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Lysosomal membrane stability in mussels

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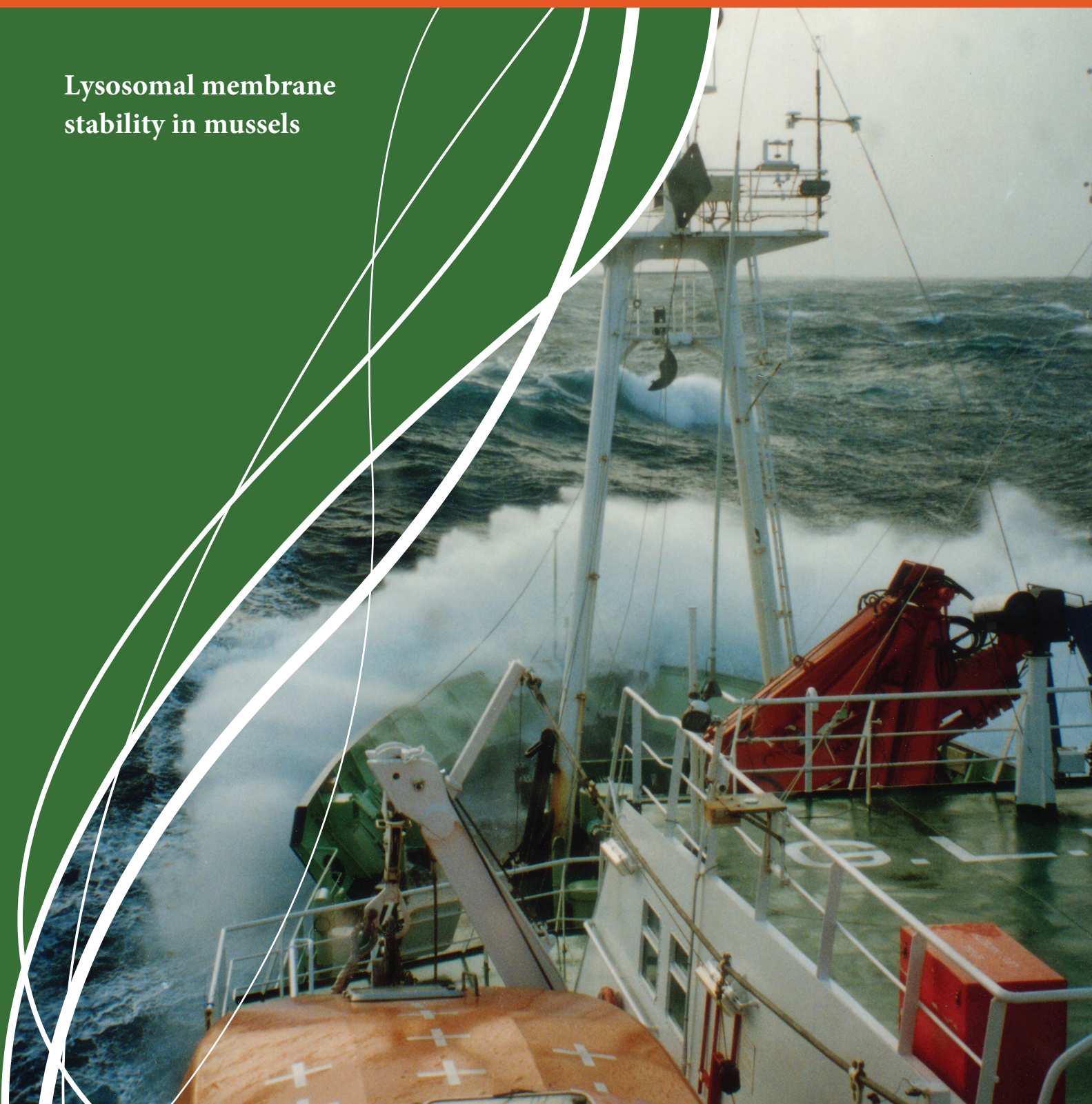the Exploration of the Sea

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This series presents detailed descriptions of methods and procedures relating to chemical and biological measurements in the marine environment. Most techniques described have been selected for documentation based on performance in ICES or other intercalibration or intercomparison exercises: they have been carefully evaluated and shown to yield good results when correctly applied. They have also been subject to review by relevant ICES working groups, but this is not to be construed as constituting official recommendation by the Council.

Contents

Abstract

In 2012, the ICES Study Group on Integrated Monitoring of Chemicals and their Effects provided a framework for integrated monitoring to the OSLO-Paris Commission. UNEP/MAP and HELCOM expert groups have also developed guidelines on integrated monitoring of chemicals and their effects for the Mediterranean and Baltic Sea. This document provides the technical information for one of the biological effects measurements, the lysosomal membrane stability (LMS), which is a part of the above mentioned integrated monitoring approaches. Lysosomes are cytoplasmic, single membrane organelles whose condition is sensitive to stress whether it be due to environmental conditions or exposure to a wide array of contaminants. Two different methodologies have been developed to assess LMS in mussels: an enzyme cytochemical method using cryostatic sections of digestive gland tissue, and an *in vivo* cytochemical method (using haemolymph cells). In this document, different aspects of the operational procedures have been standardized and harmonized, with particular reference to the *in vivo* cytochemical method. New graphical material has been added to clarify criteria of interpretation and new external quality assurance programmes for measurements of lysosomal membrane stability have been proposed. Background (BAC) and environmental (EAC) assessment criteria to assess the LMS data are provided. Additionally, a new scoring procedure to enhance the sensitivity of the LMS measurements using the *in vivo* assay is provided.

Key words: Biomarkers, lysosomal membrane stability, mussels, neutral red

1 Introduction and rationale

In the past two decades, the use of the lysosomal membrane stability (LMS) as a general stress biomarker of chemical pollution has been recommended within the framework of the pollution biomonitoring programmes of the different Regional Conventions (OSPAR, 1997; UNEP/RAMOGE, 1999). Since 2008, the Marine Strategy Framework Directive (Directive 2008/56/EC) has put emphasis on the importance of assessing key biological responses for evaluating the health of organisms and linking the observed changes to putative contaminant effects (Law *et al.*, 2010). Furthermore, LMS is one of the core biomarkers included within the mussel component of the proposed integrated assessment approach of contaminants and their effects in the NE Atlantic (Davies and Vethaak, 2012), and considered as a core biomarker in the Baltic Sea Action Plan (HELCOM, 2012) and the Mediterranean Ecosystem Approach (EcAp) (UNEP/MAP, 2014).

Most of the European countries are using mussels (i.e. *Mytilus edulis, M. galloprovincialis, M. trossulus, Perna viridis,*) as target species to monitor the environmental chemical quality of their coastal and estuarine waters (Moreira and Guilhermino, 2005; Minier *et al.,* 2006; Mubiana and Blust, 2006; Martínez-Gómez *et al.,* 2008; Brooks *et al.,* 2009). Two different methodologies have been developed to assess LMS in mussels: an enzyme cytochemical method using cryostatic sections of digestive gland tissue, and an *in vivo* cytochemical method using living cells (haemolymph cells) (UNEP/RAMOGE, 1999; Moore *et al*; 2004). In this work, different aspects of the operational procedures described in the literature for the LMS measurement, with particular reference to the *in vivo* cytochemical method, were revised and have been standardized and harmonized. New graphical material has been added to clarify criteria of interpretation and new external quality assurance programmes for LMS have been proposed. Additionally, a new scoring procedure enhancing the sensitivity of the LMS measurements when using the *in vivo* cytochemical method is provided.

1.1 Lysosomal system

Lysosomes are cytoplasmic, single membrane organelles which contain more than 40 different classes of hydrolytic enzymes (such as proteases, nucleases, lipases, etc.) with optimal activities at an acidic pH. These enzymes are able to hydrolyze essentially all biological molecules, from proteins and nucleic acids and nucleotides to complex sugars and lipids. The functions of lysosomes in different cell types and tissues of different organisms (from protozoa to mammals) may be specific and very different but in all organisms the lysosomal vacuolar system is involved in the degradation of the material taken up into the cell by endocytosis, as well as in the regulation of the catabolic rate of cellular macromolecules, proteins in particular (Moore., 1985; Viarengo, 1989; Lloyd and Mason, 1996;).The lysosomal matrix has a pH of 4.5–5.This low pH is maintained by active proton pumping due to the activity of a H+-ATPase present in the membrane of the organelle and by the acidic proteins within the lysosomal matrix (Seglen, 1983).

In the cells, lysosomes show heterogeneous shapes. This is mainly due to the fact that, after their assembling in the Golgi apparatus, they have an initial size of about 0.5 μm. These vesicles, having a pH of about 6 and an increasing amount of hydrolytic enzymes, are not functionally active and are usually named 'primary lysosomes'. When mature lysosomes directly take up components from the cytoplasm or fuse with autophagosomes or heterophagosomes, their size increases up to several μm

and the organelles, which have a pH of about 5, are actively involved in the digestion of biological macromolecules (secondary lysosomes). At the final phase of their activity a residual body containing un-degradable material (mainly lipofuscin), and with minimal hydrolytic activity, is formed ('tertiary lysosomes', i.e. residual bodies). In cells capable of exocytosis, the content of the residual bodies is released into the extracellular fluids (Lloyd and Mason, 1996).

1.2 Lysosomal reactions

Lysosomal reactions fall into essentially three categories (Hawkins, 1980; Moore, 1988a, 1990):

- Changes in lysosomal contents
- Changes in fusion events
- Changes in membrane permeability

An increase in lysosomal size indicates an enhanced rate of fusion of primary lysosomes with auto/hetero phagosomes but also may indicate increased autophagy. The major response to contaminant stress of molluscan lysosomes appears to involve enhanced autophagy and the evidence suggests that this is an evolutionarily conserved response to environmental stress (Lowe, 1988; Moore, 1990; Winston *et al.*, 1991, 1996; Kirchin *et al.*, 1992; Lowe *et al.*, 1995a, 1995b; Klionsky and Emr, 2000; Lowe and Fossato, 2000; Bursch, 2001). However normal tidal fluctuations in salinity, food, and oxygen do not induce a stress syndrome (Bayne *et al.*, 1978, 1979; Moore, 1980; Moore *et al.*, 1979, 1987; Widdows *et al.*, 1981, 1982). These autophagic changes involve an increase in the volume of the lysosomal compartment together with frequent swelling of the lysosomes and increases in hydrolase activities in mussels (Figure 1) (Nott and Moore, 1987; Lowe, 1988; Moore, 1988a; Moore *et al.*, 1996a, 1996b; Lowe and Fossato, 2000; Marigómez *et al.*, 2005a, 2005b).

The membrane Mg^{2+}- ATPase dependent H+ ion proton pump in the lysosomes is responsible for maintaining the acidic environment inside the lysosomes. Any impairment of this system will involve the efflux of the lysosomal contents into the cytosol (Ohkuma *et al.*, 1982). In their role as components of the immune system, blood cell lysosomes can release acid hydrolases that are able to degrade circulating pathogens (Grundy *et al.*, 1996a, 1996b). However, unscheduled or inappropriate release of acid hydrolases may have disastrous consequences for the functional integrity of the cell. In general, the reduction in lysosomal stability is accompanied by enlargement or swelling. Fatty change is also a frequent reaction to xenobiotics in the digestive cells, leading to apparent autophagic uptake of the unsaturated neutral lipid into the often already enlarged lysosomes (Figure 1) (Moore, 1988a).
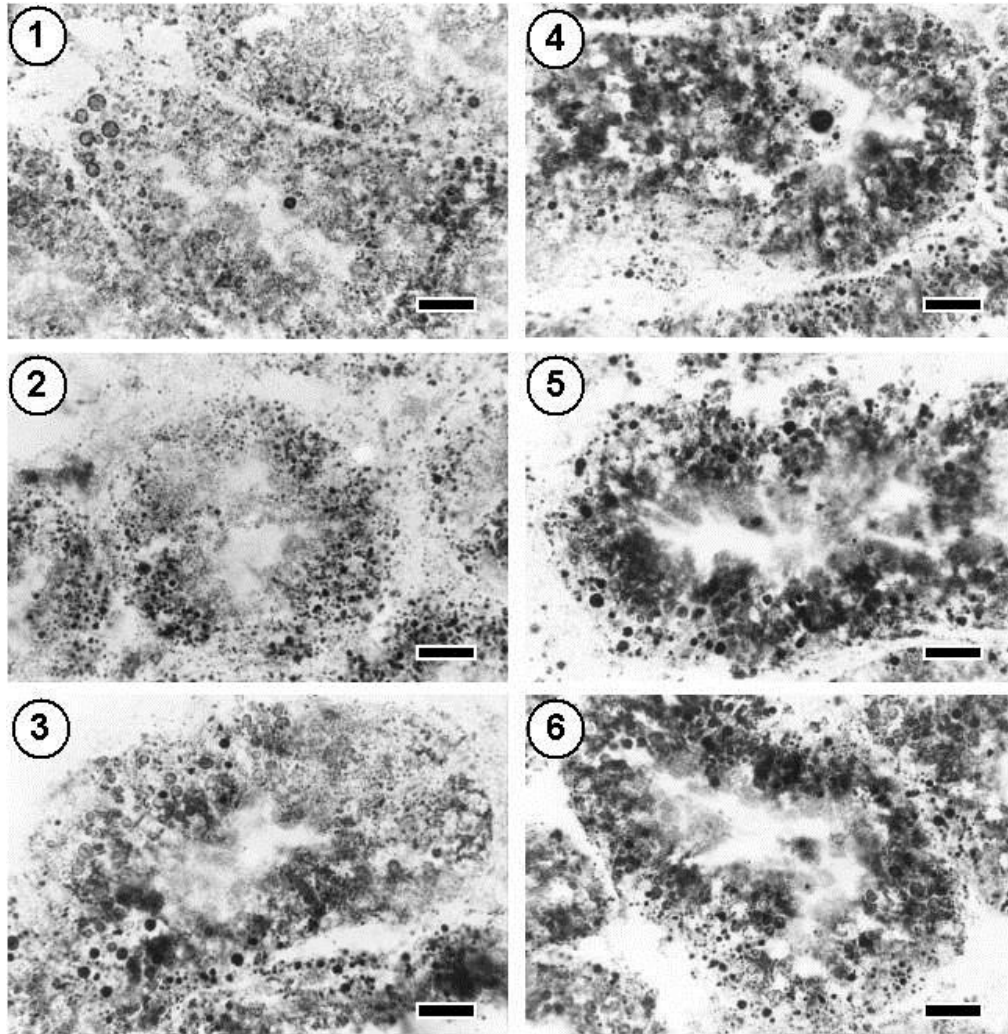
Figure 1. Serial cryostat sections of the digestive gland stained to show N-acetyl-β-hexosaminidase reactivity in the lysosomal vacuolar system of digestive cells in a digestive tubule of a mussel. Photographs 1–6: Sections pretreated at pH 4.5 and 37°C for 2–25 minutes (2, 5, 10, 15, 20, 25 minutes, respectively). Section 5 – pretreated for 20 minutes, shows maximal lysosomal staining intensity: this time of pretreatment represents the labilisation period. Section 6 – pretreated for 25 minutes, shows a decrease in staining intensity indicating a probable loss of enzyme by diffusion from fully labilised lysosomes. (Scale Bar ≡ 20 μm). (From Moore *et al.*, 2004)

1.3   Lysosomes and chemical contaminants

At the cellular level, the lysosomal system has been identified as a particular target for the toxic effects of many contaminants. Lysosomes show a high capacity for pollutant accumulation, being able to sequester and accumulate a wide number of different classes of chemical contaminants. Despite their acidic internal pH, lysosomes are able to accumulate metal cations and this seems to be mainly due to the presence in the lysosomal matrix of lipofuscins, end-products of peroxidation processes that are able to trap inside their growing granules different metal cations (Viarengo *et al.*, 1985; Viarengo, 1989). Lysosomes are also able to accumulate lipophilic organic compounds, such as aromatic hydrocarbons, PCBs, carbon tetrachloride, aminoazobenzene derived compounds, etc. (Allison, 1969; De Duve *et al.*, 1974; Rashid and Williams, 1991; Moore *et al.*, 2006). This is of particular importance in organisms, such as mussels, whose mixed function oxygenase (MFO) activity is extremely low and in which a typical cytochrome p450 is absent. Thus most aromatic compounds are not

metabolized but accumulated in these organelles – representing one of the most important sites of accumulation of organic pollutants and therefore of their action at the cellular level (Lowe *et al.*, 1995a, 1995b).

Moreover, results from earlier studies with mussels have shown that inorganic pollutants, such as Cu, Hg, and Cd, can alter lysosomal activity by affecting Ca-dependent cell signalling (Viarengo and Nicotera, 1991). It was demonstrated that the metal-induced increase in cytosolic [Ca^{2+}] concentrations is able to activate a Ca-dependent phospholipase A2 (PLA2). PLA2 binds to lysosomal membranes and activates the process of vacuole fusion with the associated increase in protein catabolism (Burlando *et al.*, 2002). Furthermore, organic contaminants known as endocrine disrupters have been shown to alter the LMS of mussel cells through activation of components of kinase-mediated cell signalling, in particular of the stress-activated Mitogen Activated Protein Kinase p38 and protein kinase C (PKC) (Canesi *et al.*, 2004).

2   Lysosomal membrane stability in mussels as stress biomarker

LMS can be altered when certain physiological or pathological conditions occur, conditions that can be induced by pollutants. Destabilization of the lysosomal membrane may result in activation and, in some cases, in liberation of hydrolytic enzymes to the cytosol. Lysosomes are extremely sensitive to minimal concentrations of toxic chemicals that penetrate into the cells. In mussels, nanomolar concentrations of both inorganic and organic pollutants are able to destabilize the lysosomal membranes and to activate protein catabolism. The change of LMS is extremely rapid in pollutant-exposed organisms (Marigómez *et al.,* 2005a; Izaguirre *et al.*, 2009).LMS is recognised as a general stress biomarker as well as a prognostic indicator for putative pathologies and as such is an integrated pathophysiological indicator of health status (Moore, 1990, 2002; Moore *et al.,*2006).

In mussels, LMS represents one of the simplest, most sensitive and low-cost biomarker to evaluate the physiological status of the organisms. Digestion in mussels is mainly an intracellular process and epithelial cells of the digestive gland serve as the major site of intracellular digestion. These cells are rich in lysosomes and are the main interface between the organism and its environment (Moore, 1990). Small granulocytes present in haemolymph are also particularly lysosome-rich cells because of their involvement in digestion processes of cellular nutrients by endocytosis and in innate immune response mechanisms in mussels (Cajaraville and Pal, 1995). Due to the fact that mussels do not have a closed circulatory system, the granulocytes move from one tissue to another.

Two different methodologies have been well developed to assess LMS in mussels:

> i)   an enzyme cytochemical method using cryostatic sections of digestive gland tissue, and
> ii)   an *in vivo* cytochemical method using haemolymph cells

Both methods to assess the LMS in mussels provide results which are consistent with data obtained using biochemical, histopathological, and physiological approaches (Bayne *et al.*, 1988; Capuzzo and Leavitt, 1988; Moore, 1988a; Widdows and Johnson, 1988). Immuno-cytochemistry, hybrido-cytochemistry (*in situ* hybridization), and *in vivo* cytochemistry using chromogenic and fluorescent molecular probes offer the potential for greatly expanding the application of the cytochemical approach to the assessment of contaminant-induced pathology, as well as greatly increasing the sensitivity of detection (Geisow *et al.*, 1981; Lekube *et al.*, 2000)

3   Mussel sampling requirements

The currently preferred target species for the LMS determination are *Mytilus edulis, M. galloprovincialis, M. trossulus and Perna viridis*. The following sampling procedures are recommended.

1) Samples should contain a minimum of ten animals;
2) The mussels should be from a standardized size class in the area to be monitored, preferably the smallest available size class (i.e. 4–5 cm)
3) Sampling should be avoided during the main spawning season;
4) Mussel byssus threads should be cut from the substrate, since pulling the animals from the rocks (threading) can result in damage to internal tissues and induce an additional stress response in mussels.
5) Mussels should be sampled from the sub-littoral part of the population since this will minimize fluctuations due to air exposure at low tide;
6) Extremes of temperature during transport are to be avoided and the animals must be maintained in a moist environment during transport to the laboratory Transport to the laboratory should avoid rough handling and mussels should be packed in an insulated container containing tissue paper soaked in sea water;
7) For transportation times of more than 4 hours, ice packs should be placed in the bottom of the insulated box.

4 Assessment of lysosomal membrane stability in tissue sections: enzyme cytochemical method

4.1 Principle

It has been observed that lysosomes contain more than 50 hydrolytic enzymes (Barret and Heath, 1977), including various phosphatases, nucleases, glycosidases, proteases, peptidases, sulfatases and lipases. For this reason, the traditional methods to mark and localise lysosomes have been based on the specificity of the lysosomal enzymes (de Duve, 1983). Latent activity of the lysosomal enzymes N-acetyl-β-hexosaminidase and β-glucuronidase have been demonstrated in the digestive cells of bivalve molluscs using naphthol AS-BI substrates and post-coupling with diazonium salts to prevent inhibition by the coupler (Moore *et al.*, 2004). The enzyme cytochemical method for the determination of LMS in mussel digestive glands is based on the activity of one of these two lysosomal hydrolases, both showing similar results.

The preparation of tissues for the examination of cell structures requires the use of specialized methodology to produce high-quality stained sections. In this section all observations are related to frozen material.

The method developed for digestive gland cells involves sacrificing the animals. However, this approach is valuable for concurrent studies with other digestive gland function studies.

4.2 Equipment

The following equipment is needed.

- High quality motorized cryostat microtome (e.g., Bright Instrument Company or Microm HM 500 OM);

- Good quality water bath (preferably shaking) up to 40 ºC;

- Cleaned Hellendahl histological staining jars;

- Good quality cleaned but untreated microscope slides with frosted glass writing area

- Good quality bright-field binocular microscope with ×10, ×25, and ×40 objectives;

- Optional use of a 580 nm green filter to enhance contrast of the purple-red reaction product.

4.3 Solutions and reagents

- <u>Lysosomal membrane labilising buffer (Solution A)</u>

  0.1M Na-citrate Buffer - 2.5% NaCl w:v, pH 4.5

- <u>Substrate incubation medium (Solution B)</u>

  20 mg of naphthol AS-BI N-acetyl-p-D-glucosaminide (SIGMA-ALDRICH® , N4006) are dissolved in 2.5 ml of 2-methoxyethanol (Merck, 859) and made up to 50 ml with Solution A, containing also 3.5 g of collagen-derived polypeptide (POLYPEP, P5115 SIGMA-ALDRICH®) as a low viscosity polypeptide to act as a section stabiliser. This solution has to be prepared just 5 minutes before use.

- <u>Diazonium dye (Solution C)</u>

0.1M Na-phosphate buffer, pH 7.4, containing 1 mg/ml of diazonium dye Fast Violet B salts (SIGMA-ALDRICH®, F1631) (Note: saturated solution)

Other dyes can also be utilised such as:

- o Fast Garnet GBC (SIGMA-ALDRICH®)
- o Fast Red Violet LB (Difco)
- o Fast Blue BB (SIGMA-ALDRICH®)
- o Fast Blue RR (SIGMA-ALDRICH®)

- ▪ Fixative (Solution D)

    Baker's calcium formol containing 2.5% NaCl (w:v)

- ▪ Mounting Medium

    Aqueous Mounting Medium (Vector Laboratories H1000, Kaiser glycerine gelatine, Difco, SIGMA-ALDRICH® or other).

- ▪ Liquid Nitrogen

4.4 Preparation of tissue sections for enzyme cytochemistry

For cytochemical examination, digestive glands from ten mussels should be cut transversely and small pieces (5 mm × 5 mm × 5 mm) (i.e., the mid-portion of the organ) are placed on metal cryostat chucks (e.g., up to five pieces of tissue in a straight row across the centre). The chucks must be pre-labelled and cooled at refrigerator temperature (4 ºC).

Each chuck is then placed for 1 minute in a small bath of *n*-hexane (aromatic hydrocarbon-free; boiling range 67–70 °C) that has been pre-cooled to −70 °C (using a surrounding bath of liquid nitrogen or a mixture of crushed solid CO_2 and acetone). The metal chuck plus the quenched (super cooled) solidified tissues are then sealed by double-wrapping in parafilm and stored at −30 °C or, preferably, at −70 °C until required for sectioning. Tissues may be stored for 6–12 months at −70 °C. By following this procedure there is no evident formation of large ice crystals and, hence, no structural damage to the subcellular components (Moore, 1976).

Cryostat sections (10 μm) are cut using a 15° knife angle in a cryostat (preferably with motorized cutting), with the cabinet temperature below −25 °C and with the haft of the knife cooled with crushed solid carbon dioxide ("dry ice"). The sections are transferred to "warm" slides (i.e. 20 °C, or room temperature), which effectively flash-dries them (Moore, 1976). The slides can then be stored in the cryostat for at least 4 hours before use.

4.5 Method for N-Acetyl-ß-hexosaminidase

Serial cryostat sections (in duplicate on the same slide), prepared as described above, are pretreated in a staining jar with Solution A at 37 ºC in order to labilise (controlled permeabilization) the lysosomes (Moore, 1976). The pre-treatment sequence commences at 30 minutes for molluscs down to 2 minutes (i.e., 30, 25, 20, 15, 10, 5, and 2 minutes). Two minutes are used as the minimal pre-treatment time since sections that have undergone zero pre-treatment may sometimes show stronger staining than short-term pretreated sections (Moore, 1976). This staining activity is believed to be largely due to non-membrane-bound acid hydrolase that can be lost by diffusion from the section when no polypeptide stabilizer is present. Such activity is frequently localized in large secondary lysosomes or digestive vacuoles that may be damaged in

sectioning. Due to this complicating factor, the zero pre-incubation is usually omitted and the 2-minute pre-treatment is taken as representing the free lysosomal activity.

Following this pre-treatment sequence, the slides are transferred to the substrate incubation medium (Solution B). Incubation time is 20 minutes at 37 °C in a staining jar, preferably in a shaking water-bath. The slides are subsequently rinsed in filtered seawater at room temperature or with a saline solution (3.0% NaCl) at 37 °C for 2 to 3 minutes before being transferred to Solution C at room temperature for 10 minutes. The slides are then rinsed rapidly in running tap water for 5 minutes, fixed for 10 minutes in Solution D at 4 °C, rinsed in distilled water, and then mounted in aqueous mounting medium.

4.6   Method for ß-Glucuronidase

The method for the demonstration of latent activity of lysosomal β-glucuronidase (Moore, 1976) is similar to the method described above, with the following exceptions. The pre-treatment to labilise the lysosomal membranes is carried out using 0.1M acetate buffer (pH 4.5) containing 2.5% NaCl (w:v), and the substrate incubation uses 14 mg naphthol AS-BI β-D-glucuronide (SIGMA-ALDRICH®). The substrate is dissolved in 0.6 ml 50mM $NaHCO_3$ which is made up to 50 ml with 0.1M acetate buffer (pH 4.5) containing 2.5% NaCl (w:v) and 3.5 g of polypeptide (SIGMA-ALDRICH® POLYPEP P5115). Rinsing and coupling solutions for β-glucuronidase are the same as those used for β-N-hexosaminidase.

4.7   Determination of Lysosomal labilisation period

The labilisation period (LP) is the time of pre-treatment required to labilise the lysosomal membranes fully, resulting in maximal staining intensity for the enzyme being assayed (Figures 1 and 2). The staining intensity can be assessed visually using microscopic examination or else measured using a scanning integrating microdensitometer or image analyser to obtain an activity plot as shown in Figure 2 (Moore, 1976). If the animal is stressed, then the peak of activity will be moved towards the *y*-axis and the decreased LP period can be determined from the *x*-axis (Figure 2).

Experience has shown that a microdensitometer is not completely necessary for accurate determination and that the labilisation period can be effectively measured by microscopic assessment of the maximum staining intensity in the pre-treatment series (Figure 1). For this procedure, each tissue section should be divided into four roughly equal sub-areas for assessment. This can be done by drawing a cross on the cover slide overlaying each section with a very fine marker pen, thus giving four sub-areas. The position and orientation of the cross should be the same on all sections (Figure 3).
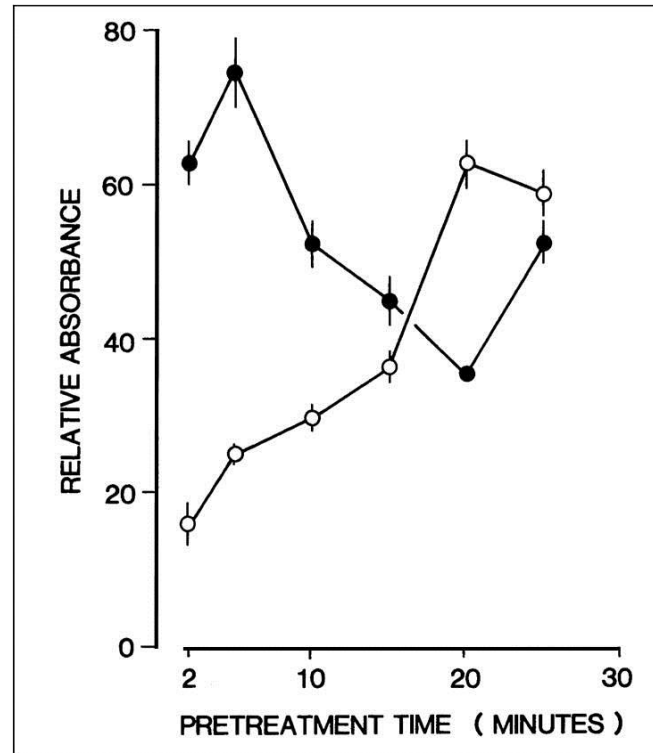
Figure 2. Microdensitometric determination of N-acetyl-β-hexosaminidase activity in sequentially pretreated (labilised) tissue sections of mussel digestive gland. Healthy cells = open circles; unhealthy cells = filled circles. Means + SE, n = 10. (From Moore *et al.*, 2004)

4.8 Data recording

Assessment of LP by visual determination should be carried out on *duplicate sections for each digestive gland* at each pre-treatment time (Figure 3). A mean or median value is obtained for each set of duplicate sections from the average of the assessments for each of the four sub-areas (i.e., sub-area 1 from all sections in the sequence, then 2, 3, and 4). Lysosomes will stain reddish-purple due to the reactivity of the substrate with N-acetyl-ß-hexosaminidase or ß-glucuronidase. The average LP for each section corresponds to the average incubation time in the acid buffer that produces maximal staining reactivity. LP for the other duplicate section is similarly obtained. For example, for figure 3, specimen 1 (replicate a) the staining intensity:

Sub-area 1 (10 min) > Sub-area 1 (30 min) > Sub-area 1 (15 min) …etc

Sub-area 2 (10 min) > Sub-area 1 (30 min) > Sub-area 1 (15 min) …etc

Sub-area 3 (15 min) > Sub-area 1 (10 min) > Sub-area 1 (30 min) …etc

Sub-area 4 (15 min) > Sub-area 1 (00 min) > Sub-area 1 (30 min) …etc

LP specimen 1 (replicate a) = (10+10+15+15)/4= 12.5 minutes

Finally, a mean value of LMS of the sample will be calculated utilizing the data obtained from the 10 animals analyzed.
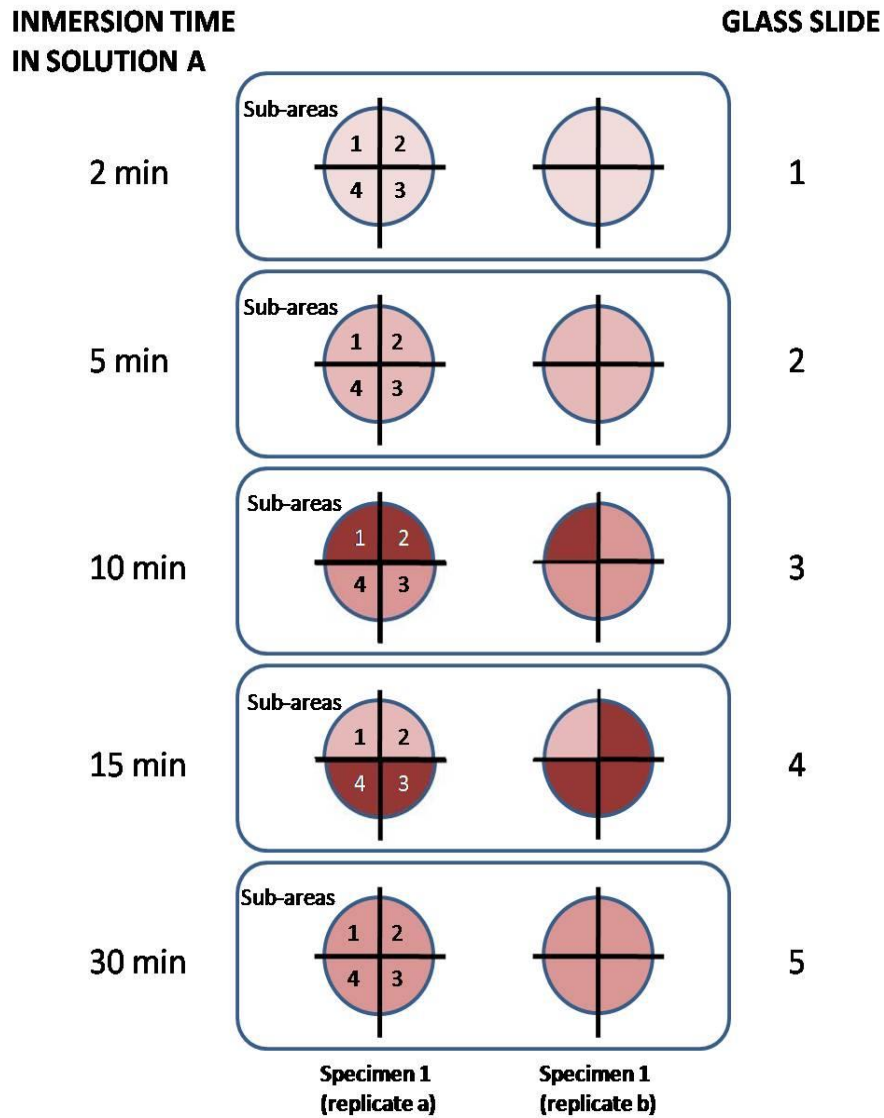
Figure 3. Virtual example of staining intensity in tissue sections (replicate a and b) of mussel digestive gland from the same specimen.

4.9   Problems in assessment of labilisation period

Determination of the labilisation period is usually quite straightforward but a complicating situation occasionally arises when the pre-treatment series shows two peaks of staining intensity along the time (Moore *et al.*, 1978a, 1978b) possibly due to differential latent properties of the subpopulations of lysosomes (Figure 2). In this situation**, the first peak of activity is used to determine labilisation period** as it is the most responsive (Figure 2).

5 Assessment of lysosomal membrane stability *in vivo* cells: neutral red retention assay

5.1 Principle

The Neutral Red Retention (NRR) assay is an *in vivo*cytochemical method based on the retention of neutral red, an amphiphilic and weak cationic dye, which freely permeates the cell membrane. Within cells the dye becomes trapped by protonisation in the lysosomes and sequestered by the lysosomal matrix, accumulating in these organelles. The degree of trapping of this lysosomotropic marker depends on the pH of the lysosome as well as the efficiency of its membrane associated proton pump (Seglen, 1983). The NRR assay reflects the efflux of the neutral red into the cytosol following damage to the membrane and, possibly, impairment of the H+ ion pump (Lowe *et al.*, 1992). Any impairment of this latter pump will result in a reduction of the retention time (RT) of the neutral red inside the lysosomes.

The rationale is that lysosomes in healthy cells take up and retain larger quantities of neutral red than those from damaged cells. The dye is visualized by using a light microscope and the progress of dye uptake into the cells and, in the case of damaged cells, leakage back into the cytosol. Any other lysosomal alterations are also determined and quantified.

The following NRR assay protocol has been specifically adapted to be used on living haemolymph granulocytes of mussels (annex 1) which are generally easy to obtain without harming the host. This NRR assay is non-destructive therefore, if the test animals are not unduly stressed during collection, they can be returned to their habitat following careful extraction of a haemolymph (blood) sample. Similarly, this assay facilitates the acquisition of time series samples e.g. during chemical exposures in the laboratory.

5.2 Equipment:

- Good quality bright-field binocular microscope (preferably inverted microscope) with ×10, ×25 and ×40 objectives;
- Optional use of a 580 nm green filter to enhance contrast of the neutral red
- Dark humidity chamber for incubation of the cells with neutral red.

5.3 Reagents and Solutions

Neutral red

Neutral red dye powder is commercially available in a range of purities and strengths. If possible, the highest strength/purity dye (≥90 %) should be used. However, what is most important is that only dye batches of similar quality and concentration are used when making comparisons between sites/treatments in an experiment or a monitoring exercise.

Filtered seawater

In mussels, the salinity of haemolymph reflects the salinity of the ambient surrounding seawater (ICES, 2010). Sea water (SW) salinity around OSPAR area ranges up to 36 PSU. In Mediterranean Sea salinity ranges up to 44 PSU. Salinity of Baltic coastal waters usually falls below 6 PSU. The use of ambient filtered SW (filter 0.45 μm) from the mussel sampling sites is recommended. Alternatively, a physiological saline adjusted to the sampling site salinity can be prepared in the laboratory. An advantage of

using a physiological saline with adjusted salinity is more robust inter-site comparisons because all samples are being compared to a common baseline.

Standard mussel physiological saline formula:

HEPES (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid) 4.77 g

Sodium chloride 25.48 g

Magnesium sulphate 13.06 g

Potassium chloride 0.75 g

Calcium chloride 1.47 g

The above salts should be dissolved in approximately 800 ml of distilled water and then made up to one litre by the addition of more distilled water. The solution should be stored in a refrigerator, raised to room temperature prior to use, and the pH checked and adjusted to 7.36 with 1M NaOH.

CAUTION: The salinity of the above physiological saline is about 30.5 PSU and therefore it needs to be adjusted to the equivalent ionic strength of the ambient seawater.

Neutral red stock solution

Prepare a 100 mM stock solution of neutral red by dissolving 28.8 mg of dye powder in 1 ml of DMSO and store in the refrigerator prior to use. The stock solution will last for about 2–3 weeks when stored in this way. However, the solution will solidify in the refrigerator and should be raised to room temperature for dilution to the working strength stock.

Neutral red working solution

For a working solution, dilute 10 µl of stock neutral red in 5 ml of physiological saline.

The working solution will last about four hours before the dye begins to precipitate out.

CAUTION: Different grades of dye will have a different effect on the lysosomes depending on their purity and strength.

Siliconizing reagent for glass and other surfaces (i.e. Sigmacote® fromSIGMA-ALDRICH®)

It retards clotting of blood or plasma. It is reusable if kept free of moisture. Its use on microtubes is recommended but only when haemolymph samples are not processed immediately after extraction. Alternatively, pre-siliconised microtubes may be purchased.

Poly-L-Lysine (P 8920 SIGMA-ALDRICH®)

Slide adhesive solution for use in adhering tissue sections to glass slides. Alternatively, pre-coated slides may be purchased. Its use is recommended though it is not essential.

5.4 Haemolymph (blood) extraction and neutral red incubation

The detailed procedure for extraction of haemolymph and incubation with the neutral red dye follows.

1) Slides should be pre-labelled and draw a circle on the reverse side of the slides before use to easily identify the area on which granulocytes will be at-

tached. Fill microtubes with siliconizing reagent, leave for 10-30 minutes, and then return siliconizing reagent to container. Keep microtubes on crushed ice. Put 2 μL of Poly-L-Lysine (0.1 % w/v, in distilled water) on the circled area of the slides and spread out with a cover slip. Leave to dry in a humidity chamber.

2) The mussel valves should be carefully prised apart along the ventral surface using a solid scalpel. The scalpel should remain in position in order to keep the valves apart. The blade width of a scalpel should be sufficient to hold the valves apart far enough to insert a hypodermic needle. However, numerous items can be used for this purpose. For example, a pipette tip can be inserted between the open valves (figure 4). Dissection scissors are also useful for this purpose because they can be simultaneously used as a support (figure 5). This is particularly useful to facilitate a steady hand during the extraction of haemolymph. Allow any water retained within the shell cavity to drain out before attempting to withdraw any haemolymph.

3) Using a new syringe and needle for each specimen, withdraw 0.1ml of haemolymph from the posterior adductor muscle using a 1ml hypodermic syringe fitted with a 21-gauge needle and containing 0.1ml of filtered SW or physiological saline. It is important that one is not tempted to withdraw more haemolymph than necessary during a successful "bleed". **Failure to maintain a 50:50 ratio of haemolymph to physiological saline can result in the "clumping" together of granulocytes** (this may also occur if the physiological saline temperature has been allowed to become too warm).

Figure 4. Haemolymph extraction. A typical "yellow" tip (100-1000 cc) or dissection scissor can be used to keep mussel valves apart and to allow that water retained within the shell cavity drain out before attempting to withdraw any haemolymph.

4) Having obtained a sample of haemolymph, remove the needle (to reduce shearing forces that may damage the cells) from the syringe and softly expel the contents of the syringe into a 1.5–2.0 ml siliconised microtubes. If cells are used immediately after extraction, non-siliconised tubes can be also used. If necessary cells can be kept in a refrigerator prior to use, but stored in siliconised microtubes for no longer than 20 minutes

5) Gently invert the microtubes in order to mix the contents and then pipette 40 μl haemolymph/SW mixture (or haemolymph/physiological saline mixture) onto each slide, using a clean pipette tip for each sample. Haemolymph-saline mixture should be dispensed in the same position where the Poly-L-lysine was added. Prepare two slides (S1 and S2) per specimen (Figure 5). It is recommended to cut about 3mm off the end of the pipette tips prior to dispensing haemolymph onto the slides. This will enlarge the tip aperture and

help decrease potential shearing forces that may damage haemocytes during pipetting.

6) Place all slides into a light-proof humidity chamber and incubate for 15–20 minutes (Figure 6). It is important that the slide preparations are kept cool (15-18 ºC) throughout the period of cell attachment and dye incubation. This can be achieved by using controlled temperature incubator or by placing a thin layer of ice water in the light-proof humidity chamber. **The slides must not be in direct contact with the ice water and should be placed on racks allowing sufficient space (approximately 3 cm) for the chilled air to circulate.**

Figure 5. Haemolymph-saline mixture should be dispensed in the same position where the Poly-L-lysine was added (circled area). After the incubation period of cell attachment excess suspension from the slide must be carefully drained.
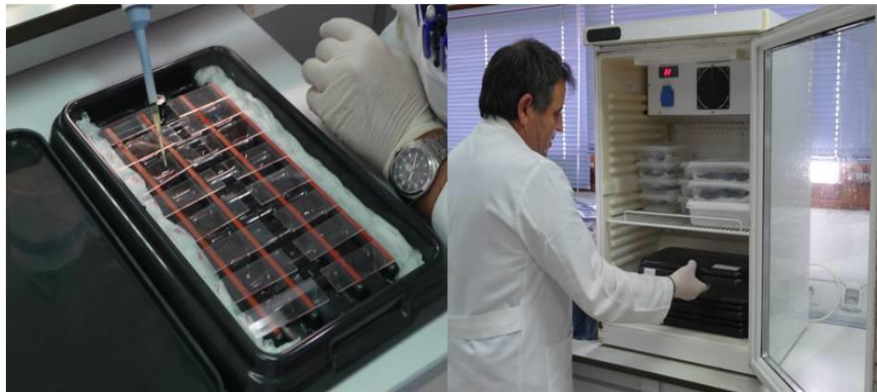

Figure 6. Application of neutral red working solution onto the haemocytes. Slides are located on racks and inside a light-proof humidity chamber. The light-proof humidity chamber should be located inside a controlled temperature incubator (18ºC) throughout the periods of cell attachment, dye incubation and during those time intervals between microscopic assessments. This is particularly important in those labs where air ambient temperature can be easily over 20 ºC. In the absence of a controlled temperature incubator, a humidity chamber chilled with ice can be used to help maintain cool temperature.

7) After 15–20 minutes, carefully drain the excess suspension from the slides by placing each slide on its side and letting the liquid run off (figure 5). Immediately pipette 40 µl neutral red (NR) working solution onto the haemocytes and allow neutral red to penetrate the cells. When applying the neutral red working solution, do not drop the solution onto the cells; touch the surface of

the slide with the pipette tip and slowly eject the dye onto the cells in the same position where cell suspension was added (figure 6).

8) After 15 minutes, carefully drain the excess neutral red working solution from the slide by placing the slide on its side and letting the liquid run off. Immediately apply ambient filtered SW or physiological saline to the slide. Analysis of the slides can be undertaken on either an upright or inverted microscope but in both cases it is necessary to apply a 22x22 coverslip.

9) One of the duplicate slides should be checked systematically under the microscope after 15, 30, 60, 90, 120, 150 and 180 minutes of incubation with NR. Neutral red is photosensitive; therefore, all slides should receive the same exposure to light under the microscope, and the light intensity should be kept as low as possible. The whole circled area on the slide should be scanned and the slide replaced in the humidity chamber as quickly as possible—ideally 1 minute per slide maximum. Cells should be examined for both structural abnormalities and NR probe retention time. Conditions should be recorded in a table at each time increment (Table 1). **The retention time of the NR probe by the lysosomes is recorded by estimating the proportion of cells displaying leakage from the lysosomes into the cytosol and/or exhibiting abnormalities in lysosomal size and colour** (Figure 7 and 8; Annex 2). Cell shape may also change as a consequence of contaminant impact.

Note 1: If the first slide does not have a sufficient number of attached haemocytes (< 200 cells) at the initial observation of 15 minutes due to a technical error, then the duplicate slide can be utilised as a "back-up" or "replacement" slide.

5.5 Determination of Neutral Red Retention Time

Samples are analysed under the microscope following 15, 30, 60, 90, 120, 150 and 180 minutes incubation. Neutral red retention time (NRRT) will correspond to the last time period recorded when there was no evidence of dye loss or lysosomal abnormalities in more than 50% of the cells (Figure 7 and 8; Annex 2), based on either a visual or a digital photographic determination. Elongated granulocytes can be considered healthy in terms of the NRR assay if they contain normal size lysosomes and they are able to retain the NR dye inside the lysosomes.
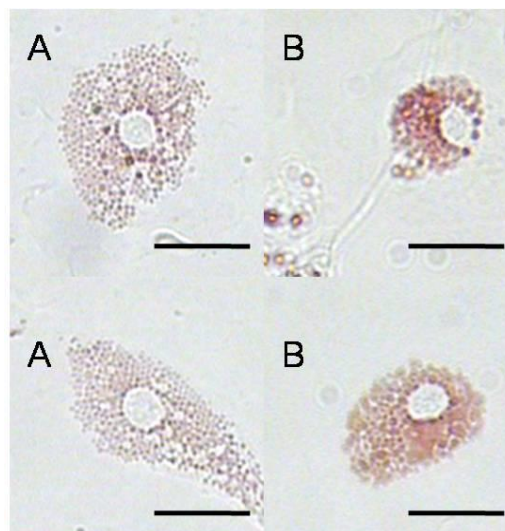

Figure 7. Mussel granulocyte cells (haemocytes) showing uptake of neutral red in lysosomes. A) Healthy cells showing the retention of neutral red within the lysosomal compartment. B) Stressed cells showing loss of neutral red into the cytosol. (Scale Bar ≡ 5 μm).

In order to minimize the length of time the cells are exposed to light under the microscope, it is possible only to make a visual estimate of the condition of the lysosomes. As this approach is potentially open to bias, it is recommended that samples are read "blind", i.e. the analyst has no prior information about the samples. A typical blood sample of 50 µl generates approximately 20 fields of view of attached cells. By quickly raster scanning the preparation under the microscope it is possible, with practice, to obtain a visual estimate of the condition of the lysosomes for the entire sample in one minute or less. The number of blood cells in individual mussels is highly variable and a field of view, using a ×25 objective lens, may contain between 20 and 50 cells; therefore, the analysis assesses the lysosomal membrane status on between 400 and 1000 cells.

Another procedure, that would be appropriate for certain types of studies and which is used in some laboratories, is to photograph fields of view, using a digital camera, and then make detailed counts of cells exhibiting dye loss at a later point in time. However, this operational procedure has drawbacks when used within the framework of biomonitoring programmes because: (1) requires the use of control samples each time a set of study samples are tested, (2) removes the capability for real-time results and (3) limits the number of cells that can be screened at one time (as each photograph normally covers 4–8 granulocytes).

5.6 Determination of percentage of lysosomal membrane stability: scoring procedure.

For additional sensitivity of the NRR assay, a scoring procedure has been developed. This procedure is alsobasedon neutral red retention time and lysosomal alterations and allows to calculate the percentage of LMS. The numerous lysosomal alteration types are illustrated in Figure 8. Briefly, samples are analysed under the microscope and scored at 15, 30, 60, 90 and 120 minutes incubation for evidence of 50% or greater of the cells exhibiting the pathologies below which are listed in increasing severity of effect.

Pathology	score
No effect	0
Enlargement but no leakage	1
Leakage but no enlargement	2
Leakage and enlargement	3
Leakage and enlarged but colourless lysosomes	4
Rounded up fragmenting cells	5

Experience using NRR assay in mussels from natural populations shows that three main case-types of samples occur:

- **Case 1**: Samples that demonstrate a predominance of small granulocytes retaining NR in their lysosomes at the first microscope observation (score 0). This type of sample usually evolves toward: i) stationary situation (Score 0); ii) swelling or enlargement of the lysosomal compartment (score 1 or 3); iii) leakage of NR into the cytosol followed by a rounding process of the cells (score 2 and 5).

- **Case 2**: Samples containing a predominance of big granulocytes (usually demonstrating different sizes at the first microscope observation (score 1). This kind of sample normally evolves toward an enhanced staining and/or enlargement of the lysosomal compartment with different strength of coloration in their lysosomes (score 3 and 4) but not towards a general leakage of NR into the cytosol (score 2 and 5).
- **Case 3**: Samples that demonstrate a predominance of small stainless granulocytes in their lysosomes at the first microscope observation (score 4). These types of samples normally remain in this condition over the duration of the test, though in some cases they evolve toward an enlargement of the lysosomal compartment.
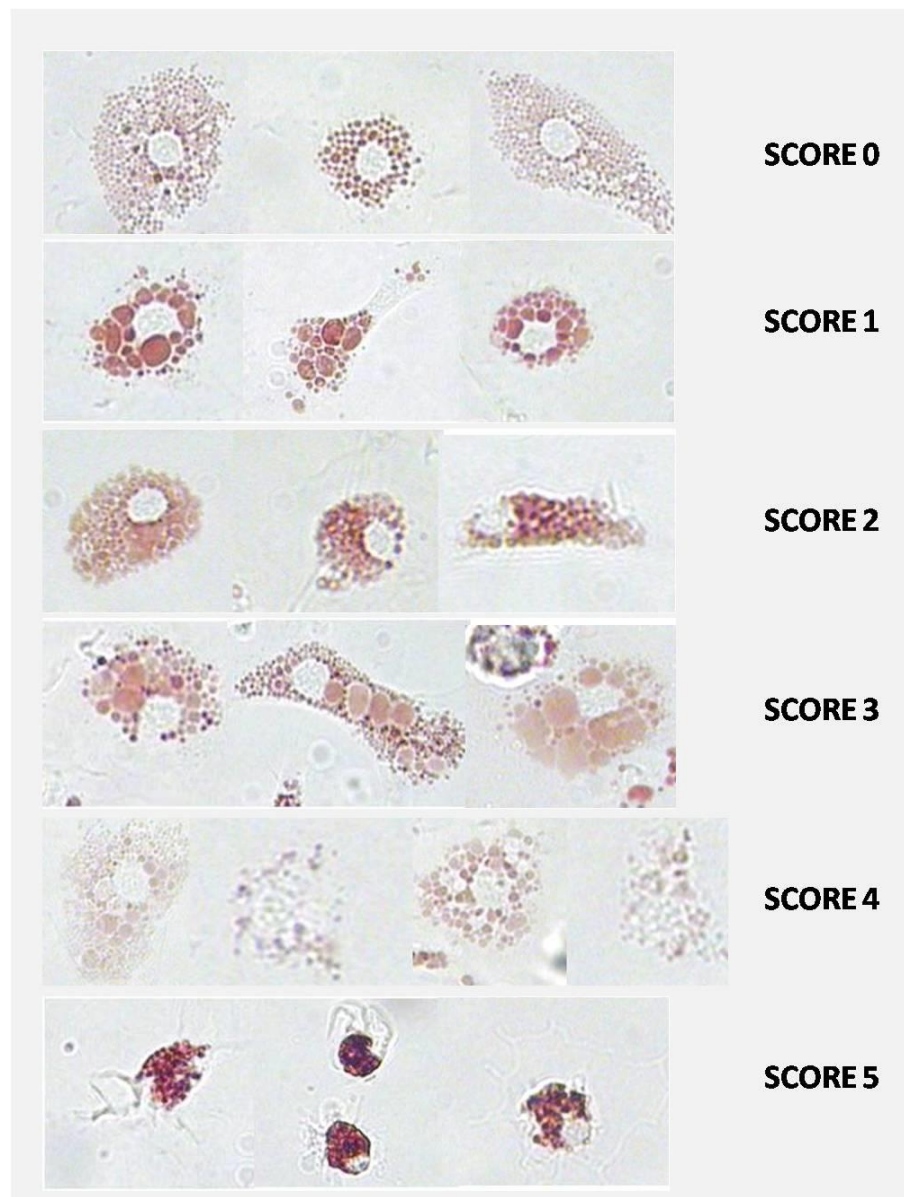

Figure 8. Illustrations of granulocytes (*M. galloprovincialis*) exhibiting different pathologies and associated scores: Score 0= no effects; Score 1= enlargement but no leakage; Score 2= leakage but no enlargement; Score 3= leakage and enlargement; Score 4= leakage and enlarged but colourless lysosomes; Score 5= rounded up fragmenting cells.

5.7   Data recording

As mentioned before, two types of data can be recorded when assessing LMS at each observation time using the NRR assay:

- Neutral red retention (NRR): Plus (+) or negative (-) sign.

- Score of the lysosomal state (figure 8)

If more than 50% of the cells show a clear cytosol and there is no evidence of lysosomal abnormalities (score =0), then a plus sign (+) should be recorded in the appropriate box of Table 1. If there is evidence of dye loss and/or lysosomal abnormalities in more than 50% of the cells (score >0), then a negative sign (-) should be recorded. The NRRT is the last time period when the lysosomal state is a plus sign (+).

If more than 50% of the cells show a clear cytosol and there is no evidence of lysosomal abnormalities (score =0), then a plus sign (+) should be recorded in the appropriate box of Table 1. If there is evidence of dye loss and/or lysosomal abnormalities in more than 50% of the cells (score >1), then a negative sign (-) should be recorded. The NRRT is the last time period when the lysosomal state is a plus sign (+) (see table 1) The test must be truncated at maximum 180 minutes, since for most healthy animals the neutral red itself becomes a toxic stress factor, regardless of the previous contaminant history of the mussels under study.

When evidence of 50% or greater of the cells exhibit no effect or one of the 5 pathological conditions described above (figure 8), then its corresponding score will be recorded in the appropriate box. The weighted score is consequently calculated by multiplying the score by the weighting factor for that time period. The scoring is truncated at 120 minutes. The total final score for the lysosomal condition is calculated as

% LMS = (1-(sum of weighted score/75))*100.

6   Interpretation of results

For assessment purposes, neutral red retention time (NRRT; min) or lysosomal labilisation period (LP; min) should be assessed against the background assessment criteria (BAC) and environmental assessment criteria (EAC) developed for the technique (Davies *et al.*, 2012) . NRRT or LP shorter than the EAC level suggests the mussel sampled are severely stressed and probably exhibiting pathology. NRRT or LP shorter than the BAC level but longer than the EAC level is considered to represent stressed but compensating organism (figure 9).
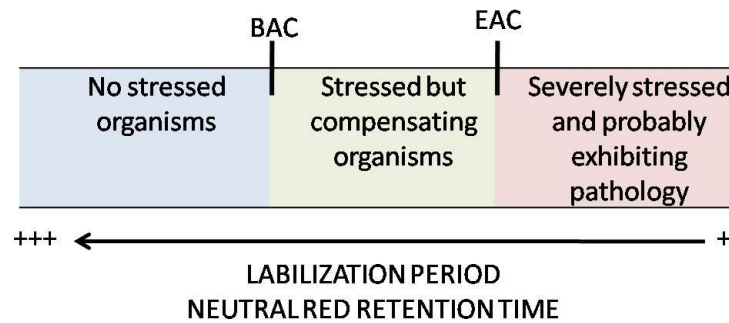

Figure 9. Lysosomal membrane stability (measured as labilization period or neutral red retention time) decrease as the animal is stressed.

Assessment criteria (BAC and EAC) of %LMS data have not been established due to the absence of the necessary number of data from different field studies to validate them effectively. The % LMS data generated alongside NRRT will lead to the generation of its corresponding assessment criteria in near future (figure 10).

Table 1. Example of fact sheet of Neutral Red Retention Assay results

Sampling site: Cartagena   Specie: *M. galloprovincialis*   Code site: 4139
Sampling date: 15-04-2013   Temp SW: 18ºC   Salinity SW: 37.8 PSU
Analysis date: 16-04-2013   Analyst: Navarro C.

Time period	15			30			60			90			120			150	180	Sum of weighted scores	%LMS	NRRT
Weighting factor	1			2			3			4			5							
Slide nº	NRR	Score	Wtd score	NRR	Score	Wtd score	NRR	Score	Wtd score	NRR	Score	Wtd score	NRR	Score	Wtd score	NRR	NRR		$(1-(WS/75))*100$	
1	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0	-	-	0	100	120
2	+	0	0	-	2	4	-	2	6	-	5	20	-	5	25	-	-	55	27	15
3	+	0	0	+	0	0	-	1	3	-	1	4	-	3	15	-	-	22	71	30
4	+	0	0	+	0	0	+	0	0	-	2	8	-	5	25	-	-	33	56	60
5	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0	+	-	0	100	150
6	+	0	0	+	0	0	+	0	0	+	0	0	-	2	10	-	-	10	87	90
7	-	4	4	-	4	8	-	5	12	-	5	16	-	5	20	-	-	60	20	0
8	+	0	0	+	0	0	+	0	0	-	3	12	-	5	25	-	-	37	51	60
9	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0	+	+	0	100	180
10	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0	-	-	0	100	120

Table 2: Background assessment criteria (BAC) and environmental assessment criteria (EAC) for LMS measurements (Davies *et al.*, 2012)

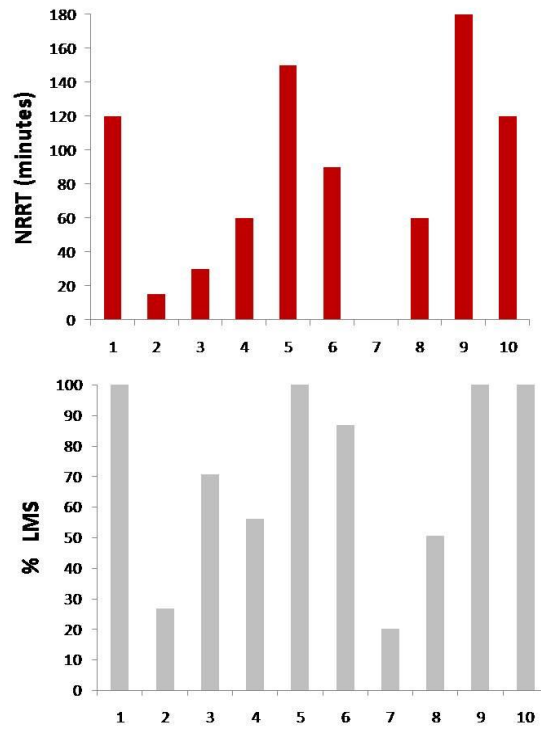| LYSOSOMAL STABILITY; MINUTES	BAC	EAC
LP (labilisation period)	20	10
NRRT (neutral red retention time)	120	50

Figure 10. Example of lysosomal membrane stability (LMS) results expressed as Neutral Red Retention Time (NRRT; minutes) and percentage of lysosomal damage (% LMS) in same specimens.

7   Quality assurance

At present QA for these assays is provided through collaboration among the laboratories conducting these assays, including workshops and ring trials. When organising a QA /QC exercises, all tests and determinations should be carried out by trained staff working to defined protocols. Any deviations from the protocols should be recorded and assessed by the laboratory manager for their potential to influence the results.

For the enzyme cytochemical technique frozen tissue samples can be used both for internal QA as Laboratory Reference Materials (LRM) and also distributed to other laboratories for external QA purposes. Frozen (quenched) tissues should be prepared for the intercomparison of the cytochemical lysosomal stability test (laboratory reference materials). The test will be performed in the lead laboratory and the frozen tissues will be sent to the participating laboratories for them to perform the test. Repeated measurements from LRMs produced by a lead laboratory will be used to monitor differences of interpretation between analysts. All samples should be coded and the test performed and assessed as a double-blind exercise. This will involve the results being returned to a second laboratory for the compilation of the data.

For the NRR assay, live mussels from the same sources can be distributed for external QA, workshops or on-line workshops, involving multiple participants, can be conducted to provide external QA data on the same samples. During the workshops, consecutive projection of images for short time periods (5-8 seconds) can also be used to assess the ability of the participants to interpret and score samples. There is considerable potential for virtual slide intercomparison to be used in the context of the NRR assay. The benefit of this approach is that all participants would be analysing the same samples from the same mussels meaning that results are directly comparable. The disadvantage of this approach is that the intercomparisson would only assess the ability to interpret results and not test the ability for the participant to undertake the assay itself.

NOTE: According to the principle of the neutral red assay, S1 and S2 should not significantly differ if S1 has been quickly scanned (≤1 min) and the light intensity has been kept as low as possible during each time observation. Therefore, S2 can be used for training purposes and for internal QA as follows:

a) Scanning S2 immediately after S1

b) Scanning S2 once the dye is lost to the cytosol and/or abnormalities in lysosomal size and colour is observed in at least 50% of the cells of the S1.

Even though the NRR assay is a semi-quantitative method, significant differences of LMS values between S1 and S2 (i.e. variation coefficient > 50%) should be interpreted as a low quality assurance of the analysis.

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Annex 1: overview of mussel haemocytes

Definition of the number of haemocytes cell types and their nomenclature in mussels is still a subject of debate. A good number of authors (Moore and Lowe, 1977; Rasmussen *et al.*, 1985; Renwrantz, 1990; Noël *et al.*, 1993; Cajaraville and Pal, 1995; Cajaraville *et al.*, 1997) have described two types of haemocytes in *M. edulis* and *M. galloprovincialis*:

1. Hyalinocytes are smaller than granulocytes and have large nuclei and reduced basophilic cytoplasm, generally without granules. Hyalinocytes are generally less abundant than granulocytes in haemolymph and have a limited ability to spread on glass slides, thus demonstrating a round shape (cell diameter 8.73 ± 0.54 μm; nucleus diameter 5.27 ± 0.27 μm).
2. Granulocytes display less basophilia and spread more than cytoplasm of hyalinocytes on glass slides. Ectoplasm and endoplasm can be easily distinguished. Granulocytes are more abundant in haemolymph than hyalinocytes. Some granulocytes present abundant acidophilic/eosinophilic granules (AG) (cell diameter 49.08 ± 0.70 μm; nucleus diameter 6.86 ± 0.09 μm). These cells have the largest size and the highest density in the mussel haemolymph. Other granulocytes present only basophilic granules (BG) (cell diameter 43.70 ± 0.0.91 μm; nucleus diameter 7.71 ± 0.12 μm). A few granulocytes contain both types of granules (BAG) (cell diameter 44.70 ± 1.34 μm; nucleus diameter 7.54 ± 0.13 μm).

On the basis of light microscopy, Moore and Lowe (1977) subdivided the granular cells in *M. edulis* into basophilic (or macrophages) and eosinophilic granulocytes. Pipe (1990) separated the granulocytes of the mussel *M. edulis* in granulocytes with smaller granules (0.2 to 0.3 μm) and granulocytes with large granules (0.5 to 1.5 μm). Nöel *et al.* (1994) demonstrated that the basophilic granulocytes of *M. edulis* observed by light microscopy correspond to the granulocytes with small granules and the acidophilic/eosinophilic granulocytes correspond to the granulocytes with large granules. Carballal *et al.*, (1997) identified hyalinocytes and three different types of granulocytes according to the staining properties of their granules in *M. galloprovincialis*. Moore and Lowe (1977) and Nöel *et al.* (1994) suggested that different granulocytes are maturing stages within a single cell line. Cheng (1981) proposed that granulocytes with large granules are the most mature cells, and probably arise from granulocytes with small granules. Cheng (1981) also proposed an ontogenic model with two cell lines, one for hyalinocytes and another for granulocytes, each originating in a different prohemocyte. For some other authors (Ottaviani *et al.*, 1998), differences in cytology and number of haemocytes were thought to be due to cellular aging and that only one cell type in two different stages, young or old, is present in haemolymph in mussels, being observed in various forms at various stages of maturation, rather than different cell types.

An experimental study demonstrated that granulocytes in control animals typically appeared with the characteristic pseudopodial projection and that treated animal granulocytes clearly demonstrated an increase in their dimensions, cell rounding and a substantial loss of pseudopods (Calisi *et al.*, 2008).

When undertaking the NRR assay, there are occasions where the presence of enlarged lysosomes increases. Initially these can appear as numerous and slightly enlarged compared with those lysosomes that are considered normal. As the assay progresses, the frequency of these lysosomes decreases, however the general size increases. The result is fewer enlarged lysosomes as those shown in Figure 8. Lyso-

somes may undergo fusion when LMS is compromised which may manifest as these enlarged lysosomes.

Overall, two main types of granulocytes (Type 1 and Type 2) can be found in most of the observations (Table 3).

Table 3. Gross comparative characteristics of granulocytes in mussels.

GRANULOCYTES IN MUSSELS	TYPE 1	TYPE 2
Cell diameter		+
Number of lysosomes	++	+
Size of lysosomes	small	big
Nucleus diameter	+	++

(++) Higher/bigger than (+); (+) Smaller/lower than (++)
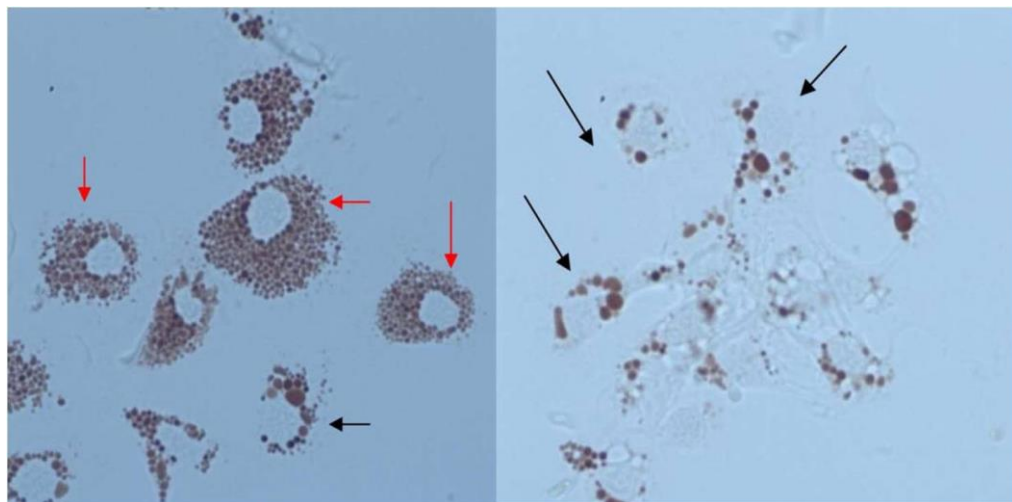
Figure 11. Main type of granulocyte cells frequently observed in haemolymph samples from *Mytilus* sp. from natural populations. Red arrow = type 1; black arrow = type 2.

It has been reported that the cellular composition of the haemolymph is different in impacted animals to that of clean animals. Dolcetti and Venier (2002) found significant increases in granular cells in the haemolymph and gills when coupled comparison between two industrial sites and the related reference site were tested. Furthermore, they found that the granular cell fraction in gills and haemolymphwas substantially lower in the reference site than at the industrial sites. This is supported by the evidence of an increase in lysosomal size, suggesting an enhanced rate of fusion of primary lysosomes with auto/hetero phagosomes. For pollutant-exposed cells, lyso-

somal enlargement and swelling usually indicates increased autophagy, which may greatly contribute to induction of cell dysfunctions, potentially leading to cell death.

Annex 2: Illustrated guidance for granulocyte pathology interpretation
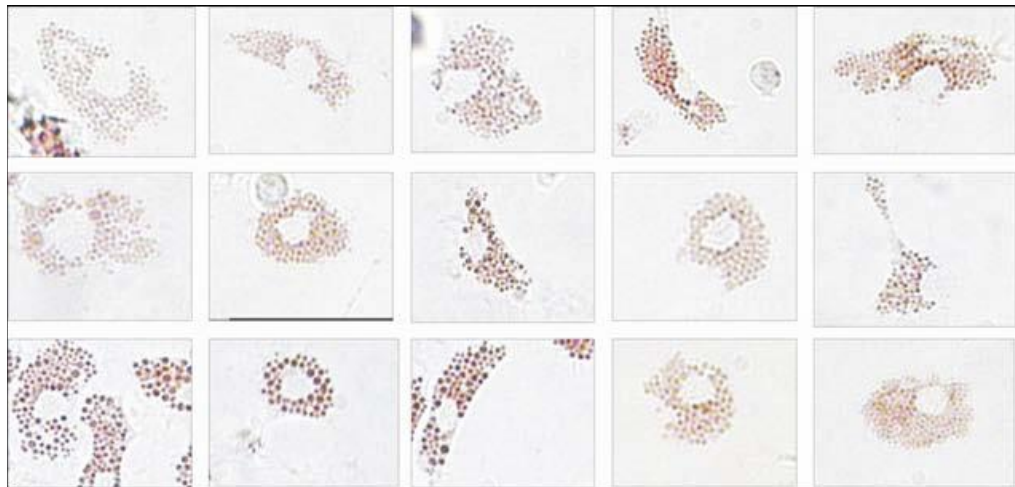
Image 1. Examples of normal granulocytes observed in *M. edulis* and *M. galloprovincialis* with small lysosomes. Neutral red is well retained inside the lysosomes (score 0).
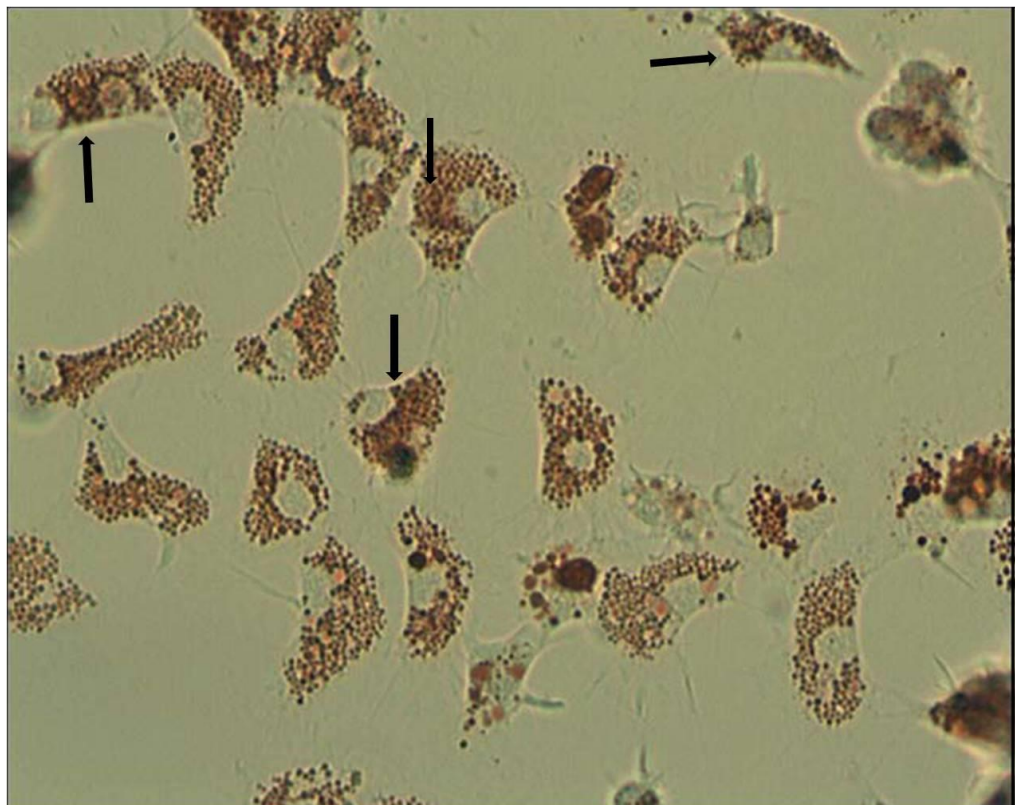

Image 2. Example of a haemolymph sample (x600) in which more than 50% of the granulocytes retain NR dye inside the lysosomes. (*Mytilusgalloprovincialis* sampled in Spanish Mediterranean Waters). In this image pseudopods are clearly well developed in most of the granulocytes. Arrow indicates cells showing evidence of leakage of NR into the cytosol but no enlargement (Score = 2).
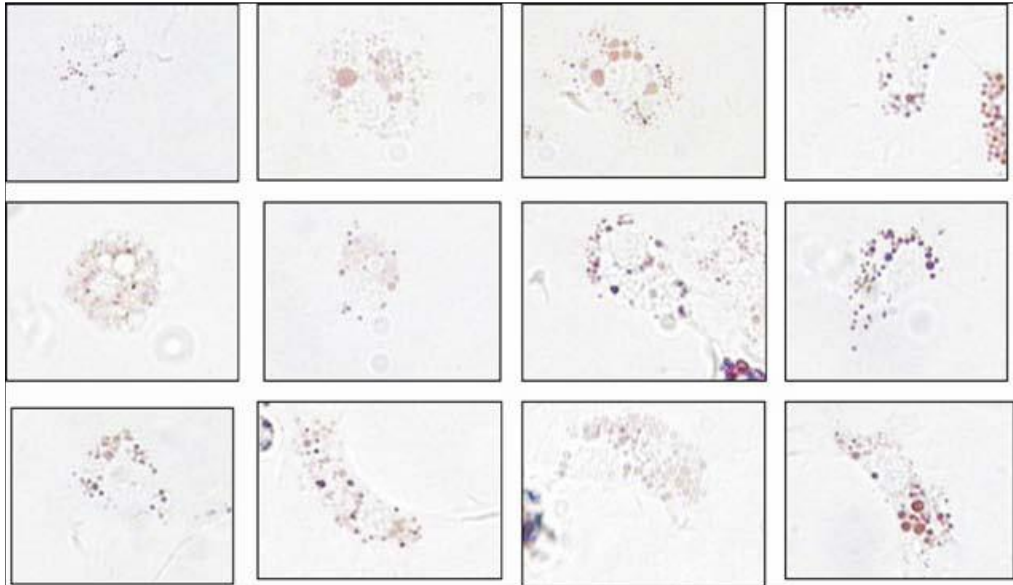
Image 3. Examples of granulocytes observed in *M. edulis* and *M. galloprovincialis* with small and enlarged but colourless lysosomes (score 4). Neutral red is not retained inside the lysosomes.

Image 4. Typical sequential process observed by using NRR assay. Rounding-up of the cells (score 5) usually follows the leakage of neutral red into the cytosol (score 2) of the granulocytes with small lysosomes.
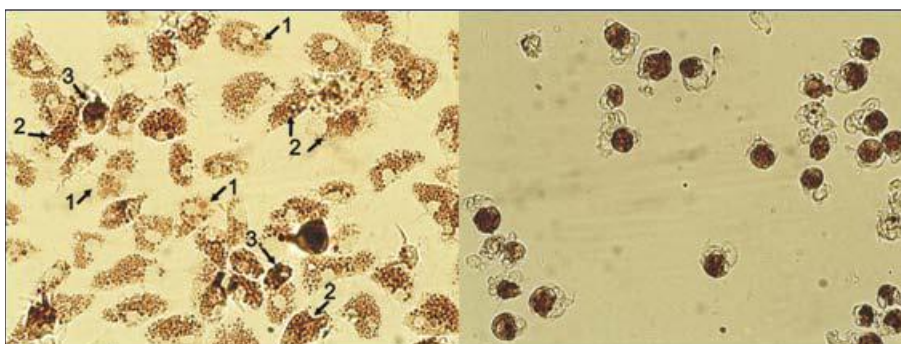
Image 5. Example of a haemolymph sample that demonstrated a predominance of granulocytes with small lysosomes retaining NR at the first microscope observation. This sample usually evolved in time toward swelling or enlargement of the lysosomal compartment, leakage of NR into the cytosol, followed in time by a rounding process of the cells.
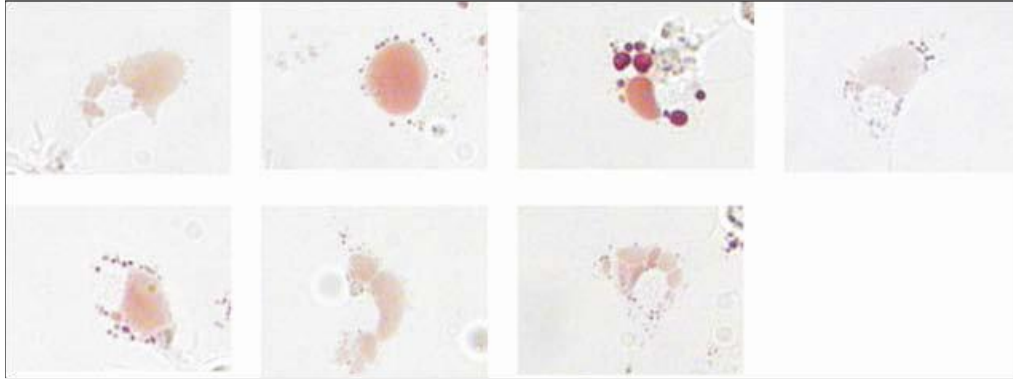
Image 6. Haemocytes showing lysosomal vacuolation, which generally occurs much later (e.g. 90 minutes) into the incubation, although once again in severely impacted animals is evident within the first 15–30 minutes of incubation (score 3).
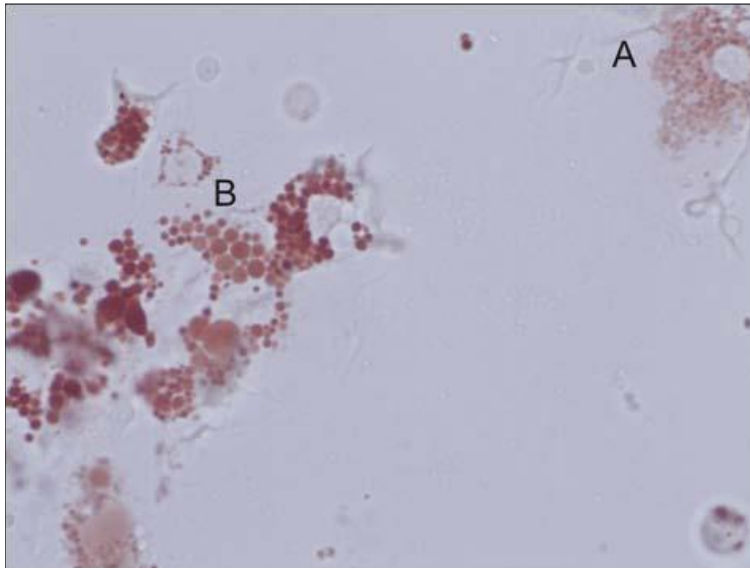

Image 7. Example of granulocyte with small lysosomes (A; score 2) and granulocyte showing lysosomal swelling and enlarged lysosomes (B; score 1).*M. galloprovincialis* from and Italian sampling site.
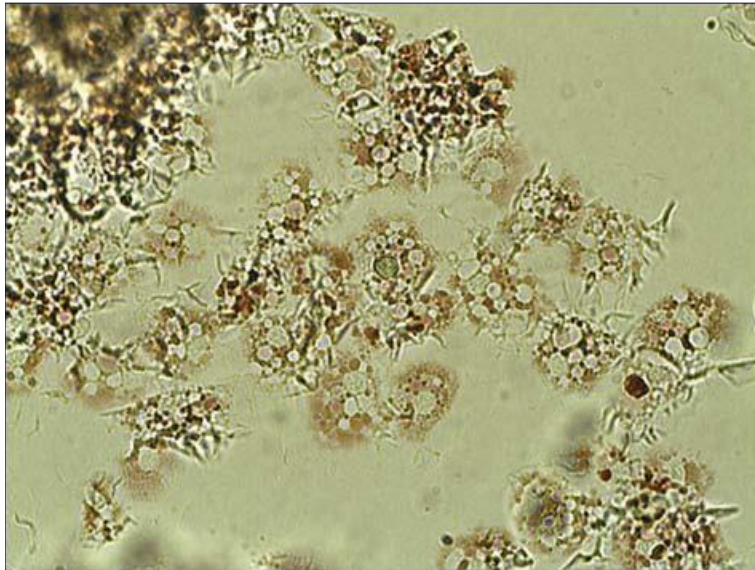
Image 8. Example of granulocytes with enlarged and colourless lysosomes (score 4). *M. edulis* **from Wadden Sea (The Netherlands) sampling site.**
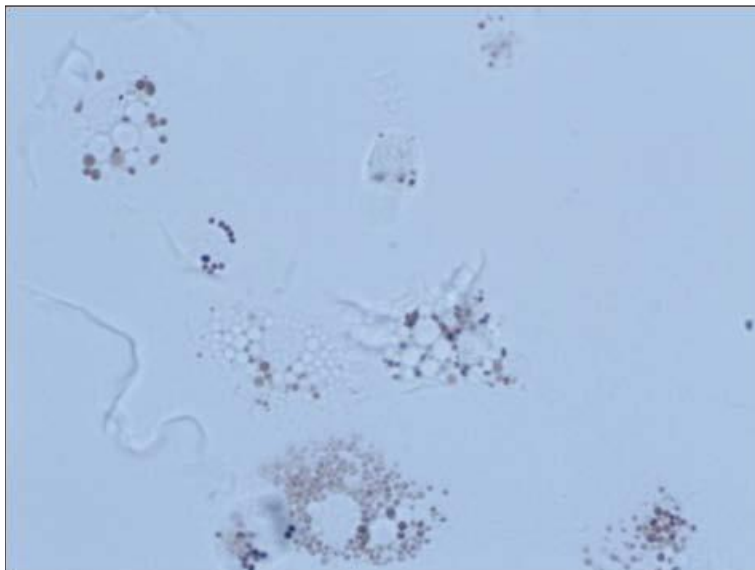
Image 9. Example of granulocytes with big vacuoles (colourless lysosomes) (score 4). *M. galloprovincialis* **from a Mediterranean Spanish sampling site.**
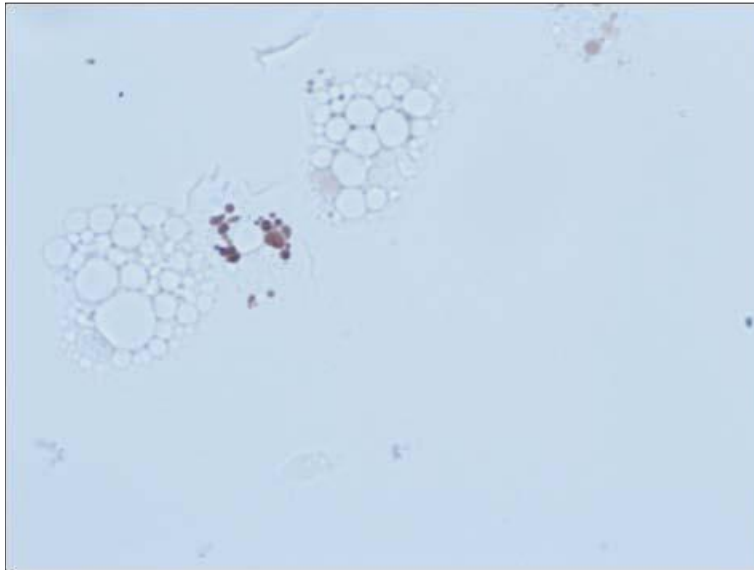
Image 10. Example of granulocytes full of vacuoles (lipid droplets or colourless lysosomes). *M. galloprovincialis* **from a Mediterranean Spanish sampling site.**
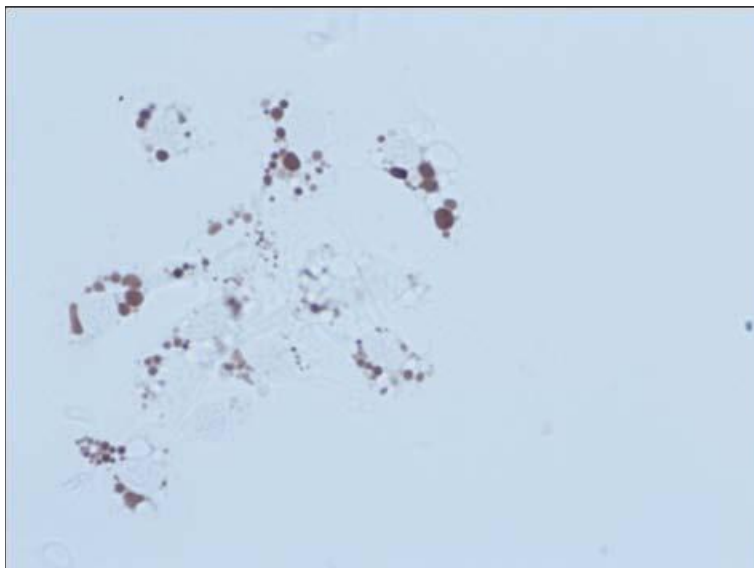

Image 11. Example of granulocytes retaining NR dye inside the lysosomes but showing some enlarged colourless lysosomes and small number of lysosomes with different staining colour (score 4). *M. galloprovincialis* **from a Mediterranean Spanish sampling site.**
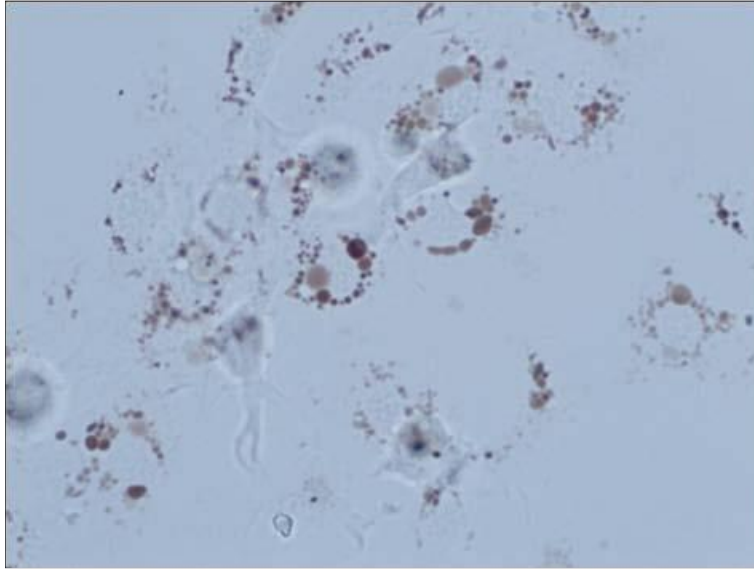
Image 12. Example of granulocytes retaining NR dye inside the lysosomes but showing vacuoles and low number of lysosomes with different staining colour. *M. galloprovincialis* **from a Mediterranean Spanish sampling site.**
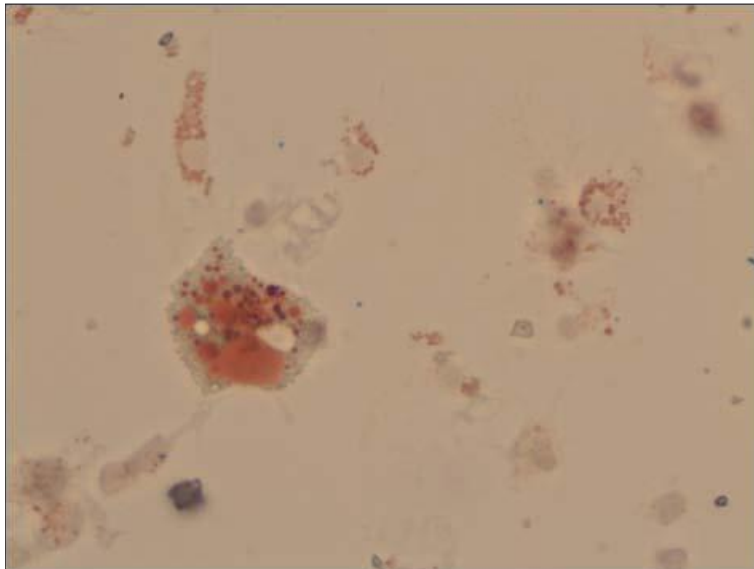

Image 13. Example of a macrogranulocyte found in a sample of haemolymph in *M. gallopro-vincialis* **from a Mediterranean Spanish sampling site. These cells are considered to be the result of a fusion of granulocytes in some pathological conditions or rejection of grafts. Multinucleate giant haemocyte have been reported in** *M. galloprovincialis* **heavily infected by a trematode,** *Proctoeces maculates* **(Carballal** *et al.***, 1997).**

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