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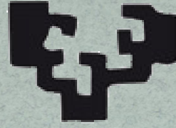

Universidad del País Vasco

Euskal Herriko Unibertsitatea

Plentziako Itsas Estazioa

Estación Marina de Plentzia

Contribution to the development of Best Available Practices (BAPs) for a multi-biomarker approach in sentinel mussels based on a better understanding of the influence of confounding factors on biomarker values and responsiveness

Esther Blanco Rayón

International PhD Thesis

2018

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International PhD Thesis submitted to the Euskal Herriko Unibertsitatea/
Universidad del País Vasco in the candidature for the degree of the
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by
Esther Blanco Rayón
2018

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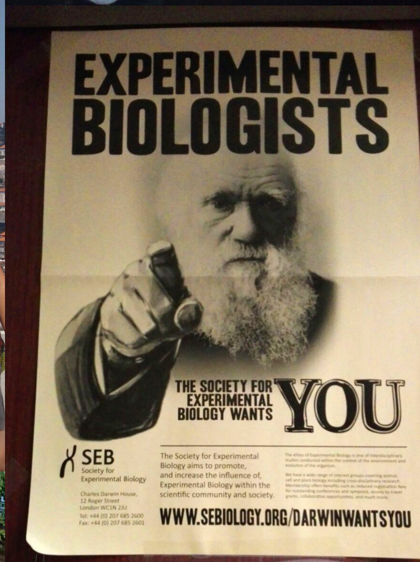
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“El talento gana partidos, pero el trabajo en equipo y la inteligencia ganan campeonatos.“ – Michael Jordan

Ahora que ya empiezo a ver la línea de meta de este gran maratón que ha sido la tesis, echas la vista atrás y ves todo lo que ha pasado y la gente que ha transcurrido por ella. Y llegas a la conclusión de que sola, no hubiese podido hacer nada, ni desde empezar como alumna interna, pasando por las rutinarias pero imprescindibles disecciones, hasta escribir el manuscrito. Por todo ello, me gustaría agradecer a ciertas personas que hayan coincidido conmigo, porque directa o indirectamente me han ayudado a ver la bandera de cuadros.

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El viaje de la tesis tiene sus altibajos, como una gran aventura, que si estás bien acompañada las penas se dividen y las alegrías se multiplican (buena cuenta de ello puede dar el Zua, jajajaja). Por ello, por aprender de vosotros y/o por la imprescindible ayuda prestada, quería agradecer a mis compañeros (doctores/doctorandos, alumnas internas y PAS): Mutri, Javi, Joshua, Cristina, Gorri, Iratxe, Alberto, Txema, Erik, La familia gitana (María, Oihane, Ane y Tif), Endika, Tamer, Denis, Laura, Nagore, Gabriela, Edurne, Merle, Irune, Asier, Cayo, Egoitz ….

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I. INTRODUCTION

"KNOWLEDGE OF THE OCEANS IS MORE THAN A MATTER OF CURIOSITY, OUR VERY SURVIVAL MAY HINGE UPON IT."
— President John F Kennedy, March 1961, Message to Congress

BIOMARKER APPROACH IN MARINE POLLUTION MONITORING

In words of the European Commission, the marine environment is a precious asset (Commission of the European Communities, 2005). Oceans and seas provide 99% of the available living space on the planet cover 71% of the Earth's surface and contain 90% of the biosphere and consequently contain more biological diversity than terrestrial and freshwater ecosystems. Moreover, marine ecosystems play a key role in climate and weather patterns and is also a great contributor to economic prosperity, social well-being and quality of life (Commission of the European Communities, 2005). However, pollution generated by anthropogenic activities threated continuously marine ecosystems worldwide (Spies et al., 1996; Bocquenè et al., 2004; Adams, 2005; Garmendia et al., 2011a,b). Coastal ecosystems are particularly impacted as they are exposed to chronic discharges and accidental events such as oil spills, threatening organism health, biodiversity and consequently ecosystem functioning (Islam and Tanaka, 2004). Nowadays, it is known that apart from pollution, marine ecosystems and organisms are affected by internal and external factors, such as ecological processes and their interactions, fisheries, a climate change, habitat modification and eutrophication (Hylland et al., 2017). Therefore, the assessment of the marine pollution has been recognised as an immediate need for sustained management and conservation of the marine resources (Islam and Tanaka, 2004). Accordingly, different strategies have been promoted worldwide to assess effects of pollutants in marine ecosystems, such as monitoring programs (Melwani et al., 2014; Hylland et al., 2017; Chiu et al., 2018).

A monitoring program is a systematic repeated analysis of environmental quality parameters in water, sediments and/or biota from a set of defined geographical locations and over a time-span of several years (Goldberg, 1975, 1986, 1980). At the beginning of the marine pollution monitoring programs, pollution has been determined by analyses of chemical compounds in water, sediments and biota. However, it is important to remember that detection and quantification of contaminants in the environment using only analytical tools is not enough to accurately determine their impact on ecosystem (OSPAR, 1998; Jha et al., 2000; Laane et al., 2012), because contaminants are present in the environment as complex mixtures, being challenging the accurately evaluation of the pollutants toxicity to the biota (Jha et al., 2000). Consequently, the assessment of the biological responses to pollutants on different biological organization is needed in ecotoxicology (OSPAR, 1998; Martinez-Gómez et al., 2017) and in marine monitoring programs such as the JAMP, MED POL and HELCOM (OSPAR, 1998; Viarengo et al., 2000; HELCOM, 2017). Likewise, the relevant role of biological effects in marine health assessment is also supported by the European Marine Strategy Framework Directive (MSFD; 2008/56/EC), including biological effects among the criteria to determine the Good Environmental Status[1] of marine waters (European Commission,

[1] According to the European Marine Strategy Framework Directive, GES means the environmental status of marine waters where these provide ecologically diverse and dynamic oceans and seas which are clean, healthy and productive within their intrinsic conditions, and the use of the marine environment is at a level that is sustainable, thus safeguarding the potential for uses and activities by current and future generations.

2008). The organism used in biomonitoring programs to assess the biological responses to pollutants is called sentinel organism (Viarengo et al., 2007; Beyer et al., 2017). Organism commonly employed as sentinel organisms in routine biomonitoring programs (Med Pol, UNEP Mediterranean Biomonitoring Program; OSPAR Convention, UNEP/RAMOGE., etc.) are molluscs (mussels, oysters, periwinkles), crustaceans (*Gammarus sp.)* and fish (*Mullus sp*., *Platichthys flesus* L., *Zoarces viviparus*, *Perca sp*.) (Viarengo et al., 2007; Hylland et al., 2017). Sentinel organisms required certain characteristics: (a) widespread distribution, (b) high trophic status, (c) ability to bioaccumulate pollutants, (d) suitability to be maintained and studied in captivity, (e) easy to be captured in sufficient numbers, (f) restricted home range, (g) well known biology, and (h) sensitivity to pollutants (Beeby, 2001; Fox, 2001; Basu et al., 2007; Masson et al., 2010). Among sentinel organisms, mussels are widely used in order to assess the biological effect of pollutants in marine monitoring (ICES, 2012; Beyer et al., 2017; Faggio et al., 2018). In order to determine the biological effects of pollutants, biomarkers (early measurable biological response to pollutants) are widely used tool in marine monitoring programs (Viarengo et al., 2007; Beyer et al., 2017), being abundant and diverse to apply in mussels (UNEP/RAMOGE, 1999; ICES, 2012).

Mussels as sentinel organisms

The popularity of mussels as sentinel organism is due to their biological and ecological characteristics, which are very appropriate for pollution monitoring and toxicological experiments (Moore et al., 2004; ICES, 2012; Beyer et al., 2017). They are filter-feeders with very low metabolic activity, which implies that the pollutants concentrations in their tissues reflect accurately the environmental pollution magnitude. Moreover, they are relatively tolerant (but not insensitive) to a wide range of environmental conditions, including moderately high levels of most types of contaminants. Mussels are also easy to collect and maintain in laboratory, making possible their very extensive use in experimentation. Furthermore, they are economically relevant sessile organisms that are widely distributed (Fig. 1). In the case of mussels in the genus *Mytilus* (from the Greek word mitilos, meaning sea mussel) are a dominant component of rocky shore communities in cool and temperate waters of the northern and southern hemisphere (Fig. 1). Three taxa (*M. galloprovincialis, M. edulis* and *M. trossulus*) are found in Europe (McDonald et al., 1991; Hilbish et al., 2000). The three taxa maintain relatively distinct genetic identities on a global scale, largely because the taxa are adapted to different biogeographic provinces (Gardner, 1996). However, in areas of environmental transition from one biogeographical province to another, hybrid zones between neighboring taxa can be formed (Gardner and Thompson, 2001; Brooks et al., 2015).

Mussels (*Mytilus spp.*) are wide distributed, extending from high intertidal to subtidal zones, from estuarine to fully marine conditions, and from sheltered to extremely wave-draining shores (Gosling, 2004). Along the intertidal zone, upper distribution limits for mussels are usually governed by physical factors, such as temperature, while predators are mainly responsible for setting lower limits (Seed and Suchanek, 1992).

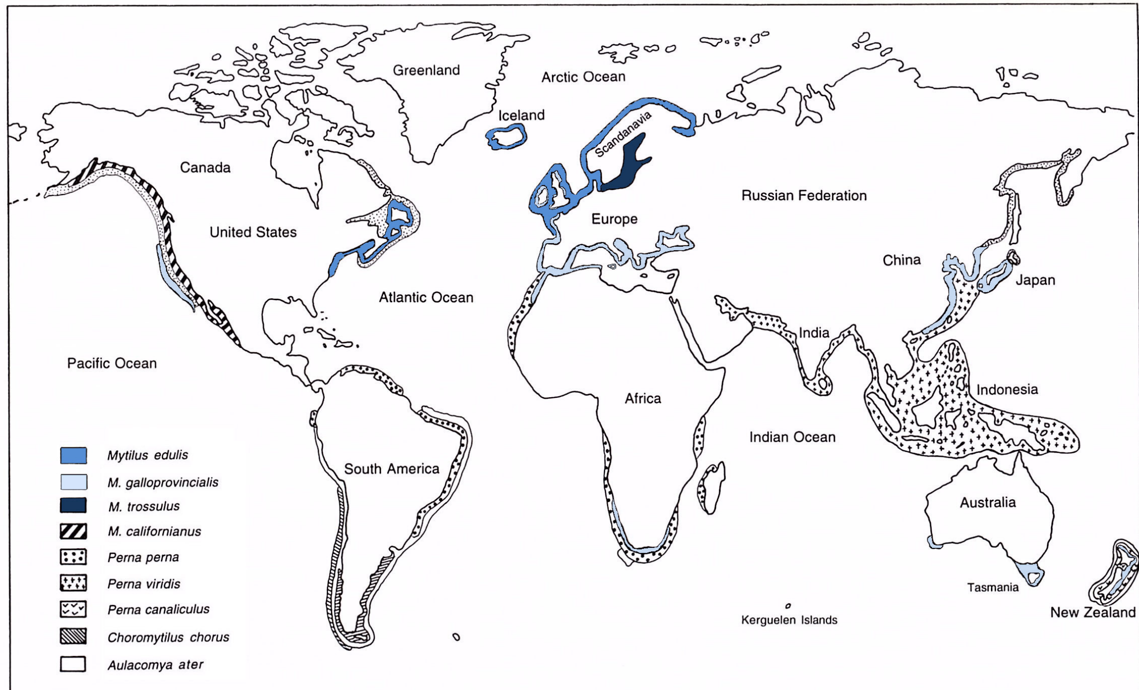

Fig. 1. Approximate global ranges of the main commercially important mussel species. In the Northern Hemisphere where the ranges of *M. edulis*, *M. trossulus* and *M. galloprovincialis* overlap variable amounts of hybridisation occurs between species pairs. In the Southern Hemisphere using shell morphology and protein markers mussels (*Mytilus*) were identified as either *M. edulis* or *M. galloprovincialsis*. However, recent results from mtDNA analysis indicate that mussels in this region are derived from two ancient migration events from the Northern Hemisphere; mussels are similar but not identical to Northern Hemisphere *M. edulis* and *M. galloprovincialis*. (adapted from Gosling, 2004)

The whole flesh of mussels is used for chemical analysis or condition factor in environmental health assessment. Moreover, different organs, such as foot, mantle, gills and digestive gland, are separately used to apply biomarkers (ICES, 2012). The foot provides some locomotory capacity to mussels, especially in the juvenile stage (Fig 2). The foot is proportionately very large and sock shapped, and is made up of layers of circular and longitudinal muscles surrounding a capacious haemolymph space (Fig. 3 A, 3B).

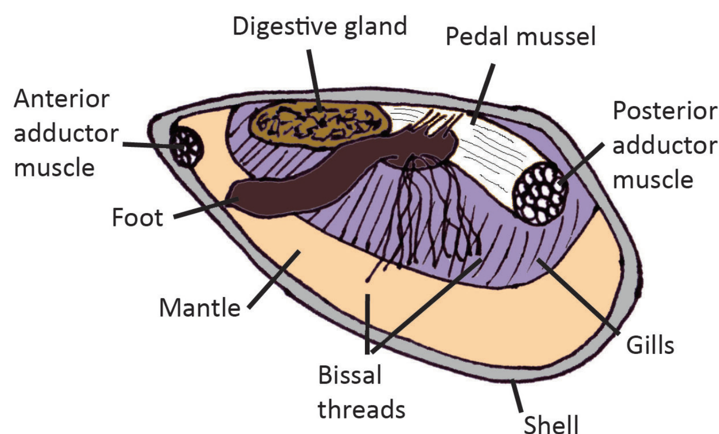

Fig.2. Scheme of the internal anatomy of the mussel *Mytilus sp.*

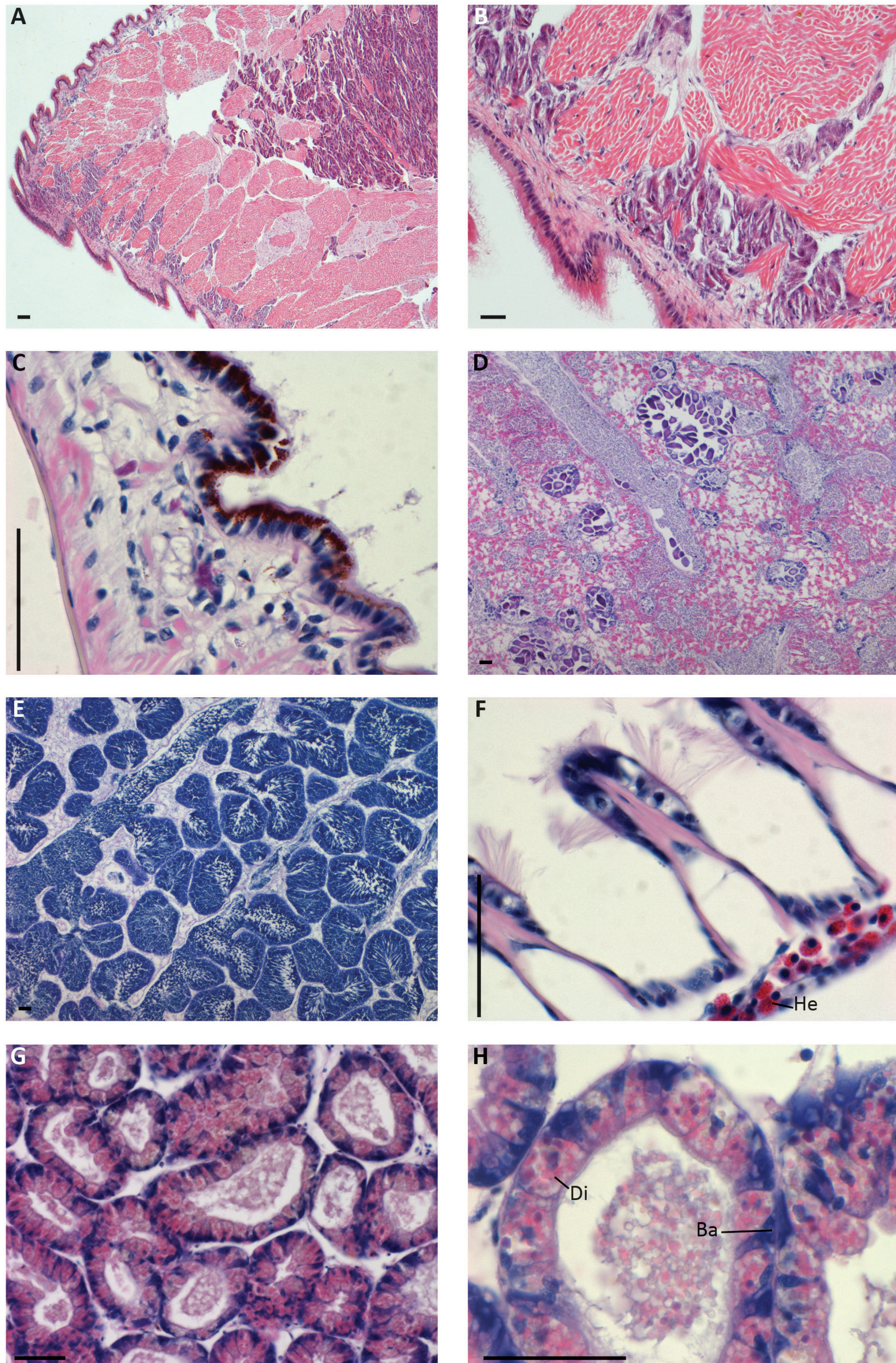

Fig. 3. Micrograph of mussel *M. galloprovincialis* stained with hematoxylin-eosin. Scale: 50 µm. A, B) Foot. C) Female gonad D) Male gonad. E) Mantle. F) Gills. G, H) Digestive gland. He: hemocyte; Ba: basophilic cell; Di: digestive cell.

In bivalves, the **mantle** consists of two lobes of tissue which completely enclose the animal within the shell (Fig 2), where connective tissue with hemolymph vessels, nerves and muscles that are particularly well developed near the margins. The edges of the mantle are usually darkly pigmented, which gives protection from the harmful effects of solar radiation (Seed, 1971) (Figs. 3C). Gametes proliferate within the mantle and are carried along ciliated channels to paired gonoducts that discharge into the mantle cavity (Fig. 3D, 3E). Once the mussels have released their gametes the mantle is thin and transparent. Gamete development is analysed in marine ecosystem health assessment because provide additional information on the health and physiology of the mussel (Cajaraville et al., 2006; ICES, 2011). Moreover, alteration in sex ratio suggest endocrine disruptors pollution in the ecosystem (Ortiz-Zarragoitia et al., 2011).

The most reliable methods for assessing the course of the reproductive cycle in bivalves are those based on histological preparation of gonad (Hines et al., 2007). From such preparations various gonad developmental stages can be identify following the criteria adapted after Kim et al. (2006) (Fig. 4):

- Stage I. Resting gonad. No trace of sexuality can be observed in this stage. It includes virgin animals where the reproductive system is rudimentary, and those animals which have completed spawning.
- Stage II. Early gametogenic gonad. These are occupied mainly by early stages of gametogenesis (small, numerous oocytes attached to the germinal epithelium in the female, and spermatogonia and spermatocytes in the male).
- Stage III. Advanced gametogenic gonad. Here there is a general increase in the mass of the gonad at the expense of the stored food in the connective tissue. This is a stage of rapid gametogenesis with approximately half of each follicle occupied by ripe gametes, and half with early stages of gametogenesis. The area occupied by gonadal tissue is about half that of the fully ripe conditions.
- Stage IV. Mature gonad. Here the gonad attains its fully ripe condition. The connective tissue is very low. Oocytes are compacted into polygonal configuration, whilst in the males the follicles are distended with morphologically ripe spermatozoa.
- Stage V. Spawing gonad. Gonadal follicles appear partially empty of gametes.
- Stage VI. Post-spawing gonad. Gonadal follicles have not containing few gametes or residual gametes.

The reproductive cycle has a degree of flexibility depending on environmental factors (temperature, food availability), making differences between mussel populations relatively close geographically and also, annual variation within the population. However, pollutants can provoke marked changes in the reproductive cycle (Ortiz-Zarragoitia et al., 2011). In general, gonad development of mussels *Mytilus* spp. starts at the turn of winter and spring when water temperature and food availability increase and spawning takes place during late spring (Gosling, 2004; Beyer et al., 2017). In the case of *M. galloprovincialis* pattern for the Basque Coast populations, mussels are at advanced gametogenesis presenting ripe gametes

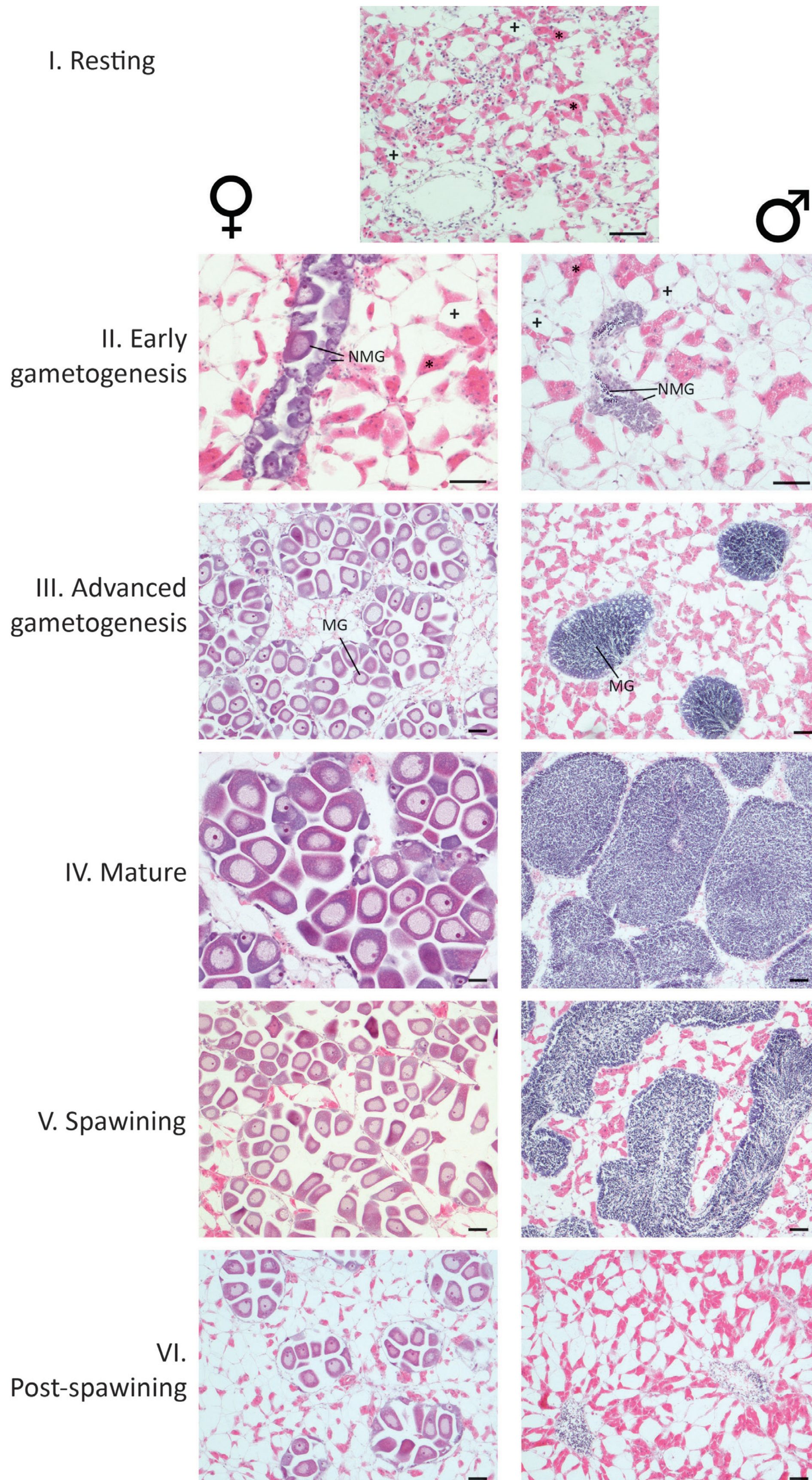

Fig. 4. Micrographs of the gamete developmental stages of female and male mussels *M. galloprovincialis* stained with hematoxylin-eosin. Scale bar: 50 µm. +: vesicle conjunctive cells, *: adipogranular cells, NMG: no mature gamete, MG: mature gamete.

in winter and the spawning period extend throughout spring and, to a lesser extent, summer (Ortiz-Zarragoitia et al., 2011; Múgica et al., 2015). Depending on the food availability, new sequences of gonad restoration and spawning occurred again in late summer, but most of the mussels reabsorbed their gonads concurrently with development of storage tissue. A new gametogenic cycle starts in late summer (Villalba, 1995; Garmendia et al., 2010; Ortiz-Zarragoitia et al., 2011). Apart from gametogenic development, the mantle is also the main site for the storage of nutrient reserves, especially glycogen (Gosling, 2004). The reserves are laid down in summer and are utilized in autumn and winter in the formation of gametes.

Mussels feed on plankton and other microscopic sea creatures which are free-floating in seawater. The **gill**s, also called ctenidia, are flat, homorhabdic (adjective; Greek homos, same; rhabdos, rod; uniform series), non-plicate and filibranch (Figs. 2). The gills have a respiratory as well as a feeding role. Their large and thin surface area and rich haemolymph supply make them well suited for gas exchange (Fig. 3F). Deoxygenated haemolymph is carried from the kidneys to the gills by way of the afferent gill vessel. Each filament receives a small branch of this vessel. The filaments are essentially hollow tubes within which the haemolymph circulates. Gas exchange takes place across the thin walls of the filaments. The oxygenated haemolymph from each filament is collected into the efferent gill vein that goes to the kidney and on to the heart. In gills the level of oxidative processes is expected to be very high, due to gills constitute the major entry site for uptake of dissolved pollutants since gills are the first organ to be exposed to waterborne pollutants (Vidal-Liñan and Bellas, 2013). Moreover, physiological status of the organism weakly influence on gills and thus, this tissue may indicate accurately the environmental health status (Soldatov et al., 2007). So, gills are widely used for biochemical biomarkers determination, such as enzymatic activity changes to pollutants (ICES, 2012).

In mussels, the feeding stars in the gills. The gills, with their different ciliary tracts, remove suspended particles from the water pumped through the mantle cavity, employing a hydromechanical and mucociliary mechanism of particle transport (Gosling, 2004). The gills divide the mantle cavity into inhalant and exhalant chambers. The water that enters through the inhalant opening is driven from the inhalant to the exhalant chambers by cilia on the gills and mantle surface, and exits by the exhalant opening. Both openings possess a muscular velum, the inner fold of the mantle, which regulates water flow through the mantle cavity. Particles in suspension enter the mouth from an oral groove at the base of the labial palps. The mouth, and the oesophagus which leads into the stomach, have a ciliated epithelial lining, which is well supplied with mucocytes that secrete both acid and neutral mucopolysaccharides, even when the animal is not feeding (Beninger and Le Pennec, 1991). The oesophagus does not have a digestive function, merely serving to propel material along ciliated tracts towards the stomach in digestive gland. As the name indicated, the main function of digestive gland is the digestion (Figs.2, 3G). Through the gills, the food arrive to the stomach in the digestive gland. The stomach is a flattened sac into which the oesophagus opens at the anterior end and from which the midgut leaves at the posterior end. The crystalline style projects from the posterior end of the stomach across the floor of the stomach to rest against the gastric

shield. Ingested particles are mixed with the liberated digestive enzymes from the crystalline style (Gosling, 2004). During the mixing and extracellular digestive processes the stomach contents come under the influence of ciliary tracts that cover large areas of the stomach.

In the digestive gland of bivalves, the stomach branches in blind-ending tubules by several ciliated ducts (Fig. 5). Within these ducts there is a continuous two-way flow (Fig. 6): materials enter the gland for intracellular digestion and absorption and wastes leave in route to the stomach and intestine.

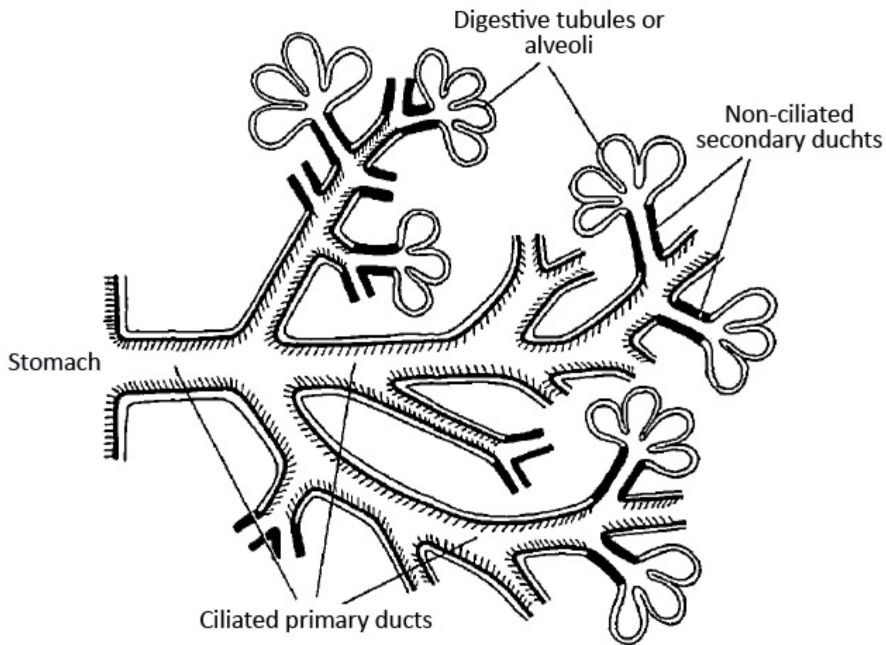

Fig. 5. The duct system of the digestive diverticula of bivalves (adapted Owen, 1955).

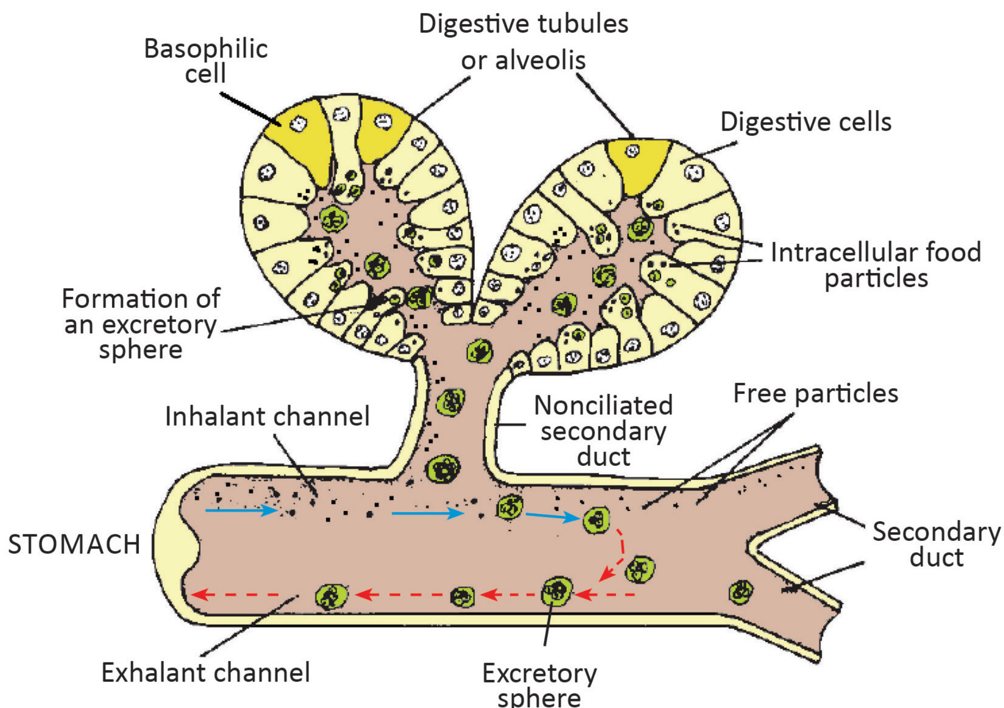

Fig. 6. A section of de digestive gland of bivalves showing absorption and intracellular digestion of material coming from the stomach (solic arrows) and outward movement of wastes (broken arrows). Adapted from Owen (1955).

The epithelium of blind-ending digestive tubules (or alveolis as have been called in this work) are composed of two cell types, digestive cells and basophilic cells (Morton, 1983) (Fig. 7). Under normal conditions, digestive cells are the most abundant type (Fig. 7) (Rasmussen et al., 1983; Cajaraville et al. 1990; Marigómez et al. 1990; Zaldibar et al., 2008). They are columnar, characterized by a well-developed endolysosomal system and responsible for intracellular digestion of food (Owen, 1972; Morton, 1983). Digestion takes place within lysosomes that contain hydrolytic enzymes. Lysosomes are highly conserved multi-functional cell organelles containing acid hydrolases (pH 4.5-5.5) that are present in almost all eukaryotic cells and which are dealing with the intracellular digestion of nutrients (Allison and Young, 1969). The endolysosomal compartment is a system of vacuoles that constantly fuse with (a) secretory vesicles pinched off from Golgi apparatus to receive new hydrolytic enzymes and; (b) autophagosomes and late endosomes (Owen, 1973). Thus, they are involved in the degradation of redundant or damaged organelles (i.e., mitochondria and endoplasmic reticulum) as part of the autophagic cellular turnover (Moore et al., 1996; Klionsky and Emr, 2000; Moore et al., 2006) and in the digestion of materials ingested by endocytosis (Moore, 1985; Cajaraville et al., 1995b).

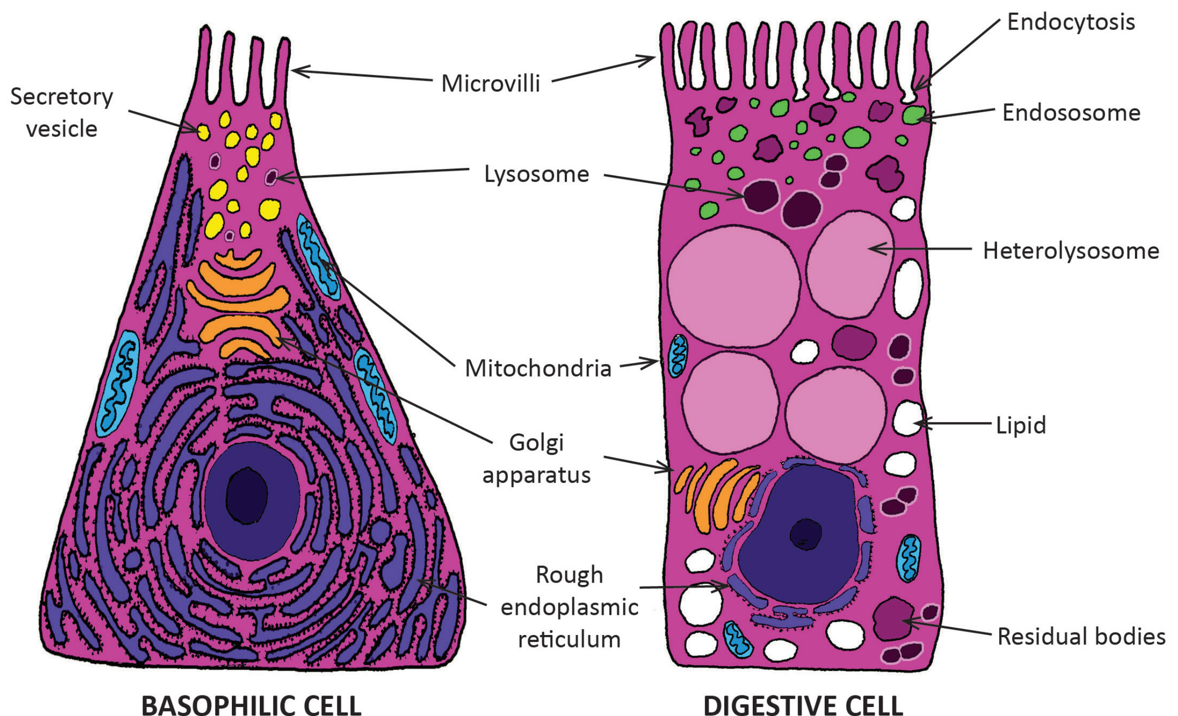

Fig. 7. Schematic view of the ultrastructure of the two cells of the digestive epithelium of bivalves (redraw from Owen 1973).

After degradation of these intracellular and extracellular substrates into building blocks, such as single sugars and amino and fatty acids, can be reutilized for synthesis of complex biomolecules after active or passive transport into the cytosol (De Duve and Wattiaux, 1966). Intracellular digestion is a dynamic process, where four phases related to feeding and tidal rhythm can be morphologically distinguished (Langton, 1975; Robinson and Langton, 1980;

Morton, 1983; Owen, 1996): Type I or holding phase; type II or absorpting phase; type III or disaggregating phase; and type IV or reconstitution phase (Fig. 8).

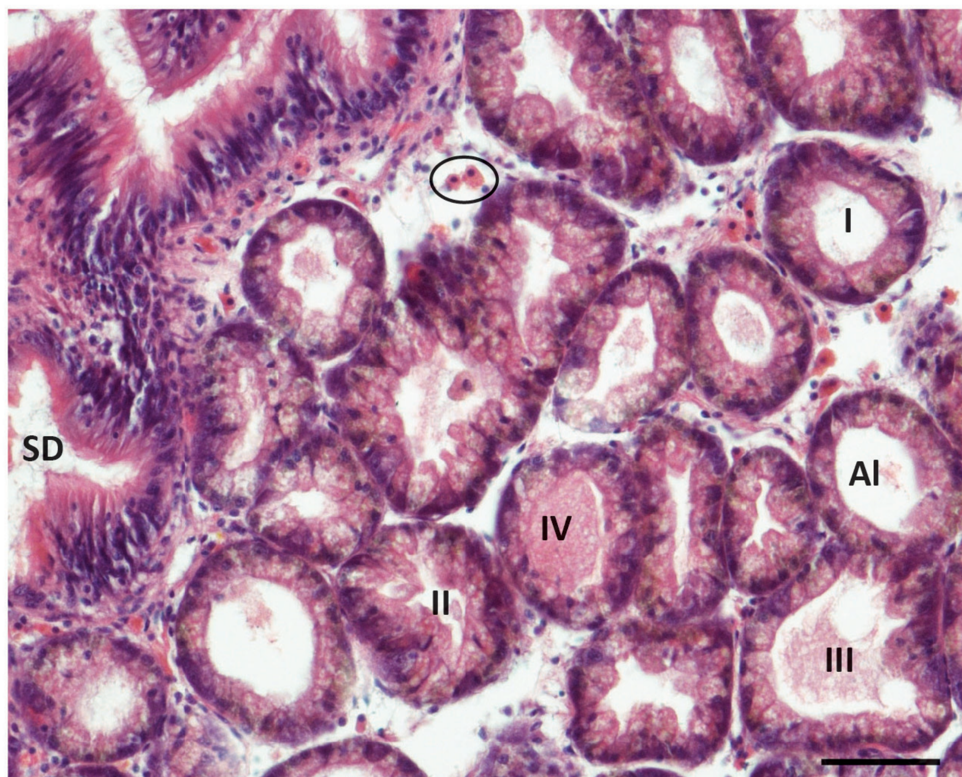

Fig. 8: Micrograph of the digestive gland of mussel *M. galloprovincialis* stained with hematoxylin-eosin. I:holding phase, II: absorpting phase, III: disintegrating phase, and IV: reconstituting phase. SD: secondary duct. Al: digestive alveoli. Circle: haemocytes. Scale: 50 µm.

Basophilic cells are pyramidal in shape (Fig. 7) and much of their cytoplasm is filled with rough endoplasmic reticulum (ER) and Golgi bodies, which indicates that these cells play a main role in protein synthesis. They may play a role in enzyme secretion for extracellular digestion in lumen of alveolus, but the exact role of secretory cells is unclear (Weinstein, 1995). Lekube et al. (2000) and Izagirre et al. (2009) suggested that lysosomal enzyme transfer might occur from basophilic to digestive cells. Thus, basophilic cells, which possess a well-developed ER, would transfer acid hydrolases to digestive cells, which have less developed ER and Golgi apparatus and an extraordinary demand of lysosomal enzymes to supply the highly developed endo-lysosomal system that to a great extent needs to be renovated every digestion/tidal cycle. Apart from the digestion process, digestive gland participates in detoxification and elimination of xenobiotics and accumulates pollutants. As such, digestive gland is one of the main target tissues in toxicology when the biomarker approach is applied (ICES, 2012). Thus, the understanding of digestive process and digestive gland functioning is relevant, in order to better interpret biomarkers.

Biomarkers

As mentioned above, mussels are widely used organisms to assess biological responses to pollutants in field and laboratory studies based on biomarkers approach (Moore et al., 2007; ICES, 2012; Beyer et al., 2017; Faggio et al., 2018). The biomarker concept was initially applied in medical diagnostics as an indicator of a particular state or disease in humans (Paone et al., 1980), and in the early 1990s, it became very appealing in environmental studies (McCarthy and Shugart, 1990; Walker, 1992; Depledge and Fossi, 1994; Peakall, 1994). Between 1990 and 2017, the total number of scientific publications concerning "environmental biomarkers" has increased by an average of 12% per year since 2000, and currently according to Web of Science (2017) more than 800 works are published every year (Fig. 9).

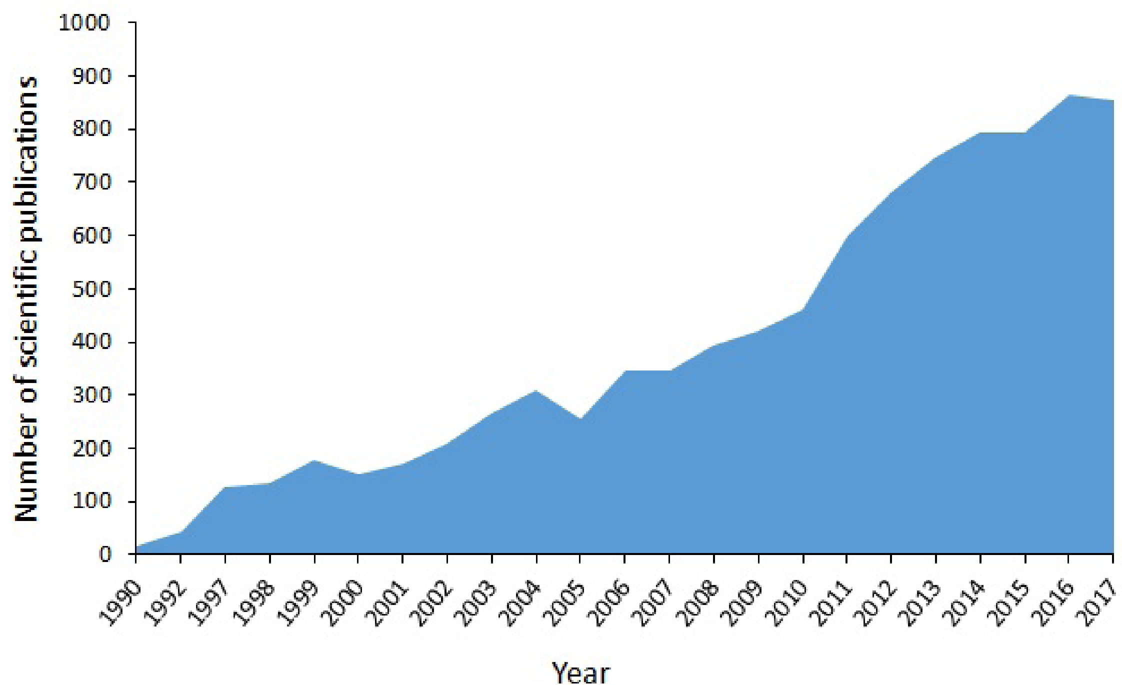

Fig. 9. Number of scientific publications published annually containing keywords environmental biomarker (Web of Science, 26/05/2018).

In environmental toxicology, biomarkers are considered measurements at biochemical, cell or tissue level, which indicate the presence of pollutants (exposure biomarkers) and/or the magnitude of its response (effects biomarkers) (McCarthy and Shugart, 1990). These early warning responses occur at low levels of biological complexity and allow the prediction, at longer-term, at more complex levels of biological complexity (Fig. 10). It is highly recommended to use a battery of biomarkers at different biological levels, such as biochemical, cellular and tissue-level, since an individual biomarker only provides partial information about the presence of pollutants as well as about their biological consequence (UNEP/RAMOGE, 1999).

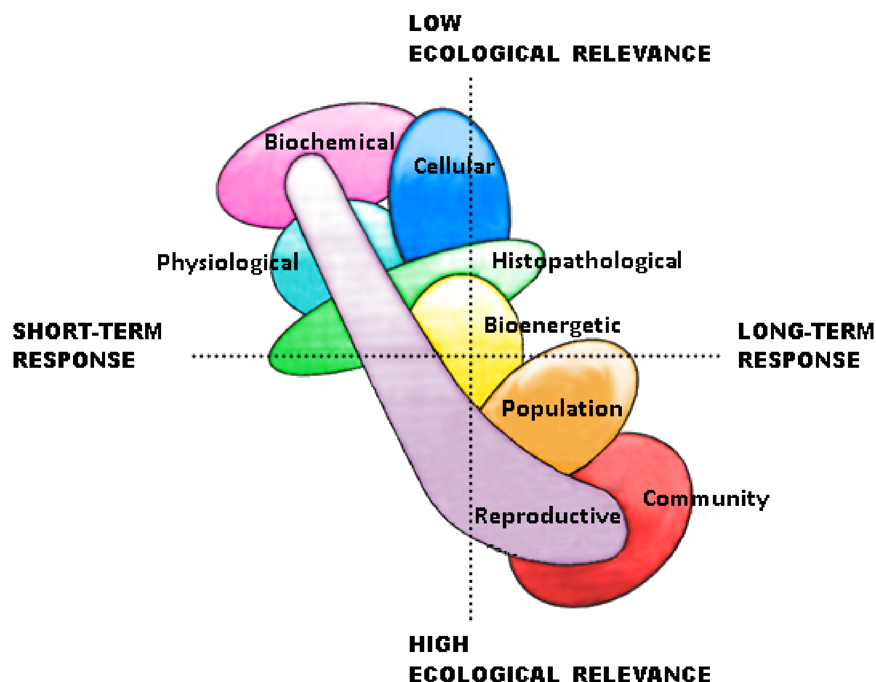

Fig. 10. Link between biological responses at different levels of biological complexity and their relevance at time-scales. (Modified by Van der Oost et al. 2003 based on Adams et al. 1989).

Biochemical biomarkers

Most pollutants are known to alter the cellular balance between prooxidant challenge and antioxidant defences, depressing or increasing antioxidant capacity (Di Giulio et al., 1989; Regoli and Giuliani, 2014). Marine organisms, such as mussels, possess a high responsiveness in enzymatic antioxidant defences after the exposure to multiple stress and thus, antioxidant enzymes activity are widely apply as biomarkers of oxidative stress (Valavanidis et al., 2006; Lacroix et al., 2015). Enzymes involved in antioxidant defenses exist as a coordinated system and include superoxide dismutase which catabolizes superoxide radicals, and catalase (CAT) and glutathione peroxidase (GPx) which degrade hydrogen peroxide and hydroperoxides, respectively (Fig. 11). Secondary enzymes in antioxidant defense include those of glutathione metabolism. Glutathione S-transferase (GST) catalyzes the conjugation of reduced glutathione to nucleophilic xenobiotics or cellular components damaged by ROS attack which leads to their detoxification. NADPHdependent glutathione reductase (GR) replenishes the GSH substrate for GPx and GST from oxidized glutathione.

Apart from oxidative stress, pollutants can also provoke neurotoxicity (Payne et al., 1996; Galgani and Bocquene, 2000). In order to detect neurotoxicity, the acetylcholinesterase (AChE) enzyme activity changes are widely used as biomarker, because the commonly observed inhibition of AChE is linked directly with the mechanism of toxic action of organophosphorus and carbamate insecticides that bind to the catalytic site of the enzyme (Guilhermino et al., 1998; Sarkar et al., 2006; ICES, 2012; Matozzo et al., 2018). AChE

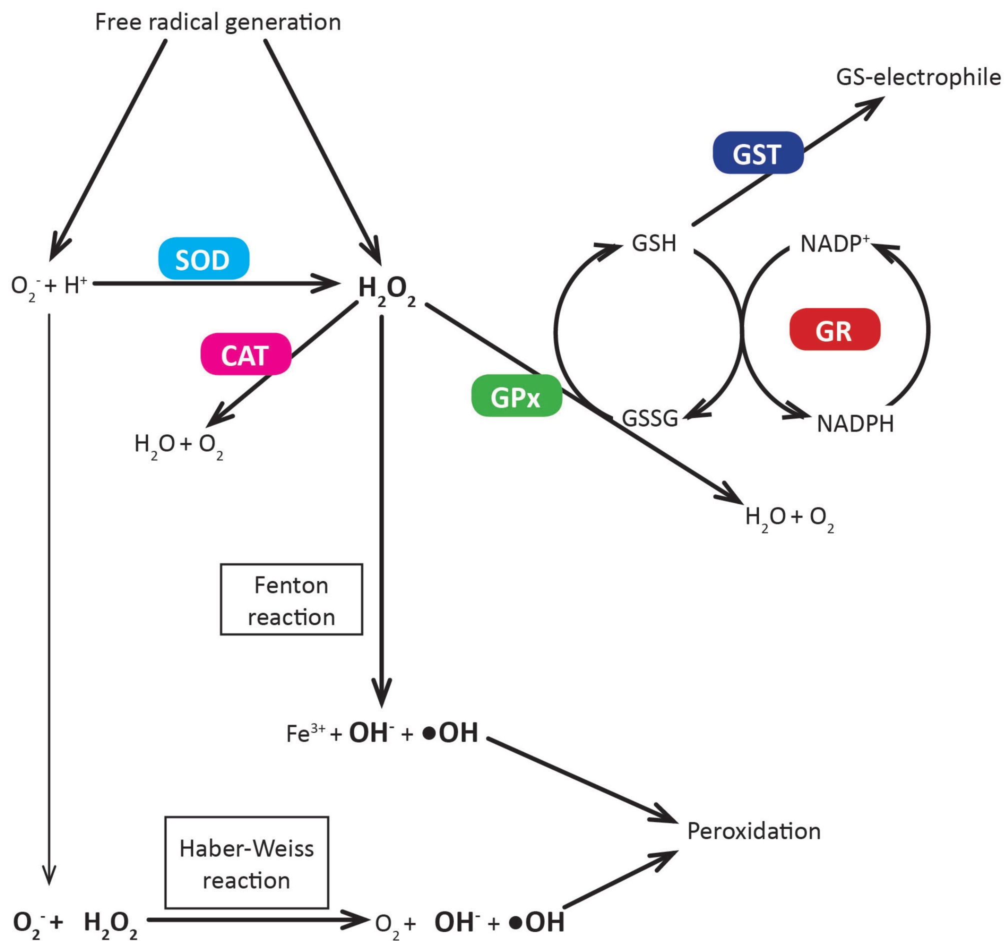

Fig. 11. Summary of the pathways for the generation of reactive oxygen species and of the actions of some of the enzymes involved in antioxidant defenses in the cell. Scheme modified from Storey 1996.

is responsible for the signal termination at cholinergic synapses by rapid hydrolysis of the neurotransmitter acetylcholine, preventing continuous nerve firings, which is vital for normal functioning of sensory and neuromuscular systems (Murphy, 1986) (Fig. 12).

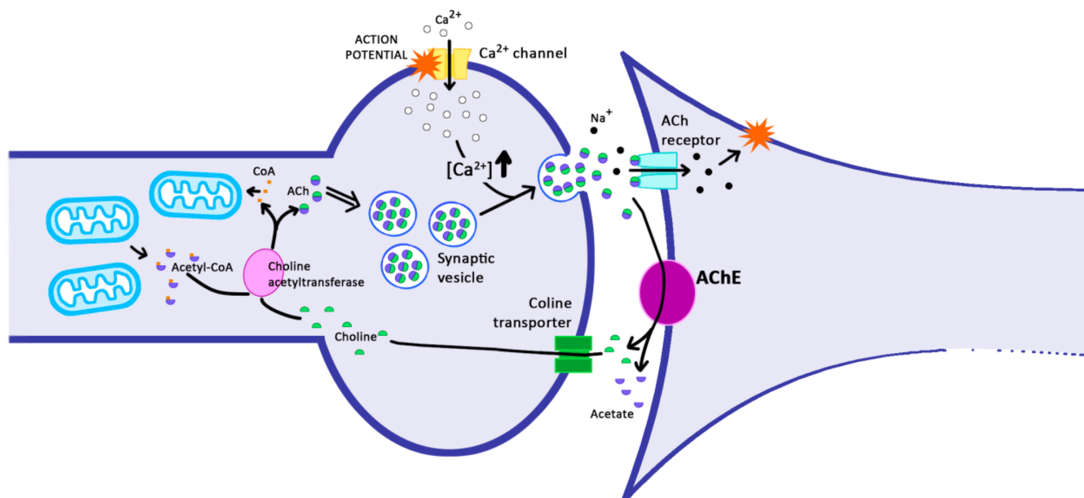

Fig. 12. Nervous transmission scheme.

Modulation of energy metabolism is a key aspect of the stress response, and therefore, energy-related biomarkers can be useful tools to assess physiological stress (Kültz, 2005; Sokolova et al., 2012; Lacroix et al., 2015). Energetic metabolism can be affected directly and/or indirectly by pollution via disruption of ATP-producing pathways and increased metabolic costs (Sokolova et al., 2012). Bioenergetic markers such as pyruvate kinase/phosphoenolpyruvate carboxykinase (PK/PEPCK) and cytochrome c oxidase (COX), are consider as proper biomarkers for the assessment of aerobic capacity. PK is a key energy enzyme of the conversion of phosphoenolpyruvate (PEP) and ADP to pyruvate and ATP in glycolysis and plays a role in regulating cell metabolism (Cameselle et al., 1980) (Fig. 13). PEPCK is involved in both gluconeogenesis and anaerobic pathways of energy production by catalyzing the reversible decarboxylation of oxaloacetate to PEP (Fig. 13). Low PK/PEPCK ratio is considered an index of low aerobic flux and relatively higher reliance on anaerobic pathways (Sokolova and Pörtner, 2001). Moreover, COX is the final enzyme in the mitochondrial electron transport chain that transfers electrons from reduced cytochrome *c* to hydrogen, thereby producing water molecules (Antonini and Brunori, 1970). COX regulates oxidative phosphorylation, proton pumping, and ATP production (Arnold, 2012) and thus, COX is considered a marker of mitochondrial density and capacity (Lucassen et al., 2003; Morley et al., 2009) (Fig. 13).

Sies (1991) defined oxidative stress as a disturbance in the prooxidant-antioxidant balance in favor of the former, leading to potential damage. Such damage is often called oxidative damage, which has been defined as the biomolecular damage caused by attack of reactive species upon the constituents of living organisms (Halliwell and Whiteman, 2004). Increased oxidative damage can result not only from more oxidative stress, but also from failure to repair or replace damaged biomolecules, such as lipids and proteins.

Radical attack on polyunsaturated fatty acids can provoke lipid peroxidation in a membrane, which can disrupt its function by altering fluidity and eventually, membrane rupture (Halliwell 1992). Therefore, lipid peroxidation has been reported as a major contributor to the loss of cell function under oxidative stress situations. The ROS induced peroxidation end products (α, β unsaturated reactive aldehydes, such as malondialdehyde - MDA, 4-hydroxy-2-nonenal - HNE, acrolein and isoprostanes) have also deleterious effects on proteins and DNA, which can promote alterations of signaling pathways and gene mutations, respectively (Esterbauer et al. 1991, Trachootham et al. 2008, Aprioku et al. 2013) (Fig. 14).

Proteins are important targets for radical reactions (Dalle-Donne et al., 2003; Žitňanová et al., 2007). Oxidative modification of proteins may occur via various mechanisms: direct oxidation of amino acid side chains, modification of side chains with lipid peroxidation products (MDA, HNE), and with products of glycation and glycoxidation. All of these mechanisms introduce a carbonyl group into a protein and may lead to the loss of its biological activity (Berlett and Stadtman, 1997). Therefore, protein carbonyls may serve as a biomarker of general oxidative

stress (Žitňanová et al., 2007). Attack on proteins can lead to the modification of amino acids, oxidation of sulfhydryl groups leading to conformational changes, altered enzymatic activity, crosslinking, peptide bond cleavage as well as carbohydrate modification in glycoproteins, loss of metal in metalloproteins, altered antigenicity, and increased proteolytic susceptibility (Stadtman, 1992; Sies, 1993).

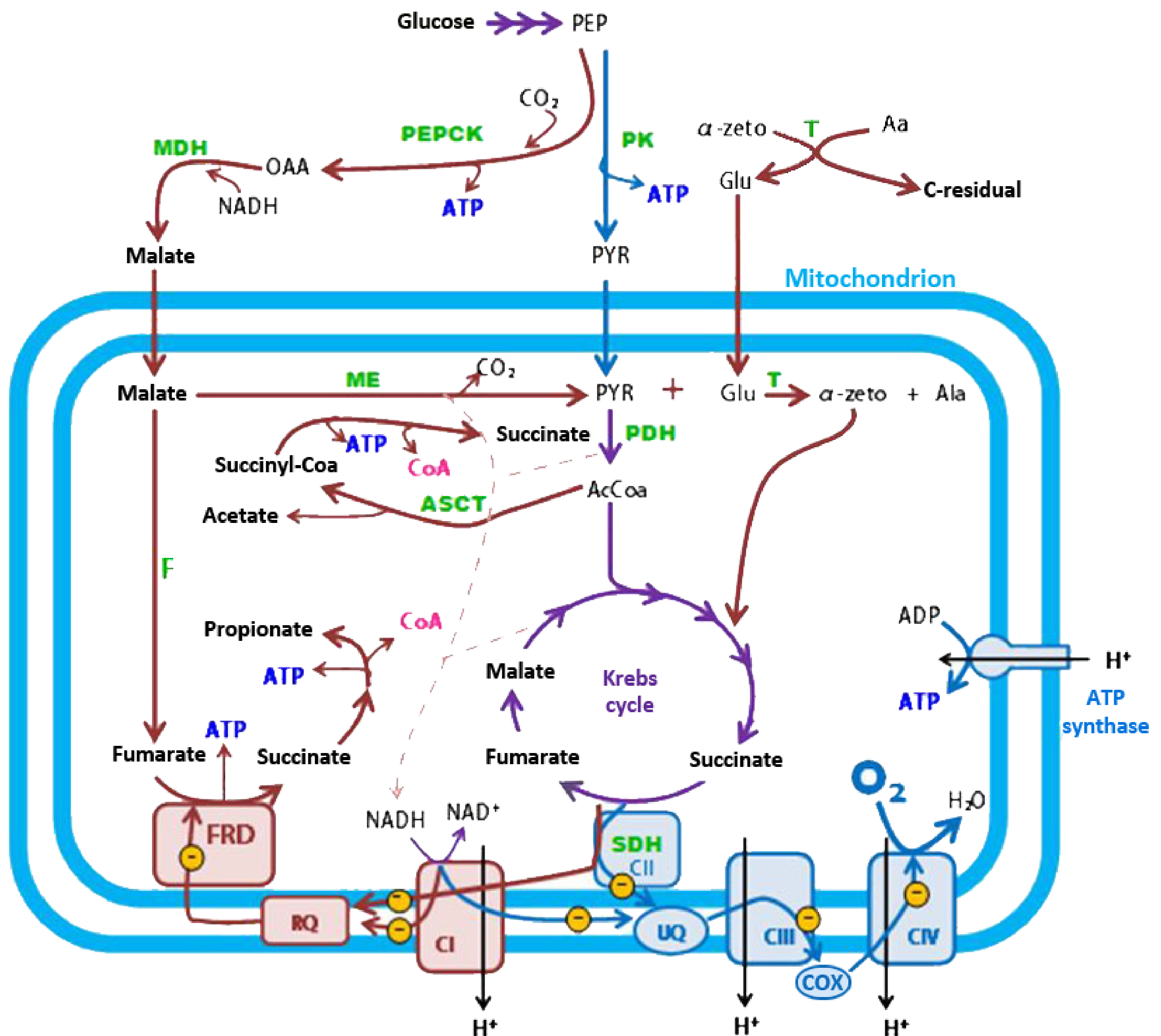

Fig. 13. Carbohydrate degradation pathways in normoxic (blue arrows) and anoxic (red arrows) conditions in bivalves. Pathways, indicated with purple arrows, are carried out in both oxygen conditions. Abbreviations: α-ceto, α-ketoglutaric acid; aa, amino acid; AcCoA, acetyl-CoA C; Ala, alanine; ASCT, acetate succinate CoA transferase; (I, II, III, IV), complex enzyme (I, II, III, IV); COX, cytochrome C oxidase; F, fumarase; FRD, fumarate reductase; Glu, glutamate; ME, malic enzyme; MDH, malate dehydrogenase; OAA, oxalacetate; PDH, pyruvate dehydrogenase; PEP, phosphoenolpiruvate; PEPCK, phosphoenolpyruvate kinase; PK, pyruvate kinase; PYR, pyruvate; RD, rodoquinone; T, transaminase; SDH, succinate dehydrogenase; and UQ, ubiquinone.

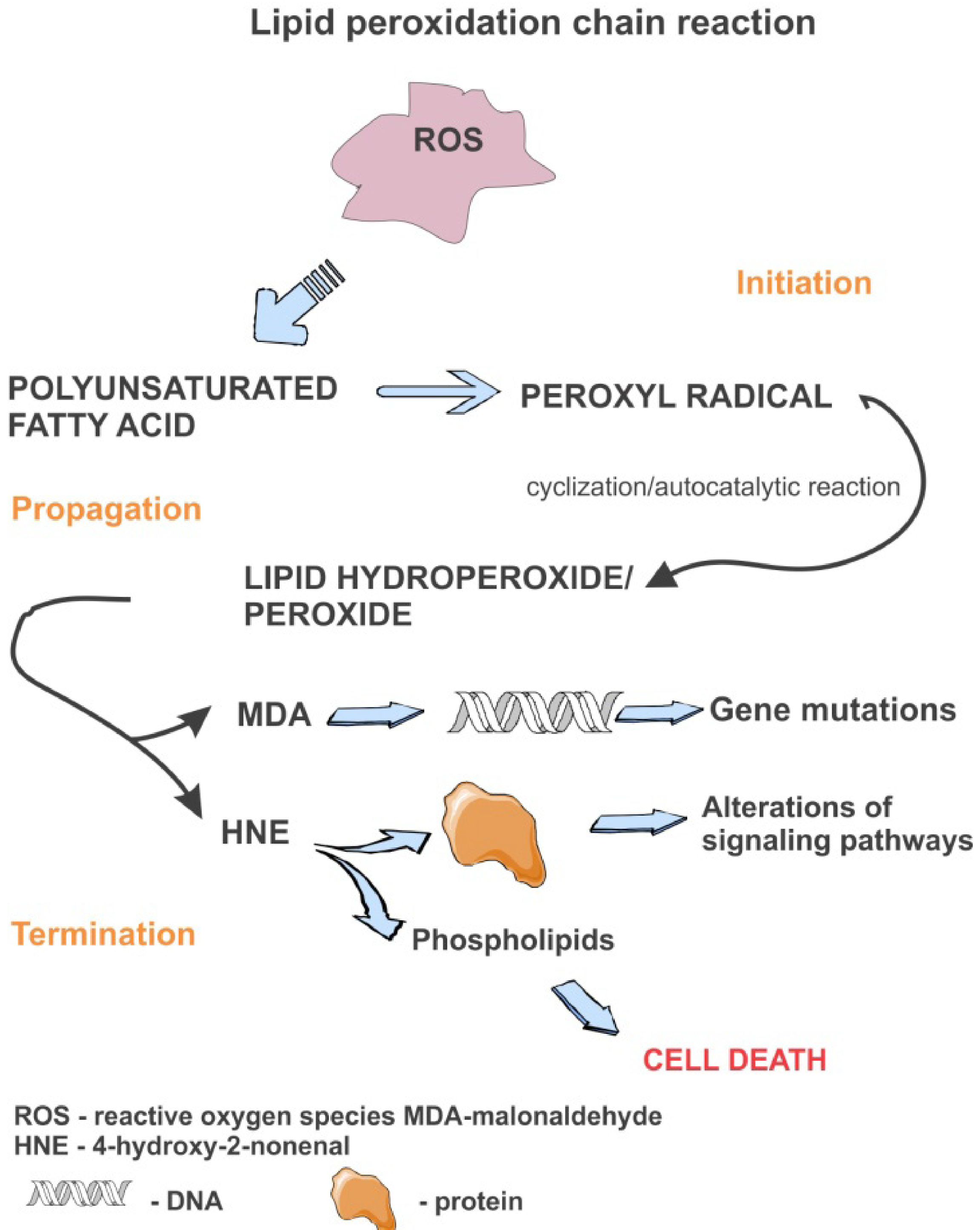

Fig. 14. ROS-induced lipid peroxidation. Image obtained from Andreazza and Scola (2015).

Lysosomal biomarkers

Lysosomal responses are widely used as effect biomarkers especially in mussels and other bivalve molluscs, whose digestive cells are very rich in lysosomes (Cajaraville et al., 1995a; UNEP/RAMOGE, 1999; Cajaraville et al., 2000; JAMP, 2003; ICES, 2012). Apart from

their main functions in intracellular digestion of ingested material (Robledo et al., 2006; Cajaraville et al., 1995a) and autophagic processes (Klionsky and Emr, 2000; Moore et al., 2006, 2007), lysosomes of digestive cells play an important role in responses to toxic compounds through the sequestration and accumulation of pollutants (Domouhtsidou and Dimitriadis 2001; Soto et al., 2002; Marigómez and Baybay-Villacorta, 2003; Moore et al., 2004; Einsporn et al., 2005; Izagirre et al., 2008; Raftopoulou and Dimitriadis, 2011). Eventually, lysosomal responses to environmental stress could be divided in three main categories (Marigómez and Baybay-Vilacorta, 2003; Marigómez et al., 2005b): (a) reduction of lysosomal membrane stability, (b) increase of lysosome size and (c) changes in lysosomal contents such as accumulation of unsaturated neutral lipids (Fig. 15).

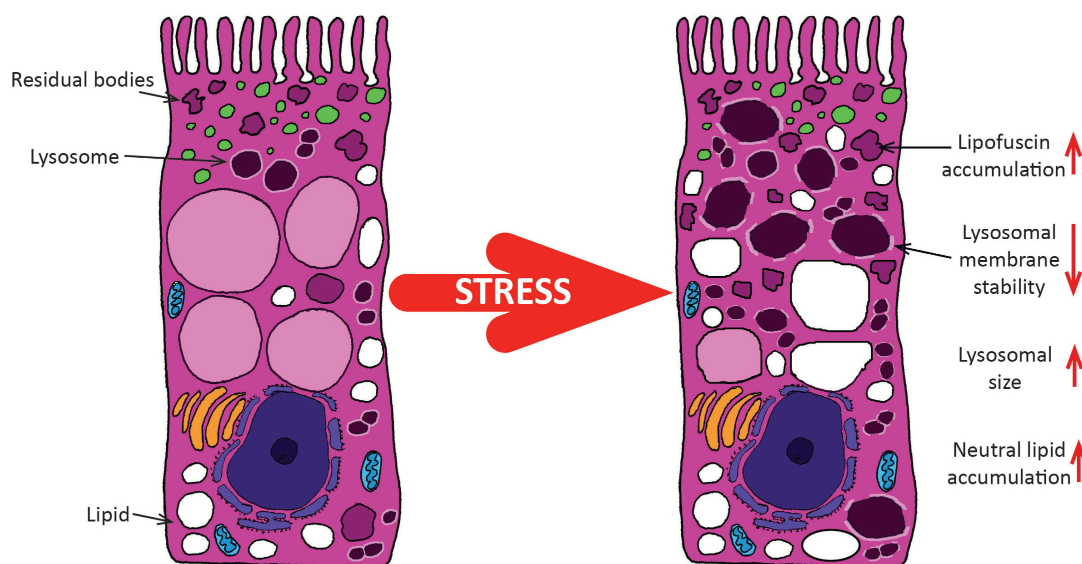

Fig. 15. Lysosomal responses to stress.

The lysosomal membrane destabilisation 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 (ICES, 2012), and considered as a core biomarker in the Baltic Sea Action Plan (HELCOM, 2012) and the Mediterranean Ecosystem Approach (EcAp) (UNEP/MAP, 2014). Thus, membrane destabilisation constitutes the main lysosomal response to a wide range of pollutants and it is a very reliable biomarker of general stress in biomonitoring studies (Regoli, 1992; Krishnakumar et al., 1994; Domouhtsidou and Dimitriadis, 2001; ICES, 2015).

Lysosomal membrane stability (LMS) test is based on the demonstration of the latent activity of lysosomal hydrolases (most commonly N-acetyl-ß-hexosaminidase) and it has been successfully applied to assess lysosomal integrity since the pioneering work by Bitensky et al. (1973) (Fig. 16A). The time needed for the increase of the permeability of the substrate into lysosomes is considered the labilisation period (LP) (UNEP/RAMOGE, 1999). Significant decreases in LP have been reported on exposure to organic chemicals (Moore, 1988; Krishnakumar et al., 1997; Moore et al., 2007) and metals (Harrison and Berger, 1982;

Regoli, 1992; Moore et al., 2007; Izagirre et al., 2014). In mussels, LP values higher than 20 to 25 min indicate a healthy status, whereas LP values <10 min indicate a disturbed health or severe stress situation (Viarengo et al., 2000; Izagirre and Marigómez, 2009) which has been reported, for example, after exposure to organochemical compounds (Moore et al., 1987).

Together with the membrane destabilisation in lysosomes, lysosomal enlargement has been included in monitoring guidelines for the assessment of biological responses to pollutants (ICES, 2012). In general terms, lysosomes become enlarged under stress conditions, which are reflected as increases in volume density of lysosomes (high volume density of lysosomes (Vv_{LYS}) and low surface-to-volume ratio (S/V_{LYS} values; inverse to lysosomal size) (Cajaraville et al., 1995; Marigómez et al., 2005). In certain cases, lysosomal enlargement is accompanied by increased the number of lysosomes (Nv_{LYS}, (more lysosomes relative to digestive cell cytoplasm), but reductions in Nv_{LYS} have also been reported. On the other hand, exposure to pollutants may also elicit an intricate response that includes different phases (Marigómez and Baybay-Villacorta, 2003): (a) transient lysosomal enlargement; (b) transient lysosomal size reduction; and finally (c) lysosomal enlargement after long-term exposure. Lysosomal structural changes (LSC) are commonly determined by image analysis of digestive gland cryotome sections where β-glucuronidase is employed as lysosomal marker enzyme (Cajaraville et al., 1991; Izagirre et al., 2008, 2009; ICES, 2012) (Fig. 16B). Overall, reference values for these lysosomal parameters vary with season, but $Vv_{LYS} > 0.002$ $\mu m^3/\mu m^3$ and $S/V_{LYS} < 5$ may be indicative of the existence of a degraded health status in mussels (Marigómez et al., 2006; Garmendia et al., 2010; ICES, 2012).

Regarding changes in lysosomal contents, intracellular accumulation of neutral lipids is considered as exposure biomarker linked mainly to organic chemical pollution (Moore, 1988; Domouhtsidou and Dimitriadis, 2001; Marigómez and Baybay-Villacorta, 2003). Increased lysosomal accumulation of neutral lipids is also possibly related to autophagy of excess lipid droplets (Moore et al., 1987a; Lowe, 1988; Moore, 1988; Krishnakumar et al., 1994) or may be attributed to lipid peroxidation in which free radicals participate in the formation of the pigment lipofuscins that represents indigestible residue of injured cells components (Donato, 1981; Halliwell and Gutteridge, 1984). The intracellular neutral lipid accumulation is commonly determined by image analysis of digestive gland cryotome sections after staining with Oil Red O (ORO; Culling, 1974) (Fig. 16C), in terms of volume density of neutral lipids with respect to the digestive epithelium volume (Vv_{NL}; $\mu m^3/\mu m^3$) (Dondero et al., 2006; Gomiero et al., 2015) .

Lipofuscin are pigments considered as end products of lipid peroxidation (Cheung et al., 2001). In response to pollutants, lipofuscins are accumulated in digestive cells (Viarengo et al., 1990). Enhanced lipofuscin accumulation has been reported in digestive cells of mussels exposed to PAHs under both laboratory and field conditions (Krishnakumar et al., 1994). The lipofuscin accumulation is commonly determined by image analysis of digestive gland

cryotome sections after staining with Schmorl's reaction (Pearse, 1972) (Fig.14D), in terms of volume density of lipofuscins with respect to the digestive epithelium volume (Vv_{LPF}; $\mu m^3/\mu m^3$).

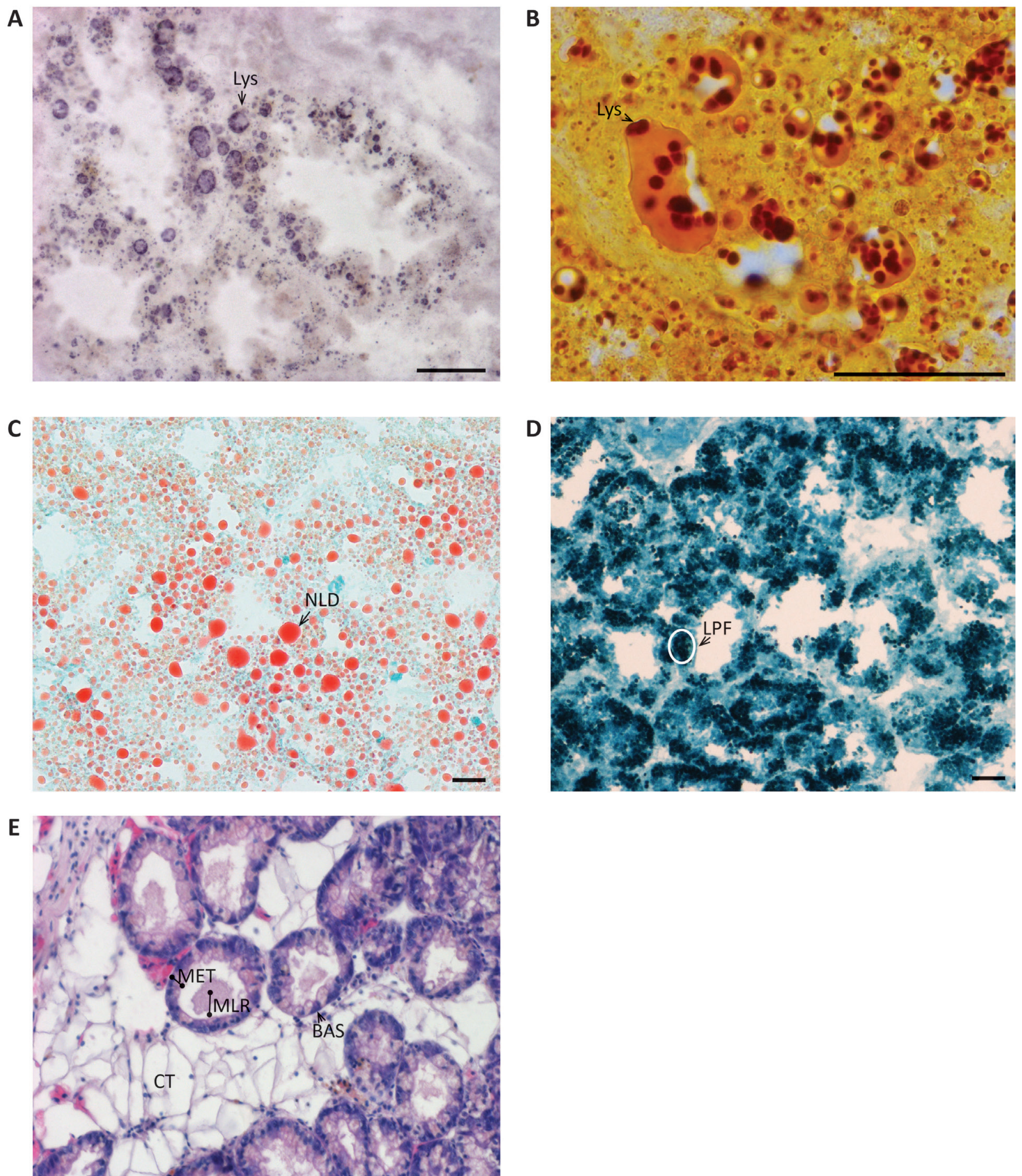

Fig. 16. Micrographs of hexosaminidase (LMS test; A) and β-glucuronidaseenzyme histochemistry (LSC test; B), Oil Red O staining (neutral lipid accumulation: NLA; C), Schmorl's staining (lipofuscins: LPF; D), and hematoxyline-eosine staining (basophilic cell: BAS; MLR: mean luminal radio; MET: mean epithelial thickness; CT: connective tissue). Scale bar: 30 µm.

Tissue-level biomarkers

The assessment of both, the cell-type composition of the digestive gland epithelium and the changes in the structure of the digestive diverticula are important tools in the study of the environmental health assessment (Marigómez et al., 1998; Cajaraville et al., 2000; Zaldibar, 2006; ICES, 2012). Typically, these tissue-level biomarkers have been applied using a stereological procedure on hematoxylin and eosin-stained, digestive gland paraffin sections (Soto et al., 2002; ICES, 2012) (Fig. 16E).

Under normal physiological conditions the digestive cells outnumber basophilic cells, but under different stress situations, the relative proportion of basophilic cells increases (Yoshino, 1976; Syasina et al., 1997; Marigómez et al., 1992, 1993; Soto et al., 2002; Zaldibar et al., 2007). The increase in the relative proportion of basophilic cells is usually associated to degenerative changes in digestive cells related to pollution effect (Thompson et al., 1974; Rasmussen et al., 1983; Cajaraville et al., 1990; Marigómez et al., 1990; ICES, 2012) (Fig. 17). This alteration in the cell type composition of the digestive epithelium may cause disturbances in food digestion and xenobiotic metabolism and accumulation (Marigómez et al., 1998). These changes were initially attributed to basophilic cell proliferation (Cajaraville et al., 1989; Lowe and Clarke, 1989; Marigómez et al., 1990), but further on it was concluded that it mainly results from digestive cell loss and basophilic cell hypertrophy (Zaldibar et al., 2007). It is a fast, inducible, and reversible response that can be measured in terms of volume density of basophilic cells (Vv_{BAS}). After exposure to pollutants, Vv_{BAS} may surpass 0.12 $\mu m^3/\mu m^3$ (Marigómez et al., 2006; Garmendia et al., 2010).

Among tissue-level biomarkers, the best documented tissular alteration by pollutants in

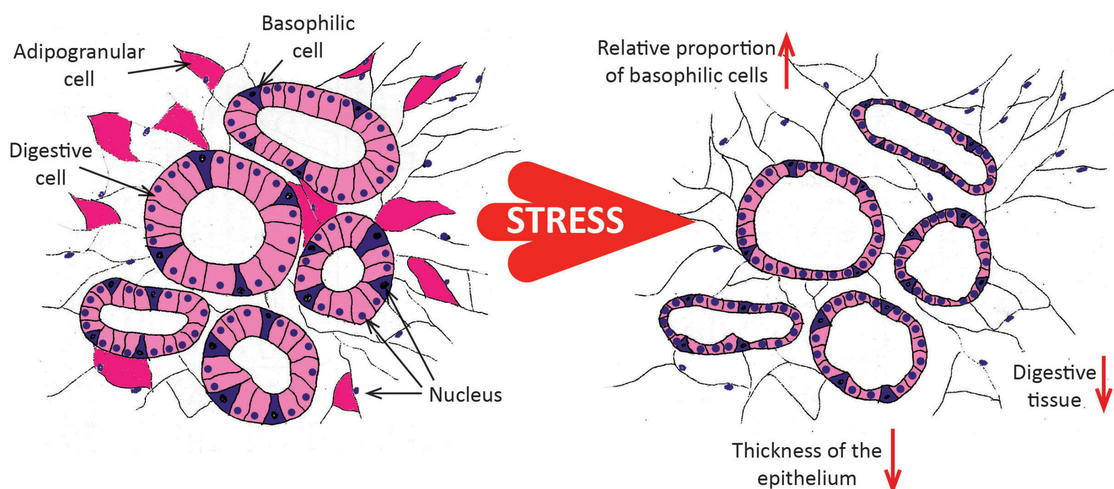

Fig. 17. Biological responses to stress at tissue-level. In stressed mussels, digestive epithelial thinning can made difficult distinguish between basophilic cells and nucleus of digestive cells.

mollusc is apparent atrophy or "thinning" of the digestive gland epithelium (Couch, 1984; Kim et al., 2006; ICES, 2012) (Fig. 17). The digestive gland of mussels is greatly dynamic and plastic. The morphology of digestive alveoli undergoes severe changes even during normal

physiological processes (i.e. trough every digestion cycle; Langton, 1975; Fig. 8). Changes in the normal phasic activity may be attributed to environmental factors, such as food availability or saline and thermal stress (Winstead, 1995) as well as exposure to pollutants. In particular, it has been widely demonstrated that molluscs exposed to pollutants exhibit a net mass loss in the digestive gland epithelium that gives rise to abnormal epithelial thinning and finally atrophy or less digestive tissue (Lowe et al., 1981; Couch, 1984; Lowe and Clarke, 1989; Vega et al., 1989; Cajaraville et al., 1992; Marigómez et al., 1993; Garmendia et al., 2010; Lopes et al., 2016). Epithelial thinning and atrophy constitute a non-specific, inducible, and slowly or not recoverable response to stressful environmental conditions that can be measured or in terms of mean luminal radio/mean epithelial thickness (MLR/MET) and connective tissue to digestive tissue (CTD) ratio (Garmendia et al., 2010, 2011; ICES, 2012). After exposure to pollutants, MLR/MET may surpass 0.7 µm/µm in spring and 1.2 µm/µm in autumn (Marigómez et al., 2006; ICES, 2012). There are not reference values for CTD.

“I AM I AND MY CIRCUMSTANCE” — José Ortega y Gasset, 1914, Meditaciones del Quijote

CONFOUNDING FACTORS AND BEST AVAILABLE PRACTICES

As it is mentioned before, *Mytilus sp.* are well recognized and widely used organism in toxicological experiments and monitoring for the assessment for the health status of marine environment, applying internationally recognized biomarkers (ICES, 2012; Faggio et al., 2018). The application of biomarkers is considered a good tool to assess the biological responses to pollutants, because their use made possible the early detection and ecological relevance determination of a pollutant exposure or stress situation in the environment, unlike chemical analysis. Thus, the used of biomarkers in mussels have been stablished in marine pollution monitoring programs worldwide (Viarengo et al., 2000; HELCOM, 2017; Chiu et al., 2018), because they are a useful tool to determined early biological effect of pollutants in sentinel organism, such as pollutants. Moreover, the use a battery biomarkers at different biological complexity levels respond to the whole suite of possible scenarios, providing a broader picture of the ecosystem health status in pollution monitoring programs (UNEP/RAMOGE, 1999).

Due to the interactions between pollutants and natural factors or between natural factors themselves, interpretation of biological responses to pollutants can be unreliable in monitoring programs. Determine the range of natural variability in biomarkers and how such variability might influence on correct interpreting of biomarkers in the assessment of the biological effects of pollution is of mayor importance (Izagirre et al., 2008; Vidal-Liñan and Bellas, 2013; Beyer et al., 2017). Seasonality affects markedly biochemical and lysosomal biomarkers (Garmendia et al., 2010; Nahrgang et al., 2013), due to the fluctuation in temperature, salinity, oxygen, gamete development, food availability and size/age. Antioxidant defense

enzymes, such as CAT, GST and GPx, show higher activity values in spring (Bochetti and Regoli, 2006; Nahrgang et al., 2013; Gonzalez-Fernandez et al., 2016), whereas lysosomes are enlarged (high Vv_{LYS} and low S/V_{LYS} values) in summer and their membrane is more destabilised (low LP values) (Etxeberria et al., 1995; Marigómez et al., 1996; Tremblay and Pellerin-Massicotte, 1997; Izagirre et al., 2008). The increased food availability in summer seems to lead to increased activity of the lysosomes, which become larger and more numerous (Etxeberria et al., 1995). Loss of lysosomal membrane stability has been related with latest stages of gametogenesis and spawning in *M. galloprovincialis* (Domouhtsidou and Dimitriadis, 2001; Garmendia et al., 2010; Lekube et al., 2014). Likewise, neutral lipid accumulation is linked with food availability and gamete development, being higher Vv_{NL} in summer and autumn (Cancio et al., 1999; Múgica et al., 2015). Lipofuscin accumulation increase typically in summer (Bochetti and Regoli, 2006; Brenner et al., 2014). Regarding tissue-level biomarkers, Vv_{BAS} does not seem to be influenced by season, whereas lower MLR/MET values have been recorded in early autumn (Garmendia et al., 2010).

Several laboratory studies have been carried out to study the temperature influence on biomarkers (Khessiba et al., 2005; Verlecar et al., 2007; Múgica et al., 2015). Increased activities of antioxidants, such as CAT, GPx, GR and GST, were recorded under long-term exposures to high temperatures (Khessiba et al., 2005; Verlecar et al., 2007). In the case of energetic biomarkers, temperature elevation reduced PK/PEPCK ratio (Múgica et al., 2015). Rising of temperature induced membrane destabilization (low LP) and lysosomal enlargement (high Vv_{LYS} and low S/V_{LYS}) in *M. galloprovincialis*, whereas tissue-level biomarkers were highly responsive only at extreme temperature of 28°C (Múgica et al., 2015).

Mussels as intertidal sessile organisms have to face to thermal stress, desiccation, anoxia, wave beating and reduce food availability during the periods of emersion. In contrast, subtidal mussels inhabit a less variable environment. The differences in biochemical and lysosomal biomarkers between mussels of different tide-level have been study by Vidal-Liñan and Bellas (2013) and Izagirre et al., (2008), respectively. GST activity in gills was higher in low tidemark level mussels (from 0.82 m tidemark level) than in high tidemark level mussels (2.32 m), whereas CAT activity in the digestive gland showed the highest values in medium high tidemark level mussles (1.82 m) and in contrast, tidemark level did not affected GPx and AChE (Vidal-Liñan and Bellas, 2013). Regarding lysosomal biomarkers, membrane of lysosomes was more stable in LT mussels (0.5 m) than in HT mussels (3 m), whereas lysosomes were more enlarged in LT mussels (Izagirre et al., 2008). Furthermore, focusing in how lysosomal biomarkers changes throughout a tidal cycle, size and number of lysosomes was low when mussels were immersed, whereas lysosomal membrane is stable (Izagirre et al., 2008). On the other hand, subtidal mussels would have almost continuous food availability, making cyclic digestion more subtle, which would possess a more stable and homogeneous endo-lysosomal system consist of abundant and small lysosomes (Izagirre et al., 2008).

The influence of salinity on biochemical and lysosomal biomarkers has been researched by few studies. Increased salinity rises CAT activity and reduces membrane stability, number

and size of lysosomes in mollusc (Bayne et al., 1981; Marigómez et al., 1991; Khessiva et al., 2005). On the other hand, it has been reported recently that mussels in resting stage of the gonad development presented low CAT and GPx, whereas mussels upon nutritive stress presented high CAT and GPx (Gonzalez-Fernandez et al., 2016). In lysosomal and tissue-level biomarkers, the influence of size/age of mussels was studied, determining that lowest LP values were recorded in older mussels and tissue-level biomarker were not influenced by age/size (Izagirre et al., 2014).

Studies about natural variability are needed to develop best available practices (BAPs) or recommendations for the correct interpretation of biomarkers in the assessment of the biological effects of pollution. Thus, for instance, after Izagirre et al. (2008, 2014) and Garmendia et al. (2010), it was determined that the BAPs for sampling is to handpick mussels between 3.5-4.5 cm shell length in early spring or autumn, in order to avoid the influence of the spawning period (normally, in late spring and late summer in Basque Coast) and low activity period in winter.

In Europe, three *Mytilus spp.* have been used in monitoring depending on region latitude: *M. galloprovincialis* (Lmk.) in Southern Europe, *M. edulis* (Lmk.) in Northern Europe, and *M.trossulus* (Gould) within the Baltic (Fig. 1). Thus, in long-scale pollution monitoring programs, the species of the mussel could be a source of variability for biological responses to pollutants. The various taxa in the *Mytilus* genus are difficult to distinguish without genetic identification (Kijewski et al., 2011; Brooks and Farmen, 2013). This can lead to misrepresentation of *Mytilus* with potential implications for biological effects assessment. Indeed, although the ability of the different *Mytilus* spp. to bioaccumulate contaminants is not fully known, few studies have reported differences in bioaccumulation and physiological level. For example, higher concentrations of heavy metals were measured in *M. trossulus* compared to *M. edulis* in mussels collected from the same field population and in the same size category (Lobel et al., 1990). On the other hand, Gardner and Thompson (2001) found that physiological responses of *M. trossulus* and *M. edulis* were similar in the same estuary. Thus, the effect of taxa on biomarkers of *Mytilus* genus is still unclear, and further research for the determination of the taxa influence on biomarkers is needed.

In studies of health status assessment based on biomarkers, BAPs for sampling and transport of mussels from the field to the laboratory are lacking. As a result, the procedures are disparate amongst studies (Table 1). Overall, whereas 96% of the studies dealt with quick responses that may occur within minutes to a few hours, only 18.5% reported to be based on in situ dissection practices and 52.3% recognized that mussels were transported to laboratory before dissection, and often without taken into account the condition of the mussels in their source site. Thus, intertidal mussels were transported in seawater at least in 19% of the cases and subtidal mussels were taken to the laboratory in air in cold boxes in 50% of the cases, with transport time varying from 1-2 hr to beyond overnight (Table 1). Noteworthy, Chandurvelan et al. (2013) concluded that transport in seawater or in air at 5°C (cold boxes) severely affects mussel physiology and for this reason transport in air at ambient temperature

Table 1. Mussel collection and sample processing used in recent coast monitoring studies based on the biomarkers approach. n.d.a: no data available; u.a.: upon arrival dissection; IT, intertidal; ST, subtidal; IT_{low}, low tide-mark level; IT_{sub}, below low tide-mark level. * Types of biomarkers depending on their endpoint and technology: (1) Functional and in vitro assays; (2) Biochemistry and molecular biology; (3) Cryotechnology and cytochemistry; (4) Histo(path)ology; (5) Biometry and physiology. ** Presumably: logical but not detailed in the source document.

Littoral zonation	Sample processing			Biomarkers *	References
	In situ	Transport (time)	Mainteinance at lab (time)		
n.d.a.	n.d.a.	n.d.a. (n.d.a.)	n.d.a. (n.d.a.)	1, 4	Bolognesi et al. 2004
				4	Cajaraville et al. 1990
				4	Dimitriadis and Papadaki 2004
				2	Fernández et al. 2010
				2	Gonzalez-Rey and Bebianno 2013
				3	Lowe and Fossato, 2000
				2	Minier et al. 2000
				3	Moore et al. 2007
				2	Nasci et al. 2002
				2,3	Orbea and Cajaraville 2006
				2,3	Orbea et al. 2002
				2,4	Ortiz-Zarragoitia and Cajaraville 2010
				2,3,4	Zorita et al. 2006
	Collection	n.d.a. (<2 hr)	without ** (u.a.)	2	Porte et al. 2001
		SW (n.d.a.)	n.d.a. (n.d.a.)	2	Micovic et al. 2009
		cold (n.d.a.)	n.d.a. (n.d.a.)	1,3,4	Dailianis et al. 2003
				3	Raftopoulou and Dimitriadis 2012
				2	Roméo et al. 2003
				1,2,3	Shaw et al. 2011
				2	Vlahogianni et al. 2007
		wet (15ºC; n.d.a.)	SW (24 hr)	1,2,5	Balbi et al. 2017
		wet (<2 hr)	without (u.a.)	2,3	Petrovic et al. 2001
				3,5	Petrovic et al. 2004
	Inmediate processing	-	-	2,4	Ortiz-Zarragoitia et al. 2011
				3	Garmendia et al. 2011
				3,4,5	Izagirre et al. 2014
				2,3,4,5	Marigómez et al. 2006
				1	Pisanelli et al. 2009
IT	Collection	cold (n.d.a.)	SW (overnight)	1,2	Emmanouil et al. 2008
			n.d.a. (n.d.a.)	2,4	Da Ros et al. 2007

Table 1. continuation

Littoral zonation	Sample processing			Biomarkers *	References
	In situ	Transport (time)	Mainteinance at lab (time)		
IT_{low}	Collection	n.d.a. (n.d.a.)	no (u.a.)	2,3	Cancio et al. 1999
		SW (n.d.a.)	n.d.a. (n.d.a.)	2	Azevedo et al. 2015
		SW (<2 hr)	without ** (u.a.)	1	Moreira and Guilhermino 2005
			without (u.a.)	2	Lima et al. 2007
		cold (n.d.a.)	n.d.a. (n.d.a.)	2	Bebianno et al. 2007
				1,2,4,5	Lacroix et al. 2015
			SW (food; 24 hr)	2,5	Gonzalez-Fernandez et al. 2015
		cold (<24 hr)		5	Albentosa et al. 2012
		cold (n.d.a.)	without (u.a.) without (< 2hr)	3,4	Lekube et al. 2014
				2,5	Bellas et al. 2013
	Freezing (mussels)	frozen (n.d.a.)	frozen	3	Etxeberria et al. 1995
	Inmediate processing	-	-	2,3	Orbea et al. 1999
				2,3,4	Orbea et al. 2006
				2,3,4	Izagirre et al. 2014
				4	Soto and Marigómez 1997
IT_{sub}	Collection	cold (n.d.a.)	n.d.a. (n.d.a.)	1,4,5	Martínez-Gómez et al. 2017
		wet (2-4 hr)	without (u.a.)	2,3	Nahrgang et al. 2013
IT plus ST	Collection	cold (n.d.a.)	without (u.a.)	3	Marigómez et al. 2005b
ST	n.d.a.	n.d.a. (n.d.a.)	n.d.a. (n.d.a.)	3	Aarab et al. 2008
				2,3,4	De los Rios et al. 2012
				2,3	Dondero et al. 2006
				5	Erk et al. 2011
				1,2	Gorbi et al. 2008
	Collection	n.d.a. (n.d.a.)	SW (<24 hr)	1	Höher et al. 2012
		cold (n.d.a.)	n.d.a. (n.d.a.)	2	Barhoumi et al. 2014
				2	Vidal-Liñán et al. 2014
		cold (<2 hr)	SW (overnight)	2,3,4	Schiedek et al. 2006
		cold (n.d.a.)	without (u.a.)	2,3	Da Ros et al. 2011
		cold and wet (< 4 hr)	without ** (u.a.)	2,3,4	Brooks et al. 2012
		SW and cold (n.d.a.)	n.d.a. (n.d.a.)	1,2	Rank et al. 2007
		SW (<3 hr)	without (u.a.)	2,3	Kopecka et al. 2006
				2	Giarratano et al. 2013
		- wet - physiol (n.d.a.) - cold - bmk (n.d.a.)	- SW (food; 24 hr) - n.d.a. (n.d.a.)	1,5	Karacik et al. 2009
	Freezing (mussels)	-	frozen	2	Sureda et al. 2011
	On board	-	without **(u.a.)	2,3,4	Zorita et al. 2007

for the shortest possible time period is recomended, at least for intertidal mussels collected at the lowest tide-mark level. In contrast, the mussels are maintained for relatively long periods (1 to several days) by other authors in the laboratory in seawater at controlled temperature and with food supply, aimed at getting the mussels acclimatized and recovered from the stress caused by collection and transport (González-Fernández et al., 2015; Balbi et al., 2017). This approach seems to be useful for some late-responses that require experimental manipulation of the mussels in the laboratory endpoints (e.g. scope-for-growth; ICES, 2006). However, other biological responses such as e.g. oxidative stress or lysosomal biomarkers are known to be elicited within a few minutes to hours by changes in oxygen and food availability or in temperature (Regoli and Pricipato, 1995; Izagirre et al., 2009b; Vidal-Liñán and Bellas, 2013). Therefore, the biomarker data measured after "recovery" or "acclimatization" would be difficult to compare with field data obtained in other studies and, moreover, they would hardly reflect the status of mussels in the field. Therefore, as previously recognized by other authors (Chandurvelan et al. 2013, Beyer et al. 2017), it is urgent advance in the standardization of procedures for collection, transport and processing of mussels used as sentinels in biomarker-based biomonitoring programs.

Another potential source of variability in field and laboratory studies is the sex. Although the influence of mussel's sex on biomarkers has been scarcely investigated, there are studies that demonstrate differences between sexes in proteomic, metabolism level and bioaccumulation (Lobel et al., 1991; Ji et al., 2013, 2014 and 2016; Liu et al., 2014). In contrast, there also studies that determine no sex influence on molecular biomarkers (Zilbergerg et al., 2011; González-Fernández et al., 2017). Taking into account the not conclusive data about sex influence on biomarkers in the literature, studies are needed in order to develop clear guidelines in mussels. For fish already exist recommendations in order to assess biomarkers (ICES, 2012), but nothing is stablished for biomarkers in mussels.

In order to determine the influence of one factor on biomarkers, laboratory experiments are very useful tool due to the possibility to maintain under control the experimental conditions. However, among experimental conditions, feeding strategy is diverse and vary in amount, type and rate in laboratory experiments with mussels, including absence of additional food supply or supply of diverse commercial food products or a variety of live microalgae either in monocultures or in mixtures; frequently, as a matter of fact, not mention is made to food type or feeding conditions (Table 2). For example, in a non-exhaustive literature mining in which 75 classical and recent manuscripts were selected, the 16% of the papers did not even provide any indication of whether mussels were fed during experimentation, the 11% maintained mussels without additional food supply (particularly during short-term experiments), the 40% of the reports used live microalgae in monoculture (29%) or in mixtures (11%), and the 33% used commercial food of diverse origin (14 manufacturers), nature (lyophilized algae, flour, ...) and components (Table 2). Alas, there are few studies which have been research about the influence of feeding strategy on biomarkers and all of them agreed that feeding strategy (in terms of fasting, food quality or quantity) affect biomarkers and their responsiveness to pollution (Ibarrola et al., 2000; Moore et al., 2007; Gónzalez-Fernández et al., 2015). Thus,

the differences in food type between experiments could hamper the comparability between them; because biomarker responsiveness could be modulate by food type. So, further research about feeding strategy influence on biomarkers is needed in order to develop BAPs to maintain mussels during short and long-term experimentation in aquatic toxicology.

Table 2. Different diets, exposure times and stressors used in recent toxicological experiments with mussels. * Types of biomarkers depending on their endpoint and technology: (1) Functional and in vitro assays; (2) Biochemistry and molecular biology; (3) Cryotechnology and cytochemistry; (4) Histo(path)ology; (5) Biometry and physiology.

FOOD TYPE	FOOD COMPOSITION /SOURCE	TIME (wk)	STRESS SOURCE	Biomarkers	REFERENCES
Not reported		<1	Cu	2	Canesi et al., 1998
			Diclofenac	1, 2	Fontes et al., 2018
			B[a]P	4	Speciale et al., 2018
		1	Cu, Hg, CH_3Hg	2	Canesi et al., 1999
			H_2O_2	2, 3	Cavaletto et al., 2002
		2	Drugs	2	González-Rey & Bebianno, 2012
				1, 2	Mezzelani et al., 2016
		4	Chemical mixtures	2	Giuliani et al., 2013
			Cd and thermal	1, 2	Nardi et al., 2017
		5	Cd	2	Serafim et al., 2002
			Crude oil WAF	1	Taban et al., 2004
		6	Cd and thermal	2, 5	Bebianno & Serafim, 1998
No additional food supply		<1	B[a]P	2	Banni et al., 2010
			TiO_2 NPs	1, 2, 3	Barmo et al., 2012
				3, 4	Jimeno-Romero et al., 2016
			PAHs	5	Guerra-Rivas et al., 2002
		1	Nickel	2	Attig et al., 2010
			B[a]P+ Cu	2	Maria & Bebianno, 2011
			Anoxia	2	Woo et al., 2011
		2	Cd-based QDs	4	Rocha et al., 2016
		3	Cd	1	Torre et al., 2013

Table 2. continuation

FOOD TYPE	FOOD COMPOSITION /SOURCE	TIME (wk)	STRESS SOURCE	Biomarkers	REFERENCES
Microalgae pure culture	*Isocrhrysis galbana*	<1	Cd thermal	3	Múgica et al., 2015
			Osmotic	4	Pipe & Moore, 1985
	Phaeodactylum tricornutum		Thermal	3	Moore et al., 2007
			PAHs and chloro-quine	3	
	I. galbana	1	ZnPT	2	Dallas et al., 2018
			4-Nonyl-phenol	2	Ricciardi et al., 2008
	P.tricornutum		Anoxia	3	Moore et al., 2007
			Paraquat	3	
			Cu and fasting	3	
	Scenedesmus subspi-catus		Drospire-none	2, 4	Cappello et al., 2017
	I. galbana	2	Thermal	2, 3, 4	Mugica et al., 2015
			Pyrene	3, 5	Okay et al., 2006
	P.tricornutum		Cu	3	Moore et al., 2007
			Pyrene	3, 5	Okay et al., 2006
	Tetraselmis sp.		Oiled food	2	Solé et al., 2007
	Chaetoceros muelleri		Polystyrene microbeads	1, 2	Paul-Pont et al., 2016
	I.galbana	3	Pesticides	2	Milan et al., 2018
	P. tricornutum		Cu, Phen and fasting	3	Moore, 2004
	I. galbana	4	Thermal	1, 2, 3	Dimitriadis et al., 2012
	Macrocystis pyrifera		Cu	3	Harrison & Berger, 1982
	I. galbana	8	Fluoran-thene	2, 5	Gonzaléz-Fernández et al., 2015
	P. tricornutum	26	Crude oil WAF	3, 5	Widdows et al, 1982
Microalgae mixture	*Chrysophyta, T. chui*	<1	Cd and *Vib-rio*	2	You et al., 2013
	I. galbana, C. gracilis, T. suecica	1	Saponin	2	Faria et al., 2018
	Isochrysis, Rhodomonas	3	Endocrine disruptors	2, 4	Ortiz-Zarragoitia & Cajaraville, 2006
	I. galbana, C. gracilis, T. suecica	4	PCB153	2	Vidal-Liñan et al., 2016
	I.galbana, R. baltica, S.costatum	30	Dispersed crude oil	1, 4	Bausant et al., 2011
Microalgae + OM	*C. neogracile, H. triquetra*	2	Fluoran-thene	1, 2, 5	Gonzaléz-Fernández et al., 2016

Table 2. continuation

FOOD TYPE	FOOD COMPOSITION /SOURCE	TIME (wk)	STRESS SOURCE	Biomarkers	REFERENCES
Microalgae + CF	*I. galbana* + SERA	3	CuO NPs	1, 2, 4	Ruiz et al., 2015
COMMERCIAL FOOD (CF)	Korall fluid	<1	Cd and crude oil WAF	3	Izagirre et al. 2009
			Cd and thermal	2, 3	Izagirre et al. 2014
	Liquifry		Cr (VI)	1, 2, 3	Barmo et al., 2011
			Pesticides	2, 3	Dondero et al., 2010
	KORAL	1	Cr (VI)	3	Franzellitti et al. 2012
	Phytofeast®	2	Drugs	2, 5	Solé et al., 2010
	Easy Reefs®	3	PE Microparticles	2, 4, 5	Brate et al., 2018
	Hawaiian Marine Imp Inc		Crude/Lubricant oil WAF	3	Cajaraville et al., 1991
			Organo-chemicals	2	Cancio et al., 1998
			Organo-chemicals	3	Marigómez & Baybay-Villacorta, 2003
	Marine Invertebrate Diet		B[a]P and Cd	3	Marigómez et al., 2005
	SERA		Fuel oil WAF	2	Lima et al., 2008
	Shellfish diet 1800		Cu	1, 2, 3, 5	Brooks et al., 2015
	AlgaMac protein+	4	Carbam-azepine	2, 5	Oliveira et al., 2017
	Algal feed		Cd	5	Chandurvelan et al., 2012
	Drymicroencapsules Myspat		Pesticides	1, 2	Patetsini et al., 2012
	Shellfish diet 1800	5	Treated produced water	1, 2, 3, 4	Brooks et al., 2011
	Hawaiian Marine Imp Inc	6	Cu, Zn , Cd	4	Soto et al., 1996
		13	Crude /Lubricant oil WAF	4	Cajaraville et al., 1990
				4, 5	Cajaraville et al., 1992a
				4	Cajaraville et al., 1992b
				3	Cajaraville et al., 1995
				3	Cajaraville et al., 1997
	Saunders-Microencapsulates	14	Metals (Hg, Ag, Pb, Cu)	4	Domouhtsidou & Dimitriadis, 2000

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II. STATE OF THE ART, HYPOTHESIS AND OBJECTIVES

STATE OF THE ART

The assessment of marine pollution is a need for sustained management and conservation of the marine resources. Accordingly, monitoring programs have been promoted worldwide as strategy to assess effects of pollutants in marine ecosystems and in the biota. The organism used in biomonitoring programs to assess the biological responses to pollutants is called sentinel organism. Among sentinel species, mussels are widely used due to their biological and ecological characteristics, which are very appropriate for pollution monitoring and toxicological experiments based on biomarkers. These early warning responses occur at low levels of biological complexity and allow the prediction, at longer-term, at more complex levels of biological complexity. Thus, the use of biomarkers in mussels has been stablished in marine pollution monitoring programs worldwide. Moreover, the use of a biomarker battery at different biological level responds to the whole suite of possible scenarios, providing a broader picture of the ecosystem health status in pollution monitoring programs.

Due to the interactions between pollutants and natural factors or between natural factors themselves, interpretation of biomarkers could be unreliable and confusing in monitoring programs. Understanding the influence of natural variability on biological responses to pollutants is of mayor importance in order to interpret the environmental health status properly. Few studies have dealt with the influence of natural variables and factors other than pollutants on biological responses, including biomarkers (season, shore zonation, temperature, salinity, oxygen, gamete developmental cycle, food availability and quality or size/age). Consequently, further research is needed to deepen in the knowledge of the natural variability of biological responses to pollutants in order to achieve a more reliable application of biomarkers in marine pollution monitoring. Within this framework, getting best available practices (BAPs) is crucial for biomarker applications in a harmonised manner.

In long-scale monitoring programs, only in Europe, three *Mytilus spp.* have been used depending on the region latitude: *M. galloprovincialis* (Lmk.) in Southern Europe, *M. edulis* (Lmk.) in Northern Europe, and *M. trossulus* (Gould) within the Baltic. Although few studies have reported differences in bioaccumulation and physiology between the mentioned species, the taxa influence on biomarkers of *Mytilus* genus is still unclear. Thus, further research for the determination of the taxa influence on biological responses to pollutants is needed for the proper interpretation of the mussel health status in long-scale biomonitoring programs. In pollution field studies based on biomarkers, BAPs for sampling and transport of mussels from the field to the laboratory are lacking and thus, the procedures are disparate amongst studies. Differences in the sampling and transport of mussels can affect biological responses to pollutants, thus hampering the comparison with field data obtained in other studies. Likewise, it could be a matter of data misinterpretation. Therefore, there is urgent demand to advance in the standardization of procedures for collection, transport and processing of mussels used as sentinels in biomonitoring programs based on biomarkers. The influence of mussel's sex on biomarkers has been scarcely investigated and the data obtained in those studies were not conclusive. Thus, studies are needed in order to develop clear guidelines in

mussels dealing with sex-related changed in biomarker values and biomarker responsiveness against environmental stimuli.

Laboratory experiments are a very useful tool to understand the responsiveness of biomarkers to pollutants and how environmental variables and factors impinge on the biological responses, In most studies laboratory experimental conditions are well fixed and described in detail. However, feeding strategy often differs markedly between laboratory experiment, regarding the amount, type and quality of food, as well as the feeding rate and regime. Alas, although a few studies have reported that the feeding strategy affects biomarkers and their responsiveness in mussels experimentally exposed to pollutants, the influence of the feeding strategy on biomarkers has been commonly neglected. So, further research is needed in order to develop BAPs for feeding mussels during short and long-term experimentation in aquatic toxicology.

HYPOTHESIS

A better understanding of the influence of confounding factors on biomarker responsiveness endorses Best Available Practices (BAPs) for the multi-biomarker approach applied to mussels as sentinels in marine pollution monitoring and as experimental animals in aquatic toxicology.

OBJECTIVES

In order to demonstrate the hypothesis, the following objectives are to be achieved:

1. To contribute to the development of Best Available Practices (BAPs) for mussel selection (e.g. regarding taxonomy) in large-scale monitoring of the coastal zone in Europe by determining species-specific differences in core biological responses to pollutant exposure in mussels *Mytilus spp*. from diverse European coastal regions.

2. To contribute to the development of BAPs for intertidal mussel collection and transport in biomarker-based environmental monitoring of the coastal zone in the Bay of Biscay by determining the influence of different sampling strategies on biomarkers.

3. To contribute to the development of BAPs for mussel selection (e.g. regarding sex and reproductive condition) in long-term monitoring of the coastal zone in the Bay of Biscay by determining sex-dependent seasonal variability in biomarker responsiveness to environmental stress in mussels.

4. To contribute to the development of BAPs for feeding mussels during short-term pollutant-exposure experiments by investigating the influence of fasting on biomarker responsiveness in mussels exposed to a model pollutant.

5. To contribute to the development of BAPs for feeding mussels during toxicological experiments in the laboratory by determining dietary food-type influence on biomarkers in mussels.

III. RESULTS

CHAPTER 1

SPECIES-SPECIFIC LYSOSOMAL RESPONSES TO COPPER EXPOSURE IN DIGESTIVE CELLS OF MUSSELS *MYTILUS* SPP. FROM DIVERSE EUROPEAN COASTAL ZONES

This chapter has been published as a main part of the following ISI paper:

- Brooks SJ, Farmen E, Heier LS, Blanco-Rayón E, Izagirre U. 2015. Differences in copper bioaccumulation and biological responses in three *Mytilus* species. Aquatic Toxicology 160, 1–12.

ABSTRACT

Mussels are typically used in biological effects studies and have a suite of biological effects endpoints that are frequently measured and evaluated for stress effects in laboratory experiments and field studies. Differences in bioaccumulation and biological responses of the three *Mytilus* species following exposure to copper (Cu) were investigated. A laboratory controlled exposure study was performed with three genetically confirmed *Mytilus* species; *M. galloprovincialis*, *M. edulis* and *M. trossulus*. Lysosomal biomarkers were assessed in all three Mytilus species following 21 d exposure to waterborne Cu concentrations of 0, 10, 100 and 500 µg Cu/L. Both 100 and 500 µg Cu/L were lethal to all three *Mytilus* spp. with 100% mortality recorded in the exposed mussels after 21 d exposure. Thus, lysosomal biomarkers were investigated only in control mussels and those exposed to 10 µg Cu/L. Inter-species differences in lysosomal enlargement, membrane destabilization and contents (neutral lipids and lipofuscins) were found to occur following Cu exposure. Overall, the study demonstrated that differences in lysosomal responses between the *Mytilus* spp. occur with potential consequences for the use of mussels as sentinels in marine pollution monitoring. Consequently, it is highly recommended that the correct species must be identified when using *Mytilus* mussels as sentinels in biological effects monitoring.

Europako hainbat kostalde eskualdeko muskuiluen (*Mytilus* spp.) espezieen araberako erantzun lisosomikoak haien liseri zeluletan kobre esposizioaren aurrean

Laburpena

Muskuiluak efektu biologikoko ikasketetan normalean erabiltzen dira eta, laborategiko esperimentuetan eta landa-azterketetan estres-efekturako sarritan neurtu eta ebaluatzen diren efektu biologikoen jomugen multzoa dute. Kobre (Cu) esposizioaren ostean hiru *Mytilus* espezieen biometaketa eta erantzun biologikoak ikertu ziren. Kontrolatutako laborategiko esposizio-ikerketa bat burutu zen, genetikoki baieztatutako hiru *Mytilus* espeziekin: *M. galloprovincialis*, *M. edulis* eta *M. trossulus*. Hiru muskuilu espezietan biomarkatzaile lisosomikoak ebaluatu ziren, 21 egunetako Cu kontzentrazio desberdineko (0, 10, 100 eta 500 µg Cu/L) esposizioaren ostean. Hiru muskuilu espezietarako 100 eta 500 µg Cu/L kontzentrazioak letalak ziren, muskuiluen %100 heriotza-tasa neurtuz 21 egunetako esposizioaren ostean. Beraz, biomarkatzaile lisosomikoak ikertzeko, 0 eta 10 µg Cu/L kontzentrazioetara esposatutako muskuiluak soilik erabili ziren. Lisosomen handiagotzean, lisosomen mintzaren desegonkortasunean eta edukietan (lipido neutroak eta lipofustinak) espezien arteko desberdintasunak aurkitu ziren Cu-aren esposizioaren ondoren. Oro har, ikerketak *Mytilus* espezieen artean zeunden erantzun lisosomikoen desbesdintasunak frogatu zituen, ondorio potentzialekin muskuiluen erabilerako itsasoko kutsaduraren jarraipenetan. Beraz, *Mytilus* espezieko muskuiluak erabiltzen direnean efektu biologikoen jarraipenerako begirale gisa, espezie egokiak identifikatzea oso gomendagarria da.

Respuestas lisosómicas específicas de especie a la exposición de cobre en células digestivas de mejillón *Mytilus* spp. de diversas zonas costeras europeas

Resumen

Los mejillones se usan normalmente en estudios de efectos biológicos y tienen un conjunto de metas en efectos biológicos que con frecuencia se miden y se evalúan para determinar los efectos del estrés en experimentos de laboratorio y estudios de campo. Se investigaron las diferencias en bioacumulación y respuestas biológicas de las tres especies de *Mytilus* tras exponerlos a cobre (Cu). Se realizó un estudio de exposición controlado en laboratorio con tres especies de *Mytilus* genéticamente confirmadas: *M. galloprovincialis*, *M. edulis* y *M. trossulus*. Los biomarcadores lisosómicos se evaluaron en las tres especies de *Mytilus* tras 21 d de exposición a diferentes concentraciones de Cu en el agua de 0, 10, 100 y 500 µg de Cu/L. Tanto 100 como 500 µg de Cu/L fueron letales para las tres especies de *Mytilus*, con un 100% de mortalidad registrada en los mejillones expuestos tras una exposición de 21 d. Por lo tanto, los biomarcadores lisosómicos se investigaron solo en mejillones control y aquellos expuestos a 10 µg de Cu/L. Se encontraron diferencias entre especies en el agrandamiento lisosomal, en la desestabilización de la membrana lisosómica y en el contenido lisosomal (lípidos neutros y lipofuscinas) después de la exposición al Cu. En general, el estudio demostró que las diferencias en las respuestas lisosómicas entre *Mytilus* spp. ocurren, con consecuencias potenciales para el uso de mejillones como centinelas en el seguimiento de la contaminación marina. Por ende, se recomienda encarecidamente identificar las especies correctas cuando se usen mejillones *Mytilus* como centinelas en el seguimiento de los efectos biológicos.

INTRODUCTION

Different species of mussels of the genus *Mytilus* are worldwide used in biological effects assessment in pollution monitoring programmes (Beyer et al., 2017). They possess the ability to bioaccumulate contaminants from the water column as well as to respond to environmental insults in a measurable way. Thus, mussels are a recommended sentinel organism of the International Corporation for the Exploration of the Seas (ICES) integrated biomonitoring scheme (ICES, 2011) and routinely used within national monitoring programmes (e.g. Mussel Watch (Goldberg et al., 1978); Artic Marine and Assessment Program (AMAP) (Dietz et al., 2000; Christensen et al., 2002); Prestige Oil Spill Biomonitoring (Marigómez et al., 2013); Biological Effects of Environmental Pollution on Marine Coastal Ecosystems (Lehtonen and Schiedek, 2006); Norwegian Water Column Monitoring (Hylland et al., 2008; Brooks et al., 2011), providing information on the health status of particular water bodies.

It has been frequently assumed that the mussels collected from the field and then used within biological effects studies are a distinct species whether being *Mytilus edulis* (Lmk.) in Northern Europe, *M. galloprovincialis* (Lmk.) in Southern Europe and *M. trossulus* (Gould) within the Baltic. However, this generalisation has been increasingly challenged by recent studies that have shown *Mytilus* species in a patchy distribution with all three *Mytilus* spp. and hybrids occurring within the same populations (Kijewski et al., 2011; Väinölä and Strelkov, 2011; Brooks and Farmen, 2013). Since environmental factors can shape the mussel's external morphology (Seed, 1968; Akester and Martel, 2000), it is not always possible to reliably distinguish between *Mytilus* species by mere visual inspection. This can therefore lead to misrepresentation of *Mytilus* with potential implications for biological effects assessment in marine pollution monitoring programmes. Indeed, although the ability of the different *Mytilus* spp. to bioaccumulate contaminants is not fully known, some studies have reported differences. For example, higher concentrations of heavy metals were measured in *M. trossulus* compared to *M. edulis* in mussels collected from the same field population and in the same size category (Lobel et al., 1990).

Biological responses or biomarkers can be defined as cellular, biochemical, molecular, or physiological changes that are measured in cells, body fluids, tissues, or organs within an organism and are indicative of xenobiotic exposure and/or effect (Lam and Gray, 2003). Differences in biomarkers between the *Mytilus* species have, to the authors knowledge, been investigated only occasionally (Brooks et al., 2015). Species differences in genotoxic response have been indicated with different background assessment criteria (BACs) for the three *Mytilus* species with respect to the frequency of micronuclei in haemocyte cells (ICES, 2011). Histological parameters of reproductive condition were different between *M. edulis, M. galloprovincialis* and their hybrids sampled from the same location in the UK (Bignell et al., 2008). Likewise, differences in reproductive strategy (i.e. spawning times) of *M. edulis* and *M. galloprovincialis* have been found to occur (Hilbish et al., 2002). Such differences in spawning are likely to impact energy budgets between the species and influence general fitness at different times of the year. Furthermore, *Mytilus* species have been

found to be differentially susceptible to parasitism (Coustau et al., 1991), which may suggest some underlying difference in general physiology. Hence, there is increasing evidence to suggest that the biological responses between the *Mytilus* species do exist, although there remains a lack of controlled laboratory investigations, which are needed to measure the full extent of these potential differences.

Thus, the main aim of this study was to determine if differences in lysosomal responses occur between the three *Mytilus* species *M. edulis*, *M. trossulus* and *M. galloprovincialis* following a relatively 21d exposure to acute waterborne Cu concentrations. As a part of the study, other researchers investigated micronuclei formation as a genotoxicity biomarker, (Barsiene et al., 2010) and glutathione (GSH) as an oxidative stress biomarker (Regoli and Principato, 1995). The part of the study herein presented deals with lysosomal biomarkers such as lysosomal membrane stability (LMS), lysosomal structural changes (LSC) and neutral lipid (NL) and lipofuscin (LPF) accumulation in the mussel digestive cells after 21 d Cu exposure. Lysosomal biomarkers are known to be responsive to copper exposure in mussels (Viarengo et al., 1985) and are ICES core biomarkers for biological affects assessment widely used in marine pollution monitoring programmes; thus, the findings will be discussed in relation to potential implications for biomonitoring.

MATERIALS AND METHODS

Collection of mussels and acclimation

Mussels were collected from three separate locations, which were known to contain one of the three *Mytilus* species. *M. edulis* were collected in late autumn from the lower intertidal zone at low tide at the Outer Oslo fjord, Norway (59.488 N 10.498 E), and brought back to the NIVA marine research station in Solbergstrand, Norway. *M. trossulus* were collected in late autumn from Tingvoll fjord, west coast of Norway (62.81 N 8.275 E), and transported within an ice-cooled cooler box to the NIVA marine research station within 4 h. *M. galloprovincialis* were collected from the low intertidal zone in winter at Mundaka in the Biscay Bay (43.410 N 2.698 W), and were carefully packed in an ice-cooled styrene box and transported by overnight airfreight to the NIVA marine research station. All mussels on arrival were placed in aquaria of flowing seawater, with the temperature maintained at 15 ± 2°C for 4–6 weeks prior to testing. This acclimation period was considered sufficient for the mussels to adapt to the controlled laboratory conditions before testing (Widdows and Bayne, 1971; Altieri, 2006). No mortalities were recorded during transport and acclimation. The mussels were fed daily with a concentrated solution of Shellfish diet 1800® containing a mixture of four marine microalgae (Reed Mariculture Inc. USA).

Exposure to copper

The three mussel species were exposed to high, mid and low stable doses of copper chloride ($CuCl_2$) solution within a flow-through seawater system. With an established filtered (10 μm) seawater flow rate of 3 L/min at 15±2°C and 33‰ salinity, a concentrated solution of $CuCl_2$ was dosed into the seawater inlet pipe to provide total nominal Cu concentrations of 10, 100 and 500 μg/L (0.16, 1.57 and 7.87 μM Cu). Fifty mussels of each group were exposed per treatment.

The three mussel species of each exposure condition were placed inside the same glass aquarium and separated by a perforated plastic screen that allowed water flow but prevented mixing of the mussels. The flow-through set-up was designed to ensure that all mussels within the same aquaria received identical Cu exposure. The mussels were fed every 2 d with Shellfish diet 1800® and sampled after 21 d exposure (additional samples were taken after 4 d for chemical analyses and additional biomarkers measured in parallel by other researchers).

Mussel sampling

Both 100 and 500 µg Cu/L were lethal to all three *Mytilus* spp. with 100% mortality recorded in the exposed mussels after 21 d exposure. Thus, only control mussels and those exposed to 10 µg Cu/L were used to investigate lysosomal biomarkers. The digestive gland and gonad were excised and placed in separate labelled cryovials before being snap frozen in liquid nitrogen. Each mussel was given a code number that was independent of the exposure dose, which enabled the biomarker analysis to be performed blind. The digestive gland was used to measure histochemistry (LMS, LSC, NL, LPF). The gonad was used to confirm *Mytilus* species. All mussels were identified to *Mytilus* species from DNA isolated from gonad tissue samples, followed by PCR amplification and gel electrophoresis. The individual species were determined before samples were selected for bioaccumulation and biomarker measurements to ensure that only pure *M. edulis*, *M. trossulus* and *M. galloprovincialis* were analysed.

In parallel, 3 replicates of 5 mussels from each treatment and species group were excised after 4 and 21 d exposure, and stored at −20°C for Cu analysis. Individual whole mussel samples were selected based on the results of the mussel speciation assessment, with three mussels measured per group. Samples were defrosted, homogenised and a sub-sample taken of approximately 5 g. Total Cu tissue concentrations were determined by inductively coupled plasma optical emission spectrometry (Fig. S1; Brooks et al., 2015). Thus, almost identical Cu tissue concentrations were recorded in control mussels of the 3 *Mytilus* spp (1-2 µg Cu/g flesh dry-wt), both after 4 and 21 d. Almost identical Cu tissue concentrations were found after 4 and 21 d in *M. galloprovincialis* exposed to 10 µg Cu/L (~6 µg/g). Twice higher Cu tissue concentrations were recorded after 21 d exposure to 10 µg Cu/L (∼17–18 µg Cu/g flesh dry-wt) than after 4 d (7–8 µg Cu/g flesh dry-wt) in *M. edulis* and *M. trossulus*. Thus, no differences in Cu tissue concentrations were observed between the *Mytilus* spp after 4 d exposure to 10 and 100 µg Cu/L (6–9 µg Cu/g flesh dry-wt); however, on exposure to 500 µg Cu/L, the Cu tissue concentration in *M. trossulus* (∼70 µg Cu/g flesh dry-wt) was 3× higher than in *M. edulis* and *M. galloprovincialis* (∼22 µg Cu/g flesh dry-wt) (Fig. S1; Brooks et al., 2015).

DNA isolation, amplification and Gel electrophoresis

Total DNA was extracted from 20 to 40 mg of gonad tissue from frozen mussels using QuickExtract DNA extraction solution (Epicentre, Madison, Winsconsin, USA) following the manufacturer's recommended protocol. Briefly, the tissue was homogenised in 0.1 mL QuickExtract DNA extraction solution by vortexing for 15 s, incubated

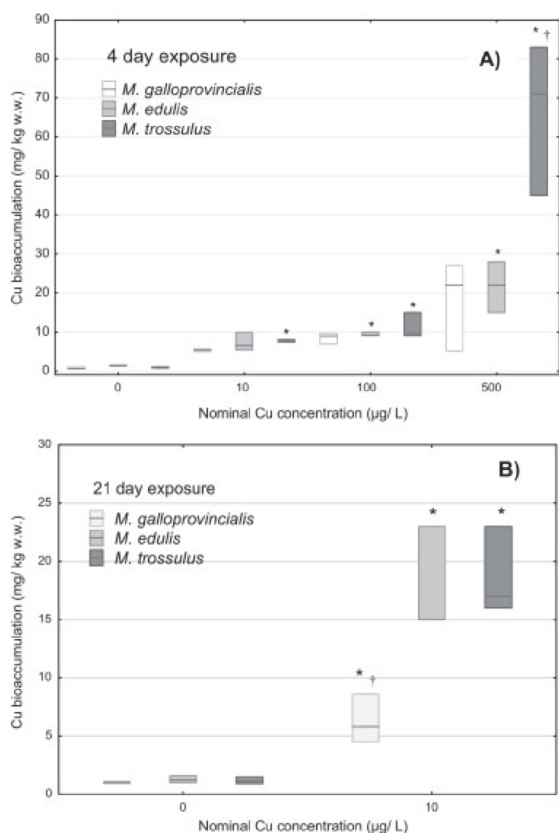

Fig. S1. Copper concentrations (median ± quartiles, n=3) measured in individual whole mussel homogenates of the different *Mytilus* spp. following (A) 4 d and (B) 21 d Cu exposure. Asterisks indicate significantly different from control group; †: significant difference from other *Mytilus* within treatment group (Brooks et al., 2015).

at 65°C for 10 min, vortexed for another 15 s, and finally incubated at 98°C for 2 min. The homogenates were then diluted 1:10 in molecular grade H_2O. For species identification, polymerase chain reaction (PCR) were used to amplify a specific 180 base pair (bp) segment for *M. edulis*, 168 bp segment for *M. trossulus* or 126 bp segment for *M. galloprovincialis* of the Glu gene (polyphenolic adhesive protein) as described by Inoue et al. (1995). The 25 µL PC reactions contained 5 µL of DNA template, 300 µM forward and reverse primers, 2x SsoFast EvaGreen Mastermix (BioRad, Hercules, CA, USA), and were subjected to a 5 min pre-heating stage at 95°C followed by 35 cycles of 30 s at 95°C, 30 s at 55°C, 30 s at 72°C, and final extension step of 10 min at 72°C. One microlitre of the PCR product was loaded onto a DNA 1000 chip (Agilent technologies, Santa Clara, California, USA) and run in a Bioanalyser instrument (Agilent technologies, Santa Clara, California, USA) for visualisation of amplicon size.

Lysosomal membrane stability (LMS)

The determination of lysosomal membrane stability (LMS) was based on the time of acid labilisation treatment required to produce the maximum staining intensity according to UNEP/RAMOGE (1999), after demonstration of hexosaminidase (Hex) activity in digestive cell lysosomes. Eight serial cryotome sections (10 µm) were subjected to acid labilisation in intervals of 0, 3, 5, 10, 15, 20, 30 and 40 min in 0.1 M citrate buffer (pH 4.5 containing 2.5% NaCl) in a shaking water bath at 37°C, in order to find out the range of pre-treatment time needed to completely labilise the lysosomal membrane, denoted as the labilisation period (LP; min). Following this treatment, sections were transferred to the substrate incubation medium for the demonstration of Hex activity. The incubation medium consisted of 20 mg naphthol AS- BI-Nacetyl- β -d glucosaminide (Sigma, N 4006) dissolved in 2.5 mL 2-methoxyethanol (Merck, 859), and made up to 50 mL with 0.1 M citrate buffer (pH 4.5) containing 2.5% NaCl and 3.5 g low viscosity polypeptide (Sigma, P5115) to act as a section stabiliser. Sections were incubated in this medium for 20 min at 37°C, rinsed in a saline solution (3.0% NaCl) at 37°C for 2 min and then transferred to 0.1 M phosphate buffer (pH 7.4) containing 1 mg/ mL diazonium dye Fast Violet B salt (Sigma, F1631), at RT for 10 min. Slides were then rapidly rinsed in running tap water for 5 min,

fixed for 10 min in Baker's formol calcium containing 2.5% NaCl at 4°C and rinsed in distilled water. Finally, slides were mounted in Kaiser's glycerine gelatin. The time of acid labilisation treatment required to produce the maximum staining intensity was assessed under the light microscope as the maximal accumulation of reaction product associated with lysosomes (UNEP/RAMOGE, 1999). Although two maximum staining peaks may be observed in some cases, the first one was always considered. Four determinations were made for each animal by dividing each section in the acid labilisation sequence into 4 approximately equal segments and assessing the LP in each of the corresponding set of segments. The mean LP value was then derived for each section, corresponding to an individual digestive gland.

Lysosomal structural changes (LSC)

The histochemical reaction for β-Gus was demonstrated as in Moore (1976) with the modifications described by Cajaraville et al. (1989). Frozen tissue sections (8 μm) from 10 mussels were put at 4°C for 30 min and then brought to room temperature before staining. Slides were incubated in freshly prepared β-Gus substrate incubation medium consisting of 28 mg naphthol AS-BI-β-Gus-glucuronide (Sigma, N1875) dissolved in 1.2 mL of 50 mM sodium bicarbonate, made up to 100 mL with 0.1 M acetate buffer (pH 4.5 containing 2.5% NaCl) and 15% of polyvinyl alcohol at 37ºC for 40 min in a shaking water bath and then transferred to a post- coupling medium containing 0.1 g Fast Garnet (GBC) (Sigma, F8761) dissolved in 100 mL of 0.1 M phosphate buffer (pH 7.4 containing 2.5% NaCl) in the darkness and at room temperature for 10 min. Afterwards, sections were fixed in Baker's formol calcium containing 2.5% NaCl at 4°C for 10 min and rinsed briefly in distilled water. Finally, sections were counterstained with 0.1% Fast Green FCF (Sigma, F7252) for 2 min, rinsed several times in distilled water and mounted in Kaiser's glycerol gelatine. Five measurements using a 100× objective lens were made in each individual using image analysis (BMS, Sevisan). The mean value of the stereological parameters was determined for each mussel digestive gland (Lowe et al., 1981): lysosomal volume density (Vv_{LYS}), lysosomal surface-to-volume ratio (S/V_{LYS}) and lysosomal numerical density (Nv_{LYS}).

Intracellular accumulation of neutral lipids (NL)

Intracellular NL were demonstrated histochemically by staining with Oil Red O (ORO) (Culling, 1974). Cryostat sections (8 μm) were transferred to a cabinet at 4°C and fixed in Baker's formol calcium containing 2.5% NaCl at 4°C for 15 min. Then sections were dried at room temperature, washed in isopropanol (60%) and rinsed in (ORO) staining solution for 20 min. The ORO stock solution is a saturated (0.3%) solution of ORO (BDH, 34061) in isopropanol. The staining solution was freshly made by dissolving 60 mL stock solution in 40 mL distilled water and filtering after a 10 min gap to stabilise the solution. The staining solution is only stable for 1–2 hr. Stained sections were differentiated in 60% isopropanol, washed in water, counterstained with 1% Fast Green FCF (Sigma, F7252) for 20 min and mounted in Kaiser's glycerine gelatine. Five measurements using a 40× objective lens were made in each individual using image analysis (BMS, Sevisan). The mean volume density of neutral lipids in digestive alveoli (Vv_{NL}) was determined for each mussel.

Lipofuscin (LPF) accumulation

LPF content of tertiary lysosomes was quantified using Schmorl's reaction (Pearse, 1972). Cryostat sections (8 µm thick) were fixed for 15 min in Baker's formol calcium containing 2.5% NaCl at 4°C. Then, they were rinsed in distilled water and placed in Schmorl's solution, which contains 1% ferric chloride and 1% potassium ferricyanide, at a ratio of 3:1. Sections were stained in this solution for 5 min. After that, they were washed with 1% acetic acid for 1 min, followed by rinsing in distilled water. The slides were mounted in Kaiser's glycerine gelatine.

Statistical analyses

IBM® SPSS® Statistics ver. 22.0.0.0 (IBM Corp., Armonk, NY, USA) and Statistica v11 software (Statsoft Inc.), were employed for the statistical analyses. Homogeneity of variance (Levene's test) and normality of data (Kolmogorov–Smirnov's) were tested before statistical analyses (Sokal and Rohlf, 1995). Statistically significant differences among species were tested according to the Duncan's post hoc test based on one-way analysis of variances (1-way ANOVA) for parametric variables (Vv_{LYS}, Sv_{LYS}, S/V_{LYS}, Nv_{LYS}), and the Mann–Whitney's U-test for non-parametric variable (LP). Furthermore, statistically significant differences between unexposed and exposed mussels for each species were tested according to the Student's t-test for parametric variables (Vv_{LYS}, Sv_{LYS}, S/V_{LYS}, Nv_{LYS}) and the Mann–Whitney's U-test for non-parametric variable (LP). A 95% significance level ($p < 0.05$) was established for all statistical analyses carried out.

RESULTS

Mytilus species determination

The method of DNA amplification and gel electrophoresis of mussel gonad tissue was successful in differentiating between the three *Mytilus* species. Single bands were identified at 180 bp for *M. edulis* 168 bp for *M. trossulus* and 126 bp *for M. galloprovincialis*. The proportion of the *Mytilus* spp. and hybrids from the different populations are shown in Table 1. As expected *M. galloprovincialis, M. edulis* and *M. trossulus* were the dominant mussel species in the populations collected from Bilbao estuary, the outer Oslo fjord and Tingvoll fjord in Norway, respectively. However, pure populations were not found at any of the sampling locations, with a single *M. trossulus* identified from the Bilbao population as well as five *M. galloprovincialis/M. edulis* hybrids. Hybrids of *M. galloprovincialis/M. edulis* (n=1) and *M. edulis/M. trossulus* (n=3) were found in the outer Oslo fjord population. Highest variability was seen in the Tingvoll fjord population with hybrids of *M. edulis/M. trossulus* (n=13) and *M. galloprovincialis/M. trossulus* (n=3) as well as *M. edulis* found in addition to the dominant *M. trossulus*.

Lysosomal membrane stability (LMS)

No significant differences were found in lysosomal membrane stability between control *Mytilus* spp., neither between exposed *Mytilus* spp. (Fig. 1). In the comparison between control and exposed groups of each species, only *M. trossulus* showed significant decrease in lysosomal membrane stability after 21 d.

Table 1. The numbers of the various *Mytilus* complex species within the collected populations.

Mytilus **complex**	**Bilbao estuary (n = 85)**	**Outer Oslo fjord (n = 78)**	**Tingvoll fjord nr Molde (n = 73)**
M. galloprovincialis	79(93%)		
M. edulis		74(95%)	1(1%)
M. trossulus	1(1%)		56(77%)
M. galloprov/M. edulis hybrid	5(6%)	1(1%)	
M. galloprov/M. trossulus hybrid			3(4%)
M. edulis/M. trossulus hybrid		3(4%)	13(18%)

NB: Only the pure *Mytilus* were selected for bioaccumulation and biomarker measurements.

Lysosomal structural changes (LSC)

Differences between species were recorded for lysosomal volume density (Vv_{LYS}) and lysosomal surface to volume ratio (S/V_{LYS}) (Fig. 2). In the control groups, Vv_{LYS} was significantly lower in *M. galloprovincialis* compared to *M. edulis* and *M. trossulus* (Fig. 2A). Moreover, higher S/V_{LYS} values were recorded in *M. galloprovincialis* than in *M. edulis* and *M. trossulus*. In exposed groups, *M. galloprovincialis* showed significantly lower Vv_{LYS} and higher S/V_{LYS} than *M. edulis* (Fig. 2B). In addition, significant decreases on Vv_{LYS} values were observed in *M. edulis* and *M. trossulus* after Cu exposure to 10 µg Cu/L for 21 d. Lysosomal numerical density (Nv_{LYS}) showed no significant differences neither between species, nor among control and exposed mussels (Fig. 2C).

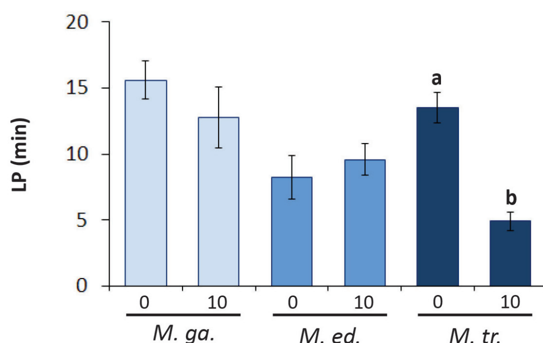

Fig. 1. Lysosomal membrane stability measured as labilisation period (LP) in the digestive gland cells of the three *Mytilus* spp., *M. galloprovincialis* (*M. gallo.*), *M. edulis* (*M. ed.*) and *M. trossulus* (*M. tr.*); following 21 d Cu exposure. Intervals indicate standard deviation; letters indicate significant differences between control and exposed mussels of the same species according to the Mann–Whitney's U-test ($p < 0.05$).

Intracellular accumulation of neutral lipids

Intracellular neutral lipid accumulation of unexposed *M. edulis* was significantly higher than unexposed *M. galloprovincialis* (Fig. 3). However, in *M. edulis*, neutral lipid accumulation tended to decrease after 10 µg Cu/L exposure for 21 d. Regarding *M. trossulus*, their Vv_{NL} values in control and exposed groups were between those of the other two species. Although following Cu exposure, neutral lipid accumulation in *M. trossulus* tended to increase.

Lipofuscin accumulation

Differences in the content of lipofuscins were observed between species and, also, between controls and exposed mussels (Fig. 4). *M. galloprovincialis* showed higher concentration of lipofuscins than *M. edulis* and *M. trossulus*. In all mussel species less lipofuscin content was observed after 10 µg Cu/L exposure for 21 d. In addition, *M. edulis* appeared to have smaller lipofuscins than the other two species.

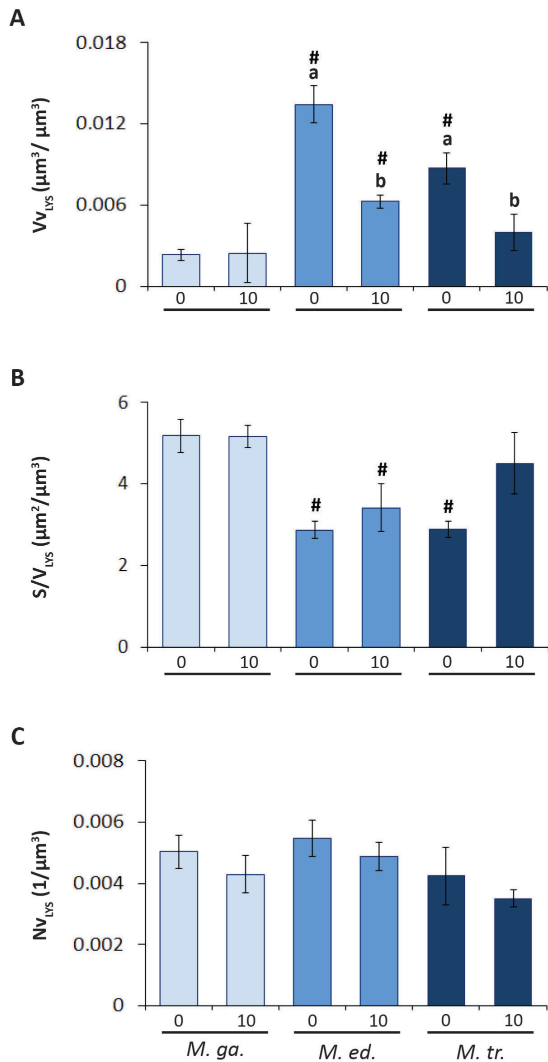

Fig. 2. Lysosomal structural changes in digestive gland of the three *Mytilus* spp. following 21 d Cu exposure. (A) Lysosomal volume density (Vv_{LYS}); (B) lysosomal surface to volume ratio (S/V_{LYS}); and (C) lysosomal numerical density (Nv_{LYS}). Intervals indicate standard error, letters indicate significant differences between control and exposed mussels of the same species according to the Duncan's test performed after one-way ANOVAs ($p < 0.05$) and hashes indicate significant differences of *M. edulis* (*M. ed.*) and *M. trossulus* (*M. tr.*) respecting *M. galloprovincialis* (*M. ga*) in each experimental condition according to the Student's t-test ($p < 0.05$).

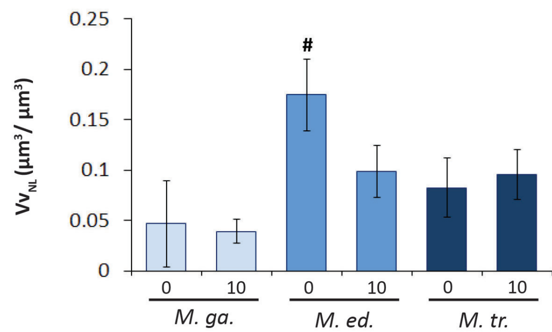

Fig. 3. Intracellular neutral lipid volume density (Vv_{NL}) in digestive gland of the three *Mytilus* spp. following 21 d Cu exposure. Intervals indicate standard error and hashes indicate significant differences of *M. edulis* (*M. ed.*) and *M. trossulus* (*M. tr.*) respecting *M. galloprovincialis* (*M. ga.*) in each experimental condition according to the Student 's t-test ($p < 0.05$).

DISCUSSION

The patchy distribution of *Mytilus* species around the European coast and elsewhere has made it difficult to assume the correct species without first confirming through genetic identification (Kijewski et al., 2011; Brooks and Farmen, 2013). For instance, of the 85 mussels analysed from the Northern coast of the Basque Country, which were initially considered to be entirely *M. galloprovincialis*, 5 individuals of *M. galloprovincialis/edulis* hybrids as well as a single *M. trossulus* individual was detected. Likewise, *Mytilus* populations from the Oslo fjord had 95% of pure *M. edulis* and 5% of hybrids. In contrast, the Tingvoll fjord nr Molde, Norway had a mixed population despite having a dominate species present. Therefore, genetic species identification should be used if specific *Mytilus* species are required, which may be of significance when for example the bioaccumulation and biomarker responses to chemical stress differ between the species.

Digestive cell lysosomes are very sensitive to a wide range of contaminants and their responses are widely used as general stress or effect biomarkers of pollution, both in field and laboratory studies (Moore, 1988; Regoli, 1992; Etxeberria et al., 1994; Marigómez et al., 1996; Marigómez and Baybay-Villacorta, 2003; ICES, 2011; Izagirre et al., 2014a).

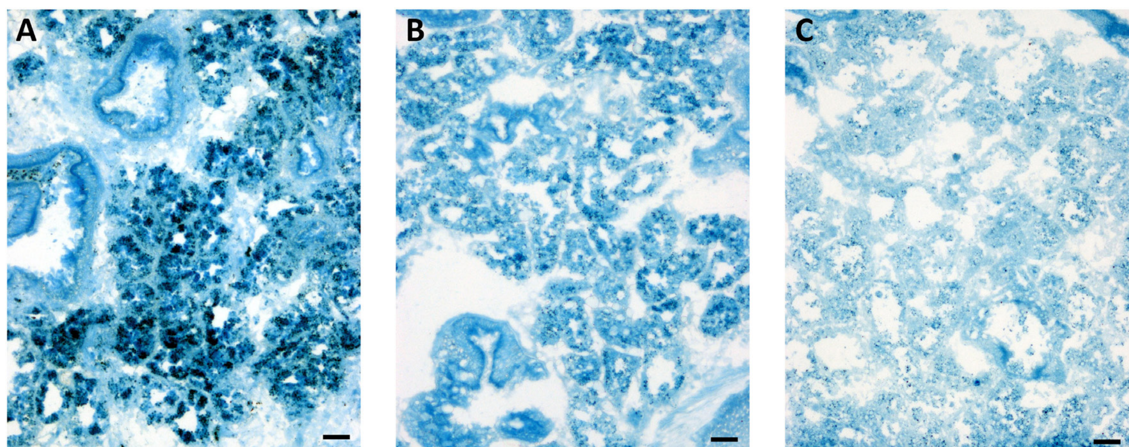

Fig. 4. Histochemistry of lipofuscins in digestive gland of mussels. (A) Control *M. galloprovincialis*; (B) control *M. edulis*; (C) 10 µg Cu/L exposed *M. edulis* for 21 d. Scale bar=50 µm.

In the present study, after 21 d exposure to 10 µg Cu/L, membrane destabilisation and lysosomal structural changes were measured. The lysosomal membrane stability test is recommended by the OSPAR Convention to assess the biological effects of contaminants (UNEP/RAMOGE, 1999). Overall, mussels are considered healthy when they exhibit LP values above 20 min and stressed when LP values are below 10 min (Viarengo et al., 2000; Dagnino et al., 2007; Izagirre and Marigómez, 2009). In the present study, low LP values were recorded in controls of the three *Mytilus* spp. Nevertheless, low LP values were also observed in natural conditions at certain moments of the process of intracellular digestion (Tremblay and Pellerin-Massicotte, 1997; Izagirre et al., 2009) and in different lysosomal populations of laboratory control *M. edulis* (Moos et al., 2012). In fact, *M. edulis* showed the lowest LP values in the control group. After Cu exposure, only LP was maintained in *M. edulis* and decreased in *M. galloprovincialis* and *M. trossulus*. However, only *M. trossulus* showed a significant decrease in LP value, which was in accordance with the other biomarkers, indicating that *M. trossulus* was the most sensitive species.

The assessment of lysosomal structural changes provides an indication of general environmental stress exhibited by the mussel (Cajaraville et al., 2000; Marigómez and Baybay-Villacorta, 2003). Overall, exposure of mussels to pollutants induces lysosomal enlargement (Moore, 1988; Etxeberria et al., 1994; Marigómez and Baybay-Villacorta, 2003). However, a decrease in lysosomal size was shown after 21 d exposure in *M. edulis* and *M. trossulus*. These results are in accordance with Etxeberria et al. (1994), who observed a significant reduction in Nv_{LYS} of *M. galloprovincialis* following 20 d exposure to 8 µg Cu/L. This response has also been observed in mussels exposed to organic pollutants (Cajaraville et al., 1995; Marigómez and Baybay-Villacorta, 2003). In fact, lysosomal size reduction itself appears to be transient since longer exposure times have been found to provoke lysosomal enlargement (Cajaraville et al., 1995). In addition to chemical stress, lysosomal biomarkers change depending on natural environmental factors, such as temperature (Etxeberria et al., 1995; Tremblay et al., 1998; Izagirre et al., 2014b; Múgica et al., 2015). For instance, lysosomal size and membrane stability show marked seasonal variability, with lysosomes more

conspicuous (high Vv_{LYS}, Sv_{LYS} and low S/V_{LYS} values) in the summer than in winter and their membranes are more destabilised (low LP values). Furthermore, low lysosomal responsiveness in winter has been previously reported (Garmendia et al., 2010; Lekube et al., 2014; Múgica et al., 2015). Since the study was performed at 15°C, which was more representative of winter for *M. galloprovincialis* and spring for *M. edulis* and *M. trossulus*, due to their different geographical origins. The mussels were therefore more likely adapted to different seasonal patterns in water temperature and food availability. This could be a reason for the lack of lysosomal responsiveness in *M. galloprovincialis* to Cu exposure and the significant differences with *M. edulis* and *M. trossulus*.

Intracellular neutral lipid accumulation in the lysosomes and cytosol of the digestive cells of mussels has been mainly linked to pollution. However, intracellular neutral lipid accumulation differs between geographical locations and can vary throughout the year, mainly due to changes in the reproductive cycle and food availability (Cancio et al., 1999; Garmendia et al., 2010). The observed differences in Vv_{NL} between *Mytilus* spp. could therefore be due to natural factors such as temperature and reproductive cycle, rather than any physiological and/or cellular differences caused by metal exposure.

Lipofuscins are pigments regarded as the end-products of lipid peroxidation (Moore, 1990; Yin, 1996; Terman et al., 1999; Brunk and Terman, 2002). Their accumulation in digestive cells is one of the best documented changes in lysosomal content in response to pollutant exposure, and therefore, lipofuscin accumulation is considered a general response to pollutants (Viarengo et al., 1990; Regoli, 1992). In the present study, lipofuscin accumulation followed the same pattern of Vv_{LYS}, with a higher amount of lipofuscins in controls than in exposed groups. This decrease is in accordance with lysosomal enlargement indicating a close relation between these two biomarkers. The reduction of lysosomes and lipofuscins could be explained by cell type replacement and tissue renewal processes, which are typical responses in long term pollutant exposures (Cajaraville et al., 1995; Zaldibar et al., 2007).

It has been shown that the responsiveness of *M. trossulus* was higher when exposed to Cu stress than the other studied species. However, differences between the initial conditions of mussels from the different geographical locations cannot be ignored and such differences were thought to influence, to a certain extent, some of their biological responses. However, in the case of *M. edulis* and *M. trossulus* similar temperatures and seasonal patterns were experienced prior to laboratory acclimation. During the experiment, water temperatures were maintained at 15°C, which was considered to be a compromise between the temperate ranges experienced by the Norwegian mussels and those from the Basque coast. However, the seawater temperature was typical of the summer months in Norway and the winter months on the Basque coast (www.seatemperature.org). Although the long acclimation period of 4–6 wk was thought to be sufficient time for the normalisation of the physiological parameters (Widdows and Bayne, 1971; Altieri, 2006), there is still a lack of knowledge of the biological responses of mussels with changes in seasonal/thermal patterns. For instance, the use of energy reserves, gonad development and food

availability could be important factors, which are known to change with the seasons (Garmendia et al., 2010). Another important factor that could modulate the biomarkers is the age of the mussels. In the present work, mussels of similar size have been used in order to normalise these differences but recent work has confirmed that mussels from different geographical areas could have different growth rates and different biomarker responses (Izagirre et al., 2014b).

In conclusion, it is clear from this study that differences in lysosomal responses occur between the three *Mytilus* species, which may influence the assessment of the environmental health status in marine pollution programmes; particularly knowing that a large extent of geographical areas around the European coast have two or three of the species studied and their hybrids (Kijewski et al., 2011; Brooks and Farmen, 2013). Future research is needed to determine the degree of influence of the species in biological responses to pollutants especially in different mussel species from the same population/sampling point.

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CHAPTER 2

BEST AVAILABLE PRACTICES FOR INTERTIDAL MUSSEL COLLECTION AND TRANSPORT IN BIOMARKER-BASED ENVIRONMENTAL MONITORING OF THE COASTAL ZONE

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ABSTRACT

Biomarkers use to be recorded in sentinel mussels for biological effects assessment in coastal pollution monitoring. There are recommended procedures (e.g., by ICES) for biomarker determination but not for sample collection and transport. However, where and when mussels are collected and how samples are processed can be major sources of variability in biomarker values. Thus, the present study aims at contributing to identify reliable practices for collection and transport of intertidal mussels in biomarker-based coastal pollution monitoring. Mussels, *Mytilus galloprovincialis*, were handpicked from the high (2.5-3.0 m) and low (0.5-1.0 m) tide-mark levels at autumn tides in Gorliz, a relatively clean locality of the Basque Coast (Bay of Biscay). Then, they were processed by 3 different ways: (a) *in situ* dissected immediately after sampling; (b) transported to the laboratory either in air or in seawater; and (c) dissected at different time intervals after sampling. Enzyme activities, lipid peroxidation, lysosomal responses and tissue-level biomarkers were investigated. Oxidative stress and lysosomal biomarkers were significantly affected by collection and transport to the laboratory, as a result of the combination of hypoxia associated to air exposure and the progression of food digestion. Consequently, it is recommended that sentinel mussels should be collected the nearest possible to the 0 tide-mark level and processed *in situ* as soon as possible, and always within 1 hr upon collection. Yet, in those particular cases in which *in situ* processing is not possible (force majeure) mussels should be transported in air at ambient temperature (never in seawater) and processed within the shortest time possible. Whatever the case may be, details on collection, transport and the time passed before dissection should always be provided in publications and reports in order to assist other researchers to properly interpret and compare results.

Eskuragarriak diren jardunbide hoberenak marearteko muskuiluen bilketa eta garraiorako kostaldean burutzen diren eta biomarkatzaileetan oinarritutako kutsadura-jarraipenetan

Laburpena

Kutsadura-jarraipen programetan efektu biologikoak aztertzeko muskuilu begiraleen biomarkatzaileak erabiltzen dira. Biomarkatzaileak zehazteko gomendutako prozedurak daude (adibidez, ICES-ekoak), baina laginak biltzeko eta garraitzeko ez. Baina muskuiluak non eta noiz jaso diren, eta laginak nola prozesatu diren biomarkatzaileen balioen aldakortasun-iturri handia izan daitezke. Hori dela eta, lan honen helburua, biomarkatzaileetan oinarritutako kutsadura-jarraipen programetarako marearteko muskuiluen bilketarako eta garraiorako praktika fidagarrien identifikazioan laguntzea da. *Mytilus galloprovincialis* muskuiluak goiko (2,5-3 m) eta beheko (0.5-1 m) istasaldi-markako mailetan bildu ziren udazkenean Gorlizen, Euskal Kostaldeko (Bizkaiko Golkoa) lokalizazio nahiko garbia. Ondoren, 3 modu desberdinetan prozesatu ziren: (a) lagindu bezain laster *in situ* disekzionatzea; (b) airean edo itsasoko uran laborategira garraiatze; eta (c) denbora tarte desberdinetan disekzionatzea laginketa egin ostean. Estres oxidatiboarekin eta neurotoxikotasunarekin erlazioa duten jarduera entzimatikoak eta lipidoen peroxidazioa, erantzun lisosomikoak eta ehun-mailako biomarkatzaileak aztertu ziren. Bilketak eta garraioak laborategira eragina dute estres oxidatiboko eta lisosoma-markatzaileetan, aire-esposizioari lotutako hipoxiaren eta digestioaren aurreratzearen konbinarioaren ondorioz. Beraz, muskuilu begiraleak ahalik eta gertuen 0 itsasaldi-markatik biltzea eta *in situ* disekzionatzea ahalik eta lazterren (beti bilketa ordubete baino gutxiagoan) gomendatzen da. Baina *in situ* prozesatzea posiblea ez denean (ezinbesteko kasuetan), muskuiluak airean eta giro tenperaturan garraiatu behar dira (inoiz ez itsasoko uran) eta ahalik eta denbora laburrenean prozesatu. Edonola ere, argitalpenetan eta txostenetan bilketari, garraioari eta disekzioa baino lehen igarotako denborari buruzko xehetasunak beti eman behar dira, beste ikertzaileen emaitzen interpretazio eta konparazio egokian laguntzeko.

Mejores prácticas disponibles para la recogida y transporte los mejillones intermareales en el seguiemiento ambiental de la zona costera basado en biomarcadores

Resumen

Los biomarcadores se suelen aplicar en mejillones centinela para la evaluación de efectos biológicos en el seguimiento de la contaminación costera. Existen procedimientos recomendados (e.g., por ICES) para la determinación de biomarcadores pero no para la recogida y el transporte de muestras. A pesar de que dónde y cuándo se recogen los mejillones y cómo las muestras son procesadas pueden ser una fuente importante de variabilidad en los valores de los biomarcadores. Por lo tanto, el presente estudio tiene como objetivo contribuir a identificar prácticas fiables para la recolegida y el transporte de mejillones intermareales en el seguimiento de la contaminación costera basada en biomarcadores. Mejillones, *Mytilus galloprovincialis*, fueron recogidos de los niveles alto (2,5-3,0 m) y bajo (0,5-1,0 m) de la altura de marea en otoño en Gorliz, una localidad relativamente limpia de la Costa Vasca (Golfo de Vizcaya). Después fueron procesados de 3 formas diferentes: (a) *in situ* diseccionadolos inmediatamente después de la recogida; (b) transportadolos al laboratorio tanto en aire como en agua de mar; y (c) diseccionadolos a diferentes intervalos de tiempo después del muestreo. Se analizaron las actividades enzimáticas, peroxidación lípidica, las respuestas lisosómicas y los biomarcadores tisulares. Los biomarcadores de estrés oxidativo y lisosómicos fueron significativamente afectados por la recogida y el tranporte al laboratorio, como resultado de la combinación de la hipoxia asociada a la exposición al aire y la progresión de la digestión. En consecuencia, se recomienda que los mejillones centinela se recojan lo más cerca posible del nivel 0 de altura de marea y se procesen *in situ* tan pronto como sea posible, y siempre dentro de 1 hr después de la recogida. Sin embargo, en aquellos casos particulares en los que el procesamiento *in situ* no es posible (fuerza mayor), los mejillones deben transportarse al aire a temperatura ambiente (nunca en agua de mar) y procesarse en el menor tiempo posible. Cualquiera que sea el caso, los detalles sobre la recojida, el transporte y el tiempo transcurrido antes de la disección siempre deben proporcionarse en publicaciones e informes para ayudar a otros investigadores a interpretar y comparar los resultados.

INTRODUCTION

Countries participating in the OSPAR agreement are obliged to perform integrative monitoring as part of their national monitoring programs (Beyer et al., 2017). ICES has developed a holistic integrated approach to biological effects monitoring that includes mussels as a key target organism for monitoring biological responses at different levels of biological complexity in combination with chemical analysis to determine the concentrations of environmental contaminants in tissues or the whole body of animals (Davies and Vethaak, 2012). Mussels are sessile filter-feeders, globally widespread, ecologically relevant for coastal environments, and responsive to a wide range of environmental stimuli including short-term and long-term chemical pollution (Moreira et al., 2004, Lima et al., 2007; Tim-Tim et al., 2009; Beyer et al., 2017). For those reasons, mussel responses are commonly applied as sensitive biomarkers to assess biological effects of pollutants in coastal and estuarine ecosystems (UNEP/RAMOGE, 1999; Cajaraville et al., 2000; OSPAR, 2000; Moreira et al., 2001, 2004; Moreira and Guilhermino, 2005; Sarkar et al., 2006; Lima et al., 2007; Tim-Tim et al., 2009; ICES, 2012; Marigómez et al., 2013).

Biomarkers commonly used in mussels include enzyme activities (CAT: catalase; GPx: glutathione peroxidase; GST: glutathione-S-transferase; GR: glutathione reductase; AChE: acetylcholinesterase; ChE: cholinesterases), metabolic by-products such as lipid peroxides (LPO), and lysosomal responses. CAT and GPx are essential antioxidant defenses used as biomarkers of oxidative stress (Livingstone, 2001; Lima et al., 2007; Vidal-Liñán and Bellas, 2013). GST catalyzes the conjugation of reduced glutathione (GSH) with xenobiotics or oxidised cellular components and GR replenishes the GSH substrate for GPx and GST (Regoli and Giuliani, 2014). LPO is indicative of the damage caused by reactive oxygen species to cell membrane lipids (Regoli and Principato, 1995; ICES, 2012). AChE activity is commonly used to detect the neurotoxic stress caused by anticholinesterase chemicals (Guilhermino et al., 1996; Sarkar et al., 2006; ICES, 2012), whereas ChE activity may be used as indicative of neurotoxicity or detoxication response depending on the species and the specific tissue used Lysosomal responses to pollutants in mussel digestive cells are widely used as effect biomarkers. Lysosomal enlargement (augmented volume density: Vv_{LYS}) has been reported in response to pollutant exposure (Etxeberria et al., 1995; Domouhtsidou and Dimitriadis, 2001; Marigómez and Baybay-Villacorta, 2003) and lysosomal membrane destabilization (reduced labilization period: LP) is recommended by OSPAR as a core biomarker for marine pollution monitoring programs (UNEP/RAMOGE, 1999; ICES, 2004). Intracellular accumulation of neutral lipids (augmented volume density; Vv_{NL}) has been related to exposure to organic xenobiotics, to non-specific stress and to nutritional status (Cancio et al., 1999; Marigómez and Baybay-Villacorta, 2003; Shaw et al., 2011; Marigómez et al., 2013). Likewise, changes in cell type composition in the digestive gland epithelium (e.g., increase in volume density of basophilic cells: Vv_{BAS}), atrophy of the digestive epithelium (augmented lumen-to-epithelium ratio: MLR/MET), inflammatory responses, and loss of digestive gland histological integrity (augmented connective-to-diverticula ratio: CTD) have been reported to occur in

response to pollutant exposure (Cajaraville et al., 1992; Marigómez et al., 2006; Garmendia et al., 2011).

Recently, efforts have been addressed to develop consensus or standard operating procedures to determine the most widely accepted biomarkers aforementioned (ICES, 2012). Moreover, factors that may exert a confounding influence to assessments of pollution responsive markers in mussels have been clearly identified; these include taxonomical position, seasonality, age, size, nutritional status and condition, gender, sexual maturity and spawning, multiple stress scenarios, and sample collection and transport (Beyer et al., 2017). Noteworthy, biomarker values may be subjected to change depending on where and when mussels are collected and how samples are processed afterwards, as reported by Moreira and Guilhermino (2005), Lima et al., (2007), Izagirre et al. (2008), Chandurvelan et al. (2013) and Vidal-Liñán and Bellas (2013). The species and the specific tissue analysed are also factors of important variability and may make comparisons between studies difficult and even impossible. The same biomarker may have different background levels in closed related species, in distinct populations of the same species inhabiting habits with different abiotic conditions (e.g. temperature, salinity), and in distinct tissues of the same animal. Moreover, according the species and tissues, the same biomarker may play be involved in distinct physiological functions. A good illustrative example is provided by cholinesterase enzymes (ChE) that are among the most used and characterized biomarkers, including in mussels. In several species, such as *M. galloprovinciallis*, the ChE activity determined in distinct tissues may be very different (Mora et al., 1999), and in the same tissue may variate along the year (Moreira and Guilhermino, 2005). Moreover, one or more ChE enzymes (and also non-specific esterases) may contribute the measured activity and this may be different in distinct tissues (Mora et al., 1999; Moreira et al., 2001). Also distinct ChE enzymes, present in different species or in distinct tissues of the same species, may have different sensitivities and/or responses towards the same environmental contaminant or mixture of contaminants, including opposite ones such as inhibition or induction (Tim-Tim et al., 2009). Furthermore, in mussels, distinct ChE enzymes may play different physiological roles, thus being indicative of different effects/responses, namely (i) degradation of the neuro transmitter acethylcholine in neuro or neuro-muscular junctions, with inhibition indicating neurotoxicity; and (ii) binding to or hydrolysing environmental contaminants (biotransformation function) and having no role in cholinerginc neurotransmission.

Collection and transport of mussels from the field involve multiple steps, which include sampling procedure as well as conditions (air or water, humidity, temperature) and duration of the transport and maintenance in laboratory before analyses. These steps may induce a stress response that will be reflected on biomarker values, rendering these less comparable, less sensitive and less reliable for coastal environmental monitoring (Chandurvelan et al., 2013).

Alas, there are few guidelines available for collection, manipulation and transport of mussels and these differ in their recommendations (Chandurvelan et al., 2013); as a result, the procedures are disparate

amongst studies (see table 1 in the general introduction (T1GI)). For example, 28 out of 65 studies reviewed herein do not provide details about the tide-mark level where the mussels were collected; whereas 18 do not indicate whether mussels were sacrificed *in situ* or transported to the laboratory before dissection (T1GI). In 18 of the 65 cases subtidal mussels were collected; *in situ* dissections were carried out in 2 of them whereas in 10 cases mussels were reported to be transported to the laboratory before dissection, in 4 cases in seawater and in 6 in air (T1GI). In 20 of the 65 cases intertidal mussels were collected, albeit the tide-mark level, or at least their gross position on the shore, was rarely detailed. *In situ* dissections were carried out in 5 of them whereas in 16 cases mussels were reported to be transported to the laboratory before dissection; in seawater in 3 cases and in air in 10 cases, most of them in cold conditions (T1GI). As a whole, whereas 96% of the studies dealt with prompt responses that may occur within minutes to a few hours, only 18.5% reported to be based on *in situ* dissection practices and 52.3% recognized that mussels were transported to the laboratory before dissection, and often without taken into account the condition of the mussels in their source site. Thus, intertidal mussels were transported in seawater at least in 19% of the cases and subtidal mussels were taken to the laboratory in air in cold boxes in 50% of the cases, with transport time varying from 1-2 hr to beyond overnight (T1GI).

Noteworthy, Chandurvelan et al. (2013) concluded that mussel physiology is severely affected by transport in seawater or in air at 5°C (cold boxes) and for this reason they recommended transport in air at ambient temperature for the shortest possible time period, at least for intertidal mussels collected at the lowest tide-mark level. In contrast, other authors maintained the mussels for relatively long periods (1 to several days) in the laboratory in seawater at controlled temperature and with food supply, aimed at getting the mussels acclimatized and recovered from the stress caused by collection and transport (González-Fernández et al., 2015; Balbi et al., 2017). This approach seems to be useful for some late-response that requires experimental manipulation of the mussels in the laboratory endpoints (e.g. scope-for-growth; ICES, 2006). However, other biological responses such as e.g. oxidative stress or lysosomal biomarkers are known to be elicited within a few minutes to hours by changes in oxygen and food availability or in temperature (Regoli and Pricipato, 1995; Izagirre et al., 2009b; Vidal-Liñán and Bellas, 2013); therefore, the biomarker data measured after "recovery" or "acclimatization" would be difficult to compare with field data obtained in other studies and, moreover, they would hardly reflect the status of mussels in the field. This overall analysis clearly reflects the urgent need to advance in the standardization of procedures for collection, transport and processing of mussels used as sentinels in biomarker-based biomonitoring programs, as previously recognized by other authors (Chandurvelan et al. 2013, Beyer et al. 2017).

The present study aims at contributing to develop reliable Best Available Practices (BAPs) for intertidal mussel collection and transport in biomarker-based environmental monitoring of estuaries and other coastal ecosystems. For this purpose, intertidal mussels were handpicked from the lowest and highest tide-mark levels at spring tides in

a reference locality of the Basque Coast (Bay of Biscay) and processed by different ways before biomarker determinations, say: (a) *in situ* dissection immediately after sampling; (b) transport to the laboratory either in air or seawater; and (c) dissection at different time intervals after sampling. CAT, GPx, GST, GR, and AChE enzymatic activities, LPO, LP, Vv_{LYS}, Vv_{NL}, Vv_{BAS}, MLR/MET and CTD were investigated. The investigation was completed with the determination of gamete maturation (Gonad Index: GI) as supporting parameter.

MATERIAL AND METHODS

Sampling strategy

Intertidal mussels (N=160) were handpicked from Gorliz (43°26'N, 2°55'W) at the lowest (LIL: 0.5 – 1.0 m) and the highest (HIL: 2.5 – 3.0 m) tide-mark levels (80 mussels each) in September 2010, and processed in different ways. Gorliz is a relatively unpolluted locality, according to the data available at the Biscay Bay Environmental Biospecimen Bank (BBEBB) Database established at PiE-UPV/EHU (Table 1). This database includes annual average tissue concentrations of selected metals (determined by Inductively Coupled Plasma - Mass Spectrometry) and total PAHs (determined by Gas Chromatography - Mass Spectrometry) in intertidal mussels collected from the low tide-mark level. In mussels collected from Gorliz in 2010, metal concentrations in the soft tissues were low according to Kimbrough et al. (2008) criteria, except for the case of Zn that was low-to-moderate (Table 1). Likewise, the tissue concentration of total PAHs was low-to-moderate, according Kimbrough et al. (2008), and the PAHs were mainly of pyrolytic origin (Table 1).

A set of mussels (N=20) was sacrificed *in situ* at each tide-mark level (IS_0). A second set (N=20 per tide-mark level) was transported "in air" in thermally insulated boxes and sacrificed in the laboratory 3 hr after sampling (A_3). A third set (N=40 per tide-mark level) was transported in seawater (directly taken from the sampling site) at ambient temperature in thermally insulated boxes (~20-24ºC). Once in the laboratory, a first subset of mussels (N=20 per tide-mark level) was sacrificed 3 hr after sampling (SW_3) and the last subset (N=20 per tide-mark level) was further on kept at 18°C in the seawater in the laboratory and sacrificed 24 hr (SW_{24}) after sampling as previously indicated.

The digestive gland, the gills, the mantle and the foot of 10 mussels per experimental group were dissected out. The digestive gland was halved. One half of the digestive gland, the gills and the mantle were fixed in 4% formaldehyde in seawater for histological analyses. The second halves of digestive gland of 5 mussels per experimental group were pooled, rapidly placed on plastic chucks, flash-frozen in cryovials with liquid nitrogen and stored at −80°C until they were processed. The digestive gland, the gills and the foot of other 10 mussels per experimental group were dissected out and individually flash-frozen in cryovials with liquid nitrogen and stored at −80°C until CAT, GPx, GST, and GR enzyme activities and LPO were determined. The foot of 15 mussels dissected per experimental group was individually flash-frozen in cryovials with liquid nitrogen and stored at −80°C until AChE activity was measured.

Table 1. Annual average tissue concentrations of metals (µg metal/g soft tissue dry-wt) and PAHs (ng PAH/g soft tissue dry-wt) recorded in mussels, *Mytilus galloprovincialis*, collected from the low intertidal level in Gorliz in 2010 (unpublished; Biscay Bay Environmental Specimen Bank data base). Superscript numbers on top of individual PAHs refer to the sums and indices given in the table where these compounds are integrated. LOD, limit of detection.

Metals (µg/g soft tissue dry-wt)		
METAL	**LOD**	**$[metal]_{tissue}$**
Ag	0.0015	1.7
As	0.01	11.2
Cd	0.0001	0.7
Cr	0.22	1.7
Cu	0.11	6.9
Ni	0.54	2.0
Pb	0.015	2.4
Zn	2.36	287.5
PAHs (ng /g soft tissue dry-wt)		
PAH	**LOD**	**$[PAH]_{tissue}$**
Acy (1)	1.00	2.8
Ace (1)	1.00	4.7
Flu (1)	0.07	9.7
Phe (1)	0.49	84.8
Ant	1.00	45.2
Flr (1)	0.34	173.1
Pyr (1)	1.00	139.6
Benz[a]A (2)	1.00	91.8
Chr (2)	1.00	82.0
B[b]F +B[k]F (2)	1.00	165.4
B[a]P (2)	1.00	77.4
B[ghi]P	1.00	43.3
Ind (2)	2.00	116.5
D[ah]A (2)	2.00	20.9
∑PAHs (16 US EPA; except Naph)		**1057.2**
$\sum_{HMW}PAHs^{\Sigma(2)}$ (carcinogenic)		554.0
$\sum_{LMW}PAHs^{\Sigma(1)}$		414.8
(%∑PAHs)[a]		(39%)
Ind/B[ghi]P[b]		2.7
$\sum_{LMW}PAHs/\sum_{HMW}PAHs$[c]		0.7
Ind/(Ind+B[ghi]P)[d]		0.7
Phe/Ant[e]		1.9
Flr/Pyr[f]		1.2

[a] < 50%: waterborne
[b] > 0.25: pyrolytic origin; power plants
[c] < 1: pyrolytic origin
[d] > 0.5: biomass/coal combustion
[e] < 10: pyrolytic origin; urban area
[f] < 10: pyrolytic origin; urban area

Enzyme activities and lipid peroxidation

CAT, GPx, GST, and GR enzyme activities and LPO were determined in gills and digestive gland, and AChE activity in foot, using a JASCO V-630 spectrophotometer. All the enzyme activities were expressed as a function of the protein concentration in the samples; this was determined in triplicate according to the Bradford's method adapted to microplate assays and using *y*-bovine globulins as standard (Guilhermino et al., 1996). All enzyme assays were performed at 25ºC. CAT activity was determined by measuring the consumption of H_2O_2 at 240 nm, according to Clairborne (1985). GPx activity was determined by measuring the decrease in NADPH using H_2O_2 as substrate at 340 nm, according to Mohadas et al. (1984). GST activity was determined by measuring the formation of thioether at 340 nm, according to an adaptation of the Habig's method to microplate and using *y*-bovine globulins as standard (Guilhermino et al., 1996). GR activity was determined by monitoring the decrease of NADPH levels at 340 nm, based on the method described by Cribb et al. (1989). LPO levels were determined in the homogenates by measuring thiobarbituric acid-reactive substances (TBARS) at 535 nm, based on the Ohkawa's method after Bird and Draper (1984) adapted by Torres et al. (2002). AChE activity was determined by measuring formation of 5-thio-2-nitobenzoic acid at 412 nm, according to the Ellman's method adapted to microplate assays (Guilhermino et al., 1996).

Lysosomal biomarkers

Lysosomal membrane stability was evaluated in serial cryotome sections (10 µm thick;

Leica CM 3000 cryotome) of digestive gland chucks after the cytochemical demonstration of hexosaminidase activity, according to a standardized procedure (UNEP/RAMOGE, 1999), based on the time of acid labialization (LP; min) required to produce the maximum staining intensity.

In order to quantify changes in lysosomal structure, cryotome sections (8 µm thick; Leica CM 3000 cryotome) of digestive gland chucks were stained for the histochemical demonstration of ß-glucuronidase activity according to Cajaraville et al. (1991). For each mussel digestive gland the following stereological parameters were determined (Lowe et al., 1981): lysosomal volume density ($Vv_{LYS}=V_{LYS}/V_C$), lysosomal surface density ($Sv_{LYS}=S_{LYS}/V_C$), lysosomal surface-to-volume ratio ($S/V_{LYS}=S_{LYS}/ V_{LYS}$) and lysosomal numerical density ($Nv_{LYS}=N_{LYS}/V_C$); where V_{LYS}, S_{LYS} and N_{LYS} are the volume, surface and number of lysosomes, and V_c the volume of digestive cells.

Intracellular accumulation of neutral lipids was determined in cryotome sections (8 µm thick; Leica CM 3000 cryotome) of digestive gland chucks after staining with Oil Red O (ORO; Culling 1974). The extent of ORO staining in the digestive gland epithelium was measured by image analysis (Marigómez and Baybay-Villacorta, 2003) to calculate the volume density of neutral lipids with respect to the digestive epithelium volume ($Vv_{NL;}$ $\mu m^3/\mu m^3$).

Tissue-level biomarkers

Fixed digestive gland samples were dehydrated in graded ethanol series, and embedded in paraffin. Sections (5 µm thick) were cut in a rotary microtome (Leitz 1512), and stained with hematoxylin-eosin. Slides were viewed at 40× magnification using a drawing tube attached to a light microscope. A Weibel graticule (multipurpose system M-168) was used, and hits of basophilic and digestive cells, luminal area and connective tissue were recorded. The volume density of basophilic cells ($Vv_{BAS;}$ in $\mu m^3/\mu m^3$) in digestive gland of mussels was determined according to Soto et al. (2002). The mean epithelial thickness of the digestive alveoli (MET; µm) was determined according to Lowe et al. (1981) together with other estimates of changes in alveolus morphology such as the mean luminal radius (MLR; µm) and the MLR-to-MET ratio (MLR/MET; µm/µm) (Vega et al., 1989). Likewise, the integrity of the digestive gland tissue was simultaneously determined as the extent of the interstitial connective tissue relative to the space occupied by digestive diverticula (connective-to-diverticula (CTD) ratio) (Brooks et al., 2011; Garmendia et al., 2011) on the basis of the same stereological data set.

Gamete developmental stages and Gonad Index

Fixed mantle (including the gonad tissue) samples were dehydrated in graded ethanol series, and embedded in paraffin. Paraffin sections (5 µm) were stained with hematoxylin-eosin and used for the determination of developmental stages of gametes and gonad index (GI) at the light microscope, according to Ortiz-Zarragoitia et al., (2011). Briefly, gamete developmental stages were distinguished and a GI value was assigned to each mussel depending on its gamete developmental stage (adapted after Kim et al., 2006), and the average GI of 10 mussels was calculated per experimental group.

Statistical analyses

The statistical analyses were made using IBM® SPSS® Statistics ver. 22.0.0.0 (IBM Corp., Armonk, NY, USA). Biological parameters were tested for normality (Kolmogorov-Smirnov's test) and homogeneity (Levene's test). CAT, GPx, GR, ChE, LPO, Vv_{NL}, Vv_{BAS}, MLR/MET and CTD ratio were analyzed by one-way analysis of variance (ANOVA), statistical differences among processing ways for each tide-mark level were establish using Duncan's test, and statistically differences between pairs of tide-mark level for each processing way were determined by the Student's t-test. For GST, LP, Vv_{LYS}, Nv_{LYS} and GI significant differences among processing ways for each tide-mark level and between pairs of tide-mark level for each processing way were determined by the Mann-Whitney's U-test. Significance for all statistical tests was established at $p<0.05$.

RESULTS

Enzyme activities and lipid peroxidation

Different enzyme activities and LPO values were recorded in gills and digestive gland both in HIL and LIL mussels processed *in situ*. CAT (Fig. 1A, 1F), GPx (Fig. 1B, 1G), and GST (Fig. 1C, 1H) activities and LPO levels (fig. 1E, 1J) were higher in the digestive gland than in gills. For mussels collected in both LIL and HIL and processed *in situ*, the comparison of the values in gills and in the digestive gland indicated no significant differences in GR activity (Figs. 1D and 1I), and significant differences in all the other enzymatic biomarkers and LPO levels (Fig. 1). Likewise, differences in enzyme activities and LPO values were found between HIL and LIL mussels processed *in situ*. GPx values in gills (Fig. 1B) and LPO values in gills and digestive gland (Fig. 1E and 1J, respectively) were higher in HIL mussels than in LIL mussels. In contrast, GST activity in gills (Fig. 1C) and GR activity in digestive gland (Fig. 1I) were lower in HIL mussels than in LIL mussels.

In the gills of LIL mussels, CAT and GR activities and LPO remained unchanged after transport to the laboratory (Figs. 1A, 1D and 1E). However, GPx rose after transport in seawater for 3 hr before dissection (Fig. 1B) and GST activity decreased after transport to the laboratory both in air and in seawater (Fig. 1C). In the gills of HIL mussels, CAT activity rose after transport in seawater for 3 hr (Fig. 1A), GPx and GST activities and LPO values remained unchanged after transport to the laboratory (Figs. 1B, 1C and 1E), and GR activity rose after transport in air for 3 hr and dropped after transport in seawater for 24 hr (Fig. 1D). In the digestive gland of LIL mussels, CAT, GPx and GST activities rose after 3 hr transport in air and GPx and GR activities decreased after transport in seawater (Figs. 1F-1I). No clear change was recorded in LPO (Fig. 1J). In HIL mussels, CAT, GPx and LPO remained unchanged during transport to the laboratory but GR was reduced in the gills and elevated in the digestive gland after transport in seawater; and GST was lowered in the digestive gland after transport in seawater (Fig. 1F-J).

Regarding AChE activity in foot, significant differences were not recorded between LIL and HIL mussels processed *in situ* (Fig. 2). Overall, a slight but significant decrease (<20%) in AChE activity was observed in LIL and HIL mussels during transport to the laboratory both in air and in seawater (Fig. 2).

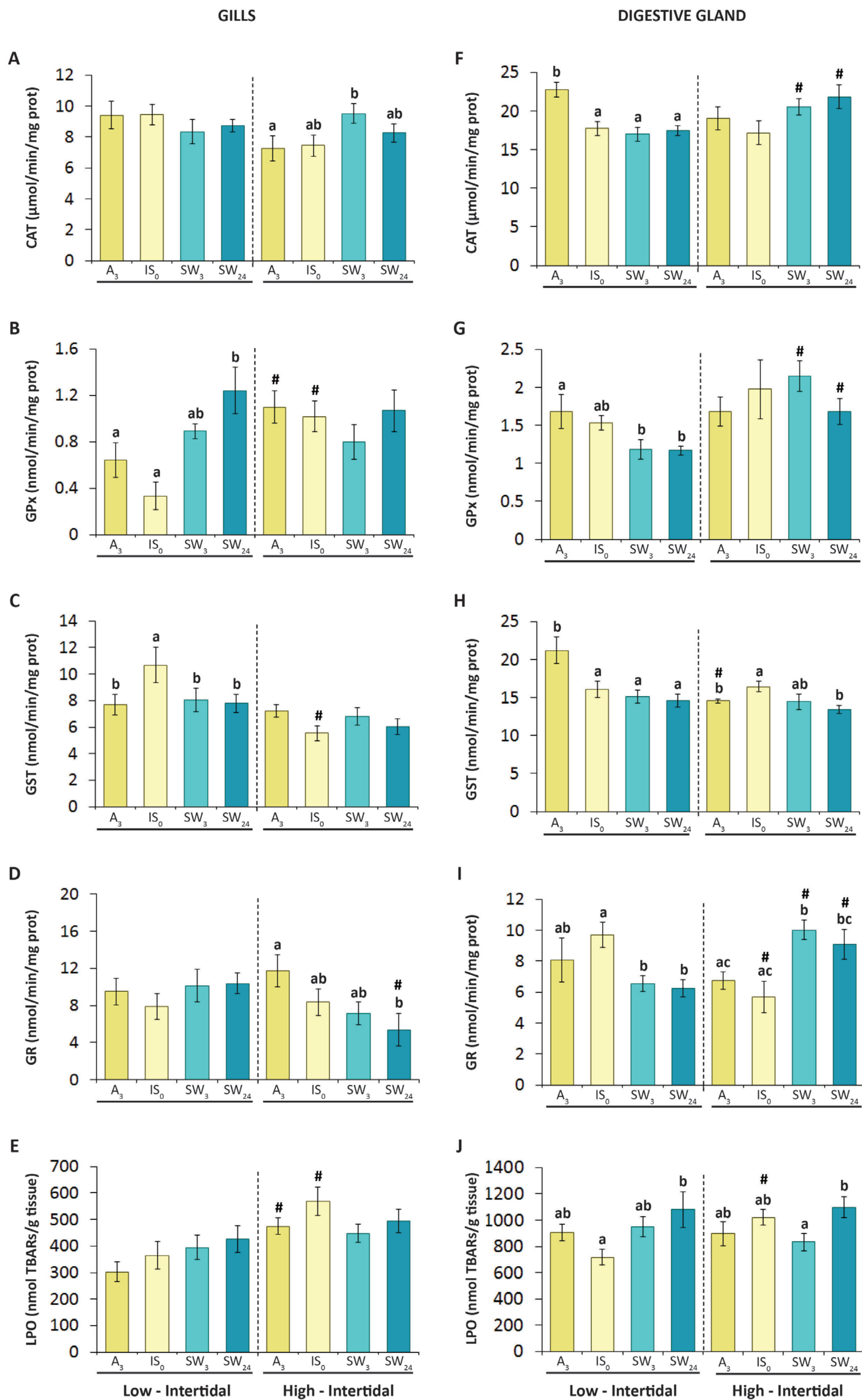

Fig. 1. Enzyme activities and metabolites in gills (1A-1E) and digestive gland (1F-1J) of intertidal mussels handpicked at the lowest and the highest tide-mark levels and processed in different ways: in situ dissected (IS_0), transported in air for 3 hr (A_3) and transported in seawater for 3 hr (SW_3) and 24 hr (SW_{24}). Intervals indicate standard error. Letters indicate significant differences among processing ways for each tide-mark level according to the Duncan test performed after a one-way ANOVA ($p<0.05$) and, Mann-Whitney's U-test ($p<0.05$) for GST only. The hashes indicate significant differences between pair of tide-mark level for each processing way according to Student's t-test and, Mann-Whitney's U-test ($p<0.05$) for GST only. CAT: catalase; GPx: glutathione peroxidase; GST: glutathione S-transferase; GR: glutathione; LPO: lipid peroxidation products.

Lysosomal responses

LP and Vv_{LYS} were seemingly lower and S/V_{LYS} was seemingly higher in HIL than in LIL mussels processed *in situ* but significant differences were not found in any case (Figs. 3A-3C). Alike, only minor changes were observed in HIL mussels after transport to the laboratory. These included a slight decrease in LP and S/V_{LYS}, together with a small increase in Vv_{LYS} after transport in air for 3 hr, and an increase in Nv_{LYS} after transport in seawater for 24 hr (Figs. 3A-3D). Changes were more marked in LIL mussels. LP and S/V_{LYS} increased and Vv_{LYS} decreased after transport both in air and in seawater (Figs. 3A-3C) and Nv_{LYS} increased remarkably after transport in seawater, especially after 3 hr (Fig. 3D). Vv_{NL} values did not exhibit any clear trend, with the exception of a certain increase in the case of HIL mussels after 3 hr transport both in air and in seawater, and especially in this latter (Fig. 3E).

Tissue-level responses

The digestive diverticula presented a normal appearance, with alveoli at different phases of intracellular digestion both in LIL and HIL mussels processed *in situ*; yet, the lumen of alveoli seemed to be dilated to some extent in all the cases (Fig. 4). Accordingly, MLR/MET values were within the normal range but moderately high (Marigómez et al., 2006; Garmendia et al., 2010) and no differences were found in tissue-level biomarkers neither between LIL mussels and HIL mussels nor after transport to the laboratory [mean±standard error (min-max)]: Vv_{BAS} = 0.09±0.01 (0.06-0.10) $\mu m^3/\mu m^3$; MLR/MET = 0.68±0.04 (0.60-0.73) µm/µm; CTD ratio = 0.37±0.05 (0.24-0.41).

The gonad tissue also presented a normal

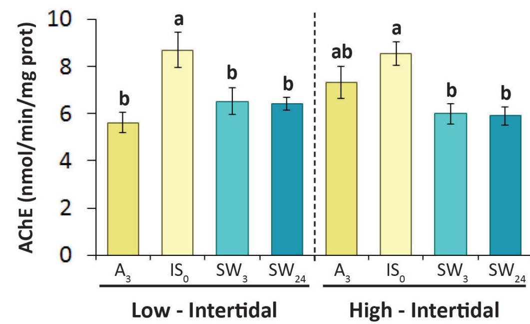

Fig. 2. Acetylcholinesterase (AChE) activity in foot of intertidal mussels handpicked at the lowest and the highest tide-mark levels and processed in different ways: in situ dissected (IS_0), transported in air for 3 hr (A_3) and transported in seawater for 3 hr (SW_3) and 24 hr (SW_{24}). Intervals indicate standard error. Letters indicate significant differences among processing ways for each tide-mark level according to the Duncan's test performed after a one-way ANOVA ($p<0.05$). The hashes indicate significant differences between pair of tide-mark level for each processing way according to the Student's t-test ($p<0.05$).

histological appearance and different gametogenic stages were identified both in LIL and HIL mussels processed *in situ*. Post-spawning and resting stages were dominant in LIL mussels (GI=1.8) whereas advanced gametogenesis, mature gonad and spawning were the dominant stages in HIL mussels (GI=3.4). After collection and transport to the laboratory some signs of spawning induction could be envisaged in both LIL and HIL mussels after transport in seawater (Fig. 5).

DISCUSSION

Enzyme activities and metabolites

In the present study, the values of CAT, GPx, GST, GR activities and LPO were higher in digestive gland than in gills, both in HIL and LIL mussels. Accordingly, CAT, GPx and GST activities were reported to be higher in digestive gland than in gills in the green mussel, *Perna viridis* (De Luca-Abbot et al.,

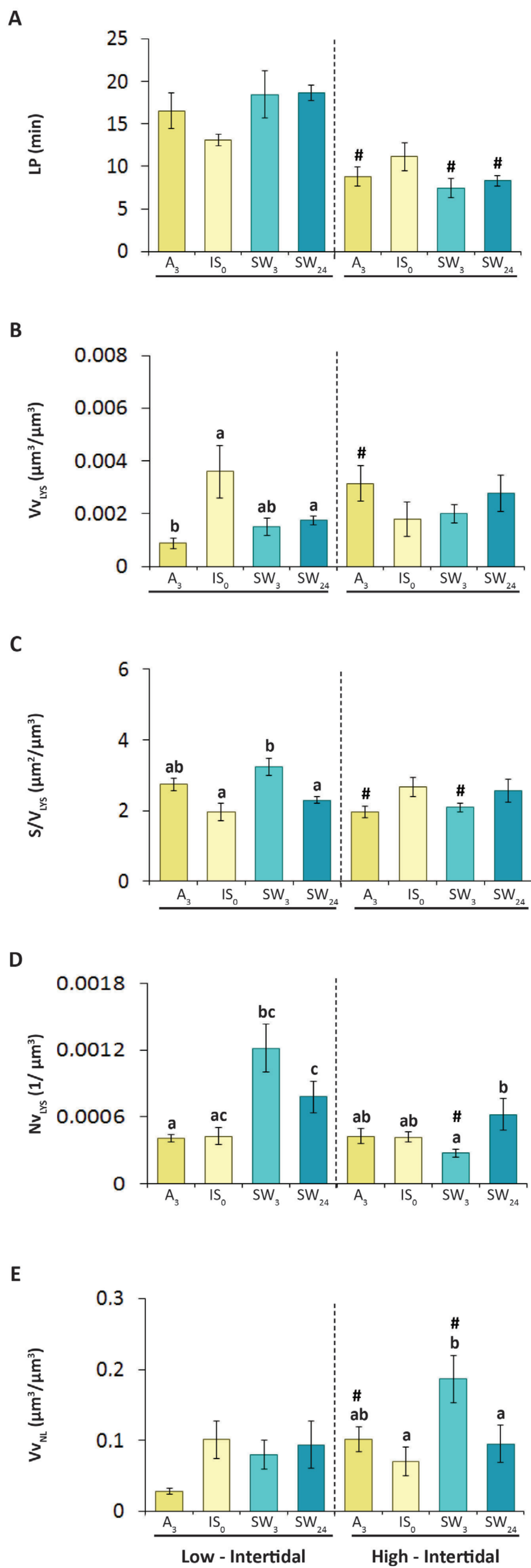

Fig. 3. Lysosomal biomarkers and intracellular neutral lipid accumulation in digestive gland of intertidal mussels handpicked at the lowest and the highest tide-mark levels and processed in different ways: in situ dissected (IS_0), transported in air for 3 hr (A_3) and transported in seawater for 3 hr (SW_3) and 24 hr (SW_{24}). Intervals indicate standard error. Letters indicate significant differences among processing ways for each tide-mark level according to Mann-Whitney's U-test (p<0.05), and to the Duncan's test performed after a one-way ANOVA (p<0.05) for S/VLYS and Vv_{NL} only. The hashes indicate significant differences between pair of tide-mark level for each processing way according to Mann-Whitney's U-test (p<0.05), and to Student's t-test (p<0.05) for S/V_{LYS} and Vv_{NL} only. LP: lysosomal membrane labilization period, Vv_{LYS}: lysosomal volume density, S/V_{LYS}: lysosomal surface density, Nv_{LYS}: lysosomal numerical density, Vv_{NL}: volume density of intracellular neutral lipids.

2005). Regarding CAT activity, as general rule it seems to be higher in digestive gland than in gills (Regoli and Principato, 1995; Power and Sheehan, 1996; Box et al., 2007; Vidal-Liñán and Bellas, 2013; Nogueira et al., 2017), which is conceivable because the digestive gland is the major target organ for xenobiotic biotransformation and oxy-radical generating enzymes (Livingstone, 2001). In contrast, GPx, GST and GR activities and LPO in gills have been reported to be similar to or higher than those recorded in parallel in the digestive gland (Regoli and Principato, 1995; Power and Sheehan, 1996; Manduzio et al., 2004; Almeida et al., 2005; Box et al., 2007; Vidal-Liñan and Bellas, 2013; Nogueira et al. 2017). Exceptionally, GPx activity was reported to be lower in gills than in digestive gland (Nogueira et al. 2017). The natural dynamics of activities of antioxidant enzymes is tightly correlated with the state of animals and with abiotic environmental factors (Soldatov et al., 2007). Thus, whilst the gills reflect environmental conditions rather than the physiological state of the mollusk, the antioxidant enzyme complex in digestive gland reacts to both exogenous and endogenous factors including e.g.

temperature, food availability, physiological or metabolic status, feeding and digestion, gonad development, spawning stress, etc. (Sheehan and Power, 1999; Lesser and Kruse, 2004; Manduzio et al., 2004; Soldatov et al., 2007). As a result, the digestive gland may show high fluctuations in antioxidant enzyme activities thus rendering interpretation of the results uneasy (Manduzio et al., 2004). However, we have observed that the values of enzyme activity are higher, and more relevantly, that the responsiveness to environmental stimuli (e.g. stress associated to transport) seems to be higher than in the gills. As a whole, environmental effects on levels of activity of antioxidant enzymes can also vary among tissues; for instance, CAT responds strongly to food availability whereas SOD varies strongly along tide-mark levels in gills but not in gonad (Dowd et al., 2013). For the particular case of CAT, both gills and digestive gland have been recommended, depending on the authors (Box et al., 2007; Vidal-Liñan and Bellas, 2013).

Intertidal mussels are subjected to chronic fluctuations in environmental factors such as oxygen and food availability; their ability to respond to anoxia during emersion or to food availability during immersion depends on how long the air exposure lasts along the tidal regime at each tide-mark level (Letendre et al., 2009). As such, HIL mussels have longer hypoxia periods and shorter

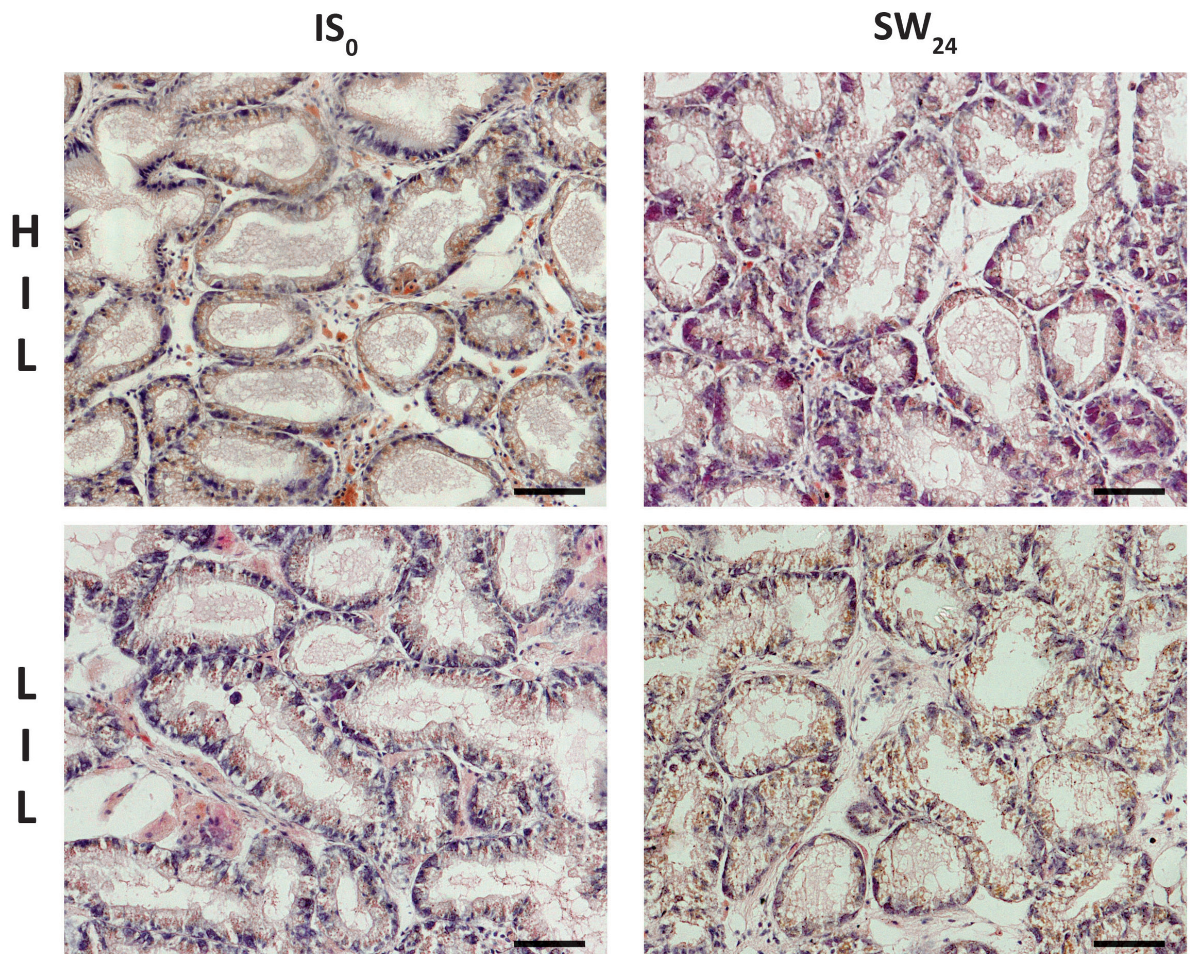

Fig. 4. Micrographs of digestive gland of intertidal mussels handpicked at the highest (HIL) and lowest (LIL) tide-mark levels and processed in different ways. IS_0: mussels were sacrificed in situ at each tide-mark level. SW_{24}: mussels were transported in seawater in thermally insulated boxes, kept at 18°C in the laboratory and sacrificed 24 hr after sampling. Scale bars: 50 µm.

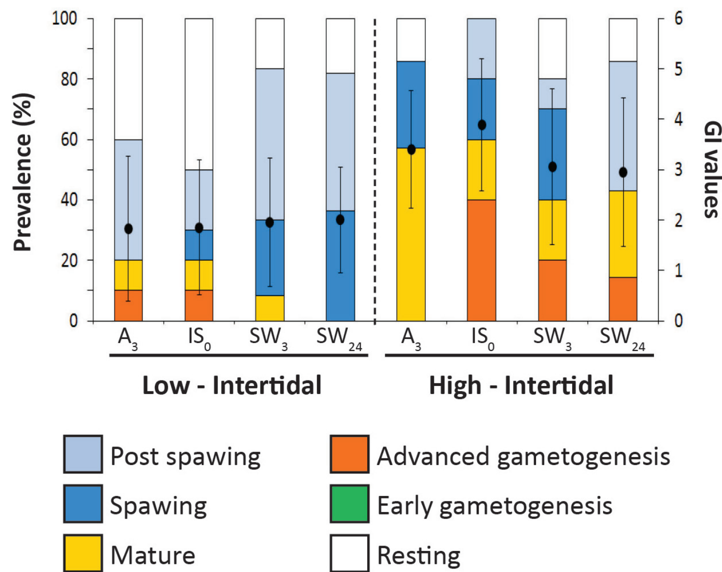

Fig. 5. Gamete development of intertidal mussels handpicked at the lowest and the highest tide-mark levels and processed in different ways: in situ dissected (IS_0), transported in air for 3 hr (A_3) and transported in seawater for 3 hr (SW_3) and 24 hr (SW_{24}). Gamete developmental stages are represented by stocked bars and gonad index (GI) values are represented by a solid line at each time endpoint. Intervals indicate standard error.

feeding time than LIL mussels but they compensate with metabolic, physiological and morphological adjustments (Charles and Newell, 1997; Gracey et al., 2008). Thus, the mussel position in the littoral zone is crucial regarding differences in the gene expression pattern and in essential metabolic and physiological processes such as antioxidant defences, feeding and digestion (Gracey et al., 2008; Izagirre et al., 2008; Letendre et al., 2009; Vidal-Liñan and Bellas, 2013). Some enzyme activities such as CAT and GST can vary largely in mussels along the intertidal zone gradient, whilst others such as GPx are more stable (Vidal-Liñan and Bellas, 2013). In agreement, the profiles of antioxidant enzyme activities and peroxidation products recorded in the present study were different depending on the position of the mussels on the shore, with higher CAT, GPx and LPO values in gills and GST in digestive gland of HIL mussels in comparison with LIL mussels, together with lower values of GST in gills and GR in digestive gland. Nevertheless, the antioxidant response profile appears to be intricate in intertidal mussels (Regoli and Giuliani, 2014). For instance, mitochondrial ROS production and antioxidant enzyme activities are enhanced during the initial periods of hypoxia associated to air exposure but lowered during prolonged hypoxia (Letendre et al., 2009; River-Ingraham et al., 2013; Nogueira et al., 2017). It also seems to be tissue-specific. Due to the immediacy of their contact with the external milieu, gills are one of the first tissues to experience hypoxic conditions, quickly decreasing mitochondrial respiratory rate and proton leak in order to save energy under conditions of low ATP turnover (Sussarellu et al., 2013). Thus, whereas hypoxia was associated in mussel gills with a burst in ROS within the first hours of O_2 depletion, ROS production resulted lowered after prolonged hypoxia (Welker et al., 2013; Hermes-Lima et al. 2015). Vidal-Liñan and Bellas (2013) found that CAT activity in the digestive gland was higher in mussels from the medium intertidal

zone than in HIL and LIL mussels, which was related to the timing of the feeding activity and the progression of food digestion. Taking into account the results of the present study, intertidal zonation is an important factor to consider when GPx and GST in gills, GR in digestive gland and LPO (in both tissues) are measured, in agreement with previous statements (Letendre et al., 2009). Moreover, using LIL mussels and recording their tide-mark level are highly recommended for environmental monitoring, as they are prone to reasonably respond to environmental changes.

Antioxidant enzyme activities were less affected by transport to the laboratory in HIL mussels than in LIL mussels. This would be in agreement with the compensation ability of HIL mussels above discussed, which might render HIL mussels less responsive than LIL mussels to further environmental changes. Thus, antioxidant enzyme activities remained unchanged after transport in air in HIL mussels, both in gills and in digestive gland. Yet, minor changes were recorded after transport in seawater in GST and GR activities: GST was lowered in SW_{24} digestive gland, and GR was slightly reduced in SW_{24} gills and markedly raised in the digestive gland after transport in seawater. Since GST may also have peroxidase activity, increases in GST during air exposure would represent a defense against oxidative stress linked to hypoxia (Manduzio et al., 2004; Almeida et al., 2005). Thus, returning to seawater would cause GST values to be reduced, at least after long immersion periods (e.g. 24 hr). Likewise, GST is known to remain high for at least 1 hr after immersion in order to excrete metabolic by-products to seawater (Almeida and Bainy, 2006), which will explain that presently GST was not lowered after 3 hr transport tin seawater. The reduction in GR in the gills can be explained in similar terms, as the result of reversing oxidative stress in the gills after immersion. GR is not a real antioxidant but it is essential to maintain the correct glutathione balance and the intracellular redox status in marine organisms and, as such, GR can be considered as an additional component of the antioxidant system (Regoli and Giuliano, 2014). This role would also explain the increase in GR in the digestive gland after transport in seawater, as intracellular digestion of the food during low tide would produce additional ROS and demand higher levels of GSH. Food availability may affect levels of ROS production and ROS detoxification, thereby influencing mussels' requirements for antioxidant enzymes (Dowd et al., 2013). For instance, ROS production is enhanced at high oxygen consumption rate, which is elevated due to the costs of filter-feeding and digestion, as well as to the increase in aerobic metabolic rate (Dowd et al., 2013). Indeed, GR in the digestive gland is largely influenced by feeding and food quality in mussels (Gonzalez-Fernandez et al., 2016).

In the gills of LIL mussels CAT and GR remained unchanged albeit GPx rose remarkably after transport in seawater and GST decreased both after transport in air and in seawater. Both the GPx elevation and the GST drop can be easily explained. CAT is known to be more efficient that GPx to metabolize peroxides; however, GPx can decompose hydroperoxides more efficiently when these are at low levels (Almeida et al., 2005). This could be the case of the gills when mussels are immersed because a part of the H_2O_2 can be directly excreted to the water, as shown for fish; these eliminate H_2O_2 by gill diffusion into the environment

until a given steady-state is reached for the H_2O_2 concentration (Wilhelm-Filho et al., 1994). Regarding GST, its decrease after transport in seawater would agree with the observations made on HIL mussels, above discussed. However, GST seems to be particularly relevant as antioxidant defense in mussel gills during air exposure (Manduzio et al., 2004; Almeida et al., 2005; Almeida and Bainy, 2006) and therefore, contrary to our observations, elevated GST activity would be expected in after transport in air.

In the digestive gland, CAT, GPx and GST activities rose after transport in air and GPx and GR decreased after transport in seawater. In agreement, in brown mussels, *Perna perna*, GST in digestive gland rose after air exposure and was prolonged for at least 1 hr after returning to seawater, maybe to accomplish the excretion of metabolites generated during the air exposure period (Almeida et al., 2005). Conversely, it was reported that CAT and GPx in digestive gland remained unchanged in *P. perna* after 4 hr air exposure (Almeida and Bainy, 2006). Yet, details about where mussels were collected from were not provided and therefore we cannot know whether those mussels would correspond to LIL mussels (with changes in the digestive gland in all the studied antioxidant enzymes) but not or to our HIL mussels (with changes in GR and GST activities in the digestive gland but not in CAT and GPx). Overall, LPO values were highly variable and no clear changes were envisaged after transport to the laboratory not in LIL nor HIL mussels. Thus, although the antioxidant defense profile of intertidal mussels is clearly influenced by collection and transport to the laboratory, the degree of oxidative damage, measured in terms of LPO, does not seem to change, at least after 3 hr transport in air and 24 hr transport in seawater. Nevertheless, the activity levels of specific antioxidant enzymes do change and therefore, whenever possible, *in situ* dissection of the mussels immediately after collection is highly recommended.

Mora et al. (1999) reported that the ChE activity in the gills of *M. galloprovincialis* was about four times higher than in the other organs, whilst the lowest activity was found in the digestive gland. ChE activity in foot values in between those recorded in gills and digestive gland and was not dissimilar between HIL and LIL mussels. In agreement, it was previously reported that ChE activity in gills of mussels was not affected by the intertidal zonation (Vidal-Liñan and Bellas, 2013). However, in the present study, ChE activity was slightly reduced after transport to the laboratory in all cases. This is difficult to explain. Yet, it might be related to the fact that for sample collection mussels are detached from their substrate by cutting the byssus and once they are placed in boxes mussels tend to move to form clumps and produce new byssus to get attached to each other and to the box wall. As a result, the activity of the byssus gland and the foot musculature would be enhanced thus slightly modifying synaptic activity and the associated AChE enzyme activity (e.g., the role of AChE in cholinergic transmission is to regulate nervous transmission by reducing the concentration of acetylcholine in the junction through; Kopecka et al., 2004). For instance, high levels of acetylcholine are known to stimulate contraction and catch of the anterior byssus retractor muscle in mussels (Zange et al., 1989), which can be associated to low AChE activities observed after collection and transport of the mussels in the present study.

Lysosomal biomarkers

In intertidal mussels, lysosomal size, numbers and membrane stability are known to depend on the vertical position of the mussels on the shore, which has been directly related to the running of the intracellular digestion cycle (Izagirre et al., 2008). Indeed, feeding regimes vary along the shore depending on the tide amplitude, thus directly affecting the process of digestion (Morton, 1983). Presently, lysosomal membrane stability in digestive cells of LIL mussels was relatively higher than in HIL mussels, more markedly after transport to the laboratory both in air and in seawater. Likewise, the endo-lysosomal system of the digestive cells was relatively enlarged in LIL mussels in comparison with that of HIL ones (higher Vv_{LYS} and lower S/V_{LYS}). Comparable profiles of lysosomal size and membrane stability have been reported in field studies for mussels collected along intertidal transects (Tremblay and Pellerin-Massicote 1997; Izagirre et al., 2008). Rather than characteristic of each tidemark, lysosomal size and membrane stability may vary within a few hours during the tide, depending on the progression of the digestion process (Izagirre et al., 2008). Consequently, the differences found herein would rather indicate that mussels were at different stages of the intracellular digestion cycle. In a preceding laboratory study, when low-tidemark mussels were exposed to air for 4 hr their endo-lysosomal system resembled that of high-tidemark mussels in both structure and membrane stability (Izagirre et al., 2008). Presently, HIL mussels are naturally subjected each tide to air exposure for 3-4 hr and present few small lysosomes with destabilized membrane, which could be associated to a late stage of digestion (Izagirre et al., 2009). In contrast, the LIL mussels that were collected at same moment had been immersed until then and, therefore, they were at an earlier stage of digestion, showing large and pleomorphic lysosomes in which the membrane is destabilized as well.

After transport to the laboratory, both in air and in seawater, opposite trends were observed in the LP values of LIL and HIL mussels. LP increased in LIL mussels and decreased in HIL mussels, even beyond the initial low LP values recorded in mussels processed *in situ*. In LIL mussels, transport also affected the size and numbers of digestive cell lysosomes, thus: (a) Vv_{LYS} was reduced after transport in seawater and more markedly after transport in air; (b) S/V_{LYS} rose after transport both in air and in seawater for 3 hr; and (c) Nv_{LYS} rose dramatically in mussels transported in seawater (both SW_3 and SW_{24}). In contrast, the digestive cell lysosomes of HIL mussels seemed to be less responsive with only two remarkable responses. First, a certain trend to lysosomal enlargement and membrane destabilization could be envisaged after transport in air for 3 hr (increased Vv_{LYS} and decreased S/V_{LYS}); which can be interpreted as the consequences of a retarded or prolonged food digestion after 7 hr emersion (4 on shore + 3 during transport). Second, a significant increase was observed in the numbers of lysosomes after transport in seawater for 24 hr but not after 3 hr. In HIL mussels, the numbers of digestive cell lysosomes augmented after 24 hr transport in seawater but, again like in the case of LIL mussels, not after 3 hr. Accordingly, it has been reported that lysosomal membrane destabilization occurs when the tide comes down within a few minutes but LP values are not quickly recovered after submersion (Tremblay and Pellerin-Massicotte, 1997; Izagirre et al., 2008). Likewise, lysosomal size is known to

increase with air exposure for about 4 hr and then to start decreasing, while the lysosomal membrane stability and the numbers of lysosomes decrease immediately after a new immersion, possibly due to the excretion of residual bodies at the end of the digestion process (Domouhtsidou and Dimitriadis, 2001; Izagirre et al., 2008). Feeding (immersion) and digestion (emersion) are kept apart in intertidal mussels, especially in HIL ones, and therefore the changes in lysosomal biomarkers might be explained by the progression of food processing, as previously argued by Izagirre et al. (2008). It is worth noting that, as above discussed, some of the changes presently found in antioxidant enzymes can be also related to feeding and digestion. In any case, these results reveal that the time and condition of transport of the mussels to the laboratory are also crucial parameters for the determination of lysosomal biomarkers, as above mentioned for enzyme activities and metabolites.

Intracellular accumulation of neutral lipids in lysosomes of mussel digestive cells has been reported to exhibit a marked seasonal variability that is related to the gametogenic cycle, with higher values from September to January and lower values from February to August (Cancio et al. 1999; Garmendia et al., 2010). In the present study, Vv_{NL} values correspond to those expected in September in the study area and did not exhibit any clear trend depending on the intertidal zonation nor after transport to the laboratory.

Tissue-level biomarkers

No differences were found between LIL and HIL mussels in Vv_{BAS} and MLR/MET and CTD ratio, neither as regards *in situ* dissected mussels nor after transport to the laboratory in air or in seawater. Certainly, responses at the histological level are not so fast as metabolic or lysosomal responses and therefore for mussels of a given tide-mark level and these responses should be *a priori* less compromised by collection and "short-term" transport (<24 hr). Presently, all the values recorded in Vv_{BAS}, MLR/MET and CTD were not statistically different amongst cases; moreover, they were always below their normal reference values. In clean localities and in experimental control conditions, Vv_{BAS} values are below 0.1 μm^3/μm^3 whilst upon exposure to pollutants they can go beyond 0.12 μm^3/μm^3 (Cajaraville et al., 1990; Marigómez et al., 2006; Garmendia et al., 2011). In the present study the average Vv_{BAS} was 0.09 μm^3/μm^3 and the Vv_{BAS} values never exceeded 0.1 μm^3/μm^3. MLR/MET values are reported to be between 0.7 μm/μm (spring-summer) and 1.2 μm/μm (winter) in mussels from reference localities, whereas upon environmental insult the values can surpass 1.6 μm/μm (Marigómez et al., 2006; Garmendia et al., 2011). Presently, the average MLR/MET (September) was 0.68 μm^3/μm^3. Maybe this value was quite at the edge but anyway it remained within the normal range; this would be in agreement with the relatively wide lumens above described upon microscopic examination and could be related to the low-to-moderate levels of Zn and PAHs found in the mussel tissues (ESM 01). Nevertheless, all the MLR/MET values were below 0.1 μm^3/μm^3. As a whole, the tissue-level biomarkers indicated an acceptable condition of the mussels and did not change as a result of transport to the laboratory. However, it is worth noting that the histological examination of the gonad revealed signs of spawning induction after transport in seawater both in HIL and LIL mussels. Spawning induction could be

critical for biomarker determination and interpretation and must be avoided (Beyer et al., 2017). This is an additional reason to elude transporting mussels to the laboratory in seawater before dissecting for biomarker determination.

Recommended Best Available Practices (BAPs)

In biomarker-based monitoring in with mussels are used as sentinels, the influence of confounding factors should be minimized by using consensus BAPs, including procedures for mussel collection and transport. Recently, great efforts have been made to recommend BAPs. Recommendations include collecting mussels: (a) from subtidal or intertidal from the lowest tide-mark level possible, (b) of similar relative shell length (e.g. 70-90% of their local maximum potential length) or age; and (c) out of their spawning season (Izagirre et al., 2008; 2014; Vidal-Liñan and Bellas, 2013; Beyer et al., 2017).

The vertical position of mussels on the shore is therefore crucial, as also envisaged in the present study, especially as regards the measurement of GST and GPx activities in gills, GR activity in digestive gland, LPO in both tissues and lysosomal biomarkers in digestive gland cells. Consequently, taking into account the present and previous studies (Izagirre et al. 2008, Vidal-Liñán and Bellas 2013), it is highly recommended that sentinel mussels used in biomarker-based monitoring must be collected from the low intertidal zone (nearest as possible to the 0 m tide-mark level).

The timing and conditions for dissecting and transport are also crucial factors. Mussels should be processed *in situ* as soon as possible and always within the first hour upon collection. This is especially crucial when enzyme activities, metabolites and lysosomal responses are the biomarker endpoints. Unfortunately, *in situ* pre-processing is not always possible (e.g. not easy-to-access for staff or equipment, adverse weather or physical conditions); then, mussels should be transported in air at ambient temperature (Chandurvelan et al., 2013) resembling as much as possible their natural conditions and physiological status at the collection site. Even so, prompt biological responses such as e.g. changes in antioxidant enzyme activities, lysosomal membrane stability and lysosomal numbers should be cautiously interpreted in this case, at least in comparative terms in relation with data obtained in other studies. In addition, transport in seawater should be always disregarded because, together with direct interferences of immersion with antioxidant defense mechanisms and intracellular digestion, spawning may be induced thus indirectly impinging into biological mechanisms underneath biomarkers. Whatever the case may be, details on collection, transport and the time passed before dissection should be always provided in publications and reports in order to assist other researchers to properly interpret and compare results, especially when the biomarker-based environmental monitoring is carried out in coastal zones subject to remarkable intertidal variability.

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CHAPTER 3

SEX AND REPRODUCTIVE STAGE RELATED SEASONAL VARIABILITY IN BIOMARKER RESPONSIVENESS TO ENVIRONMENTAL STRESS IN MUSSELS

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ABSTRACT

Mussels are widely used as biomonitors and sentinels in marine pollution assessment. However, it is need to achieve a better understanding of the influence of natural confounding factors on biomarkers and pollutant tissue levels in marine pollution monitoring programmes, in order to properly interpret biological responses. Potential confounding factors, such as sex, the reproductive cycle and sex-related variability along the reproductive cycle, have been less frequently investigated. Moreover, the scarcity of conclusive data in the literature makes difficult to generate guidelines about the influence of sex in mussels. Within this context, the aim of the present study was to contribute to the understanding of how pollutant tissue levels and biomarkers and their responsiveness to environmental insult vary with sex, with the reproductive cycle and with sex-related variability along the reproductive cycle. For this purpose, mussels were collected from a relatively clean locality and from a chronically polluted site in the Basque Coast in January, April, August and November 2014 and sexed. The gametogenic cycle, the gonad index and the flesh condition index were characterized. And the tissue concentrations of metals and PAHs and a battery of biomarkers were determined, say: cytochrome c oxidase activity, pyruvate kinase activity, phosphoenolpyruvate carboxykinase activity, protein carbonyls products, malondialdehyde and 4-hydroxy-2-nonenal, lysosomal enlargement and membrane stability, intracellular neutral lipid accumulation, cell type composition and thinning of the digestive gland epithelium and survival-in-air (Stress-on-Stress test). Overall, there exists evidence of sex and reproductive stage related differences in bioaccumulation and in the values and responsiveness to environmental insult of many of the studied biomarkers. Nevertheless, it was concluded that selecting individuals of one specific sex was not a priory necessary to carry out biomarker-based monitoring programs using mussels as sentinels. However, it is highly recommended to carry out histological analysis of gonad tissue in order to determine sex ratios and gamete developmental stages.

Sasoiko aldakortasuna erlazionatutako sexuarekin eta ugalketa-fasearekin biomarkatzaileen ingurumen estresari erantzuteko gaitasunean muskuiluetan

Laburpena

Muskuiluak biomonitore eta begirale gisa oso erabiliak dira itsasoko kutsadura-jarraipen programetan. Hala ere, nahaste-faktore naturalen eragina biometaketan eta biomarkatzaileetan hobeto ulertzea beharrezkoa da itsasoko kutsadura-jarraipen programetan, erantzun biologikoak modu egokian interpretatzeko. Potentzial nahaste-faktoreak, hala nola sexua, ugalketa-zikloa eta sexu-aldakortasuna ugalketa zikloan zehar, gutxi ikertu dira. Gainera, literaturaren datu eztabaidaezinen urritasunak muskuiluetan sexuaren eraginari buruzko jarraibideen sorrera zailtzen du. Testuinguru honetan, ikerketa honen helburua hurrengoa da: sexuak, ugalketa-zikloak eta sexu-aldakortasunak ugalketa zikloan zehar, biometaketan eta biomarkatzaileetan eta haien erantzun-gaitasunean dituzten eraginari buruzko ulermenean laguntzea. Horretarako, muskuiluak Euskal Kostaldeko (Bizkaiko Golkoa) lokalizazio nahiko garbi batean eta kronikoki kutsatutako beste batean bildu ziren 2014ko urtarrilean, apirilen, abuztuan eta azaroan, eta haien sexua bereizi zen. Ziklo gametogenikoa, gonadaren indizea eta baldintza indizea ezaugarri ziren. Metalen eta HAPen ehunen kontzentrazioak eta biomarkatzaile-multzoa zehaztu ziren: zitokromo c oxidasa jarduera, pirubato kinasa jarduera, fosfoenolpirubato karboxikinasa jarduera, proteinen karboniloen produktuak, malondialdehidoa eta 4-hidroxi-2-nonenala, lisosomen mintzaren desegonkortasuna, lisosomen egituren aldaketak, lipido neutroen metaketa, liseri epitelioaren zelulen konposaketa eta liseri albeoloen egituren aldaketak eta biziraupena airean. Oro har, sexuak eta ugalketa faseak eragina dituztela biometaketaren eta biomarkatzaileen balioen eta erantzun-gaitasunean ageri izan dira. Hala eta guztiz ere, sexu espezifiko bat hautatzea ez dela beharrezkoa ondorioztatu zen, muskuiluen biomarkatzeileetan oinarritutako itsasoko kutsadura-jarraipen programak burutzeko. Alabaina, ehun gonadalaren azterketa burutzea arren gomendatzen da, sexu-ratioak eta gametoen garapen-faseak zehazteko.

Variabilidad estacional relacionada con el sexo y la etapa reproductiva en la capacidad de respuesta a estrés ambiental de los biomarcadores en los mejillones

Resumen

Los mejillones son muy utilizados como biomonitores y centinelas en la evaluación de la contaminación marina. Sin embargo, es necesario mejorar la comprensión de la influencia de los factores de confusión naturales en los biomarcadores y los niveles de contaminantes en los programas de monitoreo de la contaminación marina, a fin de interpretar adecuadamente las respuestas biológicas. Potenciales factores de confusión, como el sexo, el ciclo reproductivo y la variabilidad relacionada con el sexo a lo largo del ciclo reproductivo, se han investigado con poca frecuencia. Además, la escasez de datos concluyentes en la literatura dificulta la generación de directrices sobre la influencia del sexo en los mejillones. En este contexto, el objetivo del presente estudio fue contribuir a la comprensión de cómo los niveles de contaminante en tejido y, biomarcadores y su capacidad de respuesta a la agresión ambiental varían con el sexo, el ciclo reproductivo y la variabilidad relacionada con el sexo a lo largo del ciclo reproductivo. Para este propósito, los mejillones fueron recogidos de una localidad relativamente limpia y de un sitio crónicamente contaminado en la costa vasca en enero, abril, agosto y noviembre de 2014 y fueron sexados. El ciclo gametogénico, el índice de gonadal y el índice de condición fueron caracterizados. Y se determinaron las concentraciones tisulares de metales y HAP, y una batería de biomarcadores: piruvato quinasa, fosfoenolpiruvato carboxinquinasa, citocromo c oxidasa, productos de carbonilos proteicos, malondialdehído, 4-hidroxi-2-nonenal, estabilidad de la membrana lisosómica, cambios estructurales lisosómicos, acumulación intracelular de lípidos neutros; y composición del tipo celular y cambios estructurales de alvéolos digestivos y supervivencia en aire. En general, existen pruebas de diferencias relacionadas con el sexo y el estado reproductivo en la bioacumulación y en los valores y la capacidad de respuesta a la agresión ambiental de muchos de los biomarcadores estudiados. Sin embargo, se concluyó que seleccionar individuos de un sexo específico no es una necesidad prioritaria para llevar a cabo programas de monitoreo basados en biomarcadores en mejillones centinelas. Sin embargo, se recomienda encarecidamente realizar un análisis histológico del tejido gonadal para determinar las proporciones de sexos y las etapas de desarrollo de los gametos.

INTRODUCTION

Mussels are widely used as biomonitors and sentinels in marine pollution assessment (Goldberg et al., 1978; Morgado and Bebianno, 2004; Beyer et al., 2017). In combination with pollutant tissue concentrations, biomarkers are an integral component of environmental monitoring, as these provide sensitive and early warning signals of environmental distress and ecosystem health disturbance caused by pollution (Bickham et al., 2000; Cajaraville et al., 2000; Schettino et al., 2012; Marigómez et al., 2013a; 2013b; Beyer et al., 2017; Breitwieser et al., 2018). However, the limited knowledge of their natural variability and baseline values at local/regional scale has contributed to controversy about the biomarkers validity for pollution monitoring programmes and environmental regulatory policies (Devier et al., 2005; Bocchetti et al., 2008; Hagger et al., 2008). Experimental laboratory research on bioaccumulation and biomarkers has usually provided highly coherent results, but in the field, with the exception of extreme case studies, the results can be controversial; most likely because native mussels are examined in a multiple stress scenario and under environmental insult (e.g. pollution levels) of lower magnitude than in the laboratory or in extreme field cases (Devier et al., 2005; Hagger et al., 2008; ICES, 2012). Consequently, it is needed to achieve a better understanding of the influence of natural confounding factors on biomarkers and pollutant tissue levels in order to properly interpret biological responses in marine pollution monitoring programmes (Petrovic et al., 2004; Balbi et al., 2017).

Aimed at establishing best available practices (BAPs) for biomarker-based biomonitoring using mussels as biomonitors and sentinels, previous studies have dealt with the influence exerted on pollutant tissue concentrations and a variety of biomarkers by confounding factors (Lobel et al., 1991; Izagirre et al., 2008, 2014; Garmendia et al., 2010; Chapters 1 and 2). Potential confounding factors, such as sex, the reproductive cycle and sex-related variability along the reproductive cycle, have been less frequently investigated. Sex is known to play a role in the genetics, physiology, morphology, and behaviour of organisms, and thus influences the uptake, fate, and effects of contaminants (Burger, 2007). On the one hand, biomarker values may vary with sex and along the reproductive cycle. Thus, *Gammarus roeseli* females had higher glutathione (GSH) concentrations than males, maybe linked to higher activities of antioxidant enzymes using GSH as a substrate or differences in the physiological status between sexes (Gismondi et al., 2012). On the other hand, biomarker responsiveness against environmental insult can be different as well. Thus, distinct physiological strategies exist between sexes in molluscs in order to cope oxidative stress, which are apparently more efficient in females (Grilo et al., 2018). For instance, in tropical topshells (*Trochus histrio*) antioxidant protective response was mainly based on catalase (CAT) for females and glutathione-S-transferase (GST) for males and, as a consequence, malonyldialdehyde (MDA) production was lower in females than in males (Grilo et al., 2018). In mussels, some studies concluded that the sex-related differences may introduce undesirable variations in biological responses to pollution when both females and males are analysed altogether (Ji et al., 2013, 2014, 2016; Lobel et al., 1991). For instance, brominated flame-retardants caused different

responses and alterations in female and male mussels, *M. galloprovincialis* (Ji et al., 2013, 2014, 2016). In contrast, sex-related differences were not found in phosphorylated p38 mitogen activated protein kinase in gills of mussel *Perna perna* (Zilbergerg et al., 2011), nor in mRNA levels of target genes (molecular biomarkers) in gills of mussels *M. galloprovincialis* exposed to fluoranthene (González-Fernández et al., 2017).

Within this context, the aim of the present study was to contribute to the understanding of how pollutant tissue levels and biomarkers and their responsiveness to environmental insult vary with sex, with the reproductive cycle and with sex-related variability along the reproductive cycle. For this purpose, mussels were collected from a relatively clean locality and from a chronically polluted site in the Basque Coast in January, April, August and November 2014, and sexed. The gametogenic cycle and the Gonad Index (GI) and the individual condition (Flesh Condition Index; FCI) were characterized, and the tissue concentrations of metals and PAHs and a battery of biomarkers (cytochrome c oxidase activity (COX), pyruvate kinase activity (PK), phosphoenolpyruvate carboxykinase activity (PEPCK), protein carbonyls products, malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE), lysosomal enlargement and membrane stability, intracellular neutral lipid accumulation, cell type composition and thinning (atrophy) of the digestive gland epithelium and survival-in-air (Stress-on-Stress test)) response were determined.

COX, PK and PEPCK are energetic biomarkers indicative of metabolic changes (Lucassen et al., 2003; Siu et al., 2003; Morley et al., 2009; Ivanina et al. 2016). MDA and HNE are biomarkers of lipid peroxidation and cell oxidative damage (Viarengo et al., 1991; Maria and Bebianno, 2011). Lysosomal enlargement and membrane destabilisation are biomarkers of health condition (UNEP/RAMOGE, 1999; Marigómez and Baybay-Villacorta, 2003; ICES, 2004; 2012). Intracellular neutral lipid accumulation occurs in response to organic xenobiotics and non-specific stress, and varies with the nutritional status of the mussels (Regoli, 1992; Cancio et al., 1999; Marigómez and Baybay-Villacorta, 2003; Shaw et al. 2011). Increase in the relative proportion of basophilic cells, atrophy of the digestive epithelium and loss of digestive gland histological integrity are known to occur in the digestive gland epithelium of molluscs under stress conditions (Couch, 1984; Cajaraville et al., 1992; Marigómez et al., 2006; Zaldibar et al., 2007; Garmendia et al., 2011). Alterations in gametogenic development and gonad histopathology are useful biological effects endpoints for marine ecosystem health assessment (Garmendia et al., 2011; ICES, 2012; Ortiz-Zarragoitia et al., 2011; Cuevas et al., 2015). The SoS response reveals the capacity of bivalves to survive on air and as such it is interpreted as a measure of resilience, which is recommended by ICES (2012) for monitoring programmes.

MATERIALS AND METHODS

Sampling strategy

Intertidal mussels, *Mytilus galloprovincialis* Lmk. of 3.5–4.5 cm length size were collected from the low tide-mark level (0.5-1 m) of a relatively clean locality (Plentzia, 43° 24’ N, 2° 56’ W) and of a chronically polluted one (Arriluze, 43° 20’ N, 3° 0’ W), both located in the Bay of Biscay (Fig. 1) in January, April, August and November 2014. Ninety

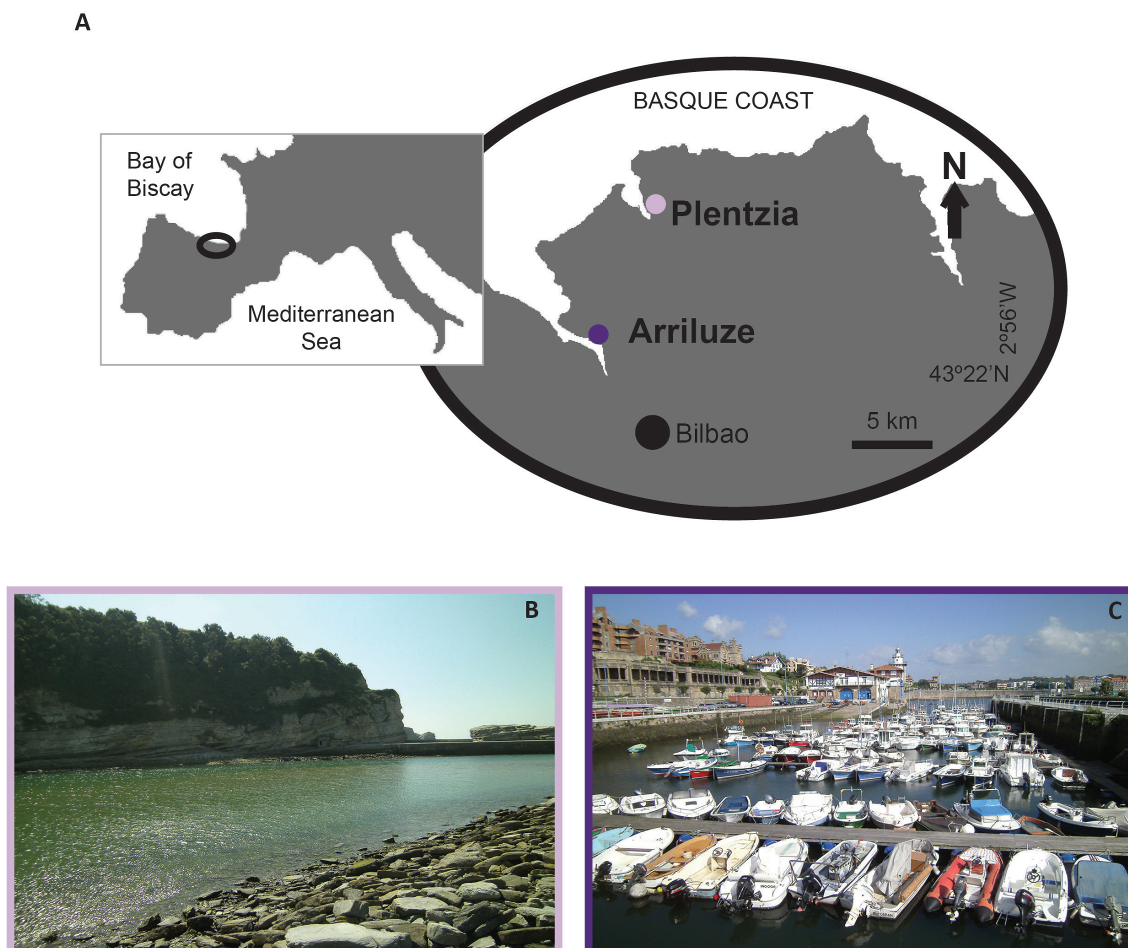


Fig. 1. (A) Map showing the locality of both sampling point (violet and purple): (B) Plentzia, a relatively clean locality, and (C) Arriluze, a chronically polluted locality.

mussels per locality and sampling time were collected. After collection, animals were immediately transferred to the laboratory in less than 1hr in air and pre-processed, as was recommended in the Chapter 2.

Per locality and sampling time, gills and one-half of the digestive gland of 30 mussels were frozen in liquid N_2 and stored at -80°C until required for biochemical and histochemical analyses. The second half of the digestive gland and the mantle of 30 mussels were fixed in 4% formaldehyde in seawater for histological analyses. Another 30 mussels were used for Stress-on-Stress (SoS) test (Viarengo et al., 1995). Additional 30 mussels were used for the determination of metal and PAH concentrations in the soft body tissues. The sex of all the mussels used for histochemical and histological analyses, for the SoS tests and for chemical analyses was determined after histological examination of mantle tissue fixed in 4% formaldehyde in seawater and routinely processed for histology, as below detailed (Table 1).

Sex, gamete developmental stages and Gonad Index

Fixed mantle samples were dehydrated in graded ethanol series, and embedded in paraffin. Sections (5 µm thick) were cut in a rotary microtome (Leica RM2125 RTS), and stained with hematoxylin-eosin to determine the sex of each mussel (n=90 per locality and sampling time) at the light microscope. GI of

the mussels used for biomarkers analysis was determined according to Ortiz-Zarragoitia et al., (2011). Briefly, gamete developmental stages were distinguished and a GI value was assigned to each mussel depending on its gamete developmental stage (adapted after Kim et al., 2006), and the average GI of 30 mussels was calculated per locality and sampling time.

Alterations on gametogenic development and gonad histopathology were recorded as biomarkers of reproductive impairment: (a) alterations in the timing of gametogenesis; (b) malformation of gametes; (c) oocyte atresia; (d) abnormal haemocytic infiltration in gonad follicles; (e) changes in sex ratio (females-to-males ratio); and (f) intersex gonads (Figueiras et al., 2002; Ortiz-Zarragoitia and Cajaraville, 2010).

Concentration of metals and PAHs in mussel tissues

For each locality and season, pools of female, male and resting mussels were homogenised and freeze-dried before being stored (at 4°C) before their analysis. For the analysis of metals, 1.5 g of each pool were digested in concentrated nitric acid, diluted with 0.1 M nitric acid and analysed by atomic absorption spectrophotometry (AAS; Perkin Elmer 2280 spectrophotometer, Boston, USA). Merck standard solutions were diluted in 0.1 M nitric acid for calibration. Seven metals were analysed, Cd, Cr, Cu, Hg, Ni, Pb, and Zn. Tissue metal concentrations are expressed as μg metal/g tissue dw.

For the analysis of 16 parental PAHs incorporated in the US EPA list of priority pollutants, 0.5 g of each pool were extracted under microwave-assisted system (MSD-2000 CEM, Matthews, NC, USA) in 15 mL acetone and processed and analysed by Gas Chromatography Mass Spectrometry (GCMS) according to Bartolomé et al. (2005) and Navarro et al. (2006). As shown in those works, the recoveries of surrogates (deuterated congeners) generally ranged from 70% to 110% of the spiked concentration (except fornaphthalene-d8 with range from 40% to 80%). Typically, samples with recoveries below 70% were re-analysed to assure an accurate result. The tissue concentration of PAHs is expressed as ng PAHs/g tissue dw.

Enzyme activities and oxidative damage

Activities of the mitochondrial enzymes cytochrome c oxidase (COX; EC 1.9.3.1), pyruvate kinase (PK; EC 2.7.1.40) and phosphoenolpyruvate carboxykinase (PEPCK; EC 4.1.1.31) were determined in gill tissue. This was thoroughly homogenized in enzyme-specific homogenization buffer (see below) using hand-held Kontes® Duall® tissue grinders (Fisher Scientific, Suwanee, GA, USA). Homogenates were sonicated 3× 10 sec each (output 7, Sonic Dismembrator Model 100, Fisher Scientific, Suwanee, GA) to ensure complete release of the enzymes, with cooling on ice (1 min) between sonications and centrifuged at 16000× g and 4°C for 25 min. The supernatant was collected and used for enzyme determination. Enzyme extracts were stored at −80°C for less than 2 wk before activity assays. For determination of enzyme activities, enzyme extracts were thawed on ice and immediately analyzed using standard spectrophotometric techniques as described elsewhere (Bergmeyer, 1985; Sidell et al., 1987; Birch Machin and Turnbull, 2001). Enzyme activities were measured at 20°C using a UV–Vis spectrophotometer (VARIAN Cary 50 Bio, Cary NC, USA).

The temperature of the reaction mixture was controlled using a water-jacketed cuvette holder. Briefly, isolation and assay conditions for the studied enzymes were as follows: (a) PK: homogenization buffer: 10 mM Tris–HCl buffer (pH 7.2), 5 mM EDTA, 1 mM DTT, 0.1 mM PMSF; assay: 50 mM Tris-HCl (pH 7.2), 50 mM KCl, 5 mM $MgSO_{4,}$ 1 mM ADP, 0.2 mg/mL NADH, 5.5 U LDH, 0.5 mM PEP; acquisition wavelength: 340 nm. (b) PEPCK: homogenization buffer: 10 mM Tris–HCl buffer (pH 7.2), 5 mM EDTA, 1 mM DTT, 0.1 mM PMSF; assay: 100 mM HEPES (pH 7.2), 2.3 mM MnCl2, 0.5 mM IDP, 5 mg/mL KHCO3, 0.2 g/mL NADH, 10U MDH, 15 mM PEP; acquisition wavelength: 550 nm. (c) COX: homogenization buffer: 25 mM potassium phosphate, pH 7.2, 10 µg phenylmethylsulfonyl fluoride (PMSF)/mL, 2 µg aprotinin/mL; assay: 20 mM potassium phosphate, pH 7.0, 16 µM reduced cytochrome c (II), 0.45 mM n-dodecyl-b-d-maltoside, 2 µg antimycin A/mL; acquisition wavelength: 550 nm. Protein concentration was measured in the same samples using Bradford's method.

Protein carbonyl groups (CO) were measured spectrophotometrically (Philipp et al., 2005). Digestive gland was ground under liquid nitrogen and homogenized in buffer containing 50 mM HEPES, 125 mMKCl, 1.1 EDTA and 0.6 mM MgSO4 (pH 7.4) and protease inhibitors [leupeptin (0.5 µg/mL), pepstatin (0.7 µg/mL), phenylmethylsulfonyl fluoride (40 µg/mL) and aprotinin (0.5 µg/mL)]. Samples were centrifuged at 100000× g for 15 min, supernatant was collected and incubated at room temperature with 10 mM 2,4-dinitrophenylhydrazine (DNP) in 2 M HCl. The blanks were incubated with HCl without DNP. After incubation, proteins were precipitated by adding 100% TCA and centrifuged at 11000 g for 10 min. The pellet was collected, washed with ethanol ethylacetate (1:1) and resuspended in 6 M guanidine hydrochloride in 20 mM in KH_2PO_4 (pH 2.5) until dissolved. The absorbance was measured at 360 nm on a spectrophotometer (Cary 50, Varian) using guanidine HCl solution as reference. The amount of carbonyls was estimated as a difference in absorbance between samples and blanks using a molar extinction coefficient of carbonyls ε = 22000 L/cm M. The amount of carbonyls was expressed as per mg total protein measured as above described for the gills.

Protein conjugates of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) were measured as biomarkers of lipid peroxidation using enzyme-linked immunosorbent assay (MDA OxiSelect™ MDA adduct ELISA Kit and HNE OxiSelect™ HNE-His adduct ELISA Kit, respectively) according to the manufacturers' protocols (Cell Biolabs, Inc., CA, USA). About 200–300 mg of digestive gland were homogenized in ice cold phosphate-buffered saline (PBS; 1:5 w:v) with protease inhibitors (50 µg aprotinin/L and 40 µM phenylmethylsulfonyl fluoride) using Kontes Duall tissue grinders (Fisher Scientific, Suwanee, GA, USA). Samples were centrifuged at 15000 *g* for 10 min at 4°C. Protein concentration was measured in the supernatant using Bradford's method.

Lysosomal biomarkers

Lysosomal membrane stability was evaluated in serial cryotome sections (10 µm thick; Leica CM3050S cryotome) of digestive gland chucks after the cytochemical demonstration of hexosaminidase activity, according to a

standardized procedure (UNEP/RAMOGE, 1999), based on the time of acid labialization (LP; min) required to produce the maximum staining intensity.

In order to quantify changes in lysosomal structure, cryotome sections (8 µm thick; Leica CM 3050S cryotome) of digestive gland chucks were stained for the histochemical demonstration of ß-glucuronidase activity according to Cajaraville et al. (1991). For each mussel digestive gland the following stereological parameters were determined (Lowe et al., 1981): lysosomal volume density ($Vv_{LYS}=V_{LYS}/V_C$), lysosomal surface density ($Sv_{LYS}=S_{LYS}/V_C$), lysosomal surface-to-volume ratio ($S/V_{LYS}=S_{LYS}/V_{LYS}$) and lysosomal numerical density ($Nv_{LYS}=N_{LYS}/V_C$); where V_{LYS}, S_{LYS} and N_{LYS} are the volume, surface and number of lysosomes, and V_c the volume of digestive cells.

Intracellular accumulation of neutral lipids was determined in cryotome sections (8 µm thick; Leica CM 3050S cryotome) of digestive gland chucks after staining with Oil Red O (ORO; Culling, 1974). The extent of ORO staining in the digestive gland epithelium was measured by image analysis (Marigómez and Baybay-Villacorta, 2003) to calculate the volume density of neutral lipids with respect to the digestive epithelium volume ($Vv_{NL;}$ $µm^3/µm^3$).

Tissue-level biomarkers

Fixed digestive gland samples were dehydrated in graded ethanol series, and embedded in paraffin. Sections (5 µm thick) were cut in a Leica RM2125 microtome, and mounted on albumin coated slides, dried at 37°C for 24 hr, and stored at room temperature until staining with hematoxylin-eosin.

After staining, slides were viewed at 40× magnification using a drawing tube attached to a light microscope. A Weibel graticule (multipurpose system M-168) was used, and hits of basophilic and digestive cells, luminal area and connective tissue were recorded. The volume density of basophilic cells ($Vv_{BAS;}$ in $µm^3/µm^3$) in digestive gland of mussels was determined according to Soto et al. (2002). The mean epithelial thickness of the digestive alveoli (MET; µm) was determined according to Lowe et al. (1981) together with other estimates of changes in alveolus morphology such as the mean luminal radius (MLR; µm) and the MLR-to-MET ratio (MLR/MET; µm/µm) (Vega et al., 1989). Likewise, the integrity of the digestive gland tissue was simultaneously determined as the extent of the interstitial connective tissue relative to the space occupied by digestive diverticula (connective-to-diverticula (CTD) ratio) (Brooks et al., 2011; Garmendia et al., 2011) on the basis of the same stereological data set.

Flesh condition index

Once in the laboratory, the shell and flesh weights for 30 mussels from each locality were measured in order to obtain the flesh condition index (FCI; Lobel and Wright, 1982) as flesh weight (mg) to shell weight (g), using the wet flesh weight (Marigómez et al. 2006).

Stress-on-Stress (SoS)

The time until 50% mortality (LT50) was calculated by means of the "survival-in-air" SoS test (Veldhuizen-Tsoerkan et al., 1991; Viarengo et al., 1995). For this purpose, upon arrival to the laboratory 30 mussels from each locality and sampling period were placed over wet paper on plastic trays at constant room

temperature (18°C). Survival was assessed daily and mussels were considered to be dead when their valves gaped and failed to close when they were physically stimulated, or did not recover when placed in seawater.

Statistical analyses

Sex ratio bias was studied using the G test of association, comparing total number of female and male mussels and normalizing for theoretical gender bias (1:1). Other statistical analyses were performed using IBM® SPSS® Statistics ver. 22.0.0.0. (IBM Corp., Armonk, NY, USA). Homogeneity of variance (Levene's test) and normality of data (Kolmogorov-Smirnov's) were tested before statistical analyses (Sokal and Rohlf, 1995). Three-way ANOVAs were made in order to determine the effect of locality, sex and season and their combination (Locality x Sex; Locality x Season; Sex x Season and Locality x Sex x Season) on biomarkers. For the case of non-parametric biomarkers (LP and GI) logarithmic transformation was applied. Further on, one-way ANOVA followed by Duncan›s post-hoc test was applied to determine the effect of season in Plentzia and Arriluze populations in the case of parametric variables (Cd, Cr, Cu, Pb, Zn, NapH, Flu, Ant, Fla, Pyr, Chr, IcdPy, DBA, BghiP, COX, PK, PEPCK, PK/PEPCK, MDA, HNE, protein carbonyls, Vv_{LYS}, S/V_{LYS}, Nv_{LYS}, Vv_{NL}, Vv_{BAS}, MLR/MET, CTD and FCI). The Mann-Whitney's U-test was used for non-parametric parameters (Ni, Acy, Ace, Phe, BaA, BbF, BkF, BaP, ∑PAHs, LP and GI). For each season, differences between Plentzia and Arriluze populations were established according to the Student's t-test for parametric variables and to the Mann-Whitney's U-test for non-parametric variables. Moreover, Student's t-test or Duncan's post-hoc test was applied to determine the differences between localities taking into account female, male or resting mussels for each season in the case of parametric biomarkers, whereas the Mann-Whitney's U-test was used for non-parametric ones. Differences in biomarkers between sexes were determined in each locality and season applying Student's t-test in winter and spring and Duncan's post-hoc test in summer and autumn. The Mann-Whitney's U-test was used for non-parametric biomarkers in all seasons. The Z-score test was applied to determine significant differences in the metals and PAH tissue concentrations between female, male and resting mussels. A 95% significance level ($p<0.05$) was established for all statistical analyses carried out. Differences between survival curves from SoS test were estimated using the non-parametric Kaplan-Meier test followed by the Tarone-Ware post-hoc test for comparisons between localities for each season and between seasons for each locality and between sexes for the various combinations of season and locality.

RESULTS

No differences in the sex ratio of the studied populations was observed (Plentzia, 1:0.98; Arriluze, 1:1.13; N=360 per locality). Besides, the sex ratio was similar, and close to "1" in all the samples studied except in Plentzia in autumn (Table 1). It is worth noting that the percentage of mussels in which sex could not be identified was very high in summer and autumn, especially in the latter (Table 1). No cases of altered gametogenesis, gamete malformation, oocyte atresia; haemocytic infiltration and intersex were recorded.

Dealing with the gametogenic development stages along the reproductive cycle, advanced gametogenesis and spawning stages were

Table 1. Sex of mussels used for chemical analysis (CA), biological responses (BR) determination and Stress on Stress (SoS) test in Plentzia and Arriluze during winter, spring, summer and autumn 2014. Sex ratio value with asterisk indicate that is different in comparison with the theoretical 1:1 between female and males, according to X^2 test ($p < 0.05$).

		Winter		**Spring**		**Summer**		**Autumn**	
		Plentzia	Arriluze	Plentzia	Arriluze	Plentzia	Arriluze	Plentzia	Arriluze
CA	Female	15	12	21	17	6	10	4	8
	Male	10	17	8	11	7	10	6	3
	Resting	0	0	0	0	16	9	9	17
BR	Female	15	16	16	12	11	7	3	5
	Male	15	14	13	17	8	16	10	9
	Resting	0	0	0	0	11	7	17	16
SoS	Female	12	17	14	11	6	13	3	6
	Male	12	11	16	18	9	16	10	9
	Resting	0	0	0	0	15	1	17	15
Sex ratio		0.88	0.93	0.73	1.15	1.04	1.40	2.60*	1.11
(p) X^2		0.573	0.748	0.135	0.517	0.884	0.156	0.007	0.752
% resting		0	0	0	0	47.19	19.10	54.43	54.55

dominant in both localities in winter and spring, respectively; and the resting stage was incipient in summer and dominant in autumn (Fig. 2). Meanwhile, advanced gametogenesis was dominant in Arriluze in summer (Fig. 2). Three-way ANOVA (d.f.(residual)=217) revealed that gonad index (GI) was affected by season (F=15.29; p<0.001; d.f.(season)=3), sex (F=92.88; p<0.001; d.f.(sex)=2) and the interaction between locality and season (F=2.84; p<0.05; d.f.(interaction)=3).

Concentration of metals and PAHs in tissues
The concentration of metals and PAHs in the soft body of mussels was different between sexes depending on the season and the locality (Table 2).

The tissue metal concentration in mussels from Arriluze was higher than in Plentzia in all seasons, except for the case of Zn in which these concentrations were only higher in spring and autumn (Table 2).

In Plentzia inter-season variability was not relevant but a marked seasonality was observed in Arriluze, where the lowest tissue metal concentrations were recorded in summer for all the metals (Table 2).

In Plentzia, the tissue concentrations of Cr and Cu in females in winter were markedly lower than in males; however, in Arriluze Cr and Cu concentrations were higher in females than in males in autumn, and to some extent in summer as well (Table 2). On the other hand, the tissue concentration of Ni in Plentzia was higher in females than in males in all the seasons except in winter, whereas in Arriluze it did not differ between sexes except in autumn when the values were lower in females than in males (Table 2).
Overall, the tissue concentrations of PAHs in the soft body of mussels were higher in Arriluze than in Plentzia in all the seasons,

Table 2. Tissue concentration of metals (µg/g) and PAHs (ng/g) in *Mytilus galloprovincialis* of different sex or reproductive condition (F: female; M: male; R; resting) from Plentzia and Arriluze during winter, spring, summer and autumn 2014. Different colors indicate significantly different groups for each chemical after the Z-score test. n/a; missing record; udl; under detection limits.

	PLENTZIA										ARRILUZE									
	Winter		Spring		Summer			Autumn			Winter		Spring		Summer			Autumn		
	F	M	F	M	F	M	R	F	M	R	F	M	F	M	F	M	R	F	M	R
Cd	0.66	0.76	0.59	0.52	0.42	0.44	0.36	0.47	0.49	0.45	1.50	1.96	2.20	1.78	1.02	0.97	1.01	1.20	1.23	1.17
Cr	1.7	2.1	2.8	5.4	1.8	1.56	1.30	1.1	1.5	1.78	2.0	2.3	3.5	3.0	2.4	1.4	2.5	2.7	1.8	2.8
Cu	5.5	8.3	7.9	6.3	7.9	5.9	5.6	5.6	5.2	5.6	16.4	22.4	20.2	14.0	10.4	8.6	12.0	16.8	9.6	14.9
Ni	1.4	1.6	2.4	1.7	2.1	1.6	1.5	2.9	1.9	6.8	2.1	2.2	3.1	2.1	2.51	2.8	1.9	2.9	5.0	3.0
Pb	2.0	2.3	2.4	1.7	1.7	1.0	1.2	1.4	1.7	1.9	5.4	6.3	7.3	3.9	3.4	2.9	3.5	5.9	4.7	6.0
Zn	299	307	247	186	148	153	109	151	167	248	281	316	346	205	170	177	169	229	202	263
Acy	udl	udl	udl	udl	udl	udl	udl	n/a	n/a	20.3	udl	udl	udl	udl	n/a	n/a	n/a	udl	30.8	n/a
Ace	15.3	27.4	17.2	n/a	14.6	12.9	16.5	29.5	16.0	20.9	49.5	69.7	27.1	16	n/a	2.7	n/a	25.7	10.2	n/a
Flu	17.9	23.2	udl	udl	udl	8.5	6.9	15.9	12.9	29.8	56.5	30.7	udl	udl	n/a	25.6	n/a	udl	14.9	n/a
Phe	udl	10.4	udl	11.8	21.1	6.6	32.2	1.1	2.6	4.6	19.3	32.1	18.8	23.6	16.0	265	20.4	*32.8*	*88.4*	*23.3*
Ant	udl	udl	udl	udl	2.2	1.7	6.0	4.4	1.8	1.3	11.0	13.2	6.3	9.5	8.8	26.7	13.7	21.6	12.8	18.0
Fla	n/a	15	68	38	27	29	92	36	59	65	610	295	111	85	195	202	147	306	323	246
Pyr	n/a	udl	44	30	23	28	53	36	44	45	517	280	88	91	138	149	112	227	241	177
BaA	35	47	14	19	6	0.1	21	12	16	12	152	128	30	23	11	20	21	41	34	32
Chr	128	78	35	44	18	21	43	33	36	27	275	242	115	92	72	66	67	170	136	143
BbF	173	77	46	41	26	56	71	38	61	63	251	107	77	60	53	57	67	158	113	111
BkF	75	100	31	15	19	16	36	26	21	25	130	98	31	34	59	62	57	91	130	65
BaP	n/a	12	9	7	7	4	45.9	n/a	5	121	215	182	16	10	14	163	17	196	135	10
IcdPy	n/a	18.9	n/a	10.4	14.7	27.9	22.7	udl	udl	udl	31.8	23.1	10.1	14.8	udl	15.6	udl	14.3	51.9	8.2
DBA	n/a	n/a	15.1	12.6	7.7	1.2	12.3	udl	udl	udl	31.8	27.6	n/a	n/a	n/a	12.2	n/a	n/a	21.6	udl
BghiP	12.2	15.6	11.2	10.3	20.6	29.3	24.0	7.0	9.7	6.9	44.0	23.0	19.9	11.5	n/a	14.0	10.5	25.9	8.6	25.6
ΣPAHs	**456**	**425**	**291**	**239**	**207**	**242**	**483**	**239**	**285**	**422**	**2394**	**1551**	**550**	**470**	**567**	**1081**	**533**	**1309**	**1320**	**859**

irrespective of the sex (Table 2). The average Fla/Pyr ratios were 1.31 for Plentzia mussels and 1.25 for Arriluze mussels. The average Fla/(Fla+Pyr) ratio was 0.56 for Plentzia mussels and 0.6 for Arriluze mussels.

Like in the case of metals, seasonality in tissue total PAH concentrations in Plentzia was not relevant; however, in Arriluze the lowest tissue total PAH concentrations were recorded in spring and the highest in winter, with autumn and summer in between (Table 2).

In Plentzia, differences between sexes in tissue total PAH concentrations were circumstantial, whilst in Arriluze in summer the total PAH tissue levels were much lower in females than in males (Table 2). Regarding individual PAH compounds, Phe tissue concentrations were lower in females than in males in winter and spring in Plentzia, and more remarkably in summer and autumn in Arriluze; a similar pattern being identified for IcdPy in summer in Plentzia and in autumn in Arriluze (Table 2). In contrast, the tissue concentrations of Chr and BbF were higher in females than in males in winter in both localities; likewise, the tissue concentration of Ace and BghiP were higher in females than in males in autumn in Arriluze (Table 2).

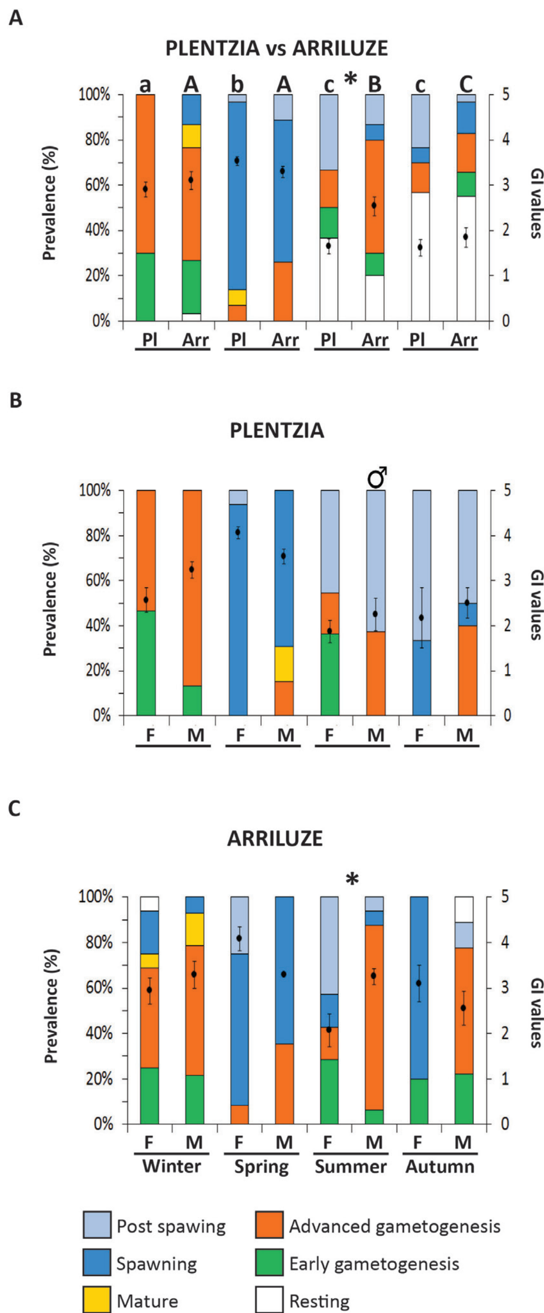


Fig. 2. Gamete development in mussels *Mytilus galloprovincialis* collected from a relatively clean locality (Plentzia) and from one chronically polluted locality (Arriluze) in winter, spring, summer and autumn 2014. Gamete developmental stages are represented by stocked bars and gonad index (GI) values are represented by a solid line at each time endpoint. Vertical segments represent standard deviation. (A) Gamete development of whole populations of Plentzia and Arriluze. Letters indicate significant differences among seasons in mussel populations of the same locality (Plentzia, lowercase letters; Arriluze, capital letters). (B, C) Gamete development of female and male mussels from Plentzia (B) and Arriluze (C). Sex symbols indicate statistical differences between among Plentzia and Arriluze mussel of the same sex (♂, between male mussels).

Energetic biomarkers

According to the three-way ANOVA performed, season (SS), sex (SX) and the interactions between locality and sex (L×SX) and between season and sex (SS×SX) contributed significantly to the variance in COX activity in gills (Table 3). To a lesser extent, COX activity also varied with the interactions between locality and season (L×SS) and between season and sex (SS×SX) ($p<0.1$; Table 3). COX activity did not show differences between Plentzia and Arriluze in any season, with the exception of those recorded between females autumn (Fig. 3A). In Plentzia, COX activity increased in summer, although in Arriluze COX activity values remained constant along the year. Moreover, COX activity was higher in males of both localities in spring than in females, whereas in Plentzia COX activity was higher in females than in resting mussels in summer and autumn (Figs. 3B and 3C).

Three-way ANOVA indicated that only L×SS affected to a limited extent the variance in PK activity in gills ($p<0.1$; Table 3). Accordingly, PK activity was significantly higher in Plentzia than in Arriluze only in winter and summer, lower in autumn than in the other seasons in Plentzia and remained unchanged among seasons in Arriluze (Fig. 4A). According to the 3-way ANOVA performed, SS and SS×SX ($p<0.05$), and to a lesser extent SX ($p<0.1$), affected PEPCK in gills (Table 3). In agreement, PEPCK activity was not dissimilar between Plentzia and Arriluze in any season but in both localities was much higher in winter than in rest of the seasons (Fig. 4B). According to the three-way ANOVA performed, SX, SS and SX×SS interaction affected significantly the PK/PEPCK ratio (Table 3). PK/PEPCK values

Table 3. Summary of the 3-way ANOVAs performed to analyse the effects of sampling locality (d.f.: 1), season (d.f.: 3), sex (d.f.: 2) and their combination ("Locality × Season", d.f.: 3; "Locality × Sex", d.f.: 2; "Season × Sex", d.f.: 4) on biomakers in mussels collected from Plentzia and Arriluze in winter, spring, summer and autumn 2014. Logarithmic transformation was applied to LP (non-parametric variable). d.f.: degrees of freedom; F: Fisher's F; (*): p<0.1; *: p<0.05; **: p<0.01; ***: p<0.001.

Parameter	*F* (Locality)	*F* (Season)	*F* (Sex)	*F* (L x SS)	*F* (L x SX)	*F* (SS x SX)	Residual d.f.
COX	0.135	4.880**	4.119*	2.572(*)	8.052**	2.043(*)	70
PK	0.572	0.152	0.603	1.778(*)	0.607	0.767	69
PEPCK	0.035	12.301***	2.325(*)	1.731	1.517	3.166*	58
PK/PEPCK	0.060	3.159*	4.145*	2.578(*)	1.067	2.593*	58
CO	0.017	13.090***	0.997	5.964**	1.475	3.212*	67
MDA	0.237	25.477***	1.059	1.542	0.654	0.756	75
HNE	42.559***	49.003***	0.878	14.223***	1.780	3.404*	75
LP	125.331***	23.478***	1.300	12.087***	0.297	0.579	195
Vv_{LYS}	22.938***	20.159***	3.114*	4.438**	1.004	0.255	175
S/V_{LYS}	42.605***	33.964***	0.876	2.276(*)	0.132	0.062	175
Nv_{LYS}	2.139	19.270***	0.212	0.085	0.027	0.057	175
Vv_{NL}	56.584***	36.253***	3.919*	9.702***	2.583(*)	0.226	209
Vv_{BAS}	1.621	13.129***	0.148	6.575***	0.012	2.715*	193
MLR/MET	2.325	16.179***	0.712	4.193**	1.410	1.460	193
CTD ratio	0.484	46.736***	0.249	11.267***	0.940	3.062*	193
FCI	117.298***	17.828***	1.065	4.396**	0.677	0.360	219

of Plentzia mussels were higher than values of Arriluze in spring when mussels of any sex/reproductive condition were compared altogether and in spring-summer when only females were compared (Fig. 4C). In both localities, PK/PEPCK showed the lowest values in winter, whereas PK/PEPCK reached the highest values in summer. Moreover, in Arriluze, male spring mussels showed higher PK/PEPCK than females (Fig. 4E). In Plentzia, PK/PEPCK values of resting mussels were higher than values of females and males in summer (Fig. 4D).

Oxidative damage biomarkers

Three-way ANOVA indicated that SS and, L×SS and SS×SX were factors exerting a significant effect on CO in digestive gland (Table 3). In summer, CO values of Plentzia mussels were higher than values of Arriluze mussels (Fig. 5A). In Plentzia, the highest CO values were recorded in spring, while in the case of Arriluze these were recorded in winter (Fig. 5A). Male mussels showed higher CO values than females in spring but lower in autumn in Plentzia (Fig. 5B). MDA in gills was affected significantly only by SS (3-way ANOVA; Table 3); no differences were found between Plentzia and Arriluze in any season and the highest values were recorded in summer, both in Plentzia and Arriluze (Fig. 5C). According to the 3-way ANOVA performed, L, SS, L×SS and SS×SX exerted a significant effect on digestive gland HNE (Table 3). HNE values recorded in mussels from Plentzia and Arriluze were different in all seasons, except in winter; the highest HNE values being always recorded in autumn (Fig. 5D). In Plentzia, females presented higher

HNE values than males in winter (Fig. 5E).

Lysosomal responses

Three-way ANOVA indicated that L, SS and their interaction L×SS were the factors exerting a significant effect on LP, Vv_{LYS} and S/V_{LYS}; whereas Vv_{LYS} was additionally affected by sex and Nv_{LYS} was only affected by season (Table 3).

LP values recorded in Plentzia mussels were higher than those recorded in Arriluze mussels in all seasons (Fig. 6A). These differences between localities were also observed for each particular sex only in many but not in all the cases. Thus, LP values differed between females in winter-summer but not in autumn, and between males in spring-autumn but not in winter, and between mussels in reproductive resting condition in autumn but not in summer (Fig. 6A). In both localities, higher LP values were found in winter-spring than in summer-autumn, more markedly in Plentzia than in Arriluze. Vv_{LYS} was lower in Plentzia than in Arriluze in spring, summer and autumn (Fig. 6B). Significant differences between localities were also evident when females, males and resting mussels were compared separately in summer; however, the Vv_{LYS} values recorded in autumn were not dissimilar between Plentzia and Arriluze for females and for resting mussels (Fig. 6B). Overall, Vv_{LYS} values were low in spring in both localities and increased, dramatically in the case of Arriluze, in summer-autumn (Fig. 6B). S/V_{LYS} values were higher in Plentzia than in Arriluze in spring, summer and autumn (Fig. 6C). When females, males and resting mussels were compared separately differences between localities were recorded for particular sex/ reproductive conditions in winter, summer

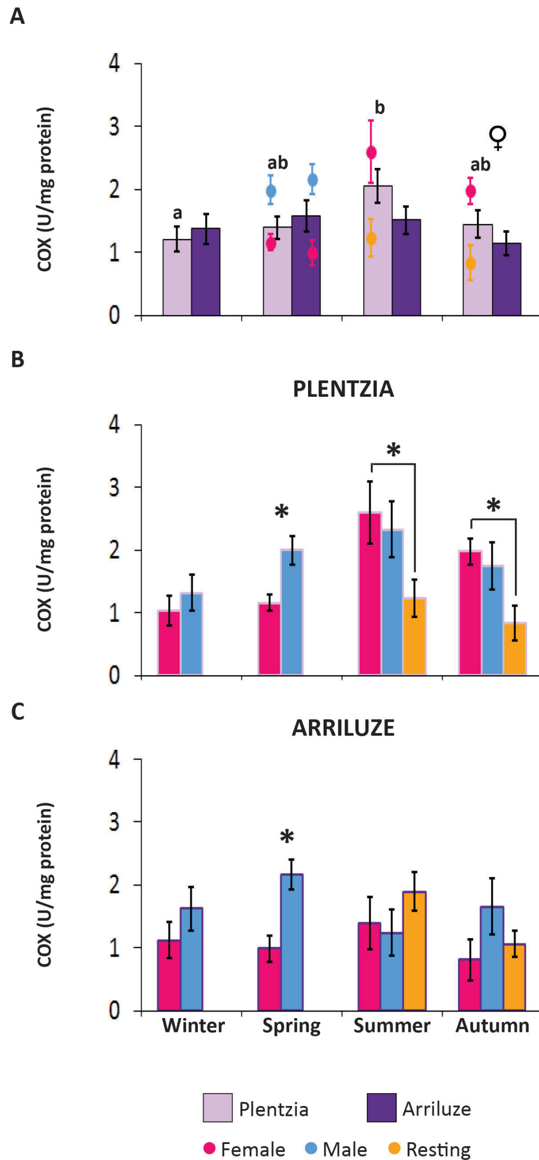


Fig. 3. Cytochrome C oxidase (COX) activity in gills of *Mytilus galloprovincialis* mussels collected from a relatively clean locality (Plentzia) and from one chronically polluted locality (Arriluze) in winter, spring, summer and autumn 2014. Intervals indicate standard error. Letters indicate significant differences among seasons in mussel populations of the same locality (Plentzia, lowercase letters) and asterisk indicate differences among sexes or resting mussels, according to Student's t-test or one-way ANOVA (p<0.05). Sex symbols indicate statistical differences among Plentzia and Arriluze mussel of the same sex (♀, between females) according to Student's t-test and one-way ANOVA (p<0.05).

and autumn (Fig. 6C). In spring, although large differences between Plentzia and Arriluze were recorded when all the mussels from each locality were considered altogether

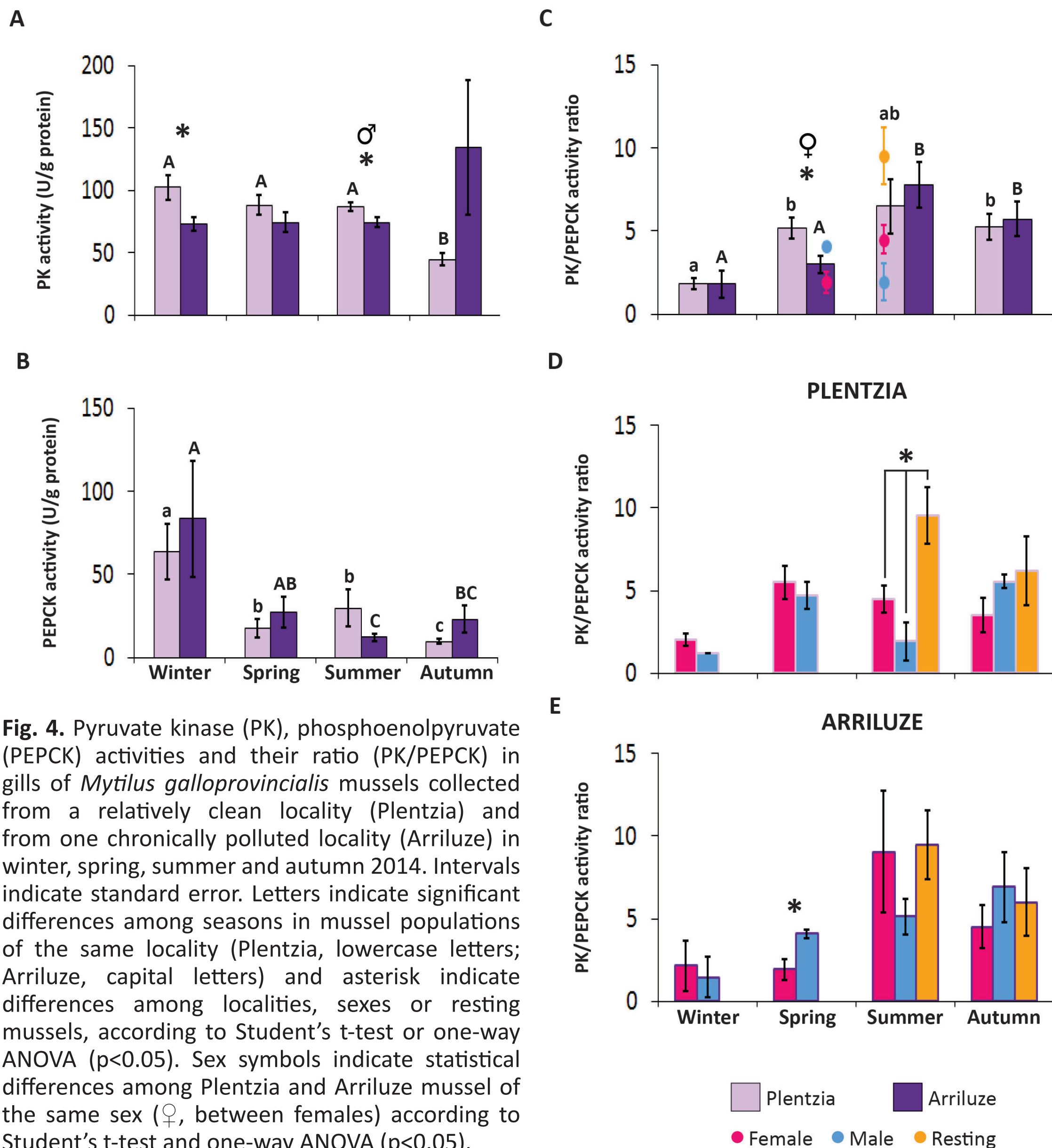


Fig. 4. Pyruvate kinase (PK), phosphoenolpyruvate (PEPCK) activities and their ratio (PK/PEPCK) in gills of *Mytilus galloprovincialis* mussels collected from a relatively clean locality (Plentzia) and from one chronically polluted locality (Arriluze) in winter, spring, summer and autumn 2014. Intervals indicate standard error. Letters indicate significant differences among seasons in mussel populations of the same locality (Plentzia, lowercase letters; Arriluze, capital letters) and asterisk indicate differences among localities, sexes or resting mussels, according to Student's t-test or one-way ANOVA (p<0.05). Sex symbols indicate statistical differences among Plentzia and Arriluze mussel of the same sex (♀, between females) according to Student's t-test and one-way ANOVA (p<0.05).

irrespective of their sex/reproductive condition, no differences were found when the comparison between localities was made using females, males and resting mussels separately (Fig. 6C). As a general rule, S/V_{LYS} exhibited a decreasing trend from spring to autumn in both localities (Fig. 6C). No differences were recorded between Plentzia and Arriluze in Nv_{LYS}, except in winter when the Nv_{LYS} values recorded in males was used for comparisons (Fig. 6D). In contrast, this parameter varied dramatically along the year, with approximately four times higher values in winter than in the other seasons (Fig. 6D). Regarding neutral lipid accumulation, L, SS, SX, L×SS and L×SX were the factors exerting a significant effect on Vv_{NL} in digestive gland, according to the three-way ANOVA performed (Table 3). Thus, Vv_{NL} values were always higher in Arriluze than in Plentzia, outstandingly in summer and autumn; which also was envisaged for females and males separately, except for females in autumn, but not when mussels in reproductive resting stage were compared (Fig. 7A). Accordingly, relevant sex-related variability has been found.

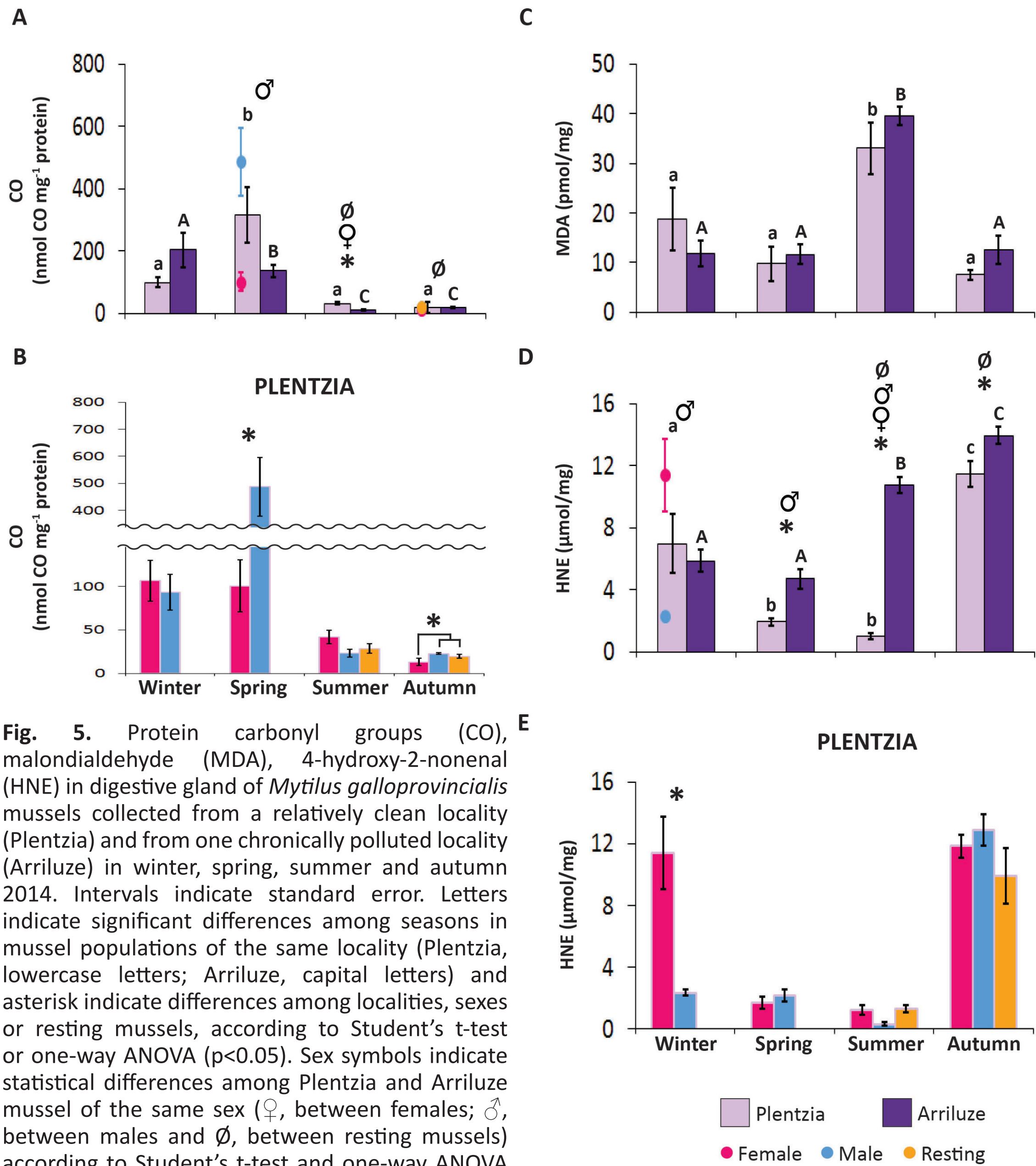
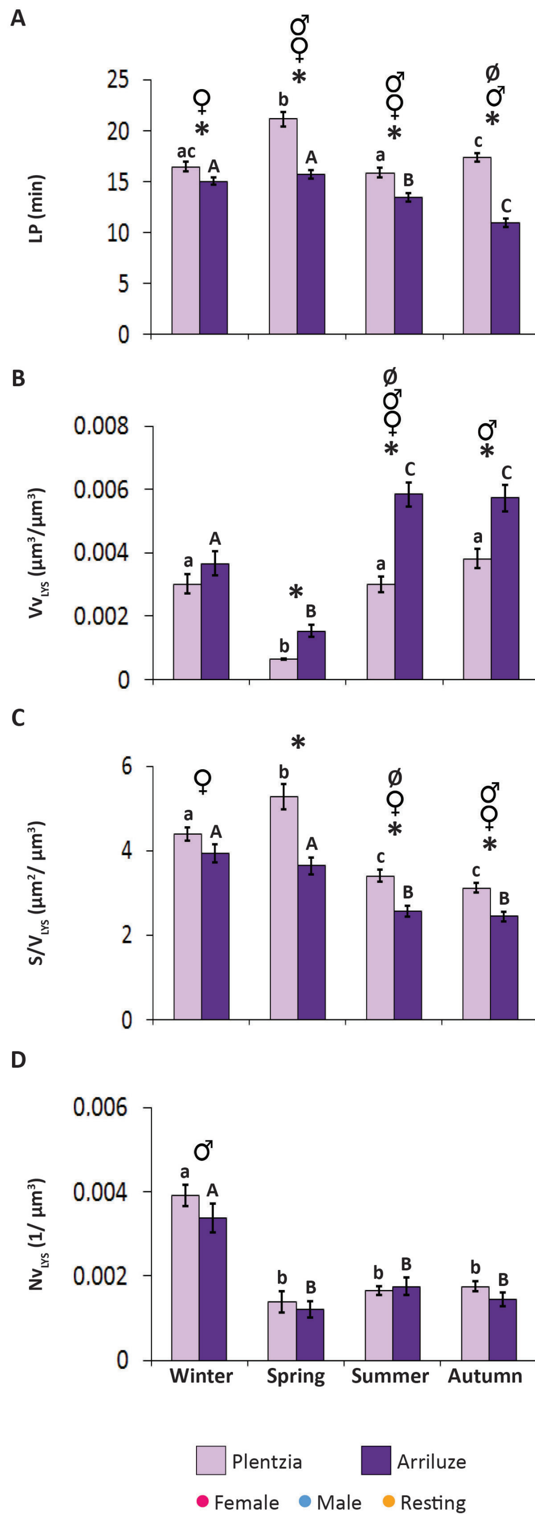


Fig. 5. Protein carbonyl groups (CO), malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE) in digestive gland of *Mytilus galloprovincialis* mussels collected from a relatively clean locality (Plentzia) and from one chronically polluted locality (Arriluze) in winter, spring, summer and autumn 2014. Intervals indicate standard error. Letters indicate significant differences among seasons in mussel populations of the same locality (Plentzia, lowercase letters; Arriluze, capital letters) and asterisk indicate differences among localities, sexes or resting mussels, according to Student's t-test or one-way ANOVA ($p<0.05$). Sex symbols indicate statistical differences among Plentzia and Arriluze mussel of the same sex (♀, between females; ♂, between males and Ø, between resting mussels) according to Student's t-test and one-way ANOVA ($p<0.05$).

With the exception of spring, Vv_{NL} values are always higher in females than in males (Figs. 7A and 7B). Likewise, Vv_{NL} values in mussels in resting reproductive condition are lower than in females and males, especially in comparison with females in summer (Figs. 7A and 7B). Overall, Vv_{NL} showed the same increasing trend from spring to autumn in both localities, much more markedly in Arriluze (Fig. 7A).

Tissue-level responses

Three-way ANOVA indicated that SS, L×SS and SX×SS were the factors exerting a significant effect on Vv_{BAS} in digestive gland (Table 3). Vv_{BAS} values were higher



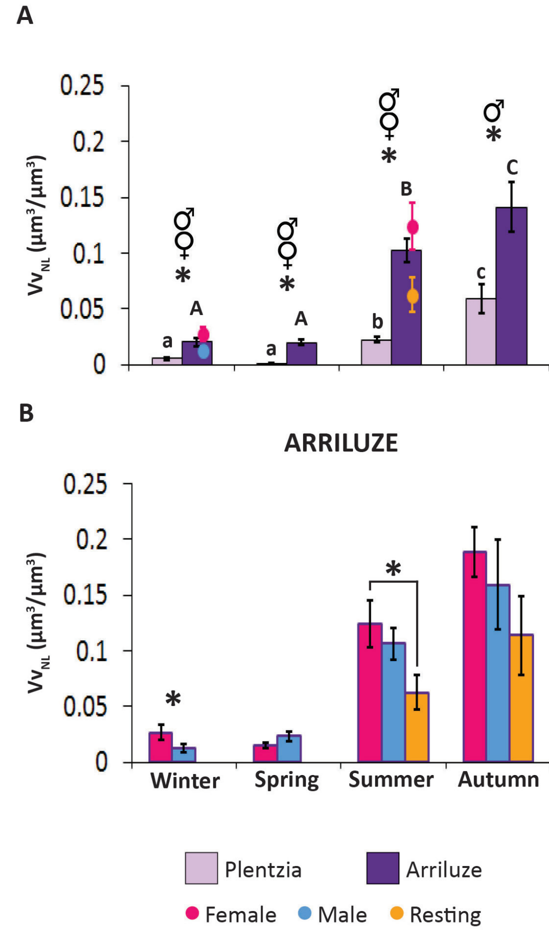


Fig. 7. Volume density of neutral lipids (Vv_{NL}) in digestive gland of *Mytilus galloprovincialis* mussels collected from a relatively clean locality (Plentzia) and from one chronically polluted locality (Arriluze) in winter, spring, summer and autumn 2014. Intervals indicate error standard. Letters indicate significant differences among seasons in mussel populations of the same locality (Plentzia, lowercase letters; Arriluze, capital letters) and asterisk indicate differences among sites in the same season or sexes, according to one-way ANOVA ($p<0.05$). Sex symbols indicate statistical differences among Plentzia and Arriluze mussel of the same sex (♀, between females and ♂, between males), according to Student's t-test and one-way ANOVA ($p<0.05$).

Fig. 6. Lysosomal biomarkers in digestive gland of *Mytilus galloprovincialis* mussels collected from a relatively clean locality (Plentzia) and from one chronically polluted locality (Arriluze) in winter, spring, summer and autumn 2014. Intervals indicate error standard. Letters indicate significant differences among seasons in mussel populations of the same locality (Plentzia, lowercase letters; Arriluze, capital letters) and asterisk indicate differences among sites in the same season, according to one-way ANOVA ($p<0.05$) and Mann-Whitney's U-test ($p<0.05$) for LP. Sex symbols indicate statistical differences among Plentzia and Arriluze mussel of the same sex (♀, between females; ♂, between males and Ø, between resting mussels) according to t-Student test and one-way ANOVA ($p<0.05$) and Mann-Whitney's U-test ($p<0.05$) for LP. LP: labilisation period. Vv_{LYS}: lysosomal volume density. S/V_{LYS}: surface/volume of lysosomes. Nv_{LYS}: lysosomal numerical density.

in Arriluze than Plentzia in spring-summer when all the mussels were considered altogether irrespective of their sex condition and in winter-summer when only female mussels were used for comparisons (Fig. 8A). Female mussels from Plentzia showed higher Vv_{BAS} values than males in winter (Fig. 8A). Similarly, female mussels from Arriluze showed higher Vv_{BAS} values than male and resting mussels in summer, though the profile was reversed in autumn (Figs. 8A and 8B). A certain seasonal trend can be envisaged in both localities, with low Vv_{BAS} values in summer and autumn in comparison with spring, and to some extent with winter, too (Fig. 8A).

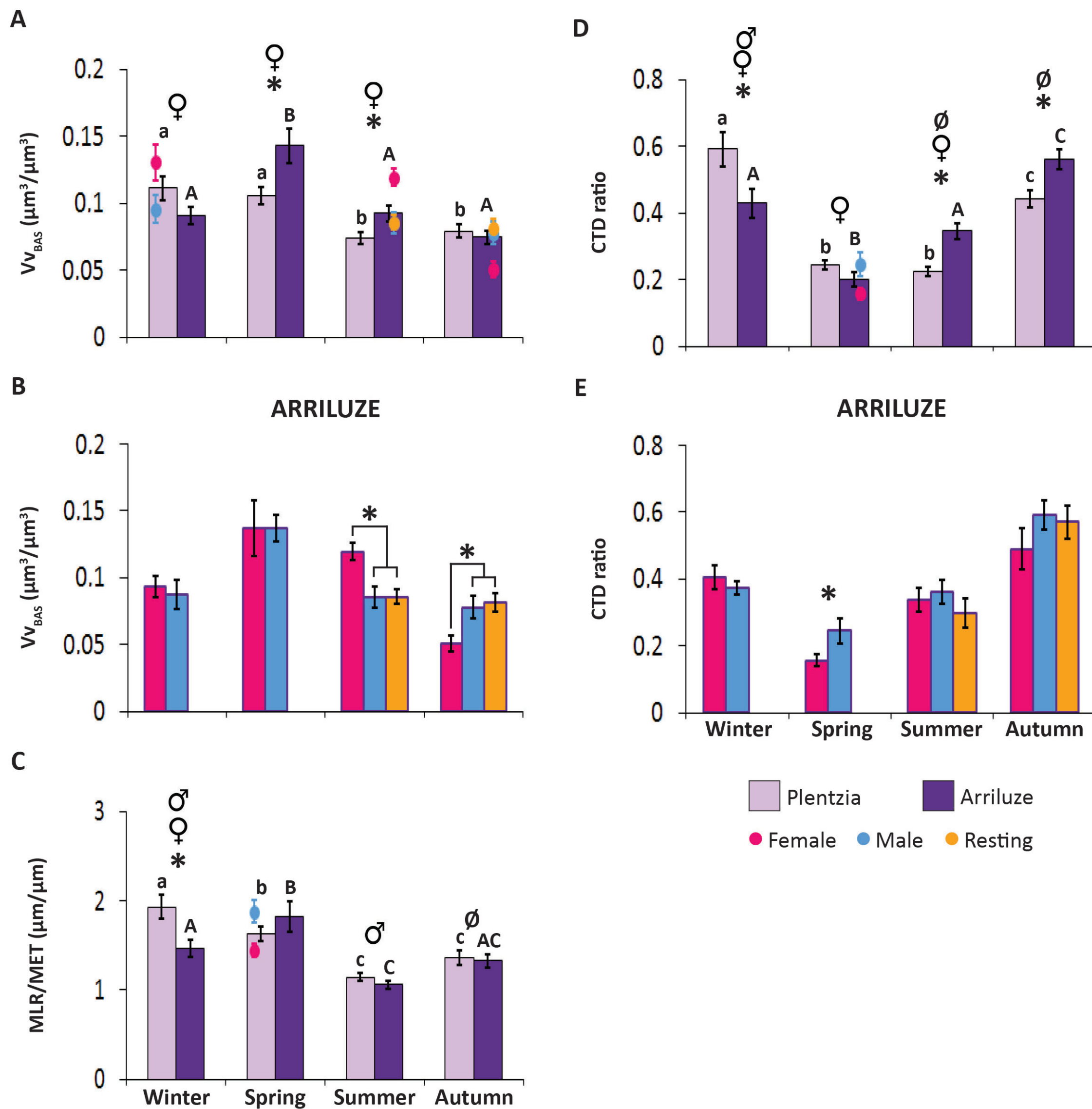


Fig. 8. Tissue-level biomarkers in digestive gland of *Mytilus galloprovincialis* mussels collected from a relatively clean locality (Plentzia) and from one chronically polluted locality (Arriluze) in winter, spring, summer and autumn 2014. Intervals indicate error standard. Letters indicate significant differences among seasons in mussel populations of the same locality (Plentzia, lowercase letters; Arriluze, capital letters) and asterisk indicate differences among sites in the same season or sexes, according to one-way ANOVA ($p<0.05$). Sex symbols indicate statistical differences among Plentzia and Arriluze mussel of the same sex (♀, between females; ♂, between males and Ø, between resting mussels), according to Student's t-test and one-way ANOVA ($p<0.05$). Vv_{BAS}: volume density of basophilic cells, MLR/MET: mean luminal radius/ mean epithelial thickness of the digestive alveoli, and CTD: connective-to-diverticula.

Three-way ANOVA revealed that MLR/MET was affected by SS and L×SS (Table 3). Plentzia mussels showed higher MLR/MET than Arriluze mussels in winter, considering both approaches, either all the mussels collected at each locality altogether or females and males separately (Fig. 8C). In addition, differences between localities were found for males in summer and for resting mussels in autumn (Fig. 8C). Female mussels from Plentzia showed lower MLR/MET values than males in spring (Fig. 8C). Overall, MLR/MET was lower in summer-autumn than in winter-spring in both localities (Fig. 8C).

According to 3-way ANOVA performed, SS, L×SS and SS×SX contributed significantly to the variance in CTD ratio (Table 3). Plentzia mussels showed higher CTD ratio values than Arriluze mussels in winter and lower in summer and autumn (Fig. 8D). The same profile is recognised when females are used for comparison in winter-summer, males in winter, and resting mussels in summer-autumn (Fig. 8D). However, with the exception of the significant differences recorded between males and females in Arriluze in spring, no significant differences between mussels of different sex/reproductive condition have been found for each locality and season (Figs. 8D and 8E). In both localities, CTD ratio values decreased in spring-summer and was maximal in autumn-winter (Fig. 8D).

Organism-level biomarkers

Three-way ANOVA indicated that L, SS and L×SS were the factors exerting an effect on FCI (Table 3). In all seasons, FCI values were higher in Arriluze than Plentzia, the

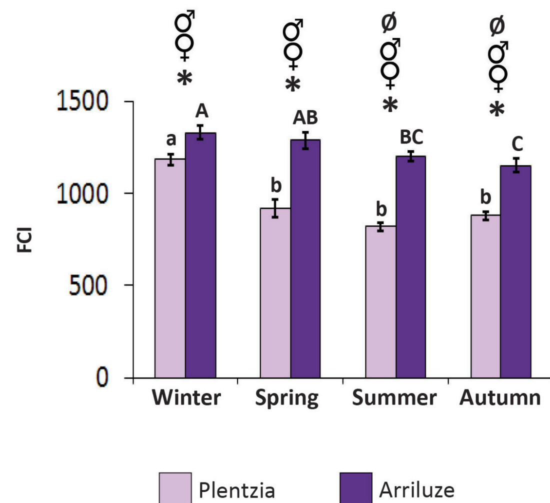


Fig. 9. Flesh Condition Index (FCI) in mussels *Mytilus galloprovincialis* collected from a relatively clean locality (Plentzia) and from one chronically polluted locality (Arriluze) in winter, spring, summer and autumn 2014. Intervals indicate standard deviation. (A) FCI of whole populations of Plentzia and Arriluze. Asterisks indicate significant differences among Plentzia and Arriluze mussel populations in the same season according to one-way ANOVA test (p<0.05). Letters indicate significant differences among mussel populations of seasons for each sampling point (Plentzia, lowercase letters; Arriluze, capital letters) according to one-way ANOVA (p<0.05). FCI of females, males and resting mussels from Plentzia (B) and Arriluze (C). Sex symbols indicate statistical differences between Plentzia and Arriluze mussel of the same sex (♀, between females; ♂, between males; and Ø, between resting mussels) according Student's t-test and one-way ANOVA (p<0.05). Asterisks indicate significant differences among sexes of the same locality and season according to Student's t-test test and one-way ANOVA (p<0.05).

maximum FCI in both localities being reached in winter (Fig. 9). This was obsevred for mussels considered altogether irrespective of their sex/reproductive condition but also for females, males and resting mussels separately.

The longest survival-in-air times were recorded in winter in both localities (Fig. 10A-10D). LT50 of Arriluze mussels was higher than in Plentzia mussels in winter, spring and summer, more markedly in the two latter (Figs. 10A-10C). However, when only females are used to compare localities,

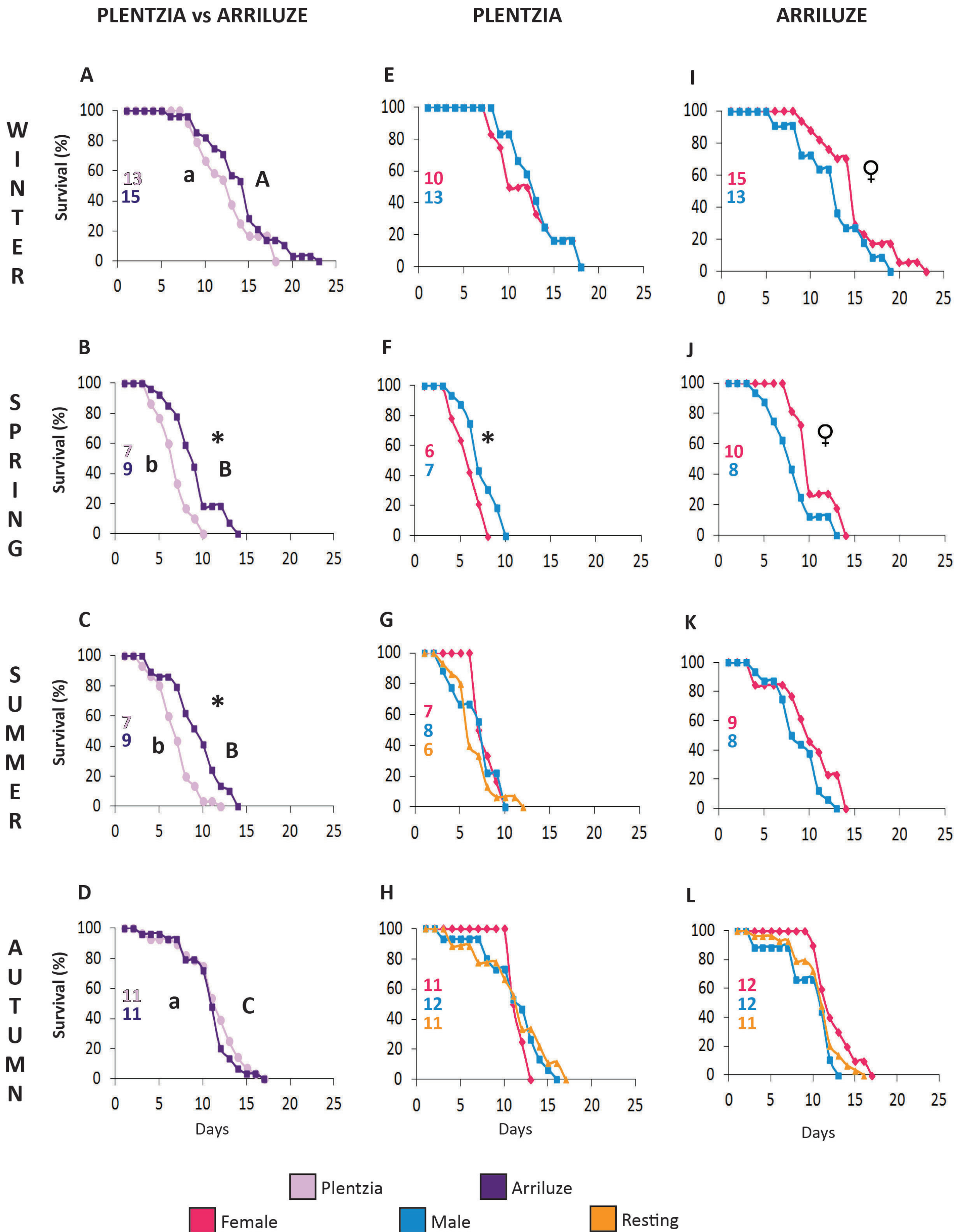


Fig. 10. Cumulative survival curves of mussels *Mytilus galloprovincialis* collected from a relatively clean locality (Plentzia) and from one chronically polluted locality (Arriluze) in winter, spring, summer and autumn 2014. Survival curves were compared using Kaplann-Meier test. (A-D) Cumulative survival curves of whole populations of Plentzia and Arriluze for each season. Asterisks indicate significant differences among Plentzia and Arriluze mussel populations in the same season. Letters indicate significant differences among mussel populations of different season for each sampling point (Plentzia, lowercase letters; Arriluze, capital letters). Survival curves of females, males and resting mussels from Plentzia (E-H) and Arriluze (I-L). Sex symbols indicate statistical differences between Plentzia and Arriluze mussel of the same sex (♀, between females). Asterisks indicate significant differences among sexes of the same locality and season. Coloured numbers are LT50 values for the sex group of the corresponding colour in adjoining graphs.

LT50 is higher in Arriluze than in Plentzia in winter and in spring but not in summer (Figs. 10E-10L). Whereas in Arriluze no differences were recorded between both sexes, females showed a certain trend to survive longer in air than males, especially in winter-summer. In contrast, males from Plentzia showed higher LT50 values in spring than females (Fig. 10F).

DISCUSION

In the present study, the gametogenic cycle followed the typical pattern for the Basque Coast mussel populations. In both localities, gametogenesis occurred during winter and spawning in spring, in agreement with previous studies (Villalba, 1995; Ortiz-Zarragoitia et al., 2010; Múgica et al., 2015). In the case of Arriluze, gametogenesis seemed to be uninterrupted as there were always follicles with gametes in advance gametogenesis stage. Gamete development and reproduction are controlled by factors such as food availability and temperature, and therefore, differences in nutritional conditions could explain the differences found between the gametogenic cycles in Plentzia and Arriluze (Villalba, 1995; Ortiz-Zarragoitia et al., 2011). However, the sex ratio was similar in both studied populations, and close to “1” in most cases. Moreover, unlike in previous works in the study area, no evidence of reproductive disturbance or pathology was recorded (Ortiz-Zarragoitia et al., 2011; Garmendia et al., 2011). Certainly, altered gametogenesis, gamete malformation, oocyte atresia, haemocytic infiltration and intersex were reported after the Prestige oil spill in mussels from the present study area (Ortiz-Zarragoitia et al. 2011); however, the tissue concentration of total and individual PAHs were much higher in that occasion than in the present study (Soriano et al., 2006; Fernández et al. 2010). It seems, therefore, that pollution levels are not extremely high even in Arriluze, as below discussed.

Tissue pollutant concentrations

Overall, tissue metals and PAHs concentrations confirmed that Plentzia was a relatively clean locality and Arriluze a moderately polluted one. Thus, the two studied mussels populations lived in localities with different pollution degree, as expected (Orbea and Cajaraville et al., 2006; Bartolome et al., 2010; Marigómez et al., 2013a).

The tissue concentration of metals was different between sexes depending on the season and the locality. Overall, the metal tissue concentrations were low-to-moderate in both localities (Devier et al., 2005), being always higher in Arriluze, the chronically polluted site. Inter-season variability was marked in Arriluze, where the lowest tissue metal concentrations were recorded in summer for all the metals. This seasonal pattern has been previously reported in other studies carried out in the Arcachon Bay and along the Basque Coast (Soto et al., 1995; Franco et al., 2002; Devier et al., 2005). The reason of the higher tissue metal concentration in winter has been related to the reduced metabolism during this season, promoting slower metal depuration (Dahlgaard, 1986).

Whereas in crustaceans females contain higher concentrations of metals than males (Chen et al., 2005), in fish pollutant bioaccumulation has been reported to be higher in males than in females (Bodiguel et al., 2009), and in molluscs controversial

results have been obtained (Hummel et al., 1989; Lobel et al., 1991). In the present study, differences between sexes in tissue pollutant concentrations occurred only at particular seasons. Thus, Cr tissue concentration in females, in comparison with males, was lower in Plentzia in winter and higher in Arriluze in autumn and summer. Likewise, Ni tissue concentration in females from Plentzia was higher than in males in all the seasons except in winter; and in females from Arriluze lower than in males in autumn. Sex-related differences in metal tissue levels can be attributed to differences in growth rate between males and females and to the greater release of contaminants during spawning (gamete release) in females, at least in some species (Bodiguel et al., 2009). Therefore, sex-related differences are driven by changes along the reproductive cycle, including different metabolic and physiological status at different gametogenic developmental stages and the consequences of spawning and post-spawning stress (Bodin et al., 2007). For instance, some contaminants are known to be eliminated through spawning in molluscs (Hummel et al., 1989; Ruiz et al., 2011; Xiu et al., 2015).

PAH tissue concentrations were higher in Arriluze than in Plentzia in all the seasons, irrespective of the sex. Based on the categories of Baumard et al. (1998) and Kimbrough et al. (2008), the tissue PAHs concentrations could be classified as "low-to-moderate" in Plentzia and "moderate-to-high" in Arriluze. The values of tissue concentration of total PAHs recorded un Plentzia mussels were comparable to those recently reported in the nearby Bidasoa estuary (Olenycz et al., 2015) and much lower than those recorded in the same area after the Prestige oil spill (Bartolomé et al., 2010). The tissue concentration of total PAHs recorded in Arriluze mussels was in between.

In both localities, Fla/Pyr and Fla/(Fla+Pyr) ratios suggested that PAHs contamination originated mostly from pyrolytic sources (combustion processes) since obtained Fla/Pyr and Fla/(Fla+Pyr) values were higher than threshold values (1 and 0.4 respectively), which separate petrogenic and pyrolytic origins (Ruiz et al., 2011; Lacroix et al., 2017).

Seasonality in the tissue concentrations of total PAHs in Plentzia was not relevant. In Arriluze, the tissue total PAH concentration tended to be lower in spring and summer. A similar seasonal pattern was observed in mussels from other temperate regions (Piccardo et al., 2001; Soriano et al., 2006; Langston et al., 2012; Olenycz et al., 2015; Solaun et al. 2015). This seasonal cycle has been linked directly to the availability of food, hydrological conditions, and internal factors such as physiological condition, age/size, and lipid content of the mussels (Hummel et al., 1990; Baumard et al., 1998). However, PAHs are hydrophobic compounds that occur mainly in tissues with high lipid content but their tissue concentration increased during autumn-winter when the lipid content in mussels is relatively low (Olenycz et al., 2015). As previously sugegsted (Baumard et al., 1999; Olenycz et al., 2015), the origin of these PAHs would include mainly atmospheric deposition and river run-off derived from enhanced organic combustion and emissions from motor vehicles in cold months leading to elevated concentrations of PAHs in suspended particulate-bound forms in coastal waters and resulting in the more intense accumulation of these compounds in filter-feeding organisms like mussels (Olenycz et al., 2015).

The tissue concentrations of total PAHs were different between sexes depending on the season and the locality: whilst in Plentzia they were similar in both sexes, in Arriluze in summer they were much lower in females than in males. Regarding individual PAH compounds, Phe tissue concentrations were also lower in females than in males in particular seasons both in Plentzia (winter and spring) and in Arriluze (summer and autumn). Likewise, IcdPy tissue concentrations were lower in females than in males both in Plentzia (summer) and in Arriluze (autumn). In contrast, the tissue concentrations individual PAHs were higher in females than in males: Chr and BbF in both localities (winter) and Ace and BghiP in Arriluze (autumn). In agreement, females of the scallop *Chlamys farreri* accumulated more chrysene than males (Xiu et al., 2015).

Both seasonal trends and sex-related differences in tissue total PAH concentrations could be attributed to seasonal and sex-related differences in the tissue lipid contents (higher in autumn-winter and in females), as well as the "depurating effects" of PAH release together with gametes though spawning, which is especially remarkable in late spring-early summer and in females (Ruiz et al., 2011; Viñas et al., 2012). Moreover, seasonal and sex-related variability has been reported in detoxifying enzymes such as GST in molluscs and e.g. EROD in crustacean and fish (Hylland et al., 1998; Xiu et al., 2015); this could explain also the seasonal and sex-related variability in tissue total PAH concentrations reported herein. Moreover, in the present work, greater differences in tissue metal and PAH concentrations were observed when comparing resting mussels with females and males, especially in Arriluze in autumn. Thus, overall it seems that tissue pollutant concentrations are greatly affected by the progressing of the gametogenic development cycle.

Energetic biomarkers

COX and PK activities in gills varied with season, only in the clean locality, with increased COX and decreased PK in summer. PEPCK activity, however, varied with season at both localities, with highest values in winter. As a result, the PK/PEPCK ratio varied with season, with low values in winter and high values in summer in both localities. COX activity indicate the mitochondrial status of the organisms (Lucassen et al., 2003; Siu et al., 2003; Morley et al., 2009), whereas PK, PEPCK and the PK/PEPCK ratio are applied to assess the aerobic capacity (Ivanina et al., 2016). Oxidative stress can promote inhibition of COX activity (Chen et al., 1998).

A seasonal pattern of variability was envisaged in both localities, characterised by a lower aerobic scope in winter than in the other seasons. Thus, low PK/PEPCK ratio was recorded in both localities and low COX activity in Plentzia, which indicates that mussels spend the winter in an inactive state, as it has been observed in other marine molluscs from temperate regions (Innes and Houlihan, 1985; Sokolova and Pörtner, 2003). The winter depression in aerobic scope caused by low seawater temperature provides mussels with an efficient survival strategy against food restriction (Sokolova and Pörtner, 2003). Seasonal variability in metabolic enzyme activities could be sex-related. COX activity was higher in males than in females in both localities in spring-summer and lower in resting mussels than

in females and males in summer-autumn in Plentzia. The PK/PEPCK ratio was recorded in males was higher than in females in Arriluze in spring and lower in summer in both localities. As a result of these intricate profiles, differences between localities were essentially recorded between females in summer-autumn for COX and in spring-summer for PK/PEPCK (this latter was so marked that remained when females and males were considered altogether). In addition, PK activity was lower in Arriluze than in Plentzia in winter-summer (especially when summer males were compared) and this was absolutely reversed in autumn.

Oxidative damage biomarkers

CO is recognised as a biomarker of oxidative damage in proteins (Dalle-Donne et al., 2003; Sureda et al., 2011). In the present study, season exerts a significant effect on CO in mussel digestive gland, together with its interactions with locality and sex. Accordingly, CO exhibited a similar seasonal trend in the two studied localities, with high values in winter-spring and low values in summer-autumn and minor locality and sex dependent deviations. The most outstanding sex-related difference was the very high CO values recorded in males from Plentzia in spring.

MDA and HNE are biomarkers of lipid peroxidation and cell oxidative damage (Viarengo et al. 1991; Maria and Bebianno 2011). MDA exhibited a remarkable seasonality whilst HNE varied with locality and season, their interaction, and season-dependent sex/reproductive condition. Thus, MDA rose in summer in both localities and sex-related differences were not observed. HNE, however, also varied with locality and sex at certain seasons; thus, HNE values were the highest in summer-autumn in both localities irrespective of the sex-condition (females, males and resting) and in Plentzia females in summer. High metabolic activity in summer has been associated with an increase in oxidative damage in mussels (Bocchetti et al., 2008). In agreement, GSH mobilization is enhanced (linked to higher activities of antioxidant enzymes) in summer in shrimps, both in males and females (Gismondi et al., 2012). There exist, however, sex-dependent seasonal differences seemingly driven by the reproductive cycle. For instance, GSH concentrations vary with the reproduction cycle in mussels (Viarengo et al., 1991; Wilhelm Filho et al., 2001) and shrimps (Gismondi et al., 2012); the highest GSH concentrations being in spring and in females, which could be related to spawning and post-spawning stress (Knapen et al., 1999). Likewise, according to Gismondi et al. (2012), *G. roeseli* females exhibit lower MDA levels than males (less cell damage) and higher LC50 after Cd-exposure (more resilience). Interestingly, female scallops have been reported to suffer from oxidative damage after spawning more than males (Xiu et al., 2015).

Overall, differences between localities in CO, MDA and HNE were sporadic and meaningless, irrespective of the sex/reproductive condition of the mussels. Antioxidant activities and oxidative damage in temperate mussels are to a large extent governed by seasonal changes in seawater temperature, dissolved oxygen and nutrients (Devier et al., 2005). As previously suggested for the Arcachon Bay (Devier et al., 2005), it seems that seasonal variability and intrinsic individual traits (e.g. sex and reproductive condition) can be important confounding

factors regarding the responsiveness of oxidative stress biomarkers to pollutant exposure.

Lysosomal biomarkers

Lysosomal enlargement (usually augmented Vv_{LYS} and reduced S/V_{LYS}) and membrane destabilisation (reduced LP) are well known effect biomarkers widely used in mussels (UNEP/RAMOGE, 1999; Marigómez and Baybay-Villacorta, 2003; ICES, 2004; 2012). In the present investigation, LP and S/V_{LYS} varied with season and locality, Vv_{LYS} varied with locality, season and sex, and Nv_{LYS} was only affected by season.

The endo-lysosomal system of mussel digestive cells is known to show significant changes during the annual cycle (Etxeberria et al., 1995; Marigómez et al., 1996; Tremblay et al., 1998; Izagirre et al., 2008; Múgica et al. 2015). Both in Plentzia and Arriluze, seasonality in lysosomal parameters was marked irrespective of the differences between these localities in pollution level and profile, above discussed. Thus, LP and S/V_{LYS} were lower in summer-autumn than in winter-spring, and Vv_{LYS} higher. In parallel, very high Nv_{LYS} was recorded in winter and low in the other seasons. This response profile reflects that lysosomal enlargement (high Vv_{LYS} and low S/Vv_{LYS}) and membrane destabilization (low LP) occur in summer-autumn, compared to winter-spring. High temperatures and high food availability may be drivers for these seasonal changes (Etxeberria et al., 1995; Tremblay et al. 1998; Izagirre et al., 2008; Múgica et al., 2015).

Beyond seasonal changes, LP was lower in Arriluze than in Plentzia in all seasons both considering all the mussels altogether irrespective of their sex/reproductive condition and considering females (winter-summer), males (spring-autumn) and mussels in reproductive resting condition (autumn). Likewise, Vv_{LYS} was higher and S/V_{LYS} was lower in Arriluze than in Plentzia in the spring-autumn period when all the mussels were considered irrespective of their sex/reproductive condition. However, differences between localities in Vv_{LYS} were more restricted to the summer season when females, males and resting mussels were compared separately. Likewise, differences in S/V_{LYS} between localities were recorded when females, males and resting mussels were compared separately except in spring, although differences between Plentzia and Arriluze in this season were outstanding when mussels were considered altogether irrespective of their sex/reproductive condition. These lysosomal responses can be associated to the higher tissue pollutant concentrations recorded in Arriluze mussels in comparison with Plentzia, especially in summer and autumn for metals and in winter for PAHs (see above).

Intracellular neutral lipid accumulation in mussel digestive gland has been used as biomarker of exposure to organochemicals and as biomarker of non-specific stress (Regoli, 1992; Marigómez and Baybay-Villacorta, 2003; Shaw et al., 2011). Presently, neutral lipid accumulation varied with locality, season, sex and their interactions.
Lipid content in bivalves varies with physiological condition and reproduction cycle, increasing markedly during gamete maturation in late winter and early spring, and decreasing afterwards until they are depleted in winter to avoid starvation when there is a deficiency of food (Zandee et al., 1980). Thus, in mussels from the Bay of

Biscay neutral lipid accumulation follows a seasonal change of variation that depends on the nutrient availability and the progression of the reproductive cycle (Cancio et al., 1999; Garmendia et al., 2010; Múgica et al., 2015). In agreement, in the present study Vv_{NL} showed a similar increasing trend from spring to autumn in both localities, much more markedly in Arriluze.

Vv_{NL} was always higher in Arriluze than in Plentzia, outstandingly in summer and autumn. This was envisaged for females and males separately, except for females in autumn, but not when mussels in reproductive resting stage were compared. Accordingly, Vv_{NL} varies depending on the sex/reproductive condition of the mussels. Thus, Vv_{NL} was always higher in females than in males except in spring, and mussels in resting reproductive condition always presented the lowest Vv_{NL} values.

Tissue-level biomarkers

Increase in the relative proportion of basophilic cells, atrophy of the digestive epithelium and loss of digestive gland histological integrity have been often used as effect biomarkers in the Bay of Biscay (Cajaraville et al., 1992; Marigómez et al., 2006; Garmendia et al., 2011). According to the results of the present study, season and their interactions with locality exerted a significant effect on Vv_{BAS}, MLR/MET and CTD ratio, whilst Vv_{BAS} was also affected by the SS×SX interaction. Seasonality in tissue-level biomarkers can be envisaged in both localities, with lower Vv_{BAS} and MR/MET in summer-autumn than in winter-spring, and low CTD ratio in spring-summer and high CTD ratio in autumn-winter, in agreement with previous reports (Garmendia et al., 2010; Múgica et al., 2015).

There existed sex and reproductive stage related differences in Vv_{BAS} responsiveness; thus, values were higher in Arriluze than in Plentzia in spring-summer irrespective of the mussels' sex condition and in winter-summer for the case of females. MLR/MET was lower in Arriluze than in Plentzia in winter irrespective of the mussel sex/reproductive condition but also for females and males separately. CTD ratio was lower in Arriluze than in Plentzia in winter and higher in summer and autumn. The same profile is recognised when females and resting mussels are used for comparison but only in winter for males.

Some apparently inconsistent differences between mussels of different sex/reproductive condition were observed in Vv_{BAS}, MLR/MET and CTD ratio. For instance, whereas females showed higher Vv_{BAS} values than males in Arriluze in summer the opposite was observed in autumn, and males showed higher MLR/MET values than females in Plentzia in spring and higher CTD ratio in Arriluze spring.

Organism-level biomarkers

Individual condition, measured in terms of FCI, reflects the physiological status of bivalves and it has been reported that it is reduced on exposure to chemical pollutants due to the increase of energy demand to face the toxic effect of pollutants. FCI varied significantly with locality, season and the interaction between locality and season. Seasonality was featured by maximum FCI values in winter in both localities, which seems to be dependent on food availability and the gamete developmental

cycle (Beyer et al., 2017). Similarly, the longest survival-in-air times were recorded in winter in both localities. In agreement, survival and condition factors in mussels have been reported to be better in winter; which has been related to exogenous factors such as water temperature and food supply and endogenous factors such as spawning (Denvier et al., 2015). Presently, FCI was always higher in Arriluze than Plentzia, irrespective of the sex/reproductive condition of the mussels, and less variable throughout the year.

The capacity of mussels to survive-in-air is recommended by ICES (2012) for monitoring programmes as a measure of resilience. Stressed mussels are expected to exhibit low capacity to survive-in-air (Viarengo et al., 1995; ICES 2012). Conversely, LT50 in Arriluze (higher chemical pollution) was higher than in Plentzia (lower chemical pollution) in the winter-summer period. However, the profile changed when comparisons were restrained to mussels of the same sex/reproductive condition. Thus, when females were compared, LT50 was higher in Arriluze than in Plentzia in winter and spring but not in summer. Sex-related variability was intricate; whereas females showed a certain trend to survive longer in air than males in Arriluze, especially in winter-summer, males seemed to be more resilient to air-exposure than females in Plentzia in spring.

Concluding remarks

The values obtained in the present study contribute to identify baseline values of pollutant tissue concentrations and a suite of biomarkers suitable for pollution monitoring in the North Iberian Peninsula. Overall, there exists evidence of sex and reproductive stage related differences in bioaccumulation and in the values and responsiveness to environmental insult of many of the studied biomarkers, though diverse patterns can be envisaged. The present results suggest that females seem to be more sensitive for some biomarkers and males for others, but this also depends on the season, and most likely it is linked to the progression of the reproductive cycle. Overall, selecting individuals of one specific sex was not a priory necessary to carry out biomarker-based monitoring programs using mussels as sentinels. However, it is highly recommended that both sex and reproductive condition should be recorded and for this purpose histological analysis of gonad tissue must be conducted in order to determine sex ratios and gamete developmental stages.

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CHAPTER 4

THE INFLUENCE OF FASTING IN SHORT-TERM EXPERIMENTATION ON BIOMARKER RESPONSIVENESS IN OIL WAF EXPOSED MUSSELS

This chapter has been presented at:

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ABSTRACT

Mussels are widely used organisms in toxicological experiments. But, standardized procedures to keep mussels are lacking for experimentation. Thus, there is a large disparity on feeding strategies to maintain mussels in short-term toxicological studies, being fasting a common procedure. However, there are evidences that some biomarkers to change during food digestion and depending on the mussels' nutritive status. Within this context, this research was designed to study the influence of fasting on biomarkers in mussels exposed to petrochemicals, contributing to develop Best Available Practices to maintain mussels during short-term toxicological experiments. Accordingly, fasted mussel and mussels fed *Isochrysis galbana* were exposed for 96 hours at various sublethal dilutions of water accommodated fraction (WAF) of a heavy fuel oil (0%, 6.25%, 12.5% and 25% WAF) in seawater. PAH levels in digestive gland and biomarker battery was determined: glutathione-S-transferase, lipid peroxidation, cytochrome C oxidase, lysosomal membrane stability, lysosomal structural changes, lipofuscin and structural changes of digestive alveoli. Furthermore, the integrated biological response index (IBR/n) was calculated. Overall, biomarker responsiveness against WAF short-term exposure changed markedly depending on whether mussels were fed or not during experimentation. Consequently, it was recommended fed mussels during short-term toxicological experiments, in order to avoid biological responses to fasting and to guarantee comparability with longer experiments and field data in monitoring programmes. Progress in the standardization of the methodology for mussels feeding in laboratory experiments is needed for a proper assessment of biological responses. With this framework, the present investigation provides the first recommendation about feeding strategy during toxicological experiments based on mussel biomarkers.

Barauaren eragina biomarkatzaileen erantzun-gaitasunean epe motzean erregai astun baten WAF-era esposatutako muskuiluetan

Laburpena

Muskuiluak esperimentu toxikologikoetan organismo oso erabiliak dira. Baina muskuiluak esperimentuetan zehar mantentzeko prozedura normalizatuak falta dira. Horrela, elikadura-estrategia desberdinak eta anitzak daude muskuiluak mantentzeko epe laburreko ikerketa toxikologikoetan, baraua ohiko prozedura izanez. Hala ere, froga desberdinen arabera, biomarkatzaile batzuk elikagaien liseriketan zehar aldatzen dira, baita muskuiluaren egoera nutritiboaren arabera. Testuinguru honetan, lan hau petrokimikoetara esposatutako muskuiluetan barauaren eragina biomarkatzaileetan ikertzeko diseinatu zen. Beraz, elikatu gabeko muskuiluak zein *Isochrysis galbana*-rekin elikatutako muskuiluak 96 ordupean itsas ur filtratuan eta erregai astun baten urari egokitutako frakzioaren (WAF) hainbat diluziopean mantendu ziren (0%, 6,25%, 12,5% eta 25% WAF). Erabilitako dosiak subletalak ziren. Liseri-guruineko HAP kontzentrazioa eta hurrengo biomarkatzaileak aztertu ziren: glutation-S transferasa, lipidoen peroxidazioa, lisosomen mintzaren desegonkortasuna, lisosomen egituren aldaketak, lipofustinen metaketa, liseri epitelioaren zelulen konposaketa eta liseri albeoloen egituren aldaketak. Horrez gain, biomarkatzaile indize integratua (IBR/n) kalkulatu zen. Oro har, elikadura estrategiaren arabera epe laburreko esposizioan biomarkatzaileen WAF-ren aurka erantzuteko gaitasuna aldatzen da. Horren ondorioz, epe laburreko esperimentu toxikologietan muskuiluak elikatzea gomendatzen da, barauko erantzun biologikoa saihesteko eta konparagarritasuna epe luzeagoko esperimentuekin eta biojarraipenekin bermatzeko. Laborategiko esperimentuetan muskuiluak elikatzeko metodologia estandarizatzeko aurrera pausuak ematea beharrezkoa da, erantzun biologikoen ebaluazio egokia burutzeko. Esparru honetan, lan honek elikadura-estrategiari buruzko lehengo gomendioa ematen du muskuiluen biomarkatzaileetan oinarritutako esperimentu toxikologikoetarako.

La influencia del ayuno sobre la capacidad de respuesta de los biomarcadores en mejillones expuestos WAF de un fuel pesado en experimentación a corto plazo

Resumen

Los mejillones son muy utilizados en experimentos toxicológicos. Pero para mantener a los mejillones durante la experimentación no existen procedimientos estandarizados. Por lo tanto, existe una gran disparidad en las estrategias de alimentación para mantenerlos en estudios toxicológicos a corto plazo, siendo común el ayuno. Sin embargo, hay evidencias de que algunos biomarcadores cambian durante la digestión de los alimentos, y que dependen del estado nutritivo de los mejillones. En este contexto, esta investigación se diseñó para estudiar la influencia del ayuno en biomarcadores en mejillones expuestos a petroquímicos, contribuyendo a desarrollar mejores prácticas disponibles para mantenerlos durante experimentos toxicológicos a corto plazo. Por consiguiente, mejillones en ayunas y mejillones alimentados con *Isochrysis galbana* se expusieron durante 96 horas a varias diluciones subletales de fracción de agua acomodada (WAF) de un fuel pesado (0%, 6,25%, 12,5% y 25% WAF) en agua de mar. Se determinaron los niveles de HAP en la glándula digestiva y la siguiente batería de biomarcadores: glutation-S-transferasa, peroxidación lipídica, citocromo c oxidasa, estabilidad de la membrana lisosómica, cambios estructurales lisosómicos, acumulación intracelular de lipofustinas, y composición del tipo celular y cambios estructurales de alvéolos digestivos. Además, se calculó el índice de integración de respuestas biológicas (IBR/n). En general, frente a la exposición a corto plazo del WAF la capacidad de respuesta de los biomarcadores cambió notablemente dependiendo de la estrategia de alimentación. En consecuencia, se recomienda alimentar a los mejillones durante experimentos toxicológicos a corto plazo, para evitar las respuestas biológicas al ayuno y para garantizar la comparabilidad con experimentos más largos y programas de seguimiento ambiental. Progresar en la estandarización de la metodología para la alimentación de mejillones en experimentos de laboratorio es necesario para una evaluación adecuada de las respuestas biológicas. En este marco, la presente investigación proporciona la primera recomendación sobre la estrategia de alimentación durante experimentos toxicológicos basados en biomarcadores en mejillón.

INTRODUCTION

Mussels are commonly used model organisms in aquatic toxicological experiments (Moore, 1988; Lima et al., 2008; Mezzelani et al., 2016; Beyer et al., 2017). The digestive gland is the major target organ, as it is involved in pollutant accumulation and detoxification and responds in a measurable manner to environmental insult (Moore, 1991; Lowe and Pipe, 1994; Cajaraville et al., 1995; Livingstone et al., 2000). Likewise, the digestive gland is the main organ involved in food processing, digestion and nutrient delivery (Morton, 1983) and, as such, these processes could interfere with the biological responses to pollutants. Indeed, lysosomal, biochemical and physiological biomarkers are known to change during food digestion and depending on the mussels' nutritive status (Moore et al., 2004; 2007; Izagirre et al., 2008; 2009; González-Fernández et al., 2015). However, little is known about how keeping mussels fed or fasted during experimentation can influence biomarker values and biomarker responsiveness to pollutant exposure. In pioneering studies, nutritional deprivation-induced autophagy was shown to exert a protective effect on lysosomal stability in copper- and phenanthrene-exposed mussels (Moore et al., 2004; 2007). More recently the nutritive status was shown to dramatically affect mRNA expression of biomarkers in mussels, as well as the total RNA content (González-Fernández et al., 2017). Thus, undernourished mussels showed lower RNA content and lower expression of mRNA biomarkers and housekeeping genes, reflecting suppression of protein turnover, together with increased catabolism, negative energy balance and enhanced autophagy (González-Fernández et al., 2017). In parallel, elevated filtration and oxygen consumption rates occurred in undernourished mussels accompanied by an increase of antioxidant enzymes activity (González-Fernández et al., 2015). As a result, the mussels' nutritive status might obviously mask biomarker responsiveness to pollutant insult (González-Fernández et al., 2017).

Alas, the regular practice can be very diverse and mussels are either fed microalgae during mid and long-term experimentation (Pipe and Moore, 1985; Okay et al., 2006; Ricciardi et al., 2008; Dimitriadis et al., 2012; González-Fernández et al., 2015) or fasted, especially in experiments shorter than 1 wk (Guerra-Rivas et al., 2002; Attig et al., 2010; Woo et al., 2011; Barmo et al., 2012). These disparate experimental designs render hardly comparable results on mussel biomarkers. Within this context, the present study aims to research the influence of fasting on the responsiveness of biomarkers in mussels exposed to petrochemicals, contributing to develop Best Available Practices (BAPs) to maintain mussels during short-term experimentation in aquatic toxicology. For this purpose, fasted mussel and mussels fed *Isochrysis galbana* were exposed to heavy fuel oil #6 WAF for 96 hr, and PAH tissue levels in digestive gland and a battery of biomarkers were investigated. Biomarkers included some of those commonly employed for biological effect assessment in marine pollution monitoring, say: enhancement of glutathione-S-transferase (GST) and cytochrome-C-oxidase (COX) enzyme activities, augmented lipid peroxidation (LPO), lysosomal enlargement (measured in terms of Vv_{LYS}, Sv_{LYS}, S/V_{LYS} and Nv_{LYS}) and membrane destabilisation (LP), lipofuscin accumulation (Vv_{LPF}), and histopathological alterations in the digestive gland epithelium

(Vv_{BAS}, MLR/MET, CTD ratio). Further on, the integrated biological response (IBR/n) index was calculated.

COX is a key enzyme in aerobic metabolism that correlates well with actual oxygen consumption rates for different tissues (Simon and Robin, 1971). Significant increases in COX enzyme activity (aerobic respiration) have been reported following exposure to hydrocarbons together with reduced anaerobic respiration (Ravindran, 1988). GST induction has been related to PAH exposure in several bivalve species (Cheung et al., 2004; Silva et al., 2005; Bebianno et al., 2007; Lima et al., 2007; Solé et al., 2007; Luchmann et al., 2011). GST enhancement would reveal an activation of phase II biotransformation pathway to defend against organic chemicals whilst inhibition would be a non-specific response to other environmental contaminants; moreover, activation of GST enzyme activity has been suggested to be transient leading to a biphasic, or bell-shaped, response in mussels (Regoli et al., 2004; Bebianno et al., 2007; Bocchetti et al., 2008; Moschino and Da Ros, 2016). When antioxidant defences are impaired, oxidative stress may produce LPO in cell constituents (Halliwell and Gutteridge, 1989). LPO is considered a major mechanism by which oxyradicals can cause impaired cellular function (Livingstone, 2001). Hydrocarbon exposure has been linked to increase LPO in bivalves (Lima et al., 2007; Bebianno and Barreira, 2009; Lüchmann et al., 2011; Verlecar et al., 2012). Lysosomal enlargement and membrane stability in mussel digestive cells are well-known biomarkers used in marine pollution monitoring (UNEP/RAMOGE, 1999; Marigómez and Baybay-Villacorta, 2003; ICES, 2004, 2012). Lipofuscins are non-degradable pigments, deriving from lipid peroxidation, which may be accumulated in lysosomes as granules, increasing with the age (Terman and Brunk, 2004). Their accumulation in digestive cell lysosomes is well documented in response to pollutant exposure (Viarengo et al., 1990; Regoli, 1992). Atrophy of the digestive gland epithelium and loss of digestive gland histological integrity are also widely used tissue-level biomarkers indicative of deteriorated health condition in mussels (Marigómez et al., 2006; Garmendia et al., 2011). Experimental exposure to the WAF of crude and lubricant-oil induced a significant increase in Vv_{BAS} and MLR/MET in mussels (Cajaraville et al., 1990; 1992b). The effect was quantitatively similar for the different types of WAF investigated and to a large extent reversible within a few days (Cajaraville et al., 1990; Marigómez et al., 1990).

MATERIAL AND METHODS

Experimental design

Mussels (*Mytilus galloprovincialis*) of 3.5–4.5 cm shell length were purchased from a nearby farm in Ria do Boiro (Galicia, Spain) in June and were acclimatized in seawater at controlled laboratory conditions (20±1°C; 16:8 L:D cycle) for a period of 48 hr in absence of food supply. Natural seawater was filtered (5 μm), sterilized with UV light and filtered again (0.45 μm). Seawater was constantly aerated (dissolved oxygen 7.6-8.3 mg/L; pH 7.8-8; salinity 33‰; temperature 18°C) and changed every day. After acclimatization, mussels were distributed in 8 experimental groups (each made of 15 mussels in 9 L glass tanks) that included combinations of fed/fasted and WAF-exposed mussels, as detailed below.

Mussels were kept either fasted (in filtered seawater) or fed *I. galbana* microalgae (20000 cells/mL per tank per day. Both fasted and fed mussels were exposed for 96 hr to 4 doses of the WAF of heavy fuel oil #6 (a complex mixture of water soluble PAHs, mainly of light molecular weight; Solé et al., 2007): 0% (control), 6.25%, 12.5% and 25% WAF. WAF was prepared according to Cajaraville et al. (1992a). Briefly, WAF was prepared in a Mariotte bottle (5 L) by adding 400 g heavy fuel oil #6 on top of 4 L seawater and stirring (vortex <20%) during 24 hr at 20°C. The system was protected from light in order to minimize evaporation and degradation of fuel components. The experimental WAF doses were selected because they had been previously determined to be sublethal for mussels (Lima et al., 2008). Accordingly, no mussel died during experimentation.

After 96 hr, gills of 15 mussels and digestive gland of 5 mussels per sample were frozen in liquid N_2 and stored at -80°C until required for biochemical and histochemical analyses. Additionally, the digestive gland and mantle of 10 mussels per experimental group were fixed in 4% formaldehyde at 4°C (24 hr) for histological examination. Gonad histology was examined to provide supporting data of mussel general condition (Ortiz-Zarragoitia et al., 2011); thus, upon microscopic examination of mantle tissue sections all the individuals in all the treatments were found to be at a comparable gametogenic stage.

PAHs in the digestive gland

Reagents and materials: (a) perdeuterated IntenalStandardPAHMixture6(naphthalene-d8,byphenyl-d10,phenanthrene-d10, pyrene-d10, bez[a]anthracene-d12, benzo[a]pyrene-d12, benzo[ghi]perylene-d12, 200 µg each/mL, 1.1 mL in toluene) and PAHs of Norwegian Standard NS 9815 were purchased from Chiron AS (Trondheim, Norway); (b) methanol and n-hexane were purchased from Macron Fine Chemicals (HPLC grade, Poland); and (c) potassium hydroxide from Merck.

Pieces of 5 digestive glands per treatment were used to perform a semiquantitative analysis of the PAHs. The method described by Navarro et al. (2006) was applied with modifications due to the shortage of samples (only 0.07-0.21 g of frozen samples at -80°C were available per analysis). In order to avoid losses, wet samples were employed for homogenization, saponification and extraction. Homogenization and extraction steps were carried out using a Precellys coupled to a Cryolys (Bertin Instruments, Montignyle-Bretonneux, France). A Precellys lysing Kit of 7 mL tubes and 2.8 mm zirconium oxide beads were used for this purpose. The method applied was based in three steps. The first step lied on a homogenization and saponification procedure using cold methanol saturated with potassium hydroxide in the Precellys tissue homogenizer (6000 rpm, 2 times 60 s with intervals of 15 s). This homogenizer was equipped with a Cryolys that helped to control the temperature (ambient air at 5°C) since it was an exothermic reaction. In this first step we added the deuterated PAHs as surrogates. Once the homogenization tubes were cooled using an ice bath, we added hexane for the second step, the liquid-liquid extraction that was carried out once again in the Precellys. As a final step, the samples were centrifuged (18000 rpm, 20 min, 4°C) to collect the hexane phase and they were preconcentrated for the analysis

using nitrogen blow-down evaporation and redissolved in 200 µL n-hexane. The GC-MS analyses were performed using a GC7890A (Agilent Technologies, Avondale, PA, USA) equipped with a 5975C MS. The column set used for gas chromatography was a DB-5ms (30 m × 0.25 mm, 0.25 µm, Agilent Technologies) capillary column. The oven temperature program started at 100°C, held for 2 min, raised to 240 °C at 16 °C /min and a second ramp to 300 °C at 4 °C /min and held for 10 min. The carrier gas was helium (Messer, Tarragona, Spain). 2 µL of the sample were injected in splitless mode at 300 °C using a 7683 Agilent autosampler. The mass spectrometer detector worked in the selected ion-monitoring (SIM) mode, and temperatures of quadrupole and source were 150 °C and 230 °C, respectively. Chemstation software (Agilent Technologies) was used for chromatographic raw data acquisition and to integrate the areas of chromatographic peaks. Blank samples doped with deuterated PAH standards (surrogates) were processed together with samples to calculate the limits of detection (LODs) based on the standard deviation of the signal and the slope of the calibration curve.

Enzyme activities and lipid peroxidation

COX was determined in digestive gland. For this purpose, the method detailed by Ivanina et al. (2011) was adapted to microplate. Briefly, digestive gland was thoroughly homogenized in 25 mM potassium phosphate (pH 7.2) with 10 µg mL^{-1} phenylmethylsulfonyl fluoride (PMSF) and 2 µg mL^{-1} aprotinin using hand-held Kontes Duall tissue grinders (Fisher Scientific, Suwanee, GA, USA). Homogenates were sonicated 3×10 s (output 7, Sonic Dismembrator Model 100, Fisher Scientific, Suwanee, GA) to ensure complete release of the enzyme, with cooling on ice (1 min) between sonications. Homogenates were centrifuged for 10 min at 20000× g and 4°C to collect debris, and supernatants (enzyme extracts) were used to assay enzyme activity (stored at -80°C for less than 2 wk before activity assays). Enzyme extracts were thawed on ice and immediately analysed using standard spectrophotometric techniques as described elsewhere (Bergmeyer 1985; Sidell et al. 1987; Birch Machin and Turnbull 2001). COX activity was measured at 20°C using a UV–Vis spectrophotometer (VARIAN Cary 50 Bio, Cary NC, USA) at 550 nm (20 mM potassium phosphate (pH 7.0), 16 µM reduced cytochrome c (II), 0.45 mM n-dodecyl-b-d-maltoside and 2 µg mL-1 antimycin A). Assays were completed within 1 hr during which enzyme extracts were maintained on ice.

GST and LPO were determined in gills. For this purpose, gills were homogenized in 100 mM phosphate buffer (pH 7.4) and centrifuged at 10000x g for 20 min at 4°C. The post-mitochondrial supernatant was used to determine GST activity by measuring the formation of thioether at 340 nm, according to an adaptation of the Habig's method to microplate and using γ-bovine globulins as standard (Guilhermino et al., 1996). GST activity was expressed in nmol of substrate conjugated per min per mg of protein. Protein content was determined by the Bradford's method (Bradford, 1976) adapted to microplates, using γ-bovine globulins as standard (Guilhermino et al., 1996). Enzymatic assays were performed at 25°C. The remaining homogenate was used to determine the endogenous LPO by measuring thiobarbituric acid-reactive substances (TBARS) at 535 nm, based on the Ohkawa's method after Bird and Draper

(1984) adapted by Torres et al. (2002). LPO levels were expressed in nmol of TBARS per g of wet tissue.

Lysosomal biomarkers

Lysosomal membrane stability was evaluated in serial cryotome sections (10 μm thick; Leica CM 3000 cryotome) of digestive gland chucks after the cytochemical demonstration of hexosaminidase activity, according to a standardized procedure (UNEP/RAMOGE, 1999), based on the time of acid labialization (LP; min) required to produce the maximum staining intensity.

In order to quantify changes in lysosomal structure, cryotome sections (8 μm thick; Leica CM 3000 cryotome) of digestive gland chucks were stained for the histochemical demonstration of ß-glucuronidase activity according to Cajaraville et al. (1991). For each mussel digestive gland the following stereological parameters were determined (Lowe et al., 1981): lysosomal volume density ($Vv_{LYS}=V_{LYS}/V_C$), lysosomal surface density ($Sv_{LYS}=S_{LYS}/V_C$), lysosomal surface-to-volume ratio ($S/V_{LYS}=S_{LYS}/V_{LYS}$) and lysosomal numerical density ($Nv_{LYS}=N_{LYS}/V_C$); where V_{LYS}, S_{LYS} and N_{LYS} are the volume, surface and number of lysosomes, and V_c the volume of digestive cells.

Lipofuscin (LPF) accumulation was determined in cryotome sections (8 μm thick; Leica CM 3000 cryotome) of digestive gland chucks after staining with Schmorl's reaction (Pearse, 1972). The extent of Schmorl positive materials (LPFs) in the digestive gland epithelium was measured by image analysis to calculate the volume density of lipofuscins with respect to the digestive epithelium volume (Vv_{LPF}; $\mu m^3/\mu m^3$).

Tissue-level biomarkers

Fixed digestive gland samples were dehydrated in graded ethanol series, and embedded in paraffin. Sections (5 μm thick) were cut in a rotary microtome (Leitz, 1512), and stained with hematoxylin-eosin. Slides were viewed at 40× magnification using a drawing tube attached to a light microscope. A Weibel graticule (multipurpose system M-168) was used, and hits of basophilic and digestive cells, luminal area and connective tissue were recorded. The volume density of basophilic cells ($Vv_{BAS;}$ in $\mu m^3/\mu m^3$) in digestive gland of mussels was determined according to Soto et al. (2002). The mean epithelial thickness of the digestive alveoli (MET; μm) was determined according to Lowe et al. (1981) together with other estimates of changes in alveolus morphology such as the mean luminal radius (MLR; μm) and the MLR-to-MET ratio (MLR/MET; μm/μm) (Vega et al., 1989). Likewise, the integrity of the digestive gland tissue was simultaneously determined as the extent of the interstitial connective tissue relative to the space occupied by digestive diverticula (connective-to-diverticula (CTD) ratio) (Brooks et al., 2011; Garmendia et al., 2011) on the basis of the same stereological data set.

Integrative Biological Response (IBR/n) index

IBR index was based on the integration of five biological responses from metabolic to tissue levels (COX, GST, LPO, LP and Vv_{BAS}) according to Beliaeff and Burgeot (2002), Broeg and Lehtonen (2006), and Marigómez et al. (2013). Calculations were based on a multivariate graphic method, according to the following procedure: (1) calculation of the mean and standard deviation for each

sample; (2) standardization of data for each sample: $x_i' = (x_i - x)/s$; where, x_i' = standardised value of the biomarker; x_i = mean value of a biomarker from each sample; x = general mean value of x_i calculated from all compared samples (data set); s = standard deviation of x_i calculated from all samples; (3) addition of the standardised value obtained for each sample to the absolute standardised value of the minimum value in the data set: $y_i = x_i'+|x_{min}'|$; (4) calculation of the radar plot triangular areas as $A_i = (0.59 \times (y_i \times y_{i+1})/2$, where "$y_i$" and "$y_{i+1}$" are the standardised values of each biomarker and its next one in the radar plot, respectively, and 0.59 is sin α (α: radial angle for a pentagonal radar plot; α=2π/5); and (5) calculation of the IBR index which is the summing-up of all the radar plot triangular areas ($IBR=\sum A_i$) (Beliaeff and Burgeot, 2002). Since the IBR value is directly dependent on the number of biomarkers in the data set, the obtained IBR value was divided by the number of biomarkers used to calculate IBR/n (Broeg and Lehtonen, 2006).

Statistical analysis

IBM® SPSS® Statistics ver. 22.0.0.0 (IBM Corp., Armonk, NY, USA) was employed for the statistical analyses. Homogeneity of variance (Levene's test) and normality of data (Kolmogorov-Smirnov's) were tested (Sokal and Rohlf, 1995). Two-way ANOVAs were made in order to determine the effect of effect of fasting, WAF exposure and their combination (Fasting × WAF) on biomarkers. For the case of non-parametric biomarkers (AChE, GST, Vv_{LYS}, S/V_{LYS}, Nv_{LYS} and CTD ratio) logarithmic transformation was applied. Further on, one-way ANOVA followed by Duncan's post-hoc test was applied to determine the effect of WAF exposure concentration in the case of parametric variables (COX, LPO, LP, Vv_{LPF}, Vv_{BAS} and MLR/MET) both for fasted and for fed mussels, whereas the Mann-Whitney's U-test was used for non-parametric biomarkers. For each WAF exposure concentration, differences between fasted and fed mussels were established according to the Student's t-test for parametric variables and to the Mann-Whitney's U-test for non-parametric variables. The Z-score test was applied to determine significant differences in the PAH tissue concentrations in the digestive gland, in the IBR/n index and for the cases of biological endpoints with small sample size for reliable Duncan's *and* Mann-Whitney's U-test (e.g., when samples were pooled or when some of a set were missing or difficult to measure), say: Vv_{LYS}, S/V_{LYS}, and Nv_{LYS}. A 95% significance level ($p<0.05$) was fixed for all statistical analyses carried out.

RESULTS

Effect of fasting/feeding on biomarker values

In control mussels, COX activity was lower in fasted than in fed ones (Fig. 1A). In contrast, GST activity was higher (Fig. 1B) and LPO and AChE values did not vary (Figs. 1C and 1D). The Student's t-test for LP values (Fig. 2A) and the Mann-Whytney U-test for Vv_{LYS}, S/V_{LYS} and Nv_{LYS} (Figs. 2B-2D) did not show significant differences between fasted and fed mussels (Fig. 2A). However, LP, Vv_{LYS} and Nv_{LYS} were seemingly higher and S/V_{LYS} lower in fasted mussels than in fed ones on the basis of the Z-score test. Lipofuscins were apparently less abundant after direct microscopic examination (Fig. 3) and hence the Vv_{LPF} values were lower (Fig. 2E) in the digestive gland epithelium of

fasted mussels than in fed mussels. According to the Student's t-test for Vv_{BAS} and MLR/MET values (Figs. 4A and 4B) and the Mann-Whitney U test for CTD ratio (Fig. 4C), fasted mussels presented lower Vv_{BAS} and higher MLR/MET and CTD ratio than fed mussels.

Effect of fasting/feeding on PAH tissue levels

As a result of 96 hr WAF exposure, PAHs such as phenanthrene (Phe), anthracene (Ant), fluoranthene (Fla) and pyrene (Pyr) were recorded in the digestive gland of exposed mussels, both fasted and fed; with Pyr and to a lesser extent Phe as the individual PAHs found at the highest concentrations (Table 1). The accumulation of these PAHs in the digestive gland of fed mussels was higher than in fasted mussels and showed a clear dose-dependent trend (Table 1). Conversely, it seemed that in fasted mussels the highest accumulation, especially for Phe, Fla and Pyr, occurred at the 6.25%WAF exposure group (Table 1).

Effect of fasting/feeding on biomarker responsiveness to WAF exposure

According to the two-way ANOVA performed, WAF exposure (W) was the only factor significantly affecting COX activity in digestive gland tissue (Table 2). In both fasted and fed mussels, COX showed a decreasing trend at increasing WAF exposure levels (Fig. 1A). However, a significant dose-dependent decrease was only found in fed mussels, in which the lowest COX activity was found in mussels exposed to 25% WAF (Fig. 1A; Table 3). Moreover, comparisons between pairs based on the Z-score test revealed that the COX activity was lower in fasted mussels exposed to 25% WAF than in the other groups of fasted mussels, and not dissimilar from the values recorded in fed mussels exposed to 25%WAF.

According to the two-way ANOVA performed, both fasting (F) and W, as well as their interaction (F×W) significantly contributed to variance in GST activity in gills (Table 2). In fasted mussels, GST

Table 1. PAH concentration (ng/g wet-wt) in the digestive gland of fasted and fed mussels *Mytilus galloprovincialis* exposed for 96 hr to different dilutions (0% (control), 6.25%, 12.5% and 25%) of heavy fuel #6 WAF. LOD, Limit of detection; n.d.a., no data available; % RSD, relative standard deviation

		C (ng/g wet-wt)			
		Phenanthrene	**Anthracene**	**Fluoranthene**	**Pyrene**
Fasted	Control	< LOD[a]	< LOD[a]	n.d.a	n.d.a
	6.25 % WAF	256[b]	< LOD[a]	111	300
	12.5 % WAF	100[c]	27[a]	64	259
	25 % WAF	129[c]	44[b]	38	186
Fed	Control	< LOD[a]	< LOD[a]	<LOD[a]	<LOD[a]
	6.25 % WAF	65[b]	< LOD[a]	18[a]	<LOD[a]
	12.5 % WAF	86[b]	19[a]	27[a]	109[a]
	25 % WAF	158[c]	49[b]	99[b]	526[b]
	LOD	*5*	*7*	*9*	*49*
	% RSD	*6*	*7*	*7*	*7*

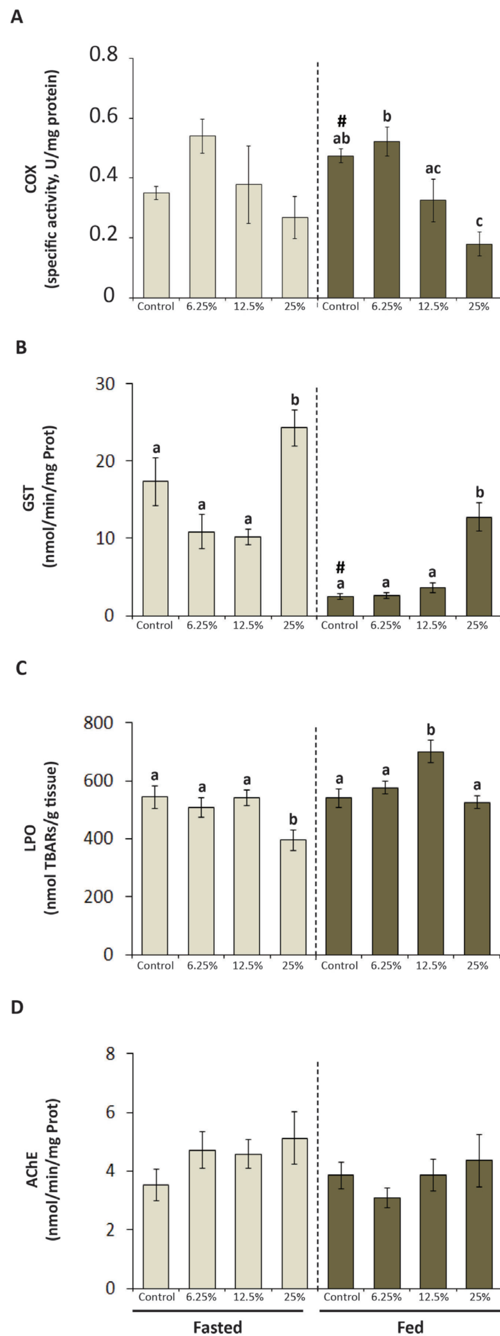

Fig. 1. Biochemical biomarkers in fasted and fed *Mytilus galloprovincialis* exposed for 96 hr to different dilutions (control, 6.25%, 12.5% and 25%) of heavy fuel #6 WAF. (A) Cytochrome c oxidase (COX) activity in digestive gland; (B) glutathione-S-transferase (GST) activity in gills; (C) lipid peroxides (LPO) in gills; and (D) acetyl cholinesterase (AChE). Intervals indicate standard error. Letters indicate significant differences among WAF exposure groups for fasted and fed mussels according to the post-hoc Duncan's test performed after a one-way ANOVA (COX, LPO) or the post-hoc Mann-Whitney's U-test after the Kruskal-Wallis' test (GST, AChE). Hashes indicate significant differences between fasted and fed control mussels according to the Student's t-test for COX or the Mann-Whitney's U-test for GST ($p < 0.05$).

activity tended to decrease after 6.25% and 12.5% WAF exposure but rose markedly in 25% WAF treated mussels (Fig. 1B; Table 3). In fed mussels, GST values were overall lower than in fasted mussels in all the experimental groups (Fig. 1B; Table 3). Nevertheless, GST showed a similar dose-dependent profile with a dramatic rose in GST activity after 25% WAF exposure (Fig. 1B).

Two-way ANOVA indicated that both F and W were factors exerting a significant effect on LPO in gills (Table 2). In fasted mussels, LPO values decreased after 25% WAF exposure (Fig. 1C; Table 3). In fed mussels, LPO values rose in 12.5%WAF exposed mussels and then returned to control values in 25% WAF exposed ones (Fig 1C; Table 3).

Two-way ANOVA revealed that AChE activity in foot was not significantly affected by F, W nor their interaction (Table 2). In agreement, no significant differences between experimental groups were found within fasted and fed mussels (Fig. 1D; Table 3).

According to the two-way ANOVA performed, W was the only factor significantly affecting LP in digestive cell lysosomes (Table 2). Both fasted and fed mussels showed the same decreasing trend in LP at increasing WAF exposure levels (Fig. 2A). The dose-response decrease in LP was significant for fasted mussels (Fig. 2A; Table 3); meanwhile, only the LP after exposure to 25%WAF was dissimilar for fed mussels (Z- score test).

Two-way ANOVA indicated that both F and the F×W interaction were the factors exerting a significant effect on Vv_{LYS} and Nv_{LYS} and that S/V_{LYS} was not affected by any of the factors analysed (Table 2). In agreement, fasted mussels did not show changes in Vv_{LYS}

Table 2. Summary of two-way ANOVAs performed to analyse the effect of fasting (d.f.: 1), WAF exposure (d.f.; 3) and their combination (Food × WAF; d.f.: 3) type on biomarkers recorded in mussels *Mytilus galloprovincialis* exposed for 96 hr to different dilutions (0% (control), 6.25%, 12.5% and 25%) of heavy fuel #6 WAF. d.f.: degrees of freedom; F: Fisher's F; (1) Logarithmic transformation of the variables (non-parametric); (*): p<0.1; *: p<0.05; **: p<0.01; ***: p<0.001

Parameter	F-ratio (Fasting)	F-ratio (WAF)	F-ratio (F ×W)	Residual d.f.
COX	0.026	5.836**	0.677	26
GST(1)	88.615***	19.511***	3.997**	102
LPO	15.501***	8.469***	2.608	104
AChE(1)	3.623	0.443	1.289	106
LP	0.709	8.690**	2.553	30
Vv_{LYS}(1)	5.322*	3.153	8.104**	14
S/V_{LYS}(1)	0.097	2.479	0.714	14
Nv_{LYS}(1)	5.680*	3.017	6.683**	14
Vv_{LPF}(1)	33.435***	0.920	0.492	24
Vv_{BAS}	14.540***	21.09	2.683	40
MLR/MET	8.023**	2.636	0.923	40
CTD-ratio(1)	<0.0001	0.187	1.824	40

and Nv_{LYS} depending on the WAF exposure levels (Figs. 2B and 2D; Table 3) whilst fed mussels exhibited a remarkable increase in these parameters on exposure to 12.5% and 25% WAF (Figs. 2B and 2D; Table 3). These changes were easily observed after direct microscopic examination, with apparently less conspicuous lysosomes in fasted mussels exposed to 12.5% and 25%WAF and abundant large lysosomes in fed mussels subjected to the same treatments (Fig. 3).

According to the two-way ANOVA performed, F was the only factor that significantly contributed to variance in Vv_{LPF}

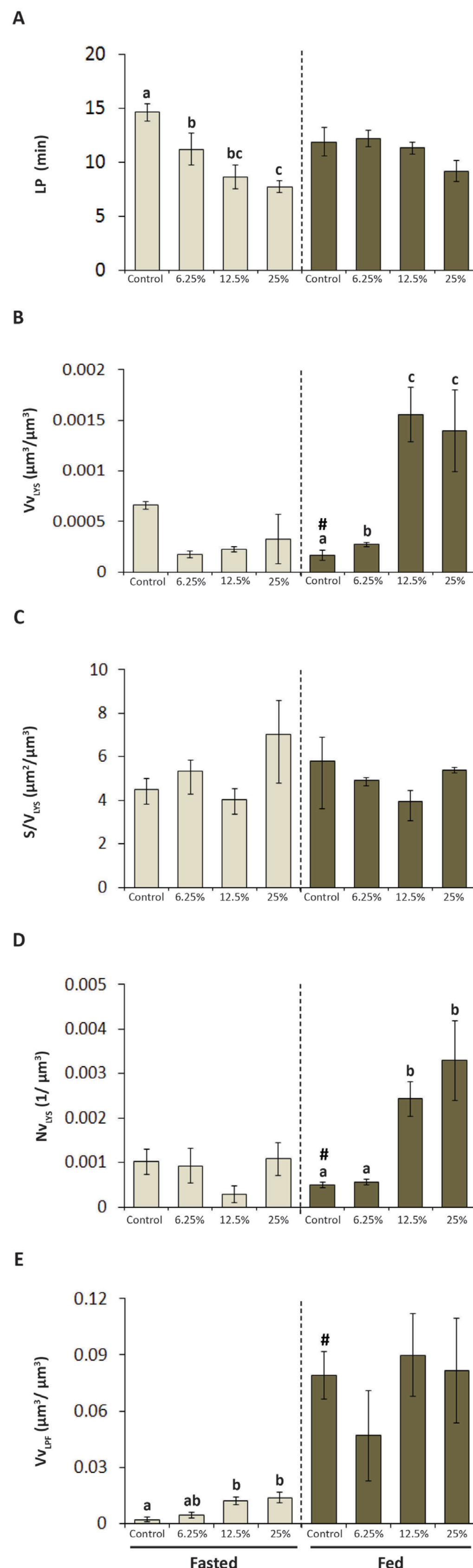

Fig. 2. Lysosomal membrane stability, lysosomal structural changes and lipofuscin content in digestive gland of fasted and fed mussels *Mytilus galloprovincialis* exposed for 96 hr to different dilutions (0%, 6.25%, 12.5% and 25%) of heavy fuel #6 WAF. (A) Lysosomal membrane labilisation period (LP); (B) lysosomal volume density (Vv_{LYS}); (C) lysosomal surface-to-volume ratio (S/V_{LYS}); (D) lysosomal numerical density (Nv_{LYS}); and (E) volume density of lipofuscins (Vv_{LPF}). Intervals indicate standard error. Letters indicate significant differences among WAF exposure groups for fasted and fed mussels according to the post-hoc Duncan's test performed after a one-way ANOVA for LP, the post-hoc Mann-Whitney's U-test after the Kruskal-Wallis' test for Vv_{LPF}, and the Z-score test for Vv_{LYS}, S/V_{LYS} and Nv_{LYS}. Hash indicates significant differences between fasted and fed control mussels according to the Mann-Whitney's U-test for VvLPF. Statistical differences were always established at p<0.05.

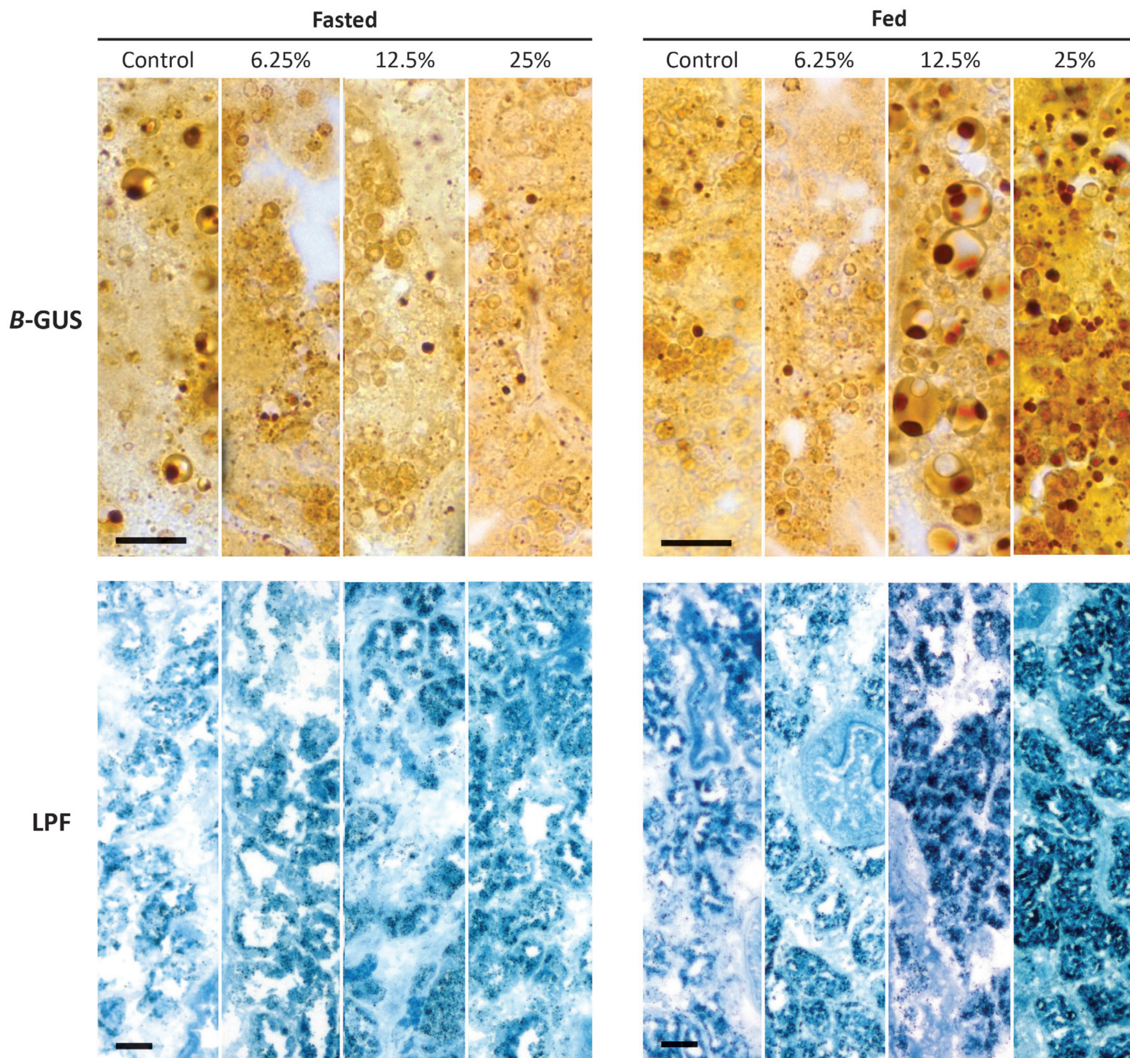

Fig. 3. Micrographs of cryotome sections of mussel digestive gland after cytochemical demonstration of β-glucuronidase enzyme activity (β-GUS, scale bar: 10 µm) and after histochemical demonstration of lipofuscins (LPF, scale bar = 50 µm) in fasted and fed mussels *Mytilus galloprovincialis* exposed for 96 hr to different dilutions (0% (control), 6.25%, 12.5% and 25%) of heavy fuel #6 WAF.

in digestive gland epithelium (Table 2). Thus, Vv_{LPF} was much lower in fasted mussels than in fed ones for all the experimental treatments and increased with WAF exposure levels (Figs. 2E and 3; Table 3). In contrast, Vv_{LPF} were much higher in fed mussels and remained unchanged amongst experimental treatments (Figs. 2E and 3; Table 3).

All the Vv_{BAS} values were below 0.12 $\mu m^3/\mu m^3$ and those of MLR/MET below 1.2 µm/µm (Fig. 4A and 4B). According to the two-way ANOVA performed, F was the only factor significantly affecting Vv_{BAS} and MLR/MET, whilst no factor contributed to the variance in CTD ratio (Table 2). The highest Vv_{BAS} value was recorded in fed mussels exposed to 25%WAF (Fig. 4A; Table 3). A certain trend in MLR/MET to decrease with WAF exposure level was envisaged in fasted mussels but not in fed mussels (Fig. 4B; Table 3). In agreement with the two-way ANOVA results, no significant differences in CTD ratio were found amongst experimental groups for fasted and fed mussels (Fig. 4C; Table 3).

Table 3. Summary of one-way ANOVAs (parametric) and Krukal-Wallis tests (non-parametric) performed to compare WAF exposure groups (d.f.(between)=3) for fasted and fed mussels *Mytilus galloprovincialis* exposed for 96 hr to different dilutions (0% (control), 6.25%, 12.5% and 25%) of heavy fuel #6 WAF. d.f.: degrees of freedom; *F*: Fisher's F; X^2: chi-square; p: probability. * Significant differences between diets according to the Z-score test (p<0.05; see Figs.) *: p<0.05; **: p<0.01; ***: p<0.001.

Parameter	FASTED		FED	
	F-ratio/X^2	d.f.$_{(residual)}$/ N	*F-ratio/* X^2	d.f.$_{(residual)}$/ N
One-way ANOVA				
LPO	4.345**	51	7.088***	53
COX	1.820	14	7.404**	12
LP	8.691***	16	2.378	14
Vv_{BAS}	1.897	19	2.655(*)	19
MLR/MET	2.752(*)	19	0.402	19
Kruskal-Wallis				
GST	19.638***	53	32.212***	57
AChE	5.460	57	1.270	57
Vv_{LYS}*	4.644	10	8.127*	11
S/V_{LYS}	4.664	10	6.026	11
Nv_{LYS}*	3.682	10	7.818*	11
Vv_{LPF}	10.376*	16	2.625	16
CTD-ratio	2.003	23	2.902	23

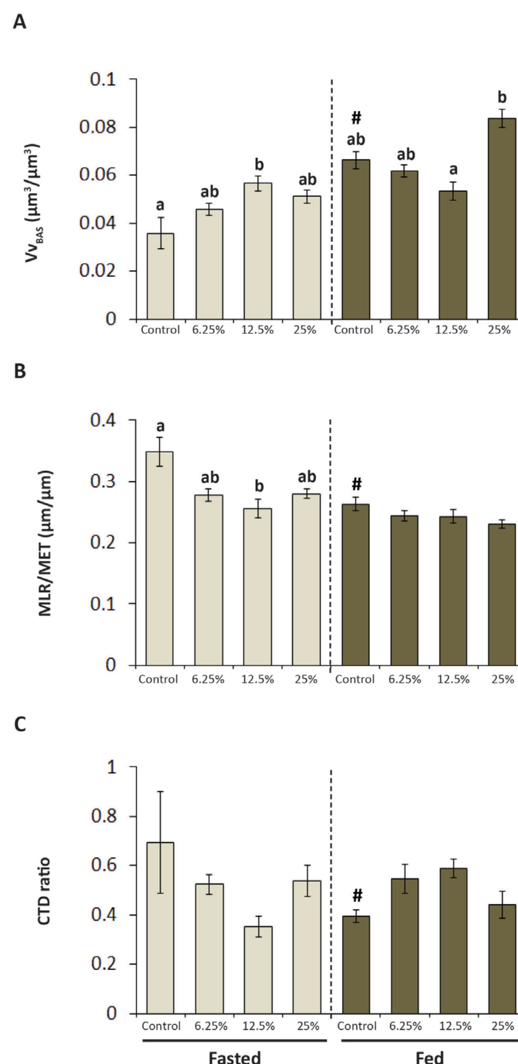

Fig. 4. Tissue-level biomarkers recorded in digestive gland of fasted and fed mussels *Mytilus galloprovincialis* exposed for 96 hr to different dilutions (0%, 6.25%, 12.5% and 25%) of heavy fuel #6 WAF. (A) Volume density of basophilic cells (Vv_{BAS}); (B) mean luminal radius to mean epithelial thickness ratio (MLR/MET); and (C) connective-tissue-to-diverticula ratio (CTD ratio). Intervals indicate standard error. Letters indicate significant differences among WAF exposure groups for fasted and fed mussels according to the post-hoc Duncan's test performed after a one-way ANOVA for VvBAS and MLR/MET or the post-hoc Mann-Whitney's U-test after the Kruskal-Wallis' test for CTD ratio. Hashes indicates significant differences between fasted and fed control mussels according to the Student's t-test for Vv_{BAS} and MLR/MET and the Mann-Whitney's U-test for CTD ratio. Statistical differences were always established at p<0.05.

IBR/n increased at increasing WAF exposure levels both in fasted and fed mussels; however, the response was more marked in the latter (Fig. 5; Z-score test). Radar plots revealed that the response profiles were not very dissimilar between fasted and fed mussels in qualitative terms but the response was quantitatively more relevant in fed mussels treated with 25% WAF (Fig. 5).

DISCUSSION

Effects of fasting/feeding on biomarker values

In contrast with feeding (living microalgae) during short-term experimentation (e.g., 96 hr), fasting in mussels led to reduced COX, Vv_{LPF} and Vv_{BAS} values, augmented GST, Vv_{LYS}, Nv_{LYS}, MLR/MET and CTD ratio values, and unchanged or highly variable LPO, AChE and LP values. Consequently, we have obtained unquestionable

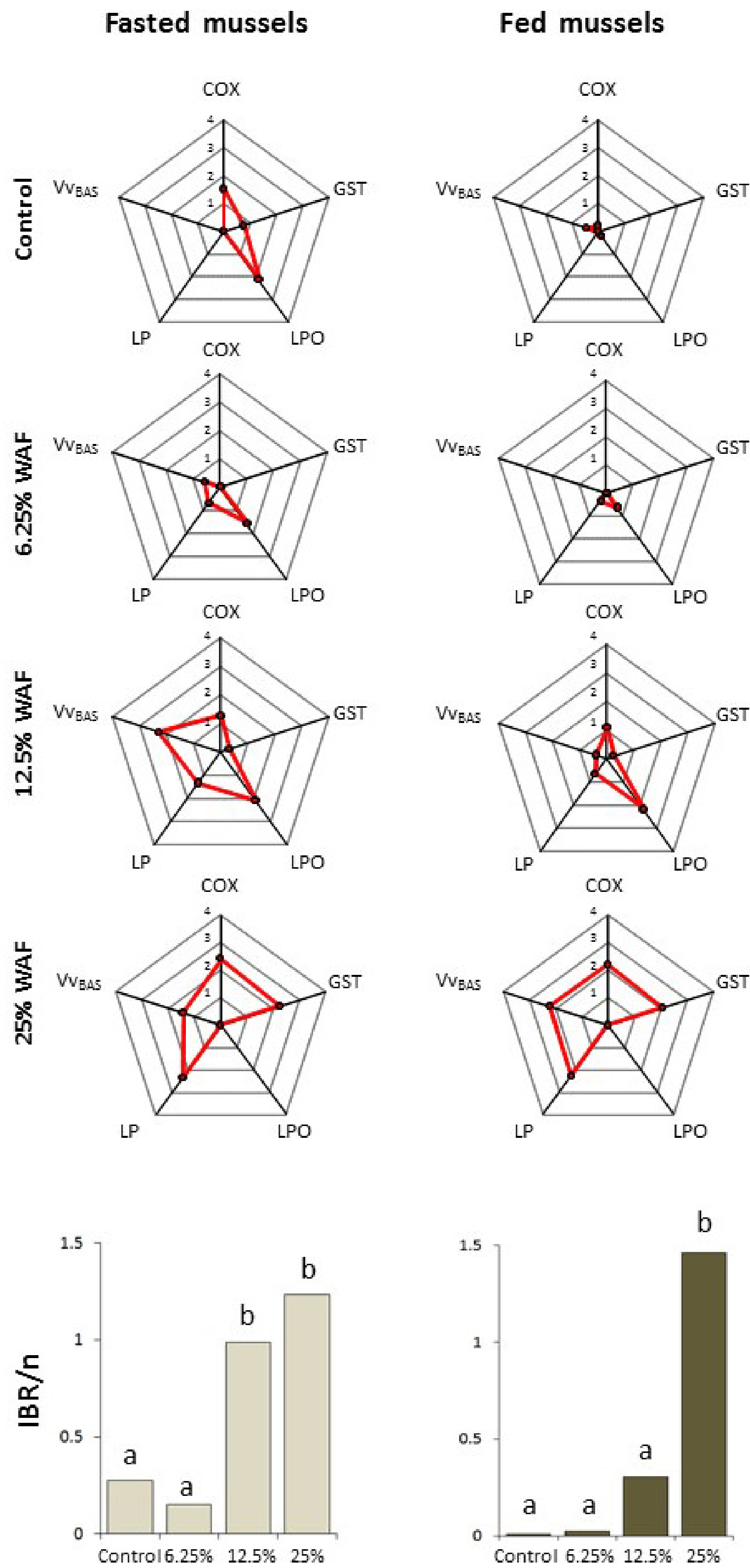

Fig. 5. Radar plots for five selected biomarkers (COX, GST, LPO, LP and Vv_{BAS}) and the corresponding IBR/n index for fasted and fed mussels *Mytilus galloprovincialis* exposed for 96 hr to different dilutions (Control, 6.25%, 12.5% and 25%) of heavy fuel #6 WAF. For IBR/n index, different letters denote statistically significant differences between WAF exposure groups for fasted and fed mussels, according to the Z-score test ($p<0.05$).

experimental evidence about how fasting during experimentation significantly affects the values of endpoints commonly used for biological effects assessment.

The observed differences can be directly attributed to feeding and digestion processes as well as to the adaptive and compensatory responses against food deprivation. In mussels, active metabolism associated with feeding includes higher ventilation rate, filtration and oxygen consumption, and concomitant stimulation of the aerobic metabolism (Thomson and Bayne, 1972; Ivanina et al., 2011). Likewise, the presence of food in the gut also increases the oxygen requirement (Thomson and Bayne, 1972). Thus, the present low COX values in fasted control mussels may be related to the decline of the aerobic metabolism in absence of food for 1-2 wk, as mussels go through a resting state. In agreement, starvation in marine crustaceans was reported to result in longer resting times associated with decreases in daily oxygen consumption (Ansell et al., 1973).

Presently, enhancement in GST activity was recorded in gills of mussels fasted for 1-2 wk (1 wk acclimatization + 96 hr experimentation). In agreement, enhanced GST activity has been reported in mussels subject to caloric restriction (González-Fernández et al., 2015), as well as in the digestive gland of Antarctic limpets after 1-2 wk food deprivation (Ansaldo et al., 2007). Likewise, GST and other antioxidant enzyme activities rose at the short-term in undernourished fish (Pascual et al., 2003; Morales et al., 2004). Overall, GST seems to augment as a result of the extra demand for antioxidant defences resulting from the enhancement of aerobic metabolism that may occur as an early response to fasting (Morales et al., 2004). Food deprivation also stimulates degradation of endogenous proteins, glycogen and lipids together with enhanced autophagy, which causes additional ROS generation and the need for activation of antioxidant enzymes (Moore, 2004; Ansaldo et al., 2007; Moore et al., 2007).

Antioxidant activities are often inversely related to LPO (Viarengo et al., 1991); however, LPO levels are known to be highly variable in mussels depending on time-curse changes in their antioxidant capacity (Richardson et al., 2008). Thus, for instance, in Antarctic limpets LPO levels rose only transiently at early stages of experimental food deprivation (Ansaldo et al., 2007). Transient changes have been also reported in undernourished gilt-head sea bream, *Sparus aurata*, in which hepatic LPO raised transiently in the mid-term (Pascual et al., 2003). As a result, we did not observe significant differences between fasted and fed mussels under the present experimental conditions; nevertheless differences at longer experimental periods cannot be disregarded.

LP values recorded in control mussels were comparable to values recorded in Galician and Basque Coasts in healthy mussels (Marigómez et al., 2006; Garmendia et al., 2011). As aforementioned, fasting results in enhanced autophagy, which may result in changes in the lysosomal size and numbers in mussel digestive cells (Moore et al., 2007). Thus, higher Vv_{LYS}, and Nv_{LYS} values were recorded in fasted mussels than in fed ones.

Augmented levels of lipofuscins in mussel digestive gland can be related to oxidative damage (Viarengo et al., 1991); however, the high Vv_{LPF} values recorded in fed mussels

in comparison with fasted mussels seem be attributed to a large extent to the presence of pigments of the microalgae that are being digested in digestive cell lysosomes (Chapter 5). Indeed, lipofuscin accumulation resulting from oxidative damage would be associated with augmented LPO levels (Regoli and Guiliani, 2014), and these remain similar in both fasted and fed mussels.

In summary, biomarker values can vary dramatically depending on whether mussels are fasted of fed during short-term experimentation. This can be interpreted as the consequence of alterations in feeding and digestion, enhanced autophagy and ROS production and the antioxidant responses associated to feeding, digestion and autophagy; as well as to the presence of microalgae (food reach in pigments) in the gut and the digestive gland. Thus, the results of experimental studies in which mussels either fed microalgae (common practice in mid and long-term experimentation but also in the short-term) or fasted (often used for short and mid-term experimentation) seem to be hardly comparable, which can be even more critical regarding biomarker responsiveness, (below discussed) than the biomarker values themselves, as herein concluded.

Effects of fasting/feeding on PAH tissue levels

Bivalves accumulate PAHs directly from the water-phase and food, especially the more-soluble PAHs than the less-soluble and heavier PAHs (Neff, 2002). Presently, both fasted and fed mussels accumulated PAHs in their tissues after 96 hr WAF exposure. Only small amount of tissues were used for the analyses because the frozen samples for chemical analyses had been incidentally lost. Remainders of cryo-processed digestive gland samples were used and a specific separation and analytical procedure was developed for this purpose. Due to analytical procedural constrains many individual PAHs could not be satisfactorily identified/quantified. It is well known that the saponification step brings negative consequences in the recuperation of the heavier PAHs but it was necessary to avoid the matrix effect caused by lipids (Navarro et al., 2006). However, Phe, Ant, Fla and Pyr were successfully recorded in the digestive gland tissue of exposed mussels thus demonstrating an effective exposure to the WAF under the present experimental conditions.

Mussels are known to have a poor ability to metabolize PAHs and to accumulate them in tissues, especially those of 3-rings (e.g., Phe and Ant) and 4-rings (e.g., Fla and Pyr) (Baumard et al., 1998, Gunther et al., 1999; Orbea et al., 2002); which is consistent with the present findings. However, the concentrations of these PAHs in digestive gland were lower in fasted mussels than in fed ones. Moreover, whereas a dose-dependent accumulation trend was observed in fed mussels, in fasted ones there was a peak of accumulation for Phe, Fla and Pyr at the lowest dose (6.25% WAF) and the tissue concentrations of these PAHs remained unchanged or lowered at highest WAF exposure levels. Gonzalez-Fernandez et al. (2016) also found differences in the tissue concentrations of Fla recorded in mussels of diverse dietary conditioning that had been exposed under experimental laboratory conditions to identical levels of the PAHs. These authors argued that the different incorporation of Fla to dietary microalgae might have depended either on the microalgae lipid contents or on differences

in the assimilation efficiency inherent to each dietary condition. Thus, it is likely that presently the uptake of PAHs was mediated by their incorporation into lipophilic compounds of the microalgae supplied as food (*I. galbana* has a high lipid content; Chapter 5), unlike in fasted mussels, in which feeding activity is presumably reduced in absence of food, as above discussed. In addition, it might be conceivable that feeding activity could be stimulated by low WAF exposure levels and inhibited by high ones. Feeding activity is stimulated by non-particulate or particulate (inert particles also elicit responses) food as the receptor system concerned is both mechanosensory and chemosensory (Thomson and Bayne, 1972). Thus, feeding enhancement in fasted mussels by chemical stimulation mediated by the presence of 6.5% WAF might have contributed to PAH accumulation whereas behavioural defence mechanisms (valve closure and prolonged resting state) most likely elicited by higher WAF exposure levels (12.5% and 25%WAF) would have counteracted PAH accumulation.

Effects of fasting/feeding on biomarker responsiveness to WAF exposure

No significant changes were recorded in foot AChE activity, S/V_{LYS} and CTD ratio in any case; however all the other biomarkers showed significant changes depending on the fasting/feeding condition, the exposure to WAF and/or their interaction. As a result, the integrated biomarker index IBR/n was higher at increasing WAF exposure levels both in fasted and fed mussels albeit the response was more marked in the latter. Overall, the response profiles were not very dissimilar between fasted and fed mussels in qualitative terms but responses were quantitatively more relevant in fed mussels, especially upon exposure to the highest dose (25% WAF).

COX activity in digestive gland tissue decreased on exposure to WAF both in fasted and fed mussels and though a significant dose-dependent response was only elicited in fed mussels similar COX values were recorded on exposure to 25% WAF irrespective of the fasting/feeding condition. GST activity was affected by fasting, WAF exposure and their interaction. Thus, although GST values were lower in fasted mussels than in fed ones in all the experimental treatments, a qualitatively similar dose-dependent profile was observed, with exposure to 25% WAF resulting in a remarkably high GST. GST induction has been associated to petroleum exposure in bivalves (Boutet et al., 2004; Pan et al., 2005; Silva et al., 2005; Lima et al., 2007; Solé et al., 2007; Banni et al., 2010; Liu et al., 2014). For instance, GST activity was significantly increased in digestive gland of oysters exposed to diesel WAF (Cheung et al., 2004; Lima et al., 2007; Luchmann et al., 2011) and positively correlated with the tissue concentration of individual PAHs in mussels (Gowland et al., 2002). Likewise, GST activity also rose in Antarctic limpet *Nacella concinna* exposed to diesel seawater contamination for 1 wk (Ansaldo et al., 2005). However, in mussel digestive gland GST activity may be promptly induced on exposure to PAHs but high enzyme activity levels may remain beyond cessation of the PAH exposure (Richardson et al., 2008). In *P. viridis*, GST activity was elevated immediately on exposure to diesel WAF at short exposure times and remained high only after exposure to the highest does of WAF (Verlecar et al., 2012). As such, the results are often not always fully consistent, which has been attributed to the intricate

response profile of GST and to the influence of not well-known confounding factors (Livingstone et al., 1995; Sole et al., 1996; Richardson et al., 2008). Thus, GST enzyme activity may be induced or inhibited in mussels from polluted areas (Bocchetti et al., 2008; Vidal-Liñán et al., 2014; González-Fernández et al., 2015). LPO changed in a different way, governed by both fasting and WAF exposure. Thus, while LPO values were lowest at the highest WAF exposure levels in fasted mussels, LPO did not drop in fed mussels and still raised in 12.5%WAF exposed mussels. A clearer LPO response to WAF exposure was observed in fed mussels than in fasted mussels. Hydrocarbon exposure has been linked to increase LPO in marine molluscs (Ansaldo et al., 2005; Lima et al., 2007; Bebianno and Barreira, 2009; Lüchmann et al., 2011; Sureda et al., 2011). For instance, in the digestive gland of mussels LPO increased following exposure to Phe (Moore, 1988) and to oil and diesel WAF (Richardson et al., 2008; Verlecar et al., 2012). However, LPO remained unaffected in mussels exposed to Fla (Gonzalez-Fernandez et al., 2016). It seems that LPO responses depend on whether the activation of antioxidant and phase II biotransformation enzymes is efficient or not to neutralize the oxidative damage elicited upon exposure to PAHs (Cheung et al., 2001; Fernández et al., 2010; Vidal-Liñán et al., 2010; Rodrigues et al., 2013).

LP decreased at increasing WAF exposure levels in both fasted and fed mussels but the dose-response relationship was more clearly elicited in fasted mussels. The decrease in LP values revealed a typical stress response in fasted and fed mussels exposed to the WAF (Izagirre and Marigómez, 2009), more markedly in the former. Indeed, LP in mussel digestive cells has been negatively correlated with the tissue concentrations of PAHs in the whole soft body (Zorita et al., 2007). Fasting and the F×W interaction exerted a significant effect on Vv_{LYS} and Nv_{LYS}; thus whilst these lysosomal parameters did not show changes with WAF exposure in fasted mussels, a remarkable increase was elicited on exposure to the highest doses (12.5% and 25% WAF). Vv_{LPF} was much lower in fasted mussels than in fed ones for all the experimental treatments; nevertheless, it increased with WAF exposure levels in fasted mussels whereas remained unchanged in fed mussels. All the Vv_{BAS} values were below 0.12 $\mu m^3/\mu m^3$ and those of MLR/MET below 1.2 µm/µm, and therefore they were not indicative of any apparent distress signal at histological level (Marigómez et al., 2006; Garmendia et al., 2010; ICES, 2012). Previous studies have been reported that Vv_{BAS} and MLR/MET increased in mussels exposed to the WAFs of several crude oils and of lubricant oil for 3-5 wk (Cajaraville et al., 1990; 1992b). However, in the present study only the highest Vv_{BAS} value recorded in fed mussels exposed to 25%WAF was close to the critical value of 0.12 $\mu m^3/\mu m^3$, indicative of general stress (Marigómez et al., 2006; ICES, 2012).

Consequently, not only biomarker values but also biomarker responsiveness can vary dramatically depending on whether mussels are fasted of fed during short-term experimentation. The differences in biomarker responsiveness can be explained by the influence of the differences in nutritional condition, antioxidant capacity and digestion activity between fasted and fed mussels, but also by the different levels of exposure reflected in the dissimilar tissues concentrations of PAHs and in the difference in GST enzyme activity. Thus, the condition

of keeping mussels fasted or fed during short- and mid-term experimentation is crucial for a correct and comparable interpretation of the changes elicited by pollutants in aquatic toxicological experimentation with marine mussels.

Concluding remarks

Fasting in short-term experimentation influenced biomarker values and biomarker responsiveness against oil WAF exposure, especially for GST, LPO, COX, LP, Vv_{LYS}, Nv_{LYS} and LPF. Overall, biomarker responsiveness upon experimental WAF exposure was more clearly envisaged in fed than in fasted mussels. Consequently, it is recommended that mussels should be fed also during short-term toxicological experimentation, thus avoiding the influence of fasting on biomarkers as well as enabling comparability with long-term experiments (in which feeding is absolutely required). More research is needed to advance in the selection of the Best Available Practices regarding feeding regimes and food type for experimental mussels; meanwhile preliminary steps forward have been given (Chapter 5).

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CHAPTER 5

DIETARY FOOD TYPE INFLUENCE ON BIOMARKERS IN MUSSELS USED AS MODELS FOR AQUATIC TOXICOLOGICAL EXPERIMENTATION

This chapter has been presented at:

- 10th Iberian and 7th Iberoamerican Congress on Environmental Contamination and Toxicology (CICTA 2015) Congress, Vila Real (Portugal) 14-17/07/2015. Blanco-Rayón E, Ücker M, Garmendia L, Marigómez I, Izagirre U. The influence of different microalgae diets on cell and tissue level biomarkers in mussel digestive gland. Oral communication.

ABSTRACT

In order to assess the influence of food type on biomarkers, mussels (*Mytilus galloprovincialis*) were maintained under laboratory conditions and fed using 4 different microalgae diets *ad libitum* for 1 week: (a) *Isochrysis galbana*; (b) *Tetraselmis chuii*; (c) *I.galbana* and *T.chuii* microalgae mixture; and (d) commercial food (Microalgae Composed Diet®, Acuinuga). Biochemical biomarkers (pyruvate kinase, phosphoenolpyruvate carboxykinase and cytochrome c oxidase) were determined in gills and protein, lipid, free glucose and glycogen total contents, lysosomal membrane stability, lysosomal structural changes, intracellular accumulation of neutral lipids and lipofuscins, and cell type composition and structural changes of digestive alveoli were determined in digestive gland of mussels. Gonad index was measured as supporter parameter. Moreover, digestive gland samples were also collected at 0 min, 5 min, 2 hr and 5 days after feeding mussels with alive microalgae diets, for digestive gland autofluorescences signal examination and lipofuscin determination. It was determined that *I. galbana* enter into digestive cells to be digested intracellulary and *T. chuii* is digested extracellularly. In general, the present results demonstrate that biomarkers are modulated by the type of diet employed. Overall, it is concluded that alive microalgae is recommended to maintain mussels during laboratory experiments. Further research is needed to optimize dietary food type, composition, regime and rations for toxicological experimentation.

Elikadura-mota dietetikoaren eragina biomarkatzaileetan esperimentazio toxikologiko akuatikoaren eredu gisa erabiltzen diren muskuiluetan

Laburpena

Janari motaren eraginak biomarkatzaileetan aztertzeko helburuarekin, muskuiluak (*Mytilus galloprovincialis*) laborategiko baldintzetan mantendu ziren eta mikroalgetan oinarritutako 4 dieta desberdin erabiliz *ad libitum* elikatu ziren aste batean zehar: (a) *Isochrysis galbana*; (b) *Tetraselmis chuii*; (c) *I.galbana* eta *T.chuii* mikroalgen nahazketa; and (d) janari komertziala (Microalgae Composed Diet®, Acuinuga). Biomarkatzaile biokimikoak (pirubato kinasa, fosfoenolpirubato karboxikinasa eta zitokromo c oxidasa) brankietan aztertu ziren eta, liseri-guruinan proteina, lipidoak, glukosa askea eta glukogeno totalaren edukiak, lisosomen mintzaren desegonkortasuna, lisosomen egituren aldaketak, lipido neutroen eta lipofuszinen metaketa, liseri-guruineko epitelioaren zelulen konposaketa eta liseri-albeoloen aldaketa morfologikoak aztertu ziren. Indize gonadala parametro laguntzaile moduan erabili zen. Horrez gain, 0 min, 5 min, 2 ordu eta 5 egun zehar elikatutako mukuiluen liseri-guruinaren laginak eskuratu ziren, liseri-guruinaren autofluoreszientziaren seinaleen azterketa eta lipofuskinaren determinazioa burutzeko. Intrazelularki liseria izateko, *I. galbana* liseri-zeluletan sartzen dela eta *T. chuii* zelularen kanpoaldean liseritzen dela zehaztu zen. Oro har, ikerketa honen emaitzek frogatzen dute erabiltzen den dietak biomarkatzaileak modulatzen dituela. Orokorki, laborategiko esperimentuetan muskuiluak mantentzeko mikroalgak biziak erabiltzea gomendatzen da. Toxikoloagiako esperimentuetan janari mota, konposizioa, erregimena eta errazioa optimizatzeko ikerketa gehiago beharrezkoa da.

Influencia del tipo de alimento dietético en biomarcadores en mejillones utilizados como modelos para la experimentación toxicológica acuática

Resumen

Con el fin de evaluar la influencia del tipo de alimento en los biomarcadores, los mejillones (*Mytilus galloprovincialis*) se mantuvieron en condiciones de laboratorio y se alimentaron con 4 diferentes dietas de microalgas *ad libitum* durante 1 semana: (a) *Isochrysis galbana*; (b) *Tetraselmis chuii*; (c) mezcla de las microalgas *I.galbana* y *T.chuii*; y (d) comida comercial (Microalgae Composed Diet®, Acuinuga). Se determinaron biomarcadores bioquímicos (piruvato quinasa, fosfoenolpiruvato carboxinquinasa y citocromo c oxidasa) en branquias; y proteínas, lípidos, glucosa libre y contenido total de glucógeno, estabilidad de la membrana lisosómica, cambios estructurales lisosómicos, acumulación intracelular de lípidos neutros y lipofustinas; y composición del tipo celular y cambios estructurales de alvéolos digestivos fueron determinados en la glándula digestiva de los mejillones. El índice gonadal se midió como parámetro de apoyo. Además, muestras de las glándulas digestivas también se recogieron a los 0 min, 5 min, 2 hr y 5 días después de comenzar a alimentar a los mejillones con dietas de microalgas vivas, para el examen de la señal de autofluorescencia de las glándulas digestivas y la determinación de lipofuscinas. Se determinó que *I. galbana* ingresa en las células digestivas para ser digerida intracelularmente y *T. chuii* es digerida extracelularmente. En general, los presentes resultados demuestran que los biomarcadores son afectados por el tipo de dieta empleada. En conclusión, las microalgas vivas son recomendadas para mantener los mejillones durante los experimentos de laboratorio. Se necesita más investigación para optimizar el tipo de alimento, la composición, el régimen y las raciones para la experimentación toxicológica.

INTRODUCTION

Mussels are widely used sentinel organisms in pollution monitoring programs to assess the biological effects of pollutants. Great efforts are addressed to produce consensus standardized procedures for biomarker determinations (UNEP/RAMOGE, 1999; ICES, 2012). Likewise, most recently attention has been paid to develop Best Available Practices approaches for mussel sampling and processing in field studies and monitoring programs (Chapter 2; Izagirre et al., 2008; Chandurvelan et al., 2013; Vidal-Liñán and Bellas, 2013). Together with field studies, laboratory experiments are crucial to gain understanding of the biological effects of pollutants and to develop a reliable tool box of biomarkers for environmental monitoring and assessment. Temperature, photoperiod, salinity, water renewal, dosing, etc., are regularly reported as they are fully recognised key conditions to perform laboratory experiments correctly; moreover, these parameters are more or less fixed or at least categorized to a certain consensus. However, less attention has been paid to the food type and the feeding strategy. Although feeding progression and food type may modulate biomarkers responsiveness (Moore et al., 2007, Izagirre et al., 2009; González-Fernández et al., 2015), to our knowledge there is not guidelines dealing with recommended food types and feeding strategies to keep mussels during laboratory experiments. Thus, a large disparity of food types and feeding strategies are applied in aquatic toxicological experiments with mussels, which include absence of additional food supply or supply of diverse commercial food products or a variety of live microalgae either in monocultures or in mixtures; frequently, as a matter of fact, no mention is made to food type or feeding conditions. For example, in a non-exhaustive literature mining in which 75 classical and recent manuscripts were selected, the 16% of the papers did not even provide any indication of whether mussels were fed during experimentation, the 11% maintained mussels without additional food supply (particularly during short-term experiments), the 40% of the reports used live microalgae in monoculture (29%) or in mixtures (11%), and the 33% used commercial food of diverse origin (14 manufacturers), nature (lyophilized algae, flour, ...) and components (Table 2 of the general introduction). Moreover, the rations and regime of food availability (e.g., continuous flow vs. pulses) are also very different. If as herein hypothesized the food type and feeding strategy influence biomarkers, as well as their measurability and responsiveness; any comparison between these experiments would be barely consistent.

Therefore, the present investigation was aimed at determining the influence of food type on a battery of biomarkers frequently analysed in mussels (*Mytilus galloprovincialis*). As a preliminary step, the distribution and fate of the microalgae of different size and biochemical composition *Isochrysis galbana*, *Tetraselmis chuii* and their mixture were investigated in the midgut. These species are commonly used as dietary food for aquatic toxicological experimentation (Table 2 of the general introduction). Mussels were, in this first experiment, fed live microalgae *ad libidum* for 5 min, 2 hr and 5 d and then the microalgae were observed on unstained cryotome sections by light and fluorescence microscopy and identified morphologically and by detecting specific chlorophyll fluorescence (Satoh et al., 2005).

Further on, the influence of food type on biomarkers was investigated in a second experiment in which mussels were maintained under laboratory conditions and fed *ad libitum* for 1 wk using 4 different dietary food types: (a) *I. galbana*; (b) *T. chuii*; (c) *I.galbana* and *T.chuii* microalgae mixture; and (d) commercial food (Shellfish Diet® microalgae blend, Acuinuga). The investigated biomarkers included cytochrome c oxidase (COX), pyruvate kinase (PK) and phosphoenolpyruvate carboxykinase (PEPCK) enzyme activities, metabolites such as protein carbonyl groups (CO), malondialdehyde (MDA) and 4-hydroxy-2-nonenal(HNE), lysosomal enlargement and membrane stability, intracellular neutral lipid accumulation, changes in cell type composition and thinning (atrophy) in the digestive gland epithelium, and altered integrity of the digestive gland tissue. COX is a marker of mitochondrial density and capacity, indicative of metabolic aerobic activity (Lucassen et al., 2003; Siu et al., 2003; Morley et al., 2009). The enzyme activity of PK and PEPCK and their ratio are related to the anaerobic capacity (Ivanina et al. 2016). Augmented protein CO groups are the consequence of protein oxidation resulting in early formation of relative stable carbonylated proteins and as such it is indicative of early oxidative damage (Dalle-Donne et al., 2003). In parallel, MDA and HNE are biomarkers of lipid peroxidation (Viarengo et al., 1991; Maria and Bebianno, 2011). Lysosomal responses to pollutants in mussel digestive cells are widely used as effect biomarkers. Particularly, reduced lysosomal membrane stability has been recognised as a core biomarker for marine pollution monitoring (UNEP/RAMOGE, 1999; ICES, 2004). Likewise, lysosomal enlargement in digestive cells is a widely used biomarker of mussel health condition (Marigómez and Baybay-Villacorta, 2003; ICES, 2012). Intracellular neutral lipid accumulation has been related to organic xenobiotic exposure, non-specific stress and nutritional status (Regoli, 1992; Cancio et al., 1999; Marigómez and Baybay-Villacorta, 2003; Shaw et al., 2011). The relative proportion of basophilic cells is known to increase in the digestive gland epithelium under stress conditions (Zaldibar et al., 2007). Likewise, atrophy of the digestive epithelium and loss of digestive gland histological integrity (augmented connective-to-diverticula ratio) occur in response to pollutant exposure (Couch, 1984; Cajaraville et al., 1992; Marigómez et al., 2006; Garmendia et al., 2011).

MATERIAL AND METHODS

Experimental design and sample processing

Intertidal mussels (*M. galloprovincialis*) of 3.5-4.5 cm shell length were collected from the low tide-mark level (0.5-1.0 m) in Plentzia (Basque Coast; 43°26'N; 2°55'W) in September 2014. Mussels were acclimatized for 7 d to laboratory conditions (18±1°C; 12:12 L:D cycle), maintained unfed in filtered (0.2 μm) seawater (dissolved oxygen: 7.6-8.3 mg/L; pH 7.8-8; salinity: 33‰).

After acclimatization, mussels were divided in 4 experimental groups (in 3 L seawater tanks with constant aeration) and kept fed *ad libitum* for 1 wk using 4 different microalgae diets: (a) *I. galbana*, (b) *T. chuii*, (c) a mixture of *I. galbana* and *T. chuii*, and (d) a commercial food (Microalgae Composed Diet®, Acuinuga SL, A Coruña, Spain). *I. galbana* is a brown free-living biflagellate marine microalga (≈4 μm Ø; Fig. 1A), commonly used as food for mussels in

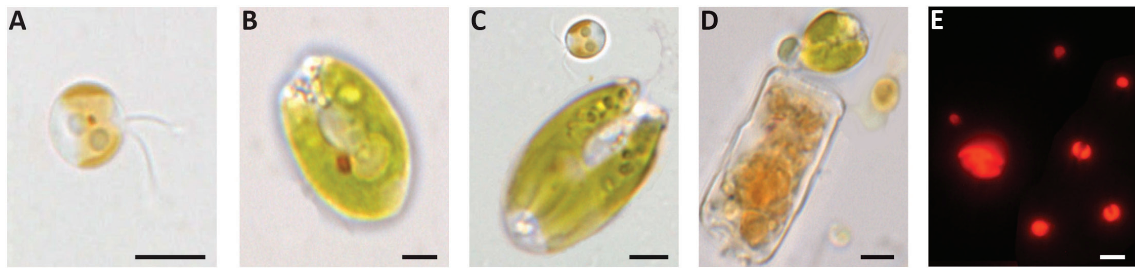

Fig. 1. Micrographs of the different microalgae diets employed in the experiment: (A) *Isochrysis galbana*; (B) *Tetraselmis chuii*;(C) a mixture of *I. galbana* and *T. chuii*; (D) a commercial food; and (E) autofluorescence signal in smear of unstained and frozen *I. galbana* and *T. chuii*. Scale bar: 2 µm.

laboratory experimentation (Table 2 in the general introduction). *T. chuii* is a green free-living tetraflagellate marine microalga (≈10 µm Ø; Fig. 1 B), also used as food for mussels in laboratory experimentation (Table 2 in the general introduction). The used commercial food (Fig. 1 D) is based on a mixture of 4 microalgae *Isochrysis sp.* (25%), *Tetraselmis sp.* (25%), *Thalassiosira sp.* (25%) and *Nannochloropsis sp.* (25%). Following the manufacturer recommendations the product was stored at -40°C before use. Once opened it was stored at <6°C for 1 wk during the realisation of the experiments.

Water and food were fully replaced every day. A strain of *I. galbana* T. Iso clone was grown in previously cleaned 30 L volume methacrylate reactors with natural filtered seawater. *T. chuii* was grown in identical conditions. Microalgae were maintained under constant white light exposure (two lamps of 36 W per reactor), room temperature (T=17°C) and filtered air flow (0.2 µm filters). Microalgae culture density was daily checked using a Beckman Coulter Counter® Z2 particle size analyser. After checking, the cultures were diluted in seawater enriched with F/2 medium (Easyalgae® Fitoplancton Marino SL, Cádiz, Spain) in order to keep an average concentration (cell/mL) of $7.8 \pm 1.4 \times 10^6$ for *I. galbana and* $11.4 \pm 3.4 \times 10^4$ for *T. chuii.* Aliquots were retrieved for water and food changes in mussel tanks (total volume: 3 L seawater+food per tank): (a) 3 L/d of *I. galbana* culture; (b) 3 L/d of *T. chuii* culture; and (c) 1.5 L/d of *I. galbana* culture and 1.5 L/d of *T. chuii* culture for the mixture diet. Finally, the commercial food (2×10^9 particles/mL, as indicated in the label; Microalgae Composed Diet®) was diluted in seawater in order to provide a particle concentration of 5×10^6 particles/mL in 3 L seawater for daily changes.

Immediately after the acclimatization period and after 5 min, 2 hr and 1 wk feeding with *I. galbana* and *T. chuii* and their mixture, the digestive gland of a few mussels was dissected, frozen in liquid nitrogen and stored at -80°C. Further on, the autofluorescence of cryotome sections (8 µm) of these digestive glands was examined under the Nikon Eclipse Ni-Series fluorescence microscope using a 485 nm excitation filter and a 645 nm emission filter, as commonly used to visualize chlorophyll in marine algae suspensions (Satoh et al., 2005). Likewise, Schmorl's staining (see below) was applied to visualize lipofuscins in the same cryotome sections (Pearse, 1972).

After 1 wk experimentation, gills and digestive gland of 5 mussels were dissected out, frozen in liquid nitrogen and stored at -80°C for biochemical and histochemical processing. Mantle and digestive gland of 10 mussels were

dissected out, fixed in formaldehyde (4% in seawater) at 4ºC and embedded in paraffin for histological analyses. No mussel died during experimentation. Gonad histology was examined to provide supporting data of mussel general condition (Ortiz-Zarragoitia et al., 2011); thus, upon microscopic examination of mantle tissue sections, all the individuals in all the treatments were found to be at a comparable gametogenic stage.

Biochemical analysis

Total lipid content was determined in the digestive gland using a chloroform extraction method (Folch et al., 1957; Iverson et al., 2001). Briefly, about 50 mg of digestive gland tissue was homogenized in chloroform/methanol mixture (2:1 v/v) using tissue: solvent proportion of 1:20 w/v. Samples were sonicated for 1 min (output 69 W, Sonicator 3000, Misonix, Farmingdale, NY, USA), incubated overnight at 4°C and centrifuged for 5 min at 13000×*g*. The supernatant was transferred in a new tube, mixed with ultrapure water (0.25 volumes of the supernatant), vortexed for 2 min and centrifuged for 5 min at 13000 *g*. The lower phase (chloroform) was transferred into a pre-weighed microcentrifuge tube and chloroform was allowed to evaporate to determine the dry mass of extracted lipids.
Tissue was powdered with mortar and pestle under liquid nitrogen and homogenized with five volumes of ice-cold 0.6 M perchloric acid (PCA) with 150 mM EDTA to maximize ATP recovery (Sokolova et al., 2000). An aliquot of the homogenate was reserved for glycogen determination, and the remaining homogenate was centrifuged to remove precipitated protein and neutralized with 5 M potassium hydroxide to pH 7.2–7.5. Precipitated potassium perchloride was removed by a second centrifugation and extracts were stored at−80°C. Concentrations of metabolites were measured in neutralized PCA extracts using standard NADH- or NADPH-linked spectrophotometric tests (Bergmeyer, 1985). Briefly, assay conditions were as follows: 38.5 mM D-glucose, TRA buffer, pH 7.6, 0.04 mM $NADP^+$, 7 mM $MgCl_2 \cdot 6H_2O$, 0.462 U glucose-6-phosphate dehydrogenase/mL, 1.8 U hexokinase/mL. Glycogen concentration was measured in PCA extracts after enzymatic hydrolysis of glycogen to D-glucose by glucoamylase (Keppler and Decker, 1984) and determined by the difference in the D-glucose levels in the tissue extract before and after glucoamylase treatment. Concentrations of glycogen, lipids and proteins were expressed in mg/g wet tissue mass.

For total protein content analysis, digestive gland was homogenized in ice-cold homogenization buffer (100 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X 100, 10% glycerol, 0.1% sodium dodecylsulfate, 0.5% deoxycholate, 0.5 µg leupeptin/mL, 0.7 µg pepstatin/mL, 40 µg PMSF /mL and 0.5 µg aprotinin/mL) using Kontes® Duall® tissue grinders (Fisher Scientific, Suwanee, GA, USA). Homogenates were sonicated 3×10 sec each (output 69 W, Sonicator 3000, Misonix), with cooling on ice between sonications, centrifuged for 10 min at 20000 *g* and 4°C, and supernatants were used for protein determination. Protein content was measured using the Bio-Rad Protein Assay kit according to the manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA, USA). Activities of the mitochondrial enzymes pyruvate kinase (PK; EC 2.7.1.40), phosphoenolpyruvate carboxykinase (PEPCK; EC 4.1.1.31) and cytochrome c oxidase (COX; EC 1.9.3.1)

were determined in gill tissue. This was thoroughly homogenized in enzyme-specific homogenization buffer (see below) using hand-held Kontes® Duall® tissue grinders (Fisher Scientific, Suwanee, GA, USA). Homogenates were sonicated 3×10 sec each (output 7, Sonic Dismembrator Model 100, Fisher Scientific, Suwanee, GA) to ensure complete release of the enzymes, with cooling on ice (1 min) between sonications and centrifuged at 16000×*g* and 4°C for 25 min. The supernatant was collected and used for enzyme determination. Enzyme extracts were stored at −80°C for less than 2 wk before activity assays. For determination of enzyme activities, enzyme extracts were thawed on ice and immediately analyzed using standard spectrophotometric techniques as described elsewhere (Bergmeyer, 1985; Sidell et al., 1987; Birch Machin and Turnbull, 2001). Enzyme activities were measured at 20°C using a UV–Vis spectrophotometer (VARIAN Cary 50 Bio, Cary NC, USA). The temperature of the reaction mixture was controlled within 0.1°C of the set value using a water-jacketed cuvette holder. Briefly, isolation and assay conditions for the studied enzymes were as follows: (a) PK: homogenization buffer: 10 mM Tris–HCl buffer (pH 7.2), 5 mM EDTA, 1 mM DTT, 0.1 mM PMSF; assay: 50 mM Tris-HCl (pH7.2), 50 mM KCl, 5 mM $MgSO_4$, 1 mM ADP, 0.2 mg/mL NADH, 5.5 U LDH, 0.5 mM PEP; acquisition wavelength: 340 nm. (b) PEPCK: homogenization buffer: 10 mM Tris–HCl buffer (pH 7.2), 5 mM EDTA, 1 mM DTT, 0.1 mM PMSF; assay: 100 mM HEPES (pH 7.2), 2.3 mM $MnCl_2$, 0.5 mM IDP, 5mg/mL $KHCO_3$, 0.2 g/mL NADH, 10U MDH, 15 mM PEP; acquisition wavelength: 550 nm. (c) COX: homogenization buffer: 25 mM potassium phosphate, pH 7.2, 10 µg phenylmethylsulfonyl fluoride (PMSF)/mL, 2 µg aprotinin/mL; assay: 20 mM potassium phosphate, pH 7.0, 16 µM reduced cytochrome c(II), 0.45 mM n-dodecyl-b-d-maltoside, 2 µg antimycin A/mL; acquisition wavelength: 550 nm. Protein concentration was measured as above described for the digestive gland.

Protein carbonyl groups (CO) were measured spectrophotometrically (Philipp et al., 2005). Digestive gland was grounded under liquid nitrogen and homogenized in buffer containing 50 mM HEPES, 125 mM KCl, 1.1 EDTA and 0.6 mM $MgSO_4$ (pH 7.4) and protease inhibitors [leupeptin (0.5 µg/mL), pepstatin (0.7 µg/mL), phenylmethylsulfonyl fluoride (40 µg/mL) and aprotinin (0.5 µg/mL)]. Samples were centrifuged at 100000 *g* for 15 min, supernatant was collected and incubated at room temperature with 10 mM 2,4-dinitrophenylhydrazine (DNP) in 2 M HCl. The blanks were incubated with HCl without DNP. After incubation, proteins were precipitated by adding 100% TCA and centrifuged at 11000 *g* for 10 min. The pellet was collected, washed with ethanol ethylacetate (1:1) and resuspended in 6 M guanidine hydrochloride in 20 mM in KH_2PO_4 (pH 2.5) until dissolved. The absorbance was measured at 360 nm on a spectrophotometer (Cary 50, Varian) using guanidine HCl solution as reference. The amount of carbonyls was estimated as a difference in absorbance between samples and blanks using a molar extinction coefficient of carbonyls ε = 22000 L/cm M. The amount of carbonyls was expressed as per mg total protein measured in the same samples using Bradford method.

Protein conjugates of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) were measured as biomarkers of lipid peroxidation

using enzyme-linked immunosorbent assay (MDA OxiSelect™ MDA adduct ELISA Kit and HNE OxiSelect™ HNE-His adduct ELISA Kit, respectively) according to the manufacturers' protocols (Cell Biolabs, Inc., CA, USA). About 200–300 mg of digestive gland were homogenized in ice cold phosphate-buffered saline (PBS; 1:5 w:v) with protease inhibitors (50 µg aprotinin/L and 40 µM phenylmethylsulfonyl fluoride) using Kontes Duall tissue grinders (Fisher Scientific, Suwanee, GA, USA). Samples were centrifuged at 15000 *g* for 10 min at 4°C. Protein concentration was measured in the supernatant using the Bio-Rad Protein Assay kit according to the manufacturer›s protocol (Bio-Rad Laboratories, Hercules, CA, USA). Supernatants were diluted with PBS to a final concentration of 1 mg protein/L.

Histochemical analyses

Lysosomal membrane stability was evaluated in serial cryostome sections (10 µm thick; Leica CM3050S cryotome) of one chuck (5 digestive glands) per sample after the cytochemical demonstration of hexosaminidase activity, according to a standardised procedure (UNEP/ RAMOGE, 1999), based on the time of acid labilisation (LP) required to produce the maximum staining intensity. LP was determined at the light microscope as the maximal accumulation of reaction product associated with lysosomes. Four determinations were made per digestive gland and their mean value corresponded to an individual digestive gland LP, expressed in min.

In order to quantify changes in lysosomal structure, digestive gland cryotome sections (8 µm thick) of one chuck (5 digestive glands) were stained for the histochemical demonstration of ß-glucuronidase activity according to Cajaraville et al. (1991). Five measurements using a 1000× magnification were made in each individual using image analysis (Sevisan S.L., Spain).The mean value of the stereological parameters was determined for each mussel digestive gland (Lowe et al. 1981): lysosomal volume density ($Vv_{LYS} = V_{LYS}/V_C$), lysosomal surface density ($Sv_{LYS} = S_{LYS}/V_C$), lysosomal surface to volume ratio ($S/V_{LYS} = S_{LYS}/V_{LYS}$) and lysosomal numerical density ($Nv_{LYS} = N_{LYS}/V_C$, where V is volume, S is surface, N is number, LYS is lysosomes and C is digestive cell cytoplasm.

Intracellular neutral lipids accumulation was determined in digestive gland cryotome sections (8 µm thick) of 5 mussels after the histochemical demonstration of neutral lipids by staining with Oil Red O (ORO; Culling 1974). Slides were viewed at 400× magnification. The extent of ORO staining in the digestive gland epithelium was measured with the aid of image analysis, as in recent contributions (Garmendia et al., 2010; Brooks et al., 2011; Múgica et al., 2015). The method is described in detail by Marigómez and Baybay-Villacorta (2003). The volume density of ORO positive reaction product (neutral lipids) with respect to the digestive epithelium volume (Vv_{NL}) was calculated by applying a stereological procedure. Vv_{NL} is expressed as $\mu m^3/\mu m^3$.

Lipofuscin (LPF) accumulation was determined in digestive gland cryostat sections (8 µm thick) fixed for 15 min in Baker buffer at 4°C. Then, they were rinsed in distilled water and stained using Schmorl's reaction (Pearse, 1972). Five measurements using a 400× magnification were made in each individual using image analysis (Sevisan S.L., Spain).The mean value of LPF volume density ($Vv_{LPF} = V_L/V_C$) was determined for each mussel digestive gland.

Histological examination

Digestive gland sections (5 µm thick) were cut in a Leica RM2125 microtome, and mounted on albumin coated slides, dried at 37°C for 24 hr, and stored at room temperature until staining with toluidine-eosin (Chapter 6). Sections were dewaxed in xylene, rehydrated in alcohols, brought to distilled water. Then, sections were rinsed in 1% toluidine in distilled water for 10 min. After washing in tap water, sections were rinsed in eosin for 15 sec, washed in tap water, dehydrated in ascending graded-ethanol series, cleared in xylene and mounted in DPX.

After staining, a stereological procedure was applied to quantify the volume density of basophilic cells (Vv_{BAS}), the mean epithelial thickness (MET; µm) and the mean luminal radius (MLR; µm) used to calculate the MLR/MET (µm/µm) ratio (Vega et al., 1989). Connective-to-diverticula ratio (CTD) was also calculated. Counts were made in 3 optical fields per mussel in 6 mussels per experimental group. Slides were viewed using a drawing tube attached to a Nikon Eclipse Ni microscope (40x objective). A Weibel graticule (multipurpose system M-168) was used, and hits of basophilic and digestive cells, luminal area and connective tissue were recorded to calculate Vv_{BAS}, MLR/MET and CTD (Brooks et al., 2011; Garmendia et al., 2011).

Statistical analysis

Statistical analyses were made using IBM® SPSS® Statistics ver. 22.0.0.0 (IBM Corp., Armonk, NY, USA). Parameters were tested for normality (Kolmogorov-Smirnov's test) and homogeneity (Levene's test).Thus, parametric statistics (one-way analysis of variance) was applied to COX, PK, PEPCK, PK/PEPCK, CO, MDA, HNE, Vv_{NL}, Vv_{LPF} Vv_{BAS}, MLR/MET and CTD, in which statistical differences among the diets were established using Duncan's *post-hoc* test. In contrast, non-parametric statistics (Mann-Whitney's U-test) was used for LP. The Z-score test (N=4) was applied for the cases of small sample size for reliable Duncan's and Mann-Whitney's U-test (e.g., when samples were pooled or when some of a set were missing or difficult to measure), say: total lipid content, glycogen, total protein content, Vv_{LYS}, S/V_{LYS}, and Nv_{LYS}. Significance for all statistical tests was established at $p<0.05$.

RESULTS

Microalgae distribution and fate in the midgut

After 7 d acclimatization in absence of food supply, some brownish granules were observed in the lumen and in the epithelium of digestive alveoli (Fig 2A). These granules were identified as LPFs (Figs. 3A and 3B), most likely related to residual bodies of digestive cells, and exhibited a weak fluorescence (Fig. 2B).

The same LPF granules with comparable background weak fluorescence were observed after feeding mussels with *I. galbana* for 5 min (Figs. 2C, 2D, 3C and 3D). Microalgae were indentified in the lumen of the stomach as well as in primary and secondary digestive ducts (Fig. 2C). These microalgae presented a strong fluorescence (Fig. 2D). In contrast, no microalgae were found, nor florescence detected in the digestive alveoli (Figs. 2C and 2D). Although microalgae pigments also resulted to be stained with the Schmorl's method, they were easily distinguished from mussel tissue LPFs because the staining intensity was much higher in the microalgae

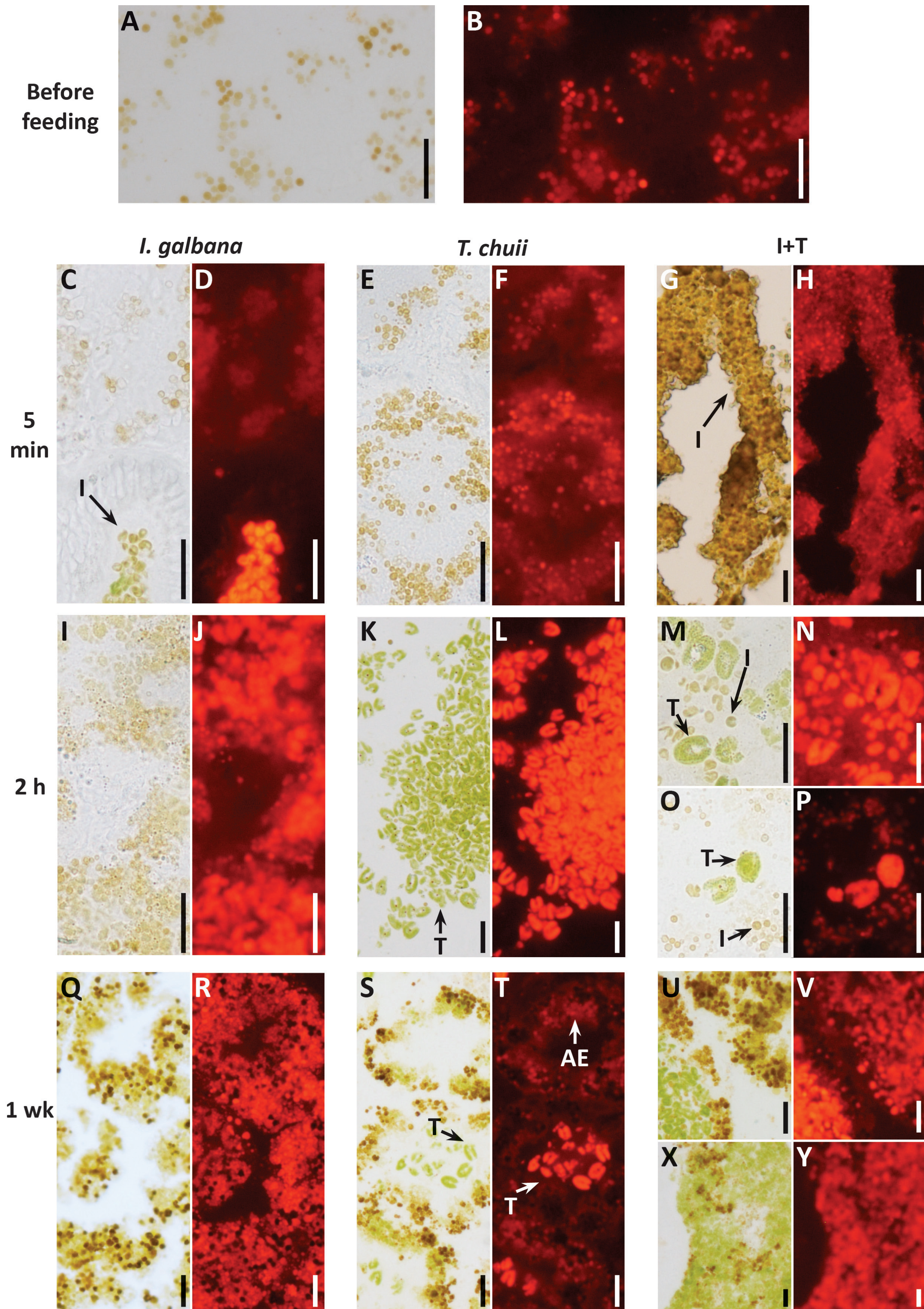

Fig. 2. Cryostat sections (8 µm) of unstained fresh tissue of mussels before feeding and after feeding during 5 min, 2 hr and 1 wk *ad libitum I. galbana*, *T. chuii* and *I. galbana* + *T. chuii* (A, C, E, G, I, K, M, O, Q, S, U, X). The same tissue section fields were examined at the fluroescence microscope with 485 nm excitation filter and 645 nm emission filter (B, D, F, H, J, L, N, P, R, T, V, Y). B, D and F: 12% light intensity; H: 6% light intensity.J, L, N, P, R, T, V and Y: 3% light intensity. Scale bar: 20 µm. I, *I. galbana*-like body; T, *T. chuii*; AE, alveolus epithelium.

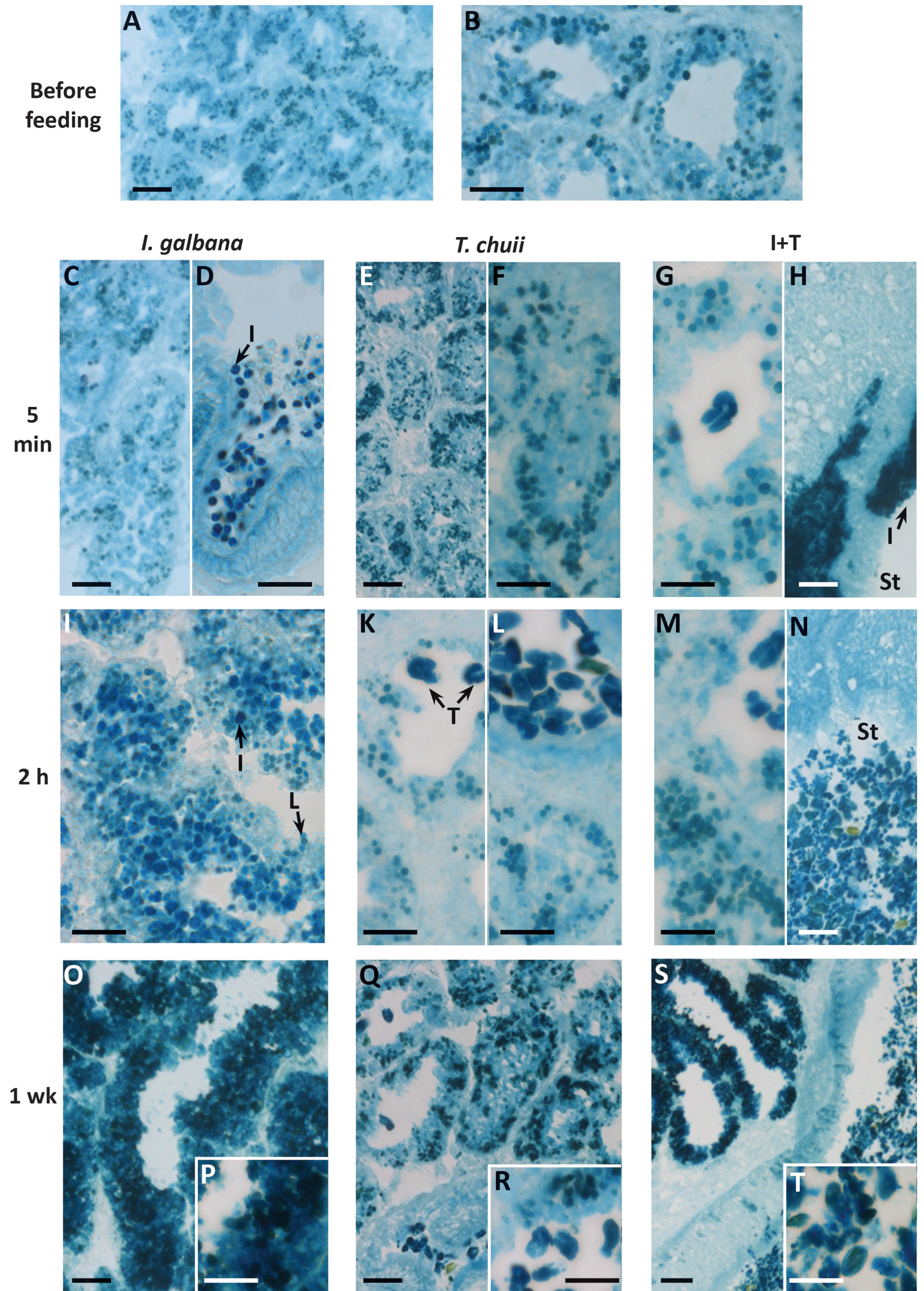

Fig. 3. Histochemistry of lipofuscins in digestive gland of mussels before feeding and after feeding *ad libitum* with *I. galbana*, *T. chuii* and *I. galbana* + *T. chuii* for 5 min, 2 hr and 1 wk. Scale bar (A, C, E, H, N, O, Q): 30 µm. Scale bar (B, D, F, G, I, K, L, M, P, R, T): 20 µm. Scale bar (S): 50 µm. I, *I. galbana*-like body; T, *T. chuii*; St, stomach; L, lipofuscin.

and also because normally these latter were easily indentified morphologically (Figs. 3C and 3D). After 2hr, microalgae-like bodies were observed within the epithelium of digestive alveoli (Fig. 2I), which exhibited a remarkable fluorescence intensity (Fig. 2J). After 1 wk, abundant dark brown bodies were found in the epithelium of digestive alveoli together with yellowish corpuscles resembling microalgae (Fig. 2Q); in parallel, Schmorl positive materials (both LPFs and microalgae, which were indistinguishable due to the enormous intensity of the Schmorl's reaction) were extremely abundant (Figs. 3O and 3P) and fluorescence intensity was increased in the epithelium though some small patchy areas, seemingly LPFs, appeared dark (Fig. 2R).

After feeding mussels *ad libitum* with *T. chuii* for 5 min, Schmorl positive brownish bodies with background weak florescence were found (Figs. 2E, 2F, 3E and 3F), similar to those found after acclimatization in absence of food (Figs. 2A, 2B, 2E and 2F). In contrast, after 2 hr feeding, abundant microalgae and their large fragments were found in the stomach but not in the digestive alveoli (Fig. 2K). These microalgae exhibited very intense fluorescence (Fig. 2L) and were highly reactive after Schmorl's staining (Figs. 3K and 3L). No change was observed in the reactivity of the alveolus epithelium after Schmorl's staining (Figs. 3K and 3L). After 1 wk, highly fluorescent microalgae and their fragments were found in the lumen of alveoli, whereas in the epithelium fluorescence was practically residual (Figs. 2S and 2T). In contrast, the amount and staining intensity of LPFs in the epithelium of digestive alveoli was increased (Figs. 3Q and 3R).

In mussels fed with the mixture of both microalgae species (I+T) for 5 min, a compact mass in which some bodies resembling *I. galbana* could be indentified in the stomach lumen together with a strong fluorescence (Figs. 2G and 2H). After 2 hr, both microalgae species were found in the lumen of the stomach (Figs. 2M and 2N) and of the alveoli (Figs. 2O and 2P). After 1 wk, the stomach lumen was full of both microalgae and microalgae fragments (Figs. 2X and 2Y); however the appearance of digestive alveoli was diverse. Some alveoli with empty lumen presented high fluorescence intensity in their epithelium (Fig. 2V) whilst other had the lumen full of *T. chuii* and fragments, and exhibited low fluorescence within the epithelium (Figs. 2U and 2V). Overall, the LPF content increased greatly in the epithelium of the digestive alveoli (Fig. 3 S).

Food type influence on biomarkers

Whilst the total protein levels were similar in all the diets, the total lipid content was higher and the glycogen levels were lower in *I. galbana* than in the other diets (Table 1). Likewise, whilst the total protein levels were similar in all the mussels irrespective of the diet, the total lipid content was lower and the glycogen levels higher in the digestive gland of mussels fed commercial food than in the other experimental groups (Table 1).

Overall, differences between diets were found in the majority of the biomarkers investigated herein (Table 2). COX activity was not significantly different between treatments (0.45-0.67 U/g prot; Table 2). PK activity was lower in mussels fed commercial food than in any other experimental group (Fig. 4A; Table 2) whereas PEPCK was much higher (Fig. 4B), thus resulting in an extremely low PK/PEPCK (Fig. 4C). CO values were not

Table 1. Total lipid, protein and glycogen content in different microalgae diets (*I. galbana*; *T.chuii*; a mixture of *I. galbana* and *T. chuii*; and a commercial food) as reported by Albentosa et al. (1996), FAO (2004) and Acuinuga Product Sheet, and in mussels fed *ad libitum* for 1 wk with them as food. Letters in superscripts indicate significant differences between groups of dietary food type according to the Z-score test (p<0.05).

	Total lipids		Proteins		Carbohydrates	
	Food (% organic matter)	Mussel (g lipid/g tissue)	Food (% organic matter)	Mussel (g protein/g tissue)	Food (% organic matter)	Mussel (*mmol glycosyl unit/L)
I. galbana	30[a]	0.40±0.11[a]	20	0.95±0.19	20[a]	6.12±2.46[a]
I. galbana + T. chuii	-	0.37±0.17[a]	-	0.68±0.10	-	7.68±5.48[a]
T. chuii	10[b]	0.32±0.09[a]	15	0.86±0.48	50[b]	1.62±1.12[a]
Commercial food**	10[b]	0.26±0.12[b]	20	0.72±0.14	45[b]	19.80±10.66[b]

*Glycogen levels
** Estimates, according to the product label, subject to some variability among batches

significantly different between treatments (124.19-226.35 nM CO/mg prot; Table 2). The response profiles of MDA and HNE were similar to each other, with lower values in mussels fed *I. galbana* alone or mixed with *T. chuii* than in those fed *T. chuii* and commercial food (Figs. 4D and 4E; Table 2).

LP could not be determined in mussels fed *I. galbana* alone or in mixture with *T. chuii* because histochemical hexosaminidase activity in digestive alveoli was not clearly discriminated from the background brownish coloration caused by LPFs and microalgae (Hex in Fig. 5). LP values were between 15 and 20 min were recorded in mussels fed *T. chuii* and commercial food (Fig. 6A), although in the former the measurements were made with high difficulty due to the presence of extensive brownish bodies in the digestive cells. Similarly, lysosomal structural changes could hardly be measured in mussels fed *I. galbana* due to the massive amount of microalgae within the epithelium of digestive alveoli (β-Gus in Fig. 5); thus, estimates of Vv_{LYS} and Nv_{LYS} in mussels fed *I. galbana* are of limited significance. Nevertheless, Vv_{LYS} and Nv_{LYS} were higher in mussels fed *I. galbana* and I+T than in those fed *T. chuii* or commercial food (Figs.

Table 2. Summary of 1-way ANOVAs (parametric) and Krukal-Wallis tests (non-parametric) performed to analyse the effect of food type (d.f. = 3) on biomarkers in mussels fed *ad libitum* for 1 wk with 4 different microalgae diets (*I. galbana*; *T.chuii*; *I. galbana + T. chuii* mixture; commercial food). d.f.: degrees of freedom; F: Fisher's F-ratio; X^2: chi-square; p: probability (significant: p<0.05). #: Significant differences between diets according to the Z-score test (p<0.05; see Figs.).

Parameter	*F/ X²*	d. f. $_{(residual)}$ / N	p
One-way ANOVA			
PK	3.242	8	0.081
PK/PEPCK	2.267	8	0.158
MDA	2.093	6	0.203
HNE	4.021	12	0.034*
Vv_{LYS}#	3.857	9	0.050*
S/V_{LYS}#	1.647	9	0.247
Nv_{LYS}#	1.169	9	0.374
Vv_{NL}	6.696	11	0.008*
Vv_{LPF}	7.825	16	0.002*
Vv_{BAS}	5.256	16	0.041*
MLR/MET	3.429	16	0.043*
Kruskal-Wallis			
COX	2.174	18	0.537
PEPCK	4.212	12	0.240
CO	1.471	16	0.689
CTD ratio	10.337	20	0.016*

COX: cytochrome C oxidase; PK: pyruvate kinase; PEPCK: phosphoenolpyruvate carboxykinase; CO: protein carbonyl groups; MDA: malondialdehyde; HNE: 4-hydroxy-2-nonenal; LP: labilisation period; Vv_{LYS}: lysosomal volume density; S/V_{LYS}: surface/volume of lysosomes; Nv_{LYS}: lysosomal numerical density; Vv_{NL}: volume density of neutral lipids; Vv_{LPF}: volume density of lipofuscins; Vv_{BAS}: volume density of basophilic cells; MLR/MET: mean luminal radius/mean epithelial thickness of the digestive alveoli; CTD ratio: connective-to-diverticula ratio.

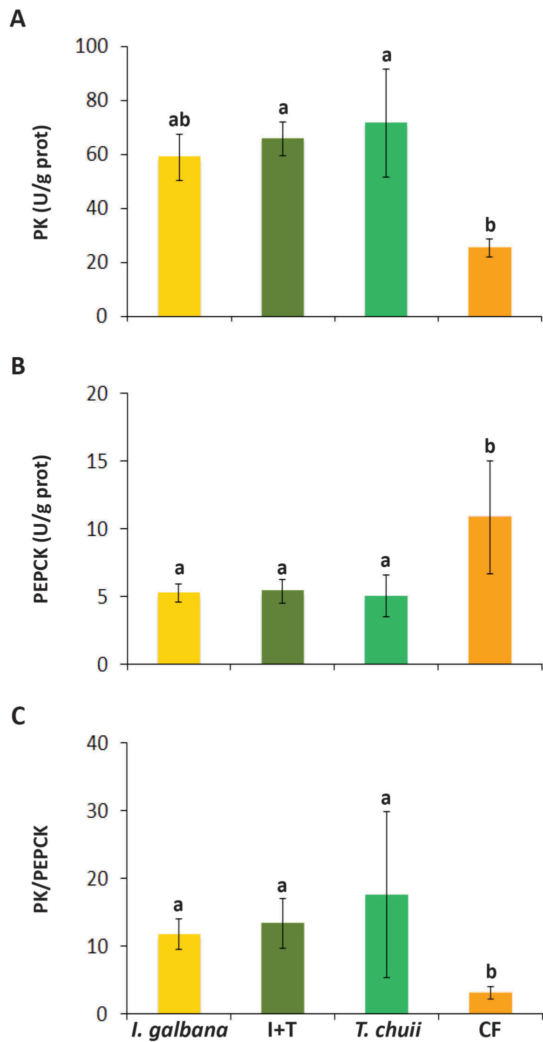

Fig.4. Cytochrome c oxidase (COX) (A), pyruvate kinaseactivity (PK) (B), phosphoenolpyruvate carboxykinase activity (PEPCK) (C), and PK/PEPCK (D) in gills of mussels fed *ad libitum* for 1 wk with 4 different diets (*I. galbana*; *T. chuii*; a mixture of *I. galbana* and *T. chuii*; and a commercial food). Intervals indicate standard error. Letters indicate significant differences among diets according to the Duncan's test performed after one-way ANOVAs (p<0.05).

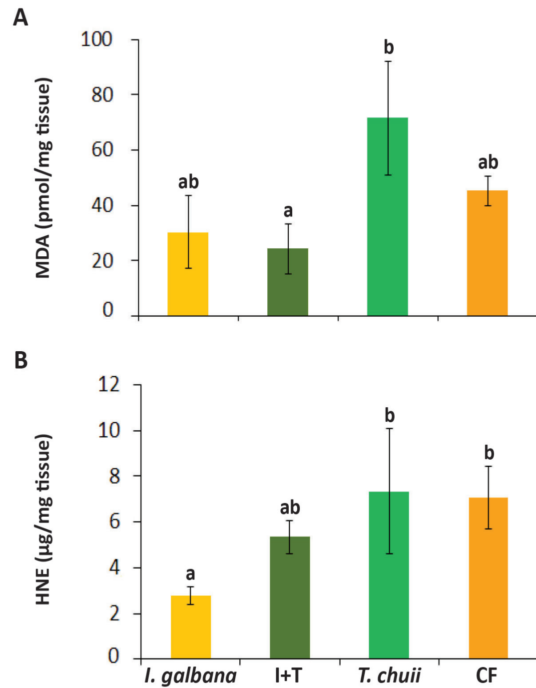

Fig. 5. Protein carbonyl groups (CO) (A), malondialdehyde (MDA)-protein conjugates (C) and 4-hydroxynonemal (HNE)-protein conjugates in digestive gland of mussels fed *ad libitum* for 1 wk with 4 different diets (*I. galbana*; *T. chuii*; a mixture of *I. galbana* and *T. chuii*; and a commercial food). Intervals indicate standard error. Letters indicate significant differences among diets according to the Duncan's test performed after one-way ANOVAs (p<0.05).

6B and 6C). In contrast, S/V_{LYS} values did not differ among experimental groups (S/V_{LYS} = 3.19-4.19 $\mu m^2/\mu m^3$; Table 2). Vv_{NL} was higher in mussels fed *I. galbana* and I+T than in mussels fed *T. chuii* or commercial food, values being especially low in the latter (NL in Fig. 5; Fig. 6D; Table 2). Vv_{LPF} was much higher in mussels fed *I. galbana* than in mussels fed commercial food, with those fed *T. chuii* and the mixture of microalgae in between (LPF in Fig. 5; Fig. 6E; Table 2).

Vv_{BAS} was lower in mussels fed *I. galbana* than in other experimental groups and the highest in mussels fed I+T and commercial food (T&E in Fig. 5; Fig. 7A; Table 2). The lowest MLR/MET values were found in mussels fed *I. galbana* and the highest in those fed commercial food, with the remaining two experimental groups in between (T&E in Fig. 5; Fig. 7B; Table 2). CTD ratio was higher in mussels fed commercial food than in the other experimental groups (Fig. 7C; Table 2).

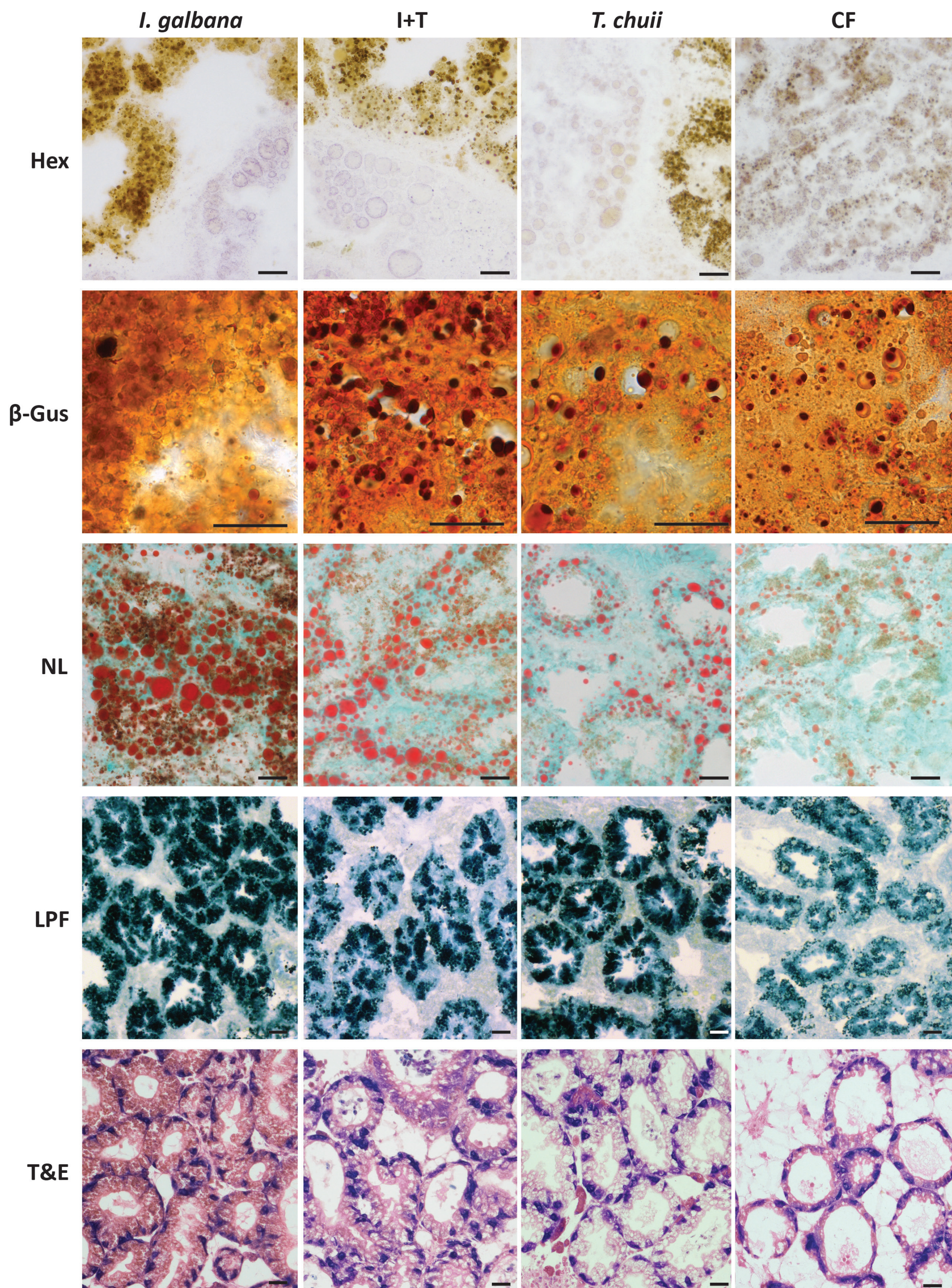

Fig. 6. Micrographs of hexosaminidase (LMS test) and β-glucuronidase enzyme histochemistry (LSC test), Oil Red O staining (neutral lipid accumulation: NLA), Schmorl's staining (lipofuscins: LPF), and T&E staining (cell-type composition and structural changes of digestive alveoli: CTC and SCDA) in digestive gland of mussels fed *ad libitum* for 1 wk with 4 different microalgae diets (*I. galbana*; *T. chuii*; a mixture of *I. galbana* and *T. chuii*; and a commercial food). Scale bar: 20 µm.

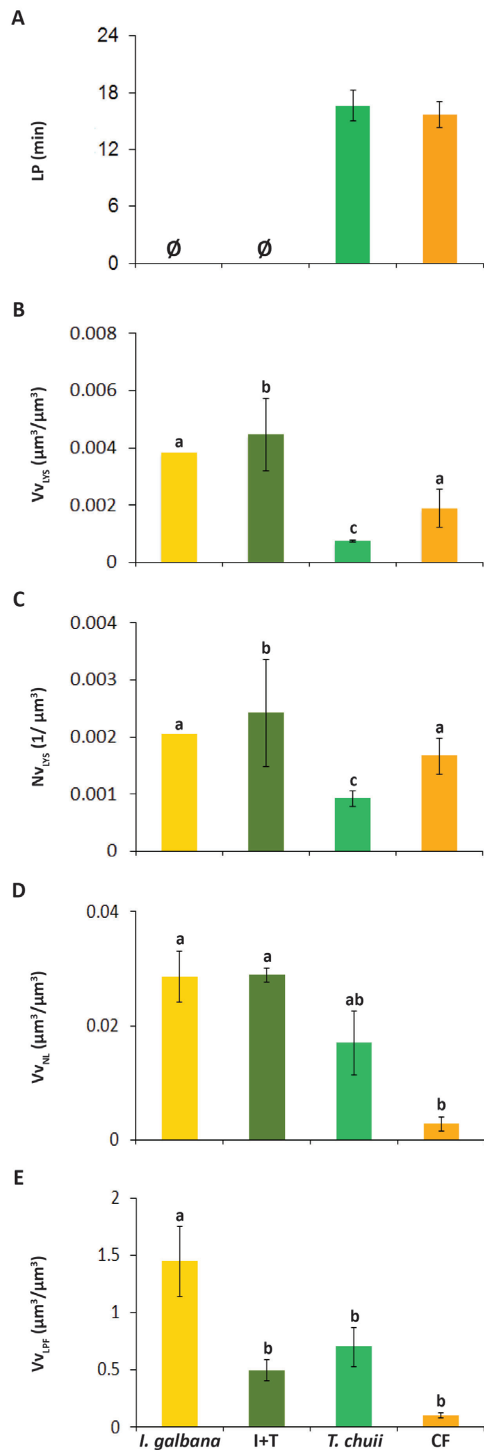

Fig. 7. Lysosomal membrane LP (A), lysosomal volume density (Vv_{LYS}) (B), lysosomal numerical density (Nv_{LYS}) (C), and volume density of neutral lipids (D) and lipofustin (E) in digestive gland of mussels fed *ad libitum* for 1 wk with 4 different diets (*I. galbana*; *T.chuii*; a mixture of *I. galbana* and *T. chuii*; and a commercial food). Intervals indicate standard error. Letters indicate significant differences among diets according to Mann-Whitney's U-test (LP), the Duncan's test performed after one-way ANOVAs (Vv_{NL}, Vv_{LPF}) or Z-score test (Vv_{LYS}, S/V_{LYS}, and Nv_{LYS}). Significance for all statistical tests was established at p<0.05. Ø, no reliable measurements.

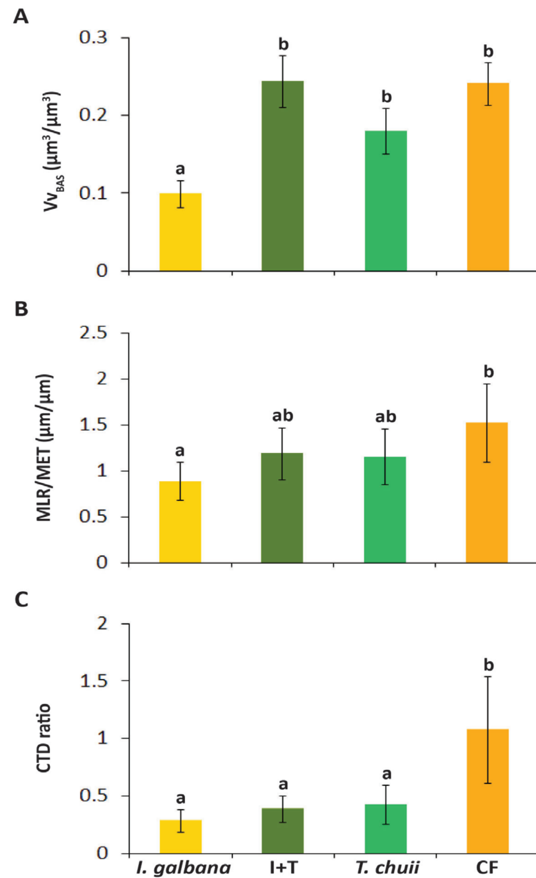

Fig. 8. Volume density of basophilic cells (Vv_{BAS})(A); Mean luminal radius to mean epithelial thickness (MLR/MET) (B); connective tissue to digestive tissue ratio (CTD ratio) (C) in digestive gland of mussels fed *ad libitum* for 1 wk with 4 different diets (*I. galbana*; *T.chuii*; a mixture of *I. galbana* and *T. chuii*; and a commercial food). Intervals indicate standard error. Letters indicate significant differences among diets according to the Duncan's test performed after one-way ANOVAs (p<0.05).

DISCUSSION

Microalgae distribution and fate in the midgut

Microalgae species used as a food source for bivalves can largely differ in cell size and morphology, digestibility, biochemical composition and toxicity. Some microalgae may have an excellent biochemical composition and mussels may ingest them but this does not necessarily imply that they will be subject to digestion (Fernández-Reiriz et al., 2015). For instance, after

testing ten species of microalgae including amongst others, several species of the genus *Tetraselmis*, only 2 species (*Isochrysis* and *Pavlova*) were digested by winged pearl oyster larvae (Martínez-Fernández et al., 2004).

Presently, after 5 min feeding, strongly fluorescent *I. galbana* reached the lumen of the stomach and digestive duct but they were only inside the digestive cells 2 hr later and beyond. This is conceivable because in intertidal mussels, the digestion cycle lasts around 4 hr and *I. galbana* has been reported to be integrally internalized (phagocytosed) in digestive cells for intracellular digestion (Izagirre et al., 2008). In agreement, digestion of *Isochrysis* sp. by giant clam veligers was observed 2 hr after the start of feeding (Southgate et al., 2017). With the exception of Izagirre (2007), the massive presence of microalgae-like spherical bodies in mussel digestive cells bivalves has not been reported previously in laboratory experiments in which *I. galbana* was employed as food (Lowe et al., 1981; Pipe and Moore 1985; Okay et al., 2006; Dimitriadis et al., 2012), even though several of these investigations were based on microscopic observations of digestive gland tissue sections. Therefore, further research is needed in order to understand the mechanism through which small and relatively easily digestible microalgae such as *I. galbana* are digested in bivalves.

In contrast, *T. chuii* took 2 hr to reach the stomach and was never found within digestive cells. Similarly, digestion of *Tetraselmis* sp. by giant clam veligers was only observed at time periods exceeding 4-8 hr after feeding (Southgate et al., 2017), unlike for the case of *Isochrysis* above commented. Moreover, although *T. chuii* reached the lumen of digestive alveoli and the digestive cells were rich in lipofuscins after 1 wk feeding, the epithelium was weakly fluorescent. Florescence intensity decays as the degree of lysed and digested phytoplankton cells increases (Aldana-Aranda et al., 1994). Therefore, it seems that *T. chuii* digestion was mainly extracellular and subject to extended gut retention times. Gut retention time (and associated absorption efficiency; Hawkins & Bayne 1984) are determined by the amount and quality of ingested food but most largely by its degree of digestibility (Bricelj et al., 1984; Chaparro et al., 2001). In adult oysters and mussels fed *Tetraselmis*, the gut retention time can go beyond 10 hr (Chaparro et al., 2001). Indeed, it has been reported that mussels have more difficulties in absorbing *Tetraselmis*, in comparison with other microalgae (e.g., absorption efficiency is half of that recorded for *Isochrysis*; Fernández-Reiriz et al., 2015). The large cell size can hamper absorption (Martínez-Fernández et al., 2004). The presence of refractory cell walls is also a well known cause for indigestibility of chlorophytes in bivalves (Bricelj et al., 1984). Cell walls can contain highly refractory components resistant to enzymatic attack and strong acid degradation, which would thus appear to be little advantage in a more prolonged retention of such cells in the gut (Bricelj et al., 1984; Rouillon and Navarro et al., 2003; Fernández-Reiriz et al., 2015).

The digestibility of the cell wall of *Tetraselmis* seems to be low in mussels, which is reflected in low absorption efficiency (Fernández-Reiriz et al., 2015). *Tetraselmis* cells are not easily digested due to their thick cellulose-rich cell wall, which renders intracellular starch granules and other components unavailable to the gut digestive enzymes (Tizon et al., 2013). Cellulase is a common molluscan enzyme; however, hydrolysis of structural cellulose is generally low in bivalves. Moreover, the cell wall of *Tetraselmis* has been

shown to be made of a pectin-like material, with galactose, galacturonic acid and unusual 2-keto sugar acids as major components, which impart an acidic character to the cell walls and are difficult to degrade (Becker et al., 1998). As a result, *Tetraselmis* is known to be less nutritious that *I. galbana* in a variety of bivalve species (Tizon et al., 2013).

Finally, it is worth noting herein that "absorption" in bivalves' gut is not intestinal and that the "physiological" concept of absorption rather refers to processes of endocytosis and phagocytosis in digestive cells and further intracellular food digestion and nutrient delivery to haemocytes. Whilst in the case of *I. galbana* the entire cells seem to be "absorbed" in the digestive diverticula, in the case of *T. chuii* only food materials derived from extracellular digestion would be taken up by digestive cells (and lead to residual products of digestion such as lipofuscins). Interestingly, the presence of *I. galbana* seemed to facilitate the distribution of *T. chuii* towards digestive alveoli, as in mussels fed with the mixture of both microalgae species the distribution profile recorded was intermediate between those found for *I. galbana* and *T. chuii.*

In summary, different microalgae present different distribution and fate in mussel digestive gland. Whereas small microalgae such as *I. galbana* readily reach digestive alveoli and are intracellularly digested in digestive cells (extracellular pre-digestion cannot be disregarded anyway), large and hardly degradable *T. chuii* are retained in the stomach and digestive ducts. As a result of the presence of microalgae in gut and digestive gland epithelium, the enzyme activities and metabolites (e.g. pigments and lipofuscins) of the microalgae or resulting from the mussel response to the microalgae can influence the determination of biochemical biomarkers, as below discussed. Likewise, in consequences of extracellular digestion of large microalgae (associated long retention times), and the interactions between intracellular digestion of small microalgae, the form and function of the digestive cell lysosomes and the digestive gland epithelium will influence lysosomal and tissue-level biomarkers, as discussed in the next section.

Food type influence on biomarkers

In agreement with the composition of the food, the total lipid content was lower in the digestive gland of mussels fed commercial food than in those fed live microalgae. In contrast, although the carbohydrate levels were similar in the commercial food and in *T. chuii*, the glycogen levels in the digestive gland of mussels fed *T. chuii* were much lower, which can be related to the lower digestibility and absorption efficiency aforementioned. A large part of the carbohydrates determined in *T. chuii* would correspond to cellulose and pectin-like material (Becker et al., 1998; Tizon et al., 2013), which remained in the gut lumen and did not contribute to the levels determined in digestive gland. The first relevant conclusion is that the nutritional condition of mussels varied significantly depending on the diet and as such, it can have a significant influence on biomarkers (Gonzalez-Fernandez et al., 2015). The commercial food (poorest diet) and *I. galbana* (richest diet) represented the two extreme nutritional conditions, as envisaged in the basic biochemical components measured in the digestive gland of mussels. The low digestibility of freeze-dried microalgae, as

used in commercial food, has been shown to cause reduced growth rates of seed in comparison with fresh microalgae diets (Albentosa et al., 1997). Accordingly, the most striking differences in biomarker values were found when commercial food and *I. galbana* were compared, with the cases of the diets based on *T. chuii* and the mixture of live microalgae in between.

The second main conclusion is that, overall, differences between diets were found in the majority of the biomarkers. Thus, as the result of low PK and high PEPCK activities, PK/PEPCK was much lower in mussels fed commercial food than in those fed live microalgae, which indicated decreased aerobic scope and increased gluconeogenesis (Storey, 1995; Brooks and Storey, 1997; Sokolova and Portner, 2001; Ivanina et al., 2010; Lacroix et al., 2015). In parallel, lipid peroxidation (high MDA and HNE values; Viarengo et al., 1991; Maria and Bebianno, 2011) was enhanced in mussels fed *T. chuii* or commercial food; intracellular digestion was reduced (low Vv_{LYS} and Nv_{LYS} and high $Vv_{BAS,}$ MLR/MET and CTD ratio; Izagirre et al., 2008; 2009; Zaldibar et al., 2008; Garmendia et al., 2010; Múgica et al., 2015) and the levels of neutral lipids (indicative of nutritional status; Cancio et al., 1999) and lipofuscins (residual product of lipid digestion or oxidation; Viarengo et al., 1997) were low, especially in mussels fed the commercial food, in agreement with the low lipid content of this diet and in the digestive gland. This profile might resemble a situation of limited nutritional status in mussels fed commercial food and to a lesser extent, in mussels fed *T. chuii*; which is known to affect biomarkers and biomarker responsiveness (Gonzalez-Fernandez et al., 2016).

In addition, the presence of the food particles, which may vary depending on the food type and regime, may interfere with the measure of the biomarkers. For instance, LP could not be determined in mussels fed *I. galbana* alone or in mixture with *T. chuii* because hexosaminidase activity (used to visualize lysosomes) in digestive cells was not clearly discriminated from the background brownish coloration of the lipofuscins and pigments of the microalgae. Likewise, Vv_{LYS} and Nv_{LYS} could hardly be measured in mussels fed *I. galbana* due to the presence of massive microalgae within digestive cells, which also hampered any reliable distinction between microalgae and lipofuscins. More subtly, biochemical determinations (e.g. MDA and HNE in digestive gland) might include the massive presence of microalgae together with mussels tissues and, though this aspect can be obviated by biochemical operators because they do not see the microalgae, it cannot be disregarded that the measurement of biochemical biomarkers includes both the mussel digestive gland tissue and the microalgae.

Concluding remarks

According to the present study, feeding is essential for short-term experiments with mussels and the type of food is a major factor influencing a variety of biomarkers. Thus, Best Available Practices regarding the selection of food type and feeding strategy (regime, rations, etc.) need to be indentified in order to achieve reliable and comparable experimental data on the biological effects of pollutants. Within this context, commercial food based on frozen or frozen-dried diets seems not to be the best option for feeding during toxicological experiments, as it was

concluded previously for experimentation dealing with aquaculture production (Albentosa et al., 1997; Langdon et al., 1999; Fernandez-Reiriz et al., 2015). Dietary microalgae should be selected on the basis of the suitable dimension (size, volume, weight) of algal cells, their high digestibility and balanced nutritional value (Fernández-Reiriz et al., 2015). Likewise, it is worth noting that not all the live microalgae affect biomarkers and their measurement in the same way. For instance, *T. chuii* has a limited digestibility and associated long retention times in the gut (Becker et al., 1998; Rouillon and Navarro, 2003; Guéguen et al., 2008) and hence appears to influence nutritional status, oxidative stress and digestion processes in mussels; thus, affecting a variety of biomarkers and their measurement. On the other hand, the massive presence of *I. galbana* within digestive cells may hamper the measurement of cytochemical biomarkers and may render less reliable the results of biochemical biomarkers (as these can be attributed to both the mussel and the microalgae). Interestingly, results of chapter 4 indicated that at dietary cell concentrations of *I. galbana* of 2×10^4 cells/mL, the occurrence of microalgae within digestive cells is negligible; however, rations over 2×10^4 cells/mL are recommended and commonly used in physiological experiments (Langdon and Önal, 1999; Okay et al., 2006; Ibarrola et al., 2017). Further research is needed to optimize dietary food type, composition, regime and rations for toxicological experimentation. Meanwhile, it is highly recommended that research papers should include always a detailed description of the food type and feeding conditions in order to get a sustained understanding on the biological responses elicited by pollutants in mussels.

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CHAPTER 6

BEST AVAILABLE PRACTICES FOR DISCRIMINATING BASOPHILIC CELLS ON DIGESTIVE GLAND TISSUE SECTIONS IN ORDER TO DETERMINATE CHANGES IN CELL-TYPE COMPOSITION IN MUSSELS

This chapter has been presented at:

- 30th European Society for Comparative Physiology and Biochemistry (ESCPB) Congress, Barcelona 4-7/09/2016. Blanco-Rayón E, Soto M, Marigómez I, Izagirre U. Improved method for high-throughput tissue-level biomarkers in mussel digestive gland through specific labeling of basophilic cells. Poster communication.

ABSTRACT

Changes in the cell type composition in the digestive gland epithelium constitute a common and recognized biological response to stress in molluscs, especially in mussels. Conventionally, these changes are identified as alterations in the relative proportion of basophilic cells, determined on tissue sections stained with hematoxylin-eosine (H&E) and measured in terms of volume density of basophilic cells (Vv_{BAS}) after stereology. However, the identification and discrimination of basophilic cells may be difficult, especially when basophilic cells loss basophilia and the perinuclear area of digestive cells gains basophilia in circumstances of environmental stress. Thus, the present study aims to explore the best available practices to identify and discriminate basophilic cells on tissue sections of mussel digestive gland. In a first step, a screening of potentially suitable different staining methods was carried out; these included immunohistochemistry of ribosomal proteins, several trichrome staining methods (Masson's, Heiddenhain's AZAN, and Mallory's) and their variants, and toluidine-based stains. Second, the selected staining was optimized, including fixation process, and compared with the conventionally used H&E staining upon testing different field and experimental conditions with mussels, *Mytilus galloprovincialis*, as target. Finally, the selected method was tried in other marine mollusc species of interest for enrivonmental monitoring and ecotoxicological studies, say: *M. edulis*, *M. trossulus*, *Crassostrea gigas* and *Littorina littorea*. Toluidine-eosin (T&E) staining after fixation in 4% formaldehyde at 4°C for 24 hr was considered the best available method to identify and discriminate basophilic cells in the digestive gland of marine molluscs. It was successfully applied to determine Vv_{BAS} values both manually and after automatic image analysis. However, Vv_{BAS} values were always higher after T&E staining than after after H&E staining, conventionally used. Thus, further research is needed to render the Vv_{BAS} values determined after T&E staining comparable to those already published employing H&E staining.

Eskuragarriak diren jardunbide hoberenak zelula basofilikoak bereizteko liseri-guruineko ebakinetan, zelulen konposaketan aldaketak zehazteko muskuiluetan

Laburpena

Aldaketak liseri-guruineko epitelioaren zelulen konposaketan ohiko eta aitortua den estresari erantzun biologikoa da moluskuetan, bereziki muskuiluan. Konbentzioz, aldaketa hauek zelula basofilikoen proportzio erlatiboaren alterazio gisa identifikatzen dira, zeinak hematoxilina-eosinarekin (H&E) tindatutako ebakinetan zehazten da eta zelula basofilikoen dentsitate bolumetrikoa (Vv_{BAS}) bezala neurtzen da estereologikoki. Hala ere, zelula basofilikoen identifikazio eta bereizketa zaila izan daitezke, batez ere, zelula basofilikoek basofilia galtzen dutenean eta liseri-zelulen nukleoaren inguruko eremuak basofilia irabazten duenean, ingurumen-estresaren ondorioz. Beraz, lan honen helburua, muskuiluen liseri-guruineko ebakinetan zelula basofilikoak identifikatzeko eta bereizteko eskuragarriak diren jardunbide hoberenak aztertzea da. Lehenengo urrats batean, potentzialki egokiak ziren kolorazio metodo desberdinen azterketa burutu zen; hauen barne, proteina erribosomikoen immunohistokimika, zenbait kolorazio metodo trikromikoak (Masson-ena, Heiddenhain-en AZAN eta Mallory-rena) eta haien aldaera, eta toluidina oinarri gisa dituztenak. Bigarrenik, aukeratutako kolorazioa optimizatu zen, fixapen prozesua barne, eta konbentzioz erabili den H&E kolorazioarekin konparatu zen, *Mytilus galloprovincialis* muskuilua jomuga gisa zelaiko eta laborategiko egoera desberdinak aztertuz. Azkenik, ingumenaren jarraipenerako eta ikerketa ekotoxikologikoentzat interesgarriak diren beste itsasoko molusku espezietan, aukeratutako kolorazio metodoa frogatu zen, esate baterako: *M. edulis*, *M. trossulus*, *Crassostrea gigas* eta *Littorina littorea*. Itsasoko moluskuen liseri-guruinako zelula basofilikoak identifikatzeko eta bereizteko eskuragarria den jardunbide hoberena, 4°C-tara 24 ordutan zehar %4 formaldehidoan fixatuz egonez gero, toluidina-eosina kolorazioa (T&E) zela ondorioztatu zen. T&E modu egokian aplikatu zen Vv_{BAS} balioak zehazteko, bai eskuz, bai irudi-analisi automatikoa erabiliz. Hala ere, T&E erabiliz gero Vv_{BAS} balioak beti altuagoak ziren H&E erabiliz gero baino. Beraz, T&E-rako Vv_{BAS} balio zehatzak zehazteko ikerketa gehiago beharrezkoa da, konparazioak ahalbidetzeko dagoeneko argitaratuta dauden eta H&E erabili zuten lanekin.

Mejores prácticas disponibles para discriminar células basófilas en secciones de tejido de glándulas digestiva con el fin de determinar cambios en la composición celular en mejillones

Resumen

Los cambios en la composición célular en el epitelio de la glándula digestiva constituyen una respuesta biológica común y reconocida al estrés en los moluscos, especialmente en los mejillones. Convencionalmente, estos cambios se identifican como alteraciones en la proporción relativa de células basófilas, determinados en secciones de tejido teñidas con hematoxilina-eosina (H&E) y medidas estereológicamente en términos de densidad volumétrica de células basófilas (Vv_{BAS}). Sin embargo, la identificación y discriminación de las células basófilas puede ser difícil, especialmente cuando las células basófilas pierden basofilia y el área perinuclear de las células digestivas gana basofilia en circunstancias de estrés ambiental. Por lo tanto, el presente estudio tiene como objetivo explorar las mejores prácticas disponibles para identificar y discriminar células basófilas en secciones de tejido de la glándula digestiva de mejillón. Primeramente, se llevó a cabo un cribado de diferentes métodos de tinción potencialmente adecuados; estos incluyeron inmunohistoquímica de proteínas ribosomales, varios métodos de tinción tricrómicos (de Masson, AZAN de Heiddenhain y de Mallory) y sus variantes, y tinciones basadas en toluidina. Posteriormente, se optimizó la tinción seleccionada, incluido el proceso de fijación, y se comparó con la tinción H&E, usada convencionalmente, evaluando diferentes condiciones de campo y experimentales con mejillones, *Mytilus galloprovincialis*, como diana. Finalmente, se probó el método seleccionado en otras especies de moluscos marinos de interés para el seguimiento medioambiental y estudios ecotoxicológicos, como son: *M. edulis*, *M. trossulus*, *Crassostrea gigas* y *Littorina littorea*. La tinción toluidina-eosina (T&E), tras fijación en formaldehído al 4% a 4°C durante 24 horas, se consideró el mejor método disponible para identificar y discriminar las células basófilas en la glándula digestiva de los moluscos marinos. Se aplicó con éxito en la determinación de los valores de Vv_{BAS} tanto manualmente como usando análisis de imágen automático. Sin embargo, los valores de Vv_{BAS} siempre fueron más altos aplicando la tinción T&E que la tinción H&E, usada convencionalmente. Por lo tanto, más investigación es necesaria para determinar valores de Vv_{BAS} específicos de la tinción T&E, posibilitando así la comparación con estudios ya publicados que hayan aplicado la tinción de H&E.

INTRODUCTION

In marine mussels, the digestive gland diverticula are organized into clusters of blind ending alveolo-tubular units linked with the stomach by primary and secondary ducts (Owen, 1955). These alveolo-tubular units are constituted by a single epithelium comprised by two cell-types: digestive and basophilic cells (Morton, 1983). Digestive cells possess a highly developed endo-lysosomal system and are responsible for the intracellular digestion of food materials (Morton, 1983; Owen, 1973). Basophilic cells have a well-developed rough endoplasmic reticulum, which confers them their characteristic basophilia (Owen, 1973; Morton, 1983). Under normal physiological conditions, the digestive cells outnumber basophilic cells (6:1) but under different stress situations, the relative proportion of basophilic cells is augmented (Rasmussen et al., 1983; Cajaraville et al. 1990, Marigómez et al. 1990, Zaldibar et al., 2008). Thus, changes in the cell type composition in the digestive gland epithelium constitute a well-known biological response against environmental stress, commonly used as biomarker for biological effects assessment in pollution monitoring and experimental ecotoxicology (Lowe and Clarke, 1989; Cajaraville et al., 1990; Marigómez et al., 1990, 2006, 2013; Soto et al. 1996, 2002; Zorita et al., 2006, Garmendia et al., 2011; Brooks et al.. 2012; ICES, 2012; de los Rios et al., 2013, 2016; Lekube et al., 2014; Lopes et al., 2016).

These changes are usually measured as alterations in the relative proportion of basophilic cells in terms of volume density (Vv_{BAS}) determined onto tissue sections of the digestive gland stained with hematoxylin-eosine (H&E; Soto et al., 2002; ICES, 2012). After H&E staining, the nucleus and, often, the perinuclear area of digestive cells, and the nucleus and the cytoplasm of basophilic cells revealed purple color, due to their affinity for hematoxylin (basophilia). Therefore, the sole identification and discrimination of basophilic cells may be difficult, especially when these cells loss basophilia and the perinuclear area of digestive cells gains basophilia in circumstances of environmental stress (Marigómez and Ireland, 1989). Hence, a more specific labeling of basophilic cells in digestive epithelium of mussel digestive gland would contribute to an easier and more reliable quantification of changes in the cell type composition in the digestive epithelium of alveoli.

In the present study, different fixation and staining methods were tried in order to improve the specific counterstaining of basophilic cells in mussels. The goal was to explore the best available practices (BAPs) to clearly identify and discriminate basophilic cells on tissue sections of mussel digestive gland. In a first step, a screening of potentially suitable different staining methods was carried out; including immunohistochemistry of ribosomal proteins, several trichrome staining methods (Masson's, Heiddenhain's AZAN, and Mallory's) and their variants, and toluidine-based stains. Second, the selected staining was optimized and compared with the conventionally used H&E staining upon testing different field and experimental conditions with mussels, *Mytilus galloprovincialis*. Finally, the selected method was applied in other marine mollusc species of interest for environmental monitoring and ecotoxicological studies, say: *M. edulis*, *M. trossulus*, *Crassostrea gigas* and *Littorina littorea*.

MATERIAL AND METHODS

In the present study the used intertidal mussels, *Mytilus galloprovincialis*, (3.5–4.5 cm length) were always collected from the low tide-mark level (0.5-1.0 m) in a relatively clean locality (Plentzia, 43° 24' N, 2° 56' W) and time below detailed.

In all the cases, the digestive gland was dissected out, fixed for 24 hr at 4°C in 4% formaldehyde containing seawater as buffer, dehydrated in a graded ethanol series and embedded in paraffin.

Sections (5 µm thick) were cut in a Leica RM2125 RTS microtome and mounted on albumin coated slides for histology and on silanised slides for immunohistochemistry. Sections were stored at room temperature (RT) until staining. Further on, sections were stained using a series of staining methods and their variants. Before staining, the sections had been dewaxed in xylene, rehydrated in a graded ethanol series and brought to distilled water (hydrated sections).

Micrographs were obtained with an Olympus camera attached to an Olympus BX50 light microscope (Olympus Ltd., Tokyo, Japan).

Screening the most suitable staining method to identify basophilic cells

Control and stressed mussels

Intertidal mussels, *M. galloprovincialis,* were collected in November 2014. Animals (n=20) were immediately (within 30 min upon collection) transferred to the laboratory air-exposed in wet boxes, at ambient temperature. Ten mussels were sacrificed and processed immediately after arrival at the Plentzia Marine Station (PiE-UPV/EHU). A second set of 10 mussels were kept exposed to air at 18°C in wet chambers during 20 d and then sacrificed and processed. This exposure-to-air mimicked the procedure and conditions of the commonly used Stress-on-Stress toxicity assay (Smaal et al., 1991; Eertman et al., 1993), thus provoking a non-specific stress (non-chemically induced).

Different staining methods were applied in order to identify basophilic cells in digestive gland tissue sections. Since basophilic cells can be identified by the basophilia that gives them their name, hematoxylin-eosin (H&E) was used, according to Martoja and Martoja (1967), as a reference stain. This basophilia has been attributed to the dominant presence of ribosomes in the cytoplasm of these cells, most relevantly as component of the vast rough endoplasmic reticulum that occupies the majority of the cell volume (Owen, 1973). For this reason, specific immunohistochemical methods for ribosomal proteins were also applied. Finally, several topographical histological stains and their existing and novel variants were also applied aimed at clearly discriminate basophilic cells from other cell types of the digestive gland

Immunohistochemistry of ribosomal proteins

Sections (hydrated) were washed in phosphate buffered saline (PBS). Endogenous peroxidase activity was quenched by shortly incubating the sections in 3% H_2O_2. Then, sections were washed in PBS and incubated at RT for 1 hr in blocking buffer, consisting of 1% bovine

serum albumin diluted in PBS. After a brief rinse in PBS, sections were incubated 1 hr at RT or overnight at 4°C with an appropriate dilution of the antibody in PBS (1:100). Anti-Ribosomal protein S16 (Sigma, AV33535) and anti-Ribosomal protein L8 (Sigma, AV40215) were applied. After several rinses in PBS, immunocomplexes were visualized with the biotin-streptavidin-peroxidase technique, using the ExtrAvidin peroxidase kit (Sigma) as follows. Sections were first incubated for 30 min at RT with the biotinylated goat anti-rabbit IgG supplied in the kit, diluted 1:20 in PBS. After several rinses in PBS sections were incubated for 20 min at RT with the streptavidin-peroxidase conjugate, also supplied in the kit, diluted 1:20 in PBS. After a brief rinse in PBS, the visualization of peroxidase activity was achieved using a chromogen solution containing either 3-amino-9-ethyl-carbazole (AEC) (0.5 mg/mL), H_2O_2 (0.03%), ethanol (10%) and 20 mM citrate buffer, pH 5.1, or 3.3′-diaminobenzidine (DAB) (60 mg/mL), H_2O_2 (0.03%) and PBS, at RT. Finally, after a brief rinse in tap water, sections were counterstained with hematoxylin or fast green, washed in running tap water and mounted in Kaiser's glycerol gelatin. Control sections were incubated only with PBS.

Topographical histological stains

Besides H&E other topographical stains were carried out, say: (a) the Masson's trichrome with aniline blue (Masson, 1929; Liliie, 1940, 1945, 1951); (b) the Goldner's variant of the Masson's trichrome using light green (Masson, 1929; Goldner, 1938; Lillie, 1940, 1951); (c) the Heidenhain's AZAN trichrome (Mallory, 1938); (d) the Mallory's trichrome (McFarlane, 1944; Masson, 1929; Mallory 1936; Puchtler and Isler, 1958) and one variant in which acid fuchsine was replaced by 1% toluidine in distilled water; and (e) the toluidine staining (Love and Liles, 1959; Pearse, 1980; Sridharan and Shankar, 2012) and its several variants, which included toluidine-eosin (T&E) and toluidine-FCF (Fast Green) staining.

In the case of T&E staining, hydrated sections were treated for 5 min in a variety of toluidine solutions of different pH: (a) 1% toluidine diluted in distilled water (pH 5.66); (b) 1% toluidine + 1% azure-II + 1 % sodium tetraborate in distilled water (pH 8.41); (c) 1% toluidine in 70% v/v ethanol (pH 4); and (d) 0.1% and 1% toluidine in McIlvaine's buffer (pH 2.8). After washing in tap water, sections were counterstained with eosin for 15 sec, washed in tap water, dehydrated in ascending grade ethanol series, cleared in xylene and mounted in DPX.

In the case of toluidine-FCF, hydrated sections were treated for 5 min in either 1% toluidine diluted in distilled water (pH 5.66) or 1% toluidine + 1% azure-II + 1 % sodium tetraborate in distilled water (pH 8.41), washed in tap water, counterstained with either 1% FCF for 1 min, washed in tap water, dehydrated in ascending grade ethanol series, cleared in xylene and mounted in DPX. In the first trials, toluidine was applied according to the classical toluidine staining procedure (Mercer, 1963; Burns, 1978). Thus, hydrated sections were placed on a hot plate and covered with one drop of toluidine until the drop was bordered by a green ring. However, the tissue integrity in t paraffin embedded sections was clearly compromised

and therefore, for all the tests we applied toluidine staining at room temperature.

Optimisation of the T&E stain

Sample fixation effect on toluidine & eosin staining

Digestive gland of intertidal *M. galloprovincialis*, collected in autumn 2017 were fixed at 4ºC in different ways: (a) in 4% formaldehyde in filtered 0.2 µm seawater (Dorothy et al. 2004; AboElkhair et al., 2009; Howat and Wilson, 2014) for 3 and 9 d; (b) in Carnoy's fixative for 1 hr (Carnoy, 1887; Howat and Wilson, 2014); and (c) in Davidson's fixative for 48 hr (Moore and Barr, 1954; Dorothy et al. 2004). After fixation, samples were processed and stained with T&E (1% toluidine diluted in distilled water), as above detailed.

Determination of toluidine rinse time

Hydrated sections were rinsed in toluidine solutions (1% in distilled water) for 5, 10, 15, 20 and 30 min, washed in tap water, rinsed in eosin for 15 sec, washed in tap water, dehydrated in ascending grade ethanol series, cleared in xylene and mounted in DPX.

H&E versus T&E staining of basophilic cells

In order to confirm that T&E staining mark the basophilic cells, consecutive 1 µm tissue sections of *M. galloprovincialis* digestive gland were stained with H&E (conventional) and T&E (new) and then examined under light microscope.

Then, how the Vv_{BAS} values and their responsiveness vary depending on the staining method (H&E vs. T&E) was investigated using mussel digestive gland samples obtained in three preceding experimental setups. The first one intertidal *M. galloprovincialis* (n=6/sample) from a relatively clean locality (Plentzia) and from one chronically polluted locality (Arriluze) were collected in October 2015, immediately transferred to the laboratory (as detailed for control mussels in the above paragraph) and sacrificed. In the second experimental setup, intertidal *M. galloprovincialis* were collected from Plentzia in September 2017, acclimatized to controlled laboratory conditions (20±1°C; 16:8 L:D cycle) for 7 d in absence of food supply and distributed in 3 experimental groups (10 mussels in 2 L tanks) exposed to 3 doses of waterborne Cd (0% (control), 80 µg/L, 800 µg/L) for 14 d (fed *I. galbana*: 20000 cells/mL/tank/day). At the end experimentation period, 6 mussels per treatment were sacrificed and their digestive gland was processed for histological analyses. The third and last experimental setup, is detailed in Chapter 5. Briefly, intertidal *M. galloprovincialis* were collected from Plentzia in September 2014 and acclimatized to controlled laboratory conditions for 7 d in absence of food supply with filtered 0.2 µm seawater. After acclimatization, mussels were fed ad libitum for 1 wk with 4 different microalgae diets (*I. galbana*, *Tetraselmis chuii*, a mixture of *I. galbana* and *T. chuii*, a commercial food). At the end experimentation period, 6 mussels per treatment were sacrificed and their digestive gland was processed for histological analyses. Additional intertidal *M. galloprovincialis* were collected occasionally from the same site in Plentzia along 2016 and 2017, for different histological processing trials (e.g. to

assess the effects of tissue fixation methods); these mussels were also processed as detailed for control mussels in the above paragraph.
In all the samples of the three preceding experimental setups, the volume density of basophilic cells (Vv_{BAS}; in $\mu m^3/\mu m^3$) was determined both after H&E and T&E staining in the digestive gland of mussels subject to diverse experimental conditions. Counts were made in 3 zones of each digestive gland and in 6 mussels per experimental group. Slides were viewed at 40× magnification using a drawing tube attached to a Nikon Optiphot microscope. A Weibel graticule (multipurpose test system M-168; Weibel, 1979) was used, and hits on basophilic cells (B), on digestive cells (D), and on other components of the digestive gland tissue and outside were recorded to calculate Vv_{BAS} as $Vv_{BAS} = B/(B+D)$.

IBM® SPSS® Statistics ver. 22.0.0.0 (IBM Corp., Armonk, NY, USA) was employed for statistical analyses of Vv_{BAS} values. Homogeneity of variance (Levene's test) and normality of data (Kolmogorov-Smirnov's test) were tested before statistical analyses (Sokal and Rohlf, 1995). Statistically significant differences between groups were tested according to the Student's t-test or the Duncan's post-hoc test based on one-way analysis of variance (1-way ANOVA; $p<0.05$).

T&E staining in other sentinel marine molluscs

Mussels, *M. edulis* (3.5-4.5 cm length), were acquired at a local shellfish supplier in Rissa (Norway) in October 2016 and maintained as stock in the laboratory in a flow-through system at 10ºC and with regular food supply. They were sacrificed and when required for histological analyses. Mussels, *M. trossulus* (1-2 cm length), were collected in Tvärminne (Finland) in November 2016, sacrificed *in situ* and processed for histology (tissue blocks were transported from Finland to PiE-UPV/EHU). Oysters, *Crassostrea gigas* (7–8 cm length), were collected from an oyster farm (San Vicente de la Barquera, Cantabria, Spain) in September 2017, transported to the laboratory, sacrificed and processed for histology. Periwinkles *Littorina littorea* (20-30 mm height), from Scrabster (Scotland) were purchased from a commercial dealer (Arrainko SL, Mercabilbao) in October 2015, transported to the laboratory, sacrificed and processed for histology. Sections were stained with T&E.

RESULTS AND DISCUSSION

Screening the most suitable staining method to identify basophilic cells

In mussel digestive gland stained with H&E, the nuclei of the majority of the cells observed and the whole basophilic cells appeared dark blue or violet (Fig. 1A), as they are acidic (basophilic) in nature. In contrast, connective tissue adipogranular cells were stained in red by the action of eosin, and connective tissue fibers and the cytoplasm of e.g. hemocytes and digestive cells in pink; unlike in the case of basophilic cells in which the cytoplasm reacted with hematoxylin (Figs. 1A and 1B). This general staining pattern remained unchanged in mussels subject to air-exposure stress for 20 d (Fig. 1B).

After immunohistochemistry using anti-RPS16 and anti-RPL8 antibodies, a general red labeling was observed, which was

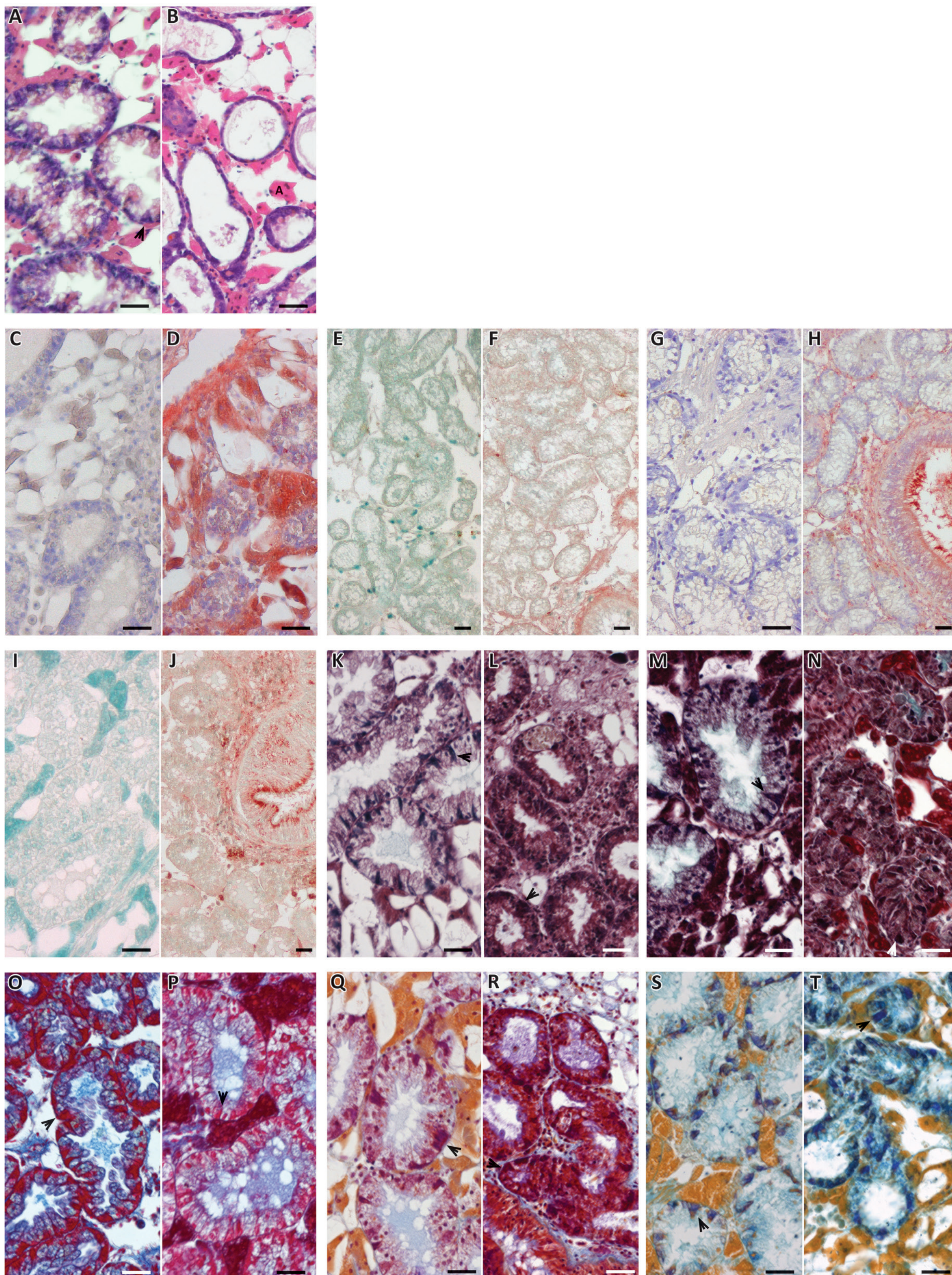

Fig. 1. Micrographs of different stainings in mussel digestive gland. Arrow: basophilic cell. A: adipogranular cell. Scale bar: 30 µm. (A, B) Hematoxylin & eosine. (C) Control section of anti-Ribosomal protein S16 antibody, counterstained with hematoxylin. (D) Immunohistochemistry, using anti-Ribosomal protein S16 antibody, counterstained with hematoxylin. (E) Control section of anti-Ribosomal protein S16 antibody, counterstained with fast green. (F) Immunohistochemistry, using anti-Ribosomal protein S16 antibody, counterstained with fast green. (G) Control section of anti-Ribosomal protein L8 antibody, counterstained with hematoxylin. (H) Immunohistochemistry, using anti-Ribosomal protein L8 antibody, counterstained with hematoxylin. (I) Control section of anti-Ribosomal protein L8 antibody, counterstained with fast green. (J) Immunohistochemistry, using anti-Ribosomal protein L8 antibody, counterstained with fast green. (K, I) Masson Trichrome with aniline blue. (M, N) Masson Trichrome with light green. (O, P) Azan Trichrome. (Q, R) Mallory Trichrome. (S, T) Sections incubated in 1% toluidine diluted in distilled water on a hot plate until appear a green ring around the drop dye, before Mallory trichrome stain without fucsina.

apparently non-specific and was especially remarkable in the connective tissue, without any clear labeling in basophilic cells (Figs. 1D, 1F, 1H, and 1J), thus it was considered unproper for identifying basophilic cells.

When Masson's trichrome with aniline blue was applied, nuclei and basophilic cells appeared stained in dark purple, the cytoplasm of e.g. digestive, duct and stomach cells and connective tissue fibers in dark pink, hemocytes, muscle fibers and connective tissue adipogranular cells in red, and collagen fibers in blue (Figs. 1K and 1L). However, the digestive gland epithelium was darker in mussels subject to air-exposure stress (Fig. 1L) than in control mussels (Fig. 1K), thus making difficult the identification of basophilic cells. The Masson's trichrome with light green provided very similar results with the exception that collagen was readily distinguished in green color (Figs. 1M and 1N). Likewise, as in the case of the aniline blue based Masson's trichrome, the digestive gland epithelium of mussels subject to air-exposure stress (Fig. 1N) was darker than in control mussels (Fig. 1M), and therefore basophilic cells were less evident.

After Heidenhain's AZAN trichrome staining, nuclei, basophilic cells and hemocytes presented a reddish color, connective tissue adipogranular cells were dark red and collagen fibers blue. The digestive gland epithelium of mussels subject to air-exposure stress (Fig. 1P) was less stained than in control mussels (Fig. 1O) and, overall, presented a distinct staining pattern. The lost of reactivity was especially marked in basophilic cells, which appeared practically devoid of any red color (Fig. 1O).

After Mallory's trichrome staining, nuclei and basophilic cells were stained in red, which rendered them difficult to be discriminated. Connective tissue fibers were stained in grey, hemocytes and muscle fibers in orange, and collagen in blue (Figs. 1Q and 1R). The color of the connective tissue adipogranular cells varied from orange (control) to bluish-grey (air-exposure) depending on mussel condition (Figs. 1Q and 1R). The digestive gland epithelium was darker in mussels subject to air-exposure stress (Fig. 1R) than in control mussels (Fig. 1Q), to the point that basophilic cells were absolutely indistinguishable. When the fuchsine was replaced by toluidine in the Mallory's trichrome method, basophilic cells were more clearly identified in purple, whereas digestive cell cytoplasm was stained in light blue and the nuclei, especially in digestive cells, and the connective tissue adipogranular cells in gold yellow (Fig. 1S). However, these results were only obtained in control mussels, whereas in mussels subject to air-exposure stress basophilic cells were not so clearly distinguished (Fig. 1T).

Regarding the T&E staining method, all the different toluidine solutions herein investigated succeeded in revealing basophilic cells in a more or less distinguishable dark blue color; however, they differed in their staining intensity (Fig. 2A-2D). Likewise, connective tissue adipogranular cells and hemocytes were always stained in red and the cell cytoplasm and connective tissue fibers in pink (Fig. 2A-2D). The weakly acidic toluidine solution (1% toluidine in distilled water) showed quite a good staining intensity, which was clear and clean (Fig. 2A). In contrast, the basic toluidine mixture (1%toluidine+1%azure-II+1%sodium tetraborate in distilled water) produced apparently a higher staining intensity but the

final result was unclean (Fig. 2B). Toluidine solutions in McIlvaine's buffer (strongly acid) were able to discriminate basophilic cells; however, they stained stronger the periphery of basophilic cells than their cytoplasm (Fig. 2C). Also, it is worth noting that the degree of reactivity was not seemingly different between solutions of 0.1% and 1% toluidine in McIlvaine's buffer. On the other hand, the moderately acid solution of 1% toluidine diluted in 70% ethanol showed a very weak staining intensity of basophilic cells (Fig. 2D). Overall, it seems that toluidine based dyes with pH above 5 stained strongly basophilic cells, suggesting that above that pH the carboxyl groups of proteins become available for the combination with dye (Herrmann et al., 1950).

Comparable results were obtained when applying toluidine in combination with FCF instead of eosin. Thus, all the toluidine solutions stained basophilic cells in dark blue; both the solution of 1% toluidine in distilled water and the mixture of toluidine, azure II, and sodium tetraborate marked strongly basophilic cells (Figs. 2E, 2F). However, this stain did not differentiate components of the digestive gland other than connective tissue adipogranular cells, which appeared colored in light green (Figs. 2E, 2F).

In view of the present results it is concluded that the T&E staining is the most adequate to identify and discriminate basophilic cells irrespective of the mussel health status (e.g. both in control mussels and in mussels subject to air-exposure stress). Moreover, the identification of components in the tissue was easy due to the obtained good counterstain with eosin. Indeed, though all the trichrome stains screened herein were suitable to clearly reveal basophilic cells, they fail in indetifying basophilic cells in stressed mussels.

Optimisation of the T&E stain

The studied fixatives are commonly employed when histology is used for environmental health assessment and for disease diagnosis of molluscs (e.g. in aquaculture or public health) (Dorothy et al., 2004; Kim et al., 2006; ICES, 2012). Interestingly, the staining pattern and reactivity of the T&E stain in mussel digestive gland varied largely depending on the fixative employed. In tissue fixed in 4% formaldehyde for 24 hr only the nucleus (and especially the nucleolus) of basophilic cells was stained (Fig. 2A); after 3 d fixation, however, also the nucleus of digestive cells was stained, though lightly (Fig. 2G). More strikingly, after 10 d fixation in 4% formaldehyde the digestive cell nucleus was much more strongly stained, eosin counterstaining was low and the nuclei were difficult to distinguish from reactive basophilic cells (Fig. 2H). In digestive gland tissue fixed with Davidson's and Carnoy's fixatives, digestive cell nucleus was already strongly reactive after T&E staining, rendering quite difficult the clear distinction of basophilic cells (Figs. 2I and 2J).

In agreement, it is known that toluidine binding endpoints change depending on the fixative employed (Love and Liles, 1959; Spicer, 1963; Wingren and Enerbäck, 1983). According to our results, fixation in 4% formaldehyde at 4°C for 24 hr seems to be the best method for identification and discrimination of basophilic cells in mussels digestive gland using T&E stain; as

no reaction was observed in the nuclei but in the nucleolus and cytoplasm of basophilic cells. This can be attributed to the richness in RNA and related acidic proteins in this particular cell type. Indeed, it has been demonstrated that formaldehyde increases the amount of toluidine-binding by the RNA component of the chromatin without effect on the DNA (Love and Liles, 1959). This can be explained because DNA-protein complexes are more resistant to the action of formalin (inactivation of amino groups

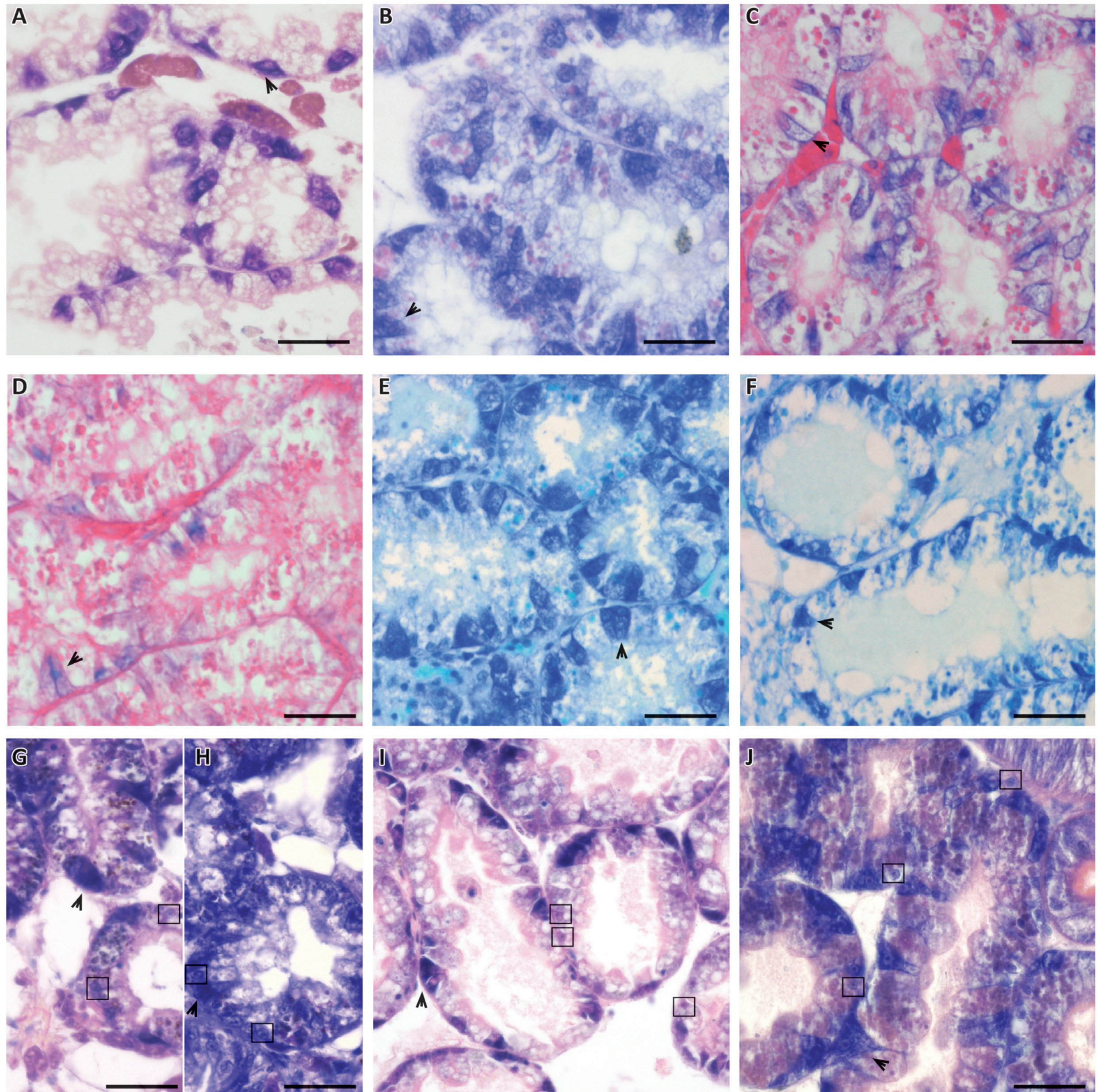

Fig. 2. Micrographs of mussel digestive gland stained with several variant of toluidine in the staining and in the fixation. Arrow: basophilic cell. Square: nucleus of non-basophilic cells. Scale bar: 30 µm. (A-D) Micrographs of mussel digestive gland fixed at 4° in 4% formaldehyde in filtered 0.2 µm seawater for 24 hr and stained with different toluidine and eosine: A) 1% toluidine diluted in distilled water, B) 1% toluidine, 1% azure II, 1 % de sodium tetraborate and distilled water, C) 0.1% toluidine diluted in MacIlvaine's buffer and D) 1% toluidine diluted in 70% alcohol. (E-H) Micrographs of mussel digestive gland fixed at 4° in 4% formaldehyde in filtered 0.2 µm seawater for 24 hr and stained with different toluidine (without or with warming) and Fast Green: (E) 1% toluidine diluted in distilled water at room temperature. (F) 1% toluidine, 1% azure II, 1 % de sodium tetraborate and distilled water at room temperature. (G-J) Micrographs with 1% toluidine diluted in distilled water and eosine; previously fixed at 4ºC in different ways: in 4% formaldehyde in filtered 0.2 µm seawater for 3 (G) and 10 (H) days; (I) in Davidson's fixative for 48 hr; and (J) in Carnoy's fixative for 1 hr.

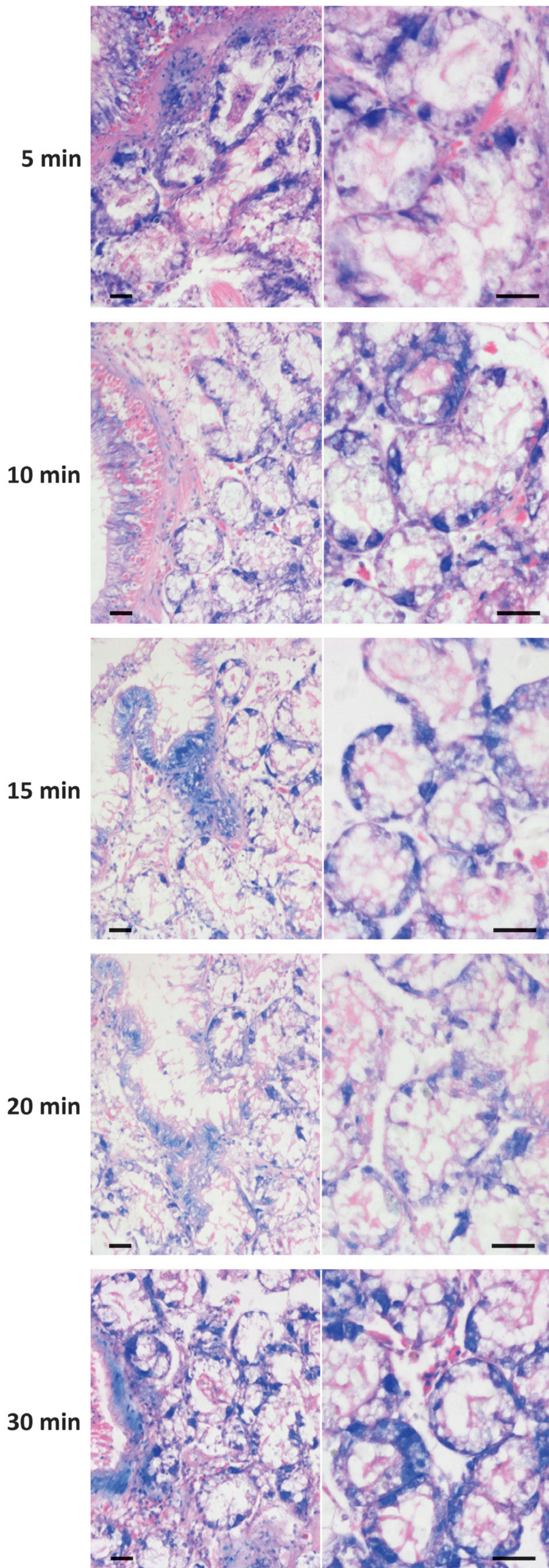

Fig. 3. Serial micrographs examined for the time determination of toluidine in toluidine & eosin staining in mussel digestive gland. 10 min was selected as proper incubation time for toluidine. Scale bar: 30 µm.

by formaldehyde an increasing number of nucleic acid phosphoryl groups to bind toluidine) than RNA-protein complexes (Fraenkel-Conrat, 1954, Love and Liles, 1959). The specific binding of toluidine to the nucleolus of basophilic cells could be due to the specific interaction of nucleic acids and proteins (Herrmann et al., 1949). Formaldehyde blocks the positively charged protein groups thus rendering toluidine more easily available for negatively charged molecules such as ribosomes and increasing the staining intensity of basophilic cells (Love and Liles, 1959; Spicer, 1963). Carnoy's fixative, in contrast, is an alcoholic fixative that does not block positively charged protein groups and therefore the resulting staining intensity of basophilic cells is much weaker.

An important factor to consider for optimizing the T&E staining is the time required to get stained in the toluidine solution for an optimal identification of basophilic cells. These resulted strongly stained already after 5 and 10 min treatment in the toluidine solution (Fig. 3). Moreover, at longer times (15 min and beyond) also other components of the digestive gland tissue exhibited a great reactivity. Thus, the stomach epithelium and connective tissue fibers were also quite strongly stained and as result, the identification of basophilic cells was less clear.

H&E *versus* T&E staining of basophilic cells

Based on the examination of consecutive 1 µm tissue sections, the same alveoli were compared after staining with H&E (conventional) and T&E (new) stains. Clearly, the same basophilic cells were stained with H&E and T&E, more clearly and cleanly after T&E staining (Fig. 4).

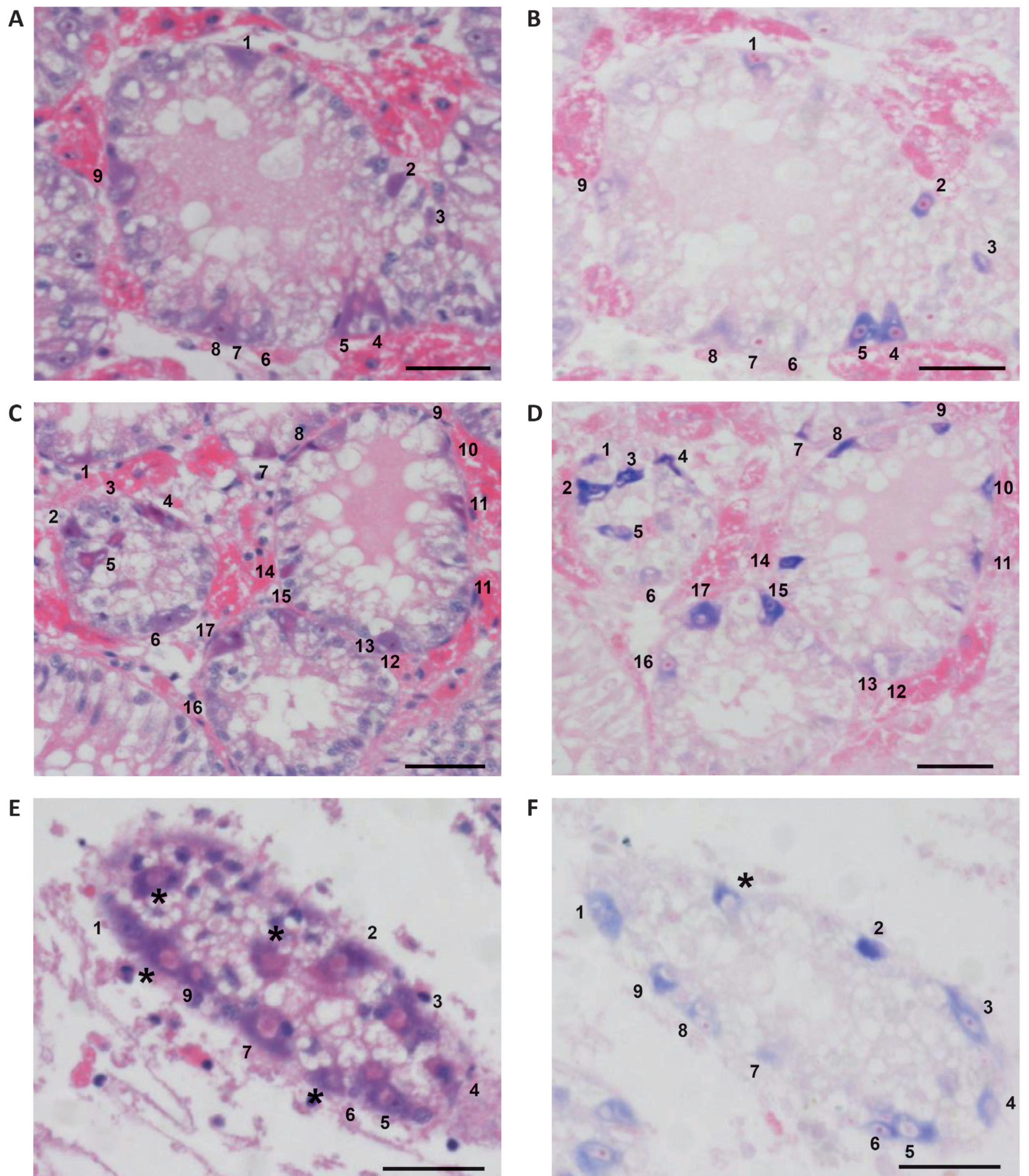

Fig. 4. Comparison of basophilic cells number between 1 µm section stained with hematoxylin-eosin (A, C, E,) or toluidine-eosin (B, D, F) in mussel digestive gland. The numbers show the basophilic cells that are marked in both stains. The asterisks show the basophilic cells that are not marked in both stains. Scale bar: 30 µm.

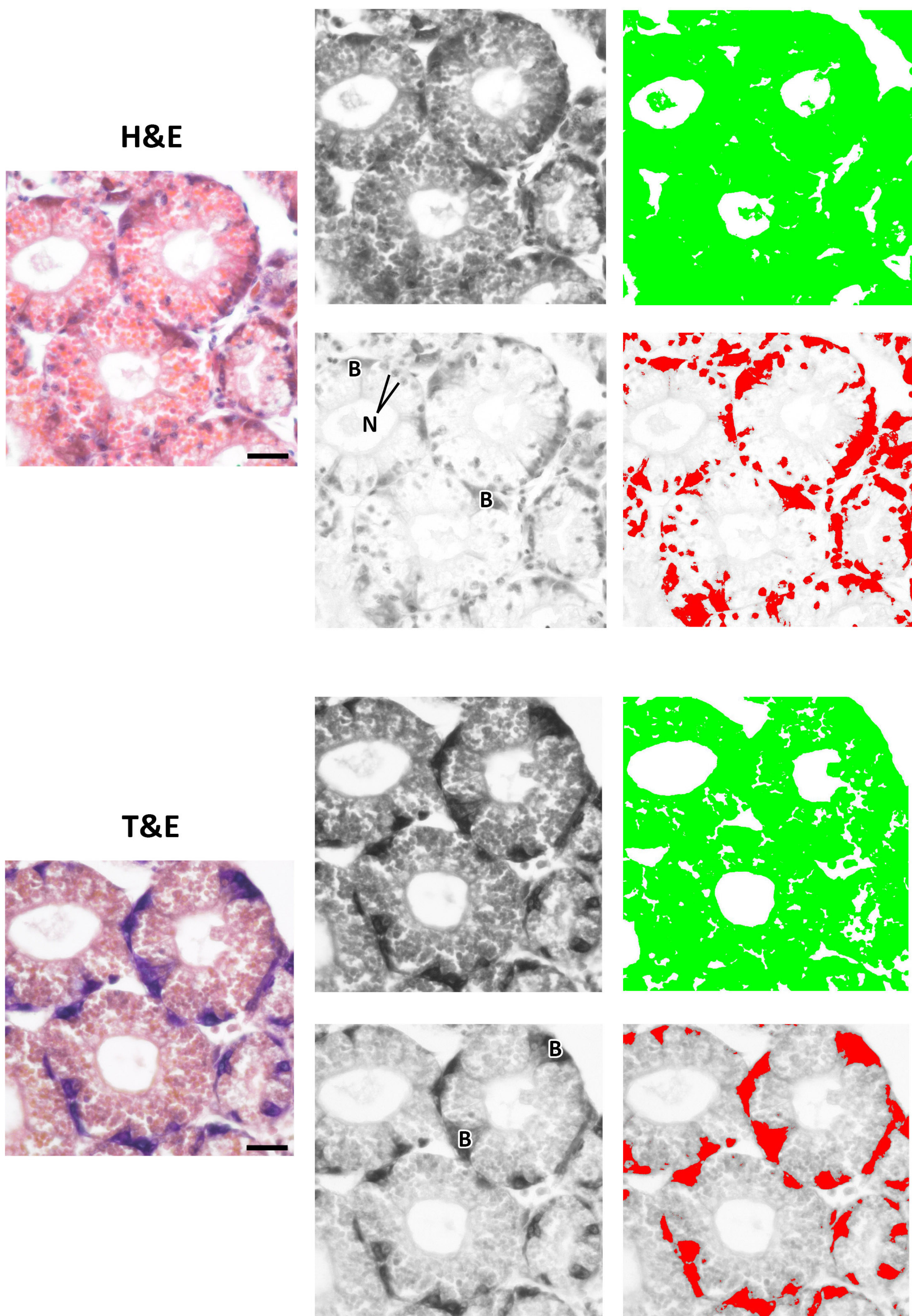

Fig. 5. Image analysis using Image J of mussel digestive gland stained with hematoxiline-eosine (H&E) and toluidine-eosine (T&E), in order to mark basophilic cells and epithelium. B: basophilic cells. N: nucleus.

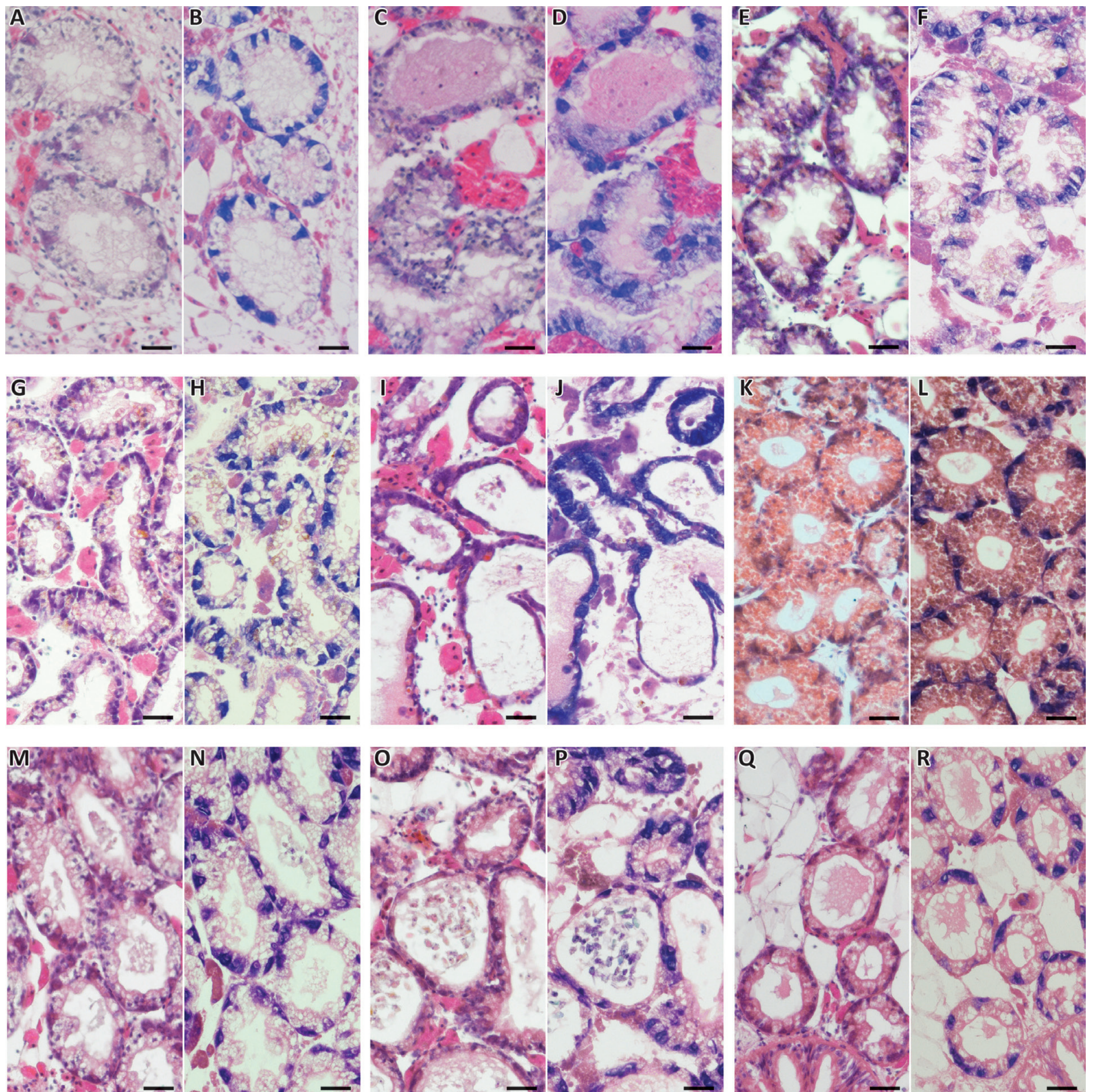

Fig. 6. Comparison of basophilic cells labeling between hematoxylin-eosin (H&E) and toluidine-eosin (T&E) in the set of samples measured. Scale bar: 30 µm. Micrograph of mussel digestive gland (MDG) from Plentzia, stained with H&E (A) and T&E (B). Micrograph of MDG from Arriluze, stained with H&E (C) and T&E (D). Digestive gland of mussels which were fed with Isochrysis galbana ad libitum for 1 week, stained with H&E (E) and T&E (F). Digestive gland of mussels which were fed with Tetraselmis chuii ad libitum for 1 week, stained with H&E (G) and T&E (H). Digestive gland of mussels which were fed with I.galbana and T.chuii microalgae mixture ad libitum for 1 week, stained with H&E (I) and T&E (J). Digestive gland of mussels which were fed with commercial food ad libitum for 1 week, stained with H&E (K) and T&E (L). Digestive gland micrographs of mussels exposed to different Cd concenterations during 14 days: control (0%) (H&E, M; T&E, N); 80 µg/L (H&E, O; T&E, P); and 800 µg/L (H&E, Q; T&E, R).

Moreover, T&E staining provides images of better quality than H&E staining in order to apply quantitative automatic image analysis for the calculation of Vv_{BAS} in environmental toxicological studies. Thus, as shown in Fig. 5.

Regarding the quantification of Vv_{BAS} in the different field and laboratory experiments used as study cases herein, it is worth noting that in all of them the T&E staining succeeded in providing a stronger reactivity in basophilic cells than H&E staining (Fig. 6). As a result, the stereological data recording was easier and faster. When mussels from Plentzia and Arriluze (field study) were compared, Vv_{BAS} did not show significant differences between mussels from both localities, however, Vv_{BAS} were higher after T&E staining than after H&E staining (Table 1; Figs. 6A–6D). In Cd-exposed mussels, Vv_{BAS} was higher in mussels exposed to 800 µg Cd/L than in control and in mussels exposed to 80 µg Cd/L, both after H&E and T&E staining; however, as in the previous case, higher Vv_{BAS} were recorded in all the experimental groups after T&E staining than after H&E staining (Table 1; Figs. 6E-6J). In mussels fed using four different diets, Vv_{BAS} results were different depending on the staining (Table 1; Figs. 6K–6R). In samples stained with H&E, only anecdotal changes were recorded (Table 1); thus, slightly lower Vv_{BAS} was found in *T. chuii* fed mussels than in those fed the commercial food (always within a narrow range of change). In contrast, in T&E stained samples, mussels fed *I. galbana* showed much lower Vv_{BAS} than the rest of the experimental groups (Table 1).

Both after H&E and T&E staining, there existed differences between experimental groups in Vv_{BAS} values; which were more marked after T&E staining than after H&E staining. As a general rule, Vv_{BAS} was increased in stressed mussels, as it would be expected according to the existing state of the art in environmental toxicology (ICES, 2012). However the Vv_{BAS} values obtained after T&E were much higher

Table 1. Vv_{BAS} mean values and error standard of the 3 set of samples measured to compare H&E and T&E stains. Capital letters indicate differences between both staining methods in the same experimental group according to Student's t-test (p<0.05). Lowercase letters indicate differences between groups stained in the same way according to Student's t-test and one-way ANOVA (p<0.05).

Sample set	Experimental group	Vv_{BAS} (µm³/µm³)	
		H&E	**T&E**
1	Plentzia	0.08 ±0.01 ^{A}	0.17 ±0.01 ^{B}
	Arriluze	0.07 ±0.01 ^{A}	0.16 ±0.02 ^{B}
2	Control	0.09 ±0.01 $^{a, A}$	0.24 ±0.02 $^{a, B}$
	80 µg/L Cd	0.12 ±0.02 $^{a, A}$	0.26 ±0.01 $^{a, B}$
	800 µg/L Cd	0.38 ±0.07 ^{b}	0.46 ±0.03 ^{b}
3	*I. galbana*	0.11±0.01 ^{ab}	0.10 ±0.02 ^{a}
	T. chuii	0.07 ±0.01 $^{a, A}$	0.18 ±0.04 $^{b, B}$
	I + T	0.09 ±0.01 $^{ab, A}$	0.24 ±0.03 $^{b, B}$
	Commercial food	0.12 ±0.01 $^{b, A}$	0.24 ±0.03 $^{b, B}$

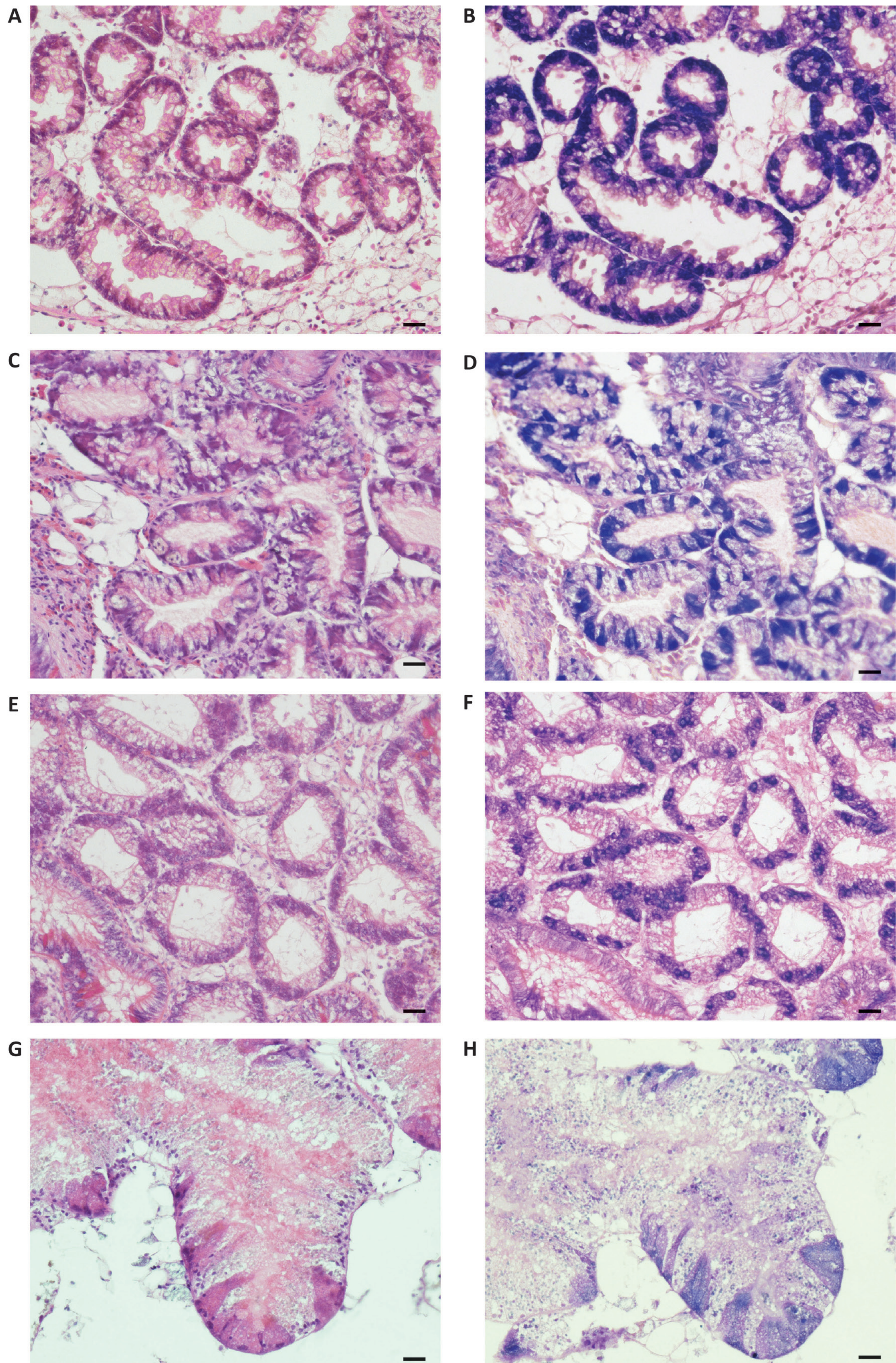

Fig. 7. Digestive gland micrographs of mussels *Mytilus edulis* (A, B), *M. trossulus* (C, D), oyster *Crassostrea gigas* (E, F) and periwinkle *Littorina littorea* (G, H) stained with hemaxylin-eosin (A, C, E, G) or toluidine-eosine (B, D, F, H). Scale bar: 30 µm.

than after the H&E method conventionally used to determine Vv_{BAS} in environmental toxicology. Thus, currently recognised Vv_{BAS} critical values (background, Vv_{BAS}<0.12 $\mu m^3/\mu m^3$; stress situation, Vv_{BAS}>0.18 $\mu m^3/\mu m^3$) (Marigómez et al., 2006; ICES, 2012) cannot be applied when T&E is used to calculate Vv_{BAS}, and therefore these critical values need to be established again for the new staining method.

T&E staining in other sentinel marine molluscs

Basophilic cells of *M. edulis, M. trossulus, C. gigas* and *L. littorea* were visualized more clearly using T&E than H&E (Fig. 7). Moreover, unlike after H&E staining, digestive cell nuclei and other confounding components of the digestive gland that might interfere with the clear identification of basophilic cells were not stained by T&E in any of the investigated species. Thus, the same approach used herein in *M. galloprovincialis* is suitable to be applied in other species of interest in marine pollution studies and in toxicological studies based on biomarkers (Bryan et al. 1983; Zaldibar et al., 2007; Kim et al., 2008; Brooks et al., 2012; David et al., 2012; Beyer et al., 2017).

Concluding remarks

T&E staining after fixation in 4% formaldehyde at 4°C for 24 hr is a highly valuable method to identify and discriminate basophilic cells in the digestive gland of marine molluscs. The reactivity and quality of staining are much better than in the case of the H&E staining used hitherto. As such, T&E staining was successfully applied to determine Vv_{BAS} values after stereology, with such a strong labeling that could be possible its use form automatic image analysis. However, current Vv_{BAS} critical levels correspond to measurements obtained after H&E staining and are therefore not applicable after T&E staining. Thus, future research is needed to adapt the environmentally relevant critical values of this parameter after calibration with studied previously carried out using H&E staining. In summary, formaldehyde fixation and T&E staining are recommended as the best available practices to identify and discriminate basophilic cells for determining changes in cell-type composition in mussel digestive gland epithelium with Vv_{BAS} as endpoint (Apendix).

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V. GENERAL DISCUSSION, CONCLUSIONS AND THESIS

GENERAL DISCUSSION

Differences in biomarkers between the *Mytilus* species have been researched only occasionally (Brooks et al., 2015). Species differences in genotoxic response have been indicated with different background assessment criteria (BACs) for the three *Mytilus* species with respect to the frequency of micronuclei in haemocyte cells (ICES, 2011). Histological parameters of reproductive condition were different between *M. edulis, M. galloprovincialis* and their hybrids sampled from the same location in the UK (Bignell et al., 2008). Likewise, differences in reproductive strategy (i.e. spawning times) of *M. edulis* and *M. galloprovincialis* have been found to occur (Hilbish et al., 2002). Such differences in spawning are likely to impact energy budgets between the species and influence general fitness at different times of the year. Hence, there is increasing evidence to suggest that the biological responses between the *Mytilus* species do exist, although there remains a lack of controlled laboratory investigations, which are needed to measure the full extent of these potential differences. Thus, the main aim of Chapter 1 was to determine differences in lysosomal responses along three *Mytilus* species, *M. edulis*, *M. trossulus* and *M. galloprovincialis,* following a 21 d exposure to waterborne Cu concentrations. This was a part of more extended study (Brooks et al., 2015). Lysosomal biomarkers, known to be responsive to copper exposure in mussels (Viarengo et al., 1985), are ICES core biomarkers for biological affects assessment in marine pollution monitoring programmes. It is clear from this study that differences in lysosomal responses occur between the three *Mytilus* species, which may influence the assessment of the environmental health status in marine pollution programmes; particularly knowing that in a large extent of geographical areas around the European coast, two or three of the species studied and their hybrids co-occur (Kijewski et al., 2011; Brooks and Farmen, 2013).

Chapter 2 aimed at contributing to develop reliable Best Available Practices (BAPs) for intertidal mussel collection and transport in biomarker-based environmental monitoring of estuaries and other coastal ecosystems. For this purpose, intertidal mussels were handpicked from the lowest and highest tide-mark levels at spring tides in a reference locality of the Basque Coast (Bay of Biscay) and processed by different ways before biomarker determinations, say: (a) *in situ* dissection immediately after sampling; (b) transport to the laboratory either in air or seawater; and (c) dissection at different time intervals after sampling. CAT, GPx, GST, GR, and AChE enzymatic activities, LPO, LP, Vv_{LYS}, Vv_{NL}, Vv_{BAS}, MLR/MET and CTD were investigated. BAPs for mussel collection and transport are needed in order to minimize the influence of confounding factors in biomarker-based monitoring in with mussels are used as sentinels. Although the procedures are disparate among studies, recently, great efforts have been made to develop some recommendations about the mussels collecting: (a) from subtidal or intertidal from the lowest tide-mark level possible, (b) of similar relative shell length (e.g. 70-90% of their local maximum potential length) or age; and (c) out of their spawning season (Izagirre et al., 2008; 2014; Vidal-Liñan and Bellas, 2013; Beyer et al., 2017; Chapter 2). The vertical position of mussels on the shore is therefore crucial, as also envisaged in the present study, especially as regards the measurement of GST and GPx activities in gills, GR activity in digestive gland, LPO in both tissues and lysosomal biomarkers in digestive

gland cells. Consequently, taking into account the present and previous studies (Izagirre et al. 2008, Vidal-Liñán and Bellas 2013), it is highly recommended that sentinel mussels used in biomarker-based monitoring should be collected from the low intertidal zone (nearest possible to the 0 m tide-mark level).

The timing and conditions for dissecting and transport are also crucial factors. Mussels should be processed *in situ* as soon as possible and always within the first hour upon collection. This is especially crucial when enzyme activities, lipid peroxidation and lysosomal responses are the biomarker endpoints. Unfortunately, *in situ* pre-processing is not always possible (e.g. not easy-to-access for staff or equipment, adverse weather or physical conditions); then, mussels should be transported in air (moist) at ambient temperature (Chandurvelan et al., 2013) resembling as much as possible their natural conditions and physiological status at the collection site. Even so, prompt biological responses such as e.g. changes in antioxidant enzyme activities, lysosomal membrane stability and lysosomal numbers should be cautiously interpreted in this case, at least in comparative terms in relation with data obtained in other studies. In addition, transport in seawater should be always disregarded because, together with direct interferences of immersion with antioxidant defence mechanisms and intracellular digestion, spawning may be induced thus indirectly impinging into biological mechanisms underneath biomarkers. Whatever the case may be, details on collection, transport and the time passed before dissection should be always provided in publications and reports in order to assist other researchers to properly interpret and compare results, especially when the biomarker-based environmental monitoring is carried out in coastal zones subject to remarkable intertidal variability.

Another potential source of variability in field and laboratory studies is the sex. The scarcity of conclusive data in the literature makes difficult to generate guidelines about the influence of sex in mussels. Thus, specific research focused in the determination of mussel sex influence on biomarkers and chemical analysis is needed. Accordingly, the goal of Chapter 3 was to contribute to develop BAPs for long-term monitoring design by investigating how sex influences metals and PAH concentration (in tissue) and biomarkers at different seasons. For this purpose, mussels were collected from a relatively clean locality and from a chronically polluted site in the Basque Coast in January, April, August and November 2014. 90 mussels were collected per locality and sampling time to analyse tissue concentration of metals and PAHs, and a battery of core and potential biomarkers, say: cytochrome c oxidase activity (COX), pyruvate kinase activity (PK), phosphoenolpyruvate carboxykinase activity (PEPCK), protein carbonyls products, malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE), lysosomal enlargement and membrane stability, intracellular neutral lipid accumulation, cell type composition and thinning (atrophy) of the digestive gland epithelium, gonad development determination, Flesh Condition Index (FCI) and Stress-on-Stress (SoS). The applied biological responses distinguished between both different environmental health status of the localities Plentzia and Arriluze in all seasons, selecting both sexes and resting mussels to conduct biomarker analysis. Additionally, both sexes and resting mussels selection strategy requires less amount of animals, time and resources in comparison with one sex

selection strategy. Therefore, the use of both female and male mussels is determined as the best available practice for a reliable and efficient heath assessment based on tissue chemical analysis and biomarkers.

BAPs are also needed for toxicological experiments. For instances, the disparate feeding strategies in toxicological experiments render hardly comparable results on mussel biomarkers (Moore et al., 2004; 2007; Izagirre et al., 2008; 2009; González-Fernández et al., 2015). Within this context, the Chapter 4 aimed to research the influence of fasting on the responsiveness of biomarkers in mussels exposed to model pollutant, contributing to develop BAPs to maintain mussels during short-term experimentation in aquatic toxicology. For this purpose, fasted mussel and mussels fed *Isochrysis galbana* were exposed to heavy fuel oil #6 WAF for 96 hr. PAH tissue levels in digestive gland and a battery of biomarkers were investigated. Biomarkers included some of those commonly employed for biological effect assessment in marine pollution monitoring, say: enhancement of glutathione-S-transferase (GST) and cytochrome-C-oxidase (COX) enzyme activities, augmented lipid peroxidation (LPO), lysosomal enlargement (measured in terms of Vv_{LYS}, Sv_{LYS}, S/V_{LYS} and Nv_{LYS}) and membrane destabilisation (LP), lipofuscin accumulation (VvLPF), and histopathological alterations in the digestive gland epithelium (Vv_{BAS}, MLR/MET, CTD-ratio). Further on, the integrated biological response (IBR/n) index was calculated. Fasting in short-term experimentation influenced biomarker values and biomarker responsiveness against oil WAF exposure, especially for GST, LPO, COX, LP, Vv_{LYS}, Nv_{LYS} and LPF. Overall, biomarker responsiveness upon experimental WAF exposure was more clearly envisaged in fed than in fasted mussels. Consequently, it is recommended that mussels should be fed also during short-term toxicological experimentation, thus avoiding the influence of fasting on biomarkers as well as enabling comparability with long-term experiments (in which feeding is absolutely required). More research is needed to advance in the selection of the BAPs regarding feeding regimes and food type for experimental mussels; meanwhile preliminary steps forward have been given in Chapter 5.

Thus, the aim of Chapter 5 was to contribute to BAPs for feeding mussels during toxicological experiments in the laboratory by determining the influence of food type on biomarkers in mussels fed *ad libitum* for 1 wk using 4 different dietary food types: (a) *I. galbana*; (b) *T. chuii*; (c) *I.galbana* and *T.chuii* microalgae mixture; and (d) commercial food. The investigated biomarkers included cytochrome c oxidase (COX), pyruvate kinase (PK) and phosphoenolpyruvate carboxykinase (PEPCK) enzyme activities, metabolites such as protein carbonyl groups (CO), malondialdehyde (MDA) and 4-hydroxy-2-nonenal(HNE), lysosomal enlargement and membrane stability, intracellular neutral lipid accumulation, changes in cell type composition and thinning (atrophy) in the digestive gland epithelium, and altered integrity of the digestive gland tissue.

According to Chapters 4 and 5, feeding is essential also for short-term experiments with mussels and the type of food is a major factor influencing a variety of biomarkers. Thus, it is necessary to identify BAPs regarding the selection of food type and feeding strategy (regime,

rations, etc.) in order to achieve reliable and comparable experimental data on the biological effects of pollutants. Within this context, commercial food based on frozen or frozen-dried diets seems not to be best option for feeding during toxicological experiments, as it was concluded previously for experimentation dealing with aquaculture production (Albentosa et al., 1997; Langdon et al., 1999; Fernandez-Reiriz et al., 2015). Dietary microalgae should be selected on the basis of the suitable dimension (size, volume, weight) of algal cells, their high digestibility and balanced nutritional value (Fernández-Reiriz et al., 2015). Likewise, it is worth noting that not all the live microalgae affect biomarkers and their measurement in the same way. For instance, *T. chuii* has a limited digestibility and associated long retention times in the gut (Becker et al., 1998; Rouillon and Navarro, 2003; Guéguen et al., 2008). For this reason it seems to influence nutritional status, oxidative stress and digestion processes in mussels; thus, affecting a variety of biomarkers and their measurement. On the other hand, the massive presence of *I. galbana* within digestive cells may hamper the measurement of cytochemical biomarkers and may render less reliable the results of biochemical biomarkers (as these can be attributed to both the mussel and the microalgae). Interestingly, results of chapter 4 indicated that at dietary cell concentrations of *I. galbana* of 2×10^4 cells/mL the occurrence of microalgae within digestive cells is negligible; however, rations over 2×10^4 cells/mL are recommended and commonly used in physiological experiments (Langdon and Önal, 1999; Okay et al., 2006; Ibarrola et al., 2017). Further research is needed to optimize dietary food type, composition, regime and rations for toxicological experimentation. Meanwhile, it is highly recommended that research papers should include always a detailed description of the food type and feeding conditions in order to get a sustained understanding on the biological responses elicited by pollutants in mussels.

As a summary, after the studies carried out in the present thesis, the next BAPs for experimentation with mussels are briefly described:

1. Mussels species could influence on biomarkers, thus in large-scale biomonitoring programs genetic species identification should be used at least at population level.

2. Low intertidal level mussels should be processed *in situ* as soon as possible and always within the first hour upon collection.

3. Use both female and male mussels is recommended for a reliable and efficient health assessment based on chemical analysis and biomarkers.

4. Histological analysis of sex ratio and gamete development is recommended in toxicological studies, as supporting parameters to check possible distress and seasonal patterns.

5. During short- and long-term toxicological experiments, mussels fed with live microalgae are recommended.

During the thesis, it was detected that the methodology for the determination of volume

density of basophilic cells (Vv_{BAS}) needed to improve because the sole identification and discrimination of basophilic cells may be difficult in tissue sections of the digestive gland stained with the traditional hematoxylin-eosin staining. In this framework, the goal was to explore the BAPs to clearly identify and discriminate basophilic cells on tissue sections of mussel digestive gland by trying different fixation and staining methods (Chapter 6). Finally, it was concluded that toluidine-eosine staining after fixation in 4% formaldehyde at 4°C for 24 hr is a highly valuable method to clearly identify and discriminate basophilic cells in the digestive gland of marine molluscs. However, future research is needed to adapt the environmentally relevant critical values of this parameter after calibration with studied previously carried out using hematoxylin-eosin staining.

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CONCLUSIONS

1. Core biological responses to pollutant exposure differ between mussels *Mytilus spp*. from diverse European coastal zones. Thus, in large-scale biomonitoring programs genetic species identification should be used at least at population level.

2. Different sampling strategies influence on biomarkers. Thus, after develop Best Available Practices (BAPs), it is recommended that sentinel intertidal mussels used in biomarker-based monitoring of the coastal zone in the Bay of Biscay must be collected from the low intertidal zone (nearest as possible to the 0 m tide-mark level) and processed *in situ* as soon as possible.

3. Dealing with BAPs for mussel selection (e.g. regarding sex and reproductive condition) in long-term monitoring of the coastal zone in the Bay of Biscay, it was concluded that the best option is to collect and analyses both sexes. Additionally, histological analysis of sex ratio and gamete development is recommended in toxicological studies.

4. Formaldehyde fixation and toluidine-eosin (T&E) staining are recommended as the BAPs to identify and discriminate basophilic cells for determining changes in cell-type composition in mussel digestive gland epithelium with volume density of basophilic cells (Vv_{BAS}) as endpoint. Future research is needed to adapt the environmentally relevant critical values of this parameter after calibration with studied previously carried out using hematoxylin-eosin staining.

5. Fasting exerts significant effects on bioaccumulation and biomarkers values and responsiveness in model pollutant exposed mussels and thus, it was determine that fed mussels is the BAPs for short-term toxicological experiments.

6. Dietary food type affects biomarkers in mussels, so it was determined fed mussel with live microalgae for maintain mussels during laboratory experiments. However, further research is needed to develop BAPs, including consensus food type and feeding strategy (regime, rations, etc.), in order to achieve reliable and comparable experimental data on the biological effects of pollutants.

THESIS

"A better understanding of the influence of confounding factors, such as mussel taxonomy, sampling strategy, sex, fasting and food composition, on biomarker values and responsiveness endorses Best Available Practices (BAPs) for a cost effective multi-biomarker approach applied to mussels as sentinels in marine pollution monitoring and as experimental animals in aquatic toxicology. BAPs will be modified and optimised, as the understanding of the mechanisms underneath biomarkers and the related confounding factors improves."

V. APPENDIX

BIOCHEMISTRY PROTOCOLS

Lipid determination
(Modified after Folch et al. 1957, Iverson et al. 2001)

1. Place about 50 mg of dried tissue powder into pre-weighed and marked 2 ml eppendorf tube.

2. Add solvent (chlorophorm/methanol mixture, 2:1 v/v) in a proportion 1:20 (tissue to solvent, w/v).

3. Sonicate the sample for 1 min.

4. Vortex vigorously for 2 min.

5. Centrifuge the sample at 1300 g for 5 min.

6. Remove supernatant into a new marked 2 ml Eppendorf tube. Add 0.5 ml of fresh solvent to the sample.

7. Repeat steps 3-5.

8. Remove supernatant to the tube with previous supernatant of the same sample.

9. Take tube with supernatants, add H_2O in the amount of ¼ of the total volume of supernatant (chlorophorm:methanol:water ratio is 8:4:3).

10. Repeat steps 4-5.

11. Transfer the lower phase (chlorophorm) in a weighed tube and evaporate chlorophorm.

12. Weigh tube with lipids residue.

Glycogen and free glucose determination

Tissue preparation

Prepare tissue following the PCA protocol. Depending on the treatment of the sample, we will measure free glucose or glycogen.

PCA extracts of tissue

1. Take 1.5 ml microcentrifuge tube, weigh on the analytical balance and record the weight. Add 250 µl of 0.6 M of ice-cold perchloric acid (PCA).

2. Weigh the tube with PCA on the analytical balances, record the weight.

3. Freeze the microcentrifuge tube with PCA in liquid nitrogen and place the tube on ice.

4. Pour some liquid nitrogen (carefully and using a ladle) into a clean mortar with pestle. Let the mortar and the pestle cold, add liquid nitrogen as needed.

5. Place a piece of tissue under the liquid nitrogen and grind it with a pestle under the liquid nitrogen. Add more nitrogen as needed.

6. Take ca. 100 mg of tissue powder with a small steel spoon pre-cooled in liquid nitrogen and place in the tube with frozen PCA. Weigh and record the weight.

7. If the weight of tissue was ca. 100 mg, add 250 µl of ice-cold PCA. If it was more than that, add 50 µl of PCA for each additional 10 mg of tissue (e.g. for tissue weight of 150 mg add 250 + 250 = 500 µl PCA, etc.). Weigh and record the weight.

8. Take 50 µl for glycogen measurement.

9. Homogenize on ice using a sonicator set at 10 for 3 times, 10 sec each, with 20 sec intervals between sonications.

10. Incubate 5 min on ice.

11. Centrifuge the sample for 2 min at maximum speed and 4°C.

12. Collect supernatant into a new clean 1.5 mml microcentrifuge tube. Weigh and record the weight.

13. Add 10% of the supernatant volume of 5 M KOH (e.g. if there was 1 g of the supernatant, add 100 µl of KOH, if there was 1.5 g of the supernatant). Mix well (vortex). Place on ice.

14. Check pH with an indicator strip. Take a drop of the liquid with a plastic stick and touch the indicator paper. If pH is between 7 and 7.5, you are fine and no further addition of KOH is needed – proceed to step 17. If pH is below 7, proceed to step 15. If pH is above 7.5, proceed to step 16.

15. Add small volumes of 1 M KOH (10 µl at a time), mix and check pH after each addition until you get pH of 7-7.5. Record volume of KOH added.

16. Add small volumes of 1 M HCl (5 µl at a time), mix and check pH after each addition until you get pH of 7-7.5. Record volume of HCl added.

17. Place on ice and incubate for 2 min.

18. Centrifuge for 10 min at maximum speed and 4°C.

19. Collect supernant without disturbing the pellet, split into 2 roughly equal volumes (ca. 250 – 300 µl) into 2 clean and labeled 500 µl tubes.

20. Place tubes in a box and store in a freezer at -80°C.

Glucose standard

1. Prepare glucose stock (500 mM): 4.5 g of glucose (Dextrose. Anhydrous; $C_6H_{12}O_6$; M.W= 180.15) in 50 ml of H_2O.

2. Prepare the standards:

Standard	Glucose	H_2O
5 mM	3 µl of the stock (500 mM)	297 µl
2.5 mM	Take 25 µl of 5mM glucose	25 µ
1.6 mM	Take 10 µl of 5mM glucose	20 µl
1 mM	Take 40 µl of 5mM glucose	160 µl
0.5 mM	Take 50 µl of 1mM glucose	50 µl
0.25 mM	Take 10 µl of 1mM glucose	30 µl
0.1 mM	Take 20 µl of 1mM glucose	180 µl
0 mM	-	100 µl

3. Measure the absorbance at 339 nm.

4. Calculate the glucose concentration using the equation of the standards.

Glucose/glycogen determination

Necessary solutions:

- Triethanolamine/magnesium sulphate buffer (TEA, 0.3 mol/l; $MgSO_4$, 4 mmol/l; pH 7.5): Dissolve 2.8 g triethanolamine hydrochloride 99% (Acros, 421645000; $C_6H_{15}NO_3HCl$; MW= 185.65) and 0.05 g of $MgSO_4 \cdot 7\ H_2O$ (Sigma, M-1880; MW = 246.5) in 40 ml of H_2O. Add 6 ml KOH (1 mol/l). Check pH, adjust if necessary, and make up to 50 ml with H_2O.

  * To do 1M KOH, mix 2.81 g of KOH (Acros, 134060010; M.W.=56.11) in 50 ml of H_2O.

- β-NADP (β-Nicotinamide adenine dinucleotide phosphate sodium salt (Chem-Impex Int'l inc., 00231, $C_{21}H_{27}N_7O_{17}P_3Na$; M.W.= 765.4): mix 0,046 g in 10 ml of H_2O (6 mM). Do aliquots of 1 ml).

- ATP (ATP Disodium, trihydrate; Fisher BioReagents, ATP Disodium, Trydrate, BP413-25, $C_{10}H_{14}N_5O_{13}P_3Na \cdot 3H_2O$; M.W= 765.4): mix 1.21 g in 10 ml of H_2O (200 mM). Do aliquots of 1 ml.

1. Prepare the reaction solution depending on the amount of wells that you will needed:

Compound	1 well (200 µl) (µl)	20 wells (µl)	96 wells
200 mM ATP	1	20	96 µl
6 mM NADP	1.32	26.4	126.72 µl
G-6P-DH	1	20	96 µl
TEA buffer	184,48	3689,6	17.7 ml

Reference of G-6P-DH: Sigma, G7877-1KU; Glucose-6-phosphate Dehydrogenase from baker's yeast (*S. cerevisiae*); 10 mg protein/ml. The volumes are taken directly from the bottle.

2. Add to each well 10 µl of sample and 188 µl of the reaction solution.
   - After measurement the values are negative or very low, increase the volume of sample: 30 µl of sample and 168 µl of the reaction solution.

3. Read absorbance at 339 nm (it would the background, A_1).

4. Add to each well 1 µl of hexokinase (Roche, 11426362001, 1500 U (1ml) from yeast overproducer).

5. Incubate during 30 min at 37°C.

6. Read absorbance at 339 nm.

Protein concentration

Take out from the fridge Bradford to temper.

1. Prepared a stock solution of 1 mg/ml of albumin from bovine serum (ABS) (Sigma, A2153). For that, mix 0.1 g ABS and 1ml homogenization buffer used to prepared tissues for this technique. To dissolve ABS maintain still at room temperature. After be dissolve, mix 10 µl of diluted ABS and 999 µl isolation buffer (IB).

2. Do standard dilutions for the curve: 0.7 (70 µl diluted ABS + 30 µl IB); 0.6 (60 µl diluted ABS + 40 µl IB); 0.5; 0.4; 0.3; 0.2; 0.1 and 0.

3. Do the dilutions of the samples: 1:10 (10 µl sample + 90 µl IB) in the case of gills or 1:50 (2 µl sample + 98 µl IB); in the case of digestive gland.

4. Add 10 µl of sample and 200 µl of Bradford (diluted solution from Protein Assay Dye Reagent Concentrate (Bio-Rad 500-0006) in each well of the plate. 2 replicates per sample.

5. Read the plate at 595 nm.

6. Calculate the protein concentration using the equation of the standards.

Homogenization for oxidative stress assays in cuvette

Solutions

- K-phosphate buffer: 400 ml ultrapure water + 1.03 g KH_2PO_4 + 5.64 g K_2HPO_4. Adjust the pH: 7.2 or 7.4.

- BHT solution: dilute 4 g of BHT in 100 ml of methanol.

Procedure

1. Depending of the protein concentration of the sample, add the appropriate proportion of tissue and 0.1 M K-phosphate buffer (pH 7.2 for AChE, and pH 7.4 for the rest of the assays) to an Eppendorf.

2. Homogenize each sample.

3. Transfer 200 µl of homogenazed tissue into an Eppendorf with 4 µl of BHT for lipid peroxidation.

4. Centrifuge the remaining homogenate at 10000 G for 20 min at 4˚C in orders to isolate Post-mithocondrial supernatant (PMS). For AChE, centrifuge at 60000 G for 3 min at 4˚C.

5. Collect the supernatant, divide in tubes and store at -80˚C.

Catalase (CAT)

Buffers

0.05 M K-phosphate buffer (pH 7)
0.03 M H_2O_2 solution
    Dilute 268 µl 30% H_2O_2 in 50 ml 0.05 M K-phosphate buffer (pH 7).

Procedure

1. Pipette 920 µl K-phosphate buffer in UV cuvette.

2. Pipette 500 µl of H_2O_2 solution.

3. Pipette 80 µl of sample.

4. Read the absorbance at 240 nm during 1 min.

$\varepsilon=40\ M^{-1}cm^{-1}$

Glutatione peroxidase (GPx)

Reagents

- 0.05 M phosphate buffer (pH 7) with 1 mM EDTA, 1 mM sodium acide and GR: dilute 0.11 g EDTA, 0.019 g sodium azide (weigh with plastic spoon) and 7.5 ml GR in 300 ml of phosphate buffer.

- 4 mM GSH solution: dilute 0.0307 g in 25 ml of 0.05 M phosphate buffer (pH 7).

- 0.8 mM NADPH solution: dilute 0.0167 g in 25 ml of 0.05 M phosphate buffer (pH 7).

- 0.5 mM H_2O_2 solution: dilute 268 µl 30% H_2O_2 in 50 ml of 0.05 M phosphate buffer (pH 7).

Procedure

1. Pipet 840 µl of 0.05 M phosphate buffer (pH 7) with 1 mM EDTA, 1 mM sodium acide and GR in cuvette.

2. Pipet 50 µl 4 mM GSH, 50 µl 0.8 mM NADPH and 10 µl 0.5 mM H_2O_2 solution.

3. Pipet 50 µl of sample.

4. Read the absorbance at 340 nm during 1 min.

$\varepsilon = 6.22 \times 10^3\ M^{-1}cm^{-1}$

Glutathione S-Transferase (GST)

Reagents

- 0.2 M K-phosphate buffer, pH 6.5.

- 10 mM GSH (in K-phosphate buffer 0.1 M, pH 6.5): Dilute 0.0251 g of GSH in 10 ml of ultrapure water. Keep on ice and always make fresh.

- 60 mM CDNB: dilute 0.0827 g CDNB (1-cloro-2,4-dinitrobenzene) in 20 ml of absolute ethanol. Add 30 ml of ultrapure water. Photosensitive so use tinfoil, keep on ice and always make fresh.

- Reaction solution: 1000 µl phosphate buffer + 30 µl GSH +30 µl CDNB (use this specific order)

Procedure

1. Leave the 1st column empty.

2. Pipette the blanks with 100 µl K-phosphate buffer

3. Pipette 30 µl of each sample (3 replicates).

4. Using multi-channel pipette add 270 µl of reaction solution to samples and blanks.

5. Read the absorbance at 340 nm, during 3 min, every 20 sec.

$\varepsilon = 9.6 \times 10^3\ M^{-1}cm^{-1}$

Glutathione Reductase (GR)

Reaction solution: dissolve 0.011 g of NADPH, 0.04 g of GSSG and 0.012 g of DTPA in 50 ml of 0.005 M K-phosphate buffer (pH 7).

Procedure

1. Pipette 720 µl of reaction solution in cuvette.

2. Pipette 40 µl of sample.

3. Read the absorbance at 340 nm during 1 min.

$\varepsilon = 6.22 \times 10^3\ M^{-1}cm^{-1}$

Acetylcholinesterase (AChE)

Reagents

- 0.1 M K-phosphate buffer (pH 7.2).

- 0.075 M acetylcholinesterase solution: dissolve 0.11 g of acetylcholinesterase (Sigma, 5751) in 5 ml in ultrapure water. Protect the solution from light with aluminum foil. Keep in the refrigerator for a maximum period of five days.

- Reaction solution: 30 ml phosphate buffer + 200 µl of acetylcholinesterase solution + 1 ml of DTNB solution.

Procedure

1. Leave the 1st column empty.

2. Pipette 50 µl of buffer or sample to make blanks or samples, respectively.

3. Add 250 µl of reaction solution.

4. Place the microplates 30 sec in agitation at 150 rev/min.

5. Read the absorbance at 414 nm for 3 min, every 20 sec.

$\varepsilon = 13.6 \times 10^3\ M^{-1}cm^{-1}$

Lipid peroxidation (LPO)

Reagents

- 12% TCA solution: dissolve 12 g of trichloroacetic acid (TCA) in 100 ml of ultrapure water.

- 60 mM Tris-HCl (pH 7.4) solution with 0.1 mM DTPA: dissolve 1.89 g of Tris-HCl and 0.0007 g of DTPA (diethylenetriaminepentaacetic acid).

- 0.73% TBA solution: dissolve 0.73 g of TBA in 100 ml of ultrapure water. Put in a hot water bath to dissolve TBA well.

Procedure

1. Pipette 180 µl of sample in a 15 ml tube and add 1 ml 12% TCA. Vortex. The blanks contain 180 µl of 0.1 M phosphate buffer (pH 7.4).

2. Pipette 820 µl of 60 mM Tris-HCl with 0.1 mM DTPA.

3. Pipette 1 ml 0.78% TBA.

4. Incubate the tubes in a hot water bath at 100°C during 1 h.

5. Transfer 2 ml of the solution to eppendorfs.

6. Centrifuge at 11500 rpm during 5 min at 25°C.

7. Place the eppendorfs in a holder covered with aluminum foil.

8. Read the absorbance at 535 nm.

$\varepsilon = 6.22x10^{5}\ M^{-1}cm^{-1}$

MDA and HNE

Tissue preparation

1. Prepare homogenization buffer, x1 PBS.

    Dilute 10 times the prepared one: Fisher BioReagents, BP399-1 Phosphate Buffered Saline, 10X Solution.

    Take 10 ml of isolation buffer and add 1mM Dithiotreitol (DTT; MW=154.3) (Sigma, D0632) (0.01861 g/100 ml) and 0.1 mM phenyl-methylsulfonyl fluoride (M.W.= 174.2; 0.0017 g/100 ml).
    In this case, we had to add 1.5 µl of DTT and 1.7 µl of 1:10 diluted 0.1 mM phenyl-methylsulfonyl fluoride.
    Cool down to 4°C. Homogenization must carried on ice.

2. Weigh tissues (the necessary to have 100 µl), homogenize (1:10 wet weight:volume) in the extraction buffer with a glass homogenizer.

3. Centrifuge extracts at 15000 or 11,5 rad for 10 minutes at 4°C.

4. Transfer in new tubes.

5. Determine protein concentration using Bradford assay (gills 1:10, 5µl sample and 45 µl of x1 PBS) or aliquot and store at -20°C.

6. Before measuring samples must be diluted with x1 PBS to have 10 µg/ml:
    - µl of sample: 10*(1/protein concentration of the sample in mg/ml).
    - µl of x1 PBS: 1000 - µl of sample.

Procedure

1. Prepare stock of reduced BSA (10 mg/ml): 0.5 g + 50 ml in 1x PBS.
    - BSA reference: Sigma A-9306; Albumin, Bovine Essentially γ-Globulin Free.
    - Or can be use: Equitech-Bio, Inc. BAH66, Bovine Serum Albumin Fatty Acid Free Powder.

1.1. Dilute stock 1000x in 1x PBS: 10 µl in 10 ml PBS (10 µg/ml reduced BSA). Store the rest of reduced BSA (10 mg/ml) to do blocking solution next day.

a) MDA STANDARD: prepared 0.5 μg/ml of MDA-BSA (STA-333, 100 μl at 1 mg/ml) by diluting the 1 mg/ml MDA-BSA std. in 10 μg/ml of reduced BSA: **2 μl MDA to 4 ml of 10 μg/ml reduced BSA.**

Series of MDA-BSA std.:

	MDA conc. (pmol/ mg)	0.5 μg/ml MDA/ BSA (μl)	10 μg/mg reduced BSA (μl)
1	120	1000	0
2	60	500 of the tube 1	500
3	30	500 of the tube 2	500
4	15	500 of the tube 3	500
5	7,5	500 of the tube 4	500
6	3,75	500 of the tube 5	500
7	1,875	500 of the tube 6	500
8	0	0	500

b) HNE std.: prepare 10 μg/ml of HNE-BSA (Cell Biolabs, inc., STA- 335100 μl at 1 mg/ ml), by diluting the 1 mg/ml HNE-BSA st. in 1xPBS: 5 μl + 495 μl PBS.
Series of HNE-BSA std.:

	HNE-BSA (μg/ml)	0.5 μg/ml MDA/ BSA (μl)	10 μg/mg reduced BSA (μl)
1	10	500	0
2	5	250 of the tube 1	250
3	2,5	250 of the tube 2	250
4	1,25	250 of the tube 3	250
5	0,625	250 of the tube 4	250
6	0,313	250 of the tube 5	250
7	0,156	250 of the tube 6	250
8	0	0	250

Note: you can vortex to mix well standards.

2. Add 100 μl of the 10 μg/ml sample or std. per well. MICROPLATES MUST BE SPECIAL FOR ELISA (Greiner bio-one 655101).

3. Incubate overnight at 4°C inside of zip-bag.

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4. Prepare blocking solution (1 mg/ml BSA in 1x PBS), 300 μl/well. For complete 2 microplates, 6 ml of BSA (10 mg/ml) + 54 ml of x1 PBS.

5. Wash samples 2 times with 200 μl/well 1x PBS. (After each step dry the plate).

6. Add 300 µl/well of the blocking solution. Incubate during 2 h at 37 °C.

7. Wash 5 times with 1x PBS + 0.05% Tween: 0.5 ml Tween (Acros, L-25017; Tween 20, pur) for 1 l 1xPBS.

8. Dilute primary antibodies:
- MDA (1:8000): 3.25 µl (directly from the tube) + 26 ml of 1 mg/ml BSA in 1x PBS
 MDA antibody reference: malon dialdehyde, US Biological M2141-04.
- HNE (1:4000): 6.5 µl (directly from the tube) + 26 ml of 1 mg/ml BSA in 1x PBS
 HNE antibody reference: Rb pAb to 4 Hydroxynonenal, 100 µg (0.85 mg/ml); ab46545 abcam.

(To prepared 60 ml of 1 mg/ml BSA from 10 mg/ml BSA: 6 ml 10 mg/ml BSA + 54 ml x1 PBS)

9. Add 100 µl/well of dilute antibody to the corresponding microplate.

10. Incubate during 1 h at room temperature on shaker.

11. Wash 5 times with PBS-Tween.

12. Dilute secondary antibody (1:16000), antirabbit: 2.5 µl + 40 ml 1 mg/ml BSA in 1x PBS.

13. Add 100 µl of diluted secondary antibody to each well.

14. Incubate during 1h at room temperature in orbital shaker.

15. Take out of the fridge the ELISA substrate (Thermo Scientific, 34028; 1-Step™ Ultra TMB-ELISA) to warm.

16. Wash 5 times with PBS-Tween.

17. Add 100 µl of ELISA substrate and incubate at room temperature until the two more concentrated standards get saturated blue color. Put under the microplates a white sheet to watch better.

18. Add 100 µl of stock solution.
- ELISA stop solution: 2M H_2SO_4 (Sigma, 339741; sulfuric acid, 99,999%; MW= 98,08; 17,822 M)

19. Measure absorbance at 450 nm.

Protein carbonyls

Tissue preparation

1. Prepare homogenization buffer:
For 200 ml, 2.4 g of HEPES (Fisher Scientific, BP-310-500; HEPES for molecular Biology fine white crystals; $C_8H_{18}N_2O_4S$; MW=238.3) and 1.9 g of KCl (Acros, 196770010, ClK: MW= 74.54). Fix the pH to 7.4.

2. When the buffer is ready, take 10 ml of homogenization buffer and add 1mM Dithiotreitol (DTT) (0.01861 g/100 ml) and 0.1 mM phenyl-methylsulfonyl fluoride (M.W.= 174.2; 0.0017 g/100 ml).

- DTT: Sigma, D0632; MW=154.3.

-Phenyl-methylsulfonyl fluoride: Sigma, P7626; phenylmethanesulfonyl fluoride; M.W.= 174.2.

3. In this case, we had to add 1.5 µl of DTT and 1.7 µl of 1:10 diluted 0.1 mM phenyl-methylsulfonyl fluoride.

4. Weigh tissues (the necessary to have 150 µl), homogenize (1:10 wet weight:volume) in the extraction buffer with a glass homogenizer.

5. Centrifuge extracts at 15000 or 11.5 rad for 10 minutes at 4°C.

6. Transfer the supernatants in new tubes.

7. Store the aliquots (min. 110 µl) at -80°C.

Procedure

1. Incubate with streptomycin sulfate at 1% in sample for 15 min at RT: 10 µl /100 µl sample.
Streptomycin sulfate: 10% Streptomycin sulfate stock solution (Santa Cruz Biotechnology, INC., Chem Cruz, SC-202821A; $C_{21}H_{39}N_7O_{12}\cdot 1.5H_2SO_4$; M.W.=728.8) should be made in 50mM potassium phosphate, pH 7.2. Do fresh for each time.

2. Centrifuge it at 6.000 X g (rcf) or 11.5 rad for 10 minutes at 4°C.

3. Aliquot supernatant into 2 epperdorfs: 50 µl (replicate) + 50 µl.

4. Add 175 µl of DNP (replicate) and 175 µl of 2M HCL (blank) per 50 µl of sample.
- 10 mM 2-4 dinitrophenylhydrazine (DNP, MW 198.1) in 2M HCL (0.099g/50ml)

5. Vortex at RT every 15 min 4 times.

6. Add 25 µl of 100% TCA.

- 100% TCA = 10 g TCA/10 ml H_2O. TCA reference: Sigma, T9159; Trichloroacetic acid; $C_2HCl_3O_2$; M.W.= 163.39.

7. Centrifuge at max. speed 10 min (or 11.000 g for 3 min) at RT.

8. Remove the supernatant and clean the pellet at least 3 times with Ethanol:Ethylacetate (50:50) (adding 125 µl each time) until the supernatant is not yellow anymore. Blank with one wash is enough.

9. Dry pellet on air until it is totally dry. (You can utilize the incubator at 45°C until dry). Keep open the lid of the tubes. For the drying process overthrow the tubes helps.

10. Add 125 µl of 6M Guanidine HCl (pH2.5), vortex and sonicate (10 s 3 times).

- Guanidine reference: Sigma, G3272; Guanidine Hydrochloride; $CH_5N_3 \cdot HCl$; M.W.= 95.53.
- 6M Guanidin HCL in 20 mM KH_2PO_4: pH 2.5 adjusted with Trichloric acid (TCA). 20 mM KH_2PO_4 MW 136.09; 1.36g/ 500ml

11. Incubate 15 min at 37°C.
12. Keep overnight at 4°C.

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13. Centrifuge 5 min at 11000 rcf.

14. Collect the supernatant.

15. Measure absorbance at 360 nm of all the samples: 2 replicates, 50 µl per well.

16. Take out from the fridge Bradford to temper and prepare the standards with Guanidine HCl and albumin from bovine serum (Sigma, A2153). The standard must be prepared fresh. If it is not possible measure protein concentration in the same day, the microplate can be store at -20°C.

For that, mix 0.1 g ABS and 1ml Guanidine HCl used to prepared tissues for this technique. To dissolve ABS maintain still at room temperature. After be dissolve, mix10 µl of diluted ABS and 999 µl Guanidine HCl.
Do standard dilutions for the curve: 0.7 (70 µl diluted ABS + 30 µl Guanidine HCl); 0.6 (60 µl diluted ABS + 40 µl Guanidine HCl); 0.5; 0.4; 0.3; 0.2; 0.1 and 0.

17. Take the sample from the microplate to do Bradford test: 10 µl of sample and 200 µl of Bradford in each well of the plate. 2 replicates per sample. It is not needed dilute the samples. 595 nm.

Mitocondrial Respiratory Enzyme Complex IV
Cytochrome-c oxidase (COX)

Buffers

-25 mM phosphatebuffer (pH 7.2) for 100 ml:
Monosodic ($Na_2H2PO_4 \cdot H_2O$): 0.11 g
Disodic ($Na_2HPO_2 \cdot 2H_2O$): 0.304 g

-200 mM phosphate buffer (pH 7.2) for 100 ml:
Monosodic ($Na_2H2PO_4 \cdot H_2O$): 0.87 g
Disodic ($Na_2HPO_2 \cdot 2H_2O$): 2.43 g

Sample preparation

1. Prepare the solution: 10 ml of 25 mM potassium phosphate and add 1mM Dithiotreitol (DTT; MW=154.3) (Sigma, D0632) (0.01861 g/100 ml) and 0.1 mM phenyl-methylsulfonyl fluoride (M.W.= 174.2; 0.0017 g/100 ml).
In this case, we had to add 1.5 µl of DTT and 1.7 µl of 1:10 diluted 0.1 mM phenyl-methylsulfonyl fluoride.

2. Weight tissue (the necessary to finish with 150-100 µl). And mix the piece of weighted tissue and the prepared solution in 1:5. Homogenize and past the liquid to a eppendorf.

3. Sonication process: 3 times, 10s each. (Power =120 W)

4) Centrifuged: 4°C, 15 min at max. speed.

5. Collect the supernatant doing aliquots of 50 µl.

6. Store at -80°C until measure.

Reagements preparation

1. Prepare 0.25 g of Sephadex G-25 (Sigma, G2580) in 2 ml of 25 mM potassium phosphate.

2. Do the column (Bio-Rad, Poly-Prep Chromatography Columns, 731-1550) with the Sephadex G-25. During precipitation process maintain the system in the fridge. Before do not have the Cyt-C with Ascorbic acid prepared, maintain a little bit of buffer in the column to no dry.

3. During precipitation process of Sephadex G-25, prepare 0.025 g of cytochrome c (Sigma, cytochrome c, from horse heart, C7752) in 2 ml of 25 mM potassium phosphate. And add a

small spatula of ascorbic acid (Sigma, A-0278; L-Ascorbic Acid; MW=176,1).

4. Once all buffer is in the tube, remove the buffer from the tube and add to the column cytochrome c.

5. Once all the buffer is in the tube, collect and do aliquots of 200 µl and store at -20°C.

6. Measure the concentration of cytochrome-C spectrophotometrically at 550 nm, to know how many you have to add in the assay. The concentration must be check after 7 days of the preparation.

- To keep the column, add 25 mM phosphate buffer several times to clean. Close and keep in the fridge.

- To reuse the column, add 4ml of buffer 4 times and ready to use.

Procedure

1. Place 2 µl of sample into 190 µl 200mM buffer and let stand for 5min to acclimate enzyme.

2. Add 1 µl of Antimycin-A stock (0.001 g/ml) (Sigma, A8674-25 mg; Antimycin A from *Streptomyces sp.*).

3. Monitor assay for 2 min to account for background activity.

4. Add cytochrome-c to a final concentration of 16µM and measure absorbance for 1-2min**. In the last case, 10 µl.

**Cyt-c Ox activity is very rapid so keep in mind when running multiple samples; may want to monitor 1 sample at a time to ensure accurate measurements

Pyrubate kinase (PK) and
phosphoenolpyrubate carboxykinase (PEPCK)

Sample preparation

Isolation buffer (pH 7.2)

Reference	Compound	For 1000 ml	For 200 ml
$C_3H_4N_2$; 68.08; Acros, 301872500	50 mM Imidazole	3.4 g	0.68 g
41.98	100 mM NaF	4.198 g	0.839 g
$C_{10}H_{16}N_2O_8$; 292.24; Sigma, EDS-500 g	5 mM EDTA	1.46 g	0.292 g
$C_{14}H_{24}N_2O_{10}$; 380.34; Acros, 409911000	5 mM EGTA	1.90 g	0.38 g

When the buffer is ready, take 10 ml of isolation buffer and add 1mM Dithiotreitol (DTT; MW=154.3) (Sigma, D0632) (0.01861 g/100 ml) and 0.1 mM phenyl-methylsulfonyl fluoride (M.W.= 174.2; 0.0017 g/100 ml).

In this case, we had to add 1.5 µl of DTT and 1.7 µl of 1:10 diluted 0.1 mM phenyl-methylsulfonyl fluoride.

1. Weigh tissues (gills) (the necessary to have 150 µl), homogenize (1:5 wet weight:volume) in the extraction buffer with a glass homogenizer.

2. Centrifuge the homogenite for 30 min at maximum velocity (4°C).

3. Collect supernatants doing aliquots of 130 µl.

Procedure

Needed solutions for PK:

Reference	Solution	Stock solution
TRIS, Amresco, 0497-1kg, $C_4H_{11}NO_3$; MW= 121.14	Tris(-HCl) pH7	1.3133 g /100 ml
Acros, 196770010, ClK, MW= 74.54	KCl	0.9319 g/ 50 ml
Sigma, M-1880; MW = 246.5	MgSO4 ·7H2O	0.615 g/10 ml
Sigma, A2754; Adenosine 5'-diphosphate sodium salt, from bacterial source; $C_{10}H_{15}N_5O_{10}P_2$; MW= 427.2	ADP, K salt	0.214 g/5 ml

Roche, 10128023001; NADH, disodium salt, grade II approx. 98%; $C_{21}H_{27}N_7O_{14}P_2Na_2$; MW= 709.4	NADH	0.04 g/10 ml
Roche, 10 127 876 001, 25 mg (5ml) from rabbit muscle	LDH (enzyme)	Need volume is taken directly from the bottle
Roche, 10108294001, phosphoenolpyrubate, $C_3H_4O_6PK$; MW=206.1	PEP (50 mM)	0.052 g/5 ml

Needed solutions for PEPCK:

References	Solution	Stock solution
Fisher Scientific, BP-310-500; HEPES for molecular Biology fine white crystals; $C_8H_{18}N_2O_4S$; MW=238.3	HEPES pH7	3.9717 g/100 ml
Sigma, M-3634; manganese chloride tetrahydrate; MW=197.9	$MnCl_2 \cdot 4H_2O$	0.2275 g/100 ml
CHEM-IMPEX INT'L INC., 00186; Inosine-5'- diphosphate trisodium salt; $C_{10}H_{11}N_4O_{11}P_2Na_3$; MW= 494.1	IDP, Na salt	0.0535 g/5 ml
Sigma, potassium hydrogencarbonate, granular, 99.7% A.C.S. reagent, 237205; M.W.=100.12	$KHCO_3$	0.25 g/5 ml
Roche, 10128023001; NADH, disodium salt, grade II approx. 98%; $C_{21}H_{27}N_7O_{14}P_2Na_2$; MW= 709.4	NADH	0.04 g/10 ml
Amresco, 0373-25, 000U	MDH	Need volume is taken directly from the bottle
Roche, 10108294001, phosphoenolpyrubate, $C_3H_4O_6PK$; MW=206.1	PEP (250 mM)	0.260 g/5 ml

1. Do measurement mixes:
- For PK:

Solution	1 well (µl)	12 wells (µl)	16 wells (µl)	24 wells (µl)	40 wells (µl)
Tris-HCl pH7	110	1320	1760	2640	4400
KCl	40	480	640	960	1600
$MgSO_4 \cdot 7H_2O$	4	48	64	96	160
ADP, K salt	2	24	32	48	80
NADH	20	240	320	480	800
LDH (enzyme)	2	24	32	48	80
PEP (50 mM)	2	Add just before measure to the well			

- For PEPCK:

Solution	1 well (µl)	12 well (µl)	16 wells (µl)	24 wells (µl)	40 wells (µl)
HEPE	70	840	1120	1680	2800
$MnCl_2 \cdot 4H_2O$	40	480	640	960	1600
IDP, Na salt	4	48	64	96	160
$KHCO_3$	2	24	32	48	80
NADH	20	240	320	480	800
MDH	12	144	192	288	480
PEP (250 mM)	12	Add just before measure to the well			

2. Added to each well to a microplate (two replicates per sample):
- For PK: 178 µl of it measurement mix + 20 µl of sample. (in green)
- For PEPCK: 148 µl of it measurement mix + 40 µl of sample. (in blue)

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.1	1.2				1.1	1.2					
B	2.1	2.2				2.1	2.2					
C	3.1	3.2				3.1	3.2					
D	…	…				…	…					
E												
F												
G												
H												

3. Measure kinetic of solution without PEP and then again after add PEP: 2 µl of PEP (50 mM) for PK measurement and 12 µl PEP (250 mM) for PEPCK measurement.

HISTOCHEMISTRY PROTOCOLS

Lysosomal structural changes test

Buffers, solutions and mediums

Sodium Bicarbonate (50 mM)
Dissolve 0.042 g of sodium bicarbonate ($NaHCO_3$) in 10 ml of distilled water. Keep it at 4°C. This solution is used to perform the incubation medium.

0.1 M acetate buffer pH 4.5 containing 2.5% NaCl (100 ml)
Dissolve 1.224 g of trihydrated crystallized sodium acetate ($NaCH_3COO\ 3H_2O$) (Sigma, S-8625) in 45 ml of distilled water and 0.63 ml of glacial acetic acid in 55 ml of distilled water. Mix both solutions and fix the pH at 4.5. Finally, add 2.5 g of NaCl. Keep it at 4°C. This solution is used to perform the incubation medium.

Incubation medium (40 ml)
Dissolve 0.0112 g of naphthol AS-BI-β-D-glucuronide (Sigma, N-1875) in 0.48 ml of 50 mM sodium bicarbonate and make up to 39.52 ml with 0.1 M acetate buffer pH 4.5 containing 2.5% NaCl. Finally, add 6 g of polyvinyl alcohol (Sigma, P-8136). Note that this final step must be done in a hot plate with continuous stirring since polyvinyl alcohol has a very low solubility. After preparing the medium, keep it in the shaking water bath at 37°C in order to settle and to temper. When this is transparent, it signs that it is tempered.

2.5% saline solution (40 ml)
Dissolve 1 g NaCl in 40 ml of distilled water. Keep it in the shaking water bath at 37°C.

Postcoupling medium (40 ml)
Dissolve 0.04 g of Fast Garnet GBC (Sigma, F-8761) in 40 ml of 0.1 M phosphate buffer pH 7.4 containing 2.5% NaCl and use immediately. Stable only for some hours at 4°C.

0.1 M phosphate buffer pH 7.4 containing 2.5% NaCl (100 ml)
Disolve 2.892 g of disodium phosphate ($Na_2HPO_4\ 12H_2O$) (Fluka, 71650) in 80.8 ml of distilled water. Dissolve 0.265 g of monosodium phosphate ($NaH_2PO_4\ H_2O$) in nearly 19.2 ml of distilled water. Mix both solutions and fix the pH at 7.4. Finally, add 2.5 g of NaCl.

Baker buffer (100 ml)
Neutralise 10 ml of 40% commercial formaldehyde solution adding some drops of sodium hydroxide. Mix the neutralised solution with 10 ml of 10% $CaCl_2$ solution.
Finally add 80 ml of distilled water and 2.5 g of NaCl. Keep it at 4°C.

Mounting medium
Kaiser glycerine gelatin (Merck, 1.09242.0100).

Methodological procedure

1. Start preparing incubation medium.

2. Let the slides air-dry at the refrigerator for 30 minutes and then 10 minutes at room temperature before starting with the staining. Switch on the shaking water bath and fix the temperature at 37°C.

3. Prepare saline solution.

4. Incubate the sections in the incubation medium at 37°C for 40 minutes in the shaking water bath.

5. Prepare Post-coupling solution.

6. Rinse the slides in the saline solution (2.5% NaCl) for 2 minutes at 37°C in the shaking water bath.

7. Transfer the slides to the post-coupling medium for 10 minutes. Keep it in the darkness at room temperature.

8. Fix the sections for 10 minutes in Baker buffer calcium at 4°C.

9. Rinse briefly the slides in distilled water.

10. Mount the slides in Kaiser's glycerol gelatin.

11. Seal the edges of the coverslip with nail's protector and if possible, do not handle the slides during a couple of days until the Kaiser's glycerol gelatin is completely dry.

Lysosomal Membrane Stability test using hexosaminidase

Solutions and chemicals for 8 couplings

Citrate buffer
2.94 g Na-citrate are dissolved in 100 ml dH_2O. The pH is fixed at 4.5 and and finally 2.5 g NaCl are added to the solution.

Substrate incubation medium (to be prepared just 5 minutes before use)
0.16 g of naphthol AS-BI N- acetyl-ß-hexosaminidine (Sigma, N4006) are dissolved in 20 ml of 2-methoxyethanol (Merck, 859) and made up to 380 ml with citrate buffer, containing also 28 g POLYPEP (Sigma, P5115; low viscosity polypeptide to act as a section stabiliser).

Diazonium dye
Mix 300 ml of phosphate buffer and 0.3 g of Fast Violet.

Baker buffer

Enzymatic determination of membrane stability

1. Before start with the staining, made the incubation medium.

2. Place the sections (cuts of 10 µm thick) in a TPX staining dishes containing citrate buffer for different times (0, 3, 5, 10, 15, 20, 30, 40 minutes) at 37°C.

3. Transfer the set of slides to the incubation medium and incubate the slides for 20 minutes at 37°C in a TPX staining dishes in a shaking water-bath.

4. Prepare the saline solution and diazodium dye.

5. Wash the slides in a saline solution (3% NaCl) at 37°C for 2 to 3 minutes.

6. Transfer the slides to diazodium dye for 10 minutes at room temperature.

7. Rapidly rinse the slides in distilled water for 5 minutes.

8. Fix the sections for 10 minutes in Baker fixative at 4°C, rinse in distilled water and mount in Kaiser›s glycerol gelatin.

9. Seal the edges of the coverslip with nail's protector and if possible, do not handle the slides during a couple of days until the Kaiser's glycerol gelatin is completely dry.

Oil Red O staining

1. Prepared the Oil Red O solution (it is stable for 2 h).

    1.1. Disolve 0.25 g ORO (Sigma, O0625) in isopropilic alcohol (50 ml) and afterwards dilute it 3:2 in H_2O (33.3 ml). Firstly, the ORO, then isopropilic alcohol and finally, H_2O.
    1.2. Mix for 15 min and filter it.

2. Prepare 60% isopropilic alcohol. 100 ml à60 ml isopropilic alcohol + 40 ml H_2O (Do not shake because alcohol evaporates, cover with film, keep in the fridge)

3. Sections in Baker buffer for 15 min at 4°C.

4. After drying the sections in the air wash then in 60% isopropilic alcohol.

5. Stain sections for 20 min in a satured Oil Red O solution.

6. Differentiate sections in 60% isopropilic alcohol for 1 min.

7. Wash them distilled water.

8. Counterstain sections with an aqueous solution of 0.1% Fast Green FCF for 30 min.

9. After returning the FG to the FG's jar and clean the samples with distilled water, the sample must be immerse in distilled water until the next step.

10. Mount them in Kaiser's glycerol gelatin.

11. Seal the edges of the coverslip with nail's protector and if possible, do not handle the slides during a couple of days until the Kaiser's glycerol gelatin is completely dry.

Lipofuscin determination
Schomorl's method

1. Take unfixed cryostat sections (8 µm) to room temperature.

2. Fix the section in Baker buffer containing 2.5% NaCl for 15 min at 4°C.

3. Rinse the sections in distilled water.

4. Immerse the sections in reaction medium for 5 min.

    Dissolve 1% ferric chloride and 1% potassium ferricyanide ($C_6FeK_3N_6$) in a ratio of 3:1. Thus, (0.33 g ferric chloride + 32. 96 ml H_2O) + (0.16 g potassium ferricyanide + 16.54 ml H_2O).

5. Rinse the sections in 1% acetic acid (1 ml acetic acid + 99 ml H_2O) for 1 min.

6. Rinse the samples in distilled water.

7. Mount the slides in Kaiser's glycerol gelatin.

8. Seal the edges of the coverslip with nail's protector and if possible, do not handle the slides during a couple of days until the Kaiser's glycerol gelatin is completely dry.

METHODS FOR HISTOLOGICAL PREPARATIONS

Sample preparation for histology

1. Fix fresh dissected samples in the selected fixative:

- **Carnoy's** (for 1 hr at 4°C): 600 ml absolute alcohol + 300 ml chloroform + 100 ml acetic acid.

- **4% buffered formalin, pH 7.2** (for 24 hr at 4°C): 900 ml natural seawater + 100 ml 37% formaldehyde OR 900 ml distilled water + 28.92 g $Na_2HPO_4 \cdot 12\ H_2O$ + 2.56 g $NaH_2PO_4 \cdot H_2O$ + 100 ml 37% formaldehyde.

- **Davidson's** (for 48 hr at 4°C): 100 ml glycerol + 100 ml acetic acid + 200 ml 37% formaldehyde + 300 ml 95° ethanol + 300 ml sodium chloride.

2. Dehydrated and embedded in paraffin the samples.

70% ethanol	45 min
96% ethanol	45 min
100% ethanol	45 min
100% ethanol	45 min
Methylbenzoate	from one night until months
Benzene	45 min
Benzene	45 min
Paraffin embedding	4 hours at 56°C

3. Made the paraffin blocks.

Protocol for hematoxyline and eosine staining using automated slide stainer

STEP	STATION	TIME	REAGENT
1	1	10’	Xilol
2	2	10’	Xilol
3	3	2’	Alc. Abs
4	4	2’	Alc. Abs
5	5	2’	96°
6	6	2’	70°
7	7	5’	Distilled H_2O
8	12	4’	Hematoxyline
9	Wash 5	4’	
10	Acid alcohol	10”	
11	Wash 4	5’	
12	Lithium carbonate	10”	Eosine
13	Wash 3	1’	
14	Eosine	1’ 30”	
15	Wash 2	1”	
16	Wash 1	2’	
17	13	2’	70°
18	14	2’	96°
19	15	2’	Alc. Abs
20	16	2’	Alc. Abs
21	17	5’	Xilol
22	18	5’	Xilol
23	Exit		

Masson trichrome with anline blue or light green
(Bio-Optica Milano s.p.a.)

Principle

Four different satins are used: Weigert's iron hematoxylin for nuclei, picric acid for erythrocytes, a mixture of acid dyes (acid fuchsin-"ponceau de xylidine") for cytoplasm and aniline blue for connective tissue or light green for collagen.

Method

1. Bring section to distilled water.

2. Rinse the section in Weigert's iron hematoxylin for 10 min.

3. Without washing, drain the slide and rinse in picric acid alcoholic solution for 4 min.

4. Wash in distilled water and rinse the section in ponceau acid fuchsin according to Mallory for 4 min.

5. Wash in distilled water and rinse the section in phosphomolybdic acid for 10 min.

6. Without washing, drain the slide and rinse in Masson aniline blue or light green for 5 min.

7. Wash in distilled water, dehydrate rapidly in ascending alcohols, clear in xylene and mount in DPX.

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Azan trichrome (Bio-Optica Milano s.p.a.)

Principle

The method associates cytological staining obtained by an acid dye (azocarmin) with a contrast stain carried out with aniline blue after staining with phosphotungstic acid. To obtain good results, it is necessary to overcolor with azocarmin and then slowly differente with aniline-alcohol, in order to avoid the predomination of the contrast staining.

Method

1. Bring section to distilled water.

2. Rinse the section in Heidenhain azocarmin for 30 min in the heater. Then, wait 5 min out of the heater and recover the dye.

3. Wash in distilled water.

4. Rinse the section in aniline-alcohol for 1 min.

5. Drain the slide and rinse the slide in acid alcohol (blocking solution) for 1 min.

6. Drain the slide and rinse the slide in phosphowolphramic acid for 30 min.

7. Without washing, drain the slide and rinse in Mallory polychromic mixture for 30 min.

8. Wash in 95° ethanol, dehydrate in ascending alcohols, clear in xylene and mount in DPX.

References

Lee-Brown RK, Laidley JWS. 1929. Some observations on the microscopical anatomy of the kidney. Journal of Urology 21, 259-274.

Mallory FB. 1938. Pathological technique. Philadelphia, WB Saunders Company.

McGregor L. 1929.The finer histology of the normal glomerulus. American Journal of Pathology 5, 545-558.

Mallory trichrome (Bio-Optica Milano s.p.a.)

Principle

In this method 3 different dyes are used: carbol fuchsin for nuclear staining, orange G for cytoplasm and aniline blue for a selective collagen staining. Selectivity in this procedure is due to different degree of affinity between dyes and tissue macromolecules. A central role is played by phosphomolibdic acid which acts as a bound between tissue structures (collagen fibrils, cell membranes) and aniline blue (amphoteric dye). Orange G, which is the second component in Mallory's polychrome solution, has no affinity to phosphomolibdic acid and is thus used to stain all remaining structures unbound to phosphotungstic acid.

Method

1. Bring section to distilled water.

2. Rinse the section in carbolfuchsin solution for 10 min.

3. Wash in distilled water.

4. Rinse the section in acid differentiating buffer for 2 min.

5. Wash quickly in tap water (2-3 seconds) and rinse the section in phosphomolibdic acid solution for 5 min.

6. Without washing, drain the slide and rinse the section in polychrome solution according to Mallory.

7. Wash in distilled water, dehydrate rapidly in ascending alcohols, clear in xylene and mount in DPX.

References

Mallory FB. 1936. The aniline blue collagen stain. Stain Technology 11, 101-102.

Masson P. 1929. Some histological methods: trichrome stainings and their preliminary technique. Journal of Technology Method 12, 75-90.

McFarlane D. 1944. An easily controlled regressive trichromic staining method. Stain Technology 19, 29-37.

Puchtler H, Isler H. 1958. The effect of phosphomolybdic acid on the stainability of connective tissues by various dyes. Journal of Histochemistry and Cytochemistry 6, 265-270.

Toluidine-eosine staining procedure

1. Fixation in 1% formaldehyde in filtered seawater at 4°C for 24 hr.

2. Dehydrated and embedded in paraffin the samples (*: waiting step).

REACTIVE	TIME (hr)
70% ethanol *	1
96% ethanol	1
96% ethanol	1
Ethanol absolute	1
Ethanol absolute	1
Denatured alcohol (IMS) 99%	1
Xylene	1
Xylene	1
Paraffin Wax (60°C)	2
Paraffin Wax (60°C)	2
Paraffin Wax (60°C)	2

3. Stain 5 μm thick paraffin sections as follows (suitable for manual staining or for using an automated slide stainer):

STEP	STATION	TIME	REAGENT
1	1	10’	Xilol
2	2	10’	Xilol
3	3	2’	Alc. Abs
4	4	2’	Alc. Abs
5	5	2’	96°
6	6	2’	70°
7	7	5’	Distilled H_2O
8	12	10’	Toluidine
9	Wash 1	1’	
10	Wash 2	2’	
11	Wash 3	2’	
12	11	15”	Eosine
13	Wash 4	5”	
14	Wash 5	1’ 30”	
15	13	1”	70°
16	14	1”	96°
17	15	1”	Alc. Abs
18	16	1”	Alc. Abs
19	17	1”	Xilol
20	18	1”	Xilol
21	Exit		

4. Mounting in DPX.