC appears to be applicable at least for some species. This would be support the assessment on a pan-regional level.

7 Review developments in MSFD related to the implementation of biological effects methods (ToR d)

During 2011 EU member states should be working towards defining Good Environmental Status (GES) for EU MSFD (Marine Strategy Framework Directive) Descriptor 8 (“concentrations of contaminants are at levels not giving rise to pollution effects”). This should be done through the identification of appropriate targets and indicators at a national level for the Descriptor. In addition member states should prepare initial assessments to be ready in 2012. The EU commission indicator relating to biological effects (8.2.1) provides a clear role for biological effects monitoring data (where appropriate and assessed against agreed thresholds) to help define GES.

Following the completion of the ICES/OSPAR WKIMON / SGIMC process in 2011 a robust integrated monitoring framework is available that will be suitable for addressing GES for Descriptor 8. This workplan completes some seven years of development work on generating integrated monitoring guidelines for OSPAR, assessment criteria for biological effects methods and an integrated assessment framework that can be used to assess monitoring data on contaminant concentrations and effects on biota together in an integrated manner. SGEH is currently investigating the transfer of the approach and guidelines to the Baltic Sea region, as seen in the report of the current meeting.

SGEH supports the integrated monitoring approach depicted in SGIMC 2011 as currently being the most appropriate approach available for determining GES for Descriptor 8. SGEH recommends its adoption by member states with some needed modifications for the Baltic Sea region.

National status and initiatives

Denmark

Denmark is at the moment preparing the initial assessment of their marine waters, taking account of existing data where available, and based on the indicative lists of characteristics, pressures and impacts set out in Table 1 and 2 of Annex III in the EU MSFD directive 2008/56/EC for establishing a framework for community action in the field of marine environmental policy.

“Effects of contaminants” on environment and wildlife will be addressed in the initial assessment, probably as a "Biological disturbance" as part of the descriptions for pressures and impacts.

In addition, national experts are involved and are giving input to for instance the HELCOM project for the development of Core Set Indicators for biodiversity and hazardous substances (HELCOM CORESET), which includes development of targets for indicators for the Baltic Sea in line with the indicators for Good Environmental Status (GES) as defined in MSF Directive, and the ensuing guidelines or criteria. In such, The CORESET project aims at ensuring coherence with and facilitating the work of the HELCOM EU member Contracting Parties in implementing the EU MSFD. This concerns also GES for descriptor 8, which states that: "Concentrations of contaminants are at levels not giving rise to pollution effects". At the moment, relevant biological effect indicators, where cause-effect relationships have been established, are under consideration for being included in the set of core indicators together with other indicators for concentration levels of selected contaminant groups in sediment and biota.

Estonia

No group members from Estonia were present in the meeting so no direct information was available. Post-meeting information from a local expert is that marine monitoring in Estonia will still be targeted to analyze the concentration of priority pollutants in the marine environment with no immediate plans to cover for biological effects.

Finland

The process of the implementation of WSFD is delayed and the European Commission are aware of this. However, the work process is now accelerated with the nomination of national experts called upon to comply and deliver a metadatabase for the basis of the national initial assessment. Regarding biological effects of contaminants, only a small amount of reports and published papers are available. However, new relevant data currently produced by the BONUS BEAST project as well as caging studies using mussels (AChE, MT, LMS, oxidative stress biomarkers, wide range of chemical contaminants) in Finnish sea areas during 2006–2010 will be added to the meta-database with the status "manuscript". In addition, other monitoring and research data, including long-term time-series on seal health and reproduction of the white-tailed eagle will be included to the database.

Germany

Germany is well aware of processes and deliverables related to MSFD. There have been numerous activities in Germany in the recent months to fulfil the national duties in the given time schedule. The initial assessments and other documents needed for MSFD are prepared in Germany at the moment. Germany considers biological effects as a part of the initial assessment.

vTI FOE was asked to contribute to the initial assessment Descriptor 8 concerning chemicals and their effects in the North Sea as well as in the Baltic Sea. vTI FOE contributed monitoring data and text on biological effects in the Baltic Sea: Fish diseases in flounder and cod, PAH metabolites in flounder, larval malformation in eelpout as well as information on the population of white-tailed eagle to underline the possibility of effects of contaminants on individuals and populations. Further on BEEP and BEAST were mentioned. However, the text is in a draft stage and vTI has not the lead for this document. So, future changes are possible.

Latvia

National experts are involved and are giving input in 8-MSFD implementation of biological effect methods in Latvia. Although regarding biological effects of contaminants only the first reports/presentations are available and mentioned methods aren't yet included in the Latvian National Monitoring programme, different biological effect methods are tested and adapted to Latvian conditions in the frame of several ongoing EC projects: BONUS BEAST, Interreg project COHIBA and ESF project HYDROTOX. Water and sediment bioassays (on different trophic level), haematological tests and biomarkers are being used for assessment of biological effects of contaminants in Latvia and for development of indicators for Good Environmental Status of the Baltic Sea.

Lithuania

According to information from the Lithuanian EPA the MSFD work has not really started yet but an expert group has been established.

Poland

National experts take part in the HELCOM CORESET project which aims to establish a Core Set of Indicators for hazardous substances and biological effects in marine organisms. In respect to work towards the Good Environmental Status (GES) Descriptor 8 of the EU MSFD, Poland is in the process of preparing an initial assessment to be ready in 2012. However it seems to be mainly targeted at measurements of priority pollutants. At this moment, it is not known to what extent biological effects will be covered in future monitoring programs. Regarding biological effects of contaminants, aside of data generated by the BEEP and BONUS Beast projects, the amount of data for Polish EEZ is relatively limited.

Sweden

Sweden participates in the MSFD related projects performed by the marine conventions HELCOM and OSPAR. There are several monitoring programs related to effects of hazardous substances currently running since more than 15 years for addressing GES for Descriptor 8. These programs include mammals (reproduction success in sea eagle and health effects in seals), fish and invertebrates. Health status in terms of various biomarkers are studied on perch and eelpout together with reproduction success in eelpout and reproduction success in small amphipods Both eelpout and amphipods are suggested as core indicators in a forthcoming HELCOM monitoring activity. The running programs have regularly been evaluated regarding their ability to establish time trends in appropriate time. Recently SEPA has initiated effort to develop assessment criteria for included variables for biodiversity and hazardous substances in terms of measurements of concentrations and effects. SEPA and FORMAS have also partly financed two BONUS programs (BEAST and BALCOFISH) in order to develop new methods and improve effect monitoring in the whole Baltic.

8 Propose a list of biological effect methods for integrated monitoring and assessments in the Baltic Sea (ToR e)

As pointed out in sections 5 and 6, HELCOM has requested the BEAST project to propose a list of biological effects methods to be included in integrated monitoring and assessment in the Baltic Sea. This was further conveyed by the HELCOM CORESET project, with a timeline of the JAB meeting in June.

Following the ToR e) agreed in 2010, SGEH members, fed by information from the BEAST project, prepared a prioritized list of biological effects methods to be considered by HELCOM JAB. The document can be found in Annex 7 of this report including supplementary information on the rationale and basis of the use of biological effects methods in monitoring of contaminants. Information and justifications for the selection of the methods can be found in Annex 8 of the report.

9 Update project planning for the BONUS call in 2011 (ToR f)

Andris Andrushaitis, Programme Manager of the Baltic Sea BONUS programme and member of SGEH attended the meeting and gave the latest information on the first call of the "new" BONUS. Strategic research agenda of the programme has been formulated. Regarding pollution and hazardous substances among the focal areas will be multilevel impacts, mixture toxicity, synergitic/antagonistic effects, multistressors, combined effects (e.g. with eutrophication), new chemicals, endocrine disruptors, and nanomaterials. The outcome of projects should include e.g. new knowledge at different biological levels, source, fate and impacts, cost-effective methods, and indicators for the use of the MSFD. The first call will open on the December 1, 2011 with the deadline of February 28, 2012. The projects will be selected using a 1-step process in May 2012 and they can be of up to 4 years in length.

As described in section 4.5, the BEAST consortium (with several new partners) submitted a project proposal for the INTERREG IV BSR call in March 2011. Regardless of the success of the proposal the its backbone and the new partner contacts achieved form a good basis for the preparation of the proposal for the coming BONUS call described above.

10 Review biological effects methods applied in ERAs, EIAs and "post-accident" studies (ToR g)

Since some of the planned experts on the subjects could not be present or contribute by correspondence to ToR g) the coverage of this rather wide topic was left to concentrate mainly on Environmental Risk Assessments (ERA) and disposal of polluted dredged materials, presented to the SGEH by Jakob Strand.

ERA includes the process of quantitatively evaluating the impact of anthropogenic activities, which can imply that stressors (e.g. chemical or physical conditions) pose a risk upon the health of the individual humans or the environmental well being of an individual, population or community of animals or plants including destruction of habitats (PIANC 2006). Assessment of pollution sources and their impact such as pollution effects is therefore an important part of ERA. In the marine environment, this issue can be relevant for ERA of activities such as dredging, aquaculture, offshore activities and constructions, but also in relation "post-accident" studies" e.g. after oil spill accidents.

In most countries around the Baltic Sea the maintenance of harbours and ports involves dredging of very large volumes of sediments and subsequent interests for disposal of the sediment in coastal or offshore waters. This can be of environmental concern if the dredged materials are highly contaminated with various kinds of hazardous substances of concern such as TBT, PAH, organochlorines and metals, which can pose a threat when disposed at sea. However, often other types of substances in sediment might also give rise to concern. Given the potential environmental consequences of dumped dredged harbour sediments, it is important to establish the potential environmental risks from exposure after disposal of the sediment at sea.

Currently, risk assessments of the dredged materials are based on chemical analyses of a limited number of well-known contaminants. Following the international guidelines, lower and upper action levels (ALs) are set by national authorities, which form the basis of the licensing process for offshore disposal (e.g. HELCOM 2004). In some countries not only the measured concentration levels, but also the total amounts of contaminants (e.g. AL at 1 kg of TBT) in the total bulk of dredged materials to be disposed at sea are used in the ERA (e.g. Danish EPA, 2007).

However, biological effect methods have also been proven to be useful for in ERA of dredged materials, and such methods can with benefits also be applied in both the hazard (potential toxicity), risk and local impact assessment of dredged polluted sediments both before it is disposed of at sea, and also in assessments of the impact on the environmental after the disposal has occurred.

Before disposal, it has been recommended to use a dedicated set of *in vitro* and *in vivo* bioassays to assess the toxicity of the sediment,, which is perceived to be best practice in the validation of the predicted risks, since different species can respond in different ways to the same dredged material (ICES WGBEC 2000; Schipper *et al.* 2010). An applicable set of *in vitro* and *in vivo* bioassays can include an *in vitro* reporter gene assay which detects and quantifies the toxic potential of dioxin-like compounds by dioxin-receptor mediated luciferase gene expression (e.g. DR-CALUX-assay), an *in vivo* toxicity assay with whole sediments for growth and survival with sediment dwelling organisms e.g. marine amphipods like *Corophium volutator*, and *in vitro* bacterial assay (e.g. Microtox assay) as described in Schipper *et al.* (2010), however, also other bioassays have been applied in e.g. the Netherlands, UK and France (ICES WGBEC 2000).

Potential *in vivo* bioassays can also include to be applied like sediment seawater elutriate and pore-water bioassays with zooplankton, and also early developmental stages of marine invertebrates (OSPAR 2007). However, confounding factors from different physical and chemical characteristics (like organic carbon content, redox etc.) should also be considered with the use of such methods (WGBEC 2000). Other promising *in vivo* sediment bioassays is to determine the level of imposex induction and impaired reproduction benthic gastropods like netted whelk (*Hinia reticulata*) and the mud snail *Potamopyrgus antipodarum* to assess the risk for chronic effects of elevated TBT levels in the sediment (ARGE Elbe 2001, Duft *et al.* 2007, Laranjeiro *et al.* 2010).

After the disposal has occurred, field indicators of relevant biological effects measurement on individual organisms and benthic communities can also be used in the assessment of the actual impact of dumped harbour sediments on local ecosystems.

For instance, some studies have found for instance that body burdens and the degree of imposex as sign of endocrine disruption caused by the antifouling agent TBT was found inversely related to the distance to the discharge site with the highest imposex frequency being found closest to the disposal sites. However, the levels of imposex found are often found to be relatively low when compared with those reported for gastropod populations from inside the moderately TBT-contaminated harbour areas (e.g. Santos *et al.* 2004). Similar observations have done at some Danish disposal sites for dredged materials (Danish EPA, pers. comm., Strand 2007).

Another example is a comprehensive Swedish study, which clearly demonstrated that the application of a set of biomarkers can be a useful approach in monitoring the impact during the time of dredging. Results showed that the resident fish eelpout was affected by remobilized pollutants from an extensive dredging in Göteborg harbour in 2002/2003. Biomarker studies showed elevated EROD/CYP1A and metallothionein levels indicated effects of PAH and metals, but also elevated levels of other more general stress responses as a decrease in lysosomal membrane stability, as well as effects on the immune system also could be observed in the fish sampled during the time of dredging (Sturve *et al.* 2005). For oxidative stress biomarkers it was concluded that TBARS is not an appropriate biomarker for pollutant mediated oxidative damage in eelpout while protein carbonyl formation does appear to be affected by xenobiotic exposure (Almroth *et al.* 2005). It was concluded that the application of a set of biomarkers is a useful approach in monitoring the impact of such anthropogenic activities on aquatic environments.

Similar results were found in a study of the impact of remobilization of contaminants from riverbed deepening of the River Elbe on local flounder populations in the German Bight. During the study period, twice statistically significant disturbances of lysosomal function were detected in fish from the River Elbe: in summer 1996 and in spring 1999 (Broeg *et al.* 2002). Similar results were found after the Elbe flood catastrophe in August 2002 (Einsporn *et al.* 2005).However, another example is a North Sea study, which was performed during the period of dumping of dredged materials, where contaminant levels of metals, TBT PCBs, PAH and TBT the sediment from this site were slightly elevated with 2–3 times higher than at the reference site. Four different bioassays with marine invertebrates and also the molecular biomarkers DNA integrity, cP450, BaP hydroxylase activity and AChE in the starfish tissues, but no significant differences were found between to the reference site. Minor pathological effects were observed in resident fish, and only very few benthic invertebrates were found at the site, but one year after dumping had ceased at the North site, a significant increase in the species richness and abundance of benthic invertebrates were observed. It was concluded that marine benthic resources at and around the dumping sites have been adversely affected by physical disturbance. However, no causal link could be established with sediment-associated contaminants from the dredged spoils (Stronkhorst *et al.* 2003).

In this context, it should also be noticed that disposal sites are often situated in erosion areas with strong currents, and the sediment materials, especially the fine sediment fractions, which often has the highest contaminant content, are often remobilisation due to resuspension and dispersed, although with the risk of being deposited again in some certain vulnerable deposition areas, and thereby also the contaminants. Hydrodynamic modelling will in such cases be a relevant tool in the impact assessment of in what areas the contaminants in the discharged dredged materials pose the highest risk to the environment, which might not necessarily be the actual disposal site.

Conclusion

ICES SGEH is of the opinion that in the Baltic Sea, biological effect methods can be applied in ERA and EIA of assessment of pollution sources and their impact in relation to where anthropogenic activities can pose a risk for the environment. This could concern activities such as dredging, aquaculture, exploitation and drilling, dumping, waste water treatment plant and industry effluents, offshore activities and constructions, but also in relation "post-accident" studies of e.g. oil spills and major flood events.

References

Almroth, B.C., Sturve, Berglund, Förlin L. 2005. Oxidative damage in eelpout (Zoarces viviparus), measured as protein carbonyls and TBARS, as biomarkers. Aquatic Toxicology 73: 171-180.

ARGE, Elbe. 2001a. Biologisches Effektmonitoring an Sedimenten der Elbe mit Potamopyrgus antipodarum und Hinia (Nassarius) reticulata (Gastropoda: Prosobranchia). http://www.argeelbe.de/wge/Download/Berichte/01BiolEffekt.pdf

Broeg, K., Köhler A.,.v. Westernhagen H. 2002. Disorder and recovery of environmental health monitored by means of lysosomal stability in liver of European flounder (Platichthys flesus L.) Marine Environmental Research 54: 569–573.

Danish EPA. 2007. Danish legislation In: Miljøministeriets bekendtgørelse nr. 1406 af 7. december 2007 om dumpning af optaget havbundsmateriale (In Danish).

Duft, M., Schulte-Oehlmann, Tillmann, Markert, Oehlmann. 2003. Toxicity of triphenyltin and tributyltin to the freshwater mudsnail potamopyrgus antipodarum in a new sediment biotest. Environmental Toxicology and Chemistry, Vol. 22, No. 1, pp. 145–152, 2003

Duft, M., C. Schmitt, J. Bachmann, C. Brandelik, U. Schulte-Oehlmann and J. Oehlmann. 2007. Prosobranch snails as test organisms for the assessment of endocrine active chemicals—an overview and a guideline proposal for a reproduction test with the freshwater mudsnail Potamopyrgus antipodarum. Ecotoxicology, 16, 169–182.

Einsporn, E., K. Broeg, A. Koehler. 2005. The Elbe flood 2002—toxic effects of transported contaminants in flatfish and mussels of the Wadden Sea. Marine Pollution Bulletin 50: 423–429

HELCOM. 2004. National instructions for dredging and disposal of dredged materials. Agenda item 4, presented by Finland at HELCOM MONAS 7/2004, Monitoring and Assessment Group, Seventh Meeting, Tallinn, Estonia, 25–29 October 2004.

Laranjeiro, F.M.G., Sousa, Takahashi, Tanabe, Barroso. 2010. Combination of Field Monitoring and Laboratory Bioassays for the Assessment of TBT Pollution in Ria de Aveiro. Interdisciplinary Studies on Environmental Chemistry. In: Biological Responses to Contaminants, Eds., N. Hamamura, S. Suzuki, S. Mendo, C. M. Barroso, H. Iwata and S. Tanabe, pp. 175–188, by TERRAPUB, 2010.

OSPAR. 2007. Background Document on Biological Effects Monitoring Techniques. Assessment and Monitoring Series. OSPAR Commission 2007.

PIANC. 2006. Environmental risk assessment of dredging and disposal operations. Report of Working Group 10 of the ENVIRONMENTAL COMMISSION 2006, Belgium.

DGE. 2006. Status of ecological assessment of dredging and relocation sites in Germany and The Netherlands. Dutch-German Exchange (DGE) on Dredged Material – Part 4 – Status of ecological assessment of dredging and relocation sites in Germany and The Netherlands, December 2006.

Santos, M.M., N. Vieira, M.A. Reis-Henriques, A.M. Santos, J.L. Gomez-Ariza, I. Giraldez, C.C. ten Hallers-Tjabbese. 2004. Imposex and butyltin contamination off the Oporto Coast (NW Portugal): a possible effect of the discharge of dredged material, Environment International 30 (2004) 793– 798

Stronkhorst, J., Ariese, van Hattum, Postma, de Kluijver, Den Besten, Bergman, Daan, Murk, Vethaak. 2003. Environmental impact and recovery at two dumping sites for dredged material in the North Sea. Environmental Pollution 124: 17–31.

Sturve, J., Berglund, Balk, Broeg, Böhmert, Massey, Savva, Parkkonen, Stephensen, Koehler, Förlin. 2005. Effects of dredging in göteborg harbor, sweden, assessed by biomarkers in eelpout (zoarces viviparus). Environmental Toxicology and Chemistry 24: 1951–1961.

11 Biological effects of perfluorinated compounds relevant to the Baltic Sea (ToR h)

Brita Sundelin presented an overview of current knowledge of biological effects of perfluorinated compounds on organisms, focusing on Baltic Sea biota. Until recently research investigating environmental fate of halogenated compounds has been focused on chlorinated and brominated chemicals. Less attention was put on fluorinated compounds (FOCs). Perfluorinated alkyd acids PFAAs comprise different groups of perfluorinated surfactants. Since the 1970s there has been a steady increase in the use of fluorinated organic compounds due to their hydrophobic and lipophobic properties and low surface tension for a great variety of industrial products. The main focus of research has so far been on the perfluorinated carboxylates (PFCAs) and sulfonates (PFSAs) which are used in a range of different applications, , e.g. as refrigerants, surfactants, and polymers, and as components of pharmaceuticals, fire retardants, lubricants adhesives, cosmetics, paper coatings and insecticides (Key *et al.* 1997, US EPA 2000; Kissa 2001).

Perfluorooctane sulfonate (PFOS) is a persistent organic compound with surfactant properties. It has been produced since the 1950s and used in a wide variety of industrial and consumer product applications (Kissa, 2001). PFOS was first documented in wildlife in 2001 (Giesy and Kannan, 2001) and is today known to be an ubiquitous environmental contaminant (Houde *et al.* 2006b, Yamashita *et al.* 2005). In May 2009 PFOS was listed under the Stockholm Convention on persistent organic pollutants (http://chm.pops.int/) Recent studies have documented the occurrence of PFOS in fish, mammals, and birds from various parts of the world, including the Arctic Ocean (Giesy and Kannan 2001; Kannan *et al.* 2001a, 2001b). No evidence has been found for metabolic or environmental degradation of PFOS (Seacat *et al.* 2002). Based on its persistence 3M announced that in 2000 it would phase out all perfluorinated sulfonates until 2004.

PFOS has been found in all trophic levels of the aquatic food chain. (Haukas *et al.*, 2007; Houde *et al.*, 2006a; Martin *et al.*, 2004). Benthic species have been suggested to be more exposed to PFOS than pelagic species from the same trophic level (Martin *et al.*, 2004). The benthic amphipod *Diporeia hoyi*, living in the Great Lakes was shown to contain an average of 280 ng PFOS/g w wt (Martin *et al.*, 2004). This concentration was two to six times higher than the concentrations in pelagic fish species in the study. Little research has yet been directed towards PFOS levels in benthic species.

The toxicology of PFOS is still under investigation. Effects documented in mammalian laboratory experiments include weight reduction, liver toxicity, juvenile mortality, immunomodulation and hormonal shifts (Lau *et al.*, 2007, DeWitt *et al.* 2009). In fish studies, levels of sex steroids, i.e. testosterone and estradiol, fluctuate significantly with increasing PFOS exposure (Oakes *et al.* 2005). There are few PFOS toxicity studies on crustaceans examining end-points in the sub lethal range, making effects of chronic low level exposures difficult to foresee. Reproduction effects have been reported in two cladoceran species (Ji *et al.*, 2008; Boudreau *et al.*, 2003) at levels ranging from 300 μg/L to 50 000 μg/L. Preliminary studies have raised the suspicion that PFOS exposure can increase the risk of malformed embryos and occurrence of unviable oocytes in *M. affinis* (unpublished data).

Perfluorooctane sulfonate (PFOS) is a widespread contaminant of environmental concern. PFOS has been found in biota globally, including remote Arctic ecosystems far from production and point source emissions (Giesy and Kannan, 2001; Kannan *et al.*, 2001; Houde *et al.*, 2006; Haukas *et al.*, 2007). Other studies have reported the occurrence of PFOS in human blood (reviewed by Lau *et al.*, 2007). PFOS and its precursors have mainly been produced due to their extraordinary capacity to lower surface tension. Applications included stain protection coating for textiles and leather, coating of paper products used for food packaging, hydraulic fluids, fire fighting foam, waxes and polishes (Kissa, 2001). However, PFOS is also an environmentally highly persistent compound as it neither hydrolyzes, photolyzes or biodegrades under naturally occurring conditions (Key *et al.*, 1998; Beach *et al.*, 2006; Hori *et al.*, 2006).

The toxicity of PFOS has been the subject of several reviews and toxicological effects have been reported on a range of vertebrate organisms including humans, monkeys, rodents, birds and fish (OECD, 2002; Beach *et al.*, 2006; Lau *et al.*, 2007). For invertebrate species there is a paucity of studies focusing on PFOS toxicity, especially those reporting chronic and sub-lethal effects. However, effects on survival and reproduction after chronic exposure have been studied in the freshwater cladocerans *Daphnia magna* (Boudreau *et al.*, 2003; Ji *et al.*, 2008) and *Moina macrocopa* (Ji *et al.*, 2008), and in the saltwater mysid *Mysidopsis bahia* (Drottar and Krueger, reported in Beach *et al.*, 2006).

In natural waters PFOS tends to sorb to sediment (3MCompany, 2003; Higgins and Luthy, 2006) why it is vital to examine the effects on sediment-dwelling organisms. The deposit-feeding amphipod *Monoporeia affinis* is a key species in soft bottoms of the Baltic Sea as well as in greater Swedish lakes. *M. affinis* is an important species in the food chain in the Baltic Sea, being both a direct and indirect food source for commercially important fish species such as herring (Aneer, 1975; Arrhenius and Hansson, 1993) and cod (Hessle, 1924; Ejdung and Elmgren, 2001; Englund *et al.*, 2008). Furthermore, the burrowing and feeding behavior of *M. affinis* in the upper sediment layer results in oxygenation of the sediments (Elmgren *et al.*, 1986), a central ecosystem service, especially after hypoxic periods (Modig and Olafsson, 2001). *M. affinis* sensitivity to contaminants and natural disturbances is well documented and reproduction variables have proven to be sensitive to contaminant exposure (Sundelin and Eriksson, 1998; Sundelin *et al.*, 2000; Wiklund and Sundelin, 2001, 2004; Jacobson *et al.*, 2008; Sundelin *et al.*, 2008a). Reproduction variables have been studied in recipients and contaminated areas and the natural variation of different embryo aberrations in *M. affinis* in reference areas is well known (Sundelin and Eriksson, 1998; Sundelin *et al.*, 2008a). Of the reproduction biomarkers, embryonic development (embryogenesis) has been identified as the most contaminant-sensitive variable, which also is an ecologically relevant variable as it affects the reproductive output (Sundelin and Eriksson, 1998). Thus, *M. affinis* is included as a sentinel in the Swedish National Monitoring Program to monitor both benthic ecology as well as effects of contaminants.

References

3MCompany. 2003. Environmental and health assessment of perfluorooctane sulfonic acid and its salts.

Aneer, G. 1975. Composition of food of the baltic herring (Clupea harengus v. membras L.), fourhorn sculpin (Myoxocephalus quadricornis L.), and eel-pout (Zoarces viviparus L.) from deep soft bottom trawling in the Askö - Landsort area during two consecutive years. Merentutkimuslait. Julk./ Havsforskningsinst. Skr 239, 146-154.

Arrhenius, F., Hansson, S. 1993. Food-Consumption of Larval, Young and Adult Herring and Sprat in the Baltic Sea. Marine Ecology-Progress Series 96, 125-137.

Beach, S.A., Newsted, J.L., Coady, K., Giesy, J.P. 2006. Ecotoxicological evaluation of perfluorooctanesulfonate (PFOS). Rev. Environ. Contam. Toxicol. 186, 133–174.

Boudreau, T.M., Sibley, P.K., Mabury, S.A., Muir, D.G.C., Solomon, K.R. 2003. Laboratory evaluation of the toxicity of perfluorooctane sulfonate (PFOS) on Selenastrum capricornutum, Chlorella vulgaris, Lemna gibba, Daphnia magna and Daphnia pulicaria. Arch. Environ. Contam. Toxicol. 44, 307–313. Yamashita, N., Kannan, K., Taniyasu, S., Horii, Y., Petrick, G., Gamo, T., 2005. A global survey of perfluorinated acids in oceans. Mar. Pollut. Bull. 51, 658–668.

DeWitt, J.C., Schnyra, A., Badr, M.Z., Loveless, S.E., Hoban, D., Frame, S.R., Cunard, R., Anderson, S.E., Meade, B.J., Peden-Adams, M.M., Luebke, R.W., Luster, M.I. 2009. Immunotoxicity of perfluorooctanoic acid and perfluorooctane sulfonate and the role of peroxisome proliferator-activated receptor alpha. Crit. Rev. Toxicol. 39, 76–94.

Ejdung, G., Elmgren, R. 2001. Predation by the benthic isopod Saduria entomon on two Baltic Sea deposit-feeders, the amphipod Monoporeia affinis and the bivalve Macoma balthica. Journal of Experimental Marine Biology and Ecology 266, 165-179.

Elmgren, R., Ankar, S., Marteleur, B., Ejdung, G. 1986. Adult Interference with Postlarvae in Soft Sediments - the Pontoporeia-Macoma Example. Ecology 67, 827-836.

Englund, G., Rydberg, C., Leonardsson, K. 2008. Long-term variation of link strength in a simple benthic food web. J. Anim. Ecol. 77, 883-890.

Giesy, J.P., Kannan, K. 2001. Global distribution of perfluorooctane sulfonate in wildlife. Environmental Science & Technology 35, 1339-1342.

Haukas, M., Berger, U., Hop, H., Gulliksen, B., Gabrielsen, G.W. 2007. Bioaccumulation of per- and polyfluorinated alkyl substances (PFAS) in selected species from the Barents Sea food web. Environ. Pollut. 148, 360–371.

Hessle, C. 1924. Bottenboniteringar i inre Östersjön. Medd. K. Lantbruksstyrelsen 250, 1-52.

Higgins, C.P., Luthy, R.G. 2006. Sorption of perfluorinated surfactants on sediments. Environmental Science & Technology 40, 7251-7256.

Hori, H., Nagaoka, Y., Yamamoto, A., Sano, T., Yamashita, N., Taniyasu, S., Kutsuna, S., Osaka, I., Arakawa, R. 2006. Efficient Decomposition of Environmentally Persistent Perfluorooctanesulfonate and Related Fluorochemicals Using Zerovalent Iron in Subcritical Water. Environ. Sci. Technol. 40, 1049-1054.

Houde, M., Bujas, T.A.D., Small, J., Wells, R.S., Fair, P.A., Bossart, G.D., Solomon, K.R., Muir, D.C.G. 2006a. Biomagnification of perfluoroalkyl compounds in the bottlenose dolphin (Tursiops truncatus) food web. Environ. Sci. Technol. 40, 4138–4144.

Houde, M., Martin, J.W., Letcher, R.J., Solomon, K.R., Muir, D.C.G. 2006b. Biological monitoring of polyfluoroalkyl substances: a review. Environ. Sci. Technol. 40, 3463–3473.

Jacobson, T., Prevodnik, A., Sundelin, B. 2008. Combined effects of temperature and a pesticide on the Baltic amphipod Monoporeia affinis. Aquatic Biology 1, 269-276.

Ji, K., Kim, Y., Oh, S., Ahn, B., Jo, H., Choi, K. 2008. Toxicity of perfluorooctane sulfonic acid and perfluorooctanoic acid on freshwater macroinvertebrates (Daphnia magna and Moina macrocopa) and fish (Oryzias latipes). Environ. Toxicol. Chem. 27 (10), 2159–2168.

Kannan, K., Koistinen, J., Beckmen, K., Evans, T., Gorzelany, J.F., Hansen, K.J., Jones, P.D., Helle, E., Nyman, M., Giesy, J.P. 2001. Accumulation of perfluorooctane sulfonate in marine mammals. Environmental Science & Technology 35, 1593-1598.

Key, B.D., Howell, R.D., Criddle, C.S. 1998. Defluorination of organofluorine sulfur compounds by Pseudomonas sp. strain D2. Environmental Science & Technology 32, 2283-2287.

Kissa, E. 2001. Fluorinated Surfactants and Repellants, 2nd ed. Marcel Decker, New York.

Lau, C., Anitole, K., Hodes, C., Lai, D., Pfahles-Hutchens, A., Seed, J. 2007. Perfluoroalkyl acids: a review of monitoring and toxicological findings. Toxicol. Sci. 99 (2), 366–394.

Martin, J.W., Whittle, D.M., Muir, D.C.G., Mabury, S.A. 2004. Perfluoroalkyl contaminants in a food web from Lake Ontario. Environ. Sci. Technol. 38, 5379–5385.

Modig, H., Olafsson, E. 2001. Survival and bioturbation of the amphipod Monoporeia affinis in sulphide-rich sediments. Marine Biology 138, 87-92.

Oakes, K.D., Sibley, P.K., Martin, J.W., MacLean, D.D., Solomon, K.R., Mabury, S.A., Van Der Kraak, G.J. 2005. Short-term exposures of fish to perfluorooctane sulfonate: acute effects on fatty acyl-CoA oxidase activity, oxidative stress, and circulating sex steroids. Environ. Toxicol. Chem. 24, 1172–1181.

OECD. 2002. Hazard assessment of perfluorooctanesulfonate (PFOS) and its salts. ENV/JM/RD (2002)17.

Seacat AM, Thomford PJ, Hansen KJ, Olsen GW, Case MT, Butenhoff JL. 2002. Subchronic toxicity studies on perfluorooctane sulfonate potassium salt in cynomolgus monkeys. Toxicol Sci 68:249–264.

Sundelin, B., Eriksson, A.K. 1998. Malformations in embryos of the deposit-feeding amphipod Monoporeia affinis in the Baltic Sea. Marine Ecology-Progress Series 171, 165-180.

Sundelin, B., Ryk, C., Malmberg, G. 2000. Effects on the sexual maturation of the sediment-living amphipod Monoporeia affinis. Environmental Toxicology 15, 518-526.

Sundelin, B., Wiklund, A.K.E., Ford, A.T. 2008a. Biological effects of contaminants: the use of embryo aberrations in amphipod crustaceans for measuring effects of environmental stressors. ICES Techniques in Marine Environmental Sciences No. 41, 21 pp.

Wiklund, A.K.E., Sundelin, B. 2001. Impaired reproduction in the amphipods Monoporeia affinis and Pontoporeia femorata as a result of moderate hypoxia and increased temperature. Marine Ecology-Progress Series 222, 131-141.

Wiklund, A.K.E., Sundelin, B. 2004. Biomarker sensitivity to temperature and hypoxia - a seven year field study. Marine Ecology-Progress Series 274, 209-214.

12 Continue compilation of effects of hazardous substances on biodiversity in the Baltic Sea (ToR i)

Because of the lack of meeting time this ToR was decided not to handled by SGEH during this meeting.

13 Assess fish diseases as an indicator of ecosystem health (ToR j)

Detailed information about fish diseases and their use as an indicator of ecosystem health can be found in Annex 14 (ICES SGEH Biological Effects methods Background Documents for the Baltic Sea region: Fish Disease Index - externally visible fish diseases, macroscopic liver neoplasms and liver histopathology) of the present report.

The SGEH Background Document is based on an OSPAR document prepared for the OSPAR Convention area that outlines concepts and methodologies for monitoring and assessment of diseases in wild fish in the frame of general and contaminant-specific biological effects monitoring under the OSPAR CEMP/JAMP. Most of its general principles, information and statements are also applicable to the Baltic Sea and, therefore, it was considered applicable for the SGEH purposes after some modifications taking account specific Baltic Sea requirements.

The procedures for the statistical analysis and assessment of disease data applying the Fish Disease Index (FDI) have been developed by ICES and are originally based on the analysis/assessment of disease data from studies in common dab (*Limanda limanda*) from the North Sea and adjacent areas. However, the analysis and assessment is constructed in a way that it can easily be adapted to be used for other fish species, and ICES WGPDMO is currently working on FDIs for flounder (*Platichthys flesus*) and cod (*Gadus morhua*) in the Baltic Sea, including the definition of BACs and EACs. This process will be finalised until the 2012 meeting of WGPDMO. Once FDIs and assessment procedures exist for Baltic Sea fish, a full background document for fish disease studies in the Baltic Sea can be written.

Germany, Poland and Russia are currently running regular fish disease monitoring studies in the Baltic Sea applying ICES guidelines and disease data have been incorporated in the fish disease database of the ICES Data Centre.

14 Start a discussion on how to evaluate the cumulative effects of impacts from a differing human sources such as contaminants, eutrophication, noise and habitat change/impacts (ToR k)

SGEH was given a task to initiate discussion on this topic on a short notice. Unfortunately, the work schedule of the group was already overloaded no serious effort could be put into this part. However, Elmira Boikova presented an overview of this item.

The reviews indicate that many ecological sub-systems of the Baltic Sea have exhibited non-linear thresholds, and that the common driver of these is climate. It further suggests that multiple drivers – climate and overfishing and/or eutrophication – have potentially eroded the resilience of the systems and hence the capacity to respond to changes in climate. These potentially synergistic drivers of regime shifts differ between sub-basins, indicating that sub-basin dynamics (coastal-offshore interactions) may be stronger than inter-basin dynamics.

A stronger focus on the sub-basin level would allow for enhanced capacity to manage conflict between stakeholders and deal with sub-basin specific multiple drivers of ecological regime shifts. The clarity of a regional management framework (e.g., common methodology and access to data for spatial planning) coupled with a stronger focus on sub-basin monitoring, research, management.

In the Baltic Sea, the extent of bottom hypoxia is interwoven with climate-modulated saline incursions and exchange across the halocline (Conley *et al.*, 2002), as well as organic loading from an enriched pelagic zone. Nutrient budgets indicate that P reflux from sediments is by far the predominant source of P (Savchuk, 2005; Artioli *et al.*, 2008), emphasizing both the role of climate and the legacy of past nutrient enrichment on current ecosystem states. However, no clear trends are observable in cyanobacteria or in situ chlorophyll concentrations. There is also evidence of a regime shift in the Baltic pelagic ecosystem, but the relative roles of climate, eutrophication and fishing pressure are still under debate (Alheit *et al.*, 2005; Østerblom *et al.*, 2007; Møllmann *et al.*, 2008).

The indicators do not suggest recovery in the Baltic despite nearly two decades of policy implementation and economic collapse in former communist countries. An important eutrophication symptom, cyanobacterial blooms are fuelled by sedimentary nutrient recycling and a positive feedback that augments P availability and may imply that the system can be expected to be relatively insensitive to policy measures. A scenario analysis (Wulff*et al.*, 2007) has shown that policy implementation could lead to recovery over a time frame of several decades (Savchuk and Wulff, in press). Thus the commonly felt urgency (HELCOM, 2006) of eutrophication abatement measures may lose its momentum and social acceptance given that a slow system response will not reveal any improvement during a decade or more.

The applicability of biochemical biomarkers as indicators of the biological effects of contamination in a given area depends on the ability to distinguish changes in enzyme activity brought about by chemical contamination from those due to natural variability. Various biotic and abiotic factors are known to affect biomarker responses. Therefore, further research is required to clarify how contaminants, both organic and inorganic, can affect the biomarker level in the Baltic Sea, and whether the signal due to chemical contaminants can be separated from background variations and used for monitoring purposes.

In addition to direct effects, pollutants can also act indirectly, for example by reducing competitive ability (Johnston and Keough, 2003; Perrett *et al.*, 2006). The effects of pollutants can also be both negative and positive (Rygg, 1985). The negative effects may be obvious, but more subtle positive effects, such as correlations between nutrient enrichment and contaminants in natural harbor sediments, may also be important. There are also studies demonstrating that competition modifies an organism's response to toxic disturbances (Johnston and Keough, 2003; Perrett *et al.*, 2006); (*Marenzellaria and Monoporeia affinis*).

Biomagnification of trace elements in the Baltic food chain - of all the trace elements examined, only mercury was biomagnified to any appreciable extent in the food chain (Atwell *et al.*, 1998; Bargagli *et al.*, 1998; Blackmore and Wang, 2004; Bowles *et al.*, 2001a,b; Kidd *et al.*, 2003; Campbell *et al.*, 2005a).

In the deposit-feeding Baltic amphipod *Monoporeia affinis* temperature was found to act synergistically with the fungicide fenarimol resulting in increased numbers of females with dead eggs.

Several studies suggest that although the contaminant levels have decreased in the Baltic ringed seals during the last decades, contaminant-mediated effects are still observed in these seals. This indicates that the health status of the Baltic Sea is far from optimal.

Question: Do we have tools to measure the accident situation in the Baltic?

15 Any other business

15.1 SSGRSP and future of SGEH

Kari Lehtonen introduced a presentation by group member Yvonne Walther, the Chair of the SCICOM Steering Group on Regional Seas Programmes (SSGRSP). Among the main visions listed by SSGRSP, the "Development of Ecosystem Health Issues – biodiversity, monitoring of contaminants and biological effects (guidelines)" and "Open further discussions on multidisciplinary initiatives" where considered as the most relevant concerning SGEH, considering especially how to continue the activities of the group after its termination at the end of 2011.

SSGRSP sees that SGEH has an important role in integrated assessments and MSFD implementation, and is asking about its future form and workplan and how e.g. collaboration with WGIAB could be strengthened. A Workshop on Benchmarking Integrated Ecosystem Assessments (WKBEMIA) is scheduled for autumn 2011 with a meeting in 30 November – 1 December 2011. Its aim is to 1) start a process on how to benchmark Integrated Ecosystem Assessment (IEA) based on results in ongoing Integrated Ecosystem Assessments Expert Groups, 2) make a brief review on the various concepts of Integrated Ecosystem Assessments including an evaluation of suitability to ICES needs in terms Science and Advice, 3) review the Integrated Ecosystem Assessments in the ongoing Regional Expert Groups, with regards to methods, models and results, 4) identify a common framework which will act as a guideline for Integrated Ecosystem assessments performed in ICES, and 5) identify the need of supporting data, processes and products.

As previously, SGEH recommends that other expert groups dealing with eutrophication, dynamics of fish communities, effects of maritime activities and biodiversity issues in the Baltic Sea area become actively involved in building a common ecosystem health assessment strategy for the Baltic. SGEH – or its possible successor starting in 2012 – could in the future act as a "focal point" for collecting information on indicators most relevant indicators of ecosystem health concerning the different fields. The basic structure and strategy in the development of kind of an "Ecosystem Health Assessment Manual". A request of assessment of indicators and assessment criteria (if available) will be distributed to relevant expert groups.

Regarding WKBEMIA, several SGEH members expressed their willingness to participate this activity and also to attend the actual meeting.

15.2 Progress in EUSBSR PA3

Kari Lehtonen presented recent developments in the EU Strategy for the Baltic Sea Region (EUSBSR), the first macroregion strategy of the EU, and especially its Priority Area 3 (PA3),"To reduce the use and impact of hazardous substances". In regard to biological effects monitoring, the BEAST project was in late 2010 nominated as one of the Flagship Projects in PA3. This is hoped to have a positive impact in promoting the theme and in applying for further funding of related projects. Other PA3 Flagships include the following projects and initiatives:

- Assess the need to clean up chemical weapons – Chemical weapons dumps in the Baltic
- Sustainable Management of Contaminated Sediments (SMOCS)
- Development of HELCOM Core Set Indicators (CORESET)
- Control of Hazardous Substances in the Baltic Sea (COHIBA)
- Innovative management of hazardous substances in the Baltic Sea region
- Reduce the use of Substances of Very High Concern (SVHC) in the Baltic Sea region
- Make the Baltic Sea region a lead in sustainable development for pharmaceuticals

SGEH welcomed these news and recommends to find ways to make best use of the Flagship status both in terms of applying funding for the current BEAST activities as well as collaboration with the other Flagship projects.

15.3 Specific presentations

15.3.1 Biomarkers in zooplankton

The presentation had been requested from Kristiina Vuori (University of Turku, Finland) who unfortunately had to postpone he participation to the meeting, and was presented to SGEH by Kari Lehtonen. Zooplankton is an important part of marine food webs channeling energy from primary production to consumers on higher trophic levels. The species composition and nutritional value of zooplankton and the accumulation of harmful substances in zooplankton may have derived consequences to the subsequent food web. Copepods are usually the dominant members of the zooplankton, and are major food organisms for small fish, seabirds and other crustaceans in the sea/ocean and in fresh water. The importance of copepods in the biogeochemical cycles of PAHs, PCBs, BDEs, non-ionic surfactants and synthetic steroids have been reported (Carls, Short & Payne, 2006, Cailleaud, Forget-Leray, Peuhiet *et al.*, 2009, Cailleaud, Budzinski, Le Menach *et al.*, 2009, Tomy, Pleskach, Ferguson *et al.*, 2009, Cailleaud, Budzinski, Lardy *et al.*, 2011). Currently, there is no available published information on the concentrations of hazardous substances in the Baltic Sea zooplankton.

The effects of various hazardous substances on copepods has been studied experimentally, but not in the Baltic Sea species. Various substances are found to cause increases in transcription of oxidative stress defence related mRNAs (e.g. Hansen, Altin, Vang *et al.*, 2008, Hansen, Altin, Booth *et al.*, 2010, Seo, Lee, Rhee *et al.*, 2006, Lee, Raisuddin, Rhee *et al.*, 2008). Field studies regarding the responses of zooplankton to hazardous substances are scarce. Cailleaud *et al.* (Cailleaud *et al.*, 2009b) have investigated biomarker responses on *Eurytemora affinis* and found significant increasing GST activity levels when hydrophobic organic contaminant (HOC) concentrations in the water column were the highest, and lower activity of AChE when HOC concentrations in the water column were the highest.

Limnocalanus macrurus is a dominant copepod zooplankton species found in the pelagic regions in the northern parts of Baltic Sea, in the Gulfs of Bothnia and Finland. It could thus be a suitable example species for studying variation and biological effects of environmental stressors in these sea areas. In a collaborative pilot study (Vuori, K.A., Kanerva, M., Nikinmaa, M., Lehtonen, K.: Studying environmental stress levels in Baltic Sea zooplankton *Limnocalanus macrurus* and *Mysis* sp.) measurements of eight oxidative stress biomarkers have been conducted from pool samples of field collected *Limnocalanus* individuals. The samples were prepared and three to five biomarkers measured onboard R/V *Aranda*. Rapid availability of results may be useful for outreach purposes. These methods and the outcomes of the pilot study could be further applied in studies of environmental stressors in key Baltic sea zooplankton species or zooplankton communities.

References

Cailleaud, K., Budzinski, H., Lardy, S., Augagneur, S., Barka, S., Souissi, S. & Forget-Leray, J. (2011) Uptake and elimination, and effect of estrogen-like contaminants in estuarine copepods: an experimental study. Environmental Science and Pollution Research 18, 226-236.

Cailleaud, K., Budzinski, H., Le Menach, K., Souissi, S. & Forget-Leray, J. (2009) Uptake and elimination of hydrophobic organic contaminants in estuarine copepods: an experimental study. Environmental Toxicology and Chemistry 28, 239-246.

Cailleaud, K., Forget-Leray, J., Peuhiet, L., Lemenach, K., Souissi, S. & Budzinski, H. (2009) Tidal influence on the distribution of hydrophobic organic contaminants in the Seine Estuary and biomarker responses on the copepod *Eurytemora affinis*. Environmental Pollution 157, 64-71.

Carls, M.G., Short, J.W. & Payne, J. (2006) Accumulation of polycyclic aromatic hydrocarbons by *Neocalanus* copepods in Port Valdez, Alaska. Marine Pollution Bulletin 52, 1480-1489.

Hansen, B.H., Altin, D., Booth, A., Vang, S.H., Frenzel, M., Sorheim, K.R., Brakstad, O.G. & Storseth, T.R. (2010) Molecular effects of diethanolamine exposure on *Calanus finmarchicus* (Crustacea: Copepoda). Aquatic Toxicology 99, 212-222.

Hansen, B.H., Altin, D., Vang, S.H., Nordtug, T. & Olsen, A.J. (2008) Effects of naphthalene on gene transcription in *Calanus finmarchicus* (Crustacea : Copepoda). Aquatic Toxicology 86, 157-165.

Lee, K.W., Raisuddin, S., Rhee, J.S., Hwang, D.S., Yu, I.T., Lee, Y.M., Park, H.G. & Lee, J.S. (2008) Expression of glutathione S-transferase (GST) genes in the marine copepod *Tigriopus japonicus* exposed to trace metals. Aquatic Toxicology 89, 158-166.

Seo, J.S., Lee, K.W., Rhee, J.S., Hwang, D.S., Lee, Y.M., Park, H.G., Ahn, I.Y. & Lee, J.S. (2006) Environmental stressors (salinity, heavy metals, H2O2) modulate expression of glutathione reductase (GR) gene from the intertidal copepod *Tigriopus japonicus*. Aquatic Toxicology 80, 281-289.

Tomy, G.T., Pleskach, K., Ferguson, S.H., Hare, J., Stern, G., Macinnis, G., Marvin, C.H. & Loseto, L. (2009) Trophodynamics of Some PFCs and BFRs in a Western Canadian Arctic Marine Food Web. Environmental Science & Technology 43, 4076-4081.

15.3.2 Development in on-line monitoring

The presentation on this subject was presented by Sergey Kholodkevich and Tatiana Kuznetsova. One of the main challenges in monitoring of different water bodies is the development of methodology for the correct assessment of ecosystem state/health. Recently the state of natural aquatic ecosystems, especially in the Baltic Sea where anthropogenic impact is progressively increased, needs permanent monitoring. This requires the development of modern methods for the assessment of ecosystem state and for real prediction of the situation in future. New methods of biomonitoring must include contemporary information about biological effects of the contaminants on organisms habiting studied aquatic ecosystems. Use of biomarker approach in such cases seems to be the most effective and reliable to obtain such information.

Biological methods based on physiological biomarkers of hydrobionts are widely used for these purposes and can provide data to find out whether environmental pollution affects marine species and to estimate the extent of such impact. Recently, the development of techniques have permitted investigations carried out by means of on-line non-invasive methods based on infrared light sensors (Depledge, Andersen, 1990 *et al.*) or on fiber-optic laser method of heart rate (HR) recordings worked out in St.-Petersburg (Kholodkevich *et al.*, 1999 etc.). Behaviour markers are also of great importance, for example, valve gape (VG) recordings are widely used in systems of water quality control (www.MusselMonitor.nl; www.mermayde.nl) based on Hall sensor.

The most well-developed and widely used systems for on-line water bodies monitoring where molluscs are used as biosensors (and their reactions as biomarkers) are the following:

- MusselMonitor (DreissenaMonitor for fresh water); (www.MusselMonitor);
- CAPMON (Depledge, Andersen, 1990);
- ClamMonitor (EPA, 2006);
- Fiber-optic method worked out at SRCES RAS (Kholodkevich *et al.*, 1999).

In all of mentioned above systems sharp HR changes or changes in VG serve as alarm-signal for information about rapid water quality changes.

Recently SRCES RAS in cooperation with IRIS and BIOTAGUARD develop such system based on simultaneously monitoring of VG and CA in *Mytilus edulis, Modiolus modiolus* and *Carcinus maenas* for the purposes of oil-production zones monitoring in the Barents and in the North Seas.

The scheme of application of such systems in industrial active zones is shown in Figure 1.

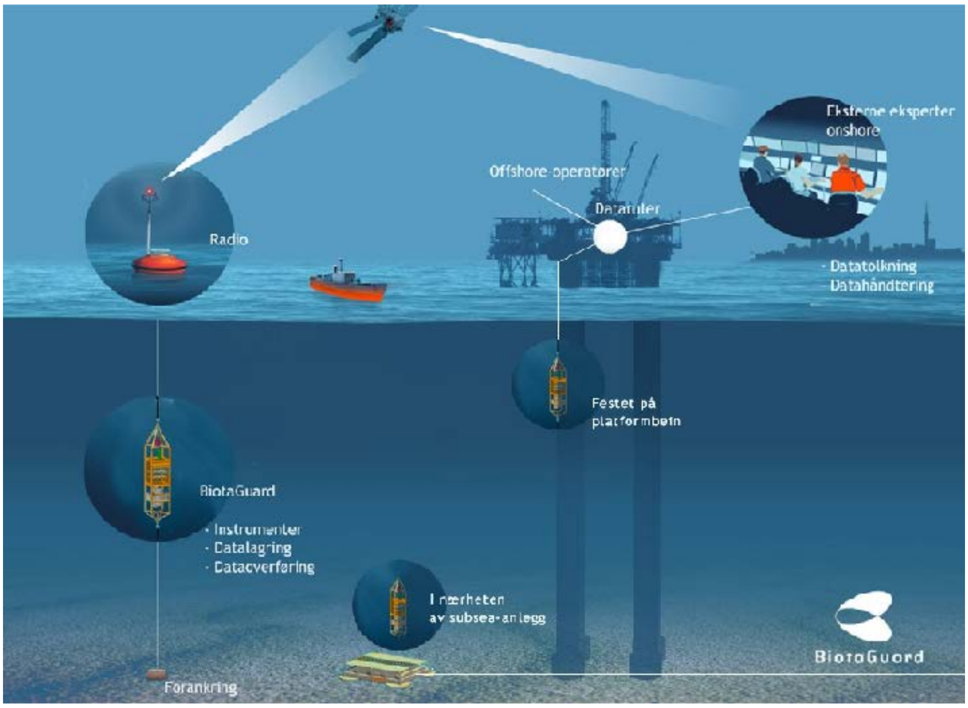

Figure 1. BiotaGuard Arctic Biomonitoring system (2008).

Technology

- Unique integration of biosensor technology and conventional chemical/physical sensor technology;
- Monitors environmental effects continuosly and in real time;
- Robust system that meets oil industry standards and criterias for the marine operations;
- Behaviour and health condition of biosensors (mussels) are monitored by measuring heart rate and valve gape behaviour. Data from biosensors and conventional sensors is transferred real time to Biota Guard Expert Center for analysis, using "e-field" technology;
- "Passive" (not instrumented) mussels are collected on as needed basis for more detailed laboratory analysis of health condition;
- The system can also accomodate other biosensors to represent specific marine conditions in other regions and waterdepths where such organisms are present;
- The technology is patent pending.

Studies by SRCES RAS

Studies during 2 years activities in the frame of the BEAST project have shown the variability of cardiac responses in mussels and crabs sampled from different in ecological status aquatoria of the Baltic Sea. This fact stimulates us to work out some tests (used as functional loading) to study the level of HR compensatory responses after such loading. Patterns of individual HRs were used for evaluation of such a responses and recovery time (Figure 2 A) needed for return (after the tests) of the HRs to their background patterns as well as SD of HRs (Figure 2 B) over a group of tested organisms were discussed as a new possible physiological biomarkers for the assessment of ecosystem status.

A B

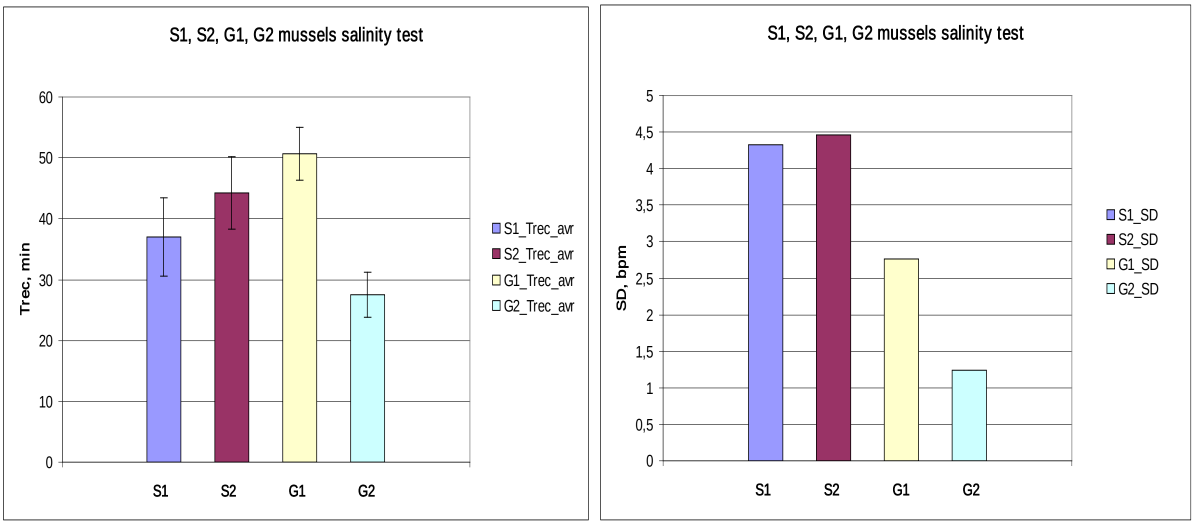

Figure 2. Data of SRCES RAS obtained in caging experiments in the Bothnian Sea expedition 2010. Abbreviations: S1, S2, G1 and G2 – different in ecological status studied sites of the Sea.

Conclusions

Results of different studies in lab and field conditions illustrate how physiological biomarkers applied to different sentinel species can provide a "diagnostic tool for stress" as non-specific response of the organism to contamination of the environment.

Evaluation of systems, indicators and assessment criteria pollution and health indices allow comparison of geographically and biologically different sites of the Baltic Sea for working out integral criteria for ecosystem health assessment.

Studies showed applicability of complex biomarker approach to ecosystem health assessment and necessity to develop systems where mussels could be used as biosensors.

It was concluded that application of a set of physiological biomarkers (i.e., valve gape and cardiac activity) is a useful approach in assessment of general biological effects of the pollution on health status of the invertebrates.

Annex 1: List of participants

Name	Address	Phone/Fax	Email
Andris Andrushaitis	BONUS Baltic Organisation Network for Funding Science EEIG, Hämeeentie 33, FI-00500 Helsinki Finland	+358 40 3528 163 +358 9 4780 0044	andris.andrusaitis@bonuseeig.fi
Maija Balode	Latvian Institute of Aquatic Ecology 8 Daugavgrivas St., LV-1048 Riga Latvia	+ 371 29471203 + 371 67601995 (fax)	maija@hydro.edu.lv
Ieva Barda	University of Latvia Faculty of Biology, 4 Kronvalda Blvd., LV- 1586 Riga Latvia	+ 371 67033878 + 371 67601995 (fax)	ieva.barda@lhei.lv
Elmira Boikova	Institute of Biology University of Latvia Salaspils, Miera 3, LV 2169 Latvia	+371 67945405 + 371 26186712 + 371 267944986 (fax)	elmira@hydro.edu.lv
Henryka Dabrowska	Sea Fisheries Institute in Gdynia ul. Kollataja 1, 31-332 Gdynia Poland	+48 58 735 6205 +48 662 595 601 +48 58 735 6110 (fax)	dabrowska@mir.gdynia.pl
Ulrike Kammann	Ulrike Kammann vTI Institute of Fishery Ecology Palmaille 9 22767 Hamburg Germany	+49 40 38905 198 +49 40 38905 261	ulrike.kammann@vti.bund.de
Sergey Kholodkevich	Scientific Research Center for Ecological Safety RAS Korpusnaya str. 18 RU-197110 St. Petersburg Russia	+7 9112273948 +7 8122307875 +7 8122354361(fax)	kholodkevich@mail.ru
Tatiana Kuznetsova	Scientific Research Center for Ecological Safety RAS Korpusnaya str. 18 RU-197110 St. Petersburg Russia	+7 9500443864 +7 8122307875 +7 8122354361(fax	kuznetsova_tv@bk.ru
Thomas Lang (by correspondece)	Johann Heinrich von Thünen-Institut Institute of Fishery Ecology Deichstrasse 12 D-27472 Cuxhaven Germany	+49 4721 38034 +49 4721 53583 (fax)	thomas.lang@vti.bund.de

Kari Lehtonen	Finnish Environment Institute Marine Research Centre POB 140 FI-00251 Helsinki Finland	+358 40 7030305 +358 9 323 3970 (fax)	kari.lehtonen@ymparisto.fi
Aleksandras Rybakovas	Institute of Ecology of Nature Research Centre Department of Aquatic Ecosystems Laboratory of Genotoxicology Akademijos str. 2 LT–08412, Vilnius–21, Lithuania	+370 5 272 98 95	arybakovas@ekoi.lt
Doris Schiedek	National Environmental Research Institute (NERI), Aarhus University P.O. 358, Frederiksborgvej 399 DK-4000 Roskilde Denmark	+45 46301962 +45 46301914 (fax)	dosc@dmu.dk
Jakob Strand	National Environmental Research Institute (NERI), Aarhus University P.O. 358, Frederiksborgvej 399 DK-4000 Roskilde Denmark	+45 46301865	jak@dmu.dk
Brita Sundelin	Department of Applied Environmental Research Stockholm University Frescativägen 54 A SE-10691 Stockholm Sweden	+46 86747235 +703136059 +46 86747338 (fax)	brita.sundelin@itm.su.se
Yvonne Walther (by correspondence)	Institute of Marine Research Utövägen 5 37137 Karlskrona Sweden	+46 45 51 42 30 +46 45 51 04 84	yvonne.walther@fiskeriverket.se

Annex 2: Draft meeting agenda and timetable of the meeting

ICES Study Group for the Development of Integrated Monitoring and Assessment of Ecosystem Health in the Baltic Sea (SGEH)

11–15 April 2011, Faculty of Geography and Earth Sciences, University of Latvia, Riga, Latvia

DRAFT AGENDA

1. opening of the meeting, including welcome address from the Director of the Institute of Biology, University of Latvia, Prof. Viesturs Melecis;
2. adoption of the agenda;
3. appointment of rapporteurs;
4. review progress in the BEAST project (ToR a);
5. develop background documents for biological effects methods in the Baltic Sea (ToR b);
6. examine the status of assessment criteria for biological effects parameters in the Baltic Sea (ToR c);
7. review developments in MSFD related to the implementation of biological effects methods (ToR d);
8. propose a list of biological effect methods for integrated monitoring and assessments in the Baltic Sea (ToR e);
9. Update project planning for the BONUS call in 2011 (ToR f);
10. review effects of perfluorinated compounds relevant to the Baltic Sea (ToR h);
11. continue compilation of effects of hazardous substances on biodiversity in the Baltic Sea (ToR i);
12. assess fish diseases as in indicator of ecosystem health (ToR j);
13. start a discussion on how to evaluate the cumulative effects of impacts from a differing human sources such as contaminants, eutrophication, noise and habitat change/impacts (ToR k);
14. any other business;
15. recommendations and action list;
16. adoption of the report and closure of the meeting.

Annex 3: Share of work

ToR	Specific items	Reporting
a) review progress in the BEAST project;	General progress WP1 WP2 WP3 Remaining work	Kari Lehtonen Brita Sundelin Ulrike Kammann Jakob Strand Kari Lehtonen
b) develop background documents for biological effects methods in the Baltic Sea;	Lysosomal membrane stability Micronuclei frequency Acetylcholinesterase activity Ethoxyresorufin-O-deethylase activity PAH metabolites Liver nodules External diseases Reproductive success (eelpout) (Comet Assay Metallothionein induction Oxidative stress (several parameters) Glutathione S-transferase activity Reproduction disorders in crustaceans Imposex and intersex in gastropods BIOASSAYS: Whole sediment, amphipods	Katja Broeg Aleksandras Rybakovas Kari Lehtonen Henryka Dabrowska Ulrike Kammann Ulrike Kammann Ulrike Kammann Jakob Strand Henryka Dabrowska, Kari Lehtonen Kari Lehtonen Kari Lehtonen Kari Lehtonen Aleksandras Rybakovas Katja Broeg, Jakob Strand Maija Balode
c) examine the status of assessment criteria for biological effects parameters in the Baltic Sea;	PAH metabolites AChE LMS MN Imposex and intersex in gastropods Reproductive success in eelpout Malformations in crustacean embryos Other? – discussion	Ulrike Kammann Kari Lehtonen Katja Broeg, Jakob Strand Aleksandras Rybakovas Jakob Strand Jakob Strand Brita Sundelin Group Effort
d) review developments in MSFD related to the implementation of biological effects methods;		Group effort (country-wise)
e) propose a list of biological effect methods for integrated monitoring and assessments in the Baltic Sea;	Also: priorisaton of the HELCOM CORESET list	Group effort
f) update project planning for the BONUS call in 2011;	Review the BEAST BSR application (March 2011)	Group effort
g) review biological effects methods applied in ERAs, EIAs and "post-accident" studies;		Kari Lehtonen, Steinar Sanni (by correspondence)
h) biological effects of perfluorinated compounds relevant to the Baltic Sea;		Brita Sundelin
i) continue compilation of effects of hazardous substances on biodiversity in the Baltic Sea;		Doris Schiedek
j) assess fish diseases as in indicator of ecosystem health;		Thomas Lang (by correspondence), Ulrike Kammann
k) EXTRA ToR FROM SSGHIE:		Group effort

start a discussion on how to evaluate the cumulative effects of impacts from a differing human sources such as contaminants, eutrophication, noise and habitat change/impacts.		
Any other business	Future of SGEH and collaborations among ICES EGs (e.g. WGIAB), new scheme	Kari Lehtonen Yvonne Walther (by corresp.) Group work
	Developments in on-line monitoring	Sergey Kholodekevich, Tatiana Kuznetsova
	Biomarkers in zooplankton	Kristiina Vuori (by corresp.), presented by Kari Lehtonen
	Progress in EUSBSR PA3	Kari Lehtonen

Annex 4: Draft work schedule

Date	Approx. Time	Agenda Item	Rapporteurs/ Contributors	Issue/Term of Reference
Monday 11th April	09:00	1	KL, MB, EB	Introduction by Chairman; Host Organisations representatives Elmira Boikova and Maija Balode; housekeeping issues; *tour de table*
	10:00	2	KL	Adoption of agenda
	10:15	3	KL	Appointment of rapporteurs
	10:30	4	KL, BS, UK, JS	**ToR a)** "Review progress in the BEAST project" 1. general progress 2. WP1 3. WP2 4. WP3 5. remaining work
	10:45-11:00			*Coffee break*
	12:30			*Lunch break*
	13:30	4		**ToR a)** contnd.
	14:45			*Coffee break*
	16:00	5	KL, AA?	**ToR f)** "Update project planning for the BONUS call in 2011" 1. general information on the call 2. outlines from BEAST General Meeting 2010 3. BEAST BSR application 2011
		6	Group Effort, EB	**ToR k)** "EXTRA ToR FROM SSGHIE: start a discussion on how to evaluate the cumulative effects of impacts from a differing human sources such as contaminants, eutrophication, noise and habitat change/impacts" (Part 1)
	17/18:00			*Close of business*
Tuesday 12th April	09:00	7	Group Effort	**ToR b)** "Develop background documents for biological effects methods in the Baltic Sea" - Presentation as in the *Share of Work* (or as fit) 1. go through status of documents (presentation & general comments)
	10:45-11:00			*Coffee break*
	12:30			*Lunch break*
	13:30	7		**ToR b)** contnd.
				1. improvement of documents (pair/team work)
	14:45-15:00			*Coffee break*
				2. presentation of final outputs
	17:30			*Close of business*
	ca. 20:00?			*Meeting dinner*

Wednesday 13th April	09:00	8	Group effort (country-wise)	**ToR d)** "Review developments in MSFD related to the implementation of biological effects methods"
	10:45-11:00			*Coffee break*
			TL (corresp.)?	**ToR j)** "Assess fish diseases as in indicator of ecosystem health"
	12:30			*Lunch*
	13:30	9	KL, SS (corresp.)	**ToR g)** "Review biological effects methods applied in ERAs, EIAs and "post-accident" studies"
	14:45-15:00			*Coffee break*
	16:00	10	BS, KL	**ToR h**) "Biological effects of perfluorinated compounds relevant to the Baltic Sea"
				Any other business Presentations as in *Share of Work*
	17:30			*Close of business*
Thursday 14th April	09:00	12	Group effort	**ToR c)** "Examine the status of Assessment Criteria for biological effects parameters in the Baltic Sea" - Presentation as in *Share of Work* (or as fit)
	10:45-11:00			*Coffee break*
	12:30			*Lunch*
	13:30	15	Group effort, DS	**ToR e)** "Propose a list of biological effects methods for integrated monitoring and assessments in the Baltic Sea" - incl. priorisationof the HELCOM CORESET list of biological effects indicators – group work
	14:45-15:00			*Coffee break*
	16:30	6	Group effort, EB	**ToR k)** EXTRA ToR FROM SSGHIE: "Start a discussion on how to evaluate the cumulative effects of impacts from a differing human sources such as contaminants, eutrophication, noise and habitat change/impacts" (Part 2) Contnd. (*if time allows*)
	17:30			*Close of business*
Friday 15th April	10:30	17	Group effort	Adoption of the report
	10:45-11:00			*Coffee break*
	12:30			*Lunch*
	14:00			Closure of the meeting

Annex 5: Supporting Information

Priority:	The activities of SGECOH will lead ICES to progress related to the ecosystem affects of fisheries, especially with regard to the application of the Precautionary Approach. Consequently these activities are considered to have a very high priority.
Scientific justification and relation to action plan:	Action Plan Nos: 1.2, 2.3, 2.6 SGEH will continue its activities, but focusing more on the effects of anthropogenic contaminants at different biological levels. Several countries are conducting or have recently completed significant studies in aspects being potentially of relevance for the integrated assessment. The integrated assessment of WGIAB would benefit from a review of progress and an evaluation of the results obtained. This shall be done to support WGIAB with all availbale information in a structured manner and to help WGIAB in selecting appropriate areas for the integrated assessment. SGEH will directly link with the Baltic Sea BONUS+ Programme project BEAST whose partners form the backbone of the group. SGEH will also link closely with the Baltic Sea BONUS+ Programme project BALCOFISH. SGEH will link with the ICES WGIAB on matters concerning methods of integrated assessments in the Baltic Sea. Key members of the now closed SGEH are also members of WGBEC, and during the annual WG meetings they reported regularly about on-going activities in the Baltic Sea in regard to research and development on biological effects and other issues in relation to Ecosystem Health. SGEH will form an even stronger link between these two ICES groups. SGEH will act as a consultant of HELCOM concerning advice on re-structuring/re-organisation/establishment of integrated biological-chemical monitoring of hazardous substances in the Baltic Sea.
Resource requirements:	The research programmes which provide the main input to this group are underway and resources already committed. The additional resources required to undertake additional activities in the framework of this group are negligible.
Participants:	The Group, apart from appointed national members, will be attended by experts involved in implementation of BONUS +/BEAST project.
Secretariat facilities:	None.
Financial:	No financial implications.
Linkages to advisory committees:	
Linkages to other committees or groups:	There is a very close working relationship the Working Group on Integrated Assessment in the Baltic (WGIAB), WGBEC & SGIMC.
Linkages to other organizations:	The work of this group is closely aligned with similar work in FAO.

Annex 6: Recommendations

Recommendation	For follow up by:
1. Interactions and collaboration among groups dealing with integrated assessments should be strengthened with the aim of harmonisation of targets and methodology.	SSGRSP and EGs within, SGIMC
2. An new expert group focused in facilitating the incorporation of contaminants and their biological effects into holistic, integrated ecosystem assessments in the whole ICES area consisting of experts from the now terminated SGEH and SGIMC as well as WGBEC should be formed.	SSGRSP and EGs within, WGBEC, SGIMC
3. Biological effects methods should be included in the monitoring and assessment toolbox of HELCOM to support (a) the Marine Strategy Framework Directive and (b) the HELCOM Holistic Assessment of the Baltic Marine Environment, thus to comply with BSAP.	HELCOM MONAS

Annex 7: CORESET Hazardous Substances - Biological Effects Indicators

CORESET Hazardous Substances - Biological Effects Indicators

The BONUS+ BEAST project has been requested to propose suitable biological effect core indicators which contribute to fulfil the main HELCOM CORESET objectives, i.e. to develop a Baltic Sea wide set of core indicators in line with the HELCOM monitoring and assessment strategy to follow the effectiveness of the implementation of the Baltic Sea Action Plan (BSAP) and the requirements of the European Union Marine Strategy Framework Directive (MSFD) Descriptor 8.

BSAP defines as main future management tasks the achievement of "good ecological status" and "healthy wildlife" with the main Ecological Quality Objectives:

- Hazardous substances within the marine environment shall not cause irreversible changes in the functioning of the ecosystem and in humans.
- Toxic substances shall not cause sub-lethal, intergenerational or transgenic effects to the health of marine organisms (e.g. reproductive disturbances).

MSFD has the overall objective of achieving or maintaining Good Environmental Status (GES) in Europe's seas by 2020. EU Member States are requested to follow a common approach to evaluate GES in applying a series of environmental targets and associated indicators.

Regarding contaminants in the marine environment, the main aim is that "concentrations of contaminants are at levels not giving rise to pollution effects" (Descriptor 8).

In both BSAP and MSFD (Descriptor 8), the achievement and maintaining of GES implies that it will not be sufficient to monitor and assess concentrations of a selection of contaminants but there is also a need to include biological effects caused by chemical contaminants present in the environment. Otherwise, it will not be possible to assess "healthy wildlife" or "sub-lethal effects".

The need for a combined and integrated monitoring strategy to fulfil MSFD requirements has also been pointed out by the Task Group established to address Descriptor 8. According to their report (2010), "the assessment of GES in relation to contaminants should be based upon monitoring programmes **covering the concentrations of chemical contaminants and also biological measurements relating to the effects of pollutants on marine organisms** in each of the assessment regions. The combination of conventional and newer general methods, responding to a variety of contaminants, and more contaminant-specific effect-based methodologies with the assessment of environmental concentrations of contaminants provides a powerful and comprehensive approach."

The Task Group further recommended that monitoring programmes should:

- include the assessment of concentrations of contaminants in environmental matrices, i.e. biota, sediment, and water as well as the quantification of biological effects of contaminants at different levels of biological organisation;
- indicate the approaching of critical contaminant levels as an early warning of the potential for effects. Where possible, this should also include effects which may be caused by synergistic or cumulative interactions between different contaminants.

The combination and integration of data from chemical and biological effects monitoring is an active area of research within the different Regional Conventions (i.e.

OSPAR, HELCOM, and Barcelona Convention-MEDPOL). In this context, considerable efforts have been made in recent years to develop indicators of ecosystem health using measures of biological effects. It has been a challenge since, with increasing level of biological complexity, the level of ecological relevance of any contaminant-related effect also increases. This is often accompanied by a decrease in the specificity of the agent causing the stress and, hence, a limited understanding of all of the factors influencing the cause and effect relationship. Nevertheless, good progress has been made on how to meet these challenges within several ICES and OSPAR expert groups such as WGBEC, WKIMON and SGIMC. Background Documents and Assessment Criteria for individual biological effect tools have been developed, together with an internationally agreed strategy for integrative sampling and assessment.

Core Indicators to assess health effects of hazardous substances in the Baltic Sea

In an anthropogenically affected ecosystem such as the Baltic Sea, cumulative or synergistic effects of contaminants are to be expected since in most cases no single-source or single-compound pollution but a mixture of contaminants is acting, and this requires an adequate monitoring strategy. Such a strategy should be based on both measurements of specific contaminants as well as measurements to assess biological effects of single substances or mixtures at a level still allowing management actions for improvement (i.e. "early warning"). By assessing biological effects against environmental target levels of response that are indicative of significant harm to the organisms concerned, it is more likely that the prevention of pollution effects occurring at the organism, population, community and ecosystem level is successful.

In the Baltic Sea region, significant progress has been made in the framework of large international research initiatives, i.e. the EU-funded project BEEP and the ongoing BONUS+ projects BEAST and BALCOFISH. Together with information from different national initiatives and monitoring activities in the Baltic Sea countries as well as improved scientific understanding of cause-and-effect relationships (C/E), sufficient data are now available which have been used to develop specific Assessment Criteria for the Baltic Sea, i.e. Background Assessment Criteria (BAC) as well as Environmental Assessment Criteria (EAC)(for details, see Table 1).

A set of Baltic Sea specific Core Indicators has been selected (for details, see Table 2), based on scientific knowledge obtained from many years of research in the Baltic Sea as well as from work done in relevant ICES and OSPAR expert groups, In addition, a set of “Candidate Indicators” has been selected.

The chosen indicators describe either effects caused by mixtures of contaminants or exposure to specific contaminants. They have also been chosen because they indicate adverse effects at different biological levels, i.e. molecular/biochemical/cellular (“early warning”) or individual/population (health and reproductive impairments) levels. Moreover, for these indicators C/E have been studied and EAC have been established (Table 2).

The chosen Core Indicators are the following:

- General stress caused by a range of contaminants ("early warning")
    - *Lysosomal membrane stability (LMS) in fish, bivalves or amphipods*
- Effects caused by genotoxic contaminants ("early warning")
    - *Induction of micronuclei (MN) in fish, bivalves or amphipods*
- Reproductive success impairments caused by a range of contaminants
    - *Embryo aberrations in fish (eelpout) or amphipods*
- General health status
    - *Fish Disease Index* based on externally visible fish diseases, macroscopic liver neoplasms and liver histopathology

In addition, two contaminant-specific biological effects indicators, *imposex in marine gastropods* and *PAH metabolites in fish*, have been included as part of the Core Indicators for TBT and PAH, respectively.

Selection criteria

All of the selected Core Indicators for biological effects (Table 2) are established methods recommended by ICES SGIMC and ICES WGBEC. They are already included in other regional monitoring programmes such as OSPAR or MEDPOL or used in National Monitoring programmes (DK, SE or DE).

For all of the listed indicators:

- sufficient research or monitoring data are now available, covering different Baltic Sea subregions;
- Baltic Sea specific BAC have been established for a range of Baltic Sea indicator species (Table 1);
- biological effects can be assessed against threshold levels (EAC) of response that are indicative of significant harm to the species under investigation (Table 1);
- monitoring guidelines including published Standard Operation Protocols (SOPs, e.g. in the ICES TIMES series) are available and widely applied;
- costs of analyses are low and samples can be collected during the same sampling campaigns as for the contaminant analyses (mostly using the same target species, size classes etc.).

In the second part of Table 1 selected “Candidate Indicators" for biological effects are listed. All these are also well-established methods but still need some elaboration since not all the selection criteria listed above are yet fulfilled in regard to the Baltic Sea. However, they are also addressed in the BEAST and BALCOFISH projects and Assessment Criteria for these indicators will be available at the end of these projects.

Inhibition of *acetylcholinesterase activity* is a common indicator of neurotoxic effects. Induction of *EROD (ethoxyresorufin-O-deethylase) activity* indicates exposure to organic compounds such as PAHs, planar PCBs and dioxins. *Intersex/vitellogenin induction in male fish* results from exposure to estrogenic substances.

Conclusion

The inclusion of biological effects indicators in a combined monitoring strategy strengthens the assessment of contaminants that are approaching critical concentration levels that give rise to pollution effects in the Baltic Sea. Their inclusion into the future HELCOM MONAS programme enables detection of combined effects of complex mixtures of hazardous substances, which is the norm for the Baltic Sea.

If using only contaminant-specific effect methods – such as imposex in marine gastropods and PAH metabolites in fish – a much larger number of indicators have to be applied to cover the effects of all relevant groups of hazardous substances potentially present in the marine environment. Most of them would still be neglected since some of the process-related chemicals released to the Baltic Sea are presently unidentified.

Effect indicators with a wider detection spectrum pinpoint the need for further investigations to identify the chemicals causing the effects observed, and in this way lead the way to direct management actions.

As stated in the MSFD Task Group 8 report, "occurrence of adverse effects at various levels of biological organisation needs to be avoided; monitoring schemes should also indicate the approaching of critical values as early warning". HELCOM has adopted a vision of an ecosystem approach defined as "the comprehensive integrated management of human activities based on the best available scientific knowledge about the ecosystem and its dynamics, in order to identify and take action on influences which are critical to the health of marine ecosystems". Inclusion of the proposed set of Core Indicators of biological effects into the future HELCOM MONAS programme will not only support the fulfilment of the BSAP but it will also be an important step towards harmonisation to the greatest possible degree between assessments of different sea areas in Europe; eventually this will allow for comparisons on larger scales and, thus, implementing an important goal of the MSFD.

Annex 8: Numerical Assessment Criteria (AC) for Baltic Sea organisms to assess biological effects

Numerical Assessment Criteria (AC) for Baltic Sea organisms to assess biological effects. Values for Background Assessment Criteria (BAC) and Environmental Assessment Criteria (EAC) are given where available or relevant. Full details of AC and how they have been derived can be found in the ICES SGIMC 2010, SGIMC 2011 and WKIMC 2009 reports on the ICES website and in the SGEH Background Documents for the individual biological effects methods. NOTE: All missing information will soon be available from the output of the ongoing projects mentioned.

Biological effect method (unit, other information)	**Target species/ tissue/endpoint**	**BAC**	**EAC**
Lysosomal membrane stability (LMS)			
minutes	Herring and eelpout		
a. Cytochemical method	- liver	15	8
	Perch and flounder		
	- liver	20	10
	All other species studied	20	10
	- liver, digestive gland		
Method modification: Acridine Orange	Amphipods	under development in BEAST	under development in BEAST
b. In vivo method (Neutral Red Retention test)	*Mytilus* spp.		
	- haemocytes	120	50
Micronuclei frequency (MN) $^0/_{00}$	*Mytilus* spp.		
	- gill cells	2.5	under development in
	- haemocytes	2.5	BEAST & GENCITOX
	Macoma balthica - gill cells	2.9	under development in BEAST & GENCITOX
	Flounder - erythrocytes	0.3	under development in BEAST & GENCITOX
	Eelpout - erythrocytes	0.4	under development in BEAST & GENCITOX
	Cod - erythrocytes	0.4	under development in BEAST
	Herring - erythrocytes	0.4	under development in BEAST & GENCITOX
	Perch - erythrocytes	0.3	under development in BEAST
	Dab - erythrocytes	0.5	Under development in GENCITOX
Intersex in fish *% prevalence*	Flounder, cod and eelpout	5	Not determined
Reproductive success in fish: eelpout *mean frequency (%)*	Malformed larvae	1	under development in BALCOFISH
	Late dead larvae	2	
	Growth retarded larvae	4	
Reproductive succes in amphipods *95th percentile of % malformed embryos*	*Monoporeia affinis*, *Pontoporeia femorata*, *Gammarus* sp., *Corophium* sp.,	2.9	5.7

	Bathyporeia sp.		
Ethoxyresorufin-*O*-deethylase activity (EROD) *[pmol/min/mg protein]* pmol/min/ mg S9 protein *pmol/min/ mg microsomal protein	Flounder (male)	24	under development in BEAST
	Eelpout	10 (Belt Sea data)	under development in BEAST
	Perch	Not determined	Not determined
	Herring	under development BEAST	under development BEAST
PAH bile metabolites *(1)ng/ml (HPLC-F)* *(2) pyrene-type µg/ml (Synchronous Scan Fluorescence at 341/383 nm)* *(3 ng/g (GC/MS)* *1-OH pyrene **1-OH phenanthrene	Herring	under development (BEAST data; 1-OH pyrene, 1-OH-phenanthrene)	under development (BEAST data; 1-OH pyrene: 20 x BAC, 1-OH phenanthrene: 200 x BAC
	Perch	under development BEEP data; FAC, 1-OH pyrene)	under development (BEEP data; FAC, 1-OH pyrene): 20 x BAC
	Cod	21 (1) * 2.7 (1) ** 1.1 (2)	483 (3) * 528 (3) ** 35 (2)
	Flounder	16 (1) * 3.7 (1) ** 1.3 (2)	745* (as for halibut) 909* (as for turbot) 29(2)
	Eelpout	under development (BEEP, BEAST and BALCOFISH data)	under development (BEEP, BEAST and BALCOFISH data); 1-OH pyrene 20 x BAC, 1-OH-phenanthrene 200 x BAC
Acetylcholinesterase activity (AChE) *[nmol/min/mg protein]*	*Mytilus* spp. - gill tissue	under development (Finnish and and German seasonal data;, cf. OSPAR temperature corrected values	under development (Finnish and German seasonal data;, cf. OSPAR temperature corrected values)
	Macoma balthica - muscle tissue (foot)	under development (Finnish seasonal data)	0.7 x BAC
	Herring - muscle tissue	under development (BEAST data) Seasonal BAC values	0.7 x BAC (cf. OSPAR AC) Seasonal BAC values
	Flounder - muscle tissue	under development (BEEP, BEAST and Polish data) Seasonal BAC values	0.7 x BAC (cf. OSPAR AC) Seasonal BAC values
	Eelpout - muscle tissue	under development (BEEP & BEAST data)	0.7 x BAC (cf. OSPAR AC) Seasonal BAC values
	Perch - muscle tissue	under development (BEEP data) Seasonal BAC values	0.7 x BAC (cf. OSPAR AC) Seasonal BAC values
Fish Disease Index	Flounder and cod	under development in	under development in

index value		ICES WGPDMO (BEEP & BEAST data, data from national monitoring in DE, PL and RU)	ICES WGPDMO (BEEP & BEAST data, data from national monitoring in DE, PL and RU)
Imposex and intersex in marine snails *index value* *(VDIS*=vas deferens *sequence index, ISI= intersex stage)* ** Equivalent index values of EAC for VDSI or ISI for relevant species. Harmonised to Class B (OSPAR) for imposex in dog whelk, which represents the most sensitive species and is also in line with WFD EQS for TBT levels.*	Dog whelk (VDSI)	0.3	2*
	Netted whelk (VDSI)	-	0.3*
	Common whelk (VDSI)	-	0.3*
	Red whelk (VDSI)	0.3	2*
	Periwinkle (ISI)	-	(not applicable)*

Annex 9: Overview of Core and selected Candidate Indicators to assess the effects of hazardous substances at different biological levels

CORE INDICATORS	**Used by** **OSPAR** **CEMP or pre-CEMP** **MEDPOL**	**1) Used in national monitoring programmes in Baltic Sea countries** **2) Research monitoring data available**	**Studied in large Baltic Sea research projects**	**Biological Indication (BI)** **Cause/effect (C/E) relationship**	**Availability of AC specific for the Baltic Sea (source)** *see Table 2*	**Monitoring Guidelines, other important info** **QA** **Method costs** **(High-Medium-Low)**
Lysosomal membrane stability	pre-CEMP MEDPOL (1º Tier)	1) DK 2) DE, FI, SE	BEEP BEAST	BI – general stress, early warning C/E – YES for different species (see Background document)	BAC and EAC (OSPAR/BEAST)	ICES TIMES 36 UNEP-MAP, 1999 QA –YES Low costs
Micronucleus test	MEDPOL (2º Tier)	2) LT, PL, SE, RU	BEEP BEAST	BI – genotoxicity effects C/E – YES for different species (see Background document)	BAC, EAC under development (BEAST)	ICES TIMES in prep. QA –YES Low costs
Reproductive success in a) fish (eelpout) b) amphipods	pre-CEMP (eelpout)	Eelpout 1) SE, DK 2) DE Amphipods 1) SE 2) DK, RU	a) BEEP BEAST BALCOFISH b) BEAST	BI – general, ecologically relevant, link to population. C/E – YES for amphipods and eelpout (see Background document)	a) BAC, EAC under development (BEAST and BALCOFISH) a) BAC and EAC	a) Swedish and Danish guidelines, ICES TIMES (in prep.) b) ICES TIMES 41 QA – YES (eelpout) QA – YES (amphipods) Medium costs

Fish Disease Index	Pre-CEMP	1) DE, PL, RU	BEEP BEAST	BI – general stress C/E ???? See Background document	BAC and EAC (source to be mentioned here?)	OSPAR JAMP, Technical Annexes 7,8,9 ICES Advice 2007 ICES TIMES 19, 38 QA – YES Medium costs

CANDIDATE INDICATORS	**Used by OSPAR** **CEMP or pre-CEMP** **MEDPOL**	**1) Used in national monitoring programmes in Baltic Sea countries** **2) Research monitoring data available**	**Studied in large Baltic Sea research projects**	**Biological Indication (BI)** **Cause/effect (C/E) relationship**	**Availability of AC specific for the Baltic Sea (source)**	**Monitoring Guidelines, other important info** **QA** **Method costs** **(High-Medium-Low)**
Intersex or vitellogenin induction in male fish		2) DE, SE, DK	BEEP BEAST BALCOFISH	BI – exposure to estrogenic contaminants	BAC, EAC under development (BEAST)	ICES TIMES 31 QA–NO Low costs
Acetylcholin-esterase inhibition	MEDPOL (2º Tier)	2) FI, LV, DE, SE	BEEP BEAST	BI – neurotoxic effects, early warning C/E – YES (see Background document)	BAC and EAC	ICES SGIMC 2010 QA –YES Low costs
Ethoxyresorufin-*O*-	pre-CEMP	1) DK, SE,	BEEP	BI – to Ah-receptor ac-	BAC, EAC under	ICES TIMES 13 and 23

deethylase (EROD) activity		2) PL, FI, Pl, EE	BEAST	tive chemicals such as PAH, planar PCB, and dioxins (see Background document)	development (BEAST)	QA-YES Low costs

Abbreviations

- BAC – Background Assessment Criteria
- EAC – Environmental Assessment Criteria (concentrations above which there is concern that negative effects might be observed in marine organisms. EAC describes the direct linkage to adverse health effects of the individuals)
- AC – Numerical Assessment Criteria
- QA – Quality Assurance measures
- CEMP – guidelines, quality assurance and assessment tools are in place - monitoring of the component is mandatory for OSPAR contracting parties
- pre-CEMP – agreed to be included as components of the CEMP, guidelines, QA tools and/or assessment tools are currently not all in place. Monitoring of the components is voluntary on a temporary basis

Country codes: DK-Denmark, EE-Estonia, FI-Finland, DE-Germany, LV-Latvia, LT-Lithuania, PL-Poland, RU-Russia, SE-Sweden

Annex 10: ICES SGEH Biological Effects methods Background Documents for the Baltic Sea region*

Lysosomal membrane stability

***ICES/OSPAR document from the ICES SGIMC Report 2010, complemented and modified by SGEH 2011 with information relevant for application in the Baltic Sea region**

Background

Lysosomal functional integrity is a generic common target for environmental stressors in all eukaryotic organisms from yeast and protozoans to humans (Cuervo, 2004), that is evolutionarily highly conserved. The stability of lysosomal membranes (lysosomal membrane stability, LMS) is a good diagnostic biomarker of individual health status (Allen and Moore, 2004; Broeg *et al.*, 2005; Köhler *et al.*, 1992, Lowe *et al.*, 2006). Dysfunction of lysosomal processes has been mechanistically linked with many aspects of pathology associated with toxicity and degenerative diseases (Cuervo, 2004; Köhler, 2004; Köhler *et al.*, 2002; Moore *et al.*, 2006a, b, Broeg 2010). Lysosomes are known to accumulate many metals and organic xenobiotics. Metals such as copper, cadmium and mercury are known to induce lysosomal destabilisation in mussels (Viarengo *et al.*, 1981, 1985a, b). LMS is strongly correlated with the concentration of PAHs and PCBs in mussel tissue (Cajaraville *et al.*, 2000; Krishnakumar *et al.*, 1994; Moore, 1990; Moore *et al.*, 2006a, b; Viarengo *et al.*, 1992, Strand *et al.*, 2009), as well as organochlorines and PCB congeners in the liver of fish (Köhler *et al.*, 1992, Broeg *et al.*, 2002). LMS of various species of mussel and fish from different climate zones clearly reflect gradients of complex mixtures of chemicals in water and sediments (Da Ros *et al.*, 2002; Pisoni *et al.*, 2004; Schiedek *et al.*, 2006, Barsiene *et al.*, 2006; Sturve *et al.*, 2005), point sources of pollution, single pollution events and accidents (Garmendia *et al.*, 2011; Einsporn *et al.* 2005; Broeg *et al.*, 2002, Broeg *et al.*, 2008, Nicholson and Lam, 2005) and also serves for the discovery of new "Hot Spots" of pollution (Bressling, 2006; Moore et. al., 1998; 2004a).

LMS can also be used as a prognostic tool, able to predict liver damage and tumour progression in the liver of various fish species (Broeg *et al.*, 1999; Diamant *et al.*, 1999; Köhler *et al.*, 2002; Köhler, 2004, Broeg, 2010). Also hepatopancreatic degeneration in molluscs, coelomocyte damage in earthworms, enhanced protein turnover as a result of radical attack on proteins, and energetic status an indicator of fitness of individuals within a population can be predicted (Allen and Moore, 2004; Kirchin *et al.*, 1992; Köhler *et al.*, 2002; Moore *et al.*, 2004a, 2006a; Nicholson and Lam, 2005; Svendsen and Weeks, 1995; Svendsen *et al.*, 2004). Recently it is tested for its prognostic potential with respect to reproductive disorders in amphipods in the Baltic Sea. For eelpout, this prognostic potential has already been demonstrated. Low membrane stabilities coincided with distinct reproductive disorders that indicated adverse effects at the population level (Broeg and Lehtonen, 2006).

Thus, LMS has been adopted by UNEP as part of the first tier of techniques for assessing harmful impact in the Mediterranean Pollution programme (MEDPOL Phase IV) and is also recommended as biomarker to be included into the OSPAR Coordinated Environmental Monitoring Programme (pre-CEMP). LMS of blue mussel from the Inner Danish waters and the Danish Belt Sea is part of the Danish monitoring programme NOVANA since 2003 (Strand *et al.* 2009). It is also under consideration for the Swedish monitoring programme (Granmo, pers. comm.). Methods applied to assess LMS are the Neutral Red Retention test (NRR) on living cells like mussel haemocytes, and the cytochemical test on serial cryostat sections performed from snap-frozen tissue. These methods are described in detail by Moore *et al.* (2004b). Currently a new method is developed for the assessment of LMS in single tissue sections of small indicator species like amphipods (Broeg and Schatz, in prep.).

Beside LMS, adverse lysosomal reactions to xenobiotic pollutants include swelling, lipidosis (pathological accumulation of lipid), and lipofuscinosis (pathological accumulation of age/stress pigment) in molluscs but not fish (Köhler *et al.* 2002; Moore, 1988; Moore *et al.*, 2006a, b; Viarengo *et al.*, 1985a). LMS in blue mussels is correlated with oxygen and nitrogen radical scavenging capacity (TOSC), protein synthesis, scope for growth and larval viability and inversely correlated with DNA damage (incidence of micronuclei), lysosomal swelling, lipidosis and lipofuscinosis (Dailianis *et al.*, 2003; Kalpaxis *et al.*, 2004; Krishnakumar *et al.*, 1994; Moore *et al.*, 2004a, b, 2006a; Regoli, 2000; Ringwood *et al.*, 2004). In fish liver, LMS is strongly correlated with a suppression of the activity of macrophage aggregates, and lipidosis (Broeg *et al.*, 2005).

A conceptual mechanistic model has been developed linking lysosomal damage and autophagic dysfunction with injury to cells, tissues and the whole animal; and the complementary use of cell-based bioenergetic computational model of molluscan hepatopancreatic cells that simulates lysosomal and cellular reactions to pollutants has also been demonstrated (Allen and McVeigh, 2004; Lowe, 1988; Moore *et al.*, 2006a, b, c). Various biomarker indices and decision support systems have been developed based on LMS as "guiding" parameter to interpret the results of other biomarkers (Figure 1) which show "bell-shaped" responses since it reflects deleterious effects of various classes of contaminants in an integrative linear manner (Dagnino *et al.*, 2007, Broeg *et al.*, 2005, Broeg and Lehtonen, 2006).

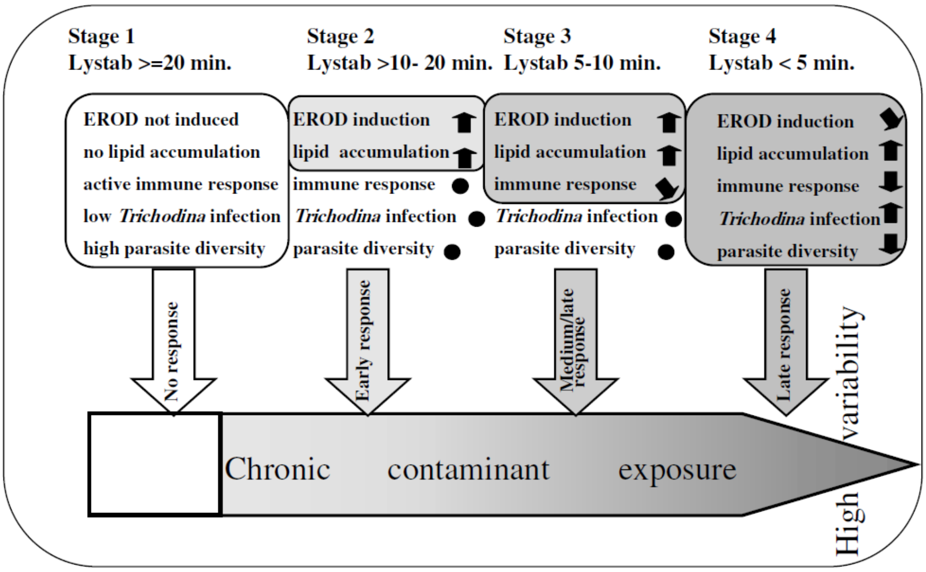

Figure 1. Progression of biological effects detected on the basis of LMS in individual flounder of the German Bight (Broeg *et al.*, 2005).

Confounding factors

LMS is an integrative indicator of individual health status and will be affected also by non-contaminant factors such as severe nutritional deprivation, severe hyperthermia, prolonged hypoxia, and liver infections associated with high densities of macrophage aggregates (Moore *et al.*, 1980; Moore *et al.*, 2007, Broeg, 2010). Processing for neutral red retention (NRR) in samples of molluscs adapted to low salinity environments should use either physiological saline adjusted to the equivalent ionic strength or else use ambient filtered seawater. The major confounding factor in respect of biomonitoring is the adverse effect of the final stage of gametogenesis and spawning in mussel, which is a naturally stressful process (Bayne *et al.*, 1978). In general, this period should be avoided anyway for sampling purposes, as most physiological processes and related biomarkers are adversely affected (Moore *et al.*, 2004b).

However, for fish, spawning has only a minimal effect on LMS and does not mask harmful chemical induced damage to LMS (Köhler, 1990, 1991). Salinity changes didn't provoke significant effects on LMS in flounder (Broeg, unpublished results).

Using the cytochemical approach, temperature stress during the tissue incubation at 37° has to be considered when working with animals from subpolar and polar regions. For these animals, temperature stress leads to a significant decrease of LMS. In this case, temperature during incubation should not be higher than 20° above the ambient temperature of the sampling location to avoid effects of too severe hyperthermia.

Ecological relevance

Lysosomal integrity is directly correlated with physiological scope for growth (SFG) and is also mechanistically linked in terms of the processes of protein turnover (Allen and Moore, 2004; Moore *et al.*, 2006a). Ringwood *et al.* (2004) have also shown that LMS in parent oysters is directly correlated with larval viability. It is also inversely correlated with reproductive disorders in eelpout (Broeg and Lehtonen, 2006). Finally, LMS is directly correlated with diversity of macrobenthic organisms in an investigation in Langesund Fjord in Norway (Moore *et al.*, 2006b), and with parasite species diversity in flounder from the German Bight (Broeg *et al.*, 1999).

Quality Assurance

Intercalibration exercises for LMS techniques have been carried out in the ICES/UNESCO-IOC-GEEP Bremerhaven Research Workshop, the UNEP-MEDPOL programme, in the framework of the EU-project BEEP and the BONUS+ project BEAST as well as for the neutral red retention method in the GEF Black Sea Environmental Programme (Köhler *et al.*, 1992; Lowe *et al.*, 1992; Moore *et al.*, 1998; Viarengo *et al.*, 2000, BEEP, 2004). The results from these operations indicated that both techniques could be used in the participating laboratories in an effective manner with insignificant inter-laboratory variability.

Comparisons of the cytochemical and the neutral red retention techniques have been performed in fish liver (ICES-IOC Bremerhaven Workshop, 1990) and in mussels experimentally exposed to PAHs (Lowe *et al.*, 1995). An AWI/Imare international workshop on "Histochemistry of lysosomal disorders as biomarkers in environmental monitoring" in Bremerhaven, 2008, demonstrated good correspondence of results obtained by the participants by applying various different assessments by computer assisted image analysis and light microscopy. In 2010, an ICES\OSPAR Workshop on Lysosomal Stability Data Quality and Interpretation (WKLYS) has been held in Alessandria, Italy (ICES, 2010). This workshop concentrated on the NRR.

Guidelines for LMS procedures are published as ICES Times Series (Moore *et al.*, 2004b) and in the UNEP/Ramoge biomarker manual (UNEP, 1999).

Background responses and Assessment Criteria

Health status thresholds for NRR and cytochemical methods for LMS have been determined from data based on numerous studies (Cajaraville *et al.*, 2000; Moore *et al.*, 2006a, Broeg *et al.*, 2005, Broeg and Lehtonen, 2006).

LMS is a biophysical property of the bounding membrane of lysosomes and appears to be largely independent of taxa. In all organisms tested to date, which includes protozoans, annelids (terrestrial and marine), molluscs (freshwater and marine), crustaceans (terrestrial and aquatic), echinoderms and fish, the absolute values for measurement of LMS (NRR and cytochemical method) are directly comparable. Furthermore, measurements of this biomarker in animals from climatically and physically diverse terrestrial and aquatic ecosystems also indicate that it is potentially a universal indicator of health status. For the cytochemical method animals are considered to be healthy if the LMS is ≥20 minutes; stressed but compensating if <20 but ≥10 minutes and severely stressed and probably exhibiting pathology if <10 minutes (Moore *et al.*, 2006a, Broeg *et al.*, 2005, Broeg and Lehtonen, 2006). Similarly for the NRR method, animals are considered to be healthy if NRR is ≥120 minutes; stressed but compensating if <120 but ≥50 minutes and severely stressed and probably exhibiting pathology if <50 minutes (Moore *et al.*, 2006a).

The use of different fish species as indicators at identical locations in the Baltic Sea showed species differences with respect to their liver LMS in the following order:

Herring < Eelpout < Dab < Flounder

At locations which are higher affected by anthropogenic impact, differences are pronounced. Potential causes are higher fishing stress and high frequencies and intensities of parasite infections in almost all livers of herring and eelpout as confounding factors. Thus, for these species the assessment criteria for the cytochemical test are defined as follows: animals are considered to have no toxically-induced stress if the LMS is ≥15 minutes; are stressed but compensating if <15 but ≥8 minutes and are severely stressed and probably exhibiting irreversible toxicopathic alterations if <8 minutes (Broeg *et al.*, in prep.).

The following species have been tested as indicator species for LMS in the different regions of the Baltic Sea:

Fish	Herring (*Clupea harengus*), flounder (*Platichthys flesus*), eelpout (*Zoarces viviparus*), dab (*Limanda limanda*).
Bivalves	Blue mussel (*Mytilus edulis, Mytilus trossulus*)
Amphipods	Gammarids, *Monoporeia affinis*

Acknowledgements

Research on the application of LMS as a biomarker for the monitoring and assessment of general toxic effects in different Baltic Sea regions and indicator organisms has been funded by the EU Project BEEP Contract n° ECK3-CT2000-00025, and the BONUS+ Project BEAST.

References

Allen, J.I. and Moore, M.N. 2004. Environmental prognostics: is the current use of biomarkers appropriate for environmental risk evaluation. Mar. Environ. Res., 58: 227–232.

Allen, J.I. and McVeigh, A. 2004.Towards computational models of cells for environmental toxicology. J. Mol. Histol., 35: 697–706.

Barsiene, J., Lehtonen, K. K., Koehler, A., Broeg, K., Vuorinen, P. J., Lang, T., Pempkowiak, J., Balk, L., Syvokiene, J., Dedonyte, V., Rybakovas, A., Repecka, R., Vuontisjärvi, H. and Kopecka, J. 2006. Biomarker responses in mussel (*Mytilus edulis*) and flounder (*Platichthys flesus*) in the Klaipeda-Butinge area (Baltic Sea). Mar. Poll. Bull., 53: 422–436.

BEEP 2004.Biological Effects of Environmental Pollution in marine coastal ecosystems (BEEP), Contract n° ECK3-CT2000-00025,final report, 238pp.

Bressling J. 2006. Natural and toxic stress indicator in blue mussel *Mytilus edulis* along an offshore-inshore gradient (Weser estuary). Master's Thesis, University of Oldenburg, pp122.

Broeg, K., Zander, S., Diamant, A., Körting, W., Krüner, G., Paperna, I. and von Westernhagen, H. 1999. The use of fish metabolic, pathological and parasitological indices in pollution monitoring. I. North Sea. Helgoland Mar. Res., 53: 171–194.

Broeg, K., Köhler, A. and von Westernhagen, H. 2002. Disorder and recovery of environmental health monitoring by means of lysosomal stability in liver of European flounder (*Platichthys flesus* L.). Mar. Environ. Res., 54: 569–573.

Broeg, K., Westernhagen, H. V., Zander, S., Körting, W., Koehler, A. 2005. The "Bioeffect Assessment Index"- A concept for the quantification of effects of marine pollution by an integrated biomarker approach, Marine pollution bulletin, 50(5), 495-503.

Broeg, K., Lehtonen, K. K. 2006. Indices for the assessment of environmental pollution of the Baltic Sea coasts: Integrated assessment of a multi-biomarker approach, Marine pollution bulletin, 53, 508-522.

Broeg, K., Kaiser, W., Bahns, S. and Köhler, A. 2008.The liver of wrasse - morphology and function as a mirror of point source chemical impact. Marine Environmental Research 66, 191-192.

Broeg, K. 2010. The activity of macrophage aggregates in the liver of flounder (*Platichthys flesus*) and wrasse (*Symphodus melops*) is associated with tissue damage Mar.Environ.Res. 69, 14-16.

Broeg, K., Schatz, S. 2011. A new method for the in situ assessment of lysosomal membrane stability using acridine orange (AO) in cryotat sections of snap-frozen organisms and tissue samples, in prep.

Broeg, K., Gschwind, M., Lang, T., Bahns, S. 2011. Lysosomal membrane stabilities in various fish species from the Baltic Sea-Species differences and potential causes. In prep.

Cajaraville, M.P., Bebianno, M.J., Blasco, J., Porte, C., Sarasquete, C. and Viarengo, A. 2000. The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian peninsula: a practical approach. Sci. Tot. Environ. 247: 295–311.

Cuervo, A.M. 2004. Autophagy: in sickness and in health. TRENDS in Cell Biology, 14, 70–77.

Dagnino A., Allen, J.I., Moore M., Broeg, K., Canesi L. and Viarengo A. 2007. Integration of biomarker data into an organism health index: development of an expert system and its validation with field and laboratory data in mussels. Biomarkers, 12(2)155-72.

Dailianis, S., Domouhtsidou, G.P., Raftopoulou, E. Kaloyianni, M. and Dimitriadis, V.K. 2003. Evaluation of neutral red retention assay, micronucleus test, acetylcholinesterase activity and a signal transduction molecule (cAMP) in tissues of *Mytilus galloprovincialis* (L.), in pollution monitoring. Mar. Environ. Res, 56: 443–470.

Da Ros, L., Meneghetti, F. and Nasci, C. 2002. Field application of lysosomal destabilisation indices in the mussel *Mytilus galloprovincialis*: biomonitoring and transplantation in the Lagoon of Venice (north-east Italy), Mar. Environ. Res., 54: 817–822.

Diamant, A., Banet, A., Paperna, I., Westernhagen, H. v., Broeg, K., Krüner, G., Körting, W., Zander, S. 1999. The use of fish metabolic, pathological and parasitological indices in pollution monitoring II. Red Sea and Mediterranean Sea, Helgoland marine research, 53, 195-208.

Einsporn, S., Broeg, K. and Köhler, A. 2005. The Elbe flood 2002-toxic effects of transported contaminants in flatfish and mussel of the Wadden Sea. Mar. Pollut. Bull., 50: 423–429.

Garmendia, L., Izagirre, U., Cajaraville, M.P., Marigómez, I. 2011. Application of a battery of biomarkers in mussel digestive gland to assess long-term effects of the Prestige oil spill in Galicia and the Bay of Biscay: Lysosomal responses.J Environ Monit. 5;13(4):901-14.

ICES. 2010. Report of the ICES\OSPAR Workshop on Lysosomal Stability Data Qual-ity and Interpretation (WKLYS), 13–17 September 2010, Alessandria, Italy. ICES CM 2010/ACOM:61. 57 pp.

Kalpaxis, D.L., Theos, C., Xaplanteri, M.A., Dinos, G.P., Catsiki, A.V. and Leotsinidis, M. 2004. Biomonitoring of Gulf of Patras, N. Peloponnesus, Greece. Application of a biomarker suite including evaluation of translation efficiency in *Mytilus galloprovincialis* cells. Environ. Res., 94: 211–220.

Kirchin, M.A., Moore, M.N., Dean, R.T. and Winston, G.W. 1992. The role of oxyradicals in intracellular proteolysis and toxicity in mussels. Mar. Environ. Res., 34: 315–320.

Köhler, A., 1990. Identification of contaminant-induced cellular and subcellular lesions in the liver of flounder (*Platichthys flesus* L.) caught at differently polluted estuaries. Aquat. Toxicol. 16: 271–294.

Köhler, A. 1991. Lysosomal perturbations in fish liver as indicator for toxic effects of environmental pollution. Comp. Biochem. Physiol., 100C: 123–127.

Köhler, A., Deisemann, H. and Lauritzen, B. 1992. Ultrastructural and cytochemical indices of toxic injury in dab liver. Mar. Ecol. Prog. Ser., 91: 141–153.

Köhler, A. and Pluta, H.J., 1995. Lysosomal injury and MFO activity in the liver of flounder (*Platichthys flesus* L.) in relation to histopathology of hepatic degeneration and carcinogenesis, Mar. Environ. Res., 39: 255–260.

Köhler, A., Wahl, E. and Söffker, K. 2002. Functional and morphological changes of lysosomes as prognostic biomarkers of toxic liver injury in a marine flatfish (*Platichthys flesus* (L)). Environ. Toxicol. Chem., 21: 2434–2444.

Köhler, A., Alpermann, T., Lauritzen, B. and Van Noorden, C.J.F., 2004. Clonal xenobiotic resistance during pollution-induced toxic injury and hepatocellular carcinogenesis in liver of female flounder. Acta Histochem., 106: 155–170.

Krishnakumar, P.K., Casillas, E. and Varanasi, U. 1994. Effect of environmental contaminants on the health of Mytilus edulis from Puget Sound, Washington, USA. I. Cytochemical measures of lysosomal responses in the digestive cells using automatic image analysis. Mar. Ecol. Prog. Ser., 106: 249–261.

Lowe, D.M. 1988. Alterations in the cellular structure of *Mytilus edulis* resulting from exposure to environmental contaminats under field and experimental conditions. Mar. Ecol. Prog. Ser.,46: 91–100.

Lowe, D.M., Moore, M.N. and Evans, B.M. 1992. Contaminant impact on interactions of molecular probes with lysosomes in living hepatocytes from dab *Limanda limanda*. Mar. Ecol. Prog. Ser., 91: 135–140.

Lowe, D. M., Fossato, V. U. and Depledge, M. H. 1995. Contaminant induced lysosomal membrane damage in blood cells of mussels *M. galloprovincialis* from the Venice Lagoon: an in vitro study. Mar. Ecol. Prog. Ser., 129: 189–196.

Lowe, D.M., Moore, M.N. and Readman, J.W. 2006. Pathological reactions and recovery of hepatopancreatic digestive cells from the marine snail *Littorina littorea* following exposure to a polycyclic aromatic hydrocarbon. Mar. Environ. Res., 61: 457–470.

Moore, M.N., Koehn R.K. and Bayne, B.L. 1980. Leucine aminopeptidase (aminopeptidase-1), N-acetyl-β-hexosaminidase and lysosomes in the mussel *Mytilus edulis* L., in response to salinity changes. J. Exp. Zool., 214: 239–249.

Moore, M.N. 1988. Cytochemical responses of the lysosomal system and NADPH-ferrihemoprotein reductase in molluscs to environmental and experimental exposure to xenobiotics. Mar. Ecol. Prog. Ser., 46: 81–89.

Moore, M.N. 1990. Lysosomal cytochemistry in marine environmental monitoring. Histochem. J., 22: 187–191.

Moore, M. N., Lowe, D. M., Wedderburn, R. J., Wade, T., Balashov. G., Büyükgüngör, H., Daurova, Y., Denga, Y., Kostylev, E., Mihnea, P., Moncheva, S.Tabagari, S., Ciocan, C., Özkoc, H., Depledge, M. H. 1998. International Mussel Watch (UNESCO/IOC) in the Black Sea: a pilot study for biological effects and contaminant residues. In: Environmental degradation of the Black Sea, (Eds. S. Beşiktepe *et al.*), pp. 273 – 289. Kluwer Academic Publishers, Amsterdam.

Moore, M.N. 2002. Biocomplexity: the post-genome challenge in ecotoxicology. Aquatic Toxicol., 59: 1–15.

Moore, M.N., Depledge, M.H., Readman, J.W. and Leonard, P. 2004a. An integrated biomarker-based strategy for ecotoxicological evaluation of risk in environmental management. Mutation Res., 552: 247–268.

Moore, M.N., Lowe, D. and Köhler, A. 2004b. Measuring lysosomal membrane stability. ICES Techniques in Marine Environmental Sciences, No. 36. ICES, Copenhagen, 31 p.

Moore, M.N., Allen, J.I. and McVeigh, A. 2006a. Environmental prognostics: an integrated model supporting lysosomal stress responses as predictive biomarkers of animal health status. Mar. Environ. Res. 61(3):278-304.

Moore, M.N., Allen, J.I. and Somerfield, P.J. 2006b. Autophagy: role in surviving environmental stress. Marine Environmental Research, 62 Suppl. 1: S420–S425.

Moore, M.N., Viarengo, A., Donkin, P. and Hawkins, A.J.S. 2007. Autophagic and lysosomal reactions to stress in the hepatopancreas of blue mussels. Aquatic Toxicology 84(1) 80-91.

Nicholson, S. and Lam, P.K.S. 2005. Pollution monitoring in Southeast Asia using biomarkers in the mytilid mussel *Perna viridis* (Mytilidae: Bivalvia). Environ. Internat., 31: 121 132.

Pisoni, M., Cogotzi, L., Frigeri, A., Corsi, I., Bonacci, S., Iacocca, A., Lancini, L., Mastrototaro, F., Focardi, S., Svelto *et al.* 2004. DNA adducts, benzo(a)pyrene monooxygenase activity, and lysosomal membrane stability in *Mytilus galloprovincialis* from different areas in Taranto coastal waters (Italy). Environ. Res., 96: 163–175.

Regoli, F. 2000. Total; oxyradical scavenging capacity (TOSC) in polluted and translocated mussels: a predictive biomarker of oxidative stress. Aquatic Toxicol.: 50, 351–361.

Ringwood, A.H., Hoguet, J., Keppler, C. and Gielazyn, M. 2004. Linkages between cellular biomarker responses and reproductive success in oysters, Crassostrea virginica. Mar Environ. Res., 58: 151–155.

Schiedek, D., Broeg, K., Barsiene, J., Lehtonen, K. K., Gercken, J., Pfeifer, S., Vuontisjärvi, H., Vuorinen, P. J., Köhler, A., Balk, L., Schneider, R. 2006. Biomarker responses and indication of contaminant effects in blue mussel (*Mytilus edulis*) and eelpout (*Zoarces viviparus*) from the western Baltic Sea. Mar. Poll. Bull., 53: 387–405.

Strand J., Dahllöf I, Larsen MM. (2009). Biologisk effektmonitering, Chaper 13 Marine områder 2007 - Tilstand og udvikling i miljø- og naturkvaliteten. NOVANA. Dahl, K. & Josefson, A.B. (Eds.), National Environmental research Institute (NERI), Aarhus University. Scientific Report from NERI, No. 707, 113pp.

Sturve, J., Balk, L., Berglund, A., Broeg, K., Böhmert, B., Koehler, A., Massey, S., Parkkonen, J., Savva, D., Stephensen, E., Förlin, L. 2005. Effects of dredging in Goteborg harbour assessed by biomarkers in eelpout (*Zoarces viviparus*). Environ. Toxicol. Chem., 24: 1951–1961.

Svendsen, C. and Weeks, J. M. 1995. The use of a lysosome assay for the rapid assessment of cellular stress from copper to the freshwater snail *Viviparus contectus* (Millet). Mar. Poll. Bull. : 31, 139–42.

Svendsen, C., Spurgeon, D.J., Hankard, P.K. and Weeks, J.M. 2004. A review of lysosomal membrane stability measured by neutral red retention: is it a workable earthworm biomarker? Ecotox. Environ. Safety, 57: 20–29.

UNEP/RAMOGE: Manual on the Biomarkers Recommended for the MED POL Biomonitoring Programme. UNEP, Athens, 1999.

Viarengo, A., Zanicchi, G., Moore, M.N. and Orunesu, M. 1981. Accumulation and detoxication of copper by the mussel *Mytilus galloprovincialis*. Aquatic Toxicol., 1: 147–157.

Viarengo, A., Moore, M.N., Mancinelli, G., Mazzucotelli, A. and Pipe, R.K. 1985a. Significance of metallothioneins and lysosomes in cadmium toxicity and homeostasis in the digestive gland cells of mussels exposed to the metal in the presence and absence of phenanthrene. Mar. Environ. Res., 17, 184–187.

Viarengo, A., Moore, M.N., Pertica, M., Mancinelli, G., Zanicchi, G. and Pipe, R.K. 1985b. Detoxification of copper in the cells of the digestive gland of mussel: the role of lysosomes and thioneins. Sci. Tot. Environ., 44: 135–145.

Viarengo, A., Moore, M.N., Pertica, M., Mancinelli, G., Accomando, R. 1992. A simple procedure for evaluating the protein degradation rate in mussel (*Mytilus galloprovincialis* Lam.) tissues and its application in a study of phenanthrene effects on protein catabolism. Comp. Biochem. and Physiol., 103B: 27–32.

Viarengo, A., Lafaurie, M., Gabrielides, G.P., Fabbri, R., Marro, A. and Romeo, M. 2000. Critical evaluation of an intercalibration exercise undertaken in the framework of the MEDPOL biomonitoring program. Mar. Environ. Res., 49: 1–18.

Annex 11: ICES SGEH Biological Effects methods Background Documents for the Baltic Sea region*

Micronucleus test

***ICES/OSPAR document from the ICES SGIMC Report 2010, complemented and modified by SGEH 2011 with information relevant for application in the Baltic Sea region**

Background

In environmental genotoxicity indication system, the micronucleus (MN) test served as an index of cytogenetic damage for over 30 years. MN consist of acentric fragments of chromosomes or whole chromosomes which are not incorporated into daughter nuclei at anaphase. These small nuclei can be formed as a consequence of the lagging of a whole chromosome (aneugenic event) or acentric chromosome fragments (clastogenic event) (Heddle, 1973; Schmid, 1975). A MN arises in cell divisions due to spindle apparatus malfunction, the lack or damage of centromere or chromosomal aberrations (Fenech, 2000).

Clastogens induce MN by breaking the double helix of DNA, thereby forming acentric fragments that are unable to adhere to the spindle fibres and integrate in the daughter nuclei, and are thus left out during mitosis. Aneuploidogenic agents are chemicals that prevent the formation of the spindle apparatus during mitosis which can generate not only whole chromatids that are left out of the nuclei, thus forming MN, but also can form multinucleated cells in which each nucleus would contain a different number of chromosomes (Serrano-García and Montero-Montoya, 2001). Furthermore, there are indications that MN additionally may be formed via a nuclear budding mechanism in the interphase. The formation of such type MN reflects in an unequal capacity of the organisms to expel damaged, amplified DNA, failed replicated or improperly condensed DNA, chromosome fragments without telomeres and centromeres from the nucleus (Lindberg *et al.*, 2007).

The MN test involves the scoring of the cells which contain one or more MN in the cytoplasm (Schmid, 1975). The assay was first developed as a routine in vivo mutagenicity assay for detecting chromosomal mutations in mammalian studies (Boller and Schmid, 1970; Heddle, 1973). Hooftman and de Raat (1982) were the first, who successfully apply the assay to aquatic species. Since these initial experiments, other studies have validated the detection of MN as a suitable biomarker of genotoxicity in a wide range of both vertebrate and invertebrate species (for review see Chaudhary *et al.*, 2006; Udroiu *et al.*, 2006; Bolognesi and Hayashi, 2011). In fish most studies have applied circulating erythrocytes (blood) cells but can also be sampled from a number of tissues, such as liver, kidney, gill or fin epithelium (Archipchuk, Garanko, 2005; Baršienė *et al.*, 2006a; Rybakovas *et al.*, 2009). The frequency of the observed MN may be considered as a suitable index of accumulated genetic damage during the cell lifespan providing a time integrated response of an organism's exposure to contaminant mixtures. Depending on the life span of each cell type and on their mitotic rate in a particular tissue, the frequency of MN may provide early warning signs of cumulative stress (Bolognesi and Hayashi 2011).

As an early warning indicator MN induction was successfully used in studies of environmental genotoxicity in gas (Gorbi *et al.*, 2008) and oil platform zones (Hyland *et al.*, 2008; Rybakovas *et al.*, 2009, Brooks *et al.*, 2011), also after the oil spills (Baršienė *et al.*, 2006b, 2006c; Santos *et al.*, 2010). Environmental genotoxicity levels in organisms from Baltic Sea, North Sea, Mediterranean and Northern Atlantic have been described in indigenous fish and mussel species inhabiting reference and contaminated sites (Wrisberg *et al.*, 1992; Bresler *et al.*, 1999; Baršienė *et al.*, 2004, 2005a, 2006b, 2006c 2008a, 2008b, 2010a; Bagni *et al.*, 2005; Bolognesi *et al.* 2006a; Magni *et al.*, 2006). The MN test was validated in laboratory with different species after exposure to a large number of various chemical agents (Fenech *et al.*, 2003; Bolognesi *et al.*, 2006b; Baršienė, Andreikėnaitė, 2007; Andreikėnaitė, 2010; Bolognesi, Hayashi, 2011).

The majority of studies to date have used haemolymph and gill cells of molluscs and peripheral blood cells of fish for the MN analysis (Bolognesi, Hayashi, 2011). There are other studies (albeit limited) available describing the use of other haemopoetic tissues, such as liver, kidney, gills, and also fins (Archipchuk, Garanko, 2005; Baršienė *et al.*, 2006a; Rybakovas *et al.*, 2009). The application of the MN assay to blood samples of fish is particularly attractive as the method is non-destructive, easy to undertake and results in an easy quantifiable number of cells present on the blood smears for microscopic analysis. However, studies must be undertaken to assess the suitability of any species or cell type analyzed.

The detected MN frequency in fish erythrocytes is approximately 6-10 times lower than in mussels and clams. The large inter individual variability associated to the low baseline frequency for this biomarker confirming the need for the scoring of a consistent number of cells in an adequate number of animals for each study point. Sampling size in most of studies conducted with mollusc species have been scoring 1000-2000 cells per animal (Izquierdo *et al.*, 2003; Hagger *et al.*, 2005; Bolognesi *et al.*, 1996, 2004, 2006a; Magni *et al.*, 2006; Baršienė *et al.*, 2006a, 2006b, 2008b, 2010; Kopecka *et al.*, 2006; Nigro *et al.*, 2006; Schiedek *et al.* 2006; Francioni *et al.*, 2007; Siu *et al.*, 2008; Koukouzika and Dimitriadis 2005, 2008) and previous reviews have suggested that when using fish erythrocytes at least 2000–4000 cells should be scored per animal (Udroiu *et al.*, 2006; Bolognesi *et al.*, 2006a). Previously scorings of 5000–10000 fish erythrocytes where used for a MN analysis (Baršienė *et al.*, 2004). Since 2009/2010, the frequency of MN in fish from the Baltic seas was mostly scored in 4000 cells. In stressful heavily polluted zones, the scoring of 5000–10000 cells in fish is still recommended.

Mussel sampling size in MN assays range from 5 to 20 mussels per site as reported in the literature (Baršienė *et al.*, 2004, 2006c, 2008b; Francioni *et al.*, 2007; Siu *et al.*, 2008). Evidence suggests that a sample size of 10 specimens per site is enough for the assessment of environmental genotoxicity levels and evaluation of the existence of genetic risk zones. In heavily polluted sites, MN analysis in 15–20 specimens is recommended, due to higher individual variation of the MN frequency. MN analysis in more than 20 mussel or fish specimens shows only a minor change of the MN means (Figure 1 in Fang *et al.*, 2009; Baršienė *et al.*, unpublished results).

Most of the studies have been performed using diagnostic criteria for MN identification developed by several authors (Heddle *et al.*, 1973, 1991; Carrasco *et al.*, 1990; Al-Sabti and Metcalfe, 1995; Fenech, 2000; Fenech *et al.*, 2003):

- the size of MN is smaller than 1/3 of the main nucleus;
- MN are round- or ovoid-shaped, non-refractive chromatin bodies located in the cytoplasm of the cell and can therefore be distinguished from artifacts such as staining particles;
- MN are not connected to the main nuclei and the micronuclear boundary should be distinguishable from the nuclear boundary.

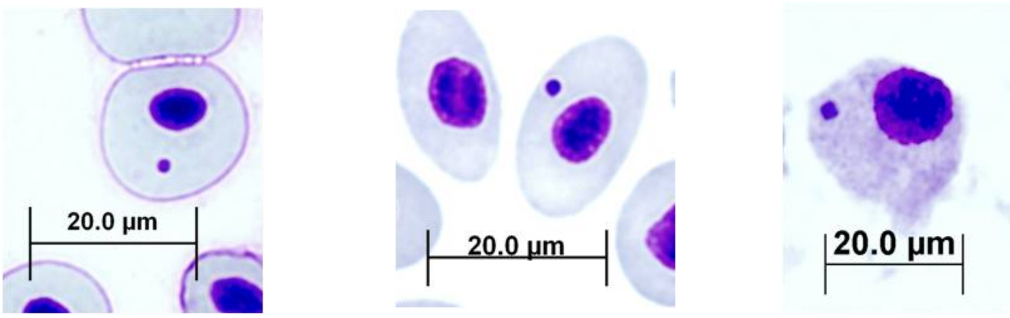

Figure 1. Micronuclei in blood erythrocytes of (a) herring (*Clupea harrengus*),(b) flounder (*Platichthys flesus*), and (c) in a gill cell of the mussel *Mytilus edulis*.

After sampling and cell smears preparation, slides should be coded. To minimize technical variation, the blind scoring of MN should be performed without knowledge of the origin of the samples. Only cells with intact cellular and nuclear membrane can be scored. Particles with colour intensity higher than that of the main nuclei were not counted as MN. The area to be scored should first be examined under low magnification to select the part of the slide showing the highest quality (good staining, non overlapping cells). Scoring of MN should then be undertaken at 1000x magnification.

Confounding factors

Earlier studies on MN formation in mussels have disclosed a significant influence of environmental and physiological factors (Dixon *et al.*, 2002). Therefore, the role of the confounding factors should be considered prior to the application of MN assay in biomonitoring programs, as well as in description of genetic risk zones, or ecosystem health assessments.

Water temperature. MN induction is a cell cycle-related process and depends on water temperature, which is a confounding factor for the mitotic activity in poikilotherm animals. Several studies have demonstrated that baseline frequencies of MN in mussels are related to water temperature (Brunetti *et al.*, 1988, 1992; Kopecka *et al.*, 2006). Baseline frequencies of MN are regarded as the incidence of MN observed in the absence of environmental risk or before exposure to genotoxins (Fenech, 1993). In fish MN frequencies showed also seasonal differences in relation to water temperature with lower MN levels in winter than in autumn (Rybakovas *et al.*, 2009). This was assumed to be an effect of higher mitotic activity and MN formation due to high water temperatures in the autumn (Brunetti *et al.*, 1988). Additionally, it has been reported that increases in water temperature (4–37ºC) can increase the ability of genotoxic compounds to damage DNA (Buschini *et al.*, 2003).

Cell type. MN may be seen in any type of cell, both somatic and germinal and thus the micronucleus test can be carried out in any active tissue. Nevertheless there are some limitations using different types of cells, for example, agranular and granular haemocytes in mussels. There are also differences between MN induction level in mussel haemolymph and gill cells, mainly because gills are primary targets for the action of contaminants. The anatomical architecture of the spleen in fish does not allow erythrocytes removal in the spleen (Udroiu *et al.*, 2006), though, in mammals this process go.

Salinity. The influence of salinity on the formation of MN was observed in mussels from the Danish coast located in the transitional zone between the Baltic and North Sea. No relationship between salinity and MN frequencies in mussels could be found for mussels from the Wismar Bay and Lithuanian coast. Similar results were found for *Macoma balthica* from the Baltic Sea in the Gulfs of Bothnia, Finland, Riga and in the Lithuanian EZ (Baršienė *et al.*, unpublished data).

Individual size. Since the linear regression analysis of animal's length and induction of MN shows that the size could be a confounding factor, sampling of organisms with similar sizes should take place (Baršienė *et al.*, unpublished data). It should also be noted that size is not always indicative of age and therefore age could also potentially affect the response of genotoxicity in the fish.

Diet. Results have shown that MN formation was not influenced in mussels who were maintained under simple laboratory conditions without feeding (Baršienė *et al.*, 2006d).

Ecological relevance

Markers of genotoxic effects reflect damage to genetic material of organisms and thus get a lot of attention (Moore *et al.* 2004). Different methods have been developed for the detection of both double- and single-strand breaks of DNA, DNA-adducts, MN formation and chromosome aberrations. The assessment of chemical induced genetic damage has been widely utilized to predict the genotoxic, mutagenic and carcinogenic potency of a range of substances, however these investigations have mainly been restricted to humans or mammals (Siu *et al.* 2004). MN formation indicates chromosomal breaks, known to result in teratogenesis (effects on offspring) in mammals. There is however limited knowledge of relationships between MN formation and effects on offspring in aquatic organisms. With a growing concern over the presence of genotoxins in the aquatic media, the application of cytogenetic assays on ecologically relevant species offers the chance to perform early tests on health in relation to exposure to contaminants.

Quality Assurance

The MN test showed to be a useful in vivo assay for genotoxicity testing. However, many aspects of its protocol need to be refined, knowledge of confounding factors should be improved and inter-species differences need further investigation. In 2009 an inter-laboratory comparison exercise was organised within the framework of the MED POL programme using the mussel *M. galloprovincialis* as test species. The results are expected by mid 2011.

Intercalibration of MN analysis in fish was done between experts from NRC and Caspian Akvamiljo laboratories, as well as between NRC experts and the University of Aveiro, Portugal (Santos *et al.* 2010). It is recommended that these relatively simple interlaboratory collaborations are expanded to include material from all the commonly used indicator species in 2011/2012.

Background response and Assessment Criteria

Baseline or background frequency of MN can be defined as incidence of MN observed in the absence of environmental risk or before exposure to genotoxins (Fenech, 1993). In fish, MN frequencies lower than 0.05‰ has been suggested by Rybakovas *et al.* (2009) as a reference level in the peripheral blood erythrocytes of the flatfish flounder (*Platichthys flesus*) and dab (*Limanda limanda*) and also cod (*Gadus morhua*) after analyzing 479 specimens from 12 offshore sites in the Baltic Sea. The frequencies of MN in marine species sampled from the Baltic Sea reference sites are summarized in Table 1.

Table 1. Reference levels of micronuclei (MN/1000 cells) in Baltic Sea species in situ.

Species	Tissue	Response MN/1000 cells	Reference
Mytilus edulis	Gills	0.37 ± 0.09	Baršienė *et al.*, 2006b
Mytilus trossulus	Gills	2.07 ± 0.32	Baršienė *et al.*, 2006b; Kopecka *et al.*, 2006
Macoma baltica	Gills	0.53 - 1.28	Baršienė *et al.*, 2008b, unpublished data (NRC, Lithuania)
Platichthys flesus	Blood erythrocytes	0.15 ± 0.03	Baršienė *et al.*, 2004
Platichthys flesus	Blood erythrocytes	0.0 ± 0.0	Kohler, Ellesat, 2008
Platichthys flesus	Blood erythrocytes	0.08 ± 0.02	Napierska *et al.*, 2009
Zoarces viviparus	Blood erythrocytes	0.02 ± 0.02	Baršienė *et al.*, unpublished data (NRC, Lithuania)
Gadus morhua	Blood, kidney erythrocytes	0.03 ± 0.02	Rybakovas *et al.*, 2009
Clupea harengus	Blood erythrocytes	0.03 ± 0.03	Baršienė *et al.*, unpublished data (NRC, Lithuania)
Scophthalmus maximus	Blood erythrocytes	0.10 ± 0.04	Baršienė *et al.*, unpublished data (NRC, Lithuania)
Perca fluviatilis	Blood erythrocytes	0.06 ± 0.02	Baršienė *et al.*, 2005a; Baršienė *et al.*, unpublished data (NRC, Lithuania)

Assessment Criteria (AC) have been established by using data available from studies of molluscs and fish in the Baltic Sea (NRC database). The background/threshold level of MN incidences is calculated as the empirical 90% percentile (P90). Until more data becomes available, values should be interpreted from existing national data sets. <u>Note: the values given here are provisional and require further validation when new data becomes available</u>.

The 90 % percentile (P90) separates the upper 10 % of all values in the group from the lower 90 %. The rationale for this decision was that elevated MN frequency would lie above the P90 percentile, whereas the majority of values below P90 belong to unexposed, weakly-medium exposed or non-responding adapted individuals. P90 values were calculated for those stations/areas which were considered being reference stations (i.e. no known local sources of contamination or those areas which were not considered unequivocally as reference sites but as those less influenced from human and industrial activity).

ACs in bivalves *Mytilus edulis, Mytilus trossulus, Macoma balthica* (data from MN analysis in 2370 specimens), in fish *Limanda limanda, Zoarces viviparus, Platichthys flesus, Gadus morhua* and *Clupea harengus* (data from MN analysis in 3239 specimens) from Baltic Sea have been calculated using NRC (Lithuania) databases (Table 2).

Table 2. Assessment criteria of MN frequency levels in bivalve mollusc and fish. BR =Background response; ER = Elevated response; n = number of specimens analysed.

Species	Size (cm)	T (ºC)	Region	Tissue	BR	ER	*n*
Mytilus edulis	1.5-3	8-18	Baltic Sea	Gills	<2.50	>2.50	1810
Mytilus trossulus	2-3	3-15	Baltic Sea	Gills	<4.50	> 4.50	230
Macoma balthica	1-3	13-18	Baltic Sea	Gills	<2.90	> 2.90	330
Zoarces viviparus	15-32	7-17	Baltic Sea	Erythrocytes	<0.38	>0.38	824
Limanda limanda	18-25	8-17	Baltic Sea	Erythrocytes	<0.49	>0.49	117
Platichthys flesus	17-39	10-17	Baltic Sea coastal	Erythrocytes	<0.29	>0.29	828
Platichthys flesus	18-40	6-18	Baltic Sea offshore	Erythrocytes	<0.23	>0.23	970
Gadus morhua	20-48	13-15	Baltic Sea	Erythrocytes	<0.38	>0.38	50
Clupea harengus	16-29	6-18	Baltic Sea	Erythrocytes	<0.39	>0.39	450

Distribution of indicator species in Baltic Sea subregions

MN test has generally been applied to organisms where other biological-effects techniques and contaminant levels are well documented. That is the case for mussels and for certain demersal fish species (as European flounder, dab or Atlantic cod), which are routinely used in biomonitoring programs and assess contamination along western European marine. However, the MN assay may be adapted for alternative sentinel species using site-specific monitoring criteria.

When selecting an indicator fish species, consideration must be given to its karyotype as many teleosts are characterised by an elevated number of small chromosomes (Udroiu *et al.*, 2006). Thus, in certain cases MN formed after exposure to clastogenic contaminants will be very small and hard to detect by light microscopy. This can be addressed to a certain extent by using fluorescent staining. After selecting target/suitable species, researchers should also ensure that other factors including age, sex, temperature and diet are similar between the sample groups. If conducting transplantation studies, consideration needs to be given to the cellular turnover rate of the tissue being examined to ensure sufficient cells have gone through cell division. For example, if using blood the regularities of erythropoiesis should be known prior to sampling.

Large-scale and long-term studies took place from 2001 to 2010 at the Nature Research Centre (NRC, Lithuania) on MN and other abnormal nuclear formations in different fish and bivalve species inhabiting various sites of the Baltic Sea. These studies revealed the relevance of environmental genotoxicity levels in ecosystem assessments. NRC established a large database on MN and other nuclear abnormalities in 8 fish species and in mussels and clams from the Baltic Sea. Fish and bivalve species were collected from 117 coastal and offshore sites. The following organisms have been tested as the target species for MN test in the different regions of the Baltic Sea:

Fish	flounder (*Platichthys flesus*), dab (*Limanda limanda,* herring (*Clupea harengus*), eelpout (*Zoarces viviparus*) plaice (*Pleuronectes platessa)* Atlantic cod (*Gadus morhua)* perch (*Perca fluviatilis*) turbot (*Scophthalmus maximus, Psetta maxima)*
Bivalves	blue mussels (*Mytilus edulis, Mytilus trossulus* Baltic clam (*Macoma baltica*)
Amphipods	Gammarids

Acknowledgements

Research on the application of the MN test as a biomarker for the monitoring and assessment of environmental genotoxicity in different Baltic Sea regions and indicator organisms has been funded by the EU Project BEEP, Contract EVK3-CT-2000-00025, BONUS+ Project BEAST and Lithuanian Science Council GENCITOX project MIP-10123.

References

Al-Sabati K, Metcalfe CD. Fish micronuclei for assessing genotoxicity in water. Mutation Research 1995; 323: 121-35.

Andreikėnaitė L. 2010. Genotoxic and cytotoxic effects of contaminants discharged from the oil platforms in fish and mussels. Summary of doctoral dissertation, 38p.,Vilnius.

Arkhipchuk V.V., Garanko N.N. 2005. Using the nucleolar biomarker and the micronucleus test on in vivo fish fin cells. Ecotoxicology and Environmental Safety. Vol. 62. P. 42-52.

Bagni G., Baussant T., Jonsson G., Baršienė J., Mascini M. 2005. Electrochemical device for the rapid detection of genotoxic compounds in fish bile samples. Analytical Letters, 38: 2639-2652.

Baršienė J., Andreikėnaitė L. 2007. Induction of micronuclei and other nuclear abnormalities in blue mussels exposed to crude oil from the North Sea. Ekologija, 53, No 3: 9-15.

Baršienė J., Andreikėnaitė L., Garnaga G., Rybakovas A. 2008b. Genotoxic and cytotoxic effects in bivalve mollusks *Macoma balthica* and *Mytilus edulis* from the Baltic Sea. Ekologija, 54: 44-50.

Baršienė J., Bjornstad A., Rybakovas A., Šyvokienė J., Andreikėnaitė L. 2010. Environmental genotoxicity and cytotoxicity studies in mussels and fish inhabiting northern Atlantic zones impacted by aluminum industry. Ekologija, 56, No 3-4: 124-131.

Baršienė J., Dedonytė V., Rybakovas A., Andreikėnaitė L., Andersen O.K. 2006a. Investigation of micronuclei and other nuclear abnormalities in peripheral blood and kidney of marine fish treated with crude oil. Aquatic Toxicology,78, Supplement 1: S99-104.

Baršienė J., Dedonytė V., Rybakovas A., Broeg K., Forlin L. , Gercken J., Kopecka J., Balk L. 2005a. Environmental mutagenesis in different zones of the Baltic Sea. Acta Zoologica Lituanica, 15: 90-95.

Baršienė J., Lazutka J., Šyvokienė J., Dedonytė V., Rybakovas A., Bjornstad A., Andersen O.K. 2004. Analysis of micronuclei in blue mussels and fish from the Baltic and the North Seas. Environmental Toxicology, 19: 365-371.

Baršienė J., Lehtonen K., Koehler A., Broeg K., Vuorinen P.J., Lang T., Pempkowiak J., Šyvokienė J., Dedonytė V., Rybakovas A., Repečka R., Vuontisjarvi H., Kopecka J. 2006c. Biomarker responses in flounder (Platichthys flesus) and mussel (Mytilus edulis) in the Klaipėda-Būtingė area (Baltic Sea). Marine Pollution Bulletin, 53: 422- 436.

Baršienė J., Rybakovas A. 2006d Cytogenetic and cytotoxic effects in gill cells of the blue mussel Mytilus edulis from the Baltic coast and after 1-3-day maintenance in laboratory. Acta Zoologica Lituanica, 16: 191-197.

Baršienė J., Rybakovas A., Forlin L., Šyvokienė J. 2008a. Environmental genotoxicity studies in mussels and fish from the Goteborg area of the North Sea. Acta Zool. Lituanica, 18: 240-247.

Baršienė J., Schiedek D., Rybakovas A., Šyvokienė J., Kopecka J., Forlin L. 2006b. Cytogenetic and cytotoxic effects in gill cells of the blue mussel Mytilus spp. from different zones of the Baltic Sea. Marine Pollution Bulletin, 53: 469-478.

Boller K., Schmid W. 1970. Chemische Mutagenese beim Sauger. Das Knochermark des Chinesischen Hamsters als in vivo-Test system. Haematologische Befunde nach Behandlung mit Trenimon. Humangenetik, 11, 35-54.

Bolognesi C., Perrone E., Roggieri P., Pampanin D.M., Sciutto A. 2006b. Assessment of micronuclei induction in peripheral erythrocytes of fish exposed to xenobiotics under controlled conditions. Aquatic Toxicology, 78, Supplement 1: S93-S98.

Bolognesi C., Perrone E., Roggieri P., Sciutto A. 2006a. Bioindicators in monitoring long term genotoxic impact of oil spill: Haven case study. Marine Environ. Res., 62: S287-S291.Bolognesi C., Hayashi M. 2011. Micronucleus assay in aquatic animals. Mutagenesis, 26: 205-213.

Bolognesi C. and Hayashi M. 2011. Micronucleus assay in aquatic animals. Mutagenesis, 26: 205-213.

Bolognesi C., Frenzilli G., Lasagna C., Perrone E., Roggieri P. 2004. Genotoxicity biomarkers in *Mytilus galloprovincialis*: wild versus caged mussels. Mutation Research. 552: 153-162.

Bolognesi C., Rabboni R. and Roggieri P. 1996. Genotoxicity biomarkers in *M. galloprovincialis* as indicators of marine pollutants. Comparative Biochemistry and Physiology, 113C, No 2: 319-323.

Bresler V., Bissinger V., Abelson A., Dizer H., Sturm A., Kratke R., Fischelson L., Hansen P.-D. 1999. Marine mollusks and fish as biomarkers of pollution stress in littoral regions of the Red Sea, Mediterranean Sea and North Sea. Helgoland Marine Research, 53: 219-243.

Brooks S.J., Harman C., Grung M., Farmen E., Ruus A., Vingen S., Godal B.F., Baršienė J., Andreikėnaitė L., Skarphedinsdottir H., Liewenborg B., Sundt R.C. 2011. Water column monitoring of the biological effects of produced water from the Ekofisk offshore oil installation from 2006 to 2009. Journal of Toxicology and Environmental Health, Part A, 74: 582-604.

Brunetti R., Gabriele M., Valerio P. and Fumagalli O. 1992. The micronucleus test: temporal pattern of the base-line frequency in *Mytilus galloprovincialis* Lmk. Marine Ecol. Prog. 83: 75-78.

Brunetti R., Majone F., Gola I and Beltrame C. 1988. The micronucleus test: examples of application to marine ecology. Marine Ecol. Prog., 44:65-68.

Buschini A, Carboni P, Martino A, Poli P, Rossi C. 2003. Effects of temperature on baseline and genotoxicant-induced DNA damage in haemocytes of *Dreissena polymorpha*. Mutatation Research, 537: 81-92.

Carrasco K.R., Tilbury K.L., Myers M.S. 1990. Assessment of the piscine micronucleus test as an *in situ* biological indicator of chemical contaminant effects. Canadian Journal Fishery and Aquatic Sciences, 47: 2123-2136.

Chaudhary, R., Pandy, S., Kushwaha, B., Gaur, K.K., Nagpure, N.S. (2006) Fish micronucleus assay: A sensitive tool for ecogenotoxicity studies. Journal of Ecophysiology and Occupational Health, 6 (3-4): 143-147.

Dixon D.R., Dixon L.R.J., Shillito B., Gwynn J.P. 2002. Background and induced levels of DNA damage in Pacific deep-sea vent palychaetes: the case for avoidance. Cah. Biol. Marine 43: 333-336.

Fenech M. 2000. The in vitro micronucleus technique. Mutation Research 455:81-95.

Fenech M., Chang W.P., Kirsch-Volders M., Holland N., Bonassi S., Zeiger E. 2003. HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. Mutation Research, 534: 65-75.

Fenech, M., 1993, The Cytokinesis-Block Micronucleus Technique: A Detailed Description of the Method and Its Application to Genotoxicity Studies in Human Populations. Mutatation Research, 285, 35-44.

Gorbi S., Lamberti C.V., Notti A., Benedetti M., Fattorini D., Moltedo G., Regoli F. 2008. An Ecotoxicological protocol with caged mussels, *Mytilus galloprovincialis*, for monitoring the impact of an offshore platform in the Adriatic sea. Marine Environmental Research, 65: 34-49.

Hagger J.A., Depledge M.H., Galloway T.S. 2005. Toxicity of tributyltin in the marine mollusc *Mytilus edulis*. Marine Pollution Bulletin, 51:811-816.

Heddle J.A. 1973. A rapid in vivo test for chromosomal damage. Mutation Research, 18: 187-190.

Heddle J.A., Cimino M.C., Hayashi M., Romagna F., Shelby M.D., Tucker J.D., Vanparys Ph. and MacGregor J.T. 1991. Micronuclei as an index of cytogenetic damage: past, present, and future. Environmental and Molecular Mutagenesis, 18: 277-291.

Hooftman R.N., Raat W.K. 1982. Induction of nuclear anomalies (micronuclei) in the peripheral blood erythrocytes of an eastern mudminnow *Umbra pygmaea* by ethyl methanesulphonate. Mutation Research, 104: 147-152.

Hylland K., Tollefsen K.-E., Ruus A., Jonsson G., Sundt R.C., Sanni S., Utvik T.I.R., Johnsen I., Pinturier L., Balk L., Baršienė J., Marigomez I., Feist S.W., Borseth J.F. 2008. Water column monitoring near oil instalations in the North Sea 2001-2004. Marine Pollution Bulletin, 56: 414-429.

Izquierdo J.I., Machado G., Ayllon F., d'Amico V.L., Bala L.O., Vallarino E., Elias R., Garcia-Vazquez E. 2003. Assessing pollution in coastal ecosystems: a preliminary survey using the micronucleus test in the *Mytilus edulis.* Ecotoxicology and Environmental Safety 55: 24-29.

Köhler A., Ellesat K. 2008. Nuclear changes in blood, early liver anomalies and hepatocellular cancers in flounder (*Platichthys flesus* L.) as prognostic indicator for a higher cancer risk? Marine Environmental Research, 66: 149-150.

Kopecka J., Lehtonen K., Baršienė J., Broeg K., Vuorinen P.J., Gercken J., Pempkowiak J. 2006. Measurements of biomarker levels in flounder (*Platichthys flesus*) and mussel (*Mytilus trossulus*) from the Gulf of Gdańsk (southern Baltic). Marine Pollution Bulletin, 53: 406-421.

Koukouzika N., Dimitriadis V.K. 2005. Multiple biomarker comparison in *Mytilus galloprovincialis* from the Greece coast: "Lysosomal membrane stability, neutral red retention, micronucleus frequency and stress on stress". Ecotoxicology, 14:449-463.

Lindberg H.K., Wang X., Järventaus H., Falck G.C.-M., Norppa H., Fenech M. 2007. Origin of nuclear buds and micronuclei in normal and folate-deprived human lymphocytes. Mutation Research, 617: 33-45.

Magni P., De Falco G., Falugi C., Franzoni M., Monteverde M., Perrone E., Sigro M., Bolognesi C. 2006. Genotoxicity biomarkers and acetylcholinesterase activity in natural populations of *Mytilus galloprovincialis* along a pollution gradient in the Gulf of Oristano (Sardinia, western Mediterranean). Environmental Pollution, 142: 65-72.

Nigro M., Falleni A., Del Barga I., Scarcelli V., Lucchesi P., Regoli F., Frenzilli G. 2006. Cellular biomarkers for monitoring estuarine environments: Transplanted versus native mussels. Aquatic Toxicology, 77: 339-347.

Rybakovas A., Baršienė J., Lang T. 2009. Environmental genotoxicity and cytotoxicity in the offshore zones of the Baltic and North Seas. Marine Environmental Research, 68: 246-256.

Santos M.M., Sole M., Lima D., Hambach B., Ferreira A.M., Reis-Henriques M.A. 2010. Validating a multi-biomarker approach with the shanny *Lipophrys pholis* to monitor oil spills in European marine ecosystems. Chemosphere, 81, 6, 685-691.

Schiedek D., Broeg K., Baršienė J., Lehtonen K.K., Gercken J., Pfeifer S., Vuontisjärvi H., Vuorinen P.J., Dedonytė V., Koehler A., Balk L., Schneider R. 2006. Biomarker responses as indication of contaminant effects in blue mussel (*Mytilus edulis*) and female eelpout (*Zoarces viviparus*) from the southwestern Baltic Sea. Marine Pollution Bulletin, 53: 387-405.

Schmid W. 1975. The micronucleus test. Mutation Research, 31:9–15.

Serrano-García L., Montero-Montoya R. 2001. Micronuclei and chromatid buds are the result of related genotoxic events. Environmental and Molecular Mutagenenesis 38:38-45.

Siu, W. H. L., J. Cao, R. W. Jack, R. S. S. Wu, B. J. Richardson, L. Xu and P. K. S. Lam. 2004. Application of the comet and micronucleus assays to the detection of B a P genotoxicity in haemocytes of the greenlipped mussel (Perna viridis). Aquatic Toxicology, 66: 381-392.

Udroiu I. 2006. The micronucleus test in piscine erythrocytes. Aquatic Toxicology, 79: 201-204.

Wrisberg M.N., Bilbo C.M., Spliid H. 1992. Induction of micronuclei in hemocytes of *Mytilus edulis* and statistical analysis. Ecotoxicology and Environmental Safety, 23: 191-205.

Annex 12: ICES SGEH Biological Effects methods Background Documents for the Baltic Sea region*

Reproductive success in fish: eelpout (*Zoarces viviparus*)

***ICES/OSPAR document from the ICES SGIMC Report 2010, complemented and modified by SGEH 2011 with information relevant for application in the Baltic Sea region**

Background

Eelpout is a benthic fish species is widely used in ecotoxicological studies and as a bioindicator of local pollution due to its stationary behaviour in coastal marine environment.

Eelpout (*Zoarces viviparus*), also called viviparous blenny, is a recommended fish indicator species for assessing the environmental conditions in the Baltic Sea and eelpout is included in the environmental monitoring programmes of several Baltic States, both in relation to biological effects, contaminants and integrated fish monitoring. For instance, Sweden and Germany have routinely measured contaminant concentrations in eelpout for >15 years, and additional samples are archived in environmental specimen banks allowing retrospective studies on chemical burdens. Similar long time-series is also available for some other biomarker studies.

The eelpout is viviparous, i.e. there is an internal fertilization of the eggs and the female fish gives birth to fully developed larvae. The eelpout's mode of reproduction (vivipari) enables the study of "reproductive success" on an individual level, and larval developmental disorders can be directly associated with e.g. the health status of the female or body burdens of toxic substances during pregnancy.

Different types of abnormal larvae development can be distinguished and be characterised into different groups, i.e. early dead embryos, late dead larvae, growth retarded larvae and deformed larvae, which can be divided into different subgroups of severe gross malformations.

Reproductive success in eelpout as a biological effects method is regarded as a general, i.e. non-specific, integrative indicator for impaired fish reproduction, and a significant biological endpoint for assessing potential population-relevant effects. Because the reproductive success in fish is a generic "stress" indicator; causal agents may, however, only be identified through a combination of chemical analyses of fish tissue and also other biological effects measurements. Several types of hazardous substances such as organochlorines, pesticides, PAH, heavy metals and organometals are known to have the potential to affect embryo and larval development in fish, generally (Bodammer 1993). Several of these substances, which may induce developmental, morphological and/or skeletal anomalies, have also been identified as endocrine disrupting substances (Davis 1997).

Reproductive success in eelpout is included in the list of parameters for biological effect monitoring for supporting programme in the HELCOM COMBINE manual for marine monitoring in the coastal zone. Part D. Programme for monitoring of contaminants and their effects (HELCOM 2006).

The method is also part of OSPAR pre-CEMP and JAMP guideline for general biological effects monitoring (OSPAR 2010).

According to the monitoring guideline, the sample size should consist of examinations of 40–50 individuals of pregnant females of eelpout per station, which should be sampled in the period between October 15 and December 1.

There is in some countries restrictions on eelpout fishery that depends on national legislations, e.g. fishing dispensation for catching pregnant females in the autumn is needed in Denmark. National concerns for impact of fishery on local populations and ethical guidelines for humane handling and fish killing should also be considered.

In the Baltic Sea region, reproductive success in eelpout is or has been used for monitoring or pre-monitoring investigations at least by labs from Denmark, Sweden, Germany and Poland and as part of integrated fish monitoring, often in combination with contaminant, biomarker studies and/or population studies.

Studies in the Baltic Sea has shown that that spatial differences occur with elevated levels of adverse developmental effects of embryo and larvae in eelpout broods have been found in populations living in contaminated areas with effluents from cities and industry. In comparison, only low levels of such effects generally occur in populations living in areas regarded as reference sites (e.g. Vetemaa *et al.* 1997, Ådjers *et al.* 2001, Sjölin *et al.* 2003, Strand *et al.* 2004, Kalmarweb 2005, Gercken *et al.* 2006) as shown in Figure 1.

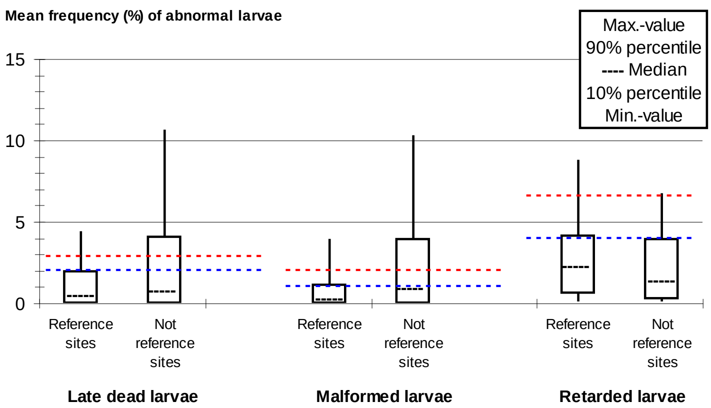

Figure 1. Comparison of data distribution of compiled monitoring data on mean frequencies of late dead, malformed and growth retarded larvae in eelpout broods from reference sites and area not regarded as reference sites. The blue dotted line refers to the 90% percentile of data from the reference sites. The red dotted line refers to significantly elevated levels compared to the 90% percentile of the reference sites.

Clear temporal developments with up- or down-going trends for the presence of different types of abnormal larvae development have not been established in monitored baseline areas with longer time-series. However, some year-to-year variations can occur.

Studies of point sources have shown that acute larval mortality also been observed in eelpout exposed to pulp mill effluents (Jacobsson *et al.* 1986). Skewed sex ratios with significant more males in the eelpout broods have also been found nearby pulp mill effluents indicating effects of endocrine disruption substances (Larsson & Förlin 2002).

Confounding factors

Other environmental stressors like increased temperature and severe oxygen depletion events may however also affect eelpout reproduction (Veetema 1999, Fagerholm 2002, Strand *et al.* 2004). There have been some indications that some specific types of abnormal larvae development like early dead embryos and late dead larvae can be induced by severe oxygen events. However, deformed larvae with severe gross malformations, which can be distinguished from the other types, seem to be more related to contaminant effects (Strand *et al.* 2004, Gercken *et al.* 2006).

Ecological relevance

The ecological relevance of reproductive success of fish is high, because of the links to reproductive disorders. Population modelling support that elevated levels of abnormal larvae developments also can be important for population sizes.

Quality Assurance

The methodology for reproductive success is well defined for studies in coastal waters and national guideline exists (Jacobsson *et al.*, 1986; Neuman *et al.*, 1999, Strand & Dahllöf, 2005). An international guideline is in preparation and to be published in the ICES TIMES series.

As method quality assurance, some international and national workshops have been held in relation to the monitoring programmes (e.g. BEQUALM 2000). A Baltic workshop has been held in 2009 as part of BONUS+-projects BALCOFISH and BEAST. National workshops in relation to NOVANA monitoring activities have also been held in Denmark (Strand 2005a).

Background response and Assessment Criteria

Assessment Criteria for reproductive success in eelpout based on below and above the background response has been proposed by ICES/OSPAR SGIMC 2010.

The derivation of assessment criteria have been based on data for either late dead lavae or deformed larvae from the Swedish and Danish monitoring programmes from several areas regarded as less polluted reference sites in the Baltic Sea, the Kattegat and the Skagerrak studies, where only low frequencies of abnormal larvae have mainly been found in areas, which were considered as reference sites, if any.

Background response values as baseline is based on 90% percentiles have been found to be <1% deformed larvae, <2% late dead larvae and <4% growth retarded larvae, respectively. Alternatively, the background response can also be based on the frequency of broods with >5% abnormal larvae development (Table 1).

Table 1. Background response for the presence of 3 types of abnormal larvae developments in eelpout, i.e. deformed larvae, late dead larvae and growth retarded larvae per station (ICES/OSPAR SGIMC 2010).

Type of abnormal larvae development	Background response, based on mean frequencies per station	Background response, based on frequency of broods with >5% abnormal larvae development
Deformed larvae	< 1% of all larvae	<5% of broods
Late dead larvae	<2% of all larvae	<5% of broods
Growth retarded larvae	<4% of all larvae	-
	Background response determined as the upper limit is the 90% percentile of response at so-called reference sites.	

However, these assessment criteria will be revised and evaluated in 2011/2012 within the BONUS+ project BALCOFISH, so that the derivation of the background response levels are performed according to the principles recommended by the OSPAR/ICES working group SGIMC.

Distribution of indicator species in Baltic Sea subregions

The eelpout inhabits coastal waters and is widely distributed and common in almost all subregions of the Baltic Sea. Eelpout occurs also from the White Sea to the southern North Sea.

Acknowledgements

The BONUS+ project BALCOFISH shall be acknowledged for the progress on the linkage between contaminants, early warning biomarkers and reproductive disorders and also on the developments of assessment criteria and evaluation of the ecological relevance using population modelling.

References

BEQUALM (2000). Fish Reproductive Success – embryo development and survival in viviparous blenny Zoarces viviparus. Biological Effects Quality Assurance in Monitoring Programmes (BEQUALM). BEQUALM Newsletter 2000.

Blekingekustens Vattenvårdsförbund (2005). Hanöbugten 2000-2002. (In Swedish).

Bodammer, J.E. (1993). The teratological and pathological effects of contaminants on embryonic and larvae fishes exposed as embryos: a brief review. Am. Fish Soc. Symp. 14: 77-84.

Davis, W.P. (1997). Evidence for developmental and skeletal responses as potential signals of endocrine disrupting compounds in fishes. In Chemically induced alterations in functional development and reproduction of fishes. Edited by Rolland, R.M., Gilbertson, M., Peterson, SETAC Press, Pensacola FL.

Fagerholm B (2002). Integrerad fiskövervakning i kustreferensområden, Fjällbacka. Årsrapport för 2001. Fiskeriverket, Kustlaboratoriet. Arbetsrapport. 10p. (In Swedish).

Gercken J., Förlin L., Andersson J. (2006). Developmental disorders in larvae of eelpout (Zoarces viviparus) from German and Swedish Baltic coastal waters. Mar Pollut Bull. 53(8-9):497-507.

HELCOM (2006). Manual for Marine Monitoring in the COMBINE Programme of HELCOM. Part D. Programme for monitoring of contaminants and their effects, updated 31.03.2006

ICES (2004). Recommended techniques for biological monitoring programmes at the national or international level – methods for fish. Report of the Working Group on Biological Effects of Contaminants. WGBEC meeting 22–26 March 2004 Ostend, Belgium.

ICES/OSPAR (2010). Report of the Joint ICES/OSPAR Study Group on Integrated Monitoring of Contaminants and Biological Effects (SGIMC), 25–29 January, Copenhagen, Denmark

Jacobsson, A., Neuman, E., Thoresson, G. (1986). The viviparous blenny as an indicator of environmental effects of harmful substances. Ambio 15(4): 236-238.

Kalmarweb (2005). Samordnad kustvattenkontroll i Kalmar län. (In Swedish).

Larsson, D.G.J, Förlin, L. (2002). Male-biased sex ratios of fish embryos near a pulp mill: Temporary recovery after a short-term shutdown. Environmental Health Perspective 110(8): 739-742.

Napierska D., Podolska M. (2006). Field studies of eelpout (Zoarces viviparus L.) from Polish coastal waters (southern Baltic Sea). Science of the Total Environment 371: 144–155.

Neuman, E., Sandström, O., Thoresson, G. (1999). Guidelines for coastal fish monitoring. National Board of Fisheries, Institute of Coastal Research, Ôregrund, Sweden, 44p

OSPAR (2008). JAMP guidelines for general biological effects monitoring. Technical Annex 10 – reproductive success in fish.

OSPAR (2010). OSPAR Coordinated Environmental Monitoring Programme (CEMP). Reference number: 2010-1.

Sjölin, A., Olsson, P., Magnusson, G. (2003). Hälsotillstånd och fortplantning hos tånglake i recipienten til Mörrums bruk hösten 2002. Toxicon rapport 8/03. (In Swedish)

Strand, J, Dahllöf, I. (2005). Biologisk effektmonitering i fisk. NOVANA teknisk anvisning for marin overvågning, kapitel 6.3. Danmarks Miljøundersøgelser. (In Danish).

Strand, J. (2005a). Biologisk effektmonitering i havsnegle og fisk. Marine workshopper 2004. Research Note from NERI No. 210, 34p. (In Danish)

Strand, J. (2005b). Bioligiske effekter i ålekvabbe og muslinger. Marine områder 2004. Status og trends i NOVANA overvågning. Faglig rapport fra DMU. www.dmu.dk. (In Danish).

Strand, J., Andersen, L., Dahllöf, I., Korsgaard, B. (2004). Impaired larval development in broods of eelpout (Zoarces viviparus) in Danish coastal waters. Fish Physiology and Biochemistry 30: 37–46

Vetemaa, M. (1999). Reproduction biology of the viviparous blenny (Zoarces vivparus L.). Rep. Nat. Board Fish. Swed. 2: 81-96, Fiskeriverket, Göteborg, Sweden.

Vetemaa, M., Förlin, L., Sandström, O. (1997). Chemical effluent impacts on reproduction and biochemistry in a North Sea population of viviparous blenny (Zoarces viviparus). J. Aquat. Ecosys. Stress Rec. 6: 33-41.

Ådjers, K., Appelberg, M., Eschbaum, R., Lappalainen, A., Lozys, L. (2001). Coastal fish monitoring in Baltic reference areas 2000. Kala- ja riistaraportteja 229: 1-14, Helsinki, Finland. Agenda item 3 at the HELCOM MONAS meeting 22 – 24 March 2004 in Tallinn, Estonia.

Summary table for reproductive success in eelpout

Evaluation Criteria	Rating	Description
Recommended indicator species	-	Eelpout (*Zoarces viviparus*)
Recommended matrix	-	Pregnant females
Recommended sample size per station	-	40 - 50 fish
Monitoring guideline (SOP) in place	Yes	Swedish and Danish monitoring guidelines, ICES guideline in prep.
ACs in place, i.e. background response (BaR) and/or EAC	Yes	BaR <1% for deformed larvae, <2% for dead larvae. EAC under development.
QA in place, i.e. ongoing intercalibrations or workshops	Yes	BALCOFISH/BEAST workshop in 2009, National workshops
Ecological relevance of effects for populations	High	Links to impaired reproduction
Persistent damage, not repairable effects	Yes	Irreversible effects
Contaminant sensitive response (Elevated effect levels in open waters, coastal waters or only point sources)	High	Coastal waters, point sources
Contaminant-specific cause-effects response	No	See below
General effect, response to several contaminant groups	Yes	Can respond to several contaminant groups like metals, OCs, PAH, EDS
Stable for confounding factors	Medium	Depending of the type of abnormal larvae development. The presence of deformed seems most related to contaminants.
Applicable indicator species in 1, 2-3 or >3 Baltic Sea subregions	Yes	Eelpout occurs in all Baltic Sea subregions
Already used in monitoring in 1, 2-3 or >3 countries	Yes	Sweden, Denmark and Germany
Available data, spatial coverage in the Baltic Sea - number of countries and stations (<10, 10-20, >20 stations)	Medium	S: 6-10 stations, DK: 10-15 st., D: 3 st.
Available data, length of time-series (<5, 5-10, >10 years)	Good	S: 1994-2010, DK: 2002-2010, D: 2003-2009
Costs of analyses per station, all required individuals (<500, 500-1500, >1500EUR)	High	~2000 EUR per station

Annex 13: ICES SGEH Biological Effects methods Background Documents for the Baltic Sea region

Reproductive success in amphipods

Background

Crustacean amphipods are regularly used in bioassays and laboratory exposure experiments for effects of contaminants. They carry their brood in an egg chamber until hatching and by analyzing the reproduction success we can score the effects of contaminant load in sediment and water. Twenty years of ecotoxicological studies in soft-bottom microcosms and studies of field populations collected in contaminated industrial areas have demonstrated toxicant-sensitive variables on the embryonic development of the Baltic amphipod species *Monoporeia affinis* and *Pontoporeia femorata* (Sundelin 1983, 1984, 1988, 1989, 1998, Eriksson *et al.*, 1996, Eriksson Wiklund *et al.*, 2005) and other amphipod species (Ford *et al.* 2003a, Sundelin *et al.* 2008, Bach *et al.* 2010).

When exposed to heavy metals, chlorinated organic compounds, pulp mill effluents or contaminated sediments in bioassays as well as in field studies, the frequency of malformed embryos has been demonstrated to be significantly higher when compared to control microcosms and reference areas (Elmgren *et al.*, 1983, Sundelin, 1983, 1984, 1988, 1989, 1991, Eriksson *et al.*, 1996, Sundelin and Eriksson, 1998, Eriksson Wiklund *et al.*, 2005)' suggesting the variable to be a general bioindicator of contaminant effects. Organic contaminants are often associated to lipids. During oogenesis large quantities of lipids are deposited into the developing oocytes (Herring 1974, Harrison, 1990, Harrison, 1997, Wouters *et al.*, 2001, Rosa and Nunes, 2003). These lipids, which consist mostly of monounsaturated fatty acids, are utilized and consumed during embryo development (Morais *et al.*, 2002, Rosa *et al.*, 2003, 2005), potentially leading to toxic effects of lipophilic contaminants increasing during embryogenesis. These effects also arise in low concentrations that do not demonstrably affect the sexual maturation, fertilization rate, fecundity (eggs/female) and rate of embryo development (time to hatching), indicating embryogenesis to be even more sensitive than other variables of the reproduction cycle.

All amphipod species show a similar direct embryo development despite differences in sexual behaviour before mating and in duration of embryogenesis that differs, mainly due to ambient temperature (Bregazzi, 1973, Lalitha *et al.*, 1991, McCahon and Pascoe, 1988). Therefore, this similar development allows for a consistent method of staging embryogenesis amongst all amphipod species and any resultant aberrations, which makes them particularly good for biomonitoring reproduction effects *in situ*. Other embryo aberrations respond to oxygen deficiency, scarcity of food quality and quantity and temperature stress (Eriksson Wiklund and Sundelin 2001, 2004, Sundelin *et al.* 2008). Multiple stressors act in concert in the environment and by analysing different types of aberrant embryo development we can discriminate between some of them. The method gives information about health status of the amphipod populations since diseases, parasite infection, sexual maturation in terms of oogenesis in females and sexual development in males and fecundity are scored.

Confounding factors

Malformed embryos seem to be comparatively insensitive to other environmental stressors but contaminant exposure. However a seven year field study showed a correlation between organic content in the sediment and malformation rate (Eriksson

Wiklund and Sundelin 2004). This could likely depend on higher concentrations of contaminants in sediments with higher load of organic content. The same study didn't show any relationship between oxygen concentrations in bottom waters, temperature and malformation rate. A negative correlation was found between females carrying a dead brood and the oxygen concentration of the bottom water. Fecundity was positively correlated with the carbon content of the sediment but negatively correlated with the temperature of the bottom water. These results confirm the findings of previous laboratory experiments (Eriksson Wiklund and Sundelin 2001). Undeveloped eggs (undifferentiated eggs) are not correlated to contaminant exposure but seem to occur due to low food resources and possibly to increased temperatures but there are no clear correlations so far (Sundelin *et al.* 2008). To meet the possible confounding factors additional variables i.e. organic content and oxygen in sediment and bottom waters should be measured on sampling stations.

Ecological relevance

Crustaceans are one of the most abundant invertebrate groups and it is relevant to include them in monitoring activities. Amphipods are regarded as particularly sensitive to contaminant exposure (Conlan 1994). Furthermore amphipods lack pelagic larvae and thus they are comparatively stationary facilitating the linkage between effects and environmental conditions. The deposit-feeding amphipod *Monoporeia affinis*, and the marine species *Pontoporeia femorata* are important benthic key stone species in the Swedish fresh and brackish water environment and are efficient bioturbators and important for the oxygenation of the sediment. They are significant food source for several fish species and other macrofauna species (Arrhenius and Hansson, 1993; Aneer, 1975). By analyzing reproduction variables as malformed embryos and other aberrant embryo development we combine the supposedly higher sensitivity of low-organization level biomarkers with the higher relevance attributed to variables giving more direct information on next-generation and population level effects (Sundelin 1983, Tarkpea *et al.*, 1999, Cold and Forbes 2004, Heuvel- Greve *et al.*, 2007, Hutchinson 2007).

Quality Assurance

Guidelines are available in ICES Techniques in Marine Environmental Sciences (TIMES) no 41. Quality assurance declaration is updated regularly at website at Swedish EPA

http://www.naturvardsverket.se/upload/02_tillstandet_i_miljon/Miljoovervakning/programomraden/kust_och_hav/kvalitetsdeklaration_embryonal_vitmarla.pdf.

Quality assurance has been practiced during training courses and workshops when different persons analyzing the embryos checked the accordance by examining the same brood. The accordance was between 90 to 97 %. Since *M. affinis* is a glacial relict of fresh water origin occurring in inland waters below the highest coastline and the method has been used and evaluated also in Swedish greater lakes as Lake Vänern and Lake Vättern (Sundelin *et al.* 2008). For method description see Sundelin *et al.* 2008 (http://ices.dk/pubs/times/times41/TIMES41.pdf).

Background response and Assessment Criteria

The reproduction success of the freshwater amphipod *Monoporeia affinis* and the marine species *Pontoporeia femorata* in terms of various embryo aberrations have been measured in Baltic proper and Bothnian Sea since an international evaluation in 1993 prioritized the method as one of the most useful for effect monitoring of contaminants in the Baltic.

The method has also been used for other amphipod species in coastal waters outside Great Britain, Gulf of Riga, Gulf of Gdansk and in the Belt Sea. The method is used in the Bonus Beast programme as a core biomarker in all areas of the beast programme.

All species of amphipods could be analyzed for embryo aberrations and health status. The same protocol and method could be used for all of them (See TIMES 41). Field studies using different species of amphipods inhabiting the same area show a similar background level of malformation rate. However assessment criteria have only recently been developed for *Monoporeia affinis* where there exist a long-term trend series since 1994 in the Bothnian Sea and Baltic proper.

Table 1. Assessment criteria for malformed embryos of Monoporeia affinis in the Baltic. Seventeen years data were used for the calculation of background response (<5.7 % malformed embryos) and assessment criteria. The limit between good and moderate status was put at a value where all (> 99 %) variation in the reference dataset is included. The yearly mean and the 99:e percentile in the reference dataset were estimated by bootstrapping. Three stations with at least 10 gravid females was put as minimum for classifying the status of the area.

Status class	Malformed embryos %
High	< 0.029
Good	0.029 < 0.057
Moderate	0.057 < 0.086
Poor	0.086 < 0.114
Bad	> 0.114

Distribution of indicator species in Baltic Sea subregions

M. affinis occurs in the whole Baltic from northern part of the Bothnian Bay to the Gulf of Gdansk and Gulf of Riga. In the Belt Sea, where salinity is too high for *M. affinis* only the related species *Pontoporeia occurs*. The marine related species *P. affinis* occurs up to the northern Bothnian Sea where it disappears due to lower salinity. The deposit-feeding amphipod *Corophium sp.*occurs in the whole Baltic. Various gammarid species occur in the whole Baltic but most species are restricted to more shallow areas than *M.affinis* and *P. femorata* and live in the seaweed belt.

Acknowledgements

We acknowledge Anders Bignert at The Swedish Museum of Natural History and Kjell Leonardsson at Umeå University for important support during the calculation of assessment criteria. We also thank the Bonus project BEAST for opportunity to validate the methods for other amphipod species and the dep. of Applied Environmental Science (ITM) for facilitating the progress of the work.

References

Bach, L., Fischer A., Strand J. 2010. Local anthropogenic contamination affects the fecundity and reproductive success of an Arctic amphipod. Marine Ecological Progress Series 419 121-128

Bregazzi, P. K. 1973. Embryological development in Tryphosella kergueleni (Miers) and Cheirimedon femoratus (Pfeffer) (Crustacea: Amphipoda). British Antarctic Survey Bulletin, 32: 63-74.

Chapman, P.M., Wang, F., Janssen, C., Persoone, G., and Allen, H. E., 1998. Ecotoxicology of metals in aquatic sediments: binding and release, bioavailability, risk assessment and remediation. Canadian Journal of Fisheries and Aquatic Sciences, 55: 2221-2243.

Cold, A., and Forbes, V. 2004. Consequences of a short pulse of pesticide exposure for survival and reproduction of Gammarus pulex. Aquatic Toxicology, 67:287-299

Conlan, K.E. 1994. Amphipod crustaceans and environmental disturbance – a review. Journal of Natural History, 28: 519-554.

Elmgren, R., Hansson, S., Larsson, U., Sundelin, B., and Boehm, P. 1983. The "Tsesis" oil spill: Acute and long-term impact on the benthos. Marine Biology, 73, 51-65.

Eriksson, A-K., Sundelin, B., Broman, D., and Näf, C. 1996. Effects on Monoporeia affinis of HPLC-fractionated extracts of bottom sediments from a pulp mill recipient. In: Environmental fate and effects of pulp and paper mill effluents. Servos *et al*., (eds) p 69-78, St Lucie Press Florida.

Eriksson Wiklund, A-K., and Sundelin, B.. 2001. Impaired reproduction of the amphipods Monoporeia affinis and Pontoporeia femorata as a result of moderate hypoxia and increased temperature. Marine Ecology Progress Series, 171: 165-180.

Eriksson Wiklund, A-K., and Sundelin, B. 2004. Biomarker sensitivity to temperature and hypoxia- a seven year field study. Marine Ecology Progress Series, 274: 209-214.

Eriksson Wiklund, A-K, Sundelin, B., and Broman, D. 2005. Toxicity evaluation by using intact sediments and sediment extracts, Marine Pollution Bulletin, 50: 660-667.

Eriksson Wiklund, Sundelin B, Rosa R (2008). Population decline of the amphipod Monoporeia affinis in Northern Europe, consequence of food shortage and competition. J Exp Mar Biol Ecol, 367; 81-90

Eriksson Wiklund AK., Dahlgren K, Sundelin B, Andersson A. (2009). Effects on benthic production of Climate induced alteration in the pelagic food web. Mar Ecol Prog Ser, 396: 13–25

Ford, A.T., Fernandes, T.F., Rider, S.A., Read, P.A., Robinson, C.D., and Davies I.M. 2003a. Measuring sublethal impacts of pollution on reproductive output in marine Crustacea. Marine Ecology Progress Series, 265: 303-309.

Gorokhova, E. Löf M, Halldórsson H, Tjärnlund U, Lindström M, Elfwing T, Sundelin (2010). Single and combined effects of hypoxia and contaminated sediments on the amphipod Monoporeia affinis in laboratory toxicity bioassays based on multiple biomarkers. Aquatic Toxicology.

van den Heuvel-Greve, M., Postina, J., Jol, J., Kooman, H., Dubbeldam, M., Schipper, C., and Kater, B. 2007. A chronic bioassay with the estuarine amphipod Corophium volutator: Test method description and confounding factors. Chemosphere, 66:1301-1309

Harrison, K. E. 1990. The role of nutrition in maturation, reproduction and embryonic development of decapod crustaceans: a review. Journal of Shellfish Research, 9: 1-28.

Harrison, K. E. 1997. Broodstock nutrition and maturation diets. In Crustacean Nutrition 6. Edited by L. R. D´Abrano, D. E. Conklin and D. M. Akiyama. The World Aquaculture Society. Baton Rouge, Louisiana, pp. 390-408.

Herring, P. J. 1974. Size, density and lipid content of some decapod eggs. Deep Sea Research, 21: 91-94.

Hutchinson, T. H. 2007. Small is useful in endocrine disrupter assessment - four key recommendations for aquatic invertebrate research. Ecotoxicology, 16: 231-238

Lalitha, M., Shyamasundari, K., and Hanumantha Rao, K. 1991. Embryological development in Orchestia platensis kroyer (Crustacea:Amphipoda). Uttar Pradesh Journal of Zoology, 11: 107-112.

McCahon, C. P., and Pascoe, D. 1988. Use of Gammarus pulex (L.) in safety evalutions tests: culture and selections of a sensitive life stage. Ecotoxicology and Environmental Safety, 15: 245-252.

Morais, S., Narciso, L., Calado, R., Nunes, M. L., and Rosa, R.. 2002. Lipid dynamics during the embryonic development of Plesionika martia (Decapoda; Pandalidae), Palaemion serratus and P. elegans (Decapoda; Pandalidae): relation to metabolic consumption. Marine Ecology Progress Series, 242: 195-204.

Rosa, R., and Nunes, M. L. 2003. Tissue biochemical composition in relation to the reproductive cycle of the deep-sea decapod, Aristeus antennatus in the Portuguese south coast. Journal of the Marine Biological Association of the United Kingdom 83:963-970.

Rosa, R., Morais, S., Calado, R., Narciso, L., and Nunes, M.L.. 2003. Biochemical changes during the embryonic development of Norway lobster, Nephrops norvegicus. Aquaculture, 221:507-522.

Rosa, R., Calado, R., Andrade, A. M., Narciso, L., and Nunes, M. L. 2005. Changes in amino acids and lipids during embryogenesis of European lobster, Homarus gammarus (Crustacea: Decapoda). Comparative Biochemistry and Physiology part B, 140: 241-249.

Sundelin, B. 1983. Effects of cadmium on Pontoporeia affinis (Crustacea: Amphipoda) in laboratory soft-bottom microcosms. Marine Biology, 74:203-212.

Sundelin, B. 1984. Single and combined effects of lead and cadmium on Pontoporeia affinis (Crustacea: Amphipoda) in laboratory soft-bottom microcosms. In: Ecotoxicological testing for the marine environment. G. Persoone, E. Jaspers, and C. Claus (Eds). State University of Ghent and Institute for Marine Scientific Research, Bredene, Belgium. Vol. 2. 588 p.

Sundelin, B. 1988. Effects of sulphate pulp mill effluents on soft-bottom organisms - a microcosm study. Water Science and Technology, 20: 175-177.

Sundelin, B. 1989. Ecological effect assessment of pollutants using Baltic benthic organisms. Thesis, Univ. of Stockholm, Dep. of Zoology 106 91 Stockholm.

Sundelin, B., and Eriksson, A-K. 1998. Malformations in embryos of the deposit-feeding amphipod Monoporeia affinis in the Baltic Sea. Marine Ecology Progress Series, 171: 165-180.

Sundelin, Rosa R, Eriksson Wiklund A-K (2008). Reproduction disorders in a benthic amphipod, Monoporeia affinis, an effect of low food quality and availability. Aquatic Biology, 2:179-190.

Swartz, R. C., DeBen, W.A., Sercu, K.A., and Lamberson, J.O. 1982. Sediment toxicity and the distribution of amphipods in Commencement Bay, Washington USA. Marine Pollution Bulletin, 13: 359-364.

Tarkpea, M., Eklund, B., Linde, M., and Bengtsson, B-E. 1999. Toxicity of conventional, elemental chlorine-free, and totally chlorine-free kraft-pulp bleaching effluents assessed by short-term lethal and sublethal bioassays. Environmental Toxicology & Chemistry, 18: 2487-2496

Wouters, R., C. Molina, P. Lavens, and Calderon, J.. 2001. Lipid composition and vitamin content of wild female Litopenaeus vannamei in different stages of sexual maturation. Aquaculture, 198: 307-323.

Annex 14: ICES SGEH Biological Effects methods Background Documents for the Baltic Sea region*

Fish Disease Index - externally visible fish diseases, macroscopic liver neoplasms and liver histopathology

***ICES/OSPAR document from the ICES SGIMC Report 2011, complemented and modified by SGEH 2011 with information relevant for application in the Baltic Sea region**

Background

Diseases of wild marine fish have been studied on a regular basis by many ICES Member Countries for more than two decades. Disease surveys are often integrated with other types of biological and chemical investigations as part of national monitoring programmes aiming at an assessment of the health of the marine environment, in particular in relation to the impact of human activities (Lang, 2002).

On an international level, fish disease data have been used for environmental assessments in the framework of the North Sea Task Force and its Quality Status Report (North Sea Task Force, 1993), the OSPAR Quality Status Reports 2000 and 2010 (OSPAR Commission, 2000, 2010a) and in the 3rd and 4th HELCOM assessments (HELCOM, 1996, 2002). Studies on externally visible diseases, macroscopic liver neoplasms and liver histopathology are on the list of techniques for general and contaminant-specific biological effects monitoring as part of the OSPAR pre-CEMP (OSPAR, 2010b).

In the Baltic Sea, fish diseases have been monitored on a more or less regular basis since the beginning of the 1980s (Lang, 2002). Baltic Sea countries currently carrying out fish disease surveys in the Baltic Sea on an annual basis are Germany (vTI Institute of Fisheries Ecology, Cuxhaven), Poland (Sea Fisheries Institute, Gdynia) and Russia (AtlantNIRO, Kaliningrad) (ICES, 2011). While Polish and Russian studies are restricted to national EEZs, the German programme covers larger areas of the southern Baltic Sea, including sampling sites in ICES Subdivision 22, 24, 25 and 26. Other Baltic Sea countries not mentioned have some experience in fish disease monitoring from studies carried out in the 1980s and 1990s, but have stopped regular activities.

Most of the regular disease surveys are so far focussed on fish species sampled in offshore areas, with the main target species flounder (*Platichthys flesus*), cod (*Gadus morhua*) and, to a lesser extent, herring (*Clupea harengus*). In the western Baltic Sea, the common dab (*Limanda limanda*) is another target species. Other common species have been examined on a more irregular basis. A wide and species-dependent range of diseases (incl. some parasite species) is being monitored, with an emphasis on externally visible lesions and parasites. Only in flounder have regular studies on liver pathology (largely related to neoplastic lesions) been included partly (Lang *et al.*, 2006). The methodologies applied largely follow ICES guidelines (Bucke *et al.*, 1996, Feist *et al.*, 2004) which can easily be adapted for other species relevant for fish monitoring in the Baltic Sea. Methodologies and diagnostic criteria involved in the monitoring of contaminant-specific liver neoplasms and liver histopathology have largely been developed based on studies with flatfish species, in Europe mainly dab and flounder, but can also be adapted to other flatfish species (e.g. plaice (*Pleuronectes platessa*) and also to bottom-dwelling roundfish species, such as viviparous blenny (*Zoarces viviparus*).

New disease trends in Baltic Sea fish species have been reviewed regularly by the ICES Working Group on Pathology and Diseases of Marine Organisms (WGPDMO) and relevant information has partly been incorporated in the HELCOM Periodic Assessments (HELCOM, 1996, 2002). However, compared to the North Sea, fish disease monitoring and assessment in the Baltic Sea is less developed and, so far, only few fish disease data from the Baltic Sea have been submitted to the ICES Environmental Databank. In the light of present developments in ICES and in HELCOM, the ICES WGPDMO recommended at its 2007 meeting that Baltic Sea countries running fish disease monitoring programmes in the Baltic Sea make attempts to submit their disease data to the ICES Environmental Databank in order to make them available for integrated assessments, such as those carried out by the ICES/HELCOM Working Group on Integrated Assessment of the Baltic Sea (WGIAB) and as part of the periodic HELCOM assessments (ICES, 2007).

In 2005, the ICES Workshop on Fish Disease Monitoring in the Baltic Sea (WKFDM) started to develop an integrative tool for the analysis and assessment of the health status of fish which was later termed 'Fish Disease Index (FDI)' (ICES, 2006a,b). In contrast to previous attempts, largely focusing on the analysis and assessment of changes in prevalence of single diseases, the FDI approach was developed with the primary aim to analyse and assess changes in spatial and temporal patterns in the overall disease status of fish, by summarising information on the prevalence of a variety of common diseases affecting the fish species as well as their severity grades and effects on the host into a robust numerical value calculated for individual fish and, as mean values, for representative samples from a population. The common dab (*Limanda limanda*) from the North Sea was selected as a model species for the construction of the FDI approach because most existing data are from fish disease surveys with the dab as primary target species. However, the FDI approach is constructed in a way that it can easily be adapted to other fish species for which disease data are available. The development of an analogous FDI approach for Baltic Sea fish species is on the agenda of the ICES Working Group on Pathology and Diseases of Marine Organisms (WGPDMO) and will be finalised during 2011 (ICES, 2011). In first instance, the efforts will focus on flounder and cod, species for which most data are available from national fish disease monitoring in the Baltic Sea.

Fish disease surveys and associated FDI data analyses and assessments according to the OSPAR and ICES requirements address four categories of diseases. These categories also are the basis for fish disease monitoring/assessment in the Baltic Sea (see Table 1). Other target species and diseases may be added when more experience and data are available.

Table 1. Categories and diseases/lesions for disease monitoring and assessment with Baltic Sea fish species (ICES, 2011; modified).

Disease Category	Diseases/Lesions	
	Flounder (*P. flesus*)	**Cod (*G. morhua*)**
Externally visible diseases	Lymphocystis Acute/healing skin ulcerations Acute/healing fin rot/erosion Epidermal hyperplasia/ papilloma *Cryptocotyle sp.* *Lepeophtheirus pectoralis*	Acute/healing skin ulcerations Acute/healing fin rot/erosion Skeletal deformities Pseudobranchial swelling Epidermal hyperplasia/papilloma *Cryptocotyle lingua* *Lernaeocera branchialis*
Macroscopic liver neoplasms	Benign and malignant liver tumours > 2 mm in diameter	Benign and malignant liver tumours > 2 mm in diameter
Non-specific liver histopathology	Non-specific degenerative/regenerative change Inflammatory lesions Parasites	Non-specific degenerative/regenerative change Inflammatory lesions Parasites
Contaminant-specific liver histopathology	Early toxicopathic non-neoplastic lesions Foci of cellular alteration Benign neoplasms Malignant neoplasms	Early toxicopathic non-neoplastic lesions Foci of cellular alteration Benign neoplasms Malignant neoplasms

Confounding factors

The multifactorial aetiology of diseases, in this context in particular of externally visible diseases, is generally accepted. Therefore, externally visible diseases have correctly been placed into the general biological effect component of the OSPAR CEMP (OSPAR, 2010b). Most wild fish diseases monitored in past decades are caused by pathogens (viruses, bacteria). However, other endogenous or exogenous factors may be required before the disease develops. One of these factors can be environmental pollution, which may either affect the immune system of the fish in a way that increases its susceptibility to disease, or may alter the number and virulence of pathogens. In addition, contaminants may also cause specific and/or non-specific changes at various levels of biological organisation (molecule, sub-cellular units, cells, tissues, organs) leading to disease without involving pathogens.

The occurrence of significant changes in the prevalence of externally visible fish diseases can be considered a non-specific and more general indicator of chronic rather than acute (environmental) stress, and it has been speculated that they might, therefore, be an integrative indicator of the complex changes typically occurring under field conditions rather than a specific marker of effects of single factors. Because of the multifactorial causes of externally visible diseases, the identification of single factors responsible for observed changes in disease prevalence is difficult, and scientific proof of a link between contaminants and externally visible fish diseases is hard to achieve. Nevertheless, there is a consensus that fish disease surveys should continue to be part of national and international environmental monitoring programmes since they can provide valuable information on changes in ecosystem health and may act as an "alarm bell", potentially initiating further more specific studies on cause and effect relationships.

A thorough statistical analysis of ICES data on externally visible diseases (lymphocystis, epidermal hyperplasia/papilloma, acute/healing skin ulceration) of dab from different North Sea regions, confirmed the multifactorial aetiology of the diseases under study since a number of natural and anthropogenic factors (stock composition, water temperature, salinity, nutrients, contaminants in water, sediments and biota) were found to be significantly related to the long-term temporal changes in disease prevalence recorded (Lang and Wosniok, 2000; Wosniok *et al.*, 2000).

The presence of macroscopic liver neoplasms and of certain types of histopathological liver lesions is a more direct indicator of contaminant effect and has been used for many years in environmental monitoring programmes around the world. Liver neoplasms (either detected macroscopically or by histopathological analysis) are likely to be associated to exposure to carcinogenic contaminants, including PAHs, and are therefore considered appropriate indicators for contaminant-specific biological effects monitoring. The study of liver histopathology (comprises the detection of more lesion categories (non-specific, neoplastic and non-neoplastic toxicopathic lesions), reflecting responses to a wider range of contaminants (including PAHs) but also to other environmental stressors and is, therefore, considered an appropriate indicator for both general and contaminant-specific biological effects monitoring.

The liver is the main organ involved in the detoxification of xenobiotics and several categories of hepatocellular pathology are now regarded as reliable biomarkers of toxic injury and representative of biological endpoints of contaminant exposure (Myers *et al.*, 1987, 1992, 1998; Stein *et al.*, 1990; Vethaak and Wester, 1996; Stentiford *et al.*, 2003; Feist *et al.*, 2004). The majority of lesions observed in field collected animals have also been induced experimentally in a variety of fish species exposed to carcinogenic compounds, PAHs in particular, providing strong supporting evidence that wild fish exhibiting these lesions could have been exposed to such environmental contaminants.

Ecological relevance

Fish diseases are considered as ecosystem health indicators, reflecting ecologically relevant effects of environmental stressors at the individual and population levels. As such, they differ from other types of indicators that reflect changes at lower levels of biological organisation (e. g. molecules, cells) and the ecological relevance of which is considered as low or unclear (e. g. biomarkers of exposure to contaminants) (ICES, 2009b).

Fish diseases may act at the individual level by adversely affecting behaviour, growth, reproduction, and survival of affected specimens. Individual effects may lead to ecologically relevant population effects (especially in epidemic situations) and ultimately to biodiversity effects at the community level. Diseases in wild fish may affect aquaculture due to transmission of pathogens. A high prevalence of a conspicuous fish disease may affect fishery profit because fish with prominent disease signs cannot be marketed. Although direct human health effects of diseases affecting wild fish are unlikely (except for a few cases), diseased fish may act as carriers of pathogens that pose a risk to human consumers.

Quality Assurance

Since the early 1980s, ICES has played a leading role in the initiation and coordination of fish disease surveys and has contributed considerably to the development of standardised methodologies. Through the work of the ICES Working Group on Pathology and Diseases of Marine Organisms (WGPDMO), its offspring, the Sub-Group/Study Group on Statistical Analysis of Fish Disease Data in Marine Stocks (SGFDDS) (1992–1994) and the ICES Secretariat, quality assurance procedures have been implemented at all stages, from sampling of fish to submission of data to the ICES Data Centre and to data assessment.

A number of practical ICES sea-going workshops on board research vessels were organised by WGPDMO in 1984 (southern North Sea), 1988 (Kattegat), 1994 (Baltic Sea, co-sponsored by the Baltic Marine Biologists, BMB) and 2005 (Baltic Sea) in order to intercalibrate and standardise methodologies for fish disease surveys (Dethlefsen *et al.*, 1986; ICES, 1989, 2006a; Lang and Mellergaard, 1999) and to prepare guidelines. Whilst first guidelines were focused on externally visible diseases and parasites, WGPDMO developed guidelines for macroscopic and microscopic inspection of flatfish livers for the occurrence of neoplastic lesions at a later stage. Further intercalibration and standardision of methodologies used for studies on liver pathology of flatfish were a major issue of the 1996 ICES Special Meeting on the Use of Liver Pathology of Flatfish for Monitoring Biological Effects of Contaminants (ICES, 1997). This formed the basis from which the quality assurance programme Biological Effects Quality Assurance in Monitoring (BEQUALM) (www.bequalm.org) developed for the application of liver pathology in biological effects monitoring (see below) (Feist *et al.*, 2004).

A fish disease database has been established within the ICES Data Centre, consisting of disease prevalence data of key fish species and accompanying information, submitted by ICES Member Countries. Submission of fish disease data to the ICES Data Centre has been formalised by the introduction of the ICES Environmental Reporting Format designed specifically for the purpose. This is used for fish disease, contaminant and biological effects data. The programme includes internal screening procedures for the validation of the data submitted providing further quality assurance.

The ICES fish disease database is extended on an annual basis to include data from other species and areas within the OSPAR and HELCOM area as well as data on studies into other types of diseases, e.g. macroscopic liver neoplasms and liver histopathology. To date, the data comprise mainly information from studies on the occurrence of externally visible diseases and macroscopic liver lesions in the common dab (*Limanda limanda*) and the European flounder (*Platichthys flesus*) from the North Sea and adjacent areas, including the Baltic Sea, Irish Sea, and the English Channel. In addition, reference data are available from pristine areas, such as waters around Iceland. In total, data on length, sex, and health status of more than 700 000 individual specimens, some from as early as 1981, have been submitted to ICES, as well as information on sampling characteristics (Wosniok *et al.*, 1999, Lang and Wosniok, 2008).

Current ICES WGPDMO activities have focussed on the development and application of statistical techniques for an assessment of disease data with regard to the presence of spatial and temporal trends in the North Sea and western Baltic Sea (Wosniok *et al.*, 1999, Lang and Wosniok, 2008). In a more holistic approach, pilot analyses have been carried out combining the disease data with oceanographic, nutrient, contaminant and fishery data extracted from the ICES Data Centre in order to improve the knowledge about the complex cause-effect relationships between environmental factors and fish diseases (Lang and Wosniok, 2000; Wosniok *et al.*, 2000). These analyses constituted one of the first attempts to combine and analyses ICES data from various sources and can, therefore, be considered as a step towards a more comprehensive integrated assessment.

Quality assurance is in place for externally visible diseases, macroscopic liver neoplasms and liver histopathology via the ongoing BEQUALM programme. Regular intercalibration and ring-test exercises are conducted. The basis for QA procedures are provided in two key publications in the ICES TIMES series (Bucke *et al.*, 1996, Feist *et al.*, 2004) and a BEQUALM CD ROM of protocols and diagnostic criteria and reporting requirements for submission of data to ICES. Guidelines on fish disease monitoring in the Baltic Sea have been prepared by ICES (2006a).

Background responses and Assessment Criteria

The development of assessment tools for externally visible diseases, macroscopic neoplasms and liver histopathology has been addressed by the ICES Working Group on Pathology and Diseases of Marine Organisms (WGPDMO) (ICES, 2006b, 2007, 2008, 2009a, 2011). Further additions were proposed at the 2009 ICES/OSPAR Workshop on Assessment Criteria for Biological Effects Measurements (WKIMC) (ICES, 2009b) (see further below).

For the analysis and assessment of fish disease data, the ICES WGPDMO developed a Fish Disease Index (FDI), using data on diseases of the common dab (*Limanda limanda*) as a model The aim of this tool is to summarise information on the disease status of individual fish into one robust and easy-to-understand and easy-to-communicate numeric figure. By applying defined assessment criteria and appropriate statistics, the FDI can be used to assess the level and temporal changes in the health status of fish populations and can, thus, serve as a tool for the assessment of the ecosystem health of the marine environment, e.g. related to the effects of anthropogenic and natural stressors. Its design principle allows the FDI to be applied to other species with other sets of diseases. Therefore, the FDI approach is applicable for wider geographical areas, e.g. as part of a convention-wide HELCOM monitoring and assessment programme.

For the calculation of the FDI, the following components are required:

- information on the presence or absence of a range of diseases monitored on a regular basis, categorised as externally visible diseases, macroscopic liver neoplasms as well as non-specific and contaminant-specific liver histopathology (see Table 1);
- for most diseases, data on three severity grades (reflecting a light, medium or severe disease status) are included;
- disease-specific weighting factors, reflecting the impact of the diseases on the host (assigned based on expert judgements);
- adjustment factors for effects of size and sex of the fish as well as for season effects.

The result of the calculation is a FDI value for individual fish which is scaled in a way that values can range from 0 to 100, with low values representing healthy and high values representing diseased fish. The maximum value of 100 can only be reached in the (purely theoretical and unrealistic) case that a fish is affected by all diseases at their highest severity grades. From the individual FDIs, mean FDIs for a sample from a fish population in a given sampling area can be calculated. Usually a sample in the present sense consists of the data collected in an ICES statistical rectangle during one cruise. All assessment is based on mean FDI values calculated from these samples. Depending on the data available, FDIs can be calculated either for single disease categories or for combinations thereof.

The assessment of the mean FDI data considers (a) long-term FDI level changes, (b) FDI trends in the recent five years time window and (c) comparing each FDI to its Background Assessment Criterion (BAC) and Environmental Assessment Criterion

(EAC) where these are defined. While assessments (a) and (b) are done on a region-wise basis, global BAC and EAC are used by assessment (c). The assessment approaches (a) and (b) do not apply any global background or reference values or assessment criteria as is often done for chemical contaminants or for biochemical biomarkers. Instead, these assessment approaches use the development of the mean FDI within the geographical units (usually ICES rectangles) over a given period of time, based on which region-specific assessment criteria are defined. The reason for choosing this approach is the known natural regional variability of the disease prevalence (even in areas considered to be pristine), making it implausible to define generally applicable background/reference values that can uniformly be used for all geographical units to be assessed. This approach is based on the availability of disease data over a longer period of time (ideally 10 observations, e.g., in the case of bi-annual monitoring over a period of five years) for every geographical area to be assessed. The assessment approach (c) ignores the known regional differences and involves globally defined Assessment Criteria (BAC, EAC; see above) with the consequence that within-region variation might be dominated by general differences in regional levels. However, by applying globally defined Assessment Criteria, the FDI can also be used for exploratory monitoring in areas not studied before or for newly installed fish disease monitoring programmes after some modification.

The final products of the assessment procedure are:

- graphs showing the temporal changes in mean FDI values in a geographical unit over the entire observation period;
- maps in which the geographical units assessed are marked with green, yellow or red smiley faces, indicating long-term changes (e.g., comparing the past five years to the preceding five-years period) in health status of the fish population (green: improvement of the health status; yellow: indifferent variation; red: worsening of the health status, reason for concern and motivation for further research on causes);
- maps in which the geographical units assessed are marked with green, yellow or red smiley faces, indicating trends in health status of the fish population during the past five years (green: improvement of the health status; yellow: indifferent variation; red: worsening of the health status, reason for concern and motivation for further research on causes);
- maps in which the geographical units assessed are marked with green, yellow or red smiley faces, indicating the level of the FDI observed at a defined point in time (green: below the BAC; yellow: between BAC and EAC; red: above the EAC, reason for concern and motivation for further research on causes).

The ICES WGPDMO applied the FDI approach and the assessment for the common dab from the North Sea using ICES fish disease data extracted from the ICES Environmental Data Centre twice in 2008 and, using an extended dataset, in 2009 (ICES, 2008, 2009a). The results have been included in the OSPAR QSR 2010 as a case study (OSPAR, 2010).

At the 2009 ICES/OSPAR Workshop on Assessment Criteria for Biological Effects Measurements (WKIMC) and the 2011 meeting of the ICES WGPDMO, Background Assessment Criteria (BAC) and Environmental Assessment Criteria (EAC) to be used for externally visible diseases, non-specific liver histopathology, macroscopic liver neoplasms and contaminant-specific liver histopathology in North Sea dab were proposed (ICES, 2009b, 2011). A common strategy was developed for externally visible fish diseases (EVD) and non-specific liver histopathology (NLH), and a modified strategy was developed for macroscopic liver neoplasm (MLN) and contaminant-specific liver histopathology (SLH). Two strategies are needed because the first two categories require an external harm entity that is to be controlled by the EAC, while the last two categories themselves already constitute measures of harm. The approach leading to a BAC for EVD and NLH is guided by the following considerations:

- No "pristine" reference area is available from which a BC (background concentration) or a BAC could be obtained and transferred to the ICES area.
- A certain number of diseases in a population seems inevitable as the vast majority of disease rates from fish disease monitoring samples is larger than zero, i.e. has FDI > 0. This suggests using a lower bound for the mean FDI as BAC (each mean FDI is calculated from data from one cruise, one ICES rectangle).
- Using the smallest historical positive FDI value produces an unstable BAC estimate.
- Preferably a small percentile of the FDI distribution should serve as BAC. The FDI value below which only a defined small proportion (e.g. 10%) of all values lies would be used as BAC.
- A BAC should be derived in this way separately for each species and sex (and the disease category).
- The BACs obtained are considered valid for the whole area from which the basic data originated.

An EAC is the threshold beyond which "unacceptable effects" must be expected. The "effect" considered for EVD and NLH is the loss in condition factor (CF) that is associated with increasing FDI. Loss in CF is defined as the difference between the mean CF for FDI = 0 and the mean CF for an FDI > 0, expressed as percentage of the mean CF for FDI = 0. The EAC is then defined as that FDI value above which the loss in CF exceeds the acceptable amount (e.g. 10%). The essential point in this approach is that a link was established between a biomarker (fish diseases) and a relevant effect, in this case the loss in condition. Therefore an EAC could be based on loss in condition. With BAC and EAC available, the FDI results can be represented in the usual three-colour scheme, also on a map (see above).

Deriving BAC and EAC for macroscopic liver neoplasm (MLN) and contaminant-specific liver histopathology (SLH) follows slightly different lines. As macroscopic liver neoplasms are themselves unacceptable effects, there is no need to employ a further effect for determining an EAC. Also, there is no point in defining a BAC, as each effect in the MLN and SLH category is unacceptable.

The suitability of these BACs/EACs for fish disease monitoring and assessment in the Baltic Sea will be evaluated in the course of 2011 (prior to the 2012 meeting of ICES WGPDMO) and modifications will be done as required.

Acknowledgements

Research on the application of the Fish Disease Index (FDI) as indicators for the monitoring and assessment of environmental stressors (incl. contaminants) in the Baltic Sea has partly been funded by the EU Project BEEP Contract n° ECK3-CT2000-00025, and the BONUS+ Project BEAST.

References

Bucke, D., Vethaak, A. D., Lang, T., and Mellergaard, S. 1996. Common diseases and parasites of fish in the North Atlantic: Training guide for identification. ICES Techniques in Marine Environmental Sciences, 19. 27 pp.

Dethlefsen, V., Egidius, E., and McVicar, A. H. 1986. Methodology of fish disease surveys. Report of an ICES Sea-going Workshop held on RV 'Anton Dohrn' 3-12 January 1984. ICES Cooperative Research Report, 140. 33 pp.

Feist, S. W., Lang, T., Stentiford, G. D. and Köhler, A., 2004. The use of liver pathology of the European flatfish, dab (Limanda limanda L.) and flounder (Platichthys flesus L.) for monitoring biological effects of contaminants. ICES Techniques in Marine Environmental Science, 28: 47pp.

HELCOM 1996. Third Periodic Assessment of the State of the Marine Environment of the Baltic Sea, 1989-1993; Background Document. Baltic Sea Environment Proceedings N0 64 B. 252 pp.

HELCOM 2002. Environment of the Baltic Sea area 1994-1998. Baltic Sea Environment Proceedings N0 82 B. 215 pp.

ICES. 1989. Methodology of fish disease surveys. Report of an ICES Sea-going Workshop held on RV U/F 'Argos' 16–23 April 1988. ICES Cooperative Research Report, 166. 33 pp.

ICES. 1997. Report of the Special Meeting on the Use of Liver Pathology of Flatfish for Monitoring Biological Effects of Contaminants. ICES CM 1997/F:2. 75 pp.

ICES. 2005. ICES Advice

http://www.ices.dk/committe/acfm/comwork/report/2005/may/Technical%20Annex%20to%20the%20review%20of%20WKIMON.pdf.

ICES. 2006a. Report of the ICES/BSRP Sea-going Workshop on Fish Disease Monitoring in the Baltic Sea (WKFDM). ICES CM 2006, BCC:02, 85 pp.

ICES. 2006b. Report of the Working Group on Pathology and Diseases of Marine Organisms (WGPDMO), ICES CM 2006/MCC:01, 104 pp.

ICES. 2007. Report of the Working Group on Pathology and Diseases of Marine Organisms (WGPDMO), ICES CM 2007/MCC:04, 92 pp.

ICES. 2008. Report of the Working Group on Pathology and Diseases of Marine Organisms (WGPDMO), ICES CM 2008/MCC:01, 128 pp.

ICES. 2009a. Report of the Working Group on Pathology and Diseases of Marine Organisms (WGPDMO), ICES CM 2009/MCC:01, 111 pp.

ICES. 2009b. Report of the ICES/OSPAR Workshop on Assessment Criteria for Biological Effects Measurements (WKIMC), ICES CM 2009/ACOM:50, 133 pp.

ICES. 2011. Report of the Working Group on Pathology and Diseases of Marine Organisms (WGPDMO), ICES CM 2011/SSGHIE:04.

Lang, T. 2002. Fish disease surveys in environmental monitoring: the role of ICES. ICES Marine Science Symposia, 215: 202–212.

Lang, T., and Mellergaard, S. 1999. The BMB/ICES Sea-Going Workshop 'Fish Diseases and Parasites in the Baltic Sea' – introduction and conclusions. ICES Journal of Marine Science, 56: 129–133.

Lang, T., Wosniok, W., Barsiene, J., Broeg, K., Kopecka, J., and Parkkonen, J. 2006. Liver histopathology in Baltic flounder (*Platichthys flesus*) as indicator of biological effects of contaminants- Marine Pollution Bulletin 53: 488-496.

Lang, T., and Wosniok, W. 2000. ICES data for an holistic analysis of fish disease prevalence. In ICES 2000, Report of the ICES Advisory Committee on the Marine Environment, 1999, pp. 193–209. ICES Cooperative Research Report, 239. 277 pp.

Lang, T. and Wosniok, W. 2008. The Fish Disease Index: a method to assess wild fish disease data in the context of marine environmental monitoring. ICES CM 2008/D:01, 13 pp.

Myers, M. S., Rhodes, L. D. and McCain, B.B. 1987. Pathologic anatomy and patterns of occurrence of hepatic neoplasms, putative preneoplastic lesions, and other idiopathic conditions in English sole (*Parophyrs vetulus*) from Puget Sound, Washington. Journal of the National Cancer Institute 78, 333–363.

Myers, M.S., Olson, O.P., Johnson, L.L., Stehr, C.M., Hom, T., and Varanasi, U. 1992. Hepatic lesions other than neoplasms in subadult flatfish from Puget Sound, WA: Relationships with indices of contaminant exposure. Mar. Environ. Res. 34, 45–51.

Myers, M.S., Johnson, L.L., Hom, T., Collier, T.K., Stein, J.E., and Varanasi, U. 1998. Toxicopathic lesions in subadult English sole (*Pleuronectes vetulus*) from Puget Sound, Washington, USA: Relationships with other biomarkers of contaminant exposure. Marine Environmental Perspectives, 45, 47–67.

North Sea Task Force. 1993. North Sea Quality Status Report 1993. Oslo and Paris Commissions, London. Olsen and Olsen, Fredensborg, Denmark. 132 pp.

OSPAR Commission. 2000a. Quality Status Report 2000, OSPAR Commission, London. 136+xiii pp.

OSPAR Commission. 2010b. OSPAR Coordinated Environmental Monitoring Programme (CEMP), Reference number 2010-1. 27 pp.

OSPAR Commission. 2010. Quality Status Report 2010, OSPAR Commission, London. 136+xiii pp.

Stein, J.E., Reichert, W.L., Nishimito, M., and Varanasi, U. 1990. Overview of studies on liver carcinogenesis in English sole from Puget Sound; evidence for a xenobiotic chemical etiology, 2, Biochemical studies. Sci. Total Environ. 94, 51–69.

Stentiford, G. D., Longshaw, M., Lyons, B. P., Jones, G., Green, M. and Feist, S. W. 2003. Histopathological biomarkers in estuarine fish species for the assessment of biological effects of contaminants. Marine Environmental Research 55, 137–159.

Vethaak, A. D. and Wester, P. W. 1996. Diseases of flounder *Platichthys flesus* in Dutch coastal and estuarine waters, with particular reference to environmental stress factors. II. Liver histopathology. Dis. Aquat. Orgs., 26, 81–97.

Wosniok, W., Lang, T., Dethlefsen, V., Feist, S. W., McVicar, A. H., Mellergaard, S., and Vethaak, A. D. 2000. Analysis of ICES long-term data on diseases of North Sea dab (*Limanda limanda*) in relation to contaminants and other environmental factors. ICES CM 2000/S:12. 15 pp.

Wosniok, W., Lang, T., Vethaak, A. D., des Clers, S., and Mellergaard, S. 1999. Statistical analysis of fish disease prevalence data from the ICES Environmental Data Centre. In: Report of the ICES Advisory Committee on the Marine Environment, 1998, pp. 297–327. ICES Cooperative Research Report, 233. 375 pp.

Annex 15: ICES SGEH Biological Effects methods Background Documents for the Baltic Sea region*

Imposex and intersex in marine snails

***ICES/OSPAR document from the ICES SGIMC Report 2010, complemented and modified by SGEH 2011 with information relevant for application in the Baltic Sea region**

Background

Imposex and intersex as signs of endocrine disruption in certain species of marine snails (*Prosobranch gastropods*) are established as specific biomarker indicators for effects caused by the endocrine disrupting substance tributyltin (TBT) in marine environments.

Imposex and intersex in marine snails is established indicators, because they reflect the effects of TBT in sensitive organisms in the marine environment very well. They can be regarded as counterpart of TBT measurements e.g. in water, sediments and biota.

Imposex refers to the irreversible development of male sexual characteristics in addition to the typical female sex characteristics, while intersex refers to the transformation of female sexual characteristics towards those of a male. The severity of imposex and intersex development in female snails can be described gradually with different stages from 0–4 (in some species have 6 stages), which also can be classified using a vas deferens sequence index (VDSI) for imposex and an intersex index (ISI) for intersex. Females with severe developed stages of imposex and intersex are sterile and unable to reproduce. VDSI and ISI can be used to describe how affected snail populations are at a station level.

The indicator is specifically related to pollution with the organotin substances TBT, and also triphenyltin (TPhT), which have been widely used as an antifouling agent biocide in ship paints. An international ban on their use has now entered into force, and from January 1, 2008, ships bearing an active TBT coating on their hulls will no longer be allowed in Community ports (782/2003/EC). Moreover, biocide use of all organic tin compounds was banned in autumn 2006 (98/8/EC) and the pesticide use of triphenyltin already in 2002 (91/414/EC). However, pollution levels giving rise to effects in gastropods can, although levels are significantly declining in the recent years, still occur in the marine environment.

Imposex and intersex in marine snails has for several years been well-established indicators in monitoring programmes in Europe, especially in the North Sea, North Atlantic and the Mediterranean Sea. The method is also part of OSPAR CEMP and therefore to be measured on a mandatory basis in the North Atlantic region (OSPAR 2010). However, the use of available and TBT sensitive species vary between regions dependent on the species distributions.

In the Baltic Sea region, imposex/intersex is used for monitoring or pre-monitoring investigations at least by labs from Denmark, Sweden and Germany. The netted whelk, periwinkle and mud snail are mainly used as indicator species in shallow coastal waters, whereas the common whelks and red whelks, whereas the common whelk and red whelk are more appropriate indicator species in deeper waters, i.e. 15–100 m depths, for instance along international shipping lanes.

Clear spatial gradients have been established in relation to areas with high ship densities, which are in line with that ship traffic is regarded as the main source of TBT in marine environments. The highest contamination levels with TBT occur generally close to harbours, marinas and shipping lanes, and similar relationships occur for the levels of imposex and intersex (e.g. Strand 2009a,b).

For red whelk, a wider spatial gradient also occur in the open waters going from the North Atlantic and the North Sea, towards the Skagerrak and into the Kattegat and the Belt Sea has been established supporting that this species are belonging to the most sensitive species in the region (Strand & Jacobsen 2002; Strand 2009a,b).

Similar to temporal trends for TBT levels in the marine environment, also the levels of imposex and intersex have in the recent years declined in the Danish waters, which are in line with the introduction of the ban of TBT as an antifouling agent in ship paints for larger vessels in 2003. However, elevated imposex levels can still be found in many areas in the Inner Danish waters (Figure 1).

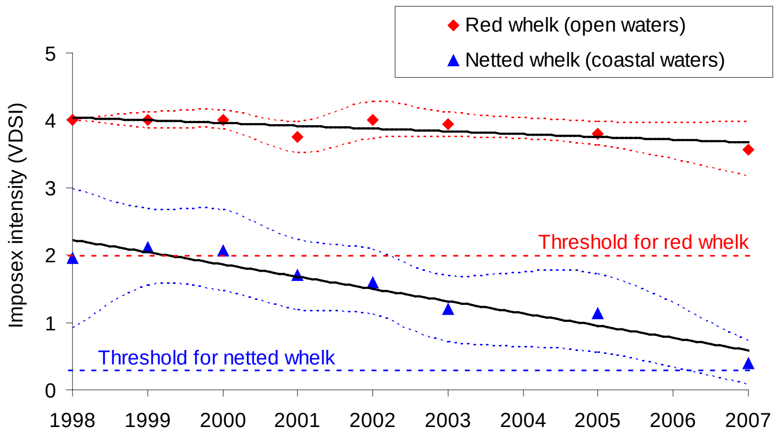

Figure 1. Temporal trends of imposex levels (intensity classified as VDSI with 4.0 as the maximum value) in two marine snail species, the netted whelk (*Hinia reticulata*) and the more sensitive red whelk (*Neptunea antiqua*) from coastal and open waters in the Danish part of the Belt Sea. Threshold values for imposex in the two species are shown for comparison (OSPAR 2008). Symbols denote median values and broken lines show 95% confidence intervals. The figure is copied from report by HELCOM (2010).

Confounding factors

The imposex and intersex effects are regarded as very specific indicator of marine pollution with tri-alkylated organotin substances TBT and TPhT. However, some studies have suggested that also few confounding factors might affect the imposex development in gastropods for instance bird colonies with intense excretions of faeces at the shore, although whether it is due to excretion of natural steroids or organotin compounds from the food, is not clear.

Ecological relevance

The ecological relevance of imposex and intersex development in marine snails is high because of the links to reproductive disorders. In severe stages, reproductive failure in female snails occurs as they are getting sterile. For instance, sterile female of the red whelk *Neptunea antiqua* has been found in the Inner Danish waters (Strand 2009).

Effects of sterile females on population structures have in the other studies also been shown in TBT contaminated areas.

Quality Assurance

OSPAR has developed international monitoring guidelines for imposex and intersex in five species of marine snails (OSPAR 2008). An ICES guideline also exists for monitoring intersex in periwinkle (Oehlmann 2004). A detailed method description for imposex in the mud snail Hydrobia can be found in Schulte-Oehlmann *et al.* (1997)

Quality Assurance in form of international workshops and intercalibrations has been organized almost yearly by QUASIMEME since 1998. National workshops have also been organized three times in relation to the Danish monitoring program NOVANA.

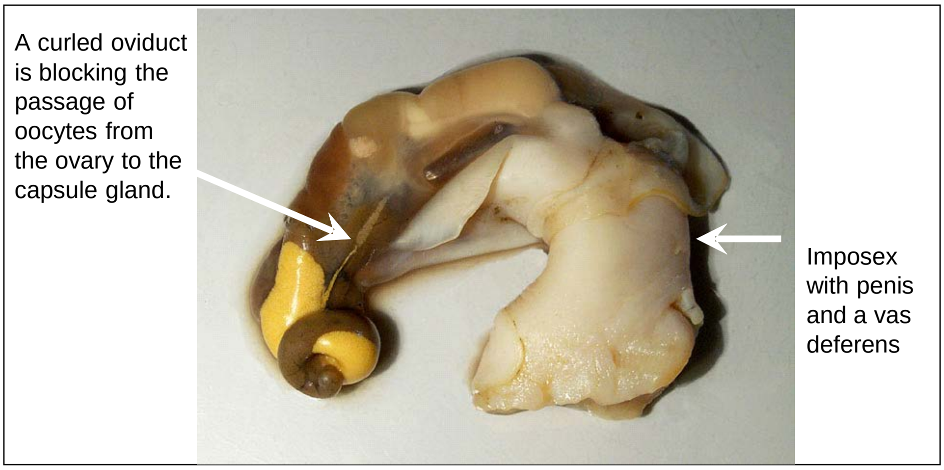

Figure 2. In severe stages of imposex development, female snails can becomes sterile due to blockage of the oviduct or the vaginal opening. This photo shows a sterile female of the red whelk *Neptunea antiqua* from the Danish part of the Belt Sea. About 10% of *Neptunea* females in the Belt Sea have a curled oviduct indicating impaired reproduction. Photo: Jakob Strand

Background responses and Assessment Criteria

Biological Assessment Criteria for imposex/intersex in marine snails for assessing the specific effects of tributyltin (TBT) exist as they have been developed by OSPAR for five species of marine gastropods used in monitoring programmes in the OSPAR region i.e. the North Sea and the North Atlantic (OSPAR 2004).

The species included are dog whelk (*Nucella lapillus*), common whelk (*Buccinum undatum*), red whelk (*Neptunea antiqua*), netted whelk (*Nassarius (Hinia) reticulata*) and periwinkle (*Littorina littorea*), which also occur in the Kattegat, the Belt Sea and in some cases also in the western Baltic Sea. The assessment criteria take interspecies differences into consideration and that dog whelk and red whelk are recognised as being particularly sensitive to TBT, and periwinkle is regarded as belonging to the less sensitive species.

According to the OSPAR monitoring guideline, the sample size should consist of examinations of 40 individual snails of the same species per station, with the exception of studies on common whelk sampled in open waters, where the recommended sample size is 100 individuals per station.

The expected baseline level for imposex/intersex has been set to <0.3 for VDSI and ISI in all species. However, the recommended target level has been suggested to be VDSI

<2 in dog whelk, which are representing the most sensitive species. Subsequently, the recommended target levels for all the listed species will correspond to the values assigned to class B in Table 1.

Intersex in periwinkle is regarded as a less sensitive biomarker, which only can be used to assess effect levels that are significantly higher than the recommended target level, i.e. class C or higher.

Schemes with integrated assessment classes has also been suggested to link TBT effects in gastropod species with five classes of ambient concentrations of TBT in water, sediment and biota, and relevant EAC- and EQS-values (Strand *et al.* 2006; OSPAR 2008; Strand 2009a).

Table 1. OSPARs Assessment Criteria for biological effects of TBT. Assessment criteria for imposex in dog whelk (regarded as representing more sensitive species) are presented alongside equivalent index values VDSI or ISI for sympatric populations of other relevant species (OSPAR 2004).

Assessment class	Dog whelk	Netted whelk	Common whelk	Red whelk	Periwinkle
	VDSI	VDSI	VDSI	VDSI	ISI
A	< 0.3			< 0.3	
B	0.3 - < 2.0	< 0.3	< 0.3	0.3 - < 2.0	-
C	2.0 <4.0	0.3 < 2.0	0.3 < 2.0	2.0 - < 4.0	< 0.3
D	4.0 - 5.0 sterility occurs	2.0 - 3.5	2.0 - 3.5	4.0 - 4+ sterility occurs	0.3 - < 0.5
E	> 5.0	> 3.5	> 3.5	-	0.5 - 1.2 sterility occurs
F	-	-	-	-	> 1.2

Distribution of indicator species in Baltic Sea subregions

The distribution and thereby the availability of these five gastropod species recommended for OSPAR monitoring is restricted by the salinity in the Baltic Sea region. However, four of these species, i.e. common whelk, red whelk, netted whelk and periwinkle are also living in the western part of the HELCOM region i.e. the Kattegat, the Belt Sea, the Sound and the western Baltic Sea. The OSPAR assessment criteria are therefore also applicable for assessing the TBT effects in gastropods in this part of the Baltic Sea region.

Only few relevant prosobranch gastropod species are occurring more widely in the Baltic Sea and until now only the mud snail (*Hydrobia ulvae*), which also occur in the more eastern part of the Baltic Sea, have also shown potential to be included as an indicator for TBT-specific effects (Schulte-Oehlmann *et al.* 1997; Gercken & Sordyl, 2007; Magnusson 2008). A similar value of VDSI < 0.3 as baseline level can also be expected for *Hydrobia*, although it needs further evaluation of the sensitivity of imposex as indicator of TBT effects in *Hydrobia* before also more target values can be set.

Acknowledgements

OSPAR is acknowledged for the development and harmonisation of criteria for the assessment of TBT–specific biological effect in marine snails.

References

Gercken, J. & Sordyl, H. (2007) Biological effect monitoring in coastal waters off Mecklenburg-Western-Pomerania. Pilotstudies 1998-2003. Report on behalf of State Office of Environment, Nature Conservation and Geology Mecklenburg-Western Pomerania, 135 p. (in German).

HELCOM (2010). Hazardous substances in the Baltic Sea An integrated thematic assessment of hazardous substances in the Baltic Sea. Baltic Sea Environment Proceedings No. 120B.

Magnusson, M. (2008). Pilotstudie inför biologisk effektövervakning av organiska tennföreningar i Östersjön 2007. Report from Institutionen för Marin ekologi vid Göteborgs universitet. (In Swedish)

Oehlmann, J. 2004. Biological effects of contaminants: Use of intersex in the periwinkle (Littorina littorea) as a biomarker of tributyltin pollution. ICES Techniques in Marine Environmental Sciences (TIMES) No. 37, 22pp.

OSPAR (2004). Provisional JAMP Assessment Criteria for TBT – Specific Biological Effects. OSPAR agreement 2004-15.

OSPAR (2008a). 2007/2008 CEMP Assessment: Trends and concentrations of selected hazardous substances in sediments and trends in TBT-specific biological effects. Assessment and Monitoring Series, OSPAR Commission 2008, 36 pp.

OSPAR (2008b). JAMP Guidelines for Contaminant-Specific Biological Effects. Technical Annex 3: TBT-specific biological effects monitoring. OSPAR Agreement 2008-09.

OSPAR (2010). OSPAR Coordinated Environmental Monitoring Programme (CEMP). Reference number: 2010-1.

Schulte-Oehlmann *et al.* (1997). Imposex and reproductive failure in Hydrobia ulvae (Gastropoda: Prosobranchia). Marine Biology 128:257-266.

Strand, J. & Jacobsen, J.A. (2002): Imposex in Two Sublittoral Neogastropods from the Kattegat and Skagerrak: the Common Whelk Buccinum undatum and the Red Whelk Neptunea antiqua. Marine Ecology Progress Series 244: 171-177.

Strand, J., Larsen, M.M., Næs, K., Cato, I. & Dahllöf, I. (2006): Tributyltin (TBT). Forekomst og effekter i Skagerrak. Uddevalla, Sweden: Rapport fra Forum Skagerrak II. 39 s. (In Danish) http://www.forumskagerrak.com/download/503/x/TBT%20web%20ver2.pdf

Strand J. (2009a). Coupling marine monitoring and environmental risk assessment of TBT: A case study using the contamination of organotin compounds in the Danish marine environment. VDM Verlag Dr. Müller, 84pp.

Strand, J. (2009b). Biologisk effektmonitering. Chapter 13 In: I: Marine områder 2008 : NOVANA. Tilstand og udvikling i miljø- og naturkvaliteten. Hjort. M & Josefson A. (Eds.). National Environmental Research Institute, Aarhus University, Scientific Report from NERI (In Danish).

Summary table for imposex and intersex in marine snails.

Evaluation Criteria	Rating	Description
Recommended indicator species	-	Hinia reticulata/nitidus, Buccinum undatum, Neptunea antiqua,Littorina littorea, Nucella lapillus,Hydrobia ulvae
Recommended matrix	-	Whole organisms, females
Recommended sample size per station	-	40 individuals per station, with exception of 100 for Buccinum
Monitoring guideline (SOP) in place	Yes	JAMP guideline for contaminant-specific effects, Annex 10 (OSPAR 2008), ICES TIMES 37 (2004)
ACs in place, i.e. background response and/or EAC	Yes	OSPAR ACs (2004, 2009), Strand *et al.* (2006)
QA in place, i.e. ongoing intercalibrations or workshops	Yes	Quasimeme intercalibrations or workshops (www.quasimeme.org)
Ecological relevance of effects for populations	High	Links to reproductive failure
Persistent damage, not repairable effects	Yes	Irreversible effects
Contaminant sensitive response (Elevated effect levels in open waters, coastal waters or only point sources)	High	Effects in open and coastal waters, and point sources
Contaminant-specific cause-effects response	High	Specific to TBT (and TPhT)
General effect, response to several contaminant groups	No	No, see above
Stable for confounding factors	Yes	Highly stable
Applicable indicator species in 1, 2-3 or >3 Baltic Sea subregions	Low - High	Whelks only in Skagerrak/Kattegat, Belt Sea, Sound and western BS, whereas Hydrobia occurs also in other Baltic Sea regions.
Already used in monitoring in 1, 2-3 or >3 countries	Yes	Denmark and Sweden are using whelks such as *Hinia*, *Buccinum* and/or *Neptunea*, whereas *Hydrobia* is used in also Sweden and Germany.
Available data, spatial coverage in the Baltic Sea - number of countries and stations (<10, 10-20, >20 stations)	High	DK: ~30 stations, S: ~15 st., D: ~5 st.
Available data, length of time-series (<5, 5-10, >10 years)	High	DK: 1998-2010, S: 2003-2010, D: 2006-2010
Costs of analyses per station, all required individuals (<500, 500-1500, >1500EUR)	Medium	500 - 1000 EUR per station

Annex 16: ICES SGEH Biological Effects methods Background Documents for the Baltic Sea region*

PAH metabolites in fish bile

***ICES/OSPAR document from the ICES SGIMC Report 2010, complemented and modified by SGEH 2011 with information relevant for application in the Baltic Sea region**

Background

Polycyclic aromatic hydrocarbons (PAH) are important environmental contaminants which may lead to increased levels of neoplastic aberrations or tumours in fish liver. Therefore monitoring of PAH and their effects are part of several international environmental programmes. PAHs do not accumulate in vertebrates, but are rapidly metabolised and excreted via bile e.g. in mammals and fish. PAH and toxic intermediates emerging during metabolic degradation can cause deleterious effects in fish. Epoxides originating from the oxidation of PAH by cytochrome P4501A1 may be further oxidised to carcinogenic diolepoxides. These dioleepoxides are known to bind to DNA and/or cause mutations which may lead to cancer. Increased levels of neoplastic aberrations or tumours were found in fish which have been exposed to PAH contaminated sediments. For this reasons PAH contamination in marine ecosystems is a cause for concern. To assess the PAH exposure of fish, concentrations of the main metabolites such as 1-hydroxypyrene, 1-hydroxyphenanthrene and 3-hydroxybenzo(a)pyrene can be determined in bile by HPLC with fluorescence detection (HPLC-F), by synchronous fluorescence scanning, gas chromatography with mass selective detection (GC/MS) and also by UPLC/MS/MS (Bayer *et al.*, 2010; Ariese *et al.* 2005). PAH metabolites in fish bile reflect the exposure of the fish to PAHs via sediment and food usually during the last days – depending on the feeding activity.

PAHs, as components of mineral oil, are geogenic and present in "unpolluted" sediments in low concentrations. Fish from "unpolluted" regions have therefore low but measurable concentrations of PAH metabolites in bile. For monitoring purposes PAH metabolites in fish are the counterpart of PAHs in sediment or water respectively.

Confounding factors

The season and the feeding status (freshly filled gall bladder or not) seem to be confounding factors for fish. Normalisation of metabolite concentration to bile pigments (expressed as absorption at 380nm or biliverdin concentration) can help to reduce variation in some data sets (Kammann 2007, Ariese *et al.* 1997). In other data sets normalisation leads to no advantage. Male and female fish tend to have different levels of PAH metabolites (Vuorinen *et al.* 2006).

Ecological relevance

PAH metabolites are sub-cellular contaminant specific markers of PAH exposure. However, PAH metabolites do not reflect directly a population relevant effect. The Assessment Criteria EAC (Environmental Assessment Criteria) indicates a significant risk for the organism related to a threshold level of PAH metabolites in the bile. In the "traffic light system" data exceeding EAC are displayed in red.

Quality Assurance

In ICES Times No. 39 there are several methods described and equally recommended. Due to different principles not all of the methods can be compared.

BONUS+ project BEAST conducted an intercalibration for PAH metabolites in fish bile in 2010. The results showed a good comparability between the participating labs as well as between the analytical methods GC-MS, HPLC-F and synchronous fluorescence scanning. A factor was used to compare synchronous fluorescence to the other two methods mentioned above.

Background response and Assessment Criteria

EAC and BAC values depend on the metabolite, the fish species as well as on the analytical method used. Both values can be used for the North Sea as well as the Baltic Sea if species fits.

The recommended way to calculate BACs is to use the 90th percentile of reference site data. Possible reference sites are Iceland and Barents Sea. Data from additional reference sites may improve the quality of the BAC in future. BACs have been calculated for dab, cod and haddock. In Figure 1 monitoring results are presented in two colours representing the proportions of the fish above and below the BAC. No EAC values were exceeded. Looking at the values regional differences seems to be much more important than species differences. If BAC should be developed for fish species which prefers more polluted habitats (coast), it could be helpful to use an species independent BAC for fish instead. SGIMC 2010 proposed a joint BAC for three fish species cod dab and haddock. A BAC value applicable for more marine fish species would be helpful regarding inter-species evaluation of monitoring data.

Even if PAH metabolites are only a marker of exposure, high levels of metabolites can be linked to deleterious effects in fish. EACs have been be identified using results from toxicological experiments linking oil exposure and PAH metabolites in fish with DNA adducts and fitness data (Morton *et al.*, 2010; Skadsheim *et al.* 2004; Skadsheim *et al.*, 2009), where the latter serves as the effect quantity for the calculation of the EAC presented in Table 1. EAC are available for some fish species only at the moment. More investigations are needed to calculate proper EAC values for Baltic fish species. EACs cannot be easily transferred to other species because they may differ in sensitivity to PAH effects.

Table 1. Background Assessment Criteria (BAC) and Environmental Assessment Criteria (EAC) for two PAH metabolites, different fish species and methods. Data partly taken from WKIMC 2009 and SGIMC 2010.

Biological Effect	Fish species	BAC [ng/ml] HPLC-F	EAC [ng/g] GC/MS
Bile metabolite 1-hydroxypyrene	dab	16	
	cod	21	483
	flounder	16 4)	
	haddock	13	
	dab, cod, haddock	17	
	turbot		909
	halibut		745
Bile metabolite 1-hydroxyphenanthrene	dab	3.7	
	cod	2.7	518
	flounder	3.7 4)	
	haddock	0.8	
	dab, cod, haddock	2.4	
	turbot		1832
	halibut		262

Table 1. (Continued)

Biological Effect	Fish species	BAC [µg/ml] Synchronuos Fluor. 341/383	EAC [µg/ml] Fixed Fluor. 341/383
Bile metabolites of pyrene-type	dab	0.15	22 1)
	cod	1.1	35
	flounder	1.3	29 2)
	haddock	1.9	35 3)
	turbot		29
	halibut		22
	herring/sprat		16

AC based on 1)halibut,
2)turbot, 3)cod and 4)dab

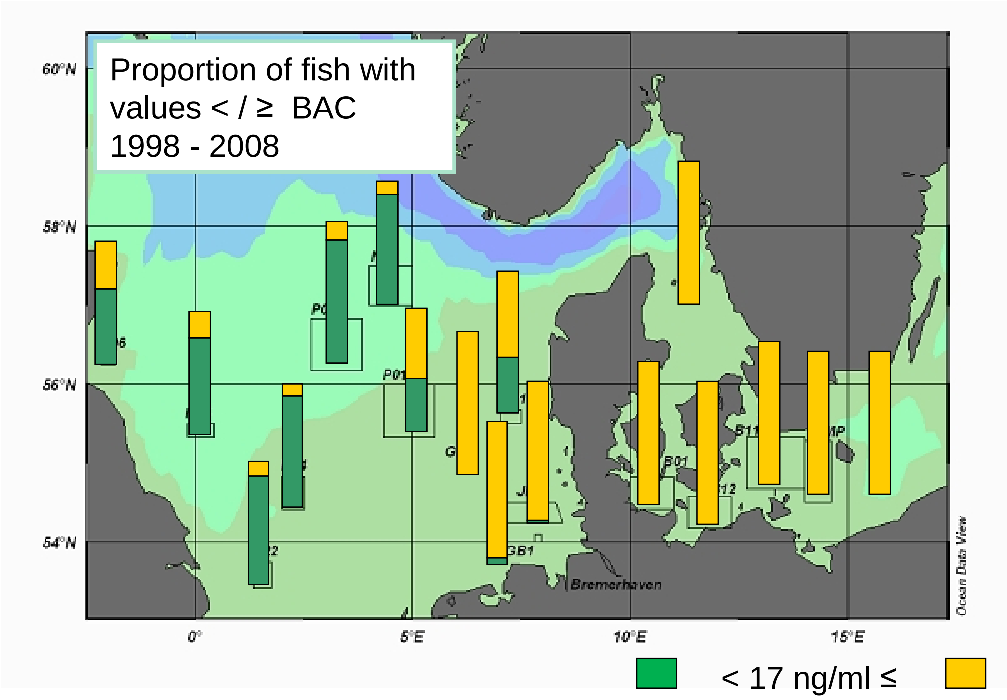

Figure 1. 1-Hydroxypyrene in bile fluids of dab, flounder and cod caught between 1998 and 2007 categorized by the species overarching BAC of 17 ng/ml. Proportion of single fish per station are categorized in relation to BAC (SGIMC 2010).

Distribution of indicator species in Baltic Sea subregions

Clear differences in PAH metabolite concentration in fish bile between the lower contaminated central North Sea and the higher contaminated western Baltic Sea have been detected. In distance of point sources there are no temporal trends detectable in dab and flounder from the North Sea and the western Baltic Sea caught during 1997 and 2004 (Kammann 2007). Lower values than in North Sea (dab, cod, flounder, haddock) and Baltic Sea (flounder, cod, herring, Vuorinen *et al.*, 2006; eelpout) have been detected in Barents Sea (cod) and near Iceland (dab). Higher concentrations are present in fish caught in harbour regions or in coastal areas (eelpout, Kammann and Gercken, 2010).

- Baltic countries with regular monitoring of PAH metabolites in fish bile: Germany, Denmark, UK
- Baltic countries with pre-monitoring stage of PAH metabolites investigations: Finland, Poland

Acknowledgements

EU BEEP project and BONUS+ BEAST project.

References

Ariese F, Beyer J, Jonsson G, Porte Visa C, Krahn MM (2005) Review of analytical methods for determining metabolites of polycyclic aromatic compounds (PACs) in fish bile. ICES Techniques in Marine Environmental Sciences No. 39

Ariese F, Burgers I, Oudhoff K, Rutten T, Stroomberg G, Vethaak D (1997): Comparison of analytical approaches for PAH metabolites in fish bile samples for marine and estuarine monitoring. Vrije Universiteit Amsterdam, Institut for Environmental Studies. R 97-9, 28 pp.

Beyer J, Jonsson G, Porte C, Krahn MM, Ariese F (2010) Analytical methods for determining metabolites of polycyclic aromatic hydrocarbon (PAH) pollutants in fish bile: A review. Environmental Toxicology and Pharmacology, 30: 224-244.

Kammann U (2007) PAH metabolites in bile fluids of dab (*Limanda limanda*) and flounder (Platichthys flesus) – spatial distribution and seasonal changes. Environ Sci Pollut Res 14(2) 102-108.

Kammann U, Gercken J (2010) PAK-Metaboliten in Aalmuttern (*Zoarces viviparus*) aus der Wismar-Bucht. Umweltwiss Schadst Forsch. 22(5): 541-546.

Morton, C.H., G. Nyhammer, B.E. Grøsvik, V. Makhotin, A. Geffen, S. Boitsov, K.A. Kvestad, A.B. Kjersem, A. Goksøyr, A. Folkvord, J. Klungsøyr, A. Svardal, S. Meier (2010): Development of Atlantic cod (Gadus morhua) exposed to produced water during early life stage. I. Effects on embryos, larvae, and juvenile fish. Mar Environ Res 70(5) 383-94.

Skadsheim, A. 2004. Cod stocks exposed to crude oils: uptake, metabolism and biomarker effects. Technical report AM-2004/005, Akvamiljo, Randaberg, Norway.

Skadsheim, A., Sanni, S., Pinturier, L., Moltu, U.-E., Buffagni, M. & Bracco, L. 2009. Assessing and monitoring long-range-transported hydrocarbons as potential stressors to fish stocks. Deep-Sea Research Part II. Vol. 56, Issues 21-22, pp. 2037-2043.

Vuorinen PJ, Keinänen M, Vuontisjärvi H, Baršienė J, Broeg K, Förlin L, Gercken J, Kopecka J, Köhler A, Parkkonen J, Pempkowiak J, Schiedek D (2006): Use of biliary PAH metabolites as a biomarker of pollution in fish from the Baltic Sea. Mar Pollut Bull 53(8-9):523-537.

Annex 17: ICES SGEH Biological Effects methods Background Documents for the Baltic Sea region*

Acetylcholinesterase inhibition

***ICES/OSPAR document from the ICES SGIMC Report 2010, complemented and modified by SGEH 2011 with information relevant for application in the Baltic Sea region**

Background

Activity of acetylcholinesterase (AChE; EC 3.1.1.7) is a classic biomarker in assessing exposure of humans to neurotoxic compounds. Likewise, in marine organisms, AChE has been shown to be a highly suitable method to similar effects in aquatic environments. In general, the methods developed are sensitive to detect neurotoxic effects of contaminant concentrations occurring in marine waters.

The AChE activity assay (Bocquené and Galgani 1990) in current use in marine monitoring is applicable to a wide range of species and has the advantage of detecting and quantifying exposure to neurotoxic substances without a detailed knowledge of the contaminants present. AChE activity is a typical biomarker that can be used in *in vitro* bioassays (e.g. chemical testing, exposure experiments) and, in a number of succesful cases, in field applications.

AChE is present in most animals and is responsible for the rapid hydrolytic degradation of the neurotransmitter acetylcholine (ACh) into the inactive products choline and acetic acid. AChE has highest specificity for ACh of any other choline ester, while butyrylcholinesterase has the highest specificity for butyrylcholine or propylthiocholine. The inhibition of AChE leads to an accumulation of ACh which, in turn, overstimulates sensitive neurons at the neuromuscular junction which results in tonic spasm and tremors. The presence of AChE has been demonstrated in a variety of tissues of marine organisms including muscle and brain tissue of fish, adductor muscle, foot tissue, haemocytes and gills of shellfish, and abdominal muscle of crustaceans (Bocquené and Galgani, 1998). In vertebrates, neurotoxic poisoning with hyperactivity, tremors, convulsions and paralysis may finally lead to death.

Being an indicator of general neurotoxic effects, AChE has traditionally been used as a specific biomarker of exposure to organophosphate and carbamate pesticides (e.g. Coppage and Braidech, 1976; Day and Scott, 1990; Bocquené and Galgani, 1998; Printes and Callaghan, 2004; Hoguet and Key, 2007). The existence of extremely low thresholds for induction of inhibitory effects on AChE suggests that detection is possible after exposure to low concentrations of insecticides (0.1 to 1 $\mu g l^{-1}$; Habig *et al.*, 1986).

During the 1990s there was a resurgence of interest concerning the use of ChEs as a biomarker. Its responsiveness to various other groups of chemicals present in the marine environment has been demonstrated, including heavy metals, detergents and hydrocarbons (Zinkl *et al.*, 1991; Payne *et al.*, 1996; Guilhermino *et al.*, 1998; Forget *et al.*, 1999; Burgeot *et al.*, 2001, Brown *et al.*, 2004). More recently, its usefulness as a general indicator of pollution stress in mussels and fish from the Baltic Sea has been suggested, and it has been used for this purpose (Schiedek *et al.*, 2006; Kopecka *et al.*, 2006, Barsiene *et al.*, 2006).

Confounding factors

The natural range of variability in AChE needs to be understood. Several chemical, hydrographic and endogenic factors affect its rates. For example, seawater temperature and salinity have been shown to have an effect on AChE rates of different organisms (Pfeifer *et al.*, 2005; Leiniö and Lehtonen, 2005; Rank *et al.*, 2007). In regard to some the key organisms in the Baltic Sea, the bivalves *Mytilus edulis* and *Macoma balthica* from the northern Baltic Sea have been shown to exhibit a two-fold alteration in enzymatic rates of AChE depending on season and following closely changes in temperature and/or amount of food available (Leiniö and Lehtonen, 2005). Seasonal variability has also been shown in coastal areas compared to offshore sites (Dizer *et al.*, 2001; Burgeot *et al.*, 2006; Bodin *et al.*, 2003).

The presence of and exposure to biotoxins or cyanobacteria/cyanobacterial extracts in mussels has been demonstrated to AChE activity (Dailianis *et al.*, 2003; Lehtonen *et al.*, 2003; Frasco *et al.*, 2005; Kankaanpää *et al.*, 2006). Anatoxin-a(s), produced by *Anabaena flos-aquae* is a well known very strong inhibitor of AChE activity (Carmichael, 1992). Toxins present in the water as a result of cyanobacteria blooms (e.g. *Anabaena flos-aquae*, *Aphanizomenon flos-aquae* and *Microcystis aeruginosa*) have also been shown to inhibit AChE activity. Thus, it is recommended that the presence of any algal blooms and their identity should be noted when the samples are collected.

In crustaceans, the hormone 20-hydroxyecdysone is the primary mechanism controlling molting and has been shown to be positively correlated with neurological activity (i.e. AChE), e.g. in the crustacean *Artemia franciscana* (Gagne and Blaise, 2004). Molting rate increases with the development, specifically peaking at the juvenile stage. The subsequent decline in AChE may also be explained by reduced molting frequencies in adult individuals.

The process and mechanisms of biological response in each organism require further investigations in specific habitats with specific chemical contamination. The mussel *Mytilus galloprovincialis* shows a great heterogeneity of esterases and a particular sensitivity to specific components such as paraoxon (Ozretic and Krajnovic-Ozretic, 1992; Brown, 2004). The alleged versatility of AChE inhibition as effect criteria after exposure to detergents may be misleading and may underestimate the contamination potential of complex mixtures (Rodrigues *et al.*, 2011).

Enzymatic polymorphism has also been demonstrated in the oyster *Crassostrea gigas*, and two forms of AChE with different sensitivity to paraoxon have been described (Bocquené *et al.*, 1997). Thus, extraction of the sensitive form now identified in some organisms would provide greater precision for determination of AChE enzymatic activity than would an overall measurement of acetylcholinesterases. In addition to polymorphisms, ChEs of some invertebrates have been showing to have some different in their properties relatively to typical forms of vertebrates. For example, ChEs of properties of both AChE and pseudocholinesterases have been found in the gastropods *Monodonta lineta* and *Nucella lapillus* (Cunha *et al.*, 2007), in the sea urchin Paracenthrotus lividus (Cunha *et al.*, 2005), in *Artemia* (Varó *et al.*, 2002) and in some strains of *Daphnia magna* (Diamantino *et al.*, 2003).

As with most indicators concerning individual responses, exploration of genetic variability and the influence of environmental factors present in specific habitats will lead to a better distinction between natural and pollutant effects.

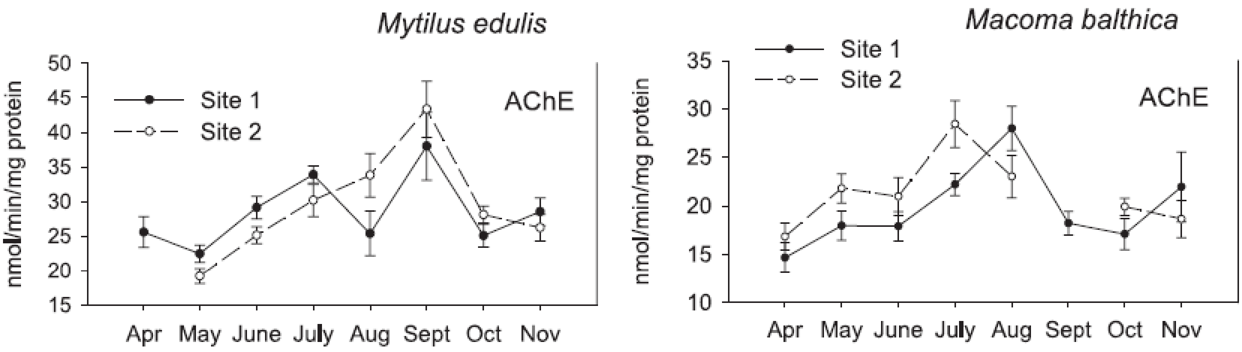

Figure 1. Seasonal variability in AChE activity in two bivalve species from the northern Baltic Sea, the hard-bottom filter feeder Mytilus edulis (left) and soft-bottom deposit feeder Macoma balthica, from inshore and offshore reference stations (Leiniö and Lehtonen 2006).

Ecological relevance

Since AChE inhibition results in continuous and excessive stimulation of nerve and muscle fibre, producing tetany, paralysis and death, it has potentially highly significant effects at different levels of biological organisation. Sublethal exposure affecting AChE can alter the behaviour and locomotive abilities of organisms (e.g. Vieira *et al.*, 2009), potentially affecting their reproduction, fitness and survival. Evidence of AChE activity modulation by various organic chemicals has been described in marine organisms and therefore the evaluation of changes in AChE activity in different species allows for the characterisation of neurotoxic effects of a wide spectre of organic and inorganic contaminants present in the marine environment as well as the effects of multiple stress.

Quality Assurance

Being one of the most applied biomarker techniques in field studies, the large experience acquired in conducting AChE measurements in general use makes it possible today to evaluate the effects of diffuse contamination in some marine organisms in marine areas globally.

A simple microplate assay technique established for *in vitro* detection of AChE inhibition by Bocquené and Galgani (1998) has been widely applied in the monitoring of coastal and offshore waters during the past decades. This technique has a specific sensitivity comparable to chemical analyses with a detection limit of 100 ng l^{-1} for carbamates and 10 ng l^{-1} for organophosphates (Kirby *et al.*, 2000). In addition, non-specific responses related to mixed pollution have been widely recorded, e.g. in the Baltic Sea (Schiedek *et al.*, 2006, Kopecka *et al.* 2006, Barsiene *et al.* 2006).

Standardisation of the sampling strategy and regular intercalibration exercises (e.g. BEQUALM, EU BEEP project, BONUS+ BEAST project) on target organisms sampled in the Northern Atlantic, Mediterranean and the Baltic Sea have been carried out and are to be continued. A QA programme is available in the BEQUALM programme and a large number of laboratories across Europe, including the Baltic Sea region, will participate in the planned activity. A major intercalibration exercise was carried out during the EU BEEP project in 2002.

Background response and Assessment Criteria

Baseline levels of AChE in different marine species have been estimated from results derived from control laboratory and field studies in the Northern Atlantic, the Mediterranean and the Baltic Sea (Table 1). Assessment Criteria should optimally be defined on regional basis using available long term data. Therefore, in order to understand and apply the AChE enzymatic activity as a biomarker of neurotoxic exposure it is of fundamental importance to gain information on the natural background levels in non-contaminated organisms. The baseline level of 35 nmol min^{-1} mg $protein^{-1}$ of the seasonal cycle in the mussel *Mytilus edulis* studied during three years along the French Northern Atlantic coast demonstrated a maximum of amplitude of 30% (Bocquené *et al.*, 2004).

Generally, it has been accepted that 20% reduction in AChE activity in fish and invertebrates indicates exposure to neurotoxic compounds (Zink *et al.*, 1987; Busby *et al.*, 1989). Depression in AChE activity by more than 20% up to 50% indicates a sublethal impact (Dizer *et al.*, 2001). In the field, several species have baseline AChE activities within the same order of magnitude among different studies (Table 1). However, differences between sea areas and seasons are also obvious, e.g. with activity values in *Mytilus* spp. varying from 25 to 54 nmol min^{-1} mg $protein^{-1}$.

According to these observations, Background Assessment Criteria (BAC) and environmental assessment criteria (EAC) were proposed. BAC are derived from reference sites and describe the threshold value for the background level. EAC are usually derived from toxicological data and indicate a significant risk for the organism. EAC were calculated by substracting 30% of the BAC values and illustrate a significant inhibition of AChE activity and a sublethal impact (Table 1).

Table 1. Assessment of AChE activity in commonly used biomonitoring organisms in the Baltic Sea.The data consists of measurements in different species collected or deployed (mussel caging) at "reference" locations in the Baltic, North and Mediterranean Seas

Organism	Tissue	Sea area	T°C or month	BAC nmol min^{-1} mg $prot^{-1}$	EAC nmol min^{-1} mg $prot^{-1}$	Reference
Platichthys flesus	Muscle	Baltic sea S	6.6	50	35	Kopecka and Pemkowiak 2008
Platichthys flesus	Muscle	Baltic sea S	22	103	72	Kopecka and Pemkowiak 2008
Platichthys flesus	Muscle	Baltic Sea E	Jun-Sept	330 275	230 193	Barsiene *et al.* 2006
Platichthys flesus	Muscle	Baltic Sea S	15 3	187 105	131 74	Kopecka *et al.* 2006
Zoarces viviparus	Muscle	Field Baltic Sea S	18	150	105	Schiedek *et al.* 2006
Macoma balthica	Foot	Baltic Sea N	Apr to Nov	15-28	11-20	Leiniö and Lehtonen 2005
Mytilus edulis	Gills	Baltic Sea N	Apr to Nov	19-43	13-30	Leiniö and Lehtonen 2005
Mytilus edulis	Gills	North Sea	5	25	18	Burgeot *et al.* 2006
Mytilus galloprovincialis	Gills	Mediterranean Sea	Sept	28	20	Bodin *et al.* 2004
Mytilus galloprovin-	Gills	Mediterranean	Apr	54	38	Bodin *et al.* 2004

cialis		Sea				
Mytilus edulis	Gills	North Atlantic	36	35	25	Bocquené *et al.* 2004

A standardised AChE measurement protocol (intercalibration) should be developed concerning the main species currently used in marine biomonitoring programmes (OSPAR, HELCOM, MEDPOL, and especially the MSFD). The method published in ICES TIMES series by Bocquené & Galgani (1998) currently forms a harmonised method can be used as a basis of a standardised procedure. Further information should be gathered to confirm baseline activities of AChE levels in different sentinels species under different environmental contrains in European waters. Modelling the background levels and response levels still require further work but for some species (e.g. *Mytilus* sp.) BAC and BEC levels can already be established for the Baltic Sea region. As with all monitoring parameters the values of BAC and EAC must be updated and revised when more data becomes available. BAC and EAC should also be established for new potential monitoring species and for specific localities.

Distribution of indicator species in Baltic Sea subregions

Of the most used indicators species, flounder are widely available in the southern-central parts of the Baltic but less so in the northern sub-basins. Perch is restricted to low-salinity areas and rivermouths. Eelpout is found all over the area but in limited numbers in the Bothnian Bay. Herring is availble in the whole area.

Acknowledgements

ICES SGIMC and OSPAR are acknowledged for the development of the document that formed the basis of the present one. Special thanks go to Thierry Burgeot, Joelle Forget-Leray, Benoit Xuereb and Gilles Bocquené (IFREMER Nantes).

References

Baršiene, J., Lehtonen, K. K., Köhler, A., Broeg, K., Vuorinen, P.J., Lang, T., Pempkowiak, J., Šyvokiene, J., Dedonyte, V., Rybakovas, A., Repecka, R., Vuontisjärvi, H.and Kopecka, J. (2006) Biomarker responses in flounder (Platichthys flesus) and mussel (Mytilus edulis) in the Klaipeda-Butinge area (Baltic Sea). Marine Pollution Bulletin 53: 422-436

Bocquené G., Chantereau S., Raffin B., Minier C., Clérendeau C., Leskowicz A., Beausir E., Burgeot T.and Ménard D. (2004). The monitoring of the biological effects of the ERIKA oil spill on the blue mussel (Mytilus edulis). Aquatic Living Ressources. 17.309-316

Bocquené G. P. (2004). Biological effects of environemental pollution in aquatic ecosystem. BEEP WEB. NOE Global change and ecosystem 6.3.(Personnal communication)

Bocquené G., Roig A. and Fournier D. (1997). Acetylcholinesterases from the common oyster Crassotrea gigas. Evidence for the presence of a soluble acetylcholinesterase insensitive to organophosphate and carbamate inhibitors. FEBS Lett 3:261-266.

Bocquené, G. and Galgani F. (1998). Biological effects of contaminants: Cholinesterase inhibition by organophosphate and carbamate compounds. Ices Techniques in Marine Environmental Sciences. No 22.12p. ICES Copenhaguen DK.

Bodin N., Burgeot T., Stanisière J.Y., Bocquené G., Ménard D., Minier C., Boutet I., Amat, A. Cherel Y. and Budzinski H. (2004). Seasonal variations of a battery of biomarkers and physiological indices for the mussel Mytilus galloprovincialis transplanted into the Northwest Mediterranean Sea. Comparative Biochemistry and Physiology. 138:411-427

Brown R.J., Galloway T.S., Lowe D., Browne M.A., Dissanayake A., Jones M.B.and Depledge M.H. (2004). Differential sensitivity of three marine invertebrates to copper assessed using multiple biomarkers. Aquat. Toxicol, 66.267-278.

Burgeot T., Faucet J , Ménard D, Grosjean P. and Bocquené G. (2006). Variations of benzo(a)pyrene hydroxylase and cholinesterase activities in mussels caged in the North Sea (German Bight and Statfjord). Special Issue. Environmental Toxicology and chemistry. 171-185.

Burgeot T., G. Bocquené, C. Porte, A. Pfhol Leszkowicz, R.M. Santella, C. Raoux, J. Dimeet, and F. Galgani, (1996). Bioindicators of pollutant exposure in the northwestern part of the Mediterranean sea. Mar. Ecol. Prog. Ser.131: 125-141.

Burgeot T., G. Bocquené, E. His, F. Vincent, O. Geffard, R. Beiras, H. Goraguer & F. Galgani (2001). Procedures for cholinesterase determination in fish and mussel. Technical annex. In: Biomarkers in marine organisms; a practical approach. Edited by P. Garrigues H. Barth, C.H. Walker and J.F. Narbonne. Eds Elsevier.487p.

Busby D.G., White L.M., Pearce P.A. and Mineau P. (1989). Fenitrothion effects on forest song-birds : a critical new look. In: Environmental effects of fenitrothion use in forestry, Ed.: Ernst, W.R. *et al.* Environment Canada Publication, no EN40-370/1989E, p.43-108.

Carmichael W.W. (1992). Cyanobacteria secondary metabolites - the cyanotoxins. J. Appl. Bacteriol. 72, 460.

Coppage D.L., Braidech T.E., (1976). River pollution by antilcholinesterase agents. Water Research, 10: 19-24.

Cunha I., Garcia L.M., Guilhermino L. (2005). Sea-urchin (Paracentrotus lividus) glutathione S-transferases and cholinesterase activities as biomarkers of environmental contamination. Journal of Environmental Monitoring 7: 288-294.

Cunha I., Mangas-Ramirez E., Guilhermino L. (2007). Effects of copper and cadmium on cholinesterase and glutathione S-transferase activities of two marine gastropods (Monodonta lineata and Nucella lapillus). Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology 145: 648-657.

Dailianis, S., Domouhtsidou, G.P., Raftopoulou, E., Kaloyianni, M.and Dimitriadis, V.K. (2003). Evaluation of neutral red retention assay, micronucleus test, acetylcholinesterase activity and a signal molecule (cAMP) in tissues of Mytilus galloprovincialis (L.), in pollution monitoring. Marine Environmental Research 56, 443-470.

Day K.E. and Scott I.M. (1990). Use of acetylcholinesterase activity to detect sublethal toxicity in stream invertebrates exposed to loww concentrations of organophosphate insecticides. Aquatic Toxicology, 18: 101-114.

Diamantino T.C., Almeida E., Soares A.M.V.M., Guilhermino, L. (2003). Characterization of cholinesterases from Daphnia magna Straus and their inhibition by zinc. Bulletin of Environmental Contamination and Toxicology 71: 219-225.

Dizer H., da Silva de Assis H.C. and Hansen., P.-D. (2001). Cholinesterase activity as a bioindicator for monitoring marine pollution in the Baltic sea and the Mediterranean sea.pp331-342. In: Biomarkers in marine organisms; a practical approach. Edited by Ph. Garrigues, H. Barth, C.H. Walker and J.F. Narbonne. Eds Elsevier.491p.

Frasco M.F., Erzen I., Stojan J., Guilhermino L. (2010). Localization and properties of cholinesterases in the common prawn (Palaemon serratus): a kinetic-histochemical study. Biological Bulletin 218: 1-5.

Frasco M.F., Fournier D., Carvalho F., Guilhermino, L. 2006. Cholinesterase from the common prawn (Palaemon serratus) eyes: catalitic properties and sensitivity to organophosphate and carbamate compounds. Aquatic Toxicology 77: 412-421.

Frasco, M.F., Fournier, D., Carvalho, F.and Guilhermon, L. (2005). Do metal inhibit acetylcholinesterase (AChE)? Implementation of assay conditions for the use of AChE activity as a biomarker of metal toxicity. Biomarkers, 10, 360-375.

Gagne F. and Blaise C. (2004). Shell protein characteristics and vitellogenin-like proteins in brine shrimp Artemia fransciana exposed to municipal effluent and 20-hydroxyecdsone. Comp. Biochem. Physiol. Part C Pharmacol.Toxicol 138. 515-522.

Galgani F. , Bocquené G and Burgeot, T. (1996). Acetylcholinesterase and ethoxyresorufin-o-deethylase in the surgeonfish Acanthurus bahianus around Martibique Island (Frencj West Indies). Biomarkers 1.1-3.

Galgani F. , Bocquené G. and Cadiou Y. (1992). Evidence of variation in cholinesterase activity in fish along a pollution gradient in the North sea. Mar. ecol. Prog. Ser. 91: 1-3),.

Garcia L.M., Castro B., Ribeiro R., Guilhermino L. (2000). Characterization of cholinesterase from guppy (Poecilia reticulata) muscle and its in vitro inhibition by environmental contaminants. Biomarkers 5: 274-284.

Habig C., Di Guilio R.T., Nomeir A.A. and Abou Donia A.M.B., (1986). Comparative toxicity, cholinergic effects and tissue levels of S,S,S-tri-nButyl Phosphoritrithioate 'DEF) to Channel catfish (Ictarus punctatus) and blue crab (Callinectes sapidus). Aquatic Toxicology, 9:193-206.

Hansson, T., Schiedek, D., Lehtonen, K.K., Vuorinen, P.J., Liewenborg, B., Noaksson, E., Tjärnlund, U., Hanson, M.and Balk, L. (2006). Biochemical biomarkers in adult female perch (Perca fluviatilis) in a chronically polluted gradient in the Stockholm recipient (Sweden). Marine Pollution Bulletin 53: 451-468

Hoguet J. and Key P.B. (2007). Activities of biomarkers in multiple life stages of the model crustacean, Palaemonetes pugio. Journal of experimental marine biology and ecology. 235-244.

Kankaanpää, H., Leiniö, S., Olin, M., Sjövall, O., Meriluoto, J. and Lehtonen, K. K. (2007). Accumulation and depuration of cyanobacterial toxin nodularin and biomarker responses in the mussel Mytilus edulis. Chemosphere 68: 1210-1217

Kirby M.F., Morris S., Hurst M. Kurby S.J., Neall P., Tylor T. and Fagg A., (2000). The use of cholinesterase activity in flounder (Platichthys flesus) muscle tissue as a biomarker of neurotoxic contamination in UK estuaries. Mar. Pollut. Bull. 9:780-791.

Kopecka, J., Lehtonen, K. K., Baršienė, J., Broeg, K., Vuorinen, P.J., Gercken, J., Balk, L. and Pempkowiak, J. (2006) Measurements of biomarker levels in flounder (Platichthys flesus) and blue mussel (Mytilus trossulus) from the Gulf of Gdańsk (southern Baltic). Marine Pollution Bulletin 53: 406-421

Lehtonen, K. K. and Leiniö, S. (2003) Effects of exposure to copper and malathion on metallothionein levels and acetylcholinesterase activity of the mussel Mytilus edulis and the clam Macoma balthica from the northern Baltic Sea. Bulletin of Environmental Contamination and Toxicology 71: 489-496

Lehtonen, K. K., Kankaanpää, H., Leiniö, S., Sipiä, V.O., Pflugmacher and S, Sandberg-Kilpi, E. (2003). Accumulation of nodularin-like compounds from the cyanobacterium Nodularia spumigena and changes in acetylcholinesterase activity in the clam Macoma balthica during short-term laboratory exposure. Aquatic Toxicology 64: 461-476

Leiniö, S. and Lehtonen, K. K. (2005) Seasonal variability in biomarkers in the bivalves Mytilus edulis and Macoma balthica from the northern Baltic Sea. Comparative Biochemistry and Physiology C 140: 408-421

Ozretið B. and Krajnovið-Ozretið M. (1992). Esterase heterogeneity in mussel Mytilus galloprovincialis: effects of organophosphate and carbamate pesticides in vitro. Comp Biochem Physiol 103:221-225.

Payne J.F., Mathieu A., Melvin W. and Fancey L.L., (1996). *Acetylcholinesterase,* an old biomarker with a new future ? field trials in association with two urban rivers and a paper mill in Newfoundland. Mar. Pollut. Bull. 32:225-231.

Pfeifer, S., D. Schiedek and Dippner J. W. (2005). Effect of temperature and salinity on *acetylcholinesterase* activity, a common pollution biomarker, in *Mytilus* sp. from the south-western Baltic Sea. J. exp. mar. biol. ecol. 320: 93-103

Printes L.B., and Callaghan A. (2004). A comparative study on the relationship between *acetylcholinesterase* activity and acute toxicity in Daphnia magna exposed to *anticholinesterase* insecticides. Environ. Toxicol.Chem.23, 1241-1247.

Rank, J., Lehtonen, K. K., Strand, J. and Laursen, M. (2007). DNA damage, acetylcholinesterase activity and lysosomal stability in native and transplanted mussels (Mytilus edulis) in areas close to coastal chemical dumping sites in Denmark. Aquatic Toxicology 84: 50-61

Rodrigues S.R., Caldeira C., Castro B.B., Gonçalvez F., Nunes B. and Antunes S.C. (2011). Cholinesterase (ChE) inhibition un pumpkinseed (Lepomis gibbosus) as environmental biomarker: ChE characterization and potential neurotoxic effects of xenobiotics. Pesticide Biochemistry and Physiology. 99 : 181-188.

Schiedek, D., Broeg, K., Baršiene, J., Lehtonen, K. K., Gercken, J., Pfeifer, S., Schneider, R., Vuontisjärvi, H., Vuorinen, P. J., Koehler, A. and Balk, L. (2006) Biomarker responses and indication of contaminant effects in blue mussel (Mytilus edulis) and female eelpout (Zoarces viviparus) from the western Baltic Sea. Marine Pollution Bulletin. 53: 387-405

Signa, G., Cartes J.E., M. Sole., Serrano, A. and Sanchez, F. (2008). Trophic ecology of the swimming crab Polybius henslowii Leach, 1820 in Galician and Cantabrian Seas: Influences of natural variability and the Prestige oil spill. Continental Shelf Research. 2659-2667.

Tim-Tim A.L., Morgado F., Moreira S., Rangel R., Nogueira A.J., Soares A.M., Guilhermino L. (2009). Cholinesterase and glutathione S-transferase activities of three mollusk species from the NW Portuguese coast in relation to the 'Prestige' oil spill. Chemosphere 77: 1465-1475.

Varó I., Navarro J.C., Amat F., Guilhermino, L. (2002). Characterisation of cholinesterases and evaluation of the inhibitory potential of chlorpyrifos and dichlorvos to Artemia salina and Artemia parthenogenetica. Chemosphere 48: 563-569.

Vieira L.R., Gravato C., Soares A.M.V.M., Morgado F., Guilhermino L. (2009). Acute effects of copper and mercury on the estuarine fish Pomatoschistus microps: linking biomarkers to behaviour. Chemosphere 76: 1416-1427.

Zinkl, J.G., Lockhart, W.L., Kenny, S.A. and Ward, F.J., (1991). The effects of cholinesterase inhibiting insecticides on fish. In: Mineau P (ed) Cholinesterase Inhibiting Insectisides. Elsevier, Amsterdam, pp. 233-254.

Zinkl, J.G.,Shea P.J., Nakampoto R.J. and Callman J., (1987). Technical and biological considerations for the analysis of brain cholinesterase of rainbow trout. Trans.Am. Soc. 116 : 570-573.

Annex 18: ICES SGEH Biological Effects methods Background Documents for the Baltic Sea region*

Ethoxyresorufin-*O*-deethylase (EROD) activity

***ICES/OSPAR document from the ICES SGIMC Report 2010, complemented and modified by SGEH 2011 with information relevant for application in the Baltic Sea region**

Background

Ethoxyresorufin-*O*-deethylase (EROD) belongs to the cytochrome P450 (CYP) family of haeme-containing enzymes, which catalyze the oxidative biotransformation of a range of organic contaminants and endogenous compounds. The biotransformation of organic contaminants results in molecular changes that lead either to inactivation of the contaminant or production of toxic metabolites as is the case of benzo(*a*)pyrene. The main form of P450 in fish is CYP1A. A fundamental feature of CYP1A is its inducibility by natural substrates and by chemical contaminants structurally similar to the substrates i.e., halogenated and non-halogenated hydrocarbons such as PCDD/Fs, dioxin-like PCBs, and PAHs. This results in an alteration of the rate of contaminant transformation catalyzed by these enzymes (Stegeman *et al.*, 1992). This phenomenon of induction i.e., increased synthesis of CYP1A protein and related enzymatic activity following the exposure forms the basis for its utility in environmental monitoring. For decades, evidence for the induction has been obtained by three methods i.e., catalytic assays, immunodetection of proteins, and detection of the synthesis of messenger RNA using cDNA (Stegeman *et al.*, 1992; Kammann *et al.*, 2008; Ortiz-Delgado *et al.*, 2008).

Since it was first suggested that CYP1A induction can be used to monitor marine petroleum pollution in the 1970s (Payne, 1976) numerous studies have employed the measurement of CYP1A-dependent activity to investigate and quantify induction and to monitor the biological effects of contaminants both in laboratory-exposed and field-collected fish (reviewed by Whyte *et al.*, 2000 and by Van der Oost *et al.*, 2003). The earliest catalytic assay involved measurement of the activity of aryl hydrocarbon hydroxylase (AHH) and employed benzo(a)pyrene as a substrate. Since the development of ethoxyresorufin, a convenient artificial substratum, the catalytic assay involves the measurement of the activity of 7-ethoxyresorufin-*O*-deethylase (EROD). EROD is a relatively simple and sensitive assay, highly specific for CYP1A. It provides a well established tool for the assessment of biological effects of contaminants.

In a review, Whyte *et al.* (2000) rank chemicals according to the level of EROD activity they induce in treated or exposed fish when compared with untreated or control fish. Contaminants that induce EROD less than tenfold above control levels are considered "weak" inducers, 10- to 100-fold are "moderate" inducers, and chemicals that elicit >100-fold induction are considered "strong" inducers. Dioxins, furans, dioxin-like PCBs, and some PAHs such as benzo(*a*)pyrene are categorized as "strong" inducers. Over 25 studies have observed induction of hepatic EROD by benzo(*a*)pyrene in 15 species of fish (Whyte *et al.*, 2000).

In respect to the Baltic Sea region, measurement of the EROD activity has been employed by some national monitoring programmes including the Swedish National Marine Monitoring Program. Within this program EROD activity has been investigated in perch (*Perca fluviatilis*) and eelpout (*Zoarces viviparus*) since 1988 (Hanson *et al.*, 2009; Ronisz *et al.*, 2005). EROD has been also examined in the Baltic Sea flounder (*Platichthys flesus*) and cod (*Gadus morhua*) within the frame of an EU founded Project

BEEP (Kopecka *et al.*, 2006, 2008; Schnell *et al.*, 2008). Available EROD data for the Baltic Sea fish have been compiled in Table 1.

Table 1. Description of EROD data for the Baltic Sea fish species (*sub-fraction S9; †microsomal subfraction).

Species	Location	Season/ water T ºC	Size (cm)	Gender	EROD activity pmol/min/mg protein	References
Platichthys flesus	Baltic Proper	Dec 2003, 7-11ºC	24-30	F M	74 – 263 * 80 – 875 *	Kopecka and Pempkowiak , 2008 [1]
Platichthys flesus	Gulf of Gdańsk	a whole year, Apr 2003 - Apr 2004	24-30	F	from 68 – 116 (Nov-Dec) to 2280 (March) *	Kopecka and Pempkowiak , 2008 [1]
Platichthys flesus	Gulf of Gdańsk	Oct 2001- Apr 2003,	24-30	F	102-212 (Fall) 1198-2198 (Spring) *	Kopecka *et al.*, 2006
Platichthys flesus	Southern Baltic	Nov 2009; 6-7 ºC	24-30	F	232 – 367 *	Dabrowska *et al.* (*in preparation)*
Perca fluviatilis	Stockholm Archipelago	Sept-Oct	19-39 (95%, 19-29)	F	42-65 *	Hansson *et al.*, 2006
Perca fluviatilis	Swedish Baltic coast,	2006, 11-19 ºC	23 - 26	F and M mixed	from 150-190 (ref.) to 220 *	Hanson *et al.*, 2010
Perca fluviatilis	Gulf of Finland	Dec 2001	21-29	F	2.7 ± 3.9 *	Pikkarainen, 2006
Gadus morhua	Bornholm Basin	Jul 1999	matured	F M	1.3- 57 † 130 †	Schneider *et al.*, 2000
Gadus morhua	Southern and western Baltic	Dec 2001; 6-10 ºC	40-50	F and M mixed	12.9 (Kiel Bight) – 110 (Słupska Bank) †	Schnell *et al.*, 2008
Zoarces viviparus	southwestern Baltic (Wismar Bay and Mecklenburg Bight)	spring and autumn 2001 and 2002	20-30	F	2 - 150 † site+season avg. 37 – 78 †	Schiedek *et al.* 2006
Zoarces viviparus	Kattegat, west coast of Sweden	whole year, Aug 1996- Jul 1997		F M	50 (Aug-Oct) - 190 (Feb-March) † 170 ± 120 †	Ronisz *et al.*, 1999

[1] The BEEP project; for both genders the lowest EROD occurred in fish from the BEEP1 site (off the Sweden coast) and highest EROD in those from the BEEP4 site (off the Hel peninsula, Gulf of Gdańsk).

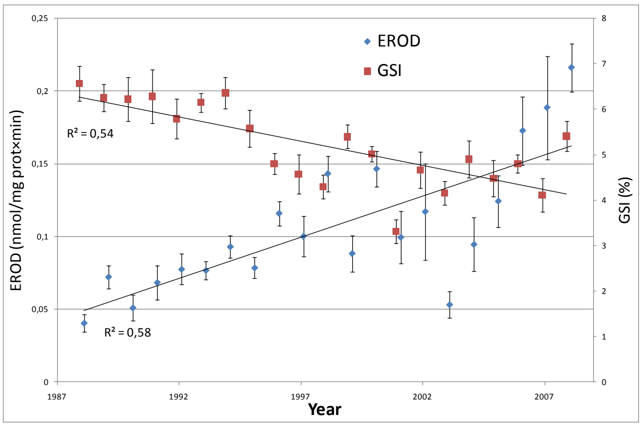

Figure 1. EROD activity and gonadosomatic index (GSI) in perch (Perca fluviatilis) at the Kvädöfjärden monitoring site at the coast of Swedish Baltic Proper between 1988–2008. Increased EROD activity and reduced GSI level indicate poorer state of the environment.

Confounding Factors

A number of endogenous and exogenous factors have been known to influence the constituent, background levels as well as the extent of EROD induction resulting from contaminant exposure. Aside from species differences, the most relevant factors are developmental stage, gender, reproductive status, season and temperature, and age. In adult specimens strong gender-related seasonal EROD differences occur. These differences are mainly due to suppression of CYP1A expression in females by the female sex hormone, 17β-estradiol, during maturation of gonads. In the field, the water temperature is the factor driving the gonadal development and maturation and it is not possible to separate the influence of these two factors. Gender differences can also result from an increase of CYP1A levels in males during reproductive season (Lindstrom-Seppa and Stegeman, 1995; Whyte *et al.*, 2000; Dabrowska *et al.*, 2000; Kirby *et al.*, 2007). The suppression of CYP1A induction in females can lead to an underestimation of exposure to AhR-responsive contaminants. 17β-estradiol also controls the induction of vitellogenin (VTG; egg yolk protein) which is produced by the liver during gonadal recrudescene. Thus an interference of environmental estrogens on CYP1A induction can be assessed. Seasonal changes in EROD induction have been observed in rainbow trout (Förlin and Haux, 1990), flounder (Van Westernhagen *et al.*, 1981; Hylland *et al.*, 1998), plaice (George and Young, 1986) and salmon (Larsen *et al.*, 1992).The main age-related factors are time of exposure/ accumulation and feeding ecology. Dietary factors can be potentially important for the induction of CYP1A. Firstly, because AhR ligands can be delivered to the organism through the food. Secondly, nutritional status can affect the functions of enzyme systems. Hylland *et al.*, (1996) reported a decreased EROD response (i.e. to control levels) in BaP-treated and starved for one month flounder.

Ecological relevance

Cytochrome P4501A is the most widely used biomarker. Information relating the toxicity of AhR-responsive contaminants to CYP1A induction in fish is mainly correlative. Ecological relevance that renders EROD as a biomarker stands from research linking EROD induction to specific biological alterations. One example is a long-term study of perch in the Baltic Sea that shows strong time trend toward increasing hepatic EROD activity which correlates with reduced gonadosomatic index (GSI; Hanson *et al.*, 2009). Furthermore, research linking the EROD induction to specific alterations at any level of organismal organisation supports the utility of this biomarker as an early warning of toxic effects.

Quality Assurance

There have been three international inter-calibrations for the method, both within BEQUALM. The inter-calibrations have pinpointed a variability in many steps of the analytical procedure, except for the enzyme kinetic analysis itself. As many factors are known to influence the EROD activity (see above) and because of a difficulty to account for all of them in the assessment process, it is advisable to include an appropriate reference group in studies that include EROD as an endpoint. It is also imperative that laboratories have internal quality assurance procedures, e.g. use internal references samples with all batches of analyses.

Baseline and Assessment criteria

Background response ranges have been developed for the Baltic Sea fish species as described in the 2010 Report of the Joint ICES/OSPAR Study Group on Integrated Monitoring of Contaminants and Biological Effects (SGIMC). The 90th percentiles of values from reference sites were used to establish 'background' and 'elevated' responses based on data obtained within the BEEP project. This information is provided in Table 2. It will allow new data to be assessed against the appropriate assessment criteria for fish species, gender, size, sampling season, and bottom-water temperature.

Table 2. EROD assessment criteria for target fish species used in European biomonitoring programmes. EROD established BRs are restricted to the sampling conditions and the size of the specimen used (*sub-fraction S9; † microsomal sub-fraction).

Species	Season	Bottom water T ºC	Size (cm)	Gender	Background Response range pmol/min/mg prot	Elevated Response range pmol/min/ mg prot)	Notes
S9 fraction							
Platichthys flesus	Nov/Dec	6-7ºC	25-30	F	≤ 267	> 267	Southern Baltic (BEAST data)
Platichthys flesus	Aug/Nov	10-18ºC	20-25	F and/or M	≤ 24	> 24	Adopted from ICES/OSPAR SGIMC
Pleuronectes platessa	Jan	5-10ºC	18.5-22.5	M	≤ 10	> 10	Adopted from ICES/OSPAR SGIMC
Microsomal fraction							
Limanda limanda	Aug/Nov	10-18ºC	20-30	F and/or M	≤ 780	> 780	Adopted from ICES/OSPAR SGIMC
Pleuronectes platessa	Sept	7-10ºC	40-60	F and/or M	≤ 255	> 255	Adopted from ICES/OSPAR SGIMC

Distribution of indicator species in Baltic Sea subregions

Of the most used indicators species, flounder are widely available in the southern-central parts of the Baltic but less so in the northern sub-basins. Perch is restricted to low-salinity areas and rivermouths. Eelpout is found all over the area but in limited numbers in the Bothnian Bay. Herring is available in the whole area.

Acknowledgements

This document has been adopted from the 2010 Report of the Joint ICES/OSPAR Study Group on Integrated Monitoring of Contaminants and Biological Effects (SGIMC) and modified for the Baltic Sea region.

References

Dabrowska H, Fisher SW, Dabrowski K, Ciereszko R, Woodin BR, Stegeman JJ. 2000. Hepatic P4501A activity, plasma sex steroids, and gonadal steroidogenesis in vitro in yellow perch exposed to 3,3’,4,4’,5-pentachlorobiphenyl. Environ. Toxicol. Chem., 19, 3052-3060.

Lindstrom-Seppa P, Stegeman JJ. 1995. Sex differences in cytochrome P450 1A induction by environmental exposure and β-naphthoflavone in liver and extrahepatic organs of recrudescent winter flounder. Mar. Environ. Res. 39, 219-223.

Hanson N, Förlin L, Larsson A. 2009. Evaluation of long-term biomarker data from perch (Perca fluviatilis) in the Baltic Sea suggests increasing exposure to environmental pollutants. Environ. Toxicol. Chem. 28, 364-373.

Hanson N, Förlin L, Larsson A. 2010. Spatial and annual variation to define the normal range of biological endpoints: an example with biomarkers in perch. Environ. Toxicol. Chem., 29, 2616-2624.

Hansson T, Schiedek D, Lehtonen KK, Vuorinen PJ, Liewenborg B, Noaksson E, Tjärnlund U, Hanson M, Balk L. 2006. Biochemical biomarkers in adult female perch (Perca fluviatilis) in a chronically polluted gradient in the Stockholm recipient (Sweden). Mar. Poll. Bull. 53, 451-468.

Kammann U, Lang T, Berkau A-J, Klempt M. 2008. Biological effect monitoring in dab (Limanda limanda) using gene transcript of CYP1A1 or EROD – a comparison. Environ. Sci. Pollut. Res., 15, 600-6005.

Kirby MF, Smith AJ, Rooke J, Neall P, Scott AP, Katsiadaki I, 2007. Ethoxyresorufin-O-deethylase (EROD) and vitellogenin (VTG) in flounder (Platichthys flesus): System interaction, crosstalk and implications for monitoring. Aquatic Toxicol., 81, 233-244.

Kopecka J, Lehtonen KK, Barsienie J, Broeg K, Vuorinen PJ, Gercken J, Pempkowiak J, 2006. Measurements of biomarker levels in flounder (Platichthys flesus) and blue mussel (Mytilus trossulus) from the Gulf of Gdańsk (southern Baltic). Mar. Pollut. Bull., 53, 406-421.

Kopecka J, Pempkowiak J. 2008. Temporal and spatial variations of selected biomarker activities in flounder (Platichthys flesus) collected in the Baltic proper. Ecotoxicol. Environ. Safety, 70, 379-391.

Lindstrom-Seppa P, Stegeman JJ. 1995. Sex differences in cytochrome P450 1A induction by environmental exposure and β-naphthoflavone in liver and extrahepatic organs of recrudescent winter flounder. Mar. Environ. Res. 39, 219-223.

Ortiz-Delgado JB, Behrens A, Segner H, Sarasquete. 2008. Tissue-specific induction of EROD activity and CYP1A protein in Sparus aurata exposed to B(a)P and TCDD. Ecotoxicol. Environ. Safety. 69, 80-88.

Payne JF. 1976. Field evaluation of benzopyrene hydroxylase induction as a monitor for marine pollution. Science, 191, 945-946.

Pikkarainen A-L. 2006. Ethoxyresorufin-O-deethylase (EROD) activity and bile metabolites as contamination indicators in Baltic Sea perch: Determination by HPLC. Chemosphere, 65, 1888-1897.

Ronisz D, Lindesjöö E, Larsson A, Bignert A, Förlin L. 2005. Thirteen years of monitoring selected biomarkers in eelpout (Zoarces viviparus) at reference site in the Fjällbacka Archipelago on the Swedish West Coast. Aquatic Ecosys. Health & Management 8, 175-184.

Ronisz D, Larsson JDG, Förlin L. 1999. Seasonal Variations in the activities of selected hepatic biotransformation and antioxidant enzymes in eelpout (Zoarces viviparous). Comp. Biochem. Physiol. C., 124, 271-279.

Schiedek D, Broeg K, Baršiene J, Lehtonen KK, Gercken J, Pfeifer S, Vuontisjärvi H, Vuorinen PJ, Dedonyte V, Koehler A, Balk L, Schneider R. 2006. Biomarker responses as indication of contaminant effects in blue mussel (Mytilus edulis) and female eelpout (Zoarces viviparus) from the southwestern Baltic Sea. Mar. Poll. Bull. 53, 387- 405.

Schneider R, Schiedek D, Peterson GI. 2000. Baltic cod reproductive impairment: ovarian organo-chlorine levels, hepatic EROD activity, muscular AChE activity, developmental success of eggs and larvae, challenge tests. ICES CM 2000/S:09.

Schnell S, Schiedek D, Schneider R, Balk L, Vuorinen PJ, Karvinen H, Lang T. 2008. Biological indications of contaminant exposure in Atlantic cod (Gadus morhua) in the Baltic Sea. Can. J. Fish. Aquat. Sci. 65, 1122-1134.

Stegeman JJ, Brouwer M, Di Giulio RT, Forlin L, Fowler B, Sanders B, Van Veld PA. 1992. Molecular responses to environmental contamination: proteins and enzymes as indicators of contaminant exposure and effects. Biomarkers: Biochemical, Physiological and Histological Markers of Anthropogenic Stress. Eds.: Huggett RJ, Kimerle RA, Mehrle PM Jr, Bergman HL, pp 235-335. Boca Raton, Fl, Lewis Publishers.

Van der Oost R, Beyer J, Vermeulen NPE. 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharmacol., 13, 57-149.

Whyte JJ, Jung RE, Schmitt CJ, Tillitt DE. 2000. Ethoxyresorufin-O-deethylase (EROD) activity in fish as a biomarker of chemical exposure. Crit. Rev. Toxicol. 30, 347-570.