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Biological effects of contaminants:
Measurement of lysosomal membrane stability

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Abstract

Lysosomes are ubiquitous cellular organelles that provide a waste disposal and macromolecular recycling system (autophagy) and also a membrane-bound compartment for intracellular digestion of food ingested by the cells. They accumulate many toxic metals and organic chemical contaminants, providing an evolutionarily primitive detoxication capacity, which if overloaded results in lysosomal damage leading to cell injury, tissue dysfunction, and reduction in animal "health status". Major reactions of lysosomes to pollutants include loss of membrane integrity, enlargement associated with autophagy, and accumulation of lipid and lipofuscin (age-pigment). These types of responses have been widely used to test for the effects of toxic contaminants in both experimental investigations and environmental impact assessments. Several methods are available to measure lysosomal functional status: these include measurement of lysosomal membrane stability in both frozen tissue sections and live cells. Protocols for the implementation of these methods are described here in practical detail for mussel/molluscan digestive gland or hepatopancreas and flatfish liver. Cytochemically determined latency of selected lysosomal marker enzymes is used as the measure of stability in frozen sections, and retention time of the chromogenic dye neutral red, as the measure of lysosomal integrity in live cells. Guidelines are included for sample handling, data analysis, and interpretation of results.

Keywords: lysosomes, metals, organic chemical contaminants, autophagy, lysosomal membrane stability, neutral red retention, lipofuscin, mussels, digestive gland, flatfish, liver.

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1   INTRODUCTION AND RATIONALE

Much of the waste generated by human activities finds its way into the oceans where some of it may present a threat to marine life and possibly to man as a consumer of seafood (Goldberg *et al.*, 1978; Moore, 2002, Moore *et al.*, 2004). An approach to the question of whether marine organisms and ecosystems are being endangered has involved the development and deployment of indices of biological effects as early warning systems of adverse environmental change (Moore, 1990, 2002; Moore *et al.*, 1987). Such methods are being used in combination with analytical chemistry on a rapidly increasing basis and on a world-wide scale (Bayne *et al.*, 1988).

The organisms of choice for this type of environmental monitoring have frequently been sedentary filter-feeding molluscs such as mussels and oysters (Lowe and Fossato, 2000). These invertebrates accumulate chemical contaminants from the sea water, resuspended sediment, and particulate food material filtered from the water. The flatfish species, dab and flounder, are the main target species for monitoring purposes in the North Sea and adjacent areas including the Baltic Sea since these species are benthic and abundant. Because they are bottom-dwelling, these flatfish species accumulate chemicals from sediments via food, skin, and gills (Bucke *et al.*, 1996).

The tissue concentrations of many environmental xenobiotics can reach very high levels, thus making both animal groups useful tools for chemical monitoring. Mussels, in particular, appear to be relatively tolerant to many metals and organic xenobiotics. This tolerance, however, does not mean that the animals are unresponsive; in fact, there is considerable evidence for pathological reactions to even low concentrations of contaminants. Such pathological reactions have been described at all levels of biological organization, ranging from the molecular to the physiology of the whole animal (Livingstone, 1988; Moore, 1988a; Stegeman and Lech, 1991; Widdows and Johnson, 1988). In flatfish species, progressive toxic liver lesions and neoplastic changes have been reported. These include foci of altered hepatocytes, adenomas and carcinomas of hepatocellular, biliary, and endothelial origin based on histopathological diagnosis (Vethaak and Wester, 1996; Köhler *et al.*, 1992, 2002; Wahl *et al.*, 1995; Feist *et al.*, 2004).

At the cellular level, the lysosomal system has been identified as a particular target for the toxic effects of many contaminants. Pathological alterations in lysosomes have been especially useful in the identification of adverse environmental impact, as many of the tissues in molluscs are extremely rich in lysosomes. Lysosomal reactions fall into essentially three categories: changes in lysosomal contents, changes in fusion events, and changes in membrane permeability (Hawkins, 1980; Moore, 1988a, 1990). The major response to contaminant stress of both molluscan and fish lysosomes appears to involve enhanced autophagy and the current evidence suggests that this is an evolutionarily conserved response to environmental stress (Bursch, 2001; Klionsky and Emr, 2000; Kirchin *et al.*, 1992; Köhler, 1989a, 1989b, 1991; Köhler *et al.*, 1992; Lowe, 1988; Lowe and Fossato, 2000; Lowe *et al.*, 1992, 1995a; Moore, 1985, 1990; Winston *et al.*, 1991, 1996). 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, 1982, 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 and fish (Köhler *et al.*, 1992; Lowe, 1988; Lowe and Fossato, 2000; Moore *et al.*, 1986, 1996a, 1996b; Moore, 1988a; Nott and Moore, 1987). In addition to these autophagic changes, molluscan lysosomes often have a considerable propensity to sequester and accumulate metals and lipophilic xenobiotics, and this is particularly evident in the

epithelial cells of the digestive gland (Moore *et al.*, 1987). In the mussel, this organ serves as the major site of intracellular digestion in the animal and, as such, is the main interface between the organism and its environment (Moore, 1990). In fish, the liver is the main organ of food conversion, detoxification, and biotransformation of xenobiotics, as is the case in mammals (Köhler *et al.*, 1992; Moore *et al.*, 1994). In addition, yolk-precursor proteins are synthesized in fish liver and reduced reproductive success is a probable consequence in the case of toxic liver injury.

2 LYSOSOMAL REACTIONS

Cytochemistry and histochemistry have been used as the main tools in the study of environmentally induced alterations in lysosomes for several reasons (Moore, 1990; Moore and Simpson, 1992). Frequently, the tissue samples are very small and cytochemistry lends itself well to dealing with this problem. In addition, there is a requirement to be able to relate functional changes in the tissues and cells to alterations in their structure; once again, cytochemistry is highly appropriate in this context. The cytochemical tests used in these investigations involved procedures for lysosomal hydrolases, lipofuscin, and lipid (Moore, 1988a; Figure 1). Another advantage of the cytochemical approach is that changes can be detected in particular target cells and lesion types, thus potentially increasing the sensitivity by many orders of magnitude as compared with the more disruptive analytical procedures involving homogenization and cell fractionation.

When marine molluscs, such as mussels, are exposed to contaminant chemicals, the lysosomes in the digestive gland epithelial cells show fairly rapid and characteristic pathological alterations (Lowe, 1988; Moore, 1988a, 1990). These include swelling of the digestive cell lysosomes (Figures 1.1 and 1.2), increased fragility of the lysosomal membrane, excessive build-up of unsaturated neutral lipid (lipidosis) in the lysosomal compartment (Figures 1.3 and 1.4), and accumulation of lipofuscin (lipofuscinosis) (Figures 1.5 and 1.6). These changes are accompanied by atrophy of the digestive epithelium, apparently involving augmented autophagic processes, although there is also evidence of increased cell deletion (analogous to apoptosis in mammals) and the relationship between the two processes, if any, is unclear (Lowe, 1988; Pipe and Moore, 1988). For instance, do the autophagic-type changes predispose the cells to deletion by programmed cell death? Programmed cell death (PCD) is divided into apoptosis (PCD Type I) and autophagy prominent cell death (PCD Type II): autophagic cell death appears to be a phylogenetically ancient phenomenon and occurs in both physiological and disease states (Bursch, 2001; Zhao *et al.*, 2001). Lysosomal changes are involved in both types of cell death and they should not be considered as mutually exclusive processes (Bursch, 2001).

Linked biochemical and cytochemical investigations have demonstrated that increased fragility of the lysosomes, induced by phenanthrene, corresponds directly with increased catabolism of cytosolic proteins (Moore and Viarengo, 1987; Viarengo *et al.*, 1992).

Experimental studies have clearly demonstrated that the lysosomal alterations described above can be induced by single toxicants such as copper (not lipidosis) and polycyclic aromatic hydrocarbons (Nott *et al.*, 1985; Viarengo *et al.*, 1985).

In fish liver, lysosomal changes comprise membrane fragility, enlargement, and accumulation of lipids (unsaturated neutral lipids, phospholipids) and lipofuscin. These changes are closely linked to toxico-pathological alterations of the liver and have clear prognostic value for cell death; they are also correlated to concentrations of lipophilic compounds and some heavy metals such as cadmium (Köhler *et al.*, 2002). Interestingly, lysosomal membrane stability

Figure 1. Cryostat sections (10 μm) of unfixed hexane-quenched (−70 °C) digestive gland of the marine mussel sampled from clean and contaminated sites. 1) Normal appearance of the digestive tubules showing lysosomes reacted for N-acetyl-β-hexosaminidase in the digestive cells (clean site). 2) Abnormally enlarged lysosomes reacted as in 1 (contaminated site). 3) Lipid droplets localized in digestive cells using oil red-O (clean site). 4) Unsaturated neutral lipid accumulation in pathologically enlarged lysosomes, together with a general increase in lipid droplets, reacted as in 3 (contaminated site). 5) Lipofuscin in secondary and tertiary lysosomes localized using the Schmorl reaction (clean site). 6) Enhanced lipofuscin content (contaminated site). Adapted from Moore (1988a). (Scale bar ≡ 20 μm).
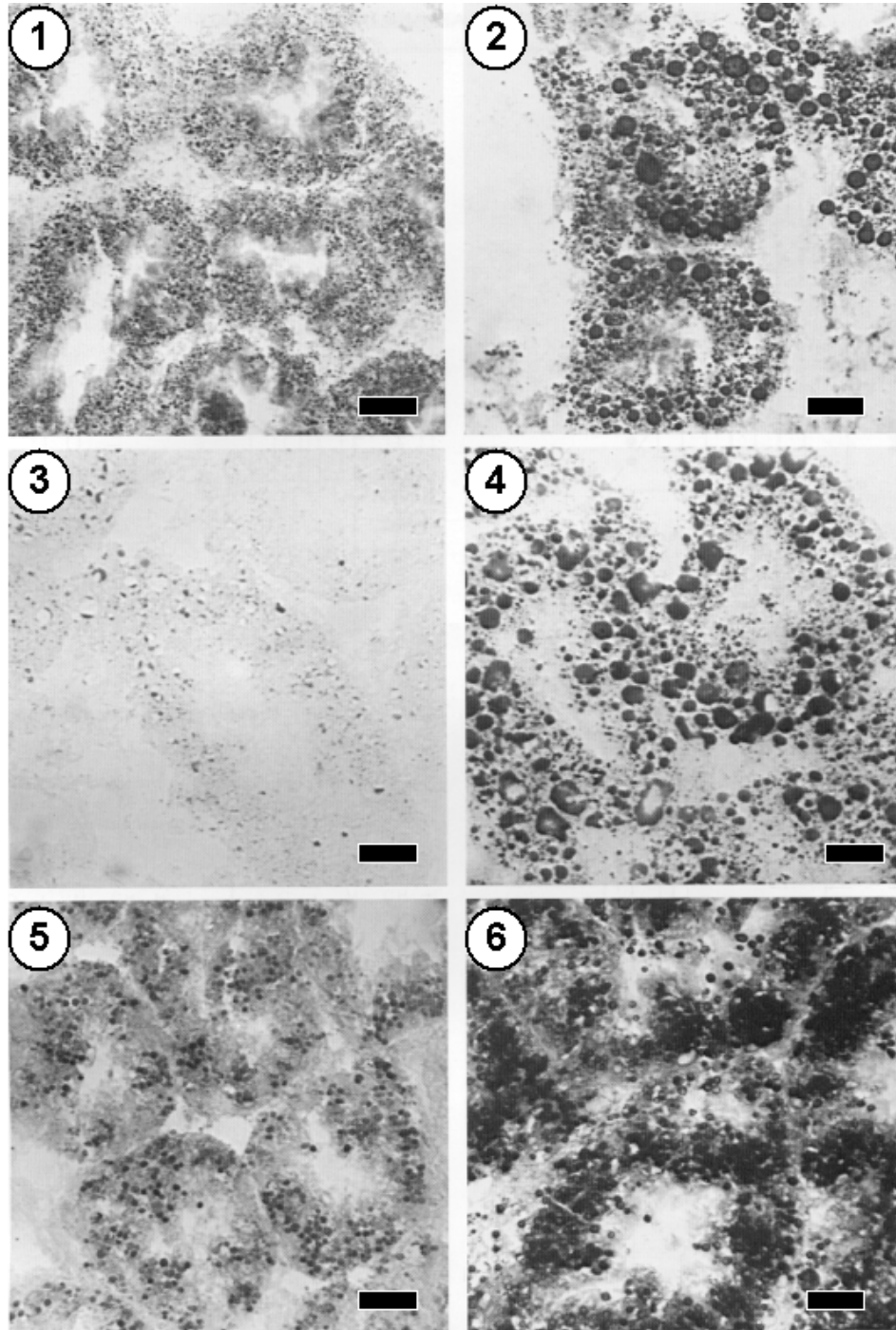
breakdown coincides with induction of cytochrome P-450 (CYP1A1) (Köhler and Pluta, 1995). It is likely that reactive free-radicals (oxygen species and xenobiotic derivatives) produced during biotransformation contribute to the damaging effects on the lysosomal membrane and build-up of lipofuscin (Kirchin *et al.*, 1992; Winston *et al.*, 1991, 1996). Lipofuscin is an end product of oxidative attack on lipids and proteins and is also an indicator of autophagy.

At first glance, these findings are perhaps surprising given that many thousands of individual toxic chemicals are often present in a contaminated situation. Lysosomal destabilization is essentially very generalized and can also be induced by non-chemical stressors such as hypoxia, hyperthermia, osmotic shock, dietary depletion, and various combinations of these (Moore, 1985). Consequently, it would appear that many adverse conditions are capable of inducing autophagic-type changes, and that this non-specificity of the lysosomal reactions is only of value as a general indicator of deterioration in the health of the animal. However, differences in the lysosomal response can be used to identify the causative agency. Specifically, patterns of lysosomal change can be used to distinguish between the effects induced by lipophilic organic xenobiotics, metals, and non-chemical stressors. These include lysosomal swelling and lipid accumulation induced by lipophilic xenobiotics but not by metals, and accumulation of metallothionein in lysosomes induced by particular metals (Köhler *et al.*, 2002; Moore, 1988a, 1990; Viarengo *et al.*, 1985). Considered as a package, the use of cytochemical tests as subcellular pathological probes can provide relatively specific information (Moore, 1990).

The types of cytochemical tests described above have been used in a range of environmental situations. The more widely used tests have been those for lysosomal membrane fragility; this has been applied to both molluscan and fish species and is based on either the demonstration of latency of lysosomal hydrolases or the retention of the amphiphilic cationic dyes such as neutral red and acridine orange (Fishelson *et al.*, 1999; Lowe *et al.*, 1992, 1995a, 1995b; Moore, 1990). Exposures to a variety of contaminant effluents such as sewage sludge, pulp-mill waste, oil spillages, and mixed wastes from industry have all been found to increase the fragility of molluscan digestive cell lysosomes as well as of fish hepatocyte lysosomes (Cajaraville *et al.*, 2000; Köhler, 1989a, 1989b, 1991; Köhler *et al.*, 1992; Lowe *et al.*, 1992, 1995a; Moore, 1985, 1988a; von Landwüst *et al.*, 1996; Wahl *et al.*, 1995; Wedderburn *et al.*, 2000). 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 and fish hepatocytes, leading to apparent autophagic uptake of the unsaturated neutral lipid into the often already enlarged lysosomes (Figures 1 and 2; Moore, 1988a; Köhler *et al.*, 2002).

In an assessment of pollutant effects organized under the auspices of the International Council for the Exploration of the Sea (ICES) and the UNESCO Intergovernmental Oceanographic Commission (UNESCO-IOC), the cytochemical approach was applied to mussels sampled from a contaminant gradient in a Norwegian fjord (Langesundefjord) together with other types of indicators of pollutant effects (Bayne *et al.*, 1988; Moore, 1988b). The results of this international multidisciplinary workshop demonstrated a considerable degree of agreement between effects determined at different levels of biological organization (Bayne *et al.*, 1988). For instance, cytochemical probes for lysosomal reactions to contaminant-induced cell injury showed clear evidence of enhanced catabolic activity apparently associated with autophagic-type changes (Figure 1), and this could be conceptually linked with structural alterations in the digestive cells, which in turn could be related to observed tissue damage (Lowe, 1988; Moore, 1988a; Widdows and Johnson, 1988). Similar effects occur in fish livers with regard to lysosomal stability, lesions, and neutral lipid (Figures 2–4; Köhler *et al.*, 2002).

Figure 2. Electronmicroscopic photographs of characteristic morphological changes of lysosomes during the progression of toxicopathic lesions in flounder (*Platichthys flesus*) liver. A) Normal small lysosomes with homogeneous structure. B) Typical lysosomes in a reversibly altered liver with high induction of CYP1A1 activity (EROD), which also contains fibrillar elements, black lipofuscin granules, and fields of digested ribosomes (arrows). C) Lysosomes that have taken up a lipid droplet (arrows), which had accumulated in the cytoplasm. D) Uptake and accumulation of phospholipid whorls into a lysosome (arrow), which has given rise to eosinophilic granules in the cytoplasm as seen at the light microscopic level (compare Figure 1.4). LY = lysosomes, M = mitochondria, LIP = lipid droplets, PL = phospholipid whorls. (Scale bar ≡ 1 μm).
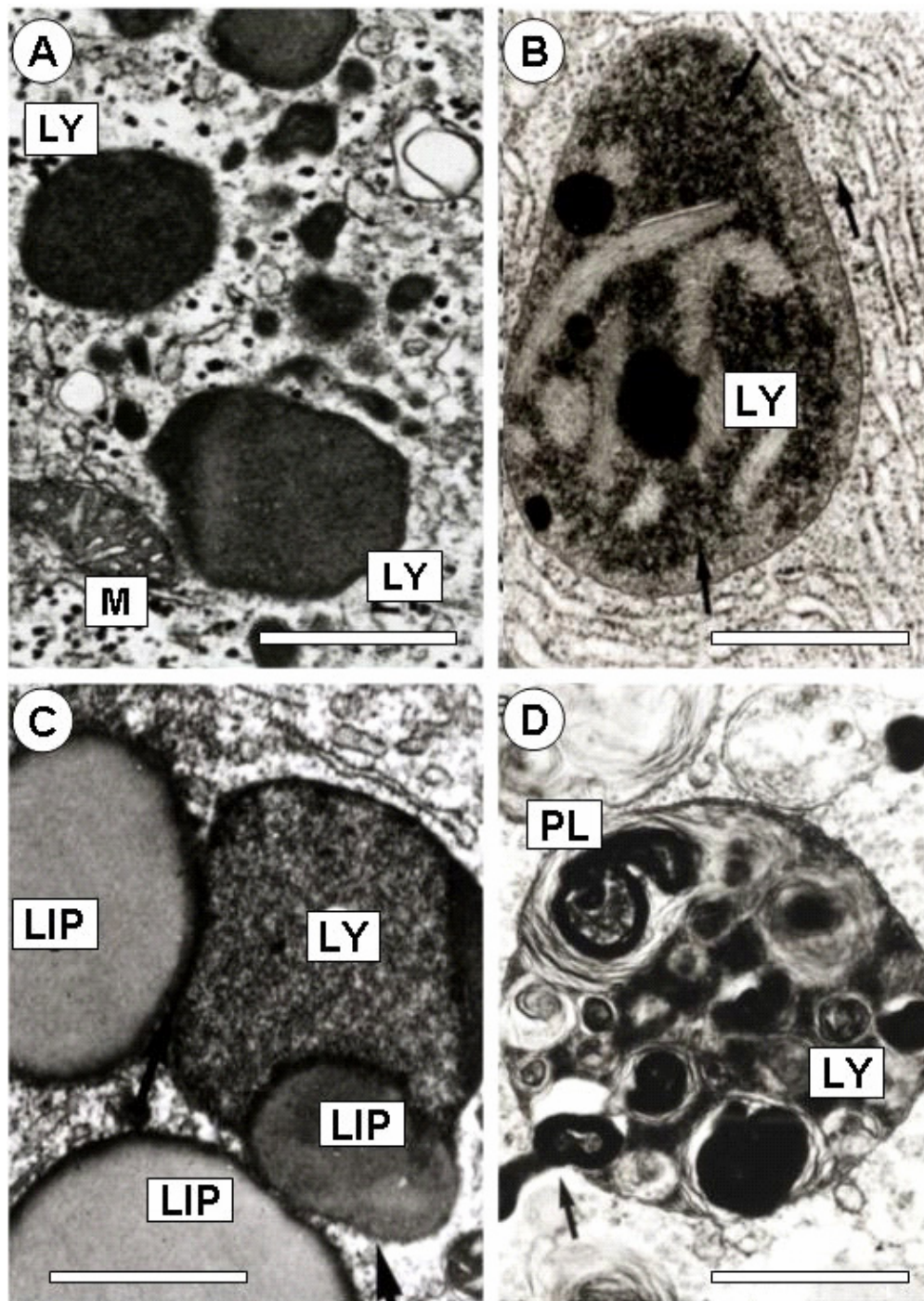
Figure 3. Early and progressive injury in flounder liver: quantitative cytochemical determination of morphological and functional responses of lysosomes in serial tissue sections. A) Unsaturated neutral lipids in hepatocytes as visualized by oil red O. B) Increase in size of lysosomes calculated as volume density. C) Number of lysosomes per unit area of liver parenchyma as numerical density (except macrophages). D) Membrane stability for N-acetyl-β-hexosaminidase measured as time intervals needed for acid labilization of the lysosomal membrane. Long intervals reflect high stability and short intervals reduced stability that is indicative of impaired membrane function.
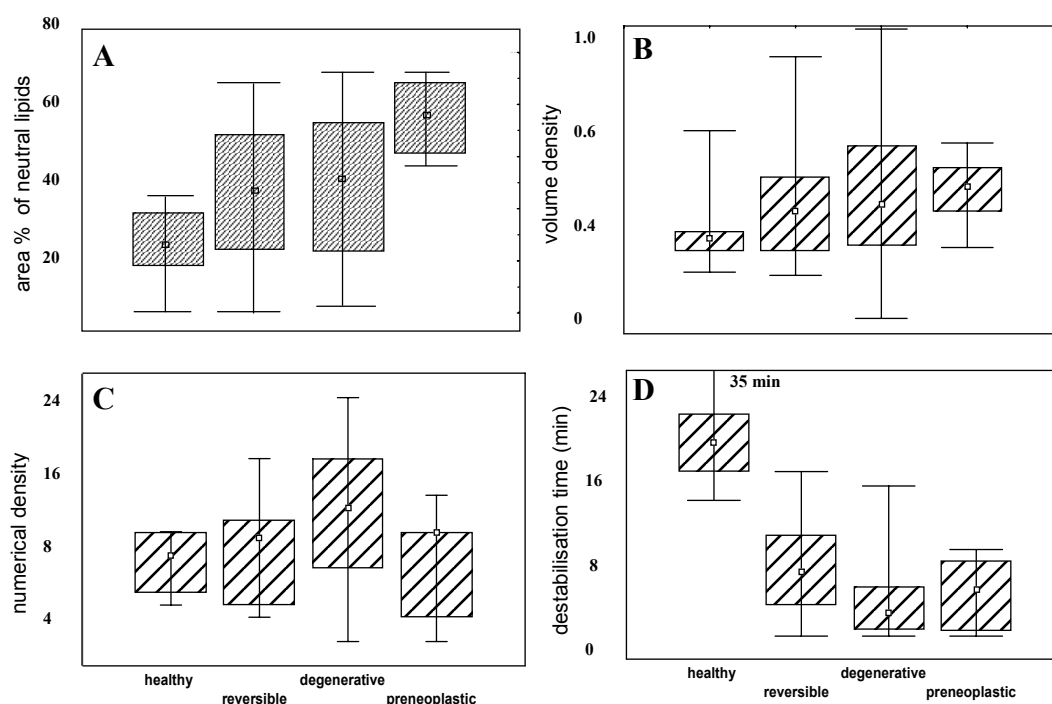
Biochemical analysis of the digestive gland demonstrated a clear increase in lipid, which was in agreement with the cytochemical demonstration of fatty change and lysosomal accumulation of unsaturated neutral lipid (Figures 1 and 2; Capuzzo and Leavitt, 1988; Moore, 1988a; Köhler *et al.*, 2002). Physiological determination of the energy available for growth and reproduction (scope for growth) also showed a decline in mussels from the contaminated sites and this could be related to a combination of reduced uptake of food and enhanced intracellular catabolism (Widdows and Johnson, 1988). More recent studies include an assessment of the health status of coastal mussels in the Black Sea (GEF International Waters Programme) supported by UNESCO-IOC (Moore *et al.*, 1999). This survey used an in vivo cytochemical determination of lysosomal fragility, based on the retention of neutral red in the lysosomes of mussel blood cells (Figure 5; Moore *et al.*, 1999). Viarengo *et al.* (2000) have implemented a laboratory intercalibration of a number of biomarkers, including lysosomal stability in tissue sections of mussels, for the UNEP Mediterranean Pollution Programme (Mediterranean Action Plan, MEDPOL).

Clearly then, the cytochemical probes are providing data which are entirely consistent with data obtained using biochemical, histopathological, and physiological approaches. Such good agreement provides strong support for the validity of the cytochemical data, particularly as a

logical conceptual framework can be devised linking pathological changes at the molecular and subcellular levels of organization to impairment of the physiological performance of the whole animal (Bayne *et al*., 1988). The inference here is that lysosomal stability/fragility is a prognostic indicator or biomarker for putative pathologies and as such is an integrated patho-physiological indicator of health status (Moore, 1990, 2002).

The advantages of using cytochemical approaches in pollutant effects assessment are several. First, cytochemistry is capable of providing information that can shed light on the molecular and subcellular mechanisms of pathological alterations induced by the contaminants. Second, cytochemistry can be applied to very small tissue samples, and thus can be applied to sections obtained from a single tissue sample and these can be readily varied to meet the particular requirements of the situation. Finally, most of the cytochemical tests used in the studies described above can be readily quantified by microdensitometry, image analysis or categorical assessment using a ranked series of photomicrographs (Moore, 1988a; Chieco *et al*., 2001).

Advances in cytochemistry, such as immuno-cytochemistry, hybrido-cytochemistry (*in situ* hybridization), and *in vivo* cytochemistry using chromogenic and fluorescent molecular probes (Figures 6 and 9) 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 (Cajaraville *et al.*, 2000; Grundy *et al.*, 1996a, 1996b; Lowe *et al*., 1992, 1995a; Moore, 1992a, 1992b; Ringwood *et al*., 1998a, 1998b, 1999). Given the increased awareness of environmental problems, particularly in a marine context, there is an urgent need for sensitive, accurate, and rapid tests for assessing evidence of biological deterioration, which will also provide direct evidence of causation. This latter point is very important given the considerable complexity of both marine ecosystems and environmental chemistry, as much of the evidence of pollution damage in the past has been circumstantial or anecdotal. It is necessary, therefore, to have the test capability to be able to give precise information about the probable cause(s) of biological damage in the future. The use of cytochemical tests in this context is by no means the complete answer; however, such tests have the potential to provide important components of a suite of tests for use in environmental monitoring.

3   PREPARATION OF TISSUE SECTIONS FOR ENZYME CYTOCHEMISTRY

For cytochemical examination, small pieces (5 mm × 5 mm × 5 mm) of freshly excised digestive gland tissues or fish livers (i.e., the mid-portion of the organ) from ten animals are placed on metal cryostat chucks (e.g., up to five pieces of tissue in a straight row across the centre). 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). For fish liver, the tissue can also be deep-frozen directly in liquid nitrogen. The metal chuck plus the quenched (supercooled) 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 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), and the slides can be stored in the cryostat for at least 4 hours before use. Cryostat sections that are required for concurrent structural or non-enzymatic cytochemistry (e.g., lipid and lipofuscin) can be fixed in Baker's calcium formol or 10% neutral formalin (+2.5% NaCl w:v).

Figure 4. Examples of PCB and organochlorine concentrations in the liver of flounders tested histochemically for lysosomal membrane stability. Impaired membrane stability consistently coincided with high levels of the polychlorinated biphenyls CB118, CB138, and CB170, and the isomers of α-HCH and β-HCH as well as the total lipid content of the liver. High stability of the lysosomal membrane (as indicated by high latency) is always associated with low concentrations of lipophilic compounds. Medium-range stability of lysosomes is related to highly variable concentrations of chemicals and lipid that are interpreted as being indicative of phenotypic differences in the individual capacity of fish livers to compensate for toxic injury.
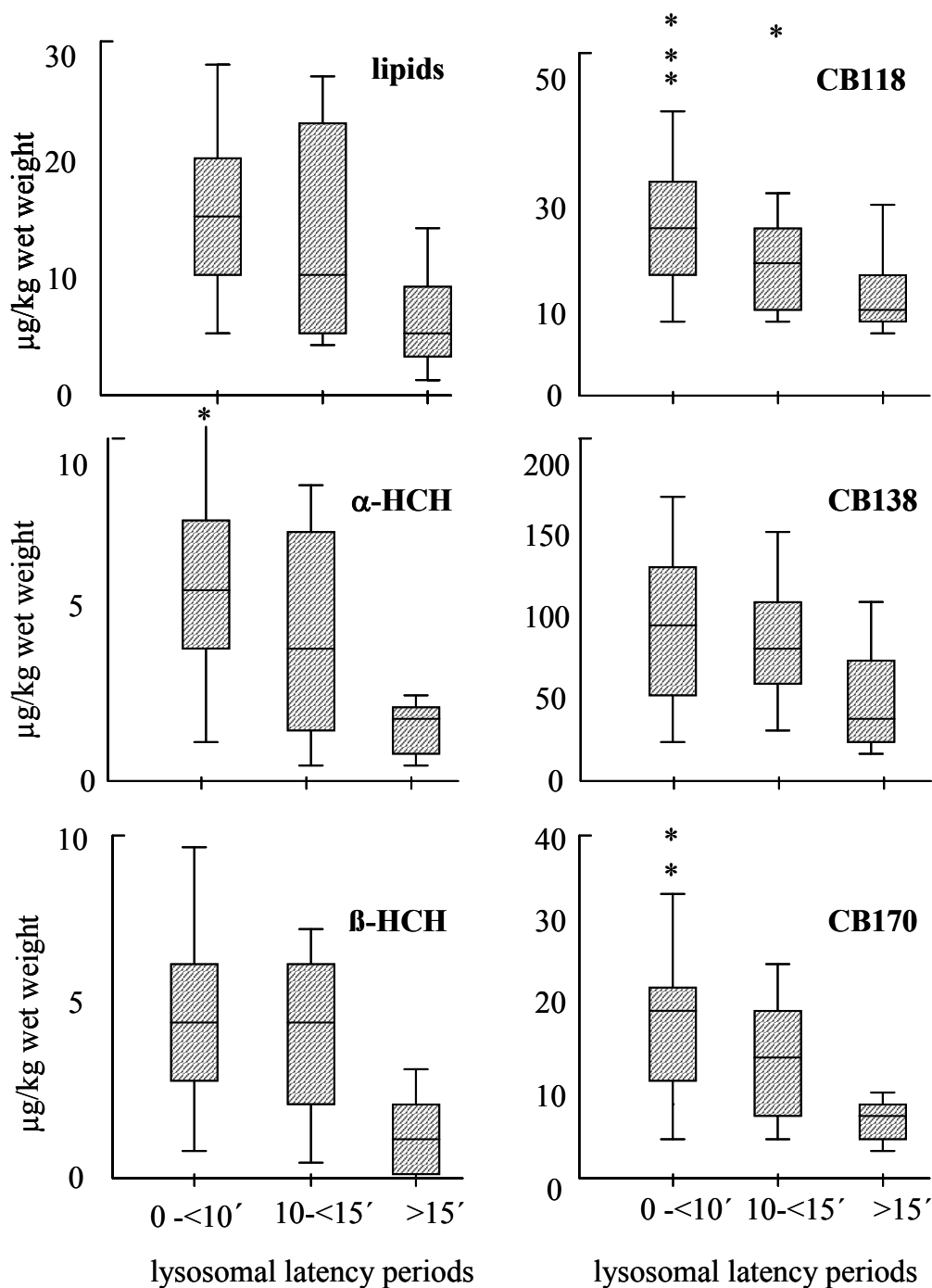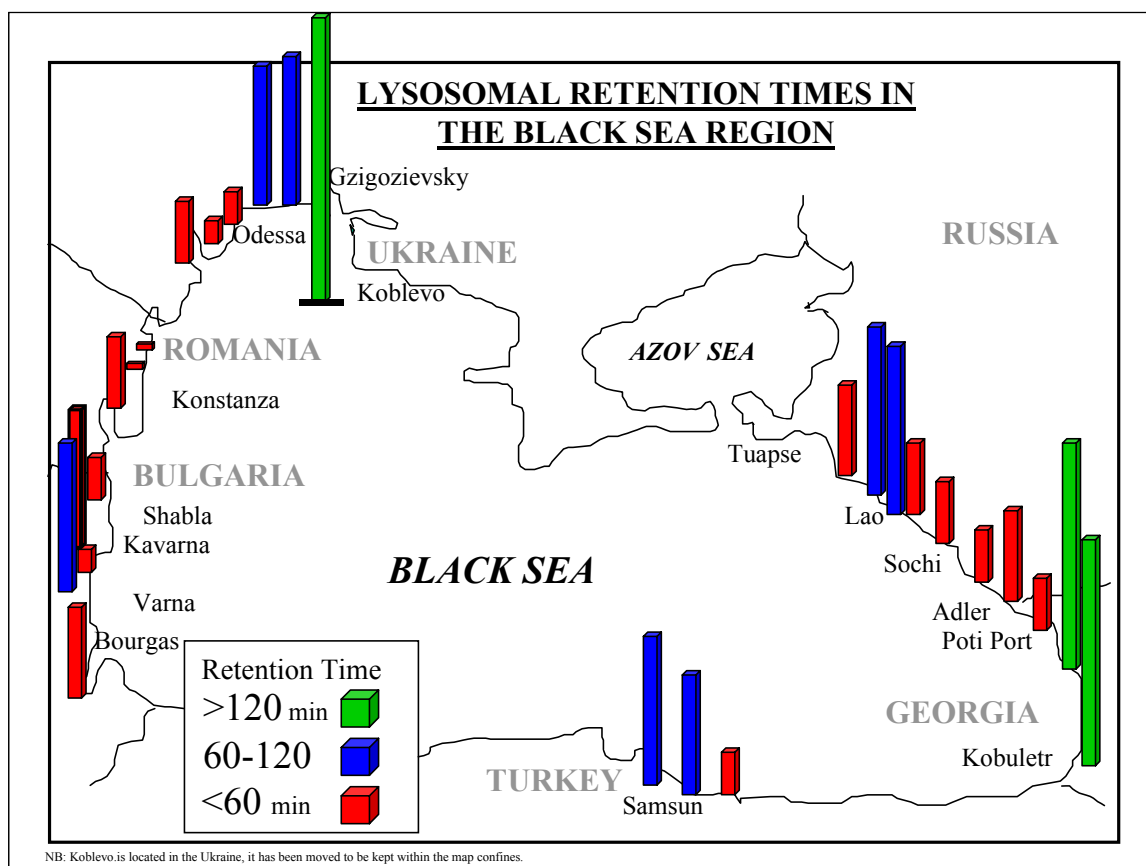
Figure 5. Summary results of the UNESCO-IOC Biological Effects Mussel Watch Programme in the Black Sea. Lysosomal stability was determined using the neutral red retention method.

4 LYSOSOMAL STABILITY IN TISSUE SECTIONS

4.1 Demonstration of Latent Activity of Lysosomal Hydrolases for Assessment of Lysosomal Stability

Latent lysosomal activity of the lysosomal enzymes N-acetyl-β-hexosaminidase and β-glucuronidase can be 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. For fish liver, we recommend the use of N-acetyl-β-hexosaminidase, β-glucuronidase or acid phosphatase.

Method for N-Acetyl-β-hexosaminidase

Serial cryostat sections (in duplicate on the same slide), prepared as described above, are pre-treated in a staining jar with 0.1M citrate buffer (pH 4.5) containing 2.5% NaCl (w:v) at 37 °C in order to labilize (controlled permeablization) 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) and 50 minutes for fish down to 2 minutes (i.e., 50, 40, 30, 25, 20, 15, 10, 5, and 2 minutes). Two minutes (molluscs) or three minutes (fish) are used as the minimal pre-treatment time since sections that have undergone zero pre-treatment may sometimes show stronger staining than short-term pre-treated 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; this contains 20 mg naphthol AS-BI N-acetyl-β-glucosaminide (Sigma) dissolved in 2.5 ml 2-methoxyethanol, which is made up to 50 ml with 0.1M citrate buffer (pH 4.5) containing 2.5% NaCl (w:v) and 3.5 g of low viscosity polypeptide (Sigma, POLYPEP P5115) to act as a section stabilizer (Bitensky *et al*., 1973; Moore, 1976). Incubation time is 20 minutes at 37 °C in a staining jar, preferably in a shaking water-bath. The slides are subsequently rinsed in 3.0% NaCl at 37 °C for 2 minutes before being transferred to 0.1M phosphate buffer (pH 7.4) containing a diazonium coupler (1 mg ml^{-1}) at room temperature for 10 minutes. Suitable diazonium salts are fast violet B (Sigma), fast red violet LB (Difco), fast garnet GBC (Sigma), fast blue BB (Sigma), and fast blue RR (Sigma). Our experience has been that fast violet B is the most suitable. The slides are then rinsed rapidly in running tap water, fixed for 10 minutes in Baker's calcium formol containing 2.5% NaCl (w:v) at 4 °C, rinsed in distilled water, and mounted in aqueous mounting medium.

Method for β-Glucuronidase

The method for the demonstration of latent activity of lysosomal β-glucuronidase (Moore, 1976) is essentially similar to the method described above, but with the following exceptions: the pre-treatment to labilize 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) as substrate 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 POLYPEP P5115) at 37 °C for 20 minutes.

Rinsing and coupling solutions for β-glucuronidase are the same as those used for β-N-hexosaminidase.

4.2 Determination of Lysosomal Labilization Period (i.e., Permeabilization Time for Latent Hydrolase)

The labilization period is the time of pre-treatment required to labilize the lysosomal membranes fully, resulting in maximal staining intensity for the enzyme being assayed (Figures 7 and 8).

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 8 (Moore, 1976). If the animal is stressed, then the peak of activity will be moved towards the *y*-axis and the decreased labilization period can be determined from the *x*-axis (Figure 8).

Our experience has shown that a microdensitometer is not necessary for accurate determination and that the labilization period can be effectively measured by microscopical assessment of the maximum staining intensity in the pre-treatment series (Figure 7). For this procedure, each tissue section should be divided into four roughly equal areas for assessment. This can be done by means of drawing a cross on the cover slide overlaying each section with a very fine marker pen, thus giving four quadrants. The position and orientation of the cross should be the same on all sections.

Figure 6. Mussel blood cells showing fluorescence (orange) for lysosomal accumulation of acridine orange (exposed to 1 µg l^{-1} for 15 minutes). Blue light (FITC) excitation; Scale Bar ≡ 10 µm.
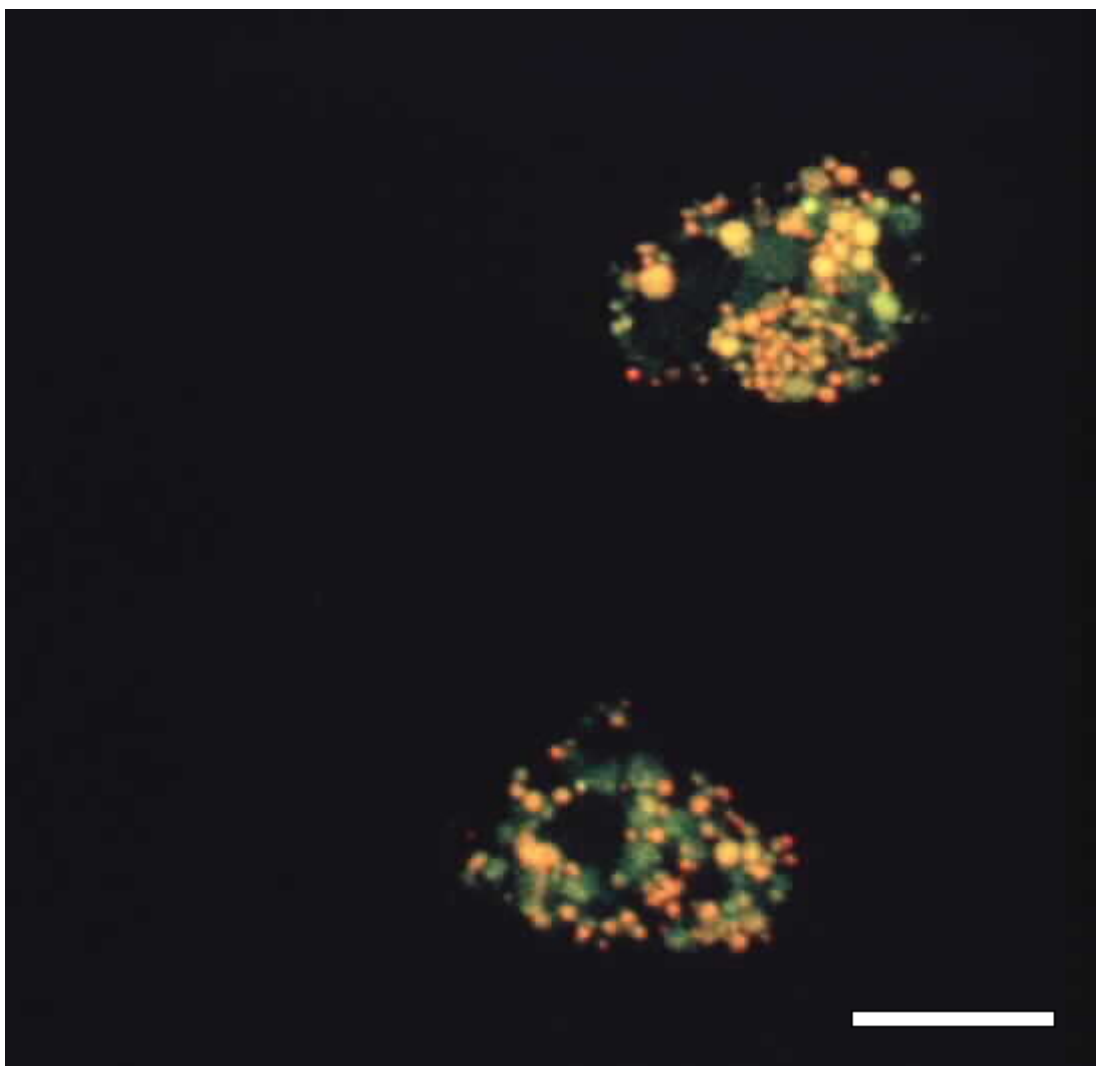
All assessments should be carried out on ***duplicate sections for each digestive gland or liver*** at each pre-treatment time. A mean or median value is obtained for each set of duplicate sections from the average of the assessments in each of the four sub-divisions (i.e., quadrant 1 from all sections in the sequence, then 2, 3, and 4). The data can be statistically tested (i.e., test data compared with references or baseline data) using the non-parametric Mann-Whitney *U*-test or Kruskal-Wallis test (Murdoch and Barnes, 1998). The Tukey *t*-test or analysis of variance can also be used with log-transformed data.

4.3 Problems in Assessment of Labilization Period

Determination of the labilization period is usually quite straightforward, but a complicating situation occasionally arises in which the pre-treatment series shows two peaks of staining intensity (Moore *et al.*, 1978a, 1978b), possibly due to differential latent properties of the sub-populations of lysosomes (Figure 8). In this situation, the first peak of activity is used to determine labilization period, as in our experience it has been the most responsive (Figure 8).

Figure 7. 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 pre-treated at pH 4.5 and 37 °C for 2–25 minutes (2, 5, 10, 15, 20, 25 minutes, respectively). Section 5), pre-treated for 20 minutes, shows maximal lysosomal staining intensity: this time of pre-treatment represents the labilization period. Section 6), pre-treated for 25 minutes, shows a decrease in staining intensity indicating a probable loss of enzyme by diffusion from fully labilized lysosomes. (Scale Bar ≡ 20 μm).
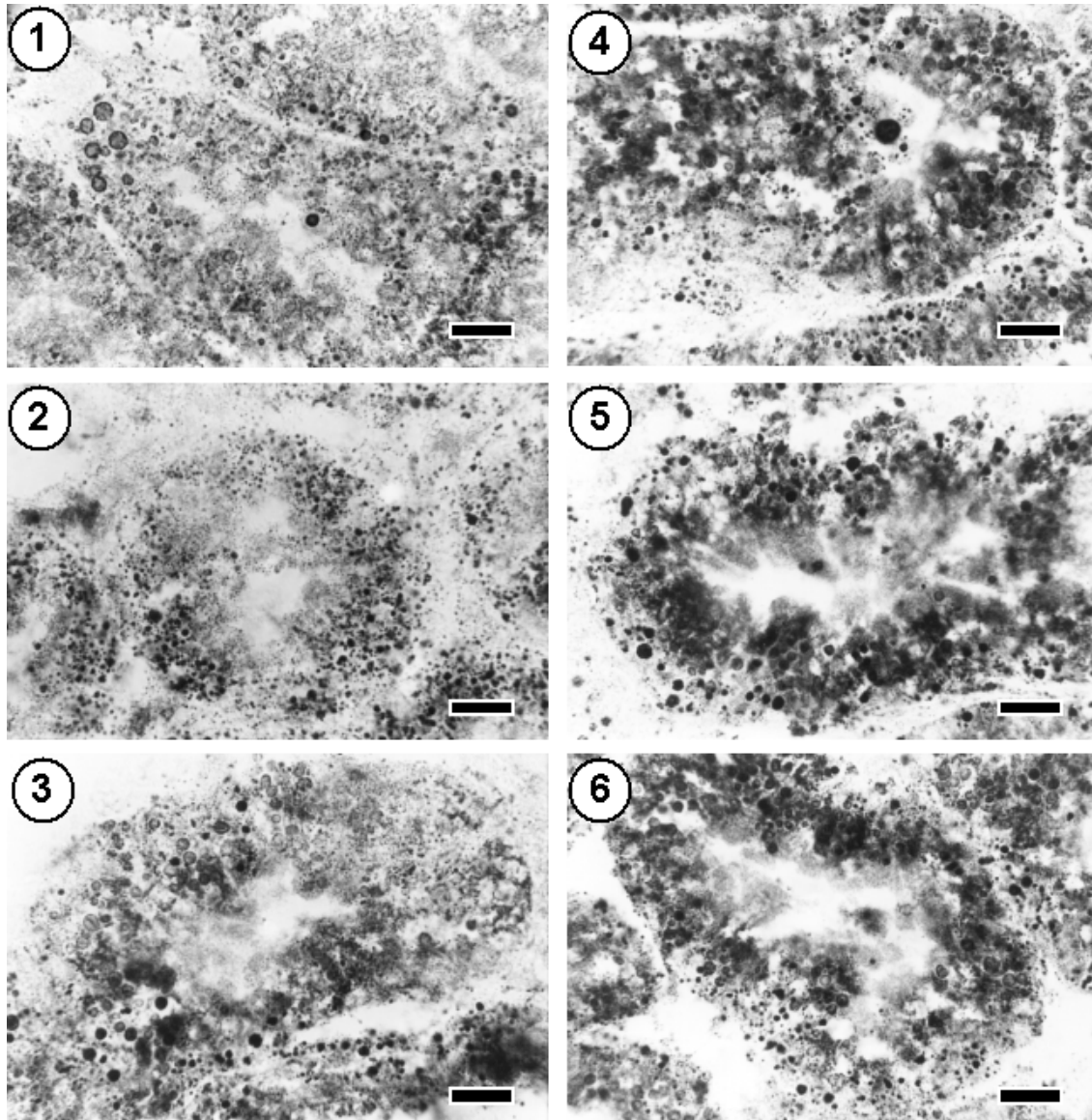
Figure 8. Microdensitometric determination of N-acetyl-β-hexosaminidase activity in sequentially pre-treated (labilized) tissue sections of mussel digestive gland. Healthy cells = open circles; unhealthy cells = filled circles. Means ± SE, n = 10.
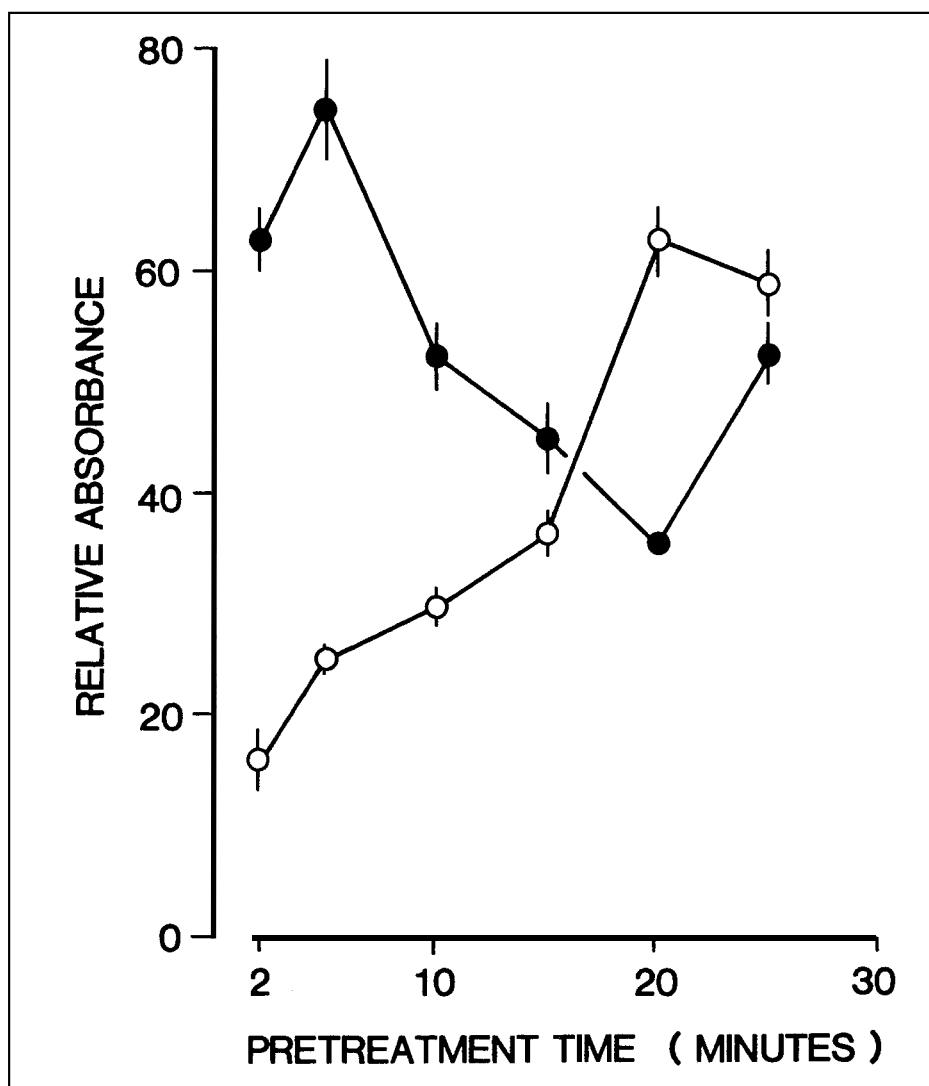
In fish liver, two peaks are also frequently observed, and the first peak of activity is strongly correlated with the degree of liver lesions.

5   *IN VIVO* CYTOCHEMISTRY: LYSOSOMAL NEUTRAL RED RETENTION

Lysosomes have a remarkable capability for accumulating a diverse range of toxic metals and organic chemicals (Figures 6 and 9; Moore, 1985, 1990). However, this concentration of toxic contaminants results in lysosomal damage and cell injury, and possible leakage of contaminants into the cytosol. The fact that lysosomes accumulate this very wide range of xenobiotics, dyes, and drugs has been used to advantage in the development of an *in vivo* cytochemical method for determining lysosomal membrane damage (Fishelson *et al*., 1999; Lowe *et al*., 1992, 1995a, 1995b).

Babich and Borenfreund (1987) published a method in which alterations in the capacity of cells to take up the dye neutral red was used as an indicator of cell damage. The rationale here was

that healthy cells could take up and retain larger quantities of the dye than damaged cells. The method involved exposing cells with the test medium and then incubating them in a neutral red solution. Following incubation, the dye remaining in the cells was extracted and measured spectrophotometrically. Lowe *et al.* (1992) reasoned that if the dye could be measured with a spectrophotometer, it could also be visualized by using a microscope. Indeed, if the lysosomally accumulated dye could be visualized, then the progress of dye uptake into the cells and, in the case of damaged cells, leakage back into the cytosol could be determined and quantified using the lysosomal retention time as a sensitive measure of effect (Figure 9). The methods developed for fish hepatocytes, mussel digestive gland cells, and oyster digestive gland cells use microscopy to assess neutral red retention, and involve sacrificing the animals followed by enzymatic digestion of tissues (Lowe *et al.*, 1992, 1995a, 1995b; Lowe and Pipe, 1994; Ringwood *et al.*, 1998a, 1998b, 1999). These approaches are valuable for concurrent studies with other liver/digestive gland function studies, and when small animals are used. In contrast, blood cells, which are generally easy to obtain without harming the host, offer a sensitive but robust lysosome-rich cell type that can be studied using *in vitro* methods, and this provides the opportunity for further contaminant effect studies (Lowe *et al.*, 1995a; Grundy *et al.*, 1996a, 1996b). 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.

Figure 9. Mussel blood cells (haemocytes) showing uptake of neutral red in lysosomes. 1) Healthy cell showing the retention of neutral red within the lysosomal compartment. 2) Stressed cell showing loss of neutral red into the cytosol. (Scale Bar ≡ 5 μm).
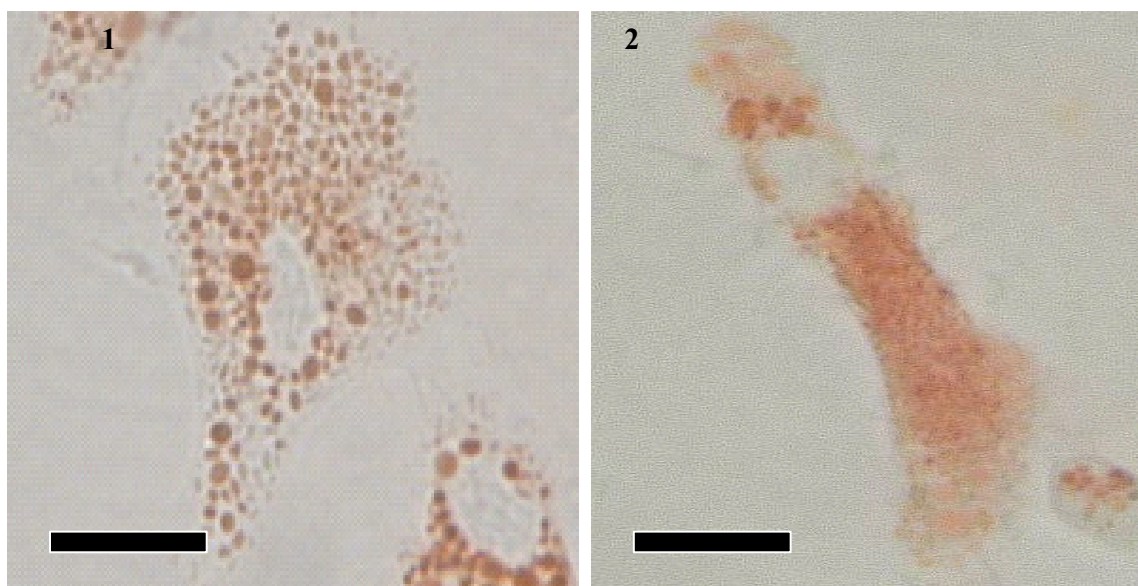
5.1 Method for Lysosomal Neutral Red Retention in Mussel Blood Cells (Cellular Dye Retention)

The neutral red retention technique for blood cell lysosomes is non-destructive; hence, if the animals under test are not unduly stressed during collection, they can be returned to their habitat following careful extraction of a blood sample. Mussel bays threads should be cut from the substrate, since pulling the animals from the rocks can result in damage to internal tissues. Extremes of temperature during transport are also to be avoided and the animals must be maintained in a moist environment during transport to the laboratory.

Stock solutions of physiological saline and neutral red should be prepared in advance and stored in a refrigerator. The neutral red stock solution will solidify in the refrigerator and should also be raised to room temperature for dilution to the working strength.

5.1.1 Haemolymph (blood) extraction

1) The mussel valves should be carefully prised apart along the ventral surface, using a solid scalpel, which should remain in position in order to keep the valves apart (see technical note 1). Allow any water retained within the shell cavity to drain out before attempting to withdraw any haemolymph.

2) Withdraw 0.1 ml of haemolymph from the posterior adductor muscle by using a 1–2 ml hypodermic syringe fitted with a 25-gauge needle and containing 0.1 ml of physiological saline.

3) Having now obtained a sample of haemolymph, remove the needle from the syringe and transfer the contents into a 1.5–2.0 ml siliconized microcentrifuge tube. Ideally, the cells should be kept in a refrigerator prior to use, but for no longer than 20 minutes.

4) Gently invert the tubes in order to mix the contents and pipette 50 µl haemolymph and physiological saline mixture onto each slide, using a clean pipette tip for each sample.

5) Place the slides in a light-proof humidity chamber and incubate for 15–20 minutes (see technical note 2).

6) Drain off excess suspension and carefully wipe around the area containing adhered cells to remove any remaining excess fluid.

Technical notes

1) The blade width of a solid scalpel should be sufficient to hold the valves apart in order to insert a hypodermic needle.

2) It is most important that the slide preparations are kept cool throughout the period of cell attachment and dye incubation. This can be achieved by having a thin layer of water ice in the light-proof humidity chamber. The slides must not be in direct contact with the ice and should be placed on racks allowing sufficient space (approximately 3 cm) for the chilled air to circulate.

3) 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.

4) Neutral red is a photosensitizer; therefore, all slides should receive the same exposure to light under the microscope, and the light intensity should be kept as low as possible.

5.1.2 Neutral red incubation

1) Pipette 40 µl neutral red (NR) working solution onto the haemocytes; wait 15 minutes to allow neutral red to penetrate the cells (see technical notes 2 and 3).

2) Gently apply a coverslip. Systematically examine the slides under a light microscope after 15 minutes and then again after a further 15 minutes. Subsequent examinations should be made at intervals of 30 minutes up to 120 minutes. The final examination should be made after 180 minutes of incubation. If possible, the whole slide should be scanned and replaced

in the chamber as quickly as possible—ideally, 1 minute per slide maximum (see technical note 4).

3) Cells should be examined for both structural abnormalities and NR probe retention time. Conditions should be recorded in a table at each time increment. 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. Cell shape may also change as a consequence of contaminant impact.

5.1.3 Determination of neutral red retention endpoint

The endpoint is when 50% or more of the cells, based on either a visual or a digital photographic determination (see below), exhibit lysosomal leakage or show abnormalities such as enlargement (Figure 9). A more objective approach, 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 removes the capability for real-time results.

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, whenever possible, samples are read "blind". A typical blood sample of 50 µl generates approximately 20 fields of view of attached cells; by quickly rastor scanning the preparation under the microscope it should be 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 anything between 20 and 50 cells; therefore, the analysis assesses the lysosomal membrane status on between 400 and 1000 cells.

5.2 Preparation of Stock Solutions

5.2.1 Mussel physiological saline

HEPES	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.

5.2.2 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.

For a working solution, dilute 10 μl of stock neutral red in 5 ml of mussel physiological saline. The working solution will last about four hours before the dye begins to precipitate out. Neutral red dye powder is commercially available in a range of purities and strengths. If possible, the highest strength/purity dye 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. Different grades of dye will have a different effect on the lysosomes depending on their purity and strength.

5.3   Data Recording

A table to record the results should be prepared as shown in Table 1. When more than 50% of the cells show a clear cytosol and there is no evidence of lysosomal abnormalities, then a plus sign should be recorded in the appropriate box. If there is evidence of dye loss and lysosomal abnormalities, then a negative sign should be recorded.

The figure used for the calculation of the retention time corresponds to the last time period recorded when there was no evidence of dye loss or lysosomal abnormalities. Thus, for animal number 1 below, the last plus is at 60 minutes, while for animal number 4 the last plus is recorded at 15 minutes. Animal 7 exhibited dye loss at 15 minutes and, therefore, is scored as zero and animal number 9 exhibited no evidence of dye loss at 180 minutes and is scored as 180 minutes. The test is truncated at 180 minutes, since for most healthy animals the neutral red itself becomes a toxic xenobiotic stress factor, regardless of the previous contaminant history of the mussels under study. Appropriate statistical tests should then be applied to the data (e.g., Tukey t-test on log-transformed data, Mann-Whitney U-test or Kruskal-Wallis test).

Table 1. Specimen data sheet for the neutral red retention test.

Lysosomal Neutral Red Retention Assay

Study Number

Treatment Group_____

Incubation Time

Mussel number	15 mins	30 mins	60 mins	90 mins	120 mins	180 mins	Retention time
1	+	+	+	-	-	-	60
2	+	+	+	-	-	-	60
3	+	+	-	-	-	-	30
4	+	-	-	-	-	-	15
5	+	+	+	-	-	-	60
6	+	+	-	-	-	-	30
7	-	-	-	-	-	-	0
8	+	+	-	-	-	-	30
9	+	+	+	+	+	+	180
10	+	+	+	+	-	-	90

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ANNEX 1

QUALITY ASSURANCE FOR LYSOSOMAL MEMBRANE STABILITY

1   THE METHODS TO BE USED

Cytochemical measurement of lysosomal membrane fragility and the cellular dye retention technique based on lysosomal uptake of Neutral Red (NR) in isolated cells (digestive gland of molluscs, liver of fish) or blood cells (as a non-destructive technique in molluscs) can be used as an alternative to the cytochemical method. However, this method, although very simple and easy to learn, requires more widespread use in other laboratories in order to fully assess its utility. Biochemical techniques are available for the measurement of membrane-linked latency of lysosomal enzymes, but these are not in general use in environmental monitoring.

2   INTERCALIBRATION STANDARDS

Frozen (quenched) tissues should be prepared for the intercalibration 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 in order for them to perform the test. 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.

An intercalibration exercise has been carried out in the UNEP-MEDPOL programme using the cytochemical technique for tissue sections, and an exercise for neutral red retention has been conducted in the GEF Black Sea Programme. The results from these operations indicated that both techniques could be used in the participating laboratories in an effective manner with insignificant interlaboratory variability.

The standards used in this intercalibration involved digestive glands from marine mussels prepared at the University of Genova (Italy). Comparisons of the cytochemical and the neutral red retention techniques have been performed in fish liver (ICES-IOC Bremerhaven Workshop, 1990) and in mussels experimentally exposed to PAHs (Lowe *et al.*, 1995).

For intercalibration of the neutral red cellular dye retention test, which is performed on live cells *in vitro*, it will be necessary to hold an intercalibration workshop for the participating laboratories at a single site, since samples cannot be exchanged between laboratories.

3   STANDARDS AND REAGENTS

3.1   Cytochemical Method

The standards and reagents for the cytochemical method are given in Moore (1988a).

Equipment:

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

2)   Good quality water bath (preferably shaking) up to 40 $^{\circ}$C;

3)   Cleaned Hellendahl histological staining jars;

4)   Good quality cleaned but untreated microscope slides with frosted glass writing area;

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

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

Reagents:

1) Naphthol AS-BI N-acetyl-β-glucosaminide (Sigma);

2) Fast Violet B (Sigma);

3) Collagen-derived polypeptide (POLYPEP, P5115, Sigma);

4) Citrate buffer 0.1M, pH 4.5, containing 2.5% sodium chloride (w:v);

5) Phosphate buffer 0.1M, pH 7.4;

6) Aqueous mounting medium (Difco, Kaiser's glycerol-gelatine, Sigma or other).

3.2 Cellular Dye Retention Test

Details of the method are described in Lowe *et al.* (1992, 1995).

Equipment:

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

2) Optional use of a 580 nm green filter to enhance contrast of the neutral red;

3) Humidity chamber for incubation of the cells with neutral red.

Reagents:

1) Neutral red (Sigma, general purpose grade).

4 SAMPLING REQUIREMENTS

4.1 Mussels

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;

3) Sampling should be avoided during the main spawning season;

4) Mussels should be sampled from the sub-littoral part of the population, since this will minimize fluctuations due to air exposure at low tide;

5) Transport to the laboratory should avoid rough handling and mussels should be packed in an insulated container containing tissue paper soaked in sea water;

6) For transportation times of more than 4 hours, ice packs should be placed in the bottom of the insulated box.

4.2 Fish

1) Flatfish are caught by 30-minute hauls with a technique appropriate for the species and are directly transferred into aerated flow-through seawater tanks in order to minimize catching and handling stress;

2) The fish should be measured for total length, dissected, and the sex determined;

3) The livers of a maximum of 25 fish of a single sex (males or females are used according to the requirements of the monitoring programme) should be removed;

4) The length of the fish selected is dependent on the specific objectives of the monitoring programmes (e.g., early effects or liver cancer).

5 SAMPLE PRESERVATION

5.1 Cytochemical Method

Mussels:

1) Digestive glands from mussels should be cut transversely into three approximately equal portions and the mid portion (up to 5 mm × 5 mm × 5 mm) used for cytochemistry immediately after dissection; they should be put on a labelled, cooled, coded chuck at refrigerator temperature (4 ^{o}C);

2) The remaining portions are available for histopathology;

3) Tissues should be prepared and stored as described by Moore (1988b).

Fish:

1) Fish livers are cut into pieces of 5 mm × 5 mm × 5 mm immediately after dissection, and put on a labelled, cooled, coded chuck at refrigerator temperature (4 ^{o}C) (Figure 10);

2) The tissue and chucks are then quenched (supercooled) in *n*-hexane cooled to −70 ^{o}C;

3) The tissues are prepared and stored as described by Köhler *et al*. (1992).

5.2 Cellular Dye Retention Method

1) This method does not need any preservation because it is performed on live cells.

6 TRAINING NEEDS

Training material includes documents, micrographs, videos, and laboratory reference material (LRM). Training workshops are also recommended and can be readily coupled with those for other biological effects methods (e.g., pathologies, metallothionein, or EROD). Interlaboratory comparison exercises are being organized through BEQUALM and BEEP.

Figure 10. Sampling procedure for interdisciplinary analysis of liver according to Feist *et al.* (2004) as developed in the EU-BEEP programme**.**
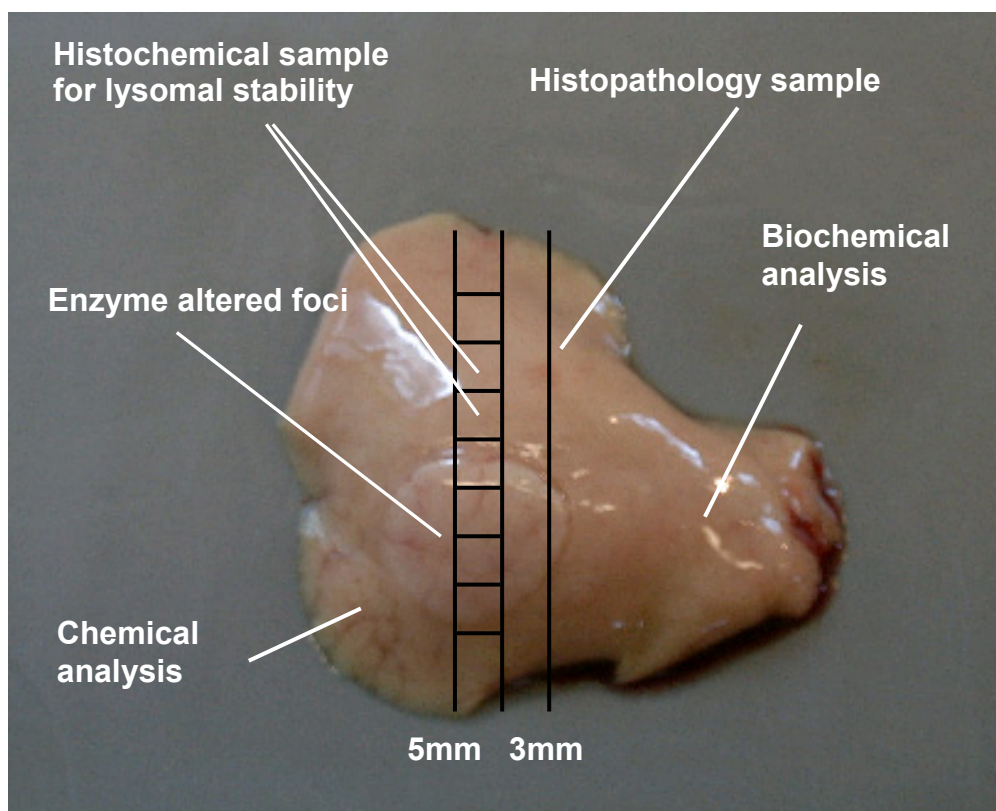
7 SPECIES AVAILABILITY

The techniques can be applied to a broad range of bivalve and gastropod molluscs as well as teleost fish. The currently preferred species are mussels, dab, flounder, dragonets, and grey mullet.

8 DEFINITION OF LIMITS

It should be possible to establish standard Shewart control charts for measurement of lysosomal stability using LRMs.

9 ACTION REQUIREMENTS WHEN LIMITS ARE EXCEEDED

Repeated measurements from LRMs produced by a lead laboratory will be used to control differences of interpretation between analysts.

10 GOOD LABORATORY PRACTICE

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.

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