ECOREGION: General advice

SUBJECT: Review and update of the JAMP Technical Annex on lysosomal stability

Advice summary

ICES advises that the revised OSPAR JAMP Technical Annex 6 on lysosomal stability provided here in Appendix 1 be adopted and that the remainder of the two OSPAR JAMP Guidelines on general and on contaminant-specific biological effects monitoring be replaced by (or updated to reflect the information provided in) the integrated monitoring guidelines (ICES, 2010a, 2011).

Request

**Review and update of the JAMP Technical Annex on lysosomal stability (OSPAR 2012/1)**

To review and update, as necessary, the Technical Annex 6 (lysosomal stability) to the JAMP Guidelines for general biological effects monitoring. This should build on the latest developments through the Workshop on Lysosomal Stability Data Quality and Interpretation and WGBEC.

**ICES advice**

Technical Annex 6 of the OSPAR JAMP Guidelines on general biological effects monitoring concerning lysosomal stability has been updated to include the latest available information relevant to the application of this method for environmental monitoring purposes and is made available in Appendix 1. The document has also been extended to incorporate the neutral red retention (NRR) method for mussels and refers to the latest OSPAR Background Document and to ICES TIMES No. 36 on the method (Moore et al., 2004), which describes the technique more fully.

The outcome of the 2010 ICES/OSPAR Workshop on the Quality and Interpretation of Lysosomal Stability Data (WKLYS) (ICES, 2010b) was the identification of some areas of inter-laboratory variability with regards to application of the methodology and assessment against criteria. ICES advises that the methodological issues are resolved through changes to the ICES TIMES No. 36 (Moore et al., 2004). This work is in progress. ICES does not recommend any changes to the assessment criteria for lysosomal stability as a result of considerations of the WKLYS 2010 report.

ICES considers that the existing OSPAR Background Document including assessment criteria (OSPAR, 2007a), the revised Technical Annex 6, and the revised ICES TIMES No. 36, should provide adequate documentation to cover the use of lysosomal stability as a monitoring technique across the OSPAR region and should help to reduce the amount of variation in the method that is being experienced by practitioners.

The two OSPAR JAMP Guidelines concerning general and contaminant-specific biological effects monitoring and their associated technical annexes that currently exist within OSPAR have been produced at various times over the last 15 years (OSPAR, 2007b, 2008). As in any other research area, there has been a development of methods and techniques, as well as increased experience, with biological effects methods. There is therefore a more or less continuous need to update and revise existing guidelines. Much effort has been put into revising OSPAR background documents/technical annexes on biological effects monitoring and developing integrated monitoring guidelines as part of the ICES/OSPAR WKIMON and SGIMC processes, resulting in ICES advice in 2010 and 2011 (ICES, 2010a, 2011). These two pieces of advice contain more up-to-date, comprehensive, and relevant information on biological effects techniques as well as information on how to integrate chemical and biological effects assessments using the monitoring data. It is recommended that either the two OSPAR JAMP Guidelines (OSPAR, 2007b, 2008) are made redundant and the guidelines on integrated monitoring (ICES, 2010a, 2011) used in their place, or that they, along with the remainder of OSPAR JAMP biological effects technical annexes, should be revised to take account of the latest information in the guidelines on integrated monitoring.

Future modifications to the OSPAR Guidelines may be required as experience with their use develops and more monitoring data with which to revise assessment criteria become available. Some mechanism for periodic update of these documents and values should be put in place.
Background

Several ICES and OSPAR documents form the basis of the background information on lysosomal membrane stability (LMS) as a technique for marine environmental monitoring in the ICES/OSPAR areas (see ‘Sources’).

The current OSPAR request is to update Technical Annex 6 of the OSPAR JAMP Guideline on General Biological Effects Monitoring (OSPAR, 2007b). This has been updated (Appendix 1) to include additional information on the application of the method in a monitoring context, the use of the LMS method in mussel species (using the neutral red retention assay, NRR), and information on quality assurance. Reference is also made to the OSPAR Background Document on lysosomal membrane stability as a global health status indicator in biomonitoring (OSPAR, 2007a), which contains the most comprehensive and up-to-date summary of the methods, their use and assessment criteria for marine monitoring purposes.

ICES also reviewed the outputs of the 2010 ICES/OSPAR Workshop on Lysosomal Stability Data Quality and Interpretation (WKLYS) (ICES, 2010b). During WKLYS 2010, a number of uncertainties were identified concerning the methods used and the assessment criteria. All of these aspects were discussed and are reported below. They are relevant to harmonize the use of the neutral red retention (NRR) assay for mussels, in terms of monitoring and intercomparison purposes across the ICES maritime area and between regional seas programmes.

Assessment criteria of LMS, using the neutral red retention (NRR) assay

Existing assessment criteria for LMS were questioned at WKLYS as many labs that attended this workshop had observed <120 min labilisation period at monitoring reference sites, which might suggest that the original background assessment concentration (BAC) value recommended for NRR was too long. After a review of monitoring data collected at reference sites by scientists within the ICES science community, it was decided not to propose amendments of the assessment criteria for NRR since a large number of laboratories reported an ability to achieve median retention times of >120 min at reference sites. There seems to be a high degree of variation in data from reference sites and the source of this needs to be reviewed in the coming years as more data becomes available.

Review of the methodology of the NRR assay

In agreement with the authors of the original ICES TIMES No. 36 (Moore et al., 2004) it was decided that the NRR assay method described in this document should be amended and improved to make it more informative and robust, particularly in relation to the following points:

- Correct the size of the needle to be used (21 gauge) for haemolymph extraction.
- Incorporate the step of tipping off the dye and replacing it with seawater as recommended in the MED POL protocol.
- Suggest the use of physiological saline adjusted to the equivalent ionic strength of the ambient water or use ambient filtered seawater from the sampling sites.
- Change the wording for the determination of endpoint to improve clarity.
- Update photographs in the original manuscript.
- Rephrase endpoint determination in manuscript with: “The test for each slide is terminated when dye loss or lysosomal change as described above are evident in greater than 50% of the granular haemocytes and the time recorded when this occurs. The retention time is therefore the last analysis point at which less than 50% of the cells exhibit dye loss or lysosomal change, i.e. the last point at which the dye was retained and there were no structural changes. The mean and median retention time is then calculated for each sample set.”

Future modifications to the method for LMS in mussels using the NRR assay

A new scoring method to record data of LMS using the NRR assay, not only based on neutral red retention time but also taking into account the observed morphological/pathological changes that occur in the lysosomes during the course of the assay to produce a ‘weighted score’ (% stability), has been proposed by one of the original method authors (David Lowe). The proposed new methodology was reviewed by ICES and, although not considered ready for adoption for national monitoring purposes (no assessment criteria, limited application to date), it has the potential to further reduce the variability of the method and improve its utility for monitoring. The new method also has the potential to provide a better understanding of how different classes of contaminants affect lysosomal membrane stability and how this is manifested. Therefore, ICES considers it beneficial to also include details of the new lysosomal scoring system in an update of ICES TIMES No. 36, so that this new improved approach can be disseminated and hopefully weighted score data generated alongside retention time data, which will lead to the generation of new assessment criteria.
Sources

ICES. 2010a. Advice 1.5.5.1. Further development of guidance on integrated monitoring and assessment of chemicals and biological effects.
ICES. 2011. Advice 1.5.5.4. Further development of guidance on integrated monitoring and assessment of chemicals and biological effects.
Appendix 1. Updated Technical Annex 6 of the OSPAR JAMP Guidelines for General Biological Effects Monitoring

Lysosomal stability

Application in monitoring

Lysosomal membrane stability (LMS) is a sub-cellular general stress response, known to be responsive to contaminant exposure, that can be monitored in marine organisms collected from the field. Two types of techniques for the measurement of LMS are recommended for monitoring purposes. A cytochemical method for use on cryopreserved tissues / organs of marine biota and an in vivo, neutral red retention (NRR) method that can be applied to live cells (mussel haemocytes). The cytochemical method can be applied to a range of species / tissue matrices, most often fish liver and mussel digestive gland. The NRR method is most suitable for use on mussel haemocytes (in a haemolymph sample). The results of the analysis are expressed in minutes, either as a lysosomal labilisation period for the cytochemical method or neutral red retention time for the NRR method. Full details on the background to this effect measurement are given in the OSPAR Background Document “Lysosomal membrane stability as a global health status indicator in biomonitoring” (OSPAR, 2007).

Target organ/organism

Fish
Liver in dab (Limanda limanda), accepting that other species (preferably those already used for contaminants monitoring) may need to be used beyond the normal geographical range of dab.

Bivalves
Haemolymph and/or digestive gland in mussels (Mytilus spp.), accepting that other species of bivalves (preferably those already used for contaminants monitoring) may need to be used beyond the geographical range of mussels.

Effect measured

Subcellular cohesion of lysosomes. An OSPAR Background Document is available on lysosomal membrane stability as a global health status indicator in biomonitoring and describes this effect measurement in more detail (OSPAR, 2007).

Means of interpretation

For assessment purposes, neutral red retention time (min) or lysosomal labilisation period (min) should be assessed against the background (BAC) and environmental (EAC) assessment criteria developed for the technique (OSPAR, 2007). Retention times or labilisation periods shorter than the EAC level suggest that the marine organisms sampled are severely stressed and probably exhibiting pathology. Dysfunction of lysosomal processes has been mechanistically linked with many aspects of pathology associated with toxicity and degenerative diseases (Cuervo, 2004; Köhler et al., 2002; Moore et al., 2006). Retention times or labilisation periods shorter than the BAC level but longer than the EAC level are considered to represent stressed, but compensating organisms.

Fish
Reduced LMS in cells from fish liver has been shown to relate to impaired liver function. It is therefore important to have an assessment of the disease status (incidence of external disease and liver pathologies) of each individual fish sampled. LMS provides useful supporting information for other physiological and molecular biomarkers in fish taken as part of an integrated contaminant and biological effects monitoring programme.

Bivalves
Reduced LMS in bivalves is known to impact on digestive gland function, immune response, and capability to effectively up-regulate proteins involved in protection from oxidative stress. This can be a significant factor contributing to the ability of organisms to tolerate stressful and polluted environments.

Additional biological effects measurements can aid the interpretation of the significance of destabilisation of lysosomal membranes in bivalves. These include: Stress on Stress, Scope for Growth (measurement of physiological status), and an assessment of the disease status of mussel sampled (histopathology).
Where monitoring is being conducted for the purposes of integrated assessment of contaminants and biological effects, sampling should be conducted according to the integrated monitoring guidelines (ICES, 2011). For other purposes the guidance in the ICES TIMES No. 26 (Moore et al., 2004) and summarised below should be followed.

**Fish**
Flatfish should be caught in short (30 min) hauls and transferred to aerated flow-through holding tanks with seawater of ambient water temperature to minimize handling stress. Individual fish should be measured, weighed, dissected, and sexed. The livers of 25 fish (gender according to the monitoring programme) are removed and cut into pieces (5 mm × 5 mm × 5 mm) and rapidly placed on a cooled (4˚C) chuck. These are then quenched in n-hexane at −70˚C and prepared and stored as described by Köhler et al. (1992).

**Bivalves**
Sampling should be avoided during the main spawning period. A minimum sample of 10 individuals from the same size class (small) should be taken from the sub-littoral (to avoid fluctuations due to aerial exposure) by cutting byssus threads to avoid damaging the internal organs of the mussel. Transportation should avoid rough handling and mussels should be packed in insulated containers containing absorbent material soaked in sea water. Transportation times should be kept to a minimum and for journeys of >4 hours ice packs should be added to the insulated boxes. For the *in vivo* NRR method no sample preservation is required and haemolymph should be removed from the mussels as described by Moore et al. (2004). For the cytochemical method digestive glands should be removed by dissection and cut transversely into 3 equal portions. The middle portion is used for analysis and the other portions are available for histopathology. Immediately after dissection this middle portion should be placed on a cooled chuck as described for fish liver and prepared and stored according to Moore (1988).

Samples should be analysed by either the cytochemical method or NRR assay according to Moore et al. (2004). At the time of writing (2012) this manuscript is under revision to improve clarity of the NRR method section.

**Cytochemical method**
The method is described by Moore et al. (2004). Protocols also exist for national programmes (e.g. Germany) (Moore, 1990; Köhler, 1991; Lowe et al., 1992) and for cooperative studies in the Mediterranean and the Baltic Sea.

**Neutral red retention method**
The analytical method is described in ICES TIMES No. 36 (Moore et al., 2004) which is currently in the process of being amended in light of methodological improvements identified during the ICES/OSPAR Workshop on Lysosomal Stability Data Quality and Interpretation (WKLYS) (ICES, 2010).

Various activities can and have been used to conduct inter-laboratory quality assurance (QA) exercises, including workshops and ring tests. For the cytochemical technique frozen tissue samples can be used both for internal QA as laboratory reference materials (LRM) and distributed between laboratories for external QA purposes. For the NRR technique, live mussels from the same sources can be distributed for external QA, or for workshops involving multiple participants conducted to provide external QA data on the same samples. Examples of such activities are provided below.

**Cytochemical method**
Intercalibration exercises for lysosomal stability techniques (in fish) have been carried out in the ICES/UNESCO–IOC–GEEP Bremerhaven Research Workshop (1990) and the UNEP–MED POL programme. A workshop was also held at the Plymouth Marine Laboratory in 1996 (organiser: Dr M. Moore) and again at Bremerhaven in 2008 aimed at harmonising methodology between participants.

**Neutral red retention method**
Intercalibration for the NRR method was carried out for mussels in the GEF Black Sea Environment Programme (Köhler et al., 1992; Lowe et al., 1992; Moore et al., 1998; Viarengo et al., 2000). An intercomparison exercise on NRR in mussels was also
conducted in the BEQUALM programme during 2001. MED POL and ICES joined forces in 2009 to carry out the first laboratory intercalibration exercise using the NRR assay, with sixteen laboratories participating. Results were presented at the Consultation Meeting to review MED POL in 2011 (UNEP/MAP, 2012). An ICES/OSPAR workshop on the quality and interpretation of lysosomal stability data (WKLYS) was conducted in 2010 (ICES, 2010).

References


