

ICES WGBEC REPORT 2011

SCICOM STEERING GROUP ON HUMAN INTERACTIONS ON ECOSYSTEMS

ICES CM 2011/SSGHIE:02

REF. SCICOM

Report of the Working Group on Biological Effects of Contaminants (WGBEC)

14–18 February 2011

Vigo, Spain



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International Council for
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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 Working Group on Biological Effects of Contaminants (WGBEC), 14–18 February 2011, Vigo, Spain. ICES CM 2011/SSGHIE:02. 163 pp.

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Executive Summary

The Working Group on the Biological Effects of Contaminants (WGBEC), chaired by Matt Gubbins (UK) and John Thain (UK), met at Estación de Ciencias Mărinas de Toralla (ECIMAT), Illa de Torallo, Vigo, Spain from 14 to 18 February 2011. There were 19 attendees representing nine countries.

A summary of the key outcomes in respect of the Terms of Reference is described below.

WGBEC includes in its membership scientists from national government institutes, academia, industry and management. The group also has a diverse membership of expertise, ranging from chemists, biologists, biochemists and environmental scientists. This is beneficial as requests in the past, particularly from OSPAR have been wide-ranging. This year there were ten items on the agenda, including two items from ICES and one from OSPAR/ICES. Priority was given to the latter items. Presentations and discussions took place in plenary, with rapporteur responsibility shared by all members of the group. All items on the agenda (covering all ToRs) were completed and are reported.

View the output from SGIMC 2010 and respond to their request for support. This agenda item was given the highest priority and considerable time was given to the completion of the tasks. WGBEC has had close links with SGIMC (previously WKI-MON) over the past seven years and have provided advice and taken on tasks to support the work and output of SGIMC. At its 2010 meeting SGIMC requested support for the completion of ten documents and reports. These were successfully completed. During discussion it was noted that the SGIMC process and work plan was nearing completion and should be finalised in 2011. Following cessation of SGIMC it was proposed that WGBEC could take on the role of reviewing assessment criteria and reviewing national applications of the integrated assessment framework to monitoring data.

Evaluate potential for collaboration with other EGs in relation to the ICES Science Plan and report on how such cooperation has been achieved in practical terms. This item was given to WGBEC in its ToR in 2010. At that meeting WGBEC examined its core business, how it fitted in with the ICES Science Plan and its potential to collaborate with other EGs within SSGHIE and other EGs outside of SSGHIE. To further investigate the potential for active collaboration between EGs, WGBEC had written to the chairs of eleven EGs. At the time of this meeting seven responses had been received actively supporting collaboration, and in some cases suggesting topics for further development.

Review of progress with publication and electronic dissemination of biological effects techniques in the ICES TIMES series. The outgoing effects editor (Matt Gubbins) reviewed progress on publications to the group. Responsibility for ICES TIMES manuscripts on biological effects methods was transferred to Ricardo Beiras (Spain) this year. Eleven manuscripts have been commissioned by WGBEC and are awaiting submission to ICES. Two (1E03, MHC07) are finalised and awaiting submission to the ICES TIMES editor. A further two that already have draft resolutions (MHC06, MHC13) are complete and were reviewed by the WG during the meeting.

Respond to requests for advice from the ICES Data Centre. No specific requests from the ICES data centre were received by the EG for attention in 2011, however it was identified that there were still some issues with biological effects data submission

that needed to be addressed. Two specific issues in relation to QA and comparability were identified and discussed. A subgroup has been formed to provide a more effective and flexible way of dealing with data centre issues, primarily by correspondence.

Review developments in biological effects monitoring activity undertaken by member states. This agenda item provided an opportunity for the group to keep abreast of biological effects monitoring activities taking place across the ICES area. During the meeting Spain, Norway, Netherlands, Belgium, Ireland, Portugal, the UK and the Baltic countries through HELCOM provided an update to the group on recent activity. This included integrated assessment of data and approaches being developed for marine monitoring in-line with OSPAR integrated approach for monitoring contaminants and biological effects.

Update on MSFD activities in relation to contaminants and biological effects: Several members of WGBEC have been heavily involved in the initial JRC Task Group activities for Descriptor 8 and much of the guidance produced reflects the views of WGBEC on the potential value of application of biological effects monitoring data to determine whether GES is being met for Descriptor 8. A round table discussion took place and developments and approaches used in each country represented were noted. WGBEC is of the view that the integrated monitoring approach devised for OSPAR by SGIMC and to be completed in 2011 is the most appropriate approach available for determining GES for Descriptor 8 and recommends its adoption by member states.

Consideration of issues of special scientific interest / value. During discussion of the future role and scope of WGBEC under agenda items 7 & 8 at the WGBEC 2010 meeting, several new work areas were raised as potential priority areas and of emerging interest to ICES and the group. Some of the areas identified for new future directions were reported on at this meeting and included: a) acidification in marine waters in relation to contaminants and biomarker response; b) effects of contaminants on primary production, including phytotoxicity; c) relationship of genetic markers to biomarkers; d) review of species differences in bioassay and biomarker responses e.g. as seen in assessment criteria currently being developed; e) immunotoxicity end points – suitability for monitoring (an intersessional meeting on the latter will be organised in October 2011).

Review progress with AQC procedures for biological effect methods and include harmonisation activities. WGBEC reviewed ICES/OSPAR WKLYS on the quality and interpretation of lysosomal stability data report and assessment criteria for LMS using NRR assay. Reports were received on the BONUS+ project BEAST intercalibration exercise for PAH metabolites in fish bile and progress with the QUASIMEME intersex/imposex intercalibrations. With the imminent adoption of the OSPAR integrated monitoring guidelines and inclusion of biological effects monitoring to determine GES for Descriptor 8 of MSFD, there is an urgent need to develop external QA for a number of additional measurements. WGBEC will endeavour to progress the development of QA intersessionally through a steering.

1 Opening of the meeting

The ICES WGBEC was hosted this year by Ricardo Beiras and José Fumega and held at Estación de Ciencias Mărinas de Toralla (ECIMAT), Illa de Torallo, Vigo, Spain. The Chair, Matt Gubbins (UK), opened the meeting at 09.30 on Monday, 14 February 2011, and thanked Ricardo Beiras for hosting the meeting and for organising the meeting arrangements and hotel accommodation, etc. The Chair then invited the participants to introduce themselves and their affiliations and describe their area of interest and field of expertise. There were nineteen participants (including one corresponding member) representing nine countries. The list of attendees is given in Annex 1.

2 Adoption of the agenda

The ToRs and a draft agenda had been circulated prior to the meeting. The Chair invited participants to examine the Terms of Reference (ToRs) and went through the agenda explaining the priority and background to the agenda items and in particular those requests from ICES and OSPAR. Attention was drawn to the request from ICES/OSPAR SGIMC (agenda item 7), this was a high priority and needed to be completed and documentation forwarded to SGIMC for their final meeting on 14 March 2011. The ToRs for the meeting can be found in Annex 2. The draft agenda was adopted by the meeting and a tentative timetable agreed, Annex 3 and 4 respectively.

3 Appointment of rapporteurs

Principle rapporteurs were appointed for the agenda items and are given in Annex 4.

4 Review developments in biological effects monitoring activity undertaken by member states; to include integrated assessment of monitoring data and approaches proposed to address Good Environmental Status under Descriptor 8 of the MSFD+ any other; (ToR c)

This agenda item provides an opportunity for the group to keep abreast of biological effects monitoring activities taking place across the ICES area. During the meeting Spain, Norway, Netherlands, Belgium, Ireland, Portugal, the UK and the Baltic countries through HELCOM provided an update to the group on recent activity. The majority of discussion concerning Descriptor 8 of the Marine Strategy Framework Directive (MSFD) was deferred to agenda item 8.

4.1 Spain

Izaskun Zorita (ES) presented the biological effects monitoring activities carried out by AZTI-Tecnalia within the Water Framework Directive for the Department of Land Action and Environment of the Basque Government since 1994 (Borja *et al.*, 2009a). The 'Littoral Water Quality Monitoring and Control Network' (hereafter, LQM) of the Basque estuarine and coastal waters comprises the analyses of hydromorphological, physico-chemical (in water, sediment and biota) and biological quality elements (phytoplankton, macroalgae, benthos and fishes). The LQM series data include 32 coastal and estuarine stations sampled, from 1995 to 2011, together with 19 more since 2002. Three of these stations, located in offshore waters, are planned to be used in the environmental status assessment within Marine Strategy Framework Directive

(MSFD). These 51 stations are distributed among the 18 water bodies of the Basque Country; as such, they constitute the surveillance and operational monitoring networks (Figure 4.1.1).

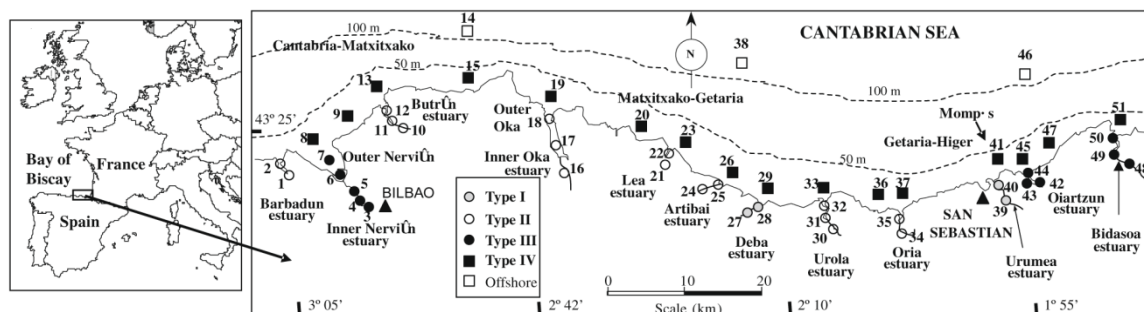


Figure 4.1.1. Sampling stations and water bodies within the Littoral Water Quality Monitoring and Control Network of the Basque Country. Typologies: Type I – small river – dominant estuaries; Type II – estuaries with extensive intertidal flats; Type III estuaries with extensive subtidal areas; Type IV – full marine soft-bottom exposed coast; and offshore – stations used within the European Marine Strategy Framework Directive.

The results of the evolution of the ecological status within the Basque estuaries and coasts indicate a progressive increase in their ecological status, reducing both "bad" and "poor" status and increasing "moderate" and "good", especially after 2001 (Borja *et al.*, 2009b).

It was pointed out that the use of ecotoxicological approaches within the WFD might assist in a more accurate ecological status assessment of the water bodies. In this respect, in the last years AZTI-Tecnalia has applied bioassays and the biomarker approach. The battery of bioassays to assess the toxicity of contaminated sediments comprises three different tests: microtox test using marine bacteria (*Vibrio fischeri*), amphipod test using two different species, *Corophium multisetosum* and *Corophium urdaibaiense* and sea urchin (*Paracentrotus lividus*) test. Among the biomarkers, imposex in gastropods (*Nassarius reticulata* and *Nassarius nitidus*) is widely used in native and caged organisms of the Basque coast as a biomarker of TBT exposure. In investigative monitorings, caged mussels (*Mytilus galloprovincialis*) have been translocated to locations with different pollution levels. The set of biological effects of pollution studied include condition index, mussel histopathology, micronuclei frequency, malonaldehyde and vitellogenin-like protein levels.

Regarding the MSFD, AZTI-Tecnalia is going to carry out sea campaigns to assess the environmental status within the south-eastern part of the Bay of Biscay. In order to respond to the qualitative descriptor 8, "Concentrations of contaminants are at levels not giving rise to pollution effects", bioaccumulation analyses together with a set of biomarkers are expected to be applied in sentinel fish species. The selection of fish species is not done yet but based on previous campaigns the benthic megrim (*Lepidorhombus whiffiagonis*) could be a possible candidate.

References

- Borja, A., Bald, J., Belzunce, M.J., Franco, J., Garmendia, J.M., Larreta, J., Muxika, I., Revilla, M., Rodríguez, J.G., Solaun, O., Uriarte, A., Valencia, V., Zorita, I., Adarraga, I., Aguirrezabalaga, F., Cruz, I., Laza, A., Marquiegui, M.A., Martínez, J., Orive, E., Ruiz, J.M., Seoane, S., Sola, J.C., Manzanos, A., 2009a. Red de seguimiento del estado ecológico de las aguas de transición y costeras de la Comunidad Autónoma del País Vasco. Report of AZTI-Tecnalia for the Basque Water Consortium, 21 volumes, 707 pp.

Borja, A., Bald, J., Franco, J., Larreta, J., Muxika, I., Revilla, M., Rodríguez, J.G., Solaun, O., Uriarte, A., Valencia, V., 2009b. Using multiple ecosystem components, in assessing ecological status in Spanish (Basque Country) Atlantic marine waters. *Marine Pollution Bulletin* 59, 54-64.

4.2 Norway

4.2.1 CEMP monitoring

Ketil Hylland (NO) presented the Norwegian CEMP using data from 2009 as an example. The programme relevant to biological effects comprises chemical measurements in blue mussels, Atlantic cod, plaice and dab from 11 locations along the Norwegian coast on an annual basis. Atlantic cod from three of the locations (Sørfjord – contaminated; Inner Oslofjord – contaminated; Bømlo – unpolluted) are also used for biological effects measurements: PAH metabolites, EROD, CYP1A and ALA-D (see Table 4.2.1). Dog whelk has been sampled at 10 locations along the Norwegian coast for imposex determination.

Table 4.2.1. Overview of the number of locations sampled for the indicated matrices in 2009 (from Green *et al.*, 2010). Sample number follows ICES guidelines.

Description	Blue mussel, soft body	Dog-whelk, soft body	Atlantic cod bile	Atlantic cod Red blood cells	Atlantic cod liver	Atlantic cod fillet	Flatfish liver	Flatfish fillet
Cd, Cu, Pb, Zn, Ag, As, Co, Cr, Ni, Sn	49				12–14		10–11	
Hg	46					13		10
TBT	14	10						
PCBs	41				14	11	11	11
HCB	41				11	11	11	9
DDT, DDE, DDD	41				11	11	11	9
HCH	41				14	11	11	11
Dioxins	8							
PBDE					10			
PFC					10			
PAHs	17							
Biological effects methods		10 Imposex	4 OH-pyrene OH-phenanthrene 3-OH-BaP	3 ALA-D	3 EROD-activity, CYP1A			

The results indicate a dramatically improved situation for imposex in dog whelks at most locations along the Norwegian coast, commencing in 2003. PAH-metabolite results indicate that PAHs are present in the inner Oslofjord with no real change over the last decade and low levels elsewhere. CYP1A and EROD results correspond and indicate a tendency for improved situations in the two polluted locations, but the activity is still higher than at the reference location.

References

Green, N. *et al.* 2010. Hazardous substances in fjords and coastal waters – 2009. Levels, trends and effects. Long-term monitoring of environmental quality in Norwegian coastal waters. NIVA report 6048-2010, 287 p.

4.2.2 Oil and gas related biomonitoring on the Norwegian Continental Shelf

Steinar Sanni (NO) gave a presentation on recent oil and gas monitoring on the Norwegian continental shelf. Biological effect monitoring offshore in Norway is to a large extent carried out within the oil and gas related biomonitoring programmes on the Norwegian Continental Shelf.

There are three such monitoring programmes:

- Sediment monitoring (SM; annually; focusing on benthos biodiversity and sediment chemistry)
- Water Column monitoring (WCM; annually, focusing on contaminants and biological effects in the pelagic vicinity of the oil field produced water discharges)
- Condition monitoring (CM; every 3 years), with varying focus covering different aspects of the environmental condition in wild caught offshore organisms.

WCM programme: In the water column monitoring, the impact assessments have been made with the use of biomarkers measured in caged mussels and cod since the BECP ELAG workshop, first on a trial basis – later in a mandatory way. Along with a core suite of biomarkers proven robust and sensitive to oil based discharges, it has been possible to test new and interesting methods within the WCM programme. This has been a valuable contribution to new experience of how biomarkers work in the field in relation to oil based discharges.

The WCM programme and results for 2006, 2008 and 2009 were presented in the present meeting based on slides by S. Brooks (NIVA) and R.C. Sundt (IRIS). In these years the programme was carried out at the Ekofisk oil field with the special purpose to evaluate the implementation of CTour cleaning technology on biological effects. The fish (Atlantic cod) and mussels were exposed for 6 weeks in cages deployed in gradients along the prevailing current directions downstream the discharge point.

Biomarkers measured were: In fish: PAH metabolites by FF, PAH metabolites by GCMS, CYP1A, GST, VTG, ZRP and DNA adducts. In mussel: PAH body burden, Pyrene Hydroxylase, Lysosomal stability, Micronucleus frequency, Lipofuscin (histology), Neutral lipid accumulation (histology). The gradients in exposure were reflected in the chemical and in parts of the biological markers. However, the predicted risk of the discharge is currently not being validated against the results, which is a key purpose of the WCM monitoring (as correctly noted by members of WGBEC).

CM programme: The Condition Monitoring does not have a fixed programme content. An extract of the objectives and results for the latest surveys (2008) was presented based on slides by B. E. Grøsvik, Institute of Marine Research, Norway.

The objectives were focused on obtaining documentation on whether discharges from O&G activities in Norwegian sea areas can be measured in effect parameters in wild caught fish, with emphasis on exposure markers in bile, and possible genotoxic (DNA-adducts) and endocrine effect signals (Vtg). The surveys also included histological gonad and fatty acid measurements. It is thus interesting that this programme also have applied biomarker methods, and that the results showed rather clear and

interesting variations in the different sea areas and between the different fish species investigated. Haddock in the area with the highest activity have consistently had elevated hepatic DNA adduct concentrations and haddock from all locations in the North Sea, including the reference areas, have higher concentrations of PAH bile metabolites than haddock from the Barents sea. No such differences have been observed for saithe or Atlantic cod (sampled in the same areas).

In a proposed objective for the Water Column Monitoring 2011 it is suggested to determine the environmental effects of produced water discharge on animals living in the water column using caged mussels, passive samplers, possibly wild caught fish, increasing the number of mussel stations, and possibly replacing the caged fish with haddock collected near platforms (if successful).

4.3 Netherlands

Klaas Klaag (NL) reported on imposex monitoring activities in the Netherlands. Initially, intersex in *Littorina littorea* was monitored at 7 coastal locations. Intersex in *Littorina* is a relatively insensitive parameter. Consequently, the ISI rapidly returned to zero. The most sensitive species in the area is *Nucella lapillus* showing imposex in reaction to very low TBT levels. *Nucella*, however, only occurs in a limited area in the Southwestern part of the Netherlands. The past 10 years *Nassarius reticulata* returned in coastal waters. This species is only slightly less sensitive than *Nucella*. In 2009 and 2010, *Nassarius* was sampled along the Dutch coast. In the southern locations 10–50% of the females showed imposex, resulting in VDSI values up to 0.47. In the two northernmost locations no imposex was found. TBT levels in the snails were low: max 3.5 µg TBT Sn/kg wet weight.

In addition to imposex in snails, routine monitoring is conducted of external diseases and liver nodules as well as PAH metabolites in dab and flounder. These biological effects methods are integrated with residue-contaminant measurements in the same fish (and contaminants in sediment, and supporting biological and hydrographical data. Evaluation of long-term data indicated a general improvement of coastal and estuarine ecosystem health during the last 15 to 20 years. At the same time the evaluation demonstrated that migration patterns play a critical role in explaining the distribution of chronic diseases such as liver neoplasms in flatfish (Vethaak *et al.*, 2009).

References

Vethaak, A.D., Pieters, J., Jol, J.G. 2009. Long-term trends in the prevalence of cancer and major diseases among flatfish in the S.E. North Sea as indicators of changing ecosystem health. *Env. Sci. Technol.* 43: 2151–2158.

4.4 Belgium

Kris Koorman (BE) gave a presentation on an approach “towards an integrated pollution monitoring strategy in the shrimp *Crangon crangon*: bridging the gap between the chemical and biological approach through gene expression profiling”.

The presentation reported on progress made within the framework of a PhD-study on effects of endocrine disruptors in the brown shrimp *Crangon crangon*.

While insects dominate the land, crustaceans dominate the water. Crustaceans, being closely related to insects, tend to be sensitive to a wide variety of chemicals. After being released in the environment, these toxicants finally end up in the seas where

they have the tendency to accumulate to often unexpectedly high concentrations in the sediment and its associated organisms.

Playing an important pivotal role in the co-ordination of development, reproduction and growth in Arthropods, the interaction of the ecdysteroid hormone with its ecdysteroid receptor (EcR) is known to be sensitive to chemicals and especially endocrine disruptors. For this reason, these interactions are currently an important target in crop protection research to develop 'safe' insecticides via *in vitro* insect cell lines. In contrast to insects, no *in vitro* testing methods for studying endocrine disruption in crustaceans are currently available. Numerous attempts to achieve a continuous crustacean cell line have so far remained unsuccessful. In this study, an established transformed insect cell line was engineered for expression of a crustacean EcR (see Hopkins (2009) for a recent review on crustacean ecdysteroids and EcR). We cloned the ecdysteroid hormone receptor (EcR) and its obligate partner protein retinoid-X-like receptor (RXR) from the brown shrimp *Crangon crangon* and transfected both in the mutant *Drosophila* L57 cell line. This cell line is characterized by a 90% inactivation of endogenous *Drosophila* EcR, and can therefore be used as a carrier for studying heterologous EcR. Our results indicate a high activity of shrimp EcR in this cell line after exposure to the ecdysteroids ponasterone A and 20-hydroxyecdysone. This proof of concept illustrates the applicability of the transformed cell line as a reporter screening tool for chemicals with crustacean endocrine disruptive effects. The reporter was further validated with some known and unknown ecdysteroid disruptors. E.g. tributyltin (TBT) showed a high potential to impair the interactions between the natural ecdysteroids and EcR. This result was expected from earlier docking simulations of the interactions of the natural ecdysteroid ponasterone A and TBT in the *in silico* reconstructed 3D-model of the ligand binding domain of the EcR.

In a next phase sex-related genes were used as biomarkers of endocrine disruption. Suppression Subtractive Hybridization-PCR (SSH-PCR) was used to isolate and sequence approximately two-thousand gene fragments originating from genes which expression levels differed strongly between male and female shrimp. This provided a set of gender specific genes. Of the 690 sequenced gene fragments 280 unique unknown sequences were derived. The other gene fragments corresponded to 80 known unique genes, including genes encoding cuticular and cytoskeletal related proteins (actins, myosin, cathepsins,...), energy metabolism related proteins (cytochrome c oxidase, ATP synthase, sugar binding proteins,...), antimicrobial proteins (crustin, carcinoïn,..) and reproduction related proteins (vitellogenin, male reproductive related protein a,...). Studies have now started with custom-made microarrays to screen for genes which are sensitive to TBT by applying chronically exposed shrimp. qPCR will be used to precisely confirm the observed expression changes and to test the applicability of these potential molecular biomarkers on field samples (paper in prep.).

The field samples were collected at 52 locations along the southern North Sea coast in between 1/09/09 and 10/11/09, thanks to the collaborations of several members of the WGCAN:

- Ingrid Tulp, IMARES, Wageningen: 5 samples from the Scheldt estuary (RV Schollevaar), 7 from the Dutch coast (RV Isis) and 14 samples from the Dutch Wadden Sea (RV Stern) through the DFS program;
- Thomas Neudecker, Institut für Seefischerei, Hamburg: 15 samples from the German Wadden Sea (chartered commercial shrimpers) through the DYFS program;

- Per Sand Kristensen, National Institute of Aquatic Resources, Charlottenlund: 7 samples from the Danish Wadden Sea using a chartered commercial shrimper.

In addition 4 samples from the Belgian Continental Shelf were collected during the monitoring campaigns aboard the RV Belgica.

All samples were analysed for tributyltin (TBT), dibutyltin (DBT), monobutyltin (MBT) and triphenyltin (TPhT) and its dealkylated analogues DPhT and MPhT in collaboration with MUMM, Oostende. In a later phase, the concentrations of per-fluorinated organics will be analyzed at the Biology Department of the Antwerp University. The results are shown in figure 4.4.1.

In order to benefit from interaction between researchers in this field and the biological effects community it was decided to invite a member of the project team and WGCAN to ICES WGBEC 2012.

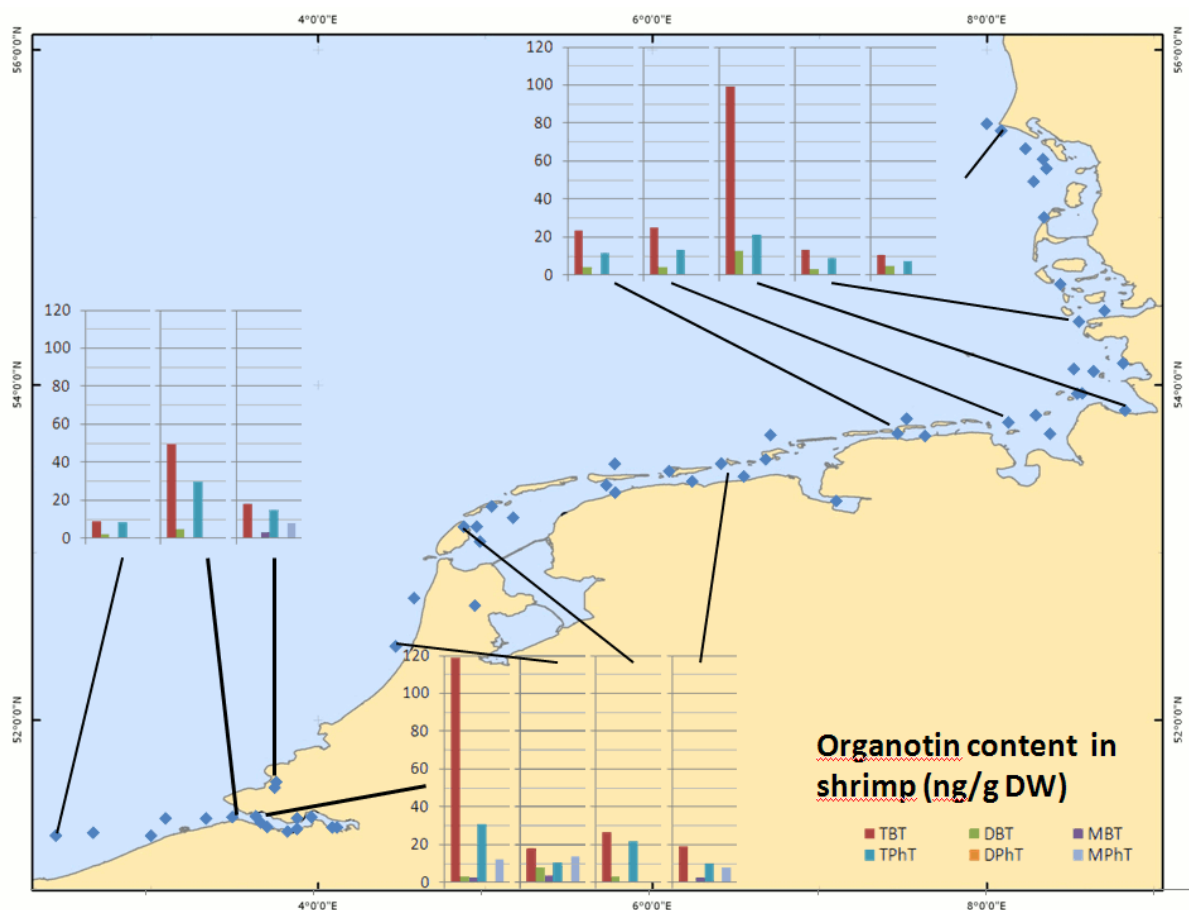


Figure 4.4.1. Shrimp caught at the Oostdyck sandbank, the most western and offshore sampling station of the Belgian continental shelf, exhibited the lowest organotin (sum of TBT and TPhT) concentrations (19 ng/g dry weight). Highest concentrations were measured in the mouth of the Scheldt (near Vlissingen-Oost harbor; 167 ng/g dw). The concentrations sharply decreased again further east at sea. Concentrations were again high in the Elbe estuary near Wehldorf (133 ng/g dw). All other nearshore and intertidal sampling stations exhibited organotin concentrations between 20–50 ng/g dw.

Action 4.4: That ICES WGBEC invite a member from WGCAN to present recent the outcome of recent studies on effects of pollutants on Crangon.

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4.5 Developments in the HELCOM area

4.5.1 SGEH

(Presented by Kari Lehtonen, FI)

ICES Study Group for the Development of Integrated Monitoring and Assessment of Ecosystem Health in the Baltic Sea (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 level of biological levels, from the molecular "early warning" level via effects on individuals and population up to ecosystem level. A 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 and Marine Strategy Framework Directive. The process is carried out in harmony with the work done in the OSPAR area (ICES SGIMC) and in close collaboration with HELCOM.

Since SGEH is focusing on the same targets as the Baltic Sea BONUS+ Programme project BEAST (Biological Effects of Anthropogenic Chemical Stress: Tools for the Assessment of Ecosystem Health) with the same life span (2009–2011) the group is largely relying on the practical work and results achieved within this project (see report section on the BEAST project).

Biological effects research in the Baltic Sea has increased significantly, especially during the past decade in amount and geographical coverage. This is important not only for the development of integrated chemical-biological monitoring of hazardous substances but also to new strategies in assessing ecosystem health in the Baltic Sea. Biological effects data (lysosomal membrane stability [general health indicator] and micronuclei [genotoxicity indicator] were already included in the HELCOM integrated thematic assessment of hazardous substances in the Baltic Sea (2010).

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/SGIMC) will be taken advantage of when developing a revision of the Baltic Sea monitoring strategy. The methodological background documents and ACs available concerning biological effects methods and parameters was agreed by the SGEH to be carefully examined in 2011 and modified as needed to be applied in the Baltic region.

With regard to the organisation and future work of Expert Groups under the SSGRSP, SGEH sees that organised collaboration especially between groups dealing with integrated ecosystem assessments in different regional sea areas (mainly WGIAB, WGHAME, WGNARS, WGEAWESS) is a key aspect to be developed, e.g. by forming a cluster of expert groups of the RSP; this would ensure that the basic approaches would be more-or-less consistent between the regions to achieve comparability of the assessments.

In the next meeting in spring 2011 SGEH will evaluate and report on Baltic Sea issues related to i) progress in the BEAST project; ii) development of background documents for biological effects methods; iii) development of assessment criteria for biological effects parameters in the Baltic region; iv) developments in MSFD related to the implementation of biological effects methods; v) list of biological effect techniques proposed to integrated monitoring and assessments; vi) planning of a project for the BONUS-169 call in 2011; vii) biological effects methods applied in ERAs, EIAs and "post-accident" studies; viii) biological effects of perfluorinated compounds; ix) examinations of effects of hazardous substances on biodiversity in the Baltic Sea; and x) fish diseases as an indicator of ecosystem health.

4.5.2 HELCOM CORESET project

(Presented by Kari Lehtonen, FI)

The target of the HELCOM CORESET project (http://www.helcom.fi/projects/on_going/en_GB/coreset/) 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 candidate core indicators for 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 has noted 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 HS 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, the 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. The Meeting invited the BEAST project experts to provide a justification and an estimate of the efforts needed for laboratory analyses of the prioritised indicators.

4.6 Update: Marine monitoring in Ireland

Michelle Giltrap (EI) was not in attendance at WGBEC this year but was able to update WGBEC by correspondence on the current marine monitoring activities.

The structure of the project is outlined in WGBEC report 2009. Tier I site sampling has been completed at 9 sites around the coast of Ireland. For Tier I, samples were

analysed for scope for growth, stress on stress, condition index, sediment toxicity testing and chemical analysis. Sediment toxicity testing included whole sediment tests with *Corophium volutator* and *Arenicola marina*, porewater and elutriate testing with *Tisbe battagliai* and *Skeletonema costatum* and the microtox test with *Vibrio fischeri*. Results from Tier I analysis informed the selection of sites for Tier II assessment which is in progress and involves analysis with a battery of biomarkers in mussels and fish, chemical analysis of water, tissue and sediment, fish and mussel histopathology, benthic monitoring, sediment bioassays and imposex/intersex analysis. Tier II biomarkers for mussels include metallothionein (MT), acetylcholinesterase (AChE), alkali labile phosphate (ALP) and comet assay. For fish, the biomarkers include CI, EROD, bile metabolites, vitellogenin, AChE, MT and comet assay. Natural reproductive cycles have been investigated for mussels from a control location on the west coast of Ireland with the use of the adipogranular scoring index/gonadal status and linked to the NR lysosomal membrane stability responses. Sampling for benthic monitoring is complete and sample analysis is currently in progress. Imposex analysis in dogwhelks will commence in April 2011. The final stages of the development of chemical methodologies for natural and synthetic steroid estrogens in water and biota are being refined at the Marine Institute, Galway. As well as Tier I/II site analysis, various caging studies have been conducted for the investigation of sewage related effects/chemical analysis in mussels. Passive samplers are currently being deployed at Tier II sites and results are to follow. A pilot *in vivo* exposure of mussels to ethinyles-tradiol has been performed and the system is now in place at the SATL. This study demonstrated a positive control for ALP biomarker in intertidal and subtidal mussels. Collaborations with the Galway and Mayo Institute of Technology (EPA funded), Athlone Institute of Technology and MI (STRIVE EPA funded) are ongoing.

Although a lot of the analyses are still to be finalized, and the data evaluated as a whole, some preliminary comments can be made in the light of the findings to date of these biomarkers in Irish estuaries.

- Reliable and unequivocal reference pristine and polluted sites were difficult to assign;
- Samples had to be collected at varying distances from putative pollution sources, which casts doubt on the validity of the calculated biomarker as an indicator of pollution status. Caging experiments may be a suggested alternative;
- Results could vary widely across biomarkers, both in accordance with, and contradicting, established pollution status of the locations;
- Biomarkers could be extremely sensitive to external forces (e.g. season, reproductive status etc), and, since the latter could not always be predicted with accuracy, it may be necessary to derive a correction factor;
- Other factors may interfere with the value obtained; there is a strong suggestion that parasite infestation of the control mussels could induce false positives.

Future Work

Continuation of selected biomarkers at Tier 2 sites annually and Tier 1 sites every 5 years or so. This will allow both the temporal (annual) variability of the biomarkers to be assessed and also provide a baseline for trend analysis.

A severe constraint is the imposition of a standard size: while this does reduce variability it limits the use of the biomarkers. Influence of size should be investigated with a view to producing a model of the response.

Similarly models should be developed to allow for sex, gonad condition, parasite prevalence etc.

Caging studies should be carried out to develop temporal and spatial models of mussel biomarker response.

Some consideration should be given to the development of biomarkers in equivalent species, which might be employed where mussels/dab/flounder are absent.

Development of guidelines for future monitoring following developments of best practice at SGIMC and in line with MSFD Descriptors.

4.7 Portugal

Presented by Lúcia Guilhermino (PT)

4.7.1 Biological effects monitoring in Portugal (marine species)

Summary

At least since 1996, there are monitoring programmes going on in Portugal based on biological effects of contaminants on marine species. In the last years, the number has been increasing and there are several published papers reporting data for diverse coastal areas and, as a whole, covering all the coast of Portugal, including the estuaries of main Rivers (Minho, Lima, Douro, Mondego, Tagus, Sado, Mira) and lagoons (ria de Aveiro and Ria Formosa). Data for estuaries of some smaller Rivers (e.g. Cávado) are also available. Some studies have been done also with open sea species. These studies have been performed mainly in the scope of research projects funded by the Portuguese Foundation for the Science and Technology, the European Commission as well as by other sources (e.g. Industry, government contracts, Universities, Research Centres and other).

In these monitoring programmes, several approaches have been used, namely: biological effects assessed directly in natural populations of native species, transplantation experiments, in situ assays, laboratory bioassays, and *in vitro* assays. In some cases, chemical analysis of environmental contaminants (e.g. PAHs, metals) in sediments, water and organisms; water quality variables (e.g. nitrates, nitrites, phosphates) and physico-chemical parameters (e.g. temperature, salinity, dissolved oxygen) have been also used. Furthermore, a considerable amount of data on primary production, structure and composition of communities, dynamics of key species also exists for some coastal areas and estuaries, some of them in the scope of national programmes including these descriptors (e.g. Water Framework Directive). The integration of relevant information from different methodologies, particularly combining ecological and ecotoxicological information may provide most important inside on the effects of environmental contamination on wild populations and ecosystems of the Portuguese coast.

Regarding monitoring programmes on biological effects of contaminants with marine species, Universities and Research Centres all over the country have been doing them in the scope of their single research strategies and projects. Some of the institutions that have been particularly active in this field are the Centre of Marine and Environmental Research (CIIMAR), the Institute of Biomedical Sciences of Abel Salazar

(ICBAS) and the Faculty of Sciences of the University of Porto; the Centre of Marine and Environmental research (CIMA) of the University of Algarve; the Centre of Environmental and Marine Studies (CESAM) and the Department of Biology of the University of Aveiro; and the University Nova de Lisboa. More recently, research groups of other institutions, such as the Institute of Oceanography of the University of Lisbon, among others, have been also doing monitoring studies based on biological effects of contaminants with marine species.

4.7.2 Monitoring based on biological effects of contaminants in the NW coast and other selected areas

CIIMAR and ICBAS of the University of Porto started the first monitoring studies based on biological effects of environmental contaminants in the 1990s taking advantage of the experience in ecotoxicology, toxicology and ecology of some research groups. Since then, several monitoring studies in the NW coast have been conducted, some of them using integrated approaches combining biological effects at different levels of biological organization (e.g. enzymes, condition indexes, population level responses), chemical analysis (in organisms, water and/or sediments), physico-chemical measurements (e.g. temperature, water dissolved oxygen, salinity, conductivity) and water quality parameters (e.g. chlorophyll, nitrates, nitrites, phosphates). Some examples of the monitoring studies on biological effects of contaminants that have been done in the NW and other areas of the Portuguese coast by CIIMAR and ICBAS are indicated in Table 4.7.2.1 Some of the studies were done in cooperation with other research institutions (e.g. CESAM, University of Coimbra, University Nova de Lisboa), and it should be worknoted that the table is not a compilation of all the studies done.

Table 4.7.2.1. Examples of monitoring studies on biological effects of contaminants done (some still going on) in the NW coast of Portugal (in some cases, including also other areas of the Portuguese coast) with marine species.

SPECIES	AREA/SITES/type of study	PARAMETERS	SOURCE
ALGAE			
<i>Fucus ceranoides</i> , <i>Fucus spiralis</i> (var <i>platycarpus</i> ; <i>spiralis</i>) <i>Fucus vesiculosus</i> var. <i>vesiculosus</i>	- NW coast (11 sites different levels of historical contamination) - 1 time period (February 2003) Monitoring wild populations	- GST - Water Physic-chemical parameters - Hg concentrations algae available for September 2002	Cairrão <i>et al.</i> 2004. <i>Aquatic Toxicology</i> 70: 277–286. Cairrão <i>et al.</i> 2007. <i>Bulletin of Environmental Contamination and Toxicology</i> 79:388–395
INVERTEBRATES			
<i>Mytilus galloprovincialis</i>	NW coast (5–11 sites with different levels of historical contamination) 1 year seasonal monitoring in natural populations over decades at regular intervals (going	Started with AChE & GST Latter: AChE, GST, glutathione levels and ratio; GR, GPx, LPO, SOD, CAT, OCT, IDH	Moreira <i>et al.</i> 2004. <i>Ecotoxicology</i> 13: 619–630. Moreira <i>et al.</i> 2005. <i>Environmental Monitoring and Assessment</i> 105: 309–325.

	on) Integrated monitoring in some periods (e.g. 2002/2003; 2005), including chemical analysis in tissues, physico-chemical & water quality variables.	PAHs metabolites; In some sites also, lysosome stability, histopathology and others Data in relation to oil spills (Corel Bulker & Prestige)	Lima <i>et al.</i> 2007. <i>Chemosphere</i> 66 : 1230–1242. Tim-Tim <i>et al.</i> 2009. <i>Chemosphere</i> 77: 1465-1475. (related with Prestige spill)
<i>Nucella lapillus</i>	NW coast (7 sites with different levels of historical pollution) 1 year: Autumn 2002 – Spring 2003	Integrated monitoring: AChE GST Physico-chemical factors	Tim-Tim <i>et al.</i> 2009. <i>Chemosphere</i> 77: 1465-1475. (related with Prestige spill)
<i>Monodonta lineata</i>	NW coast (7 sites with different levels of historical pollution) 1 year: Autumn 2002 – Spring 2003	Integrated monitoring: AChE GST Physico-chemical factors	Tim-Tim <i>et al.</i> 2009. <i>Chemosphere</i> 77: 1465-1475. (related with Prestige spill)
<i>Paracentrotus lividus</i>	NW coast, 1 year (2003) seasonal sampling, 5 sites	AChE GST Physico-chemical factors	Cunha <i>et al.</i> , 2005. <i>Journal of environmental Monitoring</i> 7: 288-294
<i>Hedistes diversicolor</i>	1 sampling period 7 sites in Sado and Mira estuaries (South Portugal) In situ and laboratory assays based on biomarkers and feeding inhibition	AChE GST LDH Oxidative stress parameters (CAT, SOD, GPx, GR, total glutathione; GSH/GSSG ratio, LPO) Post-exposure feeding inhibition Physico-chemical parameters	Moreira <i>et al.</i> , 2006. <i>Aquatic Toxicology</i> 78: 186–201
<i>Cragon crangon</i>	1 year seasonal sampling (2002/2003) Estuaries NW coast and Aveiro lagoon	AChE GST LDH Physico-chemical parameters	Quintaneiro <i>et al.</i> 2006. <i>Marine Pollution Bulletin</i> 52: 1406-1413.
<i>Carcinus maenas</i>	1 year seasonal monitoring (to be repeat at regular intervals) Integrated monitoring: biological effects, water quality variables, physico-chemical parameters; chemicals in animals and sediments 2 estuaries of the nW coast: Minho and Lima Rivers	Condition indexes AChE GST LDH IDH PAHs metabolites in tissues Chitobiase Oxidative stress parameters: GR, GPx, glutathione; LPO	Not yet published

FISH			
<i>Anguilla anguilla</i>	<p>1 year seasonal monitoring repeated periodically (going on)</p> <p>Integrated monitoring: biological effects, water quality variables, physico-chemical parameters;</p> <p>Glass and yellow eels</p>	<p>Condition indexes</p> <p>AChE</p> <p>GST</p> <p>LDH</p> <p>Na⁺/K⁺-ATPase</p> <p>Oxidative stress parameters: GR, GPx, glutathione;</p> <p>LPO</p> <p>EROD</p>	<p>Guimarães <i>et al.</i> 2009. <i>Ecotoxicology</i> 18: 385-402.</p> <p>Gravato <i>et al.</i> 2010. <i>Ecotoxicology and Environmental Safety</i> 4: 524-533.</p>
<i>Lepidorhombus boscii</i>	<p>Integrated monitoring. Biological effects, chemical analysis (2006/2007) – 6 sites in the NW coast + 1 site Algarve coast.</p> <p>Other monitoring studies</p>	<p>CYP1A1 (EROD assay)</p> <p>Genotoxicity (MN and NA)</p> <p>Bile fluorescent PAHs metabolites</p> <p>PAHs (total PAHs and EPA's 16 priority PAHs) in mussel tissues and fish liver</p> <p>Heavy metals (Cr, Ni, Pb e V) in mussel tissues and fish muscle.</p>	<p>Lima <i>et al.</i> 2008. <i>Marine Environmental Research</i> 66: 189-199.</p> <p>Santos <i>et al.</i> 2010. <i>Chemosphere</i> 81: 685-691.</p>
<i>Sparus aurata</i>	<p>Lab bioassays to monitoring the contamination of sediments</p> <p>Several sites in the Sado estuary</p>	<p>AChE</p> <p>GST</p> <p>EROD</p> <p>LDH</p>	<p>Cunha <i>et al.</i> 2007. <i>Environmental Toxicology and Chemistry</i> 26: 444-453.</p>
<i>Dicentrarchus labrax</i>	<p>Seasonal integrated monitoring biological effects, water quality variables, physico-chemical parameters;</p> <p>started 2010, going on</p> <p>estuaries of Rivers Minho, Lima and Mondego</p>	<p>Condition indexes</p> <p>AChE</p> <p>GST</p> <p>LDH</p> <p>Na⁺/K⁺-ATPase</p> <p>Oxidative stress parameters: GR, GPx, glutathione;</p> <p>LPO</p> <p>EROD</p>	<p>Not yet published</p>
<i>Pomatoschistus microps</i>	<p>Seasonal integrated monitoring: biological effects, water quality variables, physico-chemical parameters, chemical analysis tissues and sediments</p> <p>started 2006, going on</p> <p>estuaries of Rivers Minho, Lima, Cávado and Douro; Aveiro lagoon</p>	<p>Condition indexes</p> <p>AChE</p> <p>GST</p> <p>LDH</p> <p>IDH</p> <p>Oxidative stress parameters: GR, GPx, glutathione;</p> <p>LPO</p> <p>EROD</p>	<p>Monteiro, 2007. <i>Chemosphere</i> 66: 514-522.</p> <p>More recent results not yet published in the form of full paper</p>

4.7.3 Biomarker levels in the South coast

The Centre of Marine and Environmental Research (CIMA) of the University of Algarve, have studied since 1996, different kinds of biological effects as a result of contaminant exposure, namely biomarkers of susceptibility (SOD; CAT, GPX, GST), biomarkers of exposure (metallothioneins, ALP), and biomarkers of effect (CYP450, ALAD, AChE, DNA damage and lipid peroxidation) in different species: bivalve molluscs (*Mytilus galloprovincialis*, *Ruditapes decussatus*, *Scrobicularia plana*), crustaceans (*Carcinus maenas*) and fish (*Solea senegalensis*, *Dicentrarchus labrax*, *Lepidorhombus boscii* and *Trisopterus luscus*).

Mussels *Mytilus galloprovincialis* were collected at eight sites of the South Portuguese Coast (Figure 4.7.3.1) or transplanted and biomarkers of susceptibility (SOD, catalase, GPx, GST), exposure (metallothioneins, ALP) and effect (CYP450, ALAD and Acetylcholinesterase, DNA damage and lipid peroxidation) were followed long with metals (Cd, Cu, Cr, Ni, Pb, Zn) and PAHs analysed in mussels whole soft tissues or specific tissues (For results see Bebianno & Machado, 1997; Bebianno *et al.*, 2007; Maria *et al.*, 2009b; Cravo *et al.*, 2010, Company *et al.*, 2011, and other references below). Furthermore biomarkers have been used to assess the quality of the environment throughout the use of IBR index and Expert System developed by Danino *et al.*, 2007.

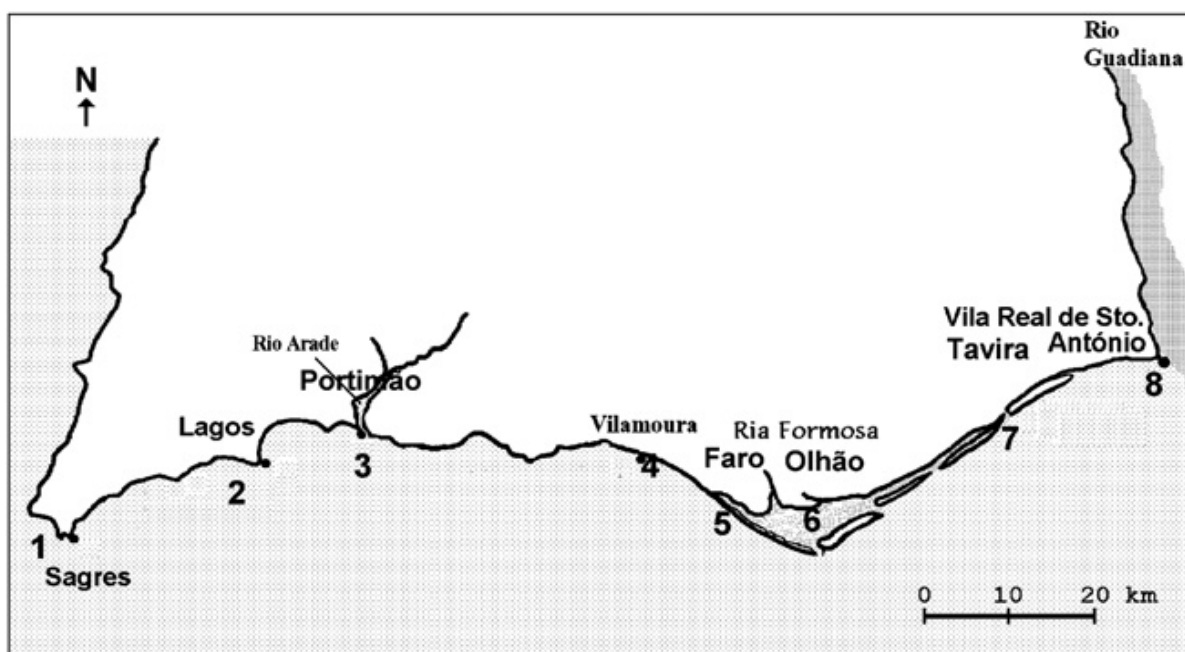


Figure 4.7.3.1. Mussels sampling sites along the South coast of Portugal.

A similar approach has been applied, since 1996 in the Ria Formosa Lagoon, where the same biomarkers were measured in the clam *Ruditapes decussatus* and compared with contaminants levels (Serafim & Bebianno, 2001; Bebianno & Serafim, 2003; G  ret *et al.*, 2003; Bebianno & Barreira, 2009). Similarly Biomarkers were also analysed in the crab *Carcinus maenas* to assess the biological effects of contaminants present in the same lagoon at different levels of biological organization (Maria *et al.*, 2009c).

Furthermore *Scrobicularia plana* and *Nereis diversicolor* have been collected from different sites of Arade Guadiana rivers in the South Coast of Portugal and biomarkers of susceptibility (SOD, CAT, GPX, GST), exposure (ALAD, AChE, MTs, ALP) and effect

(DNA damage and Lipid peroxidation) have been analysed in tissues and related with metal levels. Moreover, intersex have been identified in *Scrobicularia plana* reflecting to the presence of endocrine disruptor compounds in these systems. Gomes *et al.*, 2009; Gomes *et al.*, submitted).

In addition imposex have been identified in the whole Portuguese coast and particularly in the South Coast of Portugal putting in evidence that even after TBT ban the situation have not improved (Barroso *et al.*, 2002).

Recently the same biomarkers have also been analysed in fish namely the sole *Solea senegalensis* from the Tagus estuary (Fonseca *et al.*, 2009), *Dicentrarchus labrax* from the Arade River (Fernandes *et al.*, 2007, 2009), Ria de Aveiro Lagoon (Maria *et al.*, 2009a), four-spotted megrim (*Lepidorhombus bosci*) and the pouting (*Trisopterus luscus*) from the Northern Iberian Shelf (Fernandes *et al.*, 2008a,b).

4.7.4 Conclusions

There is already a considerable amount of data available on biological effects of environmental contaminants for the Portuguese coast as a result of monitoring programs and other complementary studies, such as in situ assays, laboratorial bioassays, in vitro assays, genetic and other molecular studies, etc. The most part of these studies have been done by initiative of individual research groups that did not received specific funds for monitoring (the work being done as a complementary part of the research conducted for other purposes). The members of the scientific community in Portugal working on biological effects of environmental contaminants hope that this situation may change in the future, particularly in relation to the Marine Strategy Framework Directive, since there is a considerable amount of expertise that can be used to generate new scientific knowledge and improve our understanding on the long-term effects of environmental contaminants, which are even more important in the actual scenarios of global climate changes, simultaneously contributing to the appropriate answer that Portugal should provide in the scope of the international compromises assumed.

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4.8 UK Coordinated Environmental Monitoring Programme

(Thomas Maes, UK)

In an attempt to streamline marine monitoring, to become more cost effective and to move forwards towards GES monitoring the UK monitoring programme is being revised to consider the following points:

- Decreasing the number of Research Vessel days at sea;
- Move to a 2 yearly rolling programme;
- Developing a risk based approach;
- Develop an inshore programme including estuaries, where contaminants are higher than off shore but effect measurements are currently limited;
- Offshore, focus on hot-spot locations and SSSI's;
- Conduct the same amount of sampling annually, which means much wider spatial coverage in a region;
- Bring coastal chemical-bio effects monitoring programme under CEMP to better integrate and bridge gap between inshore and offshore data sets;
- Link coastal CSEMP programme to existing inshore sampling (maximise efficiency and reduce field based sampling costs);
- Collect additional samples for contaminant /effects monitoring on fisheries cruises;
- Model the environmental fate of certain contaminants;
- Integrate passive samplers into monitoring strategy.

Thomas Maes (UK) presented the UK's aspirations for a more cost effective monitoring programme that could be effective in an era of reduced monitoring budget and demonstrated how the approach could also address the desired characteristics above.

4.9 National monitoring activity summary

WGBEC greatly values receiving national monitoring reports and noted that there was a lot of ongoing activity in support of HELCOM, OSPAR and WFD drivers. It was noted that much recent activity would be to support initial assessments for Descriptor 8 of the Marine Strategy Framework Directive and that some countries were already developing the thinking to alter monitoring programmes to gather the evidence required for determining good Environmental Status for Descriptor 8.

5 Review progress with the ICON (NSHEALTH) and Baltic BEAST programme; (ToR f)

5.1 Progress with ICON (NSHEALTH)

Ketil Hylland (NO) provided an update on progress with the ICON (Integrated Assessment of Contaminant Impacts on the North Sea) project. The steering group for the project is Ketil Hylland (Chair, Norway), Thomas Lang (Germany), Alistair McIntosh and Matt Gubbins (Scotland), Dick Vethaak (Netherlands), John Thain (England), Jörundur Svavarsson (Iceland). Additional members of the steering group have been Concepcion Martinez-Gomez (Spain) and Thierry Burgeot (France). There was a steering group meeting at ASC in Nantes 2010.

The main objective of ICON, a practical workshop, is to provide a demonstration programme for the framework developed through the OSPAR/ICES WKIMON proc-

ess (integrated chemical and biological monitoring). In addition the programme will allow the assessment of effects of contaminants over a range of North Sea, Icelandic and Mediterranean habitats and provide the opportunity to develop research topics and improve the underpinning science. The project was initiated by a kick-off meeting spring 2007: subsequently, samples have been collected during cruises and sampling campaigns in 2008 (all offshore locations, some inshore) and 2009 (additional inshore locations, including Iceland and UK). Analyses are just about finalised at all participating laboratories and are currently being assimilated in a database at the University of Oslo (Tables 5.1.1, 5.1.2, 5.1.3 and 5.1.4). Although there are a fixed number of locations for each (e.g. 12 for dab, 4 for haddock), the data matrix is not complete as some analyses have only been done on material from selected locations.

Table 5.1.1. Status for analyses for dab (*Limanda limanda*).

determinand	lab(s) involved	completed
chemistry	M Scotland	x
liver nodules	vTI/Cefas	x
liver histopathology	vTI/Cefas	x
external fish disease	vTI/Cefas	x
PAH-metabolites	vTI/MS/IFREMER	x
EROD	Marine Scotland	x
vitellogenin	U Le Havre	in progress
lysosomal stability (tissue)	AWI	x
DNA adducts	IFREMER/UiO	x
AChE	IEO, IFREMER	x
micronucleus	University of Vilnius	x
Comet	UiO, IFREMER	x
oxidative stress	University of Gothenburg	x

Table 5.1.2. Status for analyses for flounder (*Platichthys flesus*).

determinand	lab(s) involved	completed
chemistry	Marine Scotland	x
liver nodules	vTI/Cefas	x
liver histopathology	vTi/Cefas	x
external fish disease	vTI/Cefas	x
PAH-metabolites	vTI/IFREMER/M Scotland	x

EROD/CYP1A	MS, IFREMER	x
vitellogenin	U Le Havre	x
lysosomal stability (tissue)	AWI	
DNA adducts	IFREMER/UiO	x
AChE	IEO, IFREMER	x
oxidative stress	University of Gothenburg	x

Table 5.1.3. Status for analyses for haddock (*Melanogrammus aeglefinus*).

determinand	lab(s) involved	completed
chemistry	M Scotland	?
internal fish disease	vTI/Cefas	x
external fish disease	vTi/Cefas	x
PAH-metabolites	vTI/Cefas	x
EROD/CYP1A	MS	?
vitellogenin	UiO	not done
lysosomal stability (tissue)	?	?
DNA adducts	UiO	not done
AChE	IEO	x
gene expression	UiO	x
Comet	UiO	x
oxidative stress	University of Gothenburg	x

Table 5.1.4. Status for analyses for mussel (*Mytilus edulis* or *Mytilus galloprovincialis*).

determinand	lab(s) involved	completed
chemistry	M Scotland	?
scope for growth	IEO	Med
histopathology	UB/Cefas	x
lysosomal stability (NRR)	IEO/Cefas/IFREMER/++	x
lysosomal stability (tissue)	UB	Med
micronucleus	UiO/Cefas/IEO	
AChE	IEO/IFREMER	x
Comet	Cefas	x
stress on stress	IEO/IFREMER/Cefas	x
condition index	IEO/Cefas	x

In addition to the above, there are data for sediment chemistry and toxicity (bioassays) as well as bioavailability (using passive samplers).

It was hoped the data would be available for SGIMC 2011 to allow for the option of doing a preliminary assessment. A wrap-up conference is planned for late June 2011, possibly at EEA in Copenhagen, with half a day presentation for environmental managers and shareholders, as well as a full day for research presentations. Scientific papers from ICON will be published in a special volume of a selected journal, possibly Marine Pollution Bulletin (options are being investigated).

5.2 Progress in the BONUS+ BEAST project

Progress report by Kari Lehtonen (FI). During 2010, the BONUS+ BEAST project (2009–2011, www.environment.fi/syke/beast) fulfilled all of its milestones and deliverables planned for each of the WPs and tasks (subregions). 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, http://www.helcom.fi/publications/en_GB/publications/) concerning especially the "Biological Effects" chapter of the assessment. Related work initiated later in 2010 in the HELCOM CORESET project (http://www.helcom.fi/projects/on_going/en_GB/coreset/) Hazardous Substances component is also strongly supported by participation and inputs from several BEAST partners. Importantly, BEAST was nominated as one of the Flagship projects in the EU Strategy for the Baltic Sea Region (EUSBSR) Priority Area 3. This recognition signifies improved opportunities in receiving further funding for future BEAST activities, most immediately from the 4th call of the EU BSR Programme (BSRP) closing in March 2011.

WP 1: Field studies and experiments in selected subregions of the Baltic Sea

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, G. of Gdansk, G. of Riga, G. of Bothnia and G. 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. subcellular, cell, tissue, organ and whole organism, and represent lower-order and higher-order responses, reflecting different degrees of ecological relevance.

Four major field campaigns were successfully performed during 2010 in the Gulf of Bothnia, Gulf of Finland, Gulf of Gdansk and Belt Sea using the research vessels Walther Herwig III (DE), Aranda (FI) KBV005 (SE) and Oceania (PL). Several experimental studies were also carried out according to the project study plan.

WP 2: Application and validation of methods in monitoring and assessment in the Baltic Sea

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 and the results of the integrated data assessment (WP 3 task). The field sampling programme designed in 2009 in collaboration with WP 1 and the regional Task Leaders for the five Baltic Sea subregions under study was applied successfully.

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 and the draft handbook has been uploaded to the BEAST Central Desktop and is constantly updated with so far missing information. 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 activities and intercalibration exercises took place: 1) training and intercalibration of methods for field sampling of biomarkers and fish disease studies, 2) intercalibration exercise on measurement of PAH metabolites in fish bile, and 3) intercalibration of measurements on histochemical biomarkers). Plans have been made for further activities scheduled for 2011 and for possible follow-up activities after the end of BEAST.

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

Set-up and maintenance of the BEAST database (BonusHAZ) was 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, data have been added concerning biological effects measurements in eelpout (120 stations) and blue mussels (60 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 the Weight of Evidence approach. As a contribution to the HELCOM HAZAS, the "traffic light approach" was applied, using different biomarkers and indicators for reproductive disorders or prevalence of certain fish diseases, respectively.

In collaboration with the 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.

Action 5.2: That ICES WGBEC reviews status of integrated assessments from ICON and BONUS BEAST / SGEH and reviews the activity of relevant WGs on integrated assessments (ICES ICG-MSFD and WGHAME) at their 2012 meeting.

6 Evaluate potential for collaboration with other EGs in relation to the ICES Science Plan and report on how such cooperation has been achieved in practical terms (e.g. joint meetings, back-to-back meetings, communication between EG chairs, having representatives from own EG attend other EG meetings); (ToR i)

6.1 Background

This item was given to WGBEC in its ToR in 2010. At that meeting WGBEC examined its core business, how it fitted in with the ICES Science Plan and its potential to collaborate with other EGs within SSGHIE and other EGs outside of SSGHIE. An overview is given in Table 6.1.1.

Table 6.1.1. Overview of EGs (plus MEDPOL) with which WGBEC has had collaboration of with which WGBEC would envisage possible future interactions.

	Worked before?	Interested in joint activity?	Joint meeting?
WGPDMO	Yes	Yes	Yes
MCWG	Yes	Yes	Potential
WGMS	Yes	Yes	Potential
MPCZM	No	Potential	No
SGONS	No	No	No
WGMASC	No	No	No
WGEIM	No	Yes	Potential
WGHABD	No	Potential	No
WGEXT	No	No	No
WGFCCIFS	No	No	No
WGAGFM	Yes	Yes	Potential
WGEEL	No	Yes	Potential
WGMME	No	Yes	No
SGIMC	Yes	Yes	No
SGEH	No	Yes	Potential
MEDPOL	Yes	Yes	Yes

WGBEC were aware that the ToR given for the 2011 meeting, had been given to all EGs.

To further investigate the potential for active collaboration between EGs, WGBEC had written to the chairs of the above EGs in the table with the exception of SGONS, WGMASC, WGEXT and WGFCCIFS (no potential for collaboration was identified for these groups). Accompanying the letter was an excerpt from the WGBEC 2010 meeting report on this subject. In addition WGBEC also contacted the chair of WKMAL (Workshop on litter).

At the time of this meeting seven responses had been received.

6.2 Response from WGEEL: chairs Russell Poole and Claude Belpaire

To quote: "I'm sure there would be a very positive collaboration between the eel quality subgroup and the WGBEC. Claude Belpaire has led the eel quality initiative for the past decade or so. One of the big gaps in our knowledge is to assign an (potential) impact on the individual eel and its possible success as a spawner from dif-

ferent types, cocktails and levels of contaminants and to build this into our local stock assessments.

WGBEC could potentially be a big help to increase new and broader expert input in our eel quality discussions. The subgroup eel quality is a quite restricted one, with only 5 to 6 experts sharing between contaminant stuff and eel pathology (parasites and viral diseases). So new input will without doubt guarantee quicker advancement. As to how to collaborate.....I am not sure what back-to-back meetings mean, but having representatives from own EG attend other EG meetings seems to be quite feasible. Both directions are possible I think (one or more WG Eel experts attending WGBEC (which could include an agenda item on BEC on Eel), or vice versa WGBEC experts attending the Eel Quality Subgroup within WG Eel)."

WGBEC had discussed contaminants and eels at its meetings in 2009 and 2010. The group felt that it should pursue collaboration along the lines suggested by WGEEL, perhaps initially by correspondence with the eel quality sub group. Members of WGBEC identified to collaborate were John Thain, Jim Readman +++

Action 6.2: Chair to follow collaboration with WGEEL.

6.3 Response from WKMAL: chair Francois Galgani

To quote: "Happy that WGBEC will consider contaminant related to litter . I know some laboratories now engaged in that field trying to evaluate the "real risks" in term of ecotoxicology. There is also a technical group (GES-TSG) that i am chairing (together with G hank and S Werner and with T Maes as member)for the implementation of MSFD and ICES is now waiting for some recommendations from that group. Then they will probably go further organizing group/activities as we recommended them to organize a technical/scientific workshop on the specific microplastic issue. This could be a way to link with WGBEC?"

WGBEC had discussed aspects of contaminants and litter at its meeting in 2009, 2010 and this year. The group wanted to be involved in this work area and felt that it could contribute to the scientific of litter contaminant related effects. At this moment in time WKMAL was a one-off workshop and its continuation in whatever format had not been resolved. Therefore it was agreed that T Maes who attends both groups would act as a conduit between the two groups.

Action 6.3: Chair to write to WKMAL to propose that T Maes coordinates any contaminant related topics between the two groups.

6.4 Response from WGMME: chair Sinéad Murphy

To quote: "The WGMME would definitely be interested in cooperating with the WGBEC. In last year's WGMME report we reviewed the current contaminant loads reported in marine mammals in the ICES area, the cause-effect relationships between contaminants and health status, and the population-level effects of environmental impacts. I have cc'd Paul Jepson to this email, as he lead the subgroup that carried out the review. Unfortunately we do not have a contaminants ToR this year, but during out next meeting (on 21st Feb) we can discuss "subjects of mutual interest between our groups" etc., and the possibility of a joint meeting, or a member of the WGMME attending your meeting?"

WGBEC had in previous meetings had presentation on biomarker response in marine mammals in relation to contaminants. This is a clear area for collaboration.

Action 6.4: Chair to contact WGMME to seek potential areas for mutual interest after their meeting on 21 Feb. and also to obtain a copy of the review , ‘the cause–effect relationships between contaminants and health status, and the population-level effects of environmental impacts’ and to circulate to WGBEC group.

6.5 Response from MCWG: co-chair Katrin Vorkamp

To quote: “Thanks for getting in touch and following up on our good resolutions at Nantes, of more communication between EG chairs. Obviously, there is great potential for cooperation between WGBEC and MCWG, and we have already benefitted from WGBEC expertise, e.g. with regard to bioassays for dioxin analysis.

For our next meeting (28 Feb - 4 March) we will have an agenda point on integrated monitoring (“Contribute to ICES activities on integrated chemical and biological effects monitoring and review new information on effect directed chemical analysis”) which I believe has also been (or will be?) addressed by WGBEC? I have not received much input on this so far - we will have an update on the Belgian INRAM project (see attached) and Jocelyne Hellou of Fisheries and Oceans Canada has sent two interesting publications. Besides, Michiel Kotterman of IMARES is going to present some results on contaminant concentrations and effects in eel.

I will be happy to keep you informed about our discussions on integrated monitoring - if WGBEC has some direct input, this will be very welcome as well. I will also attach our terms of reference so you can see for yourselves if there are other areas that might be of interest to WGBEC.”

Action 6.5: Chair to forward manuscript on Extraction procedures to MCWG for review at their meeting.

6.6 Response from SGEH: chair Kari Lehtonen

To quote: “SGEH is now running its final year and the future of the group is yet unclear, it would be useful to hear WGBEC’s ideas and suggestions of how the work of SGEH could be continued and what kind of collaborations between the two groups could be achieved (if SGEH activities will be continued in some form, that is).”

The chair of SGEH is a member of WGBEC and presented the outputs from SGEH at the 2011 meeting. WGBEC have always supported and collaborated with SGEH and should this group continue then WGBEC would endeavour to continue dialogue and close collaboration, this is important and necessary for both groups.

Action 6.6: Chair to keep in contact with outputs of SGEH and liaise as appropriate and any subsequent group that is formed.

6.7 Response from SGIMC: chairs Ian Davies and Dick Vethaak

The chair Ian Davies had responded by thanking WGBEC for its valuable support over the past seven years (WKIMON and SGIMC). Many of WGBEC members were also members of SGIMC and therefore close collaboration was easily achievable and necessary.

Action 6.7: Provide documents as requested by SGIMC for their meeting on 14 March 2011 for WGBEC members to participate in the process and review output in 2012.

6.8 Response from MEDPOL: Michael Angelides, UNEP

WGBEC had collaborated with MEDPOL biological effects activities over the past four years during its WG meetings and intersessionally in workshops. The areas of

common interest were harmonisation of biological effects techniques, methods, assessment of data and AQC activities. M Angelides was supportive of these activities continuing.

Action 6.8: Continue the collaboration already established, particularly such activities becomes more important with the EU MSFD implementation. The chair to maintain contact and pursue areas of mutual interest and inclusion on WGBEC agenda.

6.9 Response from WGMS: co-chair Lucía Viñas

To quote: “I can tell you that in our last year’s report action list we put “Contact the chairs of WGBEC and MCWG to investigate collaboration on the use of passive samplers in the marine environment”, so that could be an issue in which we could work together.

Anyway, as we are going to meet in two weeks in Aberdeen if WGBEC has any proposal for collaboration we can discuss it at the meeting.”

WGBEC had recently noted the item on passive samplers in the WGMS ToR, and of particular interest was the association made on the use of passive samplers with biological effects. WGBEC in the past (2005) had communicated with WGMS on the topic of passive samplers. The group felt that it would be useful to re-establish contact on this topic; firstly passive sampler technology has become well established over the past six years and secondly, some group members are involved in combined passive sampler – mussel biological effect monitoring studies. WGBEC would consider this at its 2012 meeting and try to develop a strategy/ approach/ guidelines for combined biological effect passive sampler monitoring.

In addition WGBEC have recently produced a draft on extraction micro-scale bioassay techniques which include sediment extractions. WGBEC will pass this on to WGMS for comment.

It was noted that the topic of passive samplers and their use with biological effect techniques in monitoring programmes may also be of use to MCWG, and the chair would communicate with MCWG in this respect.

Action 6.9: Chair to communicate with WGMS on collaboration on common agenda item on passive sampling and its integration into integrated monitoring. Chair to send WGMS TIMES draft on sediment extracts for bioassays.

6.10 Response from other EGs

At the time of the meeting (14 February 2011) WGBEC had yet to receive comments from the remaining EGs listed in the above table. It is anticipated that responses may be received following the 2011 annual meetings of these groups.

Action 6.10: Chair to reestablish contact with these groups.

In addition it was identified under agenda item 4, that there would be value in communicating with the Working group on Crangon (WGCRAN) to consider recent developments in understanding pollution effects on this species in the North Sea (see Action 4.4).

WGBEC chairs will pursue the actions identified above by contacting the relevant chairs and communicate outcomes to ICES chair of SSGHIE.

7 View the output from SGIMC 2010 and respond to their request for support; (ToR h)

- a) Extraction procedures for bioassay methods
- b) Intersex in fish
- c) Background document on supporting parameters
- d) Acetylcholinesterase
- e) Mussel histology
- f) Micronucleus and comet assay
- g) In vitro YES/YAS, ER CALUX assays
- h) Sediment and elutriate bioassays for invert bioassays
- i) Sediment and elutriate bioassays with copepods
- j) Update whole sediment bioassay AC

7.1 Background

WGBEC has had close links with SGIMC (previously WKIMON) over the past seven years and have provided advice and taken on tasks to support the work and output of SGIMC. At its 2010 meeting SGIMC provided a work plan in their report and requested support in a number of areas, these are detailed below:

Effect	Task	Responsible member	When	Report to	Status January 2010
1. Extraction procedures for bioassay methods	Complete TIMES series method document	Dick Vethaak + John Thain	Imminent	WGBEC 2011	In progress, to be reviewed at SGIMC 2011
2. Intersex in fish	Review Background document	Steve Feist	WGBEC 2011	SGIMC 2011	To be reviewed at SGIMC 2011
3. Background document on Supporting parameters in fish: condition indices, SLI and SGI	develop BD	John Thain, Dick Vethaak	WGBEC 2011	SGIMC 2011	SGIMC 2011
4. Acetyl cholinesterase	To develop Background Response assessment criteria	Thierry Burgeot	WGBEC 2011	SGIMC 2011	in progress
5. Mussel histopathology	ICES Times manuscript including BAC in preparation	Steve Feist + Miren Cajaraville	WGBEC 2011, WGPDMO 2011	SGIMC 2011	will be send to SGIMC 2011
6. Micronucleus assay + comet assay	Background document and draft BAC	Brett Lyon	WGBEC2011	SGIMC 2011	Comet assay completed; micronucl. assay completed
7. New Chapter	In vitros YES/YAS, ER CALUX	JT / DV	WGBEC 2011	SGIMC	in progress

8. Chapter 8	Add Sed & SW elutriate bioassays for invert embryos. Further validate others as more data becomes available	Ricardo Beiras	2010	WGBEC 2011	To be reviewed at SGIMC 2011
9. Chapter 9	As above with copepods	As above	As above	WGBEC 2011	To be reviewed at SGIMC 2011
10. Chapter 10	Update BG and ass cri for Whole sediments with amphipods as more data becomes available	As above	As above	WGBEC 2011	To be reviewed at SGIMC 2011

During discussion it was noted that the SGIMC process and workplan was nearing completion and should be finalised in 2011. Following cessation of SGIMC it was proposed that WGBEC could take on the role of reviewing assessment criteria and reviewing national applications of the integrated assessment framework to monitoring data. It was felt that this role would be made significantly easier if the assessment criteria documentation was in a single location, rather than spread across background documents.

7.2 Progress on each task was reported as follows

a) **Extraction procedures for bioassay methods**; completed but some additional references to be added to the text (Annex 5).

b) **Intersex in fish**; (Annex 6) The document provides a useful review of the relevant methods. It was noted that this is a condition that affects male fish (ovotestis) and that there are large differences in the sensitivity of different species. The document mentions natural factors that may give rise to the development of intersex in some species, e.g. temperature. It is clearly important to be able to separate such factors from contaminant effects. Another unresolved issue, not touched upon in the document, is the fact that intersex is determined in mature fish, but the development of the condition (and presumably exposure to contaminants causing it) is thought to happen during early development. The document appropriately separates between individual and population effects, indicating 5% intersex in fish populations (dab, flounder) as an appropriate BAC. For this purpose any level of effect would be included. Derivation of an EAC could use the same prevalence (5%), but a higher level of effect in the affected individuals. There is a need for further clarification.

c) **Background document on supporting parameters**; completed (Annex 7).

d) **Acetylcholinesterase**; document completed, however it was identified that background assessment criteria needed changing to 90% ile of reference values to match the approach taken for other biological effects measurements. This was actioned by Thierry Burgeot by March (Annex 8).

e) **Mussel histology**; a manuscript was tabled at the meeting but was incomplete in some aspects. Some attention is needed particularly for the development of assessment criteria. This was addressed, post-meeting by the authors in time for the SGIMC meeting. (Annex 9).

f) **Micronucleus and Comet assay**; document completed, but needs further revision by authors. This was completed at SGIMC in March (Annex 10).

g) **In vitro YES/YAS, ER CALUX assays**; no progress had been made on the YES/YAS chapter. A document for review on DR LUC was presented, this was in lieu of a

DR/ER CALUX manuscript. There was some overlap between this and the “Extraction procedures for bioassay methods” see a) above. Dick and John to consider for duplication / completeness and send to MCWG.

h) **Sediment and elutriate bioassays for invert bioassays**; completed (Annex 11). Final edits required to the assessment criteria to be made by Ricardo Beiras (Spain) for SGIMC.

i) **Sediment and elutriate bioassays with copepods**; completed (Annex 12). To be finalised by amendments by Ricardo Beiras (Spain) for SGIMC.

j) **Update whole sediment bioassay AC**; completed (Annex 13). Finalise by amendments by Ricardo Beiras (Spain) for SGIMC.

Recommendation 7.1: That ICES/OSPAR SGIMC recommend to OSPAR that a background document on biological effects assessment criteria should be produced and that WGBEC be invited to update this annually as new data become available.

Recommendation 7.2: After the cessation of ICES/OSPAR SGIMC and ICES SGEH, WGBEC reviews background documentation, assessment criteria and applications of integrated assessment frameworks across the ICES area at its annual meetings.

8 Update on MSFD activities in relation to contaminants and biological effects; (ToR e)

During 2011 EU member states should be working towards defining Good Environmental Status for EU MSFD (Marine Strategy Framework Directive) Descriptor 8, which states that “concentrations of contaminants are at levels not giving rise to pollution effects.” This should be being done through the identification of appropriate targets and indicators at a national level for the Descriptor. In addition member states should be preparing initial assessments to be ready for 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.

Several members of WGBEC have been heavily involved in the initial JRC Task Group activities for Descriptor 8 and much of the guidance produced reflects the views of WGBEC on the potential value of application of biological effects monitoring data to determine whether GES is being met for Descriptor 8.

Following the completion of the 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.

WGBEC is of the view that the integrated monitoring approach devised for OSPAR by SGIMC in 2011 is the most appropriate approach available for determining GES for Descriptor 8 and recommends its adoption by member states.

National Status and Initiatives

In order to update the group on developments and to communicate how different countries are developing and defining Good Environmental Status (GES) with respect to biological effects, the chair invited representatives to describe the approach being

developed in each country, noting that the process was in the early stages of development.

8.1 United Kingdom

Presentation (Thomas Maes, UK): The development of indicators and targets for Good Ecological Status under the Marine Strategy Framework Directive

Concentration of contaminants

Indicator 8.1. Concentrations of contaminants in water, sediment or biota are not increasing and do not exceed environmental target levels identified on the basis of ecotoxicological data as outlined within community legislation and other obligatory agreements (such as OSPAR).

Concentrations of substances identified within relevant legislation and international obligations are below the concentrations at which adverse effects are likely to occur (e.g. EQSs within WFD; EACs within OSPAR).

Effects of contaminants

Indicator 8.2. Biological effects of contaminants are below environmental target levels considered to result in harm at organism, population, community and ecosystem levels as outlined within community legislation and other obligatory agreements.

The intensity of biological or ecological effects due to contaminants is below the toxicologically-based standards established by combined ICES/OSPAR processes within WGBEC and SGIMC.

Biological effect responses should fall below the “high and cause for concern” level as defined by ICES/OSPAR (ICES, 2009, 2010).

Pollution events

Indicator 8.3. Occurrence, origin (where possible), extent of significant acute pollution events (e.g. slicks from oil and oil products) and their impact on biota physically affected by this pollution.

As a wide range of oils and chemicals may be spilled, targets will be incident-specific and they will need to be derived at the time. Similarly, the monitoring to be undertaken will be incident- and location-specific, depending on the material spilt and the species, habitats and resources at risk.

Data on discharges, emissions and spills from offshore oil and gas installations are already reported to OSPAR. The environmental status of affected waters may be influenced by significant pollution incidents, but the scale of the impact(s) and the spatial and temporal scale of the pollution will determine whether GES at a sub-regional level (Greater North Sea or Celtic Seas for the UK) is compromised as a result of the incident.

There is no relevant target on the occurrence, origin and extent of acute pollution events at the current time, and further work is needed to develop appropriate measures.

The initial assessment will be based on the information from Charting Progress 2 and available monitoring programmes.

The need to coordinate implementation at a regional seas level is explicit within the MSFD and it is through OSPAR that this coordination will take place. At the recent

meeting of the OSPAR Coordination Group (CoG) the tight timescales for implementation of the Directive by Contracting Parties was recognised and as a result the green light was given for the creation of a new Intersessional Correspondence group on the MSFD (ICG-MSFD).

This new group provides a greater degree of flexibility for Contracting Parties to work together to find common approaches to characterising GES and establishing targets and indicators. The group has met formally once which resulted in useful and pragmatic discussions on implementation. It is hoped that the UK can use the ICG-MSFD to drive forward the process and ensure our assessment of GES across the North East Atlantic is comparable and our targets are set in a coordinated way.

Additional technical development work is continuing within each of the Committees and their respective ICG's with a recent meeting of the ICG-Marine Litter taking place during November.

(<http://www.defra.gov.uk/environment/marine/documents/interim2/201012-msfd-news.pdf>)

8.2 Spain

Spanish waters have been split into 5 distinct maritime boundaries, where two different monitoring programmes are being conducted (CEMP and MED POL). CEMP programme in the Spanish Atlantic coast is developed in mussels by rolling a regional scale spatial biomonitoring programme every three years, and fish and sediments surveys are being conducted, so far, annually. The selected biomarker techniques in the North-Atlantic boundaries considered for the GES are scope for growth (SFG) in mussels, bioassays (SET) and GST, while EROD in red mullet, Lysosomal Membrane Stability (LMS), AChE activity and Stress on Stress (SoS) in mussels are being considered to be used in the Mediterranean districts. Spanish Mediterranean monitoring programme will evolve to a rolling programme for fish and sediment sampling (every two years). Data from existing mussel networks (yearly monitoring programmes) in both North-Atlantic and Mediterranean Spanish regions will be considered in GES, but in future they may be deployed at stations with datasets of 5 year or more. All the current programmes only cover coastal areas and inner/ mid continental shelf and there is no offshore monitoring (depths > 120m). Radionuclide measurements are not included as a measurement in Spanish mussel networks and MEDPOL mussel watch data available will be used to strengthen the initial assessment.

8.3 France

The initial assessment will be based on available knowledge from existing databases containing time series and published information from running programmes. The development of targets is ongoing with consultation of stakeholders at the final stage. The definition of Good Environmental Status (GES) will be based on the indicators and recommendations of the Task Group 8 (TG8) Report. In relation to the monitoring of radionuclides, France feels that this is already covered by other existing legislation and thus will not consider further monitoring under the MSFD.

8.4 Finland

The WGBEC member (Kari Lehtonen) is not directly involved with the MSFD. Not much information available, the process is delayed and the European Commission

are aware of this. The results of the integrated assessment of the BEAST project could maybe be used for the initial assessment.

8.5 Belgium

The WGBEC member and his institute (ILVO) are not involved in the process at this stage. The organisation and coordination has been run by another institute: the Mathematic Unit of the North Sea (MUMM).

8.6 The Netherlands

The current monitoring programmes cover biomarker techniques such as the Fish Disease Index and Imposex in combination with chemical monitoring. The MSFD is been followed up by IMARES and DELTARES and progress is running according to schedule. The initial assessment is in preparation and except for descriptor 3 and 4 all necessary information is available from running programmes.

8.7 Norway

The two WGBEC members are not involved in the process, but will contact their colleagues and report back at a later stage.

8.8 Germany

Preparation of the initial assessment is taking place and on schedule. It will be rather descriptive and general without details.

Recommendation 8.1: That biological effects measurements recommended for integrated assessment by ICES/OSPAR SGIMC are used for reporting on MSFD GES descriptor 8, Commission indicator 8.2.1.

Recommendation 8.2: That WGBEC review any available initial assessments in relation to Descriptor 8 at their next meeting and make recommendations on the development of national monitoring programmes for assessing GES for Descriptor 8.

9 Consideration of issues of special scientific interest/ value; (ToR g)

- a) Acidification in marine waters in relation to contaminants and biomarker response;
- b) Effects of contaminants on primary production, including phytotoxicity;
- c) Relationship of genetic markers to biomarkers;
- d) Review of species differences in bioassay and biomarker responses e.g. as seen in assessment criteria currently being developed – also to include sources of species for testing;
- e) Lysosomal stability in fish – review use and application in fish – histochemical method;
- f) Immunotoxicity end points – suitability for monitoring.

9.1 Background

During discussion of the future role and scope of WGBEC under agenda items 7 & 8 at the WGBEC 2010 meeting, several new work areas were raised as potential priority areas and of emerging interest to ICES and the group.

The areas identified for new future directions were:

a) Impacts of contaminants on food webs and ecosystem function / processes:

Continued attention should be given to top predators such as marine mammals, but also sea birds. Special emphasis should be placed on lower levels of the trophic food web, such as the impact of contaminants on benthic, pelagic algae and microbial populations and communities and their potential impact on carrying capacity of marine and coastal waters. Over the long term, knowledge of lower food web population and community effects can also result in new indicators to be included as additional components for integrated monitoring and assessment. It was pointed out that this type of research is very challenging due to complexity/ diversity of plankton and that it will require experimental work. There are also clear interactions with eutrophication. This type of research needs modelling and energy budgeting.

b) Development of bioassays and/or biomarkers for detecting and determining the effects of contaminants on the immunocompetence and fitness of organisms:

This seems particularly relevant to clarify the contributing role of contaminants in the recently observed epizootics in marine mammals and fish.

c) Ecogenetics: There is increasing knowledge on the effects of contaminants on population genetics and for example antibacterial resistance development. So far WGBEC only considered this research field rarely, but this will deserve more attention in the future.

d) Mixture of toxicity and interactions with natural factors should receive increasing attention:

This is a very challenging field of research, but essential to clarify the role of contaminants in cumulative stress impact assessments.

e) More focus on modelling fate of contaminants and effects:

Most models are lacking an effect module on top of fate modelling. WGBEC could play a contributing role here. This should also include increased effort on expert system modelling for biomarkers based on data collected all around Europe. Such an approach was done in the late 1990s but failed due to shortage of suitable data. Hence it will be particularly worthwhile to revisit the expert system approach.

f) Genomics / proteomics / metabolomics:

Already regularly on the WGBEC agenda, this area will require increasing attention and effort. In the future the technology will make this easier and there will be much work in applying this technology in monitoring and assessment approaches.

g) Climate change including ocean acidification:

The WG already conducted some work on the effects of climate change on ecotoxicological processes and environmental quality issues. Future work should also include the changes of PH on the bioavailability, uptake and other ecotoxicological processes.

h) Plastic particles (Addressed here in agenda item 14):

The WG envisaged this increasing environmental problem as a particular urgent area for future direction, given its potential impact on food chain energetics, food web transfer of contaminants, and increased risk for contaminant exposure and effects. The influence of plastic particle presence in sediments and their confounding effects on chemical and bioassay analysis results should be assessed.

To take these areas of interest forward WGBEC identified the following subjects for further consideration at its 2011 meeting if time permitted.

Acidification in marine waters in relation to contaminants and biomarker response.

Effects of contaminants on primary production, including phytotoxicity.

Relationship of genetic markers to biomarkers.

Review of species differences in bioassay and biomarker responses eg as seen in assessment criteria currently being developed – also to include sources of species for testing.

Lysosomal stability in fish – review use and application in fish – histochemical method.

Immunotoxicity end points – suitability for monitoring.

9.2 Acidification in marine waters in relation to contaminants and biomarker response (a)

Over the past couple of centuries there has been increasing concentrations of CO₂ in the atmosphere, gradually reducing the pH of the oceans. The topic has been the subject of recent reviews (e.g. Royal Society, 2005). This process of acidification has until now presumably led to a decrease of 0.1 pH units (Blackford & Gilbert, 2008). The results from most of the experimental studies that have focused on effects on organisms indicate that a range of processes may be affected (Pörtner, 2008), including calcification (Talmage & Gobler, 2009), but that there are species differences in sensitivity (e.g. Arnold *et al.*, 2009; Widdicombe & Spicer, 2008).

Klaas Kaag (NL) described an EU-project on impacts and safety in CO₂ storage (RISCS) activities, which aims to improve the understanding of the possible environmental impacts of geological storage of CO₂ (<http://www.riscs-co2.eu/>). A mesocosm experiment was performed in 2009, comprising 3 levels of CO₂-flow. After 6 weeks exposure, no clear effects of low pH could be found. In the high CO₂-flow treatment, the pH fluctuated between 6.6 and 6.9. In the low-flow treatment the pH fluctuated between 7.5 and 8. Control pH rose to values above 9 during the experimental period. Phytoplankton production was high in all systems, but did not result in high densities of zooplankton. The smaller benthic species *Corophium volutator* and *Peringia ulvae* developed high population densities in all treatments. The experiment was initiated rather late in season and suffered extremely high temperatures during summer. Consequently, the survival of larger benthic animals added was low, but the same in all treatments. So within the extreme weather conditions experienced, no effects of CO₂ were observed. A follow-up experiment will be initiated in spring 2011, including exposure of sponges and echinoderms.

WGBEC members have comprehensive knowledge of techniques by which to evaluate responses in organisms as a result of chemical stressors. Some of the methods may be directly relevant in studies of putative effects of acidification, e.g. scope for growth and cellular energy allocation, but there is clearly a need to develop new methods, e.g. for calcification, enzymes relevant to respiration, etc. The group should keep tabs on the development of effects methods for acidification.

Recommendation 9.2: that at a future meeting WGBEC recommends methods suitable for determining the effects of acidification on marine organisms.

References

- Arnold, K.E., Findlay, H.S., Spicer, J.I., Daniels, C.L., Boothroyd, D. 2009. Effect of CO₂- related acidification on aspects of the larval development of the European lobster, *Homarus gammarus* (L.). *Biogeosciences* 6: 1747-1754.
- Blackford, J.C., Gilbert, F.J. 2007. pH variability and CO₂ induced acidification in the North Sea. *J. Mar. Syst.* 64:229-241.
- Pörtner, H. O. 2008. Ecosystem effects of ocean acidification in times of ocean warming: a physiologist's view. *Mar. Environ. Prog. Ser.* 373: 203-217.
- Royal Society, 2005. Ocean acidification due to increasing atmospheric carbon dioxide. Policy document 12/05, ISBN 0 85403 617 2; www.royalsoc.ac.uk.
- Talmage S.C., Gobler, C.J. 2009. The effects of elevated carbon dioxide concentrations on the metamorphosis, size, and survival of larval hard clams (*Mercenaria mercenaria*), bay scallops (*Argopecten irradians*), and Eastern oysters (*Crassostrea virginica*). *Limnol. Oceanogr.* 54: 2072-2080.
- Widdicombe, S., Spicer, J.I. 2008. Predicting the impact of ocean acidification on benthic biodiversity: What can animal physiology tell us? *J. Exp. Mar. Biol. Ecol.* 366: 187-197.

9.3 Effects of contaminants on primary production, including phytotoxicity (b)

Dick Vethaak (NL) introduced a discussion on effects of contaminants on primary producers using an excerpt from a chapter written by two group members (Hylland & Vethaak, 2011). Since they are at the bottom of virtually all marine foodwebs (except those based on chemoautotrophs), any effects may have dramatic consequences. Due to toxicity testing there is a large database on effects of single agents on a few species such as the diatom *Skeletonema costatum*, but there is a scarcity of studies that have targeted natural communities or used mixtures of contaminants.

One of the issues with studies of the partitioning and effects of contaminants on natural algal populations is the excretion of carbohydrates by many species of algae, which will clearly modulate bioavailability. Such dissolved organic material (DOM) will clearly compete for lipophilic contaminants and some metals with high affinity for organic material, such as Cu. In natural water samples there will be high concentrations of viruses and bacteria in addition to protists and algae, all with surfaces that will sorb contaminants. It is therefore an open question how contaminants will partition and which part of pelagic food webs will be affected. This is an area that deserves further attention, although one limitation is the lack of mesocosm facilities and the workload in running such experiments (the study referred to below involved 10–12 scientists and technicians during a period of 10 days, a total of more than 20 man-weeks).

Ketil Hylland (NO) referred to a study in which pelagic mesocosms had been used in the study of interactions between contaminants (emamectin in this case), oil (low-PAH) and nutrients (causing increased primary production). The results showed that emamectin selectively removed some grazers, but this had surprisingly transient effects on primary production, suggesting that the system was bottom-up rather than top-down regulated (Vestheim *et al.*, in prep). The results are currently being analysed and the work is under publication. A complete dataset can be presented for the group at the 2012 meeting.

One concept using communities is pollution-induced community tolerance (PICT), pioneered by Blanck *et al.* in the 1980s (reviewed in Blanck 2002). This concept is based on an assumed tolerance of communities in areas with more or less specific contaminant inputs. By testing the tolerance of communities to specific contaminants

(measured using e.g. chlorophyll as a proxy of primary production) it is possible to identify areas affected by these contaminants. This group has later concentrated on using periphyton and bacterial communities (see e.g. Paulsson *et al.*, 2002).

References

- Blanck, H (2002) A critical review of procedures and approaches used for assessing pollution-induced community tolerance (PICT) in biotic communities. *Human Ecol Risk Assess* 8:1003–1034.
- Hylland, K., Vethaak, A.D. A Marine Pelagic Community Response to Manipulation with Silicate, Oil and Contaminants. In: *Ecological Impacts of Toxic Chemicals*, Ed: Sanchez-Bayo, F., Ch 10, pp. 271-287.
- Paulsson, M., Månsson, V., Blanck, H. (2002). Effects of zinc on the phosphorus availability to periphyton communities from the river Göta Älv. *Aquat. Toxicol* 56, 103-113
- Hege Vestheim, Jorun Egge, Tom Andersen, Katherine Langford, Paolo Simonelli, T. Frede Thingstad, Ketil Hylland. A Marine Pelagic Community Response to Manipulation with Silicate, Oil and Contaminants (in prep).

9.4 Relationship of genetic markers to biomarkers (c)

Ketil Hylland (NO) introduced a discussion on two issues relevant to current approaches in ecotoxicology, i.e. (i) whether it is to be expected that there is a relationship between contaminant residues in tissues and biomarker responses in the same individual; (ii) whether it is to be expected that there is an association between levels of gene transcripts and the corresponding biomarker (protein or activity).

A biological response will clearly require an exposure of the appropriate receptor (in a wide sense) to a contaminant. There has been an assumption that there should be a relationship between contaminant residues in tissues and responses since contaminants in one compartment will be in equilibrium with other compartments, e.g. blood plasma, thereby resulting in a concentration-dependent exposure in other tissues, e.g. liver. This is of course correct, but the concentration in plasma would be very low for most lipophilic substances, even with very high concentrations in fat-rich tissues. It is even more difficult to assess “true” exposure for organisms with fatty livers, e.g. gadiids, for which one could assume that a large proportion of lipophilic contaminants reaching the organ will partition to fat rather than the metabolically active part of cells. Most marine organisms have seasonal cycles in which they mobilise resources to produce gametes or simply starve due to low food availability. Exposure to contaminants would be expected to increase dramatically during such periods, as has indeed been shown in some Arctic species. In addition, internal exposure would be expected to be high if the organism is exposed to elevated environmental concentration of the contaminant in question. There is a need for further research into possible consequences and the magnitude of true internal exposure in marine organisms.

There has been an increasing use of gene expression analyses in marine ecotoxicology over the past decade and there has been suggestions that transcriptomics should be included in biological effects monitoring. One reason is the possibility of analysing the expression of hundreds or even thousands of genes at the same time. In addition, there is not the limitation of a need for very specific methods and instruments (MT) or lack of antisera for all relevant species (vtg). Gene expression analyses are clearly very useful in short-term exposure studies with an aim to clarify mechanisms of toxicity or effect. It is less clear that gene transcript analyses can be readily interpreted in field-sampled organisms. The response time for gene expression is hours, whereas the ensuing response time for most biomarker responses is days or weeks. On the

other hand, the half-life of mRNA is hours up to a day, that of protein (biomarkers) vary widely, but generally lie in the range of days to weeks. If the objective of a study is to investigate an acute response it would appear appropriate to use gene expression, but if the objective is to assess more long-term effects in marine organisms it would be more appropriate to measure biomarkers. It is of course the biomarker response that in the end will have consequences for the cell and tissue. In addition to the above, there are post-transcriptional processes that affect mRNA contribution to translation, e.g. co-ordinate regulation of mRNA by microRNA, thus possibly invalidating the immediate signal measured using rtPCR or microarray. To conclude, although transcriptomics may contribute hundreds and thousands of datapoints for each individual, such data may not be easily interpreted for individuals sampled from a natural population and the expression may not provide information directly associated with cellular health.

It is expected that further data from integrated monitoring programmes in the ICES area will become available which include both mRNA and biomarker data from the same individuals. Analysis and interpretation of such data should take account of the points made above and allow further interpretation of the usefulness of gene expression analysis in field monitoring.

9.5 Review of species differences in bioassay and biomarker responses eg as seen in assessment criteria currently being developed – also to include sources of species for testing (d)

Ricardo Beiras (ES) presented data on a comparison in sensitivity between bivalve and sea-urchin embryo bioassays. A review of published values for bioassays for the two species using elutriates or porewater has been conducted by the University of Vigo, with 18 reference toxicants including metals, hydrocarbons, pesticides and detergents. A comparison between the sensitivity of both groups to a broad range of toxicants, including metals, PAHs, and different biocides yields a correlation $r^2=0.96$ ($p<0.01$) and a slope of the double logarithmic regression line of 1.00 with 95% confidence intervals from 0.94 to 1.06 (see Figure 9.5.1)

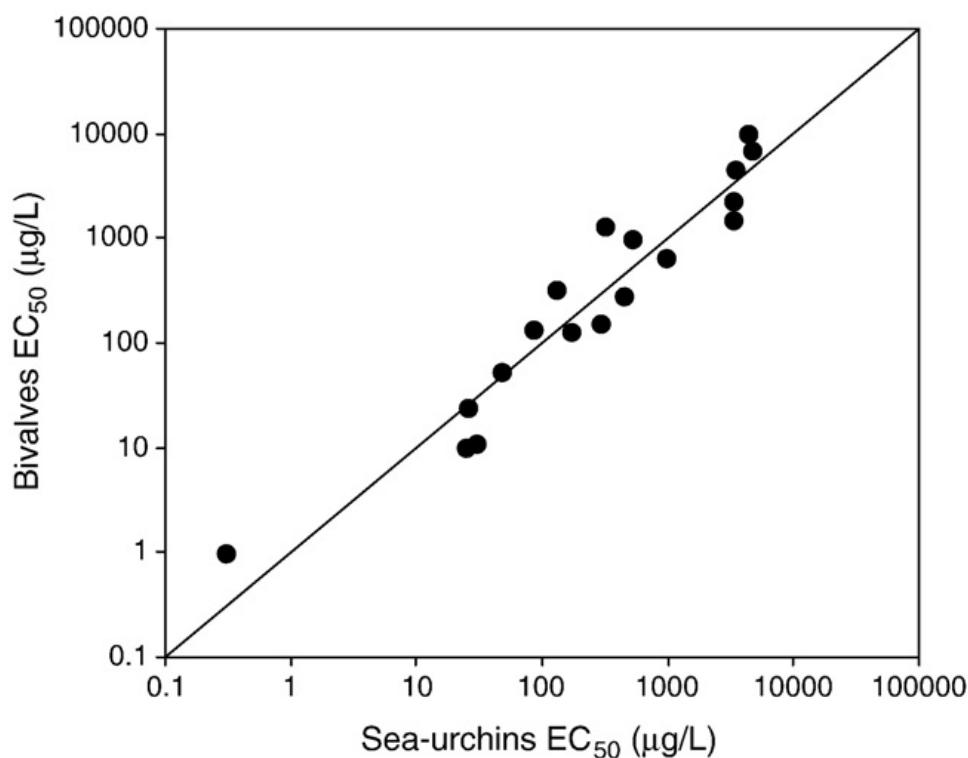


Figure 9.5.1. Relationship between the toxicity of 18 toxicants in the sea urchin bioassay and bivalve bioassays.

This makes possible to use either bivalve embryos or sea-urchin embryos, depending on biological availability, enabling to conduct the embryo-larval test in a broader period of the year and also in different geographic areas.

Ketil Hylland (NO) presented a possible strategy by which to compare the sensitivity of different fish species. Different fish species were collected by trawl and kept alive in running seawater on deck. Four to five individuals of each species were perfused on board the research vessel and hepatocytes cultured. After attaching to plates, hepatocytes were exposed to different concentrations of model toxicants, in this example copper (Cu), for 24 hrs. Different effect endpoints were then quantified reflecting modes of cytotoxicity, i.e. membrane integrity, metabolic inhibition and GSH content (Ellesat *et al.*, in prep). The results clearly indicated that flatfish hepatocytes were more sensitive to Cu than cod hepatocytes and that the effect profiles were very different (Figure 9.5.2). Some other factors will need to be taken into consideration in future studies, e.g. the size of hepatocytes (cod hepatocytes are larger than flatfish ditto), but such perfusion and exposures offer a possibility for an assessment of the sensitivity of in situ populations of the species present. Earlier studies have indicated that most species can be amenable to this treatment, but species with very fatty livers, e.g. some elasmobranchs, are challenging.

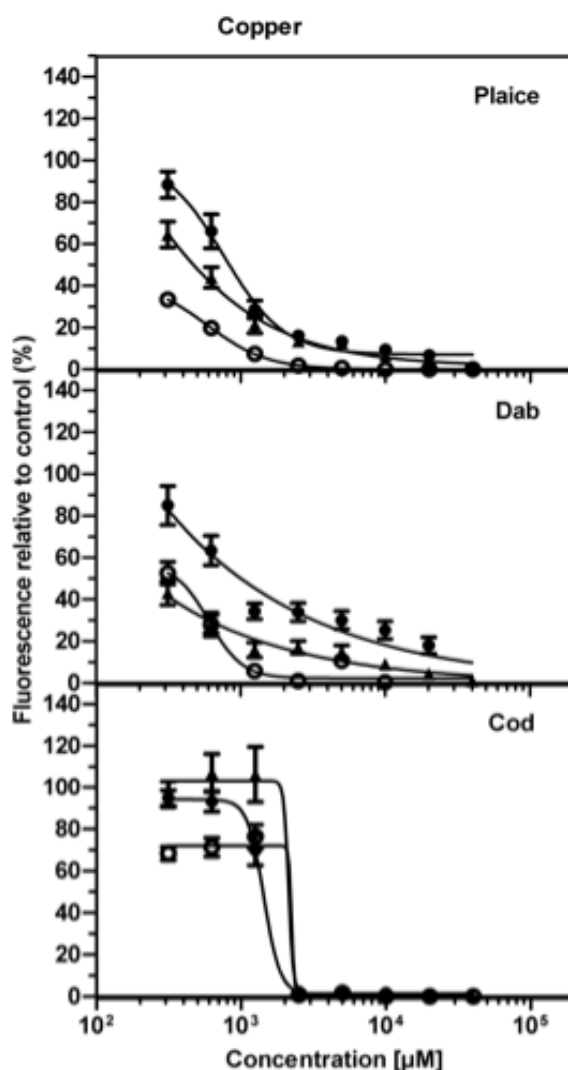


Figure 9.5.2. Response relative to control for hepatocytes taken from plaice (top), long rough dab (middle) and cod (bottom); the cells were exposed for the indicated concentrations of Cu for 24 hrs prior to analyses (circles: metabolic integrity; squares: membrane integrity; triangles: GSH concentration). Data from Ellesat *et al.* (in prep).

9.5.1 Species variation in Biomarker responses – Supplementary EACs

Steinar Sanni (NO) indicated that several oil based exposure experiments have been carried out in fish and invertebrate species in two R&D projects at IRIS Biomiljø's laboratory near Stavanger, Norway. Multivariate analysis of the exposure and biomarker results showed that the basic biomarker response patterns are similar in the different species, there are quantitative differences between the species which should be taken into account in the determination of EACs. Critical levels for fitness have been determined in larvae of the same or similar species, thus allowing that these experimental data can be used for determination of EAC levels for the most common biomarkers in all the studied species. A table of EAC values will be prepared for the SGIMC meeting in March 2011 in order to supplement the previously assigned EAC values, and is appended in Annex 14. Based on the same studies, similar data have been provided for biomarkers where ACs had not previously been determined (addressed under ToR point 7) and reported under this point. (Data source: IRIS Biomiljø)

database - BioSea I & II JIP; Total E&P NORGE and EniNorge AS, and Biomarker Bridges; PROOFNY program - Research Council of Norway).

References

Ellesat, K, Yazdani, M, Holth, TF, Hylland, K. Comparative cytotoxicity of statins and copper to primary hepatocytes of three marine fish species plaice (*Pleuronectes platessa*), long rough dab (*Hippoglossoides platessoides*) and Atlantic cod (*Gadus morhua*).

9.6 Lysosomal stability in fish – review use and application in fish – histochemical method (e)

Ketil Hylland (NO) and Steinar Sanni (NO) presented data for the use of the histological method for lysosomal membrane stability (LMS) in marine fish species.

Ketil Hylland (NO) referred to a study in which maturing Atlantic cod were exposed to two concentrations of produced water components or a pulsed exposure for 10 months. Samples were taken for LMS analysis of head kidney after 2 weeks, 10 weeks and 32 weeks. LMS was reduced in cod kept in the highest concentration after 2 weeks and this effect remained throughout the exposure period (Holth *et al.*, in prep). Effects were also seen for cod in the pulsed exposure, but not in the low exposure.

Steinar Sanni (NO) reported results from an oil-based exposure of juvenile halibut, in which histochemical lysosomal stability was analysed. The results corresponding to the critical mortality concentration for halibut larvae showed LMS value of 10 minutes, while the level measured in negative reference fish was 15 minutes. The LMS value of 10 minutes can be used tentatively as an EAC value for LMS in halibut, however which will need verification in further laboratory exposures.

Recommendation 9.6: That background documentation and assessment criteria are developed for measuring lysosomal membrane stability in fish.

References

Holth, TF, Beckius, J, Zorita, I, Cajaraville, M, Hylland, K. Long-term exposure to produced water components: effects on lysosomal alterations and peroxisome proliferation in head kidney of Atlantic cod (*Gadus morhua*).

9.7 Immunotoxicity end points – suitability for monitoring (f)

John Thain(UK) presented an overview written by Tom Hutchinson(UK) from the Cefas Weymouth Laboratory, UK. The overview looked at evaluating immune system health in marine fish and shellfish exposed to chemical toxins and other physico-chemical stressors relevant to climate change.

9.7.1 Summary

It is well established that environmental quality, including the presence of chemical pollutants, can seriously impact immune system health in a wide variety of fish and marine invertebrates and this presents an important opportunity within the use of biological effects tools to meet the challenges outlined by the European Union Marine Strategy Framework Directive (MSFD). Moreover, there is a growing body of evidence demonstrating that climate change-related physico-chemical stressors (e.g. ocean acidification) can also have an adverse impact on immune function in marine shellfish. Chemical contaminants may also interact with other water quality parameters (e.g. hypoxia) to impact the immune systems of marine organisms. A wide variety of research tools have been developed over the past 20 years for assessing both cellular and humoral immunity in teleost fish and today there are several readily

available options for validating and applying assays of immune function in marine fish health monitoring in an MSFD context. Likewise, knowledge of the immune systems of marine crustaceans and molluscs has also developed significantly over the past decade and again, subject to validation, a suite of prioritized assays of immune system health could be readily applied to monitoring marine shellfish populations potentially exposed to toxic chemicals or physico-chemical immunosuppressants.

9.7.2 Introduction

The use of biological effects tools offer enormous potential to meet the challenges outlined by the European Union Marine Strategy Framework Directive (MSFD) whereby Member States are required to develop a robust set of tools for defining 11 qualitative descriptors of Good Environmental Status (GES), such as demonstrating that “Concentrations of contaminants are at levels not giving rise to pollution effects” (GES Descriptor 8). Lyons *et al.* (2010) discusses the combined approach of monitoring chemical contaminant levels, alongside biological effect measurements relating to the effect of pollutants, for undertaking assessments of GES across European marine regions. As shown by a wide range of field and laboratory studies over the past decade, pollution effects can include the important aspect of susceptibility to disease and the adverse impacts of certain chemicals on immune system health in marine and freshwater organisms. As widely reported, a number of sentinel fish (eg dab and flounder) and aquatic invertebrates (e.g. mussels) provide ideal systems for environmental monitoring and toxicology. Like all animals, they are also subjected to a wide range of infectious diseases that can have significant effects on host ecology and physiology and are therefore a source of natural stress to populations. Anthropogenic activities, especially involving chemical contaminants that pollute the environment, can also affect population viability and in some cases their aesthetic suitability for human consumption. In combination, pathogens, chronic chemical pollution and oil spills represent a serious threat to the health of aquatic communities, to which should be added the latent and chronic impacts of climate change related physico-chemical stressors (e.g. ocean acidification) (Novas *et al.*, 2007, Bibby *et al.*, 2008; Morley *et al.*, 2010). This note briefly summarises pertinent examples of published immunotoxicology and related studies using marine finfish and shellfish pertinent to the work of ICES. It should be noted that there is also an extensive body of information on the immune function in several marine taxa (e.g. echinoderms, gastropods, urochordates, etc.) not normally included in the ICES-related biological effects monitoring programmes.

The immune system protects organisms from infection with layered defences of increasing specificity. In simple terms, physical barriers prevent pathogens such as bacteria and viruses from entering the organism. If a pathogen breaches these barriers, the innate immune system provides an immediate, but non-specific response. Innate immune systems are found in all plants and animals. If pathogens successfully evade the innate response, vertebrates possess a third layer of protection, the adaptive immune system, which is activated by the innate response. Here, the immune system adapts its response during an infection to improve its recognition of the pathogen. This improved response is then retained after the pathogen has been eliminated, in the form of an immunological memory, and allows the adaptive immune system to mount faster and stronger attacks each time this pathogen is encountered (Table 9.7.2.1).

Table 9.7.2.1. Components of the immune system.

Innate immune system	Adaptive immune system
Response is non-specific	Pathogen and antigen specific response
Exposure leads to immediate maximal response	Lag time between exposure and maximal response
Cell-mediated and humoral components	Cell-mediated and humoral components
No immunological memory	Exposure leads to immunological memory
Found in nearly all forms of life	Found only in jawed vertebrates

Both innate and adaptive immunity depend on the ability of the immune system to distinguish between self and non-self molecules. In immunology, self molecules are those components of an organism's body that can be distinguished from foreign substances by the immune system. Conversely, non-self molecules are those recognized as foreign molecules. One class of non-self molecules are called antigens (short for antibody generators) and are defined as substances that bind to specific immune receptors and elicit an immune response

9.7.3 Marine finfish

International concern of diseases in marine flatfish since the 1970s led to a number of projects to develop tools to understand immune system health in selected species used for monitoring. This effort was augmented by basic research into the evolution and function of the teleost immune system (teleosts have both innate and acquired immunity) and also for aquaculture purposes (Ellis 1977; Whyte 2007). Essentially, today there exists a very well developed toolbox of research methods for investigating finfish immune function pertinent to the defence against bacterial, fungal, viral and other pathogens. These include humoral ('soluble') factors such as antimicrobial peptides, complement, cytokines, interferons, interleukins and lectins. In addition, finfish have a battery of cellular immune functions to detect and destroy pathogens (e.g. phagocytosis and production of reactive oxygen species); (Whyte 2007). Taking cod (*Gadus morhua*), the European flounder (*Platichthys flesus*) and dab (*Limanda limanda*) as examples, there have been a number of laboratory and (semi-) field studies applying immune function assays to address the potential adverse effects of metals and organic chemical toxicants (Grinwis *et al.*, 1998 & 2000; Hutchinson *et al.*, 1996 & 2003; Pérez-Casanova *et al.*, 2010; Pulsford *et al.*, 1994; Secombes *et al.*, 1992). Seasonal studies of trends in the humoral immune system have also been reported for dab (Hutchinson & Manning 1996; see review by Bowden *et al.*, 2007). Numerous examples of studies on chemicals and innate immune functions also exist for North American marine flatfish species (e.g. English sole); (Arkoosh *et al.*, 1996) and for south east Asian species (e.g. Japanese flounder); (Nakayama *et al.*, 2008). For logistical reasons, there are fewer investigations of the impacts of chemicals on acquired immunity (antigen-induced immunological memory including antibody production), however, such studies have been conducted using turbot exposed to PCBs via the sediment (Hutchinson *et al.*, 1999). Table 9.7.3.1 gives a brief summary of biological effect endpoints that could be applied in finfish to assess water column or sediment-associated contamination with regard to immune system health.

Table 9.7.3.1. Summary of potential immune system measurements in marine finfish and shellfish species pertinent to MSFD.

Species	Contaminant exposure context	Immune system endpoints
Finfish		
Cod	Water column	Blood leucocyte counts Blood lysozyme Head kidney leucocyte counts Spleen leucocyte counts Phagocytic activity Production of reactive oxygen species
Dab	Sediment	Blood leucocyte counts Blood lysozyme Head kidney leucocyte counts Spleen leucocyte counts Phagocytic activity Production of reactive oxygen species
Flounder	Sediment	Blood leucocyte counts Blood lysozyme Head kidney leucocyte counts Spleen leucocyte counts Phagocytic activity Production of reactive oxygen species
Crustacean		
Nephrops sp	Sediment	Differential haemocyte counts Lysozyme Phagocytic activity Production of reactive oxygen species Nitrous oxide synthesis
Mollusc		
Cerastoderma sp.	Sediment	Differential haemocyte counts Lysozyme Phagocytic activity Production of reactive oxygen species Nitrous oxide synthesis
Mytilus sp.	Water column	Differential haemocyte counts Lysozyme Phagocytic activity Production of reactive oxygen species Nitrous oxide synthesis

For all the endpoints in Table 9.7.3.1, collation of data to establish normal ranges of the relevant parameters is an important priority, where necessary supplemented by further baseline studies which also take into account the reproductive status of the individuals. Validation work to define intra-laboratory and inter-laboratory variability, and define statistically robust sampling schedules, is also essential prior to deploying the immunological methods in a marine monitoring context.

9.7.4 Marine crustaceans

The defence mechanisms of crustaceans, as with other arthropods, depends completely on the innate immune system that is activated when pathogen-associated molecular patterns are recognized by soluble or by cell surface host proteins, such as lectins, antimicrobial, clotting, and pattern recognition proteins. These in turn activate cellular or humoral effector mechanisms to destroy invading pathogens. The crustacean immune system includes proteins that participate in immune defence by specific recognition of carbohydrate containing molecules (e.g. glycans, glycolipids, glycoproteins, peptidoglycans or lipopolysaccharides from Gram-negative and Gram-positive bacteria, viruses, or fungi). Key processes within the crustacean immune defence processes are agglutination, encapsulation, phagocytosis, clottable proteins, and bactericidal activity which are induced by these carbohydrate-driven recognition patterns. As a key marine example, Hernroth *et al.* (2004) showed that manganese affects several fundamental processes in the mobilisation and activation of immunoactive haemocytes. When *Nephrops norvegicus* was exposed to a 20 mg Mn²⁺ l⁻¹ for 10 days, total haemocyte count were reduced by ca. 60%. By using BrdU as a tracer for cell division, it was shown that the proliferation rate in the haematopoietic tissue did not increase, despite the haemocytepenia. A gene coding for a Runt-domain protein, known to be involved in maturation of immune active haemocytes in a variety of organisms, was identified also in haemocytes of *N. norvegicus*. The expression of this gene was >40% lower in the Mn-exposed lobsters as judged by using a cDNA probe and the in situ hybridisation technique. In response to non-self molecules, like lipopolysaccharide (LPS), the granular haemocytes of arthropods are known to degranulate and thereby release and activate the prophenoloxidase system, necessary for their immune defence. A degranulation assay, tested on isolated granular haemocytes, showed about 75% lower activity in the Mn-exposed lobsters than that for the unexposed. Furthermore, using an enzymatic assay, the activation per se of prophenoloxidase by LPS was found blocked in the Mn-exposed lobsters. Taken together, these results show that Mn exposure suppressed fundamental immune mechanisms of Norway lobsters. This identifies a potential harm that also exists for other organisms and should be considered when increasing the distribution of bioavailable Mn, as has been done through recently introduced applications of the metal. More recently, Jacobson *et al.* (2011) reported that exposure of the benthic amphipod *Monoporeia affinis* to TBT-contaminated sediments resulted in significant adverse impacts on oocyte viability and a doubling of the prevalence of microsporidian parasites in females. While TBT is known to be an immunotoxicant in mammals and fish, this marine amphipod study underlines the need to consider the reproductive status of crustaceans when undertaking assessments of immune function or disease resistance. See Table 2 for options for assessing immune system health in marine crustaceans.

9.7.5 Marine bivalve molluscs

Briefly, The internal defence system of molluscs is based on an innate, non-lymphoid immune system (Wootton & Pipe 2003) involving effective methods for cellular recognition and for discriminating foreign cells and harmful micro-organisms. The predominant mechanism of internal defence in bivalves involves phagocytosis by circulating haemocytes, followed by the release of reactive oxygen metabolites and degradative enzymes, and secretion of cytotoxic molecules. Bivalve haemocytes are also involved in nutrient digestion and transport, wound and shell repair, and excretion (Cheng 1981). Thus, any dysfunction in the immune system may have consequences for the nervous and endocrine systems. A large number of studies have

reported on the impacts of inorganic and organic chemical toxicants on immune responses and pathogens infections in species including cockles (Matozzo *et al.*, 2008), mussels (Canasi *et al.*, 2008; Novas *et al.*, 2007; Parry & Pipe 2004; Pipe & Coles 1995) and oysters (Badon-Nilles *et al.*, 2008; Chu *et al.*, 2008; Fisher *et al.*, 1999) and scallops (Hannam *et al.*, 2010). For example, Novas *et al.* (2007) studied impacts of the Prestige oil spill on mussel immunity in terms of haemocyte characterisation and synthesis of nitric oxide by haemocytes. Maximal basal production of NO by hemocytes of *M. galloprovincialis* was detected in summer, whereas the minimum values were detected in winter. In winter, the presence of IL-2 induced an increase in NO production that was not detected in summer. Three months after the Prestige oil spill (November 2002), basal NO production by the hemocytes of mussels in the Galician coast showed a progressive decrease and stopping, both in summer and in winter. The characteristic increase of NO synthesis induced by IL-2 in winter also disappeared all through 2003 and 2004. The two different nitric oxide synthases previously identified by immunoblotting between 1999 and 2002 were undetectable in both 2003 and 2004. When comparing the data obtained during 2003 and 2004 to those obtained in previous years, an increase in the proportion of SH cells was detected. Also, these cells showed a higher sensitivity to apoptosis- and necrosis-inducing agents than in earlier years. As with other marine species, establishing seasonal baselines and placing immune responses into the context of the normal reproductive cycle are both key requirements (Cao *et al.*, 2007). See Table 2 for options for assessing immune system health in marine bivalve molluscs.

Recommendation 9.7: That an intersessional technical meeting be held in autumn 2011 in the UK (led by Cefas Weymouth) to identify priority areas for technical cooperation in marine organism immunotoxicology and that WGBEC receives a report on progress of intersessional work and strategy for taking this work area forward at its 2012 meeting.

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10 Review progress with publication and electronic dissemination of biological effects techniques in the ICES TIMES series; (ToR a)

The outgoing effects editor (Matt Gubbins) reviewed progress on publications to the group. Responsibility for ICES TIMES manuscripts on biological effects methods was transferred to Ricardo Beiras (Spain) this year.

Eleven manuscripts have been commissioned by WGBEC and are awaiting submission to ICES. Two (1E03, MHC07) are finalised and awaiting submission to the ICES TIMES editor. A further two that already have draft resolutions (MHC06, MHC13) are complete and were reviewed by the WG during the meeting. Minor edits are required before going for external peer review. One manuscript (MHC02) was presented to the group as an incomplete draft that needs further work. WGBEC recognised that progress had been made by the primary author, but that co-authors were required to contribute to complete the manuscript. The WGBEC Chair will contact the co-authors to stress the importance of the manuscript in light of the imminent ICES/ OSPAR SGIMC meeting in March 2011 and encourage contribution to allow the manuscript to be completed.

Three manuscripts were identified as having made little progress over the last year and may be at risk of not being completed by the identified authors. These were MHC12, MHC14 and MHC15. For MHC 14 on micronucleus assays it was suggested that the WGBEC ICES TIMES editor contact Janina Barseine (Lithuania) to consider authoring the manuscript. For MHC12 on gonadal histology in flounder, Grant Stentiford (Cefas, UK) will be contacted to consider taking on the task. No immediate action was identified for MHC15 on YES/YAS in vitro bioassays, however the development of an OSPAR background document and assessment criteria for ICES/ OSPAR SGIMC in March 2011 by Dick Vethaak (Netherlands) may facilitate the production of a TIMES manuscript by this author.

A further three manuscripts are likely to be complete and reviewed in 2011 but do not yet have draft resolutions. It is recommended that ICES issue draft resolutions for these publications on reproductive success in eelpout, alkylphenol bile metabolites and sea urchin embryo bioassays. Table 10.1 summarises status and progress in 2011 against manuscripts in preparation for WGBEC.

Table 10.1. Status of WGBEC TIMES manuscripts.

C. Res	Method	Status / progress
2002/1E03	The report on Biological Effects of Contaminants: Oyster (<i>Crassostrea gigas</i>) Embryo Bioassay by J.E. Thain (UK)	An updated publication. Completed and reviewed by WGBEC, ready for publication.
2006/1/MHC06	The Protocol for Extraction Methods for Bioassays. Hans Klamer and John Thain (UK)	Produced during WGBEC 2011. Reviewed by group, needs editing for TIMES structure and checking against MHC13
2006/1/MHC07	The protocol for conducting EROD determinations in flatfish By M. Gubbins	An updated publication. Completed and reviewed by WGBEC, ready for publication.
2007/1/MHC02	Blue Mussel Histopathology, John Bignell, Steve Feist & Miren Cajaraville	Initial draft produced for WGBEC 2011. Reviewed at the meeting. Several sections still missing. WGBEC chair to contact co-authors and request action.
2008/1/MHC13	Protocol for measuring dioxin-like activity in environmental samples using LUC assays. Dick Vethaak (Netherlands)	Produced for the meeting. Reviewed by the group with minor edits suggested. To be passed to MCWG to review this year under agenda item L).
2008/1/MHC14	Protocols for measuring micronucleus formation in cells as an indicator of toxicant induced genetic damage. Brett Lyons & Awadesh Jha (UK).	There has been no action by authors on this manuscript. WGBEC editor to contact Janina Barseine to consider producing.
2008/1/MHC15	Protocol for measuring estrogen/androgen activity in environmental samples using YES/YAS yeast screen assays. J Thain (UK), Kevin Thomas (Norway)	No action by authors. No plan for production identified at this stage. Action required by WGBEC TIMES editor.
2008/1/MHC12	The protocol for gonadal histology in flounder. S Feist <i>et al.</i>	No further progress by author. WGBEC ICES TIMES editor to ask Grant Stentiford (Cefas) to produce.
Needed	Reproductive success in eelpout. Jakob Strand	In preparation. Expected late 2011. Draft resolution required
Needed	Alkylphenol bile metabolites. Jonsson et al	Revised manuscript reviewed by WGBEC during the meeting. Considered suitable for peer review / publication. Draft resolution required.
Needed	Sea urchin embryo bioassay. Ricardo Beiras	Manuscript has been externally peer reviewed and approved by the group. Waiting amendments by the authors. Draft resolution required.

WGBEC considered whether any new TIMES manuscripts needed to be commissioned. It was identified that following the WKLYS workshop (c.f. agenda 12) there may be a future requirement to update the manuscript on lysosomal stability by neutral red retention (Lowe *et al.*). Method documents may also be required for COMET assay (c.f. agenda 7f), immunotoxicity assays (c.f. agenda 9f) and the development of passive sampling methodologies for bioassays, which may take the form of collaboration with MCWG (c.f. agenda 6). It was also suggested that oxidative stress measurements may require a TIMES manuscript and that of these lipid peroxidation assays (LPO) would be the highest priority of these for publication.

Given the large volume of manuscripts already awaiting publication it was decided not to pursue publication of these manuscripts at this stage, but that these would be reviewed again at next year's meeting.

Recommendation 10: That draft resolutions be sought from ICES for publication of TIMES manuscripts for:

- Reproductive success in eelpout by Jakob Strand (deadline March 2012)
- Alkylphenol bile metabolites by G. Jonsson *et al.* (deadline Oct 2011)
- Sea urchin embryo bioassay by Beiras *et al.* (deadline Oct 2011)

11 Respond to requests for advice from the ICES Data Centre on reporting formats and codes for entering biological effects data into the ICES database and to consider whether further data entry guidance is required for new methods that may be required for the developing ICES/OSPAR integrated monitoring framework; (ToR b)

No specific requests from the ICES data centre were received by the Working Group for attention in 2011, however it was identified that there were still some issues with biological effects data submission that needed to be addressed. Two specific issues were identified and discussed:

How external QA data for biological effects should be entered when associated with non-QUASIMEME (e.g. BEQUALM) intercalibration exercises

Whether PAH bile metabolite data measured by different methods (e.g. synchronous scanning fluorescence or GC-MS) should be adjusted by appropriate multiplication factors to allow intercomparability before entry to the database or whether this should be addressed on assessment.

WGBEC identified a potential role for itself in assessing biological effects external QA programmes and communicating these rounds to the ICES data centre for inclusion.

The latest (2009/2010) biological effects data submissions to the ICES database were reviewed by the group. It was noted that although the quantity of biological effects data being submitted is still increasing (and the number of member states submitting data of these types is increasing) that the overall quantity and scope of data submission is still low and does not represent the level of biological effects monitoring activity taking place across the ICES region. Nine countries submitted 2538 parameter station records in 2009. Unfortunately, not many biological effects parameters are represented, with imposex and fish disease data dominating the submissions. There are likely to be many reasons for this, including lack of experience of data submission by some countries and difficulties in understanding the reporting formats. As new biological effects data types become more regularly submitted to the database, reporting format issues that have not yet been experienced are likely to arise. In the past these issues have required a considerable amount of time to address during WG meetings and not all the relevant expertise to provide the data centre with the required advice has been present.

For these reasons, WGBEC proposes to operate an intersessional subgroup covering data centre issues. The subgroup will have the objective of:

- Responding to ICES Data Centre requests for advice as they arise;
- Providing advice on data reporting to the ICES biological effects community as required;
- Improving awareness of the need to report biological effects data among the biological effects community;
- Assessing the quantity and scope of data being submitted;

- Report annually on progress to WGBEC.

It is hoped that the formation of this subgroup will provide a more effective and flexible way of dealing with data centre issues without occupying much time in plenary at WGBEC meetings. It is anticipated that issues will be dealt with primarily by correspondence. Membership of the group is to include Matt Gubbins and Thomas Maes (UK) and further individuals not present at the meeting will be contacted for participation in the subgroup.

Recommendation 11.1: That member states should be submitting the full range of their national biological effects monitoring data to the ICES data centre.

Recommendation 11.2: That an intersessional subgroup be formed to address data centre issues for biological effects data and report annually to WGBEC.

12 Review progress with AQC procedures for biological effect methods and include harmonisation activities initiated from WGBEC and within OSAPR, Baltic and MEDPOL maritime areas and to include report of Lysosomal Membrane Stability Workshop held in Alessandria, Italy; (ToR d)

12.1 Review ICES/OSPAR WKLYS on the quality and interpretation of lysosomal stability data report and assessment criteria for LMS using NRR assay

WGBEC reviewed ICES/OSPAR WKLYS on the quality and interpretation of lysosomal stability data report and assessment criteria for LMS using NRR assay.

C. Martínez-Gómez (Spain) explained that during WKLYS the main aspects of the operational procedure to harmonize the use of the Neutral Red Retention assay were identified and it was concluded that a further discussion and consensus will be necessary, in terms of monitoring and inter comparison purposes, to use this technique through the ICES/OSPAR and MED POL area. Main reason is that, apart from the analytical procedure described in UNEP/RAMOG, 1999 for MED POL programme, a new analytical procedure based on image analysis is currently being recommended in MED POL training courses to assess NRR time in mussels, being endpoints obtained in a different way. As few participants from OSPAR area were attending, the progress with the data quality and interpretation was lesser than expected. However, a substantial progress was done on identification of the potential variation sources affecting results between laboratories.

An illustrated draft document was produced to be used through the ICES/OSPAR area, with proposals for improved consistency in the interpretation of observations under microscope when NRR is used. This draft illustrated document was discussed in WKLYS with A. Viarengo, and M. Moore and it was sent A. Kohler and D. Lowe for their comments.

A new proposal for NRR assay intercalibration exercise was proposed by using virtual slides in a similar way as the BEQUALM Fish Disease Measurement programme has been utilizing successfully during the last three years. WGBEC consider there is a considerable potential for virtual slide intercalibrations to be used in the context of the neutral red retention assay. For that purpose, C. Martínez-Gómez sent a questionnaire to all WGBEC participants on January 2011 to collect key information concerning certain aspects of the application of NRR assay. Information collected during WGBEC 2011 is summarised in Table 12.1.

WKLYS identified a suite of actions (see below) that were addressed during WGBEC 2011 and for that purpose, C. Martínez-Gómez had sent a questionnaire to all WGBEC participants on January 2011 to collect key information concerning certain aspects of the application of NRR assay. Information collected during WGBEC 2011 is summarised in Table 12.1.

Action 1: Countries assessing LMS in monitoring programmes by using physiological saline should validate the use of ambient filtered seawater and provided results to WGBEC 2011 and when demonstrated the use of ambient filtered seawater should be recommended in ICES TIMES protocol.

Action 2: Countries assessing LMS in monitoring programmes should indicate what temperature is used during the dye incubation and report to WGBEC in 2011 for further discussion.

Action 3: OSPAR/ICES Member Countries that are applying NRR assay should indicate how they are determining the NRR time endpoint and report to WGBEC in 2011.

Action 4: Work should be done to harmonize a common and adequate sampling size for lysosomal NRR time for ICES/MED POL. OSPAR/ICES member that are applying NRR assay should indicate the sampling size used and report to WGBEC in 2011.

Table 12.1. Information concerning the use of NRR assay in ICES/OSPAR countries to assess LMS.

Country/Institution	Species	Sampling size	Monitoring / Research	Extraction/Incubation	ENDPOINT
Spain (IEO)	<i>M. galloprovincialis</i>	12-20	M/R	Physiological saline adjusted to salinity at the sampling site / 18-20°C	Time when >50% cells affected
UK (CEFAS)	<i>Mytilus sp.</i>	10	M/R	Physiological saline / cool ambient temperature 15°C	Time when >50% affected
Denmark (National Environmental Research) Institute	<i>M. edulis</i>	10-12	M	Physiological saline / 20°C	Time when >50% affected
Finland (Finnish Environment Institute)	<i>M. edulis</i> <i>M. trosulus</i> <i>Macoma baltica</i> <i>Carcinus maenas</i>	10-20	R	Physiological saline adjusted 5-10°C	Last time when >50% not affected
Norway (NIVA)	<i>M. edulis</i>	20	M/R	Physiological saline	Time when >50% affected
Norway (IRIS)	<i>Iceland scallop</i> <i>Pandalus borealis</i> <i>Gammarus wilkitzkii</i> <i>G. setosus</i> <i>M. edulis</i> <i>Northern shrimps</i> <i>Arctic amphipods</i>		M/R	Physiological saline Filtered SW	Time when >50% affected
Ireland (Zoology Department, Trinity College Dublin)	<i>M. edulis</i>	10	M/R	Physiological saline	Time when >50% affected

Sweden (Marine Monitoring AB).	<i>M. edulis</i>	10	M	Physiological saline Filtered SW 18°C	Time when >50% affected
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Most of the laboratories reported the use of physiological saline but also physiological saline adjusted to salinity at the sampling sites. Indeed, two laboratories also reported the use of Sea Water. C. Martínez-Gómez (IEO, Spain) reported results of a validation experiment conducted on January 2011 in her laboratory: both physiological saline (PS) and filtered sea water (SW) was assayed by using market mussels (*M. galloprovincialis*) (N=12) acclimatized in laboratory conditions for 24 hours. Results indicated no differences on NRR time between both procedures (SW 137.5 ± 55 min; PS = 132.5 ± 55 min).

All the laboratories were using room temperature but keeping slides incubation chambers with ice water on paper towels on the bottom. The sampling size ranged from 10–20 organisms among ICES/OSPAR laboratories. All laboratories, excepting the Finnish Environment Institute (K. Lehtonen; Finland), indicated that determination of the neutral red retention endpoint is recorded as the time when 50% or more of the cells exhibit lysosomal leakage or outer cell membranes are clearly contracted.

C. Martínez-Gómez reported to WGBEC that WKLYS attempted to identify the dataset that had been used to calculate and establish ACs for lysosomal membrane stability. However, this dataset is actually not available as conclusions were drawn from review of a large number of papers, and many years of practical experience (Professor M. Moore). Nonetheless, a paper describing how assessment criteria were established would be a welcome addition to the scientific literature. WKLYS participants indicated that mean NRR time use to fall into the category of Elevated Response (50–120 min) in field control samples in many of cases. WGBEC proposed that a revision should be conducted on these ACs when NRR time from field data were available in ICES database.

Recommendation 12.1.1: OSPAR/ICES Member Countries that are applying NRR assay in monitoring programmes should make available NRR time data at SGIMC. Also, NRR time data obtained within the framework of ICON workshop.

Recommendation 12.1.2: The ICES TIMES protocol should be amended/extended in relation to practical details and illustration after feedback for D. Lowe and A. Kohler have been received and discussed at SGIMC 2011.

Recommendation 12.1.3: That the need for review of NRR assessment criteria and ICES TIMES is assessed through contact with the ICES TIMES authors and presented to WGBEC at their 2012 meeting.

12.2 Quality Assurance in the BONUS+ project BEAST programme

The BONUS+ project BEAST has taken the lead to perform an intercalibration exercise for PAH metabolites in fish bile, which is needed to promote this biomarker in the international monitoring schemes. Ulrike Kammann (vTI, Germany) presented the results produced by 11 participating laboratories from 9 countries, which covered both the North Sea and the Baltic Sea regions. The intercalibration was free of charge – only the transportation cost of the samples had to be covered by the participants.

Naturally contaminated samples were prepared by mixing bile fluids of fish from various origins. Different concentration levels were obtained by mixing bile samples of dab, flounder, eelpout and some eel from North Sea, Baltic and German rivers respectively. No samples from exposure experiments were included. Every laboratory

could use its own method and protocol for PAH metabolite analysis and received 5 coded samples. The results suggest that the 4 methods described in ICES TIMES no. 39 are suitable for screening purposes but only 3 methods have shown to be comparable and produce results: GC-MS, HPLC-fluorescence and synchronous fluorescence scanning. Results produced by fixed wavelength fluorescence were not included in this evaluation. Assigned values were calculated for 1-hydroxypyrene concentrations an UV absorption. Most z-scores were within the acceptance criteria of ± 2 . Differences between blind replicates were low and acceptable for all but one participant showing the good reproducibility in the laboratories. This intercalibration underlines the ability of 1-hydroxypyrene in fish bile to act as one core parameter in environmental monitoring.

This study was performed with different methods which were not harmonized before, so that the outcome of this intercalibration can be regarded as success. It provides a good starting point for enhancement of precision of the participating laboratories in the future. The comparability of GC-MS and HPLC-F with synchronous fluorescence scanning results should be further improved. In this study a conversion factor taken from literature was applied. This conversion factor should be improved and more metabolites should be included in future intercomparisons. A continuous control of analytical quality is generally recommended.

12.3 QUASIMEME activities

Yearly, QUASIMEME organises an intersex/imposex intercalibration test (BE-1). Usually a sufficient number of labs participate in the Nucella-imposex part, but only a few in the Littorina-intersex part. As there were requests for a training workshop, QUASIMEME tried to organize one in 2009 and again in 2010, including a intercalibration test on-site. However, only few labs registered and the workshop had to be cancelled, as well as the BE-1 exercise. The exercise was not scheduled for 2011. Quasimeme decided to stop the BE-1 exercise until we receive indications that there will be more participation in the (near) future. Currently only 6 or 7 labs seem to be interested, which is not sufficient for a proper data assessment.

However, if sufficient labs express interest, the BE-1 exercise can be started up again.

Members of WGBEC were asked to urge their national labs to express interest in the imposex exercise. Costs will be approx. €1000.

The group will further discuss whether a yearly frequency for intercalibration exercises is necessary, or whether a lower frequency is more applicable.

12.4 Addition requirements for biological effects QA

It was noted by the group that with the imminent adoption of integrated monitoring guidelines by OSPAR and the inclusion of biological effects monitoring to determine GES for Descriptor 8 of MSFD, there is an urgent need to develop external QA for a number of additional measurements that are included in the integrated framework for OSPAR. To help achieve this an intersessional QA steering group (John Thain (UK), Ketil Hylland (NO), Matt Gubbins (UK)) was formed at the 2010 meeting and met immediately prior to WGBEC 2011 to discuss plans for some methods. It was agreed during the meeting that this group would progress with ring trials facilitated by sample exchange for as many methods as possible where existing QA is lacking and report back to the group in 2012.

Recommendation 12.4: That the WGBEC QA steering group initiate sample exchange to facilitate intercalibration for methods recommended for the OSPAR integrated monitoring approach and review the results of intercalibration at WGBEC.

13 Any other business

13.1 PICES

The North Pacific Marine Science Organization (PICES) 2011 annual meeting entitled 'Mechanisms of Marine Ecosystem Reorganisation in the North Pacific Ocean' will be held in Khabarovsk, Russia and is scheduled on 14–23 October 2011. The meeting is hosted by the Russian Federal Agency for Fisheries in cooperation with the government of the Khabarovsk Region and in coordination with the PICES Secretariat.

On 14 October, a one-day workshop, co-sponsored by GESAMP and IOC, will focus on Pollutants in a changing ocean: Refining indicator approaches in support of coastal management. The conveners of the workshop are Peter Kershaw (GESAMP/UK), Olga Lukyanova (PICES/Russia) and Peter Ross (PICES/Canada).

The rationale of the workshop is that many anthropogenic pollutants impact marine environmental quality, with coastal zones being particularly vulnerable. Persistent organic pollutants (POPs) are a concern because they magnify in food webs and present health risks to humans and wildlife. Other chemicals are less persistent, but may nonetheless impact the health of biota. While some pollution indicators are incorporated into monitoring and management regimes in different nations over space and time, new pollutant concerns may not yet be captured by existing protocols. These include "micro-plastics", the breakdown products of debris and other forms of structural pollutants, which can clog the gills of invertebrates and fish, and asphyxiate seabirds and marine mammals. In addition, these micro-plastics may adsorb some of the other chemical contaminants and transfer them to marine organisms. This workshop will review ways in which chemical and structural pollutants enter the marine environment, are transported through ocean currents and/or biological transport, and impact marine biota. The workshop will critically review several examples of pollution indicators used by different nations, as a basis for improving and/or expanding indicator approaches in the North Pacific Ocean. These examples will also critically evaluate the extent to which changing baselines (e.g. climate variability) may impact on source/transport/fate processes and effects on biota, and recommend means of improving the utility and reliability of current indicator / monitoring approaches in a changing world.

The objectives of this workshop are to:

- Critically review 3–5 examples of currently used indicators of marine contamination in different PICES member nations (e.g. shellfish monitoring of PAHs, metals, persistent organic pollutants, fecal bacteria; POPs in seabird eggs and marine mammals); List advantages and disadvantages for each, and describe management/policy linkages; Consider the influence of changing climate on indicator performance and ways to address this.
- Review emergent pollutant concerns and in particular, examine the topic of plastics and micro-plastics as structural pollutants and as mechanisms for the transfer of contaminants to marine biota; Examine existing and/or new opportunities to establish indicator approaches to plastic pollution, and review sampling and analytical methods.

- From these applied examples/case studies, identify opportunities for future PICES activities on the topic of marine pollution:
 - evaluate feasibility of establishing Study Group on Marine Contaminants, including terms of reference, membership, and deliverables;
 - description of the scope of PICES/FUTURE activities that focus on contaminants in the North Pacific marine environment;
 - update and revise MEQ Action Plan elements on marine contaminants;
 - identify potential interactions with IOC/ICES/GESAMP/NOWPAP/NOAA programs that focus on contaminants in the marine environment.

ICES invited Kris Cooreman, member of WGBEC, to act as a keynote speaker of this workshop. Kris Cooreman accepted the invitation.

Recommendation 13. WGBEC member Kris Cooreman should attend the PICES workshop on development of Pacific workplan on contaminants.

14 Actions / Recommendations

Action 4.4: That ICES WGBEC invite a member from WGCAN to present recent the outcome of recent studies on effects of pollutants on Crangon.

Action 5.2: That ICES WGBEC reviews status of integrated assessments from ICON and BONUS BEAST / SGEH and reviews the activity of relevant WGs on integrated assessments (ICES ICG-MSFD and WGHAME) at their 2012 meeting.

Actions relating to WGBEC collaboration with other ICES WGs as identified above. WGBEC to pursue communication with other relevant expert groups as identified below:

6.2: WGEEL – potential participation in the eel quality subgroup by WGBEC members

6.3: WKMAL – Thomas Maes to act as communication link between the two groups

6.4: WGMME – For WGBEC to review the WGMME review on contaminants and effects

6.5: MCWG – For WGBEC to request MCWG to review ICES TIMES manuscript on DR-LUC and extraction methods and respond to SGIMC for their meeting.

6.6: SGEH – To maintain contact with the SG and review their final report

6.7: SGIMC – For WG members to attend the final SGIMC meeting and contribute to the process. For WGBEC to provide SGIMC with documentation for their 2011 meeting and review output in 2012.

6.8: MEDPOL – To support and participate in any AQC initiatives that MEDPOL may organise and maintain contact / pursue mutual areas of interest on future WGBEC agendas – (Recommendation for ICES to tell MEDPOL!)

6.9: WGMS – Explore common area of interest on use of passive samplers for bioassays and send WGMS the draft TIMES manuscript on extraction methods for bioassays for comment.

6.10: Chairs to re-establish contact with other EGs already identified where no contact has yet been received.

Recommendation 7.1: That ICES/OSPAR SGIMC recommend to OSPAR that a background document on biological effects assessment criteria should be produced and that WGBEC be invited to update this annually as new data become available.

Recommendation 7.2: After the cessation of ICES/OSPAR SGIMC and ICES SGEH, WGBEC reviews background documentation, assessment criteria and applications of integrated assessment frameworks across the ICES area at its annual meetings.

Recommendation 8.1: That biological effects measurements recommended for integrated assessment by ICES/OSPAR SGIMC are used for reporting on MSFD GES descriptor 8, Commission indicator 8.2.1.

Recommendation 8.2: That WGBEC review any available initial assessments in relation to Descriptor 8 at their next meeting and make recommendations on the development of national monitoring programmes for assessing GES for Descriptor 8.

Recommendation 9.2: that at a future meeting WGBEC recommends methods suitable for determining the effects of acidification on marine organisms.

Recommendation 9.6: That background documentation and assessment criteria are developed for measuring lysosomal membrane stability in fish.

Recommendation 9.7: That an intersessional technical meeting in autumn 2011 in the UK (led by Cefas Weymouth) to identify priority areas for technical cooperation in marine organism immunotoxicology and that WGBEC receives a report on progress of intersessional work and strategy for taking this work area forward at its 2012 meeting.

Recommendation 10: That draft resolutions be sought from ICES for publication of TIMES manuscripts for:

- Reproductive success in eelpout by Jakob Strand (deadline March 2012)
- Alkylphenol bile metabolites by G. Jonsson *et al.* (deadline Oct 2011)
- Sea urchin embryo bioassay by Beiras *et al.* (deadline Oct 2011)

Recommendation 11.1: That member states should be submitting the full range of their national biological effects monitoring data to the ICES data centre

Recommendation 11.2: That an intersessional subgroup be formed to address data centre issues for biological effects data and report annually to WGBEC

Recommendation 12.1.1: OSPAR/ICES Member Countries that are applying NRR assay in monitoring programmes should make available NRR time data at SGIMC. Also, NRR data obtained within the framework of ICON workshop.

Recommendation 12.1.2: The ICES TIMES protocol should be amended/extended in relation to practical details and illustration after feedback for D. Lowe and A. Kohler have been received and discussed at SGIMC 2011.

Recommendation 12.1.3: That the need for review of NRR assessment criteria and ICES TIMES is assessed through contact with the ICES TIMES authors and presented to WGBEC at their 2012 meeting.

Recommendation 12.4: That the WGBEC QA steering group initiate sample exchange to facilitate intercalibration for methods recommended for the OSPAR integrated monitoring approach and review the results of intercalibration at WGBEC.

Recommendation 13: WGBEC member Kris Cooreman should attend the PICES workshop on development of Pacific workplan on contaminants.

15 Adoption of the report and closure of the meeting

A draft report was compiled during the week and the text and recommendations were reviewed by the group. The report when fully completed would be circulated to all members for final editing and comment subject to forwarding to the ICES Secretariat.

The meeting closed at 12:00 hr on 18 February. The Chair Matt Gubbins thanked all the participants for their contributions and gave a special thanks to Ricardo Beiras and Jose Fumega for hosting the meeting.

WGBEC will report by 1 April 2011(via SSGHIE) for the attention of SCICOM and ACOM.

16 List of Annexes

Annex 1. List of attendees

Annex 2. WGBEC Terms of Reference 2010

Annex 3. Agenda

Annex 4. Timetable and Raporteurs

Annex 5. Extraction procedures for bioassay methods

Annex 6. Intersex in fish

Annex 7. Background document on supporting parameters

Annex 8. Acetylcholinesterase

Annex 9. Mussel histology

Annex 10. Micronucleus and comet assay

Annex 11. Sediment and elutriate bioassays for invert bioassays

Annex 12. Sediment and elutriate bioassays with copepods

Annex 13. Update whole sediment bioassay AC

Annex 14. Candidate EAC values for new species

Annex 15. WGBEC draft Terms of Reference for the next meeting

Annex 1: List of participants

Name	Address	Phone/Fax	Email
Ricardo Beiras	Universidad de Vigo Campus Universitario C.P. Illa de Toralla, s/n ES-36330 Vigo (Pontevedra) Spain	TEL: +34 647 343 025 FAX: +34 986 498 626	rbeiras@uvigo.es
Stephen Brooks	Ecotoxicology and Risk Assessment Team Norwegian Institute for Water Research (NIVA) Gaustadalleen 21 NO-0349 Oslo Norway	TEL: +47 22 18 5100 FAX: +47 22 18 5200	sbr@niva.nr
Thierry Burgeot	IFREMER rue de l'Île d'Yeu B.P. 21105 F-44311 Nantes Cédex 03 France	TEL: +33 240374051 FAX: +33 240374075	tburgeot@ifremer.fr
Jose Fumega	Instituto Espanol de Oceanografia Centro Oceanografico, de Vigo Subida a Radio Faro, 50 36390 Vigo Pontevedra Espana	TEL: +34 986 492111 FAX: +34 986 498 626	jose.fumega@vi.ieo.es
Juan Bellas Bereijo	Instituto Espanol de Oceanografia Centro Oceanografico, de Vigo Subida a Radio Faro, 50 36390 Vigo Pontevedra Espana	TEL: +34 986 492111 FAX: +34 986 498 626	Juan.bellas@vi.ieo.es
Lucia Guilhermino	Universidade do Porto CIIMAR & ICBAS Laboratory of Ecotoxicology and EcologyRua dos Bragas, 177 4050-123 Porto Portugal	TEL: +351 223401815	lguilher@icbas.up.pt
Matt J. Gubbins	Marine Scotland Science Marine Laboratory 375 Victoria Road Aberdeen AB11 9DB United Kingdom	TEL: +44(0)122 429 5681	matthew.gubbins@scotland.gsi.gov.uk
Ketil Hylland	Norwegian Institute for Water Research (NIVA) Gaustadalléen 21 N-00349 Oslo Norway	TEL: +47 41451694	ketil.hylland@niva.no ketilhy@bio.uio.no

Name	Address	Phone/Fax	Email
Ulrike Kammann	Institut f. Fischereiökologie BFA f. Fischerei Palmaille 9 22767 Hamburg Germany	TEL: +49 4038905198	ulrike.kammann@ifo.bfafisch.de
Klaas Klaag	IMARES Wageningen UR Ambachtsweg 8a P.O. Box 57 1780 AB Den Helder The Netherlands	TEL: +31 317487129 FAX: +31 317487371	klaas.kaag@wur.nl
Joel Knoery	Département Biogéochimie et Ecotoxicologie Ifremer BP 21105 44311 Nantes Cedex 3	TEL: +33 (0) 2 40 37 43	Dcsmmd8@ifremer.fr
Kari Lehtonen	Finnish Institute of Marine Research P.O. Box 2 FIN-00 561 Helsinki Finland	TEL: +358 9613 94566 FAX: +358 9323 2970	lehtonen@fimr.fi
Thomas Maes	CEFAS Pakefield Road NR330HT Lowestoft	TEL. +44 (0)1502 524433	thomas.maes@cefass.co.uk
Concepción Martínez	Instituto Español de Oceanografía Centro Oceanográfico de Murcia Varadero 1, Lo Pagán 30740 San Pedro del Pinatar (Murcia) Spain	TEL: +34 968180500 FAX: +34 968184441	concepcion.martinez@mu.ieo.es
Steinar Sanni	IRIS Biomiljø Norway	TEL: +47 5187 5504 FAX: +47 5187 5540	Steinar.Sanni@iris.no
John Thain (Chair)	CEFAS Burham-on-Crouch Laboratory Remembrance Avenue Burnham-on-Crouch UK-Burnham-on-Crouch CM0 8AH United Kingdom	TEL: +44 (0)1621787239 FAX: +44 (0)1621784989	j.e.thain@cefass.co.uk
Dick Vethaak	Deltares, Unit Marine and Coastal Systems, Section Ecosystem Analysis and Assessment (ESA) Rotterdamseweg 185 2629 HD Delft The Netherlands	TEL: +31 15- 2858659 / +31 651232412	dick.vethaak@deltares.nl

Name	Address	Phone/Fax	Email
Izaskun Zorita	AZTI - Tecnia / Itsas Ikerketa Saila Herrera Kaia. Portualdea z/g 20110 - Pasaia (Gipuzkoa) Spain	TEL: 94 6574000 (Ext. 706) - FAX: 94 6572555	izorita@azti.es
Kris Cooreman	ILVO Institute for Agricultural and Fisheries Research Ankerstraat 1 8400 Oostende Belgium	TEL: +32 59569820 FAX: +3259330629 Mob: +32496252460	Kris.Cooreman@ilvo.vlaanderen.be

Annex 2: WGBEC Terms of Reference (ToRs) 2010

2010/2/SSGHIE02 The **Working Group on Biological Effects of Contaminants (WGBEC)**, chaired by Matt Gubbins*, UK, and John Thain, UK, will meet in Vigo, Spain, from 14–18 February 2011 to:

- a) Review progress with publication and electronic dissemination of biological effects techniques in the ICES TIMES series;
- b) Respond to requests for advice from the ICES Data Centre on reporting formats and codes for entering biological effects data into the ICES database and to consider whether further data entry guidance is required for new methods that may be required for the developing ICES/OSPAR integrated monitoring framework;
- c) Review developments in biological effects monitoring activity undertaken by member states and to address specifically activity to meet requirements of e.g. OSPAR, HELCOM and MEDPOL, integrated assessment of monitoring data and approaches proposed to address Good Environmental Status under Descriptor 8 of the MSFD.
- d) Review progress with AQC procedures for biological effect methods and include harmonisation activities initiated from WGBEC and within OSPAR, Baltic and MEDPOL maritime areas;
- e) Update on MSFD activities in relation to contaminants and biological effects
- f) Review progress with the ICON (NSHEALTH) and Baltic BEAST programme;
- g) Consideration of issues of special scientific interest / value
 - i) Acidification in marine waters in relation to contaminants and biomarker response.
 - ii) Effects of contaminants on primary production, including phytotoxicity.
 - iii) Relationship of genetic markers to biomarkers.
 - iv) Review of species differences in bioassay and biomarker responses eg as seen in assessment criteria currently being developed – also to include sources of species for testing.
 - v) Lysosomal stability in fish – review use and application in fish – histochemical method.
 - vi) Immunotoxicity end points – suitability for monitoring.
- h) View the output from SGIMC 2010 and respond to their request for support
- i) Evaluate potential for collaboration with other EGs in relation to the ICES Science Plan and report on how such cooperation has been achieved in practical terms (e.g. joint meetings, back-to-back meetings, communication between EG chairs, having representatives from own EG attend other EG meetings).

WGBEC will report by 1 April 2011 (via SSGHIE) for the attention of SCICOM.

Supporting information

Priority	The activities of this group will enable ICES to advise on issues relating to
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	the design, implementation and execution of regional research and monitoring programmes pertaining to hazardous substances in the marine environment. To develop procedure for quality assurance of biological effects data and to improve assessments of data relating to the biological effects of contaminants in the marine environment.
Scientific justification	<p>Term of Reference a) It is important for WGBEC to keep track of publication progress with biological effects methods it has sponsored. Protocols are needed for national and international programmes as well as the OSPAR programmes.</p> <p>Term of Reference b) Biological effect data is increasingly being submitted to the ICES database and technical queries arise and WGBEC can assist with answering queries from the ICES Data Centre.</p> <p>Term of Reference c) WGBEC has found it of value to discuss, feedback and support national monitoring programmes across the maritime areas and this is a valuable opportunity to improve and harmonise programme designs and assessment of data (e.g. OSPAR / MEDPOL / WFD / HELCOM / EU FWM);</p> <p>Term of Reference d) AQC is vital to support, report and assess data, particularly for cross maritime areas and developments and harmonisation in this area need to be taken forward in a coordinated manner.</p> <p>Term of Reference e) Some members of WGBEC have contributed to EUMSFD Task Group activities in respect of Descriptor 8 on contaminants and effects in the marine environment. It is important to update the group on this activity and to communicate how different countries are developing and defining GES in respect of biological effects.</p> <p>Term of Reference f) The ICON demonstration programme, finishing in 2010 and the on-going Baltic Beast programme underpins the integrated chemical – biological effects approach advocated by OSPAR and in the Baltic. WGBEC needs to monitor and evaluate these activities.</p> <p>Term of Reference g) There are a number of issues identified by WGBEC that are of value and special scientific interest to understanding the effects of contaminants in the marine environment e.g. acidification and primary production.. It is important that these are reviewed/assessed and taken forward, in relation to the wider aspects of environmental management and secondly in the development and application of techniques for assessment purposes.</p> <p>Term of Reference h) SGIMC has requested that WGBEC further develops specific assessment criteria for biological effects methods and develop and review OSPAR background documents for certain methods. This is required for the development of an integrated contaminant and biological effects monitoring framework.</p> <p>Term of Reference i) This is a general ToR for all Expert Groups under SSGHIE.</p>
Resource requirements	The main input to this group is from National experts. Each attendee is self-funded from their own / organisation / institute resources.
Participants	The Group is normally attended by ca. 16 members and guests.
Secretariat facilities	None required.
Financial	No financial implications.
Linkages to advisory	ACOM

committees	
Linkages to other committees or groups	There are linkages with WGSaEM, MCWG, WGMS and WGPDMO.
Linkages to other organizations	None identified

Annex 3: Agenda

The Working Group on Biological Effects of Contaminants [WGBEC]

Vigo, Spain, 14–18 February 2011

- 1) Opening of the meeting;
- 2) Adoption of the agenda;
- 3) Appointment of rapporteurs;
- 4) Review developments in biological effects monitoring activity undertaken by member states and to address specifically activity to meet requirements of e.g. OSPAR, HELCOM and MEDPOL, integrated assessment of monitoring data and approaches proposed to address Good Environmental Status under Descriptor 8 of the MSFD+ any other; (ToR c).
- 5) Review progress with the ICON (NSHEALTH) and Baltic BEAST programme; (ToR f).
- 6) Evaluate potential for collaboration with other EGs in relation to the ICES Science Plan and report on how such cooperation has been achieved in practical terms (e.g. joint meetings, back-to-back meetings, communication between EG chairs, having representatives from own EG attend other EG meetings): (ToR i).
- 7) View the output from SGIMC 2010 and respond to their request for support; (ToR h).
 - a) Extraction procedures for bioassay methods
 - b) Intersex in fish
 - c) Background document on supporting parameters
 - d) Acetylcholinesterase
 - e) Mussel histology
 - f) Micronucleus and comet assay
 - g) In vitro YES/YAS, ER CALUX assays
 - h) Sediment and elutriate bioassays for invert bioassays
 - i) Sediment and elutriate bioassays with copepods
 - j) Update whole sediment bioassay AC
- 8) Update on MSFD activities in relation to contaminants and biological: (ToR e).
- 9) Consideration of issues of special scientific interest /value
 - a) Acidification in marine waters in relation to contaminants and biomarker response.
 - b) Effects of contaminants on primary production, including phytotoxicity.
 - c) Relationship of genetic markers to biomarkers.
 - d) Review of species differences in bioassay and biomarker responses eg as seen in assessment criteria currently being developed – also to include sources of species for testing.
 - e) Lysosomal stability in fish – review use and application in fish – histochemical method.
 - f) Immunotoxicity end points – suitability for monitoring. (ToR g).

- 10) Review progress with publication and electronic dissemination of biological effects techniques in the ICES TIMES series; (ToR a).
- 11) Respond to requests for advice from the ICES Data Centre on reporting formats and codes for entering biological effects data into the ICES database and to consider whether further data entry guidance is required for new methods that may be required for the developing ICES/OSPAR integrated monitoring framework; (ToR b).
- 12) Review progress with AQC procedures for biological effect methods and include harmonisation activities initiated from WGBEC and within OSPAR, Baltic and MEDPOL maritime areas and to include report of Lysosomal Membrane Stability Workshop held in Alessandria, Italy; (ToR d).
- 13) Any other business;
- 14) Recommendations and action list;
- 15) Adoption of the report and closure of the meeting;
- 16) WGBEC will report by 1st April 2011 (via SSGHIE) for the attention of SCICOM and ACOM.

Annex 4: Timetable and rapporteurs/contributors

DATE	APPROX. TIME	AGENDA ITEM	RAPPORTEURS CONTRIBUTORS	ISSUE
Monday 14 th Feb	09:00	1	MG/JT	Introduction by Chairperson and Ricardo Beiras, housekeeping issues, <i>tour de table</i> .
	10:00	2	MG/JT	Adoption of agenda, tabling of documents
	10:15	3	MG/JT	Appointment of rapporteurs.
		6	JT	Evaluate potential for collaboration with other EGs in relation to the ICES Science Plan
	12:45			Lunch
	13:30	10	MG/RB	Review progress with publication and electronic dissemination of biological effects techniques in the ICES TIMES series
		11	MG	Respond to requests from ICES data centre
	17:18:00			Close of business.
Tuesday 15 th Feb	09:00	7	MG/JT	View the output from SGIMC and respond to their request .
			JT/SB	a) Extraction methods
			All / Subgroup?	b) Intersex in fish - REVIEW
			JT	c) Bgd on supporting parameters – REVIEW
			TB/KL	d) Acetylcholinesterase - REVIEW
			All / subgroup	e) Mussel histology - REVIEW
			CM/ F Akcha?	f) Micronuclei and COMET
	12:45			Lunch
	14:30	12	MG/JT/KH	Review progress with AQC procedures for biological effect methods and include harmonisation activities within OSPAR, Baltic and MEDPOL maritime areas.
	17:30			Close of business.
Wednesday 16 th Feb	09:00	7	RB	h) Sediment elutriate bioassay with inverts - REVIEW
			RB	i) Sediment elutriate bioassays with copepods - REVIEW
			RB / KK	j) Update sediment bioassay AC - REVIEW
				g) In vitro Yes /YAS - ??? Plan of action
			JT	a) Extraction procedures
			KH	f) COMET AC
		5	KH/KL	Review progress with ICON and BONUS BEAST
	12:45			Lunch
	14:30	12	CMG	LMS AQC
		8	TM	Update on MFSD activities in relation to contaminants and bio effects
	17:30			Close of business
Thursday 17 th Feb	09:00	9		Issues of special scientific interest a) – g)
	12:45			Lunch
	14:30	4		Review progress with national /international monitoring activities; within OSPAR / MEDPOL / WFD / HELCOM / EU MSD + any other
	16:30			ToR / venue next year
				Check report text all available / coming
		13		Any other business.
	17:30			Close of business.
Friday 18 th Feb	09:00	14		Recommendations and action list.
	10:30	15		Adoption of the report.
	12:30			Lunch
	14:00			Closure of the meeting.

Annex 5: Extraction procedures for bioassay methods

Protocols for extraction, cleanup and solvent exchange methods for *small-scale* bioassays.

Hans Klamer¹, Knut-Erik Tollefsen², Steven Brooks² John Thain³

1. Centre for Water Management. PO Box 207, 9750 AE Haren, the Netherlands.
e-mail: hans.klamer@rws.nl
2. Norwegian Institute for Water Research (NIVA). Gaustadalleen 21, 0349 Oslo, Norway.
3. Centre for Environment, Fisheries and Aquaculture Science, Weymouth Laboratory, The Nothe, Barrack Road, Weymouth, DT4 8UB, UK.

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1. Introduction

The aims of this document are as follows:

- To produce standardised protocols for bioassay extractions;
- To enhance consistency of applications between laboratories;
- To ensure applicability throughout OSPAR maritime area, including in estuarine waters;
- To ensure comparability of reported data for assessment purposes.

History:

- This document has been developed from a previous review, and relates particularly to background documents on water and sediment bioassays and *in vitro* bioassays prepared by ICES expert groups WGBEC and SGIMC. This paper describes a recommended methodology for extraction protocols for use of small scale *in vitro* and *in vivo* bioassays.

Scope:

- This procedure will be used to provide samples for measurements of toxicity in environmental samples and assessment of their potential environmental risk. Other applicable approaches include Toxicity Identification Evaluation (TIE)/ Effects Directed analysis (EDA), and toxicity tracking of effluent and produced water discharges;
- Extraction of aqueous, solid and fish bile samples;
- Preparation of extracts for *in vivo* bioassays including: Mussel and Oyster embryo, Tisbe, Daphnia, Nitocra, Acartia, Sea urchin embryo, fish embryo, algal growth, algal PAM, macrophyte germination);
- Preparation of extracts for *in vitro* bioassays (e.g. Microtox, Mutatox, YES, YAS, DR/ER/AR-CALUX, TTR, *umu*-C, Ames-II, fish cell lines).

2. Extraction protocols

In this chapter, extraction protocols will be presented covering a range of types of sample: solid, aqueous or fish bile. Depending on the bioassay that will be used, differences in extraction solvent and, in particular, sample cleanup (Section 2.5), may be applied.

Klamer *et al.*, 2005, proposed the following operational definitions of solid and aqueous samples:

- *solid samples*: particulate material, sediments, sludges, aerosols, suspended solids, and soils;
- *aqueous samples*: surface or deep waters, waste water, sediment pore water, potable water, rain, snow, ice.

Before detailed protocols are presented, the basic layout of each extraction and cleanup protocol is given below.

Solid samples

Protocol steps	Comment
1. Sample preparation	Sample sieved when necessary (e.g. sediment), dried and homogenised.
2. Extraction of crude sample	Accelerated Solvent Extraction (ASE) or Soxhlet extraction. Solvents: dichloromethane (DCM) or hexane with methanol or acetone as

	modifier.
3. Concentration of crude extract	Automatic (e.g. Turbopap® or manual) concentration to smaller volume, typically less than 5 mL. Remove co-extracted water if necessary
4. Cleanup of crude extract	Gel Permeation Chromatography (GPC) with DCM for broad-spectrum contaminant profiling. Reversed or normal phase HPLC for more selectivity. Sulfur removal may be necessary.
5. Concentration of cleaned extract	Automatic (e.g. Turbopap® or manual) concentration to smaller volume, typically less than 1 mL. Final test solvent (e.g. DMSO or methanol may be added as keeper.)

Aqueous Samples

Protocol steps	Comment
1. Sample preparation	Sample filtered and/or pH-adjusted when necessary.
2. Extraction of crude sample	Solid Phase Extraction (SPE) with resin (e.g. XAD) or cartridge containing adsorbents (C8, C18, lichrolut™, POCIS)
3. Concentration of crude extract	Automatic (e.g. Turbopap® or manual) concentration to smaller volume, typically less than 5 mL
4. Cleanup of crude extract	Gel Permeation Chromatography (GPC) with DCM for broad-spectrum contaminant profiling. Reversed or normal phase HPLC for more selectivity
5. Concentration of cleaned extract	Automatic (e.g. Turbopap® or manual) concentration to smaller volume, typically less than 1 mL. Final test solvent (e.g. DMSO or methanol may be added as keeper.)

Fish bile samples

Protocol steps	Comment
1. Sample preparation	Thaw on ice.
2. Pre-treatment of crude sample	Deconjugation with a mixture of water, sodium acetate buffer and β -glucuronidase-arylsulfatase. Total volume typically 1.5 mL.
3. Extraction of pre-treated sample	pH treatment with 100 μ L 1N HCl, extraction with 2 mL ethyl acetate.
4. Cleanup of crude extract	Precipitate any formed protein using isopropanol. Centrifugate. Repeat extraction.
5. Concentration of extract	Manual concentration to dryness of combined ethyl acetate phases using N ₂ , solvent exchange into 50 μ L DMSO.

2. 1 Protocol for extraction of dried, solid samples with Accelerated Solvent Extraction (5 g sample). Steps are numbered S.1, S.2 etc.

- S.1. Assemble the ASE cells. Add a small layer of dried silica until cellulose filter is no longer visible;
- S.2. Weigh approximately 5 gram dried sample in the ASE cells (weighing accuracy mass \pm 0.1%);
- S.3. Fill the ASE cells with dried silica and compact the content of the cells with the engraver pen. Close the cell and firmly twist the end-cap on the ASE cell;
- S.4. Extract the sample using the following ASE settings:

Solvent	Pressure [psi]	Temp [°C]	Preheat time [min]	Static time [min]	Flush volume [mL]	Purge time [sec]	Static Cycles
Hexane/Acetone 9:1 v:v	2000	100	5	5	60	90	3
DCM or DCM/modifier**	2000	45–100*	5	5	60	90	1–3*

* Set temperature to 45 to 50°C and # of cycles to 3 for use with ER-CALUX and similar tests.
 ** methanol or acetone

- S.5. If water is co-extracted, dry the extract using anhydrous sodiumsulphate. Rinse with solvent. Evaporate the extract (until approximately 2–5 mL is left), in an automatic or manual set-up;
- S.6. Proceed to solvent exchange (Section 3.3) or store the crude extract at –20°C until further use.

2.2 Protocol for extraction of aqueous samples with Solid Phase Extraction devices. Steps are numbered A.1, A.2 etc.

Extraction

- A.1. Assemble the SPE cartridge. For samples up to 20L, a single column set-up is used. A Teflon tube is filled with glass wool to remove particulates and then the SPE columns are filled with methanol and attached in series with the C8 column first, followed by the ENV+. For 100 l samples, a multi column system is used, where 6 Teflon tubes are set up as with the single column system, but then attached to a manifold, allowing one sample to pass through all 6 columns simultaneously.
- A.2. Set up the pressure system. From the pressure source, the air line passes through an air filter and then into a manifold. This allows for more than one vessel to be run at any given time, and also the airline diameter to be reduced. This line is then connected to the pressure vessel via a needle valve, ensuring the correct inlet/outlet is used (the inlet for the air is just a hole in the top of the vessel, the outlet has a pipe which goes to the bottom). From the outlet, another tube is connected which goes into the top of the single column system or manifold for the multi column set-up.
- A.3. Once the pressure lines are set up, the air line can be switched on, ensuring first that all needle valves are closed. The pressure should be no greater than 2 bar. The valve can then slowly be opened to allow a flow of approximately 40 ml min⁻¹ through the columns.
- A.4. Once all the sample has passed through the column, allow the columns to dry by passing air through them. Label each column with sample site. Wrap in hexane rinsed foil and store in a freezer at –20 °C. Samples can be stored in the freezer for up to 2 months before elution.

Elution

- A.5. Remove columns from the freezer and, while they are thawing, solvent rinse two glass sample collection tubes per column. Label the sample tubes.
- A.6. In a fume cupboard, place the columns in the vacuum unit, with a Teflon tap. Fit a length of vacuum-proof hose to the unit, attaching the other end to a

waste barrel. Another length of hose should run from the barrel to a vacuum pump.

- A.7. Wash the columns with 10 ml RO or milliQ water. This will help to remove salt from saline samples.
- A.8. Ensure columns are dry by sucking under vacuum for 10 minutes, or until there is no visible water dripping through the columns (whichever is longer).
- A.9. Place a labelled collection tube under each column in a rack.
- A.10. Elute each column with 10 ml DCM. Add 1 ml DCM to the column and allow to soak for 1 minute with the tap closed. Open the tap and allow the solvent to drip through. Repeat this 3 times with 1 ml, 4 ml and 4 ml DCM respectively.
- A.11. Remove the tube from under each column and replace it with a clean one. Repeat section A. 7 with methanol.
- A.12. Reduce the samples in volume to approximately 1 ml, and then combine the 4 fractions of each sample (C8 DCM, C8 methanol, ENV+ DCM, EMV+ methanol). For 100 l samples, there will be 6 of each type of column. Combine all fractions.
- A.13. There may be some water in the samples. This will form a layer or droplets in the DCM. If this is the case, take a glass column and packed with hexane washed anhydrous sodium sulphate. Add the samples to the top of the column. Elute with 5 ml DCM and collect in a labelled tube.
- A.14. Blow down each extract to approximately 5 ml using e.g. a Turbovap at 30 °C, 5 psi oxygen free nitrogen. From this point, aliquots of samples can be solvent exchanged into the appropriate solvent depending on the assay in question (see paragraph 3.3). Transfer sample into a glass Store extracts in freezer at -20 °C. Samples can be stored for a maximum of 1 year.

2.3 Protocol for extraction of fish bile samples. Steps are numbered B.1, B.2 etc.

The extraction procedure described below is taken from the work by Legler *et al.*, 2002.

Extraction

- B.1. Thaw bile samples.
- B.2. Transfer 100 µL of bile to glass test tubes.
- B.3. Add 700 µL sodium acetate buffer (100 mM, pH 5.0 at 37°C), followed by 600 µL distilled water and 40U of β-glucuronidase-arylsulfatase (from *H. pomatia*).
- B.4. Incubate tubes overnight (17–18 h) in a water bath (37°C, gentle shaking).

3. Cleanup

3.1 Broad-spectrum Cleanup

Cleanup procedures are applicable to all crude extracts. However: the user has to choose between two fundamentally different cleanup principles: *broad spectrum* or *target* cleanup.

Gel Permeation Chromatography with DMC as eluting solvent provides a sample with contaminants having a *broad spectrum* of physico-chemical properties. GPC separates on molecular volume and may therefore be used to easily remove, *inter alia*, humic acids and lipids. GPC column material, however, also has a secondary retention mechanism, based upon electronic interaction between the column material and

the extracted compound. This secondary mechanism is used for removal of molecular sulphur (as S₈) from the crude extract, using DCM as eluting solvent. GPC cleanup requires careful calibration using a series of different compounds. This type of cleanup has successfully been applied to very different *in vitro* bioassays: Microtox, Mutatox, (anti)DR-CALUX, (anti)ER-CALUX, *umu*-C (e.g. by Klammer *et al.*, 2005 and Houtman *et al.*, 2004).

- C 1. Set up of GPC equipment. For semi-preparative cleanup, large-diameter columns may be used in series, e.g. polystyrene-diphenylbenzene copolymer columns (PL-gel, 5 or 10 µm, 50 Å, 300x25 mm or 600x7.5 mm, preferably in a thermostatic housing at 18°C, with a PL-gel pre-column 5 or 10 µm 50x7.5mm). Use an HPLC pump with 10 ml/min dichloromethane as eluents.
- C 2. Calibration. When necessary, determine the elution profile of individual compounds by injection of 2 ml of standard solutions (concentration 0.5–10 mg/L) and assessment of retention times at peak maximum and peak shape.
- C 3. Set-up of the fraction collector. As a rule of thumb, the elution of *parathion* may be used to trigger the start of the collection of the cleaned sample, while the collection is stopped just before sulphur (as S₈) elutes (elution of the extract is monitored using a UV detector at 254 nm.) This range, however, should be carefully monitored using a several reference compounds (in DCM solution). Examples of compounds that may be included in this mixture are: sulfur, pyrene and ethyl-parathion. Depending on the particular application, other reference compounds may be needed (see e.g. Houtman *et al.*, 2004).
- C 4. Inject crude extract in 200 - 2000 µL batches, depending on the capacity of the GPC column (semi prep 25 mm column may be loaded with 2000 µL). Concentrate the collected sample fractions, proceed to solvent-exchange (see paragraph 3.3) or store at –20°C until further use.

3.2 Selective or dedicated cleanup

Selective cleanup using adsorption chromatography (e.g. reversed or normal-phase liquid chromatography, with or without modifying additives like KOH, AgNO₃).

DR CALUX

The clean-up of crude extracts for DR CALUX measurements can be done with an acid silica column combined with TBA sulfur clean-up. The protocol for the DR-CALUX cleanup is as follows:

TBA sulphite solution

Wash a 250 ml separation-funnel with hexane, fill the funnel with 100 ml HPLC water and dissolve 3.39 grams TBA
 Rinse the solution three times with 20 ml hexane.
 Dissolve 25 gram sodium sulphite in the washed solution.
 Store the solution in a dark bottle (Maximum storage time 1 to 2 weeks).

Sulfur clean-up

Add 2.0 ml TBA-sulphite solution and 2.0 ml isopropanol to the extract, mix for 1 minute on a vortex. Sulfur clean-up is complete if precipitation is visible. Add an extra 100 mg sodium sulphite if no

precipitation is present and mix during 1 minute on a vortex. Repeat the addition if necessary.

Add 5 ml of HPLC-grade water, mix for 1 minute on a vortex.

Let the layers separate during approximately 5 minutes, transfer the hexane layer to a clean collection vial.

Add 1 ml hexane to the extract and mix during 1 minute on the vortex. Let the layers separate and transfer the hexane layer to the clean collection vial. Repeat this step. Evaporate the hexane until approximately 1 ml is left.

Acid silica clean-up

Prepare a solution of hexane/diethylether (97/3; v/v)

Place a small piece of glass wool in a separation funnel. As the performance of the following steps are column-dependent (see Appendix 1 for column layout).

Fill the column with 5 grams of 33% silica and tap the column with the engraver pen. Add 5 grams of 20% silica and tap the column once more. Add a small amount of dried sodium sulphate to the top of the column.

Elute the column with 20 ml hexane/diethylether solution.

Bring the extract on the column as soon as the meniscus reaches the sodium sulphate. Wash the collection vial of the extract twice with approximately 1 ml hexane/diethylether solution.

Place a clean collection vial under the column and elute the column with 38 ml hexane/diethylether.

Evaporate the hexane until less than 1 ml is left.

Proceed to solvent exchange (see below, 3.3)

ER CALUX

This section describes the cleanup of deconjugated fish bile extract for use in the ER-CALUX assay. Steps are numbered B5, B6, etc, referring to the fish bile extraction procedure above.

- B.5. Add 100 μ L 1N HCl to each glass test tube containing the deconjugated bile sample (see B.1., above). Stir well (Vortex).
- B.6. Add 2 mL ethyl acetate to each test tube. Vortex for 1 min, followed by centrifugation for 5 min at 3,800 rpm.
- B.7. Remove the ethyl acetate fraction using a Pasteur pipette and transfer this to a new test tube. If protein formation is observed between the water and solvent phases, precipitate this protein by adding 500 μ L of isopropanol after centrifugation.
- B.8. Repeat steps B6 and B7 three times, with exception of the isopropanol-step.
- B.9. Concentrate the collected ethyl acetate fractions and evaporate to a small drop under a gentle N₂ gas flow at 37°C.
- B.10. Transfer the concentrated extract to a conical glass vial.

- B.11. Rinse the glass test tube three times with ethyl acetate, and transferred the rinses to the conical vial.
- B.12. Evaporated the ethyl acetate to dryness at 37°C under a gentle stream of nitrogen.
- B.13. Proceed to solvent exchange (see below, 3.3).

3.3 Solvent exchange

Klamer and van Loon (1998) and Bakker *et al.* (2007) developed criteria and evaluated co-solvents for bioassays. The ideal co-solvent or carrier solvent used for ecotoxicity testing should meet the following criteria: (1) effective: sufficiently high solubility of target compounds, (2) water-miscible: the carrier solvent must be water-miscible, and (3) non-toxic: the carrier solvent should have little or no adverse effects on test organisms or cells at typical test concentrations in aqueous media (usually 0.1% v/v). The authors tested 10 different solvents, with the following final ranking for the first five solvents:

Solvent	Final rank
Dimethylsulfoxide (DMSO)	1
2-Propanol	2
Acetone	2
Methanol	4
Ethanol	5

The following general solvent-exchange protocol is applicable to all five solvents:

1. Transfer the remaining cleaned extract to a conical vial and evaporate until a small meniscus of it is left (approximately 20 µl).
2. Wash the collection vial twice with at least 0.5 ml DCM or other appropriate solvent, and transfer this to the conical vial (evaporate between washes; do not let the vial fall dry).
3. Evaporate the extract until the meniscus reaches the bottom of the conical vial and then add 50 µl of co-solvent.

4 Preparation of extract test dilutions for *in vivo* bioassay

The following procedure should be employed when using the prepared extract(s) for standard *in vivo* bioassay testing. This approach is focused on microscale tests with a typical test volume of no more than 5 ml.

Once prepared using the above extraction procedure, the extract must be stored at minus 20°C degrees C until bioassayed, and should not be stored for longer than 12 weeks.

A stock solution is made with the concentrated extract using the appropriate dilution water (i.e. aerated seawater or freshwater), from which an appropriate series of concentrations will be prepared. The preparation of the stock solution is important: typically 5 ml of extract in solvent is concentrated by evaporation to 20µl. The concentration series must be made up on the day of testing and the ratio between the concentrations should not exceed 2.2 (usually log).

The stock solution must be shaken vigorously, stirred on a magnetic stirrer for at least 30 minutes or placed in the ultrasonic bath for 10 minutes to ensure that all of the chemical/compound(s) within the extract are in solution. The solvent concentration in the final test solution must not exceed 0.1 ml/L with all test concentrations containing

the same amount of solvent. A solvent control of the appropriate solvent at the same concentration must be used. All controls and test concentrations must have at least three replicates. The salinity, pH, temperature and dissolved oxygen concentration of the test concentrations must be checked prior to testing and corrected to within the specific parameters of the bioassay as appropriate.

Where possible, the concentrations selected should cover a range from low concentrations with no effect on the test organism relative to the control, intermediate effects, and complete 100% effect. Clearly, this may require an initial sighting test prior to conducting a definitive test. This will enable the calculation of the NOEC, LOEC and EC₅₀ values with greater precision.

Preparation of extracts for cell lines

DMSO is the recommended solvent for use with cell line exposures. The concentration of solvent in the final test volume should not exceed 1% (v/v).

Confounding factors

For small test volumes, evaporation of the test solution can be a problem as the volume to air-surface ratio is high, and particularly if the test temperature is high e.g. > 15 degrees C. Precautions should be taken to avoid evaporation and also the contaminant crossover that can occur in multiwell plates. In this respect, a short exposure time is desirable: Test duration is typically not greater than 48 h, although there are some exceptions, such as bioassays with algae which may need a 72 hr exposure.

The surface area to volume ratio of the test container is high and some contaminants may preferentially adhere to surfaces such as polystyrene. For this reason, glass test containers should be used in preference to plastic.

5. Conclusions

Whatever the matrix, extraction procedures generally produce small volumes and therefore small scale bioassay procedures are required for testing. In most cases, the recommended procedures are adapted from well-established protocols. The choice of test species will depend on the purpose of the study and the availability of test organism.

Bioassays frequently used for testing extracts are shown below:

	Test organism	Test volume (ml)	Number of organisms/cells per test vessel	Reference
<i>In vivo</i>	Mussel embryo	1 - 5	50 per ml	ASTM724
	Oyster embryo	1 - 5	50 per ml	ASTM724
	Sea urchin	1 - 5	40 per ml	ASTM1563
	Microalgae (freshwater and seawater)	1 - 5	5x10 ⁶ cells /L	ISO8692, ISO10253
	Macrophyte germination	1 - 5	500-1000 zygotes per ml	Brooks <i>et al.</i> , 2008
	Daphnia	1 - 5	1 per test vessel	ISO6341
	Acartia / Nitocra	5	5 per test vessel	ISO 14669
	Tisbe	5	5 per test vessel	ISO14669
	Fish embryo	2 - 5 ml	1 per 2ml test vessel	OECD draft guideline
<i>In vitro</i>	YES, YAS, anti-YES,	200 µl	0.8 x 10 ⁶ cells/ ml	Tollefsen <i>et al.</i> , 2007

	anti-YAS			
	ER calux	200 µl	5-10 x 10 ⁵ cells/ml	Legler <i>et al.</i> , 2003
	Primary cell cultures	200 µl	5x10 ⁵ cells/ml	Tollefsen <i>et al.</i> , 2003
	Cell lines	200 µl	5-10 x 10 ⁵ cells/ml	

Matrix	Procedure	Bio-assay	Reference
Sediment	ASE, DCM, acetone	ER-Calux	Houtman <i>et al.</i> 2007

In all of the above test methods, appropriate reference materials should be tested as stated in the specific test protocols.

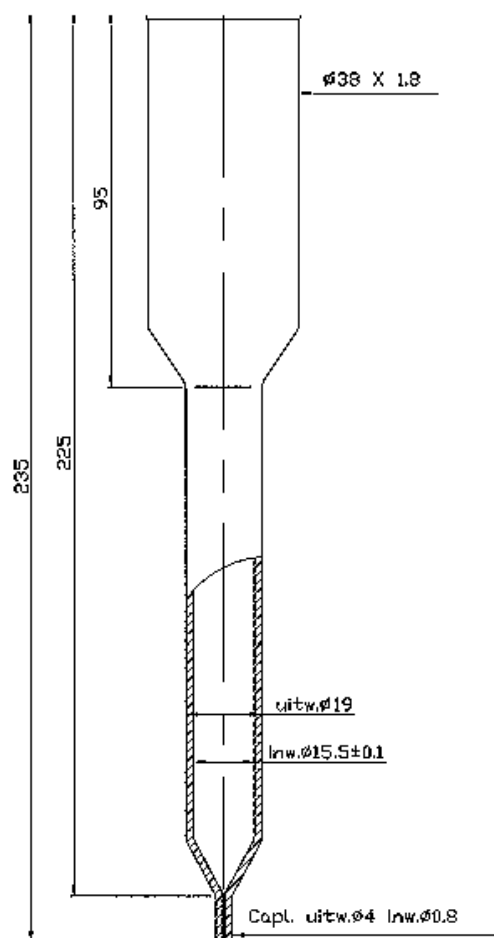
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Appendix 1: Lay-out of borosilica column for use with acid-silica cleanup



Model: A

Materiaal :
Borosilicaatglas

Benaming		Get. J de J		Datum: 07-02-02		Tek. No.			
KOLOM MODEL : A		Schaal:		Afd. IVA		01 06 08			
		Auteursrecht voorbehouden volgens de wet							
	Fac. der Exacte Wetenschappen De Boelelaan 1083 1081 HV Amsterdam Afd. Glasstechniek rijke Universiteit amsterdam	Toleranties voor		Van - tot	±	Van - tot	±	Van - tot	±
		lengte maten en		0,5 - 3	0,2	30 - 120	2	0,3 - 0,5	0,3
		diameters tenzij		3 - 6	0,5	120 - 315	5	0,5 - 0,5	0,5
		anders aangegeven		6 - 30	1	315 - 1000	10	0,5 - 0,5	0,5

Annex 6: Intersex in fish

Intersex (ovotestis) measurement in marine and estuarine fish

Compiled by G D Stentiford¹

Summary

1. *Applicability across OSPAR maritime area.*

The presence of susceptible host species utilised in monitoring programmes in marine and estuarine habitats of the OSPAR region make this an applicable measurement in field programmes. The requirement for the sampling of testis from male fish captured in such programmes and the assessment of these tissues by histology can be aligned with the sampling of other tissues currently assessed for fish diseases work (e.g. for liver cancer assessment). The epidemiological basis for the sampling of fish for intersex measurement is therefore aligned with other field sampling programmes for fish health.

2. *Status of quality assurance.*

Formal QA for the measurement of intersex in marine and estuarine fish has not been carried out under existing programmes (such as BEQUALM) but published methods are available for the grading of intersex severity in flatfish collected from monitoring programmes. These methods would be directly applicable to QA programmes. The sampling of materials from epidemiologically relevant numbers of animals is also well characterised in the literature and is outlined in this document.

3. *Influence of environmental variables.*

Although sex determination can be influenced by environmental factors and age, there has been an historic linkage between sites with the highest prevalence of intersex fish, biomarkers for exposure to endocrine disrupting chemicals (e.g. vitellogenin), and anthropogenic contaminants known to elicit development of ovotestis in a range of test species.

4. *Assessment of thresholds.*

Threshold assessment to indicate an impacted site has not previously been discussed for measurement of intersex (ovotestis) in male fish. However, based upon the reported prevalence of the condition in marine and estuarine fish from the OSPAR region, and the constraints inherent with the sampling of large populations for health effects, it would appear that a threshold of 5% prevalence (in external males) may be used to indicate impact. The epidemiological basis for this is discussed in this document.

5. *Proposals for assessment tools.*

Given background data on quality assurance techniques for intersex measurement, it seems appropriate to propose a two-tier assessment tool. Tier 1 consists of an individual sample grading system for intersex severity based on the methodology presented by Bateman *et al.* (2004). Tier 2 consists of apparent prevalence estimates based upon a sampling regime designed to detect a 5% prevalence of intersex at 95% confi-

¹ Centre for Environment, Fisheries and Aquaculture Science (Cefas), Weymouth Laboratory, Dorset DT4 8UB, UK

dence. Both of these tools can be combined to provide a population-level and individual-level assessment tool for the condition. Since intersex prevalence is likely to be negligible in non-impacted populations, survey designs are likely to be similar to that for fish disease measurement, whereby detection is based upon diseases present in a population at 5% prevalence (95% confidence). In this way, >5% prevalence would be considered the cut-off point for definition of an impacted population. The use of cohort-matching, similar to that for assessment of liver pathology in flatfish, is recommended to remove any confounding effects of age on intersex prevalence (e.g. use of fish of 4 years old) (Stentiford *et al.* 2010).

Assessment of the applicability of intersex measurement across the OSPAR maritime area

In recent years, a significant proportion of research into the biological effects of contaminants in the aquatic environment has been devoted to the study of endocrine disrupting chemicals (EDCs) of anthropogenic origin. EDCs have been widely reported to impair fertility, development, growth and metabolism in a range of animal groups (see Colborn *et al.* 1996). The effects of exposure of fish to such compounds include disturbed maturation and degeneration of the gonads, elevated concentrations of vitellogenin (egg yolk protein) in the plasma of male fish and the presence of intermediate or 'intersex' gonads (Gimeno *et al.* 1996). Using histological analysis, fish with the intersex condition are seen to possess oocytes within their normal testicular matrix (Sharpe, 1997; Bateman *et al.* 2004). Until the early 1990s intersex had only rarely been described from fish in the wild (Jafri and Ensor, 1979; Slooff and Kloowijk-Vandijk, 1982; Blachuta *et al.* 1991). However, the condition has now been detected in several wild freshwater and migratory species, including roach *Rutilus rutilus* (Jafri and Ensor, 1979; Purdom *et al.* 1994; Jobling *et al.* 1998), gudgeon *Gobio gobio* (van Aerle *et al.* 2001), barbel *Barbus plebejus* (Vigano *et al.* 2001), chub *Leuciscus cephalus* (Minier *et al.* 2000), bream *Abramis brama* (Slooff and Kloowijk-Vandijk 1982), white perch *Morone americana* (Kavanagh *et al.* 2002), stickleback *Gasterosteus aculeatus* (Gercken and Sordyl, 2002), coregonids (Mikaelian *et al.* 2002), grayling *Thymallus thymallus* (Blachuta *et al.* 1991) and Atlantic salmon *Salmo salar* (authors' pers. obs.). Furthermore, detection of elevated prevalences of intersex in some estuarine and marine species such as the European flounder *Platichthys flesus* (Allen *et al.* 1999a), Japanese flounder *Pleuronectes yokohamae* (Hashimoto *et al.* 2000), bothid flounder *Bothus pantherinus* (Amaoka *et al.* 1974), common eel *Anguilla anguilla* (Peters *et al.* 2001) and viviparous blenny *Zoarces viviparus* (Matthiessen *et al.* 2000; Stentiford *et al.* 2003) suggest that the effects of anthropogenic EDCs may extend beyond inland river systems to coastal and even offshore waters. This is supported by reports of elevated plasma vitellogenin and ovotestis in male Mediterranean swordfish *Xiphias gladius* (Fossi *et al.* 2001 and De Metrio *et al.* 2003, respectively), and the dab *Limanda limanda* (Scott *et al.* 2007; Stentiford and Feist, 2005, respectively). In terms of species of relevance to the OSPAR region, those in which intersexuality (ovotestis) have been described from marine and estuarine habitats include flounder (Allen *et al.* 1999b; Stentiford *et al.* 2003, Bateman *et al.* 2004), dab (Stentiford and Feist, 2005), viviparous blenny (Stentiford *et al.* 2003; Lyons *et al.* 2004), red mullet (Martin-Skilton *et al.* 2006) and the 3-spined stickleback (Gercken and Sordyl, 2002).

Status of quality assurance techniques for intersex measurement in marine and estuarine fish

Male fish with the intersex condition are seen to possess, at varying degrees of severity, oocytes within the testis; this being regarded as a phenotypic endpoint of endo-

crine disruption (both natural and anthropogenic) in male fish. Due to the fact that the testis may appear normal from external observations, histological examination of the testis is necessary to identify and grade individual cases of intersex and to estimate prevalence in a population. Intersex has been recorded histologically in all of those species listed above as relevant to marine and estuarine waters from the OSPAR region. It is important to consider quality assurance techniques for intersex measurement at two levels: 1. Individual (grading of intersex severity) and 2. Population (intersex prevalence).

Individual-level grading of intersex (ovotestis).

The most comprehensive assessment of ovotestis severity at the individual level has been presented by Bateman *et al.* (2004) for the European flounder. In this case, the study provided information on the different pathological manifestations of the intersex condition in flounder sampled from various estuarine and coastal waters of the United Kingdom and furthermore, described the development and application of an ovotestis severity index (OSI), calculated for individual histological sections of gonad. The development of this index provides pathologists with a robust tool for the grading of the intersex condition in flounder and potentially other fish species sampled in the OSPAR region.

The study by Bateman *et al.* (2004) utilised samples collected from monitoring programs around the United Kingdom over a four-year period (1998–2002) and assessed externally classified male flounder of above 15 cm in length. For histology, whole gonads were removed and fixed in a 10% solution of neutral buffered formalin prior to processing to wax using standard protocols. In order to assess the distribution of oocytes throughout the testis, all specimens examined, sections were cut longitudinally at a thickness of 3 to 5 μm , mounted onto glass slides, and stained using haematoxylin and eosin (H and E). Sections were analyzed by light microscopy. A total of 56 intersex cases were examined. All gonadal sections were viewed at low magnification using a x10 eyepiece and x10 objective lens, giving a total magnification of x100. Each gonadal tissue section was divided into a variable number of fields of view depending on the size of the sample. The number of fields of view comprising the whole tissue section was then used to construct a virtual grid, with each square on the grid corresponding to a field of view. Only fields of view that contained 100% tissue coverage were included in calculations of the OSI. Each field of the grid was then scored for the presence of oocytes, the distribution of these oocytes, and their stage of development (according to previously published criteria in other fish species). The overall OSI takes into consideration both the oocyte development stages present and their distribution throughout the testis (see Fig. 2 from Bateman *et al.* 2004 below).

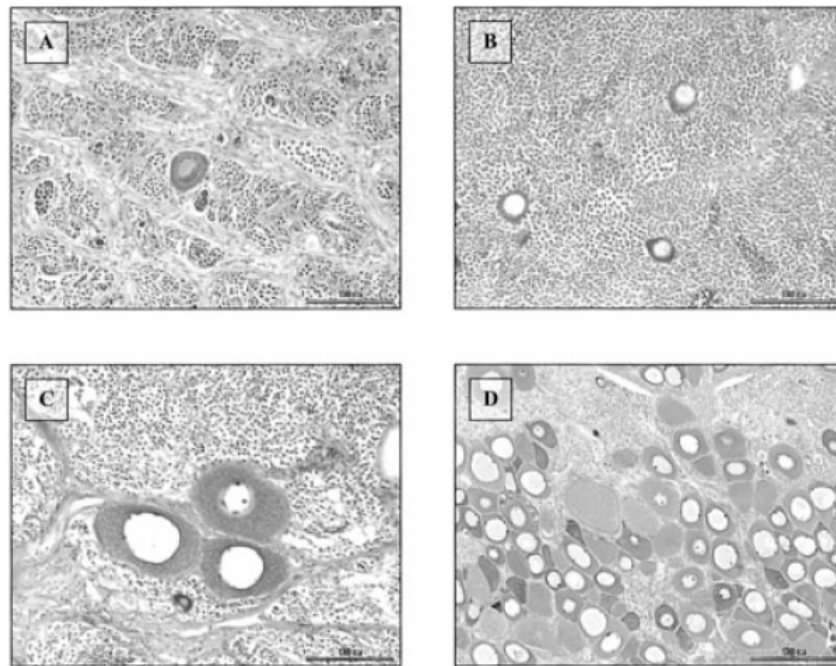


Fig. 2. Oocyte distribution patterns in ovotestis cases. (A) Focal, only single oocytes are present within field of view. (B) Diffuse distribution, more than one oocyte is present in a field of view and are not closely associated with neighboring oocytes. (C) Cluster distribution with more than one, but less than five, closely associated oocytes present within a field of view. (D) Zonal distribution, indicated by the presence of more than five closely associated oocytes within a field of view. In this case, oocytes in various stages of maturity can be seen. Haematoxylin- and eosin-stained sections. Scale bars = 100 μm .

In order to calculate the severity of the intersex condition within an individual section of gonadal material, an algorithm was formulated, incorporating the scores for development and distribution of oocytes within individual fields of view. This algorithm allowed for the calculation of the OSI for an individual section of gonad. The OSI was calculated as follows where D1 is the most advanced development stage of oocytes within a field of view (score 1–5), D2 is the distribution of oocytes within a field of view (score 1–4), and X is the total number of fields of view examined.

$$\text{OSI} = \left(\frac{\sum [D_1 \cdot D_2]}{X} \right)$$

The OSI is a sum of the severity staging for each field of view in a section of gonad. By dividing this sum by the total number of fields of view in the whole section, the mean ovotestis severity per field of view can be obtained. For intersex flounder, this gives an overall OSI of >0 up to 20 (the maximum score, whereby each field of view contains over five vitellogenic oocytes in a zonal distribution). Testing this scoring system on field collected samples of flounder, Bateman *et al.* (2004) used the OSI

scores from each gonad to create a broad grading system of: Absent (OSI = 0), stage 1 (OSI >0–5), stage 2 (OSI >5–10), and stage 3 (OSI >10–20). This was summarised as:

Table 2. Ovotestis ranking by stage based on histological appearance, proportion of fields containing oocytes, and the distribution and developmental stage of oocytes

Severity category	Histology	Proportion of fields of view with oocytes	Distribution and developmental stage of oocyte
Absent (score 0)	Testis structure is normal, with no oocytes present in section.	—	—
Stage 1 (score > 0–5)	Structure of the majority of the testis appears normal.	Generally below 50%	Single or multiple previtellogenic oocytes. Cortical alveolar or fully vitellogenic oocytes rarely are present.
Stage 2 (scores > 5–10)	Regions of the testis are altered, replacement of testicular material with oocytes.	Up to 75%	Majority of oocytes are previtellogenic, present in clusters or zones in high proportion of fields of view. Single or multiple vitellogenic oocytes.
Stage 3 (score > 10–20)	Majority of testis is disrupted, replacement of testicular material with oocytes in various stages of development.	Above 75%	Associated previtellogenic or vitellogenic oocytes through majority of section.

1. Population prevalence of intersex (ovotestis).

The second level of assessment of intersex (ovotestis) in marine and estuarine fish from the OSPAR region requires an indication of prevalence (or the total number of cases in the population, divided by the number of individuals in the population). Since it is problematic to define the number of individuals in a wild population of marine or estuarine fish, the estimation of prevalence (or so-called apparent prevalence) is therefore carried out by sampling a statistically significant number of animals from a population exceeding a presumed size (e.g. >10 000 individuals). The size of the sample required will also depend on necessity of detecting a given prevalence (e.g. 1%, 2%, 5% etc.) and the confidence level of detecting this prevalence (e.g. 90%, 95%, 99%). Whilst the majority of studies examining the presence of intersex in wild populations do not appear to have followed statistical guidelines relating to the sampling of wild populations (e.g. see Simon and Schill, 1984), it is perhaps relevant that the approach to monitoring for intersex should follow that outlined in the chapter for fish diseases and as reported in studies such as those of Stentiford *et al.* (2009, 2010). In this context, sampling is designed to detect a disease prevalence of 5% at a confidence level of 95%. Using these figures, 59 individuals should be sampled if the population size is assumed to be 10,000 individuals. By using the same confidence of detecting lower prevalence of intersex, sample sizes would need to increase to 148 individuals (for 2% prevalence) and 294 individuals (for 1% prevalence). It should be noted however that where populations exceed 100,000, 500,000 or 1,000,000 individuals, sample sizes required to detect a 5, 2 and 1% prevalence at 95% confidence are considerably larger (597, 1494 and 2985 individuals, respectively). Clearly cost and conservation limitations will relate to most monitoring schemes so that these latter numbers become somewhat unfeasible. It is for this reason that presuming a population size of 10,000 and sampling to detect 5% prevalence at 95% confidence has been chosen for much of the fish disease work (Feist *et al.* 2004).

When considering apparent prevalence of intersex in a population of marine or estuarine fish sampled from the OSPAR region, it is useful to consider the reported prevalence range for the condition in relevant species. Available data for the key monitoring species are as follows:

Flounder (<i>Platichthys flesus</i>)	Up to 20% (Allen <i>et al.</i> 1999a) Up to 9% (Allen <i>et al.</i> 1999b) Up to 8% (Minier <i>et al.</i> 2000) Up to 8.3% (Stentiford <i>et al.</i> 2003)
Viviparous blenny (<i>Zoarces viviparus</i>)	Up to 27.8% (Gercken & Sordyl, 2002) Up to 25% (Stentiford <i>et al.</i> 2003)
Dab (<i>Limanda limanda</i>)	Up to 14.3% (Stentiford & Feist, 2005)
Red mullet (<i>Mullus barbatus</i>)	Up to 14.3% (Martin-Skilton <i>et al.</i> 2006)
Stickleback (<i>Gasterosteus aculeatus</i>)	Up to 12.5% (Gercken & Sordyl, 2002)

2. Given the fact that intersex appears to exist at a range of between 0 and 27.8% in different monitoring species, a sampling regime based upon detection of 5% prevalence at 95% confidence appears appropriate. Furthermore, multi-site work in several species (e.g. flounder and dab by Stentiford *et al.* 2003 and 2005, respectively) has demonstrated that intersex is detected at some sites and not at others when this regimen is utilised. This indicates that intersex, if present, occurs at below 5% at these latter sites. As such, for monitoring purposes, it could be proposed that 5% prevalence of intersex is considered to be 'above baseline', with all sites with a prevalence above this being further assessed for intersex severity using the OSI approach of Bateman *et al.* (2004). This gives a two-tiered assessment of intersex utilising apparent prevalence in the population, and an indicator for severity in affected individuals.

Review of the environmental variables that influence the presence of intersex in marine and estuarine fish

3. Whilst the link between the formation of intersex (ovotestis) and exposure to anthropogenic contaminants considered to be 'endocrine disrupters' has been demonstrated for several fish species (e.g. Gimeno *et al.* 1996, 1997), it is also known that intersex and sex reversal are not specific markers for estrogens but rather they have many causes (including androgens, aromatase inhibitors and even water temperature shifts). Recent work has also demonstrated a potential for age to affect the occurrence and prevalence of the condition in freshwater fish species (Jobling *et al.* 2009). For certain species utilised in monitoring programmes in the OSPAR region, there is a clear historical link between those sites where anthropogenic endocrine disrupters, direct biomarkers of endocrine disruption (e.g. VTG) and the presence of intersex in populations residing in those habitats are most pronounced (for example, see links between papers by Allen *et al.* 1999a,b and Stentiford *et al.* 2003 for estuarine flounder). Extending this relationship between cause and effect to offshore populations is not so clear although data presented by Scott *et al.* (2007) showing elevated VTG in dab sampled from certain North Sea sites do correspond with data presented by Stentiford and Feist (2005) for intersex in the same species from these sites. Complications in specifically linking the presence of a chronic marker (such as intersex) with more acute phase markers (such as VTG), or the burden of anthropogenic chemicals are not unique in this instance, with similar parallels being reported in liver cancers present in a consistent, but as yet unexplainable manner in multi-year samples of dab collected from offshore sites (Stentiford *et al.* 2009, 2010). Interestingly, those estuarine and offshore sites with the highest prevalence of liver pathologies (including cancer) are also those where intersex have been reported. However, since hatchlings and juveniles are likely to inhabit different grounds to those where adults are sampled

(Dipper 1987) and it is at these early life stages at which sex is determined (and at which disruption may occur) (Gimeno *et al.* 1997; Devlin and Nagahama, 2002), the presence of fish with the intersex condition at the particular offshore sites may not necessarily reflect the presence of EDCs at the site but rather their presence at sites where hatching and early growth occurs. Future studies should be directed towards the measurement of intersex in fish of known age, or in earlier life stages residing at monitoring sites and at those sites identified at nursery grounds for the key monitoring species. Comparisons of the prevalence of the intersex condition in juvenile and adult fish of the same species may furthermore provide clarification on the population level effects of EDCs in the marine environment and on their long-term ecological effects on sensitive ecosystems. Coupled with studies on the population genetics of these species and the identification of specific spawning grounds for different adult stocks, the potential selective pressure imposed by endocrine disturbances may also be identified.

Assessment of the thresholds when the response (prevalence of intersex) can be considered to be of concern and/or require a response

4. As stated above, given the fact that intersex appears to exist at a range of between 0 and 27.8% in different monitoring species, a sampling regime based upon detection of 5% prevalence at 95% confidence appears appropriate. Furthermore, multi-site surveys in several species (e.g. flounder and dab by Stentiford *et al.* 2003 and Stentiford and Feist, 2005, respectively) have demonstrated that intersex is detected at some sites and not at others when this regimen is utilised. This indicates that intersex, if present, occurs at below 5% at these latter sites. As such, for monitoring purposes, it could be proposed that 5% prevalence of intersex is considered to be 'above baseline', with all sites with a prevalence above this being further assessed for intersex severity using the OSI approach of Bateman *et al.* (2004). This gives a two-tiered assessment of intersex utilising apparent prevalence in the population, and an indicator for severity in affected individuals. It also allows for the discounting of potential isolated cases of intersex that may occur due to genetic abnormalities or other causes.

Proposals for assessment tools

5. Given background data on quality assurance techniques for intersex measurement, it seems appropriate to propose a two-tier assessment tool. Tier 1 consists of an individual sample grading system for intersex severity based on the methodology presented by Bateman *et al.* (2004). Tier 2 consists of apparent prevalence estimates based upon a sampling regime designed to detect a 5% prevalence of intersex at 95% confidence. Both of these tools can be combined to provide a population-level and individual-level assessment tool for the condition. Since intersex prevalence is likely to be negligible in non-impacted populations, survey designs are likely to be similar to that for fish disease measurement, whereby detection is based upon diseases present in a population at 5% prevalence (95% confidence). In this way, >5% prevalence would be considered the cut-off point for definition of an impacted population. It is recommended that cohort-matching is applied when comparing fish captured from different geographic sites, similar to the manner carried out for assessment of liver pathologies (Stentiford *et al.* 2010).

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Annex 7: Background document on supporting parameters

Supporting parameters for biological effects measurements in fish and mussels

John Thain(1) and Leonard Balk (2)

1. Centre for Environment, Fisheries and Aquaculture Science, Weymouth Laboratory, The Nothe, Barrack Road, Weymouth, DT4 8UB, UK.
2. Department of Applied Environmental Science, ITM Stockholms Universitet, 106 91 Stockholm, Sweden

Measurement of supporting metrics for fish: condition indices, GSI, HIS and age

Background

1. For all biological effect techniques within the OSPAR JAMP and OSPAR integrated strategy there is a requirement to report supporting parameters, and these include species, sex, fish length, whole fish weight, liver weight and gonad size. The measurement of gonad size and liver weight is used to provide an indication of reproductive state, and liver weight may also give an indication of general health and well being. These measurements are used in indices relating gonad weight to whole body weight (Gonad Somatic Index - GSI) and liver weight to whole body weight (Liver Somatic Index - LSI or Hepato Somatic Index - HSI), explanations of these are described below. Both gonad and liver weight will change markedly throughout the year and for comparative purposes these seasonal variations must be taken into account for the interpretation of biomarker responses such as EROD and VTG for example. Additionally, the condition factor (CF) is a general indicator for fish condition, similarly the condition index (CI) for mussels.
2. ICES WGBEC recently reviewed the measurement of these metrics and their role and importance in fish monitoring programmes, and this is described below.

Summary of supporting parameters required for fish:

Parameter	Measurement	Comment
Live fish whole body weight	To 0.1g	Blotted dry
Length of fish	To nearest mm	
Liver weight	To 0.1 g	
Gonad weight	To 0.01g	In addition record sex
Gonad length	To nearest mm	In addition record sex
Age	Conducted on otoliths	At least 10 fish from each size length class.

General Overview: Organ size and related measurements

3. Organ sizes constitute a very elementary measurement . The measurements can be performed with a minimum of equipment, and the procedures are easy to undertake. At least for some species it is possible to analyse these variables on frozen material. With minimal instruction these measurements can be determined by personnel not regularly involved in biomarker analysis, although it is preferable to use personnel familiar with handling fish and able to perform simple dissection of fish.
4. Data of this type may be of relevance either in their own right, indicating adverse effects of various kinds where the toxic mechanisms are not fully understood as a

result of xenobiotic exposure and/or, partly as a supporting variable to biomarkers conducted at the whole individual, tissue, cellular and subcellular levels. As for all biomarkers in use today, there is a strong need for quality assurance when these measurements are carried out.

5. One of the most important measurements in this field may be the development of gonads among female fish. This variable is best expressed as gonad size relative to the somatic body weight (Gonad Somatic Index - GSI) and expressed as a percentage value. The best species to use are those where the gonads of juvenile and immature fish are different from adult fish and where there are distinct differences in the genders. For example, it is much easier when the morphology of the female ovary is a single structure while the male testis are paired bilaterally.

6. This offers the opportunity to investigate when the fish in relation to size and/or age are sexually immature or adult, or indeed have retarded gonad development (often termed sexually immature - SIM) as compared to normal sexual development. This can be expressed as a percentage of sexually immature females among the adult females, and represents the portion of fish with the extreme low value of the GSI value (usually below ~1%) and they have therefore a gonad with no or neglected development.

7. Analogous to the analysis of the gonad size is the liver size relative to the somatic body weight (Liver Somatic Index – LSI, or sometimes referred to as Hepato Somatic Index – HSI). It may be regarded as a parameter in its own right and also as a supporting variable for other biomarkers such as EROD.

8. Furthermore, growth (e.g. gram/year) as shown in Kiceniuk and Khan 1986; McMaster *et al.* 1991 and in Ericson *et al.* 1998, as well as the Condition Factor (CF - see reference to Foulton below) are relatively straightforward to determine and may be used as markers for adverse effects due to xenobiotic exposure. The measurement of the condition factor has not often been used in short exposure laboratory experiments, however, field observations over longer time periods indicate that it may be a valuable measure for adverse effects. (see review by van der Ost *et al.* 2003). Recent investigations related to the Fish Disease Index (WGPDMO, 2011) support this assumption.

During periods of high food intake and also in conjunction with the reproductive cycle an individual may have a higher gross weight at a particular length. This can be assessed by calculating the coefficient of condition (K) or by Fulton's condition factor (Bagenal and Tesch 1978). This is calculated as follows:

$$K = \text{weight} / (\text{length})^3$$

The condition factor reflects the nutritional state or “well-being” of an individual fish and is sometimes interpreted as an index of growth rate.

9. Feeding status in fish may be reflected in the condition factor, and may be important for a number of different responses, and as such can be included in biomonitoring investigations.

Gonad size in fish - GSI

10. The reproductive process constitutes (one of) the most essential health signals for the individual animal, and when missing or impaired indicates an obvious risk for adverse effect both genetically and for population survival. Therefore, decreased sizes of the gonad, of one or both of the genders, indicate an apparent risk for a reduced reproductive potential.

11. Gonad size is measured as a percentage of somatic body weight, gonadosomatic index (GSI*), It has been demonstrated to be a variable that can be influenced by contaminants in a number of different polluted field studies. It should be underlined that the toxicological response observed for this variable could have originated from a number of different toxicological reasons such as, tissue or cell death to more sophisticated regulatory endocrine mechanisms.

Measurement of GSI: record whole body weight of fish and gonad weight to 2 decimal places.

*GSI = (gonad weight x 100) / (total body weight[#] – gonad weight)

[#]subtract stomach content

12. Deviation in GSI levels could represent a permanent effect or impairment for the reproductive cycle for one or more years (Janssen *et al.* 1997; Vallin *et al.* 1999). Both scenarios will seriously affect reproductive potential. Examples of different pollution gradients where reduced gonads have been observed are in bleached kraft pulp mill effluents (Andersson *et al.*, 1988; Sandström *et al.*, 1988; McMaster *et al.* 1991; Balk *et al.* 1993; Förlin *et al.* 1995), including using chlorine-free processes (Karels *et al.* 2001) and general pollution (Johnson *et al.* 1988; Noaksson *et al.* 2001). Laboratory exposure experiment where effect on the GSI value have been documented include petroleum mixtures (Truscott *et al.* 1983; Kiceniuk and Khan 1986), specific PAHs (Thomas 1988; Singh 1989; Thomas and Budiantara 1995), PCB mixture (Thomas 1988), pesticides (Ram *et al.* 1986; Singh 1989), and cadmium (Singh 1989; Pereira *et al.* 1993).

13. There is no doubt that xenobiotics can affect gonad size through a number of different toxicological mechanisms. However, as for most biomarkers, a variable that shows a (annual) natural biological cycle it is essential that the normal background values are well known, and that the appropriate control material is used for comparison. For the GSI value it should be pointed out that during certain time times of the year the gonad development is very fast and that different GSI values are obtained only within a period of a few days/weeks. Analysis of the GSI in these time periods should be avoided. Baseline studies are important in order to evaluate suitable time periods for this variable (Förlin and Haux 1990; Larsen *et al.* 1992).

14. A state of complete disruption of sexual maturation reflects an extreme situation of low GSI values, e.g. a state of condition when the adult (based on age and/or size) fish are unable to develop from the prepubertal condition to the sexually mature stage. Field observations demonstrating a delay or lack of gonad development has been observed include the following species; burbot (*Lota lota*) in the northern coast of the Bothnian bay (Pulliainen *et al.* 1992), English sole (*Parophrys vetulus*) in generally polluted areas in Puget sound, USA (Johnson *et al.* 1988), perch (*Perca fluviatilis*) in the effluent water from pulp and paper mills in Baltic waters (Sandström *et al.* 1988; Sandström *et al.* 1994) as well as white sucker (*Catostomus commersoni*) in corresponding effluents in Ontario, Canada (McMaster *et al.* 1991). Studies have also shown that perch, roach (*Rutilus rutilus*), and brook trout (*Salvelinus fontinalis*) exposed to leachate from a public refuse dump in a Swedish fresh water system show corresponding adverse effects (Noaksson *et al.* 2001, Noaksson *et al.* 2002). Although the above cited field investigations are not all related to suspected PAH contamination, these kinds of disorders has been created in laboratory experiment using petroleum products and a pure naphthalene (Thomas and Budiantara 1995).

GSI Confounding factors

15. Although the measurement is robust and easy to perform there is a need to characterize and avoid confounding factors. For example female perch populations, do not naturally spawn every year and the spawning frequency is affected by water temperature as indicated in Luksiene *et al.* 2000 and Sandström *et al.* 1995. Moreover, in the closely related yellow perch (*Perca flavescens*) both photo period and temperature have been suggested to be of importance (Dabrowski *et al.* 1996). Therefore, GSI data should be interpreted with regard to the reproductive cycle for each species under investigation.

Liver size of female and/or male fish – LSI (HSI)

16. Liver size is measured in relation to somatic body weight, and is known as Liver Somatic Index (LSI* or HSI – see above).

Measurement of LSI: record whole body weight of fish and gonad weight to 2 decimal places.

*LSI = (liver weight x 100) / (total body weight[#] – gonad weight)

[#]subtract stomach content

17. LSI may be regarded as a relevant measurement since it has been documented to be affected by contaminants in a number of different polluted field studies. For example, in pollution gradients of paper and pulp mill effluents where increased LSI values were observed (Andersson *et al.* 1988, Lehtinen *et al.* 1990; Hodson *et al.* 1992; Kloepper-Sams and Owens 1993; Huuskonen and Lindström-Seppä 1995; Förlin *et al.* 1995), as well as decreased LSI levels as reported by Balk *et al.* (1993), and Förlin *et al.* (1995). Other complex effluents shown to affect liver size in various fish species are: leakage water from public refuse dumps (Noaksson *et al.* 2001, 2002) and effluent from waste water treatment plant (Kosmala *et al.* 1998).

18. Field situations where PAHs and/or organochlorines are suspected contaminants for increased liver size in various fish species are documented by: Sloff *et al.* (1983); Goksoyr *et al.* (1991); Kirby *et al.* (1999); Kirby *et al.* (1999); Beyer *et al.* (1996); Leadly *et al.* (1998); Stephensen *et al.* 2000). Laboratory experiments shown to affect liver size among different fish species from exposure to organochlorines have been documented by: Adams *et al.* (1990); Newsted and Giesy (1993); Otto and Moon (1995); Arnold *et al.* (1995); Gadagbui and Goksoyr (1996); Åkerblom *et al.* (2000), and for two-stroke outboard engine exhaust extract (Tjärnlund *et al.* 1996) and PAHs (Celandier *et al.* 1994) as well as pesticides (Singh 1989; Åkerman *et al.* 2003) and cadmium (Singh 1989).

LSI Confounding factors

19. Although there is no doubt that xenobiotics could affect liver size as a result of different toxicological mechanisms it should be emphasised that, as for most biomarkers, control/reference fish should be analyzed in close/direct parallel to the exposed site(s). In addition, seasonal variation is observed in different fish species (Koivusaari *et al.* 1981; Förlin and Haux 1990; Larsen 1992), and must be taken into account at all times. Besides the time of the year, factors (i.e., parameters) such as feeding behaviour, gender, maturity, age, size, temperature (George *et al.* 1990), photo period, parasites, among others, needs to be taken into considerations. Baseline studies are an important strategy to finally evaluate confounding factors (Balk *et al.* 1996).

20. Determination of age

It is essential for the interpretation and assessment of biological effect responses that the age of fish is known. This is particularly important for effect measurements such as fish diseases which may be more prevalent in older fish (Stentiford *et al.*2010). Age is assessed by removing the otoliths of each fish sampled, and using standard procedures. These vary with species, and sometimes location, and specific guidance should be sought from relevant experts, or ICES. Ideally age should be determined on several size ranges of fish from each site, as age length relationships can be markedly different from site to site as described by Stentiford *et al.*2010.

21. Interpretation of data

The GSI, HSI (LSI) and condition factor are described here as supporting parameters to assist the interpretation of contaminant related biological effect measurements. However, it should be noted that these supporting parameters in their own right may be influenced by a number of factors which should be described if known and these include: feeding behaviour, gender, maturity, development stage, age, water temperature, presence of parasitic infections and other disease, location and seasonality.

B: Measurement of supporting metrics for mussel: condition indices

Background

1. In Northern Europe mussels have their main spawning season in late winter to early spring e.g. February in the UK. During the onset of reproduction energy normally used in shell and somatic growth is fully utilised for gametogenesis. This is manifested by a marked increase in flesh weight relative to whole body weight which increases and reaches a maximum at spawning. Post spawning, flesh weight relative to whole body weight is at a minimum. As a consequence flesh weight relative to whole body weight or internal shell volume may be regarded as an index of condition.

2. For all biological effect techniques within the OSPAR mussel integrated strategy there is a requirement to report supporting parameters, and these include mussel length, whole body weight and condition index.

Summary of supporting parameters required for mussels: at least ten animals per site, usually within a specific size e.g. 40-45 mm or similar depending on availability at the site.

Parameter	Measurement	Comment
Live whole animal weight	To 0.1g	Must be on animals taken from full immersion i.e including water in body cavity (not gaping). Also blotted dry
Length of animal / width	To nearest mm	
Wet flesh weight	To nearest 0.1g	Flesh excised from open shell and drained / blotted dry
Dry flesh weight	To nearest 0.01g	80 degrees C for 24 hr and constant dry weight
Wet shell weight	To nearest 0.01g	Blotted dry
Dry shell weight	To nearest 0.01g	80 degrees C for 24 hr and constant dry weight
Internal shell volume	To 0.1ml	Not generally conducted but provides a very accurate measure of condition.

Condition Index

3. Condition Indices(CI) based on flesh weight relative to whole weight or shell have been used for several years, both in scientific research and in commercial fisheries and several methods are available (see Lutz, 1980, Aldrich and Crowley, 1986, Davenport and Chen, 1987). The methods may use wet flesh weight, whole weight and shell size and / or volume but these are less sensitive due to the difficulty in standardising the degree of wetness. Indices using dry flesh weight are more accurate particularly when used in relation to internal shell volume. Example of condition indices are given below:

CI "A" = $100 \times \text{Dry weight} / \text{Whole animal weight}$

CI "B" = $100 \times \text{Dry weight} / \text{Wet flesh weight}$

CI "C" = $100 \times \text{Dry weight} / \text{Internal shell volume}$

CI "D" = (Ratio of shell length:shell width) / dry weight

In general CI "A" is commonly used for convenience and ease of measurement but the most accurate assessment of condition is CI "C". Whatever condition index is used, it is high post spawning and lower post spawning when the animal is in poor condition and the flesh weight is greatly reduced relative to the whole animal weight and the volume of the internal shell cavity (Dix and Ferguson, 1984; Rodhouse *et al.*, 1984).

4. It should be noted that condition indices will vary according to body size (Lutz *et al.*, 1980). In addition, other factors such as the level of parasitic infection (Kent, 1979 and Thiessen 1987) and aerial exposure can adversely affect the condition of mussels.

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Annex 8: Acetylcholinesterase

Acetylcholinesterase assay as a method for assessing neurotoxic effects in aquatic organisms

Compiled by: Thierry Burgeot¹, Joelle Forget-Leray², Gilles Bocquené¹ and Lúcia Guilhermino³, Concha Martinez-Gomez⁴ and Kari Lehtonen⁵

¹Ifremer laboratory of ecotoxicology, BP 21105, 44311 Nantes, France

²LEMA, Le Havre University, Rue P. Lebon, 76400 Le Havre, France

³CIIMAR-LA/CIIMAR Centro Interdisciplinar de Investigação Marinha e Ambiental, Universidade do Porto, laboratório de ecotoxicologia, 4050-123, Porto, Portugal

⁴Centro Oceanográfico de Murcia, Instituto Español de Oceanografía, 30740 San Pedro Del Pinatar, Spain

⁵ Finnish Institute of Marine Research, FI 00561, Helsinki, Finland.

Background

The measurement of acetylcholinesterase (AChE; EC 3.1.1.7) activity in marine organisms has been shown to be a highly suitable method for assessing exposure to neurotoxic contaminants in aquatic environments. In general, the methods developed are sensitive to detect neurotoxic effects of contaminant concentrations occurring in marine waters. AChE activity method 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. As applied in human medicine, AChE activity is a typical biomarker that can be used in *in vitro* bioassays and 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). The highest activities have been found in the brain and muscle of fish, in the eye and muscle of prawn (Frasco *et al.*, 2010). Molluscs in general show low activity (Bocquené *et al.*, 1998). In vertebrates, neurotoxic poisoning with hyperactivity, tremors, convulsions and paralysis may finally lead to death.

Being an indicator of 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 neurotoxic insecticides (0.1 to 1 µg 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 has been demonstrated to various other groups of

chemicals present in the marine environment 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). Its usefulness as a general indicator of pollution stress in mussels from the Baltic Sea has recently been suggested and it has been used for this purpose (Schiedek *et al.*, 2006; Kopecka *et al.*, 2006, Barsiene *et al.*, 2006).

Confounding factors

It is important to know the natural limits of variability in AChE activity in the species of interest to assess the significance of the observed depression in activity. Knowledge of possible variations related to sex, size, state of gonadal maturation and the influence of seawater temperature should be systematically determined. Also, the presence of different ChEs in the same tissue having different sensitivities to anti-cholinesterase agents may act as a confounding factor; therefore, prior characterization of the enzymes present is recommended (Garcia *et al.*, 2000). AChE activity of juveniles of *Callionymus lyra* in the Atlantic sea and in *Serranus cabrilla* and *Mullus barbatus* in the Mediterranean Sea is higher than that of adults, but no differences were found between male and female in *Limanda limanda* in the Atlantic Ocean (Galvani and Bocquené, 1992).

Different biotic and abiotic factors are known to modulate AChE activity, including trace metals (cadmium copper, mercury, zinc) and variation of natural environmental factors, i.e. seawater temperature and salinity (Pfeifer *et al.*, 2005; Leiniö and Lehtonen, 2005; Rank *et al.*, 2007). In *Mytilus edulis* and *Macoma balthica* from the northern Baltic Sea, mean values of AChE values vary two-fold depending on season, following closely changes in temperature (Leiniö and Lehtonen, 2005). Seasonal variability has also been shown as different responses to natural factors 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 has been demonstrated to affect AChE activity in mussels (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. Toxins present in the water as a result of cyanobacteria blooms (e.g. *Anabaena flos-aquae*, *Aphanizomenon flos-aquae*) and *Microcystis aeruginosa* have been also 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 moulting and has been shown to be positively correlated with neurological activity (i.e. AChE) e.g. in *Artemia franciscana* (Gagne and Blaise; 2004). Moulting rate increases with the development, specifically peaking at the juvenile stage. The subsequent decline in AChE may also be explained by reduced moulting frequencies in adults.

The process and mechanisms of biological response in each organism require further investigation in specific habitats with specific chemical contamination. The mussel *Mytilus galloprovincialis* shows a great heterogeneity of esterases and a particular sensitivity to specific compounds such as paraoxon (Ozretic and Krajnovic-Ozretic, 1992; Brown 2004). The alleged versatility of AChE inhibition as an effect criterion after exposure to detergents may be misleading and may underestimate the contamination potential of complex mixtures (Rodrigues *et al.*, 2011). As for many other biomarkers, the hormesis effects cannot be ignored and represents a substantial scientific challenge. (Kefford *et al.*, 2008).

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 shown to have some differences in their properties compared to typical forms of vertebrates. For example, ChEs with properties of both AChE and pseudocholinesterases have been found in the gastropods *Monodonta lineata* and *Nucella lapillus* (Cunha *et al.*, 2007), in the sea urchin *Paracentrotus lividus* (Cunha *et al.*, 2005), in *Artemia sp* (Varó *et al.*, 2002) and in some strains of *Daphnia magna* (Diamantino *et al.*, 2003).

Exploration of genetic variability and the influence of environmental factors in specific habitats should lead to a better distinction between natural and pollutant effects.

Ecological relevance

AChE inhibition results in continuous and excessive stimulation of nerve and muscle fibres, producing tetany, paralysis and death. Sublethal exposure affecting AChE can alter the animal's behaviour and locomotive abilities (e.g. Vieira *et al.*, 2009), potentially affecting reproduction, fitness and survival. Therefore, AChE should be considered an ecologically relevant parameter, potentially affecting reproduction, fitness and survival. Evidence of modulation of AChE activity by organic chemicals, including fuel oil, has been described in marine organisms, including crustaceans (Signa *et al.*, 2008). The evaluation of the variations of AChE activity in different species allows characterisation of neurotoxic effects of a wide spectrum of organic and inorganic contaminants in the marine environment.

Quality assurance

The large experience acquired in conducting AChE measurements in the field makes it possible today to evaluate the effects of diffuse contamination in some marine organisms sampled in the Atlantic Ocean, the Baltic Sea and the Mediterranean Sea.

A microplate assay technique established for *in vitro* detection of AChE inhibition (Bocquené and Galgani, 1998) has been applied in the monitoring of coastal and off-shore waters. This technique has a specific sensitivity comparable to that of chemical analyses, with a detection limit of 100 ngL⁻¹ for carbamates and 10 ngL⁻¹ for organophosphates (Kirby *et al.*, 2000).

Standardisation of the sampling strategy and regular intercalibration exercises on specific organisms sampled in the Atlantic Ocean, Mediterranean and the Baltic Sea are necessary for the widespread use of AChE in routine pollution monitoring.

No formal quality assurance programmes are currently run within the BEQUALM programme but one major intercalibration exercise was carried out during the BEEP project (Biological Effects of Environmental Pollution in marine coastal ecosystems, EU project EVK3-2000-00543) in 2002.

Background Assessment Criteria (BAC) and Environmental Assessment Criteria (EAC)

Baseline levels of AChE activity in different marine species have been estimated from results derived from field studies in the Atlantic Ocean and the Mediterranean Sea (Table 1 below). Assessment criteria should 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 during at least two seasonal cycles. The baseline level ($35 \text{ nmol} \cdot \text{min}^{-1} \text{ mg prot}^{-1}$) of the seasonal cycle of the mussel *Mytilus edulis* studied during three years along the 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 of AChE activity by 20% to 50% indicates sublethal impact (Dizer *et al.*, 2001). In the field, several species have been found to have baseline AChE activities of the same order of magnitude in different studies/measurements (Table 1). However, differences between sea areas and seasons are obvious, e.g. with activity values in *Mytilus* spp. varying from 25 to $54 \text{ nmol min}^{-1} \text{ mg protein}^{-1}$.

According to these observations, background assessment criteria (BAC) and environmental assessment criteria (EAC) were proposed using the 10th percentile of data. BACs are estimated from data from reference sites and describe the threshold value for the background level. Environmental Assessment Criteria (EACs) are usually derived from toxicological data and indicate a significant risk to the organism. EACs were calculated by subtracting 30% from the BAC values (Table 1) and represent a significant inhibition of AChE activity. EAC values characterise a sublethal impact. BACs and EACs should be estimated for different geographical regions, and include the effect of differences in water temperature.

Table 1. Assessment of acetylcholinesterase activity after *in vitro* and *in vivo* exposure of bio-monitoring organisms in control laboratory conditions and field studies that have utilised common monitoring species collected from reference locations.

Organisms	Tissue	Reference location or control conditions	Sampling Season or month	Bottom Temperature or temperature range °C	BAC AChE 10 th Percentile (activity $\text{nmol} \cdot \text{min}^{-1} \text{ mg prot}^{-1}$)	EAC (activity $\text{nmol} \cdot \text{min}^{-1} \text{ mg prot}^{-1}$)	Ref.
Invertebrates							
<i>Mytilus gallorprovincialis</i>	Gills	Wild mussels-Mediterranean Sea in Spain	May-June	15-25	15	10	Campillo-Gonzalez (unpublished results)
<i>Mytilus gallorprovincialis</i>	Gills	Caging in field Mediterranean Sea –Carteau , France	Seasonal cycle	14-25	29	20	Bodin <i>et al.</i> , 2004
<i>Mytilus edulis</i>	Gills	Wild mussels-Atlantic ocean (N.W. Portuguese)	Seasonal cycle		26	19	L.Guilhermino (unpublished results)
<i>Mytilus edulis</i>	Gills	Wild mussels Atlantic ocean (Loire estuary)	Seasonal cycle		30	21	Bocquené <i>et al.</i> , 2004
Vertebrates							
<i>Plathichthys flesus</i>	Muscle	French Atlantic ocean (Seine Bay)		15°C	235	165	Burgeot <i>et al.</i> , 2009

<i>Plathichthys flesus</i>	Muscle	French Atlantic ocean (Ster estuary-Brittany)		15°C	335	235	Evrard <i>et al.</i> , 2010
<i>Limanda limanda</i>	Muscle	French Atlantic ocean (Seine Bay)		15°C	150	105	Burgeot <i>et al.</i> , 2009
<i>Mullus barbatus</i>	Brain	Mediterranean Sea SE Spain (Málaga-Almeria)	October	14 °C	75	52	Martínez-Gómez, unpublished results
<i>Mullus barbatus</i>	Muscle	Mediterranean Sea (France, Spain, Italy)	<i>In situ</i>	18°C	155	109	Burgeot <i>et al.</i> , 1996, Bocquené 2004

Future work

Standardised AChE measurement protocols and intercalibrations are required for the main species currently used in international marine biomonitoring programmes (OSPAR, HELCOM, MEDPOL and MSFD). An ICES TIMES series method document has been published (Bocquené & Galgani, 1998) and can be used as a basis of standardised procedure. Further information should be gathered to confirm baseline activity levels in specific habitats and different sentinel species in Europe. The BAC and EAC values must be considered as provisional and should be updated and revised when additional relevant data become available. BAC and EAC could also be derived for new species of interest and specific local studies.

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Annex 9: Mussel histology

Background Document: Histopathology of mussels *Mytilus* sp. for health assessment in biological effects monitoring.

1. Background

Mussels have long been used for the measurement of pollutants and the biological effects of contaminants in the aquatic environment (Bayne, 1976; Goldberg, 1978; Widdows and Donkin, 1992; Granmo, 1995; Salazar and Salazar, 1995). They are widespread, sessile, possess the ability to accumulate chemicals and exhibit a wide range of biological responses. They are able to tolerate wide ranging salinity conditions and are also seen attached to piers and gravelly substrates. This makes them well placed as a sentinel species in programmes designed to monitor the marine environment. Over the years numerous studies utilising mussels have demonstrated the impact of anthropogenic inputs into the aquatic environment. Early studies such as the “Mussel Watch” programme (Goldberg, 1978) were primarily designed to evaluate pollution within coastal waters by measuring levels of pollutants within tissues of mussels (and other bivalves). In comparison, relatively few studies focussed on the effect of these chemicals on their test organisms.

Over the years there has been increased emphasis placed on integrated assessments in national and international monitoring programmes within the Oslo-Paris Commission (OSPAR) region that incorporate both chemical analysis and their biological effects. A range of contaminants exist within the aquatic environment, which may elicit an assortment of biological responses. As such it is well established that integrated techniques provide a more robust approach for the overall health assessment of aquatic organisms and their environment, than the application of a single technique in isolation.

Histopathology (of aquatic organisms) is a valuable tool for providing health assessment of individuals and of populations since it incorporates measures of reproductive and metabolic condition, and allows for the detection of a range of pathogens that may affect morbidity and mortality. In addition to its role as a ‘baseline’ measure of health, histopathology has been employed to investigate the changes related to PAH, PCB and heavy metal exposure in mussels (Sunila, 1984; Lowe and Pipe, 1987; Auffret, 1988; Kluytmans *et al.*, 1988; Marigómez *et al.*, 2006). Mussel histopathology has been designated a promising technique (tissue response) for inclusion within the “mussel integrated approach”. It provides an effective set of tools for the detection and characterisation of toxicopathic pathologies, which are increasingly being used as indicators of environmental stress, in addition to disease.

Histopathology is also complementary to other techniques used to monitor the biological effects of contaminants as it can help to dissociate markers of underlying health or disease condition from those associated with exposure to contaminants. The advent of genomic and post-genomic technologies increases the potential utility of histopathology in quality assurance and quality control of sample groups for analysis (e.g. by selecting homogenous groups attributes and to control for potential variation amongst individuals). This approach should help reduce uncertainties associated with the potential confounding effects of pathogens when trying to identify the specific effects of toxicant exposure on host gene, protein and metabolite profiles (Stentiford *et al.*, 2005; Ward, *et al.*, 2006; Hines, *et al.*, 2007a). In this respect, it can be considered as a means to provide supporting information for measures (biomarkers) that specifically aim to assess historic exposure to, or effect of, a contaminant. Histo-

pathology therefore provides a 'phenotypic anchor' against which this specific data can be assessed (Stentiford *et al.*, 2005).

This ICES TIMES document provide a description of numerous health parameters that can be employed in monitoring programmes designed to assess the biological effects of contaminants. It also describes pathology that has been previously associated with contaminant exposure; and pathogens. Whilst the latter may initially seem curious in this document describing contaminant induced pathology, it is important to note that disease conditions of pathogen aetiology can result in pathology that may appear contaminant related to the untrained eye. Therefore it is essential for an individual to possess the ability to be able to distinguish between contaminant and pathogen related pathology.

2. Sampling and dissection for Formalin fixed paraffin embedded (FFPE) histology

When sampling mussels from the field, mussels should be carefully removed from their substrate by cutting the byssus threads with a pair of scissors. This will help to reduce stress that may act as a confounding factor when integrating with other sensitive biological effects techniques such as the Neutral Red Retention (NRR) assay. Mussels should be placed into a suitable insulating container and kept cool and moist during prompt transport back to the laboratory. This can be achieved by using a combination of ice-packs, wet paper towels and/or sea weed.

With integrated studies becoming more widespread, adopting a quality assurance approach is considered an important practice. So that potential post-sampling artefacts are minimised, mussels should be processed as soon as possible following removal from water. When dealing with samples distributed over a large geographical area (e.g. from national/international monitoring programmes), it may not always be possible to process samples immediately or relatively soon after. This is primarily because samples require lengthy transit to the laboratory thus delaying subsequent processing. Under these circumstances efforts should be made to keep the time from sampling until the time of processing, equivalent in duration between all samples. Currently, the number of individual mussels required for histology should be 50 although this may be refined in the final publication of the TIMES document for mussel histopathology (to be published imminently).

The dissection process is an extremely important stage in the histological process and it is crucial that it is conducted in a standardised manner. Standardised dissections ensure greater comparability between samples and simplify downstream histological analysis. It is essential to achieve good quality cross sections that are not too thick to ensure adequate penetration of tissues by the fixative. Mussels should be treated with care as not to cause any damage to any of the tissues. Any damage caused to tissues during dissection may prevent good quality cross sections being obtained.

In order to gain access to the visceral mass within the shell, hold the mussel with the posterior shell edge on a suitable work surface such as a dissection board. Insert scalpel blade into the mid-ventral byssal cavity (do not insert too far as this will damage tissues situated along the dorsal shell edge) followed by a downward movement resulting in the cutting of the posterior adductor muscle. Carefully open the two shell halves to reveal the visceral mass. Using a scalpel or scissors, remove any byssus threads that may hinder any microtomy carried out at a later stage. Do not remove byssus threads by pulling (threading) as this may cause undue stress to the mussel. Starting with one shell half at first, carefully separate the mantle tissue from the inner shell surface using the flat edge of a scalpel blade. Care should be taken as not to

“slice” the mantle with the scalpel blade itself. To an untrained individual, this can be challenging at first, however it is soon overcome. The most successful approach is to combine the use of “teasing” and “scraping”. Brush aside the partially removed visceral mass into the remaining shell half and sever the posterior retractor muscles. Once complete the empty shell half can be removed from the remaining half by disassociation of the shell ligament (a simple twist of the empty shell will suffice). In a similar manner to previous, the mantle tissue should be teased away from the inner shell surface of the remaining shell half. This process can be made easier by resting the previously dissected tissue onto a work surface whilst working with the remaining tissue. Once complete the entire visceral mass should be removed from the remaining shell and placed onto a dissection board. Using a razor blade or scalpel, a slightly angled 3mm slice across the ventral and posterior axis should be obtained towards the anterior end of the visceral mass. This will ensure that the main organs of interest (gonad, gills, mantle, digestive gland, kidney, foot) are incorporated into a single standardised section. Using forceps, carefully transfer the cross section into a histo-cassette before placing into Davidson’s Seawater Fixative or suitable alternative. The use of histo-cassettes is highly recommended due to their ability to ensure that the cross section remains intact during the fixation process. Allow fixation to proceed for a minimum of 24 hours with periodic agitation throughout. The use of a “rocker” facilitates this greatly.

3. Sampling and dissection for Histochemistry

Histochemical techniques on frozen tissue sections (obtained by cryotomy) are needed in order to evaluate lysosomal alterations described below. As such further dissection is required when incorporating these techniques.

For cryotomy, a small cube of digestive gland should be dissected from a minimum of 10 individual mussels and snap frozen onto a cryotome chuck in two rows of five, using a suitable cryo-embedding compound such as OCT. Snap freezing can be achieved using liquid nitrogen or a commercially available cryobath. For better integration of data, it is possible to obtain frozen samples from the same mussels identified for Formalin fixed paraffin embedded (FFPE) histology. Chucks should be transported to the laboratory in dry-ice (if required) and subsequently stored at -80°C.

4. Histology

Formalin fixed paraffin embedded - histology is the most widely used histological process; however resin based embedding techniques can also be employed. For FFPE histology, tissues are dehydrated through a series of graded alcohols followed by clearing and embedding within paraffin wax. Finally, tissues are placed into moulds containing molten wax that are subsequently cooled to produce a rigid support medium (block) for microtomy. See Bignell *et al.* (2011) for detailed protocols.

Using a microtome, the face of the tissue blocks are “trimmed” or “faced” in order to expose the maximum surface area of the mussel embedded within the block. Occasionally, sand or residual byssus may be encountered during sectioning, which may prevent suitable sections being obtained. Under these circumstances, it may be possible to remove these artefacts from the block face using a small sharp implement such as a pin or needle. Care should be taken not to cause any unnecessary damage to the surrounding tissues. This ensures that all areas of interest are included during sectioning. Tissue sections are obtained at 3µm to 5µm and floated onto a pre-heated water bath (35°C – 40°C) containing a suitable tissue adhesive (e.g. Sta-On, Surgipath, UK).

Alternatively, commercially available slides that have been pre-treated with saline or electrostatically charged can be used. Sections are adhered to a glass microscope slide by inserting the slide vertically into the water bath adjacent to floating section and lifting straight up. Following sectioning, slides should be dried overnight on a suitable hotplate. Alternatively, a section-dryer can be used which can decrease the time taken for slides to dry. Whatever drying method is employed, it is important to ensure that all moisture has been removed from slides prior to staining. Subsequently, sections are stained with haematoxylin and eosin (protocol provided in Annex 3) or a suitable alternative. Following staining, the end result should represent figure.X. This approach produces a uniform histological section that (a) incorporates all of the target organs of interest and (b) makes for a more simple microscopic examination due to the standardised orientation of the tissues and organs. Using a low magnification objective, the histopathologist should scan the slide for any abnormalities before further examination at higher magnifications. It is recommended to observe slides “blind” i.e. without prior knowledge to geographical location or exposure groups, in order to reduce bias that may otherwise be introduced to the interpretation.

Detailed sampling procedures are outlined in the ICES TIMES document.

5. Quality Assurance

At present there is no quality assurance scheme in place for mussel histopathology. It is envisaged that this will be run in a similar manner to the BEQUALM Fish Disease Programme currently organised by Cefas.

6. Health parameter measurements

The following parameters can be measured quantitatively or semi quantitatively with histological techniques, cell type composition in digestive gland epithelium, digestive tubule epithelial atrophy and thinning, lysosomal alterations and inflammation and are described in detail below.

7. Cell type composition in digestive gland epithelium

Under normal physiological conditions the digestive cells outnumber basophilic cells, but under different stress situations, including exposure to pollutants, the relative occurrence of basophilic cells is apparently augmented (Rasmussen *et al.*, 1985; Lowe and Clarke, 1989; Cajaraville *et al.* 1990, Marigómez *et al.* 1990, 1998; 2006; Zorita *et al.*, 2006; 2007; Garmendia *et al.*, 2011b). Changes in cell type composition in the digestive gland epithelium constitute a common response in molluscs that may lead to disturbances in food digestion and xenobiotic metabolism and accumulation Marigómez *et al.* 1998). These changes have been attributed to basophilic cell proliferation (Lowe and Clarke, 1989; Cajaraville *et al.*, 1989; Marigómez *et al.*, 1990), but it has been recently concluded that it mainly results from digestive cell loss and basophilic cell hypertrophy (Zaldibar *et al.*, 2007), which is a fast inducible and reversible response that can be measured in terms of volume density of basophilic cells ($VvBAS$). In clean localities and in experimental control conditions, $VvBAS$ is usually below $0.1 \mu\text{m}^3/\mu\text{m}^3$ but after exposure to pollutants $VvBAS$ may surpass $0.12 \mu\text{m}^3/\mu\text{m}^3$ (Marigómez *et al.*, 2006)

A stereological procedure is applied in order to quantify the volume density of basophilic cells ($VvBAS$) as a measure of digestive cells loss by counting on H/E stained digestive gland paraffin sections (Soto *et al.*, 2002). Cell counts (digestive and basophilic cells) are made in one field randomly selected per mussel ($n=10$) to complete a total of 10 counts per experimental group, with the aid of a drawing tube attached to

a light microscope using a 20x objective lens. A Weibel graticule (multipurpose test system M-168) is used, and hits on basophilic cells and on remaining digestive epithelium are recorded to calculate $VvBAS$ according to the Delesse's principle:

$$VvBAS (\mu m^3/\mu m^3) = x/(m+x);$$

where “x” is the number of hits on basophilic cells and “m” is the number of hits on digestive cells. The statistical signification of changes in $VvBAS$ volume is determined according to parametric tests (e.g. ANOVA, Duncan's test for comparison between pairs of means; $p < 0.05$). Assessment criteria should be considered as:

Background:	<0.12	$\mu m^3/\mu m^3$
Elevated:	0.12-0.18	$\mu m^3/\mu m^3$
High:	>0.18	$\mu m^3/\mu m^3$

8. Digestive tubule epithelial atrophy and thinning

The best documented cellular alteration in bivalves is apparent atrophy or “thinning” of the digestive gland epithelium. The digestive gland of mussels is greatly dynamic and plastic. The morphology of digestive alveoli undergoes severe changes even during normal physiological processes (i.e. trough every digestion cycle; Langton 1975). Changes in the normal phasic activity may be attributed to environmental factors, such as food availability or saline and thermal stress (Winstead, 1995) as well as exposure to pollutants. Particularly, it has been widely demonstrated that molluscs exposed to pollutants exhibit a net mass loss in the digestive gland epithelium that gives rise to abnormal epithelial thinning and finally atrophy (Lowe *et al.*, 1981; Couch, 1984; Lowe and Clarke, 1989; Vega *et al.*, 1989; Cajaraville *et al.*, 1992; Marigómez *et al.*, 1993; Garmendia *et al.*, 2011b). Atrophy and epithelial thinning constitute a non-specific fast inducible and slowly or not recoverable response to stressful environmental conditions that can be measured after semi-quantitative scoring (Kim *et al.*, 2006) or after quantitative morphological analysis in terms of $MPTW$ (mean proportion of tubule width; Robinson, 1983); or in terms of mean epithelial thickness (MET) and the relative parameters MLR/MET and MET/MDR (Lowe *et al.*, 1981; Vega *et al.*, 1989; Cajaraville *et al.*, 1992; Marigómez *et al.*, 1993; 2006; Garmendia *et al.*, 2011b), where MLR is the mean luminal radius and MDR the mean diverticular radius. MLR/MET ratio is more sensitive than MET alone. The alterations in these parameters are used as tissue-level biomarkers in ecosystem health assessment (Garmendia *et al.*, 2011b).

The following table describes a semi-quantitative scoring index for digestive tubule epithelial atrophy and thinning*.

Stage	Response	Description
0	None	Normal tubule thickness (0% atrophy). Lumen nearly occluded, few tubules exhibiting slight atrophy.
1	Low	Epithelium averaging less than one-half (50%) normal thickness (stage 0), most tubules show some atrophy although some tubules appear normal.
2	Elevated	Epithelium averaging about 50% of normal thickness (stage 0).
3	High	Epithelium thickness greater than one-half (50%) atrophied, most tubules affected. Some tubules extremely thin (fully atrophied).
4	Severe	Epithelium extremely thin (100% atrophied), nearly all tubules affected.

*adapted from Ellis (1996)

Most commonly, a planimetric procedure has been applied to quantify changes in size and shape of the digestive alveoli (Vega *et al.*, 1989) resulting in apparent epithe-

lial thinning. A total of 50-100 tubular profiles per sample (2 profiles per field in 5 fields per mussel in 5-10 mussels per sample) are recorded in an image analysis system attached to light microscope using a 20x objective lens. The five measurement fields are selected at given intervals throughout the tissue section, the direction of movement always following a zigzag pattern. Alternatively tubular profiles can be drawn with the aid of a drawing tube attachment to the light microscope and then digitised for data input into a computer. Other methods are also available since the final goal is just calculating the section areas of the lumen and the whole tubule profile, which can be done by image analysis systems (after data input into the computer), by hand (e.g. using millimetre paper), or by point counting onto a Weibel stereological graticle (Weibel ER, 1979). *MET*, *MLR* and *MDR* are quantified (in μm) and the ratios *MLR/MET* and *MET/MDR* (in $\mu\text{m}/\mu\text{m}$) are calculated as integrative measures of changes in the alveolar morphology, epithelial thinning included, as follows:

$$MET = 2(A_0 - A_i)/(P_0 + P_i);$$

$$MLR = \sqrt{(A_i/\pi)}; \text{ and}$$

$$MDR = \sqrt{(A_0/\pi)};$$

where A_0 is the section area of the whole tubule profile, P_0 is the perimeter of a circle with area A_0 , A_i is the section area of the lumen profile and P_i is the perimeter of the corresponding circle with area A_i . The statistical signification of changes in these parameters is determined according to parametric tests (e.g. ANOVA, Duncan's test for comparison between pairs of means; $p < 0.05$). *MLR/MET* values between 0.7 $\mu\text{m}/\mu\text{m}$ (spring-summer) and 1.2 $\mu\text{m}/\mu\text{m}$ (winter) have been recorded in *M. galloprovincialis* of reference localities in Southern Bay of Biscay, whereas after exposure to pollutants or stress in long-term laboratory manipulation *MLR/MET* surpasses 1.6 $\mu\text{m}/\mu\text{m}$ (Marigómez *et al.*, 2006).

9. Lysosomal alterations

Lysosomal responses are widely used as effect biomarkers indicative of the general stress provoked by pollution in the marine environment. Lysosomes are cell organelles containing acid hydrolases. The digestive cells of mussels possess a complex endo-lysosomal system that is primarily involved in the uptake and digestion of food materials as well as in processes of pollutant accumulation and detoxification. Endo-lysosomes and hetero-lysosomes occupy the majority of digestive cell cytoplasm and are reactive for marker hydrolases such as N-acetyl hexosaminidase, β -glucuronidase and acid phosphatase (Izagirre and Marigómez 2009a; Izagirre and Marigómez 2009b). Lysosomal responses to environmental stress fall into essentially three categories: increased lysosomal size, reduced membrane stability, and changes in lysosomal contents (Marigómez and Baybay-Villacorta, 2003).

Lysosomal enlargement

Diverse sources of environmental stress (chemical pollution, salinity changes, elevated temperature, malnutrition, reproductive stress) are known to provoke an increase in the size of digestive cell lysosomes in mussels, often accompanied by increased enzyme activity and lysosome numbers, which may compromise intracellular digestion and detoxification capacity (Moore, 1985; 1988; Lowe, 1988; Cajaraville *et al.*, 1989; 1995; Marigómez *et al.*, 1995; 2005; 2006; Domoutsidou and Dimitriadis, 2001; Garmendia *et al.*, 2011a). These lysosomal structural changes (LSC) have been commonly determined by image analysis of digestive gland cryotome sections where β -

glucuronidase is employed as lysosomal marker enzyme. The final calculations of the structural parameters are in most cases based on the equations published by Lowe *et al.* (1981). The structural parameters are lysosomal volume density (Vv), surface density (Sv), surface-to-volume ratio (S/V) and numerical density (Nv). Although the four stereological parameters altogether provide complete information about the size, size-class distribution and number of lysosomes in mussel digestive cells, Vv can be sufficient to detect changes in the size of the endo-lysosomal system and is therefore the most used parameter.

Stereological determination of lysosomal enlargement

The histochemical reaction for β -Gus is demonstrated as in Moore (1976) with the modifications described by Cajaraville *et al.* (1989). Slides are kept at 4°C for 30 min and then at RT for 5 min prior to staining. Sections (8 μ m) are incubated in freshly prepared β -Gus substrate incubation medium consisting of 28 mg naphthol AS-BI- β -glucuronide (Sigma, N1875) dissolved in 1.2 ml 50 mM sodium bicarbonate, made up to 100 ml with 0.1 M acetate buffer (pH 4.5) containing 2.5% NaCl and 15% polyvinyl alcohol, for 40 min at 37°C in a shaking water bath. After incubation, slides are rinsed in a 2.5% NaCl solution for 2 min at 37°C in a shaking water bath and then transferred to a postcoupling medium containing 0.1 g Fast garnet GBC (Sigma, F8716) dissolved in 100 ml 0.1 M phosphate buffer (pH 7.4 containing 2.5% NaCl) for 10 min in the dark and at RT. Afterwards, the sections are fixed for 10 min at 4°C in Baker's formol calcium containing 2.5% NaCl and rinsed briefly in distilled water. Finally, sections are counterstained with 0.1% Fast green FCF (Sigma, F7252) for 2 min, rinsed several times in distilled water, mounted in Kaiser's glycerine gelatine and sealed with nail varnish. Then, de visu grading and scoring can be applied to grossly determine the extent of lysosomal enlargement (Lowe, 1988), which can be straightforward and very useful in cases of extreme symptoms. However, quantifying lysosomal enlargement by hand stereology (Cajaraville *et al.*, 1989; 1992) or by image analysis (Marigómez *et al.*, 2005; Izagirre and Marigómez, 2009) can provide evidence of more subtle lysosomal responses. Slides are viewed under a light microscope fitted with a $\times 100$ objective lens. A Weibel graticule (multipurpose test system M-168) is used, and hits on digestive cell lysosomes and on digestive cell cytoplasm are recorded to calculate Vv_{LYS} , Sv_{LYS} , S/V_{LYS} , and Nv_{LYS} according to Lowe *et al.*, (1981). Five measurements are made per section in each of the 5–10 individuals per sample. The stereological formulae include a correction factor for particles with an average diameter smaller than the section thickness (Lowe *et al.* 1981). For this reason the average diameter of at least 90 lysosomes must be directly measured at the light microscope with the aid of a graded eyepiece or similar device (or directly by the image analysis system):

$$Vv_{LYS} (\mu m^3/\mu m^3) = K \times AA;$$

$$Sv_{LYS} (\mu m^2/\mu m^3) = (4/t) \times AA;$$

$$S/V_{LYS} (\mu m^{-1}) = 4/(t \times K); \text{ and}$$

$$Nv_{LYS} (\mu m^2/\mu m^3) = (4 \times AA \times n) / (t \times \pi \times \Sigma Y_i^2)$$

being

$$AA = x/m \text{ and } K = (2/(3 \times t))(\Sigma Y_i^3/\Sigma Y_i^2);$$

and where “ x ” is the number of hits on digestive cell lysosomes, “ m ” is the number of hits on digestive cells (lysosomes included), “ t ” is the section thickness (i.e., 8 μ m),

“*n*” is the number of lysosomes whose diameter has been measured; and “*Y*” are lysosomal diameters (*Y*₁, *Y*₂, ... *Y*₉₀ for *n*=90).

Lysosomal structural changes test parameters can be tested using analysis of variance. *VvLYS* and *NvLYS* data may need to be logarithmically transformed previous to the statistical analyses since the variance within individuals may depend on the mean. Parametric tests for multiple comparisons between paired means (e.g. Duncan's test) can be further applied to detect significant (*P*<0.05) differences between means.

In general terms, lysosomes become enlarged under stress conditions, which are reflected as increased in *VvLYS* and *SvLYS* values, concomitant with lowered *S/VLYS* values (Cajaraville *et al.* 1995; Marigómez *et al.*, 2005). In certain cases, lysosomal enlargement is accompanied by increased *NvLYS*, (increased numbers of lysosomes relative to digestive cell cytoplasm) but reductions in *NvLYS* have also been reported. On the other hand, exposure to pollutants may also elicit an intricate response that includes different phases (Marigómez & BayBay-Villacorta 2003): (a) transient lysosomal enlargement; (b) transient lysosomal size reduction; and finally (c) lysosomal enlargement after long-term exposure. Overall, reference values for these lysosomal parameters vary with season but *VvLYS* >0.002 $\mu\text{m}^3/\mu\text{m}^3$ and *S/VLYS* >5 may be indicative of the existence of a degraded health status in mussels that correlates with e.g. the degree of exposure to pollutants.

10. Inflammation

Inflammation affects all tissues and organs and is particularly obvious in mussels that have been adversely affected by contaminants (Auffret, 1988; Crouch, 1985). Whilst this may be true, it is important to remember that the presence of pathogens can also result in a host immune response (but not always) manifested as inflammation. Inflammation is observed as either diffuse, focal or both in appearance throughout the vesicular connective tissue and at varying degrees of severity.

Haemocytic infiltration is generally characterised by the infiltration of granulocytes possessing an eosinophilic cytoplasm into the connective tissues. Care should be taken not to confuse this with normal circulating haemocytes that are often situated around the stomach and intestine. Heavy diffuse inflammation will appear as a marked increase in the number of circulating haemocytes situated throughout the majority of connective tissues and in between organs such as the digestive diverticula and gonad. Haemocytic infiltration of the visceral mass in bivalves is generally considered to be indicative of stress, unrecognised injury or sub-microscopic agents in bivalves. Haemocytic infiltration could be interpreted as a repair process following tissue damage, albeit pathological effects could be exerted through acting as space occupying lesions. Its presence has been suggested as a qualitative or quantitative index of stress, indicative of a loss of condition. Previous studies have reported haemocytic infiltration in response to starvation and spawning stress, shell damage, and exposure to pollutants.

Brown cell (BC) aggregates (foci) are generally small and possess varying quantities of the pigment lipofuscin and are often seen in elevated numbers in mussels from contaminated environments. Comprised of serous cells, these phagocytes are mostly found within the connective tissue and possess the ability to physically remove endocytosed matter across epithelia via diapedesis. These cells are responsible for the metabolism of metal ions and can be found within the gills, which is an important organ for metal ion exchange (Marigómez *et al.* 2002). The occurrence of BC aggregates

(foci) has been considered an indicator of stress caused by xenobiotics, as well as with age and reproductive stress. BC aggregates are also observed within the gonad follicles following spawning, which is a normal event.

Large foci of inflammation termed granulocytomas (comprised of granulocytes), have previously been seen in mussels of both laboratory and field studies designed to monitor the effects of contaminants. Granulocytomas represent an inflammatory response to an irritant or pollutant, resulting in vascular occlusions. They are believed to result from chronic exposure to domestic and industrial waste products and have been reported in bivalves subjected to the impact of oil, chlorinated pesticides and heavy metals. Granulocytomas are also associated with pathogens therefore it is important to look for any indication of infection in affected individuals. These lesions can be seen at varying degrees of severity from singular foci to large numbers affecting the majority of the connective tissues. Granulocytomas can vary largely in size. In mussels, the maximum size of a known parasitically-induced granulocytoma is 400 µm, however granulocytomas of unknown aetiology can be over 800 µm (up to 1500 µm).

The following table describes a semi-quantitative scoring index for inflammation.

Stage	Response	Description
0	None	No inflammatory foci can be seen within tissues. Brown cell foci rare.
1	Low	Low numbers of inflammatory foci occupying ≤ 10% of the vesicular connective tissue (approximately 20 small foci) within standardised tissue cross section. Brown cell foci rare.
2	Elevated	Increased numbers and/or size of inflammatory foci occupying between 10% and 50% of vesicular connective tissue. Foci may displace other structures. Areas of diffuse haemocyte infiltration may also be present. Increased numbers of Brown cell foci predominately within the vesicular connective tissue, stomach and digestive gland epithelium.
3	High	Significant inflammatory response - numerous and/or large inflammatory foci (possibly with granulocytoma present) occupying ≥ 75% of vesicular connective tissue. Widespread diffuse haemocytic infiltration may be present. Increased numbers of Brown cell foci predominately within the vesicular connective tissue, stomach and digestive gland epithelium. Increased pigment density.

11. Assessment Criteria

Several parameters have been identified as suitable for the development of assessment criteria. Other histological parameters can also be measured using histopathology, although many of these fluctuate showing clear seasonal cycles (Bignell *et al.*, 2008). As such the development of assessment criteria is not deemed appropriate. Nonetheless, the collection of these data can provide additional information on the health and physiology of the mussel. Parameters include reproductive markers such as adipogranular cells, gonadal apoptosis, atresia, hermaphroditism and intersex. All health parameters are described in full detail in the ICES TIMES document (Bignell *et al.*, 2011).

The thresholds identified here have been determined using data collected as part of previous studies (Cajaraville *et al.* 1992; Marigomez *et al.* 2004; Marigomez *et al.* 2005; Marigomez *et al.* 2006; Bignell *et al.* 2008). It must be stressed that these thresholds are

preliminary and will require further review as part of a holistic assessment of these histological parameters.

Biological effect	Qualifying comments	Background	Elevated	High
Mussel histopathology	VVbas: Cell type composition of digestive gland epithelium (quantitative)	<0.12 $\mu\text{m}^3/\mu\text{m}^3$	0.12-0.18 $\mu\text{m}^3/\mu\text{m}^3$	>0.18 $\mu\text{m}^3/\mu\text{m}^3$
	MLR/MET: Digestive tubule epithelial atrophy and thinning (quantitative)	<0.7 $\mu\text{m}/\mu\text{m}$	1.2-1.6 $\mu\text{m}/\mu\text{m}$	>1.6 $\mu\text{m}/\mu\text{m}$
	VVLYS & S/VLYS: Lysosomal enlargement * (quantitative)	VvLYS <0.0002 $\mu\text{m}^3/\mu\text{m}^3$ S/VLYS > 4 $\mu\text{m}^2/\mu\text{m}^3$	0.0002 – 0.0004 $\mu\text{m}^3/\mu\text{m}^3$ S/VLYS < 4 $\mu\text{m}^2/\mu\text{m}^3$	V>0.0004 $\mu\text{m}^3/\mu\text{m}^3$ S/VLYS <<4 $\mu\text{m}^2/\mu\text{m}^3$
	Digestive tubule epithelial atrophy and thinning (semi-quantitative)	STAGE ≤ 1 (Mode)	STAGES 2-3 (Mode)	STAGE 4 (Mode)
	Inflammation (semi-quantitative)	STAGE ≤ 1 (Mode)	STAGE 2 (Mode)	STAGE 3 (Mode)

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Annex 10: Micronucleus and comet assay

Micronucleus assay as a tool for assessing cytogenetic/DNA damage in marine organisms

Authors: Janina Baršienė¹ and Brett Lyons²

The primary authors acknowledge contributions from Aleksandras Rybakovas¹, Concepción Martínez-Gómez⁵, Steinar Sanni³, Steven Brooks⁴, Beatriz Fernández⁵, and Thomas Maes⁶

¹Institute of Ecology of Nature Research Center (NRC), Vilnius, Lithuania

²Centre for Environment Fisheries and Aquaculture Science (Cefas), Weymouth, UK

³International Research Institute of Stavanger, (IRIS)–Akvamiljø, Randaberg, Norway

⁴Norwegian Institute for Water Research (NIVA), Oslo, Norway

⁵Instituto Español de Oceanografía (IEO), Centro Oceanográfico de Murcia, Spain

⁶Centre for Environment Fisheries and Aquaculture Science (Cefas), Lowestoft, UK

Background

1. Micronuclei (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 micronucleus (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 fibers 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). Thus, the scoring during interphase provides a measure of genotoxicity both in the field and also specifically through genotoxic compound exposure in the laboratory due to clastogens and/or aneugens (Al-Sabti and Metcalfe, 1995; Heddle *et al.*, 1991). In addition, there are direct indications that MN additionally may be formed via a nuclear budding mechanism in the interphase of cell division. The formation of such type MN reflects in an unequal capacity of the organisms to expel damaged, amplified, failed replicated or improperly condensed DNA, chromosome fragments without telomeres and centromeres from the nucleus (Lindberg *et al.*, 2007).

2. The micronuclei assay involves the scoring of the cells which contain one or more micronuclei 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, de Raat (1982) were the first to successfully apply the assay to aquatic species when they demonstrated the induction of micronuclei in erythrocytes of the eastern mudminnow (*Umbra pygmaea*) following waterborne exposure to the known mutagen ethyl methanesulphate (EMS). Since these initial experiments, other studies have validated the detection of micro-

nuclei as a suitable biomarker of genotoxicity in a wide range of both vertebrate and invertebrate species (for review see Chaudhary *et al.*, 2006; Udroui *et al.*, 2006; Bolognesi and Hayashi, 2011). In fish most studies have utilised 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).

3. Environmental genotoxicity levels in organisms from 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, 2008a, 2010a; Bagni *et al.*, 2005; Bolognesi *et al.* 2006b; Magni *et al.* 2006; Fernandez *et al.*, 2011). Concerns about the environmental genotoxicity in an oil and gas industrial areas of the North Sea were raised when comparatively high levels of micronuclei incidences were detected in mussels *Mytilus edulis* and Atlantic cod *Gadus morhua* caged closely to the oil platforms (Hylland *et al.*, 2008). Increased environmental genotoxicity and cytotoxicity has been described in an offshore Ekofisk oil extraction field (Rybakovas *et al.*, 2009). The Water Column Monitoring Programme indicated increased genotoxicity in caged mussels in sites that were close to the Ekofisk oil platform indicating the ability to pinpoint source discharges with genotoxic endpoints in caged mussels (Baršienė, IRIS WCM Reports 2006, 2008; Brooks *et al.*, 2011). Significant MN elevation in fish and mussels was found after exposure to the crude oil extracted from the North Sea (Baršienė *et al.*, 2006a; Bolognesi *et al.*, 2006; Baršienė, Andreikėnaitė, 2007; Andreikėnaitė, 2010) and from arctic zones (Baršienė *et al.*, unpublished data).

4. The frequency of the observed micronuclei 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 micronuclei frequency may provide early warning signs of cumulative stress (Bolognesi and Hayashi 2011). The exposure of caged mussels in the Genoa harbour, heavily polluted by aromatic hydrocarbons showed a continuous increase of micronuclei in mussel gill cells reaching a plateau after a month of caging (Bolognesi *et al.*, 2004). After 30-days caging of mussels at the Cecina estuary in Tyrrhenian coast, 2-fold increase of MN incidences in gill cells has been observed (Nigro *et al.*, 2006). The gradient-related increase in MN was found in haemocytes of mussels and liver erythrocytes of Atlantic cod caged for 5-6 months at Norwegian oil platforms in the North Sea (Hylland *et al.*, 2008, Brooks *et al.*, 2011). Furthermore recovery was detected in the Haven oilship sinking zone using the MN test in caged mussels 10 years after the oil spill (Bolognesi *et al.*, 2006b). In this respect, increase in micronuclei frequency represents a time integrated response to cumulative stress.

Short description of methodology

5. Target species

Micronuclei frequency 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, Atlantic cod or red mullet), which are routinely used in biomonitoring programmes and assess contamination along western European marine waters (see Table 1 below). 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 micronuclei 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.

In general, indigenous, ecologically and economically important fish and mollusc species could serve as indicator species for biomonitoring of environmental genotoxicity levels, for screening of genotoxins distribution or for assessments of genotoxicity effects from contaminant spills or effluent discharges. For monitoring in deep waters in northern latitudes (deeper than 1000 m), the fish Arctic rockling *Omogadus argentus* and amphipods *Eurythenes gryllus* are suitable species. In equatorial regions of the Atlantic, indicator fish species *Brachydetrius aurectus*, *Synoglossus senegalensis*, *Cynoponticus ferox* are available for the MN analysis (Baršienė IRIS reports for Deepvann and Anquilla reports).

6. Target tissues

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 haemopoietic 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 analysed. For example it is known that Atlantic cod have very low levels of MN in blood erythrocytes in specimens from reference sites, or control groups in laboratory exposures to crude oil. Furthermore it has been shown that MN induction in cod blood erythrocytes and erythrocytes from different hemopoietic tissues (liver, kidney, gill and spleen) differ significantly after 3 weeks exposure to Stajford B crude oil. In multiple laboratory exposures (108 exposure groups of cod), developing liver and kidney erythrocytes were proved to be the most sensitive endpoint and most suitable approach for the assessment of oil pollution in the northern Atlantic and North Sea (Baršienė *et al.*, 2005b, 2006a). Liver as a target organ can also be used in *in situ* exposures with turbot and halibut (caging or laboratory) (Baršienė, IRIS reports on BioSea, PROOF, WCM projects).

7. Sample and cell scoring size

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, 2010a, 2010b; 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.*, 2006). Previously scorings of 5000-10000 fish erythrocytes were used for a MN analysis (Baršienė *et al.*, 2004). Since 2009-2010, the frequency of MN in fish from the North and 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 (Venier and Zampieron, 1997; Bolognesi *et al.*, 2004; Baršienė *et al.*, 2004, 2006e, 2008a, 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 (Fig. 1 in Fang *et al.*, 2009; Baršienė *et al.*, unpublished results).

8. MN identification criteria. Most of the studies have been performed using diagnostic criteria for micronuclei 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
- Micronuclei are round- or ovoid-shaped, non-refractive chromatin bodies located in the cytoplasm of the cell and can therefore be distinguished from artefacts such as staining particles.
- Micronuclei are not connected to the main nuclei and the micronuclear boundary should be distinguishable from the nuclear boundary.

After sampling and cell smears preparation, slides should be coded. To minimize technical variation, the blind scoring of micronuclei 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 micronuclei should then be undertaken at 1000x magnification.

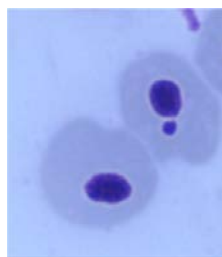


Image A. Erythrocyte of *Mullus barbatus* displaying a micronuclei formation (x 1000). Author C. Martínez-Gómez.



Image B. Gill cell of *Mytilus galloprovincialis* displaying a micronuclei (x 1000). Author B. Fernández.

Confounding factors

9. 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 programmes, 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 (4-37°C) can increase the ability of genotoxic compounds to damage DNA (Buschini *et al.*, 2003).

Types of cells

- 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) like mammals do.

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 North Sea (Karmsund zone), Wismar Bay and Lithuanian coast. Similar results were found for *Macoma balthica* from the Baltic Sea – from Gulfs of Bothnia, Finland, Riga and Lithuanian EZ (Baršienė *et al.*, unpublished data).

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.*, 2006e).

Ecological relevance

10. 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, micronuclei 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). Micronucleus formation indicates chromosomal breaks, known to result in teratogenesis (effects on offspring) in mammals. There is however limited knowledge of relationships between micronucleus 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.

Applicability across the OSPAR maritime area

Large-scale and long-term studies took place from 2001 to 2010 at the Nature Research Center (NRC, Lithuania) on micronuclei (MN) and other abnormal nuclear formations in different fish and bivalve species inhabiting various sites of the North Sea, Baltic Sea, Atlantic Ocean and Barents Sea. These studies revealed the relevance of environmental genotoxicity levels in ecosystem assessments. Nature Research Center established a large database on MN and other nuclear abnormalities in 13 fish species from the North Sea, Barents Seas and Atlantic Ocean, in 8 fish species and in mussels, scallops and clams *Macoma balthica* from the Baltic Sea. Fish and bivalve species were collected from 85 sites in the North Sea and Atlantic and from 117 coastal and offshore sites in the Baltic (Figures 1 and 2). Monitoring of MN and other nuclear abnormalities levels was performed (2–8 times) in many sites of the North and Baltic Seas. Data on MN levels in organisms inhabiting deep-sea and arctic zones are also available (Table 1).

The validation of the MN assay was done with indigenous and cultured mussels *M. edulis*, Atlantic cod, turbot, halibut and long rough dab in multiple laboratory exposures to crude oil from the North Sea and Barents Sea, to produced water discharged from the oil platforms and to other contaminants. Additional active monitoring using mussels and Atlantic cod took place in the Ekofisk, Statfjord, Troll oil platform, oil refinery zones, some northern Atlantic sites as well as in sites heavily polluted by copper or PAHs.

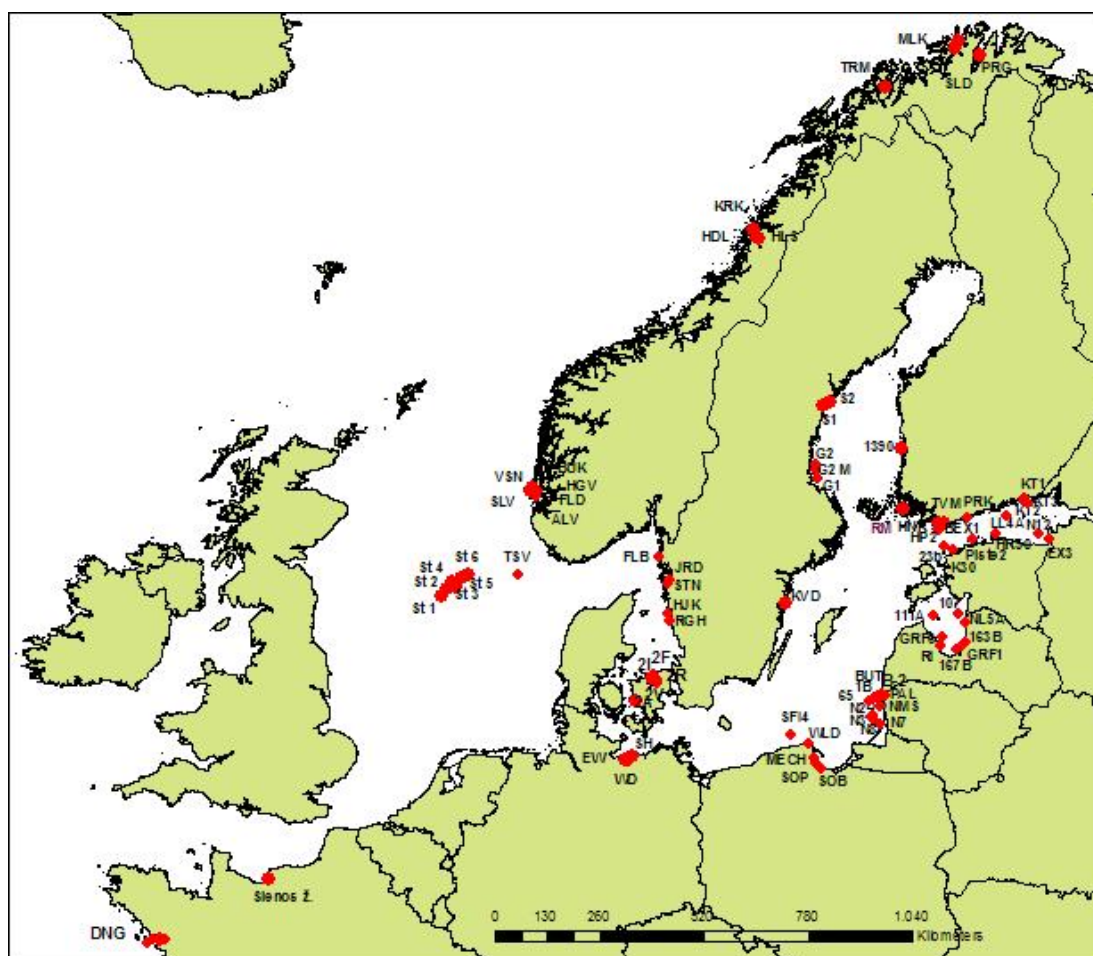


Figure 1. Sampling stations of bivalve molluscs for the micronuclei studies (NRC, Lithuania).

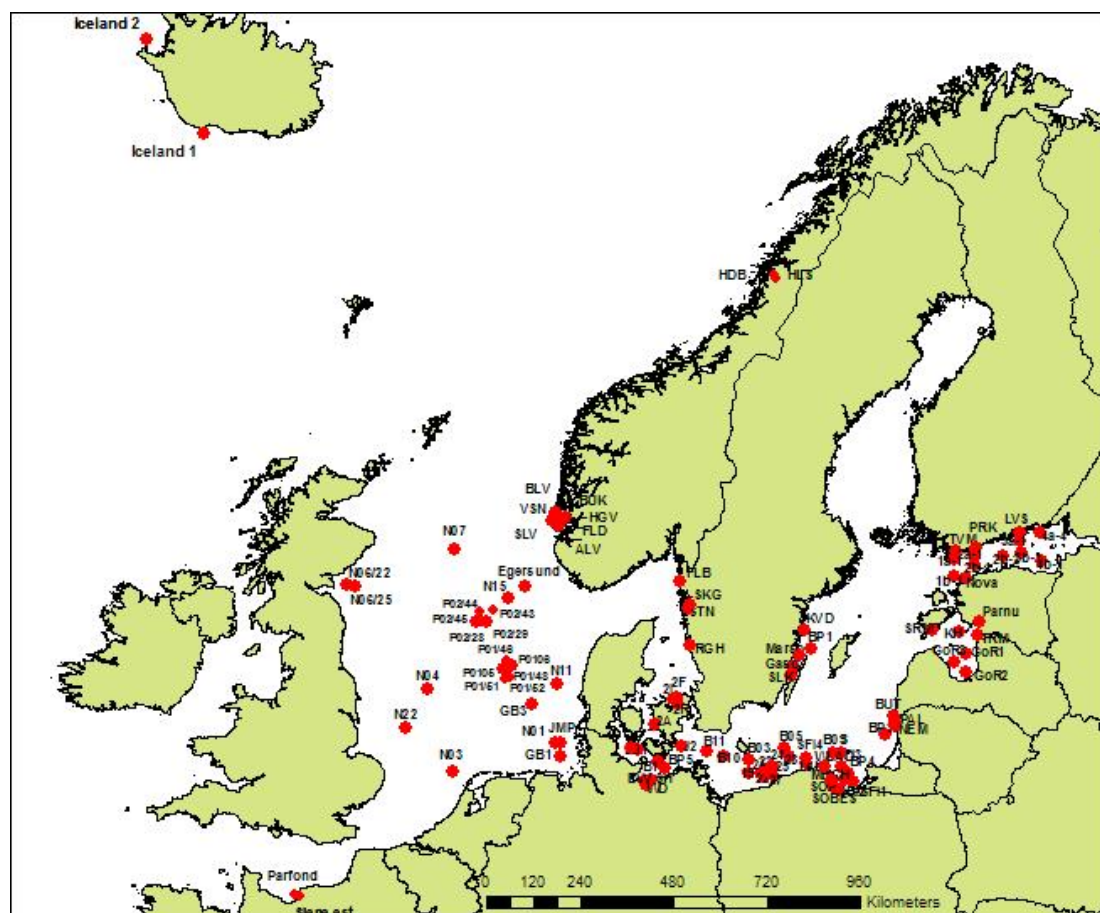


Figure 2. Sampling stations of fish species used for the micronuclei studies (NRC, Lithuania).

Background responses

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). As mentioned above, several studies have demonstrated that MN baseline frequencies depend on water temperature. In fish, MN frequencies lower than 0.05‰ (the Baltic Sea) and lower than 0.1‰ (the North Sea) 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 fishes from 12 offshore sites in the Baltic Sea (479 specimens) and 11 sites in the North Sea (291 specimens). For unpolluted sites in the Mediterranean Sea, baseline MN levels in gills of *M. galloprovincialis* have been set depending on water temperature to 1% at temperatures below 15°C, 2% between 15 and 20°C, and 3% above 20°C (Brunetti *et al.*, 1992).

The frequencies of micronuclei in marine species sampled from field reference sites are summarized in Table 1. Additionally, the frequencies of MN in blood erythrocytes of fish and in gill cells of mussels deployed to the uncontaminated sites are shown (Table 2).

Table 1. The reference levels of micronuclei (MN/1000 cells) in European marine species *in situ*.

Species	Tissue	Location	Response MN/1000 cells	Reference
<i>Mytilus galloprovincialis</i>	Gills	Adriatic and Tyrrhenian Sea	1.0 –at 15°C 2.0 – at 15-20 °C 3.0 –at above 20°C	Brunetti <i>et al.</i> , 1992
<i>M. galloprovincialis</i>	Haemolymph	Mediterranean coast	4.2± 0.7	Burgeot <i>et al.</i> 1996
<i>M. galloprovincialis</i>	Gills	La Spezia Gulf, Ligurian Sea	3.0± 2.0	Bolognesi <i>et al.</i> , 1996
<i>M. galloprovincialis</i>	Gills, Haemo-lymph	Venice Lagoon	0,73- 1,42	Dolcetti and Venier, 2002
<i>M. galloprovincialis</i>	Haemolymph	Strymonikos gulf, Mediterranean Sea	0.30; 1.30	Dailianis <i>et al.</i> 2003
<i>M. edulis</i>	Gills	Gijon coast, Spain	1.42	Izquierdo <i>et al.</i> , 2003
<i>M. galloprovincialis</i>	Gills	Strymonikos gulf, Mediterranean Sea	1.30	Dailianis <i>et al.</i> 2003
<i>M. galloprovincialis</i>	Haemolymph	Venice lagoon	0.44	Pampanin <i>et al.</i> , 2005
<i>M. galloprovincialis</i>	Gills	Tyrrhenian Sea	5.4	Nigro <i>et al.</i> , 2006
<i>M. galloprovincialis</i>	Gills	Gulf of Oristano, Mediterranean Sea	2.94 – 4.70	Magni <i>et al.</i> 2006
<i>M. galloprovincialis</i>	Haemolymph	Adriatic Sea	1.0 – 1.5	Klobučar <i>et al.</i> 2008
<i>M. galloprovincialis</i>	Haemolymph	Adriatic Sea	1.38 – 1.75	Pavlica <i>et al.</i> 2008
<i>M. galloprovincialis</i>	Gills	Gulf of Patras	≈ 2.0	Pytharopoulou <i>et al.</i> , 2008
<i>M. galloprovincialis</i>	Gills	Algerian coast	0.0-1.18	Taleb <i>et al.</i> , 2009
<i>M. galloprovincialis</i>	Haemolymph	Algerian coast	1.6-2.47	Taleb <i>et al.</i> , 2009
<i>M. galloprovincialis</i>	Gills	Western Mediterranean	1.9-2.1	Fernandez <i>et al.</i> , 2011
<i>M. edulis</i>	Haemolymph	Langesundfjord (Norway, rock)	0.90	Wrisberg <i>et al.</i> , 1992
<i>M. edulis</i>	Haemolymph	Store Belt (Denmark)	0.89	Wrisberg <i>et al.</i> , 1992
<i>M. edulis</i>	Gills	North Sea (Norwegian coast and Karmsund fjords)	1.05± 0.32	Baršienė <i>et al.</i> , 2004
<i>M. edulis</i>	Gills	North Sea (Goteborg coast)	0.71± 0.12	Baršienė <i>et al.</i> , 2008a
<i>M. edulis</i>	Haemolymph	North Sea	1.24± 0.37	Brooks <i>et al.</i> , 2011
<i>M. edulis</i>	Gills	Baltic Sea	0.37± 0.09	Baršienė <i>et al.</i> , 2006b
<i>Mytilus trossulus</i>	Gills	Baltic Sea	2.07± 0.32	Baršienė <i>et al.</i> , 2006b; Kopecka <i>et al.</i> , 2006

<i>Macoma baltica</i>	Gills	Baltic Sea	0.53-1.28	Baršienė <i>et al.</i> , 2008b, unpublished data
<i>Macoma baltica</i>	Gills	Stockholm archipelago	0.4	Smolarz, Berger, 2009
<i>Limanda limanda</i>	Blood , kidney erythrocytes	North Sea	0.02± 0.01	Rybakovas <i>et al.</i> , 2009
<i>Platyichthys flesus</i>	Blood erythrocytes	Atlantic Ocean	0.06± 0.04	Baršienė <i>et al.</i> , unpublished data
<i>Platyichthys flesus</i>	Blood erythrocytes	North Sea	0.04± 0.03	Baršienė <i>et al.</i> , 2008a;
<i>Platyichthys flesus</i>	Blood erythrocytes	Baltic Sea	0.15± 0.03	Baršienė <i>et al.</i> , 2004
<i>Platyichthys flesus</i>	Blood erythrocytes	Baltic Sea	0.0± 0.0	Kohler, Ellesat, 2008
<i>Platyichthys flesus</i>	Blood erythrocytes	Baltic Sea	0.08± 0.02	Napierska <i>et al.</i> , 2009
<i>Platyichthys flesus</i>	Blood erythrocytes	UK estuaries	0.27 – 0.66	Lyons, unpublished
<i>Zoarces viviparus</i>	Blood erythrocytes	Baltic Sea	0.02± 0.02	Baršienė <i>et al.</i> , unpublished data
<i>Gadus morhua</i>	Blood, kidney erythrocytes	North Sea	0.03± 0.02	Rybakovas <i>et al.</i> , 2009
<i>Gadus morhua</i>	Blood, kidney erythrocytes	Baltic Sea	0.03± 0.02	Rybakovas <i>et al.</i> , 2009
<i>Clupea harengus</i>	Blood erythrocytes	Baltic Sea	0.03± 0.03	Baršienė <i>et al.</i> , unpublished data
<i>Symphodus melops</i>	Blood erythrocytes	North Sea	0.08± 0.04	Baršienė <i>et al.</i> , 2004
<i>Scophthalmus maximus</i>	Blood erythrocytes	Baltic Sea	0.10± 0.04	Baršienė <i>et al.</i> , unpublished data
<i>Perca fluviatilis</i>	Blood erythrocytes	Baltic Sea	0.06± 0.02	Baršienė <i>et al.</i> , 2005a; Baršienė <i>et al.</i> , unpublished data
<i>Mugil cephalus</i>	Blood erythrocytes	Mediterranean coast, Turkey	0.82-2.07	Cavas, Ergene-Gozukara, 2005
<i>Mugil cephalus</i>	Gill cells	Mediterranean coast, Turkey	1.84-2.91	Cavas, Ergene-Gozukara, 2005
<i>Mullus barbatus</i>	Blood erythrocytes	La Spezia Gulf (Italy)	0.33 ^a	Bolognesi, 2006a
<i>Dicentrarchus labrax</i>	Blood erythrocytes	La Spezia Gulf (Italy)	0.75 ^a	Bolognesi, 2006a
<i>Pagellus mormyrus</i>	Blood erythrocytes	La Spezia Gulf (Italy)	0.4 ^a	Bolognesi, 2006a
<i>Sargus sargus</i>	Blood erythrocytes	La Spezia Gulf (Italy)	0.25 ^a	Bolognesi, 2006a
<i>Seriola dumerili</i>	Blood erythrocytes	La Spezia Gulf (Italy)	0.38 ^a	Bolognesi, 2006a

<i>Serranus cabrilla</i>	Blood erythrocytes	La Spezia Gulf (Italy)	0.0 ^a	Bolognesi, 2006a
<i>Sparus auratus</i>	Blood erythrocytes	La Spezia Gulf (Italy)	0.12 ^a	Bolognesi, 2006a
<i>Sphyraena sphyraena</i>	Blood erythrocytes	La Spezia Gulf (Italy)	0.25 ^a	Bolognesi, 2006a
<i>Trachurus trachurus</i>	Blood erythrocytes	La Spezia Gulf (Italy)	0.25 ^a	Bolognesi, 2006a
<i>Mugil cephalus</i>	Blood erythrocytes	Mediterranean Goksu Delta, Turkey	1.26± 0.40	Ergene <i>et al.</i> , 2007
<i>Mullus barbatus</i>	Blood erythrocytes	Western Mediterranean-Spain	0.10-0.16	Martínez-Gómez, 2010.
<i>Dicentrarchus labrax</i>	Blood erythrocytes	Eastern Adriatic Sea	1.25 ± 1.97	Strunjak-Perovic <i>et al.</i> 2009

^a – number of micronucleated erythrocytes per 1000 studied erythrocytes

Note: it is important to ensure that the data are normally distributed (e.g. Kolmogorov-Smirnov test) if the standard deviation is to be used to calculate MN frequency percentiles of the distribution, as this assumes that the data are normally distributed, which may not be the case.

It is important not to use the standard deviation to calculate MN frequency percentile from the distribution as it assumes a normal distribution of data, which is not the case.

Table 2. The reference levels of micronuclei (MN/1000 cells) in European marine organisms after caging in uncontaminated/reference sites *in situ*.

Species	Tissue	Location/exposure time	Response MN/1000 cells	Reference
<i>Mytilus galloprovincialis</i>	Gills	Ligurian coast/ 30 days	1.78± 1.04 ^a	Bolognesi <i>et al.</i> , 2004
<i>M. galloprovincialis</i>	Gills	Gulf of Patras/1 month	2.3-2.5	Kalpaxis <i>et al.</i> , 2004
<i>M. galloprovincialis</i>	Gills	Haven oil spill area/ 30 days	3.7± 1.62 ^a	Bolognesi <i>et al.</i> , 2006b
<i>M. galloprovincialis</i>	Gills	Cecina estuary/ 4 weeks	5.4	Nigro <i>et al.</i> , 2006
<i>M. galloprovincialis</i>	Haemolymph	Adriatic Sea/1 month	1.0	Gorbi <i>et al.</i> , 2008
<i>M. galloprovincialis</i>	Haemolymph	Tyrrhenian coast/ 1 month	0.27	Bocchetti <i>et al.</i> , 2008
<i>Mytilus galloprovincialis</i>	Haemolymph	Algerian coast/ 1 month	1.6-2.47	Taleb <i>et al.</i> , 2009
<i>M. galloprovincialis</i>	Gills	Algerian coast/ 1 month	0.0-1.18	Taleb <i>et al.</i> , 2009
<i>Mytilus edulis</i>	Gills	Visnes copper site (Norway)/3 weeks	1.87± 0.43	Baršienė <i>et al.</i> , 2006d
<i>Mytilus edulis</i>	Gills	Karmsund (Norway)/4 weeks	1.40± 0.29	Baršienė <i>et al.</i> , unpublished data
<i>Mytilus edulis</i>	Heamolymph	North Sea, oil platforms (Norway)/6 weeks	2.13± 0.48	Hylland <i>et al.</i> , 2008
<i>Mytilus edulis</i>	Heamolymph	Seiland site (Norway)/5.5 months	2.60± 0.21	Baršienė <i>et al.</i> , unpublished data
<i>Mytilus edulis</i>	Heamolymph	Ekofisk oil platform, North Sea/6 week	1.24± 0.37 (2006) 3.34± 0.28 (2008) 2.78± 0.50 (2009)	Brooks <i>et al.</i> , 2011
<i>Mytilus edulis</i>	Heamolymph	Oil refinery (France, 2004)/	3.20± 0.36	Baršienė <i>et al.</i> , unpublished data
<i>Mytilus edulis</i>	Heamolymph	Oil refinery (France, 2006)/	2.34± 0.37	Baršienė <i>et al.</i> , unpublished data
<i>Mytilus edulis</i>	Heamolymph	Oil refinery (Mongstad, 2007)/100 days	2.90± 0.40	Baršienė <i>et al.</i> , unpublished data
<i>Mytilus edulis</i>	Heamolymph	Sea Empress clean reference area (90 days)	0.75 ± 0.46	Lyons <i>et al.</i> , 1998
<i>Mytilus edulis</i>	Heamolymph	Sea Empress clean reference area (110 days)	0.81±0.36	Lyons <i>et al.</i> , 1998
<i>Crassostrea gigas</i>		Haven oil spill area/30 days	1.49± 0.79 ^a	Bolognesi <i>et al.</i> , 2006b

<i>Gadus morhua</i>	Liver erythrocytes	North Sea, oil platforms (Norway)/5 weeks	0.12± 0.05	Hylland <i>et al.</i> , 2008
<i>Gadus morhua</i>	Liver erythrocytes	North Sea, oil platforms (Norway)/6 weeks	0.27± 0.13	Baršienė <i>et al.</i> , unpublished data
<i>Boops boops</i>		Haven oil spill area/ 30 days	0.6± 0.7 ^a	Bolognesi <i>et al.</i> , 2006b
<i>Mulus barbatus</i>		Haven oil spill area/ 30 days	0.7± 0.6 ^a	Bolognesi <i>et al.</i> , 2006b
<i>Uranoscopus scaber</i>		Haven oil spill area/ 30 days	1.1± 0.5 ^a	Bolognesi <i>et al.</i> , 2006b

^a – number of micronucleated cells per 1000 studied cells

Additionally, the range of variation of the frequency of MN in blood erythrocytes of fish and gill cells of *M. galloprovincialis* is displayed in Table 3.

Table 3. The range of MN frequency fish (blood, liver, kidney erythrocytes), in mussels, clams, scallops, amphipods (haemolymph, gill and mantle cells) from different sites of the Atlantic Ocean, North Sea, Baltic Sea and Mediterranean Sea.

Species	Number of sites studied	Tissue	MN frequency range, ‰	Reference
Mussels <i>Mytilus edulis</i>	3	Haemolymph	0.89-2.87	Wrisberg <i>et al.</i> , 1992
Mussels <i>Mytilus edulis</i>	2	Haemolymph	0.90-2.32	Wrisberg <i>et al.</i> , 1992
Mussels <i>Mytilus edulis</i>	3	Mantle	≈ 3-7 ^a	Bresler <i>et al.</i> , 1999
Mussels <i>Mytilus edulis</i>	60	Gills, haemolymph	0.37-7.20	Baršienė <i>et al.</i> , 2004, 2006b, 2006c, 2008b, 2010a;; Schiedek <i>et al.</i> , 2006
Mussels <i>Mytilus trossulus</i>	5	Gills	2.07-6.70	Baršienė <i>et al.</i> , 2006b, Kopecka <i>et al.</i> , 2006
<i>Mytilus galloprovincialis</i>	13	Gills	1.8 -24	Brunetti <i>et al.</i> 1988; Scarpato <i>et al.</i> , 1990; Bolognesi <i>et al.</i> , 2004; Nigro <i>et al.</i> 2006
<i>Mytilus galloprovincialis</i>	3	Gills	2 - 12	Kalpaxis <i>et al.</i> , 2004
<i>Mytilus galloprovincialis</i>	5	Haemolymph	1.38-6.50	Pavlica <i>et al.</i> , 2008
<i>Mytilus galloprovincialis</i>	3	Gills	1.2 - 11.8	Taleb <i>et al.</i> 2009
<i>Mytilus galloprovincialis</i>		Gills	0-22	Fernandez <i>et al.</i> , 2011
Clam <i>Macoma balthica</i>	29	Gills	0.53-11.23	Baršienė <i>et al.</i> , 2008b; Baršienė <i>et al.</i> , unpublished data
Scallops <i>Chlamys islandica</i>	3	Haemolymph	3.50 to 5.83	Baršienė <i>et al.</i> , unpublished data

Amphipods <i>Eurythenes gryllus</i>	2	Haemolymph	0.35-0.52	Baršienė <i>et al.</i> , unpublished data
Dab <i>Limanda limanda</i>	3	Blood	≈ 2-5 ^b	Bresler <i>et al.</i> , 1999
Dab <i>Limanda limanda</i>	26	Blood, kidney	0.02-1.22	Rybakovas <i>et al.</i> , 2009; Baršienė <i>et al.</i> , unpublished data
Flounder <i>Platychthys flesus</i>	3	Blood	≈ 2-6 ^b	Bresler <i>et al.</i> , 1999
Flounder <i>Platychthys flesus</i>	53	Blood, kidney	0.08-1.45	Baršienė <i>et al.</i> , 2004, 2005a, 2008a; Napierska <i>et al.</i> , 2009; Baršienė <i>et al.</i> , unpublished data
Eelpout <i>Zoarces viviparus</i>	40	Blood	0.02-0.81	Baršienė <i>et al.</i> , 2005a, Baršienė <i>et al.</i> , unpublished data
Atlantic cod <i>Gadus morhua</i>	19	Liver, blood,	0.0-0.64	Rybakovas <i>et al.</i> , 2009; Baršienė <i>et al.</i> , 2010a
Wrasse <i>Symphodus melops</i>	9	Blood	0.07-0.65	Baršienė <i>et al.</i> , 2004, 2008a
Herring <i>Clupea harengus</i>	32	Blood	0.03-0.92	Baršienė <i>et al.</i> , unpublished data
Haddock <i>Melanogrammus aeglefinus</i>	3	Liver	0.06-0.75	Baršienė <i>et al.</i> , unpublished data
Turbot <i>Scophthalmus maximus</i>	4	Blood, liver, kidney	0.10-0.93	Baršienė <i>et al.</i> , unpublished data
Perch <i>Perca fluviatilis</i>	14	Blood	0.06-1.15	Baršienė <i>et al.</i> , 2005a; Baršienė <i>et al.</i> , unpublished data
Big eye grunt <i>Brachyde-trius aurestus</i>	3	Liver	0.28-0.85	Baršienė <i>et al.</i> , unpublished data
Sole <i>Synoglossus senegalensis</i>	2	Liver	0.33-0.45	Baršienė <i>et al.</i> , unpublished data
Murene <i>Cynoponticus ferox</i>	2	Liver	0.13-0.96	Baršienė <i>et al.</i> , unpublished data
Guitar ray <i>Rhinobatos irvinei</i>	1	Liver	0,50	Baršienė <i>et al.</i> , unpublished data
Arctic rockling <i>Omogadus argentus</i>	2	Liver	0.23-0.47	Baršienė <i>et al.</i> , unpublished data

^a – Frequency of micronucleated cells

^b – Frequency of micronucleated erythrocytes

Assessment Criteria

Assessment Criteria (AC) have been established by using data available from studies of molluscs and fish in the North Sea, northern Atlantic (NRC database) and Mediterranean area. The background/threshold level of micronuclei incidences is calculated as the empirical 90% percentile (P90). Until more data becomes available, values should be interpreted from existing national data sets. It should be noted that these values are provisional and require further validation when data becomes available from the ICES database.

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* and *Chlamys islandica* (data from MN analysis in 4371 specimens), in fish *Limanda limanda*, *Zoarces viviparus*, *Platichthys flesus*, *Symphodus melops*, *Gadus morhua*, *Clupea harengus* and *Melogrammus aeglefinus* (data from MN analysis in 4659 specimens) from the North Sea, Baltic Sea and northern Atlantic have been calculated using NRC (Lithuania) databases using data from five or more reference locations (Table 1).

ACs for mussel *Mytilus galloprovincialis* and fish red mullet (*Mullus barbatus*) have been estimated using available data from the Spanish Institute of Oceanography (IEO, Spain). This dataset was obtained using *M. galloprovincialis* from reference stations along the northern Iberian shelf in spring 2003 namely Cadaqués and Medas Islands. In the case of red mullet, background values were derived from the results obtained in Almeria and Málaga areas (SE Spain). Because significant sexual differences were not observed in red mullet, data of both genders were considered.

Table 5. Assessment criteria of MN frequency levels in different bivalve mollusc and fish species.
BR =Background response; ER = Elevated response; N = number of specimens analysed.

Species	Size (cm)	Temperature (°C)	Regional Area	Tissue	BR	ER	N
<i>Mytilus edulis</i>	3-4	11-17	Atlantic-North Sea	Haemolymph, gills	<2.51	>2.51	1280
<i>M. edulis</i>	1.5-3	8-18	Baltic Sea	Gills	<2.50	>2.50	1810
<i>M. edulis</i> caged for 4-6 weeks	3-4	7-9	North Sea	Haemolymph	<4.1	> 4.1	44
<i>M. edulis</i> caged for 4-6 weeks	3-4	9-16	North Sea	Haemolymph	<4.06	> 4.06	656
<i>M. trossulus</i>	2-3	3-15	Baltic Sea	Gills	<4.50	> 4.50	230
<i>Macoma balthica</i>	1-3	13-18	Baltic Sea	Gills	<2.90	> 2.90	330
<i>M. galloprovincialis</i>	3-4	13	Western Mediter-ranean	Gills	<3.87	>3.87	12
<i>Chlamys islandica</i>	4-5	2-4	North Sea	Haemolymph	<4.5	> 4.5	65
<i>Zoarces viviparus</i>	17-30	15-17	North Sea	Erythrocytes	<0.28	>0.28	226
<i>Zoarces viviparus</i>	15-32	7-17	Baltic Sea	Erythrocytes	<0.38	>0.38	824
<i>Limanda limanda</i>	19-24	8-17	North Sea	Erythrocytes	<0.52	>0.52	544

<i>Limanda limanda</i>	18-25	8-17	Baltic Sea	Erythrocytes	<0.49	>0.49	117
<i>Platichthys flesus</i>	20-28	15-17	Atlantic-North Sea	Erythrocytes	<0.33	>0.33	62
<i>Platichthys flesus</i>	17-39	10-17	Baltic Sea coastal	Erythrocytes	<0.29	>0.29	828
<i>Platichthys flesus</i>	18-40	6-18	Baltic Sea offshore	Erythrocytes	<0.23	>0.23	970
<i>Symphodus melops</i>	12-21	13-15	Atlantic-North Sea	Erythrocytes	<0.36	>0.36	158
<i>Gadus morhua</i>	20-48	13-15	Atlantic-North Sea	Erythrocytes	<0.38	>0.38	340
<i>Gadus morhua</i>	20-48	13-15	Baltic Sea	Erythrocytes	<0.38	>0.38	50
<i>Clupea harengus</i>	19-25	5-10	Atlantic-North Sea	Erythrocytes	<0.32	>0.32	60
<i>Clupea harengus</i>	16-29	6-18	Baltic Sea	Erythrocytes	<0.39	>0.39	450
<i>Melogrammus aeglefinus</i>	27-44	8-14	North Sea	Erythrocytes	<0.30	>0.30	30
<i>Mullus barbatus</i>	12-18	17	Western Mediter-ranean	Erythrocytes	<0.32	>0.32	64

Quality Assurance

The micronucleus 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 *M. galloprovincialis* 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 bioindicator species in 2011/12.

Scientific potential

MN analysis in different marine and freshwater species of bivalves and fish is carried out in many laboratories of European countries: Italy, Portugal, Spain, Turkey, Lithuania, UK, Greece, Germany, Poland, Croatia, Estonia, Russia, Norway and Ukraine. There are single laboratories in Hungary, Algeria and Egypt. Highly qualified expert groups work in Italy, Lithuania, Spain, Turkey, Portugal, UK and are able to perform analysis in both groups of animals – both in invertebrates and vertebrates.

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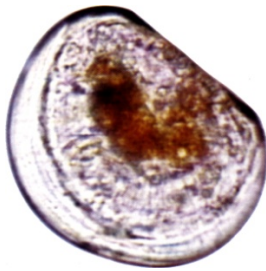
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Annexes 11, 12, and 13: Sediment and elutriate bioassays for invert bioassays, copepods and whole sediment bioassays

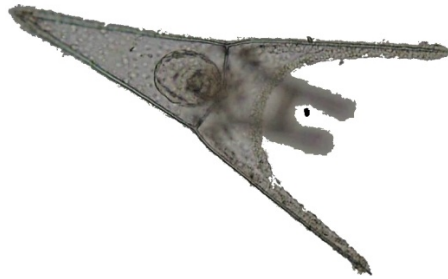
Compiled by Ricardo Beiras

Universidad de Vigo, Campus Universitario C.P.. Illa de Toralla, s/n, ES-36330 Vigo (Pontevedra), Spain

Annex 11: Sediment seawater elutriate and pore-water bioassays with early developmental stages of marine invertebrates



Bivalve D-larva

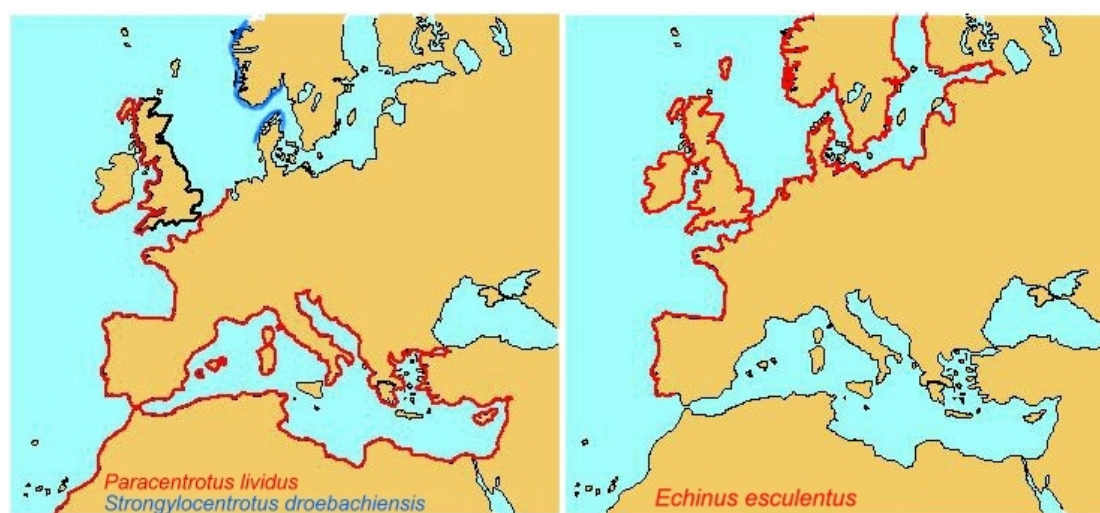


Sea-urchin pluteus larva

Background

1. Early developmental stages are generally more sensitive than adults and the weakest link in the life cycle. The embryo-larval bioassays detect a broad spectrum of toxicants at comparatively low concentrations, in the order of 1 µg/L for TBT and other antifouling products, 10 µg/L for Hg, Cu and Zn, 100 µg/L for Pb, Cd and other metals, 1 mg/L for organochlorine pesticides, detergents and refined oil, and 10 mg/L for crude oil (Kobayashi 1995, His *et al.* 1999).
2. The embryogenesis and early larval development of marine invertebrates have been frequently used as a rapid, sensitive, cost-effective biological tool for the assessment of seawater, sediment elutriates and pore water quality. Detailed descriptions of methods and applications are available for bivalves (Woelke 1961, Thain 1991, His *et al.* 1999) and sea-urchins (ASTM 1995, Carr 1998, Saco-Álvarez *et al.* 2010). Gametes are obtained from mature adults either by stripping or thermally induced spawning, fertilized in vitro in a measuring cylinder and delivered into the experimental samples. After 24 to 48 h incubation at 18 to 24°C (depending on the species), samples are fixed and microscopically observed to record the percentage of normally developed larvae and, in the case of sea-urchins, size increase.
3. Sensitivity of embryos of different species to the main pollutants of concern in the marine environment is very similar, particularly within bivalves. This allows comparison of results of embryo-larval bioassays conducted with different species. A review on the EC₅₀ values of 18 priority pollutants to bivalve vs. sea-urchin embryos reflected a correlation coefficient $r^2=0.96$ ($p<0.01$) and a slope $b=1.00$ (Beiras & Bellas 2008). Due to their abundance and broad geographical distribution or availability from commercial sources the following species are recommended: *Crassostrea gigas*, *Mytilus edulis/galloprovincialis*, *Paracentrotus lividus*. In the case of sea-urchins, other species like *Strongylocentrotus droebachiensis* and *Echinus sculentus*, extend the applicability of the assay with indigenous species to Northern countries (see figures).

4. Within bivalves, *Crassostrea gigas* and, in the U.S. *C. virginica* oysters have been most often used for embryo-larval ecotoxicological bioassays because, unlike the mussel or the native flat oyster (*Ostrea edulis*), in *Crassostrea* fertile gametes can be obtained straight from the gonad by stripping, although this method requires high percentages of embryogenesis success in the controls to guarantee comparability of the results (His *et al.* 2000). The marine mussels of the *Mytilus* genus occurring in European waters (*M. edulis* and *M. galloprovincialis*) are nearly ubiquitous, easy to collect and to maintain in aquaria. Also these species show the advantage that the adults are commonly used in marine pollution monitoring programmes, and OSPAR encourages the use of the same species for different biological tools of pollution assessment, spanning molecular, cellular and individual responses. Another advantage of the mussel embryogenesis bioassay is that this species is tolerant to a broader range of salinities, including estuarine waters down to 20 ppt (His & Beiras, 1995). The *Paracentrotus lividus* sea-urchin has a somewhat more restricted distribution, but it is easier than bivalves to feed and maintain in captivity avoiding accidental spawning. Another advantage of the sea-urchin embryogenesis bioassay is to provide a quantitative, more gradual, observer-independent and statistically treatable response: size increase (Saco-Álvarez *et al.* 2010).



5. Currently, the main limitation of the embryo-larval bioassays is the availability of reliable, good quality biological material all year round, particularly outside the natural spawning season of the different species, which changes among different European countries. The maintenance of fertile adult stocks in aquaria is feasible, particularly for sea-urchins, and conditioned bivalves should be available from aquaculture facilities, but even commercial hatcheries are unable to provide 100% reliable adult broodstocks all year round. Cryopreservation of gametes of bivalves and sea-urchins is a promising solution to provide homogeneous biological material at any time, but up to date these techniques are still on development and standard methods are not available. Combination of different species with different spawning seasons seems to be still necessary.

6. Sediment toxicity can be tested with water column organisms by either obtaining an elutriate from the sediment (mixed with control sea-water) or by directly obtaining the interstitial pore-water from the sediment. The advantages of the first method are: smaller amounts of sediment and simpler equipment are necessary, the environmental parameters of the elutriate (dissolved oxygen, pH, salinity, ammonia, sulphides) are closer to those of the natural water column than in the case of pore

water, in particular when dealing with anoxic or hypoxic sediments. These parameters are the most common source of false positives (see confounding factors), and pore water requires adjusting their values within the optimum range for the test species prior to testing. In reverse, pore-water has the advantage that no control seawater is needed and the dilution of the potential toxicants present is lower, enhancing sensitivity. The choice of the method can depend on sampling constrictions and sample availability, since when the confounding factors are taken into account both methods yield comparable results (Beiras 2001).

7. The embryo-larval bioassay generally showed higher sensitivity to polluted sediments than the amphipod bioassay (Becker *et al.* 1990, Long *et al.* 1990, Carr & Chapman 1992), although similar sensitivities have also been reported (Williams *et al.* 1986). However not rarely the concordance in estimates of toxicity using different organisms is small, and different tests indicate different patterns in toxicity (Long *et al.* 1996). Therefore, comparisons among different sediment toxicity tests must be conducted using samples representing a broad range of pollution in order to evaluate the comparability of the different tests.

Confounding factors

8. In order to avoid false positives, water quality values in the elutriate (or pore water) must be checked prior testing and they must fall within optimum ranges for the embryo development of the test species or otherwise adjusted. In the case of molluscs, His *et al.* (1999) provide a broad review on this topic. Generally speaking, full salinity, a pH higher than 7.5 and a dissolved oxygen concentration above 2 mg/L are required. This is particularly important in the case of pore waters from highly reduced sediments, which broadly depart from those values. In the case of sea urchins Saco-Álvarez *et al.* (2010) described the optimal range for salinity from 31 to 35, and from 7.0 to 8.5 for pH.

9. More often the presence of the toxic reduced compounds un-ionized ammonia and H₂S has been identified as the main sources of false positives in sediment elutriate toxicity testing (Cardwel *et al.* 1976; Matthiesen *et al.* 1998). Some threshold toxicity values for sea-urchin and bivalve embryos are available in the literature (Knezovic *et al.* 1996), but further research is strongly needed on this topic. For NH₃ Saco-Álvarez *et al.* (2010) obtained an EC₁₀ of 68.4 µg/L and a NOEC/LOEC of 40/80 µg/L using *Paracentrotus lividus*.

Regarding temperature, elutriates and pore waters are microbially rich and exposure to high temperatures during manipulation should be avoided. This includes centrifugation, when necessary. For incubation, 20°C (48 h) is recommended for mussels and *Paracentrotus lividus* urchins, and 24°C (24 h) for *Crassostrea gigas* oysters.

Ecological relevance

10. The ecological relevance is one of the strong points of the embryo-larval bioassay. Any impairment of embryo development would lead to reduced recruitment and decrease population size.

Assessment criteria

11. Marine invertebrate embryo-larval bioassays have resorted to different species and a suit of endpoints. This issues need to be discussed prior to the implementation of assessment criteria.

End-points measured

12. The end-point recorded in the standard embryo-larval bioassays is the percentage of morphologically normal larvae. The definition of morphological abnormalities change among authors and, obviously, among test species. For routine applicability's sake it is advised that only very conspicuous abnormalities were taken into account. This would reduce the time necessary to record the endpoint, and facilitate automatization and observer-independence. In bivalves normal D-shape is advised as normality criteria. This excludes larvae with protruding mantle and convex hinge. Illustrations of these abnormalities can be found in Quiniou *et al.* (2005). However, more detailed abnormalities such as the presence of indentations in the larval shell would complicate observation and in our view should not be taken into account.

13. In sea-urchins normal larvae should exhibit four fully-formed arms (two longer post-oral arms and two shorter oral arms) and a regular outer contour of the body. Pre-pluteus stages where oral arms were not yet fully separated, or larvae with missing arms, should be considered as abnormal. However more detailed abnormalities such as those related to the internal anatomy of the larvae (skeletal rods, gut) would greatly complicate observation. Their identification even depends on the position of the larva under the microscope. An alternative endpoint for the sea-urchin test was recently proposed by Saco-Álvarez *et al.* (2010), who measure the size increase in 48 h. This avoids lengthy and subjective microscopical inspection, speeding up test readings, makes automatic reading feasible, and allows a more than two-fold increase in sensitivity compared to the classical morphological endpoint.

Assessment criteria

14. Discrete approach: ICES (2008) currently recommends classification of the toxicity of a liquid sample as "elevated" when embryo abnormalities are >20% for bivalves and >10% for sea urchins, and "high concern" when they are >50% for both invertebrates.

15. Generally speaking, an elutriate can be classified as toxic when it induces a statistically significant reduction in the end-point (either normal morphology or size increase) compared to the elutriate from the reference site, for a confidence level of 95%. Percentages of response must be arcsine transformed prior to analysis using ANOVA and *a posteriori* Dunnett's test, comparing each sampling site with the reference site. The difficulty here is to establish a reference site we were sure from comprehensive analytical data that it is not polluted but was otherwise similar to the problem sites (see confounding factors). Control seawater may not be appropriate as reference because it lacks the physicochemical and microbiological properties of an elutriate, some of which may affect the response.

16. Continuous approach: Once identified as polluted, the toxicity of any sediment elutriate that causes a marked inhibition in normal development can be quantified by serial dilution with reference seawater, and calculation of the toxic units (TU). $TU = 100 / ED_{50}$, where ED_{50} is the theoretical dilution, expressed in percentage, that causes 50% abnormal larvae. This parameter can be obtained by fitting the data for the serial dilutions to standard toxicity curves (logit, probit, etc.). When data from different campaigns were pooled together for statistical analysis, they must be previously corrected by the respective controls by using Abbott's formula: $P' = (P - P_c) \times 100 / (100 - P_c)$; where P and P' are the raw and corrected abnormality percentages, and P_c is the control abnormality. Once corrected, percentages must be arcsine transformed for subsequent analysis. When using this quantitative approach with sea-urchins, larval length after 48 h, or even better, size increase from fertilized egg after

24 h, is preferred to percentage of normal larvae. This is because size increase is a more sensitive -and thus more discriminant- response than morphologically normal development (Saco-Álvarez *et al.* 2010).

17. In the case of the sea-urchin test Durán and Beiras (2010) developed quantitative assessment criteria for the size increase endpoint on the basis of the distribution of results from sites not significantly different to reference. The methodology to obtain BAC and EAC values followed OSPAR (2009). The resulting **BAC** value was PNR=0.702, which means a 30% decrease in growth (size increase) in the tested population. 18. A more detailed evaluation of the results from the sea-urchin test can be obtained by pooling the results from sites not significantly different to reference in a first dataset, and pooling toxic sites in a second dataset. Taking different percentiles from those distributions the following environmental assessment criteria (EAC) for Percent Net Response (PNR) and Toxic Units (TU) data were obtained.

18. A BAC of 22 was set for mussel larvae (Table 1).

19. EAC-values for both assays were retained at 50% as recommended earlier by ICES, either mortality (mussel embryo) or reduced growth (sea urchin embryo).

Table 1. Background response for mussel embryo bioassays (mortality); data from IEO-Vigo.

Average	90-percentile	median	10-percentile	n
14.7	22.3	8	3.2	38

Quality assurance

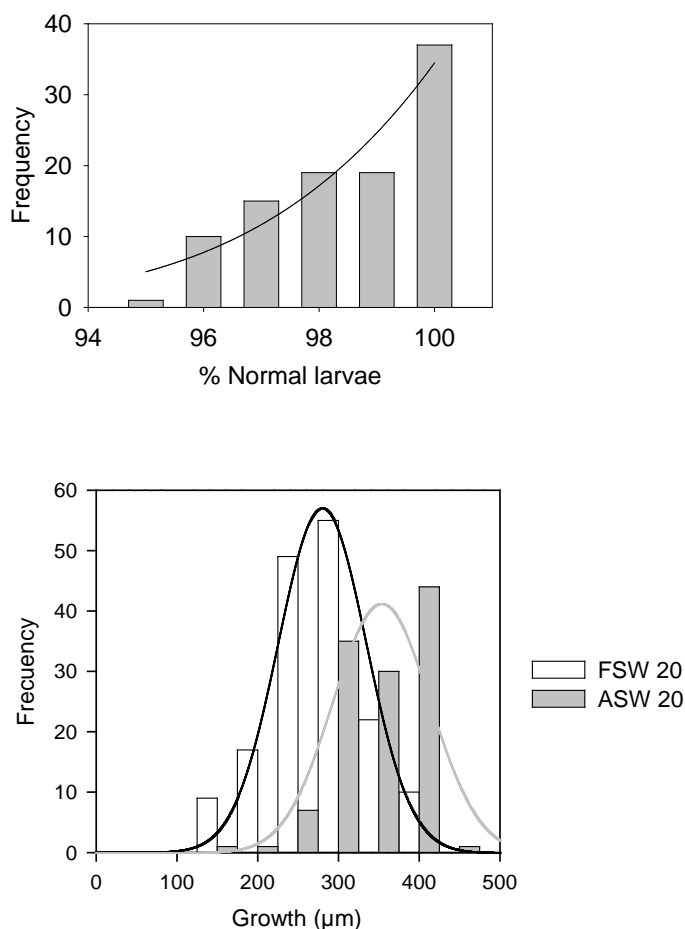
20. Sediment manipulations during sampling, storage and testing, and quality of the test organisms have been often identified as the main sources of variability in sediment toxicity bioassays. Concerning the first point, sediments intended to toxicity testing should not be frozen but stored under refrigeration in the dark inside airtight containers, and tested within one week. Some authors argue that testing can be delayed by freezing the liquid phase (elutriate or pore-water) after elimination of particles. However it must be taken into account that glass fibre filters adsorb metals and some organic filters might retain organic compounds, so refrigerated centrifugation may be preferred. After thawing, samples should be shaken and salinity checked and adjusted, if necessary.

21. Concerning the effect of homogeneous biological material, interlaboratory comparisons carried out following strict protocols are necessary. In these intercalibrations it would be desirable that not only different populations of a certain species, but also different species (oysters, mussels, clams, sea-urchins) were included.

22. The control treatment in an embryo-larval bioassay gives essential information regarding biological quality of the test organisms. Acceptability criteria must be developed concerning minimum embryogenesis success and larval length in the control for a test to be considered reliable. Those criteria must take into account both the normal seasonal variability within a certain population and interpopulation variability. In the case of bivalves, His *et al.* (1997) reported mean values in controls ranging from 75.8 to 97.0, thus suggesting a minimum of 75% normality, whereas Quiniou *et al.* (2005) arbitrarily recommend a minimum of 80% normal D-larvae in the control as acceptability criterion (see also AFNOR 2009). Preliminary results of background response levels for *Mytilus* embryo bioassays are shown in Table 1 below. Taking as acceptability criteria the 10th percentile of the distribution of all controls with natural

filtered seawater (FSW) throughout several years during the natural spawning season (april, may and june), a minimum of 68% normal D-larvae in controls is required. Nevertheless if the bioassay is carried out outside the spawning season, failure to reach the acceptability criteria is likely to occur, and a compromise between sensitivity and feasibility must be reached.

23. In the case of the *P. lividus* normal larval development, the distribution of the endpoints measured (percentage of normal larvae, and size increase) in controls with natural filtered seawater (FSW) and artificial seawater (ASW), throughout several years of tests conducted at 20°C for 48h, was the following (Saco-Álvarez *et al.* 2010):



24. From these data, and taking the 5th percentile as the acceptability criteria, a test is correct when mean response in the control exceeds 91% embryogenesis success and 218 µm size increase in FSW (natural filtered seawater) or 253 µm in ASW (artificial seawater).

25. Percentage fertilization prior to testing must always be recorded. To run a reference toxicant test may be further useful to check the biological quality of the test organisms using a chart of the reference toxicant EC₅₀ historical values.

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Annex 12: sediment seawater elutriate and pore-water bioassays with copepods (*Tisbe*, *Acartia*), mysids (*Siriella*, *Praunus*), and decapod larvae (*Palaemon*).



Tisbe battagliai

Siriella armata

Palaemon elegans

Background

1. Crustaceans, and particularly early life stages, are several orders of magnitude more sensitive to insecticides than echinoderms and mollusca (Ramamoorthy & Badaloo, 1995; Bellas *et al.* 2005). Crustaceans are also particularly sensitive to cadmium (Mariño-Balsa *et al.* 2000) compared to other marine invertebrates. Therefore when

these contaminants were suspected the inclusion of a crustacean test within the battery of bioassays is strongly recommended.

2. Acute static survival tests with benthic (*Tisbe battagliai*) and planktonic (*Acartia tonsa*) copepods have been proposed to assess the biological quality of sediment elutriates (Matthiessen *et al.* 1998). Detailed methods are available (Hatchinson & Williams 1989, UNEP 1989). The endpoint recorded may be mortality or motility after 48 to 96h incubation in the test samples at 20°C and 16 h light 8 h dark photoperiod. *Tisbe battagliai* is an abundant component of meiobenthic fauna, whereas *Acartia* and other calanoid copepods are components of the holoplankton in Atlantic waters. Both are easy to feed on microalgae. Ovigerous females can be isolated and age-controlled cultures can be obtained from the eggs. A water bioassay programme is running within BEQUALM which includes the 48h *Tisbe battagliai* acute test.

3. Mysids, particularly the American species *Mysidopsis bahia*, are recommended test organisms by US-EPA for estuarine and marine water toxicity tests (US-EPA 2002). The maintenance of fertile adult stocks in aquaria, fed on *Artemia*, is feasible. Since these organisms undergo direct development in short time periods they are suitable for life cycle assessments. Some European mysids such as *Neomysis* (for brackish waters), *Praunus* (Garnacho *et al.* 2000, Mclusky & Hagerman 1987) and *Siriella* (Pérez & Beiras 2010) have been proposed, but sensitivity intercomparisons are lacking. Also, the salinity range of tolerance for each species must be determined before recommendation for routine toxicity testing.

4. The use of decapods early life stages is less frequent (Cheung *et al.* 1997, Mariño-Balsa *et al.* 2000). The main advantages are the economic value of some species (shrimps, crabs), and the possibility to obtain ovigerous females from commercial stocks. The main restriction is to find broadly distributed species across all Europe. The *Palaemon* genus may be a potential candidate since it shows a broad geographical distribution, from Mediterranean sea to North Sea, they are easy to feed, the maintenance of fertile adult stocks in aquaria is feasible, and larval development is well known.

Confounding factors

5. In order to avoid false positives, water quality parameters in the elutriate (or pore water), specifically salinity, pH and dissolved oxygen, must be checked prior testing and they must fall within optimum ranges for the survival and motility of the test species or otherwise adjusted. This is particularly important in the case of pore waters from highly reduced sediments, which broadly depart from those values.

6. More often the presence of toxic reduced compounds, un-ionized ammonia and H₂S, have been identified as the main sources of false positives in sediment elutriate toxicity testing (Cheung *et al.* 1997). Further research is strongly needed on this topic.

Ecological relevance

7. Copepods and mysids are dominant components of holoplankton in marine ecosystems. They are primary consumers and an important food source for fish. Therefore any toxicant affecting them is a threat to the whole food web in coastal and oceanic ecosystems.

Assessment criteria

8. ICES (2008) currently recommends classification of the toxicity of a seawater sample as "elevated" when *Tisbe* mortality is >10% and "high concern" when it is >50%.

Quality assurance

9. Sediment manipulations during sampling, storage and testing, and quality of the test organisms have been often identified as the main sources of variability in sediment toxicity bioassays. Concerning the first point, sediments intended to toxicity testing should not be frozen but stored under refrigeration in the dark inside airtight containers, and tested within one week. Some authors argue that testing can be delayed by freezing the liquid phase (elutriate or pore-water) after elimination of particles. However it must be taken into account that glass fibre filters adsorb metals and some organic filters might retain organic compounds, so refrigerated centrifugation may be preferred. After thawing, samples should be shaken and salinity checked and adjusted if necessary.

10. Concerning the effect of homogeneous biological material, interlaboratory comparisons carried out following strict protocols are necessary. In these intercalibrations it would be desirable that not only different populations of a certain species, but also different species (*Tisbe*, *Tigriopus*, *Acartia*, mysids, shrimp larvae...) were included.

11. Acceptability criteria must be developed concerning minimum survival/motility in the control for a test to be considered reliable. Those criteria must take into account both the normal seasonal variability within a certain population and interpopulation variability. A stringent acceptability criteria is essential to guarantee reliable toxicity data, particularly when test organisms come from wild populations and experience a sharp change in environmental conditions in the laboratory. Results of background response levels for *Tisbe* bioassays are shown in Table 1, resulting in a BAC of 5.0.

Table 1. Preliminary results of background response levels for *Tisbe* bioassays (mortality) – data from Cefas.

Average	210-percentile	median	90-percentile	n
1.3	0.0	0.0	5.0	28

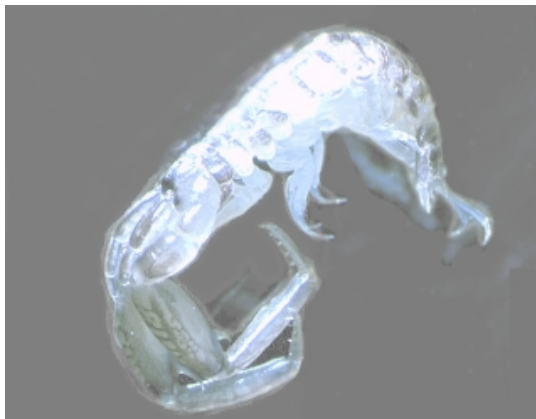
12. To run a reference toxicant test may be further useful to check the biological quality of the test organisms. The reference toxicant, ideally, should be stable in aqueous solution and not dangerous for human beings.

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Annex 13: Whole sediment bioassays with amphipods (*Corophium* sp) and *Arenicola marina*



Corophium multisetosum

Background

1. The *Rhepoxynius abronius* amphipod test is commonly used in North America to evaluate the quality of sediments intended for dredging or dumping, and very detailed protocols are available (Swartz *et al.* 1985, ASTM 1992). The endpoint is survival after 10 days incubation in the whole sediment at 20°C. These protocols can be easily adapted to the European species (*Corophium* spp). Some efforts have already

been made to compare methods and sensitivity for different amphipod species (van den Hurk *et al.* 1992, Casado-Martínez *et al.* 2006).

2. The *Corophium* genus is broadly distributed across Europe. An internationally agreed protocol for toxicity testing of offshore chemicals with *C. volutator* has been published (OSPAR 1995). ICES has also provided detailed methods (Roddie & Thain 2001). Those protocols are also suitable for other macroscopically indistinguishable *Corophium* species more abundant in Southern Europe, *C. multisetosum*. In fact ICES claims that the procedure can be used not only with any *Corophium* species but with any infaunal amphipod (Roddie & Thain 2001).

3. Other sediment dwelling species from different taxa (polychaetes, echinoderms, bivalves) may be also suitable after methodological standardisation and sensitivity comparisons with amphipods. Furthermore, *Corophium* is not tolerant to coarse grain sediments. Should sandy sediments be tested alternative species such as *Arenicola*, *Echinocardium* or *Cerastoderma* will be needed.

4. Some sublethal responses have been proposed as additional endpoints in order to enhance sensitivity, including reburial after the 10 day exposure (Bat & Raffaelli 1998), and 28-days growth (Nipper & Roper 1995). The later considerably delays the outcome of the test and may be a limitation for routine application. The use of fast growing juvenile stages might overcome this limitation.

Confounding factors

5. The presence of toxic reduced compounds such as un-ionized ammonia and H₂S in interstitial and overlying water has been identified as confounding factors in whole sediment toxicity testing (Phillips *et al.* 1997). The studies have been carried out with North America species. Further research on this topic with *Corophium* spp. is strongly needed.

6. Grain size also affects amphipod survival (De Witt *et al.* 1988). The studies have been carried out with North America species. Further research on this topic with *Corophium* spp. is strongly needed.

Assessment criteria

7. According to USEPA (1998) a sediment sample is classified as toxic when it induces an amphipod mortality 20% higher than control and the difference is statistically significant. Similarly, ICES (2008) currently recommends classification as "elevated" when *Corophium* mortality is >30% and "high concern" when it is >60%. For *Arenicola* these benchmarks go down to >10% for "elevated" and >50% for "high concern" (ICES 2008).

8. ANOVA and *a posteriori* Dunnett's test allows comparison to control and classification of sampling sites into homogeneous groups according to their toxicity. Mortality data must be arcsine transformed prior to analysis. When data from different test rounds were pooled together for statistical analysis, mortalities must be previously corrected by the respective controls by using Abbott's formula: $P' = (P - P_c) \times 100 / (100 - P_c)$; where P and P' are the raw and corrected mortality percentages, and P_c is the control mortality. In this case no control treatment is available and Tukey's rather than Dunnett's posthoc test is preferred. Again, mortality data must be arcsine transformed prior to analysis.

Quality assurance

9. Sediment manipulations during sampling, storage and testing, and quality of the test organisms have been often identified as the main sources of variability in sediment toxicity bioassays. Concerning the first point, sediments intended to toxicity testing should not be frozen but stored under refrigeration in the dark inside airtight containers, and tested within one week.

10. Concerning the effect of homogeneous biological material, interlaboratory comparisons carried out following strict protocols are necessary. The following issues have been identified as relevant for the success of the intercalibration round. Sediment samples should be homogeneous in grain size and organic content but spanning from pristine to highly polluted. Preservation of the sediment from sampling to testing should be similar for all participants, including time and temperature. Since for this species with no commercial value the test individuals must be collected from the field, they should be acclimated and maintained in laboratory long enough to assess the population health prior to testing.

11. Acceptability criteria must be developed concerning minimum survival/reburial in the control for a test to be considered reliable. Those criteria must take into account both the normal seasonal variability within a certain population and interpopulation variability. A stringent acceptability criteria is essential to guarantee reliable toxicity data, particularly when test organisms come from wild populations and experience a sharp change in environmental conditions in the laboratory. In an intercalibration round in Spain, Casado-Martínez *et al.* (2006) set acceptable maximum control mortality at 10%, following USEPA (1994). Roddie & Thain (2001) raise this threshold to 15%. Results of background response levels for *Corophium* and *Arenicola* bioassays are shown in Table 1. All laboratories show a 90th percentile for mortality higher than 10% and most above the recommended 15%, indicating that special care must be taken in avoiding any damage to the individuals during collection, maintenance and transfer into the experimental beakers.

12. The third year of a bioassay programme is running within BEQUALM from December 2006 to June 2007, and includes the 10-d *Corophium volutator* survival bioassay.

Table 1. Background response levels for whole sediment bioassays (mortality); the median 90-percentile, i.e. BAC, is 18.4%.

Test	lab	Average	10-percentile	median	90-percentile	n
Corophium	RIKZ	12.3	6.6	10.5	19.3	4
Corophium	Cefas	9.5	0.0	6.7	20.0	21
Corophium	IEOV	7.7	5.6	6.3	10.8	5
Corophium	AZTI	10.4	4.8	10.8	17.4	27
Arenicola	Cefas	4.7	0.0	0.0	13.3	20

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Annex 14: Candidate EAC values for new species

Candidate Environmental Assessment Criteria (EAC) for biomarkers in new species ⁰⁾

Fish species		Fitness EAC	Fitness EAC	FF- Nph type	FF- Py type	FF- BaP type	PAH (sum 9) met GC/MS	DNA adducts	CYP1a	EROD
English name	Latin name	µg/l PAH	nominal ppb oil	Nph PFE - µg/g (ml)	Py PFE - µg/g (ml)	BaP PFE - µg/g (ml)	OH-PAH metabolites - µg/g	nmol/mol nucleotides	abs 492 nm	pmol/min/mg protein
Atlantic cod	<i>Gadus morhua</i>	2 ¹⁾	-	>115 ²⁾	>23 ²⁾	>1,9 ²⁾	>85 ²⁾	>6,7 ²⁾	>0,29 ²⁾	-
Atlantic halibut	<i>Hippoglossus hippoglossus</i>	5,5 ³⁾	750 ³⁾	>90 ⁴⁾	>19 ⁴⁾	>2,3 ⁴⁾	>60 ⁴⁾	>5,8 ⁴⁾	-	>75 ⁴⁾
Long rough dab	<i>Hippoglossoides platessoides</i>	5,5 ⁵⁾	750 ⁵⁾	>80 ⁴⁾	>12 ⁴⁾	>2,5 ⁴⁾	>110 ⁴⁾	>4,0 ⁴⁾	-	>315 ⁴⁾
Turbot	<i>Psetta maxima</i>	(1,5) ⁴⁾	120 ⁶⁾	>85 ⁴⁾	>11 ⁴⁾	>1,7 ⁴⁾	>38 ⁴⁾	>0,8 ⁴⁾	>0,15 ⁴⁾	-
Atlantic Herring*/Sprat ^y	<i>Clupea herengus</i> */ <i>sprattus sprattus</i> ^y	5 ⁷⁾	750 ⁷⁾	>114 ⁴⁾	>16 ⁴⁾	>2,0 ⁴⁾	>149 ⁴⁾	>1,4 ⁴⁾	-	-
Atlantic Salmon	<i>Salmo salar</i>	5,2-5,4 ⁶⁾	(750) ³⁾	>31 ⁶⁾	>5,0 ⁶⁾	>0,9 ⁶⁾	>44 ⁶⁾	>1,6 ⁶⁾	>0,23 ⁶⁾	>17 ⁶⁾
* Fitness (Atlantic herring) / ^y Biomarkers(sprat) (as closely related species)										
Invertebrate species		Fitness EAC	Fitness EAC	Lysosomal stability NRRT						
English name	Latin name	µg/l PAH	nominal ppb oil	minutes NRRT						
Northern shrimp	<i>Pandalus borealis</i>	0,3 ⁸⁾	15 ⁸⁾	<60 ⁸⁾						

Blue mussel	<i>Mytilus edulis</i>	0,8 ⁹⁾	63 ⁹⁾	<60 ¹⁰⁾
Icelandic scallop	<i>Chlamys islandica</i>	0,8 ¹¹⁾	63 ¹¹⁾	<60 ¹⁰⁾

Sources of data and publications

- ⁰⁾ Norwegian Oil Industry Association (OLF); IRIS^z
- ¹⁾ Atlantic cod - larvae; Institute of Marine Research
- ²⁾ BioSea JIP (Total & Eni^z; IRIS)
- ³⁾ BioSea JIP (Total & Eni) & Biomarker Bridges (RCN^z 178408); IRIS
- ⁴⁾ BioSea JIP (Total & Eni) & Biomarker Bridges (RCN 178408); IRIS
- ⁵⁾ values taken from Atlantic halibut (as related flatfish species)
- ⁶⁾ Pink salmon - marine survival
- ⁷⁾ Biomarker Bridges (RCN 178408); IRIS

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⁸⁾ BioSea JIP (Total & Eni; IRIS)	Bechmann, RK, BK Larsen, IC Taban, LI Hellgren, P Möller, S Sanni (2010): Chronic exposure of adults and embryos of <i>Pandalus borealis</i> to oil causes PAH accumulation, initiation of biomarker responses and an increase in larval mortality. Marine Pollution Bulletin (60), 2087-2098.
⁹⁾ BioSea JIP (Total & Eni; IRIS)	Baussant, T, M Ortiz-Zarragoitia, MP Cajaraville, RK Bechmann, IC Taban, S Sanni (<i>accepted</i>): Effects of chronic exposure to dispersed oil on selected reproductive processes in adult blue mussels (<i>Mytilus edulis</i>) and the consequences for the early life stages of their larvae. Marine Pollution Bulletin.
¹⁰⁾ BioSea JIP (Total & Eni; IRIS)	Baussant, T., RK Bechmann, IC Taban, BK Larsen, AH Tandberg, A Bjørnstad, S Torgrimsen, A Nævdal, KB Øysæd, G Jonsson, S Sanni (2009): Enzymatic and cellular responses in relation to body burden of PAHs in bivalve molluscs: A case study with chronic levels of North Sea and Barents Sea dispersed oil. Marine Pollution Bulletin 58, 1796-1807.
¹¹⁾ values taken from blue mussel (as alternative mollusc species)	see ⁹⁾
^z RCN = Research Council of Norway; IRIS = International Research Institute of Stavanger - Dept. Biomiljø; Total = TOTAL E&P NORGE AS; Eni = Eni Norge AS & Eni E&P Division	

Annex 15: Draft Terms of Reference for the next meeting

The **Working Group on Biological Effects of Contaminants** (WGBEC), chaired by Matthew Gubbins, UK, and John Thain, UK, will meet in Porto, Portugal, 12–16 March 2012 to:

- a) Integrated monitoring and assessment:
 1. Respond to SGIMC 2011 and review documentation as required;
 2. Application of OSPAR integrated strategy to data sets by working group members;
 3. Review integrated assessments from ICON and BEAST;
 4. Update assessment criteria in light of new data.
- b) MSFD – review initial assessments for Descriptor 8 and advise as required on implementation of monitoring programmes for GES Commission indicator 8.2.1;
- c) Report on collaboration with other WGs as identified at 2011 meeting and any intersessional activity/representation (WGEEL, WKMAL, WGMMAL, MCWG etc.);
- d) Receive reports on marine monitoring activities being undertaken by member states;
- e) Consideration of issues of special scientific interest / value:
 1. Acidification in marine waters in relation to contaminants and bio-marker response;
 2. Effects of contaminants on primary production, including phytotoxicity;
 3. Relationship of genetic markers to biomarkers. BECOMES AC DEVELOPMENT;
 4. Review of species differences in bioassay and biomarker responses eg as seen in assessment criteria currently being developed – also to include sources of species for testing;
 5. Immunotoxicity end points – suitability for monitoring;
 6. Online monitoring.
- f) Review progress with publication and electronic dissemination of biological effects techniques in the ICES TIMES series;
- g) Review progress from the ICES database subgroup and report advice to the ICES Data Centre;
- h) Report progress from AQC subgroup and develop AQC procedures for biological effect methods including harmonisation activities initiated from WGBEC and within OSAPR, HELCOM and MEDPOL maritime areas;
- i) Review recent developments relating to contaminant effects from litter /plastic particles.

WGBEC will report by 15 April 2012 (via SSGHIE) for the attention of SCICOM.

Supporting information

Priority	The activities of this group will enable ICES to advise on issues relating to the design, implementation and execution of regional research and monitoring programmes pertaining to hazardous substances in the marine environment. To develop procedure for quality assurance of biological effects data and to improve assessments of data relating to the biological
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	effects of contaminants in the marine environment. To develop cross links and collaboration with other ICES Expert Groups in order to take forward and contribute to the implementation of the ICES Science Plan.
Scientific justification	<p>Term of Reference a) In 2011 SGIMC completed its task in developing an integrated contaminant and biological effects monitoring framework. There are still some outstanding tasks for WGBEC to complete, which may include updating and reviewing assessment criteria. In addition, it is important to review how countries are using and applying the new integrated framework and this will include the ICON and BEAST programmes.</p> <p>Term of Reference b) In 2012 initial assessments may be available for MSFD Descriptor 8. It is important that WGBEC reviews and advise as required on implementation of monitoring programmes for GES Commission indicator 8.2.1.</p> <p>Term of Reference c) WGBEC has contacted several Expert Groups within SSGHIE and areas for cross linking and collaboration have been identified. It is important that progress with collaborative activities and intersessional contacts are reviewed and reported back to SSGHIE.</p> <p>Term of Reference d) WGBEC has found it of value to discuss, feedback and support national monitoring programmes across the maritime areas and this is a valuable opportunity to improve and harmonise programme designs and assessment of data (e.g. OSPAR / MEDPOL / WFD / HELCOM/ EU MSFD).</p> <p>Term of Reference e) There are a number of issues identified by WGBEC that are of value and special scientific interest to understanding the effects of contaminants in the marine environment e.g. acidification, primary production, genetic markers, immunocompetence and online monitoring. It is important that these are reviewed/assessed and taken forward, in relation to the wider aspects of environmental management and secondly in the development and application of techniques for assessment purposes.</p> <p>Term of Reference f) It is important for WGBEC to keep track of publication progress with biological effects methods it has sponsored. Protocols are needed for national and international programmes as well as the OSPAR programmes and EU MSFD..</p> <p>Term of Reference g) Biological effect data is increasingly being submitted to the ICES database and technical queries arise and WGBEC can assist with answering queries from the ICES Data Centre. The subgroup set up to work intersessionally on any data base issues will report on its activities.</p> <p>Term of Reference h) AQC is vital to support, report and assess data, particularly for cross maritime areas and developments and harmonisation in this area need to be taken forward in a coordinated manner.</p> <p>Term of Reference i) There has been considerable interest over the past two years on the biological effects of plastic particles, particularly in relation to contaminants associated with plastic particles. It is important that this work area is reviewed and any reports and feed back from other Expert Groups is discussed at WGBEC.</p>
Resource requirements	The main input to this group is from National experts. Each attendee is self-funded from their own / organisation / institute resources.
Participants	The Group is normally attended by ca. 16 members and guests.
Secretariat facilities	None required.
Financial	No financial implications.
Linkages to advisory committees	ACOM
Linkages to other	There are direct linkages with WGSaEM, MCWG, WGMS and WGPDMO

committees or groups	and several other linkages have recently been identified and are being pursued via SSGHIE collaboration initiative.
Linkages to other organizations	None identified

Annex 16: List of actions/ recommendations

- Action 4.4: That ICES WGBEC invite a member from WGCAN to present recent the outcome of recent studies on effects of pollutants on Crangon.
- Action 5.2: That ICES WGBEC reviews status of integrated assessments from ICON and BONUS BEAST / SGEH and reviews the activity of relevant WGs on integrated assessments (ICES ICG-MSFD and WGHAME) at their 2012 meeting.
- Actions relating to WGBEC collaboration with other ICES WGs as identified above. WGBEC to pursue communication with other relevant expert groups as identified below:
 - 6.2: WGEEL – potential participation in the eel quality subgroup by WGBEC members
 - 6.3: WKMAL – Thomas Maes to act as communication link between the two groups
 - 6.4: WGMME – For WGBEC to review the WGMME review on contaminants and effects
 - 6.5: MCWG – For WGBEC to request MCWG to review ICES TIMES manuscript on DR-LUC and extraction methods and respond to SGIMC for their meeting.
 - 6.6: SGEH – To maintain contact with the SG and review their final report
 - 6.7: SGIMC – For WG members to attend the final SGIMC meeting and contribute to the process. For WGBEC to provide SGIMC with documentation for their 2011 meeting and review output in 2012.
 - 6.8: MEDPOL – To support and participate in any AQC initiatives that MEDPOL may organise and maintain contact / pursue mutual areas of interest on future WGBEC agendas – (Recommendation for ICES to tell MEDPOL!)
 - 6.9: WGMS – Explore common area of interest on use of passive samplers for bioassays and send WGMS the draft TIMES manuscript on extraction methods for bioassays for comment.
- 6.10: Chairs to re-establish contact with other EGs already identified where no contact has yet been received.
- Recommendation 7.1: That ICES/OSPAR SGIMC recommend to OSPAR that a background document on biological effects assessment criteria should be produced and that WGBEC be invited to update this annually as new data become available.
- Recommendation 7.2: After the cessation of ICES/OSPAR SGIMC and ICES SGEH, WGBEC reviews background documentation, assessment criteria and applications of integrated assessment frameworks across the ICES area at its annual meetings.
- Recommendation 8.1: That biological effects measurements recommended for integrated assessment by ICES/OSPAR SGIMC are used for reporting on MSFD GES descriptor 8, Commission indicator 8.2.1.

- Recommendation 8.2: That WGBEC review any available initial assessments in relation to Descriptor 8 at their next meeting and make recommendations on the development of national monitoring programmes for assessing GES for Descriptor 8.
- Recommendation 9.2: that at a future meeting WGBEC recommends methods suitable for determining the effects of acidification on marine organisms.
- Recommendation 9.6: That background documentation and assessment criteria are developed for measuring lysosomal membrane stability in fish.
- Recommendation 9.7: That an intersessional technical meeting in autumn 2011 in the UK (led by Cefas Weymouth) to identify priority areas for technical cooperation in marine organism immunotoxicology and that WGBEC receives a report on progress of intersessional work and strategy for taking this work area forward at its 2012 meeting.
- Recommendation 10: That draft resolutions be sought from ICES for publication of TIMES manuscripts for:
 - Reproductive success in eelpout by Jakob Strand (deadline March 2012)
 - Alkylphenol bile metabolites by G. Jonsson *et al.* (deadline Oct 2011)
 - Sea urchin embryo bioassay by Beiras *et al.* (deadline Oct 2011)
- Recommendation 11.1: That member states should be submitting the full range of their national biological effects monitoring data to the ICES data centre
- Recommendation 11.2: That an intersessional subgroup be formed to address data centre issues for biological effects data and report annually to WGBEC
- Recommendation 12.1.1: OSPAR/ICES Member Countries that are applying NRR assay in monitoring programmes should make available NRR time data at SGIMC. Also, NRR data obtained within the framework of ICON workshop.
- Recommendation 12.1.2: The ICES TIMES protocol should be amended/extended in relation to practical details and illustration after feedback for D. Lowe and A. Kohler have been received and discussed at SGIMC 2011.
- Recommendation 12.1.3: That the need for review of NRR assessment criteria and ICES TIMES is assessed through contact with the ICES TIMES authors and presented to WGBEC at their 2012 meeting.
- Recommendation 12.4: That the WGBEC QA steering group initiate sample exchange to facilitate intercalibration for methods recommended for the OSPAR integrated monitoring approach and review the results of intercalibration at WGBEC.
- Recommendation 13: WGBEC member Kris Cooreman should attend the PICES workshop on development of Pacific workplan on contaminants.